3.01 Overview and Introduction

Rob Verpoorte, Leiden University, Leiden, The Netherlands

3.01.1 Biodiversity and Chemodiversity

During evolution, a large number of species have evolved (estimations run from 10 to 100 million).[1] All of these species share more or less the basic chemistry of the primary metabolism of living cells, but on top of that they have developed a species-specific metabolism that serves the organism to survive in its ecosystem. This involves quite complex chemistry and is the basis of the huge chemodiversity in nature. At present, some 250 000 natural products are known, and some 4000 new ones are reported every year,[2] but how many more are still to be discovered we do not know. If every organism would make one unique compound, there would be some 10–100 million natural products. In part, these compounds are part of the ubiquitous primary metabolism involved in the functioning of the living cell, in part, these compounds are secondary metabolites, which means compounds that serve the producing organism to survive, that is, defense compounds, pheromones, attractants of pollinators, signal compounds between different organisms, etc.

For mankind, these natural products are quite important, they are the basis of the variety of food we have, they are involved in the resistance of plants against pests and diseases, and they are the source of, for example, medicines, agrochemicals, cosmetics, dyes, flavors, and fragrances. Moreover, plants are also used for fibers (e.g., clothing, paper, ropes), shelter (wood), fuel (wood, biofuels), and for the production of bulk products such as rubber, starch, and cellulose. Many of the applications mentioned have been discovered by our ancestors and are in fact the basis of our present life (food, shelter, health, fuel, mobility) and thus of all agricultural and major industrial activities.

The first part of Volume 3 aims at giving an overview of the chemical space available and how this concept can be used in drug development (Wetzel and Waldmann, Chapter 3.02). This approach nicely shows that natural products and synthetics cover complementary areas, whereas the chemical space of drugs is clearly overlapping both. The concept of defining the chemical space on the basis of chemical properties is thus an important tool for finding a lead in drug development. As the chemical space of natural products covers only a part of the total chemical space, a special GPS for navigating the natural products space has been described by Backlund (Chapter 3.03). This system can also be coupled to the biological space and might be very helpful to identify important hotspots for finding leads for drug development. Moreover, it helps in understanding the evolution of biosynthetic pathways in nature. Obviously, these approaches are built upon all the published knowledge on natural products. To find such information, databases for natural products, medicinal plants, and their biological activities are an important resource. Such databases are, for example, very useful for the identification of organisms of interest for specific applications or drug lead finding. Farnsworth *et al.* discusses such databases in Chapter 3.04.

3.01.2 Biodiscovery

Besides exploring chemical space *in silico*, one may also explore chemical space in an at-random approach by exploring biodiversity. Such an approach for the discovery of novel products is the aim of bioprospecting. An example how biodiscovery can be organized in a large bioprospecting program in Brazil is described by Bolzani *et al.* (Chapter 3.05).

Of the medicines presently used in Western pharmacotherapy, 25% is derived from plants, and about half of the medicines developed in the past decades are natural products, natural products derivatives, or synthetic analogues of natural products. Particularly for antibiotics and anticancer medicines, nature is the major source as shown by Cragg and Newman (Chapter 3.06). Quinn *et al.* describe in Chapter 3.07 a high-throughput screening approach for drug discovery by at-random screening of biodiversity.

Almost by definition natural products are 'drug-like' as for most natural compounds there is somewhere a target in nature, for example, an enzyme or a receptor. Some of these targets may have analogues in mammals. However, the compounds found in nature may not be the optimal structures for the application as a medicine. Optimization of the chemical structure is thus required. The statistics on the novel chemical entities that have been developed in the past decades (Cragg and Newman, Chapter 3.06) show indeed that only 6% are pure natural products, whereas 28% are natural products derivatives and 12% are synthetic analogues of natural products. Appendino (Chapter 3.08) shows with some examples how natural products can be modified resulting in interesting novel lead compounds.

Not only medicines can be developed from nature but also biopesticides are an important area for biodiscovery. All plants are resistant against the majority of pests and diseases and defend themselves, among others, with a wide range of chemicals against herbivores, microorganisms, etc. That means that they are interesting sources for developing leads for biopesticides (Gonzalez, Chapter 3.09). Kinghorn (Chapter 3.10) describes the results of biodiscovery efforts in the field of natural sweeteners.

Cosmetics are almost completely based on natural products. Some aspects of biodiscovery for cosmetic products are reviewed by Masahiro (Chapter 3.11).

3.01.3 Traditional Knowledge

Some 40 000–70 000 medicinal plant species have been described,[3] which represent an enormous potential for developing novel drugs. Heinrich (Chapter 3.12) describes how to deal with this wealth of information on plants. This is the field of ethnopharmacology, that is, all the knowledge available in countries and cultures that in many cases have no written traditions. Heinrich shows with some examples how this knowledge can be used to develop novel medicines. Such research is obviously also of great interest to devise a safe and efficient use of traditional medicines in primary health care and in low-income areas.

For two major ancient, well-documented medical systems, the Chinese medicine (Chapter 3.13) and Ayurvedic medicine (Chapter 3.14), an overview is given by De-an Guo and Mukherjee, respectively, on some of the novel chemical entities that have evolved from studies on the activity of medicinal plants. In fact, evidence-based medicinal plants is now a major target of research worldwide. Systems biology is a promising approach to better understand activities, including identifying the role of synergy and the presence of prodrugs.[3–5]

3.01.4 Food and Health

We are becoming increasingly more aware of the fact that our food may contain compounds that affect our health. Witkamp (Chapter 3.15) particularly focused on the role of plants in disease prevention, and in particular, compounds in food that may play a role in weight management and preventing type 2 diabetes. This clearly relates also to the principles of Asian medicine (see Chapters 3.13 and 3.14 by De-an Guo and Mukherjee), where the emphasis is more on restoring the homeostasis than on curing a disease or treating

symptoms as it is in Western medicine. In many cases, mildly active compounds (micromolar range) are found, which makes a clear difference with drug discovery where highly actives molecules (nanomolar range) is searched for.

Colors are another interesting aspect of natural products. In plants, colors play, for example, a role in attracting pollinators. Natural dyes are applied in food, and for dying clothes, although these have been to a great extent replaced by synthetic dyes. As a major group of plant (flower) colors, the amazing chemistry of flower colors involving anthocyanins is explained by Andersen and Monica Jordheim in Chapter 3.16.

3.01.5 Supply of Natural Products

As pure products are preferred in Western medicine, the supply of natural products is a major issue. In some cases, syntheses have been developed and synthetic compounds replaced the natural products, but also production by biotechnology is possible in certain cases. The supply of paclitaxel is a good example of the problems one may encounter in developing a natural product from a rare source.[6] Muranaka and Saito describe in Chapter 3.17 plant cell cultures as a possible production system for high-value plant products, including the genetic engineering of the plant cell factory. Zarate (Chapter 3.18) deals in more detail with the potential of genetic engineering for plant-derived products. Metabolic engineering is a tool that may be applied to cross borders between organisms for the production of compounds of interest. The basis for this is in the knowledge of the biosynthetic pathways (see Volumes 1 and 2).[7] Asakawa and coworkers (Chapters 3.19, 3.20, and 3.21) show the great potential of microorganisms to perform a wide variety of selective chemical reactions on terpenoids, thus creating novel chemodiversity or making specific products such as flavorings.

3.01.6 Chemistry of Some Common Plants and Related Products

In the abovementioned chapters, single compounds are the major focus, compounds for leads of new drugs, known drugs, their supply, etc. But much of our use of plants concerns the whole plant and the quality depends on the complexity of the compounds present, for example, the taste of our food. Therefore, in a series of chapters, the chemistry of some important well-known plant products is reviewed: beer (Verhagen, Chapter 3.22) tea (Engelhardt, Chapter 3.23) cannabis (Hazekamp, Chapter 3.24) coffee (Oestreich, Chapter 3.25), and wine (Cheynier, Chapter 3.26) showing nicely the complex chemical diversity and the biological activity of some of the compounds present in these products. Wood is a very important commodity, for example, for fuel, construction, and paper (fiber) production. The chemistry of wood in connection with these various applications is thus of interest (Lewis, Chapter 3.27).

3.01.7 Model Plant and the Future

Finally, the volume ends with the present major model plant of fundamental plant sciences: *Arabidopsis thaliana*. This plant is studied as a model for various aspects, for example, drought and salt resistance, resistance against pests and diseases, flower development, the interaction with the rhizosphere, and signal transduction systems. Functional genomics is the major approach used in these studies, linking functions with genes via transcriptomics and proteomics. The chemical characterization of the phenotype of a plant in a targeted or nontargeted way (metabolomics) is a key technology in such studies. The knowledge on secondary metabolites present in *Arabidopsis* is reviewed in the last chapter (Chapter 3.28) by Pedras. *Arabidopsis* is able to make a wide variety of compounds, many of which can also be found in other plants (e.g., flavonoids, cinnamic acid derivatives, and terpenoids), but most of the compounds mentioned in the other chapters of this volume are not found in this plant. For study of secondary metabolism, one can only study the plant(s) producing the compounds of interest. With the costs of gene sequencing going down rapidly, one may expect that more and more studies on plant-specific processes such as resistance, or

production of desired natural products will be done in the plant species concerned. The role of natural products chemistry in biology will thus increase considerably in the coming years. Fields such as chemical biology and metabolomics will play an important role in systems biology approaches to learn to understand nature's complexity. This series of *Comprehensive Natural Products Chemistry* should be an important reference work for all those entering such exciting multidisciplinary research.

References

1. S. L. Pimm; G. J. Rusell; J. L. Gittleman; T. M. Brooks., *Science* **1995**, *269*, 347–350.
2. R. Verpoorte; R. van der Heijden; H. J. G. ten Hoopen; J. Memelink, *Biotechnol. Lett.* **1999**, *21*, 467–479.
3. R. Verpoorte; H. K. Kim; Y. H. Choi, Plants as Source of Medicines: New Perspectives. In *Medicinal and Aromatic Plants – Agricultural, Commercial, Ecological, Legal, Pharmacological and Social Aspects*; R. J. Bogers, L. E. Craker, D. Lange, Eds.; Springer: Dordrecht, 2006pp 261–274.
4. R. Verpoorte; Y. H. Choi; H. K. Kim, *J. Ethnopharmacol.* **2005**, *100*, 53–56.
5. Mei Wang; R. J. A. N. Lamers; H. A. A. J. Korthout; J. H. J. van Nesselrooij; R. F. Witkamp; R. van der Heijden; R. Verpoorte; J. van der Greef, *Phytother. Res.* **2005**, *19*, 173–182.
6. G. M. Cragg; S. A. Schepartz; M. Suffness; M. R. Grever, *J. Nat. Prod.* **1993**, *56*, 1657–1668.
7. R. Verpoorte; A. W. Alfermann; T. S. Johnson, Eds., *Applications of Plant Metabolic Engineering*; Springer: Dordrecht, 2007.

Biographical Sketch

Professor Verpoorte is Head of the Division of Pharmacognosy, Section Metabolomics, Institute Biology Leiden, Leiden University. He holds a Pharmacists degree (1972) and a Ph.D. degree from Leiden University. His Ph.D. thesis was on pharmacologically active compounds from *Strychnos* species (1976). He began his career as a lecturer at Leiden University (1976–87) and became Professor and Head of the Department of Pharmacognosy in 1987. He has been a guest professor at the universities of London (UK), Uppsala (Sweden), Amiens (France), and Reims (France). From 1992 to 1998, he served as the Vice Chairman and Chairman of the committee of the Phytochemical Society of Europe.

He is the author and coauthor of more than 600 scientific papers, 3 books, and 4 patent applications. He is Editor-in-Chief of the *Journal of Ethnopharmacology and Phytochemical Reviews* and Executive Editor of *Biotechnology Letters*. He serves on the editorial board of 21 journals.

His research interests are in biosynthesis and metabolic engineering of plant secondary metabolism, metabolomics, medicinal plants, and the isolation and identification of biologically active natural products.

He received an Honorary Doctorate from the University of Amiens, France (2004). In 2007, he received the Phytochemical Society of Europe Medal.

3.02 Natural Products as Lead Sources for Drug Development

Stefan Wetzel, Hugo Lachance, and Herbert Waldmann, Max Planck Institute of Molecular Physiology, Dortmund, Germany

3.02.1 Introduction – A Historical Perspective

Since the beginning of organized social life, mankind has been on a quest to fight diseases and improve the quality of our lives. Through series of trials and errors, knowledge about medicinal herbs has been gathered and summarized in pharmacopeias dating back to antiquity.[1,2] Nowadays, natural medicines are still in use but most contemporary chemotherapeutic agents are pure, well-defined, chemical entities. The evolution from herbal remedies to drugs in clinical use today was a slow and gradual process that started with inquiring minds at the beginning of the nineteenth century.

In 1806, a young 21-year-old German pharmacist, Friedrich Wilhelm Adam Sertürner (1783–1841), reported the isolation of a white crystalline powder from opium (*Papaver somniferum*), which he named morphine after Morpheus, the Greek god of dreams.[3,4] This was in fact the first isolation of a natural product, which was commercialized in 1827 by Heinrich Emanuel Merck of Darmstadt. The isolation of morphine paved the way for the discovery of many other natural products including strychnine, colchicine, codeine, and.[5] After more than 200 years, morphine is still used for its analgesic properties but most importantly it has been a source of inspiration for the development of many natural or synthetic analogues with relevant biological activities (**Figure 1**).

Direct derivatives of morphine such as heroin, codeine, and oxycodone are used for their diverse degrees of analgesic activities through their action on the opioid receptors. The closely related analogues, the morphinanes, with reduced functionalities on the outer cyclohexane ring are also used for their analgesic effects. The removal of this outer six-membered ring produced the benzomorphanes, which also possess analgesic properties. Much simpler analogues, the 4-phenyl piperidines, lack both the outer cyclohexane ring and the bridged system of morphine and are also used for pain relief. The analogues of morphine lacking all ring systems are the simplest analogues derived from morphine; this class includes the analogue methadone, well known for its use

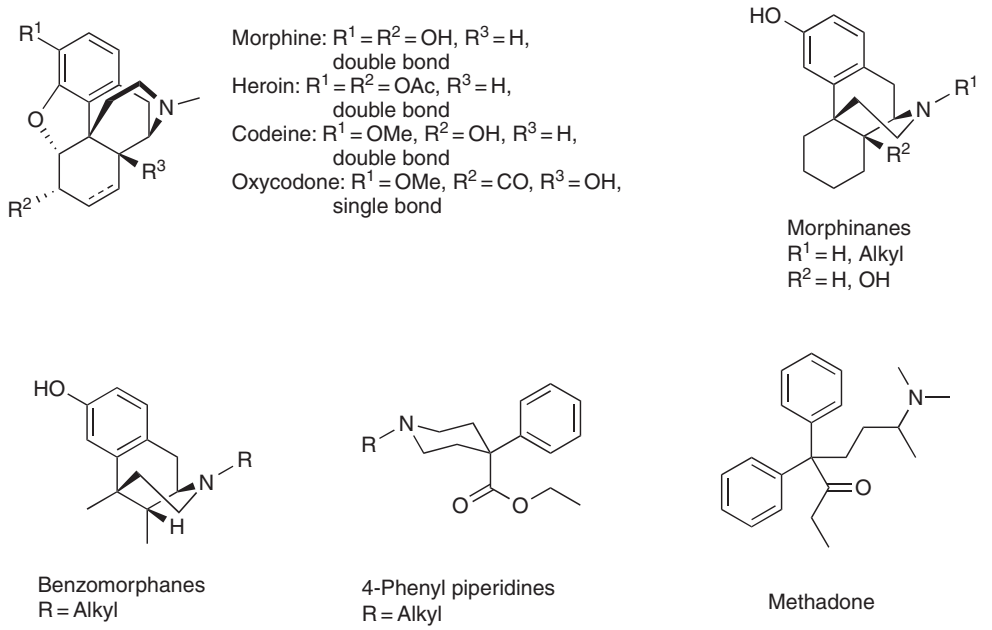


Figure 1 Structures of morphine and analogues. Reproduced with permission from S. Wetzel; A. Schuffenhauer; S. Roggo; P. Ertl; H. Waldmann, *Chimia* **2007**, *61* (6), 355–360.

in treating withdrawal symptoms associated with addiction to heroin and other opiates. Methadone is also used medically because of its mild analgesic properties for chronic pain relief.

This discovery of morphine by Sertürner was only the beginning of a long series of important discoveries that eventually developed into pharmaceutical drug development as it stands today.

When the Spanish discovered South America in 1492, they were looking for gold and spices, in addition they discovered a wild continent that was replete with natural resources. Among these, a tree from the eastern slopes of the Andes, the cinchona tree or 'quina-quina' to the natives proved to be even more valuable than gold. It would in fact be, for more than 300 years, the only cure against malaria. This remarkable power comes from the presence of the alkaloid quinine (**Figure 2**),[6] the active principle of the cinchona tree bark. The supply of cinchona bark has been an important source of conflict between European nations and was also a critical factor in the African continent exploration as well as in other parts of the world. In the early nineteenth century, the identity of the entity responsible for the curative properties of the remedy was still unknown. In that regard, the efforts of Pelletier and Caventou were rewarded in 1820 when they isolated quinine as a bitter-tasting, yellow gummy material. By 1821, instructions on how to administer quinine were available and by the mid-1830s it became the treatment of choice for malaria over the powdered bark treatment. The mass production of quinine and its use as antimalarial therapy began and it is now regarded as the first step into the pharmaceutical industrial era. The scientific community would then have to wait until 1908 for Paul Rabe to define the right atom connectivity of quinine. Vladimir Prelog determined its absolute and relative stereochemistry in 1944. From then on, a race was on to synthesize quinine, a topic that is well known to most of today's organic chemists

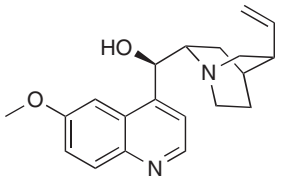


Figure 2 Quinine: Active principle of the cinchona tree bark. Reproduced with permission from S. Wetzel; A. Schuffenhauer; S. Roggo; P. Ertl; H. Waldmann, *Chimia* **2007**, *61* (6), 355–360.

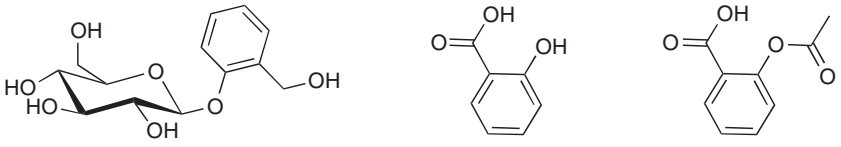


Figure 3 From willow tree concoction to Aspirin®.

and has been extensively discussed and reviewed.[6–8] As none of the numerous total syntheses are amenable to large-scale synthesis, all the quinine used today comes exclusively from the cinchona tree bark extraction.

According to the Ebers papyrus,[9,10] willow trees and other plants have been used for their analgesic, antipyretic, and anti-inflammatory properties for more than three millennia. Greek physician Hippocrates also used similar ingredients to relieve the pain of childbirth. The active ingredient of these remedies was identified by Joseph Buchner who obtained relatively pure salicin (**Figure 3**) crystals in 1828. This was followed by the isolation of an acidic component from willow extract, salicylic acid (**Figure 3**), in 1838 by Italian chemist Raffaele Piria.[11] Although very useful for their predictable reduction of pain, fever, and inflammation, their use was greatly impaired by undesirable side effects, in particular gastric irritation. In their effort to reduce these side effects, chemists at Bayer discovered acetyl salicylic acid (ASA) in 1897 (**Figure 3**). This discovery led to the commercialization of Aspirin® in 1899. This synthetically prepared analogue of salicin was quickly recognized for its reduced acidity, incidentally avoiding the gastric irritation side effects of salicylic acid and salicin. In this regard, Aspirin was an improvement on these willow-containing concoctions and was the first commercial synthetic drug. It was the beginning of the synthetic drug industry.

ASA was then investigated in great detail and the discovery in 1971 by Vane and coworkers[12,13] of the action of ASA led to the Nobel Prize for Vane in 1982 in physiology or medicine. ASA acts by inhibiting the production of prostaglandins. The enzyme responsible for the action of Aspirin was subsequently identified as cyclooxygenase 1 (COX1) and cyclooxygenase 2 (COX2), which convert arachidonic acid into prostaglandins,[14] thus, acetyl salicylic acid is a nonselective COX1 and COX2 inhibitor. It was also later found that ASA irreversibly blocks the formation of thromboxane A_2, a major component of blood clot, thus making Aspirin also a preventive treatment to reduce incidence of heart attacks.[15]

When Alexander Fleming[16] returned from vacations in August 1928 and observed unusual culture patterns in a petri dish to be disposed of, he was far from expecting it to be one of the most important scientific discoveries of the twentieth century. In one of the dish, a mold colony had grown and around this colony, *Staphylococci* colonies did not grow. He investigated the properties of what he named penicillin.[17] Despite its remarkable antibacterial properties, Fleming could not isolate the active agent due to its instability. It took 10 years before Chain *et al.*[18] took interest in penicillin and isolated it to pursue clinical trials. After a rapid succession of clinical trials, penicillin entered commercial production in 1942 and became the first natural product antibacterial chemotherapy, only second to the sulfanilamide class of antibiotics.[19] Since then, many analogues of penicillin have been isolated from other microorganisms and produced through synthesis or semisynthesis (**Figure 4**). Penicillin is the parent of all known β-lactam antibiotics. The direct analogues of penicillin are the reflection of variation in the acetyl side chain of the β-lactam moiety (**Figure 4**). This class of analogues generates molecules with varied level of antibiotic activities ranging from narrow spectrum to broad spectrum uses and is mainly active against Gram-positive bacteria. When a six-membered ring replaces the five-membered heterocycle of penicillin, the cephalosporins (**Figure 4**) are generated. These analogues are also used as antibiotic treatments against Gram-positive bacteria. The later generations of cephalosporins have an increased efficiency against the Gram-negative bacteria. The carbapenems (**Figure 4**), when a carbon replaces the sulfur atom, have the broadest spectrum of all the β-lactam antibiotics.[20,21] They are active against both Gram-positive and Gram-negative bacteria and are also stable to the β-lactamases, the main mechanisms of bacterial resistance against penicillins. The monobactams are antibiotics possessing only the β-lactam moiety, are active against Gram-negative bacteria and are considered inactive against Gram-positive bacteria.

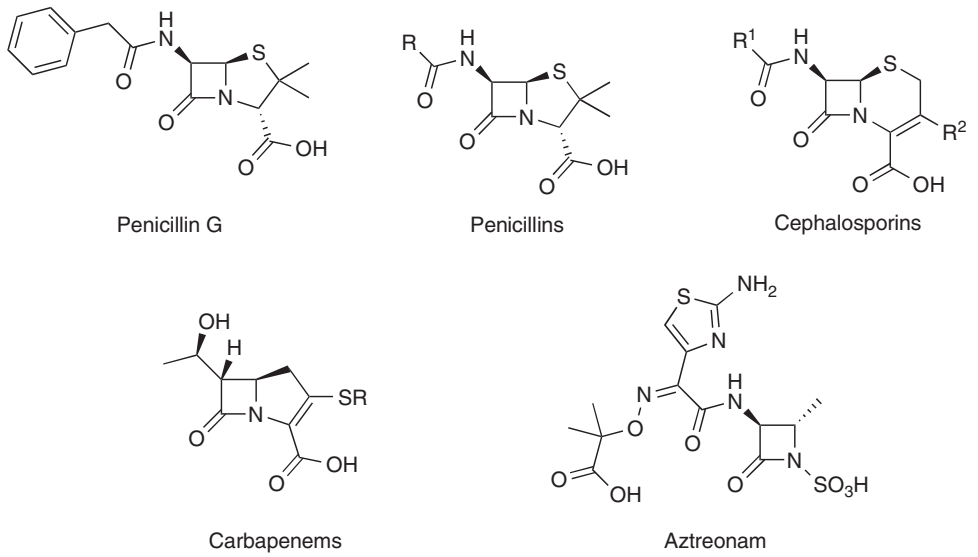


Figure 4 Structure of penicillin and other β-lactam antibiotics.

The penicillins were the first isolated and commercialized antibiotic therapy and have been a very successful class of drugs. It is thus regrettable to admit that we might be seeing the end of their era due to the widespread resistance of bacteria to this class of compounds. Fortunately, nature has provided us with other antibiotic classes such as macrolides (erythromycin), the glycopeptides (vancomycin), the aminoglycosides (streptomycin), the tetracyclines, and many more.

In the 1950s and 1960s, the medical community realized the role played by elevated levels of cholesterol in the incidence of heart diseases.[22] In the hope of finding cholesterol biosynthesis inhibitors, 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase was chosen as the intended target in the search for drug therapy. It was quickly found that a molecule of natural origin, compactin (later known as mevastatin) isolated from the fermentation broth of *Penicillium citrinum* had a powerful inhibitory effect on HMG-CoA reductase.[23,24] Unfortunately, mevastatin was never used as drug therapy due to its severe side effects. In 1978, researchers at Merck Research Laboratories isolated a new statin-related molecule from *Aspergilus terreus*, which was known later as lovastatin (**Figure 5**). Lovastatin was demonstrated to be effective in reducing cholesterol blood level (cholesterolemia) and was approved for sale by the Food and Drug Administration (FDA) in 1987. Thus it was the first cholesterol-lowering drug in the market and was the first efficient way of reducing cholesterolemia. It rapidly developed into a commercial success achieving sales of more than US$ 1 billion in the best years. It has also paved the way to the development of more mevastatin analogues. The analogue simvastatin (**Figure 5**)

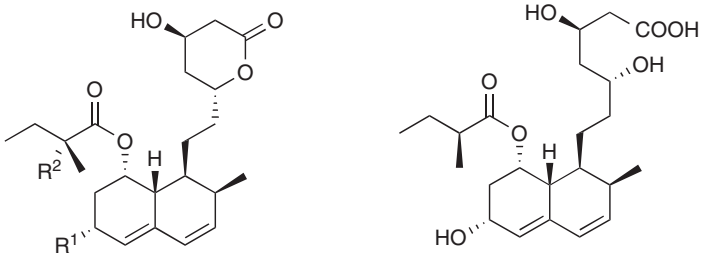


Figure 5 Naturally isolated or derived statins.

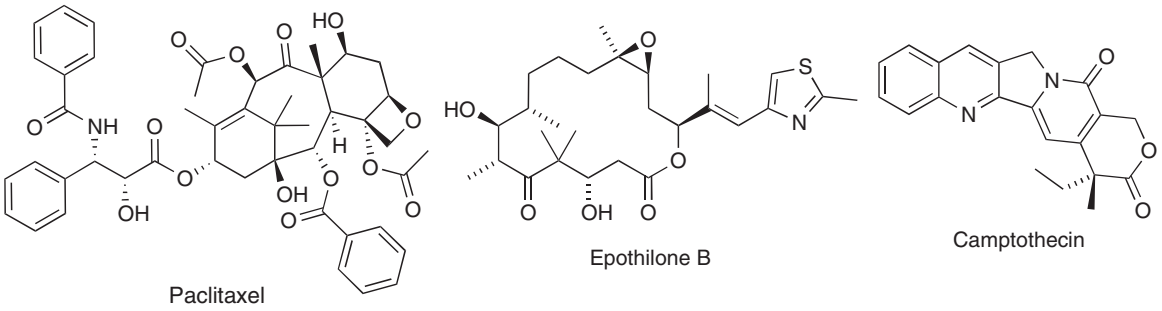


Figure 6 Structure of anticancer natural products.

(Zocor®) that is obtained by semisynthesis from lovastatin was launched in 1988 and became subsequently a huge success. In 2004 alone, Zocor sold for more than US$ 5.2 billions,[25] making it the second best selling drug on the market. During the same period, pravastatin (**Figure 5**) was also on the market (Sanko Pharma Inc. and Bristol-Myers Squibb). This closely related analogue of lovastatin is biosynthetically prepared from microbial fermentation of mevastatin.

Natural products have also played a major role in cancer therapy.[26–28] Molecules such as paclitaxel (Taxol®),[29–33] epothilone B,[34] and camptothecin[31–33] (**Figure 6**) have drastically influenced cancer research on many aspects. They have helped the scientific community to understand the disease better, provided new and efficient therapies possessing new mechanisms of action, opened new research avenues, and provided new inspiration in the development of new and future anticancer drugs.

Besides these well-known and successful examples, plenty of natural products are still brought to the market as approved drugs today. Between 2000 and 2006 more than 26 plant-derived natural products were either approved or launched in the market and many more were still in clinical trials.[35] In 2005, the global sales of plant-derived drugs were estimated to be in the order of US$18 billion and it is expected to keep increasing steadily in the coming years. These plant-derived chemotherapies consist a widerange of applications. They can be used to fight infections and to treat pain, inflammation, cardiovascular diseases, and cancer. Many other sources of natural products have been, are, and will be used to discover new potential drugs.[36–42] In the following sections diverse aspects of the processes used to discover biologically active natural products, to use them as leads in drug discovery, and to develop them into new drugs, and inspire new research avenues eventually leading to drugs will be discussed.

3.02.2 Natural Product Properties

3.02.2.1 Overview of Natural Product Property Studies

Natural products have long been seen as endowed with special properties. The systematic evaluation of natural product properties has led to a better understanding of which properties distinguish natural products from compounds originating from medicinal chemistry programs or drugs. This knowledge can be applied in the design and synthesis or acquisition of natural product-like compound collections. In 1999 Henkel *et al.* published the first comparison of properties of natural products and synthetic compounds[43] followed by Lee and Schneider in 2001 who especially addressed the drug-likeness of natural products.[44] Feher and Schmidt authored one of the most comprehensive comparisons of natural products, drugs, and combinatorial chemistry compounds in 2003 using over 40 properties.[45] Ertl and Schuffenhauer analyzed molecular properties and structural features of different natural product classes, in total for more than 130 000 molecules.[46] Recently, Grabowski and Schneider worked on the same topic with a special focus on marine natural products.[47] We will give a summary of the publications mentioned above and then present a comparison of natural product properties based on the *Dictionary of Natural Products*,[48] drugs from Drug Bank,[49] and a random selection of vendor compounds from our in-house library.

Henkel *et al.*[43] compared two natural product databases, the *Dictionary of Natural Products*[50] and the Bioactive Natural Product Database[51] with the Bayer AG in-house collection and the Available Chemicals Dictionary (ACD).[52] They found that natural products on an average contain three stereogenic centers, three times as many as in drugs and significantly more oxygen but less nitrogen than drugs and synthetic compounds. Henkel *et al.* also analyzed pharmacophoric features, that is, structural motifs linked to interactions with macromolecules including functional groups such as alcohols and isosteres. In line with their previous results, they found oxygen-containing motifs to be more abundant in natural products whereas drugs and synthetic compounds incorporate more nitrogen-containing moieties. In general, Henkel *et al.* found the properties of natural products to be more similar to those of drugs than of synthetic medicinal chemistry compounds.

Lee and Schneider[44] compared mainly properties related to the rule-of-five[53] for a set of natural products and trade drugs. The rule-of-five is an empirical set of parameters to predict compounds that are likely to be orally available drugs. It was derived from known orally available drugs by Christopher Lipinski in 1997. In short, the suitable compounds have a molecular weight below 500, an octanol–water partition coefficient (log P) of less than 5, and contain not more than five hydrogen bond donors and not more than 10 hydrogen bond acceptors. The rule-of-five has been widely used in the pharmaceutical industry, that is, for compound library design, selection of screening compounds, etc. Lee and Schneider determined the number of heteroatoms per molecule and found natural products to contain an average of 1.4 nitrogen atoms per molecule, about one less than the trade drug set. The average number of oxygen atoms was found to be four in both cases. The average calculated octanol–water partition coefficient (log P) indicates that natural products (2.9) are more lipophilic than drugs (2.1). The authors also determined how many compounds of both sets violated the rule-of-five. They discovered that only about 10% of the natural products violated the rule-of-five criteria although these had been derived exclusively from orally available drugs. The rate was similar for the trade drug set indicating that, with respect to the rule-of-five, small molecules natural products on an average were more drug-like than they were thought to be.

Feher and Schmidt[45] used marked natural products from the catalogues of three compound vendors: BioSPECS, ChemDiv, and InterBioScreen. The drug set was derived from the Chapman & Hall *Dictionary of Drugs* and the synthetic compounds from combinatorial libraries were also chosen from the databases of compound vendors, among others Maybridge, ChemDiv, and SPECS. Altogether, the analysis included about 30 000 natural products (including derivatives), 11 000 drugs, and 670 000 synthetic compounds. More than 40 molecular properties were calculated for all molecules. The most significant differences between all three sets could be found for the number of stereogenic centers, the atom distributions, types of rings, and ring fusion patterns. The average number of stereogenic centers in natural products was determined to be 6.2 as compared to 2.3 in drugs. Interestingly, this is double the number of stereogenic centers calculated by Henkel *et al.* for natural products 4 years earlier[43] although the ratio of natural products to drugs remained about the same (3:1). In contrast to Lee and Schneider[44] but in agreement with Henkel *et al.*,[43] the authors found that natural products contain twice as many oxygen atoms and only half as many nitrogen atoms as drugs. Feher and Schmidt also conducted an analysis of structural patterns, that is, rings, ring fusion patterns, and saturation. On an average, natural products contain two rings more per molecule than drugs and their degree of ring fusion is twice as high. Natural products were found to contain considerably less number of aromatic rings than drugs although their overall degree of unsaturation was higher. In line with these findings, the analysis also showed that natural products on an average contain two rotatable bonds less than drugs. Taken together, these findings imply that natural products are on an average more rigid than drugs, partially due to larger fused ring systems.

Grabowski and Schneider in 2007 compared the properties and scaffolds of drugs, pure natural products, natural product derivatives, and, particularly interesting, a collection of marine natural products.[47] The authors determined that about 10% of the drug compounds but 18% of the pure natural products and even 30% of the marine natural products violate at least two parameters of the rule-of-five. The high number of marine natural products violating the rule-of-five may be due to the higher average molecular weight (503.6 vs. 414.5 for drugs and 393.9 for pure natural products), and the higher average number of H-bond acceptors (7.4 vs. 6.4 for drugs and 6.6 for pure natural products). Natural products were found to contain one-third of the nitrogen atoms that are found in drugs (0.7 for pure natural products and 1.2 for marine natural products vs. 3.0 for drugs) but more oxygen (5.9 for pure natural products and 6.1 for marine natural products vs. 3.4 for drugs). The number of stereogenic centers was found to be 5.5 and 6.3 for pure natural products and marine natural products,

respectively, and 1.4 for drugs with a ratio of 4:1 for natural products to drugs. Interestingly, the number of rotatable bonds for drugs and pure natural products is similar (6.7 vs. 5.2) whereas marine natural products have a much higher average number of rotatable bonds (11.5) indicating a higher flexibility of the molecules. The average number of aromatic atoms reflects the trend described earlier with 12.4 for drugs and 5.1 for pure natural products. Marine natural products contain even lesser number of aromatic atoms per molecule (3.4).

Ertl and Schuffenhauer[46] analyzed the largest set of natural product structures so far, which contained 130 000 structures from the *Dictionary of Natural Products*.[54] Their analysis confirmed the data from the earlier analyses of smaller data sets. Ertl and Schuffenhauer also identified scaffolds and substituents typical for natural products produced by different classes of organisms (bacteria, fungi, plants, and animals). In the course of this analysis, the authors found that 21 000 molecules contained between 1 and 12 sugar units. Several molecules were found that exhibit more than one glycosylation pattern. Very little is known about the influence of these different glycosylation patterns on the biological effect of these compounds.

3.02.2.2 Comparison of Property Distributions of Natural Products, Drugs, and Synthetic Compounds

To allow the reader to draw his own conclusions, we calculated a range of property distributions for sets of natural products, drugs, and synthetic compounds. The resulting diagrams are shown and commented in this subsection (**Figures 7 and 8**). The natural product set was taken from the Chapman & Hall *Dictionary of Natural Products* (version 17.1),[48] which was filtered for all molecules with a structure entry yielding 191 694 compounds. For the known drugs, we took the set of small molecule drug structures from Drug Bank[49] containing 4810 structures. The set of synthetic compounds was compiled by randomly choosing 247 000 compounds from our in-house database of various compound vendors including ChemDiv, InterBioScreen, BioSPECS, and others. All structures were then cleaned and their properties calculated with Pipeline Pilot.[55]

The analysis yielded similar results as the previous analyses described. Natural products contain fewer nitrogen atoms than drugs and synthetic compounds, more oxygen, and also less sulfur. The distribution of the numbers of stereogenic centers per molecule shows that only 20% of the natural products but 40% of the drug molecules and close to 80% of the synthetic compounds have no stereogenic center. The distribution for natural products tails off slowly and stays above the line for drugs and synthetic compounds for four and more stereogenic centers. The numbers of hydrogen bond donors and acceptors do not differ much between the drug set and the natural products. In contrast, the synthetic compounds show a narrow distribution for donors and acceptors with a sharp maximum at one donor and four acceptors per molecule. Of particular interest is the fact that all synthetic compounds fall within the rule-of-five criteria of less than 5 hydrogen bond donors and less than 10 acceptors whereas a small but equal proportion of natural products and drugs violates these criteria. The analysis of the number of rings per molecule gives a similar picture: The distribution of the synthetic compounds shows a clear maximum at 3–4 rings whereas those for drugs and natural products are much broader. Natural products tend to have more rings than both, drugs and synthetic compounds but, as described earlier, aromatic rings are far more abundant in synthetic compounds and also slightly more common in drugs than in natural products. Compared to drugs, natural products incorporate almost an equal number of five-membered rings but more six-membered ones. The analysis of the number of ring assemblies, that is, fused ring systems, per molecule clearly shows that natural products generally consist of one or two larger fused ring systems. Although the maximum of the distribution for drugs is at one ring assembly per molecule like for natural products, more drugs also contain 2–4 ring assemblies than in natural products. The synthetic compounds show a clear maximum around three assemblies and, most notably, no molecules without any rings, that is, zero ring assemblies. This implies that although aliphatic molecules are found among natural products and drugs, they are not present in the synthetic compounds sold by compound vendors and often used for screening purposes. The distributions of the number of rotatable bonds per molecule are quite similar for drugs and natural products but differ significantly from the distribution for synthetic molecules that is bell shaped with a sharp maximum of five rotatable bonds.

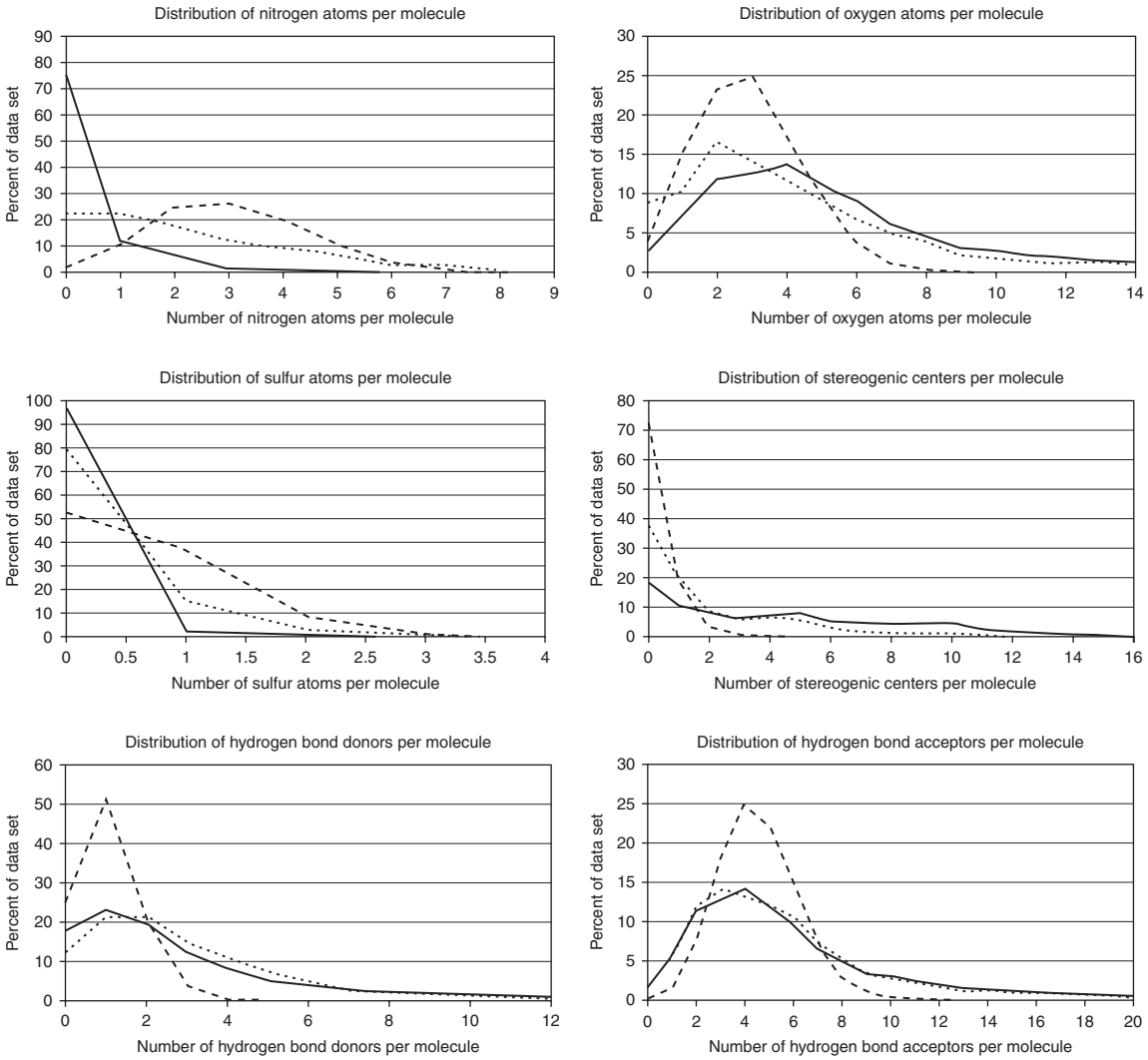


Figure 7 Property diagrams of compound collections comprising natural products (——), drugs (·····), and synthetic vendor compounds (– – – –).

3.02.2.3 Special Properties of Natural Products and Their Use in Drug Discovery

From the data presented, one can conclude that the properties of natural products are in general more similar to those of drugs than to synthetic compounds often used in high-throughput screening (HTS) libraries. Nonetheless, some properties differentiate natural products from drugs albeit not necessarily their adherence to the rule-of-five. The most prominent properties distinguishing natural products from the other compound classes are the heteroatom distribution, that is, nitrogen, oxygen, and sulfur atoms; the number of stereogenic centers; the number of rings; the fraction of aromatic rings; and the degree of ring fusion. Natural products in general contain more nitrogen and less oxygen atoms but more stereogenic centers than drugs or synthetic compounds. They comprise of more rings but less aromatic rings and exhibit a higher degree of ring fusion. These properties together suggest that natural products contain more rigid scaffolds with a defined three-dimensional structure that has evolved to bind to the corresponding protein.

It should be kept in mind that natural products are often perceived as a particular, homogenous group of diverse but related compounds such as drugs. However, natural products consist of a great variety of subclasses determined by the organism of origin, the biotope of that organism, and the natural product's molecular

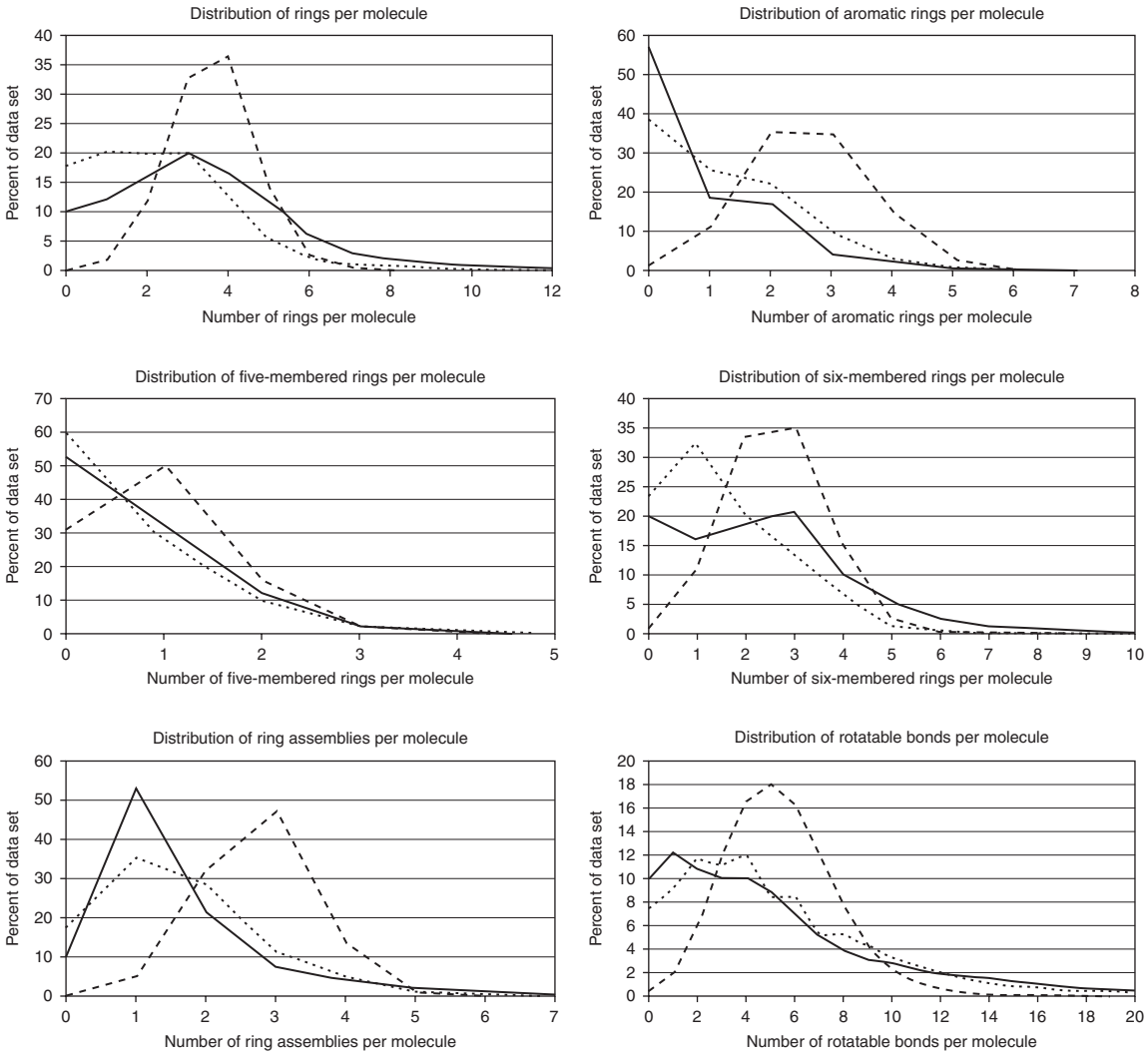


Figure 8 Property diagrams of compound collections comprising natural products (——), drugs (·····), and synthetic vendor compounds (– – – –).

function, for example, as metabolite, venom, hormone, etc. All these factors significantly influence the structure and composition of the natural products belonging to one individual subclass. The biotope, for example, may impose constraints for biosynthesis with respect to the availability of certain elements. The very low nitrogen content in natural products from plants, for example, as shown by Henkel *et al.*[43] may partly be attributable to nitrogen being a growth-limiting factor for plants. Similarly, the higher halogen content found in marine natural products could be enabled by the higher halogen content in sea water. Natural products are also far more heterogeneous in their sizes than drugs as they incorporate large molecules like peptides or macrocycles.[56] Finally, our knowledge about the different classes of natural products also varies considerably. Plant ingredients have been studied extensively and systematically for centuries whereas the analysis of marine natural products has picked up only recently. In any case, it should always be kept in mind that databases are subject to change over time and any analysis can reflect only the knowledge, that is, compounds contained in databases, at a particular point in time.

The knowledge about the molecular properties that distinguish natural products from drugs and synthetic compounds may still be highly useful and can be applied in the design of natural product-like compound collections exploiting parts of nature's diversity. Filtering available compounds according to these criteria is

also possible although the chemical space concept and especially the visualizations thereof provide more advanced methods to compare libraries and choose natural product-like compounds.

3.02.3 Chemical Space

3.02.3.1 Introduction to Natural Product Chemical Space

The chemical space comprises the total number of possible small organic molecules[57] that was estimated to exceed 10^{60} individual molecules.[58] The natural product chemical space, that is, the parts of chemical space containing natural products, is much smaller. The *Dictionary of Natural Products*, one of the most comprehensive sources of natural products, lists about 215 000 natural products and analogues in its 17.1 version from 2008,[48] and in 2004, J. Bérdy estimated that more than one million natural products are known.[59] This number has increased within the past few years so that the known natural product chemical space can be estimated to contain in the range of 10^6–10^7 compounds – a tiny but particular fraction of chemical space. Over the last decade several approaches toward charting of and navigating through chemical space have been developed. These approaches will be described in the following sections together with their view on natural product space in comparison to drug space and synthetic compound space. We will present possible applications of these concepts to exploit the natural product chemical space for hit finding.

3.02.3.2 Different Views on Chemical Space

The approaches taken to chart chemical space have explored different aspects of the molecules populating this space. Some approaches are based on the properties of the compounds, describing compound property space. Others are based on chemical structure and explore the chemical structure space. Although these approaches employ different methodologies and, therefore, yield different views on chemical space, their results are highly complementary and depending on the task at hand, one method may be more suitable than the other.

As described in the previous section, natural products have unique properties that differentiate them from drugs and synthetic compounds found in vendor databases. Although the statistics about the individual properties already give some insight, a visualization of the property space would allow a more intuitive overview and assessment of the regions of chemical space occupied by the different compounds. The first step in all these visualizations of property spaces is the reduction of the number of dimensions to two or three, that is, the number that can be visualized in a diagram. This is done via a mathematical algorithm named 'principal component analysis' (PCA). PCA is a mathematical transformation that converts an *n*-dimensional vector space to one of a smaller dimensionality, for visualization mostly two or three. When translating to a three-dimensional vector space, for example, the transformation yields a new set of three basis vectors that are linear combinations of the original ones while keeping those characteristics that contribute most to the variance of the data. In a compound property space this means that each basis consists of the sum of fractions of all the properties calculated, the so-called 'loading'. Subsequently, the properties of each compound are transformed into a set of coordinates locating this compound in the new two- or three-dimensional compound property space. The resulting diagram is a scatter plot, where each dot represents one compound.

This approach has been used by Feher and Schmidt in their 2003 publication,[45] where they calculated 40 molecular properties and used PCA to transform them to two dimensions.

The resulting diagrams show that the property space occupied by synthetic compounds from combinatorial chemistry is well confined and almost circular centered on the origin of the coordinate system. The natural products and drugs occupy almost the same space and are much more diffuse than the synthetic compound space. Moreover, the centers of the compound clouds of natural products and synthetic compounds are off the origin of the coordinate system. Thus one can say that the analysis of Feher and Schmidt is able to distinguish between synthetic compounds from combinatorial chemistry and natural products or drugs albeit probably not between the latter two.

The term 'chemography', for the art of charting chemical space, was introduced before Feher and Schmidt's publication by Oprea and Gottfries in 2001.[60,61] They developed an approach to charting chemical space that utilizes more than 60 descriptors and PCA. One of the limitations of the PCA method is its dependency on the

data set. This is due to the PCA method conserving the components contributing most to the variance of the data set. Therefore, in another data set, these components may change and the overall results cannot be compared anymore. This is true especially in the analysis of compound sets where the lack of comparability is a significant limitation. To overcome this weakness of the PCA method, Oprea and Gottfries proposed to use a reference system to map the compounds. They used a set of 423 compounds with extreme properties that populate the outer fringes of the chemical space of interest as reference compounds. The PCA is only performed with the reference compound set, which, therefore, defines the boundaries of the chemical space. The positions of all other compounds are interpolated based on the references. The authors designed the approach to resemble the Navstar global positioning system (GPS), which uses a network of satellites in geostationary orbits (far from Earth) to calculate the position of a receiver on Earth by triangulation. Since Oprea and Gottfries charted chemical space, they used the name ChemGPS for their approach.[61] They also optimized the loading of the three axes such that each axis contains mainly interpretable properties like size, hydrophobicity, or flexibility. This is a significant advantage because the position of compounds in chemical space can be directly translated back into chemical properties. In 'normal' PCAs this is often difficult because of the linear combination of fractions of many properties mapped to one axis.[62,63]

In further investigations, Larsson *et al.* applied ChemGPS in the exploration of natural product chemical space, namely natural product modulators of cyclooxygenase (COX).[64] In this study, ChemGPS was able to discriminate clusters of different activity from each other, for example, COX1 enzyme inhibition, COX2 enzyme inhibition, and reduction of the COX2 level by inhibition of its expression or translation. It also enabled the authors to identify properties that may be important for the different types of activities. Larsson *et al.* also identified some compounds that were outliers in ChemGPS. Since the properties of these compounds were outside the boundaries defined by the reference set, their position in chemical space had to be extrapolated rather than interpolated. This was not unexpected as ChemGPS was optimized for drugs and compounds from combinatorial chemistry that differ in their properties from natural products. Consequently, the authors developed a new reference system for natural products, which forms the basis of ChemGPS-NP,[65] a ChemGPS version for natural products. This revised version utilizes a set of 35 descriptors including many properties that were identified to discriminate natural products from other compound classes as described above and is based on a set of 1779 reference compounds, more than four times the number of compounds contained in the original reference set. The graphical representation generated from ChemGPS and ChemGPS-NP is a three-dimensional scatter plot. The plot is shown in illustration 1 in Chapter 3.02.

A structure-based approach to charting and comparing parts of chemical space involving PCA has been presented by Ertl and coworkers.[46,66] The authors compiled sets of 15 000 representative deglycosylated structures from the *Dictionary of Natural Products*[54] (about 113 000 unique aglycons), 15 000 bioactive molecules taken from the World Drug Index,[42] and the MDL drug data report (MDDR) database[67] (about 120 000 structures in total), and 15 000 synthetic compound selected from various vendor databases. They analyzed these compound sets for the 110 most common two-atom fragments, that is, those that were present in more than 0.3% of the molecules. The frequencies of these fragments in each data set were then used as a descriptor. After normalization, that is, calculation of relative frequencies in percent, a PCA was performed to reduce the 110 dimensions (one for each fragment) to three for visualization. The corresponding diagram is shown in **Figure 9**. It shows that synthetic compounds occupy a smaller and more well-defined space than natural products and bioactive molecules as in Feher and Schmidt's analysis. However, natural products and bioactive compounds occupy different parts of chemical space and can be differentiated by the fragment frequencies. As expected, the space occupied by bioactive compounds lies in between and overlaps with both other compound sets, natural products and synthetic compounds.

In summary, PCA-based approaches can process large numbers of compounds very rapidly. Therefore, they are well suited to visualize and compare large sets of compounds and can discriminate between different sets of compounds, for example, natural products, drugs, and synthetic compounds. However, even in the structure-based approaches like the one developed by Ertl *et al.*, it is difficult to translate the position of a compound in chemical space back into chemistry and chemical structure that is necessary to design and guide chemical synthesis.

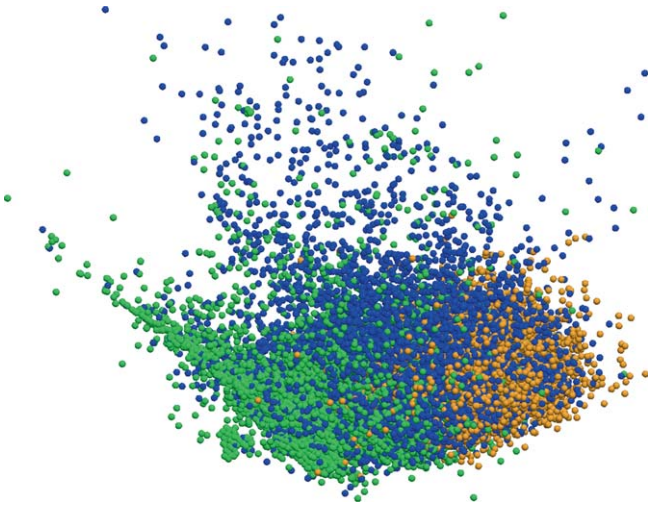


Figure 9 Scatter plot of the combined chemical spaces of natural products (green), bioactive molecules (blue), and synthetic compounds (orange). Reproduced from S. Wetzel; A. Schuffenhauer; S. Roggo; P. Ertl; H. Waldmann, *Chimia* **2007**, *61* (6), 355–360.

With a focus on chemical library design, Waldmann and coworkers developed an exclusively structure-based approach to charting of and navigating in chemical space.[68] Their method is based on a hierarchical classification of scaffolds. In the first step, the authors extracted the so-called Murcko scaffolds[69] from the compounds, that is, all rings and connecting aliphatic linker chains as well as all ring- and linker-based double bonds. These scaffolds are then deconstructed in an iterative process, one ring at a time until the scaffold cannot be pruned any further, usually when only one ring is left. This stepwise degradation process is guided by a set of rules derived from organic and medicinal chemistry knowledge in a way that each scaffold is assigned to only one smaller scaffold. The larger scaffold is called the child scaffold and the derived smaller scaffold the parent scaffold. The sequence of scaffolds for one molecule resulting from the stepwise deconstruction forms a branch with as many hierarchy levels as the number of rings contained in the compound. Combination of branches of a set of molecules, for example, natural products, yields a tree diagram, the so-called 'scaffold tree'. Waldmann and coworkers first applied their approach to the deglycosylated natural product structures in the *Dictionary of Natural Products*. This structural classification of natural products (SCONP) yielded the scaffold tree depicted in the manually drawn diagram in **Figure 10**. They analyzed the natural product chemical space using their approach and found that most natural products contained scaffolds with 2–4 rings incorporating carbocycles, O-heterocycles, and N-heterocycles in decreasing frequency.

Owing to the use of chemical structure and the substructure relationships as ordering principle, the scaffold tree diagram is intuitively understandable to chemists. Moreover, results of every analysis are structure based and, therefore, can be used to direct and design the chemical synthesis of new compound collections, in which often a scaffold is the template.

In their first analysis, they allowed only scaffolds as parents that were present as Murcko scaffolds in compounds themselves. This led to 'holes' in many cases, where one or more hierarchy levels were left unpopulated because the corresponding scaffolds were not present in compounds in the data set. Moreover, the parent–child assignments and, thus, the overall structure of the scaffold tree depended on the data set because only the present scaffolds could be parents as well.

A revised set of rules designed by Schuffenhauer *et al.* allowed all scaffolds as parents whether they were present in molecules in the data set or not.[70] Consequently, the parent–child assignment would be independent of the data set, that is, a given scaffold would always be assigned the same parent. Moreover, there would be no more holes where scaffolds in the sequence were missing. These two factors allow scaffold trees resulting from different sets of compounds to be compared to each other, one important and apparent application of such an approach.

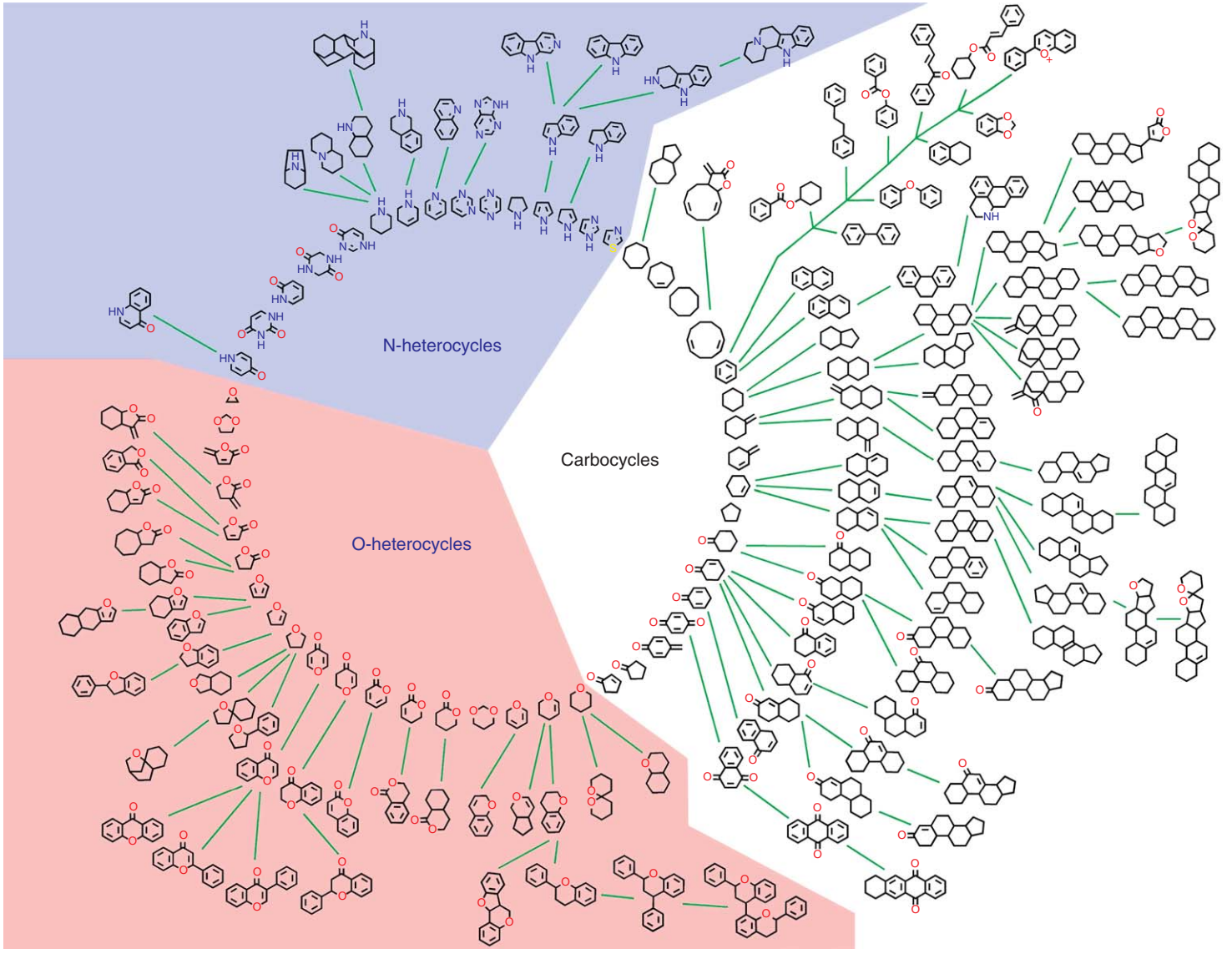


Figure 10 Scaffold tree generated from the deglycosylated structures contained in the *Dictionary of Natural Products* 14.2. For clarity, only those scaffolds are shown that represent at least 0.2% of all molecules. Reproduced from S. Wetzel; A. Schuffenhauer; S. Roggo; P. Ertl; H. Waldmann, *Chimia* **2007**, *61* (6), 355–360.

3.02.3.3 Natural Product Chemical Space Analysis as Tool for the Discovery of New Compound Classes for Medicinal Chemistry Research

As laid out in the previous subsection, there are multiple approaches to charting of and navigating through chemical space. Each method provides a different but complementary perspective on chemical space. Consequently, each method is more suitable for some applications and investigations and less suitable for others. Many more methods exist to explore and exploit the chemical space concept without charting it at all. This subsection will describe approaches to explore chemical space to discover promising regions of chemical space and, in the end, new guiding structures for medicinal chemistry programs.

One of the most apparent applications of the chemical space concept is the identification of those regions of chemical space that promise to yield biologically relevant compounds. Such analyses are of value because chemical space, like astronomical space, is mostly void of biological relevance and those small molecules that modulate protein function are scattered all over chemical space comparable to the stars in the universe.[57] Therefore, the chance of encountering such molecules by a random exploration is rather small, a phenomenon well known in serendipity-based discovery methods, for example, HTS of large compound collections where hit rates are typically below 0.1%. It has been widely recognized that the quality and outcome of HTS campaigns depend significantly on the quality of the compound library tested, for example, its biological relevance, diversity, and quality.[71–75]

Retrospective analyses can be carried out with respect to many different criteria, for example, drug-likeness, lead-likeness, and known biological relevance.[60,76–82] Natural products can be regarded as a group of compounds endowed with special properties and optimized in the course of evolution to bind to various proteins, that is, during biosynthesis, biodegradation, and while exhibiting their mode of action.[83–85] Other groups of biologically relevant compounds are those with proven bioactivity including drugs and other compounds with proven biochemical or biological activity. Chemical space analysis can be used to identify regions of chemical space occupied by such compounds, for example, natural products or drugs, in order to enrich screening libraries with structures from these regions and, thus, with biological relevance. One of the many methods to identify such structures is the natural product-likeness score developed by Ertl *et al.* that is based on the two-atom fragments described in the previous subsection.[86] The authors used a statistical model to identify those two-atom fragments that discriminate natural products from other compound classes, for example, drugs or screening compounds. Note that these fragments differentiating the compound classes from each other include those that are abundant in natural products but not in the other compounds and vice versa. From the number of such fragments present in a specific molecule, the authors calculate a natural product-likeness score. This scoring function can also be used in library acquisition or to assess natural product-likeness of newly designed libraries. Similar scoring functions exist for drug- and lead-likeness.

Another particularly interesting application of the chemical space concept in the discovery of new biologically relevant compound classes is the analysis of patent space. The patent space basically is a chemical space containing molecules that are annotated with information related to intellectual property (IP). Such an approach was published by Southall and Ajay who analyzed a kinase inhibitor patent space.[87] The authors analyzed the chemical space of 116 550 known kinase inhibitors annotated with activity and patent data for compound series that are related to each other by molecular replacements. This was achieved by adopting a method presented earlier by Sheridan,[88] who identified reoccurring transformations in drugs. In brief, the authors compared the compounds from two different series by calculating their maximum common substructure, that is, the largest part of the structure that both compounds share. The nonsimilar parts of the molecules are defined as a 'chemical replacement', that is, the moiety that needs to be changed to convert the structures from one series into the structures from the other series. Interestingly, the authors found that the number of replacements that occur more than once in the data set is limited. Moreover, they could identify several molecular replacements linking whole series of compounds from two different companies to each other. This approach can be of value to identify chemical strategies used in the design of patents in the area of interest. Additionally, a dictionary of common molecular replacements may also be used to design new libraries of promising fast-follower compounds in response to a competitor's patent.

The structure-based approach developed by Waldmann and coworkers was initially developed to chart natural product chemical space and to identify structural scaffolds abundant in nature.[68] In their publication,

the authors also presented an application to discover structurally simplified analogues of natural products with a desired biological activity. In a model case, they started from glycyrrhetinic acid, a natural product with a five-ring steroid-like scaffold that has a proven biological activity on 11β-steroid dehydrogenase type 1. From there, the authors suggest to move along the branches of the scaffold tree toward the inner rings comprising the smaller scaffolds, a method they term 'brachiation'. Waldmann and coworkers used their protein structure similarity clustering approach[89] to determine an end point of simplification, which led them to an octahydronaphtalene scaffold. A small molecule library based on this scaffold was synthesized and in a subsequent biochemical screen indeed yielded several modulators of 11β-steroid dehydrogenase type 1. Waldmann and coworkers also applied a similar approach in the discovery of phosphatase inhibitors from libraries based on indole-containing scaffolds.[90]

All the approaches described above can be applied to analyze existing (virtual) libraries. But how can we explore the unknown parts of chemical space beyond what we already know? Several attempts have been undertaken to enumerate parts of chemical space – often in a more or less comprehensive manner. A complete enumeration of the chemical space relevant to drug discovery, for example, within the rule-of-five, is not feasible with current technology.[58,91–94] The different methodologies employed include complete enumeration of all possible molecules as well as reaction-based methods utilizing catalogs of available chemicals.[93,95] These enumerated chemical spaces can then be analyzed for promising compounds, for example, by comapping them with known inhibitors of a certain enzyme in ChemGPS[60] or by identifying natural product-like, drug-like, or lead-like regions. Other methods often used are virtual screening methods like high-throughput docking or ligand-based methods, which also build on sets from known compounds exhibiting the desired activity.

A particularly interesting approach to chart unknown regions of chemical space between two known active molecules was developed by van Deursen and Reymond.[96] The authors interpret chemical space as a 'structural continuum', which they explore by transforming a given starting molecule into a target molecule. This transformation occurs stepwise by mutations of the structure, that is, atom type conversion, atom interchange, atom addition, or bond changes. A scoring based on similarity to the target molecule is applied to choose 10 structures for the next round of mutation. To these, 20 randomly selected structures are added as well. The program stores all structures generated along the way passing chemical feasibility and stability filters. This conversion is comparable to the morphing of one image into another and the molecules generated can be expected to contain features of the starting as well as the target molecule. The authors show one example where AMPA (((*S*)-2-amino-3-(3′-hydroxy-5′-methyl-isoxazol-4′-yl)-propionic acid), an agonist to the AMPA receptor, was transformed into a known antagonist but did not provide an experimental screen of intermediate structures. Albeit not in all cases, in some the biological space may also represent a continuum and in these cases the intermediate structures should contain partial activity of both, the start and target molecule. In such a case this method could be applied to design libraries for the search of compounds with a defined polypharmacology or partial agonistic or antagonistic activities.

3.02.4 Natural Product-Based Libraries

Although natural products have proven to be a prolific source of new drugs, some major issues related to their availability remain. In the event where the source is renewable or easily cultivable, isolation from the primary source is the preferred production process, but unfortunately it is not always possible to produce the desired molecule this way. In this situation, total synthesis and semisynthesis remain the only alternatives. Because in most cases the supply of the active molecules through lengthy and costly total synthesis is impractical, alternative molecules have to be found. Furthermore, many of the most biologically interesting molecules can still suffer from poor pharmacological profiles. These limitations can be manifold: limited bioavailability, *in vivo* instability, metabolite toxicity, and many others. In these cases, the preparation of modified natural products with improved stability and bioavailability while retaining the desired activity is of great interest. To rapidly and efficiently access these improved molecules, the preparation of natural product-derived or natural product-inspired libraries has been extensively pursued.[97] These natural product-based libraries can be planned using mainly two approaches, diversity-oriented synthesis (DOS)[98,99] and biology-oriented synthesis (BIOS).[100] These libraries can generate results in various ways, anticipated or not. Consequently, the screening

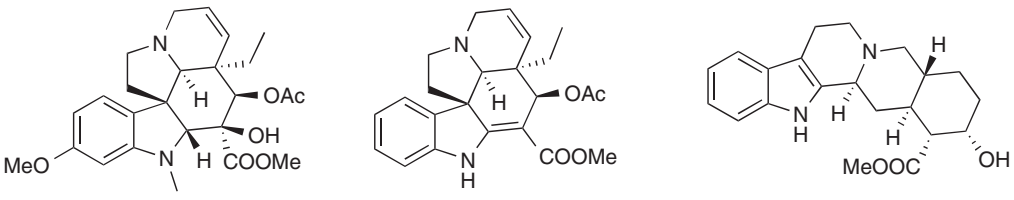


Figure 11 Indole-/indoline-containing natural products.

of such libraries can result in the discovery of simplified similarly active molecules, molecules with enhanced biological properties, or molecules with unrelated biological activities. A brief selection from the literature will be presented in the remainder of this section on natural product-based libraries. Those examples will highlight different approaches and strategies used for the preparation of libraries inspired from natural products.

The group of Arya and coworkers has designed a library based on the naturally occurring indole and indoline scaffold.[101] This class of compounds can be represented by natural products such as those shown in Figure 11.

Vindoline is the monomeric precursor of vincristine and vinblastine, both of which are currently in clinical use for their antineoplastic properties (Figure 12). Furthermore, tabersonine is the precursor of vincamine and vinpocetin, which are known vasodilators. Yohimbine can be used to treat male impotency through its selective competitive α2-adrenergic receptor antagonist activity.[102]

The preparation of the library was achieved using a (4-methoxyphenyl)diisopropylsilylpropyl linker to attach the fully protected indoline **1** on the solid support (Scheme 1). This indoline was then deprotected using piperidine and was subsequently reacted with Fmoc-protected amino acid chloride to generate the amide **2**, thus introducing the first degree of diversity. The removal of the Fmoc group was again performed using piperidine followed by spontaneous cyclization of the generated free amine. This newly generated secondary amine **3** was reacted with the second variation group using the corresponding acyl chloride and pyridine. The removal of the Alloc protecting group was achieved using palladium tetrakis(triphenylphosphine) and morpholine. The newly freed amine was reacted with the respective acyl chloride to diversify the third position. In this library, the fourth group was kept constant with the ethyl ester functionality. Furthermore, the library was generated as an epimeric mixture, which was generated in a ratio of around 8:1 during the Michael addition step.

Through this sequence, Arya and coworkers generated a 90-membered library of scaffold (**4**) based on the indoline–indole scaffold. This library is meant to be tested for its biological activity in various cellular assays.

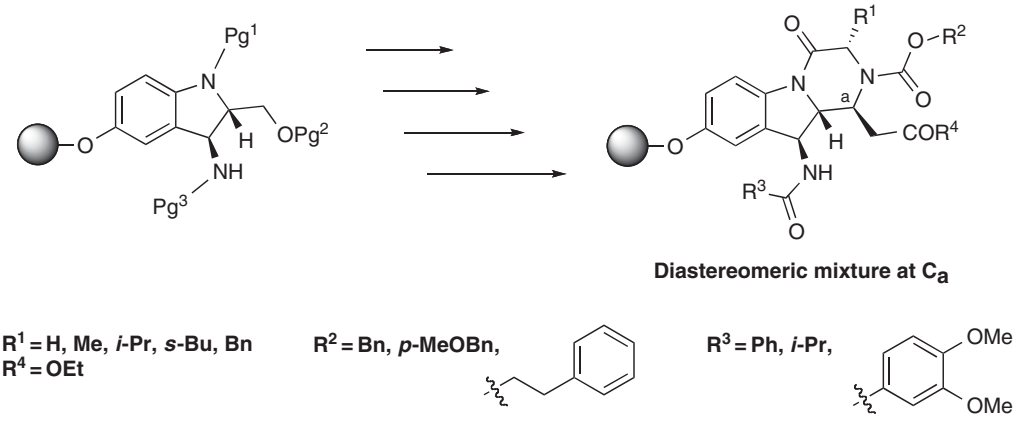
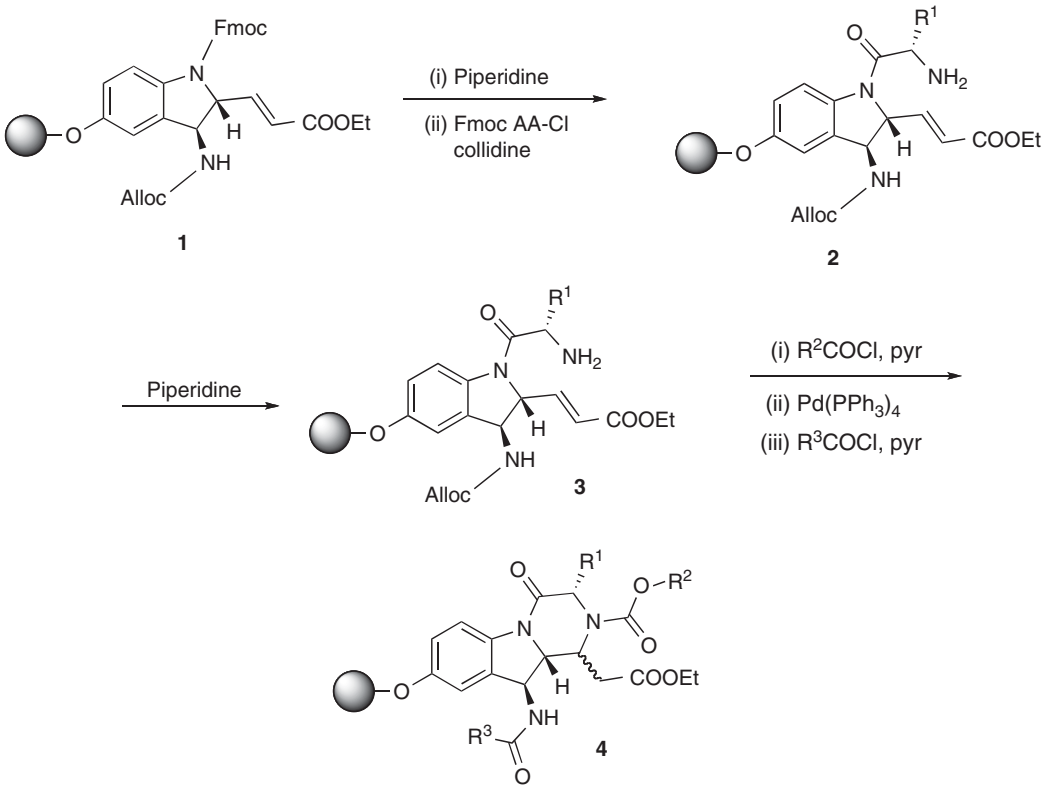


Figure 12 Proposed diversified skeleton.



Scheme 1

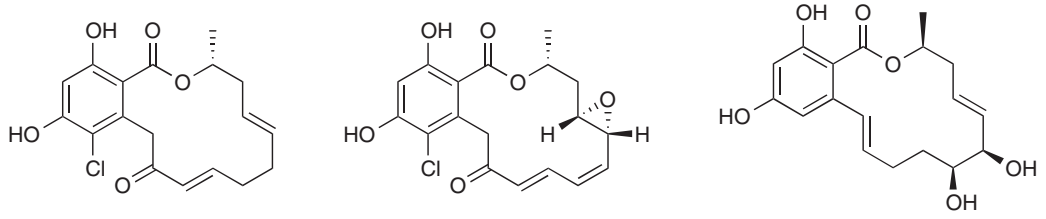


Figure 13 Structures of resorcylide natural products.

Winssinger's group has used the resorcylide scaffold (**Figure 13**) to study the effect of analogues of pochonin (**5**)[103] on the inhibition of the heat shock protein 90 (HSP90). This protein, which is a known regulator in many signaling pathways, has been associated with diseases such as cancer[104–106] and neurodegenerative diseases.[107]

Despite the remarkable activity of radicicol (**6**) against the oncogenic processes involving HSP90, its therapeutic use is hampered by its poor pharmacological profile. Based on the information available in the literature,[51,108] the library of pochonin was designed to avoid the high reactivity of both the epoxide group and the conjugated system of radicicol (**Figure 14**). The presence of an alkene in pochonin plays the role of conformational constraining group equivalent to the epoxide of radicicol.[108] The instability of the Michael-acceptor conjugated system can be reduced by using an oxime group as ketone analogue.[51]

One of the key points of this synthesis is the use of solid-supported reagents (**Scheme 2**). In the first step, the Weinreb amide alkylation, the reaction mixture was treated using a solid-supported benzoic acid to acidify the mixture. The following step, the oxime formation, was also treated with an acid resin to remove the excess hydroxylamine and amine side products formed. The solid-supported DCC reagent was used in the amidation

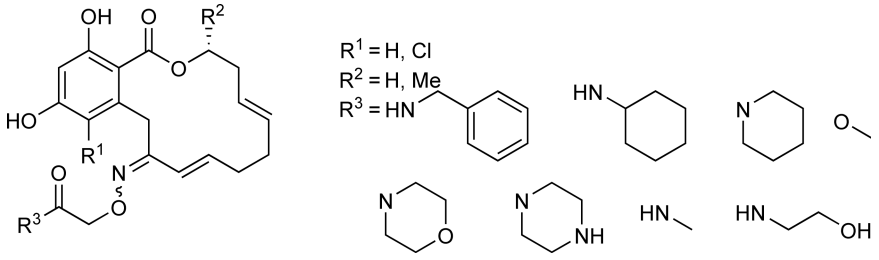
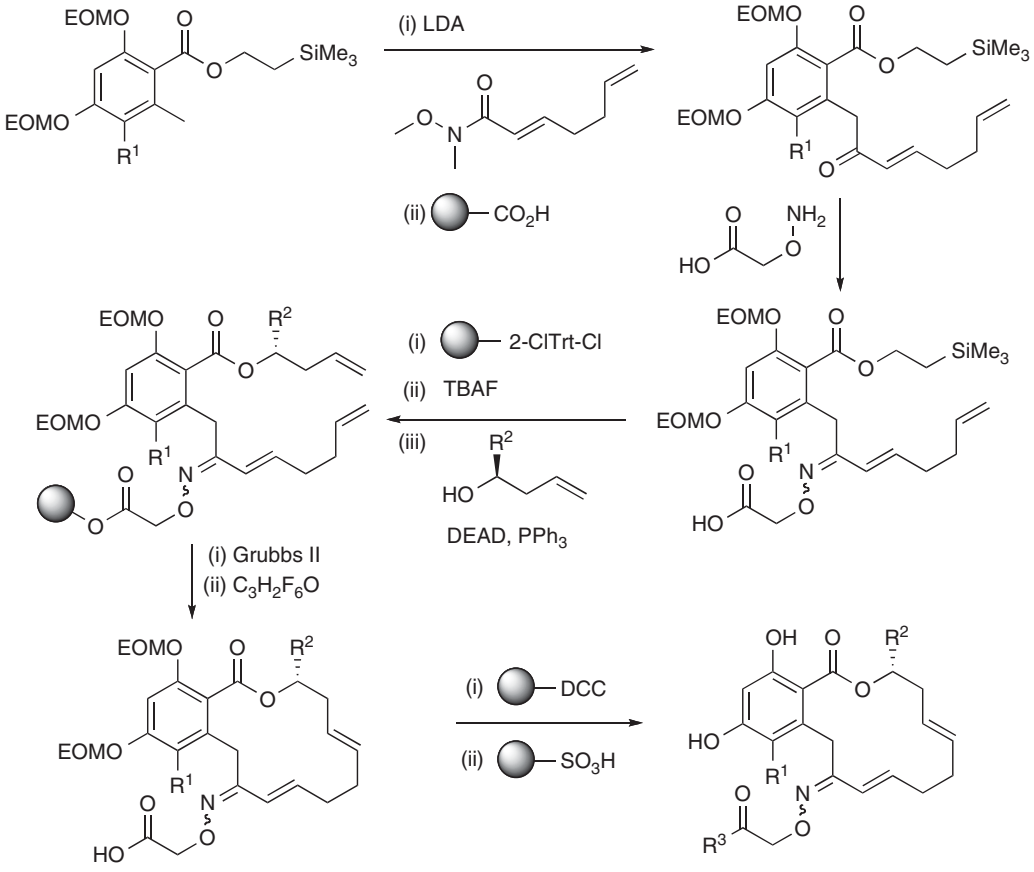


Figure 14 Pochonin library.



Scheme 2 Synthesis is the use of solid-supported reagents. Reproduced with permission from S. Barluenga; C. Wang; J. G. Fontaine; K. Aouadi; K. Beebe; S. Tsutsumi; L. Neckers; N. Winssinger, *Angew. Chem. Int. Ed. Engl*. **2008**, *47*, 4432–4435.

reaction, thus simplifying the reaction mixture purification. The final deprotection step was performed using a solid-supported sulfonic acid to remove the ethoxymethyl (EOM) protecting groups. Furthermore, the intermediate carboxylic acid was loaded onto 2-chlorotrityl resin to perform the Mitsunobu esterification and the microwave-assisted ring-closing metathesis reaction using the Grubbs second-generation catalyst. This synthesis provides a simple and quick way to access this natural product-inspired library.

This pochonin-based library was tested for its affinity to HSP90α, for the degradation of Her-2 (HSP90 client) and for its cytotoxicity against two breast cancer cell lines (SKBr3 and HCC1954) that overexpress Her-2. The results of these assays are summarized in **Table 1**.

In these screens, the best compound of the library (*E*, $R^1 = H$, $R^2 = Cl$, $R^3 =$ piperidine) showed a 10-fold improvement in activity over the library template. It is also notable that the presence of the chlorine atom on the aromatic ring is not required for the activity of these pochonine analogues. The best compound was

Table 1 Biological activities of pochonin D analogue library

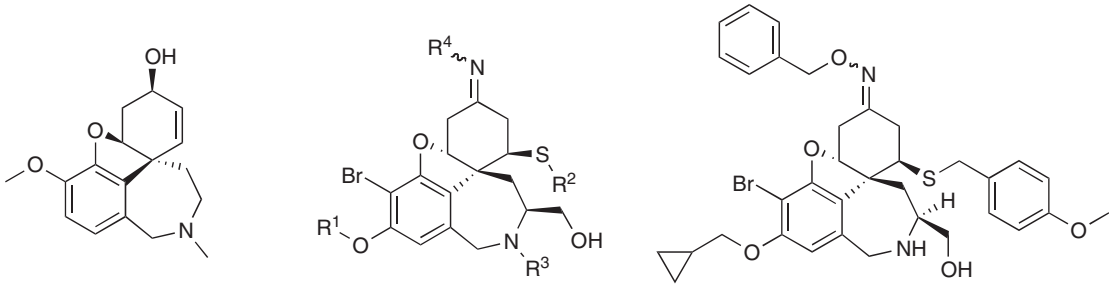
Compound (oxime E/Z, R^1, R^2, R^3)	*HSP90 affinity (μmol l^{-1})*	*Client depletion (μmol l^{-1})*	*SKBr3/HCC1954 cytotoxicity (μmol l^{-1})*
Radicicol	0.140	0.45	
Pochonin D	0.360	0.45	
E, H, Cl, piperidine	0.021	0.035	0.125/0.320
E, H, H, piperidine	0.015	0.050	0.120/0.220
E, Me, H, piperidine	0.018	0.026	0.450/0.630
E/Z, H, Cl, morpholine	0.220	>10	>10/>10
E/Z, H, Cl, piperazine	>10	>10	>10/>10
Z, H, Cl, piperidine	0.068	2.4	1.3/2.8
Z, H, H, piperidine	0.081	–	–

subsequently tested for its *in vivo* activity. In a CB17/SCID mouse model, the compound was well tolerated at 100 mg k g^{-1}. Furthermore, a tumor volume regression of 18% was observed after treatment of a BT-474 (breast tumor cell line) xerograf with the best HSP90 affinity molecule. Moreover, histological examination of the tumors by nuclear TUNEL staining (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling), and by hematoxylin and eosin staining, revealed the occurrence of massive apoptosis. These results validate the initial hypothesis that a library based on pochonin D with improved pharmacological properties could lead to more active HSP90 interacting molecules. It also shows that the best pochoxime analogue is more active than both radicicol and pochonin D.

These naturally occurring structures can also be used as biologically prevalidated starting points for the library design. Natural products can be considered privileged structure on the basis that during their synthesis by the organism of origin, these molecules must be bound to proteins at some point.[68,109–112] These molecules must also bind to proteins to exert their intended activities in the same organism or on other interacting organisms such as predators species. In the screening of such a library, two possible outcomes are possible, the library can generate hits with related biological activities, as in the previous example, or completely unrelated biological activities can be found.

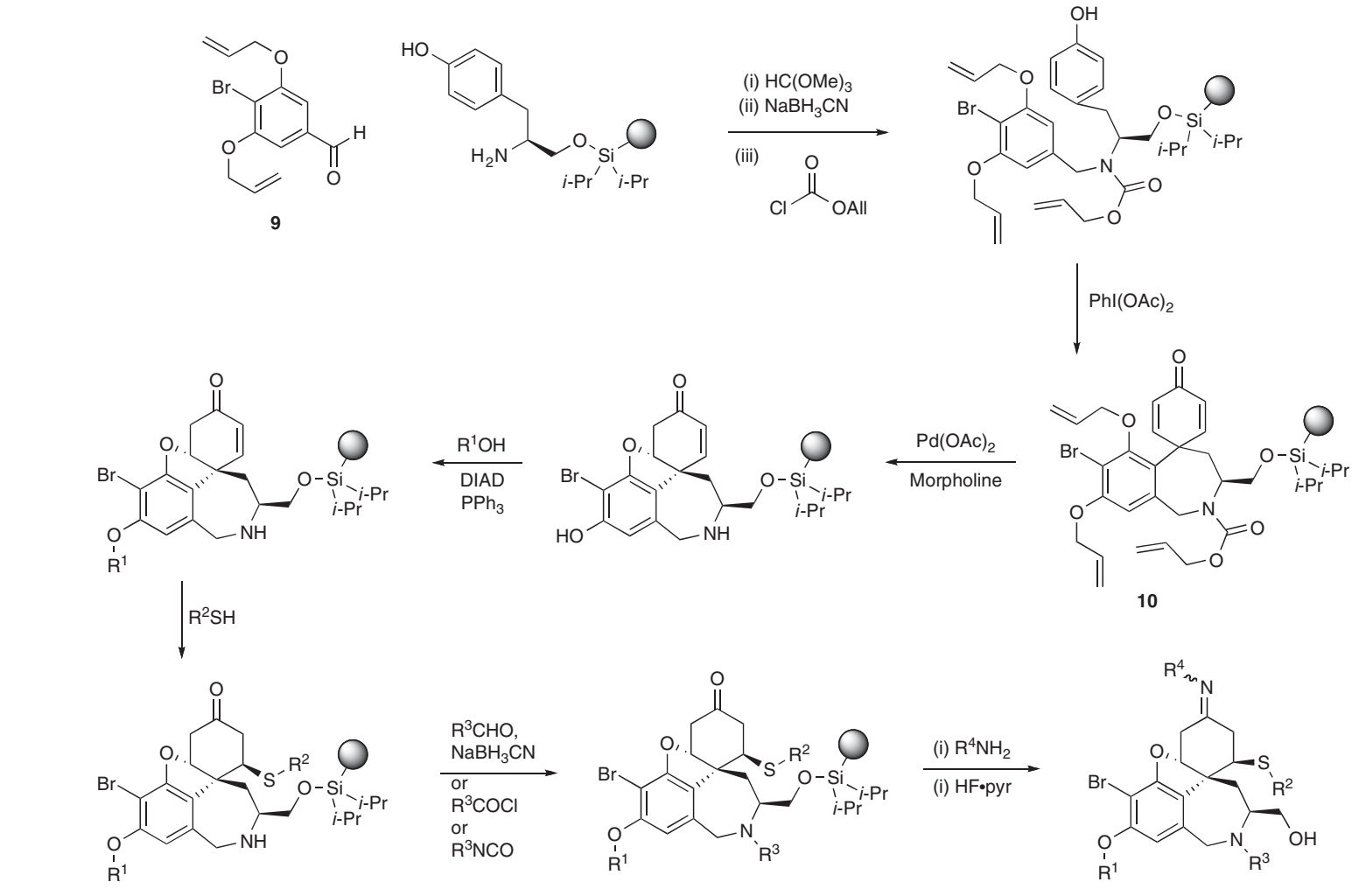
To 'divert' natural product activity toward other biological targets, one of the possible approaches is to start from a suitable natural product scaffold and apply a DOS method. This strategy was used by Shair and coworkers[113] in the generation of a 2527-membered library based on the galanthamine (7) scaffold (**Figure 15**). This structure was chosen for many reasons: It offered many points for diversity introduction, its relatively flat structure was expected to be beneficial in case of protein binding, and the preparation of the core structure would benefit from an efficient biomimetic key step. It is noteworthy that in this case the lead structure is a potent acetylcholine esterase inhibitor and that the targeted activity was the perturbation of the mammalian secretory pathway, two completely unrelated processes.

This large size library was quickly and efficiently prepared on solid support in a one-compound–one-bead strategy (**Scheme 3**). It was assembled rapidly using the polymer-supported reduced tyrosine and aromatic



Galanthamine (**7**) Library template Secramine (**8**)

Figure 15 Structure of galanthamine, library scaffold, and secramine.


9
(i) HC(OMe)3
(ii) NaBH3CN
(iii)
Cl
OAll
PhI(OAc)2
10
Pd(OAc)2
Morpholine
R1OH
DIAD
PPh3
R2SH
R3CHO,
NaBH3CN
or
R3COCl
or
R3NCO
(i) R4NH2
(i) HF•pyr

Scheme 3

aldehyde (9) under reductive aminating conditions followed by Alloc amine protection. This polymer-supported phenol was oxidized using $PhI(OAc)_2$ to produce intermediate quinone (10). All three allyl-protecting groups were removed simultaneously using palladium acetate and morpholine. The free phenol generated is spontaneously cyclized in a manner that is analogous to the proposed biosynthesis of galanthamine. After the generation of the core tetracyclic structure, diversification was first introduced using the remaining phenol under Mitsunobu reaction conditions. The second element of diversity was introduced through conjugated addition of thiolates to the α,β-unsaturated ketone. The functionalization of the secondary amine group was the site where the third point of diversity was introduced. It was achieved through reductive amination chemistry, acylation, or its reaction with isocyanates. The last diversification step was an imine formation between the remaining ketone and a series of hydroxylamines and hydrazines. This sequence quickly generated the 2527-membered library.

This library was then screened for activity on the mammalian secretory pathway through a cell-based phenotypic assay where fluorescently labeled vesicular stomatitis virus G protein tagged with green fluorescent protein (VSVG-GFP) was used to monitor the efficacy of protein trafficking from the endoplasmic reticulum (ER) to the plasma membrane via the Golgi apparatus (GA). This screen identified compound 8 (secramine) as a potent inhibitor of VSVG-GFP movement from the GA to the plasma membrane at $2\,\mu mol\,l^{-1}$. It is remarkable that the scaffold inspiration, galanthamine, has no effect on the secretory pathway even at a concentration of $100\,\mu mol\,l^{-1}$.[114]

Although this study leads to a fundamentally interesting molecule and not to a drug-type molecule, it still provides a successful proof of concept for the application of DOS to naturally occurring scaffold in order to generate biologically relevant molecules for fundamental purposes, as in this particular case, or for drug discovery applications.

The recognition of ubiquitous substructures has been used extensively for the design of libraries with valuable pharmacological properties. One example is the diketopiperazine (DKP) moiety (**Figure 16**), which inspired the pipecolic acid-based library designed by Porco, Panek, and coworkers.[115]

Porco and Panek envisioned the generation of a diketopiperazine library through dimerization of pipecolic acid moiety. The targeted pipecolic acid monomers were prepared using Panek's chiral allyl silane methodology between 2,3-aminosilanes and a variety of aldehydes followed by platinum-catalyzed hydrogenation. The two diastereomeric series were accessed using either the *syn*- or *anti*-2,3 aminosilane leading to the *cis*- and *anti*-pipecolic acid units, respectively. To increase the diversity of the library rapidly and simply, aromatic aldehydes carrying a bromine atom for transition metal-catalyzed coupling were used. This aromatic bromide group can be coupled under various conditions: Suzuki and Stille couplings were performed using a variety of boronic acids and stannanes, Sonogashira coupling was also carried out using various terminal alkynes, and the Buchwald coupling was used to introduce amine and amide functional groups. To simplify the purification of the coupling steps, a 'catch and release' strategy (**Scheme 4**) was developed using Amberlyst A26 hydroxy resin to hydrolyze the methyl ester and catch the carboxylate anion, which could then be released pure from the resin using acetic acid and water.

These monomeric pipecolic acid units can be reacted with amino acids in two steps or dimerized in one step using HATU and collidine in DMF (Equation (1)). Using these protocols, a series of diketopiperazine was

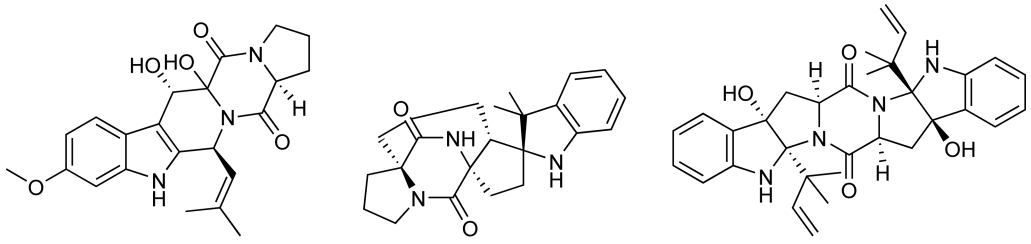
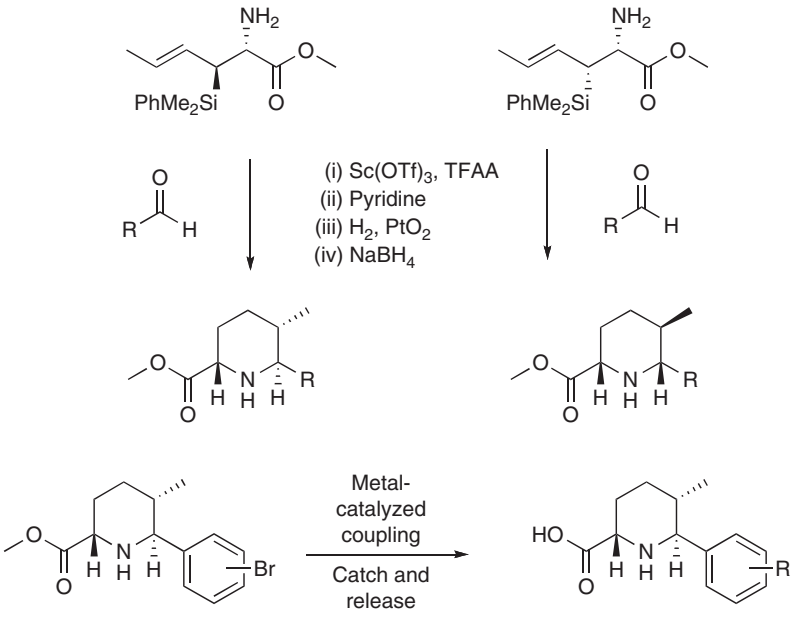
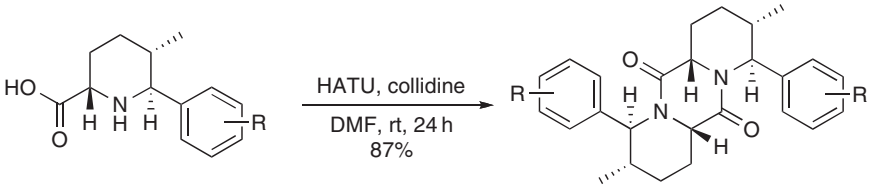


Figure 16 Diketopiperazine-containing natural products.



Scheme 4

prepared and their ClogP was calculated. A large majority of these molecules were attributed ClogP values above or around the desired value of 5.0 according to the Lipinski rules.



(1)

This library of DKPs was screened in parallel against a large panel of targets including G-protein-coupled receptors (GPCRs), ion channels, and transporters. This screening campaign yielded a significant number of highly active and specific compounds against a wide range of targets. This example of natural product-inspired library synthesis combined with a massive parallel screening campaign is demonstrating the high potential of such approaches for the discovery of new biologically active molecules and potentially new guiding structures for drug development.

Natural products have also been used as library-guiding principle identified through a tree-like analysis as discussed in the section on scaffold tree (see above) and **Figure 10**.

In this case, Waldmann and coworkers[116] have selected the α,β-unsaturated γ-lactone scaffold, a frequently observed unit. This moiety was chosen based on its presence in many natural products as shown in **Figure 17**. Furthermore, this motif is among the most common scaffolds found in nature.[68] These natural products show various bioactivities including phosphatase inhibition (fostriecin), immunosuppression (pironetin), inhibition of nuclear import (callystatin A), and cytotoxicity (goniothalamin).

To generate this library in an efficient manner, a solid-supported strategy was elaborated (**Scheme 5**).

The library synthesis strategy makes use of bromo-Wang resin, which was reacted with sodium 3-oxacrolein. Subsequently, the solid-supported aldehyde was transformed into the desired dienes by its reaction with different triphenylphosphonium salts ($R^1 = H$ or Me) under Wittig reaction conditions. The immobilized dienes were then reacted in an enantioselective hetero-Diels–Alder reaction with ethyl glyoxalate using Ti(*R*-BINOL)(O*i*-Pr)$_2$ as chiral catalyst. Under these conditions, the syn isomer of *R* configuration of the hetero-Diels–Alder product was obtained with enantiomeric excesses between 90 and 95%. This common intermediate was diversified into two main compound series. The first series containing esters or amides was

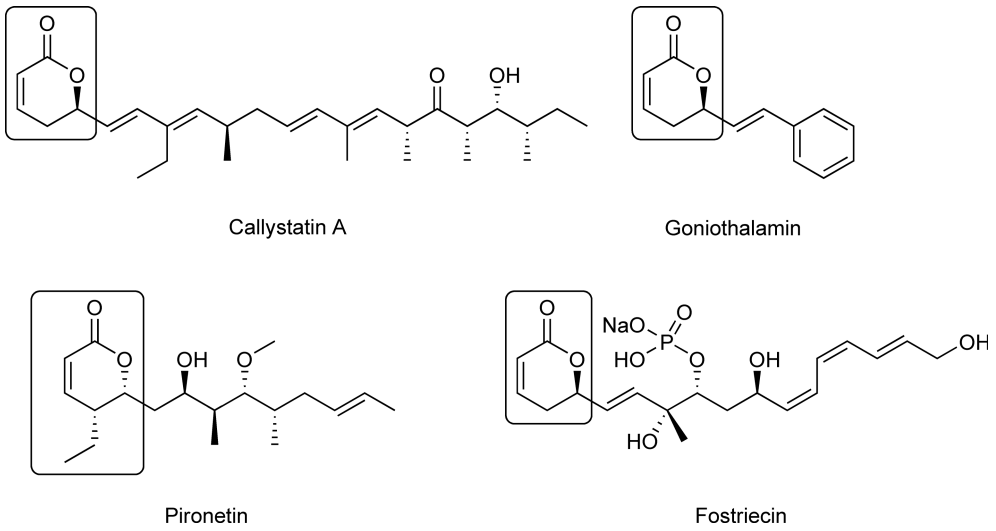


Figure 17 Structure of α,β-unsaturated γ-lactone-containing natural products.

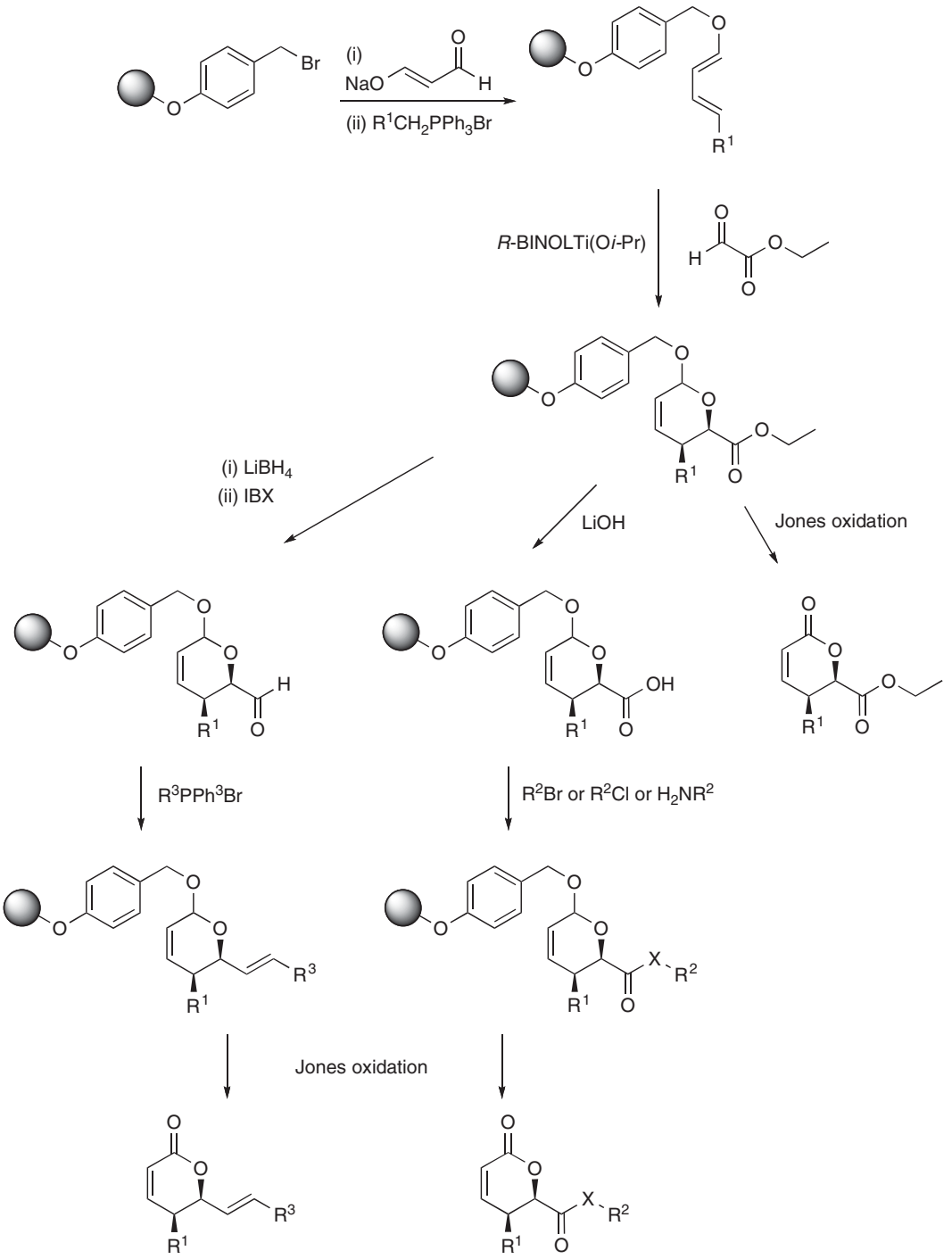
generated through lithium hydroxide hydrolysis of the ester, followed by alkylation of alkyl halides using cesium carbonate or amide formation using PyBOP as coupling reagent. The second series was obtained through reduction of the ethyl ester group with lithium borohydride to the alcohol followed by its oxidation to the aldehyde using IBX. This aldehyde was then reacted under Wittig reaction conditions with a variety of phosphonium salts to resemble more closely the natural product scaffold of fostriecin, callystatin, and goniothalamin. In both the series, the products were released using the Jones reagent with concomitant oxidation to the desired α,β-unsaturated γ-lactone. These two sequences allowed for the rapid preparation of a 50-membered library. Because pironetin is known to cause cell cycle arrest in the M phase and is a potent tubulin assembly inhibitor, this library was screened in a phenotypic cell-based assay to monitor their effect on cell cycle progression. During this screen, it was found that compounds **11** and **12** (**Figure 18**) had a marked effect on the microtubule cytoskeleton. The observed phenotype revealed many anomalies of the cytoskeleton. The most notable is the bipolar spindle formations with a high frequency of misalign chromosome and a greater pole-to-pole distance in treated cells. This observed phenotype was explored and further investigation pointed toward an inhibitory effect on the microtubule polymerization at $80\,\mu\text{mol}\,l^{-1}$.

Because some of these α,β-unsaturated γ-lactone natural products possess phosphatase inhibitory activities (fostriecin) and because phosphatases and kinases are believed to be crucially involved in the vesicular stomatitis virus (VSV) cell entry process, this small library was tested in the rVSV-GFP infectivity assay. This screen revealed that compounds, **13**, **14**, and **15** (**Figure 18**) had a significant influence on the infectivity of VSV. Further experiments support the conclusion that these small molecules act specifically on the endocytic pathway.

This combination of natural product-guided library design (BIOS), with the assay selection based on the parent natural product activities, provided high hit rates of 4 and 6% in the cell cycle progression screen and rVSV-GFP infectivity assay, respectively. Thus, it represents a successful application of the BIOS principle to discover new biologically active molecules. This approach allowed for the discovery of new modulators of cell cycle progression and of cellular viral entry, which will further open new research avenues.

3.02.5 Natural Product Drug Development

Although nature is a major source of new biologically active molecules, many challenges are found on the road toward their accession to the status of commercial drugs.[117] These roadblocks are coming from many aspects, some of which will be discussed using current examples. The following sections will deal with different aspects that need to be considered in the process of finding new natural product-based drug therapies.



Scheme 5

In today's drug industry, IP is of crucial importance because it allows the discovering companies to be compensated for their research and development investments. Today, these investments can reach as high as 1 billion dollars for a single drug. In the current economy, without this exclusivity benefit, the sheer amount of investment would be sufficient to deter many companies from innovative research. A new patent can be granted to protect three subjects: the discovery of a new chemical component, the process to access a chemical entity,

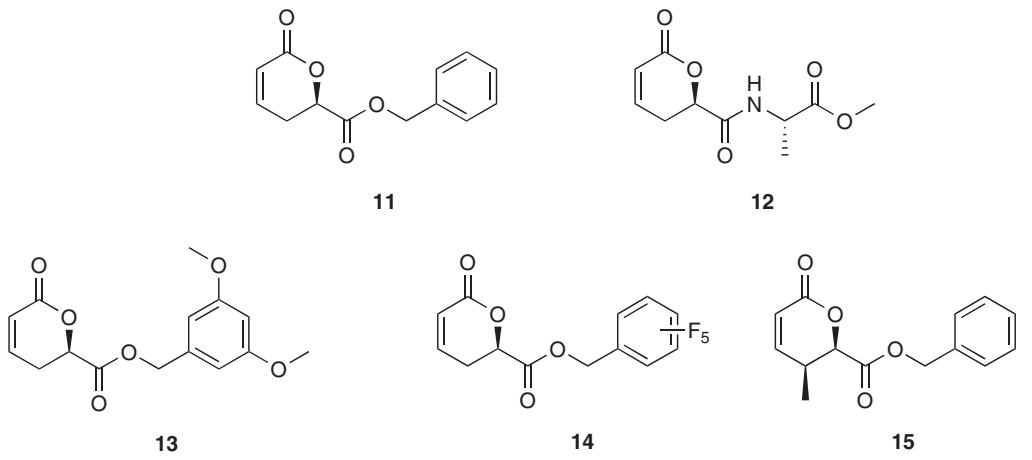


Figure 18

and a trademark. To be granted, a patent application has to fulfill minimum requirements in their novelty, their usefulness, and their nonobviousness. Failure to satisfy all of these criteria will typically result in the patent application rejection.[118,119] With natural products, other aspects need to be considered, such as traditional knowledge of indigenous people. Currently, very few local groups benefit from the commercial success of commercialized products derived from their resources, and many people have termed this phenomenon biopiracy. Biopiracy can be defined as the use of traditional knowledge or of biological resources without proper authorizations.[120] Owing to the difficulty of application of the current IP process to traditional medical knowledge, a possible separate system, the *sui generis*, is being work out.[118] Currently, 95% of patents are held in developed countries. In 1992, the Convention on Biodiversity was held in Rio and the consensus gave the sovereign states an exclusive property of their bioresources and have the freedom to trade them like any other commodities.[118,121] To demonstrate the implication of these new considerations in the IP laws, the following two examples are worth considering. In the case of neem plant extracts, their development and patenting by Japanese and US companies as biopesticides was challenged on the basis that people from India had also been using these for centuries in similar applications. In this instance, the patent was deemed valid because the actual patented invention, the neem seed extract as insecticide, possessed improved storage stability over the traditional neem-based insecticides. Thus, the patent application was significantly different to be granted protection.[122] In 1995, the US patent office granted a patent to the University of Mississippi for 'Use of Turmeric in Wound Healing'. This patent was later challenged by India upon the ground that turmeric has been in use in India for the same purpose and consequently there was nothing new in the invention. The patent was revoked based on these new evidences of prior art brought to the regulator's eyes. This successful challenge signaled to the patent offices to pay more attention to traditional practices in their prior art research.[118] Because most of today's biodiversity is concentrated in less-developed countries, it is even more important to take into account the conservation of the endangered ecosystems. It is important to protect this source of potential drugs before the next cure for cancer or malaria becomes extinct. Furthermore, it is also crucial that the exploitation of the resources is achieved in a sensible way to ensure sustainable production of the resource in terms of environmental, social, economical, and political impacts. A new, more sensible approach, bioprospecting, is aiming at limiting the impact of pharmaceutical research of plant ingredients.[123,124] This can be achieved through strong, mutually beneficial collaborations, which may include fair and equitable benefit sharing, payment of royalties, training to local participants, technology transfer, and financial contribution for diverse services.[125]

In many cases, the screening of plant extracts is the first step in natural products drug discovery. In recent years many technological improvement have sped up this process. The advent of automation and HTS have greatly influenced today's picture of plant extract screening.[126,127] Furthermore, the emergence of multiple analytical technique coupling, for example, high-pressure liquid chromatography (HPLC) coupled with

nuclear magnetic resonance and mass spectroscopy (LCMS–NMR–MS)[128] has increased the rate at which the activity of a plant extract can be traced back to its components.[129] The development of HPLC microfractionation and its adaptation to 96- and 384-well plates allowed for quick generation of easily screenable plant extracts. Coupling these automated and high performance techniques with new and innovative assays such as yeast halo assay, for example, can lead to a very efficient process.[130,131] In any case, the combination of automated purification, screening, and identification of natural products will generate huge amounts of data that require the use of computational tools for its analysis. Recently, a combined approach called forward chemical genetics has been strengthened as an efficient way of finding new drug inspiration.[132,133] This strategy uses three key elements, a set of molecules to screen (collection or library), a suitable phenotypic assay, and a means to identify the target of the active compounds in the screen. This strategy can be applied to libraries inspired by natural products, natural product compound collections, and other diverse molecule libraries. The evaluation of the activity in the phenotypic assay can be done through visual evaluation of specific cellular characteristics, visual screening. Today, automated microscopes can quickly capture images of cells and produce again a large amount of data that can then be reviewed by the experimentator or using software that can score the observed phenotypic response of the cells to the screened compounds.

Once the biological activity of a natural product extract is discovered, it is important to identify the active constituent. Today, a wide array of analytical techniques is available to organic chemists such as high-field nuclear magnetic resonance (NMR), two-dimensional NMR, high-resolution mass spectroscopy (HRMS), circular dichroism (CD), and many others. We are far from the days where derivatization of the pure compound to its simpler parts made the identification of natural products lengthy and resemble more the work of a skilled detective. Today the benchmark analysis is X-ray crystallography. But even the most trusted method of structural elucidation has its limit and the best example is diazonamide A (**Figure 19**).

The proposed structure of diazonamide A was ascertained mainly through the X-ray crystal structure of the closely related *p*-bromobenzamide of diazonamide B.[134] Because of the remarkable biological activity of this compound, many groups embarked on the total synthesis of diazonamide A. In 2001, Harran's group published their synthesis of diazonamide A and claimed that the proposed structure was wrong.[135] This example demonstrates the importance of total synthesis as a means of structural assignment proof and is also a warning to those who use natural products as inspiration for drug discovery, misassignment of natural product structures still can happen today and valuable time and resources can be wasted pursuing the wrong target.[136]

The annonaceous acetogenins are a class of fatty acid-derived natural products that can in majority be sorted in two classes, the mono-THF and the *bis*-THF depending on their structure (**Figure 20**). A large number of these natural products have interesting anticancer and pesticidal activities. Owing to their high degree of symmetry and the remote distance between the two sets of stereogenic centers, the complete and absolute stereochemistry is hard to assign. Furthermore, most of them are oils and are not easily amenable to X-ray crystallography.

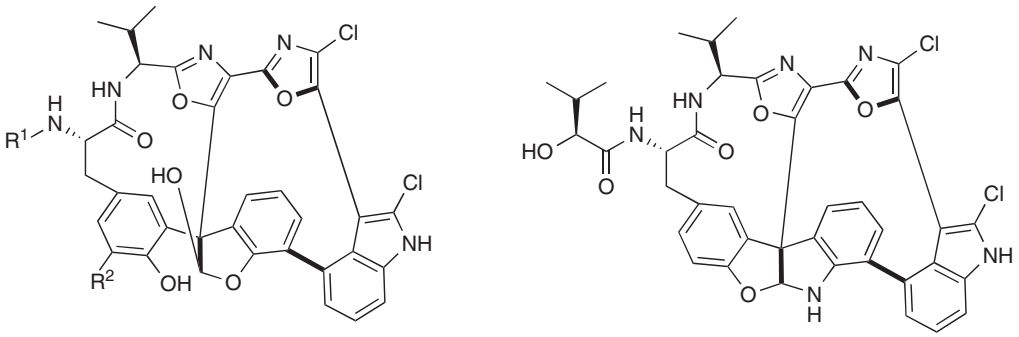


Figure 19 Proposed and revised structures of diazonamides.

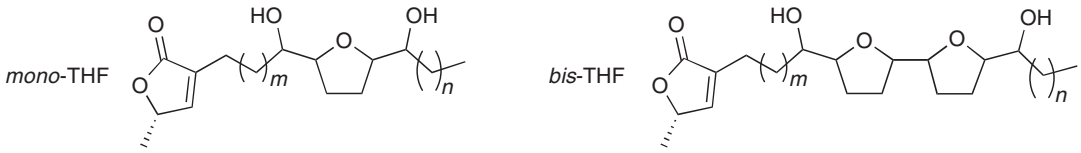


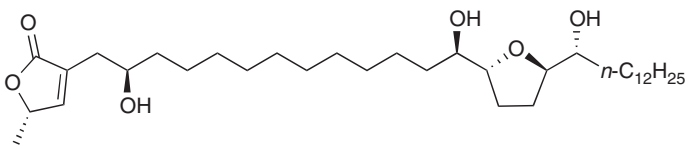
Figure 20 General subclasses of acetogenin.

The only remaining alternative to prove or disprove a stereochemical structure is to synthesize them, but synthesizing all possible stereoisomers of such a molecule is a daunting task. A molecule possessing four stereogenic centers would require the synthesis of 16 analogues with excellent control of the stereochemistry. To ascertain the stereochemistry of the acetogenin molecule murisolin, which possesses six stereogenic centers (64 possibilities), Curran and coworkers[137] have prepared a 16-membered library that accounts for 24 of the 32 possible diastereomers using a fluorous tag strategy. They then thoroughly studied these structures using a combination of ^{1}H NMR, ^{13}C NMR, 2D NMR of the compounds as well as their Mosher esters derivatives.[138] They have been able to prove the structure of murisolin A (**Figure 21**) and to disprove the stereochemical assignment of another diastereoisomer isolated.

During these studies, they generated a large amount of spectroscopic data that can now be used to assign the stereochemistry of newly isolated annonaceous acetogenins. They also came to the conclusion that it might be impossible to prove without a doubt the identity of previously isolated compounds since the original sample is not available anymore and that the available spectral data can only lead to reducing the possibility to more than one possibility.

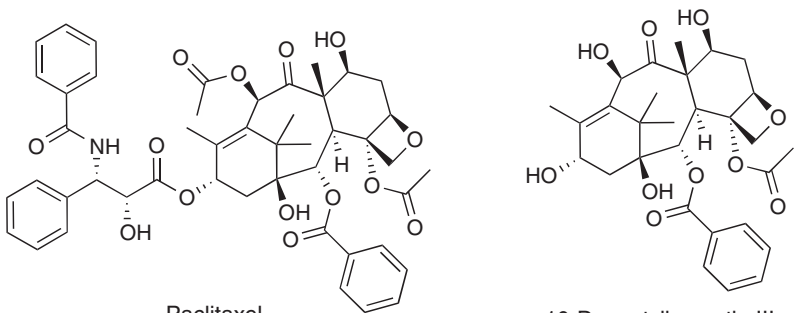
To showcase the use of natural products as drugs, paclitaxel (Taxol) is an illustrative example. Paclitaxel was first isolated in 1969 and identified in 1971[139] from the coniferous pacific yew tree (*Taxus brevifolia*), which grows on the Northwest Pacific coast of North America (**Figure 22**).

Its remarkable anticancer properties quickly attracted interest from the scientific community.[30,31,140] It then took more than 10 years before the mechanism of action of paclitaxel was elucidated.[141] It was found that Taxol is stabilizing the microtubule assembly, therefore, the cells are blocked in the late G2 mitotic phase.[142] This action renders Taxol a potent inhibitor of eukaryotic cell replication. One of the main obstacles to the development of paclitaxel as a drug was the supply of the active molecule. In each of 1991 and 1992, 1.6 million



Murisolin A

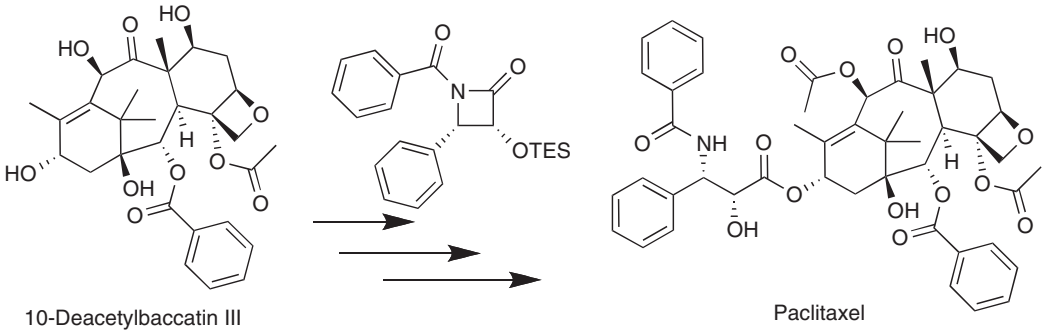
Figure 21 Structure of murisolin A.



Paclitaxel

10-Deacetylbaccatin III

Figure 22 Structures of paclitaxel and 10-deacetylbaccatin.



10-Deacetylbaccatin III

Paclitaxel

Scheme 6

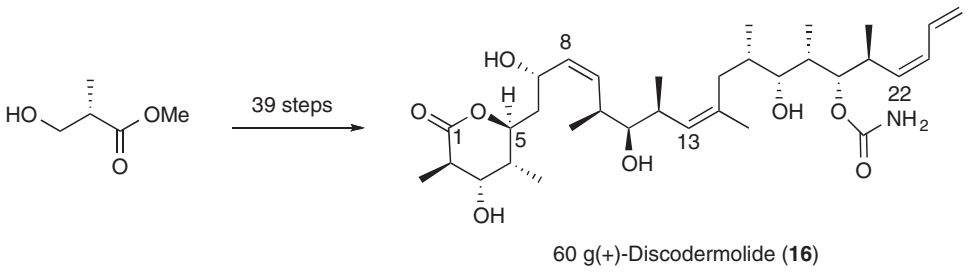
pounds of the tree bark was harvested to generate hundreds of kilograms of Taxol by Bristol-Myers Squibb (BMS) and its subsidiary.[30] This amount was only required for completing clinical trials. An even larger amount would be needed if Taxol was to be commercialized. Without a doubt, at this pace the Pacific yew tree would be extinct in a short time or the access to the drugs would need to be restricted.[143] Many total syntheses were published[144–150] but none of them proved to be amenable to large-scale production of Taxol. Fortunately, the research by French researchers, Greene, Potier, and coworkers,[151] as well as others,[152–154] led to the development of a multistep semisynthesis of paclitaxel from 10-deacetylbaccatin III (**Scheme 6**).[27] In this process the starting material can be obtained in better yield from the renewable needles of the yew tree. This mode of harvesting does not kill the tree as it does when the bark is used, thus it is a more sustainable exploitation of the resource.

With one of the best anticancer compounds in hand and a viable way of producing the required amount of the drug, BMS finally commercialized Taxol in 1993, more than 20 years after its identification from the yew tree extract. It was an instantaneous commercial success and it sold for US$ 1.6 billion in 2000. More recently, BMS was awarded the 2004 Greener Synthetic Pathways award of the Environmental Protection Agency of the United States[155] for developing a new plant cell culture process. Callus is grown on a solid medium, in fact cell suspension cultures are used that are obtained from the plant via callus cultures. This process uses calluses (cells recovering a plant wound) of a specific *Taxus* cell suspension culture and requires only simple and renewable nutrients, sugars, amino acids, vitamins, and trace elements to feed the culture in large fermentors. Paclitaxel is then isolated from the cell culture minimizing the amount of solvent, the number of purification steps, and the energy required to produce the drug.

In some cases, when it is impossible to isolate the active compound from its natural source, total synthesis is the only alternative. One such example is (+)-discodermolide (**16**), which was isolated from *Discodermidia dissoluta* by Gunasekera and coworkers.[156,157] Discodermolide was found to be a microtubule-stabilizing agent even better than Taxol and remained active even against paclitaxel- and epothilone-resistant cells.[158]

Many total syntheses of this anticancer compound have been published.[159–163] None of them was directly amenable to large-scale synthesis but chemists at Novartis have produced a hybrid synthesis made of steps from the Smith,[43,160] Paterson,[163] and Marshall[162] syntheses. This was required to access sufficient quantity of discodermolide for its development as the isolation and purification route, requiring manned submersibles, was too costly. Furthermore, attempts to isolate and cultivate a discodermolide-producing microorganism have so far generated disappointing results. Therefore, this lack of supply meant that all the discodermolide required for clinical trials needed to be obtained through total synthesis. This optimized synthesis consists of a total of 39 steps, 26 steps for the longest linear sequence,[164–168] and required 17 chromatographic purification steps to generate sufficient amount of material (60 g) to pursue early-stage human clinical trials (**Scheme 7**).

The completion of the synthesis of (+)-discodermolide in 39 steps on a 60-gram scale is a remarkable achievement. It was also the first of its kind for the pharmaceutical industry and it will certainly not be the last. This showed that when well planned, a project of that scale could be successfully achieved. This synthesis allowed Novartis to pursue the clinical trials of discodermolide but unfortunately these were halted in Phase II due to toxicity side effects.[169] Nevertheless, this synthesis remains without a doubt a remarkable achievement and opens the way for a new route of access to biologically active natural product supplies.



Scheme 7

These examples demonstrate the challenges that remain in the structural elucidation of natural products, a key point in their use as drugs. Fortunately, chemists are learning from these situations that help them improve their abilities in natural products elucidation.

3.02.6 Natural Products as Source for Leads and Clinical Candidates

Natural products have been driving chemical research, that is, synthetic methodology, structure elucidation, and analytics, for many decades.[170] Hundreds of natural products have been fully synthesized and many more analogues of these compounds have been made as described in the previous sections about natural product-derived libraries and natural product drug development. Nature herself has provided compounds for medical application for centuries – even long before chemistry and pharmacology, as we know them today, existed. In former times, these compounds were often used as extracts in contrast to modern drugs that incorporate a defined chemical entity in most cases. Because of this, nature was joined in the last decade by chemists working on the synthesis of natural products and analogues who also contributed a significant number of lead structures[39,171] and, albeit significantly fewer, drugs. This section will briefly introduce and characterize the different source of natural product leads, their exploitation, and particular characteristics. Examples will be given for various natural product-derived compounds in advanced development stages in different disease areas including the natural product domains, anticancer drugs, and antibiotics. We will also briefly sketch out the routes of development that these compounds took.

3.02.6.1 Sources of Natural Product Compounds for Drug Development

One of the main sources of natural product compounds for medicinal chemistry programs are plants. Plant extracts have been used for a long time in many traditional medicines long before the advent of modern medicine and drug discovery. Modern pharmacognosy, that is, the knowledge of natural product-based medical drugs, also contains a lot of traditional knowledge collected before HTS. In ethnopharmaceutical research,[172] traditional medical knowledge, for example, Traditional Chinese Medicine or Ayurveda, are explored to identify the active ingredients and convert them into modern drugs.[173] Although these systems are often ignored or even condemned by orthodox medicine, pharmacological effects of a growing number of compounds from Ayurveda have been proven scientifically.[174–176] In some cases modern techniques are fused with traditional medicines, for example, functional genomics with Ayurveda,[177] to elucidate active ingredients and their mode of action. There are some success stories, for example, the discovery and development of artemisine and its derivatives[178–181], an antimalaria drug, or DDB,[182] a synthetic analogue of schizandrin C that is used in China to treat chronic cases of hepatitis.[183] It has been estimated that although more than 10 000 plant natural products have an annotated medical use, only 150–200 have made it into Western medicine.[184] Thus, there may still be quite some potential drugs waiting to be discovered in and developed from plant natural products.

One relatively young source of natural products is the sea. More than 70% of our planet is covered by oceans and marine natural products resulting from the immense variety of animal, plant, and microbial life under water

quickly proved to be a viable source for many biologically relevant natural products.[185,186] Many marine organisms are sedentary and do have no shells or, in some cases, even no bones. Therefore, chemistry is a prime means of defense and the fight for survival is tough in the heavily populated marine environments like coral reefs. Consequently, many marine organisms produce very potent inhibitors of physiological functions as weapons. As described in Section 3.02.2, marine natural products differ from others, for example, from plants. This may be partly due to the biotope that provides access to some elements rare on earth, for example, iodine. Moreover, a sizeable number of marine organisms may live in close symbiosis with microorganisms, thereby greatly enhancing their chemical repertoire of available elements, reactions, and, in the end, compounds. However, this also renders determination of the true species of origin more complex. Marine natural products are promising, albeit the technological and logistical effort to obtain samples is quite large. Although different diving techniques allow divers to proceed up to 150 m for some time, small research submersibles have to be used for greater depths. This is costly and often the amount of compound contained in the organism is only a few milligrams.

Other, less conventional and underexplored sources of natural products were suggested for natural product-based drug discovery programs like, for example, beverages.[187] Several pharmacologically active ingredients (**Figure 23**) in drinks are known besides ethanol, for example, Thujone (**17**) in absinth or anandamide (**18**) in cacao, an endogenous ligand for the cannabiniod receptor.

Additional fermentation of natural products contained in food or food precursors may further increase the diversity of compounds contained in these sources. Other food ingredients like spices, herbs, and vegetables may also contain pharmaceutically active compounds. The pharmacological properties of curcumin (**19**), for example, have recently been reviewed.[188] Animals, as a source of pharmacologically active compounds, have been explored mainly in terms of hormones, for example, steroids and sexual hormones, isolated either from tissue or from excrements.

Thus one can conclude that many sources of potentially active natural products still remain to be exploited and we have barely touched upon the diversity produced by nature.

3.02.6.2 Natural Product-Derived Compounds in Advanced Development

Natural products have provided compounds for development programs in many different disease areas and, together with their derivatives, have contributed around 50% to the current pharmacopeia.[38,189] There is a plethora of natural product-derived compounds at the lead stage or even further in clinical development in the literature. Therefore, in this subsection we present a selection of natural product-derived compounds in advanced development for various disease areas including the traditional domains of natural products, that is, anticancer drugs and antibiotics. For further natural products in drug development programs, we refer the reader to a number of reviews summarizing the progress of natural product lead discovery.[38,171,172,183,185,190–197]

3.02.6.2.1 Anticancer clinical candidates and drugs

One of the most promising anticancer candidate[195] obtained in the last decade is ixabepilone (**20**), a derivative of the natural product epothilone B (**21**) isolated from the myxobacterium *Sorangium cellulosum*. Ixabepilone has been developed by BMS and is currently in clinical trials (**Figure 24**).

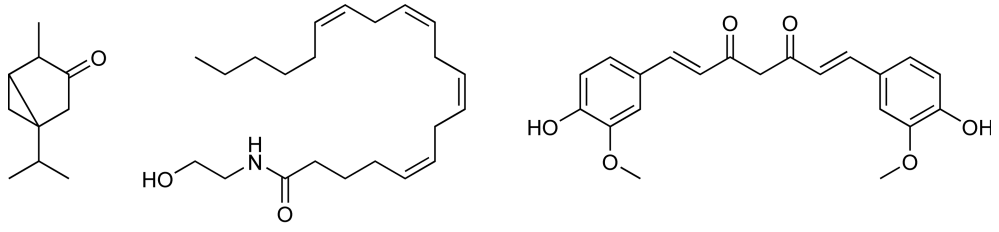


Figure 23 Structures of pharmacologically active natural products from unusual sources.

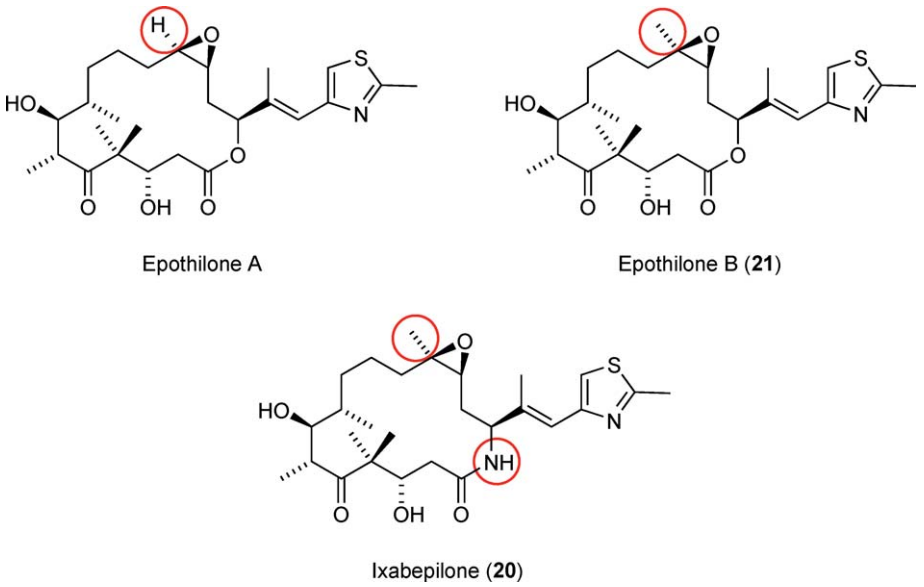


Figure 24 Structures of epothilone A, epothilone B, and ixabepilone, a drug derived from epothilone B. Note that in ixabepilone the macrocyclic lactam makes the compound much more stable to degradation *in vivo*.

Changing the macrocyclic lactone in the natural product to a lactam in the molecule improved the pharmacokinetic parameters and metabolic stability, especially plasma half-life from 5 to 13–16 h. Epothilones bind to β-tubulin, a part of the cytoskeleton, and induce its polymerization, which in turn leads to cell cycle arrest and subsequent cell death via apoptosis.[198–206] They bind, in fact, to a site overlapping with the binding site of the taxanes, a very successful plant-derived family of anticancer agents that have been described above. Epothilones are less susceptible to resistance mechanisms in cancer cells compared to taxanes and they could prove effective against taxane-resistant cancers *in vivo*.

Another group of interesting anticancer agents was derived from the natural product podophyllotoxin (**Figure 25**).[207]

This natural product is found in the roots of *Podophyllum peltatum* Linnaeus, American mandrake, a herb often found in forests, and other herbs. During early days it was already used as an alcoholic extract as cathartic in the United States. Later, it was found that the same extract produced cytological changes in human and rabbit skin upon topological application.[208] It was then used against veneral warts caused by human papilloma virus (HPV), rheumatoid arthritis, and in various other conditions in dermatology.[195] The cytotoxic properties of

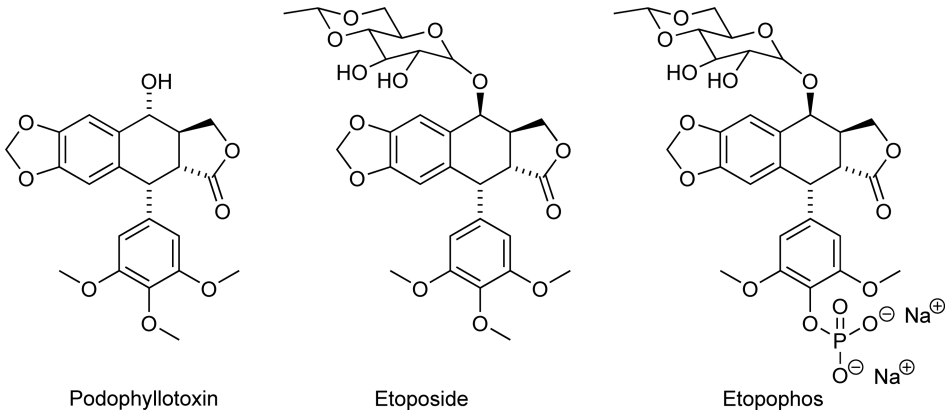


Figure 25 The natural compound podophyllotoxin and the derived leads etoposide as well as its prodrug etopophos.

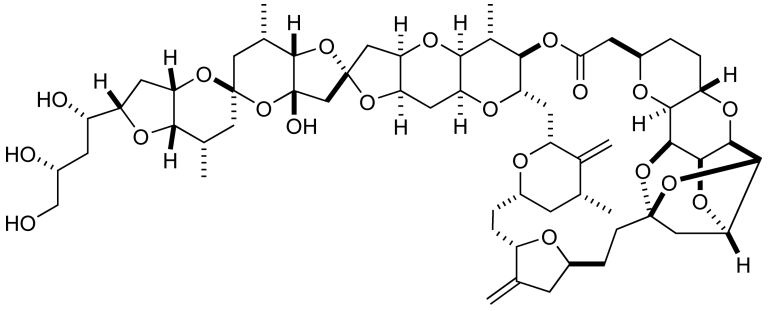
podophyllotoxin were recognized and it was found out that podophyllotoxin inhibits topoisomerase II by stabilization of the covalent topoisomerase II-DNA-cleavable complex.[209–211] Its derivative etoposide, however, was only moderately potent and weakly soluble in water. Moreover, it induced drug resistance, was metabolically unstable, and toxic, so a new development program was started to overcome these problems.[212,213] From this program resulted a prodrug of etoposide named etopophos, which showed to be effective against testicular and small-cell lung cancer as well as several other cancer types. Many more compounds derived from podophyllotoxin have been in clinical trials and research in this field is still ongoing.[195] Etophos is marketed as an anticancer drug by BMS since 1996.

Another natural product-derived tubulin inhibitor that is now in Phase III clinical trials for breast cancer in the United States is a compound currently known as E7389 (eribulin) from the Eisai Research Institute.[214–216] The compound was derived from the natural product halichondrin B isolated by the group of Daisuke Uemura in 1985 (**Figure 26**).[217,218]

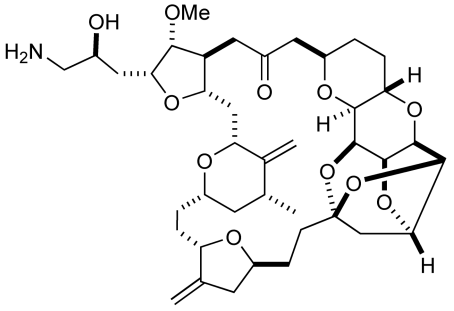
It was isolated from the sponge *Halichondria okadai* Kadota close to the coast of Japan. It was known that sponge extracts possessed remarkable antitumor activity *in vivo* and, therefore, Uemura and coworkers used a melanoma cell line to extract the halichondrins as active compounds. Eribulin itself emerged from a research effort involving the synthesis of over 200 analogues of halichondrin B. In contrast to the epothilones and podophyllotoxins, eribulin inhibits tubulin polymerization, which finally leads to cell cycle arrest and apoptosis as well.

3.02.6.2.2 Antibacterials

Many antibacterials have originated from natural products including penicillin, the first antibiotic that was discovered by Alexander Fleming in 1928.[40] Today, with increasing multidrug resistance in many bacterial strains, even against last line of defense drugs like vancomycin, new strategies in antibiotic drug discovery are



Halichondrin B



E7389 (Eribulin)

Figure 26 Structures of the natural product halichondrin B and its derived lead E7389.

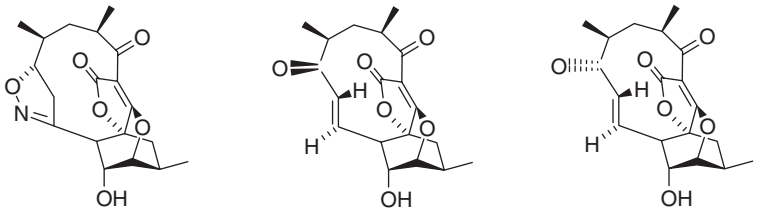


Figure 27 Structures of abyssomicin B, C, and atrop-abyssomicin C, a synthetic isomer.

needed.[219] These new strategies involve novel screening techniques, bacterial targets identified from genome analysis, and, as in the decades before, natural products as a prime source of antibacterial lead structures.[219–221]

The family of abyssomicins was discovered in 2004 in the actinomycete *Verrucosispora*, which had been cultured from a sediment sample from 300 m of depth from the Japanese sea. Actinomycetes are a group of Gram-positive bacteria that often occurs in soil. Initially three members of the family were discovered, the abyssomicins A, B, and C (**Figure 27**).

Abyssomicin C was found to inhibit the biosynthesis of *p*-aminobenzoate, a metabolite in the biosynthesis of folate only found in bacteria but not in mammals.[222,223] It has been claimed to be the first natural product from isolated bacteria that inhibits this pathway. In the following years, a total synthesis was developed by the group on Nicolaou that also yielded the atrop-abyssomicin C, an isomer of the natural product.[224–226] This isomer also showed antibacterial activity and was also discovered in the extract from the actinomycete *Verrucosispora* later together with more members of the abyssomicin family.[227] The molecular mode of action of atrop-abyssomicin C was elucidated to be the inhibition of 4-amino-4-deoxychorismate synthase PabB in *Bacillus subtilis* by covalent binding to cysteine 236 close to the active site via its Michael-acceptor system.[228] Although they are not in clinical trials yet, the abyssomicins are a promising family of antibacterial lead structures.

The mannopeptimycins (**Figure 28**) are a family of peptidoglycan antibiotics[229] that was first isolated in the late 1950s from *Streptomyces hygroscopicus*.[230] Already at that time it was found to be a potent antibiotic against Gram-positive bacteria.[231] However, due to their complex structure and focused activity it was not developed further.

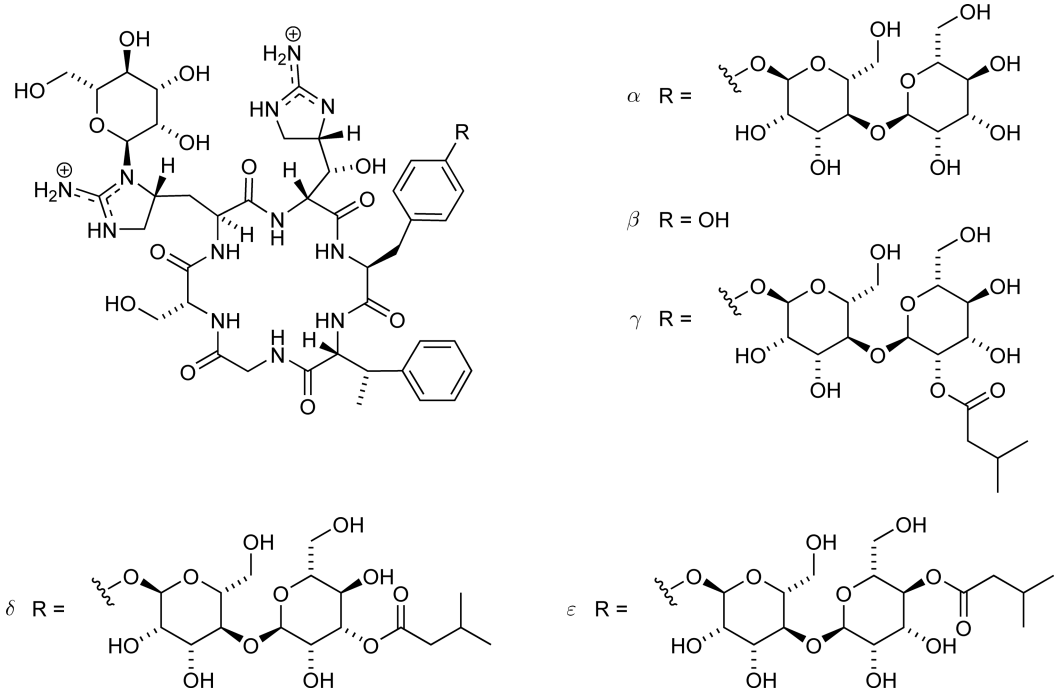


Figure 28 Structure of the mannopeptimycins α through ε.

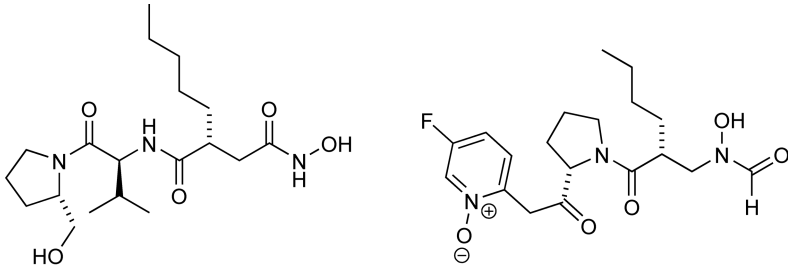


Figure 29 Structures of the natural product actinonin and its derived lead LBM-415.

Recently, in the reawakening of antibacterial research fueled by developing resistance, focus returned on the mannopeptimycins. This is due to their antibacterial activity against several important pathogens including vancomycin-resistant enterococci (VREs) and methicillin-resistant *Staphylococcus aureus* (MRSA).[232] Their mode of action was proven to inhibit the cell wall biosynthesis in Gram-positive bacteria although by a molecular target that was unknown at that time.[232] Later, it was shown that the mannopeptimycins inhibit the cell wall biosynthesis by binding the cell wall precursor lipid II. This lipidated peptidoglycan is also the target of other antibiotics, for example, vancomycin, however the mannopeptimycins were found to bind lipid II in a unique way.[233] The family of the mannopeptimycins consists of five members, all being structurally complex, macrocyclic peptidoglycans. Optimization of such a complex structural entity is quite a challenge. A first structure–activity relationship (SAR) was established by random acylation of the natural product and subsequent testing.[234] The initial SAR identified further sites of modification by chemical semisynthesis. In parallel, in a directed biosynthetic engineering approach the gene cluster for the biosynthesis of the mannopeptimycins was identified, analyzed,[235] and modified to yield a genetically engineered strain producing the desired natural product derivative.[236] Thus, the potency and the plasma stability could be optimized in a combined semisynthetic and biosynthetic approach.

In 1962 Gordon *et al.* isolated the natural product actinonin from the actinomycete *Streptomyces* Cutter C/2 from the soil in a natural product-screening campaign. They also described its antibiotic properties against *S. aureus* G, *Klebsiella pneumoniae*, and other strains of bacteria.[237] Actinonin was later described to inhibit the RNA biosynthesis albeit the molecular target was not known at the time.[238] The first total synthesis[239] was followed by the synthesis of several different classes of analogues[240–243] and a finally established SAR.[244] In further research, actinonin was proven to inhibit aminopeptidase M[245] but it was not until 2000 that its true mode of action was discovered. Chen *et al.* discovered that actinonin (**Figure 29**) is a potent peptide deformylase inhibitor. Peptide deformylase occurs exclusively in prokaryotes but not in mammalian cells.[246] This discovery increased the interest at the small company Vicuron Pharmaceuticals, where the discovery had been made and more analogues were synthesized and screened. From this campaign, LBM-415 (**Figure 29**) emerged as the most promising compound. In 2005, Vicuron was acquired by Pfizer for US$ 1.9 billion as an extension of the anti-infective R&D portfolio. In collaboration with Novartis, Vicuron is currently testing the compound in Phase I clinical trials as the first compound of the novel class of deformylase inhibitors.

3.02.7 Conclusion and Outlook

Natural products have contributed successfully to drug discovery long before the advent of modern drug development programs, HTS, and combinatorial synthesis. Still, even today about half of the drugs in the market are either natural products or have been derived from them.[38,189]

From the analysis of natural product properties, one can easily see that there are indeed molecular properties differentiating natural products from other types of molecules, for example, drugs or contemporary screening compounds from commercial sources. On the one hand, this indicates that natural products per se

are not drugs although so many drugs originated and still originate from natural products. But on the other hand, one should note that the properties of natural products also form a distribution that overlaps with the distribution of drug properties; thus there are natural products that are more drug-like and others that are farther away from being drug-like. A sizeable fraction of natural products actually could pass the drug-likeness filters often used in the assembly of screening libraries.

Moreover, natural products are structurally very diverse and do increase the diversity of most screening libraries significantly. This can be a key to success as diversity, drug-likeness, and biological relevance have been found to be more important than the sheer size of the library. Therefore, natural products can be gatekeepers to new, previously uncharted, and unexplored regions of chemical space not explored by synthetic compounds so far. This is one of the general lessons to be learned from virtually all approaches to charting chemical space whether they are descriptor based like ChemGPS or structure based like the scaffold tree. All these methods show that the natural product chemical space differs from that of drugs or synthetic compounds. But, particularly noteworthy, in most cases drugs are occupying the space between synthetic compounds and natural products partially overlapping with both of them. Exploring the chemical space occupied by natural products may therefore offer a promising route away from the heavily explored and probably already patented areas, for example, in kinase inhibitors. Besides their unique properties, natural products can be seen as biologically prevalidated structures because they have been selected during evolution to bind to various proteins. All these findings characterize natural products as promising starting points in chemical space for library design.

Natural product drug development remains and will remain a challenge, despite the advances of organic synthetic methodology, automated extraction and fractionation techniques, compound separation, and structure elucidation laid out above. However, natural products chemistry has been a driver for the development of new organic synthetic methodology and the example of the industrial-scale synthesis of discodermolide proves that the transfer of modern synthesis methodology to scales larger than the milligram scale in organic laboratories is possible. The scale-up is certainly demanding but possible and – depending on the individual dose of the drug needed – can be feasible even from an economic point of view. Natural product dereplication, that is, the identification of compounds in complex mixtures, is still a time-demanding task although probably much less than 10 years ago due to advances in separation and purification techniques as well as in analytical techniques including NMR, mass spectrometry, and combinations thereof. The bureaucratic and legal aspects of natural product drug discovery pose another hurdle to be overcome even before starting the first laboratory work. Especially the legal issues are important because any promising compound is without value if it lacks proper patent protection. Particularly, compounds found with the ethnopharmacological approach can be problematic with respect to patent protection as these compounds have been known and used for thousands of years without knowing their molecular identity, for example, in Ayurveda or Traditional Chinese Medicine. For other natural products licensing may be a problem, especially when the IP rights reside with a third world country that is lacking the proper administration to handle IP rights negotiations and contracts. Many countries like, for example, Indonesia are very professional in this respect but with others the necessary legal basis might be difficult to obtain. Nonetheless, leads derived from these compounds may still be patentable rendering these natural products an interesting source of potential leads.

Natural product-derived compounds have often been seen as 'structurally too complex' and their optimization does not fit well to today's time lines of drug development, which often allow less than one year to optimize a lead to a clinical candidate. Nonetheless, many compound libraries derived from natural products some of which are shown in Section 3.02.4 prove that it is possible to synthesize natural products and their analogues, sometimes simplified, with a reasonable effort. Available methods of library design and synthesis schemes such as, for example, the two complementary approaches, DOS and BIOS, pursue the development and synthesis of natural product-like libraries with a reasonable effort. Similarly, recent developments in engineered biosynthesis and culturing techniques open a new avenue promising more rapid access to structurally complex natural products than organic total synthesis. The optimization of natural product-derived compounds toward a clinical candidate and, in the end, a drug stays in the hands of medicinal chemists and their experience optimizing compounds for a multitude of parameters including absorption, distribution, metabolism, and excretion (ADME), pharmacodynamics, and unwanted side effects, for example, cytochrome P-450 or hERG interaction – just like any other compound in a development program.

Whether natural products are a better source for drug discovery programs than today's screening collections is a tough question to answer and probably a philosophical one. As a matter of fact, natural products cannot replace millions and millions of screening compounds needed to fuel the HTS robots. But neither can natural products be replaced by these synthetic compounds from combinatorial chemistry. Natural products are indeed one good source for potent and selective compounds – highly complementary to synthetic compounds. Thus natural products are a valuable extension of any screening library adding diversity and biologically prevalidated structures.

Although many pharmaceutical companies abandoned natural product research internally, there are signs that natural products are indeed experiencing a renaissance and that interest in them peaks in the recent years. With all the new methodology at hand, exploiting nature's diversity could become a decisive advantage in the never-ending quest for new drugs in the future and also prove economically quite feasible.

References

1. J. K. Borchart, *Drug News Perspect.* **2002**, *15*, 187–192.
2. R. S. Solecki, *Science (New Series)* **1975**, *190* (4217), 880–881.
3. R. J. Huxtable; S. K. W. Schwarz, *Mol. Interv.* **2001**, *1*, 189–191.
4. G. Lockemann, *J. Chem. Educ.* **1951**, *28*, 277–279.
5. M. Hesse, *Alkaloide*; Wiley-VCH: Weinheim, 2000.
6. T. S. Kaufman; E. A. Rúveda, *Angew. Chem. Int. Ed. Engl.* **2005**, *44*, 854–885.
7. I. Jeffrey; J. I. Seeman, *Angew. Chem. Int. Ed. Engl.* **2007**, *46*, 1378–1413.
8. A. C. Smith; R. W. Williams, *Angew. Chem. Int. Ed. Engl.* **2008**, *47*, 1736–1740.
9. A. Karenberg; C. Leitz, *Cephalalgia* **2001**, *21*, 911–916.
10. O. Temkin, *Isis* **1938**, *28*, 126–131.
11. D. Jeffreys, *Aspirin, the Remarkable Story of a Wonder Drug*; Bloomsbury Publishing: London, 2004.
12. J. R. Vane, *Nat. New Biol.* **1971**, *231*, 232–235.
13. J. R. Vane; R. M. Botting, *Thromb. Res.* **2003**, *110*, 255–258.
14. G. J. Roth; P. W. Majerus, *J. Clin. Investig.* **1975**, *56*, 624–632.
15. H. Tohgi; S. Konno; K. Tamura; B. Kimura; K. Kawano, *Stroke* **1992**, *23*, 1400–1403.
16. B. L. Ligon, *Semin. Pediatr. Infect. Dis.* **2004**, *15*, 52–57.
17. A. Fleming, *Br. J. Exp. Pathol.* **1929**, *10*, 3–13.
18. E. Chain; H. W. Florey; A. D. Gardner; N. G. Heatley; M. A. Jennings; J. Orr-Ewing; A. G. Sanders, *Lancet* **1940**, *236* (6104), 226–228.
19. E. K. J. Marshall, *Physiol. Rev.* **1939**, *19*, 240–269.
20. J. Birnbaum; F. M. Kahan; M. A. H. Kropp; L. S. Macdonald, *Am. J. Med.* **1985**, *78* (Suppl. 6A), 3.
21. T. Kumagai; S. Tamai; T. Abe; M. Hikida, *Curr. Med. Chem. Anti-Infective Agents* **2002**, *1*, 1–14.
22. J. A. Tobert, *Nat. Rev. Drug Discov.* **2003**, *21*, 517–526.
23. A. Endo; M. Kuroda; Y. Tsujita, *J. Antibiot.* **1976**, *29*, 1346–1348.
24. A. Endo; Y. Tsujita; M. Kuroda; K. Tanzawa, *Eur. J. Biochem.* **1977**, *77*, 31–36.
25. K. Maggon, *Drug Discov. Today* **2005**, *10* (11), 739–742.
26. J. Mann, *Nat. Rev. Cancer* **2002**, *2*, 143–148.
27. K. Miller; B. Neilan; D. M. Y. Sze, *Recent Pat. Anti-Cancer Drug Discov.* **2008**, *3*, 14–19.
28. J. Heilmann, *Chem. Unserer Zeit* **2007**, *41*, 376–389.
29. S. B. Horwitz, *J. Nat. Prod.* **2004**, *67*, 136–138.
30. G. M. Cragg, *Med. Res. Rev.* **1998**, *18*, 315–331.
31. M. E. Wall, *Med. Res. Rev.* **1998**, *18*, 299–314.
32. G. M. Cragg; D. J. Newman, *J. Nat. Prod.* **2004**, *67*, 232–244.
33. N. H. Oberlies; D. J. Kroll, *J. Nat. Prod.* **2004**, *67*, 129–135.
34. R. M. Wilson; S. J. Danishefsky, *J. Org. Chem.* **2006**, *71*, 8329–8351.
35. A. Saklani; S. K. Kutty, *Drug Discov. Today* **2008**, *13*, 161–171.
36. D. G. I. Kingston; D. J. Newman, *Curr. Opin. Drug Discov. Devel.* **2005**, *8* (2), 207–227.
37. D. J. Newman; G. M. Cragg, *Curr. Drug Targets* **2006**, *7* (3), 279–304.
38. D. J. Newman; G. M. Cragg, *J. Nat. Prod.* **2007**, *70* (3), 461–477.
39. D. J. Newman; G. M. Cragg, *J. Nat. Prod.* **2004**, *67*, 1216–1238.
40. I. Ojima, *J. Med. Chem.* **2008**, *51*, 2587–2588.
41. S. Banerjee; Z. Wang; M. Mohammad; F. H. Sarkar; R. M. Mohammad, *J. Nat. Prod.* **2008**, *71*, 492–496.
42. M. S. Butler, *Nat. Prod. Rep.* **2008**, *25*, 475–516.
43. T. Henkel; R. M. Brunne; H. Muller; F. Reichel, *Angew. Chem. Int. Ed. Engl.* **1999**, *38* (5), 643–647.
44. M.-L. Lee; G. Schneider, *J. Comb. Chem.* **2001**, *3* (3), 284–289.
45. M. Feher; J. M. Schmidt, *J. Chem. Inf. Comp. Sci.* **2003**, *43* (1), 218–227.
46. P. Ertl; A. Schuffenhauer, Cheminformatics Analysis of Natural Products. Lessons from Nature Inspiring the Design of New Drugs. In *Natural Products as Drugs*; F. Petersen, R. Amstutz, Eds.; Birkhaeuser Verlag: Basel, Switzerland, 2007.
47. K. Grabowski; G. Schneider, *Curr. Chem. Biol.* **2007**, *1* (1), 115–127.

48. R. Deprez-Poulain; B. Deprez, *Curr. Top. Med. Chem.* **2004**, *4* (6), 569–580.
49. D. S. Wishart; C. Knox; A. C. Guo; S. Shrivastava; M. Hassanali; P. Stothard; Z. Chang; J. Woolsey, *Nucleic Acids Res.* **2006**, *34* (Database), D668–D672.
50. J. A. Burlison; L. Neckers; A. B. Smith; A. Maxwell; B. S. J. Blagg, *J. Am. Chem. Soc.* **2006**, *128* (48), 15529–15536.
51. T. Agatsuma; H. Ogawa; K. Akasaka; A. Asai; Y. Yamashita; T. Mizukami; S. Akinaga; Y. Saitoh, *Bioorg. Med. Chem.* **2002**, *10*, 3445.
52. U. Abel; C. Koch; M. Speitling; F. G. Hansske, *Curr. Opin. Chem. Biol.* **2002**, *6* (4), 453–458.
53. C. A. Lipinski; F. Lombardo; B. W. Dominy; P. J. Feeney, *Adv. Drug Deliv. Rev.* **2001**, *46* (1–3), 3–26.
54. M. D. Burke; E. M. Berger; S. L. Schreiber, *J. Am. Chem. Soc.* **2004**, *126* (43), 14095–14104.
55. Accelrys, Pipeline Pilot. In.
56. L. A. Wessjohann; E. Ruijter; D. Garcia-Rivera; W. Brandt, *Mol. Divers.* **2005**, *9* (1–3), 171–186.
57. P. Kirkpatrick; C. Ellis, *Nature (London)* **2004**, *432* (7019), 823.
58. R. S. Bohacek; C. McMartin; W. C. Guida, *Med. Res. Rev.* **1996**, *16* (1), 3–50.
59. J. Berdy, *J. Antibiot.* **2005**, *58* (1), 1–26.
60. T. I. Oprea, Pursuing Leadlikeness in Pharmaceutical Research. In Proceedings of the Joint Meeting on Medicinal Chemistry, Vienna, Austria, 20–23 June 2005; pp 1–4.
61. T. I. Oprea; J. Gottfries, *J. Comb. Chem.* **2001**, *3* (2), 157–166.
62. T. I. Oprea; J. Gottfries; V. Sherbukhin; P. Svensson; T. C. Kuhler, *J. Mol. Graph. Model.* **2000**, *18* (4/5), 512–524.
63. T. I. Oprea; I. Zamora; A.-L. Ungell, *J. Comb. Chem.* **2002**, *4* (4), 258–266.
64. J. Larsson; J. Gottfries; L. Bohlin; A. Backlund, *J. Nat. Prod.* **2005**, *68* (7), 985–991.
65. J. Larsson; J. Gottfries; S. Muresan; A. Backlund, *J. Nat. Prod.*
66. S. Wetzel; A. Schuffenhauer; S. Roggo; P. Ertl; H. Waldmann, *Chimia* **2007**, *61* (6), 355–360.
67. F. Cachoux; T. Isarno; M. Wartmann; K. H. Altmann, *ChemBioChem* **2006**, *7* (1), 54–57.
68. M. A. Koch; A. Schuffenhauer; M. Scheck; S. Wetzel; M. Casaulta; A. Odermatt; P. Ertl; H. Waldmann, *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102* (48), 17272–17277.
69. G. W. Bemis; M. A. Murcko, *J. Med. Chem.* **1996**, *39* (15), 2887–2893.
70. A. Schuffenhauer; P. Ertl; S. Roggo; S. Wetzel; M. A. Koch; H. Waldmann, *J. Chem. Inf. Model.* **2007**, *47* (1), 47–58.
71. M. D. Burke; S. L. Schreiber, *Angew. Chem. Int. Ed. Engl.* **2004**, *43* (1), 46–58.
72. J. Sadowski; H. Kubinyi, *J. Med. Chem.* **1998**, *41* (18), 3325–3329.
73. S. L. Schreiber, *Science (Washington, DC)* **2000**, *287* (5460), 1964–1969.
74. A. V. Shah; W. P. Walters; M. A. Murcko, *J. Med. Chem.* **1998**, *41* (18), 3314–3324.
75. W. P. Walters; M. A. Ajay; Murcko, *Curr. Opin. Chem. Biol.* **1999**, *3* (4), 384–387.
76. Anonymous, *Nat. Rev. Drug Discov.* **2007**, *6* (11), 853.
77. M. M. Hann; A. R. Leach; J. N. Burrows; E. Griffen, *Comprehensive Med. Chem. II* **2006**, *4*, 435–458.
78. S. Muresan; J. Sadowski, Properties Guiding Drug- and Lead-Likeness. In *Methods and Principles in Medicinal Chemistry, Vol. 37: Molecular Drug Properties;* 2008; pp 441–461.
79. G. M. Rishton, *Drug Discov. Today* **2002**, *8* (2), 86–96.
80. G. M. Rishton, *Curr. Opin. Chem. Biol.* **2008**, *12* (3), 340–351.
81. M.-Q. Zhang; B. Wilkinson, *Curr. Opin. Biotechnol.* **2007**, *18* (6), 478–488.
82. J. H. Nettles; J. L. Jenkins; A. Bender; Z. Deng; J. W. Davies; M. Glick, *J. Med. Chem.* **2006**, *49* (23), 6802–6810.
83. B. E. Evans; K. E. Rittle; M. G. Bock; R. M. DiPardo; R. M. Freidinger; W. L. Whitter; G. F. Lundell; D. F. Veber; P. S. Anderson *et al.*, *J. Med. Chem.* **1988**, *31* (12), 2235–2246.
84. G. Mueller, *Drug Discov. Today* **2003**, *8* (15), 681–691.
85. R. Breinbauer; I. R. Vetter; H. Waldmann, *Angew. Chem. Int. Ed. Engl.* **2002**, *41* (16), 2878–2890.
86. P. Ertl; S. Roggo; A. Schuffenhauer, *J. Chem. Inf. Model.* **2008**, *48* (1), 68–74.
87. N. T. Southall; Ajay, *J. Med. Chem.* **2006**, *49* (6), 2103–2109.
88. R. P. Sheridan, *J. Chem. Inf. Comp. Sci.* **2002**, *42* (1), 103–108.
89. M. A. Koch; L.-O. Wittenberg; S. Basu; D. A. Jeyaraj; E. Gourzoulidou; K. Reinecke; A. Odermatt; H. Waldmann, *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (48), 16721–16726.
90. A. Noeren-Mueller; I. Reis-Correa, Jr.; H. Prinz; C. Rosenbaum; K. Saxena; H. J. Schwalbe; D. Vestweber; G. Cagna; S. Schunk; O. Schwarz; H. Schiewe; H. Waldmann, *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103* (28), 10606–10611.
91. P. Ertl, *J. Chem. Inf. Comp. Sci.* **2003**, *43* (2), 374–380.
92. T. Fink; H. Bruggesser; J.-L. Reymond, *Angew. Chem. Int. Ed. Engl.* **2005**, *44* (10), 1504–1508.
93. T. Fink; J.-L. Reymond, *J. Chem. Inf. Model.* **2007**, *47* (2), 342–353.
94. A.-D. Gorse, *Curr. Top. Med. Chem. (Sharjah, U.A.E.)* **2006**, *6* (1), 3–18.
95. S. C. Schuerer; P. Tyagi; S. M. Muskal, *J. Chem. Inf. Model.* **2005**, *45* (2), 239–248.
96. R. van Deursen; J.-L. Reymond, *ChemMedChem* **2007**, *2* (5), 636–640.
97. R. Breinbauer; M. Manger; M. Scheck; H. Waldmann, *Curr. Med. Chem.* **2002**, *9* (23), 2129–2145.
98. D. S. Tan, *Nat. Chem. Biol.* **2005**, *1* (2), 74–84.
99. S. L. Schreiber, *Science* **2000**, *287*, 1964–1969.
100. A. Nören-Müller; J. I. Reis-Corrêa; H. Prinz; G. Rosenbaum; K. Saxena; H. J. Schwalbe; D. Vestweber; G. Cagna; S. Schunk; O. Schwarz; H. Schiewe; H. Waldmann, *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 10606–10611.
101. Z. Gan; P. T. Reddy; S. Quevillon; S. Couve-Bonnaire; P. Arya, *Angew. Chem. Int. Ed. Engl.* **2005**, *44*, 1366–1368.
102. K.-E. Andersson, *Pharmacol. Rev.* **2001**, *53*, 417–450.
103. S. Barluenga; C. Wang; J. G. Fontaine; K. Aouadi; K. Beebe; S. Tsutsumi; L. Neckers; N. Winssinger, *Angew. Chem. Int. Ed. Engl.* **2008**, *47*, 4432–4435.
104. M. E. Gorre; K. Ellwood-Yen; G. Chiosis; N. Rosen; C. L. Sawyers, *Blood* **2002**, *100*, 3041.
105. C. Peng; J. Brain; Y. Hu; A. Goodrich; L. Kong; D. Grayzel; R. Pak; M. Read; S. Li, *Blood* **2007**, *110*, 678.
106. D. B. Solit; A. D. Basso; A. B. Olshen; H. I. Scher; N. Rosen, *Cancer Res.* **2003**, *63*, 2139.

107. W. Luo; F. Dou; A. Rodina; S. Chip; J. Kim; Q. Zhao; K. Moulick; J. Aguirre; N. Wu; P. Greengard; G. Chiosis, *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 9511.
108. Z.-Q. Yang; X. Geng; D. Solit; C. A. Pratilas; N. Rosen; S. J. Danishefsky, *J. Am. Chem. Soc.* **2004**, *126*, 7881–7889.
109. R. Breinbauer; I. R. Vetter; H. Waldmann, *Angew. Chem. Int. Ed. Engl.* **2002**, *41* (16), 2879–2890.
110. S. Wetzel; A. Schuffenhauer; S. Roggo; P. Ertl; H. Waldmann, *Chim. Int. J. Chem.* **2007**, *61*, 355–360.
111. A. Schuffenhauer; P. Ertl; S. Roggo; S. Wetzel; M. A. Koch; H. Waldmann, *J. Chem. Inf. Model.* **2007**, *47*, 47–58.
112. M. A. Koch; H. Waldmann, *Drug Discov. Today* **2005**, *10*, 471–483.
113. H. E. Pelish; N. J. Westwood; Y. Feng; T. Kirchhausen; M. D. Shair, *J. Am. Chem. Soc.* **2001**, *123* (27), 6740–6741.
114. H. E. Pelish; J. R. Peterson; S. B. Salvarezza; E. Rodriguez-Boulan; J.-L. Chen; M. Stamnes; E. Macia; Y. Feng; M. D. Shair; T. Kirchhausen, *Nat. Chem. Biol.* **2006**, *2*, 39–46.
115. S. Dandapani; P. Lan; A. B. Beeler; S. Beischel; A. Abbas; B. L. Roth; J. A. Porco, Jr.; J. S. Panek, *J. Org. Chem.* **2006**, *71*, 8934–8945.
116. T. Lessmann; M. G. Leuenberger; S. Menninger; M. Lopez-Canet; O. Muller; S. Hummer; J. Bormann; K. Korn; E. Fava; M. Zerial; T. U. Mayer; H. Waldmann, *Chem. Biol.* **2007**, *14* (4), 443–451.
117. J. D. McChesney; S. K. Venkataraman; J. T. Henri, *Phytochemistry* **2007**, *68*, 2015–2022.
118. M. Kartal, *Phytother. Res.* **2007**, *21*, 113–119.
119. M. R. Boyd, *J. Ethnopharmacol.* **1996**, *51*, 17–27.
120. G. Aguilar, *Environ. Sci. Policy* **2001**, *4*, 241–256.
121. Convention on Biological Diversity. http://www.cbd.int/ (accessed 29 August).
122. S. Udgaonkar, The Protection of Medicinal Plants in India. http://envis.frlht.org.in/sangeeta.htm (accessed 29 August).
123. T. A. Kursar; C. C. Caballero-George; T. L. Capson; L. Cubilla-Rios; W. H. Gerwick; M. R. Gupta; A. Ibañez; R. G. Linington; K. L. Mcphail; E. Ortegabarria; L. I. Romero; P. N. Solis; P. D. Coley, *BioScience* **2006**, *56*, 1005–1012.
124. T. A. Kursar; C. C. Caballero-George; T. L. Capson; L. Cubilla-Rios; W. H. Gerwick; M. V. Keller; A. Ibañez; R. G. Linington; K. L. Mcphail; E. Ortegabarria; L. I. Romero; P. D. Coley, *Biodivers. Conserv.* **2007**, *16*, 2789–2800.
125. L. P. Christoffersen; E. J. Mathur, *Ind. Biotechnol.* **2005**, *1*, 255–259.
126. P. Vuorela; M. Leinonen; P. Saikku; P. Tammela; J.-P. Rauha; T. Wennberg; H. Vuorela, *Curr. Med. Chem.* **2004**, *11*, 1375–1389.
127. S. D. Sarker; Z. Latif; A. I. Gray, *Natural Products Isolation,* 2nd ed.; Humana Press: Totowa, NJ, 2005; Vol. 20.
128. S. D. Sarker; L. Nahar, Hyphenated Techniques. In *Natural Products Isolation*; S. D. Sarker; Z. Latif; A. I. Gray, Eds. Humana Press: Totowa, NJ, 2005; Vol. 20, pp 233–267.
129. G. Lang; N. A. Mayhudin; M. I. Mitova; L. Sun; S. van der Sar; J. W. Blunt; A. L. J. Cole; G. Ellis; H. Laatsch; M. H. G. Munro, *J. Nat. Prod.*, in press.
130. N. C. Gassner; C. M. Tamble; J. E. Bock; N. Cotton; K. N. White; K. Tenney; R. P. St. Onge; M. J. Proctor; G. Giaever; C. Nislow; R. W. Davis; P. Crews; T. R. Holman; R. S. Lokey, *J. Nat. Prod.* **2007**, *70*, 383–390.
131. S. J. Kwon; M.-Y. Lee; B. Ku; D. H. Sherman; J. S. Dordick, *ACS Chem. Biol.* **2007**, *2*, 419–425.
132. R. S. Lokey, *Curr. Opin. Chem. Biol.* **2003**, *7* (1), 91–96.
133. D. P. Walsh; Y. T. Chang, *Chem. Rev.* **2006**, *106*, 2476–2530.
134. N. Lindquist; W. Fenical; G. D. Van Duyne; J. Clardy, *J. Am. Chem. Soc.* **1991**, *113*, 2303–2304.
135. J. Li; S. Jeong; L. Esser; P. G. Harran, *Angew. Chem. Int. Ed. Engl.* **2001**, *40*, 4765–4769.
136. K. C. Nicolaou; S. A. Snyder, *Angew. Chem. Int. Ed. Engl.* **2005**, *44*, 1012–1044.
137. D. P. Curran; Q. Zhang; C. Richard; H. Lu; V. Gudipati; C. S. Wilcox, *J. Am. Chem. Soc.* **2006**, *128*, 9561–9573.
138. D. P. Curran; Q. Zhang; H. Lu; V. Gudipati, *J. Am. Chem. Soc.* **2006**, *128*, 9943–9956.
139. M. C. Wani; H. L. Taylor; M. E. Wall; P. Coggon; T. A. McPhail, *J. Am. Chem. Soc.* **1971**, *93*, 2325–2327.
140. E. Leistner, *Pharm. Unserer Zeit* **2005**, *34*, 98–103.
141. P. B. Schiff; J. Fant; S. B. Horwitz, *Nature* **1979**, *277*, 665–667.
142. S. B. Horwitz, *Trends Pharmacol. Sci.* **1992**, *13*, 132–136.
143. G. M. Cragg; S. A. Schepartz; M. Suffness; M. R. Grever, *J. Nat. Prod.* **1993**, *56*, 1657–1668.
144. R. A. Holton; C. Somoza; H.-B. Kim; F. Liang; R. J. Biediger; P. D. Boatman; M. Shindo; C. C. Smith; S. Kim; H. Nadizadeh; Y. Suzuki; C. Tao; P. Vu; S. Tang; P. Zhang; K. K. Murthi; L. N. Gentile; J. H. Liu, *J. Am. Chem. Soc.* **1994**, *116*, 1597–1598.
145. R. A. Holton; H.-B. Kim; C. Somoza; F. Liang; R. J. Biediger; P. D. Boatman; M. Shindo; C. C. Smith; S. Kim; H. Nadizadeh; Y. Suzuki; C. Tao; P. Vu; S. Tang; P. Zhang; K. K. Murthi; L. N. Gentile; J. H. Liu, *J. Am. Chem. Soc.* **1994**, *116*, 1599–1600.
146. K. C. Nicolaou; Z. Yang; J. J. Liu; H. Ueno; P. G. Nantermet; R. K. Guy; C. F. Clairborne; J. Renaud; E. A. Couladouros; K. Paulvannan; E. J. Sorensen, *Nature* **1994**, *367*, 630–634.
147. S. J. Danishefsky; J. J. Masters; W. B. Young; J. T. Link; L. B. Snyder; T. V. Magee; D. K. Jung; R. C. A. Isaacs; W. G. Bornmann; C. A. Alaimo; C. A. Coburn; M. J. Di Grand, *J. Am. Chem. Soc.* **1996**, *118*, 2843–2859.
148. P. A. Wender; N. F. Badham; S. P. Conway; P. E. Floreancig; T. E. Glass; J. B. Houze; N. E. Krauss; D. Lee; D. G. Marquess; P. L. McGrane; W. Meng; M. G. Natchus; A. J. Shuker; J. C. Sutton; R. E. Taylor, *J. Am. Chem. Soc.* **1997**, *119*, 2757–2758.
149. H. Kusama; R. Hara; S. Kawahara; T. Nishimori; H. Kashima; N. Nakamura; K. Morihira; I. Kuwajima, *J. Am. Chem. Soc.* **2000**, *122*, 3811–3820.
150. T. Mukaiyama; I. Shiina; H. Iwadare; M. Saitoh; T. Nishimura; N. Ohkawa; H. Sakoh; K. Nishimura; Y.-I. Tani; M. Hasegawa; K. Yamada; K. Saitoh, *Chem. Eur. J.* **1999**, *5*, 121–161.
151. J. N. Denis; A. E. Green; D. Guénard; F. Guéritte-Voegelein; L. Mangatal; P. Potier, *J. Am. Chem. Soc.* **1988**, *110*, 5517–5519.
152. N. J. Sisti; C. S. Swindell; C. Chander, 1997.
153. N. J. Sisti; C. S. Swindell; C. Chander, 1999.
154. F. S. Gibson, 2000.
155. Agency, U. E. P. Greener Synthetic Pathways Award. http://www.epa.gov/greenchemistry/pubs/pgcc/winners/gspa04.html (accessed 29 August 2004).
156. S. P. Gunasekera; M. Gunasekera; R. E. Longley; G. K. Schulte, *J. Org. Chem.* **1990**, *55*, 4912.
157. S. P. Gunasekera; M. Gunasekera; R. E. Longley; G. K. Schulte, *J. Org. Chem.* **1991**, *56*, 1346.
158. S. P. Gunasekera; S. Cranick; R. E. Longley, *J. Nat. Prod.* **1989**, *52*, 757.

159. J. M. Minguez; S. Y. Kim; K. A. Giuliano; R. Balachandran; C. Madiraju; B. W. Day; D. P. Curran, *Bioorg. Med. Chem.* **2003**, *11* (15), 3335–3357.
160. A. B. Smith; T. J. Beauchamp; M. J. LaMarche; M. D. Kaufmann; Y. Qui; H. Arimoto; D. R. Jones, *J. Am. Chem. Soc.* **2000**, *122*, 8654–8664.
161. A. B. Smith; M. D. Kaufman; T. J. Beauchamp; M. J. LaMarche; H. Arimoto, *Org. Lett.* **1999**, *1*, 1823–1826.
162. J. A. Marshall; B. A. Johns, *J. Org. Chem.* **1998**, *63*, 7885–7892.
163. I. Paterson; G. J. Florence; K. Gerlach; J. P. Scott, *Angew. Chem. Int. Ed. Engl.* **2000**, *39*, 377–380.
164. S. J. Mickel; G. H. Sedelmeier; D. Niederer; R. Daeffler; A. Osmani; K. Schreiner; M. Seeger-Weibel; B. Bérod; K. Schaer; R. Gamboni; S. Chen; W. Chen; C. T. Jagoe; J. F. R. Kinder; M. Loo; K. Prasad; O. Repič; W.-C. Shieh; R.-M. Wang; L. Waykole; D. D. Xu; S. Xue, *Org. Process Res. Dev.* **2004**, *4*, 92–100.
165. S. J. Mickel; G. H. Sedelmeier; D. Niederer; F. Schuerch; D. Grimler; G. Koch; R. Daeffler; A. Osmani; A. Hirni; K. Schaer; R. Gamboni; A. Bach; A. Chaudhary; S. Chen; W. Chen; B. Hu; C. T. Jagoe; H.-Y. Kim; J. F. R. Kinder; Y. Liu; Y. Lu; J. McKenna; K. Prasad; T. M. Ramsey; O. Repič; L. Rogers; W.-C. Shieh; R.-M. Wang; L. Waykole, *Org. Process Res. Dev*. **2004**, *2*, 101–106.
166. S. J. Mickel; G. H. Sedelmeier; D. Niederer; F. Schuerch; G. Koch; E. Kuesters; R. Daeffler; A. Osmani; M. Seeger-Weibel; E. Schmid; A. Hirni; K. Schaer; R. Gamboni; A. Bach; S. Chen; W. Chen; P. Geng; C. T. Jagoe; J. F. R. Kinder; G. T. Lee; J. McKenna; T. M. Ramsey; O. Repič; L. Rogers; W.-C. Shieh; R.-M. Wang; L. Waykole, *Org. Process Res. Dev*. **2004**, *8*, 107–112.
167. S. J. Mickel; G. H. Sedelmeier; D. Niederer; F. Schuerch; M. Seger; K. Schreiner; R. Daeffler; A. Osmani; D. Bixel; O. Loiseleur; J. Cercus; H. Stettler; K. Schaer; R. Gamboni; A. Bach; G.-P. Chen; W. Chen; P. Geng; G. T. Lee; E. Loeser; J. McKenna; J. F. R. Kinder; K. Konigsberger; K. Prasad; T. M. Ramsey; N. Reel; O. Repič; L. Rogers; W.-C. Shieh; R.-M. Wang; L. Waykole; S. Xue, *Org. Process Res. Dev.* **2004**, *8*, 113–121.
168. S. J. Mickel; D. Niederer; R. Daeffler; A. Osmani; E. Kuesters; E. Schmid; K. Schaer; R. Gamboni; W. Chen; E. Loeser; J. F. R. Kinder; K. Konigsberger; K. Prasad; T. M. Ramsey; O. Repič; R.-M. Wang; G. Florence; I. Lyothier; I. Paterson, *Org. Process Res. Dev.* **2004**, *8*, 122–130.
169. A. Mita; A. C. Lockhart; T.-L. Chen; K. Bochinski; J. Curtright; W. Cooper; L. Hammond; M. Rothenberg; E. Rowinsky; S. Sharma, *J. Clin. Oncol.* **2004**, *22* (14S), 2025.
170. K. C. Nicolaou; S. A. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (33), 11929–11936.
171. D. J. Newman, *J. Med. Chem.* **2008**, *51* (9), 2589–2599.
172. J. Heilmann, *Chem. Unserer Zeit* **2007**, *41* (5), 376–389.
173. P. Bhushan; G. Manish, *Ann. Tradit. Chin. Med.* **2007**, *3* (Alternative Treatment for Cancer), 255–284.
174. K. Das; R. Dang; S. Bhaskaran; T. S. Roopashree, *Recent Prog. Med. Plants* **2008**, *19*, 151–191.
175. R. G. Mali; S. G. Mahajan; A. A. Mehta, *Phcog. Rev.* **2007**, *1* (2), 314–319.
176. R. S. Sangwan; N. D. Chaurasiya; P. Lal; L. Misra; R. Tuli; N. S. Sangwan, *Physiol. Plant.* **2008**, *133* (2), 278–287.
177. C. C. Deocaris; N. Widodo; R. Wadhwa; S. C. Kaul, *J. Transl. Med.* **2008**, *6*.
178. N. J. White, *Science (Washington, DC)* **2008**, *320* (5874), 330–334.
179. Y. Imakura; K. Hachiya; T. Ikemoto; S. Kobayashi; S. Yamashita; J. Sakakibara; F. T. Smith; K. H. Lee, *Heterocycles* **1990**, *31* (12), 2125–2129.
180. Y. Imakura; K. Hachiya; T. Ikemoto; S. Yamashita; M. Kihara; S. Kobayashi; T. Shingu; W. K. Milhous; K. H. Lee, *Heterocycles* **1990**, *31* (6), 1011–1016.
181. Y. Imakura; T. Yokoi; T. Yamagishi; J. Koyama; H. Hu; D. R. McPhail; A. T. McPhail; K. Lee, *J. Chem. Soc. Chem. Commun.* **1988**, *5*, 372–374.
182. G. T. Liu, *Yao Xue Xue Bao* **1983**, *18* (9), 714–720.
183. K. H. Lee, *Public Health Nutr.* **2000**, *3* (4A), 515–522.
184. J. D. McChesney; S. K. Venkataraman; J. T. Henri, *Phytochemistry (Elsevier)* **2007**, *68* (14), 2015–2022.
185. M. T. Hamann; R. Hill; S. Roggo, *Chimia* **2007**, *61* (6), 313–321.
186. B. Haefner, *Drug Discov. Today* **2003**, *8* (12), 536–544.
187. M. Tulp; L. Bohlin, *Drug Discov. Today* **2004**, *9* (10), 450–458.
188. B. B. Aggarwal; I. D. Bhatt; H. Ichikawa; K. S. Ahn; G. Sethi; S. K. Sandur; C. Sundaram; N. Seeram; S. Shishodia, *Med. Aromat. Plants – Ind. Profiles* **2007**, *45* (Turmeric), 297–368.
189. D. J. Newman; G. M. Cragg; K. M. Snader, *J. Nat. Prod.* **2003**, *66* (7), 1022–1037.
190. P. Crews; W. H. Gerwick; F. J. Schmitz; D. France; K. W. Bair; A. E. Wright; Y. Hallock, *Pharm. Biol.* **2003**, *41*, 39–52.
191. G. Mehta; V. Singh, *Chem. Soc. Rev.* **2002**, *31* (6), 324–334.
192. N. Peric-Concha; P. F. Long, *Drug Discov. Today* **2003**, *8* (23), 1078–1084.
193. O. Potterat, *Chimia* **2006**, *60* (1–2), 19–22.
194. A. de Fatima; L. V. Modolo; A. C. C. Sanches; R. R. Porto, *Mini Rev. Med. Chem.* **2008**, *8* (9), 879–888.
195. M. Gordaliza, *Clin. Transl. Oncol.* **2007**, *9* (12), 767–776.
196. M.-T. Gutierrez-Lugo; C. A. Bewley, *J. Med. Chem.* **2008**, *51* (9), 2606–2612.
197. G. M. Rishton, *Am. J. Cardiol.* **2008**, *101* (10A), 43D–49D.
198. H. Reichenbach; G. Hoefle, *Drugs R&D* **2008**, *9* (1), 1–10.
199. K. N. Bhalla, *Oncogene* **2003**, *22* (56), 9075–9086.
200. C. J. Bode; M. L. Gupta, Jr.; E. A. Reiff; K. A. Suprenant; G. I. Georg; R. H. Himes, *Biochemistry* **2002**, *41* (12), 3870–3874.
201. C. J. Bode; M. L. Gupta, Jr.; E. A. Reiff; K. A. Suprenant; G. I. Georg; R. H. Himes, *Biochemistry* **2002**, *41* (24), 7858.
202. D. M. Bollag; P. A. McQueney; J. Zhu; O. Hensens; L. Koupal; J. Liesch; M. Goetz; E. Lazarides; C. M. Woods, *Cancer Res.* **1995**, *55* (11), 2325–2333.
203. R. M. Buey; J. F. Diaz; J. M. Andreu; A. O'Brate; P. Giannakakou; K. C. Nicolaou; P. K. Sasmal; A. Ritzen; K. Namoto, *Chem. Biol.* **2004**, *11* (2), 225–236.
204. D. W. Heinz; W.-D. Schubert; G. Hoefle, *Angew. Chem. Int. Ed. Engl.* **2005**, *44* (9), 1298–1301.
205. M. Wartmann; K. H. Altmann, *Curr. Med. Chem. Anti-Cancer Agents* **2002**, *2* (1), 123–148.
206. H. Yamaguchi; J. Chen; K. Bhalla; H.-G. Wang, *J. Biol. Chem.* **2004**, *279* (38), 39431–39437.

207. Y.-Q. Liu; L. Yang; X. Tian, *Curr. Bioact. Compounds* **2007**, *3* (1), 37–66.
208. L. S. King; M. Sullivan, *Science (Washington, DC)* **1946**, *104*, 244–245.
209. B. H. Long; M. G. Brattain, *The activity of etoposide (VP-16-213) and teniposide (VM-26) against human lung tumor cells in vitro: cytotoxicity and DNA breakage.* **1984**, 63–86.
210. B. H. Long; S. T. Musial; M. G. Brattain, *Biochemistry* **1984**, *23* (6), 1183–1188.
211. A. Minocha; B. H. Long, *Biochem. Biophys. Res. Commun.* **1984**, *122* (1), 165–170.
212. Z. Xiao; K. F. Bastow; J. R. Vance; R. S. Sidwell; H.-K. Wang; M. S. Chen; Q. Shi; K.-H. Lee, *J. Med. Chem.* **2004**, *47* (21), 5140–5148.
213. X. K. Zhu; J. Guan; Y. Tachibana; K. F. Bastow; S. J. Cho; H. H. Cheng; Y. C. Cheng; M. Gurwith; K. H. Lee, *J. Med. Chem.* **1999**, *42* (13), 2441–2446.
214. D. A. Dabydeen; J. C. Burnett; R. Bai; P. Verdier-Pinard; S. J. H. Hickford; G. R. Pettit; J. W. Blunt; M. H. G. Munro; R. Gussio; E. Hamel, *Mol. Pharmacol.* **2006**, *70* (6), 1866–1875.
215. S. Newman, *Curr. Opin. Investig. Drugs (Thomson Sci.)* **2007**, *8* (12), 1057–1066.
216. T. Okouneva; O. Azarenko; L. Wilson; B. A. Littlefield; M. A. Jordan, *Mol. Cancer Ther.* **2008**, *7* (7), 2003–2011.
217. Y. Hirata; D. Uemura, *Pure Appl. Chem.* **1986**, *58* (5), 701–710.
218. D. Uemura; K. Takahashi; T. Yamamoto; C. Katayama; J. Tanaka; Y. Okumura; Y. Hirata, *J. Am. Chem. Soc.* **1985**, *107* (16), 4796–4798.
219. J. L. Martinez, *Science (Washington, DC)* **2008**, *321* (5887), 365–367.
220. M. P. Singh; M. Greenstein, *Curr. Opin. Drug Discov. Devel.* **2000**, *3* (2), 167–176.
221. G. D. Wright; A. D. Sutherland, *Trends Mol. Med.* **2007**, *13* (6), 260–267.
222. B. Bister; D. Bischoff; M. Stroebele; J. Riedlinger; A. Reicke; F. Wolter; A. T. Bull; H. Zaehner; H.-P. Fiedler; R. D. Suessmuth, *Angew. Chem. Int. Ed. Engl.* **2004**, *43* (19), 2574–2576.
223. J. Riedlinger; A. Reicke; H. Zaehner; B. Krismer; A. T. Bull; L. A. Maldonado; A. C. Ward; M. Goodfellow; B. Bister; D. Bischoff; R. D. Suessmuth; H.-P. Fiedler, *J. Antibiot.* **2004**, *57* (4), 271–279.
224. K. C. Nicolaou; S. T. Harrison, *Angew. Chem. Int. Ed. Engl.* **2006**, *45* (20), 3256–3260.
225. R. Peters; D. F. Fischer, *Angew. Chem. Int. Ed. Engl.* **2006**, *45* (35), 5736–5739.
226. K. C. Nicolaou; S. T. Harrison, *J. Am. Chem. Soc.* **2007**, *129* (2), 429–440.
227. S. Keller; G. Nicholson; C. Drahl; E. Sorensen; H.-P. Fiedler; R. D. Suessmuth, *J. Antibiot.* **2007**, *60* (6), 391–394.
228. S. Keller; H. S. Schadt; I. Ortel; R. D. Suessmuth, *Angew. Chem. Int. Ed. Engl.* **2007**, *46* (43), 8284–8286.
229. F. E. Koehn, *J. Med. Chem.* **2008**, *51* (9), 2613–2617.
230. H. He; R. T. Williamson; B. Shen; E. I. Graziani; H. Y. Yang; S. M. Sakya; P. J. Petersen; G. T. Carter, *J. Am. Chem. Soc.* **2002**, *124* (33), 9729–9736.
231. S. E. De Voe; M. P. Kunstmann, Antibiotic AC-98. U.S. Patent 68-7,685,713,495,004, 19,680,813, 1970.
232. M. P. Singh; P. J. Petersen; W. J. Weiss; J. E. Janso; S. W. Luckman; E. B. Lenoy; P. A. Bradford; R. T. Testa; M. Greenstein, *Antimicrob. Agents Chemother.* **2003**, *47* (1), 62–69.
233. A. Ruzin; G. Singh; A. Severin; Y. Yang; R. G. Dushin; A. G. Sutherland; A. Minnick; M. Greenstein; M. K. May; D. M. Shlaes; P. A. Bradford, *Antimicrob. Agents Chemother.* **2004**, *48* (3), 728–738.
234. H. He; B. Shen; P. J. Petersen; W. J. Weiss; H. Y. Yang; T.-Z. Wang; R. G. Dushin; F. E. Koehn; G. T. Carter, *Bioorg. Med. Chem. Lett.* **2004**, *14* (1), 279–282.
235. N. A. Magarvey; B. Haltli; M. He; M. Greenstein; J. A. Hucul, *Antimicrob. Agents Chemother.* **2006**, *50* (6), 2167–2177.
236. B. Haltli; Y. Tan; N. A. Magarvey; M. Wagenaar; X. Yin; M. Greenstein; J. A. Hucul; T. M. Zabriskie, *Chem. Biol. (Cambridge, MA)* **2005**, *12* (11), 1163–1168.
237. J. J. Gordon; B. K. Kelly; G. A. Miller, *Nature (London)* **1962**, *195*, 701–702.
238. M. M. Attwood, *J. Gen. Microbiol.* **1969**, *55* (2), 209–216.
239. N. H. Anderson; W. D. Ollis; J. E. Thorpe; A. D. Ward, *J. Chem. Soc. Chem. Commun.* **1974**, (11), 420–421.
240. N. H. Anderson; W. D. Ollis; J. E. Thorpe; A. D. Ward, *J. Chem. Soc. Perkin Trans. 1: Org. Bioorg. Chem. (1972–1999)* **1975**, (9), 825–830.
241. J. P. Devlin; W. D. Ollis; J. E. Thorpe; R. J. Wood; B. J. Broughton; P. J. Warren; K. R. H. Wooldridge; D. E. Wright, *J. Chem. Soc. Perkin Trans. 1: Org. Bioorg. Chem. (1972–1999)* **1975**, (9), 830–841.
242. B. J. Broughton; P. J. Warren; K. R. H. Wooldridge; D. E. Wright; W. D. Ollis; R. J. Wood, *J. Chem. Soc. Perkin Trans. 1: Org. Bioorg. Chem. (1972–1999)* **1975**, (9), 842–846.
243. J. P. Devlin; W. D. Ollis; J. E. Thorpe, *J. Chem. Soc. Perkin Trans. 1: Org. Bioorg. Chem. (1972–1999)* **1975**, (9), 846–848.
244. B. J. Broughton; P. Chaplen; W. A. Freeman; P. J. Warren; K. R. H. Wooldridge; D. E. Wright, *J. Chem. Soc. Perkin Trans. 1: Org. Bioorg. Chem. (1972–1999)* **1975**, (9), 857–860.
245. H. Umezawa; T. Aoyagi; T. Tanaka; H. Suda; A. Okuyama; H. Naganawa; M. Hamada; T. Takeuchi, *J. Antibiot.* **1985**, *38* (11), 1629–1630.
246. D. Z. Chen; D. V. Patel; C. J. Hackbarth; W. Wang; G. Dreyer; D. C. Young; P. S. Margolis; C. Wu; Z.-J. Ni; J. Trias; R. J. White; Z. Yuan, *Biochemistry* **2000**, *39* (6), 1256–1262.

Biographical Sketches

Stefan Wetzel was born in Heidelberg, Germany in 1978. From 1998 he studied chemistry at the universities of Regensburg and Heidelberg. He graduated from the University of Heidelberg in 2004 with a diploma thesis in synthetic organic chemistry from the group of Professor Haberhauer at the chair of Professor Gleiter. In 2005, Stefan Wetzel joined the department of Professor Waldmann at the Max Planck Institute of Molecular Physiology and the chemistry faculty of the Technical University Dortmund to start his doctoral work in the field of bio- and cheminformatics. His Ph.D. research project centered on novel computational approaches for the design of focused biologically relevant libraries. Two approaches were developed, one centered on the analysis of large sets of structure-related biochemical and biological data and the other based on structural similarity of protein binding sites. This work involved a variety of methods ranging from cheminformatics (analysis and visualization of large data sets) and bioinformatics (protein structure comparison and clustering) to computational chemistry (molecular modeling and structure-based ligand design) as well as biochemistry (biochemical assays). His current research interests also include computational systems biology approaches, for example, modeling of small molecule interference with molecular pathways.

Hugo Lachance was born in 1974 in Québec, Canada. He obtained his B.Sc. (pharmaceutical chemistry, COOP) in 2000 from Université de Sherbrooke. He then worked briefly as a research assistant in medicinal chemistry at Merck Frosst, Montréal, Canada. In 2001, he enrolled in the Ph.D. program at the University of Alberta, working under the supervision of Professor Dennis Hall. His research focused on the development of the Lewis acid-catalyzed enantioselective allylboration reaction and its application to new enantioselective allylboration and methallylboration reagents. He was also involved in studying its application for the synthesis of complex organic molecules. Toward the end of 2006, after obtaining his Ph.D. from the University of Alberta, he moved to the Max Planck Institute of Molecular Physiology in Dortmund, Germany to pursue postdoctoral work in chemical biology as an NSERC of Canada postdoctoral fellow. Under the guidance of Professor Herbert Waldmann, his work involves the preparation of a natural product-inspired library as well as the design and preparation of affinity probes for biological target identification.

Professor Herbert Waldmann was born in Neuwied, Germany. He studied chemistry at the University of Mainz where he received his Ph.D. in organic chemistry in 1985 under the guidance of Horst Kunz. After a postdoctoral appointment with Professor George Whitesides at Harvard University, he completed his habilitation at the University of Mainz in 1991. In 1991, he was appointed as professor of organic chemistry at the University of Bonn, and then in 1993 was appointed to full professor of organic chemistry at the University of Karlsruhe. In 1999, he was appointed as Director of the Max Planck Institute of Molecular Physiology, Dortmund and professor of organic chemistry at the University of Dortmund.

His research interests lie in the syntheses of signal transduction modulators and the syntheses of natural product-derived compound libraries and their biological evaluation, the synthesis and biological evaluation of lipidated peptides and proteins as well as protein microarray technology. He is a recipient of the Otto Bayer Award, the Max Bergmann Medal, and the GSK Award for Outstanding Achievements in Chemical Biology. He is a member of Deutsche Akademie der Naturforscher Leopoldina, Halle/Saale and since 2005 he is a Fellow of the Royal Society of Chemistry.

3.03 Topical Chemical Space Relation to Biological Space

Anders Backlund, Uppsala University, Uppsala, Sweden

3.03.1 Introduction

More than a century ago Helen Abbott concluded that

> "The evolution of chemical constituents follows parallel lines with the evolutionary course of plant forms, the one being intimately connected with the other. . ."
>
> Helen Cecilia De Silver Abbot,
> 1887, Franklin Institute lecture:
> *The chemical basis of plant forms*[1]

From this insightful suggestion follows that there ought to be a pattern of correlation between implications derived from exploration of chemical constituents and those from evolutionary studies starting with the initial attempts to characterize chemical properties, such as in the thesis by Hiortzberg[2] – one of the first in medicinal chemistry at Uppsala University – to present day exploration and charting of chemical space and in analogy from the Linnaean classification of the eighteenth century to modern phylogenetic studies of evolutionary space.

The concept of chemical space, or more properly the chemical property space, is an attempt to describe chemical information. One consistent and coherent way to pursue this is explored in the area of chemography, in which analogies are drawn to geography. Given that chemical space includes all known, and in principle also all unknown but possible, compounds, its sheer size is woeful. It has been estimated that there are well above 10^{60} possible small carbon-based compounds,[3] and the number of compounds rapidly rises to at least 10^{390} if small peptides are also included.[4] But to further complicate the endeavor, chemical space is, as Shoichet puts it "...vast but most of it is biologically uninteresting; blank, lightless galaxies exist within it into which good ideas at their peril wander."[5]

The size of chemical space makes an exhaustive exploration impossible, which is the reason why considerable effort has been put into defining which parts, or multidimensional volumes, should be prioritized and explored first and how to go about such a Herculean task.[6] Within the chemical space, subvolumes such as 'drug-like chemical space,' 'natural products chemical space,' and 'biologically relevant chemical space' have been defined.

In many fields of research such as chemical biology, pharmacognosy, and medicinal chemistry, primary attention has been given to parts of the chemical space that are believed to contain molecules with biological activities. This is usually referred to as the 'biologically relevant chemical space.' The borders of this multidimensional subvolume are defined by the properties and boundaries allowing for binding interactions between biological molecules, ranging from primary and secondary metabolites to polypeptides, enzymes, RNA, and DNA.[7]

In the field of medicinal chemistry, where during the last decade efforts were focused on a very restricted part of the chemical space as defined by Lipinski *et al.*'s[8] 'rule of five,' attention has recently (re)turned to natural products. The prime reason for this is that even though natural products often tend to invalidate the 'rule of five,' they have been found to be both more varied and more 'drug-like' than many combinatorial chemistry collections. At present, strategies including both synthetic and biosynthetic approaches are developed to produce screening libraries with a broader coverage of chemical space, and a renaissance of drug discovery inspired by natural products is predicted.[9]

A trend changing from TOS (target-oriented synthesis), attempting to explore in detail a narrow part of the chemical space, to DOS (diversity-oriented synthesis), on the contrary attempting to cover as broad a field as possible, is evident in literature. Recently, attention has (re)turned to natural products to find novel chemical scaffolds for further studies, inspiration, and possibly modification. The uniqueness of many natural product core structures and their demonstrated frequent occupation of volumes of chemical space is difficult to access.[10–15]

In a time when pharmaceutical companies have become increasingly concerned about decreasing productivity and ever increasing costs of developing new drugs, embracing new technologies may even lead to an initial increase in research and development costs.[14] Simultaneously, attention has been drawn to increasing regulatory costs.[13] Both these observations would, however, argue for a means of finding a more efficient process with regard to identifying, investigating, and developing useful lead compounds. In this process, ways to predict and model the results of experiments – to learn how to navigate, as Oprea[16] puts it – become increasingly important. The ability and the need to perform selection based on well-supported arguments, and thus avoid unnecessary and expeditious laboratory costs, turns into not only an academic issue but also a clear industrial advantage. In addition, it will enable further and more specific studies on the minute amounts of hard-to-get natural products painstakingly isolated. These suggestions fall in line with other studies, suggesting reasons for the decrease in productivity. Among these, there are problems with risky targets identified from genomic research, poor chemical libraries with 'nonnatural' properties, lack of technological integration, and – not surprising – a too small 'drug modality footprint.' The conclusion drawn from studies of survival rates in clinical studies suggests an increased attention to biologicals that have a 70% higher chance to succeed.[12]

The remainder of this chapter focuses on natural products, their use in chemical libraries, and the correlation between natural products and evolutionary biology.

3.03.2 Chemical Space

3.03.2.1 How to Explore and Navigate Chemical Space

Chemical space, as usually referred to, is an abstraction of various types of chemical information. In modern application, this is often computed information based on, for example, the structural formula of a chemical – real or virtual. This type of information can be retrieved in massive amounts by simple procedures, and forms the basis for the majority of chemical space explorations and charting endeavors published.[7,16–19] Computed information is typically retrieved from a software package such as Dragon (http://www.talete.mi.it) and is obtained from rigorous application of various algorithms resulting in a set of calculated chemical descriptors. In contrast to this computed information, there are also various types of measured information such as nuclear magnetic resonance (NMR) structure data, binding affinities, optic rotation, or color. This measured information is subject to error of observation, measurement, experimental design, and so on.[20]

In exploring chemical space, there will always be the issue of reference system, necessary, if information for a set of compounds calculated using one software is to be compared to that from another.[21,22] This is an important difference to some of the properties of biological space, where a common underlying history of evolution justifies the assumption that some aspects (e.g., phylogenies) ought to be comparable.

In the efforts of charting chemical space, Oprea and Gottfries,[18] at that time both working at AstraZeneca R&D in Mölndal, Sweden, realized that the implementation of a global map of the chemical property space would be of significant importance. If a global map could be established, it would further imply that information from different analyses could be compared in a consistent framework provided the same molecular descriptors were used. The ChemGPS developed for medicinal chemistry purposes was formed in analogy to the U.S. Navy Navstar GPS system (http://tycho.usno.navy.mil), defining a set of 423 'satellite' and 'core' chemical compound structures representing a drug-like chemical space. The corresponding chemical descriptors were calculated and subjected to principal component analysis (PCA), the results of which were used in forming a training set that defined the dimensions of the ChemGPS chemical property space.

When applying this method to a set of natural products, however, it was obvious that the comparably restricted model of chemical space defined by ChemGPS could in many cases not handle the chemical diversity encountered among natural products.[23] This initiated the work on a new model, ChemGPS-NP, tuned for navigation in biologically relevant chemical space.[24] The ChemGPS-NP global map of the chemical property space is built up from 1779 'satellite' and 'core' compounds evaluated with 35 carefully selected chemical descriptors and validated using more than 1.2 million compounds of natural and nonnatural origin. In **Figure 1**, these 1779 reference compounds, as well as the 423 compounds from the old model, are all predicted in ChemGPS-NP, forming the outline of the map. The difference in volume covered by the ChemGPS ('drug-like natural products' sensu Lipinski), as compared to the ChemGPS-NP (including natural products), gives some indication on the difference in volume between these two concepts. In **Table 1** the most important properties can be found for the first eight dimensions of ChemGPS-NP chemical property space, and in **Table 2** a complete list of the 34 + 1 chemical descriptors used (the 35th descriptor is the 'Lipinsky Alert Index'). **Figure 2** is a graphical representation of loadings for these 35 descriptors in the first three dimensions, indicating their relative contributions. A web interface, ChemGPS-NP$_{Web}$, is now available at http://chemgps.bmc.uu.se.

With respect to exploring chemical space, a very different situation is found when using tools based on measured data as compared to the computed data discussed above. While computed data can easily be generated, even for virtual (not yet found or synthesized) chemical structures in large batches, measured data are much harder to produce. To be able to measure properties, the actual compounds must exist in sufficient amounts for assays or experiments to be pursued. On the other hand, the information generated has a direct biological meaning – *in vivo* or *in vitro*. As sufficient and suitable compound libraries may be difficult to assemble from natural products, frequently DOS[25] is used to provide compounds for testing, as for example in

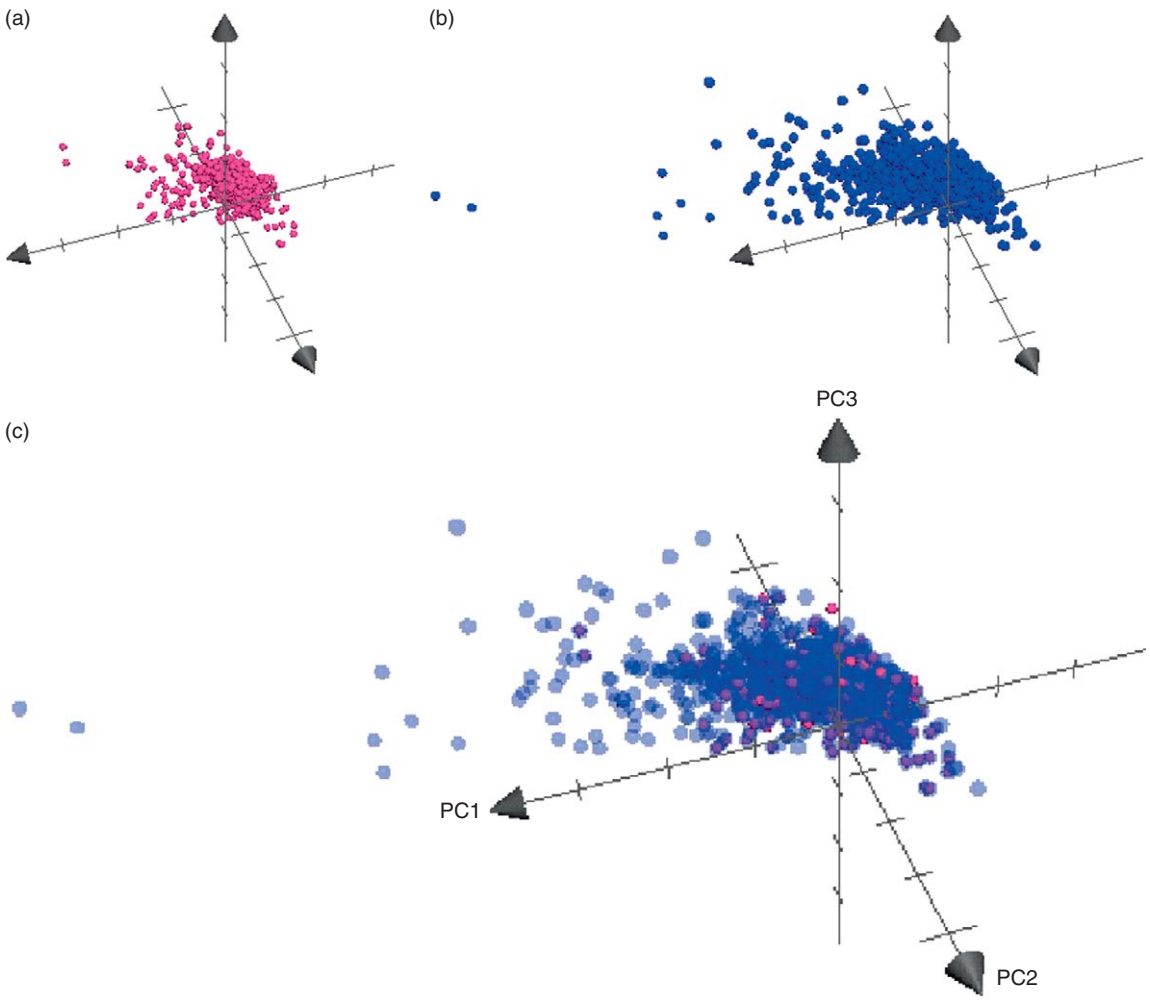


Figure 1 Score plot of the three most significant dimensions (PC1, PC2, and PC3) of the model set of compounds of (a) ChemGPS, (b) ChemGPS-NP, and (c) ChemGPS (pink) with ChemGPS-NP (blue) superimposed, illustrating the general shape of natural products chemical space and revealing its prominent parametrical asymmetry. Each sphere represents an object (a compound) of the model sets. The first three PCs explain 71% of the variance. From these plots, the difference in size and density between the 'drug-like' ChemGPS and the natural products-based ChemGPS-NP becomes evident.

Table 1 Summary of the most important contributing characteristics, for the first eight dimensions (PC1–PC8) of ChemGPS-NP chemical property space

PC	*Contributing characteristics*
1	Size, shape, polarizability
2	Aromaticity- and conjugation-related properties
3	Lipophilicity, polarity, and H-bond capacity
4	Flexibility and rigidity
5	Electronegativity, number of nitrogens, halogens, and amides
6	Number of rings, rotatable bonds, amides, and hydroxyl groups
7	Number of double bonds, oxygens, and nitrogens
8	Aromatic and aliphatic hydroxyl groups, unsaturation, LAI

LAI = Lipinsky Alert Index.

Table 2 The final 35 ChemGPS-NP descriptors, defined from an initial set of 926 descriptors by successively removing and validating the contribution of the remaining descriptors[24]

No.	*Abbreviation*	*Description*
1	MW	Molecular weight
2	Sv	Sum of atomic van der Waals volumes (scaled on C atom)
3	Se	Sum of atomic Sanderson electronegativities (scaled on C atom)
4	Sp	Sum of atomic polarizabilities (scaled on C atom)
5	Mv	Mean atomic van der Waals volume (scaled on C atom)
6	Me	Mean atomic Sanderson electronegativity (scaled on C atom)
7	nAT	Number of atoms
8	nSK	Number of nonhydrogen atoms
9	nBT	Number of bonds
10	nBO	Number of nonhydrogen bonds
11	nBM	Number of multiple bonds
12	ARR	Aromatic ratio
13	nCIC	Number of rings
14	RBN	Number of rotatable bonds
15	RBF	Rotatable bond fraction
16	nDB	Number of double bonds
17	nAB	Number of aromatic bonds
18	nC	Number of carbon atoms
19	nN	Number of nitrogen atoms
20	nO	Number of oxygen atoms
21	nX	Number of halogens
22	nBnz	Number of benzene-like rings
23	nCar	Number of aromatic carbon atoms (sp^2)
24	n_amid	Number of amides
25	nROH	Number of aliphatic hydroxyls
26	nArOH	Number of aromatic hydroxyls
27	nHDon	Number of donor atoms for hydrogen bonds (N and O)
28	nHAcc	Number of acceptor atoms for hydrogen bonds (N, O, and F)
29	Ui	Unsaturation index
30	Hy	Hydrophilic factor
31	AMR	Ghose–Crippen molar refractivity
32	TPSA(NO)	Topological polar surface area using N and O
33	TPSA(Tot)	Topological polar surface area using N, O, S, and P
34	ALOGP	Ghose–Crippen octanol–water partition coefficient
35	LAI	Lipinski Alert Index (drug-like index)

the study by Haggarty *et al.*[19] The concept of DOS (further expanded below) has been suggested by some proponents as a way of traversing both combinatorial chemistry and natural products chemical space, while opponents have declared that to be an example of the "belief that serendipity will (by a numbers game) produce diversity and rescue what the lack of intellectual input failed to produce."[26,27]

3.03.2.2 Comparing Combinatorial Chemistry and Natural Products Chemical Spaces, Library Design, and Exploration

It appears to be a coherent view that there are significant differences between natural products biosynthesized by living organisms and compounds resulting from combinatorial and medicinal chemistry synthesis performed in laboratories by man. Natural products occupy a different and larger space than that normally dealt with in, for example, medicinal chemistry,[4,18,24,28–31] which is also demonstrated in **Figure 1**.

Some of the properties that are responsible for these differences include the following. Natural products typically have a greater number of chiral centers[32] and increased molecular complexity as compared to synthetic drugs and combinatorial libraries.[33] Furthermore, they often contain fewer nitrogen, halogen, and sulfur atoms, but are noticeably rich in carbon[32] and oxygen.[33,34] Natural products also differ by having a higher

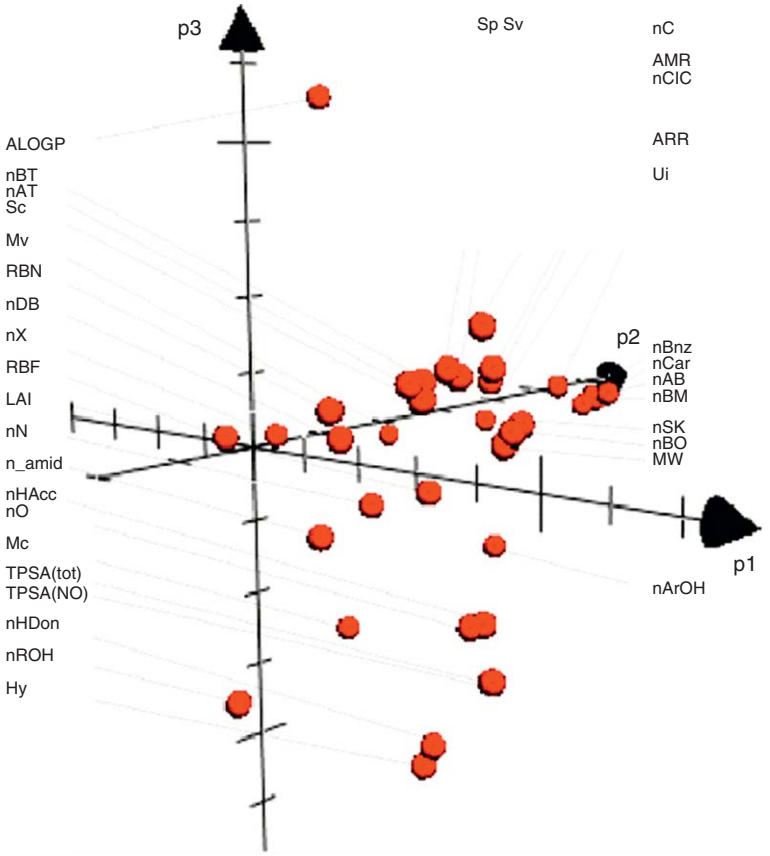


Figure 2 ChemGPS-NP loadings, indicating the contribution of the 34 + 1 descriptors used to characterize the ChemGPS-NP chemical space map.[24] Descriptor abbreviations are listed and explained in **Table 1**. Reproduced from J. Larsson; J. Gottfries; S. Muresan; A. Backlund, *J. Nat. Prod.* **2007**, *70*, 789–794. Copyright by the American Chemical Society, used with permission.

number of hydrogen bond donors and acceptors, by containing a larger number of rings, and by being more structurally rigid. Additionally, they have a broader distribution of molecular mass,[32] octanol–water partition coefficient, and diversity of ring systems compared to synthetic and medicinal chemistry compounds.[32–36]

Such insights have been used to inspire and improve the design of future screening libraries.[32,37–39] These natural compounds are tuned to function in biological systems, evolutionary prevalidated, and naturally bioavailable, which can otherwise be a problem.[23,28,40,41] Also, in the perspective that perhaps less than 1% of bacteria have yet been cultured[42] and that only fractions of the biologically relevant chemical space have been studied, and 'protein–ligand interaction space' is woefully incomplete,[31] the importance of including natural compounds is likely to increase in the future. As Chin *et al.*[43] point out "our imperfect understanding of which areas of chemical space are best suited to interact with biological space is the major bottleneck of drug discovery."

3.03.2.3 DOS – Diversity-Oriented Synthesis

Several comparisons have been made between the design and process of biosynthesis as compared to combinatorial chemistry and DOS, where the latter has been described in evolutionary terminology.

It is true that in nature a large number of unique, daunting, compounds have been produced during the course of evolution. It is important, however, to remember that evolution proceeds but without purpose toward an open end. There is no target or end point for evolution, not even to increase fitness – which is a common

misconception. Instead what happens is that random occurrences such as mutations and genetic rearrangements become fixed in a population due to exerted selection as a result of evolutionary pressure. Hence, from both a philosophical and an evolutionary perspective, nature does not 'identify a small molecule' in an anthropocentrically described combinatorial approach. Instead mutations occur and the available machinery for biosynthesis is ever so slightly modified, and eventually a new compound is synthesized. If this compound, and the precursory modifications of the biosynthetic machinery, contributes to the organism's fitness (or at least does not decrease it), it may become a remaining trait for some time. It is important to appreciate the fact that in this process it is the fitness of the entire organism, as measured by the reproductive success of its second-generation offspring, that is decisive. An amazing new antifungal compound, of tremendous value for a particular plant, will be of no use unless also the combination of the rest of the plant is 'good enough.' Here it is often that a highly reductionist view is taken, and that in comparison with the evolutionary processes it is presumably not uncommon that excellent 'evolutionary leads' may never be explored due to other circumstances.

Accepting the fact that the theoretical chemical space is unfathomly large, it appears even less possible (from a statistical perspective) to by chance produce a biologically active compound by making large random libraries in uncharted space – even if their diversity is great – than to succeed from large random libraries with low diversity in charted space. It is the factor of chance that should be reduced by intelligent selection, broad multidisciplinary understanding, and careful experimental design.

With this understanding, it is possible that the development of biosynthesis and the evolutionary exploration (and expansion) of biologically relevant chemical spaces actually took place more in the way that traditional TOS is designed, rather than DOS – that is, using core structures or introducing minor modifications or additional steps of (bio)synthesis by new species of enzymes. It must, with the present lack of other evidence, be regarded as an extremely uncommon event to suddenly evolve machinery for biosynthesis of a completely novel class of compounds. Development of such machinery takes time, evolutionary time, but once present the machinery may be switched on and off very swiftly. However, the immensely complicated regulatory cascades are difficult to identify and investigate. The key to understanding the chemical diversity found in nature is to understand the timespan (billions of years) and multitude (virtually every living cell) under which evolution proceeds. In this process, natural products are developed, refined, and validated for an optimal function in their context.[10,24,44]

With this said, it does not seem unlikely that DOS may generate chemical libraries, with a diversity more similar to the natural products found in an organism. It also appears likely that these more 'nature-like' libraries can serve as valuable sources for drug discovery and further refinement.[25,45–47] However, the reason why DOS generated libraries and natural products diversity may be at a comparable level is not the same.

Drawbacks of using natural products from natural sources, as suggested in several reviews, include potential problems with purification, isolation, and supply of material, which are all well-known obstacles in the fields of natural products chemistry, pharmacognosy, or chemical ecology. These could all be overcome by focused research, cell cultures, natural resource management etc.

However, the desire to deliberately venture outside (the known) biological space in search of possible nonnatural but natural-looking bioactive compounds[25,46,48] appears in view of the depths of chemical space difficult to defend from a rational perspective. There are, nevertheless, some published examples where small, diverse libraries have succeeded in identifying previously unknown interactions, for example, between targets and compound classes.[25] It is also obvious that there have to be biologically active chemical substances not yet 'discovered' during the course of evolution and that DOS by chance may find.[49,50] In attempting to do so, it is likely that Burke and Schreiber are correct striving to adopt a strong connection to informatics.[48]

3.03.2.4 Structures, Scaffolds, and Volumes

Comparison of chemical compounds to estimate diversity or populate the chemical space can be done in different ways. Here three popular paths are briefly discussed, by comparing structures or structure fragments, scaffolds, and volumes. The first two deal primarily in most applications with two-dimensional (2D) data, while the third also involves three-dimensional (3D) data. The distinction between structures and scaffolds may appear less clear-cut, but will be expanded below.

To a large extent the work on comparing similarity, and more recently estimating chemical diversity, has been pursued by the pharmaceutical industry. Initially, the driving force was the ability to predict[51] or expand[52] results from ongoing studies by comparing chemical similarity of compounds.[53,54] With the access to larger screening libraries, and more complex targets, a need for stringent selection and experimental design required ways to determine if a selection of compounds were diverse enough.[55] Both of these aspects are at present expanded in the ongoing exploration of chemical space and its relation to biological space.[7] In these efforts of charting, suggestions have been made that there are some volumes of chemical space that are 'unpopulated,' that is, that the chemical space is not continuous but rather discrete. This has been indicated by Xu,[56] and as clearly demonstrated by Rosén *et al.*,[57] including volumes with combinations of parameters that are chemically impossible. In the process of completing the ChemGPS-NP global property map, all prediction scores of the first eight dimensions were binned and plotted in intervals of 0,1 unit. These results are shown in **Figure 3** and give a preliminary hint that scores along each of the eight dimensions are more or less continuous (with the exception of dimension PC2, due to the influence of the aromaticity-related descriptors).

However, this does not verify that all combinations of scores are present, and even if crude 2D plots of all tested compounds (ca. 1.2×10^6) appeared quite homogeneous as demonstrated in **Figure 4**, a careful examination of 3D plots of the core assembly of compounds did reveal sparsely populated volumes of chemical space. These results are discussed further in detail by Rosén *et al.*[57]

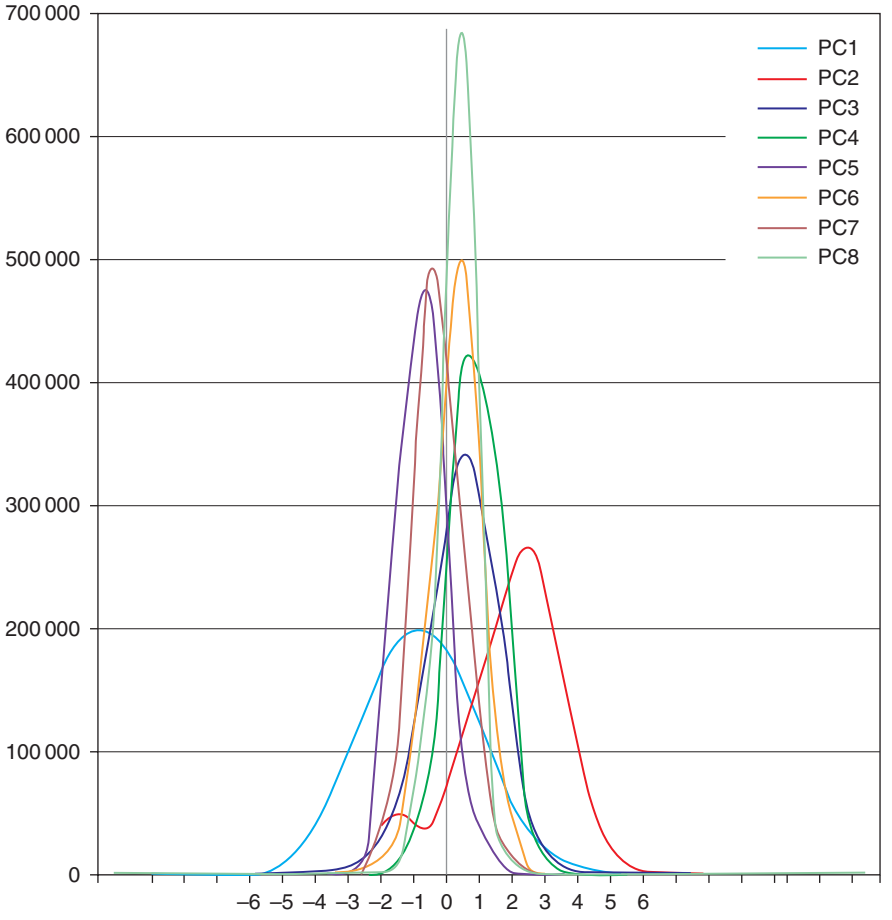


Figure 3 Distribution of prediction scores for more than 10^6 natural compounds predicted using ChemGPS-NP.[24] Only the first eight principal components are shown (PC1–PC8). Anomalous dip and skewness in PC2 is due to the strong influence of aromaticity in this dimension, separating compounds void of aromatic elements from the remainder. Vertical axis indicate number of compounds, horizontal axis shows ChemGPS-NP prediction score distribution.

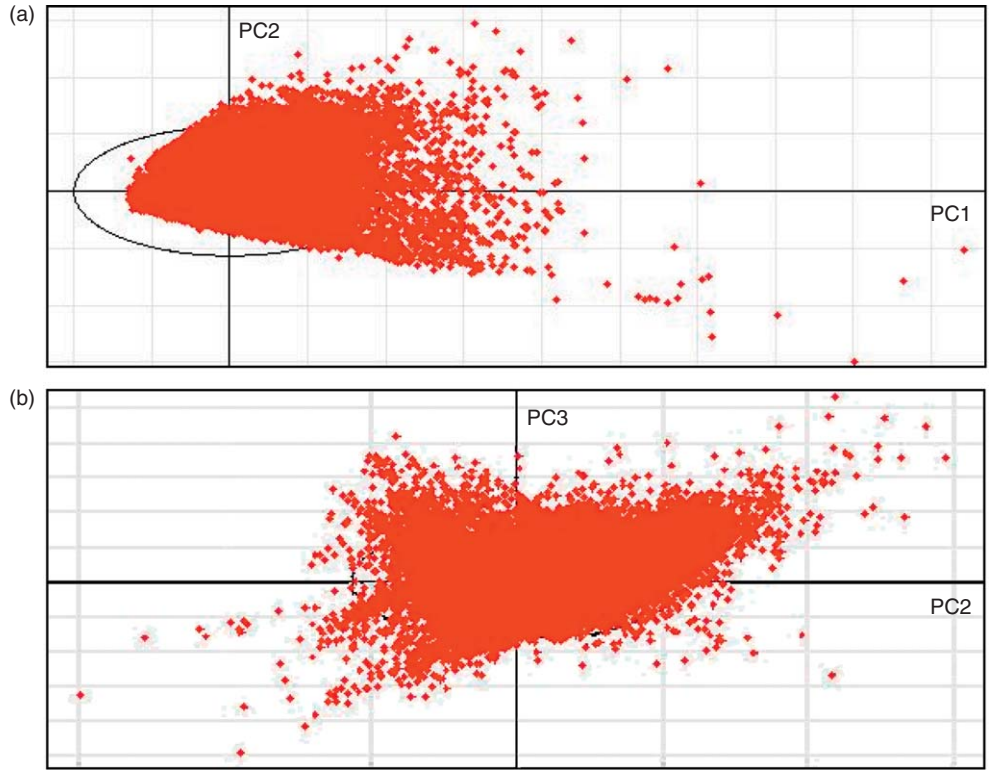
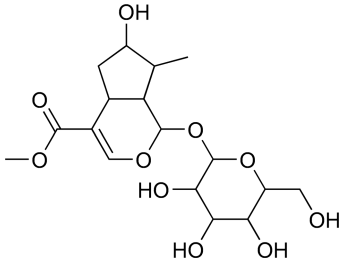


Figure 4 More than 10^6 natural products predicted in ChemGPS-NP, showing distribution along (a) PC1 and PC2 and (b) along PC2 and PC3, and clearly indicating the irregular shape of biologically relevant chemical space when plotted in ChemGPS-NP chemical property space. The main contributors to the first eight dimensions of ChemGPS-NP chemical property space are listed in **Table 2**.

3.03.2.5 Structures

The first methods for comparing similarity of compounds were based on the chemical structure representation, defining substructures or atom pairs.[51,52] Today, typically the entire structures are considered, and the graphical representation of compounds, for example, loganin,



is transformed to a format more suitable for further calculations, such as a text string in SMILES (simplified molecular input line entry system) format (http://www.daylight.com)

```
OC1C(C(OC2C3C(CC(C3C)O)C(C(OC)=O)=CO2)OC(CO)C1O)O
```

or an InChI (IUPAC International Chemical Identifier) key (http://old.iupac.org)

```
InChI=1/C17H26O10/c1-6-9(19)3-7-8(15(23)24-2)5-
25-16(11(6)7)27-17-14(22)13(21)12(20)10(4-18)26-
17/h5-7,9-14,16-22H,3-4H2,1-2H3
```

both of which can be used for continued calculations, searching in databases, and so on. In the development of the ChemGPS-NP global map of natural products chemical space,[24] all compounds were eventually handled, stored, and retrieved as SMILES.

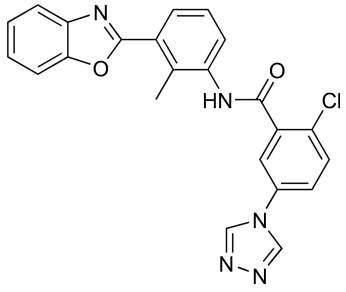
3.03.2.6 Scaffolds

Instead of using the entire structure, incorporating all details on various substituents, and comparing structural descriptors, there is another path focusing on the central core of the structure. This is commonly referred to as a topological scaffold and has been the basis for considerable scientific efforts.[29,56,58] From the perspective of natural products, scaffolds hold a particular interest as it can be assumed that there is a close coupling between scaffolds and biosynthetic pathways.[59] Determination of a structure's topological scaffold is a multistep process, and as outlined in detail by Schuffenhauer *et al.*,[59] the principles are that the structure by removal of substituents and residues is transformed into a simpler, more well-defined scaffold, which can in turn be broken down into subscaffolds. These can be used for applications such as comparison of diversity,[35,50,56] tracing of 'chemical ancestry',[58,59] or quantifying or charting chemical space.[59–62] The applications of scaffolds are further expanded in Chapter 3.02 by Wetzel and coworkers.

In many ways, structure- and scaffold-based approaches are complementary, providing different types of information. In the study by Schuffenhauer *et al.*,[59] a scaffold-based approach is used to evaluate bioassay data from a pyruvate kinase assay[63] deposited at PubChem (http://pubchem.ncbi.nlm.nih.gov) with 602 active and 50 027 inactive compounds. From this study, a set of 11 active compounds, with 2-phenyl-benzooxazole scaffolds, were identified as the most active group.

Accessing the same data set, including also 812 compounds that provided inconclusive results, it turned out that 587 of the active, 793 of the inconclusive, and 48 174 of the inactive compounds had SMILES representations for which prediction scores could be immediately calculated using the ChemGPS-NP_{Web}-server. Plotting of these data, and highlighting the 11 most active compounds identified by Schuffenhauer *et al.*, provides us with the representations in **Figure 5**.

What these results tell us is that the physical–chemical properties of active, inactive, and inconclusive compounds tested in this bioassay are largely overlapping – at least in the first three dimensions of ChemGPS-NP chemical property space. The 11 most active compounds identified by Schuffenhauer *et al.* all fall well within these parameters, with the exception of their compound 11[59] in **Figure 2**.



Compound 11

This compound has a much higher prediction score in dimension PC1, primarily depending on size parameters, as compared to the others, a result that is immediately comprehendible when comparing to the other structures. In addition to the aberrant physical–chemical properties of this compound, it is also one of the least active compounds in the privileged group identified. A more detailed chemographic interpretation of this data set is presented by Rosén *et al.*[64]

A scaffold-oriented study has been pursued on large, commonly used, data sets featuring a website (http://topology.health.unm.edu) from which scaffold topology data and other relevant information can be accessed.[61]

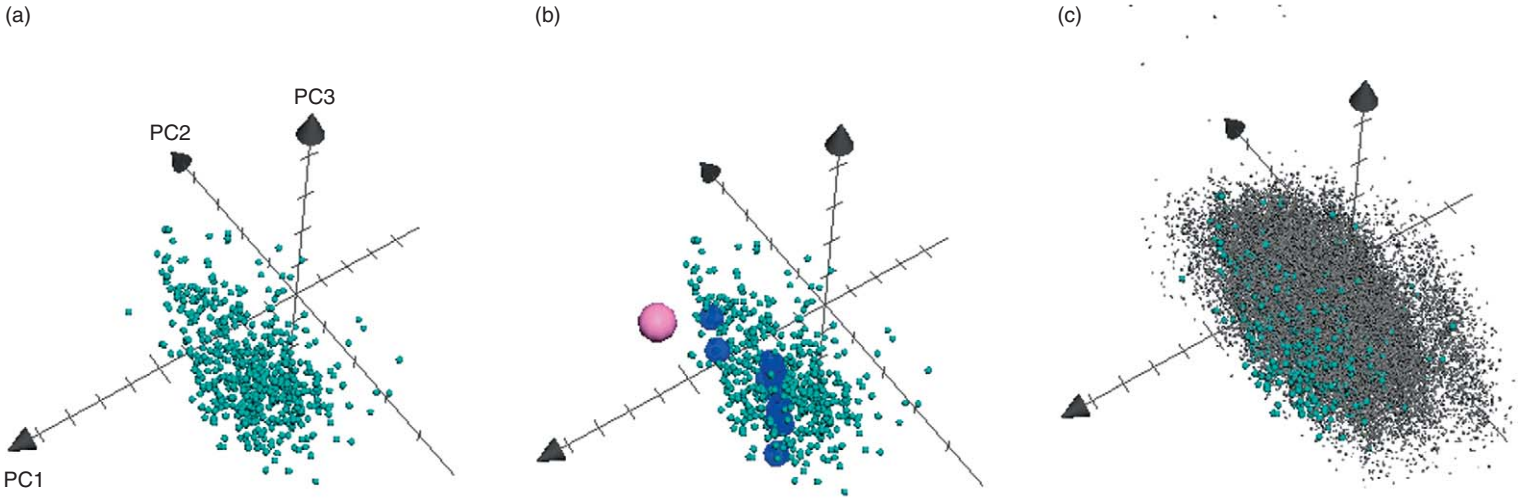


Figure 5 Structures as SMILES files downloaded from NCBI, ChemGPS-NP prediction scores calculated using the online tool ChemGPS-NP$_{Web}$ (http://chemgps.bmc.uu.se)[64] with a total processor time of 16.2 s, and results plotted with Apple system software Grapher 2.0. (a) Only confirmed active compounds (587, green), (b) active compounds and with highlighting of 2-phenyl-benzooxazoles identified as a privileged group by Schuffenhauer *et al.*[59] in their **Figure 2**, p 54 (11, blue except for one physical–chemical aberrant compound of the privileged group shown in pink), and (c) all compounds tested including nonactive (48 174, gray). The main influence in PC1 is size, in PC2 aromaticity, and in PC3 lipophilicity.[24]

3.03.2.7 Volumes

In addition to the structure- and scaffold-based approaches, 3D information can be used for comparison, similarity searches, and other means of chemical space exploration. This line of work has perhaps advanced furthest in the field of protein and enzyme structure studies and in the attempts made to investigate binding pockets, active surfaces, and their interactions with ligands.[65] As only descriptors in zero-dimensional (0D), one-dimensional (1D), and 2D (and to some extent 3D) can be calculated from SMILES, 3D, four-dimensional (4D), and other types of information require the use of other formats and descriptors. For a discussion on descriptors and 'descriptor collision', see the paper by Bologa *et al.*[66] and references therein. Although 3D descriptors may seem intuitively appealing, several studies have been published indicating that 1D, and in particular 2D, descriptors perform as well as or even better than 3D descriptors;[67–71] in one study, as much as 88% correct target prediction for 2D descriptors compared to 67% for 3D descriptors was found.[72] This was achieved even though 2D descriptors have been indicative of only a limited volume close to the molecule under study.[73,74]

The successful history of 1D and 2D descriptors has been suggested to be because they provide an appropriate level of resolution to the information at hand, avoiding overparameterization increasing the risk of character/descriptor dependencies. Also practical problems such as increasing requirements for data storage and computer processing time limiting the scope of experiments that can be performed may contribute.[17] Furthermore, for many natural products, the absolute configurations have not been determined, and in these cases there might even be that noise is introduced in the data.

VolSurf (www.moldiscovery.com) is a software that performs a transition of data from 3D energy grid maps to 2D descriptors for further analysis. With regard to the previously mentioned ChemGPS-NP global map of biologically relevant chemical space, the 1779 satellite and core compounds have also been exposed to calculation of VolSurf descriptors. When compared with the 2D Dragon descriptors used in the charting of the map, similar molecular property dimensions with regard to the most important character contributions were retrieved.[24] This in turn validated that a robust map has been established, providing similar interpretations regardless of which of the two sets of descriptors were used.

3.03.2.8 The Case of Lipophilicity and Natural Products

Some special attention needs to be given to the case of lipophilicity in natural products as compared to medicinal and combinatorial chemistry. The reasons, as will be demonstrated, are the different approaches and the implications this will have for charting chemical space. As pointed out above, natural products are generally larger but at the same time less lipophilic than compounds synthesized by man.[33,75] Several ideas have been put forward that may explain this from the water-based nature of biosynthesis systems to working methods in medicinal chemistry. In their classic paper Lipinski *et al.*[8] point out that one of the most reliable methods to improve *in vitro* activity (what is usually tested at early stages of drug discovery) is to 'incorporate properly positioned lipophilic groups.' In this way, interactions with the target-binding pocket are strengthened, while interactions with the water-based solvent systems, which are generally regarded as more complex instead, are neglected. This, despite the knowledge that a higher lipophilicity can make it chemically highly challenging to convert initial *in vitro* hits into leads with suitable properties for further development.[33,76,77] According to Lipinski,[78] this is also reflected in the difficulties faced by compound selling companies in controlling lipophilicity parameters.

Not surprisingly in the medicinal chemistry-oriented ChemGPS, the lipophilicity-related parameters are very influential and form the major contribution to the second dimension, PC2.[18] In the natural products-based ChemGPS-NP, on the other hand, these lipophilicity-related properties are not described until in the third dimension, PC3. Instead aromaticity- and conjugation-related properties are found in PC2.[24] As pointed out, natural compounds are bound to function in a generally hydrophilic environment. In order to retain supposed defense substances in solution, highly lipophilic compounds must be avoided and, hence, the variation in lipophilicity is evolutionarily reduced by a functional constraint. Features like this are among those details that some authors[45,79] suggest as problems with *in silico* approaches as compared to biophysical experiments.

3.03.2.9 Small Molecules, Peptides, and Enzymes

In most studies on the exploration of chemical space, only small molecules are regarded. This subset has been referred to as 'chemical space of small molecules' or CSSMs.[60] Strictly speaking, however, more complex molecules such as polypeptides and enzymes should also be included in the concept of chemical space.[80] This poses important questions on data handling and descriptor selection, as there are inherent differences between these groups. While the population of CSSMs are biosynthesized from biosynthetic enzymes, usually from a limited set of building blocks and in a chiral-specific manner, the polypeptides are in most, and enzymes in all, cases ribosomal products encoded by genes. This brings them closer to the evolutionary forces, as these processes act directly on the (genes coding for the) molecules and their immediate expression.[81,82] In the case of compounds in CSSM, the evolutionary forces act on the biosynthetic machinery, which may still, after substantial modifications, be able to perform the same synthesis resulting in a product indistinguishable from that of an unmodified enzyme. These differences have been explored by Larsson *et al.*[83] in a thesis on the toxic polypeptides of mistletoes. Between these two groups we can find polyketides and nonribosomal polypeptides, synthesized by large megaenzymes known as polyketide synthases and nonribosomal peptide synthetases, respectively.

With respect to properties studied, many descriptors have been developed for members of CSSM rather than enzymes. Features such as protein binding or affinity data could be regarded as spanning the gap over to tertiary structures, active site properties, and interaction surfaces.[76,84–86] This has been suggested by some authors as forming a 'binding-site chemical space,' a complementary view of CSSM.[4,7,87] A trend in more recent approaches has been to attempt spanning this width of both chemical compounds and their studied properties within the same study.[88] A cornerstone in these attempts are the advances in computational techniques as well as hardware and can in some ways be regarded as scientifically related to the concept of systems biology.

3.03.2.10 The Biological Relevance

What may then be the biological relevance of these chemical substances and their traits? In nature, virtually all (if not all) processes noticeable within and between organisms and their environment are fundamentally chemical reactions. These reactions are to an extensive degree mediated via elaborate enzymes, complex proteins, and various high- and low-molecular weight compounds, all of which are themselves synthesized 'on purpose.' Even if we at present understand only a minute fraction of these interactions, we can be confident about the fact that they have been continuously evaluated and validated by evolutionary processes. They are all there for a reason – obvious or not.

Therefore, as Vuorela *et al.*[89] put it "the interfacing of biological and chemical assessment becomes the critical issue."[89] A central dogma of medicinal chemistry and chemical biology is that compounds with similar structures have similar activities. Although there are also numerous examples to the contrary,[65,73] this appears in most cases to be true, and it is reassuring when different methods of exploration such as ethnopharmacology, database exploitation, and molecular modeling converge on a common, suggested, lead compound.[90]

3.03.3 Biological Space

3.03.3.1 The Concept of Biological Space

While there is a general agreement on the concept of chemical space, opinions are much more diverse when it comes to biological space. While some authors see biological space as a subset of chemical space including the chemistry that is related to life, others envisage a much broader view and even in some cases promote the opposite view – that chemical space is a subset of the biological. Either way, it is obvious that there is a tight link between at least parts of these two entities. If the biological space is approximated with the human genome, a highly reductionist view, its size appears quite manageable with only ca. 30 000 genes.[20,91,92] Compared to the ca. 10^{60} small carbon-based chemical substances possible to devise, this is only a minute fraction, which is presumably the reason why biological space is sometimes regarded as 'small.' However, these numbers become more overwhelming when considering possible different interactions and effects of the 10^{60} substances on not only the 30 000 human genes but also the genomes of the other millions of species found on Earth.

3.03.3.2 Exploring and Navigating Biological Space

As is the case also for the chemical space, exploration of the biological space can be done at different scales. Detailed knowledge on enzymes, structures, binding affinities, and functions,[93,94] the patterns of change in proteins during evolution,[82,95] or the overall patterns of change, speciation, and extinction that are the combined results of evolutionary forces,[42,96,97] all form small contributions to an understanding.

In this explorative process, the concept of biological diversity has become central, and in measuring and quantifying this elusive property, the advances in phylogenetic reconstruction has become instrumental. Analogous to the way chemical diversity is often defined, that is by the variation in the number of given parameters, biological diversity can also be roughly estimated. However, while chemical space is (at least in theory) populated in an unordered fashion where there is no philosophical necessity that one molecule is formed before another, this is not the case for biological space. The latter is a result of evolutionary processes, believed to have one common history on Earth, which form a generally bifurcating pattern as speciation proceeds – with countless reticulations as a result of hybridization or lateral gene transfer. There is an inherent pattern in biological space, which can be used in structuring and efficient exploration.

With the initial premise set on the first page of this chapter, that there are connections between evolution and chemistry, it can be inferred that a larger evolutionary or biological diversity should also indicate a potentially larger chemical diversity. This is one reason why the marine environment has recently attracted the attention of natural product chemists in search of novel chemical entities.[9,98,99]

3.03.3.3 Evolution

Biological space has been interpreted in different ways. Some authors use the concept of biological space in a somewhat narrow sense of 'the chemicals found in nature' while others consider not only the ligands but also the receptors. From a biologist's perspective, it can be argued that the biological space is all this and much more, including what we call the evolutionary space.

Evolutionary space can be defined as the multidimensional volume in which evolution proceeds. It is thus defined by all the parameters relevant to evolution, such as nucleotide substitutions, morphological transition series, and development of biosynthesis pathways. Analogous with the chemical space, these realms can also be navigated but by formulating evolutionary hypotheses.

3.03.3.4 Phylogenies, Phylogenetic Hypotheses, and Their Estimation

A phylogenetic hypothesis, in short a phylogeny, is an implicit hypothesis of evolutionary relationships. Such hypotheses can be erected based on intuition, as that in **Figure 6** by Haeckel,[100] but are in a modern systematic or evolutionary biology context a result of careful analysis of scientific data.

Different methods based on different philosophical underpinnings are utilized in this process, which have been further elaborated by Farris.[101] The four most commonly used methods are briefly described below, and in **Table 3** a compilation of software for phylogenetic purposes is given.

Since 1866, the results of phylogenetic analyses have often been explained as a tree diagram, a form highly intuitive to human concepts. From a philosophical standpoint, there is only one evolutionary history, and hence evolution ought to be represented as one single, bifurcating, tree diagram showing the evolution and succession of all species. To further complicate the situation, it is today widely accepted that only a bifurcating tree is not adequate for this purpose considering the well-known and studied processes of e.g. hybridization and lateral gene transfer.The crux is to figure out which of the different possible trees are 'correct,' that is, in the most exact way represent the result of the evolutionary processes. This is not a trivial problem, considering that for $\mathbf{T}$ number of organisms (taxa) the number of possible bifurcating trees $\mathbf{B}^{\mathbf{T}}$ is

$$\mathbf{B}^{\mathbf{T}} = \Pi(2i-5), \text{ for } i = 3 \text{ to } \mathbf{T}$$

This rapidly approaches very large numbers, making an exhaustive investigation of every tree impossible already from ca. 20 taxa – not to speak of the millions of species already known.

The preference of tree diagrams by human mind has been discussed by Hestmark[102] in an enlightening essay.

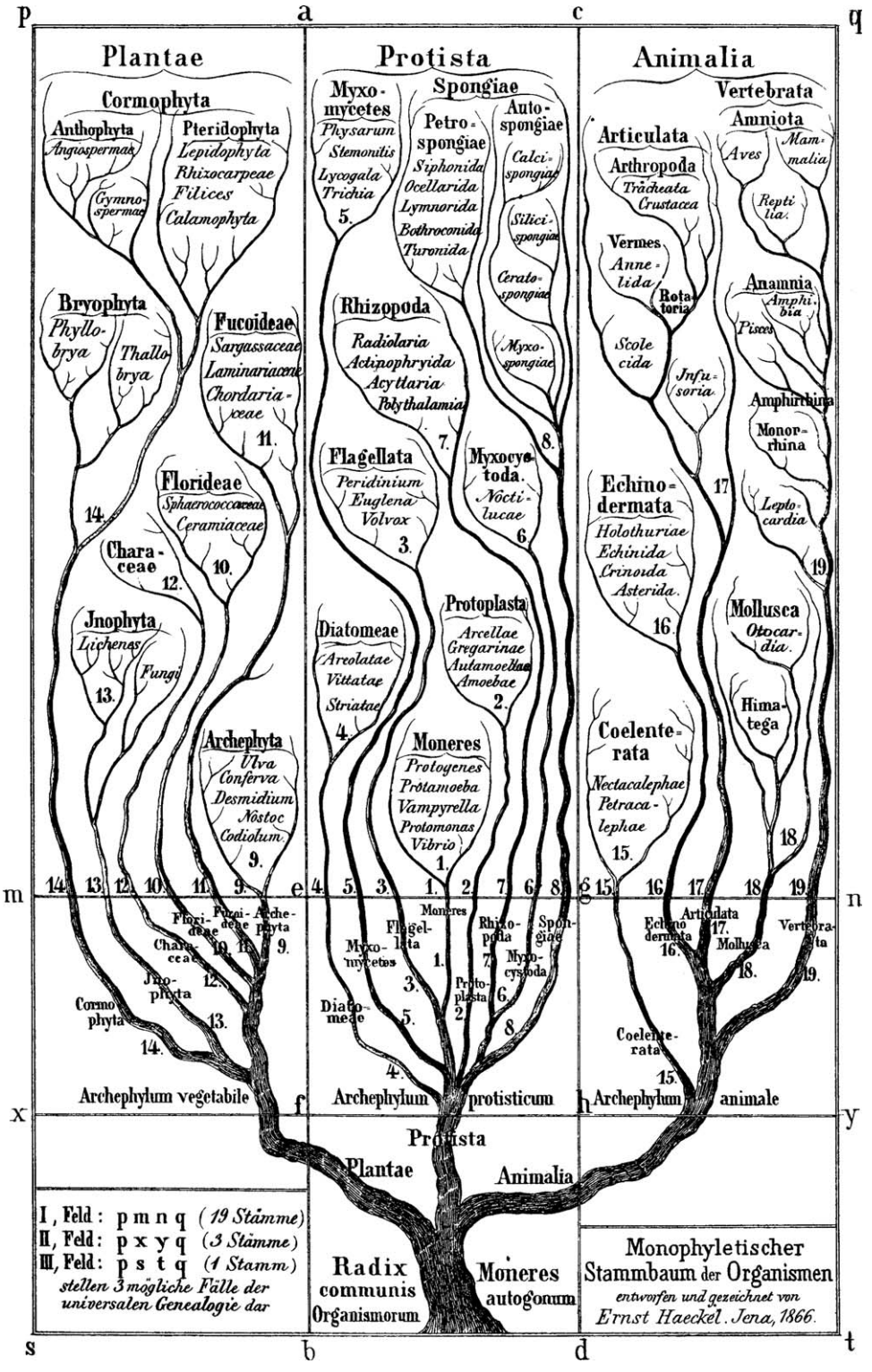


Figure 6 One of the first published phylogenetic tree by Ernst Haeckel[100] in 1866.

Table 3 An overview of popular software for phylogenetic analyses, their homepages, and the methods of analysis implemented

Software	*Homepage*	*Methods*
PAUP	http://paup.csit.fsu.edu	MP, ML, NJ
PHYLIP	http://evolution.genetics.washington.edu/phylip.html	ML, MP, NJ
MrBAYES	http://mrbayes.csit.fsu.edu	BI
SPLITSTREE	http://www.splitstree.org	BN
MEGA	http://www.megasoftware.net/m_con_select.html	NJ, MP
TNT	http://www.zmuc.dk/public/Phylogeny/TNT	MP

A very large number of software for phylogenetic reconstruction, data management, etc. are available from http://evolution.genetics.washington.edu/phylip/software.html# methods.
NJ = neighbor joining, ML = maximum likelihood, BI = Bayesian inference, MP = maximum parsimony. Note that SPLITSTREE uses Buneman networks (BN), which is a method not described here, but which allows for reticulate patterns of evolution and not exclusively bifurcating.

3.03.3.5 Neighbor Joining

Neighbor joining, as a technique for phylogenetic reconstruction, was developed by the geneticists and evolutionary biologists Kimura and Nei based on the concept of the 'theory of neutral evolution.' From this concept, it could be concluded that the vast majority of observed mutations in nucleotide sequences would be functionally neutral and thus not afflict the organism's fitness. Being not sensitive to evolutionary pressure, these neutral mutations should accumulate in a clock-like fashion as a result of chemical equilibrium.[103,104] The method as such joins the two sequences under study with least differences, the nearest neighbors, and then continues through the sample data by adding the next closest sequence. The question answered by a neighbor-joining analysis is: what is the relative similarity of my taxa?

There are several methodological drawbacks with this method, as reviewed by Farris *et al.*[105] Among the drawbacks is the lack of a clear optimality criterion although some implementations attempt a 'minimal evolution' approximation. In addition, the requirement of a more-or-less clock-like mutation rate (which has been convincingly shown not to be a ubiquitous feature[106,107]) and the fact that only a small part of the available data is used after transformation to a distance matrix are some drawbacks. On the other hand, there are situations when the neighbor-joining analysis would be the tool of choice. These include cases when data cannot be regarded as hierarchical (a prerequisite for the other methods), for example, analyses within a species, and with very large data sets when the computationally more complex methods will not be able to complete within a reasonable time.

3.03.3.6 Maximum Likelihood Analysis

Built on very different philosophical underpinnings, the maximum likelihood concept was developed by Fisher, a statistician, during the first decades of the twentieth century, as described by Aldrich and coworkers.[108] The implementation of maximum likelihood analysis for phylogenetic reconstruction was primarily done by Felsenstein.[109,110] The question answered by a maximum likelihood analysis is: what is the probability of getting my set of data (under the given model) if this tree is true?

Maximum likelihood is originally a statistical method, and using this approach for phylogenetic reconstruction implies the use of an evolutionary model. Whether this is a drawback or an advantage to the analysis is a matter of debate; however, concerns have been made over using an evolutionary model to trace evolution. In contrast to the neighbor-joining method described above, the maximum likelihood has a very clear optimality criterion – maximum likelihood – but also requires a considerably larger computational effort. A very large portion of all possible evolutionary trees have to be investigated and tested in order to find the one with the maximum likelihood.

3.03.3.7 Bayesian Inference

Bayesian inference was first introduced in phylogenetic reconstruction by Rannala and Yang[111] and later expanded by Huelsenbeck *et al.*[112] Bayesian inference and maximum likelihood analysis are somewhat similar

in nature, both applying a statistical perspective. Bayesian inference answers the question: what is the posterior probability that this tree is true under this model?

The Bayesian inference method utilizes an evolutionary model, as in the case of maximum likelihood analysis, and in a similar way it is viewed as both a drawback and an advantage. One of the major drawbacks of this is that there are few evolutionary models defined for other types of data than nucleotide and amino-acid substitutions. Attempts at analyzing information such as patterns of biosynthesis pathways or ecological/behavioral features may not be feasible. An advantage in the case of sequence data can instead be a higher sensitivity to the model, which has been employed in methodological studies of evolutionary methods. As with maximum likelihood, there is an unintuitive branch length measure – in maximum likelihood 'state change probabilities' and in Bayesian inference 'posterior probabilities.'

3.03.3.8 Parsimony Analysis

The fourth method briefly discussed in this chapter is parsimony analysis, tracing its philosophical underpinnings to the famous quotation by William of Ockham (Occam) (ca. 1285–1349), known as Occam's razor.

> "Pluralitas non est ponenda sine neccesitate"

This freely translated means 'plurality should not be posited without necessity' or 'what is explained by few is explained in vain by more.' As is true for both maximum likelihood analysis and Bayesian inference, maximum parsimony can also be seen as a conclusion of Bayes'[113] theorem of conditional probability. The main difference in interpretation lies in maximum parsimony's foci on logics rather than statistics. Maximum parsimony analysis answers the question: what is the simplest (most parsimonious) explanation to my data? Hence, the maximum parsimony analysis itself is void of evolutionary models. In a maximum parsimony analysis, the data at hand are studied in search of character state changes, for example, mutations in DNA, differences in the numbers of stamens, or presence of a particular chemical compound.

The ideas behind parsimony analysis were first developed by the German entomologist Hennig.[114,115] In the 1960s, an American botanist 'Herb' Wagner developed an algorithm for parsimony analysis, which was further implemented by Kluge and Farris.[116–118] Similar to the other three methods discussed above, maximum parsimony also has its merits and demerits. Among the merits are the lack of need for an evolutionary model, the intuitive and Euclidean branch length measure – number of character state changes – and the immediate correlation between the branch lengths and the data at hand. From the results of a maximum parsimony analysis, the initial data can be reconstructed. This is not the case for any of the other three methods. Among the drawbacks are excessive computational time (although not as long as that for maximum likelihood analysis) and a sensitivity to pronounced bias in branch lengths.

3.03.3.9 Consequences of Evolution

An immediate consequence of the relentless activities of the evolutionary forces is that the biological space, in its widest meaning, is at a constant state of change – in which our species *Homo sapiens* is but a passing flicker. Speciation is taking place as we speak, but in a pace that is difficult for our mind to grasp. At the breakup of the supercontinent Gondwanaland ~130 Mya, most of major groups of plants we know today were already developed.[119] In this process, different biological systems develop and disappear, receptors are formed and changed, and the ligands and the machinery necessary for their biosynthesis coevolve in continuous interaction. Due to the way mutational changes take place in the genome and how the genetic information is stored and processed, it is inherently more probable to lose a gene, system, or function than to gain one.[120] Hence, retaining a system also requires feedback from the evolutionary processes in terms of increased fitness. While mutational changes occur by chance, there is no element of chance in the long-term retaining and fine-tuning of a system. Evolution does not give place for unnecessary and unimportant features. Everything in nature is there for a purpose – even if that purpose may be difficult for us to unearth.

In an evolutionary short timespan of only a few million years, it has been shown that the angiosperm *Epifagus virginiana* (beechdrops) can with very high selectivity delete more than half of its chloroplast genome in the

process of turning parasitic.[121] The close relative *Nicotiana tabacum* (tobacco) features a chloroplast genome of 155 844 bp and 84 genes,[122] while the chloroplast in *E. virginiana* consists of a mere 70 028 bp and 42 genes.

One such example can be seen in the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, or rubisco, in which the history, development, and fine-tuning can be followed from possible Archaean enolizing enzymes via large subunit dimers in photosynthesizing purple and alpha bacteria to the 'modern' hexadecamer with eight large and eight small subunits.[82] From this study, as well as previous work, features such as positive selection,[81] importance of the genetic code redundancy, and rate changing separation of gene operons can be illuminated.

The rubisco and its two genes *rbcL* and *rbcS* have also become important in the breakthrough of molecular systematics of photosynthesizing organisms.[123] For the studies of angiosperms, these tools combined with the rigors of phylogenetic analysis paved the way for a general understanding of their evolution, one of the first major groups of living organisms on Earth that became the foci of an international research group.[96,97] An outline of the results from this endeavor can be seen in **Figure 7**.

As pointed out already in Section 3.03.1, the correlation between organisms and their chemistry is a result of evolution.[1] Being two sides of a coin, each will tell us something about the other. Presence of a unique compound would be an argument for a common ancestry (or a result of sharing a common endogenous parasite or symbiont).[124] In the same way close evolutionary kinship would increase the chance of encountering similar chemistry.[125] Consequently, there are also similarities between different groups of organisms, sometimes to a surprising degree. It has been suggested to use plants as models for pathogenesis of bacterial infections, as some of the systems for innate immunity are similar enough to those in humans to yield an interpretable result.[126]

3.03.3.10 Biosynthesis as a Concept

While biosynthesis and different pathways and their elucidation are described in more detail in other chapters of this series, it is important to consider some general properties in this context. Biosynthesis is performed by the actions of various enzymes producing a more or less well-defined product from one or many precursors. Some of the more specialized enzymes such as polyketide synthases have a modular design, allowing them to combine different subunits to produce a number of products in a flexible and easily controlled fashion. This modular theme, however, is also found on a smaller scale in other enzymes, where different types of binding domains can be identified.

The tools for biosynthesis are not only modular, flexible, and adjustable, but they are also at a normal state strongly regulated and perform their tasks following an often complex array of control and feedback loops. It has been suggested by some proponents that biosynthesis, and in particular that resulting in the so-called secondary metabolites, is changing and acting haphazardly. The types of arguments put forward are often based on the type of data presented by Fleming *et al.*,[127] where 400 000 microbial cultures were screened for antibiotics. An observed low frequency of activity was interpreted as a result of 'unfocused' secondary metabolism. These ideas have been challenged more recently where it is concluded that the low activity observed is not a result of absence of active compounds, but due to inappropriate assays applied that fail to detect the activity present.[128–131] This was eloquently phrased by Kingston and Newman[11]:

> "Natural products or secondary metabolites, whether from the microbial, plant or marine worlds, represent the results of evolutionary pressures to preserve and enhance the life of their producing organism. They have evolved into structurally and usually stereo chemically complex compounds with specific bioactivities."

When attempting to step out of our anthropocentric view, this appears quite logical. Instead Dobson[4] concludes that one of the greatest challenges for the future of biosynthesis research is to understand how these systems could be influenced to perform in ways better suited for our needs, for example, production of better drugs or alimentaries.

3.03.3.11 Natural Products as Drugs

The value of natural products as drugs or in the development of drugs is obvious from crude statistics, and according to Butler[132] half of the currently used drugs are natural products related by origin, synthesis, or inspiration. Of those approved during 1981–2006, as reviewed previously in a series of papers,[36] an increasing proportion of newly approved drugs are natural products. Scrutiny of defined medical indications shows that as

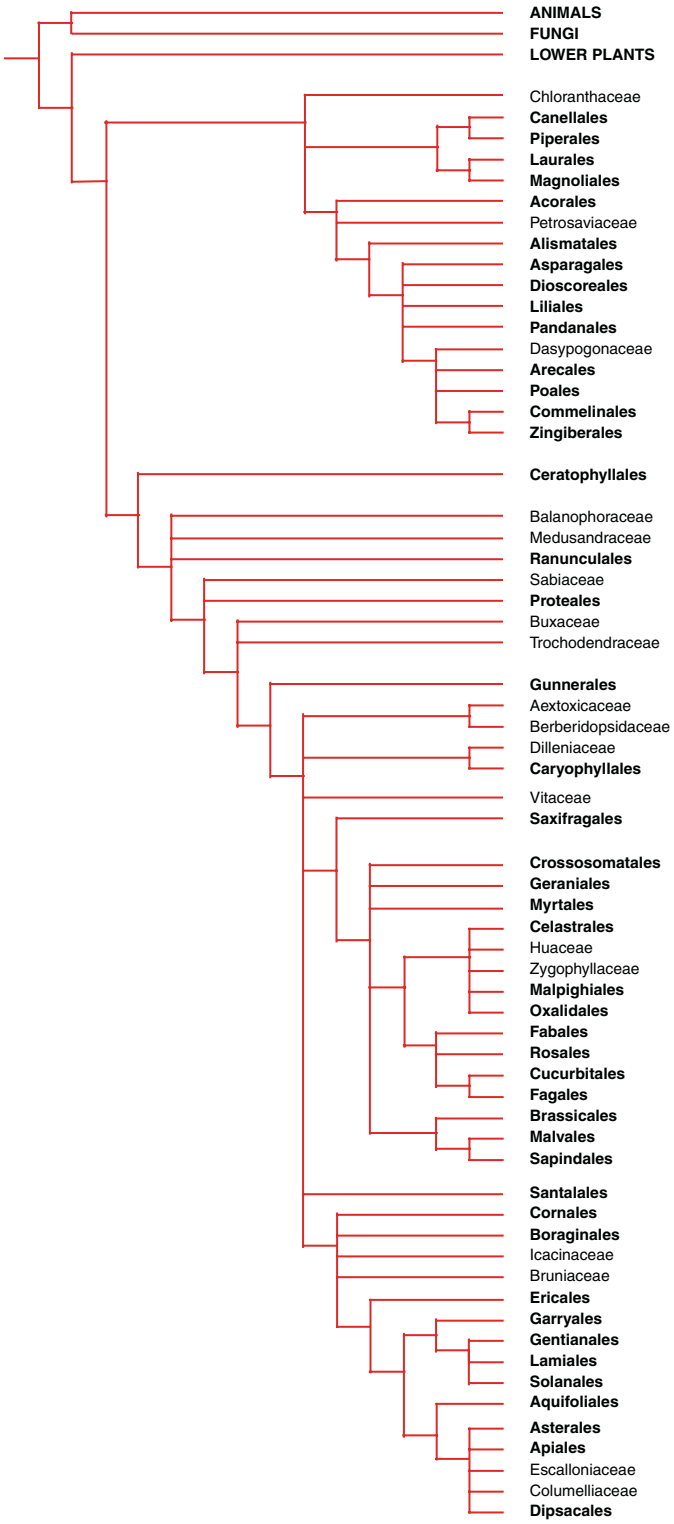


Figure 7 Proposed phylogeny of angiosperms at an ordinal level, redrawn according to APG,[97] with additional information from Larsson *et al.*[83] The hypothesis involves several separate analyses.

much as 87% of categorized human diseases were treated by natural products-based drugs.[37] In the few categories of diseases lacking newly registered drugs based on natural products, there are several well established examples already in use.

Many of these valuable natural products come from plants, which have a strong traditional standing in the field.[133] However, during the last half of the twentieth century, an increasing number of exciting natural products have been identified from marine sources. Furthermore, there is growing interest in the emerging field of microscopic fungi and bacteria, both as direct providers of compounds in biotechnological applications and as recently discovered endophytes of vascular plants and marine invertebrates.[9,36,37,43,132–135]

At the U.S. National Cancer Institute (NCI), an ambitious screening project investigated 35 000 samples already during the period 1960–82 in search of anticancer drugs, resulting in the discovery of the cytotoxic compounds paclitaxel from *Taxus brevifolia* (Taxol®), camptothecin from *Camptotheca acuminata* (Topotecan®, Irinotecan®), and homoharringtonine from *Cephalotaxus harringtonia*.

For the future development of drugs it has been suggested that an estimate of the number of potentially interesting 'druggable' targets is made. Figures from the literature have varied widely ranging from 120,[136] 218,[137] 324,[92] to 14 000 possible targets,[138] but in the latest version of DrugBank (version 2) (http://www.drugbank.ca), 1565 identified 'nonredundant' targets are presented.[139] In the light of the enormous size of chemical space, this view of the biological medicinal space appears quite modest. However, as Wishart *et al.*[138] put it "This state of affairs largely reflects the 'two solitudes' [i.e. with respect to research] of chemoinformatics and bioinformatics." It is possible that the large discrepancy may stem from the definition of the key concept 'target.' With more than 30 000 preliminary defined enzymes in the human genome, even so many as 1565 targets appear quite constrained. Instead probably a broad approach is needed, as suggested by Paolini *et al.*[30]

3.03.4 Comparing Chemical and Biological Space

3.03.4.1 Comparing Descriptors!

The fact that the natural, synthetic, and drug-like molecules to some degree represent different parts of the chemical space due to differences in physical–chemical properties is well established by several studies.[28,57,140–142] As much as 40% of the core structures found among natural products are not encountered among synthetic compounds.[140] However, this does not by necessity imply that they would require different types of chemical descriptors, nor that they would behave differently when applying different descriptors.

There is, however, a fundamental difference between the chemical space and the evolutionary space. In the latter, a single and same result is the expected outcome of different approaches to interpret evolution – as there is supposedly one single (albeit in some cases entangled and reticulate) evolutionary history for organisms on Earth. Chemical space, however, is characterized by an array of more or less well-suited molecular descriptors. When selecting a set of descriptors, this will influence the way in which the corresponding chemical space can be demonstrated. Hence, the process of selecting descriptors becomes central in chemical space exploration to a much greater and more direct extent than the selection of a particular method for phylogenetic analysis. The same holds true for the selection of exemplar compounds or training set of compounds, as compared to which organisms are included in a phylogenetic study.

As an example it can be mentioned that on the ChemGPS set of objects,[18] 2D- and 3D-based descriptors provide different maps of chemical space,[143] while this has been demonstrated to not be the case in the natural products-based ChemGPS-NP.[24] The primary principal components of ChemGPS (2D and 3D) and ChemGPS-NP are, as discussed above, not directly corresponding to each other as a result of both the selection of descriptors (72 vs 35) and reference training set of compounds (423 vs 1779).

In the frequently referred example by Feher and Schmidt,[33] coverage of volumes in chemical space for 13 506 'combinatorial compounds,' 3287 'natural compounds,' and 10 968 'drugs' is compared (**Figure 8**). For this study, a set of 10 descriptors (number of chiral centers and rotatable bonds, ratio of aromatic atoms to ring atoms, ring fusion degree, the number of hydrogen bond acceptors and donors, number of C–N, C–O, C–halogen, and C–S bonds) are used, providing an explanatory power of 54% in the first two principal components, with an additional 12% added with the third principal component. In this study, the natural products cover the largest volume, even though constituting only 11% of the investigated compounds.

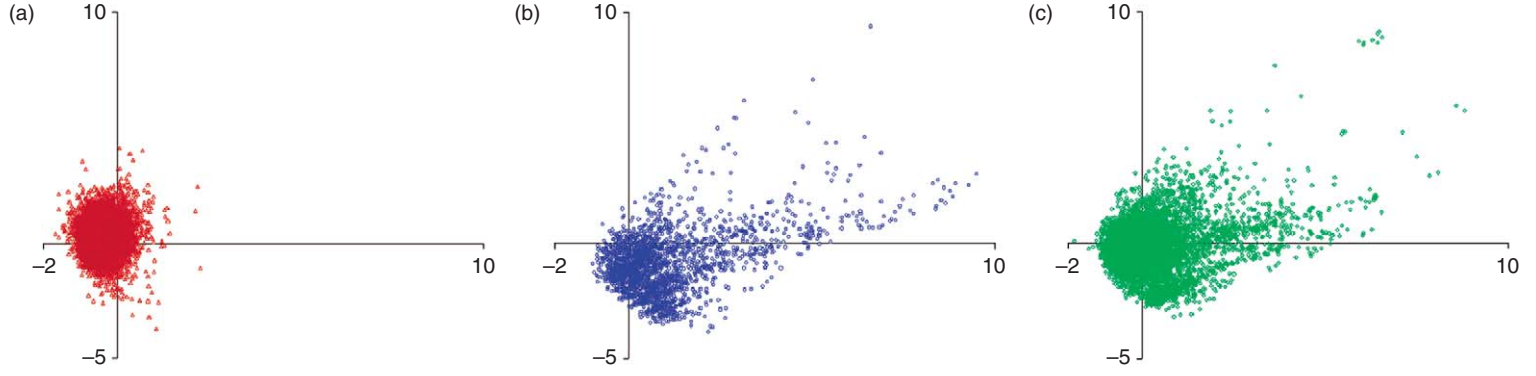


Figure 8 A plot of the first two principal components, obtained from a database containing (a) a random selection of combinatorial compounds ($n = 13\,506$), (b) natural products ($n = 3287$), and (c) drugs ($n = 10\,968$). For clarity, the data points from the three databases are plotted separately but on the same axes. This figure shows that combinatorial compounds cover a well-defined area in the diversity space given by these principal components. In contrast, natural products and drugs cover almost all of this space as well as a much larger additional volume. Drugs and natural products have (in this study) approximately the same coverage of this space. Reproduced from M. Feher; J. M. Schmidt, *J. Chem. Info. Comput. Sci.* **2003**, *43*, 218–227. Copyright by the American Chemical Society, used with permission.

3.03.5 Examples of Studies Pursued

3.03.5.1 The Example of Natural Product COX Inhibitors

Even if many natural products display a wide range of biological activities, they are not honed by evolutionary forces with the purpose of becoming drugs for use in humans. What they may contribute with, however, is an amazing chemical diversity.[10] This becomes very clear in cases such as the natural products cyclooxygenase (COX) inhibitors. The COX enzyme system of the inflammation cascade appears in at least two isoforms, COX-1 and COX-2, of which the latter is induced and involved in the complex of chronic inflammation. Part of the intriguing story, involving the development and use of aspirin and nonsteroidal anti-inflammatory drugs, has been discussed by Rishton,[144] and references therein.

Compiling data on more than 200 published COX-1 and -2 inhibitors of natural origin, their mode of inhibition, and their organism of origin provides us with an intriguing pattern – both chemographic and phylogenetic.

From a chemographic perspective, the known COX inhibitors of natural origin are a highly heterogeneous group. This has been touched upon in previous studies,[23] where it was concluded that ordinary 'medicinal chemistry' models for chemography proved insufficient to handle the chemical diversity displayed. With a more appropriate model, however, patterns are emerging,[24] which are at present under further investigation.

From a phylogenetic perspective, on the other hand, a pattern less diverse springs forward. As shown in **Figure 9**, the organisms of origin can be plotted on a phylogenetic framework – in this case the APG II[97] ordinal classification of angiosperms. From this, it is clear that several large groups of plants appear to have never been investigated with respect to COX inhibition, a knowledge that could be taken into account when designing experiments and sampling strategies. In addition to this, it appears from the patterns of already investigated compounds and the respective activities that the odds of retrieving an active compound from one or another of the classes distinguished in **Figure 9** could be greatly improved by considering the phylogenetic distributions of already known active compounds.

3.03.5.2 Chemosystematics of Cyclopeptide Alkaloids

In this study, the chemosystematic implications of the discovery of anorldianine, a cyclopeptide alkaloid, found in the species *Heisteria nitida* of the family Santalaceae are interpreted and discussed.[124] Anorldianine had previously been reported from only *Canthium arnoldianum* of the family Rubiaceae (misspelled as *Canthium anorldianum* throughout that study, hence giving the alkaloid the name anorldianine). Cyclopeptide alkaloids have been found in several families, but anorldianine has a unique substructure containing proline.[145] No extensive investigations into the physiological role of such cyclopeptides seem to have been done, but there are review reports of antibacterial and antifungal activities,[146] and vignatic acid A has been shown to be lethal to larvae of the weevil *Callosobruchus chinensis*.[147]

The structural type of cyclopeptide that anorldianine belongs to contains 14 atoms in the macrocyclic part and has thus far been found in nine families of higher plants: Olacaceae, Celastraceae, Phyllanthaceae, Pandanaceae, Fabaceae, Rhamnaceae, Urticaceae, Malvaceae, and Rubiaceae of the orders Santalales, Celastrales, Malpighiales, Pandanales, Fabales, Rosales, Malvales, and Gentianales, respectively (cf. **Figure 7** where the corresponding ordinal names are given). This pattern becomes interesting with respect to the systematic placement of Santalales to which the family Olacaceae with *Heisteria* belongs, which had at this stage not yet been possible to deduce. In the past, Santalales had been associated with a variety of plants, of which many today are placed among asterids, for example, the order Apiales,[148] and Icacinaceae which is now placed within the order Aquifoliales.[149]

Plotting the five suggested structural subgroups of 14-carbon cyclopeptides on the proposed ordinal relationships of the core eudicots[97] raises interesting implications. One of the types of cyclopeptides, type 3, has a seemingly restricted distribution, including only the two orders Santalales and Gentianales. This could

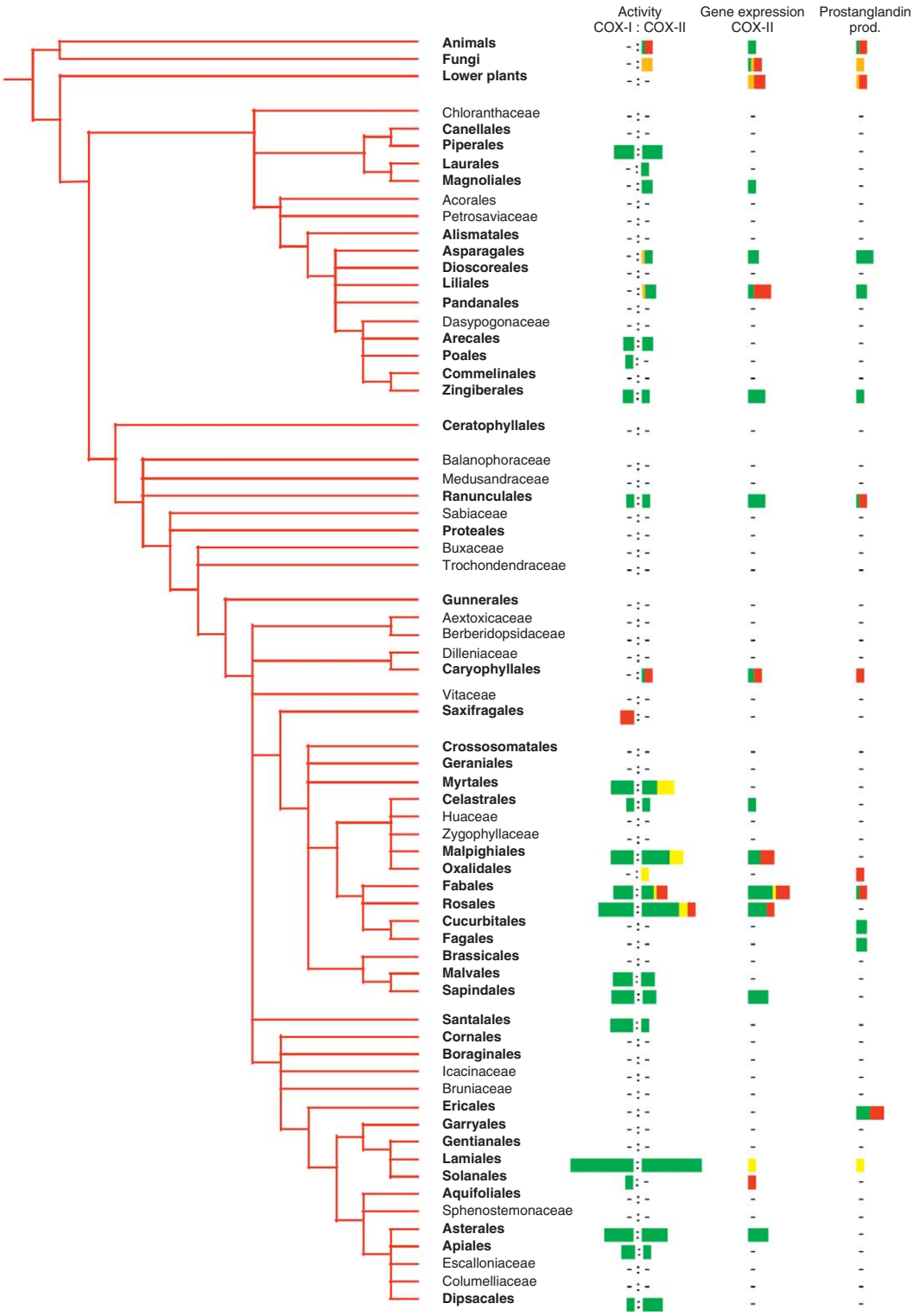


Figure 9 Information on phylogenetic relationships of organisms of origin plotted for more than 200 instances of natural products tested for activity against COX-related assays. From left to right, activity against COX-I and COX-II and against COX gene expression, as measured from change in mRNA levels, and inhibition of prostaglandin synthesis are shown. Information is compiled from several sources, and some are yet to be published. Green color denotes significant (>50%) and yellow less significant (<50%) inhibition of enzymes or prostaglandin synthesis, or decrease in gene expression. Red color denotes activation of enzymes, prostaglandin synthesis, or gene expression.

hence be interpreted as a chemosystematic argument supporting an asterid placement of the order Santalales. This argument is further strengthened taking into account that the *Heisteria olivae* species have been shown to contain scopolamine. This is a compound usually associated with another asterid family, Solanaceae, of the order Solanales.

3.03.5.3 Iridoids in Asteridae

Iridoids are a class of natural compounds frequently encountered in angiosperms of the subclass Asteridae. Biosynthesis of iridoids is basically made along the monoterpenoid part of the isopentenyl diphosphate pathways, but for the final modifications two different biosynthesis routes have been suggested. One of these routes is predominantly found among the plants informally referred to as asteridae I and the other in asteridae II.[150–152] In the plots of **Figure 10**, a sample of 387 iridoids from a wide selection of structural types is predicted in ChemGPS-NP chemical property space. Substances labeled red are the results of biosynthesis along route I, found in asteridae II, and those labeled blue are synthesized along route II, found in asteridae I. The few compounds labeled pink have been described from members of the order Ericales, which is systematically more plesiomorphic than either asteridae I or II (see **Figure 7**). It is obvious from **Figure 10** that the frequently discussed differences in biosynthesis are reflected only to a certain degree in the physicochemical properties of the resulting compounds. All substances synthesized along route II do indeed fall in one of the two main clusters, as seen in the left plot, but along with a large number of compounds from route I. A general trend for iridoids can be interpreted from the right plot, where a strong correlation between an increasing size along the first principal component 1, PC1, and a decreasing lipophilicity along the third principal component, PC3, is found. A preliminary interpretation indicates that one of the factors that render the size to increase and lipophilicity to decrease is obtained by appending additional sugar residues.

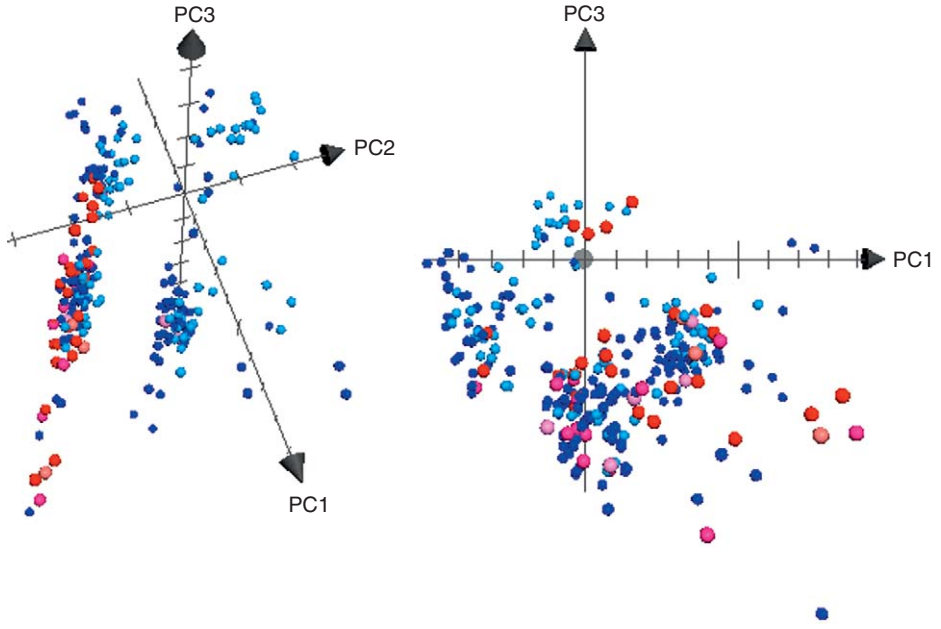


Figure 10 A sample of 387 iridoids selected from a variety of structural types. Substances labeled blue originate from biosynthesis route II and are predominantly found in members of the Asteridae subclass I, and those labeled red originate from biosynthesis route I and are found in members of the Asteridae subclass II. Principal component 1 (PC1) corresponds mainly to size, PC2 to aromaticity- and conjugation-related properties, and PC3 to lipophilicity.

3.03.5.4 Sesquiterpenes in Asteraceae

The sesquiterpene lactones (STLs) are a structure class consisting of at least 5000 compounds.[153] The majority of these, and by far the largest chemical diversity, are found in the angiosperm family Asteraceae, the sunflower family. In **Figure 11**, a set of 1391 STLs have been predicted and plotted in the ChemGPS-NP chemical property space. Substance markers are color-coded according to which phylogenetic entities the substances have been described from, and in cases with multiple biological sources the most plesiomorphic have been selected. For delimitations of phylogenetic entities of the Asteraceae, the phylogeny by Funk *et al.*[154] has been utilized.

In **Figure 11(a)**, STLs from fungi, liverworts, and conifers are shown, occupying a small and very well-defined volume. In **Figure 11(b)**, the volume populated by STLs increases as compounds from angiosperm groups outside the family Asteraceae are included. Most notable among these is the family Apiaceae, which, evolutionarily speaking, is one of the closest groups to the family Asteraceae. Already among the most plesiomorphic (primitive) Asteraceae, the subfamily Barnadesioideae included in **Figure 11(c)**, a wide range of chemical properties are covered. With the addition of the subfamily Chicorioideae in **Figure 11(d)**, the focus of the STL cluster has shifted along the PC1, indicating in general heavier and larger molecules. With the addition of the subfamily Asteroideae, in **Figure 11(e)**, the volume occupied expands further, including a set of compounds found in the tribe Senecioneae with high values in the PC3. In **Figure 11(f)**, the most apomorphic (advanced) members of the Asteraceae are included exhibiting the most diverse chemistry.

In **Figure 11(g)**, all 1391 STLs are shown, demonstrating the complete range of biological and chemical diversity. Among the STLs, there are several well-known biologically active compounds used in drugs and herbal remedies. In **Figure 11(h)**, the position of the antimalarial agent artemisinin, an STL from *Artemisia annua*, is highlighted among other gray STLs. Artemisinin has played a role in the development of new artemether drugs, and more recently its cytotoxic properties have gained importance.[36,155] In very close proximity to artemisinin in the first three dimensions of ChemGPS-NP chemical property space, we find an additional 12 compounds, all displayed in **Figure 12**.

3.03.5.5 Sesquiterpenes in *Arnica*

The genus *Arnica* was investigated for phylogenetic congruencies between data based on secondary chemistry – GC–MS data from STL-rich extracts – and nuclear ribosomal DNA (nrDNA)[156]. Polymorphism in some sequences led to cloning and for some taxa up to three paralogues were found based on nrDNA data. The complex evolutionary patterns are probably due to a history of hybridization, agamospermy, and polyploidy. Although the genus is evolutionarily complex, congruencies between the two data sets (chemistry and DNA) are evident, as seen in **Figure 13**. Multiple representatives of the same species (*Arnica montana, A. chamissonis, A. longifolia*, and *A. gracilis*) form monophyletic groups in analyses both based on chemistry and as nrDNA data. both One of the accessions was a suspected hybrid based and on morphology nrDNA data, and the same patterns are also seen in principal component analyses of chemical data. In addition, accessions of subspecies of *A. chamissonis* and *A. parryi* were also diverged according to chemical data. Congruencies between chemistry and DNA would perhaps be more evident in a group with a less-complicated evolutionary history, but nevertheless serves as a prominent example of correlation between chemistry and phylogeny.[156]

3.03.5.6 Novel Chemical Space Exploration via Natural Products

The biologically relevant parts of chemical space could be defined as those in which we find natural products, as there is a general belief that evolutionary pressure would, with time, prohibit biosynthesis of compounds that are not contributing to organism's fitness[57]. On the other hand, biologically relevant chemical space could be defined as those parts of chemical space in which substances with demonstrated biological activities are encountered. The latter definition would then include not only natural products but also semisynthesized or completely synthetic drugs, hit substances from biological assays, and so on and hence is different from the first. In an attempt to characterize the overlap or difference between these two different views of biologically relevant space, an experiment was designed by Rosén *et al.*[57]

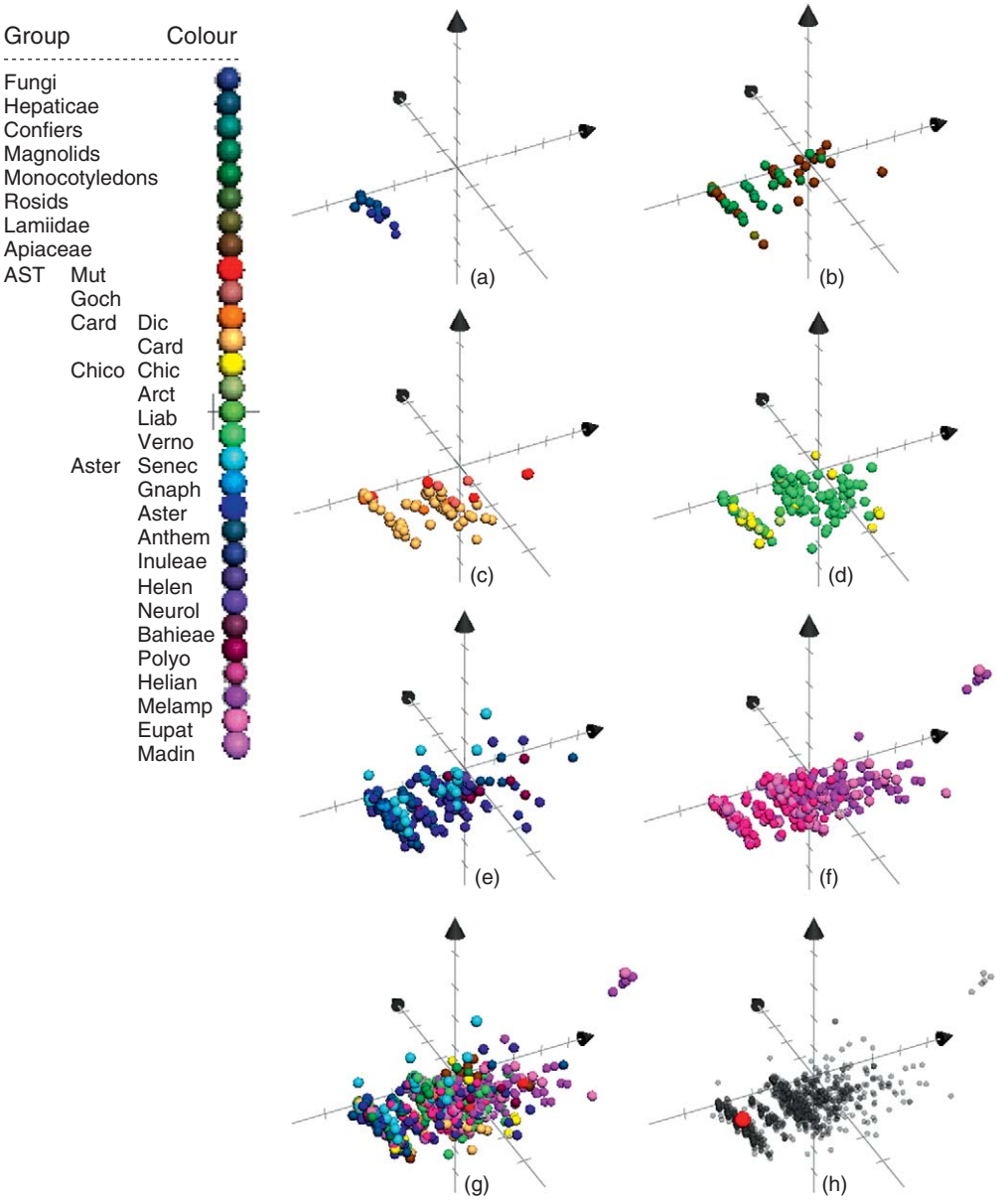


Figure 11 a–h The sesquiterpene lactones (STLs) is a structure class consisting of at least 5000 compounds,[153] a large number of which are found in the angiosperm family Asteraceae. In this figure we can follow how the physicochemical properties of a selection of 1391 sesquiterpene lactones plotted in ChemGPS-NP change during evolution. A legend of colour codes is provided in the top left area of the figure, indicating correspondence between phylogenetic entities and colouring scheme. In the family Asteraceae five subfamilies and 19 tribes are included. Prediction scores for plotting were calculated using ChemGPS-NP$_{Web}$, and plots have been made using Grapher 2.0 distributed with Apple MacOS X software. (a) In this plot STLs from fungi, liverworts and conifers are shown, occupying a small and very well defined volume. (b) The volume increases, as angiosperms are included from groups outside the family Asteraceae. (c) Already among the most plesiomorphic Asteraceae, the subfamily Barnadesioideae, a wide range is covered. (d) In the subfamily Chicorioideae the focus of the STL cluster has shifted along the first principal component indicating in general heavier and larger molecules. (e) In the subfamily Asteroideae the volume occupied expands further in both the second principal component related to aromaticity and conjugation related properties, and (f) in the first with a group of extreme STLs. Systematic and phylogenetic affinities of compounds have been based on a phylogenetic tree of the family Asteraceae by Funk and co-authors.[154] In figure (g) all 1391 STLs are shown, demonstrating complete range. In figure (h) the position of the anti-malaria agent artemisinin, a STL from *Artemisia annuua*, is highlighted.

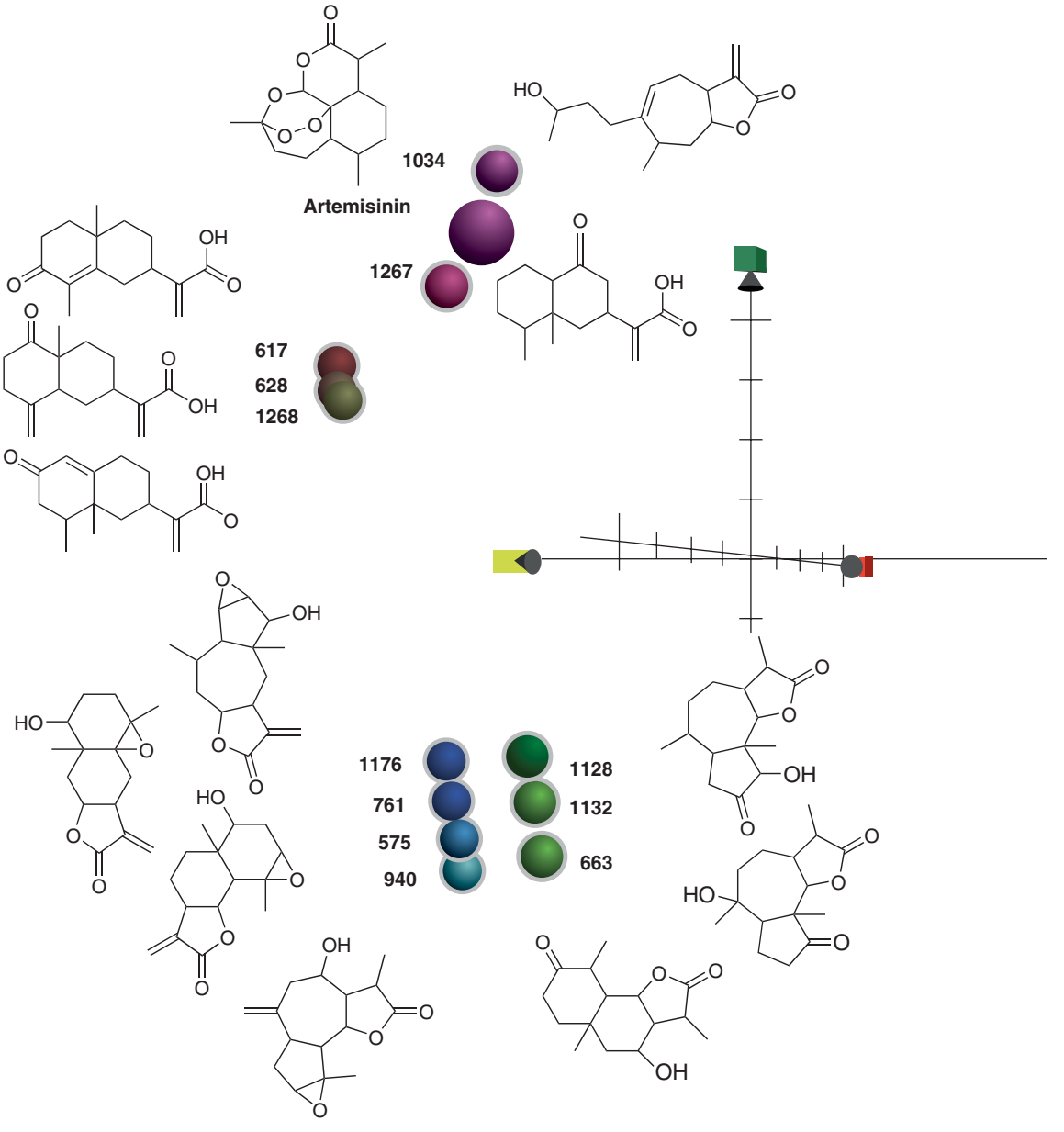


Figure 12 Among the 1391 sesquiterpene lactones predicted in **Figure 11**, one is artemisinin, a natural product from *Artemisia annua* well known for its importance as an antimalarial drug and in the development of new artemether drugs; its cytotoxic properties have gained importance recently.[36,155] In very close proximity to artemisinin in the first three dimensions of ChemGPS-NP chemical property space, we find an additional 12 very closely clustered compounds, all displayed in the figure. Interpretation of axes is PC1 (red) = size, PC2 (yellow) = aromaticity, and PC3 (green) = lipophilicity.

More than 186 000 compounds from the database 'world of molecular bioactivity' (WOMBAT v2007.2) and 160 000 substances from 'Dictionary of Natural Products' (DNP) were extracted as SMILES. Both these sets were subsequently predicted in the ChemGPS-NP chemical property space. The chemical space was then navigated in search of volumes with low density of natural products but abundant in substances showing biological activity – or maybe even more interesting, volumes with an abundance of natural products for which the corresponding biological activities have not yet been identified. Physical–chemical properties correlated to low-density regions are studied in an attempt to explain their occurrence.[57]

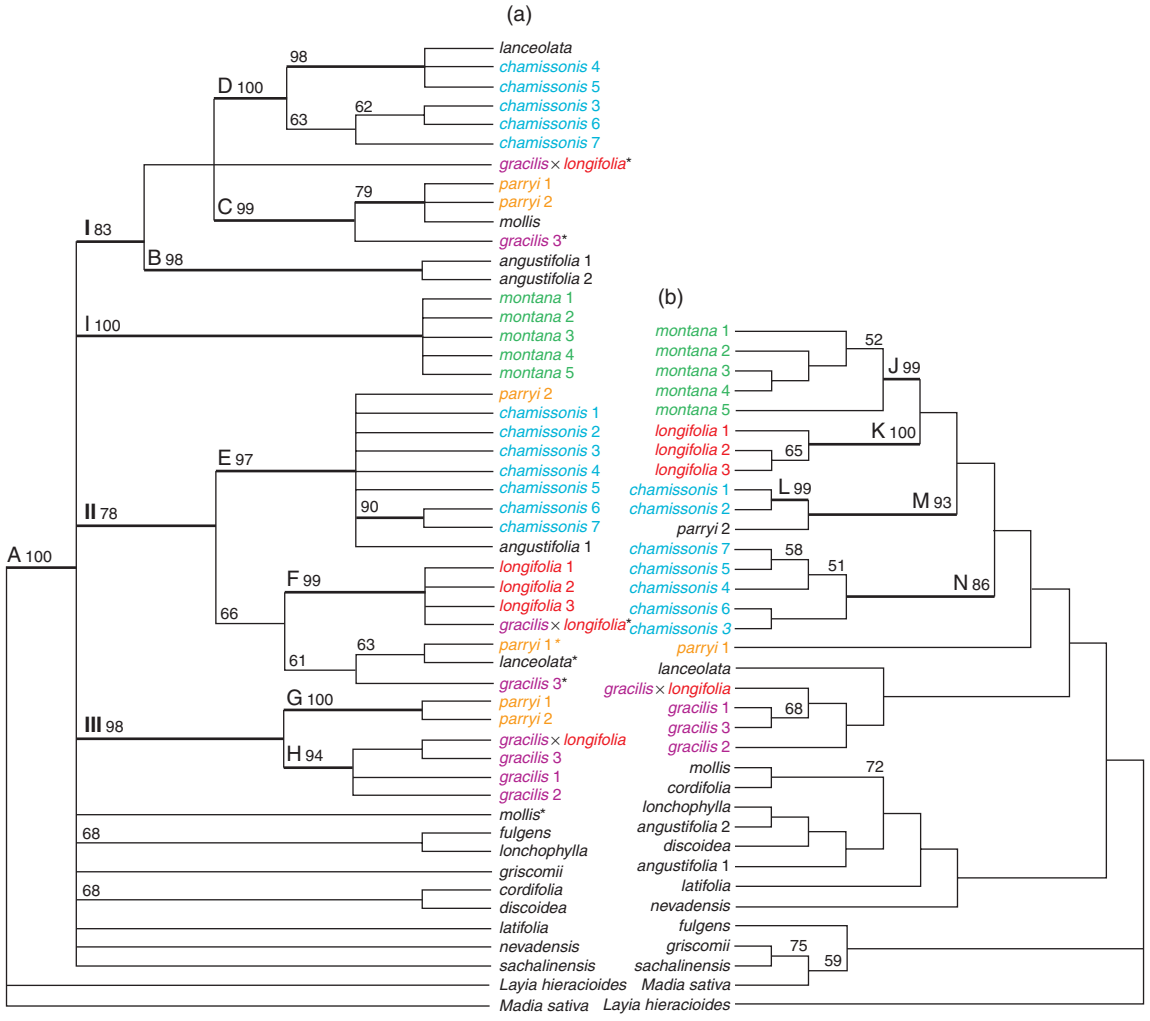


Figure 13 Phylogenetic trees based on (a) nrDNA sequence data and (b) sesquiterpene lactone data for the 33 *Arnica* accessions and accessions of *Layia hieracioides* and *Madia sativa* (outgroup taxa). Multiple accessions of the species *Ambrosia chamissonis*, *A. gracilis*, *A. longifolia*, *A. montana*, and *A. parryi* are marked by color. Branch support values as bootstrap values over 50% are indicated and branches with more than 75% bootstrap support are marked in bold. Major clades discussed by Ekenäs *et al.*[156] are marked with capital letters. A large general congruence between groups identified from molecular sequence data, and from phytochemical data, is apparent. (a) Strict consensus based on nrDNA (ITS and ETS). *No ITS sequences were possible to retrieve for these taxa. (b) The single most parsimonious trees based on a matrix of 239 peaks from GC–MS representing compounds of STL-rich extracts. Picture by C. Ekenäs.

In **Figure 14**, a 3D plot showing tPS1 (size-related parameters), tPS2 (aromaticity/conjugation), and tPS3 (lipophilicity) of ChemGPS-NP chemical property space, with a total of 353 283 bioactive compounds from WOMBAT v2007.2 (black) and natural products (green), is plotted. The different distributions of the two data sets are very obvious, with a large number of rigid compounds with a low proportion of aromaticity, but not yet tested in biological assays. This fits well with what could be expected from the general section on differences between natural products and products from chemical synthesis, a cadre to which a comparably large portion of the compounds with experimentally investigated biological activity belong.

In addition to these more general conclusions, this study by Rosén *et al.*[57] describes the development and application of a novel similarity measure based on Euclidean distance in chemical property space. From this approach, which is fundamentally different from the structure-based similarity measures such as the Tanimoto index, the closest neighbors among natural products for a set of well-known drugs are investigated.

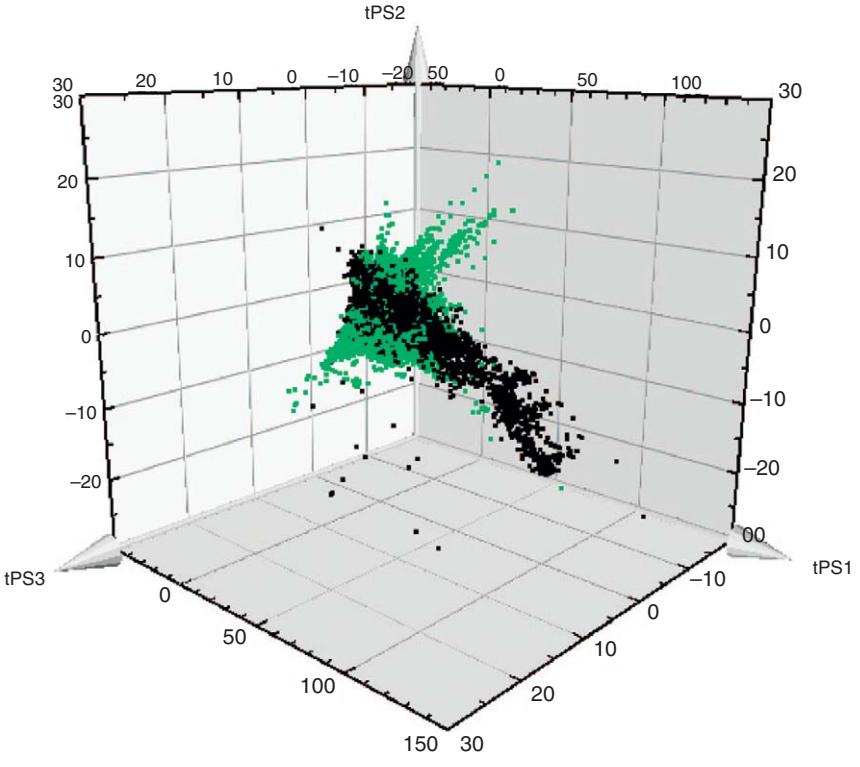


Figure 14 Predicted score plot illustrating differences in coverage of chemical property space by 186 114 instances of compounds with experimentally confirmed biological activity extracted from WOMBAT v2007.2 (black), and a small selection of 167 169 natural product compounds (green). From this, it is evident that the volumes thus far explored by experimental investigations only to some extent coincide with biologically relevant space. If the premise that all natural products are produced for a purpose is true, the above picture indicates to some extent possible directions of future research. Reproduced from J. Rosén; J. Gottfries; S. Muresan; A. Backlund; T. I. Oprea, *J. Med. Chem.* **2009**, *52*, 1953–1962, copyright by the American Chemical Society – used with permission.

3.03.6 Conclusions and Future Prospects

The exploration of the intersection of chemical property space and biological evolutionary space has only begun and presents numerous questions for the future. By *in silico* approaches, to a large extent using available data for datamining, important knowledge for the practicing natural products chemist can be gained at low cost and effort. The search for patterns of evolution – chemical and biological – and the attempts to identify and explain them can guide us in tuning our navigational skills in chemography and phylogeny. Finding safe paths will make the design of experiments, selection of samples or target, and prediction models more robust. These approaches are already having an impact on the literature in the field;[157,158] additional, possible applications are discussed in some detail in the thesis by Rosén.[159]

PATTERNS
Patterns all around you
Patterns everywhere
Patterns of behaviour
Sometimes seem unfair
Can you recognize the patterns that you find?
Patterns unfamiliar
Patterns lead you through (to)
Patterns of discovery

Tracing out the clues
Can you recognize the patterns that you find?
Stuck in your mind
In this land where stability is hard to find
You can rearrange the patterns so unkind
Don't bother asking why a pattern never cries
Old patterns never die they just go on and on
Patterns multiplying
Re-direct our view
Endless variations
Make it all seem new

DEVO/Gerald Casale, used with kind permission

Acknowledgments

I thank Josefin Rosén and Catarina Ekenäs for their comments and ideas on the previous versions of this manuscript, as well as for illustrations to sections of the applications. I am grateful to Gerald Casle and DEVO for the permission to incorporate the true-telling text of patterns, and Stefan Wetzel and Peter Ertl for their assistance in obtaining some reference literature.

References

1. H. C. D. S. Abbott, The chemical basis of plant forms. *Franklin Inst. Lecture* **1887**.
2. L. Hiortzberg, Methodo Investigandi Vires Medicamentorum Chemica. Ph.D. Dissertation in Medicinal Chemistry, Uppsala University, Uppsala, **1765**.
3. R. S. Bohacek; C. McMartin; W. C. Guida, *Med. Res. Rev.* **1996**, *16*, 3–50.
4. C. M. Dobson, *Nature* **2004**, *432*, 824–828.
5. B. K. Shoichet, *Nature* **2004**, *432*, 862–865.
6. P. Kirkpatrick; C. Ellis, *Nature* **2004**, *432*, 823.
7. C. A. Lipinski; A. L. Hopkins, *Nature* **2004**, *432*, 855–861.
8. C. A. Lipinski; F. Lombardo; B. W. Dominy; P. J. Feeney, *Adv. Drug Deliv. Rev.* **1997**, *23*, 3–25.
9. A. L. Harvey, *Curr. Opin. Chem. Biol.* **2007**, *11*, 480–484.
10. D. G. Hall; S. Manku; F. Wang, *J. Comb. Chem.* **2001**, *3*, 125–150.
11. D. G. Kingston; D. J. Newman, *Curr. Opin. Drug Discovery Dev.* **2002**, *5*, 304–316.
12. B. Booth; R. Zemmel, *Nat. Rev. Drug Discovery* **2004**, *3*, 451–456.
13. M. D. Rawlins, *Nat. Rev. Drug Discovery* **2004**, *3*, 360–364.
14. J. A. DiMasi; R. W. Hansen; H. G. Grabowski, *J. Health Econ.* **2003**, *22*, 151–185.
15. M. Pucheault, *Org. Biomol. Chem.* **2008**, *6*, 424–432.
16. T. I. Oprea, *Curr. Opin. Chem. Biol.* **2002**, *6*, 384–389.
17. D. K. Agrafiotis; V. S. Lobanov; F. R. Salemme, *Nat. Rev. Drug Discovery* **2002**, *1*, 337–346.
18. T. I. Oprea; J. Gottfries, *J. Comb. Chem.* **2001**, *3*, 157–166.
19. S. J. Haggarty; P. A. Clemons; J. C. Wong; S. L. Schreiber, *Comb. Chem. High Throughput Screening* **2004**, *7*, 669–676.
20. S. J. Haggarty, *Curr. Opin. Chem. Biol.* **2005**, *9*, 296–303.
21. J. D. Holliday; N. Salim; M. Whittle; P. Willett, *J. Chem. Info. Comput. Sci.* **2003**, *43*, 819–828.
22. A. Schuffenhauer; N. Brown; P. Selzer; P. Ertl; E. Jacoby, *J. Chem. Info. Model.* **2006**, *46*, 525–535.
23. J. Larsson; J. Gottfries; L. Bohlin; A. Backlund, *J. Nat. Prod.* **2005**, *68*, 985–991.
24. J. Larsson; J. Gottfries; S. Muresan; A. Backlund, *J. Nat. Prod.* **2007**, *70*, 789–794.
25. S. L. Schreiber, *Science* **2000**, *287*, 1964–1969.
26. S. Borman, *Chem. Eng. News* **1999**, *77*, 33–48.
27. T. I. Oprea; J. Gottfries; V. Sherbukhin; P. Svensson; T. C. Kühler, *J. Mol. Graphics Model.* **2000**, *18*, 512–524.
28. J. Bajorath, *Mol. Diversity* **2000**, *5*, 305–313.
29. M. Krier; G. Bret; D. Rognan, *J. Chem. Info. Model.* **2006**, *46*, 512–524.
30. G. V. Paolini; R. H. B. Shapland; W. P.v. Hoorn; J. S. Mason; A. L. Hopkins, *Nat. Biotechnol.* **2006**, *24*, 805–815.
31. J. J. Irwin, *Curr. Opin. Chem. Biol.* **2006**, *10*, 352–356.
32. J. Clardy; C. Walsh, *Nature* **2004**, *432*, 829–837.
33. M. Feher; J. M. Schmidt, *J. Chem. Info. Comput. Sci.* **2003**, *43*, 218–227.
34. F. L. Stahura; J. W. Godden; L. Xue; J. Bajorath, *J. Chem. Info. Comput. Sci.* **2000**, *40*, 1245–1252.
35. M.-L. Lee; G. Schneider, *J. Comb. Chem.* **2001**, *3*, 284–289.

36. D. J. Newman; G. M. Cragg, *J. Nat. Prod.* **2007**, *70*, 461–477.
37. D. J. Newman; G. M. Cragg; K. M. Snader, *J. Nat. Prod.* **2003**, *66*, 1022–1037.
38. D. S. Tan, *Nat. Chem. Biol.* **2005**, *1*, 75–84.
39. A. A. Shelat; R. K. Guy, *Curr. Opin. Chem. Biol.* **2007**, *11*, 244–251.
40. D. G. Lloyd; G. Golfis; A. J. S. Knox; D. Fayne; M. J. Meegan; T. I. Oprea, *Drug Discovery Today* **2006**, *11*, 149–159.
41. R. J. Quinn; A. R. Carroll; N. B. Pham; P. Baron; M. E. Palframan; L. Suraweera; G. K. Pierens; S. Muresan, *J. Nat. Prod.* **2008**, *71*, 464–468.
42. N. R. Pace, *Science* **1997**, *276*, 734–740.
43. Y.-W. Chin; M. J. Balunas; H. B. Chai; A. D. Kinghorn, *AAPS J.* **2006**, *8*, E239–E253.
44. P. Ertl; A. Schuffenhauer, *Prog. Drug Res.* **2008**, *66*, 219–235.
45. T. Lundqvist, *Curr. Opin. Drug Discovery Dev.* **2005**, *8*, 513–519.
46. S. Fergus; A. Bender; D. R. Spring, *Curr. Opin. Chem. Biol.* **2005**, *9*, 304–309.
47. G. E. Boldt; T. J. Dickerson; K. D. Janda, *Drug Discovery Today* **2006**, *11*, 143–148.
48. M. D. Burke; S. L. Schreiber, *Angew. Chem. Int. Ed.* **2004**, *43*, 46–58.
49. E. Meggers, *Curr. Opin. Chem. Biol.* **2007**, *11*, 287–292.
50. A. A. Shelat; R. K. Guy, *Bioorg. Med. Chem.* **2008**, *17*, 1088–1093.
51. R. E. Carhart; D. H. Smith; R. Venkataraghavan, *J. Chem. Info. Comput. Sci.* **1985**, *25*, 64–73.
52. P. Willett; V. Winterman; D. Bawden, *J. Chem. Info. Comput. Sci.* **1986**, *26*, 36–41.
53. P. Willett; J. M. Barnard; G. M. Downs, *J. Chem. Info. Comput. Sci.* **1998**, *38*, 983–996.
54. J. D. Holliday; N. Salim; M. Whittle; P. Willett, *J. Chem. Info. Comput. Sci.* **2003**, *43*, 819–828.
55. J. H.v. Drie; M. S. Lajiness, *Drug Discovery Today* **1998**, *3*, 274–283.
56. J. Xu, *J. Med. Chem.* **2002**, *45*, 5311–5320.
57. J. Rosén; J. Gottfries; S. Muresan; A. Backlund; T. I. Oprea, *J. Med. Chem.* **2009**, *52*, 1953–1962.
58. M. A. Koch; A. Schuffenhauer; M. Scheck; S. Wetzel; M. Casaulta; A. Odermatt; P. Ertl; H. Waldmann, *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 17272–17277.
59. A. Schuffenhauer; P. Ertl; S. Roggo; S. Wetzel; M. A. Koch; H. Waldmann, *J. Chem. Info. Model.* **2007**, *47*, 47–58.
60. S. N. Pollock; E. A. Coutsias; M. J. Wester; T. I. Oprea, *J. Chem. Info. Model.* **2008**, *48*, 1304–1310.
61. M. J. Wester; S. N. Pollock; E. A. Coutsias; T. K. Allu; S. Muresan; T. I. Oprea, *J. Chem. Info. Model.* **2008**, *48*, 1311–1324.
62. S. Wetzel; A. Schuffenhauer; S. Roggo; P. Ertl; H. Waldmann, *CHIMIA Int. J. Chem.* **2007**, *61*, 355–360.
63. J. Inglese; D. S. Auld; A. Jadhav; R. L. Johnson; A. Simeonov; A. Yasgar; W. Zheng; C. P. Austin, *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 11473–11478.
64. J. Rosén; A. Lövgren; T. Kogej; S. Muresan; J. Gottfries; A. Backlund, *J. Comput. Aided Mol. Des.* **2009**, *23*, 253–259.
65. H. Kubinyi, *Perspect. Drug Discovery Des.* **1998**, *9–11*, 225–252.
66. C. G. Bologa; T. K. Allu; M. Olah; M. A. Kappler; T. I. Oprea, *J. Comput. Aided Mol. Des.* **2005**, *19*, 625–635.
67. R. D. Brown; Y. C. Martin, *J. Chem. Info. Comput. Sci.* **1996**, *36*, 572–584.
68. R. D. Brown; Y. C. Martin, *J. Chem. Info. Comput. Sci.* **1997**, *37*, 1–9.
69. H. Matter, *J. Med. Chem.* **1997**, *40*, 1219–1229.
70. L. Xue; F. L. Stahura; J. W. Godden; J. Bajorath, *J. Chem. Info. Comput. Sci.* **2001**, *41*, 394–401.
71. J. Bajorath, *Nat. Rev. Drug Discovery* **2002**, *1*, 882–894.
72. J. H. Nettles; J. L. Jenkins; A. Bender; Z. Deng; J. W. Davies; M. Glick, *J. Med. Chem.* **2006**, *49*, 6802–6810.
73. Y. C. Martin; J. L. Kofron; L. M. Traphagen, *J. Med. Chem.* **2002**, *45*, 4350–4358.
74. A. Bender; R. C. Glen, *Org. Biomol. Chem.* **2004**, *2*, 3204–3218.
75. M. M. Hann; T. I. Oprea, *Curr. Opin. Chem. Biol.* **2004**, *8*, 255–263.
76. B. R. Stockwell, *Nature* **2004**, *432*, 846–854.
77. A. Ganesan, *Curr. Opin. Chem. Biol.* **2008**, *12*, 306–317.
78. C. A. Lipinski, *Drug Discovery Today* **2003**, *8*, 12–16.
79. G. M. Maggiora, *J. Chem. Info. Model.* **2006**, *46*, 1535.
80. C. Walsh, *Nature* **2001**, *409*, 226–231.
81. M. V. Kapralov; D. A. Filatov, *BMC Evol. Biol.* **2007**, *7*, 73.
82. I. Andersson; A. Backlund, *Plant Physiol. Biochem.* **2008**, *46*, 275–291.
83. S. Larsson, *Acta Universitatis Upsaliensis. Compr. Summaries of Uppsala Diss. Faculty of Pharmacy* **2007**, *49*, 1–65. (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-770).
84. L. M. Kauvar; H. O. Villar; J. R. Sportsman; D. L. Higgins; D. E. J. Schmidt, *J. Chromatogr. B* **1998**, *715*, 93–102.
85. D. C. Greenbaum; W. D. Arnold; F. Lu; L. Hayrapetian; A. Baruch; J. Krumrine; S. Toba; K. Chehade; D. Brömme; I. D. Kuntz; M. Bogyo, *Chem. Biol.* **2002**, *9*, 1085–1094.
86. J. H. Nettles; J. L. Jenkins; C. Williams; A. M. Clark; A. Bender; Z. Deng; J. W. Davies; M. Glick, *J. Mol. Graphics Model.* **2007**, *26*, 622–633.
87. A. Macchiarulo; R. Pellicciari, *J. Mol. Graphics Model.* **2007**, *26*, 728–739.
88. L. Eriksson; P. L. Andersson; E. Johansson; M. Tysklind, *Mol. Divers.* **2006**, *10*, 169–189.
89. P. Vuorela; M. Leinonen; P. Saikkuc; P. Tammelaa; J. P. Rauhad; T. Wennberge; H. Vuorela, *Curr. Med. Chem.* **2004**, *11*, 1375–1389.
90. P. Bernard; T. Scior; B. Didier; M. Hibert; J.-Y. Berthon, *Phytochemistry* **2001**, *58*, 865–874.
91. A. P. Russ; S. Lampel, *Drug Discovery Today* **2005**, *19*, 1607–1610.
92. J. P. Overington; B. Al-Lazikani; A. L. Hopkins, *Nat. Rev. Drug Discovery* **2006**, *5*, 993–996.
93. C. G. Bologa; C. M. Revankar; S. M. Young; B. S. Edwards; J. B. Arterburn; A. S. Kiselyov; M. A. Parker; S. E. Tkachenko; N. P. Savchuck; L. A. Sklar; T. I. Oprea; E. R. Prossnitz, *Nat. Chem. Biol.* **2006**, *2*, 207–212.
94. T. Blundell, *Structure* **2007**, *15*, 1342–1343.
95. T. C. Taylor; A. Backlund; K. Bjorhall; R. J. Spreitzer; I. Andersson, *J. Biol. Chem.* **2001**, *276*, 48159–48164.
96. Angiosperm Phylogeny Group, *Ann. Missouri Bot. Garden* **1998**, *85*, 531–553.

97. Angiosperm Phylogeny Group, *Bot. J. Linn. Soc.* **2003**, *141*, 399–436.
98. J. Lei; J. Zhou, *J. Chem. Info. Comput. Sci.* **2002**, *42*, 742–748.
99. D. J. Newman; R. T. Hill, *J. Indus. Microbiol. Biotechnol.* **2006**, *33*, 539–544.
100. E. Haeckel, *Generelle Morphologie der Organismen*; Georg Reiner: Berlin, 1866.
101. J. S. Farris, The Logical Basis of Phylogenetic Systematics. In *Advances in Cladistics*; N. I. Platnick, V. A. Funk, Eds.; Columbia University Press: New York, 1983; pp 1–36.
102. G. Hestmark, *Nature* **2000**, *408*, 911.
103. M. Kimura, *The Neutral Theory of Molecular Evolution*; Cambridge University Press: Cambridge, 1983.
104. M. Nei, *Molecular Evolutionary Genetics*; Columbia University Press: New York, 1987.
105. J. S. Farris; V. A. Albert; M. Källersjö; D. Lipscomb; A. G. Kluge, *Cladistics* **1996**, *12*, 99–124.
106. J. E. Richardson; F. M. Weitz; M. F. Fay; Q. C. B. Cronk; H. P. Linder; G. Reeves; M. W. Chase, *Nature* **2001**, *412*, 181–183.
107. U. Swenson; A. Backlund; S. McLoughlin; R. S. Hill, *Cladistics* **2001**, *17*, 28–47.
108. J. Aldrich, *Stat. Sci.* **1997**, *12*, 162–176.
109. J. Felsenstein, *Syst. Zool.* **1973**, *22*, 240–249.
110. J. Felsenstein, *J. Mol. Evol.* **1981**, *17*, 368–376.
111. B. Rannala; Z. Yang, *J. Mol. Evol.* **1996**, *43*, 304–311.
112. J. P. Huelsenbeck; F. Ronquist; R. Nielsen; J. P. Bollback, *Science* **2001**, *294*, 2310–2314.
113. T. Bayes, *Philos. Trans. R. Soc.* **1763**, *53*, 370–418.
114. W. Hennig, *Grundzüge einer Theorie der phylogenetischen Systematik*; Deutscher Zentralverlag: Berlin, 1950.
115. W. Hennig, *Phylogenetic Systematics*; University of Illinois Press: Urbana, 1966.
116. A. G. Kluge; J. S. Farris, *Syst. Zool.* **1969**, *18*, 1–32.
117. J. S. Farris, *Syst. Zool.* **1970**, *19*, 83–92.
118. J. S. Farris; A. G. Kluge; M. J. Eckardt, *Syst. Zool.* **1970**, *19*, 172–191.
119. C. L. Andersson; K. Bremer; E. M. Friis, *Am. J. Bot.* **2005**, *92*, 1737–1748.
120. V. Albert; A. Backlund; K. Bremer; M. W. Chase; J. R. Manhart; B. D. Mishler; K. C. Nixon, *Ann. Missouri Bot. Garden* **1994**, *81*, 534–567.
121. K. H. Wolfe; C. W. Morden; J. D. Palmer, *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 10648–10652.
122. K. Shinozaki; M. Ohme; M. Tanaka; T. Wakasugi; N. Hayashida; T. Matsubayashi; N. Zaita; J. Chunwongse; J. Obokata; K. Yamaguchi-Shinozaki; C. Ohto; K. Torazawa; B. Y. Meng; M. Sugita; H. Deno; T. Kamogashira; K. Yamada; J. Kusuda; F. Takaiwa; A. Kato; N. Tohdoh; H. Shimada; M. Sugiura, *EMBO J.* **1986**, *5*, 2043–2049.
123. G. Zurawski; M. T. Clegg, *Annu. Rev. Plant Physiol.* **1987**, *38*, 391–418.
124. H. el-Seedi; S. Larsson; A. Backlund, *Biochem. Syst. Ecol.* **2005**, *33*, 831–839.
125. A. Backlund; T. Moritz, *Biochem. Syst. Ecol.* **1998**, *26*, 309–335.
126. D. S. Guttman, *Biotechnol. Adv.* **2004**, *22*, 363–382.
127. I. D. Fleming; L. J. Nisbet; S. J. Brewer, Target Directed Antimicrobial Screens. In *Bioactive Microbial Products: Search and Discovery*; J. D. Bulock, N. J. Nisbet, Eds.; Academic Press: London, 1982; pp 107–130.
128. M. R. Berenbaum; A. R. Zangerl, Phytochemical Diversity: Adaption or Random Variation. In *Recent Advances in Phytochemistry*; J. T. Romeo, J. A. Saunders, P. Barbosa, Eds.; Plenum Press: New York, 1996; pp 1–24.
129. R. D. Firn; C. G. Jones, An Explanation of Secondary Product Redundancy'. In *Recent Advances in Phytochemistry*; J. T. Romeo, J. A. Saunders, P. Barbosa, Eds.; Plenum Press: New York, 1996; pp 295–312.
130. H. Lodish; A. Berk; L. Zipursky; P. Matsudaira; D. Baltimore; J. Darnell, *Molecular Cell Biology*, 4th ed.; W. H. Freeman: New York, 1999.
131. R. D. Firn; C. G. Jones, *Mol. Microbiol.* **2000**, *37*, 989–994.
132. M. S. Butler, *Nat. Prod. Rep.* **2005**, *22*, 162–195.
133. G. M. Cragg; D, J. Newman, *J. Ethnopharmacol.* **2005**, *100*, 72–79.
134. Y.-W. Chin; M. J. Balunas; H. B. Chai; A. D. Kinghorn, *AAPS J.* **2006**, *8*, E239–E253.
135. M. S. Butler; D. J. Newman, *Prog. Drug Res.* **2008**, *65*, 3–44.
136. A. L. Hopkins; C. R. Groom, *Nat. Rev. Drug Discovery* **2002**, *1*, 727–730.
137. P. Imming; C. Sinning; A. Meyer, *Nat. Rev. Drug Discovery* **2006**, *5*, 821–834.
138. D. S. Wishart; C. Knox; A. C. Guo; S. Shrivastava; M. Hassanali; P. Stothard; Z. Chang; J. Woolsey, *Nucleic Acids Res.* **2006**, *34*, D668–D672.
139. D. S. Wishart; C. Knox; A. C. Guo; D. Cheng; S. Shrivastava; D. Tzur; B. Gautam; M. Hassanali, *Nucleic Acids Res.* **2008**, *36*, D901–D906.
140. T. Henkel; R. M. Brunne; H. Müller; F. Reichel, *Angew. Chem. Int. Ed.* **1999**, *38*, 643–647.
141. A. Ganesan, *Curr. Opin. Biotechnol.* **2004**, *15*, 584–590.
142. K. Grabowski; G. Schneider, *Curr. Chem. Biol.* **2007**, *1*, 115–127.
143. T. I. Oprea; I. Zamora; A.-L. Ungell, *J. Comb. Chem.* **2002**, *4*, 258–266.
144. G. M. Rishton, *Drug Discovery Today* **2003**, *8*, 86–96.
145. E. Dongo; J. F. Ayafor; B. L. Sondengam, *J. Nat. Prod.* **1989**, *52*, 840–843.
146. D. C. Gournelis; G. G. Laskaris; R. Verpoorte, *Nat. Prod. Rep.* **1997**, *14*, 75–82.
147. F. Sugawara; M. Ishimoto; N. Le-Van; H. Koshino; J. Uzawa; S. Yoshida; K. Kitamura, *J. Agric. Food Chem.* **1996**, *44*, 3360–3364.
148. A. Backlund; B. Bremer, *Plant Syst. Evol.* **1997**, *207*, 225–254.
149. J. Kårehed, *Am. J. Bot.* **2001**, *88*, 2259–2274.
150. S. Rosendal Jensen, Plant Iridoids, Their Biosynthesis and Distribution in Angiosperms. In *Ecological Chemistry and Biochemistry of Plant Terpenoids*; J. B. Harborne, F. A. Tomas-Barberan, Eds.; Clarendon Press: Oxford, 1971; pp 133–158.
151. S. Rosendal Jensen, *Ann. Missouri Bot. Garden* **1992**, *79*, 284–302.
152. S. Rosendal Jensen; B. Juhl Nielsen; R. Dahlgren, *Botaniska Notiser* **1975**, *128*, 148–180.
153. T. J. Schmidt, *Stud. Nat. Prod. Chem.* **2006**, *33*, 309–392.

154. V. A. Funk; R. Bayer; S. Keeley; R. Chan; L. Watson; B. Gemeinholzer; E. Schilling; J. Panero; B. Baldwin; N. Garcia-Jacas; A. Susanna; R. K. Jansen, *Biologiske Skrifter* **2005**, *55*, 343–373.
155. T. Efferth, *Planta Medica* **2007**, *73*, 299–309.
156. C. Ekenäs; J. Rosén; S. Wagner; I. Merfort; A. Backlund; K. Andreasen, *Cladistics* **2009**, *25*, 78–92.
157. L. Bohlin; U. Göransson; A. Backlund, *Pure Appl. Chem.* **2007**, *79*, 763–774.
158. S. Larsson; L. Bohlin; A. Backlund, *Phytochem. Lett.* **2008**, *1*, 131–134.
159. J. Rosén, ChemGPS-NP and the Exploration of Biologically Relevant Chemical Space. Ph.D. Thesis in Pharmacognosy, Uppsala University, Uppsala (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-89364), 2009.

Biographical Sketch

Anders Backlund was born in 1965 in Uppsala, Sweden. After undergraduate training in biology, he defended his thesis entitled Phylogeny of Dipsacales at the Department of Systematic Botany, Uppsala University, in 1996. – Following a postdoctoral experience in molecular biology at Uppsala University, genetics at Trinity College Dublin, and structural biochemistry at the Swedish University of Agricultural Sciences, he returned to Uppsala University in 1999. There he took up the position of senior lecturer in the Division of Pharmacognosy, a part of the Department of Medicinal Chemistry.

At present, Associate Professor Backlund is leading a research group working on methods of selection based on phylogenetics and chemographics (www.fkog.uu.se/research/selection.html). From this research group, a set of publicly available doctoral theses have been produced, including Cytotoxic Compounds of Plant Origin – Biological and Chemical Diversity by Petra Lindholm, 2005 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-5728), Misteltoes and Thionins: As Selection Models in Natural Products Drug Discovery by Sonny Larsson, 2007 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-770), and ChemGPS-NP and the Exploration of Biologically Relevant Chemical Space by Josefin Rosén, 2009 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-89364.

3.04 The NAPRALERT Database as an Aid for Discovery of Novel Bioactive Compounds

James G. Graham and Norman R. Farnsworth, University of Illinois at Chicago, Chicago, IL, USA

3.04.1 Introduction

NAPRALERT is a database housed at the University of Illinois at Chicago that contains data on the geography, taxonomy, chemistry, medicinal folklore, and biological activities of natural products, including clinical trials, of their extracts and isolates. NAPRALERT stands for natural products alert; when it was started as a computer database in 1975 a prime consideration was to develop the capability to evaluate the natural products literature for the purpose of identifying new sources of commercially significant or clinically useful drugs.

Now, more than three decades later, NAPRALERT contains data on upward of 60 000 species, including more than 200 000 distinct chemical compounds of natural origin and 90 000 reports of ethnomedical uses of plants and other organisms. Data on more than 770 000 unique pharmacological records, representing more than 4000 different pharmacological activities are coded in NAPRALERT. These data have been extracted from over 200 000 scientific articles and reviews from nearly 10 000 scientific journals, representing organisms from all countries of the world.

NAPRALERT users tend to be engaged in medicinal plant research, drug development, or the botanical dietary supplement industry, and we have developed a series of standard search requests – a combination of organism-based, chemically based, or pharmacologically based queries – that can be executed online. The NAPRALERT database can be consulted at our website: http://www.napralert.org. (All internet addresses cited in this chapter were functional as of October 2008.)

Our coverage of the literature in the following areas is quite comprehensive:

- Clinical studies of natural products (including safety)
- Natural products that affect sugar metabolism
- Natural products that affect mammalian reproduction
- Extracts and compounds that affect cancer growth

- Natural products and antiviral (including HIV/AIDS) activity
- Natural products and antitubercular activity
- Natural products and tropical diseases
- Ethnomedical information on more than 20 000 species of plants
- Metabolism and pharmacokinetics
- Natural products that affect plant growth
- Review articles on organisms at the genus and/or species levels and reviews of secondary metabolites (citations only)
- Chemoprevention with natural products
- Natural insecticides
- Anti-inflammatory activity of natural products
- Analgesic activity of natural products
- Plant growth stimulators and inhibitors

NAPRALERT was designed as a fully relational database, rather than as a textual database. A growing number of digital resources, including natural product databases, are becoming available. Most of these are textual – they contain short abstracts summarizing literature reports, which refer back to a bibliographic reference that the user must read and summarize. Given the fact that even a single article in the natural product literature may contain multiple species, multiple biological activity tests conducted on multiple chemical isolates or extract types at different concentrations, the sheer abundance of data that is now available, and the inability of an individual to process it all, even with the development of the so-called 'high-quality text mining' software, provides a major hurdle to effective data extraction and manipulation, highlighting one of the unique attributes of NAPRALERT – rapid, precise, specialized data retrieval.

NAPRALERT, in contrast to a textual database, codes each of the different species, activities, compounds, extracts, and/or concentrations in a separate file, across a number of different tables. This means that multiple synchronous searches can be conducted, sorting the millions of records contained in the tables across the database in an extremely powerful way. Highly specific queries can be conducted, some of which will be illustrated here. In order to identify search criteria that will yield the most useful results, an understanding of the database architecture is critical. We will present an overview of the NAPRALERT data structure, followed by a few examples to illustrate how the database can be applied to problem solving in natural products drug discovery. In addition, we provide a brief review of other online databases.

The ability to conduct complex queries online is limited. Interested users may direct specific requests to NAPRALERT staff, whose contacts can be found on our website.

3.04.1.1 Online Searches

Three general types of online queries are organism, chemical, or pharmacologically based searches. Organism-based queries provide the user with a selection of ethnomedical, pharmacological, and/or chemical data that correspond to a particular organism of interest. Chemically based queries provide pharmacological testing results for a particular compound, or a list of organisms known to contain it. Pharmacologically based queries provide testing data for organisms or compounds that have been evaluated for a specific biological activity.

3.04.1.1.1 Organism-based queries

NAPRALERT currently cites 142 947 publications, referring to 61 116 different organisms, including 45 392 different plant species (**Table 1**). Organism-based queries can be conducted online at the family, genus, species, or even variety level. Geographic origin, by region and country, can also be searched, as well as the organism part. In cases of common names, taxonomic uncertainty, or synonymy, a 'verify names' option allows the user to search by an organism's common name or taxonomic synonym, and provides the species (binomial) nomenclature recognized as valid by the database.

Table 1 Overview of the plant data found in the NAPRALERT organism table

Class of organism	*Genera*	*Species*	*Compounds*
Monocots	962	5141	15 425
Dicots	5853	41 135	139 025
Gymnosperms	81	742	6269
Pteridophytes	210	1454	2909
Bryophytes	215	640	3045
Lichens	191	904	1399

3.04.1.1.2 Chemical queries

NAPRALERT currently cites 154 766 publications referring to 207 575 distinct chemical compounds. The chemical names used in NAPRALERT begin with a base name, which corresponds to the parent structure of a compound, usually derived from the largest ring or straight-chain carbon skeleton in the structure (see **Table 2**). Listed below are some examples.

- For oleanolic acid 3 acetate, data have been entered as oleanolic acid-3-acetate.
- For 3 acetoxy oleanolic acid, data have been entered as oleanolic acid,3-acetoxy:
- For 8 methoxy quercetin, data have been entered as quercetin,8-methoxy:

Table 2 Overview of chemical classes and number of representatives found in NAPRALERT

Class of compound	*Number*
Structure undetermined	721
Miscellaneous	1316
Alkane	1695
Alkene	1619
Alkyne	115
Alkenyne	1482
Lipid	4339
Carbohydrate	3653
Proteid	9309
Flavonoid	20 576
Polycyclic	978
Oxygen heterocycle	3896
Quinoid	5327
Benzenoid	10 166
Sulfur compound	1843
Alicyclic	1175
Nonalkaloid *N*-heterocycle	1266
Lignan	8243
Coumarin	4245
Xanthone	1713
Miscellaneous lactone	5045
Vitamin	92
Chromone	1055
Monoterpene	7156
Diterpene	23 128
Sesquiterpene	17 863
Sesterterpene	831
Triterpene	18 520
Steroid	7637
Sapogenin	2404
Cardenolide	1697

(*Continued*)

Table 2 (Continued)

Class of compound	*Number*
Carotenoid	1180
Polyprenoid	231
Alkaloid – miscellaneous	7941
Tropane alkaloid	591
Pyrrolizidine alkaloid	1025
Indolizidine alkaloid	417
Quinolizidine alkaloid	1069
Quinoline alkaloid	1554
Isoquinoline alkaloid	6753
Indole alkaloid	7251
Monoterpene alkaloid	155
Diterpene alkaloid	322
Sesquiterpene alkaloid	1530
Sesterterpene alkaloid	4
Triterpene alkaloid	152
Steroid alkaloid	1028
Peptide alkaloid	232
Lactam	426
Tetraterpenoid	22
Inorganic	599
Antibiotics (unknown structure)	1616

Substituent groups, if present, are appended to the base name. The base name approach allows for the use of wildcard searches, whereby a group of structurally related compounds can be queried in a single step. In some cases, common names of compounds widely distributed in nature (quercetin, beta-sitosterol, etc.) can be searched. A 'verify names' option is available to identify the best search parameter for the compound(s) of interest.

It should be noted that if substituents are present, the base name is followed by a comma and the substituents, followed by a colon (:). More information on substituents is listed below.

If you are interested in any compound with a base name present in the database, you can use % as a wild card. This will show you all the related compounds to the base name. For example, searching by the term oleanolic acid% will give you all the compounds present in the database that are derivatives of oleanolic acid.

3.04.1.1.2(i) Substituents

A. If substituents are present, the base name is followed by a comma followed by the substituents. For example, 2′-hydroxy flavone should be searched as flavone,2′-hydroxy:. Note that the last substituent after the base name should be followed by a colon – thus 6″-*O*-acetyl daidzin should be searched as daidzin,6″-o-acetyl:.
B. The substituents are arranged in alphabetic order after the base name; disregard prefixes such as di, tri, etc. Thus, 'dihydroxy' is alphabetized as if it began with 'h'.
   Example: 2,3-dihydroxy-4-methoxy-3′,7-dimethyl flavone – search as flavone,2-3-dihydroxy-4-methoxy-3′-7-dimethyl:
C. If the same substituent is present at different positions, they have been arranged in increasing order.
   Example: 5′,3′,4,6-tetrahydroxy flavone – search as flavone,3′-4-5′-6-tetrahydroxy:
   Example: 3′,4′,5-trihydroxy-3,6-dimethoxy flavone – search as flavone,3′-4′-5-trihydroxy-3-6-dimethoxy:
D. Hyphens separate numbers from letters and functionalities from one another.
   Example: 2,5-dimethoxy apigenin – search as apigenin,2-5-dimethoxy:
E. For hyphens within the base name, base names often include carbon numbers of unsaturations and structural details. Final vowels are dropped if the next letter following the number is a vowel. Compare these two examples – it will be seen that the final 'a' is dropped only in the second example.
   1. labda-8-13-diene
   2. labd-8-ene

F. Exceptions to the alphabetical rule for substituents are sugar moieties and parenthetical information.
   – Sugar (glycoside) groups. All glycosides are listed after the nonglycoside groups.
   – A space is left between the last group and the glycoside.
   Example: 4-methoxy flavone-7-*O*-Beta-D-glucoside – search as flavone,4-methoxy: 7-o-beta-D-glucoside
   Example: 3-*O*-Beta-D-glucopyranosyl-phytolaccagenic acid-28-*O*-Beta-D-glucopyranosyl ester – search as phytolaccagenic acid ,3-o-beta-D-glucopyranosyl: 28-o-Beta-D-glucopyranosyl ester
G. Functional groups, which are themselves substituted, are listed after the nonglycoside groups and no space is left. Parentheses enclose the substituted group, for example, flavone,4-methoxy-7-(2-hydroxy-ethyl):
H. Greek symbols are spelled out, that is, Alpha, Beta, etc.

3.04.1.1.2(ii) Prefixes

A. Prefixes that indicate an altered base structure are written immediately following the comma after the base name.
   Example: (+) dihydro kawain – search as kawain,dihydro: (+):
   Example: methyl ether of isoeugenol – search as eugenol,iso: methyl ether
   Example: isorhamnetin – search as rhamnetin,iso:
B. However, the prefixes bicyclo-, bis-, and cyclo- are not separated from the base name.
   Example: isobicyclogermacrene – search as bicyclogermacrene,iso:
C. Greek letters are spelled out in English – trans is used for E and cis is used for Z. E and Z always appear in front of the number (position) they represent.
   Example: deca-2Z,8E diene – search as deca-cis-2-trans-8-diene
   Example: cholesta-5,22Z-diene-3-Beta-ol – search as cholesta-5-cis-22-dien-3-beta-ol
D. Within a functional group parts are separated by a hyphen.
E. Example: 8-isovaleroyloxy kaurane – search as kaurane,8-iso-valeroyl-oxy:
F. Compounds with '#' sign at the end of some compounds indicates that two different compounds have been assigned the same name in different papers, for example,
   foetidin (steroid)
   foetidin# (coumarin)
   foetidin## (diterpene)
G. Compounds with an '*' indicates that they are synthetic, semisynthetic, or unnatural derivative compounds.
H. We have synonyms for most common compounds, for example, flavone,5-7-dihydroxy-8-methoxy: is synonymous with wogonin (wogonin is used in the database).
I. Some compound names are actually extracts, for example,
   sp-303
   policosanol
   silymarin
   pycnogenol

3.04.1.1.3 Pharmacological query

A pharmacologically based query typically involves searching for organisms (plant, animal, or bacterial extracts/preparations) and/or compounds that have been evaluated in a particular biological system (bacterial or cell culture, enzyme system, animal model, human clinical trial, etc.) for activity. **Table 3** provides an overview of the various kinds of data that can be selected in a NAPRALERT pharmacological query.

An important aspect of pharmacological activity queries to understand is their degree of target specificity. Often, dozens of related bioactivities (each with separate biological activity codes) are available. If a query for activity against a single pharmacological target is conducted, this will of course limit the number of compounds and/or organisms encountered in the report. A complete list of pharmacological activity tests and their corresponding activity codes can be downloaded from the NAPRALERT website for further reference.

Table 3 Overview of field names and types of data found in NAPRALERT pharmacology records

Pharmacology record type	*Number of different codes*	*Description*
Worktype	280	Type of work performed (*in vitro*, *in vivo*, human testing, etc.)
Major pharmacologic activity	16	Binary code for major pharmacological classes
Pharmacological activity code	4268	Five digit code for specific pharmacological activity studied
Alert codes	482	Provides additional experimental parameters for sorting efficiency
Extract type	475	Binary code used to identify type of extract studied
Mode of administration	107	Binary code used to identify mode of administration
Test species	264	Binary code used to describe type of animal used, if any
Animal gender	10	Sex of above animal if appropriate
Dose expression	18	Identifies type of dose (LD_{50}, MIC_{90}, etc.)
Per unit weight	43	Per unit weight (mg kg^{-1}, per plate, etc.)
Dose unit	48	Dose unit (mg, millimoles, etc.)
Qualitative result	6	Qualitative expression of result (active, equivocal, etc.)
Pathological system	6811	Alphanumeric code for disease test organism, substrate, or tissue used

3.04.1.1.4 Ethnomedical queries

Ethnomedical information provided through the organism query provides access to information on the traditional uses of a given organism. For plants, NAPRALERT currently cites 11 353 different publications containing ethnomedical data for over 15 000 distinct species – with 76 358 different reports of ethnomedical activity and 28 107 unique biological activities.

Traditional medical systems that are ancient and highly codified, including traditional Chinese medicine (TCM), ayurveda, and unani medicines, increasingly serve to guide researchers interested in the clinical potential of natural product medicines or botanical dietary supplements. NAPRALERT currently contains nearly 2500 distinct species (12 582 unique species-treatment record combinations) used in traditional medical systems in India, and over 1800 distinct species (5254 distinct species-treatment record combinations) used in TCM.

3.04.1.2 The Full Potential

The fully relational aspects of the NAPRALERT database allows for complex queries but is only available off-line. A user might be interested in identifying biological targets that meet certain criteria: activity data only from human or animal studies, for example, or *in vitro* test results limited to activity found below a certain concentration. Results of online searches of the database can contain large amounts of data, some of which may or may not be of particular interest to the user, negative biological activity testing data, for example. Off-line searches can select only data that is of interest to the user; examples are presented here to give the reader some idea of the unique data mining possibilities available with NAPRALERT.

3.04.1.2.1 Overview of the data structure

Constructing complex queries requires that the users have some basic knowledge of the data structure and the interrelationships between various types of information provided, so we present the following brief overview of NAPRALERT data structure.

All of the data in NAPRALERT derive from published literature sources in the areas of pharmacognosy, natural products drug discovery, medicinal plant research, ethnobotany/ethnomedicine, chemotaxonomy, botanical dietary supplements research, clinical and/or human studies, and related fields. Each manuscript coded in NAPRALERT contains some combination of basic experimental, chemical, geographic, demographic, taxonomic, or chemical data. These data are excerpted, coded, and entered into the database. NAPRALERT also contains a large number of review articles for which there is no original experimental data concerning the chemistry and/or biological activity of plants and/or pure compounds, for which only the citation is retrievable. For example, there are currently 59 review articles for *Cimicifuga racemosa* and 27 reviews of *Aloe vera.*

A reference to each literature source is created, which is the unique identifier (citation number) that fundamentally links all of the data from that citation, as it is distributed in the various tables across the database. There are currently more than 50 different tables utilized by NAPRALERT, but the most important to understanding how the database works include the following five: bibliography, organism, pharmacology, chemical, and worktype.

Bibliographic information, including the unique identifier (citation number) alluded to in the preceding paragraph, may contain the following: the name of the journal or publication, volume, issue, first and last pages, year of publication, language, article type (research article, scientific review, dissertation, and so on – if the data were found in an abstract, specific information identifying the abstract source and abstract number), article or chapter title, author(s), and date of entry into the database. Demographic data on the scientific institution with which the author(s) are associated, along with addresses, both standard post and e-mail, if available, are included.

Organism information is subdivided into three distinct data structures: organism, synonym, and taxon tables. The organism and synonym tables are tied together by the unique identifier (citation number) mentioned above, but the taxon table is linked to the organism and synonym tables only through nomenclature. The taxon table is the final arbiter of species names as they appear on a NAPRALERT report, and is not directly accessed by users. Online users have the capacity to freely search the database by common name as well as Latin binomial through the 'verify names' query.

The organism table, linked by the citation number to the rest of the database, may contain the following: organism number, organism class code (unique to NAPRALERT – containing various taxonomic kingdoms, it distinguishes between monocot, dicot, gymnosperm, algae, lichen, marine sponge, arthropod, echinoderm, etc.), family, genus, species, species authority, subspecies name, and subspecies authority.

Higher plant family taxonomy is based on the Engler system of classification. The subspecies record can be used to convey information about cultivar, strain, hybrid designation, and other information. In addition, the organism table contains data on the part of the organism used (leaf, stem, root, etc.), its condition (fresh, dried, frozen, etc.), and the geographical area from where the sample was collected, and whether it was cultivated or not.

The synonym table contains the unique identifier (citation number), as well as organism number found in the organism table, and contains data on common names reported for that organism, along with synonyms, either given in the text of the article or assigned by NAPRALERT staff, including author(s).

Chemical information is tied to the organism and pharmacological activity tables through the citation number. Multiple natural product isolates from a single organism can therefore be linked individually to specific pharmacological activities. Compound coding in NAPRALERT was designed to permit sorting and retrieval by compound class, functional group, as well as by a compound name and its chemical derivatives. NAPRALERT uses a unique coding system based on compound class, followed by substructure, then functional group(s). Full utilization of these parameters for complex queries is only available off-line.

Pharmacology (biochemical) information, sometimes referred to as experimental data, is linked by citation number to the rest of the database, as well as by a pharmacology record number, which is a sequentially assigned number that ties an organism to the various activity codes reported for it. The heart of the pharmacology table is the activity code, also known collectively as MPA/SPA codes – these are hexadecimal identifiers containing information on major (first two digits) and specific (final four digits) pharmacological activity. NAPRALERT has over 5000 MPA/SPA codes at present, allowing our users a great deal of specificity in terms of the kinds of queries they can perform; online users have the option to download a spreadsheet of all of the currently used MPA/SPA codes in order to refine their pharmacological activity queries (see **Table 3**).

In addition to the MPA/SPA codes, the pharmacology table may contain the following kinds of data: the extract type (not used in the case of pure compounds), a number of fields relating to the administration of an extract or compound, a number of fields relating to the results of the experiments performed, a number of fields relating to the pathological system being investigated, outlined below, as well as a catch-all known as the disease field, which is a text box available to enter any additional data, clarifications, complex descriptors of experimental design, results of clinical trials, or other miscellaneous information that is pertinent to the pharmacological activity being reported.

Fields in the pharmacology table relating to the administration of a natural product include the route of administration, animal and sex of animal (used for animal and human studies), the concentration and frequency of dosing, or quantitative measures of efficacy reported (LD_{50}, EC_{50}, IC_{90}, etc.), dose, units of the dose, units against which the dose is calibrated (grams per kilogram, micrograms per milliliter, etc.).

Fields in the pharmacology table relating to reported experimental results include qualitative assessment (strong, positive, weak, equivocal, or negative activities as defined by NAPRALERT, based on the experimental data) or quantitative assessment (pertaining particularly to anticancer studies that report on percentage of lifespan increase or tumor weight decrease) of the experimental data.

Pathological system codes can be used to define, for example, the infectious organism, cancer cell line, biochemical substrate, or tissue used in an *in vitro* or animal model test system. In the case of more complex experimental systems, the disease field can be used to clarify the nature of the study.

In addition, the pharmacology table contains data on the yield of pure isolates from crude natural product sources, as well as alert codes that provide descriptions of commonly utilized test systems, statistical significance reported for experimental results, and other data that are frequently used by NAPRALERT coders of pharmacological activity.

The worktype ties together the disparate data from the organism, chemistry, and pharmacological activity tables. Worktype is used to broadly represent the type of pharmacological activity being conducted (human, animal, or *in vitro* study; pure compound or extract tested; or ethnomedical utilization), or the methodology used in compound isolation and identification.

3.04.2 Relational Search Strategies

3.04.2.1 Practical Considerations

While database structure imposes the mechanics of a query, the nature of a query depends entirely on the interest of the user and the data at hand. NAPRALERT represents a unique database tool for natural product researchers, both because of its relational structure as well as the qualitative and quantitative nature of data it contains. Now that the reader has a basic understanding of the data and their relational structure, the database can now be manipulated in some interesting ways. We present a few examples of the kinds of off-line searches that can be conducted.

One example might be to produce a list of food plants for which potential cancer-preventive activities are reported. NAPRALERT can quickly compare 1420 edible plant records against 2038 reports of cancer-preventive activity from plants, resulting in a list of 255 plant species that are edible and have been reported to have positive results in a variety of assays, including *in vitro*, hollow fiber, organ culture, *in vivo*, and clinical/human studies (anticancer, tumor promotion inhibition, antitumor promotion activities).

A query for extracts reported to contain antibacterial activity, with data limited to test results at or below a $25\,\mu g\,ml^{-1}$ cutoff provides a list of the most bioactive of extracts. NAPRALERT currently reports 4902 organisms with antibacterial activity below cutoff, with 16 additional organisms showing antibacterial activity at the $ng\,ml^{-1}$ level. Further refinement could target a specific screening organism or model system of interest, or follow up on the known chemistry of those bioactive organisms, as well the activity of these isolates.

3.04.2.2 Chemotaxonomic Search Strategies

With its unique collection of chemical and botanical data NAPRALERT can be a useful tool to look for evidence of chemotaxonomic relationships in the plant kingdom.

Plant secondary metabolites are chemical compounds for which no apparent function in the primary metabolism of the biosynthetic organism can be discerned. Secondary metabolites have a long history of use as drugs. They tend to be of restricted taxonomic distribution, and also tend to affect the primary metabolism of the organisms that are exposed to them.

The database can be a useful search for patterns of distribution in the secondary chemistry of plant taxa at a variety of hierarchical levels, both taxonomic and chemical. Utilizing patterns of distribution for a biosynthetically distinct class of compound within the plant kingdom, certain plants could be prioritized for investigation, for example, based on indications of chemotaxonomic affiliation with a discreet plant source of a bioactive compound of interest.

To give the reader some general idea of the scope of the phytochemical information available in NAPRALERT, we found 31 694 distinct plant species in the database that have a chemical record, with 183 014 distinct compounds reported in these plants. This yields a total of 1 478 358 unique compound/species relationships.

Let us start out by exploring indole alkaloids found in nature. NAPRALERT currently identifies 2127 distinct species from which indole alkaloids have been isolated, in 893 genera and 294 families. Substructure searches can be conducted as well. We then continue our search by looking for restricted distribution of carbon skeleton-unsubstituted beta carboline alkaloids. NAPRALERT shows this subclass of indole alkaloid to be limited to 16 species in 15 genera and 14 families.

Plant secondary compounds are neither synthesized nor distributed evenly throughout an organism. NAPRALERT can be used to explore patterns of distribution within the various parts of a particular species.

One of these 16 species, the tree *Alstonia macrophylla* Wall. ex G. Don, in the Apocynaceae family, was found to contain 12 distinct unsubstituted beta carbolines in its leaves, 3 more in its root bark, 1 in its latex, and 4 more in its stem bark. Furthermore, a total of 26 indole alkaloids are reported for the plant, 13 found in leaf, 11 in bark, 1 in fruit, and 1 in latex.

3.04.2.3 Ethnomedical and Search Strategies

Consider the following scenario: A research group wishes to investigate plants as a source of new clinically useful treatments in the area of diabetes. The model that we present here begins with ethnomedical evidence. NAPRALERT is the best resource available for integrating traditional medical models not only for those with an interest in natural product drug discovery programs, but with those concerned about safe and effective botanical products, or developing an approach toward integrating rational regional traditional medical practices to health care.

Traditional medicines have historically proven very successful in identifying new drug entities: One review has identified over 100 compounds used globally as drugs today, whose current uses highly correlate with the traditional indication.[1]

The purported advantage of ethnomedical leads is based on the assumption that:

1. The traditional remedy is pharmacologically active.
2. The traditional remedy is not acutely toxic.
3. The traditional remedy is biologically available.

Our search strategy (see **Table 4**) is to select all plant species in NAPRALERT with ethnomedical records of antidiabetic use. These must have also provided positive test results in both human and animal models. They must also have no reports of acute toxicity or hepatotoxicity. Because our hypothetical query is based on a drug discovery model, these plant species must have no known chemistry that accounts for the antidiabetic activity (**Figure 1**).

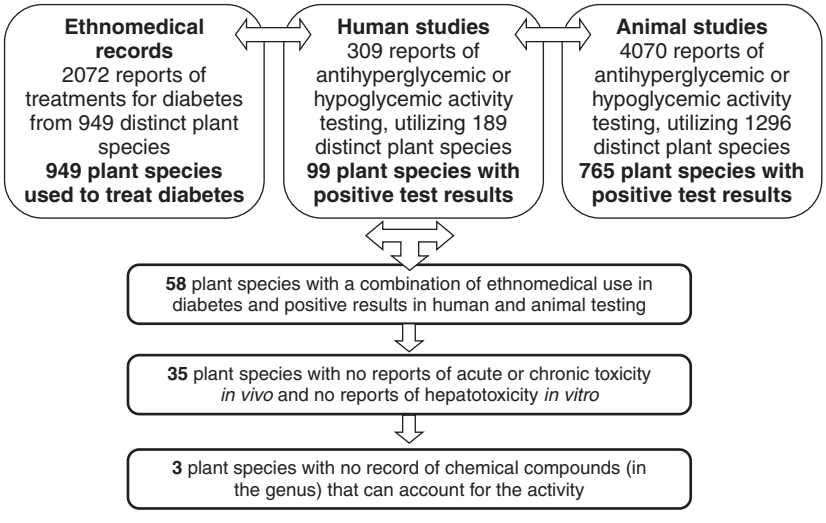


Figure 1 Organigram of combined ethnomedical search strategy.

While NAPRALERT contains more than 80 pharmacological activity codes that relate to carbohydrate metabolism, insulin secretion and metabolism, hypo- and hyperglycemia, and complications of diabetes, our search criteria utilizes only two: antihyperglycemic activity (pharmacology code 17007) and hypoglycemic activity (pharmacology code 17006).

The database returned records for 949 plant species reported to be used in ethnomedicine to treat diabetes. In addition, the database shows records for 189 species tested in humans, and 1296 plant species tested for in animals for hypoglycemic and antihyperglycemic activities. Eighteen of these species have also been clinically investigated for their antidiabetic activity.

NAPRALERT, at the time this query was conducted, produced 6451 records pertinent to our search strategy. This total excludes negative reports of bioactivity testing.

Think for a moment about the scale of this same search strategy applied to textual database.

In some cases abstracts contain qualitative and quantitative data that can be extracted from a paper relatively easily, but this is probably the exception for the natural products literature. Having been employed in systematically identifying, obtaining, extracting, coding, and entering data from the world's scientific literature on natural products for the past 30 years, we can appreciate the effort involved in just reading 6451 articles. This highlights the advantage of NAPRALERT to any similarly comprehensive textually based natural product database.

Limiting our data to ethnomedical plant species with positive test results in both human and animal experiments, 1813 possible species were reduced to 58 distinct species distributed in 54 different plant genera. These 58 species were searched for results of acute and/or chronic toxicity testing as well as reports of hepatotoxic activity. Of these, 25 species had no report of toxicity either in the species or genus. An additional 10 species had reports of toxicity in the genus but not the species.

These 35 species underwent further analysis, based on their known chemical profile. We compared each species' known chemistry with a list made up of 1576 compounds having positive antihyperglycemic or hypoglycemic activity.

Of the 35 species for which chemical comparisons were conducted, three species were identified for which there were no records of chemistry for the species that might account for the biological activity. Three taxa had no known chemistry for which to account for the biological activity. Out of 1813 possible plant species with antihypertensive and hypotensive activity *in vivo*, these three taxa represent our highest priority leads.

3.04.3 Summary and Future Prospects

3.04.3.1 Online Databases

Digital exchange of data has exploded since the launch of the INTERactive NETwork in 1983; today there are countless databases available to internet users around the globe, including a plethora of natural-product-oriented sites. This overview is by no means an exhaustive review of online natural product databases, but provides an interesting comparison of NAPRALERT with other available online natural products databases.

3.04.3.1.1 Early databases/chemical/biochemical/applied

1. The STN (Scientific & Technical Information Network) database first came online in 1984. It was licensed by STN International (http://www.stn-international.com/) through a joint collaboration of Chemical Abstracts Services (CAS), the Japan Science and Technology Corporation, and Fachinformationzentrum Karlsruhe. STN does not utilize a graphics interface – queries are conducted in text using a specialized command language.
2. NAPRALERT was available online from 1985 through 2000, with query protocols conducted by e-mail through the University of Illinois at Chicago. NAPRALERT was integrated into the STN database family in 1997.
3. The Beilstein database (http://www.beilstein.com/) is the electronic analogue of the *Beilstein Handbook of Organic Chemistry*,[2] providing data for over 10 million organic compounds. It can be searched by chemical

structure or substructure, reactions and properties, as well as by keyword, through the crossfire web interface. First available online in 1991, unlike the handbook, which is in German, the database is primarily in English.

4. The SciFinder databases, launched by Chemical Abstracts Service in 1998, (http://www.cas.org/products/scifindr/index.html) is operated through a graphics interface. SciFinderScholar, a version designed for universities and other academic institutions that lacks some supplementary features has quickly become one of the most widely used chemical registry, bibliographic, and abstracting services.
5. The *Dictionary of Natural Products* online is a searchable dictionary that contains the natural product data (http://dnp.chemnetbase.com/intro/index.jsp) that makes up a subset of the CHEMnetBASE, an online database (http://www.chemnetbase.com/) maintained by Chapman & Hall/CRC Press. The online version of the *Dictionary of Natural Products* derives from the well-known *Dictionary of Organic Compounds*,[3] which, since its inception in the 1930s has, through successive editions, always been a leading source of natural product information, the print version of which is no longer being updated.
6. MEDLINE (Medical Literature Analysis and Retrieval System) is a literature database supported by the United States National Library of Medicine. The major foci of this database are the life sciences and biomedical experiments. It covers much of the literature in biology and biochemistry, including aspects relevant to the biomedical sciences and health care. MEDLINE utilizes Medical Subject Headings (MeSH) for information retrieval, combining MeSH terms, words searches in abstract and title, author names, publication date, and other text-based parameters. PubMed (http://www.ncbi.nlm.nih.gov/ PubMed) is a part of the Entrez information retrieval system. Entrez allows users to find articles of a similar nature by linking them through an algorithm that predicts similarity of word content of abstracts and titles.
7. PubChem (http://pubchem.ncbi.nlm.nih.gov/) is a freely accessible database of chemical molecules maintained by the National Center for Biotechnology Information (NCBI), a component of the National Library of Medicine, US National Institutes of Health (NIH). It was launched in 2004 as part of the Molecular Libraries initiative of the NIH Roadmap for Medical Research to link chemical information with biomedical research data and clinical information.

3.04.3.1.2 Specialty databases/clinical/botanical dietary supplements

1. The Natural Medicines Comprehensive Database, Therapeutic Research Center, Stockton, California (http://www.naturaldatabase.com).This database provides monographs on a number of commonly available natural-product-based remedies and botanical dietary supplements. This database is available online by subscription in both a professional as well as a consumer version.

   The professional version is searchable by product name, disease condition, or by natural product/drug interaction. Each product is presented fully in monograph form, including literature citations, with data organized by common-name synonyms of the product (i.e., Ginkgo has the following synonymy: Adiantifolia, Bai Guo Ye, Baiguo, Fossil Tree, Ginkgo Folium, Herba Ginkgo Biloba, Japanese Silver Apricot, Kew Tree, Maidenhair Tree, Salisburia Adiantifolia, Yinhsing); scientific name (species binomial and family); medical condition for which the product is commonly used; safety testing (*in vitro*, *in vivo*, and clinical results); effectiveness of product (clinical review); mechanism of action; adverse reactions; possible interactions with other herbs and supplements; interactions with drugs (with a color coded rating scale); food interactions; known interactions with laboratory tests; potential for effect of product on disease conditions; dosage/administration ranges for product; and editor's comments on the product. The professional version is available in a PDA version, which can be operated on digital handheld computers (palm/pocket PC).

   A consumer version provides information for the same product range, but is presented in layman's terms, without literature citations, and the monographs are organized by common-name synonyms of the product; a general overview of the product (what is it); a generalized effectiveness rating for the product across a number of disease conditions; a generalized synopsis of mechanism of action (how does it work); a generalized synopsis of safety concerns; and interactions with drugs (the same as presented in the professional version, with color coded safety rating).

2. Food, Herbs and Supplements Database, by Natural Standard, Cambridge, Massachusetts (http://www.naturalstandard.com).

   Natural Standard is an international research collaboration that aggregates and synthesizes data on complementary and alternative therapies. The Natural Standard Food, Herbs and Supplements database is available online by subscription, and contains information on a number of commonly used natural product medicines in monograph form. Individual subscriptions for this database are not available.

   The Food, Herbs and Supplements database is searchable by product type: herbs and supplements, functional foods, vitamins and minerals, as well as brand names. Additional information is provided through the interactions and symptom checker tools. The conditions index (symptom checker tool) provides links to the herbal monographs for specific therapies, which are graded according to the scientific evidence supporting their use, and the basic interaction checker tool presents data for known drug interactions with products from the herb and supplements list.

   Professional monographs are organized by product common name, with synonyms/common names/related substances (including brand names) included. Data are presented in the areas of clinical effectiveness, dosing/toxicology, precautions/contraindications, interactions, mechanism of action, history, evidence table, with discussion of individual treatment modalities, a list of products that have been investigated clinically, along with bibliographic references, many with links to abstracts.

   The website contains a 'blog' section with news about recent developments in the area of integrative medicine research, as well as access to online continuing education modules in integrative medicine for health professionals (accredited by the Accreditation Council for Pharmacy Education).
3. HerbMed by the Alternative Medicine Foundation, Potomac, Maryland (http://www.herbmed.org/)
   The site provides monographs of commonly used botanical dietary supplements, with dynamic links to publications.
4. Herbs at a Glance – Dietary Supplement Fact Sheets, Office of Dietary Supplements, National Center for Complementary and Alternative Medicine, US National Institutes of Health (http://ods.od.nih.gov/Health_Information/Information_About_Individual_Dietary_Supplements.aspx)

   Herbs at a Glance presents overviews of a number of the most commonly used botanical dietary supplements in the United States. Presented in layman's terms, these fact sheets present an introduction, synonyms, Latin name, history, preparation, scientific evidence for its use, as well as side effects and cautions.
5. The International Bibliographic Information on Dietary Supplements (IBIDS) from the Office of Dietary Supplements, US National Institutes of Health and the Food and Nutrition Information Center (FNIC), National Agricultural Library (NAL), Agricultural Research Service, United States Department of Agriculture (USDA) (http://grande.nal.usda.gov/ibids/index.php)

   Bibliographic data and abstracts on the topic of dietary supplements from four major database sources: biomedical-related articles from MEDLINE, botanical and agricultural science from AGRICOLA (http://www.agricola.nal.usda.gov), worldwide agricultural literature through AGRIS (http://www.fao.org/Agris/), and coverage of international applied life sciences literature from CAB Abstracts and CAB Global Health (http://www.cabi.org).
6. The Cochrane Database of Systematic Reviews (http://www.cochrane.org)Cochrane Reviews are full text articles reviewing the clinical effectiveness of health care. While not limited to the use of natural product medicines, the Cochrane database contains a number (78 at present) of systematic reviews of clinical evaluations of herbal products, with data from over 2000 clinical trials. Full reviews are available for purchase online from the Cochrane Library website. Abstracts are available for free from the US National Library of Medicine.

3.04.3.1.3 Specialty databases/ethnomedicine

1. The Plants for a Future Database (Ethnobotany, including medicinal and edible plant species at http://www.pfaf.org/database/index.php) at Leeds University, with over 7000 species represented. Searchable by plant common name, family, use, geographical area, habitat, or keyword.
2. The phytochemical and ethnobotanical databases (http://www.ars-grin.gov/duke/) compiled by Jim Duke and maintained by the Agricultural Research Service, USDA. Users can search by plant, chemistry, biological activity, or ethnomedical use.

3. The Native American Ethnobotany database at the University of Michigan (http://herb.umd.umich.edu/) provides an online searchable database of foods, drugs, dyes, and fibers of Native North American peoples.
4. A number of TCM databases are available online, of which we will limit our discussion to those available in English. The traditional Chinese medicine information database, containing (http://tcm.cz3.nus.edu.sg/group/tcm-id/tcmid_ns.asp) data on formulations, herbal composition, clinical indication, and application of TCM, along with relevant literature currently contains entries for 1197 formulae, 1098 medicinal herbs, and 9852 herbal ingredients. Another example is the TCM Basics website (http://www.tcmbasics.com), which can be characterized as containing introductions to the basic theories of TCM, along with monographs of a more limited number of species. Introductions to various aspects of TCM theory and practice are presented, along with monographs of 116 herbs.
5. The traditional Indian medicines databases contain online pharmacopoeia of ayurveda, unani, and siddha (http://indianmedicine.nic.in/welcome.html) medicines. The site is hosted by the Department of Ayurveda, Yoga, Unani, Siddha and Homeopathic Medicine, Ministry of Health and Family Welfare, Government of India, and also contains historical background and introduction to the basic concepts and principles of the various forms of Indian traditional medicine, as well as formulary and pharmacopoeia.

3.04.4 NAPRALERT in Perspective

As an aid for the discovery of novel bioactive compounds, NAPRALERT represents a truly unique and invaluable resource. We hope that this overview of the database has provided the reader with a better appreciation for some of the other applications to which NAPRALERT can be applied. As for the databases mentioned above, in many cases, these can be used by researchers to complement and expand upon the information provided by NAPRALERT.

The past decade has witnessed a rapid acceleration, diversification, and expansion of publication in the area of natural products. As natural products continue to serve, and increase in impact, as resources in traditional and complementary medicine, botanical dietary supplement use, and in drug discovery, we expect that natural product databases will continue to expand and evolve right along with them. In fact, as data on natural product expands, comprehensive databases such as NAPRALERT will become increasingly valuable research tools.

References

1. D. S. Fabricant; N. R. Farnsworth, *Environ. Health Perspect.* **2001**, *109* (Suppl. 1), 69–75.
2. R. Luckenbach, Ed., *Beilstein Handbook of Organic Chemistry;* Beilstein-Institut für Literatur der Organischen Chemie. Springer-Verlag, 1996; Vol. 27.
3. J. I. G. Cadogan, *et al.,* Eds., *The Dictionary of Organic Compounds*, 6th ed.; Chapman & Hall, CRC Press, 1996.

Biographical Sketches

James G. Graham is adjunct assistant professor in the Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago. Dr. Graham is coeditor of NAPRALERT, as well as a research associate in botany at the Field Museum of Natural History in Chicago. He received his Ph.D. in 2001 from the University of Illinois at Chicago.

Norman R. Farnsworth is research professor of pharmacognosy at the College of Pharmacy, University of Illinois at Chicago (UIC), where he also directs the Program for Collaborative Research in the Pharmaceutical Sciences. Professor Farnsworth has the distinction of being senior university scholar as well as a UIC Distinguished Professor. He developed the UIC/World Health Organization (WHO) Collaborating Center for Traditional Medicine, one of the world's leading natural products and medicinal plant research centers, and also serves as director of the UIC/National Institutes of Health (NIH) Dietary Supplements Research Center.

Professor Farnsworth received his Ph.D. in 1959 from the University of Pittsburgh. He helped implement the first Ph.D. program in pharmacognosy during his tenure there and was the first to chair this program after he became a professor. He taught at the University of Pittsburgh until 1970 and then moved to the College of Pharmacy at the University of Illinois at Chicago. He pioneered natural products databases with the founding of NAPRALERT, launched at UIC in 1975.

3.04 The NAPRALERT Database as an Aid for Discovery of Novel Bioactive Compounds

James G. Graham and Norman R. Farnsworth, University of Illinois at Chicago, Chicago, IL, USA

3.04.1 Introduction

NAPRALERT is a database housed at the University of Illinois at Chicago that contains data on the geography, taxonomy, chemistry, medicinal folklore, and biological activities of natural products, including clinical trials, of their extracts and isolates. NAPRALERT stands for natural products alert; when it was started as a computer database in 1975 a prime consideration was to develop the capability to evaluate the natural products literature for the purpose of identifying new sources of commercially significant or clinically useful drugs.

Now, more than three decades later, NAPRALERT contains data on upward of 60 000 species, including more than 200 000 distinct chemical compounds of natural origin and 90 000 reports of ethnomedical uses of plants and other organisms. Data on more than 770 000 unique pharmacological records, representing more than 4000 different pharmacological activities are coded in NAPRALERT. These data have been extracted from over 200 000 scientific articles and reviews from nearly 10 000 scientific journals, representing organisms from all countries of the world.

NAPRALERT users tend to be engaged in medicinal plant research, drug development, or the botanical dietary supplement industry, and we have developed a series of standard search requests – a combination of organism-based, chemically based, or pharmacologically based queries – that can be executed online. The NAPRALERT database can be consulted at our website: http://www.napralert.org. (All internet addresses cited in this chapter were functional as of October 2008.)

Our coverage of the literature in the following areas is quite comprehensive:

- Clinical studies of natural products (including safety)
- Natural products that affect sugar metabolism
- Natural products that affect mammalian reproduction
- Extracts and compounds that affect cancer growth

- Natural products and antiviral (including HIV/AIDS) activity
- Natural products and antitubercular activity
- Natural products and tropical diseases
- Ethnomedical information on more than 20 000 species of plants
- Metabolism and pharmacokinetics
- Natural products that affect plant growth
- Review articles on organisms at the genus and/or species levels and reviews of secondary metabolites (citations only)
- Chemoprevention with natural products
- Natural insecticides
- Anti-inflammatory activity of natural products
- Analgesic activity of natural products
- Plant growth stimulators and inhibitors

NAPRALERT was designed as a fully relational database, rather than as a textual database. A growing number of digital resources, including natural product databases, are becoming available. Most of these are textual – they contain short abstracts summarizing literature reports, which refer back to a bibliographic reference that the user must read and summarize. Given the fact that even a single article in the natural product literature may contain multiple species, multiple biological activity tests conducted on multiple chemical isolates or extract types at different concentrations, the sheer abundance of data that is now available, and the inability of an individual to process it all, even with the development of the so-called 'high-quality text mining' software, provides a major hurdle to effective data extraction and manipulation, highlighting one of the unique attributes of NAPRALERT – rapid, precise, specialized data retrieval.

NAPRALERT, in contrast to a textual database, codes each of the different species, activities, compounds, extracts, and/or concentrations in a separate file, across a number of different tables. This means that multiple synchronous searches can be conducted, sorting the millions of records contained in the tables across the database in an extremely powerful way. Highly specific queries can be conducted, some of which will be illustrated here. In order to identify search criteria that will yield the most useful results, an understanding of the database architecture is critical. We will present an overview of the NAPRALERT data structure, followed by a few examples to illustrate how the database can be applied to problem solving in natural products drug discovery. In addition, we provide a brief review of other online databases.

The ability to conduct complex queries online is limited. Interested users may direct specific requests to NAPRALERT staff, whose contacts can be found on our website.

3.04.1.1 Online Searches

Three general types of online queries are organism, chemical, or pharmacologically based searches. Organism-based queries provide the user with a selection of ethnomedical, pharmacological, and/or chemical data that correspond to a particular organism of interest. Chemically based queries provide pharmacological testing results for a particular compound, or a list of organisms known to contain it. Pharmacologically based queries provide testing data for organisms or compounds that have been evaluated for a specific biological activity.

3.04.1.1.1 Organism-based queries

NAPRALERT currently cites 142 947 publications, referring to 61 116 different organisms, including 45 392 different plant species (**Table 1**). Organism-based queries can be conducted online at the family, genus, species, or even variety level. Geographic origin, by region and country, can also be searched, as well as the organism part. In cases of common names, taxonomic uncertainty, or synonymy, a 'verify names' option allows the user to search by an organism's common name or taxonomic synonym, and provides the species (binomial) nomenclature recognized as valid by the database.

Table 1 Overview of the plant data found in the NAPRALERT organism table

Class of organism	*Genera*	*Species*	*Compounds*
Monocots	962	5141	15 425
Dicots	5853	41 135	139 025
Gymnosperms	81	742	6269
Pteridophytes	210	1454	2909
Bryophytes	215	640	3045
Lichens	191	904	1399

3.04.1.1.2 Chemical queries

NAPRALERT currently cites 154 766 publications referring to 207 575 distinct chemical compounds. The chemical names used in NAPRALERT begin with a base name, which corresponds to the parent structure of a compound, usually derived from the largest ring or straight-chain carbon skeleton in the structure (see **Table 2**). Listed below are some examples.

- For oleanolic acid 3 acetate, data have been entered as oleanolic acid-3-acetate.
- For 3 acetoxy oleanolic acid, data have been entered as oleanolic acid,3-acetoxy:
- For 8 methoxy quercetin, data have been entered as quercetin,8-methoxy:

Table 2 Overview of chemical classes and number of representatives found in NAPRALERT

Class of compound	*Number*
Structure undetermined	721
Miscellaneous	1316
Alkane	1695
Alkene	1619
Alkyne	115
Alkenyne	1482
Lipid	4339
Carbohydrate	3653
Proteid	9309
Flavonoid	20 576
Polycyclic	978
Oxygen heterocycle	3896
Quinoid	5327
Benzenoid	10 166
Sulfur compound	1843
Alicyclic	1175
Nonalkaloid *N*-heterocycle	1266
Lignan	8243
Coumarin	4245
Xanthone	1713
Miscellaneous lactone	5045
Vitamin	92
Chromone	1055
Monoterpene	7156
Diterpene	23 128
Sesquiterpene	17 863
Sesterterpene	831
Triterpene	18 520
Steroid	7637
Sapogenin	2404
Cardenolide	1697

(*Continued*)

Table 2 (Continued)

Class of compound	*Number*
Carotenoid	1180
Polyprenoid	231
Alkaloid – miscellaneous	7941
Tropane alkaloid	591
Pyrrolizidine alkaloid	1025
Indolizidine alkaloid	417
Quinolizidine alkaloid	1069
Quinoline alkaloid	1554
Isoquinoline alkaloid	6753
Indole alkaloid	7251
Monoterpene alkaloid	155
Diterpene alkaloid	322
Sesquiterpene alkaloid	1530
Sesterterpene alkaloid	4
Triterpene alkaloid	152
Steroid alkaloid	1028
Peptide alkaloid	232
Lactam	426
Tetraterpenoid	22
Inorganic	599
Antibiotics (unknown structure)	1616

Substituent groups, if present, are appended to the base name. The base name approach allows for the use of wildcard searches, whereby a group of structurally related compounds can be queried in a single step. In some cases, common names of compounds widely distributed in nature (quercetin, beta-sitosterol, etc.) can be searched. A 'verify names' option is available to identify the best search parameter for the compound(s) of interest.

It should be noted that if substituents are present, the base name is followed by a comma and the substituents, followed by a colon (:). More information on substituents is listed below.

If you are interested in any compound with a base name present in the database, you can use % as a wild card. This will show you all the related compounds to the base name. For example, searching by the term oleanolic acid% will give you all the compounds present in the database that are derivatives of oleanolic acid.

3.04.1.1.2(i) Substituents

A. If substituents are present, the base name is followed by a comma followed by the substituents. For example, 2′-hydroxy flavone should be searched as flavone,2′-hydroxy:. Note that the last substituent after the base name should be followed by a colon – thus 6″-*O*-acetyl daidzin should be searched as daidzin,6″-o-acetyl:.
B. The substituents are arranged in alphabetic order after the base name; disregard prefixes such as di, tri, etc. Thus, 'dihydroxy' is alphabetized as if it began with 'h'.
   Example: 2,3-dihydroxy-4-methoxy-3′,7-dimethyl flavone – search as flavone,2-3-dihydroxy-4-methoxy-3′-7-dimethyl:
C. If the same substituent is present at different positions, they have been arranged in increasing order.
   Example: 5′,3′,4,6-tetrahydroxy flavone – search as flavone,3′-4-5′-6-tetrahydroxy:
   Example: 3′,4′,5-trihydroxy-3,6-dimethoxy flavone – search as flavone,3′-4′-5-trihydroxy-3-6-dimethoxy:
D. Hyphens separate numbers from letters and functionalities from one another.
   Example: 2,5-dimethoxy apigenin – search as apigenin,2-5-dimethoxy:
E. For hyphens within the base name, base names often include carbon numbers of unsaturations and structural details. Final vowels are dropped if the next letter following the number is a vowel. Compare these two examples – it will be seen that the final 'a' is dropped only in the second example.
   1. labda-8-13-diene
   2. labd-8-ene

F. Exceptions to the alphabetical rule for substituents are sugar moieties and parenthetical information.
   – Sugar (glycoside) groups. All glycosides are listed after the nonglycoside groups.
   – A space is left between the last group and the glycoside.
   Example: 4-methoxy flavone-7-*O*-Beta-D-glucoside – search as flavone,4-methoxy: 7-o-beta-D-glucoside
   Example: 3-*O*-Beta-D-glucopyranosyl-phytolaccagenic acid-28-*O*-Beta-D-glucopyranosyl ester – search as phytolaccagenic acid ,3-o-beta-D-glucopyranosyl: 28-o-Beta-D-glucopyranosyl ester
G. Functional groups, which are themselves substituted, are listed after the nonglycoside groups and no space is left. Parentheses enclose the substituted group, for example, flavone,4-methoxy-7-(2-hydroxy-ethyl):
H. Greek symbols are spelled out, that is, Alpha, Beta, etc.

3.04.1.1.2(ii) Prefixes

A. Prefixes that indicate an altered base structure are written immediately following the comma after the base name.
   Example: (+) dihydro kawain – search as kawain,dihydro: (+):
   Example: methyl ether of isoeugenol – search as eugenol,iso: methyl ether
   Example: isorhamnetin – search as rhamnetin,iso:
B. However, the prefixes bicyclo-, bis-, and cyclo- are not separated from the base name.
   Example: isobicyclogermacrene – search as bicyclogermacrene,iso:
C. Greek letters are spelled out in English – trans is used for E and cis is used for Z. E and Z always appear in front of the number (position) they represent.
   Example: deca-2Z,8E diene – search as deca-cis-2-trans-8-diene
   Example: cholesta-5,22Z-diene-3-Beta-ol – search as cholesta-5-cis-22-dien-3-beta-ol
D. Within a functional group parts are separated by a hyphen.
E. Example: 8-isovaleroyloxy kaurane – search as kaurane,8-iso-valeroyl-oxy:
F. Compounds with '#' sign at the end of some compounds indicates that two different compounds have been assigned the same name in different papers, for example,
   foetidin (steroid)
   foetidin# (coumarin)
   foetidin## (diterpene)
G. Compounds with an '*' indicates that they are synthetic, semisynthetic, or unnatural derivative compounds.
H. We have synonyms for most common compounds, for example, flavone,5-7-dihydroxy-8-methoxy: is synonymous with wogonin (wogonin is used in the database).
I. Some compound names are actually extracts, for example,
   sp-303
   policosanol
   silymarin
   pycnogenol

3.04.1.1.3 Pharmacological query

A pharmacologically based query typically involves searching for organisms (plant, animal, or bacterial extracts/preparations) and/or compounds that have been evaluated in a particular biological system (bacterial or cell culture, enzyme system, animal model, human clinical trial, etc.) for activity. **Table 3** provides an overview of the various kinds of data that can be selected in a NAPRALERT pharmacological query.

An important aspect of pharmacological activity queries to understand is their degree of target specificity. Often, dozens of related bioactivities (each with separate biological activity codes) are available. If a query for activity against a single pharmacological target is conducted, this will of course limit the number of compounds and/or organisms encountered in the report. A complete list of pharmacological activity tests and their corresponding activity codes can be downloaded from the NAPRALERT website for further reference.

Table 3 Overview of field names and types of data found in NAPRALERT pharmacology records

Pharmacology record type	*Number of different codes*	*Description*
Worktype	280	Type of work performed (*in vitro*, *in vivo*, human testing, etc.)
Major pharmacologic activity	16	Binary code for major pharmacological classes
Pharmacological activity code	4268	Five digit code for specific pharmacological activity studied
Alert codes	482	Provides additional experimental parameters for sorting efficiency
Extract type	475	Binary code used to identify type of extract studied
Mode of administration	107	Binary code used to identify mode of administration
Test species	264	Binary code used to describe type of animal used, if any
Animal gender	10	Sex of above animal if appropriate
Dose expression	18	Identifies type of dose (LD_{50}, MIC_{90}, etc.)
Per unit weight	43	Per unit weight (mg kg^{-1}, per plate, etc.)
Dose unit	48	Dose unit (mg, millimoles, etc.)
Qualitative result	6	Qualitative expression of result (active, equivocal, etc.)
Pathological system	6811	Alphanumeric code for disease test organism, substrate, or tissue used

3.04.1.1.4 Ethnomedical queries

Ethnomedical information provided through the organism query provides access to information on the traditional uses of a given organism. For plants, NAPRALERT currently cites 11 353 different publications containing ethnomedical data for over 15 000 distinct species – with 76 358 different reports of ethnomedical activity and 28 107 unique biological activities.

Traditional medical systems that are ancient and highly codified, including traditional Chinese medicine (TCM), ayurveda, and unani medicines, increasingly serve to guide researchers interested in the clinical potential of natural product medicines or botanical dietary supplements. NAPRALERT currently contains nearly 2500 distinct species (12 582 unique species-treatment record combinations) used in traditional medical systems in India, and over 1800 distinct species (5254 distinct species-treatment record combinations) used in TCM.

3.04.1.2 The Full Potential

The fully relational aspects of the NAPRALERT database allows for complex queries but is only available off-line. A user might be interested in identifying biological targets that meet certain criteria: activity data only from human or animal studies, for example, or *in vitro* test results limited to activity found below a certain concentration. Results of online searches of the database can contain large amounts of data, some of which may or may not be of particular interest to the user, negative biological activity testing data, for example. Off-line searches can select only data that is of interest to the user; examples are presented here to give the reader some idea of the unique data mining possibilities available with NAPRALERT.

3.04.1.2.1 Overview of the data structure

Constructing complex queries requires that the users have some basic knowledge of the data structure and the interrelationships between various types of information provided, so we present the following brief overview of NAPRALERT data structure.

All of the data in NAPRALERT derive from published literature sources in the areas of pharmacognosy, natural products drug discovery, medicinal plant research, ethnobotany/ethnomedicine, chemotaxonomy, botanical dietary supplements research, clinical and/or human studies, and related fields. Each manuscript coded in NAPRALERT contains some combination of basic experimental, chemical, geographic, demographic, taxonomic, or chemical data. These data are excerpted, coded, and entered into the database. NAPRALERT also contains a large number of review articles for which there is no original experimental data concerning the chemistry and/or biological activity of plants and/or pure compounds, for which only the citation is retrievable. For example, there are currently 59 review articles for *Cimicifuga racemosa* and 27 reviews of *Aloe vera.*

A reference to each literature source is created, which is the unique identifier (citation number) that fundamentally links all of the data from that citation, as it is distributed in the various tables across the database. There are currently more than 50 different tables utilized by NAPRALERT, but the most important to understanding how the database works include the following five: bibliography, organism, pharmacology, chemical, and worktype.

Bibliographic information, including the unique identifier (citation number) alluded to in the preceding paragraph, may contain the following: the name of the journal or publication, volume, issue, first and last pages, year of publication, language, article type (research article, scientific review, dissertation, and so on – if the data were found in an abstract, specific information identifying the abstract source and abstract number), article or chapter title, author(s), and date of entry into the database. Demographic data on the scientific institution with which the author(s) are associated, along with addresses, both standard post and e-mail, if available, are included.

Organism information is subdivided into three distinct data structures: organism, synonym, and taxon tables. The organism and synonym tables are tied together by the unique identifier (citation number) mentioned above, but the taxon table is linked to the organism and synonym tables only through nomenclature. The taxon table is the final arbiter of species names as they appear on a NAPRALERT report, and is not directly accessed by users. Online users have the capacity to freely search the database by common name as well as Latin binomial through the 'verify names' query.

The organism table, linked by the citation number to the rest of the database, may contain the following: organism number, organism class code (unique to NAPRALERT – containing various taxonomic kingdoms, it distinguishes between monocot, dicot, gymnosperm, algae, lichen, marine sponge, arthropod, echinoderm, etc.), family, genus, species, species authority, subspecies name, and subspecies authority.

Higher plant family taxonomy is based on the Engler system of classification. The subspecies record can be used to convey information about cultivar, strain, hybrid designation, and other information. In addition, the organism table contains data on the part of the organism used (leaf, stem, root, etc.), its condition (fresh, dried, frozen, etc.), and the geographical area from where the sample was collected, and whether it was cultivated or not.

The synonym table contains the unique identifier (citation number), as well as organism number found in the organism table, and contains data on common names reported for that organism, along with synonyms, either given in the text of the article or assigned by NAPRALERT staff, including author(s).

Chemical information is tied to the organism and pharmacological activity tables through the citation number. Multiple natural product isolates from a single organism can therefore be linked individually to specific pharmacological activities. Compound coding in NAPRALERT was designed to permit sorting and retrieval by compound class, functional group, as well as by a compound name and its chemical derivatives. NAPRALERT uses a unique coding system based on compound class, followed by substructure, then functional group(s). Full utilization of these parameters for complex queries is only available off-line.

Pharmacology (biochemical) information, sometimes referred to as experimental data, is linked by citation number to the rest of the database, as well as by a pharmacology record number, which is a sequentially assigned number that ties an organism to the various activity codes reported for it. The heart of the pharmacology table is the activity code, also known collectively as MPA/SPA codes – these are hexadecimal identifiers containing information on major (first two digits) and specific (final four digits) pharmacological activity. NAPRALERT has over 5000 MPA/SPA codes at present, allowing our users a great deal of specificity in terms of the kinds of queries they can perform; online users have the option to download a spreadsheet of all of the currently used MPA/SPA codes in order to refine their pharmacological activity queries (see **Table 3**).

In addition to the MPA/SPA codes, the pharmacology table may contain the following kinds of data: the extract type (not used in the case of pure compounds), a number of fields relating to the administration of an extract or compound, a number of fields relating to the results of the experiments performed, a number of fields relating to the pathological system being investigated, outlined below, as well as a catch-all known as the disease field, which is a text box available to enter any additional data, clarifications, complex descriptors of experimental design, results of clinical trials, or other miscellaneous information that is pertinent to the pharmacological activity being reported.

Fields in the pharmacology table relating to the administration of a natural product include the route of administration, animal and sex of animal (used for animal and human studies), the concentration and frequency of dosing, or quantitative measures of efficacy reported (LD_{50}, EC_{50}, IC_{90}, etc.), dose, units of the dose, units against which the dose is calibrated (grams per kilogram, micrograms per milliliter, etc.).

Fields in the pharmacology table relating to reported experimental results include qualitative assessment (strong, positive, weak, equivocal, or negative activities as defined by NAPRALERT, based on the experimental data) or quantitative assessment (pertaining particularly to anticancer studies that report on percentage of lifespan increase or tumor weight decrease) of the experimental data.

Pathological system codes can be used to define, for example, the infectious organism, cancer cell line, biochemical substrate, or tissue used in an *in vitro* or animal model test system. In the case of more complex experimental systems, the disease field can be used to clarify the nature of the study.

In addition, the pharmacology table contains data on the yield of pure isolates from crude natural product sources, as well as alert codes that provide descriptions of commonly utilized test systems, statistical significance reported for experimental results, and other data that are frequently used by NAPRALERT coders of pharmacological activity.

The worktype ties together the disparate data from the organism, chemistry, and pharmacological activity tables. Worktype is used to broadly represent the type of pharmacological activity being conducted (human, animal, or *in vitro* study; pure compound or extract tested; or ethnomedical utilization), or the methodology used in compound isolation and identification.

3.04.2 Relational Search Strategies

3.04.2.1 Practical Considerations

While database structure imposes the mechanics of a query, the nature of a query depends entirely on the interest of the user and the data at hand. NAPRALERT represents a unique database tool for natural product researchers, both because of its relational structure as well as the qualitative and quantitative nature of data it contains. Now that the reader has a basic understanding of the data and their relational structure, the database can now be manipulated in some interesting ways. We present a few examples of the kinds of off-line searches that can be conducted.

One example might be to produce a list of food plants for which potential cancer-preventive activities are reported. NAPRALERT can quickly compare 1420 edible plant records against 2038 reports of cancer-preventive activity from plants, resulting in a list of 255 plant species that are edible and have been reported to have positive results in a variety of assays, including *in vitro*, hollow fiber, organ culture, *in vivo*, and clinical/human studies (anticancer, tumor promotion inhibition, antitumor promotion activities).

A query for extracts reported to contain antibacterial activity, with data limited to test results at or below a $25\,\mu g\,ml^{-1}$ cutoff provides a list of the most bioactive of extracts. NAPRALERT currently reports 4902 organisms with antibacterial activity below cutoff, with 16 additional organisms showing antibacterial activity at the $ng\,ml^{-1}$ level. Further refinement could target a specific screening organism or model system of interest, or follow up on the known chemistry of those bioactive organisms, as well the activity of these isolates.

3.04.2.2 Chemotaxonomic Search Strategies

With its unique collection of chemical and botanical data NAPRALERT can be a useful tool to look for evidence of chemotaxonomic relationships in the plant kingdom.

Plant secondary metabolites are chemical compounds for which no apparent function in the primary metabolism of the biosynthetic organism can be discerned. Secondary metabolites have a long history of use as drugs. They tend to be of restricted taxonomic distribution, and also tend to affect the primary metabolism of the organisms that are exposed to them.

The database can be a useful search for patterns of distribution in the secondary chemistry of plant taxa at a variety of hierarchical levels, both taxonomic and chemical. Utilizing patterns of distribution for a biosynthetically distinct class of compound within the plant kingdom, certain plants could be prioritized for investigation, for example, based on indications of chemotaxonomic affiliation with a discreet plant source of a bioactive compound of interest.

To give the reader some general idea of the scope of the phytochemical information available in NAPRALERT, we found 31 694 distinct plant species in the database that have a chemical record, with 183 014 distinct compounds reported in these plants. This yields a total of 1 478 358 unique compound/species relationships.

Let us start out by exploring indole alkaloids found in nature. NAPRALERT currently identifies 2127 distinct species from which indole alkaloids have been isolated, in 893 genera and 294 families. Substructure searches can be conducted as well. We then continue our search by looking for restricted distribution of carbon skeleton-unsubstituted beta carboline alkaloids. NAPRALERT shows this subclass of indole alkaloid to be limited to 16 species in 15 genera and 14 families.

Plant secondary compounds are neither synthesized nor distributed evenly throughout an organism. NAPRALERT can be used to explore patterns of distribution within the various parts of a particular species.

One of these 16 species, the tree *Alstonia macrophylla* Wall. ex G. Don, in the Apocynaceae family, was found to contain 12 distinct unsubstituted beta carbolines in its leaves, 3 more in its root bark, 1 in its latex, and 4 more in its stem bark. Furthermore, a total of 26 indole alkaloids are reported for the plant, 13 found in leaf, 11 in bark, 1 in fruit, and 1 in latex.

3.04.2.3 Ethnomedical and Search Strategies

Consider the following scenario: A research group wishes to investigate plants as a source of new clinically useful treatments in the area of diabetes. The model that we present here begins with ethnomedical evidence. NAPRALERT is the best resource available for integrating traditional medical models not only for those with an interest in natural product drug discovery programs, but with those concerned about safe and effective botanical products, or developing an approach toward integrating rational regional traditional medical practices to health care.

Traditional medicines have historically proven very successful in identifying new drug entities: One review has identified over 100 compounds used globally as drugs today, whose current uses highly correlate with the traditional indication.[1]

The purported advantage of ethnomedical leads is based on the assumption that:

1. The traditional remedy is pharmacologically active.
2. The traditional remedy is not acutely toxic.
3. The traditional remedy is biologically available.

Our search strategy (see **Table 4**) is to select all plant species in NAPRALERT with ethnomedical records of antidiabetic use. These must have also provided positive test results in both human and animal models. They must also have no reports of acute toxicity or hepatotoxicity. Because our hypothetical query is based on a drug discovery model, these plant species must have no known chemistry that accounts for the antidiabetic activity (**Figure 1**).

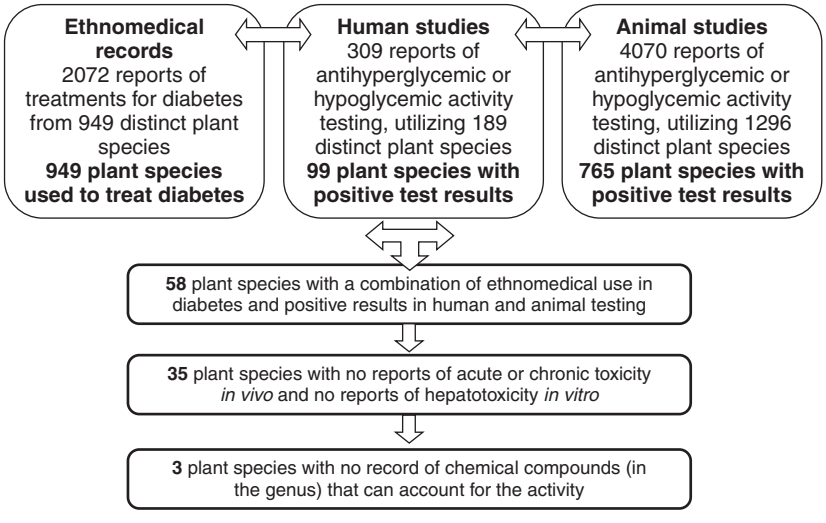


Figure 1 Organigram of combined ethnomedical search strategy.

While NAPRALERT contains more than 80 pharmacological activity codes that relate to carbohydrate metabolism, insulin secretion and metabolism, hypo- and hyperglycemia, and complications of diabetes, our search criteria utilizes only two: antihyperglycemic activity (pharmacology code 17007) and hypoglycemic activity (pharmacology code 17006).

The database returned records for 949 plant species reported to be used in ethnomedicine to treat diabetes. In addition, the database shows records for 189 species tested in humans, and 1296 plant species tested for in animals for hypoglycemic and antihyperglycemic activities. Eighteen of these species have also been clinically investigated for their antidiabetic activity.

NAPRALERT, at the time this query was conducted, produced 6451 records pertinent to our search strategy. This total excludes negative reports of bioactivity testing.

Think for a moment about the scale of this same search strategy applied to textual database.

In some cases abstracts contain qualitative and quantitative data that can be extracted from a paper relatively easily, but this is probably the exception for the natural products literature. Having been employed in systematically identifying, obtaining, extracting, coding, and entering data from the world's scientific literature on natural products for the past 30 years, we can appreciate the effort involved in just reading 6451 articles. This highlights the advantage of NAPRALERT to any similarly comprehensive textually based natural product database.

Limiting our data to ethnomedical plant species with positive test results in both human and animal experiments, 1813 possible species were reduced to 58 distinct species distributed in 54 different plant genera. These 58 species were searched for results of acute and/or chronic toxicity testing as well as reports of hepatotoxic activity. Of these, 25 species had no report of toxicity either in the species or genus. An additional 10 species had reports of toxicity in the genus but not the species.

These 35 species underwent further analysis, based on their known chemical profile. We compared each species' known chemistry with a list made up of 1576 compounds having positive antihyperglycemic or hypoglycemic activity.

Of the 35 species for which chemical comparisons were conducted, three species were identified for which there were no records of chemistry for the species that might account for the biological activity. Three taxa had no known chemistry for which to account for the biological activity. Out of 1813 possible plant species with antihypertensive and hypotensive activity *in vivo*, these three taxa represent our highest priority leads.

3.04.3 Summary and Future Prospects

3.04.3.1 Online Databases

Digital exchange of data has exploded since the launch of the INTERactive NETwork in 1983; today there are countless databases available to internet users around the globe, including a plethora of natural-product-oriented sites. This overview is by no means an exhaustive review of online natural product databases, but provides an interesting comparison of NAPRALERT with other available online natural products databases.

3.04.3.1.1 Early databases/chemical/biochemical/applied

1. The STN (Scientific & Technical Information Network) database first came online in 1984. It was licensed by STN International (http://www.stn-international.com/) through a joint collaboration of Chemical Abstracts Services (CAS), the Japan Science and Technology Corporation, and Fachinformationzentrum Karlsruhe. STN does not utilize a graphics interface – queries are conducted in text using a specialized command language.
2. NAPRALERT was available online from 1985 through 2000, with query protocols conducted by e-mail through the University of Illinois at Chicago. NAPRALERT was integrated into the STN database family in 1997.
3. The Beilstein database (http://www.beilstein.com/) is the electronic analogue of the *Beilstein Handbook of Organic Chemistry*,[2] providing data for over 10 million organic compounds. It can be searched by chemical

structure or substructure, reactions and properties, as well as by keyword, through the crossfire web interface. First available online in 1991, unlike the handbook, which is in German, the database is primarily in English.

4. The SciFinder databases, launched by Chemical Abstracts Service in 1998, (http://www.cas.org/products/scifindr/index.html) is operated through a graphics interface. SciFinderScholar, a version designed for universities and other academic institutions that lacks some supplementary features has quickly become one of the most widely used chemical registry, bibliographic, and abstracting services.
5. The *Dictionary of Natural Products* online is a searchable dictionary that contains the natural product data (http://dnp.chemnetbase.com/intro/index.jsp) that makes up a subset of the CHEMnetBASE, an online database (http://www.chemnetbase.com/) maintained by Chapman & Hall/CRC Press. The online version of the *Dictionary of Natural Products* derives from the well-known *Dictionary of Organic Compounds*,[3] which, since its inception in the 1930s has, through successive editions, always been a leading source of natural product information, the print version of which is no longer being updated.
6. MEDLINE (Medical Literature Analysis and Retrieval System) is a literature database supported by the United States National Library of Medicine. The major foci of this database are the life sciences and biomedical experiments. It covers much of the literature in biology and biochemistry, including aspects relevant to the biomedical sciences and health care. MEDLINE utilizes Medical Subject Headings (MeSH) for information retrieval, combining MeSH terms, words searches in abstract and title, author names, publication date, and other text-based parameters. PubMed (http://www.ncbi.nlm.nih.gov/ PubMed) is a part of the Entrez information retrieval system. Entrez allows users to find articles of a similar nature by linking them through an algorithm that predicts similarity of word content of abstracts and titles.
7. PubChem (http://pubchem.ncbi.nlm.nih.gov/) is a freely accessible database of chemical molecules maintained by the National Center for Biotechnology Information (NCBI), a component of the National Library of Medicine, US National Institutes of Health (NIH). It was launched in 2004 as part of the Molecular Libraries initiative of the NIH Roadmap for Medical Research to link chemical information with biomedical research data and clinical information.

3.04.3.1.2 Specialty databases/clinical/botanical dietary supplements

1. The Natural Medicines Comprehensive Database, Therapeutic Research Center, Stockton, California (http://www.naturaldatabase.com).This database provides monographs on a number of commonly available natural-product-based remedies and botanical dietary supplements. This database is available online by subscription in both a professional as well as a consumer version.

   The professional version is searchable by product name, disease condition, or by natural product/drug interaction. Each product is presented fully in monograph form, including literature citations, with data organized by common-name synonyms of the product (i.e., Ginkgo has the following synonymy: Adiantifolia, Bai Guo Ye, Baiguo, Fossil Tree, Ginkgo Folium, Herba Ginkgo Biloba, Japanese Silver Apricot, Kew Tree, Maidenhair Tree, Salisburia Adiantifolia, Yinhsing); scientific name (species binomial and family); medical condition for which the product is commonly used; safety testing (*in vitro*, *in vivo*, and clinical results); effectiveness of product (clinical review); mechanism of action; adverse reactions; possible interactions with other herbs and supplements; interactions with drugs (with a color coded rating scale); food interactions; known interactions with laboratory tests; potential for effect of product on disease conditions; dosage/administration ranges for product; and editor's comments on the product. The professional version is available in a PDA version, which can be operated on digital handheld computers (palm/pocket PC).

   A consumer version provides information for the same product range, but is presented in layman's terms, without literature citations, and the monographs are organized by common-name synonyms of the product; a general overview of the product (what is it); a generalized effectiveness rating for the product across a number of disease conditions; a generalized synopsis of mechanism of action (how does it work); a generalized synopsis of safety concerns; and interactions with drugs (the same as presented in the professional version, with color coded safety rating).

2. Food, Herbs and Supplements Database, by Natural Standard, Cambridge, Massachusetts (http://www.naturalstandard.com).

   Natural Standard is an international research collaboration that aggregates and synthesizes data on complementary and alternative therapies. The Natural Standard Food, Herbs and Supplements database is available online by subscription, and contains information on a number of commonly used natural product medicines in monograph form. Individual subscriptions for this database are not available.

   The Food, Herbs and Supplements database is searchable by product type: herbs and supplements, functional foods, vitamins and minerals, as well as brand names. Additional information is provided through the interactions and symptom checker tools. The conditions index (symptom checker tool) provides links to the herbal monographs for specific therapies, which are graded according to the scientific evidence supporting their use, and the basic interaction checker tool presents data for known drug interactions with products from the herb and supplements list.

   Professional monographs are organized by product common name, with synonyms/common names/related substances (including brand names) included. Data are presented in the areas of clinical effectiveness, dosing/toxicology, precautions/contraindications, interactions, mechanism of action, history, evidence table, with discussion of individual treatment modalities, a list of products that have been investigated clinically, along with bibliographic references, many with links to abstracts.

   The website contains a 'blog' section with news about recent developments in the area of integrative medicine research, as well as access to online continuing education modules in integrative medicine for health professionals (accredited by the Accreditation Council for Pharmacy Education).
3. HerbMed by the Alternative Medicine Foundation, Potomac, Maryland (http://www.herbmed.org/)
   The site provides monographs of commonly used botanical dietary supplements, with dynamic links to publications.
4. Herbs at a Glance – Dietary Supplement Fact Sheets, Office of Dietary Supplements, National Center for Complementary and Alternative Medicine, US National Institutes of Health (http://ods.od.nih.gov/Health_Information/Information_About_Individual_Dietary_Supplements.aspx)

   Herbs at a Glance presents overviews of a number of the most commonly used botanical dietary supplements in the United States. Presented in layman's terms, these fact sheets present an introduction, synonyms, Latin name, history, preparation, scientific evidence for its use, as well as side effects and cautions.
5. The International Bibliographic Information on Dietary Supplements (IBIDS) from the Office of Dietary Supplements, US National Institutes of Health and the Food and Nutrition Information Center (FNIC), National Agricultural Library (NAL), Agricultural Research Service, United States Department of Agriculture (USDA) (http://grande.nal.usda.gov/ibids/index.php)

   Bibliographic data and abstracts on the topic of dietary supplements from four major database sources: biomedical-related articles from MEDLINE, botanical and agricultural science from AGRICOLA (http://www.agricola.nal.usda.gov), worldwide agricultural literature through AGRIS (http://www.fao.org/Agris/), and coverage of international applied life sciences literature from CAB Abstracts and CAB Global Health (http://www.cabi.org).
6. The Cochrane Database of Systematic Reviews (http://www.cochrane.org)Cochrane Reviews are full text articles reviewing the clinical effectiveness of health care. While not limited to the use of natural product medicines, the Cochrane database contains a number (78 at present) of systematic reviews of clinical evaluations of herbal products, with data from over 2000 clinical trials. Full reviews are available for purchase online from the Cochrane Library website. Abstracts are available for free from the US National Library of Medicine.

3.04.3.1.3 Specialty databases/ethnomedicine

1. The Plants for a Future Database (Ethnobotany, including medicinal and edible plant species at http://www.pfaf.org/database/index.php) at Leeds University, with over 7000 species represented. Searchable by plant common name, family, use, geographical area, habitat, or keyword.
2. The phytochemical and ethnobotanical databases (http://www.ars-grin.gov/duke/) compiled by Jim Duke and maintained by the Agricultural Research Service, USDA. Users can search by plant, chemistry, biological activity, or ethnomedical use.

3. The Native American Ethnobotany database at the University of Michigan (http://herb.umd.umich.edu/) provides an online searchable database of foods, drugs, dyes, and fibers of Native North American peoples.
4. A number of TCM databases are available online, of which we will limit our discussion to those available in English. The traditional Chinese medicine information database, containing (http://tcm.cz3.nus.edu.sg/group/tcm-id/tcmid_ns.asp) data on formulations, herbal composition, clinical indication, and application of TCM, along with relevant literature currently contains entries for 1197 formulae, 1098 medicinal herbs, and 9852 herbal ingredients. Another example is the TCM Basics website (http://www.tcmbasics.com), which can be characterized as containing introductions to the basic theories of TCM, along with monographs of a more limited number of species. Introductions to various aspects of TCM theory and practice are presented, along with monographs of 116 herbs.
5. The traditional Indian medicines databases contain online pharmacopoeia of ayurveda, unani, and siddha (http://indianmedicine.nic.in/welcome.html) medicines. The site is hosted by the Department of Ayurveda, Yoga, Unani, Siddha and Homeopathic Medicine, Ministry of Health and Family Welfare, Government of India, and also contains historical background and introduction to the basic concepts and principles of the various forms of Indian traditional medicine, as well as formulary and pharmacopoeia.

3.04.4 NAPRALERT in Perspective

As an aid for the discovery of novel bioactive compounds, NAPRALERT represents a truly unique and invaluable resource. We hope that this overview of the database has provided the reader with a better appreciation for some of the other applications to which NAPRALERT can be applied. As for the databases mentioned above, in many cases, these can be used by researchers to complement and expand upon the information provided by NAPRALERT.

The past decade has witnessed a rapid acceleration, diversification, and expansion of publication in the area of natural products. As natural products continue to serve, and increase in impact, as resources in traditional and complementary medicine, botanical dietary supplement use, and in drug discovery, we expect that natural product databases will continue to expand and evolve right along with them. In fact, as data on natural product expands, comprehensive databases such as NAPRALERT will become increasingly valuable research tools.

References

1. D. S. Fabricant; N. R. Farnsworth, *Environ. Health Perspect.* **2001**, *109* (Suppl. 1), 69–75.
2. R. Luckenbach, Ed., *Beilstein Handbook of Organic Chemistry;* Beilstein-Institut für Literatur der Organischen Chemie. Springer-Verlag, 1996; Vol. 27.
3. J. I. G. Cadogan, *et al.,* Eds., *The Dictionary of Organic Compounds*, 6th ed.; Chapman & Hall, CRC Press, 1996.

Biographical Sketches

James G. Graham is adjunct assistant professor in the Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago. Dr. Graham is coeditor of NAPRALERT, as well as a research associate in botany at the Field Museum of Natural History in Chicago. He received his Ph.D. in 2001 from the University of Illinois at Chicago.

Norman R. Farnsworth is research professor of pharmacognosy at the College of Pharmacy, University of Illinois at Chicago (UIC), where he also directs the Program for Collaborative Research in the Pharmaceutical Sciences. Professor Farnsworth has the distinction of being senior university scholar as well as a UIC Distinguished Professor. He developed the UIC/World Health Organization (WHO) Collaborating Center for Traditional Medicine, one of the world's leading natural products and medicinal plant research centers, and also serves as director of the UIC/National Institutes of Health (NIH) Dietary Supplements Research Center.

Professor Farnsworth received his Ph.D. in 1959 from the University of Pittsburgh. He helped implement the first Ph.D. program in pharmacognosy during his tenure there and was the first to chair this program after he became a professor. He taught at the University of Pittsburgh until 1970 and then moved to the College of Pharmacy at the University of Illinois at Chicago. He pioneered natural products databases with the founding of NAPRALERT, launched at UIC in 1975.

3.05 Plant Diversity from Brazilian Cerrado and Atlantic Forest as a Tool for Prospecting Potential Therapeutic Drugs

Dulce Helena Siqueira Silva, Ian Castro-Gamboa, and Vanderlan Da Silva Bolzani,
São Paulo State University, Araraquara, São Paulo, Brazil

3.05.1 Introduction

The main aim of this chapter is to show the wealth of Brazilian chemical and biological diversity, with the efficacy of a chemo-biological background based on Brazilian scientific knowledge published in the recent literature. The current rates of plant extinction associated with local indigenous culture loss are rapidly increasing. A way of reversing this process may be to establish programs dedicated to exploring and conserving the remaining biodiversity of developing countries by involving botanists, chemists, biologists, and pharmacologists, all with the same goal: to prove the value of biodiversity by discovering as many biologically active compounds as possible through modern identification and screening methods for selecting lead molecules from the biodiversity. Well-organized inventories and advanced methodologies for studying plants extracts would result in valuable chemical and biological data of native medicinal plants many of which are still unknown. This approach would benefit the local herbal medicine market, and also contribute to the Program on Medicinal Plants launched recently by the Federal Minister of Health.

With forests covering an area almost the size of Europe, Brazil has natural environments that range from the most dense and humid Amazon and Atlantic Forest to more open and dry woodlands such as Cerrado (**Figure 1**). The challenge of this millennium for Brazilian science is to find ways to preserve the remaining areas of the various biomes, to conduct metabolomic and genomic studies of biologically active plant species and microorganisms, and to find ways to contribute to the sustainable use of this vast chemical diversity, which is still less explored.

The United Nations Conference on Environment and Development (UNCED), also known as the Earth Summit, was held in Rio de Janeiro, Brazil in June 1992, and during this meeting documents defending the sustainable use of natural resources were approved in addition to ways for defining a new strategic development model for world biodiversity. This event represented a significant advancement in the conservation of the planet's biodiversity. Brazil led the signing of these documents during the Earth Summit and counted on the support of nearly 160 countries. In February 1994, Brazil's National Congress ratified the Convention on Biological Diversity.

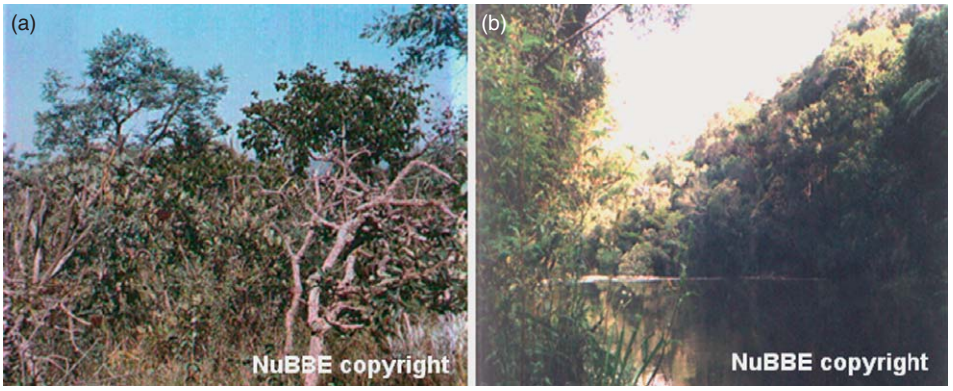


Figure 1 Overview of Cerrado (**a** – Itirapina Reserve) and Atlantic Forest (**b** – Juréia Reserve) in the São Paulo State.

Since the Rio Conference-92 there were intense efforts for compiling data and information on biological diversity in local and even global scale.[1] One of those documents was produced by the World Conservation Monitoring Centre, in conjunction with the Natural History Museum of London and other international organizations.[2] The number of species from Brazilian biodiversity was estimated by Lewinsonhn and Prado[3] (**Table 1**) taking into account the fact that the knowledge of several taxonomic groups was limited. Species of plants and terrestrial vertebrates are well documented, whereas the data for invertebrates and microorganisms are rare.

However, the measures enacted to implement the principles defended by Agenda 21 and the Convention on Biological Diversity created a gap between some international and national organizations in charge of proposing and/or managing these principles and the researchers who hold and produce high-quality scientific and technical information. Another important aspect is that biological diversity is now valued by all, in scientific and biological fields as well as in the economic and cultural areas.

Brazil is undoubtedly the most biologically diverse country, home to 20% of all the planet's species, yet these assets will probably never be accurately quantified given the country's continental size, wide continental shelf, and complex ecosystems. A considerable part of these assets has been – and still is – irreversibly wasted even before being properly understood because of habitat fragmentation, excessive exploitation of natural resources, and contamination of soil, water, and the atmosphere.

The native forests in the State of São Paulo, which originally covered more than 80% of its area, are now limited to about 13%. Modern monitoring techniques have pointed out that even with improved environmental legislation and respective enforcement policies, destruction is still rampant.

As a result of its relief and geographical position in the transition between tropical and subtropical areas, the State of São Paulo has one of the country's richest biodiversities. However, economic development has destroyed much of this wealth over time. When the Portuguese first arrived the Atlantic Forest *sensu lato*[4] and the tropical savannah, or Cerrado,[5] covered 83%[6] and 14%[7] of the state's area, respectively.

Table 1 Estimated number of species described in Brazil and in the world

Kingdom/phylum	*Brazil known species*	*Worldwide known species*
Virus	250–400	3600
Monera	1100–1350	4760
Fungi	12 500–13 500	70 500–72 000
Protista	7000–9900	75 300
Plantae	45 300–49 500	264 000–279 400
Animalia	113 000–151 000	1.287 000–1.330 000
Total estimated	178 000–2260 000	1.705240–1.766 000

Currently, a mere 12% of the original Atlantic Forest remains. Significant expanses are found only in the *Serra do Mar* range and the *Ribeira* river valley. In other areas of the state, particularly during the time when coffee farms expanded inland from 1810 to the mid-twentieth century, native forests suffered considerable degradation and are now extremely fragmented.[7] More recently, the Cerrado has been cleared at an alarming rate. From the early 1960s to the turn of the twentieth century, 90% of its area was destroyed. The 1970s were the most critical years because the federal government encouraged the expansion of sugarcane plantation under its ethanol energy program (Pro-Álcool). In the 1980s, *Citrus* groves had a similar effect. Consequently, only 230 000 ha of the original area remain, divided into 8300 fragments with more than 4000 of them less than 10 ha in size and only 47 fragments larger than 400 ha.[7] This extreme fragmentation of habitat is one of the main problems faced in the conservation of the biomes and of the species dwelling in them. The importance of the Atlantic Forest and Cerrado biomes in terms of biodiversity conservation has become even more clear since they were listed as environmental hotspots.[8]

Researchers and policymakers have a difficult time using available scientific information on biodiversity because such data are usually spread out and fragmented among hundreds of papers and publications, many of them not easily accessible (theses, dissertations, monographs, etc.) and mostly in formats not suitable for direct access. Consequently, available data not only represent a small share of this vast universe but are also underutilized.

The challenge, in this area of strategic importance to Brazil, lies in developing an environmental information system that would simultaneously (1) enhance academic knowledge on biodiversity, (2) establish mechanisms and strategies for the sustainable use of these assets, (3) improve the set of public policies to ensure the implementation of the principles defended by the Convention on Biological Diversity, and (4) establish bioprospecting incentive programs aiming at drug discovery from the huge biodiversity.

In January 1995, the State of São Paulo Environmental Secretariat (SMA) adopted Agenda 21 as its strategic planning guideline. In order to implement the commitments Brazil undertook by signing and ratifying the Convention on Biological Diversity (http://www.biodiv.org) and the United Nations Framework Convention on Climatic Change (http://unfccc.int/), the SMA established and implemented the State Program for Conservation and Sustainable Use of Biodiversity (PROBIO-SP) and the State Program for Climatic Change (PROCLIMA-SP). These linked programs spanned all areas of the SMA and had the mission of translating scientific information available in the state into rules, resolutions, decrees, and laws aiming to encourage interaction between the SMA and the scientific community, so that research was conducted to fill in any existing gaps in knowledge.

To overcome the deadlock caused by the lack of credibility of the programs proposed by the policymakers and the scientific community as well as to properly conserve and rationally use the vast assets represented by the biological diversity, the Biological Sciences Coordination and the Scientific Division of the State of São Paulo Research Foundation (FAPESP) set out to discuss these issues with leaders of the scientific communities and decided to prepare a special research program for conservation and sustainable use of biodiversity within the State of São Paulo. This program, initially called Biota-SP/FAPESP, was supported by FAPESP's biological science area with the following priorities: (1) identify the existing level of biological knowledge for each taxonomic group, from microorganisms to mammals and angiosperms, by including researchers who have worked specifically with each group and the installed conservation infrastructure, both *ex situ* (museums, herbaria, collections of microorganisms, arboreta, and botanical gardens) as well as *in situ* (conservation sites) and (2) organize and synthesize all information produced into a database. According to the goals established, Biota-SP/FAPESP would use the Internet, first on a preferential basis and later on an exclusive basis, for all communication and integration activities. In February 1999, FAPESP's Higher Board approved the establishment of the Biota/FAPESP Program – The Virtual Biodiversity Institute (www.biota.org.br), which was officially introduced on 25 March 1999.

Therefore, the Biota/FAPESP Program resulted from the State of São Paulo's scientific community in the field of knowledge encompassed by conservation and sustainable use of biodiversity. It is unique as the program was totally conceived by academic researchers. It undoubtedly represents a new concept as essential as the inventory of the state's biodiversity combined with a research program focused on conserving and ensuring the sustainable use of this biodiversity. A program such as this must not only continue the important task of describing and cataloging species, but it must also create research projects that include structural and functional

aspects of biodiversity. Such aspects must also focus on chemistry, biologically active compounds, and molecular biology of local plant species and other organisms of interest, including the spatial and temporal distribution of these organisms and their interrelationships at the chemical and various organizational levels.

The establishment of the Virtual Biodiversity Institute employing the Internet to gather the academic community around the program objectives, places the program at the forefront regarding the use of electronic networks in the field of biodiversity. The Internet enables all data produced to be entered simultaneously free of cost, and makes them available not only to the scientific community but also to organizations in charge of policymaking for the conservation and sustainable use of biodiversity.

The Standard Collection Form and a standardized model to list the species have enabled the construction of a database that contains data produced by all participating researchers. This system, called Sistema de Informação Ambiental (*SinBiota*) (http://sinbiota.cria.org.br/; Environmental Information System), does not rely on commercial systems and is easily accessible. Data can be entered online from any computer connected to the Internet with free access. To date, the project has been made digital and nearly 2 million records of the collections are available.

Plant species of the Cerrado and Atlantic Forest cataloged in this electronic network hold a tremendous resource of new secondary metabolites, which have hardly been explored. This huge biodiversity could be the only rational way to look for new bioactive compounds in short terms. The current bioprospecting efforts at the Biota Research Program has resulted in numerous biological information and opens new possibilities for finding potential leads, using traditional medicine knowledge, chemosystematic approach, and/or random search tools. Thus, a bioprospection program will certainly stimulate other Brazilian initiatives aimed to add value to Brazilian chemical diversity and, in fact, contribute to mechanisms of sustainable uses.

The first tentative bioprospecting work at Biota/FAPESP resulted in approximately 2000 extracts obtained from more than 800 species, and inspired a bioprospecting subprogram (http://www.bioprospecta.org.br), which could expand to the entire State of São Paulo. The bioprospection research in the Biota/FAPESP Program will certainly contribute in the identification of species and/or isolated substances that have future potential in the pharmaceutical, cosmetic, agrochemical, and food supplement industries. The main goal will be to join all multidisciplinary research groups in the State of São Paulo whose works are directly or indirectly associated with biodiversity, and thus, to find new bioactive secondary metabolites originating from microorganisms, macroscopic fungi, plants, invertebrates (including marine invertebrates), and vertebrates.

The State of São Paulo Flora Project identified and stored in a database approximately 8000 species of phanerogams or spermatophytes. Most of the these species are monitored by global positioning system (GPS) and, according to the number shown in this database, only few species have been studied chemically and biologically, raising a huge prospect for finding lead compounds.

The Biota bioprospecting program excelled not only in using the traditional plant knowledge from indigenous human populations but also by compiling all data already collected by the Flora Project, expanding its scope considerably. With the implementation of this bioprospection, the Biota/FAPESP Program took a significant step forward in the conservation of the State of São Paulo's biodiversity. With the systematic search for bioactive secondary metabolites, the program intends to raise funds from licensing and royalties to help create the economic mechanisms necessary to maintain and expand conservation infrastructure *in situ* (parks, reserves, etc.) and *ex situ* (museums, herbariums, microorganism collections, etc.), as well as research programs for conserving and pursuing the sustainable use of biodiversity. This represents the much talked about – and little practiced – sustainable use of biodiversity, this extraordinary natural asset we have inherited and would like to preserve for future generations. The success of the Biota/FAPESP Program in a particularly rich area from a biodiversity standpoint such as the State of São Paulo is nowadays a model for drug discovery from biodiversity, which is a natural resource of unquestionable value to Brazil.

3.05.2 Main Biomes of Brazil

Brazil stretches over a wide range of ecosystems that are home to a large number of unique species of plants and animals. About 20% of the earth's biodiversity occurs in Brazil.[9] This includes 50 000 plant species, thousands of bird species, millions of insects and microorganisms, and many unique mammals.[10] The different climate

zones of Brazil house different types of vegetation.[11] Six types of vegetation or biomes can be distinguished in Brazil: the Amazon rainforest, the Cerrado, the Caatinga, the Pantanal, the Mata Atlântica (Atlantic Forest), and the Pampas.[12] Such a diversity of biomes has its own community of diverse organisms that are well adapted to their environments.[13]

The reason for Brazil's unique wildlife is found in prehistoric times. The rock plates of the continents move slowly over the earth's surface, a process that is known as 'continental drift'. A look at the world map reveals that South America and Africa seem to fit together. About 200 million years ago, South America and Africa were indeed joined together forming one gigantic supercontinent called Pangaea. During the Cretaceous period, the rock plates that the continents sat on began to move apart. About 3 million years ago, a land bridge was formed between South America and North America.[14] Such movements may at least partly explain the occurrence of closely related species/genera in far-apart continents such as Africa and America. In terms of phytochemical data, the Amazon forest, Caatinga, Cerrado, and Atlantic Forest biomes are so far the most representative and will be briefly described in this chapter.

3.05.2.1 Amazon Forest

The Amazon rainforest covers an area of 7 million km^2 and is the largest rainforest on earth stretching over nine countries: Brazil, Colombia, Peru, Venezuela, Ecuador, Bolivia, Guyana, Surinam, and French Guyana.[15] About 60% of the Amazon forest lies in Brazil and the other 40% belongs to countries already mentioned.[9] The Amazon rainforest has an extremely high biodiversity and many species have not yet been described. Estimations of the number of plant and animal species vary widely. According to a recent publication by the World Wildlife Fund (WWF), the Amazon rainforest contains about 40 000 plant species, 427 mammal species, 1294 bird species, 378 species of reptiles, 427 species of amphibians, and 3000 species of fish. These are minimum figures as new species are continuously being discovered.[16]

The Amazon rainforest is characterized by high levels of precipitation. As a result, the landscape is green throughout the year. The Amazon rainforest can be subdivided into different vegetation types. The largest part consists of *terra firme* forests or 'dry land'.[17] This type of Amazonian forest that is not yet disturbed by humans is dominated by trees, which are able to grow as high as 50 m, and because of the lack of light, the vegetation of the forest floor is open and accessible.[18] Besides *terra firme*, the Amazonian region has two types of forest that are seasonally inundated: the *igapó* forest and the *várzea* forest.[19,20] The *igapó* forest consists of plant species growing along the black water rivers that have high concentrations of humic acid, which determine a specific kind of vegetation around it.[21,22] The *várzea* forest grows along the white water rivers that carry fertile sediments from the volcanic Andes Mountains, and has the highest concentration of species diversity of Amazonia.

Deforestation is the most important threat to the Amazon rainforest. Most of the deforestation happen illegally and the Brazilian government has huge difficulties in implementing the state of law in the Amazon basin because of the large distances to be covered.

The difficulties in protecting the Amazon rainforest from deforestation cause an irreversible loss of valuable chemical and biological information, which is fundamental for the knowledge of this wild patrimony, and thus for its conservation and sustainable use. For example, the chemical diversity of plant species of this region constitutes a powerful source of biological compounds useful in developing new drugs.

3.05.2.2 Caatinga

The Caatinga is a genuine vegetation zone in the northeast of Brazil, much dryer than the Cerrado. The name Caatinga, which means 'white forest', is derived from the Amerindian Tupi–Guarani languages.[23] This vegetation is dominated by thorny bushes, cactuses, and grasses. It is increasingly becoming clear that the Caatinga is a unique ecosystem with unique plant and animal species.[23]

In the past, the Caatinga vegetation occupied about 11% of the Brazilian national territory, but today it has been largely destroyed. The remaining plant species of this biome occur over the Brazilian states of Alagoas, Bahia Ceará, Maranhão, Minas Gerais, Paraíba, Pernambuco, Piauí, Rio Grande do Norte, and Sergipe.[24]

The Caatinga hosts one of the best-known Brazilian national parks, the Parque Nacional da Serra da Capivara, which does not only conserve many plant and animal species, especially primates, but it is also one of

the most important archeological sites of South America.[25] Plants of this region have been the subject of interesting phytochemical work developed in the northeast of Brazil, and various compounds isolated from these plants present promising bioactivities (**Figure 8**).[26]

3.05.2.3 Cerrado

The Cerrado flora should be considered a distinct vegetation type, distinguished from other physionomically similar Central and South American vegetation types by its ecology, species chemical composition, and diversity. The majority of the species belongs to the herbaceous group (76.7%) that dominates the vegetation in terms of number of species. This biome possesses a great biodiversity, and is located in the central part, extending from south of the Amazon rainforest to the Atlantic Forest in the southeast of Brazil.[27] This biome has seasonal upland vegetation on deep and well-drained soils, with a permanent water table. This situation is quite unique and has led to a great biodiversity.

The Cerrado is smaller than the Amazon forest but still impressively extensive, covering about 2 million km^2. Agriculture has expanded and encroached into this vast area, though it contributes significantly to the Brazilian economy. In an attempt to control this expansion, a significant increase in Cerrado protected areas such as nature reserves and national and state parks has been observed.[28] The chemical diversity of this ecosystem is a source of interesting secondary metabolites, several with the potential to be explored as new medicines.

3.05.2.4 Atlantic Forest or 'Mata Atlântica'

The Atlantic Forest or Mata Atlântica is a type of vegetation that can be found near the Brazilian coast between Rio Grande do Norte and Rio Grande do Sul. This biome has a high biodiversity.

This lush vegetation is a result of the rain that is carried by sea winds. Further inland is the Cerrado or the even dryer Caatinga that separates the Atlantic Forest from the other South American rainforests like the Amazon rainforest. Such isolation has resulted in a unique ecosystem with a large number of endemic species.[29]

The Atlantic Forest is very heterogeneous. The northern border of this forest is situated in the tropical state of Rio Grande do Norte while the southern border is located in the subtropical state of Rio Grande do Sul. In addition there are a number of mountain ranges like Serra do Mar and Serra da Mantiqueira that run parallel to the coast, resulting in a range of altitudes and a multitude of microclimates.[30]

The coastal region where the Atlantic Forest is located is the same area where the colonization of Brazil started. The exploitation of wood was one of the first economical activities of the Portuguese in Brazil. The most important reason for this exploitation was a red dye extracted from *Pau-brasil* wood (*Caesalpinia echinata*, Fabaceae). It represented a large part of the export in the early stages of colonization and therefore the country was named Brazil after *Pau-brasil* wood, which accumulates the pigments braziline (**1**) and brazileine (**2**). This is probably the first example of successful Western bioprospecting in Brazil. Unfortunately, this resource was not exploited in a sustainable way. Although only a small part of the Atlantic Forests remains, the Brazilian government has declared *Pau-brasil* the national tree of Brazil.[31] Notably, this biome still has a fantastic biodiversity with many unknown species, and thus possesses great potential for chemical exploration.

3.05.2.5 Conservation of Brazilian Biomes

The increasing sophisticated debates on environmental issues in Brazil have resulted in notable developments for protection of its various biomes. While conservation politics in the Amazon biome still attracts most attention, increasing concern is being devoted to the pressing need to create viable a model for environmental conservation in the Atlantic Forest (~1.2 million km^2), the Caatinga (~740 000 km^2), the Cerrado (~2 million km^2), the Pantanal (~140 000 km^2), and the South Steppes (~200 000 km^2)[32].

Priority-setting workshops for individual biomes, sponsored by the Ministry of the Environment, Global Environment Fund, and various international associations for environmental protection, have become the preferred forum for debating conservation models, which has the objective to gather academicians and

technicians to identify biodiversity 'hotpots' and priority to scale up conservation funds aiming at a strong scientific research on conservation and maintenance of these biomes.[32]

3.05.3 Development of the Brazilian Natural Products Chemistry

The interest in the natural product wealth of the country goes back to the time of its discovery by Portuguese seafarers in the sixteenth century. The first description of plants used for medicinal purposes by inhabitants was undertaken by a scientific mission brought to the northeastern part of the country by Maurice of Nassau, during the Dutch occupation in 1630. Obviously, the immense wealth of plant life in the newly discovered country drew the attention of several European scientists, who made great contributions to the description of the flora and to natural products chemistry that developed in Brazil since then.[33]

The history of Brazil is closely linked to the natural products isolated from native plant species, which determined the various disputes in the possession of the new land and, finally, by the Portuguese colonization.[34] A red dye was obtained from *Caesalpinia echinata* (Fabaceae), which had great commercial value at that time. This red pigment was widely used for dyeing of clothes and writing ink, which was already known and used in the East Indies since the Middle Ages. The name Brazil was coined due to the economic importance of this plant species, popularly called by the native Indians as *Pau-brasil*. From the wood of *Pau-brasil* braziline (**1**) was extracted. Braziline is a catecholic derivative that easily oxidizes to brazileine (**2**), a dienonic phenol derivative identified as the compound responsible for the intense red dye (**Figure 2**).[34,35] Until the end of the nineteenth century, only natural dyes were available, making this product valuable and of great interest for the settlers. In this sense, besides *Pau-brasil*, which was predatorily extracted from Brazilian territory almost to extinction, many other products aroused the interest of the Europeans in the newly discovered land. The flavonoid morin (**3**) (**Figure 2**), obtained from *Chlorophora tinctoria*, was another natural dye exported to Europe until the development of the chemistry of anilines in Germany, but is still used as an indicator of sugars in thin-layer chromatography (TLC).[36]

From *Geissospermum laeve* (Vell.) Baill. (synonym of *G. vellosii* Fr. All.), a plant known by Indians as a miraculous remedy to treat malaria, inflammations, fever, and dizziness, a compound named pereirine was isolated. In fact this compound was the first alkaloid isolated from a Brazilian plant species, which had its structural formula elucidated many years later as geissoschizoline (**4**) (**Figure 2**).[37,38]

With admirable enthusiasm, Theodor Peckolt, a pharmacist from Silesia, who lived in Brazil in 1847, analyzed over 6000 plants and published around 150 papers on plants. Although his work was very preliminary by present-day standard, he was the pioneer to isolate and identify a compound belonging to the iridoid class. He isolated a compound named agoniadin from *Plumeria lancifolia* Mart., Apocynaceae. Twenty-five years later Boorsma (1894) isolated the same iridoid from *P. acutifolia* Poir., which he, unaware of Peckolt's work, named it plumierid (**5**) (**Figure 2**), which had its structure elucidated 88 years later by Halpern and Shmid[39] (1958). The name plumierid or plumieride has been maintained for this iridoid, which possesses antifungal activity.[39,40] Medicinal and poisonous plants were extensively investigated in Brazil. From *Solanum grandiflorum* Ruiz et Pav., popularly known as wolf's fruit, a mixture of the steroidal glyco-alkaloids solasonine (**6**) and solamargine (**7**), both glycosides of the aglycone solasodine, was isolated.[41]

Chondrodendron tomentosum (Menispermaceae) and some species of *Strychnos* (Loganiaceae) were used by South American Amerindians to produce poisoned arrows for hunting and fishing.[33] Traditionally known as curare, it is an important example of traditional plants that inspired the development of a new class of anesthetic drugs. The first curare was identified from a Loganiaceae species collected in Suriname and described in 1783 by Schreber, as *Toxicaria americana*, which was further classified as *Strychnos guianensis*. Only in the nineteenth century Boehm isolated (+)-tubocurarine (**8**), the main active curare constituent of *C. tomentosum*. Other active constituents of *Chondrodendron* were subsequently isolated as (−)-curine (**9**) and isochondrondendrine (**10**)[35].

In the 1950s, modern phytochemistry in Brazil was introduced by two scientists, Walter B. Mors and Otto R. Gottlieb, who made a tremendous contribution in seeding the new generation of researchers and thus founding Brazilian modern plant science. Since then, hundreds of compounds have been isolated from plant species all over Brazilian biomes and their structures have been established. Lignans and neolignans may be the class of

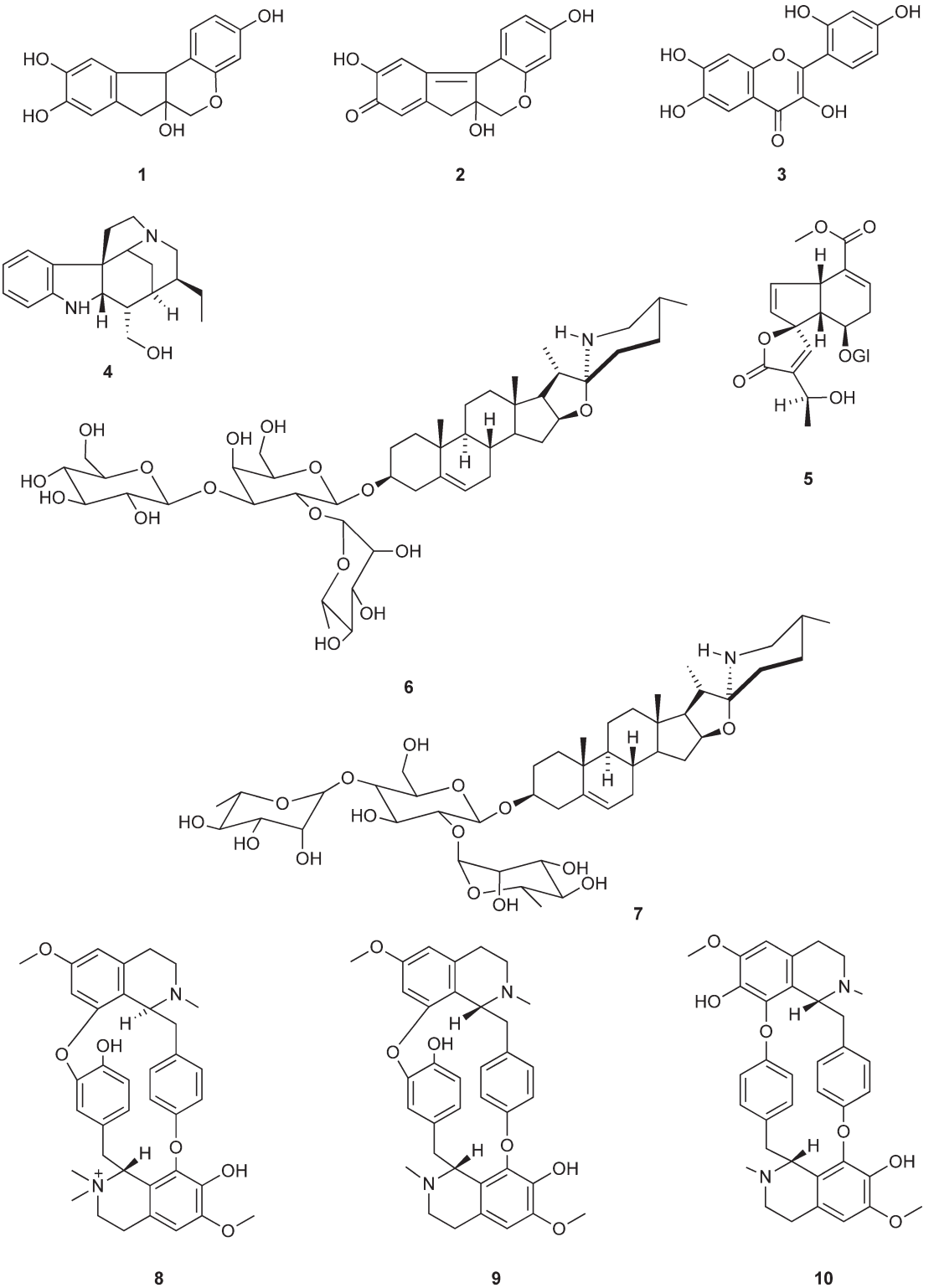


Figure 2 Secondary metabolites from historical Brazilian plants extractives.

secondary metabolites most explored, particularly from Myristicaceae[42] and Lauraceae.[43] According to the IUPAC, approximately 350 neolignans and 270 lignans have been described in the literature from Brazilian plant species of these taxa.

Arylpyrones, chromenes, and benzophenones have also been explored from Lauraceae and Myristicaceae[35] (**Figure 3**). It is known that these compounds exhibit activity on the central nervous system (CNS), which justifies their pharmacological properties as sedative. Also, several pyrones possess analgesic, antiarrhythmic, antiedemic, antimycotic, antiphlogistic properties besides potentiating barbiturate narcosis and offering protection against chemo- and electroshock.[35] Piperaceae is a very well-represented plant family in Brazil, and the secondary metabolites accumulated by this family are of chemosystematic significance, since it is considered a plant fossil.[44] Recent chemical studies on Brazilian *Piper* species have revealed the occurrence of several chromenes[45] **11–22** (**Figure 4**) and amides[46] **23–29** (**Figure 5**).

Diterpenes are another type of secondary metabolites found in many Brazilian plants. A special mention must be made of diterpenes from Velloziaceae, a very distinct vegetation of *campos ruprestres* or rocky fields, which is rich in representatives of the family Velloziaceae.[47] Although plants in this family live under conditions of high solar irradiation and low water availability, these plant species show surprising longevity.

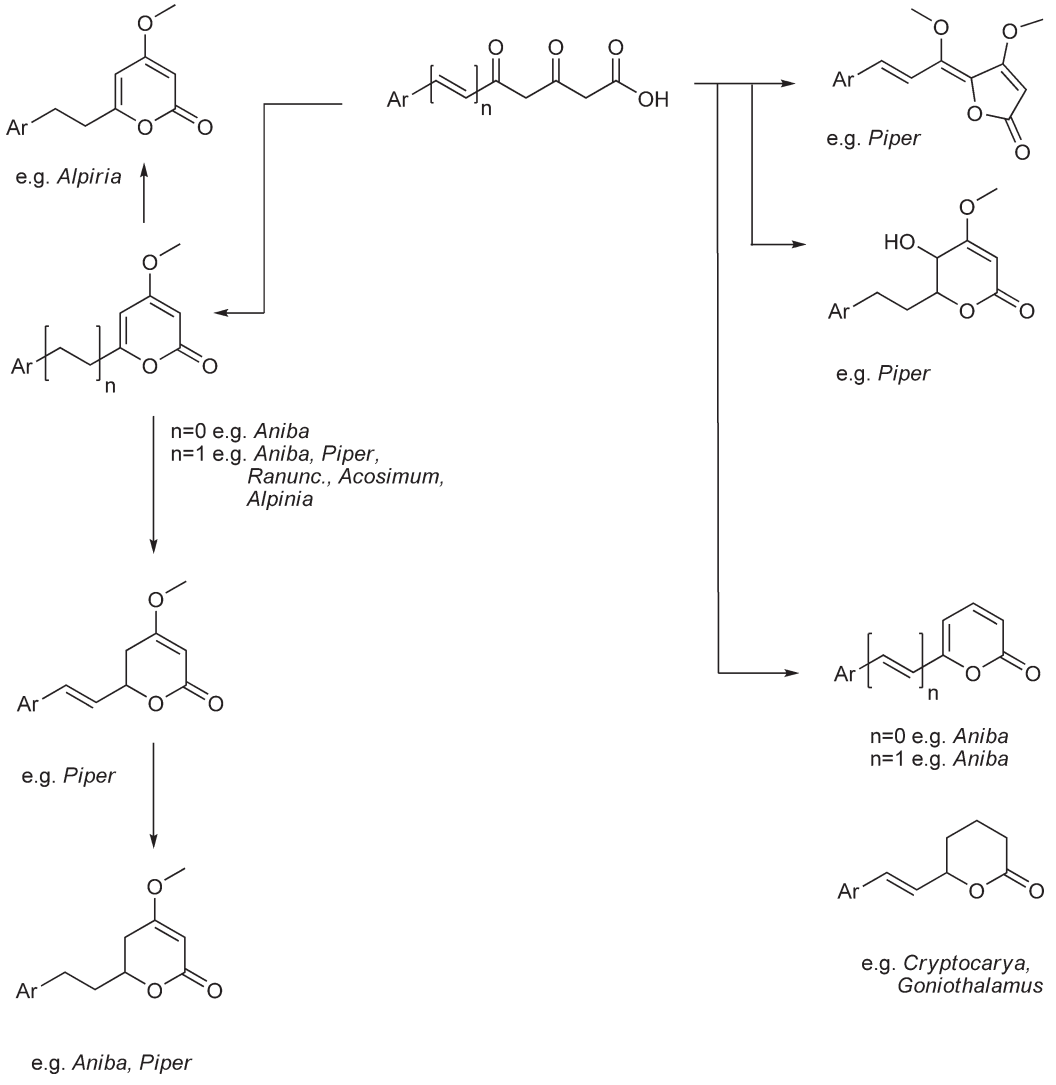
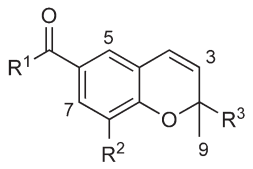
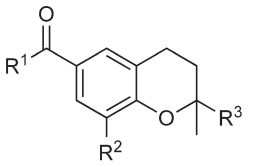


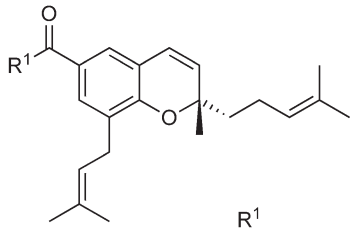
Figure 3 Biogenetic relations of arylpyrones in Brazilian Lauraceae.



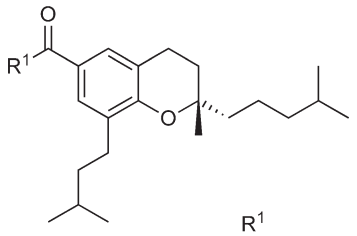
	R^1	R^2	R^3
11	OH	H	CH_3
12	OCH_3	H	CH_3
13	OCH_3	OH	CH_3
14	OCH_3		CH_3
15	OCH_3	$OCOCH_3$	CH_3



	R^1	R^2	R^3
16	OCH_3	H	CH_3
17	OCH_3	OH	CH_3
18	OCH_3		CH_3

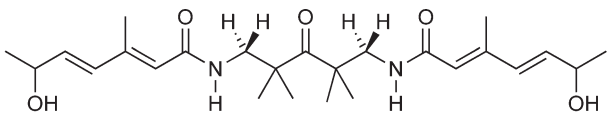


	R^1
19	OH
20	OCH_3

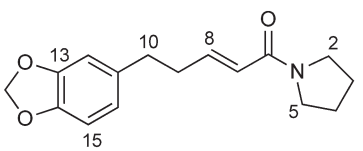


	R^1
21	OH
22	OCH_3

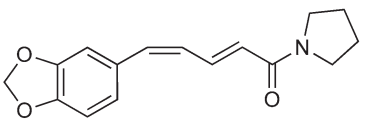
Figure 4 Chromenes isolated from *Piper* species.



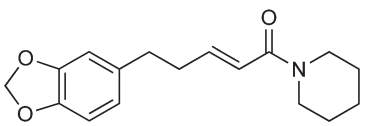
23



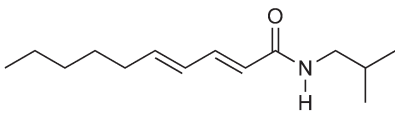
24



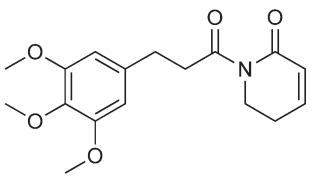
25



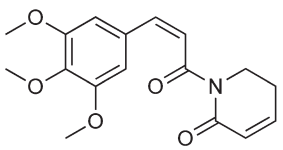
26



27

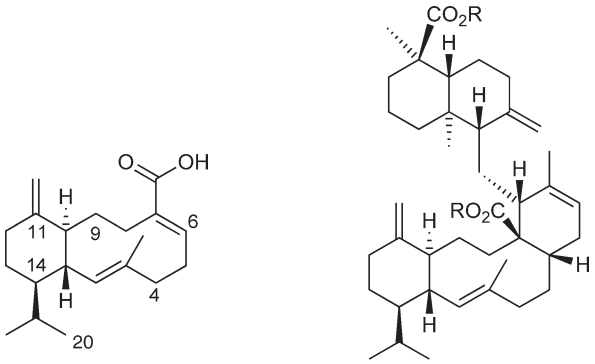


28



29

Figure 5 Amides isolated from Brazilian *Piper* species.



30

31: R = H
32: R = Me

Figure 6 Unusual diterpenes from Velloziaceae plant species endemic to *campos rupestres* (rocky fields).

The 32 Brazilian species of Velloziaceae studied to date were shown to accumulate several subclasses of diterpenes, especially those of the isopimarane,[48] clerodane,[49] cleisthantane,[50] ent-kaurane,[51] and totarane[52] subclasses. From *Vellozia magdalenae*, the unusual magdalenic acid (**30**) and bismagdalenic acid (**31**) and its dimethyl derivative (**32**) were isolated (**Figure 6**), which are typical diterpenes from the eunicellan subclass common in marine vertebrates.[53]

An increasing number of plant species from Brazilian biomes are now enjoying wide international demand, and interest in investigating this fantastic biodiversity is therefore growing, mainly because most of these plants have not yet been studied chemically and pharmacologically, thus providing an economic potential to be explored. Advanced techniques of separation and characterization of secondary metabolites have facilitated systematic research on plants of the main biomes, especially those of Cerrado and Atlantic Forest. The online combination of high-performance liquid chromatography (HPLC) with ^{1}H NMR (nuclear magnetic resonance) spectrometry allowed the identification of several complex limonoids (**33–40**, **Figure 7**) from methanol extracts of dried seeds from the mahogany tree *Switenia macrophylla* (Meliaceae).[54]

Anthracene derivatives, *rel*-8α-hydroxy-5-hydroxymethyl-2-methoxy-8aβ-methyl,7,8,8a,9-tetrahydro-1,4-anthracendione (**41**) and its derivative (**42**), *rel*-10α11β-epoxy-11α-ethoxy-8α-hydroxy-2-methoxy-8aβ-methyl,5,6,7,8,8a,9,10,10aβ-octahydro-1,4-anthracendione (**43**), *rel*-8α,11α;9α11α-diepoxy-1,4-dihydroxy-2-methoxy-8aβ-methyl-5,6,7,8,8a,9,10,10aβ-octahydro-10-anthracenone (**44**) and its acetyl derivative (**45**), auxenone (**46**), and oncocalyxonol (**47**) (**Figure 8**) are good examples of metabolites isolated from the northeast plant species. These peculiar compounds are found in *Auxemma oncocalyx* (Boraginaceae), a common tree growing in this region.[55]

Alkaloids have a long history as active compounds in highly poisonous plants, and were used since ancient times due to their potent properties on the CNS. This class of secondary metabolites occurs quite frequently in plants from several Brazilian biomes. Monoterpene indole alkaloids from Rubiaceae, Loganiaceae, and Apocynaceae were considered valuable chemotaxonomic markers to help the delimitation of the subfamilies and to disclose possible evolutionary tendencies among these taxa.[56,57] Some examples of alkaloids **48–57** isolated from Brazilian plant species are shown in **Figure 9**.[58,59]

Since the time of discovery (fifteenth century), plant species of Brazilian biomes have been continuously studied, and there are possibly thousands of secondary metabolites described in the literature. However, chemical or biological study has not been done on a large number of the Brazilian plant species and they offer an economic potential for exploration for new drugs. The current rate of extinction of plant biodiversity in Brazil will cause not only the loss of valuable therapeutic compounds but also loss of interesting genes encoding for enzymes with a potential use in plant improvement and biosynthetic studies of new natural products. A strategy for development in Brazil is to protect and promote rational exploitation of its plant biodiversity as a source of new compounds available for mankind.

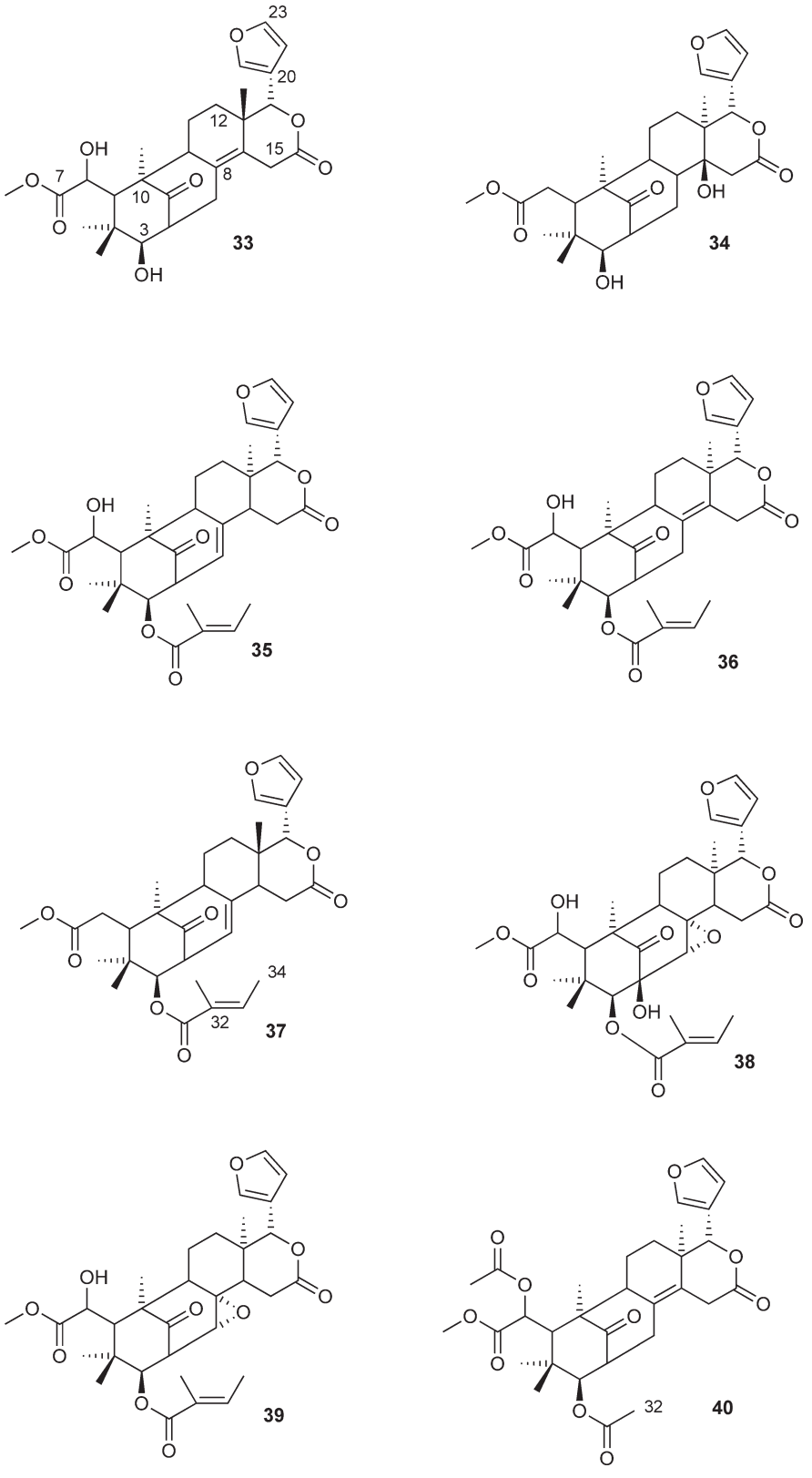
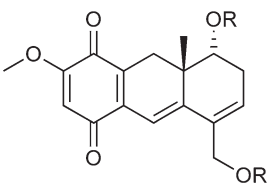
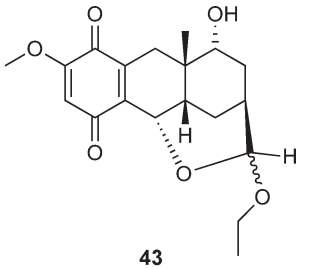


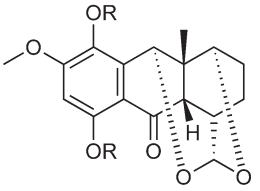
Figure 7 Limonoids from Meliaceae.



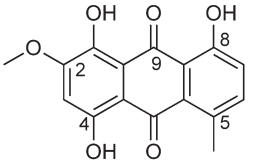
41: R = H
42: R = Ac



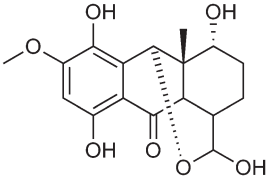
43



44: R = H
45: R = Ac

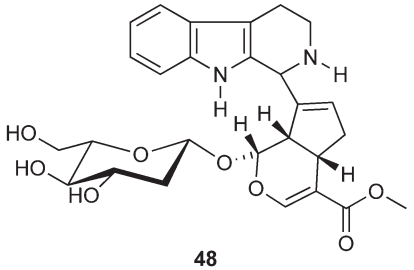


46

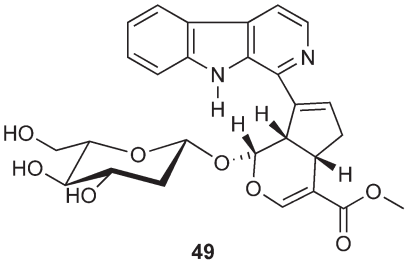


47

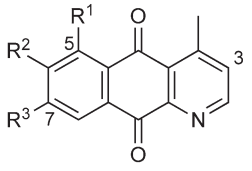
Figure 8 Representative secondary metabolites from Caatinga/Cerrado plant species.



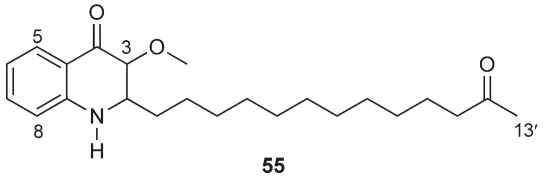
48



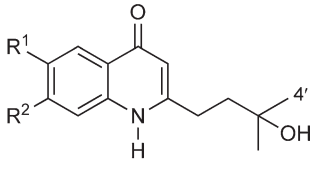
49



50 $R^1 = R^2 = R^3 = H$
51 $R^1 = R^3 = H$; $R^2 = OMe$
52 $R^1 = H$; $R^2 = R^3 = OMe$
53 $R^1 = OH$; $R^2 = OMe$; $R^3 = H$
54 $R^1 = OH$; $R^2 = R^3 = OMe$



55



56: $R^1 = H$; $R^2 = OH$
57: $R^1 = OH$; $R^2 = H$

Figure 9 Alkaloids from Brazilian plant species.

The discovery of putative new drugs in screening programs will demand the production of these compounds in larger amounts in a sustainable way. A biotechnological approach to the preservation and exploitation of plant species with potential biological activity has also been recognized as a rapid and efficient method that would contribute to sustainable economic development of nonindustrialized countries. The commercial production of some important compounds has already been achieved through *in vitro* cultivation.

3.05.4 Exploring New Approaches for Natural Product Drug Discovery in the Biota/FAPESP: Current Status of Bioprospecting in Brazil

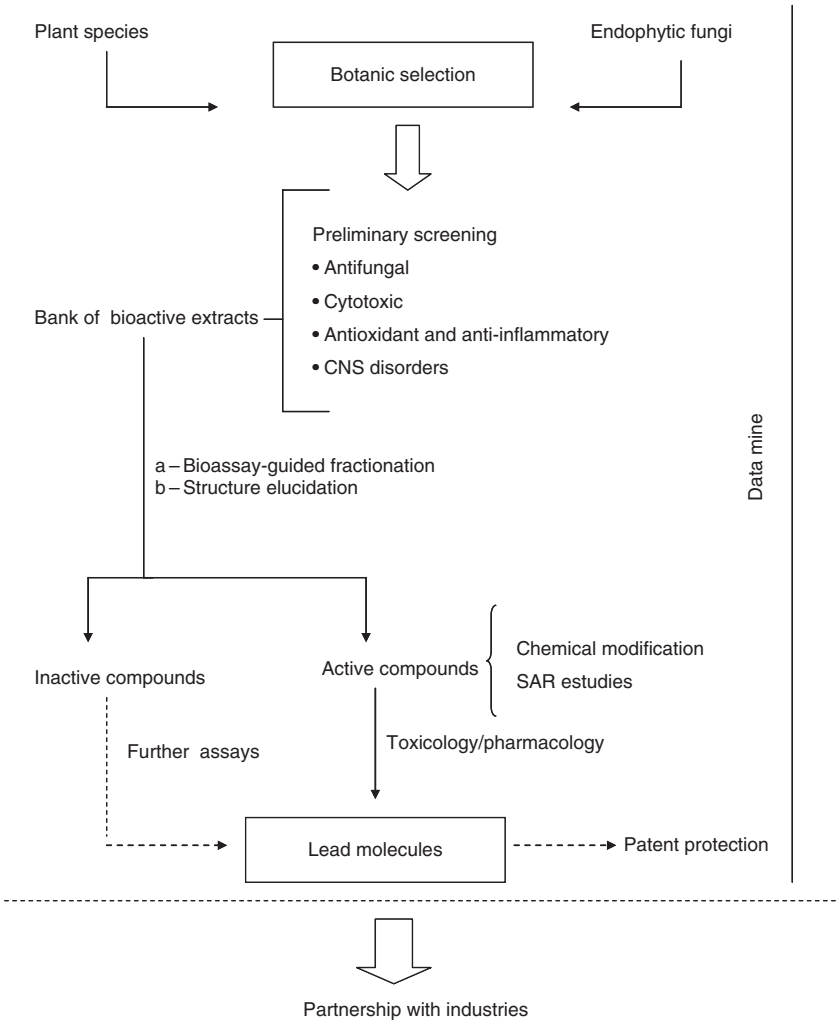
Although blessed by its great biodiversity, Brazil has been largely underexplored for new biologically active natural products, in part, due to the lack of collaborative programs aiming at drug discovery, as for example, the International Cooperative Biodiversity Groups (ICBG) founded by the National Institutes of Health (NIH), the National Science Foundation (NSF), and the United States Department of Agriculture (USDA).[26] Although Brazil has a long phytochemistry tradition, until recently, the main focus of the studies on biodiversity was on isolation and structure elucidation. There are thousands of secondary metabolites isolated from Brazilian plant species and referred in current scientific journals all over the world. However, according to INPI (Instituto Nacional de Propriedade Intelectual), the number of Brazilian patents on plants and/or bioderivatives registered during the last 30 years is approximately 284, which is very insignificant in comparison to the real potential of Brazilian biodiversity.

This scenario has changed with the creation of the current integrated and multidisciplinary collaborative program on chemistry of bioactive compounds from São Paulo State biodiversity, the so-called Biota/FAPESP Program.[60] This program that started in 2002 has been the driving force of Brazilian research on biologically active compounds, aiming at antifungal compounds from Rubiaceae plant species of Cerrado and Atlantic Forest.[61] Since then, the focus is on the search of bioactive compounds from plants of these biomes, and more recently also from endophytic fungi.

The current multidisciplinary collaborative program under development at the State of São Paulo proposes to establish a new paradigm on the search for new bioactive compounds from the great Brazilian biodiversity. Based on the popular conception of modern bioprospecting efforts, the program has been, as a major focus in drug discovery, taking into account the principle that lead compounds may be able to generate economic benefits for the country, and also help the biological conservation of Brazilian biomes.[62]

The philosophy and basic concepts of the experimental model adopted in this program are summarized in **Scheme 1**. We believe that the advances in extraction technology, chromatographic separation, analytical and spectroscopic instrumentation, and rational preliminary bioassay to screen for activities such as antifungal, anticancer, antioxidant, antimalarial, and acetylcholinesterase (AChE) inhibition will lead to synergistic outcomes. Additionally, the human resource connected with this approach will bring strong incentives to local herbal medicine industry, which surprisingly has done little with native medicinal plants.

Based on ethnopharmacology, chemosystematics, phytochemical studies, and random search, the first bioprospecting efforts accomplished during the past 6 years have resulted in a storage bank with 2000 plant extracts and more than 100 isolates of endophytic fungi, most of these from bioactive plant species already studied chemically. The plant species collected belong to approximately 88 different families (**Table 2**), which are very representative of angiosperms that occur in the two major biomes of São Paulo State (Cerrado and Atlantic Forest). The data obtained is largely the result of several field trips in the Cerrado (~387 species) and Atlantic Forest (~415 species), gathered primarily during the creation and consolidation of the Biota/FAPESP Program (1999–2003). All stored extracts (~774 from plants of Cerrado and 1226 from Atlantic Forest) were screened to preliminary bioautography with *Cladosporium cladosporioides* and *Cladosporium sphaerospermum*. From these, 800 extracts inhibited fungi growth (nystatin 0.1 mg ml^{-1} as positive control), indicating a great potential of these plant extracts in finding antifungal compounds (**Figures 10** and **11**). The AChE TLC assay method modified by Rhee was used to screen the extracts, and after developing this test in an appropriate solvent, the presence of active compounds related to the reference of AChE inhibition (galanthamine as positive control) were detected in approximately 354 extracts, representing 17.7% of the total extracts (**Figure 11**). The potential anticancer activity of the extracts was tested against a panel of cell lines using *in vitro*



Scheme 1 Experimental design for bioprospecting designed in Biota/FAPESP Program.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The preliminary antifungal screening was done against the human pathogen fungi *Candida albicans*, *Candida krusei*, *Candida parapsilosis*, and *Candida neoformans*, and showed promising activity. From the pure compounds assayed, approximately 50% showed promising activity, and these compounds are being tested in additional enzymatic and receptors bioassays. Although a large number of plants and endophytic fungi still have to be bioassayed, the Biota's preliminary results indicate that bioprospection is a promising tool to explore Brazilian biodiversity. Two lead molecules, one plant herbal medicine and the other a compound for cosmetics, identified in this program are currently in the advanced phase of pharmacological and toxicological assays, so far indicating the huge potential of the Brazilian biological resources.

3.05.5 Search for Bioactive Secondary Metabolites from Brazilian Plant Species

The phytochemical studies aimed at the biological evaluation of crude extracts, semipurified fractions, or isolated compounds have yielded natural substances belonging to several classes, including flavonoids, terpenoids, alkaloids, coumarins, arylpropanoids, and polyketides. Such compounds have been evaluated for their

Table 2 Number of plant species within families collected by biomes in the State of São Paulo

Family	*Cerrado region*	*Atlantic Forest*	*Family*	*Cerrado region*	*Atlantic Forest*
Not identified	28	12	Loganiaceae	2	1
Acanthaceae	0	3	Loranthaceae	1	3
Anacardiaceae	4	2	Malpighiaceae	13	7
Annonaceae	5	7	Malvaceae	4	2
Apocynaceae	7	14	Marcgraviaceae	0	1
Aquifoliaceae	0	2	Melastomataceae	8	5
Araliaceae	1	4	Meliaceae	0	6
Asclepiadaceae	1	0	Mimosaceae	8	0
Asteraceae	30	19	Monimiaceae	2	7
Begoniaceae	0	1	Moraceae	5	10
Bignoniaceae	23	4	Myrcinaceae	0	1
Bixaceae	2	0	Myristicaceae	1	4
Boraginaceae	0	1	Myrsinaceae	1	8
Bombacaceae	4	4	Myrtaceae	27	24
Bromeliaceae	6	1	Nyctaginaceae	2	1
Burseraceae	0	1	Ochinaceae	2	2
Campanulaceae	0	1	Olacaceae	0	3
Caryocaraceae	2	0	Palmae	0	2
Celastraceae	2	2	Piperaceae	0	23
Chrysobalanaceae	5	5	Polygonaceae	4	4
Clethraceae	0	1	Proteaceae	2	1
Clusiaceae	2	6	Ranunculaceae	0	1
Combretaceae	0	2	Rhamnaceae	3	1
Commeliaceae	0	2	Rosaceae	1	0
Costaceae	0	1	Rubiaceae	13	51
Cunoniaceae	0	1	Rutaceae	0	3
Cyatheaceae	0	1	Sabiaceae	0	1
Dilleniaceae	2	1	Sapindaceae	11	7
Ebenaceae	3	0	Sapotaceae	3	12
Ehretiaceae	0	1	Smilacaceae	1	0
Elaeocarpaceae	0	1	Solanaceae	2	10
Eythroxylaceae	8	2	Sterculiaceae	0	1
Euphorbiaceae	12	18	Styracaceae	2	0
Fabaceae	38	0	Symplocaceae	0	1
Flacourtiaceae	1	11	Theophrastaceae	0	1
Gentianaceae	0	1	Thymeleaceae	0	1
Gesneriaceae	0	4	Tiliaceae	3	1
Hippocrateaceae	2	1	Ulmaceae	1	0
Iridaceae	0	1	Valerianaceae	0	1
Lacistemataceae	4	1	Verbenaceae	6	3
Lamiaceae	14	0	Violaceae	0	1
Lauraceae	8	35	Vochysiaceae	5	2
Lecythidaseae	0	1	Winteraceae	0	1
Leguminosae	17	33			
Total	287	415			

biological properties using preliminary bioassays that allow the selection of promising samples for detailed studies using more sophisticated assays.

3.05.5.1 Antifungal Compounds

The study of Rubiaceae species from São Paulo State is one of the milestones of the bioprospecting research, as they occur widely in the Cerrado vegetation. Rubiaceae is one of the largest families of angiosperms and consists of approximately 600 genera and more than 10 000 species. It is essentially a tropical, woody family

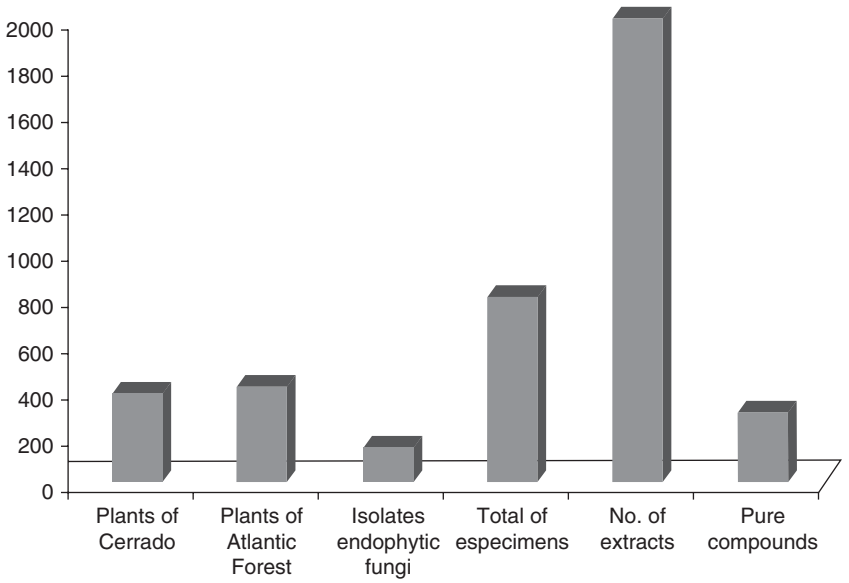


Figure 10 Number of species collected by biomes, extracts prepared, endophytic isolates, and pure compounds.

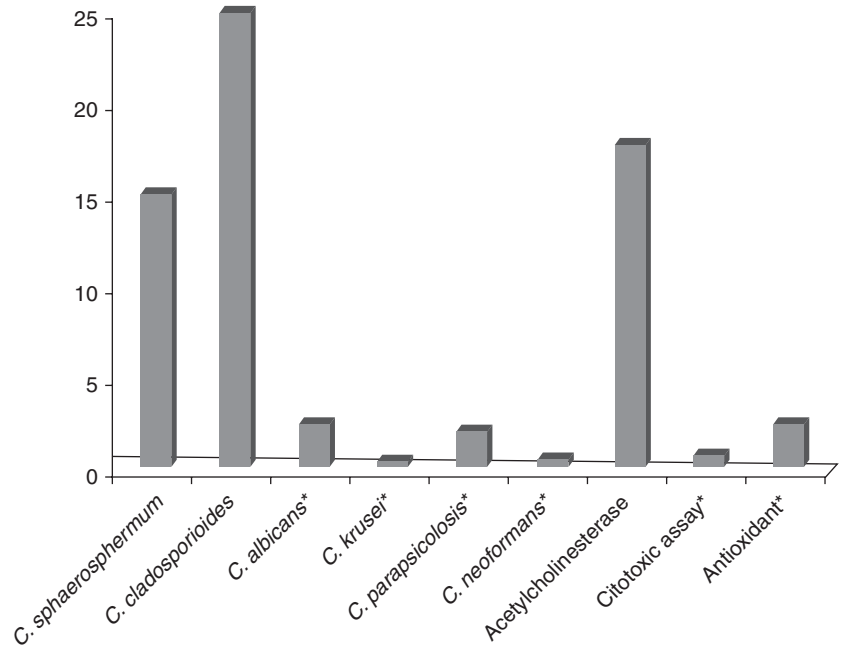


Figure 11 Preliminary screen results (%) performed in the bioprospection. *Number of extracts bioassayed is less than the total number of prepared extracts (2000).

widely distributed in Brazil's main ecosystems: Amazon, Cerrado, and Atlantic Forest. Phytochemical studies on Brazilian Rubiaceae, especially those from Cerrado and Atlantic Forest, have shown the presence of a number of antifungal secondary metabolites, mainly triterpenes, iridoids, and caffeic ester derivatives. In addition, indole and oxyndole alkaloids, coumarins, and flavonoids have also been isolated. The bioassay-guided fractionation of Rubiaceae species from Cerrado and Atlantic Forest has led to the isolation of some antifungal compounds that have been found mainly in the genera *Alibertia*, *Tocoyena*, *Chimarris*, *Randia*, and *Rudgea*.

The phytoalexin response of some selected Rubiaceae species from Atlantic Forest and Cerrado regions was previously evaluated in order to verify if the antifungal compounds present in crude extracts were phytoalexins, which means antifungal compounds whose production was elicited under stress conditions or constitutive antifungal metabolites.[63,64]

Randia spinosa is popularly used for its anti-inflammatory properties in northeast Brazil and belongs to the Ixoroideae subfamily, which has iridoids as its main secondary metabolite. These compounds were used as chemotaxonomical markers to delimit or to recognize similarities among the subfamilies of Rubiaceae. The iridoidal profile of Ixoroideae, which is essentially herbaceous, indicates this subfamily to be an ancestral group synthesizing mainly iridoids and hence suggesting affinity with the Corniflorae order.[61] The chromatographic fractionation of the stems of *Randia spinosa* led to the isolation of six iridoid glucosides: geniposide (**58**), 6α- and 6β-hydroxygeniposide (**59** and **60**), galioside (**61**), gardenoside (**62**), and the bis-iridoid randinoside (**63**).[65] These compounds proved to be inactive toward *C. cladosporioides* and *C. sphaerospermum* fungi in bioautographic TLC assays.

Tocoyena formosa, a small ornamental shrub, commonly known as 'genipapo do campo', also showed the presence of antifungal iridoids. The bioassay-guided fractionation using TLC bioautography nebulized with spore suspensions of *C. cladosporioides* and *C. sphaerospermun* as revealing agents led to the isolation of the iridoids molugoside methyl ester (**64**), formosinoside (**65**), and α- and β-gardiols (**66** and **67**). The epimeric mixture of α- and β-gardiols (**66** and **67**) showed antifungal activity.[66] Additionally, the *n*-BuOH-soluble fraction of the hexane extract of *T. formosa* stems afforded saponins, one flavonoids derivatives and four antifungal iridoids: galioside (**61**), galioside aglycone (**68**), apodanthoside (**69**) and the caffeoyl ester derivative 11-*O*-*trans*-caffeoylteucrein (**70**),[67,68] besides the iridoids gardenoside (**62**), 10-dehydrogardenoside (**71**), deacetylasperuloside (**72**), together with the fungitoxic iridoids α- and β-gardiols (**66** and **67**).[69,70] (**Figure 12**).

Other antifungal metabolites have been isolated from *Alibertia macrophylla*, whose reddish edible fruits are common in the *Alibertia* genus. The hydroalcoholic extract of its leaves was submitted to TLC bioassay, using a spore suspension of *C. sphaerospermun* as revealing agent. After incubation, the fungal growth inhibition zones appeared as three clear spots on the TLC. Further phytochemical work on this extract led to the isolation of antifungal iridoids: loganetin (**73**), 1-*epi*-loganetin (**74**), 1-β- and 1-α-hydroxydihydrocornin aglycones (**75** and **76**), in addition to the diterpene *ent*-kaurane-2β,3α,16α-triol (**77**). The pure compounds were reassayed against four fungi species: *C. sphaerospermun*, *C. cladosporioides*, the opportunist fungus *Aspergillus niger*, and the phytopathogen *Colletotrichum gloesporioides*, and compounds **75**, **76**, and **78** proved to have antifungal activity.[71–75]

In addition, seven inactive pentacyclic triterpenes were isolated: β-amyrin (**79**), α- and β-amyrinones (**80** and **81**), germanicone (**82**), lupeol (**83**), lupenone (**84**), and α-amyrin (**85**). Guided fractionation with a bioactive extract of *Alibertia edulis*, popularly known as 'marmelão' for its sweet fruits, afforded two series of inactive pentacyclic triterpenes derived from α-amyrin (**85**): uvaol (**86**), ursolic, 23-hydroxyursolic, and 23,24-dihydroxyursolic acid methyl esters (**87**–**89**); and β-amyrin (**79**): erythrodiol (**90**), oleanolic, 23-hydroxyoleanolic, and 23,24-dihydroxyoleanolic acid methyl esters (**91**–**93**).[75] Further studies on this species should be done in order to find the bioactive compounds. From an EtOH extract of *Alibertia sessilis* leaves were isolated the triterpenes ursolic (**87**), oleanolic (**91**), and *epi*-betulinic acid (**94**).

The leaves and bark of *Rudgea virbunioides*, a small tree widely distributed in the Cerrado of Brazil, are traditionally used in central Brazil to treat rheumatism, syphilis, and dyspepsia. The phytochemical work carried out on fruits of *R. virbunioides* showed the presence of the saponins trachlosperogenin (**95**) virbugenin (**96**), caffeic ester derivatives **97**–**99**, quercetin and kaempferol coumaroyl derivatives (**100** and **101**) and quercetin-3-*O*-rutinoside (**102**). This was the first time a pentacyclic pentahydroxylated triterpene was isolated from a Rubiaceae species. Virbugenin exhibited moderate activity (minimal inhibitory concentration (MIC) = 50 μg ml^{-1}, standards nystatin, MIC = 5 μg ml^{-1} and myconazol, MIC = 1 μg ml^{-1}) against *C. cladosporioides* in a test carried out on Petri dishes containing the fungus and culture medium[76] (**Figure 13**).

In some studies, as in the case of *Chiococca bracchiata*, the activity detected in the crude extract was lost during fractionation procedures, possibly due to the occurrence of synergism among the crude extract constituents. The strong antifungal activity detected in the crude extract (minimum amount required for the inhibition of fungal growth on TLC plates = 10 μg for *C. cladosporioides*, standard nystatin = 1.0 μg) was decreased proportionally during fractionation procedures, which led to the pure flavonols **103**–**108**, and the flavone apigenin (**109**), with very weak activity (detection limit values higher than 300 μg). The antifungal activity against

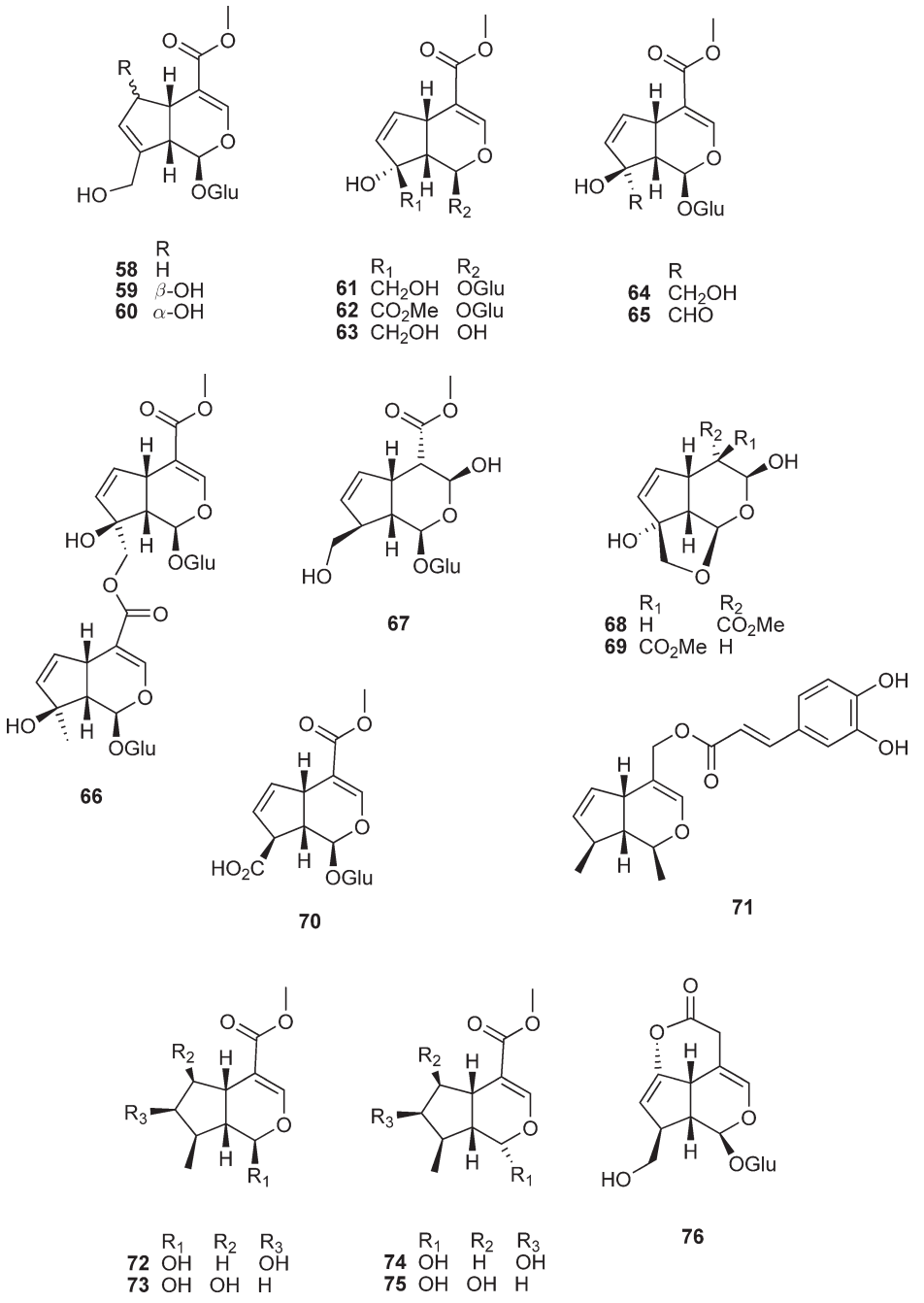
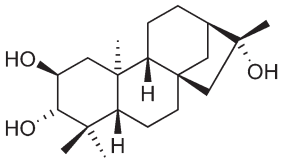


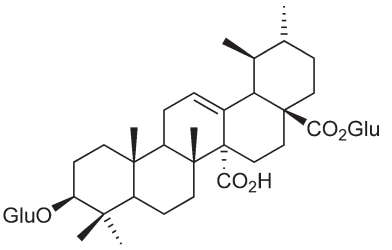
Figure 12 Selected antifungal iridoids from Rubiaceae species.

C. cladosporioides was significantly enhanced when a combination of the isolated flavonols was tested, suggesting a synergistic activity of these compounds.[77]

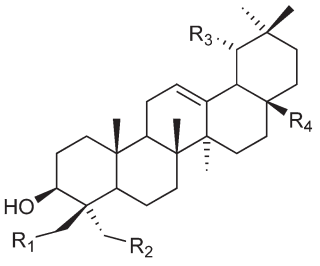
The chemical composition of *Psychotria spectabilis* includes diterpenes, solidagenone (**110**) and deoxysolidagenone (**111**); coumarin (**112**), umbelliferone (**113**), and psoralene (**114**); and flavonols, quercetin and quercetrin, which have been evaluated for their antifungal activity. Diterpenes and coumarins inhibited the growth of the phytopathogens *C. cladosporioides* and *C. sphaerospermum*[78] (**Figure 14**).



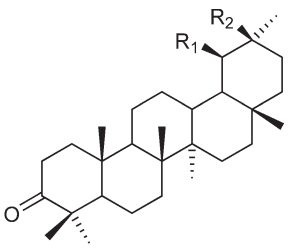
77



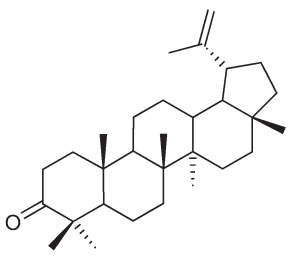
78



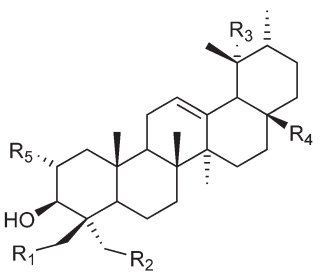
	R_1	R_2	R_3	R_4
79	H	H	H	Me
90	H	H	H	CH_2OH
91	H	H	H	CO_2Me
92	H	OH	H	CO_2Me
93	OH	OH	OH	CO_2Me



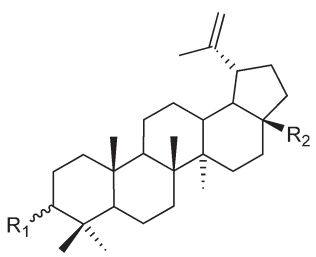
	R_1	R_2
80	Me	H; $\Delta^{12,13}$
81	H	Me; $\Delta^{12,13}$
82	H	Me; $\Delta^{18,19}$



	R_1	R_2
83	b-OH	H
84	a-OH	CO_2H



	R_1	R_2	R_3	R_4	R_5
85	H	H	H	Me	H
86	H	H	H	CH_2OH	H
87	H	H	H	CO_2Me	H
88	H	OH	H	CO_2Me	H
89	OH	OH	OH	CO_2Me	H
95	OH	OH	OH	CO_2H	OH
96	OH	OH	OH	Me	OH



94

Figure 13 Selected antifungal di- and triterpenes from Rubiaceae species.

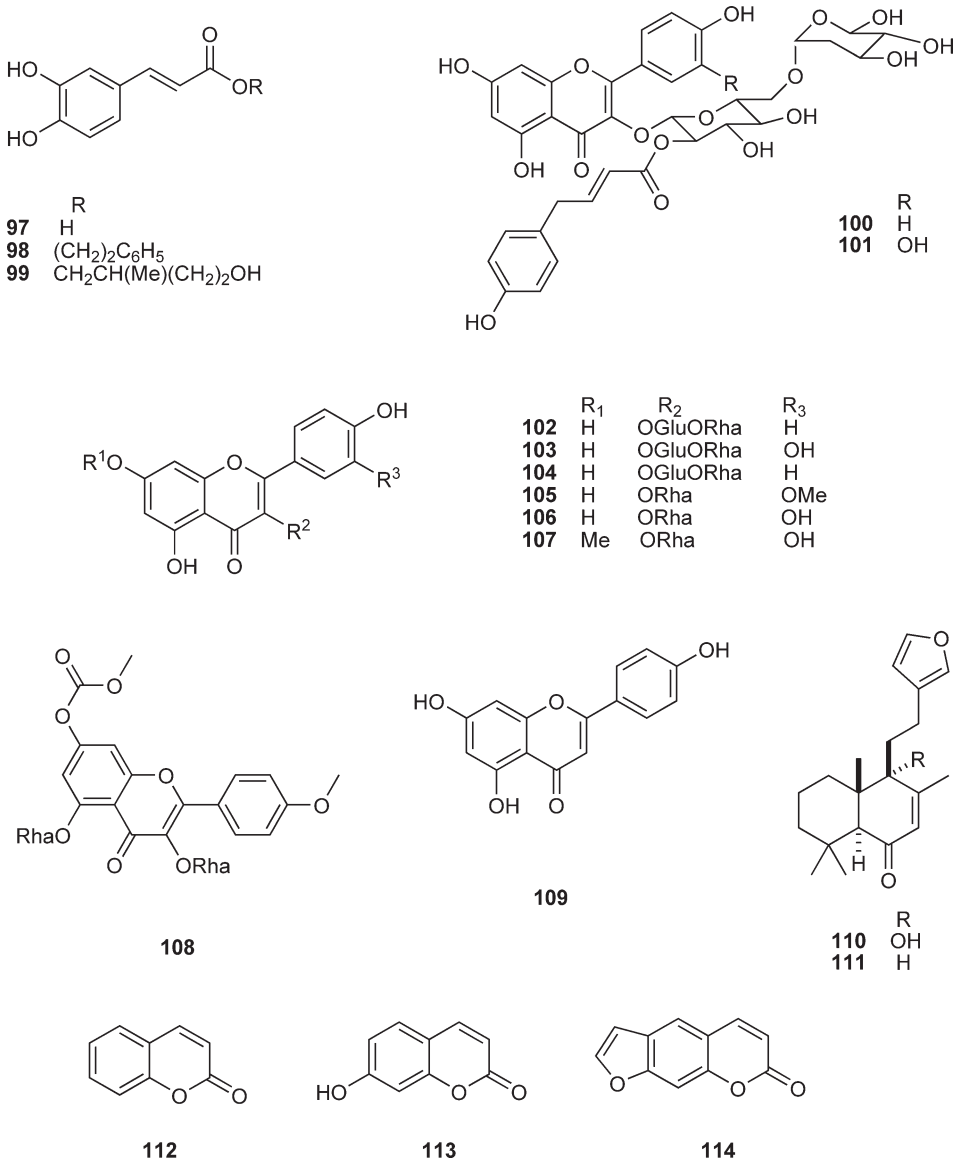


Figure 14 Selected antifungal compounds from Rubiaceae species.

Antifungal compounds have also been isolated from Apocynaceae, Celastraceae, Piperaceae, Lauraceae, Phytolaccaceae, and Styracaceae families as part of research projects carried out at the Nucley of Bioassays, Biosynthesis and Ecophysiology of Natural Products (NuBBE) laboratories. *Petiveria alliaceae* (Phytolaccaceae) is used as a traditional medicine for treating inflammation and nematode infection and owes its name to the garlic-smelling leaves and roots. Its chemical study showed the presence of alkyl and benzyl mono-, di-, tri-, and tetrasulfides **115–121** and the best activities were obtained for dipropyl disulphide (**115**) and dibenzyl disulphide (**117**), which showed MIC values as low as 0.1 μg ml^{-1} against the phytopathogen *C. sphaerospermum*.[79]

The chemical study of *Styrax ferrugineus*, used as a folk medicine against ulcer and in wound healing afforded benzofuran lignans **122–125** in addition to a coniferyl alcohol derivative, which were shown to inhibit the growth of the phytopathogens *C. cladosporioides* and *C. sphaerospermum*.[80] *Aspidosperma olivaceum* and *Malouetia arborea*, belonging to Apocynaceae, have yielded pentacyclic triterpenes **126–130**, whereas *Swartzia langsdorffii* (Fabaceae) afforded saponins **131–132** that inhibited the human pathogenic fungi *Candida albicans*, *Candida krusei*, and *Cryptococcus neoformans*[81,82] (**Figure 15**).

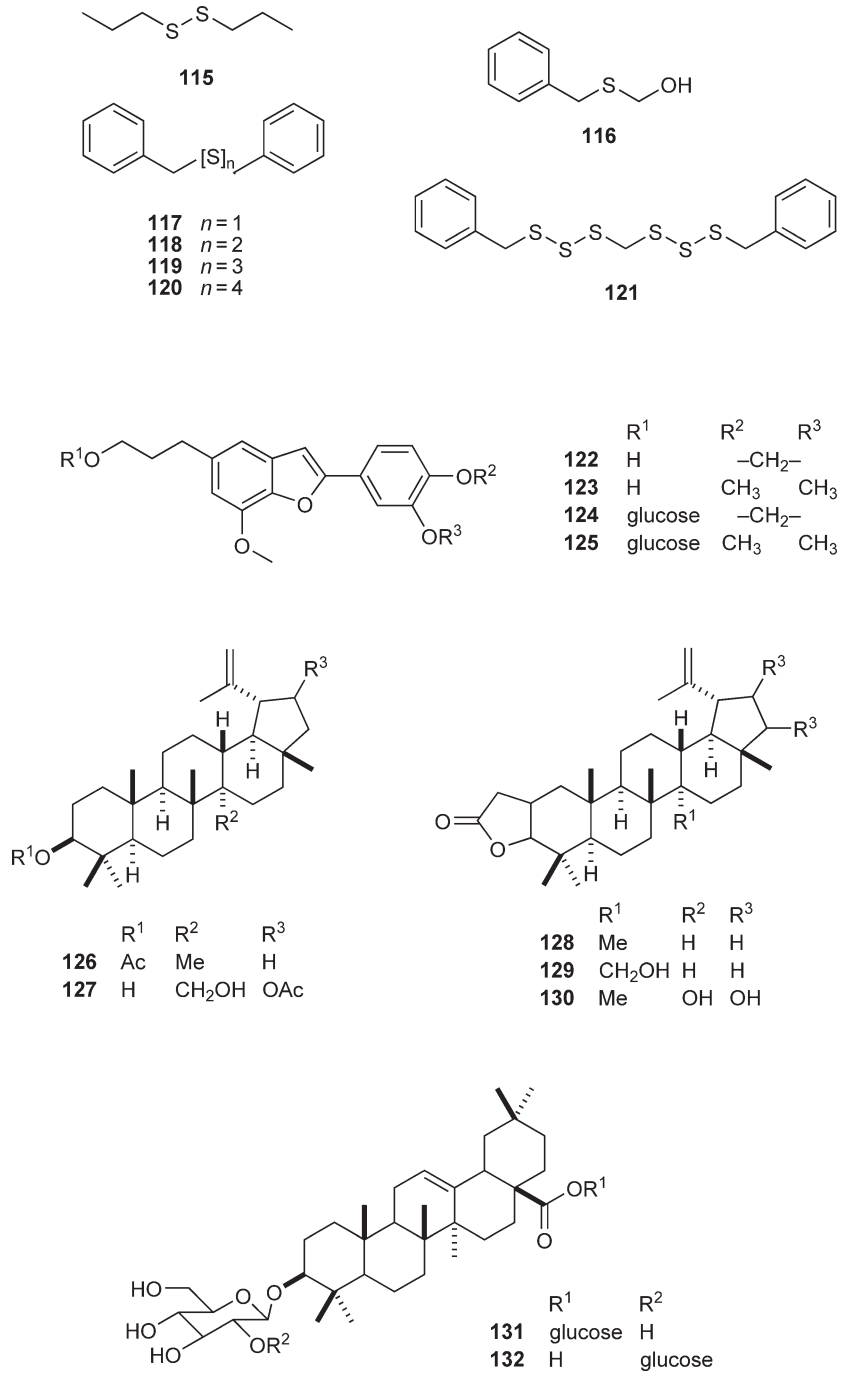


Figure 15 Selected antifungal natural compounds from Brazilian plant species.

The Piperaceae species, especially from *Piper* and *Peperomia* genera, afforded antifungal metabolites including cinnamoyl derivatives and amides. Antifungal amides bearing isobutyl, pyrrolidine, dihydropyridone, and piperidine moieties (**23–28**, **133–138**) were isolated from *Piper tuberculatum* and *Piper hispidum* and showed potent inhibition against the growth of *C. sphaerospermum*.[83,84] Additional antifungal amides, arboreumine (**22**), and *N*-[10-(13,14-methylenedioxyphenyl)-7(*E*),9(*E*)-pentadienoyl]-pyrrolidine (**139**) were obtained from *Piper arboreum*, in addition to cinnamoyl derivatives **140**, **142** from *P. tuberculatum*, which also showed potent inhibition of the growth of *C. cladosporioides* and *C. sphaerospermum*.[51] The study on the ethanol extracts of *Piper crassinervium*, *Piper aduncum*, *Piper hostmannianum*, and *Piper gaudichaudianum* showed the presence of benzoic acid derivatives crassinervic acid (**143**), aduncumene (**144**), hostmaniane (**145**,**146**), and gaudichaudianic acid (**18**), in addition to prenylated hydroquinones (**147–149**) as the major secondary metabolites. The best antifungal activities were obtained for crassinervic acid and hostmaniane against *C. cladosporioides* and *C. sphaerospermum*.[85,86] The chemical composition of essential oils from leaves, stems, and fruits of *P. tuberculatum*, *P. aduncum*, and *P. arboreum* was determined by gas chromatography–mass spectrometry (GC–MS) and comparison with literature data. There was a predominance of monoterpenes in *P. tuberculatum* and *P. aduncum* and of sesquiterpenes in *P. arboreum*. The richest essential oil composition was found in *P. aduncum*, which included linalool as the major compound. The best antifungal activity was observed for the essential oils from fruits of *P. aduncum* and *P. tuberculatum*, with MIC as low as 10 μg ml^{-1} against *C. cladosporioides* and *C. sphaerospermum*[87] (**Figure 16**).

Endophytic fungi associated with plant species studied at the Biota Program were shown to produce interesting compounds belonging to unusual classes of natural products. *Periconia atropurpurea*, an endophyte isolated from the leaves of *Xylopia aromatica*, was grown in nutrient media and, after extraction and chromatographic procedures, a coumarin (**150**), a benzaldehyde derivative (**151**), and periconicin (**152**) were obtained. The evaluation of the isolates toward phytopathogens *C. cladosporioides* and *C. sphaerospermum* evidenced a potent antifungal activity for the benzaldehyde.[88] *Colletotricum gloesporioides*, an endophyte obtained from *Cryptocarya mandioccana*, was shown to produce antifungal compounds (**153** and **154**).[89] The endophyte *Phomopsis cassiae*, isolated from *Cassia spectabilis*, was grown in agar and the ethyl acetate fraction from its ethanol extract afforded cadinane sequiterpenoids **154–159**, in addition to one benzoate derivative **160** and phomopsilactone (**161**), which evidenced moderate to strong inhibition of phytopathogens *C. cladosporioides* and *C. sphaerospermum*.[90,91] *Xylaria* sp. obtained from *Palicourea marcgravii* (Rubiaceae) was shown to produce the antifungal compounds 2-hexyl-3-methyl-butanodioic acid (**162**) and cytochalasin D (**163**), in addition to the inactive cytochalasin B (**164**), griseofulvin (**165**), and 7-dechlorogriseofulvin (**166**).[92] Studies on *Curvularia* sp., an endophyte associated with *Ocotea corymbosa* (Lauraceae), showed the presence of benzopyrane **167–169**, which showed weak antifungal activity against *C. cladosporioides* and *C. sphaerospermum*[93] (**Figure 17**).

3.05.5.2 Cytotoxic Compounds

In some studies, as in the case of *Chiococca bracchiata*, the activity detected in the crude extract was lost during fractionation procedures, possibly due to the occurrence of synergism among the crude extract constituents. In other studies, the phytochemical investigation carried out on species of the above-mentioned genera, led to the isolation of cytotoxic compounds. The phytochemical work on the leaves of *Chiococca alba*, used in folk medicine for its anti-inflammatory, diuretic, antiviral, and aphrodisiac properties,[94,95] showed the presence of albosides I–V **170–173**, which inhibited the growth of DNA repair-deficient strains of *Saccharomyces cerevisae*. *Seco*-iridoid glucoside **173** showed moderate activity against a mutant strain RS321, indicating a potential antitumoral activity, while the other iridoids proved to be inactive.[96] The bark decoctions of *Uncaria guianensis*, also belonging to Rubiaceae and popularly known as 'cat nail', are used in folk medicine for the treatment of arthritis, intestinal disorders, cancer, and some epidermal diseases. Phytochemical studies on its leaves revealed the presence of indole alkaloids isomitraphylline (**174**), 3-isoajmalicine (**175**), mitraphylline (**176**), and isomitraphylinic acid (**177**).[97] *Tocoyena formosa*, a small ornamental shrub, commonly known as 'genipapo do campo', also showed the presence of antifungal iridoids. The hydroalcoholic extract of its leaves showed moderate and nonselective activity toward the mechanism-based bioassay for potential anticancer drugs, which uses mutant strains of *Saccharomyces cerevisae*. This fact suggested the presence of cytotoxic agents, for example, antifungal agents, rather than potential anticancer agents. In addition to antifungal compounds, leaves of

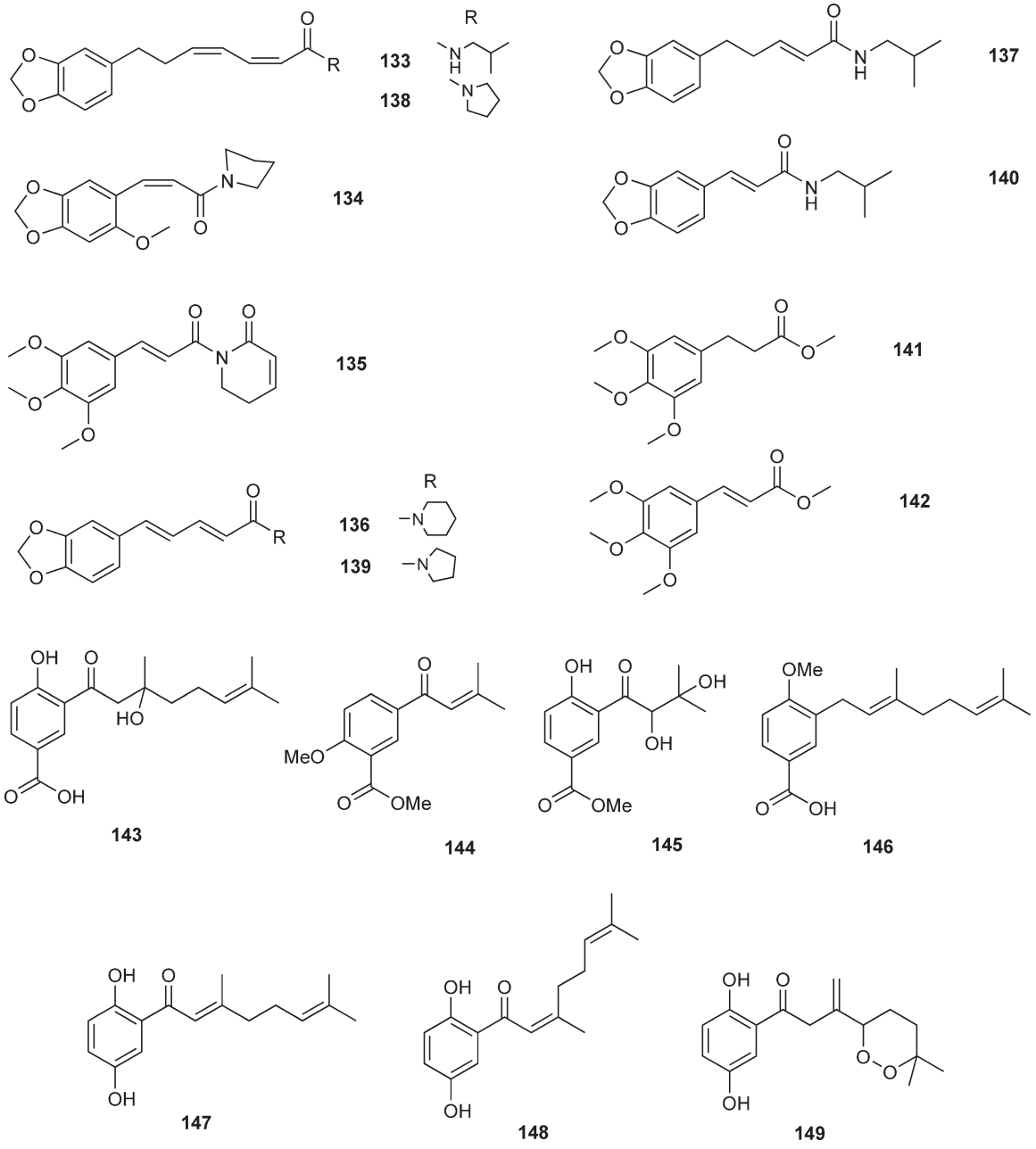


Figure 16 Antifungal compounds from Piperaceae.

Psychotria spectabilis afforded solidagenone (**110**) and psoralene (**114**), which also displayed selective cytotoxic activity against Rad 52Y mutant yeast strain of *Saccharomyces cerevisiae* and contributes to the chemical diversity of natural compounds found in Rubiaceae.[78]

Species from Lauraceae, Styracaceae, Flacourtiaceae, Celastraceae, Piperaceae, and Fabaceae have also been investigated for their cytotoxic and potential antitumor chemical components. The study on *Cinnamomum australe* (Lauraceae) leaves led to a cytotoxic cinnamoyl derivative *trans*-3′-methylsulfonylallyl *trans*-cinnamate (**178**) along with the inactive iryelliptin (**179**) and (7*R*,8*S*,1′*S*)-Δ8′-3′,5′-dimethoxy-1′,4′-dihydro-4′-oxo-7.0.2′,8.1′-neolignan (**180**).[98]

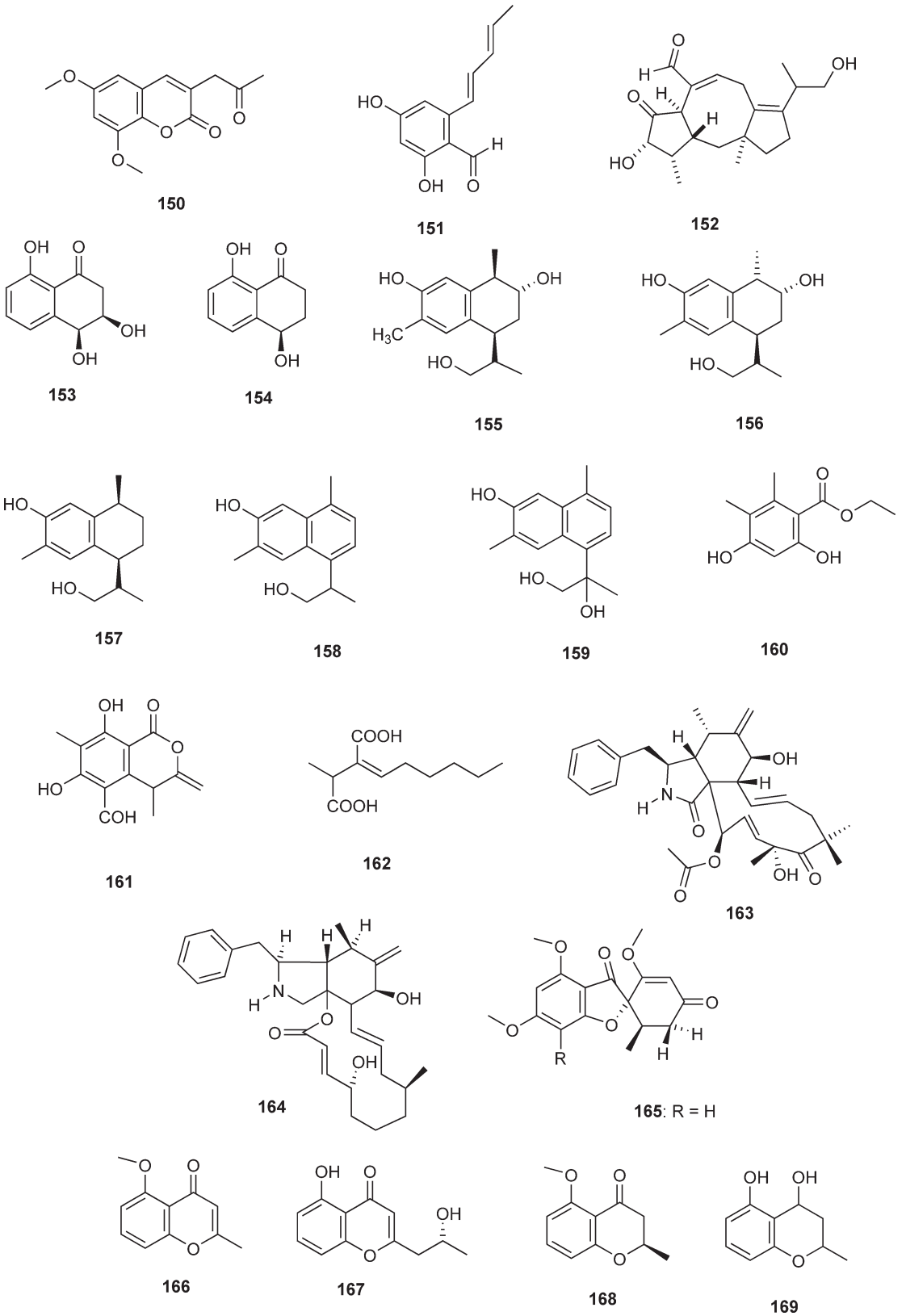


Figure 17 Compounds isolated from endophytic fungi.

Styrax camporum (Styracaceae) is known in Brazil as 'estoraque do campo' and its bark and trunk resin is used in traditional medicine to treat gastrointestinal diseases including ulcer. The chemical investigation of its leaves afforded benzofuran lignans egonol (**181**) and homoegonol (**182**), and the furofuran lignan syringaresinol (**183**), which showed cytotoxic activity against Hep-2 (larynx epidermoid carcinoma), HeLa (human cervix carcinoma), and C6 (rat glioma) tumor cell lines.[99]

Casearia sylvestris (Flacourtiaceae) is used popularly to treat gastrointestinal disorders. Clerodane diterpenes casearins G (**184**), S (**185**), and T (**186**) obtained from an acetylated bioactive extract of *Ca. sylvestris* (Flacourtiaceae) exhibited moderate but selective activity toward the DNA repair-deficient yeast *Saccharomyces cerevisiae* mutants RAD 52YK and RS.[100,101]

Sesquiterpene pyridine alkaloids (**187–188**), known as evoninate alkaloids, were obtained from *Maytenus aquifolium* and showed weak activity on mutant strains of *Saccharomyces cerevisae,* indicating a poor cytotoxic activity. Additionally, this plant species afforded quinonemethide triterpenes that have shown cytotoxic activity and have thus been quantified by HPLC.[102]

Piperaceae species have also been investigated for their cytotoxic activities. *Piper aduncum* afforded chromenes 2,2-dimethyl-2H-1-chromene-6-carboxylic acid (**189**), methyl 2,2-dimethyl-8-(3′-methyl-2′-butenyl)-2H-1-chromene-6-carboxylate (**190**), methyl 2,2-dimethyl-2H-1-chromene-6-carboxylate (**191**), methyl 8-hydroxy-2,2-dimethyl-2H-1-chromene-6-carboxylate (**192**), and the benzoic acid derivative, 3-(3′,7′-dimethyl-2′,6′-octadienyl)-4-methoxy-benzoic acid (**193**), which were shown to selectively inhibit mutant strains of *Saccharomyces cerevisae* RS 321N and RS322YK (rad 52Y), with IC_{12} values ranging from 100 to 139 μg ml^{-1}.[103] The semipurified fractions obtained from the ethanol extracts of leaves, stems, and roots of *Peperomia elongata* were tested for antimitotic activity in the sea urchin egg development test and for hemolytic activity using mice erythrocytes and have shown inhibitory activity against tumor cell lines with IC_{50} values ranging from 7.3 to 16.2 μg ml^{-1}. Lytic activity in mice erythrocytes was found at higher doses (>125 μg ml^{-1}).[104]

The chemical–biological investigation of *Cassia leptophylla* (reclassified as *Senna spectabilis*) extract from leaves showed inhibitory activity on DNA-repair deficient yeast *Saccharomyces cerevisiae* mutant strains. Reinvestigation of this species led to the isolation of piperidine alkaloids (–)-spectaline (**194**), (–)-3-*O*-acetylspectaline (**195**), (–)-7-hydroxyspectaline (**196**), and (–)-cassine (**197**) from *S. spectabilis* flowers and green fruits, which showed moderate cytotoxic activity toward a mutant strain of *Saccharomyces cerevisae*[105] (**Figure 18**).

Studies addressing the chemical constitution of endophytic fungi associated with selected plant species have also been undertaken in order to verify a possible correlation between the chemical profile of the plants and associated endophytes and explore novel sources of bioactive compounds. Nevertheless, the endophytes investigated so far, belonging to *Phomopsis*, *Curvularia*, *Xylaria*, *Periconia*, and other genera have shown distinct secondary metabolites from their host plants, including several new compounds, which is extremely attractive for the bioprospective activities carried out at NuBBE laboratories. The endophytic fungus *Phomopsis cassiae*, associated with *Cassia spectabilis* afforded ethyl 2,4-dihydroxy-5,6--dimethylbenzoate (**160**) and phomopsilactone (**161**), which showed cytotoxic activity against the human cervical tumor cell line (HeLa) in *in vitro* assays.[90,91] *Periconia atropurpurea*, isolated from the leaves of *Xylopia aromatica*, afforded a coumarin (**150**), a benzaldehyde derivative (**151**), and periconicin (**152**). Biological evaluation of the isolated compounds using two mammalian cell lines, human cervix carcinoma (HeLa) and Chinese hamster ovary (CHO) showed that compound **150** was inactive whereas the benzaldehyde (**151**) was able to induce a slight increase in cell proliferation of HeLa (37% increase) and CHO (38% increase) cell lines and periconicin showed potent cytotoxic activity against both cell lines, with IC_{50} of 8.0 μM.[88]

Studies on *Curvularia* sp., an endophyte associated with *O. corymbosa* (Lauraceae) showed the presence of benzopyran derivatives: 2-methyl-5-methoxy-benzopyran-4-one (**166**), (2′S)-2-(propan-2′-ol)-5-hydroxy-benzopyran-4-one (**167**), (2*R*)-2,3-dihydro-2-methyl-5-methoxy-benzopyran-4-one (**168**), and 2,3-dihydro-2-methyl-benzopyran-4,5-diol (**169**). The biological evaluation on HeLa and CHO cells, aiming to evaluate their potential effects on mammalian cell line proliferation, indicated that compound **167** was able to induce cell proliferation: 70% on HeLa cells and 25% on CHO cells.[93]

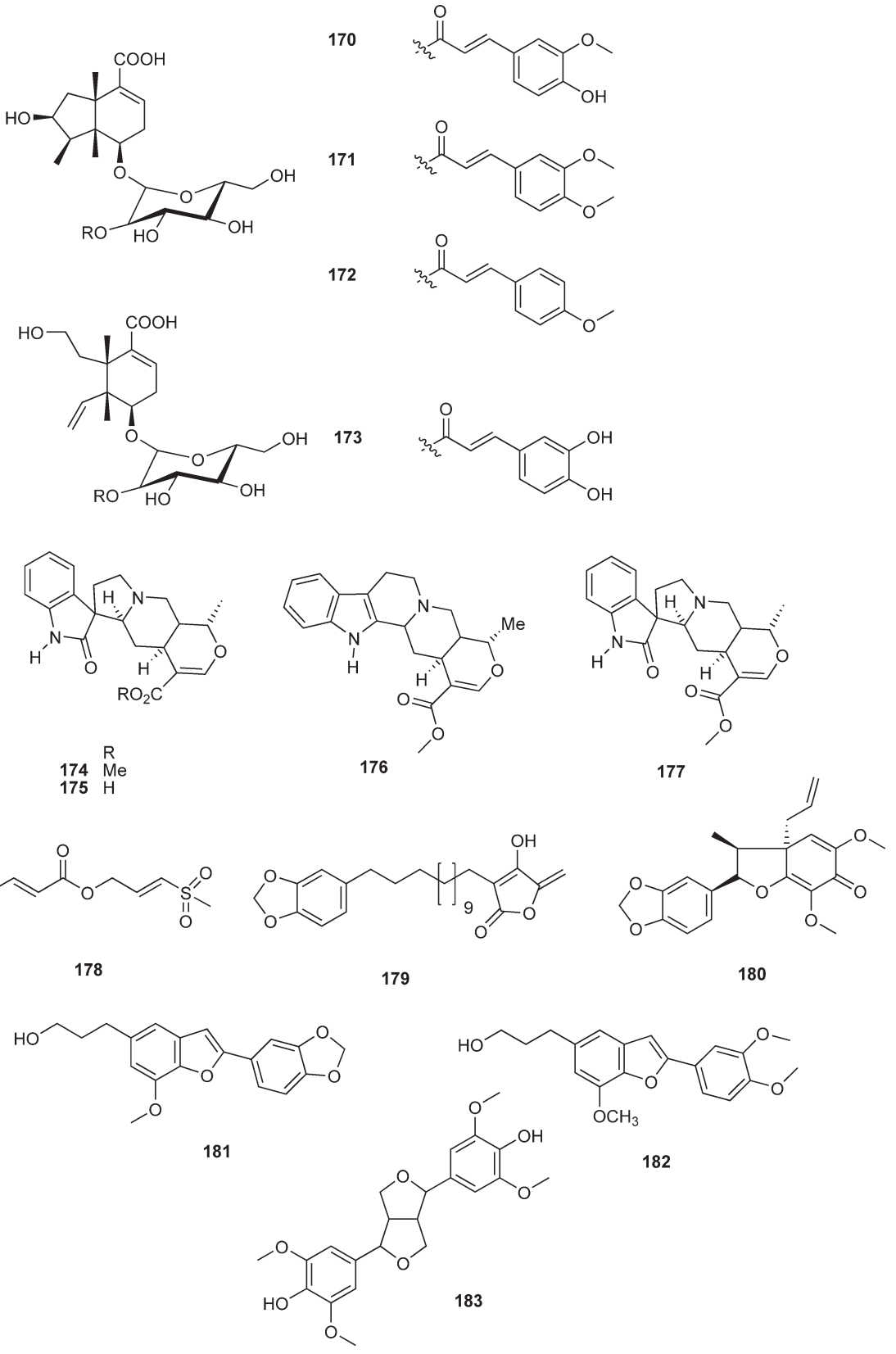
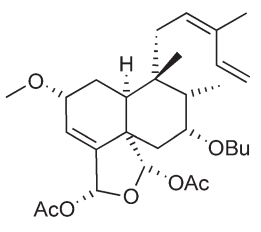
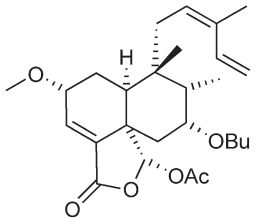


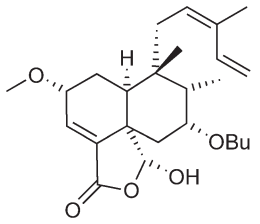
Figure 18 (Continued)



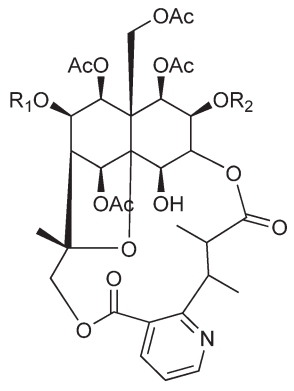
184



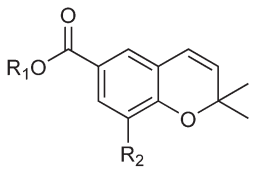
185



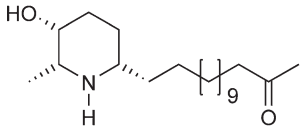
186



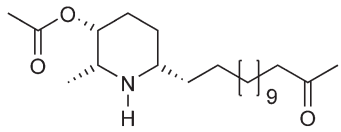
	R_1	R_2
187	COPh	AC
188	H	H



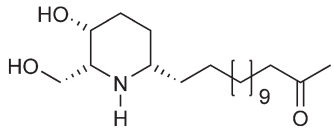
	R_1	R_2
189	H	H
190	CH_3	CH_2-CH=$(CH_3)_2$
191	CH_3	H
192	CH_3	OMe
193	CH_3	OH



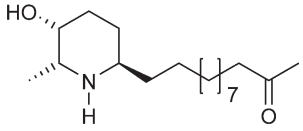
194



195



196



197

Figure 18 Cytotoxic compounds from plants of Cerrado and Atlantic Forest.

3.05.5.3 Antioxidant and Anti-Inflammatory Compounds

Chemical studies on the Amazon species *Iryanthera grandis* (Myristicaceae) yielded tocotrienols, γ-lactones, dihydrochalcones, lignans, and flavonolignans. The investigation of their antioxidant properties evidenced the potential of tocotrienols and flavonolignans toward lipoperoxidation inhibition, which stimulated the investigation of additional *Iryanthera* species.[106] *Iryanthera sagotiana* and *Iryanthera lancifolia*

have thus been selected for more in-depth study, and flavonols, dihydrochalcones, *neo*-lignans, jurueno-lides (ω-arylalkanoic acids), tocotrienols, and flavonolignans have been isolated from plant parts such as fruits, flowers, and leaves.[107] The evaluation of their lipoperoxidation-inhibitory properties using rat brain homogenates evidenced prominent activities for dihydrochalcones **198** and **199**, flavonols **200–203**, and flavonolignans **204–207**. The fruits of *Iryanthera juruensis* have also been investigated and afforded tocotrienols **208–209** and their oxidized metabolites plastoquinones **210** and **211** in addition to lignans **212–215** and ω-arylalkanoic acids **216–218**. The antioxidant potential of tocotrienols was investigated for their redox properties using cyclic voltammetry and evidenced potential similar to tocopherols, which constitute vitamin E. The presence of tocotrienols and plastoquinones in fatty acid-rich fruits suggests a possible protective role of such compounds for the germination-related tissues.[108,109]

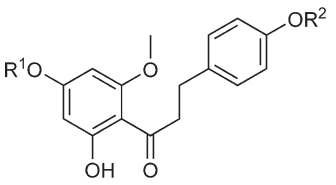
Tocotrienols (**208–209**) and flavones (**219–223**) from *I. juruensis* also shown to inhibit lipid perox-idation in a liposome model using large unilamelar vesicles (LUV).[110] In addition, tocotrienols, flavones, lignans, and plastoquinones were tested for their ability to inhibit inflammatory enzymes cyclooxy-genases 1 and 2 (COX-1 and COX-2), as the involvement of redox processes in inflammation has been established. Tocotrienols displayed potent nonselective inhibition of both enzymes, whereas plastoqui-nones inhibited COX-1 poorly and showed no inhibition of COX-2. Flavones inhibited COX-1 and COX-2 moderately and the lignans **212** and **213** showed potent and selective COX-2-inhibitory properties (**Figure 19**).

The piperidine alkaloids (**194**, **197**, and **224**) from *Senna spectabilis* have also been evaluated for their lipid peroxidation and COX enzyme-inhibitory activities and were shown to moderately inhibit liposomes from oxidation induced by Fe^{2+} or by 2,2′-azobis(2-amidinopropane) dihydrichloride (AAPH) free radical, except for the feruloyl-derived piperidine alkaloid, which presented enhanced lipoperoxidation inhibition, probably due to its phenol moiety. In addition, piperidine alkaloids showed moderate inhibition of COX-1 (∼40%) and marginal inhibition of COX-2 enzymes (<10%) at 100 ppm when compared to nonsteroidal anti-inflammatory drugs (NSAIDs) such as Aspirin, Vioxx, and Celebrex.[111]

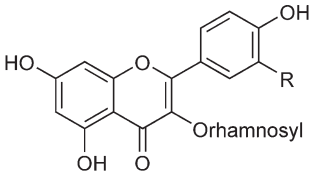
The Lauraceae family, which is closely related to Myristicaceae chemotaxonomically, has also been studied. *Cryptocarya aschersoniana* seedlings and *Nectandra grandiflora* extract from leaves yielded flavonol glucosides (**200** and **201**), in addition to protocathecuic acid (**225**), from *N. grandiflora*, which displayed radical scavenging activity toward DPPH (diphenylpycryl hydrazyl free radical).[112–114]

The Celastraceae and Hippocrateaceae families are known to accumulate pentacyclic and quinonemethide triterpenes, in addition to flavonoids. The investigation of *M. aquifolium* (Celastraceae) led to the isolation of catechins and flavonols (**226–230**), which have been evaluated for their redox properties using cyclic voltammetry, and radical scavenging activity toward DPPH, and confirmed the expected antioxidant potential normally associated with phenolic compounds, especially when reinforced by the presence of cathecol and α,β-unsaturated carbonyl moieties.[115] *Cheiloclinium cognatum* and *Salacia campestris* (Hippocrateaeceae) were shown to accumulate quinone-methide triterpenes campestrin (**231**), pristimerin (**232**), maytenin (**233**), 20α-hydroxymaytenin (**234**), 22β-hydroxypristimerin (**235**), 22β-hydroxymaytein (**236**), netzahualcoyene (**237**), netzahualcoyonol (**238**), netzahualcoyondiol (**239**), netzahualcoyone (**240**), and the aromatic 7,8-dihydro-6-oxo-netzahuacoyone (**241**), which are pentacyclic quinonemethide triterpenes with extended conjugated system through rings A and B, and in some cases, spread through rings C and/or D.[116,117] The structural features associated with antioxidant properties of such compounds include the dienone–phenol moiety, which confers great stability to the quinonemethide radical as soon as it has been formed. The use of Hippocrateaceae and Celastraceae species as folk medicines for medical purposes, including the treatment of gastric ulcer, might be related to their content in antiradical compounds such as flavonoids from *M. aquifolium* and quinonemethide triterpenes from *Cheiloclinium cognatum* and *Salacia campestris*.

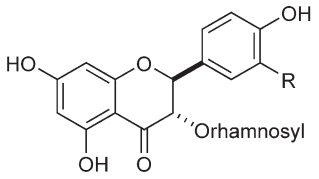
The development of analytical methodologies for detecting antioxidant compounds in complex matrices such as plant crude extracts led to the use of a tandem system high-performance liquid chromatography–ultraviolet–electrochemical detector (HPLC–UV–ECD) including two detectors: UV and electrochemical,



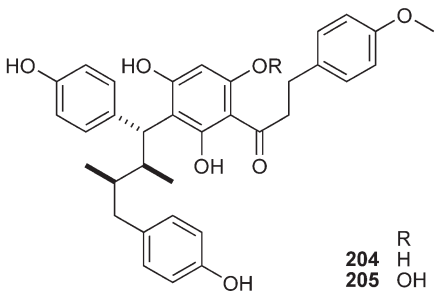
	R^1	R^2
198	H	Me
199	Me	H



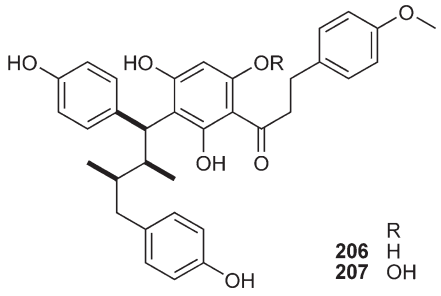
	R
200	H
201	OH



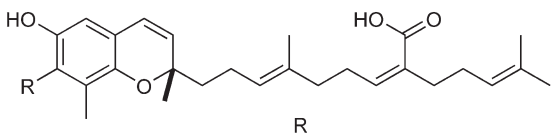
	R
202	H
203	OH



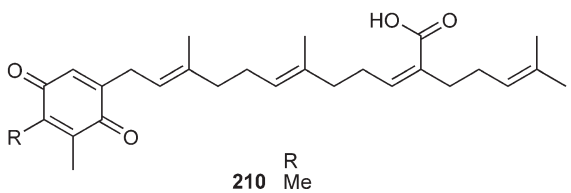
	R
204	H
205	OH



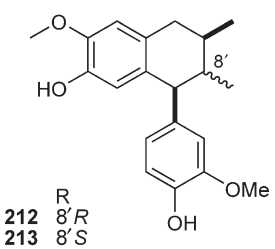
	R
206	H
207	OH



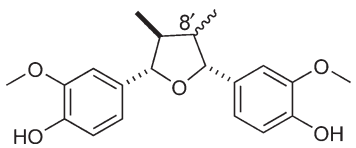
	R
208	Me
209	H



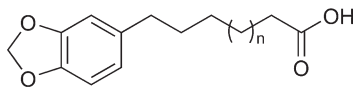
	R
210	Me
211	H



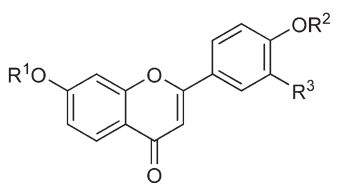
	R
212	8′*R*
213	8′*S*



	R
214	8′*R*
215	8′*S*



	R
216	*n* = 12
217	*n* = 14
218	*n* = 16



	R^1	R^2	R^3
219	Me	Me	H
220	Me	Me	Me
221	Me	Me	OH
222	H	Me	OH
223	H	H	H

Figure 19 Selected antioxidants from *Iryanthera* sp.

which enabled the comparison of UV- and ECD-detected chromatograms and the detection of redox active compounds.[118] The application of such method allowed the selection of promising plant species such as *Arrabidea samydoides* (Bignoniaceae) containing electrochemically active compounds, which yielded antioxidant xanthones (**242–247**). The subsequent evaluation of the free radical scavenging activity of the isolated compounds toward DPPH led to the quantification of the antiradical potential, and corroborated the key role of structural features as the α,β-unsaturated carbonyl and cathecol moieties.[119]

The Rubiaceae species have also afforded antioxidant compounds such as the phenyl- and arylpropanoid glucosides from *Coussarea hydrangeifolia*: hydrangeifolins I and II (**248** and **249**), in addition to caffeoylglucosides (**250** and **251**) and feruloylglucoside (**252**).[120] The study of the Amazon species *Chimarrhis turbinata* resulted in flavonoids and indole alkaloids, which have also been detected by a TLC test using β-carotene as revealing agent and by HPLC–UV–ECD. A series of kaempferol and quercetin glucosides in addition to cathechin, proanthocyanidin B3, and the new biflavonoid chimarroside (**253**) have been isolated and evaluated for their electrochemical and antiradicalar properties. The best antioxidant activities were observed for quercetin-derived compounds as expected, due to the presence of the cathecol moiety, when compared to kaempferol derivatives.[121] Indole alkaloids have also been obtained from *Chimarrhis turbinata* and showed poor antioxidant activity except for cordifoline (**263**), which presents a phenolic hydroxyl group in the extended conjugated system, thus accounting for a higher activity, comparable to the standard compound rutin.[122]

Chrysophyllum marginatum belongs to Sapotaceae, a plant family with several edible species. Its ethanol extract from the leaves showed the presence of gallic acid and flavonoids myricitrin, quercitrin, (−)-epigallocatechin, and (−)-epigallocatechin-3-*O*-gallate, which presented marked antioxidant activity, as expected for compounds containing the *o*-dihydroxyphenol moiety.[123]

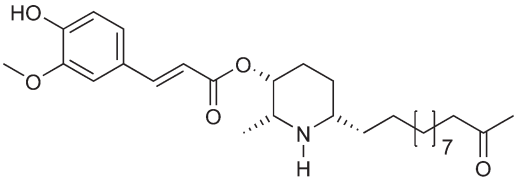
A series of flavonols has been isolated from the fruits of *Pterogyne nitens* (Fabaceae), namely pterogynoside (**254**), kaempferol, afzelin (**200**), kaempferitrin, quercetin, isoquercetrin, and rutin. Additionally, the leaves extract afforded 6-oxyflavone glucosides, nitensosides A and B (**255** and **256**), sorbifolin (**257**), sorbifolin 6-*O*-β-glucopyranoside (**258**), pedalitin (**259**), and pedalitin 6-*O*-β-glucopyranoside (**260**). The evaluation of their free radical scavenging activities toward DPPH and 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) evidenced higher antiradicalar properties for compounds possessing the cathecol moiety. In addition, the myeloperoxidase-inhibitory properties of all isolates was determined using the guaiacol method and the best activities were also observed for cathecol-derived compounds, evidencing the redox dependence for mycloperoxidase (MPO) inhibitory properties of flavonols and flavones[124,125] (**Figure 20**).

3.05.5.4 CNS-Active Compounds

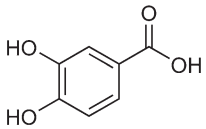
The chemical study on the leaves of *Chimarris turbinata* showed the presence of alkaloids 3,4-dehydro-strictosidine (**261**), 3,4-dehydro-strictosidine acid (**262**), cordifoline (**263**), strictosidine acid (**264**), strictosidine (**265**), 5α-carboxystrictosidine (**266**), turbinatine (**267**), desoxicordifoline (**268**), and harman-3-carboxylic acid (**269**).[122] The knowledge of the chemical profile of this genus is important in order to define its taxonomical position better, as its morphological features resemble those of the genus *Bathysa* (Rubiaceae), leading to mistakes in the classification of species belonging to these two genera. Indole alkaloids (**261–269**) were investigated for their AChE-inhibitory properties and compounds **267** and **268** exhibited moderate AChE inhibition at 0.1 and 1.0 μM. Additionally, compound **267** was evaluated in an *in vitro* rat brain assay and exhibited moderate activity ($IC_{50} = 1.86\,\mu M$), compared to the standard compound, galanthamine ($IC_{50} = 0.92\,\mu M$).

Senna spectabilis was originally investigated for its cytotoxic and potential antitumor activities, and afforded piperidine alkaloids (**194–197**) with a long methylene side chain. Owing to the similarity of the piperidine core to the acetylcholine structure, CNS-related activities were sought for this class of compounds. The antinociceptive profile of (−)-spectaline (**194**) and semisynthetic derivatives was thus investigated using chemical and thermal pain models in mice, which evidenced absence of central analgesic activity. On the other hand, the capsaicin-induced pain model evidenced potent inhibitory effect by spectaline, suggesting the involvement of vanilloid receptor systems.[126,127] Additional piperidine alkaloids **270–275** obtained by semisynthesis using (−)-spectaline (**194**) and (−)-3-*O*-acetyl-spectaline (**195**) from *S. spectabilis* were investigated for their

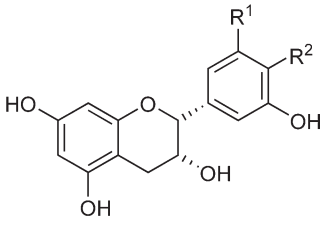
AChE-inhibitory activities. Compounds **270**, **272**, and **275** showed selective inhibitory activity on AChE when compared to butyrylcholinesterase, and all were fully efficacious in reverting scopolamine-induced amnesia in mice and suggested this class of compounds to be a new attractive target for research on Alzheimer's disease



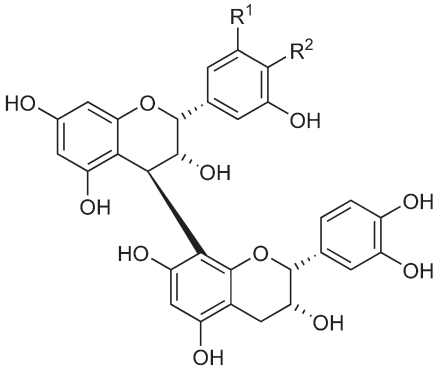
224



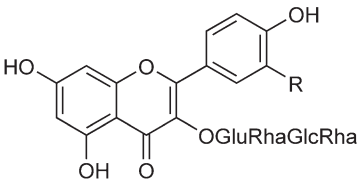
225



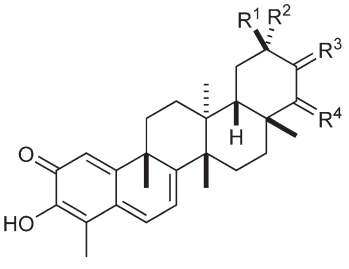
	R^1	R^2
226	OH	OMe
227	H	OH



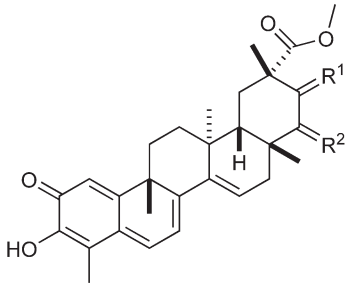
228



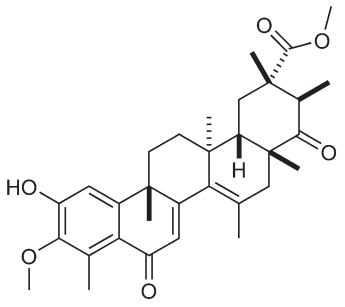
	R
229	H
230	OH



	R^1	R^2	R^3	R^4
231	H	Me	βOH, αOH	βOH, αH
232	Me	CO_2Me	H_2	H_2
233	Me	H	O	H_2
234	Me	OH	O	H_2
235	Me	CO_2Me	H_2	βOH, αH
236	Me	H	O	βOH, αH

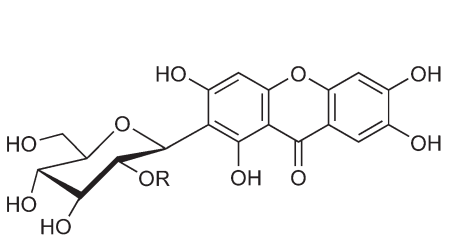


	R^1	R^2
237	H_2	H_2
238	βOH, αH	H_2
239	βOH, αH	βOH, αH
240	βOH, αH	O

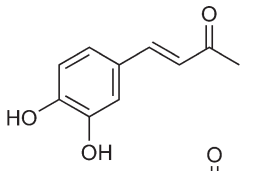


241

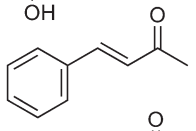
Figure 20 (Continued)



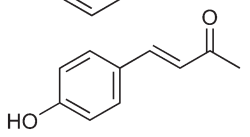
242 R =



243 R =

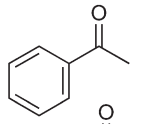


244 R =

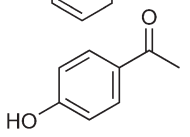


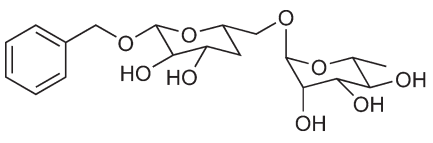
245 R = H

246 R =

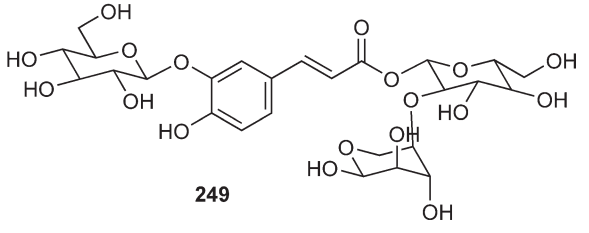


247 R =

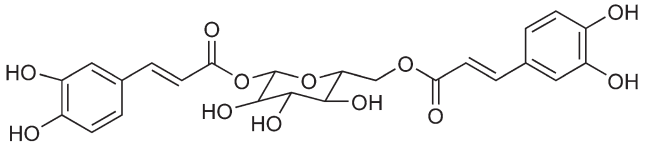




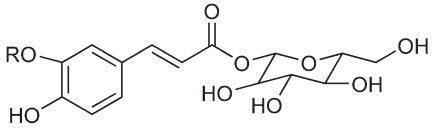
248



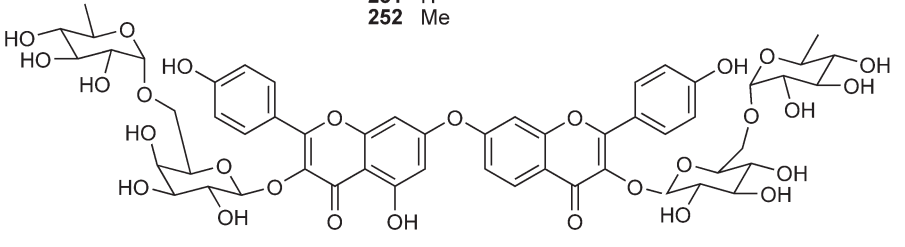
249



250

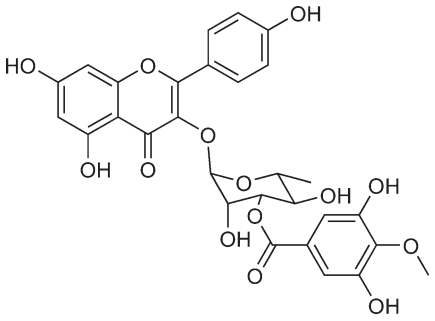


	R
251	H
252	Me

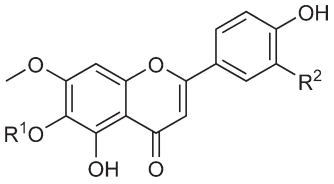


253

Figure 20 (Continued)



254



	R^1	R^2
255	OGluORha	H
256	OGluORha	OH
257	H	H
258	OGlu	H
259	H	OH
260	OGlu	OH

Figure 20 Antioxidants molecules isolated from species of Cerrado and Atlantic Forest.

(AD).[128–130] The semisynthetics (–)-3-*O*-acetyl-spectaline hydrochloride (**270**) and (–)-3-*O*-acetyl-cassine hydrochloride (**275**) obtained from natural piperidine alkaloids (**194** and **197**) are reversible noncompetitive cholinesterase inhibitors. Therefore, *in vivo* experiments with these compounds showed reversal of scopolamine-induced amnesia at 0.1–1.0 mg kg^{-1} i.p. in two behavioral tasks, and the peripheral potency ratio compares favorably with those of the ChE inhibitors in use for AD. Furthermore, the low acute toxicity of these derivatives proved to be essential to its assessment as candidate drugs for AD.[130,131] Tacrine and donepezil are both reversible noncompetitive inhibitors; however, these drugs are very toxic. Reversibility may be beneficial, because only reversible inhibitors lead to an upregulation of CNS AChE, and this is correlated with clinical improvement in AD patients. The piperidine alkaloid derivatives **270** and **275** are new candidates for AD, and have been submitted to preclinical studies with the partnership of a national pharmaceutical company. These results open new frontiers for biological molecules selected from the Biota bioprospecting program.[132]

Erythrina mulungu, also belonging to Fabaceae, has long been used as folk medicine to treat anxiety. The chemical study of its flowers showed the presence of erythrinian alkaloids, (+)-α-hydroxy-erysotrine (**276**), erythravine (**277**), and (+)-11-α-hydroxy-erythravine (**278**), which have been evaluated for anxiolytic properties in the elevated T-maze test. Additional experiments using the light–dark transition model and the elevated plus-maze suggested the alkaloids erytravine and (+)-11-α-hydroxy-erytravine to be responsible for the anxyolitic effects observed after administration of the crude extract.[133,134] Further pharmacological and toxicological assays with these alkaloids proved that they are lead candidates for CNS disorders and thus considered as one more successful example of bioprospection efforts with plants of the Brazilian biodiversity[135] (**Figure 21**).

3.05.6 Final Remarks and Conclusions

Natural biological compounds from Brazilian biodiversity will undoubtedly play a significant role in the discovery of new medicines, agrochemicals, nutraceuticals, and cosmetics. Although we are aware that the development of new bioproducts from Brazilian biodiversity is costly and time consuming, the current trends of this field in Brazil are very promising due to the large number of species that are still unexplored. This may be overcome by the opportunities for international investments in Brazil, which are nowadays greater and more attractive than ever. A recent survey from A. T. Kearney demonstrated that Brazil is reliable for new investments after United States and China. In addition to identifying new and potentially useful bioactive compounds, Biota/FAPESP Program will contribute to the generation of local natural products databases, plant collections, extraction and fractionation, isolation and structure elucidation of new derivatives, as well as pharmacology and toxicology studies. Another important debate on bioprospecting in

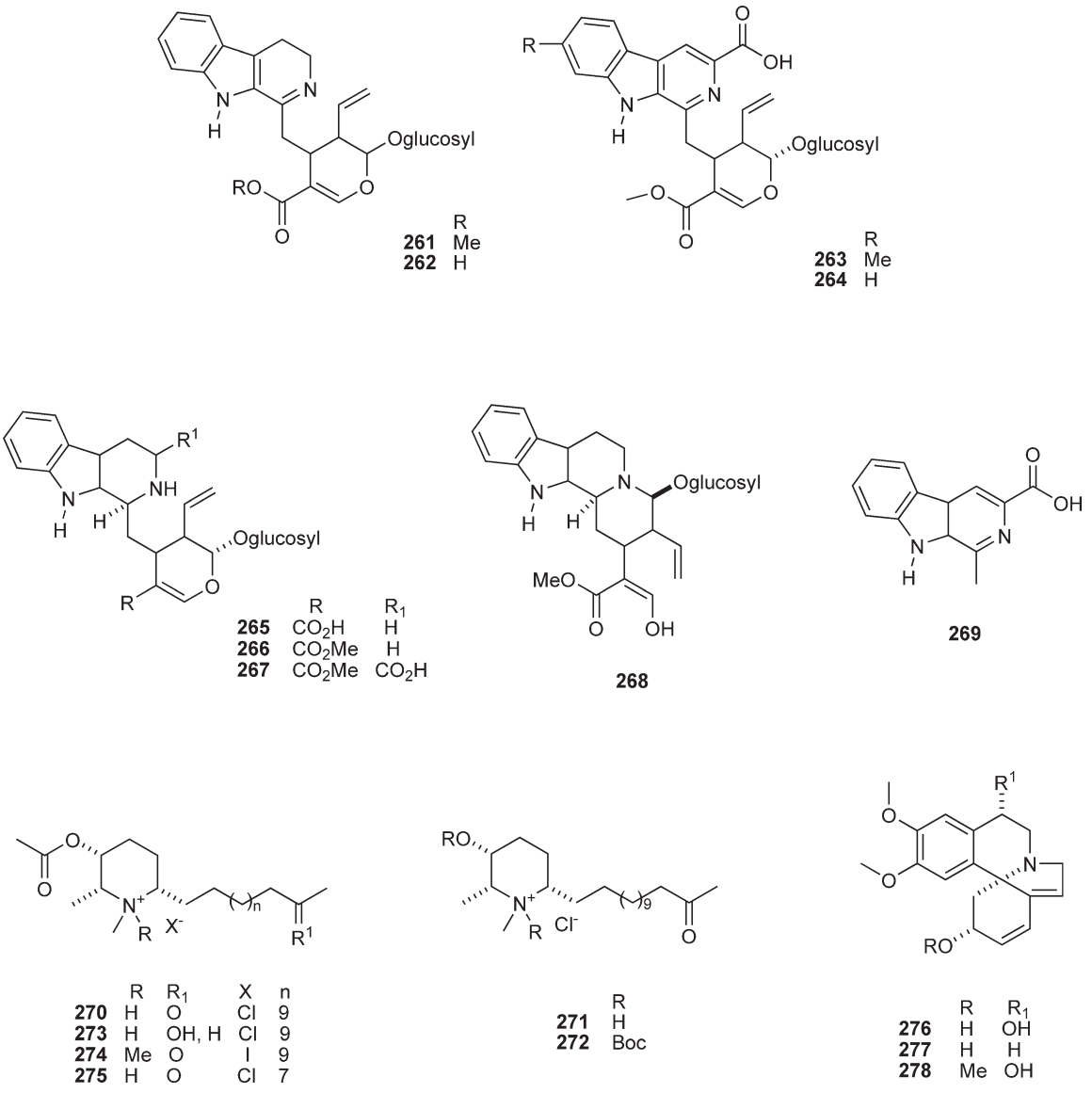


Figure 21 CNS-active compounds from Cerrado and Atlantic Forest.

Brazil is the sharing of benefits from its exploitation, including rewards to indigenous and local communities, revealing an urgent need for a short-term discussion on the intellectual property rights of the new findings emerging from the huge biodiversity. Additionally, the training of human resources, substantial infrastructure and modern equipments, and close collaborations between academia and industry represent important contributions of this reference program, which is a new model of exploitation and conservation of the biodiversity as the basis for Brazilian economic development in the globalized world.

References

1. T. M. Lewinsonhn; P. I. Prado, *Biodiversidade Brasileira – Síntese do estado atual do conhecimento*, 1st ed.; Editora Pinsky: São Paulo, 2002; Chapter 1, pp 17–25.
2. B. Groombridge, *Global Biodiversity Status of the Earth's Living Resources*; Chapman & Hall: London, 1999.
3. T. M. Lewinsonhn; P. I. Prado, *Biodiversidade Brasileira – Síntese do estado atual do conhecimento*, 1st ed.; Editora Pinsky: São Paulo, 2002; Chapter 4, pp 90–92.

4. C. A. Joly; M. P. M. Aidar; C. A. Klink; D. G. McGrath; A. G. Moreira; P. Moutinho; D. C. Nepstad; A. A. Oliveira; A. Pott; M. J. N. Rodal; E. V. S. B. Sampaio, *Ciênc. Cult.* **1999**, *51*, 331–336.
5. R. B. Cavalcanti; C. A. Joly, Biodiversity and Conservation Priorities in the Cerrado Regions. In *The Cerrados of Brazil: Ecology and Natural History of a Neotropical Savanna,* 1st ed.; P. S. Oliveira, R. J. Marquis, Eds.; Columbia University Press: New York, USA 2002; pp 351–367.
6. M.A.M. Victor, Sociedade Brasileira de Silvicultura, **1975**, p 48.
7. J. N. F. Kronka; M. A. Nalon; C. K. Matsukura; M. Pavão; J. R. Guillaumon; A. C. Cavelli; E. Giannotti; M. S. S. Ywane; L. M. P. R. Lima; J. Montes; I. H. D. Cali; P. G. Haakck, Áreas de domínio do Cerrado no Estado de São Paulo; Secretaria do Meio Ambiente ed.: São Paulo, 1998; Vol. 1.
8. N. Myers; R. A. Mittermeier; C. G. Mittermeier; G. A. B. Fonseca; J. Kent, *Nature* **2000**, *403*, 852–858.
9. L. Schulman; K. Ruokolainen; L. Junikka; I. E. Saaksjarvi; M. Salo; S. K. Juvonen; J. Salo; M. Higgins, *Biodivers. Conserv.* **2007**, *16*, 3011–3051.
10. M. P. Pinto; J. A. F. Diniz; L. M. Bini; D. Blamires; T. F. L. V.B. Rangel, *Divers. Distrib.* **2008**, *14*, 78–86.
11. C. D. Prates-Clark; S. S. Saatchi; D. Agosti, *Ecol. Modell.* **2008**, *211*, 309–323.
12. A. B. Rylands; K. Brandon, *Conserv. Biol.* **2005**, *19*, 612–618.
13. E. C. Borges; J. P. Dujardin; C. J. Schofield, *Acta Trop.* **2005**, *93*, 119–126.
14. E. Tohver; M. S. D'Agrella; R. I. F. Trindade, *Precambrian Res.* **2006**, *147*, 193–222.
15. S. S. Saatchi; R. A. Houghton; R. C. D. S. Alvala, *Glob. Chang. Biol.* **2007**, *13*, 816–837.
16. W. F. Laurance, *Trends Ecol. Evol.* **2005**, *20*, 645–648.
17. H. E. M. Nascimento; W. F. Laurance, *For. Ecol. Manage.* **2002**, *168*, 311–321.
18. D. Metcalfe; P. Meir; L. E. O. C. Aragão, *For. Ecol. Manage.* **2008**, *255*, 558–566.
19. P. Parolin; O. De Simone; K. Haase, *Bot. Rev.* **2004**, *70*, 357–380.
20. J. Tollefson, *Nature* **2008**, *452*, 134–135.
21. E. Ostrom; H. Nagendra, *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 19224–19231.
22. J. N. Moreira; M. D. Lira; M. V. F. dos Santos, *Pesqui. Agropecu. Bras.* **2006**, *41*, 1643–1651.
23. A. C. D. A. Moura, *Int. J. Primatol.* **2007**, *28*, 1279–1297.
24. T. F. L. V.B. Rangel; L. M. Bini; J. A. F. Diniz, *Appl. Geogr.* **2007**, *27*, 14–27.
25. G. Durigan; M. F. de Siqueira; G. A. D. C. Franco, *Sci. Agricola* **2007**, *64*, 355–363.
26. J. Baker; R. Borris; B. Carte; G. Cordell; D. Doerjarto; G. Cragg; M. Gupta; M. Ywu; D. Madilud; V. Tiler, *J. Nat. Prod.* **1995**, *58*, 1325–1357.
27. A. M. Z. Martini; P. Fiaschi; A. M. Amorim; J. L. Paixão, *Biodivers. Conserv.* **2007**, *16*, 3111–3128.
28. B. Wuethrich, *Science* **2007**, *315*, 1070–1072.
29. S. Saatchi; D. Agosti; K. Alger, *Conserv. Biol.* **2001**, *15*, 867–875.
30. D. Wallace; G. D. Boulter, *Phytochemistry* **1976**, *15*, 137–141.
31. G. E. Overbeck; S. C. Muller; A. Fidelis, *Perspect. Plant Ecol. Evol. Syst.* **2007**, *9*, 101–116.
32. M. Tabarelli; L. P. Pinto; J. M. C. Silva, *Conserv. Biol.* **2005**, *19*, 695–700.
33. B. Mertens; R. Poccard-Chapuis; M. G. Piketty, *Agric. Econ.* **2002**, *27*, 269–294.
34. A. C. Pinto, *Quim. Nova* **1995**, *18*, 608–615.
35. O. R. Gottlieb; W. B. Mors, *J. Agric. Food Chem.* **1980**, *26*, 196–215.
36. C. Viegas, Jr.; V. da S. Bolzani; E. J. Barreiros, *Quim. Nova* **2006**, *29*, 326–337.
37. N. P. Santos, *Quim. Nova* **2007**, *30*, 1038–1045.
38. A. C. Pinto; M. R. Almeida; J. R. Lima; N. P. Santos, *Ciência Hoje* **2007**, *40*, 1038–1042.
39. B. Halpern; O. Shmid, *Helv. Chim. Acta* **1958**, *41*, 1109.
40. K. Jewers; J. J. W. Coppen; A. H. Manchanda; H. M. Paaisley; H. M. Castillo, *Pahlavi Med. J.* **1975**, *6*, 52–56.
41. M. Motidome; M. E. leeking; O. R. Gottlieb, *An. Acad. Bras. Cienc.* **1970**, *42*, 375–376.
42. P. Romoff; M. Yoshida, *Ciênc. Cult.* **1997**, *49*, 345–353.
43. O. R. Gottlieb; M. Yoshida, Lignans. In *Natural Products of Wood Plants I*; J. W. Rowe, Ed.; Springer Series in Wood Science; Springer-Verlag: New York, 1989; pp 439–511.
44. D. W. Taylor; J. L. Rickey, *Plant Sys. Evol.* **1992**, *180*, 137.
45. J. M. Batista, Jr.; A. A. Lopes; D. L. Ambrósio; L. O. Regasini; M. J. Kato; V. da S. Bolzani; R. M. B. Cicarelli; M. Furlan, *Biol. Pharm. Bull.* **2008**, *31*, 538–540.
46. R. V. Da Silva; H. V. da S. Bolzani; M. Navickiene; M. C. M. Young; M. J. Kato; C. I. Meda; M. Furlan, *Phytochemistry* **2002**, *59*, 521–527.
47. A. Salatino; M. L. F. Salatino; D. Y. A. C. Dos Santos, *Genet. Mol. Biol.* **2000**, *23*, 931–940.
48. A. C. Pinto; M. L. Patitucci; R. S. Da Silva; P. P. S. Queiroz; A. Kelekon, *Tetrahedron* **1983**, *39*, 3351–3354.
49. A. C. Pinto; W. S. Garcez; P. P. S. Queiroz; N. G. Fiorani, *Phytochemistry* **1994**, *37*, 1115–1117.
50. A. C. Pinto; O. A. C. Antunes; C. M. Rezende; C. R. D. Correia, *Phytochemistry* **1995**, *38*, 1269–1271.
51. A. C. Pinto; S. K. Prado; R. Pinchin, *Phytochemistry* **1981**, *20*, 520–521.
52. A. C. Pinto; D. H. T. Zocher; M. C. Rezende; H. E. Gottlieb, *Nat. Prod. Lett.* **1995**, *6*, 209–213.
53. A. C. Pinto; M. G. Pizzolatti, *Tetrahedron* **1997**, *53*, 2005–2012.
54. A. B. Schefer; U. Braumann; L. Tseng; M. Spraul; M. G. Soares; J. B. Fernandes; M. F. G. F. da Silva; P. C. Vieira; A. G. Ferreira, *J. Chromatogr. A* **2006**, *1128*, 152–163.
55. W. B. Marques; H. S. dos Santos; O. D. L. Pessoa; R. Braz-Filho; T. L. G. Lemos, *Phytochemistry* **2000**, *55*, 793–797.
56. V. da S. Bolzani; M. F. G. F. Silva; A. I. Rocha; O. R. Gottlieb, *Biochem. Syst. Ecol.* **1984**, *12*, 159–166.
57. M. C. M. Young; M. R. Braga; S. M. C. Dietrich; V. da S. Bolzani; L. M. V. Trevisan; O. R. Gottlieb, *Opera Bot. Belg.* **1996**, *7*, 205–212.
58. L. T. Dusman; T. C. M. Jorge; M. C. De Souza; M. N. Eberlin; E. C. Meurer; C. C. Bocca; E. A. Basso; M. H. Sarragiotto, *J. Nat. Prod.* **2004**, *67*, 1886–1888.
59. L. Kato; R. M. Braga; I. Koch; L. S. Kinoshita, *Phytochemistry* **2002**, *60*, 315–320.

60. C. A. Joly; L. Casatti; M. C. W. de Brito; N. A. Menezes; R. R. Rodrigues; V. da; S. Bolzani, Histórico do Programa Biota-FAPESP – O Instituto Virtual da biodiversidade. In *Diretrizes para a Conservação e Restauração da Biodiversidade no estado de São Paulo*; 1st ed.; R. R. Rodrigues, Ed.; Secretaria do Meio Ambiente: São Paulo, 2008; pp 46–55.
61. V. da; S. Bolzani; M. C. M. Young; A. J. Carvalheiro; A. R. Araújo; D. H. Silva; M. N. Lopes; M. Furlan, Secondary Metabolites from Brazilian Rubiaceae Plant Species: Chemotaxonomical and Biological Significance. In *Recent Research Development in Phytochemistry,* 1st ed.; S. G. Pandaly, Ed.; Research Signpost: Trivandrum, New Delhi, 2001; pp 19–31.
62. R. D. Simpson; R. A. Sedjo; J. W. Reid, *J. Polit. Econ.* **1996**, *104*, 1548–1570.
63. M. R. Braga; M. C. M. Young; J. V. A. Ponte; S. M. C. Dietrich; V. P. Emerenciano; O. R. Gottlieb, *Biochem. Syst. Ecol.* **1986**, *14*, 507–514.
64. M. R. Braga; M. C. M. Young; J. V. A. Ponte; S. M. C. Dietrich; O. R. Gottlieb, *J. Chem. Ecol.* **1991**, *17*, 1079–1090.
65. L. Hamerski; M. Furlan; D. H. S. Silva; A. J. Cavalheiro; M. N. Eberlin; D. M. Tomazela; V. D. Bolzani, *Phytochemistry* **2003**, 397–400.
66. V. S. Bolzani; L. M. V. Trevisan; C. M. Izumisawa; A. A. L. Gunatilaka; D. G. I. Kingston; M. C. M. Young, *Phytochemistry* **1997**, *46*, 305–308.
67. V. S. Bolzani; L. M. V. Trevisan; C. M. Izumisawa; M. C. M. Young, J. Braz. Chem. Soc. 1997, 157–160.
68. L. Hamerski; C. Carbonezi; A. J. Cavalheiro; V. S. Bolzani; M. C. M. Young, *Quim. Nova,* **2005**, *28*, 601–604.
69. R. S. G. Olea; N. F. Roque; V. S. Bolzani, *J. Braz. Chem. Soc.* **1997**, *8*, 257–259.
70. M. N. Lopes; A. C. Oliveira; M. C. M. Young; V. S. Bolzani, *J. Braz. Chem. Soc.* **2004**, *15*, 468–471.
71. V. S. Bolzani; L. M. V. Trevisan; M. C. M. Young, *Phytochemistry* **1991**, *30*, 2089–2091.
72. M. C. M. Young; M. R. Braga; S. M. Dietrich; H. E. Gottlieb; L. M. V. Trevisan; V. S. Bolzani, *Phytochemistry* **1992**, *31*, 3433–3435.
73. V. C. Silva; V. S. Bolzani; M. C. M. Young; M. N. Lopes, *J. Braz. Chem. Soc.* **2007**, *18*, 1405–1408.
74. V. C. Silva; A. O. Faria; V. S. Bolzani; M. N. Lopes, *Helv. Chim. Acta* **2007**, *90*, 1781–1785.
75. C. B. Brochini; D. Martins; N. F. Roque; V. Da; S. Bolzani, *Phytochemistry* **1994**, *36*, 1293–1295.
76. M. C. M. Young; A. R. Araújo; C. A. Silva; M. N. Lopes; L. M. V. Trevisan; V. S. Bolzani, *J. Nat. Prod.* **1998**, *61*, 936–938.
77. P. J. C. Benevides; M. C. M. Young; V. S. Bolzani, *Pharm. Biol.* **2004**, *42, 8*, 565–569.
78. P. J. C. Benevides; M. C. M. Young; A. M. Giesbrecht; N. F. Roque; V. S. Bolzani, *Phytochemistry* **2001**, *57*, 743–747.
79. P. M. Pauletti; A. R. Araujo; M. C. M. Young; A. M. Giesbrecht; V. S. Bolzani, *Phytochemistry* **2000**, *55*, 597–601.
80. L. Scorzoni; T. Benaducci; A. M. F. Almeida; D. H. S. Silva; V. S. Bolzani; M. J. S. Mendes-Gianinni, *Braz. J. Microbiol.* **2007**, *38*, 391–397.
81. S. R. Marqui; R. B. Lemos; L. A. Santos; I. Castro-Gamboa; A. J. Cavalheiro; V. S. Bolzani; D. H. S. Silva; L. Scorzoni; A. M. F. Almeida; M. J. S. Mendes-Giannini; M. C. M. Young; L. M. B. Torres, Quim. Nova 2008, 828–831.
82. A. C. Alécio; V. S. Bolzani; M. C. M. Young; M. J. Kato; M. Furlan, *J. Nat. Prod.* **1998**, *61* (5), 637–639.
83. H. M. D. Navickiene; A. C. Alécio; M. J. Kato; V. S. Bolzani; M. C. M. Young; A. J. Cavalheiro; M. Furlan, *Phytochemistry* **2000**, *55* (7), 621–626.
84. A. P. Danelutte; J. H. G. Lago; M. C. M. Young; M. J. Kato, *Phytochemistry* **2003**, *64*, 555–559.
85. J. H. G. Lago; C. S. Ramos; D. C. C. Casanova; A. A. Morandin; D. C. B. Bergamo; A. J. Cavalheiro; V. S. Bolzani; M. Furlan; E. F. Guimarães; M. C. M. Young; M. J. Kato, *J. Nat. Prod.* **2004**, *67*, 1783–1788.
86. H. M. D. Navickiene; A. A. Morandim; A. C. Alécio; L. O. Regasini; D. C. B. Bergamo; A. J. Cavalheiro; M. N. Lopes; V. S. Bolzani; M. Furlan; M. J. Kato, *Quim. Nova* **2006**, *29* (3), 467–470.
87. H. L. Teles; R. Sordi; G. H. Silva; I. Castro-Gamboa; V. S. Bolzani; L. H. Pfenning; M. C. M. Young; C. M. Costa-Neto; A. R. Araújo, *Phytochemistry* **2006**, *67*, 2686–2690.
88. A. R. Araújo; M. L. Inácio; H. C. Trevisan; G. H. Silva; H. L. Teles; V. S. Bolzani; M. C. M. Young; L. H. Pfenning; A. J. Cavalheiro, *Biochem. Syst. Ecol.* **2006**, *34*, 822–824.
89. A. R. Araújo; H. L. Teles; G. H. Silva; V. S. Bolzani; M. C. M. Young; L. H. Pfenning; M. N. Eberlin; R. Haddad; C. M. Costa-Neto; I. Castro-Gamboa, *Phytochemistry* **2006**, *67*, 1964–1969.
90. G. H. Silva; H. L. Teles; H. C. Trevisan; M. C. M. Young; L. H. Pfenning; M. N. Eberlin; R. Haddad; V. S. Bolzani; A. R. Araújo, *J. Braz. Chem. Soc.* **2005**, *6b*, 1463–1466.
91. M. C. Cafêu; G. H. Silva; H. L. Teles; V. S. Bolzani; A. R. Araújo; M. C. M. Young; L. H. Pfenning, *Quim. Nova* **2005**, *28* (6), 991–995.
92. H. L. Teles; G. H. Silva; I. Castro-Gamboa; V. S. Bolzani; J. O. Pereira; C. M. Costa-neto; R. Haddad; M. N. Eberlin; M. C. M. Young; A. R. Araújo, *Phytochemistry* **2005**, *66* (18), 2363–2367.
93. O. A. Costa, *Rev. Bras. Farm.* **1978**, *8*, 124.
94. F. Dorvault, *L'officine*, 20th ed.; Vigot: Paris, 1978.
95. C. A. Carbonezi; D. Martins; M. C. M. Young; M. N. Lopes; M. Furlan; E. Rodrigues Filho; V. da S. Bolzani, *Phytochemistry* **1999**, *51*, 781–785.
96. C. A. Carbonezi; L. Hamerski; M. Furlan; V. S. Bolzani, *Quim. Nova* **2004**, *27*, 878–881.
97. C. A. Carbonezi; M. N. Lopes; D. H. S. Silva; A. R. Araújo; V. S. Bolzani; M. C. M. Young; M. R. Silva, *Quim. Nova* **2004**, *27* (2), 196–198.
98. H. L. Teles; J. P. Hemerly; P. M. Pauletti; J. R. C. Pandolfi; A. R. Araujo; S. R. Valentini; M. C. M. Young; V. S. Bolzani; D. H. S. Silva, *Nat. Prod. Res.* **2005**, *19*, 319–323.
99. M. Furlan; P. R. F. Carvalho; M. C. M. Young; D. Kingston; V. S. Bolzani, *Phytochemistry* **1998**, *49*, 1659–11652.
100. K. F. Bandeira; A. G. Tininis; V. S. Bolzani; A. J. Cavalheiro, *Phytochem. Anal.* **2006**, *17*, 168–175.
101. J. Corsino; V. S. Bolzani; S. C. França; A. M. S. Pereira; M. Furlan, *Phytochemistry* **1998**, *48*, 273–278.
102. D. C. Baldoqui; M. J. Kato; A. J. Cavalheiro; V. S. Bolzani; M. C. M. Young; M. Furlan, *Phytochemistry* **1999**, *51*, 899–902.
103. P. B. Campos; D. E. Castro; J. J. T. Gama; L. P. Martins; L. V. Costa-Lotufo; M. O. Moraes; E. F. Guimarães; M. J. Kato; M. Furlan; C. Pessoa, *Pharm. Biol.* **2007**, *45*, 1–6.
104. C. Viegas; V. S. Bolzani; M. Furlan; E. J. Barreiro; M. C. M. Young; D. Tomazela; M. N. Eberlin, *J. Nat. Prod.* **2004**, *67*, 908–910.
105. N. P. Lopes; D. H. S. Silva; M. J. Kato; M. Yoshida, *Phytochemistry* **1998**, *49*, 1405–1410.
106. D. H. S. Silva; S. C. Davino; S. B. M. Barros; M. Yoshida, *J. Nat. Prod.* **1999**, *62*, 1475–1478.

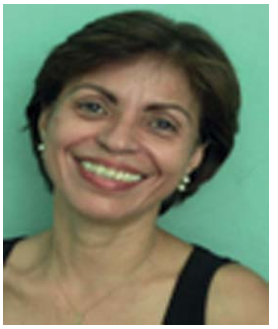
107. D. H. S. Silva; F. C. Pereira; M. V. B. Zanoni; M. Yoshida, *Phytochemistry* **2001**, *57*, 437–442.
108. D. H. S. Silva; F. C. Pereira; M. Yoshida; M. V. B. Zanoni, *Eclét. Quim.* **2005**, *30*, 15–21.
109. D. H. S. Silva; Y. Zhang; L. A. Santos; V. S. Bolzani; M. G. Nair, *J. Agric. Food Chem.* **2007**, *55*, 2569–2574.
110. C. Viegas; D. H. S. Silva; M. Pivatto; A. Rezende; I. Castro-Gamboa; V. S. Bolzani; M. G. Nair, *J. Nat. Prod.* **2007**, *70*, 2026–2028.
111. M. A. G. Ricardo; M. A. Andreo; A. J. Cavalheiro; I. Castro-Gamboa; V. S. Bolzani; D. H. S. Silva, *ARKIVOC* **2004**, *6*, 127–136.
112. A. B. Ribeiro; D. H. S. Silva; V. S. Bolzani, *Eclét. Quim.* **2002**, *27* (sp. iss, S1), 35–44.
113. A. B. Ribeiro; M. Yoshida; V. S. Bolzani; L. S. Santos; M. N. Eberlin; D. H. S. Silva, *J. Braz. Chem. Soc.* **2005**, *16*, 526–530.
114. J. Corsino; D. H. S. Silva; M. V. B. Zanoni; V. S. Bolzani; S. C. França; A. M. Pereira; M. Furlan, *Phytother. Res.* **2003**, *17*, 913–916.
115. A. H. Jeller; D. H. S. Silva; L. M. Lião; V. S. Bolzani; M. Furlan, *Phytochemistry* **2004**, *65*, 1977–1982.
116. P. R. F. Carvalho; D. H. S. Silva; V. S. Bolzani; M. Furlan, *Chem. Biodivers.* **2005**, *2*, 367–372.
117. I. Castro-Gamboa; C. L. Cardoso; D. H. S. Silva; A. J. Cavalheiro; M. Furlan; V. S. Bolzani, *J. Braz. Chem. Soc.* **2003**, *14*, 771–776.
118. P. M. Pauletti; I. Castro-Gamboa; D. H. S. Silva; M. C. M. Young; D. M. Tomazela; M. N. Eberlin; V. S. Bolzani, *J. Nat. Prod.* **2003**, *66*, 1384–1387.
119. P. R. F. Carvalho; D. H. S. Silva; V. S. Bolzani; M. Furlan, *J. Braz. Chem. Soc.* **2003**, *11*, 122–130.
120. L. Hamerski; M. D. Bomm; D. H. S. Silva; M. C. M. Young; M. Furlan; M. N. Eberlin; I. Castro-Gamboa; A. J. Cavalheiro; V. S. Bolzani, *Phytochemistry* **2005**, *66*, 1927–1932.
121. C. L. Cardoso; I. Castro-Gamboa; D. H. S. Silva; V. S. Bolzani, *J. Braz. Chem. Soc.* **2005**, *16*, 1353–1359.
122. C. L. Cardoso; I. Castro-Gamboa; D. H. S. Silva; M. Furlan; R. D. Epifanio; A. C. Pinto; C. M. Rezende; J. A. Lima; V. S. Bolzani, *J. Nat. Prod.* **2004**, *67*, 1882–1885.
123. V. C. Silva; M. N. Lopes; V. S. Bolzani, *Quim. Nova* **2006**, *29*, 493–495.
124. L. O. Regasini; J. C. R. Vellosa; D. H. S. Silva; M. Furlan; O. M. M. F. Oliveira; N. M. Khalil; I. L. Brunetti; M. C. M. Young; E. J. Barreiro; V. S. Bolzani, Phytochemistry **2008**, 1739–1744.
125. D. C. Fernandes; L. O. Regasini; J. C. R. Vellosa; V. S. Bolzani; O. M. M. F. Oliveira; D. H. S. Silva, *Chem. Pharm. Bull.* **2008**, *56*, 723–726.
126. M. S. Alexandre-Moreira; C. Viegas; A. L. P. Miranda; V. S. Bolzani; E. J. Barreiro, *Planta Med.* **2003**, *69*, 795–799.
127. C. Viegas, Jr.; M. S. Alexandre-Moreira; C. A. Fraga; E. J. Barreiro; V. S. Bolzani; A. L. P. Miranda, *Chem. Pharm. Bull.* **2008**, *56*, 407–412.
128. C. Viegas; V. S. Bolzani; E. J. Barreiro; C. A. M. Fraga, *Mini Rev. Med. Chem.* **2005**, *5*, 915–926.
129. C. Viegas; V. S. Bolzani; L. S. B. Pimentel; N. G. Castro; R. S. Costa; C. Floyd; M. S. Rocha; M. C. M. Young; E. J. Barreiro; C. A. M. Fraga, *Bioorg. Med. Chem.* **2005**, *13*, 4184–4190.
130. C. Viegas, Jr.; A. Rezende; D. H. S. Silva; I. Castro-Gamboa; V. S. Bolzani; E. J. Barreiro; A. L. P. Miranda; M. C. M. Young, *Quim. Nova* **2006**, *29*, 1279–1286.
131. N. G. Castro; R. S. Costa; L. S. B. Pimentel; A. Danuello; N. C. Romeiro; C. Viegas; E. J. Barreiro; C. A. M. Fraga; V. S. Bolzani; M. S. Rocha, *Eur. J. Pharm.* **2008**, *580*, 339–349.
132. V. da S. Bolzani; C. Viegas, Jr.; E. J. Barreiro; C. A. M. Fraga; M. C. M Young; N. G. Castro, Piperidine Derivatives, and Pharmaceutical Compositions Containing the Same Preparation Process to be Used to Treat Alzheimers Disease. World Patent Appl. BR000202, 2004; U.S. Patent Appl. 0076799, 2008.
133. O. A. Flausino; L. D. Santos; H. Verli; A. M. Pereira; V. D. Bolzani; R. L. Nunes-De-Souza, *J. Nat. Prod.* **2007**, *7*, 48–53.
134. O. A. Flausino; A. M. Pereira; V. S. Bolzani; R. L. Nunes-De-Souza, *Biol. Pharm. Bull.* **2007**, *30*, 375–378.
135. V. da S. Bolzani; R. L. N. de Souza; O. A. Flausino. Jr., Use of 11-OH-Erythravine, Erythravine, Erytratine, Pharmaceutical Compositions and Processes for Producing These Substances. World Patent Appl. 000217, 2005.

Biographical Sketches

Dulce Helena Siqueira Silva graduated from the University of Sao Paulo as a chemical engineer in 1987 and completed her Ph.D. studies with Professor Massayoshi Yoshida at the Chemistry Institute in the same university. She worked at the postdoctoral level in the Bioactive Natural Products and Phytoceuticals Laboratory, Michigan State University

(BNPP-MSU) under the supervision of Professor Muralee Nair during 2002–2003 and is Associate Professor at São Paulo State University, where she has been working since 1998. Her main interests include bioprospection of bioactive compounds in plant species, focusing on antioxidants, oxidative stress-related diseases, and chemopreventive agents from Brazilian biodiversity.

Ian Castro-Gamboa obtained his Ph.D. in 2000 from the Universidade Federal de São Carlos (UFSCar) working with chemosystematics and chemical ecology of Rutaceae species. At present, he is an Assistant Professor at the Chemistry Institute of State University – UNESP in Araraquara, São Paulo. His research interests include development of dereplication methodologies and hyphenated spectroscopic techniques as well as principal component analysis of complex natural matrixes.



Vanderlan da Silva Bolzani graduated from the Federal University of Paraíba and initiated her Ph.D. studies under the guidance of Professor Otto Richard Gottlieb at São Paulo State University. She pursued her postdoctoral studies at Virginia Polytechnic Institute (VPISU) joining the group of Professor David Kingston. Currently, she is full professor at the Chemistry Institute of State University – UNESP in Araraquara, São Paulo. Her main interests include the bioprospection of biologically active species from the Cerrado and Atlantic Rainforest especially in Rubiacae, Fabaceae, Apocynaceae, Flacourtiaceae, Fabaceae, Scrophulariaceae, Phytolaccaceae, and Styracaceae. Her other interests include the isolation and bioactivity of plant peptides and the biosynthesis of piperidine alkaloids, and looking for natural candidates as drugs and cosmetics.

3.07 The Identification of Bioactive Natural Products by High Throughput Screening (HTS)

Vicky M. Avery, David Camp, Anthony R. Carroll, Ian D. Jenkins, and Ronald J. Quinn, Griffith University, Brisbane, QLD, Australia

3.07.1 Introduction

Plants and plant extracts have been used for thousands of years to treat human disease and suffering. However, the serendipitous discovery of the antibiotic activity of an extract of the fungus *Penicillium notatus* by Fleming in 1929, and the isolation of penicillin and its development as a lifesaving antibiotic by Chain, Florey, and others during the late 1930s and early 1940s,[1] was the beginning of the 'golden age' of natural product (NP) drug discovery. The investigation of NPs as a source of therapeutic drugs reached its peak around 1970–1980.[2] In the 25 years since that time (1981–2006), approximately half of all approved small-molecule new chemical entities (NCEs) were NPs, derivatives of NPs, or synthetic compounds inspired by NPs.[3] In 2002, drugs derived in whole or in part from NPs, or based on NP scaffolds, attracted US$20.5 billion in revenue for their respective manufacturers, an increase by 8.2% since 2000.[4–6]

Up until the early 1980s, screening of NPs and NP extracts for biological activity was carried out on relatively small numbers of compounds, generally only a few at a time using *in vivo* screens. These programs required a relatively large amount of biota. For example, the Roche Research Institute of Marine Pharmacology, which operated in Sydney between 1974 and 1981, collected approximately 10 kg of wet sponge. This amount was required for screening, isolation, and structure elucidation. Six extracts from two species were screened each week against a panel of screens based on animal models and isolated tissue preparations.[7]

Over the past 20 years, the efficiency of NP-based drug discovery has increased enormously. For example, Natural Product Discovery in Brisbane (now the Eskitis Institute) began high-throughput screening (HTS) of NP extracts in 1994. By 2000, this group was screening over 100 000 extracts against a range of targets (receptors, enzymes, and mechanism-based cellular assays). Robotic liquid handling and assay-building workstations allowed 100 × 384-well plates to be screened per day. Importantly, the amount of biota collected has been reduced to 100 g. Extraction of 200 mg of dry, ground biota provides sufficient extract to undertake in excess of 150 HTS campaigns, while 10 g of biota is sufficient for the isolation and structure determination of quite minor components (0.01–0.001%) using modern (2007) NMR instrumentation and pulse sequences.[8]

The development of HTS in the late 1980s was followed by the introduction of combinatorial chemistry in the 1990s. These two new technologies revolutionized the way drug discovery was carried out and resulted in a decline in NP research by the pharmaceutical industry.

Combinatorial chemistry was seductive with its perceived promise to deliver large numbers of novel compounds. However, after 10–15 years, there is increasing concern throughout the pharmaceutical industry at the lack of druggable leads in company pipelines. It seems likely that the decreased emphasis on NPs in drug discovery has contributed to the decline in the number of new chemical entities (drugs) seen in recent years.[9,10]

Hit rates in HTS are determined by the quality, not the quantity of the library members, and the quality is determined by three factors: chemical diversity, lead-likeness, and biological relevance.[11,12] An NP program offers a source of chemical diversity unmatched by any synthetic chemical collection or combinatorial chemistry approach.[13] The chemical diversity of (synthetic) combinatorial libraries, containing maybe 10^6 compounds, pales into insignificance against the estimated 10^{63} potential drug-like compounds in the small-molecule universe.[14] Combinatorial compounds are substantially less diverse than either drugs or NPs and, interestingly, existing drugs seem to cover the joint volume in diversity space of combinatorial compounds and NPs.[15] Lead-like properties[16,17] are based on drug-like filters such as the Lipinski rules[18] and have been used to improve the quality of combinatorial libraries.[19] According to these rules, for a drug to be orally active (i.e., to be absorbed or cell permeable) it should have

1. MW ≤ 500
2. log $P \leq 5$
3. H-bond donors ≤ 5
4. H-bond acceptors (the sum of N's and O's) ≤ 10

An important caveat is that NPs and substrates for transporters are exceptions.

Analysis of the 126 000 unique entries in the *Dictionary of Natural Products*[20] against Lipinski's 'Rule-of-5' (so named because the four rules above are multiples of five) indicates that 60% are compliant.[21] Importantly, only about 10% of NPs exhibit two or more violations, the same as for trade drugs.[22] The third factor, biological relevance, is more difficult to predict with synthetic combinatorial libraries. In contrast, NPs interact with a variety of proteins during their biosynthesis, and go on to target specific proteins in order to fulfill their function. They are biologically validated compounds.[23] There is evidence that hit rates in HTS from isolated NPs are several orders of magnitude higher than from random combinatorial libraries.[24]

3.07.2 Biota Collection

Since all NPs are derived from the biosphere, any screening program must have its genesis with a biota (specimen) collection. A biota library may consist of microbial cultures (live or dried) or dried, ground macrobiota such as plants, marine invertebrates, and terrestrial invertebrates. Whatever the composition of the biota library, it is noteworthy that the UN Convention on Biological Diversity in 1992 recognized the sovereign rights of States over their own genetic resources.[25] Article 15 in particular affirms a State's authority to decide upon appropriate access to and benefit-sharing arising from the use of its genetic resources. Therefore, any organization wishing to access biota must first enter into a benefit-sharing agreement with the host nation before collection can proceed. This is of paramount importance as parties in breach of Article 15 may be in danger of being prosecuted for biopiracy.

It is also worth bearing in mind before embarking on a collection program that a mere 17 countries (Australia, Brazil, China, Colombia, Democratic Republic of Congo, Ecuador, India, Indonesia, Madagascar, Malaysia, Mexico, PNG, Peru, Philippines, South Africa, USA, Venezuela) account for more than 80% of all biodiversity (life forms).[26] These countries are termed megadiverse. In addition to extraordinary biodiversity, some megadiverse countries like Australia also have many endemic species (native species found nowhere else in the world). A greater coverage of species diversity is therefore possible only if access and benefit-sharing agreements are entered into with these countries.

3.07.3 Preparation of Natural Product Extracts for High-Throughput Screening

There are a number of ways in which biota samples can be processed into a form suitable for screening (**Figure 1**):

- crude extracts – an extract using organic or organic/aqueous solvent mixtures (many components);
- prefractionated libraries of crude extracts – crude extracts that are fractionated using conventional liquid chromatography techniques (generally containing <10 compounds);
- semipurified extracts – crude extracts that have typically undergone further generic processing using solid-phase extraction (SPE) as a chemical filter to bias the screening profile, for example, capture of basic nitrogen-containing compounds (alkaloids) using strong cation exchange (SCX) resins;
- prefractionated libraries of semipurified extracts – semipurified extracts that undergo additional fractionation via conventional liquid chromatography techniques (generally containing 1–5 compounds);
- pure NPs – comparable to compound HTS.

Generally speaking, the closer an extract approximates a pure compound, the less downstream effort is required for screening and isolating/identifying the active component. However, any approach that biases the screening set in the direction of pure compounds comes at a price. If the strategy is to screen pure compounds, then a significant effort will be required to isolate the secondary metabolites from microbial cultures or sizeable quantities (10–50 g) of macrobiota. In the latter case, a valuable resource may be consumed in an effort that succeeds only in isolating the major components. Any minor components will be lost, and these could be the most biologically active.

Screening whole extracts avoids the loss of minor components. However, crude extracts contain nuisance compounds that can interfere with various screening technologies and nonselective, ubiquitous compounds (frequent hitters) that mask the interaction of potentially novel modulators. Crude extracts may be prefractionated or refined in some manner prior to screening. Prefractionated libraries are typically obtained by eluting crude extract through a reverse-phase HPLC column and collecting between 5 and 15 fractions per extract.

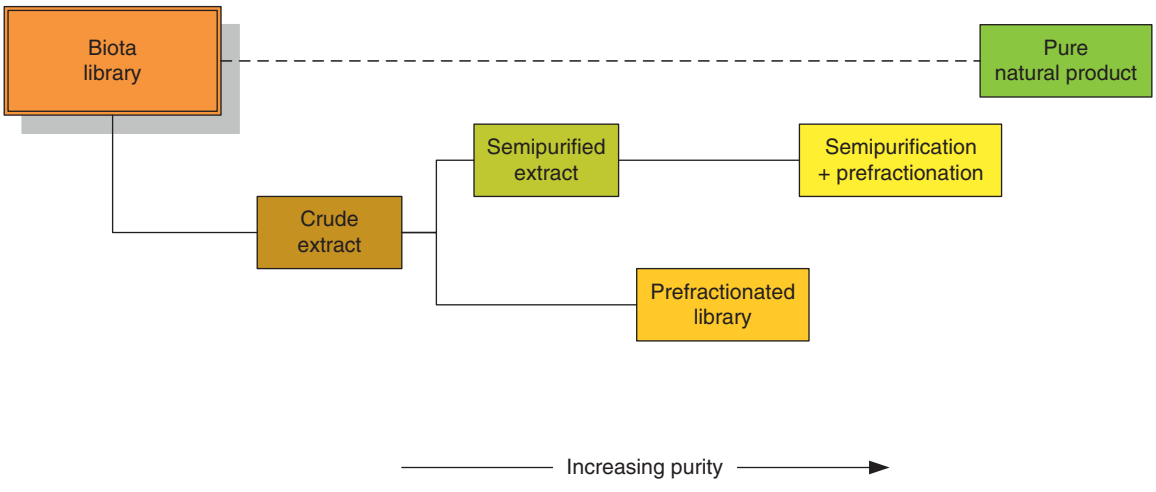


Figure 1 Possibilities for a biota screening library.

Thirty thousand extracts equates to a primary screening campaign of between 150 000 and 450 000 data points. Such large screening sets can stretch budgets. Refining crude extracts using SPE cartridges as a chemical filter into 2–3 fractions affords a viable alternative.

Using a combination of the above techniques, that is, SPE followed by prefractionation on HPLC, affords a screening set with a desired chemical profile and one that best approximates a pure compound library. For example, SCX resins can be used to capture basic nitrogen-containing compounds from a crude methanolic extract. Subsequent HPLC prefractionation can then be used to remove very polar or very lipophilic compounds if these are deemed undesirable or nondrug-like.

3.07.4 Logistics

In order to avoid continual extraction of the entire biota library for every HTS campaign, it is much more efficient to store sufficient amount of the biota extract to last 5 years (>100 screens). The two most popular ways of achieving long-term storage that maintains the integrity of the samples in DMSO are microtubes and minitubes held anywhere between −20 and 20 °C under an atmosphere of nitrogen or low relative humidity.

Microtubes are available in a range of volumes from 500 to 1000 μl and can be stored in a format-free manner within modular or purpose-built stores. All tracking is done through onboard software by dead reckoning or with the aid of 2D barcodes to provide additional assurance. The most popular 2D symbology is DataMatrix, an open source code that can generate 10 000 000 000 (10 billion) unique descriptors via a 12 × 12 numeric matrix. A slightly more condensed 14 × 14 alphanumeric matrix can afford 3 600 000 000 000 000 (3.6 quadrillion) distinctive permutations before redundancy. Manufacturers of 2D barcode microtubes guarantee that no two will ever have the same code. Screening solutions are prepared once and accessed multiple times to prepare source plates for primary screening, retest, dose response, etc.

An alternative storage mechanism is 'one-shot' 384 minitubes. The essential difference when compared to microtubes is that individual tubes, typically with a capacity of <50 μl, are arrayed into 384-well plates. Solubilized sample is introduced from freshly prepared bulk stock into a number of 384 minitube plates, which are subsequently sealed and subjected to long-term storage. Specific tubes can be punched or picked into a destination plate for retest, etc.

As alluded to earlier, absorption of water by the DMSO solution can cause precipitation and/or degradation. Precipitated components can be forced back into solution by the use of ultrasonic devices such as those manufactured by Covaris or Matrical. Degraded samples, on the contrary, cannot be rescued and are discarded. It is important to note that strong acids, such as trifluoroacetic acid, which is often used to sharpen peaks during isolation of active principles, can be a causative agent of degradation if the DMSO solution becomes appreciably aqueous in character. Acoustic auditing devices such as the Labcyte Echo are able to concomitantly measure the concentration of water in DMSO stocks and the volume of liquid. Early detection of aqueous contamination can flag a sample for potential errors in HTS and replenishment.

3.07.5 Preparation of Plates for High-Throughput Screening

HTS is most commonly performed using 384- and 1536-well microtiter plates. Extracts/pure NPs held in bulk storage must first be reformatted into HTS-friendly format plates before any biological evaluation can occur. Reformatting is where an aliquot is taken from a particular storage format, such as microtubes, and transferred to another format, such as a 384-well microtiter plate. Microtiter plates can also be reformatted in a process known as quadranting. Here, 96- or 384-well plates may be reformatted into higher density 384 (4 × 96) or 1536 (4 × 384) well plates respectively. Reformatting may be done 'just-in-time' prior to screening from the bulk sample or much earlier via the preparation of 'mother' plates. Mother plates are essentially source plates for all plate-based operations. They may be of the same concentration as the stock or be diluted depending on the screen's requirements and robotic limitations. Mother plates (or a prediluted copy known as a 'working plate') allow the extracts/pure NPs to be accessed more rapidly for just-in-time plate preparation of 'daughter' plates that are ultimately converted into assay plates.

3.07.6 High-Throughput Screening

HTS, as the name indicates, is a drug discovery process that enables a biochemical or cellular event to be reproducibly and rapidly tested against chemical entities many hundreds of thousands of times. HTS utilizes robotics (**Figure 2**), liquid handlers, data processing, considerable software, and sensitive detection systems. The objective of HTS is to rapidly identify active compounds that modulate a particular target, pathway, or biochemical/cellular event. The output from a HTS campaign provides the basis upon which drug design and elaboration are used to generate lead compounds with appropriate physicochemical properties for therapeutic indications.

HTS approaches are now being utilized more and more to facilitate ADMET/DMPK (absorption, distribution, metabolism, excretion, toxicity/drug metabolism and pharmacokinetics) activities, as the major pharmaceutical companies have adopted frontloading of these stages of the drug discovery process. Academic researchers are also increasingly making use of HTS facilities to identify probes used in chemical biology, helping to facilitate identification of new potential drug targets, and also to increase their knowledge about known targets.[27–29]

3.07.7 The Target

The target could be an enzyme, intra- or extracellular receptor, structural protein, or transcription factor. First, the target must be clearly established as playing a critical role in the molecular interactions or regulation of a pathological condition. Validation of the target may be determined by knockout experiments, or based on clinical data from individuals with gene mutations, which inactivate or block the synthesis or functionality of the target (in a knockout experiment, an organism is engineered to lack the expression and activity of one or more genes). Second, the target should preferably be expressed only in the particular cell or tissue type involved in the disease process and, finally, a human form of the target should be available. Alternatively, if the target is for a particular organism, such as a parasite or microorganism, there should preferably not be a human homologue.

3.07.8 The Assay

Ideally, an HTS assay should be relatively simple, straightforward, cost effective, comprise a minimum number of steps, capable of automation, robust, and highly reproducible with a simple readout. The assay should be able to be performed in a low volume, thus requiring small amounts of material and reagents. Importantly, the assay should reflect the biological situation to be assessed.

Assays can be generally categorized into two types: biochemical assays, which incorporate both molecular target assays and cell-free multicomponent assays, and cellular assays, which are whole-cell- or organism-based assays.

Figure 2 Examples of robotics used in high-throughput screening laboratory.

3.07.8.1 Biochemical Assays

Assays using purified enzymes, such as proteases or kinases, and assays measuring activities associated with cell extracts, membranes, or reconstituted signaling cascades are representative examples of a biochemical assay. Biochemical assays are based upon defined molecular entities compared with whole-cell assays. These may involve binding of ligand to receptor, inhibition of enzymatic pathways, interaction with structural proteins, protein–protein interactions, or protein–DNA interactions. These are well-defined interactions of well-characterized therapeutically relevant molecular targets. Generally speaking, biochemical assays are very cost effective. However, these assays provide no reflection of the potential issues the compounds being screened may face in a whole-cell/organism environment, issues such as permeability, metabolism, or competition with other cellular molecules for the target.

3.07.8.2 Cellular Assays

Cellular assays can be subdivided into 'reporter gene' and phenotypic assays that measure outputs resulting from intact cellular processes. In addition to having the potential to identify the same compounds as the biochemical assays, cellular assays identify compounds acting at additional molecular sites. Cellular systems provide a more physiologically relevant environment and expose compounds to other cellular constituents and media. These assays rapidly identify compounds that are able to cross the cell membrane, toxic compounds, and the action of metabolites.

A disadvantage of cell-based systems is that often the molecular target or 'mode of action' (MOA) needs to be determined requiring additional research. In addition, the degree of difficulty and complexity of the assay varies and may become less HTS friendly.

Cell-based assays also require an immense attention to detail not necessarily needed for biochemical assays. For example, consistency with the culture conditions may require that specific batches of the media components are purchased for the entire HTS campaign, and optimal conditions for cell growth may interfere with the detection system. In addition, costs associated with cell-based assays are usually much higher than for biochemical assays.

It is anticipated that compounds identified from cell-based HTS are likely to possess low protein binding properties, be cell permeable and nontoxic at the cellular level, therefore providing a distinct advantage over the biochemical assays. Cytotoxicity determination is essential to evaluate the relevance of a compound, whether identified from a biochemical assay or cell-based screening.

3.07.9 Essential Components for a Good HTS Assay

HTS assays, like any assay, have components that are essential for a successful outcome. These are in addition to the overall assay format being simple, robust, cost effective, and amenable to automation. Positive and negative controls are crucial. Positive controls are conditions (e.g., reference compound) that produce the same result in the assay as a desired active compound. Negative controls are usually 'vehicle'-only conditions (e.g., DMSO) or, where appropriate, small molecules demonstrated to have no activity in the assay. Controls are used to determine an assay 'window' and validate the biological response. Assay controls are critical to evaluation of assay response and the performance of any assay, and for HTS often include interplate controls to assess and correct, when possible, systematic variations in the biological response over time. Intraplate controls are essential to determine the assay window, and over the course of a screen permit the analysis of uniformity of the biological response.[30]

Performance of the assay during the screen is commonly measured using Z factor, a statistical parameter that takes into account the signal-to-background and assay signal variation.[31] Plate-based Z factor determinations provide a measure of the screen performance. Both control (Z') and sample (Z) factors are usually reported.

3.07.10 Technology

The choice of assay technology and design of the assay used for HTS are critical for a successful outcome. There is a vast array of HTS technologies that can be categorized based on their detection systems. Detection systems include absorbance, fluorescence, luminescence, and radiometric, while more recently, label-free approaches are also being utilized. A selection of the more commonly used technologies is described; however, this is by no means a comprehensive overview but rather a snapshot to illustrate the available options.

Homogeneous or 'mix and read' assay formats are generally preferred for HTS; however, there are a number of technologies based on heterogeneous assay formats, which are still widely used. ELISA (enzyme-linked immunosorbent assay) and DELFIA (dissociation enhanced lanthanide fluorescent immunoassay) are examples of heterogeneous assays. Both these technologies are based on the various assay components being sequentially added, permitted to bind, and the unbound component washed off. Usually, the detection system for an ELISA is absorbance, whereas the DELFIA relies on time-resolved fluorescence.[32]

AlphaScreen (amplified luminescent proximity-based homogeneous assay) is a homogeneous bead-based proximity technology and, as the name infers, is based upon luminescence. A donor and acceptor bead pair, both 250 nm in diameter, are coated with a layer of hydrogel providing functional groups for conjugation with the relevant biological components (**Figures 3** and **4**). Upon interaction of these two components, the beads are brought into close proximity and upon irradiation at 680 nm a photosensitizer in the donor bead converts ambient oxygen to an excited singlet state. Singlet oxygen reacts with a thioxene derivative in the acceptor bead, which activates fluorophores in the acceptor resulting in light emission (520–620 nm). Binding interactions that position the donor and acceptor beads a distance of up to 200nm apart can be detected using AlphaScreen technology, thus making this technology applicable to a diverse range of biological interactions, including RNA–protein interaction,[33] kinase-mediated phosphorylation,[34] partial agonists,[35] nuclear receptors,[36] tumor necrosis factor (TNF) receptor,[37] and protein kinase B.[38]

TR-FRET (time-resolved fluorescent resonance energy transfer) is another homogeneous proximity assay. TR-FRET relies on the transfer of fluorescence energy between the donor and acceptor beads, which are bound to labeled reaction components. For example, upon excitation at 340 nm, the donor label, europium (Eu), transfers energy (615 nm) to the acceptor label, allophycocyanin (APC), resulting in emission at 665 nm. However, the distance required for energy transfer is only 7 nm, which is significantly less than that required for AlphaScreen technology (200 nm) (**Figure 4**). This limitation restricts the use of TR-FRET to small peptide interactions, thus reducing its versatility as an HTS format.[39]

Fluorescence polarization (FP) is a homogeneous assay and is based on the difference in the mobility of fluorophores depending on whether the two components of the assay have interacted or not. FP measures the polarization of light emitted from a fluorescent probe upon excitation with plane-polarized light. The extent of polarization is dependent upon the rotation rate of the probe, which is relative to its molecular size. Free probes, which are of lower molecular weight than the bound complex, will rotate and spin more rapidly than the bound molecules. Polarized light emitted from the free probe will be minimal. Conversely, when a probe interacts with a larger target molecule, its rate of rotation will be reduced so that the resulting fluorescence will be more polarized (**Figure 5**). No separation of the bound versus unbound probe is required as these two entities can be

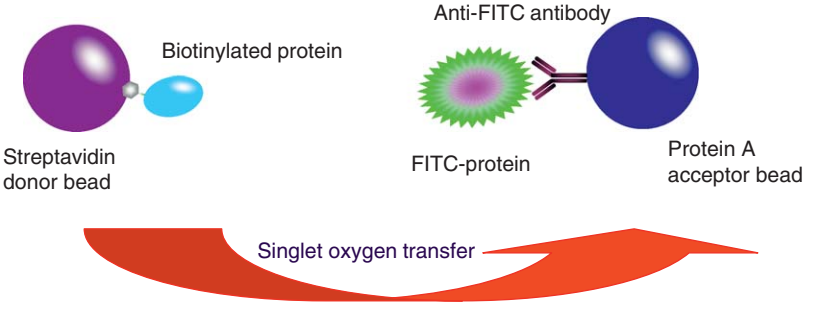


Figure 3 Assay principle of AlphaScreen technology. Interaction between the two proteins causes the donor and acceptor beads to be in close proximity (<200 nm). Excitation of donor bead (680 nm) generates singlet oxygen, which reacts with acceptor bead when in close enough proximity to produce light (520–620 nm). If the proteins do not interact, singlet oxygen is not generated and light is not produced.

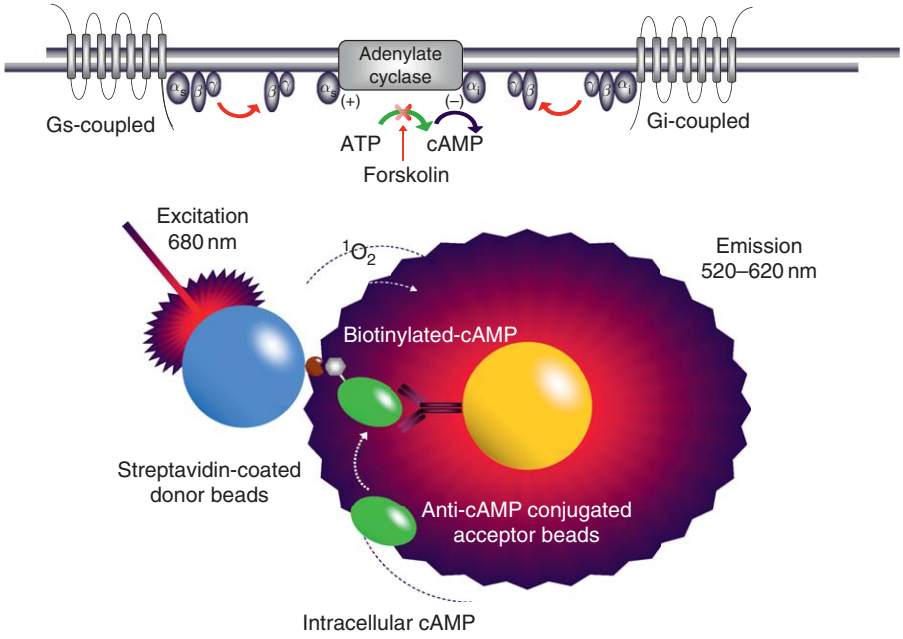


Figure 4 AlphaScreen: cAMP detection. cAMP produced intracellularly competes with the biotinylated cAMP, breaking the interaction and thus the emission of light.

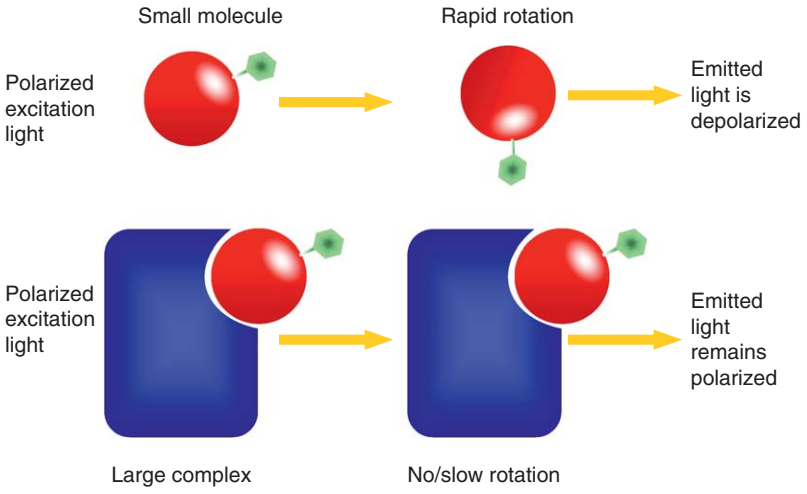


Figure 5 Fluorescence Depolarization. Small molecules have the ability to rotate rapidly in 3D space and thus result in depolarization of the emitted light. Large complexes rotate slowly or not at all and hence do not affect the polarization state of the emitted light.

distinguished based on their polarization properties. FP has been widely used for HTS and for a broad range of different targets, including nuclear hormone receptors, such as peroxisome proliferator-activated receptors (PPARα and PPARγ)[40] and glucocorticoid receptors,[41] bacterial RecA protein,[42] acetyl coenzyme A carboxylase enzymes,[43] melanoma inhibitor of apoptosis protein,[32] G protein-coupled receptors (GPCRs), such as vasopressin,[44] and the checkpoint kinase, Chk2.[45]

Scintillation proximity assay (SPA) is another homogeneous proximity assay that utilizes radiolabeled ligands. This is a simple high-throughput approach, which has broad application in screening. The scintillant-impregnated beads are coated with a variety of different surfaces allowing coupling of target molecules or membranes. Only when the radiolabeled ligand interacts with the target molecule is it close enough in proximity to elicit a light signal, therefore eliminating the need for separation of the various assay components. SPA has been used for a considerable amount of time by the Pharma industry, and although there is a clear

trend to use more nonradioactive technologies for HTS, there are still times when SPA is the best available option. SPA is frequently used as a secondary assay, thus utilizing less radiolabeled ligand. Examples of the variety of different targets for which SPA assays have been designed include insulin-like growth factor binding protein 4 (IGFBP4),[46] isoprenylcysteine carboxyl methyltransferase (ICMT),[47] nonpeptide ligand binding to gonadotropin-releasing hormone (GnRH),[48] and RNA–protein interactions.[33]

FLIPR (fluorescence imaging plate reader) technology is commonly used to measure calcium mobilization associated with activation of GPCR targets. GPCR targets which couple via Gq produce an increase in intracellular calcium, which can be measured using calcium-sensitive dyes. The FLIPR has a CCD camera imaging system, which collects the signals generated in subsecond intervals, allowing for kinetics to be determined. Traditionally, FLIPR assays were multistep with several wash steps; however, the newer 'no wash' dyes permitting 'mix and read' have increased the efficiency and quality of the data. In addition to GPCRs, ion channels that significantly increase either membrane potential or calcium permeability can also be evaluated using this technology. Examples where FLIPR has been used for HTS include platelet calcium flux,[49] low-voltage-activated T-type calcium channels (Ca_v3 T-type channels),[50] nicotinic receptors,[51] human sensory neuron-specific GPCR agonist screening,[52] and the identification of inhibitors of the norepinephrine transporter.[53]

3.07.11 High-Content Screening

HTS based on phenotypes of individual living cells, using automated imaging and analysis, can now be undertaken due to the significant technological advances in the field. Automated cellular imaging screens maximize the information obtained, thus allowing rapid parallel biological analysis of multiple factors and improving the ability to select high-quality leads.

Microscopic imaging of fluorescently labeled cells allows acquisition of a significantly greater amount and quality of information. Whereas biochemical assays simply test for binding to a particular isolated protein, observing the effect of compounds on a cellular phenotype provides more physiologically relevant information. Image-based assays also provide information regarding the cell's status and health, both for general phenotypes for overall cell health and very specific phenotypes, such as translocation of receptors to specific intracellular compartments (**Figures 6** and **7**).

Multiple features of the cell can be assessed from simple staining, such as for DNA, which allows the number of cells to be counted and measurement of cellular DNA content, providing insights into stage- and/or time-specific events. Multiplexing using multiple dyes/tags is an attribute of image-based assays, which provides a means to report on several components of the cell simultaneously and consequently their effects on one another.[54] The ability to collect information from individual cells and subpopulations, rather than the mean of

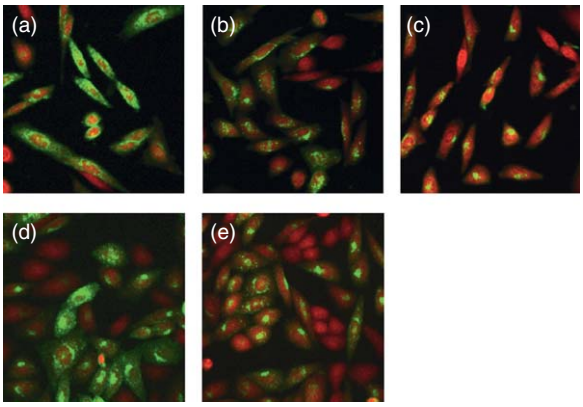


Figure 6 Internalization of the CCR5 receptor by the agonist RANTES and the antagonist met-RANTES. Cells are shown prior to treatment with agonist (a), 30 min (b) and 60 min (c) after treatment with 100 nmol l^{-1} RANTES, and 30 min (d) and 60 min (e) after treatment with 100 nmol l^{-1} met-RANTES. Cells were imaged on an Evotec Opera using a ×20 objective. Reproduced from James Longden, Greg Fechner, and Vicky Avery (2007) *Internalisation of the CCR5 Receptor by the Antagonist Met-RANTES – A Role for Endogenous Aminopeptidases*, High Content Analysis Meeting, San Francisco.

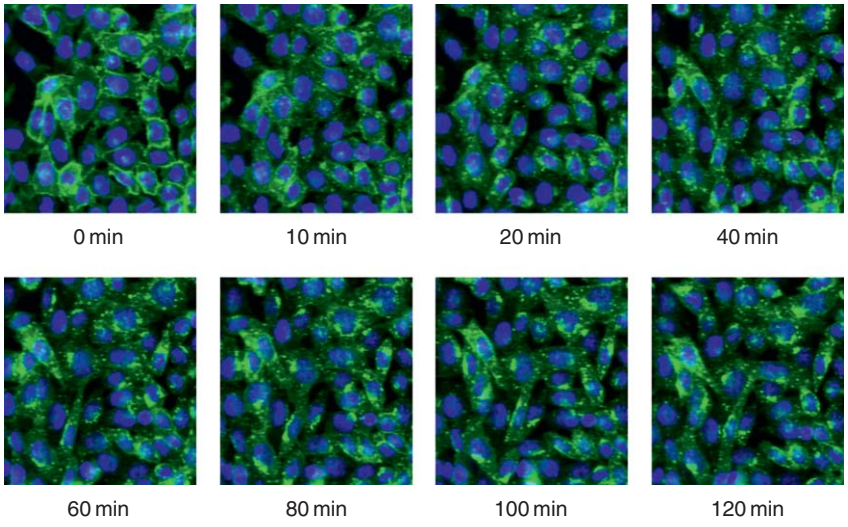


Figure 7 Internalization of CCR5-GFP induced by met-RANTES in live cells. Representative images are shown from one well of cells stimulated with 600 nmol l^{-1} met-RANTES and imaged live on the IN Cell Analyzer 3000. Cells were stained with Hoechst 33342 and treated with 100 nmol l^{-1} met-RANTES before being immediately transferred to the IN Cell Analyzer 3000 and imaged every 10 min for 120 min at 37 °C, 5% CO_2, and 75% humidity. Reprinted by permission from Macmillan Publishers Ltd: *Br. J. Pharmacol.* J. Longden; E. L. Cooke; S. J. Hill, **2008**, *153*, 1513–1527, copyright 2008.

the total population, is what sets high-content screening (HCS) apart from many other cellular assay formats. Accurate analysis of a biological process in specific subpopulations of cells can be made whether or not in mixed cell populations. These effects, if observed only in a minor population of cells, may be hidden in whole population detection analysis systems.[55,56] The extensive information that can be collected from individual cells has resulted in image-based assays being referred to as 'high-content' assays. Until quite recently, image-based assays were very low-throughput, requiring laborious manual collection of images followed by visual inspection. Significant technological advances in both automated microscopes and cell image analysis software have changed the interpretation of image-based assays, making them suitable for HTS. Such HCS[57] may also be known as chemical biology, cell-based screening, chemical genetics, or phenotypic screening. All major pharmaceutical companies have established HCS groups. The major use of this technology, within the pharmaceutical industry, is at the secondary-screen lower throughput stage. Primary screening of thousands of compounds using HCS is becoming more common, and the ability to maximize the information obtained and improve the ability to select high-quality leads suggests that it will eventually become a primary screening tool.

After acquisition, analysis of the images is necessary to obtain meaningful, quantitative information. There is the promise of even higher content information, with continual cutting edge developments in all aspects of the HCS field. The ability to undertake time-lapse studies with live cells for determining kinetics, or automated three-dimensional image acquisition and analysis, for better structural resolution or drug penetration studies is not that far off in the future.[58] Since the emergence of HCS, new biological techniques and tools, advanced instrumentation (**Figure 8**), and increasingly sophisticated and easy-to-use software tools continue to push the boundaries of image-based chemical screens. Automated image-based screening permits identification of small compounds altering cellular phenotypes and is of interest for the discovery of both new drugs and tools. However, target identification is the rate-limiting step for HCS,[59] requiring more effort to improve this part of the drug discovery process.[60,61]

3.07.12 Natural Product High-Throughput Screening

Perhaps the singular most obvious difference when screening NPs compared to small molecules is that as extracts or fractions, one is dealing with multiple components per well. There is always the potential of low-affinity compounds combining in activity to generate a 'hit' extract/fraction. It is also possible that an active

Figure 8 Evotec Opera high-throughput confocal imager.

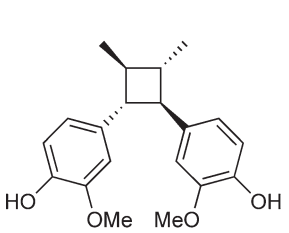
compound may be masked by interference from another compound or that the extract environment may interfere with detection, so that the active constituent may not be identified.

The design of assays for NP HTS is, in principle, the same as for conventional small-molecule screening. An additional consideration is the increased possibility of factors highly likely to cause interference, or generating false positives and/or negatives. High hit rates of nonspecific hits from NP extracts in cell-based assays offer a great challenge to investigators conducting cell-based HTS. Since extracts may contain many biologically active constituents (both pharmacologic and toxicological), care is needed to choose the extract screening concentration to increase the chance of success. Thus, evaluation of NP extract HTS data can sometimes be highly subjective in nature. Some of the issues associated with screening an extract can be addressed by fractionation, which significantly reduces many of the unwanted effects normally attributed to screening complex mixtures (see Section 3.07.3).

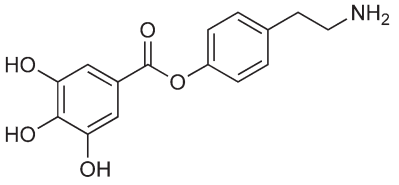
In addition to HTS resulting in the identification of NP lead-generation starting points and identification of NPs as drugs in their own right, it may provide tools to examine the role of various pathways, receptors, or proteins in disease, providing information on potentially new drug targets or increasing the knowledge about a known target for which there exists limited information.

Screening of NPs for compounds with therapeutic applications has been adopted for a broad range of therapeutic areas. Recently, the first NP inhibitor of a new cancer target, ICMT, was identified using SPA technology.[46] ICMT catalyzes the carboxyl methylation of oncogene proteins in the final methylation step of a series of post-translational modifications. Inhibition of these events is believed to provide a better approach to inhibiting cellular proliferation associated with cancer.

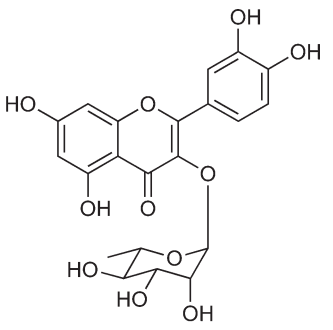
The glucocorticoid receptor (GR) is a member of the nuclear hormone receptor superfamily. Synthetic glucocorticoids are widely used as drugs to treat anti-inflammatory conditions, such as rheumatoid arthritis. Current drugs lack selectivity, thus new selective GR binders are sought. Based on FP technology, an HTS campaign undertaken against the Eskitis NP library identified an extract from *Endiandra anthropophagorum* with potent GR binding activity. As a result, endiandrin A, a new cyclobutane lignan, was isolated.[41]



Endiandrin A



Galloyl tyramine



Quercitrin

Stylissadines A and B are the most potent NP P2X7 (purinergic receptor) antagonists to be isolated to date and provide a novel class of P2X7 receptor inhibitors.[62] In addition, these compounds are the first examples of tetrameric pyrrole-imidazole alkaloids. P2X7 antagonists have a significant role to play in the treatment of inflammatory disease.

The Pim family of kinases are recognized as important regulators of apoptosis and cellular metabolism. Inhibition of these Pim kinases could have applications in the treatment of diseases such as cancer and inflammation. A recent NP HTS campaign, using AlphaScreen technology, identified an extract from the leaves of the rainforest tree *Cupaniopsis* sp. that exhibited potent activity against the Pim2 kinase. Galloyl tyramine, a new alkaloid, was identified as the active constituent in addition to a previously reported flavanoid glycoside, quercitrin.[34]

NPs represent ~80% of commercial antibacterial drugs. There is a continual need to discover novel antibacterial drugs due to antibiotic resistance limiting the use of current drugs. The search for new anti-infective agents continues to utilize NP libraries as a source of novel entities.

Novartis and Vicuron are developing novel compounds against a new target, peptide deformylase, and an essential protein unique to bacteria. HTS of an NP library led to the discovery of a novel compound, NVP-LBM-415, which is a derivative of the NP actinonin. NVP-LBM-415 is a potent antibacterial with a K_i of 0.3 nmol l^{-1}, is orally bioavailable with good tissue distribution, and is in phase I clinical trials.[63]

NP compounds in many cases have biological properties that lead to discovery of unique biology. Myriocin is a metabolite of an ascomycete, *Isaria sinclairu*, with potent immunosuppressive and antiproliferative activity. It has severe gastrointestinal (GI) side effects and therefore has limited use. An analogue, FTY720, was synthesized retaining activity and eliminating GI side effects. The MOA involves sequestration of lymphocytes from blood to the lymphatics, which is an unprecedented MOA. FTY720 is currently being developed by Novartis and is in phase II trial for multiple sclerosis (MS).[63]

The last decade has seen significant advances in the efficiency of HTS, in both the quality and quantity of information obtained, through a combination of improved technologies, reagent availability, and developments in instrumentation and robotics.

Unfortunately, there is not one single assay or detection system that is more suitable for all targets. Fluorescence technologies have played a major role in HTS during the past decade, and continue to do so. Label-free and HCS approaches have greatly improved in recent years and are now demanding greater attention.

3.07.13 Isolation of Bioactive Natural Products

The classic approach to isolation of a bioactive compound from extracts has been the iterative bioassay-guided purification.[2] In the past, this has tended to be time consuming since it is reliant on two steps, separation and bioassay. While separations are quick (10 min (HPLC) to several hours (gel permeation or countercurrent)), preparation of fractions for assay and the bioassays themselves at best might take a couple of hours or at worst might take up to a week depending on the bioassay protocol (some cell-based assays take several days) being employed. Various methods have been developed to try to dramatically shorten NP discovery timelines. We discuss below several approaches that others have taken and then highlight the approach that we have taken.

3.07.13.1 Production of Pure Natural Product Libraries

There has been a need to develop high-throughput methods to allow NP discovery to remain competitive with HTS of compound libraries.[64] One approach has been to ignore biological activity and purify as many components out of an extract as possible and determine the structures of these compounds rapidly.[21,65,66] This approach has several obvious advantages. (1) The compounds isolated can be treated the same as any other compound in a library. (2) Immediate assessment of the compounds potential can be made, thus eliminating the time delay between hit extract identification and isolation of the hit NP. (3) Lead-like or drug-like properties can be calculated for the compound and these data can be used to select compounds to be included in a screening library.[67–69] (4) Clustering of like compounds (such as by comparing Tanimoto distance)[70] can be

done and simpler analogues may be found by searching either within databases or within in-house compound libraries for follow-up testing.[71] (5) Methods to purify the compound are known, which means that access to larger quantities of the compound is only limited to availability of biota material. (6) Ugly compounds (compounds with either reactive functional groups or compounds that do not obey Lipinski criteria[18] or other defined lead-like or drug-like properties[21,65,66]) can be eliminated from the screening set prior to HTS. (7) If a number of biota produce the same active compound, it will appear only once in the screening set rather than many times. Two major disadvantages of this approach are (1) minor components may be overlooked and so never reach a screening set and (2) priority for selection of biota for purification tends to be given to biota that produce many compounds, resulting in biota that produce few compounds being ignored. For the company Analyticon this has meant that compounds from only 10 to 20% of the biota they collect are ever purified and screened.[72] A recent analysis of 2.6 million compounds gathered from 32 chemical providers (including Analyticon) showed that the NP library of 5438 compounds was unique but very low in lead-like compounds.[73]

3.07.13.2 Prefractionation

A second approach has been to generate libraries of semipurified fractions for screening. Prefractionation involves the separation of crude extracts by column chromatography, HPLC, or liquid–liquid partitioning into fractions containing many to few compounds. The company Sequoia generates 200 fractions per biota, 60% of which on average contain detectable compounds.[74] It is only these fractions that are tested. They claim that this allows biological activity of minor components to be detected since they can be screened at a similar dose to major components. Another advantage of the method is that interfering compounds such as highly lipophilic and hydrophilic compounds, pigments, and large-molecular-weight tannins and polysaccharides are removed. To some extent, the Sequoia method mimics the Analyticon approach except that fractions are still mixtures of 1–5 compounds of unknown structure. A disadvantage of both the pure compound and more particularly Sequoia's semipurified fraction approach is the severe limit to which biodiversity can be accessed in a cost-effective manner. If 200 000 wells were available for screening, the Sequoia approach would allow only ~1700 biota to be evaluated. A more conservative approach to semipurification prior to screening has been adopted by several groups, with Wyeth, Novartis, and Merlion generating fewer fractions prior to screening. Merlion claim that by reverse-phase separation of microbial extracts into four fractions (eliminating hydrophilic and hydrophobic material) prior to screening, they have been able to observe activity in fractions for more than 80% of their extracts, while the crude extracts from which they are derived were inactive.[75] Prefractionation into few fractions and at the same time eliminating highly hydrophilic and hydrophobic fractions has the advantage that the fractions will contain compounds with lead-like characteristics, a reasonably high level of biological diversity can be tested, and the concentration of compounds found within each fraction can be tested at a higher dose than would be possible in a crude extract. Dereplication can be applied directly to the bioactive fractions. Even after active fractions have been identified from HTS, there is still a need to undertake bioassay-guided purification. However, the limited number of compounds found within individual bioactive fractions makes it possible to use MS data and NMR data as well as bioassay data to guide further purification.

3.07.13.3 Dereplication

Dereplication, the use of chromatographic and spectroscopic analysis to recognize previously isolated substances present in an extract, has been used widely.[76–78] Dereplication can be used to identify ubiquitous interfering compounds such as tannins, fatty acids, saponins, or known compounds that either from the literature or from in-house knowledge are known to interact with the target of interest. Another important outcome of dereplication is the identification of multiple extracts or fractions that contain the same active component or profile and this can be used to prioritize extracts and fractions for chemical isolation. Dereplication has been particularly necessary for those groups working on microbial extracts.[79] An interesting recent development in dereplication has been the application of differential analysis of 2D NMR spectra to identify new NPs from fungal extracts.[80]

3.07.14 Small-Scale Bioprofiling

At Griffith University, small-scale bioprofiling (SSB) was introduced to provide information that could be used to determine if the active compound in an extract was likely to have conventional lead-like properties,[67–69] to semiquantitate the potency of the bioactive compound, and to predict the likely uniqueness of the bioactive component. For each screening campaign, up to 200 active extracts were processed using a rapid automated C_{18} HPLC chromatography protocol. The separated fractions (22 per extract) were then screened and the spectroscopic properties (NMR, MS, and nitrogen content) of the active fractions were analyzed (**Figure 9**). Screening of the active fractions gave the opportunity to evaluate the partially purified fraction and compare it with the crude extract. The chromatography method was tested with a variety of compounds (both lead-like and nonlead-like) to determine the relationship between elution time and clog *D* or elog *D*. Log *D* is the partition coefficient of a compound between octanol and water at a pH of 7.4. Lombardo *et al.* have developed an automated HPLC method to determine elog *D*.[81,82] An approximate linear relationship was observed and compounds eluting after fraction 17 consistently had elog *D* or clog *D* >5. NMR analysis is performed by flow probe NMR (VAST) using nondeuterated DMSO (**Figure 10**), and MS analysis is performed using positive and negative electrospray MS.

Quantitation of the nitrogen content in the active fractions is done in parallel to the MS analysis using a nitrogen detector. The intensity of the NMR signals for the active fraction in combination with the percent activity from screening provides a rough estimate of the potency of the active fraction.[83] The mass spectral analysis provides molecular size information for the bioactive fraction. Cluster analysis of spectroscopic data[84] (UV, MS, NMR) and elution times provides an indication of the uniqueness of the active fraction. The SSB process therefore helps to discriminate which bioactive extracts contain unique lead-like active compounds (clog $D < 5$, MW < 550, potency < 10 μmol l^{-1}). From these data, a decision is made for selection of extracts for scale-up purification based upon biological profiling of extracts and SSB analysis, the goal being to generate a smaller list of extracts that will deliver potent lead-like bioactive compounds from scale-up purification. Implementation of the SSB process led to an improvement in the delivery of lead-like compounds with 64% of screens yielding lead-like bioactive compounds.

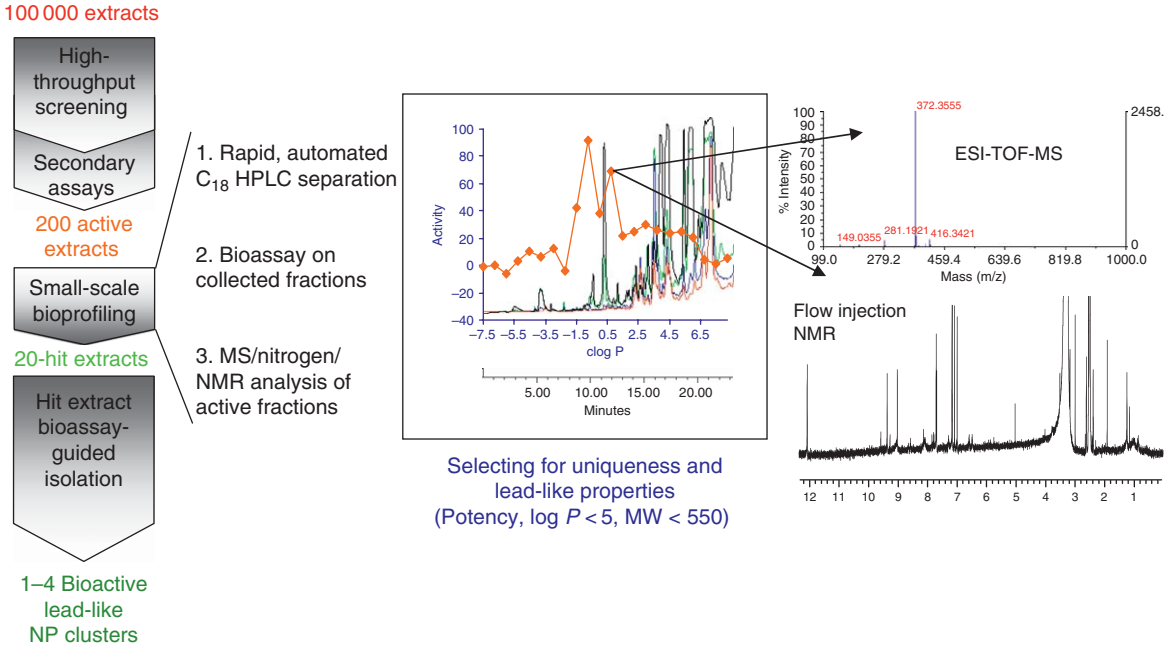


Figure 9 Illustration of the small-scale bioprofiling work flow. HPLC elution times are calibrated to log *P*. Bioassays are performed on collected fractions. Active fractions are analyzed by flow injection NMR and ESI TOF MS. Nitrogen analysis is performed in line with MS analysis.

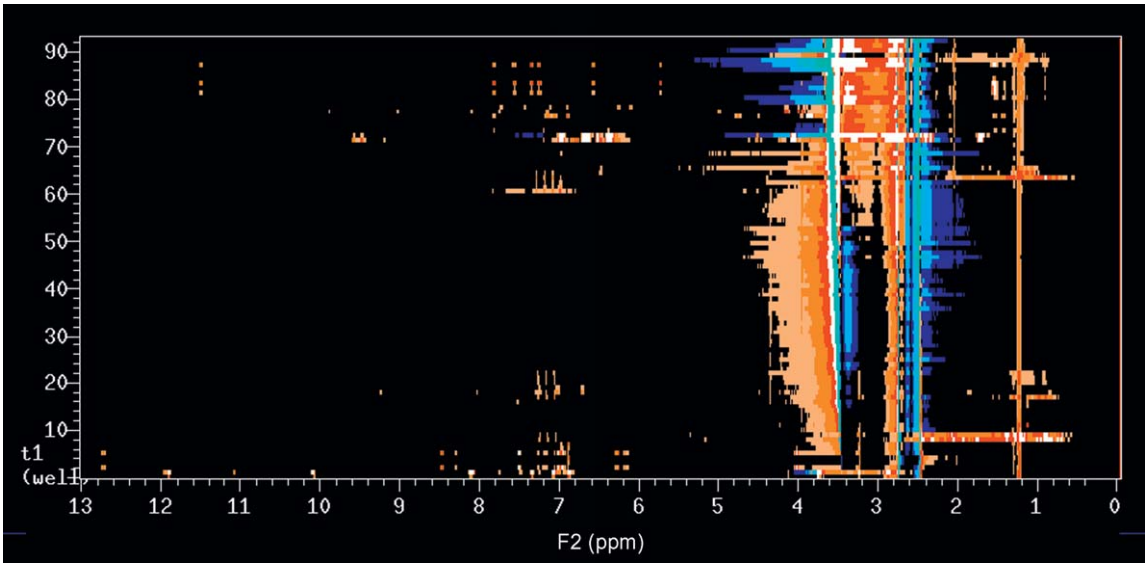
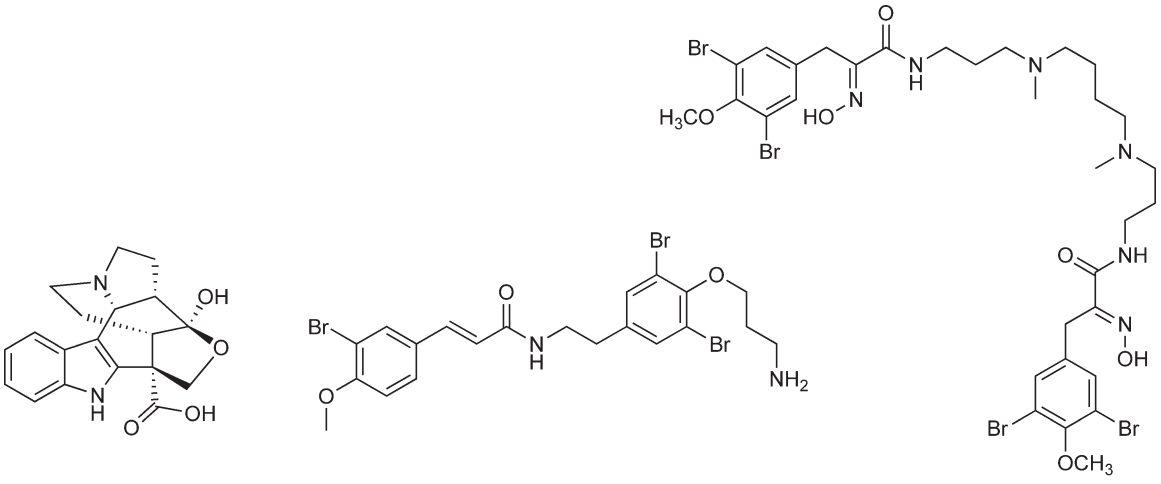


Figure 10 Pseudo-2D plot of individual wells analyzed by flow probe NMR (VAST).

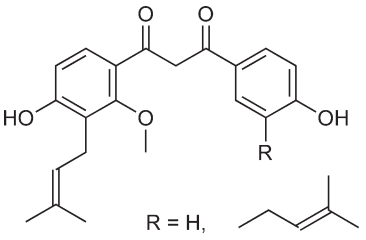
3.07.15 Scale-Up Purification

Once extracts have been selected for further purification, they are profiled on various adsorbents following the scale-up reverse-phase separation that mimics the SSB step.[85] The aim of this profiling is to find methods that can be applied to quickly eliminate >50% of nonactive components from the active fractions in one step. A matrix of adsorbants (strongly acidic and strongly basic ion exchange resin, size-exclusion gels, and diol-bonded silica) and solvent conditions have been used. Actinophyllic acid was active in a coupled enzyme assay to determine carboxypeptidase U inhibitors. The purification of actinophyllic acid from the leaves of *Alstonia actinophylia* was greatly assisted by application of successive steps of acidic and basic ion exchange chromatography as this eliminated 97% of nonactive components from the active fraction.[86] Purification of the potent glucocorticoid receptor binder, endiandrin A, from the roots of *Endiandra anthopophagorum* was achieved through successive steps of C18, sephadex LH20 gel permeation, and diol-bonded silica gel purification.[41]

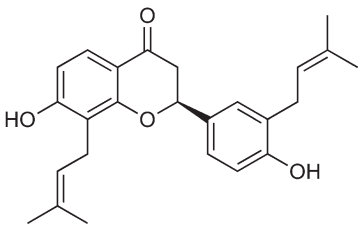


Actinophyllic acid Aplysamine 6 Spermatinamine

Improvements in pH stability and the quality of reverse-phase adsorbants have meant that the vast majority of extracts selected for scale-up purification have been successfully purified using reverse-phase HPLC alone. In many cases, only a single HPLC step has been required to purify the active component. The bioactive isoprenylated chalcones and flavanoid isolated from *Hovea parvicalyx*[87] and aplysamine 6 isolated from *Aplysina* sp. were achieved using this method.[88]



Chalcone

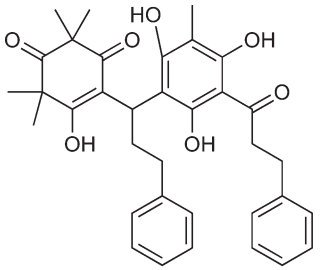


Flavanoid

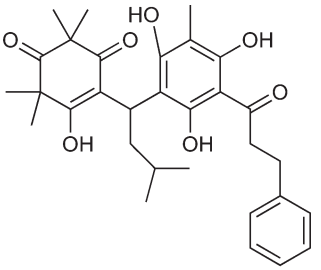
One advantage of the SSB process has been that the spectral information gained on the bioactive fraction at an early stage has allowed the use of MS- and NMR-directed fractionation to be used in place of a bioassay. The first NP inhibitor of the cancer target ICMT, spermatinamine, was isolated from the sponge *Pseudoceratina* sp. using this approach.[47]

Using chemical knowledge (MS, NMR, and chromatography) to isolate the pure compound from a larger quantity of extract is significantly faster than using bioassay-guided fractionation.[89] This has greatly reduced the time taken to isolate bioactive components. Application of these methods has provided a significant time saving while still maintaining screening access to diverse biota collections and not eliminating minor components from the screening set.

We have seen a few examples where application of solid-phase chromatography has not been successful in purifying the bioactive components from bioactive extracts. In these circumstances, we have resorted to centrifugal partition chromatography (CPC). The purification of the thyrotropin-releasing hormone receptor binding compounds corymbones A and B from the flowers of *Corymbia peltata* was achieved by using CPC with a nonaqueous solvent system heptane/dichloromethane/acetonitrile (10:3:7) in one step.[90]



Corymbone A



Corymbone B

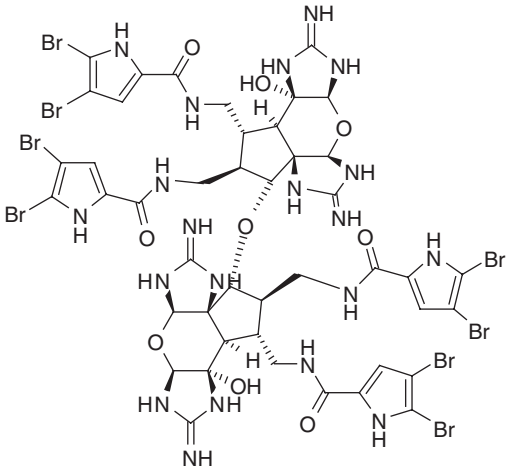
3.07.16 Structure Determination

The structure determination of unknown compounds was once considered to be a bottleneck in the NP discovery process. However, the availability of high-field NMR spectrometers and over 1000 multidimensional NMR pulse sequences has allowed the assignment of structures of complex molecules to be achieved in hours or days rather than weeks or months.

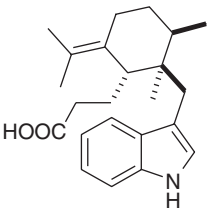
One of the driving forces behind this dramatic improvement in efficiency has been the ability to acquire quality spectroscopic data on ever smaller quantities of compound. High-resolution FTICR mass spectrometry

can provide exquisite accuracy that allows assignment of molecular formulae with high confidence on subnanogram quantities of compound.[91] Even benchtop time-of-flight (TOF) mass spectrometers can be used to generate data with accuracy better than 5 ppm in a high-throughput fashion and methods have been developed to routinely acquire high-resolution data for compounds in 96-well format.

Although MS is a powerful tool to aid structure determination, NMR spectroscopy still provides the most information to fingerprint molecules. An underlying problem that has beset NMR spectroscopy has been its low sensitivity. In the 1980s, 2D NMR pulse sequences were applied to solve the structures of NPs, but to achieve adequate quality, heteronuclear correlated spectra required >30 mg of compound and at least overnight acquisition times. The advent of inverse methods (or proton-detected heteronuclear experiments) in the late 1980s led to a major improvement that resulted in only several milligrams of compound being required to acquire multiple-bond heteronuclear correlated spectra in the same time frame. A further sensitivity gain was provided by the introduction of gradient pulse sequences. From the late 1990s until now, probes that have their electronics cryogenically cooled have delivered a further fivefold sensitivity improvement and this can be equated to a 25-fold reduction in acquisition time. The P2X7 bioactive alkaloid stylissidine A was a minor component isolated from the sponge *Stylissa flabellata* (0.003% yield). The molecular weight of this constituent was 1640 Da and this meant that only a dilute solution ($\sim$3.0 mmol l^{-1}) was available for NMR analysis. Full 2D NMR acquisition (COSY, HSQC, HMBC, and ROESY) was achieved in less than 3 days on a 600 MHz NMR spectrometer equipped with a cold probe. Prior to the introduction of the cold probe, the same quality spectra would have required 75 days acquisition.[62]



Stylissidine A



Suaveolindole

Capillary flow cell systems have further revolutionized NMR spectroscopy. These capillary probes typically have a 5 µl flow cell with an active volume inside the coil of 1.5 µl. When availability of compound is mass limited, as is typically the case for many NPs, these flow cells provide an attractive solution since 2D spectra can be acquired on microgram quantities of compound.[8,74,92] Only 300 µg (0.0018% yield) of the new antibacterial compound suaveolindole was isolated from the fruits of *Greenwayodendron suaveolens*. CapNMR analysis using a solution of suaveolindole (90 µg in 6.5 µl in CD_3OD; 20 µg in active volume inside the coil of 1.5 µl) resulted in ^1H (5 min), COSY (32 min), NOESY (2 h), HSQC (5 h), and HMBC (8 h) spectra being acquired in less than 16 h.[93]

Typically, the quantity of compound required for structure determination is similar to the amount needed for testing against biological targets from HTS. A consequence of this is that chromatography on analytical or microbore columns can provide sufficient quantity of compound for both screening and structure determination.[94] Alternatively, very minor components can be structurally elucidated without the need for massive scale-up, biota acquisition, and subsequent purification. Sensitivity improvements have also meant that pulse sequences that rely on correlations to low natural abundance nuclei such as ^{15}N can now be routinely applied

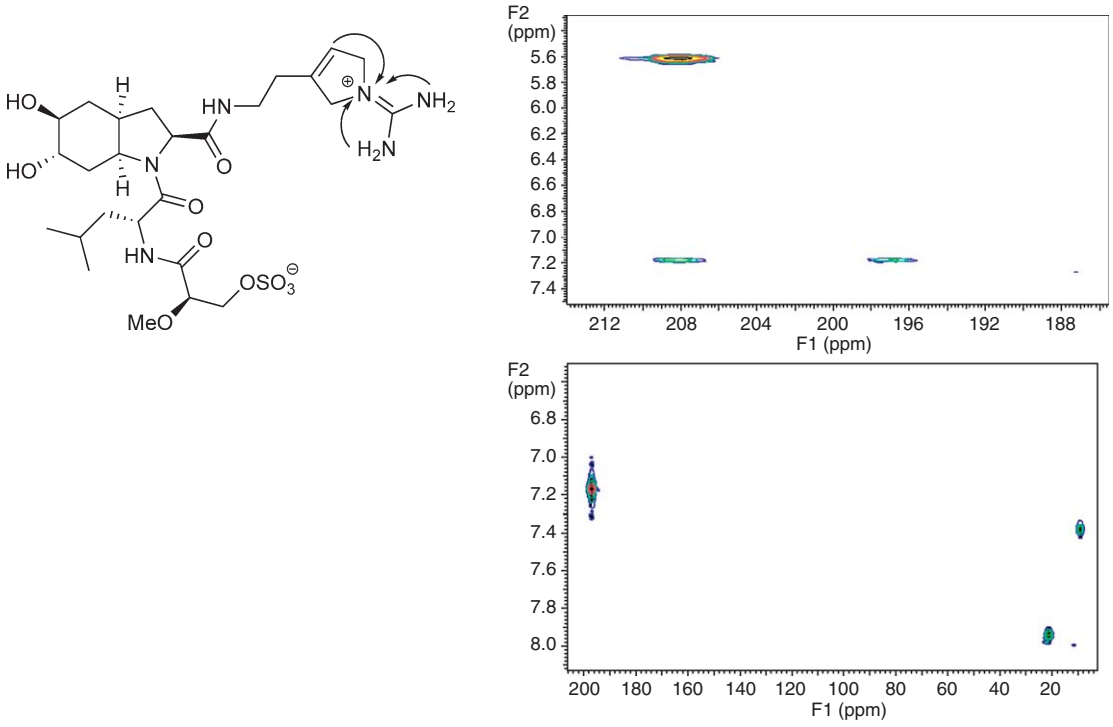


Figure 11 ^{15}N HMBC and HSQC NMR analysis of dysinosin A.

to challenging structures where only milligram quantities of compound are available.[95] Crucial ^{15}N HMBC correlations from the double-bond proton H-25 and the guanidine protons 29-NH_2 and 30-NH_2 to the nitrogen N-27 allowed the 1-*N*-amidino-*δ*-3-pyrroline moiety to be assigned in the potent FVIIa inhibitor dysinosin A (**Figure 11**) isolated from the sponge *Citronia astra*.[96]

3.07.16.1 Pulse Sequences

Although there are over 1000 pulse sequences to choose from, the structure determination of NPs is typically achieved by application of only four 2D pulse sequences: gCOSY, gHSQC, gHMBC, and either ROESY or NOESY. For overly crowded ^{1}H NMR spectra, the TOSCY or the HSQCTOCSY experiments provide useful data to assign overlapping peaks. On our 600 MHz spectrometer equipped with a cold probe, all of these experiments can be acquired in less than 2 h on 1 mg of compound (COSY 5 min, HSQC 2 min, HMBC 10 min, ROESY 80 min). Even insensitive NMR experiments such as ^{15}N HSQC and ^{15}N HMBC can yield useful data in less than 4 h. Even so, there is still a need to improve throughput of acquisition of 2D spectra and some exciting new pulse sequences have appeared recently that could revolutionize the speed of 2D acquisition.[97] Frydman *et al.*[98,99] have devised a single scan technique that relies on application of intense z-gradient pulses while at the same time selective radiofrequency pulses are linearly incremented generating a 2D data set in less than a second. A second fast method is the Hadamard technique, which relies on selective and simultaneous excitation of specific predefined frequencies employing Hadamard matrices.[100,101] If, for example, proton correlations to 15 carbons were of interest, an experiment could be set up that required only 16 increments as compared to a normal 2D experiment where the number of increments might typically be 128 or 256. There is therefore a significant time saving. The technique would be most useful where the chemical shifts of all carbons were known and many were close in chemical shift. The frequencies of the carbonyl region of peptides for instance could be selectively excited to generate a high-resolution HMBC spectrum of only the carbonyl region.

3.07.17 Automated Structure Determination

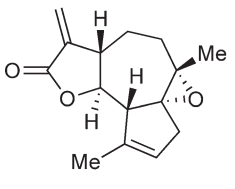
The generation of high-quality NMR data including multidimensional experiments is no longer a rate-limiting step. Rather, the analysis of the data has been the primary hurdle for the organic chemist. Both personal computer and web-based software tools have been available for the estimation and prediction of NMR spectra and now 'automated structure elucidation' based on spectral input is becoming increasingly available. Despite research in the field since the late 1970s, useable software has become available only recently, and computer programs that are able to elucidate the structure of large molecules are gaining in importance. Some of the new programs, as well as advancements in existing ones, are HOUDINI, COCON, ACDLabs Structure Elucidator (*StrucEluc*), SENECA, GENIUS, and MOLGEN. These programs rely on manual data entry (in particular, peak picking of 2D NMR data) and so one needs either extremely clean data sets with no noise or the trained eye of an analyst to discriminate between noise and real cross-peaks. Additionally, the algorithms work best if the molecular formula is known. Within these boundaries, our experience with automated structure determination is that it is possible to calculate the correct structure within 1 min of completion of data entry. The rate-limiting step is the time required to enter data, which might take 30 min. For most NPs, it is often quicker to solve the structure manually.

3.07.18 Converting a Natural Product Hit into a Drug

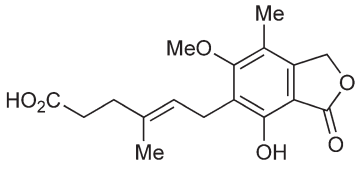
Once a bioactive NP has been isolated and its structure elucidated, there are three main options:

1. develop the NP as a drug;
2. modify the NP or synthesize a series of close analogues; and
3. use the basic structure of the NP as a starting point for the synthesis of a library of analogues. For more complex NPs such as halichondrin B, this might involve synthesis of a substructure responsible for the bioactivity. Other possibilities include incorporating the key pharmacophoric groups on a simpler scaffold or scaffolds, or identification of a key scaffold (or NP template) followed by synthesis of a combinatorial library.

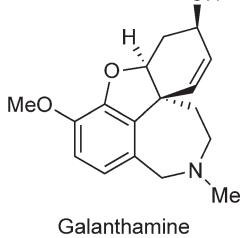
Of the 1010 new chemical entities (NCE) introduced in the 25-year period (1981–2006), 43 (<5%) were NPs, whereas approximately half (494) were NP analogues or NP inspired.[3] Examples of NPs introduced since 1999 include the anticancer drug arglabin from *Artemisia glabella*, the macrolide antibiotic and immunosuppressant rapamycin (Rapamune, Sirolimus) from *Streptomyces hygroscopicus*, the immunosuppressant Myfortic acid (mycophenolate sodium) from *Penicillium brevi-compactin*, the naturally occurring amino acid Levulan (δ-aminolevulinic acid), which is used for the treatment of actinic keratosis, Ziconitide (Prialt – a 25-residue peptide derived from the venom of the marine cone snail *Conus magus*) for neuropathic pain, and the antibiotic daptomycin.[3] Daptomycin (Cubicin) is the first approved member of a new class of antibiotics, the cyclic lipopeptides (from *Streptomyces roseosporus*).[102] Galanthamine, commonly used for the treatment of Alzheimer's disease, was originally isolated from snowdrop (*Galanthus woronowii*). It was launched as Nivalin in Austria in 1996 and as Reminyl in the UK in 2000 and in most other countries after approval by the FDA in 2001.[103] Galanthamine is available from natural sources such as daffodil bulbs (*Narcissus* spp.), or it can be synthesized.[104]



Arglabin

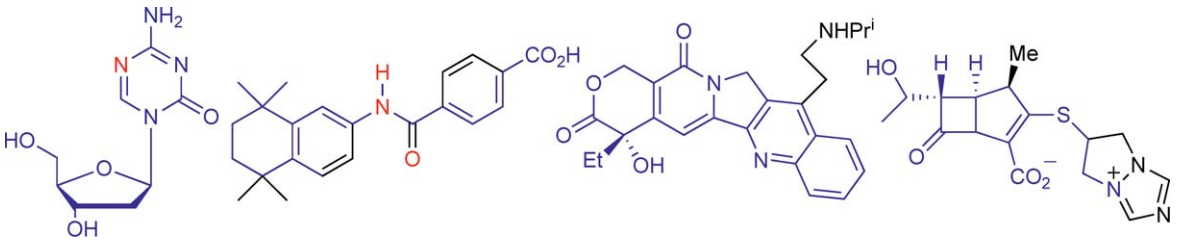


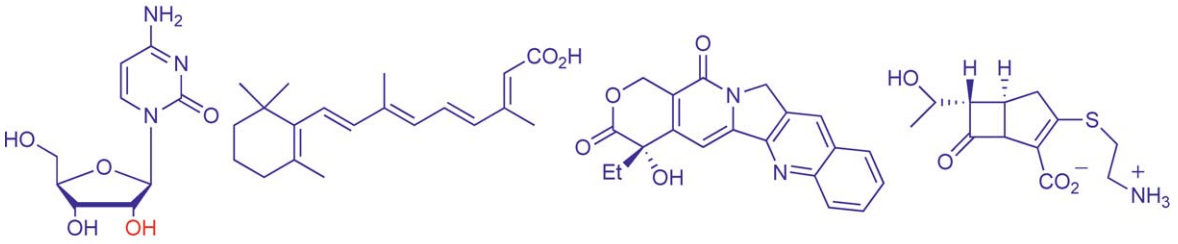
Myfortic acid



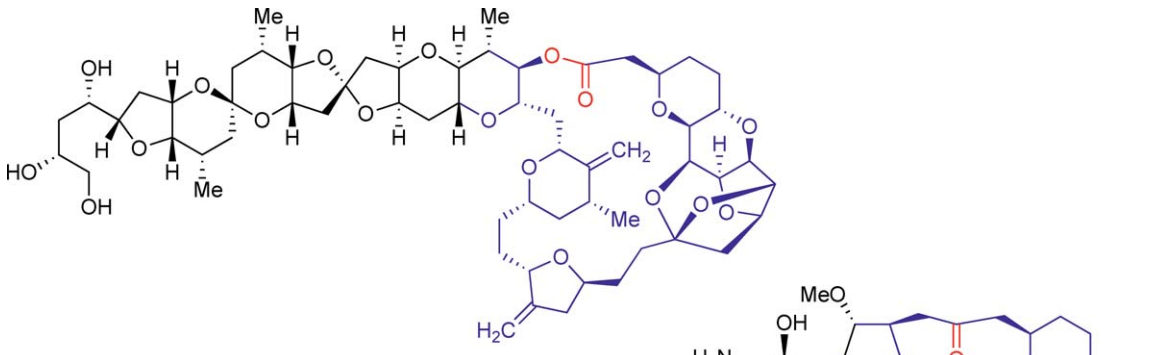
Galanthamine

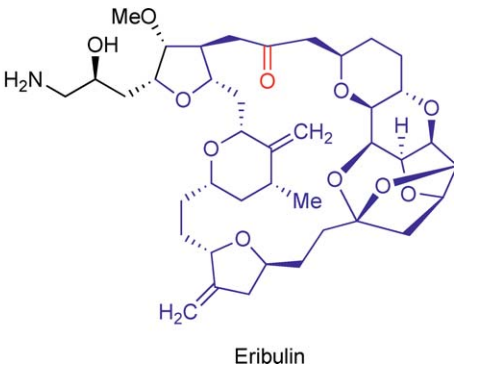
Examples of NP analogues introduced since 1999 include the anticancer drugs decitabine (an analogue of cytidine), for the treatment of myelodysplastic syndrome and chronic myelomonocytic leukemia,[105] tamibarotene (an analogue of all-*trans* retinoic acid), for the treatment of acute promyelocytic leukemia,[106] belotecan (an analogue of camptothecin), for the treatment of small cell lung cancer,[107] and the antibiotic biapenem (an analogue of the β-lactam antibiotic thienamycin).[108]





An interesting example of a complex NP where only part of the structure is required for biological activity is halichondrin B, a polyether macrolide of MW 1111.31 from the marine sponge *Halichondria*. Eribulin of MW 729.90 is a structurally simplified, fully synthetic ketone analogue that retains the subnanomolar anticancer activity of the NP *in vitro*.[109] Eribulin inhibits microtubule dynamics via a mechanistically novel mode of action and is in phase III trials for patients with breast cancer and phase II trials for non-small cell lung, pancreatic, prostate, ovarian, and other cancers.[110]



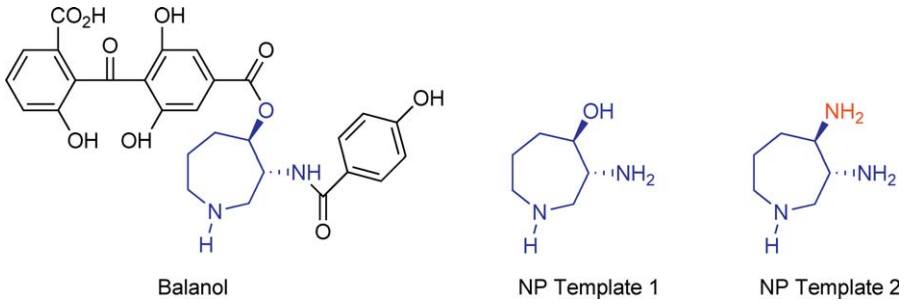


As exemplified by halichondrin B above, the major hurdle encountered in the development of a newly discovered NP lead may be its structural complexity. The lead compound may have adequate potency and selectivity but poor pharmacokinetics, so the challenge for the medicinal chemist is to turn it into a drug by appropriate modification. In many cases, this is not trivial for reasons of molecular complexity, synthetic accessibility, or 'druggability.'

One way of dealing with this difficulty is to use an NP template. This requires the recognition or identification of a readily synthesized scaffold or fragment within the NP lead structure. Once this template has been obtained, a library can be prepared by appropriate elaboration. This approach retains some of the features of the original bioactive NP structure (such as a novel ring system and conformation) but allows the medicinal chemist to introduce more 'drug-like' functional groups.

The concept of 'NP templates' is relatively recent, although historically NPs have served as 'templates' for the development of many important classes of drugs (penicillins, cyclosporins, ivermectins, statins, etc.). There has been increasing interest in the use of NP templates and scaffolds for the synthesis of combinatorial libraries and in drug discovery.[24,111–114] This is illustrated by a SciFinder search of the concept NP templates (**Figure 12**).

One of the earliest examples of an NP template that was used as the basis for a combinatorial library is that of balanol.[115] Balanol, a structurally unusual metabolite produced by *Verticillium balanoides*, inhibits protein kinase C (PKC) in the low nanomolar range, rendering it a promising lead structure.[116]



The hexahydroazepine core (NP Template 1) makes an ideal scaffold from which to synthesize a combinatorial library of analogues of balanol. However, it was found that these analogues were plasma unstable due to the ester linkage, so NP Template 2 was synthesized. The corresponding amide derivatives were plasma stable and retained their high activity.[117]

The concept of a 'privileged structure' is closely related to that of an NP template or NP scaffold. A privileged structure is a recurring ring system or scaffold in bioactive NPs that binds to a range of receptors. Examples include benzodiazepines, biphenyl (the biphenyl substructure is found in 4.3% of all known drugs),

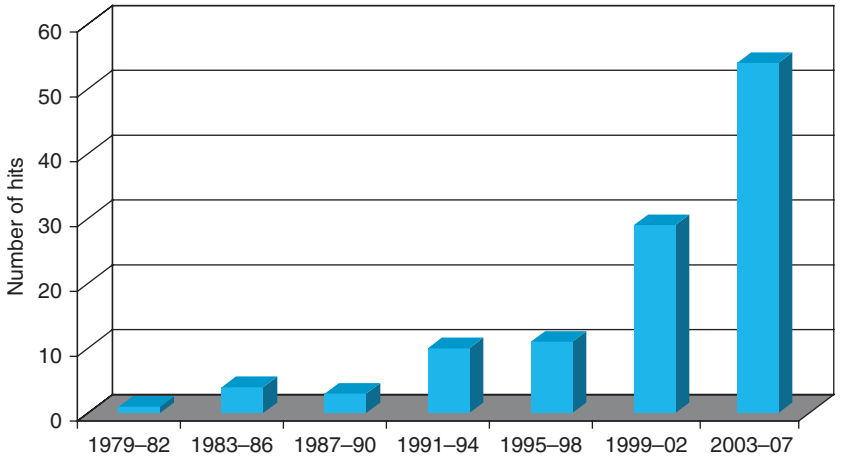


Figure 12 SciFinder hits for the concept natural product templates.

benzopyran, indole, and benzylpiperidine. The term was originally coined by Evans *et al.* as a "single molecular framework able to provide ligands for diverse receptors."[118] Medicinal chemists value privileged structures as core scaffolds around which to design combinatorial libraries.[119,120]

In conclusion, we believe that HTS of prefractionated NP extracts, pure NPs, or combinatorial libraries based on NP templates, scaffolds, or privileged structures provides an excellent chance of finding potent biological activity.

References

1. E. Lax, *The Mold in Dr. Florey's Coat: The Story of the Penicillin Miracle*; Henry Holt: New York, 2005.
2. F. E. Koehn; G. T. Carter, *Nat. Rev. Drug Discov.* **2005**, *4*, 206–220.
3. D. J. Newman; G. M. Cragg, *J. Nat. Prod.* **2007**, *70*, 461–477.
4. D. J. Newman; G. M. Cragg; K. M. Snader, *Nat. Prod. Rep.* **2000**, *17*, 215–234.
5. G. M. Cragg; D. J. Newman; K. M. Snader, *J. Nat. Prod.* **1997**, *60*, 52–60.
6. G. M. Cragg; D. J. Newman; R. B. Weiss, *Semin. Oncol.* **1997**, *24*, 156–163.
7. R. A. Quinn; P. Leone; G. Guymer; J. N. Hooper, *Pure Appl. Chem.* **2002**, *74*, 519–526.
8. J. F. Hu; E. Garo; H. D. Yoo; P. A. Cremin; L. Zeng; M. G. Goering; M. O'Neil-Johnson; G. R. Eldridge, *Phytochem. Anal.* **2005**, *16*, 127–133.
9. K. S. Lam, *Trends Microbiol.* **2007**, *15*, 279–289.
10. J. Y. Ortholand; A. Ganesan, *Curr. Opin. Chem. Biol.* **2004**, *8*, 271–280.
11. L. Arve; T. Voigt; H. Waldmann, *QSAR Comb. Sci.* **2006**, *25*, 449–456.
12. G. Wess; M. Urmann; B. Sickenberger, *Angew. Chem. Int. Ed. Engl.* **2001**, *40*, 3341–3350.
13. W. R. Strohl, *Drug Discov. Today* **2000**, *5*, 39–41.
14. R. S. Bohacek; C. McMartin; W. C. Guida, *Med. Res. Rev.* **1996**, *16*, 3–50.
15. M. Feher; J. M. Schmidt, *J. Chem. Inf. Comput. Sci.* **2003**, *43*, 218–227.
16. S. J. Teague; A. M. Davis; P. D. Leeson; T. Oprea, *Angew. Chem. Int. Ed. Engl.* **1999**, *38*, 3743–3748.
17. T. I. Oprea; A. M. Davis; S. J. Teague; P. D. Leeson, *J. Chem. Inf. Comput. Sci.* **2001**, *41*, 1308–1315.
18. C. A. Lipinski; F. Lombardo; B. W. Dominy; P. J. Feeney, *Adv. Drug Deliv. Rev.* **1997**, *23*, 3–25.
19. S. Rose; A. Stevens, *Curr. Opin. Chem. Biol.* **2003**, *7*, 331–339.
20. *Dictionary of Natural Products on CD-Rom*; Chapman and Hall, CRC Press: London, 2005.
21. R. J. Quinn; A. R. Carroll; N. B. Pham; P. Baron; M. E. Palframan; L. Suraweera; G. K. Pierens; S. Muresan, *J. Nat. Prod.* **2008**, *71*, 464–468.
22. M. L. Lee; G. Schneider, *J. Comb. Chem.* **2001**, *3*, 284–289.
23. B. M. McArdle; M. R. Campitelli; R. J. Quinn, *J. Nat. Prod.* **2006**, *69*, 14–17.
24. R. Breinbauer; I. R. Vetter; H. Waldmann, *Angew. Chem. Int. Ed. Engl.* **2002**, *41*, 2879–2890.
25. J. T. Baker; R. P. Borris; B. Carte; G. A. Cordell; D. D. Soejarto; G. M. Cragg; M. P. Gupta; M. M. Iwu; D. R. Madulid; V. E. Tyler, *J. Nat. Prod.* **1995**, *58*, 1325–1357.
26. J. Williams, *Australia: State of the Environment Report 2001 (Theme Report)*, RMIT University, CSIRO Publishing on behalf of the Department of Environment and Heritage: Commonwealth of Australia, **2001.**
27. Editorial, *Nat. Chem. Biol.* **Aug 2007**, *3* (8), 442–446.
28. R. L. Stein, *J. Biomol. Screen.* **2003**, *8*, 615–619.
29. J. Inglese; R. L. Johnson; A. Simeonov; M. Xia; W. Zheng; C. P. Austin; D. S. Auld, *Nat. Chem. Biol.* **2007**, *3*, 466–479.
30. A. Gomez-Hens; M. P. Aguilar-Caballos, *Trends Anal. Chem.* **2007**, *26*, 171–182.
31. J. H. Zhang; T. D. Chung; K. R. Oldenburg, *J. Biomol. Screen.* **1999**, *4*, 67–73.
32. S. Kadkhodayan; L. O. Elliott; G. Mausisa; H. A. Wallweber; K. Deshayes; B. Feng; W. J. Fairbrother, *Assay Drug Dev. Technol.* **2007**, *5*, 501–513.
33. N. L. Mills; A. A. Shelat; R. K. Guy, *J. Biomol. Screen.* **2007**, *12*, 946–955.
34. R. A. Davis; M. M. Simpson; R. B. Nugent; A. R. Carroll; V. M. Avery; T. Rali; H. Chen; B. Qurallo; R. J. Quinn, *J. Nat. Prod.* **2008**, *71*, 451–452.
35. L. Elster; C. Elling; A. Heding, *J. Biomol. Screen.* **2007**, *12*, 41–49.
36. N. Rouleau; R. Bosse, Homogenous Assay Development for Nuclear Receptor Using ALPHA Screen Technology. In *Handbook of Assay Development in Drug Discovery*; Lisa K. Minor, Ed.; Taylor and Francis, CRC Press: United Kingdom, 2006, Chapter 15, pp 193–207.
37. J. Wilson; C. P. Rossi; S. Carboni; C. Fremaux; D. Perrin; C. Soto; M. Kosco-Vilbois; A. Scheer, *J. Biomol. Screen.* **2003**, *8*, 522–532.
38. S. Burns; J. Travers; I. Collins; M. G. Rowlands; Y. Newbatt; N. Thompson; M. D. Garrett; P. Workman; W. Aherne, *J. Biomol. Screen.* **2006**, *11*, 822–827.
39. H. Appelblom; J. Nurmi; T. Soukka; M. Pasternack; K. E. Penttila; T. Lovgren; P. Niemela, *J. Biomol. Screen.* **2007**, *12*, 842–848.
40. R. Seethala; R. Golla; Z. Ma; H. Zhang; K. O'Malley; J. Lippy; L. Cheng; K. Mookhtiar; D. Farrelly; L. Zhang; N. Hariharan; P. T. Cheng, *Anal. Biochem.* **2007**, *363*, 263–274.
41. R. A. Davis; A. R. Carroll; S. Duffy; V. M. Avery; G. P. Guymer; P. I. Forster; R. J. Quinn, *J. Nat. Prod.* **2007**, *70*, 1118–1121.
42. A. M. Lee; T. J. Wigle; S. F. Singleton, *Anal. Biochem.* **2007**, *367*, 247–258.
43. Y. Liu; L. Zalameda; K. W. Kim; M. Wang; J. D. McCarter, *Assay Drug Dev. Technol.* **2007**, *5*, 225–235.
44. L. Albizu; G. Teppaz; R. Seyer; H. Bazin; H. Ansanay; M. Manning; B. Mouillac; T. Durroux, *J. Med. Chem.* **2007**, *50*, 4976–4985.

45. A. G. Jobson; J. H. Cardellina, II; D. Scudiero; S. Kondapaka; H. Zhang; H. Kim; R. Shoemaker; Y. Pommier, *Mol. Pharmacol.* **2007**, *72*, 876–884.
46. X. Z. Khawaja, *Anal. Biochem.* **2007**, *366*, 80–86.
47. M. S. Buchanan; A. R. Carroll; G. A. Fechner; A. Boyle; M. M. Simpson; R. Addepalli; V. M. Avery; J. N. Hooper; N. Su; H. Chen; R. J. Quinn, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 6860–6863.
48. C. E. Heise; S. K. Sullivan; P. D. Crowe, *J. Biomol. Screen.* **2007**, *12*, 235–239.
49. E. C. Liu; L. M. Abell, *Anal. Biochem.* **2006**, *357*, 216–224.
50. X. Xie; A. L. Van Deusen; I. Vitko; D. A. Babu; L. A. Davies; N. Huynh; H. Cheng; N. Yang; P. Q. Barrett; E. Perez-Reyes, *Assay Drug Dev. Technol.* **2007**, *5*, 191–203.
51. J. Dunlop; R. Roncarati; B. Jow; H. Bothmann; T. Lock; D. Kowal; M. Bowlby; G. C. Terstappen, *Biochem. Pharmacol.* **2007**, *74*, 1172–1181.
52. Y. Feng; A. R. Carroll; D. M. Pass; J. K. Archbold; V. M. Avery; R. J. Quinn, *J. Nat. Prod.* **2008**, *71*, 8–11.
53. R. Wagstaff; M. Hedrick; J. Fan; P. D. Crowe; D. DiSepio, *J. Biomol. Screen.* **2007**, *12*, 436–441.
54. D. L. Taylor; K. A. Giuliano, *Drug Discov. Today Technol.* **2005**, *2*, 149–154.
55. G. Lahav; N. Rosenfeld; A. Sigal; N. Geva-Zatorsky; A. J. Levine; M. B. Elowitz; U. Alon, *Nat. Genet.* **2004**, *36*, 147–150.
56. J. M. Levsky; R. H. Singer, *Trends Cell Biol.* **2003**, *13*, 4–6.
57. K. A. Giuliano; R. L. DeBiasio; R. T. Dunlay; A. Gough; J. M. Volosky; J. Zock; G. N. Pavlakis; D. L. Taylor, *J. Biomol. Screen.* **1997**, *2*, 249–259.
58. R. Pepperkok; J. Ellenberg, *Nat. Rev. Mol. Cell. Biol.* **2006**, *7*, 690–696.
59. U. S. Eggert; T. J. Mitchison, *Curr. Opin. Chem. Biol.* **2006**, *10*, 232–237.
60. A. E. Carpenter, *Nat. Chem. Biol.* **2007**, *3*, 461–465.
61. V. Starkuviene; R. Pepperkok, *Br. J. Pharmacol.* **2007**, *152*, 62–71.
62. M. S. Buchanan; A. R. Carroll; R. Addepalli; V. M. Avery; J. N. Hooper; R. J. Quinn, *J. Org. Chem.* **2007**, *72*, 2309–2317.
63. V. P. Gullo; J. McAlpine; K. S. Lam; D. Baker; F. Petersen, *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 523–531.
64. G. G. Harrigan; G. H. Goetz, *Comb. Chem. High Throughput Screen.* **2005**, *8*, 529–534.
65. K. U. Bindseil; J. Jakupovic; D. Wolf; J. Lavayre; J. Leboul; D. van der Pyl, *Drug Discov. Today* **2001**, *6*, 840–847.
66. U. Abel; C. Koch; M. Speitling; F. G. Hansske, *Curr. Opin. Chem. Biol.* **2002**, *6*, 453–458.
67. M. C. Wenlock; R. P. Austin; P. Barton; A. M. Davis; P. D. Leeson, *J. Med. Chem.* **2003**, *46*, 1250–1256.
68. M. M. Hann; T. I. Oprea, *Curr. Opin. Chem. Biol.* **2004**, *8*, 255–263.
69. M. S. Lajiness; M. Vieth; J. Erickson, *Curr. Opin. Drug. Discov. Dev.* **2004**, *7*, 470–477.
70. G. M. Downs; P. Willett; W. Fisanick, *J. Chem. Inf. Comp. Sci.* **1994**, *34*, 1094–1102.
71. eMolecules. http://www.emolecules.com (accessed Feb 26, 2009); PubChem. http://pubchem..ncbi.nlm.nih.gov/ (accessed Feb 26, 2009).
72. A. M. Rouhi, *Chem. Eng. News* **2003**, *81*, 84.
73. A. Monge; A. Arrault; C. Marot; L. Morin-Allory, *Mol. Divers.* **2006**, *10*, 389–403.
74. G. R. Eldridge; H. C. Vervoort; C. M. Lee; P. A. Cremin; C. T. Williams; S. M. Hart; M. G. Goering; M. O'Neil-Johnson; L. Zeng, *Anal. Chem.* **2002**, *74*, 3963–3971.
75. D. R. Appleton; A. D. Buss; M. S. Butler, *Chimia* **2007**, *61*, 327–331.
76. M. A. Strege, *J. Chromatogr. B, Biomed. Sci. Appl.* **1999**, *725*, 67–78.
77. D. Wolf; K. Siems, *Chimia* **2007**, *61*, 339–345.
78. R. K. Julian, Jr.; R. E. Higgs; J. D. Gygi; M. D. Hilton, *Anal. Chem.* **1998**, *70*, 3249–3254.
79. J. Bitzer; B. Köpcke; M. Stadler; V. Hellwig; Y.-M. Ju; S. Seip; T. Henkel, *Chimia* **2007**, *61*, 332–338.
80. F. C. Schroeder; D. M. Gibson; A. C. Churchill; P. Sojikul; E. J. Wursthorn; S. B. Krasnoff; J. Clardy, *Angew. Chem. Int. Ed. Engl.* **2007**, *46*, 901–904.
81. F. Lombardo; M. Y. Shalaeva; K. A. Tupper; F. Gao, *J. Med. Chem.* **2001**, *44*, 2490–2497.
82. F. Lombardo; M. Y. Shalaeva; K. A. Tupper; F. Gao; M. H. Abraham, *J. Med. Chem.* **2000**, *43*, 2922–2928.
83. G. K. Pierens; A. R. Carroll; R. A. Davis; M. E. Palframan; R. J. Quinn, *J. Nat. Prod.* **2008**, *71*, 820 –813.
84. G. K. Pierens; M. E. Palframan; C. J. Tranter; A. R. Carroll; R. J. Quinn, *Magn. Reson. Chem.* **2005**, *43*, 359–365.
85. J. H. Cardellina, 2nd; M. H. Munro; R. W. Fuller; K. P. Manfredi; T. C. McKee; M. Tischler; H. R. Bokesch; K. R. Gustafson; J. A. Beutler; M. R. Boyd, *J. Nat. Prod.* **1993**, *56*, 1123–1129.
86. A. R. Carroll; E. Hyde; J. Smith; R. J. Quinn; G. Guymer; P. I. Forster, *J. Org. Chem.* **2005**, *70*, 1096–1099.
87. M. S. Buchanan; A. R. Carroll; G. A. Fechner; A. Boyle; M. Simpson; R. Addepalli; V. M. Avery; P. I. Forster; G. P. Guymer; T. Cheung; H. Chen; R. J. Quinn, *Phytochemistry* **2008**, *69*, 1886–1888.
88. M. S. Buchanan; A. R. Carroll; G. A. Fechner; A. Boyle; M. Simpson; R. Addepalli; V. M. Avery; J. N. A. Hooper; T. Cheung; H. Chen; R. J. Quinn, *J. Nat. Prod.,* **2008**, *71*, 1065–1067.
89. O. Potterat, *Chimia* **2006**, *60*, 19–22.
90. A. R. Carroll; S. Urban; J. Lamb; R. Moni; G. P. Guymer; P. I. Forster; R. J. Quinn, *J. Nat. Prod.* **2008**, *71*, 881–883.
91. L. A. McDonald; L. R. Barbieri; G. T. Carter; G. Kruppa; X. Feng; J. A. Lotvin; M. M. Siegel, *Anal. Chem.* **2003**, *75*, 2730–2739.
92. M. Lambert; J. L. Wolfender; D. Staerk; S. B. Christensen; K. Hostettmann; J. W. Jaroszewski, *Anal. Chem.* **2007**, *79*, 727–735.
93. H. D. Yoo; P. A. Cremin; L. Zeng; E. Garo; C. T. Williams; C. M. Lee; M. G. Goering; M. O'Neil-Johnson; G. R. Eldridge; J. F. Hu, *J. Nat. Prod.* **2005**, *68*, 122–124.
94. C. Clarkson; D. Staerk; S. H. Hansen; P. J. Smith; J. W. Jaroszewski, *J. Nat. Prod.* **2006**, *69*, 527–530.
95. G. E. Martin; C. E. Hadden, *J. Nat. Prod.* **2000**, *63*, 543–585.
96. A. R. Carroll; G. K. Pierens; G. Fechner; P. De Almeida Leone; A. Ngo; M. Simpson; E. Hyde; J. N. Hooper; S. L. Bostrom; D. Musil; R. J. Quinn, *J. Am. Chem. Soc.* **2002**, *124*, 13340–13341.
97. R. Freeman; E. Kupce, *J. Biomol. NMR* **2003**, *27*, 101–113.
98. L. Frydman; S. Tali; A. Lupulescu, *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15858–15862.
99. L. Frydman; A. Lupulescu; T. Scherf, *J. Am. Chem. Soc.* **2003**, *125*, 9204–9217.
100. E. Kupce; R. Freeman, *J. Magn. Reson.* **2003**, *162*, 300–310.

101. E. Kupce; R. Freeman, *J. Magn. Reson.* **2003**, *162*, 158–165.
102. R. Sauermann; M. Rothenburger; W. Graninger; C. Joukhadar, *Pharmacology* **2008**, *81*, 79–91.
103. M. Heinrich; H. Lee Teoh, *J. Ethnopharmacol.* **2004**, *92*, 147–162.
104. B. Kuenberg; L. Czollner; J. Frohlich; U. Jordis, *Org. Process Res. Dev.* **1999**, *3*, 425–431.
105. E. A. Griffiths; S. D. Gore, *Semin. Hematol.* **2008**, *45*, 23–30.
106. I. Miwako; H. Kagechika, *Drugs Today (Barc)* **2007**, *43*, 563–568.
107. D. H. Lee; S. W. Kim; C. Suh; J. S. Lee; J. H. Lee; S. J. Lee; B. Y. Ryoo; K. Park; J. S. Kim; D. S. Heo; N. K. Kim, *Ann. Oncol.* **2008**, *19*, 123–127.
108. C. M. Perry; T. Ibbotson, *Drugs* **2002**, *62*, 2221–2234; discussion 2235.
109. M. J. Yu; Y. Kishi; B. A. Littlefield, *Anticancer Agents Nat. Prod.* **2005,** 241–265.
110. S. Newman, *Curr. Opin. Investig. Drugs* **2007**, *8*, 1057–1066.
111. M. S. Lesney, *Mod. Drug Discov.* **2002**, *5*, 26–30.
112. D. G. Hall; S. Manku; F. Wang, *J. Comb. Chem.* **2001**, *3*, 125–150.
113. G. Zinzalla; L. G. Milroy; S. V. Ley, *Org. Biomol. Chem.* **2006**, *4*, 1977–2002.
114. J. Mann, *Nat. Rev. Cancer* **2002**, *2*, 143–148.
115. J. Nielso; L. O. Lyngso, *Tetrahedron Lett.* **1996**, *37*, 8439–8442.
116. P. Kulanthaivel; Y. F. Hallock; C. Boros; S. M. Hamilton; W. P. Janzen; L. M. Ballas; C. R. Loomis; J. B. Jiang; B. Katz; J. R. Steiner; J. Clardy, *J. Am. Chem. Soc.* **1993**, *115*, 6452–6453.
117. C. B. Breitenlechner; T. Wegge; L. Berillon; K. Graul; K. Marzenell; W. G. Friebe; U. Thomas; R. Schumacher; R. Huber; R. A. Engh; B. Masjost, *J. Med. Chem.* **2004**, *47*, 1375–1390.
118. B. E. Evans; K. E. Rittle; M. G. Bock; R. M. DiPardo; R. M. Freidinger; W. L. Whitter; G. F. Lundell; D. F. Veber; P. S. Anderson; R. S. L. Chang; V. J. Lotti; D. J. Cerino; T. B. Chen; P. J. Kling; K. A. Kunkel; J. P. Springer; J. Hirshfield, *J. Med. Chem.* **1988**, *31*, 2235–2246.
119. D. A. Horton; G. T. Bourne; M. L. Smythe, *Chem. Rev.* **2003**, *103*, 893–930.
120. C. D. Duarte; E. J. Barreiro; C. A. Fraga, *Mini Rev. Med. Chem.* **2007**, *7*, 1108–1119.

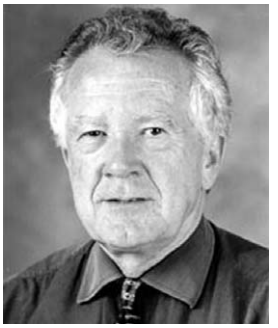
Biographical Sketches

Vicky Avery obtained her Ph.D. from Flinders University, South Australia in 1994. Following postdoctoral research at the University of Adelaide (Australian NHMRC Postdoctoral Fellowship) and in Sweden (Active Biotech AB), Vicky was appointed as an associate professor at the Eskitis Institute for Cell & Molecular Therapies at Griffith University in 2004. She gained significant industry experience during her time in Sweden (1998–2004), including being the section head, Protein Interaction and Drug Discovery; scientific project leader to identify the molecular target of 'Laquinimod,' a novel oral treatment for MS; director, Biochemistry and Molecular Biology, and director, Business Development. She was also responsible for the development of assays for the FDA to assess efficacy of a cholera vaccine, identification of compounds against CD80, which led to RhuDex®, an oral treatment for RA. Vicky specializes in high-throughput and high content screening and has been responsible for more than 50 HTS campaigns at Eskitis. More recently, the Discovery Biology team has also successfully designed & implemented HTS assays for Malaria (MMV Project of the Year, 2007) and African Sleeping Sickness (DnDi), Vicky has also played an active role in the establishment of the Bioactive Discovery (HTS) programme within the Eskitis-CRC Cancer Therapeutics collaboration.

David Camp obtained a B. App. Science from QUT in 1985 and his Ph.D. from Griffith University in 1990. After postdoctoral positions at Syntex, USA, the Australian National University, Griffith University, and a management position at Monash University, David was appointed to the Eskitis Institute for Cell & Molecular Therapies in 2001 to head the newly formed Biota and Compound Management group. Taking this opportunity he continues to improve the quality of natural product extracts in line with the emerging concepts of lead- and drug-likeness in the pharmaceutical industry. This has led to the Lead-Like Peaks (LLP) Library, a pre-fractionated library containing lead- and drug-like components compatible with high-throughput screening. The LLP library has been shown to yield more innovative leads for drug discovery than the previous crude extract library. It is available for screening by large pharma, biotechs, academe, and nonprofit organizations. More recently, David established the Queensland Compound Library (see http://www.griffith.edu.au/science/queensland-compound-library), a publicly accessible compound management and logistics facility to serve the Australasian region, where he is the foundation director.

Anthony Carroll is a graduate of the University of Sydney, where he completed his Ph.D. in 1989 on the isolation, structure elucidation, and synthesis of plant natural products under the supervision of Professor Wal Taylor. He undertook postdoctoral natural product research on marine invertebrates with Professor Paul Scheuer at the University of Hawaii and bioactive marine natural product and chemical ecology research with Professor John Coll and Associate Professor Bruce Bowden at James Cook University in Townsville. He was appointed to the Eskitis Institute for Cell & Molecular Therapies at Griffith University in 1993 and after a short period as research fellow with Professor Ron Quinn took up a position as head of the natural products chemistry group. The next 14 years was spent as a program leader in Natural Products Chemistry on an industry-based natural product drug discovery joint venture project with AstraZeneca and Griffith University. He is currently an associate professor at the School of Environment located on the Gold Coast Campus of Griffith University.

Ian Jenkins is a graduate of the University of New South Wales where he completed his Ph.D. in 1969 under the supervision of Michael Gallagher. He carried out postdoctoral work at Syntex (John Moffatt and Julien Verheyden), the University of Sussex (as an ICI Fellow with Colin Eaborn, FRS), and the ANU (Arthur Birch, FRS) before moving to Griffith University as a lecturer in chemistry in 1975. He was promoted to senior lecturer in 1981, associate professor in 1987, and professor in 1999. Ian is the author or co-author of over 150 publications on organic synthesis, reaction mechanisms, free radical chemistry, carbohydrate chemistry, organophosphorus chemistry, organometallic chemistry, and polymer chemistry. Highlights include the first synthesis of Nucleocidin (an antitrypanosomal agent and the only known naturally occurring fluorosugar derivative), the synthesis of cord factor (an immunostimulant), and seminal studies on the mechanism of the Mitsunobu Reaction. He joined the Eskitis Institute for Cell and Molecular Therapies as head of the Medicinal Chemistry Group in 1999. For the past 9 years, he and his research group have been working on the synthesis of bioactive natural products, natural product analogues and scaffolds, and the development of new synthetic methods.



Ronald Quinn obtained his Ph.D. from the University of New South Wales, Australia in 1970. Following post-doctoral research in the United States, he joined the Roche Research Institute of Marine Pharmacology in Sydney (1974–1981). He later joined Griffith University in 1982, and is currently professor and director of the Eskitis Institute for Cell & Molecular Therapies at the same university. Ron is the author or co-author of over 170 publications and patents. Research interests include developing an understanding of molecular interactions involved in biological processes through the development of Nature Bank, a unique library of 200 000 natural product fractions derived from a collection of over 40 000 samples of plants and marine invertebrates collected from mega-diverse areas. Nature Bank contains drug-like molecules separated into mixtures of a small number of compounds. Nature Bank is a state-of-the-art resource for biodiscovery (MMV Project of the Year,

2007 together with screen development – see Vicky Avery). Other research interests include understanding natural product recognition by biosynthetic enzymes and correlation with therapeutic targets as a rational approach to drug discovery and for developing concepts of biological structure space imbedded in natural product scaffolds, and the design and synthesis of receptor ligands and enzyme inhibitors.

3.08 Natural Products Drug Discovery

Giovanni Appendino, Università del Piemonte Orientale, Novara, Italy

Gabriele Fontana, Indena S.p.A., Milano, Italy

Federica Pollastro, Università del Piemonte Orientale, Novara, Italy

3.08.1 Introduction

Over the past two decades natural products drug discovery has been increasingly de-emphasized by pharmaceutical companies. Although heralded on the verge of a comeback several times,[1,2] attention for natural products has so far failed to materialize in big pharmaceutical companies (pharma), remaining relegated, with a few exceptions, to small biotech companies. Many review articles have analyzed this issue, pointing out the past success of natural products in drug discovery and their big and still largely untapped potential to provide new drugs for a host of unmet medical needs.[3–19] In this chapter, we will attempt to analyze from a pharma perspective why natural products have fallen out of favor in drug discovery despite their intrinsic utility for biomedical research. After an introduction on the current state of drug discovery, the reasons for the inbuilt utility of natural products for biomedical research will be highlighted, attempting next to explain why, despite so many advantages, it is so difficult for mainstream drug discovery to interface with natural products research. Finally, several strategies to improve natural products drug discovery and make it more efficient and attractive from a pharma side will be discussed.

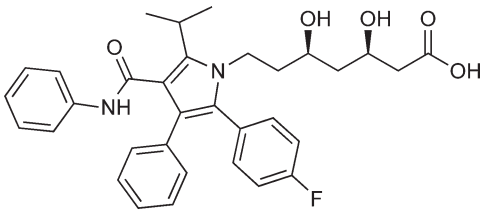
3.08.2 The Current Pharmaceutical Scenario

> A byproduct of the late-19th-century chemical business, pharmaceutical research thrived for more than a century by finding chemical combinations to treat diseases. But, after contributing substantially both to human health and drug-industry profits, it has failed to produce significant innovations in recent years.
>
> A. Johson, *Wall St. J.* 12 December 2007

Despite the introduction of a wealth of ingenious and innovative strategies to help and/or direct drug discovery (combinatorial chemistry, diversity-oriented synthesis, fragment-based drug discovery, chemical biology, *in silico* screening),[20] the number of new chemical entities (NCEs) reaching the pharmaceutical market has suffered a downward trend over most of the past decade. To explain this dismal performance, a host of arguments have been proposed, from the shortage of low-hanging drug fruits to the suitability of the much-hyped modern techniques to pick the higher-hanging fruits identified by genomics in the drug orchard.[20] Thus, aminergic G-protein coupled receptors (CPCRs) are relatively easy, highly druggable targets since their endogenous ligands are small molecules (serotonin, dopamine, adrenaline, histamine), while interfering with protein–protein interactions requires larger and more lipophilic agents and this inflation of physical properties promotes binding to unwanted targets and raises problems regarding absorption, distribution, metabolism, and excretion (ADME).[20]

Depending on the therapeutic area, the capital needed to bring a drug to the market has now skyrocketed to $0.8–1.7 billion, with cost splitting (breakdown) being approximately 10% for discovery, 15% for preclinical, 15% for manufacturing and process, 55% for clinical trials, and 5% for postmarketing.[21] Since the attrition rate of clinical development is currently estimated at an appalling 93–96%,[22] a market potential of at least $300 million is considered the lowest limit for a big pharmaceutical company to be interested in a product.[23] This combined with the lengthening of the R&D time, currently at around 12 years, is responsible for the 'blockbuster-itis' that is plaguing the pharmaceutical industry.

Faced with a looming and massive patent cliff in the first half of the next decade and with an arthritic drug pipeline, drug companies have been increasingly relying for innovation on biological big molecules (monoclonal antibodies, vaccines, and nucleic acids) and techniques (stem cells). Developments in this area have been remarkably fast. Thus, the first paper describing RNA interference (RNAi) gene silencing in mammal cells was published in 2001,[24] but six therapeutic programs based on this concept had already moved to clinical trials in 2007,[24] and also cutting-edge genetic tools like small inhibitory RNAs (sRNA) made the leap to drug candidates in record time.[24] Compared to small molecules, biologics enjoy a longer exclusivity, since clinical trials of bioequivalence are generally needed for generic versions (biosimilars),[25] but have higher costs of development and manufacturing and are therefore more expensive. A recent Swedish survey estimated that the annual cost of four biological antirheumatic drugs (etanercept, infliximab, adalimulab, and anakinara) is 50–70-fold higher than the average annual drug cost per person (€10 800–14 400 per year versus €170 per year),[26] and the financial burden of the biological anticancer agents is even higher. When generalized, these costs will become unsustainable for any national health insurance, since the total health costs in Western countries has already reached very high levels (16% of the gross domestic product (GDP) in the United States in 2005).[27] Despite these limitations, the pharmaceutical industry has embraced a 'biopharma' approach to drug discovery, diversifying its traditional focus on small molecules with a growing commitment on biologics. Thus, the term 'new molecular entities' is rapidly replacing the term NCEs in new drugs inventories. Unsurprisingly, drug companies have also started to shed their chemical workforce at an unprecedented rate. The laying off of Robert Sliskovic, the discoverer of atorvastatin (Lipitor, **1**), made headline news and was considered emblematic of the pharmaceutical industry's declining fortunes.[28] Atorvastatin is the best-selling pharmaceutical product ever, having generated over $80 billion sales to Pfizer since its introduction into the clinic, and the laying off of Sliskovic was commented in a front page article by *The Wall Street Journal*.[28] The list of major drug companies that announced programs of cost reduction and biotech refocusing in 2007 includes, apart from Pfizer, Bristol–Myers Squibb (BMS), Novartis, and Astra-Zeneca, with Merck and Lilly already adopting this model a few years earlier.[29]



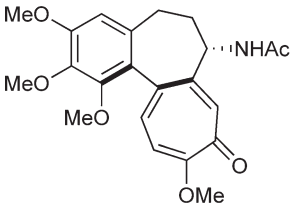
1

With a mean time of approximately 12 years from drug discovery to launch, the current problems of pharmaceutical research have their roots in choices dating to the 1990s and any strategy pursued today will have only marginal influence on the near-future late-stage development pipelines. The current downsizing of natural products drug discovery should therefore be seen as a part of a general trend of pharma research to focus on biological drugs at the expenses of small molecules. Confronted with an acute productivity crisis, mainstream pharmaceutical research will undoubtedly explore alternative strategies of discovery, with 'old' assets like natural products possibly coming of age again. Newer screening paradigms, shortened discovery times, and integration into a multifaceted drug discovery scenario will, however, be necessary to foster the long-awaited renaissance of natural products drug discovery.

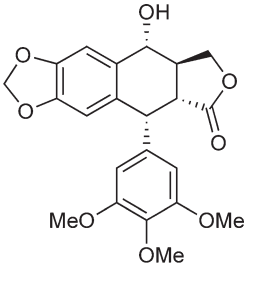
3.08.3 Why Natural Products Are Intrinsically Useful for Drug Discovery

> When you have no idea where to begin in a drug discovery program, Nature is a good starting point.
>
> L. H. Caporale, *Chem. Eng. News* 13 October 2003, p 89

A large number of biological processes involve the interaction of a small molecule (ligand) with a macromolecular target (receptor). Preeminent examples are the interaction of neurotransmitters with their protein targets, of intercalating agents with specific DNA or RNA polynucleotidic sequences, and of macromolecular carriers with the ligands they transport across biological membranes. In general, the interaction of a small molecule with a macromolecular target represents an opportunity to perturb a biological system, to study its function, and to assess its druggability, that is, its amenability to pharmaceutical exploitation.[30] In molecular terms, perturbing is equal to knowing and natural products hold a special position as molecular perturbators, since their role to reveal interesting biology, to provide the tools necessary for its study, and to generate molecular clues (hits and leads) for its ultimate therapeutic exploitation is at the very base of modern medicine. Tubulin, one of the most important anticancer targets, is a remarkable example of this process.[31] Thus, tubulin was discovered because of the availability of colchicine (**2**), a specific ligand obtained from the medicinal plant *Colchicum autumnale* L. The biological profile of tubulin was next furthered using a variety of natural products, including podophyllotoxin (**3**), and the manipulation of tubulin was eventually translated chemically with the development of *Vinca* alkaloids, taxanes, and epothilones into effective anticancer drugs. Natural products were also instrumental for the identification of hsp90 as an anticancer target and to study its function.[32] Several natural products drugs aimed at the inhibition of this chaperone are currently under clinical development, making it possible that, thanks to natural products, hsp90 biology will also soon be translated into hsp90 drugs[33](for more details, see Chapter 3.06).



2

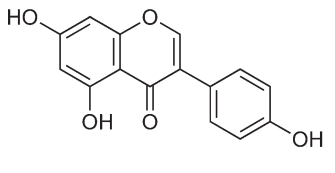


3

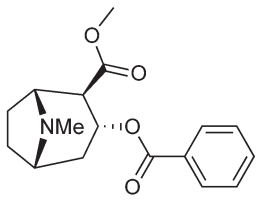
Natural products have a personality: they come with attractive names, awe-inspiring structures and dazzling biological properties, and there are clear molecular, evolutionary, and structural bases for their relevance in drug discovery. Because of their three-dimensional structural complexity and inbuilt affinity for biological surfaces, natural products are in fact privileged structures for drug discovery from both a chemical and a biological standpoint and qualify nature not only as the ultimate synthetic chemist but also as the ultimate pharmacist.

3.08.3.1 Molecular Bases for the Biomedical Relevance of Natural Products

Macromolecular biological targets (proteins, nucleic acids) 'see' small molecules as three-dimensional surfaces bearing specific binding elements (charges, polarities, and hydrogen-bonding donor and acceptor elements). Surreptitious complementarity between biogenetic enzymes and animal protein domains is the general molecular basis for the preeminence of natural products in biomedical research and drug discovery.[34] Most natural products are the results of enzymatic reactions and their shape must therefore be complementary to that of the biological protein surface that fitted their ultimate precursor(s). A biosynthetic origin therefore involves the imprinted capacity to recognize protein surfaces. All organisms, whether bacteria, plants, or animals, are the results of permutations and combinations of the same four basic nucleotides and 22 amino acids. The number of unmodified, or 'naked' proteins coded by the human genome is between 80 000 and 100 000 but the total number of different proteins produced by human cells is magnified by posttranslational modifications like glycosylation, acetylation, methylation, hydroxylation, and phosphorylation and is probably around 1 million.[35] Assuming a 10% of druggability,[36] the number of drug targets is therefore enormous, around 100 000, but only 324 of them have so far been identified for all the approved therapeutic drugs.[37] Despite the large number and structural variety of these protein targets, the number of peptide domains, which is the way secondary structures like α-helices and β-sheets can arrange themselves in space independently from the rest of the structure, is fairly limited. It has been estimated that the widely diverse function of proteins derives from the combination of only 600–8000 domains. The number of distinct protein families is therefore fairly limited[38] since a similar structural domain can be used by many proteins in more or less modified forms generated by divergent evolution, as shown, for instance, by leukotriene A4 hydrolase and thermolysin. These two enzymes catalyze different reactions (vinylogous opening of an epoxide ring and peptide hydrolysis) but share a very similar fold and profile of inhibitors.[38] Therefore, natural products are characterized by an intrinsic, biogenetically imprinted shape complementary to biological surfaces and this topological property can be translated into a reversible interaction with a druggable target totally unrelated to their original biosynthetic enzymes. In other words, since protein domains are conserved, biological surfaces homologues to those present in biogenetic enzymes might exists in animals, even though they fundamentally lack the enzyme machinery to produce secondary metabolites. For instance, the flavonoid biogenetic enzymes chalcone isomerase, chalcone synthase, and anthocyanidin synthase share a flavonoid recognition site with mammalian protein kinases and it is therefore unsurprising that the flavonoid genistein (**4**) was one of the first inhibitors of tyrosine kinases to be discovered.[39] The biosynthetic enzymes/drug targets correlation makes it possible that small-molecule ligands might exist for all the druggable human targets and neuroactive plant alkaloids represent a striking example of this assumption. Cocaine (**5**) inhibits the reuptake of mammalian biogenic amine neurotransmitters (dopamine, serotonin, noradrenaline, adrenaline), a system that has no counterpart in plants, which, in turn, also contain a host of compounds that target the cholinergic, adrenergic, dopaminergic, and gamma-aminobutyric acid (GABA)ergic systems of animals, all structures that do not fundamentally exist, even in cognate form, in plants.[34]



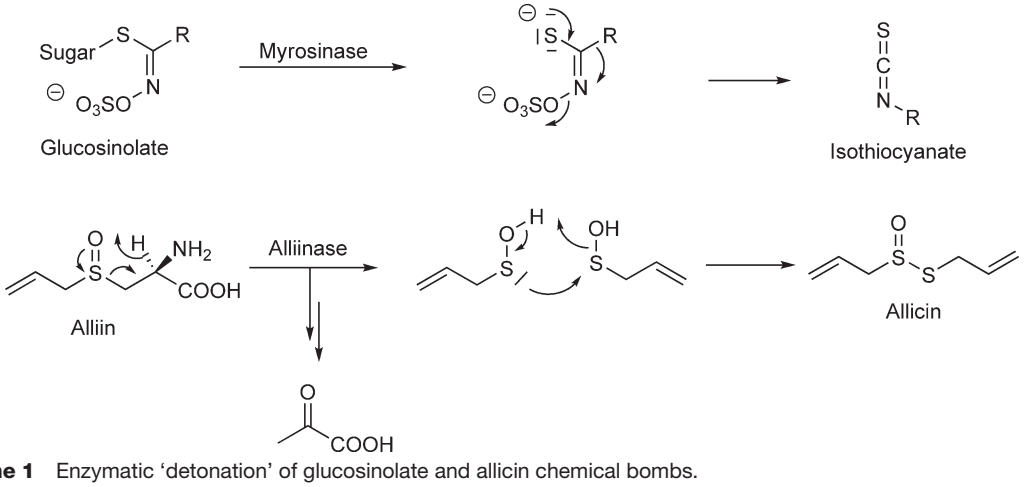
4



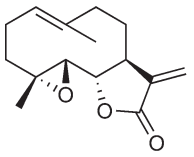
5

Some natural products are not the direct result of an enzyme reaction but rather of a spontaneous reaction cascade triggered by an enzyme reaction. In this so-called bomb-like strategy, a stable precursor is enzymatically transformed into an unstable species (chemical bomb) that undergoes an intramolecular rearrangement to a more stable but still reactive compound, capable of interacting with a variety of targets rather than with a single target. Examples from plant secondary metabolites are isothiocyanates from cruciferous plants and thiosulfinates from garlic, formed from glucosinolates and sulfoxidic amino acids by the action of specific enzymes (myrosinase and alliinase, respectively) compartmentalized in different cells or cellular stores with respect to their substrates.[40] Hydrolysis of the thioglucosidic bond of glucosinolates generates the unstable sulfate of an *N*-hydroxythioimidate, which then undergoes Lossen rearrangement to a reactive isothiocyanate,[40] while allinase triggers a β-elimination reaction that splits alliin into pyruvic acid and a sulfinic acid, which then spontaneously dimerizes to allicin, a reactive thiosulfinate ester (**Scheme 1**).[41] Compounds like isothiocyanates and thiosulfinates target nucleophilic sulfhydryl sites of a host of proteins via a polar trapping mechanism, while the microbial enediyne antibiotics cleave DNA with a radical mechanism, mediated by the formation of a 1,4-diyne (*p*-benzine) via a Bergman reaction.[42]

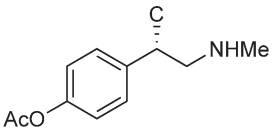
Reactive secondary metabolites have generally been perceived as nonselective and indiscriminate in their activity and therefore of little relevance for drug discovery. Lists of chemically or biologically reactive groups that should be avoided in drug discovery (aldehydes, exomethylene enones, epoxides, furans, etc.) can be found in any book on drug discovery. Nevertheless, reactive functional groups do occur in natural products and are, surprisingly, often associated with a specific activity. Thus, the exomethylene-γ-lactone parthenolide (**6**), an NF-κB inhibitor and the hallmark constituent of feverfew (*Tanacetum parthenium* L.) has been considered for clinical development for a variety of conditions, including headache, but has also raised considerable interest as a selective cytotoxic agent for stem cells,[43] while the even more reactive nitrogen mustard CpdA (Compound A, **7**) is a selective glucocorticoid receptor modifier, capable of dissecting the transactivation and the transrepression properties of these hormones.[44] CpdA is as effective as dexamethasone in counteracting acute inflammation *in vivo* but lacks the hyperglycemic side effects of glucocorticoids and, despite its reactive nature, is considered of 'great potential for therapeutic use'.[45] CpdA is a stable (*sic*) analogue of the unstable active principle of the Namibian shrub *Salsola tuberculatiformis* Botsch., a plant used in the bushman folklore as a contraceptive, and implicated in a syndrome of prolonged gestation and fetal postmaturity in sheep.[44] The active principle of this plant is a very unstable compound that quickly decomposes to synephrine (**8**) in acidic water and that has been suggested to be a precursor of the phenolic aziridine (**9**). CpdA was prepared from synephrine by treatment with acetyl chloride and its biological profile was comparable to that of the elusive active principle of the plant.[44] Although phenolic aziridines are extremely unstable *in vitro*, the active principle of *S. tuberculatiformis* and its derived aziridine are apparently stabilized *in vivo* by binding to plasma proteins.[44,46]



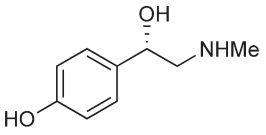
Scheme 1 Enzymatic 'detonation' of glucosinolate and allicin chemical bombs.



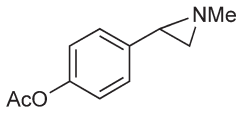
6



7

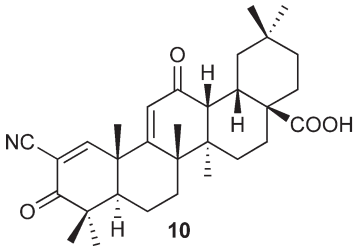


8

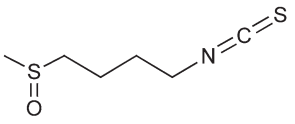


9

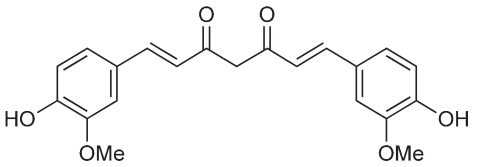
There is also growing evidence that reactive Michael acceptors can interact with biomolecules in a reversible fashion, not remaining bound to their 'proximate' targets but bouncing within a multitude of thiol-containing proteins and eventually 'landing' on key regulatory proteins having thiol group especially sensitive to electrophiles. This mechanism is the basis of the activity of CDDO (**10**), a semisynthetic homotriterpenoid nitrile considered one of the most promising anticancer agents in current clinical development.[47,48] Compounds like CDDO do not fit into the conventional models of drug action based on a single high-affinity receptor–ligand interaction, since their activity involves transient molecular interactions with multiple targets that share the critical and reactive cysteine residues.[49] If Michael acceptors that react reversibly with thiol groups have different affinity for different target proteins, then low concentrations of these compounds would preferentially interact with the most sensitive targets. A host of networks, in this case those responsible to the redox state of a cell, are therefore affected by their activity. This mechanism might well apply to a series of multifunctional chemopreventive agents like sulforafane (**11**), curcumin (**12**), and the organosulfur compounds from garlic,[49] all compounds capable of targeting multiple proteins through Michael adduct mechanisms.[50] Compounds like CDDO undoubtedly stretch the concept of ligand–receptor interaction well beyond the classic lock-and-key metaphor and it should be remarked that several tyrosine kinase inhibitors also interact in a combinatorial way with a host of their enzyme substrates, modifying the whole 'kinomic' profile of a cell rather than a single effector target and thus modulating the activity of several signaling networks.[51]



10



11

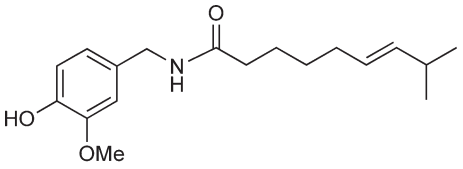


12

3.08.3.2 Evolutionary Bases for the Biomedical Relevance of Natural Products

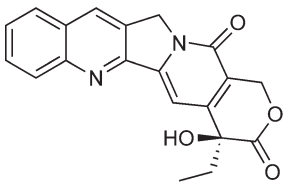
The term 'secondary' in secondary metabolites does not imply a 'less important' status but simply addresses a different functional level, just like the 'secondary' structure of a protein is equally important as the 'primary' or the 'tertiary' structure. Indeed, natural products have been detected in fossils[52,53] and presumably appeared very early in the evolution of life, possibly already in the RNA world, since many antibiotics bind specific RNA sequences.[52,53] Evolutionarily, the synthesis of natural products is a conserved and highly successful trait. Since it is not uncommon that scores of specific enzymes are involved in the biosynthesis of a single natural product, the production of secondary metabolites involves an enormous investment in terms of protein-coding DNA. Given this investment, natural products must have beneficial functions for their producers, all based on the interaction with macromolecular targets and their functional perturbation in terms of promotion or inhibition. The activity of natural products has been shaped to overcome environmental stress and provide defense against natural enemies and an adverse environment and was optimized by evolution during millions of years of environmental high throughput screening (HTS). In nature, the struggle for survival is waged at the molecular level and natural products, by reflecting eons of wisdom and refinement, have an intrinsic evolutionary and ecological meaning. They represent working examples of biological 'intelligent design' and it is therefore regrettable that the futile cat-and-mouse contest between science and creationism has not been waged at a

molecular level, spurring research into the elusive meaning of secondary metabolites. The ecological role of secondary metabolites is extremely difficult to investigate. They are like words of a language that we can read but do not understand, just like Etruscan. An important exception is capsaicin (**13**), probably the only secondary metabolite whose natural function has been elucidated in detail.[54] After survival and reproduction, dispersal of seeds is third on the list of priorities for a plant. To this purpose, peppers produce fleshy and colored fruits to attract consumers and colonize new areas. By using nonpungent peppers consumed by both mammals and birds, it was found that fruit ingestion by mammals inhibits seed germination, while, conversely, consumption by birds does not damage seed germinability, rather promotes it. Birds swallow the fruits and promote the dispersion of seeds while mammals chew the fruits with their teeth and physically damage them. Hence, mammals behave as seed predators and birds as seed dispersers, acting as living 'vessels' to carry chillies to new turfs. Capsaicin targets the vanilloid receptor TRPV1, a molecular thermometer whose activation is perceived as pungent pain by mammals but not by birds, which have a mutated form of TRPV1, insensitive to capsaicin.[55] Owing to the selective sensitivity of the mammalian version of TRPV1 to capsaicin, this compound functions therefore as a specific inhibitor of seed predation.

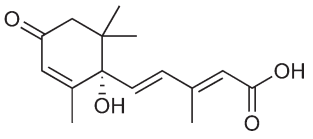


13

There are strict relationships between sensitivity to natural products and genomics, with the possibility of adaptive coevolution between the production of secondary metabolites and their target. For instance, it has been demonstrated that camptothecin (CPT, **14**)-producing plants like *Camptotheca acuminata*, *Ophiorrhiza pumila*, and *Ophiorrhiza liukiuensis* have Topo1's with point mutations that confer resistance to CPT.[56] Remarkably, one of these substitutions (Asn722Ser, human Topo1 numbering) is identical to that found in CPT-resistant human cancer cells while a phylogenetic analysis of Topo1's in CPT-producing and CPT-nonproducing plants suggests that mutations in Topo1 occurred before the CPT-producing enzymatic machinery appeared.[56] In other words, CPT was 'planned' taking advantage of a previous biochemical enzymatic unicity. Since the evolutionary history of most natural products is unknown, we lack information on the molecular milieu and the temporal range in which their production evolved. In this context, a theory alternative to surreptitious complementary has been developed to explain why animals have receptors for compounds produced by plants and, conversely, plants produce compounds for receptors they lack. According to the vestigial receptor hypothesis,[34] receptor–ligand pairs evolved in primitive organisms that predated the divergence of animals and plants. Since then, organisms that then evolved into plants lost the need of receptors and maintained that of ligands, while those that evolved into animals retained receptors but lost the need for their ligands. In this context, it is instructive to ponder that many compounds involved in cell signaling in plant cells have receptors also in animals, such as abscisic acid (**15**),[57] salicylates,[58] and genistein (**4**).[59] While being necessarily vague and simplistic, evolutionary observations and hypotheses on the function of natural products can nevertheless have interesting implications for drug discovery, as shown by the adaptation strategies developed by plants and microorganisms to overcome self-poisoning from their own metabolic products.

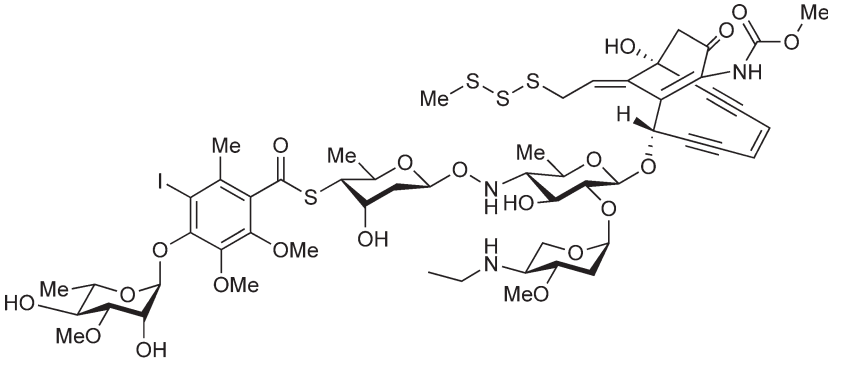


14



15

As already discussed with CPT, organisms accumulating inhibitors of basic biological processes like mitotic poisons (taxanes, *Vinca* alkaloids, colchicine) and antibiotics, must also have evolved strategies to avoid self-poisoning. These involve, apart from compartmentalization, the expression of export pumps, of antibiotic-modifying enzymes, and of target-protecting mechanisms that can mimic the molecular bases of drug resistance in the clinical setting.[60] Thus, antibiotics are produced to create, by disrupting basic biological processes, a local protective environment inhospitable to invading organisms. Clearly, antibiotic-producing microorganisms must have evolved self-resistance mechanisms prior to the production of antibiotics and, indeed, horizontal gene transfer from nonpathogenic bacteria, the major source of antibiotic resistance, has been ultimately traced to the antibiotic-producing organisms themselves.[60,61] In other words, acquired antibiotic resistance among dangerous bacterial pathogens, an increasing medical problem, relies on mechanisms primed at the stage of antibiotic production. Just like with CPT-producing plants, also in antibiotic-producing *Actinomycetes*, protection must have evolved before the production of active metabolites. A possible mechanism has been dubbed 'feed-forward' and involves inactive precursors that act as signal to prepare the organism for the later buildup of toxic levels of antibiotics.[61] More sophisticated mechanisms of self-resistance to 'endogenous' secondary metabolites have also been discovered and they might afford interesting mechanistic clues to the development of clinical resistance to them. The enediyne anticancer antibiotic calicheamicin (**16**) is an interesting case. This agent is a real 'chemical nuclease' that fragments DNA via a cycloaromatization-induced radical mechanism.[42] Because of its extraordinary toxicity, in the femtomolar range for some organisms, calicheamicin is used in the clinics only as a monoclonal antibody conjugate (Mylotarg) and the mechanism of self-resistance to this toxin has long remained a mystery. Enediyne-binding proteins (chromoproteins) stabilizing these highly unstable compounds and possibly aiding in self-protection have been detected in some ene–dyne-producing microorganisms but not in *Micromonospora calicheamicensis*, the source of calicheamicins. Recently, a gene (*calC*) conferring *in vivo* resistance to calicheamicin has been characterized in the genome of *M. calicheamicensis*.[62] The encoded CalC protein turned out to be a stoichiometric self-sacrificing agent against calicheamicin-induced double strand DNA scission, capable of quenching activated calicheamicin through a direct and specific hydrogen abstraction that mimics the action of the antibiotic on DNA. The production of CalC, along with a tightly sequestered biosynthesis and exportation system, makes it possible to escape self-toxicity from this agent and similar self-sacrificing strategies might underlie the development of resistance to enediyne also in nonproducing organisms, including cancer cells.



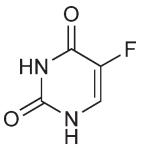
16

The examples we have described highlight how closely related genomics, metabolomics, and ecology are and how information from this fertile interface can have far-reaching clinical implications. Indeed, the evolutionary study of natural products can afford interesting clues for drug discovery in terms of both mechanism of activity and resistance to it.

3.08.3.3 Structural Bases for the Biomedical Relevance of Natural Products

The chemical complexity and diversity of natural products supercedes anything pharmaceutical companies or chemists can design, making it possible to explore areas of the biological space that are difficult, or downright

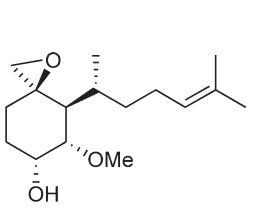
impossible, to access with compounds obtained by random synthesis campaigns. Thus, a statistical investigation of the structural complementarity of natural and synthetic compounds showed that 40% of the chemical scaffolds of natural products are absent in synthetic compounds.[63] Organic synthesis has evolved rapidly and nowadays, given enough students and funds, most natural products are within the reach of synthetic preparative chemistry. However, simple statistic considerations make it very unlikely that the constitutional and configurational structural subtleties of natural products might evolve *ex novo* from a random synthetic program. The chemical space is in fact huge but fundamentally void in biological terms. Most small biomolecules are made only by four elements (carbon, hydrogen, nitrogen, and oxygen) and have molecular weight (MW) <500 Da, while biological peptides are based on only 20 building blocks and have an average of 300 amino acids. Even with these structural constraints, the number of possible structural choices (permutations in the statistic lingo) is appalling. Thus, the number of small molecules having MW <500 Da and based on the four most common biological elements (C, H, N, and O) is 10^{60}, while the number of 300 amino acid-long linear sequences based on the 20 proteogenic amino acids is 10^{360}.[63] Life has therefore been extremely selective in molecular terms, since less than 18 000 natural products are known and, as we have seen, the human genome contains sequences of less than 100 000 proteins. Most synthetic chemical compounds are therefore biological chuff and, without opportune clues, it is extremely difficult to enter the highly selective biological space of natural products with compounds derived from a random synthetic campaign. An apparent exception is the anticancer drug 5-fluorouracil (5FU, **17**), which was synthesized over 40 years before being actually described as a natural product.[64] However, its synthesis was inspired by the RNA base uracil and was not the result of a program of random synthesis.[65] Considerations of this type, better than the much-hyped similarity between the drug space and the natural products space, support the view that natural products are special tickets in the drug discovery lottery. Since natural products have been the major source of chemical diversity for drug discovery, the drug space was filled mostly by research programs based on endogenous small molecules (neurotransmitters, hormones) or natural products and therefore this convergence might, in principle, be viewed as the mere result of past strategies of drug discoveries.



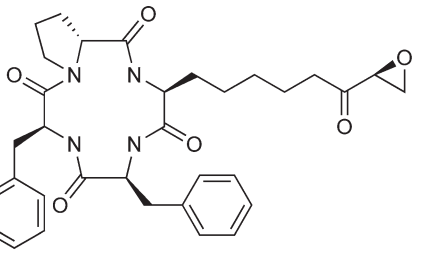
17

Synthetic libraries are straightforward to assemble but the relatively limited number of synthetic reactions and building blocks amenable to combinatorial strategies means that the resulting compounds often lack the structural complexity and diversity required to efficiently explore the biological space. For instance, rigid molecules occur rarely in combinatorial libraries since they are more difficult to assemble and the higher number of rotatable bonds means that conformational constraint, often an affinity booster, is missing from these libraries.[63] Indeed, combinatorial libraries are mainly based on flat structures. These, while very useful to explore ATP mimics and kinase ligands, are much less suitable to discover agents capable of interfering with complex processes like protein–protein interactions. Finally, synthetic libraries are often biased by the requirements of previous focused drug discovery programs and/or by the compliance to certain predefined criteria like the Lipinsky rule of 5 (RO5)[63] and the absence of reactive functional groups like epoxides, furans, and β-unsubstituted enones. Many bioactive natural products violate RO5 and feature 'undesirable' reactive groups. Thus, the alkylation of an epoxide moiety is essential for the bioactivity of important drug leads, such as the antiangiogenic methionine aminopeptidase2 (MetAP-2) inhibitor fumagillin (**18**),[66] the histone deacetylase (HDAC) inhibitor trapoxin (**19**),[67] and the exquisitely selective proteasome inhibitor epoxomicin (**20**),[68] while the anti-inflammatory agent triptolide (**21**), a putative transient receptor potential canonical (TRPC) ligand,[69] features a lineup of three adjacent epoxide groups. Other reactive, more exotic, and still biologically critical functional groups can occur in natural products drug leads, as exemplified by the hydroxamic HDAC inhibitor trichostatin A (**22**),[70] the β-lactone proteasome inhibitor lactacystin (**23**),[71] and the diazo derivative kinamycin (**24**).[72] Given a suitable molecular framework, reactive functional groups can indeed be implanted in drug leads

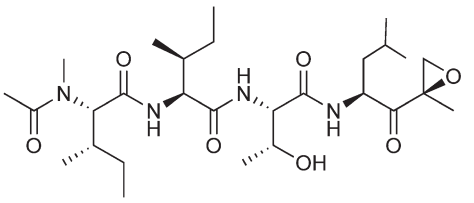
but this operation is forbidden under current 'rational' drug discovery rules, which, by doing so, precludes the exploration of a significant portion of the biological space. Finally, structural complexity aids specificity and potency in biological interactions, limiting target promiscuity,[73] while diversity is important to broaden the chemical space 'interrogated' during the biological evaluation.



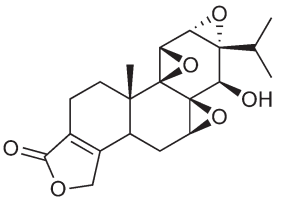
18



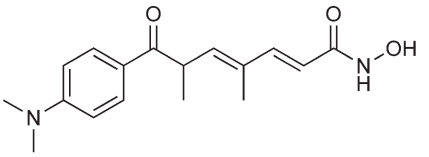
19



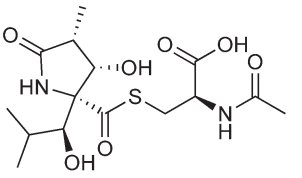
20



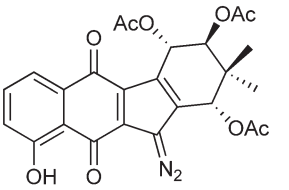
21



22



23



24

A host of molecular, evolutionary, and structural reasons therefore underlie the idea that natural products, as evolutionarily selected ligands to structurally conserved but genetically mobile protein domains, represent the most validated starting point to explore the druggable section of the chemical space. This view, while amply shared within the drug discovery environment, is nevertheless difficult to explicitly translate into numbers since structural diversity is hard to assess objectively with measurable parameters.[74] Even using simple structural elements, natural products are clearly differentiable from combinatorial chemistry products in terms of MW (414 versus 393), number of stereogenic centers (6.2 versus 0.4), and cycles (4.1 versus 3.2) and they incorporate fewer nitrogen, halogen, and sulfur atoms but are more rich in terms of oxygen and are sterically more complex, with more rings and bridgehead carbon atoms.[74] Based on these observations, a topological analysis of combinatorial libraries and natural products showed that combinatorial compounds densely populate a small area of the chemical space while natural products are more largely distributed in terms of occupancy of chemical space and are more diverse.[74]

3.08.4 Possible Reasons for the Current Downsizing of Natural Products Drug Discovery

> The pharmaceutical industry has a conception of the format through which future discoveries will be made, and natural products are not on their radar. The mavens (sic) of the pharmaceutical industry seem to think that a discovery made outside that format can't be worth much. Some of these guys would have turned down the

> Gettysburg Address because it was handwritten by an aging single author rather than turned out by some pricey word-manufacturing institute that hit upon it by chance.
>
> S. Danishefsky, *Chem. Eng. News* 13 October 2003, p 107

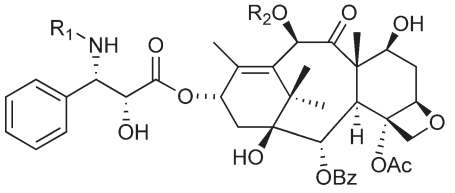
The exquisite biological specificity of natural products has laid the foundations of modern medicine. Natural products save millions of lives every year and generate annual commercial sales of billions of dollars to their developers and discoverers. Undoubtedly, natural products represent the most successful and validated strategy of drug discovery and it has been calculated that 60% of the current drugs are natural products, derivatives of natural products, or synthetic analogues of natural products.[75] Furthermore, high-speed approaches of fractionation and structure elucidation based on hyphenated techniques[76] have considerably expedited the construction of natural products libraries, alleviating the burden of dereplication.[77] Interestingly, some of these techniques, such as automated high-performance liquid chromatography (HPLC) separation with fast gradient elution, were originally developed for the production of combinatorial libraries.[78,79] Overall, the processing time of crude extracts has been substantially reduced through pretreatment, automated separation, and computer-assisted structure elucidation. In particular, iterative automated fractionation makes it possible to detect minor compounds once below the threshold of chemical and biological revelation and therefore inaccessible.[78,79] The structural complexity and the diversity of secondary metabolites are also ideal to fill the gap between the growing number of drug targets disclosed by genomics and the dismally low number of specific ligands available for them.

Nature is undoubtedly the largest and most diverse combinatorial library available but unlocking it is far from trivial since it requires multidisciplinary expertise, is more time consuming and costly than most current drug discovery approaches, and poses problems unfamiliar to corporate culture. It is therefore hardly surprising that, over the past two decades, drug discovery has gradually, and probably myopically, prematurely dismissed research into natural products as an old-fashioned delivering tool, investing instead in nonvalidated surrogates of biodiversity like combinatorial chemistry. The profitability of natural diversity to provide templates for drug discovery has been questioned and bioprospecting has lost out to high-throughput drug discovery, a process that relies on combinatorial chemistry and computational drug discovery. Thus, in 2008, Merck cut its natural products program entirely, despite a long and successful history in this area (lovastatin, caspofungin acetate) and, within the major pharmaceutical companies, only Novartis and Wyeth retain natural products research divisions with activities that go beyond the semisynthesis of antibiotics and steroids. Remarkably, less than a decade ago, it was still possible to claim that "most major pharmaceutical companies maintain important efforts in natural products' research."[80] The demise of natural products in drug discovery is even more surprising when one considers that, within the 15 small-molecule drugs approved by Food and Drug Administration (FDA) in 2007, six were natural products or derivatives of natural products.[81] This ratio is somewhat skewed by the inclusion, within the count of NCEs, of three 'old' drugs (azithromycin, topotecan, and temsirolimus) for which new indications were approved but is nevertheless remarkable. Clearly, there must be solid reasons for the pharma industry to abandon the beaten track of natural products and adventure into remote areas of the chemical space to discover new drugs.

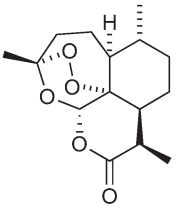
On the other hand, natural products isolation is also becoming a rare area of interest in the US and European universities. Natural products have long been pursued in academia only because of their unique chemical structure but, over the past decades, the focus has shifted on the discovery of their properties and the academic demise of natural products isolation is therefore unjustified. The academic woes of Georg Pettit, a hero of natural products drug discovery, exemplify this trend.[82] Just like taxonomy, natural products are also becoming extinct in academia, serving mostly as models for total synthesis. The shift in focus from isolation to synthesis is ironical at an age when more and more emphasis is placed on applied research since synthesis is often pursued essentially as a training ground for Ph.D. students and lacks practical application.

The transition from natural products to synthetic drugs was preceded by that from pharmacognosy to medicinal chemistry. Indeed, by the 1940s, heroic plant drugs like opium, cinchona, ipecacuhana, and mayapple had already been replaced by their isolated active constituents (morphine, quinine, emetine, and podophyllotoxin). Since the activity of most medicinal plants, even as popular as valerian and chamomile, could not be traced to a single constituent amenable to pharmaceutical development, their gradual downsizing to the health food realm was inexorable, while, in the wake of the wartime efforts to produce penicillin, large fermentation programs of drug discovery were launched, filling up the dwindling pipeline of plant natural products drugs.

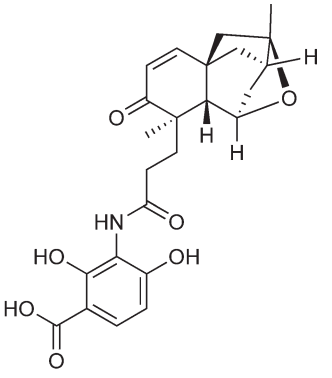
Despite several decades of success, in the past two decades microbial natural products research has also been de-emphasized in pharmaceutical companies and considered as overexploited and poised to fail to significantly enrich the yield of pharmaceuticals. Natural products are no longer considered a focal point for hit discovery, making up for only a tiny percentage of the compound archives of large pharmaceutical companies, typically comprising up to 5 million compounds. Alternative opportunities to discover novel low MW leads have taken over and drug discovery is nowadays associated with dazzling new concepts and technologies, such as functional genomics, combinatorial synthesis, structure-based ligand design, and ultrahigh-throughput screening (UHTS, >100 000 compounds per week).[20] Conversely, and despite noteworthy methodological evolution, the strategy to develop new natural products drugs has instead remained the same, involving primary screening of crude extracts, bioassay-guided fractionation, dereplication of active compounds, and isolation and structure elucidation of new bioactive constituents. Furthermore, despite the current success of natural products drugs, there is no recent record of plant-derived drug discovered by big pharmaceutical companies and modern successful plant drugs like paclitaxel (**25a**),[83,84] CPT (**14**),[84] and artemisinin (**26**)[85] originated from publicly funded research. After the success of rapamycin, chalicheamicin, tacrolimus, and epothilones, the pipeline of fermentation products also seems to have become arthritic and doubts linger if platensimycin (**27**), a lipid biosynthesis inhibitor and the most notable antimicrobial agent discovered in the past few years,[86] will ever be developed by Merck.



	R_1	R_2
25a	Bz	Ac
25b	Boc	H

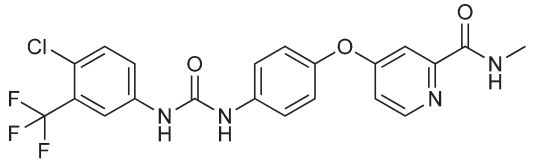


26

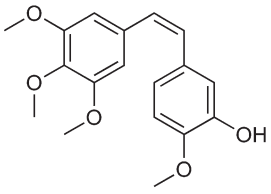


27

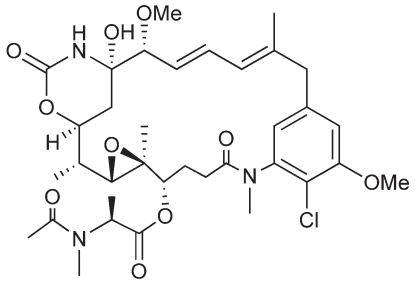
Conversely, several unsuccessful case histories of natural product-based drug discovery projects have been reported. Thus, in the early 1990s, Merck paid $1.14 million to InBio, a Costa Rican conservation group, to screen rainforest species (plants, insects, and microorganisms) for novel chemicals of interest for drug discovery.[87] Nothing useful apparently came out of this project, which was terminated in 1999. In those years, Shaman Pharmaceuticals, a company founded in 1989 to develop modern drugs from traditional medicines, went as far as late-stage clinical trials for an antiviral plant extract but then went bankrupt,[88] while the 1970s–1990s had witnessed the Herculean efforts of National Cancer Institute (NCI) to discover new natural products anticancer drugs. The program was terminated after the screening of 114 000 extracts originating from 35 000 plant samples representing 12 000–13 000 species had apparently failed to produce a single natural product-based anticancer drug.[89] For the sake of comparison, we can mention that the discovery of the kinase inhibitor sorafenib (**28**) by Bayer involved the screening of a combinatorial library of 200 000 compounds, followed by the parallel synthesis of 1000 further analogues and was accomplished in only 4 years.[90] However, since sorafenib is the first, and so far the only, drug emerging from the screening of combinatorial libraries, it is not clear how general for modern drug discovery its genesis is and if its success can be considered as a real validation of combinatorial chemistry as a delivering tool.[91] Furthermore, the clinical potential of many leads discovered during the NCI campaign was realized only after its termination. Thus, CPT derivatives and paclitaxel were introduced into the clinic almost three decades after their discovery, while a host of compounds from the original NCI program are being developed only now, such as combretastatin A4 (**29**)[92] and maytansine (**30**).[93]



28



29



30

Of great relevance to the current demise of natural products is also the growing trend toward a more rational approach to drug discovery, where ligands are designed *ex novo* or assembled by fragments and where emphasis is apparently placed on design rather than on discovery. Natural products research is also intrinsically multidisciplinary and requires the combined efforts of natural products chemists, pharmacologists, and medicinal chemists. These expertises are difficult to coordinate and focus and require a highly trained team, difficult to assemble in a pharmaceutical scenario characterized by a high personnel mobility.

For these reasons, there is no doubt that, compared to synthetic libraries, natural products and extract libraries pose a host of problems, which, albeit all singularly soluble, nevertheless conjure up to provide an overall unattractive scenario for big pharma. The future of natural products in drug discovery and their promotion to full potential will therefore critically depend on how well and quickly these issues will be solved, making the process less time and resource intensive.

3.08.4.1 Intellectual Property Issues

Natural products cannot be patented as a structure but semisynthetic analogues can, as well as their uses, isolation/manufacture processes, and specific drug formulations, although intellectual protection is obviously weaker.[94] Furthermore, the technological asymmetry between biodiversity- and technology-rich countries has raised considerable and still unresolved proprietary issues on natural products drugs.[95] According to the UN Convention on Biological Diversity (CBD), countries have sovereign rights over the biological resources within their boundaries and should establish conditions for the preservation and sustainable use of their biodiversity.[96] Source countries should be involved in projects related to their biodiversity and should share any commercial benefit resulting from its use. These claims are objectively difficult to translate in terms of pharmaceutical intellectual property (IP), whose reinforcement is, nevertheless, essential to contribute to the local development of resources and to make prospecting an engine for biodiversity conservation.[97] As a result of this ambiguity, many countries have placed barriers on the exporting of biological materials, even for noncommercial researches. CBD is not retrospective and therefore the examples of earlier natural products discovery that failed to produce commercial rewards to the source country lack legal meaning. Although the political debate on biopiracy is colored with examples from developing countries (rapamycin from Easter Island, teprotide, on which the angiotensin converting enzyme (ACE) inhibitors were molded, and tubocurarine from Brazil),[98] there are far many examples from developed countries, such as cephalosporin, rifampicin, daunomycin, and mycophenolic acid from Italy, cyclosporin A from Norway, or paclitaxel from United States, just to mention a few examples.[99]

3.08.4.2 Access to Natural Chemical Diversity

Gaining access to biodiversity from natural habitats is legally complicated, especially for broadscale corporate campaigns involving the collection of hundreds, or even thousands, of species. Developing societies that possess

important biodiversity and developed societies endowed with advanced technologies should interact on the basis of a principle of equity but, as we have seen, it is difficult to specify how.[97,98] In principle, countries with rich biological resources should be able to charge companies for bioprospecting for either drugs or genetic information that could lead to new drugs but legally binding formulas to control this 'trade' are difficult to conceive and to implement. In 2002, countries signatory of the Rio Biodiversity Convention agreed on a set of rules (Bonn Guidelines on Access and Benefit Sharing) that specify how each country should frame licenses to allow companies to access natural resources but this arrangement was fiercely opposed by many environmental and economic organizations. Thus, Jeremy Rifkin, who heads the Foundation on Economic Trends claimed that "nobody has the right to enter into exclusive deals over the products of millions of years of evolution."[100] Political sensitivity regarding access to biodiversity from developing countries is undoubtedly one of the reasons underlying the phasing out of natural products from big pharma.

Given the current downsizing of basic natural products research in drug companies, it is likely that many, if not most, of the natural products drugs of the future will originate from publicly funded research, via government organizations and academic institutions, or from venture capital small biotech companies. Taxol (paclitaxel, **25a**) was the first anticancer drug to reach a billion-dollar yearly sale and the NCI–BMS deal on the pharmaceutical development of this compound was undoubtedly highly profitable from a corporate viewpoint.[101] It was also vociferously criticized, in particular for the hijacking of the taxol name from BMS. On the other hand, the deal made rapid access to this drug possible and its price sank after it acquired a generic state. Aventis (then still Rhône–Poulenc Rhorer) developed a semisynthetic analogue of the natural product (docetaxel, **25b**) that enjoyed a longer protection status and topped the list of best-selling anticancer drugs for several years.[101] Compared to natural products, synthetic compounds undoubtedly enjoy the advantage of clarity in terms of IP but the development of taxane anticancer drugs provides an important example of how the intrinsically difficult IP protection issues associated with natural products can be solved in a pharma-profitable way. Furthermore, NIH has sponsored several bioprospecting projects of International Cooperative Biodiversity Groups (ICBG) that combine academic and industrial groups and that might serve as models for programs that combine drug discovery, conservation of environmental and genetic resources, and the establishing of sustainable economic activities.[102]

3.08.4.3 The Biodiversity Crisis

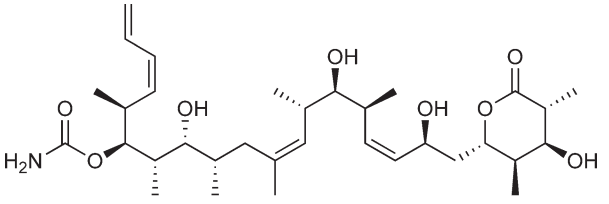
The prospect of turning natural resources to practical use has fuelled human activity for thousands of years and biodiversity is one of the most valuable but least appreciated natural resources. Some of the most important wonder drugs came from organisms not usually associated with healing, such as poisonous plants and animals, and every species has the potential to teach us something new. The calculated yearly loss of 20 000 living species therefore means that thousands of undiscovered and unique chemicals with potential utility will be lost forever, along with the genetic information necessary for their assembly.[103] The current inventory of biodiversity is very incomplete and will undoubtedly be enlarged as exotic regions and habitats are studied, with marine ecosystems being the largest unexplored habitat of life. We are monumentally uncertain as to how many living species there are on earth and even the tenet that most of the higher terrestrial plants have been discovered has been hotly debated. It has been estimated that there are at least 250 000 species of higher plants, 30 million insect species, 1.5 million species of fungi, and a similar number of algal and prokaryote species.[104] The uncertainty on these number is big since it has been reckoned that over 50 000 plants are still waiting to be discovered.[104] Whatever the case, only a fraction of the plants known have been investigated chemically or for a specific bioactivity and many of them were investigated decades ago with relatively crude techniques. Considerable disagreement also exists as to where plant biodiversity is most concentrated, although a certain consensus exists that the richest regions in terms of flowering plants are the Amazon basin, Southeast Asia, and the Mediterranean region.[105] Owing to difficulties in cultivation, only a tiny percentage of bacteria and fungi are known (12 and 5%, respectively) and most insects and nematodes living are still undiscovered.[105]

Translating biological diversity into chemical diversity has long been the aim of phytochemistry and represents the first step toward a rational utilization of bioresources. Modern developments in separation techniques and spectroscopy have expedited the 'molecular cataloguing' aspect of phytochemistry, just like technological advances and genomics have simplified the work of taxonomists. Nevertheless, and paradoxically,

both taxonomy and phytochemistry are facing a lack of academic prestige and resources that is crippling the cataloguing of biological and chemical diversity just at the time when it has become most urgent. Taxonomy and phytochemistry are enabling sciences. They do not generate new ideas or test hypotheses but make it possible to open new areas of research and translational sciences, with clearly achievable and relevant goals, which are favored by the current funding system. Thus, a project on the health benefits of a certain diet will surely have more chances of being funded than one on the phytochemistry of the plants on which the program is based.

3.08.4.4 Supply Issues

Biodiversity can be lost by natural catastrophes (fires, eruptions, diseases) or by human activity and is basically less reliable than oil-derived feedstocks to secure continuous access to a product. Indeed, resupplying of an active extract is a major drawback in natural products drug discovery and the lack of a backup sample can cause substantial delay, especially for nonfermentable biomasses like plants and marine organisms while many nonmicrobial natural products cannot be produced in bulk by isolation or can be produced only after considerable efforts, as exemplified by the development of paclitaxel (**25a**) as an anticancer agent.[101] Furthermore, many nonmicrobial natural products cannot be produced in bulk by isolation. In general, the reliability of marine feedstock as a bulk source of natural products has often been questioned, being much less than that of plants and microorganisms, and the belief that marine-derived natural products may furnish better opportunities to synthetic chemists than to medicinal chemists is still rife. The incredible ordeal represented by the 60 g synthesis of discodermolide (**31**), a marine biological analogue of paclitaxel (**25a**), gives credit to this idea. Discodermolide was prepared for preclinical studies by a total synthesis that required the combined efforts of more than 43 Novartis chemists to produce 60 g of product in an overall yield of 0.65%.[106] While a commercial chemical synthesis of discodermolide is still elusive, there is strong evidence that many marine secondary metabolites are actually of microbial origin and that the marine source simply represent a macroscopic host for the microbial colony actually producing the compound of interest.[107] Thus, it has been firmly established that tetrodotoxin, possibly the most famous marine natural product, is actually a microbial compound and that the puffer fish only accumulates it, using it as a hormone.[108] On the positive side, several techniques have been developed to gain access to natural products or natural products-like compounds, in nonnatural ways, with plant tissue cultures and combinatorial genetics being the most investigated 'rain forest' surrogates in terms of availability of natural products.[107]



31

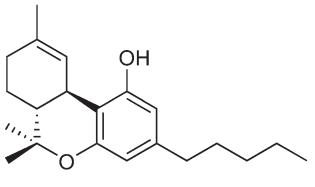
3.08.4.5 Methodological Issues

The *de novo* construction of libraries of pure natural products is prohibitively costly compared to synthetic libraries but a very big library of pure natural products (>10 000 compounds, >85% purity) has been assembled by the German biotech company AnalytiCon Discovery and made commercially available, unifying, in terms of the internal logistic of pharmaceutical companies, the screening of natural products and that of synthetic compounds.[109] However, libraries of crude extracts rather than pure compounds are typically screened in natural products-based drug discovery campaigns. Screening extracts in both biochemical and cell-based assays is operatively similar to screening libraries of synthetic compounds but the readouts are plagued by factors that occur more rarely in synthetic libraries and there is therefore great interest in the production of 'assay-friendly' libraries of extracts.

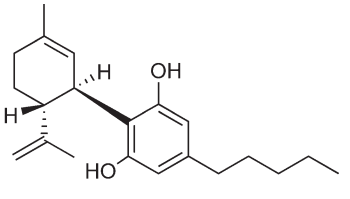
3.08.4.5.1 Entourage effects

The isolation of morphine from opium in 1805[110] was the first demonstration that the activity of a medicinal plant could be attributed to a single chemical constituent, initiating natural products chemistry and the search for similar 'quintessential' principles in other medicinal plants. This approach was successful only for highly active or poisonous medicinal plants (heroic drugs) while the activity of the majority of medicinal plants could not be traced to a single constituent (magic bullet).

There is now growing awareness that the activity of most medicinal plants is the result of the synergistic action of several constituents (magic shotgun).[111] These concepts were deftly exploited to develop Sativex, a combination of two strains of *Cannabis* characterized by a high contents of tetrahydrocannabinol (THC; **32**) and cannabidiol (CBD; **33**), used to relieve the symptoms of multiple sclerosis and which is also under clinical development for the treatment of cancer pain.[112] CBD, long considered pharmacological ballast, shows anti-inflammatory activity and modulates the psychotropic effects of THC via its CB1 reverse agonism and by interfering with the hepatic 11-hydroxylation of THC, which increases the brain penetration of this psychotropic compound.[113] The 'entourage effect' has been a deterrent for the mainstream and reductionist pharmaceutical exploitation of medicinal plants. In other words, extracts of natural origin are complex systems and we do not know how much we can simplify (fractionate) them and still have them functioning. Chronic degenerative diseases like cancer and Alzheimer's disease are multifactorial and mixtures of compounds, or compounds with a pleiotropic mechanism of activity, are in principle more useful to treat these diseases than a single compound. Indeed, cancer and HIV are treated with cocktails of drugs and not with a single agent, while synergistic combination drugs like Augmentin, an association of a β-lactam antibiotic and a lactamase inhibitor, have been developed. Nevertheless, synergies are better deduced than planned and entourage effects are unmanageable in mainstream, magic bullet style, drug discovery campaigns.



32

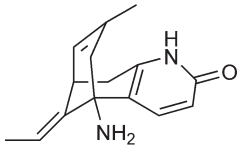


33

3.08.4.5.2 False positives/negatives and reproducibility

False positives can originate from various causes, such as nonspecific hydrophobic binding, poor solubility, the tendency to form aggregates, or the presence of denaturing agents (tannins), pigments, fluorescent compounds, nonselective and widespread ligands like linoleic acid, or functional groups that react in a nonspecific way with protein targets (aldehydes, epoxides, and Michael acceptors).[114] All these issues are more severe in extracts than in synthetic libraries, where hydrophobicity, solubility, and presence of reactive functional groups and color can be minimized at the planning stage. Conversely, extracts are generally characterized by a total lack of information on their molecular composition and, in this sense, they are black boxes. False negatives might originate from a too low concentration of an active compound in an extract, its chemical instability, the interferences with the assay readout, and/or the presence of compounds with opposite activity. Again, these issues are nonexisting or rare in synthetic libraries. Extracts are intrinsically 'dirtier' than synthetic libraries but can be cleaned by prefractionation, an operation that minimizes most of the false positive issues and increases the concentration of constituents, therefore improving the detectability of trace constituents. Several methods to remove tannins, protein-precipitating agents, and reactive chemicals from plant extracts have been developed.[115,116] False negatives might also originate from the presence of compounds with opposite bioactivity and some potent natural products could probably never have been discovered using modern HTS campaigns. Thus, fiber cannabis contains THC, a cannabinoid agonist, but also CBD, a cannabinoid reverse agonist that is much more abundant than THC.[113] Another case is *Lycopodium* extract, which, despite containing the very powerful nicotinic agent huperzin A (**34**), also contains anticholinergic compounds with, overall, little, if any, cholinergic activity.[117] Clearly, the interrogation of a novel target with a high-throughput campaign based on natural products extracts might well fail to produce any useful results, since few bioassays are robust enough to

withstand the screening of complex mixtures and previous prefractionation is therefore necessary. This operation of molecular simplification limits the possibility of false positive and negative but it is undoubtedly labor intensive, time consuming, and costly. Finally, reproducibility of activity and/or composition is often an issue, being observed in approximately 40% of plant extracts as a result of differences in geography and time of plant collection, or of the presence of microbial elicitors of the production of secondary metabolites.[118]



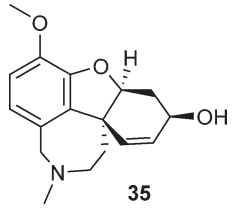
34

3.08.4.6 Dereplication

Natural product-based hit discovery campaigns suffer from a complete lack of information on the composition of the compounds to screen and assays are *per se* incapable of distinguishing between known and novel compounds. Dereplication, the identification of known compounds responsible for the activity of an extract before bioassay-guided fractionation,[119] is therefore important before screening, at least in campaigns aimed at the identification of structurally novel ligands. It is therefore possible, at least in principle, that obvious ligands are 'rediscovered' in any nondereplicated phytochemical screening. For instance, GABA is widespread within plants and its presence interferes with assays of GABAergic activity, masking the presence of both GABA inhibitors (false negative readout) and GABA mimetics (false positive readout).[120] To minimize this problem, the NCI has developed a dereplication strategy based on HPLC fractionation with diode array detection, collection of fractions into 96-well microtiter plates, and preparation of daughter plates for either biological testing or mass spectrometry–electrospray ionization (MS–ESI) detection.[121]

3.08.4.7 Advent of Combinatorial Chemistry and Progress in Synthetic Chemistry

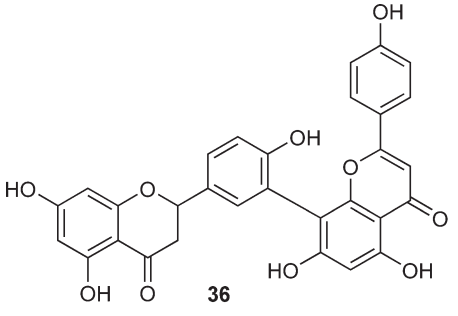
The rapid identification of protein, DNA, and RNA pharmaceutical targets has driven the need for easily prepared, chemically diverse, and target-specific small-molecule ligands.[122] HTS and combinatorial chemistry have emerged to meet this need. HTS, whose flow rate far exceeded the capacity of standard proprietary libraries, predates combinatorial chemistry and spurred its development. The design and synthesis of combinatorial libraries have focused mainly on functional group variation within members of the library, with, at least at the beginning, little, if any, stereochemical or skeletal diversification.[123] Considerable advances have been achieved in the past years in terms of purity and structural diversity of combinatorial libraries, which, however, remain dismally inferior to natural products in terms of diversity. Since it is nowadays accepted that biological relevance and chemical diversity are more important than the library size, several groups have been involved in the development of natural products-like libraries based on the combinatorial elaboration of scaffolds inspired by natural products.[123] Current pharmaceutical research needs increasingly larger number of compounds spanning as many molecular architectures as possible and phytochemical techniques minimizing manipulation and purification steps must be developed. Clearly, no magic techniques of high-throughput isolation exist and, despite all the impressive progress in isolation and structure elucidation techniques, natural products libraries will never be competitive in terms of availability and rapidity of assembly with synthetic libraries. At the same time, progress in synthetic chemistry and the spiraling of drug prices have made it possible to produce by total synthesis drugs that rival the complexity and polyfunctionalization of natural products. The anti-HIV drug enfuvirtide (Fuzeon) is a remarkable example. This 26 amino acid peptide is not produced by Roche recombinantly in engineered cells but by total synthesis, with an investment that led to a worldwide overall cost lowering of all peptide synthesis reagents, starting materials, and equipment.[124] Complex natural products like huperzine A (**34**) and galanthamine (**35**) are nowadays competitively produced by synthesis rather than by isolation,[125] and the enormous progress of the past years in synthetic methodologies and efficiency have undoubtedly made synthesis a rival of isolation for both the discovery of new drug hits and the production of bioactive natural products.



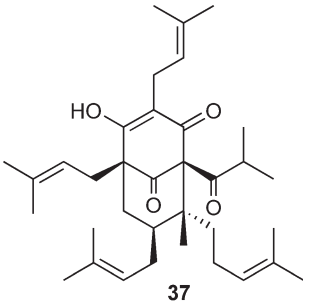
35

3.08.4.8 Poor Relevance to Noncytocidal Targets

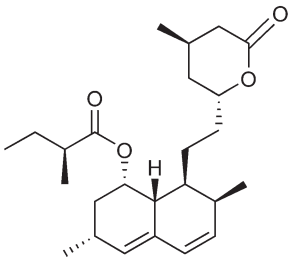
Since natural products are essentially chemical weapons, natural product-derived drugs are preeminent in the field of oncology and anti-infective therapy,[126] while chances to identify natural products leads in screening for other activities (cardiovascular, neurological, and metabolic) is undoubtedly weaker, since the source organism and human proteins did not coevolve. These low hit rates should, however, be compared to those of purely synthetic libraries and there is no shortage of examples of recent discoveries of new natural products leads and new natural product-related targets in hot areas of research like diabetes, metabolic diseases, and Alzheimer disease. A recent example is the identification of the dimeric flavone isoginkgetin (**36**) as a mechanistically new promoter of adiponectin secretion, an important antidiabetic target.[127] Adiponectin is a hormone secreted by adipocytes that increases insulin sensitivity and whose plasma level are low in diabetic and obese people. Screening of a library of drug-like synthetic compounds and natural products identified isogingketin, a constituent of gingko leaves, as a powerful inducer of adiponectin secretion, acting in a fundamentally distinct way compared to thiazolidinediones, and involving not peroxisome proliferator-activated receptor-γ (PPAR-γ) but rather AMP-activated protein kinase (AMPK).[127] Regarding the natural product-inspired discovery of new targets, a recent example is the identification of TRPC6 as the antidepressant target of the phloroglucinol hyperforin (**37**).[128] This constituent of St. John's Worth inhibits the neuronal reuptake of serotonin, dopamine, and norepinephrine, behaving as a functional biological analogue of synthetic antidepressants. However, hyperforin acts with a basically different mechanism, inducing sodium and calcium entry mediated by specific binding to TRPC6, a nonselective ion channel. Since neurotransmitter reuptake requires an efficient sodium gradient, its impairment translates into a decreased amine reuptake. The therapeutic areas of infectious diseases and oncology have undoubtedly benefited most from natural products but natural products have been successfully developed to treat human diseases in almost all therapeutic areas and it should be remarked that statins, the commercially most successful drugs ever, were molded on the microbial product lovastatin (**38**) (for more details on Natural Products of Therapeutic, see Chapter 2.19). In 2006 alone, the sales of statins were over 20 billion dollars.[129]



36



37



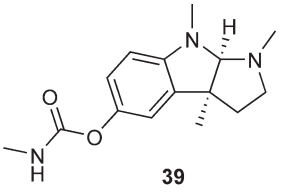
38

3.08.5 Strategies in Natural Products Drug Discovery

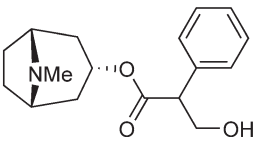
> How would penicillin have fared had the initial discovery occurred in 2007, in the absence of a clearly defined molecular target against which were screened a mind-numbing collection of low-pedigree samples, often of questionably purity?
>
> S. Danishefsky, *Chem. Eng. News* 13 October 2003, p 103

3.08.5.1 Ethnopharmacology

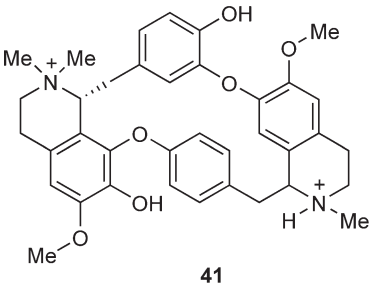
Traditional medicinal practices predate modern medicine by thousands of years. All indigenous populations have derived a pharmacopoeia unique to their environment and an enormous amount of information on the medicinal properties of plants, fungi, and animals exists in ethnic cultures.[130] By analyzing extensive databases of bioactivity such as the NCI list of 'active plants', it was calculated that plants with a traditional use in medicine were 2–5 times more likely to generate 'active (cytotoxic) extracts' compared to plants without an ethnopharmacological record.[131] Of special interest are poisonous organisms (plants, animals, mushrooms, and microorganisms), since their 'bad' properties can be potentially translated into successful therapeutic drugs.[132] Physostigmine (**39**), atropine (**40**), and tubocurarine (**41**) and botulinum toxin are important examples from the past and cyclopamine[133] (**42**) and conotoxins[134] from current research on poisonous organisms.



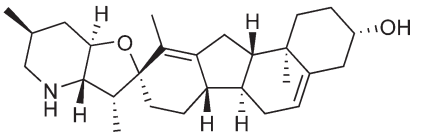
39



40



41



42

The use of medicinal plants in traditional medicine represents in principle a sort of preexisting clinical testing and a shortcut to biologically active compounds but the translation of enthobotanical knowledge into commercialized products is far from simple.[135] For one thing, many traditional medicines are not based on the Hippocratic principles of disease. Thus, traditional Chinese medicine (TCM) takes a holistic approach to treatment, emphasizing the balance and harmony of the human body. Central to its practice are concepts like *yin* and *yang*, primal and opposite forces, and the spiritual energy known as *qui*, whose block causes illness. These concepts cannot be translated into molecular terms and it is therefore hardly surprising that TCM has so far contributed so little to mainstream drug discovery.[136] Furthermore, while issues like claim validation and standardization can be addressed by current pharmaceutical expertise, others like sustainability of the source and ownership of the intellectual knowledge are unusual, or downright alien, to mainstream pharmaceutical corporate culture, as is the use of mixtures of compounds like extracts, or even of mixtures of extracts. These problems are no doubt exacerbated by the current pharmaceutical legislation, which is well suited to cope with monomolecular drugs or mixtures of active pharmaceutical ingredients (APIs) but is at a loss with complex active matrixes like extracts. For this reason, special channels have been devised in the US and European Union

pharma legislation to accommodate drug derived from 'evidence-based' ethnobotanical medicinal discovery.[137] Extracts, a fundamentally rudimental form of drug even in purified and standardized form for current pharmaceutical standards, represent an important area of drug discovery and the recent FDA approval of Veregen (polyphenon A), a standardized polyphenolic extract from green tea, for the management of genital papilloma warts represent an important example on a basically new type of natural products drug, which was approved without any evidence of mechanism of activity and on the basis of highly positive clinical results only.[138] Traditional knowledge is disappearing faster than biodiversity and many 'islands' of traditional knowledge remain to be investigated and will undoubtedly get lost forever with the current pace of globalization. The study of folk pharmacopoeias and ethnomedicine is the basis of the discovery of several important drugs and biological leads, as exemplified by digoxin, tubocurarine, ephedrine, atropine, and quinine.[139] Not only plant-derived compounds but microbial products also owe their origin to ethnopharmacology, as cogently shown by cephalosporins, whose discovery was related to the study of the so-called 'Cagliari paradox', namely the very low incidence of cholera in this Sardinian town despite the lack of a public sewage system and the habit of the inhabitants to take a bath in the polluted waters of the Su Siccu beach, later found by Brotzu to be sterile because of the presence of the antibiotic-producing mold *Cephalosporium acremomium.*[140] After their isolation by Brotzu in Cagliari, the development of cephalosporins as antibacterial agents was eventually carried out in England and their introduction into the clinic brought rich dividends to the National Research Development Corporation, a body set up in 1949 to exploit discoveries made by British universities and government laboratories.[141] The clinical translation of the original discovery by Brotzu required considerable efforts from both academy and industry but in the highly politicized context of bioprospecting, can also be perceived as a blatant case of exploitation of both tangible (genetic resources) and intangible (knowledge) indigenous resources.

While ethnopharmacology is undoubtedly an asset for natural products plant discovery, this approach has some obvious limitations, even under a Hippocratic medicinal context, since many diseases are ill defined in terms of symptoms. Thus, most cancers show little if any symptoms until the late stages of the disease and they are not specific. It is therefore difficult to translate ethnopharmacological information into clinical clues for cancer, despite a monumental attempt by Hartwell.[142] Even for diseases well defined in terms of symptoms, such as fever and malaria, traditional use might have missed important plants. A striking case is artemisinin (**26**). This antimalarial drug was discovered in a Chinese medicinal plant (*Artemisia annua* L.) that was substantially overlooked in terms of antimalarial use in the TCM.[136] Indeed, the Jesuit penetration in China in the seventeenth century was spurred by the healing of the Chinese emperor by *Cinchona*, the miracle antimalarial plant traded by Jesuits. Pure artemisinin is not orally available, although it was reported that a certain absorption takes place from crude extracts containing flavonoids,[143] and *A. annua*, even with all the limitations implicit in the translation of folklore indications into modern medicine, was not sufficiently emphasized as an antimalarial agent in TCM.[136]

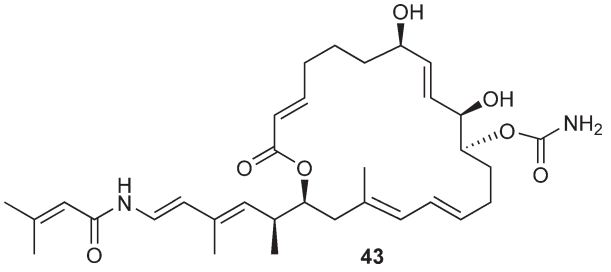
3.08.5.2 Ecology

The preservation of biodiversity goes beyond the simple cataloguing of living species but also involves the study of their physiology and the preservation of their relationships. Biodiversity is therefore strictly related to the conservation of a specific environment as a whole and it would be limiting to associate it to botanical herbaria, fungal collections, or aquaria. The study of the ecology of a species can afford interesting clues for drug discovery, as exemplified by exenatide (Byetta), a drug derived from a lizard venom and the first incretin mimetic introduced into the clinic.[144] The Gila monster (*Heloderma suspectun*), a poisonous desert reptile from the American Southwest and Northern Mexico, can withstand long periods of fasting, eating only 3 or 4 times a year. The physiological bases for this remarkable feeding behavior was traced to the presence of a salivary hormone (exendin-4) that slow down the digestion and the absorption of food.[145] Exendin-4, a 39 amino acid peptide, shows an approximately 50% analogy with glucagon-like peptide-1 (GLP-1), a hormone that increases the production of insulin when blood sugar levels are high. Exendin-4 is more potent than GLP-1 to enhance glucose-dependent insulin synthesis from pancreatic beta cells, to decrease glucagon production, and to slow down gastric emptying. Furthermore, exendin-4 has longer duration of action than GLP-1, with a half-life of

over 2 h versus less than 1 min for the human hormone, being resistant to enzymatic inactivation by dipeptidyl pepdidase-IV (DPP-1V). A synthetic form of exendin-4 (exenatide, Byetta) was approved by FDA in April 2005 for the control of type II diabetes in patients whose blood glucose cannot be controlled with oral diabetic agents (metformin, sulfonylureas, or thiazolidinediones) alone.[144] The wild population of Gila monster is declining rapidly due to habitat loss and illegal hunting for the pet trade. The project *Heloderma* has been established to save the Gila monster and related species from extinction and Eli Lilly, the company that commercializes Byetta, is making a charitable contribution to this project. Byetta is an interesting example of drug coming from a threatened species and whose clinical exploitation is actually helping its preservation. The limitation of the ecological approach to natural products drug discovery is that most targets of high-throughput screens are not easily translated into observable phenomena that can provide prospecting clues. Thus, while the observation of a fruit that does not rot can suggest the presence of antibacterial compounds, most drug targets cannot benefit from this type of observation.

3.08.5.3 Unconventional Natural Products Sources

Plants and microorganisms, especially *Actinomycetes*, are the most validated sources of natural products drugs, especially in consideration of the facility of their cultivation or fermentation. Even so, only a fraction of the known plants and microbial species have been investigated for their pharmaceutical potential and other biodiversity sources are still largely or completely unexplored and untapped.[146] In general, the taxonomic and geographical diversity of bioprospecting has constantly increased and now encompasses cyanobacteria, endophytic fungi, sponges, mollusks, seaweeds, insects, and amphibians. Particularly impressive is the bewildering variety of structurally unique natural products isolated from marine organisms, often with no counterpart in terrestrial organisms.[147] However, the difficulties of collection and scale-up of marine natural products are formidable, also because the identity of the actual biological producer is often unknown and its propagation in a commercial setting unpractical. Thus, it seems well established that, especially in sponges, the production of secondary metabolites is due to coexisting microorganisms, especially cyanobacteria, and not due to the their host.[148] The identification and fermentation of these marine microorganisms could represent a revolutionary twist in marine natural products chemistry, paving the way for the clinical exploitation of an area of the chemical space distinct from that of terrestrial natural products that has lagged far behind in terms of pharmaceutical exploitation essentially because of the lack of a sustainable supply.[149] Some environmental niches are still completely pristine in terms of bioprospecting, with Antartica being a preeminent example. Despite its harshness, this habitat supports a thriving community of invertebrates and algae that produce very interesting products, such as the polyketide palmerolide A (**43**) from the tunicate *Synoicum adareanum*.[150] Palmerolide A, so named from the Palmer Station on the Antarctic Peninsula in whose vicinity its animal source was collected, is a potent antimelanoma agent and a one-digit nanomolar inhibitor of V-ATPase, a vacuolar proton-translocating enzyme that acidifies organelles of both constitutive and regulated secretory pathways.[150–152] Extremophile microorganisms from a variety of inhospitable terrestrial and marine sources, such as acidic hot springs (acidophiles), alkaline lakes (halophiles), deep-sea vents (baro- and thermophiles), polar waters, and alpine lakes (psychrophiles) hold great promise. It is not unreasonable that, just like enzymes from extremophiles supported the discovery of PCR, also interesting drug leads might come from the study of their secondary metabolites.[153]

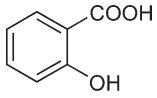


43

Apart from these exotic sources, it should also be pointed out that only a fraction of soil microorganisms can be cultured and have therefore been investigated for the production of secondary metabolites.[154] To get around this issue, genetic material coding for secondary metabolites can be obtained directly from the soil and expressed in a host organism. The secondary metabolites obtained so far from environmental DNA are rather similar to those produced by fermentable microorganisms,[155] but there have been only few studies of this type and more systematic investigations might lead to uncharted areas of the biological chemical space. Overall, there is no shortage of areas of the world and habitats where new and unusual chemodiversity can be discovered and the major limitation of these studies is that, since we know so little on the ecology of unconventional environments, there are no clues to select in biorational ways the organisms to study.

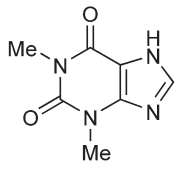
3.08.5.4 Edible Plants

Humans are daily exposed to a multitude of secondary metabolites contained in edible plants and spices. These compounds have accompanied us during evolution, playing a role in the shaping of our genome and making us not what we eat but rather what our ancestors have eaten.[156] Dietary secondary metabolites are not considered as nutrients but appear to play a role, still undefined in molecular terms, in the maintenance of health, and there is therefore great interest in their identification and in the characterization of their biological profile. Dietary compounds are the basis of the development of highly successful drugs, such as lovastatin (**38**) and salicylic acid (**44**), the archetypal statin and nonsteroid anti-inflammatory drugs, respectively. Lovastatin occurs in the red yeast of rice (*Monascus ruber*), an ingredient of Eastern cuisine used to give a red color to the Pekinese duck,[157] while salicylic acid is ubiquitous in plants.[158] Remarkably, the isolation of lovastatin from the dietary mold *M. ruber* was reported by Endo 1 year before Merck described its obtaining from *Aspergillus terreus*.[159] Other important dietary drug candidates are curcumin (**12**) from turmeric[160] and capsaicin from hot pepper (**13**),[54] while traces of pharmaceutical benzodiazepins (including diazepam) occur in common edible plants like potatoes and cherries.[161]

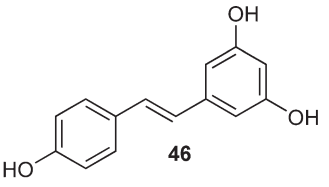


44

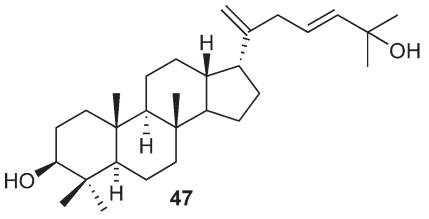
Dietary observations have afforded many clues to drug discovery. The antiasthmatic properties of theophylline (**45**), a caffeine metabolite and a minor constituent of tea, were discovered because of the improvement of breathing problems of asthmatic patients who consumed strong black coffee,[162] and resveratrol (**46**) came under the limelight because of the alleged protective effect of red wine in the fat-rich French diet (French paradox).[163] Resveratrol, a pleiotropic agent that has raised considerable interest as a sirtuin ligand, was recently granted orphan drug status for the treatment of encephalomyopathy, a rare disease.[164] Also, negative dietary correlations can afford clues to drug discovery. Thus, the potent immunosuppressant dammarane triterpenoid (**47**) was discovered because of epidemiological correlations between the incidence of cancer and the consumption of palmyrah flour (*Borassus flabellifer*), a staple food of Sri Lankan Tamils.[165] The major limitation of the many dietary clues is that the beneficial or detrimental effects of health resist a reductionistic analysis, being the results of a combination of principles and their bacterial and hepatic metabolites. Anthocyanosides are remarkable examples. They are the most abundant dietary flavonoids and show a remarkable pattern of activity *in vitro* but are also chemical chameleons, varying in structure, polarity, and overall charge according to the pH of the medium and suffering from an outmost complex enteric and hepatic metabolism as well as entourage effect in their activity.[166] Anthocyanosides such as cyanidin glucoside (**48**) have recently raised great interest as antiobesity agents, due to their inhibiting properties on the differentiation of adipocytes and their lack of toxicity.[167]



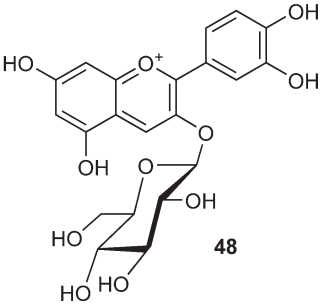
45



46



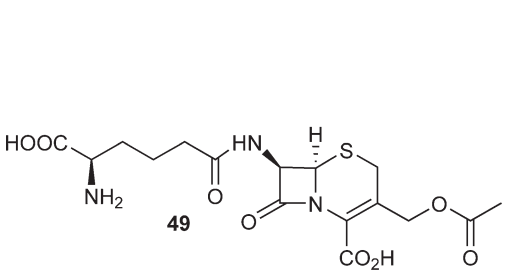
47



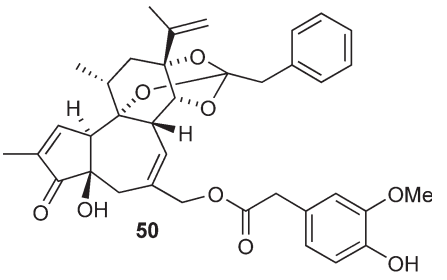
48

3.08.5.5 Derivatization, Diverted Total Synthesis, Diversity-Oriented Synthesis, and Semisynthesis

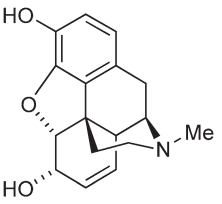
Because of toxicity, modest activity, poor solubility and stability, or overall unsatisfactory ADMET (absorption, distribution, metabolism, elimination, toxicology) profile, many natural products are of limited clinical use as such. Cephalosporin C (**49**), CPT (**14**), and curcumin (**12**) exemplify this situation in terms of suboptimal potency, toxicity, and oral bioavailability, respectively. However, natural products can be 'domesticated' by suitable chemical derivatization. In some cases, chemical modification can revert activity (iodination of the ultrapotent vanilloid resiniferatoxin (RTX; **50**),[168] *N*-methyl for *N*-allyl swap in morphine (**51**)[169]) or redirect it to unnatural targets, as observed for morphine (**51**, an opioid agonist) and its acidic rearrangement product apomorphine (**52**, a dopamine ligand).[169] However, natural products are often too complex for straightforward chemical derivatization and the exuberance of functional groups means that their reactivity is often unpredictable, with the need to develop *ad hoc* solution of specific and tailored applicability. For instance, the secondary hydroxyl of phorbol (**53**), a key element of its pharmacophore, is less reactive than the adjacent tertiary hydroxyl, which can be esterifed chemoselectively even in the presence of the primary allylic hydroxyl.[170] Patterns of reactivity like this are difficult to predict and require a careful preliminary study, with a consequent slowing down of the drug discovery campaign. Furthermore, in complex natural products, the reactivity of functional groups can be quenched by an unfavorable steric environment, as exemplified by the endocyclic double bond of paclitaxel (**25a**), which is resistant to hydrogenation,[171] or the C-9 tertiary hydroxyl of phorbol (**53**), which is characterized by total chemical inertness.[172] The manipulation of these cryptic functional groups might be of enormous biological relevance and could provide a solution to long-standing biological issues, such as the mode of binding of phorbol esters to PKC. Finally, there are limitations in the extent of the structure–activity relationships that can be studied using the functionalization pattern of a natural product. This is especially marked for apolar moieties that lack functional groups or that only bear functional groups redundant for activity. To address these issues, the concept of diverted total synthesis has been proposed by Wilson and Danishefsky.[173] The most straightforward way to assemble a complex target is by using a convergent synthesis, where smaller modules are combined sequentially en route to the target. The reactivity pattern of these small fragments is generally predictable and by feeding these modified fragments into the pipeline of the synthetic scheme, a full exploration of the structure–activity relationships can be achieved. Major applications of this strategy were described in the field of anticancer compounds, using epothilones and radicicol as leads.[173]



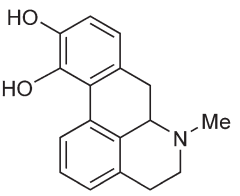
49



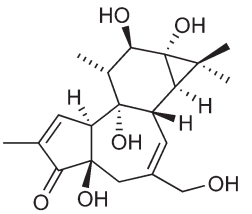
50



51

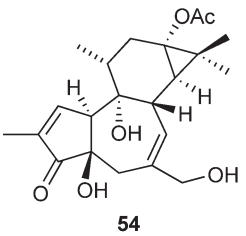


52

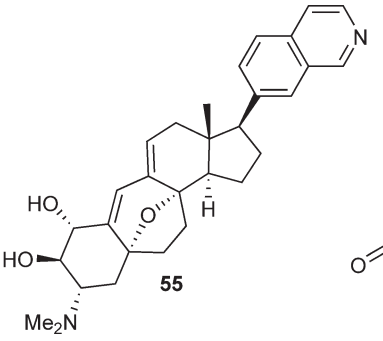


53

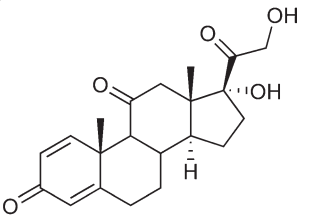
Over the previous years, there has also been an increased interest for the semisynthesis of complex natural products, with notable achievements by Wender *et al.* (prostratin (**54**) from phorbol (**53**))[174] and Baran and coworkers (cortistatin (**55**) from prednisone (**56**).[175] By elaborating easily available compounds, semisynthesis can provide a scalable access to complex structures difficult to source. It requires great ingenuity since synthetic creativity is constrained by the connectivity and configuration of the starting material. The industrial production of paclitaxel (**25a**)[171] and of ecteinascidin-743 (**57**) are examples of important industrial applications of semisynthesis to the production of natural products drugs. The marine anticancer compound ecteinascidin-743 (Yondelis), used for the treatment of soft-tissue sarcoma, was originally isolated from the marine tunicate *Ecteinascidia turbinata*. Wild harvest of this organism could not have supported its clinical development, which relied on aquaculture to afford the small amounts required at that stage. A total synthesis was reported by Corey *et al.*[176] but the supply problem was eventually solved by semisynthesis from a related microbial compound, cyanosafracin B (**58**), from *Pseudomonas fluorescens*.[177]



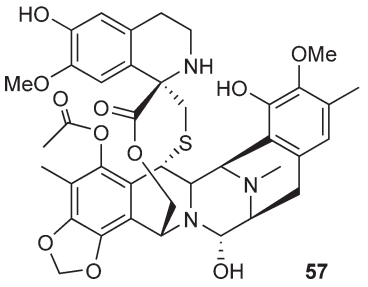
54



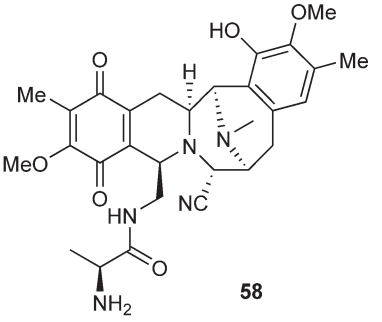
55



56

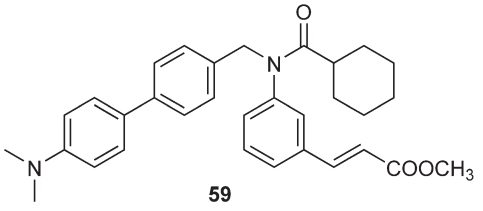


57

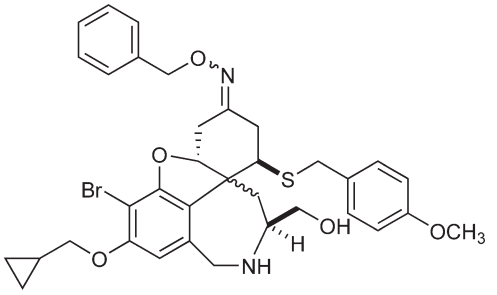


58

Many natural products are easily available in multigram amounts by isolation and boast a rich decoration of reactive functional groups as well as complex skeleton amenable to rearrangement. This chemical exuberance could be coupled to efficient technology platforms like combinatorial chemistry or diversity-oriented synthesis[178,179] to expand the pool of natural products and generate new modulators of biological activity.[180] Many attempts have also been made to combine the quality of natural products and the speed and efficiency of modern synthetic technologies by using natural products motifs as scaffolds to build combinatorial libraries. The efficiency of this process is exemplified by the discovery of fexaramine (**59**), an inhibitor of farnesoid X-receptor,[181] and of secramine (**60**), an inhibitor of protein trafficking by the Golgi apparatus.[182] These molecular probes emerged from synthetic combinatorial libraries built on the 2,2-dimethylbenzopyran motif[183] and on the tetracyclic core of galanthamine.[182]



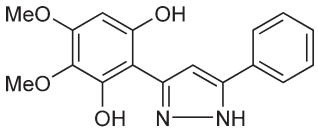
59



60

3.08.5.6 Extract Engineering

Crude extracts often contain a series of related compounds that share a common functionality that can make up for a large proportion of the extract. The crude extracts can be directly treated with a reagent specific for this functionality, generating a modified 'secondary' extract containing semisynthetic compounds that can be screened for a useful activity. In this way, the exploitable molecular diversity from a given biological source can be substantially increased. This principle was proposed by Furlan, who investigated the antifungal activity of a series of natural extracts containing flavones. Noticing the paucity of N–N motifs in natural products compared to their abundance in drugs, the extract was treated with hydrazine, affording an engineered extract where the flavone constituents had been converted to their corresponding pyrazoles by remodeling of the central C ring. Remarkably, while the natural extract lacked antifungal activity, the engineered one showed interesting activity against human fungal pathogens, traced by bioassay-directed fractionation, to the flavone-derived pyrazole (**61**).[184] This ingenuous strategy should be further investigated for its generality and holds undoubtedly great potential, although not many extracts are amenable to simple engineering.



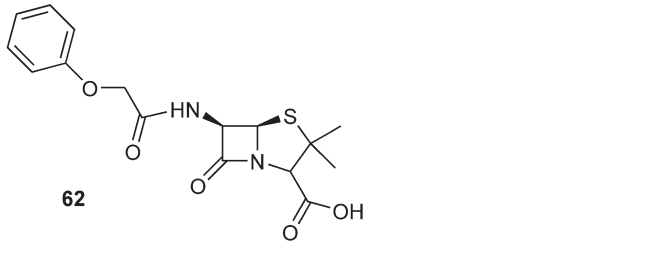
61

3.08.5.7 Engineered Biosynthesis (Mutasynthesis, Combinatorial, and Transgenic Biosynthesis)

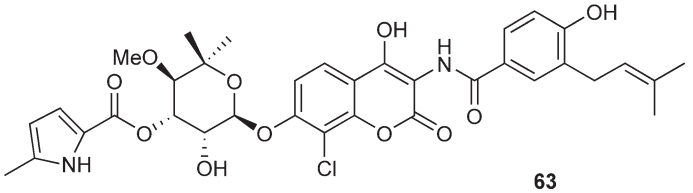
The living organisms are just a tiny fraction of those that have inhabited the earth and that went extinct during evolution. The extraordinary metabolic richness and unicity of living fossils like the gingko tree points to a chemically exuberant past that we will never be able to recapture. Millions of transient natural products were evolutionarily deselected along the pathway that eventually led to the natural products of today. Thus, hydrophobic hopanoid pentacyclic triterpenoids arose early in evolution (*Archebacteria*) as integral stabilizers

of hydrophobic membranes, followed by phytoesterols in plants, and eventually cholesterol in animal cells.[185] The very fact that squalene and squalene oxide can be cyclized in almost 100 different folding patterns to afford cyclosqualenoids gives a glimpse of the approach followed by nature to optimize natural products and generate today's chemodiversity, and of the intrinsic potential of biosynthetic pathways to generate a bewildering array of different structures.[185] Plants and microorganisms have biogenetic pathways that are expressed only under certain conditions and there is an enormous hidden chemical diversity apparent only at the genome level. We might ignore the reasons as to why most folding of squalene and squalene oxide were either never considered by nature or evolutionarily deselected but, thanks to molecular genetics, we are now in the position to randomly mutate key biogenetic enzymes, generating natural (since enzyme-derived) products in an unnatural way (molecular biology) and somehow mimicking evolution (for more details, see Chapter 2.20).

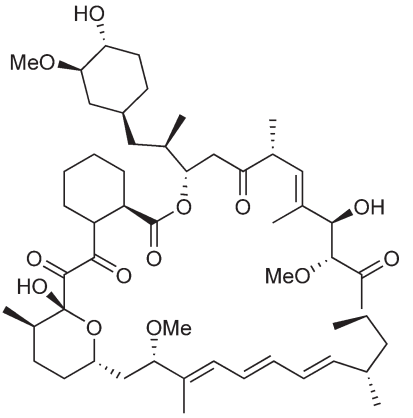
While biosynthetic engineering is still in its infancy, modification of a biosynthetic way by the addition of suitable building blocks has been pursued since the early studies on β-lactam antibiotics, as testified by the industrial production of penicillin V (phenoxymethylpenicillin, **62**) by the addition of phenoxyacetic acid to fermentation of *Penicillum chrysogenum*, a process established already in the 1950s.[185] Since the capacity to produce the natural compounds is retained, precursor-directed biosynthesis leads to a mixture of natural and unnatural compounds, resulting from competition between the natural building block and its unnatural analogue. To overcome this limitation, mutasynthesis, which is the use of microorganisms where the production of a specific building block is deficient because of an induced genetic mutation, has been developed. By blocking the biosynthesis of a specific precursor, the production of a complex compound becomes dependent on the supplementation with that specific precursor, which acts as a sort of metabolic 'vitamin'. The loose substrate specificity of many biosynthetic enzymes makes it possible to replace the natural precursor with modified versions of it. Mutasynthesis is especially suitable for modification of compounds having a modular structure. Thus, the aminocoumarin hsp90 inhibitor antibiotic chlorobiocin (**63**) consists of three elements, an aminocoumarin core, an acylated novobiose moiety, and a 3-prenyl-4-hydroxybenzoyl group (dimethylallyl-hydroxybenzoic acid, DMAHB). The introduction of the prenyl group is achieved by the dimethylallyl transferase CloQ and, by using molecular engineering, a strain of *Streptomyces roseochromogenes* was constructed where the *cloQ* gene was inactivated. Supplementation with analogues of DMAHB led to their incorporation into the biogenetic pathway and to the generation of chlorobiocin analogues.[186] A similar strategy but based on the shikimate-derived 4,5-dihydroxycyclohex-1-enecarboxylic acid was employed to generate analogues of the immunosuppressant polyketide rapamycin (**64**).[187]



62



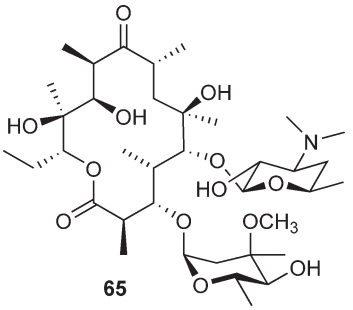
63



64

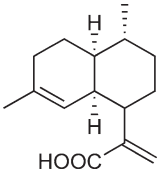
In combinatorial biosynthesis, genes from different but related biosynthetic pathways are combined to produced new compounds and this strategy has been particularly successful with polyketides.[188] These modular compounds represent the single most successful class of natural products drugs, with a lineup of compounds that encompasses first-in-the-class agents like lovastatin, erythromycin, tetracycline, doxorubicin,

amphotericin B, tacrolimus, and avermectin. Polyketides are built from a linear chain of carbon atoms generated by sequential reactions governed by polyketide synthases (PKSs), basically enzymic complexes that act like an assembly line tethering a starter unit and growing it. At the end of the process, the chain is untethered and cyclized by non-PKS enzymes (see Chapters 1.02–1.07). Additional enzymatic reactions introduce further decorations, such as sugars and methyl groups, while some PKSs also have ketone-modifying properties. Since genes in a polyketide pathway are always clustered together in contiguous DNA sequences, their isolation is easy, unlike other biogenetic pathways whose genes are dispersed in different chromosomal locations and must be isolated one at a time. Thus, a study of the biosynthesis of the polyketide antibiotic erythromycin (**65**) has resulted in the identification of some 28 domains. Repositioning the sequence of the corresponding genes enabled then to produce new 'unnatural' natural products.[189] A similar combinatorial approach was applied to the production of epothilones and to nonribosomal peptides.[190]



65

Natural products can, in principle, be also obtained from a direct biotechnological route, where all the genes involved in its biosynthesis are expressed in a fermentable host. The transgenic production of the antimalarial sesquiterpene lactone artemisinin (**26**) is currently investigated as a cheap alternative to isolation from *A. annua* L. or to total synthesis.[191] A biochemical and chemical precursor of artemisinin (artemisinic acid, **66**) has been produced in acceptable yield from the fermentation of an engineered strain of the yeast *Saccharomyces cerevisiae* where the production of farnesyl diphosphate was diverted from the triterpenoid sink to the sesquiterpene pool. The amorphadiene synthase gene and a cytochrome P-450 monooxygenase from *A. annua* were then expressed in this engineered yeast, overall resulting in the conversion of farnesyl diphosphate into artemisinic acid.[191]



66

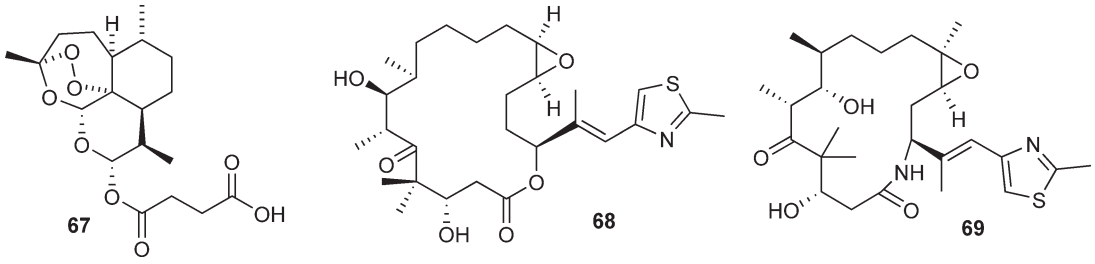
There are clearly several strategies to 'take the nature out of natural products' and produce them in a nonnatural way. Remarkably, these strategies have relevance not only for the mass production of a natural products drug but also for providing access to natural products-related chemodiversity.

3.08.6 Conclusions

> The point is not that natural products will solve all problems. It is that a lot of problems are not being solved because natural products are not being examined.
>
> S. J. Gould, *Chem. Eng. News* 13 October 2003, p 103

There is no doubt that natural products represent the best and most validated source to start a drug discovery campaign to a new druggable target but natural products can be difficult to access efficiently and effectively, unsuitable for further development due to poor ADMET properties, and plagued by IP issues. In the current

scenario of drug discovery, the dwindling use of natural products as pharmaceutical leads seems related to the intrinsically slower and more resource-intensive nature of natural products research compared to combinatorial chemistry and rational (*ab initio*) drug design. To remain competitive in drug discovery, natural products research should sharpen its tools by proper methodological evolution, interfacing with the current strategies of drug discovery, and overall, moving to higher throughput. In general, natural product-based drug discovery activities should be integrated with complementary technologies, such as combinatorial chemistry and rational drug discovery, and not be pursued alone in an independent fashion. They should also take advantage of techniques complementary to bioprospecting, such as derivatization of existing and easily available natural products, diverted total synthesis, and the high-throughput *de novo* construction of natural product-like scaffolds. Natural products have a function in the environment and nature is the functional filter that is lacking in combinatorial chemistry. A small collection of 'smart' compounds like those present in a plant extract or a fermentation broth will always be more valuable than a collection of randomly assembled synthetic compounds but the access to these 'intelligent' collections should be made technically easier and legally transparent, while the pharmacokinetic and proprietary profile of natural products could be improved by tailor-made chemical modification. The transition from paclitaxel (**25a**) to docetaxel (**25b**), from artemisinin (**26**) to artesunate (**67**),[192] or from epothilone B (**68**) to ixabepilone (**69**),[193] just to mention only recent examples, cogently demonstrates the success of this approach.



Given a promising natural product lead, there seems to be no difficulty in convincing big pharma to invest in its chemical derivatization and development. What is getting increasingly difficult is, paradoxically, to convince corporate decision makers that interesting natural products ligands, hits, leads, and even readymade drugs can originate from the study of biodiversity and of natural products libraries. It seems therefore logical to end up with a quotation from Samuel Danishefsky, possibly the most outspoken paladin for natural products in drug discovery, who, "at the risk of sounding Neanderthal," urged drug companies to "get back to the screening of natural products" and "critically examine the prevailing supposition that synthesizing zillions of compounds at a time is necessarily going to cut the costs of drug discovery or fill pharma pipelines with new drugs anytime soon."[194]

References

1. A. M. Rouhi, Chem. Eng. News 13 October 2003, 77–91.
2. I. Paterson; E. A. Anderson, *Science* **2005**, *310*, 451–453.
3. W. R. Strohl, *Drug Discov. Today* **2000**, *5*, 39–41.
4. G. Cragg; D. Newman, *Chem. Br.* **2001**, 22–26.
5. J. Meinwald; T. Eisner, *Helv. Chim. Acta* **2003**, *86*, 3633–3637.
6. D. J. Newman; G. M. Cragg; K. M. Snader, *J. Nat. Prod.* **2003**, *66*, 1022–1037.
7. A. M. Rouhi, *Chem. Eng. News* 13 October 2003, *93–103*, 104–106.
8. D. J. Newman; G. M. Cragg; D. G. I. Kingston, Natural Products as Pharmaceuticals and Sources for Lead Structures. In *The Practice of Medicinal Chemistry*; 2nd ed.; C. G. Wermuth, Ed.; Academic Press: London; 2003; pp 91–110.
9. M. S. Butler, *J. Nat. Prod.* **2004**, *67*, 2141–2153.
10. J. Clardy; C. Walsh, *Nature* **2004**, *432*, 829–837.
11. F. E. Koehnm; G. T. Carter, *Nat. Rev. Drug Discov.* **2005**, *4*, 206–220.
12. M. S. Butler, *Nat. Prod. Rep.* **2005**, *22*, 162–195.
13. D. D. Baker; M. Chu; U. Oza; V. Rajgarhia, *Nat. Prod. Rep.* **2007**, *24*, 1225–1244.
14. J. D. McChesney; S. K. Venkataraman; J. T. Henri, *Phytochemistry* **2007**, *68*, 2015–2022.
15. A. Saklani; S. K. Kutty, *Drug Discov. Today* **2008**, *13*, 161–171.

16. M. Pucheault, *Org. Biomol. Chem.* **2008**, *6*, 424–432.
17. M. S. Butler, *Nat Prod. Rep.* **2008**, *25*, 475–516.
18. O. Potterat; M. Hamburger, *Progr. Drug Res.* **2008**, *65*, 47–118.
19. D. J. Newman, *J. Med. Chem.* **2008**, *51*, 2589–2599.
20. P. J. Hajduk; J. R. Huth; S. W. Fesik, *J. Med. Chem.* **2005**, *48*, 2518–2525.
21. C. P. Adams; V. V. Brantner, *Drug Dev.* **2006**, *25*, 23–24.
22. I. Kola; J. Landis, *Nat. Rev. Drug Discov.* **2004**, *3*, 711–716.
23. L. Smith, *The Pink Sheet* 10 December 2008, 13.
24. N. Blow, *Nature* **2007**, *450*, 1117–1120.
25. C. Dombrowski, *The Pink Sheet* **2008**, *70* (23), 27–28.
26. P. Geborek; E. Nitelius; S. Noltorp; H. Petri; L. Jacobsson; L. Larson; T. Saxne; I. Leden, *Ann. Rheum. Dis.* **2005**, *64*, 1805–1807.
27. A. Catlin; C. Cowan; S. Heffler; B. Washington, *Health Aff. (Millwood)* **2007**, *26*, 142–153.
28. A. Johson, *The Wall Street Journal* 12 December 2007.
29. J. Merril, The Pink Sheet 10 December 2008, p 5.
30. P. Imming; C. Sinning; A. Meyer, *Nat. Rev. Drug. Discov.* **2006**, *5*, 821–832.
31. K. H. Altmann; J. Gertsch, *Nat. Prod. Rep.* **2007**, *24*, 327–352.
32. Y. Uehara, *Curr. Cancer Drug Targets* **2003**, *3*, 325–330.
33. P. Workman, *Curr. Cancer Drug Targets* **2003**, *3*, 297–300.
34. J. M. McPartland; G. W. Guy, The Evolution of Cannabis and Coevolution with the Cannabinoid Receptor – A Hypothesis. In *The Medicinal Uses of Cannabis and Cananbinoids*; G. W. Guy, B. A. Whittle, P. J. Robson, Eds.; Pharmaceutical Press: London, 2004; pp 71–101.
35. E. Jung; A.-L. Veuthney; E. Gasteiger; A. Bairoch, *Proteomics* **2001**, *1*, 262–268.
36. A. L. Hopkins; C. R. Groom, *Nat. Rev. Drug Discov.* **2002**, *1*, 727–730.
37. J. P. Overington; B. Al-Lazikani; A. L. Hopkins, *Nat. Rev. Drug. Discov.* **2006**, *5*, 993–996.
38. R. Breinbauer; I. R. Vetter; H. Waldmann, *Angew. Chem. Int. Ed.* **2002**, *41*, 2878–2890.
39. B. M. McArdle; M. R. Campitelli; R. J. Quinn, *J. Nat. Prod.* **2006**, *69*, 14–17.
40. B. A. Halkier; J. Gershenzon, *Ann. Rev. Plant Biol.* **2006**, *57*, 303–333.
41. E. Block, *Angew Chem Int. Ed.* **1992**, *104*, 1158–1203.
42. K. C. Nicolaou; W. M. Dai, *Ang. Chem. Int. Ed.* **1991**, *30*, 1387–1416.
43. M. L. Guzman; R. M. Rossi; L. Karnischky; X. Li; D. R. Peterson; D. S. Howard; C. T. Jordan, *Blood* **2005**, *105*, 4163–4169.
44. A. Louw; P. Swart; S. S. de Kock; K. J. van der Merwe, *Biochem. Pharmacol.* **1997**, *53*, 189–197.
45. A. Louw; P. Swart, *Endocrinology* **1999**, *140*, 2044–2053.
46. P. Dewint; V. Gossye; K. De Bosscher; W. Vanden Berghe; K. Van Beneden; D. Derocer; S. Van Calenbergh; U. Mueller-Ladner; B. Vander Cruyssen; G. Verbruggen; G. Haegeman; D. Elewaut, *J. Immunol.* **2008**, *180*, 2608–2615.
47. R. D. Couch; R. G. Browning; T. Honda; G. W. Gribble; D. L. Wright; M. B. Sporn; A. C. Anderson, *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2215–2219.
48. K. T. Liby; M. M. Yore; M. B. Sporn, *Nat. Rev. Cancer* **2007**, *7*, 357–369.
49. J. C. Espin; M. T. Garcia-Conesa; F. A. Tomás-Barberán, *Phytochemistry* **2007**, *68*, 2986–3008.
50. J. W. Fahey; T. W. Kensler, *Chem. Res. Toxicol.* **2007**, *20*, 572–576.
51. D. M. Goldstein; N. S. Gray; P. P. Zarrinkar, *Nat. Rev. Drug Discov.* **2008**, *7*, 391–395.
52. J. Jossang; H. Bel-Kassaoui; A. Jossang; M. Seuleman; A. Nel, *J. Org. Chem.* **2008**, *73*, 412–417.
53. C. Spickler; M. N. Brunelle; L. Brakier-Gingras, *J. Mol. Biol.* **1997**, *273*, 586–599.
54. G. Appendino, Capsaicin and Capsaicinoids. In *Modern Alkaloids: Structure, Isolation, Synthesis and Biology*; E. Fattorusso, O. Taglialatela-Scafati, Eds.; Wiley: Weinheim, 2007; pp 75–112.
55. G. Appendino; A. Minassi; A. Pagani; A. Ech-Chahad, *Curr. Pharm. Design* **2008**, *14*, 2–17.
56. S. Sirikantaramas; M. Yanazaki; K. Saito, *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 6782–6786.
57. S. Bruzzone; I. Moreschi; C. Usai; L. Guida; G. Damonte; A. Salis; S. Scarfi; E. Millo; A. De Flora; E. Zocchi, *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 5759–5764.
58. V. A. Halim; A. Vess; D. Scheel; S. Rosahl, *Plant Biol. (Stuttg.)* **2006**, *8*, 307–313.
59. A. A. Begum; S. Leibovitich; P. Migner; F. Zhang, *J. Exp. Bot.* **2001**, *52*, 1537–1543.
60. E. Cundliffe, *Annu. Rev. Microbiol.* **1989**, *43*, 207–233.
61. D. A. Hopwood, *Mol. Microbiol.* **2007**, *63*, 937–940.
62. S. Singh; M. H. Hager; C. Zhang; B. R. Griffith; M. S. Lee; K. Hallenga; J. L. Markley; J. S. Thorson, *ACS Chem. Biol.* **2006**, *1*, 451–460.
63. T. Henkel; R. M. Brunne; H. Mueller; F. Reichel, *Angew. Chem. Int. Ed. Engl.* **1999**, *38*, 643–647.
64. X.-H. Xo; G.-M. Yao; Y.-M. Li; J.-H. Lu; C. J. Lin; X. Wang; C.-H. Kong, *J. Nat. Prod.* **1993**, *66*, 285–288.
65. W. Sneader, *Drug Prototypes and their Exploitation*; Wiley: Chichester, 1996; p 446.
66. B. Lefkove; B. Govindarajan; J. L. Arbiser, *Expert Rev. Anti Infect. Ther.* **2007**, *5*, 373–379.
67. M. Yoshida; S. Horinouchi; T. Beppu, *Bioassays* **1995**, *17*, 423–430.
68. M. Groll; R. Huber, *Biochim. Biophys. Acta* **2004**, *1695*, 33–44.
69. X. Tao; F. Fan; V. Hoffmann; C. Y. Gao; N. S. Longo; P. Zerfas; P. E. Lpsky, *Arthritis Rheum.* **2008**, *58*, 1774–1783.
70. P. Jones; C. Steinkueler, *Curr. Pharm. Des.* **2008**, *14*, 545–561.
71. S. Omura; K. Matsuzaki; T. Fujimoto; K. Kosuge; T. Furuya; S. Fujita; A. Nakagawa, *J. Antibiot. (Tokyo)* **1991**, *44*, 117–118.
72. D. P. Arya, *Top. Heterocycl. Chem.* **2006**, *2*, 129–152.
73. C. Lipinski; A. Hopkins, *Nature* **2004**, *432*, 855–861.
74. M. Feher; J. M. Schmidt, *J. Chem. Inf. Comput. Sci.* **2003**, *43*, 218–227.
75. G. M. Cragg; D. J. Newman; K. M. Snader, *J. Nat. Prod.* **1997**, *60*, 52–60.
76. K. P. Mishra; L. Ganjum; M. Sairam; P. K. Banerjee; R. C. Sawhney, *Biomed. Pharmacother.* **2008**, *62*, 94–98.

77. G. R. Elridge; H. C. Vervoort; C. M. Lee; P. A. Cremin; C. T. Williams; S. M. Hart; M. G. Goering; M. O'Neil; M. Johnson; L. Zeng, *Anal. Chem.* **2002**, *74*, 3963–3971.
78. H. N. Woller, *Mol. Diversity* **1999**, *4*, 47–52.
79. M. Hamburger, *Chimia* **2006**, *60*, 14–18.
80. R. N. Young, *Pure Appl. Chem.* **1999**, *71*, 1655–1661.
81. A. I. Graul; E. Cruces; L. Revel; N. Serradell; E. Rosa, *Drug News Perspect.* **2008**, *21*, 44–58.
82. E. K. Wilson, Chem. Eng. News, 13 February 2006, pp 86–87.
83. G. M. Cragg, *Med. Res. Rev.* **1998**, *18*, 315–331.
84. M. E. Wall; M. C. Wani, *Cancer Res.* **1995**, *55*, 753–760.
85. E. Hsu, *Br. J. Clin. Pharmacol.* **2006**, *61*, 666–670.
86. J. Wang; S. M. Soisson; K. Young; W. Shoop; S. Kodali; A. Galgoci; R. Painter; G. Parthasarathy; Y. S. Tang; R. H. S. Cummings; K. Dorso; M. Motyl; H. Jayasuriya; J. Ondeyka; K. Herath; C. Zhang; L. Hernandez; J. Allocco; A. Basilio; J. R. Tormo; O. Genilloud; F. Vicente; F. Pelaez; L. Colwell; S. H. Lee; B. Michael; T. Felcetto; C. Gill; L. L. Silver; J. D. Hermes; K. Bartizal; J. Barrett; D. Schmatz; J. W. Becker; D. Cully; S. B. Singh, *Nature* **2006**, *441*, 358–361.
87. M. D. Coughlin, Jr.; M. Columb, *J. Transn. Law* **1993**, *31*, 337–375.
88. Challenges in Negotiating and Implementing ABS Agreements. http://www.iisd.org/pdf/2006/abs_session2_augustine.ppt
89. G. M. Cragg; M. R. Boyd; J. H. Cardellina; M. R. Grever; S. A. Schepartz; K. M. Snader; M. Suffness, *Human Medicinal Agents from Plants*; ACS Symposium Series 534; American Chemical Society: Washington, 1993.
90. T. B. Lowinger; B. Riedl; J. Dumas; R. A. Smith, *Curr. Pharm. Des.* **2002**, *8*, 2269–2278.
91. D. Pytel; T. Sliwinski; T. Poplawski; D. Ferriola; I. Maisterek, *Anticancer Agents Med. Chem.,* **2009**, *9*, 66–76.
92. G. C. Tron; T. Pirali; G. Sorba; F. Pagliai; S. Busacca; A. A. Genazzani, *J. Med. Chem.* **2006**, *49*, 3033–3044.
93. W. C. Widdison; S. D. Wilhelm; E. E. Cavanagh; K. R. Whiteman; B. A. Leece; Y. Kovtun; V. S. Goldmacher; H. Xie; R. M. Steeves; R. J. Lutz; R. Zhao; L. Wang; W. A. Blättler; R. V. Chari, *J. Med. Chem.* **2006**, *49*, 4392–4408.
94. M. R. Boyd, *J. Ethnopharmacol.* **1996**, *51*, 17–25.
95. G. A. Cordell, *Phytochemistry* **2000**, *55*, 463–480.
96. G. A. Cordell, *Phytochemistry* **1995**, *40*, 1585–1612.
97. T. D. Mays; K. D. Mazan, *J. Ethnopharmacol.* **1996**, *51*, 93–102.
98. M. A. Gollin, *Nat. Biotechnol.* **1999**, *17*, 921–922.
99. W. Snader, *Drug Prototypes and Their Exploitation*; Wiley: Chichester, 1996.
100. F. Pearce, New Scientist 18 April 2002, p 14.
101. J. Goodman; V. Walsh, *The Story of Taxol. Nature and Politics in the Pursuit of an Anti-Cancer Drug*; Cambridge University Press: Cambridge, 2001.
102. A. M. Rouhi, *Chem. Eng. News* 7 April 1997, 14–29.
103. U. Schueklenk; A. Kleinsmidt, *Dev. World Bioeth.* **2006**, *6*, 112–134.
104. S. L. Pimm; G. J. Russellm; J. L. Gittleman; T. M. Brooks, *Science* **1995**, *269*, 347–350.
105. F. Pearce, New Scientist 29 June 2006, p 11.
106. S. J. Mickel, *Curr. Opin. Drug Discov. Devel.* **2004**, *7*, 869–881.
107. M. Saleem; M. S. Ali; S. Hussain; A. Jabbar; M. Ashraf; Y. S. Lee, *Nat. Prod. Rep.* **2007**, *24*, 1142–1152.
108. J. W. Daly, *J. Nat. Prod.* **2004**, *67*, 1211–1215.
109. Analyticon Discovery. http://www.ac-discovery.com/english/go.html
110. F. W. Sertuerner, *Trommsdorff's J. Pharm.* **1805**, *13*, 234.
111. R. Mechoulam; S. Ben-Shabat, *Nat. Prod. Rep.* **1999**, *16*, 131–143.
112. M. P. Barnes, *Expert Opin. Pharmacother.* **2006**, *7*, 607–615.
113. R. G. Pertwee, *Br. J. Pharmacol.* **2008**, *153*, 199–215.
114. R. Verpoorte, *Drug Discov. Today* **1998**, *3*, 232–238.
115. M. Zhu; J. D. Phillipson; P. M. Greengrass; N. E. Bowery; Y. Cai, *Phytochemistry* **1999**, *44*, 441–447.
116. G. M. Rishton, *Drug Discov. Today* **1997**, *2*, 382–384.
117. X. Ma; C. Tan; D. Zhu; D. R. Gang; P. Xiao, *J. Ethnopharmacol.* **2007**, *113*, 15–34.
118. G. A. Cordell, *Phytochemistry* **2000**, *55*, 463–480.
119. N. CanMiddlesworth; R. J. P. Cannell, Natural Products Isolation. In *Methods in Biotechnology*; R. J. P. Cannell, Ed.; Humana Press: Totowa, NJ, 1998; Vol. 4, pp 279–327.
120. H. J. Kim; I. Baburin; S. Khom; S. Hering; M. Hamburger, *Planta Med.* **2008**, *74*, 521–526.
121. P. L. Smith; K. N. Maooney; R. G. Pothen; J. Clardy; D. E. Clapham, *J. Biol. Chem.* **2006**, *281*, 29897–29904.
122. S. L. Schreiber, *Science* **2000**, *287*, 1964–1969.
123. L. A. Marcaurelle; C. W. Johannes, *Prog. Drug Res.* **2008**, *66*, 189–216.
124. B. L. Bray, *Nat. Rev. Drug Discov.* **2003**, *2*, 587–593.
125. J. Marco-Contelles; M. do Carmo Carreiras; C. Rodríguez; M. Villaroya; A. G. Garcia, *Chem. Rev.* **2006**, *106*, 116–133.
126. M. S. Butler; D. J. Newman, *Prog. Drug Res.* **2008**, *65*, 3–44.
127. G. Liu; M. Grifman; J. Macdonald; P. Moller; F. Wong-Stall; Q. X. Li, *J. Endocrinol.* **2007**, *194*, 569–578.
128. K. Leuner; V. Kazanski; M. Müller; K. Essin; B. Henke; M. Gollasch; C. Harteneck; W. E. Muller, *FASEB J.* **2007**, *21*, 4101–4111.
129. P. Evers, SCRIP 21 May 2008, pp 31–34.
130. M. J. Plotkin, *Medicine Quest: In Search of Nature's Healing Secrets*; Viking: New York, 2000.
131. R. W. Spjut; A. R. E. Perdue, *Cancer Treat. Rep.* **1976**, *60*, 979–985.
132. Y. Gaillard; G. Pepin, *J. Chromatogr. B* **1999**, *733*, 181–229.
133. E. E. Bar; D. Stearns, *Expert Opin. Investig. Drugs* **2008**, *17*, 185–195.
134. B. M. Olivera; R. W. Teichert, *Mol. Interv.* **2007**, *7*, 251–260.
135. M. Heinrich; S. Gibbons, *J. Pharm. Pharmacol.* **2001**, *53*, 425–432.
136. W. F. Li; J. C. Jiang; J. Chen, *Arch. Med. Res.* **2008**, *39*, 246–251.
137. B. M. Schmidt; D. M. Ribnicky; P. E. Lipsky; I. Raskin, *Nat. Chem. Biol.* **2007**, *3*, 360–366.

138. G. Gross, *Hautarzt* **2008**, *59*, 31–35.
139. M. Heinrich; P. Bremner, *Curr. Drug Targets* **2006**, *7*, 239–245.
140. G. Bo, *Clin. Microbiol. Infect.* **2000**, *6* (Suppl. 3), 6–9.
141. W. Sneader, *Drug Prototypes and their Exploitation*; Wiley: Chichester, 1996; pp 489–491.
142. J. L. Hartwell, *Lloydia* **1967**, *30*, 379–426 (and the other seven articles in the series).
143. B. C. Elford; M. F. Roberts; J. D. Phillipson; R. J. Wilson, *Trans. R. Soc. Trop. Med. Hyg.* **1987**, *81*, 434–436.
144. C. Triplitt; E. J. Chiquette, *Am. Pharm. Assoc.* **2003**, *46*, 44–52.
145. J. Eng; W. A. Kleinman; L. Singh; G. Singh; J. P. Raufman, *J. Biol. Chem.* **1992**, *15*, 7402–7405.
146. A. Harvey, *Drug Disc. Today* **2000**, *7*, 294–300.
147. T. E. Adrian, *Curr. Pharm. Des.* **2007**, *13*, 3417–3426.
148. T. L. Simmons; R. C. Coates; B. R. Clark; N. Eugene; D. Gonzalez; E. Esquenazi; P. C. M. Dorrestein; W. H. Gerwick, *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 4587–4594.
149. M. Gordaliza, *Clin. Transl. Oncol.* **2007**, *9*, 767–776.
150. T. Divabalange; C. D. Amsler; J. B. McClintock; B. J. Baker, *J. Am. Chem. Soc.* **2006**, *128*, 5630–5631.
151. X. Jiang; B. Liu; S. Lebreton; J. K. Brabander, *J. Am. Chem. Soc.* **2007**, *129*, 6386–6387.
152. K. C. Nicolaou; R. Guduru; Y. P. Sun; B. Banerji; D. Y. Chen, *Angew. Chem. Int. Ed. Engl.* **2007**, *46*, 5896–5900.
153. C. Chiraldi; M. De Rosa, *Trends Biotechnol.* **2002**, *20*, 515–521.
154. C. A. Roessner; A. L. Scott, *Annu. Rev. Microbiol.* **1996**, *50*, 467–490.
155. S. F. Brady; C. J. Chao; J. Clardy, *Appl. Environ. Microbiol.* **2004**, *70*, 6865–6870.
156. G. Appendino; O. Taglialatela-Scafati, Drug-Like Compounds from Food Plants and Spices. In *Dietary Supplements of Plant Origin*; M. Maffei, Ed.; Taylor & Francis: London, 2003; pp 43–74.
157. A. Endo, *J. Antibiot.* **1979**, *32*, 852–854.
158. I. Raskin, *Plant Physiol.* **1992**, *99*, 799–803.
159. A. W. Alberts; J. Chen; G. Kuron; V. Hunt, *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 3957–3961.
160. A. Goel; A. B. Kunnumakkara; B. B. Aggarwal, *Biochem. Pharmacol.* **2008**, *75*, 787–809.
161. J. H. Medina; C. Pena; M. Piva; C. Wolfman; M. L. de Stein; C. Wasowski; C. Da Cunha; I. Izquierdo; A. C. Paladini, *Mol. Neurobiol.* **1992**, *6*, 377–386.
162. H. Salter, *Asthma. Its Pathology and Treatment*; Churchill: London, 1860; p 181.
163. J. C. Espin; M. T. Garcia-Conesa; F. A. Tomás-Barberán, *Phytochemistry* **2007**, *68*, 2986–3008.
164. *SCRIP* 3362, 16 May 2008, p 19.
165. L. Révész; P. Hiestand; L. La Vecchia; R. Naef; H. U. Naegeli; L. Overer; H. J. Roth, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1521–1526.
166. F. Galvano; L. la Fauci; P. Vitaglione; V. Fogliano; L. Vanella; C. Felgines, *Ann. Ist. Super. Sanità* **2007**, *43*, 382–393.
167. T. Tsuda, *J. Agric. Food Chem.* **2008**, *56*, 642–646.
168. P. Wahlm; C. Foged; S. Tullin; C. Thomsen, *Mol. Pharmacol.* **2001**, *59*, 9–15.
169. W. Sneader, *Drug Prototypes and their Exploitation*; Wiley: Chichester, 1996; pp 85–89.
170. N. Márquez; M. A. Calzado; G. Sánchez-Duffhues; M. Pérez; A. Minassi; A. Pagani; G. Appendino; L. Diaz; M. A. Muñoz-Fernández; E. Muñoz, *Biochem. Pharmacol.* **2008**, *75*, 1370–1380.
171. D. G. I. Kingston; P. G. Jagtap; H. Yuan; L. Samala, *Fortschr. Chem. Org. Naturst.* **2002**, *84*, 53–225.
172. G. Appendino; A. Bertolino; A. Minassi; R. Annunziata; A. Szallasi; L. De Petrocellis; V. Di Marzo, *Eur. J. Org. Chem.* 2004, 3413–3421.
173. R. M. Wilson; S. J. Danishefsky, *J. Org. Chem.* **2006**, *71*, 8329–8351.
174. P. A. Wender; J. M. Kee; J. M. Warrington, *Science* **2008**, *320*, 649–652.
175. R. A. Shenvi; C. A. Guerrero; C. C. Li; P. S. Baran, *J. Am. Chem. Soc.* **2008**, *130*, 7241–7243.
176. E. J. Corey; D. Y. Gin; R. S. Kania, *J. Am. Chem. Soc.* **1996**, *118*, 9202–9203.
177. C. Cuevas; M. Pérez; M. J. Martín; J. L. Chicharro; C. Fernandez-Rivas; M. Flores; A. Francesch; P. Gallego; M. Zarzuelo; F. de la Calle; J. García; C. Polanco; I. Rodríguez; I. Manzanares, *Org. Lett.* **2000**, *2*, 2545–2548.
178. R. J. Spandl; A. Bender; D. R. Spring, *Org. Biomol. Chem.* **2008**, *6*, 1149–1158.
179. L. A. Marcaurelle; C. W. Johannes, *Prog. Drug Res.* **2008**, *66*, 189–216.
180. G. Appendino; G. C. Tron; T. Jarevang; O. Sterner, *Org. Lett.* **2001**, *3*, 1609–1612.
181. M. Downes; M. A. Verdecia; A. J. Roecker; R. Hughes; J. B. Hogenesch; H. R. Kast-Woelbern; M. E. Bowman; J. L. Ferrer; A. M. Anisfeld; P. A. Edwards; J. M. Rosenfeld; J. G. Alvarez; J. P. Noel; K. C. Nicolaou; R. M. Evans, *Mol. Cell.* **2003**, *11*, 1079–1092.
182. H. E. Pelish; N. J. Westwood; Y. Feng; T. Kirchhausen; M. D. Shair, *J. Am. Chem. Soc.* **2001**, *123*, 6740–6741.
183. K. C. Nicolaou; J. A. Pfefferkorn; A. J. Roecker; G. Q. Cao; S. Barluenga, *J. Am. Chem. Soc.* **2000**, *122*, 9939–9953.
184. S. N. López; I. A. Ramallo; M. Gonzalez Sierra; S. A. Zacchino; R. L. E. Furlan, *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 441–444.
185. J. Kennedy, *Nat. Prod. Rep.* **2008**, *25*, 25–34.
186. U. Galm; S. Heller; S. Shapiro; M. Page; M. S. M. Li; L. Heide, *Antimicrob. Agents Chemother.* **2004**, *48*, 1307–1312.
187. K. J. Weissman, *Trends Biotechnol.* **2007**, *25*, 139–142.
188. J. K. Borchardt, Mod. Drug Discov. July/August 1999, 22–29.
189. Y. Volcegursky; Z. Hu; R. McDaniel, *Mol. Microbiol.* **2000**, *37*, 752–762.
190. D. E. Cane; C. T. Walsh; C. Khosla, *Science* **1998**, *282*, 63–68.
191. D. K. Ro; E. M. paradise; M. Ouellet; K. J. Fischer; K. L. Newman; J. M. Ndungu; K. A. Ho; R. A. Eachus; T. S. Ham; J. Kirby; M. C. Chang; S. T. Withers; Y. Shiba; R. Sarpong; J. D. Keasling, *Nature* **2006**, *440*, 940–943.
192. G. Li; X. Guo; R. Jin; Z. Wang; H. Jian; Z. Li, *J. Tradit. Chin. Med.* **1982**, *2*, 125–130.
193. F. Y. Lee; R. Barzilleri; C. R. Fairchild; S. H. Kim; B. H. Long; C. Reventos-Suarez; G. D. Vite; W. C. Rose; R. A. Kramer, *Clin. Cancer Res.* **2001**, *7*, 1429–1437.
194. Quoted in S. Borman, Chem. Eng. News 14 January 2002, 23–24.

Biographical Sketches

Giovanni Appendino was born in Carmagnola, Italy, in 1995. After graduating from the University of Torino in 1979, he did post-Laurea work with Professor Pierre De Clercq (University of Gent, Belgium), working on the total synthesis of gibberellic acids. In 1983, he became lecturer and in 1998 associated professor at his *alma mater*. Since 2000, he is full professor of organic chemistry at the Università del Piemonte Orientale, Faculty of Pharmacy and since 2006, chief scientific adviser of Indena S.p.A., Milano. Professor Appendino's research interests are in the realm of bioactive natural products (isolation, chemical modification, and total synthesis). He has published over 250 original articles in this area and in 1991 he received the Rhône–Poulenc Rorer Award of the Phytochemical Society of Europe for his studies on isoprenoids.

Gabriele Fontana was born in Magenta, Italy, in 1967. After he graduated from the University of Milano in 1992 and obtained his Ph.D. in Chemistry in 1996, he was research assistant at the University of Newcastle Upon Tyne (UK) till 1998 under the guidance of Professor Roger J. Griffin. He then moved to Glaxo-Wellcome, Italy, as medicinal chemistry scientist under the direction of Dr. Romano di Fabio. In September 2000 he joined Indena SpA, Milan, Italy, where he became head of medicinal chemistry in 2008.

Federica Pollastro was born in Novara, Italy, in 1976. After obtaining her Laurea Diploma in 2006 at the Università del Piemonte Orientale, Faculty of Pharmacy, she is currently a Ph.D. student in Professor Appendino's group in Novara, working on the medicinal chemistry of bioactive natural products.

3.09 Natural Product-Based Biopesticides for Insect Control

Azucena Gonzalez-Coloma, Instituto de Ciencias Agrarias-CCMA, Madrid, Spain

Matías Reina, Carmen E. Diaz, and Braulio M. Fraga, Instituto de Productos Naturales y Agrobiologia, Tenerife, Spain

3.09.1 Introduction

The direct use of natural products as pesticides or as leads for pesticides has been reviewed previously.[1–4] This short review will highlight methods and strategies and the rationale behind the use of natural products as insecticides with a more detailed discussion of new promising leads, including a few examples from the authors' research.

The use of botanical insecticides dates back two millennia. The use of plant products in Europe goes back to more than 150 years ago, until the discovery of synthetic insecticides (organochlorines, organophosphates, carbamates, pyrethroids), which replaced the botanical insecticides. Overuse of these synthetic insecticides has led to problems such as acute and chronic pollution, negative effects on wildlife (fish, birds), disruption of biological control and pollination, groundwater contamination, and resistance to pesticides.[5,6]

Despite the concerted effort being made to breed or engineer plants with increased resistance to pests and disease, there will always be a need for crop protection, partly for mass-produced crops and partly for niche areas such as horticulture, greenhouses, organic farming, households, and gardens where biopesticides are particularly prevalent. There is a need for environmentally friendly and consumer-friendly products that also preferably exhibit novel modes of action to mitigate resistance problems.

The development of crop protectants is similar to drug development and is presently based on synthesizing novel molecules that interact with well-defined targets found in the pest. The difference with drug development is that the compounds will be used on a large scale and must be free of all environmental toxicity. Also the products should be relatively stable and should be safe for human use (e.g., nontoxic, rapid breakdown). Toxicity is the major hurdle that needs to be overcome in the development of novel pesticides. Most compounds are eliminated due to adverse toxic effects. Screening nontoxic plants for activity reduces the risk of discovering toxic biopesticides. The chance of finding novel biopesticides is increased by screening plants that are used for food, cosmetics, or spices, or plants that have traditionally been used as crop protectants.

Plants have an excellent track record in providing novel leads for crop protection, particularly in the field of insecticides. This can be attributed to the evolution of secondary metabolites for host plant protection against insects, pathogens, and plant competitors. Our ancestors were quite successful in exploring and exploiting this natural treasure. The documented use of plant extracts and powdered plant parts as insecticides goes back at least as far as the Roman Empire. There are reports of the use of pyrethrum (*Tanacetum cinerariaefolium*, Asteraceae) as early as 400 BC. The first pure botanical insecticide used as such dates back to the seventeenth century when it was shown that nicotine obtained from tobacco leaves was lethal to plum beetles. Around 1850, a new plant insecticide known as rotenone was introduced. Rotenone is a flavonoid derivative extracted from the roots of two different *Derris* spp. (Fabaceae) and *Lonchocarpus* spp. (Fabaceae). The ground seeds of Sabadilla, a plant of South American origin known as *Schoenocaulon officinale* (Liliaceae), are one of the plant insecticides exhibiting the least toxicity to mammals.[7]

Currently, there are a number of botanical insecticides that are being marketed worldwide. Some examples are neem (*Azadirachta indica*), rotenone, and ryania, which is obtained from the roots and stems of a native South American plant known as *Ryania speciosa* (Flacourtiaceae). The active compounds isolated from the botanical pesticides may also eventually provide basic structures contributing to the development of new pesticides. Recent reviews have been published in this connection.[1–4,6,8]

The main markets for botanical pesticides are organic agriculture, horticulture, green houses, parks, gardens, and households. Organic agriculture is a market with a high demand for biopesticides, as organic growers cannot use conventional agrochemicals. This market is currently expanding owing to consumers' demand for improved food safety and the environmental problems associated with the use of synthetic pesticides. With an annual average growth of 30%, organic farming in the EU is one of the most dynamic agricultural sectors. Many more farmers have come onboard since the enactment of Community Legislation regulating organic production (Council Regulation 2092/91/EEC of 24 June 1991). One of the overarching objectives of the Common Agricultural Policy (CAP) is the achievement of sustainable agricultural production in Europe, which requires environmentally friendly pest control measures.

Botanical pesticides also feature the advantage of being compatible with other low-risk options that are acceptable for insect management, which include, inter alia, the use of pheromones, oils, detergents, entomopathogenic fungi, predators, and parasitoids. This significantly increases the likelihood of botanical pesticides being integrated into integrated pest management (IPM) programs.

New products need to be developed to meet the demands of this growing market, and to this end a systematic approach to finding new plant-derived products needs to be developed. Different sources can be considered, such as traditionally used plants, readily available plants, or agricultural waste products. Extracts from these plants need to be screened for activity and then isolated and active molecules identified. Cultivation methods then need to be developed in the case of plants exhibiting interesting activity. Environmentally friendly extraction methods should be applied to achieve the final products. The successful development of biocides from discarded citrus peels in the United States is an excellent example of how such an approach can work.

However, only a handful of botanical insecticides are in use today on commercially significant vegetable and fruit crops. In this chapter, plant products currently in use will not be reviewed (recent reviews on this topic can be found in Copping and Duke[1] and Isman[6]), but rather new sources and trends for future use and potential commercialization will be discussed.

3.09.2 Commercial Insecticides of Plant Origin

3.09.2.1 4-Allyl-2-Methoxyphenol (Eugenol)

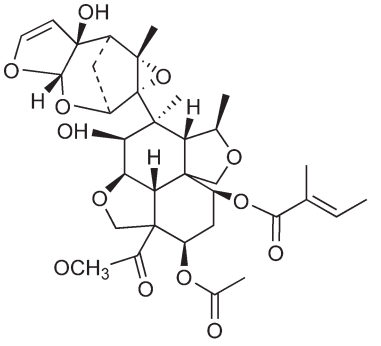
Eugenol is found in a wide range of plants, including laurel (*Laurus* species), and in clove oil. Clove oil is predominantly composed of 4-allyl-2-methoxyphenol, but also contains a small amount of acetyl 4-allyl-2-methoxyphenol. 4-Allyl-2-methoxyphenol is a strong deterrent for most insect species, although in a few cases it can be an attractant. It is sold by a large number of different suppliers under different trade names and is targeted at the home garden market. 4-Allyl-2-methoxyphenol is an irritant and should be used with care. As it is a naturally occurring plant-based phenolic, it is not expected to be hazardous to nontarget organisms or to the environment.

3.09.2.2 Azadirachtin/Dihydroazadirachtin

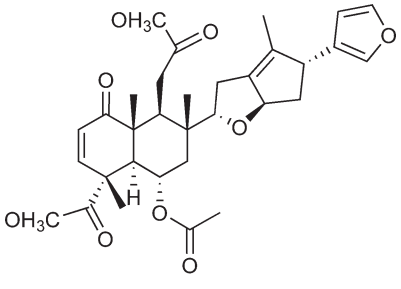
Azadirachtin is extracted from the neem tree (*A. indica* A. Juss). The tree is an attractive broad-leaved evergreen, which is thought to have originated in Burma. It is now grown in the more arid subtropical and tropical zones of Southeast Asia, Africa, the Americas, Australia, and the South Pacific Islands. The neem tree provides many useful compounds used as pesticides. The most significant neem limonoids are azadirachtin, salanin, meliantriol, and nimbin.[9] Products containing azadirachtin can be used in a wide range of crops, including vegetables (such as tomatoes, cabbage, and potatoes), cotton, tea, tobacco, coffee, protected crops and ornamentals, and in forestry. Azadirachtin has several effects on phytophagous insects and is thought to disrupt insect molting by antagonizing the effects of ecdysteroids. This effect is independent of feeding inhibition, which is another observed effect of the compound.[1,10] The antifeedant/repellent effects are dramatic, with many insects avoiding treated crops, although other chemicals in the seed extract, such as salanin, have been shown to be responsible for these effects. Azadirachtin is sold by a large number of different companies as an emulsifiable concentrate (EC) under a wide range of trade names. Azadirachtin-based products are widely used in India and are increasingly popular in North America, where they have found a place for garden use and in organic growing. Azadirachtin is considered to be nontoxic to mammals and is not expected to have any adverse effects on nontarget organisms or on the environment.[1,11,12]

Dihydroazadirachtin is a reduced form of the naturally occurring azadirachtin obtained from the seed kernels of the neem tree. It is effective against a wide range of insect pests. The two compounds are functionally identical in their antipupation properties. Dihydroazadirachtin has both antifeedant and insect growth regulator (IGR) properties. Products based on dihydroazadirachtin are not widely used outside the Indian subcontinent, although it is registered as a technical powder and an end-use product for indoor and outdoor use in the United States. Dihydroazadirachtin exhibits low toxicity to mammals, and risk to the environment is not expected because, under approved use conditions, it is not persistent, is relatively short-lived in the environment, and is metabolized by ubiquitous microorganisms in the soil and aquatic environments.[1]

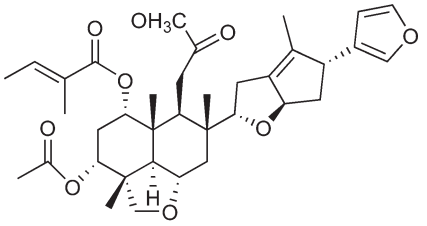
The toxicological data for neem-based preparations show that the nonaqueous extracts appear to be the most toxic, the unprocessed materials, seed oil and the aqueous extracts being less toxic. For all preparations, a reversible effect on the reproductive capacity of both male and female mammals seems to be the most important toxic effect subsequent to subacute or chronic exposure.[13] This is the reason why an array of azadirachtin- and neem extract-based insecticides and pesticides are available on the market today.



Azadirachtin



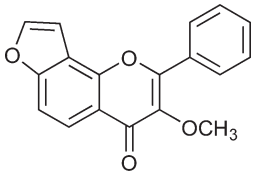
Nimbin



Salanin

3.09.2.3 Karanjin

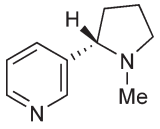
Karanjin is extracted from *Derris indica* (Lam.) Bennet (synonym *Pongamia pinnata* (L.) Pierre). Karanjin is a potent deterrent to many different genera of insects and mites in a wide range of crops. Karanjin has a dramatic antifeedant/repellent effect, with many insects avoiding treated crops. It suppresses the effects of ecdysteroids and thereby acts as an IGR and antifeedant. There are claims that it inhibits cytochrome P-450 in susceptible insects and mites. Karanjin has not achieved wide acceptance as an insecticide. There is no evidence of allergic or other adverse effects, and it is not expected that karanjin-based products will have any adverse effects on nontarget organisms or on the environment.[1]



Karanjin

3.09.2.4 Nicotine

Nicotine is the main bioactive component of the tobacco plants *Nicotiana tabacum* L., *N. glauca* Graham, and, particularly, the species *N. rustica* L. It is also present in a number of other plants belonging to the families Lycopodiaceae, Crassulaceae, Leguminosae, Chenopodiaceae, and Compositae. The average nicotine content of the leaves of *N. tabacum* and *N. rustica* is 2–6% dry weight. It is used for the control of a wide range of insects, including aphids, thrips, and whitefly, on protected ornamentals and field-grown crops, including orchard fruit, vines, vegetables, and ornamentals.



Nicotine

It was once prepared from the extracts of the tobacco plant but is now often obtained from waste of the tobacco industry, or it is synthesized. Nicotine is a nonsystemic insecticide[14] that binds to the cholinergic acetylcholine nicotinic receptor (nAch) in the nerve cells of insects, leading to a continuous firing of this neuroreceptor.[15] Nicotine has been used for many years as a fumigant for the control of many sucking insects. Nicotine is very toxic to humans by inhalation and by skin contact. It is toxic to birds, fish, and other aquatic organisms, and is toxic to bees, but has a repellent effect. In the United Kingdom, nicotine is subject to regulation under the Poisons Act. The use of nicotine as a pesticide is banned in South Africa, severely

restricted in Hungary, canceled in Australia and New Zealand, as well as not being registered in numerous African, Asian, and European countries.[1]

3.09.2.5 Phenethyl Propionate

Phenethyl propionate is also used as an herbicide and as an insecticide/insect repellent and sold under a wide range of trade names in combination with other plant-derived natural products (plus eugenol plus geraniol). The major use is in homes and gardens.[1]

3.09.2.6 Plant-Derived Oils

A wide range of plant oils are being sold for insect and mite control. Among these are canola oil, refined edible vegetable oil obtained from the seeds of two species of rape plants (*Brassica napus* L. and *B. campestris* L.) of the family Cruciferae (mustard family), jojoba oil, derived from jojoba seeds, oleoresin, derived from *Capsicum* spp., oil of anise, soybean oil, and eucalyptus oil. More recently, hexa-hydroxyl, sold as a granular formulation (GR) containing 2.90% eugenol and 0.60% thyme oil as the active ingredients, and BugOil, made from the essential oils (EOs) of three plant species, thyme (*Thymus vulgaris* L.), wintergreen (*Gaultheria procumbens* L.), and African marigold (*Tagetes erecta* L.), have been commercialized. Few of these oils have been fully characterized chemically. Various claims are made for the mode of action, including insect repellency caused by altering the outer layer of the leaf surface, acting as an insect irritant, and preventing gas exchange (suffocation) and water loss by covering the insect's body.[16] The potassium salts of plant oils (soft soaps) are also sold as insecticides under a wide range of trade names by many different manufacturers. Insecticidal soaps have not been chemically fully characterized and are contact insecticides, causing a breakdown of the target pest's cuticle, leading to dehydration and, ultimately, death. They cause the rapid knockdown of phytophagous insects, but, because they are broken down rapidly once sprayed, they will not prevent subsequent reinvasion. They are often used in conjunction with insect predators, being used to bring the populations down to manageable levels prior to release.[1]

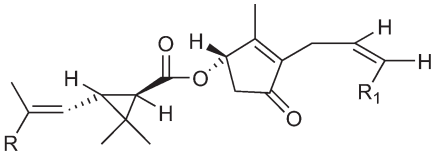
3.09.2.7 Plant-Derived Acids

A number of acids of plant origin are sold for insect control. These include citric acid, recommended for use against a wide range of insects, fatty acids (often oleic acid), and formic acid, used to control varroa (*Varroa destructor*) and tracheal mites in honeybees. The mode of action of citric acid is not identified with certainty. Formic acid is a severe irritant and acts by directly killing the mites without disrupting bee behavior or life span substantially. Oleic acid interferes with the cell membrane constituents of the target organism, leading to a breakdown of the integrity of the membrane and subsequent death.[1]

3.09.2.8 Pyrethrins, Chrysanthemates, and Pyrethrates

Pyrethrins, chrysanthemates, and pyrethrates are extracted from the flower of *T. cinerariaefolium* (Trevisan). The extract is refined using methanol or supercritical carbon dioxide. The dried, powdered flower of *T. cinerariaefolium* has been used as an insecticide from ancient times. The species was identified in antiquity in China, and it spread to the west via Iran (Persia), probably via the Silk Routes in the Middle Age, known as 'Persian insect powder'.[17] Records of use date from the early nineteenth century when it was introduced to the Adriatic coastal regions of Croatia (Dalmatia) and some parts of the Caucasus. Subsequently, it was grown in France, the United States, and Japan. Plants producing these compounds are now widely grown in East African countries, especially in Kenya (1930), in Ecuador, Papua New Guinea (1950), and in Australia (1980). The pyrethrins include pyrethrin I, cinerin I, jasmolin I, pyrethrin II, cinerin II, and jasmolin II. They have been shown to bind to and activate the voltage-sensitive sodium channels of nerve, heart, and skeletal muscle cell membranes in insect nervous systems, prolonging their opening and thereby causing knockdown and death.

They are nonsystemic insecticides with contact action. Initial effects include paralysis, with death occurring later. They have some acaricidal activity.[18] They are approved for use in organic production. Pyrethrins have moderate mammalian toxicity, and there is no evidence that the addition of synergists increases this toxicity. The compounds show low toxicity to birds, but are highly toxic to fish and honeybees (although they exhibit a repellent effect on bees).[1]

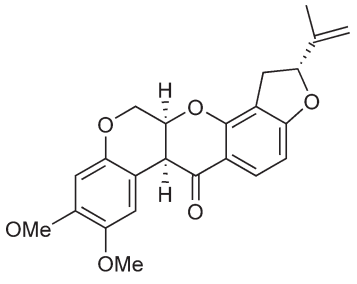


R = —CH_3 (chrysanthemates) or —CO_2CH_3 (pyrethrates)

R_1 = —$CH{=}CH_2$ (pyrethrin) or —CH_3 (cinerin) or —CH_2CH_3 (jasmolin)

3.09.2.9 Rotenone

Rotenone, also known as derris root, tuba root, and aker tuba (for the plant extract) and barbasco, cube, haiari, nekoe, and timbo (for the plants), is obtained from *Derris*, *Lonchocarpus*, and *Tephrosia* species, which were used originally in Asia and South America as fish poisons. The four major active ingredients are rotenone, deguelin, rotenolone, and tephrosin acting as inhibitors of NADH-ubiquinone oxidoreductase activity depending on the overall molecular configuration and the E-ring substituents.[19] Rotenone is used to control a wide range of arthropod pests. It is an inhibitor of site I respiration within the electron transport chain of susceptible insects and is a selective, nonsystemic insecticide with contact and stomach action and secondary acaricidal activity.[20] Rotenone has been cleared for use in organic farming when insect pressure is very high. Rotenone has a high mammalian toxicity, with the estimated lethal dose for humans being 300–500 mg kg^{-1}. It is more toxic when inhaled than when ingested and is very toxic to pigs. It is not toxic to bees, but combinations with pyrethrum are very toxic. It is very toxic to fish and must not be used near water courses.[1]

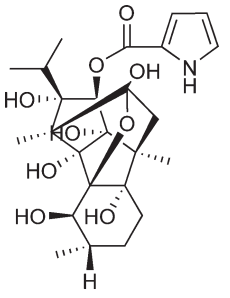


Rotenone

3.09.2.10 Ryania Extract

The alkaloids from the stem of *Ryania* species, particularly *R. speciosa* Vahl, represent the first successful discovery of a natural insecticide. The collaboration between Rutgers University and Merck in the early 1940s followed the lead from the use of *Ryania* species in South America for euthanasia and as rat poisons. This collaborative work revealed that *Ryania* alkaloid extracts were insecticidal. Ryanodine and related alkaloids affect muscles by binding to the calcium channels in the sarcoplasmic reticulum. This causes calcium ion flow into the cells, and death follows very rapidly.[21] *Ryania* extracts have had limited use as insecticides, but they do give effective control of selected species. The size and complexity of the natural compound means that it can be

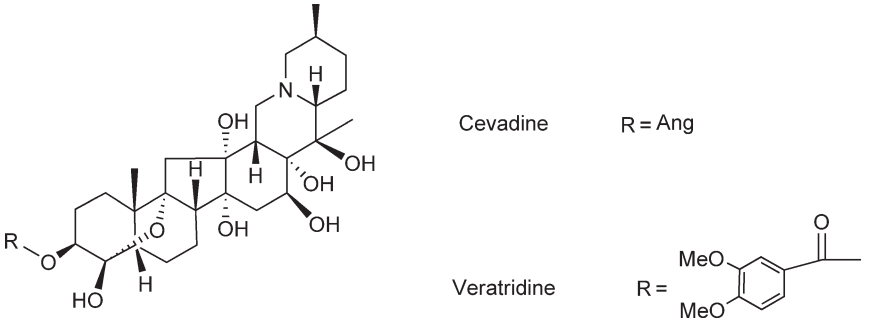
used economically only to treat infested crops, and it has no systemic activity. The rapidity of its effect is an advantage in the control of boring insects. More recently, a new class of insecticides has been discovered that provides exceptional control through action on a novel target, the ryanodine receptor, for example, Rynaxypyr™, anthranilic diamides, and substituted phthalic acid diamides with potent insecticidal activity. These substances activate ryanodine-sensitive intracellular calcium release channels in insects.[22–24] *Ryania* extracts are moderately toxic to mammals, but very toxic to fish.



Ryanodine

3.09.2.11 Sabadilla

Sabadilla is an insecticidal preparation from the crushed seeds of the liliaceous plant *S. officinale* Gray (formerly *Veratrum sabadilla* Retr.), which was used by native people of South and Central America as an insecticide for many years. Sabadilla has been used commercially since the 1970s. The seeds of *S. officinale* contain a mixture of alkaloids (veratrine) consisting of an approximately 2:1 mixture of cevadine and veratridine, in combination with many minor components, all of which are esters of the alkamine veracine. The product is produced by grinding the seeds of the plant and subsequent concentration. The seeds contain between 2 and 4% alkaloids. Cevadine, veratridine, and related ceveratrum alkaloids have a mode of action that is similar to that of the pyrethrins, in that they activate the voltage-sensitive sodium channels of nerve, heart, and skeletal muscle cell membranes, although the binding site appears to be different from that of the pyrethroids.



They are nonsystemic insecticides with contact action. Initial effects include paralysis, with death occurring later.[1] Sabadilla powder is not used widely in crop protection, but it is approved for use in organic farming systems. This powder has a low mammalian toxicity, but it is an irritant to mucous membranes. Sabadilla powder is not active against beneficial insects and may be used in insect control strategies that use them.[25]

3.09.2.12 Starch Syrup

A new insecticide prepared from reduced starch syrup has just been made available by Kyoyu Agri. It is sold under the trade name YE-621 and works by obstructing the spiracles of insect pests, causing suffocation. YE-621

is potentially effective against insect pests that are resistant to chemical-based insecticides. It is nontoxic to humans and beneficial insects and/or natural predators. The main component of YE-621 is starch syrup mainly from corn and potatoes.[1,26]

3.09.3 New Insecticide Sources

3.09.3.1 Plant Essential Oils

Plant EOs are produced commercially from cultivated plants mainly from the Lamiaceae family. EOs are complex mixtures of monoterpenes, sesquiterpenes, and aromatic compounds. Steam distillation of aromatic plants yields EOs used in perfumery, traditional medicine, pharmaceutical preparations, herbal beverages, and as natural flavorings.[6,27]

Since the middle ages, EOs have been widely used for bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, medicinal, and cosmetic applications, and today, they are particularly vital to the pharmaceutical, health, cosmetic, agricultural, and food industries. While *in vitro* physicochemical assays characterize most of these as antioxidants, recent work shows that in eukaryotic cells EOs can act as prooxidants affecting inner cell membranes and organelles such as mitochondria. Depending on the type and concentration, they exhibit cytotoxic effects on living cells, but are usually nongenotoxic.[28] Plant EOs and their components have low mammalian toxicity, but not all compounds found in plant EOs are safe. Estragole and (+)-fenchone found in the EO of *Foeniculum vulgare* are highly effective against *Sitophilus oryzae*, *Callosobruchus chinensis*, and *Lasioderma serricorne* adults and are known to be carcinogenic.[29] Similarly, safrole and β-asarone have been included in the list of carcinogenic compounds.

Some aromatic plants have been traditionally used for the protection of stored commodities due to their fumigant and contact toxicity effects. Fumigant toxicity tests conducted with EOs of plants (mainly belonging to Apiaceae, Lamiaceae, Lauraceae, and Myrtaceae) and their components (cyanohydrins, monoterpenoids, sulfur compounds, thiocyanates, and others) have largely focused on beetle pests such as *Tribolium castaneum*, *Rhyzopertha dominica*, *S. oryzae*, and *Sitophilus zeamais*.[8] Promising results have been obtained from a few EOs tested as repellents against head lice, *Pediculus humanus capitis* (Phthiraptera: Pediculidae), an ectoparasite preying on humans that causes pediculosis capitis, although *in vitro* tests and clinical trials often produce contradictory results. A handful of fixed extracts and several EOs and their individual components have also been tested as contact pediculicides or fumigants.[30] There is also renewed interest in the use of EOs as antimalarials in the form of biocidal (insect repellent) preparations against mosquitoes to prevent infection.[31]

The swift results obtained from some of these oils suggest neurotoxic action. There is evidence of some common oil components such as thujone,[32] thymol,[33] and menthol and borneol[34] interfering with the octopamine receptor[35,36] and γ-aminobutyric acid (GABA)-gated chloride channels. Moreover, several reports indicate that monoterpenoids raise insect mortality by inhibiting acetylcholinesterase enzyme (AChE) activity.[8,37] However, it has been shown that the insecticidal effects of some EOs cannot be explained by the action of their major components, suggesting that their insecticidal action is the result of a synergistic effect.[38,39]

Variations in the composition of EOs due to factors such as seasonal fluctuations, differences in the region of origin, extraction method used (steam or hydro-distillation, solvent extraction, and maceration), and the plant part used for extraction have been reported.[38–41] Therefore, careful attention should be paid to the presence of oil chemotypes for a given plant species.

Since EOs can often be extracted from cultivated plants, are readily available, and do not require further purification, there is an increasing interest in the study of their insecticidal effects and other properties. **Table 1** shows the publications on this topic for the years 2006–08 (April) as proof of this renewed interest.

3.09.3.2 Monoterpenes

Monoterpenes are the main components of plant EOs and, like these oils, have also been tested for their insecticidal effects. Some mosquito repellents include *p*-menthane-3,8-diol from mint as the active ingredient, and citronellal is also used in mosquito coils. A number of veterinary products for flea and tick control on

Table 1 Insecticidal essential oils (EOs) for the period 2006–08

Plant species	*Target insect*	*Action*	*Reference*
Achillea biebersteinii, A. wilhelmsii	*Sitophilus granarius, Tribolium confusum*	Fumigant toxicity	Calmasur *et al.*[115]
Acorus gramineus	*Lycoriella ingenua*	Toxic	Park *et al.*[116]
Allium sativum	*Lycoriella ingenua*	Toxic	Park *et al.*[117]
Alpinia calcarata	*Callosobruchus maculatus*	Fumigant toxicity and repellent	Abeywickrama *et al.*[118]
Apium graveolens	*Aedes aegypti*	Adulticidal	Chaiyasit *et al.*[119]
Armoracia rusticana	*Lycoriella ingenua*	Toxic	Park *et al.*[117]
Artemisia annua	*Tribolium castaneum*	Fumigant toxicity, repellent	Goel *et al.*[120]
A. herba-alba, A. monosperma	*Bemisia tabaci, Aphis gossypii, Thrips tabaci*	Toxic	Soliman *et al.*[121,122]
A. sieberi	*Callosobruchus maculatus, Sitophilus oryzae, Tribolium castaneum*	Fumigant toxicity	Negahban *et al.*[123]
A. vulgaris	*Thrips palmi*	Repellent	Yi *et al.*[124]
	Tribolium castaneum	Fumigant toxicity	Wang *et al.*[125]
A. princeps	*Sitophillus oryzae, Bruchus rugimanus*	Fumigant toxicity	Liu *et al.*[126]
A. nilagirica	*Aedes aegypti, Anopheles stephensi, Culex quinquefasciatus*	Larvicidal	Verma *et al.*[127]
Carum carvi	*Lycoriella ingenua*	Fumigant toxicity	Park *et al.*[128]
	Aedes aegypti	Adulticidal	Chaiyasit *et al.*[119]
Chamaecyparis formosensis	*Aedes aegypti, A. albopictus*	Larvicidal	Kuo *et al.*[129]
Chenopodium ambrosioides	*Lycoriella ingenua*	Toxic	Park *et al.*[116]
Chloroxylon swietenia	*Helicoverpa armigera*	Antifeedant	Kiran *et al.*[130]
	Anopheles gambiae, Culex quinquefasciatus, Aedes aegypti	Fumigant toxicity	
	Spodoptera litura	Toxic	Kiran *et al.*[131]
Cinnamomun cassia	*Chrysomya megacephara*	Ovicidal	Shen *et al.*[132]
C. camphora	*Resseliella oculiperda*	Repellent	Van Tol *et al.*[133]
	Sitophillus oryzae, Bruchus rugimanus		Liu *et al.*[126]
C. zeylanicum	*Musca domestica*	Knock down and mortality	Samarasekera *et al.*[134]
Citrus reticulate	*Lycoriella ingenua*	Toxic	Park *et al.*[128]
Convallaria majalis	*Ixodes ricinus*	Repellent	Thorsell *et al.*[135]
Coriandrum sativum	*Thrips palmi*	Fumigant toxicity	Yi *et al.*[124]
Croton nepetaefolius	*Aedes aegypti*	Larvicidal	Morais *et al.*[136]
C. argyrophyloides			
C. sonderianus			
C. zenhtneri			
Cryptomeria japonica	*Aedes aegypti, A. albopictus*	Larvicidal	Cheng *et al.*[137]
	Lepisma saccharina	Repellent and insecticide	Wang *et al.*[138]
Cuminum cyminum	*Lycoriella ingenua*	Toxic	Park *et al.*[128]
	Tribolium castaneum	Fumigant toxicity	Chaubey *et al.*[139]
Cupressus sempervirens	*Aedes aegypt*	Adulticidal	Chaiyasit *et al.*[119]
	Thrips palmi	Fumigant toxicity	Yi *et al.*[124]
Curcuma zedoaria	*Aedes aegypti*	Adulticidal	Chaiyasit *et al.*[118]
C. longa	*Wild mosquitoes, anthropophilic black flies*	Repellent	Tawatsin *et al.*[140]
Cymbopogon citratus	*Lycoriella ingenua*	Toxic	Park *et al.*[128]
	Musca domestica	Knock down and mortality	Samarasekera *et al.*[134]
C. martini	*Callosobruchus chinensis, Tribolium castaneum*	Repellent	Kumar *et al.*[141]
C. schoenanthus	*Callosobruchus maculatus*	Toxic	Ketoh *et al.*[142]
C. nardus	*Musca domestica*	Knock down and mortality	Samarasekera *et al.*[134]

(*Continued*)

Table 1 (Continued)

Plant species	*Target insect*	*Action*	*Reference*
Cymbopogon	*Ixodes ricinus*	Repellent	Thorsell *et al.*[135]
Digitalis purpurea	*Wild mosquitoes*	Repellent	Tawatsin *et al.*[140]
	Anthropophilic black flies		
Eucalyptus grandis	*Aedes aegypti larvae*	Larvicidal	Lucia *et al.*[143]
E. intertexta, E. sargentii, E. camaldulensis	*Callosobruchus maculatus, Sitophilus oryzae, Tribolium castaneum*	Fumigant toxicity	Negahban and Moharramipour[144]
E. tereticornis	*Anopheles stephensi*	Larvicidal, adulticidal	Senthil-Nathan *et al.*[145]
E. cinerea, E. viminalis	*Pediculus humanus capitis (permethrin-resistant)*	Repellent	Toloza *et al.*[146]
E. globulus, E. smithii	*Lycoriella ingenua*	Toxic	Park *et al.*[116]
Flourensia oolepis	*Tribolium castaneum*	Contact toxin	Garcia *et al.*[147]
	Myzus persicae, Leptinotarsa decemlineata	Antifeedant	
Foeniculum vulgare	*Tribolium castaneum*	Fumigant toxicity	Chaubey *et al.*[139]
Hyssopus officinalis	*Thrips palmi*	Fumigant toxicity	Yi *et al.*[124]
Hyptis spicigera	*Callosobruchus maculatus*	Fumigant toxicity repulsive, insecticidal	Noudjou *et al.*[148] Sanon *et al.*[149]
Illicum verum	*Chrysomya megacephara*	Ovicidal	Shen *et al.*[132]
	Aedes aegypti	Adulticidal	Chaiyasit *et al.*[119]
Juniperus oxycedrus	*Lycoriella ingenua*	Toxic	Park *et al.*[128]
J. virginiana	*Resseliella oculiperda*	Repellent	Van Tol *et al.*[133]
Laurus novocanariensis	*Myzus persicae, Rhopalosiphum padi*	Antifeedant	Rodilla *et al.*[39]
L. nobilis	*Tribolium confusum*	Fumigant toxicity	Isikber *et al.*[150]
Lavandula angustifolia	*Resseliella oculiperda*	Repellent	Van Tol *et al.*[133]
	Ixodes ricinus		Jaenson *et al.*[151]
L. luisieri	*Leptinotarsa decemlineata, Myzus persicae*	Antifeedant	Gonzalez-Coloma *et al.*[38]
Lippia gracilis	*Aedes aegypti*	Larvicidal, adulticidal	Silva *et al.*[152]
L. turbinata, L. polystachya	*Culex quinquefasciatus*		Gleiser and Zygadlo[153]
Litsea cubeba	*Aedes aegypti, Anopheles stephensi, Culex quinquefasciatus*	Repellent	Amer *et al.*[154]
Maclura pomifera	*Culex pipiens*	Repellent	Schultz *et al.*[49]
Matthiola longipetala	*Tribolium confusum*	Growth inhibitor	Hammami *et al.*[155]
Melaleuca viridiflora	*Thrips palmi*	Fumigant toxicity	Yi *et al.*[124]
	Cadra cautella	Larvicidal, fumigant toxicity	Sim *et al.*[156]
M. leucadendron, M. quinquenervia	*Aedes aegypti, Anopheles stephensi, Culex quinquefasciatus*	Repellent	Amer *et al.*[154]
Mentha piperita, M. spicata	*Culex quinquefasciatus, Aedes aegypti, Anopheles tessellatus*	Fumigant toxicity	Samarasekera *et al.*[157]
M. pulegium	*Pediculus humanus capitis*	Repellent	Toloza *et al.*[146]
	Thrips palmi	Fumigant toxicity	Yi *et al.*[124]
	Dermatophagoides farinae, D. pteronyssinus	Toxic	Rim and Jee[158]
Micromeria fruticosa	*Tetranychus urticae, Bemisia tabaci*	Fumigant toxicity	Calmasur *et al.*[159]
Myristica fragrans	*Culex quinquefasciatus, Aedes aegypti, Anopheles tessellatus*	Fumigant toxicity	Park *et al.*[128]
Myrtus communis	*Phlebotomus papatasi,*	Repellent	Yaghoobi-Ershadi *et al.*[160]
	Thrips palmi	Fumigant toxicity	Yi *et al.*[124]
Nepeta cataria	*Blattella germanica, Musca domestica, Aedes aegypti*	Repellent	Schultz *et al.*[49]
	Anopheles stephensi, Culex quinquefasciatus	Repellent	Amer *et al.*[154]
N. racemosa	*Tetranychus urticae, Bemisia tabaci*	Fumigant toxicity	Calmasur *et al.*[159]
Ocimum canum	*Anopheles gambiae*	Toxic	Njan-Nloga *et al.*[161]

(*Continued*)

Table 1 (Continued)

Plant species	*Target insect*	*Action*	*Reference*
O. basilicum	*Thrips palmi, Sitophilus oryzae*	Fumigant toxicity, insecticidal	Yi *et al.*[124]
O. sanctum	*Aedes aegypti, Anopheles stephensi, Culex quinquefasciatus*	Larvicidal	Popović *et al.*[162]
			Verma *et al.*[127]
Origanum acutidens	*Lasioderma serricorne, Sitophilus granarius, Ephestia kuehniella*	Fumigant toxicity	Caglar *et al.*[163]
O. onites	*Thaumetopoea wilkinsoni*	Larvicidal	Cetin *et al.*[164]
	Culex pipiens		Cetin and Yanikoglu[165]
O. marjorana	*Thrips palmi*	Fumigant toxicity	Yi *et al.*[124]
O. minutiflorum	*Culex pipiens*	Larvicidal	Cetin and Yanikoglu[165]
O. vulgare	*Tetranychus urticae, Bemisia tabaci*	Fumigant toxicity	Calmasur *et al.*[159]
Pelargonium graveolens	*Ixodes ricinus*	Repellent	Jaenson *et al.*[151]
Pilocarpus spicatus	*Rhodnius prolixus*	Toxic	Mello *et al.*[166]
Pimenta racemosa	*Blatella germanica*	Toxic	Leyva *et al.*[167]
Pimpinella anisum	*Lycoriella ingenua*	Toxic	Park *et al.*[117]
Piper betle	*Musca domestica*	Fumigant – acute toxicity	Mohottalage *et al.*[168]
P. nigrum	*Callosobruchus maculatus, Sitophilus zeamais, Rhizopertha dominica,Tribolium castaneum*	Fumigant toxicity	Gragasin *et al.*[169]
P. longum	*Aedes aegypti*	Adulticidal	Chaiyasit *et al.*[119]
P. aduncum, P. hispidinervum	*Sitophilus zeamais*	Insecticidal	Vidal-Estrela *et al.*[170]
Plectrancthus glandulosus	*Anopheles gambiae*	Toxic	Njan-Nloga *et al.*[161]
Pogostemon cablin	*Preris rapae, Plutella xylostella*	Insecticidal	Zeng *et al.*[171]
Psidium spp.	*Wild mosquitoes, anthropophilic black flies*	Repellent	Tawatsin *et al.*[140]
Rosmarinus officinalis	*Thrips palmi*	Fumigant toxicity	Yi *et al.*[124]
	Tribolium confusum		Isikber *et al.*[150]
	Cadra cautella	Larvicidal, fumigant toxicity	Sim *et al.*[155]
Salvia hydrangea	*Sitophilus granarius, Tribolium confusum*	Toxic	Kotan *et al.*[172]
S. officinalis	*Leptinotarsa decemlineata*	Toxic	Kostic *et al.*[173]
	Thrips palmi	Fumigant toxicity	Yi *et al.*[124]
	Sitophilus oryzae	Toxic	Popović *et al.*[162]
Satureja spinosa, S. parnassica, S. thymbra, S. montana	*Culex pipiens*	Larvicidal	Michaelakis *et al.*[174]
Schizonepeta tenuifolia	*Lycoriella ingenua*	Toxic	Park *et al.*[116]
Syzygium aromaticum	*Ixodes ricinus*	Repellent	Thorsell *et al.*[135]
Thuja occidentalis	*Thrips palmi*	Fumigant toxicity	Yi *et al.*[124]
T. vulgaris	*Musca domestica*	Fumigant toxicity, adulticidal	Park *et al.*[128]
		Larvicidal	Pavela[175]
Viola odorata	*Aedes aegypti, Anopheles stephensi, Culex quinquefasciatus*	Repellent	Amer *et al.*[154]
X. aethiopica	*Sitophilus zeamais*	Acute toxicity	Kouninki *et al.*[176]
Zanthoxylum piperitum	*Aedes gardnerii, Anopheles barbirostris, Armigeres subalbatus, Culex tritaeniorhynchus, C. gelidus, C. vishnui group, Mansonia uniformis*	Repellent	Kamsuk *et al.*[177]
Z. armatum	*Aedes aegypti, Anopheles stephensi, Culex quinquefasciatus*	Larvicidal	Tiwary *et al.*[178]
Z. piperitum	*Lycoriella ingenua*	Toxic	Park *et al.*[116]

IGR, insect growth regulation effects.

domestic pets contain *d*-limonene from citrus peels as the active ingredient. Another important use of EO components is for the fumigation of beehives to control the honeybee parasite varroa (*Varroa Jacobson* and *V. destructor*) and the tracheal mite (*Acarapis woodi*). Thymol[42–45] and menthol[46,47] are used to control these mites. Other monoterpenes have also been tested: linalyl acetate, (*R*)-myrtenyl acetate, (*S*)-perillyl acetate, although thymyl acetate exhibited high toxicity against *V. destructor* and significantly lower toxicity against *A. mellifera*.[48] Camphor and eucalyptol are also used for this purpose.[47] Several monoterpenoids exhibit toxicity against stored product and urban pests, are good spatial repellents, and could be used in pest control.[49]

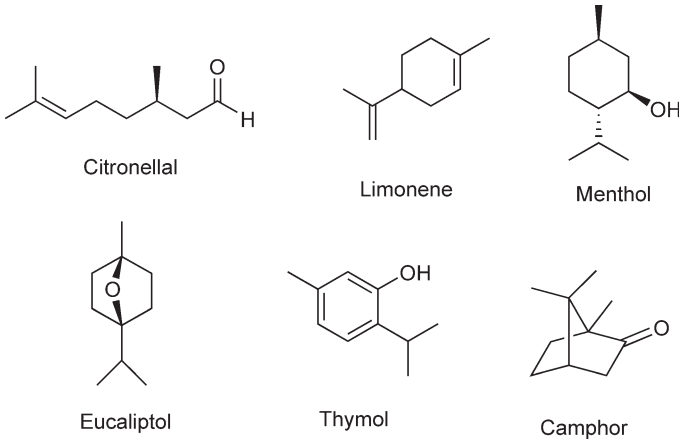


Table 2 provides an overview of the latest publications on insecticidal monoterpenes for the period 2006–08 (in part). Most of the compounds are known structures and have been studied as part of broader EO research.

3.09.3.3 Sesquiterpenes

Sesquiterpenes feature a different set of characteristics, which also have an influence on insect activity, most effectively as contact irritants.[49] Many species of the Celastraceae family such as the Chinese bittersweet (*Celastrus angulatus*) are widely distributed and used as traditional insecticides in China. These plants contain dihydro-β-agarofuran sesquiterpenoids based on a tricyclic 5,11-epoxy-5β,10α-eudesman-4(14)-ene skeleton. The compact tricyclic scaffold seems to be a prerequisite for antifeedant or insecticidal activity as are the substitutions at C-1, C-6, and C-8. Nicotinic diacid substituent may also be involved in the antifeedant activity, possibly through neuronal action of nicotinic diacid.[50] An emulsifiable mixture of celangulins has been developed for insect control.[51] This functions as a digestive poison acting on the midgut tissue of the target insect larvae. Celangulins have structure-dependent effects on insect voltage-gated sodium channels[52] and inhibit carboxylesterase activity.

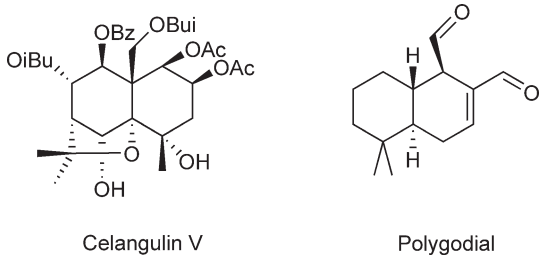


Table 2 Insecticidal monoterpenes for the period 2006–08 (in part)

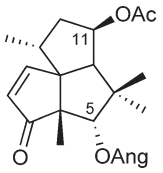
Monoterpenes	*Type*	*Target insect*	*Action*	*Reference*
Borneol	Camphane	*Sitophilus oryzae*	Fumigant	Rozman *et al.*[179]
Camphor	Camphane	*Pseudaletia unipuncta*	Toxic	Isman *et al.*[180]
		Rhyzopertha dominica		Rozman *et al.*[179]
3-Carene	Carane	*Aedes aegypti*	Larvicidal	Cheng *et al.*[137]
Carvacrol	Menthane	*Thaumetopoea wilkinsoni*	Larvicidal	Cetin *et al.*[164]
R-Carvone	Menthane	*Resseliella oculiperda*	Repellent action	Van Tol *et al.*[133]
1,8-Cineole	Menthane	*Pediculus humanus capitis*	Toxic, fumigant	Picollo *et al.*[181]
		(permethrin-resistant), Sitophilus oryzae	toxicity	Rozman *et al.*[179]
			Antifeedant	Rodilla *et al.*[39]
		Myzus persicae, Rhopalosiphum padi	Fumigant toxicity,	Abeywickrama
		Callosobruchus maculatus	repellent	*et al.*[118]
Citronellal	Linear	*Musca domestica*	Toxic	Mohottalage *et al.*[168]
		Resseliella oculiperda	Repellent	Van Tol *et al.*[133]
Citronellol	Linear	*Ixodes ricinus*	Repellent	Thorsell *et al.*[135]
(*R*)-Fenchone	Fenchane	*Resseliella oculiperda*	Repellent action	Van Tol *et al.*[133]
Geraniol	Linear	*Ixodes ricinus*	Repellent	Thorsell *et al.*[135]
Geranyl acetate	Linear	*Musca domestica*	Knock down and mortality	Samarasekera *et al.*[134]
Linalool	Linear	*Rhyzopertha dominica*	Fumigant	Rozman *et al.*[179]
		Myzus persicae, Rhopalosiphum padi	Antifeedant	Rodilla *et al.*[39]
		Thrips palmi	Fumigant toxicity	Yi *et al.*[124]
		Resseliella oculiperda	Repellent	Van Tol *et al.*[133]
L-Menthol	Menthane	*Culex quinquefasciatus, Aedes aegypti,*	Toxic	Samarasekera
		Anopheles tessellatus		*et al.*[157]
Menthone	Menthane	*Lycoriella ingenua*	Toxic	Park *et al.*[116]
α-Pinene, β-Pinene	Pinane	*Aedes aegypti larvae*	Larvicidal	Lucia *et al.*[143]
β-Pinene		*Myzus persicae, Rhopalosiphum padi*	Antifeedant	Rodilla *et al.*[39]
		Sitophilus zeamais	Acute toxicity	Kouninki *et al.*[176]
Piperitone	Menthane	*Callosobruchus maculates*	IGR	Ketoh *et al.*[142]
Pulegone	Menthane	*Lycoriella ingenua*	Toxic	Park *et al.*[116]
Limonene	Menthane	*Lycoriella ingenua*	Toxic	Park *et al.*[116]
(+)-Terpinen-4-ol	Menthane	*Sitophilus zeamais*	Acute toxicity	Kouninki *et al.*[176]
		Pediculus humanus	adulticidal, ovicidal	Priestley *et al.*[182]
μ-Terpineol	Menthane	*Trichoplusia ni (Noctuidae)*	Toxic	Isman *et al.*[180]
Terpinolene	Menthane	*Aedes albopictus*	Larvicidal	Cheng *et al.*[137]
Thymol	Menthane	*Sitophilus oryzae*	Fumigant	Rozman *et al.*[179]
		Thaumetopoea wilkinsoni	Larvicidal	Cetin *et al.*[164]
		Trichoplusia ni		Wilson and Isman[183]
α-Terpineol	Menthane	*Resseliella oculiperda*	Repellent action	Van Tol *et al.*[133]
(*Z,E*)-Nepetalactone	Iridoid	*Musca domestica*	Toxic	Schultz *et al.*[49]
(*E,Z*)-Nepetalactone		*Blatella germanica*		
Nepetaparnone	Iridoid	*Mosquito*	Larvicidal	Gkinis *et al.*[184]
Nepetanudone				

IGR, insect growth regulation effects.

Naturally occurring sesquiterpenoid dialdehydes of the drimane series such as polygodial, warburganal, and muzigadial isolated from *Polygonum* and *Warburgia* spp. (Polygonaceae) have been thoroughly researched owing to their strong antifeedant activities and considerable attention has been devoted to the synthesis of these compounds.[53] The reactivity of the unsaturated dialdehyde functionality toward biological nucleophiles is considered to account for the antifeedant activity of these substances.[53] The antifeedant activity of polygodial acetal derivatives (propylene and ethylene) is consistent with the proposed adduct formation with amino groups.[54] However, the lack of correlation between reactivity toward nucleophiles and the antifeedant effects of

polygodial and warburganal suggests that their insect antifeedant action may depend on other properties as indicated by the activity of ketoaldehydes and 3-hydroxydrimanes.[55]

The silphinenes are tricyclic sesquiterpenes isolated from *Senecio palmensis* (Asteraceae) that have antifeedant and toxic effects on insects and structural similarity to the known GABA antagonist picrotoxinin. C-5, C-11, and C-5-substituted silphinenes were active antifeedants against several insect (*Spodoptera littoralis*, *Leptinotarsa decemlineata*) and aphid species. All insects tested responded to at least one silphinene analog and/or GABA modulator (picrotoxinin/thymol), suggesting a shared GABA-mediated taste regulation mode of action for these species.[56,57] Furthermore, it has recently been shown that silphinenes interact with the GABA receptor of *Drosophila melanogaster* larvae in a manner different from pycrotoxinin (PTX), and that *rdl* resistance (resistant via an altered GABA receptor) in the field may have little effect on silphinene efficacy.[58]



Silphinene deriv.

Mixtures that include both monoterpenes (acting as a good spatial repellent) and sesquiterpenes (good contact repellent) are extremely effective via both modes of action and show potential for residual repellent action from a natural product.[49]

Table 3 shows the latest publications on insecticidal sesquiterpenes. The number of compound hits is similar to that of monoterpenes; however, new structures are described and these are mostly antifeedants in contrast to the monoterpenes shown in **Table 2**, which are all known and mostly toxic (fumigants). Therefore, sesquiterpenes can be considered as an interesting source of molecular models with potentially useful insect antifeedant properties.

Table 3 Insecticidal sesquiterpenes for the period 2006–08 (in part)

Sesquiterpenes	*Type*	*Target insect*	*Action*	*Reference*
Nerolidol	Linear	*Pediculus humanus*	Adulticide, ovicidal	Priestley *et al.*[182]
Polygodial derivatives	Drimane	*Spodoptera littoralis, Leptinotarsa decemlineata, Myzus persicae, Rhopalosiphum padi*	Antifeedant	Moreno-Osorio *et al.*[54]
(+)-Pterocarpol	Eudesmane	*Reticulitermes speratus* *Spodoptera litura*	Antifeedant	Morimoto *et al.*[185]
1α-Tigloyloxy-8β*H*,10β*H*-eremophil-7(11)-en-8α,12-olide	Eremophilanolide	*Senecio poepigii*	Antifeedant	Reina *et al.*[186]
6-Hydroxyeuryopsin, 6-acetyloxy-1(10)-epoxyeuryopsin	Furanoeremophilane	*Leptinotarsa decemlineata*	Antifeedant	Burgueño-Tapia *et al.*[187]
Cacalol acetate	Cacalolide	*Leptinotarsa decemlineata*	Antifeedant	Burgueño-Tapia *et al.*[187]
Aguerin B, chlorojanerin, janerin, cynaropicrin	Guaianolide	*Sitophilus granarius, Trogoderma granarium, Tribolium confusum*	Antifeedant	Cis *et al.*[188]
Artesin, taurin, artemin	Eudesmanolide	*Spodoptera littoralis*	Antifeedant	Susurluk *et al.*[189]

(*Continued*)

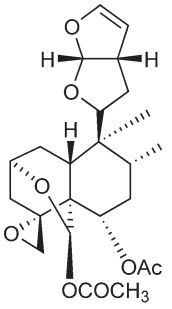
Table 3 (Continued)

Sesquiterpenes	*Type*	*Target insect*	*Action*	*Reference*
Aureane	Bisabolane	Aphids??	Toxic	Baser *et al.*[190]
Traginone	Norsesquiterpene	Aphids	Toxic	Baser *et al.*[190]
Pogostone	Norsesquiterpene	*Preris rapae, Plutella xylostella*	Toxic	Zeng *et al.*[171]
Caryophyllene oxide	Caryophyllane	*Aedes aegypti larvae*	Toxic	Silva *et al.*[152]
		Leptinotarsa decemlineata *Spodoptera littoralis*	Antifeedant	Rodilla *et al.*[39]
Celangulatins A and B Celangulins IV and V Celangulatins C–F	Eudesmane	*Mythimna separata*	Toxic	Wang *et al.*[191] Ji *et al.*[192]
Clavigerins A–C	Bergamotane	*Tineola bisselliella Anthrenocerus australis*	Antifeedant	Perry *et al.*[193]
Elemol	Elemane	*Culex pipiens*	Larvicidal	Schultz *et al.*[49]
Geijerene, pregeijerene	Norsesquiterpene	*Helicoverpa armigera*	Antifeedant and toxic	Kiran *et al.*[130]
		Anopheles gambiae	Fumigant toxicity	Kiran and Devi[194]
		Culex quinquefasciatus *Aedes aegypti*		
		Spodoptera litura	Antifeedant, oviposition deterrent	Kiran *et al.*[132]
Germacrene D	Germacrane	*Anopheles gambiae*	Fumigant toxicity	Kiran and Devi[194]
		Culex quinquefasciatus *Aedes aegypti*		
Hugonianene A	Himachalene	*Anopheles gambiae*	Larvicidal	Baraza *et al.*[195]
(±)-, (+)-, (−)-Gossypol	Cadinane	*Helicoverpa zea*	Toxic, IGR	Stipanovic *et al.*[196]
Tavulin, tanachin, tamirin	Germacranolide	*Spodoptera littoralis*	Antifeedant	Susurluk *et al.*[189]
Tutin, 2-*iso*-butenoyl-tutin	Tutin group	*Mythimna separata*	Antifeedant	Li *et al.*[197]
Nepetaparnone, nepetanudone	Iridoid	*Mosquito*	Larvicidal	Gkinis *et al.*[184]

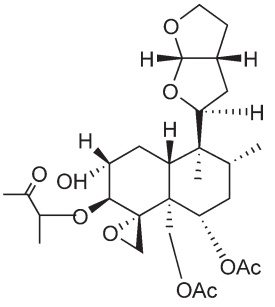
IGR, insect growth regulation effects.

3.09.3.4 Diterpenes

Clerodane diterpenoids have been found in hundreds of plant species from a number of different families. Several genera from the Verbenaceae and Lamiaceae families have been identified as rich sources of neoclerodane diterpenoids. These metabolites have attracted considerable attention for their biological activity, which includes piscicidal, trypanocidal, and antibacterial properties. The insect antifeedant property of clerodane diterpenes is the most extensively studied bioactivity of these compounds.[59] *Scutellaria* and *Ajuga* genera (Lamiaceae) produce some of the most potent clerodane antifeedants. In *Scutellaria*, jodrellin B (occurring in *S. albida*, *S. galericulata*, *S. grossa*, *S. polyodon*, and *S. woronowii*) and scutecyprol B (found in *S. columnae*, *S. cypria*, *S. grossa*, and *S. rubicunda*) exhibit the highest antifeedant index against *S. littoralis*.[60,61] From *Ajuga pseudoiva* leaves, 14,15-dihydro-ajugapitin displayed the highest activity.[62] Furthermore, the genus *Teucrium* is one of the richest sources of clerodane diterpenes.[63]

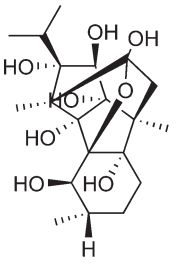


Jodrellin B

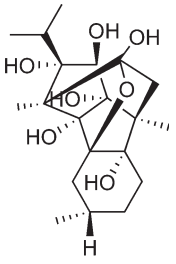


14,15-Dihydro-ajugapitin

Ryanodane diterpenes are compounds that are structurally related to the known insecticide ryanodine (see Section 3.09.2.10). Several ryanodane diterpenes, including ryanodol, cinnzeylanol, cinnzeylanone, and cinnzeylanine, have been isolated from the Macaronesian paleoendemism *Persea indica* (Lauraceae).[64,65] Ryanodol and didehydroryanodol, in contrast to ryanodine and didehydroryanodine, have low toxicity to mice and limited activity at the mammalian ryanodine receptor but are potent knockdown agents for injected houseflies or cockroaches, suggesting a possible difference in the target sites of mammals and insects.[66] The antifeedant activity of these compounds has been evaluated, showing the importance of the 11-hemiketal group for the antifeedant effects of ryanodane diterpenes. The comparative antifeedant effects of several nonalkaloidal and alkaloidal ryanoids supported the hypothesis of a ryanodol-specific mode of action in insects.[64,67] The insect-selective insecticidal and antifeedant effects of ryanodanes hold a promising future for their use as biopesticides. However, their availability is a problem that would need to be addressed prior to potential exploitation (**Table 4**).



Ryanodol



Cinnzeylanol

3.09.3.5 Triterpenes

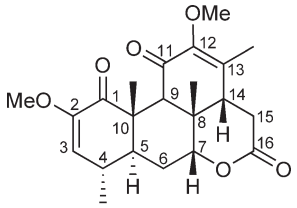
Quassinoids, the bitter compounds of the Simaroubaceae family, are a group of structurally complex and highly oxygenated degraded triterpenes. They are divided into five groups according to their basic skeleton: C-18, C-19, C-20, C-22, and C-25. In recent years, attention has been focused on quassinoids because several of them have shown promising biological activities. Some quassinoids present insecticidal and antifeedant effects in insects. Quassin was first used as an insecticide at the end of the seventeenth century, with the application of

Table 4 Insecticidal diterpenes for the period 2006–08 (in part)

Diterpenes	*Class*	*Target insect*	*Action*	*Reference*
Hugorosenone	Rosane	*Anopheles gambiae*	Larvicidal	Baraza *et al*.[198]
4-*epi*-Abieta-7,13-dien-3-one Abieta-7,13-dien-3-one	Abietane	*Mythimna separata, Pieris rapae*	Antifeedant Insecticidal	Yan *et al*.[199]
6,10-(*E,E*)-Thymifodioic acid (*2E,6E*)-2-(4-methylpent-3-enyl)-6-[3-(2-oxo-2,5-dihydrofuran-3-yl)-propylidene]-hept-2-ene-dioic acid	Linear	*Tenebrio molitor*	IGR	Hikawczuk *et al*.[200]
Neoclerodane derivatives	Neoclerodane	*Tribolium castaneum herbst*	Antifeedant	Hikawczuk *et al*.[201]
Parnapimarol	Pimarane	*Mosquito*	Larvicidal	Gkinis *et al*.[184]
14-*O*-Methyl-ryanodanol	Ryanodane	*Aedes aegypti*	Larvicidal	Barreiros *et al*.[202]
Ajuganipponin A Bajugamarins A_1, B_2, A_2, F_4 Bjugamacrin B, ajugacumbin A, ajugatakasin A, ajugacumbin B	Neoclerodane	*Spodoptera littoralis*	Antifeedant	Coll and Tandron [203]
ent-3β-(3-methyl-2-butenoyl)oxy-15-beyeren-19-oic acid	Beyerane	*Spodoptera littoralis*	Antifeedant	Wellsow *et al*.[204]
A mixture (*4R,19R*) and (*4R,19S*) diastereoisomers of coleon A	Abietane	*Spodoptera littoralis*	Antifeedant	Wellsow *et al*.[204]
Rhodojaponin-III	Grayanoid	*Pieris rapae*	Antifeedant , IGR	Zhong *et al*.[205]

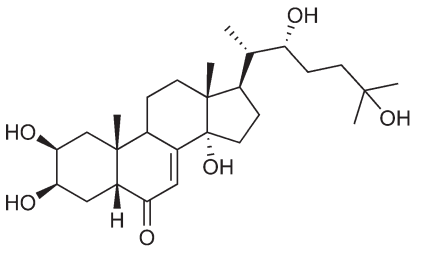
IGR, insect growth regulation effects.

plant extracts from *Quassia amara*. More recent studies also reveal this activity in other species and/or other quassinoids.[68]

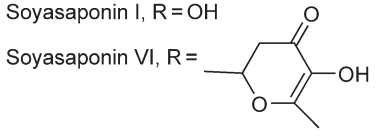


Quassin

Saponins are widely distributed among plants and have a wide range of biological properties. *Cestrum parqui* (Solanaceae) is a shrub from Chile, and toxicity comes from the saponic fraction of the plant. *Cestrum parqui* saponins, for example, are toxic to *Schistocerca gregaria, S. littoralis*, and *Tribolium confusum*. This toxicity may also be the result of interference with ecdysone metabolism by interfering with dietary cholesterol.[69,70] Alfalfa saponins exhibited deterrent and toxic effects against the pea aphid *Acyrthosiphon pisum*.[71] The larvicidal effect of aqueous extracts of the African plants *Hemidesmus indicus* roots, *Gymnema sylvestre*, and *Eclipta prostrata* on *Culex quinquefasciatus* larvae has been attributed to their high saponin content.[72] Insecticidal soyasaponins have been isolated from field pea (*Pisum sativum*) extracts.[73] The total saponins from the roots and shoots of three *Medicago* species (*M. arabica, M. hybrida*, and *M. murex*) included in the diet of *L. decemlineata* larvae reduced their feeding and growth and survival rates.[74]

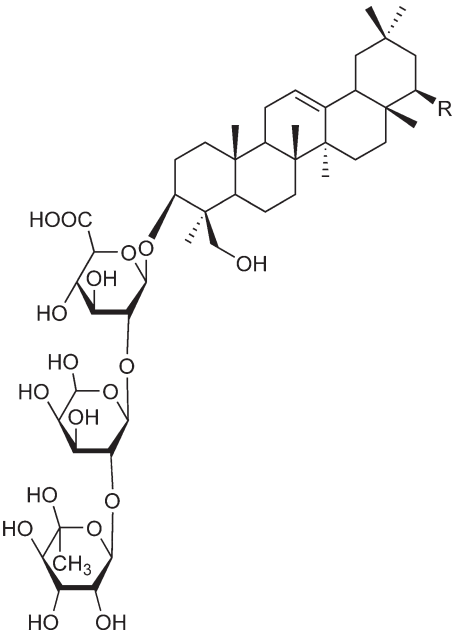


Ecdysone

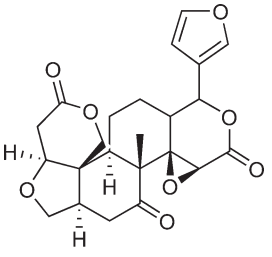


Soyasaponin I, R = OH

Soyasaponin VI, R =



The search for limonoids started way back when scientists started looking for the factor responsible for bitterness in citrus, which has a negative impact on citrus fruit and the juice industry worldwide. The term limonoids was derived from limonin, the first tetranortriterpenoid obtained from citrus bitter principles. Compounds from this group exhibit a range of biological activities (insecticidal, antifeedant, and growth regulating) on insects as well as antibacterial, antifungal, antimalarial, anticancer, and other activities. Although hundreds of limonoids have been isolated from several different plants, their occurrence in the plant kingdom is exclusively confined to plant families of the Rutales order, most abundant in Meliaceae and Rutaceae and less frequent in Cneoraceae and *Harrisonia* sp. of Simaroubaceae. Limonoids are highly oxygenated modified triterpenoids with a prototypical structure derived from a precursor with a 4,4,8-trimethyl-17-furanylsteroid skeleton. All naturally occurring citrus limonoids contain a furan ring attached to the D ring at C-17 as well as oxygenated functional groups at C-3, C-4, C-7, C-16, and C-17. There are fewer structural variations in limonoids found in Rutaceae as compared with Meliaceae, and these are generally limited to the modification of A and B rings. The limonoids of Meliaceae are more complex with a very high degree of oxidation and rearrangement in structure.[9]



Limonin

Other triterpene classes and derivatives, including lanostanes, friedelanes, and cyloartanes, also exhibit insect growth regulation effects[75–78] and therefore merit further investigation. **Table 5** shows the reported insecticidal triterpenes for the period 2006–08.

3.09.3.6 Alkaloids

Alkaloids research contributes to our understanding of their ecological role and provides essential information on the structural requirements accounting for their insecticidal activity. While the direct use of these substances

Table 5 Insecticidal triterpenes for the period 2006–08 (in part)

Triterpenes	*Class*	*Target insect*	*Action*	*Reference*
2α-Hydroxyfriedel-3-one, 2,3-*seco*-friedelan-2-al-3-oic acid, 3β- and 3α-hydroxyfriedelane, 3α-hydroxyfriedel-2-one, 4β-hydroxyfriedel-3-one, 3,4-*seco*-friedelan-4-oxo-3-oic-acid, friedelin-2,3-lactone, 3α-hydroxyfriedel-2-one	Friedelane	*Spodoptera littoralis*	Toxic, IGR	Moiteiro *et al*.[77]
β,24,25-Trihydroxycycloartane, beddomei lactone	Cycloartane	*Cnaphalocrocis medinalis*		Senthil-Nathan *et al*.[76]
Spirocaracolitones	Friedelin derivative	*Sitophilus oryzae*	Antifeedant	Omar *et al*.[206]
α-Euphol, α-euphorbol, obtusifoliol and 31-nor-lanostenol derivatives	Lanostane	*Spodoptera littoralis*	IGR	Mazoir *et al*.[75]
iso-Onoceratriene, 3-keto-22-hydroxyonoceradiene, onoceradienedione, lansiolic acid, lansiolic acid A, humilinolides C and D, gedunin	Limonoid	*Sitophilus oryzae*	Antifeedant	Omar *et al*.[206]
Musidunin, musiduol	Limonoid	*Pectinophora gossypiella* *Spodoptera frugiperda*	Antifeedant	Nihei *et al*.[207]
Unidentified saponin		*Schistocerca gregaria*	Toxic	Ikbal *et al*.[70]
Zanhic acid tridesmoside, medicagenic acid glycosides	Oleanane	*Acyrthosiphon pisum*	Antifeedant	Goławska[71]
Dehydrosoyasaponin I soyasaponins	Oleanane	*Sitophilus oryzae*	Antifeedant and insecticidal	Taylor and Fields [73]

IGR, Insect growth regulation effects.

has recently diminished, they continue to serve as leads for synthetic analogues and are also indispensable biochemical tools in mode-of-action studies. However, the development of novel insecticides of commercial importance based on these prototypes is not readily predictable. Alkaloids are typically produced as a cocktail of metabolically related compounds and occasionally co-occur with other nonalkaloidal substances, all modulating the toxicological properties of an individual component. Consequently, it would be fair to assume that a single natural compound is not optimized for a particular biological activity. Progress in the research on natural insecticides, botanicals in particular, has been surveyed from time to time.[7,79,80] Specifically, Ujváry[15] has reviewed tobacco, lobeline, quinolizidine, unsaturated amides, veratrum, solanum, physostigmine (eserine), ryanodine, *Aconitum* and *Delphinium* alkaloids, rocaglamide, cocaine, methylxanthines, isoquinoline-type alkaloids, dioncophyllines, *Erythrina*, *Stemona*, *Tripterygium*, and *Haplophyton* alkaloids, and polyhydroxy alkaloids, covering their insecticidal mode of action. Here, a few insights into insecticidal alkaloids are given.

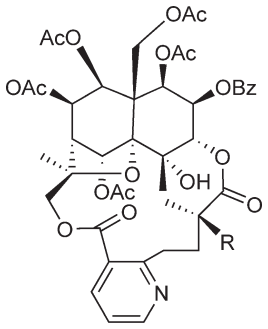
Table 6 shows the latest reports on insecticidal alkaloids. Most of these publications are related to previously known compounds except for harmaline[81] whose insecticidal effects are described for the first time.

Dihydroagarofuran sesquiterpene esters and alkaloids are the main compounds exhibiting insect antifeedant and insecticidal activities that have already been isolated from the species of Celastraceae. Insecticidal properties of *Tripterygium wilfordii* roots have been cited in the literature since 1931, and the sesquiterpene pyridine alkaloids wilforine and wilfordine were identified as its active components.[82] Several macrolide pyridine alkaloids have recently been isolated from *Euonymus* spp. and *Maytenus* spp. (Celastraceae). The number and orientation of the ester groups and the existence of pyridine alkaloids have a pronounced impact on the insecticidal activity of these dihydro-β-agarofuran sesquiterpene polyol alkaloids.[50,83] Accordingly, the structure of the nicotinic diacid and the components of the dihydro-β-agarofuran skeleton may affect the antifeedant potency of these macrolide alkaloids and could be involved in the potential neuronal action of the nicotinic diacid.

Table 6 Insecticidal alkaloids for the period 2006–08

Alkaloids	*Type*	*Target insect*	*Action*	*Reference*
Senecionine, integerrimine, seneciphylline Spartioidine	Alkaloid (PA)	*Leptinotarsa decemlineata, Myzus persicae, Spodoptera littoralis*	Antifeedant	Domínguez *et al*.[208]
Monocrotaline, acetylusaramine		*S. littoralis*	Antinutritional	
Harmaline	β-Carboline	*Plodia interpunctella*	IGR	Rharrabe *et al*.[81]
Delphigraciline, 14-Hydroxyhetisinone N-oxide	Diterpene	*Spodoptera littoralis*	Antifeedant, toxic	Reina *et al*.[209]
8-Methoxykarakoline	Norditerpene	*Spodoptera littoralis*	Antifeedant, toxic	Reina *et al*.[209]
Matrine, oxymatrine	Quinolizidine–matrine	*Coptotermes formosanus,*	Antifeedant, toxic	Mao *et al.*[210]
Matrine, sophocarpine, sophoramine, sophoridine		Clostera anastomosis	Antifeedant, antinutrirional	Yang *et al.,*[211]
Caffeine	Purine	*Lymantria dispar*	Antifeedant	Shields *et al.*[212]
Strychnine	Indole			
Berberine, aristolochic acid	Benzylisoquinoline			
Sparteine	Quinolizidine pyridine			
Nicotine Scopolamine, atropine	Tropane			
Wilfortrine, wilforgine, wilfordine, wilforine	Sesquiterpene pyridine	*Mythimna separata, Agrotis ypsilon*	Antifeedant, toxic	Shi *et al*.[213]
16-Hydroxystemofoline 13-Demethoxy-11(S^*),12(R^*)-dihydroprotostemonine	Stemofoline	*Heliothis virescens*	Insecticidal	Tang *et al*.[214]

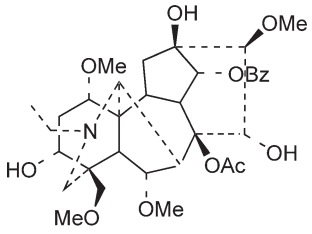
IGR, insect growth regulation effects.



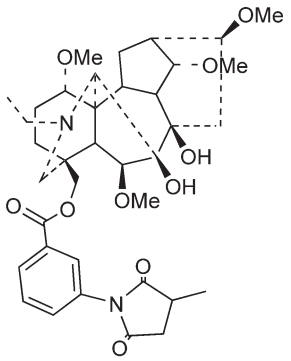
Wilforine; R = H
Wilfordine; R = OH

Diterpenoid alkaloids are well-known compounds of pharmacological interest. Aconitine, the major and one of the most toxic C-19 norditerpene alkaloids isolated from *Aconitum napellus*, and methyllycaconitine, the principal toxic alkaloid of many *Delphinium* spp. but not found in *Aconitum* species, are among the most toxic ones.[15] The insecticidal effects of C-19 diterpene alkaloids and their effects on insect nicotine acetylcholine receptors (nAChR) were already known. Recent studies on the antifeedant effects of C-19 norditerpenoid (NDAs) and C-20 diterpenoid (DAs) alkaloids isolated from *Aconitum, Delphinium*, and *Consolida* (Ranunculaceae) species showed that NDAs are better insect antifeedants and postingestive toxicants than

the related DAs. Their antifeedant or insecticidal potencies did not coincide with their reported nAChR-binding activity but did correlate with the agonist/antagonist insecticidal/antifeedant model proposed for nicotinic insecticides. Among the most potent antifeedants are the NDAs 1,14-diacetylcardiopetaline, 18-hydroxy-14-*O*-methylgadesine, and 14-*O*-acetyldelectinine and the DA 19-oxodihydroatisine.[84]



Aconitine

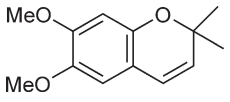


Methyllycaconitine

Table 6 shows the latest publications on the topic for the period 2006–08 (April).

3.09.3.7 Isoflavonoids, Chromenes, Coumarins, Iridoids, Lignans, and Phenylpropanoids

Precocenes have notable effects on insect development and can specifically induce destruction of corpora allatal cells, thus preventing the synthesis of juvenile hormones. As juvenile hormones have wide-ranging physiological roles in insects, from metamorphosis to reproduction, the effects of precocenes are also diverse. Precocene II and related compounds had morphogenetic, metabolic, and antifeedant effects on several insect species.[85–87]



Precocene II

Lignans and biogenetically related secondary metabolites derived from phenylpropanoid precursors play a significant role in protecting plants from insects. They mostly act as regulators of insect feeding, but in a few cases they can also exert an influence on the specific physiological functions of insects. The mode of action of such compounds is mostly unknown. One possible mechanism might be interaction with and disruption of the endocrine system, which is crucial for the proper development of insects and is dependent on the action of molting hormones (ecdysteroids).[88] These compounds also affect feeding, excretion, and *Trypanosoma cruzi* interactions with *Rhodnius prolixus*.[89] A structure–activity study revealed that natural lignan lactones with methoxy and/or methylenedioxy substituents showed significant activity that is strong enough to affect plant–insect interactions. The presence of polar substituents, especially hydroxyl or glycosyl groups, often reduces the activity. Nonpolar substituents such as methoxy or methylenedioxy groups enhance the activity not only in lignans but also in simple phenylpropanoids.[90]

Coumarins are scantly studied insecticides and there is potential to exploit this chemically simple group of natural products.[91] Iridoids are known to deter feeding or decrease the growth rate of many generalist insect herbivores. For example, catalpol-affected *T. castaneum* growth probably related to the inhibitory activity of this iridioid against DNA polymerase.[92] Phenylpropanoid derivatives accumulate in plants in response to insect herbivory and therefore are antiherbivore substances.[93] **Tables 7** and **8** show the latest reports on the insecticidal effects of the above mentioned type of compounds.

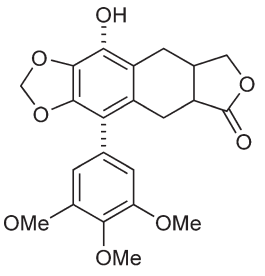
Table 7 Flavonoids, lignans, chromones, coumarins, etc. for the period 2006–08 (in part)

Flavonoids, lignans, etc.	*Type*	*Target insect*	*Action*	*Reference*
Precocene II	Chromene	*Archips podana*	Modification of the insect sensory system	Triseleva[215]
Isovitexin-2′-*O*-β-[6-*O*-*E*-*p*-coumaroylglucopyranoside]	Flavonoid	*Helicoverpa armigera*	Antifertility	Caasi-Lit *et al*.[216]
(−)-Homopterocarpin (−)-Methoxyhomopterocarpin	Isoflavonoid – pterocarpans	*Spodoptera litura* *Reticulitermes speratus*	Antifeedants	Morimoto *et al*.[185]
Quercetin glycoside, tannins	Flavonoid, tannins	*Spodoptera frugiperda*	Insecticidal IGR	Gallo *et al*.[217]
Kaempherol glycosides	Flavonoid	*Sitophilus oryzae*	Insecticidal	Taylor *et al*.[218]
Tanetin (6-hydroxykaempferol 3,7,4′-trimethyl ether), 6-hydroxykaempferol 3,6-dimethyl ether	Flavone	*Spodoptera littoralis*	Antifeedant	Susurluk *et al*.[189]
Rutin	Flavone	*Anticarsia gemmatalis*	Antinutritional	Hoffmann-Campo *et al*.[219]
(−)-Kusunokinin	Lignane	*Anticarsia gemmatalis*	Toxic	Messiano *et al*.[220]
Yangambin	Lignane	*Chrysomya megacephala*	Inhibition of postembryonic development, morphological alteration, and oviposition reduction	De Oliveira-Cabral *et al*.[221]
Geniposidic acid, 10-Hydroxyloganin, deacetyldaphylloside, monotropein	Iridoid	*Kalotermes flavicollis,* *Crematogaster scutellaris*	Toxicity	Tzakou *et al*.[222]
Khellin, visnagin, ammiol	Chromone	*Spodoptera littoralis*	Antifeedant	Sayed *et al*.[223]
2-Methyl-5,6,7-trimethoxychromone	Chromone	*Spodoptera litura*	Antifeedant	Morimoto and Komai[224]
Coumarin	Coumarin	*Rhyzopertha dominica,* *Sitophilus zeamais,* *Oryzaephilus surinamensis*	Insecticidal	Moreira *et al*.[225]
Murraxocin	Coumarin	*Plecoptera reflexa, Clostera cupreata, Crypsiptya coclesalis*	Toxic	Sharma *et al*.[91]
6-Hydroxy-7-isoprenyloxycoumarin, 6-Methoxy-7-isoprenyloxycoumarin, 6,7-Methylenedioxycoumarin, 5-methoxy-6,7-methylenedioxycoumarin, 6-Methoxy-7-(2-hydroxyethoxy)coumarin	Coumarin	*Spodoptera frugiperda*	Antifeedant, toxic, IGR	Vera *et al*.[226]
Scopoletin	Coumarin	*Spodoptera littoralis*	Antifeedant	Susurluk *et al*.[189]
Emodin	Anthraquinone	*Anopheles gambiae* *Bemisia tabaci*	Larvicidal, toxic	Georges *et al*.[227]

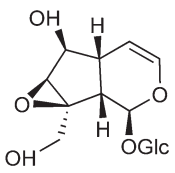
IGR, insect growth regulation effects.

Table 8 Aromatic derivatives and organosulfur compounds for the period 2006–08 (in part)

Aromatic derivatives, organosulfur compounds	*Type*	*Target insect*	*Action*	*Reference*
[4-(Prop-2-enyl) phenyl angelate4-(3-methyloxiranyl) phenyl 2-methylbutyrate]	Phenylpropanoid	Aphids??	Toxic	Baser *et al.*[190]
Anisole	Phenylpropanoid	*Pediculus humanus capitis (permethrin-resistant)*	Toxic	Toloza *et al*.[146]
trans-Anethole	Phenylpropanoid	*Lycoriella ingenua* *Trichoplusia ni*	Toxic	Park *et al*.[117] Wilson and Isman[183]
Safrole	Phenylpropanoid	*Musca domestica*	Toxic	Mohottalage *et al*.[168]
Methyl salicylate	Phenyl ester	*Trichoplusia ni*	Toxic	Wilson and Isman[183]
p-Anisaldehyde	Phenylpropanoid	*Lycoriella ingenua*	Toxic	Park *et al*.[117]
Remirol 7-Acetyl-4,6-dimethoxy-2, 3-dihydrobenzofuran	Dihydrobenzofurane	*Spodoptera litura*	Antifeedant	Morimoto and Komai[224]
Syringin	Phenylpropanoid glucoside	*Sitophilus granarius, Trogoderma granarium, Tribolium confusum*	Antifeedants	Cis *et al*.[188]
Eugenol	Phenylpropanoid	*Ixodes ricinus*	Repellent	Thorsell *et al*.[135]
Eugenyl acetate		*Musca domestica*	Toxic, knock down	Mohottalage *et al*.[168]; Samarasekera *et al*.[134]
Cinnamaldehyde	Phenylpropanoid	*Musca domestica,*	Knock down, toxic	Samarasekera *et al*.[134]
		Chrysomya megacephara	Ovicidal	Shen *et al*.[132]
Phenethyl alcohol	2-phenylethanol	*Ixodes ricinus*	Repellent	Thorsell *et al*.[135]
Diallyl disulfide	Organosulfur	*Lycoriella ingenua*	Toxic	Park *et al*.[117]
Dimethyl disulfide	Organosulfur	*Baltella germanica*	Toxic, fumigant	Gautier *et al*.[228]



Podophyllotoxin



Catalpol

3.09.4 Sustainable Production: Culture Methods

The main problem faced in the exploitation of natural compounds of plant origin as biopesticides is to ensure their sustainable supply at low cost. Biopesticides and botanicals tend to be more expensive than synthetics, and

some are not produced in great quantity or are no longer commercially available (e.g., nicotine). Several sources of plant material may be used for botanical pesticide extraction. The simplest route is extraction from plants harvested from wild plant resources. However, wild plant resources may be limited and hence may not permit sustainable production. Moreover, some plants containing these compounds are endangered species due to overexploitation.

An alternative is plant cultivation using conventional agricultural methods. Traditional cultivation permits the sustainable production of plant material in the amount required for biopesticide production and the ongoing improvement of production levels through breeding and selection of superior genotypes. The investment and the long periods of time required to establish plantations as well as environmental factors such as adverse weather conditions, pests, and diseases are the main disadvantages. It may also be that plants with interesting activities only grow in certain regions and are difficult to cultivate outside of their local ecosystems.[94] Additionally, some interesting compounds accumulate in specialized tissues, such as pyrethrins in flower heads of chrysanthemum, resulting in high labor costs related to harvest and extraction.[95]

For plant species with interesting activity, sustainable and reproducible cultivation methods should be developed as a clear alternative to traditional agriculture or wild plant collection. In the last few decades, great progress has been made in plant cell cultivation for the production of botanical insecticides.[95,96] Plant cell culture is not affected by changes in environmental conditions and the plant material can be maintained indefinitely in a defined production system. Despite considerable efforts, there are still problems in large-scale production by means of plant cell cultures due to low yields, cell line instability, and low economical viability.

As an alternative to plant cell cultures, the use of organ cultures such as fast-growing hairy roots obtained after transformation with *Agrobacterium rhizogenes* offers new opportunities for a sustainable *in vitro* production of specific metabolites when the main location of metabolite biosynthesis is in the roots. These cultures are genetically stable for long periods of time in contrast to what has been observed in many plant cell cultures and can produce metabolites at levels comparable with those of intact plants. Recent developments in bioreactor systems indicate that the industrial exploitation of this hairy root technology may be possible.[97–99] Studies on the production of some commercially important botanical insecticides by means of hairy root cultures have been carried out. Some examples are azadirachtin (*A. indica*[100]), tiophenes (*Tagetes patula*[101]), phytoecdysteroids (*Ajuga reptans* var. *atropurpurea*,[102] *Ajuga turkestanica*[103]), and nicotine (*N. rustica*[104]).

Additionally, this biotechnological method can be used as a source for the discovery of new pesticides in roots of rare and endangered species that would otherwise be inaccessible. For example, we have investigated *Salvia broussonetii*, a Canarian endangered endemic species that produces triterpenes in the aerial parts.[105,106] The phytochemical study of these roots permitted the isolation of diterpenes such as the dehydroabietane derivative 14-deoxycoleon U, which proved to be a potent antifeedant against *L. decemlineata*, and demethylcryptojaponol, which was also toxic to this insect. Additionally, the diterpenes isolated from this root culture showed strong selective cytotoxicity to insect Sf9 cells.[107]

Aeroponically grown plants in controlled environments can also be a sustainable source of metabolites from roots and aerial parts.[108] This artificial system allows the control of the root nutrient and water regimes, and also offers full access to the roots throughout the life of the plant. At the present time, aeroponic culture provides opportunity for biomass production on a commercial scale and is being applied to the production of medicinal crops.[109] As part of our ongoing studies on the sustainable production of natural biopesticides from endemic species, an aeroponic system for *P. indica* has been developed. The aerial part and stems of this species are characterized by their content of insecticidal ryanodane- and isoryanodane-type diterpenes.[64,65,110] Aeroponic culture of this protected tree in a controlled environment allowed investigation of the production of ryanodanes in aerial parts and roots. *S. palmensis*, an endemic Canarian species found on the islands of Tenerife and La Palma, is also being cultivated. The aerial part contains mostly silphinene-type tricyclic sesquiterpenes.[56,57,64,110] We have adapted this species to aeroponic culture and *in vitro* culture of transformed roots with *A. rhizogenes*, and silphinenes were produced in aerial parts and roots using both culture systems.

3.09.5 The New Biopesticide Market

The demand for nature-based biopesticides is rising steadily in all parts of the world. This is because of increased public awareness of the environment, and the pollution potential and health hazards related to many conventional pesticides. Extensive and systematic research has enhanced the effectiveness of biopesticides. Also, the techniques for their mass production, storage, transport, and application have improved in recent years.

Biopesticides are safer than conventional pesticides, which often are hazardous chemicals. They offer much more activity targeted to the desired pests as opposed to conventional pesticides, which often affect a broad spectrum of pests as well as birds and mammalian species. Often they are effective in very small quantities, thereby offering lower exposure. They decompose quickly. Lastly, they can supplement conventional pesticides when used in IPM programs. Such programs offer high crop yields while dramatically reducing conventional pesticide use.

Globally, the biopesticides market is worth €158 million. The European market has doubled in size in recent years, but the EU can only meet 45% of the demand for biopesticides. As consumers ask for greener alternatives, and as organophosphates are phased out, older pesticide licenses are not being renewed. This is creating a growing market for biopesticides.

Market trends:

- The synthetic pesticides market is expected to show a declining trend at the rate of 1.5% per annum. At the same time, the biopesticide market is growing and expected to reach more than a billion dollars in the next 5 years.
- Key developments expected in the coming years are more R&D in biopesticides, an increase in genetically modified crops, the application of IPM concepts, and a widening of organic farming.
- Biopesticides today represent about 2.5% of the overall pesticides market, and are expected to grow to about 4.2% by 2010.
- Orchard crops hold the largest share of biopesticides use at 55%.

However, the major constraint could be the changing and demanding regulations governing their registration and release.[111] Progress is being made toward achieving harmonization of requirements; however, the differences in detail required, and in the interpretation of the data, may undermine these efforts and continue to raise the hurdles against the development of new biopesticides.

3.09.5.1 Registration of Natural Products as Crop Protection Agents

3.09.5.1.1 Requirements for the United States

For registration, the Environmental Protection Agency (EPA) separates pesticides into two general categories: conventional chemical pesticides and biochemical and microbial pesticides. Natural products generally fall into the second category, and the EPA has specified test requirements for registration in the United States in 'Guidelines for Biorational Pesticides' (Subdivision M of CFR 158).[112] Biochemical pesticides are distinguished from conventional chemical pesticides by their natural occurrence and nontoxic mode of action to the target pest. Thus, insect pheromones and plant growth regulators, such as auxins and gibberellins, are defined as biochemical pesticides; active pesticide ingredients from common food sources such as garlic and cinnamon are also defined this way. However, plant-extracted pesticidal materials, although of natural origin do not necessarily always have a nontoxic mode of action. In some cases, the mode of action cannot be elucidated, and the best available scientific information and knowledge then have to be used to make the most appropriate decision. Semiochemicals (pheromones, either naturally occurring or synthetic) were also recognized by EPA as having low risks associated with their use. EPA has favored biopesticides under the reduced-risk pesticide policy, has agreed to waivers to many of the study requirements, and has agreed not to establish tolerances for many of the biopesticides.

3.09.5.1.2 Requirements for Europe

Europe uses the OECD (Organisation for Economic Co-operation and Development) definition of biopesticides, which includes pheromones, insect and plant growth regulators, plant extracts, transgenic plants and macroorganisms, as well as microorganisms.[113] Regulatory control of biopesticides in Europe has been based on precedents and standards established in the same way as for chemicals. With the development of the European Registration Directive 91/414/EEC and the Biocidal Products Directive covering requirements for chemicals and microorganisms, attempts have been made to harmonize the requirements and the interpretation of registration data throughout Europe.[111,114] The Directive covers biopesticides, and data requirements are listed in Part A (chemicals, pheromones, plant extracts) and Part B (microorganisms – bacteria, fungi, protozoa, viruses, and viroids) of Annexes II and III. The data requirements set out in these annexes appear to be very similar to those already agreed for chemicals, the requirements being fairly extensive to ensure that they cover all possible risk scenarios.

3.09.6 Conclusions

The main barriers to the commercialization of botanical insecticides are sustainability of the resource, standardization of chemically complex extracts, and regulatory approval. Additionally, finding new natural insecticides is not easy or is not currently being granted financial support as can be concluded from the lower number of publications on natural products with insecticidal properties (and mostly known ones) for the period 2006–08 (April) in contrast to the large number of publications on insecticidal EOs.

Plant EOs and/or their components have a broad spectrum of activity against insect and mite pests, plant pathogenic and other fungi, and nematodes. As such, they have considerable potential as crop protectants and for pest management in other situations (e.g., urban pest control). Current information indicates that they are safe to the user and the environment with few exceptions. However, the EOs that are most effective against pests are often the most phytotoxic. The latter property requires serious attention when formulating products. Moreover, selectivity among invertebrates is not well documented.

Among new natural products with promising insecticidal properties, it is believed that, in addition to limonoids, attention should focus on the β-dihydroagarofuran sesquiterpenes and related pyridine alkaloids, silphinene-type sesquiterpenes, drimanes, ryanodane diterpenes (more so than their pyrrole derivatives), lignans, flavonoids, and phenylpropanoids, among others. However, new single compound-based natural insecticides are difficult to produce because compound isolation and identification takes time and effort, the alternative being the production of standardized extracts once the active compounds are identified. New extraction methods to produce standardized enriched extracts and biotechnological/traditional cultivation methods are needed to produce new botanical biopesticides with commercial potential.

Like other alternative pest management products, EO-based pesticides and enriched standardized extracts will not be a panacea for crop protection, but there should be substantial market niches, particularly certified organic farming and urban pest control.

Regulatory approval in industrial nations is costly and time consuming. However, there is a growing demand for organic production of food, and the number of pest management products that can be used in this production is limited and it is here that botanical biopesticides can play an important role partially meeting such demand.

Abbreviations

CAP	Common Agricultural Policy
DA	diterpenoid
EC	emulsifiable concentrate
EO	essential oil
EPA	Environmental Protection Agency
GR	granular formulation

IGR	insect growth regulator
IPM	integrated pest management
nAChR	insect nicotine acetylcholine receptor
NDA	norditerpenoid
OECD	Organisation for Economic Co-operation and Development
PTX	pyerotoxinin

References

1. L. G. Copping; S. O. Duke, *Pest Manag. Sci.* **2007**, *63*, 524–554.
2. I. Ujváry, *Pesticidy* **2005**, *3*, 31–37.
3. I. Ujváry, Natural Product Pesticides. In *Encyclopedia of Agrochemicals*; J. R. Plimmer, D. W. Gammon, N. N. Ragsdale, Eds.; Wiley: NJ, 2003; Vol. 3, pp 1090–1104.
4. I. Ujváry, Pest Control Agents from Natural Products. In *Handbook of Pesticide Toxicology*, 23rd ed.; R. I. Krieger, Ed.; Academic Press: San Diego, 2001; pp 109–179.
5. J. R. Coats, *Annu. Rev. Entomol.* **1994**, *39*, 489–515.
6. M. B. Isman, *Annu. Rev. Entomol.* **2006**, *51*, 45–66.
7. R. W. Addor, Insecticides. In *Agrochemicals from Natural Products*; C. R. A. Godfrey, Ed.; Marcel Dekker: New York, 1995; pp 1–62.
8. S. Rajendran; V. Sriranjini, *J. Stored Prod. Res.* **2008**, *44*, 126–135.
9. A. Roy; S. Saraf, *Biol. Pharm. Bull.* **2006**, *29*, 191–201.
10. H. Schmutterer, *The Neem Tree; Source of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes*; VCH: Weinheim, Germany, 1995; p 696.
11. M. Jacobsen, Pharmacology and Toxicology of Neem. In *Focus on Phytochemical Pesticides, Vol. 1: The Neem Tree*; M. Jacobsen, Ed.; CRC Press: Boca Raton, FL, 1989; pp 133–153.
12. G. Brahmachari, *Chembiochem* **2004**, *5*, 408–421.
13. S. J. Boeke; M. G. Boersma; G. M. Alink; J. J. A. Van Loon; A. Van Huis; M. Dicke; I. M. C. M. Rietjens, *J. Ethnopharmacol.* **2004**, *94*, 25–41.
14. K. Chamberlain; A. A. Evans; R. H. Bromilow, *Pestic. Sci.* **1996**, *47*, 265–271.
15. I. Ujváry, Nicotine and Other Insecticidal Alkaloids. In *Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor*; I. Yamamoto, J. E. Casida, Eds.; Springer-Verlag: Tokyo, 1999; pp 29–60.
16. L. G. Copping, *The Manual of Biocontrol Agents*, 3rd ed.; BCPC Publications: Alton, Hants, UK, 2004; p 702.
17. C. D. S. Tomlin, *The Pesticide Manual*, 13th ed.; BCPC: Alton, Hants, UK, 2003; p 1344.
18. J. E. Casida; G. B. Quistad, *Pyrethrum Flowers; Production, Chemistry, Toxicology and Uses*; Oxford University Press: Oxford, UK, 1994.
19. N. Fang; J. E. Casida, *J. Agric. Food Chem.* **1999**, *47*, 2130–2136.
20. H. Fukami; M. Nakajima, Rotenone and the Rotenoids. In *Naturally Occurring Insecticides*; M. Jacobson, D. G. Crosby, Eds.; Marcel Dekker: New York, 1971; pp 71–79.
21. J. L. Sutko; J. A. Airey; W. Welch; L. Ruest, *Pharmacol. Rev.* **1997**, *49*, 53–98.
22. G. P. Lahm; T. M. Stevenson; T. P. Selby; J. H. Freudenberger; D. Cordova; L. Flexner; C. A. Bellin; C. M. Dubas; B. K. Smith; K. A. Hughes; J. G. Hollingshaus; C. E. Clark; E. A. Benner, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 6274–6279.
23. D. Cordova; E. A. Benner; M. D. Sacher; J. J. Rauh; J. S. Sopa; G. P. Lahm; T. P. Selby; T. M. Stevenson; L. Flexner; S. Gutteridge; D. F. Rhoades; L. Wu; R. M. Smith; Y. Tao, *Pestic. Biochem. Physiol.* **2006**, *84*, 196–214.
24. P. Lümmen; U. Ebbinghaus-Kintscher; C. Funke; R. Fischer; T. Masaki; N. Yasokawa; M. Tohnishi, *ACS Symp. Ser.* **2007**, *948*, 235–248.
25. T. S. Bellows, Jr.; J. G. Morse, *Can. J. Entomol.* **1993**, *125*, 987–994.
26. Anon, *Outlooks Pest Manag.* **2006**, *17*, 142–143.
27. J. J. W. Coppen, *Flavours and Fragrances of Plant Origin;* Non-Wood Forest Products (FAO); FAO: Rome, Italy, 1995; Vol 1, pp 1020–3370, 111p.
28. F. Bakkali; S. Averbeck; D. Averbeck; M. Idaomar, *Food Chem. Toxicol.* **2008**, *46*, 446–475.
29. D. H. Kim; Y. J. Ahn, *Pest Manag. Sci.* **2001**, *57*, 301–306.
30. C. Rossini; L. Castillo; A. González, *Phytochem. Rev.* **2008**, *7*, 51–63.
31. T. Burfield; S. L. Reekie, *Int. J. Aromather.* **2005**, *15*, 30–41.
32. K. M. Höld; N. S. Sirisoma; T. Ikeda; T. Narahashi; J. E. Casida, *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 3826–3831.
33. C. M. Priestley; E. M. Williamson; K. A. Wafford; D. B. Sattelle, *Br. J. Pharmacol.* **2003**, *140*, 1363–1372.
34. A. C. Hall; C. M. Turcotte; B. A. Betts; W. Y. Yeung; A. S. Agyeman; L. A. Burk, *Eur. J. Pharmacol.* **2004**, *506*, 9–16.
35. E. Enan, *Comp. Biochem. Physiol.* **2001**, *130C*, 325–337.
36. M. Kostyukovsky; A. Rafaeli; C. Gileadi; N. Demchenko; E. Shaaya, *Pest Manag. Sci.* **2002**, *58*, 1101–1106.
37. P. J. Houghton; Y. Ren; M. J. Howes, *Nat. Prod. Rep.* **2006**, *23*, 181–199.
38. A. Gonzalez-Coloma; D. Martín-Benito; N. Mohamed; M. C. García-Vallejo; A. C. Soria, *Biochem. Syst. Ecol.* **2006**, *34*, 609–616.
39. J. M. Rodilla; M. T. Tinoco; J. C. Morais; C. Gimenez; R. Cabrera; D. Martín-Benito; L. Castillo; A. González-Coloma, *Biochem. Syst. Ecol.* **2008**, *36*, 167–176.
40. F. J. Muller-Riebau; B. M. Berger; O. Yegen; C. Cakir, *J. Agric. Food Chem.* **1997**, *45*, 4821–4825.
41. J. Sanz; A. C. Soria; M. C. Garcia-Vallejo, *J. Chromatogr. A* **2004**, *1024*, 139–146.

42. I. Floris; A. Satta; P. Cabras; V. L. Garau; A. Angioni, *J. Econ. Entomol.* **2004**, *97*, 87–191.
43. A. Gregorc; I. Planinc, *Am. Bee J.* **2005**, *145*, 672–675.
44. S. Adamczyk; R. Lázaro; C. Pérez-Arquillué; P. Conchello; A. Herrera, *J. Agric. Food Chem.* **2005**, *53*, 10085–10090.
45. B. Emsen; E. Guzmán-Novoa; P. G. Kelly, *Am. Bee J.* **2007**, *147*, 535–539.
46. K. S. Delaplane, *J. Econ. Entomol.* **1992**, *85*, 2118–2124.
47. J. D. Ellis; K. S. Delaplane, *J. Apic. Res.* **2007**, *46*, 256–259.
48. C. Fassbinder; J. Grodnitzky; J. Coats, *J. Apic. Res.* **2002**, *41*, 83–88.
49. G. Schultz; C. Peterson; J. Coats, *ACS Symp. Ser.* **2006**, *927*, 168–181.
50. J. M. Gao; W. J. Wu; J. W. Zhang; Y. Konishi, *Nat. Prod. Rep.* **2007**, *24*, 1153–1189.
51. Y. Li; Y. Liu; Z. Song, *Agrochemicals* **2006**, *45*, 148–150.
52. Z. N. Hu; B. J. He; Y. Z. Du; A. X. Liu; W. J. Wu, *Acta Entomol. Sin.* **2007**, *50*, 788–794.
53. B. J. M. Jansen; A. de Groot, *Nat. Prod. Rep.* **2004**, *21*, 449–477.
54. L. Moreno-Osorio; M. Cortes; V. Armstrong; M. Bailen; A. Gonzalez-Coloma, *Z. Naturforsch* **2008**, *63c*, 215–220.
55. J. Justicia; E. Oltra; A. F. Barrero; A. Guadaño; A. Gonzalez-Coloma; J. M. Cuerva, Eur. J. Org. Chem. 2005, 712–718.
56. A. Gonzalez-Coloma; F. Valencia; N. Martín; J. J. Hoffmann; L. Hutter; J. A. Marco; M. Reina, *J. Chem. Ecol.* **2002**, *28*, 117–129.
57. M. Reina; M. Nold; J. C. Orihuela; O. Santana; A. González-Coloma, *J. Nat. Prod.* **2002**, *65*, 448–453.
58. J. R. Bloomquist; D. R. Boina; E. Chow; P. R. Carlier; M. Reina; A. Gonzalez-Coloma, *Pestic. Biochem. Physiol.* **2008**, *91*, 17–23.
59. E. A. Klein Gebbinck; B. J. M. Jansen; A. de Groot, *Phytochemistry* **2002**, *61*, 737–770.
60. M. Bruno; F. Piozzi; S. Rosselli, *Nat. Prod. Rep.* **2002**, *19*, 357–378.
61. S. Rosselli; A. Maggio; F. Piozzi; M. S. J. Simmonds; M. Bruno, *J. Agric. Food Chem.* **2004**, *52*, 7867–7871.
62. H. Ben Jannet; F. Harzallah-Skhiri; Z. Mighri; M. S. J. Simmonds; W. M. Blaney, *Fitoterapia* **2000**, *71*, 105–112.
63. J. Coll; Y. A. Tandron, *Phytochem. Rev.* **2008**, *7*, 25–49.
64. A. Gonzalez-Coloma; M. Reina; C. Gutiérrez; B. M. Fraga, Natural Insecticides: Structure Diversity, Effects and Structure-Activity Relationships. A Case Study. In *Studies in Natural Products Chemistry. Bioactive Natural Products*; Atta-Ur-Rahman, Ed.; Elsevier: Amsterdam, 2002; Vol. 26, pp 849–879.
65. B. M. Fraga; D. Terrero; C. Gutiérrez; A. González-Coloma, *Phytochemistry* **2001**, *56*, 315–320.
66. A. L. Waterhouse; I. N. Pessah; A. O. Francini; J. E. Casida, *J. Med. Chem.* **1987**, *30*, 710–716.
67. A. Gonzalez-Coloma; C. Gutiérrez; H. Hübner; H. Achenbach; D. Terrero; M. Fraga, *J. Agric. Food Chem.* **1999**, *47*, 4419–4424.
68. M. M. B. Almeida; A. M. C. Arriaga; A. K. L. Dos Santos; T. L. G. Lemos; R. Braz-Filho; I. J. Curcino Vieira, *Quim. Nova* **2007**, *30*, 935–951.
69. C. Ikbal; B. H.-K. Monia; T. Mounir; H. Wassila; R. Najet; B. A. Dorsaf; D. Mejda; B. H. M. Habib, *Int. J. Agric. Res.* **2007**, *2*, 275–281.
70. C. Ikbal; B. Habib; B. J. Hichem; B. H. Monia; B. H. M. Habib; M. Zine, *Pak. J. Biol. Sci.* **2007**, *10*, 3822.
71. S. Gołławska, *J. Chem. Ecol.* **2007**, *33*, 1598–1606.
72. V. Gopiesh Khanna; K. Kannabiran, *J. Biotechnol.* **2007**, *6*, 307–311.
73. W. G. Taylor; P. G. Fields, *ACS Symp. Ser.* **2006**, *927*, 194–209.
74. M. Szczepanik; Z. Bialy; M. Jurzysta, *Allelopathy J.* **2004**, *14*, 177–186.
75. A. Mazoir; M. Benharref; M. Bailén; M. Reina; A. González-Coloma, *Phytochemistry* **2008**, *69*, 1328–1338.
76. S. Senthil-Nathan; M. Y. Choi; C. H. Paik; H. Y. Seo, *Pestic. Biochem. Physiol.* **2007**, *88*, 260–267.
77. C. Moiteiro; M. J. Marcelo-Curto; N. Mohamed; M. Bailén; R. Martínez-Díaz; A. González-Coloma, *J. Agric. Food Chem.* **2006**, *54*, 3566–3571.
78. C. L. Céspedes; J. R. Salazar; M. Martínez; E. Aranda, *Phytochemistry* **2005**, *66*, 2481–2493.
79. M. Jacobson, D. G. Crosby, Eds. *Naturally Occurring Insecticides*; Marcel Dekker: New York, 1971; p 585.
80. J. A. Klocke, Plant Compounds as Sources and Models of Insect-Control Agents. In *Economic and Medicinal Plant Research*; H. Wagner, H. Hikino, N. R. Farnsworth, Eds.; Academic Press: London, 1989; Vol. 3, pp 103–144.
81. K. Rharrabe; A. Bakrim; N. Ghailani; F. Sayah, *Pestic. Biochem. Physiol.* **2007**, *89*, 137–145.
82. L. M. Liao, Sesquiterpene Pyridine Alkaloids. In *Alkaloids*; G. A. Cordell, Ed.; Academic Press: New York, 2003; Vol. 60, pp 287–344.
83. M. J. Núñez; A. Guadañol; L. Bazzocchil; A. Jimenez; A. G. Ravelo; A. González-Coloma, *J. Nat. Prod.* **2004**, *67*, 14–18.
84. M. Reina; A. González-Coloma, *Phytochem. Rev.* **2007**, *6*, 81–95.
85. J. Hardie; N. Gao; T. Timár; P. Sebok; K. I. Honda, *Arch. Insect Biochem. Physiol.* **1996**, *32*, 493–501.
86. Z. Chen; R. D. Madden; J. W. Dillwith, *J. Insect Physiol.* **2005**, *51*, 411–416.
87. M. Szczepanik; R. B. Obara; A. Szumny; B. Gabryś; A. Halarewicz-Pacan; J. Nawrot; C. Wawrzeńczyk, *J. Agric. Food Chem.* **2005**, *53*, 5905–5910.
88. J. Harmatha; L. Dinan, *Phytochem. Rev.* **2003**, *2*, 321–330.
89. E. S. Garcia; P. Azambuja, *Toxicon* **2004**, *44*, 431–440.
90. J. Harmatha; J. Nawrot, *Entomol. Exp. Appl.* **2002**, *104*, 51–60.
91. R. Sharma; D. S. Negi; W. K. P. Shiu; S. Gibbons, *Phytother. Res.* **2006**, *20*, 607–609.
92. C. R. Pungitore; M. J. Ayub; M. García; E. J. Borkowski; M. E. Sosa; G. Ciuffo; O. S. Giordano; C. E. Tonn, *J. Nat. Prod.* **2004**, *67*, 357–361.
93. M. M. Izaguirre; C. A. Mazza; A. Svatoš; I. T. Baldwin; C. L. Ballará, *Ann. Bot.* **2007**, *99*, 103–109.
94. F. Bourgaud; A. Gravot; S. Milesi; E. Gontier, *Plant Sci.* **2001**, *161*, 839.
95. A. Hitmi; A. Coudret; C. Barthomeuf, *Crit. Rev. Biochem. Mol. Biol.* **2000**, *35*, 317–337.
96. J. George; H. P. Bais; G. A. Ravishankar, *Crit. Rev. Biotechnol.* **2000**, *20*, 49–77.
97. M. I. Georgiev; A. I. Pavlov; T. Bley, *Appl. Microbiol. Biotechnol.* **2007**, *74*, 1175–1185.
98. S. Srivastava; A. K. Srivastava, *Crit. Rev. Biotechnol* **2007**, *27*, 29–43.
99. S. Guillon; J. Trémouillaux-Guiller; P. K. Pati; M. Rideau; P. Gantet, *Curr. Opin. Plant Biol.* **2006**, *9*, 341–346.
100. E. J. Allan; J. P. Eeswara; A. P. Jarvis; A. J. Mordue; E. D. Morgan; T. Stuchbury, *Plant Cell Rep.* **2002**, *21*, 374–379.
101. T. Rajasekaran; G. A. Ravishankar; B. Obul Reddy, *Indian J. Biotechnol.* **2004**, *3*, 92–96.

102. T. Matsumoto; N. Tanaka, *Agric. Biol. Chem.* **1991**, *55*, 1019–1025.
103. D. M. Cheng; G. G. Yousef; M. H. Grace; R. B. Rogers; J. Gorelick-Feldman; I. Raskin; M. A. Lila, *Plant Cell Tissue Organ Cult.* **2008**, *93*, 73–83.
104. J. D. Hamill; A. J. Parr; R. J. Robins; M. J. C. Rhodes, *Plant Cell Rep.* **1986**, *5*, 111–114.
105. A. G. Gonzalez; J. L. Bretón; B. M. Fraga, Chem. Commun. **1971,** 567–568.
106. A. G. Gonzalez; J. L. Bretón; B. M. Fraga, *An. Quim.* **1972**, *68*, 709–722.
107. B. M. Fraga; C. E. Díaz; A. Guadaño; A. González-Coloma, *J. Agric. Food Chem.* **2005**, *53*, 5200–5206.
108. F. Martín-Laurent, *For. Ecol. Manage.* **1999**, *122*, 199–207.
109. A. L. Hayden, *Hortscience* **2006**, *41*, 536–538.
110. A. Gonzalez-Coloma; C. Gutiérrez; R. Cabrera; M. Reina; C. E. Díaz; B. M. Fraga, Agroquímicos naturales de endemismos canarios. Un valor añadido a la biodiversidad. In *Interacciones químicas entre organismos: Aspectos básicos y perspectivas de aplicación*; A. L. Anaya, R. Cruz-Ortega, F. J. Espinosa-Garcia, Eds.; UNAM Plaza Y Valdés: México, 2001; pp 581–605.
111. M. C. Neale, *Pest Manag. Sci.* **2000**, *56*, 677–680.
112. US Environmental Protection Agency. Office of Pesticides and Toxic Substances, Subdivision M of the Pesticide Testing Guidelines: Microbial and Biochemical Pest Control Agents, **1989**, http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/885_Microbial_Pesticide_Test_Guidelines/Series/.
113. OECD Monograph No. 106. Data Requirements for Registration of Biopesticides in OECD Member Countries: Survey Results. Paris, **1996**, http://www.olis.oecd.org/olis/1996doc.nsf/LinkTo/NT00000C1A/$FILE/02E63126.PDF.
114. M. C. Neale; P. Newton, Registration/Regulatory Requirements in Europe. In *Biopesticides: Use and Delivery*; F. R. Hall, J. J. Menn, Eds.; Humana Press: New Jersey, 1999; pp 453–472.
115. O. Calmasur; S. Kordali; O. Kaya; I. Aslan, *J. Plant Dis. Prot.* **2006**, *113*, 37–41.
116. I. K. Park; L. S. Kim; I. H. Choi; Y. S. Lee; S. C. Shin, *J. Econ. Entomol.* **2006**, *99*, 1717–1721.
117. I. K. Park; K. S. Choi; D. H. Kim; I. H. Choi; L. S. Kim; W. C. Bak; J. W. Choi; S. C. Shin, *Pest Manag. Sci.* **2006**, *62*, 723–728.
118. K. Abeywickrama; A. A. C. K. Adhikari; P. Paranagama; C. S. P. Gamage, *Can. J. Plant Sci.* **2006**, *86*, 821–827.
119. D. Chaiyasit; W. Choochote; E. Rattanachanpichai; U. Chaithong; P. Chaiwong; A. Jitpakdi; P. Tippawangkosol; D. Riyong; B. Pitasawat, *Parasitol. Res.* **2006**, *99*, 715–721.
120. D. Goel; R. Goel; V. Singh; M. Ali; G. R. Mallavarapu; S. Kumar, *J. Nat. Med.* **2007**, *61*, 458–461.
121. M. M. M. Soliman, *Acta Phytopathol. Entomol. Hung.* **2006**, *41*, 395–406.
122. M. M. M. Soliman, *Arch. Phytopathol. Plant Prot.* **2007**, *40*, 128–138.
123. M. Negahban; S. Moharramipour; F. Sefidkon, *J. Stored Prod. Res.* **2007**, *43*, 123–128.
124. C. G. Yi; B. R. Choi; H. M. Park; C. G. Park; Y. J. Ahn, *J. Econ. Entomol.* **2006**, *99*, 1733–1738.
125. J. Wang; F. Zhu; X. M. Zhou; C. Y. Niu; C. L. Lei, *J. Stored Prod. Res.* **2006**, *42*, 339–347.
126. C. H. Liu; A. K. Mishra; R. X. Tan; C. Tang; H. Yang; Y. F. Shen, *Bioresour. Technol.* **2006**, *97*, 1969–1973.
127. R. Verma Prashant; T. Subburaju; N. Balakrishnan, *J. Nat. Remedies* **2006**, *6*, 157–161.
128. I. K. Park; J. N. Kim; Y. S. Lee; S. G. Lee; Y. J. Ahn; S. C. Shin, *J. Econ. Entomol.* **2008**, *101*, 139–144.
129. P. M. Kuo; F. H. Chu; S. T. Chang; W. F. Hsiao; S. Y. Wang, *Holzforschung* **2007**, *61*, 595–599.
130. S. R. Kiran; P. S. Devi; K. J. Reddy, *Curr. Sci.* **2007**, *93*, 544–548.
131. S. R. Kiran; A. S. Reddy; P. S. Devi; K. J. Reddy, *Pest Manag. Sci.* **2006**, *62*, 1116–1121.
132. L. R. Shen; H.-Y. Li; Y. G. Zhou; S. Gu; Y. G. Lou, *Chin. J. Appl. Ecol.* **2007**, *18*, 2343–2346.
133. R. W. H. M. Van Tol; H. J. Swarts; A. Van der Linden; J. H. Visser, *Pest Manag. Sci.* **2007**, *63*, 483–490.
134. R. Samarasekera; K. S. Kalhari; I. S. Weerasinghe, *J. Essent. Oil Res.* **2006**, *18*, 352–354.
135. W. Thorsell; A. Mikiver; H. Tunín, *Phytomedicine* **2006**, *13*, 132–134.
136. S. M. Morais; E. S. B. Cavalcanti; L. M. Bertini; C. L. L. Oliveira; J. R. B. Rodrigues; J. H. L. Cardoso, *J. Am. Mosq. Control Assoc.* **2006**, *22*, 161–164.
137. S. S. Cheng; M. T. Chua; E. H. Chang; C. G. Huang; W. J. Chen; S. T. Chang, *Bioresour. Technol.* **2009**, *100*, 465–470.
138. S. Y. Wang; W. C. Lai; F. H. Chu; C. T. Lin; S. Y. Shen; S. T. Chang, *J. Wood Sci.* **2006**, *52*, 522–526.
139. M. K. Chaubey, *Electron. J. Environ. Agric. Food Chem.* **2007**, *6*, 1719–1727.
140. A. Tawatsin; U. Thavara; U. Chansang; P. Chavalittumrong; T. Boonruad; P. Wongsinkongman; J. Bansidhi; M. S. Mulla, *J. Am. Mosq. Control Assoc.* **2006**, *22*, 306–313.
141. R. Kumar; M. Srivastava; N. K. Dubey, *J. Food Prot.* **2007**, *70*, 172–178.
142. G. K. Ketoh; H. K. Koumaglo; I. A. Glitho; J. Huignard, *Fitoterapia* **2006**, *77*, 506–510.
143. A. Lucia; P. Gonzalez-Audino; E. Seccacini; S. Licastro; E. Zerba; H. Masuh, *J. Am. Mosq. Control Assoc.* **2007**, *23*, 299–303.
144. M. Negahban; S. Moharramipour, *J. Appl. Entomol.* **2007**, *131*, 256–261.
145. S. Senthil-Nathan, *Bioresour. Technol.* **2007**, *98*, 1856–1860.
146. A. C. Toloza; J. Zygadlo; G. Mougabure-Cueto; F. Biurrun; E. Zerba; M. I. Picollo, *J. Med. Entomol.* **2006**, *43*, 889–895.
147. M. Garcia; A. González-Coloma; O. J. Donadel; C. E. Ardanaz; C. E. Tonn; M. E. Sosa, *Biochem. Syst. Ecol.* **2007**, *35*, 181–187.
148. F. Noudjou; H. Kouninki; L. S. T. Ngamo; P. M. Maponmestsem; M. Ngassoum; T. Hance; E. Haubruge; F. Malaisse; M. Marlier; G. C. Lognay, *J. Essent. Oil Res.* **2007**, *19*, 597–601.
149. A. Sanon; Z. Ilboudo; C. B. Dabire; R. C. H. Nebie; I. O. Dicko; J. P. Monge, *Int. J. Pest Manag.* **2006**, *52*, 117–123.
150. A. A. Isikber; M. H. Alma; M. Kanat; A. Karci, *Phytoparasitica* **2006**, *34*, 167–177.
151. T. G. T. Jaenson; S. Garboui; K. Pålsson, *J. Med. Entomol.* **2006**, *43*, 731–736.
152. W. J. Silva; G. A. A. Dória; R. T. Maia; R. S. Nunes; G. A. Carvalho; A. F. Blank; P. B. Alves; R. M. Marçal; S. C. H. Cavalcanti, *Bioresour. Technol.* **2008**, *99*, 3251–3255.
153. R. M. Gleiser; J. A. Zygadlo, *Parasitol. Res.* **2007**, *101*, 1349–1354.
154. A. Amer; H. Mehlhorn, *Parasitol. Res.* **2006**, *99*, 478–490.
155. S. Hammami; I. Khoja; H. Ben Jannet; M. Ben Halima; Z. Mighri, *J. Essent. Oil Bearing Plants* **2007**, *10*, 162–167.
156. M. J. Sim; D. R. Choi; Y. J. Ahn, *J. Econ. Entomol.* **2006**, *99*, 593–598.
157. R. Samarasekera; I. S. Weerasinghe; K. D. P. Hemalal, *Pest Manag. Sci.* **2008**, *64*, 290–295.
158. I. S. Rim; C. H. Jee, *Korean J. Parasitol.* **2006**, *44*, 133–138.

159. O. Calmasur; I. Aslan; F. Sahin, *Ind. Crops Prod.* **2006**, *23*, 140–146.
160. M. R. Yaghoobi-Ershadi; A. A. Akhavan; E. Jahanifard; H. Vatandoost; G. Amin; L. Moosavi; A. R. Zahraei Ramazani; H. Abdoli; M. H. Arandian, *Iran. J. Public Health* **2006**, *35*, 7–13.
161. A. M. Njan-Nloga; P. Saotoing; J. C. Tchouankeu; J. Messi, *J. Entomol.* **2007**, *4*, 444–450.
162. Z. Popović; M. Kostić; S. Popovic; S. Skorić, *Biotechnol. Biotechnol. Equip.* **2006**, *20*, 36–40.
163. Ö. Caglar; O. Calmaşur; I. Aslan; O. Kaya, *Fresenius Environ. Bull.* **2007**, *16* (11A), 2007, 1395–1400.
164. H. Cetin; F. Erler; A. Yanikoglu, *Pest Manag. Sci.* **2007**, *63*, 830–833.
165. H. Cetin; A. Yanikoglu, *J. Vector Ecol.* **2006**, *31*, 118–122.
166. C. B. Mello; C. D. Uzeda; M. V. Bernardino; D. Mendonça-Lopes; A. Kelecom; P. C. A. Fevereiro; M. S. Guerra; A. P. Oliveira; L. M. Rocha; M. S. Gonzalez, *Braz. J. Pharmacol.* **2007**, *17*, 514–520.
167. M. Leyva; J. E. Tacoronte; M. D. C. Marquetti, *Rev. Cubana Med. Trop.* **2007**, *59* (2).
168. S. Mohottalage; R. Tabacchi; P. M. Guerin, *Flavour Fragr. J.* **2007**, *22*, 130–138.
169. C. B. Gragasin; A. M. Wy; B. P. Roderos; M. A. Acda; A. D. Solsoloy, *Philipp. Agric. Sci.* **2006**, *89*, 212–216.
170. J. L. Vidal-Estrela; M. Fazolin; V. Catani; M. S. De Lima, *Pesqu. Agropecu. Bras.* **2006**, *41*, 217–222.
171. Q. Q. Zeng; Y. W. Cai; Z. Yan; X. G. Wang; Y. S. Wang, *J. Plant Res. Environ.* **2006**, *15*, 21–25.
172. R. Kotan; S. Kordali; A. Cakir; M. Kesdek; Y. Kaya; H. Kilic, *Biochem. Syst. Ecol.* **2008**, *36*, 360–368.
173. M. Kostic; S. Dražić; Z. Popoviá; S. Stanković; I. Sivčev; T. Živanović, *Biotechnol. Biotechnol. Equip.* **2007**, *21*, 426–430.
174. A. Michaelakis; S. A. Theotokatos; G. Koliopoulos; N. G. Chorianopoulos, *Molecules* **2007**, *12*, 2567–2578.
175. R. Pavela, *J. Essent. Oil Bearing Plants* **2007**, *10*, 346–356.
176. H. Kouninki; T. Hance; F. A. Noudjou; G. Lognay; F. Malaisse; M. B. Ngassoum; P. M. Mapongmetsem; L. S. T. Ngamo; E. Haubruge, *J. Appl. Entomol.* **2007**, *131*, 269–274.
177. K. Kamsuk; W. Choochote; U. Chaithong; A. Jitpakdi; P. Tippawangkosol; D. Riyong; B. Pitasawat, *Parasitol. Res.* **2007**, *100*, 339–345.
178. M. Tiwary; S. N. Naik; D. K. Tewary; P. K. Mittal; S. Yadav, *J. Vector Borne Dis.* **2007**, *44*, 198–204.
179. V. Rozman; I. Kalinovic; Z. Korunic, *J. Stored Prod. Res.* **2007**, *43*, 349–355.
180. M. B. Isman; J. A. Wilson; R. Bradbury, *Pharm. Biol.* **2008**, *46*, 82–87.
181. M. I. Picollo; A. C. Toloza; G. Mougabure-Cueto; J. Zygadlo; E. Zerba, *Fitoterapia* **2008**, *79*, 271–278.
182. C. M. Priestley; I. F. Burgess; E. M. Williamson, *Fitoterapia* **2006**, *77*, 303–309.
183. J. A. Wilson; M. B. Isman, *Can. Entomol.* **2006**, *138*, 578–589.
184. G. Gkinis; E. Ioannou; A. Quesada; C. Vagias; O. Tzakou; V. Roussis, *J. Nat. Prod.* **2008**, *71*, 926–928.
185. M. Morimoto; H. Fukumoto; M. Hiratani; W. Chavasiri; K. Komai, *Biosci. Biotechnol. Biochem.* **2006**, *70*, 1864–1868.
186. M. Reina; A. González-Coloma; D. Domínguez-Díaz; R. Cabrera; C. Giménez Marin∼o; M. L. Rodríguez; L. Villarroel, *Nat. Prod. Res.* **2006**, *20*, 13–19.
187. E. Burguen∼o-Tapia; A. González-Coloma; D. Martín-Benito; P. Joseph-Nathan, *Z. Naturforsch. C* **2007**, *62*, 362–366.
188. J. Cis; G. Nowak; W. Kisiel, *Biochem. Syst. Ecol.* **2006**, *34*, 862–867.
189. H. Susurluk; Z. Caliskan; O. Guerkan; S. Kirmiziguel; N. Goeren, *Ind. Crops Prod.* **2007**, *26*, 220–228.
190. K. H. C. Baser; N. Tabanca; K. Nese; E. Bedir; I. A. Khan; D. E. Wedge, *Pure Appl. Chem.* **2007**, *79*, 539–556.
191. M. Wang; W. Wu; J. Zhu; Z. Ji; W. Zhou, *Nat. Prod. Res.* **2006**, *20*, 653–658.
192. Z. Ji; W. Wu; H. Yang; B. Shi; M. Wang, *Nat. Prod. Res.* **2007**, *21*, 334–342.
193. N. B. Perry; E. J. Burgess; L. M. Foster; P. J. Gerard; M. Toyota; Y. Asakawa, *J. Nat. Prod.* **2008**, *71*, 258–261.
194. S. R. Kiran; P. S. Devi, *Parasitol. Res.* **2007**, *101*, 413–418.
195. L. D. Baraza; C. C. Joseph; M. H. H. Nkunya, *Nat. Prod. Res.* **2007**, *21*, 1027–1031.
196. R. D. Stipanovic; J. D. López; M. K. Dowd; L. S. Puckhaber; S. E. Duke, *J. Chem. Ecol.* **2006**, *32*, 959–968.
197. M. L. Li; J. Cui; R. H. Qin; J. M. Gao; Y. B. Zhang; X. R. Guo; W. Zhang, *Heterocycles* **2007**, *71*, 1155–1162.
198. L. D. Baraza; C. C. Joseph; J. J. E. Munissi; M. H. H. Nkunya; N. Arnold; A. Porzel; L. Wessjohann, *Phytochemistry* **2008**, *69*, 200–205.
199. H. Yan; R. Feng; L. Chen; A. Chen; G. Li; X. Zhang, *Xibei Zhiwu Xuebao* **2007**, *27*, 163–167.
200. V. E. J. Hikawczuk; J. R. Saad; O. S. Giordano; C. Garcia; T. Martin; V. S. Martin; M. E. Sosa; C. E. Tonn, *J. Nat. Prod.* **2008**, *71*, 190–194.
201. V. E. J. Hikawczuk; M. A. Lopez-Verrilli; E. J. Borkowski; M. E. Sosa; O. S. Giordano; J. R. Saad; C. E. Tonn, *Nat. Prod. Res.* **2006**, *20*, 813–819.
202. M. L. Barreiros; J. P. David; J. M. David; L. M. Xavier-Lopes; M. S. de Sa; J. F. O. Costa; M. Z. Almeida; L. P. de Queiroz; A. E. G. Sant'Ana, *Phytochemistry* **2007**, *68*, 1735–1739.
203. J. Coll; Y. A. Tandron, *Nat. Prod. Commun.* **2006**, *1*, 183–189.
204. J. Wellsow; R. J. Grayer; N. C. Veitch; T. Kokubun; R. Lelli; G. C. Kite; M. S. J. Simmonds, *Phytochemistry* **2006**, *67*, 1818–1825.
205. G. Zhong; J. Liu; Q. Weng; M. Hu; J. Luo, *Pest Manag. Sci.* **2006**, *62*, 976–981.
206. S. Omar; M. Marcotte; P. Fields; P. E. Sanchez; L. Poveda; R. Mata; A. Jimenez; T. Durst; J. Zhang; S. MacKinnon; D. Leaman; J. T. Arnason; B. J. R. Philogéne, *J. Stored Prod. Res.* **2007**, *43*, 92–96.
207. K. I. Nihei; Y. Asaka; Y. Mine; Y. Yamada; M. Iigo; T. Yanagisawa; I. Kubo, *J. Nat. Prod.* **2006**, *69*, 975–977.
208. D. M. Domínguez; M. Reina; A. Santos-Guerra; O. Santana; T. Agulló; C. López-Balboa; A. A. Gonzalez-Coloma, *Biochem. Syst. Ecol.* **2008**, *36*, 153–166.
209. M. Reina; R. Mancha; A. González-Coloma; M. Bailen; M. L. Rodríguez; R. A. Martinez-Díaz, *Nat. Prod. Res.* **2007**, *21*, 1048–1055.
210. L. Mao; G. Henderson, *J. Econ. Entomol.* **2007**, *100*, 866–870.
211. Z. Yang; B. Zhao; L. Zhu; J. Fang; L. Xia, *Front. For. China* **2006**, *1*, 190–195.
212. V. D. C. Shields; E. J. Rodgers; N. S. Arnold; D. Williams, *Naturwissenschaften* **2006**, *93*, 127–130.
213. B.-J. Shi; Z.-Q. Ji; J.-W. Zhang; W.-J. Wu, *Kun Chong Xue Bao* **2007**, *50*, 795–800.
214. C.-P. Tang; T. Chen; R. Velten; P. Jeschke; U. Ebbinghaus-Kintscher; S. Geibel; Y. Ye, *J. Nat. Prod.* **2008**, *71*, 112–116.
215. T. A. Triseleva, *Biol. Bull.* **2007**, *34*, 463–467.
216. M. T. Caasi-Lit; G. J. Tanner; M. Nayudu; M. I. Whitecross, *Photochem. Photobiol.* **2007**, *83*, 1167–1173.

217. M. B. C. Gallo; W. C. Rocha; U. S. Da Cunha; F. A. Diogo; F. C. Da Silva; P. C. Vieira; J. D. Vendramim; J. B. Fernandes; M. F. D. G. F. Da Silva; L. G. Batista-Pereira, *Pest Manag. Sci.* **2006**, *62*, 1072–1081.
218. W. G. Taylor; P. G. Fields; D. H. Sutherland, *J. Agric. Food Chem.* **2007**, *55*, 5491–5498.
219. C. B. Hoffmann-Campo; J. A. R. Neto; M. C. N. De Oliveira; L. J. Oliveira, *Pesqui. Agropecu. Bras.* **2006**, *41*, 1453–1459.
220. G. B. Messiano; L. Vieira; M. B. Machado; L. M. X. Lopes; S. A. Bortoli; J. Zukerman-Schpector, *J. Agric. Food Chem.* **2008**, *56*, 2655–2659.
221. M. M. De Oliveira-Cabral; P. M. Mendonça; C. M. Da Silva-Gomes; J. M. Barbosa-Filho; C. Da Silva-Dias; M. J. Soares; M. M. De Carvalho-Queiroz, *J. Med. Entomol.* **2007**, *44*, 249–255.
222. O. Tzakou; P. Mylonas; C. Vagias; P. V. Petrakis, *Z. Naturforsch. C* **2007**, *62*, 597–602.
223. H. M. Sayed; M. H. Mohamed; S. F. Farag; G. A. Mohamed; P. Proksch, *Nat. Prod. Res.* **2007**, *21*, 343–350.
224. M. Morimoto; K. Komai, *ACS Symp. Ser.* **2006**, *927*, 182–193.
225. M. D. Moreira; M. C. Picanço; L. C. D. A. Barbosa; R. N. C. Guedes; M. R. De Campos; G. A. Silva; J. C. Martins, *Pesqui. Agropecu. Bras.* **2007**, *42*, 909–915.
226. N. Vera; S. Popich; L. Luna; R. Cravero; M. G. Sierra; A. Bardón, *Chem. Biodiv.* **2006**, *3*, 21–26.
227. K. Georges; B. Jayaprakasam; S. S. Dalavoy; M. G. Nair, *Bioresour. Technol.* **2008**, *99*, 2037–2045.
228. H. Gautier; J. Auger; C. Legros; B. Lapied, *J. Pharmacol. Exp. Ther.* **2008**, *324*, 149–159.

Biographical Sketches

Azucena Gonzalez-Coloma completed her Ph.D. in Plant Biochemistry in 1985 at the Universidad Complutense de Madrid, under the supervision of Professor C. V. Cordova. Then, she spent 4 years as a postdoctoral researcher with Professor Phil Rundel's group at The Environmental Biology Department, UCLA, before joining the Instituto de Productos Naturales, CSIC with Professor B. M. Fraga in 1989. In 1991, she began her independent career at the CSIC. In 2007, she was promoted to the post of Investigador Científico at the Instituto de Ciencias Agrarias-CCMA, CSIC.

Matías Reina Artiles received his Ph.D. in Chemistry in 1979 at the University of La Laguna, Tenerife, under the supervision of Professor A. G. Gonzalez and Professor Dr. G. de la Fuente. He was an assistant professor in Organic Chemistry for the period 1974–76. He became Titulado Superior de Investigación, CSIC at the Instituto de Productos Naturales y

Agrobiología in 1976. In 1985, he spent one and a half years as a postdoctoral researcher at the University René Descartes in Paris, with Professor D. Mansuy. He began his independent career at the CSIC in 1991, and currently he is Investigador Titular OPIS at the Instituto de Productos Naturales y Agrobiología. His research interests range from the chemistry of alkaloids to biomimetic transformations of natural products.

Carmen Elisa Diaz completed her Ph.D. in Pharmacy in 1986 at the University of La Laguna in Tenerife, under the supervision of Professor Braulio M. Fraga. In 1987, she obtained a CSIC postdoctoral fellowship and became Científico Titular at the Instituto de Productos Naturales y Agrobiología, CSIC in 1988. She then spent 1 year as a postdoctoral researcher with Professor B. V. Charlwood's group at King's College University of London in 1989, before joining the Chemistry and Biotechnology of Natural Products's group at the Instituto de Productos Naturales y Agrobiología, CSIC.

Braulio M. Fraga was born in Tenerife (1944) and received his Ph.D. in Chemistry at the University of La Laguna (1970), where he lectured in Organic Chemistry for several years. In 1971, he was honored with the Young Researcher Award from the Spanish Royal Society of Chemistry. He obtained a permanent position in the Spanish Council for Scientific Research as a Tenured Scientist in 1972 and was later appointed Research Scientist (1986) and Research Professor (1987). He was director of the Institute of Natural Products (Tenerife) from 1988 to 1991 and has been the representative of the Spanish Council for Scientific Research in the Canary Islands since 1991. He had previously been appointed Professor of Organic Chemistry at the University of Valencia (1981). His research interests range from chemistry to biotransformation of natural products, especially in the field of terpenes. He has authored more than 180 scientific publications.

3.10 Natural Products as Sweeteners and Sweetness Modifiers

A. Douglas Kinghorn, Young-Won Chin, and Li Pan, The Ohio State University, Columbus, OH, USA

Zhonghua Jia, Givaudan Flavors Corporation, Cincinnati, OH, USA

3.10.1 Introduction

The most widely used sweetener in the world is sucrose (table sugar), a disaccharide (α-D-glucopyranosyl-(1→2)-β-fructofuranoside), which is produced from sugarcane and sugar beet.[1] However, a high daily intake of sucrose has been reported to be involved in the development of several health problems, most notably dental caries.[2] Accordingly, there has been an increasing demand for new highly sweet, noncaloric, and noncariogenic sucrose substitutes in the market. For example, the sweetener market is generally recognized as accounting currently for approximately $1 billion in sales in the United States alone. Sweet-tasting sucrose substitutes, which may be of either synthetic or natural origin, need to possess at least equal sensory properties to sucrose. Such compounds can be categorized into 'intense' or 'low-calorie sweeteners', which are 50–100 to several thousand times more

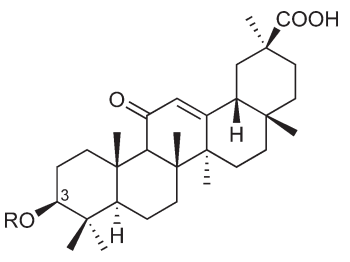
intensely sweet than sucrose,[3–5] and 'bulk' or 'reduced-calorie' sweeteners, such as certain monosaccharides, disaccharides, and polyols, which are approximately equal to sucrose in sweetness intensity.[6,7]

Synthetic sweeteners including acesulfame-K, alitame, aspartame, cyclamate, neotame, saccharin, and sucralose are currently available as potently sweet substitutes of sucrose in most western countries, but the regulations for each sweetener vary from country to country.[3,8–14] Five synthetic sweeteners, acesulfame-K, aspartame, neotame, saccharin, and sucralose, are presently approved for use in the United States, with cyclamate no longer utilized, owing to concerns about its safety.[7,11,15]

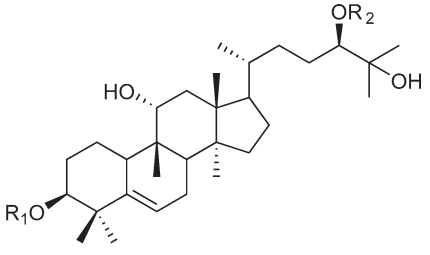
In addition to the synthetic sweeteners mentioned above, a number of highly sweet natural compounds are known to exist, which are mostly terpenoids, flavonoids (Chapter 3.16), and proteins (Chapters 5.01–5.21), and this area has been subjected to previous review.[16–24] So far, all of the known natural product sweet-tasting substances and sweetness modifiers have been discovered from green plants, as opposed to other types of organisms, such as lower plants, microbes, and marine fauna. Some of these plant-derived substances have been launched commercially in the market and are used as low-calorie sucrose substitutes, as will be mentioned in the next section. Besides these naturally occurring sweet-tasting compounds, a number of naturally occurring sweetness modifiers, either inducers or inhibitors of sweetness perception, are known to influence the sweet taste response.[23,25] In the following parts of this chapter, after sequential sections on naturally occurring sweet compounds with commercial use and how such compounds may be discovered, sweet substances in the terpenoid and steroid, phenylpropanoid, dihydroisocoumarin, flavonoid, proanthocyanidin, benzo[*b*]indeno[1,2-*d*]pyran, amino acid, and protein categories will be described. Next, the structural classes of naturally occurring sweetness inducers and sweetness inhibitors will be discussed in turn, prior to some concluding remarks. The literature for this chapter has been surveyed until the middle of 2008.

3.10.2 Commercially Used Highly Sweet Natural Products

Only a relatively few sweet-tasting plant-derived natural products have been launched commercially as sucrose substitutes to date. These natural products are used in one or more countries either in the pure form or as refined extracts, and include glycyrrhizin (**1**), mogroside V (**2**), phyllodulcin (**3**), rebaudioside A (**4**), stevioside (**5**), and thaumatin (**6**). Many of these compounds have served as lead compounds for extensive structural modification, in attempts to produce analogues that either possess better hedonic attributes or are more potently sweet tasting. A number of naturally occurring 'bulk' or 'reduced-calorie' sweeteners are commercially available as either foods or food additives. These substances include the monosaccharides fructose and D-tagatose; the disaccharides isomaltulose and trehalose; the monosaccharide polyols erythritol, mannitol, sorbitol, and xylitol; and the disaccharide polyols lactitol and maltitol. As reduced-calorie sweeteners and their hydrogenated derivatives have been dealt with in depth recently,[4–6] they will not be further described in this chapter.

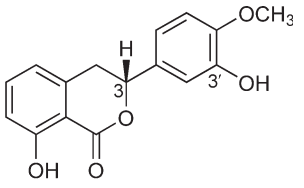


1 R = β-glcA^2-β-glcA
7 R = β-xyl
8 R = β-glcA

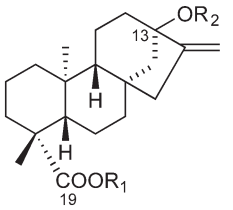


	R_1	R_2
2	β-glc^6-β-glc	β-glc^2-β-glc (6: β-glc)

glc: D-glucopyranosyl
xyl: D-xylopyranosyl



3



glcA: D-glucuronopyranosyl

	R_1	R_2
4	β-glc	β-glc^2-β-glc ^{3}| β-glc
5	β-glc	β-glc^2-β-glc
9	β-gal	β-glc^2-β-glc^2-β-glc^4-α-glc
10	$(CH_2)_3SO_3Na$	β-glc^2-β-glc ^{3}| β-glc

Ala-Thr-Phe-Glu-Ile-Val-Asn-Arg-Cys-Ser-Tyr-Thr-Val-Trp-Ala-Ala-Ala-Ser-Lys-Gly-
1 5 10 15 20
Asp-Ala-Ala-Leu-Asp-Ala-Gly-Gly-Arg-Gln-Leu-Asn-Ser-Gly-Glu-Ser-Trp-Thr-Ile-Asn-
21 25 30 31 40
Val-Glu-Pro-Gly-Thr-Asn-Gly-Gly-Lys-Ile-Trp-Ala-Arg-Thr-Asp-Cys-Tyr-Phe-Asp-Asp-
41 45 50 55 60
Ser-Gly-Ser-Gly-Ile-Cys-Lys-Thr-Gly-Asp-Cys-Gly-Gly-Leu-Leu-Arg-Cys-Lys-Arg-Phe-
61 65 70 75 80
Gly-Arg-Pro-Pro-Thr-Thr-Leu-Ala-Glu-Phe-Ser-Leu-Asn-Gln-Tyr-Gly-Lys-Asp-Tyr-Ile-
81 85 90 95 100
Asp-Ile-Ser-Asn-Ile-Lys-Gly-Phe-Asn-Val-Pro-Met-Asn-Phe-Ser-Pro-Thr-Thr-Arg-Gly-
101 105 110 115 120
Cys-Arg-Gly-Val-Arg-Cys-Ala-Ala-Asp-Ile-Val-Gly-Gln-Cys-Pro-Ala-Lys-Leu-Lys-Ala-
121 125 130 135 140
Pro-Gly-Gly-Gly-Cys-Asn-Asp-Ala-Cys-Thr-Val-Phe-Gln-Thr-Ser-Glu-Tyr-Cys-Cys-Thr-
141 145 150 155 160
Thr-Gly-Lys-Cys-Gly-Pro-Thr-Glu-Tyr-Ser-Arg-Phe-Phe-Lys-Arg-Leu-Cys-Pro-Asp-Ala-
161 165 170 175 180
Phe-Ser-Tyr-Val-Leu-Asp-Lys-Pro-Thr-Thr-Val-Thr-Cys-Pro-Gly-Ser-Ser-Asn-Tyr-Arg-
181 185 190 195 200
Val-Thr-Phe-Cys-Pro-Thr-Ala
201 207

6

Glycyrrhizin (**1**), also known as glycyrrhizic acid, is an oleanane-type triterpenoid diglycoside isolated from the roots of *Glycyrrhiza glabra* L. (licorice root; Leguminosae) and other species of the genus *Glycyrrhiza*.[26–28] The compound was first isolated in crystalline form about a century ago by Tschirch and Cederberg,[29] with the structure finalized several years later and involving more than one research group, as reviewed by Hodge and Inglett.[30] Glycyrrhizin (**1**) has been reported to be 93–170 times sweeter than sucrose, depending on concentration.[28] In Japan, extracts containing >90% w/w pure glycyrrhizin from *G. glabra* roots are used to sweeten foods and other products, such as cosmetics and medicines.[7,27,28] The ammonium salt of glycyrrhizin has generally recognized as safe (GRAS) status in the United States and is used primarily as a flavor enhancer.[7,28] Several attempts have been made to use various glycosylation methods in order to enhance the sweetness intensity of glycyrrhizin (**1**). The group of the late Professor Osama Tanaka[31] at Hiroshima University in Japan conducted the glycosylation of the aglycone glycyrrhetic acid to afford various glycyrrhizin monoglycoside analogues employing a chemical and enzymatic glycosylation procedure. A coupling reaction using mercury(II) cyanide ($Hg(CN)_2$) for chemical glycosylation was effective, leading to a significant enhancement of sweetness in the analogues obtained, especially 3-*O*-β-D-xylopyranoside (**7**) and 3-*O*-β-D-glucuronide (glycyrrhetic acid monoglucuronide (MGGR), **8**), with sweetness intensities rated as 544 and 941 times sweeter than sucrose, respectively. Such chemically modified products of glycyrrhizin were also found to have improved hedonic taste qualities.[20] MGGR (**8**), being more than five times sweeter than glycyrrhizin (**1**), as well as being readily soluble in water, is now used commercially as a sweetening agent in Japan for certain dairy products and soft drinks.[28,32]

Mogroside V (**2**) is a cucurbitane-type triterpenoid glycoside isolated from the fruits of *Siraitia grosvenorii* (Swingle) C. Jeffrey ex A.M. Lu & Zhi Y. Zhang (Cucurbitaceae), and was isolated initially in 1983 by Takemoto *et al.*[33] This plant is of Chinese origin and is known as 'lo han guo'. It has certain traditional uses such as to treat colds, sore throats, and minor gastrointestinal complaints.[28] Previous Latin binomials found in the phytochemical literature for this species are *Momordica grosvenorii* Swingle and *Thladiantha grosvenorii* (Swingle) C. Jeffrey. An extract of the dried fruits of *S. grosvenorii*, containing mogroside V (**2**) as the major sweet principle, is used in Japan as a sweetener in certain foods and beverages. The sweetness intensity of mogroside V has been rated as 250–425 times sweeter than sucrose, depending on concentration.[28] In a recent study, mogroside V (**2**) was confirmed as being the major constituent of the sweet-tasting ripe fruits of *S. grosvenorii*, whereas other cucurbitane glucosides are prevalent in unripe fruits and have a bitter taste.[34] The transglucosylation of mogroside V has been conducted, using cyclodextrin glucanotransferases and starch as donor substrate, and products showing sugar chain elongation were found to be less intensely sweet than the starting glycoside.[35] There is now a substantial body of literature on potential food and beverage applications of *S. grosvenorii*, particularly by Chinese authors.

Phyllodulcin (**3**), a dihydroisocoumarin-type sweetener, occurs in glycosidic form in the leaves of *Hydrangea macrophylla* Seringe var. *thunbergii* (Siebold) Makino (Saxifragaceae) ('Amacha') and other species of the genus *Hydrangea*. This compound was first isolated in 1916 by Asahina and Ueno,[36] with the structure determined in the following decade by Asahina and Juntaro, and the absolute configuration finally established as 3*R* in 1959.[37] Crushing or fermenting the leaves induces enzymatic hydrolysis of the native glycosides present to produce the sweet aglycone phyllodulcin (**3**; 400 times sweeter than 2% sucrose).[28] The fermented leaves of *H. macrophylla* var. *thunbergii* are used to prepare a sweet ceremonial tea in Japan, especially at 'Hamatsuri', a Buddhist religious festival.[28]

Rebaudioside A (**4**) and stevioside (**5**) are *ent*-kaurane-type diterpene (steviol) glycosides based on the aglycone steviol isolated from the leaves of the Paraguayan plant *Stevia rebaudiana* (Bertoni) Bertoni (Asteraceae),[20,38,39] with stevioside being the most abundant sweet compound in this plant part. Stevioside (**5**) was initially isolated in 1900 by the Paraguayan chemist Rebaudi, as reported by Bertoni,[40] but its structure was finalized only in 1963.[41] Rebaudioside A (**4**) was isolated and structurally determined in 1976 by Tanaka and co-workers[42] at Hiroshima University in Japan. The sweetness intensity of stevioside (**5**) has been estimated as 210 times sweeter than sucrose, although this value varies with concentration. However, rebaudioside A (**4**) (the second most abundant *S. rebaudiana* steviol glycoside with a sweetness intensity rated as about 240 times sweeter than sucrose) is considerably more pleasant tasting and more highly water soluble than stevioside (**5**), and thus better suited for use in food and beverages. Extracts of *S. rebaudiana* containing stevioside and/or purified stevioside are permitted as food additives in Japan, South Korea, Brazil, Argentina, and Paraguay, and are used as botanical dietary supplements elsewhere, in particular in the United States.[39]

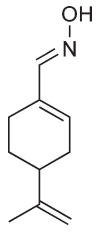
In Japan, the largest market for the *S. rebaudiana* sweeteners to date, three different forms of stevia sweetener products are commercially available, namely 'stevia extract', 'sugar-transferred stevia extract' (also known as 'enzymatically modified stevia extract' and 'glucosyl stevia'), and 'rebaudioside A-enriched stevia extract'.[43] 'Stevia extract' is a powder or granule made by several industrial steps and standardized so as to contain more than 80% of steviol glycosides, inclusive of dulcoside A (3–5%), rebaudioside A (20–25%), rebaudioside C (**20**) (5–10%), and stevioside (50–55%).[43] 'Sugar-transferred stevia extract', a complex mixture of compounds, is made by transglycosylation of steviol glycosides present in commercially available 'stevia extract' with a cyclomaltodextringlucanotransferase (CGTase)-starch system prepared from *Bacillus macerans*, followed by treatment with β-amylase.[20,43,44] Over the years, there have been many attempts to improve the taste qualities of the major *S. rebaudiana* sweet steviol glycoside, stevioside (**5**), because of its sensory limitations.[20,45–49] Several systematic studies on the structure–sweetness relationship of steviol glycosides have been conducted.[20,43,50] For example, the sweetness and pleasantness of stevioside (**5**) may be increased by treating stevioside-galactosyl ester (Sgal), prepared by removal of the 19-*O*-glucosyl group of stevioside, and replacing it with a β-galactosyl group. Transglucosylation of the intermediate with soluble starch using CGTase prepared from *B. macerans* then affords a mixture of mono-, di-, tri-, and tetra-α-glycosylated compounds. The product with four glucosyl units attached at the C-13 position showed an enhanced sweetness (**9**, Sgal-2).[48] A rebaudioside A analogue (**10**) with a (sodiosulfo)propyl group at C-19 in place of a β-glucosyl moiety showed improved sweetness qualities over the parent compound.[46] Stevioside (**5**) has been converted synthetically to rebaudioside A (**4**) by removal

of the terminal glucose unit at C-13 using amylase and then reintroducing synthetically two glucose units at different linkage positions to the remaining glucose moiety.[51] 'Rebaudioside A-enriched extract' is made from improved varieties of *S. rebaudiana*, which produce more rebaudioside A (**4**) than the native Paraguayan species.[52] Products incorporating *S. rebaudiana* sweeteners are used in more than 100 different food applications in Japan, in particular for salted foods such as Japanese-style pickles and dried seafoods, but also for beverages, yoghurt, ice cream, and sherbet.[43] In Korea, pure stevioside has become an important sucrose substitute and is used principally to sweeten 'soju' (a traditional distilled liquor made from sweet potatoes), soy sauce, pickles, and medicines.[53]

Currently, efforts are being made to introduce the sweet *S. rebaudiana ent*-kaurane (steviol) glycosides for use as sucrose substitutes in the United States and Europe. In the United States, rebaudioside A (**4**) was accorded GRAS status in late 2008 to sweeten foods and soft drinks and as a tabletop sweetener.[54] The existing literature has been surveyed and some additional studies have been performed for rebaudioside A (**4**) and, in some cases, stevioside (**5**), with regard to compound stability,[55] microbial hydrolysis,[56] genetic toxicity,[57] subchronic toxicity,[58] reproductive toxicity,[59] and toxicokinetics and metabolism in rats.[60] In humans, the pharmacokinetics after oral absorption[61] and also potential effects on adults with type 2 diabetes mellitus[62] and on healthy adults with normal and low-normal blood pressure[63] have been investigated. When taken together, these studies have led to the conclusion that rebaudioside A (**4**) (now also known as 'rebiana') seems to be appropriate for the sweetening of foods and beverages when purified to food-grade specifications.[64] In 2008, an acceptable daily intake (ADI) was established for 'steviol glycosides' at 0–4 mg kg^{-1} body weight for adults based on steviol, by the Food and Agriculture Organization of the United Nations/World Health Organization Joint Expert Committee on Food Additives (JECFA).[65] According to Renwick,[66] the estimated intake of rebaudioside A through normal use would not exceed a daily amount of steviol of 2 mg kg^{-1} body weight. In a further toxicological investigation to have appeared in the literature very recently, in a 90-day subchronic study, dietary supplements of high-dose levels of rebaudioside A (**4**) to Sprague–Dawley rats were not associated with any toxicity signs.[67]

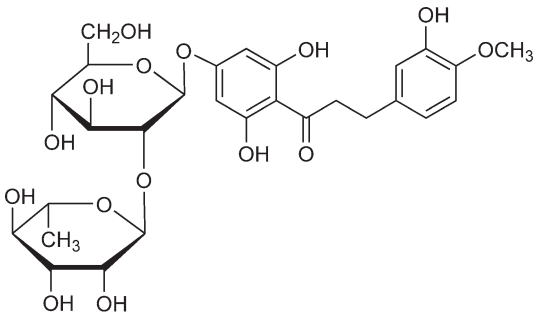
Thaumatin (**6**) is a protein sweetener isolated from the fruits of *Thaumatococcus danielli* Benth. (Marantaceae), and has been in use for several years as a sweetener and flavoring agent.[18,28,68–70] Five different thaumatin analogues (thaumatins I, II, III, a, and b) are now known, and thaumatins I and II are the major forms with both having 207-amino-acid residues.[18] The molecular weights of thaumatins I and II are 22 209 and 22 293 Da, respectively.[70] The three-dimensional (3D) structure of thaumatin I, based on X-ray analysis, has been reported.[71,72] The sweetness of thaumatin I has been rated between 1600 and 3000 times in comparison with sucrose on a weight basis, making this one of the most sweet natural substances so far discovered. Talin protein, the trade name of the commercial form of thaumatin protein as an aluminum ion adduct, was first approved as a food additive in Japan in 1979, and is an approved sweetener in Australia and, when used in limited levels, in countries of the European Union.[7] Talin protein has GRAS status as a flavor enhancer for use in chewing gum in the United States[28] and is used extensively worldwide as a flavoring ingredient.[7]

Perillartine (**11**) is a semisynthetic compound utilized on a limited basis in Japan, mainly as a replacement for maple syrup or licorice for the flavoring of tobacco.[16,28] Perillartine is an *α-syn*-oxime and can be synthesized from perillaldehyde, a monoterpenoid constituent of the volatile oil of *Perilla frutescens* (L.) Britton (Lamiaceae). This compound has a limited solubility in water and possesses a concomitant bitter taste along with sweetness.[16,28]



11

Neohesperidin dihydrochalcone (NHDC; **12**) is another semisynthetic compound and is a dihydrochalcone glycoside prepared from a flavanone constituent of *Citrus aurantium* L. (Rutaceae) (Seville orange).[73] It is permitted for use as a sweetener in a wide range of foodstuffs in countries of the European Union, as well as in Turkey and Switzerland, and has GRAS status as a flavor ingredient in the United States.[7,73]



12

It is necessary for low-calorie food ingredients to undergo rigorous testing in order to receive official sanction for marketing as a low-calorie sweetener in a western country, with considerations such as safety (acute and chronic toxicity; reproductive toxicology; carcinogenicity; mutagenicity), metabolism, stability, and other attributes such as the establishment of an ADI. Kemp[7] has provided an excellent chapter that describes the regulatory processes for new sweeteners in North America and Europe and summarizes current knowledge on 11 low-calorie sweeteners used in various countries around the world.

3.10.3 Discovery of Natural Sweeteners

The general approaches to the discovery of new sweetening agents from plant sources used by the group of the senior author of this review when at the University of Illinois at Chicago have been described previously.[17,21,74–76] This work led to the discovery of several new intensely sweet compounds of the terpenoid and flavonoid types, as mentioned in Section 3.10.4. A key aspect of our work was the accession of candidate sweet-tasting plants, and for this purpose three basic strategies were used, comprising scrutiny of scientific and popular texts, collecting plants in the field after making inquiries in market places, and performing organoleptic evaluations. For the first of these, the book *Index Kewensis* may be mentioned in particular. This is a listing of plant Latin binomials, with words such as 'dulcificum', 'dulcis', 'glycyrrhiza', 'mellosa', and 'saccharum' all implying either a sweet taste or a sweet smell for a particular species.[75–77] Although fieldwork for sweet-tasting plant has paid dividends in the search for new candidate sweet-tasting plants, ethnobotanical investigators must now arrange for approved 'prior informed consent' in order to make inquiries with members of indigenous populations who may be knowledgeable about the sensory and other properties of local plants. This is as a consequence of the 1992 United Nations Convention on Biological Diversity held in Rio de Janeiro, also known as the Rio Convention.[78] Another aspect of the passage of this convention is that source countries have been recognized as having a sovereign right over their own genetic resources, so that prior to any plant collections ever taking place, it is necessary for the investigator to develop detailed agreements pointing to an equitable sharing of benefits.[75–78] Although indiscriminate organoleptic testing of plants for the presence or absence of a sweet taste cannot be recommended, this approach has led to interesting results in the past. For example, when Soejarto *et al.*[79] carefully tasted 110 dried herbarium species of the genus *Stevia* (Asteraceae), collected previously from North and South America, several of these were found to be somewhat sweet tasting, including a 62-year-old specimen of *S. rebaudiana* (Bertoni) Bertoni collected in Paraguay. In a phytochemical study of these same samples, stevioside (**5**) was detected in both a *S. rebaudiana* sample and a Mexican species, *Stevia*

phlebophylla A. Gray, where it occurred in only trace amounts. Steviol (*ent*-kaurane) glycosides were absent in the other 108 *Stevia* species analyzed.[80]

The laboratory stage of a sweetener discovery protocol requires the use of a preliminary plant extraction protocol, producing extracts of various polarities. These should not be tasted for sweetness until negative results in both a mouse acute toxicity and a bacterial mutagenicity assay are demonstrated. It was found in our previous work that it is very rare indeed for a plant part to be sweet owing to its content of one or more highly sweet compounds. It is more usual for any inherent sweetness to be a result of high levels of sugars and polyols[81,82] or of phenylpropanoids such as *trans*-anethole[83] and *trans*-cinnamaldehyde.[84] In fact, as an empirical observation, if the combined amount of saccharides and polyols exceeds 5% w/w in a given plant part, the resultant sweetness can generally be considered as being due to the presence of these 'bulk' sweeteners. A suitable dereplication procedure using gas chromatography–mass spectrometry (GC–MS) has been developed for this purpose to rule out the sweetness contribution from saccharides and polyols in candidate sweet-tasting plants.[82]

For plant materials found to contain considerable amounts of sugars and polyols, these common sweet substances may be removed before assessing the residual material for the presence or absence of sweetness. A rapid, effective screening protocol utilizing a solid-phase extraction (SPE) technique permits the facile removal of sugars and polyols. A suitable SPE cartridge that may be employed is reversed-phase octadecyl silica gel (C_{18}) eluted initially with water, followed by 30, 50, 70, and 100% MeOH. The free sugars will be eluted with water together with some types of amino acids, small organic acids, and other materials. A ^{1}H NMR spectroscopic measurement of the water eluant can readily reveal if there are any interesting, highly polar molecules coeluted in this fraction. Together with the water eluant, the MeOH-containing fractions can be lyophilized after removal of the organic solvents before tasting. If sweetness is detected in any of these fractions, the polarity of the elution solvents may serve as an indicator of the type of compounds present. For example, sweet-tasting glycosides (e.g., saponins, diterpene glycosides, and flavonoid glycosides) would be found in the 30, 50, or 70% MeOH eluants, depending on the nature of the aglycones and the numbers of sugar units in the molecules. The above SPE procedure has the ability to partially purify complex plant extracts into several well-defined fractions based on the polarity of the compounds in a short period of time. Additionally, such a procedure will facilitate subsequent sensory evaluation as it will separate any bitter-tasting molecules coexisting in the plant material from other interesting tastants. If sweetness is detected in any of the nonsugar fractions, a scale-up isolation procedure is warranted. Sequential solvent partition using hexane/petroleum ether, ethyl acetate, and *n*-butanol may be carried out on the positive leads obtained. Subsequently, sensory-guided fractionation will be conducted using a combination of chromatographic techniques, inclusive of passage over reversed-phase macroresins, such as Diaion HP-20, as well as Sephadex gels and silica gel-based sorbents, until pure sweet-tasting molecules are obtained. The loading capacity of HP-20 is much higher than that of a C_{18} cartridge, so this procedure can be easily scaled up to generate samples for taste evaluation and subsequent fine chromatographic purification.

In our sweetener discovery work, purified plant secondary metabolites were subjected to mouse acute toxicity testing and mutagenicity evaluation prior to being tasted for sweetness and then evaluated for sweetness potency in comparison with sucrose.[74–76] This approach will require approval of both the relevant Animal Care Committee and the Institutional Review Board responsible for human subjects. Moreover, a minimum of 50–100 mg of each pure sweet compounds is required for safety testing, a quantity that is not always readily obtainable from the plant material on hand.[74–76]

Efforts have been made to circumvent the use of human subjects in the screening of samples of natural products for sweetness. For example, a combination of electrophysiological and behavioral assays on the Mongolian gerbil has been used to predict the sweetness of plant extracts of varying polarities with reasonable accuracy.[85] However, this is a somewhat time-consuming method, using specialized equipment, and the Mongolian gerbil does not respond to natural product sweeteners in the same manner as humans.[86] It is now possible to screen pure compound libraries for sweetness and other tastes in a less time-consuming fashion, using receptor-binding procedures (see Section 3.10.7).[87,88] Future screening of natural products should not necessarily be focused on only green plants, and such compounds may well occur also in microorganisms, insects, and marine organisms. In addition, more primitive plants may also afford new sweet substances. For instance,

Asakawa[89] has indicated that the moss *Fissidens japonicus* Dozy & Molk. (Fissidentaceae) is sweet tasting and contains nonsugar constituents that are so far structurally uncharacterized.

3.10.4 Structural Types of Highly Sweet Natural Products

In this section, the presently known highly sweet substances of natural origin are described. Sweet-tasting compounds of natural origin are listed in **Table 1**, and the same type of arrangement used in earlier reviews and book chapters on natural noncaloric sweeteners has been expounded upon.[19,23,24] Many of the sweet compounds obtained from plants are glycosides.[22] A few semisynthetic compounds that have exhibited a significant improvement in sweetness potency or pleasantness relative to the relevant natural product prototype sweet molecule are included in **Table 1**. Values of sweetness intensity relative to sucrose on a weight basis (sucrose = 1) are provided for the compounds listed, where such data are

Table 1 Highly sweet compounds from plants

Compound type/name[a]	*Plant name*	*Sweetness potency*[b]	*Reference(s)*
Monoterpenoids			
Perillartine (**11**)[c]	*Perilla frutescens* (L.) Britton (Lamiaceae)	370	90, 91
Sesquiterpenoids			
Acyclic glycoside			
Mukurozioside IIb (**13**)	*Sapindus rarak* DC. (Sapindaceae)	~1	82, 92
Bisabolanes			
(+)-Hernandulcin (**14**)	*Lippa dulcis* Trevir. (Verbenaceae)	1500	93, 94
4β-Hydroxyhernandulcin (**15**)	*L. dulcis*	NS[d]	101
Diterpenoids			
Diterpene acid			
4β,10α-Dimethyl-1,2,3,4,5,10-hexahydrofluorene-4α,6α-dicarboxylic acid (**16**)[e]	Pine tree[f]	1300–1800[g]	103
***ent*-Kaurene glycosides**			
Cussoracoside C (**17**)	*Cussonia racemosa* Baker (Araliaceae)	NS[d]	111
Dulcoside A (**18**)	*Stevia rebaudiana* (Bertoni) Bertoni (Asteraceae)	30	106
Rebaudioside A (**4**)	*S. rebaudiana*	242	42
Rebaudioside B (**19**)	*S. rebaudiana*	150	42
Rebaudioside C (**20**)	*S. rebaudiana*	30	104
Rebaudioside D (**21**)	*S. rebaudiana*	221	105
Rebaudioside E (**22**)	*S. rebaudiana*	174	105
Rebaudioside F (**23**)	*S. rebaudiana*	NS[d]	108
Rubusoside (**24**)	*Rubus suavissimus* S.K. Lee (Rosaceae)	115	109
Steviolbioside (**25**)	*S. rebaudiana*	90	42
Steviol 13-*O*-β-D-glucoside (**26**)	*R. suavissimus*	NS[d]	109, 110
Stevioside (**5**)	*S. rebaudiana*	210	40, 41
Suavioside A (**27**)	*R. suavissimus*	NS[d]	109
Suavioside B (**28**)	*R. suavissimus*	NS[d]	109
Suavioside G (**29**)	*R. suavissimus*	NS[d]	109
Suavioside H (**30**)	*R. suavissimus*	NS[d]	109
Suavioside I (**31**)	*R. suavissimus*	NS[d]	109
Suavioside J (**32**)	*R. suavissimus*	NS[d]	109
Labdane glycosides			
Baiyunoside (**33**)	*Phlomis betonicoides* Diels (Lamiaceae); *Phlomis medicinalis* Diels (Lamiaceae)	500	112, 113
Phlomisoside I (**34**)	*P. betonicoides*; *P. medicinalis*; *Phlomis younghushbandii* Mukerjee (Lamiaceae)	NS[d]	112, 113

(*Continued*)

Table 1 (Continued)

Compound type/name[a]	*Plant name*	*Sweetness potency*[b]	*Reference(s)*
Gaudichaudioside A (**35**)	*Baccharis gaudichaudiana* DC. (Asteraceae)	55	117
Triterpenoids			
Cucurbitane glycosides			
Bryodulcoside[h]	*Bryonia dioica* Jacq. (Cucurbitaceae)	NS[d]	119
Bryoside (**36**)	*B. dioica*	NS[d]	119
Bryonoside (**37**)	*B. dioica*	NS[d]	119
Carnosifloside V (**38**)	*Hemsleya carnosiflora* C. Y. Wu et Z. L. Chen (Cucurbitaceae)	51	121
Carnosifloside VI (**39**)	*H. carnosiflora*	77	120
Isomogroside V (**40**)	*Siraitia grosvenorii*[i] (Swingle) C. Jeffrey ex A. M. Lu & Zhi Y. Zhang (Cucurbitaceae)		125
Mogroside IV (**41**)	*S. grosvenorii*	233–392[g]	124
Mogroside V (**2**)	*S. grosvenorii*	250–425[g]	33, 124
11-Oxomogroside V (**42**)	*Siraitia siamensis* (Craib) C. Jeffrey ex S. Q. Zhong & D. Fang (Cucurbitaceae)	NS[d]	123, 124
Scandenoside R6 (**43**)	*Hemsleya panacis-scandens* C.Y. Wu et Z. L. Chen (Cucurbitaceae)	54	121
Scandenoside R11 (**44**)	*H. panacis-scandens*	NS[d]	122
Siamenoside I (**45**)	*S. grosvenorii*; *S. siamensis*	563	123, 124
Cycloartane glycosides			
Abrusoside A (**46**)	*Abrus precatorius* L.; *A. fruticulosus* Wall. (Fabaceae)	30	126, 129
Abrusoside B (**47**)	*A. precatorius*; *A. fruticulosus*	100	126, 129
Abrusoside C (**48**)	*A. precatorius*; *A. fruticulosus*	50	126, 129
Abrusoside D (**49**)	*A. precatorius*; *A. fruticulosus*	75	126, 129
Abrusoside E (**50**)	*A. precatorius*	NS[d]	128, 130
Abrusoside E methyl ester (**51**)[c]	*A. precatorius*	150	130
Dammarane glycosides			
Cyclocarioside A (**52**)	*Cyclocarya paliurus* (Batal.) Iljinsk. (Juglandaceae)	200	132
Cyclocaryoside I (**53**)	*C. paliurus*	250	133
Gypenoside XX[j] (**54**)	*Gynostemma pentaphyllum* (Thunb.) Makino (Cucurbitaceae)	NS[d]	134
Oleanane glycosides			
Albiziasaponin A (**55**)	*Albizia myriophylla* Benth. (Fabaceae)	5	135
Albiziasaponin B (**56**)	*A. myriophylla*	600	135
Albiziasaponin C (**57**)	*A. myriophylla*	NS[d]	135
Albiziasaponin D (**58**)	*A. myriophylla*	NS[d]	135
Albiziasaponin E (**59**)	*A. myriophylla*	NS[d]	135
Apioglycyrrhizin (**60**)	*Glycyrrhiza inflata* Batalin (Fabaceae)	300	136
Araboglycyrrhizin (**61**)	*G. inflata*	150	136
Glycyrrhizin (**1**)	*Glycyrrhiza glabra* L. (Fabaceae)	93–170[g]	136
Periandrin I (**62**)	*Periandra dulcis* Mart. ex Benth.; *P. mediterranea* (Vell.) Taub. (Fabaceae)	90	139
Periandrin II (**63**)	*P. dulcis*; *P. mediterranea*	95	137
Periandrin III (**64**)	*P. dulcis*; *P. mediterranea*	92	138
Periandrin IV (**65**)	*P. dulcis*; *P. mediterranea*	85	137
Periandrin V (**66**)	*P. dulcis*	220	140
Secodammarane glycosides			
Pterocaryoside A (**67**)	*Pterocarya paliurus* Batalin (Juglandaceae)	50	141
Pterocaryoside B (**68**)	*P. paliurus*	100	141
Steroidal saponins			
Osladin (**69**)	*Polypodium vulgare* L. (Polypodiaceae)	500	142–145
Polypodoside A (**70**)	*Polypodium glycyrrhiza* Eat. (Polypodiaceae)	600	146, 148
Polypodoside B (**71**)	*P. glycyrrhiza*	NS[d]	147

(*Continued*)

Table 1 (Continued)

Compound type/name[a]	*Plant name*	*Sweetness potency*[b]	*Reference(s)*
Telosmoside A_8 (**72**)	*Telosma procumbens* Merr. (Asclepiadaceae)	NS[d]	149
Telosmoside A_9 (**73**)	*T. procumbens*	NS[d]	149
Telosmoside A_{10} (**74**)	*T. procumbens*	NS[d]	149
Telosmoside A_{11} (**75**)	*T. procumbens*	NS[d]	149
Telosmoside A_{12} (**76**)	*T. procumbens*	NS[d]	149
Telosmoside A_{13} (**77**)	*T. procumbens*	NS[d]	149
Telosmoside A_{14} (**78**)	*T. procumbens*	NS[d]	149
Telosmoside A_{15} (**79**)	*T. procumbens*	1000	149
Telosmoside A_{16} (**80**)	*T. procumbens*	NS[d]	149
Telosmoside A_{17} (**81**)	*T. procumbens*	NS[d]	149
Telosmoside A_{18} (**82**)	*T. procumbens*	NS[d]	149
Phenylpropanoids			
trans-Anethole[k] (**83**)	*Foeniculum vulgare* Mill. (Apiaceae) *Illicium verum* Hook f. (Illiciaceae) *Myrrhis odorata* Scop. (Apiaceae) *Osmorhiza longistylis* DC. (Apiaceae) *Piper marginatum* Jacq. (Piperaceae) *Tagetes filicifolia* Lag. (Asteraceae)	13	83
trans-Cinnamaldehyde (**84**)	*Cinnamomum osmophloeum* Kaneh. (Lauraceae)	50	84
Dihydroisocoumarin			
Phyllodulcin[l] (**3**)	*Hydrangea macrophylla* Seringe var. *thunbergii* (Siebold) Makino (Saxifragaceae)	400	36, 37, 150
Flavonoids			
Dihydrochalcone glycosides			
Glycyphyllin (**85**)	*Smilax glycyphylla* Hassk. (Liliaceae)	NS[d]	152, 153, 156
Naringin dihydrochalcone[c] (**86**)	*Citrus paradisi* Macfad. (Rutaceae)	300	73, 156
Neohesperidin dihydrochalcone[c] (**12**)	*Citrus aurantium* L. (Rutaceae)	1000	73, 156
Phlorizin (**87**)	*Lithocarpus litseifolius* Chun (Fagaceae); *Symplocos lancifolia* Siebold et Zucc. (Symplocaceae)	NS[d]	154
Trilobatin (**88**)	*L. litseifolius*; *Symplocos microcalyx* Hayata (Symplocaceae)	NS[d]	154
Dihydroflavonols and dihydroflavonol glycosides			
3-Acetoxy-5,7-dihydroxy-4′-methoxyflavanone (**89**)	*Aframomum hanburyi* K. Schum.; *Aframomum pruinosum* Gagnep. (Zingiberaceae)	NS[d]	157, 158
2*R*,3*R*-(+)-3-Acetoxy-5,7,4′-trihydroxyflavanone (**90**)	*A. hanburyi*	NS[d]	157
(2*R*,3*R*)-Dihydroquercetin 3-*O*-acetate (**91**)	*T. dodoneifolia* (Hook. & Arn.) Cabrera (Asteraceae); *Hymenoxys turneri* K.F. Parker (Asteraceae)	80	159, 162
Dihydroquercetin 3-*O*-acetate 4′-methyl ether[e] (**92**)	*T. dodoneifolia*	400	159
(2*R*,3*R*)-2,3-Dihydro-5,7,3′,4′-tetrahydroxy-6-methoxy-3-*O*-acetylflavonol (**93**)	*H. turneri*	25	162
(2*R*,3*R*)-2,3-Dihydro-5,7,3′,4′-tetrahydroxy-6-methoxyflavonol (**94**)	*H. turneri*	15	162
(2*R*,3*R*)-2,3-Dihydro-5,7,4′-trihydroxy-6-methoxy-3-*O*-acetylflavonol (**95**)	*H. turneri*	20	162
Huangqioside E (**96**)	*Engelhardtia chrysolepis* Hance (Juglandaceae)	NS[d]	161
Neoastilbin (**97**)	*E. chrysolepis*	NS[d]	160

(*Continued*)

Table 1 (Continued)

Compound type/name[a]	*Plant name*	*Sweetness potency*[b]	*Reference(s)*
Proanthocyanidins			
Cinnamtannin B-1 (**98**)	*Cinnamomum sieboldii* Meisn. (Lauraceae)	NS[d]	163
Cinnamtannin D-1 (**99**)	*C. sieboldii*	NS[d]	163
Selligueain A (**100**)	*Selliguea feei* Bory (Polypodiaceae); *Polypodium decumanum* Willd. (Polypodiaceae); *Polypodium triseriale* Sw. (Polypodiaceae)	35	164, 167
Unnamed (**101**)	*Arachniodes sporadosora* (Kuntze) Nakaike; *A. exilis* Ching (Aspidiaceae)	NS[d]	164
Unnamed (**102**)	*A. sporadosora*; *A. exilis*	NS[d]	164
Benzo[*b*]indeno[1,2-*d*]pyran			
Hematoxylin (**103**)	*Haematoxylum campechianum* L. (Fabaceae)	120	169
Amino acid			
Monatin (**104**)	*Sclerochiton ilicifolius* A. Meeuse (Acanthaceae)	1200–1400[g]	171
Proteins			
Brazzein (**105**)	*Pentadiplandra brazzeana* Baill. (Capparaceae)	2000	175
Curculin (**106**)	*Curculigo latifolia* Dryand. (Hypoxidaceae)	550	178
Mabinlin[m] (**107**)	*Capparis masakai* Levl. (Capparaceae)	NS[d]	179, 180
Monellin (**108**)	*Dioscoreophyllum cumminsii* Diels (Menispermaceae)	3000	181
Neoculin (**109**)	*Curculigo latifolia* Dryand. (Hypoxidaceae)	4000	183
Pentadin[n]	*Pentadiplandra brazzeana* Baillon (Capparaceae)	500	184
Thaumatin[o] (**6**)	*Thaumatococcus danielli* Benth. (Marantaceae)	1600	68, 185

[a] The structures of the compounds are shown in the text (**1**–**6**, **11**–**109**).
[b] Values of relative sweetness are on a weight comparison basis to sucrose (=1.0), and are taken from either the original literature report of the sweet compound concerned or from later reports, and represent consensus figures.
[c] Semisynthetic derivative of the natural product.
[d] NS = sweetness potency not given.
[e] Synthetic sweetener based on the natural product lead compound.
[f] Plant Latin binomial not given in the original reference.
[g] Relative sweetness varied with the concentration of sucrose.
[h] Complete structure and stereochemistry not determined.
[i] Formerly named *Momordica grosvenorii* Swingle and *Thladiantha grosvenorii* (Swingle) C. Jeffrey.
[j] Although a known compound, the sweet taste becomes evident only after the initial compound isolation.[22]
[k] Identified as a sweet-tasting constituent of these six species. However, this compound has a wider distribution in the plant kingdom.
[l] The plant of origin may be crushed or fermented in order to generate phyllodulcin (**3**).
[m] The structure of mabinlin II is shown in the text.
[n] The amino acid sequence of pentadin has not yet been determined.
[o] The structure of thaumatin I is shown in the text.

available. However, it is to be noted that sweetness intensity values for a given sweet molecule vary with concentration as well as the organoleptic method used. A more detailed discussion of sensory testing methods is provided in Section 3.10.7.

It may be seen from **Table 1** that the principal groups of highly sweet-tasting compounds of plant origin are terpenoids, flavonoids, and proteins, although compounds of other chemical classes have also been found to be highly sweet, inclusive of an amino acid, a benzo[*b*]indeno[1,2-*d*]pyran, a dihydroisocoumarin, phenylpropanoids, proanthocyanidins (Chapter 6.18), and steroidal saponins (Chapter 4.16). Within the terpenoid and flavonoid categories, a number of subgroups are represented. Among the terpenoids, there are several subclasses of diterpenoids (Chapter 1.17) and triterpenoids (Chapter 1.18), whereas both the dihydrochalcones and the dihydroflavonols are known to be sweet among the flavonoids. Accordingly, 20 major structural types of plant-derived sweeteners have been found to date. Altogether, about 100 structurally characterized natural products and 6 semisynthetic or synthetic compounds are included in **Table 1**, and these were obtained from species representative of more than 25 separate plant families. The distribution of plant families containing sweet-tasting compounds, according to a Dahlgren's

superorder organizational scheme, has been found to be random.[17] However, certain plant families biosynthesize natural sweeteners of more than one structural class, as exemplified by the family Asteraceae, which produces such compounds of both the *ent*-kaurane diterpenoid and the dihydroflavonol types.[17] It may be seen from **Table 1** that species of the same genus occasionally biosynthesize the same sweet-tasting constituent. Also, all three structural variants known to date of the oleanane-type glycosides (viz., the albiziasaponins, glycyrrhizin derivatives, and the periandrins) are all biosynthesized from plants of the family Fabaceae.

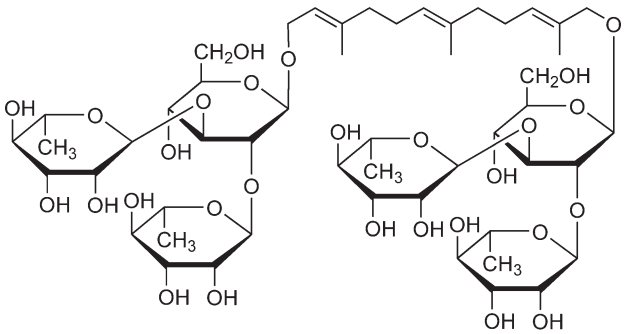
3.10.4.1 Terpenoids and Steroids

3.10.4.1.1 Monoterpenoids

As mentioned earlier, perillartine (**11**) has been known for many years as a highly sweet semisynthetic analogue prepared from the naturally occurring monoterpenoid (Chapter 1.15) perillaldehyde, a constituent of the volatile oil of *P. frutescens* (L.) Britton (Lamiaceae).[90,91] Although this compound is the only member of the monoterpenoid group of compounds so far known to be potently sweet, its poor solubility and sweetness qualities have precluded any significant commercial development.[16,28] However, owing to its inherent sweetness, perillartine remains of current interest in the literature, both for its potential applications and as a standard substance in sweetener research.

3.10.4.1.2 Sesquiterpenoids

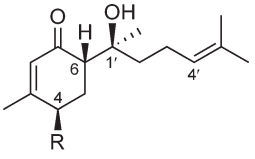
3.10.4.1.2(i) Acyclic Mukuroziosides IIb (**13**) is an acyclic sesquiterpene glycoside isolated and characterized initially from the pericarps of *Sapindus mukorossi* Gaertn. (Sapindaceae).[92] As a result of work performed at the University of Illinois at Chicago, this compound was isolated from the fruits of *Sapindus rarak* DC. (Sapindaceae) collected in Indonesia, where it was found to occur in a high yield (6.8% w/w). This is the first identification of an acyclic sesquiterpene glycoside with a sweet taste from a plant source, and it possesses a sweetness potency approximately equal to that of sucrose.[82]



13

3.10.4.1.2(ii) Bisabolane (+)-Hernandulcin (**14**) is a highly sweet bisabolane-type sesquiterpenoid, (Chapter 1.16), which was first purified and characterized at the University of Illinois at Chicago from a sweet-tasting herb collected in Mexico, *Lippia dulcis* Trevir. (Verbenaceae), a plant known to the Aztecs.[93,94] The sweetness potency of this substance was rated as 1500 times sweeter than 0.25 mol l^{-1} sucrose on a weight basis, but this compound was also found to possess some bitterness and a somewhat unpleasant aftertaste.[93] Of the four possible diastereomers for the structure of this compound, it was found after total synthesis that only the 6*S*,1′*S* configuration of hernandulcin shows intense sweetness.[95] Three primary structural units involved in the mediation of the sweet taste of this rather simple molecule have been resolved (i.e., the C-1′ hydroxyl group, the C-6 carbonyl, and the C-4′, C-5′ double bond).[96] Souto Bachiller *et al.*[97] have demonstrated that there are at least two different

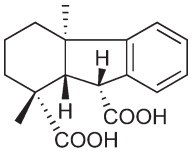
chemotypes of *L. dulcis*, with the Puerto Rican type containing (+)-hernandulcin as the major component (33% w/w) of its volatile oil and the Mexican type containing only trace amounts of this sesquiterpenoid. Hernandulcin has been produced both by total synthesis[21,98,99] and from both shoot and hairy root cultures of *L. dulcis*[21] and subjected to microbial biotransformation.[100] A second sesquiterpene-type analogue in this series, namely 4β-hydroxyhernandulcin (**15**), was isolated in the laboratory of the senior author of this chapter from a sample of *L. dulcis* collected in Panama. However, the sweetness potency of this compound relative to sucrose was not evaluated because of the paucity of availability of **15**.[101] Recently, six further bisabolane analogues of hernandulcin have been isolated and characterized by Japanese workers from the aerial parts of *L. dulcis*, although these were not evaluated for the presence or absence of a sweet taste.[102] Now that nearly 25 years have elapsed since hernandulcin (**14**) was first discovered, this structurally simple highly sweet substance remains of interest as a tool for sweetener research, although it is probably too unstable and unpleasant tasting for commercial development.



14 R = H
15 R = OH

3.10.4.1.3 Diterpenoids

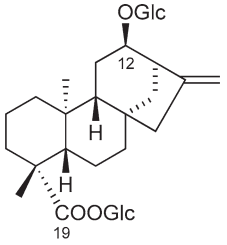
3.10.4.1.3(i) Diterpene acid In 1971, Tahara *et al.*[103] described four stereoisomers of 4β,10α-dimethyl-1,2,3,4,5,10-hexahydrofluorene-4α,6-dicarboxylic acid derived from pine tree resin. One of these compounds, **16**, was found to be highly sweet, but also bitter tasting. There has been very little follow-up to this initial literature report on this sweet-tasting diterpene acid.



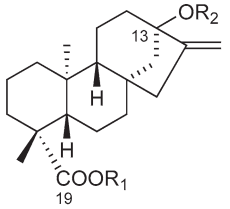
16

3.10.4.1.3(ii) *ent*-Kaurane As mentioned earlier in this chapter, two steviol glycosides, rebaudioside A (**4**) and stevioside (**5**), have commercial applications in various forms, and there is considerable interest in extending these uses further.[39,43,53,54] Several additional sweet diterpene glycosides of the *ent*-kaurane type were isolated from two plant species, *S. rebaudiana*[42,104–106] and *Rubus suavissimus* S. K. Lee (Rosaceae),[107] in the 1970s and 1980s. Dulcoside A (**18**) and rebaudioside C (**20**) are the major constituents of the leaves of *S. rebaudiana*, but occur in somewhat lower yields (0.4–0.7 and 1–2% w/w, respectively) when compared with stevioside (**5**) and rebaudioside A (**4**).[104–106] Other less abundant sweet principles of *S. rebaudiana* leaves are rebaudioside B (**19**),[42] rebaudioside D (**21**),[105] rebaudioside E (**22**),[105] and steviolbioside (**25**).[42] It is possible that rebaudioside B and steviolbioside are actually artifacts of extraction as opposed to being actual natural products. More recently, a ninth sweet-tasting principle has been obtained from *S. rebaudiana* leaves, namely rebaudioside F (**23**), which contains a β-xylose unit as part of the C-13 saccharide substituent.[108] Rubusoside (=desglucosylstevioside) (**24**) is the main *ent*-kaurene glycoside from *R. suavissimus* leaves (a sweet-tasting species originally published in the literature as *Rubus chingii* Hu[107]) and its sweetness potency was rated as 115 times sweeter than sucrose, but also with the perception of some bitterness and an unpleasant aftertaste.[109] Additional *ent*-kaurene-type diterpene glycosides were isolated as minor constituents of

R. suavissimus leaves, namely suaviosides A, B, G, H, I, and J (**27**–**32**) and steviol 13-*O*-β-D-glucoside (steviol monoside) (**26**).[109,110] However, their sweetness intensities have not been determined. No other species of the genus *Stevia* or *Rubus* appears to biosynthesize sweet-tasting *ent*-kaurene glycosides to any significant degree.[21] Like stevioside (**5**), rubusoside (**24**) was subjected to extensive structural modification by the group of the late Professor Osamu Tanaka at Hiroshima University in order to improve on its quality of taste.[20,44,48,49] Several *ent*-kaurene glycosides were isolated in 2002 by Yamasaki *et al.*[111] from the Madagascan plant *Cussonia racemosa* Baker (Araliaceae), and one of these compounds, cussoracoside C (**17**), bearing a β-glucose unit at C-12, was stated to be sweet tasting, although its relative potency compared with sucrose was not documented.

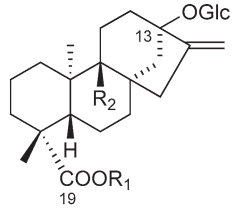


17

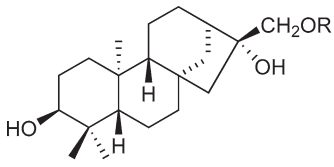


	R_1	R_2
18	β-glc	β-glc^2-α-rha
19	H	β-glc^2-β-glc
		$\vert^3$
		β-glc
20	β-glc	β-glc^2-α-rha
		$\vert^3$
		β-glc
21	β-glc^2-β-glc	β-glc^2-β-glc
		$\vert^3$
		β-glc
22	β-glc^2-β-glc	β-glc^2-β-glc
23	β-glc	β-glc^2-β-xyl
		$\vert^3$
		β-glc
25	H	β-glc^2-β-glc

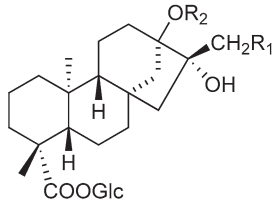
rha: L-rhamnopyranosyl



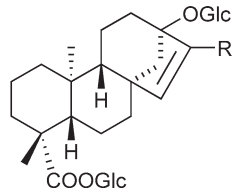
	R_1	R_2
24	β-glc	H
26	H	H
28	β-glc	OH



27 R = β-glc



	R_1	R_2
29	H	β-glc
31	OH	H



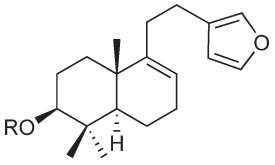
30 R = CHO
32 R = CH_2OH

Rebaudioside A (**4**) has a branched trisaccharide unit at C-13 and is sweeter and more pleasant tasting than stevioside (**5**), with a C-13 sophorosyl disaccharide moiety. Removal of the C-19 sugar unit of rebaudioside A, so as to produce rebaudioside B (**19**), results in a less potently sweet-tasting compound. Rebaudioside C (**20**),

having a terminal glucose unit at C-13 replaced by rhamnose, is not only less sweet than rebaudioside A (**4**), but is somewhat bitter. Sauvioside A (**27**) is unusual among the *ent*-kaurane sweet glycosides in that it contains no C-16, C-17-exomethylene group. Sauvioside B (**28**), which differs from rubusoside (**24**) only in the presence of a C-9 hydroxy group, has only half of the resultant sweetness potency (**Table 1**).[19,109]

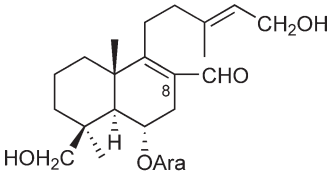
There is now a very large technical and patent literature on *S. rebaudiana* and its sweet steviol glycoside constituents. This information refers principally to methods for the purification of these substances, procedures for taste improvement, and biological test results.

3.10.4.1.3(iii) Labdane Two furanolabdane-type diterpene glycosides, baiyunoside (**33**) and phlomisoside I (**34**), were isolated as sweet constituents from the roots of a Chinese plant, *Phlomis betonicoides* Diels (Lamiaceae).[112,113] Baiyunoside (**33**) was rated about 500 times sweeter than sucrose, whereas the sweetness intensity of phlomisoside I (**34**) was not determined. Both **33** and **34** were also isolated from a second species, *Phlomis medicinalis* Diels (roots), whereas phlomisoside I (**34**) occurred in the roots of *Phlomis younghushbandii* Mukerjee. The specimens of *P. medicinalis* and *P. younghushbandii* investigated were collected in Tibet.[114] The sweet-tasting compound phlomisoside I (**34**) has a C-3 neohesperidyl group, whereas when this sugar unit is replaced by a sophorosyl group moiety as in phlomisoside II, the compound is bitter tasting.[112,113] In Japan, Nishizawa *et al.*[115,116] at Tokushima Bunri University have prepared a large number of synthetic analogues of baiyunoside (**33**), with some of these found to be sweeter than the natural product.



33 R = β-glc^2-β-xyl
34 R = α-rha^2-β-glc

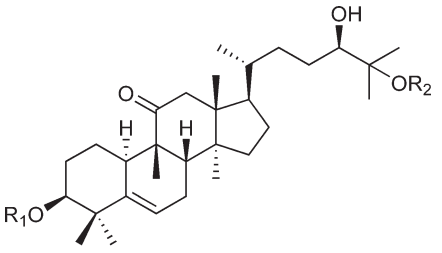
Another labdane-type diterpene glycoside, namely gaudichaudioside A (**35**), was isolated from the aerial parts of a species collected in Paraguay, *Baccharis gaudichaudiana* DC. (Asteraceae) (local name 'chilca melosa'), in work carried out at the University of Illinois at Chicago.[117] It was found that gaudichaudioside A was 55 times sweeter than 2% w/w sucrose solution and gave only a very low perception of bitterness.[117] Several closely related compounds with the same carbon skeleton as gaudichaudioside A were isolated but were not highly sweet. Instead, these derivatives exhibited other taste properties (sweet-bitter, bitter, and neutral tasting).[117] For example, when the C-8 aldehyde group of gaudichaudioside A (**35**) was replaced with a $-CH_2OH$ group, as in gaudichaudioside B, a fleeting sensation of sweetness lasting only a few seconds occurred when tasted, followed by prolonged bitterness.[117] *Baccharis* species are somewhat bitter tasting, so the occurrence of a sweet-tasting labdane glycoside, such as compound **35** in *B. gaudichaudiana*, seems to be an anomaly.



35
Ara: L-arabinopyransyl

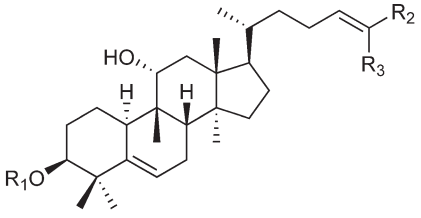
3.10.4.1.4 Triterpenoids

3.10.4.1.4(i) Cucurbitane Many cucurbitane-type triterpenoid glycosides have been isolated as sweet principles from several plants of the family Cucurbitaceae, and this is now one of the largest groups of natural highly sweet compounds. Two cucurbitane-type glycosides, bryoside (**36**) and bryonoside (**37**), have been reported from the roots of *Bryonia dioica* Jacq. as sweet principles, although their sweetness intensities relative to sucrose were not reported.[118,119] The structure of bryonoside (**37**) was revised by Arihara and co-workers[119] in 1992. The structure of a third sweet compound from *B. dioica*, bryodulcoside, has not yet been resolved.[119]

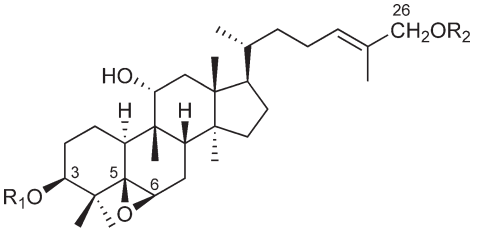


	R_1	R_2
36	β-glc^2-α-rha	β-glc
37	β-glc^2-α-rha	β-glc^6-β-glc

Two species of the genus *Hemsleya*, namely *H. carnosiflora* C.Y. Wu et Z.L. Chen and *H. panacis-scandens* C.Y. Wu and Z.L. Chen, have afforded between them three sweet cucurbitane-type triterpene glycosides, carnosifllosides V (**38**) and VI (**39**), and scandenoside R6 (**43**).[120,121] In addition, several other cucurbitane-type triterpenoid glycosides, scandenosides R8–R11, were isolated from *H. panacis-scandens*.[122] Of these, only scandenoside R11 (**44**) was reported to be sweet tasting, but its sweetness potency was not stated.[122]

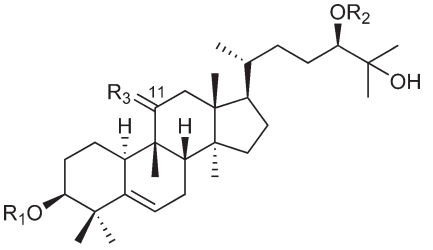


	R_1	R_2	R_3
38	β-glc	CH_2O-β-glc^2-β-glc	CH_3
39	β-glc	CH_2O-β-glc^6-β-glc	CH_3
43	β-glc	CH_3	CH_2O-β-glc^2-β-glc



	R_1	R_2
44	β-glc	β-glc^6-β-glc

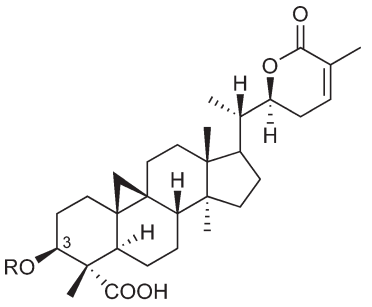
Several highly sweet cucurbitane-type triterpene glycosides have been isolated from the dried fruits of the Chinese medicinal plant *S. grosvenorii* (Swingle) C. Jeffrey ex A.M. Li & Zhi Y. Zhang, a plant mentioned already in this chapter (Section 3.10.2).[33–35,123,124] Mogrosides IV (**41**) and V (**2**) and siamenoside I (**45**) are the major sweet principles of this plant species and their sweetness intensities were rated as 233–392, 250–425, and 563 times sweeter than sucrose, respectively.[124] Siamenoside I (**45**) was also isolated as a minor constituent from another species of the genus *Siraitia*, *S. siamensis* (Craib) C. Jeffrey ex S.Q. Zhong & D. Fang, together with 11-oxomogroside V (**42**), with the sweetness intensity of the latter compound unreported.[123,124] Recently, Jia and Yang[125] have described a further sweet-tasting glycoside from *S. grosvenorii*, namely isomogroside V (**40**).



	R_1	R_2	R_3
40	β-glc^4-β-glc	β-glc^2-β-glc $\vert^6$ β-glc	α-OH, β-H
41	β-glc^6-β-glc	β-glc^2-β-glc	α-OH, β-H
42	β-glc^6-β-glc	β-glc^2-β-glc $\vert^6$ β-glc	=O
45	β-glc	β-glc^2-β-glc $\vert^6$ β-glc	α-OH, β-H

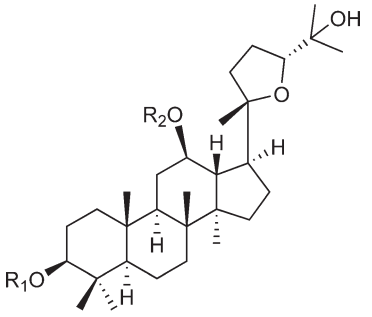
Analysis of many cucurbitane glycosides has indicated that at least three sugar units need to be present in the molecule for the exhibition of sweetness, with glycosides of aglycones containing 11α-hydroxy, 11β-hydroxy, and 11-keto functionalities being highly sweet, neutral tasting, and less highly sweet or bitter, respectively.[19,121,124]

3.10.4.1.4(ii) Cycloartane Abrusosides A–E (**46–50**) are prototype triterpenoid sweeteners of the cycloartane type and were isolated at the University of Illinois at Chicago from a sample of the leaves of *Abrus precatorius* L. (Fabaceae) collected in Florida.[126–128] Of these, compounds **46–49** were isolated from a second species of the genus, *A. fruticulosus* Wall. from Thailand.[129] The aglycone of these compounds, namely abrusogenin, was identified as having a novel carbon skeleton, as confirmed by single-crystal X-ray crystallography of abrusogenin methyl ester.[127] Abrusosides A–E differ structurally from one another in the type of saccharide unit affixed to the C-3 position. The sweetness intensities of the ammonium salts of abrusosides A–D were evaluated as 30, 100, 50, and 75 times sweeter than 2% w/w sucrose solution, respectively.[126] The sweetness intensity of abrusoside E per se was not determined, whereas the semisynthetic monomethyl ester (the 6″-methyl-β-D-glucuronopyranosyl-(1→2)-β-D-glucopyranosyl derivative) of abrusoside E (**51**) was found to exhibit about 150 times the sweetness potency of 2% sucrose, making it the sweetest compound in this series.[130] When the aglycone carboxylic acid group was methylated, as in abrusoside E dimethyl ester, no sweetness was perceived.[130] Abrusogenin methyl ester has been synthesized in our laboratories.[131] Thus far, the abrusosides seem to be the only sweet constituents from the genus *Abrus*.



46 R = β-glc
47 R = β-glcA-6-CH_3^2-β-glc
48 R = β-glc^2-β-glc
49 R = β-glcA^2-β-glc
50 R = β-glc^2-β-glcA
51 R = β-glc^2-β-glcA-6-CH_3

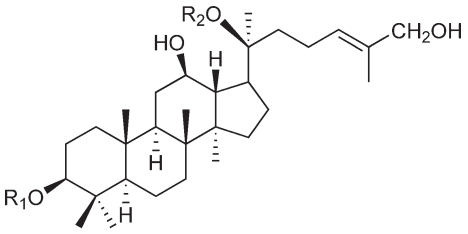
3.10.4.1.4(iii) Dammarane Cyclocarioside A (**52**), a dammarane-type triterpenoid glycoside sweet principle from the leaves of *Cyclocarya paliurus* (Batal.) Iljinsk. (Juglandaceae), was isolated and characterized from a plant used in the People's Republic of China as a treatment for diabetes.[132] Later, another sweet-tasting principle, cyclocarioside I (**53**), was isolated from the same plant along with two other compounds with the same dammarane-type triterpenoid aglycone structure.[133] Cyclocarioside I was shown to exhibit about 250 times the sweetness potency of sucrose.[133]



	R_1	R_2
52	α-araf-5-Ac	α-rha
53	α-araf	β-qui

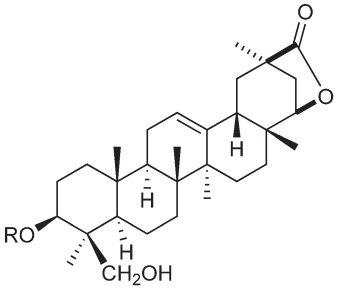
qui: D-quinovosyl
araf: D-arabinofuranosyl

From the crude extract of the vine of *Gynostemma pentaphylum* (Thunb.) Makino (Cucurbitaceae), a plant used to make a sweet tea ('Amachazuru') in Japan, gypenoside XX (**54**) was isolated by Takemoto *et al.*[134] in Tokushima. Although the sweetness of this compound was not reported when it was first characterized, it was later stated to be sweet.[22] The relative sweetness potency of gypenoside XX (**54**) to sucrose has not appeared in the literature.



	R_1	R_2
54	β-glc^2-β-glc | 6 α-rha	β-glc^6-β-glc

3.10.4.1.4(iv) Oleanane Five oleanane-type triterpene saponins, namely albiziasaponins A–E (**55**–**59**), have been reported by Yoshikawa and co-workers from Kyoto Pharmaceutical University as sweet principles of stems of *Albizia myriophylla* Benth. (Fabaceae), a traditional medicinal plant collected in Thailand, used as a substitute for Glycyrrhizae Radix (licorice root) as a sweetening agent. A lactone ring was attached to the C-20,22 positions in ring E of the aglycone portion of albiziasaponins A and C–E (**55**, **57**–**59**). Albiziasaponin B (**56**), which has a C-29 carboxyl group instead, was rated as about 600 times sweeter than sucrose.[135]

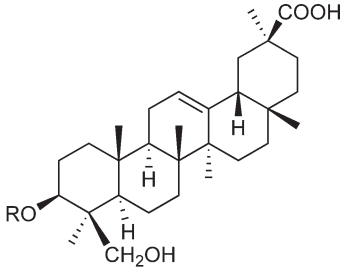


55 R = β-glcA^2-β-glcA^2-α-rha

57 R = β-glcA^2-β-glc^2-α-rha
|
3
β-glc

58 R = β-glcA^2-β-glc^2-α-rha
|
3
β-xyl

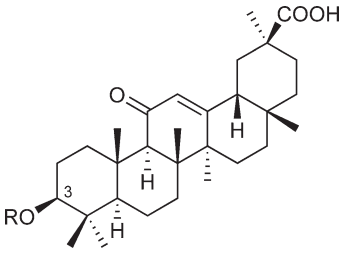
59 R = β-glcA^2-β-glcA^2-α-rha
|
3
β-xyl



56 R = β-glcA^2-β-glcA^2-α-rha

As mentioned earlier, glycyrrhizin (**1**) and its ammonium salts are available commercially for sweetening and flavoring purposes, and glycyrrhetic acid 3-*O*-D-glucuronide (MGGR, **7**) is a promising new intense sweetener.[27,28,32] Apioglycyrrhizin (**60**) and araboglycyrrhizin (**61**) have been isolated from the roots of *Glycyrrhiza inflata* Batalin (Fabaceae) by Kitagawa and colleagues.[136] Glycyrrhizin has a C-3-affixed diglucuronate unit, whereas apioglycyrrhizin (**60**) has a β-D-apiofuranosyl-(1→2)-β-D-glucuronopyranosyl group and araboglycyrrhizin (**61**) an α-L-arabinopyranosyl-(1→2)-β-D-glucuronopyranosyl group at the C-3 position of the aglycone glycyrrhetic acid. The sweetness intensities of apioglycyrrhizin (**60**) and araboglycyrrhizin (**61**) were rated as 300 and 150 times sweeter than sucrose, respectively.[136] In a published review of 13 glucuronide

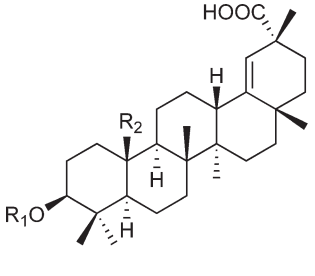
saponins from licorice, it was pointed out that 11-deoxoglycyrrhizin is bitter, thereby showing the requirement for the presence of the C-11 carbonyl group for the mediation of sweetness in glycyrrhizin (**1**) and its sweet derivatives.[27]



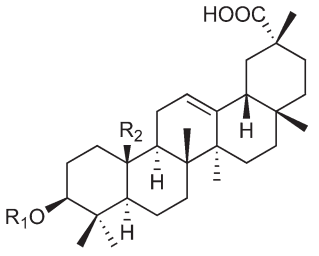
60 R = β-glcA^2-β-api
61 R = β-glcA^2-α-ara

api: D-apiofuranosyl

Periandrins I–IV (**62–65**) were characterized in the 1980s as oleanane-type triterpenoid glycoside sweeteners from the roots of *Periandra dulcis* Mart. ex Benth. (Fabaceae) (Brazilian licorice) by Hashimoto *et al.*[137–139] at Kobe Pharmaceutical University in Japan, and the sweetness potency was determined as about 90 times sweeter than sucrose for each compound. Previously, the sweet principle of Brazilian licorice roots was thought to be glycyrrhizin (**1**).[16] Periandrins I–IV (**62–65**) were also found in another species, *Periandra mediterranea* (Vell.) Taub.[137–139] A fifth compound in this series, periandrin V (**66**), was isolated from the roots of *P. dulcis* at the University of Illinois at Chicago, and was found to be based on the same aglycone as periandrin I (**62**). The terminal D-glucuronic acid residue of periandrin I was substituted by a D-xylose moiety in periandrin V. Periandrin V (**66**) exhibited 220 times the sweetness of 2% sucrose and was accordingly ranked as the sweetest substance obtained so far in the periandrin series.[140]



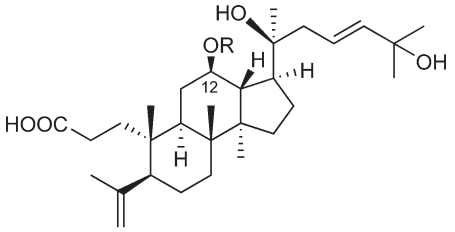
	R_1	R_2
62	β-glcA^2-β-glcA	CHO
64	β-glcA^2-β-glcA	CH_2OH
66	β-glcA^2-β-xyl	CHO



	R_1	R_2
63	β-glcA^2-β-glcA	CHO
65	β-glcA^2-β-glcA	CH_2OH

3.10.4.1.4(v) Secodammarane Two new sweet secodammarane glycosides, pterocaryosides A (**67**) and B (**68**), were isolated and structurally determined from the leaves and stems of *Pterocarya paliurus* Batalin (Juglandaceae), at the University of Illinois at Chicago.[141] *Pterocarya paliurus* Batal. is a preferred taxonomic name for *C. paliurus* (Batal.) Iljinsk (see Section 3.10.4.1.4(iii)). The leaves of *P. paliurus* are used by local

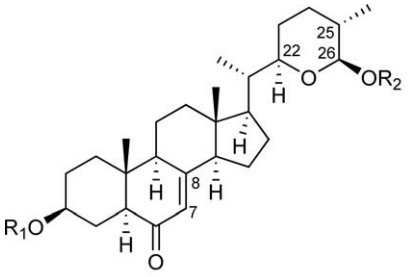
populations in Hubei Province of the People's Republic of China to sweeten cooked foods. Pterocaryoside A (**67**), which has a β-quinovose unit attached to the C-12 position, is 50 times sweeter than sucrose, whereas pterocaryoside B (**68**), with an α-arabinose unit at C-12, is 100 times sweeter than sucrose.[141] These are the first highly sweet secodammarane glycosides to have been isolated and structurally characterized, and represent interesting lead compounds for potential synthetic optimization.



67 R = β-qui
68 R = α-ara

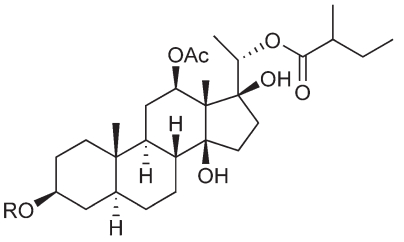
3.10.4.1.5 Steroidal saponins

The steroidal saponin osladin (**69**) was isolated as a sweet principle from the fern *Polypodium vulgare* L. (Polypodiaceae) nearly 40 years ago by Czech workers.[142] However, the original structure proposed was later revised because when this compound was synthesized by Nishizawa and Hamada[143–145] it was not sweet at all. The correct structure of osladin (**69**) was characterized by single-crystal X-ray crystallography and the stereochemistry of osladin was reassigned as 22*R*, 25*S*, and 26*R*. The actual sweetness potency of osladin was revised to 500 times, rather than 3000 times, sweeter than sucrose, as originally published.[143–145] Polypodosides A (**70**) and B (**71**) were isolated at the University of Illinois at Chicago from the rhizomes of the North American fern *Polypodium glycyrrhiza* Eat. (Polypodiaceae) as additional highly sweet steroidal glycosides.[146,147] The aglycone on which these compounds are based, polypodogenin, is the $\Delta^{7,8}$-derivative of the aglycone of osladin. The structure of polypodoside A (**70**) was also revised as 22*R*, 25*S*, 26*R*, by a chemical interconversion procedure, in collaboration with Nishizawa of Tokushima Bunri University.[148] Polypodoside A (**70**) shows a high sweetness potency and was rated as 600 times sweeter than sucrose.[146] In order to exhibit sweetness, steroidal saponins of this type must be bidesmosidic, with saccharide substitution at both C-3 and C-26.[19] Polypodoside C, a third compound in the polypodoside series, has an L-acofriopyranosyl (3-*O*-methylrhamnosyl) unit attached at C-26, in place of the L-rhamnosyl moiety of polypodoside B (**71**), and is devoid of sweetness.[19,147]



	R_1	R_2	Other
69	β-glc^2-α-rha	α-rha	7,8-dihydro
70	β-glc^2-α-rha	α-rha	-
71	β-glc	α-rha	-

Telosmosides A_8–A_{18} (**72–82**), pregnane-type steroidal saponins, were isolated by Yamasaki and co-workers[149] at Hiroshima University as sweet principles of the stems of *Telosma procumbens* Merr. (Asclepiadaceae). This plant has been used as a traditional medicinal plant in certain Asian countries and employed as a licorice substitute in Vietnam. Several unusual sugars such as D-cymarose, D-oleandrose, D-digitoxose, D-thevetose, and 6-deoxy-3-*O*-methyl-D-allose were found in the saccharide moieties attached at the C-3 position of the common aglycon of these compounds. Telosmoside A_{15} (**79**) was reported to exhibit a sweetness intensity 1000 times greater than that of sucrose.[149]

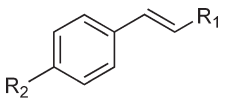


72 R = β-dig^4-β-cym^4-β-ole^4-β-glc
73 R = β-dig^4-β-ole^4-β-the^4-β-glc
74 R = β-dig^4-β-cym^4-β-ole^4-β-ole^4-β-ole
75 R = β-dig^4-β-cym^4-β-ole^4-β-ole^4-β-the
76 R = β-dig^4-β-cym^4-β-ole^4-β-ole^4-β-glc
77 R = β-dig^4-β-dig^4-β-ole^4-β-ole^4-β-the
78 R = β-dig^4-β-cym^4-β-ole^4-β-ole^4-β-ole^4-β-glc
79 R = β-dig^4-β-cym^4-β-ole^4-β-ole^4-β-the^4-β-glc
80 R = β-dig^4-β-cym^4-β-ole^4-β-ole^4-β-glc^4-β-glc
81 R = β-dig^4-β-cym^4-β-ole^4-β-ole^4-β-alm^4-β-glc
82 R = β-dig^4-β-cym^4-β-ole^4-β-the^4-β-glc^4-β-glc

dig: D-digitoxosyl
alm: 6-deoxy-3-*O*-methyl-D-allosyl
cym: D-cymarosyl
the: D-thevetosyl
ole: D-oleandrosyl

3.10.4.2 Phenylpropanoids

The phenylpropanoids *trans*-anethole (**83**) and *trans*-cinnamaldehyde (**84**) are used as flavoring agents in foods in the United States and many other countries.[16] In work performed at the University of Illinois at Chicago, *trans*-cinnamaldehyde (**84**) was isolated from *Cinnamomum osmophloeum* Kaneh. (Lauraceae) as a sweet principle,[84] whereas *trans*-anethole (**83**) was isolated as the volatile oil constituent responsible for the sweet taste of several plant species, as listed in **Table 1**.[83] These two compounds occur widely in the plant kingdom. As previously indicated, it is necessary to rule out their presence in any candidate sweet plant when searching for new natural product sweeteners, by preliminary analysis using GC–MS.[83,84]



	R_1	R_2
83	CH_3	OCH_3
84	CHO	H

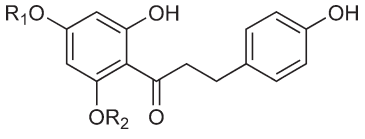
3.10.4.3 Dihydroisocoumarins

The leaves of *H. macrophylla* var. *thunbergii*, containing the dihydroisocoumarin 3*R*-phyllodulcin (**3**), were mentioned earlier in the chapter as having a limited use in Japan.[28,36,37] It has been demonstrated that 3*R*-phyllodulcin occurs naturally in unprocessed leaves of its plant of origin as a 5:1 enantiomer with the previously undescribed compound 3*S*-phyllodulcin.[150] Also reported were several new 3*R*- and 3*S*-phyllodulcin 3′-*O*-glycosides, although the presence or absence of a sweet taste in these three new phyllodulcin analogues was not disclosed.[150] Much work has been performed on the synthesis of dihydroisocoumarin sweeteners, using phyllodulcin (**3**) as a lead compound. For example, Merlini *et al.*[151] have recently summarized their research data on the effects of the structural modification of this compound on sweetness, wherein 120 compounds containing an isovanillyl unit were produced.

3.10.4.4 Flavonoids

3.10.4.4.1 Dihydrochalcones

Glycyphyllin (**85**), phlorizin (**87**), and trilobatin (**88**) are dihydrochalcone glycosides reputed to be sweet and were isolated from *Smilax glycyphylla* Hassk. (Smilacaceae),[16,152,153] *Symplocos lancifolia* Siebold et Zucc.,[154] and *Symplocos microcalyx* Hayata (Symplocaceae),[154] respectively. Trilobatin (**88**) was isolated as a major sweet compound along with phlorizin (**87**) from the leaves of *Lithocarpus litseifolius* Chun (Fagaceae).[155] According to Horowitz and Gentili,[156] glycyphyllin is bittersweet, with the bitterness predominating. Naringin dihydrochalcone (**86**) and neohesperidin dihydrochalcone (**12**) are semisynthetic dihydrochalcone glycosides and can be obtained as by-products of the citrus industry.[73,156] Neohesperidin dihydrochalcone (NHDC; **12**; 250–1800 times sweeter than sucrose, depending on concentration) is sweeter than compound **86**, and has acceptable hedonic properties, and is used in a wide variety of foodstuffs as a sweetener and flavor ingredient, as mentioned earlier.[71,73,156] There have been several attempts to synthesize improved sweet-tasting dihydrochalcones, with such compounds requiring 3-hydroxy-4-alkoxy substitution in ring B.[73,156]

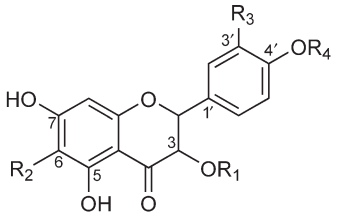


	R_1	R_2
85	H	α-rha
86	β-glc^2-α-rha	H
87	H	β-glc
88	β-glc	H

3.10.4.4.2 Dihydroflavonols

The seeds of *Aframomum hanburyi* K. Schum. (Zingiberaceae) are used as an antidote and ingredient in certain medicinal preparations in Cameroon. From an acetone extract of the seeds of this plant, two sweet dihydroflavonols, 3-acetoxy-5,7-dihydroxy-4′-methoxyflavanone (**89**) and 2*R*,3*R*-(+)-3-acetoxy-5,7,4′-trihydroxyflavanone (**90**), were isolated.[157] 3-Acetoxy-5,7-dihydroxy-4′-methoxyflavanone (**89**) was previously isolated from a different species, *Aframomum pruinosum* Gagnep.[158] However, the sweetness intensities of these compounds were not indicated.[157,158] The previously known (2*R*,3*R*)-dihydroquercetin 3-*O*-acetate (**91**), which was rated as 80 times sweeter than sucrose, was isolated at the University of Illinois at Chicago as a sweet principle from the young leaves of *Tessaria dodoneifolia* (Hook. & Arn.) Cabrera (Asteraceae), collected in Paraguay.[159] The sweetness of this compound was increased to 400 times that of sucrose by methylation at the

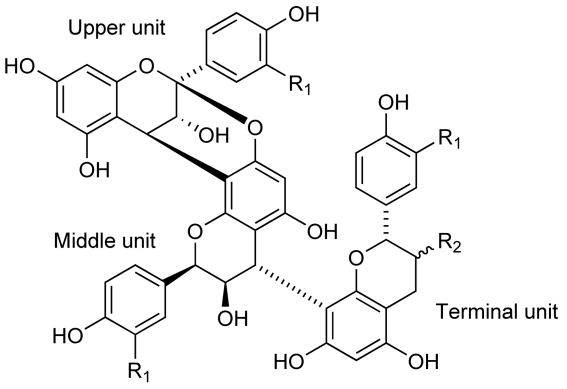
C-4′ hydroxyl to form a synthetic isovanillyl derivative (**92**).[159] Two dihydroflavonols, huangqioside E (**96**) and neoastilbin (**97**), were purified from *Engelhardtia chrysolepis* Hance (Juglandaceae).[160,161] However, their sweetness intensities were not evaluated. Compound **91** and three additional sweet dihydroflavonols (**93–95**) with a C-6 methoxy group were isolated from the leaves of *Hymenoxys turneri* K.F. Parker (Asteraceae), collected in Texas.[162] Compound **93**, the 6-methoxylated analogue of compound **91**, showed less than 50% of its sweetness potency.[19,162]



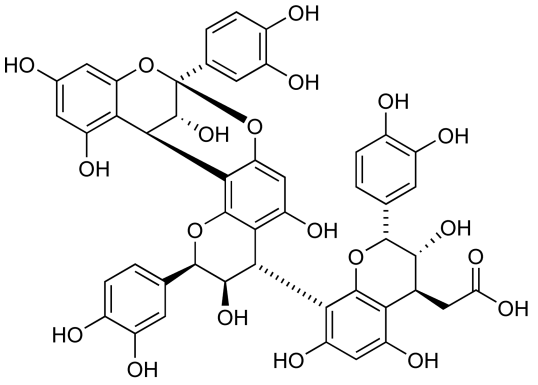
	R_1	R_2	R_3	R_4	Other
89	Ac	H	H	CH_3	2*R*,3*R*
90	Ac	H	H	H	2*R*,3*R*
91	Ac	H	OH	H	2*R*,3*R*
92	Ac	H	OH	CH_3	-
93	Ac	CH_3O	OH	H	2*R*,3*R*
94	H	CH_3O	OH	H	2*R*,3*R*
95	Ac	CH_3O	H	H	2*R*,3*R*
96	α-rha^3-β-glc	H	OH	H	2*R*,3*R*
97	α-rha	H	OH	H	2*S*,3*S*

3.10.4.5 Proanthocyanidins

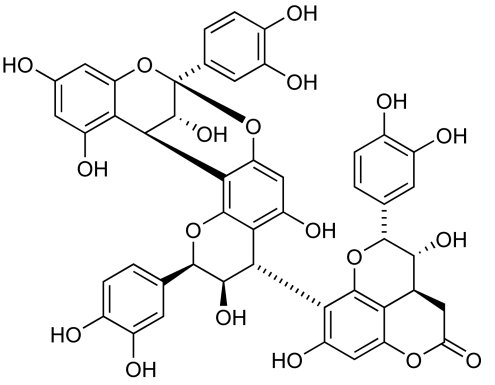
Several doubly linked ring-A proanthocyanidins are known to be sweet tasting. For example, two proanthocyanidins, cinnamtannin B-1 (**98**) and cinnamtannin D-1 (**99**), isolated from the roots of *Cinnamomum sieboldii* Meisn. (Lauraceae) showed sweet properties.[163] Other sweet-tasting proanthocyanidins with carboxylic acid (**101**) and lactone (**102**) functionalities were isolated from the ferns *Arachniodes sporadosora* (Kuntze) Nakaike and *Arachniodes exilis* Ching (Aspidiaceae).[164] However, none of these proanthocyanidins was ever quantitatively rated for its sweetness intensity relative to sucrose. A sweet-tasting proanthocyanidin, selligueain A (**100**), was isolated at the University of Illinois at Chicago from the rhizomes of the fern *Selliguea feei* Bory (Polypodiaceae), collected in Indonesia.[165] Selligueain A may be distinguished from previously known sweet-tasting doubly linked ring-A trimeric proanthocyanidins **98** and **99**, as it has an afzelechin residue rather than an epicatechin moiety as the lower terminal unit of the molecule. When evaluated by a small human taste panel, selligueain A (**100**) showed 35 times the sweetness of a 2% sucrose solution and was not perceived as astringent when in solution.[165] A further doubly linked ring-A proanthocyanidin, selligueain B, was also isolated from the rhizomes of *S. feei*, but was not perceived as sweet tasting.[166] As a result of the investigation of selligueain A (**100**) and related compounds, stringent structural requirements seem to be necessary for proanthocyanidins of this type to exhibit a sweet taste. In this connection, it is notable that an epimer of selligueain A (epiafzelechin-(4β→8,2β→*O*→7)-epiafzelechin-(4β→8)-epiafzelechin) was astringent without any hint of sweetness.[165,166] Bohlin and co-workers[167] have demonstrated that selligueain A (**100**) is present in low yields in two *Polypodium* species collected in Honduras, and that this sweet-tasting compound is also an elastase inhibitor in human neutrophils. Moreover, Subarnas and Wagner[168] have reported the analgesic and antiinflammatory activities of selligueain A (**100**) in two *in vivo* models.



	R_1	R_2
98	OH	α-OH
99	OH	β-OH
100	H	β-OH



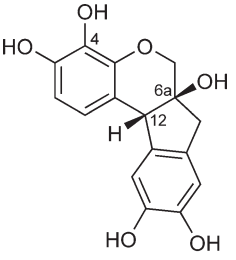
101



102

3.10.4.6 Benzo[*b*]indeno[1,2-*d*]pyrans

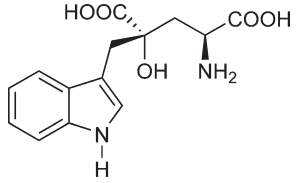
From the extract of the heartwood of *Haematoxylum campechianum* L. (Fabaceae), a sweet principle was isolated, namely (+)-hematoxylin (**103**). This compound has been used for a long time as a microscopic staining reagent, but the sweetness of this compound was not recognized previously. Also, in the same study, brazilin, the 4-deoxy derivative of (+)-hematoxylin and a constituent of *Caesalpinia echinata* Lam. (Fabaceae), was found not to be sweet.[169] It was concluded that requirements for sweetness of compound **103** include the C-4 hydroxy group and the *cis* junction of the cyclopentene and pyran rings.[19,169] In a follow-up study, (+)-hematoxylin (**103**) was rated as 120 times sweeter than 3% sucrose, whereas its synthetic (−)-enantiomer was only 50 times sweeter.[169,170]



103

3.10.4.7 Amino Acids

A highly sweet amino acid, (−)-monatin (**104**), was isolated from an African plant, *Sclerochiton ilicifolius* A. Meeuse (Acanthaceae).[171] Monatin (**104**) was rated as being comparable in sweetness to the synthetic amino acid 6-chloro-D-tryptophan, which showed a sweetness intensity 1300 times that of sucrose. Monatin (**104**) appears to be the only native plant amino acid with a highly sweet taste to have been discovered. This compound has been synthesized in chiral form.[172,173] A structure–sweet-tasting activity relationship study on synthetic analogues of monatin has been carried out in the laboratory of Merlini at the University of Milan. The 2*R*,4*R* isomer, rather than the natural 2*S*,4*S* isomer, is the sweetest of three of the four stereoisomers of monatin found to be sweet tasting.[174]



104

3.10.4.8 Proteins

Several plant-derived proteins, including brazzein (**105**),[175–177] curculin (**106**),[18,178] mabinlin (**107**),[179,180] monellin (**108**),[181,182] neoculin (**109**),[183] pentadin,[184] and thaumatin (**6**),[18,28,68–70,185] have been reported as sweeteners, with thaumatin mentioned earlier in this chapter as having commercial use as a sweetener and a flavor enhancer. The amino acid sequence of at least one form of each of these proteins is provided in this chapter, and information on their species of origin is given in **Table 1**. In a book chapter, Crammer[186] has summarized the recent literature for the plant proteins, including their subtypes, so this information is not repeated here. The genes for the production of curculin, mabinlin, monellin, and thaumatin have been expressed in microorganisms and solid-phase synthesis has been used to produce mabinlin and monellin.[182] The two most recently discovered sweet-tasting plant proteins are brazzein and neoculin, and these will be briefly described in turn. Brazzein (**105**), isolated from the fruits of a West African climbing vine, *Pentadiplandra brazzeana* Baill. (Capparaceae), by Ming and Hellekant at the University of Wisconsin, has 54-amino-acid residues and a molecular weight of 6473 Da, making it a relatively small protein compared to other sweet proteins such as curculin (12 491 Da), mabinlin (12 441 Da), monellin (11 086 Da), and thaumatin (22 209 Da).[175,177] Brazzein has four disulfide bridges and promising thermostability, as its sweetness was not destroyed even after 4 h exposure at 80 °C.[176] Most of the other protein sweeteners are unstable to heat and inappropriate for use at high temperature. The sweetness potency of brazzein (**105**) was rated as 2000 times greater than that of 2% sucrose, so this protein offers considerable potential as a new naturally occurring sweetener, and there are plans for its commercialization.[187] Markley and co-workers[187] have designed a new protocol for the production of brazzein by *Escherichia coli* as a fusion protein, and the potential mode of interaction of this sweet protein with the sweet taste receptor has been investigated by computer homology modeling.[188] Neoculin (**109**), a heterodimer of an acidic, glycosylated subunit of 113-amino-acid residues and a basic subunit that is the monomeric curculin itself, was isolated from the fruit of *Curculigo latifolia* Dryand. (Hypoxidaceae).[183] This protein tastes sweeter (40 000 times) than sucrose on a molar basis and converts sourness to sweetness. Interestingly, neoculin exhibits its potent sweetness at a weakly acidic pH and interacts with the hT1R3 human sweet taste receptor.[189,190]

PyrGlu-Asp-Lys-Cys-Lys-Lys-Val-Tyr-Glu-Asn-Tyr-Pro-Val-Ser-Lys-Cys-Gln-Leu-Ala-Asn-
1 5 10 15 20
Gln-Cys-Asn-Tyr-Asp-Cys-Lys-Leu-Asp-Lys-His-Ala-Arg-Ser-Gly-Glu-Cys-Phe-Tyr-Asp-
21 25 30 35 40
Glu-Lys-Arg-Asn-Leu-Gln-Cys-Ile-Cys-Asp-Tyr-Cys-Glu-Tyr
41 45 50 54

105

Asp-Asn-Val-Leu-Leu-Ser-Gly-Gln-Thr-Leu-His-Ala-Asp-His-Ser-Leu-Gln-Ala-Gly-Ala-
1 5 10 15 20
Tyr-Thr-Leu-Thr-Ile-Gln-Asn-Asn-Cys-Asn-Leu-Val-Lys-Tyr-Gln-Asn-Gly-Arg-Gln-Ile-
21 25 30 35 40
Trp-Ala-Ser-Asn-Thr-Asp-Arg-Arg-Gly-Ser-Gly-Cys-Arg-Leu-Thr-Leu-Leu-Ser-Asp-Gly-
41 45 50 55 60
Asn-Leu-Val-Ile-Tyr-Asp-His-Asn-Asn-Asn-Asp-Val-Asn-Gly-Ser-Ala-Cys-Cys-Gly-Asp-
61 65 70 75 80
Ala-Gly-Lys-Tyr-Ala-Leu-Val-Leu-Gln-Lys-Asp-Gly-Arg-Phe-Val-Ile-Tyr-Gly-Pro-Val-
81 85 90 95 100
Leu-Trp-Ser-Leu-Gly-Pro-Asn-Gly-Cys-Arg-Arg-Val-Asn-Gly
101 105 110 114

106

Glu-Leu-Trp-Arg-Cys-Gln-Arg-Gln-Phe-Leu-Gln-His-Gln-Arg-Leu-Arg-Ala-Cys-Gln-Arg-
1 5 10 15 20
Phe-Ile-His-Arg-Arg-Ala-Gln-Phe-Gly-Gly-Gln-Pro-Asp
21 25 30 33

A chain

Glu-Pro-Arg-Arg-Pro-Ala-Leu-Arg-Gln-Cys-Cys-Asn-Gln-Leu-Arg-Gln-Val-Asp-Arg-Pro-
1 5 10 15 20
Cys-Val-Cys-Pro-Val-Leu-Arg-Gln-Ala-Ala-Gln-Gln-Val-Leu-Gln-Arg-Gln-Ile-Ile-Gln-
21 25 30 35 40
Gly-Pro-Gln-Gln-Leu-Arg-Arg-Leu-Phe-Asp-Ala-Ala-Arg-Asn-Leu-Pro-Asn-Ile-Cys-Asn-
41 45 50 55 60
Ile-Pro-Asn-Ile-Gly-Ala-Cys-Pro-Phe-Arg-Ala-Trp
61 65 70 72

B chain

107

Arg-Glu-Ile-Lys-Gly-Tyr-Glu-Tyr-Gln-Leu-Tyr-Val-Tyr-Ala-Ser-Asp-Lys-Leu-Phe-Arg-
1 5 10 15 20
Ala-Asp-Ile-Ser-Glu-Asp-Tyr-Lys-Thr-Arg-Gly-Arg-Lys-Leu-Leu-Arg-Phe-Asn-Gly-Pro-
21 25 30 35 40
Val-Pro-Pro-Pro
41 44

A chain

(Thr)-Gly-Glu-Trp-Glu-Ile-Ile-Asp-Ile-Gly-Pro-Phe-Thr-Gln-Asn-Leu-Gly-Lys-Phe-Ala-Val-
1 5 10 15 20
Asp-Glu-Glu-Asn-Lys-Ile-Gly-Gln-Tyr-Gly-Arg-Leu-Thr-Phe-Asn-Lys-Val-Ile-Arg-Pro-
21 25 30 35 40
Cys-Met-Lys-Lys-Thr-Ile-Tyr-Glu-Glu-Asn
41 45 50

B chain

108

```
Asp-Ser-Val-Leu-Leu-Ser-Gly-Gln-Thr-Leu-Tyr-Ala-Gly-His-Ser-Leu-Thr-Ser-Gly-Ser-
1                5                  10                  15                  20
Tyr-Thr-Leu-Thr-Ile-Gln-Asn-Asn-Cys-Asn-Leu-Val-Lys-Tyr-Gln-His-Gly-Arg-Gln-Ile-
21               25                  30                  35                  40
Trp-Ala-Ser-Asp-Thr-Asp-Gly-Gln-Gly-Ser-Gln-Cys-Arg-Leu-Thr-Leu-Arg-Ser-Asp-Gly-
41               45                  50                  55                  60
Asn-Leu-Ile-Ile-Tyr-Asp-Asp-Asn-Asn-Met-Val-Val-Trp-Gly-Ser-Asp-Cys-Trp-Gly-Asn-
61               65                  70                  75                  80
X-Gly-Thr-Tyr-Ala-Leu-Val-Leu-Gln-Gln-Asp-Gly-Leu-Phe-Val-Ile-Tyr-Gly-Pro-Val-
80               85                  90                  95                 100
Leu-Trp-Pro-Leu-Gly-Leu-Asn-Gly-Cys-Arg-Ser-Leu-Asn
111              115                 110         113
```

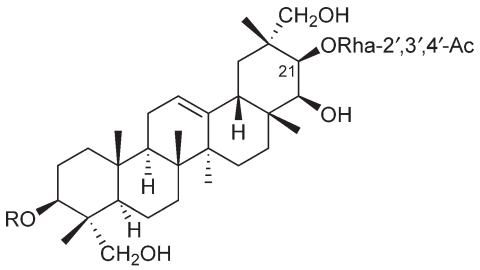
Acidic subunit (NAS) of neoculin

109

3.10.5 Naturally Occurring Sweetness Inducers

3.10.5.1 Triterpenoids

Five oleanane-type triterpenoid glycosides, strogins 1–5, were isolated from the leaves of the Malaysian plant *Staurogyne merguensis* Kuntze (Acanthaceae) by Kurihara and co-workers.[191] Strogins 1, 2, and 4 (**110–112**) show a persistent sweetness-inducing activity, in response to tasting cold water, which lasts for at least an hour.[192] In its country of origin, *S. merguensis* grows wild and local populations have used the leaves to sweeten rice during cooking.[191] The sweetness-inducing activities of strogins 1–5 were measured by a psychometric method.[191–193] Thus, the compounds were held in the mouth by a small taste panel for 3 min at a concentration of 1 mmol l^{-1} and then expectorated. The subjects then tasted water and the induced sweetness activity was determined by comparison with 0.05–0.4 mol l^{-1} standard sucrose solutions. Strogins 1, 2, and 4 also showed a sweet taste, lasting less than a minute, with strogin 1 (**110**) tasting sweeter than strogin 2 (**111**) or 4 (**112**). In contrast, strogins 3 and 5 were neither sweet tasting nor sweetness enhancing.[191,192] The sweetness-inducing activity of strogin 1 (**110**) reduced the antisweet activity of gymnemic acid (see Section 3.10.6), and was not reduced by the presence of Ca^{2+} and Mg^{2+} cations, unlike miraculin (**115**) (see Section 3.10.5.3).[192]

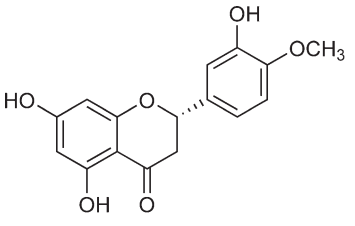


110 R = β-glcA^2-β-xyl
111 R = β-glcA
112 R = β-glcA^2-β-glc

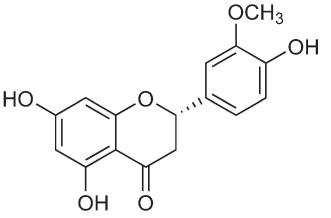
3.10.5.2 Flavonoids

Recently, several flavonoids have been reported to enhance sweetness or to improve taste in the patent literature. For example, the flavanone hesperetin (**113**), the aglycone of hesperidin, a glycoside found in citrus fruits, has been demonstrated as a sweetness-enhancing agent.[194] Homoeriodictyol (**114**), a naturally occurring

structurally related substance to compound **113**, was found to exhibit a 6% sweetness-enhancing activity when present at 100 ppm and evaluated with a 5% w/v sucrose solution.[195] When dissolved in water at 100 ppm, compound **114** exhibited a sweet, vanillin-like, phenolic taste.[195] Both hesperetin (**113**) and homoeriodictyol (**114**) occur in *Eriodictyon californicum* Decne. (Hydrophyllaceae) ('Herba Santa').[196]



113



114

3.10.5.3 Proteins

Miraculin (**115**) is a protein isolated from the fruits of the West African plant *Richardella dulcifica* (Schumacher & Thonn.) Baehni (Sapotaceae) (miracle fruit)[18,186,197,198] and has the property of making sour or acidic materials taste sweet. Miraculin is a homodimer of two glycosylated 191-amino-acid polypeptides linked by disulfide bonds, having a molecular weight of about 24 000 Da, with the monomeric form shown (**115**).[199] It was found that at acidic pH this protein converts a sour taste to a sweet taste, by an unknown molecular mechanism, whereas at neutral pH it tastes flat. The compound has no sweet taste per se. Miracle fruit concentrate was formerly on the market in the United States, but was removed because prior FDA approval for the scientific claims made had not been realized.[28] Although miraculin so far has not been expressed by *E. coli*,[186] this protein has been produced in transgenic lettuce[200] and tomatoes.[201]

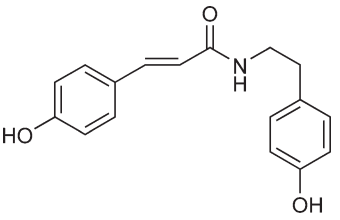
Asp-Ser-Ala-Pro-Asn-Pro-Val-Leu-Asp-Ile-Asp-Gly-Glu-Lys-Leu-Arg-Thr-Gly-Thr-Asn-
1 5 10 15 20
Tyr-Tyr-Ile-Val-Pro-Val-Leu-Arg-Asp-His-Gly-Gly-Gly-Leu-Thr-Val-Ser-Ala-Thr-Thr-
21 25 30 31 40
Pro-Asn-Gly-Thr-Phe-Val-Cys-Pro-Pro-Arg-Val-Val-Gln-Thr-Arg-Lys-Glu-Val-Asp-His-
41 45 50 55 60
Asp-Arg-Pro-Leu-Ala-Phe-Phe-Pro-Glu-Asn-Pro-Lys-Glu-Asp-Val-Val-Arg-Ala-Ser-Thr-
61 65 70 75 80
Asp-Leu-Asn-Ile-Asn-Phe-Ser-Ala-Phe-Met-Pro-Cys-Arg-Trp-Thr-Ser-Ser-Thr-Val-Ser-
81 85 90 95 100
Arg-Leu-Asp-Lys-Tyr-Asp-Glu-Ser-Thr-Gly-Gln-Tyr-Phe-Val-Thr-Ile-Gly-Gly-Val-Lys-
101 105 110 115 120
Gly-Asn-Pro-Gly-Pro-Glu-Thr-Ile-Ser-Ser-Trp-Phe-Lys-Ile-Glu-Glu-Phe-Cys-Gly-Ser-
121 125 130 135 140
Gly-Phe-Tyr-Lys-Leu-Val-Phe-Cys-Pro-Thr-Val-Cys-Gly-Ser-Cys-Lys-Val-Lys-Cys-Gly-
141 145 150 155 160
Asp-Val-Gly-Ile-Tyr-Ile-Asp-Gln-Lys-Gly-Arg-Arg-Arg-Leu-Ala-Leu-Ser-Asp-Lys-Pro-
161 165 170 175 180
Phe-Ala-Phe-Glu-Phe-Asn-Lys-Thr-Val-Tyr-Phe
181 185 190

115

Curculin (**106**)[18,178] and neoculin (**109**),[18,183,189,190] proteins isolated from the fruits of *C. latifolia* (see Section 3.10.4.8), also have sweetness-inducing activity. These proteins have a sweet taste that dissipates before the sweetness-inducing effect on water becomes evident.

3.10.5.4 Miscellaneous Compounds

The plant constituent *N-trans*-coumaroyltyramine (**116**), found in various plants inclusive of *Berberis vulgaris* L. (Berberidaceae),[202] has also been found to be a sweetness-inducing agent.[195] This compound was rated as being sweet when evaluated at a concentration of 100 ppm by a taste panel, and demonstrated a 6% sweetness-enhancing activity when evaluated in the same manner as compound **114** described above.[195] The effects of the caffeic acid conjugates cynarin and chlorogenic acid in turning water sweet have been documented.[25,203]



116

As relatively small percentage increases in sweetness enhancement by a given ingredient of foods and beverages may be important, it can be expected that additional naturally occurring compounds of this type will be discovered in the near future, especially now that screening via receptor binding is possible.[87,88]

3.10.6 Naturally Occurring Triterpenoid Sweetness Inhibitors

It has been known for some years that a number of synthetic compounds and certain enzymes suppress the sweet taste in humans and animals.[28,204–211] In addition, three plant species in particular, *Gymnema sylvestre* (Retz.) Schult. (Asclepiadaceae), *Hovenia dulcis* Thunb. (Rhamnaceae), and *Ziziphus jujuba* Mill. (Rhamnaceae), have been studied extensively for their sweetness-inhibitory (antisweet) constituents.[25] In recent years, additional sweetness-inhibiting agents have been isolated from *G. sylvestre* and *H. dulcis*, as well as three other plant species, *Gymnema alterniflorum* (Lour.) Merr. (Asclepiadaceae), *Stephanotis lutchuensis* Koidz. var. *japonica* (Asclepiadaceae), and *Styrax japonica* Sieb. et Zucc. (Styracaceae). The presently known oleanane- and dammarane-type triterpenoid sweetness-inhibitory agents from these species are reported in **Table 2**. In addition to antisweet triterpenoids, a 35-amino-acid peptide called gurmarin has been isolated from the leaves of *G. sylvestre* and has also been found to exhibit a sweetness-inhibitory effect.[210,211]

The sweetness-inhibitory activity of plant triterpenoids has been evaluated by placing 5 ml of a 50% or 1 mmol l^{-1} solution of the compound under consideration in the mouth for 2–3 min. On expectorating, the mouth is washed with distilled water. Subsequently, different concentrations of sucrose (0.1–1 mmol l^{-1}) are tasted. The maximum concentration of sucrose at which complete suppression of sweetness is perceived is then recorded for each tastant.[23,25,212] In practice, antisweet compounds of plant origin have been ranked in terms of sweetness-inhibitory potency by comparison with gymnemic acid I (**120**).[23]

Since the initial reports of sweetness-inhibitory oleanane-type gymnemic acids from the leaves of *Gymnema sylvestre*, plant species of the family Asclepiadaceae have served as the sources of several sweetness-inhibitory compounds. The initial isolation and structural characterization of these compounds was very challenging, and these early investigations have been reviewed.[23,25] In 1989, gymnemic acids I–VI (**120–125**) were isolated, with a common gymnemagenin (**191**) oleanane-type aglycone structure and a glucuronic acid moiety.[213–215] Gymnemic acid I (**120**) is the compound with which all other 'antisweet' compounds are compared (**Table 2**). This compound is structurally β-D-glucopyranosiduronic acid, (3β,4α,16β,21β,22α)-28-(acetyloxy)-16,22,23-trihydroxy-21-[(2*S*)-2-methyl-1-oxobutoxy]olean-12-en-3-yl. A different series of antisweet compounds, namely gymnemasaponins III–V (**117–119**), were then isolated.[212] These nonacylated compounds

Table 2 Sweetness inhibitors from plants

Compound name[a]	*Plant name*	*Sweetness-inhibitory potency*[b]	*Reference(s)*
Gymnemasaponin III (**117**)	*Gymnema sylvestre* (Retz.) Schult. (Asclepiadaceae)	0.125	212
Gymnemasaponin IV (**118**)		0.125	212
Gymnemasaponin V (**119**)		0.125	212
Gymnemic acid I (**120**)		1	213
Gymnemic acid II (**121**)		1	213
Gymnemic acid III (**122**)		0.5	213
Gymnemic acid IV (**123**)		0.25	214
		0.5	213
Gymnemic acid V (**124**)		0.5	215
Gymnemic acid VI (**125**)		0.5	215
Gymnemic acid VIII (**126**)		NS[c]	216
Gymnemic acid IX (**127**)		NS[c]	216
Gymnemic acid X (**128**)		0.5	217
Gymnemic acid XI (**129**)		1	217
Gymnemic acid XII (**130**)		1	217
Gymnemic acid XIII (**131**)		0.5	217
Gymnemic acid XIV (**132**)		0.5	217
Gymnemic acid XV (**133**)		1	218
Gymnemic acid XVI (**134**)		1	218
Gymnemic acid XVII (**135**)		1	218
Gymnemic acid XVIII (**136**)		1	218
21β-*O*-Benzoylsitakisogenin-3-*O*-β-D-glucopyranosyl (1→3)-β-D-glucuronopyranoside (**137**)		1	219
Alternoside I (**138**)	*Gymnema alterniflorum* (Lour.) Merr. (Asclepiadaceae)	0.25	222
Alternoside II (**139**)		0.25	222
Alternoside III (**140**)		0.25	222
Alternoside IV (**141**)		0.25	222
Alternoside V (**142**)		0.25	222
Alternoside XI (**143**)		0.25	223
Alternoside XII (**144**)		0.25	223
Alternoside XIII (**145**)	*Gymnema alterniflorum* (Asclepiadaceae)	0.25	223
Alternoside XIV (**146**)		0.25	223
Alternoside XV (**147**)		0.25	223
Alternoside XVI (**148**)		0.25	223
Alternoside XVII (**149**)		0.25	223
Jujuboside B (**150**)	*Hovenia dulcis* Thunb. var. *tomentella* Makino (Rhamnaceae)	0.25	225
Hoduloside I (**151**)		0.25	225
Hoduloside II (**152**)		0.125	225
Hoduloside III (**153**)		0.125	225
Hoduloside IV (**154**)		0.125	225
Hoduloside V (**155**)		0.125	225
Hoduloside VII (**156**)		0.25	226
Hoduloside VIII (**157**)		0.25	226
Hoduloside IX (**158**)		0.25	226
Hoduloside X (**159**)		NS[c]	226
Hovenoside I (**160**)		0.125	225
Saponin C_2 (**161**)		0.125	225
Saponin E (**162**)		0.125	225
Saponin H (**163**)		0.0625	225

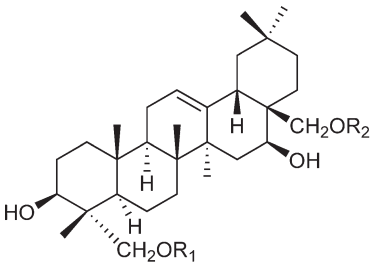
(*Continued*)

Table 2 (Continued)

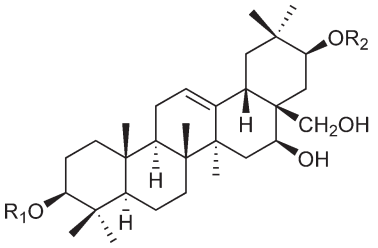
Compound name[a]	*Plant name*	*Sweetness-inhibitory potency*[b]	*Reference(s)*
Sitakisoside I (**164**)	*Stephanotis lutchuensis* Koidz. var. *japonica* (Asclepiadaceae)	0.25	227
Sitakisoside II (**165**)		0.25	227
Sitakisoside III (**166**)		0.25	227
Sitakisoside IV (**167**)		0.25	227
Sitakisoside V (**168**)		0.5	227
Sitakisoside VI (**169**)		0.25	228
Sitakisoside VII (**170**)		0.25	228
Sitakisoside VIII (**171**)		0.25	228
Sitakisoside IX (**172**)		0.25	228
Sitakisoside XI (**173**)	*Stephanotis lutchuensis* Koidz. var. *japonica* (Asclepiadaceae)	0.25	229
Sitakisoside XII (**174**)		0.25	229
Sitakisoside XIII (**175**)		0.25	229
Sitakisoside XVI (**176**)		0.25	229
Sitakisoside XVIII (**177**)		0.25	229
Jujubasaponin II (**178**)	*Ziziphus jujuba* Mill. (Rhamnaceae)	0.5	230
Jujubasaponin III (**179**)		0.5	230
Jujubasaponin IV (**180**)		0.25	230
Jujubasaponin V (**181**)		0.25	230
Jujubasaponin VI (**182**)		0.25	230
Jujuboside B (**150**)		0.25	230
Ziziphin (**183**)		0.5	230, 231
Zizyphus saponin I (**184**)		0.125	230
Zizyphus saponin II (**185**)		0.125	230
Zizyphus saponin III (**186**)		0.25	230
Jegosaponin A (**187**)	*Styrax japonicus* Siebold et Zucc. (Styracaceae)	0.25	232
Jegosaponin B (**188**)		0.25	232
Jegosaponin C (**189**)		0.25	232
Jegosaponin D (**190**)		0.25	232

[a] The structures of the compounds are shown in the text (**117**–**190**).
[b] As compared with gymnemic acid I (**120**) (×1).
[c] NS = sweetness-inhibitory potency not given.

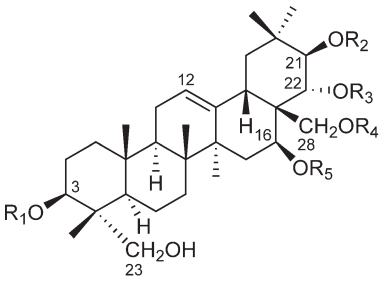
show slightly less potent sweetness-inhibitory activities compared with the previously isolated gymnemic acid I (**120**). Subsequently, the additional sweetness-inhibitory gymnemic acids VIII–XVIII (**126**–**136**) and 21β-*O*-benzoylsitakisogenin-3-*O*-β-D-glucopyranosyl (1→3)-β-D-glucuronopyranoside (**137**) have been isolated from *G. sylvestre*.[216–219] Gymnemic acids XIII (**131**) and XIV (**132**) were previously named gymnemic acids VIII and IX when they were isolated by Yoshikawa *et al.*[217] However, Liu *et al.*[216] independently isolated different compounds designated as gymnemic acids VIII (**126**) and IX (**127**) from the same plant species. Therefore, for clarification purposes, gymnemic acids VIII and IX were renamed as gymnemic acids XIII (**131**) and XIV (**132**), respectively.[218] The antisweet potencies of gymnemic acids XIII (**131**) and XIV (**132**) were rated as about half the potency of gymnemic acid I (**120**). The sweetness-inhibitory potencies of gymnemic acids XV–XVIII (**133**–**136**) and compound **137** were judged to be as about the same as that of gymnemic acid I (**120**).[218,219] There is an extensive literature on *Gymnema sylvestre* exclusive of its sweetness-inhibiting properties, such as its potential antidiabetic and antiobesity effects.[220,221] Preparations containing *G. sylvestre* leaves are sold in health food stores in the United States as a botanical dietary supplement.

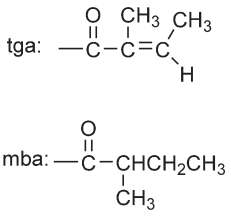


	R_1	R_2
117	β-glc	β-glc^6-β-glc
118	β-glc^6-β-glc	β-glc
119	β-glc^6-β-glc	β-glc^6-β-glc



137 $R_1 = \beta$-glcA^3-β-glc R_2 = Bz



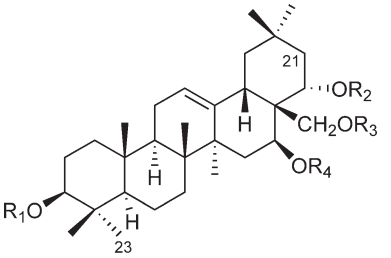


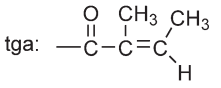
	R_1	R_2	R_3	R_4	R5
120	β-glcA	tga	H	Ac	H
121	β-glcA	mba	H	Ac	H
122	β-glcA	mba	H	H	H
123	β-glcA	tga	H	H	H
124	β-glcA	tga	tga	H	H
125	β-glcA^3-β-glc	tga	H	H	H
126	β-glcA^3-β-OG	mba	H	H	H
127	β-glcA^3-β-OG	tga	H	H	H
128	β-glcA	H	H	Ac	H
129	β-glcA	tga	H	tga	H
130	β-glcA^3-β-glc	tga	H	Ac	H
131	β-glcA	H	H	mba	H
132	β-glcA	H	H	tga	H
133	β-glcA	mba	tga	H	H
134	β-glcA	H	tga	H	tga
135	β-glcA	Bz	H	H	H
136	β-glcA	H	H	Bz	H
191	H	H	H	H	H

Gymnema alterniflorum is an evergreen tree growing in the forests of Taiwan and the southern part of mainland China. The roots of this plant have been used for detoxification purposes and for the treatment of edema and fever.[222] Several oleanane-type triterpenoid glycosides, alternosides I–V and XI–XVII (**138–149**), have been isolated as sweetness inhibitors from the roots of *G. alterniflorum*.[222,223] Complete hydrolysis of alternosides I–V (**138–142**) and XIII–XVII (**145–149**) yielded a known oleanane-type triterpenoid, chichipegenin (**192**).[223,224] There is no functional group at the C-21 and C-23 positions of the alternosides, as commonly present in the gymnemic acids. The antisweet effects of alternosides I–V and XI–XVII (**138–149**) have been evaluated using a 1 mmol l^{-1} solution of each compound, and were found to completely suppress the sensation of sweetness induced by a 0.2 mol l^{-1} sucrose solution in all cases. The sweetness-inhibitory potencies of alternosides I–V and XI–XVII (**138–149**) were rated as about half those of gymnemic acids XIII (**131**) and XIV (**132**).[217]

Subsequent to the isolation of the dammarane-type triterpenoid glycosides jujuboside B (**150**), hodulosides I–V (**151–155**), hovenoside I (**160**), and saponins C2, E, and H (**161–163**) as sweetness inhibitors from the leaves of *H. dulcis* Thunb. var. *tomentella* Makino,[225] hodulosides VII–X (**156–159**) were isolated as sweetness-inhibitory agents.[226] Hodulosides I (**151**) and II (**152**) have hovenolactone (**193**) as their aglycone, the same compound as for saponins E (**162**) and H (**163**). Hodulosides III–V and VII–X (**153–159**) are based on two different dammarane-type aglycone structures, however.[225,226] The sweetness-inhibitory potencies of hodulosides are shown in **Table 2**. The sweetness-inhibitory potency of hoduloside X (**159**) was not determined.[226]

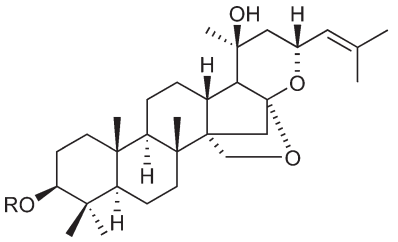
From the stems of *Stephanotis lutchuensis* var. *japonica*, an evergreen woody climber growing in forests near the warm coastal areas of Japan, several oleanane-type sweetness-inhibitory triterpenoid glycosides, namely sitakisosides I–IX, XI–XIII, XVI, and XVIII (**164–177**),[227–229] have been isolated. Some sitakisosides such as *N*-sitakisosides VI (**169**), VII (**170**), XI (**173**), XII (**174**), and XIII (**175**) afforded sitakisogenin (**194**),[228,229] whereas hydrolysis of sitakisosides II (**165**) and XVIII (**177**) yielded marsglobiferin (**195**).[227,229] In turn, hydrolysis of sitakisoside VIII (**171**) afforded 3β,16β,21β,28β-tetrahydroxyoleanan-12-en-22-one (**196**) as the aglycone.[228] Sitakisoside IX (**172**) has a gymnestrogenin-type aglycone structure (**197**).[228] The sweetness-inhibitory potencies of the sitakisosides are about 25% of that of gymnemic acid I, except for the most potent analogue sitakisoside V (**165**; 50% of the activity of gymnemic acid I (**120**)) (**Table 2**).





	R_1	R_2	R_3	R_4
138	β-glcA^3-β-glc	Ac	α-rha	H
139	β-glcA^3-β-glc	H	α-rha	Ac
140	β-glcA^3-β-glc	tga	α-rha	H
141	β-glcA	Ac	α-rha	H
142	β-glcA	H	α-rha	Ac
143	β-glcA^3-β-glc	tga	H	H
144	β-glcA^3-β-glc	H	tga	H
145	β-glcA^3-β-glc	H	H	tga
146	β-glcA^3-β-glc	tga	β-glc	H
147	β-glcA^3-β-glc	tga	β-fuc	H
148	β-glcA^3-β-glc	tga	β-xyl	H
149	β-glcA	tga	α-rha	H
192	H	H	H	H

fuc: D-fucosyl



150 R = α-ara^3-β-glc^2-β-xyl
|^2
α-rha

153 R = α-ara^2-β-qui
|^3
β-glc

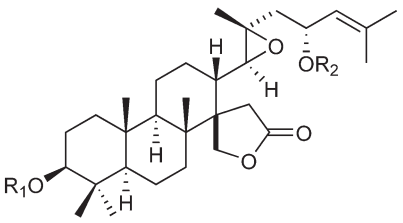
154 R = α-ara^2-β-glc
|^3
β-glc

155 R = β-glc^2-α-rha
|^3
β-glc

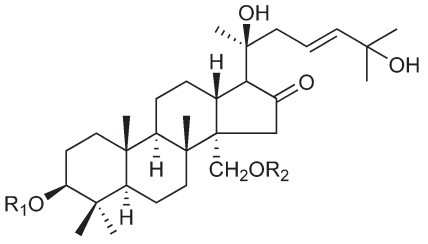
160 R = α-ara^2-β-xyl
|^3
β-glc

161 R = α-ara^2-α-rha
|^3
β-glc

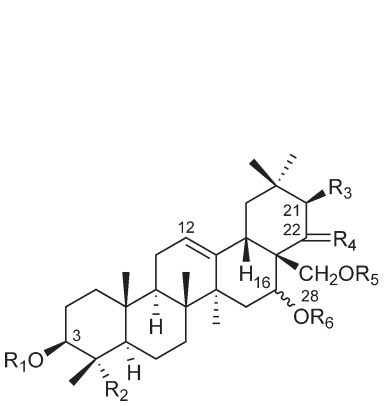
ara: L-arabinopyranosyl

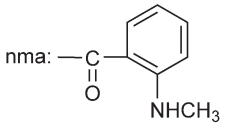


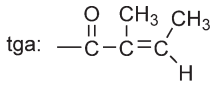
	R_1	R_2
151	β-glc^2-α-rha	β-glc
152	β-glc^2-α-rha |^3 β-glc	H
162	β-glc^2-α-rha	H
163	β-glc	H
193	H	H

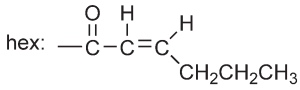


	R_1	R_2
156	α-ara^2-α-rha	β-glc
157	α-ara	β-glc^6-β-xyl
158	α-ara^2-α-rha	β-glc^6-β-xyl
159	α-ara^2-α-rha |^3 β-glc	β-glc



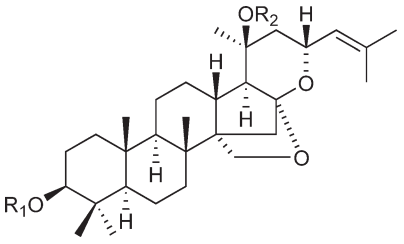






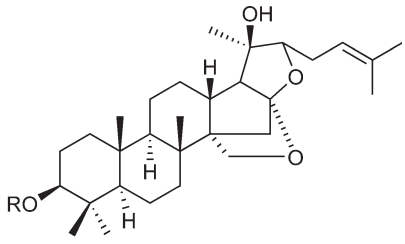
	R_1	R_2	R_3	R_4	R_5	R_6
164	β-glc^6-β-glc^6-β-xyl	CH_3	H	α-*O*-nma, β-H	H	β-OH
165	β-glc^6-β-glc^6-β-xyl	CH_3	*O*-nma	α-OH, β-H	H	β-OH
166	β-glc^6-β-glc^6-β-xyl	CH_3	H	α-OH, β-H	nma	β-OH
167	β-glc^6-β-glc^6-β-glc	CH_3	H	α-*O*-nma, β-H	H	β-OH
168	β-glc^6-β-glc^6-β-xyl	CH_3	H	α-*O*-tga, β-H	H	β-OH
169	β-glc^6-β-glc^6-β-xyl	CH_3	*O*-β-glc^6-nma	H_2	H	β-OH
170	β-glc^6-β-glc^6-β-xyl	CH_3	*O*-β-glc^4-nma	H_2	H	β-OH
171	β-glc^6-β-glc^6-β-xyl	CH_3	*O*-nma	O_2	H	β-OH
172	β-glc^6-β-glc^6-β-xyl	CH_2OH	*O*-β-glc^6-nma	H_2	H	β-OH
173	β-glc^6-β-glc^6-β-xyl	CH_3	*O*-β-glc^4-tga	H_2	H	β-OH
174	β-glc^6-β-glc^6-β-xyl	CH_3	*O*-β-glc^6-tga (4 → glc)	H_2	H	β-OH
175	β-glc^6-β-glc^6-β-xyl	CH_3	*O*-β-glc^3-nma	H_2	H	β-OH
176	β-glc^6-β-glc^6-β-xyl	CH_3	H	α-OH, β-H	tga	β-OH
177	β-glc^6-β-glc^6-β-xyl	CH_3	OH	α-OH, β-H	nma	β-OH
187	β-glcA^3-β-gal^2-α-rha (2 → β-glc)	CH_3	*O*-tga	α-OAc, β-H	H	α-OH
188	β-glcA^3-β-gal^2-α-rha (2 → β-glc)	CH_3	*O*-tga	α-OH, β-H	Ac	α-OH
189	β-glcA^3-β-gal^2-α-rha (2 → β-glc)	CH_3	OH	α-tga, β-H	Ac	α-OH
190	β-glcA^3-β-gal^2-α-rha (2 → β-glc)	CH_3	*O*-hex	α-OH, β-H	Ac	α-OH
194	H	CH_3	OH	H_2	H	β-OH
195	H	CH_3	OH	α-OH, β-H	H	β-OH
196	H	CH_3	OH	O_2	H	β-OH
197	H	CH_2OH	OH	H_2	H	β-OH
198	H	CH_3	OH	α-OH, β-H	H	α-OH

In the late 1980s, ziziphin (**183**) was isolated from the Chinese jujube tree *Ziziphus jujuba* P. Miller as the first recognized antisweet principle of this plant.[23,25] Ziziphin (**183**) has the same dammarane-type aglycone structure as hodulosides III–V (**153–155**). Yoshikawa *et al.*[230] isolated nine additional antisweet compounds, namely jujubasaponins II–VI (**178–182**), ziziphin (**183**), and zizyphus saponins I–III (**184–186**), from the leaves of *Ziziphus jujuba* (**Table 2**). Among them, three acylated compounds, ziziphin (**183**) and jujubasaponins II (**178**) and III (**179**), showed the most potent antisweet activity, equivalent to 50% of that of gymnemic acid I (**120**)[231] (**Table 2**).



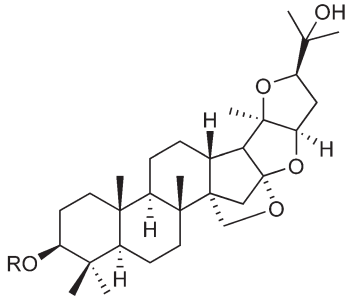
	R_1	R_2
178	α-ara^2-α-rha	α-rha^2-Ac
179	α-ara^2-α-rha	α-rha^3-Ac
183	α-ara^4-α-rha	α-rha^2-Ac $\vert^3$ Ac
184	α-ara^3-β-glc $\vert^2$ 6-deoxy-α-tal	H
185	α-ara^3-β-glc $\vert^2$ α-rha	H
186	α-ara^3-β-glc^2-β-xyl $\vert^2$ α-rha	H

tal: D-talosyl



180 R = β-gal^2-α-rha
$\vert^3$
β-glc

181 R = β-glc^2-α-rha
$\vert^3$
β-glc



182 R = β-gal^2-α-rha

Styrax japonicus Siebold et Zucc. (Styracaeae) is a deciduous tree distributed in Japan, Korea, and mainland China. Recently, jegosaponins A–D (**187–190**), four new oleanane-type saponins, were isolated from the fresh fruits of this tree as sweetness inhibitors.[232] The structures of jegosaponins A–D (**187–190**) are based on the aglycone barringtogenol C (**198**) and they all have the same tetraglycoside chain at C-3, with different acylated groups at C-21, C-22, and C-28. The antisweet activities of jegosaponins A–D (**187–190**) are about half those of gymnemic acids III (**122**), IV (**123**), and VI (**124**).[232]

3.10.7 Sensory Evaluation of Natural Products for Sweetness and Sweetness-Modifying Properties

Sensory evaluation using the human tongue as a detector is a crucial step in the discovery of natural sweeteners and sweetness modifiers. The human tasting stage can be divided into raw material screening, sensory-guided fractionation, and sensory evaluation of purified natural sweeteners. After a careful safety evaluation (see Section 3.10.3), tasting can be carried out on the samples of candidate sweet-tasting plants extracted with MeOH or MeOH–water, sometimes at an elevated temperature. Then, additional dried extracts prepared by partitioning the initial MeOH or MeOH–water extract with solvents of various polarities and thoroughly removing the residual solvent in each case may also be tasted for the presence or absence of sweetness. For relatively clean samples, that is, certain fruit extracts, the above-mentioned solvent partition steps may be omitted, thus avoiding the tedious solvent removal steps prior to human tasting.

Pure natural product compounds need to be subjected to a rigorous safety evaluation as a prerequisite to human tasting. Thus, toxicological evaluation may include acute toxicity evaluation in mice and bacterial mutagenicity testing.[74–76,84] Once approved for human tasting, pure samples are typically dissolved in water for preliminary evaluation. For some samples with poor solubility in water, samples may be solubilized with the aid

of ethanol and then diluted with distilled water before tasting. Caution should be taken to keep the quantity of ethanol to a minimum as this solvent has an inherent sweetness that may interfere with sensory evaluation. Samples that are completely devoid of sweetness, that are strongly bitter, or that have a strong off-taste will be eliminated at this stage.

Samples of further interest are evaluated as to their relative sweetness, taste profile, and temporal profile when compared to a sucrose standard. The relative sweetness is utilized to indicate the potency of the natural sweetener concerned. Many natural sweeteners are high-potency sweeteners that are at least 50–100 times sweeter than sucrose. The sweetening power of highly potent sweeteners varies due to many factors and decreases relative to that of sucrose as concentration increases.[233] Relative sweetness can be best determined using a ranking test.[234] The taste panelists involved should be prescreened for their sensitivity and trained to respond to other common tastes (bitter, sour, salty, umami, etc.). The panel size should be at least eight. The sample concentration needs to be adjusted so that the perceived sweetness would be in the proper range within that of the sucrose references. A prescreened sample is presented randomly to the panel together with a series of sucrose standards in coded cups. The panel is instructed to taste each sample and then rinse the mouth thoroughly with water. All tasting should be carried out at ambient temperature. The panel is asked to rank the samples from low to high with respect to perceived sweetness. The relative sweetness of the sample can then be determined after statistical analysis of the sensory data. In lieu of a formal sensory evaluation, relative sweetness can be estimated by bench tasting using paired comparison with a smaller panel.[117,126]

The relative sweetness of natural sweeteners may be evaluated against different concentrations of sucrose. It is not uncommon to determine the relative sweetness of natural sweeteners at or near the sucrose threshold; generally, this is around 0.5% w/v. The natural sweetener (2*R*,3*R*)-dihydroquercetin 3-*O*-acetate (**91**) isolated from the Paraguayan plant *T. dodoneifolia* was rated as being 80 times sweeter than a 2% w/v sucrose solution (**Table 1**).[159] The semisynthetic, intensely sweet NHDC (**12**) has been thoroughly studied by several groups.[235,236] At or near threshold, compound **12** was determined to be 1800 times sweeter than sucrose. At 1 and 5% sucrose levels, the sweetness potency of **12** was rated as 600 and 250 times sweeter than sucrose, respectively, indicating that the perceived sweetness intensity of the compound decreases as concentration increases.[237] Another example is telosmoside A_{15} (**79**), a natural pregnane-type sweetener isolated from the Vietnamese plant *Telosma procumbens* (**Table 1**).[149] This molecule was dissolved in 7% ethanol solution and tasted at different concentrations against a series of sucrose references ranging from 3.2 to 9.6% (w/v). Telosmoside A_{15} (**79**) at a concentration of 0.008% was iso-sweet to 8% sucrose and thus determined to be 1000 times sweeter than 8% sucrose. As indicated above, the taste and temporal profiles are also important factors associated with natural sweeteners. Compared to sucrose, which exhibits a characteristic time–intensity profile, many of the natural high-intensity sweeteners show a slow onset, a lingering aftertaste, bitterness, or a metallic off-taste. These characteristics can be indicated during sensory evaluation by an experienced panel.

There are increasing health concerns about the high intake of calorie-rich sugar-sweetened food, which can contribute to obesity, diabetes, and other chronic diseases, in addition to dental caries.[2,238] Accordingly, it has been a long-time goal of the food and beverage industry to reduce the sucrose content in their products without sacrificing food palatability. Sugar replacement to reduce the caloric consumption can be achieved via the addition of the highly potent artificial or natural sweeteners. One characteristic often associated with high-potent sweeteners is their synergy when combined with other sweeteners.[239] Synergy refers to the total sweetness intensity of a mixture when greater than the theoretical sum of the intensities of the individual components. However, many artificial and natural sweeteners have off-tastes and different taste profiles from that of sucrose.

Another alternative is to utilize sweetness enhancers to enhance the perception of the sweet taste, and thus be able to reduce the quantity of sugar content in food products. The ideal sweetness enhancer would have no intrinsic taste and aroma but would increase the sweetness of sucrose without imparting any negative effect on other flavor profiles.[240] However, most (if not all) of the sweetness enhancers reported so far have some intrinsic sweetness, for example, hesperetin (**113**)[194] and the 4-hydroxydihydrochalcones.[241] Therefore, it is important to distinguish if the enhancement of sweetness is from true synergy or merely the additive effect from the intrinsic sweetness of the ingredients. The preliminary screening of sweetness-enhancing activity for botanical extracts, chromatographic fractions, or isolated compounds can be carried out by a small, sweetness-sensitive taste panel. Samples are added to an aqueous sugar solution, for example at 2% (w/v), and then administered to

the panel along with a positive control (2% sugar) in coded beakers. The panel members are then asked to compare their sweetness. If the samples are evidently sweeter than the control, further purification and sensory evaluation are warranted. As there is the possibility that the samples of interest may have intrinsic sweetness, the formal sensory evaluation procedure needs to determine if the elevation of the sweetness is due to an additive effect or true synergy. Evidence has shown that there is a positive correlation between the sweetness-enhancing effect and the intrinsic sweetness of the test samples. However, the sample size may be too small for a definite conclusion to be made.[195]

The relative sweetness of pure samples can be determined using the ranking method discussed above. The test sample at a certain concentration (say, 100 ppm) in water is ranked versus a series of sucrose (say, 0.5, 1.0, 1.5, 2.0% w/v) references. The concentration range of the references chosen depends on the sweetness of the test samples. The sweetness-enhancing evaluation can be carried out in a 5% sucrose solution because the change in sweetness can be most easily detected at this concentration.[195] The sample sweetness in 5% sugar solution can be determined using a ranking test or a paired comparison versus 5, 6, 7, and 8% sucrose reference solutions. The difference between the actual measured sweetness of the test sample in a 5% sugar solution and the calculated sweetness of a pure 5% sucrose solution plus the measured sweetness of the sample (at 100 ppm) will reveal if the elevation of the sweetness is from additive effects or a true synergy.

The time- and material-consuming process of sensory evaluation is limited to those samples cleared for human tasting, and sometimes this is precluded by the demonstration or presumption of toxicity for a given sample under consideration. In the past few years, considerable progress has been made in research on human/mammalian taste receptors.[87,88,242,243] The sweet receptor is a G-protein-coupled receptor (GPCR) and is composed of two proteins, T1R2 and T1R3, expressed on the surface of taste bud cells.[244,245] Sweet receptor-based assay systems have been used in high-throughput screening of molecules for sweeteners and sweetness enhancers or modifiers.[88] Receptor-based assay systems have many potential advantages over the classical human tasting method owing to their speed, sensitivity, and selectivity, and thus can aid in the discovery of novel natural sweeteners and sweetness modifiers. However, human taste perception is a very complex process and sensory evaluation can give an overall characterization of the sweeteners owing to its holistic approaches. The combination of an *in vitro* assay with human panel sensory evaluation would be ideal for the discovery of novel natural sweeteners and sweetness enhancers.

3.10.8 Interactions of Natural Products at the Sweet Receptor

Before the recent discovery of the mammalian/human sweet receptor, proposals for the structure–activity relationships (SAR) of classes of sweeteners were based on the analysis of their structures and the activities of various derivatives. Many synthetic analogues of natural sweeteners have been made to study how structural variation influences their sweetness activities. Such approaches led to the identification of essential structural features (glucophores) necessary for the sweetness and potency of these molecules. Through indirect mapping, several models of the hypothetical ligand binding sites for the sweet receptor have been developed.[246] The consensus feature of these models is the presence of AH–B groups, in which the AH group is a hydrogen donor and the B group is an electronegative center.[247] According to this theory, all sweet-tasting compounds contain a hydrogen bond donor (AH) and a hydrogen bond acceptor (B), separated by a distance of 2.5–4.0 Å, that react with a complementary AH–B pair on the receptor. For example, plant-derived sweeteners such as phyllodulcin (**3**) and NHDC (**12**) owe their sweetness to the presence of the so-called isovanillyl glucophoric (3-hydroxy-4-methoxyphenyl) group. The adjacent hydrogen donor (–OH) and hydrogen acceptor (OCH_3) of the isovanillyl group satisfy the requirements of the AH–B theory. For instance, the sweet principle (2*R*,3*R*)-dihydroquercetin 3-*O*-acetate (**91**), from the young leaves of *T. dodoneifolia*, was rated as 80 times sweeter than sucrose while the sweetness of this compound was increased fivefold by methylation at the C-4′ hydroxyl to form a synthetic isovanillyl derivative (**92**).[159] Interestingly, (2*R*,3*R*)-dihydroquercetin (taxifolin) itself is not sweet but bitter.[248] These hypothetical models became generally accepted for many of the small-molecule synthetic and natural product sweeteners, but not for all of them, indicating that these sweet molecules may have different binding sites on the receptor. Additionally, such models have been unable to explain the sweetness of sweet proteins. It has been postulated that there may be more than one type of sweet receptor.[249]

At the present time, it is clear that the detection of sweet taste is mediated by a heterodimeric receptor comprised of T1R2 and T1R3 proteins.[243,244] The sweet receptor belongs to class C type of GPCRs, which also include several metabotropic glutamate receptors, the umami receptor, and the bitterness receptor. These receptors are characterized by a large clam shell-shaped extracellular N-terminal domain linked to a hydrophobic domain with the seven-transmembrane topology common to all GPCRs. This N-terminal domain is responsible for ligand binding and has a characteristic structure known as the 'Venus flytrap' module. These membrane-bound proteins are difficult to crystallize; hence, a 3D structure has not been solved so far for the sweet taste receptor, making it difficult to use structure-based methods to study the SAR and design new sweeteners. The sweet taste receptor is similar to the dimeric metabotropic glutamate receptor mGluR1 and the crystal structures of the extracellular ligand-binding region of mGluR1 have been determined.[250] Several 3D homology models of sweet receptor have been built using the known structure of the N-terminal domain of mGluR1 as a template.[245,251,252] With the new knowledge gained from molecular biology and homology modeling studies, it is evident that the human sweet receptor has multiple active sites.[245,249,253,254] The artificial sweeteners aspartame and neotame were found to interact at the N-terminal domain of human T1R2 whereas the binding site of cyclamate was localized to the human T1R3 transmembrane domain.[254,255] The well-known sweetness blocker lactisole was found to interact with the transmembrane domain of human T1R3 to inhibit the sweet taste.[254,256]

Sweet proteins may act via a mechanism different from that of low-molecular-weight sweeteners. Chimera studies have indicated that the sweet protein brazzein (**105**) interacts with the cysteine-rich domain of human T1R3.[257] A wedge model for sweet protein binding to the receptor was proposed based on extensive modeling of the human sweet receptor and docking studies of both sweet proteins and small sweet molecules.[245] The above findings also shed some light on the synergy effect between different sweeteners. If two sweeteners act via the same mechanism, then they will compete for the same binding site and behave in an additive way. It has long been known that aspartame and cyclamate are synergistic in sensory experiments.[258] Recent findings have revealed that these two sweeteners have separate orthostatic binding sites[254] and a cooperative binding effect may well explain their synergy.[259]

With the discovery of the sweet receptor, our understanding toward the SAR of sweet molecules increases significantly. Homology modeling, molecular docking studies, and molecular biology have yielded useful information regarding the binding sites of the sweet receptor. These results may be used as a guide to design new and better sweeteners. Despite these advances, there are still many unanswered questions regarding the details of the binding activities. Some of these questions may have to wait until a 3D structure is finally established for the sweet receptor.

3.10.9 Conclusions

In this chapter, information has been provided concerning the botanical source, structure, and sweetness potencies relative to sucrose of more than 100 highly sweet natural products. Also mentioned are seven known sweetness enhancers from organisms, and over 80 antisweet plant constituents. These substances are chemically quite diverse and represent the terpenoid, flavonoid, and protein classes of compounds, in particular. A number of sweet compounds described have present use or future commercial potential as sucrose substances, and these are expected to increase in the near future to meet a public demand for ingredients of natural origin in foods and beverages in western countries. The approval of natural sweet substances varies from country to country, and of paramount concern in the approval process is the need for demonstrated safety. Not all of the commercially used sweeteners are innocuous in terms of their potential toxicity. For example, glycyrrhizin (**1**) has an adrenocorticomimetic effect and may lead to abnormal fluid retention (hypokalemia) and hypertension when ingested in licorice-flavored confectionary or when used in drug formulations.[26,260,261] Therefore, it is necessary for an upper limit to be placed on the amount of glycyrrhizin (**1**) ingested daily.[28] Because almost all natural sweeteners of plant origin have hedonic limitations in their quality of taste, many efforts have been made to produce more pleasant-tasting modified analogues either synthetically or enzymatically, and several key references in this regard have been cited in the present chapter.

Although ideally low-calorie sweeteners should have no significant biological activities other than a sweet effect, the recent work by Konoshima[262] on the potential cancer chemopreventive activity of these compounds is worthy of mention. Cancer chemoprevention has been described as "a strategy of cancer control by administration of synthetic compounds to reverse or suppress the process of carcinogenesis".[263] In a model of the inhibition of Epstein–Barr virus early antigen (EBV-EA) induction, both stevioside (**5**) and mogroside V (**2**) were shown to exhibit potent activity in this assay and were more active than several other natural sweeteners. Furthermore, stevioside and mogroside V showed significant anticarcinogenic effects in a follow-up *in vivo* model of two-stage carcinogenesis in mice.[262]

The search for highly sweet substances has proven to be fascinating, and scientific reports of new substances of this type have attracted wide attention. While several groups in Japan and the United States, in particular, reported frequently on the isolation and structural characterization of new sweet principles from green plants in the last quarter of the twentieth century, such reports have recently declined in frequency. The principal reason for this seems to be the fact that many if not all of the more obvious candidate sweet plant leads have already been discovered. Indeed, it is unlikely that another organism will be found with, for example, the profound sweet taste exhibited by the leaves of the plant *S. rebaudiana*. However, it is entirely possible that additional sweet-tasting or sweetness-inducing plants are used by local populations for sweetening purposes, and are as yet undiscovered, in more remote geographical locations. The search for new sweet-tasting compounds from plants by fieldwork has become more complex than previously, as a result of the passage of the United Nations Convention on Biological Diversity in Rio de Janeiro in 1992, so it is now necessary to obtain 'prior informed consent' and to develop benefit-sharing agreements with the source country before accessing indigenous traditional knowledge and accessing plant material. Therefore, this approach now requires a great deal of preplanning and may have an uncertain outcome. Sweetener discovery from natural sources may best be done with a multidisciplinary team consisting of taxonomists, natural products chemists, and biologists.[21,74,75] The prospects of a greatly increased knowledge on the occurrence of sweet-tasting and sweetness-modifying natural products, not only from plants, but also from other terrestrial and marine organisms, may be expected in the future. This is due to the recent availability of receptor-binding assays, which can be applied to libraries of pure natural products and then be followed by sensory testing using human taste panels, as discussed in Section 3.10.7.

A question that often arises is why do plants produce low-calorie sweet-tasting compounds at all? There is no generally agreed upon answer to this question. However, it has been postulated that secondary metabolites of plants and other organisms accumulate under the pressure of natural selection to bind to specific receptors and thus help in the survival of the producing organism.[264] Therefore, one might suppose that bitter-tasting compounds would be preferred for organism survival rather than sweet-tasting compounds, in order to ward off predators, by being less palatable when chewed. If the organoleptic results obtained by Soejarto *et al.*[79] on the taste properties of the leaves of more than 100 *Stevia* species are typical, then this group of plants was found to be overwhelmingly bitter tasting, with only a few specimens somewhat sweetish, including a sample of *S. rebaudiana*. The bitterness of the vast majority of the *Stevia* species represented would be expected to be due to constituents such as sesquiterpene lactones[265] and *ent*-atisane diterpenoids[266] that are known to be biosynthesized in this genus. Accordingly, the production of such high concentration levels of sweet-tasting steviol glycosides in just one species (*S. rebaudiana*) of the group evaluated in this manner seems to be genetically illogical. However, given that two glycosidic constituents of this plant (rebaudioside A (**4**) and stevioside (**5**)) have wide use as noncaloric sucrose substitutes, this is very much to the benefit of humankind.

Abbreviations

ADI	acceptable daily intake
CGTase	cyclomaltodextringlucanotransferase
EBV-EA	Epstein–Barr virus early antigen
GC–MS	gas chromatography–mass spectrometry
GPCR	G-protein-coupled receptor
GRAS	generally recognized as safe
JECFA	Joint Expert Committee on Food Additives
MGGR	glycyrrhetic acid monoglucuronide

NHDC	neohesperidin dihydrochalcone
SAR	structure–activity relationships
SPE	solid-phase extraction

References

1. T. J. M. Cooper, Sucrose. In *Optimising Sweet Taste in Foods*; W. J. Spillane, Ed.; CRC Press: Boca Raton, FL, 2006; pp 135–145.
2. T. H. Grenby, *Chem. Br.* **1991**, *27*, 342–345.
3. V. B. Duffy; G. H. Anderson, *J. Am. Diet. Assoc.* **1998**, *98*, 580–587.
4. L. O'Brien Nabors, Ed., *Alternative Sweeteners: Third Edition, Revised and Expanded*; Marcel Dekker: New York, 2001.
5. G.-W. von Rymon Lipinski, Reduced-Calorie Sweeteners and Caloric Alternatives. In *Optimising Sweet Taste in Foods*; W. J. Spillane, Ed.; CRC Press: Boca Raton, FL, 2006; pp 252–280.
6. M. E. Embuscado, Polyols. In *Optimising Sweet Taste in Foods*; W. J. Spillane, Ed.; CRC Press: Boca Raton, FL, 2006; pp 153–174.
7. S. E. Kemp, Low-Caloric Sweeteners. In *Optimising Sweet Taste in Foods*; W. J. Spillane, Ed.; CRC Press: Boca Raton, FL, 2006; pp 175–251.
8. G.-W. von Rymon Lipinski; L. Hanger, Acesulfame K. In *Alternative Sweeteners: Third Edition, Revised and Expanded*; L. O'Brien Nabors, Ed.; Marcel Dekker: New York, 2001; pp 13–30.
9. M. H. Auerbach; G. Locke; M. E. Hendrick, Alitame. In *Alternative Sweeteners: Third Edition, Revised and Expanded*; L. O'Brien Nabors, Ed.; Marcel Dekker: New York, 2001; pp 31–40.
10. H. H. Butchko; W. W. Stargel; C. P. Comer; D. A. Mayhew; S. E. Andress, Aspartame. In *Alternative Sweeteners: Third Edition, Revised and Expanded*; L. O'Brien Nabors, Ed.; Marcel Dekker: New York, 2001; pp 41–61.
11. B. A. Bopp; P. Price, Cyclamate. In *Alternative Sweeteners: Third Edition, Revised and Expanded*; L. O'Brien Nabors, Ed.; Marcel Dekker: New York, 2001; pp 63–85.
12. W. W. Stargel; D. A. Mayhew; C. P. Comer; S. E. Andress; H. H. Butchko, Neotame. In *Alternative Sweeteners: Third Edition, Revised and Expanded*; L. O'Brien Nabors, Ed.; Marcel Dekker: New York, 2001; pp 129–145.
13. R. L. Pearson, Saccharin. In *Alternative Sweeteners: Third Edition, Revised and Expanded*; L. O'Brien Nabors, Ed.; Marcel Dekker: New York, 2001; pp 147–165.
14. L. A. Goldsmith; C. M. Merkel, Sucralose. In *Alternative Sweeteners: Third Edition, Revised and Expanded*; L. O'Brien Nabors, Ed.; Marcel Dekker: New York, 2001; pp 185–207.
15. Anon, Artificial sweeteners: no calories. . .sweet! FDA Consumer Magazine, July–August 2006 (http://www.fda.gov; accessed 23 July 2008).
16. A. D. Kinghorn; D. D. Soejarto, *CRC Crit. Rev. Plant Sci.* **1986**, *4*, 79–120.
17. A. D. Kinghorn; D. D. Soejarto, *Med. Res. Rev.* **1989**, *9*, 91–115.
18. Y. Kurihara, *Crit. Rev. Food Sci. Nutr.* **1992**, *32*, 231–252.
19. A. D. Kinghorn; F. Fullas; R. A. Hussain, Structure-Activity Relationships of Highly Sweet Natural Products. In *Studies in Natural Product Chemistry, Vol. 19: Structure and Chemistry (Part E)*; Atta-ur-Rahman, Ed.; Elsevier Science Publishers: Amsterdam, 1995; pp 3–41.
20. O. Tanaka, *Pure Appl. Chem.* **1997**, *69*, 675–683.
21. A. D. Kinghorn; N. Kaneda; N.-I. Baek; E. J. Kennelly; D. D. Soejarto, *Med. Res. Rev.* **1998**, *18*, 347–360.
22. A. D. Kinghorn; N.-C. Kim; D. S. H. L. Kim, Terpenoid Glycoside Sweeteners. In *Naturally Occurring Glycosides: Chemistry, Distribution and Biological Properties*; R. Ikan, Ed.; John Wiley & Sons: Chichester, UK, 1999; pp 399–429.
23. N.-C. Kim; A. D. Kinghorn, Sweet-Tasting and Sweetness-Modifying Constituents of Plants. In *Studies in Natural Product Chemistry, Vol. 27: Bioactive Natural Products (Part H)*; Atta-ur-Rahman, Ed.; Elsevier Science Publishers: Amsterdam, 2002; pp 3–57.
24. N.-C. Kim; A. D. Kinghorn, *Arch. Pharm. Res.* **2002**, *25*, 725–746.
25. R. Suttisri; I.-S. Lee; A. D. Kinghorn, *J. Ethnopharmacol.* **1995**, *47*, 9–26.
26. G. R. Fenwick; J. Lutomski; C. Nieman, *Food Chem.* **1990**, *38*, 119–143.
27. I. Kitagawa, *Pure Appl. Chem.* **2002**, *74*, 1189–1198.
28. A. D. Kinghorn; C. M. Compadre, Less Common High-Potency Sweeteners. In *Alternative Sweeteners: Third Edition, Revised and Expanded*; L. O'Brien Nabors, Ed.; Marcel Dekker: New York, 2001; pp 209–233.
29. A. Tschirch; H. Cederberg, *Arch. Pharm.* **1907**, *245*, 97–111.
30. J. E. Hodge; G. M. Inglett, Structural Aspects of Glycosidic Sweeteners Containing (1′→2)-Linked Disaccharides. In *Symposium: Sweeteners*; G. E. Inglett, Ed.; Avi Publishing Company, Inc.: Westport, CT, 1974; pp 216–234.
31. K. Mizutani; T. Kuramoto; Y. Tamura; N. Ohtake; S. Doi; M. Nakamura; O. O. Tanaka, *Biosci. Biotechnol. Biochem.* **1994**, *58*, 554–555.
32. K. Mizutani; T. Kambara; H. Masuda; Y. Tamura; T. Ikeda; O. Tanaka; H. Tokuda; H. Nishino; M. Kozuka; T. Konoshima; M. Takasaki, Glycyrrhetic Acid Monoglucuronide (MGGR): Biological Activities. In *Toward Natural Medicine Research in the 21st Century*; H. Ageta, N. Aimi, Y. Ebizuka, T. Fujita, G. Honda, Eds.; Elsevier: Amsterdam, 1998; pp 225–235.
33. S. Takemoto; Arihara; T. Nakajima; M. Okuhira, *Yakugaku Zasshi* **1983**, *103*, 1151–1154.
34. D. Li; T. Ikeda; Y. Huang; J. Liu; T. Nohara; T. Sakamoto; G.-I. Nonaka, *J. Nat. Med.* **2007**, *61*, 307–312.
35. S. Yoshikawa; Y. Murata; M. Sugiura; T. Kiso; M. Shizuma; S. Kitahata; H. Nakano, *J. Appl. Glycosci.* **2005**, *52*, 247–252.

36. Y. Asahina; E. Ueno, *J. Pharm. Soc. Jpn.* **1916**, *408*, 146; *Chem. Abstr.* **1916**, *10*, 1524.
37. H. Arakawa; M. Nakazaki, *Chem. Ind.* **1959**, 671.
38. J. R. Hanson; B. H. De Oliveira, *Nat. Prod. Rep.* **1993**, *10*, 301–309.
39. A. D. Kinghorn; C. D. Wu; D. D. Soejarto, Stevioside. In *Alternative Sweeteners: Third Edition, Revised and Expanded*; L. O'Brien Nabors, Ed.; Marcel Dekker: New York, 2001; pp 167–183.
40. M. S. Bertoni, *An. Cie. Parag., Ser. I* **1905**, 1–14.
41. E. Mosetig; U. Beglinger; F. Dolder; H. Lichti; P. Quitt; J. A. Waters, *J. Am. Chem. Soc.* **1963**, *85*, 2305–2309.
42. H. Kohda; R. Kasai; K. Yamasaki; K. Murakami; O. Tanaka, *Phytochemistry* **1976**, *15*, 981–983.
43. K. Mizutani; O. Tanaka, Use of *Stevia rebaudiana* Sweeteners in Japan. In Stevia: *The Genus Stevia*; A. D. Kinghorn, Ed.; Taylor & Francis: London, 2002; pp 178–195.
44. K. Ohtani; K. Yamasaki, Methods to Improve the Taste of the Sweet Principles of *Stevia rebaudiana*. In Stevia: *The Genus Stevia*; A. D. Kinghorn, Ed.; Taylor & Francis: London, 2002; pp 138–159.
45. S. Kamiya; F. Konishi; S. Esaki, *Agric. Biol. Chem.* **1979**, *43*, 3553–3557.
46. G. E. DuBois; L. A. Bunes; P. S. Dietrich; R. A. Stephenson, *J. Agric. Food Chem.* **1984**, *32*, 1321–1325.
47. S. Esaki; R. Tanaka; S. Kamiya, *Agric. Biol. Chem.* **1984**, *48*, 1831–1834.
48. K. Mizutani; T. Miyata; R. Kasai; O. Tanaka; S. Ogawa; S. Doi, *Agric. Biol. Chem.* **1989**, *53*, 395–398.
49. H. Ishikawa; S. Kitahata; K. Ohtani; C. Ikuhara; O. Tanaka, *Agric. Biol. Chem.* **1990**, *54*, 3137–3143.
50. Y. Fukunaga; T. Miyata; N. Nakayasu; K. Mizutani; R. Kasai; O. Tanaka, *Agric. Biol. Chem.* **1989**, *53*, 1603–1607.
51. N. Kaneda; R. Kasai; K. Yamasaki; O. Tanaka, *Chem. Pharm. Bull.* **1977**, *25*, 2466–2467.
52. M. Shibasato, *Jpn. Fudo Saiensu* **1995**, *34* (12), 51–58.
53. J. Kim; Y. H. Choi; Y.-H. Choi, Use of Stevioside and Cultivation of *Stevia rebaudiana* in Korea. In Stevia: *The Genus Stevia*; A. D. Kinghorn, Ed.; Taylor & Francis: London, 2002; pp 196–202.
54. B. E. Erickson, *Chem. Eng. News,* **2009**, *57*, 18.
55. I. Prakash; G. E. DuBois; J. F. Clos; K. L. Wilkens; L. E. Fosdick, *Food Chem. Toxicol.* **2008**, *46* (7S), S75–S82.
56. A. G. Renwick; S. M. Tarka, *Food Chem. Toxicol.* **2008**, *46* (7S), S70–S74.
57. D. J. Brusick, *Food Chem. Toxicol.* **2008**, *46* (7S), S83–S91.
58. L. L. Curry; A. Roberts, *Food Chem. Toxicol.* **2008**, *46* (7S), S11–S20.
59. L. L. Curry; A. Roberts; N. Brown, *Food Chem. Toxicol.* **2008**, *46* (7S), S21–S30.
60. A. Roberts; A. G. Renwick, *Food Chem. Toxicol.* **2008**, *46* (7S), S31–S39.
61. A. Wheeler; A. C. Boileau; P. C. Winkler; J. C. Compton; I. Prakash; X. Jiang; D. A. Mandarino, *Food Chem. Toxicol.* **2008**, *46* (7S), S54–S60.
62. K. C. Maki; L. L. Curry; M. S. Reeves; P. D. Toth; J. M. McKenney; M. V. Farmer; S. L. Schwartz; B. C. Lubin; A. C. Boileau; M. R. Dicklin; M. C. Carakostas; S. M. Tarka, *Food Chem. Toxicol.* **2008**, *46* (7S), S47–S53.
63. K. C. Maki; L. L. Curry; M. C. Carakostas; S. M. Tarka; M. S. Reeves; M. V. Farmer; J. M. McKenney; P. D. Toth; S. L. Schwartz; B. C. Lubin; M. R. Dicklin; A. C. Boileau; J. D. Bisognano, *Food Chem. Toxicol.* **2008**, *46* (7S), S40–S46.
64. M. C. Carakostas; L. L. Curry; A. C. Boileau; D. J. Brusick, *Food Chem. Toxicol.* **2008**, *46* (7S), S1–S10.
65. Anonymous, Summary and Conclusions, Joint FAO/WHO Expert Committee on Food Additives. 69th Meeting, Rome, Italy, 17–26 June 2008, 21 pp.
66. A. G. Renwick, *Food Chem. Toxicol.* **2008**, *46* (7S), S61–S69.
67. A. I. Nikiforov; A. K. Eapen, *Int. J. Toxicol.* **2008**, *27*, 65–80.
68. H. Van der Wel; K. Loeve, *Eur. J. Biochem.* **1972**, *31*, 221–225.
69. J. D. Higginbotham, Talin Protein (Thaumatin). In *Alternative Sweeteners*; L. O'Brien Nabors, R. C. Gelardi, Eds.; Marcel Dekker: New York, 1986; pp 103–134.
70. B. F. Gibbs; I. Alli; C. Mulligan, *Nutr. Res.* **1996**, *16*, 1619–1630.
71. C. M. Ogata; P. F. Gordon; A. M. De Vos; S.-H. Kim, *J. Mol. Biol.* **1992**, *228*, 893–908.
72. T.-P. Ko; J. Day; A. Greenwood; A. McPherson, *Acta Crystallogr. D, Biol. Crystallogr.* **1994**, *50D*, 813–825.
73. F. Borrego; H. Montijano, Neohesperidin Dihydrochalcone. In *Alternative Sweeteners: Third Edition, Revised and Expanded*; L. O'Brien Nabors, Ed.; Marcel Dekker: New York, 2001; pp 87–104.
74. A. D. Kinghorn; E. J. Kennelly, *J. Chem. Educ.* **1995**, *72*, 676–680.
75. A. D. Kinghorn; D. D. Soejarto, *Pure Appl. Chem.* **2002**, *74*, 1169–1174.
76. A. D. Kinghorn; N.-C. Kim, Discovering New Natural Sweeteners. In *Optimising Sweet Taste in Foods*; W. J. Spillane, Ed.; CRC Press: Boca Raton, FL, 2006; pp 292–306.
77. R. A. Hussain; A. D. Kinghorn; D. D. Soejarto, *Econ. Bot.* **1988**, *42*, 267–283.
78. W. V. Reid; S. A. Laird; C. A. Meyer; R. Gámez; A. Sittenfeld; D. H. Janzen; M. A. Gollen; C. Juma, Eds., *Biodiversity Prospecting: Using Genetic Resources for Sustainable Development*; World Resources Institute: Washington, DC, 1993.
79. D. D. Soejarto; A. D. Kinghorn; N. R. Farnsworth, *J. Nat. Prod.* **1982**, *45*, 590–595.
80. A. D. Kinghorn; D. D. Soejarto; N. P. D. Nanayakkara; C. M. Compadre; H. C. Makapugay; J. M. Hovanec-Brown; P. J. Medon; S. K. Kamath, *J. Nat. Prod.* **1984**, *47*, 439–444.
81. R. A. Hussain; Y.-M. Lin; L. J. Poveda; E. Bordas; B. S. Chung; J. M. Pezzuto; D. D. Soejarto; A. D. Kinghorn, *J. Ethnopharmacol.* **1990**, *28*, 103–115.
82. M.-S. Chung; N.-C. Kim; L. Long; L. Shamon; W.-Y. Ahmad; L. Sagrero-Nieves; L. B. S. Kardono; E. J. Kennelly; J. M. Pezzuto; D. D. Soejarto; A. D. Kinghorn, *Phytochem. Anal.* **1997**, *8*, 49–54.
83. R. A. Hussain; L. J. Poveda; J. M. Pezzuto; D. D. Soejarto; A. D. Kinghorn, *Econ. Bot.* **1990**, *44*, 174–182.
84. R. A. Hussain; J. Kim; T.-W. Hu; J. M. Pezzuto; D. D. Soejarto; A. D. Kinghorn, *Planta Med.* **1986**, *52*, 403–404.
85. W. Jakinovich, Jr.; C. Moon; Y.-H. Choi; A. D. Kinghorn, *J. Nat. Prod.* **1990**, *53*, 190–195.
86. E. Vasquez; W. Jakinovich, Jr.; N. P. D. Nanayakkara; R. A. Hussain; M.-S. Chung; A. D. Kinghorn, *J. Agric. Food Chem.* **1993**, *41*, 1305–1310.
87. C. S. Zuker; N. J. P. Ryba; G. A. Nelson; M. A. Hoon; J. Chandrashekar; Y. Zhang, Cloning, Sequences, and Expression of Mammalian Sweet Taste Receptors, and Use for Taste Modulator Screening. U.S. Patent 7,402,400 B2, 2008, 76pp.

88. X. Li; G. Servant, Functional Characterization of the Human Sweet Taste Receptor: High-Throughput Screening Assay Development and Structural Function Relation. In *Sweetness and Sweeteners*; D. K. Weerasinghe, G. E. DuBois, Eds.; ACS Symposium Series 979; Oxford University Press: New York, 2008; pp 368–385.
89. Y. Asakawa, *Planta Med.* **2008**, *74*, 898–899.
90. S. Furukawa, *Tokyo Kagaku Kaishi* **1920**, *41*, 706–728.
91. E. M. Acton; H. Stone; M. A. Leaffer; S. M. Oliver, *Experientia* **1970**, *26*, 473–474.
92. R. Kasai; H. Fujino; T. Kuzuki; W.-H. Wong; C. Goto; N. Yata; O. Tanaka; F. Yasuhara; S. Yamaguchi, *Phytochemistry* **1986**, *25*, 871–876.
93. C. M. Compadre; J. M. Pezzuto; A. D. Kinghorn; S. K. Kamath, *Science* **1985**, *227*, 417–419.
94. C. M. Compadre; R. A. Hussain; R. L. Lopez de Compadre; J. M. Pezzuto; A. D. Kinghorn, *J. Agric. Food Chem.* **1987**, *35*, 273–279.
95. K. Mori; M. Kato, *Tetrahedron* **1986**, *42*, 5895–5900.
96. C. M. Compadre; R. A. Hussain; R. L. Lopez de Compadre; J. M. Pezzuto; A. D. Kinghorn, *Experientia* **1988**, *44*, 447–449.
97. F. A. Souto Bachiller; M. De Jesus Echevarría; O. E. Cárdenas González; M. F. Acuña Rodriguez; P. A. Meléndez; L. Romero Ramsey, *Phytochemistry* **1997**, *44*, 1077–1086.
98. J. H. Kim; H. J. Hyun; H. Seung, *Tetrahedron* **2003**, *59*, 7501–7507.
99. F. G. Gatti, *Tetrahedron Lett.* **2008**, *49*, 4997–4998.
100. H.-J. Yang; H. J. Kim; Y.-A. Whang; J.-K. Choi; I.-S. Lee, *Nat. Prod. Sci.* **1999**, *5*, 151–153.
101. N. Kaneda; I.-S. Lee; M. P. Gupta; D. D. Soejarto; A. D. Kinghorn, *J. Nat. Prod.* **1992**, *55*, 1136–1141.
102. M. Ono; T. Tsuru; H. Abe; M. Eto; M. Okawa; F. Abe; J. Kinjo; T. Ikeda; T. Nohara, *J. Nat. Prod.* **2006**, *69*, 1417–1420.
103. A. Tahara; R. Nakata; Y. Ohtsuka, *Nature* **1971**, *233*, 619–620.
104. I. Sakamoto; K. Yamasaki; O. Tanaka, *Chem. Pharm. Bull.* **1977**, *25*, 844–848.
105. I. Sakamoto; K. Yamasaki; O. Tanaka, *Chem. Pharm. Bull.* **1977**, *25*, 3437–3439.
106. M. Kobayashi; S. Horikawa; I. H. Degrandi; J. Ueno; H. Mitsuhashi, *Phytochemistry* **1977**, *16*, 1405–1408.
107. T. Tanaka; H. Kohda; O. Tanaka; F.-H. Chen; W.-H. Chou; J.-L. Leu, *Agric. Biol. Chem.* **1981**, *45*, 2165–2166.
108. A. N. Starratt; C. W. Kirby; R. Pocs; J. E. Brandle, *Phytochemistry* **2002**, *59*, 367–370.
109. K. Ohtani; Y. Aikawa; R. Kasai; W.-H. Chou; K. Yamasaki; O. Tanaka, *Phytochemistry* **1992**, *31*, 1553–1559.
110. S. Hirono; W.-H. Chou; R. Kasai; O. Tanaka; T. Tada, *Chem. Pharm. Bull.* **1990**, *38*, 1743–1744.
111. L. R. R. Harinantenaina; R. Kasai; K. Yamasaki, *Chem. Pharm. Bull.* **2002**, *50*, 268–271.
112. T. Tanaka; O. Tanaka; Z.-W. Lin; J. Zhou; H. Ageta, *Chem. Pharm. Bull.* **1983**, *31*, 780–783.
113. T. Tanaka; O. Tanaka; Z.-W. Lin; J. Zhou, *Chem. Pharm. Bull.* **1985**, *33*, 4725–4780.
114. M. Katagiri; K. Ohtani; R. Kasai; K. Yamasaki; C.-R. Yang; O. Tanaka, *Phytochemistry* **1994**, *35*, 439–442.
115. H. Yamada; M. Nishizawa, *Tetrahedron* **1992**, *48*, 3021–3044.
116. M. Nishizawa; H. Yamada, *Synlett* **1995**, 785–793.
117. F. Fullas; R. A. Hussain; E. Bordas; J. M. Pezzuto; D. D. Soejarto; A. D. Kinghorn, *Tetrahedron* **1991**, *47*, 8515–8522.
118. P. J. Hylands; J. Kosugi, *Phytochemistry* **1982**, *22*, 1379–1384.
119. K. Oobayashi; K. Yoshikawa; S. Arihara, *Phytochemistry* **1992**, *31*, 943–946.
120. R. Kasai; K. Matsumoto; R. Nie; T. Morita; A. Awazu; J. Zhou; O. Tanaka, *Phytochemistry* **1987**, *26*, 1371–1376.
121. R. Kasai; K. Matsumoto; R. L. Nie; J. Zhou; O. Tanaka, *Chem. Pharm. Bull.* **1988**, *36*, 234–243.
122. H. Kubo; K. Ohtani; R. Kasai; K. Yamasaki; R.-L. Nie; O. Tanaka, *Phytochemistry* **1996**, *41*, 1169–1174.
123. R. Kasai; R.-L. Nie; K. Nashi; K. Ohtani; J. Zhou; G.-D. Tao; O. Tanaka, *Agric. Biol. Chem.* **1989**, *53*, 3347–3349.
124. K. Matsumoto; R. Kasai; K. Ohtani; O. Tanaka, *Chem. Pharm. Bull.* **1990**, *38*, 2030–2032.
125. Z. Jia; X. Jang, In *234th American Chemical Society National Meeting*; Boston, MA, 19–23 August 2007, Abstract AGFD-112.
126. Y.-H. Choi; R. A. Hussain; J. M. Pezzuto; A. D. Kinghorn; J. F. Morton, *J. Nat. Prod.* **1989**, *52*, 1118–1127.
127. Y.-H. Choi; A. D. Kinghorn; Z. Shi; H. Zhang; B.-K. Teo, *J. Chem. Soc., Chem. Commun.* **1989**, 887–888.
128. E. J. Kennelly; L. Cai; N.-C. Kim; A. D. Kinghorn, *Phytochemistry* **1996**, *41*, 1381–1383.
129. F. Fullas; Y.-H. Choi; A. D. Kinghorn; N. Bunyapraphatsara, *Planta Med.* **1990**, *56*, 332–333.
130. E. J. Kennelly; R. Suttisri; A. D. Kinghorn, Novel Sweet-Tasting Saponins of the Cycloartane, Oleanane, Secodammarane, and Steroidal Types. In *Saponins Used in Food and Agriculture, Vol. 405: Advances in Experimental Medicine and Biology*; G. R. Waller, K. Yamasaki, Eds.; Plenum Press: New York, 1996; pp 13–24.
131. N.-C. Kim; A. D. Kinghorn; D. S. H. L. Kim, *Org. Lett.* **1999**, *1*, 223–224.
132. D. J. Yang; Z. C. Zhong; Z. M. Xie, *Yao Hsueh Hsueh Pao* **1992**, *27*, 841–844.
133. R. G. Shu; C. R. Xu; L.-N. Li, *Acta Pharm. Sin.* **1995**, *30*, 757–761.
134. T. Takemoto; S. Arihara; T. Nakajima; M. Okuhira, *Yakugaku Zasshi* **1983**, *103*, 1015–1023.
135. M. Yoshikawa; T. Morikawa; K. Nakano; Y. Pongpiriyadacha; T. Murakami; H. Matsuda, *J. Nat. Prod.* **2002**, *65*, 1638–1642.
136. I. Kitagawa; M. Sakagami; F. Hashiuchi; J. L. Zhou; M. Yoshikawa; J. Ren, *Chem. Pharm. Bull.* **1989**, *37*, 551–553.
137. Y. Hashimoto; H. Ishizone; M. Ogura, *Phytochemistry* **1980**, *19*, 2411–2415.
138. Y. Hashimoto; Y. Ohta; H. Ishizone; M. Kuriyama; M. Ogura, *Phytochemistry* **1982**, *21*, 2335–2337.
139. Y. Hashimoto; H. Ishizone; M. Suganuma; M. Ogura; K. Nakatasa; H. Yoshioka, *Phytochemistry* **1983**, *22*, 259–264.
140. R. Suttisri; M.-S. Chung; A. D. Kinghorn; O. Sticher; Y. Hashimoto, *Phytochemistry* **1993**, *34*, 405–408.
141. E. J. Kennelly; L. Cai; L. Long; L. Shamon; K. Zaw; B.-N. Zhou; J. M. Pezzuto; A. D. Kinghorn, *J. Agric. Food Chem.* **1995**, *43*, 2602–2607.
142. J. Jizba; L. Dolejs; V. Herout; F. Sorm, *Tetrahedron Lett.* **1971**, 1329–1332.
143. H. Yamada; M. Nishizawa; C. Katayama, *Tetrahedron Lett.* **1992**, *33*, 4009–4010.
144. H. Yamada; M. Nishizawa, *Synlett* **1993**, 54–56.
145. M. Nishizawa; H. Yamada, Intensely Sweet Saponin Osladin: Synthetic and Structural Study. In *Saponins Used in Food and Agriculture, Vol. 405: Advances in Experimental Medicine and Biology*; G. R. Waller, K. Yamasaki, Eds.; Plenum Press: New York, 1996; pp 25–36.
146. J. Kim; J. M. Pezzuto; D. D. Soejarto; F. A. Lang; A. D. Kinghorn, *J. Nat. Prod.* **1988**, *51*, 1166–1172.

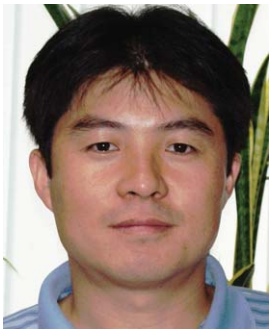
147. J. Kim; A. D. Kinghorn, *Phytochemistry* **1989**, *28*, 1225–1228.
148. M. Nishizawa; H. Yamada; Y. Yamaguchi; S. Hatakeyama; I.-S. Lee; E. J. Kennelly; J. Kim; A. D. Kinghorn, *Chem. Lett.* **1994**, 1555–1558; 1979.
149. V. D. Huan; K. Ohtani; R. Kasai; K. Yamasaki; N. V. Tuu, *Chem. Pharm. Bull.* **2001**, *49*, 453–460.
150. M. Yoshikawa; T. Murakami; T. Ueda; H. Shimoda; J. Yamahara; H. Matsuda, *Heterocycles* **1999**, *50*, 411–418.
151. A. Bassoli; L. Merlini; G. Morini, *Pure Appl. Chem.* **2002**, *74*, 1181–1187.
152. C. R. A. Wright; E. H. Rennie, *J. Chem. Soc., Trans.* **1881**, *39*, 237–240.
153. E. H. Rennie, *J. Chem. Soc., Trans.* **1886**, *49*, 857–865.
154. T. Tanaka; K. Kawamura; H. Kohda; K. Yamasaki; O. Tanaka, *Chem. Pharm. Bull.* **1982**, *30*, 2421–2423.
155. R.-L. Nie; T. Tanaka; J. Zhou; O. Tanaka, *Chem. Pharm. Bull.* **1982**, *46*, 1933–1934.
156. R. M. Horowitz; B. Gentili, Dihydrochalone Sweeteners. In *Symposium: Sweeteners*; G. E. Inglett, Ed.; Avi Publishing Company, Inc.: Westport, CT, 1974; pp 182–193.
157. A. Tsopmo; M. H. Tchuendem; J. F. Ayafor; F. Tillequin; M. Koch; H. Anke, *Nat. Prod. Lett.* **1996**, *9*, 33–37.
158. J. F. Ayafor; J. D. Connolly, *J. Chem. Soc., Perkin Trans. 1* 1981, 1750–1754.
159. N. P. D. Nanayakkara; R. A. Hussain; J. M. Pezzuto; D. D. Soejarto; A. D. Kinghorn, *J. Med. Chem.* **1988**, *31*, 1250–1253.
160. R. Kasai; S. Hirono; W.-H. Chou; O. Tanaka; F.-H. Chen, *Chem. Pharm. Bull.* **1988**, *36*, 4167–4170.
161. R. Kasai; S. Hirono; W.-H. Chou; O. Tanaka; F.-H. Chen, *Chem. Pharm. Bull.* **1991**, *39*, 1871–1872.
162. F. Gao; H. Wang; T. J. Mabry; A. D. Kinghorn, *Phytochemistry* **1990**, *29*, 2865–2869.
163. S. Morimoto; G.-I. Nonaka; I. Nishioka, *Chem. Pharm. Bull.* **1985**, *33*, 4338–4345.
164. N. Tanaka; R. Orii; K. Ogasa; H. Wada; T. Murakami; Y. Sakai; C. M. Chen, *Chem. Pharm. Bull.* **1991**, *39*, 55–59.
165. N.-I. Baek; M.-S. Chung; L. Shamon; L. B. S. Kardono; S. Tsauri; K. Padmawinata; J. M. Pezzuto; D. D. Soejarto; A. D. Kinghorn, *J. Nat. Prod.* **1993**, *56*, 1532–1538.
166. N.-I. Baek; E. J. Kennelly; L. B. S. Kardono; S. Tsauri; K. Padmawinata; D. D. Soejarto; A. D. Kinghorn, *Phytochemistry* **1994**, *36*, 513–518.
167. M. Vasaenge; B. Liu; C. J. Welch; W. Rolfsen; L. Bohlin, *Planta Med.* **1997**, *63*, 511–517.
168. A. Subarnas; H. Wagner, *Phytomedicine* **2000**, *7*, 401–405.
169. H. Masuda; K. Ohtani; K. Mizutani; S. Ogawa; R. Kasai; O. Tanaka, *Chem. Pharm. Bull.* **1991**, *39*, 1382–1384.
170. A. Arnoldi; A. Bassoli; G. Borgonovo; L. Merlini, *J. Chem. Soc., Perkin Trans. 1* **1995**, 2447–2453.
171. R. Vleggaar; L. G. J. Ackerman; P. S. Steyn, *J. Chem. Soc., Perkin Trans. 1* **1992**, 3095–3098.
172. K. Nakamura; T. J. Baker; M. Goodman, *Org. Lett.* **2000**, *2*, 2967–2970.
173. O. Tamura; T. Shiro; A. Toyao; H. Ishibashi, *J. Chem. Soc., Chem. Commun.* **2003**, 2678–2679.
174. A. Bassoli; G. Borgonova; G. Busnelli; G. Morini; L. Merlini, *Eur. J. Med. Chem.* **2005**, *40*, 2518–2525.
175. D. Ming; G. Hellekant, *FEBS Lett.* **1994**, *355*, 106–108.
176. M. Kohmura; M. Ota; H. Izawa; D. Ming; G. Hellekant; Y. Ariyoshi, *Biopolymers* **1996**, *38*, 553–556.
177. G. Hellekant, Brazzein. In *Sweeteners*, 3rd ed.; R. Wilson, Ed.; Blackwell: Oxford, UK, 2007; pp 47–50.
178. H. Yamashita; S. Theerasilp; T. Aiuchi; K. Nakaya; Y. Nakamura; Y. Kurihara, *J. Biol. Chem.* **1990**, *265*, 15770–15775.
179. Z. Hu; M. He, *Yunnan Zhi Wu Yan Jiu* **1991**, *5*, 207–212.
180. M. Kohmura; Y. Ariyoshi, *Biopolymers* **1998**, *46*, 215–223.
181. G. Frank; H. Zuber, *Hoppe-Seyler's Z. Physiol. Chem.* **1976**, *357*, 585–592.
182. M. Kohmura; T. Mizukoshi; N. Nio; E.-I. Suzuki; Y. Ariyoshi, *Pure Appl. Chem.* **2002**, *74*, 1235–1242.
183. Y. Shirasuka; K.-I. Nakajima; T. Asakura; H. Yamashita; A. Yamamoto; S. Hata; S. Nagata; M. Abo; H. Sorimachi; K. Abe, *Biosci. Biotechnol. Biochem.* **2004**, *68*, 1403–1407.
184. H. Van der Wel; G. Larson; A. Hladik; C. M. Hladik; G. Hellekant; D. Glaser, *Chem. Senses* **1989**, *14*, 75–79.
185. R. B. Iyengar; P. Smits; F. Van der Ouderaa; H. Van der Wel; J. Van Brouwershaven; P. Ravenstein; G. Richters; P. D. Van Wassanaar, *Eur. J. Biochem.* **1979**, *96*, 196–204.
186. B. Crammer, Recent Trends of Some Natural Sweet Substances from Plants. In *Selected Topics in the Chemistry of Natural Products*; R. Ikan, Ed.; World Scientific: Singapore, 2008; pp 189–208.
187. F. M. Assadi-Porter; S. Patry; J. L. Markley, *Prot. Exp. Purific.* **2008**, *58*, 263–268.
188. D. E. Walters; G. Hellekant, *J. Agric. Food Chem.* **2006**, *54*, 10129–10133.
189. A. Koizumi; K.-i. Nakajima; T. Asakura; Y. Morita; K. Ito; A. Shmizu-Ibuka; T. Misaka; K. Abe, *Biochem. Biophys. Res. Commun.* **2007**, *358*, 585–589.
190. K.-i. Nakajima; Y. Morita; A. Koizumi; T. Asakura; T. Terada; K. Ito; A. Shimizu-Ibuka; J.-i. Maruyama; K. Kitamoto; T. Misaka; K. Abe, *FASEB J.* **2008**, *22*, 2323–2330.
191. A. Hiura; T. Akanabe; K. Ohtani; R. Kasai; K. Yamasaki; Y. Kurihara, *Phytochemistry* **1996**, *43*, 1023–1027.
192. D. Sugita; R. Inoue; Y. Kurihara, *Chem. Senses* **1998**, *23*, 93–97.
193. K. Kurihara; L. M. Beidler, *Nature* **1969**, *222*, 1176–1179.
194. J. Ley; G. Kindel; S. Paetz; T. Riess; M. Haug; R. Schmidtmann; G. Krammer, Use of Hesperetin for Enhancing the Sweet Taste. PCT Int. Pat. Appl. WO2007014879 A1, 2007, 97pp.
195. J. P. Ley; M. Blings; S. Paetz; G. Kindel; K. Freiherr; G. E. Krammer; H.-J. Bertram, Enhancers for Sweet Taste from the World of Non-Volatiles: Polyphenols as Taste Modifiers. In *Sweetness and Sweeteners*; D. K. Weerasinghe, G. E. DuBois, Eds.; ACS Symposium Series 979; Oxford University Press: New York, 2008; pp 400–409.
196. J. P. Ley; G. Krammer; G. Reinders; I. L. Gatfield; H.-J. Bertram, *J. Agric. Food Chem.* **2005**, *53*, 6061–6066.
197. K. Kurihara; L. M. Beidler, *Science* **1968**, *161*, 1241–1242.
198. J. N. Brourer; H. van der Wel; A. Francke; G. L. Henning, *Nature* **1968**, *220*, 373–374.
199. S. Theerasilp; Y. Kurihara, *J. Biol. Chem.* **1988**, *263*, 11536–11539.
200. H.-J. Sun; M.-L. Cui; B. Ma; H. Ezura, *FEBS Lett.* **2006**, *580*, 620–626.
201. H.-J. Sun; H. Katoaka; M. Yano; H. Ezura, *Plant Biotechnol. J.* **2007**, *5*, 768–777.
202. H. Tomosaka; Y.-W. Chin; A. A. Salim; W. J. Keller; A. D. Kinghorn, *Phytother. Res.* **2008**, *22*, 979–981.
203. L. M. Bartoshuk; C.-H. Lee; R. Scarpellino, *Science* **1972**, *178*, 988–990.

204. T. Imoto; A. Miyasaka; R. Ishima; K. Akasaka, *Comp. Biochem. Physiol. A* **1991**, *100*, 309–314.
205. J. I. Fletcher; A. J. Dingley; R. Smith; M. Connor; M. J. Christie; G. F. King, *Eur. J. Biochem.* **1999**, *264*, 525–533.
206. W. S. Zawalich, *Comp. Biochem. Physiol. A* **1973**, *44*, 903–909.
207. Y. Hiji, *Nature* **1975**, *256*, 427–429.
208. Y. Hiji; H. Ito, *Comp. Biochem. Physiol. A* **1977**, *58*, 109–113.
209. W. Jakinovich, Jr., *Science* **1983**, *219*, 408–410.
210. V. Vlahopoulos; W. Jakinovich, Jr., *J. Neurosci.* **1986**, *6*, 2604–2610.
211. G. W. Muller; J. C. Culberson; G. Roy; J. Ziegler; D. E. Walters; M. S. Kellogg; S. S. Schiffman; Z. S. Warwick, *J. Med. Chem.* **1992**, *35*, 1747–1751.
212. K. Yoshikawa; S. Arihara; K. Matsuura, *Tetrahedron Lett.* **1991**, *32*, 789–792.
213. K. Yoshikawa; K. Amimoto; S. Arihara; K. Mutsuura, *Tetrahedron Lett.* **1989**, *30*, 1103–1106.
214. M. Maeda; T. Iwashita; Y. Kurihara, *Tetrahedron Lett.* **1989**, *30*, 1547–1550.
215. K. Yoshikawa; K. Amimoto; S. Arihara; K. Matsuura, *Chem. Pharm. Bull.* **1989**, *37*, 852–854.
216. H.-M. Liu; F. Kiuchi; Y. Tsuda, *Chem. Pharm. Bull.* **1992**, *40*, 1366–1375.
217. K. Yoshikawa; M. Nakagawa; R. Yamamoto; S. Arihara; K. Matsuura, *Chem. Pharm. Bull.* **1992**, *40*, 1779–1782.
218. K. Yoshikawa; Y. Kondo; S. Arihara; K. Matsuura, *Chem. Pharm. Bull.* **1993**, *41*, 1730–1732.
219. W. Ye; X. Liu; Q. Zhang; C.-T. Che; S. Zhao, *J. Nat. Prod.* **2001**, *64*, 232–235.
220. J. Yamahara; H. Matsuda; T. Murakami; H. Shimada; M. Yoshikawa; R. Karahara; Y. Hiji, *Wakan Iyakugaku Zasshi* **1996**, *13*, 295–299.
221. Y. Hichi, Foods Containing Gymnemic Acids for Preventing Obesity. Jpn. Kokai Tokyo Koho JP 0,643,421, 1994, 8pp.
222. K. Yoshikawa; H. Ogata; S. Arihara; H.-C. Chang; J.-D. Wang, *Chem. Pharm. Bull.* **1998**, *46*, 1102–1107.
223. K. Yoshikawa; K. Takahashi; K. Matsuchika; S. Arihara; H.-C. Chang; J.-D. Wang, *Chem. Pharm. Bull.* **1999**, *47*, 1598–1603.
224. P. W. Khong; K. G. Lewis, *Aust. J. Chem.* **1975**, *28*, 165–172.
225. K. Yoshikawa; S. Tumura; K. Yamada; S. Arihara, *Chem. Pharm. Bull.* **1992**, *40*, 2287–2291.
226. K. Yoshikawa; N. Nagai; M. Yoshida; S. Arihara, *Chem. Pharm. Bull.* **1993**, *41*, 1722–1725.
227. K. Yoshikawa; H. Taninaka; Y. Kan; S. Arihara, *Chem. Pharm. Bull.* **1994**, *42*, 2023–2027.
228. K. Yoshikawa; H. Taninaka; Y. Kan; S. Arihara, *Chem. Pharm. Bull.* **1994**, *42*, 2455–2460.
229. K. Yoshikawa; A. Mizutani; Y. Kan; S. Arihara, *Chem. Pharm. Bull.* **1997**, *45*, 62–67.
230. K. Yoshikawa; N. Shimono; S. Arihara, *Chem. Pharm. Bull.* **1992**, *40*, 2275–2278.
231. Y. Kurihara; K. Ookubo; H. Tasaki; H. Kodama; Y. Akiyama; A. Yagi; B. Halpern, *Tetrahedron* **1988**, *44*, 61–66.
232. K. Yoshikawa; H. Hirai; M. Tanaka; S. Arihara, *Chem. Pharm. Bull.* **2000**, *48*, 1093–1096.
233. R. J. Alexander, *Sweeteners: Nutritive*; Eagan Press: St. Paul, MN, 1998.
234. M. Meilgaard; G. V. Civille; B. T. Carr, *Sensory Evaluation Techniques*, 2nd ed.; CRC Press: Boca Raton, FL, 1991.
235. D. G. Guadagni; V. P. Maier; J. H. Turnbaugh, *J. Sci. Food Agric.* **1974**, *25*, 1199–1205.
236. G. E. DuBois; G. A. Crosby; R. A. Stephenson, *J. Med. Chem.* **1981**, *24*, 408–428.
237. R. M. Horowitz; B. Gentili, Dihydrochalcone Sweeteners from Citrus Flavanones. In *Alternative Sweeteners; Second Edition, Revised and Expanded*; L. O'Brien Nabors, R. C. Gelardi, Eds.; Marcel Dekker: New York, 1991; pp 97–115.
238. V. B. Duffy; M. Sigman-Grant; M. A. Powers; D. Elmore; E. F. Myers; D. Quagliani; M. Spano; K. F. Stitzel; S. Taylor; R. Earl; S. Connor, *J. Am. Diet. Assoc.* **2004**, *104*, 255–275.
239. S. S. Schiffman; E. A. Sattely-Miller; B. G. Graham; B. J. Booth; K. M. Gibes, *Chem. Senses* **2000**, *25*, 131–140.
240. R. W. Bryant; K. S. Atwal; I. Bakaj; M. T. Buber; S. Carlucci; R. Cerne; R. Cortes; H. R. Devantier; C. J. Hendrix; S. P. Lee; R. J. Palmer; C. Wilson; Q. Yang; F. R. Salemme, Development of Transient Receptor Potential Melanostatin 5 Modulators for Sweetness Enhancement. In *Sweetness and Sweeteners*; D. K. Weerasinghe, G. E. DuBois, Eds.; ACS Symposium Series 979; Oxford University Press: New York, 2008; pp 386–399.
241. G. Krammer; J. Ley; T. Riess; M. Haug; S. Paetz, Use of 4-Hydroxydihydrochalcones and their Salts for Enhancing an Impression of Sweetness. PCT Int. Pat. Appl. WO2007107586 A1, 2007, 87pp.
242. G. Nelson; M. A. Hoon; J. Chandrashekar; Y. Zhang; N. J. P. Ryba; C. S. Zuker, *Cell* **2001**, *106*, 381–390.
243. X. Li; L. Staszewski; H. Xu; K. Durick; M. Zoller; E. Alder, *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 4692–4696.
244. R. F. Margolskee, *J. Biol. Chem.* **2002**, *277*, 1–4, and references therein.
245. G. Morini; A. Bassoli; P. A. Temussi, *J. Med. Chem.* **2005**, *48*, 5520–5529.
246. S. C. Eggers; T. E. Acree; R. S. Shallenberger, *Food Chem.* **2000**, *68*, 45–49.
247. R. S. Shallenberger; T. E. Acree, *Nature* **1967**, *206*, 480–482.
248. J. P. Ley, *Chem. Percept.* **2008**, *1*, 58–77.
249. P. Temussi, The Sweet Taste Receptor: A Single Receptor with Multiple Sites and Modes of Action. In *Advances in Food and Nutrition Research*; S. L. Taylor, Ed.; Elsevier: Amsterdam, 2007; Vol. 53, pp 199–239.
250. N. Kunishima; Y. Shimada; Y. Tsuji; T. Sato; M. Yamamoto; T. Kumasaka; S. Nakanishi; S. Jingami; K. Morikawa, *Nature* **2000**, *407*, 971–977.
251. M. Max; Y. G. Shanker; L. Huang; M. Rong; Z. Liu; F. Campagne; H. Weinstein; S. Damak; R. F. Margolskee, *Nat. Genet.* **2001**, *28*, 58–63.
252. P. A. Temussi, *FEBS Lett.* **2002**, *526*, 1–3.
253. M. Cui; P. Jiang; E. Maillet; M. Max; R. F. Margolskee; R. Osman, *Curr. Pharm. Des.* **2006**, *12*, 4591–4600.
254. H. Xu; L. Staszewski; H. Tang; E. Adler; M. Zoller; X. Li, *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 14258–14263.
255. P. Jiang; M. Cui; B. Zhao; L. A. Snyder; L. M. Benard; R. Osman; M. Max; R. F. Margolskee, *J. Biol. Chem.* **2005**, *280*, 34296–34305.
256. P. Jiang; M. Cui; B. Zhao; Z. Liu; L. A. Snyder; L. M. J. Benard; R. Osman; R. F. Margolskee; M. Max, *J. Biol. Chem.* **2005**, *280*, 15238–15246.
257. P. Jiang; Q. Ji; Z. Liu; L. A. Snyder; L. M. Benard; R. F. Margolskee; M. Max, *J. Biol. Chem.* **2004**, *279*, 45068–45075.
258. S. S. Schiffman; B. J. Booth; B. T. Carr; M. L. Losee; E. A. Sattely-Miller; B. G. Graham, *Brain Res. Bull.* **1995**, *38*, 105–120.
259. G. E. DuBois, *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 13972–13973.

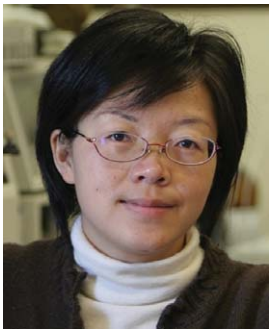
260. G. J. de Klerk; M. G. Nieuwenhuis; J. J. Beutler, *Br. Med. J.* **1997**, *314*, 731–732.
261. T. G. J. Van Rossum; F. H. De Jong; W. C. J. Hop; F. Boomsma; S. W. Schalm, *Neth. J. Gastroenterol. Hepatol.* **2001**, *16*, 789–795.
262. T. Konoshima; M. Takasaki, *Pure Appl. Chem.* **2002**, *74*, 1309–1316.
263. M. B. Sporn; N. M. Dunlop; D. L. Newton; J. M. Smith, *Fed. Proc.* **1976**, *35*, 1332–1338.
264. D. H. Williams; M. J. Stone; P. R. Hauck; S. K. Rahman, *J. Nat. Prod.* **1989**, *52*, 1189–1208.
265. C. M. Cerda-García-Rojas; R. Pereda-Miranda, The Phytochemistry of Stevia: A General Survey. In Stevia: *The Genus Stevia*; A. D. Kinghorn, Ed.; Taylor & Francis: London, 2002; pp 86–118.
266. R. Mata; V. Rodríguez; P. Pereda-Miranda; N. Kaneda; A. D. Kinghorn, *J. Nat. Prod.* **1992**, *55*, 660–666.

Biographical Sketches

Dr. A. Douglas Kinghorn is Professor and Jack L. Beal Chair at the College of Pharmacy, The Ohio State University, Columbus, OH. He received his Ph.D. (1975) and D.Sc. (1990) degrees from the School of Pharmacy, University of London. From 1976 to 2004, he was a faculty member at the College of Pharmacy of the University of Illinois at Chicago where he was also a Senior University Scholar. He began working on the discovery of small-molecule natural sweeteners from plants in 1980, particularly of the terpenoid and flavonoid types. Dr. Kinghorn has served as Editor of the *Journal of Natural Products* since 1994 and as Series Editor of *Progress in the Chemistry of Organic Natural Products* since 2007.



Dr. Young-Won Chin was a research scientist at the College of Pharmacy, The Ohio State University (2007–2008), where he was also a postdoctoral research associate (2004–2007). He now holds a research position at the College of Pharmacy, Seoul National University, where he received the Ph.D. degree in 2003.



Dr. Li Pan received her Ph.D. degree from Chengdu Institute of Biology, Chinese Academy of Sciences, in 2006. She is currently a postdoctoral research associate at the College of Pharmacy, The Ohio State University.

Dr. Zhonghua Jia has been a research scientist at Givaudan Flavors Corporation, Cincinnati, OH since 2005. He received his Ph.D. degree from the School of Pharmaceutical Sciences Toho University, Japan, in 1997, and performed postdoctoral work at the Department of Chemistry and Biochemistry, Texas Tech University and the Complex Carbohydrate Research Center, University of Georgia. He has focused on the chemistry of triterpenoid saponins from traditional Chinese medicine in the past, whereas his current research interests are on natural flavor molecules.

3.11 Chemistry of Cosmetics

Masahiro Ota and Mineyuki Yokoyama, Shiseido Co., Ltd., Yokohama, Japan

3.11.1 Introduction

Cosmetics have a deep relation to natural products. It is true that there are many cosmetic products that have catchphrases or selling points claiming 100% pure natural ingredients; however, it is impossible to make cosmetics without natural products, regardless of such catchphrases. Hence, as regards natural products, no one is in doubt about the notion that they are something that cannot be done away with in cosmetics. The role of natural products in medicines is as one of the sources of lead compounds in the research and development of modern medicines, but in cosmetics natural products themselves are combined as ingredients, although there may be similar cases in medical research process too. In general the categories of cosmetics are limited only by people's imagination. As for the definition of cosmetics, we can suggest some categories from the point of view of a cosmetics company's research worker, as shown in **Table 1**.[1] It may indicate that cosmetics encompass a wider variety of items than you would have expected. It will be unreasonable to expect us to discuss all categories of cosmetics in this chapter owing to the limitation on space. Hence, first we would like to discuss the history of cosmetics and their relationship with natural products. We outline the natural products that are being used for cosmetics manufacture, conforming to the regulations under the Pharmaceutical Affairs Law in Japan. Furthermore, we would like to focus on the cosmetics categories called advanced cosmetics or cosmeceuticals, which have drug-like benefits and belong to the category of quasi-drugs, products that fall between drugs and cosmetics, especially skin-whitening cosmetics, antiaging ones, and hair growth promoters. Finally, plant cell/tissue culture technology is described, together with the illustration of some important natural products used in cosmetics.

3.11.2 History of Cosmetics and Natural Products

The ancient Chinese pharmacopoeia, *The Divine Farmer's Herb-Root Classic*, attributed to Shen Nong (3494 BC), who tasted and tested plants, includes 365 medicines derived from minerals, plants, and animals[2]. They are classified into three kinds depending on their effect. Of these, 120 items are categorized as natural and nonpoisonous. Another 120 items are a little poisonous and are used for prevention of illness. The remaining 125 items are poisonous and are used for treatment.[3,4] This kind of classification, owing to its virulence and

Table 1 Broad categorization of cosmetics

Classification		*Usage*	*Main products*
For skin	Skin care cosmetics	Cleansers	Face cleansing creams and foams
		Conditioners	Lotions, packs, massage creams
		Protectors	Milky lotions, moisture creams
	Makeup cosmetics	Base makeup	Foundations, face powders
		Point makeup	Lipstick, blushers, eye shadow, eye liners
		Nail care	Nail enamels, nail polish removers
	Body cosmetics	Bath	Soaps, liquid cleansers, bath preparations
		Sun cares and suntans	Sunscreen creams, sun oils
		Antiperspirants and deodorants	Deodorant sprays
		Bleaching, depilatory	Bleaching creams, depilatory creams
		Insect repellents	Insect repellent lotions and sprays
For hair and scalp	Hair care cosmetics	Cleansing	Shampoos
		Treatments	Rinses, hair treatments
		Hair styling	Hair mousses, hair liquids, pomades
		Permanent waves	Permanent wave lotions
		Hair colors and bleaches	Hair colors, hair bleaches, color rinses
	Scalp care cosmetics	Hair growth promoters	Hair growth promoters, hair tonics
		Treatments	Scalp treatments
Oral	Oral care cosmetics	Toothpastes	Toothpastes
		Mouthwashes	Mouthwashes
	Fragrances	Fragrances	Perfumes, Eau de Colognes

efficacy, is believed to have been one of the sources of information in selecting suitable natural products for cosmetics during a period when scientific toxicity assessment of today was nonexistent. The ancient European pharmacopoeia *De Materia Medica*, by Pedanius Dioscorides, comprises the description of around 600 plants and is known as the root of western herbs. It was the only representative pharmacopoeia until modern medicine was reconstructed in Europe. Ayurveda is traditional Indian medicine and was established around 3000–2000 BC. The word 'Ayurveda' is a *tatpurusha* (compound word) composed of the word *ayus* meaning 'life' or 'longevity' and the word *veda*, which refers to a system of 'knowledge'. Hence 'Ayurveda' roughly translates as the 'knowledge of a long life'. The classic in Ayurveda, *Charaka Sambita*, attributed to Charaka includes 500 plants and their applications.[5] The oldest Japanese pharmacopoeia in existence, Ishin-hou, is composed of 30 volumes referring to Chinese literature. It describes as good manners keeping one's skin-white using a mixture of *Aurantii nobilis pericarpium*, Benincasae semen, and peach branch. In those days, beauty culture and natural products were closely related, and it can be assumed that a fair-skinned face was already as preferable as it is nowadays.

Much of the philosophy of traditional medicine making use of natural products, especially plants, brought a health benefit to people both in the East and in the West. People today use natural products in their daily life, inspired by the experience, knowledge, and wisdom of their ancestors. All over the world, when did people begin to use them in cosmetics, based on these facts? It is difficult to answer this question properly, but archaeological excavations have revealed that they were used in the Paleolithic era. Thus we can assume that cosmetics have so long a history as the development and prosperity of humankind. Egyptians and Arabians have used ointment cosmetics since 4000 years ago. According to some sources, around 2920 BC cosmetics were developed from materials like tar or mercury, and around 1930 BC perfumes were already being traded in Egypt. It is presumed that Egyptian civilization brought about the development of cosmetics in those days. Japan's traditional cosmetics in the Edo period are believed to have been composed of three basic colors—red, white, and black. Especially, red cosmetics were important in glamorizing facial appearance. Cosmetics use spread among the general public and became an essential behavior in daily life, not being limited to the upper classes such as the aristocracy. Thus, as the demand for cosmetics increased, the cultivation of safflower, biennial herb of Compositae, which is an ingredient of red cosmetics, increased. But as the extracts from home-grown safflower were insufficient and expensive, safflower grown in Egypt was used as an alternative, which

was introduced to Japan through India, Central Asia, and China. Both in the East and in the West, women have always longed for a white complexion. In Japan, right from ancient times, clay, corn flour, light powder (calomel), white lead, and chalk were used by women to make their face appear white. Originally, calomel was developed in ancient China, and lead white (basic lead carbonate) was first made around the fourth century BC in Greece. As light powder was effective in the treatment of syphilis and was promulgated as something to get rid of lice, it was known as a medicine rather than as a cosmetic in China. Bactericidal properties of mercury have been empirically exploited. Regarding black color cosmetics, there was a custom tooth dye in black, which is called ohaguro in Japan. The origin of the use of ohaguro is uncertain. Some people say that the custom traveled from a race in the South that dyes their teeth black, while others say that it is a practice that has Japan as its home country.[6] Powdered gall and water were used for dying the teeth black. Powdered gall originates from gall made in the bark of anacardiaceous tree, containing tannin. People of those times understood that the powdered gall was effective not only for dying the teeth, but also for curing bleeding from the gums. Actually vasoconstrictive effect and hemostatic action were attributed to the presence of tannin.

Although today fragrance is one of the categories of cosmetics, the original type of fragrance, incense, has a long history in Japan, coming out first on record in *Chronicles of Japan*, in the year 595. Incense, which is essential for the ceremony of purging the Buddhist altar, had started to be used in Japan since the introduction of Buddhism in the beginning of the sixth century. It had much religious significance, but according to the historical materials written in 747, Ehi incense, which was a mixture of six or seven kinds of incenses, used to be burned with clothes or Buddhist scriptures and was used as bug repellent, being inducted into daily necessities. The ingredients of Ehi incense included agalloch of Thymelaeaceae; wood sandal of Santalaceae evergreen tree; clove, which is the floral bud of *Syzygium aromaticum* or *Eugenia caryophyllata*; spikenard oil, which is the extract of dry root or dry rhizome of valerianaceous plants; musk, which is extracted from the fragrance pouch of the male musk deer and dried; and ambergris, which is a waxy secreted material obtained from sperm whale. In ancient China, a preparation that combined 10–30 kinds of crude drugs such as soybean, red azuki bean, chalk, root of the crow gourd, sandalwood, and musk was used as washing charge. Much strong effervescent material called saponin was mixed with bean powder, such as adzuki beans, to impart the cleansing effect. In addition, honey locust, a Leguminosae plant, was also used, as it contains saponin in the fruit rind.[7] As compared with modern cosmetics, these classic ancient cosmetics may be inferior in quality or functionality, but we can infer that people of those times had learned the basic functions and actions of natural products empirically, which had helped in the preparation of cosmetics since then.

3.11.3 Pharmaceutical Affairs Law in Japan and Its Relevance to Natural Products

According to the Pharmaceutical Affairs Law in Japan, cosmetics are stipulated as articles that are applied to the human body for the purpose of cleansing, beautifying, promoting the attractiveness, improving the appearance, or maintaining the skin or hair in a healthy condition without affecting structure or function[8,9]. Their biological activity on the human body is required to be gentle and mild. In addition, quasi-drug, one of the cosmetics categories, exists as a unique system of the Pharmaceutical Affairs Law in Japan, occupying an intermediate position between drugs and cosmetics. Natural products are indispensable; as a practical matter, various crude drugs or extracts have been used in cosmetics. New components added to cosmetics had needed original examination for the approval system that existed before the flexible regulation of 2001 under the Pharmaceutical Affairs Law in Japan, but the flexible regulation of 2001, which reached the point where each cosmetics ingredient whose safety and stability are guaranteed by the manufacturing enterprise can be combined, became nearer to the regulation of the European–American types.

Table 2 provides a compendium of natural products that have been approved as cosmetic ingredients in the official book compiled by the Ministry of Health, Labour and Welfare, Japan before the flexible regulation of 2001. When you look at the individual entries, plant species and extract process materials, such as the extracting solvent, are limited to the actual official book details. A large portion of products in **Table 2** has come from plant materials, indicating the diversity of plants. It is something that shows how many botanical constituents

Table 2 Natural products as cosmetic ingredients

Acanthopanax senticosus extract	*Cumin extract*	*Japanese mugwort water*	*Peach core grain*	*Seaweed extract*
Almond extract	Defatted rice bran	Japanese raisin extract	Peach juice	Shiitake extract
Aloe	Duku extract	Japanese valerian extract	Peach leaf extract	Silk extract
Althea extract	Echinacea leaf extract	Jojoba oil	Peach seed extract	Sophora root extract
Angelica extract	Eucalyptus extract	Jujube extract	Peanut oil	Soy extract
Apple extract	Evening primrose oil	Juniper extract	Pellitory extract	Soybean lysophospholipid solution
Apricot kernel extract	Fennel	Kiwi extract	Peony root extract	Spearmint oil
Arnica extract	Fermented rice bran extract	Lagerstroemia speciosa extract	Peppermint extract	Sponge gourd extract
Artemisia capillaris extract	Filipendula extract	Lavender extract	Perilla extract	Stevia extract
Asiasarum root extract	Gambir extract	Lemon extract	Persimmon leaf powder	Strawberry juice
Aspalathus linearis extract	Ganoderma extract	Lettuce extract	Phellodendron bark extract	Styrax resin extract
Avocado extract	Garlic extract	Lily extract	Pine extract	Sunflower seed oil
Balm mint extract	Gentian extract	Lime juice	Placental extract	Sweet brier extract
Barley extract	Geranium herb extract	Linden extract	Plankton extract	Sweet clover extract
Beech extract	Ginger tincture	Lithospermum root extract	Pleurotus sajor-caju culture solution	Swertia herb extract
Birch extract	Ginkgo extract	Logwood extract	Polyporus sclerotium extract	Swertia pseudochinensis extract
Bitter orange peel extract	Ginseng extract	Loquat leaf extract	Potato starch	Tea seed extract
Burdock root extract	Grape extact	Low acid value candelilla wax	Prune extract	Terminalia extract
Burnet extract	Grape leaf extract	Lysine cocoate solution	Pueraria root extract	Thyme extract
Butcher broom extract	Grape seed oil	Mallow extract	Rape seed oil	Tiencha extract
Calamus rhizome extract	Grapefruit extract	Malt extract	Raspberry extract	Tomato extract
Calendula extract	Green tea extract	Matricaria oil	Rehmannia root extract	Tormentilla extract
Capsicum tincture	Gynostemma pentaphyllum extract	Mentha herb powder	Restharrow extract	Tsubaki oil
Carrot extract	Hayflower extract	Milk thistle extract	Rice bran extract	Turmeric extract
Celery extract	Hazelnut oil		Rice bran oil	Ume powder

Centella extract
Chamomile extract
Chinese caterpillar fungus
Chinese milk vetch extract
Chinese quince extract
Chlorella extract
Cinchona extract
Cinnamon bark extract
Citrus unshiu peel extract
Clove extract
Cnidium rhizome extract
Coix extract
Coltsfoot extract
Comb extract
Comfrey extract
Corn extract
Cornflower extract
Crataegus extract
Crataegus fruit extract
Cucumber extract
Hestnut rose extract
Hinoki powder
Hoelen extract
Honeysuckle extract
Hop extract
Hop powder
Horse chestnut extract
Horsetail extract
Houttuynia extract
Houttuynia herb powder
Hydrangea extract
Hydrolyzed milk protein
Hydrolyzed prune
Hypericum extract
Isodonis extract
Ivy extract
Japanese angelica root extract
Japanese coptis extract
Japanese cypress water
Japanese knotweed radix extract
Mucuna birdwoodiana extract
Mugwort extract
Mukurossi peel extract
Mulberry bark extract
Mulberry leaf extract
Murraya koenigii extract
Nettle extract
Nuphar extract
Okura extract
Olive oil
Oolong tea extract
Ophiopogon tuber extract
Orris extract
Oyster extract
Paeonia extract
Palm fatty acid
Palm oil
Papain
Papaya powder
Parsley extract
Pea extract
Rice germ oil
Rice starch
Romanchamomile extract
Rose extract
Rose fruit extract
Rose hips oil
Rosemary extract
Royal jelly
Rye flour powder
Safflower extract
Saffron extract
Sage extract
Sambac flos extract
Sambucus extract
Sandalwood extrtact
Saponaria extract
Sasa albo-marginata extract
Sasanqua oil
Saxifrage extract
Scutellaria root extract
Uva ursi fluid extract
Walnut shell extract
Watercress extract
Water-soluble collagen
Wax gourd seed extract
Wheat flour
Wheat germ extract
White nettle extract
Wild rose extract
Wild thyme extract
Witch hazel extract
Xanthan gum
Yarrow extract
Yeast extract
Yuzu extract
Zanthoxylum fruit extract

have been utilized so far in cosmetics. But the virulent plants like aconiti tuber, ephedra herba, atropa bella-donna, and the digitalis, or plants containing the medicinal components, are not included in this list. It was felt that the plants that keep a distance from medicine should be chosen. In other words, things for which safety is not a problem and which are appropriate as cosmetics ingredients are chosen. Stating that the plant material image is good does not imply that it is possible to use whatever we want after the flexible regulation of 2001, because companies must guarantee safety, stability, and quality, which are ascertained more strictly and more responsibly.

3.11.4 Skin-Whitening Cosmetics

Skin-whitening cosmetics is one category of advanced cosmetics and it decreases pigmentation (generally known as blotch, freckle) of the skin caused by the solar ultraviolet (UV, wavelength in the range of 400 to 10 nm) rays. People, especially women, have always longed for skin that takes on a transparent impression—being brightly white without blotch, dark brown spots, and being somber. Especially it is said that in Asia, because the change of the color tone of the skin is considered a symptom of skin deterioration, people strongly tend to desire a uniform skin color tone more than any other race. As regards the market target, Japan being the center of research and development emphasizes the fact that it is Asia so far for skin-whitening cosmetics. But since a major European–American cosmetic company has stressed the development of such products, development and market competition has intensified. The largest primary determinant of human skin color is melanin pigment produced by the melanocyte, which exists in the epidermal basal layer. Inside the melanocyte, tyrosine, one of the amino acids, works as the substrate, producing the melanin pigment by the activation of the enzyme tyrosinase. Melanogenesis progresses through the pathway shown in **Figure 1**.[10]

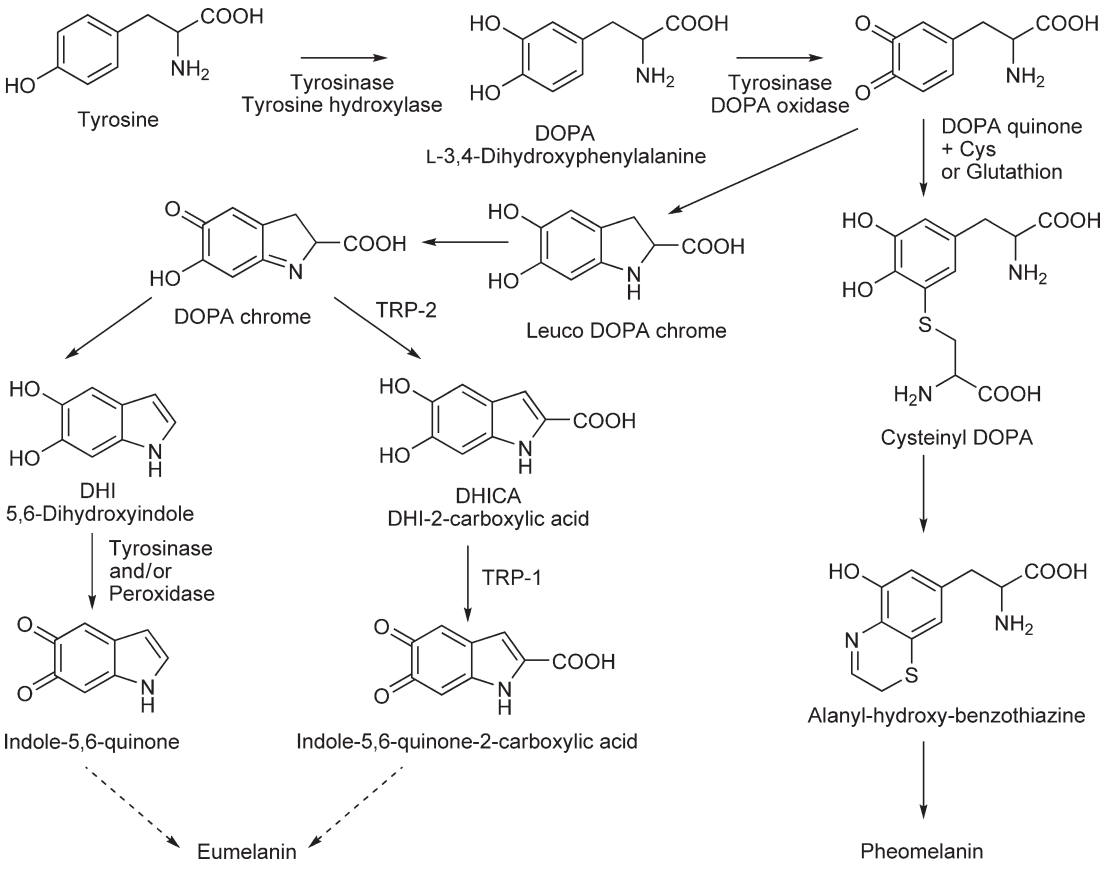


Figure 1 Melanogenesis pathway.

Figure 2 Bilberry.

Tyrosinase is involved in early stages of the pathway and is considered the target molecule in the development of skin-whitening cosmetics, which means that the key is how the product inhibits this enzymatic activity. Arbutin has been developed as a tyrosinase inhibitor and approved as an active ingredient of quasi-drugs. It is also a well-known naturally occurring compound contained in bilberry (**Figure 2**), pear, or the genus *Arctostaphylos*. Arbutin, β-D-glucopyranoside of hydroquinone (**Figure 3**), is effective in the topical treatment of various cutaneous hyperpigmentations characterized by hyperactive melanocyte function. As shown in **Figure 4**, it causes a concentration-dependent reduction in cellular tyrosinase activity of cultured human melanocytes at final concentrations between 1×10^{-5} and 1×10^{-3} mol l^{-1}. Its potency is about one hundredth of that of hydroquinone,

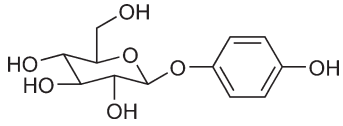


Figure 3 Chemical structure of arbutin.

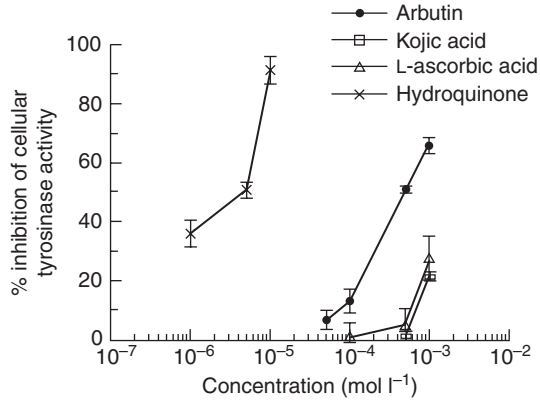


Figure 4 Inhibitory effects of arbutin (•) on tyrosinase activity in human melanocytes. Cultures of 10 000 cells cm^{-2} were incubated with these agents for 3 days. Tyrosinase activity was measured using L-DOPA (1×10^{-3} mol l^{-1}) as the substrate. The results are expressed as percentage of inhibition with respect to the untreated control.

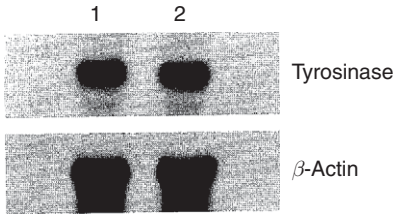


Figure 5 Effect of arbutin on tyrosinase mRNA level. Melanocytes were cultured for 2 days in the medium with (lane 2) or without (lane 1) 1×10^{-3} mol l^{-1} arbutin.

therapeutic drug for vitiligines, but is higher than that of kojic acid or ascorbic acid, which are also known as skin-whitening agents. The regulation of tyrosinase gene expression was studied to facilitate our understanding of the effect of arbutin on the synthesis and expression of tyrosinase. There was significant difference in the expression level of tyrosinase mRNA caused by the presence of 1×10^{-3} mol l^{-1} arbutin (**Figure 5**). Melanin production was significantly inhibited by arbutin, as determined by measuring eumelanin radicals with an electron spin resonance spectrometer. The study of the kinetics and mechanism of inhibition of tyrosinase confirms the reversibility of arbutin as a competitive inhibitor of this enzyme. The use of −L-tyrosine orL-dihydroxyphenylalanine (L-DOPA) as a substrate suggests a mechanism involving competition with arbutin for the L-tyrosine binding site at the active site of tyrosinase. These results suggest that the depigmenting mechanism of arbutin in humans involves inhibition of melanosomal tyrosinase activity, rather than the suppression of the expression and synthesis of tyrosinase.[11] Ellagic acid (**Figure 6**), developed as an active ingredient of quasi-drugs, is also one of the well-known inhibitors of tyrosinase. It was confirmed that ellagic acid inhibits tyrosinase dose-dependently and noncompetitively, unlike arbutin. As shown in **Figure 7**, tyrosinase activity was reduced with decreasing copper concentration, when

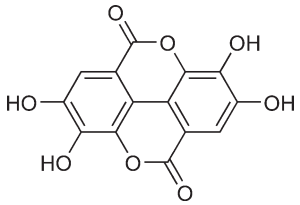


Figure 6 Chemical structure of ellagic acid.

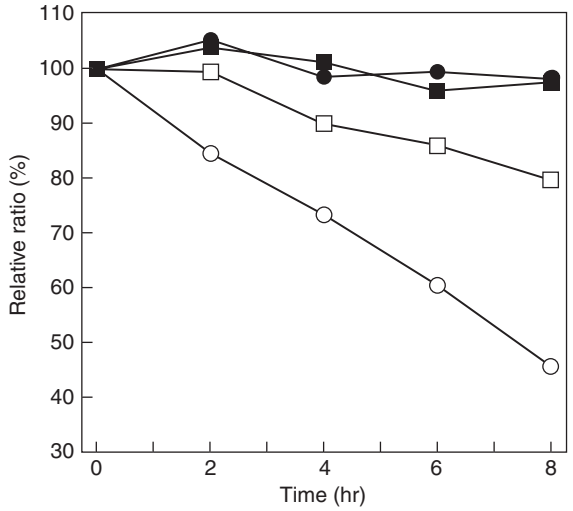


Figure 7 Correlation between mushroom-derived tyrosinase activity and copper content during incubation with ellagic acid. Enzyme activity (circles) and copper content (squares) of tyrosinase incubated in the presence of (open) or absence of (closed) 250 μmol ellagic acid are shown. Data are expressed as a percentage of control.

Figure 8 Chinese tamarisk.

mushroom-derived tyrosinase, a metalloprotein containing copper, was incubated with ellagic acid.[12] Since ellagic acid is known to chelate some specific metal ions, it is presumed to react specifically with the copper located at the active site of the tyrosinase molecule. Ellagic acid is a naturally occurring polyphenol, which is found widely distributed in plants such as tara, green tea, eucalyptus, and geranium. These two active ingredients are examples of natural-origin compounds being used as skin-whitening agents.

The extract from Chinese tamarisk, a deciduous tree (*Tamarix chinensis* Lour, **Figure 8**), showed tyrosinase inhibitory effect at the final dry residual concentration of between 0.001% and 0.003%, using B16 melanoma cell (**Figure 9**). Melanin content was also inhibited at the same concentration without the occurrence of cell cytotoxicity. Fifty percent ethanol extract of Chinese tamarisk was approved as a cosmetic additive agent of

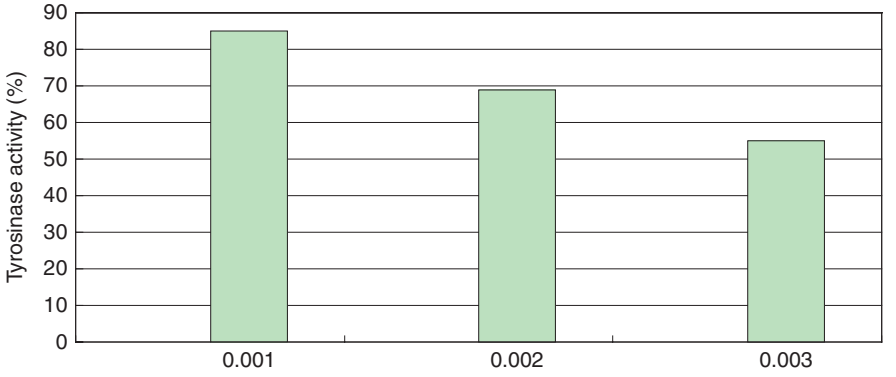


Figure 9 Tyrosinase inhibitory effect of Chinese tamarisk extract on melanogenesis of B16 melanoma. B16 melanoma cells were cultured for 3 days in the medium with 0.001–0.003% extract.

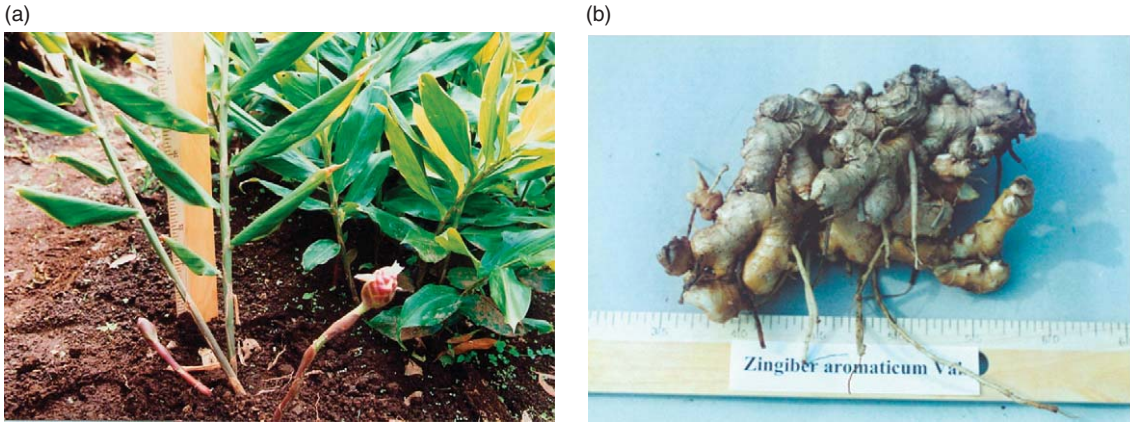


Figure 10 *Zingiber aromaticum* Valeton: (a) aerial part, (b) rhizome.

quasi-drugs in Japanese Pharmaceutical Affairs Law. Chinese tamarisk blossoms with racemiferous light pink flowers twice a year, in late spring and late summer, which attracts people's attention. Originally it had been used as a medicine effective for diuresis, detoxification, and colds in China. It came over to Japan in the eighteenth century as a medicinal plant effective in the treatment of measles. The extract of Chinese tamarisk is considered to be suitable as a cosmetics ingredient, having both adequate skin-whitening effect and a beautiful-flower image to meet the requirement of cosmetics. Furthermore, our research evaluated a novel plant extract that exhibited a new action mechanism against melanogenesis, not the tyrosinase inhibitory effect.

The extract prepared from the rhizome of *Zingiber aromaticum* Valeton, Zingiberaceae (**Figure 10**), exhibited no direct tyrosinase inhibitory effect but caused a decrease in tyrosinase production, which is melanogenesis on account of inhibition of the expression of tyrosinase gene.[13] *Zingiber aromaticum* is found widespread from India to Southeast Asia, grows to around 1.5 m tall, and is called 'Imoniga ginger' in Japan or 'puynag' and 'lempuyang' in Indonesia. The rhizome part of this plant has been used as a vital ingredient in folk medicines. Imoniga ginger extract was added to the B16 melanoma cell, which was cultured for 3 days and then evaluated for melanin content. As shown in **Figure 11**, the addition of the extract decreased melanogenesis in a dose-dependent manner. Melanin content decreased to 34% in the presence of dry residue concentration 0.002%, revealing a significant depression of melanogenesis. The tyrosinase activity inside the cell also decreased at the same time in the B16 melanoma cell system. On the other hand, the direct enzymatic activity of tyrosinase was studied using mushroom tyrosinase and L-DOPA as the substrates. No significant differences were observed by the addition of the extract, which indicated that the action mechanism of depigmentation by this extract is not tyrosinase inhibition, but some other effect

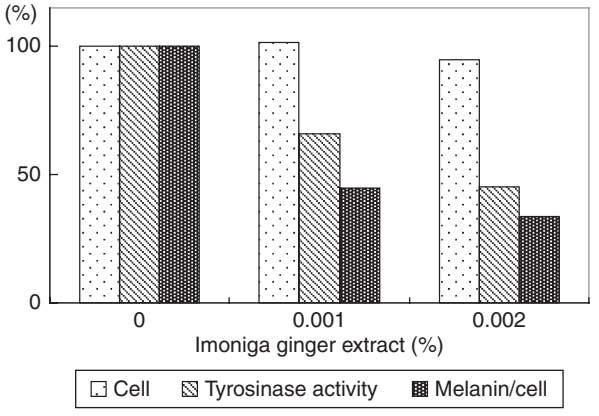


Figure 11 Effect of Imoniga ginger extract on melanogenesis of B16 melanoma. B16 melanoma cells were cultured for 3 days in the medium with (0.001%, 0.002%) or without Imoniga ginger extract.

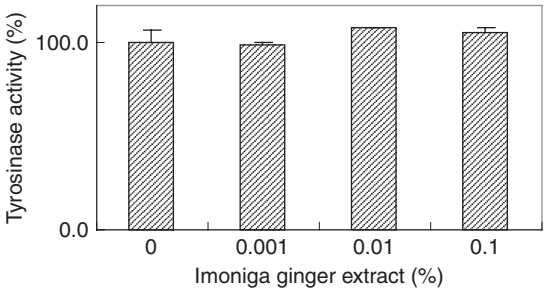


Figure 12 Effect of Imoniga ginger extract on mushroom tyrosinase.

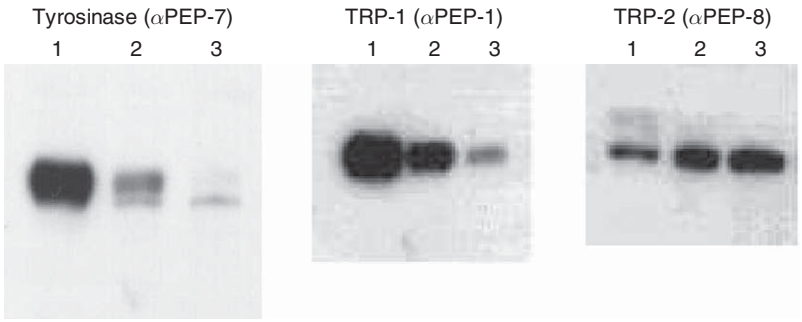


Figure 13 Effect of Imoniga ginger extract on tyrosinase, TRP-1, and TRP-2 protein levels. B16 melanoma cells were cultured for 3 days in the medium with (lane 2, 0.001%; lane 3, 0.003%) or without (lane 1) Imoniga ginger extract. A 20 μg portion of cell extracts per lane was used for SDS polyacrylamide gel electrophoresis.

(**Figure 12**). The regulation of tyrosinase-related protein (TRP)-1 and TRP-2 gene expression was studied to understand the action mechanism of Imoniga ginger on melanogenic inhibition. There was no significant difference in the expression of TRP-2 by the addition of the extract, but the expression of TRP-1 decreased in the presence of 0.001% and 0.003% extracts (**Figure 13**). Furthermore, the expression level of tyrosinase mRNA exhibited dose-dependent decrease in the presence of 0.001% and 0.005% Imoniga ginger extract, while no specific effect of the addition of the extract was observed on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. Promoter assays were performed by constructing luciferase reporter plasmid, which combined human tyrosinase promoter region to a vector (Pica Gene Basic Vector2 or PGv-B2). Tyrosinase promoter activity was significantly decreased by the addition of the extracts. As described above, Imoniga ginger extract had no direct inhibitory effect on tyrosinase activity, unlike common melanogenesis inhibitors based on tyrosinase inhibitory action, such as Chinese tamarisk. In addition, it was inferred that the extract had an inhibitory effect related to the expression or posttranslational modification of tyrosinase from the fact that tyrosinase activity inside the cell decreased. Furthermore, it was demonstrated to cause the amount of the tyrosinase protein to decrease, resulting in a decrease in tyrosinase mRNA expression and promoter activity. These results suggested that the action mechanism of this extract would be a transcriptional suppression of tyrosinase gene. The decrease in TRP-1 suggested that microphthalmia-associated transcription factor (MITF) regulating TRP-1 expression would be influenced by the extracts. On the other hand, TRP-2 was not affected by the extracts, which would be consistent with the reported theory that TRP-2, unlike TRP-1, is not involved in the control of the MITF expression.

3.11.5 Antiaging Cosmetics

Various senile changes appear on the skin with aging, and hence prevention of aging and improvement of deteriorated skin are major goals of skin care cosmetics. Skin aging is roughly classified into two categories, based on the factors that cause them: chronological aging, which is age-dependent, and photo aging owing to the solar UV ray.

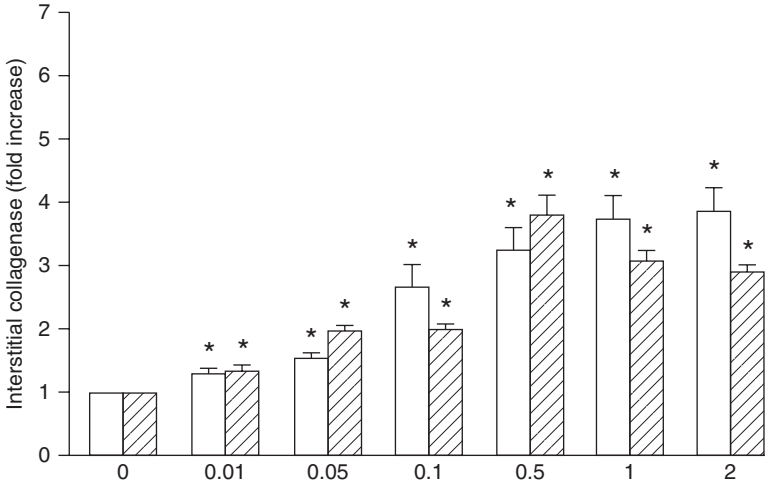


Figure 14 Low-dose UVB induces collagenase protein and activities in human skin *in vivo*. Interstitial collagenase protein (□), determined by Western blot, and activity (▨). Band intensities were quantified by laser densitometry. Results are means ± SEM, $n = 10$, $^{*}P < 0.025$ Versus no UVB control.

These two primary factors do not exist separately, but photo aging makes ends meet on chronological aging. Especially on sun-exposed regions the aging effects are intertwined in a complicated way, and wrinkles and sag keep developing. The wrinkles or the laxation of the face, which is a major sun-exposed part in human body, usually appears on such regions as around the eyes, mouth, forehead, and back of the neck, which are involved in facial expressions. Exposure to UV over the long term causes qualitative and quantitative structural damage of the skin in these regions. It is reported that even low-dose ultraviolet B (UVB, medium wave, which is wavelength in the range of 320 to 280 nm) induces interstitial collagenase, which is the enzyme that breaks the peptide bonds in collagen and may be involved in degrading various components of the skin (**Figure 14**).[14]

As shown in **Figure 15**, skin is organized in three layers: epidermis, dermis, and subcutaneous tissue.[1] The dermis contains a macromolecular network structure, called the extracellular matrix (ECM), which has an impact on structural formation of the skin. The basic components of ECM are glycosaminoglycans, or acidic

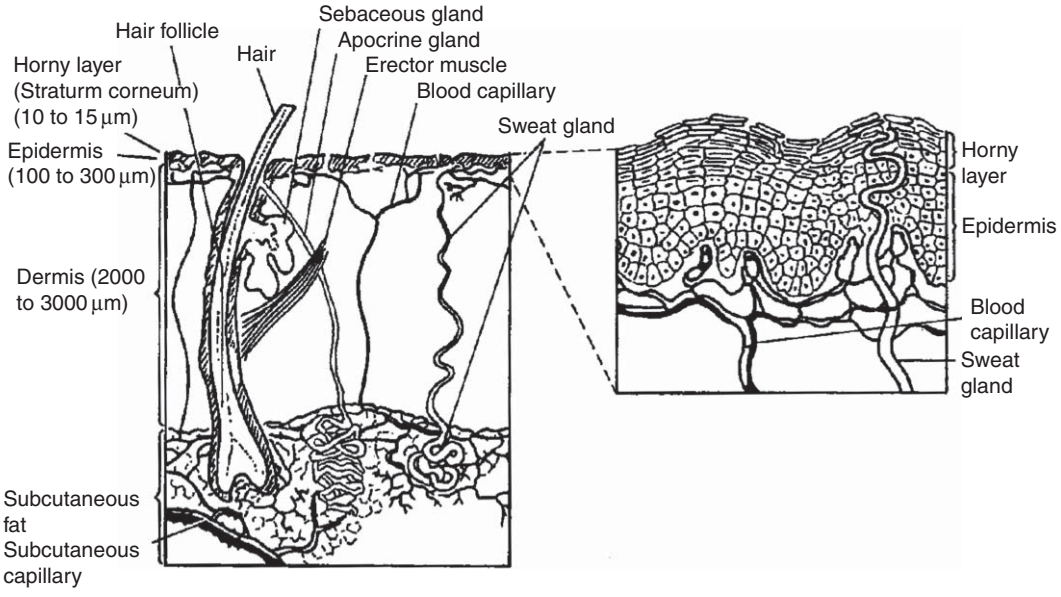


Figure 15 Schematic diagram of skin.

Table 3 Crude drugs used traditionally to keep the skin vitalized

Nomenclature	*Part*
Cistanche salsa	Fleshy caulome
Lycium chinense	Fruit
Asparagus cochinchinensis	Tuberous root
Polygonatum officinale	Rhizome
Atractylodes ovata	Rhizome
Benincasa hispida	Seed
Polygala tenuifolia	Root or root bark
Crocus sativus L.	Style
Nelumbo nucifera	Fruit
N. nucifera	Stamen
Vigna radiata	Seed

mucopolysaccharides, and fibrous proteins. Collagen, one of the fibrous proteins, is the principal component of ECM and plays a critical role in maintaining the form of the skin tissues. The production of type I collagen, which comprises around 80% of the total collagen, was examined for the plants that have traditionally been used to keep skin vitalized; the results are listed in **Table 3**. The enzyme-linked immunosorbent assay (ELISA) was carried out by applying plant extracts to the cell culture medium and measuring the quality of type I collagen, making use of the antibody specifically recognizing the collagen C-terminal peptide. As shown in **Figure 16**, 70% ethanol extract of Asparagi radix, tuberous root of *Asparagus cochinchinensis* Merrill Liliaceae (**Figure 17**), significantly promoted type I collagen production in a dose-dependent manner and consequently showed that the traditional use of Asparagi radix can be evaluated as collagen production *in vitro*. In general, skin collagen content has been observed to decrease with age (**Figure 18**).[15] Promoting collagen production may make a contribution to the alleviation of the negative effects of not only photo aging but also chronological aging.

Fibroblast cell, which synthesizes and secretes collagen and other ECM in dermis, plays an important role in the structural formation of connective tissue. Bupleuri radix, the root of *Bupleurum falcatum* L. Apiaceae (**Figure 19**), one of the widely used crude drugs in Traditional Chinese Medicine (TCM) or Kampo medicine, which is Japanese study and adaptation of TCM, revealed fibroblast proliferative activity and hyaluronan production. Furthermore, saikosaponin derivatives (**Figure 20**), oleanane saponins derived from *B. falcatum* L., were evaluated for fibroblast proliferative effect. As shown in **Figure 21**, saikosaponin b1 (SSb1) and saikosaponin b2 (SSb2) showed the effect in a dose-dependent manner. On the other hand, saikosaponin a (SSa),

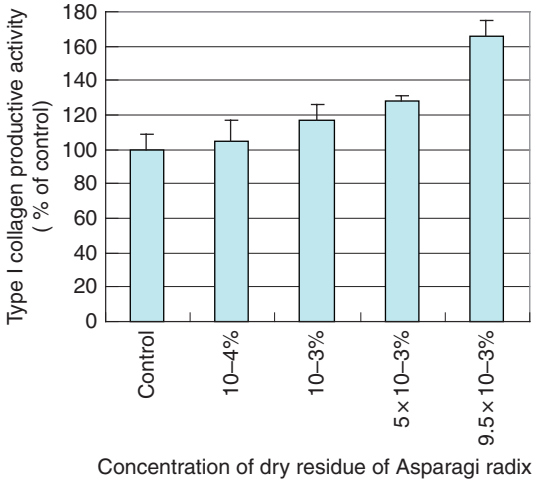


Figure 16 Type I collagen-producing activity of 70% ethanol extract of Asparagi radix.

Figure 17 *Asparagus cochinchinensis* Merrill, Liliaceae.

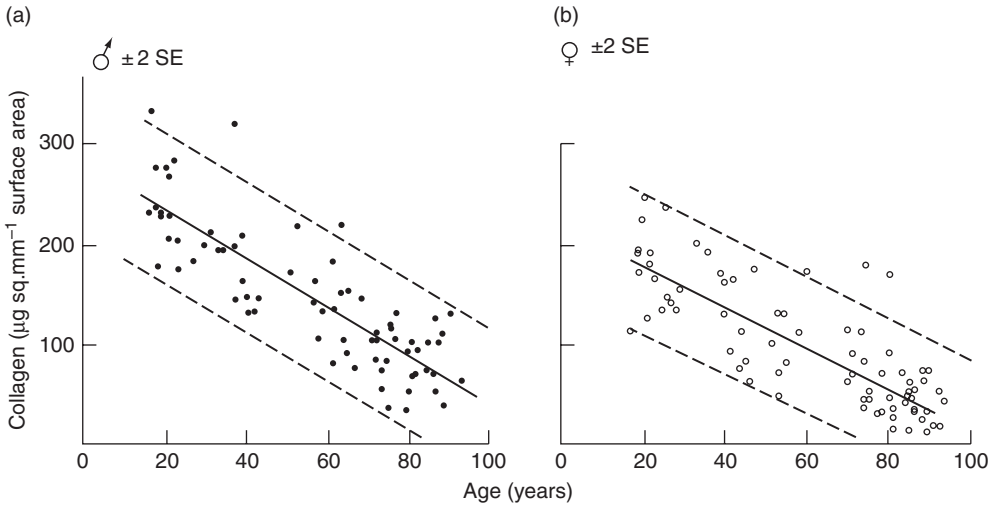


Figure 18 The relationship of skin collagen content to age in male (a) and female (b). Females have less collagen than males at all ages but the rate of decrease is the same in both sexes.

saikosaponin d (SSd), and saikosaponin c (SSc) were inactive. Adding epidermal growth factor (EGF) stimulated the fibroblast proliferative effect of SSb1 and SSb2. Consequently, fibroblast proliferative effect appears to be associated with the presence of a double bond at C-13 or hydroxymethylene group at C-17 within the chemical structure of those five saikosaponins, as SSb1 and SSb2 were metabolized by the cleavage of the 13-ether bond of SSa and SSd respectively.

Fibulins, a seven-member protein family, are secreted glycoproteins that are featured by repeated EGF-like domains and a unique C-terminal fibulin-type module. Fibulins are widely prevalent and are often involved with blood vessels and elastic tissues. Recently, it was shown that fibulin-5 decreased and disappeared with age and that it was significantly reduced in sun-exposed skin (**Figure 22**). On screening some medicinal plants, winged bean's extract (nomenclature: *Psophocarpus tetragonolobus* (L.) D.C.) was found to exhibit a promoting effect on fibulin-5 mRNA expression level and thus is expected to protect the skin from damage by UV light.

Figure 19 *Bupleurum falcatum* L. Apiaceae.

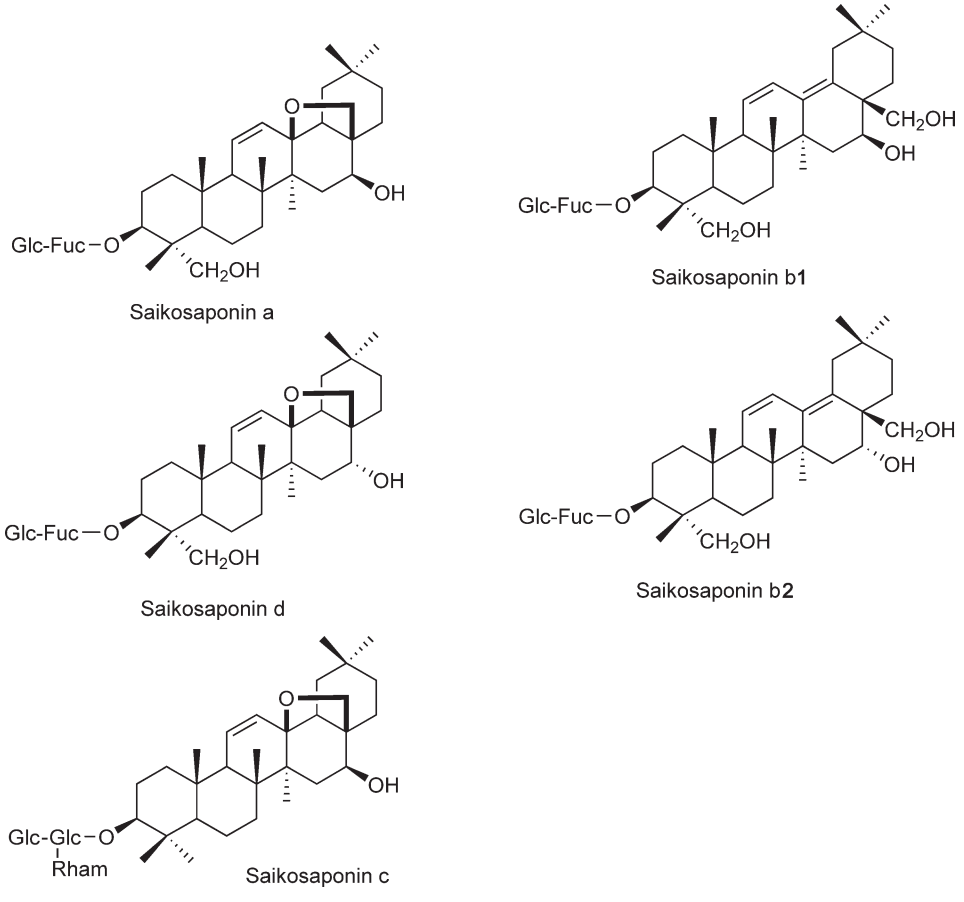


Figure 20 Saikosaponin derivatives.

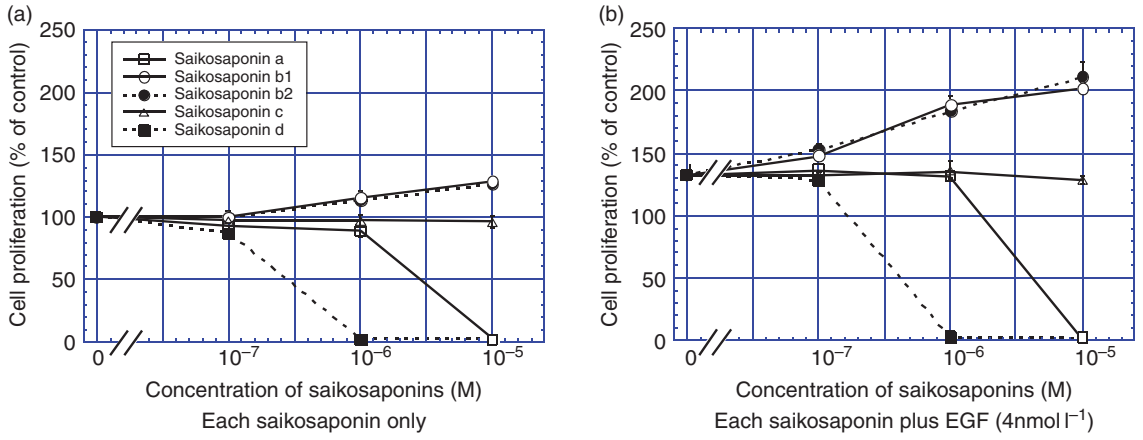


Figure 21 Fibroblast proliferative effect on saikosaponins alone and saikosaponins plus EGF. Saikosaponin b1 (•) and saikosaponin b2 (○) showed fibroblast proliferative effect.

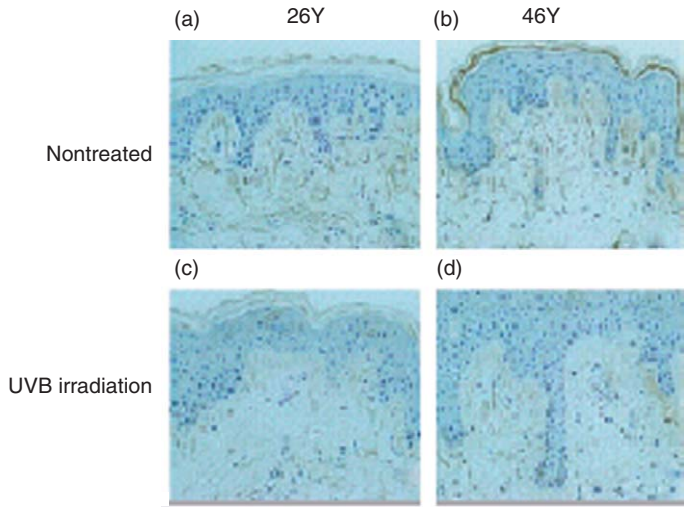


Figure 22 Reduction of fibulin-5 in the dermis after UVB irradiation. The effect of UVB irradiation on fibulin-5 distribution in buttock skin from two male volunteers was examined. Fibulin-5 was significantly reduced in dermis by UVB irradiation (c, d), compared to nontreated skin (a, b).

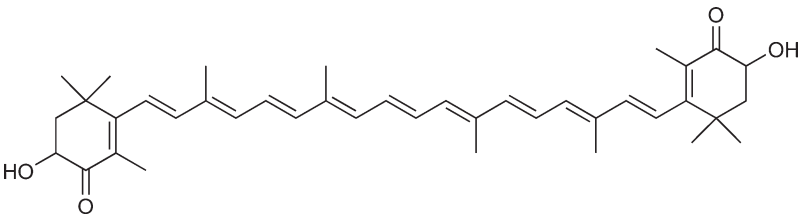


Figure 23 Chemical structure of astaxanthin.

Another unique natural product is astaxanthin (**Figure 23**), a carotenoid, which is becoming popular not only in health food products but also as a cosmetics ingredient, in recent years.[16] Astaxanthin is found in marine natural products, for example, some fish such as salmon and trout, or some shellfish like krill, shrimp, crayfish, and crustaceans or marine microalgae, and yeast. It has been demonstrated to have approximately a 1000-fold

stronger antioxidation effect than vitamin E on lipid peroxidation and 550 times stronger antioxidation effect than vitamin E against singlet oxygen oxidation. Based on such powerful antioxidation properties, astaxanthin is reported to play a key role in many pharmacological activities. Some clinical studies have been carried out by both internal and external application to evaluate the efficacy of astaxanthin. A cream containing 0.07% astaxanthin showed moisturizing effect and improvement of crow's feet by external application over a period of 3 weeks. Ingestion of 2 mg astaxanthin twice a day for 6 weeks resulted in significant improvement of skin in diagnosis based on inspection and manipulation by a dermatologist, measuring moisture content and elasticity. The improvement effect after 6 weeks is attributed to a collagen astaxanthin, which is regenerated in dermis, protecting oxidative cross-linking and preventing degradation of collage by way of causing to disappear the singlet oxygen excited by UV radiation.[17] This is an example of the fact that natural products derived from not only plant sources but also marine resources exhibit rich diversity.

3.11.6 Hair Growth Promoters

Much of the natural products are made use of in hair care products, especially as hair growth stimulant or for hair loss replacement. In the past, based only on traditional folk methods, the hair growth stimulant had been combined with other products, but today it is developed based on evidence of modern biological molecular approaches. Since the early times, some of the typical plants have been used as hair growth stimulants, for example, swertiae herba as a blood circulation promoter or hinokitiol as a bactericide. Male pattern alopecia can be caused by many factors, which include hereditary predisposition and rogenic hormone, and blood circulation disorders owing to stress, local scalp tonus, nutritional deficit, adverse drug effect, and aging. Hence the active constituent of any hair growth formula should have a combined effect on these factors. Plant extracts often used in hair growth formulas are categorized as follows: Blood circulation-promoting drugs and locally stimulating ones are combined to increase peripheral blood flow. As an example, active constituents of blood circulation promoters are Swertiae herba (*Swertia japonica* Makino, **Figure 24**) extract and cepharanthine. Swertiae herba extract comes from a Gentianaceae plant containing a bitter glycoside, which is effective for capillary dilation and in promoting blood circulation, supplying the energy to hair follicle cells. Sefarantine, an alkaloid, extracted from the root of *Stephania cephalantha* Hayata (**Figure 25**), is known to show vasodepressor effect. Capsicum tincture, ginger tincture, and cantharis tincture exhibit focused stimulation action. Capsicum

Figure 24 *Swertia japonica* Makino.

Figure 25 *Stephania cephalantha* Hayata.

tincture is an ethanol extract from the fruit of *Capsicum annuum* Linneus, whose pungent component, capsaicin, stimulates hair roots to grow. Zinger tincture obtained from the ethanol extraction of the rhizome of *Zingiber officinale* Roscoe contains zingerone and shogaol, which promote hair growth by stimulating hair roots. Glycyrrhizin and its derivatives as antiphlogistic agents, or hinokitiol, an antimicrobial constituent, are also used in hair care products. Glycyrrhizin is obtained from the root of *Glycyrrhiza uralensis* Fischer or *Glycyrrhiza glabra* Linne, both Leguminosae plants. Hinokitiol is an essential oil obtained by refining from *Chamaecyparis obtuse*. It is generally well known that ginseng extract obtained from the roots of *Panax ginseng* (Araliaceae), a herbaceous perennial, is a cellular stimulant and that Polygoni multiflori radix, the tuberous root of *Polygonum multiflorum* Thunb (**Figure 26**), has inhibitory effects on sebum-filled hair follicles. At the present stage of research, the mechanism of hair growth and the cause of epilation having been elucidated, hair growth formulas are not always being developed. Among the previously mentioned various factors determining hair growth, one of the scientifically proven factors is androgenic hormone. Testosterone, a type of androgen, is converted to dihydrotestosterone (DHT), which has a powerful androgen action owing to 5α-reductase, the enzyme that converts testosterone into DHT inside the papillary cell of the hair follicle. It is assumed that DHT binds with the androgen receptor inside the cell and it becomes the trigger of the incidence of male pattern alopecia.

Figure 26 *Polygonum multiflorum* Thunb.

Hence inhibiting the activity of 5α-reductase is one of the approaches for stimulating hair growth.[18] The enzyme 5α-reductase is composed of two isozymes: type I providing an optimum pH of 6–9 and type II providing an optimum pH of 5.5.[19] Type II 5α-reductase is found in the mustache or in the frontal hair papilla causing male pattern alopecia. On the one hand, since the androgen receptor does not figure in the occipital hair papillary cell, the occipital hair remains even for persons with male-type alopecia. Therefore, it is clear that the adjustment of the androgen sensitivity against steroid type II 5α-reductase has a significant role in the control of male pattern alopecia. Moreover, the fact that finasteride, marketed as Propecia, a specific inhibitor of 5α-reductase type II, is effective in the treatment of male pattern alopecia also bears testimony to this theory. Many natural products have been studied, leveraging this androgen action as target to evaluate their effectiveness against male pattern alopecia. Many crude drugs have been reported to have 5α-reductase inhibitory effect, and some of them are listed in **Table 4**, including not only plant material but also hoptoad secretion.[20] From the wide variety of herbal medicines listed, it can be inferred how much competitive research and development in this field has taken place. We discovered the steroid 5α-reductase inhibitory effect of cuachalalate, a Mexican herbal medicine, and found some active compounds. Cuachalalate (nomenclature: *Juliania adstringens* Schltdl., **Figure 27**), a tree around 6 m tall, grows only at altitudes of 200–300 m in Acapulco district in the southern Mexican Pacific Ocean bank. The bark has proliferated in the herbal medicinal market of the American Indian. It is traditionally known that the infusion or the powder of the bark accelerates wound healing when applied to a wound site and that it is effective in the treatment of digestive system cancer and fever. In addition, there is a myth concerning an improvement of alopecia condition, on which we focused our attention and evaluated the 5α-reductase inhibiting effect of cuachalalate extract. The inhibitory activity was measured with the cell culture system to detect the produced androstanedione by high-performance liquid chromatography (HPLC) after adding the tritium-labeled androstenedione into the culture medium. The Chang liver cell of normal human liver cell origin was used to detect type I 5α-reductase inhibitory activity and Hs68 fibroblast of human neonatal foreskin origin was used to detect type II 5α-reductase inhibitory activity. The substrate was mixed in the culture supernatant, and then the tritium-labeled product was isolated by reversed-phase HPLC. The conversion rate of the enzyme activity was calculated by measuring the radiation intensity. Specific steroid type II 5α-reductase inhibitory effect of cuachalalate was demonstrated, as shown in **Figure 28**. To search for 5α-reductase inhibitory compounds, the cuachalalate's dry bark was extracted with ethanol and then the obtained extract was fractionated with HP-20 column adsorbent (**Figure 29**). Next, the active fraction was isolated with silica gel column chromatography, and eluted with hexane–ethyl acetate solvent system. Triterpenes, including four novel ones, were isolated as active constituents (**Figure 30**). Some compounds specifically inhibited type II 5α-reductase activity as compared to type I 5α-reductase activity. Schinol (compd.1), 3α-hydroxy-masticadienolic acid, a Euphan structured triterpene, and a novel spiro-type compound (compd.9) showed high specificity for type II 5α-reductase activity. The resulting IC_{50} values of these two compounds were 100 mmol l^{-1} for type I 5α-reductase activity and 300 nmol l^{-1} for type II 5α-reductase activity, indicating more than 300 times higher specificity.[21,22]

The structure–activity correlation of schinol was examined further. Masticadienonic acid, which is a 3-keto variant of schinol, exhibited an inhibitory activity a few tenths of that of schinol, whereas the compound substituted with 3β-hydroxyl group possessed much the same inhibitory activity as schinol. When the 26-carboxyl group of schinol was converted to a methyl ester group or to a hydroxyl group, the inhibitory activities disappeared. When the 24-double bond was reduced, it was found that type II 5α-reductase inhibitory activity decreased by a 20th, but type I 5α-reductase inhibitory activity occurred almost at the same concentration as type II 5α-reductase inhibitory activity. Thus, the structure–activity relationship of type II 5α-reductase inhibitory activity of schinol was significantly determined by the 3-hydroxyl group, 26-carboxyl group, and 24-double bond – especially the double bond played a critical role in the specificity of type II 5α-reductase. The other two, namely, 26-carboxyl group and 24-double bond, would contribute toward making the terminal structure rigid, and could be thought of as modifying the structure to make it fit easily to the substrate-binding site of type II 5α-reductase molecules. It is unmistakable that androgen is significantly involved in male pattern alopecia, based on the fact that male pattern alopecia never occur in capons. Products that treat male pattern alopecia by reducing the effect of androgen have been tried for a long time. Finasteride received FDA's approval in 1997 for the treatment of male pattern alopecia as a type II 5α-reductase-specific inhibitor and has been marketed since. Its action is related to some male-specific conditions like the prostatic

Table 4 Crude drugs reported to have 5α-reductase inhibitory effect

General name	*Nomenclature*	*Family*
Polygonum tuber	*Polygoni multiflorum* Thub.	Polygonaceae
Panax rhizome	*Panax japonicus* C. A. Meyer	Araliaceae
Rugosa rose	*Rosa rugosa* Thunb.	Rosaceae
Myricae cortex	*Myrica rubra* Sieb. et Zucc.	Myricaceae
Bistort	*Polygonum bistorta* L	Polygonaceae
Lygodii spora	*Lygodium japonicum* Sw.	Lygodiaceae
Mutan cortex	*Paeonia moutan* Sims	Paeoniaceae
Suberect spatholobus	*Spatholobus suberectus* Dunn	Leguminosae
Scurfy pea	*Psoralea corylifolia* L.	Leguminosae
Corni fructus	*Cornus officinalis* Sieb. et Zucc.	Cornaceae
Schisandrae fructus	*Schisandra chiinensis* Baill	Schisandraceae
Black pepper	*Piper nigrum* L.	Piperaceae
Tailed pepper	*Piper cubeba* L.	Piperaceae
Gambir	*Uncaria gambir* Roxbourgh	Ruiaceae
Foeniculi fructus	*Foeniculum vulgare* Mill	Apiaceae
Polygalae radix	*Polygala tenuifolia* Willd.	Polygalaceae
Glycyrrhizaei radix	*Glycyrrhiza uralensis* Fisch Ex DC.	Leguminosae
Pharbitidis semen	*Pharbitis hederacea* Chois.	Convolvulaceae
Galla rhois	*Rhus japonica* L.	Anacardiaceae
Paeoniae radix	*Paeonia lactiflora* Pallas var. triocarpa Stern.	Paeoniaceae
Plantaginis semen	*Plantago asiatica* L.	Plantaginaceae
Bufonis venenum	*Bufo buo gargarizans* Cantor	Bufonidae
Rhei rhizoma	*Rheum palmatum* L.	Polygonaceae
Caryophylli flos	*Syzygium aromaticum* (L.) Merr. et Perry	Myrtaceae
Arecae semen	*Areca catechu* L.	Arecaceae
Resina pini	*Pinus massoniana* Lamb	Pinaceae
Ageratum	*Ageratum conyzoides* L.	Asteraceae
Geranii herba	*Geranium thunbergii* Sieb. et Zucc.	Geraniaceae
Prunellae spica	*Prunella vulgaris* L. subsp. Asiatica Hara	Lamiaceae
Bupleuri radix	*Bupleurum falcatum* L.	Apiaceae
Rosae fructus	*Rosa multiflora* Thunb.	Rosaceae
Coicis semen	*Coix lachryma-jobi* L. var. ma-yuen (Roman) Stapf	Poaceae
Perillae herba	*Perilla frutescens* (L.) Britton var. acuta Kudo	Lamiaceae
Picrasmae lignum	*Picrasma quassioides* Benn.	Simarubaceae
Schizonepetae herba	*Ocimum basilicum* L.	Lamiaceae
Catalpae fructus	*Catalpa ovata* G. Don	Bignoniaceae
Dichroae radix	*Dichroa febrifuga* Lour.	Saxifragaceae
Valerianae radix	*Valeriana fauriei* Briquet	Valerianaceae
Common mallow	*Malva sylvestris* L.	Malvaceae
Leonuri herba	*Leonurus japonicus* Houttuyn	Lamiaceae
Arctii fructus	*Arctium lappa* L.	Asteraceae
Pot marigold	*Calendula officinalis* L.	Asteraceae
Greater celandine	*Chelidonium majus* L. var. *asiaticum* Hara	Papaveraceae
Japanese honey locust	*Gleditsia japonica* Miq.	Leguminosae
Gardeniae fructus	*Gardenia jasminoides* Ellis	Rubiaceae
Fragrant orange-colored olive	*Osmanthus fragrans* var. *aurantiacus*	Oleaceae
Guarana	*Paullinia cupana* H. B. K.	Sapindaceae

enlargement in addition to male pattern alopecia, and finasteride was originally the remedy for prostatic enlargement. But it has been approved as the internal use remedy for male-type alopecia, based on the fact that alleviation of that condition had been observed as a side effect, at low dosage. Systemic action might be brought on through oral administration, but the side effect on male function must be paid attention to. Working at only the hair follicle, the ideal hair growth promoter is something the effect of which only prevents dehairing and does not have any side effect. Blood circulation accelerators such as vitamin E derivatives and nicotinic acid benzyl are combined in usual hair growth formula products. It is important to prescribe a component that brings

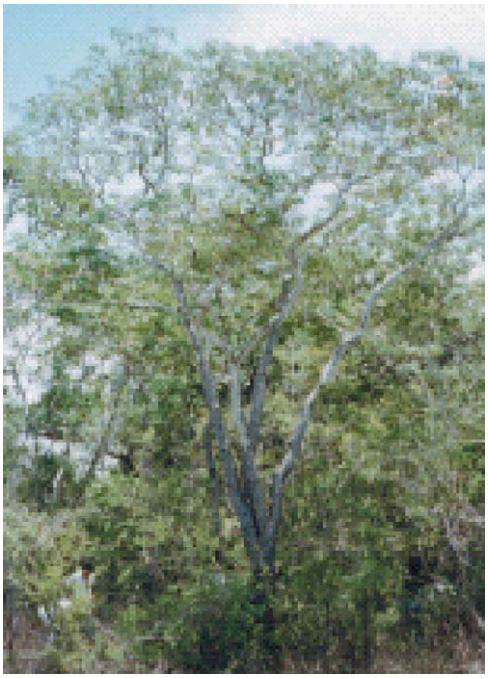


Figure 27 *Juliania adstringens* Schltdl.

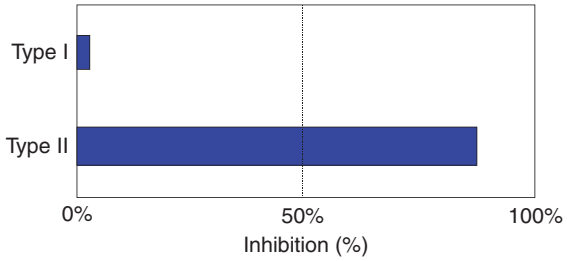


Figure 28 5α-Reductase inhibitory effect of cuachalalate ethanol extract (dry residue concentration 0.01%).

about a balance of the androgen besides the other ingredients of the hair growth formula product. For these reasons, cuachalalate extract that contains a 5α-reductase inhibitory component such as schinol or other triterpenes, which possesses a few tenths of type II 5α-reductase inhibitory activity compared to that of finasteride and almost do not inhibit type I 5α-reductase, would be a reasonable material to be prescribed in hair growth formula products classified as quasi-drug in Pharmaceutical Affairs Law.

3.11.7 Plant Cell/Tissue Culture Technology for Natural Products in Cosmetics

3.11.7.1 Potential of Plant Cell/Tissue Culture for Cosmetic Application

Most of the plant components used as cosmetics ingredients are of natural origin. However, many herbal medicinal companies are concerned about the stable supply of the natural herbs in the future for many reasons, such as the changing climate, the urbanization of the herbal growing districts, political instability, especially in

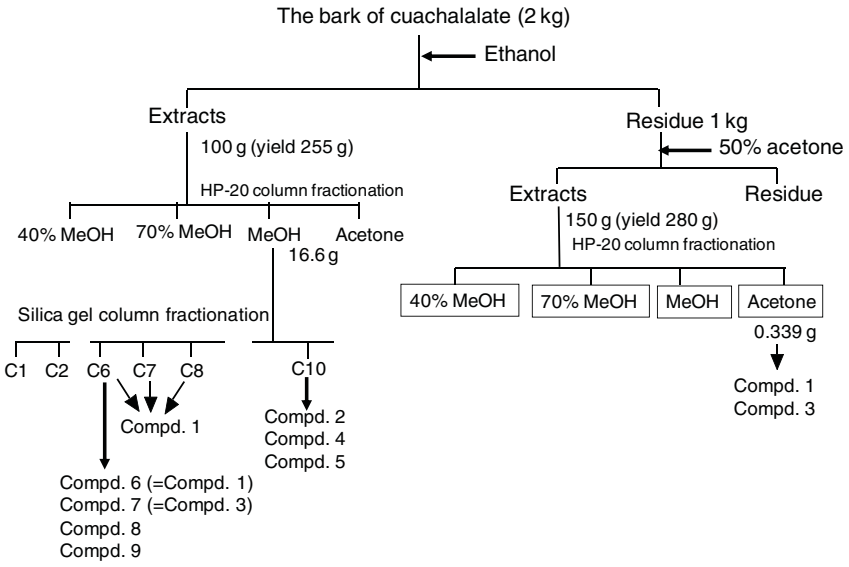


Figure 29 Fractionation process of cuachalalate extract.

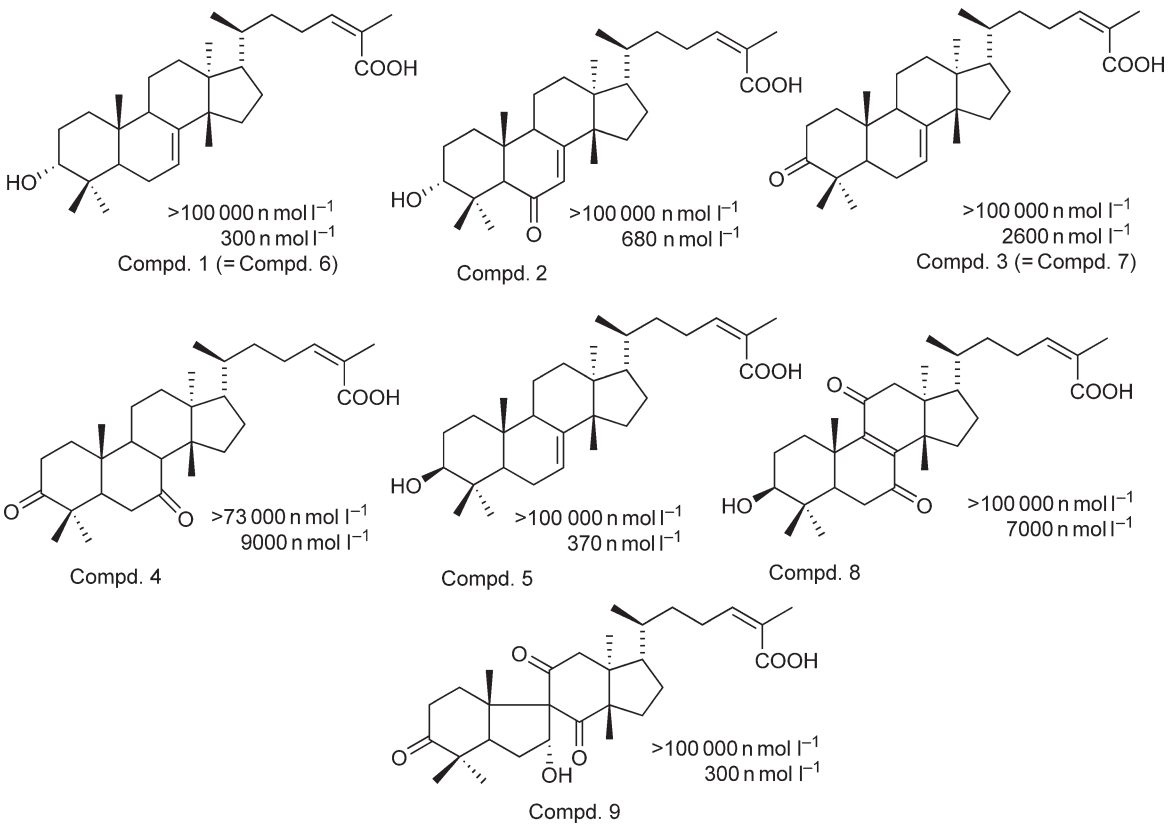


Figure 30 Isolated compounds from cuachalalate extract (upper: IC_{50} of type I 5α-reductase, lower: IC_{50} of type II 5α-reductase).

the middle of the Eurasian Continent, and ratification of the CBD treaty. Hence, the technology of the phytogenic component production will eventually become important for cosmetics.

In 1983, plant cell culture was successfully established by Mitsui Chemicals Inc. in Japan for the production of shikonin, which had been known to have fungicidal and wound healing action in East Asia.[22] Although that represented an outstanding and epochal work in the industrialization of biotechnology, unfortunately the plant cell culture technique has not become the tide in the manufacture of natural products till the present day. In most cases, plant cell culture is incapable of producing the secondary plant metabolites, to which most of the useful components belong. Micropropagation and root culture techniques have been used for real commercial products. In addition, biotransformation technique may offer a promising prospect for production in large amounts.

3.11.7.2 Micropropagation

Micropropagation technique is essentially established nowadays and could overcome the genetic segregation of the plants germinating from seeds; field-selected elite strains could be efficiently propagated with micropropagation techniques. Micropropagation techniques are of three types based on the way of propogation: first, the propagation from shoots with cytokinin like benzyladenine or kinetin; second, multiple shoot differentiation from dedifferentiating tissue, callus, with an auxin like indole acetic acid; and finally, the embryo differentiation from callus. The former two methods need the rooting process with an auxin like indole acetic acid and with naphthaleneacetic acid thereafter. Nowadays, the method of propagation from shoots is the most preferred one, because the latter two methods present the possibility of genetic variation owing to the dedifferentiated phase, callus.

In the 1980s, micropropagation technique was first tried to adopt a tank culture in conformation with fermentation technology, for example, for a perfume plant *Pelargonium graveolens* at Kanebo Cosmetics in Japan. However, liquid culture never gained popularity because manufacturing cost was not expected to be lowered to guarantee successful commercialization; it is unlikely to increase the density of cultured shoots in a tank. Nowadays, micropropagation is used as a process for a rapid *in vitro* multiplication of shoots selected as the elite strains from the field, before getting them back to grow in the field.

Although rose is one of the most important aromatic plants in the commercial market, only a few species are scented among the 200 species of rose.[23] *Rosa damascena* Mill is preferred for high-quality rose oil used in perfumery, cosmetics, and food markets. It is crucially important for a company to retain the monopoly of the elite strain when it generates the elite strain by breeding. Kaur *et al.* reported the molecular evaluation of *R. damascena* by means of random amplified polymorphic DNA (RAPD) analysis.[24] Among the 58 primers they used, one decamer primer OPV-4 could distinguish six oil-rich varieties of *R. damascena*. The elite strain of *R. damascena* could be efficiently proliferated by micropropagation technique.[25]

Demand for *Aloe vera* has been growing in both health care and cosmetics markets in the world. The speed of field propagation of *A. vera* by means of axillary shoots is rather low, in addition to the characteristic male sterility being a barrier in seed propagation.[26] Therefore, several groups have reported the micropropagation of *A. vera*.[26–28] The regenerated plants are morphologically similar to the mother plants. *Lavandula viridis*, whose essential oils are important for the cosmetic market, was investigated as to the variation in quality of the essential oil between three types of the plants from the same clone: field-grown plants, *in vitro* shoot cultures, and micropropagated plants.[29] It was demonstrated that the same major components were found without significant compositional variation. Like this, micropropagation could be the dependable technique to multiply an elite strain.

The Maruzen pharmaceutical company in Japan has undertaken a grand project using the micropropagation technique for *G. glabra*. *Glycyrrhiza glabra* is one of the most important plants in cosmetics, food, and pharmaceutical markets. The major component glycyrrhizin is massively used as a sweetener in food industry and also frequently compounded in cosmetics and pharmaceuticals for the reason that it has strong bioactivities such as anti-inflammation and a chemopreventive activity on cutaneous oxidative stress.[30] *Glycyrrhiza glabra* grows naturally in the middle region of the Eurasian continent of the Middle East, China, and Mongolia. However, the future supply is uncertain due to various circumstances, such as political instability in the Middle East, fear of *G. glabra* depletion with overharvesting, and climate change. Maruzen Company planned to cultivate *G. glabra* in NSW, Australia, whose latitude is almost the same as one of the

Table 5 Micropropagation

Plants	*Reference(s)*
Aloe vera	27, 28
Aloe barbadensis	30
Pelargonium graveolens	32
Lavandula viridis	33
Rose damascena	24
Glycyrrhiza glabra	34
Stevia rebaudiana	35, 36
Lawsonia inermis	37
Cunila galioides	38
Artemisia judaica	39

regions in the Eurasian continent where *G. glabra* grows naturally, although it is in the Southern Hemisphere. They screened 20 elite clones including glycyrrhizin, which is more than 5% of the many plants introduced from Turkey and Russia.[31] They multiplied the elite clones by micopropagation technique and sent 200 000 aseptic juvenile plants to the cultivating place in Australia. The *G. glabra* plants were further propagated there and are being cultivated, after acclimatization, in the oceanic space of 741 acres (**Figure 31**). Nowadays, most of the medicinal plants used for cosmetics are of wild origin. In the future, however, cultivated plants will gradually replace natural plants. This heralds a new era in the way natural components are produced.

Some examples of micropropagation of plants that could be used as cosmetics ingredients are shown in **Table 5**.

3.11.7.3 Root Culture

Root culture has been investigated with two different types: the hairy root that is induced with Ri plasmid of *Agrobacterium rhizogens* and the adventitious root that is not transformed. Hairy roots generally grow faster because they differentiate new roots in succession and, thus, possibly have an advantage for industrialization over adventitious roots. However, it might take longer to achieve public acceptance on using the genetically transformed tissue in industry.

Inomata and Yokoyama[40] succeeded in obtaining the transformed roots from *P. ginseng*, which is an important plant used as a cosmetics ingredient. They induced more than 100 transformed roots and selected 34 clones to be subcultured in liquid Linsmaier and Skoog media.[42] R52 clone was finally selected because of a unique quality: superior growth rate with high ginsenoside productivity, and stability of these features during subculture. The strain R52 produced ginsenosides as much as 5-year-old native roots would ($17\,mg\,g^{-1}$ dry wt.), the level of which remained almost constant throughout the cultures. The advantage of R52 over native roots is higher ginsenoside productivity ($12\,mg\,l^{-1}\,day^{-1}$). Kim *et al.*[42] examined the optimal condition for ginsenosides production using adventitious root culture to achieve a productivity of $2.6\,mg\,g^{-1}$ dry wt. In Korea, root culture of *P. ginseng* was first successfully achieved for commercial production at Microplants Co., Ltd., and five other companies have contended for this art so far. Most products (several thousands) developed are in health care foods. However, dozens of cosmetics, including cultured ginseng extract, are also produced, especially at IHKCOS Co., Ltd.

The most successful research using adventitious roots for cosmetics ingredients is saikosaponin production by root culture of *B. falcatum* L. The root of *B. falcatum* L, known as Bupleuri radix, is a galenical formulated in a variety of TCMs. Among the more than 10 different saikosaponins,[43] SSa and SSd are especially known as pharmacologically active components, possessing properties such as antiallergic activity, analgesic action, and anti-inflammatory action.[44–46] SSb1 and SSb2, which are produced artificially from SSa and SSd, have been recognized as unique biologically active substances for skin cells, as described in Section 3.11.5.[47–49] Kusakari *et al.*[50] overcame the defect of slower growth of adventitious roots by regulating lateral root differentiation. They found that the formation of the lateral roots, which was induced in the presence of auxin (indolebutyric acid), was strongly

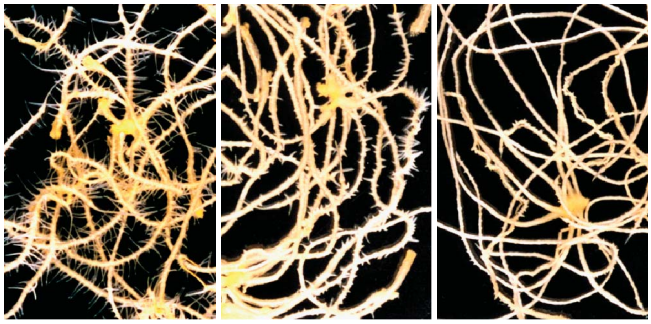


Figure 32 Effect of sucrose on the lateral root formation in *Bupleurum falcatum* root culture. The roots were cultured for 14 days in B5 medium containing sucrose at various designated densities.

suppressed as the sugar concentration was increased (**Figure 32**). This effect may be involved in the scavenging effect of sugar on hydroxyl radical[51] because lateral root initiation in *B. falcatum* root culture was promoted by stresses such as drought or heat, and active oxygen species that the addition of hydrogen peroxide or methylviologen contributed.[52] As saikosaponin was accumulated only on lateral roots and original roots were losing their function to root tissues during the culture in that culture system, the prompt increase of lateral root formation was crucially important. However, as sucrose is also an energy source, a two-step culture was adopted, with the addition of 1% sucrose at the beginning of the culture and 6% sucrose thereafter at 14 days when lateral roots had emerged. This means adding sugar greatly improved the productivity, affording 0.8 $g\,l^{-1}$ of SSa and SSd. In addition, they developed a new type of tank for commercial production (**Figure 33**) and have been producing the extract containing SSb1 and SSb2, which were produced by converting SSa and SSd in the extract by regulating pH. Saikosaponin-containing extract was compounded in a new brand of cosmetics called 'Bioperformance' by Shiseido Co., Ltd., successfully marketed in Europe. Saikosaponin-containing extract has been used in cosmetics for more than 10 years; this extract was the first repeat product manufactured by biotechnological means.

3.11.7.4 Biotransformation Techniques with Plant Cell Culture

Biotransformation refers to the technique of converting various substrates to more useful products using freely suspended, immobilized plant cells.[53–55] Biotransformations by plant cell cultures include a wide range of reactions, such as glucosylation, glucosyl esterification, hydroxylation, oxidoreductions between alcohols and ketones, reduction of carbon double bonds, hydrolysis, isomerization, epoxidation, dehydrogenation, methylation, demethylation, and others.[54] From the point of view of industrialization, however, glucosylation and hydroxylation seem feasible because only those reactions have brought about a yield of more than 1 $g\,l^{-1}$ (**Table 6**). The reason why these two types of reaction produce much higher yield of products than others is the fact that biotransformation is involved in detoxification of xenobiotics; introduced xenobiotics must all be detoxified as glucosides and hydroxides for the plant (cells) to survive.

The glucoside of a phenol that has been most successfully used as a cosmetics ingredient is arbutin. Arbutin, the glucoside of hydroquinone, has been found to be effective for depigmentation without adverse effects and developed as a whitening agent at Shiseido Co., Ltd. Hydroquinone itself acts as a decolorant and has been used as a depigmenting cosmetic in some countries, but it does not seem to be popular nowadays owing to a strong adverse action. Yokoyama and Inomata[62] investigated extensively and developed the technique for the manufacture of arbutin by biotransformation using *Catharanthus roseus* cells. Their work was an epochal trial in that the feasibility of biotransformation was investigated at the earliest time. Hydroquinone generates superoxide anion in a neutral aqueous or more readily in a weak basic solution that is in similar condition as that of cytoplasm.[63] The superoxide is meant to be reduced to hydrogen peroxide and then the two active oxygen species react to generate the most deleterious active oxygen, the hydroxyl radical. Putative scheme for the evolution of the three active oxygen species from hydroquinone is illustrated in **Figure 34**. Other phenolic

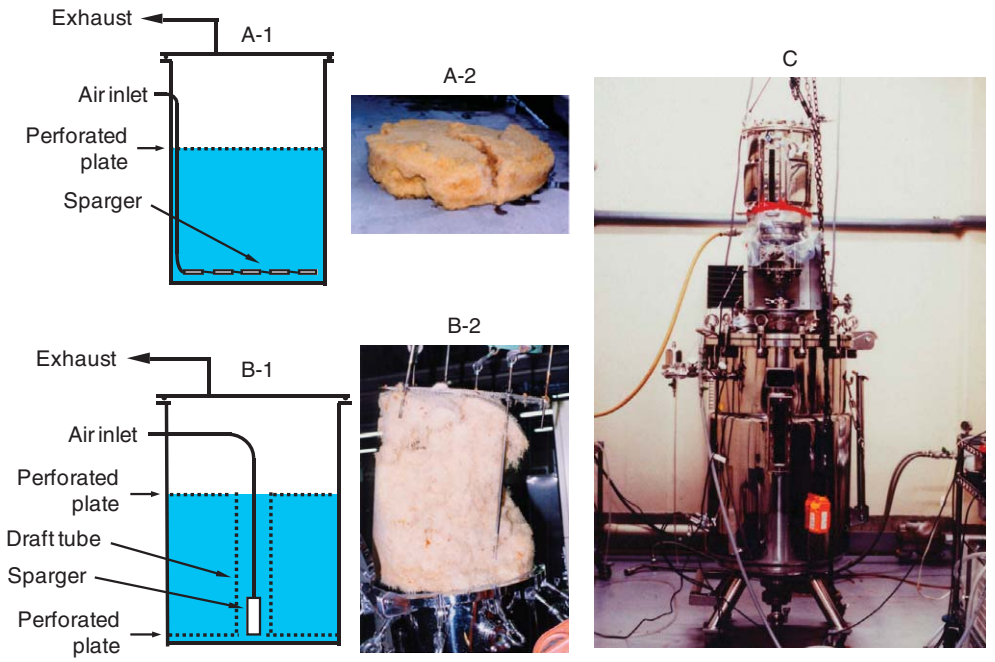


Figure 33 Culture of *Bupleurum falcatum* roots with a simple air lift type (A-1) or a draft type (B-1) tank. The photos at the center show the harvesting of the cultured roots of *B. falcatum* with the simple air lift type tank (A-2) or the draft one. The right-hand side view (C) shows 200 l scale tank with 20 l seed culture tank attached.

Table 6 Examples of high yield with bitotrasformation techniques

Product	*Substrate*	*Type of reaction*	*Plant species*	*Yield (g l^{-1})*	*Reference*
Arbutin	Hydroquinone	Glucosylation	*Rauwolfia serpentina*	18	56
			Catharanthus roseus	9.2	57
p-Hydroxyphenyl-*O*-primeveroside	Hydroquinone	Glucosylation	*R. serpentina*	5.8	58
Serotonin	Tryptamine	Hydroxylation	*Peganum harmala*	2.5	59
Skimmin	Umbelliferone	Glucosylation	*Datura innoxia*	1.6	60
Salicylic acid-*O*-glucoside	Salicylic acid	Glucosylation	*Mallotus japonicus*	1.1	61

substances also are believed to have similar pathways for generating active oxygen species. Such toxic signals seem necessary to induce hydroquinone glucosyltransferase, which converts hydroquinone to arbutin. The rate of hydroquinone consumption in the early stages (by day 1) increases in proportion as the initial concentration of hydroquinone goes up to 12 mmol l^{-1}.[64] By nature, however, too great an amount of hydroquinone damages the cells and causes their death. To evade the excessive toxicity that deteriorates cells, two methods have been researched: one is to search for the substances that suppress the cell-deteriorating oxidation and the other is to control the concentration of hydroquinone in the medium. As regards substances, antioxidants such as ascorbic acid, gallic acid, cysteine, and tea tannin at 200 mg ml^{-1} were effective as we had anticipated.[65] In terms of practical use, however, it was astounding to find that sucrose or glucose remarkably improved the damaged cells to enhance arbutin production by as much as two- to threefold.[63] The exogenously added sugar was not metabolized and remained unchanged, contrary to common sense. This is explained by the fact that the system of metabolism of cells was all set for the glucosylation of hydroquinone. It is also very important to control the

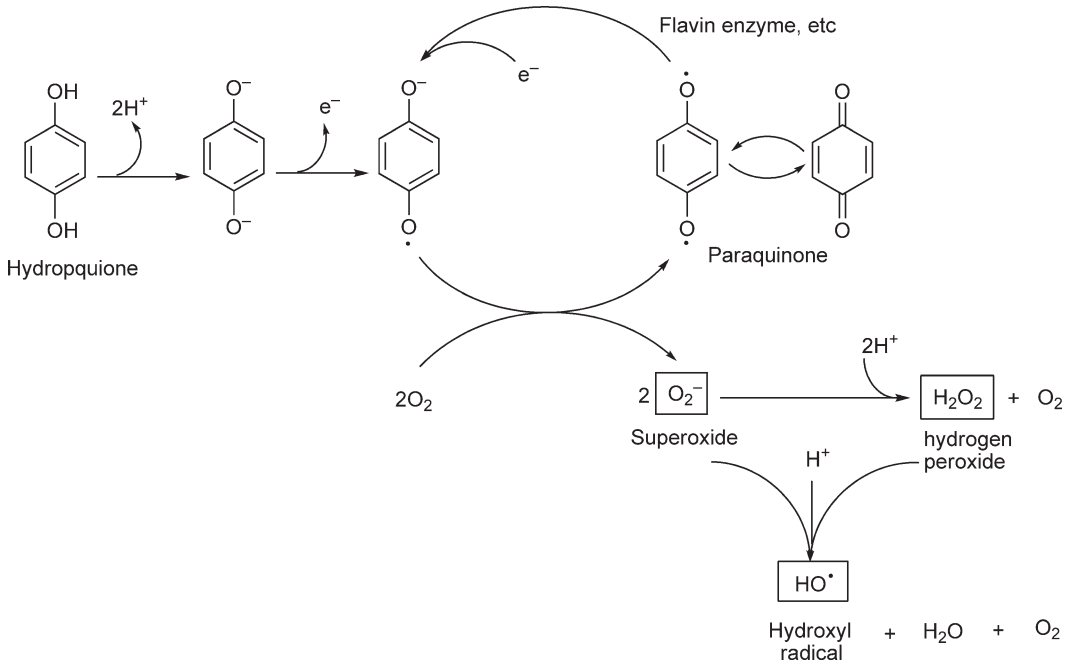


Figure 34 Putative scheme of evolution of various kinds of active oxygen species by the addition of hydroquinone.

concentration of hydroquinone in the medium to level up the production of arbutin. Sugar is completely safe and is also a specific hydroxyl radical scavenger *per se*.[66] Inomata *et al.*[67] achieved the productivity of 9.2 $g\,l^{-1}$ (45% of cell dry weight) by way of the continuous addition of hydroquinone maintaining the concentration at almost zero levels in the medium. In regard to the strain of *C. roseus*, the strain having a larger vacuole was much superior to one having a smaller vacuole.[68] Biotransformation is usually performed using the cells in late exponential stage. This choice is the result of a bargain between the two determinative factors: the maturation (developing vacuole) of cells and the concentration of the remaining sugars in the medium. Matured cells are absolutely suitable for biotransformation of themselves.[69] This conclusion is provable by the fact that the strain equipped with larger vacuoles in the cell produces much more arbutin than strains with smaller vacuoles, as described above.[68]

Hydroxylation, as well as glucosylation, is the reaction that could yield more than 1 $g\,l^{-1}$ of products.[54] The reason why hydroxylation is important for cosmetics ingredients is that terpenoids seem to be easily hydrated to various types of products.[54,55] Monoterpenes, such as geraniol, citronellol, linalool, and menthol, and sesquiterpenes are commonly used in cosmetics as aroma chemicals. Therefore, if the usability of such hydrated monoterpenoids can be determined, the extract of the plant cells containing hydrated monoterpenoids will become a unique ingredient. Furusawa *et al.*[70] demonstrated the advantage of biotransformation applied to flavor industy sector. Nootkatone is the most important grapefruit aroma, which has been found to be effective in consuming body fat[71,72] and marketed successfully by Shiseido in Japan. Nootkatone was chemically synthesized from valencene obtained from the essential oil of valencia oranges in three steps with $AcOOCMe_3$ and chromic acid in low yield[73] or by other methods.[74] In both cases, toxic heavy metals are involved, which gives rise to anxiety regarding safety. Furusawa *et al.*[70] tried unique materials for biotransformation: *Chlorea* sp. They investigated three *Chlorea* sp.: *C. fusuca*, *C. pyrenoidosa*, and *C. vulgaris*. All the three species converted valencene to nootkatone by more than 80%, especially *C. vulgaris*, by 100%. Nootkatone was presumed to be formed via nootkatol, which was the product formed from valencene at lower velocity and as well was the substrate for nootkatone at higher velocity (**Figure 35**). This is why the reaction is better suited for the formation of nootkatone.

The examples of biotransformation, which could be used for cosmetics ingredients, are listed in **Table 7**.

Table 7 Biotransformation

Substrate	*Type of reaction*	*Plant species*	*Reference*
Digitoxin	Hydroxylation	*Digitalis lanata*	75
β-Methyldigoxin	Hydroxylation	*D. lanata*	76
Geraniol	Oxidation of OH	*Rosa centifolia*	77
Geraniol	Oxidation of OH	*Vitis vinifera*	78
10-Hydroxylinalool 10-Hydroxycitronellol 7,8-Dihydro-10-hydroxygeraniol 7,8-Dihydro-10-hydroxycitronellol 7,8-Dihydro-10-hydroxylinallol	Reduction of C–C	*Catharanthus roseus*	79
Geranyl acetate	Oxidation of OH	*R. centifolia*	77
Nerol	Oxidation of OH	*R. centifolia*	77
Neryl acetate	Oxidation of OH	*R. centifolia*	77
Neral and geranial (mixture)	Reduction of C=O, Acetylation	*V. vinifera*	80
Citronellol	Oxidation of OH	*R. centifolia*	77
Citronellal	Reduction of C=O	*Lavandula augustifolia*	81
Citronellyl acetate	Oxidation of OH	*R. centifolia*	77
Citral	Reduction of C=O	*L. augustifolia*	81
Linalool	Hydroxylation	*Nicotiana tabacum*	82
Linalyl acetate	Hydrolysis Hydroxylation	*N. tabacum*	82
Linalyl acetate	Hydrolysis hydroxylation	*Papaver bracteatum*	83
(−)-Menthol	Glucosylation Hydroxylation	*Eucalyptus perriniana*	84
(+)-Menthol	Glucosylation	*E. perriniana*	85
(+)-Menthol	Glucosylation	*Mentha* sp.	86
(+)-Menthone	Hydroxylation reduction of C=O	*N. tabacum*	87
(−)-Menthone	Hydroxylation	*Mentha cell lines*	88
(±)-Cravoxime	Hydroxylation Reduction of C=O	*N. tabacum*	89
β-Terpineol (c-4-p-Menth-81-en-r-1-ol)	Hydroxylation	*N. tabacum*	90
α-Terpineol	Hydroxylation	*N. tabacum*	91
β-Terpinyl acetate	Hydroxylation	*N. tabacum*	91
α-Pinene	Hydroxylation	*R. centifolia*	77
Steviol	Glucosylation	*E. perriniana*	92
Steviol	Glucosylation	*Coffea arabica*	92
18β-Glycyrrhetinic acid	Glucosylation Hydroxylation	*Glycyrrhiza glabra*	93
18β-Glycyrrhetinic acid	Glucosylation Hydroxylation	*G. glabra*	94
18β-Glycyrrhetinic acid	Glucosylation	*C. arabica*	94
18β-Glycyrrhetinic acid	Glucosylation Malonylation	Transformed *Panax ginseng*	95
Benzoic acid	Glucosylation	*G. echinata*	96
Benzoic acid	Glucosylaion	*Aconitium japonicum* *Coffea arabica,* *Dioacoreophyllum cumminsii,* *N. tabacum*	96
Benzylacetate	Hydrolysis	*Spirodela oligorrhiza*	97
Salicyl alcohol	Glucosylation	*D. innoxia,* *Perilla frutescens,* *Gardenia jasminoides*	98
Salicylic acid	Glucosylation	*Mallotus japonicus*	60
Coniferyl alcohol	Gulcosylation	*P. ginseng* (roots)	99
Vanillin	Glucosylation	*C. arabica*	100

(*Continued*)

Table 7 (Continued)

Substrate	*Type of reaction*	*Plant species*	*Reference*
Vanillin	Glucosylation	*D. innoxia*	101
Capsaicin	Glucosylation	*C. arabica*	102
Aromatic ketones (acetophenon, etc.)	Reduction of C=O	*Daucus carota* *N. tabacum* *G. jasminoides*	103
Umbelliferone	Glucosylation	*Datura innoxia* *P. frutescens* *C. roseus* *L. erythrorhizon* *Bupleurum falcatum* *G. jasminoides*	98
Esculetin	Glucosylation	*L. erythrorhizon*	98
Quercetin	Glucosylation, Methylation	*Cannabis sativa*	104
Naringenin	Glucosylation	*P. frutescens,* *B. falcatum*	98
Naringenin	Glucosylation	*Swertia jasminica*	105
Naringenin	Glucosylation	*Duboisia myoporoides*	106
Naringenin	Glucosylation	*Citus paradisi*	107
Naringenin	Glucosylation	*Citus aurantium*	108
Liquiritigenin	Glucosylation	*Datura innoxia* *P. frutescens* *C. roseus* *L. erythrorhizon* *B. falcatum* *G. jasminoides*	98

3.11.7.5 Miscellaneous

Many valuable, volatile components are secondarily formed by microorganisms, and have been used in food industry products such as wine, pickles, and vanilla. Fermentation produces latent aroma. In cosmetics industry, fermentation has not been used as a way to produce a new perfume presumably because fermented odor is not suitable for cosmetics. Oris oil, which is newly produced after the rhizomes of *Iris pallida* are stored for a couple of years, is one of the superlatives in perfume and the most expensive. The characteristic component is γ-iron. γ-Iron is believed to be produced through oxidation, not fermentation. Terajima *et al.*[109] examined the effects of stress on latent aroma formation from Somei-Yoshino (*Prunus* × *yedoensis* Matsum. Cv. Yedoensis). They proposed three kinds of stress made up of physical stress (crushed after drying) or chemical stress (immersed in an acidic or salt aqueous solution, or in an organic solution) to leaves or flowers of *Prunus* × *yedoensis* and found that the characteristic odorants are produced differently under each stress. In this way, latent aroma production can be controlled.

Plant extracts are usually used after filtration because insoluble matter could impart to the products turbidity and inconvenience of use. However, Iida and Yokoyama[110] showed the advantages of the plant cells kept in the extracts. The extracts caused the plant tissues to break down and the cells covered with cell walls to be detached from each other by digesting with pectinase. Naturally such a material contains proteins, lipids, and minerals along with the crushed plant, unlike the extract, which contains mainly lipids.[110] An outstanding character of the raw material containing cells is the presence of useful hydrophobic compounds like β-carotene, making it much more stable. This could exhibit a different biological activity as compared to the extract of the original plant. New cosmetics containing *Aloe arborescens*, *Vaccinium vitis-idaea*, and *Eriobotrya japonica* have successfully been marketed in Japan.

3.11.8 Conclusion

Prior to the 1990s, it could be assumed that what the users had demanded from the function of skin care cosmetics is a fundamental effect based on moisture-retaining properties, emphasizing the feeling or image of cosmetics. Since then, with advanced molecular biology or genetic technology, physiological function of the skin has been elucidated at the molecular level, and hence active cosmetics, which means that ingredients have high functionality based on latest scientific data rather than mere moisturizing effect, have been predominant in the market. In the past, only the images such as naturally occurring and safe had been appreciated in the role of natural products in cosmetics. Just combining natural products simply gave rise to reasonable concepts of cosmetic products, and consequently the existence value of natural products was recognized. But over the last decade, only such an existence value was proving insufficient to survive in the market. When developing the plant extract that should be added to the cosmetics, it was also necessary that some new concept arise, in addition to such an image. Especially in the field of functional cosmetics, the so-called cosmeceuticals, publicizing some information such as a new characteristic or a new pharmacological action as the product's concept gives the product further charm needed to allure the customer. While highlighting the fact that natural products have been deeply connected with cosmetics since olden days, each plant extract that was introduced in this chapter is an example of the discovery of a new pharmacological action. It is very important to establish not only the pharmacological effect and the concept of the cosmetics, but also the technology that supplies the effective ingredient for stability. Plant tissue culture techniques have the advantage of providing cosmetics with consistent quality through extracts or components from plants. In the present day, when we are facing the crisis of climate change like global warming, such advantages will not be ignored in cosmetics and medicines. Plant tissue culture techniques can be classified mainly as micropropagation, root culture, and biotransformation. Micropropagation techniques are applied in many plants as the method of propagation of the elite strains of the plant before transplanting in soil. Root culture techniques could offer higher density than that offered by micropropagation, as well as the stable production of components. However, this necessitates further development of the equipment to handle the large quantities of roots required for commercial production. The simple system of the tank that we had developed should be referred to. Biotransformation is a unique way for the production of useful components in respect of higher yield, although such higher yields of more than 1 g l^{-1} are limited to glucosylation and hydroxylation. In future, as the natural circumstances are likely to change, plant tissue culture will become a prosperous art.. Cosmetics are products made to attract image-conscious users, while various technologies are condensed into the actual product. In the future, more and more natural products based on new concepts and possessing new pharmacological actions or based on plant tissue culture techniques would be developed, with the expectation that they will boost the functionality of cosmetics.

Abbreviations

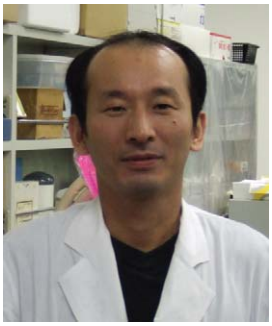
DHT	dihydrotestosterone
DOPA	dihydroxyphenylalanine
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HPLC	high-performance liquid chromatography
MITF	microphthalmia-associated transcription factor
RAPD	random amplified polymorphic DNA
TCM	Traditional Chinese Medicine
TRP	tyrosinase-related protein
UV	ultraviolet
UVB	ultraviolet B

References

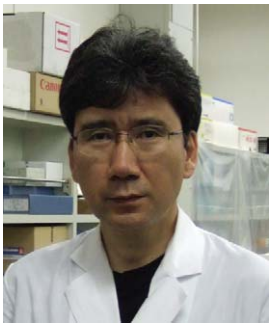
1. T. Mitsui, *New Cosmetic Science*; Elsevier, 1997.
2. T. Namba, *Outline of Pharmacognosy a Textbook*; Nankodo Co., Ltd., 1993.
3. M. Hotta, *Useful Plants of the World*; Heibonsha, 1989.
4. T. S. C. Li, *Chinese and Related North American Herbs*; CRC Press, 2002.
5. D. Frawley, *The Yoga of Herbs*; Lotus Press, 1986.
6. R. Corson, *Fashion in Makeup*; Pola Research Institute of Modern Culture, 1982.
7. T. Murata, *Nippon no keshou*; Pola Research Institute of Modern Culture, 1989.
8. *International Comparison Lists of Cosmetic Ingredients*; Yakuji Nippo Ltd., 1996.
9. P. Elsner, *Cosmetics Controlled Efficacy Studies and Regulation*; Springer, 1999.
10. R. H. Champion, *Textbook of Dermatology*; Oxford Blackwell Scientific Publication, 1992.
11. K. Maeda; M. Fukuda, *J. Pharmacol. Exp. Ther.* **1996**, *276*, 765–769.
12. H. Shimogaki; Y. Tanaka; H. Tamai; M. Masuda, *Int. J. Cosmet. Sci.* **2000**, *22*, 291–303.
13. N. Ota; K. Sato; O. Moro; M. Ota; O. Ifuku, *J. Jpn. Cosmet. Sci. Soc.* **2001**, *25*, 8–12.
14. G. J. Fisher; S. C. Datta; H. S. Talwar; Z.-Q. Wang, *Nature* **1996**, *379*, 335–339.
15. S. Shuster, *Br. J. Dermatol.* **1975**, *93*, 639–643.
16. M. Guerin; M. E. Huntley; M. Olaizola, *Trends Biotechnol.* **2003**, *21*, 210–216.
17. E. Yamashita, *Fragrance J.* **2006**, *34*, 21–27.
18. S. Itami, *Fragrance J.* **2007**, *35*, 12–16.
19. S. Itami, *J. Invest. Dermatol.* **1991**, *96*, 57–60.
20. H. Matsuda, *Fragrance J.* **2007**, *35*, 41–48.
21. Y. Nakazawa, *J. Jpn. Biochem. Soc.* **1999**, 766.
22. Y. Nakazawa, *J. Jpn. Biochem. Soc.* **1998**, 729.
23. S. Takahashi; Y. Fujita, In *Production of Useful Plant Metabolites by Plant Cell Culture Technology*; A. Komamine, Ed.; CMC: Tokyo, 1990; pp 147–153.
24. S. Gudin, *Plant Breeding Reviews*; John Wiley and Sons, Inc., 2000; Vol. 17, pp 159–189.
25. P. K. Pati; M. Sharma; P. S. Ahuja, *Acta Hortic.* **2001**, *547*, 147–158.
26. L. Natali; I. C. Sanchez; A. Cavallini, *Plant Cell Tissue Organ Cult.* **1990**, *20*, 71–74.
27. S. Ahmed; A. H. Kabir; M. B. Ahmed; M. A. Razvy; S. Ganesan, *Sjemenarstvo* **2007**, *24*, 121–128.
28. D. Hashemabadi; B. Kaviani, *Afr. J. Biotechnol.* **2008**, *7*, 1899–1902.
29. J. M. F. Nogueira; A. Romano, *Phytochem. Anal.* **2002**, *13*, 4–7.
30. S. Rahman; S. Sultana, *J Enzyme Inhib. Med. Chem.* **2007**, *22*, 363–369.
31. Y. Tamura; S. Nakamura, *Yakuyoushokubutukenkyu* **2004**, *1*, 1–6.
32. A. K. Kumar; D. Patnaik, *In-Vitro* System of Micropropagation of Rose Scented *Pelargonium Graveolens*, of Bourbon Type. U.S. Patent 10/453,016, 6 March 2003.
33. J. M. F. Nogueira; A. Romano, *Phytochem. Anal.* **2002**, *13*, 4–7.
34. Y. Tamura; M. Oda; S. Nakamura; K. Mizutani; T. Ikeda; K. Goto, In Proceedings of the 45th Annual Meeting of the Japanese Society of Pharmacognosy, 1998; pp 2P–26.
35. Y. Tamura; S. Nakamura; H. Fukui; M. Tabata, *Plant Cell Rep.* **1984**, *3*, 180–182.
36. Y. Tamura; S. Nakamura; H. Fukui; M. Tabata, *Plant Cell Rep.* **1984**, *3*, 183–185.
37. G. R. Rout; G. Das; S. Samantaray; P. Das, *Rev. Biol. Trop.* **2001**, *49*, 3–4.
38. F. Fracaro; S. Echeverrigaray, *Plant Cell Tissue Organ Cult.* **2001**, *64*, 1–4.
39. C. Z. Liu; S. J. Murch; M. EL-Demerdash; P. K. Saxena, *Plant Cell Rep.* **2003**, *21*, 525–530.
40. S. Inomata; M. Yokoyama, In *Biotechnology in Agriculture and Forestry, Vol. 38: Plant Protoplasts and Genetic Engineering VII*. Y. P. S. Bajaj, Ed.; Springer: Berlin, Heidelberg, 1996; pp 253–269.
41. E. M. Linsmaier; F. Skoog, *Physiol. Plant.* **1965**, *18*, 100–127.
42. J. H. Kim; E. J. Chang; H.-I. Oh, *Asia Pac. J. Mol. Biol. Biotechnol.* **2005**, *13*, 87–91.
43. H. Ishii; M. Nakamura; S. Seo; K. Tori; T. Tozyo; Y. Yoshimura, *Chem. Pharm. Bull.* **1980**, *28*, 2367–2373.
44. M. Yamamoto; A. Kumagai; Y. Yamamura, *I. Arzneimittelforschung* **1975**, *25*, 1021–1023.
45. M. Yamamoto; A. Kumagai; Y. Yamamura, *II. Arzneimittelforschung* **1975**, *25*, 1240–1243.
46. T. Kita; T. Hata; E. Ito; R. Yoneda, *J. Pharmacobio-dyn.* **1980**, *3*, 269–280.
47. T. Nishiyama; I. Horii; Y. Nakayama; T. Ozawa; T. Hayashi, *Matrix* **1990**, *10*, 412–419.
48. T. Nishiyama; N. Akutsu; Y. Nakayama, *Seikagaku* **1999**, *71*, 889.
49. Y. Nakayama; N. Akutsu; T. Nishiyama, *Seikagaku* **1999**, *71*, 889.
50. K. Kusakari; M. Yokoyama; S. Inomata, *Plant Cell Rep.* **2000**, *19*, 1115–1120.
51. K. Asada, *Active Oxygen (in Japanese)*; Kyoritsu Press: Tokyo, 1987; pp 23–40.
52. M. Yokoyama; S. Yamaguchi; K. Kusakari, *Plant Biotechnol.* **2003**, *20*, 331–334.
53. E. Reinhard; A. W. Alferman, In *Advances in Biochemical Engineering;* A. Fiechter, Ed.; Springer: New York, 1980; Vol.16, p 49.
54. M. Yokoyama, In *Plant Cell Culture Secondary Metabolism. Toward Industrial Application*. M. Misawa,F. DiCosmo, Eds.; CRC Press: Boca Raton, FL, 1996; pp 79–121.
55. K. Ishihara; H. Hamada; T. Hirata; N. Nakajima, *J. Mol. Catal. B: Enzym.* **2003**, *23*, 145–170.
56. R. Lutterbach; J. Stöckigt; H. Kolshorn, *J. Nat. Prod.* **1993**, *56*, 1421.
57. S. Inomata; M. Yokoyama; S. Seto; M. Yanagi, *Appl. Microbiol. Biotechnol.* **1991**, *36*, 315–319.
58. R. Lutterbach; J. Stöckigt, *Helv. Chim. Acta* **1992**, *75*, 2009–2011.
59. D. Courtois; D. Yvernel; B. Florin; V. Petiard, *Phytochemistry* **1988**, *27*, 3137–3142.
60. Y. Umetani; S. Tanaka; M. Tabata, In Proceedings of the 5th International Congress Plant Tissue and Cell Culture; Tokyo, Japan; K. Fujiwara, Ed.; 1982; pp 383–384.

61. Y. Umetani; E. Kodakari; T. Yamamura; S. Tanaka; M. Tabata, *Plant Cell Rep.* **1990**, *9*, 325–327.
62. M. Yokoyama; S. Inomata, In *Biotechnology in Agriculture and Forestry, Vol. 41: Medicinal and Aromatic Plants X*; Y. P. S. Bajaj, Ed.; Springer-Verlag: Berlin, Heidelberg, 1998; pp 67–80.
63. M. Yokoyama; S. Inomata; S. Seto; M. Yanagi, *Plant Cell Physiol.* **1990**, *31*, 551–555.
64. M. Yokoyama; M. Yanagi, In *Plant Cell Culture in Japan*; A. Komamine, M. Misawa, F. DiCosmo, Eds.; CMC Press: Tokyo, 1992; pp 79–91.
65. M. Yokoyama, Manufacture of Phenolic Glucosides. Japanese Patent 259,091, 1991.
66. K. Asada; K. Kiso, *Eur. J. Biochem.* **1973**, *3*, 253–257.
67. S. Inomata; M. Yokoyama; S. Seto; M. Yanagi, *Appl. Microbiol. Biotechnol.* **1991**, *36*, 315–319.
68. S. Inomata; M. Yokoyama; Y. Wachi, *Plant Tissue Cult. Lett.* **1994**, *11*, 211–217.
69. M. Yokoyama; S. Inomata; M. Yanagi; Y. Wachi, *Plant Cell Mol. Biol. Lett.* **1996**, *13*, 285–290.
70. M. Furusawa; T. Hashimoto; Y. Noma; Y. Asakawa, *Chem. Pharm. Bull.* **2005**, *53*, 1513–1514.
71. S. Haze; K. Sakai; Y. Gozu, *Jpn. J. Pharmacol.* **2002**, *90*, 247–253.
72. J. Shen; A. Niijima; M. Tanida; Y. Horii; K. Maeda; K. Nagai, *Neurosci. Lett.* **2005**, *380*, 289–294.
73. C. W. Wilson; P. E. Shaw, *J. Agric. Food Chem.* **1978**, *24*, 1430–1432.
74. J. A. R. Salvador; J. H. Clark, *Green Chem.* **2002**, *4*, 352–356.
75. W. Kreis; E. Reinhard, *Planta Med.* **1986**, 418–419.
76. M. Heins; J. Wahl; H. Lerch; F. Kaiser; E. Reinhard, *Planta Med.* **1978**, *33*, 57–62.
77. B. Corbier; C. Ehret, In *Flavors and Fragrances.* Proceedings of the 10th International Congress of Essential Oils; Elsevier Science Publishers B.V.: Amsterdam, 1988; p 731.
78. F. Cormier; C. Ambid, *Plant Cell Rep.* **1987**, *6*, 427–430.
79. J. Balsevich, *Planta Med.* **1985**, *51*, 128–132.
80. C. Ambid; M. Moisseeff; J. Fallot, *Plant Cell Rep.* **1982**, *1*, 91–93.
81. G. Lappin; J. Tampion; J. D. Stride, In *Secondary Metabolism in Plant Cell Cultures*; P. Morris, A. H. Scragg, A. Stafford, M. W. Fowler, Eds.; Cambridge University Press: Cambridge, 1986; pp 113–116.
82. T. Hirata; T. Aoki; Y. Hirano; T. Ito; T. Suga, *Bull. Chem. Soc. Jpn* **1981**, *54*, 3527–3529.
83. I. Hook; R. Lecky; B. McKenna; H. Sheridan, *Plant Physiol. Biochem.* **1990**, *29*, 2143–2145.
84. T. Furuya; Y. Orihara; H. Miyatake, *J. Chem. Soc. Perkin Trans. I* **1989**, 1711–1719.
85. Y. Orihara; H. Miyatake; T. Furuya, *Phytochemistry* **1991**, *30*, 1843.
86. R. G. Berger; F. Drawert, German Patent 3,718,340 (DE A1), 1988.
87. T. Suga; T. Hirata; H. Hamada; S. Murakami, *Phytochemisty* **1988**, *27*, 1041.
88. D. Aviv; A. Dantes; E. Krochmal; E. Galun, *Planta Med.* **1983**, *47*, 7–10.
89. T. Suga; T. Hirata; M. Futatsugi, *Phytochemisty* **1984**, *23*, 1327.
90. T. Suga; Y. M. Lee; T. Hirata, *Bull. Chem. Soc. Jpn.* **1983**, *56*, 784.
91. T. Suga; T. Aoki; T. Hirata; Y. S. Lee; O. Nishimura; M. Utsumi, *Chem. Lett.* **1980**, 229.
92. Y. Orihara; K. Saiki; T. Furuya, *Phytochemistry* **1991**, *30*, 3989.
93. H. Hayashi; H. Fukui; M. Tabata, *Phytochemistry* **1990**, *29*, 2149–2152.
94. Y. Orihara; T. Furuya, *Phytochemistry* **1990**, *29*, 3123.
95. Y. Asada; H. Saito; T. Yoshikawa; K. Sakamoto; T. Furuya, *Phytochemistry* **1993**, *34*, 1049–1052.
96. M. Ushiyama; S. Kumagai; T. Furuya, *Phytochemistry* **1989**, *28*, 3335–3339.
97. P. Pawtowicz; A. Siewiński, *Phytochemistry* **1987**, *26*, 1001.
98. M. Tabata; Y. Umetani; Y. Ooya; S. Tanaka, *Phytochemistry* **1988**, *27*, 809.
99. M. Ushiyama; T. Furuya, *Phytochemistry* **1989**, *28*, 3009–3013.
100. T. Kometani; H. Tanimoto; T. Nishimura; S. Okada, *Biosci. Biotechnol. Biochem.* **1993**, *57*, 1290–1293.
101. H. J. Scholten; M. J. Schans; I. P. M. Somhorst, *Plant Cell Tissue Organ Cult.* **1991**, *26*, 173.
102. T. Kometani; H. Tanimoto; T. Nishimura; I. Kanbara; S. Okada, *Biosci. Biotechnol. Biochem.* **1993**, *57*, 2192–2193.
103. Y. Naoshima; Y. Akakabe, *Phytochemistry* **1991**, *30*, 3595.
104. R. Breamer; Y. Tsoutsias; M. Hurabielle; M. Paris, *Planta Med.* **1987**, *53*, 225–226.
105. H. Miura; K. Kawashima; Y. Kitamura; M. Sugii, *Shoyakugaku Zasshi* **1986**, *40*, 40.
106. H. Miura; Y. Kitamura; M. Sugii, *Plant Cell Physiol.* **1986**, *40*, 113.
107. E. Lewinsohn; E. Berman; Y. Mazur; J. Gressel, *Phytochemistry* **1986**, *25*, 2531–2535.
108. E. Lewinsohn; E. Berman; Y. Mazur; J. Gressel, *Plant Sci.* **1989**, *61*, 23–28.
109. Y. Terajima; K. Yomogida; T. Namba; T. Katsuki, In *Volatile Components from the Flowers and Leaves of Yoshino Cherry.* Proceedings of the TEAC; Yamaguchi, Japan, 30 October–1 November, 2004.
110. T. Iida; M. Yokoyama, In *The Current Technique of Enzyme Application and the Development*; A. Masuo, Ed.; CMC: Tokyo, 2001; pp 117–124.

Biographical Sketches



Masahiro Ota was born in Tokyo, Japan, where he completed his B.Sc. and M.Sc. degrees at Tokyo University of Science. He joined Shiseido Co., Ltd., where he has researched natural products for cosmetics ingredients, especially for antiaging and skin-whitening products and hair growth promoters. He studied under Professor P. J. Houghton at King's College London (2004–05). He joined Horticultural Diploma Course at Royal Botanic Gardens Kew (2005). Presently his research interests are concerned with the relationship between skin aging and blood vessels, and the development of cosmetics ingredients based on new concepts.



Mineyuki Yokoyama was born in Yokohama, Japan, where he completed his B.Sc. at the University of Shizuoka and M.Sc. at Tokyo University of Education. He obtained his Ph.D. in 1981 on the physiological research on greening of the primary leaves of *Phaseolus vulgaris* at the University of Tshukuba under the supervision of Professor H. Suzuki. After 2 years of postdoctoral studies at Plant Virus Research Institute, the Ministry of Agriculture, Forestry and Fisheries, Tsukuba, he moved to the Research Center, Shiseido Co., Ltd. in 1983 as a research scientist. Thereafter, he has been engaged in the research on biotechnology as a senior scientist during 1983–99 and as a principal senior scientist since 1999. His research interests are concerned with the development of new plant materials for cosmetics using plant tissue culture technique. He found the importance of stress involvement when plants (or cultured tissue) go into a cell-differentiating phase. He succeeded in establishing the system for a stable and high-level yield of saikosaponins to be compounded in new cosmetics products. That research helped him find novel stress-inducing components and apply them to regulate the growth of plants cultivated in the field.

3.12 Ethnopharmacology and Drug Discovery

Michael Heinrich, University of London, London, UK

3.12.1 Introduction

> Artemisinin, triptolide, celastrol, capsaicin, and curcumin are poster children for the power and promise of turning traditional medicines into modern drugs. However, their stories highlight the ongoing interdisciplinary research efforts that continue to be necessary to realize the pharmaceutical potential of traditional therapeutics.[1]

As highlighted by Corson and Crews,[1] drug development in its modern understanding focuses on pure chemical entities, and local and traditional knowledge remains an essential starting point for such research and development (R&D).

There can be no doubt that observational knowledge about the effect of a plant, an animal, or a microorganism on other organisms offers ideal opportunities to limit the huge diversity of possible leads to more promising ones (knowledge-based drug discovery). Such observational knowledge is exemplified by the discovery of penicillin (Alexander Fleming, 1928) and by the discovery of desmoteplase, a protein recently isolated from vampire bats (which need it to prevent their prey's blood from coagulating), which was developed to treat the effects of strokes.[2]

The ethnopharmacological approach is unique in natural product research in that it requires *input from the social and cultural sciences*. It is essential to distinguish two parts of these development activities: the field-based study of local resources, or the documentation of practitioners' healing practices, and the bioscientific study of this knowledge and of the products used.

In many regions of the world, knowledge was or still is mostly passed on orally from one generation of healers to the next. This knowledge has been the focus of researchers who have been called ethnobotanists or ethnopharmacologists. On the contrary, there are *written* records from practitioners from cultures such as the

Chinese, Arabic, Asian Indian, Mexican Indian (Aztec), and, of course, European traditions who wrote down their knowledge.

In 1896, the term 'ethnobotany' was coined by the American botanist William Harshberger describing the study of plant use by humans. The term is generally based on a detailed observation and analysis of the use of plants used in a society and of all beliefs and cultural practices associated with such use. Ethnobotany and ethnopharmacology investigate the relationship between humans and plants in all its complexity. Ethnobotanists live with members of a community, share their everyday lives, and, of course, respect the cultures of the host. Ethnobotanists have a responsibility not only to the scientific community but, equally important, also to indigenous cultures. A complex set of methods are used that are derived from the social and cultural sciences (including taking detailed field notes, quantitatively assessing reported uses, cognitively and symbolically analyzing plant usage) and the natural sciences (collecting plant samples – voucher specimens – that allow precise determination of the plant species). Ethnobotanical studies have many theoretical and applied interests; in fact, only a very few are in any way directly linked with projects in the area of drug discovery.[3–7]

Ethnopharmacology as a specifically designated field of research has had a relatively short history. The term 'ethnopharmacology' was first used in 1967 as the title of a book on hallucinogens: *Ethnopharmacological Search for Psychoactive Drugs*.[8] However, it would be meaningless to limit this discussion to the period after 1967. Medicinal plants are an important element of indigenous medical systems in many parts of the world, and these resources are usually regarded as part of the traditional knowledge of a culture; thus, any study that focuses on the documentation and systematic study of local and traditional uses of a plant or a group of plants can be considered to have ethnopharmacological relevance. Explorers, missionaries, merchants, but also knowledgeable experts in the respective healing, tradition, describe the uses of such medicinal plants; all this is the basis for ethnopharmacology-based drug development. Although such knowledge has been widely used for centuries as a starting point for drug development, once an initial lead is found, many researchers no longer consider this knowledge to be relevant. Unfortunately, the oral tradition of medical knowledge is often simply ignored as in a classic review of the drug development process, W. Sneader's *Drug Discovery: A History*.[9]

Clearly, natural products remain one of the most important sources (or maybe even the most important one) of new drug leads. As Chin *et al.*[10] have pointed out, more than half of all new chemical entities launched in the market are natural products or their derivatives or mimetics. This review is thus not about drug discovery from natural sources, a topic that has received considerable attention in recent years,[10–17] but specifically on the link between local/traditional knowledge (or what could also be called botanical therapeutics[18]) and drug development.[19–22]

3.12.2 'Old' Drugs – New Medicines

Drug development and discovery as we know it today is an outcome of the Enlightenment in Europe and the rapid expansion of pharmaceutical industries, which started in the second half of the nineteenth century. Up to this point, medical treatment strictly relied on crude materials obtained from nature and their extracts that were processed and formulated into medicines.[23] The nineteenth century was when researchers began to characterize pure chemical entities in medically used or toxic plants and other organisms.

3.12.2.1 The Late Eighteenth and the Nineteenth Century

The study of the botanical origin of the arrow poison curare, its physiological (as well as toxic) effects, and the compound responsible for these provides a fascinating example of an early ethnopharmacological approach. Curare was used by 'certain wild tribes in South America for poisoning their arrows'.[24] Many early explorers documented this usage. Particularly well known are the detailed descriptions of the process used by Alexander von Humboldt in 1800 to prepare poisoned arrows in Esmeralda, Venezuela, on the Orinoco River. There, von Humboldt met inhabitants who were celebrating their return from an expedition to obtain the raw material for making the poison. Von Humboldt then describes the 'chemical laboratory' used:

> He [an old Indian] was the chemist of the community. With him we saw large boilers (Siedekessel) made out of clay, to be used for boiling the plant sap; plainer containers, which speed up the evaporation process because of their large surface; banana leaves, rolled to form a cone-shaped bag [and] used to filter the liquid which may contain varying amounts of fibres. This hut transformed into a laboratory was very tidy and clean (von Humboldt,[24] p 88)

As early as 1800, von Humboldt had to face one of the classical problems of ethnopharmacology:

> We are unable to make a botanical identification because this tree [which produces the raw material for the production of curare] only grows at quite some distance from Esmeralda and because [it] did not have flowers and fruit. I had mentioned this type of misfortune previously, that the most noteworthy plants cannot be examined by the traveller, while others whose chemical activities are not known [i.e. which are not used ethnobotanically] are found covered with thousands of flowers and fruit.

Later, the botanical source of curare was identified as *Chondrodendron tomentosum* Ruiz et Pavon, which produces the so-called tube curare (named because of the bamboo tubes used as storage containers). Other species of the Menispermaceae (*Chondrodendron* spp., *Curarea* spp., and *Abuta* spp.) and species of the Loganiaceae (*Strychnos* spp.) are also used in the production of curares.

The first systematic studies on the pharmacological effects were conducted by the French physiologist Claude Bernard (1813–78). It is worth looking at his description of the pharmacological effects of curare in some detail. "If curare is applied into a living tissue via an arrow or a poisoned instrument, it results in death more quickly if it gets into the blood vessels more rapidly. Therefore death occurs more rapidly if one uses dissolved curare instead of the dried toxin" (Bernard,[25] p 92). "One of the facts noted by all those who reported on curare is the lack of toxicity of the poison in the gastrointestinal tract. The Indians indeed use curare as a poison and as a remedy for the stomach" (Bernard,[25] p 93). Bernard was also able to demonstrate that the animals did not show any nervousness and any sign of pain. Instead, the main sign of death induced by curare is muscular paralysis. If the blood flow in the hind leg of a frog is interrupted using a ligature, but without interrupting the innervation, and it is poisoned via an injury of the hind leg, it retains its mobility and the animal does not die from curare poisoning (Bernard,[25] p 115).These and subsequent studies allowed a detailed understanding of the pharmacological effects of curare in causing respiratory paralysis. The most important compound responsible for this activity was isolated for the first time from *C. tomentosum*, and in 1947 the structure of the bisbenzylisoquinoline alkaloid D-tubocurarine was established. Finally, tubocurarine's structure was established unequivocally using nuclear magnetic resonance (NMR) in the 1970s, showing that it has only one quaternary nitrogen. In many European countries, tubocurarine is currently used only sporadically, but in France, for example, it is still used for muscle relaxation during surgery.

The use of medicinal plants was always an important part of all medical systems of the world, and Europe was no exception. Little is known about popular traditions in medieval and early modern Europe. Our knowledge starts with the availability of written (printed) records on medicinal plant use by common people. As pointed out by Griggs,[26] a woman in the seventeenth century was a 'superwoman' capable of administering "any wholesome receipts or medicines for the good of the family's health" (p 88). A typical case is foxglove (*Digitalis purpurea* L., Scrophulariaceae), reportedly used by an English housewife to treat dropsy, and then more systematically by the physician William Withering (1741–99). He transformed the orally transmitted knowledge of British herbalism into a form of medicine that could be used by medical doctors. Prior to that, herbalism was more of a clinical practice interested in the patient's welfare, and less of a systematic study of the virtues and chemical properties of medicinal plants.

Below are listed examples of natural products first identified during the early years of the nineteenth century and briefly summarize information on subsequent research to fully characterize these compounds and to establish their structures. All these activities were automatically based on the common medical use of these species. Today, they would thus be considered ethnopharmacologically driven.

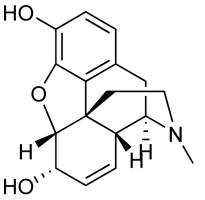
Examples of pure compounds first isolated during the early nineteenth century:

- 1804 – *Morphine* (**1**) from the opium poppy (*Papaver somniferum* L., Papaveraceae) was first identified by F. W. Sertürner (Germany). It took until 1817 for it to be chemically characterized as an alkaloid. Its structure was established in 1923 by J. M. Gulland and R. Robinson England

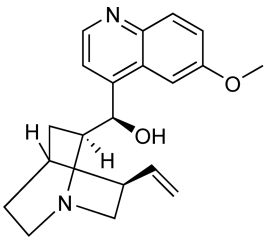
- 1817 – *Emetine* from ipecacuanha (*Cephaelis ipecacuanha* (Brot.) A. Rich., Rubiaceae) was fully characterized as late as 1948 and used as an emetic as well as in cough medications
- 1817 – *Strychnine* from *Strychnos* spp., Loganiaceae, was used as a tonic and stimulant
- 1820 – *Quinine* (**2**) was first isolated from *Cinchona* spp. (Rubiaceae) by Pierre Joseph Pelletier and Joseph Bienaime Caventou of France: the structure was elucidated in the 1880s by various laboratories
- 1821 – *Caffeine* (**3**) from the coffee tree (*Coffea arabica* L. and *C. canephora* Pierre ex. Froehn, Rubiaceae); its structure was elucidated in 1882
- 1826 – *Coniine*, a highly poisonous natural product, was first isolated from hemlock (*Conium maculatum* L., Apiaceae). Its properties had been known for years (Socrates sentenced to death by drinking a mixture containing poison hemlock). It was the first alkaloid to have its structure elucidated (1870). Some years later, it was synthesized (1889)
- 1833: *Atropine* from belladonna (*Atropa belladonna* L., Solanaceae) used at the time for asthma; today, the compound is still used in ophthalmology
- 1846: L. Thresh isolated *capsaicin* from *Capsicum frutescens* L., s.l. Its structure was partly elucidated in 1919 by E.K. Nelson

(modified after Heinrich *et al.*[27] based on Sneader[28] and others)

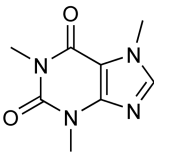
Morphine, for example, derived from the opium poppy (*P. somniferum*, Papaveraceae), was first identified by F. W. Sertürner (Germany) in 1804 and first chemically characterized in 1817 as an alkaloid. Its structure was finally established in 1923 by J. M. Gulland and R. Robinson in Manchester. There can be no doubt that this development was driven by local and traditional knowledge. The opium poppy was and is still used widely as both a medicine and a recreational drug of abuse. The opium poppy (family Papaveraceae) is an annual plant native to Asia. It is cultivated widely for food (the seed and seed oil), for medicinal purposes, and as a garden ornamental. It has been used since time immemorial as a painkiller, sedative, cough suppressant, and antidiarrheal and is featured in ancient medical texts, myths, and histories.



1 Morphine



2 Quinine



3 Caffeine

Quinine from Cinchona bark (*Cinchona pubescens* Vahl. and others) was first isolated by Pierre Joseph Pelletier and Joseph Bienaime Caventou of France in 1820 and the structure was elucidated in the 1880s by various laboratories. These two researchers were also instrumental in isolating many of the alkaloids listed above.

Salicin, from willow bark (*Salix* spp., Salicaceae), was first isolated by Johannes Buchner in Germany. It was derivatized first to yield salicylic acid (1838, Rafaele Pirea, France) and later, by the company Bayer in 1899, to yield acetyl salicylic acid, or *aspirin* – a compound previously known but which had not been studied pharmaceutically.

3.12.2.2 The First Half of the Twentieth Century

3.12.2.2.1 Antibiotics as a new model

Penicillin was further developed by Howard Florey and Ernst Chain in the late 1930s. One of the most important events that influenced the use of ethnopharmacology-driven drug development in the last century was the serendipitous discovery of the antibacterial properties of fungal metabolites such as benzylpenicillin by Alexander Fleming in 1928 at St. Mary's Hospital (London, Paddington). These natural products changed

forever the perception and use of plant-derived metabolites as medicines by both scientists and the lay public.[27] From this point onward, in terms of drug discovery, plant-derived drug leads, generally based on local and traditional knowledge, competed with the chemosystematic diversity of microorganisms. This diversity resulted in tremendous discoveries most importantly as anti-infective agents. Clearly, and with only a few exceptions, microorganism-based drug discovery cannot be ethnopharmacologically driven.

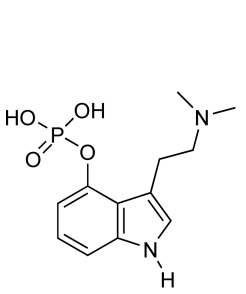
Another important development came with the advent of synthetic chemistry in the field of pharmacy. Many of these studies involved compounds that were synthesized because of their potential as coloring materials.[28] The first successful use of a synthetic compound as a chemotherapeutic agent was achieved by Paul Ehrlich in Germany (1854–1915), who used methylene blue in the treatment of mild forms of malaria in 1891. Unfortunately, this finding could not be extended to the more severe forms of malaria common in the tropics. Many further studies on the therapeutic properties of dyes and of other synthetic compounds followed. The later twentieth century also saw a rapid expansion in the knowledge of secondary natural products, their biosynthesis, and their biological and pharmacological effects. There is now a better understanding of the genetic basis of the reactions that give rise to such compounds, and also the biochemical (and in many cases genetic) basis of many important illnesses. This has opened up new opportunities and avenues for drug development.

This is important in the context of our discussion here because it highlights the fact that during this period alternative strategies offered novel ways to discover and develop new drugs and drug leads. Serendipity and more random approaches ultimately led to a strategy where the essential goal was an increase in the total number of samples to be screened, resulting in high-throughput technologies.

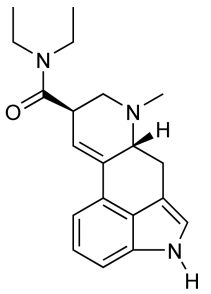
3.12.2.3 Do We Need Ethnopharmacology-Driven Drug Development? 1945 Until the 1990s

3.12.2.3.1 Compounds with an effect on the central nervous system

One of the most famous examples of a drug development project driven by traditional knowledge is the discovery of psilocybin and derivates from the hallucinogenic mushroom *Psilocybe*, which for centuries has been used by the Mazatec Indians in Oaxaca, Mexico. This drug development project of the 1940s and 1950s was only possible thanks to the collaboration of two ethnobotanists and two chemists. R. G. Wasson (1898–86) had been trained as a journalist and in literature studies. Thanks to his wife Valentina Pavlovna Guercken, he became interested in ethnobotany. This brought him in contact with the American ethnobotanist Richard Evans Schultes (1916–01), who, while doing his Ph.D. dissertation in the Mazatec region, learned about the use of hallucinogenic mushrooms commonly known by the Aztec name 'teonanacatl'. While continuing to work they devoted much of their spare time to the study of these 'enthogens'. R. G. Wasson ultimately became the first outsider to participate in a nightlong *velada*, a 'stay-awake' in the community of Huautla de Jimenez, Mexico. These experiences were publicized very widely and in 1957 they were even reported in detail in *Life* magazine.



4 Psilocybin



5 LSD

The last two persons who were involved in the discovery of the new leads were Swiss chemist Albert Hofmann (1906–08) and natural product chemist Robert F. Raffauf (1916–01). Phytochemical studies indicated that the pharmacological activity is due to relatively simple alkaloids, especially psilocybin (**4**), which is a phosphate salt in the fungi, and the *in vivo* active metabolite psilocin. Hofmann developed a semisynthetic derivative – lysergic acid diethylamide (LSD) (**5**), which was to be developed as a psychoactive medication and

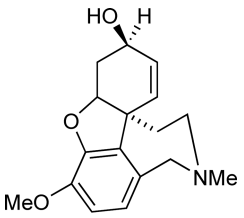
which also shows structural similarities to the ergot alkaloids. The compound is structurally also closely related to other indole alkaloids like ergotamine from the sclerotia of *Claviceps purpurea* (ergot), a compound also developed on the basis of local (European) knowledge. The expectations for developing new drugs based on this ethnomycological information were ultimately not met, but the compound became one of the most problematic drugs of abuse. The species that yield these compounds are popularly used as mind-altering drugs (e.g., *Lophophora williamsii* (Lem. ex Salm-Dyck) Coult., a Cactaceae, and the 'magic mushrooms' (*Psilocype* and related genera) discussed above). In regions of study, drastic sociocultural changes were the result of these research projects, especially because of the popularization of this sacred and specialized information and the subsequent influx of nonnatives.

Galanthamine (syn. galantamine, **6**) is a natural product known from several members of the amaryllis family (Amaryllidaceae) and the idea for developing a natural product from these species seems to be based on the local use of one of these species in a remote part of Europe[29] (ethnobotanical information). Today, galanthamine (esp. under its brand names Reminyl and Nivalin) is commonly used in the treatment of Alzheimer's disease. This example highlights both the uncertainties and problems of linking information about local and traditional uses with a compound's development. Broadly, speaking the development of galanthamine into a widely used Alzheimer's drug can be divided into three main periods:

- Early, development in Eastern Europe for use in the treatment of poliomyelitis
- Preclinical, development in the 1980s into an Alzheimer's medication
- Clinical, development in the 1990s

In the context of this review, the first phase is of particular relevance. The early development of galanthamine in Eastern Europe for use in the treatment of poliomyelitis started with the alkaloid's isolation from the garden snowdrop (*Galanthus* spp., most notably *G. woronowii*), but today the compound is obtained from other members of the same plant family like the daffodil (*Narcissus* spp.) and the snowflake (*Leucojum* spp., esp. *L. aestivum*) as well as being made synthetically.

Galanthus species are native to many parts of Europe including Bulgaria, the eastern parts of Turkey, and the Caucasus mountain range. Overall, little is known about the local and traditional uses of this genus in Europe. A. Plaitakis and R. C. Duvoisin[30] hypothesize that Homer's 'moly' might have been the snowdrop, *Galanthus nivalis*. In his epic poem the Odyssey, he described 'moly' and its use by Odysseus as an antidote against Circe's poisonous drugs. Thus the description of 'moly' as an antidote in Homer's Odyssey may represent the oldest recorded use of *Galanthus*, but the evidence is scanty. The 'classical' medicobotanical texts of the sixteenth century (i.e., Fuchs, Bock, and Brunfels) do not mention the snowdrop (*G. nivalis*) and make only cursory reference to *Leucojum*. Interestingly, the German pharmacognosist G. Madaus[31] does not mention *Galanthus* or *Leucojum* and only discusses *Narcissus pseudonarcissus*, giving some isolated uses that have no direct association with the Central nervous system (CNS), whereas Marzell[32] does not discuss any of the three genera. In F. Köhler 'Arzneipflanzen',[33] practically no medical use is given for species of the three genera. Thus, it is certain that *Galanthus* and other genera of the Amaryllidaceae were not commonly used European medicines. On the contrary, this clearly does not exclude local and traditional uses in rural regions of Europe and Asia.



6 Galanthamine

According to unconfirmed reports, in the 1950s, a Bulgarian pharmacologist noticed the use of the common snowdrop growing in the wild by rubbing on their foreheads to ease nerve pain. Also, some of the earlier publications indicate extensive use of snowdrop in Eastern Europe, such as Romania, Ukraine, Balkan Peninsula, and Eastern Mediterranean countries. However, we were unable to trace down any relevant

ethnobotanical literature. In the first pharmacological publication on galanthamine (**6**), no reference is made to the traditional use of snowdrop in the Caucasian region by the Russian authors.[34] An interesting note comes from the London pharmacognosist E. J. Shellard[35] and was published as a letter to the editor of the *Pharmaceutical Journal* (UK): He recalls a presentation in 1965 by "a Russian pharmacognosist reporting about a peasant women living at the foot of the Caucasian mountains (Southern Russia, Georgia) who, when their young children developed symptoms of an illness which, as he described them, was obviously poliomyelitis, they gave them a decoction of the bulbs of the Caucasian snowdrop (*Galanthus woronowii* Los) [sic] and the children completely recovered without showing any signs of paralysis".[35] This is one of the few, secondhand reports currently available recording the use of snowdrop prior to the development of galanthamine as a licensed medicine (see **Table 1**). Systematic exploration by the author with colleagues from central Europe and Russia resulted in one additional, but still secondhand review. According to Teodora Ivanova of the Bulgarian Academy of Sciences (personal communication, 2008), an alcoholic extract of *L. aestivum* L. was used by her grandparents and other older people in the eastern parts of Bulgaria. The extract was reported to be used in the prevention or treatment of memory loss, but because this record postdates the introduction of galanthamine as an Alzheimer's medication onto the worldwide market, this report may not actually be a secondary outcome of the species' use to extract galanthamine for clinical use.

Most of the early investigation on galanthamine was conducted in Bulgaria and the USSR during the coldest period of the Cold War. In the early 1950s, the Russian pharmacologist Mashkovsky worked with galanthamine isolated from *G. woronowii*. In 1951, M. D. Mashkovsky and R. P. Kruglikojva-Lvov used an *ex vivo* system (rat smooth muscle) to prove its acetylcholine esterase (AChE)-inhibiting properties. Consequently, this is the first published work that proves AChE-inhibiting properties of galanthamine. In 1952, N. F. Proskurnina and A. P. Yakovleva established and published the chemical structure of galanthamine as an alkaloid with a tertiary nitrogen atom, again based on material isolated from *G. woronowii*. Also, the compound's physicochemical characteristics were determined.[36] In 1955, Mashkovsky published a second paper on the

Table 1 Historical development of galanthamine as a clinically used drug

Year	*Development step of galanthamine*
Early 1950s	Russian pharmacologist discovers that local villagers living at the foot of the Ural mountains use wild Caucasian snowdrop to treat (what he considers to be) poliomyelitis in children
1952	Galanthamine was first isolated from *G. woronowii*
1956	D. Paskov suggested that galanthamine can be extracted from the leaves of *Galanthus*
Late 1950s	Various preclinical studies on the pharmacology of galanthamine were carried out. For instance, i. Galanthamine was found to have antagonistic effects against nondepolarizing neuromuscular blocking agents. This has been shown in experiments on neuromuscular preparation of cats *in situ*, in experiments *in vitro* on frog rectus abdominis muscle, etc. ii. *In vivo* and *in vitro* experiments were done in rats for determining the effects of galanthamine on the brain Galanthamine was registered as a medicine under the trade name 'Nivalin' and is commercially available in Bulgaria
Early 1960s	The first data on anticholinesterase activity of galanthamine was reported from an *in vivo* study in an anesthetized cat
1980s	Preclinical development: Researchers searching for novel treatments of Alzheimer's disease started investigating the therapeutic effects of galanthamine
1990s	Clinical development of galanthamine into a medication for Alzheimer's disease
1996	Sanochemia Pharmazeutika obtained the first patent on the synthetic process of galanthamine
1997	Sanochemia began collaboration with a Belgium-based company (Janssen Pharmaceutica) and an emerging British company (Shire Pharmaceuticals Group plc)
2000	Galanthamine licensed in the first countries (Iceland, Ireland, Sweden, UK) for the treatment of Alzheimer's disease
Currently	Galanthamine has been approved for use in the United States, many European countries, and many Asian countries. Controversies remain over the therapeutic benefits of acetylcholinesterase inhibitors, since they delay the onset of more severe symptoms and offer no curative treatment

Adapted from M. Heinrch; H. L. Teoh, *J. Ethnopharmacol.* **2004**, 92, 147–162.

cholinesterase-inhibiting properties of galanthamine. Unfortunately, Mashkovsky does not indicate the source of the galanthamine used, but most probably Mashkovsky worked again with galanthamine isolated from *G. woronowii*. In 1956, the Bulgarian pharmacologist D. Paskov discovered galanthamine in the European daffodil and in the common snowdrop, *G. nivalis*. Paskov suggested extracting galanthamine from the leaves of *G. nivalis*. In 1957, this scientist, who trained in Russia under Mashkovsky, published his results from the study of *L. aestivum* (summer snowflake) and its content of galanthamine, which was to become the main source of the compound. In 1960, a full chemical synthesis was published. This was a biomimetic laboratory process with a yield below 1% and had been designed as proof of structure, not for industrial production.[29]

The indication polyomyelitis, which was the main indication in the Eastern Block from 1950 until a few years ago, came as a result of the data that galanthamine enhances nerve impulses transmission at the synapses. In the form of hydrobromide salt, it became commercially available as a registered product under the trade name 'Nivalin'. Furthermore, galanthamine shows extremely potent antagonizing action against curare (D-tubocurarin; Nikolev, personal communication, 2003).

Many preclinical studies were carried out in animals for testing the pharmacological activity of galanthamine. After a few years, some researchers demonstrated the penetration of galanthamine through the blood–brain barrier, and thus effects on the CNS became of particular interest. Based on the knowledge of galanthamine in both peripheral nervous system and CNS, many countries in Eastern Europe had used it as an acknowledged treatment in myasthenia gravis and muscular dystrophy, residual poliomyelitis paralysis symptoms, trigeminal neurologia, and other forms of neuritides.

Overall, this is not only an example of the successful ethnobotany-driven development of a natural product into a clinically important drug, but also highlights that it is often difficult to establish the link between local and traditional uses and drug development. Ethnobotany gave an essential, initial hint, but at this point the evidence where the initial ethnobotanical information comes from remains scanty.

A second case relates to a pharmaceutical product that in many countries is not considered to be a medicine, while in others it has been one of the best selling herbal medicines – a special extract obtained from the leaves of *Ginkgo biloba* L. The most important use of *Ginkgo* is in age-related disorders. It is especially used to prevent or reduce memory deterioration and milder forms of dementia including the early stages of Alzheimer's disease. It enhances cognitive processes, and experimental evidence points to improvements in blood circulation to the brain and anti-inflammatory and antioxidant effects.

The species is a living fossil and has survived in China, where it is found mainly in monasteries in the mountains and in palace or temple gardens. In Asia, *Ginkgo* is an object of veneration, and is considered a sacred tree of the East; it has been seen by some as a symbol of changelessness, possessing miraculous power, bearer of hope and of the immeasurable past, a symbol of love, and unity of opposites. Because of all its properties, it is associated with longevity. Buddhist monks cultivated the tree from about AD 1100 for its many good qualities. It was spread by seed to Japan (around AD 1192, associated with Buddhism) and Korea. In the oldest Chinese literature, *Ginkgo* is not mentioned, but in the eleventh century (Sung dynasty) it appeared in the literature as a plant native to Eastern China. When *Ginkgo* was transplanted in the residence of Prince Li Wen-ho in the first half of the eleventh century, came from the south and by transplanting it in his residence, it became famous and spread through propagation. From that time on, *Ginkgo* has been depicted in Chinese paintings and appeared in poetry. Scientists thought that *Ginkgo* had become extinct, but in 1691, Engelbert Kaempfer, a German naturalist, discovered *G. biloba* trees in Japan, and in 1730 it was brought to Europe (Utrecht).

The earliest known medicinal use dates back to 2800 BC and is described as the pseudofruits of *G. biloba*. There are many historic and modern medical uses of the pseudofruit. Interestingly, the leaves are much less frequently used in Eastern Asia One use is to treat chilblains (reddening, swelling, and itching of the skin due to frostbite) and as a throat spray for asthma. Europeans were fascinated by this tree since they first discovered it[37] because it symbolizes longevity and its leaves have a unique structure. It fascinated poets and scientists alike, including the famous German poet and natural historian J. W. von Goethe:

This leaf from a tree in the East,
Has been given to my garden.
It reveals a certain secret,
Which pleases me and thoughtful people.

Does it represent One living creature
Which has divided itself?
Or are these Two, which have decided,
That they should be as One?
To reply to such a Question,
I found the right answer:
Do you notice in my songs and verses
That I am One and Two?[38]

Ginkgo contains two major types of pharmacologically active constituents – diterpene lactones, for example, ginkgolides A, B, and C and bilobalide, as well as flavonoids, the most important being the biflavone glycosides such as ginkgetin, isoginkgetin, and bilobetin, which also contribute to its activity. Ginkgolic acids are present in the fruit but normally only in very minor amounts in the leaf. Based on some not very well documented uses in traditional Chinese medicine (TCM), a German company, Dr. Willmar Schwabe Pharmaceuticals, first developed a poorly characterized ethanolic and later a 'special' extract – extract *G. biloba* (EGb) 761 – which is based on an ethyl acetate extraction and subsequent fractionation. The extract was developed into a highly successful phytomedicine. Unfortunately, the history of development of this extract is not well documented, and little information seems to be available within the company. Initial research in the mid-1960s identified flavonoid glycosides as active constituents of *G. biloba* leaf extracts. In 1971, the first patent on the complete extraction and standardization was filed in Germany and a year later in France.[39] These patents describe the process for obtaining a 'mixture of vasoactive substances' and formed the basis for the highly successful clinical development for the indications listed above.

This example is of interest, because it highlights that the symbolic importance both in Asian and European countries was a driving force for developing this into a medication. There may not have been a direct link between the traditional use and modern European medical use, but species association with longevity presumably has provided the ideas for pharmacological experiments, which ultimately resulted in the development of a 'rational phytomedicine'.[40] Also, this example is the first one that highlights the development of a standardized extract for use as a medicine based on traditional knowledge systems (in this case, TCM) into an over-the-counter herbal medical product. In later years, numerous similar development projects resulted in novel phytomedicines including *Hypericum perforatum* L. (St. John's Wort, Hypericaceae) used for mild to moderate depression, *Harpagophytum procumbens* (Burch) DC. (Devil's Claw, Pedaliaceae) used for chronic pain, and *Piper methysticum* G. Forst. (kava kava, Piperaceae) for relieving anxiety. *P. methysticum*, for example, originates from many Pacific islands. Best known is its religious and/or symbolic use.[41] It is consumed under very strict sociocultural control. On many islands, for example, the local leader is the only or at least the first one to drink it. It is often prepared by chewing the root and rootstock and then spitting the mixture into a large bowl. According to local Pacific traditions, *P. methysticum* is the ideal species to overcome social tensions and to help to (re-)establish proper social relations. This offered a clear and direct lead for developing a phytomedicine, which for many decades, but especially since the 1960s, has been used as a mild stimulant and has been a widely acclaimed treatment for this condition. However, in 2001, kava kava-containing drugs were withdrawn from practically all markets due to suspected hepatotoxic effects. In this therapeutic area as in many other areas, ethnopharmacology-driven drug development continues to be an exciting opportunity. Recently, over 150 plant species in various preparations and mixtures with the potential for R&D on developing new drug leads for age-related cognitive disorders were found by systematically assessing the information available in Swiss university libraries.[42]

3.12.2.3.2 Anticancer agents developed between 1950 and 1980

Etoposide (Vepesid, **8**) and teniposide (VM-26, **9**) are well-known topomerase II inhibitors. Both are semisynthetic derivatives of podophyllotoxin first isolated from *Podophyllum peltatum* L., a native American remedy for warts, and is used as a purgative. Ethanolic extracts of the rhizomes are known as Resina podophylli (podophyllin). This resin was included in many pharmacopoeias for the topical treatment of warts and benign tumors (*condylomata acuminate*) (and as Podophyllum Resin is still included in some pharmacopoeias like the *British Pharmacopoeia*).[43] It is highly irritating and unpleasant and therefore can only be used topically.

Podophyllotoxin (**7**) was first isolated in 1880 and its structure was proposed in 1932. Clearly, this usage was one of the reasons for the species' selection for anticancer screens. This natural product is also found in other *Podophyllum* species like *P. hexandrum* Royle (syn. *P. emodi*, Berberidaceae) from India and China.

The second case is the vinca alkaloids – vinblastine (**10**), vincristine (**11**), and navelbine (**12**) – from *Catharanthus roseus* (L.) G. Don (formerly called *Vinca roseus*, Madagascar periwinkle, Apocynaceae).[44] As the name indicates, the species is originally from Madagascar, but researchers at the National Cancer Institute (NCI) of the United States actually worked with samples collected in the Caribbean, where the plant was used locally to treat diabetes. By the early twentieth century, it was used as an oral hypoglycemic agent (to lower blood sugar levels) in South Africa, Southern Europe, and the Philippines to treat diabetic ulcers in the British West Indies and in Brazil to control hemorrhages and scurvy. It was the role of the plant as an antidiabetic agent in the Caribbean that led to the discovery of its effective anticancer activity. In 1952, a patient from Jamaica sent a sample of the plant to Dr. Clark Noble, a canadian researcher, who forwarded it to his brother Dr. Robert L. Noble (at the University of Western Ontario) and Dr. J. B. Collip, researchers who helped refine insulin.[44] This prompted a small scientific study, which found that rats given tea, which was made from crushed Madagascar periwinkle from which 'vinblastine' was isolated, had a significantly lowered white blood cell count. Although this mixture was fatal to the rats, this action prompted the interest of the researchers to assess the action of the Madagascar periwinkle against leukemia[44] – a disease caused by an abnormal increase in white blood cells, first reported in 1958. The active principle was identified vinblastine, a new alkaloidal compound. Vinblastine was licensed in the United States and approved for use in cancer treatments in 1961. Prior to this, industrial processes for isolation had to be developed, a task taken on by Eli Lilli Co. under the scientific leadership of the chemist Gordon Svoboda and collaborators, who were also instrumental in identifying a related alkaloid from the same plant, vincristine, which was licensed as a drug 2 years later.[37] Vince alkaloids bind to β-tubulin and inhibit microtubule assembly. Vindesine and vinorelbine are novel vinca alkaloid derivatives with improved clinical features for tumor therapy.[45]

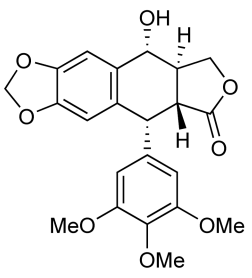
The previous example highlights how difficult it is to establish retrospectively whether a compound has had local and traditional uses and specifically whether vinca alkaloids are directly linked to the therapeutic uses of the compound in biomedicine.[46] The most recent clinically significant discovery from the NCI screening program is taxol (**13**), from *Taxus brevifolia* Nutt. (Taxaceae). It has been argued many times that this discovery was not ethnobotany driven, but considerable evidence highlights the importance of *T. brevifolia* in native American medicine. Even though the initial sample was collected as part of a random sampling approach, *T. brevifolia* has been reported to be used by a variety of western Indian groups (USA and Canada) as a medicine and also for producing a variety of other useful products (canoes, brooms, combs). Very diverse ethnopharmaceutical uses of the root and the bark are recorded and include several reports for stomachache and only in case of the Tsimshian tribe (British Columbia, Canada) in the treatment of cancer.[47] Thus, unbeknown to researchers, the Tsimshian selected a plant with a high cultural salience in many western North American cultures. This example highlights the fact that species used to isolate medicines are highly likely to have traditional uses.[48]

It showed activity in the NCI's cancer screening platform, and the core compound taxol was first isolated in the mid-1960s by Monroe Wall (1916–2002), Mansukhlal C. Wani, and coworkers. After some initial research, the project was halted in 1971. In 1977, its activity against a melanoma cell line and in the human xenograft model led to the start of preclinical development. Initially, there were problems in acquiring large amounts of the compound, but solutions to these problems and the report of taxol's unique mode of action by promotion of tubulin polymerization and stabilization of microtubules against depolymerization increased the interest. Clinical studies started in 1984. Prior to this, studies on the compound's toxicology and the pharmacological mechanism of action were conducted. It took a further 10 years before taxol was approved by the FDA in the treatment of anthracyclin-resistant, metastasis-forming breast cancers. Taxol has excellent activity against ovarian and breast cancers, but it also has serious side effects. In the meantime, the compound has been approved for a variety of other cancers and now semisynthetic derivatives are also employed.[49] Although it is generally considered to be a metabolite of *Taxus* sp. and associated endophytic fungi, taxol was also found in shells and leaves as well as in cell cultures of *Corylus avellana* L. (the hazelnut shrub, Betulaceae). In addition to taxol, 10-deacetylbaccatin III, baccatin III, paclitaxel C, and 7-epipaclitaxel were also identified and quantified in shells and leaves. The finding of these compounds in shells, which often are waste products of mass production in the food industries, may open new avenues of supply for this anticancer agent.[50]

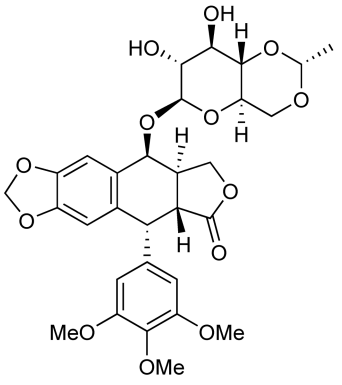
Even though the initial sample was collected as part of a random sampling approach, local and traditional uses clearly predate the R&D activities of the NCI and associated researchers. The fact that the local and traditional knowledge on *T. brevifolia* was not known to these researchers may indicate that it is the outcome of a random screen, but clearly the fact that ethnopharmacologically preselected species were developed highlights that such local and traditional knowledge is an excellent starting point for drug development.

Camptotheca acuminata Decne (Xi Shu, tree of joy, Nyssaceae) is widely used in TCM and, therefore, was included in 1958 in a screening program at the NCI where it gave positive results. Wood and bark (20 kg) were collected for extraction; These extracts were shown to be active against a mouse leukemia life prolongation assay in which it was unusual to find activity. The fractionation and anticancer testing was a very slow process and finally resulted in the isolation and structure elucidation (in 1966) of camptothecan (**15**), a highly unsaturated quinoline alkaloid with a unique (at the time) structure as an α-hydroxylactone. *C. acuminata* was shown to be extremely active in the life prolongation assay of mice treated with leukemia cells and in solid tumor inhibition. These activities encouraged the NCI to initiate clinical trials with the water-soluble sodium salt. While the results of some studies conducted in the United States were disappointing, in a clinical trial in China with 1000 patients the sodium salt showed promising results, for example, against head, neck, gastric, intestinal, and bladder carcinomas.[28]

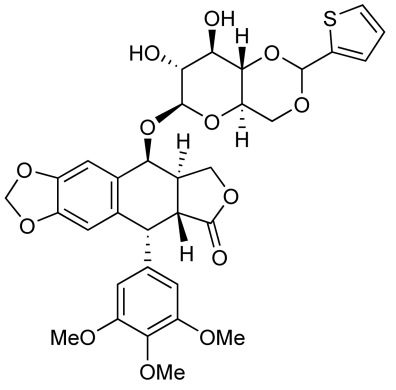
As these examples show, the taxanes (taxol, **13**, and taxotere, **14**), agents derived from podophyllotoxin (etoposide and teniposide), the vinca alkaloids (vinblastine, **10**, and navelbine, **12**), and the camptothecine (**15**)-derived anticancer agents (topotecan, **16**, and irinotecan, **17**) all exemplify a similar situation. The drugs, which yielded the anticancer agents (and ultimately their derivatives), were all important medicines in their respective cultures. Although this may have not been recognized at the time of initial discovery, it is an astounding fact that all species of plants have a tradition of medical use. Researchers may not have known it at the time of their research, but they followed a path healers in the various cultures had taken many generations before them.



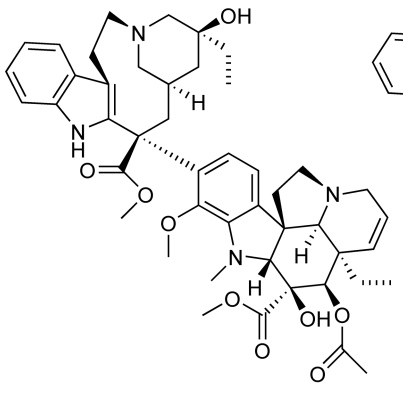
7 Podophyllotoxin



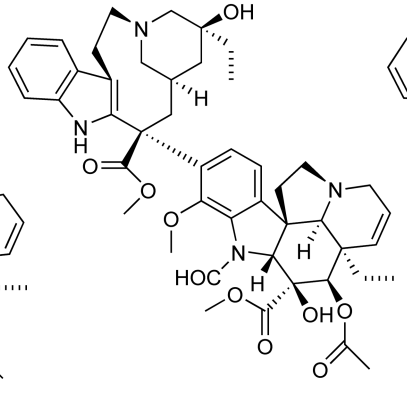
8 Etoposide



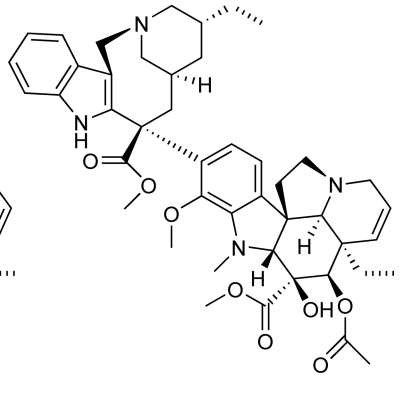
9 Teniposide



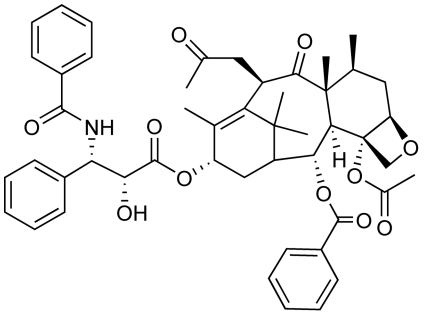
10 Vinblastine



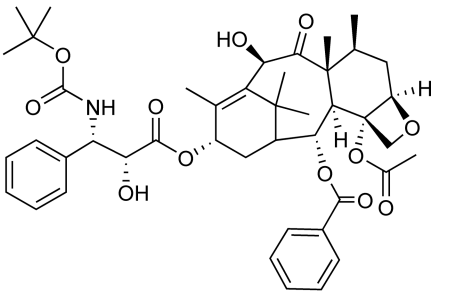
11 Vincristine



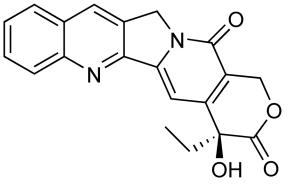
12 Navelbine



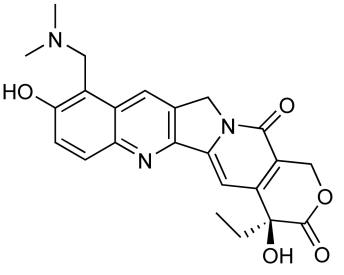
13 Taxol



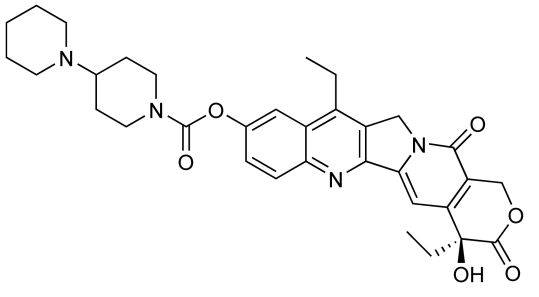
14 Taxotere



15 Camptothecine



16 Topotecan



17 Irinotecan

3.12.2.4 The Changing Legal Framework: The Convention on Biological Diversity (1992)

In recent years, more direct benefits for the providers (the states and their peoples) have become a core element of discussion. Ethnobiological research and any other research involving the use of biological resources of a country are today based on agreements and permits, which in turn are based on international and bilateral treaties. The most important of these is the Convention of Rio or the Convention on Biological Diversity (CBD),[51] which looks at the rights and tasks associated with biodiversity at an international level:

> The objectives of this Convention, to be pursued in accordance with its relevant provisions, are the conservation of biological diversity, the sustainable use of its components and the fair and equitable sharing of the benefits arising out of the utilisation of genetic resources, including by appropriate access to genetic resources and by appropriate transfer of relevant technologies, taking into account all rights over those resources and to technologies, and by appropriate funding.

The rights of indigenous peoples and other keepers of local knowledge is addressed in article 8j:

> (j) Subject to its national legislation, respect, preserve and maintain knowledge, innovations and practices of indigenous and local communities embodying traditional lifestyles relevant for the conservation and sustainable use of biological diversity and promote their wider application with the approval and involvement of the holders of such knowledge, innovations and practices and encourage the equitable sharing of the benefits arising from the utilization of such knowledge, innovations and practices.

This and the subsequent treaties significantly changed the basic conditions for ethnopharmacological research. Countries that provide resources for natural product research and drug development have well-defined rights, which specifically includes sharing benefits that may potentially arise from such research.

Especially in case of ethnopharmacological research, the needs and interests of the populations a researcher is collaborating with also become an essential part of the research.[5] As pointed out many times, 'there is an inextricable link between cultural and biological diversity'. This principle was first formulated at the First International Congress on Ethnobiology in Belem in the year 1988. No generally agreed upon standards have so far been accepted, but the importance of obtaining the informants' prior informed consent and ascertaining appropriate benefit-sharing agreements has been stressed by numerous authors (e.g., Posey[52]), even though the exact requirements of such arrangements sometimes remain contentious.

Numerous other agreements (like TRIPS (trade-related aspects of intellectual property rights), WTO (World Trade Organization) agreements, cf. www.wto.org) are also of relevance, but it is beyond the scope of this chapter to address the complexity of national and international agreements.

3.12.2.5 The Revolution of Molecular Biology: From the 1990s Until Today

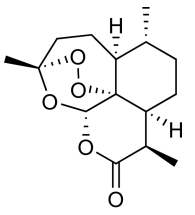
The previous examples (Sections 3.12.2.1–3.12.2.3) also highlight the shift from organism- or cell-based screening system, which was the mainstay of drug development until about the 1980s, to a more biochemical–mechanistic approach. This chapter highlights projects that have come into fruition in the last years and that extensively use modern molecular–biological approaches. Also, these examples emphasize the central role of the Convention of Biological Diversity and related agreements in the drug discovery and development process.

3.12.2.5.1 Antiparasitic and insecticidal agents

Quinine has been one of the first biologically active natural products to have been isolated and has had a tremendous impact on drug development programs (see above). Similarly, the discovery of artemisinin and its analogues as potent antimalarial agents has been among the prime examples of ethnopharmacology-driven drug discovery. Recently, the alkaloid cryptolepine from the west African *Cryptolepis sanguinolenta* (Lindl.) Schltr., used traditionally in the treatment of malaria, has received considerable attention. In 2005, these examples were reviewed by C. W. Wright.[53] This is an area of drug discovery where *direct* ethnopharmacological links have been well documented. For hundreds of years, *Artemisia annua* L. (Asteraceae, Qing Hao) has been used in TCM. The leaves were harvested in the summer, before the plant comes into flower, and dried for later use. It is generally used in the treatment of fever, malaria, colds, diarrhea, as a digestive, and, externally, as a vulnerary.

Artemisia annua has been known since the *Zhou Hou Bei Ji Fang* – (*Handbook of Prescriptions for Emergency Treatment*) of Ge Hong of AD 340 as a treatment for fevers. In 1967, a group of Chinese scientists started a search for new antimalarial drugs from Chinese medicine. Only in 1977 did a Chinese research group isolate the active principle, the sesquiterpene lactone artemisinin,[54] which proved to be very potent against the malarial parasite *Plasmodium falciparum* and especially against chloroquine-resistant malaria.[55] The development of this sesquiterpene lactone with a highly unusual endoperoxide moiety was based directly on traditional and local knowledge. Clinical trials in China in a large number of patients showed that artemisinin (**18**) was highly effective in clearing parasitemia and reducing symptoms in patients with malaria, including some with chloroquine-resistant malaria and/or cerebral malaria.[53] However, for many years, lack of funding was a major problem in this area (see below). Interestingly, the compound also shows considerable promise as an

anticancer agent.[45] In an attempt to overcome the problem of the recrudescence (1 month after the treatment, many patients have a recurrence of the illness), a number of derivatives of artemisinin (**18**) have been developed (ethers, such as artemether and arteether, and esters, such as sodium artesunate and sodium artenlinate). Although the compound is used as a first-line treatment, combination therapies are generally considered to be the best available choice. One core problem that has plagued the treatment of tropical diseases remains the limited access of the poor to such effective treatments[56] and a continuous lack of funding for natural product-based drug development. However, such locally based drug development projects would also offer unique advantages once the results of preclinical and clinical work were implemented locally.[57,58]



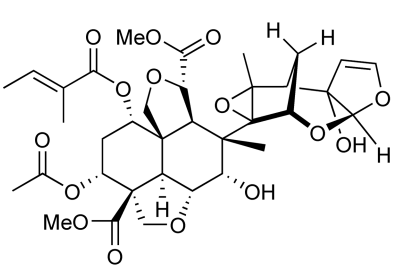
18 Artemisinin

Although it was not developed based on the concepts of molecular biology, *Azadirachta indica* A. Juss. (syn. *Melia azadirachta*, *Antelaea azadirachta*), or neem, has become a classical case of a drug development process rife with controversies regarding the species' traditional use. It is a principal species used within Indian Ayurvedic medical traditions and today is a pan(sub-)tropically grown tree. Neem is thought to have originated in the northeastern region of India (Assam) and in Burma/Myanmar. The exact location of origin is uncertain. It has been attributed to the entire Indian subcontinent and others to dry forest regions throughout all South and Southeast Asia, including Pakistan, Sri Lanka, Thailand, Malaysia, and Indonesia. The introduction of neem to East Africa is thought to have arisen during the construction of the Kenya–Uganda railways. Indian migrant workers are believed to have brought neem seed with them in order to cultivate this important medicinal plant.

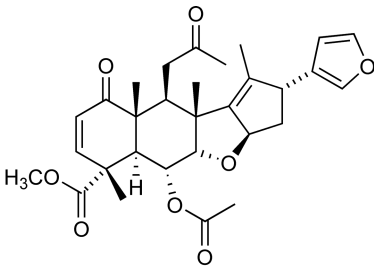
The species is drought resistant and thrives in arid conditions with an annual rainfall between 400 and 1200 mm. It can grow between 0 and 1500 m above sea level but is intolerant to freezing, extended periods of cold, and waterlogged soils. Neem trees can reach a height of 25–30 m and provide valuable shade with its dense canopy of pinnate leaves. Consequently, it is a species that has become planted or naturalized in many countries.

The neem tree possesses a kaleidoscope of medicinal uses that are found in all parts of the plant. As part of Ayurvedic medicine, the leaves (5–10) are chewed for 15 days in late winter in order to maintain a healthy body. Tonics prepared by boiling the leaves, often with other herbal constituents, are useful against intestinal worms, fevers, and internal ulcers. Externally, the juice of the leaves is applied to the skin for the treatment of boils and eczema. The twigs are used extensively in dental hygiene to brush the teeth and incorporated into pastes or mouth washes for sale on markets. Neem fruits are used against leprosy, intestinal worms, and urinary diseases. Neem oil (Margosa) is a chemically diverse mixture that includes the isoprenoid azadirachtin and a complex mixture called nimbidin (which contains nimbin,[59] **20**) plus numerous fatty acids such as oleic and palmitic acids. The oil is used for chronic skin complaints, leprosy, and ulcers; it is commercially marketed as a natural botanical insecticide. Azadirachtin is the main insecticidal ingredient of neem.[38]

Many controversies surround the development of this traditional insecticide and medicine: In 1992, the U.S. company W. R. Grace applied for a patent to extract seeds of the neem tree in a simple manner. The plant material is extracted with a lipophilic solvent (e.g., ethyl ether) instead of with a watery one, as it has been done for many centuries in India, resulting in an increased stability. However, is this really an innovation? American patent law does not recognize oral traditions like the Indian ones and approval of such a patent would have, for example, resulted in the exclusion of Indian companies from the U.S. market. This patent and some related ones have been revoked, but the overall conflict continues.[60]



19 Azadirachtin

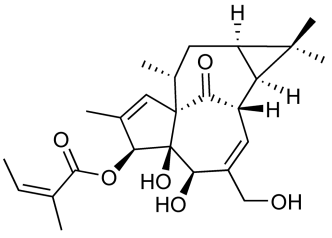


20 Nimbin

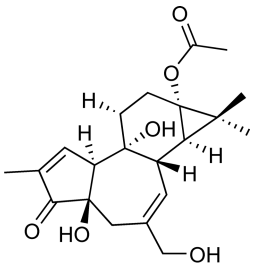
3.12.2.5.2 Antiviral and anticancer agents

Peplin Ltd. in Queensland, Australia, currently manufactures ingenol 3-angelate (or PEP005; **21**), an unusual diterpene ester isolated from *Euphorbia peplus* L. (Euphorbiaceae) or petty spurge/radium weed/cancer weed. Most advanced are studies on the topical use for treating actinic keratoses and nonmelanoma skin cancer. In addition, it was developed for intravesicular treatment of bladder cancer systemically against leukemia. *E. peplus* was widely used in Europe and Morocco[62] to treat warts and other skin conditions. The species was introduced into Australia and many other temperate countries. During the 1970s and 1980s, members of the Australian public used the sap from *E. peplus* to treat skin cancers and solar keratoses.[63] A. C. Green and G. L. Beardmore[63] reported that in Brisbane, Australia, *E. peplus* is the second most commonly used plant product treating these conditions. Only *Aloe vera* was used more frequently (35 reports). Overall, there were 164 persons (out of 2095 respondents) who indicated that they self-treated for skin cancers and solar keratoses. Of these, 75 used herbal medicines, whereas 8 used *E. peplus*.[63] Another commonly used treatment was *Carica papaya* (8 reports). Although this is a relatively small number, it clearly served as a starting point to investigate the species' medical effects[64], proving that this R&D project was clearly ethnopharmacologically driven. Ingenol 3-angelate (PEP005) had an initial LD_{90} of 180–220 $\mu mol\,l^{-1}$ against a range of human and mouse cell lines. *In vivo* experiments using various tumors transplanted into mice indicated that a topical application for 3 days of 42 nmol formulated as an isopropanol-based gel was the most effective. The compound induced an acute erythema. Mechanistic studies indicated a rapid disruption of the plasma membrane, swelling of mitochondria, and cell death via primary necrosis. Experimental evidence exists that, at a second stage, neutrophil-mediated antibody-dependent cellular toxicity plays an important role. *In vitro*, ingenol 3-angelate has potent antileukemic effects in a large number of cell lines, inducing apoptosis in myeloid leukemia cell lines and primary acute myeloid leukemia cells at nanomolar concentrations.[65] It was then established that this activity is correlated with the expression of PKC-δ (protein kinase δ). Interestingly, it induced a translocation pattern of PKC-δ different from that of the well-known tumor copromoter PMA (phorbol 12-myristate-13-acetate (also known as PTA)). At low concentrations (10 $nmol\,ml^{-1}$), ingenol 3-angelate induces a rapid translocation of PKC-δ *simultaneously* to the internal membranes and the nuclear membranes. PMA, on the contrary, causes PKC-δ first to translocate to the plasma membrane and then to the nuclear membrane.[64] In addition, ingenol 3-angelate modulates the activity of targets in the nuclear factor kappaB (NF-κB) pathway. This activity is complex and time dependent. Up to 6 h after application of ingenol 3-angelate, a biphasic activation of p65 and, to a lesser degree, C-Rel, was observed.[64] As of 2008, phase III clinical trials of topical use are planned.

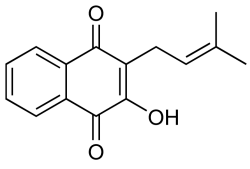
This example offers some amazing insights into the complexity of modern drug discovery, especially as it relates to the ethnopharmacological links of the research. Without doubt, this discovery was driven by local and traditional knowledge. It is based on European 'indigenous' knowledge, which clearly had been passed on from generation to generation and both the plant and its usage traveled with the Europeans to Australia. As claimed by the researchers and the company involved in the discovery,[66] the initial idea goes back to usage in Brisbane, Australia. If, hypothetically, this would have been a species brought back by the Europeans from India or what is now Spanish speaking America, this discovery would certainly spark a fierce discussion about the ownership of traditional knowledge. At a pharmacological–clinical level, this discovery highlights the potential to move from one therapeutic field (in this case, topical uses for various forms of skin cancer and precancerous conditions) to other therapeutic uses linked only indirectly with the original use.



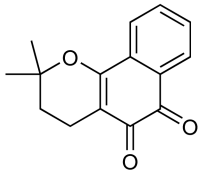
21 Ingenol 3-angelate



22 Prostratin



23 Lapachol



24 β-Lapachone

Another promising, structurally related, lead is derived from a second Euphorbiaceae, *Homalanthus nutans*, a small rainforest tree used by Samoan healers to treat hepatitis. Its extracts exhibited potent activity in an *in vitro*, tetrazolium-based assay to detect cytopathic effects on HIV-1.[67] It yielded a unique non-tumor-promoting protein kinase C (PKC) activator, prostratin (**22**), a 12-deoxyphorbol ester, which protects T-lymphoblastoid CEM-SS and C-8166 cells from death due to HIV-1 infection.[3] The compound was first isolated and its structure reported in 1992; thus, this discovery predates that of peplin. Williams *et al.*[68] demonstrated that prostratin effectively activates HIV gene expression in latently infected Jurkat cells and that it acts by stimulating IKK (IκB kinase)-dependent phosphorylation and degradation of IκBα, leading to the rapid nuclear translocation of NF-κB and activation of the HIV-1 long terminal repeat.[69] Ultimately, prostratin induces the HIV virus to leave cells and thus makes a silent virus accessible to medication. Both ingenol 3-angelate and prostratin rapidly inhibit the HIV virus from infecting cells at an early point in infection.[68] Prostratin has been offered for licensing by the NCI as a candidate anti-AIDS drug, with a significant portion of the potential license income to be returned to the Samoan people.

Betulinic acid, a pentacyclic triterpene found in many higher plants including *Betula* spp. (where it is the most abundant secondary metabolite), was first shown to specifically inhibit the growth of melanoma cell lines. Traditionally, extracts from the *Betula* species have been used topically to treat a variety of inflammatory skin conditions. Species of the genus have been used in North America especially for a variety of gastrointestinal conditions (e.g., removing bile from the intestines, diarrhea, dysentery), as a blood purifier and diuretic, as a general tonic, and as an ointment for persistent scabs and rashes (Cree), gonorrhea (Cree. Iroquois), skin rashes (Algonquin, Cree), and infections (Micmac).[47] Members of the genus are also very widely used in Europe. Historically, uses for dropsy, wounds, and gout were reported, and today it is used popularly to promote hair growth and as a diuretic/cleansing agent.[70] *Betula* species are currently at the focus of a variety of projects on novel anticancer agents. No direct ethnobotanical link seems to exist between the traditional uses (i.e., as an anticancer agent) and modern biomedical research. This is not surprising, because only few species have recorded uses as anticancer agents. However, many of these uses imply that the extract will modulate the cell cycle, a property that is explored in the development of novel anticancer agents. *Betula* effectively induces apoptosis in neuroectodermal tumors and was shown to be a potent trigger of cell death in human leukemia-derived cell lines.[71,72] This activity is linked to the activation of NF-κB in a variety of cell lines. Consequently, combination therapies with NF-κB inhibitors would not be of therapeutic benefit,[73] but the drug may have potential if it is used in appropriate combinations. Its potential as an antiviral agent is also under investigation.[74]

The last example is a cure for cancer and tumors from South America. Red Lapacho tea is a canopy tree indigenous to the Amazonian rainforest, which for the first time during the 1960s attracted considerable

attention in Brazil and Argentina. Traditionally, the botanical drug is widely used in local and traditional phytomedicine, usually ingested as a decoction prepared from the inner bark of the tree to treat numerous conditions like bacterial and fungal infections, fever, syphilis, malaria, trypanosomiasis, and stomach and bladder disorders.

As early as 1873, biomedical uses of Red Lapacho (Pau D'Arco) were reported. In 1967, after reports in the Brazilian press, it came to the light of international attention as a 'wonder drug'. Also in the 1960s, the NCI looked at *T. impetiginosa* in considerable detail. Two main bioactive components have been isolated from *T. impetiginosa*: lapachol (**23**) and β-lapachone (**24**). β-Lapachone is considered to be the main antitumor compound, and proapoptotic effects were observed *in vitro*. Some mechanistic studies on this compound's molecular effects have been conducted. The botanical (drug) material available on international markets today seems to have varying quality and composition, making a specific assessment of the products' therapeutic claims problematic. Currently, no drug lead based on this species seems to be under development. The bioscientific evidence for products derived from *T. impetiginosa* is insufficient and highlights both the potential of such new leads and the risks of overstating a (botanical) drug's therapeutic potential based on limited (generally *in vitro*) data.[75]

3.12.2.5.3 Anti-inflammatory natural products

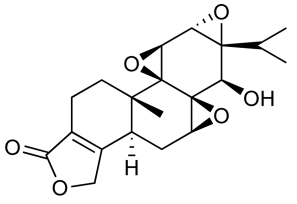
Several compounds are currently under development that may result in clinically approved medications for use in chronic inflammatory conditions. Preparations of *Tripterygium wilfordii* Hook.f. (Celastraceae) are part of the Chinese traditional herbal traditions (Radix Tripterygü)[76] and were first mentioned in the *Ben Cao Gang Mu Shi Yi* (1765, *Information about Medicinal Drugs: A Monographic Treatment*), the classic herbal encyclopedia produced by Li Shizhen (AD 1517–93) during the Ming dynasty. In TCM, it has the functions of dispelling the wind, dehumidification, promoting blood circulation and removing obstruction in channels, subsiding the swelling, relieving pain, killing insects, and detoxifying.[77] Preclinical and clinical development has focused on potential uses against cancer, chronic nephritis, hepatitis, systemic lupus erythematosus, ankylosing spondylitis, and a variety of skin conditions.[78] In TCM, a patient who has rheumatism would be regarded as having wind, be wet in the body as well as the blood, and her/his Qi being hindered. Dispelling the wind, dehumidification, promoting blood circulation and removing obstruction in channels and reducing the swelling and thus relieving pain are used to treat rheumatism.[76,77] Also, in TCM theory, the kidney is in charge of water, that is, is responsible for metabolizing human body water. Therefore, a Chinese doctor would use the functions of promoting blood circulation and removing obstruction in channels as well as inducing diuresis to alleviate edema to cure nephropathy.

Triptolide (**25**), a deterpenoid epoxide, is essential for the anti-inflammatory and immunosuppressive activities of extracts. As far as one can ascertain, based on uses in TCM, the drug was first further developed in China and then came to the attention of the international research community.

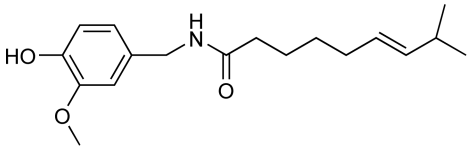
Triptolide inhibited inducible NO synthase (iNOS) gene expression by downregulating NF-κB's DNA-binding activity and the Jun N-terminal kinase (JNK) or stress-activated protein kinase (SAPK) pathway.[79] In other studies,[80] the extract of *T. wilfordii* or triptolide was shown to inhibit lipopolysaccharide (LPS)- and cytokine-induced expression of cyclooxygenase (COX)-2, MMP-3, and MMP-13 in articular chondrocytes, to inhibit the interleukin (IL)-1-, IL-17-, and tumor necrosis factor-α (TNF-α)-induced expression of the aggrecanase gene in human chondrocytes (triptolide), and to suppress the expression of adhesion molecules E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1).

An exciting example of research driven by traditional knowledge is the discovery of the transient receptor potential vanilloid type 1 protein (TRPV1). These channels were originally cloned while researchers were looking for a molecular target of the pungent compound capsaicin (**26**) from Mexican hot chili/chilli (spicy varieties of *Capsicum annuum* L. and *C. frutescens* L.) and the phorboid resiniferatoxin (RTX, **27**) from species of the genus *Euphorbia*.[81] Of course, chilli and paprika have long been used in Meso- and South American cultures, popular as a spice but also as a medicine including for chronic inflammatory conditions. *Capsicum annuum* (which often is less pungent than *C. frutescens*) originated from Mesoamerica and *C. frutescens* from the western Amazonian region or Bolivia,[82] but today both are part of a universal culture and are generally considered to be an integral part of the medical and culinary traditions on the Indian subcontinent. Chilli is a typical Balkan (Hungarian) spice. Multiple medical uses were recorded during the Aztec period, including as a remedy for dental problems, infections of the ear, and various types of wounds as well as digestive problems. Consequently,

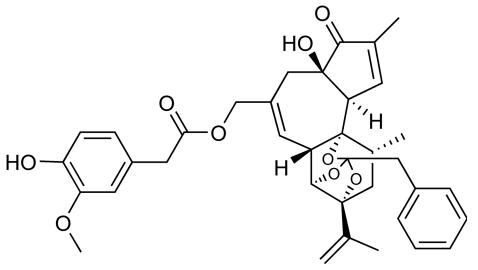
chillies were also an important element of tribute requested by the Aztec rulers. During the colonial period, these uses continued and developed further. Now, records of chilli's use as an aphrodisiac appeared. More recently, *C. frutescens*[83] has been used as a rubefacient to locally stimulate blood circulation. In chemical and pharmacological terms, the development of *Capsicum* spp. is linked to another traditional medicinal plant, *Euphorbia resinifera* Berg (Euphorbiaceae), a large, leafless cactuslike perennial and a native of the Anti-Atlas Mountains of Morocco, which yields euphorbium. Probably, it was King Juba II of Mauretania (50 BC–AD 23) and his physician Euphorbius who discovered the medicinal potential of the resin. Euphorbium has had a medical history of more than 2000 years. This makes RTX one of the most ancient drugs still in use today. Some of its uses, like its application on nerves to suppress chronic pain or on dental cavities to mitigate tooth ache, can be linked directly to the biochemical studies discussed below.[84] The pharmacological interest in this species goes back to the discovery that its key constituent RTX has effects on the transient receptor potential (TRP) channel, similar to capsaicin; this links the history of the drug development of these two botanical drugs.



25 Triptolide



26 Capasaicin



27 Resiniferatoxin

Both RTX and capsaicin contain a vanilloid (i.e., 3-methoxy-4-hydroxy-benzyl) substructure known to be essential for the potent activity in typical assays of such receptors.[85,86] The first modern biological studies in the 1950s and 1960s on capsaicin are attributed to the Hungarian pharmacologist Miklos (Nicholas) Jancsó, who died in 1967 and did not see the outcome of his work, which was published by his wife Aurelia Jancsó-Gábor and his pupil Janos Szolcsányi. In 1975 and based on structure–activity relationship studies using capsaicin analogues (capsaicinoids) and fine-tuned dose–response curves in their activities, they first postulated the existence of a specific receptor for capsaicin.[84,87,88] Ultimately, these studies transformed the compound from a culinary curiosity to an important pharmacological model and molecular tool for the study of neurogenic inflammation and pain.[86] Empirical evidence for the possibility of desensitization to capsaicin has potential in diverse diseases such as chronic intractable pain, vasomotor rhinitis, or an overactive bladder[89] (**Table 2**).

Considerable evidence has accumulated bringing attention to the fact that transient receptor potential cation channels (TRPC) function as a molecular integrator not only of the effect of capsaicin but also of a multitude of noxious stimuli including heat, pollutants with negative electric charge, acids, and endogenous proinflammatory substances.[90] The first endovanilloid (i.e., a substance in humans acting like a vanilloid) was the lipid mediator anandamide identified in, 1999, which is also essential as an endogenous cannabinoid receptor ligand. Anandamide is structurally related to capsaicin because both compounds have an amide bond and an aliphatic side chain. Ultimately, these data provide strong evidence for links between the cannabinoid receptor-mediated signaling cascade and TRPC.[86] Thus, the discovery of a receptor for capsaicin has had wide biochemical and pharmacological implications.

Therefore, it is an ideal situation in which to develop anti-inflammatory and nociception-modulation drugs. In 2007, an exciting anesthetic drug lead based on two compounds – a lidocain derivative QX-314 and capsaicin – was developed. Binshtok *et al.*[91] used a combination of these two chemicals to target *only* pain-sensing neurons, or nocireceptors while leaving other types, such as motor neurons, untouched. QX-314, a charged derivative of

Table 2 Capsicum and TRP – an interwoven history

7000 BC–5000	Archaeological records of *Capsicum annuum*'s use presumably as a food and medicine in the Teohucán Valley, in Puebla, and in Tamaulipas, México. This includes coprolites and carbonized seeds. These may have been the first cultivated chillis
Ca. 2000 BC	Archaeological records of *Capsicum frutescens* in the graves of Huaca Prieta
Ca. fifteenth century	Chilli (*C. frutescens)* is used widely in Mesoamerican Indian cultures and is discovered by the Spanish conquistadores. The Aztec term is adopted into Spanish
1542	Introduction of *C. frutescens* into India by the Portuguese
Sixteenth century	Spread of varieties of *C. annuum* in the eastern Mediterranean, the Near East, and south-central Europe (Hungary)
1543	Indianischer Pfeffer (*C. frutescens*) is mentioned in Leonhard Fuchs' *New Kreüterbuch*. Subsequently, the plant is incorporated into numerous cultures
1846	Capsaicin is first isolated by L. Thresh
1850	Turnbull demonstrates that *Capsicum* extract provides instant relief from toothache, highlighting the therapeutic potential of the species. This line of research is not followed up, however
Twentieth century	In Europe, *C. frutescens* (fruit) is used topically for rheumatism
1919	E. K. Nelson elucidates the structure of capsaicin
1949	Jancso′ demonstrates that capsaicin produces pain and neurogenic inflammation
1977	*Drosophila* TRP channel is identified
1989	Szallasi and Blumberg demonstrate that RTX from *Euphorbia resinifera* is an ultrapotent capsaicin analogue
1990	[3H]-RTX binding sites are described
1997	Vanilloid receptror 1 (TRPV1) is cloned
1999	Vanilloid receptorlike channel (TRPV2) is cloned
2000	TRPV1-deficient mice are developed
2002	TRPV3 and TRPV4 are cloned
2002–03	Cold-sensitive TRPs are cloned

Modified and expanded based on J. B. Calixto; C. A. Kassuya; E. André; J. Ferreira, *Pharmacol. Ther.* **2005**, *106*, 179–208.

lidocaine, blocks electrical activity in neurons but cannot permeate the cell membranes and induce this anesthetic effect. The excitability of primary sensory nociceptor (pain-sensing) neurons was selectively blocked by introducing the membrane-impermeant compound QX-314 through the pore of the noxious heat-sensitive TRPV1 channel using capsaicin for facilitating selective membrane passage.[91] Thus, the active medication would be composed of a pharmacologically active one and one that facilitates this compound's membrane transport. Is this an ethnopharmacology-driven drug development? Again, it is a complex picture. The concept of a compound targeting the TRPV1 channel is certainly based on the traditional (and very widely distributed) knowledge about chilli's pungent effects. Detailed molecular understanding of how these ion channels work allowed the development of the strategy to transport the active constituent in a piggyback fashion. Numerous other natural product-derived modulators of these TRP channels are known.[86]

Two final examples highlight the potential of developing novel anti-inflammatory drug leads using a proinflammatory transcription factor NF-κB as a molecular target. NF-κB is one of the principal inducible transcription factors in mammals and has been shown to play a pivotal role in the mammalian innate immune response and chronic inflammatory conditions such as rheumatoid arthritis. The signaling mechanisms of NF-κB involve an integrated sequence of protein-regulated steps. Many mechanisms are potential key targets for intervention in treating inflammatory conditions. Curcumin is a core compound in turmeric (*Curcuma longa*, Zingiberaceae) endemic to peninsular India, especially the provinces of Tamil Nadu, West Bengal, and Maharashtra. Turmeric has a small branched rhizome that is bright yellow on the interior. It is used in medicine and widely used in Indian cuisine, for dyeing cloth, and in traditional medicine. In local and traditional medicines, turmeric is considered to be a strong antiseptic and is used to heal wounds, infections, jaundice, urinary diseases, and ulcers and to reduce cholesterol levels. Turmeric, in the form of a paste, has been used to treat external conditions such as psoriasis (anti-inflammatory) and athlete's foot (antifungal). Therefore, the link with NF-κB signaling is an obvious one, and curcumin has repeatedly shown its inhibitory effects against the signaling cascade of activated NF-κB.[92]

Finally, parthenolide from *Tanacetum parthenium*, Asteraceae (feverfew), is a potent inhibitor of NF-κB at low micromolar concentrations. Feverfew has long been used as a bitter tonic and antipyretic. Since the 1990s, some efforts have focused on its use as a potential treatment for migraines. Although parthenolide is not a good drug choice due to its nonspecific cytotoxicity, it parthenolide has been studied in great detail from a biochemical–mechanistic perspective. It prevents IκBα and IκBβ degradation and acts against the IKK complex, specifically IKKβ by modifying cysteine 179.[93,94] Parthenolide discovery is based on the systematic screening of Mexican Indian medicinal plants used in the context of acute or chronic inflammatory conditions where several sesquiterpene-containing species showed activity.[95,96] Parthenolide had not been reported from these species, in fact, but was selected as a model compound for the class. Since that time, numerous members of the sesquiterpene family have been identified as inhibitors of NF-κB.

3.12.2.5.4 Antiobesity and antidiabetes drugs

In the 1990s, Fanie R. van Heerden and colleagues at the Council for Scientific and Industrial Research (CSIR) of South Africa isolated two hunger-suppressing pregnane glycosides (**28**, **29**) from *Hoodia gordonii* (Masson) Sweet ex Decne, established their chemical structure, and patented it in 1997.[97] Research had already started during the early 1960s focusing on the nutritional value and also any possible long-term toxic effects of food from the veld. The appetite suppressant effect of the plant extracts had already been established in 1983. Without doubt, this discovery was driven by traditional knowledge. *Hoodia pilifera* (L.f.) Plowes (Apocynaceae) and *H. gordonii* are succulent, slow-growing desert plants in southern Africa. Their indigenous names include ghaap, guaap, or ngaap. *H. pilifera* has been known to quench thirst since the nineteenth century, at least.[98] The discovery has specifically been linked to the Khoi-San people, but it seems to have been known also in other groups.

Very quickly, this patent arose the interest of the industry, and a small U.K.-based company (Phytopharm) took a lead further developing it. Key was the extracts' and compounds' hunger suppressant and later their antidiabetic effects. In 1998, clinical studies for treating obesity were started and was licensed to Pfizer. The ultimate goal of this R&D effort was a fully licensed medicine on the basis of a characterized extract with a defined amount of the active constituent for the treatment of obesity. Considerable clinical and preclinical research went into developing the drug, but Pfizer unexpectedly returned the license to Phytopharm in July 2003. In late 2004, the food giant Unilever stepped in with the strategic goal to develop a slimming food.[99] So far, only limited information about the extracts' characteristics and their pharmacological effects or clinical effectiveness has been published.[100]

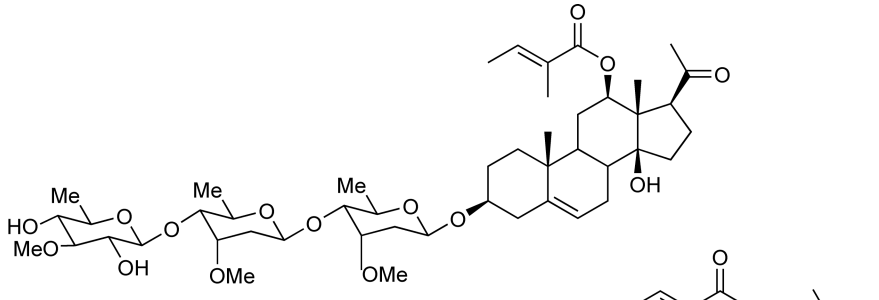
However, this is the biomedical side. Two other issues are essential, and they highlight the responsibilities of researchers and the industry in ethnopharmacology-driven drug discovery. Because *H. gordonii* is a traditional medicinal and food plant of the San but had been patented without their prior consent, the San of the Kalahari Desert and other stakeholders raised concern about this lack of intellectual and financial recognition. The San and the CSIR finally signed a benefit-sharing agreement in 2004. This was, in fact, one of the first benefit-sharing agrements and gave the San a share of royalties derived from the sale of products containing the patented extract. Specifically, the following agreement was reached:

- CSIR will pay the San 8% of all milestone payments it receives from its licensee, U.K.-based Phytopharm plc
- CSIR will pay the San 6% of all royalties that it receives once the drug is commercially available
- CSIR will make study bursaries and scholarships available to the San community
- CSIR and the San people agree to collaborate in future bioprospecting for the benefit of both parties[101]

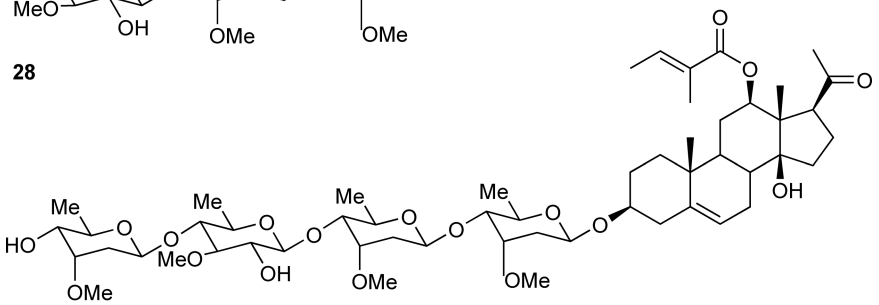
This agreement between the San and the CSIR made further development of the product possible. As of today (2008), a second more detailed agreement is due to be signed soon.

The second issue relates to the supply side. As pointed out above, *H. gordonii* are succulent, slow-growing desert plants. The chemical structure of the pregnane glycosides makes synthesis impossible. Also, the commercial goal has been the development of an extract earlier as a medicine and now as a food supplement. With the huge number of obese people in North America, Europe, and other parts of the world, the demand for the botanical drug will be extremely high. Consequently, the commercial production of the plant on farms in the deserts of South Africa and Namibia had to be developed. This has now been achieved, and it is hoped that sufficient material will be available within a few years.

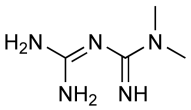
Another now-classical example, the biguanide metformin, which is a semisynthetic derivative of an active natural product, galegine, a guanidine isolated from *Galega officinalis* L. (Fabaceae, s.str.), is used to treat diabetes. In medieval times, this species was used to relieve intense urination in diabetic people. It also provides an interesting example that although traditional systems of knowledge may lack diagnostic and technical tools to identify certain diseases in a modern biomedical way, such a diagnosis is based on specific signs (or symptoms) a disease produces. Similarly, patients today are diagnosed in one of the primary health care centers and the MDs in these centers normally also prescribe appropriate medication. In many countries like Mexico[102] or India,[103] once a diagnosis is made, patients often go to either local healers or to vendors of herbal and other health care products. From an ethnopharmacological perspective, it is important to understand that diabetes is one at the interface of conventional biomedical and local (or traditional) treatment. Thus, diabetes is for which many of the traditional treatments were, in fact, developed in the last decades by local healers.



28



29



30 Metformin

The potential of novel antidiabetic medications is enormous. In Mexico alone, for example, a total of 306 species of *G. officinalis* have been used to treat this disease. *Opuntia* spp. (cactus pears or prickly pears, Cactaceae) are an essential element of Mesoamerican botanical history. Ripe fruits and nopals (or nopalitos, tender cladodes) have been used as food and medicine for centuries. Ill-defined extracts from *Opuntia* spp. are now widely available over the Internet as a treatment for diabetes and related metabolic disorders for which chemically and pharmacologically characterized extracts are currently under development. Seven other species from México – *Cecropia obtusifolia* Bertol. (Cecropiaceae), *Equisetum myriochaetum* Schlecht & Cham (Equisetaceae), *Acosmium panamense* (Benth.) Yacolev (Fabaceae), *Cucurbita ficifolia* Bouché (Cucurbitaceae), *Agarista mexicana* (Hemsl.) Judd. (Ericaeae), *Brickellia veronicaefolia* (Kunth) A. Gray (Asteraceae), and *Parmentiera aculeata* (*Kunth*) *Seem.* (Bignoniaceae) – also been studied in detail but have not yet resulted in usable, licensed drugs or nutraceuticals.[102]

3.12.2.5.5 Examples of other drug leads

Numerous examples of new wonder drugs regularly hit the media. It is unlikely that they stand up to such claims, and they regularly highlight the problems associated with poorly defined and characterized starting material. Two examples highlight the core issues.

Cordyceps sinensis[104] is a medicinal fungus of TCM. It is a parasite on the larvae of moths (Lepidoptera) of the genera *Hepialus* and *Thitarodes* endemic to alpine habitats (3600–5000 m in elevation) on the Tibetan plateau in southwestern China. In China, *C. sinensis* has a long history of medicinal use. It is thought to have been discovered 2000 years ago with the first formally documented use coming from the *Bencao Congxin* (New Compilation of Materia Medica) in the Qing dynasty in 1757. Overall, little primary ethnomedical data describing the medical uses of *C. sinensis* exist in the literature. Current ethnomedical reports are limited to the use as a general tonic in China and as an aphrodisiac in Nepal. *Cordyceps sinensis* first gained worldwide attention when it was revealed that several Chinese runners who broke world records in 1993 had included this fungus as part of their training program.[104]

Although there are a wide range of reported uses of *Cordyceps* in the literature, the reports that extracts of this fungus may alter apoptotic homeostasis are most intriguing. The reports of clinical trials suggest that *C. sinensis* potentially contains agents that may inhibit apoptosis. These clinical results have stimulated work to assess the ability of *C. sinensis* to inhibit apoptosis *in vitro*; however, the results of these studies are conflicting. The effects may be due to the extracts' ability to scavenge reactive oxygen species or due to the downregulation of apoptotic genes and the modulation of apoptosis (including downregulation of Fas, Fas ligand, and TNF-α expression) or the induction of apoptosis/cytotoxicity. These conflicting data may be linked to the variability of the strains used and the lack of a consensus strain, variability in the extraction procedures used and/or the need to potentially activate a prodrug present in the extract into an active constituent.[104] Overall, this example highlights once more problems in developing new drugs without proper characterization of the complex biological starting material.

'Lingzhi' is the Chinese name of a basidiomycete white rot fungus, *Ganoderma lucidum* (Japanese: Munnertake, Sachitake, and Reishi; Korean: Youngzhi) and related species, which have been used for medicinal purposes for centuries particularly in China, Japan, and Korea. As is often the case with such widely used species, recorded uses vary widely are used to treat migraine, hypertension, arthritis, bronchitis, asthma, anorexia, gastritis, hemorrhoids, diabetes, hypercholesterolemia, nephritis, dysmenorrhea, constipation, lupus erythematosus, hepatitis, and cardiovascular problems.[105] According some researchers,[76] it is used for dizziness, insomnia, palpitations, dyspnea, consumptive cough, and asthma. It is practically impossible to establish how widespread the respective uses have been. Whatever the specific use, the cultural importance of this species has been the driving force for developing potential leads from this taxon. Phytochemical research has focused on bioactive 'Lingzhi' polysaccharides and triterpenes, especially ganodermic acid. Extracts from *Ganoderma* have been investigated as potential antitumor and antiviral agents and less so as possible antibacterial agents for antibacterial activity (against Gram-positive bacteria). Some extracts markedly inhibited intracellular signaling and invasive behavior of cancer cells, whereas others were inactive. Also, immunomodulatory effects were observed, which had an impact on various types of cancers.[105] It is too early to assess whether this will result in a successful new drug, but the fact that the extract is the active constituent of this species highlights the need for detailed chemical analysis or metabolomic profiling (cf. Section 3.12.3.3) and for the selection of the most potent extract(s).

3.12.2.6 Ethnopharmacological Information Today

Information on the local and traditional use of plants is scattered in a multitude of sources, and very often such sources are not easily accessible to an international (English-speaking) community because they are written in the national languages of the respective countries.

A well-known and very useful source is a database – NAPRalert, discussed in another chapter of this volume.[106] In addition to many articles in technical journals, there are many monographic treatments available summarizing data for a particular region or country, as well as many ethnobotanical monographs, that can be used as a starting point for research, such as the following:

- Africa[107–112] incl. the Indian Ocean islands[113,114]
- South America[115–117] and North[47,118–120] America, including Mexico[7,48,121,122] and the Caribbean[123]

- Asia (India,[124–128] China,[80,129] Southeast Asia[130])
- Europe and the Circum-Mediterranean,[131–133] which in many cases is based on historical studies[70,134,135]
- Australia (for which relatively little information is available) and Oceania[136,137]

The best known research facility is the Indian National Institute of Science Communication and Information Resources (NISCAIR) of the CSIR, New Delhi, India.

Ethnobotanical studies are normally conducted with goals that are quite different from the ones in drug development. Therefore, compilations like the foregoing have been used as a starting point in an ethnobotanically driven drug discovery project. However, such information also has a multitude of other uses, as, for example, indigenous groups who want to learn about (often historic) plant use in the cultures and in general the noneconomical benefits of such projects are much higher than the potential but highly uncertain economic gains. The complex and controversial discussion whether such studies should be conducted at all is beyond the scope of this chapter, but its contentiousness will require a continued and open dialogue between all stakeholders. The complex problem has been eloquently highlighted by the late Darrell Posey, an American anthropologist and biologist, who labeled it as the commodification of the Sacred through Intellectual Property Rights.[139]

Ethnobotanical data are generally collected using a series of well-defined methods.[140–142] Despite these clear standards, many projects suffer from poor botanical documentation or from inadequate anthropological methodologies. Here, we describe general requirements for such projects. In the first instance, an appropriate community or region needs to be selected. All projects can be started only after appropriate permits from relevant national and regional institutions have been obtained (see Section 3.12.2.4). Such projects often last for about 1 year, but there are also examples of shorter projects. In the context of drug development, fieldwork needs to focus on collecting information on the plant's medicinal use, as well as plants known to be toxic. Essential parts of the process are gathering general ethnographical (background) data, collecting information about how these plants are used, preparing dried herbarium specimens, and collecting samples for further analysis. Complete sets of voucher specimens need to be deposited both in one or more international herbaria that are regionally accessible.[143] Identification generally requires the help of specialists for specific taxa from these institutions, and, of course, the taxonomic validity of the identification needs to be checked using the *Index Kewensis* (which is at the Royal Botanic Gardens, Kew, U.K.), for example.

Interviews can be conducted either with specialists in local and traditional medicine or with a broader subset of the general population. Specialists can include herbalists, midwives, experts in home remedies (i.e., specialists in treating common illnesses who may not have a specialized status as a healer), bone setters, diviners, and other forms of spiritualist healers. Specialists collect samples known in the region. An important distinction needs to be made between the theoretical and the practical materia medica. The practical knowledge is composed of the prescriptions and plants for which actual evidence for their usage can be collected. The theoretical materia is composed of those preparations that were used historically but that have been replaced by other treatments, by preparations that are known but not used, and by written documents that list potential local sources of preparations (for details on this distinction and some conceptual discussions, see Lev and Amar[144]). In a more structured interview, the specialists are asked about the uses, preparations, applications of the plants gathered, as well as their concepts about healing. It is essential to transcribe the words in the local language. Information from each healer about the use of one species or preparation for one illness is classed as one use report. For a rapid and simple analysis, these use reports can then be summed up for the various use groups (see below) and taxa. Overall, this results in a set of data that allow a (semi-)quantitative analysis of the data. Many other forms of semiquantification and analysis of the data exist.

In general, this first phase serves to gain an overview of commonly used species and the main concepts of treatment. All this information needs to be stored in appropriate databases. In the case of the abovementioned project, for example, the database consists of 4488 use reports on 614 plant species, contributed by 72 informants.[143] Early on, important decisions about the database's structure and availability need to be made.[145] For example, it has to be decided who will ultimately be in control of the data that are collected and stored in the database and where it will be held. Will it be in the public domain and possibly available over the Web, or private with limited password-controlled access?

It is beyond the scope of this discussion to provide technical details about which database management system one wants to select. These range from tailor-made ones specific for one project to a simple Access- or Excel-based system. The selection clearly also depends on factors such as the operating system, potential size of database, number of users, and available funds. Currently, relational databases, which use multiple tables of related data, offer one of the best alternatives. The relationships between these tables represent the 'real-world' multidimensions. A surprisingly large problem is the lack of adequate data standards within a single project. This is obviously required for data consistency, exchange of data, and comparative analyses.

In our own work and in order to analyze the cultural importance of the species used and for a cross-cultural comparison, we generally separate the use reports into a series of categories of use, grouping the illnesses into relatively well-defined ethnomedical categories normally based on the human body's organ system like gastrointestinal, respiratory, and dermatological conditions.

Many criteria exist for selecting possible taxa for further pharmacological and phytochemical analysis. Clearly, already well-studied taxa will often be excluded (dereplication). On the contrary, I have for many years argued that more commonly used species should have priority for further research. The selection may also be driven by preexisting priorities (e.g., specific therapeutic goals of the project).

For further laboratory-based analysis, samples will normally not be processed 'on site' and it requires storage of the sample to be used for extraction in an alcoholic solvent or drying of the samples. It is often argued that one should mimic the traditional modes of extraction, but, for example, if the traditional extraction involves fresh plant material, it will be difficult to replicate this in the laboratory if only air-dried material is available. Various extraction solvents have been suggested and used and once more the strategy to be used in a project will depend on its specific requirements and goals of the project.[146] The main general recommendation is to start a dialogue between the scientists involved in the field work and those involved in the pharmacology and phytochemistry well before the collection of the samples starts.

Currently, there is an exciting discussion about which ways to follow on the basis of such information. Many groups follow a systematic *in vitro* screening approach, which in recent years has become multitarget (many such studies have been published, e.g., in the *Journal of Ethnopharmacology*). However, few of these extracts or compounds are then taken further.

An alternative approach has been proposed by Graz *et al.*[58] and by Raza.[147] The latter argues for a role of physicians at all stages of the drug development process from the initial fieldwork (where she/he interprets traditional terminologies using biomedical modern counterparts, identifies the disease for which a local and traditional remedy is used, and examines patients consuming herbal remedies) to clinical studies on herbs as well as the study of their potential interaction with modern medicines. Graz *et al.* suggest designing clinical studies appropriate for traditional medicines and for use in the field. Core methods are the retrospective assessment of treatment outcome and population surveys, the prognosis–outcome method (with modern physicians observing progress of patients treated by a traditional healer), or the dose-escalating prospective study (detecting a dose–response phenomenon in humans). In each case, clinical data are generated at an early stage and allow a much more detailed understanding of the local and traditional medicines used as well as of the treatments and their outcome in general. Arguably, such strategies will work best for diseases prevalent in the regions of study (e.g., infectious diseases) and thus may not be as useful, for example, for those diseases that are currently at the center of most commercial drug development programs.

This short overview cannot be a comprehensive review of the relevant methods, but offers some general strategic hints highlighting the complexity and multidisciplinarity of such projects.

3.12.3 Today's Core Challenges

3.12.3.1 The Stakeholders

Until the implementation of the CBD (cf. Section 3.12.2.4), the main stakeholders were scientists (generally in large scientific research institutions like the US NCI, the pharmaceutical industry, and some university-based researchers), medical doctors, and their legal representatives.

With the changes in the legal framework, indigenous groups in the 'provider countries', NGOs and, most importantly, the provider countries themselves entered the scene. Few of these groups had or have an

understanding of the process of drug discovery and its duration, but they are united by an interest in protecting the rights of those who represent the providers. Clearly, there is a need for a dialogue between all groups involved.

The example of galanthamine (Section 3.12.2.3) points to another core challenge. Drug development has always been a lengthy process and the initial development of this drug started in the Soviet Union shortly after World War II. When the compound became of interest for treating Alzheimer's disease 40 years later, the Soviet Union had disappeared and, consequently, one has to ask whether it will be possible to develop a system that could withstand such political changes.

3.12.3.2 Neglected People and Diseases

There can be no doubt that diseases for which no industrial R&D activities exist remain a truly neglected area of medical science and practice. There is no standard global definition of neglected diseases. 'Neglect' has become one of the most commonly used words to describe certain diseases primarily, if not exclusively, affecting poor populations in developing countries. The key elements are diseases affecting principally poor people in poor countries, for which health interventions – and R&D – are seen as inadequate. Ten neglected ('tropical') diseases have been listed by the World Health Organization Special Programme for Research and Training in Tropical Diseases (WHO/TDR). These are leishmaniasis, schistosomiasis, onchocerciasis, lymphatic filariasis, Chagas disease, malaria, leprosy, African trypanosomiasis, tuberculosis (TB), and dengue. Other diseases commonly considered to be neglected include hookworm, roundworm, or diarrheal illnesses, Buruli ulcer, congenital syphilis, and trachoma. Despite various bacterial threats, such as multiply drug-resistant strains, and emerging pathogens like mycoplasma, most large pharmaceutical companies have abandoned antibacterial drug discovery. Bacterial and mycoplasmatic diseases are therefore also considered to be neglected.[148,149]

As pointed out in a joint policy document by the London School of Economics and Political Sciences and the Wellcome Trust,[150] in the context of neglected diseases the (commercial) Intellectual Property (IP)-driven innovation model has some limitations. There is no public control over industry's R&D agenda, which (being commercially driven) may not coincide with the areas of greatest public health need. Limited public control over the pricing of the final product, when this occurs, can also result in reduced patient access if purchase funds are tight since fewer daily doses can be purchased at the higher monopoly price than at the lower competitive price.[150] Natural products offer much more realistic opportunities for developing such low-cost innovative drugs. The classical example of a drug used against neglected diseases is *A. annua* and the sesquiterpene lactone qinghaosu derived from it (see above). The advantages of drug development projects based on plants traditionally used in the treatment of these conditions are the direct link between the traditional use, the drug development project, and hopefully the opportunity to develop these products at lower costs. Lastly, some of these products, if proven to be safe and efficacious, may be used as phytomedicines produced locally.

3.12.3.3 Extracts as Medicines?

In recent years, novel opportunities have been subsumed under the idea of the 'omics revolution. Metabolomics, for example, ideally will qualitatively and quantitatively analyze all metabolites in an organism (e.g., a medicinal plant) or a complex drug. As pointed out by Verpoorte *et al.*,[151,152] this is a very ambitious goal, and it is questionable whether this is a realistic goal. This approach allows a systematic investigation of complex mixtures and specifically to link phytochemical analysis with other strategies (such as *in vitro* or *in vivo* screening for biological activity or toxicity, morphological plant diversity, and ecological parameters). Specifically, as it relates to the study of medicinal and food plants, the main challenge is to understand the complex effects of such extracts. This may offer unique and novel opportunities to develop new medicines based on local and traditional knowledge, but the true potential of such an approach remains to be seen.

Our group investigated two poorly studied traditional preparations of cannabis (*Cannabis sativa* L., Cannabidaceae, various cultivars), the water extracts and tinctures, in order to evaluate the overall metabolite profiles and the relative amount of Δ^9-tetrahydrocannabinol (THC) with respect to Δ^9-THC-acid and other cannabis constituents using a combination of NMR analysis (diffusion-edited ^{1}H NMR

(1D DOSY) and ^{1}H NMR with suppression of the ethanol and water signals) and *in vitro* cell assays (inhibition of NF-κB activation). Depending on the extraction procedure, the extracts were highly variable with respect to constituents including Δ^{9}-THC and Δ^{9}-THC-acid. With this method, it was possible, without any evaporation or separation step, to distinguish between tinctures from different cannabis cultivars. This case highlights the potential of optimizing an extract based on the effects of a specific target (or potentially a series of targets).[153,154] Here it serves as an example of developing extracts into medicines (see also Section 3.12.2.3) and the specific case of cannabis is discussed in much greater detail in another chapter of this volume.[155] In another example, Boelsma *et al.*[156] investigated the effect of *G. biloba* extract EGb 761 on skin blood flow in healthy volunteers using laser Doppler flowmetry and the accompanying changes in urinary metabolites in urine using a combination of NMR spectroscopy and multivariate data analysis (MVDA). Following EGb 761 treatment, the overall mean skin blood flow was significantly reduced as compared with placebo. NMR/MDVA analyses showed that urinary metabolic patterns differed depending on the change in baseline blood flow after treatment. The results highlight the usefulness of metabolic fingerprinting as a tool for understanding biochemical changes and associated functional changes and, therefore, have implications for drug development.

3.12.3.4 Let Food Be Your Medicine and Let Medicine Be Your Food

As it becomes obvious from the above, and as pointed out by others, the borderline between food and medicine is blurred.[157–159] Similarly, anthropologists[160–162] have argued that there exist strong links between food and medicines in indigenous societies. Today, we are very conscious about this, and this chapter highlights that the decision whether an ethnopharmacology-driven research project has a new food supplement or a new medical product as its ultimate goal is often arbitrary. The case of *Hoodia* demonstrates this very clearly. In legal terms, in many countries a product is considered to be a medicine if it makes specific claims for treating or preventing a certain illness and a health food if it has general health beneficial effects as well as alleviating a specific illness or syndrome. Consequently, ethnopharmacology-driven drug development has a broader scope for applications than approaches based on medicinal chemistry, for example. Arguably, especially in the case of Europe (and presumably also North America and Australia/New Zealand), from an industrial perspective, the greatest opportunities lie in developing novel food supplements/health foods/traditional herbal medical products or 'cosmeceuticals' based on local and traditional knowledge.

3.12.4 Conclusion: People, Plants, and the Future of Medicines

This chapter reviewed some of the many medicines and drug substances that are based on local and traditional knowledge. Such a review needs to be examplatory and selective. Overall, it highlights that oral and written local/traditional knowledge has provided many unique novel leads and that such an ethnopharmacological approach continues to be a fascinating and particularly valuable strategy. As we pointed out recently, the world's societies are in a continuous process of globalizing selected elements of local knowledge[157] and equitable benefit sharing as well as the development of mechanisms to safeguard such knowledge for future generations[163] in the regions where this knowledge developed will have to be an essential element of any R&D strategy.

Ethnopharmacology and drug development can be understood only if a truly multidisciplinary approach is taken and this is one of the most exciting and promising challenges of the field – it requires a dialogue not only between disciplines but also between cultures. Ethnopharmacology-driven drug development uses a unique *knowledge-based strategy*, which will hopefully result in many more new medicines for use by all humans. The needs of those who require such new and better medications most and who can least afford them have to come at the forefront of decision makers in industry and politics. Locally and traditionally (mostly plant based) used medicines offer unique opportunities provided that there exists the willingness to support such research, which generally is at the border between basic and applied research.

Abbreviations

CBD	Convention on Biological Diversity (1992) also known as Rio Convention
COX	cyclooxygenase
ICAM 1	intercellular adhesion molecule 1
IKK	IκB kinase
IL	interleukin
iNOS	inducible NO synthase
IP	Intellectual Property
JNK	Jun N-terminal Kinase or Stress Activated Protein Kinase
LPS	lipopolysaccharide
MMP (3/13)	Matrix metallopeptidase (3/13)
NCI	National Cancer Institute
NF-κB	nuclear factor kappaB
PKC	protein kinase C
TCM	traditional Chinese medicine
THC	tetrahydrocannabinol
TNF-α	tumor necrosis factor α
TRIPS	Trade-Related Aspects of Intellectual Property Rights
TRPC	transient receptor potential cation channels
TRPV	transient receptor potential vanilloid type [1–4] protein
VCAM-1	vascular cell adhesion molecule-1
WTO	World Trade Organization

References

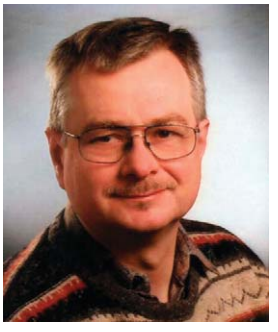
1. T. W. Corson; C. M. Crews, *Cell* **2007**, *130*, 769–774.
2. C. Reddrop; R. X. Moldrich; P. M. Beart; M. Farso; G. T. Liberatore; D. W. Howells; K.-U. Petersen; W.-D. Schleuning; R. L. Medcalf, *Stroke* **2005**, *36*, 1241–1246.
3. P. A. Cox; M. Heinrich, *Pharm. News* **2001**, *8* (3), 55–59.
4. R. F. Ellen; K. Fukui, Eds., *Redefining Nature: Ecology, Culture and Domestication (Explorations in Anthropology)*; Berg: Oxford, 1996.
5. L. Maffi, Ed., *On Biocultural Diversity: Linking Language, Knowledge and the Environment*; Smithsonian Books: Washington, DC, 2001.
6. B. Berlin, *Ethnobiological Classification. Principles of Categorization of Plants and Animals in Traditional Societies*; Princeton University Press: Princeton, NJ, 1992.
7. B. Berlin; E. A. Berlin, *Medical Ethnobiology of the Highland Maya of Chiapas, Mexico*; Princeton University Press: Princeton, NJ, 1997.
8. D. Efron; S. M. Farber; B. Holmstedt; N. L. Kline; R. H. L. Wilson, *Ethnopharmacologic Search for Psychoactive Drugs*; Government Printing Office: Washington, DC, Public Health Service Publications No. 1645, reprint, 1970 (orig. 1967).
9. W. Sneader, *Drug Discovery. A History*; John Wiley & Sons Ltd.: Chichester, 2005.
10. Y.-W. Chin; M. J. Balunas; H. B. Chai; A. D. Kinghorn, *AAPS J.* **2006**, *8* (2), Article 28.
11. G. M. Cragg; D. J. Newman, *J. Ethnopharmacol.* **2005**, *100*, 72–79.
12. D. J. Newman; G. M. Cragg; K. M. Sneader, *J. Nat. Prod.* **2003**, *66*, 1022–1037.
13. I. Raskin; D. M. Ribnicky; S. Komarnytsky; N. Ilic; A. Poulev; N. Borisjuk; A. Brinker; D. A. Moreno; C. Ripoll; N. Yakoby; J. M. O'Neal; T. Cornwell; I. Pastor; B. Fridlender, *Trends Biotechnol.* **2002**, *20*, 522–531.
14. J. D. Phillipson, *Phytochemistry* **2007**, *68*, 2960–2972.
15. L. Bohlin; U. Göransson; A. Backlund, *Pure Appl. Chem.* **2007**, *79*, 763–774.
16. I. Paterson; E. A. Anderson, *Science* **2005**, *310*, 451–453.
17. F. E. Koehn; G. T. Carter, *Nat. Rev. Drug Discov.* **2005**, *4*, 206–220.
18. B. M. Schmidt; D. M. Ribnicky; P. E. Lipsky; I. Raskin, *Nat. Chem. Ecol.* **2007**, *3*, 360–366.
19. M. Heinrich; M. Robles; J. E. West; B. R. Ortiz de Montellano; E. Rodriguez, *Annu. Rev. Pharmacol. Toxicol.* **1998**, *38*, 539–565.
20. M. Heinrich; S. Gibbons, *J. Pharm. Pharmacol.* **2001**, *53*, 425–432.
21. P. A. Cox; M. J. Balick, *Sci. Am.* **1994**, *270* (6), 82–87.
22. M. Wang; R. J. A. N. Lamers; H. A. Korthout; J. H. J. van Nesselrooij; R. F. Witkamp; R. van der Heijden; P. J. Voshol; L. M. Havekes; R. Verpoorte; J. van der Greef, *Phytother. Res.* **2005**, *19* (3), 173–182.
23. C. Hartwich, *Die Bedeutung der Entdeckung von Amerika für die Drogenkunde*; Springer: Berlin, 1892.

24. A. von Humboldt (Hrsg. H. Beck), *Die Forschungsreise in den Tropen Amerikas.* Studienausgabe Bd. 2, Teilband 3; Wissenschaftliche Buchgesellschaft: Darmstadt, 1997.
25. C. Bernard, Physiologische Untersuchungen über einige amerikanische Gifte. Das Curare. Übs. In *Ausgewählte physiologische Schriften*; C. Bernard, N. Mani, Übs.; Huber Verlag: Bern (frz. orig. 1864), 1966; pp 84–133.
26. B. Griggs, *Green Pharmacy. A History of Herbal Medicine*; J. Norman & Hobhouse: London, 1981.
27. M. Heinrich; J. Barnes; S. Gibbons; E. M. Williamson, *Fundamentals of Pharmacognosy and Phytotherapy*; Churchill Livingstone (Elsevier): Edinburgh, London, 2004; ISBN 0 443 07132 2.
28. W. Sneader, *Drug Prototypes and Their Exploitation*; John Wiley & Sons: Chichester, 1996.
29. M. Heinrich; H. L. Teoh, *J. Ethnopharmacol.* **2004**, *92*, 147–162.
30. A. Plaitakis; R. C. Duvoisin, *Clin. Neuropharmacol.* **1983**, *6*, 1–5.
31. G. Madaus, *Lehrbuch der biologischen Heilmittel. Nachdr. d. Ausg. Leipzig 1938*; Mediamed: Ravensburg, 2002.
32. H. Marzell, *Geschichte und Volkskunde der deutschen Heilpflanzen*; Reich Verlag: St. Goar, 2002 (orig. 1938).
33. G. Pabst, *Köhler's Medizinal-Pflanzen in naturgetreuen Abbildungen mit kurz erläuterndem Texte: Atlas zur Pharmacopoea Germanica, Austriaca, Belgica, Danica, Helvetica, Hungarica, Rossica, Suecica, Neerlandica, British pharmacopoeia, zum Codex medicamentarius sowie zur Pharmacopoeia of the United States of America*; Köhler: Gera-Untermhaus, 1889.
34. M. D. Mashkovsky; R. P. Kruglikova-Lvova, *Farmakol. Toxicol. (Moskow)* **1951**, *14*, 27–30 (in Russian).
35. E. J. Shellard, *Pharm. J.* **2000**, *264*, 883.
36. D. S. Paskov, *Series Exp. Biol. Med. (Sofia),* **1957**, *1*, 29–35.
37. M. Heinrich; A. Pieroni; P. Bremner, Medicinal Plants and Phytomedicines. In *The Cultural History of Plants*; G. Prance (Consulting Editor), M. Nesbitt (Scientific Editor); Routledge (Taylor & Francis): New York, 2005; pp 205–238.
38. W. A. Goethe, West-östlichen Divan (Book 'Suleika') first published in 1819. http://www.xs4all.nl/∼kwanten/goethe.htm (accessed 5 January 2008).
39. F. V. de Feudis, *Ginkgo biloba Extract EGb 761: Pharmacological Activities and Clinical Applications*; Elsevier: Paris.
40. V. Schulz; V. R. Hänsel; V. E. Tyler, *Rational Phytotherapy. A Physician's Guide to Herbal Medicine*, 4th ed.; Springer: Berlin, 2001.
41. E. E. V. Collocott, *J. Polynesian Soc.* **1927**, *36*, 21–47.
42. M. Adams; F. Gmünder; M. Hamburger, *J. Ethnopharmacol.* **2007**, *113*, 363–381.
43. *The British Pharmacopoeia*; The Stationary Office: London, 2001.
44. R. L. Noble, *Biochem. Cell Biol.* **1990**, *68*, 1344–1351.
45. T. Efferth; C. H. Li; V. S. B. Konkimalla; B. Kaina, *Trends Mol. Med.* **2007**, *13*, 353–361.
46. M. Heinrich; P. Bremner, *Curr. Drug Targets* **2006**, *7*, 239–246.
47. D. E. Moerman, *Native American Ethnobotany*; Timber Press: Portland, OR, 1998.
48. D. E. Moerman, Native North American Food and Medicinal Plants: Epistemological Considerations. In *Plants for Food and Medicine*; N. L. Etkin, D. R. Harris, H. D. V. Prendergast, P. J. Houghton, Eds.; Royal Botanic Gardens, Kew: Richmond, UK, 1998; pp 69–74.
49. M. Suffness, *Taxol®: Science and Application*; CRC Press: Boca Raton, FL, 1995.
50. L. Ottaggio; F. Bestoso; A. Armirotti; A. Balbi; G. Damonte; M. Mazzei; M. Sancandi; M. Miele, *J. Nat. Prod.* **2008**, *71*, 123–126. DOI: 10.1021/np0704046.
51. Secretariat of the Convention on Biological Diversity, *Handbook of the Convention on Biological Diversity;* Earthscan: London, 2001; ISBN 0-85383 737 7.
52. D. A. Posey, Kayapó Ethnoecology and Culture. In *Studies in Environmental Anthropology*, K. Plenderleith, Ed.; Routledge: London and New York, 2002; Vol. 6.
53. C. W. Wright, *J. Ethnopharmacol.* **2005**, *100*, 67–71.
54. Co-ordinating Group of Research on the structure of Qing Hau Sau, , *K'O Hsueh Tung Pao* **1977**, *22*, 142 (Chem. Abstr. **1977**, *87*, 98788).
55. Qinghaosu Antimalarial Co-ordinating Research Group, , *Chin. Med. J.* **1979**, *92*, 811–816.
56. J. N. White, *Science* **2008**, *320*, 330–340.
57. M. S. Mueller; I. B. Karhagomba; H. M. Hirt; E. Wemakor, *J. Ethnopharmacol.* **2000**, *73*, 487–493.
58. B. Graz; E. Elisabetsky; J. Falquet, *J. Ethnopharmacol.* **2007**, *113*, 382–386.
59. J. C. T. Silva; G. N. Jham; R. D'arc; L. Oliveira; L. Brown, *J. Chromatogr. A* **2007**, *1151*, 203–210.
60. S. Kadidal, *Biodivers. Conserv.* **1998**, *7*, 27–39.
61. D. E. Allen; G. Hatfield, *Medicinal Plants in Folk Traditions An Ethnobotany of Britain and Ireland*; Timber Press: Portland, OR, USA and Cambridge, UK, 2004.
62. J. Bellakhdar, *La pharmacopée marocaine traditionelle*. Ibis Press: Paris, 1997.
63. A. C. Green; G. L. Beardmore, *Australas. J. Dermatol.* **1988**, *29*, 127–130.
64. S. Ogbourne; M. P. Hampson; J. M. Lord; P. Parson; P. A. de Witte; A. Suhrbier, *Anticancer Drugs* **2007**, *18*, 357–362.
65. P. Hampson; H. Chahal; F. Khanim; R. Hayden; A. Mulder; L. K. Assi; C. M. Bunce; J. M. Lord, *Blood* **2005**, *106*, 1362–1368.
66. www.peplin.com (accessed 8 May 2008).
67. K. R. Gustafson, J. H. Cardellina II, J. B. McMahon, R. J. Gulakowski, J. Ishitoya, Z. Szallasi, *et al.*, *J. Med. Chem.* **1992**, *35*, 1978–1986.
68. S. Williams; L.-F. Chen; H. Kwon; D. Fenard; D. Bisgrove; E. Verdin; W. C. Greene, *J. Biol. Chem.* **2004**, *279* (40), 42008–42017.
69. D. Warrilow; J. Gardner; G. A. Darnell; A. Suhrbier, *AIDS Res. Hum. Retroviruses* **2006**, *22* (9), 854–864.
70. M. de Cleen; M. C. Lejeune, *Compendium of Ritual Plants in Europe Vol. 1: Trees and Shrubs*; mens & cultuur uitgevers n.v.: Ghent, Belgium, 2003.
71. A. Sami; M. Taru; K. Salme; J. Yli-Kauhaluoma, *Eur. J. Pharm. Sci.* **2006**, *29*, 1–13.
72. J. H. Kessler; F. B. Mullauer; G. M. de Roo; J. P. Medema, *Cancer Lett.* **2007**, *251*, 132–145.
73. H. Kasperczyk; K. La Ferla-Bruehl; M. A. Westhoff; L. Behrend; R. M. Zwacka; K.-M. Debatin; S. Fulda, *Oncogene* **2005**, *24*, 6945–6956.
74. A. J. Vlietnick; T. De Bruyne; S. Apers; L. A. Pieters, *Planta Med.* **1998**, *64*, 97–109.

75. J. R. Gómez-Castellanosa; J. M. Prieto-García; M. Heinrich, *J. Ethnopharmacol.* **2009**, *121*, 1–13.
76. Z. Zhongzhen, Ed., *An Illustrated Chinese Materia Medica in Hong Kong School of Chinese Medicine*; Hong Kong Baptist University: Hong Kong, 2004.
77. Z. Zhongzhen, Prof. Hong Kong Baptist University, Personal communication, 7 December 2007.
78. A. M. Brinker; J. Ma; P. E. Lipsky; I. Raskin, *Phytochemistry* **2007**, *68*, 732–766.
79. B. Wang; L. Ma; X. Tao; P. E. Lipsky, *Arthritis Rheum.* **2004**, *50*, 2995–3003.
80. S. Ahmed; J. Anuntiyo; C. J. Malemud; T. M. Haqqi, *eCAM* **2005**, *2* (3), 301–308.
81. V. Di Marzo, *Eur. J. Biochem.* **2004**, *271*, 1813.
82. B. Wolters; Agave bis Zaubernuss*, Heilpflanzen der Indianer Nord- und Mittelamerikas*; U. Freund Verl.: Greifenberg, Germany, 1996.
83. J. Long-Solis, *Capsicum y cultura. La historia del Chilli*; Fondo de Cultura Económica: México, 1986.
84. G. Appendino; A. Szallasi, *Life Sci.* **1997**, *60*, 681–696.
85. D. N. Cortright; A. Szallasi, *Eur. J. Biochem.* **2004**, *271*, 1814–1819.
86. J. B. Calixto; C. A. Kassuya; E. André; J. Ferreira, *Pharmacol. Ther.* **2005**, *106*, 179–208.
87. J. Szolcsányi; A. Jancsó-Gábor, *Arzneimittelforschung* **1975**, *25*, 1877–1881.
88. G. Appendino; A. Minassi; A. Pagani; A. Ech-Chahad, *Curr. Pharm. Des.* **2008**, *14*, 2–17.
89. A. Szallasi; G. Appendino, *J. Med. Chem.* **2004**, *47*, 2717–2722.
90. A. Ferrer-Montiel; C. García-Martínez; C. Morenilla-Palao; N. García-Sanz; A. Fernández-Carvajal; G. Fernández-Ballester; R. Planells-Cases, *Eur. J. Biochem.* **2004**, *271*, 1820–1826.
91. A. M. Binshtok; B. P. Bean; C. J. Woolf, *Nature* **2007**, *449* (7162), 607–610.
92. P. M. Bremner; M. Heinrich, *Phytochem. Rev.* **2005**, *4*, 21–37.
93. S. P. Hehner; M. Heinrich; P. M. Bork; M. Vogt; F. Ratter; V. Lehmann; K. Schulze-Osthoff; W. Dröge; M. L. Schmitz, *J. Biol. Chem.* **1998**, *273*, 1288–1297.
94. P. M. Bremner; M. Heinrich, *J. Pharm. Pharmacol.* **2002**, *54*, 453–472.
95. P. M. Bork; M. L. Schmitz; C. Weimann; M. Kist; M. Heinrich, *Phytomedicine* **1996**, *3*, 263–269.
96. P. M. Bork; M. L. Schmitz; M. Kuhnt; C. Escher; M. Heinrich, *FEBS Lett.* **1997**, *402*, 85–90.
97. F. R. van Heerden; R. Vleggaar; R. M. Horak; R. A. Learmonth; V. Maharaj; R. D. Whittal, Pharmaceutical Compositions Having Appetite-Suppressant Activity. PCT/GB98/01100, **1998**.
98. F. R. van Heerden; R. M. Horak; V. J. Maharaj; R. Vleggaar; J. V. Senabe; P. J. Gunning, *Phytochemistry* **2007**, *68*, 2545–2553.
99. H. Tomlinson, Prickly Solution to Slimmers' Woes. *The Guardian,* **16** December 2004; p 20.
100. O. L. Tulp; N. A. Harbi; J. Mihalov; A. DerMarderosian, *FASEB J.* **2001**, *15*, A404.
101. http://www.cljhealth.com/hoodia-research-study.htm (accessed 8 December 2007).
102. A. Andrade-Cetto; M. Heinrich, *J. Ethnopharmacol* **2005**, *99* (3), 325–348.
103. P. K. Mukherjee; K. Maiti; K. Mukherjee; P. J. Houghton, *J. Ethnopharmacol.* **2006**, *106*, 1–28.
104. E. J. Buenz; B. A. Bauer; T. W. Osmundson; T. J. Motley, *J. Ethnopharmacol.* **2005**, *96*, 19–29.
105. R. Paterson; M. Russell, *Phytochemistry* **2006**, *67*, 1985–2001.
106. N. R. Farnsworth, This volume.
107. J. M. Watt; M. G. Breyer-Brandwijk, *The Medicinal and Poisonous Plants of Southern and Eastern Africa*, 2nd ed.; Livingstone: London, 1962.
108. B. Oliver-Bever, *Medicinal Plants in Tropical West Africa*; Cambridge University Press: Cambridge, 1986.
109. B.-E. Van Wyk; B. van Oudtshoorn; N. Gericke, *Medicinal Plants of South Africa*; Briza Publications: Pretoria, South Africa, 1997.
110. H. D. Neuwinger, *African Ethnobotany: Poisons and Drugs*; Chapman & Hall: London, 1994.
111. H. D. Neuwinger, *African Traditional Medicine. A Dictionary of Plant Use and Application*; Medpharm Scientific Publication: Stuttgart, Germany, 2000.
112. F. Haerdi, *Die Eingeborenen-Heilpflanzen des Ulanga-Distriktes Tanganjikas (Ostafrika)*; Verlag für Recht und Gesellschaft: Basel, 1964; *Acta Tropica* (Suppl. 8).
113. A. Gurib-Fakim; T. Brendler, *Medicinal and Aromatic Plants of Indian Ocean Islands*; Medpharm Scientific Publication: Stuttgart, Germany, 2004.
114. A. G. Miller; M. Morris; D. Alexander (design); R. Atkinson, (Ed.), *Ethnoflora of the Soqotra Archipelago*; Royal Botanic Garden Edinburgh: Edinburgh, UK, 2004.
115. R. E. Schultes; R. F. Raffauf, *The Healing Forest*; Dioscorides Press: Portland, OR, 1991.
116. W. Wilbert, *Fitoterpia Warao*; Fundación La Salle de Ciencias Naturales: Caracas, 1997.
117. H. García Barriga, *Flora Medicinal de Colombia. Botánica Médica*; Tercer Mundo Editores: Bogotá, 1992.
118. B. Wolters, *Agave bis Zaubernuß: Heilpflanzen der Indianer Nord- und Mittelamerikas*; Urs Freund Verlag: Greifenberg, Germany, 1996.
119. N. J. Turner; L. C. Thompson; M. T. Thompson; A. Z. York, *Thompson Ethnobotany. Knowledge and Usage of Plants by the Thompson Indians of British Columbia*; Royal British Columbia Museum: Victoria, BC, Canada, 1990; Memoir No. 3.
120. M. Heinrich, *Ethnobotanik der Tieflandmixe und phytochemische Untersuchung von Capraria biflora*; Kramer: Stuttgart, 1989; Dissertationes Botanicae No. 144.
121. V. A. Argueta, coordinador, *Atlas de las plantas de la medicina tradicional Mexicana;* Instituto Nacional Indigenista: México, DF, 1994; 3 Vols.
122. M. Martinez, *Las Plantas Medicinales de México*; Ed Botas: México, 1969.
123. J. F. Morten, *Atlas of Medicinal Plants of Middle America, Bahamas to Yucatan*; C. Thomas: Springfield, IL, 1981.
124. L. D. Kapoor, *Handbook of Ayurvedic Medicinal Plants*; CRC Press: Boca Raton, FL, 1990.
125. E. M. Williamson, *Major Herbs of Ayurveda*; Churchill Livingstone: Edinburgh, 2002.
126. Anonymous, *The Wealth of India*; CSIR: New Delhi, India. 11 volumes with two supplements dealing with the Raw Materials, and 9 volumes covering the Industrial Products, 1950–1976.
127. R. N. Chopra; I. C. Chopra; K. L. Handa, *Indigenous Drugs of India*, 2nd ed.; U.N. Dhur and Sons Pvt. Ltd: Calcutta, 1958; p 679.
128. J. A. Parrotta, *Healing Plants of Peninsular India*; CABI Publishing: New York, 2001.

129. D. Benski; A. Gamble, Eds., *Chinese Herbal Medicine. Materia Medica*; Eastland Press: Seattle, WA, 1993.
130. C. G. Steenis, General Ed., *Flora Malesiana: Being an Illustrated Systematic Account of the Malesian Flora, Including Keys for Determination, Diagnostic Descriptions, References to the Literature, Synonymy, and Distribution, and Notes on the Ecology of Its Wild and Commonly Cultivated Plants/Republik Indonesia, Kementerian Pertanian;* Different publishers since Vol. 6; Indonesian Institute of Science: Jakarta, 1948.
131. P. F. Quer, *Plantas medicinales, El Dioscórides renovado*; Ed. Labor: Barcelona, 1962.
132. D. Rivera; C. Obón, *La Guía de Incafo de las Plantas Útiles y Venenosas de la Península Ibérica y Baleares*; INCAFO: Madrid, 1991.
133. J. J. Bellakhdar, *La pharmacopoée marocaine traditionelle. Médecine Arabe ancienne et savouirs populaires*; Ibis Press: Paris, 1997.
134. H. O. Lenz, *Botanik der alten Griechen und Römer*; Thienemann Verlag: Gotha, Germany, 1859.
135. E. Lev; Z. Amar, *Historical Arabic and Jewish Materia Media of the Eastern Mediterranean. Practical Materia Medica of the Medieval Eastern Mediterranean According to the Cairo Genizah*; Brill: Leiden, the Netherlands and Boston, MA, 2008.
136. J. Sterly, *Arzneipflanzen der Polynesier*; D. Reimer: Berlin, 1972.
137. B. Zepernick, *Heilpflanzen der Einwohner Melanesiens. Beiträge zur Ethnobotanik des südwestlichenen Pazifiks*; Arbeitsstelle für Ethnomedizin. Hamburger Reihe zur Literatur und Sprachwissenschaft Bd. 7: Hamburg, 1970.
138. www.niscair.res.in (New Delhi 110012, India) (last accessed 17 June 2009).
139. D. A. Posey, *J. Ethnopharmacol.* **2002**, *83*, 3–12.
140. C. M. Cotton, *Ethnobotany*; Wiley & Sons: Chichester, 1997.
141. G. M. Martin, *Ethnobotany*; Chapman & Hall: London, 1995.
142. M. Heinrich, *Ethnobotanik und Ethnopharmazie. Eine Einführung*; Wissenschaftliche Verlagsgesellschaft: Stuttgart, 2001.
143. M. Leonti; H. Vibrans; O. Sticher; M. Heinrich, *J. Pharm. Pharmacol.* **2001**, *53*, 1653–1669.
144. E. Lev; Z. Amar, *Practical Materia Medica of the Medieval Eastern Mediterranean According to the Cairo Genizah*; Brill: Leiden, the Netherlands and Boston, MA, 2008.
145. M. Heinrich; S. Edwards; M. Leonti; D. Moerman, *J. Ethnopharmacol.* **2009**, *124*, 1–17.
146. P. Bremner; M. Heinrich, *Phytochem. Rev.* **2005**, *4*, 21–37.
147. M. Raza, *J. Ethnopharmacol.* **2006**, *104*, 297–301.
148. D. J. Payne; M. N. Gwynn; D. J. Holmes; D. L. Pompliano, *Nat. Rev. Drug Discov.* **2007**, *6*, 29–40.
149. D. M. Shlaes, *Curr. Opin. Pharmacol.* **2003**, *3*, 470–473.
150. London School of Economics and Political Sciences/Wellcome Trust, The New Landscape of Neglected Disease Drug Development. LSE/Wellcome Trust. http://www.wellcome.ac.uk/stellent/groups/corporatesite/@msh_publishing_group/documents/web_document/wtx026593.pdf (2005) (last accessed 17 June 2009).
151. R. Verpoorte; Y. H. Choi; H. K. Kim, *J. Ethnopharmacol.* **2005**, *100*, 53–56.
152. R. Verpoorte; Y. H. Choi; H. K. Kim, *Phytochem. Rev.* **2007**, *6*, 3–14.
153. M. Politi; W. Peschel; N. Wilson; M. Zloh; J. M. Prieto; M. Heinrich, *Phytochemistry* **2008**, *69*, 562–570.
154. M. Heinrich, *Phytochem. Lett.* **2008**, *1*, 1–5.
155. A. Hazekamp; R. V. Cannabbis, This volume.
156. E. Boelsma; R. J. A. N. Lamers; F. F. J. Hendrick; R. A. J. Nesselroj; J. Roza, *Planta Med.* **2004**, *70*, 1052–1057.
157. M. Heinrich; J. Prieto, *Ageing Res. Rev.* **2008**, *7*, 249–274 (DOI: 10.1016/j.arr.2007.08.002).
158. N. L. Etkin; D. R. Harris; H. D. V. Prendergast, P. J. Houghton, Eds., *Plants for Food and Medicine*; Royal Botanic Gardens, Kew: Richmond, UK, 1998.
159. D. E. Moerman, *J. Ethnopharmacol.* **1996**, *52*, 1–22.
160. N. L. Etkin; P. J. Ross, *Soc. Sci. Med.* **1982**, *16*, 1559–1573.
161. N. Etkin, *Eating on the Wild Side. The Pharmacologic, Ecologic and Social Implications of Using Noncultigens*; University of Arizona Press: Tucson, AZ, 1994.
162. N. Etkin; P. J. Ross, *J. Ethnopharmacol.* **1991**, *32*, 25–36.
163. P. A. Cox, *Science* **2000**, *287* (5450), 44.

Biographical Sketch



Professor Dr. Michael Heinrich is the head of the Centre for Pharmacognosy and Phytotherapy, The School of Pharmacy, University of London. He is a pharmacognosist,

biologist (Dr. rer nat. habil, University Freiburg 1989, 1997, Dipl. Biol., 1985), and anthropologist (M. A., Wayne State University, 1982), with many years of research experience in many aspects of medicinal and food plants (esp. bioactive natural products), as well as at the interface of cultural and natural sciences with a particular interest in the cultural basis of medicinal plant use in Lowland Mexico and other countries. Current research interests include medicinal and food plants of the Mediterranean basin, Mexico and adjacent countries, anti-inflammatory natural products focusing on transcription factors as molecular targets, quality and standardization of herbal medical products used in Europe, cognitive aspects of medicinal plant usage, and the history of European plant-derived medicines. He has authored approximately 160 peer-reviewed full publications on the above topics. He is Reviews Editor of *Journal of Ethnopharmacology*, Associate Editor of the *Journal of Pharmacy and Pharmacology*, and Section Editor of *Phytochemistry Letters*.

3.13 Chinese Traditional Medicine

Min Yang, Sijia Tao, Shuhong Guan, Xiaohui Wu, Pingping Xu, and De-an Guo, Shanghai Institute of Materia Medica, Shanghai, China

3.13.1 Introduction

Traditional Chinese Medicine (TCM) has a long history of development and application in China and, recently, is beginning to play a role in western health care as a complementary and alternative medicine modality. A large number of human clinical data on the efficacy and the toxicity of TCM were gathered for the treatment of many diseases over thousands of years. The oldest monograph of the TCM is *Shenlong Bencaojing*, the author and the age of this monograph is not detailed. It is said that the book was written during the Warring States Period or during the Qin and Han Dynasties. In all, 365 medicines were recorded, including 252 plant medicines, 67 animal medicines, and 46 mineral drugs. In the Ming Dynasty, another classic of the TCM was generated by the great pharmaceutical scientist Li Shizhen in ancient times, which is the *Compendium of Materia Medica (Bencao Gangmu)* that was cherished as the best wealth by the later generations. The Chinese indigenous medicine before the sixteenth century was summarized systematically and in 1892 medicines were recorded, among which 1095 were plant medicines. Now, the commonly used TCM are embodied in *Chinese Pharmacopoeia* (2005 edition).[1] Totally, 1146 Chinese medicines were recorded, including 551 materia medica (*Zhongyaocai*) and decoctions (*Zhongyao Yinpian*) (439 are plant medicines), 31 plant oils, fats, and extracts, and 564 prescriptions and single preparations.

The basic theory and principles of TCM were raised by Huangdi Neijing (Inner Canon of Huangdi or the Yellow Emperor's Medicine Classic), which was written 2000 to 3000 years ago. Based on the Chinese philosophy of yin–yang and five elements, the basic theory of TCM includes five-zang organs and six fu organs, vital energy (*qi*), blood, and meridians (*jingluo*). TCM has an overall treatment concept that differs from western medicine. It emphasizes holistic and synergistic principles and harmony with the universe. According to the holistic viewpoint of TCM, the balance and interaction of all the components are considered more important than the effect of any individual component in TCM formulations. This is because other components in the TCM formulation may be used to suit the patient's *yin* and *yang* conditions or to reduce the drug resistance, toxicity, or side effects of the main components. Recently, there has also been a change in drug design with a step toward developing a combination of drugs in western medicine, the so-called cocktail therapy. It originated from the triple cocktail treatment of AIDS, also known as highly active antiretroviral therapy (HAART). The key to its success in some patients lies in the drugs combination ability to disrupt HIV at different stages in its replication. TCM drug treatment typically consists of a complex prescription of

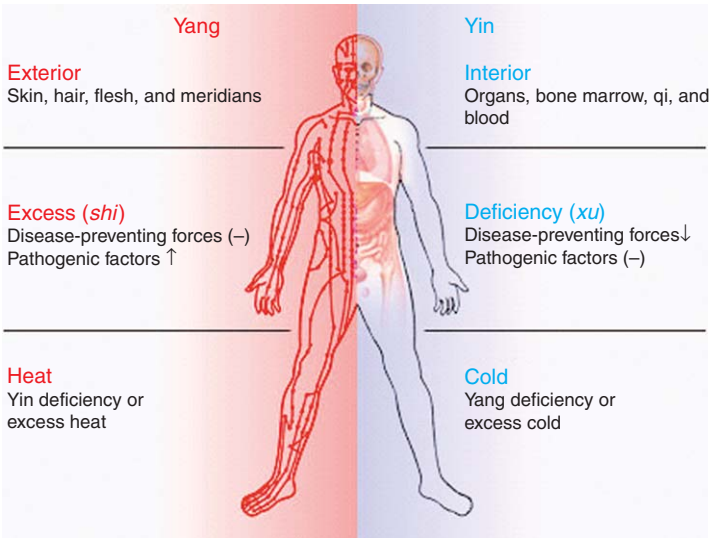


Figure 1 Summary on the eight principles of TCM symptoms and signs. Source: Shen-Nong Web, available from http://www.shen-nong.com/eng/exam/diagnosis_eightprinciples.html.

multiple components based on differentiation of symptoms and signs (*zheng*), including *yin*, *yang*, exterior (*biao*), interior (*li*), cold (*han*), heat (*re*), deficiency (*xu*), and excess (*shi*) (**Figure 1**). This quite agrees with the recently emerging personalized medicine, which is now a hot topic in western medicine. TCM treats the root cause of diseases rather than decrease the symptoms immediately. Therefore, it might take months or years for patients to recover and this is suitable particularly in the treatment of chronic diseases and rare illnesses. However, the four basic diagnostic methods in TCM, including inspection (*wang*), listening and smelling (*wen* 闻), inquiry (*wen* 问), and palpation (*qie*), are largely determined by the experience and knowledge of the physicians and easily affected by environmental factors. Therefore, it is necessary to build an objective diagnostic standard and it is of great importance to deepen the study of TCM syndrome and diagnostic methods by modern biomedicine technologies. The main differences between TCM and western medicine are shown in **Table 1**.

TCM not only consists of plants, but also includes the medicinal uses of animals and minerals. The processing (*pao zhi*) and prescriptions (*fang ji*) are also very unique and critical in the application of TCM. Over the past 100 years, uses of TCM dramatically decreased due to the growing popularity of western medicine. Therefore, a broader understanding of medical knowledge and reasoning on TCM is necessary. However, it is one of the two mainstream medical practices in the Chinese health care system. TCM represents 22% of the total medication revenue in hospitals; however, it also represents 15% in health centers and 33% in health clinics.[2] According to the report of the China Chamber of Commerce for Import & Export of Medicines & Health Products (CCCMHPIE), the TCM herbal medicine export is almost US$1.2 billion in 2007, which

Table 1 Main differences between TCM and western medicine

	TCM	*Western medicine*
Material base	Natural products	Single chemical synthesis product
Mechanism of action	Holistic and synergistic principles, multiple targets	Single target, selectivity, specificity
Treatment protocols	Determine the treatment based on differentiation of symptoms and signs	Indiscrimination
Purpose	Recover function of human body, regulate symptoms and signs, treat diseases from the root cause	Recover organs, treat disease directly, decrease the symptoms immediately
Side effect	Not obvious	Obvious
Preponderance	Chronic disease, rare illness	Acute disease, emergency treatment

represents 20–50% of the herbal medicine market share worldwide depending on different definitions and calculations.[2] Furthermore, TCM has been widely used for therapeutic interventions in diseases, such as cancers,[3–7] asthma and allergy,[8] Parkinsonism,[9] Alzheimer's disease (AD),[10–12] drug addiction,[13,14] and metabolic syndrome,[15] thereby allowing us to identify promising compounds for treatment of those diseases using TCM. However, the value of TCM has not yet been fully recognized worldwide due to the lack of definitive information of active ingredients in almost all TCM preparations.

Over the past decades, development of TCM has followed two separate paths, either toward complementary medicine or toward western medicine. Anyway, there is no question that TCM has become one of the most important resources for screening of lead compounds. Modern pharmaceutical sciences, such as phytochemistry, pharmacognosy, phytotherapy, pharmacokinetics, and pharmacology, provide the scientific methodology and technology to systematically investigate the scientific basis of TCM. The studies on the active constituents of TCM not only develop directly new drugs or lead compounds, but also provide the material basis and biomarkers for modernization of TCM. Some medicinal uses of natural products and derivatives from TCM have been recently reviewed.[3,16] **Figure 2** compiles the structures of some TCM-based drugs that are being used in therapy or being applied in clinical trials against various diseases, especially for cancer therapy.

Malaria is one of the most severe communicable diseases in the world. Artemisinin combination treatments (ACTs) are now first-line drugs for uncomplicated falciparum malaria and are recommended by the World Health Organization (WHO) to treat especially multidrug-resistant forms of malaria. Artemisinin (**1**), called qinghaosu in Chinese, is a sesquiterpene lactone that bears a peroxide grouping and, unlike most other western antimalarials, lacks a nitrogen-containing heterocyclic ring system. The compound was isolated in 1971 by Chinese chemists from the herb *Artemisia annua* L. (Qinghao) (Asteraceae), which has been used for many centuries in TCM for treatment in fever and malaria. Qinghaosu has been used successfully in large number of malaria patients, including those with both chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*. Artemether (**2**) and the water-soluble sodium artesunate (**3**) are the semisynthetic derivatives of qinghaosu. They are well established worldwide for use in malaria therapy. Thus, qinghaosu and its derivatives offer promise as a totally new class of antimalarials.[16–18] Artesunate has also been tried for cancer treatment during the past decade.[19,20]

Arsenic trioxide is the most important commercial compound of arsenic and the main starting material for arsenic chemistry with high toxicity. However, it is also the main active ingredient of a traditional Chinese mineral remedy named Pishuang for a variety of ailments. From the mid-twentieth century, researchers at Shanghai and Harbin, China, have found that arsenic trioxide can induce remissions in up to 70% of a rare blood cancer called acute promyelocytic leukemia (APL) patients. In 1996, Jeffrey Mervis[21] gave a special report on this research in *Science* entitled 'Cancer Research: Ancient Remedy Performs New Tricks'. Subsequently, randomized clinical trials in the United States resulted in FDA's approval of arsenic trioxide for relapsed or refractory APL in September 2000.[22]

Xishuguo, fruits of the Chinese 'happy tree' (Xishu), *Camptotheca acuminate* Decne. (Davidiaceae), produce a valuable natural product namely camptothecin (**4**), which was reported to be applied in tumor therapy by inhibiting the ligation of DNA after topoisomerase I-mediated strand breaks.[23,24] Besides other antitumor drugs are also found in TCM, such as podophyllotoxin (**5**, found in *Podophyllum emodi* Wall var. *chinensis* Sprague or *Dysosma versipellis* (Hance) M. Cheng (Berberidaceae) (Guijiu)),[3,25] β-elemene (**6**, isolated from *Curcuma aromatica* Salisb. or *C. wenyujin* Y. H. Chen et C. Ling (Zingiberaceae) (Wenyujin)),[26,27] cantharidin (**7**, from *Mylabris phalerata* Pallas or *M. cichorii* L. (Meloidae) (Banmao)),[28] oridonin A (**8**, from *Rabdosia rubescens* (Hamst.) C. Y. Wu et Hsuan (Lamiaceae) (Donglingcao)),[29] and ginsenoside Rg_3 (**9**, from *Panax ginseng* (Ginseng) or *P. notoginseng* (Sanqi) (Araliaceae)).[30] Among these TCMs, Guijiu and Banmao are traditionally used in the therapy of carbuncle abscess and tumescence. Recently, Wenyujin, Banmao, and Donglingcao are used for treating cancer at clinics.

Two novel antihepatitis drugs, bifendate (**10**)[31] and bicyclol (**11**),[32] were semisynthesized from schizandrin C (**12**),[33] which was isolated from *Schisandrae chinensis* (Turcz.) Baill. (Magnoliaceae) (Wuweizi), a Chinese herb used in the therapy of viral hepatitis. Huperzine A (**13**), an alkaloid isolated from a Chinese herbal medicine *Huperzia serrata* (Thunb. ex Murray) Trev. (Lycopodiaceae) (Qiancengta), has been reported to be a potent, highly specific, and reversible inhibitor of acetylcholinesterase (AChE) and used for treatment of Alzheimer's

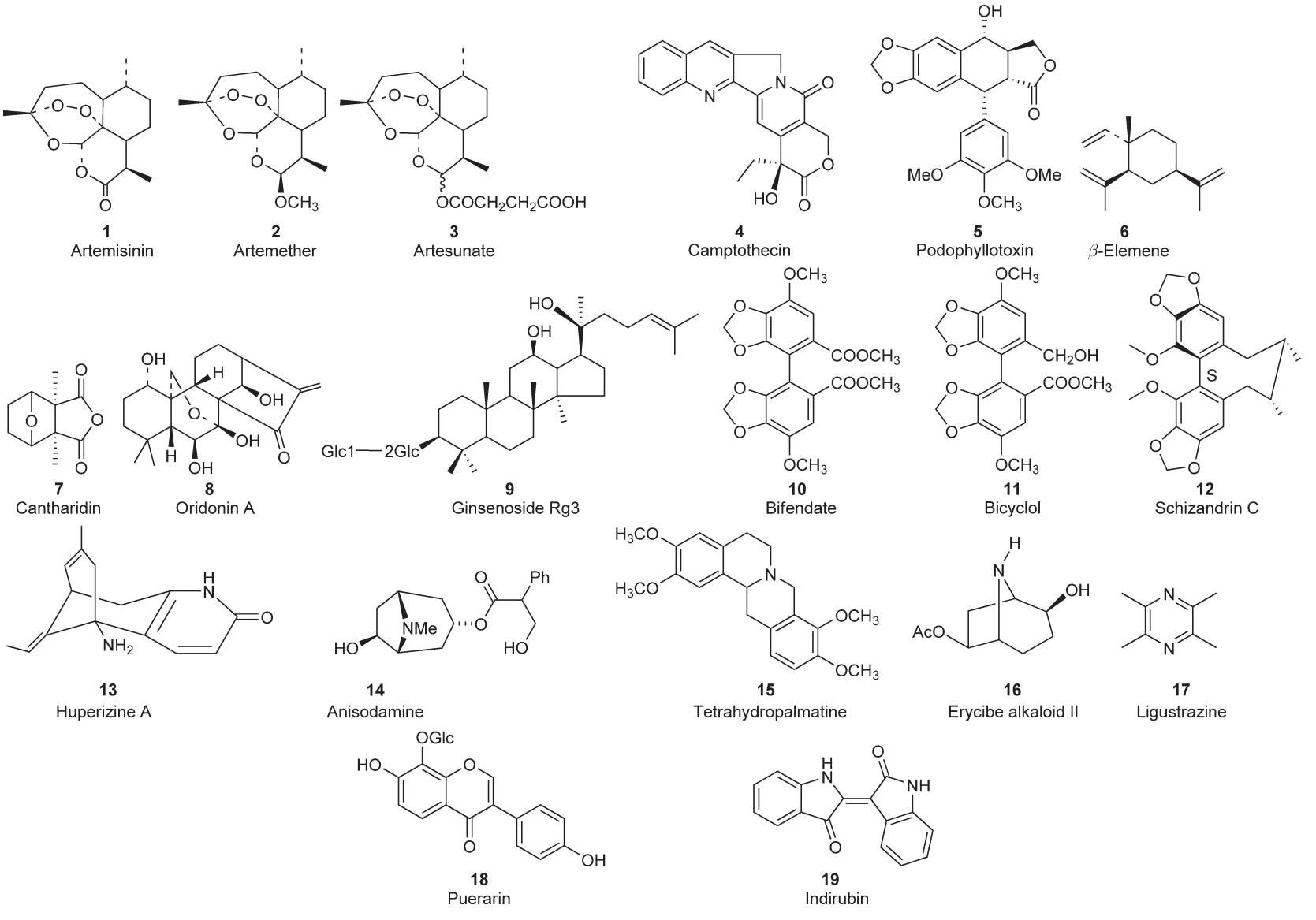


Figure 2 Structures of TCM-derived products used in western medicine.

disease (AD).[34–36] Anisodamine (**14**), a naturally occurring atropine derivative isolated from the plant *Anisodus tanguticus* (maxim.) Pasch. (Solanaceae) (Shanlangdang) by scientists in China, has been used to improve the blood flow in the microcirculation and treat organophosphorous (OP) poisoning and snakebites.[37,38] Tetrahydropalmatine (**15**) was isolated from *Corydalis ambigua* W. T. Wang (Papaveraceae) (Yanhusuo) and used for analgesia.[39] Erycibe alkaloid II (**16**, baogongteng A), a naturally occurring tropane muscarinic agonist isolated from the Chinese medicinal plant *Erycibe obtusifolia* Benth. (Convolvulaceae) (Baogongteng), has been used for treatment of cataracta glauca.[40,41] Ligustrazine (**17**), an active component of *Ligusticum chuanxiong* Hort. (Apiaceae) (Chuanxiong), has been studied and developed to be a new drug for therapy of acute cerebral thrombosis.[42,43] Puerarin (**18**), an isoflavone glycoside in *Pueraria lobata* (Willd.) Ohwi (Fabaceae) (Gegen), is known as an antioxidant and vascular protective drug.[44] Indirubin (**19**) was identified from *Radix isatidis* (Banlangen) as an antileukemic drug with no inhibition of the bone marrow.[45,46] The activities of anisodamine, erycibe alkaloid II, and indirubin are seemingly not connected with the traditional use of Chinese medicines.

About 140 new drugs have been developed from TCM.[47] The popularity of TCM in China and throughout the world caused systematic investigations on a scientific basis of TCM. Here, we review the work on phytochemical investigations of 10 of the most popular TCM mainly regarding the treatment of cardiovascular and cerebrovascular diseases, cancers, gynecological diseases, and immunological diseases.

3.13.2 Radix et Rhizoma Notoginseng (Sanqi, Tianqi, or Sanchi)

Sanqi, the radix and rhizome of *Panax notoginseng* (Burk.) F. H. Chen (Araliaceae), is one of the most commonly used and highly researched species of the *Panax* genus. This species has been an important herbal remedy in TCM for thousands of years, where it has been used primarily in the treatment of cardiovascular diseases, inflammation, different body pains, trauma, and internal and external bleeding due to injuries. In 1970s, it was found that Sanqi contained similar constituents as those of *Panax ginseng* C. A. Meyer and this attracted the attention of many investigators. Large numbers of systematic studies were performed involving modern pharmaceutical disciplines, including phytochemistry, pharmacology, and clinical application. Ng[48] reviewed the research findings on the pharmacological activities of Sanqi. The main active components were found to be saponins with the protective actions against cerebral ischemia, beneficial effects on the cardiovascular system,[49–51] hepatoprotective,[52] antioxidant, renoprotective, and estrogen-like activities.[48] Besides, polysaccharides with immunopotentiating activity,[53] proteins with antifungal,[54,55] ribonuclease[56] and xylanase[57] activity, and a triacylglycerol (trilinolein) with antioxidant activity[58,59] have been reported. The pharmacological activities are quite in agreement with the traditional use of Sanqi.

Here, we summarize the reported saponins from *P. notoginseng* in **Figure 3**. Over 70 compounds were isolated from the different parts of Sanqi.[30,60–90] Most of these compounds are 20(*S*)-protopanaxadiols and 20(*S*)-protopanaxatriols possessing dammarane skeleton. No oleanolic acid saponins were found, which can differentiate Sanqi from Ginseng. Except for the common ginsenosides of the *Panax* genus, some exclusive notoginsenosides were also isolated from Sanqi. The contents of ginsenosides Rg_1 and Rb_1 are higher than the others.

3.13.3 Radix et Rhizoma Salviae Miltiorrhizae (Danshen)

Danshen (or Tanshen), the dried root of *Salvia miltiorhiza* Bunge (Lamiaceae), has been widely used for the treatment of cardiovascular and cerebrovascular diseases and widely accepted as a health product in the Western countries in recent years.[91] Phytochemical studies on its chemical components and biological activities resulted in the lipophilic diterpenoid tanshinones and hydrophilic caffeic acid derivatives. Both groups contribute to the biological activities of Danshen. About 70 tanshinones (**Figure 4**) and 30 caffeic acid derivatives (**Figure 4**) were isolated from this plant. Some reviews are focused on the recent progress of the chemical, pharmacological, and analytical studies on this herb.[92–95]

Research has been mainly confined to the lipophilic constituents before the 1990s. Most of the tanshinones possess phenanthraquinone and naphthaquinone chromophores and show antibacterial,[96] antioxidant,[97] and

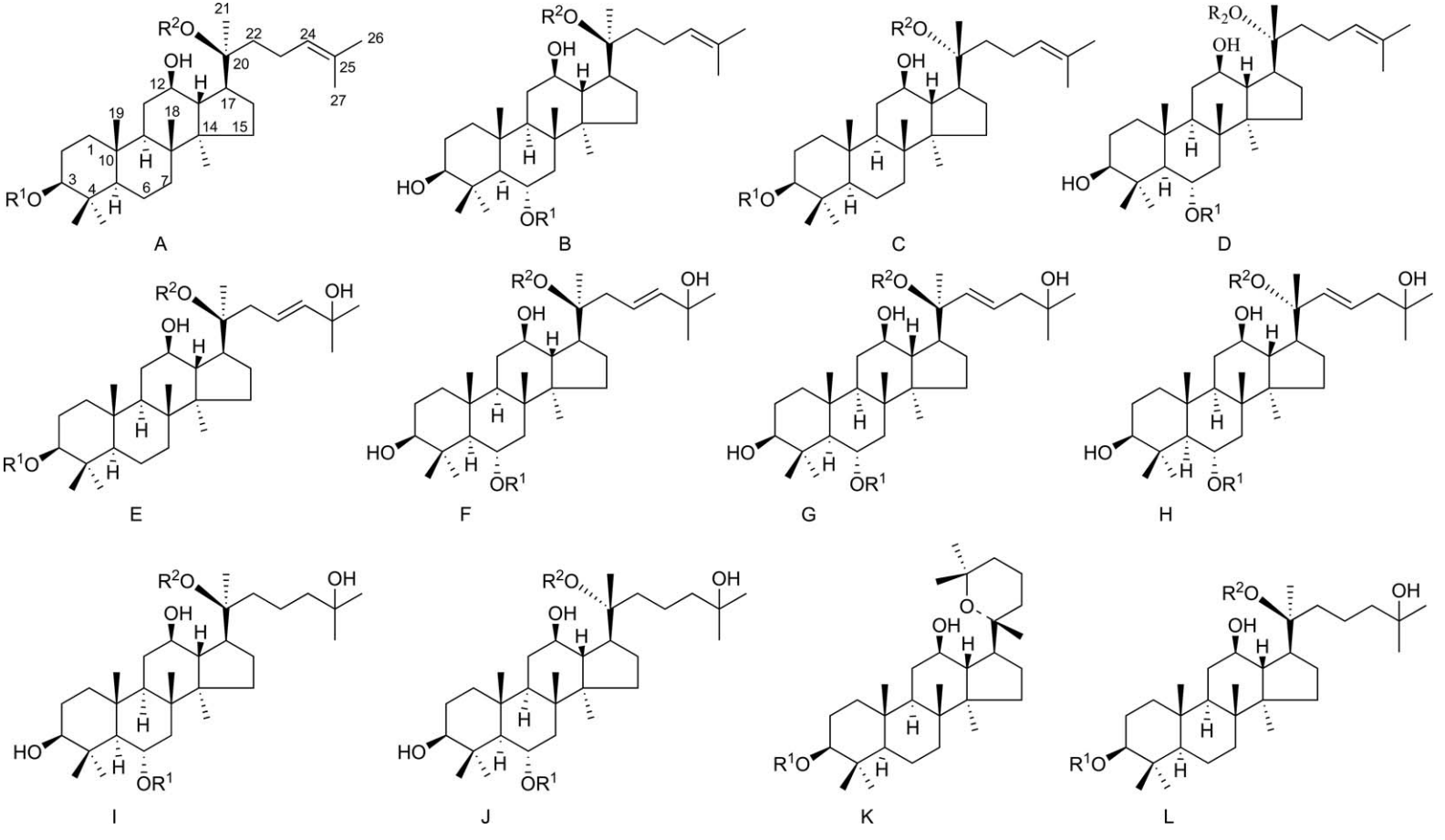
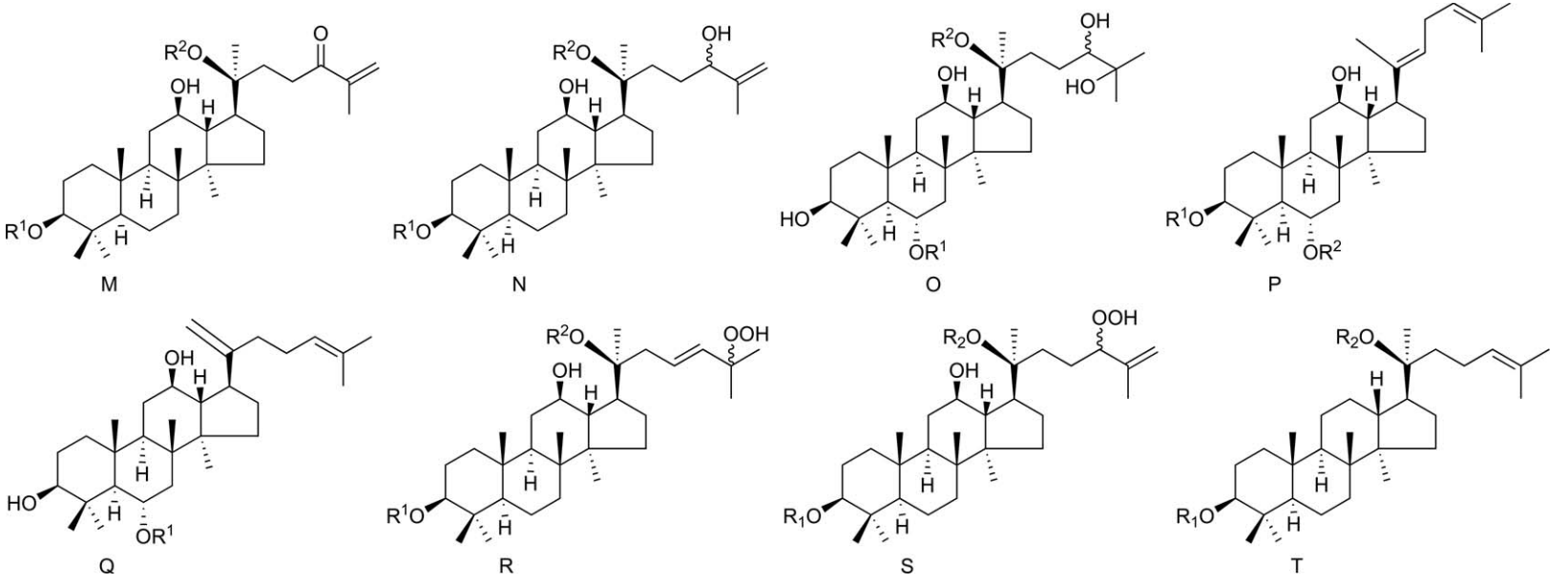


Figure 3 (*Continued*)



Type		Name	R^1	R^2	Reference(s)
A	**20**	Ginsenoside Rd	Glc^2–^1Glc	Glc	60, 61
	21	Ginsenoside Rb_1	Glc^2–^1Glc	Glc^6–^1Glc	60, 61
	22	Ginsenoside Rb_2	Glc^2–^1Glc	Glc^6–$^1Ara(p)$	60
	23	Ginsenoside Rh_2	Glc	H	30
	9	Ginsenoside Rg_3	Glc^2–^1Glc	H	30
	24	Ginsenoside F_2	Glc	Glc	62, 63
	25	Ginsenoside Ra_3	Glc^2–^1Glc	Glc^6–$^1Glc^3$–^1Xyl	64
	26	Ginsenoside Rb_3	Glc^2–^1Glc	Glc^6–^1Xyl	65
	27	Ginsenoside Rc	Glc^2–^1Glc	Glc^6–$^1Ara(f)$	65, 66
	28	Ginsenoside CK	H	Glc	67, 68
	29	Ginsenoside MC	H	Glc^6–$^1Ara(f)$	67, 69
	30	Notoginsenoside D	Glc^2–$^1Glc^2$–^1Xyl	Glc^6–$^1Glc^6$–^1Xyl	64
	31	Notoginsenoside Fa	Glc^2–$^1Glc^2$–^1Xyl	Glc^6–^1Glc	70
	32	Notoginsenoside Fc	Glc^2–$^1Glc^2$–^1Xyl	Glc^6–^1Xyl	71, 72

(continued)

Figure 3 (*Continued*)

Type	Name	R^1	R^2	Reference(s)
33	Notoginsenoside Fc	Glc	Glc^{6}–^{1}Ara(f)	67
34	Notoginsenoside K	Glc^{6}–^{1}Glc	Glc	61
35	Notoginsenoside L	Glc^{2}–^{1}Xyl	Glc^{6}–^{1}Glc	73
36	Notoginsenoside O	Glc	Glc^{6}–^{1}Xyl^{3}–^{1}Xyl	74
37	Notoginsenoside P	Glc	Glc^{6}–^{1}Xyl^{4}–^{1}Xyl	74
38	Notoginsenoside Q	Glc^{2}–^{1}Glc^{2}–^{1}Xyl	Glc^{6}–^{1}Xyl^{4}–^{1}Xyl	74
39	Notoginsenoside R_4	Glc^{2}–^{1}Glc	Glc^{6}–^{1}Glc^{6}–^{1}Xyl	61
40	Notoginsenoside S	Glc^{2}–^{1}Glc^{2}–^{1}Xyl	Glc^{6}–^{1}Ara(f)^{5}–^{1}Xyl	74
41	Notoginsenoside T	Glc^{2}–^{1}Glc^{2}–^{1}Xyl	Glc^{6}–^{1}Glc^{3}–^{1}Xyl	74
42	3-*O*-[β-D-Glucopyranosyl(1→6) -β-D-glucopyranosyl]-20-*O*-β-D -glucopyranosyl-3β, 12β, 20 (*S*)-trihydroxydammar-24-ene	Glc^{6}–^{1}Glc	Glc	60
43	3-O-β-D-Glucopyranosyl 20-*O*- [α-L-arabino-pyranosyl (1→2)-β-D -glucopyranosyl] 3β, 12β, 20 (*S*)-trihydroxydammar-24-ene	Glc	Glc^{6}–^{1}Ara(p)	60
44	Gypenoside IX	Glc	Glc^{6}–^{1}Xyl	75, 76
45	Gypenoside XIII	H	Glc^{6}–^{1}Xyl	76
46	Gypenoside XV	Xyl^{2}–^{1}Glc	Glc^{6}–^{1}Xyl	77
47	Gypenoside XVII	Glc	Glc^{6}–^{1}Glc	76, 77
48	Malonyl-ginsenoside Rb_1	Glc^{2}–^{1}Glc^{6}–Malonyl	Glc^{6}–^{1}Glc	78, 79
49	Malonyl-ginsenoside Rd	Glc^{2}–^{1}Glc^{6}–Malonyl	Glc	80
50	(20*S*)-Protopanaxadiol	H	H	81

(continued)

Figure 3 (*Continued*)

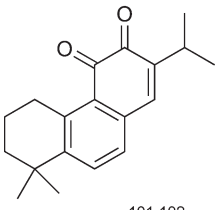
Type		Name	R^1	R^2	Reference(s)
B	**51**	Ginsenoside F_1	H	Glc	62, 63
	52	Ginsenoside Re	Glc^2–^{1}Rha	Glc	60, 82, 83
	53	Ginsenoside Rg_1	Glc	Glc	60, 82, 83
	54	Ginsenoside Rg_2	Glc^2–^{1}Rha	H	60, 82
	55	Ginsenoside Rf	Glc^2–^{1}Glc	H	66, 80
	56	Ginsenoside Rh_1	Glc	H	60, 81, 82, 83
	57	20-*O*-glucoginsenoside Rf	Glc^2–^{1}Glc	Glc	80
	58	Malonyl-ginsenoside Rg_1	Glc^6–Malonyl	Glc	84
	59	Notoginsenoside M	Glc^6–^{1}Glc*	Glc	73
	60	Notoginsenoside N	Glc^4–^{1}Glc*	Glc	73
	61	Notoginsenoside R_1	Glc^2–^{1}Xyl	Glc	60, 82, 83
	62	Notoginsenoside R_2	Glc^2–^{1}Xyl	H	60, 82, 83
	63	Notoginsenoside R_3	Glc	Glc^6–^{1}Glc	60
	64	Notoginsenoside R_6	Glc	Glc^6–^{1}Glc*	80, 85
	65	Chikusetsusaponin L_5	H	Glc^6–^{1}Ara(p)^4–^{1}Xyl	84
	66	Yesanchinoside E	Glc^2–^{1}Rha	Glc^6–^{1}Glc	84
C	**67**	(20*R*)-Ginsenoside Rg_3	Glc^2–^{1}Glc	H	30, 86
	68	(20*R*)-Ginsenoside Rh_2	Glc	H	86
	69	Notoginsenoside Ft_1	Glc^2–^{1}Glc^2–^{1}Xyl	H	30
	70	(20*R*)-Protopanaxadiol	H	H	81
D	**71**	(20*R*)-Ginsenoside Rh_1	Glc	H	82
E	**72**	Notoginsenoside A	Glc^2–^{1}Glc	Glc^6–^{1}Glc	64
	73	Yesanchinoside H	Glc^2–^{1}Glc	Glc^2–^{1}Xyl	84

(continued)

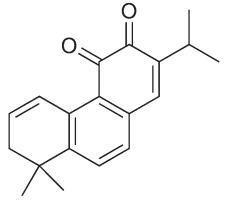
Figure 3 (*Continued*)

Type		Name	R^1	R^2	Reference(s)
F	**74**	6-*O*-β-D-glucopyranosyl 20-O-β-D-glucopyranosyl 3β, 6α, 12β, 20 (*S*), 25-pentahydroxydammar-23-ene	Glc	Glc	60
G	**75**	(20*S*)-Dammar-22-ene-3β, 6α, l2β, 20, 25-pentol 6-0-β-D-glucopyranoside	Glc	H	81
H	**76**	(20*R*)-Dammar-22-ene-3β, 6α, l2β, 20, 25-pentol 6-0-β-D-glucopyranoside	Glc	H	81
I	**77**	25-Hydroxy-20(*S*)-ginsenoside-Rh_1	Glc	H	82
J	**78**	25-Hydroxy-29(*R*)-ginsenoside-Rh_1	Glc	H	82
K	**79**	Notoginsenoside R_7	Glc	–	87
L	**80**	Notoginsenoside Ft_2	$Glc^2–^1Glc^2–^1Xyl$	H	30
M	**81**	Notoginsenoside B	$Glc^2–^1Glc$	$Glc^6–^1Glc$	64
N	**82**	Notoginsenoside Ft_3	$Glc^2–^1Glc^2–^1Xyl$	H	30
O	**83**	Notoginsenoside J	Glc	Glc	88
P	**84**	Ginsenoside Rh_3	Glc	H	84
	85	Ginsenoside Rh_4	H	Glc	82, 83
Q	**86**	Notoginsenoside T_5	$Glc^2–^1Xyl$	–	62, 89
R	**87**	Notoginsenoside E	$Glc^2–^1Glc$	Glc	62, 88
	88	Notoginsenoside H	$Glc^2–^1Xyl$	Glc	88
	89	Notoginsenoside K	$Glc^2–^1Glc$	$Glc^6–^1Glc$	64
S	**90**	Notoginsenoside II	$Glc^2–^1Glc$	Glc	62, 90
	91	Notoginsenoside C	$Glc^2–^1Glc$	$Glc^6–^1Glc$	64
T	**92**	Notoginsenoside I	$Glc^2–^1Glc$	$Glc^6–^1Glc$	88
U	**93**	Notoginsenoside G	$Glc^2–^1Glc$	Glc	88

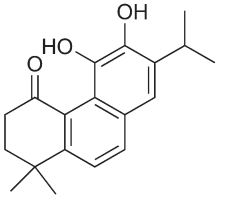
Figure 3 Compounds isolated from *Panax notoginsenoside* (Sanqi).



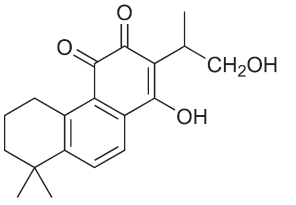
Miltirone (**94**)[101,102]



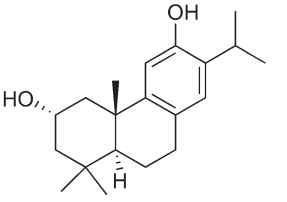
1, 2-Didehydromiltirone (**95**)[101]



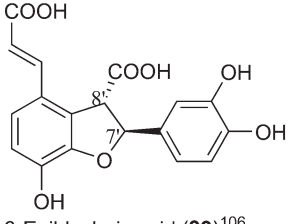
Miltiodiol (**96**)[103]



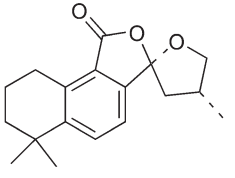
Tanshinone V (**97**)[104]



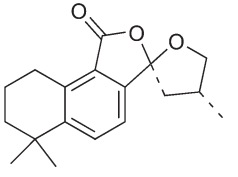
Salviol (**98**)[105]



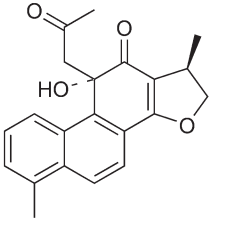
8-Epiblechnic acid (**99**)[106]



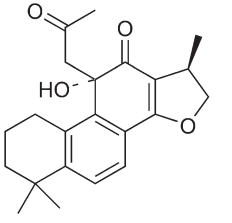
Cryptoacetalide (**100**)[107]



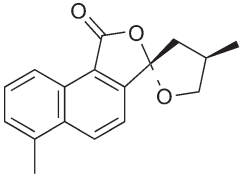
Epicryptoacetalide (**101**)[107]



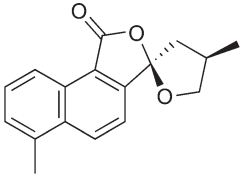
Danshenol A (**102**)[108]



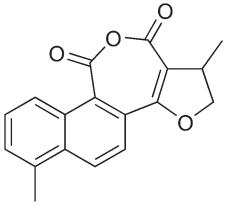
Danshenol B (**103**)[108]



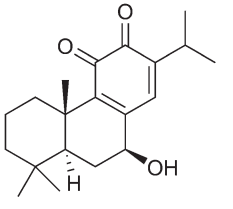
Danshenspiroketallactone (**104**)[109]



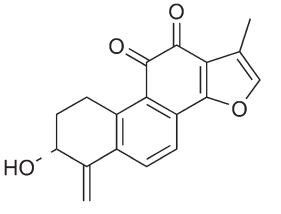
Epi-danshenspiroketallacto ne (**105**)[110]



1,2-Dihydro-1,6- dimethyl furo[3,2]naphth[2,1-e]oxe pine-10,12-dione (**106**)[111]

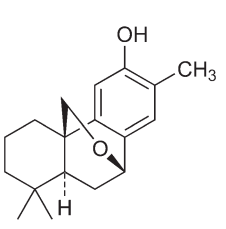


7-Hydroxy-8,13-abietadiene-11,12-dione (**107**)[112]

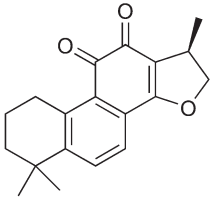


3a-Hydroxymethylenetan shinquinone (**108**)[113]

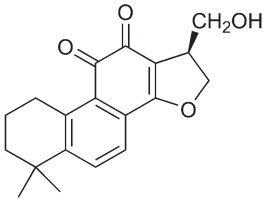
Figure 4 (*Continued*)



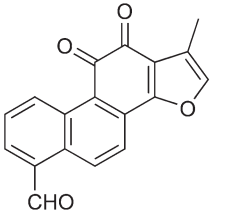
Norsalvioxide (**109**)[103]



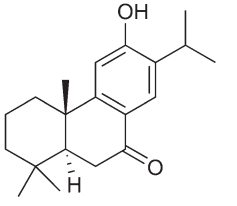
Cryptotanshinone (**110**)[114,115]



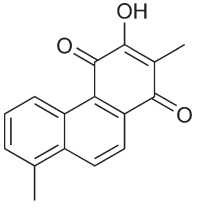
17-Hydroxycryptotanshinone (**111**)[116,117]



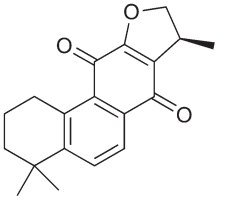
Formyltanshinone (**112**)[112]



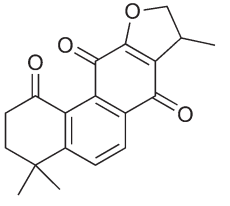
Isomicropinic acid (**113**)[112]



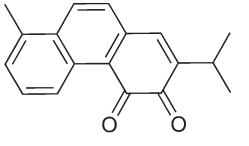
Tanshinquinone C (**114**)[118]



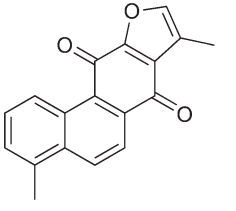
Isocryptotanshinone (**115**)[119,120]



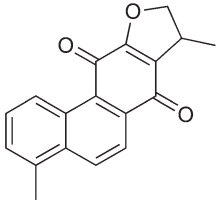
1-Ketoisocryptotanshinone (**116**)[117]



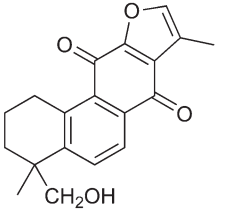
2-Isopropyl-8-methyl-3,4-phenanthraquinone (**117**)[121]



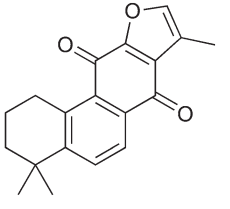
Isotanshinone I (**118**)[119,122]



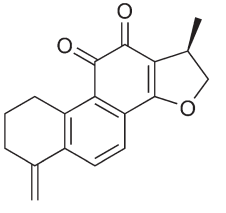
Dihydroisotanshinone I (**119**)[120]



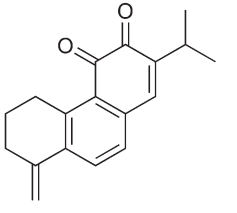
Isotanshinone IIB **(120)**[119]



Isotanshinone IIA (**121**)[119,120]



Methylenedihydrotanshinquinone (**122**)[112]



4-Methylenemiltirone **(123)**[112]

Figure 4 (*Continued*)

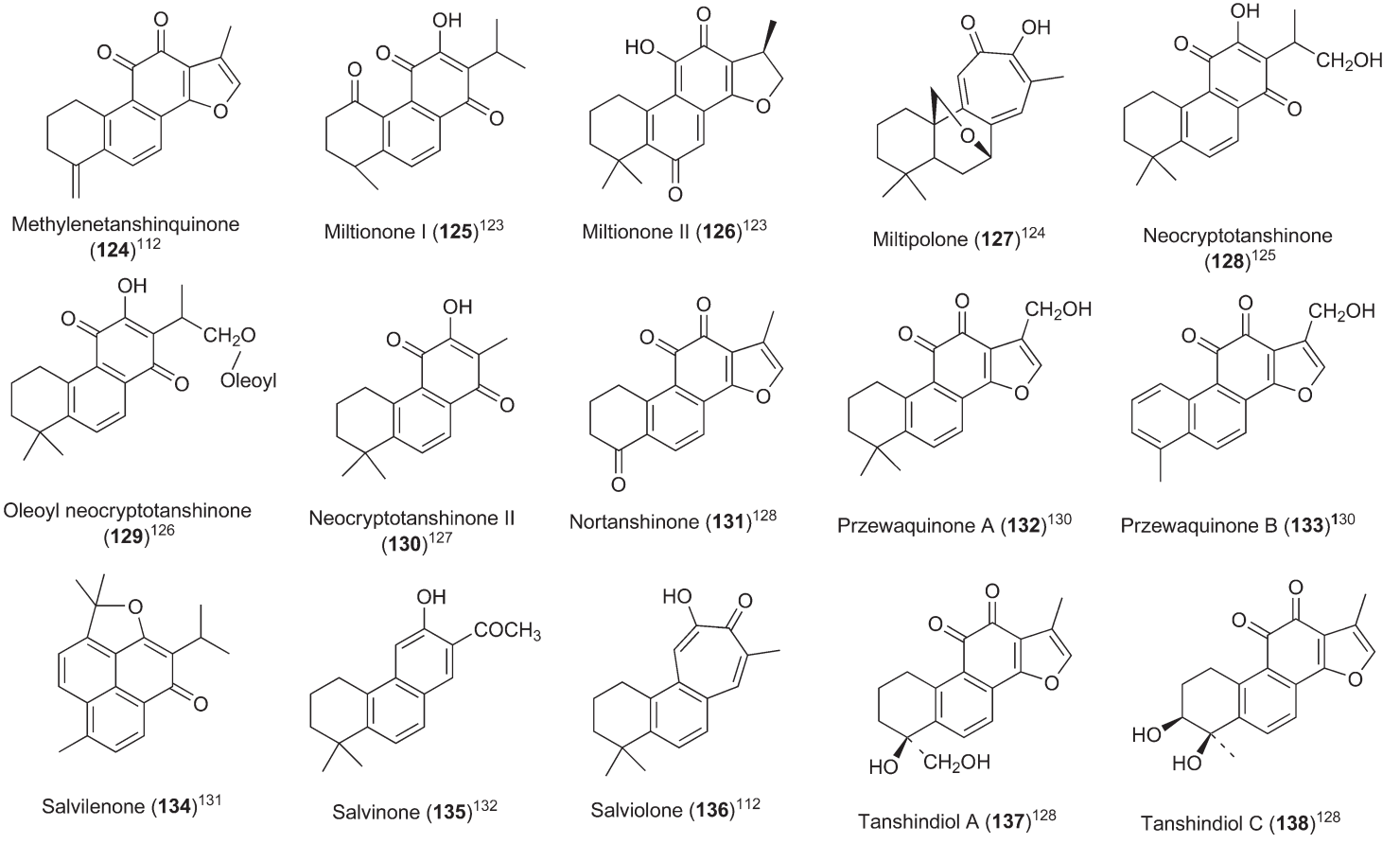
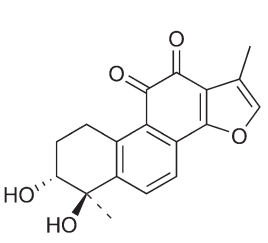
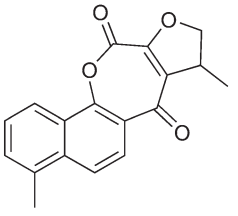


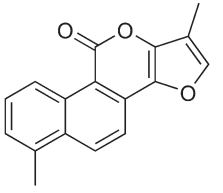
Figure 4 (*Continued*)



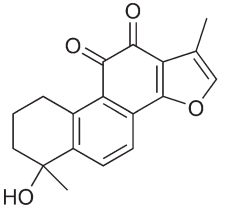
Tanshindiol B (**139**)[128]



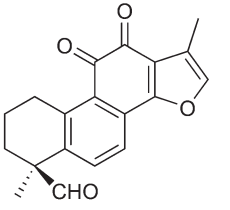
Tanshinketolactone (**140**)[133]



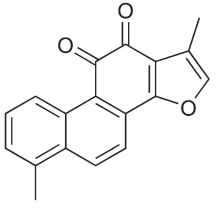
Tanshinlactone (**141**)[134]



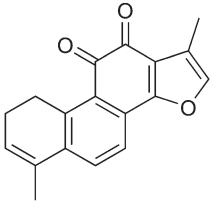
Tanshinol B (**142**)[135]



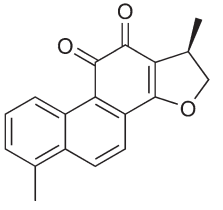
Tanshinonal (**143**)[113]



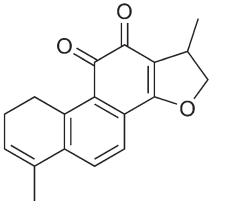
Tanshinone I (**144**)[119,121]



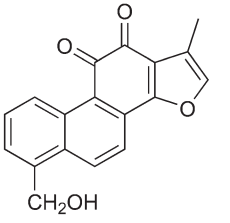
1,2-Dihydrotanshinone I (**145**)[112]



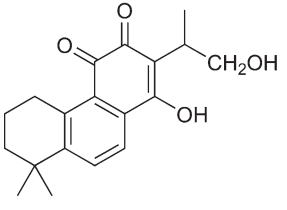
15,16-Dihydrotanshinone I (**146**)[111]



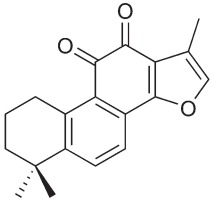
Trijuganone B (**147**)[112]



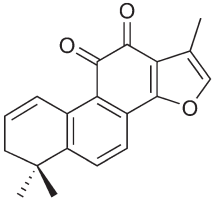
Tanshinol A (**148**)[135]



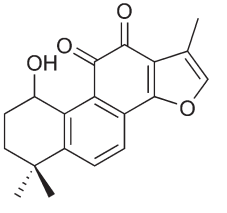
Tanshinone V (**149**)[104]



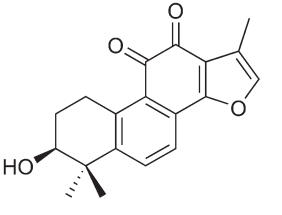
Tanshinone IIA (**150**)[136]



1,2-Didehydrotanshinone IIA (**151**)[112]



Hydroxytanshinone (**152**)[136]



3-Hydroxytanshinone IIA (**153**)[128,129]

Figure 4 (*Continued*)

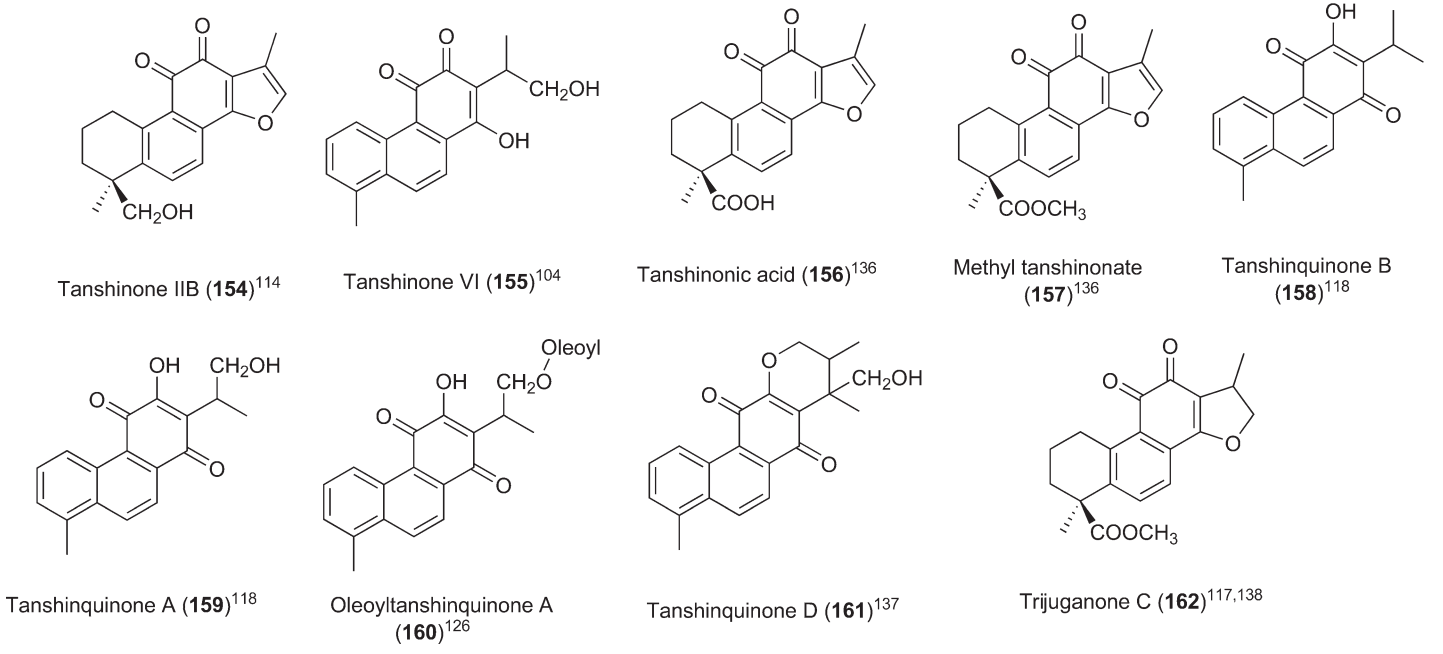


Figure 4 Tanshinones isolated from Danshen.

antineoplastic[98] activities. Tanshinone IIA (**150**) is the most abundant lipophilic compound in Danshen. Tanshinone I (**144**), cryptotanshinone (**110**), and 15,16-dihydrotanshinone I (**51**) are also the main constituents of the plant. Thus, the biological studies have prominently focused on these relatively abundant compounds. Cryptotanshinone (**17**) and 15,16-dihydrotanshinone I (**146**) generate superoxide radicals and thus show strong antibacterial activity against Gram-positive bacteria.[96] Tanshinone IIA (**150**), tanshinone I (**144**), cryptotanshinone (**110**), and 15,16-dihydrotanshinone I (**146**) were reported to be effective coronary artery dilators[99] and can prevent myocardial ischemia.[100] Tanshinone IIA also shows cytotoxic activity and induces differentiation and apoptosis and may be a promising chemotherapy drug to destroy cancer cells.[98]

Since the 1980s, the water-soluble constituents of Danshen have been studied by the Chinese and Japanese scientists and nearly 30 phenolic acids were isolated from this plant. The structures of these phenolic acids, including caffeic acid monomers and oligomers, are summarized in **Figure 5**. The oligomers of caffeic acid are also called salvianolic acids, which have attracted particular attention of medicinal chemists and clinicians due to their variety of pharmacological activities such as antioxidant, antiblood coagulation, and cell protection.[95,139,140] Salvianolic acid B (**187**, lithospermic acid B[141]) is more abundant than other water-soluble constituents in Danshen. Along with its Mg^{2+} salt (**188**), salvianolic acid B was reported to show multiple activities such as antioxidant,[142] kidney function regulation,[143] cardiovascular effects,[144] and anti-HIV activity.[145]

These studies on tanshinones and salvianolic acids not only provide evidence for the traditional uses of Danshen, but also lead to promising use for treatment of new diseases.

3.13.4 Ganoderma (Lingzhi)

Lingzhi (*Ganoderma lucidum* (Leyss.ex Fr.) Karst, Polyporaceae), a well-known TCM, has been used clinically in China and other Asian countries for several thousand years.[148,163–165] It was classified as one of the first class of traditional Chinese medicinal materials in *Shennong Bencaojing*. Ancient Chinese believed that it could cure various diseases and worshipped it as an 'immortal herb'. It is recorded in the *Chinese Pharmacopoeia*.

It was claimed to possess antimicrobial,[166,167] antiviral activities, including antihuman immunodeficiency virus (HIV),[168] antiaging activity,[169–171] antioxidant activity,[172,173] anti-inflammatory activity,[174] immunomodulating activity,[175–186] anti-HUC-PC growth properties,[187] antitumor activity through inhibiting proliferation and inducing apoptosis of cancer cells,[148,164,188–195] reducing tumor invasiveness,[196–198] immunomodulating effect,[199–204] and modulating signaling.[205,206] It was also reported that the cytotoxicity of doxorubicin (DOX) combined with Ganoderma triterpenes (GTS) or lucidenic acid N has a synergistic effect in HeLa cells, and the molecular targets of GTS was identified by two-dimensional gel electrophoresis-based comparative proteomics.[207] The aqueous extracts of *G. lucidum* could provide beneficial effects in treating type 2 diabetes mellitus (T2DM) by lowering the serum glucose levels through the suppression of the hepatic PEPCK gene expression[208] and other mechanisms.[209] It could significantly decrease the galactitol accumulation[210] and inhibit tyrosinase activity (skin care).[211] It could be used to treat arthritis[212–214] and hypoglycemosis. It has an effect on the blood vessel system[215] and protects against hepatic injury in rats.[216] It has long been a popular oriental medicine for treating liver diseases. Triterpenoid-rich extract inhibited PDGF-BB-activated HSC proliferation possibly through blocking PDGFbetaR phosphorylation, thereby indicating its efficacy for preventing and treating hepatic fibrosis.[217] The ganoderic acids possessed antihepatitis B activity.[218,219] It also has an effect on hepatic damage through antimutagenic activity.[220] The peptides and proteoglycans of *G. lucidum* could protect against liver injury in mice.[221,222] The proteoglycans of *G. lucidum* also have an ameliorative effect on carbon tetrachloride-induced liver fibrosis.

Ganoderma lucidum extracts could stimulate glucose uptake in L6 rat skeletal muscle cells.[223] It also exerts a potent chemopreventive effect,[224] and related to its neuroprotective role has a potential for therapeutic treatment of Parkinson's disease.[225] *Ganoderma lucidum* might be a useful ingredient in the treatment of androgen-induced diseases, including benign prostatic hyperplasia and prostate cancer.[226,227] The extracts of several species of *Ganoderma* were cytotoxic to both drug-sensitive and drug-resistant SCLC cells, and were proapoptotic, induced gene-expression patterns that were similar to SCLC cells treated with chemotherapeutic drugs, and could reverse resistance to chemotherapeutic drugs.[228]

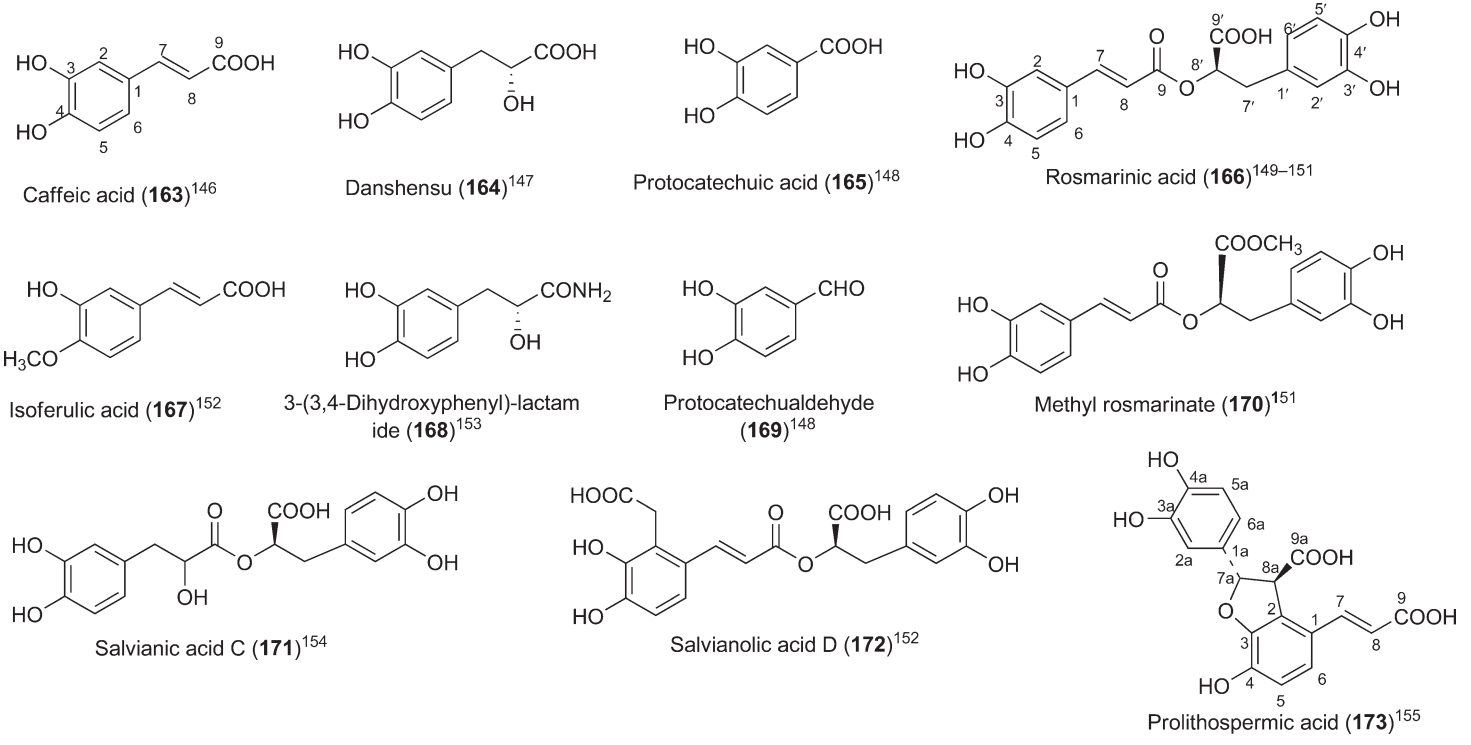
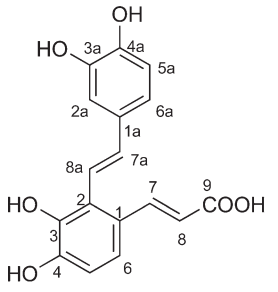
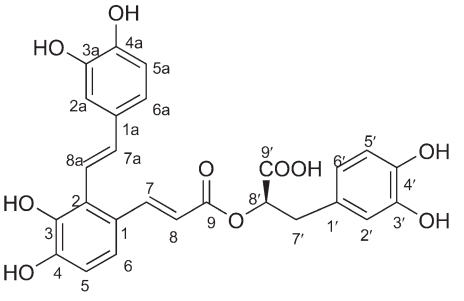


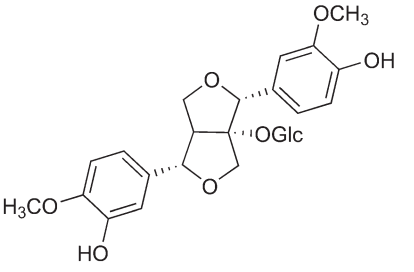
Figure 5 (*Continued*)



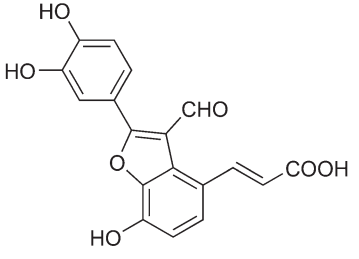
Salvianolic acid F (**174**)[156]



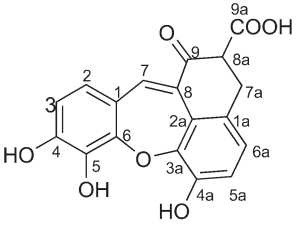
Salvianolic acid A (**175**)[157]



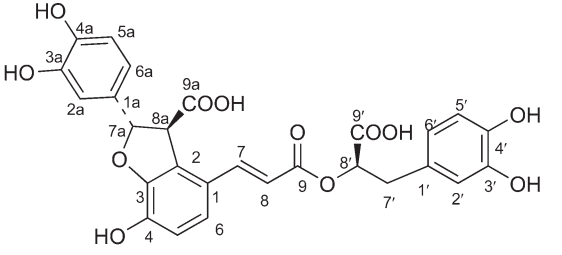
1-Hydroxypinoresinol-1-O-β-D-glucoside (**176**)[158]



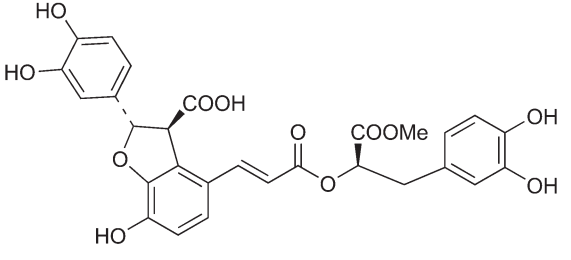
Salvinal (**177**)[156,159]



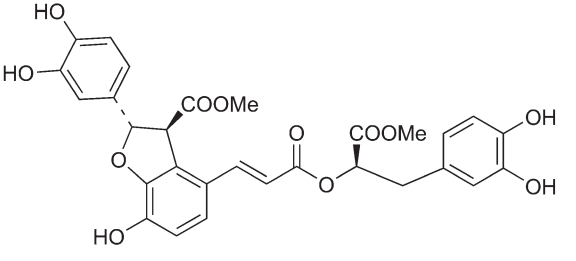
Salvianolic acid G (**178**)[160]



Lithospermic acid (**179**)[151]

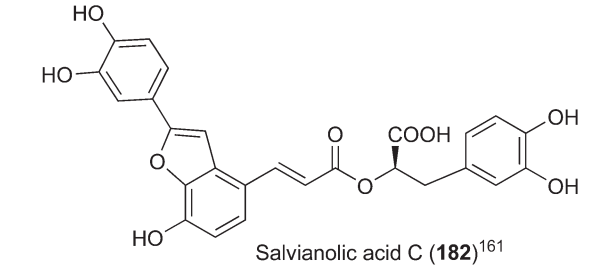


Lithospermic acid monomethyl ester (**180**)[151]

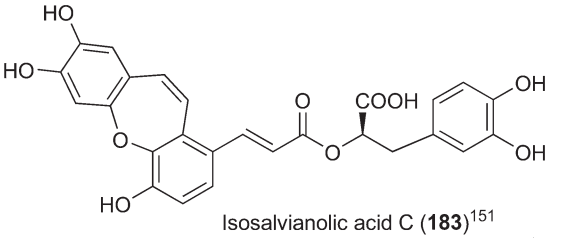


Lithospermic acid dimethyl ester (**181**)[151]

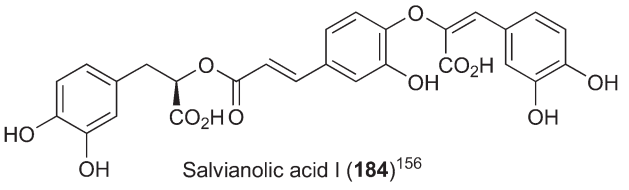
Figure 5 (*Continued*)



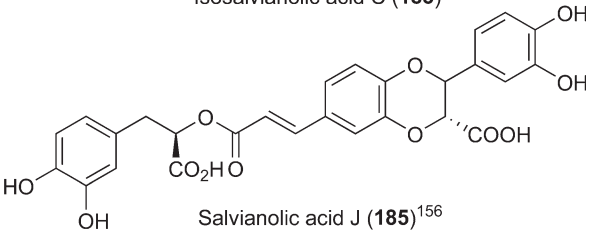
Salvianolic acid C (**182**)[161]



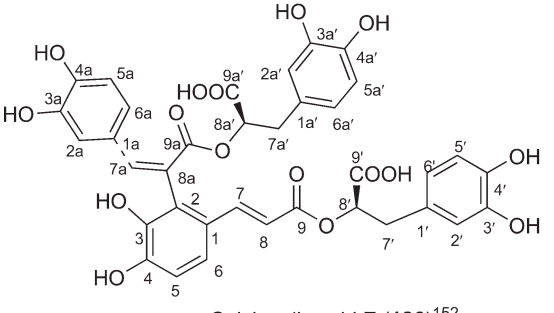
Isosalvianolic acid C (**183**)[151]



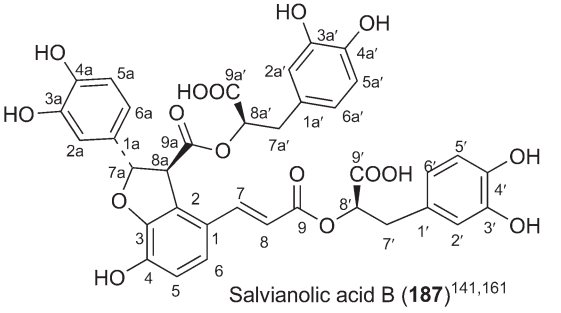
Salvianolic acid I (**184**)[156]



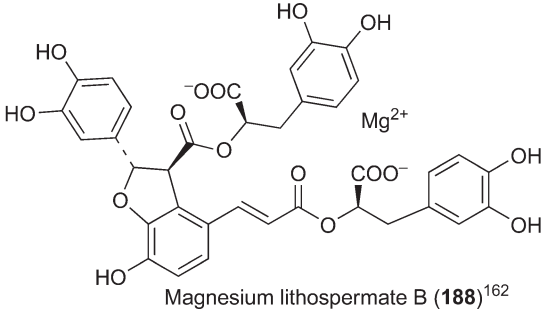
Salvianolic acid J (**185**)[156]



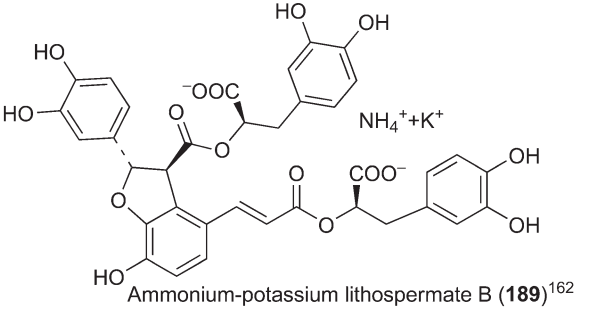
Salvianolic acid E (**186**)[152]



Salvianolic acid B (**187**)[141,161]



Magnesium lithospermate B (**188**)[162]



Ammonium-potassium lithospermate B (**189**)[162]

Figure 5 Phenolic acids isolated from Danshen.

There are many kinds of components in *G. lucidum*, including triterpenes, polysaccharides, sterols,[174] proteins,[229] alkaloids,[230] long-chain fatty acids,[190] glycopeptides,[172] polysaccharide peptides,[231] and peptides.[232] The main groups of bioactive compounds in *G. lucidum* seem to be triterpenes and polysaccharides.[162–232] More than 200 highly oxygenated and pharmacologically active lanostane-type triterpenoids have been isolated from the fruiting bodies, spores, and mycelia of *G. lucidum* (see **Figure 6** and **Table 2**).[233–282]

Ganoderic acid D (**203**) (GAD) is one of the major components in GTS. It could bind six isoforms of the protein family, annexin A5, and aminopeptidase B. The possible network associated with GAD target-related proteins was constructed, and the possible contribution of these proteins to the cytotoxicity of GAD is discussed.[283]

Ganoderic acid DM (**292**) could inhibit prostate cancer cell growth and block osteoclastogenesis.[284] Ganoderic acid DM especially suppressed the expression of c-Fos and nuclear factor of activated T cells c1 (NFATc1). This suppression leads to the inhibition of dendritic cell-specific transmembrane protein (DC-STAMP) expression and reduces osteoclast fusion.[285]

The effect of lucidenic acids (A, B, C, and N) isolated from a new *G. lucidum* (YK-02) on induction of cell apoptosis and the apoptotic pathway in HL-60 cells were investigated. Lucidenic acid B (**395**) (LAB) did not affect the cell cycle profile; however, it increased the number of early and late apoptotic cells but not necrotic cells. This finding may be critical to the chemopreventive potential of LAB.[286]

Ganoderol B (**299**) with 5-α-reductase inhibitory activity and the ability to bind to the androgen receptor (AR) can inhibit androgen-induced LNCaP cell growth and suppress regrowth of the ventral prostate induced by testosterone in rats. The downregulation of AR signaling by ganoderol B provides an important mechanism for its antiandrogenic activity.[287]

Ganoderic acid Me (**315**) (GA-Me) is a lanostane triterpenoid purified from *Ganoderma lucidum* mycelia. GA-Me could inhibit both tumor growth and lung metastasis of Lewis lung carcinoma in C57BL/6 mice. Compared with the control group, natural killer (NK) cells activity was significantly enhanced by intraperitoneal administration of GA-Me (28 mg kg^{-1}). Results of an ELISA and RT-PCR showed that the expression of interleukin-2 (IL-2) and interferon-gamma (IFN-γ) were also increased ($p < 0.05$). Additionally, the expression of nuclear factor-kappaB (NF-κB) was upregulated after the treatment of GA-Me, which might be involved in the production of IL-2. In conclusion, the findings of this study imply that GA-Me can effectively inhibit tumor growth and lung metastasis by increasing the immune function.[288]

Ganoderic acid T (**302**) (GA-T) is a lanostane triterpenoid purified from methanol extract of *G. lucidum* mycelia, which was found to exert cytotoxicity in various human carcinoma cell lines in a dose-dependent manner, while it was less toxic to normal human cell lines. Animal experiments *in vivo* also showed that GA-T suppressed the growth of human solid tumors in athymic mice. GA-T induced apoptosis of metastatic lung tumor cells through an intrinsic pathway related to mitochondrial dysfunction and p53 expression, and it may have potential as a chemotherapeutic agent.[289]

Ganoderiol F (**308**) (GolF) was found to induce senescence of cancer cell lines. GolF induced growth arrest of cancer cell lines HepG2, Huh7, and K562, but exerted much less effect on hepatoma Hep3B cells and normal lung fibroblast MRC5 cells, and no effect on peripheral blood mononuclear cells. GolF treatment of the cancer cells, with the exception of Hep3B, resulted in prompt inhibition of DNA synthesis and arrest of cell progression cycle in G1 phase. GolF was found to inhibit activity of topoisomerases *in vitro*, which may contribute to the inhibition of cellular DNA synthesis. Activation of the mitogen-activated protein kinase EKR and upregulation of cyclin-dependent kinase inhibitor p16 were found in early stages of GolF treatment and were presumed to cause cell cycle arrest and trigger premature senescence of HepG2 cells. The growth arrest and senescence induction capability on cancer cells suggest anticancer potential of GolF.[290]

3.13.5 Radix et Rhizoma Glycyrrhizae (Licorice, Gancao)

Licorice (Gancao), derived from the dried roots and rhizomes of *Glycyrrhiza* species (Fabaceae), appears as a main component in about 60% of all TCM prescriptions.[291] Therefore, Gancao maybe the most popular herbal medicine in TCM. Of about 30 species that belong to the *Glycyrrhiza* genus, only three species, *Glycyrrhiza uralensis* Fisch., *G. inflata* Bat., and *G. glabra* L., are officially used as Gancao according to the Chinese

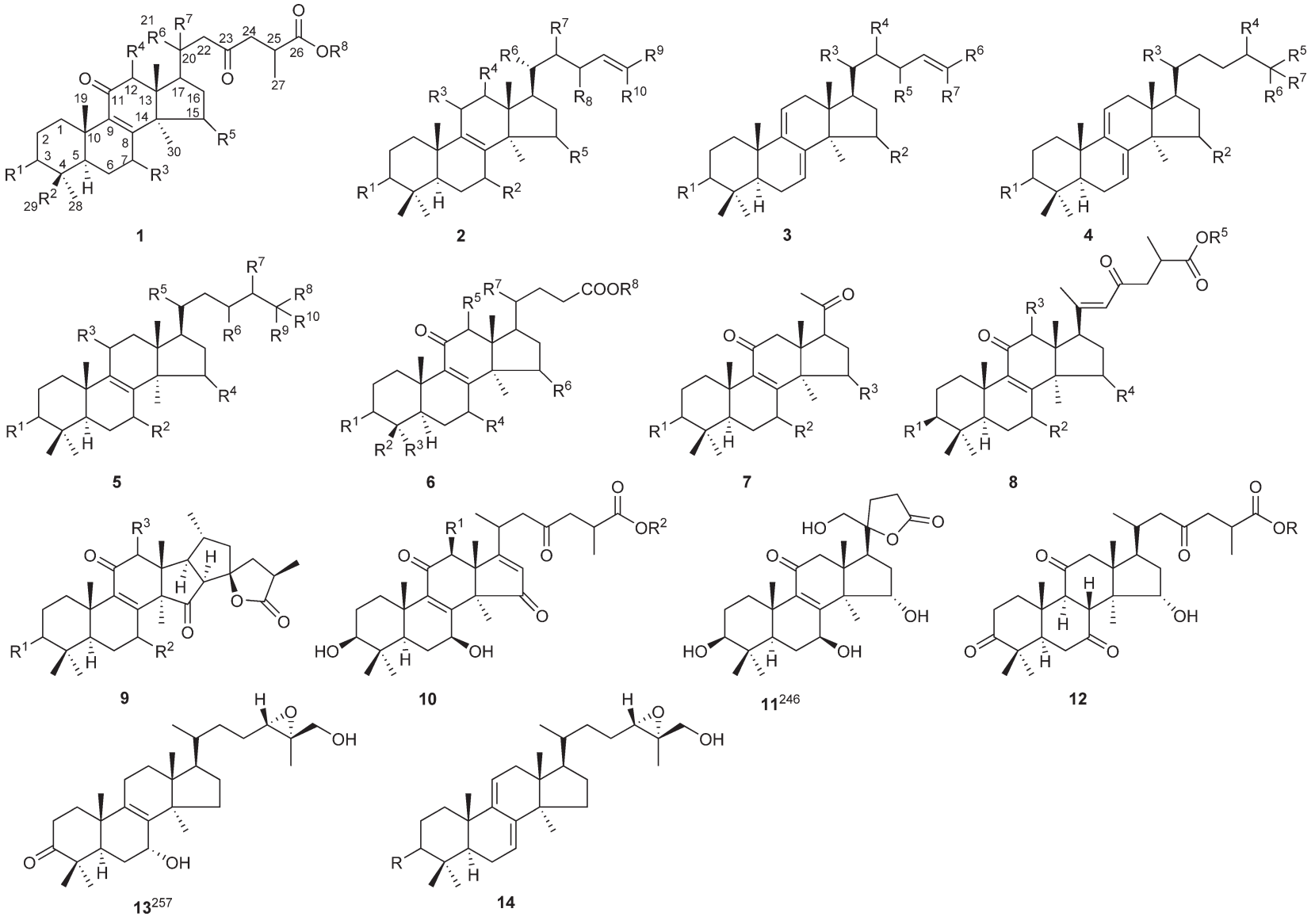


Figure 6 Fourteen skeletons of triterpenoids of *Ganoderma lucidum*.

Table 2 Triterpenoids isolated from *Ganoderma lucidum*

No.	Skeleton	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	R^9	R^{10}	Reference(s)
190	1	OH	CH_3	OH	OH	=O	CH_3	H	CH_3			234
191		OCHO	CH_3	OH	OH	=O	CH_3	H	H			234
192		=O	CH_3	=O	β OH	H	CH_3	H	H			235
193		β OH	CH_3	β OH	β OH	=O	CH_3	H	H			236
194		=O	CH_3	=O	β OH	=O	CH_3	H	H			236
195		=O	CH_3	β OH	H	H	CH_3	H	H			236
196		β OH	CH_3	β OH	H	H	CH_3	H	CH_3			236
197		β OH	CH_3	β OH	H	α OH	CH_3	H	H			236
198		=O	CH_3	β OH	H	H	CH_3	H	H			236
199		=O	CH_3	OH	H	α OH	α CH_3	H	H			241
200		OH	CH_3	OH	H	α OH	α CH_3	H	H			241
201		β OH	CH_3	=O	β OAc	β OH	α CH_3	H	H			243
202		β OAc	CH_3	=O	β OAc	β OAc	α CH_3	H	H			243
203		=O	CH_3	β OH	H	=O	α CH_3	H	H			243
204		β OH	CH_3	β OH	β OH	=O	α CH_3	H	H			243
205		β OH	CH_3	β OH	β OAc	=O	α CH_3	H	H			243
206		β OH	CH_3	=O	β OAc	β OH	α CH_3	H	H			243
207		β OH	CH_3	β OH	β OH	=O	CH_3	OH	H			249
208		=O	CH_3	β OH	α OH	=O	CH_3	=O	CH_3			252
209		=O	CH_3	β OH	H	=O	CH_3	OH	CH_3			252
210		=O	CH_3	=O	H	=O	CH_3	OH	CH_3			252
211		β OH	CH_3	=O	H	α OH	CH_3	H	CH_3			252
212		β OH	CH_3	α OH	H	α OH	CH_3	H	CH_3			252
213		β OAc	CH_3	α OH	H	α OAc	CH_3	H	CH_3			252
214		=O	CH_3	=O	H	α OH	CH_3	H	H			253
215		=O	CH_3	=O	H	α OH	CH_3	H	CH_3			253
216		β OH	CH_2OH	H	H	α OH	CH_3	H	H			253
217		β OH	CH_3	H	H	α OH	CH_3	H	CH_3			253
218		β OH	CH_3	=O	β OAc	=O	CH_3	H	H			254
219		β OH	CH_3	β OH	β OAc	=O	CH_3	H	H			254
220		β OH	CH_3	β OH	H	α OH	CH_3	H	CH_3			255
221		=O	CH_3	β OH	H	=O	CH_3	H	CH_3			96
222		=O	CH_3	=O	β OAc	=O	CH_3	H	CH_3			96
223		β OH	CH_3	β OH	H	α OH	CH_3	OH	H			97
224		β OH	CH_3	β OH	H	α OH	CH_3	OH	CH_3			97
225		=O	CH_3	β OH	β OH	=O	CH_3	H	H			98
226		β OH	CH_3	β OH	H	=O	CH_3	OH	CH_3			100
227		=O	CH_3	H	H	α OH	CH_3	H	CH_3			100
228		β OH	CH_3	H	H	α OH	CH_3	H	CH_3			259

229		=O	CH_3	H	H	=O	CH_3	H	CH_3		259
230		=O	CH_3	β OH	H	α OH	α CH_3	H	CH_3		260
231		β OH	CH_3	β OH	H	=O	α CH_3	H	CH_3		260
232		β OH	CH_3	=O	OAc	=O	α CH_3	H	CH_3		260
233		=O	CH_3	β OAc	H	α OAc	α CH_3	H	CH_3		260
234		β OAc	CH_3	β OAc	H	=O	α CH_3	H	CH_3		260
235		β OAc	CH_3	=O	OAc	=O	α CH_3	H	CH_3		260
236		=O	CH_3	=O	H	=O	α CH_3	H	CH_3		260
237		=O	CH_3	β OAc	H	α OAc	CH_3	H	CH_3		262
238		=O	CH_3	=O	H	=O	α CH_3	H	H		270
239		β OH	CH_3	=O	H	α OH	α CH_3	H	H		270
240		β OH	CH_3	α OH	H	α OH	α CH_3	H	H		111
241		=O	CH_3	H	H	α OH	CH_3	H	H		112
242		β OH	CH_3	H	H	α OH	CH_3	H	H		112
243		β OH	CH_3	β OH	OH	=O	CH_3	H	H		113
244		=O	CH_3	β OH	OH	=O	CH_3	H	CH_3		113
245		=O	CH_3	=O	OAc	=O	CH_3	H	H		272
246		β OH	CH_3	=O	OAc	=O	CH_3	H	H		272
247		β OH	CH_3	=O	OH	=O	CH_3	H	CH_3		272
248		β OH	CH_3	β OH	H	=O	CH_3	H	H		277
249		β OH	CH_3	β OH	H	=O	α CH_3	H	H		243, 250, 270
250		β OH	CH_3	β OH	H	α OH	α CH_3	H	H		250, 270
251		=O	CH_3	α OH	H	α OH	α CH_3	H	H		250, 270
252		β OH	CH_3	=O	β OAc	=O	CH_3	H	CH_3		250, 255
253		=O	CH_3	β OH	H	α OH	CH_3	H	CH_3		255, 259, 262
254		=O	CH_3	=O	H	=O	CH_3	H	CH_3		255, 259, 262
255		β OH	CH_3	β OH	H	=O	CH_3	H	CH_3		255, 259, 262
256		=O	CH_3	β OH	H	=O	CH_3	H	H		257, 258, 268, 277
257		β OH	CH_3	β OH	H	α OH	CH_3	H	H		258, 262, 268, 277
258		β OH	CH_3	β OH	H	=O	CH_3	H	H		235, 254, 257
259		=O	CH_3	=O	H	=O	CH_3	H	H		235, 236
260		=O	CH_3	=O	=O	=O	CH_3	H	H		235, 236
261		=O	CH_3	β OH	H	α OH	CH_3	H	H		262, 277
262		=O	CH_3	=O	H	=O	CH_3	H	CH_3		236, 252
263		=O	CH_3	=O	β OAC	=O	CH_3	H	H		236, 254
264		β OH	CH_3	β OH	H	α OH	CH_3	H	H		236, 254, 268
265		β OH	CH_3	β OH	β OH	=O	CH_3	H	CH_3		236, 259
266		β OH	CH_3	=O	β OAc	=O	α CH_3	H	H		239, 243
267		=O	CH_3	β OH	H	α OH	α CH_3	H	H		239, 243, 250
268		β OH	CH_3	=O	=O	=O	α CH3	H	H		251
269	2	β OH	=O	=O	H	=O	CH_3	OH	H	COOH	CH_3	239
270		=O	=O	H	H	α OH	α CH_3	H	H	COOH	CH_3	241
271		=O	β OH	=O	H	α OH	α CH_3	H	β OH	COOH	CH_3	250

(*Continued*)

Table 2 (Continued)

No.	Skeleton	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	R^9	R^{10}	Reference(s)
272		=O	α OH	=O	H	α OH	α CH_3	H	β OH	COOH	CH_3	250
273		β OH	β OH	=O	H	=O	α CH_3	H	β OH	COOH	CH_3	250
274		β OH	=O	=O	H	=O	α CH_3	H	β OH	COOH	CH_3	250
275		=O	H	=O	H	α OH	α CH_3	H	β OH	COOH	CH_3	250
276		β OH	β OH	=O	β OH	=O	CH_3	H	β OH	COOH	CH_3	250
277		β OH	=O	=O	β OH	=O	CH_3	H	β OH	COOH	CH_3	250
278		=O	H	=O	H	α OH	CH_3	H	OH	COOH	CH_3	256
279		=O	H	=O	H	α OH	CH_3	H	OH	$COOCH_3$	CH_3	256
280		α OAc	α OAc	H	H	α OH	CH_3	H	H	COOH	CH_3	264
281		α OAc	α OH	H	H	α OAc	CH_3	OAc	H	COOH	CH_3	264
282		α OAc	α OAc	H	H	α OH	CH_3	OAC	H	COOH	CH_3	264
283		α OAc	α OCH_3	H	H	H	CH_3	H	H	COOH	CH_3	264
284		α OAc	α OCH_3	H	H	α OH	CH_3	OAc	H	COOH	CH_3	265
285		α OAc	α OH	H	H	α OH	CH_3	OAc	H	COOH	CH_3	265
286		α OAc	α OCH_3	H	H	α OH	CH_3	H	H	COOH	CH_3	265
287		α OH	α OCH_3	H	H	H	CH_3	OAc	H	COOH	CH_3	265
288		=O	α OH	H	H	H	CH_3	H	H	CHO	CH_3	274
289		β OH	=O	H	H	H	CH_3	H	H	COOH	CH_3	280
290		β OH	=O	H	H	H	CH_3	H	H	CH_2OH	CH_3	281
291		β OH	=O	H	H	H	CH_3	H	H	CHO	CH_3	281
292		=O	=O	H	H	H	α CH_3	H	H	COOH	CH_3	241, 242
293		=O	=O	=O	H	=O	CH_3	H	β OH	COOH	CH_3	282
294	3	α OAc	α OAc	α CH_3	β OAc	H	H	CH_3				239
295		α OH	H	α CH_3	β OAc	H	H	CH_3				239
296		α OAc	H	α CH_3	β OAc	H	H	CH_3				239
297		=O	H	CH_3	H	H	CHO	CH_3				254
298		=O	H	CH_3	H	H	CH_2OH	CH_3				254
299		β OH	H	CH_3	H	H	CH_2OH	CH_3				254
300		=O	H	CH_3	H	H	COOH	CH_3				254
301		β OH	H	CH_3	H	H	COOH	CH_3				254
302		α OAc	α OAc	α CH_3	β OAc	H	COOH	CH_3				266, 269
303		α OH	H	α CH_3	β OAc	H	COOH	CH_3				266
304		α OAC	H	α CH_3	β OAc	H	COOH	CH_3				266
305		α OAC	α OH	α CH_3	β OAc	H	COOH	CH_3				266
306		α OH	α OAc	α CH_3	β OAc	H	COOH	CH_3				266
307		α OH	α OH	α CH_3	β OH	H	CH_2OH	CH_3				266
308		=O	H	α CH_3	H	H	CH_2OH	CH_2OH				267
309		OH	H	α CH_3	H	H	CH_2OH	CH_2OH				267
310		H	H	α CH_3	H	H	CH_3	CH_2OH				267

311		H	H	α CH_3	β OAc	H	COOH	CH_3	269
312		α OAc	H	α CH_3	β OAc	H	COOH	CH_3	269
313		β OH	α OAc	CH_3	OAc	H	COOH	CH_3	275
314		=O	α OH	CH_3	H	H	COOH	CH_3	280
315		α OAc	α OAc	CH_3	H	H	COOH	CH_3	233, 240, 263, 264
316		α OH	α OAc	CH_3	H	H	COOH	CH_3	233, 240
317		β OH	α OAC	CH_3	H	H	COOH	CH_3	233, 240
318		β OAc	α OH	CH_3	H	H	COOH	CH_3	233, 240
319		=O	α OAc	CH_3	H	H	COOH	CH_3	233, 240
320		α OAc	α OH	CH_3	β OAc	H	COOH	CH_3	233, 240
321		β OH	α OAc	CH_3	β OAc	H	COOH	CH_3	233, 240
322		α OAc	α OAc	CH_3	β OAc	H	COOH	CH_3	233, 240
323		β OAc	α OAc	CH_3	β OAc	H	COOH	CH_3	233, 240
324		α OH	α OAc	CH_3	H	=O	COOH	CH_3	233, 240
325		α OAc	α OAc	CH_3	H	=O	COOH	CH_3	233, 240
326		α OAc	α OH	CH_3	H	=O	COOH	CH_3	233, 240
327		α OAc	α OH	CH_3	α OH	H	COOH	CH_3	233, 240
328		α OH	H	CH_3	H	H	COOH	CH_3	233, 240
329		α OAc	H	CH_3	H	H	COOH	CH_3	233, 240
330		β OAc	α OAc	CH_3	H	H	COOH	CH_3	233, 240, 263
331		α OAc	α OH	CH_3	H	H	COOH	CH_3	233, 240, 264
332		α OH	α OH	CH_3	H	H	COOH	CH_3	233, 240, 275
333		β OH	α OH	CH_3	H	H	COOH	CH_3	233, 240, 275
334		α OH	α OH	CH_3	α OH	H	COOH	CH_3	233, 240, 276
335		β OH	α OH	CH_3	β OH	H	COOH	CH_3	233, 240, 276
336		α OAc	α OAc	CH_3	α OH	H	COOH	CH_3	233, 240, 276
337		β OAc	α OAc	CH_3	α OH	H	COOH	CH_3	233, 240, 276
338		α OH	α OH	CH_3	β OAc	H	COOH	CH_3	233, 240, 276
339		β OH	α OH	CH_3	β OAc	H	COOH	CH_3	233, 240, 276
340		α OAc	α OH	CH_3	OAc	H	COOH	CH_3	265, 275
341	4	=O	H	CH_3	α OH	CH_2OH	CH_3	β OH	235
342		β OH	H	α CH_3	OH	CH_2OH	CH_3	OH	237
343		β OH	H	α CH_3	α OH	CH_3	CH_2OH	OH	243
344		=O	H	α CH_3	α OH	CH_3	CH_2OH	OH	243
345		=O	H	α CH_3	α OH	CH_2OH	CH_3	OH	244
346		β OH	H	α CH_3	$\Delta^{24(25)}$	CHO	CH_3	$\Delta^{24(25)}$	247
347		=O	H	α CH_3	OH	CH_3	CH_3	OH	247
348		=O	H	α CH_3	α OH	CH_3	CH_3	OH	278
349		=O	H	α CH_3	$\Delta^{24(25)}$	CH_2OH	CH_2OH	$\Delta^{24(25)}$	243, 244
350		=O	H	α CH_3	α OH	CH_3	CH_3	OH	244, 273
351		β OH	H	α CH_3	α OH	CH_3	CH_3	OH	244, 273, 278
352		β OH	H	α CH_3	$\Delta^{24(25)}$	CH_2OH	CH_3	$\Delta^{24(25)}$	247, 273, 278
353		=O	H	α CH_3	$\Delta^{24(25)}$	CH_2OH	CH_3	$\Delta^{24(25)}$	273, 278

(*Continued*)

Table 2 (Continued)

No.	Skeleton	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	R^9	R^{10}	Reference(s)
354		=O	H	α CH_3	α OH	CH_2OH	CH_3	OH				273, 278
355		=O	α OH	α CH_3	□[24(25)]	CH_2OH	CH_2OH	□[24(25)]				237, 243
356		=O	H	α CH_3	OH	CH_2OH	CH_3	OH				237, 247
357		β OH	H	α CH_3	□[24(25)]	CH_2OH	CH_2OH	□[24(25)]				237, 278
358	5	=O	=O	H	H	α CH_3	H	OH	CH_2OH	CH_3	α OH	241
359		=O	=O	H	H	α CH_3	H	α OH	CH_3	CH_3	OH	244
360		β OH	β OH	=O	H	α CH_3	H	□[24(25)]	COOH	CH_3	H	244
361		=O	β OH	=O	α OH	α CH_3	=O	H	COOH	CH_3	H	244
362		β OH	β OH	=O	=O	α CH_3	=O	H	COOH	CH_3	H	244
363		=O	β OH	=O	=O	α CH_3	=O	H	COOH	CH_3	H	244
364		=O	H	=O	=O	α CH_3	=O	H	COOH	CH_3	H	244
365		=O	=O	H	H	α OH	H	□[24(25)]	CHO	CH_3	□[24(25)]	247
366		β OH	=O	H	H	α OH	H	□[24(25)]	CHO	CH_3	□[24(25)]	247
367		=O	β OH	=O	α OH	α OH	=O	H	COOH	CH_3	H	247
368		=O	α OH	=O	α OH	α OH	=O	H	COOH	CH_3	H	247
369		=O	β OH	=O	=O	α OH	=O	H	COOH	CH_3	H	247
370	6	=O	CH_3	CH_3	OH	OH	=O	CH_3	H			234
371		β OCHO	CH_3	CH_3	OH	OH	=O	CH_3	H			234
372		=O	CH_3	CH_3	=O	β OAc	=O	CH_3	H			236
373		β OH	CH_3	CH_2OH	β OH	H	α OH	=	H			245
374		B OH	CH_3	CH_3	βOH	H	=O	α OH	H			246
375		=O	CH_3	CH_3	=O	H	=O	α OH	CH_3			246
376		=O	CH_3	CH_3	OH	H	=O	α CH_3	H			248
377		β OH	CH_3	CH_3	OH	H	=O	α CH_3	H			248
378		β OH	CH_3	CH_3	OH	β OH	=O	α CH_3	H			248
379		β OH	CH_3	CH_2OH	β OH	H	=O	CH_3	CH_3			252
380		β OH	CH_3	CH_2OH	=O	H	=O	CH_3	CH_3			252
381		β OH	CH_3	CH_2OH	=O	β OH	=O	CH_3	CH_3			252
382		=O	CH_3	CH_3	=O	α OH	=O	CH_3	CH_3			252
383		β OH	CH_3	CH_3	=O	β OH	=O	CH_3	CH_3			252
384		β OH	CH_3	CH_3	α OH	H	α OH	CH_3	CH_3			252
385		β OH	CH_3	CH_3	=O	β OAc	=O	CH_3	CH_3			252
386		=O	CH_3	CH_3	=O	β OAc	=O	H	CH_3			255
387		β OH	CH_3	CH_3	=O	β OAc	=O	H	CH_3			255
388		=O	CH_3	CH_3	=O	H	=O	CH_3	CH_3			255
389		=O	CH_3	CH_3	β OH	H	=O	CH_3	CH_3			255
390		=O	CH_2OH	H	β OH	H	α OH	CH_3	H			256
391		=O	CH_3	CH_3	=O	H	=O	α CH_3	H			270
392		=O	CH_3	CH_3	=O	OAc	=O	CH_3	H			272

393		β OH	CH_3	CH_3	=O	OAc	=O	CH_3	H	272
394		β OH	CH_3	CH_3	β OH	β OH	=O	CH_3	H	257, 258
395		=O	CH_3	CH_3	β OH	β OH	=O	CH_3	H	257, 258, 268
396		=O	CH_3	CH_3	=O	=O	=O	CH_3	H	257, 268
397		=O	CH_3	CH_3	β OH	α OH	=O	CH_3	H	257, 268
398		=O	CH_3	CH_3	β OH	H	=O	CH_3	H	258, 261, 268
399		=O	CH_3	CH_3	β OH	H	=O	CH_3	H	236, 257
400		=O	CH_3	CH_3	β OH	OH	=O	CH_3	H	236, 261
401		β OH	CH_3	CH_3	β OH	OH	=O	CH_3	H	236, 261
402		β OH	CH_3	CH_3	=O	H	=O	α CH_3	H	251
403		β OH	CH_3	CH_3	=O	H	=O	α CH_3	H	251
404	7	β OH	β OH	α OH						256
405		β OH	β OH	=O						257
406		=O	β OH	=O						257
407	8	=O	OH	H	α OH	H				236
408		β OH	OH	H	=O	H				236
409		β OH	OH	H	α OH	H				236
410		=O	OH	H	=O	H				236
411		=O	β OH	β OH	=O	CH_3				252
412		β OH	=O	β OAc	=O	H				282
413	9	=O	β OH	β OH						238
414		β OH	β OH	β OH						238
415	10	H	H							279
416		H	CH_3							279
417		OAc	H							279
418	12	H								249
419		CH_3								249
420	14	=O								274
421		β OH								274

Pharmacopoeia.[1] Gancao is used traditionally for the treatment of peptic ulcers, hepatitis C, and pulmonary and skin diseases.[292] Modern clinical and experimental studies showed that it has a variety of pharmacological activities, such as antiulcer, anti-inflammatory,[293] antispasmodic,[294] antioxidative,[295] antiallergic, antiviral,[296] antidiabetic, anticancer,[297] antidepressive,[298] hepatoprotective,[299] expectorant, and memory-enhancing[300] activities.[292,301] Flavonoids and triterpene saponins are considered to be responsible for the bioactivities of licorice. In this section, we summarize the isolated flavonoids (**Figure 7**) and triterpenoids (**Figure 8**) of the three officially used *Glycyrrhiza* species.

3.13.6 Herba Epimedii (Yinyanghuo)

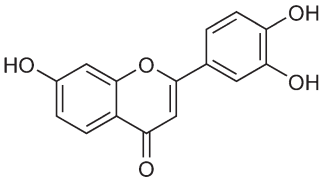
Yinyanghuo (Herba Epimedii), derived from the aerial part of the *Epimedium* species (Berberidaceae), has been used in China for over 2000 years as antirheumatic, to nourish the *kidney* and reinforce *yang*, and to strengthen the bones and muscles. Five species are used officially as Yinyanghuo in TCM according to the Chinese Pharmacopoeia. They are *E. brevicornum* Maxim., *E. sagittatum* (Sieb. et Zucc.) Maxim., *E. pubescens* Maxim., *E. wushanense* T.S. Ying, and *E. koreanum* Nakai. Earlier chemical and pharmacological investigations on Yinyanghuo afforded a series of flavonoids, which have been reported to show multiple biological activities such as androgenic, antioxidant, antidepressant-like actions, to enhance the osteogenic differentiation, and to increase osteoblastic proliferation.[343–348] These studies gave support to the traditional use of Yinyanghuo. Here, we summarized the flavonoids and some other phenolic compounds in **Figure 9**.

3.13.7 Flos Carthami (Honghua)

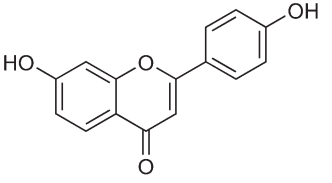
The dried flower of *Carthamus tinctorius* L., safflower (Asteraceae), named Honghua in Chinese, is a common TCM widely used in the treatment of coronary heart diseases, stroke, gynecological ailments, angina, and hypertension.[400–402] Ye *et al.*[403] researched the protection of the aqueous extract of safflower on ox-LDL-induced injury in rat cardiac microvascular endothelial cells. The results showed that the aqueous extract has antioxidant activity. Hiramatsu *et al.*[404] suggested that the petal extract of safflower, containing carthamin (**746**) as one of its major active components, has free radical scavenging activity and a neuroprotective effect. Studies by Song-Ja Bae *et al.*[405] suggested that phenolic compounds in the safflower seeds may be useful as potential cancer chemopreventive agents. Phytochemical investigations led to a variety of compounds such as flavonoids (**709–745**) (**Figure 10**), chalcone pigments (**746–759**) (**Figure 11**), lignans (**760–767**) (**Figure 12**), compounds containing nitrogen (**768–783**) (**Figure 13**), polyacetylenes (**784–823**) (**Figure 14**), and other compounds (**Figure 15**). Hydroxysafflor yellow A (**749**) is the major and most active antioxidant from safflower and has been clinically prescribed in China to treat patients with cerebral ischemia.[406] A number of investigations were carried out to determine its mechanism.[406–409] Tracheloside (**767**), a lignan glycoside isolated from the seeds of *C. tinctorius*, was proved to have antiestrogenic activity against cultured Ishikawa cells.[410]

3.13.8 Radix Isatidis (Banlangen)

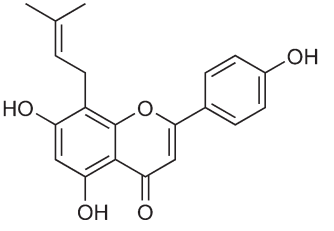
Banlangen is one of the most commonly used TCMs being valued to have antipyretic, antiviral, and detoxifying activities and traditionally used for the treatment of seasonal febrile diseases, pestilence, mumps, eruptive diseases, inflammatory diseases with redness of skin, and sore throat.[467,468] The main source of Banlangen has been identified as *Isatis indigotica* Fort. (Brassicaceae) and is recorded in the Chinese Pharmacopoeia (2005 edition). Pharmacological studies showed that Banlangen has widely useful activities including antivirus, antibacterial, antiendotoxic, antitumor, anti-inflammatory, and immune regulatory effects.[469–471] The species *I. indigotica* is a biennial herbaceous plant, distributed widely in the Changjiang river valley. Besides the roots, the dried leaves are also commonly used in TCM, named Daqingye (*Folium isatidis*) in Chinese. Therefore, a number of studies regarding this plant were carried out and over 100 compounds were isolated over the past decades. The structures of these constituents are listed in **Figure 16**, which includes alkaloids, lignans, steroids,



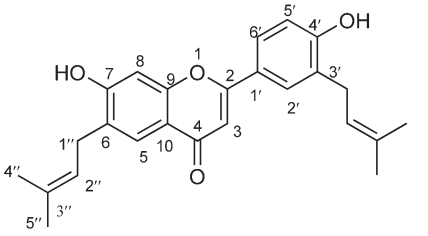
3′,4′,7-Trihydroxyflavone (**422**)[302]



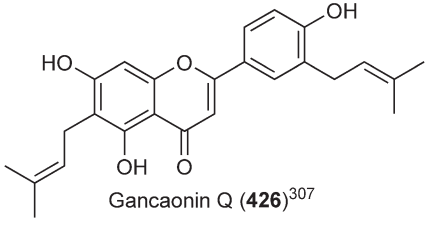
4′,7-Dihydroxyflavone (**423**)[303–306]



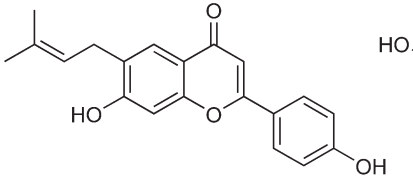
Licoflavonone C (**424**)[303]



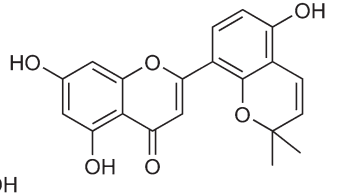
Licoflavonone B (**425**)[303]



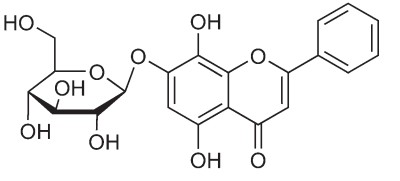
Gancaonin Q (**426**)[307]



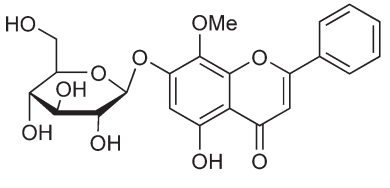
Licoflavone A (**427**)[303,304]



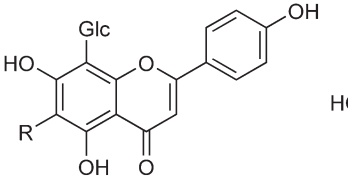
Allolicoisoflavone B (**428**)[308]



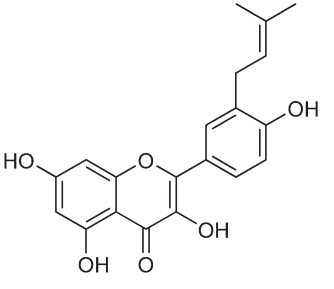
Glychionide A (**429**)[309]



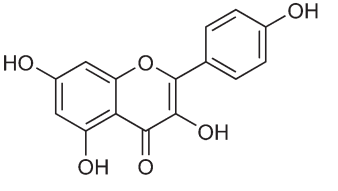
Glychionide B (**430**)[309]



Vicenin-2 (**431**)[310]: R = Glc
Isoschaftoside (**434**)[310]: R = Ara



Isolicoflavonol (**432**)[311]



Kaempferol (**433**)[308]

Figure 7 (*Continued*)

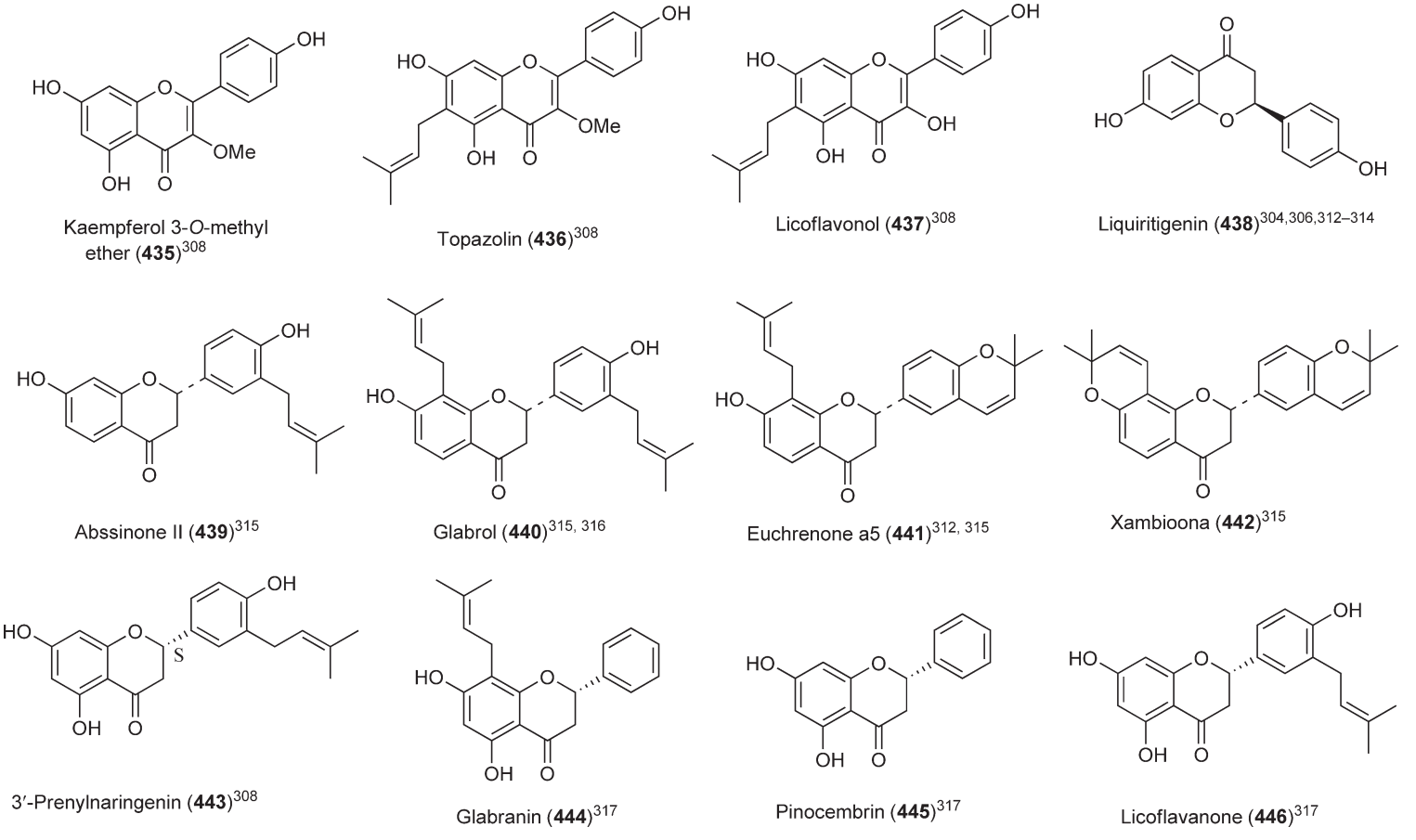


Figure 7 (*Continued*)

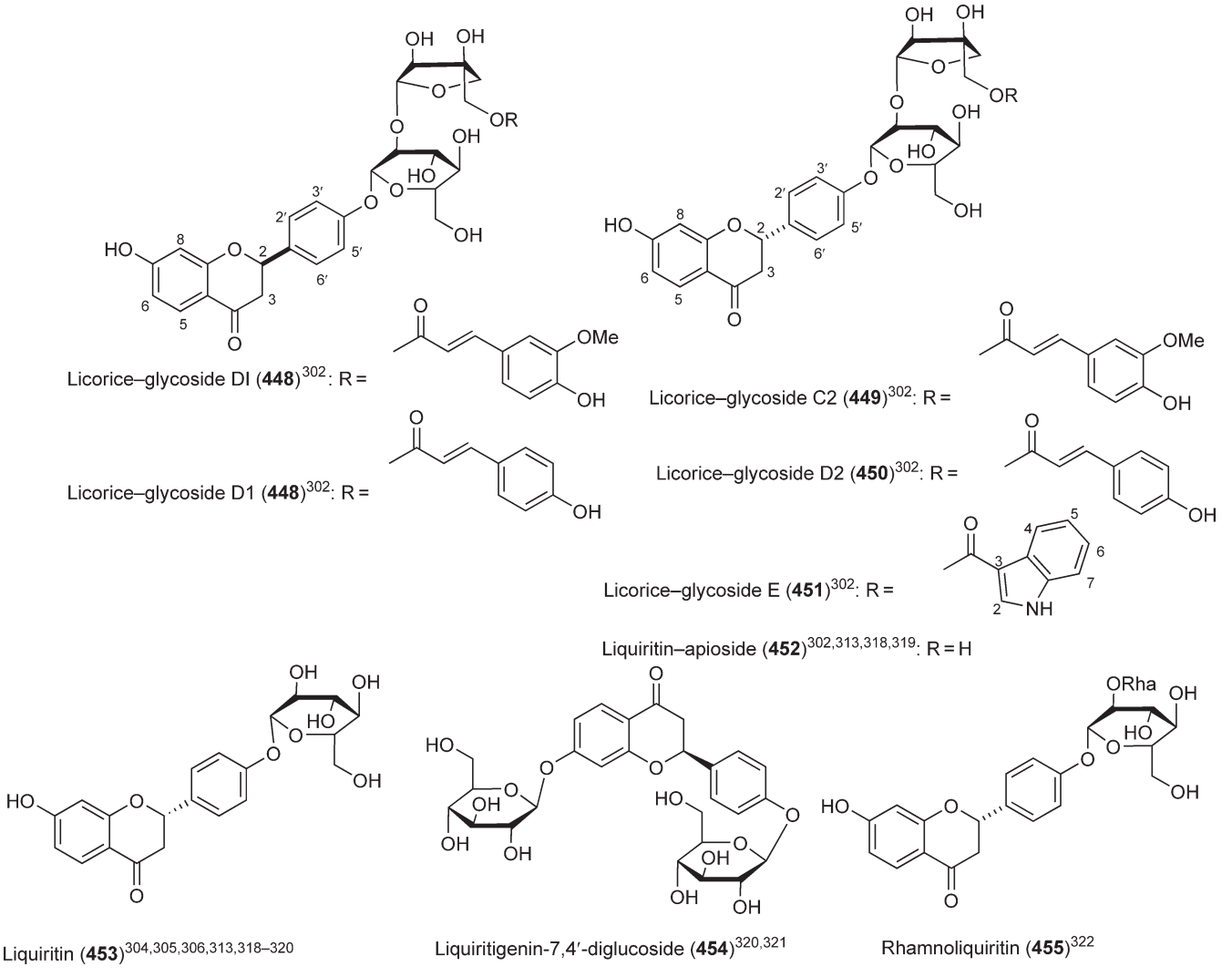
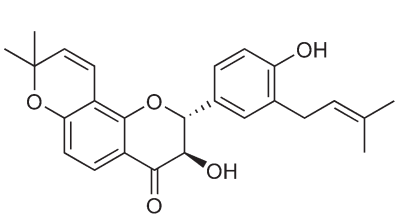
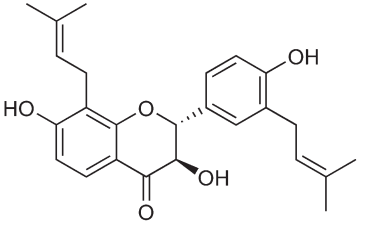


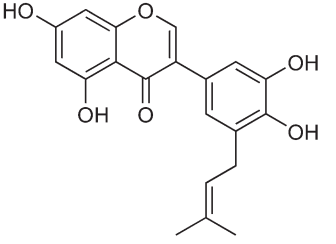
Figure 7 (*Continued*)



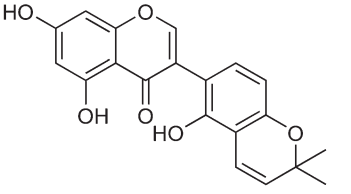
Kanzonol Z (**456**)[312]



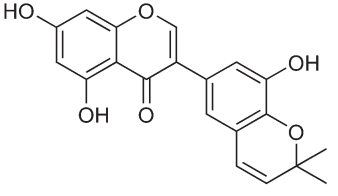
3-Hydroxyglabrol (**457**)[315,316]



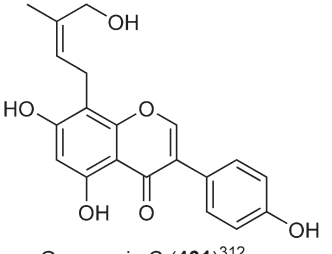
Glycyrrhisoflavone (**458**)[311]



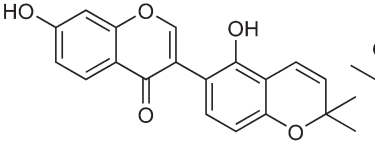
Licoisoflavone B (**459**)[312,323]



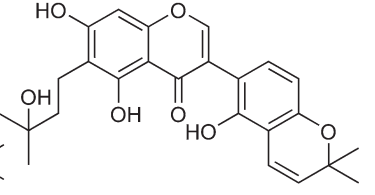
Semilicoisoflavone (**460**)[312]



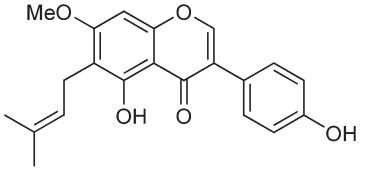
Gancaonin C (**461**)[312]



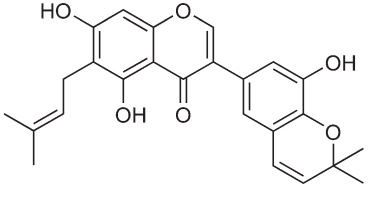
Glabrone (**462**)[312]



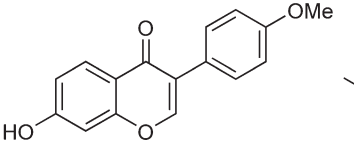
Kanzonol T (**463**)[323]



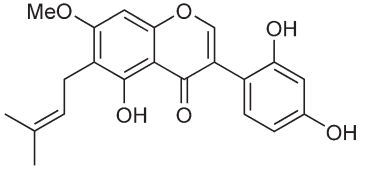
Gancaonin G (**464**)[323]



Gancaonin H (**465**)[323]

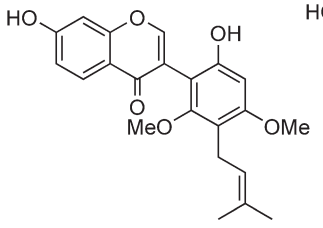


Formononetin (**466**)[304,324]

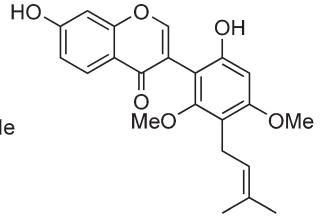


7-*O*-Methylluteone (**467**)[308]

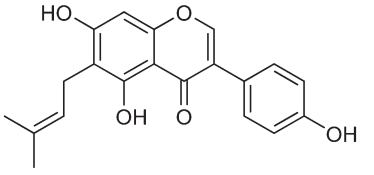
Figure 7 (*Continued*)



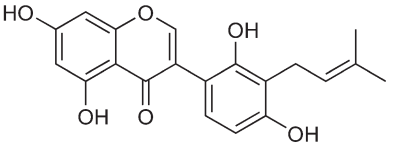
Glicoricone **(468)**[314]



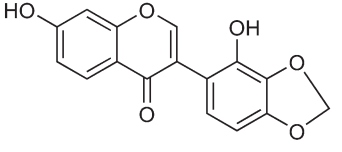
Licoricone **(469)**[314]



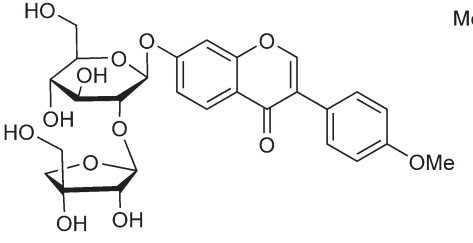
Wighteone (**470**)[317]



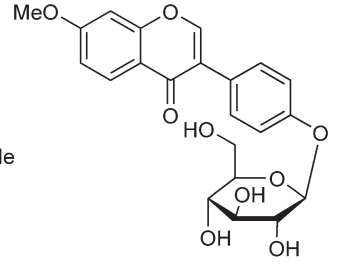
Licoisoflavone A **(471)**[325]



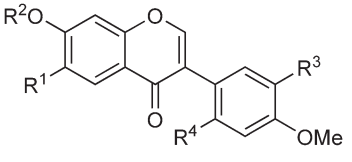
Glyzaglabrin (**472**)[326]



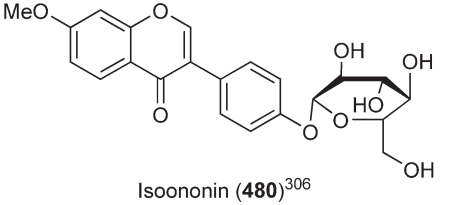
Glycyroside (**473**)[302]



Isoonotin (**474**)[320]



Licoagroside A **(475)**[310]: R^1 = OMe, R^2 = H, R^3 = OH, R^4 = Oglc;
Ononin **(476)**[304,310,318,319]: R^1 = R^3 = R^4 = H, R^2 = Glc;
Calycosin 7-*O*-glucoside (477)[310]: R^1 = R^4 = H, R^2 = Glc, R^3 = OH;
Wistin **(478)**[310]: R^1 = OMe, R^2 = Glc, R^3 = R^4 = H;
Afrormosin 7-*O*-(6″-malonylglucoside) **(479)**[310]: R^1 = OMe, R^2 = Glc^6–OOCCH$_2$COOH, R^3 = R^4 = H



Isoononin (**480**)[306]

Figure 7 (*Continued*)

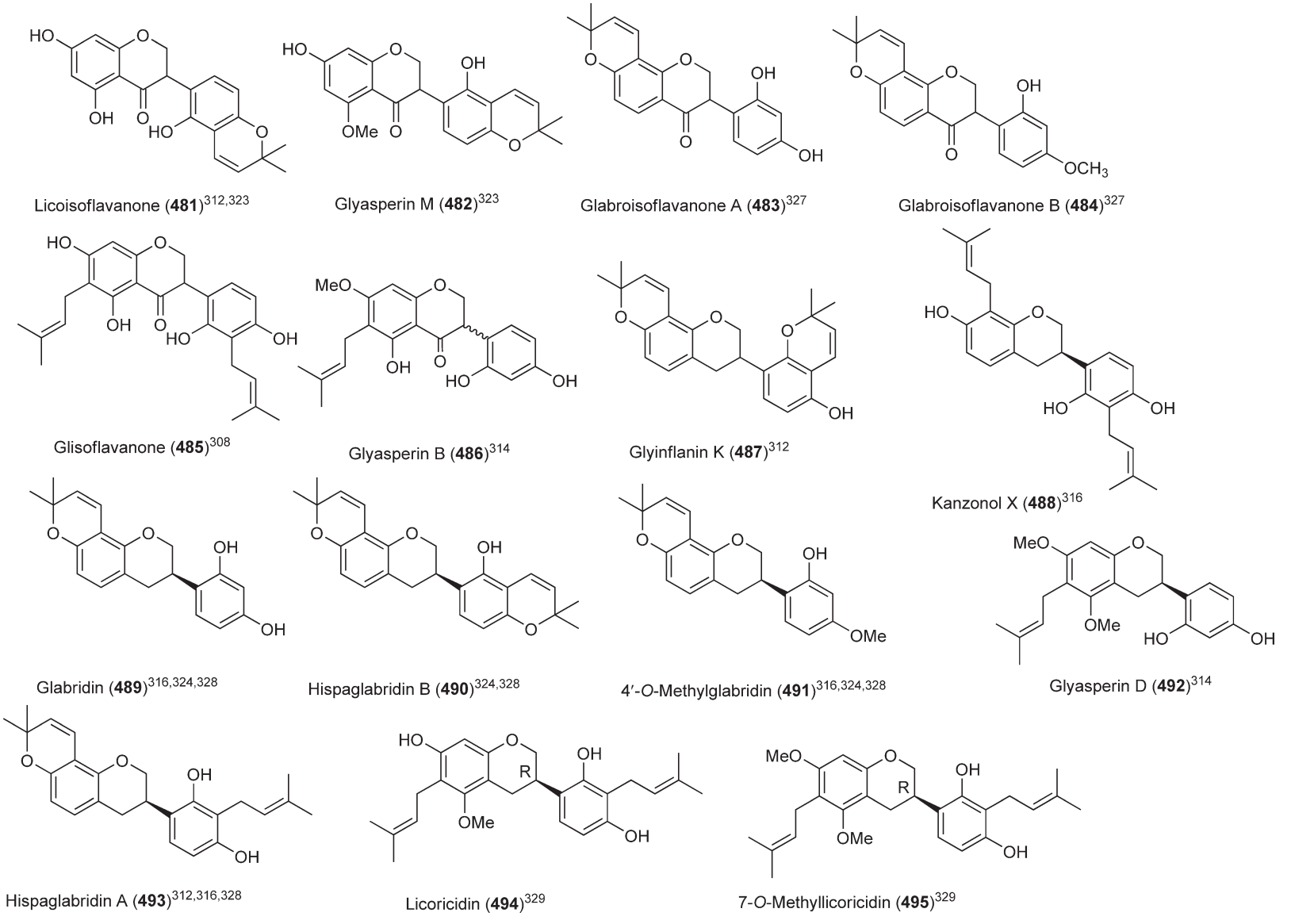
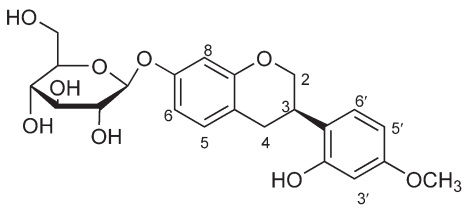
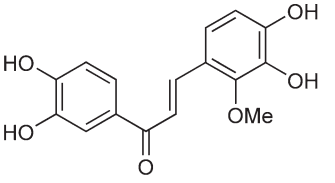


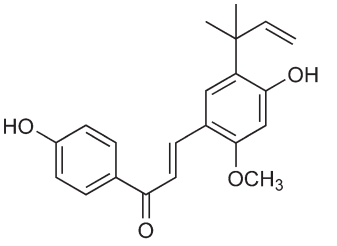
Figure 7 (*Continued*)



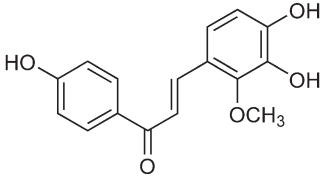
(3*R*)-Vestitol 7-*O*-glucoside (**496**)[302]



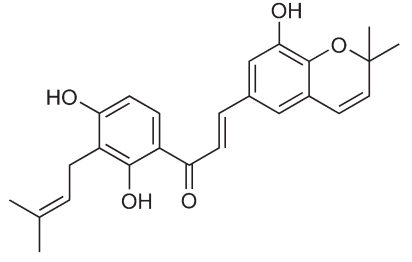
Tetrahydroxymethoxychalcone (**497**)[302]



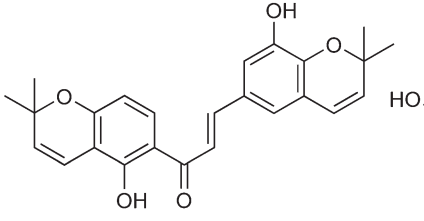
Licochalcone A (**498**)[304,305,311,330,331]



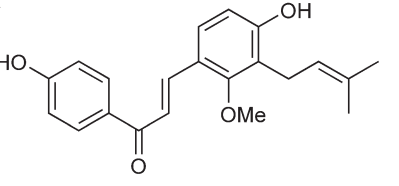
Licochalcone B (**499**)[304,305,330]



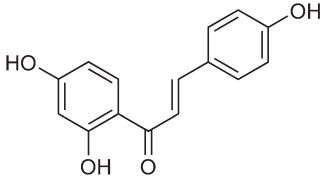
Prenylatedpyranochalcone (**500**)[316]



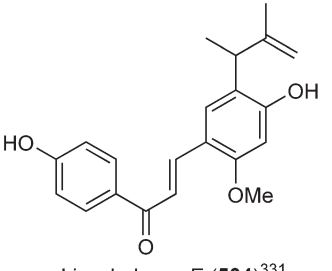
Glyinflanin G (**501**)[316]



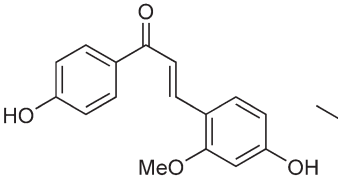
Licochalcone C (**502**)[310,331]



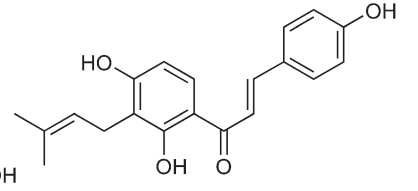
Isoliquiritigenin (**503**)[304,306,313,324,331,332]



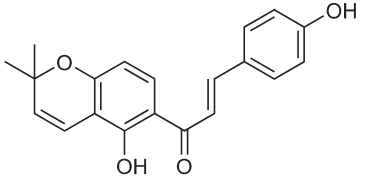
Licochalcone E (**504**)[331]



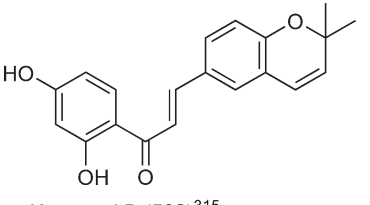
Echinatin (**505**)[304]



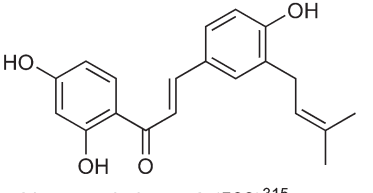
Isobavachalcone (**506**)[315]



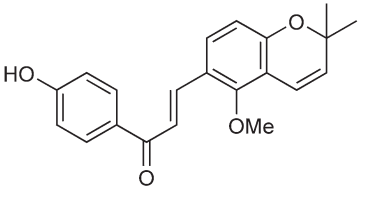
4-Hydroxylonchocarpin (**507**)[315]



Kanzonol B (**508**)[315]

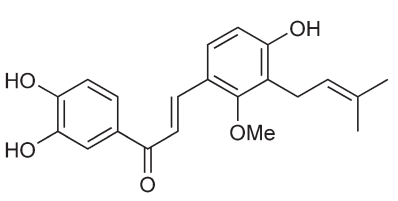


Licoagrochalcone A (**509**)[315]

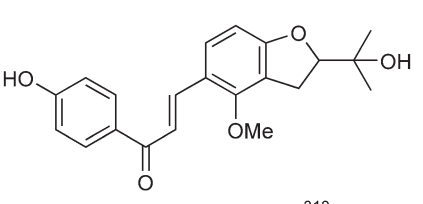


Licoagrochalcone B (**510**)[310]

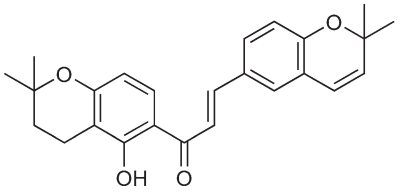
Figure 7 (*Continued*)



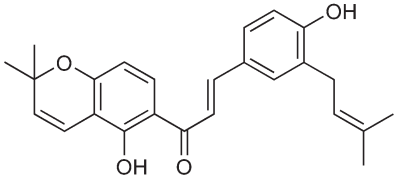
Licoagrochalcone C (**511**)[310]



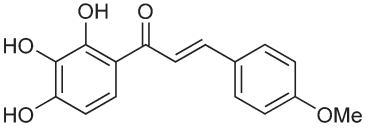
Licoagrochalcone D (**512**)[310]



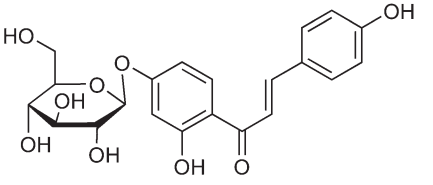
1,2-Dihydroparatocarpin A (**513**)[324]



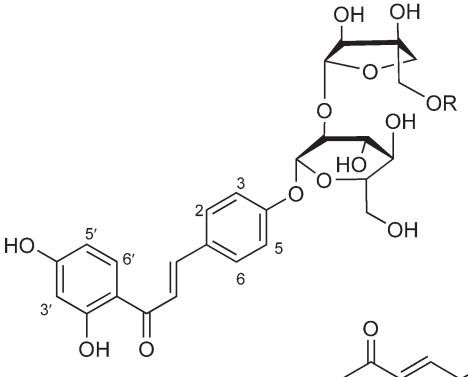
Paratocarpin B (**514**)[324]



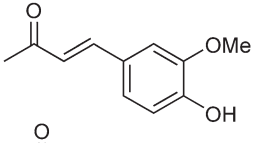
2,3,4-Trihydroxy-4′-methoxychalcone (**515**)[332]

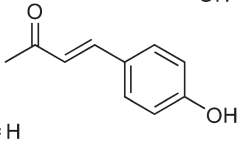


Neoisoliquiritin (**516**)[302,318,320]



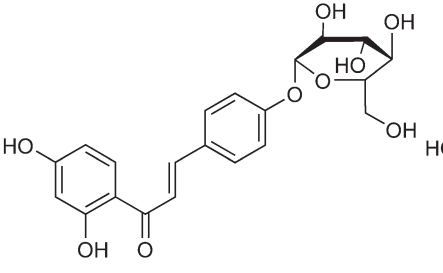
Licorice–glycoside A (**517**)[302]: R =



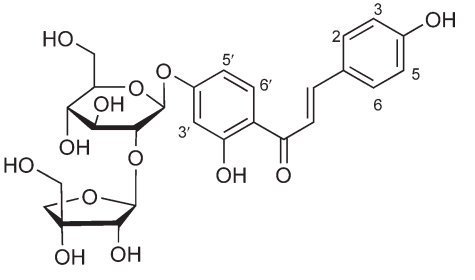


Licorice–glycoside B (**520**)[302]: R =

Isoliquiritin apioside (**521**)[302,318]: R = H

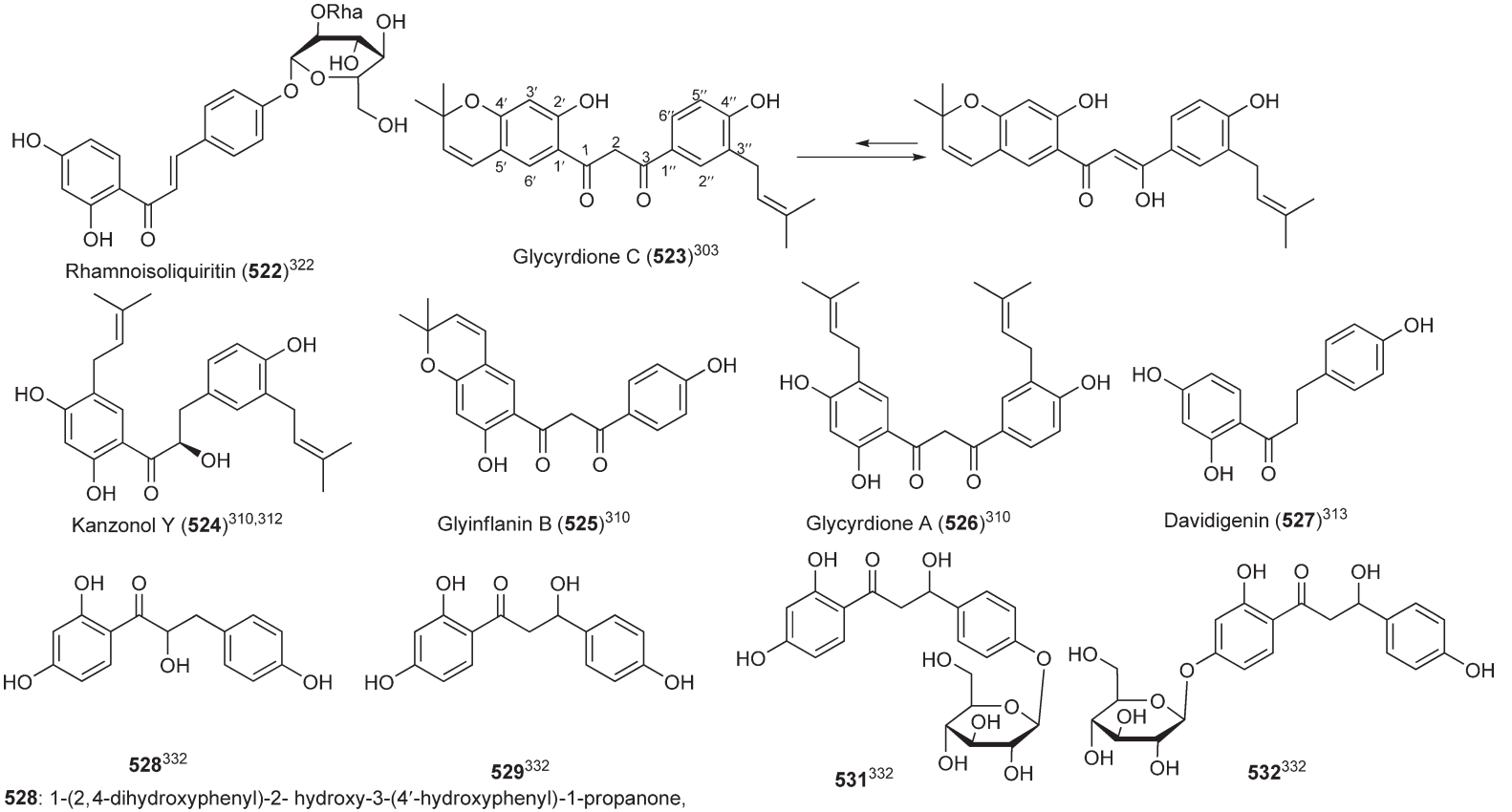


Isoliquiritin (**518**)[17,19,302,304,313]



Licuraside (**519**)[312,318]

Figure 7 (*Continued*)



528: 1-(2,4-dihydroxyphenyl)-2- hydroxy-3-(4′-hydroxyphenyl)-1-propanone,

529: 1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4′-hydroxyphenyl)-1-propanone,

530: 1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4′-hydroxyphenyl-4′-*O*-β-D-glucopyranoside)-1-propanone,

531: 1-(2,4-dihydroxyphenyl-4-*O*-β-D-glucopyranoside)-3-hydroxy-3-(4′-hydroxyphenyl)-1-propanone

Figure 7 (*Continued*)

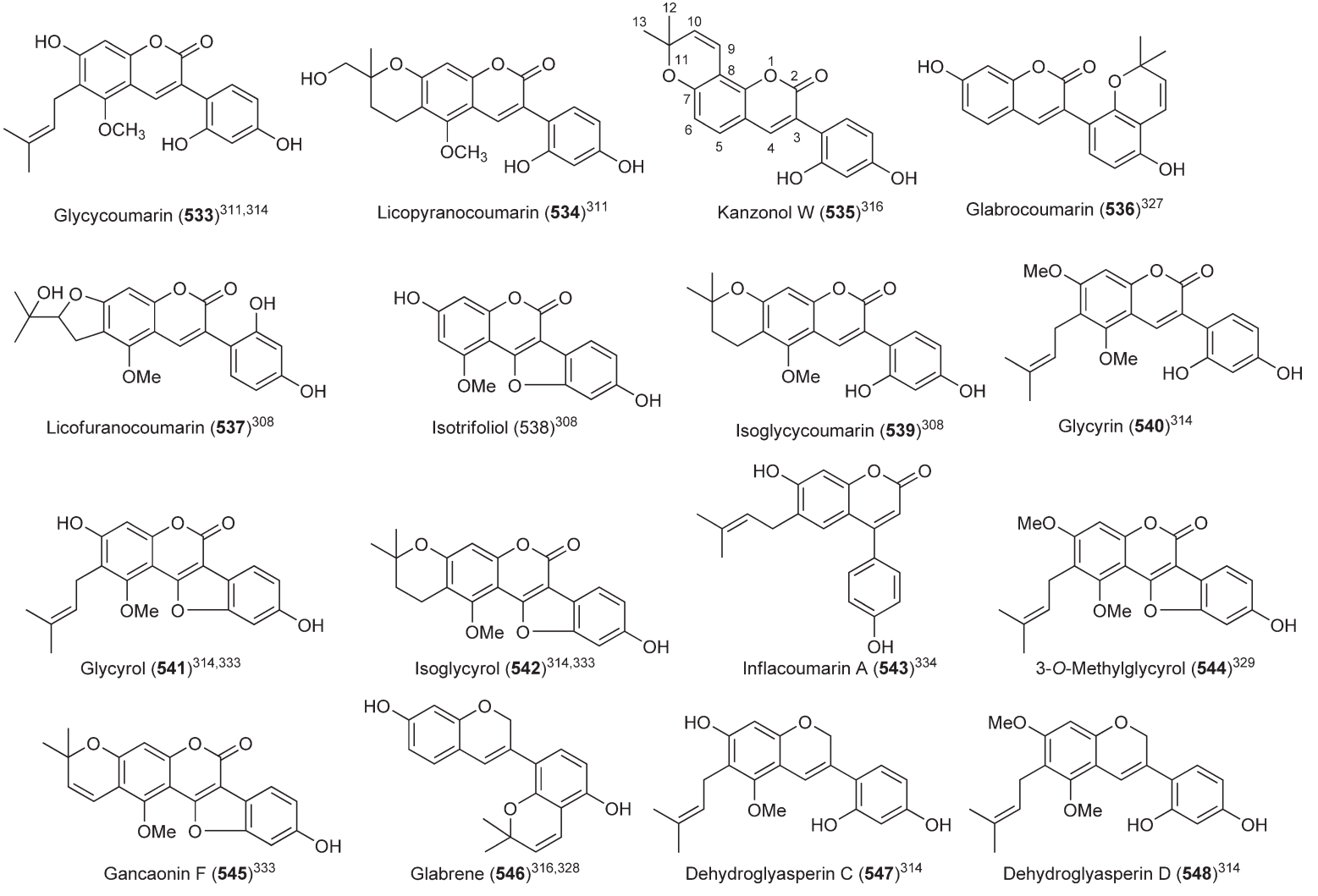


Figure 7 (*Continued*)

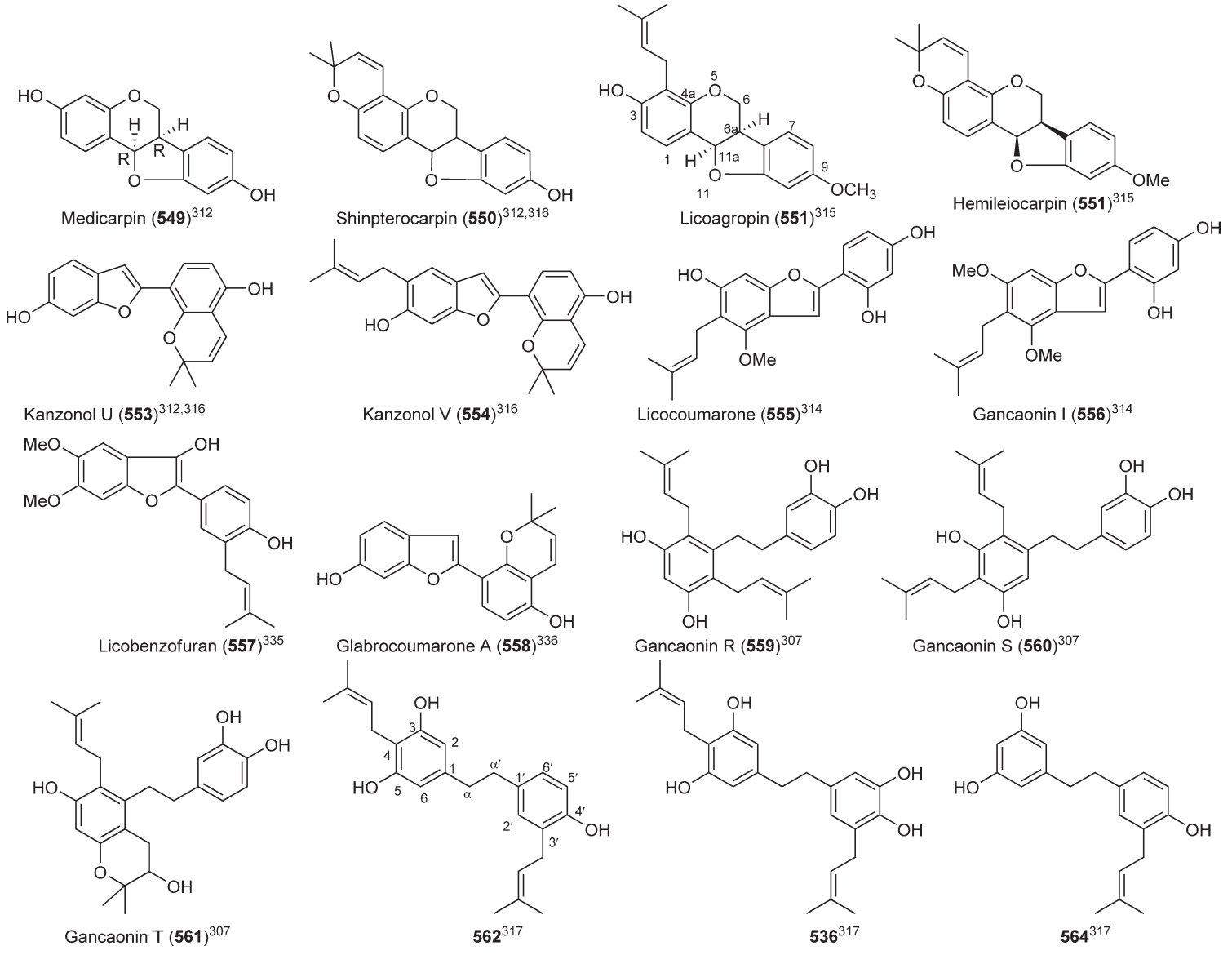
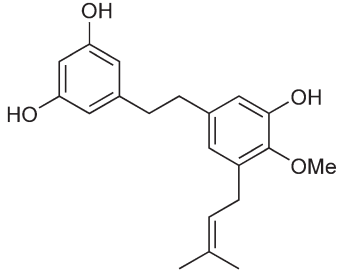


Figure 7 (*Continued*)

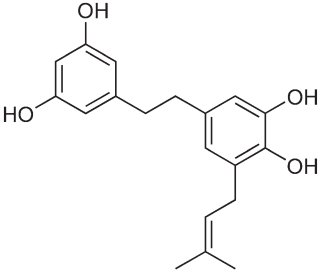
562: α,α′-dihydro-3,5,4′-trihydroxy-4,5′-diisopentenylstilbene,

563: α,α′-dihydro-3,5,3′,4′-tetrahydroxy-4,5′-diisopentenylstilbene,

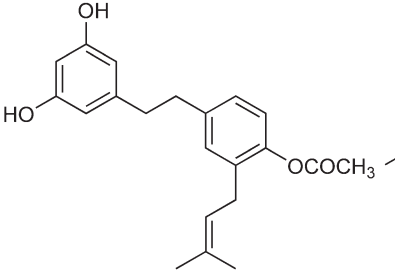
564: α,α′-dihydro-3,5,4′-trihydroxy-5′-isopentenylstilbene



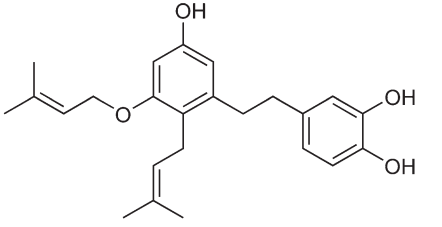
565[317]



566[337]



567[337]



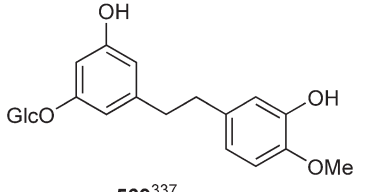
568[337]

565:α,α′-dihydro-3,5,3′-trihydroxy4′-methoxyl-5′-isopentenylstilbene,

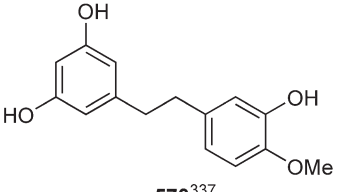
566:α,α′-dihydro-3,5,3′, 4'-tetrahydroxy-5′-isopentenylstilbene,

567:α,α′-dihydro-3,5-dihydroxy-4′-acetoxy-5′-isopentenylstilbene,

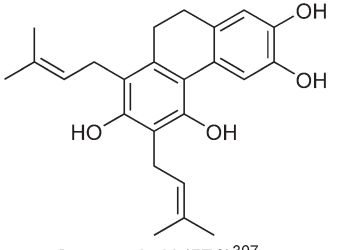
568:α,α′-dihydro-3,3′,4′-trihydroxy-5-*O*-isopentenyl-6-isopentenylstilbene



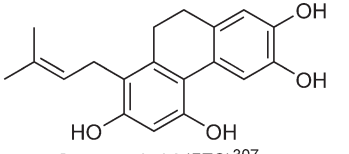
569[337]



570[337]



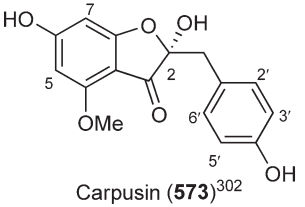
Gancaonin U (**571**)[307]



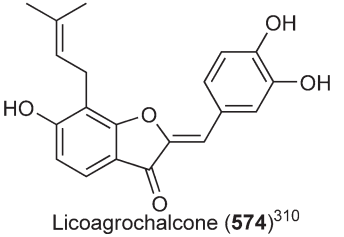
Gancaonin V (**572**)[307]

569: α,α′-Dihydro-3,3′-dihydroxy-5β-d-*O*-glucopyranosyloxy-4′-methoxystilbene,

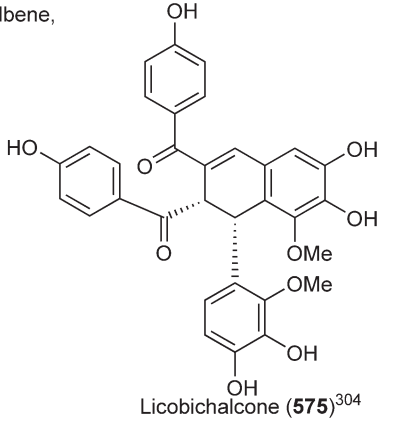
570: α,α′-Dihydro-3,5,3′-trihydroxy-4′-methoxystilbene



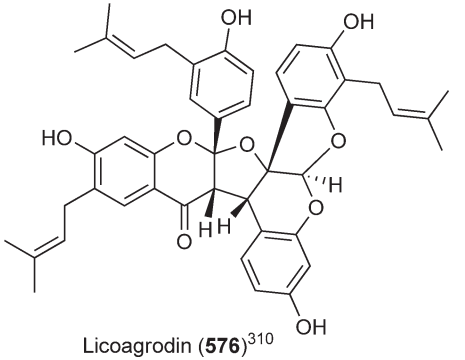
Carpusin (**573**)[302]



Licoagrochalcone (**574**)[310]

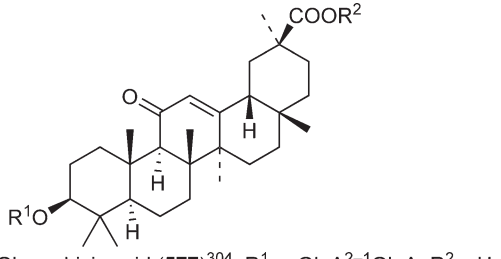


Licobichalcone (**575**)[304]

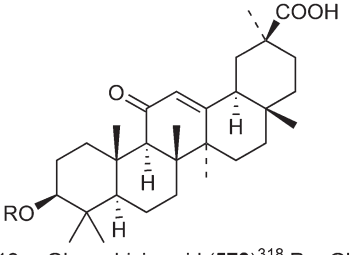


Licoagrodin (**576**)[310]

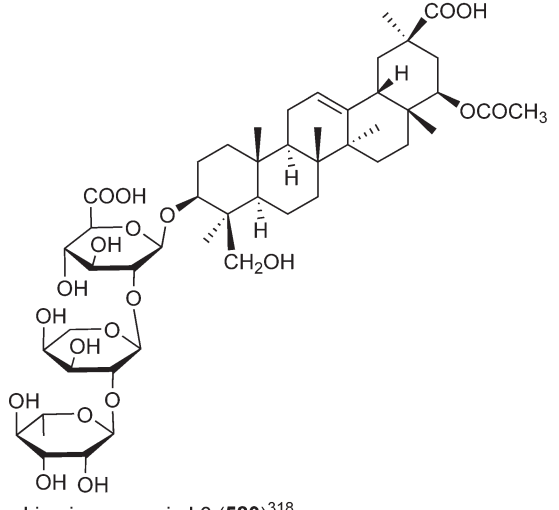
Figure 7 Phenolic compounds isolated from Gancao.



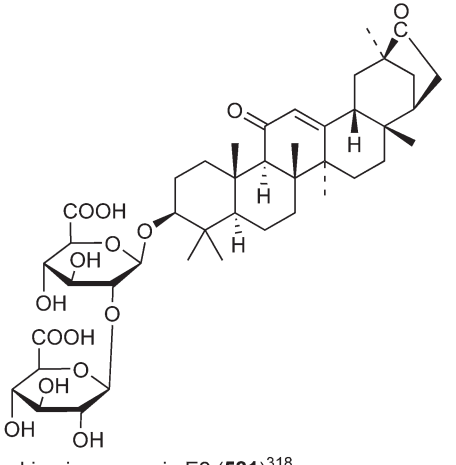
Glycyrrhizic acid (**577**)[304]: R^1 = $GluA^{2-1}GluA$, R^2 = H;
Licorice–saponin A3 (**578**)[318]: R^1 = $GluA^{2-1}GluA$, R^2 = Glu



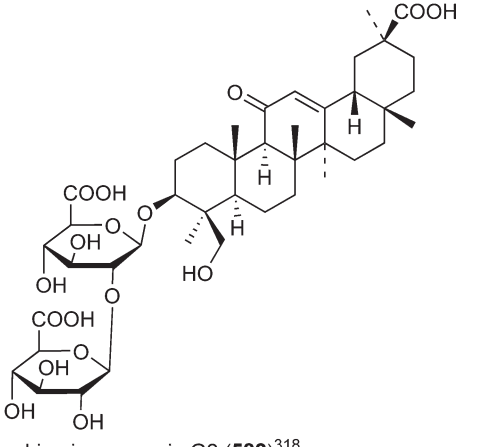
18-α-Glycyrrhizic acid (**579**)[318] R = $GluA^{2}$–$^{1}GluA$



Licorice–saponin L3 (**580**)[318]

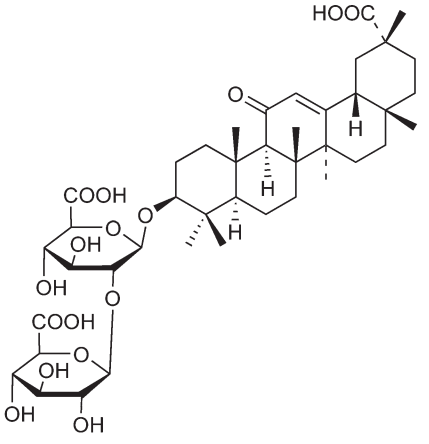


Licorice–saponin E2 (**581**)[318]

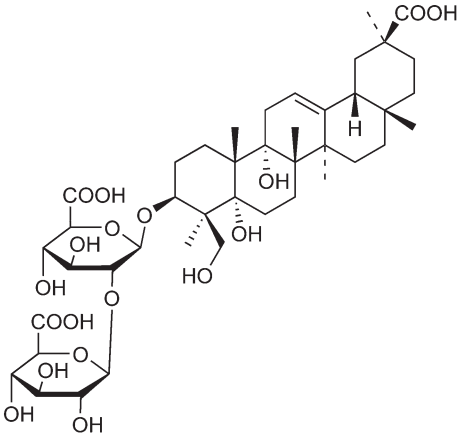


Licorice–saponin G2 (**582**)[318]

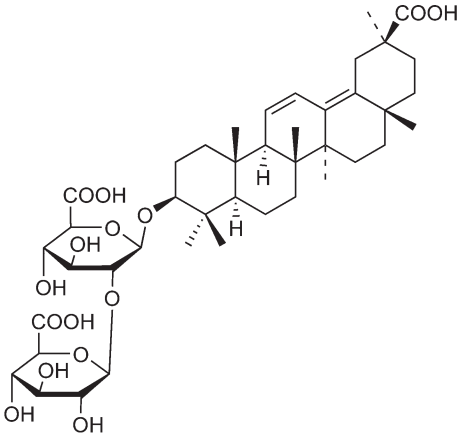
Figure 8 (*Continued*)



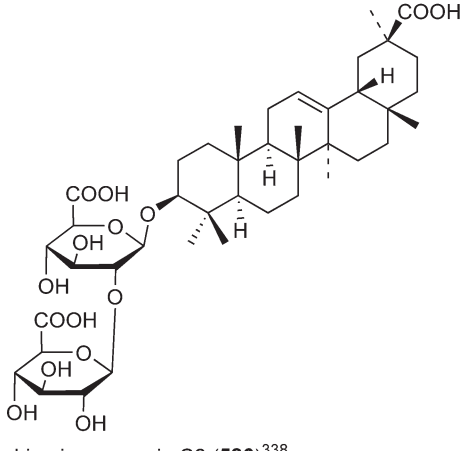
Licorice–saponin H2 (**583**)[318]



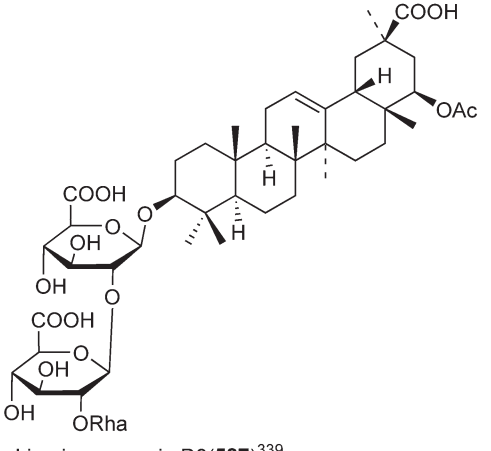
Licorice–saponin J2 (**583**)[318]



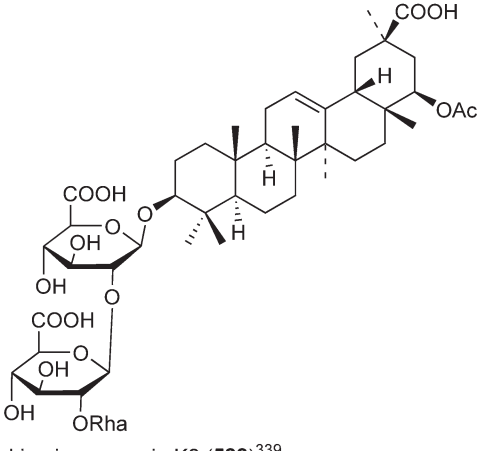
Licorice–saponin C2 (**585**)[338]



Licorice–saponin C2 (**586**)[338]

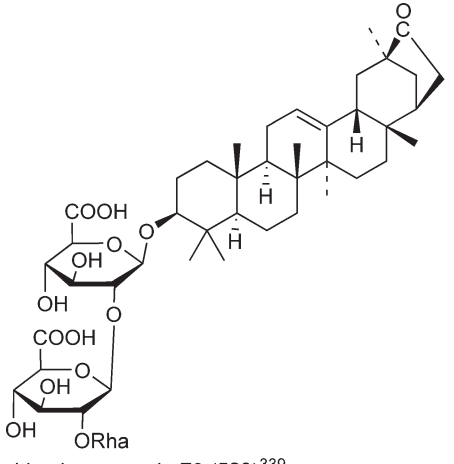


Licorice–saponin D3(**587**)[339]

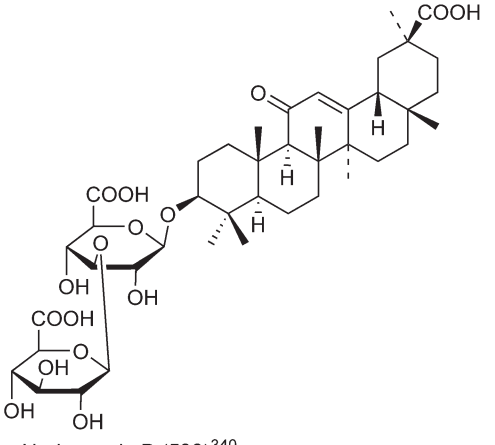


Licorice–saponin K2 (**588**)[339]

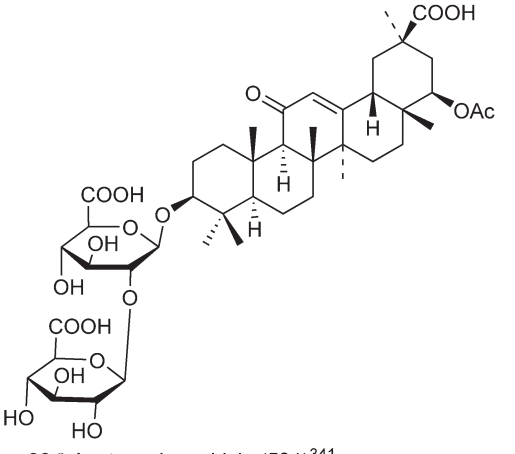
Figure 8 (*Continued*)



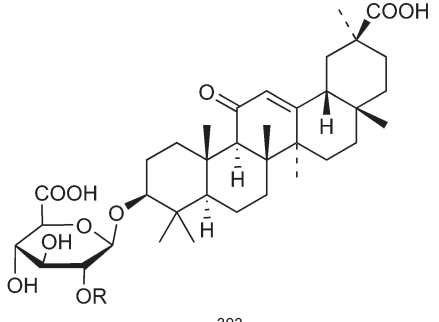
Licorice–saponin F3 (**589**)[339]



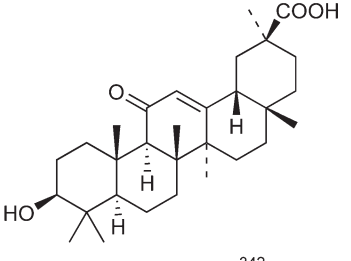
Uralsaponin B (**590**)[340]



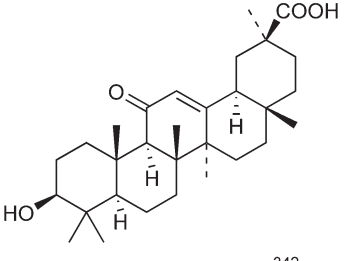
22β-Acetoxyglycyrrhizin (**591**)[341]



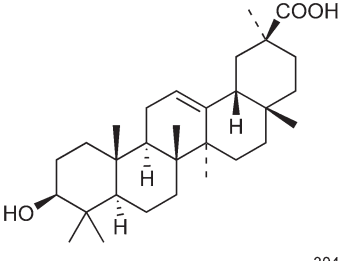
Apioglycyrrhizin (**592**)[303]: R = Api,
Glycyrrhetinic acid (**594**)[303]: R = Ara



Glycyrrhetinic acid (**594**)[342]

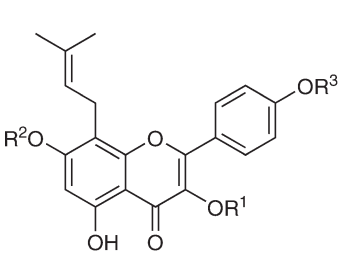


18α-Glycyrrhetic acid (**595**)[342]

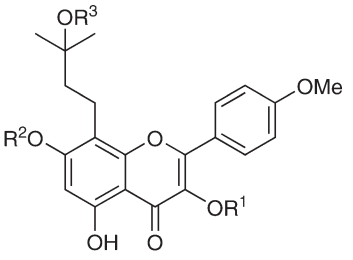


11-Deoxyglycyrrhetinic acid (**596**)[304]

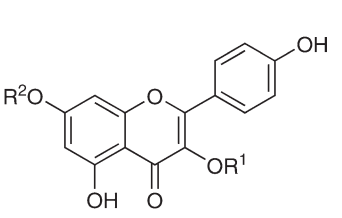
Figure 8 Triterpenoids isolated from Gancao.



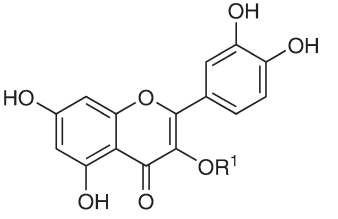
I



II



III



IV

Type		Compounds	Substituents			Reference(s)
			R^1	R^2	R^3	
I	**597**	Desmethylicaritin	H	H	H	348
	598	Epimedoside B (ikarisoside A, baohuoside II)	Rha	H	H	350
	599	Ikarisoside B	Rha^2–^{1}Glc	H	H	351
	600	Ikarisoside F	Rha^2–^{1}Xyl	H	H	352
	601	Baohuoside III	Rha^2–^{1}Rha	H	H	351, 353
	602	Epimedoside C	H	Glc	H	354
	603	Epimedoside A	Rha	Glc	H	355
	604	Hexandraside E	Glc	Glc	H	354, 356
	605	Epimedoside E	Rha^2–^{1}Xyl	Glc	H	357
	606	Ikarisoside C (diphylloside A)	Rha^2–^{1}Glc	Glc	H	352, 358
	607	Diphylloside B (baohuoside V)	Rha^2–^{1}Rha	Glc	H	353

Figure 9 (*Continued*)

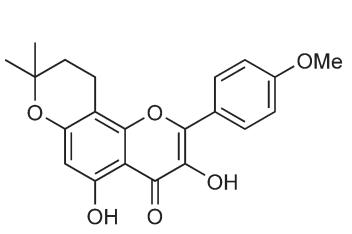
Type	Compounds	Substituents			Reference(s)
		R^1	R^2	R^3	
608	Rouhuoside (maohuoside B)	$Rha^{4}–^{1}Glc$	Glc	H	359
609	Icaritin	H	H	Me	348
610	Icariside II (baohuoside I)	Rha	H	Me	354, 360, 361
612	Sagittatoside A	$Rha^{2}–^{1}Glc$	H	Me	358, 362
613	Sagittatoside B	$Rha^{2}–^{1}Xyl$	H	Me	352, 363
614	Baohuoside VII	$Rha^{4}–^{1}Glc$	H	Me	364
615	2″-*O*-Rhamnosylicariside II	$Rha^{2}–^{1}Rha$	H	Me	352, 353
616	Sagittatoside C	$Rha(^{3}–OAc)^{2}–^{1}Rha$	H	Me	362
617	3‴-Carbonyl-2″-β-L-quinovosylicariside II	$Rha^{2}–^{1}Rha(^{3}=O)$	H	Me	365
618	Epimedokoreanoside II	$Rha(^{4}–OAc)^{3}–^{1}Glc$	H	Me	366
619	Korepimedoside C (Epimedin I)	$Rha(^{4}–OAc)^{3}–^{1}Glc$	Glc	Me	367, 368
620	Epimedoside (korepimedoside A)	$Rha(^{4}–OAc)^{3}–^{1}Glc(^{6}–OAc)$	H	Me	368, 369
621	Icariside I	H	Glc	Me	354, 360
622	Icariin	Rha	Glc	Me	370–373
623	Acetylicariin	Rha	$Glc^{2}–$OAc	Me	374
624	Epimedin A	$Rha^{2}–^{1}Glc$	Glc	Me	360, 375
625	Epimedin B	$Rha^{2}–^{1}Xyl$	Glc	Me	354, 360
626	Epimedin C (Baohuoside VI)	$Rha^{2}–^{1}Rha$	Glc	Me	360, 353
627	4‴-*O*-Xylosylicariin	$Rha^{4}–^{1}Xyl$	Glc	Me	376
628	Hexandraside F	$Rha^{3}–^{1}Glc$	Glc	Me	357
629	Sempervirenoside B	$Rha(^{4}–OAc)^{3}–^{1}Glc$	Glc	Me	377
630	Epimedokoreanoside I	$Rha(^{4}–OAc)^{3}–^{1}Glc(^{6}–OAc)$	Glc	Me	368, 378
631	Demethyl-epimedokoreanoside I	$Rha(^{4}–OAc)^{3}–^{1}Glc(^{6}–OAc)$	Glc	H	368
632	Caohuoside E	$Rha(^{4}–OAc)^{3}–^{1}Glc(^{2}–OAc)$	Glc	Me	379
633	Caohuoside A	$Rha(^{4}–OAc)^{3}–^{1}Glc(^{6}–OAc)^{3}–$OAc	Glc	Me	380

(continued)

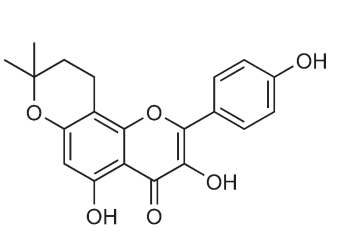
Figure 9 (*Continued*)

Type		Compounds	Substituents			Reference(s)
			R^1	R^2	R^3	
	634	Caohuoside B	Rha(^{4}–OAc)^3–^{1}Glc(^{6}–OAc)^4–OAc	Glc	Me	368
	635	Epimedin K (korepimedoside B)	Rha(^{4}–OAc)^3–^{1}Glc(^{6}–OAc)^2–OAc	Glc	Me	369
	636	4H-1-Benzopyran-4-one, 7-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-3,5-dihydroxy-2-(4-hydroxyphenyl)-8-(3-methyl-2-buten-1-yl)-	H	Glc^2–^{1}Glc	H	368
II	**637**	Icaritin-3-*O*-α-rhamnoside	Rha	H	H	381
	638	Wanepimedoside A	Rha^2–^{1}Rha	H	H	351
	639	Brevicornin	H	H	Me	382
	640	Caohuoside D	H	Glc	Me	383
III	**641**	Kaempferol	H	H		357
	642	Kaempferol-3-*O*-rhamnoside	Rha	H		357
	643	Kaempferol-3-*O*-β-D-galactoside (Trifoliin)	Gal	H		357, 384
	644	Astragalin	Glc	H		385, 381
	645	Kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranoside	Rha–Rha	H		357
	646	Kaempferol-3,7-*O*-dirhamnoside	Rha	Rha		356
	647	Sagittatin A	Rha^2–^{1}Xyl	Rha		386
	648	Sagittatin B	Rha(^{4}–OAc)^2–^{1}Xyl	Rha		386
IV	**649**	Quercetin	H			355
	650	Quercitrin	Rha			357, 376
	651	Hyperin	Gal			373, 376
	652	Isoquercetin	Glc			387
	653	3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranoside (Horridin)	Rha^2–^{1}Rha			357
	654					

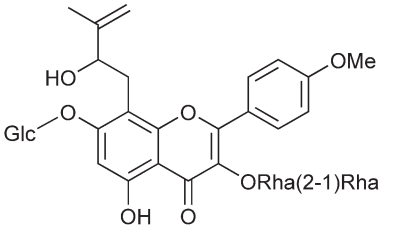
Figure 9 (*Continued*)



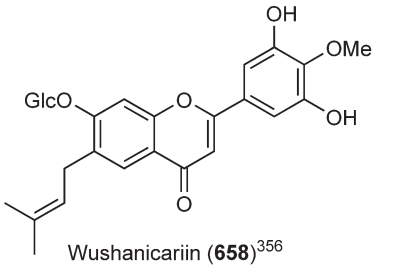
β-Anhydroicaritin (**655**)[348,382]



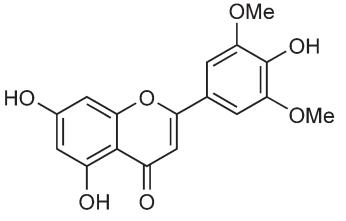
Des-*O*-β-anhydroicaritin (**656**)[354]



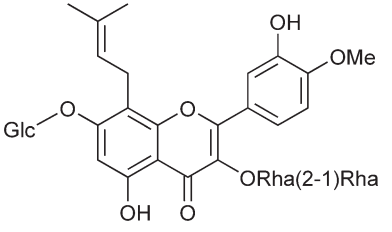
Sagittasine A (**657**)[357]



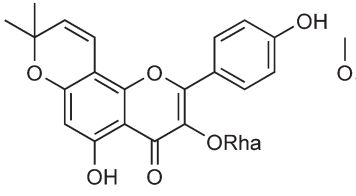
Wushanicariin (**658**)[356]



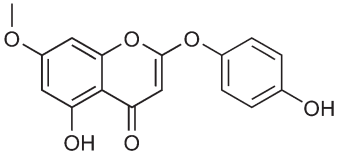
Tricin (**659**)[382,388]



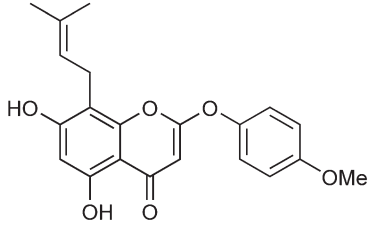
Sagittasine B (**660**)[357]



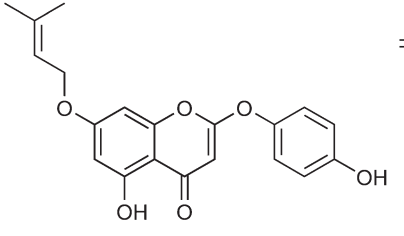
Acuminatin (**661**)[355]



6-Demethoxy-7-methylcapillarisin (**662**)[389]

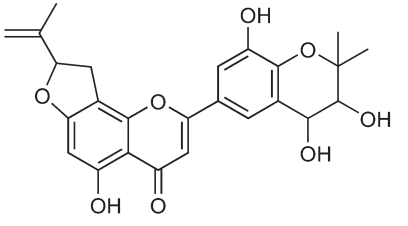


663[389]

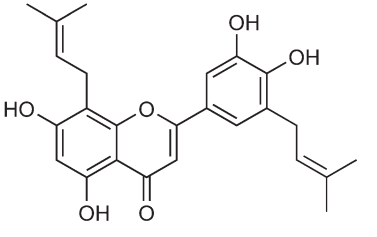


664[389]

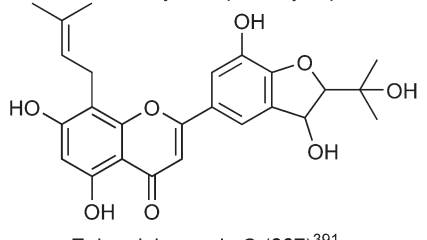
663: 6-Demethoxy-4'-methyl-8-isopentenylcapillarisin,
664: 6-Demethoxy-7-isopentenylcapillarisin



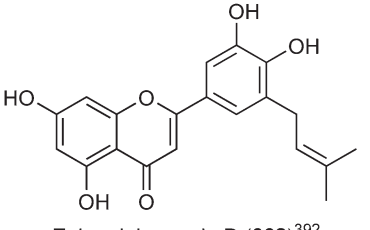
Epimedokoreanin A (**665**)[363]



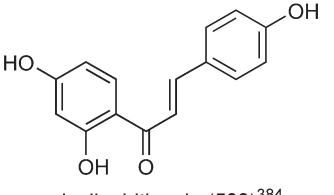
Epimedokoreanin B (**666**)[390,391]



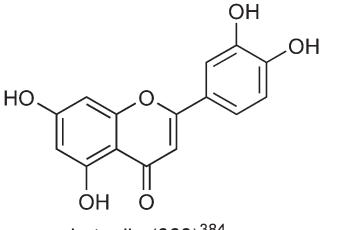
Epimedokoreanin C (**667**)[391]



Epimedokoreanin D (**668**)[392]



Isoliquiritigenin (**503**)[384]



Luteolin (**669**)[384]

Figure 9 (*Continued*)

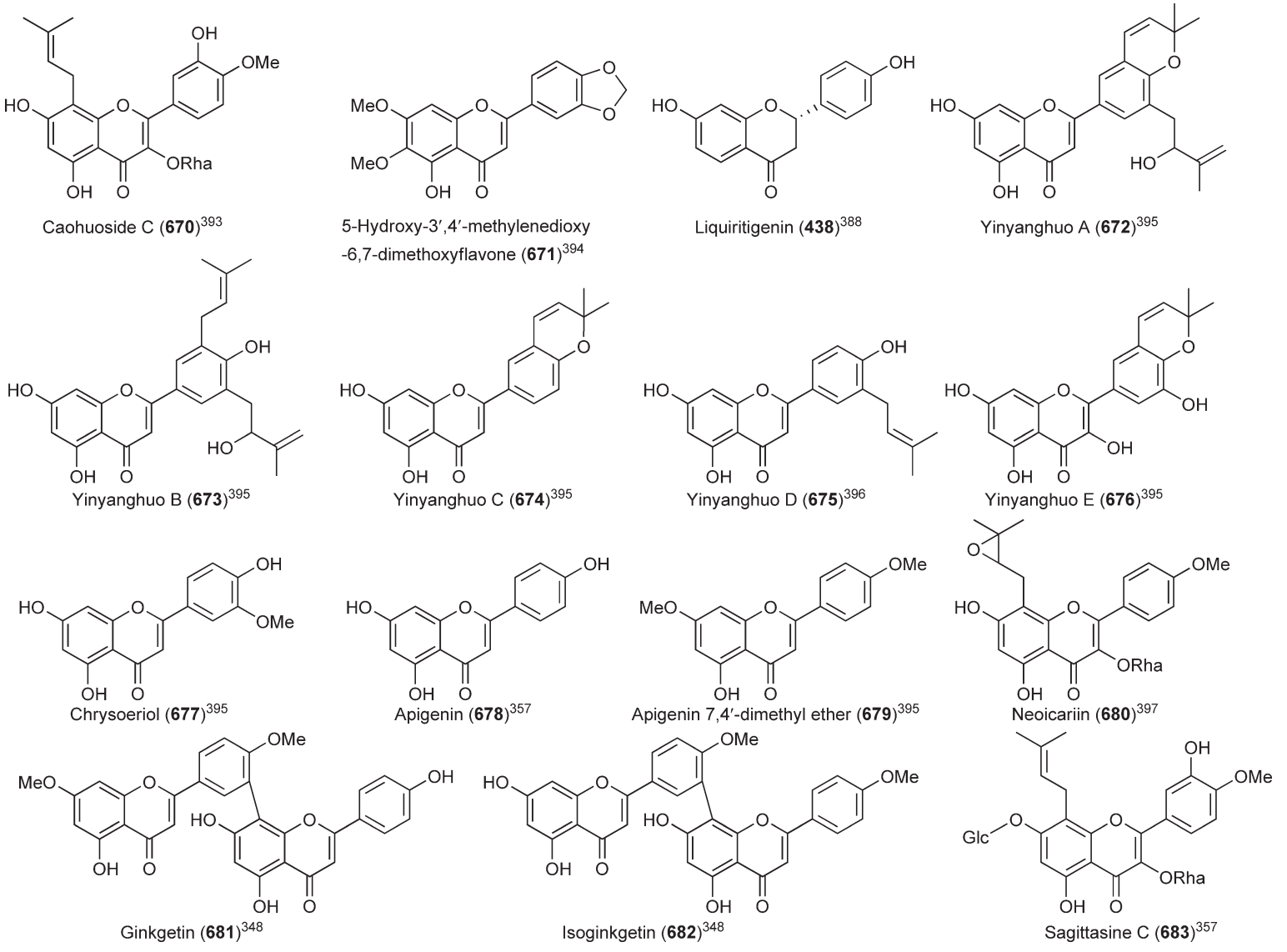
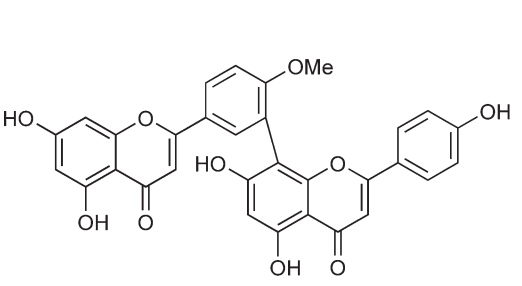
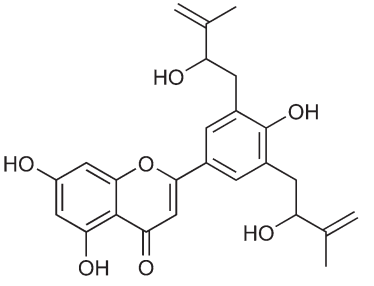


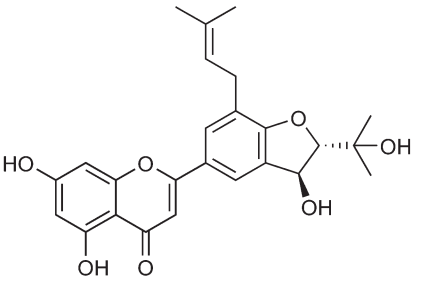
Figure 9 (*Continued*)



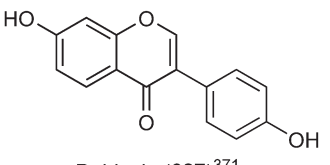
Bilobetin (**684**)[348]



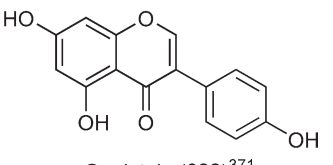
Breviflavone A (**685**)[396]



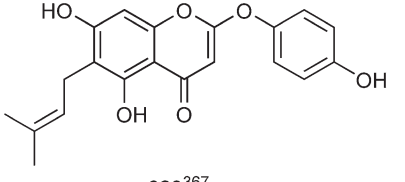
Breviflavone B (**686**)[396]



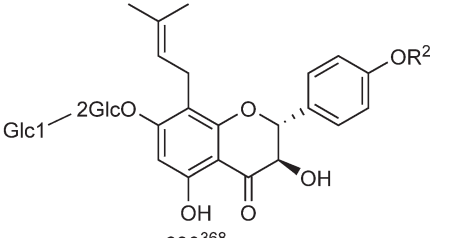
Daidzein (**687**)[371]



Genistein (**688**)[371]



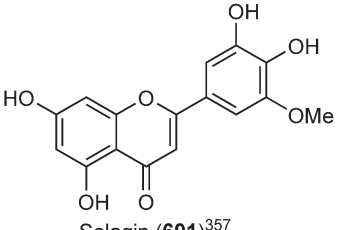
689[367]



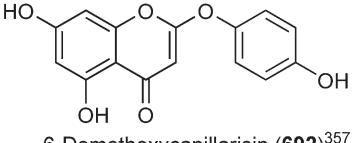
690[368]

689: 5,7-Dihydroxy-2-(*p*-hydroxyphenoxy)-6-prenylchromone[367]

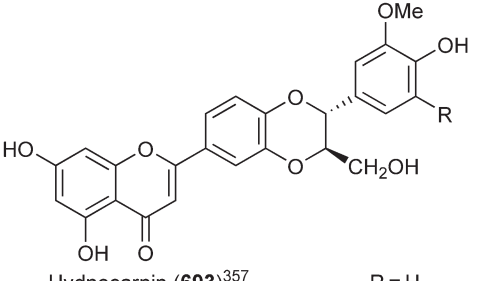
690: 4H-1-Benzopyran-4-one, 7-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-2,3-dihydro-3,5-dihydroxy-2-(4-hydroxy -phenyl) -8-(3-methyl-2-buten-1-yl)-, (2*R*,3*R*)-[368]



Selagin (**691**)[357]



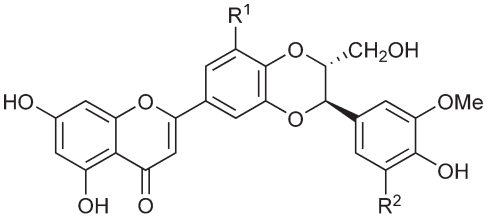
6-Demethoxycapillarisin (**692**)[357]



Hydnocarpin (**693**)[357] R = H

5″-Methoxyhydnocarpin (**694**)[357] R = OMe

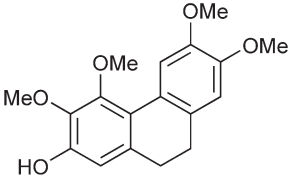
Figure 9 (*Continued*)



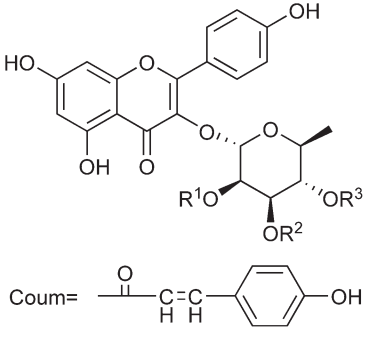
Hydnocarpin D (**695**)[357] $R^1 = R^2 = H$

5′-Methoxyhydnocarpin D (**696**)[357] $R^1 = OMe$, $R^2 = H$

5′,5′-Dimethoxyhydnocarpin (**697**)[357] $R^1 = R^2 = OMe$

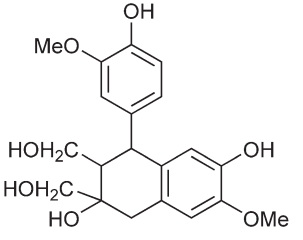


9,10-Dihydro-3,4,6,7-tetramethoxy-2-phenanthrenol (**698**)[398]



Coum=

698, 699, 700, 701, 702



703

698: Kaempferol-3-*O*-(2′′,4′′-di-E-*p*-coumaroyl)-α-L-rhamnopyranoside[357] $R^1 = R^3 =$ E-Coum, $R^2 = H$

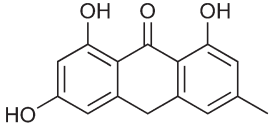
699: Kaempferol-3-*O*-(2′′-*E*-*p*-coumaroyl, 4′′-*Z*-*p*-coumaroyl)-α-L-rhamnopyranoside[357] $R^1 =$ E-Coum, $R^2 = H$, $R^3 =$ Z-Coum

700: Kaempferol-3-*O*-(2′′-*Z*-*p*-coumaroyl, 4′′-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside[357] $R^1 =$ Z-Coum, $R^2 = H$, $R^3 =$ E-Coum

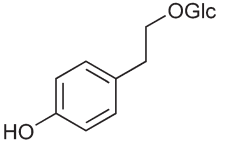
701: Kaempferol-3-*O*-(3′′,4′′-di-E-*p*-coumaroyl)-α-L-rhamnopyranoside[357] $R^1 = H$, $R^2 = R^3 =$ E-Coum

702: Kaempferol-3-*O*-(3′′-*Z*-*p*-coumaroyl, 4′′-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside[357] $R^1 = H$, $R^2 =$ Z-Coum, $R^3 =$ E-Coum

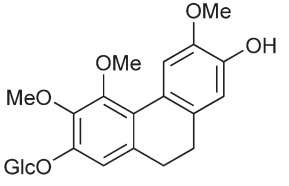
703: 2,3-Naphthalenedimethanol, 1,2,3,4-tetrahydro-3,7-dihydroxy-1-(4-hydroxy-3-methoxyphenyl)-6-methoxy-[385]



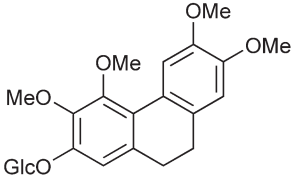
Emodin (**704**)[388]



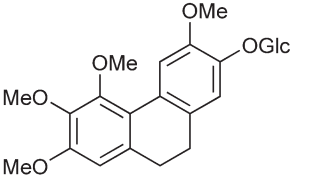
Salidroside (**705**)[355]



Icaricide A7 (**706**)[367]



Epimedoicarisoside A (**707**)[354]



Icariside A1 (**708**)[399]

Figure 9 Phenolic compounds isolated from Yinyanghuo.

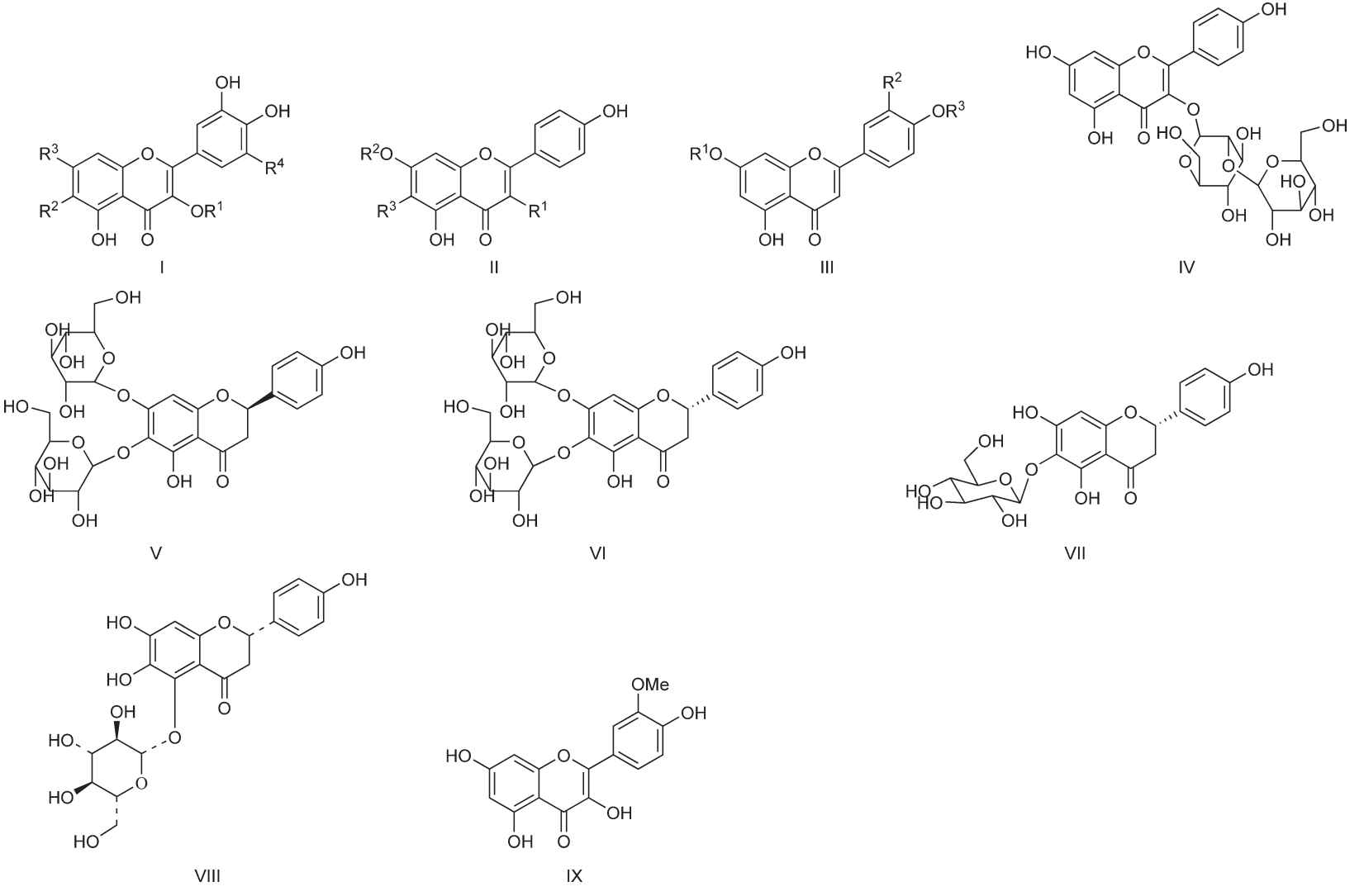


Figure 10 (*Continued*)

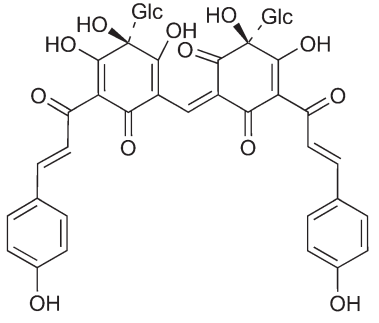
Type		Compound	R^1	R^2	R^3	R^4	Reference(s)
I	**709**	Quercetin	H	H	OH	H	411
	710	Rutin	Rha–Glc	H	OH	H	411
	711	Quercetin-7-*O*-glucoside	H	H	OGlc	H	411
	712	Quercetin-3-*O*-glucoside	Glc	H	OH	H	411
	713	Quercetin-3-*O*-galactoside	Gal	H	OH	H	412
	714	Quercetin 3,7-diglucoside	Glc	H	OGlc	H	413
	715	Quercetin 3-α-L-rhamnoside-7-β-D-glucuronide	Rha	H	OGluA	H	414
	716	Myricetin	H	H	OH	OH	415
II	**717**	Kaempferol	OH	H	H		411
	718	Kaempferol-3-*O*-glucoside	OGlc	H	H		411
	719	Kaempferol 3-*O*-β-D-rutinoside	ORha–Glc	H	H		411, 416
	720	Kaempferol 7-β-D-glucopyranoside	OH	Glc	H		417
	721	Kaempferol 3-β-D-glucoside-7-β-D-glucuronide	OGlc	GluA	H		414
	722	6-Hydroxykaempferol	OH	H	OH		411, 413
	723	6-Hydroxykaempferol 3-*O*-β-D-glucoside	OGlc	H	OH		413
	724	6-hydroxykaempferol-7-*O*-glucoside	OH	Glc	OH		418
	725	6-Hydroxykaempferol 3-*O*-β-D-rutinoside	ORha–Glc	H	OH		419
	726	6-Hydroxykaempferol 3,6-di-*O*-β-D-glucoside	OGlc	H	OGlc		413

(continued)

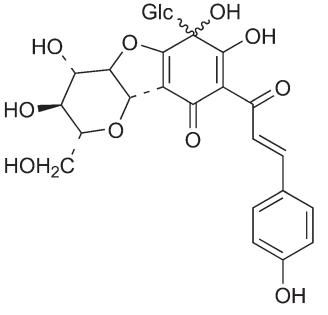
Figure 10 (*Continued*)

Type		Compound	R^1	R^2	R^3	R^4	Reference(s)
	727	6-Hydroxykaempferol 6,7-di-*O*-β-D-glucoside	OH	Glc	OGlc		420
	728	6-Hydroxykaempferol 3-*O*-β-rutinoside-6-*O*-β-D-glucoside	ORha–Glc	H	OGlc		413
	729	6-Hydroxykaempferol 3,6,7-tri-*O*-β-D-glucoside	OGlc	Glc	OGlc		413
	730	6-Hydroxykaempferol 3,6-di-*O*-β-D-glucoside-7-*O*-β-D-glucuronide	OGlc	GluA	OGlc		414
	731	Apigenin	H	H	H		413
	732	Scutellarein (6-Hydroxyapigenin)	H	H	OH		413
III	**733**	Acacetin	H	H	Me		421
	734	Acacetin 7-*O*-α-L-rhamnopyranoside	Rha	H	Me		417
	735	Acacetin 7-*O*-β-D-glucoside	Glc	H	Me		422
	736	Acacetin-7-*O*-β-D-glucuronide	GluA	H	Me		423
	737	Acacetin-7-*O*-[β-D-Apiofuranosyl-(1→6)-β-D-glucopyranoside]	Glc–Api	H	Me		417
	738	Luteolin	H	OH	H		424
	739	Luteolin-7-*O*-β-D-glucopyranoside	Glc	OH	H		424, 425
IV	**740**	Kaempferol 3-*O*-β-D-sophoroside					426
V	**741**	(2*R*)-4',5-Dihydroxyl-6,7-di-*O*-β-D-glucopyranosyl flavanone					427
VI	**742**	(2*S*)-4',5-Dihydroxyl-6,7-di-*O*-β-D-glucopyranosyl flavanone					420
VII	**743**	(2*S*)-4',5,6,7-Tetrahydroxyflavavone 6-*O*-β-D-glucopyranoside					428
VIII	**744**	Neocarthamin					429
IX	**745**	Isorhamnetin					430

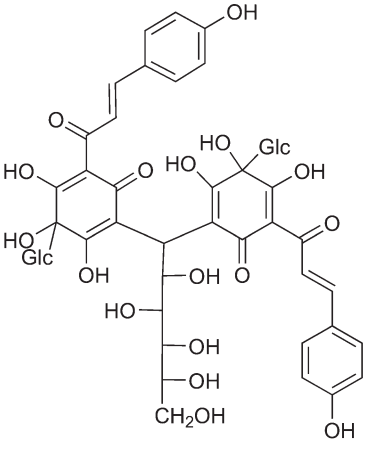
Figure 10 Structures of flavonoids isolated from safflower.



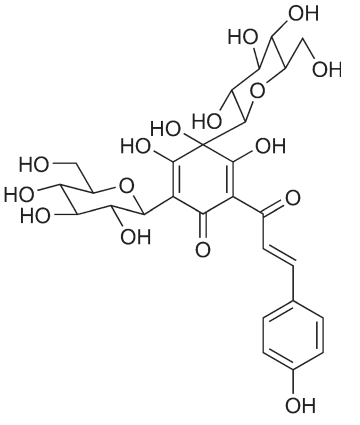
Carthamin (**746**)[429]



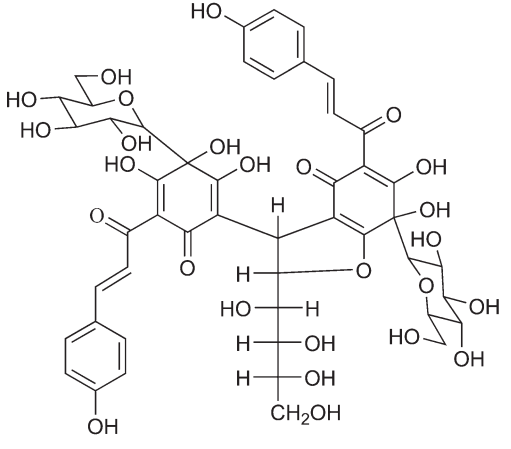
Safflor yellow A (**747**)[431]



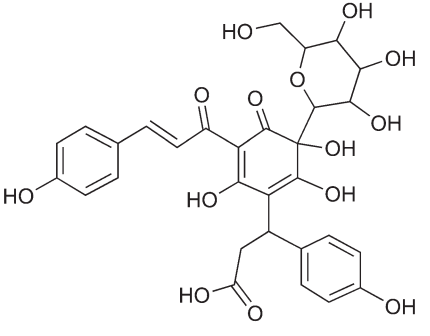
Safflor yellow B (**748**)[432]



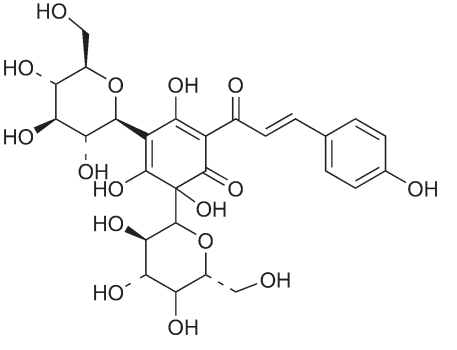
Hydroxysafflor yellow A (**749**)[433]



Anhydrosafflor yellow B (**750**)[434]

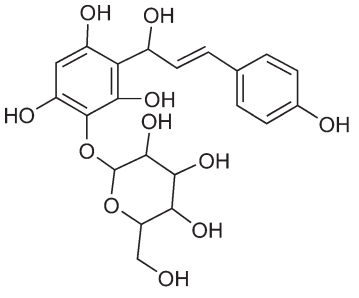


Safflomin C (**751**)[435]

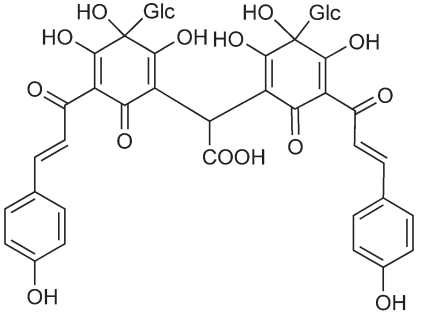


Saffloside A (**752**)[436]

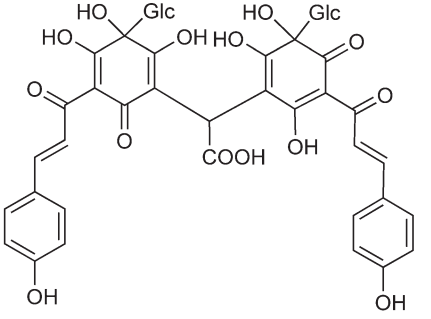
Figure 11 (*Continued*)



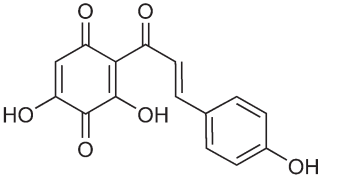
Precarthamin 2 (**753**)[437]



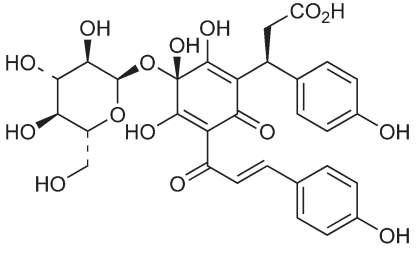
Precarthamin 1 (**754**)[438]



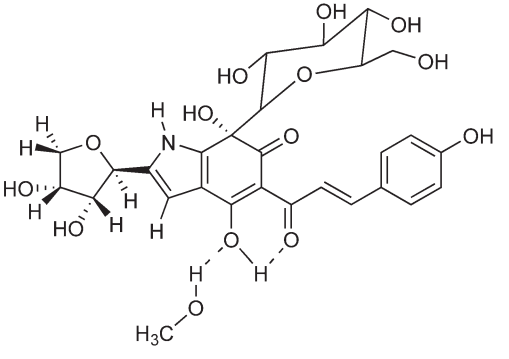
Precarthamin 3 (**755**)[439]



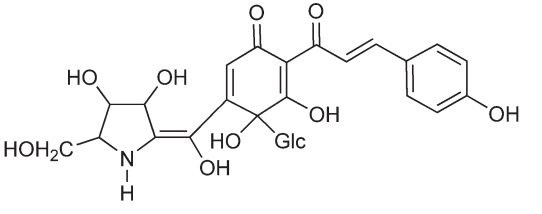
Carthamone (**756**)[429]



Isosafflomin C (**757**)[440]

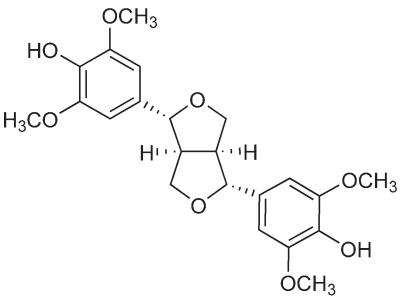


Cartormin (**758**)[441]

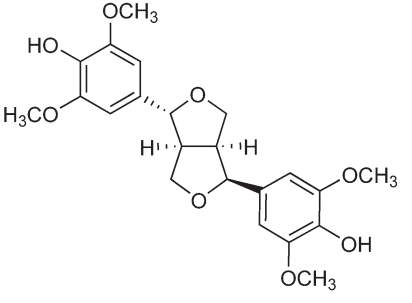


Tinctormine (**759**)[433, 442]

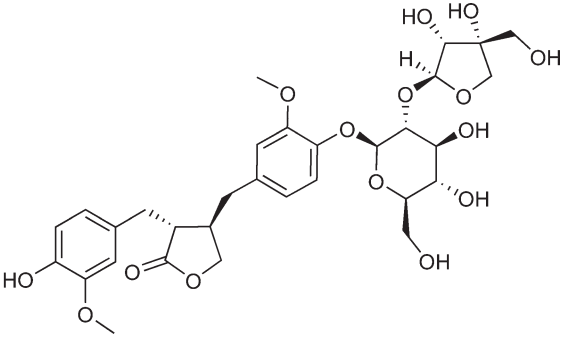
Figure 11 Structures of chalcone pigments isolated from safflower.



Syringarenol (**760**)[443]

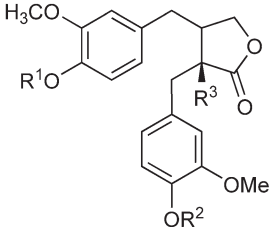


Lirioresinol-A (**761**)[443]



762

762: Matairesinol 4′-O-β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside[444]



Matairesinol (**763**)[422]: R^1 = H, R^2 = H, R^3 = H;

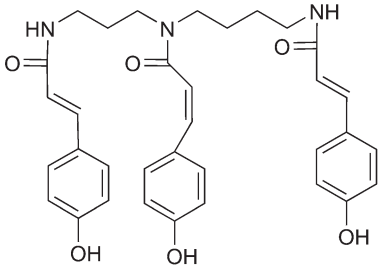
Matairesinol-4′-*O*-β-D-glucoside (**764**)[445]: R^1 = H, R^2 = Glucose, R^3 = H;

Matairesinol monoglucoside (**765**)[421]: R^1 = Glucose, R^2 = H, R^3 = H;

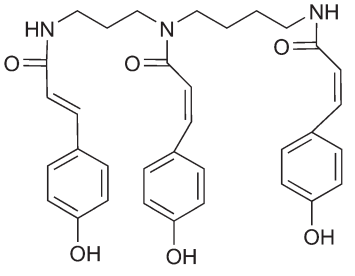
8′-Hydroxyarctigenin (**766**)[422]: R^1 = Me, R^2 = H, R^3 = OH;

8′-Hydroxyarctignin-4′-O-β-D-glucoside (Tracheloside) (**767**)[446]: R^1 = Me, R^2 = Glucose, R^3 = OH

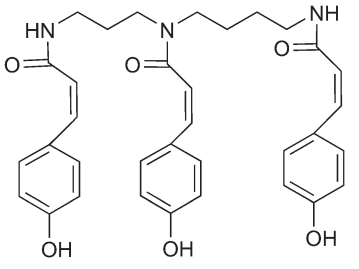
Figure 12 Structures of lignans isolated from safflower.



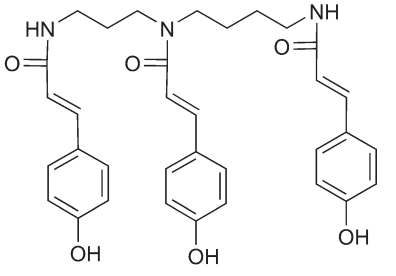
Safflospermidine A (**768**)[447]



Safflospermidine B (**769**)[447]

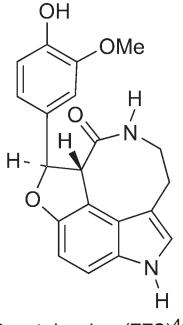


770

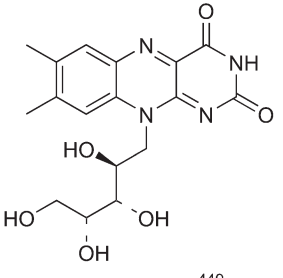


771

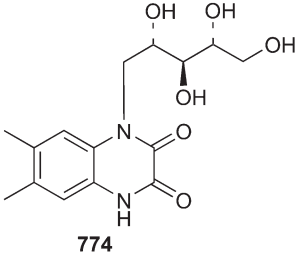
770: N_1,N_5,N_{10}-(*Z*)-tri-*p*-coumaroylspermidine[447]
771: N_1,N_5,N_{10}-(*E*)-tri-*p*-coumaroylspermidine[447]



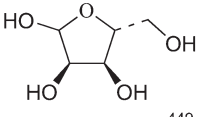
Serotobenine (**772**)[448]



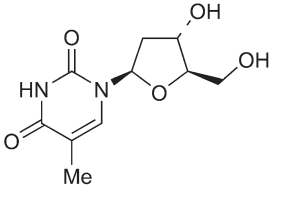
Riboflavin (**773**)[449]



774

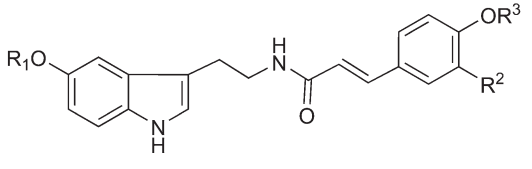


Ribofuranose (**775**)[449]



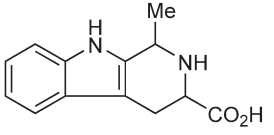
Thymine-2-desoxyribofuranoside (**776**)[412]

774: 1,4-Dihydro-6,7-dimethyl-1-ribityl-2,3-quinoxalinedione[449]

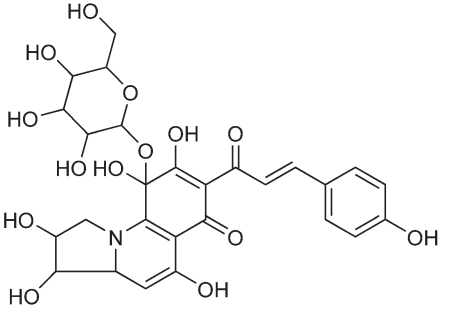


N-Feruloylserotonin (moschamine) (**775**)[421]: $R^1 = H$, $R^2 = OMe$, $R^3 = H$
N-(*p*-Coumaroyl)serotonin (**776**)[421]: $R^1 = H$, $R^2 = H$, $R^3 = H$
N-Feruloylserotonin-5-*O*-β-D-glucoside (**777**)[421]: R^1 = glucose, $R^2 = OMe$, $R^3 = H$
N-(*p*-Coumaroyl)serotonin-5-*O*-β-D-glucoside (**778**)[421]: R^1 = glucose, $R^2 = H$, $R^3 = H$

Figure 13 (*Continued*)



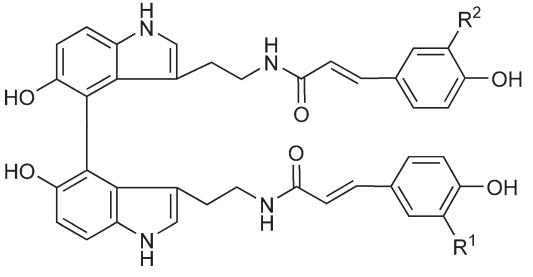
779



780

779: 2,3,4,9-tetrahydro-1-methy1-1-H-pyrido [3,4-β] indole-3-carboxylic acid[412];

780: 9-(β-D-glucopyranosyloxy)-2,3,3a,9-tetrahydro-2,3,5,8,9-pentahydroxy-7-[3-(4-hydroxyphenyl)-1-oxo-2-propen-1-yl]-Pyrrolo [1,2-a]quinolin-6(1H)-one[450]

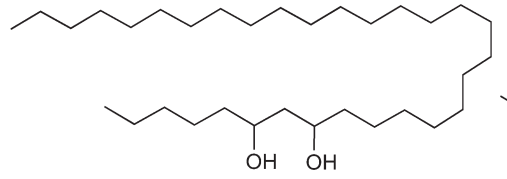


4,4″-Bis(*N*-*p*-coumaroylserotonin) (**781**)[451]: $R^1 = H$, $R^2 = H$

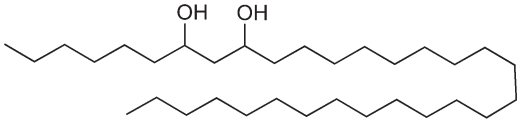
4,4″-Bis(N-feruloyl)serotonin (**782**)[451]: $R^1 = OMe$, $R^2 = OMe$

4-[*N*-(*p*-Coumaroyl)serotonin-4″-yl]-*N*-feruloylserotonin (**783**)[451]: $R^1 = OMe$, $R^2 = H$

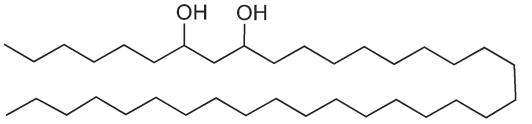
Figure 13 Structures of nitrogen-containing compounds isolated from safflower.



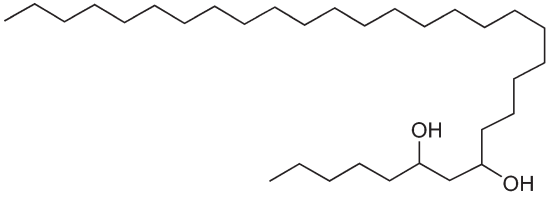
syn-Dotriacontane-6,8-diol (**784**)[452]



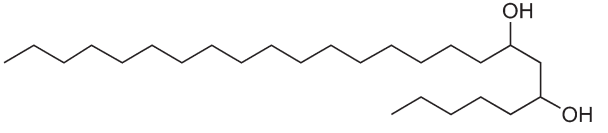
syn-Dotriacontane-7,9-diol (**785**)[453]



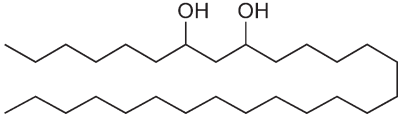
syn-Hexatriacontane-7,9-diol (**786**)[453]



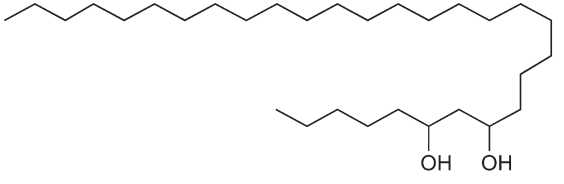
syn-Hentriacontane-6,8-diol (**787**)[452]



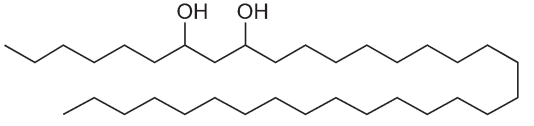
syn-Pentacosane-6,8-diol (**788**)[452]



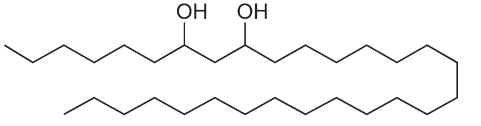
syn-Octacosane-7,9-diol (**789**)[452]



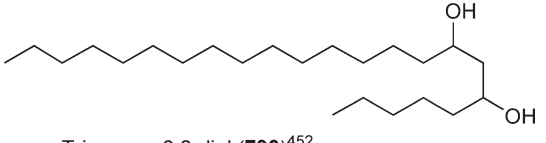
syn-Triacontane-6,8-diol (**790**)[452]



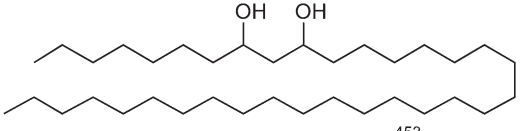
s*yn*-Tetratriacontane-7,9-diol (**791**)[452]



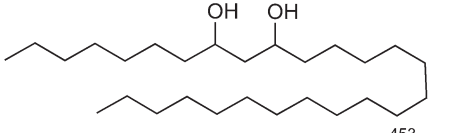
syn-Triacontane-7,9-diol (**792**)[453]



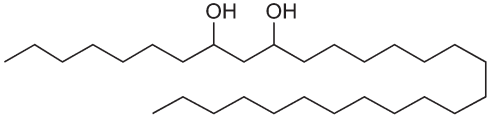
syn-Tricosane-6,8-diol (**793**)[452]



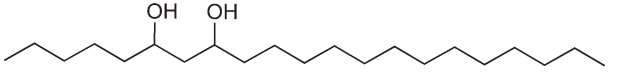
s*yn*-Pentatriacontane-8,10-diol (**794**)[453]



s*yn*-Heptacosane-8,10-diol (**795**)[453]

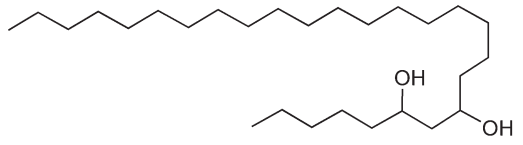


syn-Nonacosane-8,10-diol (**796**)[453]

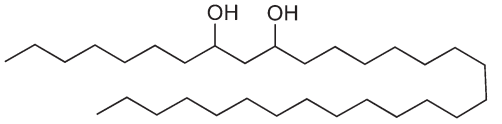


syn-Heneicosane-6,8-diol (**797**)[452]

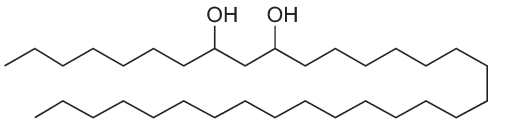
Figure 14 (*Continued*)



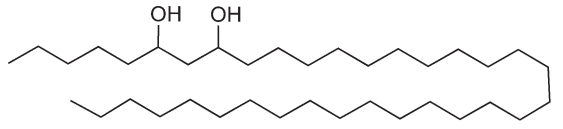
syn-Heptacosane-6,8-diol (**798**)[452]



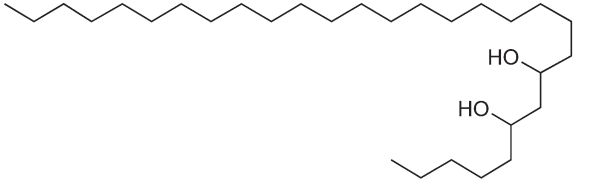
syn-Hentriacontane-8,10-diol (**799**)[453]



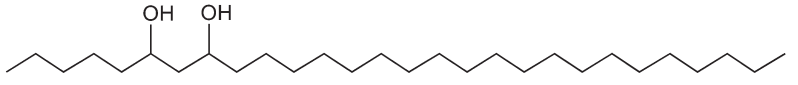
syn-Tritriacontane-8,10-diol (**800**)[453]



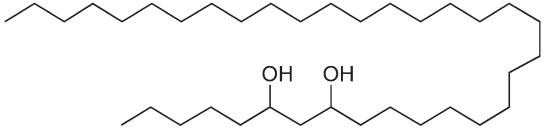
syn-Hexatriacontane-6,8-diol (**801**)[453]



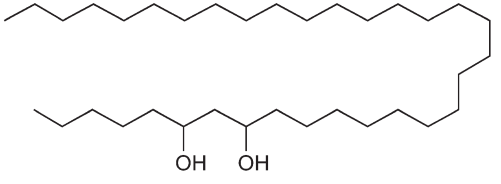
syn-Nonacosane-6,8-diol (**802**)[452]



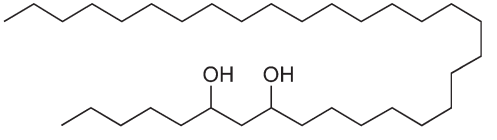
syn-Octacosane-6,8-diol (**803**)[453]



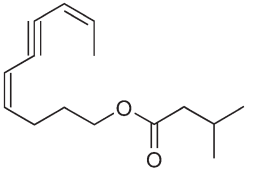
syn-Pentatriacontane-6,8-diol (**804**)[452]



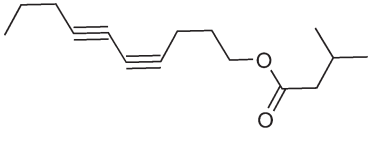
syn-Tetratriacontane-6,8-diol (**805**)[453]



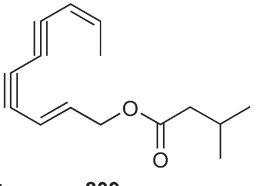
syn-Tritriacontane-6,8-diol (**806**)[452]



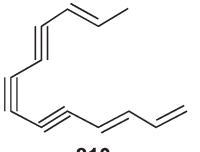
cis-8-Decene-4,6-diyn-1-ol isovalerate (**807**)[454]



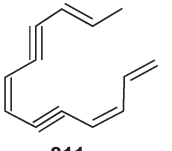
4, 6-Decadiyn-1-ol isovalerate (**808**)[454]



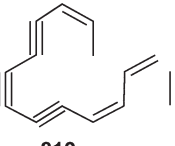
809



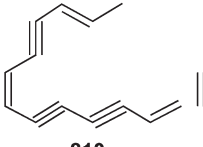
810



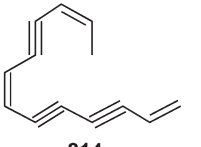
811



812



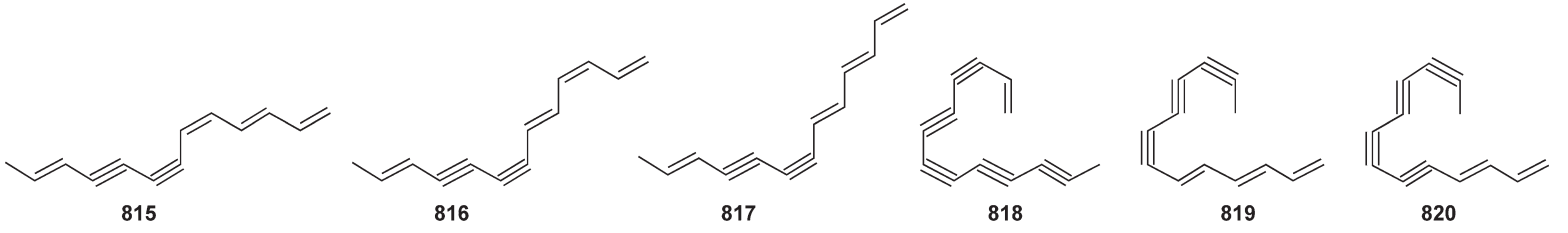
813



814

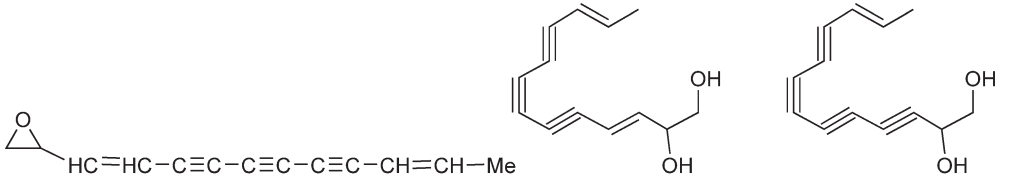
809: *trans*-2, *cis*-8-Decadiene-4,6-diyn-1-ol isovalerate,[454] **810**: (3*E*,11*E*)-Trideca-1,3,11-triene-5,7,9-triyne,[455]
811: (3*Z*,11*E*)-Trideca-1,3,11-triene-5,7,9-triyne,[455] **812**: (3*Z*,11*Z*)-Trideca-1,3,11-triene-5,7,9-triyne,[456]
813: (11*E*)-Trideca-1,11-diene-3,5,7,9-tetrayne,[456] **814**: (11*Z*)-Trideca-1,11-diene-3,5,7,9-tetrayne[456]

Figure 14 (*Continued*)



815 **816** **817** **818** **819** **820**

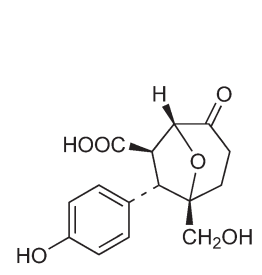
815: (3*E*,5*Z*,11*E*)-Trideca-1,3,5,11-tetraene-7,9-diyne,[456] **816**: (3*Z*,5*E*,11*E*)-Trideca-1,3,5,11-tetraene-7,9-diyne,[456]
817: (3*E*,5*E*,11*E*)-Trideca-1,3,5,11-tetraene-7,9-diyne,[456] **818**: 1-Tridecene-3,5,7,9,11-pentayne,[457]
819: 1,3,5-Tridecatriene-7,9,11-triyne,[457] **820**: (3*E*)-Trideca-1,3-diene-5,7,9,11-tetrayne[456]



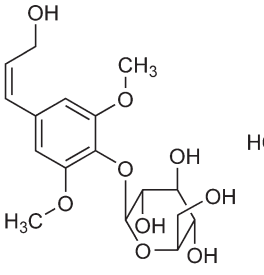
821 **822** **823**

821: 12,13-Epoxy-2,10-Tridecadiene-4,6,8-triyne,[458] **822**: (2*R*,3*E*,11*E*)-3,11-Tridecadiene-5,7,9-triyne-1,2-diol,[459]
823: (2*R*,11*E*)-11-Tridecene-3,5,7,9-tetrayne-1,2-diol[460]

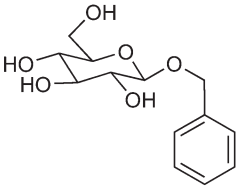
Figure 14 Structures of alkenes isolated from safflower.



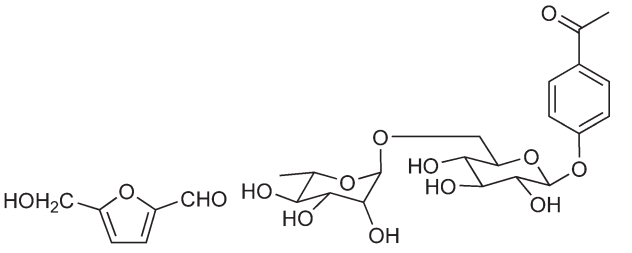
Cartorimine (**824**)[461]



Syringin (**825**)[413]

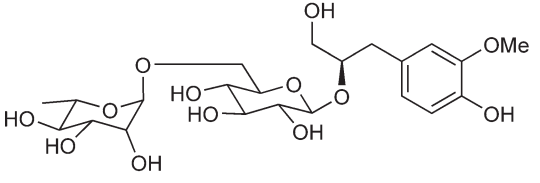


Benzyl-*O*-β-D-gluco-pyranoside (**826**)[443]

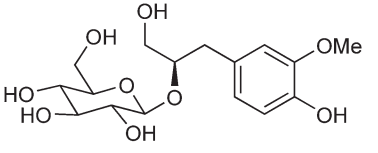


827

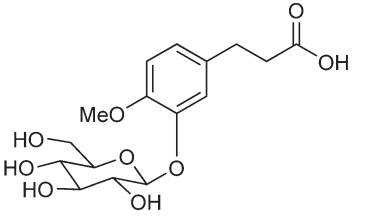
827: 5-Hydroxymethyl-2-furaldehyde,[443] Carthamoside B_7[462]



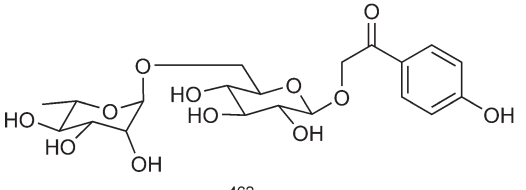
Carthamoside B_4 (**828**)[462]



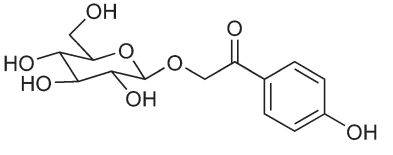
Carthamoside B_5 (**829**)[462]



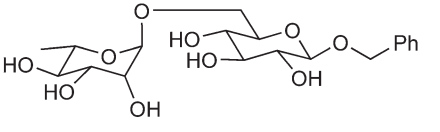
Carthamoside B_6 (**830**)[462]



Carthamoside B_8 (**831**)[462]

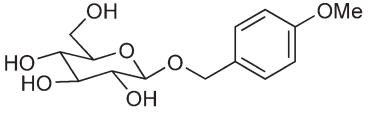


4′-(Hydroxyphenacyl)-β-D-glucopyranoside (**832**)[462]

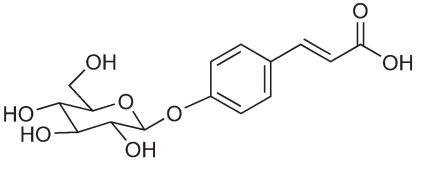


833

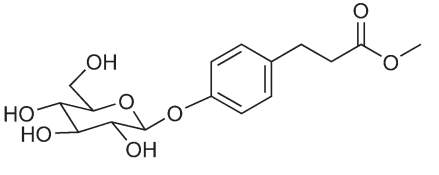
833: Benzyl-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside[462]



834



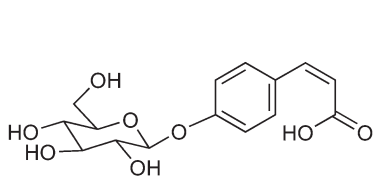
835



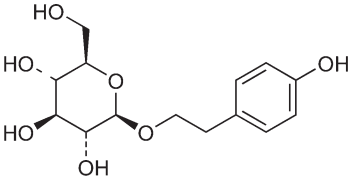
836

834: 4-(Methoxybenzyl)-*O*-β-D-glucopyranoside,[462] **835**: 4-*O*-β-D-Glucosyl-*trans*-*p*-coumaric acid[427]
836: Methyl-3-(4-*O*-β-D-glucopyranosylphenyl) propionate[427]

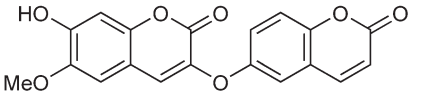
Figure 15 (*Continued*)



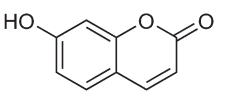
4-*O*-β-D-Glucosyl-*cis*-*p*-coumaric acid (**837**)[427]



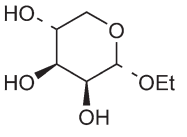
Salidroside (**838**)[425]



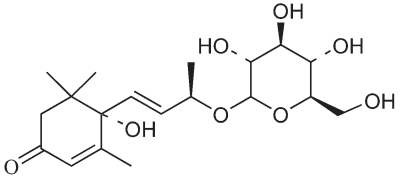
Daphnoretin (**839**)[430]



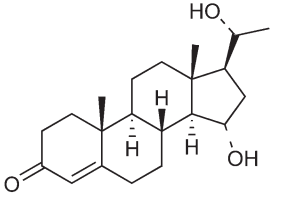
Umbelliferone (**840**)[430]



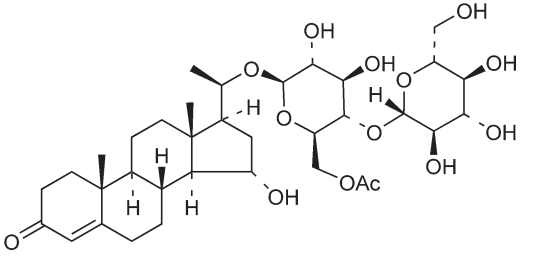
Ethyl-α-D-lyxopyranoside (**841**)[412]



Roseoside (**842**)[463]

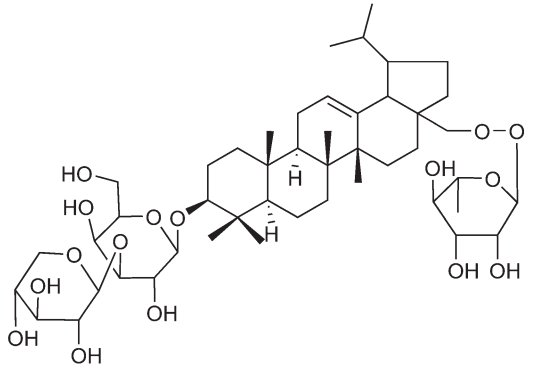


843

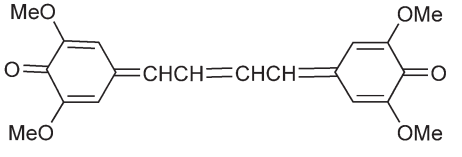


844

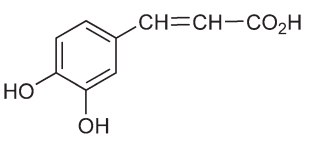
843: 15α,20*R*-Dihydroxypregn-4-en-3-one[464] **844**: (15α,20*R*)-Dihydroxypregn-4-en-3-one 6′-*O*-acetyl-20-β-cellobioside[444]



845[465]



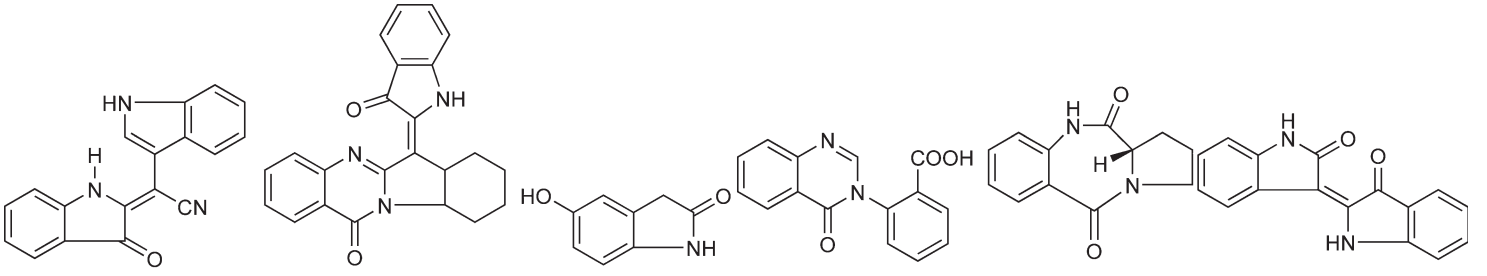
Kinobeon A (**846**)[466]



Caffeic acid (**847**)[413]

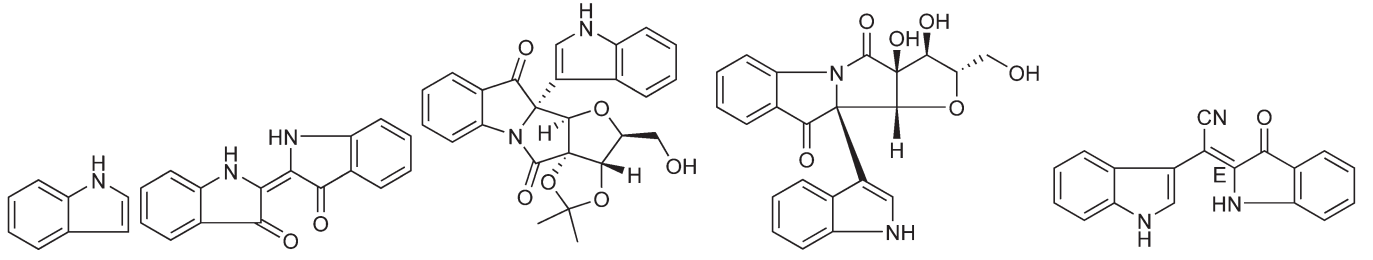
845: 3β-*O*-[β-D-xylopyranosyl(1⟶3)-*O*-β-D-galactopyranosyl]-lup-12-ene-28-oic acid-28-*O*-α-L-rhamnopyranosyl ester[465]

Figure 15 Other compounds isolated from safflower.



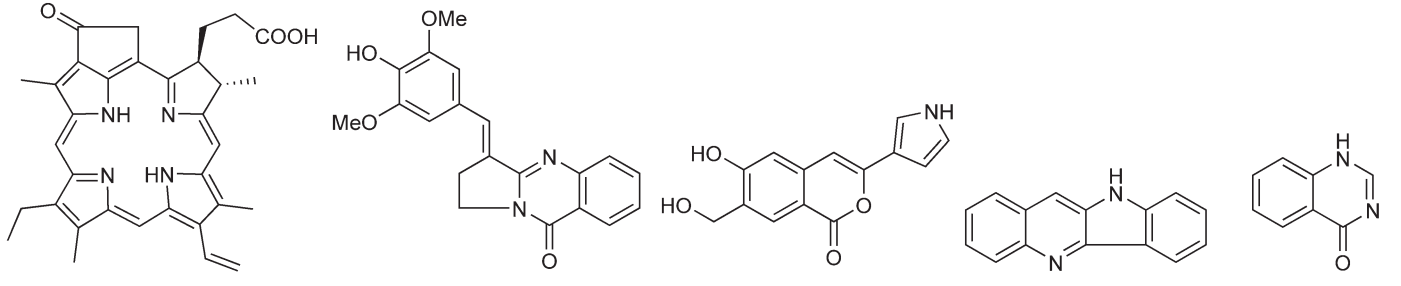
848[472] Qingdainone (**849**)[473] 5-Hydroxyoxindole (**850**)[474] **851**[475] Indirubin (**19**)[476]

848: *E*-2-[(3′-indole)cyanomethylene]-3-indolinone, **851**: Benzoic acid, 2-(4-oxo-3(4*H*)-quinazolinyl)-,
852: 1*H*-Pyrrolo[2,1-*c*][1,4]benzodiazepine-5,11(10*H*,11a*H*)-dione, 2,3-dihydro-, (11*aS*)-



1*H*-Indole (**853**)[476] Indigo (**854**)[476] **855**[477] Isatisine A (**856**)[477] **857**[478]

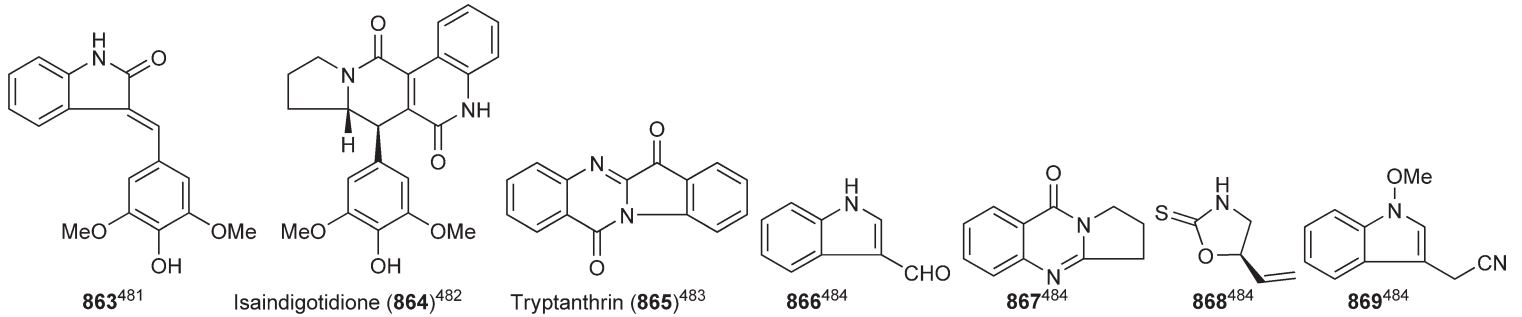
855: 9α,13α-Dihydroxylisopropylidenylisatisine A,
857: 1*H*-Indole-3-acetonitrile, α-(1,3-dihydro-3-oxo-2*H*-indole-2-ylidene)-, (αE)-



Pyrophaeophorbid A (**858**)[479] Isaindigotone (**859**)[479] Indigotiisocoumarin A (**860**)[480] Quindoline (**861**)[481] **862**[481]

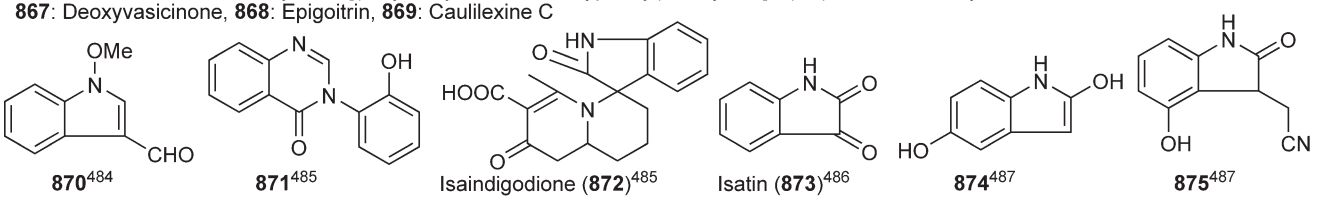
862: 4(3*H*)-Quinazolinone

Figure 16 (*Continued*)



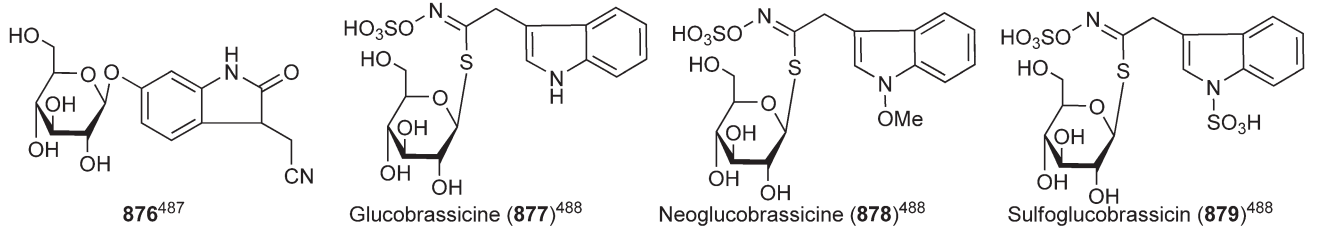
863[481] Isaindigotidione (**864**)[482] Tryptanthrin (**865**)[483] **866**[484] **867**[484] **868**[484] **869**[484]

863: 2*H*-Indol-2-one, 1,3-dihydro-3-[(4-hydroxy-3,5-dimethoxyphenyl)methylene]-, (3*E*)-; **866**: 3-Formylindole, **867**: Deoxyvasicinone, **868**: Epigoitrin, **869**: Caulilexine C



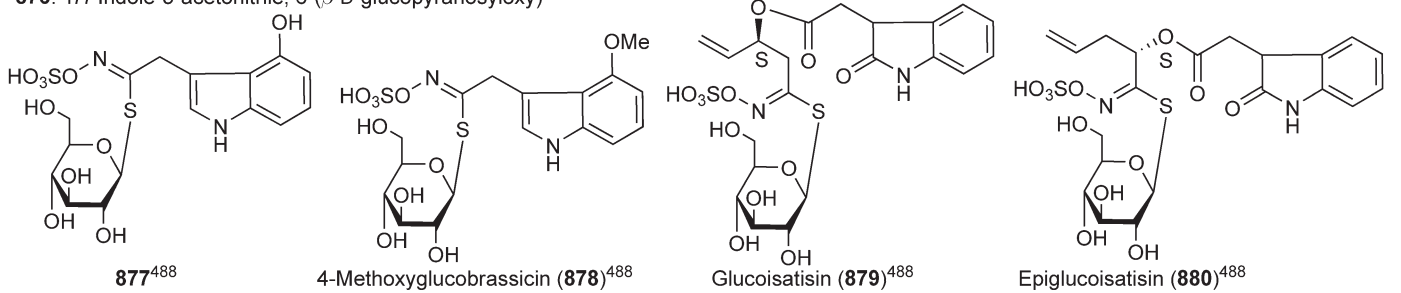
870[484] **871**[485] Isaindigodione (**872**)[485] Isatin (**873**)[486] **874**[487] **875**[487]

870: 1-Methoxyindole-3-carboxaldehyde, **871**: 3-(2′-Hydroxyphenyl)-4(3*H*)-quinazolinone, **874**: 1H-Indole-2,5-diol, **875**: 1*H*-Indole-3-acetonitrile, 2,3-dihydro-4-hydroxy-2-oxo-



876[487] Glucobrassicine (**877**)[488] Neoglucobrassicine (**878**)[488] Sulfoglucobrassicin (**879**)[488]

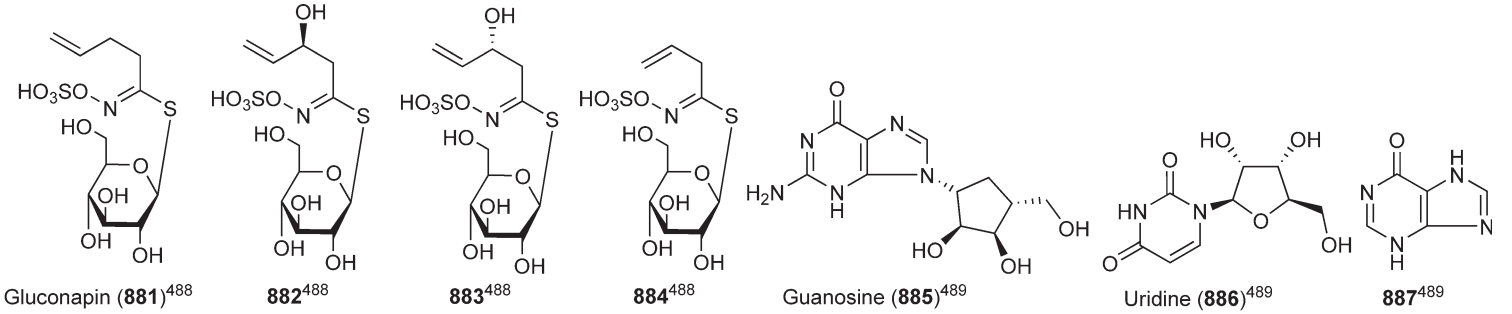
876: 1*H*-Indole-3-acetonitrile, 6-(β-D-glucopyranosyloxy)-



877[488] 4-Methoxyglucobrassicin (**878**)[488] Glucoisatisin (**879**)[488] Epiglucoisatisin (**880**)[488]

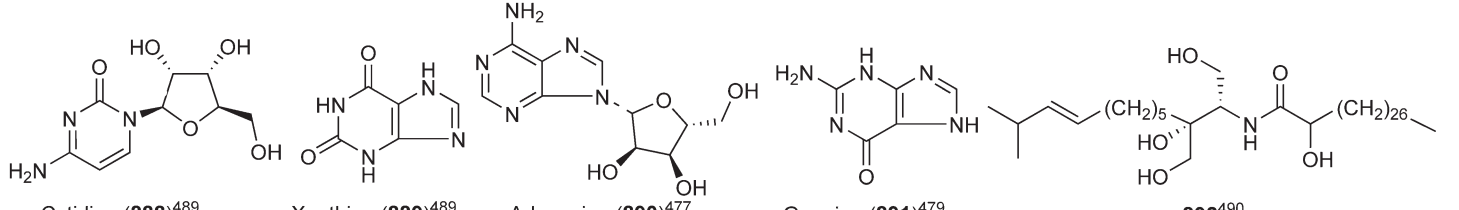
877: 4-Hydroxyglucobrassicin

Figure 16 (*Continued*)



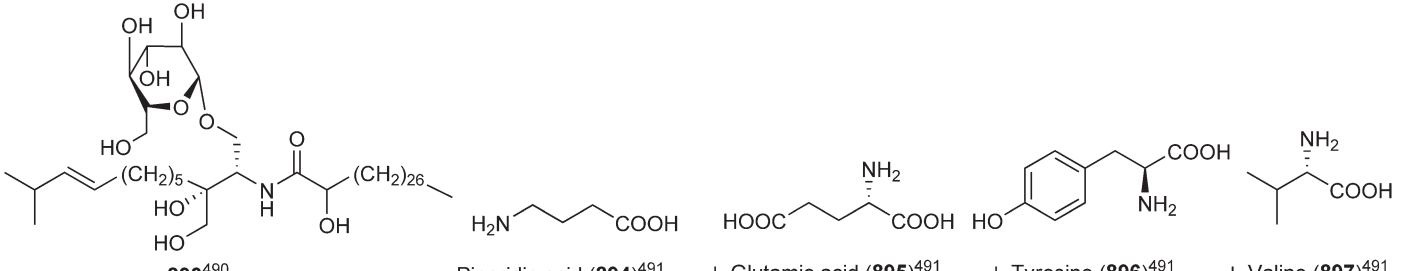
Gluconapin (**881**)[488] **882**[488] **883**[488] **884**[488] Guanosine (**885**)[489] Uridine (**886**)[489] **887**[489]

882: Epiprogoitrin, **883**: Progoitrin, **884**: Sinigrine, **887**: Hypoxanthine



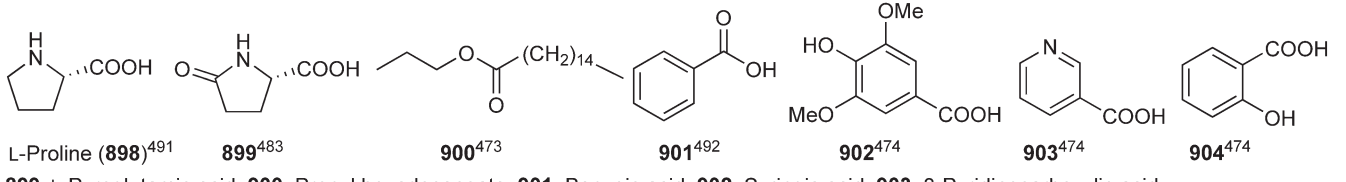
Cytidine (**888**)[489] Xanthine (**889**)[489] Adenosine (**890**)[477] Guanine (**891**)[479] **892**[490]

892: (2*S*,3*R*)-3-Hydroxymethyl-*N*-(2′-hydroxynonacosanoyl)-trideca-9*E*-sphingenine



893[490] Piperidic acid (**894**)[491] L-Glutamic acid (**895**)[491] L-Tyrosine (**896**)[491] L-Valine (**897**)[491]

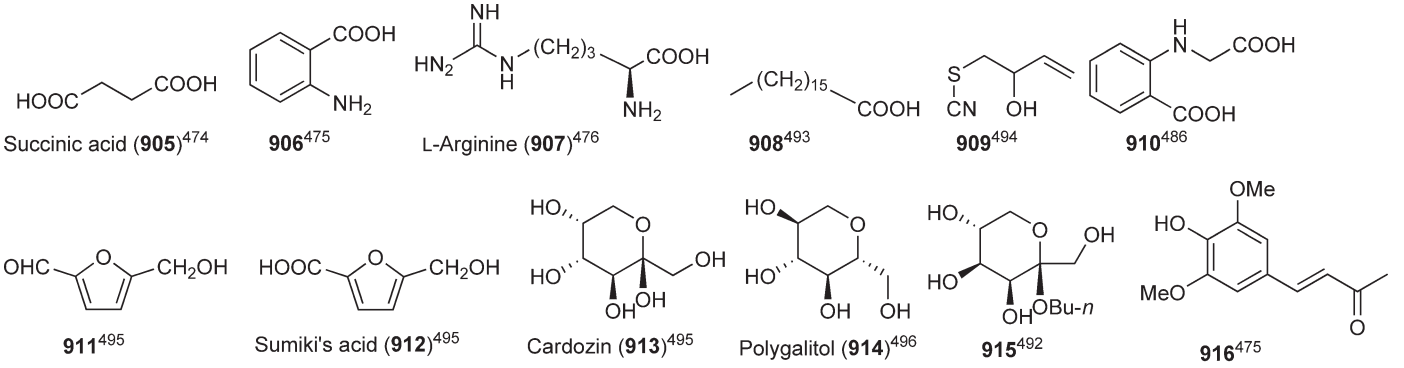
893: 1-*O*-β-D-glucopyranosyl-(2*S*,3*R*)-3-hydroxymethyl-*N*-(2′-hydroxynonacosanoyl)-trideca-9*E*-sphingenine



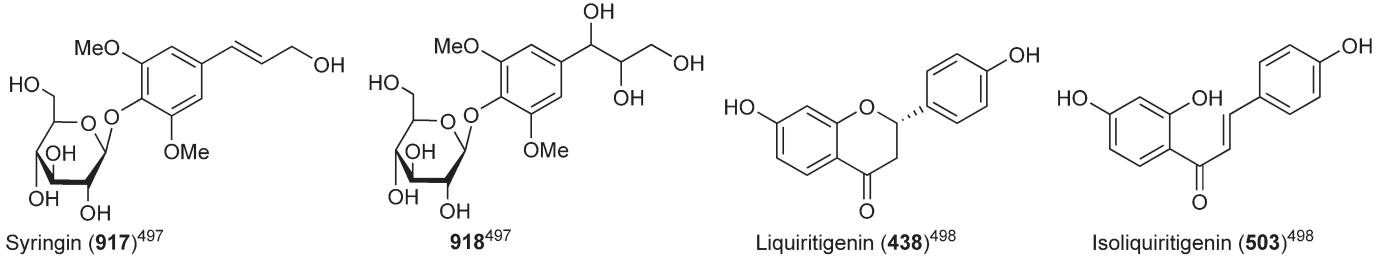
L-Proline (**898**)[491] **899**[483] **900**[473] **901**[492] **902**[474] **903**[474] **904**[474]

899: L-Pyroglutamic acid, **900**: Propyl hexadecanoate, **901**: Benzoic acid, **902**: Syringic acid, **903**: 3-Pyridinecarboxylic acid, **904**: Salicylic acid

Figure 16 (*Continued*)



911: Hydroxymethylfurfuraldehyde; **915**, β-*n*-Butyl-D-tagatopyranoside, **916**: 3-Buten-2-one, 4-(4-hydroxy-3,5-dimethoxyphenyl)-



918: 4-(1,2,3-trihydroxypropyl)-2,6-dimethoxyphenyl-1-*O*-β-D-glucopyranoside

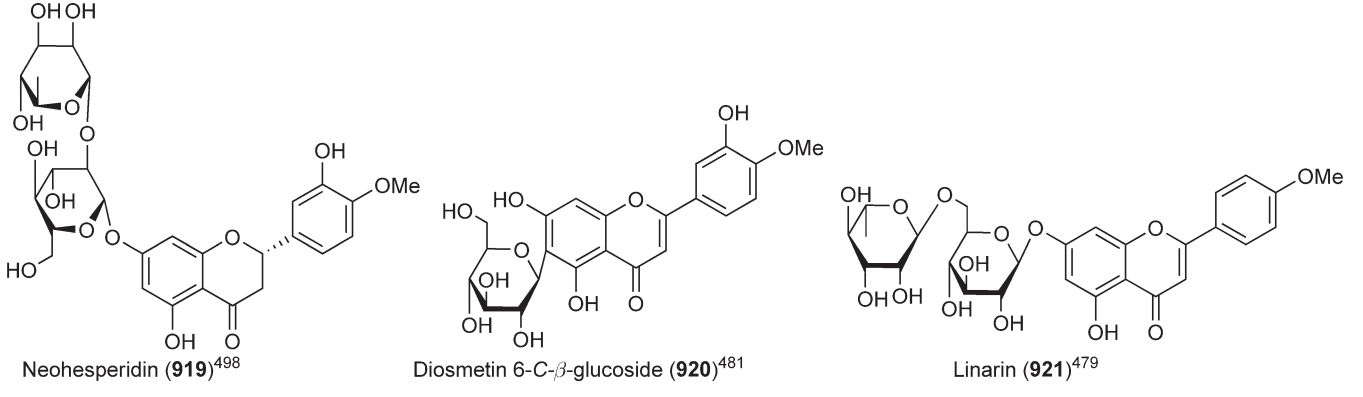
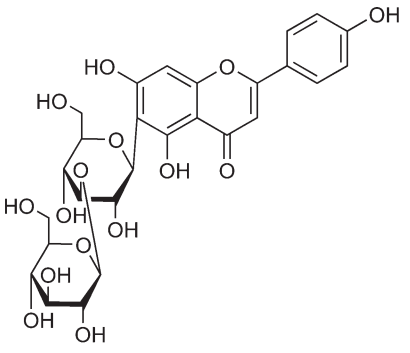
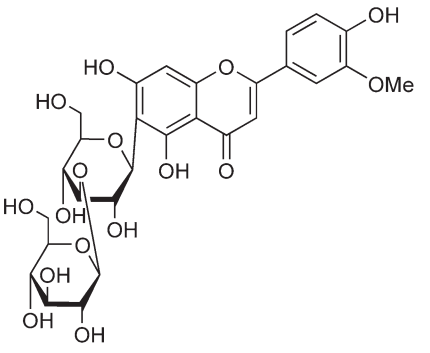


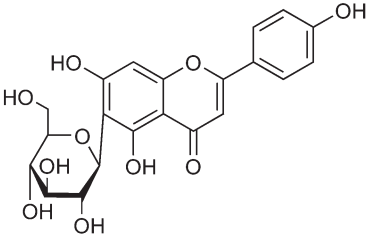
Figure 16 (*Continued*)



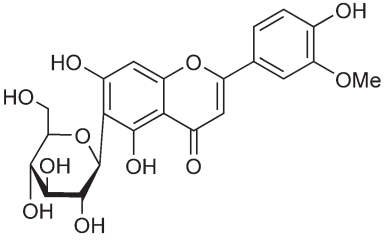
Isovitexin-3″-*O*-glucopyranoside (**922**)[499]



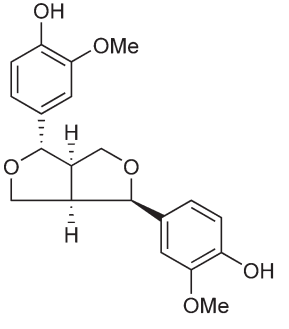
Isoscoparin-3″-*O*-glucopyranoside (**923**)[499]



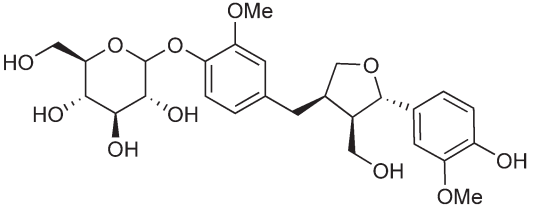
Homovitexin (**924**)[499]



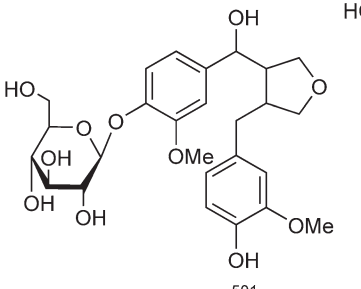
Isoscoparin (**925**)[499]



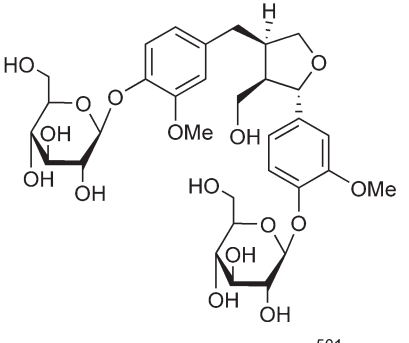
(+)-Epipinoresinol (**926**)[500]



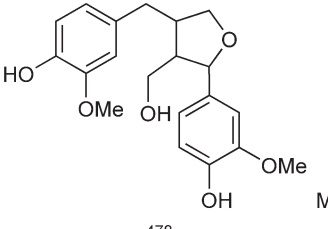
Lariciresinol 4-*O*-β-D-glucopyranoside (**927**)[500]



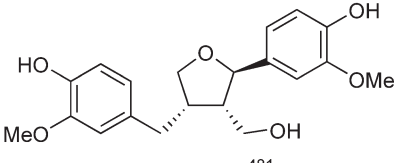
Indigoticoside A (**928**)[501]



Clemastanin B (**929**)[501]



930[478]



(–)-Lariciresinol (**931**)[481]

932: 3-Furanmethanol, tetrahydro-2-(4-hydroxy-3-methoxyphenyl)-4-vanillyl-

Figure 16 (*Continued*)

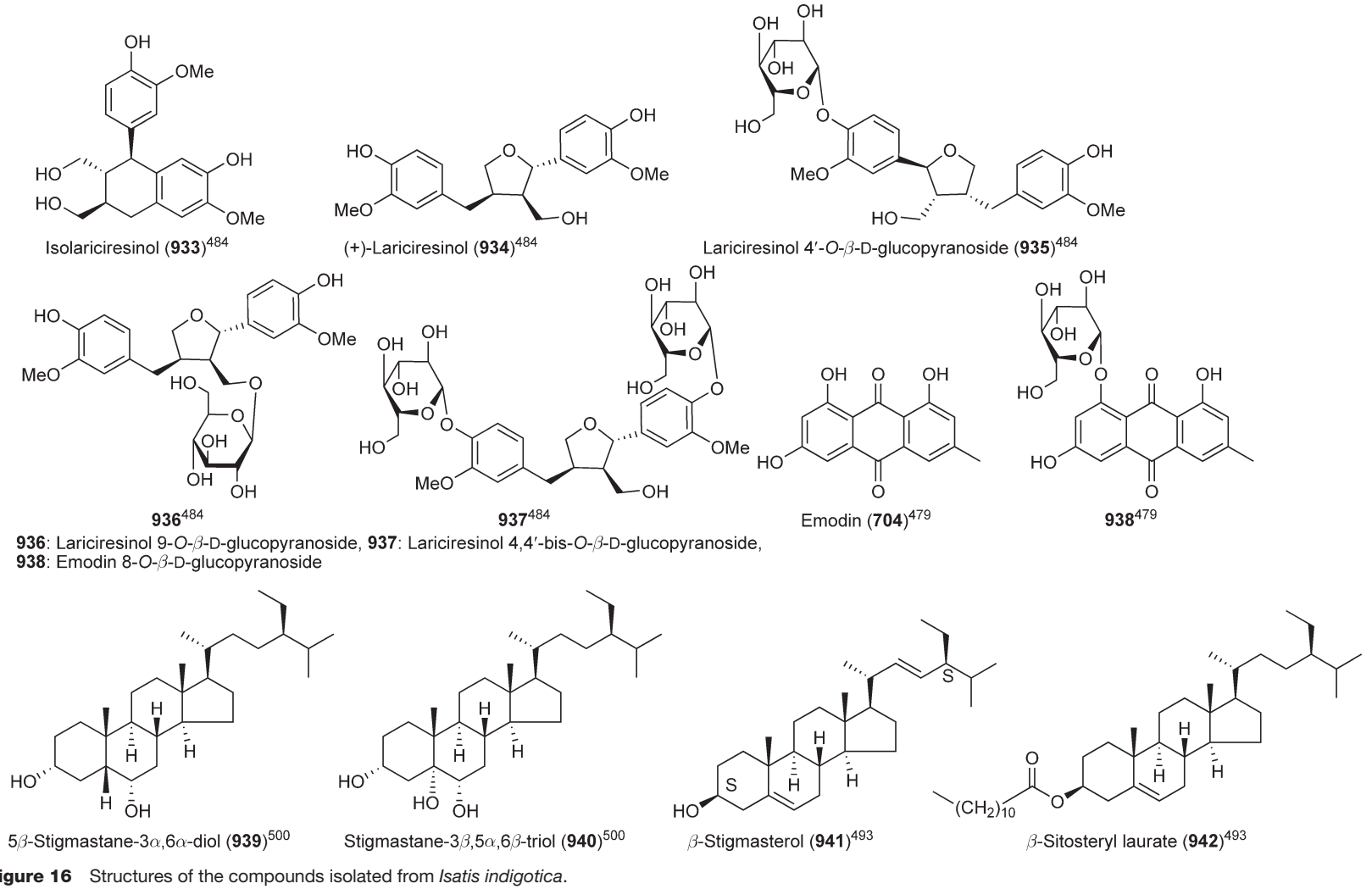


Figure 16 Structures of the compounds isolated from *Isatis indigotica*.

purines, adenosine, organic acids, flavonoids, anthraquinones, and some other compounds. Indirubin (**19**) was identified as an antileukemic drug with no inhibition of the bone marrow.

3.13.9 Radix Astragali (Huangqi)

Huangqi (Radix Astragali), the dried roots of *Astragalus membranaceus* (Fisch.) Bge. or *A. membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao (Fabaceae), is a well-known TCM, and used as adjunctive therapy in the treatment of colds and influenza, chronic diarrhea, edema, abnormal uterine bleeding and diabetes mellitus, and as a cardiotonic agent.[502,503] Both pharmacology and clinical practices indicate that Huangqi exhibits hepatoprotective, immune modulation, antiviral, cardiotonic, and antiaging activities and was also used for adjunct cancer therapy.[503,504] The main constituents of the root of Huangqi include flavonoids, saponins, polysaccharides, amino acids, and other components. In **Figure 17** we summarize the saponins and flavonoids isolated from this plant.

3.13.10 Herba Cistanches (Roucongrong)

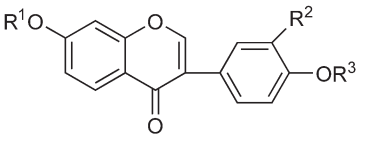
Roucongrong (Herba Cistanche), the dried succulent stems of the *Cistanche* plants (Orobanchaceae) the so-called 'Ginseng of the deserts', has been considered as a superior tonic and used for the treatment of kidney deficiency, impotence, female infertility, morbid leucorrhea, profuse metrorrhagia, and senile constipation.[526] Among *Cistanche* species, only *C. deserticola* Y. C. Ma and *C. tubulosa* (Schrenk) Wight are recorded in the Chinese Pharmacopoeia (2005 edition). However, in recent years, the wild *C. deserticola* and *C. tubulosa* are on the verge of extinction due to overharvesting. Thus, there should be an awareness in protecting *C. deserticola* and its growing environment; therefore, the plant is considered as one of the Class II plants requiring protection in China.

Studies on *Cistanche* species started in the 1980s. A number of compounds were isolated from this genus, including the essential oil, phenylethanoid glycosides, monoterpenes, lignans, and other compounds. The group of Pengfei Tu provided a number of reports[527–534] regarding the chemical constituents and biological activities of Roucongrong. Recently, they reviewed the chemical constituents and the analysis methods of *Cistanche* genus.[535] Here, we just describe the nonvolatile compounds (**Figures 18–20**) isolated from *C. deserticola* and *C. tubulosa*, which are used officially as Roucongrong in TCM. Phenylethanoid glycosides have been reported to be one type of the major active components and demonstrate antioxidation, neuroprotection, enhancing immune and sexual, hepatoprotection, and antiradiation activities.[527,536,537]

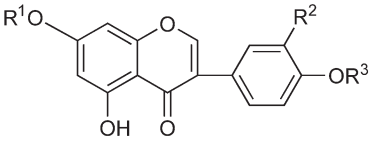
Carbohydrates are also found abundantly in *Cistanche* species, and polysaccharides have been considered as the active principle, which improve body immunity, and possess antiaging and antitumor properties.[538–540] Nevertheless, galactitol, one of the monosaccharides, has been reported to be the main active component with laxative activity.[532,533] Several papers report the isolation and structural elucidation of carbohydrates from *Cistanche* species.[528–530,541,542] The detailed structural information is not listed here.

3.13.11 Gamboge (Tenghuang)

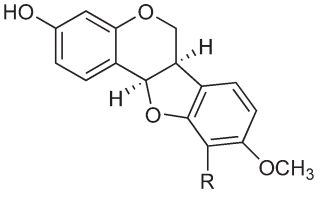
Gamboge (Tenghuang in Chinese), the resin of *Garcinia hanburyi* Hook f. (Clusiaceae), well known as a natural fresh orange-yellow pigment, is used in TCM for removing stasis, detoxification, hemostasis, and as an anthelmintic.[567] Tenghuang was not a popularly used herb in China. However, in recent years, this resin has been a focus of intense research in phytochemical, pharmacological, synthetic, and biological communities owing to its antitumor activities.[569–572] Studies on the bioactive components of the extracts yielded the chemical structures possessing a unique 4-oxa-tricyclo[4.3.1.$0^{3,7}$]dec-2-one scaffold built into a caged xanthone backbone.[573] These compounds exhibit potent antitumor activity and have been referred to 'caged *Garcinia* xanthones'. Over 100 compounds have been reported from *Garcinia* species to date. Here, we describe the compounds (**Figure 21**) isolated from *G. hanburyi*. Gambogic acid (GA), the best representative of this class of



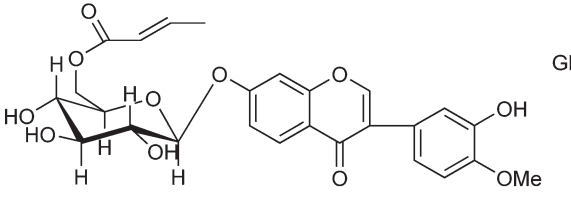
	R^1	R^2	R^3
944[505]: Calycosim	H	OH	Me
476[506]: Ononin	Glc	H	Me
466[506]: Formononetin	H	H	Me
945[507]	Glc	OH	Me
687[508]: Daidzein	H	H	H



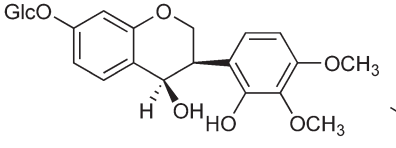
	R^1	R^2	R^3
946[507]	Glc	OH	Me
947[507]	H	OH	Me
688[508]: Genistein	H	H	H
948[508]: Biochanin	H	H	Me



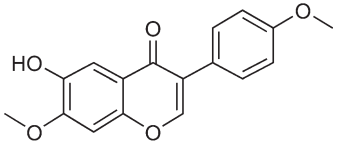
	R
949[506]: Demethylhomopterocarpin	H
950[509]: Astrapterocarpan	Me



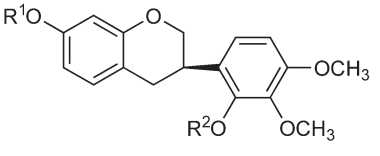
952[510]



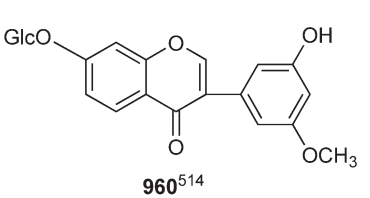
953[511]



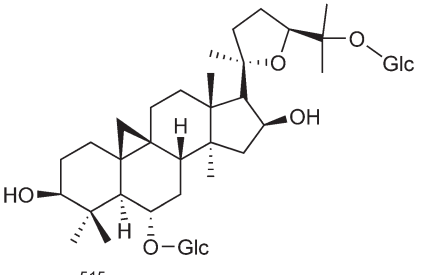
954[512]: Afrormosin



	R^1	R^2
955[513]	Glc	H
956[513]	H	H
957[513]	Ac	Ac
958[513]	H	Ac
959[513]	H	Me

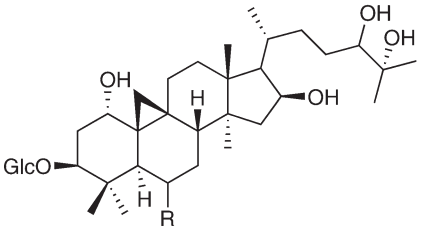


960[514]

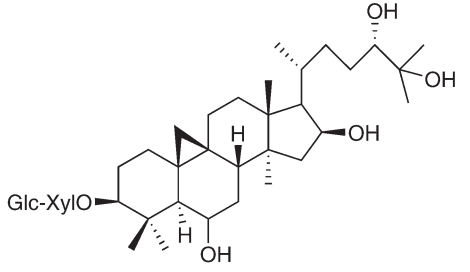


961[515]: Astramembranoside A

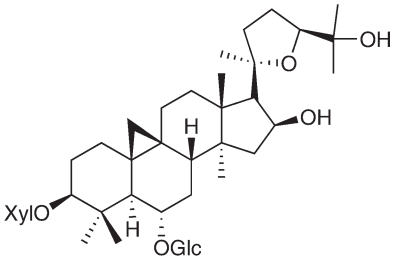
Figure 17 (*Continued*)



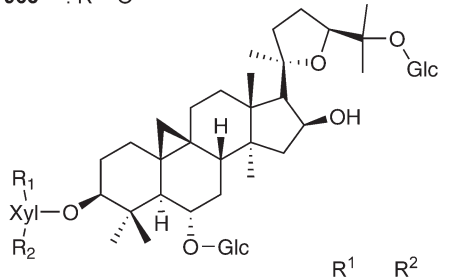
962[511]: R = OH
963[511]: R = O



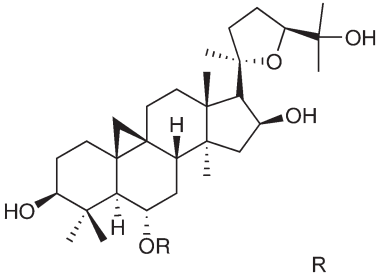
964[515]: Astramembranoside B



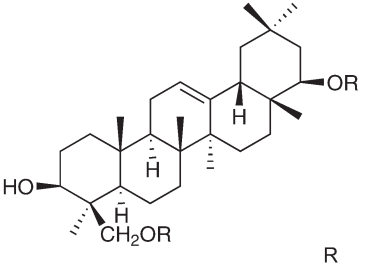
965[516]: Astragaloside IV



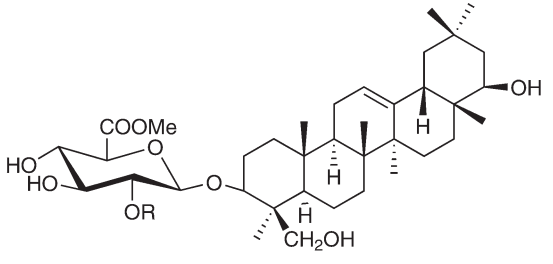
	R^1	R^2
966[517]: Agrostragaloside III	Ac	Ac
967[517]: Agrostragaloside IV	Ac	H
968[517]: Astragaloside VII	H	H



	R
969[518]: Cycloastragenol	H
970[518]:	Glc



	R
971[518]: Soyasapogenol B	H
972[518]:	Me



	R
973[518]	H
974[518]	Xyl

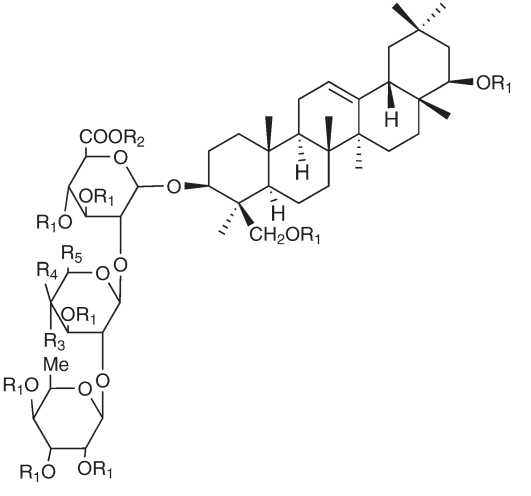
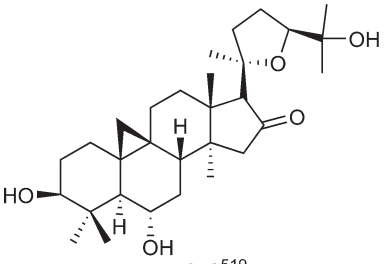
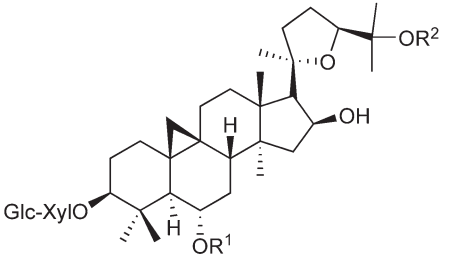


Figure 17 (*Continued*)



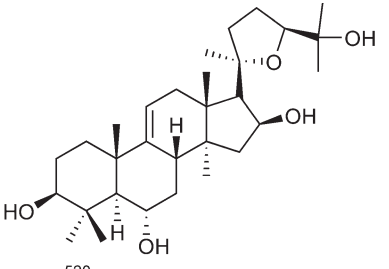
979[519]

	R^1	R^2	R^3	R^4	R^5
975[518]: Astragaloside VIII	H	H	OH	H	H
976[518]	H	Me	OH	H	H
977[518]	Me	Me	OMe	H	H
978[518]: Azukisaponin V	H	H	OH	H	CH_2OH

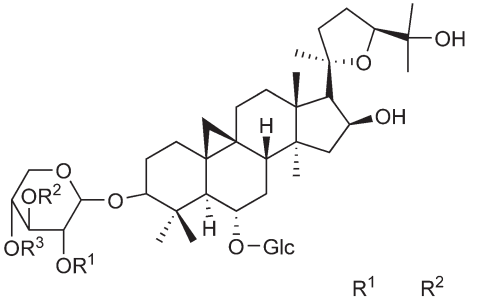


980[519]: Astragaloside V
981[519]: Astragaloside VI

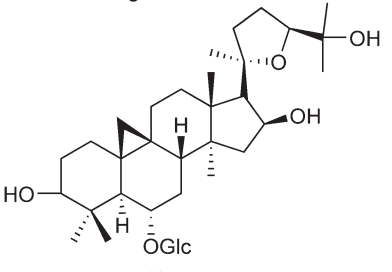
	R^1	R^2
980[519]: Astragaloside V	H	Glc
981[519]: Astragaloside VI	Glc	H



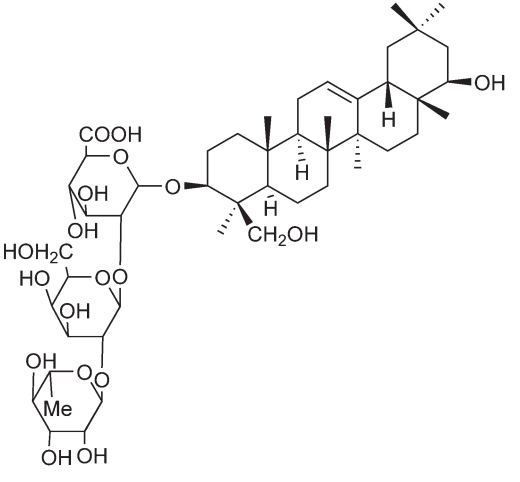
982[520]: Astragenol



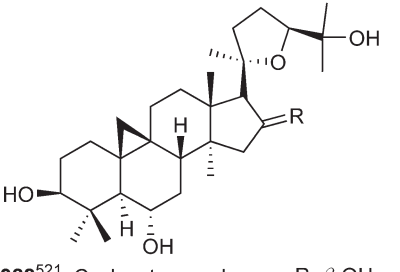
	R^1	R^2	R^3
983[520]: Isoastragaloside I	Ac	H	Ac
984[520]: Astragaloside II	Ac	H	H
985[520]: Isoastragaloside II	H	Ac	H



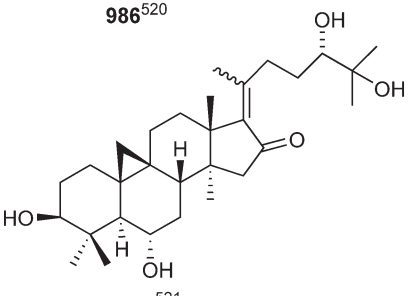
986[520]



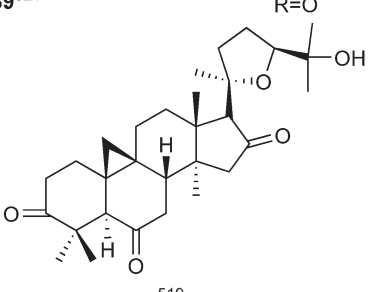
987[520]: Soyasaponin I



988[521]: Cycloastragenol R=β-OH
989[521] R=O

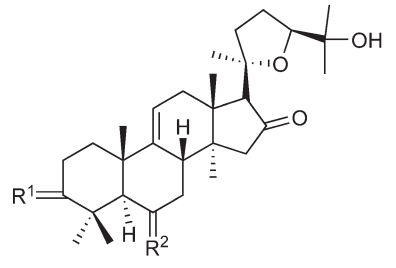


990[521]

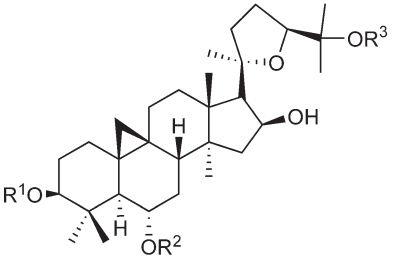


991[519]

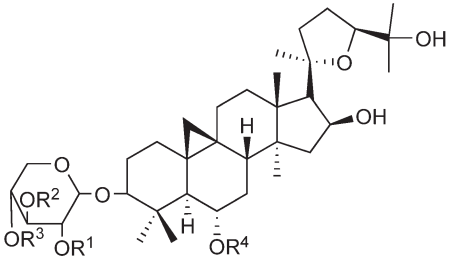
Figure 17 (*Continued*)



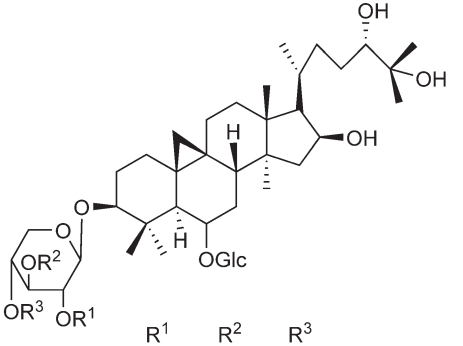
	R^1	R^2
992[521]:	β-OAc	α-OAc
993[521]	O	O



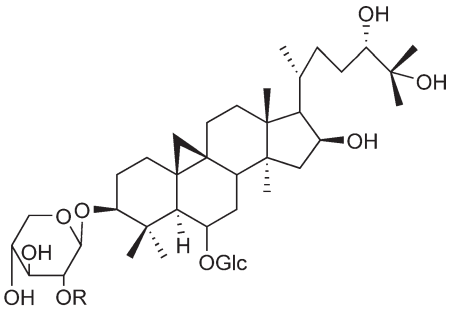
	R^1	R^2	R^3
1000[513]	Xyl	H	Glc
1001[513]	Xyl	H	H
1002[513]	H	H	Glc



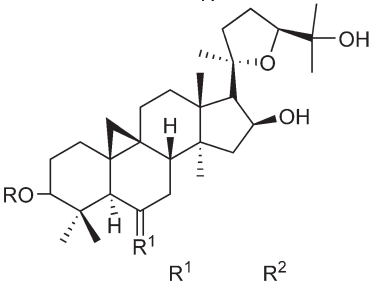
	R^1	R^2	R^3	R^4
994[522]:				
Astragaloside I	Ac	Ac	H	Glc
995[522]:				
Astragaloside III	Glc	H	H	H
996[522]:				
Astragaloside IV	H	H	H	Glc
997[523]	Ac	Ac	H	Xyl



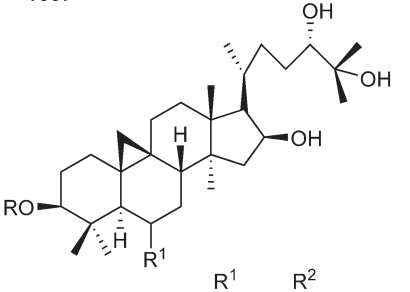
	R^1	R^2	R^3
1003[522]	Ac	Ac	H
1004[522]	H	H	Ac



	R
998[524]	Ac
999[524]	H

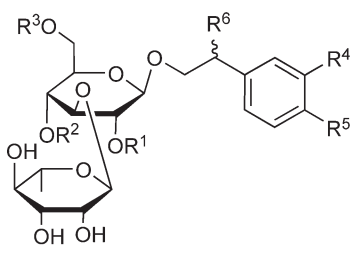


	R^1	R^2
1005[525]	Glc	O
1006[525]	H	O
1007[525]	H	OH

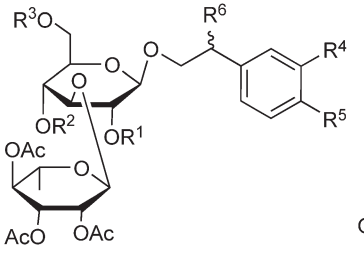


	R^1	R^2
1005[525]	Glc	O
1006[525]	H	O
1007[525]	H	OH

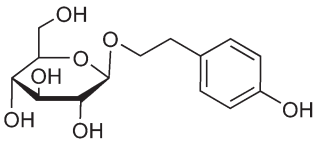
Figure 17 Phenolic compounds and saponins isolated from *Astragalus membranaceus*.



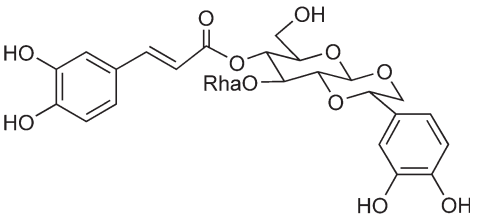
I



II



Salidroside (**15**)



Crenatoside (**34**)

Type	*Compound name*	*R*^{1}	*R*^{2}	*R*^{3}	*R*^{4}	*R*^{5}	*R*^{6}	*Reference(s)*
I **1007**	2'-Acetylacteoside	Ac	Cf	H	OH	OH	H	531, 543–545
1008	Acteoside	H	Cf	H	OH	OH	H	531, 544–547
1009	Cistanoside A	H	Cf	Glc	OMe	OH	H	544, 546, 548
1010	Cistanoside B	H	Fr	Glc	OMe	OH	H	546, 549
1011	Cistanoside C	H	Cf	H	OMe	OH	H	543, 549
1012	Cistanoside D	H	Fr	H	OMe	OH	H	543
1013	Cistanoside E	H	H	H	OMe	OH	H	550
1014	Cistanoside G	H	H	H	H	OH	H	551
1015	Cistanoside H	Ac	H	H	OH	OH	H	549, 552
1016	Decaffeoylacteoside	H	H	H	OH	OH	H	531, 552
1017	Echinacoside	H	Cf	Glc	OH	OH	H	531, 544–546, 548
1018	Isoacteoside	H	H	Cf	OH	OH	H	544, 531, 553
1019	Isosyringalide 3'-α-L-rhamnopyranoside	H	Cm	H	OH	OH	H	554
1020	Osmanthuside B	H	Cm	H	H	OH	H	543

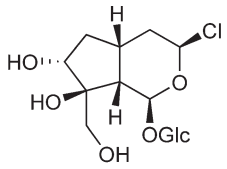
(continued)

Figure 18 (*Continued*)

Type		Compound name	R^1	R^2	R^3	R^4	R^5	R^6	Reference(s)
	1021	Syringalide A-3'-α-L-rhamnopyranoside	H	Cf	H	H	OH	H	531, 553, 554
	1022	Tubuloside A	Ac	Cf	Glc	OH	OH	H	544, 531, 553
	1023	Tubuloside B	Ac	H	Cf	OH	OH	H	544, 545
	1024	Cistantubuloside A	H	Cf	Glc	H	OH	H	555
	1025	Cistantubuloside B_1/B_2	H	Cm/c-Cm	Glc	OH	OH	H	555
	1026	Kankanoside F	H	H	Glc	OH	OH	H	556
	1027	Kankanoside G	H	H	Cf	H	OH	H	556
	1028	Cistantubuloside C_1/C_2	H	Cf	Glc	OH	OH	OH (S/R)	555
II	**1029**	Tubuloside C	Ac	Cf	Glc	OH	OH	H	544
	1030	Tubuloside D	Ac	Cm	Glc	OH	OH	H	544
	1031	Tubuloside E	Ac	Cm	H	OH	OH	H	554
	1032	Salidroside							531, 551, 555
	1033	Crenatoside							547

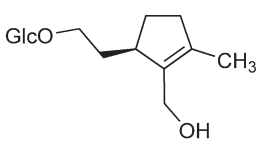
Cf, trans-caffeoyl; Fr, ferulic acid; Cm, *trans*-coumaroyl; c-Cm, *cis*-coumaroyl; Glc, β-Glucopyranose; Rha, α-L-rhamnopyranose; Ac, acetyl.

Figure 18 Phenylethanoid glycosides isolated from Herba Cistanches.



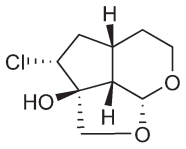
1034[559]

1034: Kankanoside C



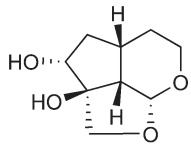
1035[559]

1035: Kankanoside D



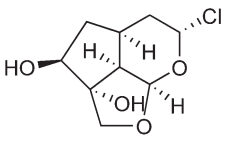
1036[560]

1036: Cistachlorin



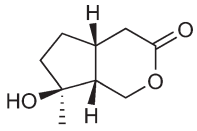
1037[557,560]

1037: Cistanin



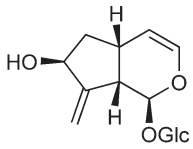
1038[559]

1038: Kankanol



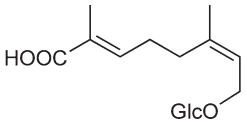
1039[559]

1039: Argyol

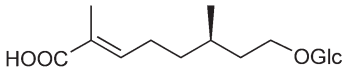


1040[559]

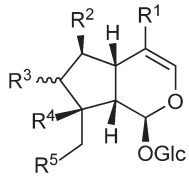
1040: Antirrhide



(2E,6Z)-8-β-D-Glucopyranosyloxy-2,6-dimethyl-2,6-octadienoic acid (**1041**)[559]



Kankanoside E (**1042**)[559]



No.	*Compound name*	*R*[1]	*R*[2]	*R*[3]	*R*[4]	*R*[5]	*Reference(s)*
1043	Ajugol	H	OH	H	OH	H	516
1044	Bartsioside	H	H	═		OH	516
1045	6-Deoxycatalpol	H	H	—O—		OH	554, 561, 567, 568
1046	Gluroside	H	H	H	OH	H	516
1047	Leonuride	H	OH	H	OH	H	516
1048	Kankanoside B	H	H	e-OH[a]	OH	OH	559
1049	Kankanoside A	CH_3	H	H	OH	H	559
1050	8-Epideoxyloganic acid	COOH	H	H	H	H	516
1051	8-Epiloganic acid	COOH	H	a-OH[b]	H	H	534, 547, 548, 557, 558, 562, 563
1052	Geniposidic acid	COOH	H	═		OH	534, 561
1053	Mussaenosidic acid	COOH	H	H	OH	H	534, 561
1054	Adoxosidic acid	COOH	H	H	H	OH	534
1055	Kankanoside A	CH_3	H	H	OH	H	559

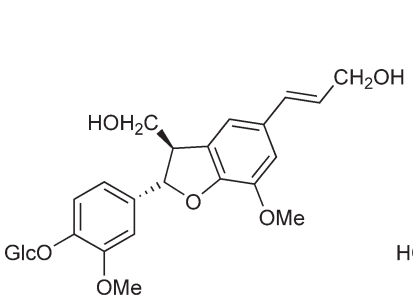
[a] 'e', equatorial bond.
[b] 'a', axial bond.

Figure 19 Monoterpene constituents isolated from Herba Cistanches.

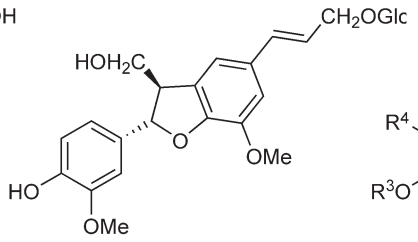
constituents, has the most potential as a broad-spectrum anticancer drug candidate. In China, it is now in phase II clinical trial as a new anticancer drug candidate.[574]

3.13.12 Conclusion

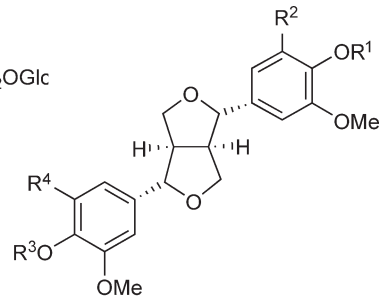
TCM has been used for the treatment of diseases in China for thousands of years. Its physical foundation, mode of action, and prescription compatibility of TCM pharmacodynamic action were not determined in complete detail. Applying modern disciplines while studying TCM has resulted in large numbers of active compounds, some of which were developed to produce new drugs for the treatment of some important diseases such as



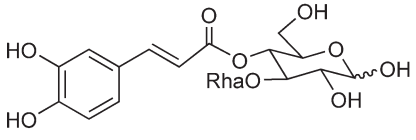
1056



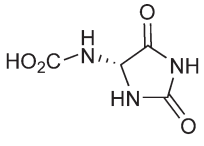
1057



No.	*Compound name*	R^1	R^2	R^3	R^4	*Reference(s)*
1058	Dehydrodiconifery alcohol 4-*O*-β-D-glucoside					554
1059	Dehydrodiconifery alcohol γ'-*O*-β-D-glucoside					554
1060	Liriodendrin	Glc	OMe	Glc	OMe	549, 550, 554, 564, 565
1061	(+)-Pinoresinol	H	H	H	H	551
1062	(+)-Pinoresinol-*O*-β-D-glucopyranoside	H	H	Glc	H	554
1063	(+)-Syringaresinol-*O*-β-D-glucopyranoside	H	OMe	Glc	OMe	550, 554, 561



Cistanoside F (**1064**)[566]



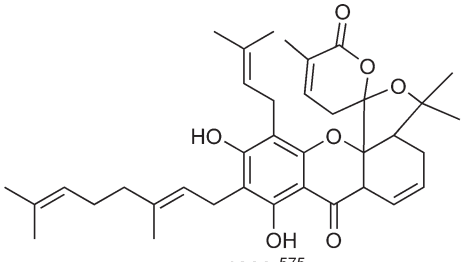
(2,5-Dioxo-4-imidazolidiny)-carbamic acid (**1065**)[567]

Figure 20 Lignans and other compounds isolated from Herba Cistanches.

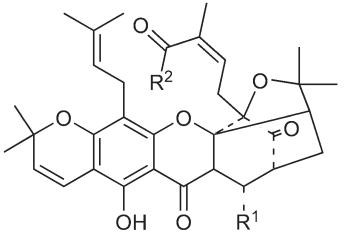
malaria, vascular diseases, and cancer. The results also include the identification and characterization of the active compounds from TCM, which is important for the improvement of TCM. However, till date, a majority of TCM has not been studied chemically and pharmacologically in detail and most of the TCM-derived compounds were found not to be as active as the original TCM preparation. This points to the possibilities of prodrugs and synergism being involved. Therefore, it is a huge and challenging task to develop evidence-based Chinese medicines and possible new leads from TCM. Combined chemical and biological studies on the effective TCM provide an opportunity for the development of new drugs and the modernization of TCM.

Here we mention some examples on the chemical studies of 10 popular Chinese medicines mainly involved in the treatment of cardiovascular and cerebrovascular diseases, cancers, gynecological diseases, and immunological diseases. The results of combined studies of chemistry and biology provided clear evidence for the efficacy of TCM. Some information may lead to new application of the medicines.

Although TCM exceedingly depends on experience of physicians, its theories and principles seem to be similar to personalized medicine and cocktail therapy of the western practice. The pattern of western medical practice has changed from disease treatment alone to the combination of prevention, health care, treatment, and recovery. On this point, TCM has its particular advantage, as it has a unique system with special etiology and theories for treatment. For the future development of Chinese medicine, efficacy and safety are the two critical elements. Accordingly, the combination of TCM with modern technology, improved academic thoughts, and up-to-date scientific knowledge is essential for the modernization of TCM. This modernization should not abandon the direction of traditional theory and experience. It is necessary to make a standard, quantitative, and correct description for the concept and basic theory of TCM using modern experimental methods. On the other hand, active extracts of TCM and their combination may be used to produce innovative Chinese medicine, and they are listed as biomedicine in China.



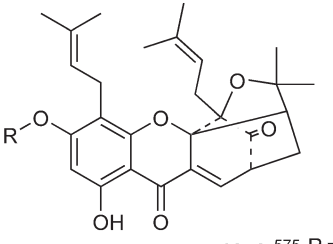
Gambospiroene (**1066**)[575]



Methyl 8,8a-dihydromorellate (**1067**)[575] $R^1 = H$ $R^2 = OMe$

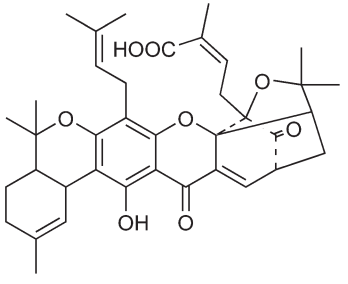
Moreollic acid (**1068**)[576] $R^1 = OMe$ $R^2 = OH$

8,8*a*-Dihydro-8-hydroxymorellic acid (**1069**)[575] $R^1 = OH$ $R^2 = OH$

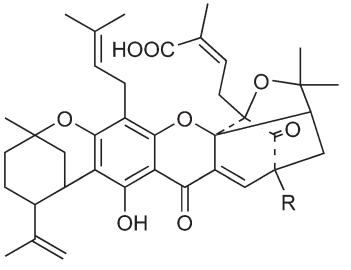


3-*O*-Geranylforbesione (**1070**)[575] R = Geranyl

Forbesione (**1071**)[577] R = H

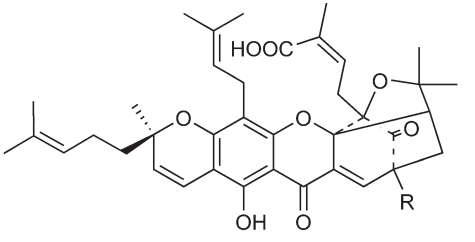


Gambogefic acid (**1072**)[575]



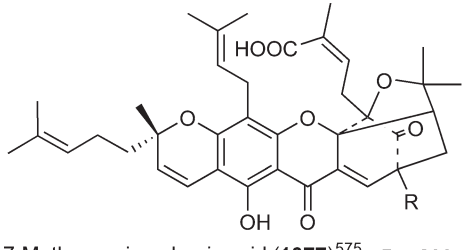
7-Methoxygambogellic acid (**1073**)[575] R = OMe

Gambogellic acid (**1074**)[576] R = H



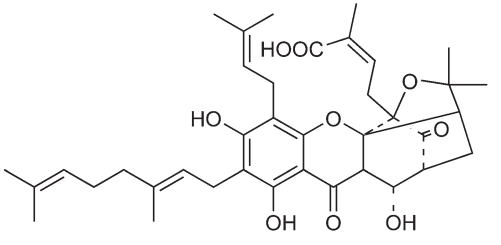
7-Methoxygambogic acid (**1075**)[575] R = OMe

R-Gambogic acid (**1076**)[578] R = H

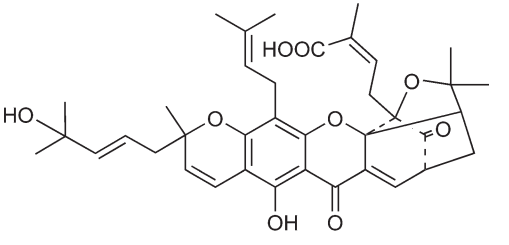


7-Methoxyepigambogic acid (**1077**)[575] R = OMe

S-Gambogic acid (**1078**)[578] R = H

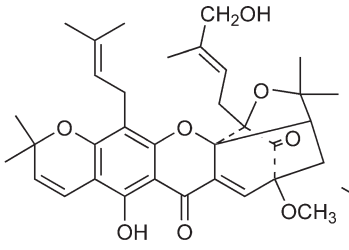


8,8a-Dihydro-8-hydroxygambogenic acid (**1079**)[575]

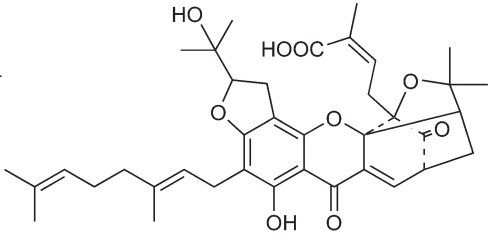


Oxygambogic acid (**1080**)[575]

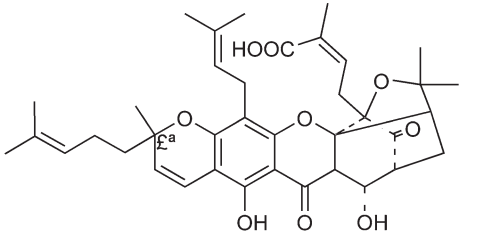
Figure 21 (*Continued*)



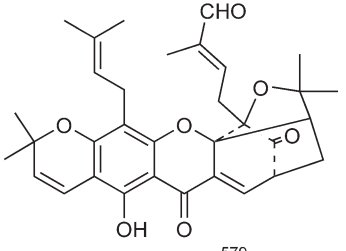
7-Methoxyisomorellinol (**1081**)[575]



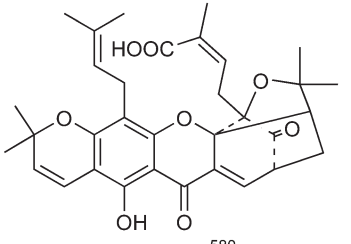
Gambogenific acid (**1082**)[575]



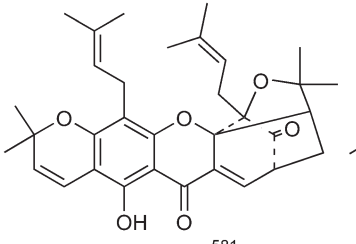
1083 (**1084**) 8,8a-Dihydro-8-hydroxygambogic acid and its isomer[575]



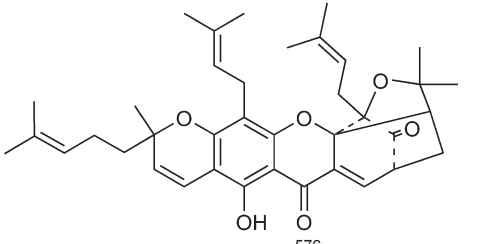
Isomorellin (**1085**)[579]



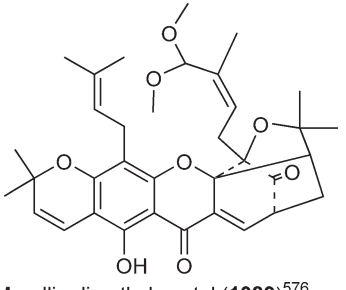
Morellic acid (**1086**)[580]



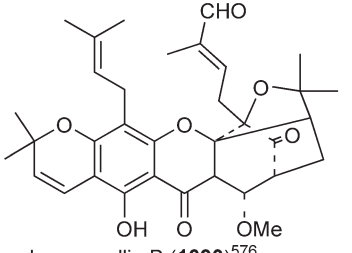
Desoxymorellin (**1087**)[581]



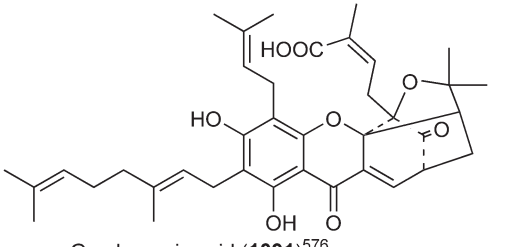
Gambogin (**1088**)[576]



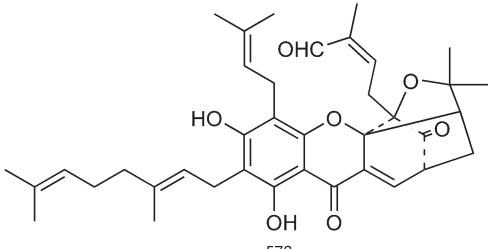
Morellin dimethyl acetal (**1089**)[576]



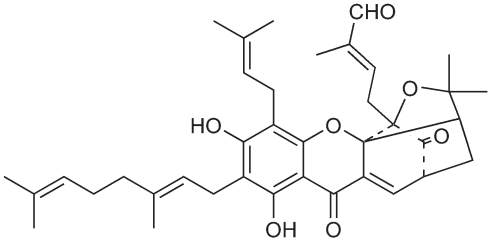
Isomoreollin B (**1090**)[576]



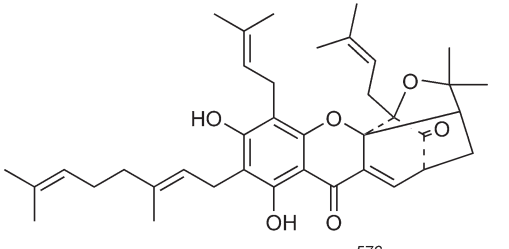
Gambogenic acid (**1091**)[576]



Gambogenin (**1092**)[576]



Isogambogenin (**1093**)[576]



Desoxygambogenin (**1094**)[576]

Figure 21 (*Continued*)

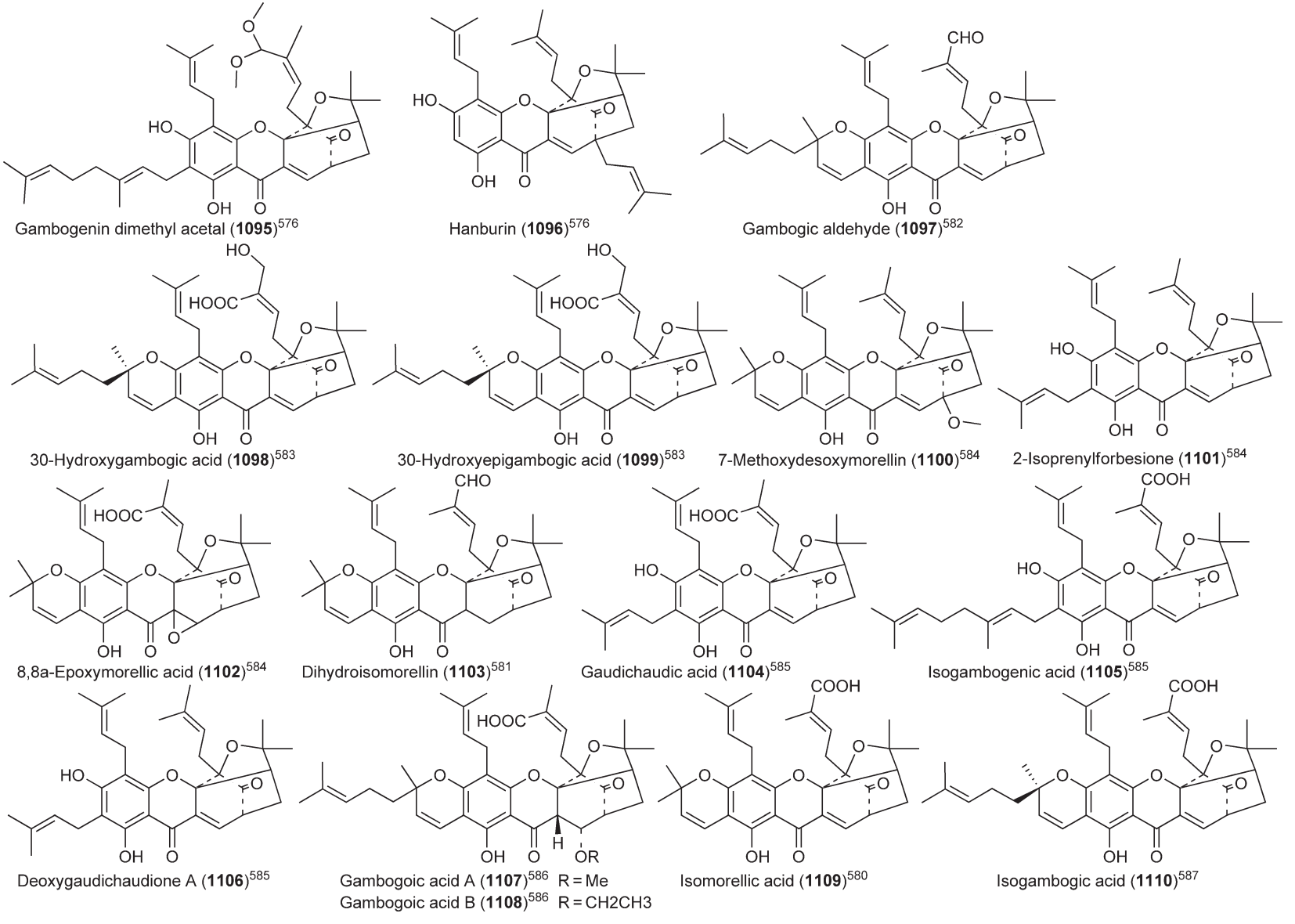


Figure 21 (*Continued*)

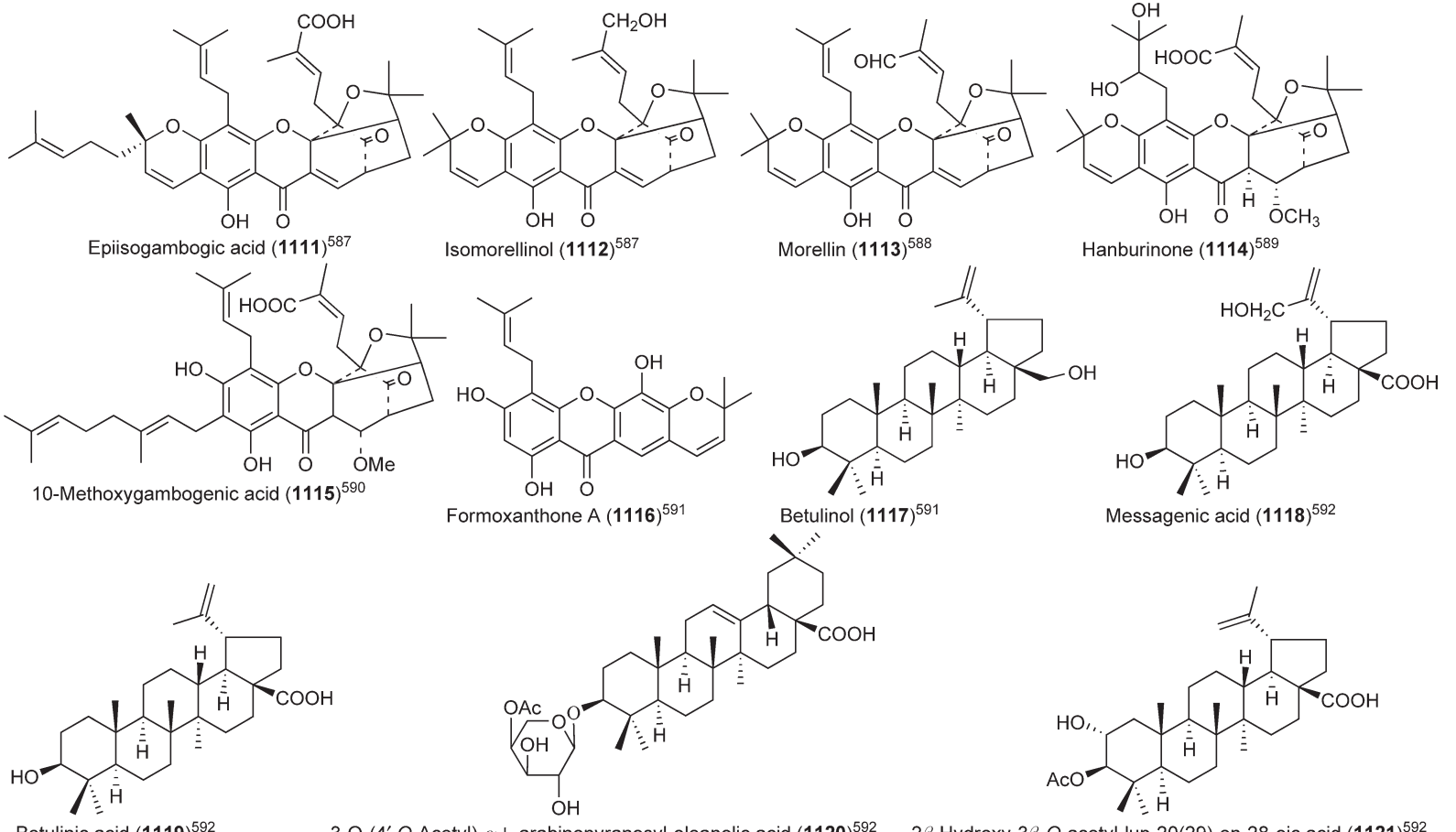


Figure 21 Structures of compounds isolated from *Garcinia hanburyi*.

In summary, TCM has made big contributions for a variety of clinical treatments of different kinds of diseases and symptoms in China for thousands of years. Numerous bioactive constituents have been obtained from TCM and the traditional uses of some Chinese medicines have been validated by chemical and biological studies. Further research may broaden the clinical use of TCM. Some natural bioactive constituents and their synthetic analogues indicate that TCM holds much promise for the discovery of new drug candidates. Therefore, TCM has held, and will hold, an important position in primary health care in China, and even throughout the world.

Glossary

yin–yang In Chinese philosophy and religion, two principles, one negative, dark, and feminine (*yin*) and one positive, bright, and masculine (*yang*), from whose interaction all things are produced and all things are dissolved.
jingluo Meridians, classical loci in acupuncture. They are main and collateral channels, regarded as a network of passages, through which vital energy circulates and along which acupoints (ACUPUNCTURE POINTS) are distributed.

Abbreviation

TCM Traditional Chinese Medicine

References

1. Chinese Pharmacopeia Commission, , *Pharmacopeia of People's Republic of China*.; Chemical Industry Press: Beijing, 2005; Vol. 1.
2. J. Xu; Y. Yang, *Health Policy* **2009**, *90* (2–3), 133–139.
3. T. Efferth; P. C. H. Li; V. S. B. Konkimalla; B. Kaina, *Trends Mol. Med.* **2007**, *13* (8), 353–361.
4. K. Y. Tan; C. B. Liu; A. H. Chen; Y. J. Ding; H. Y. Jin; F. Seow-Choen, *Tech. Coloproctol.* **2008**, *12*, 1–6.
5. F. Guo; C. J. Xu, *Zhongguo Zhongxiyi Jiehe Za Zhi (Chin. J. Integr. Tradit. West. Med.)* **(2005)**, *25* (12), 1140–1144.
6. J. H. Cheng; W. S. Liu; Z. M. Li; Z. G. Wang, *Chin. J. Integr. Med.* **2007**, *13* (4), 269–274.
7. Y. Xu; Y. F. Yang, *Zhongguo Zhongxiyi Jiehe Za Zhi (Chin. J. Integr. Tradit. West. Med.)* **2008**, *28* (2), 182–186.
8. X. M. Li; L. Brown, *J. Allergy Clin. Immunol.* **2009**, *123* (2), 297–306.
9. G. Li, *J. Tradit. Chin. Med.* **1995**, *15* (3), 163–169.
10. J. F. Chi; J. Z. Niu; S. Q. Xu; J. Li; J. F. Wang; J. P. Liu, *Zhongxiyi Jiehe Xue Bao (J. Chin. Integr. Med.)* **2007**, *5* (3), 247–254.
11. G. Sun; J. Ren; Q. Sun, *J. Tradit. Chin. Med.* **1999**, *19* (4), 304–312.
12. L. Yan; B. Liu; W. Guo; G. Li; Y. Li; H. Gao; H. Cui; L. Sun; M. Wang, *J. Tradit. Chin. Med.* **2000**, *20* (2), 83–86.
13. J. B. Jordan; X. Tu, *Am. J. Chin. Med.* **2008**, *36* (3), 437–447.
14. J. Shi; Y. L. Liu; Y. X. Fang; G. Z. Xu; H. F. Zhai; L. Lu, *Zhongguo Yaoli Xue Bao (Acta Pharmacol. Sin.)* **2006**, *27* (10), 1303–1308.
15. J. Yin; H. Zhang; J. Ye, *Endocr. Metab. Immune Disord. Drug. Targets* **2008**, *8* (2), 99–111.
16. D. Y. Zhu, *Prog. Chem.* **2009**, *21* (1), 24–29.
17. D. L. Klayman, *Science* **1985**, *228* (4703), 1049–1055.
18. T. Kuhn; Y. Wang, *Prog. Drug Res.* **2008**, *66*, 383, 385–422.
19. T. Efferth; H. Dunstan; A. Sauerbrey; H. Miyachi; C. R. Chitambar, *Int. J. Oncol.* **2001**, *18*, 767–773.
20. T. G. Berger; D. Dieckmann; T. Efferth; E. S. Schultz; J. O. Funk; A. Baur; G. Schuler, *Oncol. Rep.* **2005**, *14*, 1599–1603.
21. J. Mervis, *Science* **1996**, *88* (2), 578–588.
22. K. H. Antman, *Oncologist* **2001**, *6* (2), 1–2.
23. Y. Pommier, *Nat. Rev. Cancer* **2006**, *6*, 789–802.
24. S. Kusari; S. Zühlke; M. Spiteller, *J. Nat. Prod.* **2009**, *72* (1), 2–7.
25. R. W. Jiang; J. R. Zhou; P. M. Hon; S. L. Li; Y. Zhou; L. L. Li; W. C. Ye; H. X. Xu; P. C. Shaw; P. P. H. But, *J. Nat. Prod.* **2007**, *70* (2), 283–286.
26. J. W. de Kraker; M. C. R. Franssen; A. de Groot; T. Shibata; H. J. Bouwmeester, *Phytochemistry* **2001**, *58* (3), 481–487.
27. F. Q. Yang; S. P. Li; Y. Chen; S. C. Lao; Y. T. Wang; T. T. X. Dong; K. W. K. Tsim, *J. Pharm. Biomed. Anal.* **2005**, *39* (3–4), 552–558.
28. T. Efferth; R. Rauh; S. Kahl; M. Tomicic; H. Böchzelt; M. E. Tome; M. M. Briehl; R. Bauer; B. Kaina, *Biochem. Pharmacol.* **2005**, *69* (5), 811–818.
29. Y. Lu; C. Sun; Y. Pan, *J. Sep. Sci.* **2006**, *29* (2), 314–318.

30. J. T. Chen; H. Z. Li; D. Wang; Y. J. Zhang; C. R. Yang, *Helv. Chim. Acta* **2006**, *89*, 1442–1448.
31. S. Y. Pan; R. Yang; H. Dong; Z. L. Yu; K. M. Ko, *Eur. J. Pharmacol.* **2006**, *552* (1–3), 170–175.
32. G. T. Liu, *Med. Chem.* **2009**, *5* (1), 29–43.
33. S. X. Cheng; J. B. Chang; L. B. Qu; R. F. Chen, *Bioorg. Med. Chem. Lett.* **2004**, *14* (7), 1665–1667.
34. R. Wang; H. Yan; X. C. Tang, *Acta Pharmacol. Sin.* **2006**, *27* (1), 1–26.
35. H. Y. Zhang; C. Y. Zheng; H. Yan; Z. F. Wang; L. L. Tang; X. Gao; X. C. Tang, *Chem. Biol. Interact.* **2008**, *175* (1–3), 396–402.
36. A. R. Desilets; J. J. Gickas; K. C. Dunican, *Ann. Pharmacother.* **2009**, *43* (3), 514–518.
37. Q. B. Li; R. Pan; G. F. Wang; S. X. Tang, *J. Nat. Toxins* **1999**, *8* (3), 327–330.
38. J. M. Poupko; S. I. Baskin; E. Moore, *J. Appl. Toxicol.* **2007**, *27* (2), 116–121.
39. X. Z. Zhu, *Mem. Inst. Oswaldo Cruz.* **1991**, *86* (2), 173–175.
40. Z. Xiang; J. E. Zhou; Z. N. Chen; L. P. Wang; H. N. Wang; T. R. Yao; J. X. Xie; G. Y. Xu; D. N. Yi, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **1989**, *24* (2), 105–109.
41. Y. Y. Niu; L. M. Yang; H. Z. Liu; Y. Y. Cui; L. Zhu; J. M. Feng; J. H. Yao; H. Z. Chen; B. T. Fan; Z. N. Chen; Y. Lu, *Bioorg. Med. Chem. Lett.* **2005**, *15* (21), 4814–4818.
42. X. C. Cheng; X. Y. Liu; W. F. Xu; X. L. Guo; Y. Ou, *Bioorg. Med. Chem.* **2007**, *15* (10), 3315–3320.
43. J. W. Ho; M. Jie, *Cardiovasc. Hematol. Agents Med. Chem.* **2007**, *5* (4), 273–277.
44. X. H. Meng; C. Ni; L. Zhu; Y. L. Shen; L. L. Wang; Y. Y. Chen, *Vascul. Pharmacol.* **2009**, *50* (3–4), 110–115.
45. R. Hoessel; S. Leclerc; J. A. Endicott; M. E. Nobel; A. Lawrie; P. Tunnah; M. Leost; E. Damiens; D. Marie; D. Marko; E. Niederberger; W. Tang; G. Eisenbrand; L. Meijer, *Nat. Cell. Biol.* **1999**, *1* (1), 60–67.
46. T. Maugard; E. Enaud; P. Choisy; M. D. Legoy, *Phytochemistry* **2001**, *58* (6), 897–904.
47. J. T. Zhang, *Therapie* **2002**, *57* (2), 137–150.
48. T. B. Ng, *J. Pharm. Pharmacol.* **2006**, *58* (8), 1007–1019.
49. B. Y. Gao; X. J. Li; L. Liu; B. H. Zhang, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **1992**, *27* (9), 641–644.
50. J. X. Wu; J. J. Sun, *Zhongguo Yaoli Xue Bao (Acta Pharmacol. Sin.)* **1992**, *13* (6), 520–523.
51. X. Li; J. X. Chen; J. J. Sun, *Zhongguo Yao Li Xue Bao (Acta Pharmacol. Sin.)* **1990**, *11* (1), 26–29.
52. L. Shi; P. S. Fan; L. Wu; J. X. Fang; Z. X. Han, *Zhongguo Yaoli Xue Bao (Acta Pharmacol. Sin.)* **1990**, *11* (1), 29–32.
53. H. Gao; F. Wang; E. J. Lien; M. D. Trousdale, *Pharmacol. Res.* **1996**, *13*, 1196–1200.
54. S. K. Lam; T. B. Ng, *Int. J. Biochem. Cell Biol.* **2001**, *33*, 287–292.
55. S. K. Lam; T. B. Ng, *Planta Med.* **2002**, *68*, 1024–1028.
56. S. K. Lam; T. B. Ng, *Biochem. Biophys. Res. Commun.* **2001**, *285*, 419–423.
57. S. K. Lam; T. B. Ng, *Life Sci.* **2002**, *70*, 3049–3058.
58. T. B. Ng; F. Liu; H. X. Wang, *J. Ethnopharmacol.* **2004**, *93*, 285–288.
59. P. Chan; G. N. Thomas; B. Tomlinson, *Zhongguo Yaoli Xue Bao (Acta Pharmacol. Sin.)* **2002**, *23* (12), 1157–1162.
60. W. G. Ma; M. Mizutani; K. E. Malterud; S. L. Lu; B. Ducrey; S. Tahara, *Phytochemistry* **1999**, *52*, 1133–1139.
61. H. X. Sun; F. Qin; Y. P. Ye, *Vaccine* **2005**, *23*, 5533–5542.
62. J. P. Song; J. Zeng; X. M. Cui; Y. Dai; Z. Y. Jing; X. M. Zhang; J. M. Zhou; Y. B. Ma; J. J. Chen, *J. Yunnan Univ.* **2007**, *29* (3), 287–290.
63. S. Yahara; O. Tanaka, *Chem. Pharm. Bull.* **1976**, *24* (9), 2204–2208.
64. M. Yoshikawa; T. Murakami; T. Ueno; K. Yashiro; N. Hirokawa; N. Murakami; J. Yamahara; H. Matsuda; R. Saijoh; O. Tanaka, *Chem. Pharm. Bull.* **1997**, *45* (6), 1039–1045.
65. J. B. Wan; F. Q. Yang; S. P. Li; Y. T. Wang; X. M. Cui, *J. Pharm. Biomed. Anal.* **2006**, *41* (5), 1596–1601.
66. D. S. Kim; Y. J. Chang; U. Zedk; P. Zhao; Y. Q. Liu; C. R. Yang, *Phytochemistry* **1995**, *40* (5), 1493–1497.
67. B. H. Jiang; C. Z. Wang; Y. Han; X. M. Hu; L. X. Zheng; Y. Q. Zhao, *Zhongyaocai (J. Chin. Med. Mater.)* **2004**, *27* (7), 489–491.
68. H. Koizumi; S. Sanada; Y. Ida; J. Shoji, *Chem. Pharm. Bull.* **1982**, *30* (7), 2393–2398.
69. H. Hasegawa; J. H. Sung; S. Matsumiya; M. Uchiyama, *Planta Med.* **1996**, *62* (5), 453–457.
70. Z. M. Qian; J. B. Wan; Q. W. Zhang; S. P. Li, *J. Pharm. Biomed. Anal.* **2008**, *48* (5), 1361–1367.
71. F. Y. Gan; G. Z. Zheng, *Chin. Pharm. J.* **1992**, *27* (3), 138.
72. L. B. Zhang; J. R. Wei; M. X. Wu, *Chin. J. Pharm. Anal.* **2001**, *21* (5), 310–312.
73. M. Yoshikawa; T. Morikawa; K. Yashiro; T. Murakami; H. Matsuda, *Chem. Pharm. Bull.* **2001**, *49* (11), 1452–1456.
74. M. Yoshikawa; T. Morikawa; Y. Kashima; K. Ninomiya; H. Matsuda, *J. Nat. Prod.* **2003**, *66* (7), 922–927.
75. J. X. Wei; S. M. Cao, *Zhongguo Zhong Yao Za Zhi (China J. Chin. Mater. Med.)* **1992**, *17* (2), 96–98.
76. H. Z. Li; Y. Y. Zhang; C. R. Yang, *Tianran Chanwu Yanjiu Yu Kaifa (Nat. Prod. Res. Dev.)* **2006**, *18* (4), 549–554.
77. J. X. Wei; Y. G. Chen; S. M. Cao, *Zhongguo Zhong Yao Za Zhi (China J. Chin. Mater. Med.)* **1992**, *17* (10), 611–613.
78. G. C. Kite; M. J. R. Howes; C. J. Leon; M. S. J. Simmonds, *Rapid Commun. Mass Spectrom.* **2003**, *17*, 238–244.
79. E. C. Y. Chan; S. L. Yap; A. J. Lau; P. C. Leow; D. F. Toh; H. L. Koh, *Rapid Commun. Mass Spectrom.* **2007**, *21*, 519–528.
80. J. C. Bao; G. Liu; D. L. Cong; C. X. Zhang, *Zhong Cheng Yao (Chin. Trad. Pat. Med.)* **2006**, *28* (2), 246–253.
81. P. Zhao; Y. Q. Liu; C. R. Yang, *Phytochemistry* **1996**, *41* (5), 1419–1422.
82. R. W. Teng; H. Z. Li; J. T. Chen; D. Z. Wang; Y. N. He; C. R. Yang, *Magn. Reson. Chem.* **2002**, *40*, 483–488.
83. H. X. Sun; Z. G. Yang; Y. P. Ye, *Int. Immunopharm.* **2006**, *6*, 14–25.
84. J. H. Liu; X. Wang; S. Q. Cai; K. Kamatsu; Ts. Namba, *J. Chin. Pharm. Sci.* **2004**, *13* (4), 225–237.
85. M. D. Nguyen; R. Kasai; K. Ohtani; A. Ito; T. N. Nguyen; K. Yamasaki; O. Tanaka, *Chem. Pharm. Bull.* **1994**, *42* (1), 115–122.
86. Y. G. Chen; E. Y. Zhan; H. F. Chen; X. Q. Duan; L. Q. Guo, *Zhongyaocai (J. Chin. Med. Mater.)* **2002**, *25* (3), 176–178.
87. P. Zhao; Y. Q. Liu; C. R. Yang, *Acta Bot. Yunnan.* **1993**, *15* (4), 409–412.
88. M. Yoshikawa; T. Morikawa; K. Yashiro; M. Yoshikawa, *Chem. Pharm. Bull.* **1997**, *45* (6), 1056–1062.
89. R. W. Ten; H. Z. Li; D. Z. Wang; C. R. Yang, *Helv. Chim. Acta* **2004**, *87* (5), 1270–l278.
90. F. Qiu; Z. Z. Ma; S. X. Xu; X. S. Yao; C. T. Che; Y. J. Chen, *J. Asian Nat. Prod. Res.* **2001**, *3* (3), 235–240.
91. T. O. Cheng, *Int. J. Cardiol.* **2006**, *110*, 411.
92. L. Zhou; Z. Zuo; M. S. Chow, *J. Clin. Pharmacol.* **2005**, *45* (12), 1345–1359.
93. X. B. Wang; S. L. Morris-Natschke; K. H. Lee, *Med. Res. Rev.* **2007**, *27* (1), 133–148.

94. Y. G. Li; L. Song; M. Liu; Z. B. Hu; Z. T. Wang, *J. Chromatogr. A* **2009**, *1216*, 1941–1953.
95. R. W. Jiang; K. M. Lau; P. M. Hon; T. C. W. Mark; K. S. Woo; K. P. Fung, *Curr. Med. Chem.* **2005**, *12*, 237–246.
96. D. S. Lee; S. H. Lee; J. G. Noh; S. D. Hong, *Biosci. Biotechnol. Biochem.* **1999**, *63*, 2236–2239.
97. X. L. Niu; K. Ichimori; X. Yang; Y. Hirota; K. Hoshiai; M. Li; H. Nakazawa, *Free Radic. Res.* **2000**, *33*, 305–312.
98. S. L. Yuan; X. J. Wang; Y. Q. Wei, *Chin. J. Cancer* **2003**, *22*, 1363–1366.
99. C. C. Chen; H. T. Chen; Y. P. Chen; H. Y. Hsu; T. C. Hsieh, *Taiwan. Yao Xue Za Zhi* **1986**, *38*, 226–230.
100. A. Yagi; N. Okamura; K. Tanonaka; S. Takeo, *Planta Med.* **1994**, *60*, 405–409.
101. T. Hayashi; H. Kakisawa, *Chem. Commun.* **1970**, *5* (5), 299.
102. L. Z. Lin; X. M. Wang; X. L. Huang; Y. H. Huang; B. J. Huang, *Yaoxue Xuebao (Acta Pharm. Sin.)* **1988**, *23* (4), 273–275.
103. G. Haro; K. Takenori; O. I. Midori; K. Hiroshi; W. J. Zhao; J. Chen; Y. T. Guo, *Tetrahedron Lett.* **1988**, *29* (36), 4603–4606.
104. A. Yagi; K. Fujimoto; K. Tanonaka; K. Hirai; S. Takeo, *Planta Med.* **1989**, *55* (1), 51–54.
105. T. Hayashi; T. Handa; M. Ohashi; H. Kakisawa; H. Y. Hsū; Y. P. Chen, *Chem. Commun.* **1971**, *8* (11), 541.
106. M. Nagai; M. Noguchi; T. Iizuka; K. Otani; K. Kamata, *Biol. Pharm. Bull.* **1996**, *19* (2), 228–232.
107. F. Asari; T. Kusumi; G. Z. Zheng; Y. Z. Cen; H. Kakisawa, *Chem. Lett.* **1990**, *19* (10), 1885–1888.
108. Y. Tezuka; R. Kasimu; P. Basnet; T. Namba; S. Kadota, *Chem. Pharm. Bull.* **1997**, *45* (8), 1306–1311.
109. D. Y. Kong; X. J. Liu; M. K. Teng; Z. H. Rao, *Yaoxue Xuebao (Acta Pharm. Sin.)* **1985**, *20* (10), 747–751.
110. H. W. Luo; S. X. Chen; J. N. Lee; J. K. Snyder, *Phytochemistry* **1988**, *27* (1), 290–292.
111. H. M. Chang; T. F. Choang; K. Y. Chui; P. M. Hon; C. M. Lee; T. C. W. Mak; H. N. C. Wong, *J. Chem. Res. Synop.***1990**, (4), 114.
112. H. M. Chang; K. P. Cheng; T. F. Choang; H. F. Chow; K. Y. Chui; P. M. Hon; F. W. L. Tan; Y. Yang; Z. P. Zhong, *J. Org. Chem.* **1990**, *55* (11), 3537–3543.
113. N. Okamura; M. Sato; A. Yagi; K. Tanonaka; S. Takeo, *Planta Med.* **1992**, *58* (6), 571–573.
114. K. Takiura; K. Koizumi, *Chem. Pharm. Bull.* **1962**, *10* (2), 112–116.
115. A. C. Bailfie; R. H. Thomson, *J. Chem. Soc. C* **1968**, *3*, 48–52.
116. A. Ulubelen; A. H. Meriçli; F. Meriçli, *Pharmazie* **2004**, *59* (4), 301–303.
117. M. Yang; A. H. Liu; S. H. Guan; J. H. Sun; M. Xu; D. A. Guo, *Rapid Commun. Mass Spectrom.* **2006**, *20* (8), 1266–1280.
118. Q. N. Fang; P. L. Zhang; Z. P. Xu, *Acta Chim. Sin.* **1976**, *34* (3), 197–208.
119. H. Kakisawa; T. Hayashi; T. Yamazaki, *Tetrahedron Lett.* **1969**, *10* (5), 301–304.
120. Y. M. Han; H. Oh; M. K. Na; B. S. Kim; W. K. Oh; B. Y. Kim; D. G. Jeong; S. E. Ryu; D. E. Sok; J. S. Ahn, *Biol. Pharm. Bull.* **2005**, *28* (9), 1795–1797.
121. M. Onitsuka; M. Fujiu; N. Shinma; H. B. Maruyama, *Chem. Pharm. Bull.* **1983**, *31* (5), 1670–1675.
122. H. Zhang; C. Yu; J. Y. Jia; S. W. S. Leung; Y. L. Siow; R. Y. K. Man; D. Y. Zhu, *Zhongguo Yaoli Xue Bao (Acta Pharmacol. Sin.)* **2002**, *23* (12), 1163–1168.
123. Y. Ikeshiro; I. Mase; Y. Tomita, *Phytochemistry* **1989**, *28* (11), 3139–3141.
124. G. Haro; H. Kakisawa, *Chem. Lett.* **1990**, *19* (9), 1599–1602.
125. A. R. Lee; W. L. Wu; W. L. Chang; H. C. Lin; M. L. King, *J. Nat. Prod.* **1987**, *50* (2), 157–160.
126. H. C. Lin; H. Y. Ding; W. L. Chang, *J. Nat. Prod.* **2001**, *64* (5), 648–650.
127. H. C. Lin; W. L. Chang, *Phytochemistry* **2000**, *53* (8), 951–953.
128. H. W. Luo; B. J. Wu; M. Y. Wu; Z. G. Yong; M. Niwa; Y. Hirata, *Phytochemistry* **1985**, *24* (4), 815–817.
129. M. Haiza; J. N. Lee; J. K. Snyder, *J. Org. Chem.* **1990**, *55*, 5008–5013.
130. B. J. Yang; M. K. Qian; G. W. Qin; Z. X. Chen, *Yaoxue Xuebao (Acta Pharm. Sin.)* **1981**, *16* (11), 837–841.
131. T. Kusumi; T. Ooi; T. Hayashi; H. Kakisawa, *Phytochemistry* **1985**, *24* (9), 2118–2120.
132. N. Wang; H. W. Luo; M. Niwa; J. Ji, *Planta Med.* **1989**, *55* (4), 390.
133. H. C. Lin; W. L. Chang; P. L. Wu; T. S. Wu, *J. Chin. Chem. Soc.* **1996**, *43* (2), 199–200.
134. H. W. Luo; J. Ji; M. Y. Wu; Z. G. Yong; M. Niwa; Y. Hirata, *Chem. Pharm. Bull.* **1986**, *34* (8), 3166–3168.
135. S. Y. Ryu; Z. S. No; S. H. Kim; J. W. Ahn, *Planta Med.* **1997**, *63* (1), 44–46.
136. H. Kakisawa; T. Hayashi; I. Okazaki; M. Ohashi, *Tetrahedron Lett.* **1968**, *9* (28), 3231–3234.
137. H. W. Luo; B. J. Wu; M. Y. Wu; Z. G. Yong; Y. Jin, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **1985**, *20* (7), 542–544.
138. X. Z. Lu; H. W. Luo; J. Ji; H. Cai, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **1991**, *26* (3), 193–196.
139. G. H. Du; J. T. Zhang, *Yi Yao Dao Bao* **2004**, *23*, 355–360.
140. G. H. Du; J. T. Zhang, *Yi Yao Dao Bao* **2004**, *23*, 435–440.
141. A. Watzke; S. J. O'Malley; R. G. Bergman; J. A. Ellman, *J. Nat. Prod.* **2006**, *69*, 1231–1233.
142. G. T. Liu; T. M. Zhang; B. E. Wang; Y. W. Wang, *Biochem. Pharmacol.* **1992**, *43*, 147–152.
143. T. Yokozawa; H. Y. Chung; T. W. Lee; H. Oura; T. Tanaka; G. Nonaka; I. Nishioka, *Chem. Pharm. Bull.* **1989**, *37*, 2766–2769.
144. T. Yokozawa; H. Y. Chung; H. Oura; G. I. Nonaka; I. Nishioka, *Nat. Med.* **1995**, *49*, 164–167.
145. I. S. Abd-Elazem; H. S. Chen; R. B. Bates; R. C. C. Huang, *Antiviral Res.* **2002**, *55*, 91–106.
146. T. Yokozawa; J. J. Zhou; X. L. Sun; M. Hattori; T. Namba; T. Tanaka; I. Nishioka; H. Oura, *Nat. Med.* **1994**, *48*, 330–333.
147. D. C. Zhang; W. L. Wu; X. K. Liu; D. J. Pan, *Res. Chin. Pat. Drug* **1982**, *1*, 24–25.
148. Y. L. Pan; L. Zhang; G. N. Chen, *Analyst* **2001**, *126*, 1519–1523.
149. M. Petersen; M. S. J. Simmonds, *Phytochemistry* **2003**, *62*, 121–125.
150. M. L. Scarpati; G. Oriente, *Ric. Sci.* **1958**, *28*, 2329–2333.
151. H. Kohda; O. Takeda; S. Tanaka; K. Yamasaki; A. Yamashita; T. Kurokawa; A. Ishibashi, *Chem. Pharm. Bull.* **1989**, *37*, 1287–1290.
152. C. B. Ai; L. N. Li, *Planta Med.* **1992**, *58*, 197–199.
153. H. S. Kang; H. Y. Chung; J. H. Jung; S. S. Kang; J. S. Choi, *Arch. Pharmacol. Res.* **1997**, *20*, 496–500.
154. Z. X. Chen; W. H. Gu; H. Z. Huang; X. M. Yang; C. J. Sun; W. Z. Chen; Y. L. Dong; H. L. Ma, *Acta Pharm. Bull.* **1981**, *16*, 536–537.
155. X. Z. Lu; W. H. Xi; J. X. Shen; H. Naoki, *Chin. Chem. Lett.* **1991**, *2*, 301–302.
156. L. N. Li, *Medicinal and Aromatic Plants-Industrial Profiles*; Harwood Academic Publishers: Amsterdam, 2000; Vol. 14, pp 81–91.
157. L. N. Li; R. Tan; W. M. Chen, *Planta Med.* **1984**, *50*, 227–228.
158. H. S. Kang; H. Y. Chung; D. S. Byun; J. S. Choi, *Arch. Pharm. Res.* **2003**, *26*, 24–27.

159. Z. Yang; P. M. Hon; K. Y. Chui; Z. L. Xu; H. M. Chang; C. M. Lee; Y. X. Cui; H. N. C. Wong; C. D. Poon; B. M. Fung, *Tetrahedron Lett.* **1991**, *32*, 2061–2064.
160. C. B. Ai; L. N. Li, *Chin. Chem. Lett.* **1991**, *2*, 17–18.
161. C. B. Ai; L. N. Li, *J. Nat. Prod.* **1988**, *51*, 145–149.
162. T. Tanaka; S. Morimoto; G. Nonaka; I. Nishioka; T. Yokozawa; H. Y. Chung; H. Oura, *Chem. Pharm. Bull.* **1989**, *37*, 340–344.
163. G. Stanley; K. Harvey; V. Slivova; J. Jiang; D. Sliva, *Biochem. Biophys. Res. Commun.* **2005**, *330*, 46–52.
164. D. Sliva, *Leuk. Res.* **2006**, *30*, 767–768.
165. R. R. M. Paterson, *Phytochemistry* **2006**, *67*, 1985–2001.
166. S. Y. Yoon; S. K. Eo; Y. S. Kim; C. K. Lee; S. S. Han, *Arch. Pharm. Res.* **1994**, *17*, 438–442.
167. H. Wang; T. B. Ng, *Peptides* **2006**, *27*, 27–30.
168. H. W. Kim; M. J. Shim; E. C. Choi; B. K. Kim, *Arch. Pharm. Res.* **1997**, *20*, 425–431.
169. Y. H. Shieh; C. F. Liu; Y. K. Huang; J. Y. Yang; I. L. Wu; C. H. Lin; S. C. Li, *Am. J. Chin. Med.* **2001**, *29*, 501–507.
170. N. P. Sudheesh; T. A. Ajith; K. K. Janardhanan, *Biogerontology* **2009**, *10* (5), 627–636.
171. C. S. Lai; M. S. Yu; W. H. Yuen; K. F. So; S. Y. Zee; R. C. Chang, *Brain Res.* **2008**, *1190*, 215–224.
172. Y. Wu; D. Wang, *J. Proteome Res.* **2009**, *8* (2), 436–442.
173. J. W. Yuen; M. D. Gohel, *J. Ethnopharmacol.* **2008**, *118* (2), 324–330.
174. T. Akihisa; Y. Nakamura; M. Tagata; H. Tokuda; K. Yasukawa; E. Uchiyama; T. Suzuki; Y. Kimura, *Chem. Biodivers.* **2007**, *4* (2), 224–231.
175. P. V. Jeurink; C. L. Noguera; H. F. Savelkoul; H. J. Wichers, *Int. Immunopharmacol.* **2008**, *8* (8), 1124–1133.
176. C. Ma; S. H. Guan; M. Yang; X. Liu; D. A. Guo, *Phytomedicine* **2008**, *15* (4), 268–276.
177. H. Y. Hsu; K. F. Hua; W. C. Wu; J. Hsu; S. T. Weng; T. L. Lin; C. Y. Liu; R. S. Hseu; C. T. Huang, *J. Cell. Physiol.* **2008**, *215* (1), 15–26.
178. K. C. Cheng; H. C. Huang; J. H. Chen; J. W. Hsu; H. C. Cheng; C. H. Ou; W. B. Yang; S. T. Chen; C. H. Wong; H. F. Juan, *BMC Genom.* **2007**, *8*, 411.
179. X. Pang; Z. Chen; X. Gao; W. Liu; M. Slavin; W. Yao; L. L. Yu, *J. Food Sci.* **2007**, *72* (6), S435–S442.
180. Z. Ji; Q. Tang; J. Zhang; Y. Yang; W. Jia; Y. Pan, *J. Ethnopharmacol.* **2007**, *112* (3), 445–450.
181. W. Cheuk; J. K. Chan; G. Nuovo; M. K. Chan; M. Fok, *Int. J. Surg. Pathol.* **2007**, *15* (2), 180–186.
182. K. F. Hua; H. Y. Hsu; L. K. Chao; S. T. Chen; W. B. Yang; J. Hsu; C. H. Wong, *J. Cell Physiol.* **2007**, *212* (2), 537–550.
183. Y. Yin; W. Fu; M. Fu; G. He; L. Traore, *Asia Pac. J. Clin. Nutr.* **2007**, *16* (1), 258–260.
184. X. L. Zhu; A. F. Chen; Z. B. Lin, *J. Ethnopharmacol.* **2007**, *111* (2), 219–226.
185. K. I. Lin; Y. Y. Kao; H. K. Kuo; W. B. Yang; A. Chou; H. H. Lin; A. L. Yu; C. H. Wong, *J. Biol. Chem.* **2006**, *281* (34), 24111–24123.
186. Y. L. Lin; S. S. Lee; S. M. Hou; B. L. Chiang, *Mol. Pharmacol.* **2006**, *70* (2), 637–644.
187. J. W. Yuen; M. D. Gohel; D. W. Au, *Nutr. Cancer* **2008**, *60* (1), 109–119.
188. C. I. Müller; T. Kumagai; J. O'kelly; N. P. Seeram; D. Heber; H. P. Koeffler, *Leuk. Res.* **2006**, *30*, 841–848.
189. J. Mahajna; N. Dotan; B. Z. Zaidman; R. D. Petrova, *Nutr. Cancer* **2009**, *61* (1), 16–26.
190. M. Fukuzawa; R. Yamaguchi; I. Hide; Z. Chen; Y. Hirai; A. Sugimoto; T. Yasuhara; Y. Nakata, *Biol. Pharm. Bull.* **2008**, *31* (10), 1933–1937.
191. K. C. Kim; H. J. Jun; J. S. Kim; I. G. Kim, *Int. J. Mol. Med.* **2008**, *21* (4), 489–498.
192. B. Z. Zaidman; S. P. Wasser; E. Nevo; J. Mahajna, *Int. J. Oncol.* **2007**, *31* (4), 959–967.
193. J. Jiang; V. Slivova; D. Sliva, *Int. J. Oncol.* **2006**, *29* (3), 695–703.
194. K. C. Kim; J. S. Kim; J. K. Son; I. G. Kim, *Cancer Lett.* **2007**, *246* (1–2), 210–217.
195. C. I. Müller; T. Kumagai; J. O'Kelly; N. P. Seeram; D. Heber; H. P. Koeffler, *Leuk. Res.* **2006**, *30* (7), 767–768.
196. D. Sliva, *Integr. Cancer Ther.* **2003**, *2* (4), 358–364.
197. C. J. Weng; C. F. Chau; K. D. Chen; D. H. Chen; G. C. Yen, *Mol. Nutr. Food Res.* **2007**, *51* (12), 1472–1477.
198. A. Thyagarajan; J. Zhu; D. Sliva, *Int. J. Oncol.* **2007**, *30* (4), 963–1939.
199. X. Chen; Z. Hi; X. X. Yang; M. Huang; Y. Gao; W. Tang; S. Y. Chan, *Int. Immunopharmacol.* **2006**, *6*, 499–508.
200. W. K. Chan; C. C. Cheung; H. K. Law; Y. L. Lau; G. C. Chan, *J. Hematol. Oncol.* **2008**, *21* (1), 9.
201. G. G. Yue; K. P. Fung; P. C. Leung; C. B. Lau, *Phytother. Res.* **2008**, *22* (10), 1282–1291.
202. Y. Nonaka; H. Ishibashi; M. Nakai; H. Shibata; Y. Kiso; S. Abe, *Biosci. Biotechnol. Biochem.* **2008**, *72* (6), 1399–1408.
203. Y. Nonaka; H. Shibata; M. Nakai; H. Kurihara; H. Ishibashi; Y. Kiso; T. Tanaka; H. Yamaguchi; S. Abe, *Biosci. Biotechnol. Biochem.* **2006**, *70* (9), 2028–2034.
204. J. T. Xie; C. Z. Wang; S. Wicks; J. J. Yin; J. Kong; J. Li; Y. C. Li; C. S. Yuan, *Exp. Oncol.* **2006**, *28* (1), 25–29.
205. J. Jiang; B. Grieb; A. Thyagarajan; D. Sliva, *Int. J. Mol. Med.* **2008**, *21* (5), 577–584.
206. C. J. Weng; C. F. Chau; Y. S. Hsieh; S. F. Yang; G. C. Yen, *Carcinogenesis* **2008**, *29* (1), 147–156.
207. Q. X. Yue; F. B. Xie; S. H. Guan; C. Ma; M. Yang; B. H. Jiang; X. Liu; D. A. Guo, *Cancer Sci.* **2008**, *99* (7), 1461–1470.
208. S. W. Seto; T. Y. Lam; H. L. Tam; A. L. Au; S. W. Chan; J. H. Wu; P. H. Yu; G. P. Leung; S. M. Ngai; J. H. Yeung; P. S. Leung; S. M. Lee; Y. W. Kwan, *Phytomedicine* **2009**, *16* (5), 426–436.
209. C. Y. He; W. D. Li; S. X. Guo; S. Q. Lin; Z. B. Lin, *J. Asian Nat. Prod. Res.* **2006**, *8* (8), 705–711.
210. S. Fatmawati; K. Kurashiki; S. Takeno; Y. U. Kim; K. Shimizu; M. Sato; K. Imaizumi; K. Takahashi; S. Kamiya; S. Kaneko; R. Kondo, *Phytother. Res.* **2009**, *23* (1), 28–32.
211. C. C. Chien; M. L. Tsai; C. C. Chen; S. J. Chang; C. H. Tseng, *Mycopathologia* **2008**, *166* (2), 117–120.
212. E. K. Li; L. S. Tam; C. K. Wong; W. C. Li; C. W. Lam; S. Wachtel-Galor; I. F. Benzie; Y. X. Bao; P. C. Leung; B. Tomlinson, *Arthritis Rheum.* **2007**, *57* (7), 1143–1150.
213. Y. W. Ho; J. S. Yeung; P. K. Chiu; W. M. Tang; Z. B. Lin; R. Y. Man; C. S. Lau, *Mol. Cell Biochem.* **2007**, *301* (1–2), 173–179.
214. B. Y. Xi; W. C. Kwok; M. L. E. Kwok; T. L. Shan; L. P. Chung; Y. Y. Bing; K. L. C. Wai, *Immunopharmacol. Immunotoxicol.* **2006**, *28* (2), 197–200.
215. X. Zhou; J. Lin; Y. Yin; J. Zhao; X. Sun; K. Tang, *Am. J. Chin. Med.* **2007**, *35* (4), 559–574.
216. X. Wang; X. Zhao; D. Li; Y. Q. Lou; Z. B. Lin; G. L. Zhang, *Biol. Pharm. Bull.* **2007**, *30* (9), 1702–1706.
217. G. J. Wang; Y. J. Huang; D. H. Chen; Y. L. Lin, *Phytother. Res.* **2009**, *23* (6), 833–839.

218. Y. Q. Li; S. F. Wang, *Biotechnol. Lett.* **2006**, *28* (11), 837–841.
219. Y. Li; Y. Yang; L. Fang; Z. Zhang; J. Jin; K. Zhang, *Am. J. Chin. Med.* **2006**, *34* (2), 341–349.
220. B. Lakshmi; T. A. Ajith; N. Jose; K. K. Janardhanan, *J. Ethnopharmacol.* **2006**, *107* (2), 297–303.
221. Y. Shi; J. Sun; H. He; H. Guo; S. Zhang, *J. Ethnopharmacol.* **2008**, *117* (3), 415–419.
222. X. J. Yang; J. Liu; L. B. Ye; F. Yang; L. Ye; J. R. Gao; Z. H. Wu, *World J. Gastroenterol.* **2006**, *12* (9), 1379–1385.
223. K. H. Jung; E. Ha; M. J. Kim; Y. K. Uhm; H. K. Kim; S. J. Hong; J. H. Chung; S. V. Yim, *Acta Biochim. Pol.* **2006**, *53* (3), 597–601.
224. N. Kashimoto; M. Hayama; K. Kamiya; H. Watanabe, *Oncol. Rep.* **2006**, *16* (6), 1181–1187.
225. L. W. Chen; Y. Q. Wang; L. C. Wei; M. Shi; Y. S. Chan, *CNS Neurol. Disord. Drug Targets* **2007**, *6* (4), 273–281.
226. J. Liu; S. Tamura; K. Kurashiki; K. Shimizu; K. Noda; F. Konishi; S. Kumamoto; R. Kondo, *Chem. Biodivers.* **2009**, *6* (2), 231–243.
227. B. Z. Zaidman; S. P. Wasser; E. Nevo; J. Mahajna, *Mol. Biol. Rep.* **2008**, *35* (2), 107–117.
228. D. Sadava; D. W. Still; R. R. Mudry; S. E. Kane, *Cancer Lett.* **2009**, *277* (2), 182–189.
229. P. V. Jeurink; C. L. Noguera; H. F. Savelkoul; H. J. Wichers, *Int. Immunopharmacol.* **2008**, *8* (8), 1124–1133.
230. P. G. M. Yang; J. J. Liou, *Taiwan Tangye Gongsi Yanjiuso Yanjiu Huibao* **2002**, (177–178), 45–53.
231. S. Q. Lin; S. Z. Wang; Z. B. Lin; S. G. Lin, *Zhong Hua Yi Yao Za Zhi* **2006**, *6* (3), 272–274.
232. H. He; J. Sun; B. Xie, *Zhongguo Nongye Ke Xue* **2006**, *39* (12), 2603–2607.
233. R. Chyr; M. S. Shiao, *J. Chromatogr.* **1991**, *542*, 327–336.
234. J. Luo; Z. B. Lin, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **2002**, *33* (3), 197–200.
235. R. Y. Chen; D. Q. Yu, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **1991**, *26* (4), 267–273.
236. Y. Komoda; H. Nakamura; S. Ishihara; M. Uchida; H. Kohda; K. Yamasaki, *Chem. Pharm. Bull.* **1985**, *33* (11), 4829–4835.
237. H. Sato; T. Nishitaoba; S. Shirasu; K. Oda; S. Sakamura, *Agric. Biol. Chem.* **1986**, *50* (11), 2887–2890.
238. R. Y. Chen; D. Q. Yu, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **1991**, *26* (6), 430–436.
239. M. Hirotani; C. Ino; T. Furuya, *Phytochemistry* **1993**, *33* (2), 379–382.
240. M. S. Shiao, *J. Chin. Chem. Soc.* **1992**, *39*, 669–674.
241. F. S. Wang; H. Cai; J. S. Yang; Y. M. Zhang; C. Y. Hou; J. Q. Liu; M. J. Zhao, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **1997**, *32* (6), 447–450.
242. H. Cai; F. S. Wang; J. S. Yang; Y. M. Zhang; K. Hu; Y. J. Zhao; Y. Bai, *Zhongguo Shouyi Xue Bao* **1997**, *17* (5), 511–513.
243. S. El-Mekkawy; M. R. Meselhy; N. Nakamura; Y. Tezuka; M. Hattori; N. Kakiuchi; K. Shimotohno; T. Kawahata; T. Otake, *Phytochemistry* **1998**, *49* (6), 1651–1657.
244. B. S. Min; N. Nakamura; H. Miyashiro; K. W. Bae; M. Hattori, *Chem. Pharm. Bull.* **1998**, *46* (10), 1607–1612.
245. Y. Mizushina; N. Takahashi; L. Hanashima; H. Koshino; Y. Esumi; J. Uzawa; F. Sugawara; K. Sakaguchi, *Bioorg. Med. Chem.* **1999**, *7* (9), 2047–2052.
246. T. S. Wu; L. S. Shi; S. C. Kuo, *J. Nat. Prod.* **2001**, *64*, 1121–1122.
247. J. J. Gao; B. S. Min; E. M. Ahn; N. Nakamura; H. K. Lee; M. Hattori, *Chem. Pharm. Bull.* **2002**, *50* (6), 837–840.
248. J. Luo; Z. B. Lin, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **2001**, *36* (8), 595–598.
249. J. Y. Ma; Q. Ye; Y. J. Hua; D. C. Zhang; R. Cooper; M. N. Chang; J. Y. Chang; H. Sun, *J. Nat. Prod.* **2002**, *65*, 72–75.
250. B. S. Min; J. J. Gao; N. Nakamura; M. Hattori, *Chem. Pharm. Bull.* **2000**, *48* (7), 1026–1033.
251. S. H. Guan; M. Yang; X. Liu; J. M. Xia; X. M. Wang; H. Jin; D. A. Guo, *Nat. Prod. Commun.* **2006**, *1* (3), 177–181.
252. T. Nishitoba; H. Sato; S. Sakamura, *Phytochemistry* **1987**, *26* (6), 1777–1784.
253. T. Nisitoba; H. Sato; S. Sakamura, *Agric. Biol. Chem.* **1985**, *49* (12), 3637–3638.
254. A. Morigiwa; K. Kitabatake; Y. Fujimoto; N. Ikekawa, *Chem. Pharm. Bull.* **1986**, *34* (7), 3025–3028.
255. T. Kikuchi; S. Matsuda; S. Kadota; Y. Murai; Z. Ogita, *Chem. Pharm. Bull.* **1985**, *33* (6), 2624–2627.
256. T. Nishitoba; H. Sato; S. Sakamura, *Agric. Biol. Chem.* **1986**, *50* (3), 809–811.
257. T. Nishitoba; H. Sato; S. Sakamura, *Agric. Biol. Chem.* **1985**, *49* (5), 1547–1549.
258. T. Nishitoba; H. Sato; T. Kasai; H. Kawagishi; S. Sakamura, *Agric. Biol. Chem.* **1985**, *49* (6), 1793–1798.
259. T. Kikuchi; S. Matsuda; Y. Murai; Z. Ogita, *Chem. Pharm. Bull.* **1985**, *33* (6), 2628–2631.
260. M. Hirotani; T. Furura; M. Shiro, *Phytochemistry* **1985**, *24* (9), 2055–2061.
261. T. Nishitoba; T. H. Sato; T. Kasai; H. Kawagishi; S. Sakamura, *Agric. Biol. Chem.* **1984**, *48* (11), 2905–2907.
262. T. Kubota; Y. Asaka; I. Miura; H. Mori, *Helv. Chim. Acta* **1982**, *65* (2), 611–618.
263. M. S. Shiao; L. J. Lin; S. F. Yen; C. S. Chou, *J. Nat. Prod.* **1987**, *50* (5), 886–890.
264. T. Nishitoba; H. Sato; S. Shirasu; S. Sakamura, *Agric. Biol. Chem.* **1987**, *51* (2), 619–622.
265. T. Nishitoba; H. Sato; S. Sakamura, *Agric. Biol. Chem.* **1987**, *51* (4), 1149–1153.
266. M. Hirotani; I. Asaka; C. Ino; T. Furuya; M. Shiro, *Phytochemistry* **1987**, *26* (10), 2797–2803.
267. M. Arisawa; A. Fujita; M. Saga; H. Fukumura; T. Hayashi; M. Shimizu; N. Morita, *J. Nat. Prod.* **1986**, *49* (4), 621–625.
268. T. Nishitoba; H. Sato; S. Shirasu; S. Sakamura, *Agric. Biol. Chem.* **1986**, *50* (8), 2151–2154.
269. M. Hirotani; C. Ino; T. Furuya; M. Shiro, *Chem. Pharm. Bull.* **1986**, *34* (5), 2282–2285.
270. T. Kikuchi; S. Kanomi; S. Kadota; Y. Murai; K. Tsubono; Z. I. Ogita, *Chem. Pharm. Bull.* **1986**, *34* (9), 3695–3712.
271. T. Kikuchi; S. Kanomi; Y. Murai; S. Kadota; K. Tsubono; Z. I. Ogita, *Chem. Pharm. Bull.* **1986**, *34* (10), 4030–4036.
272. T. Kikuchi; S. Kanomi; Y. Murai; S. Kadota; K. Tsubono; Z. I. Ogita, *Chem. Pharm. Bull.* **1986**, *34* (10), 4018–4029.
273. M. Arisawa; A. Fujita; T. Hayashi; M. Shimizu; N. Morita, *J. Nat. Prod.* **1988**, *51* (1), 54–59.
274. T. Nishitoba; H. Sato; K. Oda; S. Sakamura, *Agric. Biol. Chem.* **1988**, *52* (1), 211–216.
275. M. S. Shiao; L. J. Lin; S. F. Yeh, *Phytochemistry* **1988**, *27* (3), 873–875.
276. L. J. Lin; M. S. Shiao, *J. Nat. Prod.* **1988**, *51* (5), 918–924.
277. H. Kohda; W. Tokumoto; K. Sakamoto; M. Fujii; Y. Hirai; K. Yamasaki; Y. Komoda; H. Nakamura; S. Ishihara; M. Uchida, *Chem. Pharm. Bull.* **1985**, *33* (4), 1367–1374.
278. A. Fujita; M. Arisawa; M. Saga; T. Hayashi; N. Morita, *J. Nat. Prod.* **1986**, *49* (6), 1122–1125.
279. S. H. Guan; M. Yang; X. M. Wang; J. M. Xia; Z. M. Zhang; X. Liu; D. A. Guo, *Magn. Reson. Chem.* **2007**, *45*, 789–791.
280. C. J. Li; Y. M. Li; H. H. Sun, *Nat. Prod. Res.* **2006**, *20* (11), 985–991.
281. A. G. Gonzalez; F. Leon; A. Rivera; C. M. Munoz; J. Bermejo, *J. Nat. Prod.* **1999**, *62*, 1700–1701.
282. S. H. Guan; J. M. Xia; M. Yang; X. M. Wang; X. Liu; D. A. Guo, *J. Asian Nat. Prod. Res.* **2008**, *10* (7–8), 705–710.

283. Q. X. Yue; Z. W. Cao; S. H. Guan; X. H. Liu; L. Tao; W. Y. Wu; Y. X. Li; P. Y. Yang; X. Liu; D. A. Guo, *Mol. Cell Proteom.* **2008**, *7* (5), 949–961.
284. J. Liu; J. Shiono; K. Shimizu; A. Kukita; T. Kukita; R. Kondo, *Bioorg. Med. Chem. Lett.* **2009**, *19* (8), 2154–2157.
285. I. Miyamoto; J. Liu; K. Shimizu; M. Sato; A. Kukita; T. Kukita; R. Kondo, *Eur. J. Pharmacol.* **2009**, *602* (1), 1–7.
286. C. L. Hsu; Y. S. Yu; G. C. Yen, *J. Agric. Food Chem.* **2008**, *56* (11), 3973–3980.
287. J. Liu; K. Shimizu; F. Konishi; S. Kumamoto; R. Kondo, *Bioorg. Med. Chem.* **2007**, *15* (14), 4966–4972.
288. G. Wang; J. Zhao; J. Liu; Y. Huang; J. J. Zhong; W. Tang, *Int. Immunopharmacol.* **2007**, *7* (6), 864–870.
289. W. Tang; J. W. Liu; W. M. Zhao; D. Z. Wei; J. J. Zhong, *Life Sci.* **2006**, *80* (3), 205–211.
290. U. M. Chang; C. H. Li; L. I. Lin; C. P. Huang; L. S. Kan; S. B. Lin, *Life Sci.* **2006**, *79* (12), 1129–1139.
291. Y. C. Wang; Y. S. Yang, *J. Chromatogr. B* **2007**, *850*, 392.
292. M. N. Asl; H. Hosseinzadeh, *Phytother. Res.* **2008**, *22*, 709–724.
293. M. Menegazzi; R. Di Paola; E. Mazzon; T. Genovese; C. Crisafulli; M. Dal Bosco; Z. Zou; H. Suzuki; S. Cuzzocrea, *Pharmacol. Res.* **2008**, *58* (1), 22–31.
294. G. Chen; L. Zhu; Y. Liu; Q. Zhou; H. Chen; J. Yang, *Phytother Res.* **2009**, *23* (4), 498–506.
295. J. Vaya; P. A. Belinky; M. Aviram, *Free Radic. Biol. Med.* **1997**, *23* (2), 302–313.
296. C. Fiore; M. Eisenhut; R. Krausse; E. Ragazzi; D. Pellati; D. Armanini; J. Bielenberg, *Phytother. Res.* **2008**, *22* (2), 141–148.
297. E. H. Jo; S. H. Kim; J. C. Ra; S. R. Kim; S. D. Cho; J. W. Jung; S. R. Yang; J. S. Park; J. W. Hwang; O. I. Aruoma; T. Y. Kim; Y. S. Lee; K. S. Kang, *Cancer Lett.* **2005**, *230* (2), 239–247.
298. D. Dhinqra; A. Sharma, *Prog. Neuropsychopharmacol. Biol. Psychiatry* **2006**, *30* (3), 449–454.
299. C. H. Lee; S. W. Park; Y. S. Kim; S. S. Kang; J. A. Kim; S. H. Lee; S. M. Lee, *Biol. Pharm. Bull.* **2007**, *30* (10), 1898–1904.
300. D. Dhingra; M. Parle; S. K. Kulkarni, *J. Ethnopharmacol.* **2004**, *91* (2–3), 361–365.
301. Q. Y. Zhang; M. Ye, *J. Chromatogr. A* **2009**, *1216*, 1954–1969.
302. T. Hatano; M. Takagi; H. Ito; T. Yoshida, *Phytochemistry* **1998**, *47* (2), 287–293.
303. K. Kajiyama; S. Demizu; Y. Hiraga; K. Kinoshita; K. Koyama; K. Takahashi; Y. Tamura; K. Okada; T. Kinoshita, *J. Nat. Prod.* **1992**, *55* (9), 1197–1203.
304. S. L. Yang; Y. L. Liu, *Acta Bot. Sin.* **1988**, *30* (2), 176–182.
305. H. Bai; W. Li; K. Koike; D. Q. Dou; Y. P. Pei; Y. J. Chen; T. Nikaido, *Chem. Pharm. Bull.* **2003**, *51* (9), 1095–1097.
306. B. Jayaprakasam; S. Doddaga; R. Wang; D. Holmes; J. Goldfarb; X. M. Li, *J. Agric. Food. Chem.* **2009**, *57* (3), 820–825.
307. T. Fukai; Q. H. Wang; T. Nomura, *Phytochemistry* **1991**, *30* (4), 1245–1250.
308. T. Hatano; Y. Aga; Y. Shintani; H. Ito; T. Okuda; T. Yoshida, *Phytochemistry* **2000**, *55*, 959–963.
309. J. R. Li; Y. Q. Wang; Z. Z. Deng, *J. Asian Nat. Prod. Res.* **2005**, *7* (4), 677–680.
310. W. Li; Y. Asada; T. Yoshikawa, *Phytochemistry* **2000**, *55*, 447–456.
311. T. Hatano; T. Yasuhara; K. Miyamoto; T. Okuda, *Chem. Pharm. Bull.* **1988**, *36* (6), 2286–2288.
312. T. Fukai; B. S. Cai; K. Maruno; Y. Miyakawa; M. Konishi; T. Nomura, *Phytochemistry* **1998**, *49* (7), 2005–2013.
313. Y. J. Sato; J. X. He; H. Nagai; T. Tani; T. Akao, *Biol. Pharm. Bull.* **2007**, *30* (1), 145–149.
314. M. Kuroda; Y. Mimaki; Y. Sashida; T. Mae; H. Kishida; T. Nishiyama; M. Tsukagawa; E. Konishi; K. Takahashi; T. Kawada; K. Nakagawa; M. Kitahara, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4267–4272.
315. Y. Asada; W. Li; T. Yoshikawa, *Phytochemistry* **1998**, *47* (3), 389–392.
316. T. Fukai; B. S. Cai; T. Horikoshi; T. Nomura, *Phytochemistry* **1996**, *43* (5), 1119–1124.
317. D. M. Biondi; C. Rocco; G. Ruberto, *J. Nat. Prod.* **2003**, *66* (4), 477–480.
318. I. Kitagawa; K. Hori; E. Uchida; W. Z. Chen; M. Yoshikawa; J. L. Ren, *Chem. Pharm. Bull.* **1993**, *41* (9), 1567–1572.
319. T. Nakanishi; A. Inada; K. Kambayashi; K. Yoneda, *Phytochemistry* **1985**, *24* (2), 339–341.
320. H. J. Zhang; Y. Liu; R. Y. Zhang, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **1994**, *29* (6), 471–474.
321. S. Yahara; I. Nishioka, *Phytochemistry* **1984**, *23* (9), 2108–2109.
322. H. C. Van; P. Braeckman; M. Vandewalle, *Planta Med.* **1971**, *20* (3), 278–282.
323. T. Fukai; L. Tantai; T. Nomura, *Phytochemistry* **1996**, *43* (2), 531–532.
324. Y. W. Chin; H. A. Jung; Y. Liu; B. N. Su; J. A. Castoro; W. J. Keller; M. A. Pereira; A. D. Kinghorn, *J. Agric. Food. Chem.* **2007**, *55* (12), 4691–4697.
325. K. Takeshi; S. Tamotsu; S. Shoji, *Chem. Pharm. Bull.* **1978**, *26* (1), 141–143.
326. D. K. Bhardwaj; R. Singh, *Curr. Sci.* **1977**, *46* (21), 753.
327. T. Kinoshita; Y. Tamura; K. Mizutani, *Chem. Pharm. Bull.* **2005**, *53* (7), 847–849.
328. P. A. Belinky; M. Aviram; S. Mahmood; J. Vaya, *Free Radic. Bio. Med.* **1998**, *24* (9), 1419–1429.
329. X. R. Chang; Q. H. Xu; D. Y. Zhu; G. Q. Song; R. S. Xu, *Zhongcaoyao (Chin. Trad. Herb. Drugs* **1981**, *12* (12), 530.
330. T. Saitoh; S. Shibata, *Tetrahedron Lett.* **1975**, *50*, 4461–4462.
331. G. Yoon; Y. D. Jung; S. H. Cheon, *Chem. Pharm. Bull.* **2005**, *53* (6), 694–695.
332. M. M. Rafi; B. C. Vastano; N. Q. Zhu; C. T. Ho; G. Ghai; R. T. Rosen; M. A. Gallo; R. S. Dipaola, *J. Agric. Food Chem.* **2002**, *50*, 677–684.
333. T. Fukai; Q. H. Wang; T. Kitagawa; K. Kusano; T. Nomura; Y. Iitaka, *Heterocycles* **1989**, *29* (9), 1761–1772.
334. Q. E. Wang; R. S. C. Lee; X. R. Wang, *J. Chromatogr. A* **2004**, *1048* (1), 51–57.
335. F. Kiuchi; X. Chen; Y. Tsuda, *Heterocycles* **1990**, *31* (4), 629–636.
336. T. Kinoshita; K. Kajiyama; Y. Hiraga; K. Takahashi; Y. Tamura; K. Mizutani, *Chem. Pharm. Bull.* **1996**, *44* (6), 1218–1221.
337. D. M. Biondi; C. Rocco; G. Ruberto, *J. Nat. Prod.* **2005**, *68* (7), 1099–1102.
338. I. Kitagawa; K. Hori; T. Taniyama; J. L. Zhou; M. Yoshikawa, *Chem. Pharm. Bull.* **1993**, *41* (1), 43–49.
339. I. Kitagawa; K. Hori; M. Sakagami; J. L. Zhou; M. Yoshikawa, *Chem. Pharm. Bull.* **1993**, *41* (8), 1337–1345.
340. R. Y. Zhang; J. H. Zhang; M. Wang, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **1986**, *21* (7), 510–515.
341. L. Y. Jinwei; J. I. Nakajima; N. Kimura; K. Saito; S. Seo, *Nat. Prod. Commun.* **2007**, *2* (3), 243–248.
342. C. Sabbioni; R. Mandrioli; A. Ferranti; F. Bugamelli; M. A. Saracino; G. C. Forti; S. Fanali; M. A. Raggi, *J. Chromatogr. A* **2005**, *1081*, 65–71.
343. Y. K. Wang; Z. Q. Huang, *Pharm. Res.* **2005**, *52*, 174.

344. Y. Pan; L. D. Kong; X. Xia; W. Y. Zhang; Z. H. Xia; F. X. Jiang, *Pharmacol. Biochem. Behav.* **2005**, *82*, 686.
345. H. X. Ning; Z. C. Xin; G. T. Lin; L. Banie; T. F. Lue; C. S. Lin, *Urology* **2006**, *68*, 1350.
346. K. M. Chen; B. F. Ge; H. P. Ma; X. Y. Liu; M. H. Bai; Y. Wang, *Pharmazie* **2005**, *60*, 939–942.
347. F. H. Meng; Y. B. Li; Z. L. Xiong; Z. M. Jiang; F. M. Li, *Phytomedicine* **2005**, *12*, 189–193.
348. H. Wu; E. J. Lien; L. L. Lien, *Prog. Drug Res.* **2003**, *60*, 1–57.
349. P. Y. Sun; Y. Xu; Y. Wen; Y. P. Pei; Y. J. Chen; S. Noriko; T. Tadahiro, *Zhongguo Yaowu Huaxue Zazhi (Chin. J. Med. Chem.)* **1998**, *8* (2), 122–126.
350. H. J. Chun; S. Jeong; W. H. Woo; I. K. Kim, *Bull. Korean Chem. Soc.* **2001**, *22* (10), 1159–1162.
351. W. K. Li; R. Y. Zhang; P. G. Xiao, *Phytochemistry* **1996**, *43* (2), 527–530.
352. B. L. Guo; J. G. Yu; P. G. Xiao, *Zhongguo Zhong Yao Za Zhi (China J. Chin. Mater. Med.)* **1996**, *21* (5), 290–292.
353. W. K. Li; J. Q. Pan; M. J. Lu; P. G. Xiao; R. Y. Zhang, *Phytochemistry* **1996**, *42* (1), 213–216.
354. X. H. Zheng; L. Y. Kong, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **2002**, *33* (11), 964–967.
355. P. Y. Sun; Y. Xu; Y. Wen; Y. P. Pei; Y. J. Chen; S. Noriko; T. Tadahiro, *Zhongguo Yaowu Huaxue Zazhi (Chin. J. Med. Chem.)* **1998**, *8* (4), 281–284.
356. W. M. Yan; Y. Fu; Y. Ma; Y. W. Li; X. Z. Zhang; F. Xin, *Zhongguo Zhongyao Zazhi (China J. Chin. Mater. Med.)* **1998**, *23* (12), 735–736.
357. G. J. Wang; T. H. Tsai; L. C. Lin, *Phytochemistry* **2007**, *68* (19), 2455–2464.
358. W. K. Li; J. Q. Pan; M. J. Lu; R. Y. Zhang; P. G. Xiao, *Phytochemistry* **1995**, *39* (1), 231–233.
359. Y. S. Li; Y. L. Liu, *J. Nat. Prod.* **1990**, *53* (5), 1337–1339.
360. M. Q. Wang; X. Peng; Q. F. Gan, *Xiandai Zhongyao Yanjiu Yu Shijian* **2005**, *19* (2), 39–42.
361. M. L. Cai; H. Ji; P. Li; M. Q. Wang, *Zhongguo Tianran Yaowu* **2004**, *2* (4), 235–238.
362. C. S. Wu; Y. X. Sheng; Y. H. Zhang; J. L. Zhang; B. L. Guo, *Rapid Commun. Mass Spectrom.* **2008**, *22* (18), 2813–2824.
363. W. K. Li; P. G. Xiao; R. Y. Zhang, *Phytochemistry* **1995**, *38* (3), 807–808.
364. X. J. Chen; H. Ji; Q. W. Zhang; P. F. Tu; Y. T. Wang; B. L. Guo; S. P. Li, *J. Pharm. Biomed. Anal.* **2008**, *46* (2), 226–235.
365. H. Y. Zhang; T. Z. Zhao; W. P. Yin; M. J. Wu, *Chin. Chem. Lett.* **2006**, *17* (10), 1328–1330.
366. P. Pachaly; C. Schoenherr-Weissbarth; K. S. Sin, *Planta Med.* **1990**, *56* (3), 277–280.
367. P. Y. Sun; Y. Wen; Y. Xu; Y. P. Pei; Y. J. Chen; S. Noriko; T. Tadahiro, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **1998**, *33* (12), 919–922.
368. H. Y. Zhao; L. Fan; L. Zhou; J. Han; B. R. Wang; D. A. Guo, *Helv. Chim. Acta* **2007**, *90* (11), 2186–2195.
369. P. Y. Sun; Y. J. Chen; Y. Wen; Y. P. Pei; Z. H. Liu; X. S. Yao; T. Takeda; Y. Ogihara, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **1996**, *31* (8), 602–606.
370. C. H. Yang; H. K. Liu; C. L. Wu, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **1980**, *11* (10), 444.
371. G. Zhang; L. Qin; Y. Y. Shi, *J. Bone Miner. Res.* **2007**, *22* (7), 1072–1079.
372. Y. Pan; L. D. Kong; Y. C. Li; X. Xia; H. F. Kung; F. X. Jiang, *Pharmacol. Biochem. Behav.* **2007**, *87* (1), 130–140.
373. P. Y. Sun; J. F. Zhao; Y. Wen; Y. P. Pei; Y. Xu; O. Yukio; G. F. Zhang; Z. X. Wang; Y. J. Chen; T. Tadahiro, *J. Shenyang Pharm. Univ. (Shenyang Yaoke Daxue Xuebao)* **1995**, *12* (4), 266–269, 306.
374. J. P. Dou; Z. Q. Liu; S. Y. Liu, *Anal. Sci.* **2006**, *22* (3), 449–452.
375. Y. Ito; F. Hirayama; K. Suto; K. Sagara; T. Toshida, *Phytochemistry* **1988**, *27* (3), 911–913.
376. Y. S. Li; Y. L. Liu, *Phytochemistry* **1990**, *29* (10), 3311–3314.
377. T. Ueda; K. Nakajima; M. Chin; H. Mihashi, *Jpn. Kokai Tokkyo Koho* **1992**, 9pp.
378. W. K. Li; P. G. Xiao; G. Z. Tu; L. B. Ma; R. Y. Zhang, *Phytochemistry* **1995**, *38* (1), 263–265.
379. W. K. Li; P. G. Xiao; M. C. Liao; J. Q. Pan; M. J. Lu; R. Y. Zhang, *Chem. J. Chin. Univ. (Gaodeng Xuexiao Huaxue Xuebao)* **1995**, *16* (12), 1892–1895.
380. P. Y. Sun; Y. J. Chen; N. Shimizu; T. Takeda, *Chem. Pharm. Bull.* **1998**, *46* (2), 355–358.
381. W. K. Li; P. G. Xiao; R. Y. Zhang, *Tianran Chanwu Yanjiu Yu Kaifa (Nat. Prod. Res. Dev.)* **1994**, *6* (4), 12–18.
382. B. L. Guo; W. K. Li; J. G. Yu; P. G. Xiao, *Phytochemistry* **1996**, *41* (3), 991–992.
383. W. K. Li; P. G. Xiao; M. C. Liao; R. Y. Zhang, *Chem. J. Chin. Univ. (Gaodeng Xuexiao Huaxue Xuebao)* **1995**, *16* (10), 1575–1576.
384. W. K. Li; P. G. Xiao, *Tianran Chanwu Yanjiu Yu Kaifa (Nat. Prod. Res. Dev.)* **1994**, *6* (3), 4–8.
385. Y. Cheng; N. L. Wang; X. L. Wang; D. W. Zhang; W. X. Huang; X. S. Yao, *J. Shenyang Pharm. Univ. (Shenyang Yaoke Daxue Xuebao)* **2006**, *23* (10), 644–647, 657.
386. Y. Oshima; M. Okamoto; H. Hikino, *Planta Med.* **1989**, *55* (3), 309–311.
387. H. Huang; M. J. Liang; X. M. Zhang; C. Zhang; Z. Y. Shen; W. D. Zhang, *J. Sep. Sci.* **2007**, *30* (18), 3207–3213.
388. W. K. Li; P. G. Xiao; J. Q. Pan; M. J. Lu; R. Y. Zhang, *Chin. Pharm. J. (Zhongguo Yaoxue Zazhi)* **1995**, *30* (8), 455–456.
389. Y. L. Huang; J. C. Ou; C. F. Chen; C. C. Chen, *J. Nat. Prod.* **1993**, *56* (2), 275–278.
390. T. Wang; D. W. Zhang; J. C. Zhang; M. S. Yang; P. G. Xiao, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **2006**, *37* (10), 1458–1462.
391. W. K. Li; R. Y. Zhang; P. G. Xiao, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **1994**, *29* (11), 835–839.
392. W. K. Li; J. Q. Pan; M. J. Lu; R. Y. Zhang; M. C. Liao; P. G. Xiao, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **1996**, *31* (1), 29–32.
393. W. K. Li; P. G. Xiao; M. C. Liao; R. Y. Zhang, *Chem. J. Chin. Univ. (Gaodeng Xuexiao Huaxue Xuebao)* **1995**, *16* (2), 230–233.
394. Q. L. Wu; Y. Q. Zhao; Z. L. Li, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **1995**, *26* (9), 451–452.
395. C. C. Chen; Y. L. Huang; C. M. Sun; C. C. Shen; F. N. Ko; C. M. Teng, *J. Nat. Prod.* **1996**, *59* (4), 412–414.
396. S. P. Yap; P. Shen; M. S. Butler; Y. H. Gong; C. J. Loy; E. L. Yong, *Planta Med.* **2005**, *71* (2), 114–119.
397. C. S. Yao; X. H. Zhang; W. Zhang; Y. H. Shen; Y. L. Xu, *Tianran Chanwu Yanjiu Yu Kaifa (Nat. Prod. Res. Dev.)* **2004**, *16* (2), 101–103.
398. W. K. Li; J. Q. Pan; R. Y. Zhang; P. G. Xiao, *J. Chin. Pharm. Sci.* **1998**, *7* (3), 161–163.
399. P. Y. Sun; W. Ye; J. F. Zhao; Y. P. Pei; Z. X. Wang; Y. J. Chen; Y. Ogihara; T. Takeda, *Chem. Pharm. Bull.* **1995**, *43* (4), 703–704.
400. H. Z. Zhang; Z. H. Dong; J. She, *Modern Study of Traditional Chinese Medicine*; Xue Yuan Press: Beijing, 1998; Vol. 3, p 2057.
401. Compilation Group, *Quan-guo-Zhong-cao-yao-hui-bian (Compendium of Chinese Traditional Herbal Drugs)*; People's Health Press: Beijing, 1992; Vol. 2, p 386.

402. Jiangsu New Medical College, *A Dictionary of the Traditional Chinese Medicines*; Shanghai People's Publishing House: Shanghai, 1986; p 992.
403. J. X. Ye; R. X. Liang; L. Wang; B. Yang; R. S. An, *Zhongguo Zhongyao Zazhi (China J. Chin. Mater. Med.)* **2008**, *33* (21), 2513–2517.
404. M. Hiramatsu; T. Takahashi; M. Komatsu; T. Kido; Y. Kasahara, *Neurochem. Res.* **2009**, *34* (4), 795–805.
405. S. J. Bae; S. M. Shim; Y. J. Park; Y. Y. Lee; E. J. Chang; S. W. Choi, *Food Sci. Biotechnol.* **2002**, *11* (2), 140–146.
406. J. Tian; G. Li; Z. Liu; F. Fu, *Pharmacol.* **2008**, *82* (2), 121–126.
407. D. B. Ji; L. Y. Zhang; C. L. Li; J. Ye; H. B. Zhu, *Vascul. Pharmacol.* **2009**, *50* (3–4), 137–145.
408. D. B. Ji; M. C. Zhu; B. Zhu; Y. Z. Zhu; C. L. Li; J. Ye; H. B. Zhu, *J. Cardiovasc. Pharmacol.* **2008**, *52* (2), 191–202.
409. H. B. Zhu; L. Zhang; Z. H. Wang; J. W. Tian; F. H. Fu; K. Liu; C. L. Li, *J. Asian Nat. Prod. Res.* **2005**, *7* (4), 607–613.
410. H. H. Yoo; J. H. Park; S. W. Kwon, *Biosci. Biotechnol. Biochem.* **2006**, *70* (11), 2783–2785.
411. M. N. Kim; F. L. Scao–Bogaert; M. Paris, *Planta Med.* **1992**, *58*, 285–286.
412. H. B. Yin; Z. S. He; Y. Ye, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **2001**, *32* (9), 776–778.
413. M. Hattori; X. L. Huang; Q. M. Che; Y. Kawata; Y. Tezuka; T. Kikuchi; T. Namba, *Phytochemistry* **1992**, *31* (11), 4001–4004.
414. K. Kohei; T. Takashi; S. Katsura; T. Hisatomo; M. Takeshi; O. Toshikatsu, *Biosci. Biotechnol. Biochem.* **2000**, *64* (8), 1588–1599.
415. M. Jin; Y. Q. Wang; J. S. Li; X. K. Wang, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **2003**, *34* (4), 306.
416. V. V. S. Murti; P. V. Raman; T. R. Seshadri; R. S. Thakur, *J. Sci. Ind. Res., Sec. B: Phys. Sci.* **1962**, *21B*, 80–83.
417. K. M. Ahmed; M. S. Marzouk; E. A. el-Khrisy; S. A. Wahab; S. S. el-Din, *Pharmazie* **2000**, *55*, 621–622.
418. Y. M. Li; Q. M. Che, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **1998**, *33* (8), 626–628.
419. E. J. C. Gamez; L. Luyengi; S. K. Lee; L. F. Zhu; B. N. Zhou, *J. Nat. Prod.* **1998**, *61*, 706–708.
420. F. Li; Z. S. He; Y. Ye, *Chin. J. Chem.* **2002**, *20*, 699–702.
421. S. Sakamura; Y. Terayama; S. Kawakatsu; A. Ichihara; H. Saito, *Agric. Biol. Chem.* **1980**, *44* (12), 2951–2954.
422. G. H. Kang; E. J. Chang; S. W. Choi, *J. Food Sci. Nutr.* **1999**, *4* (4), 221–225.
423. J. Y. Lee; E. J. Chang; H. J. Kim; J. H. Park; S. W. Choi, *Arch. Pharm. Res.* **2002**, *25* (3), 313–319.
424. N. M. Ismailov; A. A. Kuliev, *Doklady Akad. Nauk Azerbaidzhanskoi SSR* **1985**, *41* (3), 61–63.
425. R. Omar, *Bull. Fac. Pharm. (Cairo Univ.)* **2002**, *40* (1), 79–83.
426. N. I. Baek; Y. H. Kim; E. M. Ahn; M. H. Bang; J. Y. Nam; B. M. Kwon, *Han'guk Nonghwa Hakhoe Chi.* **1998**, *41* (2), 197–200.
427. Y. Z. Zhou; H. Chen; L. Qiao; N. Xu; J. Q. Cao; Y. H. Pei, *J. Asian Nat. Prod. Res.* **2008**, *10* (5), 429–433.
428. M. B. Zhao; Y. Ito; P. F. Tu, *J. Chromatogr. A* **2005**, *1090* (1–2), 193–196.
429. T. R. Seshadri; R. S. Thakur, *Curr. Sci.* **1960**, *29*, 54–55.
430. T. A. Suleimanov, *Chem. Nat. Compd. (translation of Khim. Prirodnykh Soedinenii)* **2004**, *40* (1), 13–15.
431. Y. Takahashi; N. Miyasaka; S. Tasaka; I. Miura; S. Urano; M. Ikura; K. Hikichi; T. Matsumoto; M. Wada, *Tetrahedron Lett.* **1982**, *23* (49), 5163–5166.
432. Y. Takahashi; K. Saito; M. Yanagiya; M. Ikura; K. Hikichi; T. Matsumoto; M. Wada, *Tetrahedron Lett.* **1984**, *25* (23), 2471–2474.
433. M. R. Meselhy; S. Kadota; Y. Momose; N. Hatakeyama; A. Kusai; M. Hattori; T. Namba, *Chem. Pharm. Bull.* **1993**, *41* (10), 1796–1802.
434. K. Kazuma; K. Sato; T. Matsumoto; T. Okuno, *Tennen Yuki Kagobutsu Toronkai Koen Yoshishu* **1996**, *38*, 7–12.
435. J. Onodera; H. Obara; R. Hirose; S. Matsuba; N. Sato; S. Sato; M. Suzuki, *Chem. Lett.***1989**, (9),1571–1574.
436. H. Z. Guo; D. A. Guo; H. Cheng; J. H. Peng, *Faming Zhuanli Shenqing Gongkai Shuomingshu* **2003**, 8pp.
437. K. Kazuma; E. Shirai; T. Okuno; K. Umeo; T. Matsumoto, *Tennen Yuki Kagobutsu Toronkai Koen Yoshishu* **1994**, *36*, 619–623.
438. K. Kazuma; E. Shirai; M. Wada; K. Umeo; A. Sato; T. Matsumoto; T. Okuno, *Biosci. Biotechnol. Biochem.* **1995**, *59* (8), 1588–1590.
439. T. Kumazawa; S. Sato; D. Kanenari; A. Kunimatsu; R. Hirose; S. Matsuba; H. Obara; M. Suzuki; M. Sato; J. Onodera, *Chem. Lett.* **1994**, *23* (12), 2343–2344.
440. A. Kunimatsu; S. Matsuba; S. Sato; T. Kumazawa; Y. Ozawa; M. Funamizu; H. Obara; J. Onodera; N. Harada; K. Furuhata, *Tennen Yuki Kagobutsu Toronkai Koen Yoshishu* **1997**, *39*, 565–570.
441. H. B. Yin; Z. S. He, *Tetrahedron Lett.* **2000**, *41*, 1955–1958.
442. R. Meselhy; S. Kadota; Y. Momose; M. Hattori; T. Namba, *Chem. Pharm. Bull.* **1992**, *40* (12), 3355–3357.
443. Y. Z. Zhou; H. Chen; L. Qiao; D. F. Hao; H. M. Hua; Y. H. Pei, *Zhongguo Yaowu Huaxue Zazhi (Chin. J. Med. Chem.)* **2007**, *17* (6), 380–382.
444. A. Nagatsu; H. L. Zhang; T. Watanabe; N. Taniguchi; K. Hatano; H. Mizukami; J. Sakakibara, *Chem. Pharm. Bull.* **1998**, *46* (6), 1044–1047.
445. E. O. Kim; J. H. Oh; S. K. Lee; J. Y. Lee; S. W. Choi, *Food Sci. Biotechnol.* **2007**, *16* (1), 71–77.
446. S. Nishibe; A. Sakushima; S. Hisada; I. Inagaki, *Phytochemistry* **1972**, *11* (8), 2629.
447. J. S. Jiang; L. Lu; Y. J. Yang; J. L. Zhang; P. C. Zhang, *J. Asian Nat. Prod. Res.* **2008**, *10* (5), 447–451.
448. H. Sato; H. Kawagishi; T. Nishimura; S. Yoneyama; Y. Yoshimoto; S. Sakamura; A. Furusaki; S. Katsuragi; T. Matsumoto, *Agric. Biol. Chem.* **1985**, *49* (10), 2969–2974.
449. F. Li; Z. S. He; Y. Ye, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **2004**, *35* (3), 247–249.
450. Q. M. Che, *Faming Zhuanli Shenqing Gongkai Shuomingshu* **1998**, 4pp.
451. H. L. Zhang; A. Nagatsu; J. Sakakibara, *Chem. Pharm. Bull.* **1996**, *44* (4), 874–876.
452. T. Akihisa; H. Oinuma; T. Tamura; Y. Kasahara; K. Kumaki; K. Yasukawa; M. Takido, *Phytochemistry* **1994**, *36* (1), 105–108.
453. T. Akihisa; A. Nozaki; Y. Inoue; K. Yasukawa; Y. Kasahara; S. Motohashi; K. Kumaki; N. Tokutake; M. Takido; T. Tamura, *Phytochemistry* **1997**, *45* (4), 725–728.
454. F. Bohlmann; C. Zdero, *Chem. Ber.* **1970**, *103* (9), 2853–2855.
455. S. Kogiso; K. Wada; K. Munakata, *Agric. Biol. Chem.* **1976**, *40* (10), 2085–2089.
456. R. G. Binder; R. E. Lundin; S. Kint; J. M. Klisiewicz; A. C. J. Waiss, *Phytochemistry* **1978**, *17* (2), 315–317.
457. K. Ichihara; M. Noda, *Agric. Biol. Chem.* **1975**, *39* (5), 1103–1108.
458. K. Ichihara; M. Noda, *Biochim. Biophys. Acta, Lipids Lipid Metab.* **1977**, *487* (2), 249–260.
459. C. A. Thomas; E. H. Allen, *Phytopathology* **1970**, *60* (2), 261–263.

460. E. H. Allen; C. A. Thomas, *Phytopathology* **1971**, *61* (9), 1107–1109.
461. H. B. Yin; Z. S. He; Y. Ye, *J. Nat. Prod.* **2000**, *63*, 1164–1165.
462. Y. Z. Zhou; H. Chen; L. Qiao; X. Lu; H. M. Hua; Y. H. Pei, *Helv. Chim. Acta* **2008**, *91*, 1277–1285.
463. J. S. Jiang; P. F. Xia; Z. M. Feng; P. C. Zhang, *Zhongguo Zhongyao Zazhi (China J. Chin. Mater. Med.)* **2008**, *33* (24), 2911–2913.
464. R. Palter; R. E. Lundin; G. Fuller, *Naturwissenschaften* **1969**, *56*, 37.
465. R. N. Yadava; N. Chakravarti, *J. Enzyme Inhib. Med. Chem.* **2008**, *23* (4), 543–548.
466. A. Kobayashi; Y. Sakamoto; K. Kawazu; S. Wakayama; K. Kusaka; T. Kanehira; M. Kudo; T. Akita, *Tennen Yuki Kagobutsu Toronkai Koen Yoshishu* **1992**, *34*, 626–633.
467. G. W. Qin; R. S. Xu, *Med. Res. Rev.* **1998**, *18* (6), 375–382.
468. J. Y. Peng; G. R. Fan; Y. T. Wu, *J. Chromatogr. A* **2005**, *1091*, 89–93.
469. S. Li; W. S. Chen; C. Z. Qiao; S. Q. Zheng, *J. Second Mil. Med. Univ.* **2000**, *21*, 204.
470. Y. Wang; C. Z. Qiao; S. Liu; H. M. Zhang, *Zhongguo Zhongyao Zazhi (China J. Chin. Mater. Med.)* **2000**, *25*, 327.
471. H. Xu; J. G. Fang; Y. H. Liu, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **2003**, *34*, 10.
472. W. S. Chen; B. Li; W. D. Zhang; G. J. Yang; C. Z. Qiao, *Chin. Chem. Lett.* **2001**, *12* (6), 501–502.
473. Y. Peng; L. P. Zhang; H. Song; W. S. Pan; Y. Q. Sun, *Zhongguo Yaowu Huaxue Zazhi (Chin. J. Med. Chem.)* **2005**, *15* (6), 371–372.
474. W. Li; F. K. Chen; X. W. Yin; X. Q. Liu, *J. Shenyang Pharm. Univ. (Shenyang Yaoke Daxue Xuebao)* **2005**, *22* (1), 15–16, 44.
475. X. Y. Wu; Y. H. Liu; W. Y. Sheng; J. Sun; G. W. Qin, *Planta Med.* **1997**, *63* (1), 55–57.
476. J. Wu; Y. X. Xie, *Shizhen Guoyi Guoyao* **2006**, *17* (10), 2067–2068.
477. J. F. Liu; Z. Y. Jiang; R. R. Wang; Y. T. Zheng; J. J. Chen; X. M. Zhang; Y. B. Ma, *Org. Lett.* **2007**, *9* (21), 4127–4129.
478. B. Li; W. S. Chen; H. M. Zhang; W. D. Zhang; G. J. Yang; C. Z. Qiao, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **2003**, *38* (6), 430–432.
479. X. Y. Wu; G. W. Qin; K. K. Cheung; K. F. Cheng, *Tetrahedron* **1997**, *53* (39), 13323–13328.
480. F. N. Wang; R. L. Zhang; L. J. Wu, *J. Shenyang Pharm. Univ. (Shenyang Yaoke Daxue Xuebao)* **2005**, *22* (3), 187–188, 212.
481. J. F. Liu; X. M. Zhang; D. Q. Xue; Z. Y. Jiang; Q. Gu; J. J. Chen, *Zhongguo Zhongyao Zazhi (China J. Chin. Mater. Med.)* **2006**, *31* (23), 1961–1965.
482. Y. H. Liu; G. W. Qin; S. P. Ding; X. Y. Wu, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **2001**, *32* (12), 1057–1060.
483. J. L. Ruan; J. H. Zou; Y. L. Cai, *Zhongguo Zhongyao Zazhi (China J. Chin. Mater. Med.)* **2005**, *30* (19), 1525–1526.
484. L. Zuo; J. B. Li; J. Xu; J. Z. Yang; D. M. Zhang; Y. L. Tong, *Zhongguo Zhongyao Zazhi (China J. Chin. Mater. Med.)* **2007**, *32* (8), 688–691.
485. Y. H. Liu; G. W. Qin; S. P. Ding; X. Y. Wu, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **2002**, *33* (2), 97–99.
486. Q. H. Li, *Zhiwu Xuebao* **1987**, *29* (1), 67–72.
487. B. Li; W. S. Chen; S. Q. Zheng; G. J. Yang; C. Z. Qiao, *Yao Xue Xue Bao (Acta Pharm. Sin.)* **2000**, *35* (7), 508–510.
488. T. Mohn; M. Hamburger, *Planta Med.* **2008**, *74* (8), 885–888.
489. R. Liu; B. Yuan; Z. G. Liu; X. Q. Li; Z. L. Xiong; F. M. Li, *Zhongyaocai (J. Chin. Med. Mater.)* **2005**, *28* (9), 772–774.
490. X. Li; D. D. Sun; J. W. Chen; L. W. He; H. Q. Zhang; H. Q. Xu, *Fitoterapia* **2007**, *78* (7–8), 490–495.
491. S. H. Zhang, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **1983**, *14* (6), 247–248, 246.
492. J. G. Fang; S. B. Wang; H. Xu; Y. H. Liu; Y. W. Liu, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **2004**, *35* (8), 845–846.
493. D. D. Sun; X. Li; J. W. Chen; L. W. He; J. Wu, *Huaxi Yaoxue Zazhi* **2007**, *22* (5), 487–488.
494. Q. S. Huang; K. Yoshihira; S. Natori, *Planta Med.* **1981**, *42* (3), 308–310.
495. H. L. Liu; L. J. Wu; H. Li; J. Wang, *J. Shenyang Pharm. Univ. (Shenyang Yaoke Daxue Xuebao)* **2002**, *19* (2), 93–95, 100.
496. H. Xu; J. G. Fang; S. B. Wang; Y. W. Liu, *Chin. Pharm. J. (Zhongguo Yaoxue Zazhi)* **2003**, *38* (6), 418–419.
497. B. Li; W. S. Chen; Y. Zhao; H. M. Zhang; J. X. Dong; C. Z. Qiao, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **2005**, *36* (3), 326–328.
498. Y. He; J. Lu; R. C. Lin, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **2003**, *34* (9), 777–778.
499. G. H. Gao; X. Y. Deng; J. Liu; F. M. Li, *J. Shenyang Pharm. Univ. (Shenyang Yaoke Daxue Xuebao)* **2007**, *24* (12), 748–750.
500. D. Q. Xue; J. F. Liu; X. M. Zhang; X. Gu; Z. Y. Jiang; J. J. Chen; Q. Wang, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **2006**, *37* (9), 1304–1309.
501. J. Y. Peng; G. R. Fan; Y. T. Wu, *J. Chromatogr. A* **2005**, *1091* (1–2), 89–93.
502. H. Chang; P. But, *Pharmacology and Applications of Chinese Materia Medica*; World Scientific: Singapore, 1987, 1041pp.
503. Astragalus Membranaceus, *Altern. Med. Rev.* **2003**, *8* (1), 72–77.
504. Y. H. Kuo; W. J. Tsai; S. H. Loke; T. S. Wu; W. F. Chiou, *J. Ethnopharmacol.* **2009**, *122*, 28–34.
505. X. Du; Y. Bai; H. Liang; Z. Wang; Y. Zhao; Q. Zhang; L. Huang, *Magn. Reson. Chem.* **2006**, *44*, 708–712.
506. Y. H. Kuo; W. J. Tsai; S. H. Loke; T. S. Wu; W. F. Chiou, *J. Ethnopharmacol.* **2009**, *122*, 28–34.
507. X. G. Du; Y. J. Bai; H. Liang; Z. Y. Wang; Y. Y. Zhao; Q. Y. Zhang; L. Q. Huang, *Magn. Reson. Chem.* **2006**, *44*, 708–712.
508. P. Shen; M. H. Liu; T. Y. Ng; Y. H. Chan; E. L. Yong, *J. Nutr.* **2006**, *136* (4), 899–905.
509. S. Ohkawara; Y. Okuma; T. Uehara; T. Yamagishi; Y. Nomura, *Eur. J. Pharmacol.* **2005**, *525* (1–3), 41–47.
510. Y. H. Pei; R. F. Li; H. W. Fu; J. Wang; Y. Z. Zhou, *Fitoterapia* **2007**, *78*, 602–604.
511. W. Liu; J. Chen; W. J. Zuo; X. Li; J. H. Wang, *Chin. Chem. Lett.* **2007**, *18*, 1092–1094.
512. Y. Shirataki; M. Takao; S. Yoshida, *Phytother. Res.* **1997**, *11*, 603–605.
513. Z. Q. He; J. A. Findlay, *J. Nat. Prod.* **1991**, *54* (3), 810–815.
514. Z. Z. Cao; Y. Cao; Y. J. Yi; Y. P. Wu; Z. K. Len; D. Li; N. L. Owen, *Chin. Chem. Lett.* **1998**, *9* (6), 537–538.
515. J. S. Kim; M. H. Yean; E. J. Lee; H. S. Jung; J. Y. Lee; Y. J. Kim; S. S. Kang, *Chem. Pharm. Bull.* **2008**, *56* (1), 105–108.
516. M. Du; X. J. Wu; J. Ding; Z. B. Hu; K. N. White; C. J. Branford-White, *Biotechnol. Lett.* **2003**, *25* (21), 1853–1856.
517. Y. Zhou; M. Hirotani; H. Rui, *Phytochemistry* **1995**, *38* (6), 1407–1410.
518. I. Kitagawa; H. K. Wang; M. Yoshikawa, *Chem. Pharm. Bull.* **1983**, *31* (2), 716–722.
519. I. Kitagawa; H. K. Wang; M. Saito; M. Yoshikawa, *Chem. Pharm. Bull.* **1983**, *31* (2), 709–715.
520. I. Kitagawa; H. K. Wang; M. Saito; A. Takagi; M. Yoshikawa, *Chem. Pharm. Bull.* **1983**, *31* (2), 698–708.
521. I. Kitagawa; H. K. Wang; A. Takagi; M. Fuchida; I. Miura; M. Yoshikawa, *Chem. Pharm. Bull.* **1983**, *31* (2), 689–697.
522. M. Hirotani; Y. Zhou; H. K. Lui; T. Furuya, *Phytochemistry* **1994**, *36* (3), 665–670.
523. M. T. Baretta; G. Ruberto, *Planta Med.* **1997**, *63* (3), 280–282.

524. M. Hirotani; Y. Zhou; H. K. Rui; T. Furuya, *Phytochemistry* **1994**, *37* (5), 1403–1407.
525. Y. L. Ma; Z. K. Tian; H. X. Kuang; C. S. Yuan; C. J. Shao; K. Ohtani; R. Kasai; O. Tanaka; Y. Okada; T. Okuyama, *Chem. Pharm. Bull.* **1997**, *45* (2), 359–361.
526. Xinjiang Institute of Traditional Chinese and Ethnologic Medicines Ed., *Culture Techniques of Xinjiang Staple Medicinal Plants*; Xinjiang Science and Technology Press: Xinjiang, 2004; pp 84–88.
527. X. Geng; L. Song; X. Pu; P. Tu, *Biol. Pharm. Bull.* **2004**, *27* (6), 797–801.
528. X. M. Wu; P. Tu, *Pharmazie* **2004**, *59* (10), 815–816.
529. X. M. Wu; P. F. Tu, *Beijing Daxue Xuebao (J. Peking Univ.)* **2004**, *36* (1), 24–26.
530. X. M. Wu; X. M. Gao; K. W. K. Tsimc; P. F. Tu, *Int. J. Biol. Macromol.* **2005**, *37*, 278–282.
531. Z. H. Song; P. F. Tu; Y. Y. Zao; J. H. Zheng, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **2000**, *31* (11), 808–810.
532. P. F. Tu; Y. P. He; Z. C. Lou, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **1994**, *25*, 451–452.
533. P. F. Tu; Y. P. He; Z. C. Lou, *Tianran Chanwu Yanjiu Yu Kaifa (Nat. Prod. Res. Dev.)* **1997**, *9*, 7–10.
534. Z. H. Song; S. H. Mo; Y. Chen; P. F. Tu; Y. Y. Zhao; J. H. Zheng, *Zhongguo Zhongyao Zazhi (China J. Chin. Mater. Med.)* **2000**, *25*, 728–730.
535. Y. Jiang; P. F. Tu, *J. Chromatogr. A* **2009**, *1216*, 1970–1979.
536. C. Z. Liu; X. Y. Cheng, *Plant Cell Rep.* **2008**, *27* (2), 357–362.
537. X. F. Tian; X. P. Pu, *J. Ethnopharmacol.* **2005**, *97* (1), 59–63.
538. Y. Sun; D. J. Wang; X. M. Liu; L. Shi; H. Q. Zhang, *Chin. Pharmacol. Bull.* **2002**, *18*, 84–87.
539. Q. L. Zeng; J. H. Mao; Z. L. Lu, *J. Zhejiang Med. Univ.* **1998**, *27*, 108–111.
540. D. J. Xue; M. Zhang; X. H. Wu; X. D. Chen; Y. C. Zhang, *Zhongguo Zhongyao Zazhi (China J. Chin. Mater. Med.)* **1995**, *20*, 6.
541. D. Xue; M. Zhang; X. Wu; X. Chen; Y. Zhan, *Zhongguo Zhongyao Zazhi (China J. Chin. Mater. Med.)* **1995**, *20* (11), 687–689, 704.
542. Q. Dong; J. Yao; J. N. Fang; K. Ding, *Carbohydr. Res.* **2007**, *342*, 1343–1349.
543. H. Kobayashi; H. Karasawa; T. Miyase; S. Fukushima, *Chem. Pharm. Bull.* **1984**, *32*, 3880–3885.
544. H. Kobayashi; H. Oguchi; N. Takizawa; T. Miyase; A. Ueno; K. Usmanghani; M. Ahmad, *Chem. Pharm. Bull.* **1987**, *35* (8), 3309–3314.
545. N. S. Du; H. Wang; Y. H. Yi, *Tianran Chanwu Yanjiu Yu Kaifa (Nat. Prod. Res. Dev.)* **1993**, *5*, 5–8.
546. H. Kobayashi; H. Karasawa; T. Miyase, *Chem. Pharm. Bull.* **1984**, *32*, 3009–3014.
547. M. H. Cheng; F. S. Liu; J. P. Xu, *Zhongguo Zhongyao Zazhi (China J. Chin. Mater. Med.)* **1993**, *18*, 424–425.
548. N. S. Du; P. W. Zhou; J. Wang, *J. Chin. Pharm. Univ.* **1993**, *24*, 46–48.
549. W. H. Xu; S. X. Qiu; J. H. Zhao; Y. Xu; K. W. Wan; Q. X. Yan; C. Y. Ji; H. Qin, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **1994**, *25* (10), 509–513.
550. H. Kobayashi; H. Karasawa; T. Miyase, *Chem. Pharm. Bull.* **1985**, *33*, 1452.
551. H. Karasawa; H. Kobayashi; N. Takizawa, *Yakugaku Zasshi* **1986**, *106*, 721–724.
552. H. Karasawa; H. Kobayashi; N. Takizawa; T. Miyase, *Yakugaku Zasshi* **1986**, *106* (7), 562–566.
553. Q. Xiong; S. Kadota; T. Tani; T. Namba, *Biol. Pharm. Bull.* **1996**, *19*, 1580–1585.
554. F. Yoshizawa; T. Deyama; N. Takizawa; K. Usmanghani; M. Ahmed, *Chem. Pharm. Bull.* **1990**, *38*, 1927.
555. P. F. Tu; Z. H. Song; H. M. Shi; Y. Jiang; Y. Y. Zhao, *Helv. Chim. Acta* **2006**, *89* (5), 927–935.
556. M. Yoshikawa; H. Matsuda; T. Morikawa; H. Xie; S. Nakamura; O. Muraoka, *Bioorg. Med. Chem.* **2006**, *14*, 7468–7475.
557. C. H. Xu; J. S. Yang; R. M. Lv, *Zhongcaoyao (Chin. Trad. Herb. Drugs)* **1999**, *30*, 244–246.
558. P. Z. Yu; C. Q. Hu; E. J. Meehan; L. Q. Chen, *Chem. Biodivers.* **2007**, *4*, 508–513.
559. H. H. Xie; T. Morikawa; H. S. S. Matsuda; S. K. Nakamura; O. S. Muraoka; M. S. Yoshikawa, *Chem. Pharm. Bull.* **2006**, *54*, 669–675.
560. H. Kobayashi; H. Karasawa; T. Miyase, *Chem. Pharm. Bull.* **1984**, *32*, 1729–1734.
561. H. Kobayashi; H. Karasawa; T. Miyase; S. Fukushima, *Chem. Pharm. Bull.* **1985**, *33*, 3645–3650.
562. H. Kobayashi; J. Komatsu, *Yakugaku Zasshi* **1983**, *103*, 508–511.
563. S. F. Ruo; Y. Ying; Y. H. Liu, *Bull. Chin. Mater. Med.* **1986**, *11*, 681.
564. L. Lei; F. Q. Yang; T. Y. Zhang; P. F. Tu; L. J. Wu; Y. Ito, *J. Chromatogr. A* **2001**, *912*, 181–185.
565. L. Li; R. Tsao; R. Yang; C. M. Liu; J. C. Young; H. H. Zhu, *Food Chem.* **2008**, *108*, 702–710.
566. K. Hayashi, *Nat. Med.* **2004**, *58* (6), 307–310.
567. Z. H. Xu; J. S. Yang; R. M. Lu; Y. Lu; J. G. Zhou; Q. T. Zheng; S. S. Yang, *J. Chin. Pharm. Sci.* **1999**, *8* (2), 61–63.
568. K. Venkataraman, *Proc. Ind. Natl. Acad. Sci. U.S.A.* **1973**, *39A*, 365.
569. S. Kasibhatla; K. A. Jessen; S. Maliartchouk; J. Y. Wang; N. M. English; J. Drewe; L. Qiu; S. P. Archer; A. E. Ponce; N. Sirisoma; S. Jiang; H. Z. Zhang; K. R. Gehlsen; S. X. Cai; D. R. Green; B. Tseng, *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 12095.
570. M. K. Pandey; B. Sung; K. S. Ahn; A. B. Kunnumakkara; M. M. Chaturvedi; B. B. Aggarwal, *Blood* **2007**, *110*, 3517.
571. Y. Qin; L. Meng; C. Hu; W. Duan; Z. Zuo; L. Lin; X. Zhang; J. Ding, *Mol. Cancer Ther.* **2007**, *6*, 2429.
572. N. Lu; Y. Yang; Q. D. You; Y. Ling; Y. Gao; H. Y. Gu; L. Zhao; X. T. Wang; Q. L. Guo, *Cancer Lett.* **2007**, *258*, 80.
573. Q. B. Han; Y. Zhou; C. Feng; G. Xu; S. X. Huang; S. L. Li; C. F. Qiao; J. Z. Song; D. C. Chang; K. Q. Luo; H. X. Xu, *J. Chromatogr. B* **2009**, *877*, 401–407.
574. Z. T. Zhou; J. W. Wang, *Chin. J. N. Drugs* **2007**, *16*, 79.
575. S. J. Tao; S. H. Guan; W. Wang; Z. Q. Lu; G. T. Chen; N. Sha; Q. X. Yue; X. Liu; D. A. Guo, *J. Nat. Prod.* **2009**, *72*, 117–124.
576. J. Asano; K. Chiba; M. Tada; T. Yoshii, *Phytochemistry* **1996**, *41*, 815–820.
577. Y. W. Leong; L. J. Harrison; G. J. Bennett; H. T. W. Tan, *J. Chem. Res.* **1996**, 392–393.
578. Q. B. Han; L. Yang; Y. Liu; Y. L. Wang; C. F. Qiao; J. Z. Song; L. J. Xu; D. J. Yang; S. L. Chen; H. X. Xu, *Planta Med.* **2006**, *72*, 281–284.
579. P. M. Nair; K. Venkataraman, *Ind. J. Chem.* **1964**, *2*, 402–404.
580. C. G. Karanjgaonkar; P. M. Nair; K. Venkataraman, *Tetrahedron Lett.* **1966**, *7*, 687–691.
581. H. B. Bhat; P. M. Nair; K. Venkataraman, *Ind. J. Chem.* **1964**, *2*, 405–409.
582. L. L. Wang; Z. L. Li; Y. P. Xu; X. Q. Liu; Y. H. Pei; Y. K. Jing; H. M. Hua, *Chin. Chem. Lett.* **2008**, *19*, 1221–1223.
583. Q. B. Han; L. Yang; Y. L. Wang; C. F. Qiao; J. Z. Song; H. D. Sun; H. X. Xu, *Chem. Biodivers.* **2006**, *3*, 101–105.

584. V. Reutrakul; N. Anantachoke; M. Pohmakotr; T. Jaipetch; S. Sophasan; C. Yoosook; J. Kasisit; C. Napaswat; T. Santisuk; P. Tuchinda, *Planta Med.* **2007**, *73*, 33–40.
585. Q. B. Han; Y. L. Wang; L. Yang; T. F. Tso; C. F. Qiao; J. Z. Song; L. J. Xu; S. L. Chen; D. J. Yang; H. X. Xu, *Chem. Pharm. Bull.* **2006**, *54*, 265–267.
586. S. X. Cai; H. Z. Zhang; Y. Wang; B. Tseng; S. Kasibhatla; J. A. Drewe, *PCT Int. Appl.* **2000**, 123.
587. L. J. Lin; L. Z. Lin; J. M. Pezzuto; G. A. Cordell, *Magn. Reson. Chem.* **1993**, *31*, 340–347.
588. B. S. Rao, *J. Chem. Soc.* **1937**, 853–855.
589. Y. Sukpondma; B. Rukachaisirikul; S. Phongpaichit, *Chem. Pharm. Bull.* **2005**, *53*, 850–852.
590. F. Feng; W. Y. Liu; Y. S. Chen; Q. L. Guo; Q. D. You, *J. Asian Nat. Prod. Res.* **2007**, *9*, 735–741.
591. S. B. Lee; C. M. Chen, U.S. Patent Appl. Publ. CODEN: USXXCO US 2005261363 A1 20051124, 2005, 22pp.
592. L. L. Wang; Z. L. Li; D. D. Song; L. Sun; Y. H. Pei; Y. K. Jing; H. M. Hua, *Planta Med.* **2008**, *74* (14), 1735–1740.

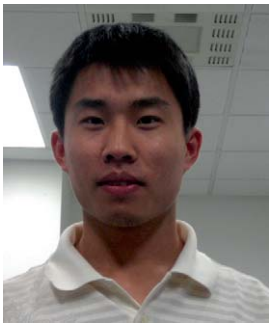
Biographical Sketches

Min Yang is an associate professor at Shanghai Institute of Materia Medica, Chinese Academy of Sciences. He obtained his Ph.D. in 2004 from Lanzhou University and obtained postdoctoral training at the School of Pharmaceutical Sciences, Peking University from 2004 to 2006. His research focusses on the analysis and metabolism of bioactive natural products and quality control of TCMs.

Sijia Tao is a Ph.D student under D. A. Guo at Shanghai Research Center for Modernization of TCM, Shanghai Institute of Materia Medica, Chinese Academy of Sciences. She has majored in pharmaceutical chemistry and her research project is entitled 'Phytochemical and Metabolic Studies of *Garcinia Hanburyi*'.

Shuhong Guan is an Associate Professor in the Shanghai Research Center for TCM modernization, Shanghai Institute of Materia Medica, Chinese Academy of Sciences. She did her under- and postgraduate training in Changchun University of TCM, obtained doctorate training in Germany, and received Ph.D. degree in Peking University. Her research is focused on the investigation of principle components of TCM and establishment of modern quality control techniques of TCM.

Xiaohui Wu is a postgraduate student under De-an Guo in Shanghai Institute of Materia Medica, Chinese Academy of Sciences. He obtained his undergraduate training in TongJi Medical College in Wuhan. His doctorate research project is entitled 'Studies on the chemical constituents and metabolism of *Catsia tora*'.



Pingping Xu is a postgraduate student under De-an Guo in Shanghai Institute of Materia Medica, Chinese Academy of Sciences. He obtained his undergraduate training in Shandong

University of Traditional Chinese Medicine. His doctorate research project is entitled "Studies on the chemical constitutes and metabolism of *Caesalpinia sappan L.*".

De-an Guo, professor of pharmacognosy, currently the director of Shanghai Research Center for TCM Modernization, Shanghai Institute of Materia Medica, Chinese Academy of Sciences. He received his Ph.D. in 1990 from the Beijing Medical University. He was a postdoctoral fellow at the Texas Tech University from 1993 to 1996. His major research interest includes bioactive principles of Chinese herbal medicines, biomedical and pharmaceutical analysis of marker compounds or active constituents in herbal medicines and their related products, biotransformation of natural products, pharmacokinetics of herbal complex systems, and herbal quality control.

3.14 Ayurveda in Modern Medicine: Development and Modification of Bioactivity

Pulok K. Mukherjee, M. Venkatesh, and Arunava Gantait, Jadavpur University, Kolkata, India

3.14.1 Introduction

Ayurveda is one of the oldest systems of medicine followed among the countries of the world. Its origin can be traced back to 4500 BC, based on the ancient knowledge contained in Rigveda–Atharvaveda. It deals with the totality of individual and social health including preventive and curative aspects.[1] In fact Ayurveda is a way of life based on emphasis on certain diet, lifestyle, and yoga practices suitable for an individual according to his/her constitution. The basic concept of Ayurveda is based on the fact that the universe is made up of five elements: earth, air, water, fire, and space. Human beings are composed of these same elements. These five elements interact and in humans they occur as three doshas (vata, pitta, and kapha), called 'tridoshas'. When the doshas are out of balance, the body does not function properly and disease will follow. The treatment strategy for Ayurveda is based on these concepts (**Table 1**).[2]

Until the discovery of modern medicines, any system of medicine that relieved the patient of their ailments was considered to be a therapeutic system without further investigation. Subsequently, with the development of modern medicine, the systems that did not give scientifically validated results and an immediate remedy were set aside. Thus Naturopathy, Ayurveda, Chinese Medicine, and Homeopathy all were devalued during the onward march of modern medicine. In spite of these setbacks, the interest in Ayurveda is now increasing worldwide along with the incorporation of modern diagnostic tests and scientific validations of the system. But one common thing is

Table 1 Component of 'tridoshas' in Ayurveda

Component of tridoshas	*Implication*
Vayu (vata)	Explains the biological phenomenon controlled by central and autonomic nervous system. Diseases may be developed due to 'vayu' alone or in combination with 'pitta' and 'kapha.'
Pitta	The expression of energy in human beings that helps daily activities, such as tissue building/blood pigmentation, digestion, and so on.
Kapha	A function of heat regulation which provides nutrition to body tissues and includes the formation of various body fluids, such as mucus, sinovial fluid, and so on.

that both modern medicine and Ayurveda attempt to give relief to the suffering patients. In almost all the traditional systems of medicine, the medicinal plants play a major role and constitute their backbone. The *Indian Materia Medica* includes about 2000 drugs of natural origin, almost all of which are derived from different traditional systems and folklore practices.[3] In fact several allopathic drugs used in the treatment of significant ailments, such as digitoxin, reserpine, withanolide, taxol, silymarin, and so on, have been developed from ayurvedic medicinal plants. The traditional system of medicines used worldwide like Ayurveda in India, Kampo in Japan, Traditional Chinese Medicine in China, Unani medicine of Greco–Arabian origin, and Tibetan medicine has a long and impressive history of effectiveness. The increasing use of traditional therapies demands more scientifically sound evidence for the principles behind therapies and for effectiveness of medicines. Modern research has now confirmed the usefulness and safety of these botanicals. With the developing technology and research capabilities, many studies have been conducted worldwide in the area of ayurvedic herbals leading to scientific evidence for the activities of the age-old system of medicine. Recent advances in the analytical and biological sciences, along with innovations in genomics and proteomics, can play an important role in validation of these therapies. In the last 50 years, the teaching and training specialties of Ayurveda are more focused toward diagnosis, treatment, and drug development and have developed into 16 specialties. The treatments are enriched by accepting and adopting the outcomes of experience. There are several formulations available from the ayurvedic formulary and Pharmacopoeia of India, which has been explored to a wide extent for treatment of several disorders and have potential market as well. A list of the most important ayurvedic formulations available in the Indian market has been produced in **Table 2**.[4] In this context, this chapter attempts to give an insight into various scientific techniques for the development and modification of bioactivity of those modern medicines from the ayurvedic herbals.

Table 2 Several ayurvedic formulations used extensively in present-day practice as prescribed in the Ayurvedic Formulary of India[4]

Name of formulation	*Intended use*
Triphala choorna	Increased frequency and turbidity of urine, diseases of eye, diseases of skin, dyspepsia, loss of sense of taste, intermittent fever
Chawanprash	Cough, asthma, debility due to chest injury, hoarseness of voice, pthisis, heart disease, digestive impairment, urinary disease, diseases of semen
Aswagandha aristha	Syncope, epilepsy, psychosis, cachexia, piles, digestive impairment
Asokaristha	Dysmenorrhea, pain in female genital tract, leucorrhea, fever, bleeding disorder, piles, loss of sense of taste, excessive flow of urine, inflammation
Draksharistha	Cough, digestive impairment, chest wound, pthisis, laxative, weakness, disease of throat
Bhaskar lavan	Digestive impairment, pain/colic, malabsorption syndrome, spleen disease, diseases of abdomen, piles, constipation, fistula-in-ano, edema, rheumatism, angina pectoris
Baiswanar choorna	Flatulence with gurgling sound, abdominal lump, duodenal ulcer, rheumatism, heart disease, diseases of urinary bladder, spleen disease, anorectal disease, constipation, disease of the limbs
Chandraprava vati	Constipation, distension of abdomen due to obstruction to passage of urine and stools, cyst, anemia, jaundice, dysuria, piles, urinary obstruction, lower backache, itching, splenomegaly, gynecological disorder, loss of sense of taste
Sankha vati	Digestive impairment, malabsorption syndrome, loss of sense of taste, duodenal ulcer, pthisis
Dasamularista	Loss of sense of taste, emesis, malabsorption syndrome, abdominal lump, tissue wasting, piles, fistula-in-ano, anemia, excessive flow of urine, gravel in urine, infertility, emaciation, weakness
Punarnavasava	Hyperacidity, abdominal lump, inflammation, diseases of liver

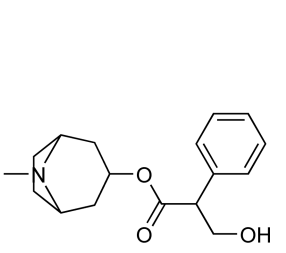
(*Continued*)

Table 2 (Continued)

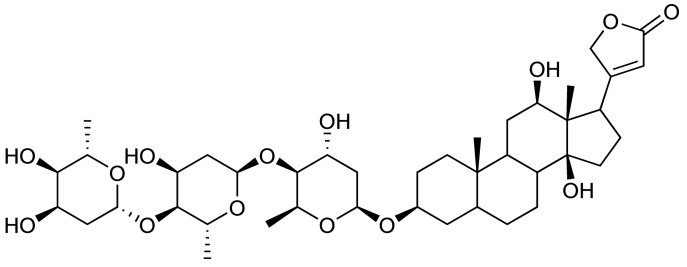
Name of formulation	*Intended use*
Jatamansyarka	Digestive impairment, loss of sense of taste, halitosis, epilepsy, psychosis
Sarsapadi pralepa	Cyst, goiter, cervical lymphadenitis
Manikya pisti	Loss of immunity, heart disease, deficiency of semen, digestive impairment, weakness, low intelligence
Laghvananda rasa	Digestive impairment, anemia, fever, vertigo, excessive vaginal discharge, loss of sense of taste, abdominal lump
Dhanvantara taila	Neurological disease, paralysis, quadriplegia, tissue wasting, diseases of children
Maha Pancagavya ghrta	Diseases of abdomen, fistula-in-ano, intermittent fever, edema, epilepsy, piles, jaundice, anemia, abdominal lump, cough
Mukta bhasma	Cough, asthma, chronic fever, dentitional fever, weakness of heart, mental disorder
Pippalyadi lauha	Digestive impairment, emesis, hiccup, asthma, low-grade fever
Bilvadi leha	Loss of sense of taste, digestive impairment, emesis, excessive salivation

3.14.2 Plant-Based Pharmaceuticals from Ayurveda

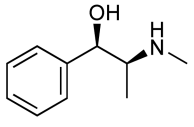
Throughout the history of drug development, plants are an important source for the discovery of novel therapeutically active compounds. In India, around 25 000 effective plant-based formulations are used in traditional and folk medicine. It is estimated that more than 7800 manufacturing units are involved in the production of natural health products and traditional plant-based formulations in India, which requires more than 2000 tons of medicinal plant raw material annually.[5] The diversity in ayurvedic plants is a source of templates for structure optimization programs designed to make new chemical entities. Many conventional drugs originate from plant sources: a century ago, most of the few effective drugs were plant based. Examples include atropine [1], digoxin [2], ephedrine [3], morphine [4], physostigmine [5], quinine [6], reserpine [7], sennoside [8], glycyrrhizin [9], and psoralen [10].[6] A simple flowchart for explaining the study of plants used in traditional medicine is shown in **Figure 1**.[7] Combining the strengths of the knowledge base of complementary alternative medicines such as Ayurveda with the dramatic power of combinatorial sciences and high-throughput screening will help in the generation of structure–activity libraries. The development of drugs from ayurvedic plants continues, with drug companies engaged in large-scale pharmacological screening of herbs.[8] There is a revival of interest in ayurvedic herbal products at a global level: herbs such as turmeric, neem, ginger, holy basil, and ashwagandha are a few examples of what is gaining popularity among modern physicians. A list of ayurvedic plants with their therapeutic potentials, phytoconstituents, and uses are given in **Table 3**.



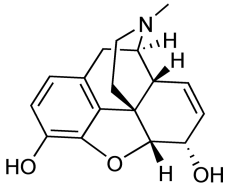
Atropine [**1**]



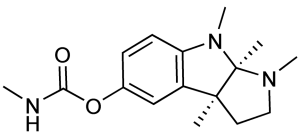
Digoxin [**2**]



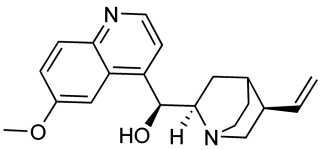
Ephedrine [**3**]



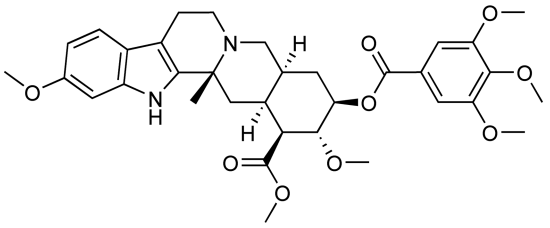
Morphine [**4**]



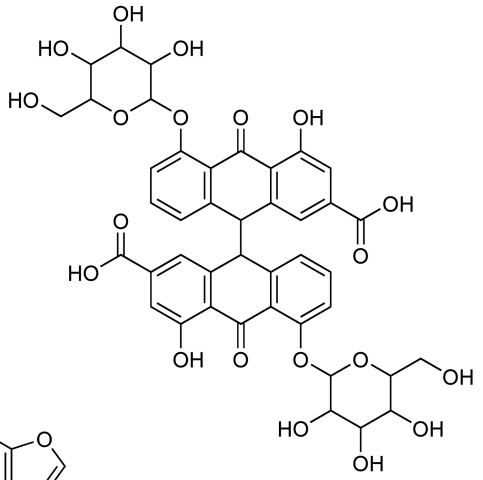
Physostigmine [**5**]



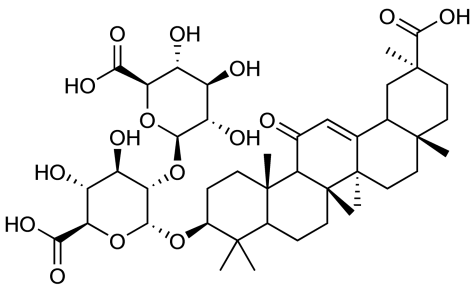
Quinine [**6**]



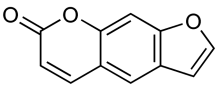
Reserpine [**7**]



Sennoside [**8**]



Glycyrrhizin [**9**]



Psoralen [**10**]

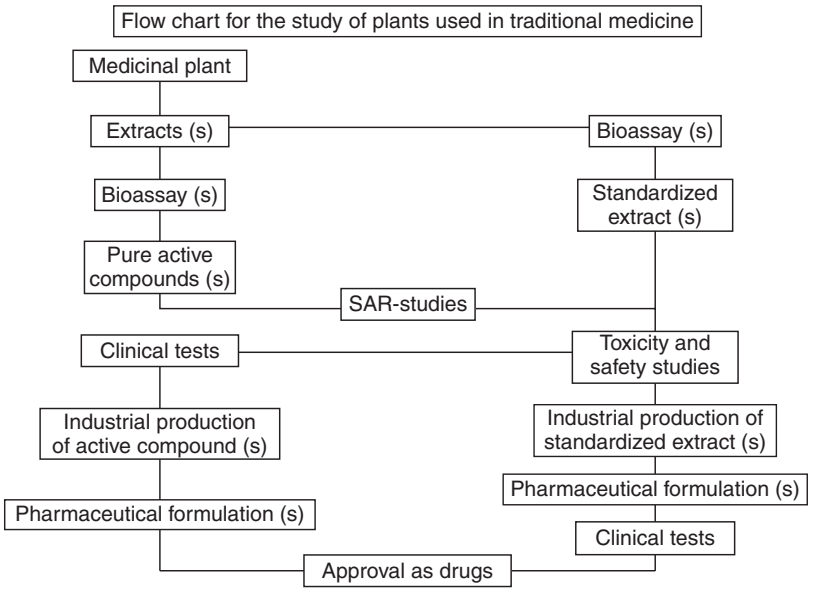
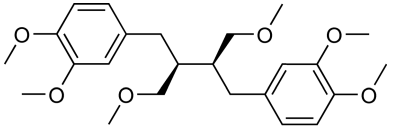
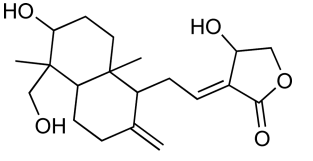
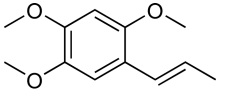
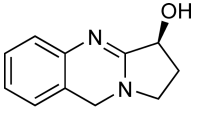


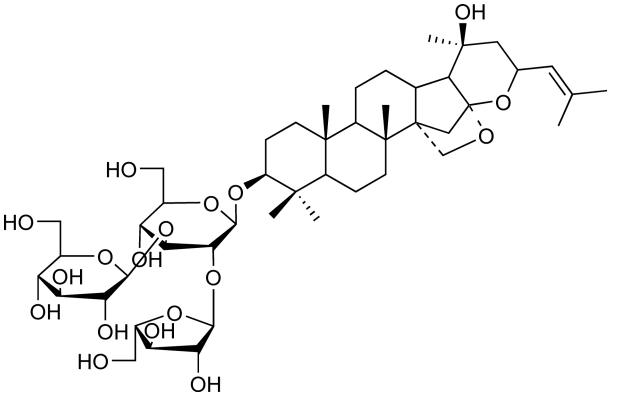
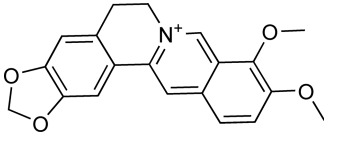
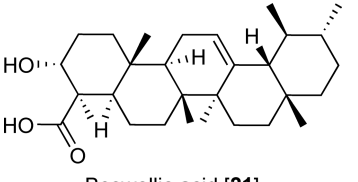
Figure 1 Flowchart for study of plants used in traditional medicine. Reproduced with permission from L. Pieters; A. J. Vlietinck, *J. Ethnopharmacol.* **2005**, *100*, 58.

Table 3 List of ayurvedic plants with their therapeutic potentials, phytoconstituents, and uses

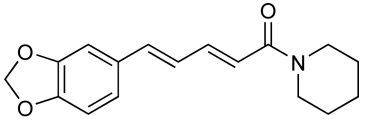
Ayurvedic source	*Biological name*	*Family*	*Parts used*	*Active constituent*	*Pharmacological activity (reference)*
Haridra	*Curcuma longa* Linn.	Zingiberaceae	Rhizome	Curcumin	Anti-inflammatory[9]
Bhumyamalaki	*Phyllanthus amarus* Schum. and Thonn.	Euphorbiaceae	Whole plant	Phyllanthin [**15**]  Phyllanthin [**15**]	Hepatoprotective[9]
Kaalmegha	*Andrographis paniculata* Wall. ex Nees.	Acanthaceae	Aerial part	Andrographolide [**16**]  Andrographolide [**16**]	Hepatoprotective[9]
Vacha	*Acorus calamus* Linn.	Araceae	Rhizome	β-Asarone [**17**]  β-Asarone [**17**]	CNS Active[9]
Vasaka	*Adhatoda vasica* Nees.	Acanthaceae	Leaves	Vasicine [**18**]  Vasicine [**18**]	Bronchodilator, expectorant[9]

(*Continued*)

Table 3 (Continued)

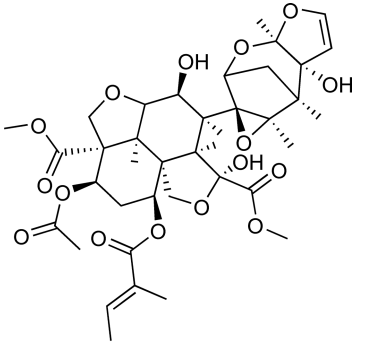
Ayurvedic source	*Biological name*	*Family*	*Parts used*	*Active constituent*	*Pharmacological activity (reference)*
Brahmi	*Bacopa monnieri* (Linn.) Penn.	Scrophulariaceae	Whole plant	Bacoside A_3 [**19**] 	Brain Tonic[9]
Daruharidra	*Berberis aristata* DC.	Berberidaceae	Stem	Berberine [**20**]  Berberine [**20**]	Diaphoretic, anti-inflammatory[9]
Shallaki	*Boswellia serrata* Roxb.	Burseraceae	Gum resin	Boswellic acid [**21**]  Boswellic acid [**21**]	Antiarthritic[9]
Guggul	*Commiphora mukul* (Hook. ex Stocks) Engl.	Burseraceae	Oleo-gum-resin	Guggulsterone-Z	Hypolipidemic[9]

Aamalaki	*Emblica officinalis* Gaertn.	Euphorbiaceae	Fruit	Gallic acid	Carminative, cerebral and G.I. tonic[9]
Yashtimadhu	*Glycyrrhiza glabra* Linn.	Papilionaceae	Root	Glycyrrhizin	Antitussive[9]
Pippali	*Piper longum* Linn.	Piperaceae	Fruit	Piperine [**22**]	Cough and cold[9]



Piperine [**22**]

Ashwagandha	*Withania somnifera* Linn.	Solanaceae	Root	Withanolides	Adaptogen[9]
Lashuna	*Allium sativum* Linn.	Liliaceae	Bulb	Alliin	Hypocholesterolemic and antibiotic[10]
Suuchi	*Atropa belladonna* auct.	Solanaceae	Leaf and root	Atropine	Parasympatholytic[10]
Nimba	*Azadirachta indica* A. Juss.	Meliaceae	Leaf and stembark	Azadirachtin [**23**]	Antimicrobial, anthelmintic[10]



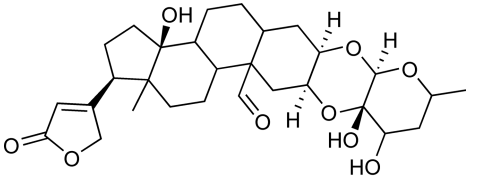
Azadirachtin [**23**]

Svarnapatri	*Cassia angustifolia* Vahl.	Caesalpiniaceae	Leaves	Sennoside	Purgative[10]

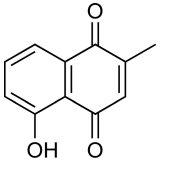
(*Continued*)

Table 3 (Continued)

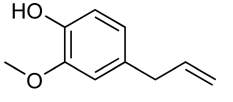
Ayurvedic source	*Biological name*	*Family*	*Parts used*	*Active constituent*	*Pharmacological activity (reference)*
Alarka	*Calotropis procera* (Ait.) R.Br.	Asclepiadaceae	Leaf, root, stembark	Calotropin [24]	Antitumor[11]
Asana	*Pterocarpus marsupium* Roxb.	Papilionaceae	Heartwood, stembark	Pterostilbene	Antidiabetic[10]
Atibala	*Abutilon indicum* Linn. Sweet.	Malvaceae	Root	Asparagine	Diuretic[10]
Somaraaji	*Psoralea corylifolia* Linn.	Papilionaceae	Seed	Psoralen	Used in leucoderma, cytotoxic *in vitro*[10]
Bibhitaka	*Terminalia bellerica* Roxb.	Combretaceae	Fruit	Gallic acid	Antioxidant[10]
Chitraka	*Plumbago zeylanica* Linn.	Plumbaginaceae	Root	Plumbagin [25]	Abortifacient, antiovulatory[10]
Lavanga	*Eugenia caryophyllata* Thunb.	Myrtaceae	Flower bud	Eugenol [26]	Antibacterial and antiseptic[10]



Calotropin [**24**]

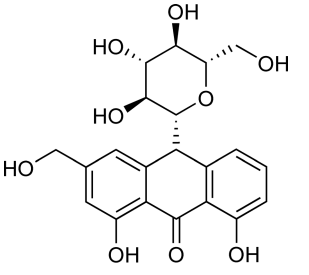


Plumbagin [**25**]



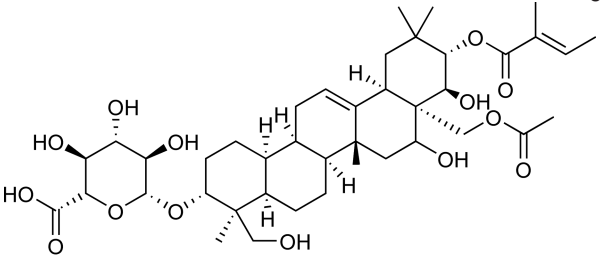
Eugenol [**26**]

Kanyaasaara	*Aloe barbadensis* Mill.	Liliaceae	Leaf	Aloin [**27**]	Purgative[10]



Aloin [**27**]

Meshashringi	*Gymnema sylvestre* B. Br.	Asclepiadaceae	Root, Leaf	Gymnemic acid [**28**]	Inhibits plasma glucose level[10]

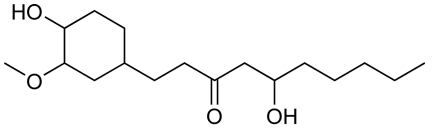


Gymnemic acid [**28**]

Raktamaricha	*Capsicum annuum* Linn.	Solanaceae	Fruit	Capsaicin	Stimulant, hypoglycemic[10]

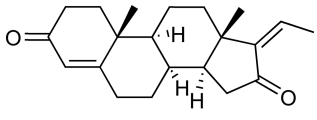
(*Continued*)

Table 3 (Continued)

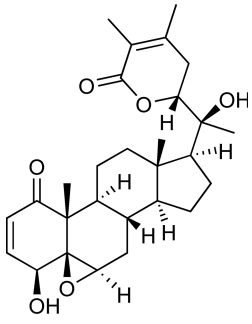
Ayurvedic source	*Biological name*	*Family*	*Parts used*	*Active constituent*	*Pharmacological activity (reference)*
Aardraka	*Zingiber officinale* Rosc.	Zingiberaceae	Rhizome	Gingerol [29]  Gingerol [**29**]	Anti-inflammatory, antipyretic, analgesic[10]
Mandukaparni	*Centella asiatica* (Linn.) Urban.	Umbelliferae	Aerial part	Asiaticoside	Antibacterial[11]
Sarpagandha	*Rauwolfia serpentine* Benth. ex Kurz.	Apocynaceae	Root	Reserpine	Antihypertensive[11]

Plant-based drugs may be used directly as crude drugs, or their chief constituents/active principles might be isolated by various chemical processes and employed as medicines. Ayurvedic knowledge and experiential database can provide new functional leads to solve the main hurdles of drug development for ayurvedic compounds viz. identity of active constituent, prodrugs, synergy, and toxicology. These records are particularly valuable, since these medicines have been effectively tested for thousands of years on people. Ayurveda has been developed in a time-tested manner; some milestones in the development of Ayurveda are summarized in **Figure 2**. Efforts are underway to establish a pharmacoepidemiological evidence base regarding the safety and practice of ayurvedic medicines. Randomized controlled clinical trials for rheumatoid and osteoarthritis, hepatoprotectives, hypolipidemic agents, asthma, Parkinson's disease, and many other disorders have reasonably established clinical efficacy.[12–14] Exemplary evidence-based researches and approaches have now resulted in wider acceptance of ayurvedic medicines.

Sushruta-Samhita, a Sanskrit text on Ayurveda written in 600 BC, noted that the plant *Commiphora mukul* Hook. (family: Burseraceae) was useful in the treatment of obesity and equivalent ailments. The first appearance of this plant in modern scientific literature was in a thesis published from Varanasi in India in 1966. It was shown that the crude gummy guggul obtained from the plant significantly lowers the serum cholesterol levels in rabbits and protects them from cholesterol-induced atherosclerosis. The major bioactive constituents from this plant have been reported to be guggulsterone Z [**11**] and E. This was followed by clinical trials in human and approval was obtained from the National Drug Regulatory Authority in India for carrying out clinical trials with the drug guggulipid. After about 20 years the drug has been marketed in India and other countries for its cholesterol-reducing property. *Withania somnifera* (family: Solanaceae), commonly known as Ashwagandha, is used as an adaptogen traditionally in India. This is also known as 'Indian Ginseng'. Primarily its roots are used for their medicinal purposes, but the leaves and berries can also be used. Ashwagandha is high in the content of withanolides [**12**], which are steroidal lactones. The withanolides are believed to directly stimulate the body's immune system and stop inflammation. Withanolides are currently being explored for their brain regenerative properties.[15]



Guggulsterone-Z [**11**]



Withanolide D [**12**]

Flavopiridol is a synthetic drug but the basis of it is rohitukine [**13**], isolated from *Dysoxylum binectariferum* Hook. (family: Meliaceae), which is phylogenetically related to the ayurvedic plant *D. malabaricum* Bedd., which is used for rheumatoid arthritis. The successful introduction of plants into modern therapeutics indicates that other discoveries are waiting to be made.[16]

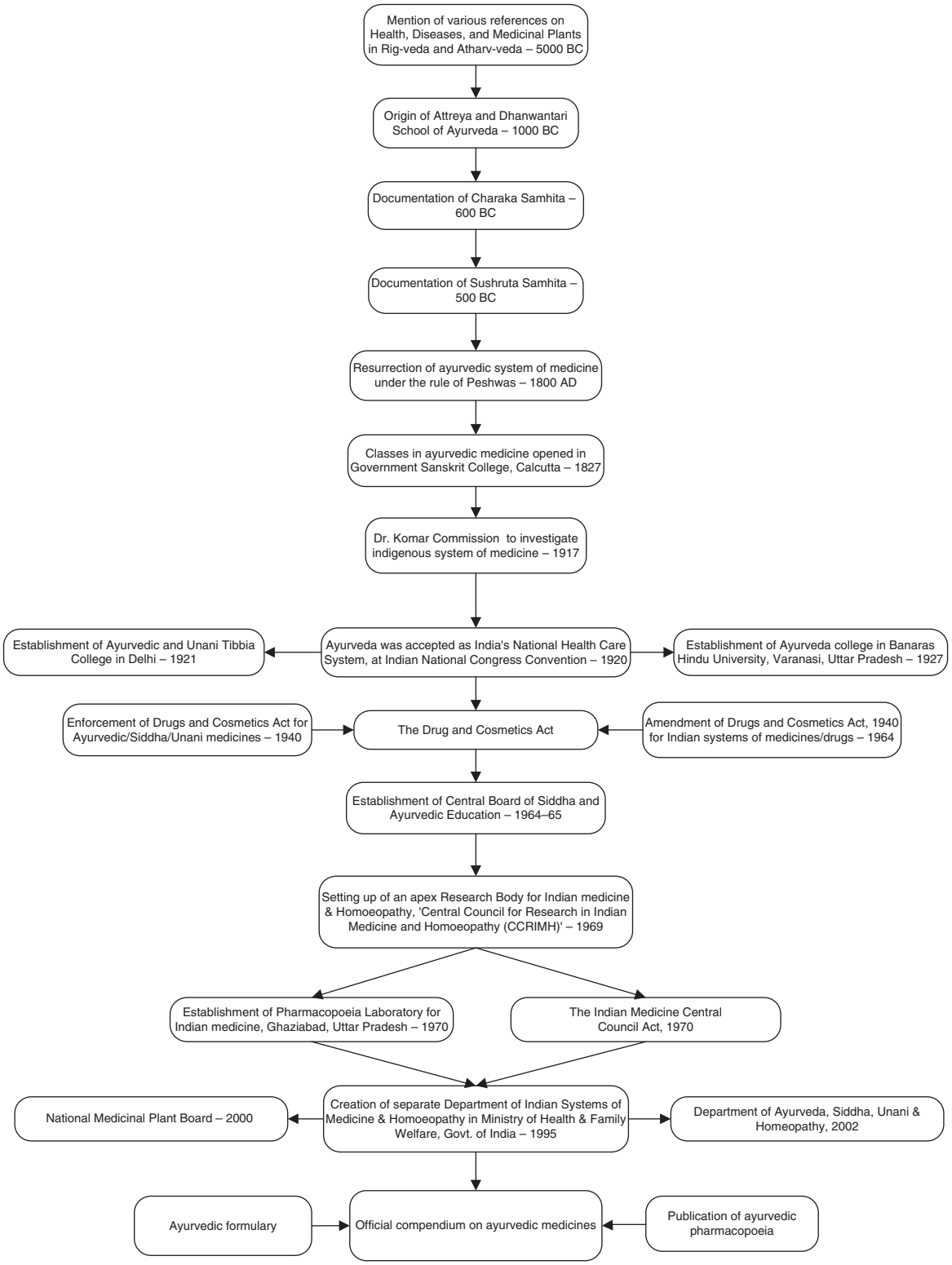
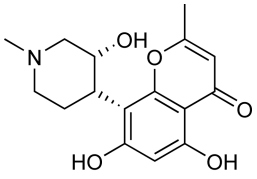


Figure 2 A few milestones in the development of Ayurveda. Reproduced with permission from P. K. Mukherjee; A. Wahile, J. Ethnopharmacol. **2006**, *103*, 27.



Rohitukine [**13**]

3.14.3 Techniques for Development of Bioactivity of Ayurvedic Medicines

3.14.3.1 Bioassay-Guided Isolation and Characterization

Apart from proper cultivation, collection, extraction, and standardization of raw material, the evaluation of herbal medicine should be performed in a better way to get fruitful results. There are many approaches for the search of new biologically active principles in higher plants. The first approach is by randomly testing the plant constituents for the available activities. In the second approach plant extracts are tested for one or more pharmacological activities followed by the isolation and the subsequent structure–activity relationship (SAR) studies of the active fraction of the extract. Integrated approaches for development of ayurvedic drugs may be ascribed as explained in **Figure 3**.[17] Primary screening of the herbs will be made based on the ayurvedic claim, which will be preceded by phytochemical profiling leading to exploration of the bioactive chemical entity. This can further lead to various high-throughput screening techniques for evaluating their therapeutic potential, and ultimately formulation of the natural health products is being made through a holistic approach. This approach can further be explored through clinical trial, various pharmacovigilance studies, herbal therapeutics, and pharmacokinetics.[18]

The detection of bioactive phytomolecules is the starting point for a search for potentially useful compounds. Most natural product chemists are more concerned with the isolation and structural elucidation of

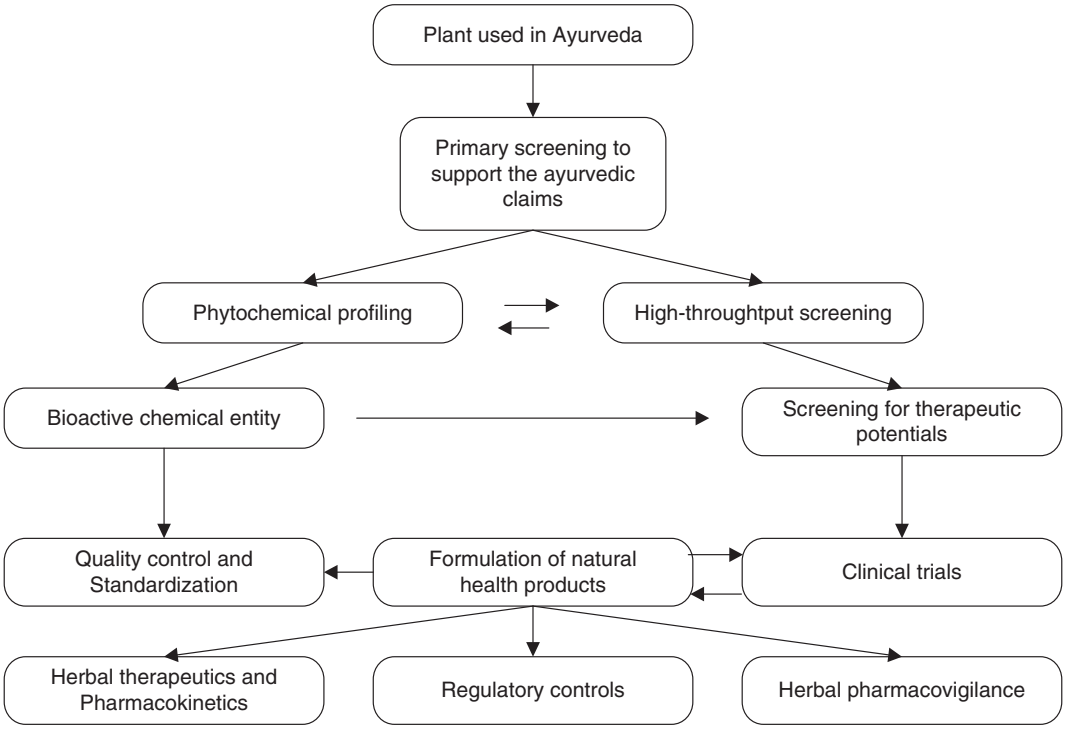


Figure 3 Integrated approaches for ayurvedic drug development.

secondary metabolites than with their bioactivity. Modern advances in separation and spectroscopic techniques have provided tools for purification and structural analysis that have reached extraordinary levels of sensitivity and sophistication. With the aid of these tools, natural product chemists have forayed into bioassay-guided isolation of metabolites followed by their identification by means of general characterization techniques, such as ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR), and mass spectroscopy.

3.14.3.2 Reverse Pharmacology

Generally, it takes a long time for a new drug to be marketed after its discovery as it passes through a series of phases after the discovery. Most of the leads developed may drop out because of toxicity or due to failure during clinical trials. In the reverse pharmacology approach, the existence of a drug for years was used to prove their traditional claim through systematic clinical trials. Ayurveda-based drug discovery uses this 'Reverse Pharmacology' approach, in which drug candidates are first identified based on large-scale use in the population, and then validated in clinical trials. Reverse pharmacology cuts the time and cost required for drug discovery from traditional medicine.[19]

Clinical experiences and observations on available data become a starting point for drug development from ayurvedic sources, in contrast to the conventional drug development. Randomized controlled clinical trials for rheumatoid and osteoarthritis, hepatoprotective and hypolipidemic agents, asthma, Parkinson's disease, and many other disorders have reasonably well established the clinical efficacy of a series of ayurvedic drugs. Exemplary evidence-based research made ayurvedic medicines widely acceptable. Thus, the ayurvedic knowledge database allows drug researchers to start from a well-tested and safe botanical material and by using this knowledge, the conventional drug discovery begins from patients instead of laboratories. The reverse pharmacology approach first confirms the activity of ayurvedic drugs, after which further studies should correlate this to components correlated with activity. This method will emphasize the safety and efficacy.

Reverse pharmacology is an alternative path for drug discovery. The reverse approach in pharmacology has been quite successfully applied in the past. The drawback was the long time-lag from the observational therapeutics to a new drug. Drugs like reserpine, obtained from *Rauwolfia serpentina*, emerged only after 20 years of work even though its antihypertensive property was demonstrated long ago. It is the need of the time to document unknown, unintended, and desirable novel prophylactic and therapeutic effects in observational therapeutics. Several new classes of drugs have accidentally emerged by adopting this path.[20] In reverse pharmacology limited clinical trials can be attempted for both safety and efficacy. Since not many new molecules are being developed, the scope of using this approach for validating the traditional knowledge is tremendous and many studies are planned for the near future.[21] The advantage of this technique is the liberty to conduct limited trials and also prove safety and efficacy in clinical and preclinical studies. In addition, efficacy can be modulated as per clinical needs. The conventional drug discovery approach of screening thousands of molecules and their biological targets is time-consuming and expensive, whereas reverse pharmacology makes it less time-consuming and less expensive, with lower risks.

3.14.3.3 Ayurgenomics

3.14.3.3.1 Functional genomics

Better understanding of the human genome has helped in understanding the scientific basis of individual variation. Pharmacogenetics is the study of the hereditary basis for differences in response of populations to a drug. The same dose of a drug will result in elevated plasma concentrations for some patients and low concentrations for others. Some patients will respond well to the drugs, while others will not. A drug might show adverse effects in some patients, but not in others. The importance of such individual variations in health and disease is an important basic principle of Ayurveda and was underlined by 'Charaka' some 4000 years ago as "Every individual is different from another and hence should be considered as a different entity". Diseases according to Ayurveda can arise from the body/mind because of several internal factors or intrinsic causes. Treatment in the ayurvedic concept is aimed at the patient as a whole considering all its aspects, which consist of salubrious use of drugs, diets, and practices. The main concept is based on 'dosha–dhatu–mala' theory, which is concerned with 'tridoshas' as explained in **Table 1**.[22]

Large differences among racial groups also occur for glutathione S-transferase (GST), an enzyme involved in detoxification of environmental toxins. CYP2D6 (a variant of the enzyme, cytochrome (CYP) P450), an enzyme that metabolizes at least 30 or 40 commonly used drugs, shows great variability in individuals: some individuals are poor metabolizers, while others are rapid metabolizers. Studies indicated that the differences in response to disease and drugs differ from population to population, and truly from individual to individual.[19,21] Ayurgenomics describes the basis of such individual variations and it has clear similarities with the pharmacogenomics that is expected to be the basis of designer medicine. Understanding the possible relationship between 'prakruti' (nature) and genome will be important. Functionally, this will involve creation of three organized databases that are capable of intelligently communicating with each other to give a customized prescription: these are human constitution (genotype), disease constitution (phenotype), and drug constitution. Nearly 5800 clinical signs and symptoms are available in ayurvedic texts. Effects of season, time, and environmental conditions according to ayurvedic chronobiological principles need to be considered to give advice on lifestyle modifications followed by dietary advice.

3.14.3.4 Ayurinformatics

Globally, there is a need to build libraries for ayurvedic phytoconstituents. Although some institutions have small plant extract libraries, they are not in the public domain. Such libraries could serve as a powerful tool and source of extracts to be screened for biological activities using high-throughput assays. In recent years, a considerable body of information has accumulated on the chemical constituents of ayurvedic herbs. This is reflected in the appearance of a number of new electronic databases, which contain both structural details of several thousand herbal constituents and accompanying information on their uses in Ayurveda. Although obscure at first, many of the therapeutic categories found in *Ayurveda Materia Medica* are interpretable in Western terminology and a variety of texts are now available in English. All the main classical works on Ayurveda, such as *Charaka Samhita*, *Sushruta Samhita*, *Ashtanga Sangraha*, and *Astanga Hrdaya*, deal with drugs, their composition, and action in addition to the other aspects of the medical system. Some of the ayurvedic books, known as *Nighantugranthas*, such as *Dhanvantarinighantu*, *Kaiyadevanighantu*, *Bhavaprakasanighantu*, *Rajanighantu*, and so on, deal mainly with a single drug, describing their habitat, characteristics, and therapeutic action.

The ayurvedic drugs are derived from different vegetable, animal, and plant sources. Ayurvedic formulations, which are predominantly derived from plants, are known as 'kasthausadhi', where the formulations are being made from extract or juice of plants' parts. These include several ayurvedic formulations like 'aristra', 'avleha', 'grafa', 'churna', and 'taila'. Formulations which are predominantly derived from metal and minerals are known as 'rasausadhi', where the formulations are made mainly from minerals and in combinations of minerals and plants; these include 'bhasma', 'pisti', 'lauha', 'kapibadkva', 'rasayana', and so on. A detailed description of all these formulations has been provided under Section 3.14.5.1. There are many authentic books on both groups of compound formulations. While *Sarngadhara Samhita*, *Cakradatta*, *Bhaisajya Ratnavali*, *Sahasrayogam*, *Bharat Bhaisajya Ratnakara*, and so on deal with both the groups of formulations, others like *Rasendra Sarasangraha*, *Rasarathna Samuccaya*, *Rasaprakasam Sudhakara*, *Ayurvedaprakasa*, *Rasatarangini*, *Rasayogasagara*, and so on deal only with the rasausadhi group of formulations.[4] Ayurveda is based on experiences as if these were experimental results. It has been divided into eight major disciplines known as 'astanga ayurveda', major component of which includes kaya chikitsa (medicine), salya chikitsa (surgery), salakya chikitsa (ENT treatment), bala chikitsa (pediatric treatment), jara chikitsa (treatment related to genetics), rasayana chikitsa (treatment with chemicals), vajikarama chikitsa (treatment with rejuvenation and aphrodisiacs), graham chikitsa (planetary effects), and visha chikitsa (toxicology). In the last 50 years the teaching and training specialties have focused toward diagnosis, treatment, and drug development as explained in **Table 4**.[17]

3.14.3.4.1 Traditional knowledge digital library

Since time immemorial, Ayurveda has been considered as a rich traditional knowledge of ways and means practiced to treat diseases afflicting the people.[23] This knowledge has generally been passed down by word of mouth from generation to generation. Some of these practices have been described in ancient classical and other literature, often inaccessible to the common man. A number of countries are evincing interest in ayurvedic

Table 4 Major disciplines and specialties in Ayurveda

Sanskrit name (as explained in ancient text)	*English*
Ayurveda siddhanta	Fundamental principles of Ayurveda
Ayurveda samhita	Ayurvedic text
Sharira rachna	Anatomy
Sharira Kriya	Physiology
Dravya guna vigyan	Materia medica and pharmacology
Ras–shastra	Chemistry
Bhaishajya kalpana	Pharmaceuticals
Kaumar bharitya	Pediatrics
Prasuthi tantra	Obstetrics and gynecology
Swasth vritla	Social and preventive medicine
Kayachikitsa	Internal medicine
Rog nidan	Pathology
Salya tantra	Surgery
Salkya tantra	Eye and ENT
Mano roga	Psychiatry
Panchkarma	Detoxification of body

plants and medicinal use described in ancient texts and treatises. Documentation of this existing knowledge on ayurvedic systems of medicine has become imperative to safeguard the sovereignty of this traditional knowledge and to protect them from being misused in patenting on nonpatentable inventions. Although this knowledge is in the public domain, the patent office does not have a mechanism to access this information to deny patenting rights. It is impossible to obtain patents for all such medicinal uses. It is also extremely costly and time-consuming to fight patents granted to others. Thus, bringing such knowledge into an easily accessible format to forestall wrongful patents was thought out to be a way out. The Traditional Knowledge Digital Library (TKDL) is an original proprietary database, which is fully protected under national and international laws of intellectual property rights. At the core of the project is the innovative approach in the form of Traditional Knowledge Resource Classification (TKRC) that enables conversion of 140 000 pages of information, containing 36 000 formulations described in 14 texts of Ayurveda, into patent-compatible format in various languages, viz. translation of Sanskrit slokas into not only Hindi but also English, French, German, Spanish, and Japanese.[23] TKDL, based on a novel way of decodification software, allows automatic conversion of information from Sanskrit into various languages. The information includes names of plants, ayurvedic description of diseases under their modern names, therapeutic formulations, and so on. The target users of the TKDL database are primarily the patent examiner(s) in national and regional international patent offices worldwide and international search authorities (ISAs) under the patent cooperation treaty (PCT) of the World Intellectual Property Organization (WIPO). During the current year, the second phase of TKDL (Ayurveda) has been initiated. Approximately 65 000 formulations will be taken up from 45 selected ayurvedic books, of which 23 000 will be transcribed after excluding the duplicate references. The activity on identification of the formulations has been initiated. So far more than 34 000 formulations have been identified from the Ayurveda texts, and they have been checked for duplicates. Transcription of 25 000 formulations has been completed from 14 texts of the targeted 45 texts.

3.14.4 Further Development from Phytochemical Leads

3.14.4.1 Biosynthesis of Phytomolecules from Ayurvedic Plants

Plant tissue culture technique has become an important tool in the hands of the plant biotechnologists. A number of research investigations have been reported for the production of biologically active constituents using plant tissue culture techniques. *Cassia senna* Linn. (Caesalpineaceae) is an important medicinal plant, which has been widely used in Ayurveda. The active chemical components of the plant are anthraquinone glycosides – sennosides, especially sennosides A and B, which are responsible for the purgative action. A protocol for tissue culture of

C. senna is established in different morphogenetic media and *in vitro*-grown tissues/cells were analyzed for their biosynthetic potential.[24] The results of the study indicate that the *in vitro*-cultured partially organized cells of *C. senna* inherited the biosynthetic potential, which can be exploited for production of sennosides on a large scale under proper growth conditions. The whole venture to explore the cultures of ayurvedic medicinal plants for bioactive constituents was undertaken all over the world and soon it blossomed into a new technology that has affected the phytochemical industry to a large extent. Commercial viability and economic feasibility still remain the decisive factors in the industrial production of such metabolites from the cultures. The range of metabolites produced by the callus and cell suspension cultures includes alkaloids, glycosides, flavonoids, and others. The cell suspension cultures are particularly capable of synthesizing such molecules and are regarded as potentially suitable systems for producing the metabolites of high economic value. They produce the bioactive molecules equivalent to or higher in yields to the plants from which they are derived.[25] Plant cell culture provides an alternative method for production of plant secondary metabolites.

3.14.4.2 Combinatorial Chemistry and Natural Products

Even though the medical uses of plants are at times scary for a new entrant to the field, for multidisciplinary research it provides a great opportunity for the identification of new pharmacophores and new targets. Also the novel structures found offer new opportunities for combinatorial chemistry. In this approach, an active natural product can be used as the central scaffold and a large numbers of analogs for structure–activity studies can be generated. With this parallel synthetic approach and similar other combinatorial approaches, a library of natural product-like compounds can be obtained. Polyketides constitute some of the structurally diverse natural products exhibiting a broad range of activities (e.g., tetracyclines, doxorubicin). With the advanced knowledge in biosynthesis of bacterial aromatic polyketide, polyketide synthase enzymes, the potential for generating novel molecules with enhanced bioactivities or novel bioactivities is high.[26] Thus application of combinatorial biosynthetic and/or combinatorial chemical techniques for the generation of molecular diversity for testing with high-throughput screens may be applied.

3.14.5 Formulation in Ayurveda and Its Value Addition

3.14.5.1 Ayurvedic Formulations

Drug delivery systems for ayurvedic drugs are classified according to their method of preparation. They are described in the Ayurvedic Formulary of India (AFI), an official publication of the Government of India[4]:

1. *Asavas* and *Aristas*: These preparations are made by soaking the herb in sugar solution or jaggery for a specified period of time. Thus it undergoes fermentation, producing alcohol, which extracts active principles and acts also as a preservative. Examples include *ahiphenasava* containing *Glycyrrhiza glabra* Linn. as main constituent, *draksharista* containing *Vitis vinifera* Linn. as major ingredient, and *devadarvarista* with *Cedrus deodara* Loud. as major ingredient.[4]
2. *Arka*: A liquid preparation obtained by distillation of certain liquids or herbs soaked in water using the distillation apparatus. For example, *ajamodarka*, which is used as a digestive, contains *Apium graveolens* as the main ingredient.
3. *Avaleha* or *leha* and *paka*: These are semisolid preparations, prepared with the addition of jaggery, sugar, or sugar candy and boiled with prescribed juice of the herbs or its decoction. "Kutajavaleha" is an example, used in treating hyperacidity, anemia, and diarrhea; its major ingredient is *Holarrhena antidysenterica*.
4. *Churna*: Powder of herb(s), where a single herb or combinations of herbs are made into a coarse powder ('javkut'); for example, *narasimha churna* is used in the treatment of cough, pthisis, and fever and contains *Tinospora cordifolia* Miers and *Semecarpus anacardium* Linn. as the main ingredients.
5. *Guggulu*: An exudate obtained from the plant *Commiphora weightii*. Preparation having the exudate as the main effective ingredient is known as 'guggulu'. Among five different varieties, *Mahisaksa* and *Kanaka guggulu* are usually preferred for medicinal preparation. Examples include *kaisora guggulu* (contains mainly *T. cordifolia* Miers) and *kancanara guggulu* (contains mainly *Bauhinia variegata* Linn.).

6. *Ghritas* (*snehakalpa*): Preparation in which ghee (clarified butter derived from milk) is boiled with prescribed decoction of drugs according to the formula as prescribed in ayurvedic text.[4] This process ensures absorption of the active therapeutic principles of the ingredients used. For example, *asoka ghrita* is used in the treatment of pelvic pain, lower backache, and anemia and contains *Saraca asoca* de Wilde as the major herb.
7. *Taila*: Preparations in which oil is boiled with prescribed decoction of drugs according to the formula.[4] This process ensures absorption of the active therapeutic principles of the ingredients of the plant. Examples are *prasarini taila* (major ingredient, *Paederia foetida* Linn.) and *bhringaraja taila* (major ingredient, *Eclipta alba* Linn.).
8. *Dravakas:* Liquid preparations obtained from *lavanas* (rock salts) and *ksaras* by distillation process with or without any addition of fluids. Ksaras are alkaline substances obtained from the ash of drugs. The drugs are cut into small pieces, dried, kept in an earthen pot, and burnt to ash. *Sankha dravaka* is used in treating diseases of the abdomen and spleen and contains *Calotropis procera* R.Br. and *Euphorbia nerrifolia* Linn. along with other ingredients.
9. *Lepa*: Topical applications in the form of a paste. The drugs are made into a fine powder. Before use on the body, it is mixed with some liquid or other medium indicated in each preparation and made into a soft paste. Water, cow's urine, oil, and ghee are some of the media used for mixing.[4] *Avalgujadi lepa* (contains *Psoralea corylifolia* Linn.) and *pathyadi lepa* (contains *Terminalia chebula* Retz. along with other ingredients) are some of the examples of this category.
10. *Vati* and *Gutika*: Medicinal preparations in the form of tablets or pills. They are made of one or more drugs of plant, animal, or mineral origin. *Khadiradi gutika* is an example to mention. It contains *Acacia catechu* Willd. and is used in the treatment of halitosis, diseases of the teeth, and dental cavities (caries).
11. *Vartti*, *Netrabindu*, and *Anjana*: Preparations used externally for the eye. *Nalikeranjana* (containing *Berberis aristata* DC and *Glycyrrhiza glabra* Linn.) and *tamradi gutika* (containing *Glycyrrhiza glabra* Linn. and *Saussurea lappa* C.B. Clarke along with other ingredients) are examples of this category.
12. *Bhasma and Pishti*: In Ayurveda, use of both bhasma (residue after incineration–calcined preparation) as well as pishti (powdered gem or metal) along with appropriate herbs is recommended for treatment of critical ailments. The procedures for preparing these medicines are time-consuming and complicated. 'Bhasma' is a calcined preparation in which the gem or metal is converted into ash. Gems or metals are purified to remove impurities and treated by triturating and macerating in herbal extracts. The dough so obtained is then calcinated to obtain the ashes through the way of 'bhasmikaran'. Bhasmikaran is a process by which a substance that is otherwise bioincompatible is made biocompatible by certain 'samskaras' or processes. The objectives of samskara include elimination of harmful matters from the drug and modification of undesirable physical properties to enhance the therapeutic action. For example, 'loha bhasma' (ash made from iron) is the main ingredient of preparations like 'lauha kalpas'.
13. *Rasa Yoga*: Contains mineral drugs as main ingredients, and it may be in pill or powder form. Examples are 'amlapittantaka rasa' (contains *T. chebula* Retz.) and 'anandabhairava rasa' (contains *Piper nigrum* Linn. and *Piper longum* Linn.).

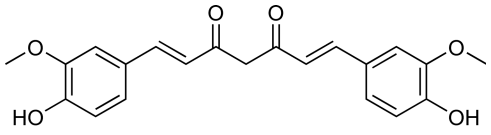
3.14.5.2 Value-Added Delivery System

The effectiveness of any ayurvedic medication is dependent on delivery of an effective level of the therapeutically active compound(s). However, a severe limitation exists in their bioavailability when administered orally or by topical application. To overcome this limitation of absorption, developing value-added herbal drug delivery systems with a better absorption profile is of prime importance. Value-added formulation, as its name indicates, is a formulation with added value, which gives better therapeutic efficacy of its main chemical constituents inside the body. The development of value-added herbal formulations having better absorption and utilization profiles in our body is of paramount importance. To minimize drug degradation and loss during the consumption of ayurvedic drugs and to increase their bioavailability, various drug delivery and drug targeting systems are currently under development.[27]

Liposomal drug delivery systems have changed the therapeutic spectrum of herbal drug molecules. Liposomes provide a means to alter pharmacokinetic and toxicity profiles of potent herbal molecules and to achieve effective utilization of ayurvedic drugs. Flavonoids are well-known phytoconstituents having a vast

array of biological activities. Liposomal dosage forms of different natural products have proved the efficacy of this value-added delivery system, for example, paclitaxel, sphingosomal vincristine, soy isoflavones, *Centella asiatica* extract, oleanolic acid, quercetin, *Dioscorea villosa* (wild yam) root extract, and *Panax ginseng* root extract. A similar spectrum of ayurvedic products may provide improved pharmacokinetic profiles of these herbals.

As an example phytosomes can be mentioned, which are advanced forms of herbal products that are better absorbed, utilized, and as a result produce better effects than conventional herbal extracts. Phytosomes, which are another form of liposome, are produced via a patented process whereby the individual components of an herbal extract are bound to phospholipid, unlike liposomes, in which many phospholipid molecules enclose the drug without binding. The choline head of the phosphatidylcholine molecule binds to these compounds while the fat-soluble phosphatidyl portion comprising the body and tail envelops the choline-bound material. The phytosome process also intensifies the action of herbal compounds by improving absorption, increasing biological activity, and enhancing delivery to the target tissue. The effectiveness of *Centella asiatica* L. selected triterpenes (CAST) has been improved by complexing with soy phospholipids; this enhances the oral bioavailability of incompletely absorbed molecules by promoting interaction with bile salts. The resulting complex, Centella Phytosome®, is a new molecular entity whose improved activity is demonstrated by comparison with CAST in the uncomplexed form.[28] Similarly phytosomes were prepared from curcumin [**14**], naringenin, and quercetin and were found to have an improved pharmacokinetic profile.[29–31] Thus the technology is a beneficial novel drug delivery system which will help in drug development and modernizing the potential ayurvedic phytomolecules.



Curcumin [**14**]

3.14.5.2.1 Nanotechnology

In recent years, nanoparticle technology has emerged as a strategy to tackle formulation problems associated with poorly water-soluble and poorly water- and lipid-soluble drugs. Nanotechnology is an area of science devoted to the manipulation of atoms and molecules, leading to the construction of structures in the nanometer scale size, which retain unique properties.

In a study, ellagic acid-loaded nanoparticles were prepared following an emulsion–diffusion–evaporation method by using poly(lactide-co-glycolide) (PLGA) and polycaprolactone (PCL) employing didodecyldimethyl ammonium bromide (DMAB) and polyvinyl alcohol (PVA) as stabilizers. The antioxidant potential of the DMAB-stabilized nanoparticulate formulations was evaluated against cyclosporine A (CyA)-induced nephrotoxicity in rats. From the studies, it was evident that ellagic acid nanoparticles were able to prevent the cyclosporine A-induced nephrotoxicity at three times lower dose, suggesting improved oral bioavailability of EA.[32]

He *et al.*[33] studied the silymarin-loaded solid lipid nanoparticles (SM-SLNs) developed using Compritol 888 ATO, soybean lecithin, and poloxamer 188. Two kinds of SM-SLNs were prepared using a hot and cold homogenization method. The particle size distribution, zeta potential, drug loading (DL), and entrapment efficiency (EE) were investigated in detail. The *in vitro* release of both SM-SLNs preparations was studied by a bulk equilibrium reverse dialysis bag. It showed that a prolonged drug release can be achieved from the SM-SLNs produced by cold homogenization (cold-SM-SLNs). The relative bioavailability of the cold-SM-SLNs was 2.79 fold higher compared to the SM suspension. The results indicated that the cold-SM-SLNs can improve the oral bioavailability of SM.

Bisht *et al.* in 2007 synthesized a polymeric nanoparticle encapsulated formulation of curcumin – nanocurcumin – utilizing the micellar aggregates of cross-linked and random copolymers of nisopropylacrylamide (NIPAAM), with *N*-vinyl-2-pyrrolidone (VP) and poly(ethyleneglycol) monoacrylate (PEG-A). Physicochemical characterization of the polymeric nanoparticles by dynamic laser light scattering and transmission electron microscopy confirms a narrow size distribution in the 50 nm range. Nanocurcumin, unlike free curcumin, is readily dispersed in aqueous media. Nanocurcumin demonstrates comparable *in vitro* therapeutic efficacy to free curcumin against a panel of human pancreatic cancer cell lines.[34]

3.14.6 Quality Control

With the different regulatory situations in different countries and due to several quality control issues, successful establishment of ayurvedic drugs is becoming more complicated. Several challenges were involved in the quality control of the plant materials starting from the field to the market, which may be solved by use of various analytical tools. Variability in plant material, adulterations or mistakes in plant identification, microbial contamination, mycotoxins, heavy metals, and pesticides are some of the most frequently encountered problems.

Several identification tests such as macroscopic study, microscopic study, and organoleptic identification solve these problems to some extent. Each type of quality requirements needs other tools; for example, biomarkers are needed in case of unknown active compounds, and biomarkers must be validated in connection with bioassay. Identity of plant material by metabolic fingerprinting or DNA fingerprinting (**Figure 4**) may be more fruitful in the final identification of the plant and the estimation of the plant biomarkers will help in the standardization of these ayurvedic plant products. Chemical contaminants require targeted analysis, as these plants may be contaminated by the pesticides, ground water, and soil that are used for their cultivation; special tests are required to identify and quantify the presence of such impurities in the products. As these contaminants are unavoidable in cultivated products, limit tests and special tests for expected heavy metals should be performed. Throughout these quality control steps analytical tools play a very significant role.[35] The standard procedure which can be used for quality control of herbals has been explained further in **Figure 4**. Some important analytical tools helpful in the analysis of the ayurvedic medicines, which can be used for development of quality products with reliable scientific data on storage, identification, handling, and processing of crude ayurvedic plant extracts or materials, have been discussed in the following section.

Standardized manufacturing processes and suitable analytical tools are very much required to establish the quality of herbal drugs like those used in Ayurveda. Among these tools, separation techniques like high-performance thin layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC) play an important role.[1,36] Ayurvedic herbs and herbal preparations are particularly difficult to standardize. For marketing an ayurvedic product, investigation of the chemical and biochemical composition of a plant material is necessary. Fingerprint analysis by HPTLC or HPLC is one of the powerful tools to link the botanical identity to the chemical constituents of the plant. In combination with microscopy, the fingerprint provides a means for checking the identity of the plant. From the constituents, a number of marker compounds can be chosen to standardize the plant material. Biomarker is an important concept for which the chromatographic techniques are used to standardize the active extract.[37,38]

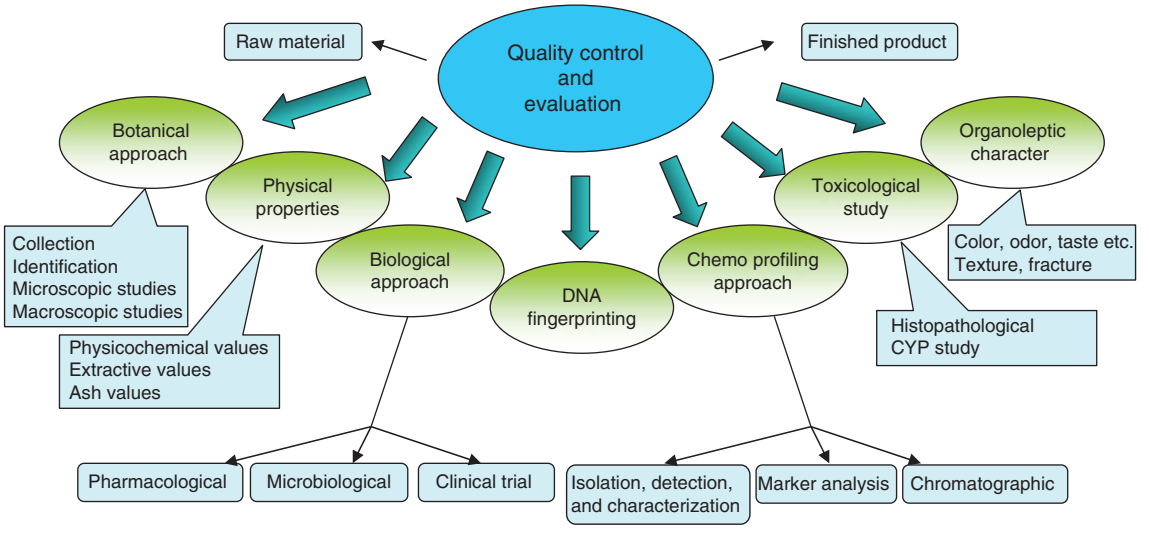


Figure 4 Standard procedure for quality control of herbals.

3.14.6.1 High-Performance Thin Layer Chromatography

HPTLC can be employed for quantitative determination of marker compound. For example, gingerol content in ginger was determined using this technique.[39] Curcumin is a major bioactive marker present in *Curcuma longa* Linn. (family: Zingiberaceae). The amount of curcumin present is quantified by using HPTLC technique (**Figure 5**). A thin layer chromatography (TLC) with standard curcumin (S) along with two test samples (T1 and T2) by using solvent system chloroform:ethanol:glacial acetic acid (95:5:1) gave several spots with a spot corresponding to standard curcumin (R_f: 0.5). The corresponding graph obtained after the scan showed the presence of a standard peak of curcumin in both test samples. Thus marker profiling has been used in the quality control of several ayurvedic medicinal plants, namely their identification, quantification, and standardization.

3.14.6.2 High-Performance Liquid Chromatography

HPLC is another important technique used for the quantification of the marker constituents. HPLC is the method of choice owing to its high versatility, precision, and relatively low cost. For example, 'triphala' is an antioxidant-rich herbal formulation used in anemia, jaundice, and so on and contains fruits of *Emblica officinalis*, *Terminalia chebula*, and *Terminalia belerica* (1:1:1). A simple HPLC method for the separation and quantitative determination of the major antioxidant polyphenols from triphala was developed by Singh *et al.*[40] The results indicate that triphala contains a number of phenolics that may be responsible for the therapeutic activity. The HPLC method developed assisted in the standardization of triphala.

3.14.6.3 Nuclear Magnetic Resonance Spectroscopy

An ayurvedic drug must have a specific chemical structure or contain essential structural features in order to elicit the desired pharmacological activity. Ayurvedic natural products are unique in that they have to be purified from complex matrices. This places additional demands on the processes of purification and complicates the structural analysis. In this context, NMR itself has intrinsic differentiation capability, that is, the chemical shift scale not only disperses protons that belong to the same molecule, but also those of other molecular species. In addition, multidimensional NMR enables dispersion within the multiple dimensions and adds significantly to the differentiation power of NMR.

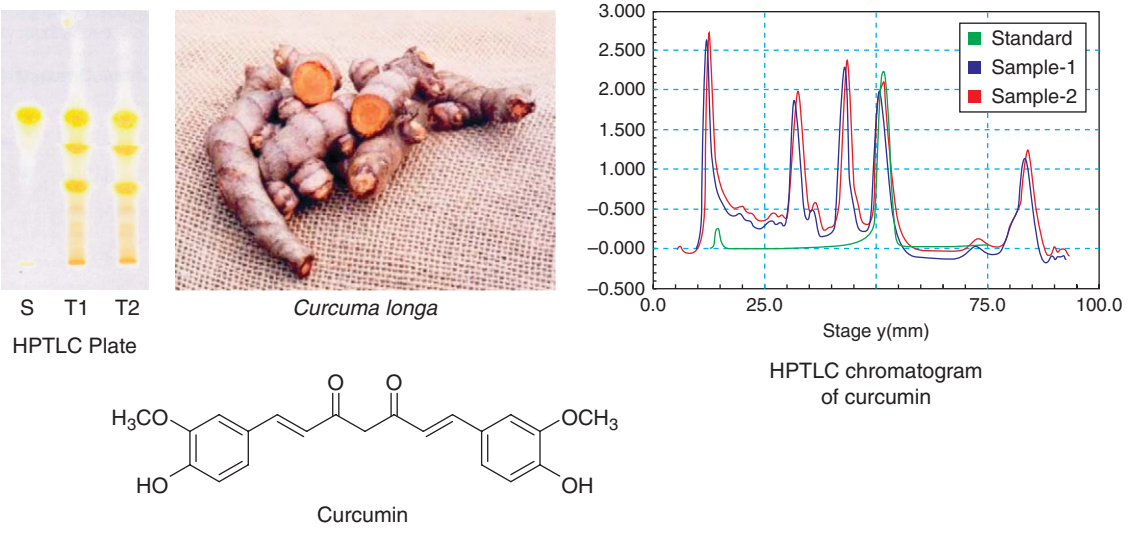


Figure 5 Marker profiling of curcumin in *Curcuma longa*.

3.14.6.4 Combined Analytical Approach for Chemical Screening

In traditional Indian medicine, Gentianaceae plants are used to cure depression. Their bitter character is mainly due to the presence of monoterpene glycosides. The xanthone content in this plant is potentially interesting as new antidepressant drugs. To find new xanthones numerous gentians have been screened chemically by both LC/UV and LC/MS. Different plant parts were extracted successively with dichloromethane and methanol. Without purification, crude extracts were directly separated on a reverse-phase column with acidic acetonitrile–water gradient. By using LC/UV methods, the compounds like belidifolin, isoscoparin, and swertia japonin were suspected from the spectra and by using LC/MS, the presence of swertia japonin is confirmed. Thus, by a combination of techniques, several phytochemicals can be used to characterize a minute quantity of the sample.[41]

3.14.6.5 Biometric and Chemometric Methods

Analysis of ayurvedic herbs and formulations remain challenging issues for analytical chemists due to the complex interplay of so many constituents. Biometric and chemometric methods can help in the measurements made on a chemical or biological system or process to the state of the system via application of mathematical or statistical methods and thus can help in the analysis of the phytoconstituents and measuring their therapeutic benefits in different ways. Chemometric research spans a wide area of different methods which can be applied in the analysis of herbal products and their formulations through various instrumental techniques as discussed earlier. This approach includes collecting reliable data (optimization of experimental parameters, design of experiments, calibration, and signal processing) and analyzing information through statistics, pattern recognition, modeling, and structure–property relationship estimations.

Biometrics is the application of statistics to a wide range of topics in biology. In the screening and development of natural products from traditional resources it encompasses the design of biological experiments, especially in medicine and agriculture; the collection, summarization, and analysis of data from those experiments; and the interpretation of, and inference from, the results. For example multivariate analysis has been used to compute quantitative estimates of 'tridosha' and 'prakriti' to provide a basis for biostatistical analysis of this ancient Indian science, which is a promising field of alternative medicine. Similarly, other tools such as biometric analysis solve the major problem in identification of plant material and their quality control.

3.14.7 Safety of Ayurvedic Preparations

Herbal products are generally considered to be safe. However, studies show that these herbs generally lack the stringent regulation of therapeutic products. As an increasing number of people include herbal products in their diet, it is important that users and health care professionals are aware of any consequences and possible side effects involved with their use, particularly when used in combination with conventional therapeutic products. The medicinal plants used in Ayurveda may markedly affect the disposition of concurrently used conventional drugs.[42]

CYP 450 isoenzymes are a superfamily of hemoprotein enzymes found on the membrane of endoplasmic reticulum. They are predominantly present in the liver and are responsible for biotransformation of drugs, including phytomolecules. They render the phytomolecules ionic and more water soluble, so that they can be excreted. This process may also lead to limited bioavailability of these molecules.[9,43] Drug interactions involving the CYP 450 isoforms concern one of two processes, enzyme induction and inhibition.

3.14.7.1 Enzyme Induction

On repeated administration, phytomolecules can induce CYP 450 enzymes, leading to an increase in rate of drug metabolism ultimately resulting in reduced efficacy of the drug.

3.14.7.2 Enzyme Inhibition

CYP enzymes can be inhibited by phytomolecules both reversibly and irreversibly. Enzyme inhibition leads to decrease in rate of hepatic biotransformation of the phytomolecules, causing increased serum concentration and toxicity. *Valeriana officinalis* and garlic tablets and capsule formulation were studied for their CYP inhibition effect on human CYP 3A4, CYP 2C19, and CYP 2D6. Of these only *V. officinalis* has shown some significant inhibition and garlic preparation did not show significant inhibition.[44–46] Safety parameters have been studied with various medicinal plants and their isolated constituents for their CYP enzyme activity (**Table 5**). The Central Council for Research in Ayurveda and Siddha (CCRAS) under the government of India has been involved in evaluating the safety profile of ayurvedic medicines by using the CYP 450 enzyme inhibition studies.

3.14.8 Ongoing Research in India on Ayurveda

The Department of Indian Systems of Medicine and Homoeopathy (ISM&H) was created in March, 1995, and renamed as Department of Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homoeopathy (AYUSH) in November, 2003, with a view to provide focused attention to the development of education and research in Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homoeopathy systems. The Department continued to lay emphasis on upgrading of AYUSH educational standards, quality control, and standardization of drugs, improving the availability of medicinal plant material, research and development, and awareness generation about the efficacy of the systems domestically and internationally. The council has taken up several research programmes which include survey of medicinal plants, pharmacognosy, cultivation of medicinal plants including tissue culture, phytochemistry, pharmacology, toxicity, and drug standardization.[2,54]

Central councils have their own research institutes, laboratories, and dispensaries throughout India, which work on the development and propagation of the respective system and thereby develop lead compounds from the tradition for the treatment of deadly ailments. The individual laboratories and institutes performing research and development work on development and evaluation of ayurvedic drugs are listed in **Table 6**. Apart from that there are 289 ayurvedic and siddha colleges (run either by Government or private sector), contributing to the research and development in India.

3.14.9 Conclusion

Ayurvedic drugs present the unique nature of a complex mixture of different secondary metabolites, and their combination ratio varies depending on environmental conditions. Sometimes the constituents responsible for the pharmacological activity are not known or identified. This is even more complicated with polyherbal formulations. For commercialization, an authentic supply of raw material should be ensured to avoid adulteration. Thus a proper standardization method is essential for promoting an ayurvedic drug to modern medicine. The concept of marker analysis in standardizing ayurvedic drugs is a challenge, considering the complexity of materials involved. Another issue with ayurvedic drugs is documentation of their safety and toxicity. Obviously these drugs have their potential of being used in therapy for so many years, but documenting their safety profiles as well as pharmacovigilance and related aspects are a major breakthrough. Studies of the pharmacokinetic and pharmacodynamic parameters relating to an ayurvedic drug are important to promote it through modernization. Also bioavailability of each constituent has to be measured. In the coming years, rapidly increasing efforts in the field of studies of ayurvedic medicine will result in evidence-based ayurvedic medicines as well as new leads to drug development.

Table 5 CYP activity of several plants used in Indian systems of medicine and their isolated constituents

Plant	*Family*	*Part(s) of plant/ constituent tested*	*Type of extract/class of compound*	*Study method*	*Isoforms used*	*Result*
Alpinia galangal	Zingiberaceae	Rhizome	Methanolic	Radiometry	CYP3A4	Inhibition[47]
Andrographis paniculata	Acanthaceae	Aerial part	Methanolic	Radiometry	CYP3A4 and CYP2D6	Inhibition[47]
Glycyrrhiza glabra	Leguminosae	Stem	Methanolic	Radiometry	CYP3A4 and CYP2D6	Inhibition[47]
Phyllanthus amarus	Euphorbiaceae	Aerial part	Alcoholic	Fluorescent spectrophotometry	CYP1A1, 1A2, 2B1/2, 2E1, 1A, 2A, 2B, 2D, 3A	Inhibition[48]
Piper nigrum (black pepper)	Piperaceae	Fruit and leaf	Methanolic and ethanolic	Radiometry	CYP3A4 & CYP2D6	Inhibition[47]
Valeriana officinalis	Valerianaceae	Root	Aqueous, ethanol, acetonitrile	Fluorimetry	CYP3A4	Inhibition[49]
Zingiber aromaticum	Zingiberaceae	Rhizome	Methanolic and ethanolic	Radiometry	CYP3A4 and CYP2D6	Inhibition[47]
CYP activity of isolated constituents						
Mentha piperita	Labiatae	(-)-Menthol	Monoterpenes	Spectro-fluorimetry	CYP2B1	Inhibition[50]
Curcuma longa	Zingiberaceae	Curcumin	Dieruloylmethane (polyphenolic)	Fluorometric assay	CYP1A2, 3A4, 2D6, 2C9, 2B6	Inhibition[51]
Zingiber aromaticum	Zingiberaceae	Kaempferol-3,4′-di-*O*-methyl ether	Kaempferol glycoside	Radiometry	CYP3A4	Inhibition[52]
Piper nigrum	Piperaceae	Piperine	Alkaloid		CYP3A4	Inhibition[53]

Table 6 Government institutes dealing with the research and development of the traditional systems of medicine in India

Council	*Institute*	*Area of research*
Central Council for Research in Ayurveda and Siddha	Regional Research Institute, Bangalore (Karnataka) Regional Research Institute, Guwahati (Assam) Central Research Institute, Gwalior (Madhya Pradesh) Regional Research Institute, Itanagar (Arunachal Pradesh) Regional Research Institute, Jhansi (Uttar Pradesh) Regional Research Institute, Nagpur (Maharashtra) Regional Research Institute, Tarikhet (Uttaranchal) Regional Research Institute (Drug Research), Trivandrum (Kerala)	Survey of medicinal plants
	Captain Srinivasa Murti Drug Research Institute, Chennai Central Research Institute, Kolkata Regional Research Institute, Trivandrum Regional Research Institute, Bangalore Central Research Institute, Gwalior Central Research Institute, Cheruthruthy Central Research Institute (Siddha), Chennai 12 research centers 60 units and dispensaries	Pharmacological, toxicological, and standardization studies
Council for Scientific and Industrial Research and regional laboratories	Regional Research Laboratory, Jammu Central Drug Research Institute, Lucknow Central Institute of Medicinal and Aromatic Plants, Lucknow National Botanical Research Institute, Lucknow Indian Institute of Chemical Biology, Kolkata, and others	Cultivation of medicinal plants, quality control, and investigation of medicinal plants and pharmacology, including development of agrobiotechnological approaches

Acknowledgment

The authors wish to express their gratitude to Drugs and Pharmaceuticals Research Programme (DPRP) of Technology Development and Transfer Division, Department of Science & Technology, Government of India, New Delhi for financial support to the School of Natural Product Studies, Jadavpur University, Kolkata, India. Thanks are also due to Dr. Achintya Mitra, Research Officer (Ayurveda), Central Research Institute of Ayurveda, Kolkata, for his valuable suggestions.

Abbreviations

AFI	Ayurvedic Formulary of India
AYUSH	Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homoeopathy
CAST	*Centella Asiatica* L. selected triterpenes
CCRAS	Central Council for Research in Ayurveda and Siddha
CyA	cyclosporine A
CYP	cytochrome
DL	drug loading
DMAB	didodecyldimethyl ammonium bromide
EE	entrapment efficiency
ENT	ear, nose, throat
GST	glutathione S-transferase
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin layer chromatography
ISA	international search authorities
NIPAAM	*N*-isopropylacrylamide
NMR	nuclear magnetic resonance
PCL	polycaprolactone
PCT	patent cooperation treaty
PEG-A	poly (ethyleneglycol) monoacrylate
PLGA	poly (lactide-co-glycolide)
PVA	polyvinyl alcohol
SAR	structure–activity relationship
SM-SLN	silymarin-loaded solid lipid nanoparticle
TKDL	Traditional Knowledge Digital Library
TKRC	Traditional Knowledge Resource Classification
VP	*N*-vinyl-2-pyrrolidone
WIPO	World Intellectual Property Organization

References

1. P. K. Mukherjee, *Quality Control of Herbal Drugs*, 1st ed.; Eastern Publishers (Business Horizons Ltd.): New Delhi, 2002.
2. P. K. Mukherjee, *Drug Inf. J.* **2001**, *35*, 623–631.
3. S. Rajan; M. Sethuraman; P. K. Mukherjee, *Phytother. Res.* **2002**, *16* (2), 98–116.
4. Anonymous, *The Ayurvedic Formulary of India*, Part 1, 2nd ed.; The Controller of Publications: Delhi, 2003.
5. http://www.fao.org/DOCREP/005/AA021E/AA021e00.htm (accessed 19 July 2008).
6. P. K. Mukherjee; S. Rai; V. Kumar; K. Mukherjee; P. J. Hylands; R. C. Hider, *Expert Opin. Drug Discov.* **2007**, *2*, 633–657.
7. L. Pieters; A. J. Vlietinck, *J. Ethnopharmacol.* **2005**, *100*, 58.
8. A. H. Gilani; A. Rahman, *J. Ethnopharmacol.* **2005**, *100*, 43–49.
9. P. K. Mukherjee; K. Mukherjee, Evaluation of Botanical-Perspectives of Quality Safety and Efficacy. In *Advances in Medicinal Plants*, 1st ed.; N. D. Prajapati, T. Prajapati, S. Jaypura, Eds.; Asian Medicinal Plants and Health Care Trust: Jodhpur, Rajasthan, India, 2005; Vol. 1, pp 87–110.
10. C. P. Khare, *Indian Medicinal Plants*; Springer-Verlag: Berlin/Heidelberg, 2007.
11. A. P. Singh, *A Treatise on Phytochemistry*; Emedia Science Ltd: UK, 2002.

12. P. K. Mukherjee; K. Maiti; K. Mukherjee; P. J. Houghton, *J. Ethnopharmacol.* **2006**, *106* (1), 1–28.
13. K. Maiti; A. Gantait; K. Mukherjee; B. P. Saha; P. K. Mukherjee, *J. Nat. Remedies,* **2006**, *6* (1), 1–13.
14. S. Rai; A. Wahile; K. Mukherjee; B. P. Saha; P. K. Mukherjee, *J. Ethnopharmacol.* **2006**, *104*, 322–327.
15. P. K. Mukherjee, *Clin. Res. Regul. Aff.* **2003**, *20*, 249–263.
16. P. K. Mukherjee; V. Kumar; M. Mal; P. J. Houghton, *Phytomedicine* **2007**, *14* (4), 289–300.
17. P. K. Mukherjee; A. Wahile, *J. Ethnopharmacol.* **2006**, *103*, 25–35.
18. E. L. Ghisalberti, Detection and Isolation of Bioactive Natural Products. In *Bioactive Natural Products-Detection, Isolation, and Structural Determination*; S. M. Colegate, R. J. Molyneux, Eds.; CRC Press: New York, 2007; p 19.
19. B. Patwardhan, *Drug Discovery and Development: Traditional Medicine and Ethnopharmacology*, 1st ed.; New India Publishing Agency: New Delhi, 2007; pp 284, 307.
20. A. D. B. Vaidya, *Indian J. Pharmacol.* **2006**, *38*, 311–315.
21. B. Patwardhan; A. D. B. Vaidya; M. Chorghade, *Curr. Sci.* **2004**, *86*, 789–799.
22. V. V. S. Sastri, *Tridosha Theory*; Arya Vaidya Sala: Kottakkal, India, 2002; pp 3–6.
23. http://www.tkdl.res.in (accessed 19 July 2008).
24. N. Shrivastava; T. Patel; A. Srivastava, *Curr. Sci.* **2006**, *90* (11), 1472–1473.
25. P. K. Mukherjee; R. Verpoorte, *Indian J. Pharm. Edu.* **2003**, *37*, 187–198.
26. R. S. Gokhale; S. Y. Tsuji; D. E. Cane; C. Kosla, *Science* **1999**, *284* (5413), 482–485.
27. P. K. Mukherjee; K. Maiti; K. Mukherjee; M. Venkatesh, *Ind. J. Pharm. Ed. Res.* (in press).
28. http://www.indena.com/pdf/centella.pdf (accessed 19 July 2008).
29. K. Maiti; K. Mukherjee; A. Gantait; K. H. N. Ahamed; B. P. Saha; P. K. Mukherjee. *Iran J. Pharmacol. Ther.* **2005**, *4* (2), 84–90.
30. K. Maiti; K. Mukherjee; A. Gantait; B. P. Saha; P. K. Mukherjee, *Int. J. Pharm.* **2007**, *330* (1–2), 155–163.
31. K. Maiti; K. Mukherjee; A. Gantait; B. P. Saha; P. K. Mukherjee, *J. Pharm. Pharmacol.* **2006**, *58*, 1227–1233.
32. K. Sonaje; J. L. Italia; G. Sharma; V. Bhardwaj; K. Tikoo; M. N. V. Ravi Kumar, *Pharm. Res.* **2007**, *24*, 899–908.
33. J. He; S. Hou; W. Lu; L. Zhu; J. Feng, *J. Biomed. Nanotechnol.* **2007**, *3*, 195–202.
34. S. Bisht; G. Feldmann; S. Soni; R. Ravi; C. Karikar; A. Maitra; A. Maitra, *J. Nanobiotechnol.* **2007**, *5*, 1–18.
35. P. K. Mukherjee, *Drug Inf. J.* **2002**, *63* (3), 635–644.
36. P. K. Mukherjee; A. Wahile; V. Kumar; S. Rai; K. Mukherjee, *Drug Inf. J.* **2006**, *40*, 131–139.
37. P. K. Mukherjee; V. Kumar; M. Mal; P. J. Houghton, *Pharm. Biol.* **2007**, *45* (8), 651–666.
38. T. Tamizhmani; P. K. Mukherjee; S. Manimaran; T. Subburaju; B. Suresh, *Hamdard Med.,* **2003**, *XLVI* (4), 95–100.
39. S. Rai; K. Mukherjee; M. Mal; A. Wahile; B. P. Saha; P. K. Mukherjee, *J. Sep. Sci.* **2006**, *29*, 2292–2295.
40. D. P. Singh; R. Govindarajan; A. K. S. Rawat, *Phytochem. Anal.* **2008**, *19*, 164–168.
41. K. Hostettmann; J. L. Wolfender, Application of LC/MS and LC/NMR in the Search for New Bioactive Compounds from Plants of the America. In *Chemistry, Biological and Pharmacological Properties of Medicinal Plants from the Americas*; K. Hostettmann, M. P. Gupta, A. Marston, Eds.; CRC Press: New York, 1998; pp 25.
42. S. Ponnusankar; P. Venkatesh; M. Venkatesh; S. C. Mandal; P. K. Mukherjee, *The Pharma Rev.* December **2007**, 113–126.
43. B. S. Kalra, *Indian J. Med. Sci.* **2007**, *61*, 102–116.
44. J. Strandell; A. Neil; G. Carlin, *Phytomedicine* **2004**, *11*, 98–104.
45. S. K. Banerjee; P. K. Mukherjee; S. K. Maulik, *Phytother. Res.* **2003**, *17* (2), 97–106.
46. S. Mandlekar; J. L. Hong; A. T. Kong, *Curr. Drug Metab.* **2006**, *7*, 661–675.
47. T. Usia; H. Iwata; A. Hiratsuka; T. Watabe; S. Kadota; Y. Tezuka, *Phytomedicine* **2006**, *13*, 67–73.
48. K. B. Harikumar; R. Kuttan, *Biol. Pharm. Bull.* **2006**, *29*, 1310–1313.
49. T. Lefebvre; B. C. Foster; C. E. Drouin; A. Krantis; J. T. Arnason; J. F. Livesey; S. A. Jordan, *J. Pharm. Pharm. Sci.* **2004**, *7*, 265–273.
50. A. C. A. X. De-Oliveira; A. A. Fidalgo-Neto; F. J. R. Paumgartten, *Toxicology* **1999**, *135*, 33–41.
51. R. Appiah-Opong; J. N. M. Commandeur; B. van Vugt-Lussenburg; N. P. E. Vermeulen, *Toxicology* **2007**, *235*, 83–91.
52. T. Usia; H. Iwata; A. Hiratsuka; T. Watabe; S. Kadota; Y. Tezuka, *J. Nat. Prod.* **2004**, *67*, 1079–1083.
53. K. B. Rajinder; H. Glaeser; L. Becquemont; U. Klotz; K. G. Suresh; F. Martin, *J. Pharmacol. Exp. Ther.* **2002**, *302*, 645–650.
54. http://www.ccras.nic.in/ (accessed 19 July 2008).

Biographical Sketches

Dr. Pulok K. Mukherjee is Director of School of Natural Product Studies at Jadavpur University, Kolkata, India. He completed his M. Pharm in 1993 and PhD in pharmacy in 1997. He is a fellow of the Royal Society of Chemistry, UK, and Institute of Chemist, India. He has published over 100 research papers and review articles in peer reviewed journals, on topics connected with chemistry and biological activity of ayurvedic plants and their constituents. He has published and edited several books. He has received several awards and honors for his contribution in the field of natural product research. His research interest is in the promotion and development of natural resources based on their quality, safety, and efficacy from the Indian system of medicine.

M. Venkatesh is a Ph.D. student at the School of Natural Product Studies, Jadavpur University, Kolkata, working on development of value-added herbal formulations for the improvement of bioactivity of the leads from Indian medicinal plants. After obtaining his B. Pharm and M. Pharm degree he worked in a pharma industry. His expertise is on the development of novel dosage form using plant polyphenols and evaluating their bioavailability profile.

Arunava Gantait is a PhD student at the School of Natural Product Studies, Jadavpur University, Kolkata, working on development of quality control and standardization of herbal medicinal products. He completed his graduate and postgraduate studies in pharmacy from the same university. He has experience in developing the phytochemical profile of medicinal plants and presently working on the standardization of botanicals used in Ayurveda through marker profiling.

3.15 Biologically Active Compounds in Food Products and Their Effects on Obesity and Diabetes

Renger F. Witkamp, Wageningen University and TNO Quality of Life, Wageningen, The Netherlands

3.15.1 Introduction

It is obvious that diet affects our health in many ways. In fact, terms like functional foods and nutritional bioactives are pleonasms, since food is biologically active and functional by definition. The results of food can be regarded as positive or negative, but more often they are mixed and complex, since our diet is subtly acting on a multitude of physiological and interacting processes. Moreover, the effects take place at a rate that ranges from very gradual to acute. From a chemical point of view, food can be considered as a highly complex mixture of molecules of many different classes. In addition, these molecules are generally present in a changing and often unstable matrix and in an extremely wide concentration range. To make it even more complex, food is often subjected to different forms of processing and storage, which can make the chemical nightmare complete.

When nutrition developed as a scientific discipline, the emphasis was originally on the prevention of deficiencies, focusing on what were found to be essential components of the diet. Pharmacology on the contrary, having strong roots in experimental physiology, developed into a field in which biologically active compounds of either natural or synthetic origin were investigated for their properties to change organ and body functions. The focus was on single compounds, selectivity, and potency. As a result, nutrition and pharmacology developed rather separately in the Western world. Compared to this, the gap between nutrition and medicine has been much smaller in many other parts of the world. During the last decades, nutrition and pharmacology are again moving toward each other. The pharmacological discipline acknowledges that it can learn from nutrition when it comes to understanding the subtle regulation of metabolic diseases and the complexity of pathological disturbances. Pharmacologists are also increasingly realizing that the one disease–one target–one drug concept does not always lead to successful cures, in particular for chronic and degenerative diseases. This has led to new developments in drug discovery including systems-based approaches,[1,2] the principles of multitarget pharmacology,[3] and dirty or promiscuous[4,5] drugs. Vice versa, nutrition science is realizing that the principles of pharmacokinetics and pharmacodynamics provide tools for understanding the effects of both essential and nonessential components in our diet. The increased scientific and commercial interest in functional foods and food supplements has further intensified research and

development in this area. Many food companies are actively engaged in finding new bioactive compounds that can be used in food products. Some discovery programs in the food industry resemble approaches used in the pharmaceutical world, starting with molecular targets that are not infrequently derived from drug targets.[6,7]

Weight management and intervention strategies in metabolic complications of obesity, including type 2 diabetes and cardiovascular disease, clearly represent the most prominent areas of interaction and overlap between nutrition and pharmacology. They also embody fields of high interest from a public health and economic perspective. Obesity has reached epidemic proportions and is far more than a cosmetic problem.

The associated comorbidities including cardiovascular disease, type 2 diabetes, osteoarthritis, and many other health problems present a growing burden to society. This chapter focuses on the role of natural products in weight management and type 2 diabetes. Many of the compounds and preparations discussed here are, or will be, regulated as food products or food ingredients. As will be described in the next section, these compounds or preparations often go beyond conventional food products and should be classified as functional foods or dietary supplements. The arena of food and dietary supplements also represents an area where many positive health effects are being claimed, often without solid scientific evidence. This holds even more true for weight management, where there is a lot of emotion and high commercial interests involved. The number of bioactives that are claimed or just supposed to be useful in relation to weight management is enormous. In many cases, claims are anecdotal or based on *in vitro* data only. As will become clear from the following sections, human eating behavior and thus weight management are extremely complex and overweight is a multifactorial problem that goes far beyond biomedicine alone. As a consequence, animal studies also have limited value in predicting whether an intervention is ultimately effective in humans. Therefore in this chapter, only those compounds or mixtures will be discussed in detail for which there is at least some evidence that they are effective in humans. However, even this is sometimes difficult and also clearly represents a general problem to the regulatory authorities involved in claim evaluation. For example, how are historical and ethnopharmacological reports, sometimes going back for centuries, to be weighed against intervention studies or epidemiological data? As will also become clear in this chapter, in many cases there is not just one single molecule responsible for a given effect. On the contrary, it is often the combination that causes the effects. Therefore, preparations, mixtures, and single compounds are described throughout this chapter. Furthermore, one compound or one preparation can also have different actions. This implicates that the classification followed in the chapter might sometimes look somewhat arbitrary.

3.15.2 Some Basic Aspects of Food Composition

Traditionally, food components have been classified into macronutrients, which are present as bulk components, and micronutrients, which are present in lower amounts. There is not always a strict division between these two categories. Obviously, the bulk carbohydrates, proteins, fats, and water present in the diet are necessary for energy supply, homeostasis, growth, and development. Likewise, some minerals including calcium, chlorine, magnesium, phosphorus, sodium, and sulfur could be classified as macrominerals, since they are present and needed in relatively high amounts in the daily diet. Micronutrients include vitamins, many other minerals, and phytochemicals. Nutritionists also classify food components into the so-called nonnutrient compounds such as soluble and insoluble fibers. However, it is important to realize that this classification is mainly based on the nutritional role of the molecules and their average demand. Macronutrients, micronutrients, and nonnutrient compounds can all affect health in a positive or negative way. The metabolism of carbohydrates, fats, and proteins is interconnected in many ways and these processes in turn can be affected by nonnutrient components of the diet. Many micronutrients are pivotal to the homeostatic regulation of metabolism, growth, immunological processes, and hormonal and nervous regulation. Accurate regulation of these processes is required to maintain the fine balance between optimal health and early onset of (diet-related) disease. With our increasing knowledge on food chemistry and biology, it has become clear that molecules that were originally classified as macronutrients can have very specific effects at low concentrations. The fat fraction of the diet, for example, is far more heterogeneous than previously recognized, containing several bioactive lipids including n-3 polyunsaturated fatty acids, conjugated fatty acids, sterols, medium-chain fatty acids, diacylglycerols, sphingolipids, and phospholipids. The activity of many of these bioactive lipids is now

regarded as potentially beneficial.[8] On the contrary, it has become clear that some saturated fatty acids and trans conjugated fats increase the risk of cardiovascular disease.[9] Many compounds present in food apparently follow a hormesis behavior when it comes to their effects on health. Hormesis describes the phenomenon in which a mild stress, including exposure to toxins, can induce a protective response toward subsequent stresses.[10,11] Examples include several vitamins (A, D, K, etc.), mineral nutrients, dietary restriction, alcohol (ethanol), natural dietary and some synthetic pesticides, resveratrol, etc.[10,11] Another example is unfiltered coffee. The diterpenoids cafestol and kahweol, present in unfiltered coffee, are thought to protect against carcinogenesis due to their induction of glutathione *S*-transferase (GST).[12] At the same time, cafestol is one of the most potent cholesterol-increasing compounds that may be present in our diet, thereby contributing to an increased risk of cardiovascular disease.[13] In nutrition, the term 'bioactive compound' (or bioactive) is frequently used. It will be clear that bioactivity can have different meanings and that basically almost any molecule in the diet has some bioactivity. However, for practical reasons, this term will be used here to define a compound without significant energetic share, which in low concentrations has advantageous influence on health or functioning. Another term that is often used in this context is that of nutraceutical, which is a combination of nutrient and pharmaceutical. This term was coined around 1980 by Dr. Stephen DeFelice, who defined nutraceutical as any substance that is a food or a part of a food and provides medical or health benefits, including the prevention and treatment of disease.[14]

Last but not least, although the technical aspects of food storage and processing fall beyond the scope of this chapter, it is very important to realize that the ultimate composition of our diet can be very different from that of the fresh ingredients. Domestic methods of food processing have been developed over the centuries to make the final product more attractive in flavor, appearance, taste, and consistency. Cooking methods are an important factor affecting not only the food chemical composition, but also the intake of bioactive compounds under normal dietary conditions. These issues are for example reviewed in Ruiz-Rodriguez *et al.*[15]

3.15.3 The Regulatory Categories Conventional Foods, Functional Foods, and Dietary Supplements

3.15.3.1 Introduction

Although the gap between pharma and food is becoming narrower during the last few years, both disciplines have developed rather separately in the Western world. Historically, this has not always been the case. As Hippocrates stated in 500 BC, "Let food be your medicine and let medicine be your food. Only nature heals, provided it is given the opportunity." In many so-called traditional medicinal systems such as Ayurveda[16,17] and Traditional Chinese Medicine (TCM),[18] there is still no fundamental difference, and nutrition is a normal part of disease prevention therapy. During the last decades, the view on nutrition has changed even in the Western world. Food is no longer regarded as something to keep alive. In addition to its social effects, there is a considerable interest in food and nutrition to stay healthy or even become healthier. The primary goal of nutrition research is now to optimize health and to prevent, delay, or ameliorate the severity of disease. When it comes to health effects, it is clear that food differs from pharma. Maintaining and optimizing health requires quantification of homeostatic robustness and minimal deviations from normal. It is a well-known physiological fact that any organism will try to maintain a situation of homeostasis as long as possible, using various compensation mechanisms when its system is being disturbed. Quantification of phenotypic effects of dietary exposure relies on biomarkers, but the biomarkers used thus far are less than entirely suitable. It is quite understandable that especially in nutrition, single biomarkers are often unable to provide sufficient information and specificity and that biomarker profiles are necessary.[19] In addition, it is becoming increasingly apparent that early markers of disease will differ from markers of the later stages of disease progression. This concept is illustrated in **Figure 1**.

In general, for pragmatic reasons, health has been defined as the absence of evidence of disease and it is widely recognized that such a definition is inadequate. To address these issues, a series of omics technologies and functional analysis are now being applied, which need to be optimized and made widely available for use in nutrition research (see Section 3.15.10).

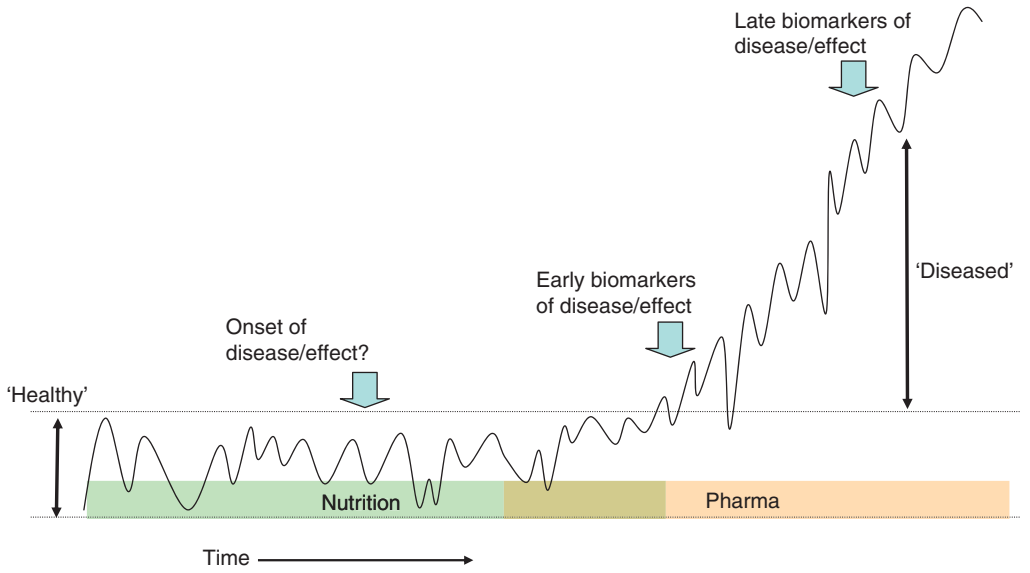


Figure 1 Schematic depiction of the concept of physiological balance and the significance of biomarker patterns for the various stages of development in time from normality (homeostatis) via dysfunction, to disease. An organism maintains its homeostasis as long as possible by changes in its metabolic pathway dynamics. Modern nutrition aims at early detection of dysfunction and the onset of disease. This requires repeated measurements, analysis of several pathways at each time point, and pattern recognition techniques (see also Section 3.15.10).

Driven by science but certainly also by commercial considerations, new product categories have evolved in the area between pharma and food. These include *functional foods* and *food supplements*, which will be described briefly in the next sections.

3.15.3.2 Functional Foods

Different types and forms of functional foods exist. The EU working definition provides a good description: food can be regarded as functional if it is satisfactorily demonstrated to beneficially affect one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being, or reduction of risk of disease. According to some, the so-called convenience foods should also be called functional foods. Most functional foods so far do not bear health claims but nutrition claims. Examples are low-fat, no added sugar, and high-fiber products. Health claims are of a different category. They suggest that health benefits can result from consuming the product. Recent EU legislation now also allows claims relating to reduction of disease risk or to children's development or health (Article 14 claims).[20,21] However, there are strict regulations and the European authorities (EFSA, http://www.efsa.eu) require solid scientific evidence that the claim is substantiated by taking into account the totality of the available data and by weighing of the evidence. Weighing is done according to a set of criteria as outlined in **Table 1**.

From a scientific point of view, claim support can be very complicated. How can we prove that a product improves health when the consumer is apparently healthy? What is health and (how) can we measure this? Possible answers to these questions may be related to protection against (possible) risk factors, the slowing down of (normal) degenerative processes, or showing that the flexibility of homeostasis improves. Another difficult issue is the relation between a biomarker and the likeness of getting a disease. New molecular and analytical technologies could provide some of the answers, since they allow detection of patterns of biomarkers, often called fingerprints. This may give a more holistic picture of health and resistance against disease. It is obvious that the requirements for health claims have become more stringent, and that the difference between health and disease has become smaller.

Table 1 Overview of basic criteria for the scientific substantiation of health claims on food products according to the PASSCLAIM consensus[22]

1. The food or food component to which the claimed effect is attributed should be adequately characterized
2. Substantiation of a claim should be based on data obtained in humans, primarily from intervention studies. The design of the study should include the following considerations:
   a. Study groups are representative of the target group
   b. Appropriate controls are included
   c. An adequate duration of exposure and follow-up is included to demonstrate the intended effect
   d. Characterization of the study groups' background diet and other relevant aspects of lifestyle has been performed
   e. The amount of the food or food component is consistent with its intended pattern of consumption
   f. The influence of the food matrix and dietary context on the functional effect of the component is considered
   g. Monitoring of subjects' compliance concerning intake of food or food component under test has been performed
   h. The statistical power to test the hypothesis is adequate
3. When the true end point of a claimed benefit cannot be measured directly, studies should use accepted and validated markers. Such markers are
   a. biologically valid in that they have a known relationship to the final outcome and their variability within the target population is known
   b. methodologically valid with respect to their analytical characteristics
4. Within a study, the target variable should change in a statistically significant way and the change should be biologically meaningful for the target group and consistent with the claim to be supported
5. A claim should be scientifically substantiated taking into account the totality of the available data and by weighing of the evidence

3.15.3.3 Food (Dietary) Supplements

A dietary (food) supplement is officially intended to supply nutrients (vitamins, minerals, fatty acids, amino acids, etc.) that are supposed to be missing or not consumed in sufficient quantity in a person's diet.

In reality a great number of dietary supplements are now containing (mixtures of) herbal products.

Food supplements often look like drugs in having the form of a tablet, capsule, liquid, etc. Most products described as supplements are regulated as foods, but some may be regarded by law as medicinal products. This differs from one country to another. Regulations require that supplements are demonstrated to be safe, both in quantity and quality. Some vitamins are essential in small quantities but dangerous in large quantities. In Europe, it is also an established notion that food supplements should not be labeled with drug claims. New EU legislation states that with regard to health claims dietary supplements should meet the general regulations set for other food products.[20–22] Compared to the European Union, the regulatory framework on dietary supplements in the United States is less stringent. In the United States, dietary supplements are regulated by the 1994 Dietary Supplement Health and Education Act (http://vm.cfsan.fda.gov/%7Edms/dietsupp.html). This means that dietary supplements can be marketed without FDA approval and without any scientific evidence to substantiate safety or efficacy. Only after a supplement has been marketed and shown to be unsafe – in other words, once serious injury or illness has occurred – can the FDA take action[23] to remove the product from the market.

3.15.4 Obesity: From Prevention to Metabolic Complications

3.15.4.1 Introduction

Weight management and the consequences of obesity represent typical fields where nutrition and pharmacology are interacting and overlapping. Obesity and its metabolic complications such as type 2 diabetes are being regarded as a worldwide epidemic.[24] Even in developing countries, the percentage of overweight and obese people is rapidly increasing.[25,26] Obesity is defined as a state of excess body fat that frequently results in impairment of health. According to the WHO, it may be expressed in adults in terms of the body mass index (BMI: weight in kg/height in meters squared). A BMI of between 18.5 and 25 is considered within the normal range, a BMI of 25–30 represents overweight, and a BMI of >30 is considered to represent obesity. Extreme obesity is defined as a BMI of $\geq$40 and is associated with a substantially greater health risk than a BMI of 30.

Although BMI remains a good and simple general indicator for risk and population studies, other methods such as waist circumference or waist/hip ratio are often better,[27,28] for example, in children or people with a different stature. This is due to the fact that not body weight by itself, but fat distribution determines health risks. For any given amount of total body fat, the subgroup of individuals with a predominant excess of intraabdominal or visceral adipose tissue is at substantially higher risk of becoming insulin resistant or getting any of the other features of metabolic syndrome.[29] The increased access to food with high-energy density and the reduced need for physical activity are the main drivers for the obesity epidemic. Obesity may be regarded as an ecological problem for populations that are living in an increasingly obesogenic environment.[30] However, it has also become clear that weight management in our society is not to be considered as a simple biological or psychological problem. The desire to eat is one of the strongest of human instincts. In ancient times, our ancestors had to survive during periods of famine, and certain genes have evolved to regulate efficient intake and utilization of fuel stores. Such genes were termed thrifty genes in 1962.[31] People who become obese have a lifelong tendency both to defend their excess weight and to continue to gain extra body fat.[32] In addition, genetic factors play a role in determining the differences between people.[33]

3.15.4.2 Appetite and Eating Behavior. Why are Many People Overeating?

Humans eat not only to satisfy their appetite, but also for many other reasons, including sensory hedonics, sensory stimulation, reduction of stress, social pressure, and boredom.[34–37] The processes that determine eating behavior are often divided into sensory, cognitive, postingestive, and postabsorptive processes. Sensory effects are generated through the taste, smell, temperature, and texture of food. Cognitive effects may also play a role, for example, in the beliefs of the consumer about the properties of the foods being eaten and their effects. Postingestive factors include the effects of gastric distension and rate of gastric emptying and the release of hormones by the gastrointestinal (GI) tract. These include the pancreatic hormone glucagon, cholecystokinin (CCK) from the duodenum, glucagon-like peptide-1 (GLP-1), peptide YY (PYY), etc.[38,39] The postabsorptive phase of satiety results from the action of metabolites after absorption and passage into the bloodstream. Circulating levels of glucose, amino acids, and lipids may act directly on the brain or through peripheral receptors leading to the termination of eating. Blood glucose concentration activates glucoreceptors in the hypothalamus, either acting to upregulate hunger when blood glucose levels fall, or upregulate satiety when glucose concentrations rise. In the longer term, deposition of fat may lead to control of appetite by neuronal and hormonal signals. Leptin, a protein secreted by white fat cells, acts on the leptin receptors in the hypothalamus. Doing so, leptin provides a feedback mechanism between adipose tissue and the brain. Leptin inhibits neuropeptide Y (NPY), the most potent peptide to stimulate feeling.[40] According to the most current views, endocrine biochemical signals are not regarded as major drivers for meal onset.[37] So far, the only exception seems to be ghrelin, which is an orexigenic (stimulating eating) peptide hormone, surging just before meals and suppressed by ingested nutrients. By contrast, meal termination is a process that is delicately regulated by various signals originating from the stomach and gut,[37–39] and is often referred to as satiation. In order to achieve efficient nutrient digestion and absorption, the gut is equipped with an extensive signaling system that regulates GI motility, secretion of enzymes, and food intake. Eating is typically stopped long before gastric capacity is reached. When food is diluted with noncaloric bulking agents, the volume ingested increases to maintain constant caloric intake.[38] Satiation signals arise from multiple sites in the GI system, including the stomach, proximal small intestine, distal small intestine, colon, and pancreas. Satiation signals interact with adiposity signals, in which leptin plays an important role.[37,38] This delicate regulatory mechanism is controlled by the hypothalamus, which is continuously informed about the nutritional, energetic, and environmental status of the body through peripheral and central orexigenic or anorexigenic messages. Gastric satiation is mainly volumetric, with signals arising primarily from mechanical distention.[38] Intestinal satiation, on the contrary, is mainly nutritive. A central role in the sensing of nutrients in the GI tract is played by the so-called enteroendocrine cells. By their shape and location these cells are excellently equipped to sense chemical structures in the lumen and to pass the information to small blood vessels and nerve terminals. Their apical side is in contact with the luminal contents and their basolateral side is in contact with the vasculature of the lamina propria, neural cells, and with distant enterocytes.[38,41] Several satiation-inducing peptides are being released by enteroendocrine cells, including CCK, bombesin, glucagon, GLP-1, GLP-2, apolipoprotein A-IV, amylin,

somatostatin, enterostatin, and PYY. Enteroendocrine cells are thus the primary sensor in the GI on the crossroad between food and nutritional physiology. Different types of enteroendocrine cells are being distinguished, depending on their location along the GI tract and (main) secreted products. Until recently, luminal sensing mechanisms were poorly explored. However, there is now increasing evidence for the role of G-protein-coupled receptors (GPCRs) as molecular sensors on the surface of enteroendocrine cells that are responsive to luminal contents.[42,43] Some of these GPCRs are still called orphan receptors, which means that their principal ligands are not yet known. However, considerable progress is being made with deorphanizing these receptors. For example, Overton *et al.*[44] describe the GPCR GPR119 for the lipid derivative oleoylethanolamine (OEA), and its potential use in the discovery of small-molecule hypophagic agents. GPR119 is expressed predominantly in the human and rodent pancreas and GI tract. Very recently, Tanaka *et al.*[45] reported that the GPR120 receptor is involved in mediating lipid-induced CCK release in the mouse (*in vivo*) and in the (murine) enteroendocrine STC-1 cell line. Of interest is also the recent publication of Ryberg *et al.*,[46] who described the deorphanization of the GPR55 receptor, suggesting that it is a novel cannabinoid (CB) receptor. This receptor is highly expressed in the human jejunum and ileum (in addition to the brain and parts of the immune system). Remarkably, GPCRs belonging to the taste receptors are also expressed on enteroendocrine cells.[43,47] For example, Jang *et al.*[47] showed that human duodenal L cells express sweet taste receptors, the taste G protein gustducin, and several other taste transduction elements. Nutrient signaling to the brain is a combination of endocrine and neural processes. The vagal afferent nerve fibers form the enteric nervous system (ENS), which communicates to the brain via the vagus nerve. Nerve terminals in the mucosa contain specific receptors that recognize satiation peptides.[48]

3.15.4.3 The Role of the Endocannabinoid System

The appetite-inducing properties of marijuana (*Cannabis sativa*) and its main psychoactive component Δ^9-tetrahydrocannabinol (THC) have already been known since centuries. Following the cloning of the first CB receptor (now called CB_1) in 1990,[49] research on the endocannabinoid system has spectacularly increased, especially during the last decade. The endocannabinoid system is clearly a highly pleiotropic system. It has become clear that it plays a major role in the central and peripheral regulation of eating behavior, food intake, and energy metabolism. In addition, endocannabinoids have been found to be involved in well-being, stress, the immune response, bone formation, etc. A complete review of the many roles of the CB system falls outside the scope of this chapter. However, many excellent reviews have been published on this topic during the last few years.[37,50–68] So far, two CB receptors, CB_1 and CB_2, have been described. In addition, the previously named orphan receptor GPR55 is likely to be called a CB receptor soon.[46] The CB_1 receptor is possibly the most abundantly expressed GPCR in the central nervous system. The CB_2 receptor was initially considered as linked to the immune system, being largely expressed in several immune cells and tissues. However, it has become clear that this characteristic is not strict, as CB_1 receptors also have important functions in peripheral tissues, and CB_2 receptors are also present in some brain regions. For example, the first-in-class CB_1 blocker rimonabant was initially developed for its central, appetite-reducing, properties. In the mean time, it has become clear that the positive effects of the compound on plasma lipids and insulin resistance are due to its peripheral actions.[55,69] The endogenous ligands of the CB receptor have been named endocannabinoids. Anandamine (*N*-arachidonoylethanolamine (AEA)) and 2-arachidonoylglycerol (2-AG) were the first compounds discovered and these are still the most investigated. In the meantime, several other compounds have been discovered with affinity for the CB receptors. Many of these are actually fatty acid amides or esters, as depicted in **Figure 2**.

It has been found that endocannabinoids are not stored in secretory vesicles but are synthesized *de novo* on demand and rapidly broken down after synthesis. It has also become clear that many of the compounds that had originally been called endocannabinoids are promiscuous in their targets.[56,61,70] For example, the endocannabinoid OEA was found to act predominantly on the peroxisome proliferator-activated receptor (PPAR)-α receptor.[71–73] Interestingly, it has also been found that plants produce similar alkylamides.[74–76] Gertsch[74] and Gertsch *et al.*[75] found that *Echinacea* plants contained dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides as well as trienoic and dienoic acid derivatives that bind to the CB_2 receptor and are able to inhibit tumor necrosis factor-α (TNF-α) release. Structurally similar amides of fatty acids and primary amines have been found in a

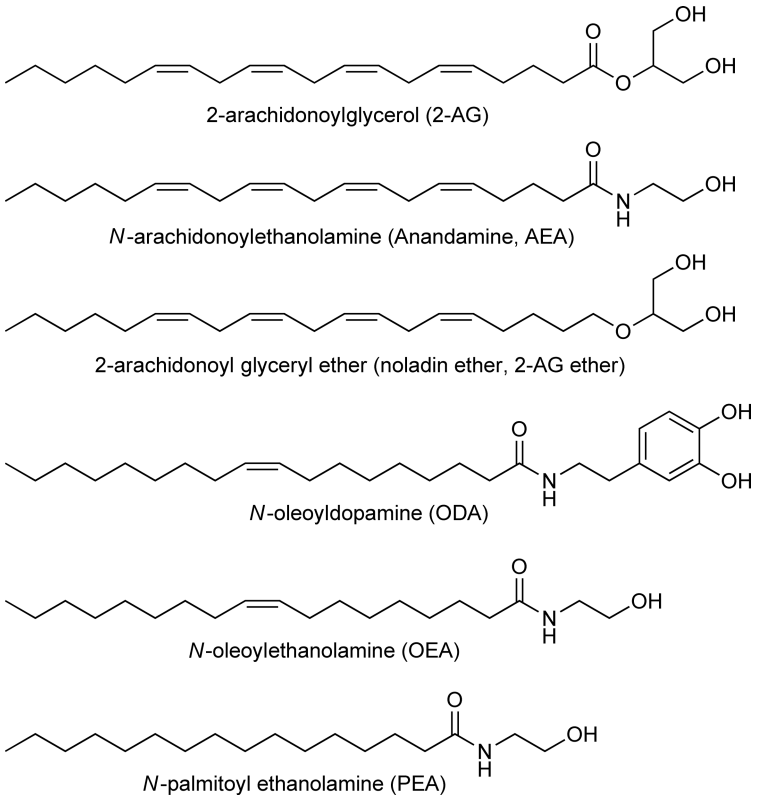


Figure 2 Chemical structures of some endocannabinoids and related compounds: *N*-arachidonoylethanolamine (anandamine, AEA), 2-arachidonoylglycerol (2-AG), 2-arachidonylglyceryl ether (noladin ether, 2-AG ether), *N*-oleoyl dopamine (ODA), oleoylethanolamine (OEA), and palmitoyl ethanolamine (PEA).

variety of plant families, including the Asteraceae, Brassicaceae, Leguminosae, Piperaceae, and Rutaceae.[77] CBs are involved in both the central and peripheral regulation of appetite and eating behavior.[51,54] In the GI tract, nerve terminals in the lamina propria have been shown to contain CB receptors and there appears to be a cross talk between satiation peptides and the endocannabinoid system.[37,78] Burdyga *et al.*[78] were the first to show that CCK decreased CB_1 expression on vagal afferent neurons. In rats, the expression of CB_1 in the vagal afferent neurons was shown to be increased by food deprivation and decreased by a following subsequent refeeding period. Several experiments have proved that energy status profoundly stimulates the intestinal synthesis of both the endocannabinoids anandamine and OEA. In rats, starvation strongly stimulates the intestinal synthesis of anandamine and this effect is reversed by feeding. The anorectic endocannabinoid, OEA, is synthesized in larger quantities in fed rats than in starving rats. Cani *et al.*[79] showed that the CB_1 antagonist rimonabant and OEA were able to suppress both ghrelin and GLP-1 in rats. At present, it is unknown whether endocannabinoids are released by enteroendocrine cells as messenger molecules or whether CBs and lipids with CB-like properties in the diet can directly interact with nerve fibers in gut tissue. In addition to CB receptors, there is also evidence for the presence of vanilloid transient receptor potential vanilloid receptor subtype 1 (TRPV1) and melanin-concentrating hormone-1 receptors[80] on nerve afferents.

3.15.4.4 Pathological Complications of Obesity

3.15.4.4.1 Obesity and the metabolic syndrome

Obesity is far more than a cosmetic problem. Several epidemiological studies have documented that excessive fat accumulation is associated with serious diseases and leads to increased morbidity and mortality. Although the relationship is not linear, health risks increase with severity of obesity and include hypertension, insulin

resistance and type 2 diabetes mellitus, and cardiovascular disease (angina pectoris, claudicatio intermittens, venous thromboses and their major consequences such as pulmonary embolism). This cluster of complications is often called the metabolic syndrome.[81] According to the International Diabetes Federation,[82] a person is defined as having the metabolic syndrome when he/she has central obesity (waist circumference >94 cm for Europid men and >80 cm for Europid women), plus any two of the following four factors: raised triacylglycerol level (>150 mg dl^{-1}, or specific treatment for this lipid abnormality); reduced high-density lipoprotein (HDL) cholesterol (<80 mg dl^{-1} in males and <50 mg dl^{-1} in females, or specific treatment for this lipid abnormality); raised blood pressure (systolic >130 mm Hg or diastolic >85 mm Hg, or treatment of previously diagnosed hypertension); and raised fasting plasma glucose (>100 mg dl^{-1}, or previously diagnosed type 2 diabetes). Excess body fat, particularly visceral fat (Section 3.15.4.1), is one contributing cause of the metabolic syndrome. In abdominally obese individuals with the metabolic syndrome, weight reduction will reduce all of the metabolic risk factors. The core 'metabolic risk factors' are atherogenic dyslipidemia, elevated blood pressure, elevated plasma glucose, and a prothrombotic and a proinflammatory state, and each of these 'risk factors' has several components. Typically, in the early stages, the metabolic risk factors are often only marginally increased. With time, particularly when obesity increases and other exacerbating factors come into play, the risk factors become categorically increased. Throughout this period, atherogenesis proceeds and in many individuals, atherosclerotic cardiovascular disease becomes apparent. Eventually, the condition culminates in type 2 diabetes, which further raises the risk of atherosclerotic cardiovascular disease. Although the complications of obesity tend to cluster, differences between patients can be large. In addition, the metabolic syndrome as a cluster does not fully predict cardiovascular mortality. Therefore, some experts argue whether metabolic syndrome should indeed be called a syndrome.[83,84] Increasing evidence suggests that visceral obesity, when going together with obesity, also increases the risk of other complications including osteoporosis and cognitive decline. Overweight and obesity in middle age have also been associated with future risk of dementia.[85]

Another important and serious complication of obesity and diabetes is nonalcoholic fatty liver disease (NAFLD), which is nowadays the major reason for abnormal liver function in the Western world.[86] Evidence is accumulating that in obese persons, adipose tissue activates inflammatory responses in fat and liver, with associated increases in the production of cytokines and chemokines.[87–93]

Immune cells including monocytes and macrophages are recruited and/or activated, and together these cause local insulin resistance. Proinflammatory and proatherogenic mediators are produced in the adipose tissue and liver and associated immune cells. This creates a systemic inflammatory diathesis that promotes insulin resistance in skeletal muscle and other tissues, and atherogenesis in the vasculature associated with an increased risk of cardiovascular disease in adults and with less favorable cardiovascular risk factor status in children and adolescents.

3.15.4.4.2 Type 2 diabetes

The International Diabetes Federation estimates that 246 million adults worldwide now have diabetes mellitus. Type 2 diabetes mellitus (T2DM) accounts for 90–95% of all diabetes. It was previously called noninsulin-dependent diabetes or adult-onset diabetes. It is generally characterized by insulin resistance and relative insulin deficiency.[94] Obesity and T2DM are closely linked although genetic and/or environmental factors are also involved since still many obese human subjects do not progress to the diabetic state. The incidence of T2DM is escalating to epidemic proportions and by 2025, the figure is expected to reach 380 million. Diabetes accounts for around 6% of total global mortality, with 50% of diabetes-associated deaths being attributed to cardiovascular disease.[24] Furthermore, obesity and diabetes mellitus are increasingly being diagnosed in younger individuals.[24] T2DM significantly increases the risk of cardiovascular morbidity and mortality. Long-term complications in patients with T2DM include cardiovascular disease, blindness, neuronal damage, renal failure, and diabetic foot disease. Nonetheless, it is generally accepted that two features are particularly critical for obesity to elicit type 2 diabetes.[95] First, impaired responsiveness of skeletal muscle to insulin is a primary condition in obesity and a precondition for the onset of type 2 diabetes. Second, a defect required for progression from insulin resistance to type 2 diabetes is the failure of β-cells to secrete the required levels of insulin that maintain normal fasting blood glucose levels. In addition to muscle, the liver and adipose tissue also become insulin resistant. It has been established that elevated levels of circulating free fatty acid (FFA) play an important role in glucose uptake. This is now often called the lipotoxicity phenomenon.[96] In addition to the effects of circulating FFAs, deposition of fatty acids into nonadipose fat stores, including muscle, might contribute to insulin resistance in obesity. However,

a similar increase in muscle triglyceride during exercise correlates with high insulin sensitivity.[95] Guilherme *et al.*[95] in their excellent review stress the importance of adipose tissue in controlling whole-body metabolism by sequestering fat. Interestingly, a lack of adipose tissue also leads to elevated circulating concentrations of triglycerides and fatty acids and can also cause insulin resistance. The presence of adipose tissue is also required for normal secretion of adipokines such as leptin and adiponectin, which enhance insulin sensitivity. In conclusion, normal insulin sensitivity and glucose homeostasis require functional adipose tissue in proper proportion to body size. The development of the inflammatory state in adipose tissue is associated with insulin resistance in skeletal muscle, as reviewed by Hotamisligil.[88] Adipocytes and macrophages secrete monocyte chemoattractant protein-1 (MCP-1) and other attractants for inflammatory cells, as well as large amounts of TNF-α and other cytokines.The action of such cytokines has two dramatic effects on adipocyte function: an increase in lipolysis and a decrease in triglyceride synthesis.

3.15.4.5 Current Medical Intervention Strategies

3.15.4.5.1 Weight management

Nonpharmacological options for treatment include nutritional education and modification (usually caloric restriction) and lifestyle modification, including increased activity and exercise. Unfortunately, long-term effects of diet or exercise on weight are often disappointing (see also Section 3.15.4.5.2). With respect to durable weight reduction, bariatric surgery is the most effective long-term treatment for obesity with the greatest chances for amelioration and even resolution of obesity-associated complications.[97,98] Two main groups of techniques are being used. Malabsorptive procedures induce decreased absorption of nutrients by shortening the functional length of the small intestine.[97] Restrictive operations reduce the storage capacity of the stomach. As a result, early satiety arises, leading to a decreased caloric intake. Liposuction, not to be confused with bariatric surgery, removes only subcutaneous fat, which carries little metabolic risk. With liposuction, energy intake is unaffected and body weight will rise again to achieve energy balance. Pharmacological options are considered only as an adjunct to dietary measures and physical exercise. It goes without doubt that drug treatment alone cannot cure obesity. Effective management, including drugs when needed, must be lifelong and focused on weight-loss maintenance in a similar fashion to the effective treatment of hypertension or diabetes.

Currently, two compounds are licensed for pharmacological weight management in most countries.[99–102] The first one is the intestinal lipase inhibitor Orlistat, which causes malabsorption of 30% of dietary fat. It leads to 5–10% weight loss in 50–60% of patients, and in clinical trials the loss (and related clinical benefit) is largely maintained for up to at least 4 years.[99–101] Typical side effect of the compound is steatorrhea. The second compound, sibutramine inhibits the reuptake of norepinephrine and serotonin, promoting and prolonging satiety. It produces 5–10% weight loss in 60–70% of patients, and in clinical trials it was found to be well tolerated for at least 2 years.[99–101] A third drug, the CB_1 antagonist rimonabant, was approved by the European Authorities in 2006, being the first compound in a new class of drugs. Expectations were high and several other CB_1 blockers were in clinical testing at that time. Rimonabant produces weight loss and weight-independent improvements of some cardiovascular risk factors. These include an increase in HDL cholesterol and adiponectin and a decrease in triglycerides, in the peak size of LDL cholesterol particles, fasting insulin, leptin, and C-reactive protein. However, already during the approval procedure concerns were raised about possible central side-effects, including depression and suicidal ideation. This was particularly relevant for patients who already had a history of depression. It has been suggested that this was related to the fact that the compound might dampen the feedback systems for pleasurable responses. Further clinical trials and experience with the compound finally led to the decision of the European Medicines Agency (EMEA) to recommended suspension of the marketing authorisation by the end of 2008. Around that time several other companies stopped their clinical development programs on CB_1 blockers. However, CB_1 antagonists or reverse agonists predominantly acting peripherally, or compounds with lower receptor affinity remain of interest. This includes the plant cannabinoid Δ^9-tetrahydrocannabivarin (THCV), discussed in Section 3.15.6.3.3.

Currently, many different preparations of natural origin are already in use as dietary supplement for weight loss. For example, Pillitteri *et al.*[23] performed a survey on the use of dietary supplements for weight loss in the United States. In this nationally representative survey of adults, 33.9% who had ever made a serious weight-loss attempt reported using a dietary supplement to lose weight. Use of dietary supplements was more common among women,

younger adults, minorities, and those with less education and lower incomes. In addition, the food industry is intensively looking for new ingredients that can be added to functional food products in order to produce more satiation, reduce fat absorption, or change fat deposition. These will be discussed further in the following sections.

3.15.4.5.2 Type 2 diabetes: General intervention strategies

T2DM is increasingly being regarded as a multifactorial disease with different clinical symptom patterns. As described in Section 3.15.4.4.2, T2DM is often, but not necessarily, associated with obesity. If this is the case, lifestyle changes, dietary measures, weight reduction, and increased physical activity will generally be very effective.[103] However, although people with diagnosed disease may be better motivated to change their behavior, long-term changes in lifestyle still remain difficult. Zivkovic *et al.*[104] have recently reviewed dietary possibilities for metabolic syndrome with special emphasis on nonalcoholic fatty liver disease. If weight can be reduced to desirable levels and if regular exercise can be sustained, all risk factors of the syndrome will improve and progression to more advanced stages will be slowed. Nutrition recommendations in diabetes are, for example, described in the position statement of the American Diabetes Association,[105] which is regularly updated. Modest but regular physical activity as part of weight management programs and T2DM therapy is important. Although it is relatively inefficient for losing weight, regular exercise appears to be crucial in the prevention of weight gain, the successful maintenance of weight loss and, most importantly, the promotion of general health.[106] The detrimental effects of high plasma levels of FFA that occur in obesity are increasingly being recognized as contributing factors to T2DM[95,96] and exercise helps to lower these. In severe obesity, an effective lifestyle change requires a multidisciplinary team. Moreover, many people are not able to fully reverse the existing metabolic risk factors with lifestyle modification, and as risk factors worsen with advancing age, there is an increased need for drugs to manage particular risk factors. A pharmacotherapeutic management program takes into account lifestyle, general cardiovascular and renal status, liver functions, blood pressure, etc. A complete discussion of the therapeutic considerations and options in T2DM and the other complications of the metabolic syndrome falls outside the scope of this chapter. For a review, see Grundy.[87] Briefly, current pharmacotherapy often involves oral antidiabetic drugs and sometimes insulin. Oral antidiabetic drugs include metformin, sulfonylureas, and PPAR-γ agonists. Sulfonylureas, for example, glicazide, glipizide, and tolbutamide, act to increase the production of endogenous insulin by the β-cells in the islets of Langerhans. Metformin seems to lower hepatic glucose output, which decreases insulin resistance and plasma glucose levels. Metformin has originally been derived from the plant *Galega officinalis* (see Section 3.15.9.3).[107] The compound has been available for many years and is relatively inexpensive. It is widely used for the treatment of diabetes and can be combined with a sulfonylurea. Thiazolidinediones (TZDs), which include pioglitazone and rosiglitazone, are used to improve insulin sensitivity and reduce hyperglycemia. These drugs act as agonists of the nuclear receptor PPAR-γ, which is predominantly expressed in adipose tissue, but also occurs in other cell types including macrophages, hepatocytes, and endothelial and vascular smooth muscle cells. TZDs reduce the secretion of FFAs and adipokines such as TNF-α, other inflammatory cytokines, resistin, and plasminogen activator inhibitor-1 (PAI-1). Recent and future treatment options include GLP-1 analogues and dipeptidyl peptidase-IV (DPP-IV) inhibitors,[108–110] and PPAR-α/PPAR-γ dual agonists.[109,111] In addition, CB_1 antagonists are now being repositioned in T2DM.[55,69,112] Several natural products have been used, which will be discussed in Section 3.15.9.

3.15.5 Natural Compounds in Weight Management and Diabetes – Introduction and Classification

Weight management represents a highly challenging area for scientists, consumers, and industry. With regard to the industry, nutritional weight loss and weight management are regarded as a multibillion market worldwide. The major problem is that our physiology is not adapted to the obesogenic environment and that we are extremely well equipped to store as much energy as possible and to use it very efficiently.

As mentioned before, many preparations claim to decrease appetite, decrease energy absorption, or stimulate energy expenditure. In many cases however, at its best *in vitro* data are available and there is perhaps no other area in nutrition where there are so many anecdotes and so little solid evidence. Having said this, it seems logical that the clues that affect satiety and satiation do reside in nutrition and eating habits. There are

interesting preparations and promising results available. There are possibly many others to be discovered from traditional forms of medicine, especially when ethnopharmaceutical research and metabolomics are combined to their full potential.[113–116]

For reasons of simplicity, current approaches to weight management will be divided here into

– natural compounds and preparations suppressing appetite or stimulating satiation (feeling of fullness) – described in Section 3.15.6
– natural compounds or preparations affecting lipid (energy) absorption – described in Section 3.15.7
– natural compounds affecting lipid metabolism or energy expenditure – described in Section 3.15.8

Despite this categorization, it will be obvious that some compounds fall into more than one class. In addition, several herbal preparations consist of mixtures with different active ingredients. It is often not apparent which single molecule is responsible for a given effect. Even more, it is often the combination that is responsible for the effects. Therefore, preparations, mixtures, and single compounds are described in the following sections.

3.15.6 Natural Compounds and Preparations for Appetite Regulation

3.15.6.1 Introduction and General Mechanisms

There are numerous natural compounds, dietary supplements, and functional foods for which an effect on satiety or satiation is being claimed. In some cases, there is scientific support for such claims. In many cases however, there is no or hardly any evidence, and numerous other compounds have not been systematically investigated at all. In this section, some of the most promising and well-known developments will be discussed.

They will be divided into peripherally acting preparations, that is, in the GI tract itself, and centrally acting compounds and preparations.

The first category includes

– proteins and peptides
– lipids
– pinolenic acid
– fatty acid amides
– protease inhibitors
– lipase inhibitors

Centrally acting compounds and preparations prepared from

– *Hoodia gordonii*
– *Caralluma fimbriata*
– CBs

3.15.6.2 Peripherally Acting Compounds and Preparations

3.15.6.2.1 Proteins and peptides

Several studies suggest that dietary proteins suppress food intake and delay the return of hunger more than fats or carbohydrates, in a manner not related to energy content alone.[117,118] Some protein sources contain specific amino acid sequences or proteins themselves that may elicit direct effects on satiety. In this respect, dairy proteins and, to a lesser extent, meat proteins have received most attention so far. However, several lines of research also suggest that plant-derived proteins and peptides can exert induction of satiation. Some specific amino acid motifs may, at least partly, have their effect via inhibition of proteases in the GI tract, which may be an evolutionary developed mechanism (see Section 3.15.6.2.5).

Evidence is accumulating that peptides are also being recognized by G-protein-coupled receptors on enteroendocrine cells. Choi *et al.*[119] found that protein hydrolysates induced CCK transcription via the GPR93 receptor on STC-1 cells. Meat hydrolysate and amino acids were also shown to affect GLP-1 secretion in the NCI-H716 cell line.[120] For the peptide receptors, there are two endogenous ligand proteins of around 8 kDa that have been described to

specifically bind gut receptors and induce CCK: the luminal CCK releasing factor (LCRF).[121] There are some commercial preparations based on peptide mixtures that claim to induce satiation. Following the recent progress in the discovery of GI receptors and the developments in genomics, there is probably more to come in this area.

3.15.6.2.2 Lipids

Lipids are digested mainly into monoglycerides and FFAs. These products are absorbed into the enterocytes by diffusion and travel to the endoplasmic reticulum, where they are resynthesized back into triglycerides. Fatty acids with a chain length of C10 or greater than C10 are packed into chylomicrons, removed from the enterocyte by exocytosis, and absorbed into the lymph. Through the lymph system they enter the systemic circulation. It has been shown that the FFAs, and not the triglycerides, are the stimulus for satiation feedback mechanisms.[48,122,123] So far, most of the studies concern the effect of fatty acids on the release of CCK and GLP-1. CCK is mainly produced in the upper parts of the GI tract by enteroendocrine cells of the I-type.[38] The chain length appears to be a major determinant for FFA to induce satiation. Fatty acids with chain lengths up to 10 carbon atoms induce no more CCK release than vehicle.[122,124,125] GLP-1 is produced and secreted from enteroendocrine L-cells in the small intestine and colon. After food intake, plasma levels of GLP-1 rapidly increase. This peptide is responsible for effects such as inhibition of gastric emptying, stimulation of insulin release, inhibition of glucagon release, and inhibition of appetite, thereby inhibiting food ingestion. It is suggested that peripheral actions of GLP-1 are mediated by the vagus nerve.[126] Recently, Tanaka *et al.*[45] showed that long-chain fatty acids such as α-linolenic acid (C18:3) stimulated the G-protein-coupled fatty acid receptor GPR120 in the murine STC-1 cell line, leading to CCK release. They also provided further evidence for the involvement of Ca^{2+} influx through L-type Ca^{2+} channels upon stimulation. The GPR120 receptor is widely expressed in the intestine. Another GPCR, GPR40, shows many similarities to GPR120 but its role is not yet clear. In another study by the same group,[127] rat GPR120 was cloned and characterized. It showed similar tissue distribution and ligand properties to those of mouse GPR120, and 85 and 98% sequence identity with the human and mouse GPR120 proteins, respectively. At least in rat, stimulation by α-linolenic acid also induced GLP-1. Another important feedback peptide in lipid sensing is PYY. Its role has recently been reviewed by Grudell and Camilleri.[128] PYY is a 36 amino acid linear peptide that is mainly secreted from enteroendocrine L cells of the distal small intestine and colon. It is released upon stimulation by FFAs, but also by glucose, bile salts, amino acids, and other gut peptides including vasoactive intestinal peptide (VIP), CCK, gastrin, and GLP-1. PYY release may also be mediated via a neural reflex involving the vagus nerve, as a liquid meal infused into the duodenum of rats leads to increased circulating PYY levels, even before nutrients of the meal have reached the distal small intestine. PYY is the principal mediator of the 'ileal brake' reflex, which is a feedback mechanism that slows gastric emptying and intestinal transit in response to nutrients (fat, protein, and carbohydrates) in the distal small intestine. The ileal brake is regarded as a biological salvage mechanism to ensure fat absorption is maximized.[122] In the past, this has been without question advantageous to survival. There is evidence that the degree of saturation of FFAs affects satiety. Lawton *et al.*[129] studied the satiety in human volunteers after different fat mixes. Polyunsaturated fatty acids (PUFAs) resulted in greater satiety ratings than both monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs). Very recently, Parra *et al.*[130] published the results of a study that enrolled 232 overweight and obese volunteers. They received an energy-restricted balanced diet supplemented with either low-dose (260 mg day^{-1}) or high-dose (1300 mg day^{-1}) omega-3 fatty acid, obtained from dietary fish and fish oil food supplements. The intervention was for 8 weeks and appetite measurements were taken during the last 2 weeks of the study. Subjects who ate a dinner rich in long-chain omega-3 fatty acids felt less hunger and more full immediately and 2 h after food intake than their counterparts fed with diet low in long-chain omega-3 fatty acids. Blood sample analysis also showed that a higher omega-3 concentration and an improved omega-3 to omega-6 ratio were associated with higher satiety.

3.15.6.2.3 Pinolenic acid

Pinolenic acid (5,9,12-octadecatrienoic acid; see **Figure 3**) is an example of a fatty acid that has received special attention with regard to its potential satiation-inducing properties. It is found in high concentration in oil from the nuts of the Korean pine (*Pinus koraiensis*).

Seeds of the Korean pine and other pine trees contain high levels of poly- and unsaturated fatty acids, which may be of interest because of their lipid-lowering properties in general.[131] The Korean pine seems to produce

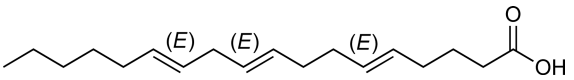


Figure 3 Chemical structure of pinolenic acid – (5*E*,9*E*,12*E*)-octadeca-5,9,12-trienoic acid).

relatively much pinolenic acid.[132] Pinolenic acid was shown to stimulate the release of CCK-8 from cultured STC-1 cells.[133] In a randomized, placebo-controlled, double-blind crossover trial involving 18 overweight postmenopausal women, a Korean pine preparation significantly induced CCK-8 levels compared to placebo 30 min after a pine nut FFA preparation and 60 min after a triglyceride (TG) preparation. GLP-1 was higher 60 min after pine nut FFA compared to placebo. The appetite sensation prospective food intake was 36% lower after pine nut FFA relative to placebo. This study suggests that Korean pine nut may work as an appetite suppressant through an increasing effect on satiety hormones and a reduced prospective food intake. The mechanism through which pine nut FFA and TG are able to induce CCK-8 and GLP-1 remains unknown.[133] It is speculated that the fatty acids in pine nut oil interact with chylomicron formation or transport, and thereby influence the release of CCK-8.[133]

3.15.6.2.4 Fatty acid amides

As described in Section 3.15.4.3, some fatty acid amides including OEA are known to reduce food intake and body weight gain. These fatty acid amides have been linked to the endocannabinoid system. However, for OEA, it was demonstrated that the compound had no interaction with CB_1 or CB_2 and acts as PPAR-α agonist instead.[71–73] OAE is an endogenous lipid produced primarily in the small intestine and has been identified to play an important role in the regulation of animal food intake and body weight.[134] It has also been suggested that in addition to appetite regulation, OEA may regulate body weight by altering peripheral lipid metabolism, including by increasing lipolysis in adipocytes and enhancing fatty acid uptake in enterocytes.[134] Recently, Overton *et al.*[44,135] presented evidence that OEA is an endogenous ligand of the orphan receptor GPR119, a GPCR expressed predominantly in the human and rodent pancreas and GI tract and also in rodent brain. They suggest that the reported effects of OEA on food intake may be mediated, at least in part, by the GPR119 receptor. Other potential ligands for the GPR119 receptor include different phospholipids and oleoyldopamine.[135] GPR119 might represent a novel and attractive potential target for the therapy of obesity and related metabolic disorders. An alternative way to achieve stimulation of satiating mechanisms in the GI tract would be by inhibition of the enzyme fatty acid amide hydrolase (FAAH), which is involved in the breakdown of amides.[136]

3.15.6.2.5 Protease inhibitors

Protease inhibitors are thought to be produced by plants as a natural mechanism to defend themselves against damage by insects and herbivores.[137,138] Some plants, including potato, produce high concentrations of protease inhibitors in storage organs and seeds; 10–50% of the total protein content can be devoted to this purpose alone. As a result, the potato tuber is a poor protein source to herbivores because it appears indigestible. However, herbivores, both mammals and insects, have developed mechanisms to overcome this by means of endogenous regulatory peptides, which can trigger the release of higher concentrations of proteases and proteases with insensitivity to the inhibitors.[139,140] Plants and animals are thus engaged in an evolutionary battle and the plant's strategy is to be unattractive as a food source in a way that goes beyond a 20% reduction in food intake due to satiety. It has been hypothesized that plants that express peptides that block the CCK-inducing receptors (antagonists) in combination with protease inhibitors would have an evolutionary advantage. In this way, plants would achieve low proteolysis by a combined inhibition of the enzymes and the feedback mechanism.[139,140] Oral administration of proteinase inhibitor II (PI2) from potato has indeed been shown to reduce energy intake in man.[141,142] A potato protein extract standardized to its active compound, PI2, is now being commercialized. It is claimed that this extract acts via a stimulation of CCK.[143] Statements on efficacy made by the company (Kemin Industries, Des Moines, IA, USA) on their website regarding efficacy in human trials could not be verified.

3.15.6.2.6 Inhibition of pancreatic lipase

Hydrolysis of dietary triacylglycerols by lingual, gastric, and pancreatic lipase is essential for their absorption by enterocytes.[144,145] Pancreatic lipase produced by the pancreatic acinar cells is one of the

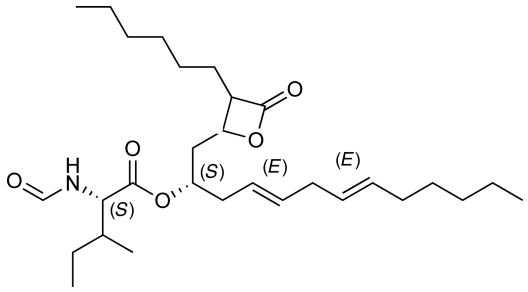


Figure 4 Chemical structure of lipstatin – [(2*S*,4*E*,7*E*)-1-(3-hexyl-4-oxooxetan-2-yl)trideca-4,7-dien-2-yl] (2*S*)-2-formamido-3-methylpentanoate.

exocrine enzymes of pancreatic juice and is essential for digestion of dietary fats in the intestinal lumen. Lipases, in particular pancreatic lipase, are interesting targets for prevention and treatment of obesity.[144,145] The marketed medicinal product orlistat (Section 3.15.4.5.1) is a potent lipase inhibitor and has shown to be effective in reducing body weight.[101] Orlistat is the tetrahydro derivative of the natural compound lipstatin, which is produced by the microorganism *Streptomyces toxytricini*. The isolation and characterization of lipstatin were described by scientists from Roche in 1987.[146,147] Roche later developed orlistat. The lipstatin molecule has a β-lactone structure incorporated into a hydrocarbon backbone (**Figure 4**)

The pharmaceutical industry is working on new lipase inhibitors, including cetilistat.[144]

There are several other potentially interesting lipase inhibitors from bacterial or plant origin. These include various saponins, polyphenols, and terpenes from higher plants and various structures from microorganisms. For a recent overview, see Birari and Bhutani.[144] Some preparations have been investigated in more detail and will be described in the following sections.

3.15.6.2.6(i) Thylakoids A recent study of Albertsson *et al.*[148] suggested that thylakoids, the photosynthetic membranes of chloroplasts isolated from green leaves, suppress appetite in rats during intake of a food containing 42% fat, a level of fat found in the everyday energy intake in the Western diet. In addition, the concentration of circulating triacylglycerols was reduced. It was proposed that the appetite suppression occurred through the retardation of intestinal fat digestion without causing steatorrhea. The lipolysis appears to be only temporarily blocked.

3.15.6.2.6(ii) Pomegranate leaf extract Extracts of the leaves of pomegranate (*Punica granatum)* are known for their antioxidant properties[149] and are of interest in diabetes.[150] A recent study suggested that pomegranate leaf extract (PLE) can also inhibit the development of obesity and hyperlipidemia in high-fat diet-induced obese mice. The effects appeared to be partly mediated by inhibiting the pancreatic lipase activity and suppressing energy intake.[151] The flowers of pomegranate have been used in unani and ayurvedic medicines specifically for the treatment of diabetes. A recent review suggests that this might be related to the dual PPAR-α/PPAR-γ activator properties of compounds in the pomegranate flower.[152]

3.15.6.2.6(iii) Salacia root *Salacia* species (Celasteraceae) are widely distributed in India, Sri Lanka, China, and other southeast Asian countries. *Salacia* roots have been used in ayurvedic medicine for diabetes and obesity since antiquity, and have been extensively consumed in Japan, the United States, and other countries as a food supplement for the prevention of obesity and diabetes.[153] *Salacia* contains many different components, depending on the species and its source. It is also an interesting example of a multitarget preparation as the different components interact with different key processes. One of its actions is the inhibition of pancreatic lipase.[153]

3.15.6.3 Centrally Acting Preparations and Compounds that Reduce Appetite

3.15.6.3.1 Hoodia gordonii

Studies initiated and conducted by the Council for Scientific and Industrial Research (CSIR, South Africa) in the early 1980s identified extracts from *Hoodia* species, in particular *H. pilifera* and *H. gordonii*, that possess appetite-suppressing properties.[154] *Hoodia* species are succulents growing in arid areas of southern Africa. Both *H. pilifera* and *H. gordonii* were historically reported to be eaten by the Xhomani San Bushmen to suppress their appetite, although *H. gordonii* appeared to have been less popular.[154] In addition to *H. gordonii* and *H. pilifera*, there are 11 other *Hoodia* species reported,[155] with some grown as ornamentals in gardens. *Hoodia gordonii* is presently the only sought-after species for trade due to the claim of its anorectic activity. Growing *H. gordonii* outside its natural habitat (the Kalahari Desert) has proven to be extremely difficult so far. In addition, its cycle of maturation is very slow. As a result, *H. gordonii* is now listed as an endangered species and its export out of southern Africa is strictly controlled. This has led to fraudulation because of the high demand for *H. gordonii* for weight-loss products.[155] Because of the many fake preparations that have entered the market, methods have been developed for the qualitative and quantitative analysis of *Hoodia* products.[155,156] The oxypregnane steroidal glycoside P57, 3β-[β-D-thevetopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranosyloxy]-12-β-tigloyloxy-14β-hydroxy-pregn-5-en-20-one (P57AS3) (**Figure 5**), was isolated as the only compound reported to have appetite-suppressant activity.[154–157]

van Heerden *et al.*[154] originally reported the isolation of two molecules. The other molecule consisted of the same steroid core and the tiglate side group, the only difference being in the glycosylation. In the mean time, other reports[155–157] have identified a total of 24 steroid glycosides related to P57 with sugar chains ranging from two to five (deoxy- and/or O-methoxy-)sugars. All groups also reported the aglycone to be present in dried *H. gordonii* material. In 1997 the rights on *Hoodia*/P57 were licensed by Phytopharm (UK) from CSIR, and around 1998 Pfizer started to investigate the compound for its potential to be developed into a medicinal product. For unknown reasons, Pfizer terminated the program and returned the rights. In 2004, the food company Unilever obtained the rights to develop P57 for weight management in functional food products. However, this project was terminated in November 2008. So far, little has been published on the activity of P57/*Hoodia* in animals or man. The reports from the CSIR[154] and the patent literature confirm a decrease in food consumption and weight loss in rats. In a study published by Maclean and Luo[158] in 2004, it was found that intraventricular injection of P57 to rats reduced ATP content in hypothalamic regions that are associated with central nutrient sensing. Phytopharm (www.phytopharm.com) disclosed the results of a double-blind 15-day trial in which 19 overweight males were randomized to P57 or placebo. Nine subjects in each group completed the study. There was a statistically significant decrease in calorie intake and body fat and no serious adverse events. In 2004, a new ingredient notification was submitted to the FDA with some more detail, though most anecdotal on the safety and efficacy of *Hoodia*.[159]

3.15.6.3.2 Caralluma fimbriata

Caralluma is a genus that belongs to the same family as *Hoodia*, the Asclepiadaceae. Some species such as *C. negevensis* and *C. russeliana* grow wild in the east African–Arabian region, whereas others such as

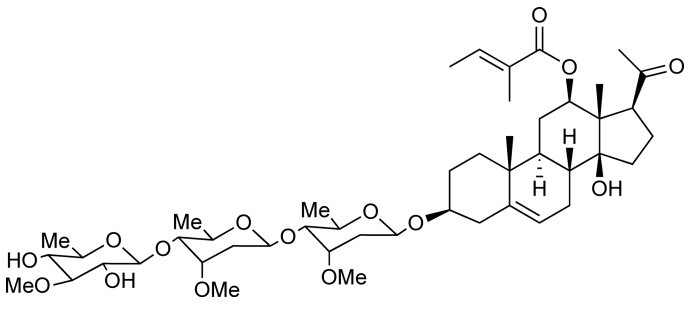


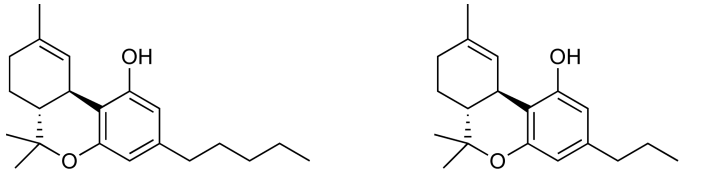
Figure 5 Chemical structure of P57AS3 from *Hoodia*.

C. stalagmifera, *C. indica*, and *C. fimbriata* are endogenous to the Indian region. The species have been traditionally used for different medicinal properties. For example, *C. negevensis* has been reported to be used by the Bedouins to treat chronic lung diseases.[160] The Indian *Caralluma* species are used against different diseases, including diabetes and inflammatory diseases.[161] The species of *Caralluma* found in India are edible and form a part of the Ayurvada tradition. *Caralluma fimbriata* has been used as an appetite suppressant and has also been used to treat diabetes, pain, fever, and inflammation.[162,163] Like *Hoodia*, the plant has a tradition of use over many centuries with claims in folklore about its appetite-suppressant activity. It grows wild all over India and is also planted as a roadside shrub and boundary marker in gardens. *Caralluma* species in general have been described to contain pregnane glycosides, flavone glycosides, megastigmane glycosides, bitter principles, saponins, and various other flavonoids,[160–162,164,165] although thus far no specific studies on this appear to be published for *C. fimbriata*. Like with *Hoodia*, the appetite-suppressing action of *Caralluma* could possibly be attributed to the pregnane glycosides. Like *H. gordonii*, *C. fimbriata* has also attracted commercial interest.[166–168] Gencor Pacific (Hong Kong) develops preparations of *Caralluma* as a dietary supplement for weight loss under the trade name Slimaluma^{TM}. The company describes data on clinical efficacy and safety of the preparation on their website. A new dietary ingredient notification for *C. fimbriata* extract is listed on the FDA website.[169] So far, the only report on the clinical efficacy of *C. fimbriata* in the scientific literature is by Kuriyan *et al.*[163] In this study, effects of a *Caralluma* extract were investigated in 50 overweight ($BMI > 25\,kg\,m^{-2}$) individuals by a placebo-controlled randomized trial. Individuals received 1 g of *Caralluma* extract per day or placebo for 60 days. Several parameters were tested. Waist circumference and hunger levels over the observation period showed a significant decline in the experimental group when compared to the placebo group. Although there was a trend toward a greater decrease in body weight, BMI, hip circumference, body fat, and energy intake between assessment time points in the experimental group, these were not significantly different between experimental and placebo groups. The authors conclude that *Caralluma* extract appears to suppress appetite and reduce waist circumference when compared to placebo over a 2-month period.

3.15.6.3.3 Compounds acting on the endocannabinoid system

Fatty acid amides have been described in Section 3.15.6.2.4. Some of these at least partly interact with the endocannabinoid system, presumably mainly in the GI tract. Some other compounds, for example from *Cannabis* itself, may reduce appetite also via central mechanisms. The appetite-inducing properties of CBs like THC (**Figure 6**) have been known for centuries. It is of interest to see whether there might be plant sources for natural CB_1 antagonists. Indeed, it has been shown that Δ^9-tetrahydrocannabivarin (THCV) (**Figure 6**), which is a constituent of *C. sativa*, in variable amounts has CB_1-blocking properties and might share some properties with the synthetic CB_1 blockers such as rimonabant.[170]

Recent experiments have shown that THCV shares the ability of the CB_1 blocker AM251 to reduce food intake and body weight of nonfasted and fasted 'nonobese' mice when administered once and of dietary-induced obese mice when given repeatedly over 28 days.[170] It has also been found that like AM251, THCV can reduce the body fat content and plasma leptin concentration and increase the 24-h energy expenditure and thermic response to food of dietary-induced obese mice.[170]



Δ^9-tetrahydrocannabinol (THC) Δ^9-tetrahydrocannabivarin (THCV)

Figure 6 Chemical structures of Δ^9-tetrahydrocannabinol (Δ^9-THC) and Δ^9-tetrahydrocannabivarin (Δ^9-THCV).

3.15.7 Natural Compounds and Preparations Claiming to Affect Fat Absorption

3.15.7.1 Chitosan

Chitosan is a cationic polysaccharide, produced from chitin, a substance derived from the exoskeleton of crustaceans. Chitosan is used as a biopolymer for many different applications, including many in drug delivery. It is also widely advocated as a food supplement to lose weight, the proposed mechanism being that fat absorption from the GI tract is reduced. Dwyer *et al.*[171] concluded that there is little evidence of benefit and also mentioned some adverse GI symptoms. A similar conclusion was drawn by Pittler and Ernst.[172] Ni Mhurchu *et al.*[173] performed a systematic review of the literature and concluded that there may be some evidence that chitosan is more effective than placebo in the short-term treatment of overweight and obesity. However, they also concluded that the majority of the trials to date have been of poor quality and results have been variable. Results obtained from high-quality trials indicate that the effect of chitosan on body weight is minimal and unlikely to be of clinical significance.

3.15.7.2 Glucomannan

Glucomannan is derived from the root of *Amorphophallus konjac* (Konjac plant or elephant yam), which is native to the warm and tropical parts of Asia. Glucomannan from the Konjac plant is a glucose-mannose (**Figure 7**) polysaccharide in which 5-10% of the sugars are acetylated. The molecule is structurally related to glucomannan from guar gum (see Section 3.15.7.3).

Macroscopically, Konjac glucomannan is a soluble, fermentable, and highly viscous fiber, which is traditionally also used for culinary purposes in Japan and China. It is claimed that glucomannan preparations promote weight loss, probably by stimulating satiety and/or reducing fat absorption. Pittler and Ernst[172] describe one double-blind randomized controlled trial (RCT) including patients with body weight >20% over their ideal. The report suggests significantly greater weight loss in the treatment group than in the placebo group. However, more and independent studies are needed. Glucomannan seems to be well tolerated.

3.15.7.3 Guar Gum

Guar gum is a dietary fiber from the Indian bean *Cyamopsis tetragonolobus*. Like glucomannan, it is a galactomannan consisting of a (1→4)-linked β-D-mannopyranose backbone with branch points from their 6-positions linked to α-D-galactose (that is, 1→6-linked α-D-galactopyranose). There are between 1.5 and 2 mannose residues for every galactose residue. Compared to other gums, it has a relatively high viscosity. As a thickener/stabilizer it is also an EU allowed food additive (E412). Like with glucomannan, it has also been claimed for guar gum that it stimulates satiety and/or reduces fat absorption. Pittler and Ernst[172] assessed the efficacy of guar gum in their meta-analysis. Twenty double-blind, placebo-controlled RCTs were included, and the data from 11 trials were statistically pooled. The results of the meta-analysis suggest that guar gum is not effective in reducing body weight. The agreement between the individual RCTs confirms the overall result of the meta-analysis. Adverse events reported in the reviewed trials predominately relate to the GI system.

3.15.7.4 Plantago Psyllium and Pectins

The psyllium extract from the seeds of *Plantago* is also a water-soluble fiber. In one randomized placebo-controlled trial identified by Pittler and Ernst,[174] there was no significant change in body weight in either the treatment or placebo group. Pectins (E440) are acid polysaccharides present in nearly all fruits, especially apples, quinces, and oranges. Pectin is commercially produced from apple pulp and orange peels. In spite of the claims, there is little evidence for its effects in humans.

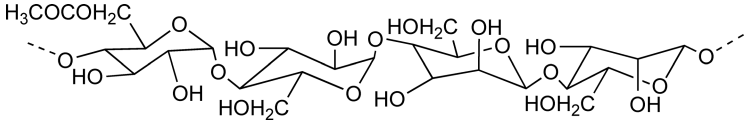


Figure 7 Chemical structure of glucomannan.

3.15.8 Natural Compounds Affecting Lipid Metabolism or Energy Expenditure

3.15.8.1 Introduction

In addition to achieving reduced energy intake, metabolic strategies are being investigated to reach weight reduction or redistribution of adipose tissue. The latter may be useful to change dangerous fat, that is, visceral fat, into less risk bearing fat depots (see Sections 3.15.4.1 and 3.15.4.4.1). In principle, mechanisms include increasing blood flow and hence delivery of fats to sites of metabolism and/or stimulating fat metabolism either directly or through changes in gene expression. An example of how to measure these effects by using genomics-based methods is described in Section 3.15.10. The energy expended through everyday nonexercise activity, called nonexercise activity thermogenesis (NEAT), has a considerable potential impact on energy balance and weight gain.[175] Systems that regulate NEAT according to energy balance may be linked to neural circuits that modulate sleep, addiction, and the stress response and are potential targets for the treatment of obesity. Some compounds and preparations that have been suggested to affect lipid metabolism or NEAT will be discussed in the next section.

3.15.8.2 Green Tea Extract (Epigallocatechin-3-Gallate)

In recent years, there has been an increased interest in the health benefits of polyphenols, particularly flavonoids, which are found in many plant-derived foods. Flavanols are the predominant flavonoids found in tea, wine, cocoa, berries, apples, and onions. They include the catechins epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC). Tea, in particular green tea in which EGCG is the most abundant catechin, has been investigated predominantly for its potential to prevent cancer and cardiovascular disease. However, there is also evidence suggesting that green tea catechins, particularly EGCG (**Figure 8**), may have an additional metabolic role in reducing body fat.[176]

Epidemiological evidence from humans indicates that habitual tea consumption (predominantly green tea) for >10 years is associated with a smaller waist circumference and waist-to-hip ratio, and a lower percentage of body fat.[177–179] Consumption of green tea extracts has been shown to increase fat oxidation and energy expenditure, particularly if combined with a metabolic stimulant such as caffeine, and reduce total and abdominal fat.[177,178]

Several mechanisms have been attributed to green tea or the tea catechins, as reviewed, for example, by Moon *et al.*,[177] Wolfram,[178] and Wolfram *et al.*[180] Reported effects of EGCG include

1. Blocking adipocyte proliferation and differentiation by inhibition of the extracellular signal-regulated kinase (ERK)- and cyclin-dependent kinase (CDK)-dependent signaling pathways. EGCG also inhibits adipocyte differentiation by activating AMP-activated protein kinase (AMPK). This kinase plays an important role in energy homeostasis. In addition, the expression of the transcription factor PPAR-γ in adipose tissue might be downregulated by EGCG. PPAR-γ is responsible for adipocyte differentiation.
2. Inhibition of lipogenic enzymes, leading to a decrease in fatty acid and TG synthesis.

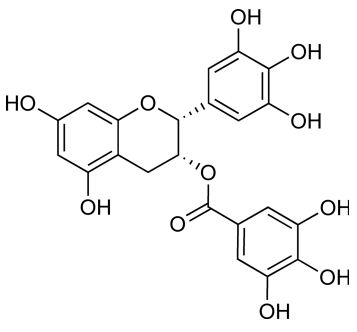


Figure 8 Chemical structure of epigallocatechin gallate (EGCG).

3. Stimulation of thermogenesis by inhibiting catechol-*O*-methyltransferase. By reducing the enzymatic degradation of norepinephrine, sympathetic stimulation of thermogenesis is prolonged.[181]
4. Effects on nutrient absorption. Glucose and fat absorption appears to be reduced by the action of EGCG. These effects are assumed to be caused by inhibition of gastric and pancreatic lipase activity. Glucose uptake in skeletal muscle is supposed to be elevated by increased translocation of the glucose transporter GLUT4.

In addition to the effects of catechins, weight-loss effects of tea can also be due to the effects of caffeine present. Caffeine is further discussed in Section 3.15.8.6.

3.15.8.3 Citrus aurantium

Citrus aurantium (bitter orange) contains a number of phytochemicals, including *p*-octopamine and synephrine alkaloids. Synephrine (**Figure 9**) is an α-adrenergic agonist but also has some β-adrenergic properties.[182]

Its recent popularity is supposed to be due to the ban on ephedrine and ephedra preparations. Food supplements containing *C. aurantium* are supposed to increase energy expenditure or lipolysis or reduce appetite. Although sympathicolytic drugs may exert this type of action at a cellular level, there is no support for efficacy in humans. A systematic review by Bent *et al.*[183] found only one randomized placebo-controlled trial involving 20 subjects treated with *C. aurantium* for 6 weeks. This trial demonstrated no statistically significant benefit for weight loss. Since that review, there was a report of two small studies: one with 8 subjects randomized to *C. aurantium* or placebo and the other an open-label study with 20 subjects. The first showed weight gain in the *C. aurantium* group and the second showed only a 0.8-kg weight loss, which was not statistically or clinically significant.[99] Some concern has been raised about its cardiovascular safety and altogether the efficacy is being questioned.[99,171,182]

3.15.8.4 Garcinia cambogia

Garcinia cambogia contains hydroxycitric acid, an inhibitor of citrate-cleavage enzyme (ATP-citrate lyase) that inhibits fatty acid synthesis from carbohydrate. Hydroxycitrate was studied by Hoffmann-LaRoche in the 1970s and was shown to reduce food intake and cause weight loss in rodents.[99]

Although there have been reports of successful weight loss in small studies in humans, some of which included other herbs, the largest and best-designed placebo-controlled study demonstrated no difference in weight loss compared with a placebo.[172] Overall, the evidence for *G. cambogia* is not compelling.[171,172,184].

3.15.8.5 Yerba Maté (Ilex paraguariensis)

Yerba maté (*I. paraguariensis*) is an evergreen tree that is endogenous to South America. It is taken as a tea and available in a number of dietary supplements that contain relatively high amounts of caffeine.[185] The use, chemistry, biological activities, and some technological aspects have recently been reviewed by Heck and De Mejia.[186] In a combination preparation also containing guarana (*Paullinia cupana*) and damiana (*Turnera diffusa*), it was tested in patients with a BMI of 26–30. The results of that study indicated that this combination preparation might potentially be effective in lowering body weight. It was shown by ultrasound scanning that the combination prolongs gastric emptying time. Adverse events were not reported.[172,186]

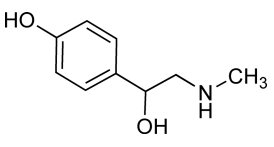


Figure 9 Chemical structure of synephrine.

3.15.8.6 Caffeine

Caffeine is a common ingredient of many food supplements and part of the regular diet of numerous people. It is also an ingredient of preparations discussed elsewhere in this chapter such as green tea (Section 3.15.8.2), Yerba maté (Section 3.15.8.5), and coffee. Caffeine inhibits phosphodiesterase, increasing cyclic adenosine monophosphate and enhancing the activity of certain excitatory neurotransmitters. However, caffeine's primary neurobiological effect may be through its activity as an antagonist at the adenosine receptor, impairing the presynaptic inhibitory function of adenosine, thereby increasing excitatory neurotransmitter release.[187] The weight-loss effects of coffee[187,188] are considered as primarily due to the thermogenic effect caused by caffeine. However, the effect of caffeine is only temporal, which is possibly related to tolerance.[187] The effects of coffee without caffeine in relation to diabetes are discussed in Section 3.15.9.3.

3.15.8.7 Ephedra sinica

Ephedra sinica or *ma-huang* is an evergreen shrub native to central Asia.[172] Ephedrine, the primary active constituent, has been studied alone and in combination with caffeine. The thermogenic properties of ephedrine are mainly due to its action as a sympathicomimetic compound. It has been suggested that in particular the combination of ephedrine and caffeine is effective in reducing weight.[187,189] Meta-analysis of the effect of *Ephedra* and ephedrine[172,190] suggests that a modest short-term weight loss can be reached. However, because of safety concerns, EU authorities and the FDA (http://www.fda.gov/bbs/topics/NEWS/2004/NEW01050.html) have banned *Ephedra*-containing supplements from the market in 2004.[191] Although there may be reasons for concern, the discussion continues to what extent all reported effects can be directly attributed to *Ephedra*.[191]

3.15.8.8 Hydroxy Methylbutyrate

β-Hydroxy-β-methylbutyrate (HMB) is a metabolite of leucine. It has been reported to act as anticatabolic compound through the inhibition of protein breakdown. HMB is available as a dietary supplement and is primarily used as ergogenic agent in sports, particularly among bodybuilders and strength/power athletes. In sports, the aim is to induce changes in body composition and it is suggested to be effective for this purpose.[192] However, no recent data were found on the effect on weight management. Pittler and Ernst[172] are quite positive about the possible effects of the compound in this respect. They identified four RCTs reported in three articles. Two double-blind RCTs reported significant between-group differences with respect to fat mass with at least a trend toward an increase in lean body mass reported from all trials. Although the trials were not fully perfect in all respect, the results were considered encouraging. No adverse effects were reported.

3.15.8.8.1 n-3 polyunsaturated fatty acids

The n-3 polyunsaturated fatty acids (PUFAs), primarily eicosapentaenoic acid and docosahexaenoic acid, can be found in some cold water fish (e.g., mackerel, salmon, and cod). A well-known effect of n-3 PUFAs is a reduction of plasma triacylglycerols.[8] There is a clear dose–response relationship and the effects are persistent even after 2 years.[8] The effects of n-3 PUFAs on appetite and satiation have been described in Section 3.15.6.2.2. There appears to be only limited evidence for a direct antiobesity effect of n-3 PUFAs.

3.15.8.8.2 Conjugated linoleic acid

Conjugated linoleic acid (CLA) is a collective term for a group of linoleic acid isomers with conjugated double bonds. Depending on the position and geometry (*cis* or *trans*) of the double bonds, several isomers of CLA are being distinguished. Most of the published data concern one of the two major forms, *cis*-9, *trans*-11-CLA (c9, t11-CLA) or *trans*-10, *cis*-12-CLA (t10, c12-CLA). In addition, a number of minor isomers have been described (i.e., t7, t9-CLA; c9, c11-CLA; t9, t11-CLA; c10, c12-CLA; t10, t12-CLA; t11, t13-CLA; and c11, c13-CLA). The major dietary sources of CLA are ruminant (e.g., cattle, goat, and sheep) meat and dairy products. In these species, the 9-*cis*,11-*trans*-CLA isomer (**Figure 10**) is naturally produced through the fermentation, in the rumen, of unsaturated fatty acids by the bacterium *Butyrivibrio fibrisolvens*.[8]

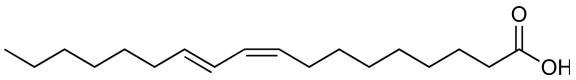


Figure 10 Chemical structure of the 9-*cis*,11-*trans* isomer of conjugated linoleic acid (CLA): (9*Z*,11*E*)-octadeca-9,11-dienoic acid.

The intake of CLA from a typical diet is estimated at several hundred milligrams per day in various countries.[8] There are also other CLAs present in other sources such as some plant oils and seaweeds.[8] CLA is receiving considerable attention because of its supposed weight-reducing, antiatherosclerotic, antidiabetic, and hypotensive properties. The fat-lowering properties of CLA were originally discovered in rodents and the debate continues to what extent they can be extrapolated to humans. Several mechanisms of action have been suggested, including a decrease of energy intake, an increase of energy expenditure, a reduction of adipocyte size, an inhibition of preadipocyte differentiation, an increased apoptosis of adipocytes, an inhibition of lipogenesis in the liver and adipose tissue, and an increase in fat oxidation in the liver and adipose tissue. These issues have been the subject of a number of recent reviews.[193–195] The authors of these reviews remain skeptical about the health effects of CLA in humans. Some studies suggest that the *trans*-10, *cis*-12 isomer induces insulin resistance in obese subjects. In addition, no major effect of CLA on plasma lipids was detected in human studies. On the contrary, some recent papers seem to be more positive.[187,196,197] Sneddon *et al.*[197] reported that supplementation with CLA plus n-3 long chain polyunsaturated fatty acids (LC-PUFA) prevented increased abdominal fat mass and raised fat-free mass and adiponectin levels in younger obese individuals without deleteriously affecting insulin sensitivity. Young and older lean, and older obese individuals were unaffected, apart from increased fasting glucose in older obese men. Gaullier *et al.*[196] performed a study aiming to evaluate the effect of CLA per region and safety in healthy, overweight, and obese adults. A total of 118 subjects were included in a double-blind placebo-controlled trial. CLA significantly decreased body fat mass at months 3 and 6 compared with placebo. The reduction in fat mass was located mostly in the legs. The waist–hip ratio also decreased significantly compared with placebo. Lean body mass increased within the CLA group with bone density unaffected. All changes were independent of diet and physical exercise. Safety parameters including blood lipids and inflammatory and diabetogenic markers remained within the normal range and adverse events did not differ between the groups.

3.15.9 Natural Compounds in Type 2 Diabetes

3.15.9.1 Introduction

A remarkably wide array of food supplements, medicinal plants, and their active constituents play a role in the prevention and treatment of diabetes. Currently, specific food supplements and different herbal preparations are frequently being used by diabetic patients.[198] Even more important, there are probably many more compounds and preparations to be discovered from nature.[114] Several traditional health systems, including TCM[199] and Ayurveda,[200] are potentially rich sources of new compounds. About 800 plant species have been reported to possess antidiabetic properties,[200] but only a few of these plants have been studied and validated for their hypoglycemic properties using diabetic animal models and even fewer in clinical studies using human subjects. Some of these will be discussed briefly in Section 3.15.9.6. In addition to having a direct glycemic effect, the inflammatory component of the metabolic syndrome (Sections 3.15.4.4.1 and 3.15.4.4.2) provides an interesting target for intervention. Finally, some other supplements and the common beverage coffee will be discussed in relation to diabetes.

3.15.9.2 The Inflammatory Component

The process of chronic inflammation that occurs in obesity[87,90,92] is now commonly regarded as a suitable target for treatment of the complications of the metabolic syndrome. Clues to the involvement of inflammation in diabetes date back to more than a century ago, when high doses of sodium salicylate were demonstrated to

diminish glycosuria (zucker) in diabetic patients having the milder form of the disease.[92,201] Although the pathophysiology of diabetes and the difference between what is now distinguished as type 1 and 2 diabetes were not known at that time, the descriptions of the patients correspond well with type 2 diabetes.[202] Doses needed were high. From time to time, interest in therapy with salicylates awakened. For example, in 1971, Hyams *et al.*[203] described the beneficial effects of 3-methyl salicylic acid, given orally, on glucose tolerance and insulin levels in diabetic patients. Due to the high doses and side effects produced, the authors conclude that 3-methyl salicylate should not be considered as therapeutic agent in diabetes. However, the potential of salicylic acid was not forgotten and following the discovery of the role of inflammation in adipose tissue,[89,93] the interest in targeting this process increased. Further studies demonstrated that high-dose aspirin ($\sim$7.0 g day^{-1}) improved multiple metabolic measures in patients with type 2 diabetes, including substantial reductions in fasting and postprandial glucose, triglycerides, and FFAs.[204]

The high dose of aspirin needed was shown to be due to the necessity to inhibit nuclear factor-κB (NF-κB), which requires higher doses than COX-2. Shoelson *et al.*[91,92] suggested that nonacetylated salicylates, delivered as sodium salicylate, salsalate, and trilisate, might be better alternatives as they would be better tolerated. Recently, it was shown in young obese persons at risk for developing diabetes that salsalate (**Figure 11**) at a dose of 4.0 g day^{-1} divided in two doses indeed improved glycemia, insulin resistance, and inflammatory profiles.[201]

Remarkably, some other drugs that are commonly used in diabetes or (other) complications of obesity have been shown to have antiinflammatory properties. This is now more and more regarded as part of their beneficial therapeutic effects such as on the liver or vascular wall. For example, statins and TZDs have also been shown to have antiinflammatory properties.[91] Based on this, several other compounds that have antiinflammatory properties could be of interest, provided that they reach the primary sites of inflammation: adipose tissue, pancreas, or liver. A more comprehensive approach would involve targeting a network of responses instead of one single pathway or molecule.[88] The best examples for this approach would be c-Jun amino-terminal kinase (JNK) and inhibitor of nuclear factor-κB (NF-κB) kinase (IKK) pathways. The inhibition of IKK has been shown to be important in the effects of high-dose salicylates on glucose metabolism in both obese mice and diabetic persons.[88] In this context, the effects obtained with resveratrol in obese mice are of great interest. Baur *et al.*[205] showed that resveratrol was able to shift the pathophysiology of mice on a high-calorie diet toward that of mice on a standard diet. This was associated with a significant increase in survival. Resveratrol produced changes associated with longer lifespan, including increased insulin sensitivity and reduced insulin-like growth factor-1 (IGF-1) levels. Numerous natural compounds have been tested *in vitro* for their ability to reduce the inflammatory reaction in adipose tissue. Examples include procyandins from grape seed[206] and various spice-derived components, including curcumin[207] and capsaicin.[208] In addition, the antiinflammatory effects of compounds acting on the CB_2 receptor[74,75] have shown promising results in our laboratory.

3.15.9.3 Coffee

Coffee might be regarded as an example of a multicomponent mixture with potentially beneficial effects in (pre) diabetes. However, these effects may also be partly opposed. Although caffeine has been shown to impair insulin sensitivity and glycemic response,[209,210] a large body of evidence has emerged documenting a protective association of decaffeinated coffee consumption with risk for developing type 2 diabetes.[188,209,211,212] Coffee is a complex mixture of more than thousand chemicals, including substantial amounts of caffeine (except in decaf coffee) and chlorogenic acid. Coffee induces some temporary weight loss,[187,188] which is primarily due to the thermogenic effect caused by caffeine. With regard to the effects on glucose homeostasis, most attention has

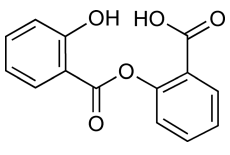


Figure 11 Chemical structure of salsalate.

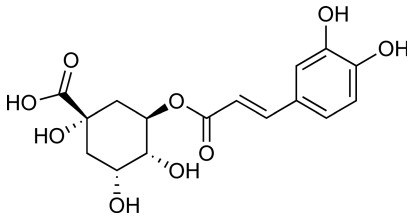


Figure 12 Chemical structure of chlorogenic acid.

been paid to chlorogenic acid and the lignans. The precise mechanisms by which chlorogenic acid (**Figure 12**) improves glycemic control have not been completely elucidated.[211,212] Chlorogenic acid may delay glucose absorption in the intestine through inhibition of glucose-6-phosphate translocase 1 and reduction of the sodium gradient-driven apical glucose transport. Decaffeinated coffee consumption appeared to delay glucose absorption and (possibly as a result) stimulate the secretion of the incretin hormone GLP-1, in a recent metabolic study in humans. Chlorogenic acid can also decrease hepatic glucose output through inhibition of glucose-6-phospatase. Finally, the compound may act as antioxidant or as metal chelating agent.

The lignans present in coffee have also been considered since they may affect glucose metabolism through their antioxidant and (anti) estrogenic properties.[212]

Considerable attention has also been paid to advanced glycation end products (AGEs) present in coffee preparations. Advanced glycation end products are formed by a Maillard-like mechanism from sugars and proteins.[213] AGEs are connected to various diseases including osteoarthritis and diabetes.[214] AGEs can bind to a receptor called RAGE (receptor of AGEs), which can activate the well-known transcription factor NF-κB. Also in coffee, other Maillard reaction products (MRPs) products called 'melanoidins' contribute the antioxidant activity of coffee brews.[215]

When it comes to health effects of coffee, attention should be paid to the possible presence of cafestol. Cafestol is normally not present in filtered coffee. As mentioned in Section 3.15.2, cafestol is one of the most potent cholesterol-increasing compounds that may be present in our diet, thereby contributing to an increased risk of cardiovascular disease.[13]

3.15.9.4 Chromium Picolinate

Chromium picolinate is an organic compound of trivalent chromium and picolinic acid, a naturally occurring derivative of tryptophan. The picolinic form increases Cr absorption from the GI tract, although this remains quite low, around 3%.[216] Chromium picolinate is a popular dietary supplement and it is claimed to reduce the desire for sweets and sugar, thus helping to lose weight. Trivalent chromium is reported to enhance insulin action. However, the exact molecular mechanism of chromium action on insulin is not clear.[216] For several years, it was supposed that chromium acts as part of the glucose tolerance factor (GTF). More recently, other mechanisms such as functioning via the low-molecular-weight chromium-binding substance (LMWCr) have been proposed.[216] Chromium deficiency so far has been diagnosed only in persons receiving total parenteral nutrition. The use of chromium in diabetes patients has been the subject of much debate, but the American Diabetes Association states that benefit from chromium supplementation in individuals with diabetes or obesity has not been clearly demonstrated and therefore cannot be recommended.[105] In a recent study by Martin *et al.*[217] in subjects with type 2 diabetes who were taking sulfonylurea preparations and used chromium picolinate or placebo for 6 months, it was found that insulin sensitivity increased in the chromium group. Supplementation with chromium picolinate attenuated body weight gain and visceral fat accumulation. With regard to its effects on weight management, Pittler and Ernst[172] concluded that chromium picolinate may result in modest reduction of body weight of obese persons, but that these effects are clinically not meaningful. Side effects have not been observed. Taken together, it is likely that the debate on chromium may continue for a while.

3.15.9.5 Fatty Acids

There is some evidence for an antidiabetic effect of n-3 PUFAs. Epidemiological studies suggest that there is a low prevalence of diabetes in populations with a high intake of n-3 PUFAs.[8] It is not clear whether this is related to the reduction of plasma triglycerides, which is seen with increased consumption of n-3 PUFAs.

3.15.9.6 Examples of Food and Medicinal Plant Species with Reported Antidiabetic Properties

A remarkably high number of plants with hypoglycemic activity or other beneficial effects in diabetes are known from traditional medicinal sources.[107,114,198–200,218] Interestingly, one of the most common prescription drugs used, metformin, is originally derived from a plant, *Galega officinalis*.[107] This plant, also known as professor-weed, goat's rue, or French lilac, has been used to treat diabetes in medieval Europe. It was found to contain high concentrations of guanidine, which became the basis of the biguanidines, including metformin.

Although T2DM is often referred to as a lifestyle-related disease of this era, it was known in ancient times. Already around the year 700 BC, diabetes was diagnosed in India by tasting the patient's urine for sweetness as well as from other symptoms. Ayurvedic sources dating back to the fourth and fifth century BC describe two types of diabetes: a genetically based disorder and one resulting from dietary indiscretion.[107] Like its Indian counterpart, Chinese medical books written as early as 3000 BC spoke of diabetes and therapies for this disease.[107] In addition to TCM[199] and ayurveda,[200,218] there are several interesting reviews on plants used in other traditional health systems. These include Jewish,[219] North American,[220,221] Mexican,[222–224] Moroccan,[225–228] and many others. Altogether, it is estimated that there are thousands of species that could have interesting properties in diabetes but have not been investigated using today's techniques and standards. On the one hand, this represents an enormous treasure box and, on the other hand, it is very obvious that there is far more to study than there is capacity.[114]

Within the scope of this chapter, just a few examples can be briefly described. They may serve as illustrations and have been chosen here because they have been the subject of relatively much attention recently. In addition, they represent examples of some of the many different pathways that might be involved in diabetes and its metabolic complications. However, it should be clear that this list is by no means complete and thorough discussion would require several volumes of this book. By selecting just six sources there is always the risk that highly relevant species might be overlooked. For comprehensive lists of compounds or preparations that have been linked to diabetes, readers are referred to for example Li *et al.*[199] (for TCM), Grover *et al.*[218] (for ayurveda), Mukherjee *et al.*[229] (for ayurveda),[229] or Yeh *et al.*[198] (general).The fact that many traditional preparations with reported positive effects are mixtures of several plants and other ingredients makes the discussions even more complex.

3.15.9.6.1 Aloe barbadenis (Aloe vera)

Aloe has been used for centuries as an oral treatment for T2DM. Products used are the gel (the leaf pulp) or the juice (latex) from the leaves.[198] For a recent review on the botany, constituents, pharmacology, and toxicology, see Boudreau and Beland.[230] Polysaccharides, acemannans, are now supposed to be responsible for the major effects of aloe in diabetes. In addition to animal data, there have been a few studies in T2DM patients from which it appears that oral use of aloe gel decreases fasting blood glucose and hemoglobin A1c levels.[198,200] The results suggest potential effects of aloe in T2DM but more research is definitely needed.

3.15.9.6.2 Eugenia jambola

Eugenia jambolana (black plum, Jamun) is indigenous to India, Thailand, and the Philippines. It has been used for centuries in India to treat diabetes. Its antihyperglycemic effects have been demonstrated in many animal studies. Recently, Sharma *et al.*[231] showed that flavonoid-rich fractions of the seeds produced hypoglycemic and hypolipidemic effects in streptozotocin-induced diabetic rats. This was suggested to be mediated through a stimulation (16%) of insulin release from pancreatic islets, upregulation of both PPAR-α and PPAR-γ, and increased capacity of preadipocytes to differentiate. No published data from controlled human trials could be found.

3.15.9.6.3 Gymnema sylvestre

Gymnema sylvestre is another commonly used herb in ayurveda. Its seeds and leaves have been reported to cause a loss of sweet taste. There are no clear data on the mechanism(s) of the active components. From animal studies, it has been suggested that *Gymnema* preparations inhibit glucose uptake from the intestine, increase glucose uptake, or increase insulin release.[198,200]

At least two nonrandomized controlled clinical trials have been published and available, both from the same investigator group. According to Mentreddy[200] and Yeh *et al.*,[198] there is some evidence for a beneficial effect of *G. sylvestre* in diabetes. The results should be regarded as inconclusive given the limited data.

3.15.9.6.4 Momordica charantia

The plant *Momordica charantia* is commonly known as bitter melon or bitter gourd. It is widely used in Asia, Africa, and the Caribbean as a vegetable as well as medicinal product. Again, it has a long history of use in TCM, ayurveda, and in other traditional systems. It has a remarkable wide spectrum of reported effects.[232] In animal studies, *Momordica* preparations were reported to increase tissue glucose uptake, liver muscle glycogen synthesis, glucose oxidation, and to decrease hepatic gluconeogenesis.[198,200] Active components were thought to be charantin, vicine, and polypeptide-p (an unidentified insulin-like protein similar to bovine insulin). Recently, Tan *et al.*[233] described the isolation of a series of triterpenoids, cucurbitane glysosides, from *Momordica* and their possible molecular mechanism of action.

Two compounds shown in **Figure 13** and their aglycones stimulated the translocation of the insulin-responsive glucose transporter GLUT4 to the plasma membrane in muscle and adipocyte cell lines. Intriguingly, these compounds also activated the AMP-activated protein kinase (AMPK) pathway, a major regulatory pathway for GLUT4 translocation. *In vivo* studies in mice showed a significant enhancement of glucose clearance and increases in fatty acid oxidation after acute administration of compound B. There are some data on its effect from two small RCTs with T2DM and from two open-label trials.[198,200] Again, data suggest a potential effect but more information is needed.

3.15.9.6.5 Smallanthus sonchifolius

Smallanthus sonchifolius, commonly called yacon, is indigenous to the Andean highlands. It has become popular in Japan and the Philippines, where the roots and leaves of yacon are widely used for managing diabetes. They are also used as a source of natural sweeteners and syrups suitable for persons suffering from digestive

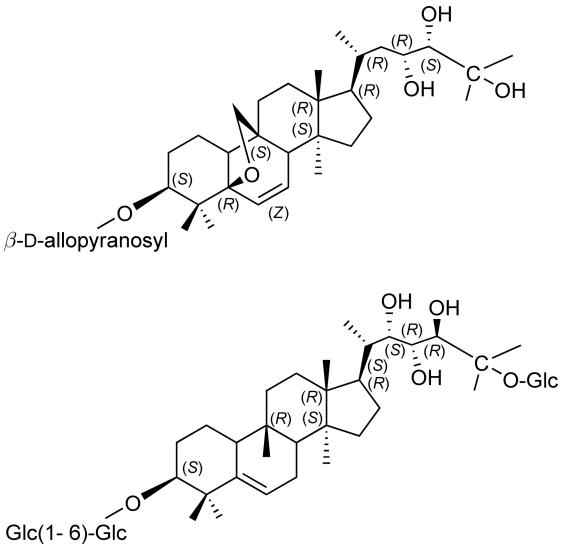


Figure 13 Structures of two of the main active triterpenoids found in *Momordica* that were found to stimulate GLUT4 activity.[233]

problems.[234] The roots contain high amounts of low-polymerized fructooligosaccharides. Yacon saccharides, particularly β-(2→1) fructooligosaccharides, appear to be a good candidate for modulating the metabolic syndrome and dyslipidemia. Fructooligosaccharides have also prebiotic activity.[234] It was also suggested that dicaffeoylquinic in yacon extracts may be the main active compounds involved in the reduction of plasma glucose by inhibiting α-glucosidase.[235] Finally, the leaves contain melampolide-type sesquiterpene lactones, which have been shown to possess antiinflammatory activity, via the transcriptional factor NF-κB or nitric oxide (NO).[236] Hypoglycemic effects of aqueous extracts of the leaves of yacon were seen in normal, transiently hyperglycemic, and STZ-induced diabetic rats.[200] Valentová *et al.*[237] investigated the safety of yacon in humans in combination supplements with silymarin (from 'milk thistle'). They conclude that this supplement appears to be promising in the prevention of diseases with a proatherogenic lipoprotein profile and liver steatosis.

3.15.9.6.6 Salacia roots

Salacia has already been mentioned in Section 3.15.6.2.6(iii) for its action on pancreatic lipase, leading to a reduced absorption of fat from the GI tract. In addition, recent pharmacological studies have demonstrated that *Salacia* roots modulate multiple targets: PPAR-α-mediated lipogenic gene transcription, angiotensin II/angiotensin II type 1 receptor, α-glucosidase, aldose reductase, and pancreatic lipase. These multitarget actions may mainly contribute to *Salacia* root-induced improvement of type 2 diabetes and obesity-associated hyperglycemia, dyslipidemia, and related cardiovascular complications seen in humans and rodents.[153]

3.15.9.6.7 Guggulsterone

Guggulsterone (4,17(20)-pregnadiene-3,16-dione, **Figure 14**) is the active component of gugulipid. This preparation is derived from the gum resin (guggulu) of the tree *Commiphora muku*. This gum resin has been used for centuries in ayurvedic medicine to treat obesity, arthritis, and hyperlipidemia.

Guggelsterone is currently attracting considerable interest[238–240] as an effective antagonist of farnesoid X receptor (FXR). Via this pathway it can regulate bile acid synthesis and carbohydrate metabolism. It has also been shown to inhibit adipocyte differentiation and lipid storage. The recent paper of Lv *et al.*[238] suggests that guggelsterone protects pancreatic β-cells and inhibits NO and prostaglandin E2 (PGE2) production, which would be an interesting additional mechanism in diabetes.

3.15.10 Nutrigenomics – Finding Effects, Pathways, and New Molecules

3.15.10.1 Nutrient–Gene Interactions – the Possible Solution to Analyzing Complex Effects

Food represents an extremely complex and highly variable mixture of compounds. Effects of food are subtle and may develop very slowly. Moreover, several genetic and environmental factors determine the ultimate effects. In the past, only a few dietary compounds and a handful of relevant biochemical pathways could be investigated. Moreover, the emphasis was on what were regarded as macronutrients and micronutrients, and the prevention of deficiencies and diseases. In the mean time, nutrition science has discovered the importance of what were previously called nonnutritive components of food. In addition, the concept of what should be considered bioactive molecules has changed. One of the most interesting developments in this respect is probably that in the lipid-like compounds. In addition to the triglycerides and FFAs, our food contains many different lipid-derived structures, intermediates, steroid-like compounds, etc. As it has become clear from the previous sections, already the class of the FFAs is far more heterogeneous in its biological activities than

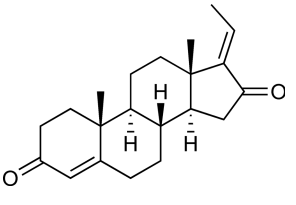


Figure 14 Chemical structure of guggelsterone.

originally assumed. To make it even more complex, bioactives in food are variable, often instable, and present in mixtures. The paradigm shift that dietary intake is not just to avoid deficiency diseases, but also serves to optimize health or to avoid chronic disease has changed nutrition research considerably. A major question is how health-promoting effects of the diet or its components should be measured. Can health be quantified and is it possible to demonstrate a health-improving effect? Is this by reduction of risk? or should the system be challenged or perturbated to be able to measure an effect in terms of the ability of the system to respond?[241]

Postgenomic technologies such as transcriptomics, proteomics, and metabolomics take a more holistic perspective. These approaches are increasingly being used to investigate how our diet interacts with organisms, or more specifically our genes, proteins, and metabolism. Therefore, the rapid technological developments and the fast progress in biochemistry and molecular biology have enabled considerable progress in nutrition science. For a recent review of the technological applications of the 'omics' technologies in nutrition, see Kussmann *et al.*[242] Compared to similar approaches that are mostly followed in pharmacology and toxicology, the assessment of the metabolic response to complex foods by 'omics' technologies is quite different. Food delivers hundreds or thousands of compounds simultaneously and causes thereby an organ-specific response changing over time and in space, in which individual organs or even cell types within an organ react differently to the nutritional challenge. In this respect, there are many more similarities with the application of 'omics' technologies in ethopharmacology and modern pharmacognosy.[113,115,243–245] Following the fashion of giving the different 'omics' areas different names, the term nutrigenomics has become popular. Nutrigenomics is thus defined as the discipline that is involved in studying the response of individuals to food compounds using postgenomic and related technologies (e.g., genomics, transcriptomics, proteomics, metabol/nomics etc.).[241,246–254] The long-term aim of nutrigenomics is to understand how the body responds to foods using an integrated approach termed 'systems biology'. The huge advantage in this approach is that the studies can examine people (i.e., populations, subpopulations – based on genes or disease – and individuals), food, life stage, and lifestyle without preconceived ideas.[115,243,255] This approach can also provide the link with personalized nutrition, since it is obvious that the benefits of some dietary choices are not the same for everyone.[246,252,256]

3.15.10.2 Nutrigenomics, Some Examples

The number of nutrition studies, not only in animals but also in humans, in which transcriptomics, proteomics, metabolomics, or different combinations have been used is steadily increasing, in spite of the complexity and high costs. In the near future, this should allow us to obtain signatures, for example gene expression changes, by comparative analysis of different treatments or dietary interventions. In human studies, the accessibility and availability of material presents a technical challenge. However, it has been shown that shifts in the metabolic status can be analyzed at the level of the transcriptome, using peripheral blood mononuclear cells (PBMCs), isolated from the buffy coat of blood samples taken. This technique has rapidly evolved during the last few years to analyze gene expression profiles in relation to diseases and other factors. With regard to nutrition for example, it was shown that high-protein and high-carbohydrate breakfasts differentially changed the PBMC transcriptome.[257] The approach was also successfully used to study the effect of fasting on PPAR-α target genes involved in fatty acid β-oxidation.[258] Recently, our laboratory showed that subcutaneous adipose tissue can also be used to investigate changes of gene expression profiles in response to dietary lipid intervention.[259] In this study, it was found that gene expression profiles of lean and overweight persons were distinctly different, mainly with respect to genes involved in inflammation and lipid metabolism. In lean subjects, consumption of a PUFA-enriched spread resulted in changes in expression of genes related to energy metabolism. Remarkably, genes that responded to PUFA intervention in overweight persons were mostly linked to inflammation (**Figure 15**).

3.15.10.3 New Compound Discovery for Nutrition – How to Find the Needles in the Haystack

The technological developments in genomics and systems biology have not only changed the possibilities to analyze the effects of nutrients, but have also increased the possibilities to find new compounds. Even more, it is now increasingly becoming possible to study multitarget concepts and to find bioactive mixtures instead of single compounds.[6,244] Also in the pharmaceutical industry this has become a more general trend. In previous

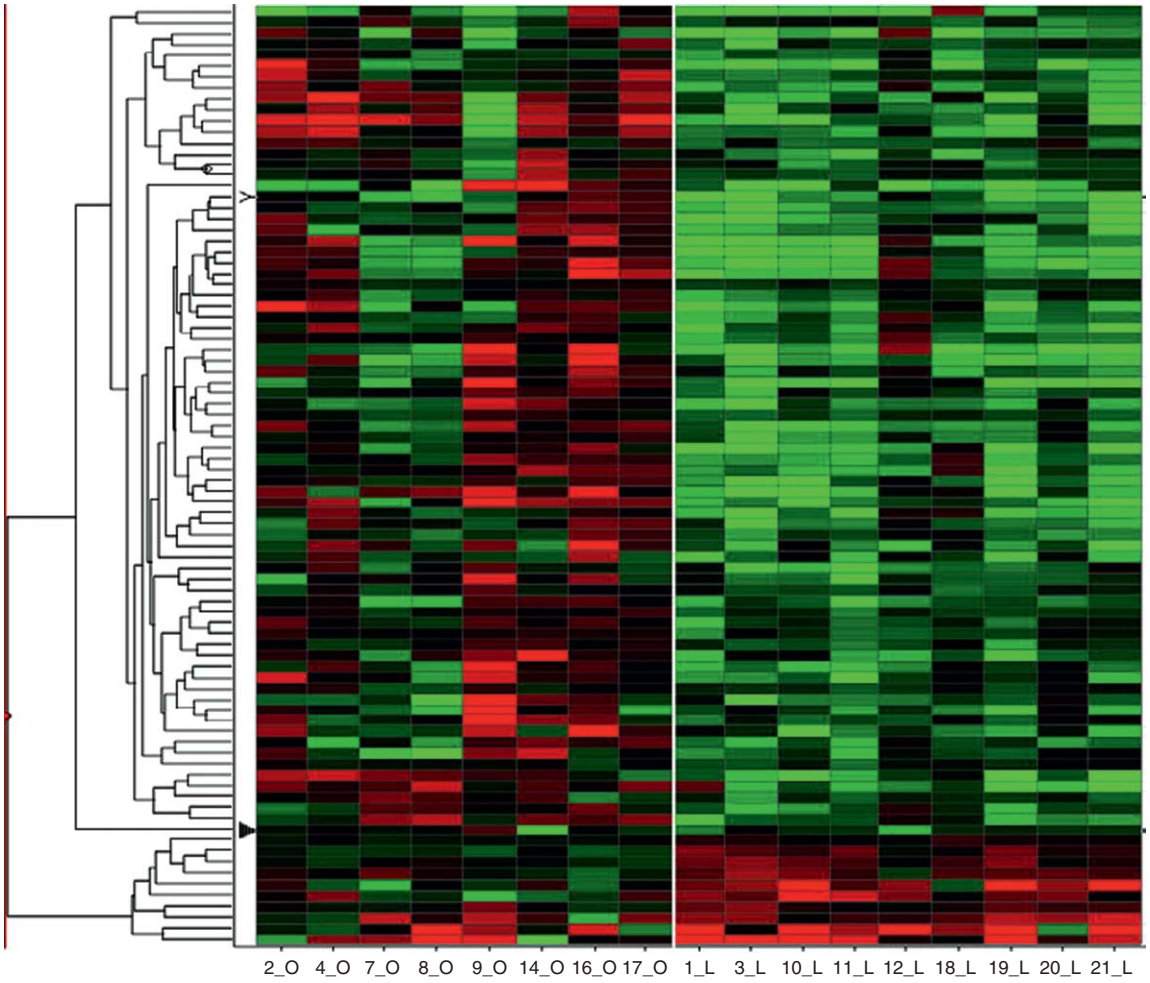


Figure 15 Differential response of gene expression profiles in subcutaneous adipose tissue of 10 lean (BMI 18–25 kg m $^{-2}$) and 10 overweight (BMI 27.5–35 kg m $^{-2}$) men in response to intervention with a control or a PUFA-rich dietary spread. In lean subjects (right), gene clusters involved in energy metabolism were downregulated in response to the PUFA-rich spread, whereas a variable response was seen in overweight (left) subjects.[259]

years, much emphasis was placed on a steady increase in capacity (quantitative increase) via various strategies in the fields of automation and miniaturization, and the past years have seen a steady shift toward higher content and quality (quality increase) for these biological test systems.[260] In the food industry, this is even more relevant and compound discovery has to drift away from the reductionistic approach (defined individual steps in a given pathway) to the holistic assessment of metabolic pathways and processes.[6] A further boost has come from the developments in plant metabolomics.[116] The diversity in plant secondary metabolites is enormous. Plants have evolved through continuous interaction with challenging and predominantly hostile environments and plant metabolites generally confer a specific bioactivity related to their biochemical structures.[244] From the previous sections, it has become clear how complex and multifactorial weight management and metabolic diseases are. Since our metabolic system has evolved in constant interaction with plants and other natural resources that were surrounding us, it is logical that natural compounds will provide us the molecular leads to adapt our physiology to the changed environment. The application of the full range of omics techniques will allow really innovative strategies to help discover the complex multicomponent and multipathway interactions and synergy.[243] It will also enable us to explore the full potential of the so-called traditional medicinal systems.[115] With regard to new compound discovery, ethopharmacological databases can be incorporated in the so-called reverse screening strategies.[6]

3.15.11 Conclusions

Although the principle of homeostasis has been a cornerstone of Western physiology for more than a century, the enormous complexity of biological systems has shifted the focus of pharmacology toward the concept of 'one disease–one target–one drug'. This approach has indeed provided many potent drugs, especially for the treatment of acute conditions such as infectious diseases, but also revealed major drawbacks. It is based on trying to influence a system by interacting with a single protein that is often part of a complex pathway and involved in a cascade of reactions and feedback loops. The reality is that most diseases are multifactorial, which means that treating a single target provides a partial treatment and in the majority of cases no cure or side effects. Weight management and therapeutic intervention strategies in metabolic diseases are the perfect examples of such multifactorial disturbances. In fact, it is still not clear whether we are dealing with a disease, and when or where it starts. Weight management by trying to influence satiety and satiation is a battle against one of the most dominant human instincts. Man is equipped with a highly efficient system to absorb and store a maximum amount of energy. Our brain not only possesses several parallel systems to optimize these processes, but it also rewards us with taste, joy, and the pleasure of eating. Voluntary efforts to reduce weight are resisted by potent compensatory biologic responses. In the presence of a continuous nutritional surplus, this once advantageous metabolic state could set the stage for excess adiposity and its associated problems. In parallel, the ability to fight off infections has also led to selection of strong immune responses, particularly after massive population declines during periods of infectious disease epidemics and pandemics.[88]

So far, attempts to develop successful pharmacological intervention strategies aiming to achieve long-term weight management have not been successful. The same applies to the nutritional approaches, including those based on food supplements and functional foods. For society, the problem has become enormous and in theory, financial gains can be huge in this multibillion dollar market. In this context, the citizen petition of 28 April 2008 of a consortium consisting of the American Dietetic Association, The Obesity Society, Shaping America's Health, and GlaxoSmithKline Consumer Healthcare is interesting.[261] As the petition states, there is no credible scientific evidence that would support any type of a claim accompanying a weight-loss supplement. The fact that GlaxoSmithKline recently (2007) received approval in a number of countries for marketing orlistat as the first OTC weight-loss drug has to be considered among its concern for the public's health. However, it is clear any breakthrough in this area will require new scientific approaches and concepts. These should be based on multipathway and multitarget strategies. Whether or not we are dealing with a syndrome, a cluster of diseases, or a permanent imbalance in energy homeostasis, obesity and its complications can be considered a natural disease that developed in a hostile environment. The clue to solving this lies in understanding the interaction between nature and our metabolism and immune system. Therefore, it seems quite obvious that natural compounds will prove to be of major importance to provide us the leads.

Abbreviations

BMI	body mass index
CCK	cholecystokinin
CLA	conjugated linoleic acid
EGCG	epigallocatechin gallate
FFA	free fatty acid
GI	gastrointestinal
GLP-1	glucagon-like peptide-1
GPCR	G-protein-coupled receptor
MUFA	monounsaturated fatty acid
OEA	oleoylethanolamine
PBMC	peripheral blood mononuclear cell
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acid
PYY	peptide YY
RCT	randomized controlled trial

STZ	streptozotocin
SFA	saturated fatty acid
T2DM	type 2 diabetes mellitus
TCM	Traditional Chinese Medicine
THC	Δ^9-tetrahydrocannabinol
THCV	Δ^9-tetrahydrocannabivarin
TNF-α	tumor necrosis factor-α
TZD	thiazolidinedione

References

1. J. van der Greef; R. N. McBurney, *Nat. Rev. Drug Discov.* **2005**, *4*, 961–967.
2. J. van der Greef; T. Hankemeier; R. N. McBurney, *Pharmacogenomics* **2006**, *7*, 1087–1094.
3. G. R. Zimmermann; J. Lehár; C. T. Keith, *Drug Discov. Today* **2007**, *12*, 34–42.
4. R. Morphy; C. Kay; Z. Rankovic, *Drug Discov. Today* **2004**, *9*, 641–651.
5. A. L. Hopkins; J. S. Mason; J. P. Overington, *Curr. Opin. Struct. Biol.* **2006**, *16*, 127–136.
6. J. Schwager; M. H. Mohajeri; A. Fowler; P. Weber, *Curr. Opin. Biotechnol.* **2008**, *19*, 66–72.
7. M. Tulp; J. G. Bruhn; L. Bohlin, *Drug Discov. Today* **2006**, *11*, 1115–1121.
8. K. Nagao; T. Yanagita, *Prog. Lipid Res.* **2008**, *47*, 127–146.
9. A. Erkkila; V. D. F. de Mello; U. Risérus; D. E. Laaksonen, *Prog. Lipid Res.* **2008**, *47*, 172–187.
10. J. A. Baur; D. A. Sinclair, *Nat. Rev. Drug Discov.* **2006**, *5*, 493–506.
11. D. P. Hayes, *Eur. J. Clin. Nutr.* **2007**, *61*, 147–159.
12. L. G. Higgins; C. Cavin; K. Toh; M. Yamamoto; J. D. Hayes, *Toxicol. Appl. Pharmacol.* **2008**, *226*, 328–337.
13. M. L. Ricketts; M. V. Boekschoten; A. J. Kreeft; G. Hooiveld; C. J. A. Moen; M. Muller; R. R. Frants; S. Kasanmoentalib; S. M. Post; H. M. G. Princen; J. G. Porter; M. B. Katan; M. H. Hofker; D. D. Moore, *Mol. Endocrinol.* **2007**, *21*, 1603–1616.
14. V. Brower, *Nat. Biotechnol.* **1998**, *16*, 728–731.
15. A. Ruiz-Rodriguez; F. Marín; A. Ocaña; C. Soler-Rivas, *Phytochem. Rev.* **2008**, *7*, 345–384.
16. A. D. B. Vaidya; T. P. A. Devasagayam, *J. Clin. Biochem. Nutr.* **2007**, *41*, 1–11.
17. S. Zisman; D. L. Goldberg; M. Veniegas, *Alternat. Complement. Therap.* **2003**, *9*, 191–197.
18. C. F. Chau; S. H. Wu, *Trends Food Sci. Tech.* **2006**, *17*, 313–323.
19. J. Van der Greef; E. Davidov; E. Verheij; J. Vogels; R. van der Heijden; A. S. Adourian; M. Oresic; E. W. Marple; S. Naylor, *The Role of Metabolomics in Systems Biology, a New Vision for Drug Discovery and Development*; Kluwer Academic Publishing: Boston, MA, 2003.
20. N. G. Asp; S. Bryngelsson, *J. Nutr.* **2008**, *138*, 1210S–1215S.
21. P. J. H. Jones; N. G. Asp; P. Silva, *J. Nutr.* **2008**, *138*, 1189S–1191S.
22. P. J. Aggett; J. M. Antoine; N. G. Asp; F. Bellisle; L. Contor; J. H. Cummings; J. Howlett; D. J. G. Müller; C. Persin; L. T. J. Pijls; G. Rechkemmer; S. Tuijtelaars; H. Verhagen; J. Lucas; C. Shortt, *Eur. J. Nutr.* **2005**, *44* (suppl. 1), I/5–I/30.
23. J. L. Pillitteri; S. Shiffman; J. M. Rohay; A. M. Harkins; S. L. Burton; T. A. Wadden, *Obesity* **2008**, *16*, 790–796.
24. T. Lancet, *Lancet* **2008**, *371*, 1723–1723.
25. R. Nugent, *Ann. N. Y. Acad. Sci.* **2008**, *1136*, 70–79.
26. W. P. T. James, *J. Intern. Med.* **2008**, *263*, 336–352.
27. T. S. Han; N. Sattar; M. Lean, *Br. Med. J.* **2006**, *333*, 695–698.
28. A. Behn; E. Ur, *Curr. Opin. Cardiol.* **2006**, *21*, 353–360.
29. J.-P. Despres; I. Lemieux, *Nature* **2006**, *444*, 881–887.
30. G. Egger; B. Swinburn, *Br. Med. J.* **1997**, *315*, 477–480.
31. M. V. Chakravarthy; F. W. Booth, *J. Appl. Physiol.* **2004**, *96*, 3–10.
32. M. W. Schwartz; K. D. Niswender, *J. Clin. Endocrinol. Metab.* **2004**, *89*, 5889–5897.
33. J. M. Friedman, *Nat. Med.* **2004**, *10*, 563–569.
34. C. De Graaf; W. A. M. Blom; P. A. M. Smeets; A. Stafleu; H. F. J. Hendriks, *Am. J. Clin. Nutr.* **2004**, *79*, 946–961.
35. D. J. Mela, *Appetite* **2006**, *47*, 10–17.
36. S. J. Torres; C. A. Nowson, *Nutrition* **2007**, *23*, 887–894.
37. E. Valassi; M. Scacchi; F. Cavagnini, *Nutr. Metab. Cardiovasc. Dis.* **2007**, 1–10.
38. D. E. Cummings; J. Overduin, *J. Clin. Invest.* **2007**, *117*, 13–23.
39. K. E. Foster-Schubert; D. E. Cummings, *Endocr. Rev.* **2006**, *27*, 779–793.
40. R. A. H. Adan; L. J. M. J. Vanderschuren; S. E. la Fleur, *Trends Pharmacol. Sci.* **2008**, *29*, 208–217.
41. S. Choi; M. Lee; A. L. Shiu; J. Y. Sek; G. W. Aponte, *Am. J. Physiol. Gastrointest. Liver Physiol.* **2007**, *292*, G98–G112.
42. G. Dockray, *Curr. Opin. Pharmacol.* **2004**, *4*, 557–560.
43. E. Rozengurt; C. Sternini, *Curr. Opin. Pharmacol.* **2007**, *7*, 557–562.
44. H. A. Overton; A. J. Babbs; S. M. Doel; M. C. T. Fyfe; L. S. Gardner; G. Griffin; H. C. Jackson; M. J. Procter; C. M. Rasamison; M. Tang-Christensen; P. S. Widdowson; G. M. Williams; C. Reynet, *Cell Metab.* **2006**, *3*, 167–175.

45. T. Tanaka; S. Katsuma; T. Adachi; T.a. Koshimizu; A. Hirasawa; G. Tsujimoto, *Naunyn Schmiedebergs Arch. Pharmacol.* **2008**, *377*, 523–527.
46. E. Ryberg; N. Larsson; S. Sjogren; S. Hjorth; N. O. Hermansson; J. Leonova; T. Elebring; K. Nilsson; T. Drmota; P. J. Greasley, *Br. J. Pharmacol.* **2007**, *152*, 1092–1101.
47. H. J. Jang; Z. Kokrashvili; M. J. Theodorakis; O. D. Carlson; B. J. Kim; J. Zhou; H. K. Hyeon; X. Xu; S. L. Chan; M. Juhaszova; M. Bernier; B. Mosinger; R. F. Margolskee; J. M. Egan, *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 15069–15074.
48. H. E. Raybould, *Curr. Opin. Pharmacol.* **2007**, *7*, 570–574.
49. L. A. Matsuda; S. J. Lolait; M. J. Brownstein; A. C. Young; T. I. Bonner, *Nature* **1990**, *346*, 561–564.
50. U. Pagotto; C. Cervino; V. Vicennati; G. Marsicano; B. Lutz; R. Pasquali, *Int. J. Obes.* **2006**, *30*, S39–S43.
51. M. A. Storr; K. A. Sharkey, *Curr. Opin. Pharmacol.* **2007**, *7*, 575–582.
52. K. K. Isoldi; L. J. Aronne, *J. Am. Diet. Assoc.* **2008**, *108*, 823–831.
53. P. V. Piazza; M. Lafontan; J. Girard, *Diabetes Metab.* **2007**, *33*, 97–107.
54. D. Cota; M. H. Tschop; T. L. Horvath; A. S. Levine, *Brain Res. Rev.* **2006**, *51*, 85–107.
55. J. M. Perkins; S. N. Davis, *Curr. Diab. Rep.* **2008**, *8*, 12–19.
56. V. Di Marzo; T. Bisogno; L. De Petrocellis, *Chem. Biol.* **2007**, *14*, 741–756.
57. D. G. Demuth; A. Molleman, *Life Sci.* **2006**, *78*, 549–563.
58. G. J. Sanger, *Br. J. Pharmacol.* **2007**, *152*, 663–670.
59. A. A. Coutts; A. A. Izzo, *Curr. Opin. Pharmacol.* **2004**, *4*, 572–579.
60. V. Di Marzo; I. Matias, *Nat. Neurosci.* **2005**, *8*, 585–589.
61. V. Di Marzo; S. Petrosino, *Curr. Opin. Lipidol.* **2007**, *18*, 129–140.
62. V. Di Marzo, *Nat. Rev. Drug Discov.* **2008**, *7*, 438–455.
63. I. Matias; V. Di Marzo, *Trends Endocrinol. Metab.* **2007**, *18*, 27–37.
64. A. H. Lichtman; B. F. Cravatt, *J. Clin. Invest.* **2005**, *115*, 1130–1133.
65. T. Bisogno; A. Ligresti; V. Di Marzo, *Pharmacol. Biochem. Behav.* **2005**, *81*, 224–238.
66. R. G. Pertwee, *Int. J. Obes.* **2006**, *30*, S13–S18.
67. I. Matias; T. Bisogno; V. Di Marzo, *Int. J. Obes.* **2006**, *30*, S7–S12.
68. I. Bab; A. Zimmer, *Br. J. Pharmacol.* **2007**, *153*, 182–188.
69. A. J. Scheen, *Best Pract. Res. Clin. Endocrinol. Metab.* **2007**, *21*, 535–553.
70. S. P. H. Alexander; D. A. Kendall, *Br. J. Pharmacol.* **2007**, *152*, 602–623.
71. J. Fu; S. Gaetani; F. Oveisi; J. Lo Verme; A. Serrano; F. Rodriguez De Fonseca, *Nature* **2003**, *425*, 90–93.
72. M. Guzman; J. Lo Verme; J. Fu; F. Oveisi; C. Blazquez; D. Piomelli, *J. Biol. Chem.* **2004**, *279*, 27849–27854.
73. J. Fu; F. Oveisi; S. Gaetani; E. Lin; D. Piomelli, *Neuropharmacology* **2005**, *48*, 1147–1153.
74. J. Gertsch, *Planta Med.* **2008**, *74*, 638–650.
75. J. Gertsch; R. Schoop; U. Kuenzle; A. Suter, *FEBS Lett.* **2004**, *577*, 563–569.
76. K. Woelkart; R. Bauer, *Planta Med.* **2007**, *73*, 615–623.
77. A. Kilaru; E. B. Blancaflor; B. J. Venables; S. Tripathy; K. S. Mysore; K. D. Chapman, *Chem. Biodivers.* **2007**, *4*, 1933–1955.
78. G. Burdyga; S. Lal; A. Varro; R. Dimaline; D. G. Thompson; G. J. Dockray, *J. Neurosci.* **2004**, *24*, 2708–2715.
79. P. D. Cani; M. L. Montoya; A. M. Neyrinck; N. M. Delzenne; D. M. Lambert, *Br. J. Nutr.* **2004**, *92*, 757–761.
80. G. Burdyga; A. Varro; R. Dimaline; D. G. Thompson; G. J. Dockray, *Neuroscience* **2006**, *137*, 1405–1415.
81. S. M. Grundy; J. I. Cleeman; S. R. Daniels; K. A. Donato; R. H. Eckel; B. A. Franklin; D. J. Gordon; R. M. Krauss; P. J. Savage; S. C. Smith, Jr.; J. A. Spertus; F. Costa, *Circulation* **2005**, *112*, 2735–2752.
82. International Diabetes Federation (IDF), *IDF Consensus Worldwide Definition of the Metabolic Syndrome*; Brussels, Belgium, 2006. http://www.idf.org/webdata/docs/MetS_def_update2006.pdf (accessed 6 June 2009).
83. R. Kahn, *Lancet* **2008**, *371*, 1892–1893.
84. N. Sattar; A. McConnachie; A. G. Shaper; G. J. Blauw; B. M. Buckley; A. J. de Craen; I. Ford; N. G. Forouhi; D. J. Freeman; J. W. Jukema; L. Lennon; P. W. Macfarlane; M. B. Murphy; C. J. Packard; D. J. Stott; R. G. Westendorp; P. H. Whincup; J. Shepherd; S. G. Wannamethee, *Lancet* **2008**, *371*, 1927–1935.
85. R. Whitmer; D. Gustafson; E. Gunderson; E. Barrett-Connor; M. N. Haan; K. Yaffe, *Neurology* **2007**, *68*, A167–A168.
86. H. Tilg; G. S. Hotamisligil, *Gastroenterology* **2006**, *131*, 934–945.
87. S. M. Grundy, *Nat. Rev. Drug Discov.* **2006**, *5*, 295–309.
88. G. S. Hotamisligil, *Nature* **2006**, *444*, 860–867.
89. G. S. Hotamisligil; N. S. Shargill; B. M. Spiegelman, *Science* **1993**, *259*, 87–91.
90. C. Nathan, *Mol. Med.* **2008**, *14*, 485–492.
91. S. E. Shoelson; L. Herrero; A. Naaz, *Gastroenterology* **2007**, *132*, 2169–2180.
92. S. E. Shoelson; J. Lee; A. B. Goldfine, *J. Clin. Invest.* **2006**, *116*, 1793–1801.
93. B. M. Spiegelman; L. Choy; G. S. Hotamisligil; R. A. Graves; P. Tontonoz, *J. Biol. Chem.* **1993**, *268*, 6823–6826.
94. American Diabetes Association (ADA), *Diabetes Care* **2008**, *31*, S55–S60.
95. A. Guilherme; J. V. Virbasius; V. Puri; M. P. Czech, *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 367–377.
96. S. Mittra; V. S. Bansal; P. K. Bhatnagar, *Drug Discov. Today* **2008**, *13*, 211–218.
97. J. Couzin, *Science* **2008**, *320*, 438–440.
98. M. J. F. Bult; T. van Dalen; A. F. Muller, *Eur. J. Endocrinol.* **2008**, *158*, 135–145.
99. G. A. Bray; F. L. Greenway, *Pharmacol. Rev.* **2007**, *59*, 151–184.
100. M. Lean; N. Finer, *Br. Med. J.* **2006**, *333*, 794–797.
101. D. Rucker; R. Padwal; S. K. Li; C. Curioni; D. C. W. Lau, *Br. Med. J.* **2007**, *335*, 1194–1199.
102. K. G. Hofbauer; J. R. Nicholson; O. Boss, *Annu. Rev. Pharmacol. Toxicol.* **2007**, *47*, 565–592.
103. C. L. Gillies; K. R. Abrams; P. C. Lambert; N. J. Cooper; A. J. Sutton; R. T. Hsu; K. Khunti, *Br. Med. J.* **2007**, *334*, 299–302.
104. A. M. Zivkovic; J. B. German; A. J. Sanyal, *Am. J. Clin. Nutr.* **2007**, *86*, 285–300.
105. American Diabetes Association, *Diabetes Care* **2008**, *31*, S61–S78.
106. D. R. Bensimhon; W. E. Kraus; M. P. Donahue, *Am. Heart J.* **2006**, *151*, 598–603.

107. A. Y. Oubre; T. J. Carlson; S. R. King; G. M. Reaven, *Diabetologia* **1997**, *40*, 614–617.
108. M. M. J. Combettes, *Curr. Opin. Pharmacol.* **2006**, *6*, 598–605.
109. S. Jain; S. Saraf, *Diabetes and Metabolic Syndrome: Clinical Research and Reviews*, in press (available online)
110. B. Panunti; A. A. Jawa; V. A. Fonseca, *Drug Discov. Today Dis. Mech.* **2004**, *1*, 151–157.
111. C. Fiévet; J.-C. Fruchart; B. Staels, *Curr. Opin. Pharmacol.* **2006**, *6*, 606–614.
112. I. Matias; M. P. Gonthier; P. Orlando; V. Martiadis; L. De Petrocellis; C. Cervino, *J. Clin. Endocrinol. Metab.* **2006**, *91*, 3171–3180.
113. M. Heinrich, *Phytochem. Lett.* **2008**, *1*, 1–5.
114. R. Verpoorte, *J. Ethnopharmacol.* **2008**, *115*, 161–162.
115. M. Wang; R. J. A. N. Lamers; H. A. A. J. Korthout; J. H. J. Van Nesselrooij; R. F. Witkamp; R. Van Der Heijden; P. J. Voshol; L. M. Havekes; R. Verpoorte; J. Van Der Greef, *Phytother. Res.* **2005**, *19*, 173–182.
116. R. D. Hall; I. D. Brouwer; M. A. Fitzgerald, *Physiol. Plant* **2008**, *132*, 162–175.
117. G. H. Anderson; S. E. Moore, *J. Nutr.* **2004**, *134*, 974S–979S.
118. W. A. M. Blom; A. Lluch; A. Stafleu; S. Vinoy; J. J. Holst; G. Schaafsma; H. F. J. Hendriks, *Am. J. Clin. Nutr.* **2006**, *83*, 211–220.
119. S. Choi; M. Lee; A. L. Shiu; S. J. Yo; G. Halldén; G. W. Aponte, *Am. J. Physiol. Gastrointest. Liver Physiol.* **2007**, *292*, G1366–G1375.
120. R. A. Reimer, *J. Endocrinol.* **2006**, *191*, 159–170.
121. Y. U. Wang; V. Prpic; G. M. Green; J. R. Reeve, Jr.; R. A. Liddle, *Am. J. Physiol. Gastrointest. Liver Physiol.* **2002**, *282*, G16–G22.
122. J. McLaughlin, *Biochem. Soc. Trans.* **2007**, *35*, 1199–1202.
123. H. E. Raybould, *Am. J. Physiol. Gastrointest. Liver Physiol.* **1999**, *277*, 751–755.
124. J. T. McLaughlin; R. B. Lomax; L. Hall; G. J. Dockray; D. G. Thompson; G. Warhurst, *J. Physiol. (Lond.)* **1998**, *513*, 11–18.
125. S. S. Sidhu; D. G. Thompson; G. Warhurst; R. M. Case; R. S. P. Benson, *J. Physiol. (Lond.)* **2000**, *528*, 165–176.
126. E. Naslund; P. M. Hellstrom, *Physiol. Behav.* **2007**, *92*, 256–262.
127. T. Tanaka; T. Yano; T. Adachi; T. A. Koshimizu; A. Hirasawa; G. Tsujimoto, *Naunyn Schmiedebergs Arch. Pharmacol.* **2008**, *377*, 515–522.
128. A. B. Grudell; M. Camilleri, *Curr. Opin. Endocrinol. Diabetes Obes.* **2007**, *14*, 52–57.
129. C. L. Lawton; H. J. Delargy; J. Brockman; F. C. Smith; J. E. Blundell, *Br. J. Nutr.* **2000**, *83*, 473–482.
130. D. Parra; A. Ramel; N. Bandarra; M. Kiely; J. A. Martínez; I. Thorsdottir, *Appetite* **2008**, *51*, 676–680.
131. G. Asset; B. Staels; R. Wolff; E. Baugé; Z. Madj; J.-C. Fruchart; J. Dallongeville, *Lipids* **1999**, *34*, 39–44.
132. R. Wolff; F. Pédrono; E. Pasquier; A. Marpeau, *Lipids* **2000**, *35*, 1–22.
133. W. Pasman; J. Heimerikx; C. Rubingh; R. van den Berg; M. O'Shea; L. Gambelli; H. Hendriks; A. Einerhand; C. Scott; H. Keizer; L. Mennen, *Lipids Health Dis.* **2008**, *7*, 10.
134. Y. Yang; M. Chen; K. E. Georgeson; C. M. Harmon, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2007**, *292*, R235–R241.
135. H. A. Overton; M. C. T. Fyfe; C. Reynet, *Br. J. Pharmacol.* **2008**, *153*, S76–S81.
136. V. Di Marzo; M. Maccarrone, *Trends Pharmacol. Sci.* **2008**, *29*, 229–233.
137. J. Beekwilder; B. Schipper; P. Bakker; D. Bosch; M. Jongsma, *Eur. J. Biochem.* **2000**, *267*, 1975–1984.
138. C. A. Ryan, *Annu. Rev. Phytopathol.* **1990**, *28*, 425–449.
139. M. A. Jongsma; P. L. Bakker; J. Peters; D. Bosch; W. J. Stiekema, *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 8041–8045.
140. M. A. Jongsma; C. Bolter, *J. Insect Physiol.* **1997**, *43*, 885–895.
141. A. J. Hill; S. R. Peikin; C. A. Ryan; J. E. Blundell, *Physiol. Behav.* **1990**, *48*, 241–246.
142. L. Pouvreau; H. Gruppen; S. R. Piersma; L. A. M. van den Broek; G. A. van Koningsveld; A. G. J. Voragen, *J. Agric. Food Chem.* **2001**, *49*, 2864–2874.
143. S. Komarnytsky; I. Raskin; Method for Producing Plant Extracts Enriched with Protease Inhibitors for Regulation of Appetite and Food Intake in Humans. U.S. Patent Appl. WO 2007075448, 5 July 2007.
144. R. B. Birari; K. K. Bhutani, *Drug Discov. Today* **2007**, *12*, 879–889.
145. M. Mukherjee, *J. Mol. Catal. B Enzym.* **2003**, *22*, 369–376.
146. E. Hochuli; E. Kupfer; R. Maurer, *J. Antibiot.* **1987**, *40*, 1086–1091.
147. E. K. Weibel; P. Hadvary; E. Hochuli, *J. Antibiot.* **1987**, *40*, 1081–1085.
148. P.-A. Albertsson; R. Köhnke; S. C. Emek; J. Mei; J. F. Rehfeld; H.-E. Åkerlund; C. Erlanson-Albertsson, *Biochem. J.* **2007**, *401*, 727–733.
149. K. N. Chidambara Murthy; G. K. Jayaprakasha; R. P. Singh, *J. Agric. Food Chem.* **2002**, *50*, 4791–4795.
150. S. R. Katz; R. A. Newman; E. P. Lansky, *J. Med. Food* **2007**, *10*, 213–217.
151. F. Lei; X. N. Zhang; W. Wang; D. M. Xing; W. D. Xie; H. Su; L. J. Du, *Int. J. Obes.* **2007**, *31*, 1023–1029.
152. Y. Li; Y. Qi; T. H. W. Huang; J. Yamahara; B. D. Roufogalis, *Diabetes Obes. Metab.* **2008**, *10*, 10–17.
153. Y. Li; T. H.-W. Huang; J. Yamahara, *Life Sci.* **2008**, *82*, 1045–1049.
154. F. R. van Heerden; R. Marthinus Horak; V. J. Maharaj; R. Vleggaar; J. V. Senabe; P. J. Gunning, *Phytochemistry* **2007**, *68*, 2545–2553.
155. B. Avula; Y. H. Wang; R. S. Pawar; Y. J. Shukla; I. A. Khan, *J. AOAC Int.* **2007**, *90*, 1526–1531.
156. H. G. Janssen; C. Swindells; P. Gunning; W. Wang; C. Grun; K. Mahabir; V. J. Maharaj; P. J. Apps, *Anal. Chim. Acta* **2008**, *617*, 200–207.
157. S. Dall'Acqua; G. Innocenti, *Steroids* **2007**, *72*, 559–568.
158. D. B. MacLean; L. G. Luo, *Brain Res.* **2004**, *1020*, 1–11.
159. Food and Drug Administration, *New Dietary Ingredient Notification on Hoodia gordonii*; Center for Food Safety and Applied Nutrition: USA. http://www.fda.gov 23 (accessed 23 Mar 2004).
160. A. Braca; A. Bader; I. Morelli; R. Scarpato; G. Turchi; C. Pizza; N. De Tommasi, *Tetrahedron* **2002**, *58*, 5837–5848.
161. O. Kunert; B. V. A. Rao; G. S. Babu; M. Padmavathi; B. R. Kumar; R. M. Alex; W. Schühly; N. Simic; D. Kühnelt; A. V. N. A. Rao, *Helv. Chim. Acta* **2006**, *89*, 201–209.
162. E. Abdel-Sattar; A. A. Ahmed; M. E. F. Hegazy; M. A. Farag; M. A. A. Al-Yahya, *Phytochemistry* **2007**, *68*, 1459–1463.
163. R. Kuriyan; T. Raj; S. K. Srinivas; M. Vaz; R. Rajendran; A. V. Kurpad, *Appetite* **2007**, *48*, 338–344.
164. E. Abdel-Sattar; M. R. Meselhy; M. A. A. Al-Yahya, *Planta Med.* **2002**, *68*, 430–434.

165. A. Bader; A. Braca; N. De Tommasi; I. Morelli, *Phytochemistry* **2003**, *62*, 1277–1281.
166. R. Rajendran; K. Rajendran, Pregnane Glycoside Compositions and Caralluma Extract Products and Uses Thereof. U.S. Patent Application 2,005,202,103, 18 March 2005.
167. R. Rajendran; K. Rajendran, Caralluma Extract Products and Processes for Making the Same. U.S. Patent 7,060,308, 13 June, 2006.
168. L. Shatkina; R. Shatkina; S. Gurevic; Meal Replacement Products Having Appetite Suppressing qualities. International Patent Application WO2,006,045,112, 27 April 2006.
169. Food and Drug Administration, *New Dietary Ingredient Notification on Caralluma fimbriata.* Center for Food Safety and Applied Nutrition: USA. http://www.fda.gov (accessed 24 Aug 2004).
170. R. G. Pertwee, *Br. J. Pharmacol.* **2007**, *153*, 199–215.
171. J. T. Dwyer; D. B. Allison; P. M. Coates, *J. Am. Diet. Assoc.* **2005**, *105*, S80–S86.
172. M. H. Pittler; E. Ernst, *Am. J. Clin. Nutr.* **2004**, *79*, 529–536.
173. C. Ni Mhurchu; C. A. Dunshea-Mooij; D. Bennett; A. Rodgers, *Cochrane Database Syst. Rev.* **2005**, CD003892.
174. M. H. Pittler; E. Ernst, *Int. J. Obes.* **2005**, *29*, 1030–1038.
175. C. M. Novak; J. A. Levine, *J. Neuroendocrinol.* **2007**, *19*, 923–940.
176. T. Murase; A. Nagasawa; J. Suzuki; T. Hase; I. Tokimitsu, *Int. J. Obes.* **2002**, *26*, 1459–1464.
177. H. S. Moon; H. G. Lee; Y. J. Choi; T. G. Kim; C. S. Cho, *Chem. Biol. Interact.* **2007**, *167*, 85–98.
178. S. Wolfram, *J. Am. Coll. Nutr.* **2007**, *26*, 373S–388S.
179. A. M. Hill; A. M. Coates; J. D. Buckley; R. Ross; F. Thielecke; P. R. C. Howe, *J. Am. Coll. Nutr.* **2007**, *26*, 396S–402S.
180. S. Wolfram; Y. Wang; F. Thielecke, *Mol. Nutr. Food Res.* **2006**, *50*, 176–187.
181. Q. Shixian; B. VanCrey; J. Shi; Y. Kakuda; Y. Jiang, *J. Med. Food* **2006**, *9*, 451–458.
182. S. Haaz; K. R. Fontaine; G. Cutter; N. Limdi; S. Perumean-Chaney; D. B. Allison, *Obes. Rev.* **2006**, *7*, 79–88.
183. S. Bent; A. Padula; J. Neuhaus, *Am. J. Cardiol.* **2004**, *94*, 1359–1361.
184. J. M. Hollander; J. I. Mechanick, *J. Am. Diet. Assoc.* **2008**, *108*, 495–509.
185. K. W. Andrews; A. Schweitzer; C. Zhao; J. M. Holden; J. M. Roseland; M. Brandt; J. T. Dwyer; M. F. Picciano; L. G. Saldanha; K. D. Fisher; E. Yetley; J. M. Betz; L. Douglass, *Anal. Bioanal. Chem.* **2007**, *389*, 231–239.
186. C. I. Heck; E. G. De Mejia, *J. Food Sci.* **2007**, *72*, R138–R151.
187. E. M. R. Kovacs; D. J. Mela, *Obes. Rev.* **2006**, *7*, 59–78.
188. J. A. Greenberg; C. N. Boozer; A. Geliebter, *Am. J. Clin. Nutr.* **2006**, *84*, 682–693.
189. K. Diepvens; K. R. Westerterp; M. S. Westerterp-Plantenga, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2007**, *292*, R77–R85.
190. P. G. Shekelle; M. L. Hardy; S. C. Morton; M. Maglione; W. A. Mojica; M. J. Suttorp; S. L. Rhodes; L. Jungvig; J. Gagne, *J. Am. Med. Assoc.* **2003**, *289*, 1537–1545.
191. M. Maglione; K. Miotto; M. Iguchi; L. Hilton; P. Shekelle, *Expert Opin. Drug Saf.* **2005**, *4*, 879–884.
192. G. J. Wilson; J. M. Wilson; A. H. Manninen, *Nutr. Metab.* **2008**, *5*, 1.
193. A. Quignard-Boulange; P. Clouet; B. Schmitt, *Eur. J. Lipid Sci. Tech.* **2007**, *109*, 935–944.
194. S. Tricon; P. Yaqoob, *Curr. Opin. Clin. Nutr.* **2006**, *9*, 105–110.
195. Y. Wang; P. J. Jones, *Am. J. Clin. Nutr.* **2004**, *79*, 1153S–1158S.
196. J. M. Gaullier; J. Halse; H. O. Høivik; C. Syvertsen; M. Nurminiemi; C. Hassfeld; A. Einerhand; M. O'Shea; O. Gudmundsen, *Br. J. Nutr.* **2007**, *97*, 550–560.
197. A. A. Sneddon; F. Tsofliou; C. L. Fyfe; I. Matheson; D. M. Jackson; G. Horgan; M. S. Winzell; K. W. J. Wahle; B. Ahren; L. M. Williams, *Obesity* **2008**, *16*, 1019–1024.
198. G. Y. Yeh; D. M. Eisenberg; T. J. Kaptchuk; R. S. Phillips, *Diabetes Care* **2003**, *26*, 1277–1294.
199. W. L. Li; H. C. Zheng; J. Bukuru; N. De Kimpe, *J. Ethnopharmacol.* **2004**, *92*, 1–21.
200. S. R. Mentreddy, *J. Sci. Food Agric.* **2007**, *87*, 743–750.
201. A. Fleischman; S. E. Shoelson; R. Bernier; A. B. Goldfine, *Diabetes Care* **2008**, *31*, 289–294.
202. R. Willamson, *Br. Med. J.* **1901**, *1*, 760–762.
203. D. E. Hyams; A. N. Howard; I. E. Evans; S. H. H. Davison, *Diabetologia* **1971**, *7*, 94–101.
204. R. S. Hundal; K. F. Petersen; A. B. Mayerson; P. S. Randhawa; S. Inzucchi; S. E. Shoelson; G. I. Shulman, *J. Clin. Invest.* **2002**, *109*, 1321–1326.
205. J. A. Baur; K. J. Pearson; N. L. Price; H. A. Jamieson; C. Lerin; A. Kalra; V. V. Prabhu; J. S. Allard; G. Lopez-Lluch; K. Lewis; P. J. Pistell; S. Poosala; K. G. Becker; O. Boss; D. Gwinn; M. Wang; S. Ramaswamy; K. W. Fishbein; R. G. Spencer; E. G. Lakatta; D. Le Couteur; R. J. Shaw; P. Navas; P. Puigserver; D. K. Ingram; R. De Cabo; D. A. Sinclair, *Nature* **2006**, *444*, 337–342.
206. X. Terra; J. Valls; X. Vitrac; J. M. Mérrillon; L. Arola; A. Ardèvol; C. Bladé; J. Fernández-Larrea; G. Pujadas; J. Salvadó; M. Blay, *J. Agric. Food Chem.* **2007**, *55*, 4357–4365.
207. H. M. Woo; J. H. Kang; T. Kawada; H. Yoo; M. K. Sung; R. Yu, *Life Sci.* **2007**, *80*, 926–931.
208. J. H. Kang; C. S. Kim; I. S. Han; T. Kawada; R. Yu, *FEBS Lett.* **2007**, *581*, 4389–4396.
209. S. Bidel; G. Hu; J. Tuomilehto, *Cent. Eur. J. Med.* **2008**, *3*, 9–19.
210. L. L. Moisey; S. Kacker; A. C. Bickerton; L. E. Robinson; T. E. Graham, *Am. J. Clin. Nutr.* **2008**, *87*, 1254–1261.
211. B. K. Bassoli; P. Cassolla; G. R. Borba-Murad; J. Constantin; C. L. Salgueiro-Pagadigorria; R. B. Bazotte; R. da Silva; H. M. de Souza, *Cell Biochem. Funct.* **2008**, *26*, 320–328.
212. R. M. van Dam, *Nutr. Metab. Cardiovasc. Dis.* **2006**, *16*, 69–77.
213. S. Muscat; J. Pelka; J. Hegele; B. Weigle; G. Munch; M. Pischetsrieder, *Mol. Nutr. Food Res.* **2007**, *51*, 525–535.
214. R. Nagai; Y. Fujiwara; K. Mera; M. Otagiri, *Mol. Nutr. Food Res.* **2007**, *51*, 462–467.
215. C. Delgado-Andrade; F. J. Morales, *J. Agric. Food Chem.* **2005**, *53*, 1403–1407.
216. W. T. Cefalu; F. B. Hu, *Diabetes Care* **2004**, *27*, 2741–2751.
217. J. Martin; Z. Q. Wang; X. H. Zhang; D. Wachtel; J. Volaufova; D. E. Matthews; W. T. Cefalu, *Diabetes Care* **2006**, *29*, 1826–1832.
218. J. K. Grover; S. Yadav; V. Vats, *J. Ethnopharmacol.* **2002**, *81*, 81–100.
219. Z. Yaniv; A. Dafni; J. Friedman; D. Palevitch, *J. Ethnopharmacol.* **1987**, *19*, 145–151.

220. L. M. McCune; T. Johns, *J. Ethnopharmacol.* **2007**, *112*, 461–469.
221. L. M. McCune; T. Johns, *J. Ethnopharmacol.* **2002**, *82*, 197–205.
222. J. S. García-Alvarado; M. J. Verde-Star; N. L. Heredia, *J. Herbs Spices Med. Plants* **2001**, *8*, 37–89.
223. E. Hernandez-Galicia; A. Aguilar-Contreras; L. Aguilar-Santamaria; R. Roman-Ramos; A. A. Chavez-Miranda; L. M. Garcia-Vega; J. L. Flores-Saenz; F. J. Alarcon-Aguilar, *Proc. West. Pharmacol. Soc.* **2002**, *45*, 118–124.
224. A. Andrade-Cetto; M. Heinrich, *J. Ethnopharmacol.* **2005**, *99*, 325–348.
225. A. Tahraoui; J. El-Hilaly; Z. H. Israili; B. Lyoussi, *J. Ethnopharmacol.* **2007**, *110*, 105–117.
226. A. Ziyyat; A. Legssyer; H. Mekhfi; A. Dassouli; M. Serhrouchni; W. Benjelloun, *J. Ethnopharmacol.* **1997**, *58*, 45–54.
227. M. Eddouks; M. Maghrani; A. Lemhadri; M. L. Ouahidi; H. Jouad, *J. Ethnopharmacol.* **2002**, *82*, 97–103.
228. H. Jouad; M. Haloui; H. Rhiouani; J. El Hilaly; M. Eddouks, *J. Ethnopharmacol.* **2001**, *77*, 175–182.
229. P. K. Mukherjee; K. Maiti; K. Mukherjee; P. J. Houghton, *J. Ethnopharmacol.* **2006**, *106*, 1–28.
230. M. D. Boudreau; F. A. Beland, *J. Environ. Sci. Health C* **2006**, *24*, 103–154.
231. B. Sharma; C. Balomajumder; P. Roy, *Food Chem. Toxicol.* **2008**, *46*, 2376–2383.
232. J. K. Grover; S. P. Yadav, *J. Ethnopharmacol.* **2004**, *93*, 123–132.
233. M.-J. Tan; J.-M. Ye; N. Turner; C. Hohnen-Behrens; C.-Q. Ke; C.-P. Tang; T. Chen; H.-C. Weiss; E.-R. Gesing; A. Rowland; D. E. James; Y. Ye, *Chem. Biol.* **2008**, *15*, 263–273.
234. J. Lachman; B. Havrland; E. C. Fernandez; J. Dudjak, *Plant Soil Environ.* **2004**, *50*, 383–390.
235. S. Terada; K. Ito; M. Taka; N. Ogose; N. Noguchi; Y. Koide, *Nat. Med.* **2003**, *57*, 89–94.
236. S. S. Hong; S. A. Lee; X. H. Han; M. H. Lee; J. S. Hwang; J. S. Park; K. W. Oh; K. Han; M. K. Lee; H. Lee; W. Kim; D. Lee; B. Y. Hwang, *Chem. Pharm. Bull. (Tokyo)* **2008**, *56*, 199–202.
237. K. Valentová; D. Stejskal; J. Bartek; S. Dvorácková; V. Kren; J. Ulrichová; V. Simánek, *Food Chem. Toxicol.* **2008**, *46*, 1006–1013.
238. N. Lv; M.-Y. Song; E.-K. Kim; J.-W. Park; K.-B. Kwon; B.-H. Park, *Mol. Cell. Endocrinol.* **2008**, *289*, 49–59.
239. N. L. Urizar; A. B. Liverman; D. T. Dodds; F. V. Silva; P. Ordentlich; Y. Yan; F. J. Gonzalez; R. A. Heyman; D. J. Mangelsdorf; D. D. Moore, *Science* **2002**, *296*, 1703–1706.
240. N. L. Urizar; D. D. Moore, *Annu. Rev. Nutr.* **2003**, *23*, 303–313.
241. B. van Ommen; J. Keijer; R. Kleemann; R. Elliott; C. A. Drevon; H. McArdle; M. Gibney; M. Müller, *Genes Nutr.* **2008**, *3*, 51–59.
242. M. Kussmann; S. Rezzi; H. Daniel, *Curr. Opin. Biotechnol.* **2008**, *19*, 83–99.
243. R. Verpoorte; Y. H. Choi; H. K. Kim, *J. Ethnopharmacol.* **2005**, *100*, 53–56.
244. L. F. Shyur; N. S. Yang, *Curr. Opin. Chem. Biol.* **2008**, *12*, 66–71.
245. G. Ulrich-Merzenich; H. Zeitler; D. Jobst; D. Panek; H. Vetter; H. Wagner, *Phytomedicine* **2007**, *14*, 70–82.
246. L. Afman; M. Müller, *J. Am. Diet. Assoc.* **2006**, *106*, 569–576.
247. R. M. Elliott; I. T. Johnson, *Obes. Rev.* **2007**, *8*, 77–81.
248. L. R. Ferguson, *Mol. Diagn. Ther.* **2006**, *10*, 101–108.
249. J. Kaput; J. Noble; B. Hatipoglu; K. Kohrs; K. Dawson; A. Bartholomew, *Nutr. Metab. Cardiovasc. Dis.* **2007**, *17*, 89–103.
250. F. C. Lau; M. Bagchi; C. Sen; S. Roy; D. Bagchi, *Curr. Genomics* **2008**, *9*, 239–251.
251. E. C. M. Mariman, *Biotechnol. Appl. Biochem.* **2006**, *44*, 119–128.
252. M. Muller; S. Kersten, *Nat. Rev. Genet.* **2003**, *4*, 315–322.
253. C. M. Williams; J. M. Ordovas; D. Lairon; J. Hesketh; G. Lietz; M. Gibney; B. van Ommen, *Genes Nutr.* **2008**, *3*, 41–49.
254. J. Ovesná; O. Slabý; O. Toussaint; M. Kodíček; P. Maršík; V. Pouchov; T. Vaněk, *Br. J. Nutr.* **2008**, *99*, ES127–ES134.
255. S. Naylor; A. W. Culbertson; S. J. Valentine, *Curr. Opin. Biotechnol.* **2008**, *19*, 100–109.
256. J. López-Miranda; P. Pérez-Martinez; C. Marin; F. Fuentes; J. Delgado; F. Pérez-Jiménez, *J. Mol. Med.* **2007**, *85*, 213–226.
257. M. J. van Erk; W. A. M. Blom; B. van Ommen; H. F. J. Hendriks, *Am. J. Clin. Nutr.* **2006**, *84*, 1233–1241.
258. M. Bouwens; L. A. Afman; M. Muller, *Am. J. Clin. Nutr.* **2007**, *86*, 1515–1523.
259. M. J. Van Erk; W. J. Pasman; W. H. M.; B. van Ommen; H. F. J. Hendriks; H. H. F. J., *Genes Nutr.* **2008**, *3–4*, 127–137.
260. L. M. Mayr; P. Fuerst, *J. Biomol. Screen.* **2008**, *13*, 443–448.
261. American Dietetic Association; The Obesity Society; Shaping America's Health; GlaxoSmithKline Consumer Healthcare Administration, *Citizen Petition Requesting FDA to Treat Weight Loss Claims for Dietary Supplements as Disease Claims*, FDA, 17 April, 2008.

Biographical Sketch

Renger Witkamp (1959) studied Biology and Pharmacy at the Utrecht University (the Netherlands). He received his Ph.D. in Pharmacology in 1992 and worked as an associate professor at the Utrecht University until 1996. Subsequently, he moved to TNO, the Netherlands' Organization for Applied Research. At TNO, he worked in several positions, mainly in pharmacology and analytical chemistry. As the director of TNO Pharma (2003–06) he was also responsible for business development relations with the pharmaceutical industry. In 2006, he was appointed as professor of Nutrition and Pharmacology at the division of Human Nutrition of Wageningen University, which is a new academic chair. This is combined with a position at TNO for 2 days per week. In his present position, he is working in the food–pharma interface by applying pharmacological concepts to nutrition and vice versa. His research interests focus on natural bioactives that influence appetite regulation and the development of obesity and its metabolic complications.

3.16 Chemistry of Flavonoid-Based Colors in Plants

Øyvind M. Andersen and Monica Jordheim, University of Bergen, Bergen, Norway

3.16.1 Introduction

Plants exhibit two fundamentally different types of colors, one based upon the physical and optical properties of the plant cell and tissue structures, and the other upon the presence of pigments and their copigments. These two mechanisms for coloration may, and often do, co-exist in the same tissues, and the perceived color will thus depend on the combined effect of the two distinct phenomena. When considering only the number of pigment classes that are commonly recognized in plants, it is found to be fairly modest.[1,2] This does not mean that the main classes answer for all natural plant pigments, but they do include the majority of all known pigments of general occurrence. Chlorophylls can easily be recognized by their characteristic green color, similar to many carotenoids with their deep yellow to orange-red hues. These latter colors are produced by the flavonoid groups, chalcones, and aurones (**Figure 1**) in a restricted number of plants. Even more pronounced are the anthocyanins (**Figure 1**, **Table 5**), a special class of flavonoids, which are responsible for the often intense, orange to blue colors of most flowers, leaves, and fruits (see Chapter 6.18). The betacyanins (**Figure 1**) with restricted distribution mainly in Caryophylalles, show superficial color similarities to anthocyanins, although, their occurrence seem to be mutually exclusive. Less noticeable are the flavones and flavonols (**Figure 1**), which provide rather pale yellow colors, often masked by other pigments and often seen only by the insect eye. This chapter is concerned with plant pigmentation, which is based upon anthocyanins, chalcones, and aurones. Strack and Wray[3] have described anthocyanins as 'the most important group of watersoluble plant pigments visible to the human eye'. Anthocyanin pigmentation is the major part of this chapter, while colors of chalcones and aurones are mainly treated in Section 3.16.4.

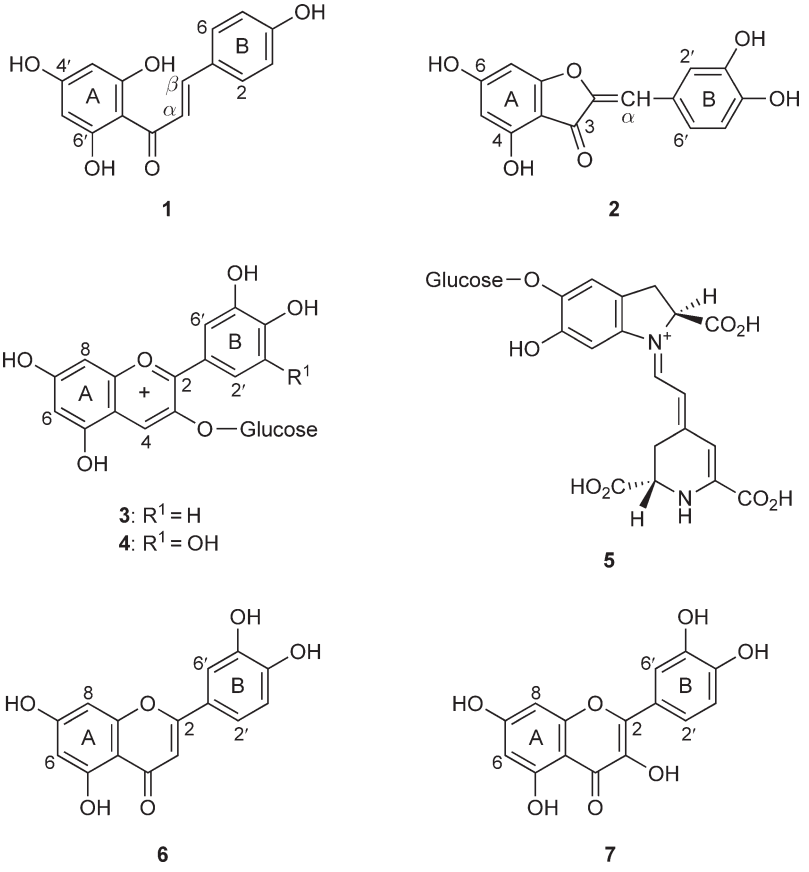


Figure 1 Examples of the colored flavonoid groups, chalcone: isosalipurpol (**1**), aurone: aureusidin (**2**), anthocyanins: cyanidin 3-glucopyranoside (**3**) and delphinidin 3-glucopyranoside (**4**), flavone: luteolin (**6**) and flavonol: quercetin (**7**), and the betalain: betacyanin (**5**).

In nature, anthocyanins are known for providing colors and patterns in flowers, fruits, and seeds to attract or repel pollinators and seed dispersers, thereby enhancing the survival of plants (Sections 3.16.6.1 and 3.16.6.2). The exact functions of anthocyanins and other flavonoid pigments in leaves, seedlings, roots, and stems are far from completely known, although their protective roles against various abiotic stresses and active defensive functions against pathogens, insects, and herbivores have been discussed in many papers (Section 3.16.6.3), especially in recent years. Once anthocyanins are formed in a given organ they operate through evolution to increase the number of individuals producing these compounds, which may explain the range of species-specific anthocyanins, which are found in petals, fruits, and leaves of various genera of higher plants.[4] Up to August 2008 a total of 644 anthocyanins isolated from plants have been identified appropriately;[4–7] however, only a minor fraction have been subjected to further analysis at the molecular level besides their structure elucidation. In general, the anthocyanins are treated as a homogeneous group of pigments. In the European Union's directive for a list of colorants permitted in food, anthocyanins (and grape color extract and grape skin extract) have been given just one common code (E163), in contrast to carotenoids and carotenoid containing sources. The importance of specific anthocyanin structures for various functions under *in vivo* conditions is reflected in the following example.

When compiling the reported anthocyanin content of blue flowers (**Table 1**), it is clear that nearly all anthocyanins are based on just one anthocyanidin, delphinidin (**Table 5**). The majority of these pigments contain aromatic acyl group(s) (**Figure 8**), and those without are reported together with copigments. When considering flowers of all colors, delphinidin derivatives constitute just around 22% of the different anthocyanins, which have been identified.

In recent years there has been worldwide interest in the extended use of anthocyanins as color additives as a consequence of perceived consumer preferences as well as legislative action, which has continued the delisting of approved artificial dyes. The main disadvantages of these pigments seem to be their relative low tinctorial strength and stability, which varies considerably between individual anthocyanins.

Over the past two decades considerable evidences reported that adequate fruit and vegetable consumption has a role in maintaining health and preventing various diseases. Some of these protective effects seem to be caused by the content of anthocyanins and other flavonoids, or their degradation products.[39–44] Certain studies concerned with the absorption of anthocyanins in humans indicate, however, that anthocyanins are only partially bioavailable, in nanomolar concentrations observed in plasma and urinary yields, commonly less than 0.1% of the oral dose.[45,46] Therefore, *if* intake of anthocyanins has a positive health effect, and *if* the various anthocyanins have different effects or properties (bioavailability, stability, etc.) of vital importance, then of course both the qualitative and quantitative content of various fruits and vegetables should be more closely considered. The range of anthocyanin structures found in the human diet (**Tables 2** and **3**) constitute only 20% of all the various anthocyanins, which have been isolated from plants (mainly flowers).[5] More than 55% of the different anthocyanins that occur in vegetables are acylated with aromatic acyl groups, while the corresponding number in fruits is only 21%. Among the commonly eaten fruits, only some grape and gooseberry cultivars have been reported to contain anthocyanins with aromatic acylation as major pigments,[112,124] while the majority of the vegetables contain considerable amounts of such pigments (**Tables 2** and **3**).

Continual improvements in methods and instrumentation (e.g., HPLC, LC–MS, and NMR) used for separation and structure elucidation of anthocyanins have made it easier to use smaller quantities of material, and to achieve results at increasing levels of precision (see Chapters 9.02, 9.06, and 9.11). Discovery of new anthocyanins regularly turn up in plant sources, which already have been well investigated before. When nearly no anthocyanins were reported to be acylated with malonic acid (**Figure 8**) two decades ago, malonyl units are now the most common acylation agent of anthocyanins occurring in 158 different anthocyanins. This chapter makes no attempts to cover methods used for analysis of anthocyanins and other flavonoids, however, the most recent techniques used for extraction, separation, identification, and quantification of these compounds have been treated thoroughly in several recent reviews.[125–133] A complete observation of anthocyanin color should include the molecular structure as well as the environment. Gonnet[134] has specified that an adequate description of anthocyanin color variation caused by pH differences requires that spectral variations considered should be those affecting the entire spectral curve (not only visible λ_{max}), that three color attributes (hue, saturation, and lightness) should be used to describe color (e.g., CIELAB parameters), and that these should refer to the light source and the condition of the observer. Recently, the influence of concentration, pH, and solvent on the

Table 1 Anthocyanin content in blue flowers

Family	*Species*	*Anthocyanin*[a]	*Reference(s)*
Monocotyledoneae			
Hyacinthaceae	*Hyacinthus orientalis* cv.	Dp3-[6-(cum)glc]-5-[6-(mal)glc]	8
Iridaceae	*Crocus antalyensis*[b]	Dp3-glc-5-[6-(mal)glc], Dp3-glc-7-glc, Pt3-glc-7-glc	9
Liliaceae	*Dianella nigra, D. tasmanica*[a]	Dp3-[6-(cum)glc]-7-[6-(cum)glc]-3′-[6-(cum)glc]-5′-[6-(cum)glc], Dp3-glc-7-[6-(cum)glc]-3′-[6-(cum)glc]-5′-[6-(cum)glc], Dp3-glc-7-glc-3′-[6-(cum)glc]-5′-[6-(cum)glc], 2-ace-1,5-di-OH-3-Me-8-[6-(xyl)glc]-naphthalene	10
	Muscari armeniacum	Dp3-[6-(cum)glc]-5-[4-(rha)-6-(mal)glc]	11
	Ophiopogon jaburan[c]	Pt3-[2-(glc)-6-(rha)glc]-3′-glc, Ka3-gal-4′-glc, Ka3,4′-di-glc	12
	Triteleia bridgesii[d]	Dp3-[6-(cum)glc]-5-glc, Dp3-[6-(cum)glc]-5-[6-(mal)glc], Dp3-[6-(4-(glc)cum)glc]-5-[6-(mal)glc]	13
Dicotyledoneae			
Campanulaceae	*Campanula medium*	Dp3-[6-(rha)glc]-7-[6-(4-(6-(4-(glc)hba)glc)hba)glc]	14
Compositae	*Cichorium intybus*	Dp3-[6-(mal)glc]-5-[6-(mal)glc], Dp3-[6(mal)glc], Dp3-[6-(mal)glc]-5-glc, Dp3-glc-5-glc, 3-cum quinic acid	15,16
	Felicia amelloides	Dp3-[2-(rha)glc]-7-[6-(mal)glc], 7-O-MeAp6-*C*-[2-(rha)glc]-4′-glc (ratio 1:18),7-*O*-methylisovitexin	17
	Senecio cruentus	Dp3-[6-(mal)glc)]-7-[6-(4-(6-(caf)glc)caf)glc]-3′-[6-(caf)glc]	18
Convolvulaceae	*Evolvulus pilosus*	Dp3-[6-(4-(6-(4-(glc)caf)glc)caf)glc]-5-[6-(mal)glc], Dp3 -[6-(4-(6-(4-(glc)caf)glc)caf)glc]-5-glc	19
	Ipomoea tricolor	Pn3-[6-(4-(6-(3-(glc)caf)glc)caf)-2-(6-(3-(glc)caf)glc)glc]-5-glc	20
Cornaceae	*Cornus alba* cv.[e]	Dp3-gal-3′-glc-5′-glc, Dp3-gal-3′-glc, Cy3-gal-3′-glc	21
Gentianaceae	*Gentiana* cv.	Cy3-glc-5-[6(caf)glc], Dp3-glc-5-[6-(caf)glc]-3′-glc, Dp3-glc-5-[6-(cum)glc]-3′-glc, Dp3-glc-5-[6-(cum)glc], Dp3-glc-5-[6-(caf)glc]-3′-[6-(cum)glc], Dp3-glc-5-[6-(cum)glc]-3′-[6-(caf)glc], Dp3-glc-5-[6-(caf)glc]-3′-[6-(caf)glc]	22
Goodeniaceae	*Leschenaultia* cv.	Dp3-[6-(mal)glc]-7-[6-(4-(6-(4-(glc)caf)glc)caf)glc]	23
Hydrophyllaceae	*Phacelia campanularia*	Dp3-[6-(4-(6-(4-(glc)caf)glc)caf)glc]-5-[6-(mal)glc]	19
Labiatae	*Salvia patens*	Dp3-[6-(cum)glc]-5-[6-(mal)glc], Ap7,4′-di-glc	24
	Salvia uliginosa	Dp3-[6-(cum)glc]-5-[4-(ace)-6-(mal)glc] Ap7-[4-(glc)glc], Ap7-[4-(glc)glc]-4′-glc	25
Leguminosae	*Clitoria ternatea*	Dp3,3′,5′-trigly (16 acylated ternatins)	26–30
	Lupinus cv.	Dp3-[6-(mal)glc], Ap7-[6-(mal)glc]	31
	Vicia villosa[d]	Dp3-rha-5-glc, Mv3-rha-5-glc, Pt3-rha-5-glc	32
Nymphaeaceae	*Nymphaea caerulea*	Dp3′-[2-(gao)-6-(ace)gal], Dp3′-[2-(gao)gal]	33

Ranunculaceae	*Aconitum chinense*[d]	Dp3-[6-(rha)glc]-7-[6-(4-(6-(hba)glc)hba)glc]	34
	Anemone coronaria[d]	Dp3-[2-(2-(caf)glc)-6-(mal)gal]-7-[6-(caf)glc]-3′-glu, Dp3-[2-(2-(caf)glc)gal]-7-[6-(caf)glc]-3′-glu, Dp3-[2-(2-(caf)glc)-6-(3-(2-(tar)mal)gal]-7-[6-(caf)glc]-3′-glu, Dp3-[2-(2-(caf)glc)-6-(3-(2-(tar)mal)gal]-7-[6-(caf)glc], Cy3-[2-(2-(caf)glc)-6-(3-(2-(tar)mal)gal]-7-[6-(caf)glc]-3′-glu	35
	Delphinium hybridum	Dp3-[6-(rha)glc]-7-[3-(3-(6-(4-(6-(hba)glc)hba)glc)glc)-6-(4-(6-(hba)glc)hba)glc]	36
Rhamnaceae	*Ceanothus papillosus*	Dp3-[6-(rha)glc]-7-[6-(cum)glc]-3′-[6-(cum)glc], Dp3-[6-(rha)glc]-7-[6-(cum)glc]-3′-glc, Ka3-[2-(xyl)rha]	37
Solanaceae	*Browallia speciosa* cv.[d]	Dp3-[6-(4-(caf)rha)glc]-5-[2-(cum)glc]	38

[a] In some cases copigments also identified.
[b] Blue perianth segments.
[c] Seed coats.
[d] Purple-blue.
[e] Fruits.

See **Table 9** for anthocyanin-flavonoid conjugates and **Table 10** for metalloanthocyanins.

Cy, cyanidin; Dp, delphinidin; Hi, hirsutidin; Mv, malvidin; Pg, pelargonidin; Pn, peonidin; Pt, petunidin; CCy, 5-carboxypyranocyanidin; CPg, 5-carboxypyranopelargonidin; CMv, 5-carboxypyranomalvidin; Ap, apigenin; Ka, kaempferol; ace, acetic acid; caf, caffeic acid; cum, *p*-coumaric acid; fer, ferulic acid; mal, malonic acid; gao, gallic (tri-OH-benzoyl) acid; hba, *p*-OH-benzoic acid; sin, sinapic acid; tar, tartaric acid; ara, arabinose; gal, galactose; glc, glucose; glu, glucuronic acid; gly, glycoside; rhamnose, rha; xyl, xylose.

Table 2 Qualitative and quantitative anthocyanin content of selected vegetables used in the human diet

Vegetables	*Major*[a] anthocyanins[b]	*Content (mg 100 g^{-1})*		*Reference(s)*
		FW	*DW*	
Bean, black (*Phaseolus vulgaris*)	Dp-, Mv-, Pt3-glc	24–45	214–278	47–50
Bean, red (*Phaseolus vulgaris*)	Cy3-glc, Cy3-[2-(xyl)glc], Pg3-glc	7	27–74	47,48,51
Cabbage, red (*Brassica oleracea*)	Cy3,5-di-glc, Cy3-[2-(glc)glc]-5-glc, Cy3-[2-(2-(sin)glc)glc]-5-glc, Cy3-[6-(sin)-2-(2-(sin)glc)glc]-5-glc	6–363		47,52–54
Carrot, black (*Daucus carrota*)	Cy3-[2-(xyl)gal], Cy3-[2-(xyl)-6-(glc)gal], Cy3-[2-(xyl)-6-(6-(sin)glc)gal], Cy3-[2-(xyl)-6-(6-(fer)glc)gal], Cy3-[2-(xyl)-6-(6-(cum)glc)gal]	44	4–1799	55–57
Chicory (*Cichorium intybus*)	Cy3-glc, Cy3-[6-(mal)glc], Dp3-[6-(mal)glc]	126–590		58–60
Corn (*Zea mays*)	Cy3-glc, Cy3-[6-(mal)glc], Cy3-[3,6-di-(mal)glc], Pg3-glc, Pn3-glc	54–1734	1680–1878	61,62
Eggplant (*Solanum melongena*)	Dp3-[6-(rha)glc], Dp3-[6-(rha)glc]-5-glc, Dp3-[6-(4-(*Z*/*E*-cum)rha)glc]-5-glc	8–86		47,52,63
Lentil (*Lens culinaris*)	Dp3-[2-(glc)ara]			64
Lettuce, red leaf (*Lactuca sativa*)	Cy3-[6-(mal)glc]	2–5		47,51,52,65
Onions (*Allium cepa*)	Cy3-glc, Cy3-[3-(glc)glc], Cy3-[6-(mal)glc], Cy3-[6-(mal)-3-(glc)glc]	15–49		47,52,66,67
Potatoes (*Solanum* spp.)	12 Pt-, Mv-, Pn-, Pg- and Dp3-[6-(rha)glc]-5-glc monoacylated with cum, fer or caf	2–40		52,68–70
Radish, red (*Raphanus sativus*)	Pg3-[2-(glc)-6-(cum)glc]-5-[6-(mal)glc], Pg3-[2-(glc)-6-(fer)glc]-5-[6-(mal)glc]	32–100		47,51,52,71
Rice, black (*Oryza sativa*)	Cy3-glc	10–493		72
Rhubarb (*Rheum rhabarbaru*)	Cy3-glc, Cy3-[6-(rha)glc]	4		52,73
Shamrock, purple (*Oxalis triangularis*)	Mv3-[6-(rha)glc]-5-glc, Mv3-[6-(4-(mal)rha)glc]-5-glc	195		74,75
Soybean, black (*Glycine* spp.)	Cy3-glc, Dp3-glc		158–2040	76,77
Sweet potato (*Ipomoea batatas*)	10 Cy- and Pn3-[2-(glc)glc]-5-glc mono- or diacylated with fer, caf, cum, or hba	180–184	611–625	62

[a] Major: Compounds estimated to occur in relative anthocyanin amounts higher than 10%. In some papers there exist no discrimination between major and minor compounds.
[b] See **Table 1** for abbreviations.
FW, fresh weight; DW, dry weight.

Table 3 Qualitative and quantitative anthocyanin content of selected fruits used in the human diet

Fruits	*Major*[a] anthocyanin[b]	*Content (mg 100 g^{-1})*		*Reference(s)*
		FW	*DW*	
Acai, jussara (*Euterpe* sp.)	Cy3-glc, Cy3-[6-(rha)glc]	30–293	730–2956	42,78–80
Acerola (*Malpighia* sp.)	Cy3-rha, Pg3-rha	4–60	261–528	79–84
Apple (*Malus sylvestris* spp.)	Cy3-gal	1–50	3–4	47,52,85–91
Baguacu (*Eugenia umbelliflora*)	Cy-, Dp-, Mv-, Pn- and Pt3-glc	342		91
Black currant (*Ribes nigrum*)	Cy3-glc, Cy3-[6-(rha)glc], Dp3-glc, Dp3-[6-(rha)glc]	236–587	744–1072	90,92–94
Black raspberry (*Rubus occidentalis*)	Cy3-glc, Cy3-[6-(rha)glc], Cy3-[2-(xyl)glc]	18–687	87–973	47,95
Blackberry(*Rubus fruticosus*)	Cy3-glc	70–300		47,96,97
Blood orange (*Citrus sinensis*)	Cy3-glc, Cy3-[6-(mal)glc], Dp3-glc	18–84		98–101
Blueberries (*Vaccinium* spp.)	Cy-, Dp-, Mv-, Pn-, Pt3-glc, 3-gal and 3-ara	110–823	2221–3146	47,90,94,97,102,103
Cherries (*Prunus* spp.)	Cy3-glc, Cy3-[6-(rha)glc]	66–144		47,52,104
Chokeberry(*Aronia* sp.)	Cy3-ara, Cy3-gal	410–1480	177–1052	53,90,93,94,102,105
Cowberry/lingonberry (*Vaccinium vitis-idaea*)	Cy3-ara, Cy3-gal	49–174	225–355	52,90,92,94,106–108
Cranberries (*Vaccinium oxycoccos*)	Cy3-glc, Cy3-ara, Cy3-gal, Pn3-ara, Pn3-gal	112–169	395–399	47,90,94,109
Crowberry (*Empetrum* sp.)	Cy-, Dp-, Mv-, Pn-, Pt3-gal and 3-ara	360	2379–4180	52,90,110
Elderberries (*Sambucus* spp.)	Cy3-glc, Cy3-[2-(xyl)glc], Cy3-[2-(xyl)glc]-5-glc, Cy3-[2-(xyl)-6(-Z/E-cum)glc]-5-glc	280–1005		93,94,111
Gooseberry (*Ribes uva-crispa*)	Cy3-xyl, Cy3-[6-(rha)glc], Cy3-[6-(cum)glc], Cy3-[6-(caf)glc], Pn3-glc	3–46	81–85	52,90,112,113
Grapefruit (*Citrus paradisis*)	Cy3-glc	6		52
Grapes *Vitis* spp.	Cy-, Dp-, Mv-, Pn- and Pt3-glc,	16–790	113	47,52,97,114,115
Litchi (*Litchi chinensis*)	Cy3-[6-(rha)glc],	48–177		116,117
Mango (*Mangifera indica*)	7-MeCy3-gal	$(0.2–3.8)\times10^{-4}$		118,119
Nectarine (*Prunus persica* var. *nucipersica*)	Cy3-glc	2–8		47,52
Passion fruits (*Passiflora* spp.)	Cy3-glc, Cy3-[6-(mal)glc], Dp3-glc			120
Peach (*Prunus persica*)	Cy3-glc	4–50		47,52,121
Pear (*Pyrus* spp.)	Cy3-gal	7		122
Plum (*Prunus domestica*)	Cy3-glc, Cy3-[6-(rha)glc], Pn3-glc, Pn3-[6-(rha)glc]	5–1833		47,52,121,123
Red currant (*Ribes rubrum*)	Cy3-[6-(rha)glc], Cy3[2(-xyl)glc], Cy3[2-(xyl)-6-(rha)glc],	1–21	108–118	52,90,93,94,
Red raspberry (*Rubus idaeus*)	Cy3-glc, Cy3-[6-(rha)glc]	2–109	3–594	47,52,90,95,102
Strawberry (*Fragaria* × *ananassa*)	Pg3-glc	18–52	184–235	47,52,90,102

[a] Major: Compounds estimated to occur in relative anthocyanin amounts higher than 10%. In some papers there exist no discrimination between major and minor compounds.
[b] See **Table 1** for abbreviations.
FW, fresh weight; DW, dry weight.

colors analyzed *in vitro* by CIELAB parameters,[131] and the molar absorptivities and visible λ_{max} values of various anthocyanins have been compiled.[135] Some of the drawbacks with respect to standardization of color analysis of pigments like anthocyanins are reflected in the variation, sometimes inconsistent, between the data shown within both of these compilations. Therefore, although the perception of the final anthocyanin pigmentation in plants depends on various factors, the reality is that UV–visible absorption spectra (visible λ_{max} values in particular) have been used as the common tool in most papers to describe and compare anthocyanin colors, as exemplified throughout this chapter. **Table 4** presents a compilation of molar absorption values of various anthocyanins reported after 1990 useful for quantitative determinations. Values reported before 1990 seems to be elevated in discrepancy.[138] On the basis of **Table 4**, we recommend for general use in measurements of anthocyanin concentration (antioxidant effects, etc.) a molar absorptivity value of 22 000 for

Table 4 Molar absorptivity values and visible absorption maxima of selected anthocyanins reported after 1990

Pigment[a]	*Molar absorptivity (ε)*	*$\lambda_{vis\text{-}max}$ (nm)*	*Solvent*	*Reference*
Pelargonidin (Pg)				
Pg	18 420	505	A-aq., pH 1.0	136
	19 780	524	MeOH, 0.1% HCl	136
Pg3-glc	15 600	496	A-aq., pH 1.0	136
	14 300	498	B-aq., pH 1.0	137
	21 021	497	B-aq. pH 1.0	138
	17 330	508	MeOH, 0.1% HCl	136
	23 800	502	MeOH, 0.01 v/v HCl	138
Pg3-(di-caf-glc)-[2-(glc)glc]-5glc	28 000	512	aq., pH 0.8	139
Pg3-[6-(rha)glc]-5-glc+[cum]	32 080	504	A-aq., pH 1.0	136
	39 591	511	MeOH, 0.1% HCl	136
Pg3-[2-(glc)glc]-5-glc	19 000	498	aq., pH 0.8	139
	25 370	497	A-aq., pH 1.0	136
	30 690	506	MeOH, 0.1% HCl	136
Pg3-[2-(glc)glc]-5-glc+[fer]	24 140	506	A-aq., pH 1.0	136
	29 636	507	MeOH, 0.1% HCl	136
Pg3-[2-(glc)glc]-5-glc-caf	19 000	498	aq., pH 0.8	139
Pg3-[2-(glc)glc]-5-glc+[cum]	28 720	506	A-aq., pH 1.0	136
	34 889	508	MeOH, 0.1% HCl	136
Pg3-[2-(glc)glc]-5-glc+[cum]+[mal	33 010	508	A-aq., pH 1.0	136
	39 785	508	MeOH, 0.1% HCl	136
Pg3-[2-(glc)glc]-5-glc+[fer]+[mal]	31 090	508	A-aq., pH 1.0	136
	39 384	508	MeOH, 0.1% HCl	136
CPg3-glc	21 500	495	MeOH, 0.01 v/v HCl	140
Cyanidin (Cy)				
Cy3-glc	18 800	512	10% EtOH, pH 1.5	141
	20 000	510	B-aq. pH 1.0	142
	16 520		B-aq. pH 1.1	143
	20 000	510	B-aq. pH 1.0	138
Cy3-[2-(glc)glc]-5-glc	19 260		B-aq. pH 1.1	143
Cy3-[2-(2-(sin)glc)-6-(sin)glc]-5-glc	23 460		B-aq. pH 1.1	143
Cy3-gal	23 450	508	B-aq. pH 1.0	138
	21 630	519	MeOH, 0.01 v/v HCl	138
CCy3-gal	20 840	506	MeOH, 0.01 v/v HCl	138
Peonidin (Pn)				
Pn3-glc	15 100	510	B-aq. pH 1.0	137
	14 100	512	10% EtOH, pH 1.5	141
Delphinidin (Dp)				
Dp3-glc	23 700	520	10% EtOH, pH 1.5	141
Petunidin (Pt)				
Pt3-glc	21 300	515	B-aq. pH 1.0	137
	18 900	520	10% EtOH, pH 1.5	141
	23 370	527	MeOH, 0.01 v/v HCl	138

(*Continued*)

Table 4 (Continued)

Pigment[a]	*Molar absorptivity (ε)*	$\lambda_{vis\text{-}max}$ *(nm)*	*Solvent*	*Reference*
Malvidin (Mv)				
Mv	16 000	538	MeOH, 0.01% HCl	144
Mv3-glc	23 400	517	B-aq. pH 1.0	137
	25 150	529	MeOH, 0.01 v/v HCl	138
	20 200	520	10% EtOH, pH 1.5	141
CMv3-glc	12 900	532	MeOH, 0.01% HCl	144

[a] See **Table 1** for abbreviations.
A-aq., aqueous buffer 0.025 mol l^{-1} KCl; B-aq., aqueous buffer 0.2 mol l^{-1} KCl – 0.2 mol l^{-1} HCl.

simple nonacylated anthocyanins dissolved in methanolic solutions containing 0.1% conc. hydrochloric acid. With respect to acylated anthocyanins, the values are considerably higher and depend largely on the structure.

A rather detailed description of the various structural elements influencing anthocyanin colors is presented in Section 3.16.2 under various headings. In Section 3.16.2.8, we have summarized copigmentation mechanisms. The primary anthocyanin structure, copigmentation, and pH are shown to be the most important factors influencing anthocyanin colors and stability, however, the exact mechanisms involved are poorly understood. Many isolated anthocyanins, which are nearly colorless in slightly acidic aqueous solvents, express their colors in plant vacuoles, which are indeed slightly acidic. Over the past decades, the question of how blue colors can be produced in flowers has been raised. Our understanding today is that anthocyanin monomers contain multiple structural features, which in combinations contribute to the formation of different supramolecular complexes. Anthocyanins are within the cells most often found dissolved uniformly in vacuolar solutions (see Section 3.16.3). Some anthocyanins have been reported in intensively colored intravascular bodies recently called AVIs (anthocyanic vacuolar inclusions). Although it is generally accepted that anthocyanins as other flavonoids are synthesized on the cytoplasmic surface of the endoplasmic reticulum membrane, the mechanisms for transportation and anthocyanin accumulation in the cells are more indecisive – even the structures of the AVIs are partly unknown.

Anthocyanin pigmentation has been very useful in genetic experiments, including the well-known studies of Gregor Mendel on inheritance of genes responsible for pea seed coat colors. Nowadays, the flavonoid biosynthetic pathway has been almost completely elucidated (Section 3.16.5). Since flower colors are among the key determinants influencing consumer choices, new varieties are of high commercial value. In recent years the intense search for a blue rose and other new anthocyanin flower colors has demanded the need for molecular bioengineering. By introducing new genes in plants encoding for novel enzyme activities, transcription factors, or inactivation of endogenous genes used in anthocyanin biosynthesis, several new varieties with modified flower colors and plant coloration have been created. The interest in and demand for natural food colorants and pharmacologically interesting natural compounds have also encouraged new research initiatives aimed at the development of more efficient means of harvesting anthocyanins. The production of anthocyanins in plant tissue cultures and by microorganisms is treated separately in Sections 3.16.7.1 and 3.16.7.2.

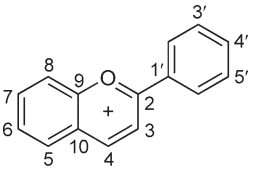
3.16.2 Color Variation Owing to Anthocyanin Structure

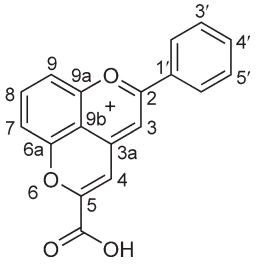
The anthocyanins are responsible for cyanic colors ranging from salmon pink through red and violet to dark blue in most flowers, fruits, and leaves of angiosperms. They are sometimes present in other plant tissues such as roots, tubers, stems, bulbils, and are also found in various gymnosperms, ferns, and some bryophytes. The term anthocyanin was initially coined to designate the substance responsible for the color of the cornflower (from the Greek words *anthos* (flower) and *kyanos* (blue). At present the actual number of anthocyanins reported with complete structure elucidation is 644.[4–7] The anthocyanins differ with respect to their aglycone (anthocyanidin), nature of glycosyl and potential aliphatic and aromatic acyl moieties, and their substitution positions.[4] During the last 15 years one new methylated anthocyanidin (7-*O*-methylcyanidin from mango, *Mangifera indica*), seven new deoxyanthocyanidins, and a novel type of anthocyanidins called pyranoanthocyanidins have been reported

(**Table 5**). In addition, new types of anthocyanins called flavanol–anthocyanidin heterodimers and anthocyanin–flavonoid conjugates as well as some new metalloanthocyanins have been identified.

In 1962, Hayashi summarized the major factors that caused the wide range of flower colors as a result of the presence of anthocyanin pigments: (1) The co-existence of several anthocyanins, (2) variation in the cellular concentration of anthocyanins, (3) the pH of the cell, (4) the phenomenon of copigmentation, (5) the colloidal condition of the cell sap, and (6) association of anthocyanins with metals.[145] Considerable efforts have later been made to improve explanations for the color variations expressed by anthocyanins.[4,146] Today various assets with the anthocyanin structure including (1) nature and concentration of the anthocyanidins, (2) anthocyanin

Table 5 Structures of naturally occurring anthocyanidins





	Substitution pattern						
Anthocyanidins[a]	*3*	*5*	*6*	*7*	*3′*	*4′*	*5′*
Common anthocyanidins							
Pelargonidin (Pg)	OH	OH	H	OH	H	OH	H
Cyanidin (Cy)	OH	OH	H	OH	H	OH	H
Delphinidin (Dp)	OH	OH	H	OH	OH	OH	OH
Peonidin (Pn)	OH	OH	H	OH	OMe	OH	H
Petunidin (Pt)	OH	OH	H	OH	OMe	OH	OH
Malvidin (Mv)	OH	OH	H	OH	OMe	OH	OMe
Rare methylated anthocyanidins							
5-MethylCy	OH	OMe	H	OH	OH	OH	H
7-MethylCy	OH	OH	H	OMe	OH	OH	H
7-MethylPn (Rosinidin)	OH	OH	H	OMe	OMe	OH	H
5-MethylDp (Pulchellidin)	OH	OMe	H	OH	OH	OH	OH
5-MethylPt (Europinidin)	OH	OMe	H	OH	OMe	OH	OH
5-MethylMv (Capensinidin)	OH	OMe	H	OH	OMe	OH	OMe
7-MethylMv (Hirsutidin)	OH	OH	H	OMe	OMe	OH	OMe
6-Hydroxylated anthocyanidins							
6-HydroxyPg	OH	OH	OH	OH	H	OH	H
6-HydroxyCy	OH	OH	OH	OH	OH	OH	H
6-HydroxyDp	OH	OH	OH	OH	OH	OH	OH
3-Deoxyanthocyanidins							
Apigeninidin (Ap)	H	OH	H	OH	H	OH	H
Luteolinidin (Lt)	H	OH	H	OH	OH	OH	H
Tricetinidin (Tr)	H	OH	H	OH	OH	OH	OH
7-MethylAp	H	OH	H	OMe	H	OH	H
5-MethylLt	H	OMe	H	OH	OH	OH	H
5-Methyl-6-hydroxyAp (Carajurone)	H	OMe	OH	OH	H	OH	H
5,4′-Dimethyl-6-hydroxyAp (Carajurin)	H	OMe	OH	OH	H	OMe	H
5-Methyl-6-hydroxyLt	H	OMe	OH	OH	OH	OH	H
5,4′-Dimethyl-6-hydroxyLt	H	OMe	OH	OH	OH	OMe	H
Pyranoanthocyanidins		*6a*	*7*	*8*			
5-CarboxypyranoPg (CPg)	OH	O-	H	OH	H	OH	H
5-CarboxypyranoCy (CCy)	OH	O-	H	OH	OH	OH	H

[a] See **Figure 2** for riccionidins A and B, sphagnorubins A–C, and rosacyanins A1, A2, and B.
The numbering of the structures on the left and right is used for anthocyanins and pyranoanthocyanins, respectively.

glycosidation and acylation, (3) flavanol–anthocyanidin heterodimers, (4) anthocyanin–flavonoid conjugates, (5) metal complexes, (6) anthocyanidin secondary structures (equilibrium forms), (7) nature and concentration of copigmentation including intra- and/or inter-molecular association mechanisms, (8) tertiary organization in the so-called AVIs (anthocyanic vacuolar inclusions) have been examined for their impact on anthocyanin coloration. In addition, external factors like pH, salts, temperature, involvement by pigment matrix/solvents, and so on, have been found to influence anthocyanin colors. Inter- and intramolecular copigmentation is supposed to be the most common mechanism in anthocyanin stabilization *in vivo*, and in the formation of most blue flower colors.[4,146–148] The following section describes various factors influencing anthocyanin colors.

3.16.2.1 Anthocyanidin Skeleton

The anthocyanidins (anthocyanin aglycones) are derivatives of 2-phenylbenzopyrylium (flavylium cation). The numbering of the left structure in **Table 5** is used for most anthocyanins, including anthocyanidins having the classical C_{15} skeleton. The pyranoanthocyanins, which have at least one additional C_3 unit, are based on the skeleton represented by the structure on the right in **Table 5**. While thirty-two naturally occurring monomeric anthocyanidins have been properly identified (**Table 5**), most of the identified anthocyanins are based on cyanidin (31%), delphinidin (22%), and pelargonidin (18%), respectively,[5] which only differ by the hydroxylation pattern of their B-rings. The other three common anthocyanidins (peonidin, malvidin, and petunidin), which contain methoxyl group(s) on their B-rings, constitute together the aglycones of 21% of the reported anthocyanins. This means that the rest of the anthocyanins, which have been identified (8%), are based on as many as 24 different anthocyanin aglycones. Although anthocyanin aglycones have been reported to occur *in vivo*, these findings have normally been treated as artifacts formed during the extraction and isolation stages. Recently, the natural presence of cyanidin, peonidin, and pelargonidin in extracts of beans has been suggested after careful consideration of the process of extraction and purification followed by LC–MS for identification purposes.[149] Otherwise, the 3-deoxyanthocyanidins found in *Sorghum bicolor*, spagnorubins in peat moss (*Sphagnum* spp.), and rosacyanins from petals of *Rosa hybrida* are the only anthocyanidins found in their nonglycosidated forms in plants (**Figure 2**).[7]

Although the perception of the final flower color based on anthocyanins depends on various factors, an UV–visible absorption spectrum of an anthocyanin gives a fair idea about its color. A typical anthocyanin exhibits a broad absorption maximum in the visible spectral region and has one less intense maximum in the UV region at about 275 nm (**Figure 3**). However, spectroscopic properties of anthocyanidins and anthocyanins are highly influenced by substituents and changes made to solvent and pH. This latter effect is shown by apigeninidin (**Table 5**), for which the absorption maximum (λ_{max}) is reported at wavelengths from 468 to 547 nm.[150,151] To understand the effect of a specific hydroxyl or methoxyl substituent on the color of an anthocyanidin, we have compiled from literature a standardized set of spectroscopic absorption data obtained at room temperature and with 0.01% (or 0.1%) hydrochloric acid in methanol as the solvent (**Table 6**). The interpretations (**Figure 4**) are based on the calculated shift difference in observed λ_{max} values between anthocyanidins differing at exactly one specific position. For instance, the difference in λ_{max} of 7-hydroxyflavylium (441 nm) and 3,7-dihydroxyflavylium (488 nm) is 47 nm caused by the hydroxyl group in the 3-position, while the difference between similar values of 4′-hydroxyflavylium (453 nm) and 3,4′-dihydroxyflavylium (484 nm) is 31 nm again caused by the 3-hydroxyl group. From **Table 6** we thus are able to obtain altogether seven $\Delta\lambda_{max}$ values caused by the effect of the 3-hydroxyl group, which have an average value of $\Delta\lambda_{max} = 37$ nm (**Figure 4**). Although the data behind the $\Delta\lambda_{max}$ values in **Figure 4** of the various anthocyanidin OH-substituents are somewhat scarce with respect to some positions, the following general conclusions given in Sections 3.16.2.1–3.16.2.3 may be drawn. As seen in **Table 6**, the replacement of an −OH moiety for an −OMe group leads to only minor hypsochromic effects on λ_{max}, meaning minute reddening effect on the color. This effect is just a couple of nanometers for the common anthocyanidins (**Table 5**), peonidin (3′-*O*-methylation), petunidin (3′-*O*-methylation), and malvidin (3′,5′-di-*O*-methylation). Anthocyanidins with 5-, 7-, or 4′-*O*-methylation are very rare.[4] The hypsochromic effect of methylation in such compounds seems to be from 5 to 10 nm. Toki *et al.*[13] have for the series cyanidin (3,5,7,3′,4′-pentahydroxyflavylium), peonidin (3,5,7,4′-tetrahydroxy-3′-methoxyflavylium), 7-*O*-methylcyanidin (3,5,3′,4′-tetrahydroxy-7-methoxyflavylium), and rosinidin (3,5,4′-trihydroxy-7,3′-dimethoxyflavylium) reported $\lambda_{vis\text{-}max} = 538$, 537, 532, and 530 nm, respectively, in accordance with these trends. When several substitutions on the aglycone skeleton exist, synergetic effects between substituents in the various positions have to be considered.

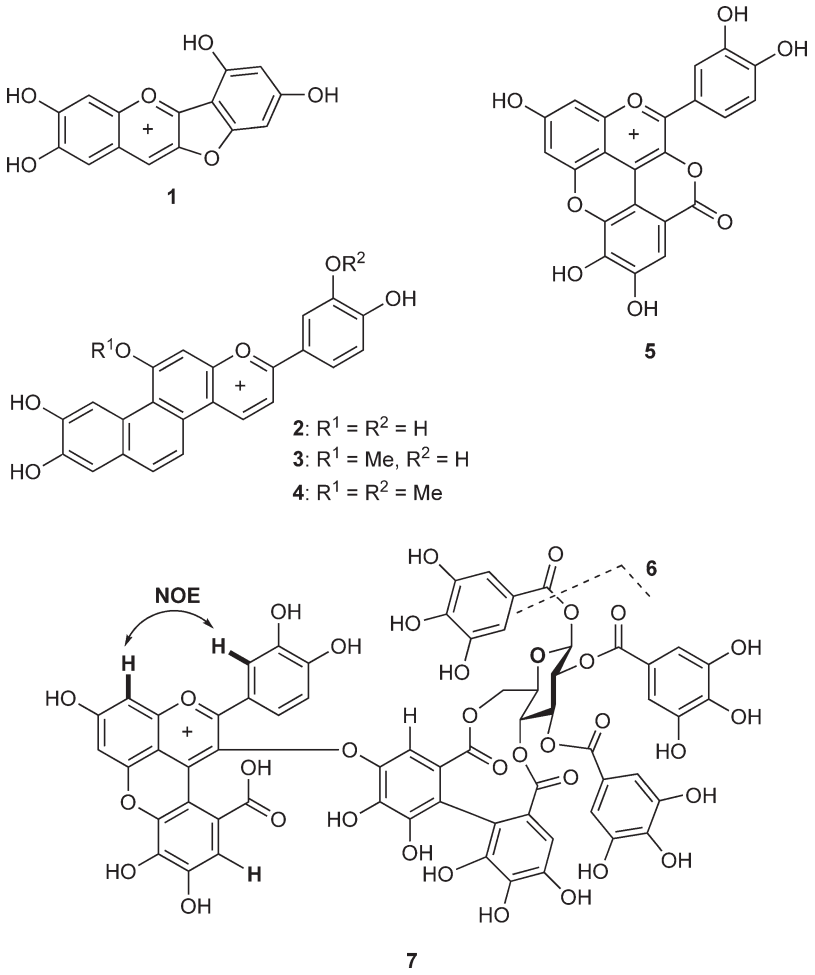


Figure 2 Structures of some rare anthocyanidins. Riccionidin A (**1**), sphagnorubins A-C (**2–4**), rosacyanin B (**5**), rosacyanin A1 (**6**), and A2 (**7**). In structure **7** the NOE between H-2′ and H-8 in the NMR spectrum is highlighted. Other anthocyanidin structures are found in **Table 5**.

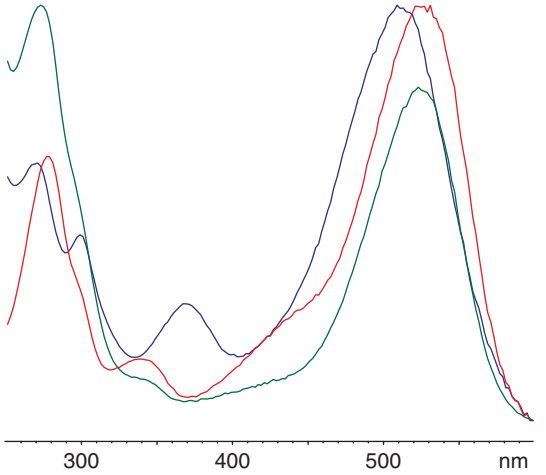


Figure 3 UV–visible spectra recorded on-line during HPLC analysis for petunidin 3-glucoside (red), petunidin 3,5-diglucoside (green), and 5-carboxypetunidin 3-*O*-β-glucoside (blue). See **Table 5** for anthocyanidin structures. See Jordheim *et al.*[138] for experimental conditions.

Table 6 Colors and absorption maxima of selected anthocyanidins dissolved in 0.01% (or 0.1%) conc. HCl in MeOH

Anthocyanidin (trivial name)	*Substitution pattern*	*Color*	$\lambda_{UV\text{-}max}$ *(nm)*	$\lambda_{vis\text{-}max}$ *(nm)*	*Reference(s)*
One O-substituent					
6-Hydroxyflavylium	6-OH	Light-yellow		388	152
7-Hydroxyflavylium	7-OH	Yellow		441	152
4′-Hydroxyflavylium	4′-OH	Yellow		453	152
Two O-substituents					
5,7-Dihydroxyflavylium	5,7-diOH	Yellow		461	152
7,4′-Dihydroxyflavylium	7,4′-diOH	Yellow		476	152
3′,4′-Dihydroxyflavylium	3′,4′-diOH	Orange		480	152
3,4′-Dihydroxyflavylium	3,4′-diOH	Orange		484	152
3,7-Dihydroxyflavylium	3,7-diOH	Orange		488	152
Three O-substituents					
3,4′-Dihydroxy-8-methoxyflavylium	3,4′-diOH; 8-OMe	Yellow		467	152
7-*O*-Methylapigeninidin[a]	5,4′-diOH; 7-OMe	Yellow	279	476	153
7,3′-Dihydroxy-4′-methoxyflavylium	7,3′-diOH; 4′-OMe	Yellow		477	152
Apigenidin[a]	5,7,4′-triOH	Orange		486	152
3,7,4′-Trihydroxyflavylium	3,7,4′-triOH	Orange-red		508	152
Four O-substituents					
Carajurin[a]	6,7-diOH; 5,4′-diOMe	Yellow	285	469	154
Carajuron[a]	6,7,4′-triOH; 5-OMe	Yellow	295	475	154
3,8,3′,4′-Tetrahydroxyflavylium	3,8,3′,4′-tetraOH	Orange-red		500	152
Luteolinidin[a]	5,7,3′,4′-tetraOH	Orange-red		502	152
Pelargonidin[a]	3,5,7,4′-tetraOH	Red	270	520	150,151
Fisetinidin	3,7,3′,4′-tetraOH	Red		526	151,152
Five O-substituents					
5-*O*-Methyl-6-hydroxyluteolinidin[a]	6,7,3′,4′-tetraOH; 5-OMe	Orange	302	492	154
Aurantinidin[a]	3,5,6,7,4′-pentaOH	Orange-red	286	499	150
Tricetinidin[a]	5,7,3′,4′,5′-pentaOH	Orange-red	281	513	150
Rosinidin[a]	3,5,4′-triOH; 7,3′-diOMe	Magenta	276	524[b]	155
Peonidin[a]	3,5,7,4′-tetraOH; 3′-OMe	Magenta	277	532[b]	150
7-*O*-Methylcyanidin[a]	3,5,3′,4′-tetraOH; 7-OMe	Magenta	273	533[b]	150
Cyanidin[a]	3,5,7,3′,4′-pentaOH	Magenta	277	535[b]	150,152
Six O-substituents					
Hirsutidin[a]	3,5,4′-triOH; 7,3′,5′-triOMe	Magenta		536	150
Capensinidin[a]	3,7,4′-triOH; 5,3′,5′-triOMe	Magenta	273	538	150
Europinidin[a]	3,5,7,4′-tetraOH; 3′,5′-diOMe	Purple	270	542	150
Malvidin[a]	3,5,7,4′-tetraOH; 3′,5′-diOMe	Purple	275	542	150
Petunidin[a]	3,5,7,4′,5′-pentaOH; 3′-OMe	Purple	276	543	150,151
Pulchellidin[a]	3,7,3′,4′,5′-pentaOH; 5-OMe	Purple	278	543	150
Delphinidin[a]	3,5,7,3′,4′,5′-hexaOH	Purple	277	546	150,151

[a] Naturally occurring.
[b] In the series rosinidin, 7-*O*-methylcyanidin, peonidin, and cyanidin, Toki *et al.*[29] have reported $\lambda_{vis\text{-}max}$ = 530, 532, 537, and 538 nm, respectively.

3.16.2.1.1 3-Deoksyanthocyanidins – lack of 3-hydroxyl on the anthocyanidin C-ring

A hydroxyl substituent in position 3 on the C-ring of the flavylium cation strongly favors shift of the absorption maximum to longer wavelengths (bathochromic shift) (**Figure 4**). This indicates that the 3-deoxyanthocyanidins (**Table 5**), which lack this 3-hydroxyl group, have a large hypsochromic shift (around

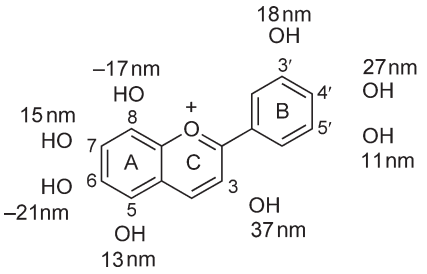


Figure 4 The numbers represent calculated shift differences (nm) between visible λ_{max} values in absorption spectra of anthocyanidins differing at exactly one specific position. For example, the introduction of an OH-group in the 7-position gives on average a bathochromic effect of 15 nm in the absorption spectrum, while an OH-group in the 6-position gives on average a hypsochromic shift of −21 nm.

37 nm) giving yellow, orange, and bright red plant colors. The 3-deoxyanthocyanidins constitute the few anthocyanins of ferns and bryophytes,[156–159] and have been found rarely in a few diverse angiosperm taxa; including some species belonging to Poaceae, *Arrabidaea chica* (Bignoniaceae), and abundantly in New World species of Gesneriaceae (e.g., the ornamental *Sinningia cardinalis)*. In fact, the 3-deoxyanthocyanidins occurred in 18 out of 21 species studied in the subfamily Gesnerioideae (Gesneriaceae) on the American continent, and in none of the 25 cyanic species in the subfamily Cyrtandroideae (Gesneriaceae) of the Old World.[160] The high frequency of 3-deoxyanthocyanidins in the Gesnerioideae has been linked with the pattern of ornithophily in this group (see Section 3.16.6.1). The bright orange-red colors produced by 3-deoxyanthocyanins are effective as bird-attracting colors, and production of these compounds is therefore believed to have evolved separately in the subfamily Gesnerioideae.

In recent years, a series of new 3-deoxyanthocyanidins have been reported (**Table 5**). 7-*O*-Methylapigeninidin, has been isolated in low concentration from grains and leaf sheaths of *Sorghum caudatum* (Poaceae).[153] A similar 3-deoxyanthocyanidin has been detected in grains of *S. bicolor* after incubation with the fungus *Colletotrichum sublineolum*.[161] In addition to plasma desorption mass spectrometry data, bathochromic shift analyses indicated that the structure of the compound was consistent with that of 5-*O*-methylluteolinidin. The spectrum of this phytoalexin, which showed greater fungitoxicity than luteolinidin, has its absorption maximum at 495 nm in pure methanol. Although the synthesis of the deoxyanthocyanidin carajurin, 6,7-dihydroxy-5,4′-dimethoxy-flavylium, isolated from leaves of *A. chica* was published in 1953,[162] the structure of this pigment was considered to be only partially described.[163,164] Later two groups nearly simultaneously confirmed the structure of carajurin – even by presenting a crystal structure.[154,165] The structure of carajurone was revised to be 6,7,4′-trihydroxy-5-methoxy-flavylium.[165] Additionally, the two new 3-deoxyanthocyanidins, 6,7,3′-trihydroxy-5,4′-dimethoxy-flavylium and 6,7,3′,4′-tetrahydroxy-5-methoxy-flavylium were isolated from the leaves,[154,165] which are traditionally used by some indigenous populations of South America for body painting and for dyeing fibers. The 3-deoxyanthocyanidins are relative stable toward pH changes,[166,167] and some 3-deoxyanthocyanidins have recently been demonstrated to be more cytotoxic to cancer cells than their anthocyanidin analogues.[168]

3.16.2.1.2 O-Substituents on the anthocyanidin B-ring

According to **Figure 4**, hydroxyl substituents on the anthocyanidin B-ring give comparable effects on the visible absorption maximum as a 3-OH substituent, though to a lesser extent. $\Delta\lambda_{max}$ for 4′-OH, 3′-OH, and 5′-OH is 27, 18, and 11 nm, respectively. Some representative studies reporting the distribution pattern of anthocyanins in various genera ensuing this correlation between substitution pattern on the anthocyanidin B-ring and flower color follows.

The qualitative and relative quantitative anthocyanin content of petal-like tepals of 17 different tulip (*Tulipa*) species and 25 cultivars have been analyzed as a background for carrying out breeding programs directed in particular toward flower colors.[169] Correlations between colors described by CIELab coordinates and anthocyanin content of individual samples were performed by multivariate analysis. Altogether five anthocyanins were identified as the 3-rutinosides of delphinidin, cyanidin, and pelargonidin, and the 3-[2‴-acetylrutinosides] of

cyanidin and pelargonidin. All tepals classified with hue angles described as 'blue nuances' were from the cultivars. They contained delphinidin 3-rutinoside (3 OH-substituents on the B-ring) as the major anthocyanin, and no or just traces of pelargonidin derivatives. The species and cultivars having 'magenta nuances' showed similar anthocyanin content with increased relative proportions of cyanidin 3-rutinoside (2 OH-substituents on the B-ring) at the expense of delphinidin 3-rutinoside. Orange-colored tepals were to a large extent correlated with high relative proportions of the pelargonidin derivatives (1 OH-substituent on the B-ring). Acetylation of anthocyanins furnished a weak color effect opposite to the blueing effect previously reported for anthocyanins with aromatic acyl groups.[170]

The impact of pigment structure, composition and concentration, pigment to copigment ratio, and pH on colors of *Pelargonium* flowers was investigated as background for any attempt to modify flower color via genetic manipulation.[171] The major factors responsible for color variation were shown to be the *types* and *relative levels* of pigments present. Variations in pH and copigment levels were not found to contribute significantly. Flowers with colors ranging from cream and pink to deep purple, including salmon, orange, and red, were studied. While either flavonols or carotenoids were responsible for cream/yellow coloration, all other colors resulted from anthocyanin mixtures. The major anthocyanins of various *Pelargonium* species and cultivars were identified as the 3,5-diglucosides and 3-glucoside-5-[6-(acetyl)glucosides] of the six common anthocyanidins.

Approximately twenty similar anthocyanidin 3,5-diglucosides with a cinnamic acid (**Table 5**, **Figure 8**) derivative located on the 6-position of the 3-sugar and possible malonyl or acetyl units (**Figure 8**) connected to the 5-sugar, have been isolated from flowers of *Hyacinthus orientalis*.[172–174] A survey of the anthocyanins in the floral organs (perianth, anthers, and ovaries) revealed that the dominant anthocyanin was delphinidin derivatives in four cultivars with blue flowers and cyanidin- or pelargonidin derivatives in cultivars with red or pink flowers.[8] Different patterns of anthocyanins were observed in each floral organ.

Around 35 different anthocyanins have been reported to occur in one or more species in the family Ranunculaceae.[4] Flowers of species in the genera *Delphinium* (blue),[36,175] *Consolida* (blue-violet), and *Aconitum* (purplish-blue) contain similar anthocyanins with polyacyl substitution based on *p*-hydroxybenzoylglucose residues at the 7-hydroxyl of delphinidin, in addition to a simpler glycosyl moiety at the 3-position.[34,176] Red flowers of *Delphinium hybridum* share a similar 3,7-disubstitution pattern based on pelargonidin instead of delphinidin.[177,178]

In *Salvia* and other genera belonging to Labiatae the red, scarlet, and pink-colored flower varieties contained pelargonidin derivatives, the blue ones delphinidin derivatives, while the amethyst- and grape-violet-colored ones were based on cyanidin derivatives.[24,25,179,180]

3.16.2.1.3 *O*-Substituents on the anthocyanidin A-ring – 6-hydroxyanthocyanidins

Regarding the anthocyanidin A-ring, the situation with respect to color effects of hydroxyl groups in the various positions is more intricate. While an OH-substituent in position 7 or 5 induces shifts to longer wavelengths ($\Delta\lambda_{max} = 15$ and 13 nm, respectively), an OH-substituent in position 6 or 8 implies even larger shifts to shorter wavelengths (hypsochromic shifts) with $\Delta\lambda_{max} = -21$ and -17 nm, respectively (**Figure 4**). All natural anthocyanins have $-$OH, $-$OMe, or $-O$-glycoside in their 5- and 7-positions. However, natural anthocyanins with 6-OH have very limited distribution, mainly within the genus *Alstroemeria*. In addition, aurantinidin has been reported to occur in *Impatiens aurantiaca* (Balsaminaceae),[181] however, this report has not been confirmed. The flower color, hue, and color intensity of fresh tepals of 28 Chilean *Alstroemeria* species and 183 interspecific hybrids have been described by parameters of CIELab.[182] Compared with flowers containing exclusively cyanidin 3-glycosides (**Figure 1**), the hues of flowers with 6-hydroxycyanidin 3-glycosides (**Table 5**) were more reddish. The relationship between flower color and anthocyanin content in 45 *Alstroemeria* cultivars showed that the major anthocyanins of outer perianths were cyanidin 3-rutinoside and 6-hydroxycyanidin 3-rutinoside in cultivars with red flowers, 6-hydroxydelphinidin 3-rutinoside in those that were red-purple, and delphinidin 3-rutinoside in purple ones.[183] The same group has also isolated the 3-(glucoside) and 3-[6-(rhamnosyl)glucoside] of 6-hydroxypelargonidin (aurantinidin) from extracts of the orange-red flowers of the *Alstroemeria* cultivars 'Oreiju', 'Mayprista,' and 'Spotty-red.'[184] The position of the 6-hydroxyl of 6-hydroxyanthocyanins has been unambiguously assigned by homo- and heteronuclear NMR techniques.[185]

3.16.2.1.4 Pyranoanthocyanidins

The group of pyranoanthocyanins (**Table 5**) has gained much attention during the last 10 years, mostly because of their color evolution in wine during maturation [186] (see Chapter 3.26). There are also some reports on the identification of pyranoanthocyanins from juices and other processed foodstuff.[187–193] Only the reports of rosacyanins from *R. hybrida* petals,[194,195] 5-carboxypyranopelargonidin 3-glucoside from strawberry fruits and 5-carboxypyranocyanidin 3-glucosides from outer scales of red onion (*Allium cepa*) are from fresh plant material (see **Figure 2** and **Table 5**).[140,196] The additional ring unit of the pyranoanthocyanins linking C-4 and the C-5 hydroxyl group of the flavylium nucleus, influences the various chromophores, and both bathochromic and hypsochromic effects have been observed.

The first pyranoanthocyanidin (rosacyanin B) found to occur in intact plants, was isolated in small amounts together with red cyanidin 3,5-diglucoside from the mauve petals of *R. hybrida* cv. 'M'me Violet.'[194] Rosacyanin B, which contained no sugar, however a galloyl moiety linked to the 5-OH and 4-position of cyanidin, was reported to be very stable in acidic alcoholic solutions. Under neutral or weakly acidic aqueous conditions it was precipitated before forming the colorless hemiketal form. Recently, Fukui *et al.*[195] showed that rosacyanin B in fact was connected in the 3-position to ellagitannins in two pigments named rosacyanin A1 and A2 (**Figure 2**). In comparison to cyanidin ($\lambda_{vis\text{-}max}$ at 531 nm in 0.1% conc. HCI in methanol), rosacyanin A1 and A2 possess bathochromic shifts with corresponding $\lambda_{vis\text{-}max}$ values at 585 nm giving more blue-colored solutions. The authors suggested that these colors were due to horizontal or vertical stacking. However, no nuclear Overhauser effects (NOEs) was observed between signals of the tellimagrandin 1 moiety and the cyanidin nuclei in rosacyanin A1. NOE effects were observed between cyanidin A-8 and B-ring protons (**Figure 2**) supporting a longer distance between the protons of the cyanidin nucleus and tellimagrandin 1 than between A-8 and B-2′/B-6′ of the cyanidin nucleus. Rosacyanin B was not very stable under neutral conditions, but the rosacyanin A's were blue or violet in a wide pH range (pH 1–7) (**Table 7**). Fukui and co-workers have indicated the possibility of preparing a blue rose based on the accumulation of large amounts of rosacyanins in the petals. Similar to the rosacyanins, the Port wine created portisins showed bathochromic shifts ($\lambda_{vis\text{-}max}$ values around 575 nm) compared to the spectra of their mother anthocyanins.[197]

Other types of pyranoanthocyanins created during wine maturation, including vitisins, hydroxyphenyl-pyranoanthocyanins, and vinylflavanol-pyranoanthocyanins, showed hypsochromic shifts compared to the absorption spectra of their mother anthocyanins. This results in more orange coloration. Similar hypsochromic shifts ($\lambda_{vis\text{-}max}$ values around 507 nm in 0.1% conc. HCl in methanol) have also been observed for 5-carboxypyranocyanidin 3-glucosides (vitisin A-type) isolated from acidified, methanolic extracts of the edible scales as well as from the dry outer scales of red onion (*A. cepa*),[196] and for 5-carboxypyranopelargonidin 3-glucoside isolated in small amounts from strawberries (*Fragaria* × *ananassa*) (**Figure 2** and **Table 5**).[140] By comparing UV–visible absorption spectra, 5-carboxypyranopelargonidin 3-glucoside showed in contrast to ordinary

Table 7 Visible absorption maxima of pelargonidin 3-*O*-β-glucopyranoside (Pg3-glc), 5-carboxypyranopelargonidin 3-*O*-β-glucopyranoside (CPg3-glc),[140] and rosacyanin A1[195] at various pH values

pH	*Pg3-glc*[a] λ_{max} *(nm)*	*CPg3-glc*[a] λ_{max} *(nm)*	*Rosacyanin A1*[b] λ_{max} *(nm)*
1	496.5	484.0	567.0
2			565.5
3	502.5	480.0	557.5
4			554.5
5	510.0	490.5	555.0
6	521.5	493.5	557.0
7	540.0	503.5	564.0
8	549.5	533.0	573.0
9	553.0	549.5	

[a] See **Table 5** for structure.
[b] See **Figure 1** for structure.

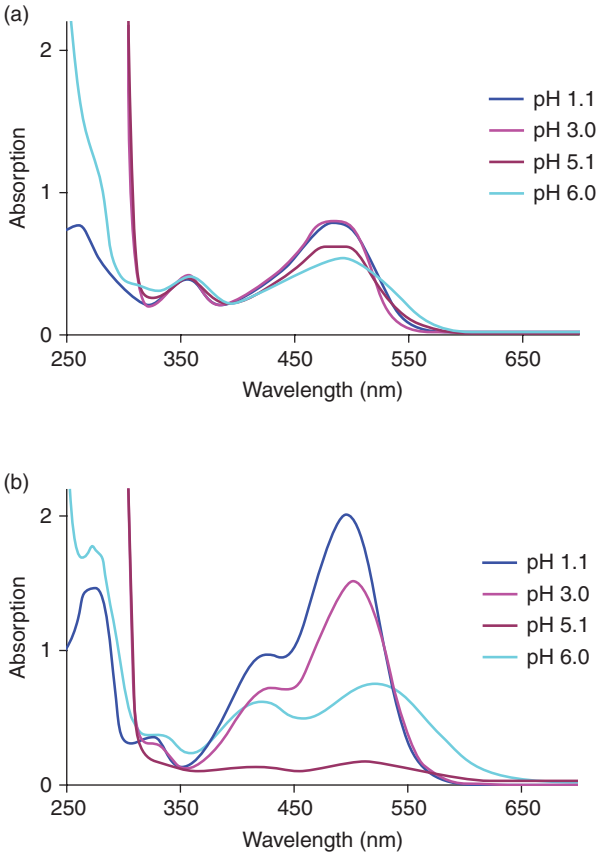


Figure 5 UV–visible absorption spectra of 5-carboxypyranopelargonidin 3-glucoside (0.10 mmol l^{-1}) (a) and pelargonidin 3-glucoside (0.10 mmol l^{-1}) (b) in four buffered solutions with pH ranging from 1.1 to 6.0.[140] While the main pigment of strawberries (b) is nearly colorless at pH 6.1, the 5-carboxypyrano-analogue retains most of its color at this pH.

pelargonidin 3-glucoside, a characteristic local absorption peak around 360 nm, a hypsochromic shift (8 nm) of the visible absorption maximum, and lack of a distinct UV absorption peak around 280 nm. This hypsochromic effect is shown in **Figure 3**. The similarities between the absorption spectra of 5-carboxypyranopelargonidin 3-glucoside in various acidic and neutral buffer solutions implied restricted formation of the unstable colorless equilibrium forms (**Table 7**, **Figure 5**), which are typical for most anthocyanins in weakly acidic solutions.[140,198] This is because the substitution at position 4 of the flavylium cation affects the distribution of the charge throughout the molecule. As a result, positions 2 and 4 become less reactive toward nucleophilic attack (hydration), which increases the stability of this type of anthocyanins in weakly acidic and neutral aqueous solutions.[199] Another consequence of the existence of colored flavylium cations of 5-carboxypyranopelargonidin 3-glucoside in a broad pH range is that the molar absorptivity of this pigment varied little with pH, contrary to similar values obtained for pelargonidin 3-glucoside.[140] At pH 5.1, the ϵ-value of 5-carboxypyranopelargonidin 3-glucoside (6250) was nearly four times the corresponding value of pelargonidin 3-glucoside (1720), which indicated that 5-carboxypyranopelargonidin derivatives may be beneficial as colorants of solutions with pH around 5. Similarly, Vivar-Quintana *et al.*[187] have reported that vitisin-like pigments made the major contribution to the color of wine at pH 4.

3.16.2.2 Anthocyanin Glycosides

Anthocyanins bear glycosyl units in the anthocyanidin 3-, 5-, 7-, 3′-, 4′-, or 5′-position. With exemption of the 3-deoxyanthocyanins, nearly all anthocyanins have a sugar located at the 3-position. The only reported exceptions are the 3′-[2-(galloyl)galactoside] and 3′-[2-(galloyl)-6-(acetyl)galactoside] of delphinidin

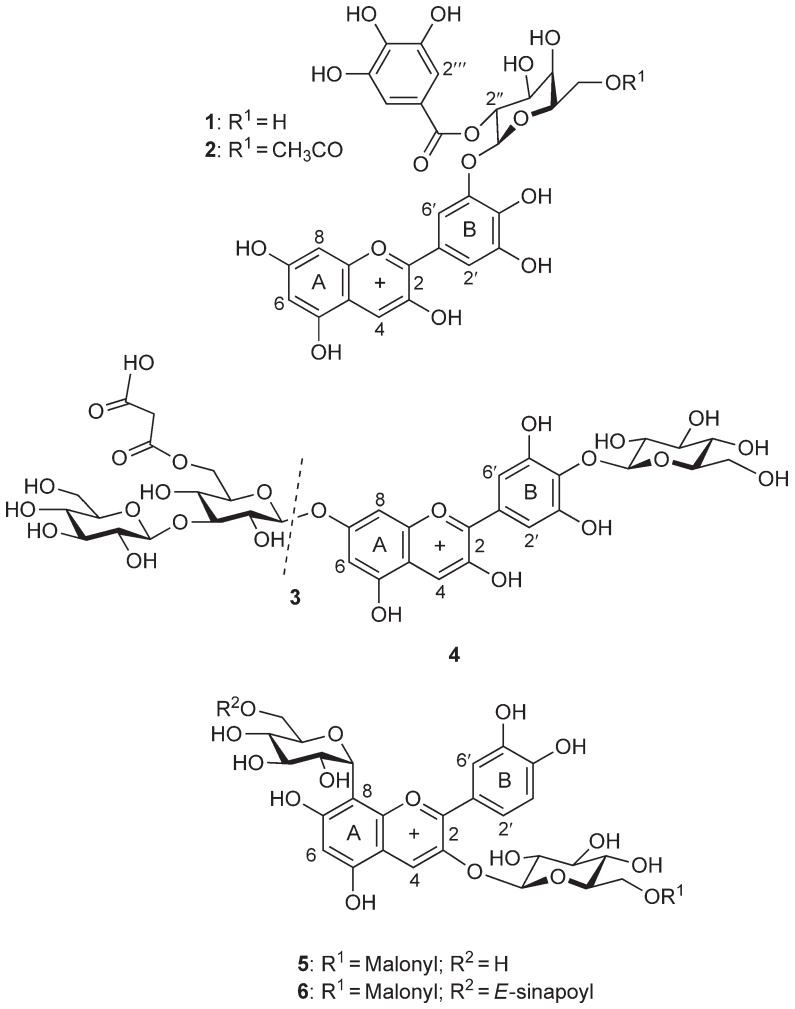


Figure 6 Structures of delphinidin 3′-[2-(galloyl)galactoside] (**1**) and delphinidin 3′-[2-(galloyl)-6-(acetyl)galactoside] (**2**) isolated from blue flowers of the African water lily *Nymphaea caerulea*,[33] cyanidin 4′-glucoside (**3**), and cyanidin 7-[3-(glucosyl)-6-(malonyl)glucoside]-4′-glucoside (**4**) from red onion (*Allium cepa*),[200] and cyanidin 3-*O*-[6-*O*-(malonyl)glucoside]-8-*C*-glucoside (**5**), and cyanidin 3-*O*-[6-*O*-(malonyl)-glucoside]-8-*C*-[6-*O*-(*trans*-sinapoyl)-glucoside] (**6**) isolated from the purple flowers of *Tricyrtis formosana*.[202,203]

(**Figure 6**) isolated from blue flowers of the African water lily *Nymphaea caerulea*,[33] and the 4′-glucoside and 7-[3-(glucosyl)-6-(malonyl)glucoside]-4′-glucoside of cyanidin (**Figure 6**) from red onion (*A. cepa*).[200] Several anthocyanidin 5-monoglycosides and anthocyanidin 7-monoglycosides without sugar in their 3-positions have been reported to occur naturally,[201] however, they may be classified as tentative structures due to limited experimental data for exact identification of the linkage positions of the sugar groups. The sugar(s) is normally connected to the anthocyanidin through an *O*-linkage. However, both cyanidin 3-*O*-[6-*O*-(malonyl)-*β*-glucopyranoside]-8-*C*-*β*-glucopyranoside and cyanidin 3-*O*-[6-*O*-(malonyl)-*β*-glucopyranoside]-8-*C*-[6-*O*-(*trans*-sinapoyl)-*β*-glucopyranoside] (**Figure 6**) have been isolated from the purple flowers of *Tricyrtis formosana* cultivar Fujimusume (Liliaceae) together with four known cyanidin derivatives.[202,203] Eight 3-deoxyanthocyanidin *C*-glycosides have recently been made from their respective flavone 6-*C*-glycosides.[204] Apigeninidin 6,8-di-*C*-*β*-glucoside with two *C*–*C* linkages between the sugar moieties and the aglycone, was found to be far more stable toward acid hydrolysis than pelargonidin 3-*O*-glucoside, which has the common anthocyanidin *C*–*O* linkage between the aglycone and the sugar.

The monosaccharide units found in anthocyanins are represented by glucose, galactose, rhamnose, arabinose, xylose, and glucuronic acid. Glucosyl moieties have been identified in more than 90% of the various anthocyanins, while the most unusual glycosyl moiety in anthocyanins, glucuronosyl, is limited to 11 anthocyanins.[5] Most anthocyanins contain one, two, or three monosaccharide units, however, as much as seven units have been found in ternatin A1 (*Clitoria ternatea*) and cyanodelphin (*D. hybridum*).[28,36] Altogether 287 different anthocyanins contain one or more disaccharides out of 12 different disaccharides.[5] The most common disaccharides, sophorosyl and rutinosyl, have been found in 84 and 76 anthocyanins, respectively. Only 20 different anthocyanins contain a trisaccharide among the 8 trisaccharides, which have been reported.[5] No tetrasaccharide has yet been found in an anthocyanin. See Andersen and Jordheim[4] for distribution of the various anthocyanin glycosyl moieties.

The addition of a sugar residue to the anthocyanidin 3-position produces in general a hypsochromic effect of 10–14 nm in the visible region of the absorption spectra, depending on the solvent and nature of the aglycone. The nature of the glycosyl unit has no effect as long as it is not acylated. The addition of a second sugar residue in a new aglycone position of anthocyanidin 3-glycosides, produces with one exemption, the 5-position, a hypsochromic effect of 8–12 nm in the visible region of the absorption spectra (**Figure 7**). UV–visible spectra have been recorded on-line during HPLC for delphinidin 3-galactoside-3′,5′-diglucoside, delphinidin 3-galactoside-3′-glucoside and cyanidin 3-galactoside-3′-glucoside isolated from bluish white berries of Siberian dogwood, *Cornus alba* 'Sibirica.'[21] When the spectra of delphinidin 3-galactoside-3′-glucoside and cyanidin 3-galactoside-3′-glucoside were compared with analogous spectra of the corresponding anthocyanidin 3-galactosides, hypsochromic shifts (about 8–10 nm), and increased $A_{440}/A_{\text{Vis.max}}$ ratios were observed. The corresponding hypsochromic shift for delphinidin 3-galactoside-3′,5′-diglucoside was 16 nm. The UV–visible data for cyanidin 3-galactoside-3′-glucoside are quite similar to that of cyanidin 3,4′-diglucoside,[200] cyanidin 3,5,3′-triglucoside,[205] and cyanidin 3,7,3′-triglucoside.[206] Thus, whether the glucosyl is located either in the anthocyanidin 3′-, 4′-, or 5′-position, it seems to have the same characteristic hypsochromic shift effect on the UV–visible maxima and diagnostic hyperchromic effect on the absorbances around 440 nm.

Compared with spectra of cyanidin 3-glucoside, cyanidin 4′-glucosides from red onions showed hypsochromic shifts (12 nm) of the visible $\lambda_{\max}$, and hyperchromic effects on wavelengths around 440 nm, similar to pelargonidin 3-glycosides.[200] These spectra characteristics were nearly identical for cyanidin 4′-glucoside, cyanidin 3,4′-diglucoside, cyanidin 3-[3-(glucosyl)-6-(malonyl)glucoside]-4′-glucoside, and cyanidin 7-[3-(glucosyl)-6-(malonyl)glucoside]-4′-glucoside, showing that an extra sugar residue in the 3- or 7-position has really no effect when there also is a sugar residue in the 4′-position. As indicated above, when the sugar moiety is added to the 5-position, the visible $\lambda_{\max}$ shows hypsochromic shifts by only a couple of nanometers, if at all. However, the two most common classes of anthocyanins, the 3- and 3,5-diglucosides, have differences in intensity around 440 nm, which is of diagnostic value (Harborne;[207] **Figure 3**). Thus will the anthocyanidin 3,5-diglycosides have only about 50% of the absorbance measured for anthocyanidin 3-glycosides at this wavelength.

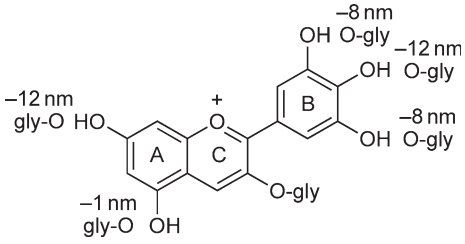


Figure 7 The numbers represent observed shift differences (nm) of visible $\lambda_{\max}$ values in absorption spectra of anthocyanidin glycosides obtained after addition of a second sugar residue in a new aglycone position of the corresponding anthocyanidin 3-glycoside. For example, cyanidin 3,7-diglucoside will have its visible $\lambda_{\max}$ at 12 nm shorter wavelength compared to similar absorption spectrum of cyanidin 3-glucoside.

3.16.2.3 Anthocyanidin Acylglycosides

More than 66% of the reported anthocyanins with well characterized structures have one or more acyl moieties linked to their sugar unit(s).[5] The colors of these pigments in plants are highly affected by the nature, number, and linkage positions of the acyl groups. As many as 319 different anthocyanins have aromatic acylation, which include various hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, sinapic, and 3,5-dihydroxycinnamic acids) and two hydroxybenzoic acids (*p*-hydroxybenzoic and gallic acids) (**Figure 8**). These acyl groups may participate in intramolecular copigmentation of the anthocyanidin nucleus with huge impact on the colors revealed by the plants, especially in flowers (see Section 3.16.2.8). The structural variation between the various anthocyanins found in fruits and vegetative tissues are limited compared to the variation found among flowers. Considering the anthocyanins eaten in a typical European diet (**Tables 2** and **3**), around 55% of the different anthocyanins in vegetables contain aromatic acyl groups, while the corresponding number in fruits is only around 20%. The different distribution of anthocyanins acylated with aromatic acyl groups may reflect the different functions of this type of anthocyanins in fruits and flowers.

Malonic acid, which is identified in 25% of the various anthocyanins, is the most frequently occurring acyl moiety of anthocyanins. This acyl unit constitutes the aliphatic acyl moieties together with acetic, malic, oxalic, succinic, and tartaric acids (**Figure 8**), which have been identified in altogether 205 anthocyanins.[5] Tartaric acid has the most limited distribution among the acylation agents, identified in only four anthocyanins isolated from flowers of *Anemone coronaria* (Ranunculaceae).[35,208] The only anthocyanins found conjugated with sulfate, malvidin 3-glucoside-5-[2-(sulfato)glucoside] and malvidin 3-glucoside-5-[2-(sulfato)-6-(malonyl)glucoside], have been isolated from violet flowers of *Babiana stricta* (Iridaceae).[209] As many as four different acyl groups located at four different glycosyl moieties have been identified in Lobelinin B isolated from flowers of *Lobelia erinus* (Lobeliaceae).[210]

More than 86% of the acylated anthocyanins have one or more acyl moieties located to the 6-position(s) on the monosaccharide(s), while 13 and 11% of the anthocyanins have an acyl group in the 2- and 4-position, respectively. The location of the acyl group to the 3-position is only found in five anthocyanins, either in family Gramineae,[211] Alliaceae,[211,212] Liliaceae,[213] Aceraceae,[214] or Compositae.[215] The location of the acyl group to the sugar 5-position is even more restricted including three different anthocyanins in either family Gramineae or Commelinaceae.[216–218] In these latter cases the sugar is an α-L-arabinofuranosyl. Restricted distribution of

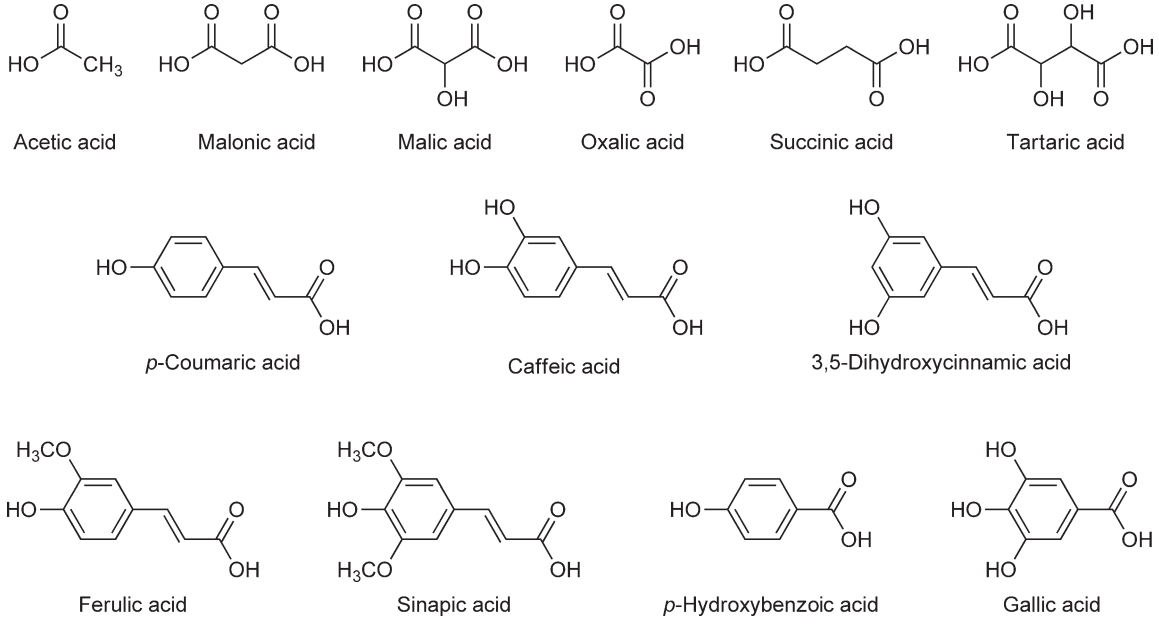


Figure 8 Structures of the aromatic and aliphatic acyl units, which have been found connected to a glycosyl moiety of acylated anthocyanins.

any sugar or acyl unit of anthocyanins, and rare linkage positions, might have chemotaxonomic relevance. Details regarding distribution of acyl and sugar moieties of anthocyanins, including some chemotaxonomic considerations, have been treated elsewhere.[4,219]

3.16.2.4 Anthocyanidin Equilibrium Forms and Stability

Anthocyanins are outstanding in the way each anthocyanidin may be involved in a series of equilibria giving rise to different forms (secondary structures), which exhibit their own properties including color expression.[198,220–228] The secondary structures have been examined/proposed using pH-jump methods, UV–visible, and fluorescence spectroscopy, and NMR spectroscopy. The experimental proofs for accurate structural assignments of other aglycone forms than the flavylium cation, have been incomplete for most anthocyanins. The knowledge about distribution of the individual aglycone secondary structures is limited for most anthocyanins both under *in vitro* and *in vivo* conditions. The color and distribution of the various secondary structures is highly linked to the stability of the various anthocyanin molecules.[143,229]

When a common anthocyanidin mono- or diglycoside is dissolved in water, secondary structures (**Figure 9**) are formed according to different acid–base, hydration and tautomeric reactions. **Figure 9** shows some possible anthocyanin transformations in aqueous solution, however, other reactions may be involved. **Table 8** containing visible λ_{max}-values of the six common anthocyanidin 3-glucosides in buffered aqueous solutions at different pH values recorded after 1 h, reflects the impact of variation of secondary structures in the pH range of 1–11. The flavylium cation (**Figure 9**, **1**) with reddish nuances is the predominant form in relative strong acidic aqueous solutions (below pH 2). Under more mildly acidic pH conditions, the anthocyanin solution is typically only slightly colored. The amount of colored forms drops down to 10% or less for the six common anthocyanidin 3-glucosides based on comparison of their molar absorptivities at visible λ_{max} at pH 5 and 1.[229] This is caused by displacement of the hydration equilibrium of the flavylium cation toward colorless hydroxy adducts (called carbinol bases, pseudobases, hemiacetals, or hemiketals) formed by a nucleophilic reaction with water mainly in the 2-position (**Figure 9**, **8**). The presence of a 4-adduct has also been presented (**Figure 9**, **9**). The hemiketal will to some extent be rapidly converted into its open-chain isomer, *cis*-retrochalcone (**Figure 9**, ***Z*-10**),[230] and finally *trans*-retrochalcone (**Figure 9**, ***E*-10**), which also are nearly colorless forms. For malvidin 3,5-diglucoside the ratio between the hemiketal and *cis*-retrochalcone forms is 4:1 at room temperature in weakly acidic aqueous solutions. A further pH increase to 6 leads to uncharged tautomeric quinonoidal bases (**Figure 9**, **2–4**) (anhydrobases) with purple colors derived from the flavylium cation by deprotonation, and finally to anionic structures with bluish nuances (**Figure 9**, **5–7**).

Color stability of nonacylated anthocyanins has been found to vary tremendously in aqueous solutions depending on pH.[229] Although initially detected after 1 h in aqueous solutions, no color was observed for instance for malvidin 3-glucoside after one day storage at pH 6 and 6.5. Opening of the pyrylium ring and chalcone formation have been postulated as the first degradation step of anthocyanins;[231,232] however, hydrolysis of the glycosidic moiety and aglycon formation has also been proposed as the initial reaction.[233] In a recent study of heat-treated elderberry and strawberry pigment isolates, the presence of chalcone glycosides and the absence of aglycones at pH 3.5 demonstrated pH-dependent degradation pathways of the anthocyanins.[234] Supposedly, the first step of thermal degradation at pH 3.5 was not anthocyanin deglycosylation, but opening of the pyrylium ring and chalcone glycoside formation. Recently, the hemiketal forms of the 3-glucosides of delphinidin, petunidin, and malvidin and cyanidin 3-galactoside dissolved in deuterated methanolic solutions were characterized as two epimeric 2-hydroxy-hemiketals on the basis of assignments of both proton and carbon NMR signals together with chemical shift considerations.[198] No 4-hydroxy-hemiacetal form was detected for any of the pigments. For each anthocyanin dissolved in deuterated methanol, the equilibrium between each of the two epimeric hemiketals and the corresponding flavylium cation was confirmed by the observed positive exchange cross-peaks in the 2D ^{1}H NOESY spectra. The molar proportions of the flavylium cation and the two hemiketal forms of the four pigments in deuterated methanol were very similar (70:30) for all pigments, even during storage for weeks. No other secondary structures were observed in this study. The reason for the stability of the anthocyanin pigments in the NMR solvent (deuterated methanol) might be the lack of conversion of hemiketals into chalcone forms. The same supposed mechanism might be the reason for high color stability of even simple anthocyanidin mono- and disaccharides under *in vivo* conditions in plants.

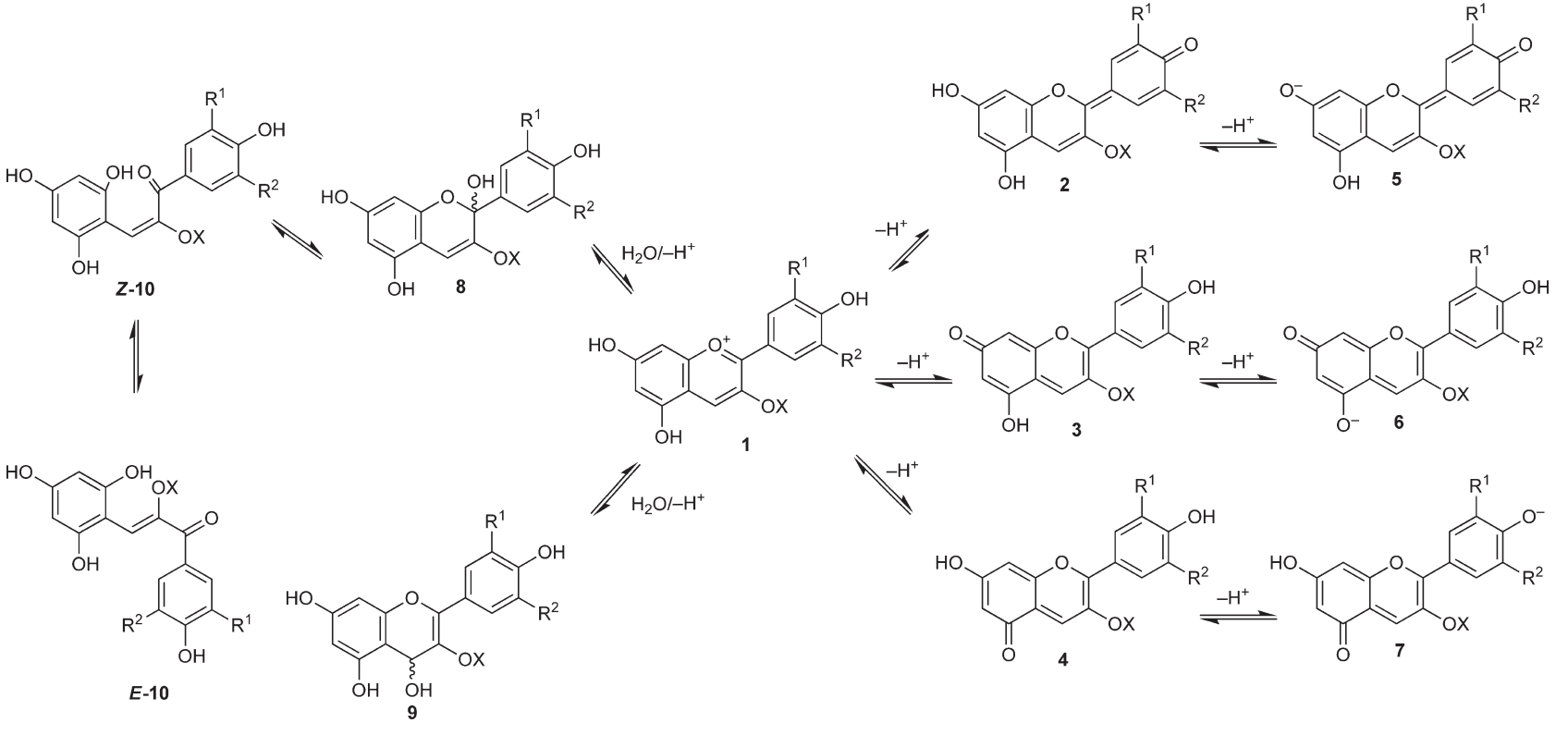


Figure 9 The scheme shows some possible anthocyanin transformations in aqueous solution. X = glycoside, R^1 and R^2 can be hydroxyl and/or methoxyl groups, depending on the type of aglycone. Other transformations may be involved.

Table 8 Visible λ_{max} values (nm) for chloride salts of the six common anthocyanidin[a] 3-glucosides (1.0×10^{-4} mol l^{-1})

pH	*Pg3-glc*	*Cy3-glc*	*Pn3-glc*	*Dp3-glc*	*Pt3-glc*	*Mv3-glc*
1.0	498	510	510	514	515	517
2.4	501	512	516	521	521	525
3.1	504	517	518	525	525	528
4.0	507	520	522	528	529	533
5.0	515	523	527	530	531	535
6.0	519	528	532	558	565	537
6.5	525	539	537	567	569	559
7.0	540	554	554	576	584	576
7.3	547	562	568	574	589	586
7.7	551	571	571	577	590	593
8.1	553	570	574	574	588	594
8.6	553	539	571	542	543	595
9.0	555	540	573	547	542	596
9.5	554	542	573	552	543	597
9.8	553	541	573	546	598	
10.6	554	569	575	595		
11.5	588					

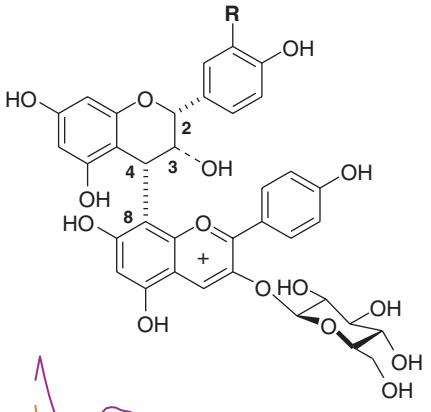
[a] See **Table 5** for structures.
One hour after dissolution in buffered aqueous solutions at various pH values in room temperature.[229]

The pH-dependent reaction from flavylium cation toward colorless hemiketals in slightly acidic aqueous solutions is affected by the type, position, and number of substituent groups attached to the aglycone.[139,235] When the substituent groups are long enough to adopt a folded conformation over the pyrylium ring of the anthocyanidin, the reactive sites (C-2 and C-4) may be protected against nucleophilic water attack, thus favoring the existence of the colored forms. When a covalently linked anthocyanin–flavone *C*-glycoside isolated from purple leaves of *Oxalis triangularis* (Oxalidaceae) dissolved in deuterated methanol and trifluoroacetic acid (95:5) was observed by NMR 45 min after sample preparation, the pigment occurred mainly as flavylium cation (38%) and two equilibrium forms assigned to be quinonoidal bases (54%).[236] More simple anthocyanins are normally considered to be on the flavylium cation form in this acidified deuterated methanolic solvent.[198] The NMR results indicated the presence of vertical π–π stacking between the B-ring of the flavone unit and the A-ring of each of the two quinonoidal bases.[236] It was not possible to discriminate between inter- or intramolecular association mechanisms. Only minor amounts of the two hemiketal forms were present. After five days of storage at 27 °C, the hemiketals (39%) and flavylium cation (38%) constituted the main forms of the pigment. More examples related to the effect of copigmentation on secondary anthocyanidin structures are given in Section 3.16.2.8.

The deep-red color of the Dragon's blood is a natural resin obtained from *Dracaena draco* and *D. cinnabaris* (Dracaenaceae).[237] The resin is known to appear in injured parts of the tree and has been used over the centuries for medicinal and artistic purposes. The compound 7,4′-dihydroxy-5-methoxyflavylium (dracoflavylium) was identified as the major red colorant of this resin. It was concluded that the red color was due to a stable quinonoidal base, which was the major species at pH 4–7. As for the *Oxalis* pigment described above, here we have a second example where the quinonoidal form of the pigment is the major species under slightly acidic conditions. In this latter case the methoxyl group in the 5-position is most probably of significant importance for stabilization of the quinonoidal forms. Similar to the other flavylium compounds, 7,4′-dihydroxy-5--methoxyflavylium was involved in a complex network of chemical reactions in which the different forms can be reversibly interconverted by changing the pH.

3.16.2.5 Flavanol-Anthocyanidin Heterodimers – 'Blueing Effect'

Most reported anthocyanins are monomeric in nature, however, more recently new types of flavonoids consisting of an anthocyanidin moiety covalently linked to another flavonoid unit, have been reported. Anthocyanins resulting from direct condensation between an anthocyanidin unit and a flavanol have been



Compound	On-line HPLC (diode array detection)	
	$\lambda_{Vis\text{-}max}$ (nm)	$A_{440}/A_{Vis\text{-}max}$ (%)
1	518, 438	81
2	518, 433	70
3	516, 434	69
4	520, 432	68
Pg 3-glc	504	43

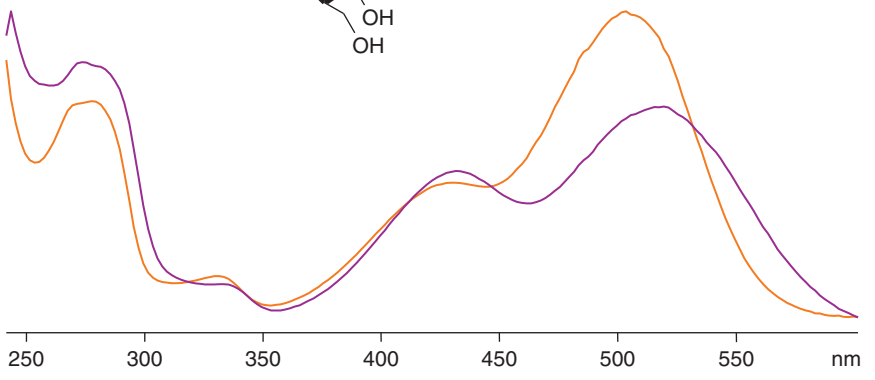


Figure 10 UV–Vis spectroscopy data recorded on-line during HPLC of catechin(4$\alpha \rightarrow$8)pelargonidin 3-glucoside (R = OH), **1**, epicatechin(4$\alpha \rightarrow$8)pelargonidin 3-glucoside (R = OH), **2**, afzelechin(4$\alpha \rightarrow$8)pelargonidin 3-glucoside (R = H), **3**, epiafzelechin(4$\alpha \rightarrow$8)pelargonidin 3-glucoside (R = H), **4**, and pelargonidin 3-glucoside from strawberries.[239] The UV–Vis spectra display epiafzelechin(4$\alpha \rightarrow$8)pelargonidin 3-glucoside (**4**) (purple) and pelargonidin 3-glucoside (orange). The colorless flavan-3-ol derivatives in the dimers provide a substantial bathochromic copigment effect on the anthocyanin.

assumed to be formed exclusively during storage and processing in plant-derived foods including wines.[238] However, this type of pigments seems also to appear naturally, although in small quantities, in extracts of unprocessed plants. In extracts of fresh strawberries four purple-colored pigments (**Figure 10**) were characterized by spectroscopic methods to be catechin (4$\alpha \rightarrow$8) pelargonidin 3-glucoside (**1**), epicatechin (4$\alpha \rightarrow$8) pelargonidin 3-glucoside (**2**), afzelechin (4$\alpha \rightarrow$8) pelargonidin 3-glucoside (**3**), epiafzelechin (4$\alpha \rightarrow$8) pelargonidin 3-glucoside (**4**).[239] The stereochemistry at the 3- and 4-positions of the flavan-3-ols was elucidated after assumption of the *R*-configuration at C-2. Because of rotational hindrance around the linkage between C-8 of the anthocyanidin moiety and C-4 of the flavanol, conformational isomers (two rotamers) of each heterodimer were identified in the NMR solvent.

The UV–visible spectra of the flavanol–anthocyanin heterodimers recorded on-line during HPLC analysis showed two visible absorption maxima at 516–520 nm and 432–438 nm (**Figure 10**). The purple colors of the heterodimers were different from the scarlet color of pelargonidin 3-glucoside, which constitute the monomeric anthocyanin unit of these heterodimers. For comparison, when a hydroxyl group is located at the 8-position of the anthocyanin as in 8-hydroxyanthocyanidin, a shift of the visible absorption maximum to lower wavelengths (red shift) is experienced (see Section 3.16.2.1). However, when a flavanol is linked to the 8-position of the anthocyanidin, as in the flavanol–anthocyanin heterodimers, a shift to longer wavelengths (12–16 nm) (**Figure 10**) is observed. The flavanol–anthocyanin heterodimers represent other types of structures thus enhancing bluish colors in plants.

The same four heterodimers as reported by Fossen *et al.*[239] together with afzelechin-(4 → 8)-pelargonidin 3-rutinoside were tentatively identified in extracts of the strawberry cultivar 'Camarosa.'[240] Similarly, (epi)catechin-cyanidin 3,5-diglucoside has been identified in the extract of purple corn, (epi)catechin-peonidin 3-glucoside and (epi)catechin-malvidin 3-glucoside in extract of grape skin, while (epi)catechin-cyanidin

3-glucoside, (epi)gallocatechin-delphinidin and (epi)catechin linked to cyanidin, petunidin, and peonidin have been reported to occur in extracts of various beans (*Phaseolus coccineus*, *P. coccineus*, and *P. vulgaris*).[49,149,240] Putative flavanol–anthocyanin condensation products have also been detected in a concentrate from black currant (*Ribes nigrum*) fruits and in extracts of the fig (*Ficus carica*).[241,242]

3.16.2.6 Anthocyanin-Flavonoid Conjugates – 'Blueing Effect'

In a few cases anthocyanins have been found to be covalently linked to another flavonoid unit, either flavone- or flavonol-glycoside, through a disubstituted dicarboxylic acid (**Table 9**). When the visible maxima in the UV–visible spectra of the anthocyanin–flavone/flavonol conjugates are compared with similar spectra of the same monomeric anthocyanins, bathochromic shifts (11–28 nm) are observed in all cases (**Table 9**). These bathochromic effects reveal intramolecular (and/or intermolecular) association between the anthocyanidin and flavonol units, which produce 'more bluish' colors than expressed by their monomeric counterparts. It is interesting to note that this effect is pronounced regardless of anthocyanidin type (delphinidin, cyanidin, or malvidin). The various conjugates, which have been reported are explained below.

Two anthocyanin–flavone *O*-glycoside conjugates have been isolated from blue-violet flowers of *Eichhornia crassipes* (Pontederiaceae) by Toki *et al.*[243,244] The major *Eichhornia* anthocyanin A has apigenin 7-glucoside attached with an ester bond to one end of malonic acid, and delphinidin 3-gentiobioside linked with a similar bond to the other end. The minor *Eichhornia* anthocyanin B has a similar structure with apigenin 7-glucoside replaced with luteolin 7-glucoside. The three-dimensional structure of these pigments were suggested from the observation of negative Cotton effects at λ_{max} (535 and 547 nm, respectively). The chromophore (delphinidin) and the copigment (flavone) occupy a folding conformation as a binary complex.[243,244] The existence of intramolecular hydrophobic interactions between the chromophoric skeleton and the flavone group was indicated by reduction in the hydration constant when compared with the parent delphinidin 3-glycoside.[246] *Eichhornia* anthocyanin A exhibited remarkable color stability in aqueous solution at mildly acidic pH values.

Recently, a covalently linked anthocyanin–flavone *C*-glycoside has been isolated from purple leaves of *O. triangularis* (Oxalidaceae).[236] This pigment has an apigenin 6-*C*-sophoroside molecule attached with an ester bond to one end of malonic acid, and malvidin 3-*O*-rutinoside-5-*O*-glucoside linked to the other end (**Table 9**). See more about the distribution of the various equilibrium forms of this pigment in Section 3.16.2.4. The existence of other anthocyanin–flavone conjugates has been indicated in salvia, *Salvia patens*,[24] and the blue flower color of garden lupine Russel hybrids (*Lupinus* sp.) has been proposed to be due to copigmentation of the malonylated glucosides of delphinidin and apigenin – possibly linked *in vivo* covalently through a common malonic acid residue.[31]

Two anthocyanin–flavonol conjugates have been isolated from the pale-purple flowers of chive (*Allium schoenoprasum*).[211] These pigments, which constituted more than 65% of the total anthocyanin content, were based on either cyanidin 3-glucoside or cyanidin 3-[3-(acetyl)glucoside] esterified to one end of malonic acid, and kaempferol 3-[2-(glucosyl)glucoside]-7-glucosiduronic acid connected to the other end. The chemical shifts of the anthocyanidin H-4 in the two complexes were 0.3 ppm upfield compared to the same shifts of the monomeric anthocyanins without connection to a flavonol moiety, indicating intramolecular association between the anthocyanidin and flavonol moieties. Two similar anthocyanin–flavonol pigments have been isolated from the blue *Agapanthus* flowers (Agapanthaceae).[245] In these structures the succinate was involved instead of malonate to connect delphinidin 3-[6-(*p*-coumaloyl)glucoside]-7-glucoside to either kaempferol 3,4′-di-glucoside-7-xyloside or kaempferol 3,7,4′-tri-glucoside. An anthocyanin–flavonol conjugate has also been suggested for orchicyanin I, which has been isolated from several orchids.[247] This pigment has been given a hypothetical structure, cyanidin oxalyl-3,5-diglucoside-kaempferol 7-glucoside.[248]

3.16.2.7 Metalloanthocyanins – 'Blueing Effect'

In a few extraordinary cases anthocyanins and flavones/flavonols in complexation with metal ions have been reported to be efficient in producing blue flower colors (**Table 10**). Previous investigations of most of these complexes (commelinin, protocyanin, protodelphin, and hydrangea blue pigment) have recently been reviewed by Takeda[253], while Ellestad[258] similarly has reviewed experimental results obtained over the past 30 years for

Table 9 Anthocyanin–flavonoid conjugates reported from plants

Anthocyanin-flavonoid conjugate	*λ_{max} in the visible region*[a]	*Plant*	*Reference*
(6″-(delphinidin 3-[6″-(glucosyl)glucoside])) (6″-(apigenin 7-glucoside))malonate[M]	548 (538) nm[b]	*Eichhornia crassipes* (water hyacinth) blue-violet flowers	243
(6″-(delphinidin 3-[6″-(glucosyl)glucoside])) (6″-(luteolin 7-glucoside))malonate[m]	548 (537) nm[b]		244
(4′^{V}-(malvidin 3-[6″-(rhamnosyl)glucoside]-5-glucoside)) (6‴-(apigenin 6-*C*-[2″-(glucosyl)glucoside]))malonate[m]	558 (530) nm[c]	*Oxalis triangularis* (purple shamrock) purple leaves	236
(6″-(cyanidin 3-[3″-(acetyl)glucoside])) (4′^{V}-(kaempferol 3-[2″-(glucosyl)glucoside]-7-glucosiduronic acid))malonate[M]	540 (522) nm[c]	*Allium schoenoprasum* (chive) pale-purple flowers	236
(6″-(cyanidin 3-glucoside)) (4′^{V}-(kaempferol 3-[2-(glucosyl)glucoside]-7-glucosiduronic acid))malonate[m]	538 (522) nm[b]		
(6‴-(delphinidin 3-[6″-(*p*-coumaroyl)glucoside]-7-glucoside)) (6′^{V}-(kaempferol 3,4′-diglucoside-7-xyloside))succinate[M]	548 (526) nm[c]	*Agapanthus praecox* sp. *orientalis* (African lily) blue flowers	245
(6‴-(delphinidin 3-[6″-(*p*-coumaroyl)glucoside]-7-glucoside)) (6′^{V}-(kaempferol 3,7,4′-triglucoside))succinate[M]	548 (526) nm[c]		

[a] Values in brackets correspond to data recorded for the monomeric anthocyanin.
[b] In 0.1% HCl–MeOH.
[c] In on-line HPLC solvent.
[M] Major.
[m] Minor.

Table 10 Metalloanthocyanins from plants producing blue flower colors

Anthocyanin	*Composition*	*Color expression*	*Plant*	*Reference(s)*
Commelinin	Delphinidin 3-[6-(*p*-coumaryl)glucoside]-5-[6-(malonyl)glucoside] (malonylawobanin) $\times$ 6, 7-methoxyapigenin 6-*C*-,4′-*O*-diglucoside (flavocommelin) $\times$ 6, $Mg^{2+} \times 2$.	Self-association between the anthocyanin moieties. The blue flower-color development and the stability of the color were explained by metal complexation of the anthocyanin and intermolecular hydrophobic association.	*Commelina communis* (dayflower)	249–251
Protodelphin	Malonylawobanin $\times$ 6, apigenin 7,4′-diglucoside $\times$ 6, $Mg^{2+} \times 2$.	Restricted chiral and structural recognition controlled the entire self-assembly of the metalloanthocyanin and was responsible for the blue flower color.	*Salvia patens* (blue salvia)	24,252,253
Protocyanin	Cyanidin 3-[6-(succinyl)glucoside]-5-glucoside] $\times$ 6, apigenin 7-glucuronide-4′-[6-(malonyl)glucoside] $\times$ 6, Fe^{2+}, Mg^{2+}, Ca^{2+}.	The blue color is caused by LMCT interaction between succinylcyanin and Fe^{3+}.	*Centaurea cyanus* (cornflower)	253,254,255
Meconopsis metalloanthocyanin complex	Cyanidin derivative, two or more equivalents of kaempferol derivatives, 1/6 equivalents of Fe^{3+} and excess of Mg^{2+}.	Ferric ions essential for blue color development by chelating the *ortho*-dihydroxy group of the cyanidin B-ring. The flavonols might stack on both sides of cyanidin. Final composition is not known.	*Meconopsis grandis*, (Himalayan blue poppy)	256
Hydrangea metalloanthocyanin complex	Delphinidin 3-glucoside, caffeoylquinic acid, or *p*-coumaroylquinic acid, Al^{3+}.	Al^{3+} complexes with the *ortho*-dihydroxy group of the delphinidin B-ring and the carboxyl and α-hydroxyl groups of the quinic acid moiety. Final composition is not known.	*Hydrangea macrophylla* (hydrangea) blue sepals	170,253,257

elucidating the self-assembly of the same metalloanthocyanins. This latter review focuses also on the role of the pendant sugars in directing the observed stacking chirality, and end up with speculation on the biological significance of the stacking chirality of the pigments in flower petals and its importance as to the possibility that insects might be sensitive to reflected circularly polarized light from flowers. A short description of the various metalloanthocyanins, which have been reported is as follows.

An anthocyanin with hydroxyl groups in *ortho*-position to each other on the B-ring of the anthocyanidin forms a metal complex with aluminum ion (Al^{3+}), leading to bathochromic and hyperchromic shift effects in the absorption spectrum. An interesting example here is the flower color of *Hydrangea macrophylla*. When grown in neutral to basic soils, hydrangea has its sepals colored red by the anthocyanin, delphinidin-3-glucoside. However, these sepals can become blue when the shrubs are grown in acidic soil. Here the Al^{3+} ion is soluble and can be absorbed and transported to the sepals, where Al^{3+} complexes with the anthocyanidin resulting in the blue color.[257,259,260] Under alkaline conditions the Al^{3+} ion becomes insoluble and the sepals turn out to be red. Sepal color of hydrangeas is, however, not determined by the acidity of the soil alone. It is also affected by copigments, amounts of Al^{3+}, and vacuolar pH.[253,257,260,261] The metal-complex pigment in hydrangeas is suggested to consist of delphinidin 3-glucoside, copigments (5-*O*-caffeoylquinic acid, and/or 5-*O*-*p*-coumaroylquinic acid), and sufficient Al^{3+} in an aqueous solution around pH 4.0, although neither its structure nor composition is completely known. Complexation of Al^{3+} with various synthetic and natural anthocyanins has been investigated in aqueous solutions within the pH range 2–5.[262,263] Shown by UV–visible spectroscopic data the complexes involved not only the colored forms, but also colorless forms of the pigments. ^{1}H NMR analysis confirmed conversion of anthocyanins (dissolved in deuterated methanol) from the red flavylium form into deep-purple quinonoidal forms upon coordination with Al^{3+}.[262] From relaxation kinetics measurements (pH jump), complexation constants of Al^{3+} and several synthetic and natural anthocyanins have been calculated.[262–264]

Commelinin from blue flowers of *Commelina communis* has been found to consist of six molecules of delphinidin 3-[6-(*p*-coumaryl)glucoside]-5-[6-(malonyl)glucoside] (malonylawobanin) copigmented with six flavone (flavocommelin) molecules complexed with two Mg^{2+} ions (**Figure 11**).[251] Self-association was shown to exist between the anthocyanidin moieties. The blue flower-color development and the stability of the color were explained by

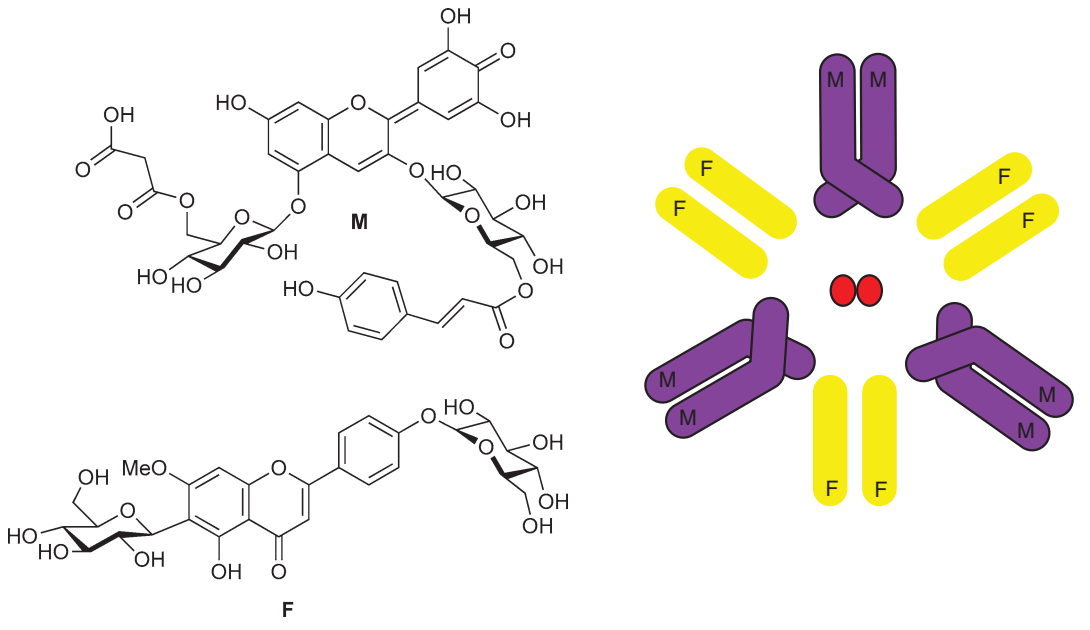


Figure 11 The metalloanthocyanin commelinin responsible for the blue coloration of flowers of *Commelina communis*.[251] Commelinin consists of six molecules of delphinidin 3-[6-(*p*-coumaryl)glucoside]-5-[6-(malonyl)glucoside] (malonylawobanin) (**M**, purple) copigmented with six flavone molecules (**F**, yellow) complexed with two magnesium atoms (red).

metal complexation of the anthocyanidin and intermolecular hydrophobic association. The octaacetate derivative of the flavone part of this molecule has been determined by X-ray diffraction,[265] and in the crystal the flavone molecules were arranged parallel to each other according to the periodicity of the crystal lattice. Intermolecular stacking of the flavone skeletons was, however, not observed, and the hydrophilicity of the glucose moieties was suggested as an important factor governing the self-association of the anthocyanidin moieties.

The structure of protocyanin from cornflower, *Centaurea cyanus*, was suggested to be similar to that of commelinin, composing of six molecules each of apigenin 7-glucuronide-4′-[6-(malonyl)glucoside] and succinylcyanin, complexed with Mg^{2+} and Fe^{3+} ions.[254,255,266,267] It has been proposed that the molecular stacking of the aromatic units in protocyanin prevent hydration of the anthocyanidin nucleus.[268] The blue color of protocyanin was found to be caused by ligand to metal charge transfer (LMCT) interaction between succinylcyanin and Fe^{3+}, which is a different mechanism from that known to operate for commelinin. Recently it has been shown that the additional presence of two Ca^{2+} ions was essential for the formation of protocyanin.[269,270]

Protodelphin, which also is similar to commelinin, has been isolated from flowers of *S. patens*.[24,252] Protodelphin includes six molecules malonylawobanin, two Mg^{2+} ions, and six molecules of another flavone than commelinin, apigenin 7,4′-diglucosides. Takeda *et al.*[24] resynthesized the natural blue pigment *in vitro* by adding the three components together. Mg^{2+} could be substituted *in vitro* by other divalent metal cations (e.g., Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+}).

The blue petal color of the Himalayan blue poppy, *Meconopsis grandis*, has been proposed to be based on a new type of anthocyanin complex containing a cyanidin derivative, two or more equivalents of flavonol (kaempferol) derivatives, 1/6 equivalents of Fe^{3+} and excess of Mg^{2+} ions.[256] The ferric ions chelated the *ortho*-dihydroxy group of the B-ring of the anthocyanidin and were essential for blue color development. The flavonols might be stacked on both sides of cyanidin and stabilized by a copigmentation effect. The experiments indicated that the malonyl group of the anthocyanin was not required for blue color development. The full structure of this complex has not yet been solved, however, it was suggested that it may represent a new type of metal pigment complex similar to that responsible for the blue flower color of hydrangea.[256]

Finally with respect to reports on metalloanthocyanins, the blue petals of *Phacelia campanularia* may be developed by intra- and inter-molecular stackings and the existence of a very small amount of metal ions.[19] The involvement of copigments seems not to be vital in this complex.

3.16.2.8 Copigmentation 'Blueing Effect'

Copigmentation of anthocyanins is one of the most important factors for producing anthocyanin coloration in plants. In this chapter the term *copigment* is used broadly to cover any molecule influencing the anthocyanin chromophore, including self-association of several anthocyanidin nucleus. The exact mechanisms for copigmentation of anthocyanins are poorly understood, as indicated with some examples below. Several models for copigmentation of anthocyanins have been proposed, however, their complex nature demand improved experimental basis in most cases. It is difficult to separate between intra- and intermolecular association (including self-association phenomenon), and the exact orientation of the copigment in relation to the anthocyanin chromophore in the associated complexes is only rarely measured experimentally. In **Figure 12** we have sketched the main associations of the various models, which have been proposed for copigmentation between nonacylated anthocyanins, monoacylated anthocyanins, di-, and polyacylated anthocyanins as well between anthocyanins and other aromatic molecules (intermolecular copigmentation). Copigmentation complexes involving metal ions have been described in Section 3.16.2.7. Structural elements of anthocyanins described in Sections 3.16.2.3–3.16.2.6 have, of course, relevance for the copigmentation phenomenon. The research carried out on copigmentation of anthocyanins by the groups of Professors Tadeo Kondo, Kumi Yoshida, and late Toshio Goto at Nagoya University, Japan has really been outstanding.

3.16.2.8.1 Nature of copigmentation of anthocyanins

The colorless and weakly colored hemiketal and chalcone forms are the prevalent forms of most nonacylated and monoacylated anthocyanins in aqueous solutions in the pH range 2–6. Since this also includes the pH range of most plant vacuoles, plants should expose, based on this fact alone, rather faint anthocyanin coloration in many situations in which this certainly is not the case. Therefore, in plants the colored forms of these

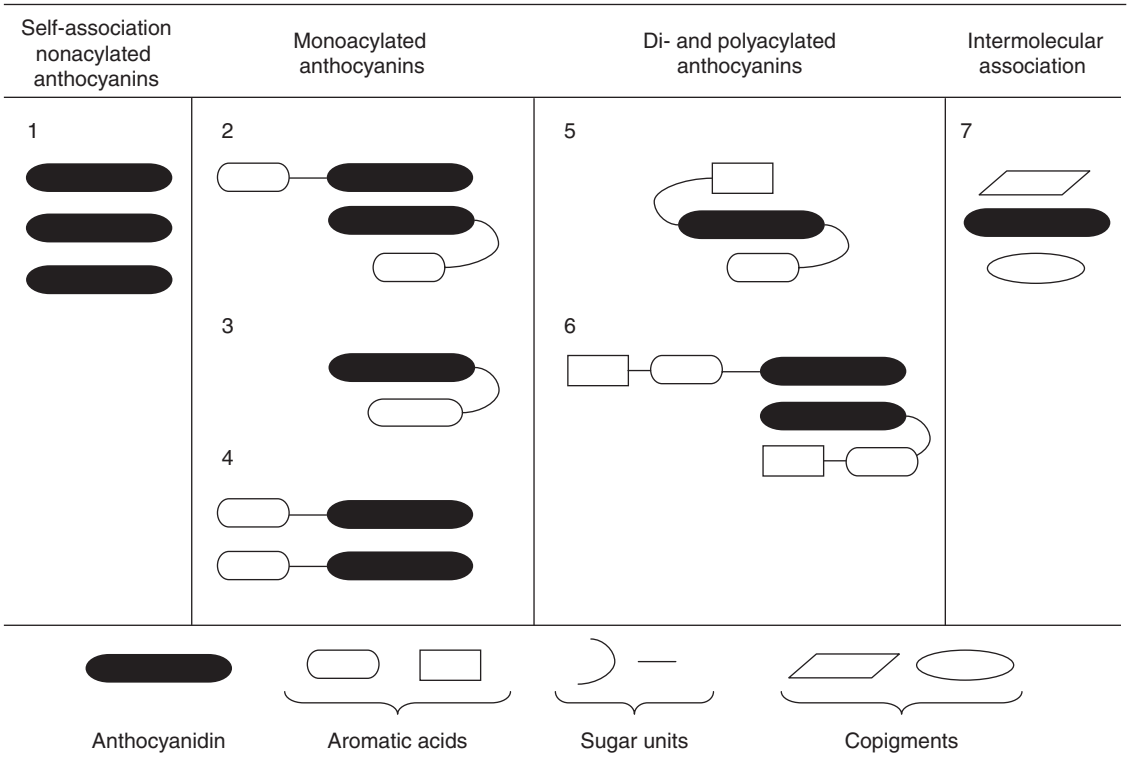


Figure 12 Model sketches showing the main molecular interactions, which have been proposed for copigmentation between nonacylated anthocyanins (self-association) (1), monoacylated anthocyanins (2–4), di- and polyacylated anthocyanins (5–6), and between anthocyanins and other aromatic molecules (intermolecular copigmentation) (7). Both intra- and intermolecular associations as well as self-association contribute to the models presented for monoacylated and di- and polyacylated anthocyanins, respectively.

anthocyanins (flavylium cation and/or quinonoidal bases) must be stabilized to some extent in the cells. When it comes to anthocyanins, which are diacylated or polyacylated with aromatic acids, it has been shown that these do not readily undergo loss of color even at pH > 5.[28,30,271] This suggested a specific role for the aromatic acyl units in stabilization of this type of anthocyanins.

Many anthocyanins are indeed proposed or found to be associated noncovalently with auxiliary molecules (copigments), which both modify their color expression and increase their stability. The copigmentation phenomenon is observed as a bathochromic shift (blueing effect) since the absorption wavelengths around visible λ_{max} are shifted to longer wavelengths compared to similar absorptions of the anthocyanin without copigment. In most cases the color is also intensified (hyperchromic effect). The magnitude of the copigmentation effects has been shown to be influenced by the nature of the anthocyanidin and the copigment, the concentration of the anthocyanin, the copigment:anthocyanin molar ratio, as well as pH and temperature.[147,272–276] Organic acids like benzoic and cinnamic acids, other flavonoid types and anthocyanins themselves, alkaloids, primary metabolites like polysaccharides, peptides and nucleotides, and metals, have all been found capable of inducing copigmentation effects.[277–279] According to Asen[280] the anthocyanin concentration requires to be above 3.5×10^{-5} mol l^{-1} before copigmentation reactions are possible, however, the significance of the concentration depends most probably on the nature of the copigment–anthocyanin complex. The copigmentation complexes are disrupted by dilution, which can be used to distinguish copigmentation phenomenon. Copigmentation of malvidin 3,5-diglucoside appears to be an exothermic process with unfavorable entropy change in the case of cinnamic acids, chlorogenic acid, and (+)-catechin, and a temperature increase will thus favor dissociation of these copigmentation complexes resulting in loss of color.[275,281] At relative low pH values, where the flavylium cation dominates, copigmentation reactions are normally weaker than at pH values where also the quinonoidal equilibrium forms exist.[282]

3.16.2.8.2 Proposed mechanisms

Although strong attractive interactions between π-systems have been known for almost a century, they still do not have a clear explanation.[283] They control such diverse phenomena as the vertical base–base interactions which stabilize the double helical structure of DNA, the tertiary structures of proteins, complexation in porphyrin aggregations, and so forth, and in our case most probably copigmentation of anthocyanins. Several theories have been proposed as mechanisms for copigmentation of anthocyanins. The theory of horizontal stacking, which is based on hydrogen bonding of the hydroxyl and carbonyl groups on the aromatic nuclei (and sugar moieties),[272,282,284] has especially been used to describe intermolecular interactions. In more recent papers this theory has generally been replaced by the proposal of vertical stacking between the anthocyanidin nucleus and copigment(s) (e.g., de Freitas and Mateus; Dangles and Brouillard; Goto *et al.*).[193,281,285] However, the nature of this vertical stacking is still under discussion. Mori *et al.*[19] have recently described the proposed vertical stacking structures and mechanisms of intramolecular charge-transfer suggested for many polyacylated anthocyanins as obscure.

It is generally accepted that vertical associations between copigments and anthocyanins in slightly acidic to neutral solvents or vacuoles protect the anthocyanidin nucleus from hydration, especially in position 2, making the percentage of colorless forms of the anthocyanidins smaller than expected according to the pH. However further details here lead to various models and sometimes opposing proposals. Brouillard and Dangles[146] have discussed copigmentation of anthocyanins in detail. In their review they express that hydrophobic contributions in addition to dispersion forces (especially π–π overlap) between pigment and copigment, provide the major driving force for copigmentation. Da Silva *et al.*[286] has opposed this and have instead proposed the generality of charge transfer (strictly a charge-shift), from the copigment to the flavylium cation, as a major driving force for the stabilization of anthocyanin–copigment complexes. Thus, polyphenols with lower ionization potential (e.g., the flavonol rutin) should serve as stronger copigments than those with higher ionization potentials (e.g., benzoic acids). However, Hunter and Sanders[283] have previously in a more general context reported that π–π interactions are not due to any attractive electronic interaction between the two π-systems, but occur when the attractive interactions between π-electrons and the σ-framework outweigh unfavorable contributions such as π-electron repulsion. Their model implies that the donor–acceptor concept can be misleading when used to describe π–π interactions: It is the properties of the atoms in the regions of molecular contact that control the strength and geometry of interactions, rather than the overall molecular oxidation or reduction potentials.

3.16.2.8.2(i) Nonacylated anthocyanins Nonacylated anthocyanins are normally not related to copigmentation effects. However, anthocyanidin-3,5-diglucosides in their quinonoidal forms at pH 7 have been suggested as being vertically stacked with the A rings on top of each other in a left or right-handed screw axis, supported by data obtained by circular dichroism (CD) and NMR measurements.[285,287–290] This association mechanism, which is called *self-association* (**Figure 12**), is relatively weak in nature. The CD data for the 3,5-diglucosides of cyanidin and pelargonidin did show aberrant properties compared to the other anthocyanidin-3,5-diglucosides examined.[287,291] The vertical stacking mechanism has also been suggested for flavylium cations, however, the CD intensities of the flavylium cations of all six common anthocyanidin-3,5-diglucosides were small compared with those of the quinonoidal bases.[289] After analyses of the 3-glucosides of malvidin, delphinidin, and peonidin in wine-like solutions (12% ethanol, pH 3.6), the existence of anthocyanin self-association and its influence on the apparent hydration constant of the anthocyanins with subsequent modification in the color of the solutions was recognized in all cases.[279] The authors observed that the greater the degree of methoxylation of the anthocyanin B-ring, the greater was the magnitude of the self-association. For malvidin 3-glucosides it has been suggested that the flavylium cation can be stabilized by self-aggregation or by complexation with the chalcone *Z*-form in moderate acidic environment.[292] On the basis of studies on temperature and concentration dependencies of proton chemical shifts of cyanidin 3-[2-(xylosyl)-6-(glucosyl)galactoside] it has been shown that not all nonacylated anthocyanins were protected by self-association.[293]

3.16.2.8.2(ii) Monoacylated anthocyanins Although the effect is normally not as strong as for anthocyanins with several aromatic acyl groups, the presence of one aromatic acyl group hinders hydrolysis of the red flavylium cationic form to colorless hemiketal forms, allowing preferential formation of the blue quinonoidal bases, thereby resulting in pigments remaining colored in mildly acidic or neutral media. Altogether 179

different anthocyanins have been reported to be monoacylated with an aromatic acyl unit, and although just a few of them have been examined with respect to their association mechanisms, three different mechanisms (**Figure 12**) have been proposed related to monoacylated anthocyanins.

The most common mechanism used to explain copigmentation effects of monoacylated anthocyanins with aromatic acyl groups includes an intramolecular copigmentation process bringing together the chromophoric part (anthocyanidin) and the aromatic acyl group which belong to the same anthocyanin in a folded conformation.[293,294] This has been demonstrated by the observation of long-range NOEs in NMR spectra. In some cases the chemical shifts of for instance the cinnamic acid protons, which lie markedly upfield with respect to the analogous methyl cinnamate, have been taken as evidence for copigmentation.

Some monoacylated anthocyanins are more stable than others in neutral aqueous solutions. Yoshida *et al.*[295] have reported that the monoacylated anthocyanin, cyanidin 3-[6-(6-(sinapoyl)glucosyl)glucoside], isolated from the tuber of purple yam *Dioscorea alata*, is unusually stable even in neutral aqueous solutions. The stability is ascribed both to the intramolecular stacking of the sinapoyl unit and the chiral self-association of the anthocyanidin nuclei. The position of the acyl group on the anthocyanin, the position of the sugar moiety, and the length of the sugar spacer were reckoned as relevant factors for good stacking. Two processes of association were also observed for four monoacylated anthocyanins isolated from cell cultures of the wild carrot (*Daucus carota* ssp. *carota*). The formation of strong intramolecular π-complexes that involved both the double bond and aromatic ring of sinapic acid, and intermolecular association of these π-complexes into larger aggregates upon decreasing the temperature and/or increasing the concentration.[293] These aggregates dissociated upon diluting the solution, while the intramolecular π-complexes were disrupted only upon increasing the temperature above 30 °C.

The third mechanism is explained by intermolecular association of two anthocyanins as a dimer.[296,297] The two anthocyanidin nuclei and the two aromatic acyl groups are associated in a type of self-association. Nuclear Overhauser enhancement (NOESY) NMR was used for analyses of petanin (petunidin 3-[6-(4-*E*-*p*-coumaroyl)rhamnosyl)glucoside]-5-glucoside) from blue potatoes in acidified methanolic solutions. Intra- and inter-molecular NOESY cross-peaks were observed, and the corresponding proton–proton distance bounds were used in distance geometry calculations to determine distances between the units of the complex. The orientation of two self-associated petanin aglycones was found to be head-to-tail along both the long and the short aglycone axis, while the two associated coumaroyl groups were found to be head-to-tail along the long coumaroyl group axis. Lack of observed NOESY cross-peaks between protons of the coumaroyl group and the aglycone indicated absence of the intramolecular coumaroyl group–aglycone association, which has been suggested for other acylated anthocyanins. Noncoplanarity between the planes of the benzopyrylium and the phenyl rings was also shown. It was suggested that the dimer might protect the aglycone from hydration, and thereby prevent formation of hemiketals and chalcones. It was indicated that the dimer could be part of a tetramer. Some of the measured associations disappeared when the temperature was increased.

3.16.2.8.2(iii) Di- and polyacylated anthocyanins Since Goto *et al.*[298] in 1982 reported the structure of gentiodelphin from the blue petals of *Gentiana makinoi*, 144 more anthocyanins have been identified containing two or more aromatic acyl units, including several being responsible, at least partly, for blue coloration of petals (**Table 1**). Most di- and polyacylated anthocyanins are remarkably stable in neutral or weakly acidic aqueous solutions,[28,30,271] and both the shifts to more bluish colors and increased anthocyanin stability have been ascribed to two different mechanisms (**Figure 12**). The most common model describes intramolecular copigmentation involving a sandwich-type complex in which two aromatic acyl moieties stack above and below the anthocyanidin nucleus, thus providing protection against nucleophilic water attack.[146,147,219] The second model is based on studies of the dicaffeoyl anthocyanin, phacelianin, isolated from blue petals of *P. campanularia*.[19] It was suggested that the pigment chromophores of phacelianin might stack intermolecularly in an anticlockwise manner in the blue-colored vacuoles. At the same time the caffeoyl residues were suggested to stack intramolecularly in the anthocyanidin nucleus. The authors also indicated that small amounts of metal ions might be involved in the blue coloration of *Phacelia* petals.

In a detailed review by Honda and Saito[219] progress in the chemistry of polyacylated anthocyanins as flower color pigments has been outlined. It was recognized that both the blueing effect and stabilization of flower

colors depended on the number of aromatic acids presented in the polyacylated anthocyanins. After classification of the polyacylated anthocyanins into seven types by the substitution pattern of the acyl functions, it was concluded that anthocyanins with the aromatic acyl groups in glycosyls in both the 7- and 3′-positions were considered to make the most stable colors in the flowers. This conclusion was also supported through studies of the diacylated anthocyanin gentiodelphin, a pigment from the blue flower of *G. makinoi*, and its two mono-deacyl derivatives.[299] The acyl residue in the 3′-position on the B-ring contributed more to blue color development than the acyl residue in the 7-position on the A-ring.

Red-purple colors in the flowers of orchids have been shown to be derived from altogether 15 cyanidin and peonidin glycosides, with aromatic acylated sugars attached both at the 7- and 3′-positions.[300–307] Intramolecular associations of these planar molecules provided stable colors without the need for any copigment or metal cation.[308] Figueiredo *et al.*[308] proposed that the glycosyl-acyl 'side chains' attached to both positions 3′ and 7 of the chromophore favored a better overlap and stronger interaction with the π-system of the central chromophore, than what was observed for other acylated anthocyanins. They supported the assessment by molecular calculations, which gave minimum energy conformation for a 'sandwich' type with the 3′-chain folded 'over' and the 7-chain folded 'under' the chromophore. Similar acylation of glycosyls in anthocyanidin 7- and 3′-positions has also been reported for anthocyanins in Commelinaceae,[218] Compositae,[309] Liliaceae,[310] and Rhamnaceae.[37] The final example in this context concerns three acylated delphinidin 3,7,3′,5′-tetraglucosides from berries of two *Dianella* species (Liliaceae). These pigments showed exceptional blueness at *in vivo* pH values due to effective intramolecular copigmentation involving *p*-coumaryl-glucose units (GC) at the aglycone 7-, 3′-, and 5′-positions.[10] Evidences showed that the effectiveness of the copigmentation could be ranked as 3′,5′-GC > 7-GC > 3-GC.

The Morning Glory flowers (*Ipomoea/Pharbitis nil*) exist in a wide range of color forms. There is a good correlation between scarlet flower color and the occurrence of pelargonidin derivatives.[176,302] Lu *et al.*[311] have shown that the flower color of *P. nil* gradually shifts to more bluish colors with increasing numbers of caffeic acid residues in the polyacylated pelargonidin glycosides. Blue flower colors, attractive to bee pollinators, are generally based on delphinidin (**Table 1**). However, some exceptional cases are found, for instance in *I. tricolor* and *P. nil*, where the blue flower colors are caused by the 'Heavenly Blue Anthocyanin,' HBA, pigment.[208,307,312] HBA, a peonidin 3-sophoroside-5-glucoside with three caffeylglucosyl residues,[20] is among the largest monomeric anthocyanins, which has been isolated. Yoshida *et al.*[313] have shown that the color change of *I. tricolor*, while flowering, was due to vacuolar pH changes from 6.6 to 7.7, at which the quinonoidal base anion of HBA was formed and stabilized by intramolecular stacking. HBA was actually found to be more stable at physiological pH (pH 7.5) than in strong acidic or weakly acidic solutions.[137] Anthocyanins are normally considered to be more stable in strong acidic than neutral aqueous media. It has also been reported that polyacylated anthocyanins like HBA are more tolerant to UV-B than nonacylated anthocyanins.[314] These results suggest that petal anthocyanins might play some biological role in protecting petal tissues from solar radiation.

3.16.2.8.2(iv) Intermolecular associations Intermolecular copigmentation describes the interaction between the anthocyanidin nucleus and another colorless molecule (copigment), which is not bound covalently to the anthocyanin molecule.[315] This mechanism is proposed to play a major role in the stabilization of anthocyanins lacking acyl moieties. When considering the anthocyanin content in fruits and berries in **Table 3**, it is clear that very few of them contain anthocyanins with aromatic acylation. In these cases intermolecular interaction is the most probable means of copigmentation. An electronic delocalization on a planar system seems to be required for a molecule to act as a copigment. No evidence of the existence of interactions taking place between a copigment and the colorless forms of anthocyanins has been reported, which suggests π–π overlap (vertical stacking) between aromatic residues in the intermolecular associations.[146] Intermolecular copigmentation interactions are specific in nature, and by varying the copigment a variety of colors may be produced. Some examples involving intermolecular copigmentation in flowers are explained below. Intermolecular copigmentation is very important for the metalloanthocyanin complexes, which have been reported (Section 3.16.2.7)

The blue flower color of *Ceanothus papillosus* (Rhamnaceae) has been proposed to arise from a supramolecular complex of high stoichiometry including anthocyanins and the flavonol kaempferol 3-[2-(xylosyl)rhamnoside] (Bloor,[37] Tabell 1). This copigmentation effect appeared to be quite specific, and did not occur to the same

extent with other more common flavonols. An extraordinary, long wavelength visible absorption maximum at 680 nm was produced, which conferred additional blueness. The blue color of the petals of the blue marguerite daisy (*Felicia amelloides*) has been found to arise from copigmentation between delphinidin 3-[2-(rhamnosyl)glucoside]-7-[6-(malonyl)glucoside] and the flavone *C*-glycoside swertisin 2″-*O*-rhamnoside-4′-*O*-glucoside (Bloor,[17] Tabell 1). The visible spectrum of the upper epidermal peel showed the characteristic triple maxima shape of many violet or blue flowers with specific absorption maxima at 550, 585, and 632 nm. The flavones were present at high concentration in the petal; the molar ratio of flavone to anthocyanin was estimated to be at least 18:1, and the anthocyanin concentration in the petal sap was *c.* 1.8 mmol l^{-1}.

3.16.2.8.2(v) Cis (Z)- and trans (E)-configuration of cinnamic acids Around 20 anthocyanins acylated with hydroxycinnamic acids have been reported to occur in both the *cis* (*Z*)- and *trans* (*E*)-configuration, however, this number is most probably somewhat underestimated due to lack of proper determination of this configuration during structure elucidation of the cinnamic acids. George *et al.*[316] have compared the pairs of 3-[6-(*E*/Z-*p*-coumaryl)glucoside]-5-[6-(malonyl)glucosides] of malvidin and delphinidin. They observed that the *cis* isomers exhibited ϵ values about 1.5 times greater than the *trans* isomers, in both pairs. It was calculated that the *cis* forms were less prone to undergo hydration reactions forming the colorless anthocyanin forms. On the basis of computed structures the more co-planar arrangement allowed by the *cis* isomers was postulated as the rationale supporting the enhanced color stability.[316] When considering the color effect of this type of intramolecular copigmentation *in vivo*, one should bear in mind that the *trans* isomer seems to predominate, and that the conversion between the two isomers is rare. When Yoshida *et al.*[314] studied the *E,Z*-isomerization reaction and stability of several types of acylated anthocyanins under the influence of UV irradiation, their interest was focused on the reason why isomerization reaction of some acyl residues was prevented in living plant cells. They concluded that the stability of anthocyanins under irradiation highly depended on molecular stacking. They proposed that light energy absorbed by cinnamoyl residues might be transferred to the anthocyanidin nucleus and released without any isomerization reaction or degradation of pigments. Thus, the flower color may be stable for a long time under strong solar radiation.

3.16.2.8.2(vi) Sugar moieties The anthocyanins contain sugar(s) that contribute to hydrogen bondings, which constrain the possibilities for orientations of the anthocyanin–copigment complex. The crucial role of the hydrogen bondings of the sugar moieties in studies of anthocyanin copigmentation is mostly overlooked due to experimental limitations. The different effects of D- and L-glucose in experiments related to the metalloanthocyanin, protodelphin are highlighted in Section 3.16.2.7. This blue pigment consists of the anthocyanin malonylawobanin (M), the flavone apigenin 7,4′-di-*O*-β-D-glucoside (F), and Mg^{2+} ions; $M_6F_6Mg_2$.[252] Mixing of malonylawobanin, with synthetic apigenin 7,4′-di-*O*-β-D-glucoside and apigenin 7,4′-di-*O*–β-L-glucoside yielded protodelphin containing only the D-glucosyl, while the L-glucosyl was completely excluded. Three flavone molecules in protodelphin were associated to form a helical structure (minus form), similar to a propeller with three blades. They were bound at the pivot point by a strong hydrogen-bonding network among the hydroxyl groups at C-2 and C-3 of the 4′-*O*-β-D-glucosides. Two sets of this helical flavone structure fit closely into the vacant space formed from the metal complex of six molecules of M and two Mg^{2+} ions. Replacement of D- by L-glucosyl at the 4′-OH position of apigenin inverted the helical structure of the three associated flavones (plus form), with the consequence that it did not fit into the vacant space. The authors concluded that restricted chiral and structural recognition controlled the entire self-assembly of the metalloanthocyanin, and was responsible for the blue flower color.

3.16.3 Anthocyanin Localization in Plant Tissue

Several decades ago microscopic examinations have shown a compartmentalized and sharply delimited location of anthocyanins and other flavonoids.[317] With bi-colored roses for instance, anthocyanins are invariably concentrated on the inner and carotenoids on the outer side of the petal. In many flowers, flavonoid colors are enriched in epidermal cells while adjacent sub-epidermal cells are colorless. However, the shoot meristems of many angiosperms consist of three layers of cells, designated L1, L2, and L3 cells.[318] The L1 cells give rise to

the epidermal layer, the L2 cells to the sub-epidermis, and the L3 cells to the internal tissues. Each of the cell layers in petals generally originates from one of these three layers, and the layers of anthocyanin-producing cells differ among species; L2 cells are used in *Petunia* and *Antirrhinum*, and all three of them (L1–L3 cells) in *Pharbites*.[319] In leaves, anthocyanins may be found in the upper epidermis, lower epidermis, palisade mesophyll, spongy mesophyll, and trichomes, either in one cell type or in almost any combination of them.[320]

It is generally accepted that anthocyanins as other flavonoids are synthesized on the cytoplasmic surface of the endoplasmic reticulum membrane.[321,322] Although the biosynthetic pathways for flavonoids and their regulation have been closely studied (see Section 3.16.5.1 and references therein), the mechanisms for anthocyanin accumulation in the cells are more indecisive.

3.16.3.1 From *Anthocyanoplasts* to *Anthocyanic Vacuolar Inclusions*

Inside cells, the anthocyanins are most often found dissolved uniformly in vacuolar solutions. However, Pecket and Small[323] listed 26 dicotyledon and 7 monocotyledon families in which the presence of pigmented bodies, which they called *anthocyanoplasts*, had been noted. These spherical bodies were described as membrane-bound organelles that provide intense coloration in the vacuoles of mature plant cells. Such pigmented bodies have been described as 'blue spherules' in epidermal rose petal cells,[324] 'blue crystals' in *Consolida ambigua* petals,[325] 'crystals,' and 'ball-like structures' in *Matthiola incana* petals,[326] 'red crystals' in mung bean hypocotyl,[327] and as 'intravacuolar spherical bodies' in *Polygonum cuspidatum* seedlings.[328] Similar structures were found to occur in the leaves of various Brassicaceae,[329,330] in grapes,[331] and in the tubers of *Ipomoea batatas*.[332] It was then indicated that these globular inclusions may be protein matrices,[332,333] and that they possess neither a membrane boundary nor an internal structure.[333–335] Recent anatomical observations of anthocyanin-rich cells in apple skin carried out by light and electron microscopy showed that the skin with fully developed red color had more layers of anthocyanin-containing epidermal cells than those of green skin.[336] The anthocyanins were frequently found in clusters or in agglomerations that were round in shape in the epidermal cells of the red skin. There was no distinct envelope membrane on the anthocyanin granule in the vacuoles. The anthocyanins seemed to be synthesized around the tonoplast and condensed on the inward side of the vacuole.

However, not much was documented about the chemical nature and the functional significance of these inclusions in petal cells before Markham *et al.*[337] reported intensively colored intravascular bodies in petals of lisianthus (*Eustoma grandiflorum*) and blue-gray carnations (*Dianthus caryophyllus*), which they named AVIs. The AVIs occurred predominantly in the adaxial epidermal cells, and their presence was shown to have major influence on flower color by enhancing both intensity and blueness. This latter effect was especially dramatic in blue-gray carnations where the normally pink 3,5-diglucoside and 3-glucoside of pelargonidin produced a blue-gray coloration. In contrast, epidermal cells of pink carnation petals lacked AVIs but contained vacuoles that were homogeneously pigmented pink with the same pelargonidin derivatives. The absolute level of anthocyanins in the blue-gray tissue as measured spectrophotometrically, was four times that in the pink tissue. This much higher level of anthocyanins in the blue-gray petals was associated almost entirely with the AVIs as little color was seen in the surrounding vacuolar solution. The presence of AVIs thus appeared to be the predominant factor that accounted for the observed color difference.

In lisianthus, the presence of large AVIs produced marked color intensification in the inner zone of the petal by concentrating anthocyanins above levels that would be possible in vacuolar solutions.[337] The electron microscopy studies on lisianthus epidermal tissue failed to detect a membrane boundary in AVI bodies, and the isolated AVIs were shown to have a protein matrix. Bound to this matrix were four cyanidin- and delphinidin acylated 3,5-diglycosides, which were relatively minor anthocyanins in the whole petal extracts where acylated delphinidin triglycosides predominated. Flavonol glycosides were not found to be bound to the AVIs. The specificity of this 'anthocyanin trapping' was confirmed by the presence in the surrounding vacuolar solution of only delphinidin triglycosides, accompanied by the full range of flavonol glycosides. 'Trapped anthocyanins' were shown to differ from solution anthocyanins only in that they lack a terminal rhamnose on the 3-linked galactose. On a closer look by light and electron microscopy on the epidermal cells of different regions of the lisianthus petal, Zhang *et al.*[338] observed that the AVIs occurred on three different forms: vesicle-like, rod-like, and irregular shaped. Again no membrane encompassing the AVIs was observed, however, the AVI itself consisted of membranous and thread structures throughout. The results strongly suggested the existence of

mass transport for anthocyanins from biosynthetic sites in the cytoplasm to the central vacuole. The anthocyanins were found to accumulate first as vesicle-like bodies in the cytoplasm, which themselves were contained in prevacuolar compartments (PVCs). The vesicle-like bodies seemed to be transported into the central vacuole through the merging of the PVCs and the central vacuole in the epidermal cells.[338]

Analogous 'anthocyanin trapping' as reported for lisianthus has also been reported by Conn *et al.*,[339] who found that AVIs in two lines of grapevine (*Vitis vinifera*) cell suspension culture appeared as dark red-to-purple spheres of various sizes in vacuoles due to their interaction with anthocyanins. Compared with the total anthocyanin profile, the profile of the AVI-bound anthocyanins showed an increase of approximately 28–29% in acylated (*p*-coumarylated) anthocyanins in both lines. At the subcellular level in maize (*Zea mays*) it has recently been found that light induces an alteration in the way the anthocyanins were distributed within vacuolar compartments.[340] In sorghum (*S. bicolor*) 3-deoxyanthocyanidins accumulate as inclusions in leaf cells under fungal attack, and function as phytoalexins by inhibiting infection in a site-specific response.[341–343] The cytological response commences when colorless 3-deoxyanthocyanidin inclusions (0.1 mm diameter) accumulate exclusively in those leaf cells, which are under fungal attack. These inclusions become orange to red in color and accumulate at sites of physical contact between host and pathogen. Dark red inclusions of up to 20 mm appear by coalescence. The progressive color shift of the 3-deoxyanthocyanidins from faint orange to dark red during defense response is most likely caused by changes in local, subcellular pH. It has been shown that the 3-deoxyanthocyanidin, luteolinidin, when self-organized as pigmented inclusions, mediates disruption of plant and fungal plasma membranes as well as reconstructed bilayer liposomes.

3.16.4 Colors of Aurones and Chalcones

3.16.4.1 Introduction

Carotenoids play the principal role in yellow to orange floral pigmentation.[1,344] Anthocyanins in their natural environment (vacuoles, AVIs) do not provide yellow coloring of plants. Among the flavonoids involved in yellow to orange plant colors are the aurones and chalcones, and to some degree flavonols. The chalcones and aurones have, however, limited distribution in the plant kingdom as colorants. Some striking examples include yellow and red quinochalcones from safflower (*Carthamus tinctorius*, Asteraceae), which have been used as textiles dyes throughout history. Likewise are colored kamalachalcones the pigment basis of kamala, an orange-colored exudate of *Mallotus philippensis* (Euphorbiaceae) fruits used as dye to produce yellow to orange colors on wool, mohair, and silk. It has further been found that the only reported colored plant nectar in nature, which has been revealed in three bird/gecko-pollinated plant species in Mauritius, are based on a red-colored aurone (see Section 3.16.6). Chalcones and aurones are nevertheless best known for providing yellow flower colors to some popular ornamental plants in family Asteraceae and snapdragon (*Antirrhinum majus*, Scrophulariaceae). They are also found in the bark, wood, leaves, and seedlings of a variety of plants.[201]

The chalcones, and the closely related dihyrochalcones, are unique among the flavonoids by lacking a central heterocyclic C-ring (**Figure 13**). Furthermore, their nomenclature is based on a unique numbering system having the primed positions on the A-ring, as opposed to the B-ring in all cyclic flavonoids. Altogether, around 700 different chalcone structures have been reported, including aglycones, glycosides, chalcone conjugates, quinochalcones, chalcone dimers, and oligomers, as well as chalcone Diels–Alder adducts.[6,345] In addition nearly 250 dihydrochalcones have been identified. Both numbers of structures and structural complexity of new chalcone and dihydrochalcone aglycones have advanced considerably during the last decade. However, the occurrence of complicated glycosidic patterns among the chalcones are lacking compared to those of other flavonoid groups such as flavonols and flavones.[346] Most chalcone monoglycosides are *β*-glucopyranosides, and only a few disaccharides are encountered with any frequency. The majority of the chalcone glucosides found in nature are based on just a few aglycones such as isoliquiritigenin (4,2′,4′-trihydroxychalcone), chalconaringenin (4,2′,4′,6′-tetrahydroxychalcone) and okanin (3,4,2′,3′,4′-pentahydroxychalcone). Around 25 chalcone glycosides are acylated with either aromatic or aliphatic acyl groups.

The name 'aurone' comes indeed from the Latin word 'aurum' (= gold) because of the golden-yellow colors.[347] The systematic name of the skeleton is 2-benzylidene-3(2*H*)-benzofuran-3-one, also called

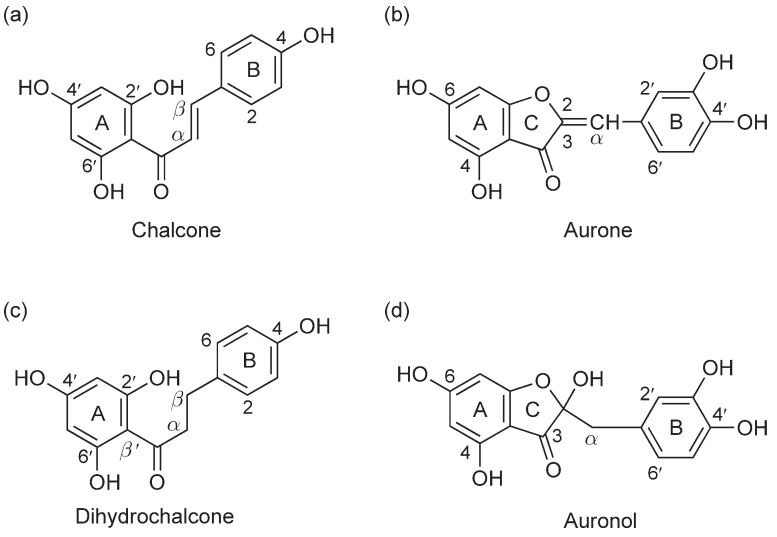


Figure 13 Structure examples, ring labeling, and atom numbering of chalcones (a), aurones (b), dihydrochalcones (c), and auronols (d).

2-benzylidenecoumaran-3-one. The compounds in the subgroup, auronols, are based on the 2-hydroxy-2-benzylcoumaran-3-one skeleton (**Figure 13**). Positions in the aurones are identified using the 'normal' flavonoid nomenclature, however, the 4-position in aurones is biosynthetically equivalent to the 5-position in 'normal' flavonoids. There are two possible geometric isomers of aurones with respect to the C2–Cα double bond. The aurones comprise the smallest group in the flavonoid family including just above 100 different structures as aglycones, aglycone dimers, and glycosides.[6,345] The majority of the aurone glycosides are β-glucopyranosides or α-rhamnopyranosides, and acylation has just been found in some maritimetin 6-*O*-glucosides.

The chalcones and aurones often occur together in plants. They have been referred to as the 'anthochlor' pigments because of their alkali-induced bathochromic shifts.[346] These specific shifts are and have been important tools in their structural elucidation.[347,348] Chalcones can be converted into aurones in the presence of weak base and atmospheric oxygen. Conversion of chalcones into aurones by enzyme extracts from plant tissue has also been demonstrated.[349] The main focus on chalcones and aurones in this section will be on their role as plant pigments, and examples of their natural presence will be given. Some examples of their UV-absorbing character in pollination is presented in Section 3.16.6.1.

3.16.4.2 Occurrences and Colors

The UV–visible spectra of chalcones and aurones are characterized by intense Band I and diminished Band II absorptions.[347] For chalcones the most intense band usually occurs in the range of 340–390 in methanolic solutions, although chalcones lacking B-ring oxygenation may have their Band I absorptions at considerably shorter wavelengths. Band II is usually a minor peak in the 220–270 nm region. As with flavones and flavonols, increased oxygenation of both the A- and B-rings usually results predominantly in bathochromic shifts of Band I (**Table 11**). Going from 2′,4′-dihydroxychalcone ($\lambda_{max} = 345$ nm) via 4,2′,4′-trihydroxychalcone ($\lambda_{max} = 369$ nm) to 3,4,2′,4′-tetrahydroxy ($\lambda_{max} = 379$ nm), considerable bathochromic shifts caused by extra hydroxyl groups on the B-ring are experienced. The same effect is revealed for the A-ring when comparing absorption spectra of 4,4′-trihydroxychalcone ($\lambda_{max} = 348$ nm) and 4,2′,4′-trihydroxychalcone ($\lambda_{max} = 369$ nm). With respect to the A-ring, an interesting effect is observed when comparing absorption spectra of 3,4,2′,4′-tetrahydroxychalcone ($\lambda_{max} = 379$ nm) with those of 3,4,2′,4′,6′-pentahydroxychalcone ($\lambda_{max} = 378$ nm) and 3,4,2′,3′,4′- pentahydroxychalcone ($\lambda_{max} = 384$ nm). The former with the phloroglucinol pattern (2′,4′,6′-trihydroxy-) shows no effect on the wavelength of the absorption maximum, while the latter with the

Table 11 Visible λ_{max} values in absorption spectra of selected chalcones dissolved in methanol or ethanol

Compound	λ_{max} *(nm)*	*Reference*
Chalcone		
2′,4′-dihydroxy	345[a]	350
2′,4′-dihydroxy-4-methoxy	362[a]	350
4′,4′-dihydroxy	348[a]	350
4,2′,4′-trihydroxy (isoliquiritegenin)	369	351
4,2′,4′,6′-tetrahydroxy (chalconaringenin, isosalipurpol)	369	351
4′-*O*-glucoside	368	351
3,4,2′,4′-tetrahydroxy (butein)	379	352
4′-*O*-glucoside	380	353
4′-*O*-malonylglucoside	380	353
4′-*O*-sophoroside	377	353
4′-*O*-malonylsophoroside	379	353
3,4,2′,3′,4′-pentahydroxy (okanin)	384[b]	354
4′-*O*-[2″-(caffeoyl)-6″-(acetyl)glucoside]	380	355
4′-*O*-[2″-(caffeoyl)-6″-(coumaroyl)glucoside]	380	355
3,2′,3′,4′-tetrahydroxy-4-methoxy (methylokanin)		
4′-*O*-[6″-(coumaroyl)glucoside]	373	355
4′-*O*-[6″-(acetyl)glucoside]	372	355
4′-*O*-[2″-(caffeoyl)-6″-(coumaroyl)glucoside]	360	355
3,4,2′,4′,6′-pentahydroxy	378	351
4′-*O*-glucoside	378	351
Chalcone dimer		
Kamalachalcone A	344	356
Kamalachalcone B	345	356
Quinochalcone		
2,2,6-tri-isoprenyl-cyclohex-5-ene-1,3-dione (munchiwarin)	422	357
Quinochalcone dimer		
Precarthamin	406	358
Anhydrosafflor B	410	359
Carthamin	519	360
Dihydrochalcone		
4,2′,4′,6′-Tetrahydroxy-4,3′-dimethoxy	284	361

[a] In EtOH.
[b] In 98% EtOH.

pyrogallol pattern (2′,3′,4′-trihydroxy-) has a small bathochromic shift effect compared to the absorption spectrum of 3,4,2′,4′-tetrahydroxychalcone. Glycosyl substitution on the aglycones shows no or very weak hypsochromic shift effects on the spectra.

The aurones produce 'stronger' yellow colors than chalcones due to their absorbances at longer wavelengths. The majority of aurones show four absorption maxima.[362] Two (sometimes one) of these absorption bands are usually found in the 370–430 nm region due to resonance contribution of the carbonyl group with the different conjugated systems in the aurone molecules, although some of the simpler aurones absorbs at much shorter wavelengths (**Table 12**). The effect of hydroxyl- and methoxyl groups of aurones on UV–visible absorption spectra have been described in detail by Geissmann and Harborne.[362] The following hydroxyl groups give bathochromic effects: 7-OH, 2′-OH, 4′-OH in the presence of 6-OH, and 3′-OH in the presence of 4′-OH. While the presence of a 4-OH or 3′-OH, or a 5-OH in a 6-hydroxyaurone, does not change the spectra appreciably, a 6-OH has a pronounced hypsochromic effect. An *O*-glycosyl in the 6-position causes a small bathochromic effect (**Table 12**) compared to the spectra of analogous 6-hydroxyaurones. Introduction of *O*-glycosyls in other hydroxyl positions of aurones, has only minute effects on the spectra.

Table 12 Visible λ_{max} values in absorption spectra of selected aurones dissolved in methanol or ethanol

Compound	*λ_{max} (nm)*	*Reference*
Aurone		
4-Hydroxy	389[a]	362
4-Methoxy	387[a]	362
6-Hydroxy	344[a]	362
2′-Hydroxy	402[a]	362
3′-Hydroxy	381[a]	362
4′-Hydroxy	405[a]	362
5,6-Dihydroxy	347[a]	362
6,4′-Dihydroxy (hispidol)	388	347
4,6,4′-Trihydroxy	393	351
6,3′,4′-Trihydroxy (sulfuretin)	399[a]	351
6-*O*-Glucoside	404[a]	351
6-di-*O*-Glucoside	402	363
4,6,3′,4′-Tetrahydroxy (aureusidin)	398	364
4-*O*-Glucoside (cernuoside)	404	364
6-*O*-glucoside (auresin)	407	364
6-*O*-rhamnoside	404	365
4,6-di-*O*-glucoside	411	364
5,6,3′,4′-tetrahydroxy	395[a]	362
6,7,3′,4′-tetrahydroxy (maritimetin)	412	347
6-*O*-glucoside (maritimein)	419[a]	362
7-*O*-glucoside	404	366
6-*O*-[6-(coumaroyl)glucoside]	412	366
6-*O*-[6-(acetyl)glucoside]	411	366
6,3′,4′-dihydroxy-7-methoxy	406[a]	362
6-*O*-glucoside (letopsin)	411[a]	362
7,3′,4′-trihydroxy-6-methoxy	413[a]	362
6,7,3′,4′-tetramethoxy	404[a]	362
6-hydroxy-7,3′,4′-trimethoxy	401[a]	362
7-hydroxy-6,3′,4′-trimethoxy	411[a]	362
4,6,3′,4′,5′-pentahydroxy (bracetin)	403[a]	350
4-*O*-glucoside	409[a]	350
6-*O*-glucoside	408[a]	350
Aurone dimer		
2×(4,6,3′,4′-Tetrahydroxy)(C5′ → C5)(aulacomniumbiaureudsidin)	411	367
4,6,3,4-Tetrahydroxy(C5′ → C6)5,7,3,4-tetrahydroxyflavanone(capylopusaurone)	402	368
Auronol		
2,4,6,3′,4′,5′-Hexahydroxy (amaronol A)	333	369
2,4,6,3′,5′-Hexahydroxy-4′-methoxy (amaronol B)	335	369

[a] In EtOH.

Owing to some loss of conjugation, the dihydrochalcones and auronols have as expected absorbances at shorter wavelengths than corresponding chalcones and aurones. Lusianin (4,2′,4′,6′-tetrahydroxy-4,3′-dimethoxydihydrochalcone) isolated from the orchid *Lusia volucris*, shows UV absorption peaks at 205, 215, and 284 nm in methanol,[361] while the pale yellow amaronols A and B (2,4,6,3′,4′,5′-hexahydroxyauronol and 2,4,6,3′,5′-pentahydroxy-4′-methoxyauronol) isolated from the bark of *Pseudolarix amabilis*, have similar UV spectra with absorption peaks at 212, 288, and 333/335 nm in methanol.[369]

The real *in vivo* colors based on chalcones and aurones are of course influenced by the matrix of these pigments, including intermolecular associations with solvent and other molecules in their surroundings, as well as physical parameters. However, the impact of these factors has hardly been treated in papers reporting anthochlor colors. Some examples where plant colors are related to chalcone or aurone structures are as follows.

3.16.4.2.1 Chalcone and aurone monomers

Chalcones and aurones are best known for their provision of yellow flower colors to some popular ornamental plants such as *Dahlia*, *Coreopsis*, *Cosmos* (Asteraceae) and snapdragon (*A. majus*, Scrophulariaceae). Yellow coloration of *Dahlia variabilis* flowers is mainly due to the presence of 4′-malonylglucosides of the 6′-deoxychalcones isoliquiritigenin and butein (3,4,2′,4′-tetrahydroxychalcone),[370–372] while accumulation of butein 4′-glucoside and the aurone sulfuretin 6-glucoside are responsible for the yellow petal color of some *Cosmos* species.[373,374] In 1957 Shimokoriyama isolated two chalcones, okanin and okanin-4′-glucoside, from flowers of *Coreopsis tinctoria*.[354] Chalcones were indeed found to occur in floral tissue of all the 46 *Coreopsis* species of North America.[375] Recently, altogether 11 flavonoids, including several chalcones, flavanones, and flavonols, were reported to occur in flower extracts of *C. tinctoria*.[376] The yellow snapdragon is one of the best-known sources for aurones. Small amounts of the 4′-*O*-glucosides of chalconaringenin and 3,4,2′,4′,6′-pentahydroxychalcone serve as direct precursors of the 6-glucosides of the aurones aureusidin (4,6,3′,4′-tetrahydroxyaurone) and bracteatin (4,6,3′,4′,5′-pentahydroxyaurone), which are the main pigments responsible for the yellow flower color.[377–383] Yellow snapdragon has become the model species for the study of aurone biosynthesis.[379,384] Aureusidin 6-*O*-glucoside is also the main yellow pigment in the orange petals of *Mussaenda hirsutissima* (Rubiaceae),[364] where it co-exists with aureusidin 4,6-di-*O*-glucoside and aureusidin 4-*O*-glucoside (cernuoside). Okanin derivatives are in general typical for species in the genus *Bidens* (Asteraceae), where additionally butein, sulfuretin (6,3′,4′-trihydroxyaurone) and maritimetin (6,7,3′,4′-tetrahydroxyaurone) derivatives are reported.[355,366,385,386] Isoliquiritigenin glycosides generate yellow flower colors in Leguminosae, however not exclusively.[387,388] The glycosidic patterns of these chalcones are rather simple compared to the glycosyl moieties of other flavonoid groups found in this family.

3.16.4.2.2 Chalcone and aurone dimers

Dimeric and oligomeric structures of chalcones are most commonly found in family Ochnaceae, and in particular from species in genera *Lophira* and *Ochna*, but they are also represented in Anacardiaceae.[345] Two chalcone dimers with unusual structures, kamalachalcone A and B (**Figure 14**) have among other compounds been isolated from kamala,[356] an orange-colored exudate from grandular trichomes on the surface of the fruits of *M. philippensis* (Euphorbiaceae).

Kamala has been used as a dye to produce yellow to orange colors on wool, mohair, and silk. Kamalachalcone A has been described as a yellow powder, while kamalachalcone B has been described as an orange powder,[356] however, they have approximately the same maximum wavelengths, 344 and 345 nm, respectively, measured in methanolic solutions. In kamalachalcone B an acetophenone was connected with the chalcone moiety through a methylene group. We may speculate in that the orange color of kamalachalcone B powder is due to intramolecular association between the acetophenone with the dimeric structure, or alternatively that the methylene–acetophenone group improves the chromophore by increasing the planarity

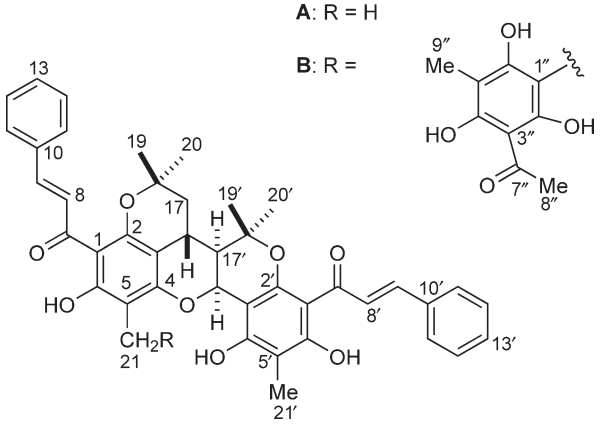


Figure 14 Structures of kamalachalcone **A** and **B** isolated from exudate of the fruits of *Mallotus phillippensis*.[356]

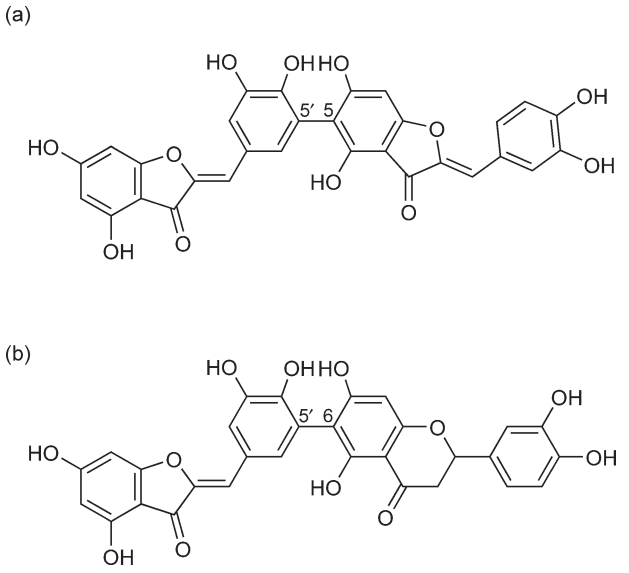


Figure 15 Structures of aulacomniumbiaureusidin (a) (biaurone) isolated from *Aulacomnium* species and campylopusaurone (b) (auroneflavanone biflavonoid) isolated from the mosses *Campylopus clavatus* and *Campylopus holomitrium*.[367,368]

within the dimeric structure. More recently acetone extracts of kamala, yielded two intense yellow kamalachalcones (C and D), which are characterized by fused benzopyran rings.[389]

The first biaurone found in nature was isolated from the gametophytes of the mosses *Aulacomnium androgynum* and *A. palustre*.[367] The dimer of two aureusidin (4,6,3′,4′-tetrahydroxyaurone) molecules with a C–C bond from C-5′ → C-5 constitutes the bright-green pigment named aulacomniumbiaureusidin (**Figure 15**(**a**)). The chromophore of this dimer ($\lambda_{max} = 411$ nm) in methanol was improved compared to the chromophore of its monomeric units (both aureusidin), which had λ_{max} at 398 nm in methanol. In comparison, the bright-yellow aurone heterodimer (**Figure 15**(**b**)) isolated from the moss *Campylopus* sp., gave rise to an absorption maximum at 402 nm in methanol.[368] In this aureusidin–eridodictyol heterodimer the flavanone moiety (eriodictyol with $\lambda_{max} = 324$ nm in methanol) did not influence the absorption maximum of the aureusidin moiety at all.

3.16.4.2.3 Quinochalcones

Quinochalcones is a small group consisting of eight aglycones and ten *C*-glycosides (both monomers and dimers).[345] In the field of plant colors they have a pronounced position as major pigments in the flowers of safflower (*C. tinctorius*, Asteraceae). The botanical genus name *Carthamus* derives from the Arabic verb *qurtum* 'dye,' in reference to the usage of safflower flowers for textile dyeing, while the botanical species name *tinctorius* is an adjective corresponding to the noun *tinctor* 'dyer.' The flowers has been used for coloring textiles in ancient times in Egypt, Persia, India, and China, while the use of this dye in cotton textiles started in Europe in the eighteenth century. In food the flowers sometimes serve as a color substitute for saffron, and recently the dye from the extract has been used in cosmetics. The flowers are used for treatment of various diseases, especially in Chinese medicine.

The flowers of safflower (*C. tinctorius*, Asteraceae) are yellow just after flowering and changes gradually to red within some days. Altogether 11 different quinochalcones have been identified in the flowers.[359,390] The color transition is mainly due to the enzymatic conversion of yellow quinochalcones (precarthamin and anhydrosafflor yellow B) into a red quinochalcone, carthamin, which accumulates in mature petals.[359,391–393] The conversion from yellow precarthamin ($\lambda_{max} = 406$ nm) and anhydrosafflor yellow B ($\lambda_{max} = 410$ nm) into red carthamin ($\lambda_{max} = 519$ nm) involves the removal of a carboxyl or a glucosyl moiety, respectively

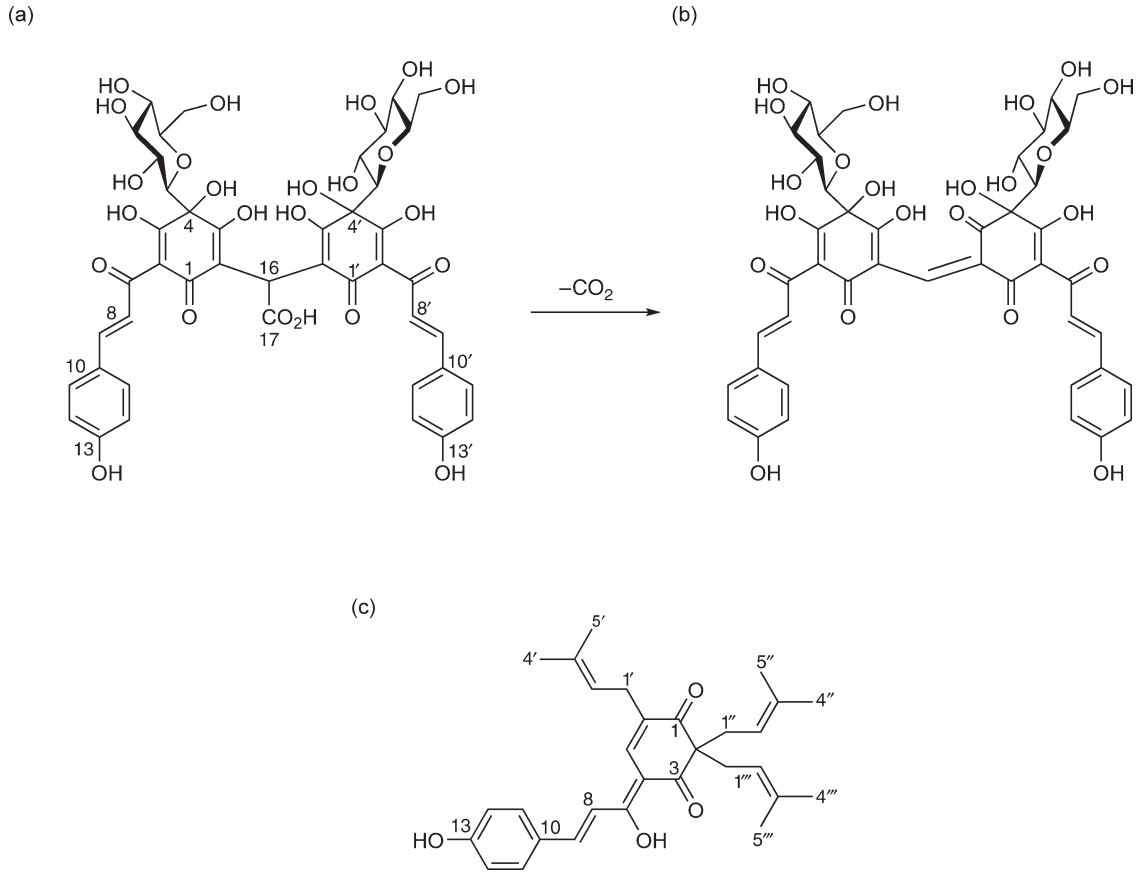


Figure 16 Structures of precarthamin (a) and carthamin (b) isolated from flowers of safflower.[359,391] When the carboxyl group is enzymatically removed from (a), the pigment changes color from yellow to red. (c) Represents the extraordinary planar structure of the orange chalcone, munchiwarin, isolated from roots of *Crotalaria trifoliastrum* (Leguminosae).[390]

(**Figure 16**). The red color of carthamin is caused by the double bond created at the bridging carbon between the two quinochalcone monomers, which increases the chromophore.

Another interesting colored quinochalcones include munchiwarin (**Figure 16**) isolated from roots of *Crotalaria trifoliastrum* (Leguminosae).[357] This orange pigment ($\lambda_{max} = 422$ nm in methanol) is the only known natural product possessing a 2,2,6-tri-isoprenyl-cyclohex-5-ene-1,3-dione ring system. The crystal structure shows a long conjugated system from the phenol to a keto-stabilized resorcinol group with three isopentenyl units attached. The conjugated unit is rather planar with a mean deviation from the best plane of only 0.09 Å; hence the orange color of the substance. The planarity of the structure is additionally supported by a hydrogen bond between the hydroxy group at the C-7 position and the carbonyl at position 3.

3.16.5 Biosynthesis of Flavonoids

The biosynthesis of flavonoids is most probably the best characterized pathway leading to any group of secondary metabolites (see Chapter 6.18). Floral pigmentation including anthocyanins has been used to help elucidation of fundamental genetic principles since the days of Mendel, and knowledge acquired through understanding of the various steps in flavonoid biosynthesis is used today in genetic engineering to expand the floriculture gene pool. Flower colors are among the key determinants influencing consumer choices, and new varieties have commercial value. A brief overview of the biosynthetic pathway leading to chalcones, aurones, anthocyanins, and

3-deoxyanthocyanidins (**Figure 17**), including a few examples of modern molecular bioengineering in the field, will be given in this section. An excellent detailed description of the various steps in biosynthesis of flavonoids, and advances in molecular biology and biotechnology of flavonoids, have been given by Davies and Schwinn.[394] Other relevant reviews covering biosynthesis of anthocyanins and other plant pigments[322,395–400] and manipulation of flower colors[401–403] expose important progress made within these fields in recent years.

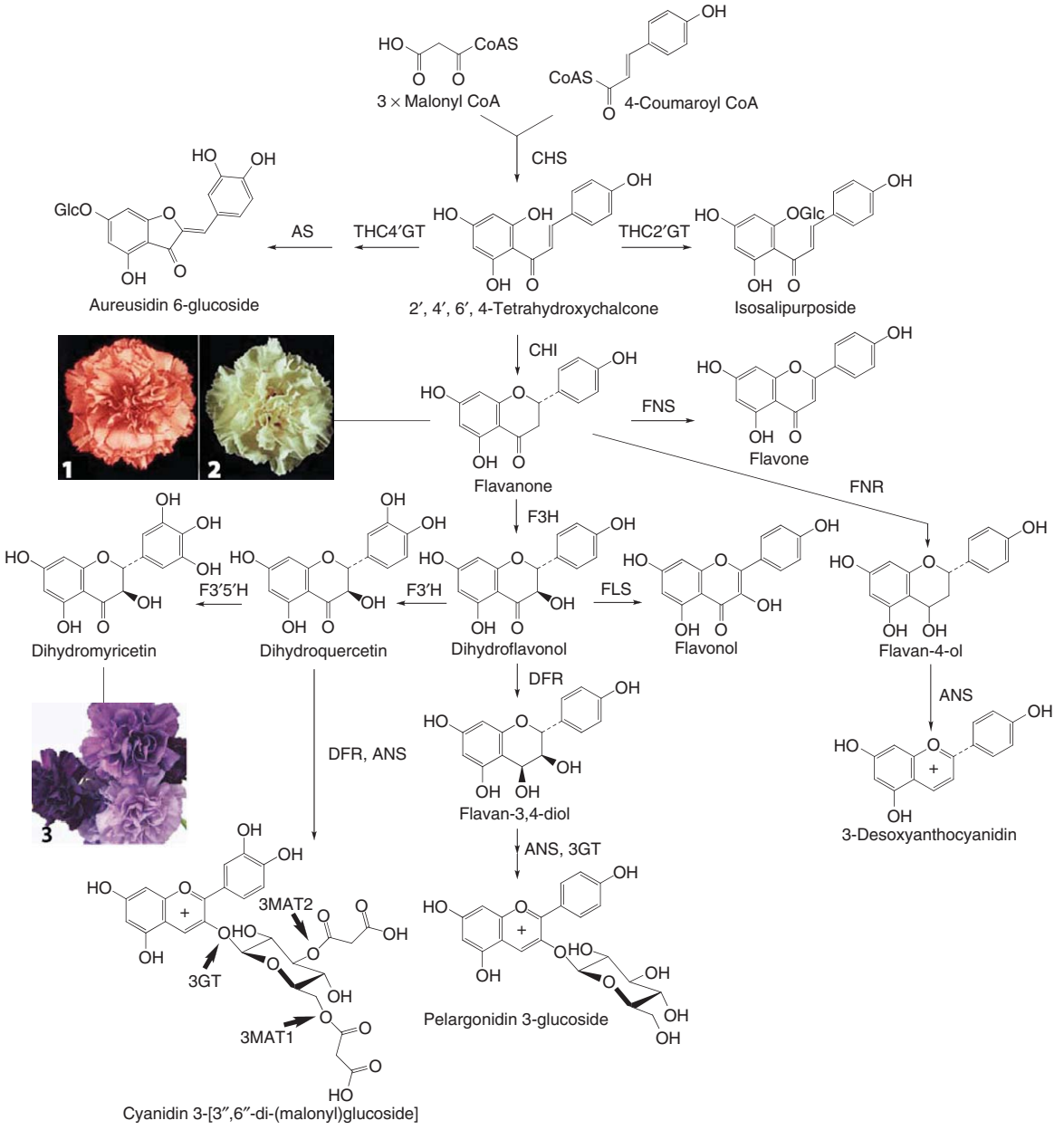


Figure 17 General biosynthesis scheme, which leads to most of the flavonoid classes. The pictures of red **(1)** and **(2)** white carnations show the difference in color achieved when the activity of the flavanone 3β-hydroxylase (F3H) has been inhibited. Photos courtesy: A. Zuker; T. Tzfira; H. Ben-Meir; M. Ovadis; E. Shklarman; H. Itzhaki; G. Forkman; S. Martens; I. Neta-Sharir; D. Weiss; A. Vainstein, *Mol. Breed.* **2002**, *9*, 33–41. Overexpression of the flavonoid 3′,5′-hydroxylase (F3′5′H enzyme) produced purple to violet transgenic flower colors due to the induction of the synthesis of delphinidin derivatives **(3)**. Photos courtesy: Y. Tanaka; A. Ohmiya, *Curr. Opin. Biotechnol.* **2008**, *19*, 190–197. See text in Section 3.16.5.1 for explanations of the abbreviations used for the enzymes involved in the various steps.

3.16.5.1 Biosynthetic Steps Leading to Chalcones, Aurones, Anthocyanins, and 3-Deoxyanthocyanins

It is generally accepted that flavonoids are synthesized in the cytosol, and the involved enzymes are connected to the membrane of the endoplasmic reticulum.[321,322] The pathway starts with formation of the C_{15} backbone by chalcone synthase (CHS), which catalyzes synthesis of 4,2′,4′,6′-tetrahydroxychalcone (THC) from one molecule of coumaroyl-CoA and three molecules of malonyl CoA (**Figure 17**). This polyketide synthase displays high flexibility with respect to various starters.[397]

Chalcones are precursor in biosynthesis of aurones (**Figure 17**), which is catalyzed by a homologue of plant polyphenol oxidase.[379] The final biosynthetic mechanism for forming aurones from chalcones has recently been clarified.[382,404] It has been revealed that the chalcones in snapdragon (*A. majus*) flowers are 4′-*O*-glucosylated in the cytoplasm by chalcone 4′-*O*-glucosyltransferase and then transported to the vacuole. Within the vacuoles they are enzymatically converted into aurone 6-*O*-glucosides by an aurone synthase (AS), which in snapdragon has the name aureusidin synthase (AUS). This metabolic pathway is unique, because for all other flavonoids the carbon backbone is completed before transport to the vacuole.

In the biosynthesis of anthocyanins (and other flavonoid groups) the unstable chalcone THC, is converted stereospecifically into the flavanone, (2*S*)-naringenin, by chalcone isomerase (CHI) (**Figure 17**). This was the first enzyme involved in flavonoid biosynthesis to be described,[405] and is today one of the best-characterized enzymes involved in plant secondary metabolism. In the absence of CHI the isomerization of THC occurs spontaneously, yielding a racemic mixture of (2*R*/2*S*)-naringenin.[395] (2*S*)-flavanones are *in vivo* the exclusive substrates of the downstream enzymes of the flavonoid pathway, and thus, CHI guarantees the efficient formation of biologically active (2*S*)-flavonoid isomers. Mutants lacking CHI activity accumulate only trace amounts of flavonoids.[406] Flavanones are converted into dihydroflavonols by hydroxylation in position 3 catalyzed by flavanone 3β-hydroxylase (*F3H* or *FHT*). This enzyme is classified as a soluble 2-oxoglutarate-dependent dioxygenase according to its requirement of the co-factors 2-oxyoglutarate, molecular oxygen, ferrous iron (Fe(II)), and ascorbate. Dihydroflavonols (dihydrokaempferol, dihydroquercetin, and dihydromyricetin) are reduced to flavan-3,4-diols/leucoanthocyanidins (leucopelargonidin, leucocyanidin, or leucodelphinidin, respectively) by dihydroflavonol 4-reductase (DFR) in the course of anthocyanidin and/or catechin biosynthesis. The various DFR has different substrate specificity, which finally affects the type of anthocyanidins produced by each species (more about substrate specificity of petunia DFR in Section 3.16.5.2). Anthocyanidin synthase (ANS) catalyzes the final oxidation of a colorless flavan-3,4-diol (leucoanthocyanidin) to an anthocyanidin. Similar to F3Hs and flavonol synthases (FLSs), ANSs belong also to the 2-oxoglutarate-dependent oxygenases. The formed anthocyanidin is relatively unstable. It is readily glucosylated by glucosyltransferase (GT), and in some cases acylated by aromatic/aliphatic acyltransferase (AT) and/or methylated by methyltransferase (MT).

The biosynthesis of 3-deoxyanthocyanins is on the other hand thought to occur through the formation of flavan-4-ols by the activity of flavanone 4-reductase (FNR) and finally through the action of ANS (**Figure 17**). Recent studies on FNR of recombinant *S. cardinalis* showed that this enzyme both has DFR and FNR activity,[407] which is in accordance with the ability of the recombinant DFR enzymes of *Malus domestica*, *Pyrus communis*, and *Z. mays* to produce 3-deoxyflavonoids.[408,409]

3.16.5.2 New Anthocyanin Flower Colors by Molecular Bioengineering

Classical breeding methods including continuous crossing/selection and in some cases mutations, have been used to develop new cultivars with flowers varying in both colors and patterns. However, most species lack a particular color due to the absence of a biosynthetic gene or because of the substrate specificity of an enzyme in the pathway. The search for the blue rose is just one example. Over the past two decades knowledge about flower coloration at the biochemical and molecular level has made it possible to achieve new varieties by genetic engineering. Today virtually all the genes that encode the enzymes of anthocyanin biosynthesis have been isolated. By introducing new genes in plants encoding for novel enzyme activities and transcription factors or inactivation of endogenous genes used in anthocyanin biosynthesis, new varieties with modified flower colors and plant coloration have been created. A few examples are depicted below.

Pelargonidin glycosides are not found in petunias (see cross references in Andersen and Jordheim[4]), which is the main reason for the absence of orange- to nearly scarlet-colored *Petunia* species in nature. The enzyme DFR in *Petunia* has strict substrate specificity, and is unable to convert dihydrokaempferol into the substrate for pelargonidin, namely leucopelargonidin. An orange petunia was, however, created two decades ago,[410] and represents the first product of successful manipulation of flower color by gene technology. This was achieved by producing the maize DFR enzyme, which was able to convert dihydrokaempferol in a white petunia variety accumulating this substrate.

Flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H), which are members of the cytochrome P450 family, play key roles in the determination of the substitution pattern of the B-rings of the anthocyanidins. These enzymes have generally broad substrate specificity, and are able to catalyze hydroxylation of flavanones, dihydroflavonols, flavonols, and flavones. F3′H is necessary for the synthesis of 3′-hydroxylated anthocyanidins (e.g., cyanidin), while F3′5′H participates in the synthesis of 3′5′-hydroxylated anthocyanidins (e.g., delphinidin). Thus, will the development of blue roses, carnations, chrysanthemums, or tulips by molecular breeding, include introduction of F3′5′H activity for production of delphinidin derivatives in the petals, which are not produced in native flowers. Florigene Ltd. (Australia) and Suntory Ltd. (Japan) have successfully developed transgenic violet carnations by introduction of petunia F3′5′H and DFR genes into a DFR-deficient white carnation.[411] The petals of these carnations predominantly contained delphinidin derivatives. Under the name Moondust they were the first transgenic floricultural crop to be sold. However, blue to violet flower colors are known to depend on more factors than just their content of delphinidin derivatives (see Section 3.16.2). After a closer look on Moondust, Fukui *et al.*[412] concluded that the following reasons accounted for the bluish hue of the transgenic carnation flowers: (1) accumulation of the delphinidin-type anthocyanins as a result of flavonoid 3′,5′-hydroxylase gene expression, (2) the presence of a flavone derivative as a strong copigment, and (3) an estimated relatively high vacuolar pH of 5.5.

3.16.6 Functions of Flavonoid Pigments in Plants

In nature, flavonoid pigments are involved in a wide range of known and most probably unknown functions. They are integrated into the plant's strategies for survival by providing pigmentation for flowers, fruits, and seeds to attract pollinators and seed dispersers (Sections 3.16.6.1 and 3.16.6.2), serving protective roles as shields against abiotic stresses like UV–B radiation, temperature variation, mineral stress, and so on, and active defensive roles against pathogens, insects, and herbivores (Section 3.16.6.3) (see Chapter 4.08). The functions of flavonoid pigments in leaves, seedlings, roots, and stems are, however, less obvious than those reported for flowers and fruits. Understandably, most plant physiologists and ecologists are more inclined to consider the physiological and ecological roles of the pigments than to concern themselves with their chemical nature, as a number of excellent reviews and papers in this field attest (e.g., Chalker-Scott; Harborne and Grayer; Simmonds; Gould; Gould and Lister; and references therein).[413–417] In a recent paper data have been reported which suggest that specific polyacylated anthocyanins in flower petals can screen harmful UV–B efficiently.[418] See more about the mechanism in Section 3.16.2.8.

3.16.6.1 Flavonoid Pigments in Pollination

The importance of flavonoid pigments in flowers for attracting bees, butterflies, birds, and other animals to ensure pollination is well established.[414] The pollination syndrome hypothesis (e.g., Vogel, Faegri and van der Pijl, Fenster *et al.*)[419–421] has provided an important conceptual framework for how plants and pollinators interact. It has been assumed that pollinators are the primary selective agents influencing factors like flower color, while transitions to different colors represent adaptation to different suites of pollinators. However, in recent years alternative interpretations have also been suggested, including the possibilities that flower color transitions are nonadaptive, or reflect natural selection on pleiotropic effects of genetic variants that affect flower color.[422]

Bird pollination (ornithophily) appears to have evolved independently in a variety of plant genera, usually from bee pollination.[423,424] Ornithophilous flowers, which are typically red or orange, have elongated floral tubes, reduced floral limbs, exserted stigmas, and copious dilute nectar. Some phenotypic convergences in plants with this pollination syndrome have recently been reviewed by Cronk and Ojeda.[425] Thus far, only one gene, flavonoid-3′-hydroxylase (F3′H) in morning glories (*Ipomoea*/*Pharbitis*) has been linked with shifts to

ornithophily.[426] The ancestral color in *Ipomoea* is blue or purple based on cyanidin and peonidin glycosides (see cross references in Andersen and Jordheim[4]), and together with other traits this indicates an adaptation to bee pollination.[427] Blue and mauve flower colors, attractive to bee pollinators, are generally based on delphinidin, petunidin, or malvidin, however, the blue or purple colors of the peonidin and cyanidin derivatives of *Ipomoea* spp. are most probably caused by the intramolecular association with caffeic acid residues in these polyacylated molecules. In one clade including *I. quamoclit* and five other species, there has been a shift to red flowers containing pelargonidin derivatives implying hummingbird pollination. The F3′H gene, which is required for the production of cyanidin rather than pelargonidin, has been downregulated in the *I. quamoclit* lineage.

In the genus *Mimulus* (monkeyflowers) two closely related species, *M. lewisii* and *M. cardinalis* display great differences in floral characteristics. The former is pollinated mainly by bumblebees and has pink flowers, higher proportion of pelargonidin derivatives, nectar guides, and the dominant allele *YUP*, which prevents carotenoid deposition. The latter is associated with hummingbird pollination, red flowers, higher proportion of pelargonidin derivatives and the recessive allele *yup*, which allows carotenoid deposition.[415,428,429] When the *yup* allele of *M. cardinalis* is introgressed into the *M. lewisii* background, hummingbird visitation increases dramatically, whereas bee visitation is considerably lowered.[430] This suggests that an adaptive divergence in pollination syndrome can be initiated by a major change in flower color alone.[425] However, a recent study indicates that the evolutionarily recent appearance of red-pigmented flowers in the 'yellow monkeyflower' section of *Mimulus* was not associated with a transition to 'red-flower' pollinators such as hummingbirds.[431]

Although floral traits including color have been associated with particular pollination mechanisms as far back as in the work of the Neapolitan botanist Federico Delpino (1833–1905), the following example may illustrate some difficulties in the process of revealing exact pollination mechanisms, even today. In 1998 Olesen *et al.*[432] published an article entitled *Mauritian Red Nectar Remains a Mystery*. They reported that the unique presence of scarlet-red nectar in three bird-pollinated plant species in Mauritius was based on an aurone (**Figure 18**, **1**). The authors stated that the three endogenous species, *Nesocodon mauritianus* (Campanulaceae),

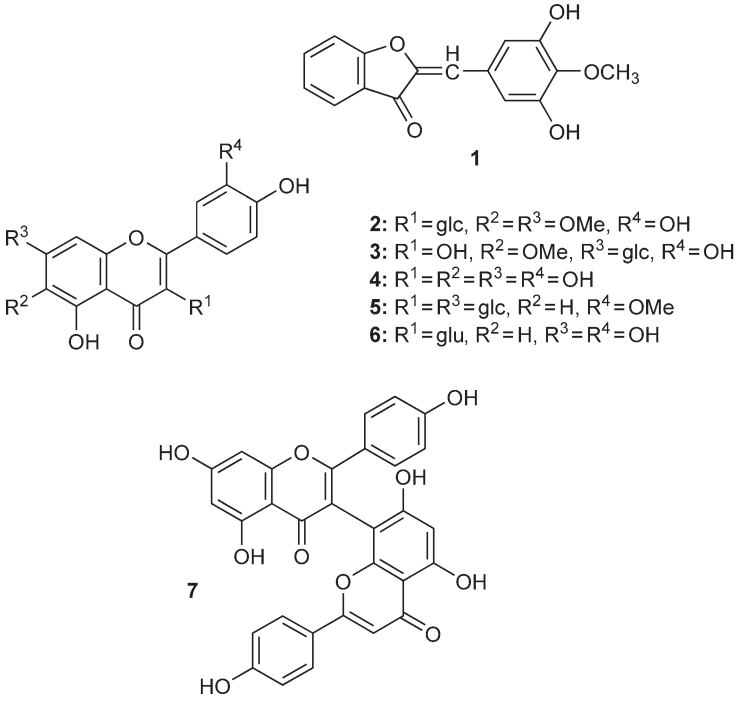


Figure 18 Structures of 3′,5′-dihydroxy-4′-methoxyaurone (**1**), 6,7-dimethoxy-3′,4′,5-trihydroxyflavone-3-*O*-glucoside (**2**), 6-methoxy-3′,4′,3,5-tetrahydroxyflavone 7-*O*-glucoside (**3**), 3,5,6,7,3′,4′-hexahydroxyflavone (**4**), isorhamnetin (5,3′-dihydroxy-4′-methoxyflavone) 3,7-diglucoside (**5**), quercetin (5,7,3′,4′-tetrahydroxyflavone) 3-*O*-glucuronide (**6**), and biapigenin (dimeric flavone) (**7**).

Trochetia boutoniana, and *T. blackburniana* (Malvaceae), were the only ones in the world that produce a colored nectar. They envisaged three explanations for the evolution of the unique coloration: (1) the pigment was an attractant for an endemic recently extinct original pollinator; (2) the red color was an honest signal to pollinators, thereby improving their foraging efficiency and consequently providing an advantage to the red nectar containing plant species; and (3) the red pigment was associated with a deterrent against nectar robbers. Olesen *et al.*[432] considered explanations (2) and (3) as unlikely.

A year later, by using knowledge-based computational structure–activity relationship models, explanation (3) was on the other hand supported.[433] In this paper it was hypothesized that the aurone responsible for the uniquely red nectar functions as a repellant of nectar-robbing or herbivorous mammalian species. Recently, a new dimension was brought into this mystery.[434] It was reported that at least two of the three red nectar-producing species were visited and pollinated by endemic lizards (**Figure 19**). Experimental evidence reports that *Phelsuma* geckos preferred colored over clear nectar in artificial flowers. Hansen *et al.* expressed that colored nectar could additionally function as an honest signal that allows pollinators to assert the presence and judge the size of a reward prior to flower visitation, and to adjust their behavior accordingly, leading to increased pollinator efficiency according to explanation (2). It was reported by Olesen *et al.*[432] that the nectar's pH was as high as 9.2 (the known pH range of all species is 3–10), and when placed in acid, the red nectar turned yellow. No other chemical data were supplied with the pigment. The red pigment of the nectar is a tri-*O*-substituted aurone with a substitution pattern, which has not been reported for any aurone before (**Figure 18**, **1**). The author's suggest that the red color of the pigment is due to the anionic form of the aurone. We suggest that this form is achieved by deprotonation of the phenolic groups under the relative basic conditions in the nectar, and will thus have an increased chromophore giving red color instead of the yellow color of the aurone under acidic conditions.

3.16.6.1.1 Nectar guides

Many flowers contain visible dots, stripes, and patterns. The foxglove (*Digitalis purpurea*), for instance, has a pink bell-shaped corolla pigmented with cyanidin and peonidin 3,5-diglucosides. Higher concentrations of the same pigments inside the bell makes patterns, called nectar guides or honey guides, which helps pollinating insects to the stigma and style. Not surprisingly, the nectar guides in general are displayed predominately on the exposed 'facial' surface of the flower, where the pollinator makes its landing. Other flowers have UV patterns invisible to humans but visible to insects, again with the purpose of guiding pollinating insects. In radial flowers, the UV-absorbing pigments responsible for the UV demarcation are often concentrated in the center of the flower. The petals of the black-eyed Susan (*Rudbeckia hirta*, Compositae) was found to contain three flavonols

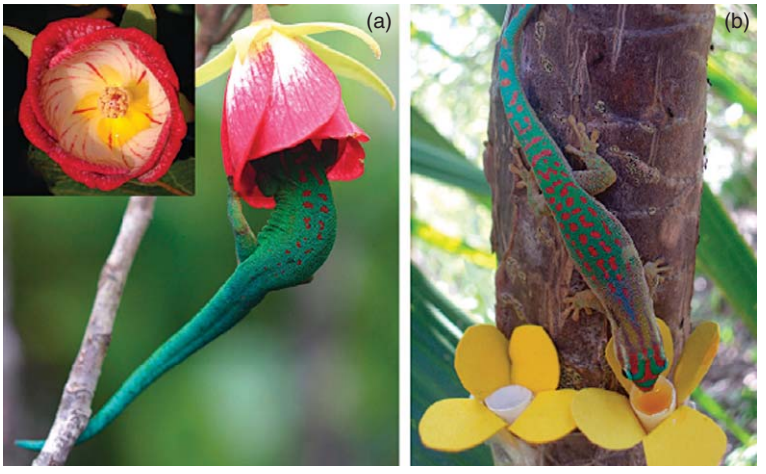


Figure 19 *Phelsuma* geckos and colored nectar. (a) *Phelsuma cepediana* nectar-feeding at *Trochetia blackburniana*. (b) *Phelsuma ornate* choosing between clear and colored nectar at experimental flowers. Photos courtesy: D. M. Hansen; K. Beer; C. B. Muller, *Biol. Lett.* **2006**, *2*, 165–168.

(6,7-dimethoxy-3′,4′,5-trihydroxyflavone-3-*O*-glucoside, 6-methoxy-3′,4′,3,5-tetrahydroxyflavone 7-*O*-glucoside, and 3,5,6,7,3′,4′-hexahydroxyflavone) (**Figure 18**, **2**–**4**) with restricted distribution to the petal bases.[435] These compounds, which showed intense spectral absorptions from 340 to 380 nm, created petal zones of orientation value to the pollinating insect. This was the first time ultraviolet (UV) absorption in a nectar guide was interpreted in chemical terms. The first reports in the field of chemical basis for nectar guides have been reviewed thoroughly by Harborne and Grayer.[414] More recently it has been reported that the corolla of *Brassica rapa* (Cruciferae) has an UV-absorbing zone in its center, containing isorhamnetin 3,7-diglucoside (**Figure 18**, **5**).[436] This flavonol is present at 13-fold greater amounts in the basal parts of the petals than in the apical regions, which is presumed to contribute to the visual attractiveness of *B. rapa* flowers to insect pollinators. The flower of *Hypericum calycinum*, which appears uniformly yellow to humans, bears a UV pattern, presumably visible to insects. Two categories of pigments, flavonoids (the flavonol quercetin 3-*O*-glucuronide and the dimeric flavone, biapigenin) (**Figure 18**, **6**–**7**) and smaller amounts of dearomatized isoprenylated phloroglucinols, were responsible for the UV demarcations of this flower.[437]

The chalcones and aurones, as other flavonoids, absorb strong UV-light giving pattern in petals, which would otherwise be seen as dull or translucent by insect eyes. In wild-type *A. majus*, aurones are produced only in the inner epidermis, accumulating in the hinge (face) region of the petal lobe and in two stripes within the throat. This yellow region is surrounded by magenta anthocyanins, which provide the majority of the color in the petal but are usually absent from the two aurone-producing regions.[438] The pattern of aurone and anthocyanin pigmentation is thought to provide a nectar guide for pollinating bumblebees, and their biosynthesis has evolutionary importance concerning plant–pollinator interaction. Several studies have reported that the loci regulates yellow flower coloration of *A. majus*.[382,438–440] In *Helianthus* (Asteraceae) honey guides in some species resulted from UV absorbance by the chalcone coreopsin and the aurone sulfurein, whereas in *H. annuus* the honey guides resulted from absorbance by quercetin 3- and 7-glucosides.[441] However, the occurrence of yellow flavonoids in other flowers may not be directly correlated with the presence of UV nectar guides. A detailed study of the distribution of chalcones in *Coreopsis bigelovii* flowers revealed that these pigments were present in epidermal cells on both upper and lower surfaces.[441]

In general in plants, nectar guides are prominent in those flowers, which are pollinated by bees. It has also been suggested that carnivore plants use contrasting stripes or UV marks on their pitchers to lure insects. However, after recent experiments with visual signaling it was emphasized that insect traps did not need to sport contrasting colors to be attractive.[442] It might be sufficient that the pitchers are just different from their background.

The chemical basis of UV–visible absorptions in nectar guides has remained remarkably unexplained in many plants. One reason for this is related to analytical difficulties when small amount of material is available. However, the field of nectar guides and pollination may be seen from other more complex angles. Insects and vertebrates have been shown to have multiple classes of photoreceptors that contribute to vision, for example, the honeybee has trichromatic vision based on UV, blue, and green photoreceptors.[443] Perception of color will thus require the integration of information from all the primary receptors, however, the UV receptors have often been singled out for special consideration. It has also been postulated, although related to carotenoids, that insects may sense patterns of polarized light as reflected from the flowers and, in fact, use this as a signal for pollination of a given plant.[444] Finally we will draw attention to AVIs, which are discussed in Section 3.16.3.1. The occurrence of AVIs in many flowers is most probably of vital importance for the presence of nectar guides.

3.16.6.2 Flavonoid Pigments in Seed Dispersal

Herbivorous and frugivorous animals rely on color for identification of edible tissues and for judgment of vegetable ripeness. A gardener will thus experience that yellow- or amber-colored mutants of red raspberries mostly are ignored by birds. The distinctive colors of many fruits and ‘fruit-similar’ structures are derived from anthocyanins, which render the fruit attractive for seed dispersing animals. Other classes of flavonoids contribute occasionally to yellow, orange, red, or brown colors in fruits (see Harborne[445]). The anthocyanins may be present throughout the fruit (European bilberries, *Vaccinium myrtillus*), while in other cases it is limited to the skin (lowbush blueberries, *V. angustifolium*) and juice (blood orange, *Citrus sinensis*). Anthocyanin colors are as for other flavonoid colors primarily determined genetically, although environmental factors such as pH,

temperature, light conditions, and availability of nutrition can have effect on pigment composition and on the final hue of the fruit.

The qualitative and quantitative anthocyanin content of most of the common fruits used in the human diet is now determined (**Table 3**), however, some variation in content between different varieties and cultivars are very common. On the basis of principal component analysis of the content of 15 different anthocyanins in 30 samples of bilberries (*V. myrtillus*) of various origins, a clear separation between a group composed of Norwegian and Swedish berries and a group of berries with Italian or Romanian origin was revealed.[446] Cyanidin glycosides were slightly better represented in all the samples of the first group, while delphinidin glycosides were better represented in the latter. Recently, the variation of the content of the same 15 anthocyanins in berries from 179 individual bilberry plants in 20 populations on a south–north axis of about 1000 km in Finland were analyzed.[447] A significantly lower content of the total anthocyanins was observed in the berries of the southern region compared to those in the central and northern regions. Differences in the proportions of anthocyanins were also observed.

Burns and Dalen[448] postulated that red-orange autumn foliage of Canadian shrub species would accentuate the conspicuousness of black-colored fruits to birds. Experimental manipulation of fruit and background foliage colors confirmed that the black-red contrast was indeed an effective enhancer of fruit-removal rates by avian dispersers. Although fruit colors are traditionally viewed as an adaptation to seed dispersers, the selective pressure on fruit coloration are not well understood.[449] Most bird species exhibit inconsistent and transient color choices with high variability within and between individuals. Cazetta *et al.*[449] suggest that fruit colors differ between habitats because fruit colors that have strong chromatic contrasts against background can increase plants' reproductive success, particularly under variable light conditions.

In the Gymnospermae, anthocyanin pigmentation is most commonly observed in the reproductive structures (the strobili or cones),[450] which is quite interesting since anthocyanins are mainly associated with flower color in the Angiospermae. From flowers and cones of species in the Pinaceae, variation between simple 3-glucosides of cyanidin and delphinidin and their methylated analogues, peonidin, petunidin, and malvidin (**Table 5**), have been reported.[451–453] These pigments are the only reports of methylated anthocyanins being found outside of the Angiospermae. For some Pinaceae species (e.g., Norway spruce, *Picea abies*) two types of clones were found.[452,453] One type contained methylated anthocyanidins (peonidin and petunidin), while the other did not, which suggested that the methylating genes have evolved recently. Anthocyanins have been reported to play various roles in protecting plants (Section 3.16.6). Several observations suggest that anthocyanins may lack this protective function in conifer cones. First, the anthocyanins are restricted to the outer cone scales. Second, the anthocyanins are only present for a short period of time early in development and disappear once the pollen and egg cells are formed.[453]

In the Gymnospermae family Podocarpaceae, anthocyanins are as well located mainly in seed-bearing structures, where they have a comparable role to angiosperm fruit pigments. A typical ripe female ovule of white pine (*Dacrycarpus dacrydioides,* Podocarpacaeae) consists of an orange-red receptacle, atop a bluish seed and two dark-blue undeveloped ovules, which must be among the most outstanding anthocyanin-colored structures in nature. It gives the appearance of an angiosperm fruit, and the anthocyanins obviously render the structure more readily detectable and aid in animal dispersion of the seed. While pelargonidin 3-neohesperidoside (2-(rha)glc) was the major pigment in the receptacles, cyanidin 3-glucoside and delphinidin 3-glucoside constituted the major anthocyanins in the seeds and undeveloped ovules.[454] Since the undeveloped ovules are nonmature seeds, it is expected that the anthocyanin content in seeds and ovules are rather similar, however, the relative proportions of these two pigments were different. The receptacles of *Podocarpus* species, which mainly contain cyanidin 3-neohesperidoside are more reddish in color than the receptacles of white pine,[156,455] which contain pelargonidin 3-neohesperidoside, again in accordance with the colors of the receptacles. In fact, anthocyanins containing neohesperidosides are very rare,[4] and the 3-neohesperidosides of cyanidin and pelargonidin have not been found outside the genera *Podocarpus* and *Dacrycarpus*. Finally, we want to highlight the extraordinary color similarities of the receptacles of several *Podocarpus* species, which are mainly located in the Southern hemisphere, and the arils of *Taxus baccata*, Pinaceae, mainly located in the Northern hemisphere. While the receptacles are colored by hydrophilic anthocyanins, the arils are colored by lipophilic carotenoids (rhodoxanthin, etc.).

As the most visible role of anthocyanins is to impart colors, the adaptive significance of anthocyanins in fruits, seeds, and fruit-similar structures is invariably attributed to the attraction of seed dispersers. However, as suggested in Section 3.16.6, anthocyanins in vegetative tissue may also have other functions, for instance in plant defense. Finally, here we include one report related to fruit color polymorphism. This phenomenon occurs in at least 19 plant families;[456–458] however, the ecological and evolutionary dynamics of fruit color polymorphisms remain poorly known because patterns and agents of selection have rarely been identified. *Acacia ligulata* populations are composed of two or three color morphs, producing red, yellow, or (more rarely) orange arillate diaspores.[459] Seed production differences between these morphs were found to be a function of both intrinsic plant characters (fruit production) and predispersal seed predation.[460] Thus, it was suggested that pleiotropic effects might be a common feature of fruit color polymorphisms, and that the most obvious selective agents (i.e., seed dispersers) may not always be the most important.

3.16.6.3 Roles of Anthocyanins in Vegetative Tissue, Mainly Leaves

The functions of red colorants in vegetative tissue have puzzled scientists for more than a century. The presence of colored flavonoids in young leaves, seedlings, roots, and stems has not been looked upon as obvious, as the presence of colored flavonoids in fruits and flowers. Lee and Collins[320] have studied the distribution of anthocyanins (and betacyanins) in leaves (expanding, mature, and senescing) of tropical plants. At both expanding and senescing stages they found anthocyanins primarily in the mesophyll. In their opinion was the presence of anthocyanins in the mesophyll of so many species inconsistent with the hypothesis of protection against UV damage and fungal pathogens. Dominy *et al.*[461] have noted that a common location for most of the anthocyanin in young leaves is just above the lower epidermis and well away from photosynthetic tissue,[145,462] and express that this would appear to offer little benefit for either photoprotection or photoinhibition. Gould and Lister[417] have pointed out that the vacuolar location of the colored forms of the anthocyanins precludes any major role in free-radical scavenging in planta, since almost all free radicals originate from organelles, the plasma membrane, and the apoplasm. Cytoplasmic antioxidants, and the extremely efficient enzyme superoxide dismutase, should be more optimally located to scavenge organelle-derived reactive oxygen.

However, there exist increasing evidences that anthocyanins, particularly when they are located at the upper surface of the leaf or in the epidermal cells, also play roles in the physiological survival of plants. It has been outlined that foliar anthocyanins accumulate in young, expanding foliage, in autumnal foliage of deciduous species, in response to nutrient deficiency, temperature changes, or UV radiation exposure, and in association with damage or defense against browsing herbivores or pathogenic fungal infections. The functions have in this context mainly been hypothesized around the anthocyanins as compatible solutes contributing to osmotic adjustment to drought and frost stress, as antioxidants, and as UV and visible light protectants. Johnson *et al.*[463] placed the function of anthocyanins in leaf, or in their case in stems, into a fundamental punch line in their title 'better red than dead,' which may illuminate the importance of anthocyanin coloration also in vegetative tissue. In this chapter the different responses have been separated under subtitles, although they in many cases may be related to each other. The chapter is far from being exhaustive with respect to literature coverage. Its nature is more introductory with selected examples from the most recent publications in the field. For further reading reviews by Chalker-Scott,[413] Harborne and Williams,[148] Gould and Lee,[464] Dominy,[461] Simmonds,[415] Close and Beadle,[465] Gould and Lister,[417] and Manetas[466] are highly recommended.

3.16.6.3.1 Photoprotection

Historically, the first scientific reference concerning the role of anthocyanins in vegetative tissues is attributed to Haberlandt,[353] who assumed a kind of photoprotective role of the red leaf colorants. According to Karageorgou *et al.*[467] this function is still preferred among physiologists. The anthocyanins are thought to be working either as sunscreens by attenuation of excess visible light, which reduce excitation load in the underlying mesophyll cells, or/and by their detoxification of oxy-radicals produced during photosynthesis. However, the literature is far from consistent here. While laboratory trials indicate that red leaves are less prone to undergo photoinhibition than green leaves,[468–471] field studies have failed to show any actual photoprotective superiority of red leaves.[472–478] Some recent specific results reflecting correlation between anthocyanin content in vegetative tissue and their potential function(s) are discussed.

In 2002 the first report on anthocyanins was published, which proved this type of pigments to function as photoprotectors of light-sensitive defensive compounds in plants.[477] Silver beachwood (*Ambrosia chamissonis*) located along the sunny Pacific coast of North America, contains high amounts of thiarubrines in stems and leaf petioles. Thiarubrines are red plant pigments that decompose easily to colorless thiophenes when exposed to sunlight (**Figure 20**). They are in tissue compartmentalized in laticifers that are surrounded by anthocyanin-containing cells. In leaves and stems of seedlings the anthocyanins were identified as mainly cyanidin 3-*O*-[6″-*O*-(malonyl)glucoside] and cyanidin 3-*O*-glucoside (**Figure 20**), while none of these anthocyanins was detected in roots. To correlate anthocyanin distribution with thiarubrine photoprotection, changes in thiarubrine A and thiophene A levels were measured in seedlings and roots exposed to light. In roots, thiarubrine A levels decreased by 94 and 100% after 30 min and 4 h of irradiation, respectively, with a concomitant threefold increase in thiophene A levels. In leaves and stems, thiarubrine A levels did not change appreciably during light exposure. To confirm the photoprotective function of anthocyanins, solutions of cyanidin 3-*O*-glucoside were used to filter visible light incident on a solution of thiarubrine A. Anthocyanin solutions with concentrations higher than 0.1 mmol l^{-1} completely prevented thiarubrine photoconversion. The conclusion is that when the light-screening sheath of anthocyanins is absent and the laticifers containing red thiarubrines are exposed to light, rapid bleaching of the thiarubrine content occurs. Without a mechanism for photoprotection including anthocyanins, sunlight would rapidly convert the red thiarubrines in *A. chamissonis* into colorless thiophenes.

The red-to-blue colors of juvenile leaves is most commonly caused by anthocyanins appearing within vacuoles of epidermal and/or mesophyll cells within hours to days during seedling germination. It has been

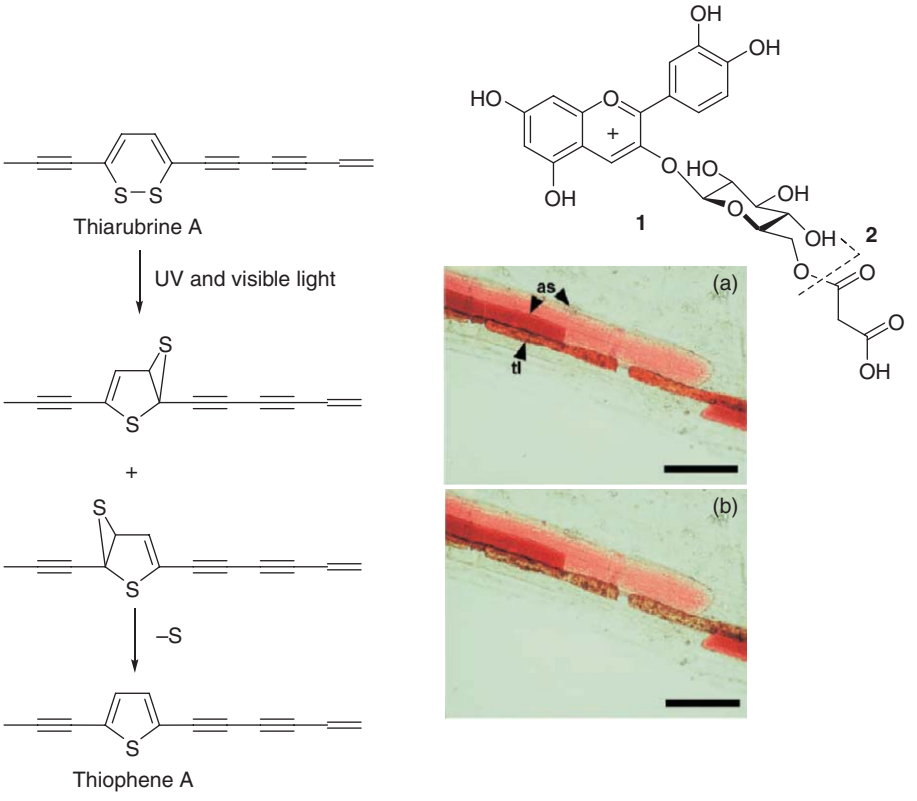


Figure 20 Chemical structures of thiarubrines and anthocyanins occurring in *Ambrosia chamissonis*. *Left*: thiarubrine A is converted into its photoproduct thiophene A by exposure to UV and visible light. *Pictures*: Anatomy of thiarubrine photoprotection in *A. chamissonis*. (a), (b) Thiarubrine laticifers (**tl**) and anthocyanin sheath (**as**) cells before (a) and after (b) 2 min irradiation. The discoloration and granular appearance of the thiarubrine laticifer after light exposure is visible. Bars = 200 μm. Photos courtesy: J. E. Page; G. H. N. Towers, *Can. Planta* **2002**, *215*, 478–484. *Top right*: structures of cyanidin 3-[6-(malonyl)glucoside] (**1**) and cyanidin 3-glucoside (**2**).

argued that juvenile leaves contain anthocyanins to protect themselves in early development stages. In developing leaves, Hughes *et al.*[478] found that anthocyanin disappearance occurred when: *c.*80% of mature leaf thickness had been attained, *c.*50% of mature photopigment concentrations was developed, and after differentiation of the mesophyll into palisade and spongy layers. The loss of anthocyanins during leaf development may thus correspond to a decreased need for photoprotection, as photosynthetic maturation allows leaves to utilize higher light intensities. Gould *et al.*[479] on the other hand surveyed 1000 leaves from a forest population of *Quintinia serrata*, which displayed natural polymorphism in leaf color. Red leaves contained cyanidin 3-glucoside and cyanidin 3-galactoside, while green leaves lacked anthocyanins, but had otherwise similar pigment profiles. The anthocyanins were most commonly located in the vacuoles of photosynthetic cells, and most abundant in older leaves on trees found at the uppermost level of a mature forest with south-facing gaps. It was therefore indicated that anthocyanins most probably were associated with photosynthesis. However, the anthocyanins did not serve any auxiliary phytoprotective role. Their function was to protect shade-adapted chloroplasts from brief exposure to high-intensity sunflecks.

Leaf color in some individuals of *Cistus creticus* turns transiently to red during winter, while neighboring individuals occupying the same site remain green. Kytridis *et al.*[480] have analyzed the accumulation of leaf anthocyanins in the two phenotypes. The frequency of red individuals was considerably higher in fully exposed sites, pointing to a photoprotective function of leaf anthocyanins. Red leaves were among other factors also characterized by lower nitrogen contents at all sampling dates throughout the year. The nitrogen content of leaves is strongly correlated with photosynthetic capacity,[481] and a link between the lower nitrogen levels and the lower linear electron transport rates in the red phenotype of *C. creticus* was assumed.[480] Lower nitrogen levels may leave the red phenotype more vulnerable to photoinhibition and oxidative stress, due to lack of inadequate photochemical and nonphotochemical sinks for excess excitation energy. On the basis of correlative evidences it was thus assumed that the anthocyanins in red leaves were an adaptation to compensate for this deficiency, with the aim of reducing the risk of photodamage.

3.16.6.3.2 Antioxidant activity

Pure anthocyanins and purified anthocyanin extracts have been shown to have strong antioxidant activity in many *in vitro* assays. Anthocyanins, as other flavonoids, have been shown to act as scavengers of various oxidizing species, that is, superoxide anion, hydroxyl radical, or peroxy radicals. They may act as quenchers of singlet oxygen or they may react with metal ions and thereby indirectly decrease hydroxyl radical production. Anthocyanins do not react specifically with a single species, and so a number of different evaluation methods (assays) have been developed. This makes comparison of the various studies very problematic, and the antioxidant-related effects difficult to interpret for *in vivo* conditions. Regarding anthocyanins in living vegetative cells, the purpose whether they scavenge or quench reactive oxygen species is inadequately known. A growing body of results indicates that anthocyanins contribute to control the levels of reactive oxygen in plant cells.[417,482–485] However, not all results are of the same kind: While Gould *et al.*[486] have proposed that cytosolic and organelle-bound antioxidants, rather than the vacuolar anthocyanins, may offer the first line of defense against oxidative stress in leaves, Kytridis and Manetas[484] have concluded that leaf vacuolar anthocyanins may afford a detoxifying sink for some reactive oxygen species when the chloroplastic, the first line of antioxidative defense, is surpassed. Although not optimally located in relation to the chloroplastic source of oxy-radical production, this latter function is more possible for anthocyanins located in mesophyll than in epidermal vacuoles. Here are some of the more recent results in the field.

Red leaf lettuce (*Lactuca sativa*) (Lollo Rosso) has been grown under three types of plastic films that varied in transparency to UV radiation.[487] Exposure to increased levels of UV radiation during cultivation caused the leaves to redden and considerably increased concentrations of cyanidin glycosides and other phenolics. Red coloration was found mainly in the outer leaves and toward the extremities of the inner leaves, where the leaves were exposed to most light. Neil and Gould[488] have examined the potential of anthocyanins to extenuate photooxidative injury in a similar type of leaves, both by shielding chloroplasts from excess high-energy quanta, and by scavenging reactive oxygen species. To distinguish between the impacts of these two putative mechanisms, superoxide ($O_2^{\bullet -}$) concentration and chlorophyll oxidation were measured for chloroplast suspensions under various light and antioxidant-supplemented environments. A red cellulose filter, which had optical properties approximated that of anthocyanins, was used to shield irradiated chloroplasts. The outcome was a

33% decline in rate of $O_2^{\bullet -}$ generation and 37% reduction in chlorophyll bleaching. Colorless and blue tautomers of cyanidin 3-*O*-[6″-*O*-(malonyl)glucoside] at pH 7 removed up to 17% of $O_2^{\bullet -}$ generated by chloroplasts, indicating that cytosolic anthocyanins can serve as effective antioxidants. Red flavylium cation forms, typical of vacuolar anthocyanins at lower pH values, also showed strong reducing potentials as indicated by cyclic voltammetry potentials, which declined by 40% after 15 min exposure to $O_2^{\bullet -}$.

Shao *et al.*[485] have looked at antioxidant capability, among other factors, in leaves of the wild type *Arabidopsis thaliana* L. and tree mutants deficient in anthocyanin biosynthesis during treatment with temperatures ranging from 25 to 45 °C (see Chapter 3.28). High temperatures are harmful to plant development, and influence the formation and functions of the photosynthetic apparatus in plants. In comparison to the wild type, the mutants lacking anthocyanins had lower activities of superoxide dismutase, ascorbate peroxidase, and inferior scavenging capability to DPPH (1,1-diphenyl-2-picrylhydrazyl) radical under heat stress. In addition H_2O_2 accumulated in the leaf vein and mesophyll cells of the mutants at 40 °C. The same group has also investigated antioxidative capability within the same type of leaves under photooxidation stress induced by methyl viologen (5 μm) in light.[489] In comparison with the wild-type plant, photooxidation resulted in significant decreases in the contents of total phenolics and flavonoids, total antioxidative capability, and chlorophyll fluorescence parameters, and to increase in cell-membrane leakiness in the three mutants, which were deficient in anthocyanin biosynthesis.

3.16.6.3.3 Antiherbivory activity

It is generally accepted that flavonoids, along with other plant polyphenols, play a role in protecting plants from both insect and mammalian herbivory (see Chapter 4.08). Among the flavonoids, attention has been mainly centered on polymeric flavolans or proanthocyanidins but some research has been concerned with monomeric flavones, flavonols, and isoflavones [148] (see Chapter 6.18). The roles of colored anthocyanins are in this context still under discussion. As physiologists seem to prefer the photoprotecitve role for anthocyanins in leaves, the antiherbivory theory has been championed by ecologists.[461,490,491] The fact that the anthocyanins are located in vacuoles of epidermis and the mesophyll away from the photosynthesis apparatus and the chloroplastic source of oxy-radical production, has among other factors supported antiherbivory hypotheses. Here are more recent hypotheses, which propose that nongreen plant coloration based on anthocyanins has evolved as a defense against herbivores.

Hamilton *et al.*[490] have proposed that leaf colors function to signal the defensive strength of an individual plant to herbivorous insects. It was predicted that tree species suffering greater insect damage would, on an average, invest more in autumn-color signaling than less troubled species. Protective anthocyanin coloration promotes handicap signals, which indicate plant fitness. Karageorgou *et al.*[467] have examined whether the assumed handicap signal is honest and, accordingly, costly, by seeking a correlation between anthocyanin and total phenolic levels in 11 plants exhibiting variation in the expression of the red character, either between individuals or between modules on the same individual. On the basis of the results they concluded that for senescing leaves the redness was both honest and costly. They did not find the same results for young, developing leaves, and questioned the handicap signal hypothesis in this case. Young leaf redness fits more to alternative hypotheses that red leaf color is less easily perceived by folivorous insect photoreceptors, or that red leaf color undermines insect camouflage.[467]

Lev-Yadun *et al.*[491] have earlier proposed that the diversity of plant coloration undermines the crypsis of their herbivorous predators. Many color patterns in plants undermine the camouflage of invertebrate herbivores, especially insects, thus exposing them to predation and causing them to avoid plant organs with unsuitable coloration, to the benefit of the plants. In antiherbivory strategy dark colors can camouflage leaves against the exposed soil and litter of forest floors,[492,493] or they can mimic dead leaves.[494] Red leaves might appear dark or dead to a potential herbivore, since most nonmammalian folivores lack red light receptors.[461]

The anthocyanins are in contrast to certain other phenolic compounds reckoned to be nontoxic to higher animal species. However, cyanidin 3-glucoside, which is the most abundant foliar anthocyanin, has been reported to inhibit the growth of larvae of the tobacco budworm, *Heliothis virescens*, an important pest of cotton and other crops.[495] Recently, Johnson *et al.*[496] have examined resistance due to anthocyanins from commercial petunia flowers (*Petunia hybrida*) for insecticide or antifeedant activity against corn earworm (*Helicoverpa zea*) and cabbage looper (*Trichoplusia ni*). The petunia flowers studied contained a star pattern, with colored and white sectors. Corn earworm larvae ate in most cases significantly less colored sectors than white sectors in no-choice bioassays. The studies demonstrated that the colored sectors of these petunia cultivars slowed the

development of the larvae, and indicated that anthocyanins play some part in flower defense in petunia. Herbivory and fungal infection of Chinese cabbage (*B. rapa* ssp. *pekinensis*) leaves have been found to increase the total amount of anthocyanins.[497] However, anthocyanin-rich extracts did not influence the feeding behavior or survival rate of aphids, nor inhibit larval growth of the fruitworm.[498–500]

3.16.6.3.4 Anthocyanin induction caused by different stressors

An assortment of intrinsic and environmental factors has been linked to anthocyanin induction, accumulation, or inhibition in vegetative tissue. Plants are most probably equipped with specific pathways to activate anthocyanin synthesis to cope with different stressors.[413] More recent examples of different stressors which have been studied are deficiencies in phosphorous,[501,502] nitrogen,[503,504] increased level of metals,[505] drought, heat, cold, and salinity,[506–510] wounding,[511] pathogen infection,[512] and fungal elicitors.[512] Temporal variation of anthocyanins may also be related to the severity of induced photoinhibition (see Section 3.16.6.3.1),[513] and oxidative stress injury caused by enhanced solar UV–B radiation (see Section 3.16.6.3.2).[514] Here are some selected illustrations.

Schaefer *et al.*[515] have found that anthocyanins can reduce fungal growth in fruits. They reported that the risk of fruit-rot in grape varieties infected with *Botrytis cinerea* decreased with increasing anthocyanin content. Anthocyanins did also inhibit growth rates of nine fruit-rot fungi on agar plates. Based on the phenomena that different stressors initiate anthocyanin production, Chalker-Scott[413,506] has provided a generalized role for the anthocyanins as osmoregulators in plant cells, since most types of suboptimal environments induce water stress, either directly or indirectly. She indicated that since developing leaves lack cell wall modifications to induce cross-resistance, they must rely on vacuolar substances to modify water relations. The high water solubility of anthocyanins makes them easy to accumulate in vacuoles, and they may in this manner serve to decrease leaf osmotic potential. The resulting depression of leaf water potential might increase water uptake and/or reduce transpirational losses. The often transitory nature of foliar anthocyanin accumulation may in this manner allow plants to respond quickly and temporarily to environmental variability rather than through more permanent anatomical or morphological modifications.

To understand the response of plants to varying nitrogen (N) levels, a growth system has recently been developed where N was the growth-limiting factor.[504] An *Arabidopsis* whole genome microarray was used to evaluate global gene expression under different N conditions. Plants went obviously purple in color under severe N limiting conditions. The genes involved in anthocyanin biosynthesis, such as leucoanthocyanidin dioxygenase and dihydroflavonol reductase, were upregulated just over twofold under mild N stress, but increased to about 14- and 18-fold under severe N stress. CHS, which participates in the early stages of the biosynthetic pathway to all flavonoids was upregulated only under severe N stress.

3.16.7 Anthocyanin Production

The main current methods for producing anthocyanins rely on plant extraction, a process that often is subjected to seasonal variability, low purity, poor yields, and high expenditures. Over the past decade interest in and demand for natural food colorants and pharmacologically interesting natural compounds have encouraged new research initiatives aimed at the development of more efficient means of harvesting anthocyanins. Among these are various attempts to produce anthocyanins from plant cell and tissue cultures. The construction of *Escherichia coli* recombinant strains and the development of fermentation approaches that has allowed relatively high yield anthocyanin production from this microorganism,[516] is very promising. Anthocyanins may also be produced by synthesis, or by hemisynthesis from other types of flavonoids;[4] however, restrictions with respect to legislation limits the applications of these compounds. In the past, the leading techniques employed to elucidate biosynthetic pathways in plants have consisted of feeding experiments with radioactive or isotope-labeled precursors. Isotope-labeling methods lead to selective enhancement of signals from nuclei with low natural abundance. With the development of plant cell culture methodologies, it has become feasible to reveal biosynthetic pathways by isolating and characterizing the participating enzymes. Alternatively, if isotope labeled compounds like anthocyanins are made, their content in tissue and derived metabolites can be measured quantitatively by hetero-nuclear NMR.

3.16.7.1 Production of Anthocyanins in Plant Tissue Cultures

When growth procedures are optimized, cell culture systems have the potential of producing both higher anthocyanin concentrations within reduced time, and another selection of anthocyanins relative to production in whole plants. To improve production of anthocyanins, efforts have mainly been devoted to the optimization of biosynthetic pathways by both process and genetic engineering approaches. The productivity in the cultures is, however, determined by synthetic capacity, storage capacity, and the capacity to metabolize the compounds in the transport and detoxification processes.[517] In a general review, Ramachandra Rao and Ravishankar[517] have dealt with the production of high-value secondary metabolites including anthocyanins through plant cell cultures, shoot cultures, root cultures, and transgenic roots obtained through biotechnological means. In an overview of the status and prospects in the commercial development of plant cell cultures for production of anthocyanin, Zhang and Furusaki[518] have focused on strategies for enhancement of anthocyanin biosynthesis to achieve economically viable technology. The potential of manipulation and optimization of postbiosynthetic events have been reviewed by Zhang *et al.*[519] These events, including chemical and enzymatic modifications, transport, storage or secretion, and catabolism or degradation, were outlined with anthocyanin production in plant cell cultures as case studies.

Production of anthocyanins in plant cell and tissue cultures has been reported for more than 30 species including *D. carota*, *Fragaria* × *ananassa*, *Vaccinium* spp., *Vitis hybrida*, *Solanum tuberosum*, *Malus sylvestris*, *Aralia cordata*, *Perilla frutescens*, *I. batatas*, *Euphorbia millii*, *Strobilanthes dyeriana*, *Hibiscus sabariffa*, *Dioscorea cirrhosa*, and so on(see examples in **Table 13**).[518,551,552] The production has shown to be influenced by a variety of environmental stimuli such as light irradiation, UV light, low temperature, oxygen level, hormones, fungal elicitors, low nutrient levels, and so forth.[517,518,551] Increased level of O_2 supply and light irradiation have, for instance, shown independently positive influence on the production of anthocyanins in suspended cultures of *P. frutescens* cells in a bioreactor.[553] However, a combination of irradiation with a higher oxygen supply reduced the production. In *Vaccinium pahalae* cell cultures, anthocyanin yield was enhanced by increasing sucrose concentration in the liquid suspension medium and by manipulating the initial inoculum density.[546] *Catharanthus roseus* flowers and cell cultures have been shown to accumulate the same type of anthocyanins, however, the differentiated petal cells showed a higher capacity for anthocyanin accumulation than the undifferentiated cell suspension cells.[533] It is also interesting to note that the anthocyanin production within cultures of this species was located to only a fixed percentage of the cells, and that all these cells had about the same concentration of anthocyanins.[554] The anthocyanin production seemed to be ruled by a feedback mechanism giving physiological maximum anthocyanin concentration. Bioreactor-based systems for mass production of anthocyanins from cultured plant cells have been described for several species.[520,553,555–559]

A cell culture system has the potential advantage of facilitating selective production of certain anthocyanins. The nine acylated anthocyanins produced by flowers of *H. orientalis* regenerated *in vitro*, were identical to those of field-grown flowers.[560] However, the concentration of cyanidin 3-[6-(*p*-coumaryl)glucoside]-5-[6-(malonyl)glucoside] was considerably higher in the regenerated flowers. Lower concentration of 2,4-dichlorophenoxyacetic acid in the medium used for strawberry suspension cultures has, for instance, limited cell growth and enhanced both anthocyanin production and anthocyanin methylation.[551] The ratio of peonidin-3-glucoside to the total anthocyanin content increased significantly under these conditions. A methylated anthocyanin like peonidin 3-glucoside is normally not found in intact strawberries, and although the activity of anthocyanin methyltransferase was not measured by Nakamura *et al.*,[551] the results indicated that lower 2,4-dichlorophenoxyacetic acid concentrations enhanced the activity of anthocyanin methyltransferase. Do and Cormier[561] have reported that increased osmotic potential in the medium resulted in a significant intracellular accumulation of peonidin-3-glucoside in *V. vinifera* cells. Similarly, jasmonic acid has been reported to increase the peonidin 3-glucoside content considerably, while the other major anthocyanins only experienced smaller increments.[562]

To improve understanding of the ways in which cinnamic acid groups alter the color retention of anthocyanins, a series of anthocyanins that differed systematically in their acyl group was needed. When cinnamic acids were fed to wild carrot suspension cultures, the proportion of acylated to nonacylated anthocyanins increased.[563] With high relevance for future metabolic studies, *V. vinifera* cells grown in a bioreactor have been used for production of isotopically ^{13}C-labeled phenolic substances such as

Table 13 Qualitative and quantitative anthocyanin content in cell cultures of various plants

Plant species	*Anthocyanins*[a]	*Yield*	*Reference(s)*
Aralia cordata	Cy3-[2-(xyl)gal], Pn3-[2-(xyl)gal]	7.0–17.2% DW	520–522
Ajuga reptans	Cy3,5-di-glc, Cy3-[2-(6-(cum)glc)-6-(cum)glc]-5-glc, Cy3-[2-(6-(cum)glc)-6-(cum)glc]-5-[6-(mal)glc], Cy3-[fer-cum(2-(glc)glc)]-5-[mal-glc], Dp3,5-di-glc, Dp3-[di-fer(2glc-glc)]-5-glc, Dp3-[2-(6-(fer)glc)-6-(fer)glc]-5-[6-(mal)glc], Dp3-[2-(6-(fer)glc)-6-(cum)glc]-5-[6-(mal)glc]	1–3% DW	523–528
Catharanthus roseus	Hi3-[6-(cum)glc], Hi3-glc, Mv3-[6-(cum)glc], Mv3-glc, Pt3-[6-(cum)glc], Pt3-glc	0.6–2.8 mmol l^{-1}	529–533
Daucus carota	Cy3-glc, Cy3-[2-(xyl)-6-(glc)gal], Cy3-[2-(xyl)gal], Cy3-[2(xyl)-6-(6(sin)glc)gal], Cy3-[2(xyl)-6-(6(fer)glc)gal], Cy3-[2(xyl)-6-(6(cum)glc)gal], Cy3-[2(xyl)-6-(6(3,4,5-tri-MeOHcin)glc)gal], Cy3-[2(xyl)-6-(6(di-MeOHcin)glc)gal], Cy3-[6-(6(sin)glc)gal], Cy3-gal	5.4–23.7% DW	534–537
Euphorbia millii	NR	64 mg l^{-1} day^{-1} 4% DW	538,539
Fragaria × *ananassa*	Cy3-glc, Pg3-glc, Pg3-[6-(mal)glc], Pn3-glc	0.9 mg g^{-1} FW 30.2 mg l^{-1} day^{-1}	518,540–542
Glehnia littoralis	Cy3-[6-(6-(fer)glc)-2-(xyl)glc]	14% DW	543
Ipomoea batatas	Cy3-[2-(glc)glc]-5-glc, Cy3-[2-(6-(cum)glc)glc]-5-glc,Cy3-[6-(caf)-2-(glc)glc]-5-glc, Pn3-[6-(caf)-2-(glc)glc]-5-glc, Cy3-[2-(6-(hba)glc)-6-(caf)glc]-5-glc, Cy3-[2-(6-(caf)glc)-6-(caf)glc]-5-glc, Cy3-[2-(6-(fer)glc)-6-(caf)glc]-5-glc, Pn3-[2-(6-(fer)glc)-6-(caf)glc]-5-glc, Pn3-[2-(6-(caf)glc)-6-(caf)glc]-5-glc, Pn[2-(6-(hba)glc)-6-(caf)glc]-5-glc	NR	4,544[b]
Perilla frutescens	Cy3-[6(cum)glc]-5-glc, Cy3-[6(cum)glc]-5-[6-(mal)glc], Cy3-[6-(fer)glc]-5-[6-(mal)glc]	24% DW	539,545
Vaccinium spp.	Cy3-ara, Cy3-gal, Pn3-gal,^{14}C-ANC (after feeding medium with ^{14}C-sucrose)	max 70 g l^{-1}	546,547
Vitis spp.	Cy3-glc, Cy3-cum-glc, Mv3,5-di-glc, Pn3-glc, Pn3,5-di-glc, Pn3-ace-glc, Pn 3-caf-glc, Pn 3-[6(cum)glc]	1.0–16% DW	539,540,548,549
Zea mays	Cy3-[3,6-di-(mal)glc], Cy3-[6-(mal)glc], Cy3-glc, Pn3-glc	NR	550[b]

[a] See **Table 1** for abbreviations.
[b] Not reported from cell culture but isolated from the species.
FW, fresh weight; DW, dry weight; NR, not reported.

anthocyanins.[555,564] The enrichment of labeling (between 40 and 65%) obtained for all compounds, should be sufficient to investigate their absorption and metabolism in humans. Similarly, ^{14}C-L-phenylalanine has been incorporated into a range of polyphenolic compounds when fed to cell cultures.[565,566] Experiments with *V. pahalae* berries and *V. vinifera* suspension cultures, using [^{14}C]-sucrose as the carbon source, have demonstrated a 20–23% efficiency of ^{14}C incorporation into the flavonoid-rich fractions.[567] All in all there has, however, been limited success in achieving processes, which are commercially viable, using plant tissue and cell cultures for anthocyanin production – in part because of some unique engineering challenges inherent in mass cultivation of plant cultures.

3.16.7.2 Production of Anthocyanins by Microorganisms

Both prokaryotic and eukaryotic microbes have been used for the expression of genes that are able to convert fed precursors or endogenously produced substrates into valuable end products. The first report of plant-specific anthocyanins produced by a microorganism involved *E. coli* cells.[516] In order to produce stable, glycosylated anthocyanins from colorless flavanones such as naringenin and eriodictyol, a four-step metabolic pathway that contained plant genes from heterologous origins: flavanone 3-β-hydroxylase from *M. domestica*, DFR from *Anthurium andraeanum*, ANS also from *M. domestica*, and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (3GT) from *P. hybrida,* was constructed. Using two rounds of polymerase chain reaction each of the four genes was first placed under control of the *trc* promoter and its own bacterial ribosome-binding site. Then they were cloned sequentially into vector pK184. *E. coli* cells containing the recombinant plant pathway were able to take up either naringenin or eriodictyol and convert these compounds into the corresponding glycosylated anthocyanins, pelargonidin 3-glucoside or cyanidin 3-glucoside. The formed anthocyanins were present at low concentrations.

More recently it was, however, reported that the recombinant *E. coli* cells successfully could achieve milligram level production of the same two anthocyanins, pelargonidin 3-glucoside (1.0 mg l^{-1}) and cyanidin 3-glucoside (2.1 mg l^{-1}) from their respective flavanone precursors.[568] Cyanidin 3-glucoside was produced at even higher yields (16.1 mg l^{-1}) from the flavan-3-ol precursor, (+)-catechin. It was demonstrated that availability of the glucosyl donor, UDP glucose, was the key metabolic limitation, while product instability at normal pH was identified as a barrier. It is known that common anthocyanidin 3-glucosides rapidly will break down in weakly acidic and neutral aqueous solutions,[229] and production optimization of anthocyanidin 3-glucoside from flavan-3-ol precursors in *E. coli* cells was demonstrated by adjusting the pH to mimic the acidic condition of plant vacuole and stabilize the anthocyanin compounds.[568] A translational fusion of ANS and 3GT was created to mimic the enzyme complex, which may exist in the plant cells, to facilitate the transportation of the unstable intermediate anthocyanidins from ANS to 3GT. The metabolic network of the host *E. coli* BL21 was rationally manipulated to channel carbon flux into the UDP-glucose biosynthetic pathway, a key precursor in anthocyanin biosynthesis. As a result, production of as much as 79 mg l^{-1} pelargonidin 3-glucoside and 71 mg l^{-1} cyanidin 3-glucoside was achieved from their precursor (flavan-3-ols) without supplementation with extracellular UDP glucose.

Glossary

afzelechin/epiafzelechin flavan-3-ol (flavonoid) epimers, 5,7-dihydroxy-2-(4-hydroxy)phenyl-3,4-dihydro-2*H*-chromen-3-ols

anthocyanidin anthocyanin aglycone

apigenin flavone (flavonoid), 5,7-dihydroxy-2-(4-hydroxy)phenylchromen-4-one

bathochromic shift change of spectral band position in the absorption spectrum of a molecule to a longer wavelength (lower frequency)

catechin/epicatechin flavan-3-ol (flavonoid) epimers, 5,7-dihydroxy-2-(3,4-dihydroxy)phenyl-3,4-dihydro-2*H*-chromen-3-ols

chemotaxonomy classification of organisms according to demonstrable differences and similarities in their biochemical compositions, here according to anthocyanin content

epimer two epimers are diastereomers, which differ in configuration of only one stereogenic center
flavone flavonoid class based on the backbone of 2-phenylchromen-4-one
flavonol flavonoid class based on the backbone of 3-hydroxy-2-phenylchromen-4-one
HPLC high performance liquid chromatography
hyperchromic effect increase in absorbance of a spectral band in the absorption spectrum of a molecule
hypsochromic shift change of spectral band position in the absorption spectrum of a molecule to a shorter wavelength (higher frequency)
kaempferol a flavonol (flavonoid), 3,5,7,4′-tetrahydroxy-2-phenylchromen-4-one
LC–MS liquid chromatography–mass spectroscopy
NMR nuclear magnetic resonance spectroscopy
NOESY nuclear Overhauser effect spectroscopy, a two-dimensional homo-nuclear magnetic resonance (NMR) technique, which is based upon coupling between protons through space. The method can provide information about the molecular geometry and linkages between anthocyanin sub-units
photoinhibition reduction in a plant's (or other photosynthetic organism's) capacity for photosynthesis caused by exposure to strong light (above the saturation point)

References

1. T. W. Goodwin, *Chemistry and Biochemistry of Plant Pigments*, 2nd ed.; Academic Press: New York, 1976; Vol. 1, p 870.
2. Ø. M. Andersen; G. W. Francis, Techniques of Pigment Identification. In *14 Plant pigments and their Manipulation*; K. Davies, Ed.; Annual Plant Reviews, Blackwell Publishing: London, 2004; pp 293–341.
3. D. Strack; V. Wray, Methods in Plant Biochemistry. In *Plant Phenolics*; J. B. Harborne, Ed.; Academic Press: New York, 1989; Vol. 1, pp 325–356.
4. Ø. M. Andersen; M. Jordheim, The Anthocyanins. In *Flavonoids: Chemistry, Biochemistry and Applications*; Ø. M. Andersen, K. R. Markham, Eds.; CRC Press: Boca Raton, 2006; pp 471–553.
5. Ø. M. Andersen; M. Jordheim, **2009**, Personal database.
6. N. C. Veitch; R. J. Grayer, *Nat. Prod. Rep.* **2008**, *25*, 555–611.
7. Ø. M. Andersen, Recent Advances in the Field of Anthocyanins. In *Polyphenols-Recent Advances in Research*; V. Lattanzio, F. Daayf, Eds.; Blackwell: London, 2008; pp 167–201.
8. K. Hosokawa, *J. Plant Physiol.* **1999**, *155*, 285–287.
9. R. Nørbæk; T. Kondo, *Phytochemistry* **1999**, *51*, 1113–1119.
10. S. J. Bloor, *Phytochemistry* **2001**, *58*, 923–927.
11. K. Yoshida; H. Aoki; K. Kameda; T. Kondo, *ITE Lett. Batter. New Technol. Med.* **2002**, *3*, 35–38.
12. K. Yoshitama; T. Kawasoe; N. Ishikura, *J. Plant Res.* **1993**, *106*, 223–227.
13. K. Toki; N. Saito; T. Honda, *Phytochemistry* **1998**, *48*, 729–732.
14. K. Brandt; T. Kondo; H. Aoki; T. Goto, *Phytochemistry* **1993**, *33*, 209–212.
15. K. Takeda; J. B. Harborne; R. Self, *Phytochemistry* **1986**, *25*, 1337–1342.
16. R. Nørbæk; K. Nielsen; T. Kondo, *Phytochemistry* **2002**, *60*, 357–359.
17. S. J. Bloor, *Phytochemistry* **1999**, *50*, 1395–1399.
18. T. Goto; T. Kondo; T. Kawai; H. Tamura, *Tetrahedron Lett.* **1984**, *25*, 6021–6024.
19. M. Mori; T. Kondo; K. Toki; K. Yoshida, *Phytochemistry* **2006**, *67*, 622–629.
20. T. Kondo; T. Kawai; H. Tamura; T. Goto, *Tetrahedron Lett.* **1987**, *28*, 2273–2276.
21. Ø. Bjorøy; T. Fossen; Ø. M. Andersen, *Phytochemistry* **2007**, *66*, 640–645.
22. K. Hosokawa; E. Fukushi; J. Kawabata; C. Fujii; T. Ito; S. Yamamura, *Phytochemistry* **1997**, *45*, 167–171.
23. N. Saito; F. Tatsuzawa; Y. Yazaki; K. Shinoda; A. Shigihara; T. Honda, *Phytochemistry* **2007**, *68*, 673–679.
24. K. Takeda; M. Yanagisawa; T. Kifune; T. Kinoshita; C. F. Timberlake, *Phytochemistry* **1994**, *35*, 1167–1169.
25. T. Ishikawa; T. Kondo; T. Kinoshita; H. Haruyama; S. Inaba; K. Takeda; R. J. Grayer; N. C. Veitch, *Phytochemistry* **1999**, *52*, 517–521.
26. N. Terahara; N. Saito; A. T. Honda; K. Toki; Y. Osajima, *Heterocycles* **1990**, *31*, 1773–1776.
27. N. Terahara; N. Saito; T. Honda; K. Toki, *Tetrahedron Lett.* **1990**, *30*, 5305–5308.
28. N. Terahara; N. Saito; T. Honda; K. Toki; Y. Osajima, *Tetrahedron Lett.* **1990**, *31*, 2921–2924.
29. N. Terahara; O. Masahiro; T. Matsui; Y. Osajima; N. Saito; K. Toki; T. F. Honda, *J. Nat. Prod.* **1996**, *59*, 139–144.
30. T. Kondo; M. Ueda; T. Goto, *Tetrahedron* **1990**, *46*, 4749–4756.
31. K. Takeda; J. B. Harborne; P. G. Watermann, *Phytochemistry* **1993**, *34*, 421–423.
32. G. Catalano; T. Fossen; Ø. M. Andersen, *J. Agric. Food Chem.* **1998**, *46*, 4568–4570.
33. T. Fossen; Ø. M. Andersen, *Phytochemistry* **1999**, *50*, 1185–1188.
34. K. Takeda; S. Sato; H. Kobayashi; Y. Kanaitsuka; M. Ueno; T. Kinoshita; H. Tazaki; T. Fujimori, *Phytochemistry* **1994**, *36*, 613–616.
35. N. Saito; K. Toki; H. Moriyama; A. Shigihara; T. Honda, *Phytochemistry* **2002**, *60*, 365–373; Blackwell Publishing: London, 2004; pp 1–352.

36. T. Kondo; K. Suzuki; K. Yoshida; K. Oki; M. Ueda; M. Isobe; T. Goto, *Tetrahedron Lett.* **1991**, *32*, 6375–6378.
37. S. J. Bloor, *Phytochemistry* **1997**, *45*, 1399–1405.
38. K. Toki; N. Saito; Y. Irie; F. Tatsuzawa; A. Shigihara; T. Honda, *Phytochemistry* **2008**, *69*, 1215–1219.
39. C. Gerhauser, *Planta Med.* **2008**, *74*, 1608–1624.
40. S. C. Forester; A. L. Waterhouse, *J. Agric. Food Chem.* **2008**, *56*, 9299–9304.
41. B. Xu; S. K. Chang, *J. Agric. Food Chem.* **2008**, *56*, 8365–8373.
42. S. U. Mertens-Talcott; J. Rios; P. Jilma-Stohlawetz; L. A. Pacheco-Palencia; B. Meibohm; S. T. Talcott; H. Derendorf, *J. Agric. Food Chem.* **2008**, *56*, 7796–7802.
43. Q. Wang; M. Xia; C. Liu; H. Guo; Q. Ye; Y. Hu; Y. Zhang; M. Hou; H. Zhu; J. Ma; W. Ling, *Life Sci.* **2008**, *83*, 176–184.
44. M. Ding; R. Feng; S. Y. Wang; L. Bowman; Y. Lu; Y. Qian; V. Castranova; B. H. Jiang; X. Shi, *J. Biol. Chem.* **2006**, *281*, 17359–17368.
45. C. Manach; G. Williamson; C. Morand; A. Scalbert; C. Remesy, *Am. J. Clin. Nutr.* **2005**, *81*, 230S–242S.
46. R. L. Prior; X. Wu; L. Gu, *J. Sci. Food Agric.* **2006**, *86*, 2487–2491.
47. X. Wu; G. R. Beecher; J. M. Holden; D. B. Haytowitz; S. E. Gebhardt; R. L. Prior, *J. Agric. Food Chem.* **2006**, *54*, 4069–4075.
48. M.-G. Choung; B.-R. Choi; Y.-N. An; Y.-H. Chu; Y.-S. Cho, *J. Agric. Food Chem.* **2003**, *51*, 7040–7043.
49. G. A. Macz-Pop; A. M. Gonzalez-Paramas; J. J. Perez-Alonso; J. C. Rivas-Gonzalo, *J. Agric. Food Chem.* **2006**, *54*, 536–542.
50. G. R. Takeoka; L. T. Dao; G. H. Full; R. Y. Wong; L. A. Harden; R. H. Edwards; J. D. J. Berrios, *J. Agric. Food Chem.* **1997**, *45*, 3395–3400.
51. X. Wu; R. L. Prior, *J. Agric. Food Chem.* **2005**, *53*, 3101–3113.
52. J. M. Koponen; A. M. Happonen; P. H. Mattila; A. R. Toerroenen, *J. Agric. Food Chem.* **2007**, *55*, 1612–1619.
53. R. Lo Scalzo; A. Genna; F. Branca; M. Chedin; H. Chassaigne, *Food Chem.* **2008**, *107*, 136–144.
54. G. Hrazdina; H. Iredale; L. R. Mattick, *Phytochemistry* **1977**, *16*, 297–299.
55. G. Elham; H. Reza; K. Jabbar; S. Parisa; J. Rashid, *Pak. J. Biol. Sci.* **2006**, *9*, 2905–2908.
56. F. C. Stintzing; A. S. Stintzing; R. Carle; B. Frei; R. E. Wrolstad, *J. Agric. Food Chem.* **2002**, *50*, 6172–6181.
57. D. Kammerer; R. Carle; A. Schieber, *Eur. Food Res. Technol.* **2004**, *219*, 479–486.
58. P. Bridle; R. S. Loeffler; T. Timberlake; F. Colin; R. Self, *Phytochemistry* **1984**, *23*, 2968–2969.
59. N. Mulinacci; M. Innocenti; S. Gallori; A. Romani; F. F. Vincieri, *SP – Roy. Soc. Chem.* **2001**, *269*, 174–178.
60. M. Innocenti; S. Gallori; C. Giaccherini; F. Ieri; F. F. Vincieri; N. Mulinacci, *J. Agric. Food Chem.* **2005**, *53*, 6497–6502.
61. Y. S. Moreno; G. S. Sanchez; D. R. Hernandez; N. R. Lobato, *J. Chromatogr. Sci.* **2005**, *43*, 483–487.
62. B. A. Cevallos-Casals; L. Cisneros-Zevallos, *J. Agric. Food Chem.* **2003**, *51*, 3313–3319.
63. T. Ichiyanagi; Y. Kashiwada; Y. Shida; Y. Ikeshiro; T. Kaneyuki; T. Konishi, *J. Agric. Food Chem.* **2005**, *53*, 9472–9477.
64. G. R. Takeoka; L. T. Dao; H. Tamura; Le.A. Harden, *J. Agric. Food Chem.* **2005**, *53*, 4932–4937.
65. M.-A. Yamaguchi; S. Kawanobu; T. Maki; I. Ino, *Phytochemistry* **1996**, *42*, 661–663.
66. N. Terahara; M.-A. Yamaguchi; T. Honda, *Biosci. Biotechnol. Biochem.* **1994**, *58*, 1324–1325.
67. T. Fossen; Ø. M. Andersen; D. O. Øvstedal, *J. Food Sci.* **1996**, *64*, 703–706.
68. T. Fossen; Ø. M. Andersen, *J. Hort. Sci. Biotechnol.* **2000**, *75*, 360–363.
69. T. Fossen; D. O. Øvstedal; R. Slimestad; Ø. M. Andersen, *Food Chem.* **2003**, *81*, 433–437.
70. L. E. Rodriguez–Saona; M. M. Giusti; R. E. Wrolstad, *J. Food Sci.* **1998**, *63*, 458–465.
71. M. M. Giusti; H. Ghanadan; R. E. Wrolstad, *J. Agric. Food Chem.* **1998**, *46*, 4858–4863.
72. S. N. Ryu; S. Z. Park; C.–T. Ho, *Yaowu Shipin Fenxi* **1998**, *6*, 729–736.
73. R. E. Wrolstad; D. A. Heatherbell, *J. Food Sci.* **1968**, *33*, 592–594.
74. E. A. Pazmino-Duran; M. M. Giusti; R. E. Wrolstad; M. B. A. Gloria, *Food Chem.* **2001**, *75*, 211–216.
75. T. Fossen; S. Rayyan; M. H. Holmberg; H. S. Nateland; Ø. M. Andersen, *Phytochemistry* **2005**, *66*, 1133–1140.
76. M.-G. Choung; I.-Y. Baek; S.-T. Kang; W.-Y. Han; D.-C. Shin; H.-P. Moon; K.-H. Kang, *J. Agric. Food Chem.* **2001**, *49*, 5848–5851.
77. K. Yoshida; Y. Sato; R. Okuno; K. Kameda; M. Isobe; T. Kondo, *Biosci. Biotechnol. Biochem.* **1996**, *60*, 589–593.
78. S. Neida; S. Elba, *Arc. Latinoam. Nutr.* **2007**, *57*, 94–98.
79. E. Sousa de Brito; M. C. Pessanha de Araujo; R. E. Alves; C. Carkeet; B. A. Clevidence; J. A. Novotny, *J. Agric. Food Chem.* **2007**, *55*, 9389–9394.
80. V. Vera de Rosso; S. Hillebrand; M. Cuevas; B. Elyana; O. Florinda; P. Winterhalter; A. Z. Mercadante, *J. Food Comp. Anal.* **2008**, *21*, 291–299.
81. E. das M. Penha; R. H. Moretti, *Alimentaria* **2000**, *311*, 37–41.
82. V. L. A. G. Lima; E. A. Melo; M. I. S. Maciel; D. E. S. Lima, *Ciencia e Tecnol. de Alimentos* **2003**, *23*, 101–103.
83. A. L. A. Vendramini; L. C. Trugo, *J. Braz. Chem. Soc.* **2004**, *15*, 664–668.
84. T. Hanamura; T. Hagiwara; H. Kawagishi, *Biosci. Biotechnol. Biochem.* **2005**, *69*, 280–286.
85. B. H. Sun; F. J. Francis, *J. Food Sci.* **1967**, *32*, 647–649.
86. Z. Ju; Y. Yuan; C. Liu; S. Zhan; M. Wang, *Postharvest Biol. Technol.* **1996**, *8*, 83–93.
87. T. M. Rababah; K. I. Ereifej; L. Howard, *J. Agric. Food Chem.* **2005**, *53*, 4444–4447.
88. S. F. Hagen; K. A. Solhaug; G. B. Bengtsson; G. I. A. Borge; W. Bilger, *Postharvest Biol. Technol.* **2006**, *41*, 156–163.
89. J. E. Lancaster, *Crit. Rev. Plant Sci.* **1992**, *10*, 487–502.
90. M. P. Kaehkoenen; A. I. Hopia; M. Heinonen, *J. Agric. Food Chem.* **2001**, *49*, 4076–4082.
91. E. M. Kuskoski; J. M. Vega; J. J. Rios; R. Fett; A. M. Troncoso; A. G. Asuero, *J. Agric. Food Chem.* **2003**, *51*, 5450–5454.
92. M. P. Kaehkoenen; J. Heinaemaeki; V. Ollilainen; M. Heinonen, *J. Sci. Food Agric.* **2003**, *83*, 1403–1411.
93. X. Wu; L. Gu; R. L. Prior; S. McKay, *J. Agric. Food Chem.* **2004**, *52*, 7846–7856.
94. K. R. Maeaettae-Riihinen; A. Kamal-Eldin; P. H. Mattila; A. M. Gonzalez-Paramas; A. R. Toerroenen, *J. Agric. Food Chem.* **2004**, *52*, 4477–4486.
95. S. Y. Wang; H. S. Lin, *J. Agric. Food Chem.* **2000**, *48*, 140–146.
96. H.-J. Fan-Chiang; R. E. Wrolstad, *J. Food Sci.* **2005**, *70*, C198–C202.
97. J. C. Mi; L. R. Howard; R. L. Prior; J. R. Clark, *J. Sci. Food Agric.* **2004**, *84*, 1771–1782.

98. E. Maccarone; P. Rapisarda; F. Fanella; E. Arena; L. Mondello, *Ital. J. Food Sci.* **1998**, *10*, 367–372.
99. H. S. Lee, *J. Agric. Food Chem.* **2002**, *50*, 1243–1246.
100. P. Rapisarda; S. E. Bellomo; S. Fabroni; G. Russo, *J. Agric. Food Chem.* **2008**, *56*, 2074–2078.
101. M. A. Zoide; S. G. Papunidze; M. R. Banidze; A. G. Kalandia, *Pivo i Napitki* **2006**, *6*, 26–27.
102. F. S. Hosseinian; T. Beta, *J. Agric. Food Chem.* **2007**, *55*, 10832–10838.
103. L. Gao; G. Mazzo, *J. Food Sci.* **1994**, *59*, 1057–1059.
104. T. Sugawara; K. Igarashi, *Nippon Shokuhin Kagaku Kogaku Kaishi* **2008**, *55*, 239–244.
105. A. W. Strigl; E. Leitner; W. Pfannhauser, *Z. Lebensm. -Untersuch. -Forsch.* **1995**, *201*, 266–268.
106. Ø. M. Andersen, *J. Food Sci.* **1985**, *50*, 1230–1232.
107. M. Saario, *J. Food Qual.* **2000**, *23*, 453–463.
108. A. T. Hukkanen; S. S. Poeloenen; S. O. Kaerenlampi; H. I. Kokko, *J. Agric. Food Chem.* **2006**, *54*, 112–119.
109. R. L. Prior; S. A. Lazarus; G. Cao; H. Muccitelli; J. F. Hammerstone, *J. Agric. Food Chem.* **2001**, *49*, 1270–1276.
110. K. Ogawa; H. Sakakibara; R. Iwata; T. Ishii; T. Sato; T. Goda; K. Shimoi; S. Kumazawa, *J. Agric. Food Chem.* **2008**, *56*, 4457–4462.
111. J. Lee; C. E. Finn, *J Sci. Food Agric.* **2007**, *87*, 2665–2675.
112. M. Jordheim; F. Måge; Ø. M. Andersen, *J. Agric. Food Chem.* **2007**, *55*, 5529–5535.
113. G. E. Pantelidis; M. Vasilakakisa; G. A. Manganaris; G. Diamantidisa, *Food Chem.* **2007**, *102*, 777–783.
114. I. Revilla; S. Perez–Magarin; M. L. Gonzalez–SanJose; S. Beltra, *J. Chromatogr. A* **1999**, *847*, 83–90.
115. V. Amico; E. M. Napoli; A. Renda; G. Ruberto; C. Spatafora; C. Tringali, *Food Chem.* **2004**, *88*, 599–607.
116. H. S. Lee; L. Wicker, *Food Chem.* **1991**, *40*, 263–270.
117. P. Sarni-Manchado; E. Le Roux; C. Le Guerneve; Y. Lozano; V. Cheynier, *J. Agric. Food Chem.* **2000**, *48*, 5995–6002.
118. N. Berardini; I. Schieber; I. Klaiber; U. Beifuss; R. Carle; J. Conrad, *Naturforsch., B: Chem. Sci.* **2005**, *60*, 801–804.
119. N. Berardini; R. Fezer; J. Conrad; U. Beifuss; R. Carle; A. Schieber, *J. Agric. Food Chem.* **2005**, *53*, 1563–1570.
120. L. Kidøy; A. M. Nygaard; Ø. M. Andersen; A. T. Pedersen; D. W. Aksnes; B. T. Kiremire, *J. Food Comp. Anal.* **1997**, *10*, 49–54.
121. B. A. Cevallos-Casals; D. H. Byrne; L. Cisneros-Zevallos; W. R. Okie, *Acta Hort.* **2002**, *2*, 589–592.
122. M. C. Dussi; D. Sugar; R. E. Wrolstad, *J. Am. Soc. Hort. Sci.* **1995**, *120*, 785–789.
123. J. Los; J. Wilska-Jeszka; M. Pawlak, *Pol. J. Food Nutr. Sci.* **2000**, *9*, 35–38.
124. A. Z. Merkandante; F. O. Bobbio, Anthocyanins in Foods: Occurrence and Physicochemical Properties. In *Food Colorants*; C. Socaciu, Ed.; CRC Press: Boca Raton, 2007; pp 241–276.
125. C. Santos-Buelga; G. Williamson, *Methods in Polyphenol Analysis*, The Royal Society of Chemistry: Cambridge, **2003**; pp 1–383.
126. Ø. M. Andersen; G. W. Francis, Techniques of Pigment Identification. In *Plant Pigments and their Manipulation, Annual Plant Reviews*; K. Davies, Ed.; Blackwell Publishing: London, 2004; pp 293–341.
127. G. Mazza; J. E. Cacace; C. D. Kay, *J. AOAC Int.* **2004**, *87*, 129–145.
128. Ø. M. Andersen; T. Fossen, Characterization of Anthocyanins by NMR. In *Handbook of Food Analytical Chemistry: Pigments, Colorants, Flavors, Texture, and Bioactive Food Components*; R. E. Wrolstad, T. E. Acree, E. A. Decker, M. H. Penner, D. S. Reid, S. J. Schwartz, C. F. Shoemaker, D. Smith, P. Sporns, Eds.; John Wiley & Sons, Inc.: Hoboken, NJ, 2005; pp 47–69.
129. L. E. Rodriguez-Saona; R. E. Wrolstad, Extraction, Isolation, and Purification of Anthocyanins. In *Handbook of Food Analytical Chemistry: Pigments, Colorants, Flavors, Texture, and Bioactive Food Components*; R. E. Wrolstad, T. E. Acree, E. A. Decker, M. H. Penner, D. S. Reid, S. J. Schwartz, C. F. Shoemaker, D. Smith, P. Sporns, Eds.; John Wiley & Sons, Inc.: Hoboken, NJ, 2005; pp 7–17.
130. A. Marston; K. Hostettmann, Separation and Quantification of Flavonoids. In: *Flavonoids: Chemistry, Biochemistry and Applications*; Ø. M. Andersen, K. R. Markham, Eds.; CRC Press, Taylor & Francis Group: Boca Raton, 2006; Chapter 1, pp 1–36.
131. T. Fossen; Ø. M. Andersen, Spectroscopic Techniques Applied to Flavonoids. In *Flavonoids: Chemistry, Biochemistry and Applications*; Ø. M. Andersen, K. R. Markham, Eds.; CRC Press, Taylor & Francis Group: Boca Raton, 2006; pp 37–142.
132. M. M. Giusti; P. Jing, Analysis of Anthocyanins. In *Food Colorants: Chemical and Functional Properties*; C. Socaciu, Ed.; CRC Press, Taylor & Francis Group: Boca Raton, 2007; pp 479–506.
133. G. R. Takeoka; L. T. Dao, Anthocyanins. In *Methods of Analysis for Functional Foods and Nutraceuticals*, 2nd ed.; J. Hurst, Ed.; CRC Press, Taylor & Francis Group: Boca Raton, 2008; pp 247–276.
134. J. F. Gonnet, *Food Chem.* **1998**, *63*, 409–415.
135. M. M. Giusti; R. E. Wrolstad, Characterization and Measurement of Anthocyanins by UV-visible Spectroscopy. In *Handbook of Food Analytical Chemistry: Pigments, Colorants, Flavors, Texture, and Bioactive Food Components*; R. E. Wrolstad, T. E. Acree, E. A. Decker, M. H. Penner, D. S. Reid, S. J. Schwartz, C. F. Shoemaker, D. Smith, P. Sporns, Eds.; John Wiley & Sons, Inc.: Hoboken, NJ, 2005; pp 19–31.
136. M. M. Giusti; L. E. Rodriguez-Saona; R. E. Wrolstad, *J. Agric. Food. Chem.* **1999**, *47*, 4631–4637.
137. K. Yoshida; M. Mori; M. Kawachi; R. Okuno; K. Kameda; T. Kondo, *Tetrahedron Lett.* **2003**, *44*, 7875–7880.
138. M. Jordheim; K. Aaby; T. Fossen; G. Skrede; Ø. M. Andersen, *J. Agric. Food. Chem.* **2007**, *55*, 10591–10598.
139. O. Dangles; N. Saito; R. Brouillard, *J. Am. Chem. Soc.* **1993**, *115*, 3125–3132.
140. Ø. M. Andersen; T. Fossen; K. Torskangerpoll; A. Fossen; U. Hauge, *Phytochemistry* **2004**, *65*, 405–410.
141. F. J. Heredia; E. M. Francia-Aricha; J. C. Rivas-Gonzalo; I. M. Vicario; C. Santos-Buelga, *Food Chem.* **1998**, *63*, 491–498.
142. T. Fossen; L. Cabrita; Ø. M. Andersen, *Food Chem.* **1998**, *63*, 435–440.
143. K. Torskangerpoll; Ø. M. Andersen, *Food Chem.* **2004**, *89*, 427–440.
144. N. Mateus; V. A. P. de Freitas, *J. Agric. Food Chem.* **2001**, *49*, 5217–5222.
145. K. Hayashi, The Anthocyanins. In *The Chemistry of Flavonoid Compounds*; T. A. Geissman, Ed.; Pergamon Press: New York, 1962; pp 248–285.
146. R. Brouillard; O. Dangles, Flavonoids and Flower Colour. In *The Flavonoids, Advances in Research Since 1986*; J. B. Harborne, Ed.; Chapman & Hall: London, 1994; pp 565–588.
147. T. Goto; T. Kondo, *Angew. Chem.* **1991**, *103*, 17–33.
148. J. B. Harborne; C. A. Williams, *Phytochemistry* **2000**, *55*, 481–504.
149. G. A. Macz-Pop; J. C. Rivas-Gonzalo; J. J. Perez-Alonso; A. M. Gonzalez-Paramas, *Food Chem.* **2006**, *94*, 448–456.

150. J. B. Harborne, *Comparative Biochemistry of the Flavonoids*, Academic Press: New York, 1967; p 383.
151. E. Nilsson, *Arkiv för Kemi* **1969**, *31*, 111–119.
152. G. Bendz; O. Martensson; E. Nilsson, *Arkiv för Kemi* **1967**, *27*, 65–77.
153. E. Pale; M. Kouda-Bonafos; M. Nacro; M. Vanhaelen; R. Vanhaelen-Fastré; R. Ottinger, *Phytochemistry* **1997**, *45*, 1091–1092.
154. B. Devia; G. Llabres; J. Wouters; L. Dupont; M. T. Escribano-Bailon; S. de Pascual-Teresa; L. Angenot; M. Tits, *Phytochem. Anal.* **2002**, *13*, 114–119.
155. K. Toki; N. Saito; A. Shigihara; T. Honda, *Phytochemistry* **2008**, *75*, 1503–1509.
156. R. K. Crowden; S. J. Jarman, *Phytochemistry* **1974**, *13*, 1947–1948.
157. E. E. Swinny, *Z. Naturforsch., C: Biosci.* **2001**, *56*, 177–180.
158. G. Bendz; O. Martensson; L. Terenius, *Acta Chem. Scand.* **1962**, *16*, 1183–1190.
159. S. Kunz; G. Burkhardt, *Phytochemistry* **1994**, *35*, 233–235.
160. J. B. Harborne, *Phytochemistry* **1966**, *5*, 589–600.
161. S.-C. Lo; I. Weiergang; C. Bonham; J. Hipskind; K. Wood; R. L. Nicholson, *Physiol. Mol. Plant Pathol.* **1996**, *49*, 21–31.
162. L. Ponniah; T. R. Seshadri, *Proc. Ind. Acad. Sci.* **1953**, *A38*, 77–83.
163. C. F. Timberlake; P. Bridle, Anthocyanins. In *The Flavonoids*; J. B. Harborne, T. J. Mabry, Eds.; Academic Press: New York, 1975; Chapter 5.
164. G. Mazza; E. Miniati, *Anthocyanins in Fruits, Vegetables and Grains*; CRC Press: Boca Raton, 1993.
165. B. Zorn; A. J. García-Piñeres; V. Castro; R. Murillo; G. Mora; I. Merfort, *Phytochemistry* **2001**, *56*, 831–835.
166. G. Mazza; R. J. Brouillard, *Agric. Food Chem.* **1987**, *35*, 422–426.
167. J. M. Awika; L. W. Rooney; R. D. Waniska, *J. Agric. Food Chem.* **2004**, *52*, 4388–4394.
168. C. H. Shih; S.-O. Siu; R. Ng; E. Wong; L. C. M. Chiu; I. K. Chu; C. Lo, *J. Agric. Food Chem.* **2007**, *55*, 254–259.
169. K. Torskangerpoll; R. Nørbæk; E. Nodland; D. O. Øvstedal; Ø. M. Andersen, *Biochem. Syst. Ecol.* **2005**, *33*, 499–510.
170. K. Takeda; R. Kubota; C. Yagioka, *Phytochemistry* **1985**, *24*, 1207–1209.
171. K. A. Mitchell; K. R. Markham; M. R. Boase, *Phytochemistry* **1998**, *47*, 355–361.
172. K. Hosokawa; Y. Fukunaga; E. Fukushi; J. Kawabata, *Phytochemistry* **1995**, *38*, 1293–1298.
173. K. Hosokawa; Y. Fukunaga; E. Fukushi; J. Kawabata, *Phytochemistry* **1995**, *39*, 1437–1441.
174. K. Hosokawa; Y. Fukunaga; E. Fukushi; J. Kawabata, *Phytochemistry* **1995**, *40*, 567–571.
175. F. Hashimoto; M. Tanaka; H. Maeda; S. Fukuda; K. Shimizu; Y. Sakata, *Biosci. Biotechnol. Biochem.* **2002**, *66*, 1652–1659.
176. N. Saito; K. Toki; S. Ozden; T. Honda, *Phytochemistry* **1996**, *41*, 1599–1605.
177. N. Saito; K. Toki; A. Suga; T. Honda, *Phytochemistry* **1998**, *49*, 881–886.
178. K. Honda; K. Tsutsui; K. Hosokawa, *Sci. Hort.* **1999**, *82*, 125–134.
179. M. S. Haque; D. N. Ghoshal; K. K. Ghoshal, *Proc. Ind. Nat. Sci. Acad. Part B Biol. Sci.* **1981**, *47*, 204–209.
180. N. Saito; J. B. Harborne, *Phytochemistry* **1992**, *31*, 3009–3015.
181. S. Clevenger, *Can. J. Biochem. Physiol.* **1964**, *42*, 154–155.
182. R. Nørbæk; L. P. Christensen; K. Brandt, *Plant Breed.* **1998**, *117*, 63–67.
183. F. Tatsuzawa; N. Murata; K. Shinoda; R. Suzuki; N. Saito, *J. Jpn. Soc. Hortic. Sci.* **2003**, *72*, 243–251.
184. F. Tatsuzawa; N. Saito; N. Murata; K. Shinoda; A. Shigihara; T. Honda, *Phytochemistry* **2003**, *62*, 1239–1242.
185. A. M. Nygård; D. W. Aksnes; Ø. M. Andersen; A. K. Bakken, *Acta Chem. Scand.* **1997**, *51*, 108–112.
186. J. Oliveira; V. De Freitas; A. M. S. Silvia; N. Mateus, *J. Agric. Food Chem.* **2007**, *55*, 6349–6356.
187. A. M. Vivar-Quintana; C. Santos-Buelga; J. C. Rivas-Gonzalo, *Anal. Chim. Acta* **2002**, *458*, 147–155.
188. M. Schwarz; V. Wray; P. Winterhalter, *J. Agric. Food Chem.* **2004**, *52*, 5095–5101.
189. S. Hillebrand; M. Schwarz; P. Winterhalter, *J. Agric. Food Chem.* **2004**, *52*, 7331–7338.
190. M. J. Rein; V. Ollilainen; M. Vahermo; J. Yli-Kauhaluoma; M. Heinonen, *Eur. Food Res. Technol.* **2005**, *220*, 239–244.
191. V. Cheynier, Flavonoids in Wine. In *Flavonoids: Chemistry, Biochemistry and Applications*; Ø. M. Andersen, K. R. Markham, Eds.; CRC Press: Boca Raton, 2006; pp 263–318.
192. V. Cheynier; M. Duenas-Paton; E. Salas; C. Maury; J.-M. Souquet; P. Sarni-Manchado; H. Fulcrand, *Am. J. Enol. Vitic.* **2006**, *57*, 298–305.
193. V. de Freitas; N. Mateus, *Environ. Chem. Lett.* **2006**, *4*, 175–183.
194. Y. Fukui; T. Kusumi; K. Masuda; T. Iwashita; K. Nomoto, *Tetrahedron Lett.* **2002**, *43*, 2637–2639.
195. Y. Fukui; K. Nomoto; T. Iwashita; K. Masuda; Y. Tanaka; T. Kusumi, *Tetrahedron* **2006**, *62*, 9661–9670.
196. T. Fossen; Ø. M. Andersen, *Phytochemistry* **2003**, *62*, 1217–1220.
197. N. Mateus; A. M. S. Silva; J. C. Rivas-Gonzalo; C. Santos-Buelga; V. de Freitas, *J. Agric. Food Chem.* **2003**, *51*, 1919–1923.
198. M. Jordheim; T. Fossen; Ø. M. Andersen, *J. Agric. Food Chem.* **2006**, *54*, 9340–9346.
199. J. Bakker; C. F. Timberlake, *J. Agric. Food Chem.* **1997**, *45*, 35–43.
200. T. Fossen; R. Slimestad; Ø. M. Andersen, *Phytochemistry* **2003**, *64*, 1367–1374.
201. J. B. Harborne; H. Baxter, *The Handbook of Natural Flavonoids*; John Wiley & Sons: New York, 1999; Vol. 2, pp 193–205.
202. N. Saito; F. Tatsuzawa; K. Miyoshi; A. Shigihara; T. Honda, *Tetrahedron Lett.* **2003**, *44*, 6821–6823.
203. F. Tatsuzawa; N. Saito; K. Miyoshi; K. Shinoda; A. Shigihara; T. Honda, *Chem. Pharm. Bull.* **2004**, *52*, 631–633.
204. Ø. Bjorøy; S. Rayyan; T. Fossen; K. Kalberg; Ø.M. Andersen, *Phytochemistry* **2009**, *70*, 278–287.
205. N. Saito; J. B. Harborne, *Phytochemistry* **1983**, *22*, 1735–1740.
206. K. Yoshitama; K. Abe, *Phytochemistry* **1977**, *16*, 591–593.
207. J. B. Harborne, *Comparative Biochemistry of the Flavonoids*; Academic Press: New York, 1967; p 383.
208. K. Toki; N. Saito; A. Shigihara; T. Honda, *Phytochemistry* **2001**, *56*, 711–715.
209. K. Toki; N. Saito; T. Ueda; T. Chibana; A. Shighihara; T. Honda, *Phytochemistry* **1994**, *37*, 885–887.
210. T. Kondo; J. Yamashiki; K. Kawahori; T. Goto, *Tetrahedron Lett.* **1989**, *30*, 6055–6058.
211. T. Fossen; R. Slimestad; D. O. Øvstedal; Ø. M. Andersen, *Phytochemistry* **2000**, *54*, 317–323.
212. Ø. M. Andersen; T. Fossen, *Phytochemistry* **1995**, *40*, 1809–1812.
213. M. Nakayama; M.-A. Yamaguchi; O. Urashima; Y. Kan; Y. Fukui; Y. Yamaguchi; M. Koshioka, *Biosci. Biotechnol. Biochem.* **1999**, *63*, 1509–1511.

214. T. Fossen; Ø. M. Andersen, *Phytochemistry* **1999**, *52*, 1697–1700.
215. M. Nakayama; M. Koshioka; M. Shibata; S. Hiradate; H. Sugie; M. Yamaguchi, *Biosci. Biotechnol. Biochem.* **1997**, *61*, 1607–1608.
216. J. D. Hipskind; R. Hanau; B. Leite; R. L. Nicholson, *Physiol. Mol. Plant Pathol.* **1990**, *36*, 381–396.
217. E. Idaka; Y. Ohashi; T. Ogawa; T. Kondo; T. Goto, *Tetrahedron Lett.* **1987**, *28*, 1901–1904.
218. A. J. Baublis; M. D. Berber-Jiménez, *J. Agric. Food Chem.* **1995**, *43*, 640–646.
219. T. Honda; N. Saito, *Heterocycles* **2002**, *56*, 633–692.
220. E. Sondheimer, *J. Am. Chem. Soc.* **1953**, *75*, 1507–1508.
221. L. Jurd, *J. Org. Chem.* **1963**, *28*, 987–991.
222. R. Brouillard; J. E. Dubois, *J. Am. Chem. Soc.* **1977**, *99*, 1359–1364.
223. A. Cheminat; R. Brouillard, *Tetrahedron Lett.* **1986**, *27*, 4457–4460.
224. T. V. Mistry; Y. Cai; T. H. Lilley; E. Haslam, *J. Chem. Soc., Perkin Trans. 2* **1991**, *8*, 1287–1296.
225. H. Santos; D. L. Turner; J. C. Lima; P. Figueiredo; F. S. Pina; A. L. Macanita, *Phytochemistry* **1993**, *33*, 1227–1232.
226. N. Terahara; H. Suzuki; K. Toki; H. Kuwano; N. Saito; T. Honda, *J. Nat. Prod.* **1993**, *56*, 335–340.
227. J. Bakker; P. Bridle; T. Honda; H. Kuwano; N. Saito; N. Terahara; C. F. Timberlake, *Phytochemistry* **1997**, *44*, 1375–1382.
228. F. Pina, *J. Chem. Soc., Faraday Trans.* **1998**, *94*, 2109–2116.
229. L. Cabrita; T. Fossen; Ø. M. Andersen, *Food Chem.* **2000**, *68*, 101–107.
230. R. Brouillard; J. Lang, *Can. J. Chem.* **1990**, *68*, 755–761.
231. P. Markakis; G. E. Livingstone; G. R. Fillers, *Food Res.* **1957**, *22*, 117–130.
232. G. Hrazdina, *Phytochemistry* **1971**, *10*, 1125–1130.
233. J. B. Adams; M. H. Ongley, *J. Food Technol.* **1973**, *8*, 139–145.
234. E. Sadilova; R. Carle; F. C. Stintzing, *Mol. Nutr. Food Res.* **2007**, *51*, 1461–1471.
235. C. F. Timberlake; P. J. Bridle, *Sci. Food Agric.* **1967**, *18*, 473–478.
236. T. Fossen; S. Rayyan; M. H. Holmberg; M. Nimtz; Ø. M. Andersen, *Phytochemistry* **2007**, *68*, 652–662.
237. M. J. Melo; M. Sousa; A. J. Parola; J. S. Seixas de Melo; F. Catarino; J. Marcalo; F. Pina, *Chemistry* **2007**, *13*, 1417–1422.
238. S. Remy–Tanneau; C. Le Guerneve; E. Meudec; V. Cheynier, *J. Agric. Food Chem.* **2003**, *51*, 3592–3597.
239. T. Fossen; S. Rayyan; Ø. M. Andersen, *Phytochemistry* **2004**, *65*, 1421–1428.
240. A. M. Gonzalez-Paramas; F. Lopes da Silva; P. Martin-Lopez; G. Macz-Pop; S. Gonzalez-Manzano; C. Alcalde-Eon; J. J. Perez-Alonso; M. T. Escribano-Bailon; J. C. Rivas-Gonzalo; C. Santos-Buelga, *Food Chem.* **2006**, *94*, 428–436.
241. G. J. McDougall; S. Gordon; R. Brennan; D. Stewart, *J. Agric. Food Chem.* **2005**, *53*, 7878–7885.
242. M. Duenas; J. J. Perez-Alonso; C. Santos-Buelga; T. Escribano-Bailon, *J. Food Comp. Anal.* **2007**, *21*, 107–115.
243. K. Toki; N. Saito; S. Tsutsumi; C. Tamura; A. Shigihara; T. Honda, *Heterocycles* **2004**, *63*, 899–902.
244. K. Toki; N. Saito; K. Iimura; T. Suzuki; T. Honda, *Phytochemistry* **1994**, *36*, 1181–1183.
245. S. J. Bloor; R. Falshaw, *Phytochemistry* **2000**, *53*, 575–579.
246. P. Figueiredo; M. Elhabiri; K. Toki; N. Saito; O. Dangles; R. Brouillard, *Phytochemistry* **1996**, *41*, 301–308.
247. W. Uphoff, *Experientia* **1982**, *38*, 778–780.
248. D. Strack; E. Busch; E. Klein, *Phytochemistry* **1989**, *28*, 2127–2139.
249. S. Mitsui; K. Hayashi; S. Hattori, *Proc. Jpn. Acad.* **1959**, *35*, 169–174.
250. T. Goto; T. Kondo; H. Tamura; S. Takase, *Tetrahedron Lett.* **1983**, *24*, 4863–4866.
251. T. Kondo; K. Yoshida; A. Nakagawa; T. Kawai; H. Tamura; T. Goto, *Nature* **1992**, *358*, 515–518.
252. T. Kondo; K. Oyama; K. Yoshida, *Angew. Chem., Int. Ed.* **2001**, *40*, 894–897.
253. K. Takeda, *Proc. Jpn. Acad., Ser. B* **2006**, *82*, 142–154.
254. E. Bayer, *Chem. Berichte* **1958**, *91*, 1115–1122.
255. T. Kondo; M. Ueda; H. Tamura; K. Yoshida; M. Isobe; T. Goto, *Angew. Chem., Int. Ed.* **1994**, *33*, 978–979.
256. K. Yoshida; S. Kitahara; D. Ito; T. Kondo, *Phytochemistry* **2006**, *67*, 992–998.
257. T. Kondo; Y. Toyama-Kato; K. Yoshida, *Tetrahedron Lett.* **2005**, *46*, 6645–6649.
258. G. A. Ellestad, *Chirality* **2006**, *18*, 134–144.
259. K. Takeda; T. Yamashita; A. Takahashi; C. F. Timberlake, *Phytochemistry* **1990**, *29*, 1089–1091.
260. K. Yoshida; Y. Toyama; K. Kameda; T. Kondo, *Plant Cell Physiol.* **2003**, *44*, 262–268.
261. Y. Toyama-Kato; T. Kondo; K. Yoshida, *Heterocylces* **2007**, *72*, 239–254.
262. O. Dangles; M. Elhabiri; R. Brouillard, *J. Chem. Soc., Perkin Trans. 2* **1994**, *12*, 2587–2596.
263. M. Elhabiri; P. Figueiredo; K. Toki; N. Saito; R. Brouillard, *J. Chem. Soc., Perkin Trans. 2* **1997**, *2*, 355–362.
264. M. C. Moncada; S. Moura; M. J. Melo; A. Roque; C. Lodeiro; F. Pina, *Inorg. Chim. Acta* **2003**, *356*, 51–61.
265. Y. Ohsawa; S. Ohba; S. Kosemura; S. Yamamura; A. Nakagawa; K. Yoshida; T. Kondo, *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.* **1994**, *C50*, 645–648.
266. N. Saito; S. Mitsui; K. Hayashi, *Proc. Jpn. Acad.* **1961**, *37*, 485–490.
267. K. Takeda; S. Tominaga, *Bot. Mag. Tokyo* **1983**, *96*, 359–363.
268. T. Kondo; M. Ueda; M. Isobe; T. Goto, *Tetrahedron Lett.* **1998**, *39*, 8307–8310.
269. M. Shiono; N. Matsugaki; K. Takeda, *Nature* **2005**, *436*, 791.
270. K. Takeda; A. Osakabe; S. Saito; D. Furuyama; A. Tomita; Y. Kojima; M. Yamadera; M. Sakuta, *Phytochemistry* **2005**, *66*, 1607–1613.
271. N. Saito; K. Abe; T. Honda; C. F. Timberlake; P. Bridle, *Phytochemistry* **1985**, *24*, 1583–1586.
272. S. Asen; R. N. Stewart; K. H. Norris, *Phytochemistry* **1972**, *11*, 1139–1144.
273. P. Scheffeldt; G. Hrazdina, *J. Food Sci.* **1978**, *43*, 517–520.
274. R. Brouillard; M. C. Wigand; O. Dangles; A. Cheminat, *J. Chem. Soc., Perkin Trans. 2* **1991**, *8*, 1235–1241.
275. A. Bakowska; A. Z. Kucharska; J. Oszmianski, *J. Food Chem.* **2003**, *81*, 349–355.
276. M. Gomez-Miguez; S. Gonzalez-Manzano; M. T. Escribano-Bailon; F. J. Heredia; C. Santos-Buelga, *J. Agric. Food Chem.* **2006**, *54*, 5422–5429.
277. G. Mazza; R. Brouillard, *Phytochemistry* **1990**, *29*, 1097–1102.

278. J. M. D. Markovic; N. A. Petranovic; J. M. Baranac, *J. Agric. Food Chem.* **2000**, *48*, 5530–5536.
279. S. Gonzalez-Manzano; C. Santos-Buelga; M. Duenas; J. C. Rivas-Gonzalo; T. Escribano-Bailon, *Eur. Food Res. Technol.* **2008**, *226*, 483–490.
280. S. Asen, *Acta Hort.* **1976**, *63*, 217–223.
281. O. Dangles; R. Brouillard, *Can. J. Chem.* **1992**, *70*, 2174–2189.
282. M. Williams; G. Hrazdina, *J. Food Sci.* **1979**, *44*, 66–68.
283. C. A. Hunter; J. K. M. Sanders, *J. Am. Chem. Soc.* **1991**, *112*, 5525–5534.
284. L.-J. Chen; G. Hrazdina, *Phytochemistry* **1981**, *20*, 297–303.
285. T. Goto; H. Tamura; T. Kawai; T. Hoshino; N. Harada; T. Kondo, *Ann. N. Y. Acad. Sci.* **1986**, *471*, 155–173.
286. P. F. Da Silva; J. C. Lima; A. A. Freitas; K. Shimizu; A. L. Macanita; F. H. Quina, *J. Phys. Chem.* **2005**, *109*, 7329–7338.
287. T. Hoshino; U. Matsumoto; T. Goto, *Tetrahedron Lett.* **1980**, *21*, 1751–1754.
288. T. Hoshino; U. Matsumoto; N. Harada; T. Goto, *Tetrahedron Lett.* **1981**, *22*, 3621–3624.
289. T. Hoshino, *Phytochemistry* **1986**, *25*, 829–832.
290. T. Hoshino; U. Matsumoto; T. Goto; N. Harada, *Tetrahedron Lett.* **1982**, *23*, 433–436.
291. T. Goto; H. Tamura; T. Kondo, *Tetrahedron Lett.* **1987**, *28*, 5907–5908.
292. C. Houbiers; J. C. Lima; M. H. Santos; A. L. Macanita, *J. Phys. Chem.* **1998**, *102*, 3578–3585.
293. E. G. Gakh; D. K. Dougall; D. C. Baker, *Phytochem. Anal.* **1998**, *9*, 28–34.
294. V. Glassgen; S. Wray; J. W. Dieter, *Phytochemistry* **1992**, *31*, 1593–1601.
295. K. Yoshida; T. Kondo; T. Goto, *Tetrahedron Lett.* **1991**, *32*, 5579–5580.
296. W. Nerdal; Ø. M. Andersen, *Phytochem. Anal.* **1991**, *2*, 263–270.
297. W. Nerdal; Ø. M. Andersen, *Phytochem. Anal.* **1992**, *3*, 182–189.
298. T. Goto; T. Kondo; H. Tamura; H. Imagawa; A. Iino; K. Takeda, *Tetrahedron Lett.* **1982**, *23*, 3695–3698.
299. K. Yoshida; Y. Toyama; K. Kameda; T. Kondo, *Phytochemistry* **2000**, *54*, 85–92.
300. F. Tatsuzawa; N. Saito; M. Yokoi; A. Shigihara; T. Honda, *Phytochemistry* **1994**, *37*, 1179–1183.
301. F. Tatsuzawa; N. Saito; M. Yokoi; A. Shigihara; T. Honda, *Phytochemistry* **1996**, *41*, 635–642.
302. N. Saito; M. Ku; F. Tatsuzawa; T. S. Lu; M. Yokoi; A. Shigihara; T. Honda, *Phytochemistry* **1995**, *40*, 1523–1529.
303. F. Tatsuzawa; N. Saito; M. Yokoi; A. Shigihara; T. Honda, *Phytochemistry* **1998**, *49*, 869–874.
304. F. Tatsuzawa; N. Saito; H. Seki; R. Hara; M. Yokoi; T. Honda, *Phytochemistry* **1997**, *45*, 173–177.
305. C. A. Williams; J. Greenham; J. B. Harborne; J.-M. Kong; L.-S. Chia; N.-K. Goh; N. Saito; K. Toki; F. Tatsuzawa, *Biochem. Syst. Ecol.* **2002**, *30*, 667–675.
306. F. Tatsuzawa; N. Saito; H. Seki; M. Yokoi; T. Yukawa; K. Shinoda; T. Honda, *Biochem. Syst. Ecol.* **2004**, *32*, 651–664. (Di and poly acyl-copigments).
307. N. Saito; K. Toki; K. Uesato; A. Shigihara; T. Honda, *Phytochemistry* **1994**, *37*, 245–248.
308. P. Figueiredo; F. George; F. Tatsuzawa; K. Toki; N. Saito; R. Brouillard, *Phytochemistry* **1999**, *51*, 125–132.
309. K. Yoshitama; M. Kaneshige; N. Ishikura; F. Araki; S. Yahara; K. Abe, *J. Plant Res.* **1994**, *107*, 209–214.
310. P. A. Hedin; P. L. Lamar, III; A. C. Thompson; J. P. Minyard, *Am. J. Bot.* **1968**, *55*, 431–437.
311. T. S. Lu; N. Saito; M. Yokoi; A. Shigihara; T. Honda, *Phytochemistry* **1991**, *31*, 289–295.
312. T. Sen Lu; N. Saito; M. Yokoi; A. Shigihara; T. Honda, *Phytochemistry* **1992**, *31*, 659–663.
313. K. Yoshida; T. Kondo; Y. Okazaki; K. Katou, *Nature* **1995**, *373*, 291.
314. K. Yoshida; R. Okuno; K. Kameda; M. Mori; T. Kondo, *Biochem. Eng. J.* **2003**, *14*, 163–169.
315. R. Brouillard, *Phytochemistry* **1983**, *22*, 1311–1323.
316. F. George; P. Figueiredo; K. Toki; F. Tatsuzawa; N. Saito; R. Brouillard, *Phytochemistry* **2001**, *57*, 791–795.
317. R. N. Stewart; K. H. Norris; S. Asen, *Phytochemistry* **1975**, *14*, 937–942.
318. E. Huala; I. M. Sussex, *Plant Cell* **1993**, *5*, 1157–1165.
319. K. Hosokawa, *Biosci. Biotechnol. Biochem.* **1999**, *63*, 930–931.
320. D. W. Lee; T. M. Collins, *Int. J. Plant Sci.* **2001**, *162*, 1141–1153.
321. B. S. Winkel, *Annu. Rev. Plant Biol.* **2004**, *55*, 85–107.
322. E. Grotewold, *Annu. Rev. Plant Biol.* **2006**, *57*, 761–780.
323. R. C. Pecket; C. J. Small, *Phytochemistry* **1980**, *19*, 2571–2576.
324. H. Yasuda, *Cytologia* **1974**, *9*, 107–112.
325. S. Asen; R. N. Stewart; K. H. Norris, *Phytochemistry* **1975**, *14*, 2677–2682.
326. V. Z. Hemleben, *Naturforschung* **1981**, *36c*, 925–927.
327. C. Nozzolillo; N. Ishikura, *Plant Cell Rep.* **1988**, *7*, 389–392.
328. H. Kubo; M. Nozue; K. Kawasaki; H. Yasuda, *Plant Cell Physiol.* **1995**, *36*, 1453–1458.
329. C. J. Small; R. C. Pecket, *Planta* **1982**, *154*, 97–99.
330. C. Nozzolillo; J. Anderson; S. Warwick, *Polyphenols Actu.* **1995**, *12*, 25–26.
331. F. Cormier; C. B. Do, XXVII, *Vitis vinifera* L. (Grapevine): In Vitro Production of Anthocyanins. In *Biotechnology in Agriculture and Forestry, Medicinal and Aromatic Plants*; Y. P. S. Bajaj, Ed.; Springer Verlag: Berlin, 1993; Vol. 24, pp 373–386.
332. M. Nozue; H. Kubo; M. Nishimura; H. Yasuda, *Plant Cell Physiol.* **1995**, *36*, 883–889.
333. M. Nozue; K. Yamada; T. Nakamura; H. Kubo; M. Kondo; M. Nishimura, *Plant Physiol.* **1997**, *115*, 1065–1072.
334. C. Nozzolillo, *Polyphenols Actu.* **1994**, *11*, 16–18.
335. F. Cormier, Food Colourants from Plant Cell Cultures. In *Functionality of Food Phytochemicals – Recent Advances in Phytochemistry*; T. Johns, J. T. Romeo, Eds.; Plenum Press: London and New York, 1997; Vol. 31, p 8.
336. R.-N. Bae; K.-W. Kim; T.-C. Kim; S.-K. Lee, *Hort. Sci.* **2006**, *41*, 733–736.
337. K. R. Markham; K. S. Gould; C. S. Winefield; K. A. Mitchell; S. J. Bloor; M. R. Boase, *Phytochemistry* **2000**, *55*, 327–336.
338. Y. Zhang; S. Shi; M. Zhao; Y. Jiang; P. Tu, *Biochem. Syst. Ecol.* **2006**, *34*, 766–769.
339. S. Conn; W. Zhang; C. Franco, *Biotechnol. Lett.* **2003**, *25*, 835–839.
340. N. G. Irani; E. Grotewold, *BMC Plant Biol.* **2005**, *5*, 1–15.
341. B. A. Snyder; R. L. Nicholson, *Science* **1990**, *248*, 1637–1639.

342. M. E. Aguero; A. Gevens; R. L. Nicholson, *Physiol. Mol. Plant Pathol.* **2002**, *61*, 267–271.
343. K. A. Nielsen; C. H. Gotfredsen; M. J. Buch-Pedersen; H. Ammitzboll; O. Mattsson; J. O. Duus; R. L. Nicholson, *Physiol. Mol. Plant Pathol.* **2004**, *65*, 187–196.
344. G. Britton, *The Biochemistry of Natural Pigments*; Cambridge University Press: Cambridge, 1983.
345. N. Veitch; R. J. Grayer, Chalcones, Dihydrochalcones and Aurones. In *Flavonoids: Chemistry, Biochemistry and Applications*; Ø. M. Andersen, K. R. Markham, Eds.; CRC Press: Boca Raton, 2006; pp 1003–1100.
346. J. B. Harborne, Biochemistry of Plant Pollination. In *Introduction to Ecological Biochemistry*; J. B. Harborne, Ed.; Academic Press: London, 1993; pp 36–70.
347. E. C. Bate-Smith; T. A. Geissman, *Nature* **1951**, *167*, 688–689.
348. A. N. Kesari; R. Kumar Gupta; G. Watal, *Phytochemistry* **2004**, *65*, 3125–3129.
349. M. Shimokoriyama; S. Hattori, *J. Am. Chem. Soc.* **1953**, *75*, 1900–1904.
350. K. R. Markham, *Techniques of Flavonoid Identification*; Academic Press: London, 1982.
351. T. Nakayama, *J. Biosci. Bioeng.* **2002**, *94*, 487–491.
352. H. P. Bhartiya; P. Dubey; S. B. Katiyar; P. C. Gupta, *Phytochemistry* **1979**, *18*, 689.
353. G. Haberlandt, 1914. *Physiological Plant Anatomy,* 2nd ed.; Today and Tomorrow's Printers & Publishers: New Delhi, 1990; pp 117–119.
354. M. Shimokoriyama, *J. Am. Chem. Soc.* **1957**, *79*, 214–220.
355. H. Karikome; K. Ogawa; Y. Sashida, *Chem. Pharm. Bull.* **1992**, *40*, 689–691.
356. T. Tanaka; T. Ito; M. Iinuma; Y. Takahashi; H. Naganawa, *Phytochemistry* **1998**, *48*, 1423–1427.
357. S. W. Yang; G. A. Cordell; H. Lotter; H. Wagner; B. C. Mouly; A. V. N. Appa Rao; P. S. Rao, *J. Nat. Prod.* **1998**, *61*, 1274–1276.
358. M.-H. Cho; Y.-S. Paik; T.-R. Hahn, *J. Agric. Food Chem.* **2000**, *48*, 3917–3921.
359. K. Kazuma; T. Takahashi; K. Sato; H. Takeuchi; T. Matsumoto; T. Okuno, *Biosci., Biotechnol., Biochem.* **2000**, *64*, 1588–1599.
360. J.-B. Kim; M.-H. Cho; T.-R. Hahn; Y.-S. Paik, *Agric. Chem. Biotech.* **1996**, *39*, 501–505.
361. P. L. Majumders; S. Lahiri; N. Mukhoti, *Phytochemistry* **1995**, *40*, 271–274.
362. T. A. Geissmann; J. B. Harborne, *J. Am. Chem. Soc.* **1956**, *78*, 832–837.
363. E. A. Julian; D. J. Crawford, *Phytochemistry* **1971**, *11*, 1841–1843.
364. J. B. Harborne; A. R. Girija; H. M. Devi; N. K. M. Lakshmi, *Phytochemistry* **1983**, *22*, 2741–2742.
365. P. Mohan; T. Joshi, *Phytochemistry* **1989**, *28*, 2529–2530.
366. Y. Sashida; K. Ogawa; M. Kitada; H. Karikome; Y. Mimaki; H. Shimomura, *Chem. Pharm. Bull.* **1991**, *39*, 709–711.
367. H. Hahn; T. Seeger; H. Geiger; H. D. Zinsmeister; K. R. Markham; H. Wong, *Phytochemistry* **1995**, *40*, 573–576.
368. H. Geiger; K. R. Markham, *Phytochemistry* **1992**, *31*, 4325–4328.
369. X.-C. Li; H. N. ElSohly; A. C. Nimrod; A. M. Clark, *J. Nat. Prod.* **1999**, *62*, 767–769.
370. D. E. Giannasi, *Mem. New York Bot. Gard.* **1975**, *26*, 1–125.
371. C. G. Nordström; T. Swain, *Arch. Biochem. Biophys.* **1956**, *60*, 329–344.
372. J. B. Harborne; J. Greenham; J. Eagles, *Phytochemistry* **1990**, *29*, 2899–2900.
373. T. A. Geissman; L. Jurd, *J. Am. Chem. Soc.* **1954**, *76*, 4475–4476.
374. A. Inazu, *Bull. Fac. Agric., Tamagawa Uni.* **1993**, *33*, 75–140.
375. D. J. Crawford; E. B. Smith, *Am. J. Bot.* **1983**, *70*, 355–362.
376. H. Zhang; L. Wang; S. Deroles; R. Bennett; K. Davies, *BMC Plant Biol.* **2006**, *6*, 1–14, 29.
377. E. C. Jorgensen; T. A. Geissmann, *Arch. Biochem. Biophys.* **1955**, *55*, 389–402.
378. J. B. Harborne, *Phytochemistry* **1963**, *2*, 327–334.
379. T. Nakayama; K. Yonekura-Sakakibara; T. Sato; S. Kikuchi; Y. Fukui; M. Fukuchi-Mizutani; T. Ueda; M. Nakao; Y. Tanaka, *Science* **2000**, *290*, 1163–1166.
380. T. Nakayama; T. Sato; Y. Fukui; K. Yonekura-Sakakibara; H. Hayashi; Y. Tanaka; T. Kusumi; T. Nishino, *FEBS Lett.* **2001**, *499*, 107–111.
381. T. Sato; T. Nakayama; S. Kikuchi; Y. Fukui; K. Yonekura-Sakakibara; T. Ueda; T. Nishino; Y. Tanaka; T. Kusumi, *Plant Sci.* **2001**, *160*, 229–236.
382. E. Ono; M. Fukuchi-Mizutani; N. Nakamura; Y. Fukui; K. Yonekura-Sakakibara; M. Yamaguchi; T. Nakayama; T. Tanaka; T. Kusumi; Y. Tanaka, *Proc. Nat. Acad. Sci. U.S.A.* **2006**, *103*, 11075–11080.
383. M. Hatayama; E. Ono; K. Yonekura-Sakakibara; Y. Tanaka; T. Nishino; T. Nakayama, *Plant Biotechnol. Tokyo* **2006**, *23*, 373–378.
384. K. E. Schwinn; K. Davies, *Flavonoids in Plant Pigments and their Manipulation – Annual Plant Reviews*; K. Davies, Ed.; Blackwell Publishing/CRC Press: Boca Raton, 2004; Vol. 14, Chapter 4, pp 91–149.
385. B. Hoffmann; J. Hölzl, *Plant Med.* **1988**, *54*, 52–53.
386. F. R. Ganders; B. A. Bhom; S. P. McCormick, *Syst. Bot.* **1990**, *15*, 231–239.
387. I. Kitagawa; W.-Z. Chen; K. Hori; M. Kobayashio; J. Ren, *Chem. Pharm. Bull.* **1998**, *46*, 1511–1517.
388. T. Hatano; H. Kagawa; T. Yasuhara; T. Okuda, *Chem. Pharm. Bull.* **1988**, *36*, 2090–2097.
389. M. Furusawa; Y. Ido; T. Tanaka; T. Ito; K.-I. Nakaya; I. Ibrahim; M. Ohyama; M. Iinuma; Y. Shirataka; Y. Takahashi, *Helv. Chim. Acta* **2005**, *88*, 1048–1058.
390. Y. Jin; X.-L. Zhang; H. Shi; Y.-S. Xiao; Y.-X. Ke; X.-Y. Xue; F.-F. Zhang; X.-M. Liang, *Rapid Commun. Mass Spectrom.* **2008**, *22*, 1275–1287.
391. K. Saito; Y. Takahashi; M. Wada, *Biochim. Biophys. Acta* **1983**, *756*, 217–222.
392. J. Onodera; K. Kawamoto; S. Matsuba; S. Sato; H. Kojima; Y. Kaneya; H. Obara, *Chem. Lett.* **1995**, *10*, 901–902.
393. K. Kazuma; E. Shirai; M. Wada; K. Umeo; A. Sato; T. Matsumoto; T. Okuno, *Biosci. Biotechnol. Biochem.* **1995**, *59*, 1588–1590.
394. K. M. Davies; K. E. Schwinn, Molecular Biology and Biotechnology of Flavonoid Biosynthesis. In *Flavonoids: Chemistry, Biochemistry and Applications*; Ø. M. Andersen, K. R. Markham, Eds.; CRC Press: Boca Raton, 2006; pp 143–218.
395. G. Forkmann; W. Heller, *Compre. Nat. Prod. Chem.* **1999**, *1*, 713–748.

396. H. Ben-Meir; A. Zuker; D. Weiss; A. Vainstein, Molecular Control of Floral Pigmentation: Anthocyanins. In *Breeding for Ornamentals: Classical and Molecular Approaches*; A. Vainstein, Ed.; Kluwer Academic Press: The Notherlands, 2002; pp 253–272.
397. K. Springob; J.-I. Nakajima; M. Yamazaki; K. Saito, *Nat. Prod. Rep.* **2003**, *20*, 288–303.
398. N. G. Irani; J. M. Hernandez; E. Grotewold, *Recent Adv. Phytochem.* **2003**, *37*, 59–78.
399. Y. Tanaka; A. Ohmiya, *Curr. Opin. Biotechnol.* **2008**, *19*, 190–197.
400. S. de Pascual–Teresa; M. T. Sanchez–Ballesta, *Phytochem. Rev.* **2008**, *7*, 281–299.
401. J. Mol; E. Grotewold; R. Koes, *Curr. Plant Sci. Biotechnol. Agric.* **1999**, *36*, 597–600.
402. S. Chandler; Y. Tanaka, *Crit. Rev. Plant Sci.* **2007**, *26*, 169–197.
403. Y. Tanaka; N. Sasaki; A. Ohmiya, *Plant J.* **2008**, *54*, 733–749.
404. H. Okuhara; K. Ishiguro; C. Hirose; M. Y. Gao; M. Yanaguchi; Y. Tanaka, *Plant Cell Physiol.* **2004**, *45*, S133.
405. E. Moustafa; E. Wong, *Phytochemistry* **1967**, *6*, 625–632.
406. B. W. Shirley; W. L. Kubasek; G. Storz; E. Bruggemann; M. Koornneef; F. M. Ausubel; H. M. Goodman, *Plant J.* **1995**, *8*, 659–671.
407. C. S. Winefield; D. H. Lewis; E. E. Swinny; H. Zhang; S. H. Arathoon; T. C. Fischer; H. Halbwirth; K. Stich; C. Gosch; G. Forkmann; K. M. Davies, *Physiol. Plant* **2005**, *124*, 419–430.
408. T. C. Fischer; H. Halbwirth; B. Meisel; K. Stich; G. Forkmann, *Arch. Biochem. Biophys.* **2003**, *412*, 223–230.
409. H. Halbwirth; S. Martens; U. Wienand; G. Forkmann; K. Stich, *Plant Sci.* **2003**, *164*, 489–495.
410. P. Meyer; I. Heidmann; G. Forkmann; H. Saedler, *Nature* **1987**, *330*, 677–678.
411. J. Mol; E. Cornish; J. Mason; R. Koes, *Curr. Opin. Biotechnol.* **1999**, *10*, 198–201.
412. Y. Fukui; Y. Tanaka; T. Kusumi; T. Iwashita; K. Nomoto, *Phytochemistry* **2003**, *63*, 15–23.
413. L. Chalker-Scott, *Photochem. Photobiol.* **1999**, *70*, 1–9.
414. J. B. Harborne; R. Grayer, The Anthocyanins. In *The Flavonoids*; J. B. Harborne, Ed.; Chapman & Hall: London and New York, 1994; pp 589–618.
415. M. S. J. Simmonds, *Phytochemistry* **2003**, *64*, 21–30.
416. K. S. Gould, *J. Biomed. Biotechol.* **2004**, *5*, 314–320.
417. K. S. Gould; C. Lister, Flavonoid Functions in Plants. In *Flavonoids: Chemistry, Biochemistry and Applications*; Ø. M. Andersen, K. R. Markham, Eds.; CRC Press: Boca Raton, 2006; pp 397–442.
418. M. Mori; K. Yoshida; Y. Ishigaki; T. Matsunaga; O. Nikaido; K. Kameda; T. Kondo, *Bioorg. Med. Chem.* **2005**, *13*, 2015–2020.
419. S. Vogel, *Bot. Stud.* **1954**, *1*, 1–338.
420. K. Faegri; L. Van der Pijl, *The Principles of Pollination Ecology*; Pergamon: Oxford, 1966; p 248.
421. C. B. Fenster; W. S. Armbruster; P. Wilson; M. R. Dudash; J. D. Thomson, *Annu. Rev. Ecol. Evol. Syst.* **2004**, *35*, 375–403.
422. M. D. Rausher, *Int. J. Plant Sci.* **2008**, *169*, 7–21.
423. F. G. Stiles, *Ann. Missouri Bot. Gard.* **1981**, *68*, 323–351.
424. J. D. Thomson; P. Wilson; M. Valenzuela; M. Malzone, *Plant Species Biol.* **2000**, *15*, 11–29.
425. Q. Cronk; I. Ojeda, *J. Exp. Bot.* **2008**, *59*, 715–727.
426. R. A. Zufall; M. D. Rausher, *Nature* **2004**, *428*, 847–850.
427. A. McDonald, *Anal. Inst. Biol. UNAM Ser. Bot.* **1991**, *62*, 65–86.
428. S. M. Wilbert; D. W. Schemske; H. D. Bradshaw, Jr., *Biochem. Syst. Ecol.* **1997**, *25*, 437–443.
429. J. Ramsey; H. D. J. Bradshaw; W. Schemske, *Evolution* **2003**, *57*, 1520–1534.
430. H. D. J. Bradshaw; D. W. Schemske, *Nature* **2003**, *426*, 176–178.
431. A. M. Cooley; G. Carvallo; J. H. Willis, *Ann. Bot.* **2008**, *101*, 641–650.
432. J. M. Olesen; N. Rønsted; U. Tolderlund; C. Cornett; P. Mølgaard; J. Madsen; C. G. Jones; C. E. Olsen, *Nature* **1998**, *393*, 529.
433. H. S. Rosenkranz; G. Klopman, *Naturwissenschaften* **1999**, *86*, 404–405.
434. D. M. Hansen; K. Beer; C. B. Muller, *Biol. Lett.* **2006**, *2*, 165–168.
435. W. R. Thompson; J. Meinwald; D. Aneshansley; T. Eisner, *Sci. (N. Y.)* **1972**, *177*, 528–530.
436. K. Sasaki; T. Takahashi, *Phytochemistry* **2002**, *61*, 339–343.
437. M. Gronquist; A. Bezzerides; A. Attygalle; J. Meinwald; M. Eisner; T. Eisner, *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 13745–13750.
438. K. M. Davies; G. B. Marshall; J. M. Bradley; K. E. Schwinn; S. J. Bloor; C. S. Winefield; C. R. Martin, *Physiol. Plant* **2006**, *128*, 593–603.
439. G. Forkmann; G. Stotz, *Z. Naturforsch.* **1981**, *36c*, 411–416.
440. R. Spribille; G. Forkmann, *Phytochemistry* **1982**, *21*, 2231–2234.
441. J. B. Harborne; D. M. Smith, *Biochem. Syst. Ecol.* **1978**, *6*, 287–291.
442. H. M. Schaefer; G. Ruxton, *Biol. Lett.* **2008**, *4*, 153–155.
443. D. Peitsch; A. Fietz; H. Hertel; J. de Souza; D. F. Ventura; R. Menzel, *J. Comp. Physiol.* **1992**, *A170*, 23–40.
444. M. Simonyi; Z. Bikadi; F. Zsila; J. Deli, *Chirality* **2003**, *15*, 689–698.
445. J. B. Harborne, Fuctions of Flavonoids in Plants. In *Chemistry and Biochemistry of Plant Pigments*, 2nd ed.; T. W. Goodwin, Ed.; Academic Press: New York, 1976; Vol. 1, pp 736–778.
446. E. M. Martinelli; A. Baj; E. Bombardelli, *Anal. Chim. Acta* **1986**, *191*, 275–281.
447. A. K. Latti; K. R. Riihinen; P. S. Kainulainen, *J. Agric. Food Chem.* **2008**, *56*, 190–196.
448. K. C. Burns; J. L. Dalen, *Oikos* **2002**, *96*, 463–469.
449. E. Cazetta; H. M. Schaefer; M. Galetti, *Evol. Ecol.* **2009**, *23*, 233–244.
450. G. J. Niemann, Distribution and Evolution of the Flavonoids in Gymnosperms. In *The Flavonoids*; J. B. Harborne, Ed.; Chapman & Hall: London and New York, 1988; pp 469–478.
451. F. S. Santamour, Jr., *For. Sci.* **1966**, *12*, 429–431.
452. Ø. M. Andersen, *Biochem. Syst. Ecol.* **1992**, *20*, 145–148.
453. R. J. Griesbach; F. S. Santamour, Jr., *Biochem. Syst. Ecol.* **2003**, *31*, 261–268.
454. Ø. M. Andersen, *Acta Chem. Scand. Ser. B.* **1988**, *B42*, 462–468.

455. Ø. M. Andersen, *Phytochemistry* **1989**, *28*, 495–497.
456. N. Forde, Relationships between Birds and Fruits in Temperate Australia. In *The Dynamic Partnership: Birds and Plants in Southern Australia*; H. A. Ford, D. C. Paton, Eds.; Government Printer: Adelaide, South Australia, 1986; pp 42–58.
457. M. F. Willson, *Curr. Ornithol.* **1986**, *3*, 223–279.
458. M. F. Willson; A. K. Irvine; N. G. Walsh, *Biotropica* **1989**, *21*, 133–147.
459. D. J. O'Dowd; A. M. Gill, Seed Dispersal Syndromes in Australian *Acacia.* In *Seed Dispersal*; D. R. Murray, Ed.; Academic Press: San Diego, USA, 1986; pp 87–122.
460. K. D. Whitney; M. L. Stanton, *Ecology* **2004**, *85*, 2153–2160.
461. N. J. Dominy, *Oikos* **2002**, *98*, 163–176.
462. A. Zuker; T. Tzfira; H. Ben-Meir; M. Ovadis; E. Shklarman; H. Itzhaki; G. Forkman; S. Martens; I. Neta-Sharir; D. Weiss; A. Vainstein, *Mol. Breed.* **2002**, *9*, 33–41.
463. E. G. Johnson; B. A. Vaughan, 59th Southeast Regional Meeting of the American Chemical Society, Greenville, USA, 24–27 October 2007; American Chemical Society: Washington, DC, 2007.
464. K. S. Gould; D. W. Lee, Anthocyanins in Leaves. In *Advances in Botanical Research*; Academic Press: London, 2002; Vol. 37, pp 1–212.
465. D. C. Close; C. L. Beadle, *Bot. Rev.* **2003**, *69*, 149–161.
466. Y. Manetas, *Flora* **2006**, *201*, 163–177.
467. P. Karageorgou; C. Buschmann; Y. Manetas, *Flora* **2008**, *203*, 648–652.
468. T. S. Feild; D. W. Lee; N. M. Holbrook, *Plant Physiol.* **2001**, *127*, 566–574.
469. N. M. Hughes; W. K. Smith, *Am. J. Bot.* **2007**, *94*, 784–790.
470. M. Krol; G. R. Gray; V. M. Hurry; G. Öquist; L. Malek; N. P. A. Huner, *Can. J. Bot.* **1995**, *73*, 1119–1127.
471. M. Mendez; D. G. Jones; Y. Manetas, *New Phytol.* **1999**, *144*, 275–282.
472. I. C. Dodd; C. Critchley; G. S. Woodall; G. R. Stewart, *J. Exp. Bot.* **1998**, *49*, 1437–1445.
473. P. Karageorgou; Y. Manetas, *Tree Physiol.* **2006**, *26*, 613–621.
474. A. Kyparissis; G. Grammatikopoulos; Y. Manetas, *Tree Physiol.* **2007**, *27*, 849–857.
475. D. W. Lee; J. O'Keefe; N. M. Holbrook; T. S. Feild, *Ecol. Res.* **2003**, *18*, 677–694.
476. Y. Manetas; T. Petropoulou; G. K. Psaras; A. Dirinia, *Funct. Plant Biol.* **2003**, *30*, 275–282.
477. J. E. Page; G. H. N. Towers, *Can. Planta* **2002**, *215*, 478–484.
478. N. M. Hughes; C. B. Morley; W. K. Smith, *New Phytol.* **2007**, *175*, 675–685.
479. K. S. Gould; K. R. Markham; R. H. Smith; J. J. Goris, *J. Exp. Bot.* **2000**, *51*, 1107–1115.
480. V.-P. Kytridis; P. Karageorgou; E. Levizou; Y. Manetas, *J. Plant Physiol.* **2008**, *165*, 925–959.
481. K. Hikosaka, *J. Plant Res.* **2004**, *117*, 481–494.
482. S. Grace; B. A. Logan; A. Keller; B. Demmig-Adams; W. W. Adams, *Plant Physiol.* **1995**, *108*, 36.
483. J. P. Foot; J. A. Caporn; J. A. Lee; T. W. Ashenden, *New Phytol.* **1996**, *133*, 503–511.
484. V.-P. Kytridis; Y. Manetas, *J. Exp. Bot.* **2006**, *57*, 2203–2210.
485. L. Shao; Z. Shu; S.-L. Sun; C.-L. Peng; X.-J. Wang; Z.-F. Lin, *J. Integr. Plant Biol.* **2007**, *49*, 1341–1351.
486. K. S. Gould; J. Mckelvie; K. R. Markham, *Plant Cell Environ.* **2002**, *25*, 1261–1269.
487. P. Garcia-Macias; M. Ordidge; E. Vysini; S. Waroonphan; N. H. Battey; M. H. Gordon; P. Hadley; P. John; J. A. Lovegrove; A. Wagstaffe, *J. Agric. Food Chem.* **2007**, *55*, 10168–10172.
488. S. O. Neill; K. S. Gould, *Funct. Plant Biol.* **2003**, *30*, 865–873.
489. L. Shao; Z. Shu; C.-L. Peng; Z.-F. Lin; C.-W. Yang; Q. Gu, *Funct. Plant Biol.* **2008**, *35*, 714–724.
490. W. D. Hamilton; S. P. Brown, *Proc. R. Soc. Lond. Ser. B* **2001**, *268*, 1489–1493.
491. S. Lev-Yadun; A. Dafni; M. A. Flaishman; M. Inbar; I. Izhaki; G. Katzir; G. P. Ne'eman, *BioEssays* **2004**, *26*, 1126–1130.
492. P. Blanc, *Biologie des plantes des sous-bois tropicaux*, Thèse de Doctorat d'Etat, Univeristé Pierre et Marie Curie (Paris 6), Paris, 1989.
493. T. J. Givnish, *Func. Ecol.* **1990**, *4*, 463–474.
494. B. C. Stone, *Biotropica* **1979**, *11*, 126.
495. P. A. Hedin; J. N. Jenkins; D. H. Collum; W. H. White; W. L. Parrott; M. W. MacGown, *Experientia* **1983**, *39*, 799–801.
496. E. T. Johnson; M. A. Berhow; P. F. Dowd, *J. Chem. Ecol.* **2008**, *34*, 757–765.
497. M. Rostas; R. Bennett; M. Hilker, *J. Chem. Ecol.* **2002**, *28*, 2449–2463.
498. C. F. Quiros; M. A. Stevens; C. M. Rick; M. L. J. Kok-Yokomi, *Am. Soc. Hort. Sci.* **1977**, *102*, 166–171.
499. M. B. Isman; S. S. Duffey, *Entomol. Exp. Appl.* **1982**, *31*, 370–376.
500. W. L. Gonzales; C. C. Ramirez; N. Olea; H. M. Niemeyer, *Entomol. Exp. Appl.* **2002**, *103*, 107–113.
501. M. C. Trull; M. J. Guiltinan; J. P. Lynch; J. Deikman, *Plant Cell Environ.* **1997**, *20*, 85–92.
502. N. P. Shaikh; M. B. Adjei; J. M. Scholberg, *Commun. Soil Sci. Plant Anal.* **2008**, *39*, 1006–1015.
503. U. Diaz; V. Saliba-Colombani; O. Loudet; P. Belluomo; L. Moreau; F. Daniel-Vedele; J. F. Morot-Gaudry; U. Maselaux-Daubresse, *Plant Cell Physiol.* **2006**, *47*, 74–83.
504. Y.-M. Bi; R.-L. Wang; T. Zhu; S. J. Rothstein, *BMC Genomics* **2007**, *8*, 281.
505. M. M. Posmyk; A. Dobranowska; K. M. Janas, *Ecol. Chem. Eng.* **2005**, *12*, 1107–1112.
506. L. Chalker-Scott, *Adv. Bot. Res.* **2002**, *37*, 103–106.
507. C. Nozzolillo; P. Isabelle; Ø. M. Andersen; M. Abou-Zaid, *Can. J. Bot.* **2002**, *80*, 796–801.
508. R. A. Khavari-Nejad; M. Bujar; E. Attaran, *Tasks Veget. Sci.* **2006**, *40*, 127–134.
509. F. Eryilmaz, *Biotech. Biotechnol. Equip.* **2006**, *20*, 47–52.
510. E. Giraud; L. H. M. Ho; R. Clifton; A. Carroll; G. Estavillo; Y.-F. Tan; K. A. Howell; A. Ivanova; B. J. Pogson; A. H. Millar; J. Whelan, *Plant Physiol*. **2008**, *147*, 595–610.
511. F. Ferreres; M. I. Gil; M. Castaner; F. A. Tomas-Barberan, *J. Agric. Food Chem.* **1997**, *45*, 4249–4254.
512. R. A. Dixon; M. J. Harrison; C. J. Lamb, *Annu. Rev. Phytopathol.* **1994**, *32*, 479–501.
513. D. C. Close; N. W. Davies; C. L. Beadle, *Aust. J. Plant Physiol.* **2001**, *28*, 269–278.
514. T. Balakumar; B. Gayathri; P. R. Anbudurai, *Biol. Plant.* **1997**, *39*, 215–221.

515. H. M. Schaefer; M. Rentzsch; M. Breuer, *Nat. Prod. Commun.* **2008**, *3*, 1267–1273.
516. Y. Yan; J. Chemler; L. Huang; S. Martens; M. A. Koffas, *Appl. Environ. Microbiol.* **2005**, *71*, 3617–3623.
517. S. Ramachandra Rao; G. A. Ravishankar, *Biotechnol. Adv.* **2002**, *20*, 101–153.
518. W. Zhang; S. Furusaki, *Biotechnol. Bioprocess. Eng.* **1999**, *4*, 231–252.
519. W. Zhang; S. Furusaki; C. Franco, *Sci. China, Ser. B: Chem.* **1999**, *42*, 345–350.
520. Y. Kobayashi; M. Akita; K. Sakamoto; H. Liu; T. Shigeoka; T. Koyano; M. Kawamura; T. Furuya, *Appl. Microbiol. Biotechnol.* **1993**, *40*, 215–218.
521. Y. Asada; K. Sakamoto; T. Furuya, *Phytochemistry* **1994**, *35*, 1471–1473.
522. K. Sakamoto; K. Iida; K. Sawamura; K. Hajiro; Y. Asada; T. Yoshikawa; T. Furuya, *Phytochemistry* **1993**, *33*, 357–360.
523. N. Terahara; A. Callebaut; R. Ohba; T. Nagata; M. Ohnishi–Kameyama; M. Suzuki, *Phytochemistry* **1996**, *42*, 199–203.
524. N. Terahara; A. Callebaut; R. Ohba; T. Nagata; M. Ohnishi–Kameyama; M. Suzuki, *Phytochemistry* **2001**, *58*, 493–500.
525. A. Callebaut; G. Hendrickx; A. M. Voets; J. C. Motte, *Phytochemistry* **1990**, *29*, 2153–2158.
526. A. Callebaut; M. Decleire; K. Vandermeiren, *Biotechnol. Agric. For.* **1993**, *24*, 1–22.
527. A. Callebaut; N. Terahara; M. De Haan; M. Decleire, *Plant Cell, Tiss. Org. Cult.* **1997**, *50*, 195–201.
528. F.-W. How; M. A. Smith, *Zhonghua Nongye Yanjiu,* **2003**, *52*, 291–296.
529. K. H. Knobloch; G. Bast; J. Berlin, *Phytochemistry* **1982**, *21*, 591–594.
530. A. B. Ohlsson; T. Berglund, *Plant Cell Tiss. Org. Cult.* **2001**, *64*, 77–80.
531. R. Filippini; R. Caniato; A. Piovan; E. M. Cappelletti, *Fitoterapia* **2003**, *74*, 62–67.
532. A. Piovan; R. Filippini; D. Favretto, *Rapid Commun. Mass Spectrom.* **1998**, *12*, 361–367.
533. A. Piovan; R. Filippini, *Phytochem. Rev.* **2007**, *6*, 235–242.
534. L. Rajendran; G. A. Ravishankar; L. V. Venkataraman; K. R. Prathiba, *Biotechnol. Lett.* **1992**, *14*, 707–712.
535. L. Rajendran; G. Suvarnalatha; G. A. Ravishankar; L. V. Venkataraman, *Appl. Microbiol. Biotechnol.* **1994**, *42*, 227–231.
536. M. S. Narayan; L. V. Venkataraman, *J. Food Sci.* **2002**, *67*, 84–86.
537. D. C. Baker; D. K. Dougall; W. E. Glaessgen; S. C. Johnson; J. W. Metzger; A. Rose; H. U. Seitz, *Plant Cell Tissue Organ. Cult.* **1994**, *39*, 79–91.
538. R. Hamade; Y. Kinoshita; Y. Yamamoto; M. Tanaka; Y. Yamada, *Biosci. Biotechnol. Biochem.* **1994**, *58*, 1530–1531.
539. J. J. Zhong; J. T. Yu; T. Yoshida, *World J. Microbiol. Biotechnol.* **1995**, *11*, 461–467.
540. T. Mori; M. Sakurai; J. Shigeta; K. Yoshida; T. Kondo, *J. Food Sci.* **1993**, *58*, 788–792.
541. W. Zhang; M. Seki; S. Furusaki; A. P. J. Middelberg, *J. Ferment. Bioeng.* **1998**, *86*, 72–78.
542. S. Asano; S. Ohtsubo; M. Nakajima; M. Kusunoki; K. Kaneko; H. Katayama; Y. Nawa, *Food Sci. Technol. Res.* **2002**, *8*, 64–69.
543. H. Miura; Y. Kitamura; T. Kenaga; K. Mizobe; T. Shimizu; M. Nakamura; Y. Kato; T. Yamada; T. Maitani; Y. Goda, *Phytochemistry* **1998**, *48*, 279–283.
544. M. Nozue; J. Kawai; K. Yoshitama, *J. Plant Physiol.* **1987**, *129*, 81–88.
545. H. Tamura; M. Fujiwara; H. Sugisawa, *Agric. Biol. Chem.* **1989**, *53*, 1971–1973.
546. M. A. L. Smith; D. L. Madhavi; Y. Fang; M. M. Tomczak, *J. Plant Physiol.* **1997**, *150*, 462–466.
547. G. G. Yousef; D. S. Seigler; M. A. Grusak; R. B. Rogers; C. T. G. Knight; T. F. B. Kraft; J. W. Erdman, Jr.; Lila, M. A. *J. Agric. Food Chem.* **2004**, *52*, 1138–1145.
548. T. Yamakawa; K. Ishida; S. Kato; T. Kodama; Y. Minoda, *Agric. Biol. Chem.* **1983**, *47*, 997–1001.
549. S. Lofty; A. Fleuriet; T. Ramos; J. J. Macheix, *Plant Cell Rep.* **1989**, *8*, 93–96.
550. T. Fossen; R. Slimestad; Ø. M. Andersen, *J. Agric. Food Chem.* **2001**, *49*, 2318–2321.
551. M. Nakamura; M. Seki; S. Furusaki, *Enzyme Microb. Technol.* **1998**, *22*, 404–408.
552. N. Terahara; I. Konczak–Islam; M. Nakatani; O. Yamakawa; Y. Goda; Y. Honda, *Phytochemistry* **2000**, *54*, 919–922.
553. J. Zhong; M. Yoshida; D. Fujiyama; T. Seki; T. Yoshida, *J. Ferment. Bioeng.* **1993**, *75*, 299–303.
554. R. D. Hall; M. M. Yeoman, *New Phytol.* **1986**, *103*, 33–43.
555. V. Aumont; F. Larronde; T. Richard; H. Budzinski; A. Decendit; G. Deffieux; S. Krisa; J.-M. Merillon, *J. Biotechnol.* **2004**, *109*, 287–294.
556. A. Decendit; K. G. Ramawat; P. Waffo; G. Deffieux; A. Badoc; J.-M. Merillon, *Biotechnol. Lett.* **1996**, *18*, 659–662.
557. H. Kurata; A. Mochizuki; N. Okuda; M. Seki; S. Furusaki, *Enzyme Microb. Technol.* **2000**, *26*, 621–629.
558. H. Honda; K. Hiraoka; E. Nagamori; M. Omote; Y. Kato; S. Hiraoka; T. Kobayashi, *J. Biosci. Bioeng.* **2002**, *94*, 135–139.
559. J. E. Meyer; M.-F. Pepin; M. A. L. Smith, *J. Biotechnol.* **2002**, *93*, 45–57.
560. K. Hosokawa; Y. Fukunaga; E. Fukushi; J. Kawabata, *Phytochemistry,* **1996**, *42*, 671–672.
561. C. B. Do; F. Cormier, *Plant Cell, Tiss. Organ Cult.* **1991**, *24*, 49–54.
562. C. Curtin; W. Zhang; C. Franco, *Biotechnol. Lett.* **2003**, *25*, 1131–1135.
563. D. K. Dougall; D. C. Baker; E. G. Gakh; M. A. Redus; N. A. Whittemore, *Carbohydr. Res.* **1998**, *310*, 177–189.
564. S. Krisa; P. W. Teguo; A. Decendit; G. Deffieux; J. Vercauteren; J.-M. Merillon, *Phytochemistry* **1999**, *51*, 651–656.
565. P. S. Wharton; R. L. Nicholsen, *New Phytol.* **2000**, *145*, 457–469.
566. X. Vitrac; S. Krisa; A. Decendit; J. Vercauteren; A. Nuhrich; J.-P. Monti; G. Deffieux; J.-M. Merillon, *J. Biotechnol.* **2002**, *95*, 49–56.
567. M. A. Grusak; R. B. Rogers; G. G. Yousef; J. W. Erdman, Jr.; M. A. Lila, *In Vitro Cell Dev. Biol. Plant* **2004**, *40*, 80–85.
568. Y. Yan; Z. Li; M. A. G. Koffas, *Biotechnol. Bioeng.* **2008**, *100*, 126–140.
569. C. A. Williams, Flavone and Flavonol *O*-Glycosides. In *Flavonoids: Chemistry, Biochemistry and Applications*; Ø. M. Andersen, K. R. Markham, Eds.; CRC Press: Boca Raton, 2006; pp 749–856.
570. T. J. Marbry; K. R. Markham; M. B. Thomas, The Ultraviolet Spectra of Chalcones and Aurones. In *The Systematic Analysis of Flavonoids by Ultraviolet Spectroscopy*; Springer-Verlag: New York, 1970; pp 227–252.
571. G. Forkmann; S. Martens, *Curr. Opin. Biotechnol.* **2001**, *12*, 155–160.
572. M. R. Van Calstren; F. Cormier; C. B. Do; R. R. Laing, *Spectroscopy* **1991**, *9*, 1–15.

Biographical Sketches

Øyvind M. Andersen, a full Professor of Chemistry since 1993, has specialized in the chemistry of flavonoids. He received his Dr. Philos. degree in 1988 from University of Bergen. He along with Dr. Ken R. Markham is the editor of the book *Flavonoids: Chemistry, Biochemistry and Applications* and author of over 100 international journal articles, ten invited book chapters and five patents in the field of anthocyanins and other flavonoids. He has supervised over 40 M.Sc. and Ph.D. students in natural product chemistry, and the activities of Dr. Andersen's research group have led to the establishment of several flavonoid-based companies. His research projects concentrate on structure elucidation of new compounds, methodology within NMR spectroscopy and chromatography, and bioprospecting. Some of the latter projects treat comparable behavior and functions of individual anthocyanins with the underlying aim of exploring their pharmaceutical potential and use as colorants in food. Dr. Andersen received the Groupe Polyphenols Award in 2006.

Monica Jordheim born in 1979 has at present a post doctoral position at Department of Chemistry, University of Bergen, Norway. She completed here M.Sc. in 2003 and her Ph.D. in 2007 at the University of Bergen, both in natural product chemistry, with anthocyanins as her main research field. From 2005 she has been teaching organic analytical chemistry at Bachelor and Master levels and supervised M.Sc. students. Her publications focus on structure elucidation of anthocyanins, the stability and reducing capacity of anthocyanins, and the complexity of their purity determinations using various chromatographic techniques and advanced NMR spectroscopy. She has co-authored the chapter along with Dr. Andersen entitled *Anthocyanins* in the book *Flavonoids: Chemistry, Biochemistry and Applications.*

3.17 Production of Pharmaceuticals by Plant Tissue Cultures

Toshiya Muranaka, Yokohama City University, Yokohama, Japan, RIKEN Plant Science Center, Yokohama, Japan

Kazuki Saito, Chiba University, Chiba, Japan, RIKEN Plant Science Center, Yokohama, Japan

3.17.1 Introduction

Plants produce 'primary metabolites' for their basic life functions, which include cell division and elongation, respiration, cell differentiation, storage, and respiration. In addition, plants produce a wide variety of 'secondary metabolites' for survival under biotic and abiotic stress. The chemical diversity of plant species-specific secondary metabolites is believed to be the consequence of natural selection during evolution.[1] Owing to their biological activities, plant secondary metabolites have been used for centuries in traditional medicines, and are valued today as pharmaceuticals, food additives, and industrial raw materials (see Chapter 3.18).

Chemical synthesis is sometimes successful in producing certain metabolites of plant origin, such as menthol and salicylate, but because most of these compounds have complex chemical structures and their bioactivities are critically defined by their chirality, chemical synthesis can require many complicated reaction steps. Moreover, the recovery rates are not always high, which leads to high costs for the synthesis of these natural products. For these reasons, the extraction of useful secondary metabolites from medicinal plants is in great demand. Desirable secondary metabolites, however, are usually found in low abundance in plant tissues. For example, approximately 10 000 kg of dry bark is required to produce 1 kg of paclitaxel, an active pharmaceutical ingredient possessing antitumor activity.[2] Furthermore, extraction from intact plants is highly variable depending on the source plant, location, and harvest season, and the harvesting process sometimes degrades the compound.

Plant tissue culture provides a renewable and environmentally friendly alternative for the production of secondary plant metabolites, and recent advances in genetic engineering and gene discovery offer new opportunities to enhance both the quality and quantity of these compounds. In this chapter, we discuss methods for plant tissue culture including classical plant cell cultures, hairy root cultures, transgenic cultures, and shoot cultures, followed by a discussion of methods to produce pharmaceuticals via plant tissue culture and end the chapter with a summary.

3.17.2 Methods of Plant Tissue Culture

3.17.2.1 Plant Cell Cultures

When plant tissue segments, called 'explants', are aseptically cultured on plant tissue culture medium such as Murashige and Skoog,[3] Linsmaier and Skoog,[4] and B5,[5] along with an appropriate carbon source and phytohormones, undifferentiated cell clusters called 'calli (singular: callus)' are usually obtained. The calli are subcultured in an appropriate liquid medium as suspension cells. The basic technology for plant cell culture was established in the 1960s, but it has long been thought that calli or suspension cells produce negligible or trace amounts of secondary metabolites, unlike differentiated cells or specialized organs. Zenk's group extensively demonstrated that cell cultures have the ability to accumulate reasonable amount of useful secondary metabolites such as anthraquinones (*Morinda citrifolia*).[6,7] Yamada's group demonstrated that selection of high-producing cell lines is important to achieve high and stable cell line for desired secondary-producing metabolites by using *Euphorbia millii* cells (anthocyanin)[8,9] and *Coptis japonica* cells (berberine).[10] Somaclonal variation regarding producibility of secondary metabolites is often observed in cell culture. The mechanism underlying somaclonal variation is still not well understood, but in rice cell cultures, the transposable element Tof17 is activated, resulting in the variation.[11] The 'selection of elite clones' led to the first successful commercialization of shikonin production from *Lithospermum erythrorhizon* cell cultures by Mitsui-Petrochemicals, Japan.[12] The compounds were used in cosmetics such as lipstick in the 1980s. Phyton Biotech, founded in 1990 in Ithaca, USA, first achieved the commercial production of paclitaxel after the acquisition of Phyton GmbH in Ahrensburg, Germany, with the largest cGMP (current good manufacturing practice) plant cell culture facility (from Phyton Biotech home page, (http://www.phytonbiotech.com/index.htm). Recently, Samyang Genex, Korea, has also commercialized a process for the production of paclitaxel after selecting a high-producing stable cell line from more than 300 cell lines of various kinds of *Taxus* trees (from Samyang home page, (http://www.genex.co.kr/eng/). In **Table 1**, plant cell culture of medicinal plants is listed as 'cell culture'.

3.17.2.2 Hairy Root Cultures

When *Agrobacterium rhizogenes*, a Gram-negative soil bacterium, infects plants, adventitious roots called 'hairy roots' are induced from the infected site.[110,111] This event occurs due to the transfer of the particular DNA region called transfer DNA (T-DNA) comprising the loci between the TR and TL regions of the root-inducing (Ri) plasmid of the bacterium into the plant genome. The basic molecular mechanism of T-DNA trimming from the Ri plasmid, transfer to plant cells, and integration into the plant genome is known, although the functions of several genes on the T-DNA have not yet been elucidated. The hairy roots are aseptically cultured *in vitro* without added phytohormones.

As described in Section 3.17.2.1, undifferentiated plant cell cultures are successfully used to produce valuable secondary metabolites such as shikonin and paclitaxel, but many researchers have realized that plant cell culture is not always successful for the purpose of their production. Even if the cultured cells produced the desired compounds, the concentration was often very low compared to that of intact plants.

Several secondary metabolites of pharmaceutical interest are accumulated in plant roots. Hairy root cultures, in contrast to undifferentiated cell cultures, can usually synthesize the same compounds as the roots of the intact plant. In 1986, three laboratories independently demonstrated the production of secondary metabolites by hairy root cultures, including the production of tropane alkaloids by *Atropa belladonna*[43] and *Scopolia*,[112] and nicotine by *Nicotiana rustica*.[113]

Agrobacterium rhizogenes-mediated transformation has several features desirable for the production of secondary metabolites. The rapid and efficient induction of hairy roots from explant tissues in a wide variety of plant species, including medicinal plants, has been reported. These hairy roots are characterized by a high growth rate and high root branching without added phytohormones. Furthermore, they often produce secondary metabolites for a long period of time, unlike intact roots. For these reasons, switching from undifferentiated cell culture to hairy root culture is considered an attractive alternative for the production of many valuable secondary metabolites that originally accumulated in root tissues. In **Table 1**, the hairy root culture of medicinal plants is listed as 'HR'.

Table 1 Tissue cultures on medicinal plants

Plant species	*Metabolites*	*Plant tissue*	*Reference*
Ajuga multiflora	20-Hydroxyecdysone	HR	13
Ajuga reptans	Anthocyanins	Cell culture	14
Ajuga reptans	Phytoecdysteroids (20-hydroxyecdysone, norcyasterone B, cyasterone, isocyasterone)	HR	15
Ajuga reptans	20-Hydroxyecdysone	HR	16
Ajuga reptans	Ecdysteroids	Root culture	17
Ajuga turkestanica	20-Hydroxyecdysone	HR	18
Ambrosia artemisiifolia	Thiarubrine	HR	19
Ambrosia artemisiifolia	Thiarubrine	HR	20
Ambrosia maritima	Thiophene A and thiophene A diol	HR	21
Ambrosia tenuifolia	Sesquiterpene lactone (psilostachyinolide)	Cell culture	22
Ambrosia tenuifolia	Sesquiterpene lactone (altamisine)	Cell culture	23
Amsonia elliptica	Indole alkaloids	HR	24
Anthemis nobilis	Essential oil	Crown-gall tissue	25
Anthemis nobilis	Geranyl isovalerate	HR	26
Artemisia absinthium	Essential oils	HR	27
Artemisia absinthium	Essential oils	HR	28
Artemisia annua	Artemisinin	HR	29
Artemisia annua	Artemisinin	HR	30
Artemisia annua	Compound(s) structurally related to artemisinin	HR (green)	31
Artemisia annua	Artemisinin	Shoot cultures	32
Artemisia annua	Artemisinin	Shoot cultures	33
Artemisia annua	Artemisinin	Shoot cultures	34
Artemisia annua		Shooty teratoma	35
Artemisia dracunculus	Essential oils (phenylpropene, allylanisole)	Cell culture	36
Astragalus membranaceus	Tritepene saponins (astragalosides)	HR	37
Astragalus membranaceus	Agroastragaloside II	HR	38
Astragalus membranaceus	Tritepene saponins (astragalosides)	HR	39
Astragalus membranaceus	Tritepene saponins (astragalosides)	HR	40
Atropa baetica	Tropane alkaloids	HR	41
Atropa belladonna	Tropane alkaloids	Cell culture	42
Atropa belladonna	Tropane alkaloids	HR	43
Atropa belladonna	Calystegines	HR	44
Atropa belladonna	Tropane alkaloids	Shoot cultures	45
Azadirachta indica	Azadirachtin	Cell culture	46
Azadirachta indica	Azadirachtin-related limonoids	Cell culture	47
Azadirachta indica	Azadirachtin	HR	48
Azadirachta indica	Azadirachtin	HR	49
Beta vulgaris	Ferulic acid conjugates, betacyanins	Cell culture	50
Beta vulgaris	Betalain	Cell culture	51
Beta vulgaris	Betacyanin	Cell culture	52
Beta vulgaris	Betaxanthin	HR	53
Brugmansia candida	Tropane alkaloid	HR	54
Brugmansia candida	Tropane alkaloid	Root culture	55
Calystegia sepium	Calystegines are nortropane alkaloids	Root culture	56
Campanula glomerata	Polyacetylenes	HR	57
Campanula medium	Polyacetylenes	HR	58
Camptotheca acuminata	Camptothecin	Cell culture	59
Camptotheca acuminata	Camptothecin, 10-hydroxycamptothecin	HR	59
Cassia didymobotrya	Anthraquinones	Cell culture	60
Cassia obtusifolia	Anthraquinones	Cell culture	61
Cassia obtusifolia	Anthraquinones	HR	62
Cassia obtusifolia	Anthraquinones	HR	63
Cassia podocarpa	Anthraquinones	Cell culture	64
Cassia tora	Anthraquinones	Cell culture	65
Catharanthus roseus	Indole alkaloids	Cell culture	66
Catharanthus roseus	Indole alkaloids	HR	67

(*Continued*)

Table 1 (Continued)

Plant species	*Metabolites*	*Plant tissue*	*Reference*
Catharanthus roseus	Catharanthine, ajmalicine	HR	68
Catharanthus roseus	19(*S*)-epimisiline	HR	69
Coptis japonicus	Berberine	Cell culture	70
Coptis japonicus	Berberine	Cell culture	10
Coreopsis tincroria	Phenylpropanoids	Cell culture	71
Coreopsis tincroria	Phenylpropanoids	HR	72
Coreopsis tincroria	Allylphenol	HR	73
Coreopsis tincroria	Phenylpropanoids	Root culture	74
Datura stramonium	Tropane alkaloid	HR	75
Digitalis lanata	Cardenolides	HR	76
Digitalis pupurea	Cardiac glycosides	Cell culture	77
Duboisia leichhardtii	Tropane alkaloid	Cell culture	78
Duboisia leichhardtii	Tropane alkaloid	HR	79
Hyoscyamus niger	Tropane alkaloid	HR	75
Leontopodium alpium	Hydroxycinnamic acid esters	Cell culture	80
Leontopodium alpium	Hydroxycinnamic acid esters	HR	81
Leontopodium alpium	Essential oil	HR	82
Lippia dulcis	Hernandulcin	HR (green)	83
Lippia dulcis	Sesquiterpene (hernandulcin)	Shoot cultures	84
Ophiorrhiza pumila	Anthraquinones	Cell culture	85
Ophiorrhiza pumila	Camptothecin	HR	86
Panax ginseng	Ginsenoside	Cell culture	87
Panax ginseng	Ginsenoside	Cell culture	88
Panax ginseng	Ginsenoside	HR	89
Panax hybrid	Ginsenoside	HR	90
Panax quinquefolium	Ginsenoside	Cell culture	91
Platycodon grandiflorum	Polyacetylenes	HR	92
Rubia cordifolia	Anthraquinone	Cell culture	93
Rubia peregrina	Anthraquinone	HR	94
Rubia tinctorum	Anthraquinone	HR	95
Rubia tinctorum	Anthraquinone (nordamnacanthal)	HR	96
Rubia tinctorum	Phytochelatins, desglycyl peptides	Root culture	97
Scoparia dulcis	Diterpenoids	Cell culture	98
Scoparia dulcis	Diterpenoids (scopadulciol)	Cell culture	99
Swainsona galegifolia	Swainsonine	HR	100
Swainsona galegifolia	Swainsonine	Root culture	100
Taxus baccata	Taxol	Cell culture	101
Taxus canadensis	Taxol	Cell culture	102
Taxus cuspidata	Taxol	Cell culture	103
Taxus x media	Taxol	HR	104
Trigonella foenum	Trigonelline	Cell culture	105
Trigonella foenum	Diosgenin	HR	106
Valeriana officinalis	Valepotriates	HR	107
Valeriana officinalis	Iridoid diester	HR	108
Valeriana wallichii	Valepotriates	Cell culture	109

3.17.2.3 Transgenic Hairy Root Cultures

As described in Section 3.17.2.2, hairy root formation is induced as a consequence of the transfer of T-DNA from the Ri plasmid of *A. rhizogenes* to the host plant genome.[111] *Agrobacterium rhizogenes* can transfer the T-DNA of binary vector plasmids '*in trans*', thereby enabling the production or conversion by 'transgenic' hairy roots containing other foreign genes carried on a binary vector.[114] The procedure for hairy root induction and cultures is shown in **Figure 1**. As summarized in **Table 2**, transgenic hairy roots are often used for metabolic engineering. Some examples will be described in Section 3.17.3.

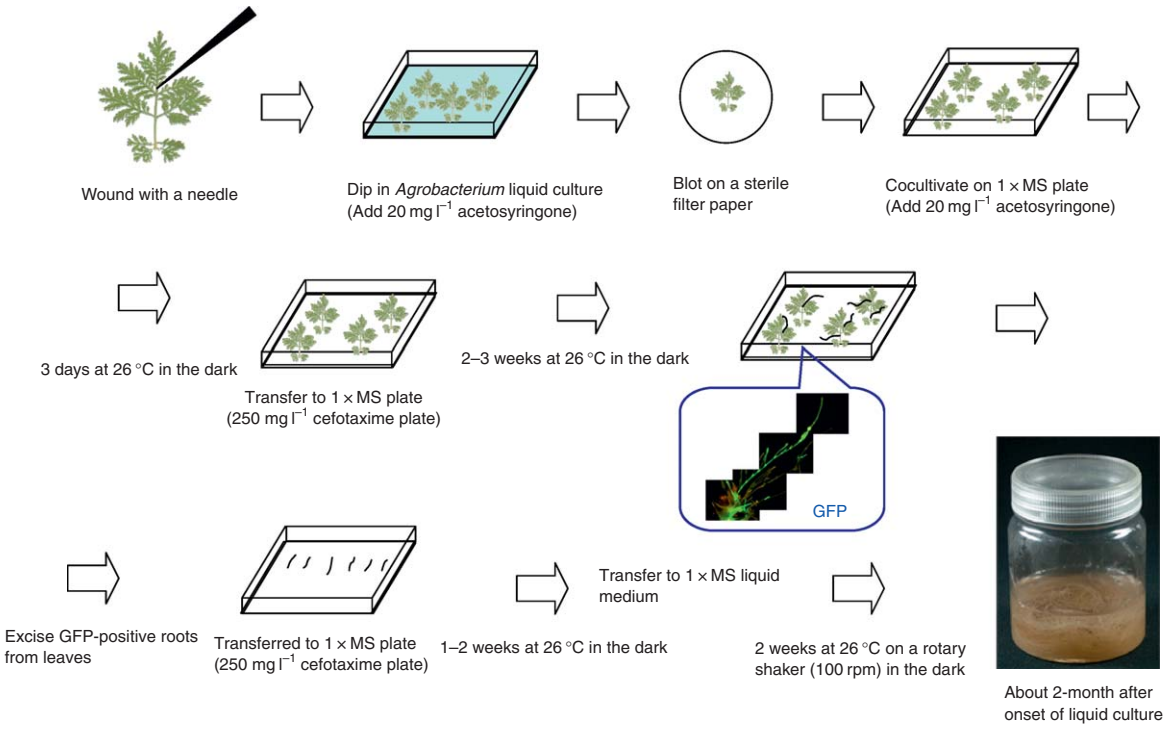


Figure 1 General scheme to induce transgenic hairy roots.

Table 2 Transgenic cell/tissue cultures for metabolic engineering

Plant species	*Metabolites*	*Transgenic tissue*	*Introduced gene*	*Reference*
Artemisia annua	Artemisinin	Transgenic HR	Farnesyl diphosphate synthase	115
Atropa baetica	Tropane alkaloids	Transgenic HR	Hyoscyamine-6-β-hydroxylase	116
Atropa belladonna	Tropane alkaloids	Transgenic HR	Hyoscyamine-6-β-hydroxylase	117
Atropa belladonna	Tropane alkaloids	Transgenic HR	Tropinone reductase	118
Atropa belladonna	Tropane alkaloids	Transgenic root	Putrescine *N*-methyltransferase	119
Catharanthus roseus	Serpentine	Transgenic HR	Feedback-resistant anthranilate synthase alpha subunit	120
Catharanthus roseus	Horhammericine	Transgenic HR	Feedback-resistant anthranilate synthase alpha subunit	121
Datura stramonium	Tropane alkaloid	Transgenic HR	Putrescine:SAM *N*-methyltransferase	122
Eschscholzia californica	Benzylisoquinoline alkaloids	Transgenic cell	(*S*)-scoulerine 9-*O*-methyltransferase (SMT)	123
Eschscholzia californica	Benzylisoquinoline alkaloids	Transgenic cell	Norcoclaurine 6-*O*-methyltransferase	124
Hyoscyamus muticus	Tropane alkaloid	Transgenic HR	Putrescine:SAM *N*-methyltransferase	122
Panax ginseng	Triterpene and phytosterol	Transgenic root	Squalene synthase	125

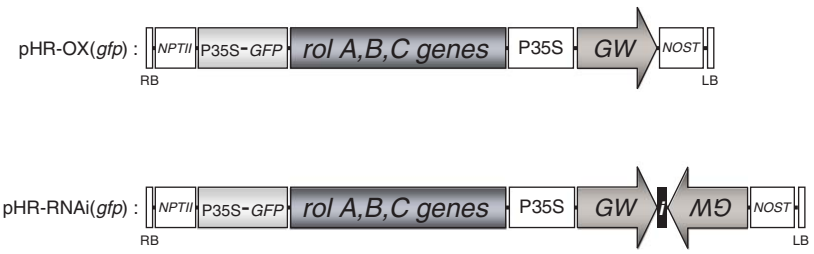


Figure 2 'All-in-one' rol-type binary vectors.[126]

Although *A. rhizogenes*-mediated delivery of binary T-DNA is indeed useful for metabolic engineering, Seki *et al.*[126] pointed out several disadvantages:

1. The frequency of codelivery of the Ri plasmid-derived and the binary vector-derived T-DNA is often very low without antibiotic selection pressure, whereas a significant reduction in the total number of hairy roots occurs under antibiotic selection, as reported by several researchers.
2. Apart from the well-studied *rol* genes (*rol A*, *rol B*, and *rol C*), which are particularly important in the induction of hairy roots, at least 16 more open reading frames (ORFs), whose functions are not yet well characterized, are present on the Ri T-DNA (TL-DNA in the agropine-type Ri plasmid).[127] These additional uncharacterized genes may cause the bias of normal root metabolism and/or physiology.
3. The Ri plasmids of the *A. rhizogenes* agropine-type strains, which are the most widely used, contain two independently transferable T-DNAs, TL-DNA and TR-DNA, which are not always transferred together into the plant genome. This nonuniformity of gene transfer would increase in inter-line genetic variation.[128]

To overcome these disadvantages, a binary vector set for efficient target gene overexpression and RNA interference (RNAi) in transformed (hairy) roots has been recently developed.[126] The vectors pHR-OX and pHR-RNAi contain a cluster of *rol* (rooting locus) genes, together with a single GATEWAY conversion cassette (in pHR-OX vectors) or inverted repeats of the GATEWAY conversion cassette separated by an intronic sequence (in pHR-RNAi vectors), flanked by a dual CaMV 35S promoter and nopaline synthase polyadenylation signal, on the same T-DNA (**Figure 2**). Transformation experiments with pHR-OX vectors using *Arabidopsis*, potato, and tobacco as model plants revealed that inoculation with *Agrobacterium tumefaciens* harboring these vectors results in the induction of numerous independently transformed roots from explants in a short period of time, and subsequent establishment of competent root culture lines. An experiment focused on the sterol biosynthetic pathway in *Arabidopsis* validated the utility of pHR-RNAi vectors for gene function analysis in cultured roots. The use of the vector system may facilitate identification of the regulatory or biosynthetic genes for the production of valuable secondary metabolites in plant roots.[126]

3.17.2.4 Shoot Cultures

The aerial parts of plants, especially *in vitro*-differentiated shoots, are known to produce a wide range of compounds. The *in vitro* shoot cultures generally require the presence of plant hormones, and several examples of shoot cultures for producing secondary metabolites are described in **Table 1** as 'shoot culture'. *Agrobacterium tumefaciens*-induced shoot-differentiated tumors called 'shooty teratoma' have the advantage of growing independently of growth regulators. Brasileiro *et al.* screened six *A. tumefaciens* strains for highly efficient shoot-differentiated tumors from poplar, wild cherry, and walnut. As a result, *A. tumefaciens* strain 82.139 was found to be the most effective for shoot-differentiated tumor induction and culture.[129] These cultures would be useful in cases in which hairy roots do not form the secondary metabolites found in the aerial parts of plants such as in *Artemisia* species.

3.17.3 Production of Pharmaceuticals by Plant Tissue Culture

So far, thousands of research papers on the production of pharmaceuticals in plant tissue culture have been published, and representative research papers are listed in **Tables 1** and **2**. Large-scale cultures for metabolite production are also listed in **Table 3**. In this section, several methods for the production of pharmaceuticals by plant tissue culture are described.

3.17.3.1 Alkaloids

3.17.3.1.1 Monoterpene indole alkaloids

Monoterpene indole alkaloids (MIAs) are a large class of plant alkaloids of major pharmacological interest (see Chapter 1.25). *Catharanthus roseus* produces the powerful anticancer drugs vinblastine and vincristine, which are derived from the dimerization of the MIAs, vindoline and catharanthine. Alkaloid formation by cell and hairy root cultures of *C. roseus* was first reported in 1969 and 1989,[145] respectively. Because of the importance of these compounds, hundreds of research papers related to the tissue culture of this plant have been published, but production of vincristine and vinblastine by cell or hairy root cultures remains unsuccessful. Instead, precursor indole alkaloids such as catharanthine, ajmalicine, and vindoline were produced in these cultures. The gene for deacetylvindoline-4-*O*-acetyltransferase (DAT), responsible for the terminal step of vindoline biosynthesis, was cloned from the plant[146] and expressed in *C. roseus* hairy root cultures. Neither vindoline nor the dimeric

Table 3 Production by bioreactor

Plant species	*Metabolites*	*Production, culture period*	*Plant tissue*	*Reactor type*	*Reference*
Artemisia annua	Artemisinin	578 mg l^{-1}, 20 days	HR	Inner-loop airlift bioreactor	130
Artemisia annua	Artemisinin	536 mg l^{-1}, 20 days	HR	Modified inner-loop airlift bioreactor	131
Artemisia annua	Camphor	3.41 mg%FW, 15 days	Shoot cultures	Bioreactor (1 l)	132
Artemisia annua	Artemisinin	47 mg l^{-1}, 25 days	Shoot cultures	Inner-loop airlift bioreactor	133
Astragalus membranaceus	Astragaloside IV	8.46 mg l^{-1}, 20 days	HR	Airlift bioreactor	134
Astragalus membranaceus	Polysaccharide	300 mg l^{-1}, 20 days	HR	Airlift bioreactor	134
Atropa belladonna	Tropane alkaloids	50 mg l^{-1}, 30 days	HR	Stirred bioreactors with a stainless-steel net	135
Azadirachta indica	Azadirachtin	71 mg l^{-1}	Cell culture	Centrifugal impeller bioreactor	136
Beta vulgaris	Betacyanin	238 mg l^{-1}, 15 days	HR	Airlift bioreactor	137
Catharanthus roseus	Catharanthine	61 mg l^{-1}, 35 days	HR	Two-stage culture	138
Coptis japonicus	Berberine	1000 mg l^{-1}, 14 days	Cell culture	One-stage culture	139
Ophiorrhiza pumila	Camptothecin	1.2 mg l^{-1}, 56 days	HR	Bioreactor (3 l)	140
Panax ginseng	Ginsenoside	50 mg l^{-1}, 42 days	Embryogenic tissues	Airlift reactor	141
Taxus baccata	Paclitaxel	43 mg l^{-1}, 16 days	Cell culture	Stirred bioreactor	142
Taxus chinensis	Paclitaxel	17 mg l^{-1}, 12 days	Cell culture	Airlift loop reactor	143
Taxus yunnanensis	Paclitaxel	38 mg l^{-1}, 20 days	Cell culture	Two-stage culture	144

FW, fresh weight.

alkaloids were altered in the transgenic hairy root. Instead, their MIA profiles were altered, suggesting that further expression of vindoline pathway genes could lead to important changes in alkaloid profiles.[147] Hirata *et al.* reported trace amounts of vinblastine in multiple shoot cultures,[148] but routine production of the dimeric alkaloid by shoot culture is still difficult.[149]

Camptothecin (CPT) is one of most important MIAs of plant origin, though its skeleton belongs to the quinoline group by rearrangement. The compound is produced in many distantly related plants, including *Camptotheca acuminata*, *Nothapodytes foetida*, and *Ophiorrhiza* species. Its semisynthetic water-soluble analogues topotecan and irinotecan are used as clinical antitumor agents throughout the world. Despite rapid growth in the market, CPT is still extracted from the seeds of *C. acuminata* and the bark of *N. foetida*. *Camptotheca acuminata* hairy roots produce and secrete CPT as well as a natural derivative, 10-hydroxycamptothecin, into the medium. The cultures were able to synthesize the alkaloids at levels equal to, and sometimes greater than, the roots *in planta*.[150] No studies, however, have been reported on hairy root cultures of *N. foetida*, the main species used for the production of CPT. In the genus *Ophiorrhiza* (Rubiaceae), widely distributed throughout tropical and temperate Asia, CPT has been detected in four species: *Ophiorrhiza pumila*, *Ophiorrhiza liukiuensis*, *Ophiorrhiza kuroiwai*, and *Ophiorrhiza mungos*. Although *Ophiorrhiza* species are not used as commercial sources of CPT, intensive tissue culture studies has been performed to produce the compounds and molecular genetic studies to identify the biosynthetic genes.[151] *Camptotheca acuminata* cell suspension cultures were reported in the 1970s, but the level of CPT production in these cultures was insufficient for a commercial production.[152] Saito *et al.* developed the first feasible CPT production system in *O. pumila* hairy root cultures. In these hairy roots, CPT accumulated at levels similar to those in the roots of the original plants and was excreted into the culture medium in large quantities.[86] These hairy root cultures have been grown in 3 l bioreactors for the production of CPT.[140] Biosynthetic studies have been carried out using these hairy roots.[153,154]

3.17.3.1.2 Benzylisoquinoline alkaloids

Papaver somniferum (opium poppy) accumulates several benzylisoquinoline alkaloids (BIAs). The most important compounds are the narcotic analgesic morphine and the cough suppressant codeine. Both BIAs were detected in cell cultures,[155,156] but the concentration of the alkaloids was not very high. When hairy root cultures were established for this plant,[157,158] the total alkaloid content was higher in the hairy roots than in the untransformed roots. The hairy root clones accumulated three times more codeine than intact roots, and morphine and sanguinarine were found in the liquid culture medium.[158] The gene for codeinone reductase, the penultimate step in morphine biosynthesis,[159] was overexpressed in *P. somniferum*. In the transgenic plants, levels of both morphine and codeine were increased. In addition, thebaine, which is upstream of codeinone reductase in the pathway, was also increased.[160] Large-scale expression data suggest that overexpression of one alkaloid biosynthetic gene might cause coordinated transcriptional induction of other pathway genes.[161] It would be interesting to test the overexpression of the gene in a hairy root system to examine whether the same results will occur.

Coptis japonica accumulates berberine, an antimicrobial agent, in the root. Sato and Yamada isolated high berberine-accumulating cell lines based on the 'selection of elite clones' described in Section 3.17.2.1.[10] By using the high berberine-accumulating cell lines, the genes for berberine biosynthesis were cloned. As transgenic work is difficult in *C. japonica*, the functions of these genes were analyzed by overexpression and RNAi cell lines in *Eschscholzia californica* (California poppy).[123,124]

3.17.3.1.3 Nicotine and tropane alkaloids

The Solanaceae family produces a range of biologically active alkaloids that include nicotine and tropane alkaloids. Tropane alkaloids such as hyoscyamine and scopolamine, which are found mainly in *Atropa*, *Duboisia*, *Hyoscyamus*, and *Scopolia* species, together with their semisynthetic derivatives, are useful as parasympatholytics that competitively antagonize acetylcholine. The degree of difficulty of tissue culture is dependent upon the particular plant species; both *Nicotiana* species and *A. belladonna* belong to a group of plants that are 'easy' to tissue culture. Because these two plant species produce the important alkaloids nicotine and tropane, respectively, various *in vitro* methods have been performed on them.

Increasing the activity of ornithine decarboxylase in *N. rustica* can result in increased nicotine production.[162] N-methylation of putrescine catalyzed by putrescine *N*-methyltransferase (PMT) is the first committed step in the biosynthesis of both tropane and nicotine alkaloids. Overexpression of tobacco PMT cDNA increased the

nicotine content in *Nicotiana sylvestris*, whereas suppression of endogenous PMT activity severely decreased the nicotine content.[123] To control metabolic flow effectively, it is important to regulate many genes for enzymes in the pathway. In the early 1990s, the overexpression of hyoscyamine-6-β-hydroxylase (H6H) in *A. belladonna* was reported to efficiently convert hyoscyamine to scopolamine in transgenic hairy roots.[163] The *H6H* gene alone, or in combination with other genes, has been further transferred to various tropane alkaloid-producing plant species. Transgenic root cultures of *Hyoscyamus muticus* with the *H6H* gene were able to produce over 100 times more scopolamine than the control culture.[164] While overexpression of the *PMT* gene in *H. muticus* and *Datura metel* resulted in only slight changes in tropane alkaloid levels,[122] when both the *PMT* and *H6H* genes were simultaneously overexpressed in the hairy roots of *Hyoscyamus niger*, a significantly high production of scopolamine was achieved.[165]

3.17.3.2 Terpenoids

More than 20 000 terpenoids, belonging to a big chemical family, were isolated from various plants. These plant terpenoids of a wide chemical variety are produced in the cytosol, plastids, and mitochondria. All terpenoids are biosynthesized from common C5 isoprene units, namely isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Unlike vertebrates, plants synthesize IPP and DMAPP via two different pathways, the cytosolic mevalonate (MVA) pathway and the plastidial 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway. Sesquiterpenes, triterpenes, and sterols are biosynthesized via the MVA pathway, while monoterpenes, diterpenes, carotenoids, and chlorophyll side chains are biosynthesized via the MEP pathway. Despite the compartmentalization of these two pathways, the existence of metabolic flow between them has been reported.[166–168] Since no biosynthetic pathway for IPP or DMAPP has been found in mitochondria, cytosolic C5 isoprene units are believed to be precursors of the ubiquinone side chains that are synthesized in mitochondria.

3.17.3.3 Sesquiterpenoids

Artemisinin, a sesquiterpene lactone obtained from *Artemisia annua* (Asteraceae), is a new and highly effective antimalarial drug (see Chapter 1.16). *Artemisia annua* has a long history of use in traditional Chinese medicine and this plant is currently the only source of artemisinin; therefore, extensive molecular genetic and chemical studies to find the gene for biosynthesis of this sesquiterpenoid have been undertaken. Recently, three enzymes (1) amorpha-4, 11-diene synthase (ADS), a sesquiterpene synthase;[169] (2) CYP71AV1, a P-450 monooxygenase oxidizing amorpha-4,11-diene to artemisinic acid;[170,171] and (3) artemisinic aldehyde Δ11(13) reductase[172] have been shown to have key roles in artemisinin biosynthesis. Transformation protocols to obtain hairy roots containing artemisinin from this plant have been reported.[173,174] Because artemisinin biosynthetic genes are highly expressed in trichomes, and only expressed in trace amounts in root tissue, identification of artemisinin from root extracts by nuclear magnetic resonance (NMR) and mass spectrometry analysis is required. Liu *et al.* reported the production of artemisinin in shoot cultures and tested various types of bioreactors for artemisinin production from shoot cultures, finding that nutrient mist bioreactors produced more than multiplate radius-flow bioreactors or modified airlift bioreactors.[175]

3.17.3.4 Triterpenoids

Triterpenes exhibit wide structural diversity and biological activity, and many of these saponin glycosides are economically important as natural medicines. *Panax ginseng* is the most popular medicinal plant and has long been recognized as a herb of great value. This plant contains a complex of ginsenosides, triterpene saponins, such as Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1. In 1985, Nitto-Denko, Japan, succeeded in large-scale cell culture of *P. ginseng* for commercial purposes.[176] Hairy root cultures of *P. ginseng* have been established and tested for glycosylation of exogenously applied substrates (phenylpropionic acid),[177] cryopreservation,[178] and large-scale culture for ginsenoside production.[179] Genetic engineering of saponin production in *P. ginseng* has been reported.[125,180] Glycyrrhizin, a natural sweetener accumulated in roots and stolons of *Glycyrrhiza* species, is also a triterpene saponin. Compared to ginsenoside, efforts to produce glycyrrhizin through tissue cultures,

such as cell or hairy root cultures, have all been unsuccessful. Glycyrrhizin is probably derived from the triterpene, β-amyrin. Very recently, a novel CYP88 family P-450, named CYP88D6, was identified as a β-amyrin 11-oxidase in the glycyrrhizin pathway.[181] CYP88D6 and additional biosynthetic genes could be used to engineer the production of glycyrrhizin in plant tissue culture (see Chapter 1.18).

3.17.4 Perspectives

In this chapter, a brief history and recent advances in the production of pharmaceuticals by plant tissue cultures were summarized. Research on each step in a tissue culture protocol for the growth of tissues and the production of desired compounds is still important for success. Furthermore, several challenging trials for metabolic engineering will be required to enhance the quality and quantity of the tissue culture-derived metabolites, including the overexpression of a rate-limiting enzyme in an early pathway to increase the overall downstream compounds, introduction of a new branch into the pathway via different plant sources, and accumulation of a pathway intermediate by the knockout or knockdown of a key step. Furthermore, in addition to direct metabolic engineering with isolated biosynthetic genes, the regulation of biosynthetic activity by transcription factors and/or reconstruction of the entire biosynthetic pathway will be interesting. Although a large amount of genome and gene expression information is available for several plant species, such information is still limited for many medicinal plants. To apply this abundant information to medicinal plants of interest, it is important to determine the general concepts and methodology.

Abbreviations

ADS	amorpha-4,11-diene synthase
BIA	benzylisoquinoline alkaloid
CPT	camptothecin
DAT	deacetylvindoline-4-*O*-acetyltransferase
DMAPP	dimethylallyl diphosphate
HR	hairy root
IPP	isopentenyl diphosphate
MEP	2-*C*-methyl-D-erythritol-4-phosphate
MIA	monoterpene indole alkaloid
MS	Murashige and Skoog
MVA	mevalonate
ORF	open-reading frame
PMT	putrescine *N*-methyltransferase
RNAi	RNA interference
Ri	root-inducing
T-DNA	transfer DNA

References

1. M. Benderoth; S. Textor; A. J. Windsor; T. Mitchell-Olds; J. Gershenzon; J. Kroymann, *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 9118–9123.
2. D. G. I. Kingston, *Trends Biotechnol.* **1994**, *12*, 222–227.
3. T. Murashige; F. Skoog, *Physiol. Plant.* **1962**, *15*, 473–479.
4. E. M. Linsmaier; F. Skoog, *Physiol. Plant.* **1965**, *18*, 100–127.
5. O. L. Gamborg; R. A. Miller; K. Ojima, *Exp. Cell Res.* **1968**, *50*, 151–158.
6. K. Inoue; H. Nayeshiro; H. Inouye; M. Zenk, *Phytochemistry* **1981**, *20*, 1693–1700.
7. M. H. Zenk, *Phytochemistry* **1991**, *30*, 3861–3863.
8. Y. Yamamoto; R. Mizuguchi; Y. Yamada, *Theor. Appl. Genet.* **1982**, *61*, 113–116.
9. Y. Yamamoto; N. Kadota; R. Mizuguchi; Y. Yamada, *Agric. Biol. Chem.* **1983**, *47*, 1021–1026.

10. F. Sato; Y. Yamada, *Phytochemistry* **1984**, *23*, 281–285.
11. H. Hirochika; K. Sugimoto; Y. Otsuki; H. Tsugawa; M. Kanda, *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 7783–7788.
12. H. Deno; C. Suga; T. Morimoto; Y. Fujita, *Plant Cell Rep.* **1987**, *6*, 197–199.
13. O. T. Kim; M. Manickavasagm; Y. J. Kim; M. R. Jin; K. S. Kim; N. S. Seong; B. Hwang, *J. Plant Biol.* **2005**, *48*, 258–262.
14. A. Callebaut; G. Hendrickx; A. M. Voets; J. C. Motte, *Phytochemistry* **1990**, *29*, 2153–2158.
15. T. Matsumoto; N. Tanaka, *Agric. Biol. Chem.* **1991**, *55*, 1019–1025.
16. N. Uozumi; S. Makino; T. Kobayashi, *J. Ferment. Bioeng.* **1995**, *80*, 362–368.
17. J. Tomas; F. Camps; J. Coll; E. Mele; J. Messeguer, *Phytochemistry* **1993**, *32*, 317–324.
18. D. M. Cheng; G. G. Yousef; M. H. Grace; R. B. Rogers; J. Gorelick-Feldman; I. Raskin; M. A. Lila, *Plant Cell Tissue Organ Cult.* **2008**, *93*, 73–83.
19. M. L. Gomezbarrios; F. J. Parodi; D. Vargas; L. Quijano; M. A. Hjortso; H. E. Flores; N. H. Fischer, *Phytochemistry* **1992**, *31*, 2703–2707.
20. S. G. Bhagwath; M. A. Hjortso, *J. Biotechnol.* **2000**, *80*, 159–167.
21. S. AbouZid; Y. Orihara, *Planta Med.* **2007**, *73*, 1327–1329.
22. M. E. Goleniowski; G. L. Silva; V. S. Trippi, *Phytochemistry* **1990**, *29*, 2889–2891.
23. M. Goleniowski; V. S. Trippi, *Plant Cell Tissue Organ Cult.* **1999**, *56*, 215–218.
24. M. Sauerwein; K. Ishimaru; K. Shimomura, *Phytochemistry* **1991**, *30*, 1153–1155.
25. M. L. Fauconnier; M. Jaziri; M. Marlier; J. Roggemans; J. P. Wathelet; G. Lognay; M. Severin; J. Homes; K. Shimomura, *J. Plant Physiol.* **1993**, *141*, 759–761.
26. T. Omoto; I. Asai; K. Ishimaru; K. Shimomura, *Phytochemistry* **1998**, *48*, 971–974.
27. A. I. Kennedy; S. G. Deans; K. P. Svoboda; A. I. Gray; P. G. Waterman, *Phytochemistry* **1993**, *32*, 1449–1451.
28. S. Nin; A. Bennici; G. Roselli; D. Mariotti; S. Schiff; R. Magherini, *Plant Cell Rep.* **1997**, *16*, 725–730.
29. P. J. Weathers; R. D. Cheetham; E. Follansbee; K. Teoh, *Biotechnol. Lett.* **1994**, *16*, 1281–1286.
30. C. Z. Liu; Y. C. Wang; F. Ouyang; H. C. Ye; G. F. Li, *Biotechnol. Lett.* **1997**, *19*, 927–929.
31. M. Jaziri; K. Shimomura; K. Yoshimatsu; M. L. Fauconnier; M. Marlier; J. Homes, *J. Plant Physiol.* **1995**, *145*, 175–177.
32. D. P. Fulzele; A. T. Sipahimalani; M. R. Heble, *Phytother. Res.* **1991**, *5*, 149–153.
33. H. J. Woerdenbag; J. F. J. Luers; W. Vanuden; N. Pras; T. M. Malingre; A. W. Alfermann, *Plant Cell Tissue Organ Cult.* **1993**, *32*, 247–257.
34. N. B. Paniego; A. M. Giulietti, *Plant Cell Tissue Organ Cult.* **1994**, *36*, 163–168.
35. B. Ghosh; S. Mukherjee; S. Jha, *Plant Sci.* **1997**, *122*, 193–199.
36. C. M. Cotton; L. V. Evans; J. W. Gramshaw, *J. Exp. Bot.* **1991**, *42*, 365–375.
37. M. Hirotani; Y. Zhou; H. Lui; T. Furuya, *Phytochemistry* **1994**, *36*, 665–670.
38. M. Hirotani; Y. Zhou; H. K. Rui; T. Furuya, *Phytochemistry* **1994**, *37*, 1403–1407.
39. Y. Zhou; M. Hirotani; H. Rui; T. Furuya, *Phytochemistry* **1995**, *38*, 1407–1410.
40. I. Ionkova; T. Kartnig; W. Alfermann, *Phytochemistry* **1997**, *45*, 1597–1600.
41. R. Zarate, *Plant Cell Rep.* **1999**, *18*, 418–423.
42. L. K. Simola; S. Nieminen; A. Huhtikangas; M. Ylinen; T. Naaranlahti; M. Lounasmaa, *J. Nat. Prod.* **1988**, *51*, 234–242.
43. H. Kamada; N. Okamura; M. Satake; H. Harada; K. Shimomura, *Plant Cell Rep.* **1986**, *5*, 239–242.
44. G. Rothe; U. Garske; B. Drager, *Plant Sci.* **2001**, *160*, 1043–1053.
45. B. D. Benjamin; P. C. Roja; M. R. Heble; M. S. Chadha, *J. Plant Physiol.* **1987**, *129*, 129–135.
46. A. Wewetzer, *Phytoparasitica* **1998**, *26*, 47–52.
47. K. N. Raval; S. Hellwig; G. Prakash; A. Ramos-Plasencia; A. Srivastava; J. Buchs, *J. Biosci. Bioeng.* **2003**, *96*, 16–22.
48. E. J. Allan; J. P. Eeswara; A. P. Jarvis; A. J. Mordue; E. D. Morgan; T. Stuchbury, *Plant Cell Rep.* **2002**, *21*, 374–379.
49. R. K. Satdive; D. P. Fulzele; S. Eapen, *J. Biotechnol.* **2007**, *128*, 281–289.
50. M. Bokern; S. Heuer; V. Wray; L. Witte; T. Macek; T. Vanek; D. Strack, *Phytochemistry* **1991**, *30*, 3261–3265.
51. R. R. Leathers; C. Davin; J. P. Zryd, *In Vitro Cell. Dev. Biol.-Plant* **1992**, *28P*, 39–45.
52. T. Akita; Y. Hina; T. Nishi, *Biosci. Biotechnol. Biochem.* **2000**, *64*, 1807–1812.
53. J. Hempel; H. Bohm, *Phytochemistry* **1997**, *44*, 847–852.
54. A. M. Giulietti; A. J. Parr; M. J. C. Rhodes, *Planta Med.* **1993**, *59*, 428–431.
55. J. Nino; C. M. Gallego; Y. M. Correa; O. M. Mosquera, *Plant Cell Tissue Organ Cult.* **2003**, *74*, 289–291.
56. Y. Scholl; B. Schneider; B. Drager, *Phytochemistry* **2003**, *62*, 325–332.
57. N. Tanaka; E. Matsuura; N. Terahara; K. Ishimaru, *J. Plant Physiol.* **1999**, *155*, 251–254.
58. H. Tada; T. Nakashima; H. Kunitake; K. Mori; M. Tanaka; K. Ishimaru, *J. Plant Physiol.* **1996**, *147*, 617–619.
59. X. W. Pan; Y. Y. Shi; X. Liu; X. Gao; Y. T. Lu, *Plant Growth Regul.* **2004**, *44*, 59–63.
60. B. Botta; G. Dallolio; F. Ferrari; B. Monacelli; G. Pasqua; R. Scurria; G. Dellemonache, *J. Plant Physiol.* **1989**, *135*, 290–294.
61. S. Takahashi; S. Kitanaka; M. Takido; Y. Ebizuka; U. Sankawa; M. Hoson; M. Kobayashi; S. Shibata, *Planta Med.* **1978**, *33*, 389–392.
62. T. Asamizu; K. Akiyama; I. Yasuda, *Yakugaku Zasshi* **1988**, *108*, 1215–1218.
63. H. Z. Guo; Z. Z. Chang; R. J. Yang; D. Guo; J. H. Zheng, *Phytochemistry* **1998**, *49*, 1623–1625.
64. P. P. Rai, *J. Nat. Prod.* **1988**, *51*, 492–495.
65. M. Tabata; N. Hiraoka; M. Ikenoue; Y. Sano; M. Konoshima, *Lloydia-J. Nat. Prod.* **1975**, *38*, 131–134.
66. B. D. Patterson; D. P. Carew, *Lloydia* **1969**, *32*, 131–140.
67. L. Toivonen; J. Balsevich; W. G. W. Kurz, *Plant Cell Tissue Organ Cult.* **1989**, *18*, 79–93.
68. F. Vazquezflota; O. Morenovalenzuela; M. L. Mirandaham; J. Coellocoello; V. M. Loyolavargas, *Plant Cell Tissue Organ Cult.* **1994**, *38*, 273–279.
69. S. R. Peraza-Sanchez; M. M. Gamboa-Angulo; C. Erosa-Lopez; I. Ramirez-Erosa; F. Escalante-Erosa; L. M. Pena-Rodriguez; V. M. Loyola-Vargas, *Nat. Prod. Lett.* **1998**, *11*, 217–224.
70. Y. Yamada; F. Sato, *Phytochemistry* **1981**, *20*, 545–547.
71. J. Reichling; U. Thron, *Pharm. Weekbl.-Sci.* **1989**, *11*, 83–86.

72. J. Reichling; U. Thron, *Planta Med.* **1990**, *56*, 488–490.
73. K. H. Horz; J. Reichling, *Phytochemistry* **1993**, *33*, 349–351.
74. U. Thron; L. Maresch; R. Beiderbeck; J. Reichling, *Z. Naturforsch., C:-Biosci.* **1989**, *44*, 573–577.
75. M. Jaziri; M. Legros; J. Homes; M. Vanhaelen, *Phytochemistry* **1988**, *27*, 419–420.
76. K. Yoshimatsu; M. Satake; K. Shimomura; J. Sawada; T. Terao, *J. Nat. Prod.* **1990**, *53*, 1498–1502.
77. J. Staba, *J. Pharm. Sci.* **1962**, *51*, 249–254.
78. Y. Yamada; T. Endo, *Plant Cell Rep.* **1984**, *3*, 186–188.
79. Y. Mano; H. Ohkawa; Y. Yamada, *Plant Sci.* **1989**, *59*, 191–201.
80. D. Hennessy; I. Hook; H. Sheridan; A. McGee, *Phytochemistry* **1989**, *28*, 489–490.
81. I. Hook, *Plant Cell Tissue Organ Cult.* **1994**, *38*, 321–326.
82. N. Comey; I. Hook; H. Sheridan; J. Walsh; P. James, *J. Nat. Prod.* **1997**, *60*, 148–149.
83. M. Sauerwein; T. Yamazaki; K. Shimomura, *Plant Cell Rep.* **1991**, *9*, 579–581.
84. M. Sauerwein; H. E. Flores; T. Yamazaki; K. Shimomura, *Plant Cell Rep.* **1991**, *9*, 663–666.
85. M. Kitajima; U. Fischer; M. Nakamura; M. Ohsawa; M. Ueno; H. Takayama; M. Unger; J. Stockigt; N. Aimi, *Phytochemistry* **1998**, *48*, 107–111.
86. K. Saito; H. Sudo; M. Yamazaki; M. Koseki-Nakamura; M. Kitajima; H. Takayama; N. Aimi, *Plant Cell Rep.* **2001**, *20*, 267–271.
87. T. Furuya; H. Kojima; K. Syono; T. Ishii; K. Uotani; M. Nishio, *Chem. Pharm. Bull.* **1973**, *21*, 98–101.
88. N. Fujioka; H. Kohda; K. Yamasaki; R. Kasai; O. Tanaka; Y. Shoyama; I. Nishioka, *Planta Med.* **1989**, *55*, 576–577.
89. T. Yoshikawa; T. Furuya, *Plant Cell Rep.* **1987**, *6*, 449–453.
90. D. Washida; K. Shimomura; Y. Nakajima; M. Takido; S. Kitanaka, *Phytochemistry* **1998**, *49*, 2331–2335.
91. A. Mathur; Y. N. Shukla; M. Pal; P. S. Ahuja; G. C. Uniyal, *Phytochemistry* **1994**, *35*, 1221–1225.
92. J. C. Ahn; B. Hwang; H. Tada; K. Ishimaru; K. Sasaki; K. Shimomura, *Phytochemistry* **1996**, *42*, 69–72.
93. H. Suzuki; T. Matsumoto; Y. Mikami, *Agric. Biol. Chem.* **1984**, *48*, 603–610.
94. A. H. Lodhi; B. V. Charlwood, *Plant Cell Tissue Organ Cult.* **1996**, *46*, 103–108.
95. K. Sato; T. Yamazaki; E. Okuyama; K. Yoshihira; K. Shimomura, *Phytochemistry* **1991**, *30*, 1507–1509.
96. R. Vanderheijden; R. Verpoorte; S. S. Hoekstra; J. H. C. Hoge, *Plant Physiol. Biochem.* **1994**, *32*, 399–404.
97. H. Kubota; K. Sato; T. Yamada; T. Maitani, *Plant Sci.* **1995**, *106*, 157–166.
98. T. Hayashi; K. Okamura; M. Kawasaki; N. Morita, *Phytochemistry* **1993**, *33*, 353–356.
99. T. Hayashi; K. Gotoh; K. Kasahara, *Phytochemistry* **1996**, *41*, 193–196.
100. T. M. Ermayanti; J. A. McComb; P. A. Obrien, *J. Exp. Bot.* **1994**, *45*, 633–639.
101. T. J. Hirasuna; L. J. Pestchanker; V. Srinivasan; M. L. Shuler, *Plant Cell Tissue Organ Cult.* **1996**, *44*, 95–102.
102. M. Hezari; R. E. B. Ketchum; D. M. Gibson; R. Croteau, *Arch. Biochem. Biophys.* **1997**, *337*, 185–190.
103. A. G. Fettneto; F. Dicosmo; W. F. Reynolds; K. Sakata, *Biotechnology* **1992**, *10*, 1572–1575.
104. M. Furmanowa; K. Syklowska-Baranek, *Biotechnol. Lett.* **2000**, *22*, 683–686.
105. S. S. Radwan; C. K. Kokate, *Planta* **1980**, *147*, 340–344.
106. A. Merkli; P. Christen; I. Kapetanidis, *Plant Cell Rep.* **1997**, *16*, 632–636.
107. F. Granicher; P. Christen; I. Kapetanidis, *Plant Cell Rep.* **1992**, *11*, 339–342.
108. F. Granicher; P. Christen; P. Kamalaprija; U. Burger, *Phytochemistry* **1995**, *38*, 103–105.
109. H. Becker; S. Herold, *Planta Med.* **1983**, *49*, 191–192.
110. M. D. Chilton; D. A. Tepfer; A. Petit; C. David; F. Cassedelbart; J. Tempe, *Nature* **1982**, *295*, 432–434.
111. O. Nilson; O. Olsson, *Physiol. Plant.* **1997**, *100*, 463–473.
112. Y. Mano; S. Nabeshima; C. Matsui; H. Ohkawa, *Agric. Biol. Chem.* **1986**, *50*, 2715–2722.
113. M. J. C. Rhodes; J. Payne; R. J. Robins, *Planta Med.* **1986**, *52*, 226–229.
114. R. B. Simpson; A. Spielmann; L. Margossian; T. D. McKnight, *Plant Mol. Biol.* **1986**, *6*, 403–415.
115. D. H. Chen; C. J. Liu; H. C. Ye; G. F. Li; B. Y. Liu; Y. L. Men; X. Y. Chen, *Plant Cell Tissue Organ Cult.* **1999**, *57*, 157–162.
116. R. Zarate; N. El Jaber-Vazdekis; B. Medina; A. G. Ravelo, *Biotechnol. Lett.* **2006**, *28*, 1271–1277.
117. D. J. Yun; T. Hashimoto; Y. Yamada, *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11799–11803.
118. U. Richter; G. Rothe; A. K. Fabian; B. Rahfeld; B. Drager, *J. Exp. Bot.* **2005**, *56*, 645–652.
119. G. Rothe; A. Hachiya; Y. Yamada; T. Hashimoto; B. Drager, *J. Exp. Bot.* **2003**, *54*, 2065–2070.
120. E. H. Hughes; S. B. Hong; S. I. Gibson; J. V. Shanks; K. Y. San, *Metab. Eng.* **2004**, *6*, 268–276.
121. C. A. M. Peebles; S. B. Hong; S. I. Gibson; J. V. Shanks; K. Y. San, *Biotechnol. Bioeng.* **2006**, *93*, 534–540.
122. E. Moyano; K. Jouhikainen; P. Tammela; J. Palazon; R. M. Cusido; M. T. Pinol; T. H. Teeri; K. M. Oksman-Caldentey, *J. Exp. Bot.* **2003**, *54*, 203–211.
123. F. Sato; T. Hashimoto; A. Hachiya; K. Tamura; K. B. Choi; T. Morishige; H. Fujimoto; Y. Yamada, *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 367–372.
124. T. Inui; K. Tamura; N. Fujii; T. Morishige; F. Sato, *Plant Cell Physiol.* **2007**, *48*, 252–262.
125. M. H. Lee; J. H. Jeong; J. W. Seo; C. G. Shin; Y. S. Kim; J. G. In; D. C. Yang; J. S. Yi; Y. E. Choi, *Plant Cell Physiol.* **2004**, *45*, 976–984.
126. H. Seki; K. Ohyama; T. Nishizawa; S. Yoshida; T. Muranaka, *Plant Biotechnol.* **2008**, *25*, 347–355.
127. J. L. Slightom; M. Durand-Tardif; L. Jouanin; D. Tepfer, *J. Biol. Chem.* **1986**, *261*, 108–121.
128. J. Batra; A. Dutta; D. Singh; S. Kumar; J. Sen, *Plant Cell Rep.* **2004**, *23*, 148–154.
129. A. C. M. Brasileiro; J. C. Leple; J. Muzzin; D. Ounnoughi; M. F. Michel; L. Jouanin, *Plant Mol. Biol.* **1991**, *17*, 441–452.
130. C. Liu; Y. Wang; C. Guo; F. Ouyang; H. Ye; G. Li, *Bioprocess Eng.* **1998**, *19*, 389–392.
131. C. Z. Liu; Y. C. Wang; F. Ouyang; H. C. Ye; G. F. Li, *Biotechnol. Lett.* **1998**, *20*, 265–268.
132. D. P. Fulzele; M. R. Heble; P. S. Rao, *J. Biotechnol.* **1995**, *40*, 139–143.
133. C. Z. Liu; Y. C. Wang; C. Guo; F. Ouyang; H. C. Ye; G. F. Li, *Plant Sci.* **1998**, *135*, 211–217.
134. M. Du; X. J. Wu; J. Ding; Z. B. Hu; K. N. White; C. J. Branford-White, *Biotechnol. Lett.* **2003**, *25*, 1853–1856.
135. K. T. Lee; T. Suzuki; T. Yamakawa; T. Kodama; Y. Igarashi; K. Shimomura, *Plant Cell Rep.* **1999**, *18*, 567–571.
136. G. Prakash; A. K. Srivastava, *Process Biochem.* **2007**, *42*, 93–97.

137. K. S. Shin; H. N. Murthy; J. Y. Ko; K. Y. Paek, *Biotechnol. Lett.* **2002**, *24*, 2067–2069.
138. K. H. Jung; S. S. Kwak; C. Y. Choi; J. R. Liu, *J. Ferment. Bioeng.* **1994**, *77*, 57–61.
139. T. Morimoto; Y. Hara; Y. Kato; J. Hiratsuka; T. Yoshioka; Y. Fujita; Y. Yamada, *Agric. Biol. Chem.* **1988**, *52*, 1835–1836.
140. H. Sudo; T. Yamakawa; M. Yamazaki; N. Aimi; K. Saito, *Biotechnol. Lett.* **2002**, *24*, 359–363.
141. I. Asaka; I. Ii; M. Hirotani; Y. Asada; T. Furuya, *Biotechnol. Lett.* **1993**, *15*, 1259–1264.
142. S. Bentebibel; E. Moyano; J. Palazon; R. M. Cusido; M. Bonfill; R. Eibl; M. T. Pinol, *Biotechnol. Bioeng.* **2005**, *89*, 647–655.
143. Y. J. Yuan; Z. J. Wei; Z. L. Wu; J. C. Wu, *Biotechnol. Lett.* **2001**, *23*, 1659–1662.
144. C. H. Zhang; J. Y. Wu; G. Y. He, *Appl. Microbiol. Biotechnol.* **2002**, *60*, 396–402.
145. T. Endo; A. Goodbody; M. Misawa, *Planta Med.* **1987**, *53*, 479–482.
146. B. St-Pierre; P. Laflamme; A. M. Alarco; V. D. Luca, *Plant J.* **1998**, *14*, 703–713.
147. M. Magnotta; J. Murata; J. X. Chen; V. De Luca, *Phytochemistry* **2007**, *68*, 1922–1931.
148. K. Hirata; M. Horiuchi; M. Asada; T. Ando; K. Miyamoto; Y. Miura, *J. Ferment. Bioeng.* **1992**, *74*, 222–225.
149. R. K. Satdive; D. P. Fulzele; S. Eapen, *Biotechnol. Prog.* **2003**, *19*, 1071–1075.
150. A. Lorence; F. Medina-Bolivar; C. L. Nessler, *Plant Cell Rep.* **2004**, *22*, 437–441.
151. S. Sirikantaramas; T. Asano; H. Sudo; M. Yamazaki; K. Saito, *Curr. Pharm. Biotechnol.* **2007**, *8*, 196–202.
152. K. Sakato; H. Tanaka; N. Mukai; M. Misawa, *Agric. Biol. Chem.* **1974**, *38*, 217–218.
153. Y. Yamazaki; H. Sudo; M. Yamazaki; N. Aimi; K. Saito, *Plant Cell Physiol.* **2003**, *44*, 395–403.
154. Y. Yamazaki; M. Kitajima; M. Arita; H. Takayama; H. Sudo; M. Yamazaki; N. Aimi; K. Saito, *Plant Physiol.* **2004**, *134*, 161–170.
155. W. H. J. Tam; F. Constabel; W. G. W. Kurz, *Phytochemistry* **1980**, *19*, 486–487.
156. C. L. Siah; P. M. Doran, *Plant Cell Rep.* **1991**, *10*, 349–353.
157. S. U. Park; P. J. Facchini, *J. Exp. Bot.* **2000**, *51*, 1005–1016.
158. V. Le Flem-Bonhomme; D. Laurain-Mattar; M. A. Fliniaux, *Planta* **2004**, *218*, 890–893.
159. B. Unterlinner; R. Lenz; T. M. Kutchan, *Plant J.* **1999**, *18*, 465–475.
160. P. J. Larkin; J. A. C. Miller; R. S. Allen; J. A. Chitty; W. L. Gerlach; S. Frick; T. M. Kutchan; A. J. Fist, *Plant Biotechnol. J.* **2007**, *5*, 26–37.
161. P. J. Facchini; V. DeLuca, *Plant J.* **2008**, *54*, 763–784.
162. J. D. Hamill; R. J. Robins; A. J. Parr; D. M. Evans; J. M. Furze; M. J. C. Rhodes, *Plant Mol. Biol.* **1990**, *15*, 27–38.
163. T. Hashimoto; D. J. Yun; Y. Yamada, *Phytochemistry* **1993**, *32*, 713–718.
164. K. Jouhikainen; L. Lindgren; T. Jokelainen; R. Hiltunen; T. H. Teeri; K. M. Oksman-Caldentey, *Planta* **1999**, *208*, 545–551.
165. L. Zhang; R. X. Ding; Y. R. Chai; M. Bonfill; E. Moyano; K. M. Oksman-Caldentey; T. F. Xu; Y. Pi; Z. N. Wang; H. M. Zhang; G. Y. Kai; Z. H. Liao; X. F. Sun; K. X. Tang, *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 6786–6791.
166. H. Kasahara; A. Hanada; T. Kuzuyama; M. Takagi; Y. Kamiya; S. Yamaguchi, *J. Biol. Chem.* **2002**, *277*, 45188–45194.
167. N. Nagata; M. Suzuki; S. Yoshida; T. Muranaka, *Planta* **2002**, *216*, 345–350.
168. A. Hemmerlin; J. F. Hoeffler; O. Meyer; D. Tritsch; I. A. Kagan; C. Grosdemange-Billiard; M. Rohmer; T. J. Bach, *J. Biol. Chem.* **2003**, *278*, 26666–26676.
169. P. Mercke; M. Bengtsson; H. J. Bouwmeester; M. A. Posthumus; P. E. Brodelius, *Arch. Biochem. Biophys.* **2000**, *381*, 173–180.
170. D. K. Ro; E. M. Paradise; M. Ouellet; K. J. Fisher; K. L. Newman; J. M. Ndungu; K. A. Ho; R. A. Eachus; T. S. Ham; J. Kirby; M. C. Y. Chang; S. T. Withers; Y. Shiba; R. Sarpong; J. D. Keasling, *Nature* **2006**, *440*, 940–943.
171. K. H. Teoh; D. R. Polichuk; D. W. Reed; G. Nowak; P. S. Covello, *FEBS Lett.* **2006**, *580*, 1411–1416.
172. Y. Zhang; K. H. Teoh; D. W. Reed; L. Maes; A. Goossens; D. J. H. Olson; A. R. S. Ross; P. S. Covello, *J. Biol. Chem.* **2008**, *283*, 21501–21508.
173. P. J. Weathers; S. Elkholy; K. K. Wobbe, *In Vitro Cell. Dev. Biol.-Plant* **2006**, *42*, 309–317.
174. C. Z. Liu; Y. Zhao; Y. C. Wang, *Appl. Microbiol. Biotechnol.* **2006**, *72*, 11–20.
175. C. Z. Liu; C. Guo; Y. C. Wang; F. Ouyang, *Process Biochem.* **2003**, *39*, 45–49.
176. K. Ushiyama; H. Oda; Y. Miyamoto, Proceedings of 6th International Congress of Plant Tissue and Cell Culture, IAPTC, Minneapolis, 1986; p 252.
177. T. Yoshikawa; Y. Asada; T. Furuya, *Appl. Microbiol. Biotechnol.* **1993**, *39*, 460–464.
178. K. Yoshimatsu; H. Yamaguchi; K. Shimomura, *Plant Cell Rep.* **1996**, *15*, 555–560.
179. J. Palazon; A. Mallol; R. Eibl; C. Lettenbauer; R. M. Cusido; M. T. Pinol, *Planta Med.* **2003**, *69*, 344–349.
180. J. Y. Han; Y. S. Kwon; D. C. Yang; Y. R. Jung; Y. E. Choi, *Plant Cell Physiol.* **2006**, *47*, 1653–1662.
181. H. Seki; K. Ohyama; S. Sawai; M. Mizutani; T. Ohnishi; H. Sudo; T. Akashi; T. Aoki; K. Saito; T. Muranaka, *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 14204–14209.

Biographical Sketches

Professor Toshiya Muranaka completed his master's degree at the Graduate School of Agriculture, Kyoto University in 1985. In the same year, he entered Sumitomo Chemical Co., Ltd. and conducted research on the production of secondary metabolites by hairy root cultures. He obtained his Ph.D. in agriculture from Kyoto University in 1993. In 1997, he conducted research on posttranscriptional regulation of plastid genes in green algae at the University of California, Berkeley as a NEDO Fellow. In April 2001, he joined RIKEN Plant Science Center as a team leader. In April 2007, he was appointed as a full professor at Yokohama City University. His research interests are metabolic regulation of terpenoids, especially sesquiterpenoids and triterpenoids based on molecular genetics, biochemistry, and organic chemistry.

Professor Kazuki Saito obtained his Ph.D. in bioorganic chemistry and biochemistry from the University of Tokyo in 1982. After his stay at Keio University in Japan and Ghent University in Belgium (at Professor Marc Van Montagu's laboratory), he has been appointed as a full professor at the Graduate School of Pharmaceutical Sciences, Chiba University, Japan, since 1995. Since 2005, he has also been appointed as a group director at RIKEN Plant Science Center to direct the Metabolomics Research Group and the Metabolic Function Group. He is one of the well-recognized leading scientists in plant metabolism. His research interests are metabolomics-based functional genomics, systems biology, biochemistry, molecular biology, and biotechnology of primary and secondary metabolism in plants.

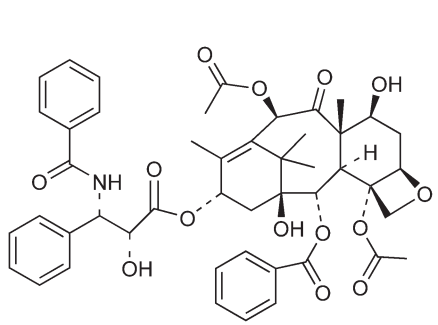
3.18 Plant Secondary Metabolism Engineering: Methods, Strategies, Advances, and Omics

Rafael Zárate, The University of La Laguna, Tenerife, Spain

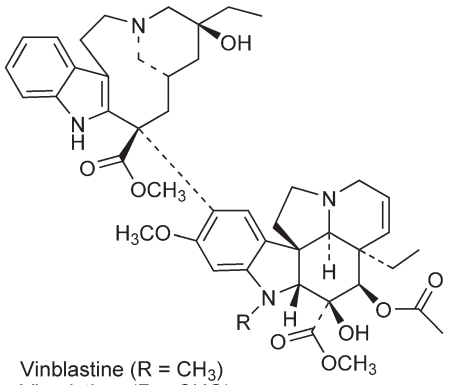
3.18.1 Introduction

Plants display a great genotypic and phenotypic diversity and play a major and irreplaceable role in generating and maintaining life and ecosystems, and are also known for their ability to spread out and occupy any territory. The number of higher plants has been estimated to be approximately 250 000–300 000 worldwide,[1,2] with only a marginal number of them being fully studied at the phytochemical level (8–10%). This number becomes even less when considering studies carried out at the molecular level. Furthermore, plants constitute one of the main sources of materials for mankind employed for multiple applications, such as construction, fabrication of tools, shelter, energy production, and food. Equally or more importantly, higher plants are also described as chemical factories able to synthesize unlimited numbers of highly complex and unusual chemical substances whose structures can be considered unlimited. Plants, being sessile organisms, interact and communicate with their immediate environment, that is, other plants, pathogens, animals, etc., by chemical means. Mainly owing to their inability to move, and the need to interact with and protect against other organisms (symbiosis, attraction, defense, pollination, etc.), plants have acquired and evolved, through millions of years of evolution, specialized biosynthetic networks also called secondary metabolic pathways, producing an extraordinarily vast array of molecules allowing them to survive and prosper in a multitude of challenging ecological niches. Therefore, the vast potential offered by natural resources for the discovery and development of new therapeutics of great benefit to mankind is clear; nonetheless, these unique gene resources may be lost forever through extinction; consequently, direct actions should be taken to reverse the current apathy in the protection of biodiversity worldwide in order to maintain and sustain the natural sources that are still largely unexplored.

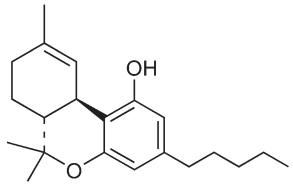
Secondary metabolites are complex small-molecule natural products produced by diverse organisms; here mainly plant secondary metabolites are considered. These include isoprenoids, flavonoids, polyketides, alkaloids, etc. (**Figure 1**), and many of them are used and exploited as pharmaceuticals, flavors, fragrances,



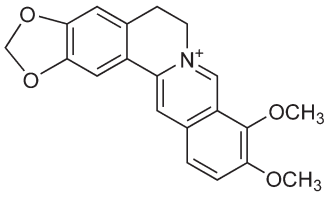
Paclitaxel



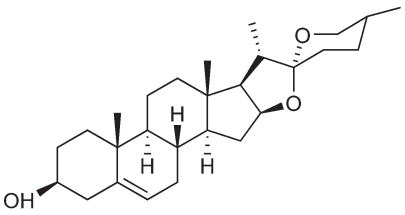
Vinblastine (R = CH_3)
Vincristine (R = CHO)



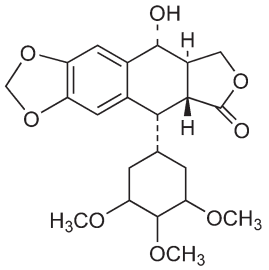
Tetrahydrocannabinol = THC



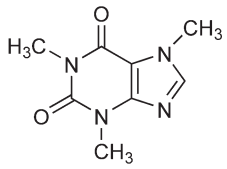
Berberine



Diosgenin



Podophyllotoxin



Caffeine

Figure 1 Molecular structures of important medicinal natural products derived from plants. Paclitaxel (**1**), antitumor compound originally extracted from *Taxus* spp. plants; vincristine (**2**) and vinblastine (**3**), anticancer products extracted from *Catharanthus roseus*; tetrahydrocannabinol (**4**), psychoactive principle obtained from the flowers and leaves of *Cannabis sativa*; berberine (**5**), an isoquinoline alkaloid present in many members of the *Ranunculaceae* (*Coptis*, *Hydrastis*) and *Berberidacea* (*Berberis*) families, with anti-inflammatory, antibacterial and antiamoebic properties; diosgenin (**6**), a steroid sapogenin extracted from the tubers of *Dioscorea* ssp. used for the commercial synthesis of progesterone, cortisone, and pregnenolone; podophyllotoxin (**7**), anticancer product extracted from the rhizomes of *Podophyllum peltatum* and *P. hexandrum*; caffeine (**8**), stimulant alkaloid present mainly in *Coffea arabica*, *Camellia sinensis, Ilex paraguariensis*, and *Paulina cupana* (guarana).

insecticides, dyes, food additives, toxins, etc. It is estimated that around 200 000 natural products are known and each year around 4000–5000 new compounds are elucidated. Moreover, the importance of natural products has increased tremendously; thus, of all drugs used in western medicine, around 40–45% are natural products or compounds derived from them, and of these, 25% are obtained from plants.[3] Moreover, the dominant role of natural products or derivatives as anticancer compounds (60%) and drugs for infectious diseases (75%) is even more evident.[4,5] Unmistakably, through evolution, nature has been fabricating and selecting natural products, which wait to be taken, assessed, and exploited.

It is well known that the yield of plant natural products is frequently low and depends primarily on the physiological and developmental stage of the plant. Often the yield obtained ranges from 0.001% to the best cases of 10–20%, with the majority of pharmaceutically important secondary metabolites being obtained from wild or cultivated plants; accordingly, often the producing plant is brought into cultivation to secure supply. Although some attempts have been made to chemically synthesize metabolites, in most cases this has not been economically feasible, and plants remain as the major source of these molecules. In other cases, more abundant precursor molecules are obtained in large amounts from the plant and then after simple chemical modifications through semisynthesis, the final active secondary metabolite is obtained; that is, baccatin III, a precursor of Taxol, which is highly abundant in the leaves of *Taxus baccata* or *T. wallichiana*, is extracted and following chemical modifications, the final product is obtained in large quantities to satisfy the world demand.[6] Furthermore, the renaissance of natural products as drug candidates has been claimed mainly after combinatorial chemistry failed to provide the chemical entities thought to be obtained through such approach, and by emphasizing their potential in drug discovery, particularly owing to their extraordinary specificity and potency gained through evolutionary selection, compared with artificially designed molecules.[7]

Secondary metabolites have also been produced through plant biotechnology by employing different types of *in vitro* cultures, such as callus, suspended cells, organ cultures, as well as hairy roots, which has received much attention as a useful technology for the production of valuable plant bioactive metabolites with different degrees of success.[8,9] Furthermore, plant cell culture has also been a tool to elucidate biosynthetic pathways and to quantify the flux of biosynthetic intermediates through a pathway, as well as to identify enzymes and encoding genes participating in the biosynthetic route, and to determine the rate-limiting step(s) within a pathway. However, the early directions of plant biotechnology, which mostly focused on *in vitro* cell and tissue culture and the production of important products,[10] are now advancing into new objectives. The current state of plant biotechnology research permits the use of a number of different approaches including high-throughput methodologies for functional analysis at the levels of transcripts, proteins, and metabolites, and methods for genome modification by both homologous and site-specific recombination. Plant biotechnology allows for the transfer of a greater variety of genetic information in a more precise and controlled manner, and these are being applied, for instance, to manipulate secondary metabolite biosynthetic networks, aiming at attaining larger product yields after the establishment of transgenic plants or plant cell cultures with an improved productivity of the desired compound(s).

Secondary metabolite biosynthetic networks are generally complex, with the involvement of numerous factors. For instance, the biosynthetic pathway of the terpenoid indole alkaloids (TIAs) in *Catharanthus roseus* is very complex; it includes many steps and different cell organs and cell types,[11,12] demonstrating the difficulties in designing and succeeding on the metabolic engineering of the final products of such a pathway (vincristine, vinblastine). Contrarily, there also exist examples of simpler biosynthetic routes with fewer catalytic steps. For instance, the biosynthesis of resveratrol is controlled by a unique enzyme encoded by a single gene (resveratrol synthase),[13–15] suggesting the ease and the potential for its manipulation.

Ever since Bailey[16] defined metabolic engineering as "the improvement of cellular activities by manipulation of enzymatic, transport and regulatory functions of the cell with the use of recombinant DNA technology," this discipline has grown as a tool for manipulating biosynthetic pathways leading to secondary metabolites, and a large body of published literature sustains the progress made and suggests the directions to be followed for accomplishing a continuous production of plant natural products through metabolic engineering.[17–19]

In contrast, with the current advances in molecular biology and DNA recombinant technology, the reconstruction of genetic circuits has been practiced and reviewed by Sprinzak and Elowitz,[20] indicating that synthetic circuits can be used as simple *in vivo* models to explore the relation between the structure and function of a genetic circuit. For building synthetic circuits, the genetic components that are to be employed must be well characterized, should function similarly in different systems, and act independently of other cellular processes. These circuits have been built to study transcription factors as well as micro-RNAs and their regulation. Furthermore, attempts to capture plant biosynthetic routes and insert them into heterologous fermentable microorganisms like yeast, bacterium, or fungus represent a valuable alternative to circumvent the often encountered low yields of plant natural products. For instance, the engineering of taxol biosynthetic genes in *Saccharomyces cerevisiae*, the engineering of *Escherichia coli* for production of functionalized terpenoids,

or the production of artemisinic acid in yeast have been reported.[21–23] Similarly, transferring biosynthetic networks from one plant species to another more amenable to manipulation has also been conducted, for example, the metabolic engineering of the isoprenoid biosynthesis for the production of taxadiene, the first committed precursor of paclitaxel in *Arabidopsis thaliana* by overexpressing the taxadiene synthase gene, generating 30-fold larger production of taxadiene;[24] likewise, a combination of nine genes from different fungi and plants catalyzing condensation and desaturation reactions for fatty acid synthesis was expressed in a host plant (*Brassica juncea*), resulting in the production of significant amounts of arachidonic acid (25%) and eicosapentaenoic acid (15%) in *B. juncea* total seed fatty acids.[25]

In the following subheadings, up-to-date information on the most common plant transformation methodologies, as well as the different approaches undertaken for the engineering of plant secondary metabolism, followed by a review on the current advances realized with three major biosynthetic pathways, that is, artemisinin pathway, tropane alkaloid pathway, and morphinan alkaloid pathway, together with the influence of the -omics technologies on plant metabolic engineering are presented. Besides, some comments on future trends and expected progress in plant natural products metabolic engineering are also drawn. Furthermore, an attempt has been made to present plant biotechnology, particularly the genetic engineering of biosynthetic networks, as a powerful alternative and an amenable tool for the controlled production of bioactive natural products.

3.18.2 Techniques for the Genetic Manipulation of Plants

Gene transfer into plants is a vital component in any genetic modification project, and thus different methods for transient or stable genetic transformation of plants or plant cells have been described.[26–28] These include particle bombardment, *Agrobacterium*-mediated transformation, floral dip transformation, use of viral vectors, protoplast transformation, ultrasound, agrodrench, and microinjection. These are the main techniques for the genetic transformation of plants, and many of them have also been practiced for the manipulation of secondary metabolite pathways in an attempt to alter the biosynthesis of target compounds.[29,30] The two most commonly utilized plant genetic manipulation techniques are described here, and for a comprehensive description of the different methods refer to the available literature.[12,26,28,31]

3.18.2.1 Agrobacterium-Mediated Transformation

Two different species of *Agrobacterium* are commonly used for genetic transformation of plants: *A. tumefaciens* and *A. rhizogenes*. These are Gram-negative soil bacteria that belong to the family Rhizobiaceae and are able to infect different plant hosts, most often dicotyledons or less frequently monocotyledons,[32] and even yeast and animal cells.[33–35] *Agrobacterium tumefaciens* and *A. rhizogenes* are the causal agents of the plant diseases crown gall and hairy root, respectively. Diseases are caused by the presence of bacterial DNA, the transferred DNA (T-DNA), within the plant cells. The T-DNA controls the synthesis of plant growth regulators, auxin and cytokinin, in the infected cells resulting in the induction of tumor or hairy roots, as well as the biosynthesis of other types of compounds, such as opines or octopines serving as nutrients for the infecting bacterium.[36] These bacteria are considered natural metabolic engineers because of their ability to transfer genes into the target plant cells and other organisms (fungi, yeast, bacterium, animal cells) and thus are capable of genetically crossing kingdoms.[37,38]

Genetic transformation occurs after bacterial infection of the plant cells or tissues. Following infection, the T-DNA, which can be engineered to carry the genes of interest, gets inserted into the plant nuclear DNA. Furthermore, other elements of the bacterial plasmids, the Ti-plasmid from *A. tumefaciens* (tumor inducer) or the Ri-plasmid from *A. rhizogenes* (root inducer), also participate in the transformation process. Both plasmids show large functional homologies and appear to have evolved from a common ancestor.[39] These plasmids also possess a virulence region, containing various silent *vir* genes, which do not penetrate the plant genome, but are indispensable for the T-DNA transfer. These genes are switched on by interacting with phenolic-type compounds (**Figure 2**), such as lignin precursors and acetosyringone, wound tissue metabolites, demonstrating the need of tissue wounding for efficient infection to take place.[40] Furthermore, the *rol*- and *onc*-genes encode enzymes for the production of plant growth regulators by the infected plant cell, as well as other opine and

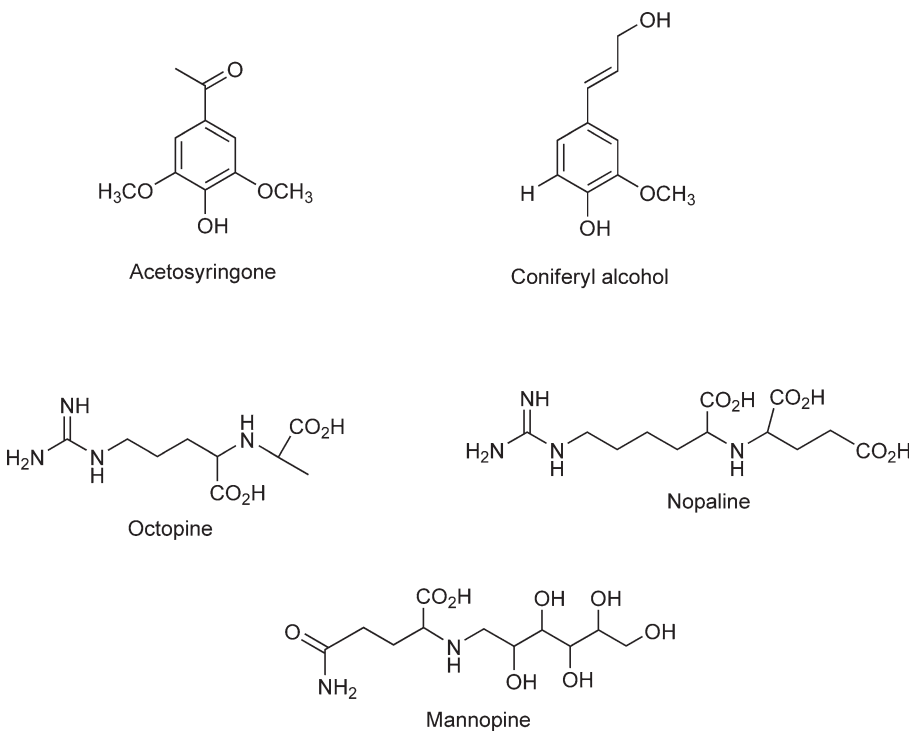


Figure 2 Chemical structures of representative opines produced following *Agrobacterium* infection, and phenolic compounds (acetosyringone, coniferyl alcohol) acting as inducers of the *vir* regulon present in wounded plant tissue.

octopine synthase genes that activate the synthesis and catabolism of different classes of opines and octopines (**Figure 2**). These are unique natural metabolites, pseudo amino acids, which serve as a nutrient source of carbon and nitrogen for the pathogenic bacteria.[41]

Transformation by *Agrobacterium* requires several conditions, that is, an acidic pH (5.0–6.0), the presence of phenolic compounds produced after tissue wounding, and, more recently, it has been described that light also increases the success of *Agrobacterium* transformation.[42] Nonetheless, it is not fully understood how the T-DNA integrates into the plant nuclear genome, but it seems to resemble illegitimate recombination, proceeding analogously in dicot and monocot plants.[36] Insertion of the T-DNA in the plant genome occurs at random positions, but showing preferences for transcriptionally active regions. Contrary to other gene insertion techniques such as particle bombardment, the plant transgenic cell lines generated via *Agrobacterium* often contain one copy or a low copy number of the T-DNA, although cell lines with multiple T-DNA copies can also be found.

3.18.2.2 Particle Bombardment

This is a direct DNA delivery technique also referred to as biolistics (biology + ballistics) developed by Sanford and co-workers.[43–45] High-velocity particles or microprojectiles coated with DNA are employed to deliver exogenous genetic material into the target cell or tissue, which is subsequently *in vitro* cultured and regenerated to produce mature transformed plants.

The gold or tungsten particles coated with DNA on their surface are of small size (0.5–5 μm), which when propelled and accelerated are able to penetrate the target cell or tissue. The microprojectiles are DNA coated following for instance the $CaCl_2$ protocol, with the addition of spermidine to protect the DNA.[43] Nevertheless, a recent report describes the successful use of *Agrobacterium* as coating material.[46] The microprojectiles are propelled under partial vacuum, employing helium or CO_2 pressure, to produce the necessary blast to boost the coated particles. Particle penetration can be controlled by altering different parameters and the instrument

setup. These include particle size, pressure applied to drive the particles, distance between sample holder and target, use of a retaining screen employed to diffuse the particle before hitting the target, as well as the biological stage of the cells or tissues to be transformed.

Following bombardment, most of the coated particles are either degraded or inactivated, and many do not reach the target plant cell nucleus. Only DNA can be expressed after penetrating the nucleus and on getting stably integrated in the genome. In contrast to *Agrobacterium*-mediated transformation, gene insertion using biolistic technique does not show a preference for insertion sites and when reaching a transcriptionally active region, it is expressed at a high rate; if it integrates in a nonactive region, gene expression may be reduced or absent. Biolistic technique, unlike *Agrobacterium*-mediated transformation, produces higher copy numbers of the inserted DNA, which often results in gene silencing at higher frequencies.

Biolistics offer unique advantages over conventional techniques such as *Agrobacterium*-mediated transformation.[47] For instance, (1) it does not show host specificity, and potentially can transform any plant species, being successfully applied for the transformation of recalcitrant species such as many monocots and some dicots; (2) it has the ability to transfer foreign DNA directly into regenerable cells, tissues, or organs; (3) the instrument allows fine-tuning and control of various parameters, permitting precise targeting of DNA-coated particles to specific cells or tissue areas; (4) the DNA size is not a limitation and in principle it is possible to bombard almost any plasmid, although larger plasmids tend to disintegrate after bombardment resulting in a poor transformation efficiency; (5) it permits simultaneous bombardment of different gene constructs, finding accounts whereby even 12 different gene constructs were bombarded and expressed in plant cells;[48] (6) this technique has been successfully applied for the transformation of not only plants but also animals, bacteria, yeast, and fungi.[49–52]

3.18.3 Strategies for the Genetic Engineering of Secondary Metabolic Pathways

Genetic engineering of metabolic pathways implies the modification of biosynthetic routes through manipulation of the gene(s), which code for the enzymes involved in the various steps of the pathway, thus permitting the redirection of complex biosynthetic networks. Furthermore, the advances in biochemistry, molecular biology, and genetics applied to secondary metabolism have allowed the isolation, characterization, cloning, and expression of many genes involved in a specific biosynthetic route, making them amenable to control, and as more genes and more biosynthetic pathways are being characterized a higher level of control and success is being gained.[19] The possible manipulation of these genes consists of either overexpression of a gene, when production of new compounds or improvement of existing metabolites is aimed, or gene silencing, when halting the accumulation of a specific reaction intermediate or end product is desired. These can be achieved by applying different approaches and these are briefly presented.

3.18.3.1 Overexpression of Transgenes

This represents one of the earlier approaches in secondary metabolite pathway engineering, aiming to boost or tailor the yield of specific compounds. Initially, the overexpression of a single native gene in a given pathway was the strategy of choice, although the heterologous expression of transgenes was also common.

Thus, the manipulation of flavonoid and anthocyanin biosynthesis was one of the first reported examples, since the pathway was well characterized, and the outcomes of the modification could be easily visualized by differences in flower pigments after insertion of a chalcone synthase or chalcone reductase gene in *Petunia*.[53,54] Similarly, the metabolic engineering of the medicinal plant *Atropa belladonna* was achieved by overexpression of a single *H6H* gene (hyoscyamine 6β-hydroxylase (H6H)), involved in the tropane alkaloid pathway, resulting in plants with elevated scopolamine yields with low levels of atropine, increasing the pharmaceutical significance of this transgenic plant.[55]

3.18.3.2 Multiple Expression of Transgenes

It has been cited that the secondary metabolic pathways are generally highly complex, involving, for instance, many biosynthetic steps, different cell types, and different cellular compartments, as well as the transport of pathway intermediates, as in the formation of TIAs, such as the antitumor compounds vinblastine and vincristine in *C. roseus*.[9] Nonetheless, there are also examples of simpler biosynthetic routes with fewer catalytic steps. The biosynthesis of resveratrol, a natural product with antioxidant, anti-inflammatory, antiplatelet, and cancer preventive properties, is controlled by a unique enzyme encoded by a single gene (resveratrol synthase).[13–15]

Considering the complexity of the pathways where multiple genes are involved, it seems reasonable to attempt to manipulate more than one gene simultaneously and thus apply different control points at various biosynthetic steps; this is not only advantageous but in some instances necessary to induce higher secondary metabolite yields. Thus, there are many instances whereby multiple transgenes have been heterologously overexpressed in an attempt to boost the production of a particular metabolite. For example, the manipulation of the biosynthesis of the tropane alkaloid scopolamine has been attempted by overexpressing two genes of the pathway, *PMT* and *H6H*, which encode the enzymes putrescine methyl transferase (PMT) and H6H, respectively. The established *Hyoscyamus niger* transgenic hairy roots produced ninefold higher amounts of scopolamine compared with controls.[56] Another interesting example is the introduction of an entire biosynthetic route such as that of the provitamin A pathway into rice.[57] Four transgenes from plants and bacteria, together with a transient peptide, were inserted and expressed in rice endosperm, managing to reproduce the β-carotene biosynthetic pathway into this stable crop, obtaining yellow-colored rice grains with a high carotenoid content, increasing the nutritional properties of this stable crop, and contributing to the reduction of the incidence of vitamin A deficiency directly linked to multiple ailments.

3.18.3.3 Gene Silencing

An alternative approach is the downregulation of a particular gene in a pathway so as to halt a particular biosynthetic step, permitting the accumulation of a desired product or inhibiting a competitive branch within a biosynthetic network. The reduction in the level of mRNA of a particular gene was initially achieved by antisense technology, by co-suppression, or even via synthesis of an antibody against the target enzyme. Furthermore, more recently, the technology of RNA interference (RNAi) is being widely used as a powerful tool for inducing post-transcriptional gene silencing via sequence-specific degradation of target mRNA,[58] by introducing a homologous double-stranded RNA (dsRNA) to precisely silence a gene. This dsRNA is responsible for the interfering activity after being cleaved by the enzyme complex Dicer into multiple molecules of 21–23 bp short interfering RNA (siRNA). These then form an RISC complex (RNA-induced silencing complex), which after activation targets a homologous transcript by base pairing interactions and cleaves the mRNA impeding transcription and gene expression.[59–61] Some examples are presented below to illustrate the success and potential of this technology.

RNAi technology has been applied for reducing the levels of tobacco products contributing to health hazard, such as specific nitrosamines, involved in cancer development, generated through nitrosation of pyridine alkaloids during the curing and processing of tobacco. For instance, nitrosonornicotine (NNN) is formed by nitrosation of nornicotine produced through enzymatic N-demethylation of nicotine in *Nicotiana tabacum*.[62] The recently isolated major nicotine demethylase gene of tobacco was employed to establish transgenic tobacco plants carrying an RNAi construct designed to inhibit the expression of this gene. Selected transgenic lines showed a sixfold decrease in nornicotine amounts, and the cured leaves had a dramatic decrease in NNN and tobacco-specific nitrosamines, lowering the levels of animal carcinogens present in tobacco.

RNAi technology has also been used to inhibit a particular step of the isoquinoline alkaloid pathway, for the accumulation of reticuline.[63] In isoquinoline alkaloid biosynthesis, (*S*)-reticuline is a branch-point intermediate involved in the biosynthesis of many types of isoquinoline alkaloids, that is, morphine, codeine, papaverine, berberine, and sanguinarine. Furthermore, (*S*)-reticuline is an attractive substrate for the formation of different compounds with antimalarial and anticancer activities, although the amounts of this intermediate are scarce since it is efficiently converted in the pathway.[64] In established *Eschscholzia californica* suspension cell cultures,

the berberine bridge enzyme (BBE) was knocked down by RNAi, with the BBE mRNA and enzyme activity being successfully suppressed in transgenic cell lines. Thus, the amounts of the final biosynthetic product sanguinarine were drastically reduced, and reticuline was clearly enhanced with maximum levels of 310 μg g fresh wt.$^{-1}$; besides, the release of this compound into the liquid medium was also observed with values of 6 mg in 20 ml of culture medium from 1 g of growing cells. This gene silencing also resulted in an apparent formation of a methylated derivative of reticuline, laudanine, hardly detected in the control cultures.[63]

Similarly, although not aiming to manipulate the production of secondary metabolites, but to avoid the accumulation of a particular allergenic protein, RNAi has been the technology of choice to reduce the level of the immunodominant Ara h2 protein in peanut, which constitutes one of the most serious allergies worldwide. Peanut hypocotyls were transformed with an Ara h2-specific RNAi cassette, resulting in the induction of 44% of plants with stable integration of the transgene, and a significant reduction in Ara h2 content in transgenic seeds. Allergenicity studies showed a significant decrease in the IgE binding capacity, demonstrating a clear reduction of peanut allergy by applying the RNAi technology.[65]

3.18.3.4 Transcription Factors as a Powerful Tool for the Engineering of Biosynthetic Pathways

Common strategies to modify complex metabolic pathways consist of altering one or more genes, by inducing their overexpression or by downregulation. Considering that many metabolic pathways in plants show coordinated metabolic regulation via transcriptional control, an alternative approach for the genetic engineering of pathways comprises the manipulation of transcription factors followed by evaluation of its effects upon the overall metabolic design. This novel strategy offers an attractive potential since the manipulation of a single gene coding for a particular transcription factor results in the simultaneous modulation of multiple genes, a response unfeasible by any other means. Currently, this is the major focus of attention for an active manipulation of metabolic pathways at multiple control points.[66,67]

Transcription factors, present in animals and plants, are regulatory proteins that control the expression of specific groups of genes within a biosynthetic network, through sequence-specific DNA binding, particularly to motifs present within the promoter region of the genes, or by protein–protein interactions, or even by acting over other transcription factors, functioning as repressors or activators of gene expression causing a decrease or increase in mRNA levels of the affected genes through the participation of RNA polymerase II.[68] New transcription factors are being isolated and characterized at a steady pace, which will further assist the development of this engineering tool for the modification of biosynthetic pathways.[69] However, the function of only a small fraction of these transcription factors has been established.

Based on available information regarding the control of branched flavonoid biosynthetic pathways in maize, it has been reported that duplication and divergence of transcription factor genes occurs and can result in the control of new metabolic pathways. This duplication and activation of new metabolic pathways is a consequence of mutations that partially impair function, resulting in a loss of the activation of one or more genes within a metabolic pathway. Then, pathway intermediates accumulate and are converted into new compounds by broad-specificity enzymes.[70]

Anthocyanins, a group of flavonoids synthesized via the phenylpropanoid pathway, exhibiting important physiological properties in plants and with broad medicinal activities, have been largely investigated. This biosynthetic network was the first target for genetic engineering since the pathway was well known, and the results could easily be observed by changes in flower color. The initial discovery of plant transcription factors was achieved in maize, characterizing two transcription factors, that is, C1 and R, which participate in the anthocyanin–flavonoid pathway.[71,72] These are ectopically expressed in unpigmented *in vitro* maize cells, inducing metabolic and structure differentiation leading to the biosynthesis and accumulation of anthocyanins,[73,74] indicating their application for redirecting biosynthetic networks. Analogously, in soybean seeds (*Glycine max*), the phenylpropanoid pathway was genetically engineered by expressing the maize C1 and R transcription factors, resulting in increased isoflavone levels. The expression of these genes decreased genistein but increased daidzein levels, with a small increase in total isoflavones. Nonetheless, co-suppression of flavanone 3-hydroxylase, which blocks the anthocyanin branch of the pathway, together with the C1 and R expression, generated higher levels of isoflavone.[75]

Further work on the same pathway, but aiming to alter the level of proanthocyanidins in *A. thaliana* by ectopic expression of the transcription factor Arabidopsis *TT2-MYB* caused the simultaneous expression of the BANYULS gene, encoding anthocyanidin reductase, AHA 10, which encodes a P-type proton pump, and TT12, encoding a transporter involved in proanthocyanidin biosynthesis. When *TT2-MYB* expression was coupled with the expression of *PAP1*, a positive regulator of anthocyanin biosynthesis, accumulation of proanthocyanidins was observed, but only in specific cells where the BANYULS promoter was naturally expressed.[76] Also working with *A. thaliana*, another important metabolic pathway involved in the conversion of γ-tocopherol into α-tocopherol (tocopherol species with the highest vitamin E activity) was modulated by the overexpression of synthetic zinc-finger transcription factors (*ZFP*-TFs), and designed to upregulate the expression of the endogenous *Arabidopsis* γ-tocopherol methyltransferase gene (*gmt*), the gene responsible for such bioconversion. Different *ZFP* DNA binding domains were constructed and some were tightly bound to 9 bp DNA sequences located in either the promoter or coding region of the *GMT* gene. Seed-specific expression of four *ZFP*-TFs yielded several transgenic *Arabidopsis* lines with a heritable increase in α-tocopherol in this plant compartment.[77]

Attempts to manipulate the biosynthetic pathway of the TIAs, the anticancer drugs vincristine and vinblastine, in *C. roseus* using transcription factors have also been made. Thus, the *ORCA*-3 (octadecanoid-responsive *Catharanthus roseus* APETALA domain protein) transcription factor whose expression is induced by methyl jasmonate[78] has been overexpressed in transgenic *C. roseus* suspension cultures inducing the expression of two genes, *AS* and *DXS* (anthranilate synthase subunit-α and D-1-deoxyxylulose 5-phosphate synthase), involved in primary metabolism, and five genes directly involved in the TIA pathway, that is, *TDC*, *STR*, *CPR*, *SGD*, and *D4H* (tryptophan decarboxylase, strictosidine synthase, cytochrome P-450 reductase, strictosidine glucosidase, and desacetoxy vindoline 4 hydroxylase, respectively), but none of the other three known genes, resulting in enhanced accumulation of several TIA network intermediates after feeding with loganin, but not the target metabolites vincristine and vinblastine.[79] *Orca-3* was a regulator of both primary and secondary metabolism biosynthetic genes involved in this pathway but its overexpression did not suffice to obtain higher yields of vincristine and vinblastine, indicating that other transcription factors might be involved and their modification would likely allow a full control of this complex biosynthetic route.[72]

Suppression of multiple genes within a biosynthetic route can also be achieved using transcription factors. It has been demonstrated that in strawberry, *FaMYB1*, a ripening regulated strawberry gene and member of the *MYB* family of transcription factors, suppresses anthocyanin and flavonol accumulation in transgenic tobacco. The flowers of transgenic tobacco plants overexpressing *FaMYB1* exhibited a severe decline in pigmentation with reduced levels of the anthocyanin cyanidin-3-rutinoside and the flavonols quercetin glycosides. It was found that expression of late flavonoid biosynthetic genes and their enzyme activities were inversely affected by *FAMYB1* overexpression. Thus, the anthocyanidin synthase gene expression was clearly reduced, together with a moderate drop in the dihydroflavonol 4-reductase gene expression. These results also indicate that this transcription factor may play a role in regulating the biosynthesis of anthocyanins and flavonols during strawberry fruit maturation, and/or regulate metabolite levels in various branches of the flavonoid biosynthetic pathway.[80] Recently, it has been reported that the overexpression of the Arabidopsis *R2R3-MYB* transcription factor *ATMYB60* functions as a transcriptional repressor of anthocyanin biosynthesis in lettuce (*Lactuca sativa*). Wild-type lettuce normally accumulates anthocyanin, chiefly cyanidin, and traces of delphinidin, and develops a red pigmentation. However, the production and accumulation of anthocyanin pigments in *ATMYB60*-overexpressing lettuce was inhibited. The complete absence or reduction of dihydroflavonol 4-reductase transcripts in *ATMYB60*-overexpressing lettuce was observed, thus establishing a correlation between the overexpression of AtMYB60 and the inhibition of anthocyanin accumulation.[81]

These data demonstrate that the expression of specific transcription factors can more efficiently redirect the metabolism of plant cells by acting simultaneously and coordinately on different metabolic events. Furthermore, the identification of transcription factor genes provides tools for modulating both the amount and distribution of secondary metabolites; therefore, new transcription factors are being pursued and most of the efforts are focused on this direction. An elegant updated review highlights all these potentials.[67]

3.18.3.5 Novel Gene Promoters and Optimization

The main approaches for plant metabolic engineering have already been described. Another approach that is gaining increasing attention is the optimization of gene promoter, as well as the design of synthetic novel promoters and the discovery of new ones.

The presence of a promoter is vital to drive the transcription of a gene; moreover, the selection of a promoter that would confer constitutive, spatial, and/or temporal gene expression is of major importance in plant biotechnology applications. The most commonly used gene promoter for the transcription of endogenous or foreign genes in plants is that of the cauliflower mosaic virus, that is, CaMV-35S. This offers a constitutive high transcription activity in many kinds of plants, as well as in many plant organs and tissues, but when many genes are being overexpressed in the same plant host, the repetitive use of this promoter to tune the activity of all the genes would likely result in gene silencing, the so-called gene co-suppression, due to the presence of multi-copies of this promoter gene.[82]

Most plant core promoters consist of CAAT and TATA boxes for recognition and binding of DNA-dependant RNA polymerase II, located upstream of the transcription initiation site, which would trigger transcription. On the other hand, organ-, tissue-, or environmental condition-specific promoters harbor a specific DNA sequence called *cis*-element located upstream of the core promoter. Furthermore, the transcription factors bind to this *cis*-element, affecting RNA polymerase activity and gene expression.[83]

Genetic control through *cis* engineering is a reality with synthetic promoters being designed in order to gain a more precise control and a stronger gene expression.[84] For example, several studies report how changes in promoter architecture, and particular designs of *cis*-motifs, enhance gene activity, regulate multiple transgenes, and avoid gene silencing in plant cells.[85,86] The value of using synthetic promoters for the elucidation of synergistic regulatory interactions, the participation of individual *cis*-motifs, and their biotechnological applications has been highlighted.[84,87] Another attractive tool is achieving inducible transgene expression in both directions. There are reports on the description of naturally occurring bidirectional promoters in various organisms, expressing two genes concomitantly, and on modifying a unidirectional promoter into a bidirectional promoter to, for example, simultaneously express sense and antisense transcripts to mediate gene silencing.[88–90]

On the other hand, when evaluating gene function, traditional approaches include either gene knockout or strong overexpression, without the possibility of moderately modulating such gene expression. Nonetheless, with the current advances in molecular biology and genetics, when different moderate expression levels of a gene are to be evaluated compared with the wild-type expression level, the design of promoter libraries is the tool.[87] This approach consists of inserting a library of promoters in front of a particular gene, whereby individual promoters may differ in their spacer sequences or bear slight differences from the consensus sequence of the vegetative promoter, inducing moderate gene activities that would assist in elucidating gene functions.

Two different methods for creating synthetic promoter libraries have been described.[87] One is built based on the known fact that regions flanking the promoter consensus sequences affect promoter strength, and if this area is kept intact and the surrounding nucleotides are randomized, the synthetic library would display variations in promoter strength. Their construction can be carried out through a single PCR step, using oligonucleotides with randomized promoter sequences preceding a region with homology to the target gene. The second methodology is based on the same principle, but instead of using oligonucleotides with randomized promoter sequences and with homology to the target gene, a derivative of the λP_L bacteriophage promoter is employed, whose sequence is mutated by mutagenic PCR. Both methods have shown to be quite potent, giving different promoter strengths within three orders of magnitude with small increments of promoter strength necessary for studying gene function. Furthermore, this methodology is becoming routine when working on systems biology, being an attractive tool for all types of quantitative and optimization studies.

3.18.3.6 Compartmentalization and Transport

It is well known that the synthesis of plant secondary metabolites is a highly regulated process, both developmentally and spatially, which involves the participation of multiple phenomena, such as gene expression and silencing, self-regulation by endogenous mechanisms, chemical modifications, and storage. Furthermore, the

involvement of intracellular compartments is also very important, for example, endoplasmic reticulum, vacuoles, plastids, nucleus, and cytoplasm, locations where biosynthetic routes partly occur, as well as the transport of intermediates and end products within cells, tissues, or even organs. These points should be considered when attempting to successfully genetically engineer complex biosynthetic networks. Accordingly, large efforts have been made to shed some light on many of these aspects, although more emphasis should be given to the importance of cell compartments and transport either within the cell or even to other cell types within a system, to enable to devise more rational strategies for metabolic engineering, aspects that are presented next.

The involvement of cell compartments and transport in secondary metabolism cannot be clearly divided, because in many events these two are necessarily implicated and participate almost simultaneously. For instance, it is known that caffeine is synthesized in the aerial parts of the coffee plant and then accumulates in the coffee beans, starting from adenine nucleotides through multiple steps catalyzed by several enzymes. The final series of steps involve methylation of xanthosine by *N*-methyltransferase, yielding 7-methylxanthosine, whose ribose residue is removed by 7-methylxanthosine nucleosidase. The resulting 7-methylxanthine is methylated at the 3-*N*-position by *N*-methyltransferase, producing 3,7-dimethylxanthine (theobromine), which is again methylated at the 1-*N*-position to give 1,3,7-trimethylxanthine (caffeine) through sequential three-step methylation of xanthine derivatives at positions 7-*N*, 3-*N*, and 1-*N* by the enzyme 7-methylxanthine methyltransferase (CaMXMT).[91] Transcripts of *CAMXMT* were shown to accumulate in young leaves and stems containing buds, demonstrating the involvement of this enzyme, which appears to be expressed specifically in the aerial parts. Then caffeine is transported and stored in coffee beans, which hardly show any xanthosine methyltransferase activity.

Another illustrative example is the biosynthesis of morphine in the opium poppy. The isoquinoline alkaloids morphine, codeine, and thebaine are found in both roots and aerial parts of the plant, and specifically accumulate in vesicles within laticifers. It has been reported that in the biosynthesis of morphine alkaloids in *Papaver somniferum*, in developing root tips, both *O*-methyltransferase and *O*-acetyltransferase enzyme activities are found in the pericycle of the stele, whereas the BBE is localized in parenchyma cells of the root cortex. Furthermore, laticifers are not found in developing root tips, and, likewise, codeinone reductase (COR) was not detected. Two cell types, parenchyma within the vascular bundle and laticifers, are sites of the biosynthesis of isoquinoline alkaloids in this species. The early stages of morphine biosynthesis occur in parenchyma cells surrounding laticifers, and then at late stages, possibly at the level of either salutaridinol-7-*O*-acetate or thebaine the synthesis moves into the laticifer, which is the storage site of thebaine, codeine, and morphine. These results reveal the existence of cell-specific localization, which gives a coherent picture of the spatial distribution of alkaloid biosynthesis in opium poppy. Moreover, the role of intercellular transporters of alkaloid intermediates as well as intracellular transport into vesicles within laticifers adds a potential level of regulation to morphine biosynthesis.[92]

The biosynthesis of the TIAs in *C. roseus* is a clear example of a complex metabolic route and compartmentalization in which different steps of the pathway occur in different cellular compartments (plastids, chloroplasts, cytosol, vacuoles) and some of the later steps occur in different cells.[11] Thus, *tdc* (tryptophan decarboxylase) and *str* (strictosidine synthase) mRNAs were present in the epidermis of stems, leaves, and flower buds, appearing in most protoderm and cortical cells around the apical meristem of root tips. In contrast, *d4h* (desacetoxyvindoline 4-hydroxylase) and *dat* (deacetylvindoline 4-*O*-acetyltransferase) mRNAs were associated with the laticifer and idioblast cells of leaves, stems, and flower buds. It was concluded that the elaboration of the major leaf alkaloids involves the participation of at least three cell types and requires the intercellular translocation of a pathway intermediate.[93]

On the other hand, in the rational metabolic engineering of transgenic plants (*Arabidopsis*, tobacco, linseed) for the biosynthesis of omega-3 polyunsaturated fatty acids, mainly by manipulation of exogenous different fatty acid desaturases and acyl-CoA elongases, with the aim of producing around 20% of omega-3 polyunsaturated fatty acid yields, the importance of compartmentalization and precursors supply has also been shown. Thus, the existence of different bottlenecks preventing the achievement of the mentioned aim was reported.[94] These bottlenecks might be caused partly by inefficient non-native enzymes in the host system, or also by suboptimal acyl-exchange mechanisms between the acyl-CoA and lipid class pools. It was shown that a lack of essential elongase substrates in the acyl-CoA pool halted the elongation of the fatty acid into a longer chain

molecule, required for further biosynthesis of the target fatty acids. Therefore, the fine-tuning of the fatty acid flux between the acyl-CoA, phospholipid, and triacylglycerol pools will be essential to maximize polyunsaturated fatty acid yields in seed oils. In addition, efficient substrate channeling and lipid biosynthesis could depend on specific endoplasmic reticulum subdomain localization of key endogenous enzymes, and this organization could be compromised in heterologous systems. Several suggestions to overcome these bottlenecks were also made, such as the identification of improved desaturases, the identification of specific acyl-exchange mechanisms, and controlling the flux of long-chain polyunsaturated fatty acids into triacylglycerols.

It is well known that plants produce a large array of diverse secondary metabolites, with the involvement of compartmentalization and transport in their biosynthesis, and are also subject to exogenous toxins, including agrochemicals (e.g., pesticides) and toxic compounds secreted by other plants or pathogenic microbes. Thus, transport, disposal, and detoxification of toxic or nontoxic endogenous and exogenous compounds are vital processes for plant survival and development. Several possible mechanisms of detoxification include modification of toxic compounds by endogenous enzymes,[95] sequestration into vacuole,[96,97] and transport out of the cell.[98,99] Regarding transport of biosynthetic precursors or final products or exogenous substances, several mechanisms have been found to be operating in plants. Vacuoles can occupy up to 40–90% of the inner cell volume, playing a major role in the repository of secondary metabolites as well as in detoxification of compounds. Two major mechanisms are proposed for the vacuolar transport of secondary metabolites: H^+-gradient-dependent secondary transport via H^+-antiport with the participation of the multidrug and toxic compound extrusion (MATE) transporters, and directly energized primary transport by multidrug resistance protein (MDR)-type ATP-binding cassette (ABC) transporters.[100,101]

MATE is a large gene family whose proteins have a common topology consisting of 12 transmembrane domains. This large gene family has been divided into three groups based on amino acid sequence similarities. Members of the two more related groups are found in prokaryotes, whereas the third group is composed exclusively of proteins from eukaryotes. Members of this third group of MATE proteins are present in *S. cerevisiae* and *Schizosaccharomyces pombe* and are abundant in *Arabidopsis* with at least 56 members.[102] These types of transporters are more commonly involved in detoxifying unwanted toxic compounds, although they have also been found to participate in many plant processes. Thus, the AtDTX1 protein, which has been identified and characterized from *Arabidopsis*, has been determined to be localized in the plasma membrane and functions as an efflux transporter able to detoxify lipophyllic cations and cadmium. Moreover, the role of MATE-related proteins in plant development has also been suggested.[103] In the same plant, the *TT12* (*transparent testa12*) gene encodes a multidrug and toxic compound extrusion secondary transporter-like protein, which controls the vacuolar sequestration of flavonoids in the vacuoles of the seed coat endothelium.[104]

Recently, another MATE family gene (*AltSB*) has been reported to confer aluminum tolerance in sorghum.[105] Crop yields are significantly reduced by aluminum toxicity in highly acidic soils, which comprise up to 50% of the world's arable land. This protein is an aluminum-activated citrate transporter, responsible for the major sorghum aluminum tolerance via enhanced root citrate exudation.

Another transport mechanism is that of the ABC-transporter superfamily, which utilizes ATP as the energy donor. This is one of the largest protein families present in animals, bacteria, fungi, and plants. For instance, following the completion of the *Arabidopsis* genome,[106] it was determined that more than 100 ABC-transporter proteins were present in this organism. Initially, these transporters were found to be implicated in detoxification processes, although recent reports indicate that the function of this type of protein family is not restricted to detoxification processes alone. In plants, ABC transporters have been shown to be involved in membrane transport and also in plastid–nucleus communication mechanism; they also participate in stomatal movements, chlorophyll biosynthesis, formation of Fe/S clusters, and likely ion fluxes, thereby playing a major role in plant growth and development.[101] On the other hand, ABC transporters in mammals have been found to confer multidrug resistance in cancer cells exposed to antitumor drugs and also upon overexpression of these transporters.[107]

The participation of these transporters in plant secondary metabolites has been demonstrated. For instance, in *Arabidopsis*, a transporter protein named AtMRP1 has been identified, whose gene encodes a glutathione *S*-conjugate pump implicated in detoxification, transporting the conjugates out of the cytosol.[108] This ABC transporter participates in the transport of glutathione *S*-conjugates of xenobiotics and endogenous substances,

including herbicides and anthocyanins, respectively. Furthermore, AtMRP1 is a structural homologue of yeast YCF1 (active in the transport of organic GS-conjugates and heavy metals) and mammalian MRP1 (having a similarly broad substrate range).

Berberine, a benzylisoquinoline alkaloid, is preferentially accumulated in the rhizome of *Coptis japonica*. On the contrary, gene expression of berberine biosynthetic enzymes has been observed specifically in root tissues, which suggests that berberine after being synthesized in the roots is transported to the rhizome, the main repository. The uptake of exogenously applied berberine was investigated in cultured *C. japonica* cells.[109] These were able to capture berberine from the liquid nutrient medium and transport it exclusively to the vacuoles. It was shown that this uptake depended on the growth phase of the culture but was independent of the nutrient medium employed. Treatment with several inhibitors suggested that berberine uptake depended on the level of ATP. On the other hand, some inhibitors of P-glycoprotein, an ABC transporter involved in multidrug resistance in cancer cells, strongly inhibited berberine uptake, whereas a specific inhibitor of glutathione biosynthesis and vacuolar ATPase (bafilomicyn A1) had little effect. These results suggest that ABC proteins of the MDR type are involved in the uptake of berberine from the liquid medium. These authors, also employing berberine-producing cultured *C. japonica* cells, isolated a cDNA encoding an MDR)-type ABC transporter (*Cjmdr1*), which is also highly expressed in rhizomes. Functional analysis of *Cjmdr1* in a *Xenopus* oocyte expression system surprisingly showed that CjMDR1 transported berberine in an inward direction, resulting in a higher accumulation of berberine in *Cjmdr1*-injected oocytes than in the controls. Furthermore, using inhibitors of ABC proteins, such as vanadate, nifedipine, and glibenclamide, as well as ATP depletion, it was shown that this CjMDR1-dependent berberine uptake was clearly inhibited, suggesting that CjMDR1 functioned as an ABC transporter. Conventional membrane separation methods showed that CjMDR1 was localized in the plasma membrane of *C. japonica* cells. *In situ* hybridization indicated that *Cjmdr1* mRNA was expressed preferentially in the xylem tissues of the rhizome. These findings strongly suggest that CjMDR1 is involved in the translocation of berberine from the root to the rhizome.[110]

On the other hand, another *A. thaliana* ABC transporter that confers antibiotic resistance to transgenic plants was identified and its gene (*Atwbc19*) characterized.[111] Its overexpression in *A. thaliana* conferred kanamycin resistance, offering the possibility of substituting the commonly used selectable markers of antibiotic resistance of bacterial origin such as neomycin phosphotransferase type II gene (*nptII*), thus avoiding the concerns about horizontal gene transfer from transgenic plants back to bacteria, which may result in antibiotic resistance. Unlike *nptII*, which confers broad tolerance to several antibiotics, *atwbc19* was very effective and specific to kanamycin tolerance. Moreover, the mechanism of resistance was novel. Using transgenic tobacco plants, it was demonstrated that the subcellular localization of Atwbc19 occurs in the vacuolar lumen; thus, kanamycin is actively sequestered in the vacuole as a substrate of this ABC transporter and prevented from interfering with ribosomal RNA in the cytosol, mitochondria, or chloroplasts, and thereby its toxicity is mitigated. Because ABC transporters are endogenous to plants, the use of *Atwbc19* as a selectable marker in transgenic agriculturally important plants may provide an alternative to current bacterial marker genes in terms of the risk of horizontal transfer of resistance genes.

These results and the involvement of specific transporter proteins indicate the importance of cell compartmentalization and transport of molecules within the cell through different cell organelles, to other cell types, or even organs, and the research involved has also been elegantly presented in recent publications summarizing these issues.[112–114] Therefore, as presented, the importance of these two points is high and needs to be taken into consideration when effective genetic engineering approaches are being designed for the enhancement of target compounds.

3.18.4 Metabolic Engineering of Plant Biosynthetic Networks

In the previous sections, the most common methodologies for plant transformation and the various approaches to modulate metabolic networks were presented. In this section, three examples of different metabolic pathways are developed, the artemisinin pathway, the tropane alkaloid pathway, and the morphinan alkaloid pathway, to reveal the advances and progress made in the metabolic engineering of pathways for the improvement of product yields or even the accumulation of new metabolites.

3.18.4.1 Artemisinin Pathway

Malaria is a devastating disease occurring mainly in underdeveloped or developing tropical countries, producing 300 000–500 000 new infections and about 1–3 million deaths each year, affecting mainly young children under 5 and pregnant women.[115] Besides, the causal agent of the disease, the *Plasmodium falciparum* parasite, is becoming increasingly resistant to many drug therapies, such as the well-established chloroquine. Artemisinin is currently effective against these drug-resistant strains, and new therapeutic approaches are being pursued and evaluated, including combination therapies based on derivatives of artemisinin, that is, dihydroartemisinin, artesunic acid, artelinic acid, artemether, and arteether (**Figure 3**), each offering a different mechanism of action that would prevent development of drug resistance by the parasite.

Artemisinin, a sesquiterpene lactone of the cadinane series (isoprenoid), contains a rare endoperoxide bridge, infrequently found in secondary metabolites, which appears essential for its antimalarial and anticancer activities, as well as a lactone group (**Figure 3**). It is found in the Chinese plant *Artemisia annua* (Asteraceae), which has been used for many centuries in traditional Chinese medicine for the treatment of fever and malaria. The plant, also known as sweet wormwood or annual wormwood, is well spread, being found in Europe, North and South America, as well as China and Asia.

The artemisinin yields from plants range typically from 0.05 to 0.3%, but as much as 1.0–1.5% artemisinin has been reported.[116] Maximum concentrations occur in the leaves, stem, flowers, seeds, small green stems, and minute amounts are present in old stems and roots; at the optimum time of harvesting at flowering stage or earlier, accumulation is specifically in glandular trichomes. Other major sesquiterpenes present in the plant are artemisinic acid (arteannuic acid) (0.2–0.8%), which can be efficiently converted into artemisinin by a simple chemical process, and arteannuin B. At present, plants constitute the major source of artemisinin and related compounds with attractive antimalarial activities, but these are subject to climate changes, attacks by insects, bacteria, and fungi, and other parameters, which can influence yield. On the other hand, the efforts toward the total synthesis of artemisinin have not become commercially exploitable due to the low yields obtained, together with the chemical complexity and high costs.[117,118] In addition, attempts to *in vitro* produce artemisinin and related compounds, as well as the manipulation of the artemisinin biosynthetic pathway to increase and modulate the production of these metabolites, have been made in plants and microorganisms and will be discussed next.

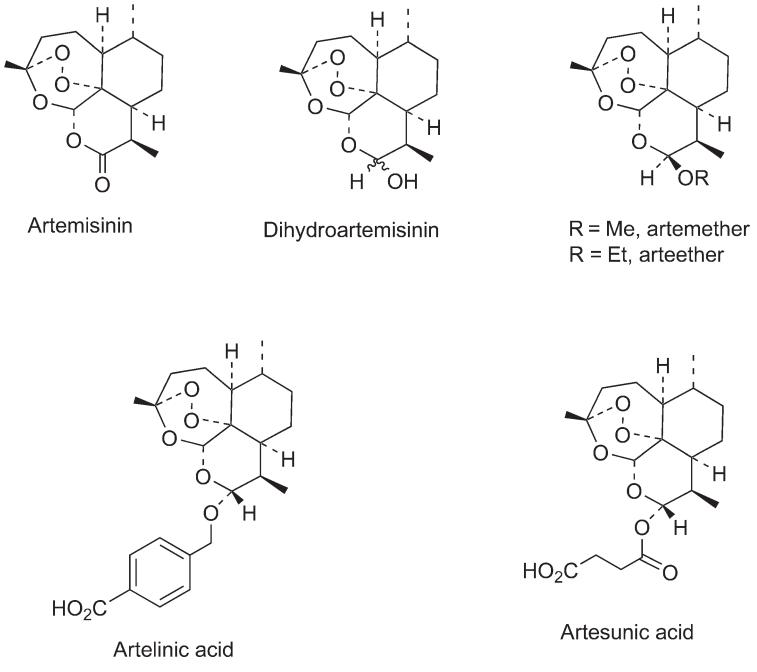


Figure 3 Molecular structures of artemisinin and derivatives.

The initial attempts for the production of this valuable metabolite comprised the establishment of *in vitro* plant tissue or suspension cultures, and studying the biosynthesis in different culture systems and conditions, including variations on nutrient regimes, to achieve moderate yields of the target compound.[119–122] Moreover, artemisinin production was also increased through biotransformation of precursors employing green callus cultures and leaf tissue homogenates. Feeding with borneol, pinene, and a combination of artemisic acid and arteannuin B increased artemisinin production significantly.[123]

Cell suspension cultures of *A. annua* have also been established for the production of artemisinin and related compounds although the production of this metabolite was reported to be absent in this culture system.[124] Contrarily, in another attempt, suspension cultures of *A. annua* were established after *A. tumefaciens* infection, as well as untransformed control suspension cultures. *Agrobacterium tumefaciens*-transformed suspension cultures grew faster and accumulated artemisinin (0.2 g per 100 g dry wt.) with lower yields by the untransformed suspension culture. This compound was also released into the liquid nutrient medium in both cultures, 38.6 and 7.5 mg ml^{-1}, respectively, suggesting the usefulness of this transformed suspension culture over callus cultures.[125]

Other types of cultures displaying cell or morphological differentiation have also been investigated for the production of artemisinin. On induction of rooting, shoot cultures of *A. annua* accumulated artemisinin and arteannuin B (0.95 mg and 6.57 mg% fresh weight, respectively).[126] When rooting was reduced or absent, artemisinin production was clearly lower, with maximum amounts accumulating in a shoot system when root induction was the highest (0.287% dry weight),[127] suggesting that cellular and/or morphological differentiation was a prerequisite in order to induce higher product accumulation. Similarly, hairy roots, induced after *A. rhizogenes* infection of *A. annua* explants, have also been studied. Nutrient media optimization of this culture system resulted in an artemisinin yield of 14 mg l^{-1}.[128] Furthermore, a larger boost in artemisinin production was achieved following elicitation of hairy roots with a homogenate of *Aspergillus oryzae*, with the artemisinin yield increased to 550 mg l^{-1}.[129] Elicitation of hairy roots was also attempted using chitosan, methyl jasmonate, and yeast extract, achieving the highest artemisinin increase, sixfold compared with controls, with chitosan (1.84 mg g dry $wt.^{-1}$) followed by methyl jasmonate (fivefold increase, 1.52 mg g dry $wt.^{-1}$) and yeast extract (0.95 mg g dry $wt.^{-1}$).[130] Stimulation of artemisinin production in *A. annua* hairy roots has also been conducted employing the *A. annua* endophytic fungus *Colletotrichum* sp.,[131] achieving a maximum production of 13 mg l^{-1}, a 44% increase over the control. Similarly, enhancement of artemisinin yields in *A. annua* hairy roots has been reported by feeding cultures with (22*S*,23*S*)-homobrassinolide, a synthetic analogue of brassinosteroids, which are a group of steroidal lactones with high and diverse phytophysiological effects such as plant growth promotion, enhancement of rooting capacity, disease resistance, and stress tolerance.[132] The maximum production of artemisinin was 14 mg l^{-1}, a 57% increase over the control.

Also working with *A. annua* hairy root cultures, the effect of light irradiation was investigated. It was found that under an illumination of 3000 lux for 16 and 8 h in the dark, an optimal artemisinin yield of 244.5 mg l^{-1} and a dry weight of 13.8 g l^{-1} were achieved.[133] Moreover, exposure to different types of light irradiations (white, red, blue, yellow, or green) was evaluated for optimization of artemisinin production. Red light at 660 nm gave the highest artemisinin content (31 mg g dry $cells^{-1}$) and hairy roots biomass (5.73 g dry wt cells l $medium^{-1}$), which were, respectively, 67 and 17% higher than those obtained under white light.[134]

Bioreactors have also been employed for the culture of *A. annua* hairy roots for the production of artemisinin. Two different classes of bioreactors, that is, nutrient mist (gas-phase, the roots are intermittently exposed to ambient air, or another gas mixture, and the nutrient liquid) and bubble column (liquid-phase, the roots are submerged in the nutrient medium), were assessed for growth and artemisinin accumulation. Hairy roots grown in the nutrient mist reactor produced nearly three times as much artemisinin as roots grown in the bubble column reactor, 2.64 and 0.98 μg g dry $wt.^{-1}$, respectively.[135] Exploitation of *A. annua* shoot cultures has also been practiced in three different bioreactors, a modified airlift bioreactor, a multiplate radius-flow bioreactor, and an ultrasonic nutrient mist bioreactor. Shoots growing in the multiplate radius-flow bioreactor and nutrient mist bioreactor showed excellent growth; however, vitrified shoots were observed in the modified airlift bioreactor where shoots were totally immersed in the nutrient medium. Artemisinin production by shoot cultures was the largest in the ultrasonic nutrient mist bioreactor (48.2 mg l^{-1} after 25 days), representing 1.4–3.3-fold higher yield compared with the other two bioreactors.[136]

Regarding the metabolic pathway leading to the synthesis of this type of compounds, it is known that terpenoids are derived from C5 isoprene units joined in a head-to-tail fashion.[137] The initial steps in the biosynthesis of terpenoids originated through the mevalonate pathway from three acetyl CoA units, taking place in the cytoplasm, involving the condensation of geranyl diphosphate with isopentenyl diphosphate (IPP), generating farnesyl diphosphate (FPP) from which many compounds of sesquiterpenoid nature are synthesized (**Figure 3**). Moreover, for the production of artemisinin, the pathway also comprises a second branch in a different cell compartment, the plastids, that is, the deoxyxylulose phosphate (DXP) pathway, producing dimethylallyl diphosphate (DMAPP), which after condensation with IPP and through various metabolic steps yields the first direct metabolite amorpha-4,11-diene, a precursor of artemisinin (**Figure 3**). Currently, 12 genes related to artemisinin biosynthesis have been cloned from *A. annua*, with their complete or partial mRNA sequences being available in the GenBank database.

It has been shown that artemisinin is a complex molecule whose chemical synthesis is not economic and commercially unfeasible; thus, genetic engineering of the pathway (**Figure 4**) leading to the synthesis of this valued product has been attempted in various organisms, that is, plants, yeasts, and bacteria, in order to establish a continuous and high supply of this metabolite to satisfy the world demand.[138,139] Thus, the genes encoding important enzymes of the artemisinin pathway, such as farnesyl diphosphate synthase (FPS), amorpha-4,11-diene synthase (ADS), as well as the squalene synthase gene (SQS), have been cloned from *A. annua*.[140–143] Besides, as early as 1999, employing *A. tumefaciens*-mediated transformation, transgenic *A. annua* plants expressing the GFP marker gene were established.[144] Two different *A. thaliana* genes involved in flowering have also been overexpressed in *A. annua* in order to determine the possible relationship between artemisinin accumulation and flowering. The flowering promoting factor1 (*fpf1*) from *A. thaliana* was transferred into *A. annua* plants via *A. tumefaciens*. Under short-day conditions, the flowering time of *fpf1* transgenic plants was about 20 days earlier than the nontransformed plants; however, no significant differences were detected in artemisinin content between the flowering transgenic plants and the nonflowering nontransgenic plants. These results showed that flowering is not a necessary factor for increasing the artemisinin content; furthermore, there may be no direct linkage between flowering and artemisinin biosynthesis.[145] Similarly, the early flowering gene *CONSTANS* (*CO*) from *A. thaliana* was also transferred into *A. annua* using the *A. tumefaciens*-mediated transformation system. Although the flowering time of the *CO* transgenic *A. annua* plants was about 2 weeks earlier than that of the nontransgenic plants under short-day conditions, no significant difference in artemisinin content was found between the flowering transgenic plant and the nonflowering nontransgenic plant. These results showed that the usually observed increase in artemisinin content before plant flowering under natural conditions is not a direct consequence of flowering itself, and perhaps there is even no direct relationship between flowering and artemisinin biosynthesis.[146]

In another instance, the endogenous FPS gene (*fps*) was overexpressed in high-yield *A. annua* plants via *A. tumefaciens*, to increase the artemisinin content.[147] The FPS activity in the transgenic plants was two- to threefold larger than the controls, obtaining the highest artemisinin content of 0.9% (dry weight), which was 34% greater than that of nontransgenic *A. annua* plants. These results clearly demonstrate the regulatory function of FPS upon artemisinin biosynthesis.

It has been mentioned above that the capitate glands on the leaf surface, together with specialized chloroplasts of the capitate glands,[148] seem to play a major role in the artemisinin biosynthesis. Accordingly, a higher cytokinin content may increase the presence of these elements and also that of artemisinin; thus, an isopentenyl transferase gene (*IPT*), participating in cytokinin biosynthesis in *A. tumefaciens*, was transferred into *A. annua* via *A. tumefaciens* under the control of the CaMV35S promoter. In the resultant transgenic *A. annua* plants, cytokinin, chlorophyll, and artemisinin were clearly enhanced. Cytokinin contents were two- to threefold larger, while chlorophyll increased 20–60% and, more importantly, artemisinin augmented 30–70% compared with controls, establishing a direct correlation between the contents of cytokinin, chlorophyll, and artemisinin, and also indicating the relationship between endogenous cytokinin levels and artemisinin production.[149] Besides, also employing an *A. tumefaciens*-mediated transformation system, a cDNA encoding FPS (*fds* placed under a CaMV 35S promoter) was transferred into *A. annua*. The established transgenic plants displayed an artemisinin content of 10 mg g dry wt.$^{-1}$, about 2–3 times higher than that in the controls.[150] Recently, the involvement of glandular trichomes of *A. annua* in artemisinin biosynthesis[151] where the Δ11(13) double bond originating in amorpha-4,11-diene is reduced was studied, and this is thought to occur in artemisinic aldehyde, although other intermediates have been suggested. In order to understand

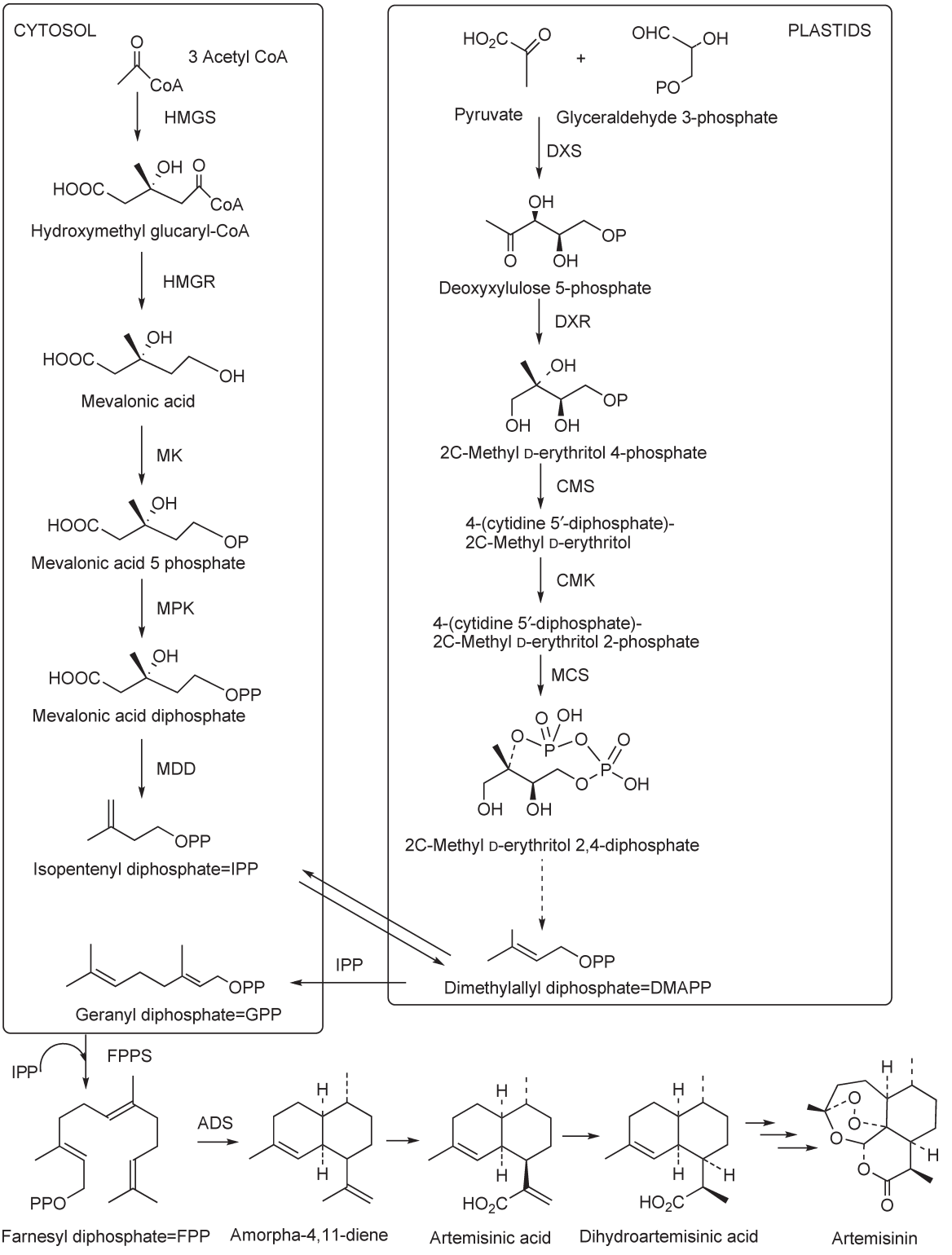


Figure 4 Biosynthetic route of artemisinin and precursors, also indicating the known enzymes participating in different metabolic steps. HMGS: 3-hydroxy-3-methylglutaryl CoA synthase; HMGR: 3-hydroxy-3-methylglutaryl CoA reductase; MK: mevalonate kinase; MPK: mevalonate-5-phosphate kinase; MDD: mevalonate diphosphate dicarboxylase; GPPS: geranyl diphosphate synthase; FPPS: farnesyl diphosphate synthase; DXS: deoxyxylulose 5-phosphate synthase; DXR: deoxyxylulose 5-phosphate reductoisomerase; CMS: 2-*C*-methyl erythritol 4-phosphate cytidyl transferase; CMK: cytidine-2-*C*-methyl erythritol kinase; MCS: 2-*C*-methyl erythritol 2,4-cyclodiphosphate synthase; ADS: amorpha-4,11-diene synthase.

double bond reduction in artemisinin biosynthesis, extracts of *A. annua* flower buds were investigated and were found to display artemisinic aldehyde Δ11(13) double bond reductase activity. Moreover, the isolation of a cDNA clone corresponding to this enzyme (*Dbr-2*), which encodes a member of the enoate reductase family similar to plant 12-oxophytodienoate reductases, and being highly expressed in glandular trichomes was achieved. This recombinant *DBR2* gene was characterized and was moderately specific for artemisinic aldehyde. Co-expression of *DBR2* and FPS (*FPS2*), amorpha-4,11-diene synthase (*ADS*), amorpha-4,11-diene monooxygenase (*CYP71AV1*), cytochrome P-450 reductase (*CPR*) genes in yeast resulted in the accumulation of dihydroartemisinic acid. Two yeast strains were studied, one expressing *FPS2*, *ADS*, *CYP71AV1*, and *CPR*, and the other in addition expressing *DBR2*; the former strain accumulated artemisinic acid to a level of 29.4 mg l culture^{-1} and the latter strain accumulated artemisinic acid to a level of 11.8 mg l^{-1} and, in addition, dihydroartemisinic acid was found at a level of 15.7 mg l culture^{-1}. Both acids can be chemically converted to artemisinin, particularly the dihydroartemisinic acid, which only requires a stream of oxygen under the appropriate conditions, resulting in an attractive alternative for the production of the target antimalarial compound.[151]

In this regard, the production of artemisinin or immediate precursors by genetically modified microbes has been extensively studied[152] by transferring artemisinin biosynthetic genes into genetically altered microbial hosts such as *E. coli* and *S. cerevisiae* (**Figure 4**). These have been established for terpene production, as many catalytic steps for terpene biosynthesis are conserved among many higher plants; these two microbial hosts also offer the possibility to produce pharmaceuticals in large-scale fermentations. Hence, a transformed *E. coli* containing a heterologous nine-gene biosynthetic pathway for the production of the terpene amorpha-4,11-diene, a precursor of the antimalarial drug artemisinin, was established showing that amorphadiene evaporates from the fermentor but could be trapped using a condenser. The amorphadiene yield was determined to be 0.5 g l^{-1}.[153] This host microorganism has also been genetically engineered to produce functionalized terpenoids using plant P-450s, that is, *CYP71AV1* that after codon optimization coupled with N-terminal transmembrane engineering, and of *Candida tropicalis CPR* gene by the *A. annua* one, resulted in a 12-fold higher artemisinic alcohol production (5.6 mg l^{-1}).[154] Also the heterologous expression of a germacrene A synthase from a glandular trichome cDNA library from *A. annua* was studied in *E. coli*, catalyzing the cyclization of FPP to germacrene A, demonstrating the potential of the use of *A. annua* glandular trichomes as a starting material for studying isoprenoid biosynthesis in this plant species.[155]

Eukaryotic heterologous expression systems for plant genes, such as that of *S. cerevisiae*, offer greater advantages over bacterial systems as the possibility of glycosylation and secretion, as well as encode membrane-bound proteins that show difficulties functionalizing in bacterial hosts, which are also unable to perform glycosylation, and the appropriate protein folding necessary for the activation of many enzymes, as compared with yeast.[156]

Thus, *S. cerevisiae* has been employed for the characterization and expression of *A. annua* genes involved in the artemisinin biosynthetic route. Consequently, a cytochrome P-450 monooxygenase (CYP71AV1) from *A. annua* was identified by an expressed sequence tag (EST) approach, and was shown to be a multifunctional enzyme catalyzing three steps of the biosynthetic network, by oxidizing artemisinic alcohol to generate artemisinic acid via artemisinic aldehyde intermediates. Artemisinic acid thus produced is easily transported out and retained on the outside of the engineered yeast, implying that a simple extraction and purification process can be implemented to obtain the pure compound. Therefore, the cloning of *CYP71AV1* offers an opportunity to improve the supply of artemisinin via production of a close intermediate by genetic engineering of microorganisms or plants.[157]

Amorpha-4,11-diene biosynthesis has also been attempted in yeast. The *ADS* (amorpha-4,11-diene synthase) gene from *A. annua* was transferred to yeast cells on an episomal plasmid and by homologous recombination.[158] Both systems showed functionally expressed *ADS* gene and produced 600 and 100 μg l^{-1} of amorpha-4,11-diene, respectively, indicating that the availability of the substrate pool (FPP) was the limiting factor. Similarly, engineered *S. cerevisiae* yeast has been constructed for the production of a more advance intermediate within the artemisinin pathway, that is, artemisinic acid, through multigene transfer to this host.[23] The mevalonate pathway to yield artemisic acid was expressed in yeast using different genes and three steps. First, the FPP biosynthetic pathway was engineered to have a higher FPP production and decrease its use for sterols; second, the *ADS* (amorpha-4,11-diene synthase) gene from *A. annua* was introduced into the higher FPP-producing strain to convert FPP to amorpha-4,11-diene; and third, the novel cytochrome P-450 monooxygenase (CYP71AV1) from

A. annua, which participates in the three-step oxidation of amorphadiene to artemisinic acid was cloned and expressed in the amorphadiene producer yeast strain. Following this approach, amorphadiene was increased 500-fold compared with previous data; moreover, the target compound, artemisinic acid, was highly accumulated ($100\ mg\ l^{-1}$) in a short period of time (4–5 days) compared with several months for *A. annua* plants, suggesting the usefulness of yeast expression systems as an alternative for the production of these bioactive compounds.

3.18.4.2 Tropane Alkaloid Pathway

Alkaloids are low-molecular-weight nitrogen-containing basic substances, classified according to the amino acid providing both the nitrogen atoms, and the fundamental part of the skeleton. In alkaloids, the nitrogen atom and in general the carbon skeleton of the amino acid are largely retained intact in the final structure. Regarding classification, various groups of alkaloids are distinguished, for example, piperidine, quinoline, pyrrolidine, indole, and tropane. In addition, different metabolic pathways provide different building blocks for the final alkaloid structure; thus, tropane alkaloids are derived mainly from ornithine or less frequently from arginine.

Tropane alkaloids occur chiefly in the Solanaceae family, as well as in the families Orchidaceae, Brassicaceae, and Euphorbiaceae[159,160] and include mainly atropine (±hyoscyamine), scopolamine, and the narcotic anesthetic cocaine. Regarding the pharmacological activities of hyoscyamine and scopolamine, these alkaloids are classified as anticholinergics (although the term antimuscarinics is preferred) by competition with acetylcholine for the muscarinic site of the parasympathetic nervous system (postganglionic cholinergic nerve endings), preventing the transmission of nerve impulses. Acetylcholine binds to two types of receptors, that is, muscarinic or nicotinic. The structural similarity between acetylcholine and muscarine is known, thus hyoscyamine and scopolamine are able to occupy the muscarinic receptor site through the spatial relationship between the nitrogen atom and the ester linkage of the molecules, with the side chain also playing a role in the binding, explaining the difference in activities between the two enantiomeric forms.[161]

Both alkaloids have (+) and (−) forms but only the (−) hyoscyamine and (−) scopolamine are active. The biosynthetic pathway of tropane alkaloids is not totally understood (**Figure 4**), especially at the enzymatic level. In this pathway, the final step of the biosynthesis is the bioconversion of hyoscyamine into scopolamine via 6β-hydroxyhyoscyamine, a reaction catalyzed by the enzyme H6H. Hyoscyamine is the ester of tropine and (*S*)-tropic acid. The (*S*)-tropic acid moiety derives from the amino acid L-phenylalanine, while the bicyclic tropane ring derives from L-ornithine primarily, or L-arginine via tropinone. Tropinone is stereospecifically reduced to form either tropine, which is incorporated into hyoscyamine, or pseudotropine, which proceeds to calystegines, a group of nor-tropane derivatives that were first found in the Convolvulaceae family.[162]

Tropane alkaloids are mainly biosynthesized in the roots of the producing plants, where they mostly accumulate, and are then transferred to the aerial parts.[163,164] Their amounts and ratios vary in stems, leaves, roots, and seeds, depending also on the developmental stage of the plant.[165,166] In order to satisfy the world demand, these alkaloids are entirely obtained from cultivated plants; besides, chemical synthesis has proved to be difficult and not economically feasible. Accordingly, biotechnology has been applied as an alternative to obtain the desired tropane alkaloids.[167]

It has been mentioned earlier that tropane alkaloids are mostly synthesized in the roots of the producing plants, although there are accounts of the presence of *h6h* gene activity in other organs, such as the anthers in *Atropa* belladonna,[168] as well as roots, stems, and leaves of *Anisodus acutangulus*[169] where these alkaloids might be produced. Accordingly, hairy root cultures known for their capacity to grow indefinitely on a nutrient medium without the need of adding plant growth regulators, together with their genomic stability,[170,171] have been established as the main system for obtaining hyoscyamine and scopolamine. Hairy roots, induced by means of *A. rhizogenes*-guided infection of plant material, of several species of Solanaceae have been established for tropane alkaloid studies, such as *Atropa baetica*, *Datura metel*, *A. belladonna*, *Hyoscyamus niger*, and *H. albus*.[172–175]

Metabolic engineering of the tropane alkaloid network has also been attempted. Despite the fact that the total elucidation of this metabolic pathway has not yet been fulfilled, many enzymes and the sequence of their coding genes have been reported,[167] with seven enzymes described, that is, ADC (arginine decarboxylase), ODC (ornithine decarboxylase), PMT, MPO (methyl putrescine oxidase), TR-I (tropinone reductase I), TR-II (tropinone reductase II), and H6H, participating at different points within the biosynthetic route (**Figure 5**). The advances made have permitted the understanding of their roles in the tropane alkaloid biosynthetic

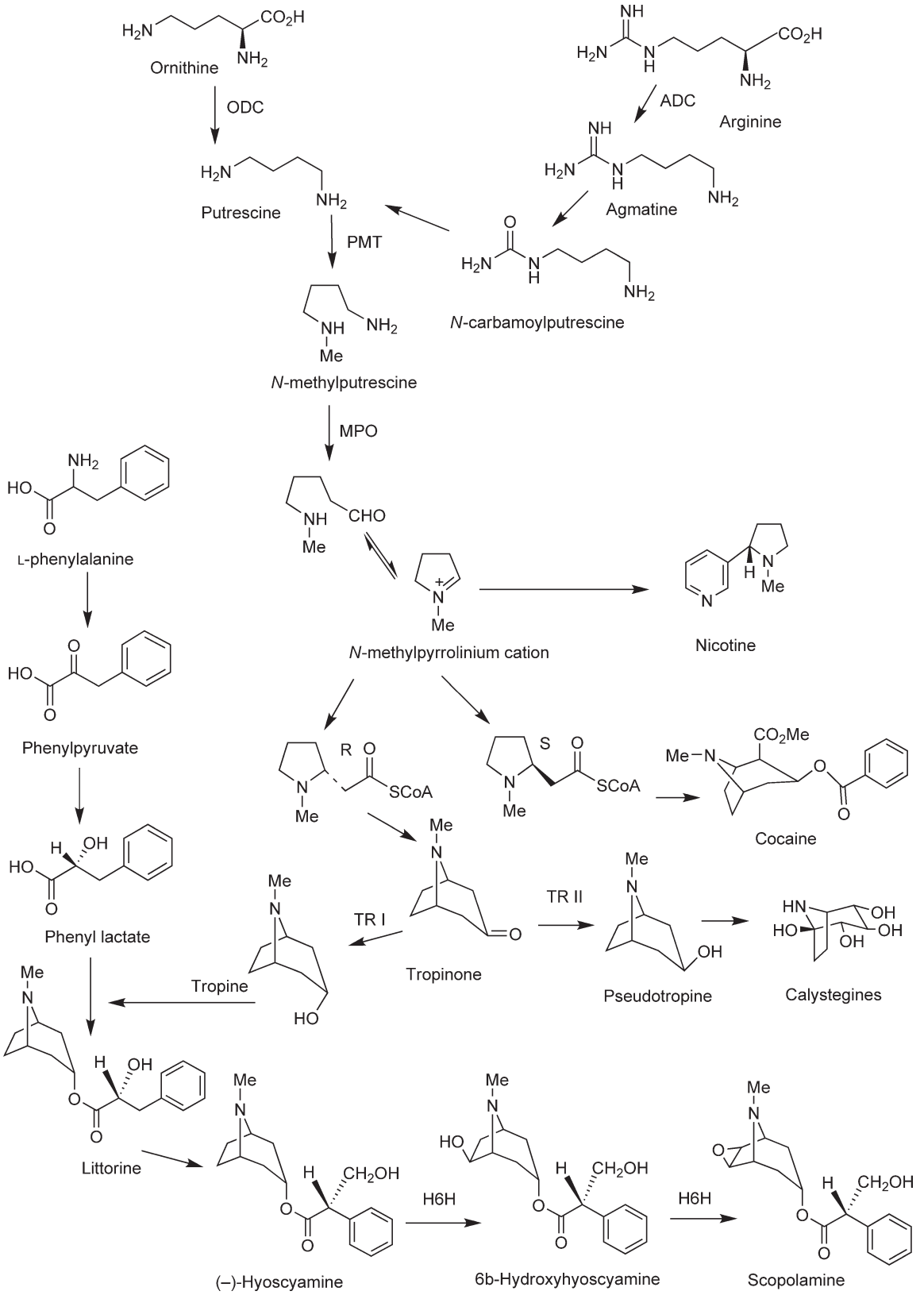


Figure 5 Tropane alkaloid biosynthetic pathway scheme, showing the different characterized enzymes participating in the network. ADC: arginine decarboxylase; ODC: ornithine decarboxylase; PMT: putrescine methyl transferase; MPO: methyl putrescine oxidase; TRI–II: tropinone reductase I and II; H6H: hyoscyamine 6β-hydroxylase.

pathway, and their manipulation has been attempted using different culture systems resulting in many cases in major product yields. Next, the genetic manipulation of these enzymes and the efforts for improving the production of the valuable tropane alkaloid scopolamine and hyoscyamine are presented.

The first step in the biosynthesis of tropane alkaloids comprises the formation of the intermediate putrescine. Polyamines and putrescine are found in plant cells and are implicated in cell division, growth, root, fruit, and flower development, and in different stress phenomena. It is well known that plants synthesize polyamines from ornithine and arginine, unlike other eukaryotes like mammals, which synthesize polyamines from ornithine alone. In plants, putrescine is synthesized either directly from ornithine, a reaction catalyzed by ODC, or indirectly from arginine via agmatine catalyzed by ADC (**Figure 5**).

In plants, the profile of *ADC* and *ODC* gene expression is tissue dependent and changes in different processes such as cell division, cell proliferation, or stress responses.[176,177] For instance, in mature tobacco plants, *ADC* and *ODC* were preferentially expressed in roots and floral tissue.[178] Moreover, in apple cells, it appears that ADC rather than ODC is the primary pathway for putrescine biosynthesis,[179] while in rice the reverse is true, with ODC being more important than ADC for putrescine synthesis.[180]

It has been mentioned earlier that tropane alkaloids can be synthesized from both ornithine and arginine, although the arginine route is favored, demonstrated for instance in *H. albus* hairy roots where the activity of ADC was twice that of ODC.[181] Regarding the genetic engineering of these enzymes, the first report was the expression of an oat *adc* cDNA in tobacco plants, resulting in an altered phenotype in transgenic lines, demonstrating the effect of the high toxic levels of putrescine or its catabolytes.[182] Analogously, the *ADC* gene has been overexpressed in *A. thaliana* transgenic plants, resulting in a higher putrescine level and alteration of the gibberellin metabolism, generation of a dwarf phenotype, and late flowering.[183] Likewise, the *ODC* gene from *Datura stramonium* was overexpressed in tobacco plants, with the transgenic lines exhibiting 25- and 5-fold increase in ODC enzyme activity in leaves and flower buds, respectively. However, the increase in putrescine levels was only 1.5–2.1-fold in leaves and 1.1–1.3-fold in flower buds, suggesting a metabolic control at different biosynthetic steps.[184] In order to demonstrate the influence of the two decarboxylases on the tropane alkaloid pathway in a Solanaceous species, *D. stramonium* hairy roots were treated with two specific inhibitors of ADC and ODC, that is, DFMA (DL-α-difluoromethylarginine) and DFMO (DL-α-difluoromethylornithine), respectively. The suppression of ADC led to an 80% decrease in hyoscyamine, as well as other intermediates in hyoscyamine biosynthesis, such as hygrine, tropinone, and tropine,[185,186] while the inhibition of ODC did not show the same effect on hyoscyamine content,[185] but produced an increase in the activity of ADC and a weak variation of metabolite contents.[186] These results indicate that in *D. stramonium*, both routes are possible, acting as two different metabolic controls by manipulating ADC and ODC enzyme activity. Another example of a useful effect of *ADC* and *ODC* genetic engineering is that of *D. innoxia*-transformed calli overexpressing both genes. A high level of hyoscyamine as well as an increase in the frequency of plant regeneration was recorded.[187]

Putrescine is an important intermediate in the biosynthesis of tropane alkaloids acting also as a precursor for polyamines, spermine and spermidine, and for the biosynthesis of nicotine.[181,188] This is catalyzed by the enzyme PMT, constituting the first specific step in the biosynthesis of tropane alkaloids, cocaine, and nicotine.[189] Putrescine is methylated by PMT via SAM (*S*-adenosylmethionine) relocating the methyl group from SAM to an amino group of putrescine (**Figure 5**). This enzyme has been isolated from the roots of both *Nicotiana tabacum* and *D. stramonium*,[190] and the activity of this enzyme is restricted to the roots of Solanaceous plants as found in *A. belladonna*,[191] although in *N. tabacum* leaves mRNA *pmt* transcript levels have also been detected.[192]

In *D. stramonium*, a close relationship between PMT activity, the morphological state of cultures, and the biosynthesis of hyoscyamine has been suggested. It was found that when dedifferentiation was induced with a mixture of kinetin, 2,4-dichlorophenoxyacetic acid, and α-naphthalene-acetic acid, the PMT activity and hyoscyamine biosynthesis were lost.[193]

Several *pmt* cDNAs have been isolated and heterologously expressed in other organisms. For instance, in *Anisodus tanguticus* hairy roots, the sequence has 1017 bp encoding a protein of 338 amino acids with high homology with other known PMTs. This cDNA was also cloned in *E. coli* exhibiting a SAM-dependent *N*-methyltransferase activity.[194] Likewise, a 1332 bp *pmt* cDNA from *Solanum tuberosum* was cloned in *E. coli* and yielded an active 334 amino acid enzyme with a high homology with *N. tabacum*, *H. niger*, and *A. belladonna* PMTs.[195]

Genetic engineering of this enzyme has been carried out in order to increase tropane alkaloid contents or nicotine amounts. Transgenic *D. metel* cultures overexpressing *pmt* exhibited enhancement of both hyoscyamine and scopolamine (1.46- and 3.35-fold, respectively) although the transgenic cultures aged faster than the controls.[196] Likewise, *H. muticus* transgenic culture lines gained a high capacity to biosynthesize hyoscyamine, with an average 2.3-fold higher than controls and 10-fold higher than *D. metel* transgenic cultures, while scopolamine contents were similar to controls.[196] These results demonstrate the presence of clear differences in the metabolic control of this pathway between two related tropane alkaloid-producing plant species. Similar results were obtained in transgenic hairy root cultures of *D. metel* overexpressing tobacco *PMT* gene, which displayed an increased production of hyoscyamine and scopolamine (1.4- and 2.1-fold, respectively).[197]

Analogously, *PMT* gene was overexpressed in *H. niger* transgenic hairy roots. These showed higher *pmt* transcript levels than the control; nonetheless, the *pmt* transgenic lines produced nicotine, hyoscyamine, and scopolamine at similar levels compared with controls, indicating that the increase in *pmt* transcripts was not sufficient to generate higher scopolamine contents, and also that the pathway in *H. niger* was downstream-limited requiring the stimulation of other metabolic steps.[198] *Scopolia parviflora* was also genetically engineered to overexpress tobacco *pmt* mRNA, resulting in high PMT protein levels and enhanced hyoscyamine and scopolamine amounts.[199]

It has been suggested that the genetic engineering of a sole key enzyme in a given pathway does not always result in the enhancement of a desired end product. In the case of *A. belladonna*, the overexpression of *pmt* resulted in a fivefold increase in *pmt* transcript levels unlike the tropane alkaloid profile (hyoscyamine and scopolamine) as well as the biosynthetic precursors tropine, pseudotropine, and tropinone, which were not affected.[200,201] In a similar fashion, the overexpression of tobacco *PMT* gene in *Dubosia* hybrid hairy roots produced amounts of tropane alkaloids similar to controls.[197,202] These results seem to indicate the presence of different or additional points of control in the tropane alkaloid metabolic pathway.

Further down in the pathway, another known enzyme is MPO (*N*-methylputrescine oxidase), which catalyzes the formation of *N*-methylpyrrolinium cation, an intermediate of tropane alkaloids as well as nicotine (**Figure 5**). The oxidative deamination of *N*-methylputrescine by MPO gives the corresponding amino aldehyde (4-methyl aminobutanal) that has the potential to be transformed spontaneously into a cyclic imine via Schiff base formation, given the *N*-methyl-Δ^1-pyrrolinium cation.[137] This enzyme has been isolated from *H. niger* and its molecular weight determined by gel filtration (135 kDa); SDS–PAGE analysis showed that the enzyme is a dimer.[203] MPO has also been studied in other plant species such as *D. stramonium*[204] and *N. tabacum*.[205] Concerning tropane alkaloid biosynthesis, there was no correlation between alkaloid production and *H. niger* MPO activity.[203] Reports also showed that MPO activity was lost when tissue de-differentiation was induced with exogenously applied plant growth regulators, although MPO activity could be restored by subculturing the de-differentiated lines in a plant growth regulator-free medium,[193] but there are no records on the genetic manipulation of this enzyme, whose gene sequence has not been studied.

Two other enzymes of this pathway, TR-I and TR-II, have been identified and their corresponding gene sequences are known. These enzymes carry out stereospecifically the reduction of tropinone yielding tropine (3α-hydroxytropine) in the case of TR-I, subsequently leading to the formation of tropane alkaloids, and pseudotropine (3β-hydroxytropine) – the precursor of calystegines, a subgroup of the tropane alkaloid class[206] – in the case of TR-II (**Figure 5**). Both enzymes have been isolated from many Solanaceous species. For instance, TR-I and TR-II were isolated from *D. innoxia* roots and a crude extract favored the production of pseudotropine over tropine.[207] Also, from the transformed root cultures of *D. stramonium*, the two tropinone reductases were obtained, with TR-I showing fivefold higher activity than TR-II; TR-I displayed a pronounced pH dependency, while TR-II was more tolerant to different pH values.[208] Moreover, the characterization of these enzymes and the sequences of the two genes have been reported.[209–211]

The engineering of tropane alkaloids by modification of these two enzymes has not received much attention although two reports describe their manipulation. The overexpression of the *TRI* and *H6H* genes from *H. niger* in tobacco plants has been reported.[212] After feeding transgenic and control tobacco plants with the TR-I substrate tropinone, the reaction product tropine was detected only in leaves of transgenic plants, with no correlation with *trI* transcript level and tropine amounts. Surprisingly, transgenic tobacco plants accumulated 3–13-fold more nicotine than wild-type plants, together with the presence of considerable amounts of nor-nicotine, myosmine, anabasine, and anatabine, but at lower levels in wild-type plants. This indicates that the

overexpression of *TRI* and *H6H* perturbs the normal nicotine biosynthesis when foreign genes taken from a different metabolic pathway are introduced in tobacco.[212] In the second example, transgenic *A. belladonna* hairy roots overexpressing either *TRI* or *TRII* were established. TR-I-transformed root lines displayed higher tropine contents and reduced pseudotropine, resulting in a decrease of 30–90% of calystegines with respect to controls. Regarding hyoscyamine and scopolamine, the two end products of the pathway, hyoscyamine was significantly accumulated (threefold) and scopolamine was increased fivefold in TR-I-transformed roots, suggesting that either the activity of H6H enzyme was enhanced or more hyoscyamine was available for the H6H enzyme, which bioconverted it into scopolamine. Contrarily, the overexpression of *TRII* led to enhanced pseudotropine, which was metabolized into calystegines, which also appeared in high concentrations.[213]

The last two metabolic steps of the tropane alkaloid network are catalyzed by the same enzyme, that is, H6H (**Figure 5**), a bifunctional 2-oxoglutarate-dependent dioxygenase, which also requires for its activity molecular oxygen, ascorbate, and Fe^{2+}. Hyoscyamine is first hydroxylated generating 6β-hydroxyhyoscyamine, which is subsequently epoxidized to form the end product scopolamine, the 6,7-epoxide of *S*-hyoscyamine. Furthermore, H6H hydroxylases only the *l*-isomer of hyoscyamine, with the *d*-isomer being unaffected.[214,215] The hydroxylase activity of H6H has been reported to be 40 times stronger than its epoxidase activity, demonstrated by producing an active H6H enzyme as a fusion protein with a maltose binding protein in *E. coli*.[216]

The *H6H* gene has been successfully used in the genetic engineering of these metabolic steps for the enhancement of scopolamine in either hairy roots or plants. Thus, the first example of a successful metabolic modification of *h6h* in a Solanaceous plant was reported by Yun *et al.*[55] Hyoscyamine-rich *A. belladonna* plants were transformed with an *H6H* transgene from *H. niger* under the control of the cauliflower mosaic virus 35S promoter through *Agrobacterium*-mediated transformation. The resulting transgenic plants showed no differences in growth and development compared with controls. More importantly, the alkaloid profile of transgenic plants and their progenies was reverted, showing almost exclusively scopolamine in leaves and stems, unlike hyoscyamine, which was the major alkaloid in leaves, stems, and main roots (over 92%) of the wild-type and control plants.

In a similar fashion, transgenic hairy root cultures of several Solanaceous species overexpressing *H6H* have been established and showed a clear increase in scopolamine contents. Hairy root cultures of *A. belladonna* harboring the *H. niger H6H* gene confirmed by PCR analysis showed an increase in the amount of scopolamine as well as H6H enzyme activity compared with wild-type hairy roots.[217] Equally, the *H6H* gene from *H. niger* was also introduced into *H. muticus* via *A. rhizogenes* infection. The largest yield of scopolamine (17 mg l^{-1}) was over 100 times larger than the control, although hyoscyamine still remained as the major alkaloid. Expression analysis indicated that the enhancement in*H6H* expression was proportional to the increase in scopolamine, and was the main reason for the variation of the scopolamine/hyoscyamine ratio.[218] Likewise, the overexpression of *H. niger H6H* gene was carried out in *Duboisia* hybrid (*D. myoporoides* × *D. leichhardtii*) hairy roots and regenerated plants derived from them. The best hairy root line produced 74.50 mg l^{-1} scopolamine, a threefold increment compared with controls, which paralleled the increase in *h6h* transcript levels, confirming the direct relationship between the expression level of *H6H* and scopolamine contents. Regarding the regenerated plants, there was no clear scopolamine increase when compared with controls.[219] Another transformed tropane alkaloid-producing plant was *Scopolia parviflora*. Hyoscyamine and scopolamine accumulated at high concentrations in the transgenic hairy roots overexpressing *H6H*. The best transgenic line yielded 8.12 mg g dry wt.$^{-1}$ of scopolamine, representing a threefold increase compared with wild-type roots.[220]

Also non-hyoscyamine-producing species such as *N. tabacum* has been transformed by the insertion of the *H6H* gene from *H. niger* and subsequent establishment of hairy roots. Following the same approach, hairy roots of *H. muticus* were also established. Hyoscyamine was fed to these hairy roots; the transgenic tobacco hairy roots showed a more efficient uptake of hyoscyamine and a higher rate of bioconversion of hyoscyamine into scopolamine (40–45%) than those of *H. muticus*. In *N. tabacum* hairy roots, scopolamine was abundantly secreted; up to 85% of the produced scopolamine was detected in the culture medium. This fact could be explained because scopolamine appears as a foreign compound in *N. tabacum* cells and is therefore secreted to the medium to thus avoid a potential toxic effect in this non-tropane alkaloid-producing species.[221]

Recently, the *h6h* cDNA from *H. niger* was overexpressed in *A. baetica* hairy roots following *A. rhizogenes* infection.[222] The best clone yielded 5.6 mg g dry wt.$^{-1}$ of scopolamine, some part of which was released into the

liquid medium. The scopolamine production was ninefold larger than *A. baetica* intact plants. Furthermore, a unique and important feature of *A. baetica* transgenic hairy root cultures was that hyoscyamine was almost totally converted into scopolamine, unlike other published reports of *h6h*-overexpressing transgenic hairy roots where hyoscyamine was still the major alkaloid or larger amounts of hyoscyamine remained unconverted. Furthermore, in *A. baetica*, a positive correlation between scopolamine increase and *h6h* gene expression enhancement was also established. Elicitation of this transgenic *A. baetica* hairy roots using either methyl jasmonate or acetylsalicylic acid resulted in a conspicuous increase in scopolamine. The best results represented a 15.2-fold (9.5 mg g dry wt.$^{-1}$) and 11.6-fold (7 mg g dry wt.$^{-1}$) increase, respectively compared with intact plants. At the molecular level, not only *h6h*, which was overexpressed, but other two genes of the pathway, *pmt* and *tri*, appeared at higher levels following elicitation.[223]

In another account, two genes were separately expressed in *Duboisia leichhardtii* hairy roots using the *A. rhizogenes* infection system. The *h6h* gene of plant origin employed to boost scopolamine production and *hchl* (4-hydroxycinnamoyl-CoA hydratase/lyase) gene of bacterial origin employed to reduce lignin levels were introduced independently. Although no expression of HCHL was detected in any clone, expression of the exogenous *H6H* gene was distinguished from the endogenous gene by detection of an amplified untranslated region of the *parAt* promoter employed. H6H- and HCHL-positive controls did not show differences in root morphology, although the alkaloid profiles differed between both clones. In HCHL-positive clones, hyoscyamine was always superior to scopolamine. Tropane alkaloids production was variable among the H6H clones, and the best H6H clone yielded a greater than 95% conversion rate from hyoscyamine to scopolamine, 38.2 mg l^{-1} scopolamine when fresh roots amounting to ca. 9 mg were cultured for 6 weeks.[224]

Genetic engineering by manipulating simultaneous genes can also be practiced in order to achieve larger production of a target compound. This approach was implemented with the tropane alkaloid pathway in order to increase scopolamine production. Accordingly, *H. niger h6h* and *tri* genes were jointly introduced into *N. tabacum* plants, a nonproducing tropane alkaloid species, using particle bombardment. Tropinone and hyoscyamine, the substrates of TR-I and H6H enzymes, respectively, were fed to the leaves of these transgenic plants. The TR-I product tropine was detected in transgenic plants after feeding with tropinone. The H6H reaction products 6β-hydroxyhyoscyamine and scopolamine were found only in hyoscyamine-fed leaves of transgenic plants. There was no correlation between *h6h* transcript levels and 6β-hydroxyhyoscyamine and scopolamine contents. It was also observed that the expression of these transgenes in tobacco plants altered the normal nicotine profile.[212]

Similarly, trying to further boost scopolamine contents, *h6h* and *pmt* genes were overexpressed in *H. niger* hairy roots. The best transgenic line produced 411.2 mg l^{-1} scopolamine, more than ninefold compared with controls and twofold larger when compared with *h6h* single-transgenic hairy root lines that produced 184.4 mg l^{-1} scopolamine.[198] These results suggest that the transgenic lines harboring both *pmt* and *h6h* genes forced the metabolic flux to accumulate much more scopolamine than those transgenic lines that overexpressed a single gene. Furthermore, *h6h* seems to be more important than *pmt* gene as described earlier where *pmt* overexpression in *H. niger* did not result in a significant scopolamine enhancement.[198]

3.18.4.3 Morphinan Alkaloid Pathway

Opium is obtained from the annual herb *Papaver somniferum* (*Papaveraceae*), which shows solitary flowers of white, pink, or dull red-purple color. Opium is the air-dried latex, obtained by transversally or longitudinally cutting the unripe capsules of the opium poppy, thus opening the latex tubes through which latex will exudate. Capsules are the site of alkaloid accumulation, and stem and roots are more likely the organs of alkaloid biosynthesis. However, for industrial production, the entire plant tops are harvested and dried, and then extracted for their alkaloid content. Furthermore, poppy straw accounts for most of the medicinal opium alkaloid production.[137] Synthesis of the opiate core structure is still uneconomical and therefore the opium poppy plant is the only source of these important medicinal compounds, and biotechnology has also been applied in order to understand the mechanism(s) of biosynthesis and to establish biological systems with increased metabolite yields, which might substitute the opium plant as the only source of benzylisoquinoline alkaloids.

Opium has been known and used for 4000 years or more as an analgesic, sleep inducer (narcotic), and for the treatment of coughs, and over 40 different alkaloids have been identified although at present mainly six are largely used in medicine (morphine, codeine, thebaine, papaverine, narceine, noscapine). Morphine, codeine,

and thebaine are most abundant in aerial organs (latex) and sanguinarine is the major alkaloid in roots, although substantial amounts of morphine also accumulate in this organ. Regarding pharmacological properties, morphine is a potent analgesic and narcotic, mostly indicated for relief of severe pain. On the other hand, codeine, the 3-*O*-methyl ether derivative of morphine obtained mainly by semisynthesis from morphine, is the most widely used of the opium alkaloids; it is used as a less potent analgesic and as antitussive for the treatment of cough. Thebaine does not show analgesic activity, but its main value is as substrate for the semisynthesis of other morphin type of drugs. Papaverine is structurally very different from the morphine alkaloids, with little or no analgesic or hypnotic properties but possesses spasmolytic and vasodilator activity and is used as a muscle relaxant. Sanguinarine is an antimicrobial agent.

The opium alkaloids, that is, morphine, codeine, and thebaine, belong to the benzylisoquinoline type of alkaloids, derived from the amino acid tyrosine, which is converted into both dopamine and 4-hydroxyphenylacetaldehyde, which are the precursors of norcoclaurine, the first benzylisoquinoline in the pathway leading to the various opium alkaloids (see **Figure 6**). Reticuline, present further downstream within the pathway, has been established as the precursor of these morphinan alkaloids, being also the central point leading to the biosynthesis of benzophenanthridine and protoberberine alkaloids, and (*S*)-reticuline conversion into its (*R*)-epimer is required to initiate the morphinan alkaloid biosynthetic route[225] (**Figure 6**). Although the enzymatic synthesis of morphine has been almost totally elucidated, the genes involved in the morphinan branch of the pathway have not been fully characterized. Nonetheless, nine genes have been characterized, permitting the genetic engineering of the biosynthetic network.

In order to study morphinan alkaloid production, the initial biotechnological attempts consisted of the establishment of *in vitro* cultures of *P. somniferum*. Soon it was realized, as proven by some early work, that cytodifferentiation was crucial for morphinan alkaloid.[226] Moreover, this prerequisite of cytodifferentiation was further proved in cultured cells of *P. somniferum* by the induction of differentiation of meristemoids producing high frequency of buds and shoots, recovering the ability to biosynthesize morphinan alkaloids that was lost in the undifferentiated cultures. Tissue that differentiated only tracheary elements produced morphinan alkaloids, with codeine as the main component.[227] Moreover, in another account, when embryogenesis and rhizogenesis were induced by the right balance of auxins and cytokinins in opium poppy cultures, morphinan alkaloid production was recuperated. Codeine, thebaine, and papaverine accumulated in the roots, whereas morphine was detected only in aerial parts. Moreover, codeine and thebaine were detected only in the rhizogenous but not in embryonic callus, which suggests that root organogenesis is casually related to alkaloid biosynthesis.[228]

A recent report demonstrated the implication of three cell types in opium poppy alkaloid biosynthesis. These compounds accumulate in the cytoplasm or latex of specialized laticifers, which accompany vascular tissues throughout the plant. It was shown that three key enzymes, (*S*)-*N*-methylcoclaurine-3′-hydroxylase (CYP80B1), BBE, and COR, are restricted to the parietal region of sieve elements adjacent or proximal to laticifers. The results demonstrate that the biosynthesis and accumulation of alkaloids in opium poppy involves cell types not implicated previously in plant secondary metabolism, and dramatically extend the function of sieve elements beyond the transport of solutes and information molecules within plants. Thus these results indicate the requirement of cell differentiation and transport for morphinan alkaloid production to take place.[229,230] Similarly, in meadow rue (*Thalictrum flavum* ssp *glaucum*), cell type-specific localization of transcripts encoding nine consecutive enzymes involved in protoberberine alkaloid biosynthesis that catalyze the conversion of L-dopa to (*S*)-canadine was determined. It was reported that the predictive proteins showed extensive sequence identity with corresponding enzymes involved in the biosynthesis of related benzylisoquinoline alkaloids such as those of opium poppy. RNA gel blot hybridization analysis showed that gene transcripts for each enzyme were most abundant in rhizomes but lower levels were also detected in roots and other organs. In rhizomes, gene transcripts encoding all nine enzymes were restricted to the protoderm of leaf primordia. Nonetheless, in roots, gene transcripts of these nine enzymes were localized to immature endodermis, pericycle and, in some cases, adjacent cortical cells. These results showed that cell type-specific localization of protoberberine alkaloid biosynthesis and accumulation are temporally and spatially separated in *T. flavum* rhizomes and roots. Furthermore, despite the close phylogeny between corresponding biosynthetic enzymes, distinct and different cell types are involved in the biosynthesis and accumulation of benzylisoquinoline alkaloids in *T. flavum* and *P. somniferum*, suggesting that the evolution of alkaloid metabolism involves not only the recruitment of new biosynthetic enzymes, but also changing the expression into other cell types.[231]

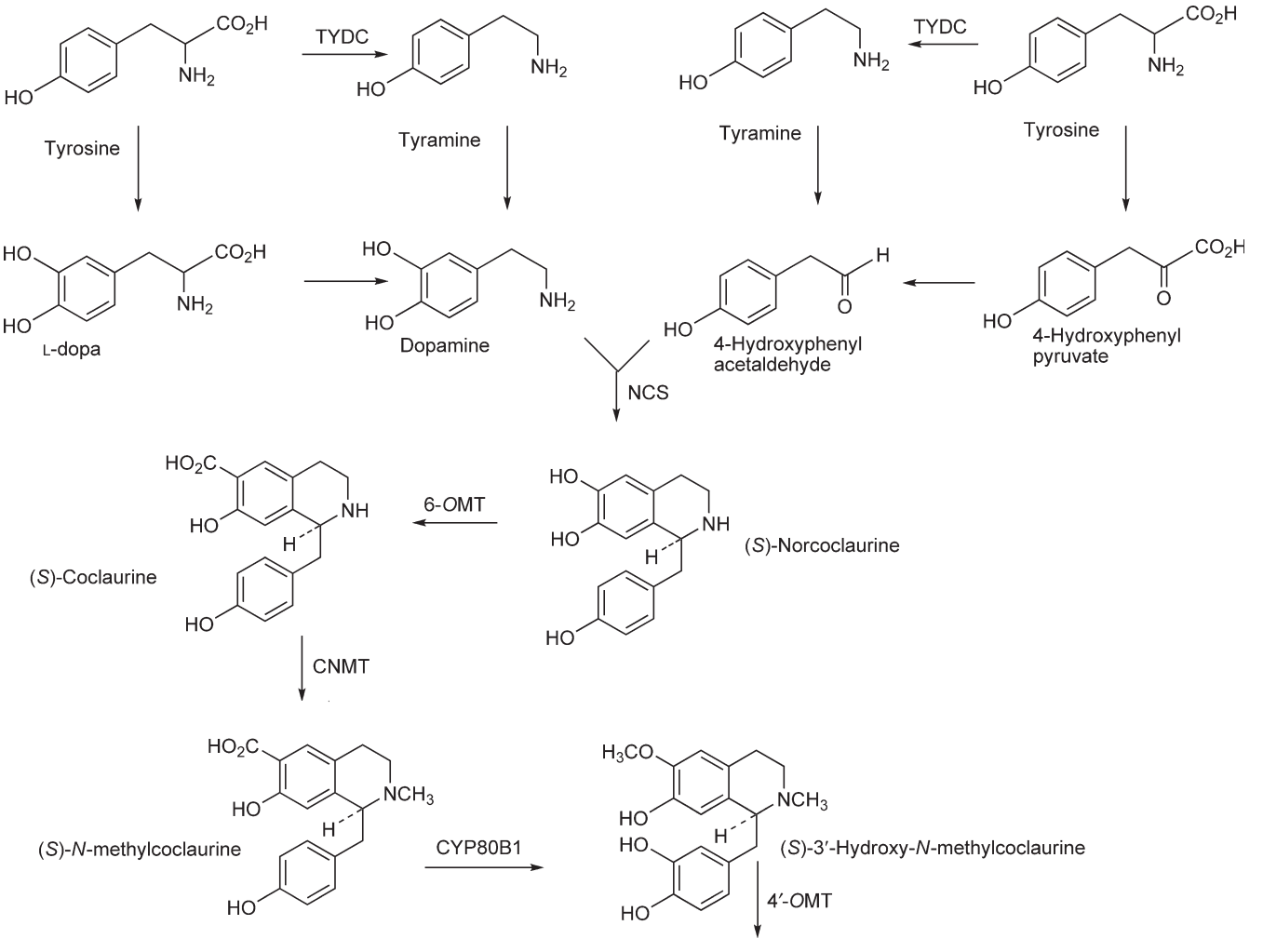


Figure 6 (Continued)

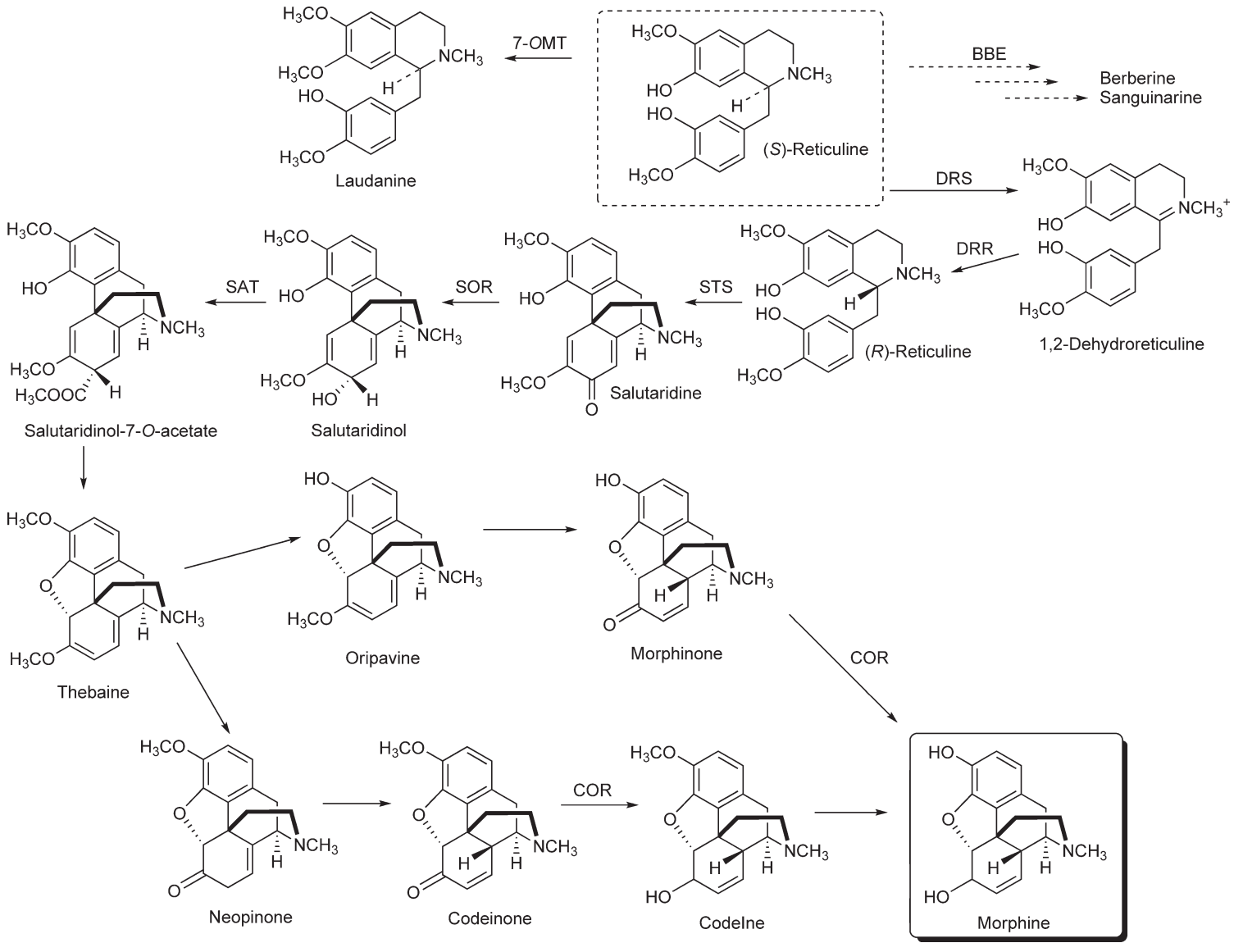


Figure 6 Scheme of the biosynthetic pathway of the morphinan alkaloids indicating the known enzymes participating in the network. TYDC: tryptophan decarboxylase; NCS: norcoclaurine synthase; 6-*O*MT: 6-*O*-methyltransferase; CNMT: coclaurine-*N*-methyltransferase; CYP80B1: (S)-*N*-methylcoclaurine 3′-hydroxylase; 4′-*O*MT: 4′-*O*-methyltransferase; 7-*O*MT: 7-*O*-methyltransferase; BBE: berberine bridge enzyme; DRS: 1,2-dehydroreticuline synthase; DRR: 1,2-dehydroreticuline reductase; STS: salutaridine synthase; SOR: salutaridine-NADPH 7-oxidoreductase; SAT: salutaridinol 7-*O*-acetyltransferase; COR: codeinone reductase.

Considering that cytodifferentiation and organogenesis are crucial elements for morphinan alkaloid biosynthesis, and that roots play a major role in the biosynthesis and accumulation of some of these metabolites, hairy root cultures have also been investigated. Both wild-type and transgenic hairy roots of *P. somniferum* and *E. californica* were established. Regarding morphinan alkaloids, *P. somniferum* hairy roots were able to produce noscapine and sanguinarine with lower amounts of morphine as determined by HPLC analysis.[232] In another account, in *A. rhizogenes*-mediated transformation of opium poppy hairy roots, the total alkaloid content was higher in the transformed roots (0.46% dry weight) than in the untransformed roots (0.32% dry weight). The transformed roots accumulated three times more codeine (0.18% dry weight) than intact roots (0.05% dry weight). Moreover, morphine (0.255% dry weight) and sanguinarine (0.014% dry weight) were found but only in the liquid culture medium.[233]

With the knowledge gained on the understanding of this interesting metabolic network, together with the identification of several genes encoding various enzymes involved in the pathway, attempts to engineer and redesign the benzylisoquinoline metabolic pathway have been performed by applying different approaches such as gene overexpression, gene silencing, or use of heterologous regulatory factors.

Morphinan alkaloid production has been enhanced by overexpressing COR in transgenic *P. somniferum* plants. This enzyme controls the penultimate step in morphine synthesis (**Figure 6**); opium poppy was transformed with constitutively expressed cDNA of COR (*PsCor1.1*). Significant increases in capsule alkaloid content in glasshouse and field trials over 4 years were recorded. The morphinan alkaloid contents on a dry weight basis were between 15 and 30% greater than those in control high-yielding genotypes and control nontransgenic segregants, representing a 22% increase in morphine, 58% increase in codeine, and 75% increase in thebaine. Increases in morphine and codeine were expected from an increase in COR; however, increases in thebaine were not expected given that this intermediate occurs prior to COR in the pathway (**Figure 6**). The analyses of tissues other than capsules (leaf, roots, pollen, and seed) indicated that there were no major changes in alkaloid types or amounts across tissues. Only codeine was significantly increased in the lower and upper stem, despite the fact that the introduced gene was driven by a constitutively expressing promoter. Moreover, transgenic leaves had approximately 10-fold greater levels of *Cor* transcript compared with nontransgenic controls.[234] In a similar fashion, the overexpression of the cytochrome P-450-dependent monooxygenase (*S*)-*N*-methylcoclaurine 3′-hydroxylase encoding gene (*CYP80B3*) has also been attempted in opium poppy. The transgenic plants displayed a 450% increase in the amount of total alkaloid in latex, and this boost occurred either without changing the ratio of the individual alkaloids, or together with an overall increase in the ratio of morphine.[235] In order to determine that the altered alkaloid profile was due to the presence of this transgene, the authors transformed *P. somniferum* plants with an *antisense-cyp80b3* cDNA, which resulted in an overall reduction in the amount of total alkaloids, varying between 16 and 78% compared to wild-type latex. The salutaridinol 7-*O*-acetyltransferase (*SalAT*)-encoding gene has also been overexpressed in opium poppy.[236] The transgenic plants exhibited an increase in capsule morphine, codeine, and thebaine on a dry weight basis. Moreover, there was no correlation between the increased alkaloid content and leaf *SalAT* transcripts; nonetheless, this comparison was based on transcript levels in total leaf, whereas the alkaloids are being synthesized only in a small proportion of cells in leaf, stem, and capsule. In the same report, poppy plants were transformed with a gene construct designed to produce a hairpin RNA molecule and trigger RNAi-induced degradation of *SalAT* mRNA. In these silenced plants, *SalAT* transcripts were reduced (12% compared with control), but not eliminated, resulting in a novel accumulation of the alkaloid salutaradine, 23% of total alkaloid, which was not detected in the parental genotype.[236]

Downregulation of genes of the morphinan alkaloid biosynthetic route has also been performed either applying the antisense or the RNAi technology. Thus, the BBE cDNA (*bbe*) from *P. somniferum* (**Figure 6**) was transformed in antisense orientation into seedling explants of an industrial elite line from which transgenic plants were obtained, and whether this downregulation would result in reduced or blocked benzophenanthridine alkaloid biosynthesis in roots of transgenic plants was studied. The resulting transgenic plants displayed an altered alkaloid profile in latex but not in roots. Several pathway intermediates from all biosynthetic branches, for example, reticuline, laudanine, laudanosine, dehydroreticuline, salutaridine, and (*S*)-scoulerine, were increased compared with controls. Moreover, the major alkaloids in the latex of transgenic plants were morphine, codeine, and thebaine, with oripavine being drastically reduced.[237] Knockdown of *BBE* by RNAi has been conducted in cultured cells of the related species of the Californian poppy (*E. californica*) with the aim

to accumulate the important key intermediate reticuline.[238] Both *bbe* mRNA accumulation and enzyme activity were effectively suppressed in transgenic cells. In these transgenic cells, the end products of isoquinoline alkaloid biosynthesis, such as sanguinarine, were considerably reduced and reticuline was accumulated at a maximum level (310 μg g fresh wt.$^{-1}$). In addition, cultured transgenic cells also secreted significant amounts of reticuline into the medium, with a maximum level of 300 mg l^{-1} culture medium. These cells also produced a methylated derivative of reticuline, laudanine, which could hardly be detected in control cells.[238]

The genetic engineering of the opium poppy whereby morphine was replaced by the non-narcotic alkaloid reticuline by RNAi has been reported.[64,239] Silencing of the COR (*COR*) in opium poppy plants was achieved employing a chimeric hairpin RNA construct designed to silence all members of the multigene *COR* family. The precursor alkaloid (*S*)-reticuline, seven enzymatic steps upstream of codienone, accumulated at the expense of morphine, codeine, oripavine, and thebaine. (*S*)-Reticuline is a potential substrate for the synthesis of various bioactive compounds (antimalarial or anticancer) but its availability is limited, though not in these silenced poppy plants, which show (*S*)-reticuline as the major metabolite. The eight enzymes long branch leading to morphine can be downregulated in response to the loss of the penultimate enzyme, COR. This represents the most dramatic example of gene silencing-induced feedback in secondary metabolism ever reported.[64] These authors suggested three possible processes as responsible for the results observed, even though the full chain of events was still unelucidated: (1) the build-up of COR substrates – codeinone and neopinone – might switch negative feedback on earlier enzyme or transport step(s); (2) or might inhibit transcription of those genes and (3) loss of COR enzyme from a larger enzyme complex might disable other enzyme reactions associated with this complex. The initial expectation of this research was the accumulation of thebaine and oripavine following silencing of COR. However, the unexpected accumulation of (*S*)-reticuline demonstrates that the morphinan alkaloid pathway can be coordinately regulated independent of the benzylisoquinoline pathway.

In a previous subheading, the importance and effectiveness of the use of regulatory or transcription factors as a tool to engineer biosynthetic routes were highlighted. Furthermore, bearing in mind the complexity of the morphinan alkaloid biosynthetic pathway, where many factors control the many metabolic steps, together with the participation of specific transporters, as well as the crucial involvement of specific tissues and cell types, the opium pathway has been engineered by transactivation using heterologous regulatory factors in an attempt to effectively and concomitantly affect the expression of several genes participating in this pathway aiming at attaining higher alkaloid yields.

Accordingly, genes encoding regulatory factors isolated from *Arabidopsis*, soybean, and corn have been screened to identify those that modulate the expression of genes encoding for enzymes involved in the biosynthesis of morphinan alkaloids in opium poppy, and ultimately being capable of increasing the production of morphinan alkaloids downstream from reticuline in the opiate branch of the benzylisoquinoline pathway.[240] This resulted in enhanced levels of *PsCOR* (COR), *Ps4′OMT* (*S*-adenosyl-L-methionine:3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase), and *Ps6OMT* ((*R*-*S*)-norcoclaurine 6-*O*-methyltransferase) transcripts by 10-fold to more than 100-fold in transgenic *P. somniferum* callus. Nonetheless, despite the transactivation of these pathway genes, no morphinan alkaloids were detected in the opium poppy callus cells. This was not surprising given previous reports that *in vitro* cultured undifferentiated poppy cells do not produce morphinan alkaloids. Thus, regenerated plants were induced from the established calli, and alkaloid analysis of leaves showed no increase in codeine and morphine, but significant enhancement of thebaine. Furthermore, the levels of the morphinan alkaloids were also analyzed in the senesced capsules taken from opium poppy lines transgenic to other regulatory factors, establishing that most of the transgenic plants demonstrated significant increases in codeine, morphine, and/or thebaine levels. Therefore, the utility of several heterologous regulatory factors in enhancing alkaloid accumulation in opium poppy was demonstrated.[240]

Mutant opium poppy plants have also been achieved by chemical mutagenesis generating the *top1* mutant. This mutant was unable to accumulate morphine and codeine, but accumulated thebaine and oripavine.[241] The alkaloid phenotype resulted from the mutation of a single genetic locus, and the only visible phenotypic change in the *top1* mutant was its latex appearing yellowish-orange as compared with the white color of wild-type plants. It was confirmed that there was a block in both arms of the bifurcated pathway at thebaine and oripavine (**Figure 6**), most probably due to a defect in the enzyme thebaine demethylase, which might be responsible for the demethylation of both compounds, although this has not been proved. Moreover, microarray

analysis revealed that 10 genes were significantly underexpressed in *top1*. These include a component of the signal-recognition particle that mediates protein trafficking, a flippase ATP-dependent transmembrane transporter, and a homologue to ftsH protein, a transmembrane ATP-dependent metalloprotease. Three other clones encoded proteins with similarity to known enzymes: phosphoenolpyruvate carboxykinase, aspartate aminotransferase, and aldose 1-epimerase. This mutation generates changes in gene expression, proteins, and secondary metabolites, and a few possible explanations were given: the gene encoding 6-O-demethylation likely responsible for the two substrates thebaine and oripavine was affected, or a gene that regulates its function or expression was affected; alterations in a structural or transport component preventing the arrival of these two substrates to the right cell compartment where O-demethylation takes place. This mutant offers an agricultural potential for the supply of thebaine and oripavine by a morphine-free plant, thus reducing possible illicit production offered by morphine-producing varieties.

3.18.5 Influences of Omics Technologies on Metabolic Engineering of Plants

One cannot rule out the powerful applications of the new -omics techniques in plant metabolic engineering and how these would further assist in the genetic manipulation approach, also helping to elaborate the finest engineering design, in order to attain the most favorable results. Several omics techniques are considered here, that is, genomics, proteomics, and metabolomics, providing a brief description, followed by examples of their application for the metabolic engineering of biosynthetic networks and how these have aided the progress achieved.

Genomics is the comprehensive analysis of the genetic content of an organism, also often refers to genome-wide studies of mRNA expression (transcriptomics).[242] Genomics has rapidly developed by the parallel advances in gene sequencing technologies such as high-throughput DNA sequencers. Furthermore, sequencing efforts are also being applied to identify ESTs, that is, single sequence reads on randomly selected cDNA clones, more easily generated because these are just gene fragments and not entire gene sequences. Nonetheless, ESTs provide a picture of the mRNA sequences expressed at a particular time and event, but intron and regulatory DNA sequences cannot be resolved using this methodology. Despite these minor drawbacks, the sequencing of ESTs is increasing at an impressive pace, and these are being used to monitor gene expression profiles.[243] Moreover, an extension of genomics has been the development of transcriptomics, which provides the gene expression patterns, revealing the identity and level of expression (mRNA) of each expressed gene in a particular sample.[244] The DNA microarray technique is commonly employed to assess gene expression profiles with the drawback that this technique needs and analyzes previously identified genes compiled in a microchip. However, with the advances made on ESTs and the larger availability of small gene fragments, another technique of choice to determine the transcriptome of a particular sample is SAGE (serial analysis of gene expression). This technology has the advantage of revealing absolute gene expression values and is not limited to previously identified cDNAs, as it occurs with the DNA microarray technique.[245] Moreover, global transcriptome analysis is a powerful tool that can be used to study regulation of secondary metabolite biosynthetic networks determining the level of expression of the genes involved.

Subsequent to the development of genomics and transcriptomics, with the knowledge gained of many genes or even of the entire genome of an organism, and their mRNA levels, the next needed step was the understanding of the resulting products, that is, proteins, following gene expression. Thus, proteomics, the study of the proteome, the compilation of all proteins expressed from the genome in all isoforms, polymorphisms, and post-translational modifications, commenced and constituted a powerful tool. This is rapidly developing, and has largely been driven by technological development of the highly sophisticated and expensive equipment needed (2D-electrophoresis, HPLC–MS, MS, etc.),[246] with the goal of a rapid and quantitative characterization of proteins, appearing in a particular biological scenario. The application of this technology in the study and development of plant secondary metabolism biosynthesis has been reviewed.[247]

Metabolites are molecules or end products derived from cellular regulatory processes (biosynthetic networks); their levels can be considered as the ultimate response of biological systems to genetic or environmental changes, providing a comprehensive insight into the end results of a particular biological condition. Thus, the

collection of metabolites synthesized by a biological system constitutes its 'metabolome.' Metabolomics provides the simultaneous identification and quantification of plant metabolomes, and as mentioned above for the other omics technologies also employing highly sophisticated and precise state-of-the-art extraction and analytical equipment. Until recently, most analyses granted the profile of selected classes of compounds, or to fingerprint metabolic changes without sufficient analytical resolution to determine metabolite levels and identities individually. Nevertheless, the current advances provide stronger tools to recognize the identities of almost all metabolites. Furthermore, for metabolomic analysis, special attention should be given to the methods employed for tissue extraction, sample preparation, data acquisition, and data mining.[248] It has been established that the challenge of metabolomics is to find changes in the metabolic networks that are functionally correlated with the physiological and developmental phenotype of a cell, tissue, or organism. This knowledge guards huge potential applications such as for the redesign of plant secondary metabolism for the production of particular target molecules.

Omics analyses require parallel development of bioinformatics tools, which allow tackling and handling the immense amount of raw data gained. The bioinformatics tools comprise first the creation of extensive and powerful databases such as those already available – protein sequence, nucleic acid sequence, and EST databases – followed by the development of potent software packages for the identification of genes and/or proteins and comparison of expression profiles, gene sequences, etc. This is of immense interest and large progress has been made.[249,250]

The exploration of the extraordinary complexity of the plant biochemical machinery is being conducted with the implementation of all these technologies, which has permitted to identify key enzymes and their encoding genes, which could then be amenable to manipulation for the controlled production of target metabolites or for improving crop plants. Thus, a proteomics approach was applied to comparatively study *Cannabis sativa* plant tissues to identify specific tissue-expressed proteins involved in the biosynthesis of cannabinoids.[251] Leaves, flowers, and glands, which possess different cannabinoid levels, were evaluated, reporting a clear different protein profile with little correlation among the proteins when comparing leaf and flower samples, with most of the proteins involved in primary metabolism. Flower and gland proteomes showed that less than half of the proteins expressed in flowers were also expressed in glands. Nevertheless, none of the identified proteins, particularly those from the glands, where higher cannabinoid amounts accumulate, were involved in cannabinoid biosynthesis, suggesting that the majority of detected proteins belonged to primary metabolism. The failure to identify cannabinoid biosynthetic enzymes, in particular an expected polyketide synthase, might be due to the low levels at which this enzyme is expressed, preventing its detection, and most likely due to it being overlapped by a much more abundant primary metabolism protein. The effect of jasmonic acid treatment of rice seedlings was also monitored by proteomics. It revealed 66 and 68 differentially expressed protein spots in shoot and root, respectively, compared with controls. MS analysis identified 52 in shoots and 56 in roots nonredundant proteins, belonging to 10 functional categories. Proteins involved in photosynthesis (44%), cellular respiration (11%), and protein modification and chaperone activity (11%) were highly represented in the shoot, whereas proteins related to antioxidant system (18%), cellular respiration (17%), and defense (15%) were highly represented in the root. Furthermore, transcriptomics analysis identified 107 and 325 induced genes and 34 and 213 suppressed genes in the shoot and root, respectively. Most genes encode for proteins involved in secondary metabolism, energy production, protein modification and chaperone, transporters, and cytochrome P-450.[252] Likewise, in *C. roseus*, a proteomic approach was undertaken aiming at the identification of novel proteins involved in the TIA biosynthesis employing a cell suspension culture able to accumulate strictosidine, ajmalicine, and vindolinine. After day 3, there was an increasing number of protein spots, but on day 13 it changed back to a similar profile as observed at the start of the experiment. Out of 88 proteins, 58 were identified including two isoforms of strictosidine synthase, which catalyzes the formation of strictosidine in the alkaloid biosynthesis; tryptophan synthase needed for the supply of the alkaloid precursor tryptamine; 12-oxophytodienoate reductase, which is indirectly involved in alkaloid biosynthesis as it catalyzes the last step in the biosynthesis of the regulator jasmonic acid. Unique sequences were also found, which may relate to unidentified biosynthetic proteins.[253]

It is known that following attacks by a large selection of herbivores, plants respond by substantial changes in their gene activity and expression of genes involved in plant defense signaling and secondary metabolism. *Arabidopsis thaliana* was employed to evaluate the effect of herbivore attack on its transcriptome.[254] The leaf

transcriptome was monitored after larval attack using a 70-mer oligonucleotide microarray covering 26 090 gene-specific elements. It was reported that almost 3000 array elements were differentially expressed, with half of them showing a twofold increase at two different sampling times. Many of the induced genes belonged to stress response, secondary metabolism, and signaling pathways. It was concluded that *Arabidopsis* responded to larval attack by reprogramming its transcriptome, and also groups of transcription factors that can be switched on by multiple forms of biotic and abiotic stress were identified. In another instance, glycosylation, which plays a major role in the chemical diversity of flavonoids, was studied in *A. thaliana* mutant plants. The application of a transcriptome co-expression analysis combined with a reverse genetics approach allowed to identify a gene belonging to the large gene family of 1 glycosyltransferase (*UGT*), which is important in determining the flavonoid composition of *Arabidopsis*. Thus, a flavonol 7-*O*-rhamnosyltransferase UGT89C1 was determined to be involved in the accumulation of C-7 rhamnosylated flavonols in *Arabidopsis* organs, consistent with the abundance of *UGT89C1* transcripts in floral buds. These results demonstrated that the integration of transcriptome co-expression analysis together with a reverse genetic approach is a versatile tool for understanding a multigene family involved in a metabolic pathway in *Arabidopsis*.[255]

Furthermore, a functional genomic approach was taken by combining targeted metabolite analysis with cDNA-amplified fragment length polymorphism (cDNA-AFLP)-based transcript profiling of jasmonate-elicited tobacco cells.[256] The major advantage of this technique is that no pre-existing gene sequence databanks are needed, and it can also discriminate between isoforms that often play distinct roles in primary and secondary metabolism. Thus, a transcriptome of nearly 600 jasmonate-modulated genes was composed and compared with the obtained jasmonate-induced shifts in tobacco metabolites. The gene inventory revealed the presence of all, except one, of the genes known to be involved in nicotine biosynthesis. Moreover, the transcriptome also revealed numerous jasmonate-induced genes involved in signal transduction, such as transcription factors, GTP-binding proteins, receptors, kinases, and phosphatases. It was determined that most of the represented families of transcription factors was the AP2-family, and their upregulation occurred before the upregulation of nicotine biosynthetic genes, indicating the potential of AP2 factors as activators of tobacco secondary metabolism. Similarly, the same approach (cDNA-AFLP) was applied to the plant species *C. roseus*,[257] which produces anticancer agents. A genomewide transcript profiling by cDNA-AFLP combined with metabolic profiling of elicited *C. roseus* cell cultures generated a collection of known and previously undescribed transcript tags and metabolites associated with the TIA pathway. It was possible to isolate 417 differentially expressed transcript tags as well as to identify 178 metabolites. Using the cDNA-AFLP technique, it was possible to monitor in one single experiment all but two of the known genes involved in TIA biosynthesis that were differentially expressed. Moreover, tags corresponding to genes encoding enzymes involved in the cytosolic mevalonate pathway or transcription factors were also located, as well as tags involved in other metabolic networks such as *S*-adensyl methionine and phenolic compound synthesis. These authors also found that all the known TIA genes visualized by cDNA-AFLP were induced by feeding methyl jasmonate to the cultured cells. The constructed correlation networks in the *C. roseus* report permitted to identify those genes most likely to be involved in TIA metabolism; thus, several of the large number of CYP450 enzymes present in *Catharanthus* were picked up such as the tabersonine 16 hydroxylase (T16H), as well as several AP2-domain transcription factors (ORCA, CRG358, CRG144), proving that this comprehensive profiling approach offers high potential for gene discovery to dissect secondary metabolism in nonmodel plant systems.[257]

As mentioned above, one of the aims of metabolomics analysis is to provide a clear picture of the whole metabolic state of the plant in a particular scenario. Accordingly, proton nuclear magnetic resonance (^{1}H-NMR) metabolomics was utilized to investigate the interplay between primary and secondary metabolism in cultured opium poppy cells treated with a fungal elicitor.[258] Metabolite fingerprinting and compound-specific profiling showed the extensive reprogramming of primary metabolic pathways in association with the induction of alkaloid biosynthesis in response to elicitor treatment. Detectable and dynamic changes in the metabolome of elicitor-treated cells, especially in cellular pools of carbohydrates, organic acids, and nonprotein amino acids, were detected after elicitor treatment. Furthermore, specific flux modulations were detected throughout primary metabolism, including glycolysis, the tricarboxylic acid cycle, nitrogen assimilation, phospholipid/fatty acid synthesis, and the shikimate pathway, all of which generate secondary metabolic precursors. There are also records on the differential mechanistic and elicitor-specific (yeast elicitor or methyl jasmonate) responses in phenylpropanoid and isoflavonoid biosynthesis in *Medicago truncatula* cell cultures.[259]

Metabolomics revealed novel pathways and differential mechanistic responses in these two pathways. Three phases of intracellular response to yeast elicitor were established: (1) a transient response mainly in (iso)-flavonoid metabolites such as formononetin and biochanin-A that peaked at 12–18 h following elicitation and then declined; (2) a sustained response through 48 h for compounds such as medicarpin and daidzin; and (3) a lesser delayed and protracted response starting at 24 h post-elicitation, for example, genistein diglucoside. In contrast, the response to methyl jasmonate differed significantly from that to yeast elicitor. Both elicitors showed the accumulation of the phytoalexin medicarpin, but coordinated increases in isoflavonoid precursors were observed only for yeast elicitor- and not methyl jasmonate-treated cells. Conversely, methyl jasmonate treatment resulted in a correlated decline in isoflavone glucosides. Three novel methylated isoflavones, 7-hydroxy-6,4′-dimethoxyisoflavone (afrormosin), 6-hydroxy-7,4′-dimethoxyisoflavone (alfalone), and 5,7-dihydroxy-4′,6-dimethoxy isoflavone (irisolidone), were induced by yeast elicitor, the first two derived from formononetin. The results highlighted the metabolic flexibility within the isoflavonoid pathway, suggesting also new pathways for complex isoflavonoid metabolism, and indicate differential mechanisms for medicarpin biosynthesis depending on the nature of elicitation. In another instance, to fully understand the effects of overexpressing an enzyme of the artemisinin biosynthesis, metabolomics was employed for determining the metabolic fingerprinting of *A. annua* transgenic plants overexpressing FPS compared with controls at different developmental stages, representing one of the few examples where this type of assessment is conducted for a full understanding of the modification of a particular gene within a pathway.[260] Chiefly, artemisinin and its biosynthetic precursors, artemisinic acid, dihydroartemisinic acid, and arteannuin B, were assessed. The highest concentration of artemisinin was revealed at the pre-flower budding stage (stage 3) for both transgenic and controls plants; moreover, it was reported that the overexpression of FPS increases artemisinic acid, dihydroartemisinic acid, and more importantly arteannuin B, but no artemisinin, suggesting the existence of a possible bottleneck in the conversion from artemisinic acid or dihydroartemisinic acid to artemisinin. Furthermore, the rapid increase of arteannuin B in the transgenic plants might suggest the presence of a rate-limiting step in the transformation of arteannuin B to artemisinin.

In an elegant report, metabolomics and transcriptomics were combined to investigate the effects of the genetic engineering of the tyrosine-derived cyanogenic glucoside dhurrin pathway in *A. thaliana*.[261] Plants expressing the entire biosynthetic pathway for dhurrin were accomplished by insertion of *CYP79A1*, CYP71E1, and *UGT85B1* genes from *Sorghum bicolor*. These accumulated 4% dry weight dhurrin with marginal inadvertent effects on plant morphology, free amino acid pools, transcriptome, and metabolome, demonstrating a positive outcome of the metabolic engineering strategy. However, insertion of the *CYP79A1* and *CYP71E1* genes resulted in undersized plants, transcriptome alterations, accumulation of numerous glucosides derived from detoxification of dhurrin pathway intermediates, together with the loss of the UV protectants sinapoyl glucose and sinapoyl malate, and kaempferol glucosides.

3.18.6 Future Directions

The engineering of medicinal plants for the production of valuable natural products has been attained.[262] However, the major barrier for the successful metabolic engineering of pathways is the limited knowledge of secondary metabolic pathways; in general, because of the fact that each pathway possesses its own enzyme machinery and genes encoding enzymes, a faster understanding of the numerous and different secondary metabolite biosynthetic pathways is difficult to achieve. Nonetheless, a larger number of biosynthetic networks are being elucidated and this is progressing steadily.[263] Besides, taking advantage of the various applications of genomic technologies, an impressive and increasing number of cDNAs encoding different biosynthetic enzymes have been identified, which is assisting the progress in metabolic engineering.

On the other hand, it should be considered that for positive metabolic engineering to be realized, natural product biosynthesis must be considered as a system of many interacting parts (promoters, transcription factors, enzymes, transporters, intracellular structures such as vesicles and membranes, etc.) all needing particular attention when attempting to successfully manipulate biosynthetic routes. Thus, the combined expertise gained from the areas of biology, chemistry, instrumentation, and bioinformatics is vital for attaining further success.

Moreover, little is known about the intra- and intercellular translocation of intermediates within a pathway and how specific transporters and/or the symplastic movement of metabolites influence the final outcome of a particular secondary metabolite yield. It has also been addressed here that the identification, employment, and exploitation of transcription factors, which have been demonstrated to be involved in the coordinated regulation of pathway enzymes and other metabolic components, are extremely useful tools to engineer metabolic networks and, therefore, further efforts should be made to increase the number of known transcription factors.

At present, plant metabolic engineering hitherto proceeds basically by trial and error when working with chosen genes, rather than by intelligent system design. Therefore, a comprehensive understanding of the different points of metabolic regulation such as those at the transcriptional, cellular, and biochemical levels, taking also into account all possible interconnections, is vital to achieve a rational and controlled engineering of secondary metabolism networks. Further efforts should be directed toward these points with the application of a number of tools and approaches: studying the diverse regulatory mechanisms governing gene expression and transcriptional regulators; identifying and characterizing the different transport mechanisms; and looking at the different postbiosynthetic events and mechanism involved.

More recently, new approaches are being applied to further unravel plant secondary metabolism at all levels, from genes to metabolites and from genome via transcriptome and proteome to the metabolome. Besides, functional genomics,[256] developed to quantitatively evaluate the spatial and temporal accumulation of specific mRNAs, proteins, and metabolites, is also being applied. Such an integrated approach involving all these technologies is what has been named as systems biology,[264] which can be defined as the study of the diverse mechanisms involved in complex biological processes as integrated systems of many components such as DNA, RNA, proteins, and cells.

With the implementation of all these new approaches, the knowledge of and possibility to control plant secondary metabolism will clearly increase in the near future, allowing tailoring many important secondary metabolite pathways aiming chiefly at achieving higher product yields. Success will be reached in the near future, and further control of metabolic pathways will also be achieved in the coming years, permitting the tailor-made design of the production of bioactive secondary metabolites to gain increased yields to finally satisfy the world demand for these important medicinal compounds.

Acknowledgments

The author acknowledges funding from Instituto Canario de Investigación del Cáncer (ICIC = Canary Islands Institute for Cancer Research), Tenerife, Spain.

References

1. R. F. Thorne, *Bot. Rev.* **1992**, *58*, 225–348.
2. V. H. Heywood; R. K. Brummitt; A. Culham; O. Seberg, *Flowering Plant Families of the World*, 2nd ed.; Firefly Books Ltd.: ON, Canada, 2007.
3. A. D. Kinghorn, M. F. Balandrin, Eds., *Human Medicinal Agents from Plants*; American Chemical Society (ACS Symposium Series, 534): Washington, 1993.
4. D. J. Newman; G. M. Cragg; K. M. Snader, *J. Nat. Prod.* **2003**, *66*, 1022–1037.
5. G. M. Cragg; D. G. I. Kingston; D. J. Newman, *Anticancer Agents from Natural Products*; CRC Press, Taylor & Francis Group: Boca Raton, FL, 2005.
6. M. Jacoby, *Chem. Eng. News* **2005**, *83* (25), 3.
7. I. Paterson; E. A. Anderson, *Science* **2005**, *310*, 451–453.
8. K. M. Oksman-Caldentey; D. Inzé, *Trends Plant Sci.* **2004**, *9*, 433–440.
9. M. Wink; A. W. Alfermann; R. Franke; B. Wetterauer; M. Distl; J. Windhövel; O. Krohn; E. Fuss; H. Garden; A. Mohagheghzadeh; E. Wildi; P. Ripplinger, *Plant Genet. Res.* **2005**, *3*, 90–100.
10. S. R. Rao; G. A. Ravishankar, *Biotechnol. Adv.* **2002**, *20*, 101–153.
11. R. van der Heijden; D. I. Jacobs; W. Snoeijer; D. Hallard; R. Verpoorte, *Curr. Med. Chem.* **2004**, *11*, 1241–1253.
12. R. Zárate; R. Verpoorte, *Phytochem. Rev.* **2007**, *6*, 475–491.
13. R. Hain; B. Bieseler; H. Kindl; G. Schroeder; R. Stocker, *Plant Mol. Biol.* **1990**, *15*, 325–335.
14. J. D. Lim; S. J. Yun; I. M. Chung; C. Y. Yu, *Mol. Breed.* **2005**, *16*, 219–233.
15. G. Schröder; J. W. Brown; J. Schöder, *Eur. J. Biochem.* **1988**, *172*, 161–169.

16. J. E. Bailey, *Science* **1991**, *252*, 1668–1675.
17. C. Khosla; J. D. Keasling, *Nat. Rev. Drug Discov.* **2003**, *2*, 1019–1024.
18. B. Wilkinson; J. Micklefield, *Nat. Chem. Biol.* **2007**, *3*, 379–386.
19. S. Wu; J. Chappell, *Curr. Opin. Biotechnol.* **2008**, *19*, 1–8.
20. D. Sprinzak; M. B. Elowitz, *Nature* **2005**, *438*, 443–448.
21. J. M. De Jong; Y. L. Liu; A. P. Bollon; R. M. Long; S. Jennewein; D. Williams; R. B. Croteau, *Biotechnol. Bioeng.* **2006**, *93*, 212–224.
22. M. C. Y. Chang; R. A. Eachus; W. Trieu; D. K. Ro; J. D. Keasling, *Nat. Chem. Biol.* **2007**, *3*, 274–277.
23. D. K. Ro; E. M. Paradise; M. Ouellet; K. J. Fisher; K. L. Newman; J. M. Ndungu; K. A. Ho; R. A. Eachus; T. S. Ham; J. Kirby; M. C. Chang; S. T. Withers; Y. Shiba; R. Sarpong; J. D. Keasling, *Nature* **2006**, *440*, 940–943.
24. O. Besumbes; S. Sauret-Güeto; M. A. Phillips; S. Imperial; M. Rodríguez-Concepción; A. Boronat, *Biotechnol. Bioeng.* **2004**, *88*, 168–175.
25. G. Wu; M. Truksa; N. Datla; P. Vrinten; J. Bauer; T. Zank; P. Cirpus; E. Heinz; X. Qiu, *Nat. Biotechnol.* **2005**, *23*, 1013–1017.
26. I. Potrykus, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1991**, *42*, 205–225.
27. G. Hansen; M. S. Wright, *Trends Plant Sci.* **1999**, *4*, 226–231.
28. A. Lorence; R. Verpoorte, Gene Transfer and Expression in Plants. In *Methods in Molecular Biology, Recombinant Gene Expression. Reviews and Protocols*; P. Balbás, A. Lorence, Eds.; Humana Press Inc: USA, 2004, Vol. 267; pp 329–350.
29. R. Zárate; M. M. Yeoman, Application of Recombinant DNA Technology to Studies on Plant Secondary Metabolism. In *From Soil to Cell – A Broad Approach to Plant Life*; L. Bender, A. Kumar, Eds.; Gießen Electronic Library: Germany, 2001, pp 82–96.
30. R. Zárate; N. El Jaber-Vazdekis; E. Cequier-Sánchez; F. Gutiérrez-Nicolás; A. G. Ravelo, Biotechnology for the Production of Plant Natural Products. In *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Elsevier Science Publishers: The Netherlands, 2008; Vol. 34, pp 309–392.
31. P. A. Lessard; H. Kulaveerasingam; G. M. York; A. Strong; A. J. Sinskey, *Metab. Eng.* **2002**, *4*, 67–79.
32. Y. Ishida; H. Saito; S. Ohta; Y. Hiei; T. Komari; T. Kumashiro, *Nat. Biotechnol.* **1996**, *14*, 745–750.
33. P. Bundock; A. den Dulk-Ras; A. Beijersbergen; P. J. J. Hooykaas, *EMBO J.* **1995**, *14*, 3206–3214.
34. C. B. Michielse; P. J. J. Hooykaas; C. A. M. J.J. Hondel; A. F. J. Ram, *Curr. Genet.* **2005**, *48*, 1–17.
35. T. Kunik; T. Tzfira; Y. Kapulnik; Y. Gafni; C. Dingwall; V. Citovsky, *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 1871–1876.
36. P. J. J. Hooykaas, In *Metabolic Engineering of Plant Secondary Metabolism*; R. Verpoorte, A. W. Alfermann, Eds.; Kluwer Academic Publishers: The Netherlands, 2000; pp 51–67.
37. M. D. Chilton; M. H. Drummond; D. J. Merlo; D. Sciaky; A. L. Montoya; M. P. Gordon; E. W. Nester, *Cell* **1977**, *11*, 263–271.
38. D. Tepfter, *Physiol. Plant.* **1990**, *79*, 140–146.
39. V. P. Sinkar; F. F. White; M. P. Gordon, *J. Biosci.* **1987**, *11*, 47–57.
40. L. S. Melchers; A. J. G. Regensburg-Tuink; R. A. Schilperoort; P. J. J. Hooykaas, *Mol. Microbiol.* **1989**, *3*, 969–977.
41. Y. Dessaux; A. Petit; J. Tempé, *Phytochemistry* **1993**, *34*, 31–38.
42. M. Zambre; N. Terryn; J. de Clercq; S. de Buck; W. Dillen; M. van Montagu; D. van der Straeten; G. Angenon, *Planta* **2003**, *216*, 580–586.
43. T. M. Klein; E. D. Wolf; R. Wu; J. C. Sanford, *Nature* **1987**, *327*, 70–73.
44. J. C. Sanford; T. M. Klein; E. D. Wolf; N. Allen, *Trends Biotechnol.* **1987**, *6*, 299–302.
45. J. C. Sanford, *Trends Biotechnol.* **1988**, *6*, 299–302.
46. M. Cordero-Mesa; S. Jiménez-Bermúdez; F. Pliego-Alfaro; M. A. Quesada; J. A. Mercado, *Aust. J. Plant Physiol.* **2000**, *27*, 1093–1110.
47. M. J. Leech; D. Burtin; D. Hallard; F. Hillou; B. Kemp; N. Palacios; P. Rocha; D. O'Callaghan; R. Verpoorte; P. Christou, Particle Gun Methodology as a Tool in Metabolic Engineering. In *Metabolic Engineering of Plant Secondary Metabolism*; R. Verpoorte, A. W. Alfermann, Eds.; Kluwer Academic Publishers: The Netherlands, 2000; pp 69–86.
48. M. Z. Hadi; M. D. McMullen; J. J. Finner, *Plant Cell Rep.* **1996**, *15*, 500–505.
49. F. D. Smith; P. R. Harpending; J. C. Sanford, *J. Gen. Microbiol.* **1992**, *138*, 239–248.
50. T. T. Wang; Y. J. Choi; B. H. Lee, *Crit. Rev. Biotechnol.* **2001**, *21*, 177–218.
51. J. O'Brien; S. C. R. Lummis, *Methods* **2004**, *33*, 121–125.
52. V. Olmedo-Monfil; C. Cortes-Penagos; A. Herrera-Estrella, *Meth. Mol. Biol.* (*Recombinant Gene Expression*, 2nd ed.) **2004**, *267*, 297–313.
53. K. M. Davies; S. J. Bloor; G. B. Spiller; S. C. Deroles, *Plant J.* **1998**, *13*, 259–266.
54. R. A. Dixon; C. L. Steele, *Trends Plant Sci.* **2002**, *4*, 394–400.
55. D. J. Yun; T. Hashimoto; Y. Yamada, *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11799–11803.
56. L. Zhang; R. Ding; Y. Chai; M. Bonfill; E. Moyano; K. M. Oksman-Caldentey; T. Xu; Y. Pi; Z. Wang; H. Zhang; G. Kai; Z. Liao; X. Sun; K. Tang, *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 6786–6791.
57. X. Ye; S. Al-Babili; A. Klöti; J. Zhang; P. Lucca; P. Beyer; I. Potrykus, *Science* **2000**, *287*, 303–305.
58. P. M. Waterhouse; C. A. Helliwell, *Nat. Rev. Genet.* **2004**, *4*, 29–38.
59. P. M. Waterhouse; M. Wang; E. J. Finnegan, *Trends Plant Sci.* **2001**, *6*, 297–301.
60. P. M. Waterhouse; M. Wang; T. Lough, *Nature* **2001**, *411*, 834–842.
61. D. Baulcombe, *Nature* **2004**, *431*, 356–363.
62. R. S. Lewis; A. M. Jack; J. W. Morris; V. J. M. Robert; L. B. Gavilano; B. Siminszky; L. P. Bush; A. J. Hayes; R. E. Dewey, *Plant Biotechnol. J.* **2008**, *6*, 346–354.
63. N. Fujii; T. Inui; K. Iwasa; T. Morishige; F. Sato, *Transgenic Res.* **2007**, *16*, 363–375.
64. R. S. Allen; A. G. Millgate; J. A. Chitty; T. Jenniffer; J. A. C. Miller; A. J. Fist; W. L. Gerlach; P. J. Larkin, *Nat. Biotechnol.* **2004**, *22*, 1559–1566.
65. H. W. Dodo; K. N. Konan; F. C. Chen; M. Egnin; O. M. Viquez, *Plant Biotechnol. J.* **2008**, *6*, 135–145.
66. J. Memelink; J. W. Kijne; R. van der Heijden; R. Verpoorte, Genetic Modification of Plant Secondary Metabolite Pathways Using Transcriptional Regulators. In *Plant Cells*; J. J. Zhong, Ed.; Advances in Biochemical Engineering Biotechnology 72; Springer: Berlin/Heidelberg, 2001; pp 106–128.
67. E. Grotewold, *Curr. Opin. Biotechnol.* **2008**, *19*, 138–144.

68. P. Broun, *Curr. Opin. Plant Biol.* **2004**, *7*, 202–209.
69. S. H. Shiu; M. C. Shiu; W. H. Li, *Plant Physiol.* **2005**, *139*, 18–26.
70. E. Grotewold, *Trends Plant Sci.* **2005**, *10*, 57–62.
71. H. K. Dooner; T. P. Robbins; R. A. Jorgensen, *Annu. Rev. Genet.* **1991**, *25*, 173–199.
72. P. Gantet; J. Memelink, *Trends Pharmacol. Sci.* **2002**, *23*, 563–569.
73. E. Grotewold, *Plant Cell* **1998**, *10*, 721–740.
74. W. Bruce; O. Folkerts; C. Garnaat; O. Crastra; B. Roth; B. Browen, *Plant Cell* **2000**, *12*, 65–80.
75. O. Yu; J. Shi; A. O. Hession; C. A. Maxwell; B. McGonigle; J. T. Odell, *Phytochemistry* **2003**, *63*, 753–763.
76. S. B. Sharma; R. A. Dixon, *Plant J.* **2005**, *44*, 62–75.
77. A. L. van Eenennaam; G. Li; M. Venkatramesh; C. Levering; X. Gong; A. C. Jamieson; E. J. Rebar; C. K. Shewmaker; C. C. Case, *Metab. Eng.* **2004**, *6*, 101–108.
78. L. van der Fits; J. Memelink, *Plant J.* **2001**, *25*, 43–53.
79. L. van der Fits; J. Memelink, *Science* **2000**, *289*, 295–297.
80. A. Aharoni; C. H. Ric De Vos; M. Wein; Z. Sun; R. Greco; A. Kroon; J. N. M. Mol; A. P. O'Connell, *Plant J.* **2001**, *28*, 319–332.
81. J. S. Park; J. B. Kim; K. J. Cho; C. I. Cheon; M. K. Sung; M. G. Choung; K. H. Roh, *Plant Cell Rep.* **2008**, *27*, 985–994.
82. A. J. Hamilton; S. Brown; Y. Han; M. Ishizuka; A. Lowe; A. G. Solis; D. Grierson, *Plant J.* **1998**, *15*, 737–746.
83. K. Yoshida; A. Shinmyo, *J. Biosci. Bioeng.* **2000**, *90*, 353–362.
84. M. Venter, *Trends Plant Sci.* **2007**, *12*, 118–124.
85. S. Bhullar; S. Chakravarthy; S. Advani; S. Datta; D. Pental; P. K. Burma, *Plant Physiol.* **2003**, *132*, 988–998.
86. S. Sawant; K. Kiran; R. Mehrotra; P. C. Chandra; S. Ansari; P. Singh; N. Lodhi; R. Tuli, *J. Exp. Bot.* **2005**, *56*, 2345–2353.
87. K. Hammer; I. Mijakovic; P. R. Jensen, *Trends Biotechnol.* **2006**, *24*, 53–55.
88. J. S. Keddie; M. Tsiantis; P. Piffanelli; R. Cella; P. Hatzopoulos; D. J. Murphy, *Plant Mol. Biol.* **1994**, *24*, 327–340.
89. N. D. Trinklein; S. F. Aldred; S. J. Hartman; D. I. Schroeder; R. P. Otillar; R. M. Myers, *Genome Res.* **2004**, *14*, 62–66.
90. M. Xie; Y. He; S. Gan, *Nat. Biotechnol.* **2001**, *19*, 677–679.
91. M. Ogawa; Y. Herai; N. Koizumi; T. Kusano; H. Sano, *J. Biol. Chem.* **2001**, *276*, 8213–8218.
92. M. Weid; J. Ziegler; A. M. Kutchan, *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 13957–13962.
93. B. St-Pierre; F. Vazquez-Flota; V. De Luca, *Plant Cell* **1999**, *11*, 887–900.
94. I. A. Graham; T. Larson; J. A. Napier, *Curr. Opin. Biotechnol.* **2007**, *18*, 142–147.
95. D. P. Dixon; I. Cummins; D. J. Cole; R. Edwards, *Curr. Opin. Plant Biol.* **1998**, *1*, 258–266.
96. R. Yelin; D. Rotem; S. Schuldiner, *J. Bacteriol.* **1999**, *181*, 949–956.
97. G. Liu; R. Sanchez-Fernandez; Z.-S. Li; P. A. Rea, *J. Biol. Chem.* **2001**, *276*, 8648–8656.
98. M. Putman; H. W. Veen; W. N. Konings, *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 672–693.
99. H. I. Zgurskaya; H. Nikaido, *Mol. Microbiol.* **2000**, *37*, 219–225.
100. K. Yazaki, *Curr. Opin. Plant Biol.* **2005**, *8*, 301–307.
101. E. Martinoia; M. Klein; M. Geisler; L. Bovet; C. Forestier; U. Kolukisaoglu; B. Müller-Röber; B. Schulz, *Planta* **2002**, *214*, 345–355.
102. A. C. Diener; R. A. Gaxiola; G. R. Fink, *Plant Cell* **2001**, *13*, 1625–1637.
103. L. Li; Z. He; G. K. Pandey; T. Tsuchiya; S. Luan, *J. Biol. Chem.* **2002**, *277*, 5360–5368.
104. I. Debeaujon; A. J. M. Peeters; K. M. Léon-Kloosterziel; M. Koornneef, *Plant Cell* **2001**, *13*, 853–871.
105. J. V. Magalhaes; J. Liu; C. T. Guimaraes; U. G. P. Lana; V. M. C. Alves; Y. H. Wang; R. E. Schaffert; O. A. Hoekenga; M. A. Piñeros; J. E. Shaff; P. E. Klein; N. P. Carneiro; C. M. Coelho; H. N. Trick; L. V. Kochian, *Nat. Genet.* **2007**, *39*, 1156–1161.
106. Arabidopsis Genome Initiative, *Nature* **2000**, *408*, 796–815.
107. M. Gottesmann; I. Pastan, *Ann. Rev. Biochem.* **1993**, *62*, 385–427.
108. Y. P. Lu; Z. S. Li; P. A. Rea, *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 8243–8248.
109. K. Sakai; N. Shitan; F. Sato; K. Ueda; K. Yazaki, *J. Exp. Bot.* **2002**, *53*, 1879–1886.
110. N. Shitan; I. Bazin; K. Dans; K. Obata; K. Kigawa; K. Ueda; F. Sato; C. Forestier; K. Yazaki, *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *100*, 751–756.
111. A. Mentewab; C. N. Stewart, Jr., *Nature* **2005**, *23*, 1177–1180.
112. P. J. Facchini; B. St-Pierre, *Curr. Opin. Plant Biol.* **2005**, *8*, 657–666.
113. D. A. Bird; V. R. Franceschi; P. J. Facchini, *Plant Cell* **2003**, *15*, 2626–2635.
114. S. U. Park; P. J. Facchini, *J. Exp. Bot.* **2000**, *51*, 1005–1016.
115. E. Korenromp; B. Nahlen; T. Wardlaw; M. Young, *World Malaria Report: World Health Organization (WHO), Roll Back Malaria (RBM) and United Nations Children's Fund (UNICEF).* Geneva, 2005.
116. J. F. S. Ferreira; J. C. Laughlin; N. Delabays; P. M. de Magalha, *Plant Genet. Res.* **2005**, *3*, 206–229.
117. M. A. Avery; W. K. M. Chong; C. Jennings-White, *J. Am. Chem. Soc.* **1992**, *114*, 974–979.
118. M. G. Constantino; M. Beltrame; G. V. J. da Silva, *Synth. Commun.* **1996**, *26*, 321–329.
119. X. C. He; M. Y. Zeng; G. F. Li; Z. Liang, *Acta Bot. Sin.* **1983**, *25*, 87–90.
120. M. Agarwal; K. Raka, *Adv. Plant Sci.* **2004**, *17*, 371–378.
121. R. Usha; P. M. Swamy, *Indian J. Plant Physiol.* **2002**, *7*, 159–162.
122. C. Z. Liu; C. Guo; Y. Wang; F. Ouyang, *World J. Microbiol. Biotechnol.* **2003**, *19*, 535–538.
123. P. K. Chen; C. Lukonis; L. Go; G. R. Leather, *Proc. – Plant Growth Regul. Soc. Am.* **1991**, *18*, 2–8.
124. N. B. Paniego; A. M. Giulietti, *Plant Cell Tissue Organ Cult.* **1994**, *36*, 163–168.
125. T. Sharma; V. K. Dixit, *Indian J. Pharm. Sci.* **2006**, *68*, 448–455.
126. D. P. Fulzele; A. T. Sipahimalani; M. R. Heble, *Phytother. Res.* **1991**, *5*, 149–153.
127. J. F. S. Ferreira; J. Janick, *Plant Cell Tissue Organ Cult.* **1996**, *44*, 211–217.
128. J. W. Wang; R. X. Tan, *Biotechnol. Lett.* **2002**, *14*, 1153–1156.
129. C. Z. Liu; Y. C. Wang; F. Ouyang; H. C. Ye; G. F. Li, *Biotechnol. Lett.* **1997**, *9*, 927–929.
130. W. Putalun; W. Luealon; W. De-Eknamkul; H. Tanaka; Y. Shoyama, *Biotechnol. Lett.* **2007**, *29*, 1143–1146.
131. J. W. Wang; Z. Zhang; R. X. Tin, *Biotechnol. Lett.* **2001**, *23*, 857–860.
132. J. W. Wang; F. X. Kong; R. X. Tan, *Biotechnol. Lett.* **2002**, *24*, 1573–1577.

133. C. Z. Liu; C. Guo; Y. C. Wang; F. Ouyang, *Process Biochem.* **2002**, *38*, 581–585.
134. Y. Wang; H. Zhang; B. Zhao; X. Yuan, *Biotechnol. Lett.* **2001**, *23*, 1971–1973.
135. Y. Kin; B. E. Wislouzyl; P. J. Weathers, *Plant Cell Rep.* **2001**, *20*, 451–455.
136. C. Z. Liu; C. Guo; Y. C. Wang; F. Ouyang, *Process Biochem.* **2002**, *39*, 45–49.
137. P. M. Dewick, *Medicinal Natural Products: A Biosynthetic Approach*;John Wiley & Sons Ltd. West Sussex, 2002.
138. C. Liu; Y. Zhao; Y. Wang, *Appl. Microbiol. Biotechnol.* **2006**, *72*, 11–20.
139. Q. Zeng; F. Qiu; L. Yuan, *Biotechnol. Lett.* **2008**, *30*, 581–592.
140. Y. Matsushita; W. Y. Kang; B. V. Charlwood, *Gene* **1996**, *172*, 207–209.
141. P. Mercke; M. Bengtsson; H. J. Bouwmeester; M. A. Brodelius, *Arch. Biochem. Biophys.* **2000**, *381*, 173–180.
142. T. E. Wallaart; H. J. Bouwmeester; J. Hille; L. Poppinga; N. C. A. Maijers, *Planta* **2001**, *212*, 460–465.
143. Y. Liu; H. C. Ye; H. Wang; G. F. Li, *Acta Bot. Sin.* **2003**, *45*, 608–613.
144. D. H. Chen; H. C. Yen; G. F. Li, *Acta Bot. Sin.* **1999**, *41*, 490–493.
145. H. Wang; L. Ge; H. C. Ye; K. Chong; B. Y. Liu; G. F. Li, *Planta Med.* **2004**, *70*, 347–352.
146. H. Wang; Y. Liu; K. Chong; B. Y. Liu; H. C. Ye; Z. Q. Li; F. Yan; G. F. Li, *Plant Biol.* **2007**, *9*, 442–449.
147. J. L. Han; B. Y. Liu; H. C. Ye; H. Wang; Z. Q. Li; G. F. Li, *J. Integr. Plant Biol.* **2006**, *48*, 482–487.
148. S. O. Duke; R. N. Paul, *Int. J. Plant Sci.* **1993**, *154*, 107–118.
149. G. Sa; M. Mi; Y. He-Chun; L. Ben-Ye; L. Guo-Feng; C. Kang, *Plant Sci.* **2001**, *160*, 691–698.
150. D. Chen; H. Ye; G. Li, *Plant Sci.* **2000**, *155*, 179–185.
151. Y. Zhang; K. H. Teoh; D. W. Reed; L. Maes; A. Goossens; D. J. H. Olson; A. R. S. Ross; P. S. Covello, *J. Biol. Chem.* **2008**, *283*, 21501–21508.
152. Q. Zeng; F. Qiu; L. Yuan, *Biotechnol. Lett.* **2008**, *30*, 581–592.
153. J. D. Newman; J. Marshall; M. Chang; F. Nowroozi; E. Paradise; D. Pitera; K. L. Newman; J. D. Keasling, *Biotechnol. Bioeng.* **2006**, *95*, 684–691.
154. M. C. Y. Chang; R. A. Eachus; W. Trieu; D. K. Ro; J. D. Keasling, *Nat. Chem. Biol.* **2007**, *3*, 274–277.
155. C. M. Bertea; A. Voster; F. W. A. Verstappen; M. Maffei; J. Beekwilder; H. J. Bouwmeester, *Arch. Biochem. Biophys.* **2006**, *448*, 3–12.
156. B. Huang; J. Guo; B. Yi; X. Yu; L. Sun; W. Chen, *Biotechnol. Lett.* **2008**, *30*, 1121–1137.
157. K. H. Teoh; D. R. Polichuk; D. W. Reed; G. Nowak; P. S. Covello, *FEBS Lett.* **2006**, *580*, 1411–1416.
158. A. L. Lindahl; M. E. Olsson; P. Mercke; O. Tollbom; J. Schelin; M. Brodelius; P. E. Brodelius, *Biotechnol. Lett.* **2006**, *28*, 571–580.
159. J. J. William; H. L. Li, *J. Nat. Prod.* **1970**, *30*, 1–4.
160. W. C. Evans, Tropane Alkaloids of the Solanaceae. In *The Biology and Taxonomy of the Solanaceae,* Ser. 7; J. G. Hawkes, R. N. Lester, A. D. Skelding, Eds.; Academic Press: London, 1979; pp 241–254.
161. P. N. Bennett; M. J. Brown, *Clinical Pharmacology*, 9th ed.; Churchill Livingstone: Edinburgh, 2003.
162. A. Goldmann; M. L. Milat; P. H. Ducrot; J. Y. Lallemand; M. Maille; A. Lepingle; I. Charpin; D. Tepfer, *Phytochemistry* **1990**, *29*, 2125–2127.
163. T. Hashimoto; A. Hayashi; Y. Amano; J. Cono; H. Iwanari; S. Usada; Y. Yamada, *J. Biol. Chem.* **1991**, *266*, 4648–4653.
164. T. Kanage; H. Kajiya; Y. Amano; T. Hashimoto; Y. Yamada, *Plant Physiol.* **1994**, *105*, 483–490.
165. G. Samuelsson, Ed., Alkaloids. In *Drugs of Natural Origin*; Swedish Pharmaceutical Society, Swedish Pharmaceutical Press: Stockholm, 1999; pp 438–448.
166. R. Zárate; B. Hermosin; M. Cantos; A. Troncoso, *J. Chem. Ecol.* **1997**, *23*, 2059–2066.
167. K. M. Oksmann-Caldentey; R. Arroo, Regulation of Tropane Alkaloid Metabolism in Plants and Plant Cell Culures. In *Metabolic Engineering of Plant Secondary Metabolism*; R Verpoorte, A. W. Alfermann, Eds.; Kluwer Academic Publisher: Netherlands, 2000; pp 253–281.
168. K. Suzuki; D. J. Yun; X. Y. Chen; Y. Yamada; T. Hashimoto, *Plant Mol. Biol.* **1999**, *40*, 141–152.
169. G. Kai; J. Chen; L. Li; G. Zhou; L. Zhou; L. Zhang; Y. Chen; L. Zhao, *J. Biochem. Mol. Biol.* **2007**, *40*, 715–722.
170. M. D. Chilton; D. A. Tepfer; A. Petit; F. Casee-Delbart; J. Tempé, *Nature* **1982**, *295*, 432–1432.
171. L. Joao; T. A. Brown, *J. Exp. Bot.* **1994**, *45*, 641–647.
172. R. Zárate, *Plant Cell Rep.* **1999**, *18*, 418–423.
173. R. M. Cusido; J. Palazón; M. T. Piñol; M. Bonfill; C. Morales, *Planta Med.* **1999**, *65*, 144–148.
174. H. Kamada; N. Okamura; M. Satake; H. Harada; K. Shimomura, *Plant Cell Rep.* **1986**, *5*, 239–242.
175. T. Hashimoto; Y. Yukimune; Y. Yamada, *J. Plant Physiol.* **1986**, *124*, 61–75.
176. A. Bouchereau; A. Aziz; F. Larher; J. Martin-Tanguy, *Plant Sci.* **1999**, *140*, 103–125.
177. R. L. Malmberg; M. B. Watson; G. L. Galloway; W. Yu, *Crit. Rev. Plant Sci.* **1998**, *17*, 199–224.
178. J. Wang; M. Sheehan; H. Brookman; M. P. Timko, *Plant Sci.* **2000**, *158*, 19–32.
179. Y. J. Hao; H. Kitashiba; C. Honda; K. Nada; T. Moriguchi, *J. Exp. Bot.* **2005**, *56*, 1105–1115.
180. O. Lepri; L. Bassie; G. Safwat; P. Thu-Hang; P. Trung-Nghia; E. Holtta; P. Christou; T. Capell, *Mol. Genet. Genomics* **2001**, *266*, 303–312.
181. T. Hashimoto; Y. Yukimune; Y. Yamada, *Planta* **1989**, *178*, 131–137.
182. L. Masgrau; T. Altabella; R. Farrás; D. Flores; A. J. Thompson; T. Besford; A. Tiburcio, *Plant J.* **1997**, *11*, 465–473.
183. R. Alcaráz; J. L. García-Martínez; J. C. Cuevas; A. F. Tiburcio; T. Altabella, *Plant J.* **2005**, *43*, 425.
184. M. J. Mayer; A. J. Michael, *J. Biochem.* **2003**, *134*, 765–772.
185. N. J. Walton; R. J. Robins; A. C. J. Peerless, Planta 1990,*182*, 136–141.
186. R. J. Robins; A. J. Parr; N. J. Walto, Planta 1991,*183*, 196–201.
187. N. Alka; K. S. Vinad; P. Deepshikha; M. V. Rajam; P. S. Srivastava, *J. Plant Biochem. Biotechnol.* **2004**, *13*, 127–130.
188. A. F. Tiburcio; A. W. Galston, *Phytochemistry* **1985**, *25*, 107–110.
189. N. Hibi; T. Fujita; M. Hatano; T. Hashimoto; Y. Yamada, *Plant Physiol.* **1992**, *100*, 826–835.
190. F. Feth; H. A. Arfmann; V. Wray, *Phytochemistry* **1985**, *24*, 921–923.
191. K. I. Suzuki; Y. Yamada; T. Hashimoto, *Plant Cell Physiol.* **1999**, *40*, 289–297.
192. N. Sachan; D. L. Falcone, *Phytochemistry* **2002**, *61*, 797–805.

193. R. J. Robins; E. G. Bent; M. J. C. Rhades, *Planta* **1991**, *185*, 385–390.
194. T. Liu; P. Zhu; K. D. Cheng; C. Ming; H. X. Zhu, *Planta Med.* **2005**, *71*, 987–989.
195. O. Stenzel; M. Teuber; B. Dräger, *Planta* **2006**, *223*, 200–212.
196. E. Moyano; K. Jouhikainen; P. Tammela; J. Palazón; R. M. Cusidó; M. T. Piñol; T. H. Teeri; K. M. Oksamnn-Caldentey, *J. Exp. Bot.* **2003**, *54*, 203–211.
197. E. Moyano; M. Bonfill; J. Cusidó; J. Palazón; M. T. Piñol, *Recent Res. Dev. Biochem.* **2004**, *5*, 317–327.
198. L. Zhang; R. Ding; Y. Chai; M. Bonfill; E. Moyano; K. M. Oksman-Caldentey; T. Xu; Y. Pi; Z. Wang; H. Zhang; G. Kai; Z. Liao; X. Sun; K. Tang, *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 6786–6791.
199. O. S. Lee; Y. M. Kang; H. Y. Jung; J. Y. Min; S. M. Kang; C. S. Karigar; D. T. Prasad; J. D. Bahk; M. S. Choi, *In Vitro Cell. Dev. Biol. Plant* **2005**, *41*, 167–172.
200. F. Sato; T. Hashimoto; A. Hachiya; K. I. Tamura; K. B. Choi; T. Mrishige; H. Fujimoto; Y. Yamada, *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 367–372.
201. G. Rothe; A. Hachiya; Y. Yamada; T. Hashimoto; B. Dräger, *J. Exp. Bot.* **2003**, *54*, 2065–2070.
202. E. Moyano; S. Fornalé; J. Palazón; R. M. Cusidó; N. Bagni; M. T. Piñol, *Phytochemistry* **2002**, *59*, 697–702.
203. T. Hashimoto; A. Mitani; Y. Yamada, *Plant Physiol.* **1990**, *93*, 216–221.
204. R. J. Robins; A. J. Parr; E. G. Bent; M. J. C. Rhodes, *Planta* **1991**, *183*, 185–195.
205. W. R. McLauchlan; A. M. Raymond; D. M. Evans, *Planta* **1993**, *191*, 440–445.
206. B. Dräger, *Phytochemistry* **2006**, *67*, 327–337.
207. M. M. Couladis; J. B. Friesen; M. E. Landgrebe; E. Leete, *Phytochemistry* **1991**, *30*, 801–805.
208. A. Portsteffen; B. Dräger; A. Nahrdtedt, *Phytochemistry* **1992**, *31*, 1135–1138.
209. K. Nakajima; T. Hashimoto; Y. Yamada, *Prod. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 9591–9595.
210. K. Nakajima; Y. Hashimoto; Y. Yamada, *Plant Physiol.* **1993**, *103*, 1465–1466.
211. R. Keiner; H. Kaiser; K. Nakajima; T. Hashimoto; B. Dräger, *Plant Mol. Biol.* **2002**, *48*, 299–308.
212. P. Rocha; O. Stenzel; A. Parr; N. Walton; P. Christou; B. Dräger; M. J. Leech, *Plant Sci.* **2002**, *162*, 905–913.
213. U. Richter; G. Rothe; A. K. Fabian; B. Rahfeld; B. Dräger, *J. Exp. Bot.* **2005**, *56*, 645–652.
214. J. Matsuda; S. Okabe; T. Hashimoto; Y. Yamada, *J. Biol. Chem.* **1991**, *266*, 9460–9464.
215. T. Hashimoto; Y. Yamada, *Plant Physiol.* **1986**, *81*, 619–625.
216. T. Hashimoto; J. Matsuda; Y. Yamada, *FEBS Lett.* **1993**, *329*, 35–39.
217. T. Hashimoto; D. J. Yun; Y. Yamada, *Phytochemistry* **1993**, *32*, 713–718.
218. K. Jouhikainen; L. Lindaren; T. Jokelainen; R. Hiltunen; T. H. Teeri; K. M. Oksman-Caldentey, *Planta* **1999**, *208*, 545–551.
219. J. Palazón; E. Moyano; R. M. Cusidó; M. Bonfill; K. M. Oksmann-Caldentey; M. T. Piñol, *Plant Sci.* **2003**, *165*, 1289–1295.
220. Y. M. Kang; O. S. Lee; H. Y. Jung; S. M. Kag; B. H. Lee; C. Karigan; T. Prosad; J. D. Bahk; M. S. Choi, *J. Microbiol. Biotech.* **2005**, *15*, 91–98.
221. S. T. Häkkinen; E. Moyano; R. M. Cusido; M. T. Piñol; K. M. Oksmann-Caldentey, *J. Exp. Bot.* **2005**, *56*, 2611–2618.
222. R. Zárate; N. El Jaber-Vazdekis; B. Medina; A. G. Ravelo, *Biotechnol. Lett.* **2006**, *28*, 1271–1277.
223. N. El Jaber-Vazdekis; M. L. Barres; A. G. Ravelo; R. Zárate; *J. Nat. Prod.* **2008**, *71*, 2026–2031.
224. L. Rahman; Y. Kitamura; J. Yamagushi; M. Mukai; K. Akiyama; H. Yamamoto; T. Muranaka; T. Ikenaga, *Enzyme Microb. Technol.* **2006**, *39*, 1183–1189.
225. P. J. Facchini; J. M. Hagel; D. K. Liscombe; N. Loukanina; B. P. MacLeod; N. Samanani; K. G. Zulak, *Phytochemistry Rev.* **2007**, *6*, 97–124.
226. T. Yoshikawa; T. Furuya, In *Plant Tissue Culture,* Proceedings of the 5th International Congress on Plant Tissue and Cell Culture; A. Fujiwara, Ed.; Japanese Association for Plant Tissue Culture: Tokyo, Japan, 1982.
227. T. Yoshikawa; T. Furuya, In *Plant Med.* **1985**, *2*, 110–113.
228. M. A. Kassem; A. Jacquin, *J. Biomed. Biotechnol.* **2001**, *1*, 70–78.
229. D. A. Bird; V. R. Franceschi; P. J. Facchini, *Plant Cell* **2003**, *15*, 2626–2635.
230. N. Samanani; J. Alcantara; R. Bourgault; K. G. Zulak; P. J. Facchini, *Plant J.* **2006**, *47*, 547–563.
231. N. Samanani; S. U. Park; P. J. Facchini, *Plant Cell* **2005**, *17*, 915–926.
232. S. U. Park; P. J. Facchini, *J. Exp. Bot.* **2000**, *51*, 1005–1016.
233. V. Le Flem-Bonhomme; D. Laurain-Mattar; M. A. Fliniaux, *Planta* **2004**, *218*, 890–893.
234. P. J. Larkin; J. A. C. Miller; R. S. Allen; J. A. Chitty; W. L. Gerlach; S. Frick; T. M. Kutchan; A. J. Fist, *Plant Biotechnol. J.* **2007**, *5*, 26–37.
235. S. Frick; R. Kramell; T. M. Kutchan, *Metab. Eng.* **2007**, *9*, 169–176.
236. R. S. Allen; J. A. C. Miller; J. A. Chitty; A. J. Fist; W. L. Gerlach; P. J. Larkin, *Plant Biotechnol. J.* **2008**, *6*, 22–30.
237. S. Frick; J. A. Chitty; R. Kramell; J. Schmidt; R. S. Allen; P. J. Larkin; T. M. Kutchan, *Transgenic Res.* **2004**, *13*, 607–613.
238. N. Fujii; T. Inui; K. Iwasa; T. Morishige; F. Sato, *Transgenic Res.* **2007**, *16*, 363–375.
239. J. E. Page, *Trends Biotechnol.* **2005**, *23*, 331–333.
240. A. G. Millgate; B. J. Pogson; I. W. Wilson; T. M. Kutchan; M. H. Zenk; W. L. Gerlach; A. J. Fist; P. J. Larkin, *Nature* **2004**, *431*, 413–414.
241. N. R. Apuya; J. H. Park; L. Zhang; M. Ahyow; P. Davidow; J. van Fleet; J. C. Rarang; M. Hippley; T. W. Johnson; H. D. Yoo; A. Trieu; S. Krueger; C. Y. Wu; Y. P. Lu; R. B. Flavell; S. C. Bobzin, *Plant Biotechnol. J.* **2008**, *6*, 160–175.
242. P. S. Lee; K. H. Lee, *Curr. Opin. Biotechnol.* **2000**, *11*, 171–175.
243. M. S. Boguski; G. D. Schuler, *Nat. Genet.* **1995**, *10*, 369–371.
244. V. E. Velculescu; L. Zhang; W. Zhou; J. Vogelstein; M. A. Basrai; D. E. Bassett, Jr.; P. Hieter; B. Vogelstein; K. W. Kinzler, *Cell* **1997**, *88*, 243–251.
245. J. G. Gibbings; B. P. Cook; M. R. Dufault; S. L. Madden; S. Khuri; Turnbull; C. J. Dunwell, *Plant Biotechnol. J.* **2003**, *1*, 271–285.
246. M. J. Dutt; K. H. Lee, *Curr. Opin. Biotechnol.* **2000**, *11*, 176–179.
247. D. I. Jacobs; R. van der Heijden; R. Verpoorte, *Phytochem. Anal.* **2000**, *11*, 277–287.
248. O. Fiehn, *Plant Mol. Biol.* **2002**, *48*, 155–171.
249. D. Samson; F. Legeai; E. Karsenty; S. Reboux; J. B. Veyrieras; J. Just; E. Barillot, *Nucleic Acids Res.* **2003**, *31*, 179–182.

250. D. Edwards; J. Batley, *Trends Biotechnol.* **2004**, *22*, 232–237.
251. T. J. Raharjo; I. Widjaja; S. Roytrakul; R. Verpoorte, *J. Biomol. Technol.* **2004**, *15*, 97–106.
252. K. Cho; G. K. Agrawal; J. Shibato; Y. H. Jung; Y. K. Kim; B. H. Nahm; N. S. Jwa; S. Tamogami; O. O. Han; K. Kohda; H. Iwahashi; R. Rakwal, *J. Proteome Res.* **2007**, *6*, 3581–3603.
253. D. I. Jacobs; M. Gaspari; J. van der Greef; R. van der Heijden; R. Verpoorte, *Planta* **2005**, *221*, 690–704.
254. J. Ehlting; S. G. Chowrira; N. Mattheus; D. S. Aeschliman; G. I. Arimura; J. Bohlmann, *BMC Genomics* **2008**, *9*, 154–174.
255. K. Yonekura-Sakakibara; T. Tohge; R. Niida; K. Saito, *J. Biol. Chem.* **2007**, *282*, 14932–14941.
256. A. Goossens; S. T. Häkkinen; I. Laakso; T. Seppänen-Laakso; S. Biondi; V. De Sutter; F. Lammertyn; A. M. Nuutila; H. Söderlund; M. Zabeau; D. Inzé; K. M. Oksman-Caldentey, *Proc. Natl. Acad. Sci. U.S.A* **2003**, *100*, 8595–8600.
257. H. Rischer; M. Oresic; T. Seppänen-Laakso; M. Katajamaa; F. Lammertyn; W. Ardiles-Diaz; M. C. E. van Montagu; D. Inzé; K. M. Oksman-Caldentey; A. Goossens, *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 5614–5619.
258. K. G. Zulak; A. M. Weljie; H. J. Vogel; P. J. Facchini, *BMC Plant Biol.* **2008**, *8*, 5–24.
259. M. A. Farag; D. V. Huhman; R. A. Dixon; L. W. Sumner, *J. Plant Physiol.* **2008**, *146*, 387–402.
260. C. Ma; H. Wang; X. Lu; G. Xu; B. Liu, *J. Chromatogr. A* **2008**, *1186*, 412–419.
261. C. Kristensen; M. Morant; C. E. Olsen; C. T. Ekstrøm; D. W. Galbraith; B. L. Møller; S. Bak, *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 1779–1784.
262. S. Gómez-Galera; A. M. Pelacho; A. Gené; T. Capell; P. Christou, *Plant Cell Rep.* **2007**, *26*, 1689–1715.
263. E. Picherky; D. R. Gang, *Trends Plant Sci.* **2000**, *5*, 439–445.
264. K. M. Oksmand-Caldentey; K. Saito, *Curr. Opin. Biotechnol.* **2005**, *16*, 174–179.

Biographical Sketch

Rafael Zárate was born on 7 April 1963 in La Orotava, Tenerife, Spain and graduated from The University of La Laguna, Tenerife with a B.Sc. (Biology) degree in 1989. Later, he moved to The University of Edinburgh where he conducted his Ph.D. studies (1994) on plant biotechnology and natural product synthesis in *in vitro* culture systems under the supervision of Professor M.M. Yeoman.

His first postdoc position was back in Spain at the University of Seville, Department of Plant Biology, jointly with the Spanish National Research Council, IRNASE-CSIC Institute (1995–1998), under the supervision of Dr. A. Aparicio. He was involved in conducting research on strategies and protocols for rescuing endangered plant species of Grazalema Natural Park, as well as implementing and initiating the biotechnology of the native species *Atropa baetica*, a major producer of tropane alkaloids.

This was followed by a second postdoc position as a Marie Curie Research Fellow (1998–2000) at the Department of Pharmacognosie, Rijks Universiteit Leiden, The Netherlands, under the supervision of Professor R. Verpoorte. The research involved studying the formation of new pharmaceutical compounds by genetic engineering of plants, in particular the medicinal plant *Catharanthus roseus*, devising a protocol for the genetic transformation of mature plants, and studying the overexpression of genes involved in the terpenoid indole alkaloid pathway.

He then returned to the Canary Islands to its original University at the Instituto Universitario de Bio-Orgánica, with another postdoc Marie Curie fellowship (2000–2001), investigating the metabolic engineering of the medicinal plant *A. baetica*, under the supervision of Professor A.G. Ravelo. He was able to implement and establish within this research

group and University plant biotechnology as a tool for studying natural products formation, being the leader of this research topic. During this period, he also became a member of the Canary Island Cancer Research Institute (ICIC), for which he run some research projects on the application of biotechnology for the production of natural products with anticancer activities. Then he was awarded a Ramón y Cajal postdoc research tenure funded by the Spanish Ministry of Science and Education for a 5-year period. He conducted research on the genetic engineering of medicinal plants, that is, *A. baetica* and some *Maytenus* species, together with species of the endemic Canary flora, and also worked as a lecturer of postgraduate courses on plant biotechnology applied to natural products. During his tenure as a researcher, he has managed to co-author many publications in the field of plant biotechnology and natural products, appearing mainly in international journals, as well as co-authored several book chapters.

3.19 Biotransformation of Monoterpenoids

Yoshiaki Noma and Yoshinori Asakawa, Tokushima Bunri University, Yamashiro-cho, Tokushima, Japan

3.19.1 Introduction

Monoterpenoids are distributed in higher plants, algae, fungi, and even in some insects and mammals, but they are very rare in mosses, ferns, and lichens. A great number of lipophilic or hydrophobic monoterpenoids have been detected in or isolated from solvent extracts and essential oils from the organisms mentioned above. Vegetables, fruits, and spices as well as many supplements and processed foods contain monoterpenoids; however, their fate in human and other animal bodies has not yet been fully investigated systematically. Recent progress in the development of analytical instruments has made it easy to analyze the chemical structures of very minor components, and the field of essential oil chemistry has dramatically developed.

Since monoterpenoids, in general, show characteristic odor and taste, they have been used as cosmetic materials, food additives, insecticides, and insect repellent and attractant drugs. In order to obtain much more functionalized substances from monoterpenoids, various chemical reactions and microbial transformation of commercially available and cheap synthetic monoterpenoids have been carried out. On the other hand, insect larvae and mammals have been used for direct biotransformation of monoterpenoids to study their fate and safety or toxicity in these organisms.

It has been 50 years since the hydroxylation of α-pinene (**130**) (see **Scheme 46**) was reported in 1960 in the black fungus *Aspergillus niger*.[1] During these years, many investigators have studied the biotransformation of a number of monoterpenoids by using various kinds of bacteria, fungi, insects, and mammals. Among fungi,

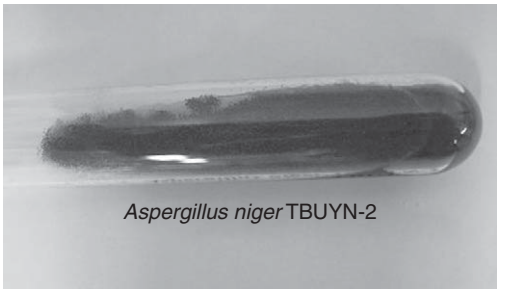


Figure 1 The fungus *Aspergillus niger* TBUYN-2.

A. niger, as shown in **Figure 1**, is one of the most popular bioreactors and is used for biotransformation of not only monoterpenoids but also sesqui- and diterpenoids and aromatic compounds. Here the biotransformation of monoterpenoids is summarized based on previously published work listed in the reference list.

3.19.2 Metabolic Pathways of Monoterpenoids

3.19.2.1 Acyclic Monoterpenoids

3.19.2.1.1 Acyclic monoterpene hydrocarbons

3.19.2.1.1(i) β-Myrcene (1) The microbial biotransformation of β-myrcene (**1**) was described in *Diplodia gossypina* ATCC 10936 in 1985.[2] The main reaction was the hydroxylation reaction on trisubstituted double bond (**Scheme 1**). On oxidation, myrcene (**1**) gave the diol (**2**) (yield up to 60%) and also a by-product (**3**) that had one carbon atom less than the parent compound (yield 1–2%).

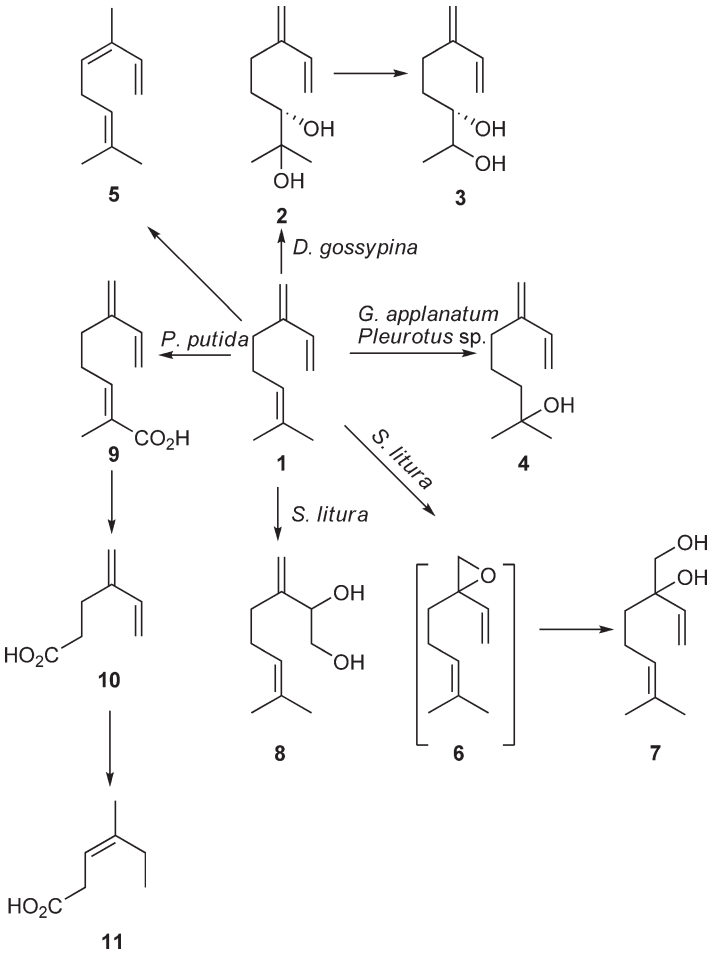
One of the publications dealing with the bioconversion of β-myrcene (**1**)[3] described its transformation to a variety of oxygenated metabolites, with *Ganoderma applanatum*, *Pleurotus flabellantus*, and *Pleurotus sajor-caju* possessing the highest transformation activity. One of the major metabolites was myrcenol (**4**) (2-methyl-6-methylene-7-octen-2-ol), giving a fresh flowery impression and dominating sensory impact of the mixture (**Scheme 1**).

The larvae of common cutworm, *Spodoptera litura*, biotransformed β-myrcene (**1**) to myrcene-3(10)-glycol (**7**) via myrcene-3(10) epoxide (**6**) and myrcene-1,2-glycol (**8**) (**Scheme 1**).[4]

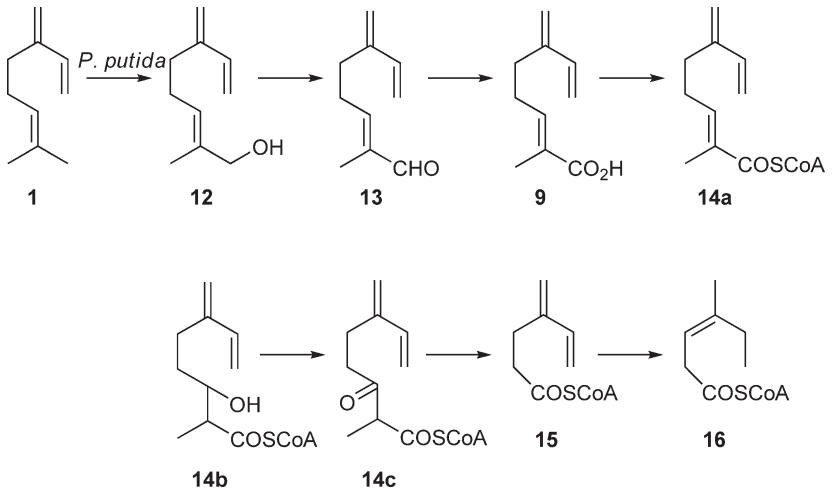
On the contrary, *Pseudomonas putida* that was able to utilize β-myrcene (**1**) as the sole carbon and energy source was isolated from soil. *Pseudomonas putida* accumulated (*E*)-2-methyl-6-methylen-2,7-octadienoic acid (**9**), 4-methylene-5-hexenoic acid (**10**), and (*E*)-4-methyl-3-hexenoic acid (**11**), together with compound (**5**) in the culture broth (**Scheme 1**). Sodium nitrate as a nitrogen source enhanced the productivity of (*E*)-2-methyl-6-methylen-2,7-octadienoic acid (**9**). Resting cells grown on glycerol also produced this acid (**9**) dominantly from β-myrcene (**1**) (**Scheme 1**).[5]

A degradation pathway from β-myrcene (**1**) to (*E*)-4-methyl-3-hexenonyl-CoA (**16**) is proposed as shown in **Scheme 2**. β-Myrcene (**1**) has terminal conjugated double bonds and allylic gem-dimethyl groups in its molecule. *Pseudomonas putida* S4-2 oxidized sequentially the allylic *trans*-methyl group of β-myrcene (**1**) to (*E*)-2-methyl-6-methylene-2,7-octadienol (**12**), (*E*)-2-methyl-6-methylen-2,7-dienal (**13**), and (*E*)-2-methyl-6-methylen-2,7-octadienoic acid (**9**), which seems to be degraded via β-oxidation subsequently to form 4-methylene-5-hexenoic acid (**10**). The mechanism of the formation of (*E*)-4-methyl-3-hexenoic acid (**11**) remains to be solved. It is, however, assumed that the reduction and shift of the double bonds of 4-methylene-5-hexenoic acid (**10**) might have occurred resulting in (*E*)-4-methyl-3-hexenoic acid (**11**).[5]

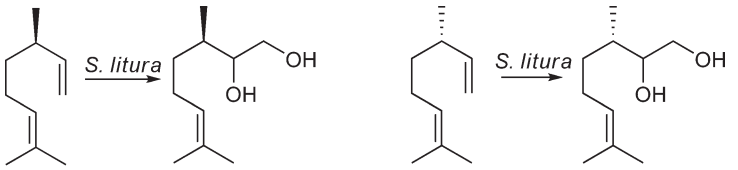
3.19.2.1.1(ii) Citronellene (17 and 17′) (−)-Citronellene (**17**) and (+)-citronellene (**17′**) were biotransformed by the cutworm *S. litura* to (3*R*)-3,7-dimethyl-6-octene-1,2-diol (**18**) and (3*S*)-3,7-dimethyl-6-octene-1,2-diol (**18′**), respectively (**Scheme 3**).[6]



Scheme 1 Biotransformation of β-myrcene (**1**) by *Diplodia gossypina, Ganoderma applanatum*, *Pleurotus* species, *Spodoptera litura*, and *Pseudomonas putida*.



Scheme 2 Proposed metabolic pathway of β-myrcene (**1**) by *Pseudomonas putida*.



17 18 17′ 18′

Scheme 3 Biotransformation of (−)-citronellene (**17**) and (+)-citronellene (**17′**) by *Spodoptera litura.*

3.19.2.1.2 Acyclic monoterpene alcohols and aldehydes

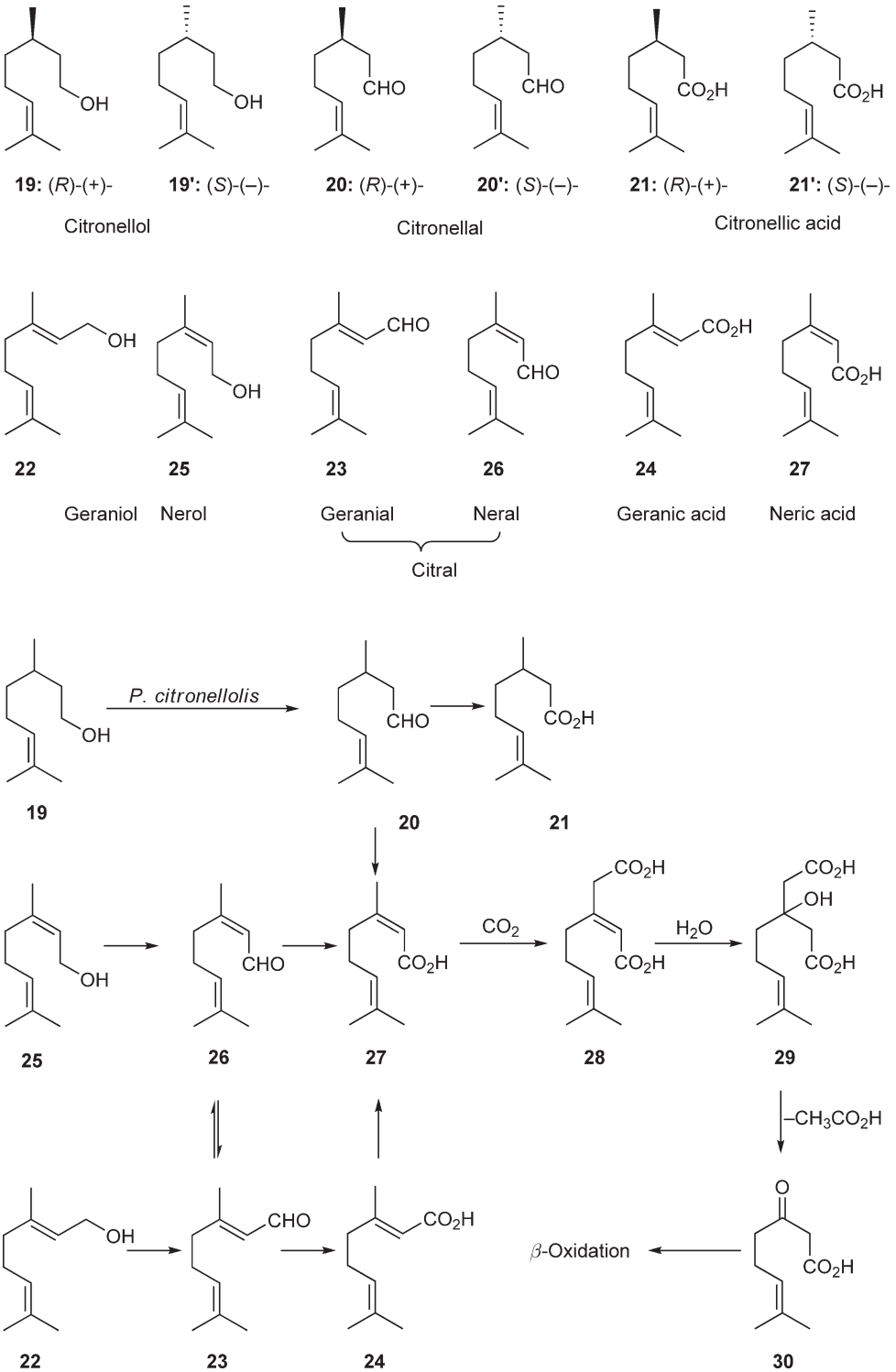
3.19.2.1.2(i) Geraniol (22), nerol (25), citral (23 and 26), citronellol (19 and 19′), and citronellal (20 and 20′) The microbial degradation of the acyclic monoterpene alcohols citronellol (**19**), nerol (**25**), geraniol (**22**), citronellal (**20**), and citral (an equal mixture of **23** and **26**) was reported in the early 1960s.[7–10] *Pseudomonas citronellolis* metabolized citronellol (**19**), citronellal (**20**), geraniol (**22**), and geranic acid (**24**). The metabolism of these acyclic monoterpenes is initiated by the oxidation of the primary alcohol group to the carboxyl group, followed by carboxylation of the C-10 methyl group (β-methyl) by a biotin-dependent caboxylase.[7] The carboxymethyl group is eliminated at a later stage as acetic acid. Further degradation follows the β-oxidation pattern. The details of the pathway are shown in **Scheme 4**.[11]

The microbial transformation of citronellal (**20**) and citral (**23** and **26**) was reported in *Pseudomonas aeruginosa.*[12] This bacterium, capable of utilizing citronellal (**20**) or citral (**23** and **26**) as the sole carbon and energy source, has been isolated from soil by the enrichment culture technique. It metabolized citronellal (**20**) to citronellic acid (**21**) (65%), citronellol (**19**) (0.6%), dihydrocitronellol (**31**) (0.6%), 3,7-dimethyl-1,7-octanediol (**32**) (1.7%), and menthol (**33**) (0.75%) (see **Scheme 5**). The metabolites of citral (**23** and **26**) were geranic acid (**24**) (62%), 1-hydroxy-3,7-dimethyl-6-octen-2-one (**34**) (0.75%), 6-methyl-5-heptenoic acid (**35**) (0.5%), and 3-methyl-2-butenoic acid (**36**) (1%) (**Scheme 5**). *Pseudomonas convexa* converted in a similar way citral (**23** and **26**) to geranic acid (**24**).[13] The biotransformation of citronellol (**19**) and geraniol (**22**) by *P. aeruginosa*, *P. citronellolis,* and *Pseudomonas mendocina* was also reported by another group.[14]

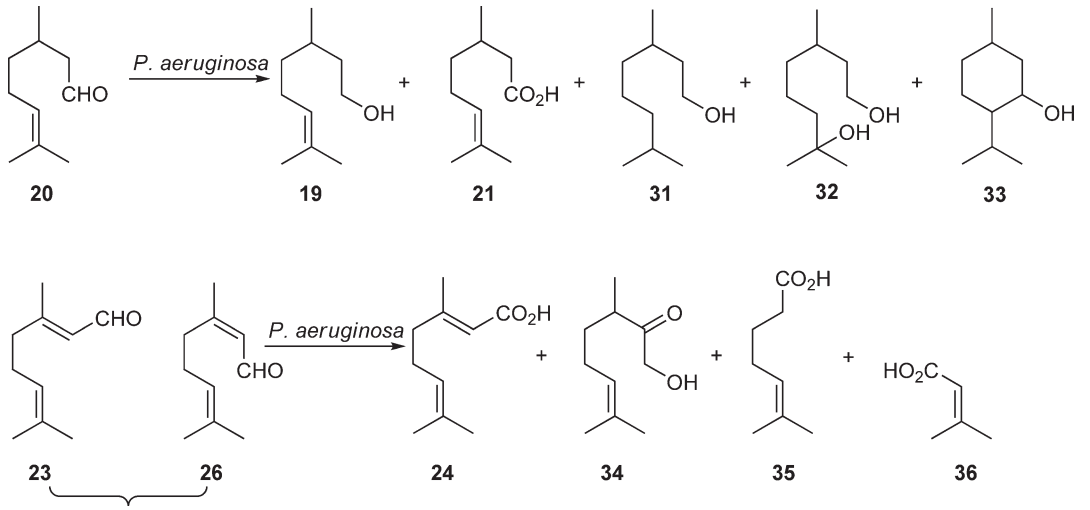
A research group in Czechoslovakia patented the cyclization of citronellal (**20**) with subsequent hydrogenation to menthol (**33**) by *Penicillium digitatum* in 1952. Unfortunately, the optical purities of the intermediates pulegol (**37**) and isopulegol (**38**) were not determined and presumably the resulting menthol (**33**) was a mixture of enantiomers (**Scheme 6**). Therefore, it cannot be excluded that this extremely interesting cyclization is the result of a reaction primarily catalyzed by the acidic fermentation conditions and only partially dependent on enzymatic reactions.[15]

Based on previous data,[16,17] two pathways for the degradation of geraniol (**22**) by *P. incognita* were proposed by Madyastha[11] (**Scheme 7**). Pathway A involves an oxidative attack on the 2,3-double bond resulting in the formation of an epoxide. Opening of the epoxide yields 2,3-dihydroxygeraniol (**39**), which upon oxidation forms 2-oxo-3-hydroxygeraniol (**40**), which is then decomposed to 6-methyl-5-hepten-2-one (**41**) by an oxidative process. Pathway B is initiated by the oxidation of the primary alcoholic group to geranic acid (**24**) and further metabolism follows the mechanism as proposed earlier for *P. citronellolis.*[7,8] In the case of nerol (**25**), the *Z*-isomer of geraniol (**22**), degradative pathways analogous to pathways A and B as in geraniol (**22**) are observed. It was also noticed that *P. incognita* metabolizes acetates of geraniol (**22**), nerol (**25**), and citronellol (**19**) much faster than their respective alcohols.[18]

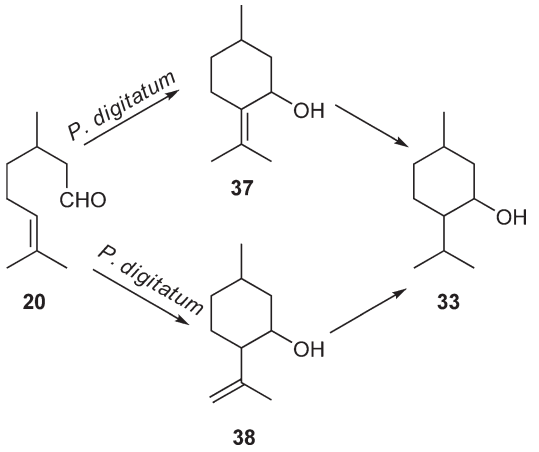
Euglena gracilis Z converted citral (**23** and **26**, 56:44, peak area in GC) to geraniol (**22**) and nerol (**25**), respectively, of which geraniol (**22**) was further transformed to (+)- and (−)-citronellol (**19** and **19′**). On the other hand, when either geraniol (**22**) or nerol (**25**) was added, both compounds were isomerized to each other and, then, geraniol (**22**) was transformed to citronellol. These results showed that *Euglena* could distinguish between the stereoisomers geraniol (**22**) and nerol (**25**) and hydrogenated selectively geraniol (**22**). (+)-, (−)-, and Racemic (±)-citronellal (**20**, **20′**, and **20** and **20′**) were also transformed to the corresponding (+)-, (−)-, and racemic (±)-citronellol (**19**, **19′**, and **19** and **19′**) as the major products and (+)-, (−)-, and racemic citronellic acids (**21**, **21′**, and **21** and **21′**) as the minor products, respectively (**Scheme 8**).[19]



Scheme 4 Structures of citronellols (**19** and **19′**), citronellals (**20** and **20′**), citronellic acids (**21** and **21′**), gerniol (**22**), nenol (**25**), citral (**23** and **24**), geranic acid (**249**), and neric acid (**27**), and biotransformation of citronellol (**19**), geraniol (**22**), and nerol (**25**) by *Pseudomonas citronellolis*.



Scheme 5 Biotransformation of citronellal (**20**) and citral (**23** and **26**) by *Pseudomonas aeruginosa*.

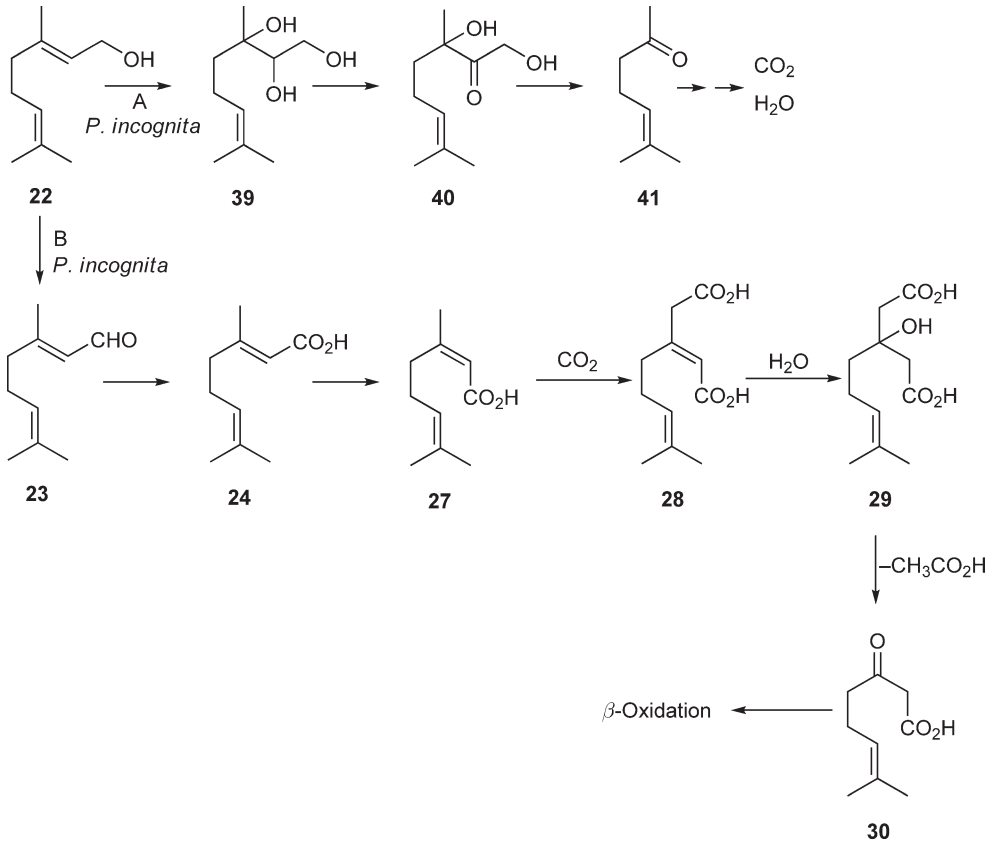


Scheme 6 Biotransformation of citronellal (**20**) to menthol (**33**) by *Penicillium digitatum.*

Dunaliella tertiolecta also reduced citral (geranial (**23**) and neral (**26**) 56:44) and (+)-, (−)-, and racemic (±)-citronellal (**20**, **20′**, and **20** and **20′**) to the corresponding alcohols, namely, geraniol (**22**) and nerol (**25**), and (+)-, (−)-, and racemic (±)-citronellol (**19**, **19′**, and **19** and **19′**).[20,21]

Citral (a mixture of geranial (**23**) and neral (**26**), 56:44, peak area in GC) is readily transformed to geraniol (**22**) and nerol (**25**), respectively, of which geraniol (**22**) is further hydrogenated to (+)-citronellol (**19**) and (−)-citronellol (**19′**). Geranic acid (**24**) and neric acid (**27**) are also formed from **23** and **26** as the minor products, respectively. On the other hand, when either **22** or **25** is used as a substrate, both compounds are isomerized to each other and, then, **22** is transformed to citronellol (**19** or **19′**). These results showed the *Euglena* could distinguish between the stereoisomers **22** and **25** and hydrogenated selectively **22** to citronellol (**19** or **19′**). (+)-, (−)-, and (±)-Citronellal (**20**, **20′**, and an equal mixture of **20** and **20′**) are also transformed to the corresponding citronellol and *p*-menthane-*trans*- and *cis*-3,8-diols (**42a**, **42b**, **42a′**, and **42b′**) as the major products, which are well known as mosquito repellents and plant growth regulators,[22,23] and (+)-, (−)-, and (±)-citronellic acids (**21**, **21′**, and an equal mixture of **21** and **21′**) as the minor products, respectively.

Streptomyces ikutamanensis Ya-2-1 also reduced citral (geranial (**23**) and neral (**26**), 56:44) and (+)-, (−)-, and racemic citronellal (**20**, **20′**, and **20** and **20′**) to the corresponding alcohols, namely, geraniol (**22**) and nerol (**25**), (+)-, (−)-, and racemic citronellol (**19**, **19′**, and **19** and **19′**). Compounds **22** and **25** were isomerized to



Scheme 7 Metabolism of geraniol (**22**) by *Pseudomonas incognita*.

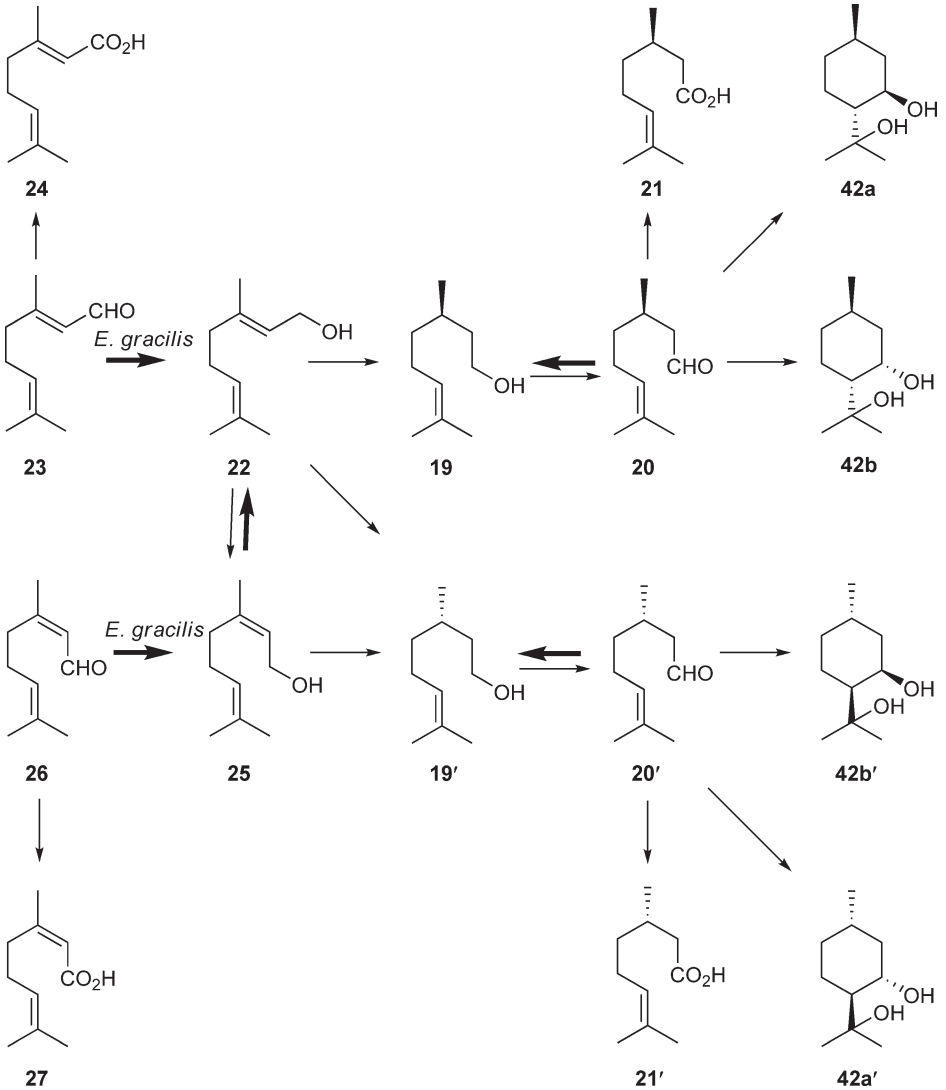
each other. Furthermore, terpene alcohols (**19′**, **25**, and **22**) were epoxidized to 6,7-epoxygeraniol (**43**), 6,7-epoxynerol (**44**), and 2,3-epoxycitronellol (**45**). On the contrary, (+)-citronellol (**19**) and racemic citronellol (**19** and **19′**) were not converted at all (**Scheme 9**).[24]

A strain of *A. niger*, isolated from garden soil, was able to transform geraniol (**22**), citronellol (**19** and **19′**), and linalool (**63**) (see **Scheme 15**) to their respective 8-hydroxy derivatives. This reaction was called 'ω-hydroxylation'.[25,26]

Fermentation of citronellyl acetate by *A. niger* resulted in the formation of a major metabolite, 8-hydroxycitronellol (**55**), accounting for ~60% of the total transformation products, accompanied by 38% citronellol (**19**). Fermentation of geranyl acetate by *A. niger* gave geraniol (**22**) and 8-hydroxygeraniol (**48**) (50 and 40% of the total transformation products, respectively).

One of the most important examples of fungal bioconversion of monoterpene alcohols is the biotransformation of citral by *Botrytis cinerea*, which is a fungus of great interest in winemaking.[27] In an unripe state of maturation, the infection of grapes by *B. cinerea* is very much feared, as the grapes become moldy ('gray rot'). With fully ripe grapes, however, the growth of *B. cinerea* is desirable; then the fungus is called 'noble rot' and the infected grapes deliver famous sweet wines such as Sauternes of France or Tokay Aszu of Hungary.[28]

One of the first reports in this area dealt with the biotransformation of citronellol (**19**) by *B. cinerea*.[28,29] The substrate was mainly metabolized by ω-hydroxylation. The same group also investigated the bioconversion of citral (**23** and **26**).[30] A comparison was made between grape must and a synthetic medium. When using grape must, no volatile bioconversion products were found. With a synthetic medium, biotransformation of citral (**23** and **26**) was observed yielding predominantly nerol (**25**) and geraniol (**22**) as reduction products and as minor compounds some ω-hydroxylation products. Finally, the bioconversion of geraniol (**22**) and nerol (**25**) was described by the same group.[31] When using grape must, a complete bioconversion of geraniol (**22**) was observed mainly yielding ω-hydroxylation products.

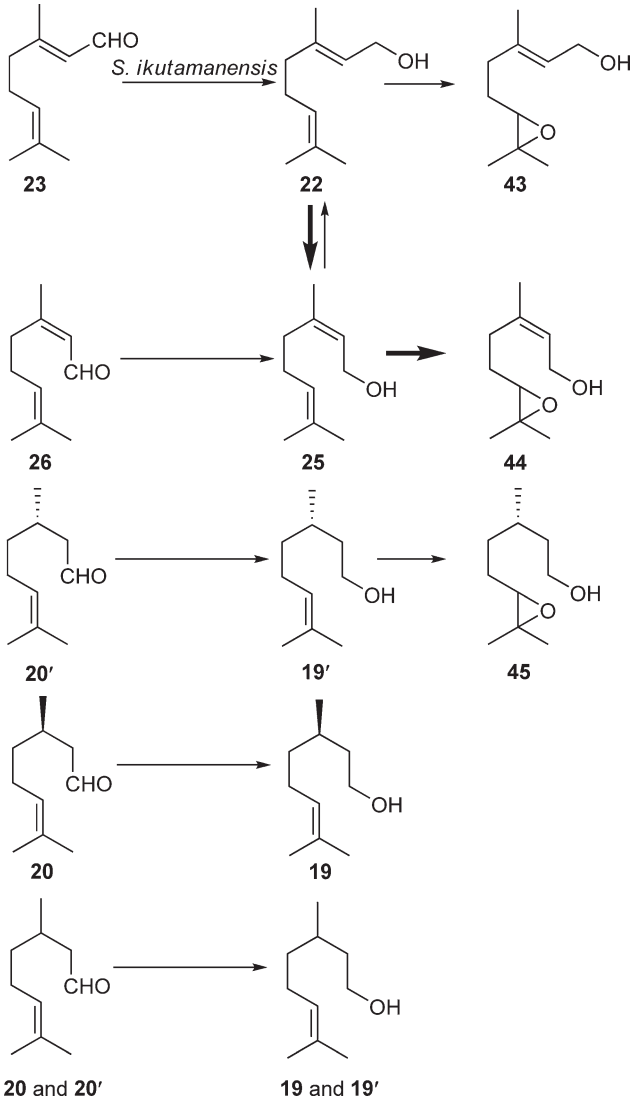


Scheme 8 Metabolic pathways of citral (**23** and **26**) by *Euglena gracilis* Z.

The most important metabolites from geraniol (**22**), nerol (**25**), and citronellol (**19**) are summarized in **Scheme 4**.

In the same year, the biotransformation of these monoterpenes by *B. cinerea* in model solutions was described by another group.[27] Although the major metabolites found were ω-hydroxylation compounds, it is important to note that some new compounds that were not described by the previous group were detected (see **Scheme 9**). Geraniol (**22**) was mainly transformed to (*2E,5E*)-3,7-dimethyl-2,5-octadiene-1,7-diol (**46**), (*E*)-3,7-dimethyl-2,7-octadiene-1,6-diol (**47**), and (*2E,6E*)-2,6-dimethyl-2,6-octadiene-1,8-diol (**48**) and nerol (**25**) was transformed to (*2Z,5E*)-3,7-dimethyl-2,5-octadiene-1,7-diol (**49**), (*Z*)-3,7-dimethyl-2,7-octadiene-1,6-diol (**50**), and (*2E,6Z*)-2,6-dimethyl-2,6-octadiene-1,8-diol (**51**). Furthermore, a cyclization product (**52**) that was not previously described was formed. Finally, citronellol (**19**) was converted to *trans*- (**56**) and *cis*-rose oxide (**57**) (a cyclization product not identified by the other group), (*E*)-3,7-dimethyl-5-octene-1,7-diol (**53**), 3,7-dimethyl-7-octene-1,6-diol (**54**), and (*E*)-2,6-dimethyl-2-octene-1,8-diol (**55**) (**Scheme 10**).

One of the latest reports in this area described the biotransformation of citronellol (**19**) by the plant pathogenic fungus *Glomerella cingulata* to 3,7-dimethyl-1,6,7-octanetriol.[32]

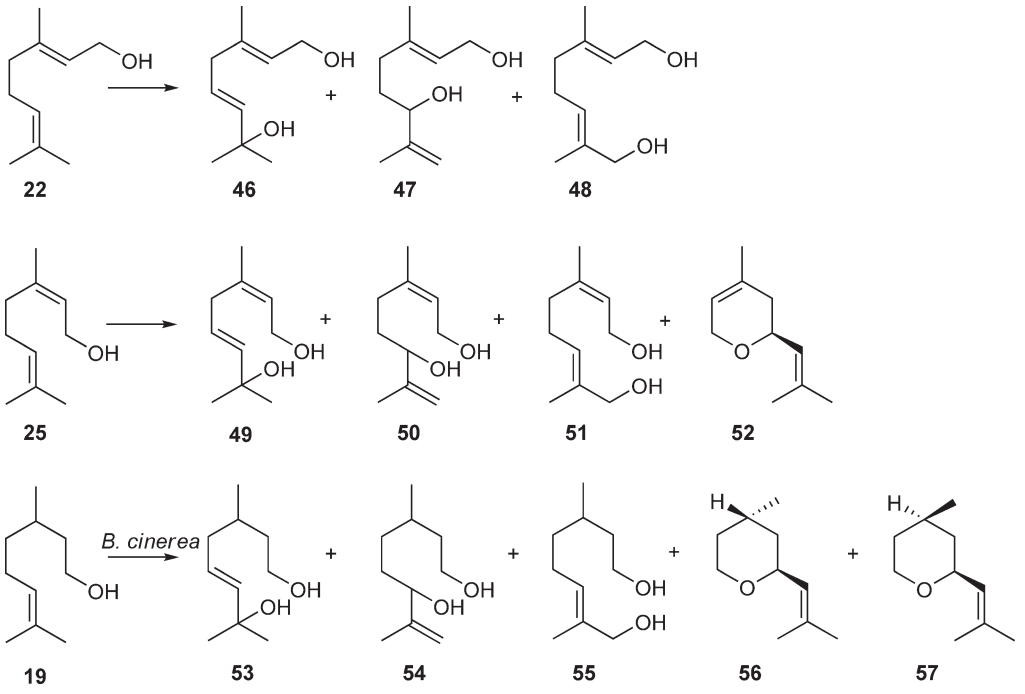


Scheme 9 Reduction of monoterpene aldehydes (**20**, **20′**, **23**, and **26**) and epoxidation of monoterpene alcohols by *Streptomyces ikutamanensis* Ya-2-1.

The ability of the fungal spores of *P. digitatum* to biotransform monoterpene alcohols, such as geraniol (**22**) and nerol (**25**), and the mixture of aldehydes, that is, citral (**23** and **26**), has been discovered only very recently by Demyttenaere *et al.*[33–36] Spores of *P. digitatum* were inoculated on solid media. After a short incubation period, the spores germinated and a mycelial mat was formed. After 2 weeks, the culture had completely sporulated and bioconversion reactions were started. Geraniol, nerol, or citral was sprayed onto the sporulated surface culture. After 1 or 2 days, the period during which transformation took place, the cultures were extracted. Geraniol and nerol were transformed into 6-methyl-5-hepten-2-one (**58**) by sporulated surface cultures of *P. digitatum*. Spores retained their activity for at least 2 months. An overall yield of up to 99% could be achieved.

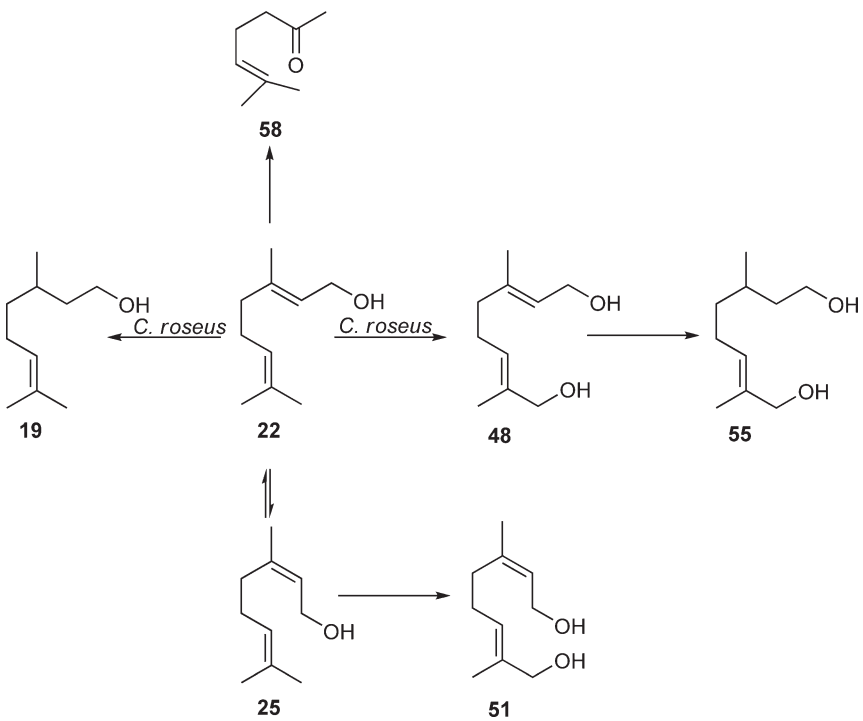
The bioconversion of geraniol (**22**) and nerol (**25**) was also performed with sporulated surface cultures of *A. niger*. Geraniol (**22**) was converted to linalool (**63**), α-terpineol (**80**) (see **Scheme 17**), and limonene (**95**) (see **Scheme 27**), and nerol (**25**) was converted mainly to linalool (**63**) and α-terpineol (**80**).[34]

The biotransformation of geraniol (**22**) and nerol (**25**) by *Catharanthus roseus* suspension cells was carried out. It was found that the allylic positions of geraniol (**22**) and nerol (**25**) were hydroxylated and reduced double



Scheme 10 Biotransformation of citronellol (**19**), geraniol (**22**), and nerol (**25**) by *Botrytis cinerea.*

bonds and ketones (**Scheme 11**). Geraniol (**22**) and nerol (**25**) are isomerized to each other. Geraniol (**22**) and nerol (**25**) were hydroxylated at C-10 to 8-hydroxygeraniol (**48**) and 8-hydroxynerol (**51**), respectively. 8-Hydroxygeraniol (**48**) and geraniol (**22**) were hydrogenated to 10-hydroxycitronellol (**39**) and citronellol (**19**) (**Scheme 11**), respectively.[37]

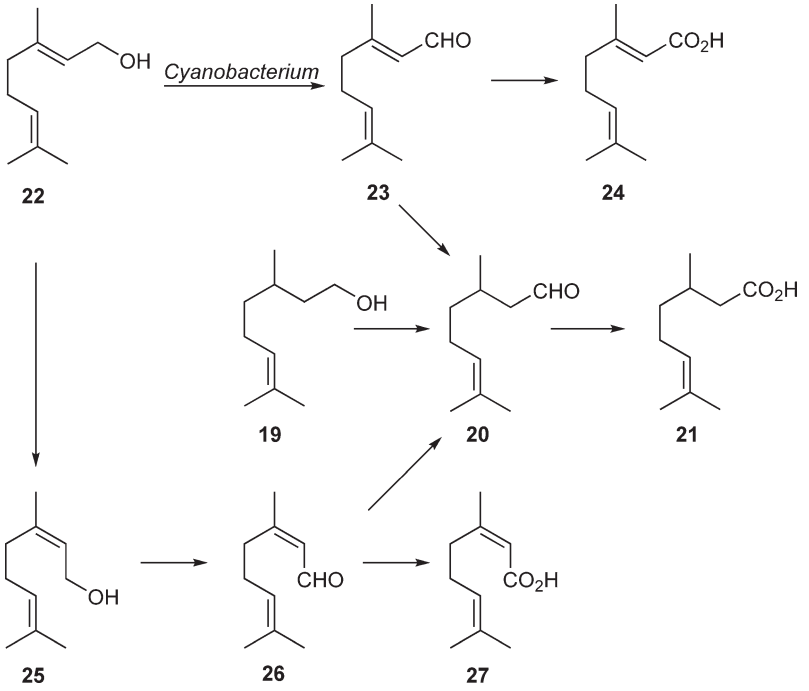


Scheme 11 Biotransformation of geraniol (**22**) and nerol (**25**) by suspension cells of *Catharanthus roseus*.

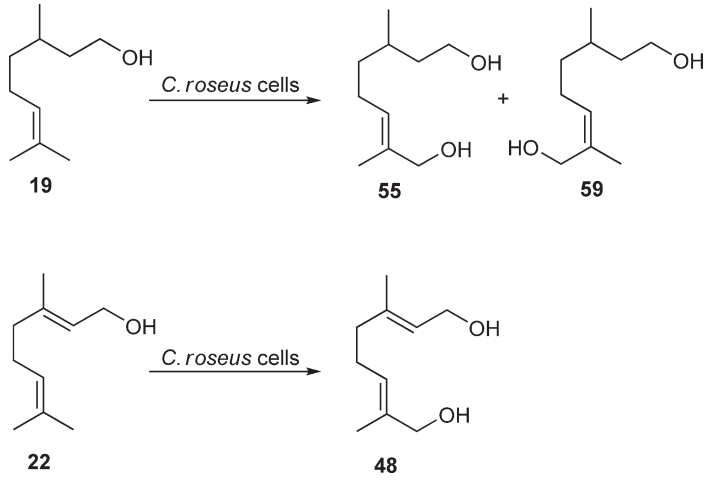
Cyanobacterium converted geraniol (**22**) to geranic acid (**24**) via geranial (**23**), followed by hydrogenation to form citronellic acid (**21**) via citrorellal (**20**). Furthermore, the substrate (**22**) was isomerized to nerol (**25**), followed by oxidation, reduction, and further oxidation to form neral (**26**), citronellal (**20**), citronellic acid (**21**), and nerolic acid (**27**) (**Scheme 12**).[38,39]

Plant suspension cells of *C. roseus* converted geraniol (**22**) to 8-hydroxygeraniol (**48**). The same cells converted citronellol (**19**) to 8- (**55**) and 10-hydrocitronellol (**59, Scheme 13**).[39]

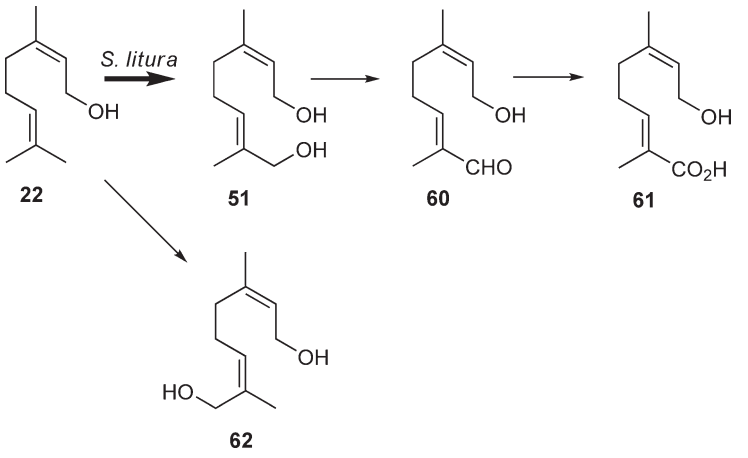
Nerol (**25**) was converted by *S. litura* to 8-hydroxynerol (**51**), 1-hydroxy-3,7-dimethyl-(2*Z*,6*E*)-octadienal (**60**), 1-hydroxy-3,7-dimethyl-(2*E*,6*E*)-octadienoic acid (**61**), and 10-hydroxynerol (**62**) (**Scheme 14**).[40]



Scheme 12 Biotransformation of citronellol (**19**) and geraniol (**22**) by *Cyanobacterium*.



Scheme 13 Biotransformation of citronellol (**19**) and geraniol (**22**) by suspension cells of *Catharanthus roseus*.



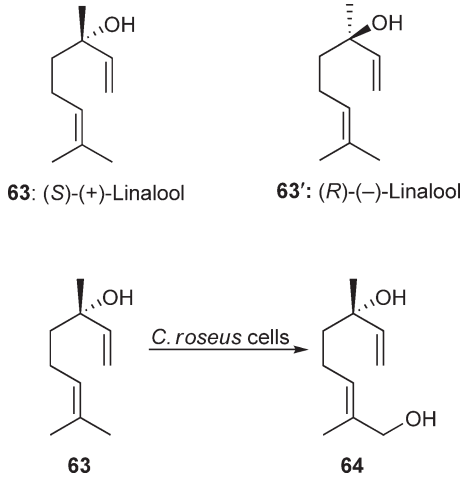
Scheme 14 Biotransformation of nerol (**25**) by *Spodoptera litura*.

3.19.2.1.2(ii) Linalool (63) and linalyl acetate (63 and 63′-Ac) (+)-Linalool (**63**, (*S*)-3,7-dimethyl-1,6-octadiene-3-ol) and its enantiomer (**63′**, (*R*)-3,7-dimethyl-1,6-octadiene-3-ol) occur in many essential oils, where it is often the main component. (*S*)-(+)-Linalool (**63**) makes up 60–70% of coriander oil. (*R*)-(−)-Linalool (**63′**), for example, occurs at a concentration of 80–85% in Ho oils from *Cinnamomum camphora*; rosewood oil contains ~80%.[41]

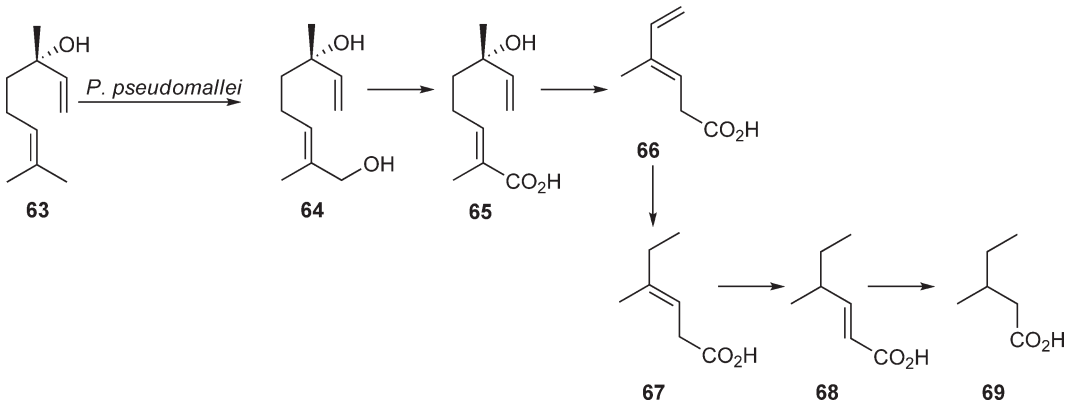
Catharanthus roseus converted (+)-linalool (**63**) to 8-hydroxylinalool (**64**) (**Scheme 15**).[39]

The biodegradation of (+)-linalool (**63**) by *Pseudomonas pseudomallei* (strain A), which grows on linalool as the sole carbon source, was described in 1973 (**Scheme 16**).[42]

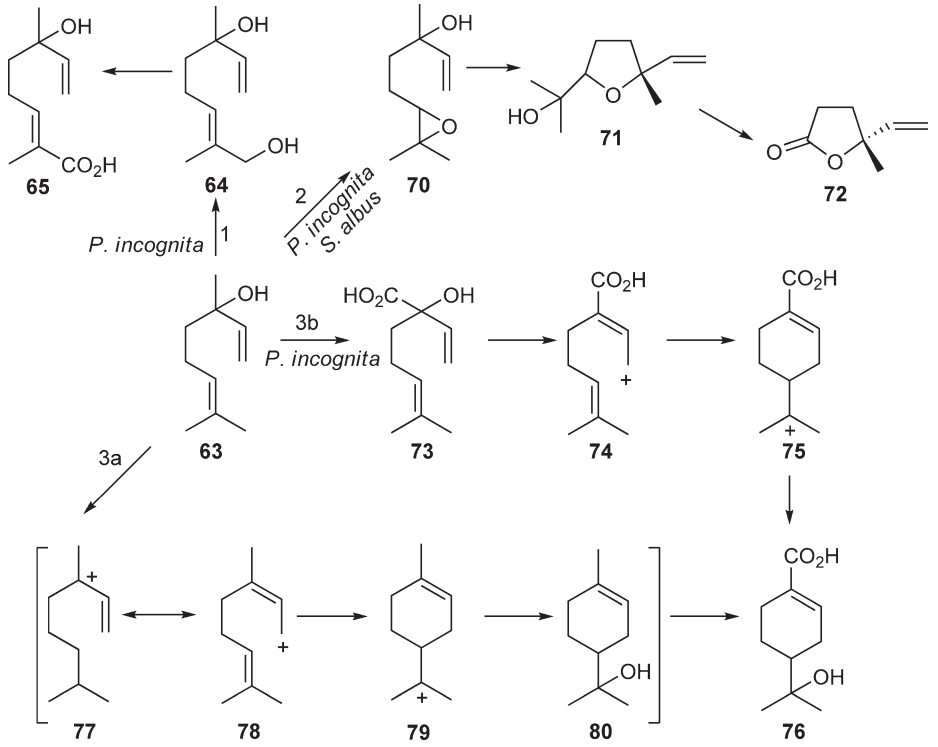
Madyastha *et al.*[16] isolated a soil Pseudomonad, *Pseudomonas incognita*, by an enrichment culture technique with linalool as the sole carbon source. This microorganism, the 'linalool strain' as it was called, was also capable of utilizing limonene (**95**), citronellol (**19**), and geraniol (**22**) but failed to grow on citral (**23** and **26**), citronellal (**20**), and 1,8-cineole (**128**). Fermentation was carried out in shake cultures containing 1% linalool (**63**) as the sole carbon source. It was suggested by the authors that linalool (**63**) was metabolized by at least three different pathways of biodegradation. One of the pathways appeared to be initiated by specific oxygenation of C-8 methyl group of linalool (**63**), leading to the formation of 8-hydroxylinalool (**64**), which was further oxidized to linalool-8-carboxylic acid (**65**). The presence of furanoid linalool oxide (**71**) as shown in **Scheme 17** and 2-methyl-2-vinyltetrahydrofuran-5-one (**72**) as the unsaturated lactone in the fermentation medium suggested



Scheme 15 Biotransformation of linalool (**63**) by suspension cells of *Catharanthus roseus*.



Scheme 16 Degradative metabolic pathway of (+)-linalool (**63**) by *Pseudomonas pseudomallei*.



Scheme 17 Biotransformation of linalool (**63**) by *Pseudomonas incognita* and *Streptomyces albus* NRRL B1865.

another mode of utilization of linalool (**63**). The formation of these compounds was believed to proceed through the epoxidation of the 6,7-double bond giving rise to 6,7-epoxylinalool (**70**), which upon further oxidation yielded furanoid linalool oxide (**71**) and 2-methyl-2-vinyltetrahydrofuran-5-one (**72**).

The presence of oleuropeic acid (**76**) in the fermentation broth suggested a third pathway. Two possibilities were proposed: (1) water elimination giving rise to a monocyclic cation (**79**), yielding α-terpineol (**80**), which upon oxidation gave oleuropeic acid (**76**); (2) oxidation of the C-10 methyl group of linalool (**63** and **63′**) before cyclization, giving rise to oleuropeic acid (**76**). The last pathway was also called the 'prototropic cyclization'.[11]

Racemic linalool (**63** and **63′**) is cyclized into *cis*- and *trans*-linalool oxide by various microorganisms such as *Streptomyces albus* NRRL B1865, *Streptomyces hygroscopicus* NRRL B3444, *Streptomyces cinnamonnensis* ATCC 15413, *Streptomyces griseus* ATCC 10137, and *Beauveria sulfurescens* ATCC 7159.[43]

Aspergillus niger isolated from garden soil biotransformed linalool and its acetates to linalool (**63**), 2,6-dimethyl-2,7-octadiene-1,6-diol (8-hydroxylinalool) (**64a**), α-terpineol (**80**), geraniol (**22**), and some unidentified products in trace amounts.[25,26]

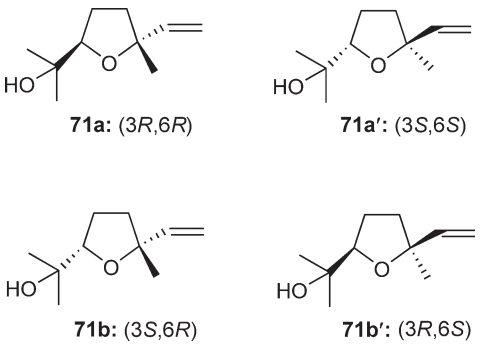
The biotransformation of linalool (**63**) by *B. cinerea* was carried out and transformation products such as (*E*)- (**64a**) and (*Z*)-2,6-dimethyl-2,7-octadiene-1,6-diol (**64b**), *trans*- (**71a**) and *cis*-furanoid linalool oxide (**71b**) (**Scheme 18**), *trans*- (**82a**) and *cis*-pyranoid linalool oxide (**82b**) (**Scheme 19**) and their acetates (**82a-Ac** and **82b-Ac**), 3,9-epoxy-*p*-menth-1-ene (**81**), and 2-methyl-2-vinyltetrahydrofuran-5-one (**72**) (unsaturated lactone) (**Scheme 20**) were identified.[44] Quantitative analysis, however, showed that linalool (**63**) was predominantly (90%) metabolized to (*E*)-2,6-dimethyl-2,7-octadiene-1,6-diol (**64a**) by *B. cinerea*. The other compounds were only found as by-products in minor concentrations.

The bioconversion of both (*S*)-(+)-linalool (**63**) and (*R*)-(−)-linalool (**63′**) was investigated in *D. gossypina* ATCC 10936.[45]

The biotransformation of (±)-linalool (**63** and **63′**) by *A. niger* ATCC 9142 with submerged shaking culture yielded a mixture of *cis*- (**71b**) and *trans*-furanoid linalool oxide (**71a**) (15–24% yield) and *cis*- (**71b**) and *trans*-pyranoid linalool oxide (**82a**) (5–9% yield).[46] The biotransformation of (*R*)-(−)-linalool (**63**) by *A. niger* ATCC 9142 yielded almost pure *trans*-furanoid linalool oxide (**71a**) and *trans*-pyranoid linalool oxide (**82a**) (enantiomeric excess (ee) > 95) (**Scheme 21**). These conversions were purely biocatalytic, since in acidified water (pH < 3.5) almost 50% linalool (**63**) was recovered unchanged and the rest was evaporated. The biotransformation was also carried out with growing surface cultures.

Streptomyces ikutamanensis Ya-2-1 also converted (+)- (**63**), (−)- (**63′**), and racemic linalool (**63** and **63′**) via the corresponding 2,3-epoxides (**70a**, **70b**, and **70ab**) to *trans*- (**71a** and **71b**) and *cis*-furanoid linalool oxides (**71a′** and **71b′**) (**Scheme 22**).[24]

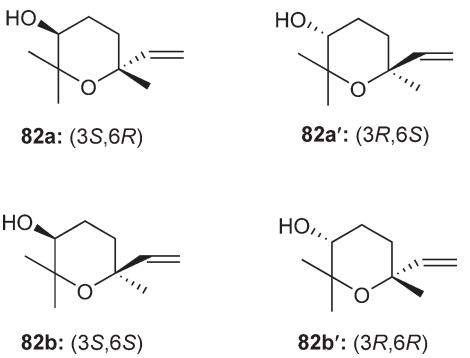
Biotransformation of racemic *trans*-pyranoid linalool oxide (**82a** and **82a′**) and racemic *cis*-pyranoid linalool oxide (**82b** and **82b′**) has been carried out using the fungus *G. cingulata* (**Scheme 23**). *trans*- (**82a**) and



71a: (3*R*,6*R*) **71a′:** (3*S*,6*S*)

71b: (3*S*,6*R*) **71b′:** (3*R*,6*S*)

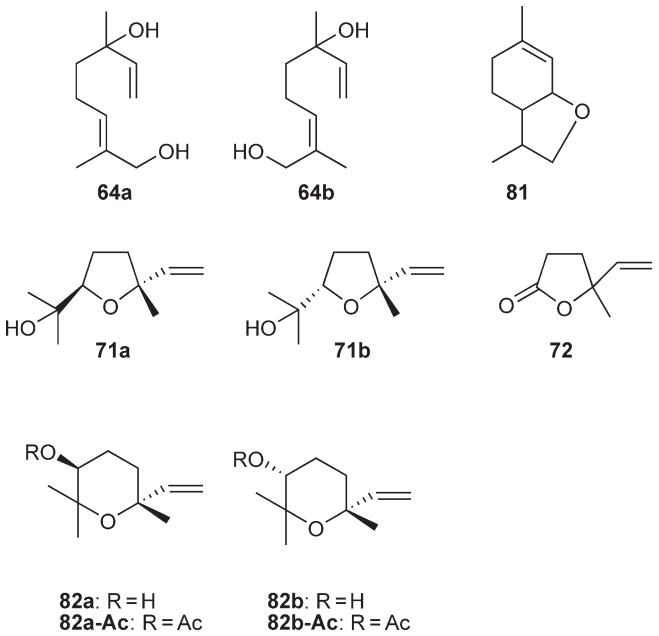
Scheme 18 Four stereoisomers of furanoid linalool oxides (**71a–71b′**).



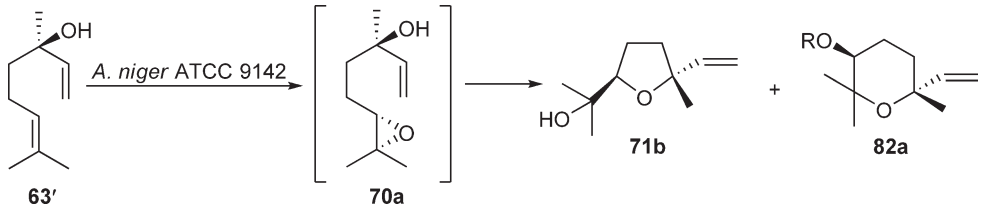
82a: (3*S*,6*R*) **82a′:** (3*R*,6*S*)

82b: (3*S*,6*S*) **82b′:** (3*R*,6*R*)

Scheme 19 Four stereoisomers of pyranoid linalool oxides (**82a–82b′**).



Scheme 20 Biotransformation products from linalool (**63**) by *Botrytis cinerea*.



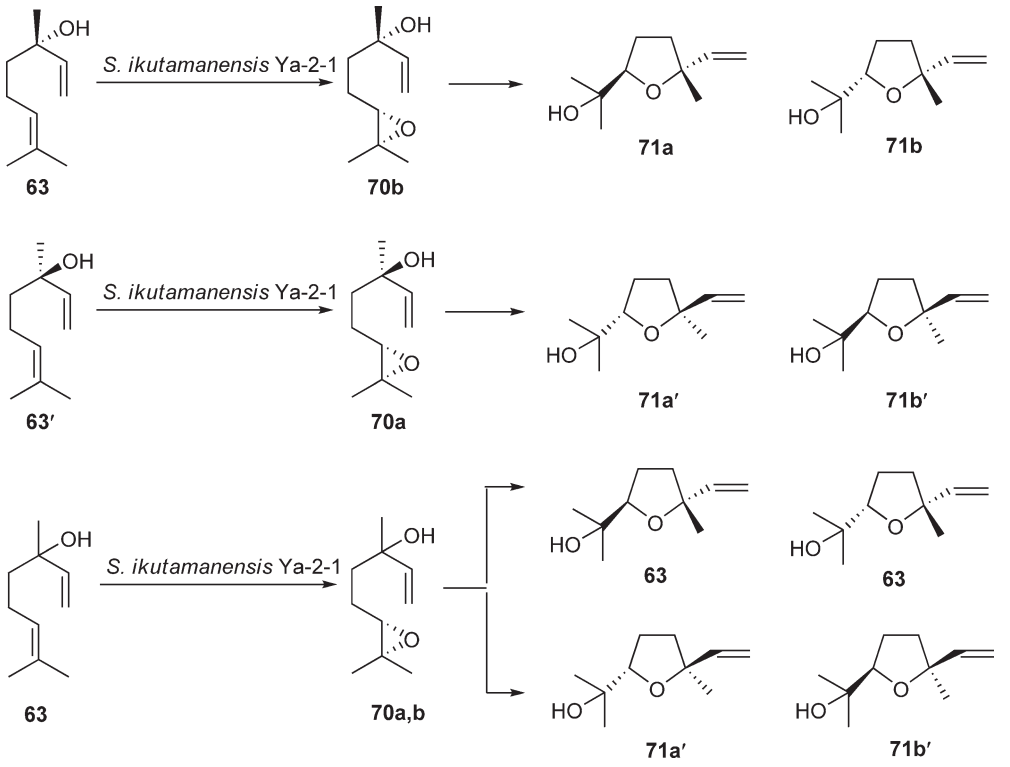
Scheme 21 Biotransformation of (*R*)-(−)-linalool (**63′**) by *Aspergillus niger* ATCC 9142.

cis-Pyranoid linalool oxide (**82b**) were transformed to *trans*- (**83a**) and *cis*-linalool oxide-3-malonate (**83b**), respectively. In the biotransformation of racemic *cis*-linalool oxide-pyranoid, (+)-(3*R*,6*R*)-*cis*-pyranoid linalool oxide (**82a** and **82a′**) was converted to (3*R*,6*R*)-pyranoid-*cis*-linalool oxide-3-malonate (**83**). (−)-(3*S*,6*S*)-*cis*-Pyranoid linalool oxide (**82a**) was not metabolized. On the contrary, in the biotransformation of racemic *trans*-pyranoid linalool oxide (**82b** and **82b′**), (−)-(3*R*,6*S*)-*trans*-linalool oxide (**82b′**) was transformed to (3*R*,6*S*)-*trans*-linalool oxide-3-malonate (**83b′**). (+)-(3*S*,6*S*)-*trans*-Pyranoid linalool oxide (**82b**) was not metabolized. These facts showed that *G. cingulata* recognized the absolute configuration of the secondary hydroxyl group at C-3. On the basis of this result, it has become apparent that the optical resolution of racemic pyranoid linalool oxide proceeded in the biotransformation with *G. cingulata*.[47]

Linalool (**63**) and tetrahydrolinalool (**84**) were converted by suspension cells of *C. roseus* to 1-hydroxylinalool (**64a**) (from linalool (**63**)) and 3,7-dimethyloctane-3,5-diol (**85**), 3,7-dimethyloctane-3,7-diol (**86**), and 3,7-dimethyloctane-3,8-diol (**87**) (from tetrahydrolinalool (**84**)) (**Scheme 24**).[39,48]

(±)-Linalyl acetate (**63** and **63′-Ac**) was hydrolyzed to (+)-(*S*)-linalool (**63**) and (±)-linalyl acetate (**63** and **63′-Ac**) by *Bacillus subtilis*, *Trichoderma* species, *Absidia glauca*, and *Gibberella fujikuroi* as shown in **Scheme 25**. But (±)-dihydrolinalyl acetate (**88**) was not hydrolyzed by the above microorganisms.[49]

3.19.2.1.2(iii) Dihydromyrcenol (90) Dihydromyrcenol (**90**) was converted by *S. litura* to 1,2-epoxydihydromyrcenol (**91**) as the major product and 3β-hydroxydihydromyrcerol (**92**) as the minor product. Dihydromyrcenyl acetate (**89**) was converted to 1,2-dihydroxydihydromyrcenyl acetate (**93**) and



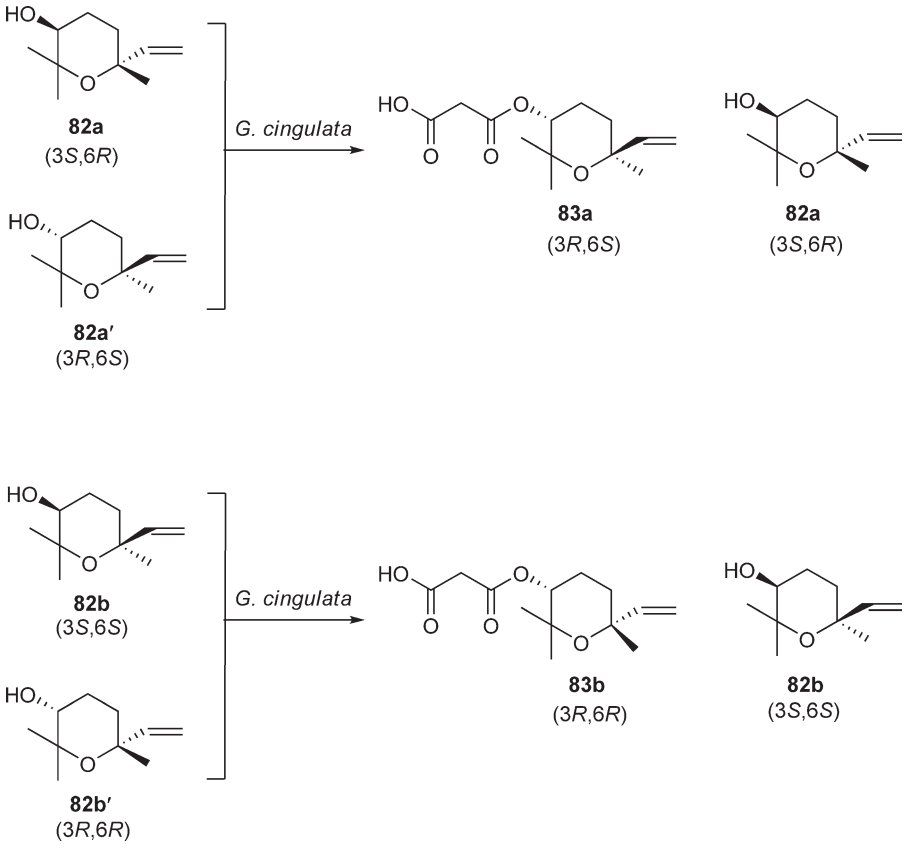
Scheme 22 Metabolic pathway of (*S*)-(+)- (**63**), (*R*)-(−)- (**63′**), and racemic linalool (**63** and **63′**) by *Streptomyces ikutamanensis* Ya-2-1.

3-hydroxydihydromyrcenyl acetate (**94**) together with dihydromyrcenol (**90**), 1,2-epoxydihydromyrcenol (**91**), and 3β-hydroxydidyromyrcerol (**92**) (**Scheme 26**).[50,51]

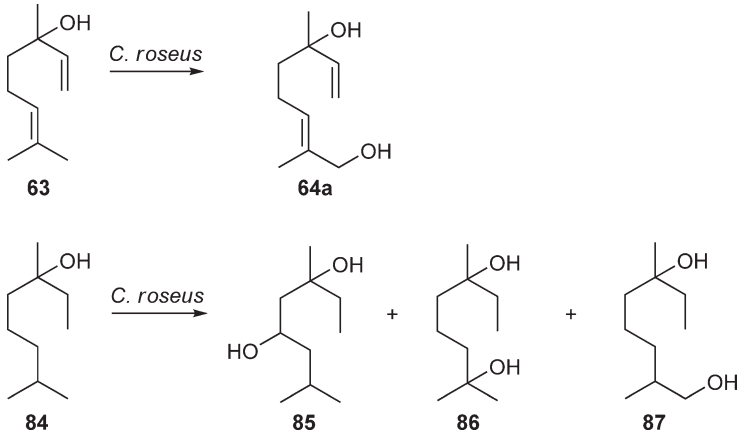
3.19.2.2 Cyclic Monoterpenoids

3.19.2.2.1 Monocyclic monoterpene hydrocarbons

3.19.2.2.1(i) Limonene (95 and 95′) Limonene is the most widely distributed terpene in nature after α-pinene (**130**).[52] (4*R*)-(+)-Limonene (**95**) is present in *Citrus* peel oils at a concentration of over 90%; a low concentration of (4*S*)-(−)-limonene (**95′**) is found in oils from the *Mentha* species and conifers.[41] The first microbial biotransformation of limonene was carried out by using a soil Pseudomonad. The microorganism was isolated by an enrichment culture technique with limonene as the sole source of carbon.[53] The microorganism was also capable of growing on α-pinene (**130**), β-pinene (**377**), 1-*p*-menthene (**139**), and *p*-cymene (**150**). The optimal level of limonene for growth was 0.3–0.6% (v/v) although no toxicity was observed at 2% levels. Fermentation of limonene (**95**) by this bacterium in a mineral salt medium resulted in the formation of a large number of neutral and acidic products such as dihydrocarvone (**105**), carvone (**104**), carveol (**100**), 8-*p*-menthene-1,2-*cis*-diol (**97b**), 8-*p*-menthen-1-ol-2-one (**102**), 8-*p*-menthene-1,2-*trans*-diol (**97a**), and 1-*p*-menthene-6,9-diol (**101**). Perillic acid (**118**), β-isopropenyl pimeric acid (**115**), 2-hydroxy-8-*p*-menthen-7-oic acid (**119**), and 4,9-dihydroxy-1-*p*-menthen-7-oic acid (**114**) were isolated and identified as acidic compounds. Based on these data, three distinct pathways for the catabolism of limonene (**95**) by the soil Pseudomonad were proposed by Dhavalikar *et al.*,[54] involving allylic oxygenation (pathway 1), oxygenation of the 1,2-double bond (pathway 2), and progressive oxidation of the 7-methyl group to perillic acid (**118**) (pathway 3) (**Scheme 27**).[52] Pathway 2 yields (+)-dihydrocarvone (**105**) via the only intermediate limonene epoxide (**96**) and 8-*p*-menthen-1-ol-2-one (**102**) as an oxidation product of limonene-1,2-diol (**97**). The third and main pathway leads to perillyl alcohol (**116**), perillaldehyde (**117**), perillic acid (**118**), constituents of various essential



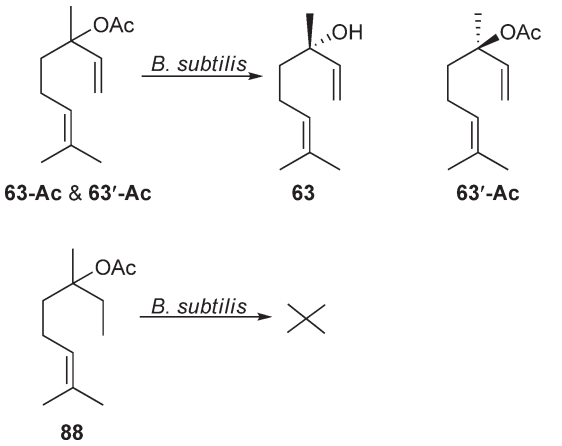
Scheme 23 Biotransformation of racemic *trans*-pyranoid linalool oxide (**82a** and **82a′**) and racemic *cis*-pyranoid linalool oxide (**82b** and **82b′**) by *Glomerella cingulata.*



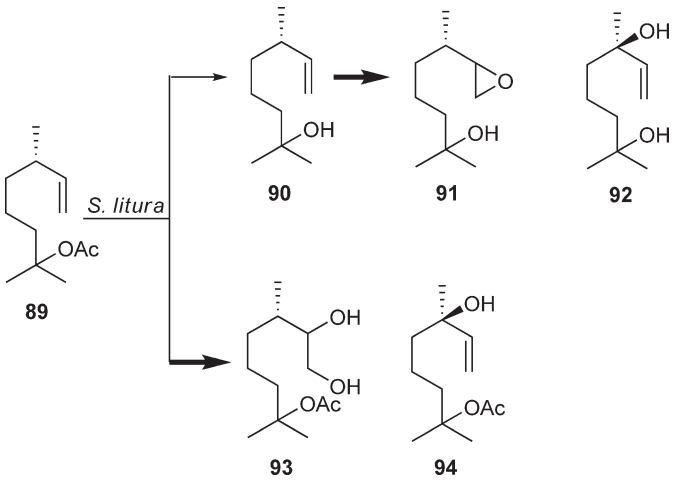
Scheme 24 Biotransformation of linalool (**63**) and tetrahydrolinalool (**84**) by suspension cells of *Catharanthus roseus*.

oils and used in the flavor and fragrance industry,[55] 2-oxo-8-*p*-menthen-7-oic acid (**120**), β-isopropenyl pimelic acid (**115**), and 4,9-dihydroxy-1-*p*-menthen-7-oic acid (**114**).

(+)-Limonene (**95**) was biotransformed by *A. niger* via limonene-1,2-epoxide (**96**) to 8-*p*-menthene-1,2-*trans*-diol (**97a**). On the other hand, the same fungus biotransformed (+)-carvone (**104**) via (−)-isodihydrocarvone (**105**) and 1α-hydroxydihydrocarvone (**102**) to (+)-8-*p*-menthene-1,2-*trans*-diol (**97a**) (**Scheme 28**).[56,57]



Scheme 25 Hydrolysis of (±)-linallyl acetate (**63-Ac**) by *Bacillus subtilis* and other microorganisms.

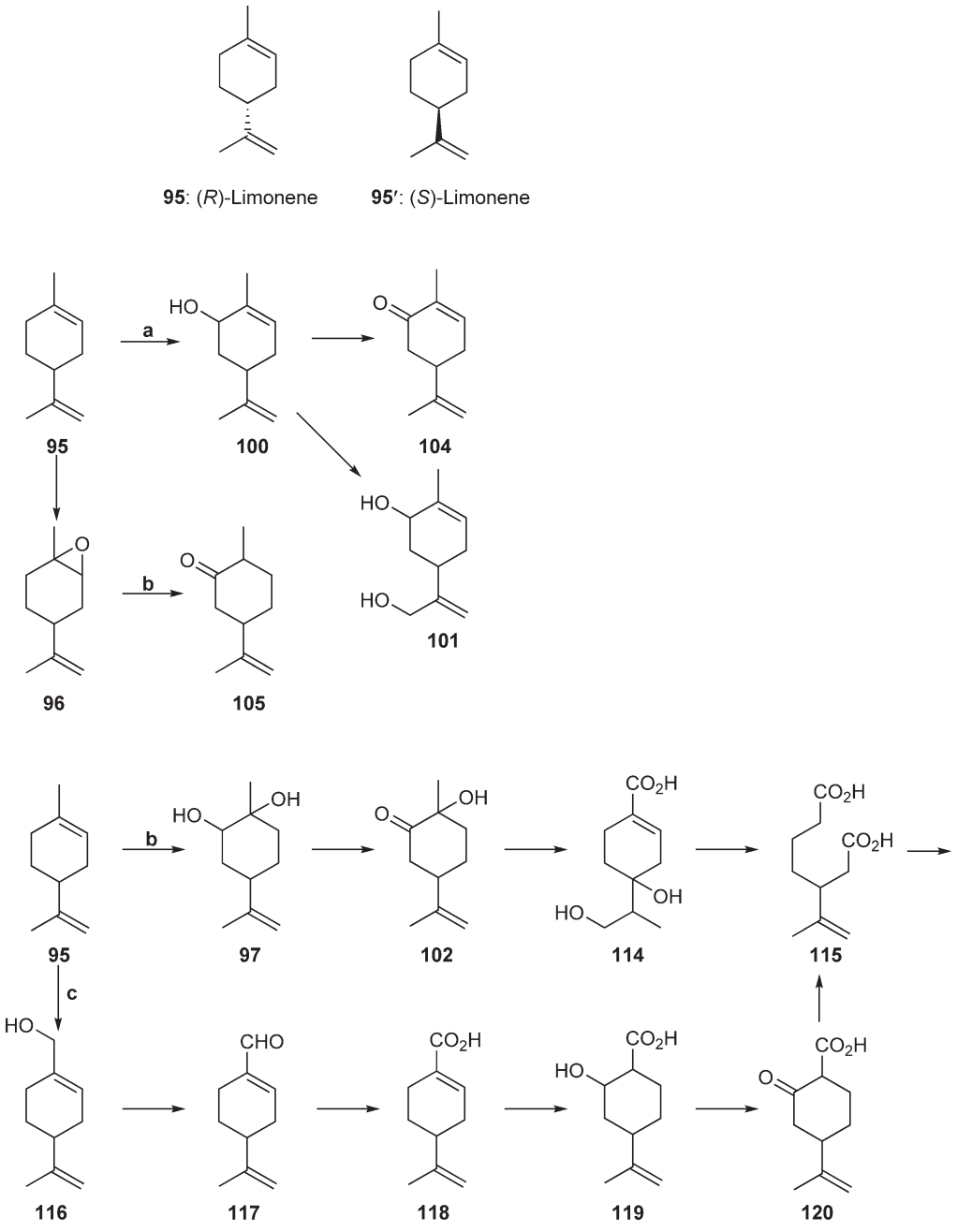


Scheme 26 Biotransformation of dihydromyrcenyl acetate (**89**) and dihydromyrcenol (**90**) by *Spodoptera litura*.

A soil Pseudomonad formed 1-hydroxydihydrocarvone (**102**) and 8-*p*-menthene-1,2-*trans*-diol (**71**) from (+)-limonene (**95**) (**Scheme 28**). Dhavalikar and Bhattacharyya[53] considered that the formation of 1-hydroxydihydrocarvone (**102**) is from dihydrocarvone (**105**).

Pseudomonas gladioli was isolated by an enrichment culture technique from pine bark and sap using a mineral salt broth with limonene as the sole carbon source.[58,59] Fermentation was performed during 4–10 days in shake flasks at 25 °C using a pH 6.5 mineral salt medium and 1.0% (+)-limonene (**95**). The major products were identified as (+)-α-terpineol (**80**) and (+)-perillic acid (**118**). This was the first report of the microbial conversion of limonene to (+)-α-terpineol (**80**).

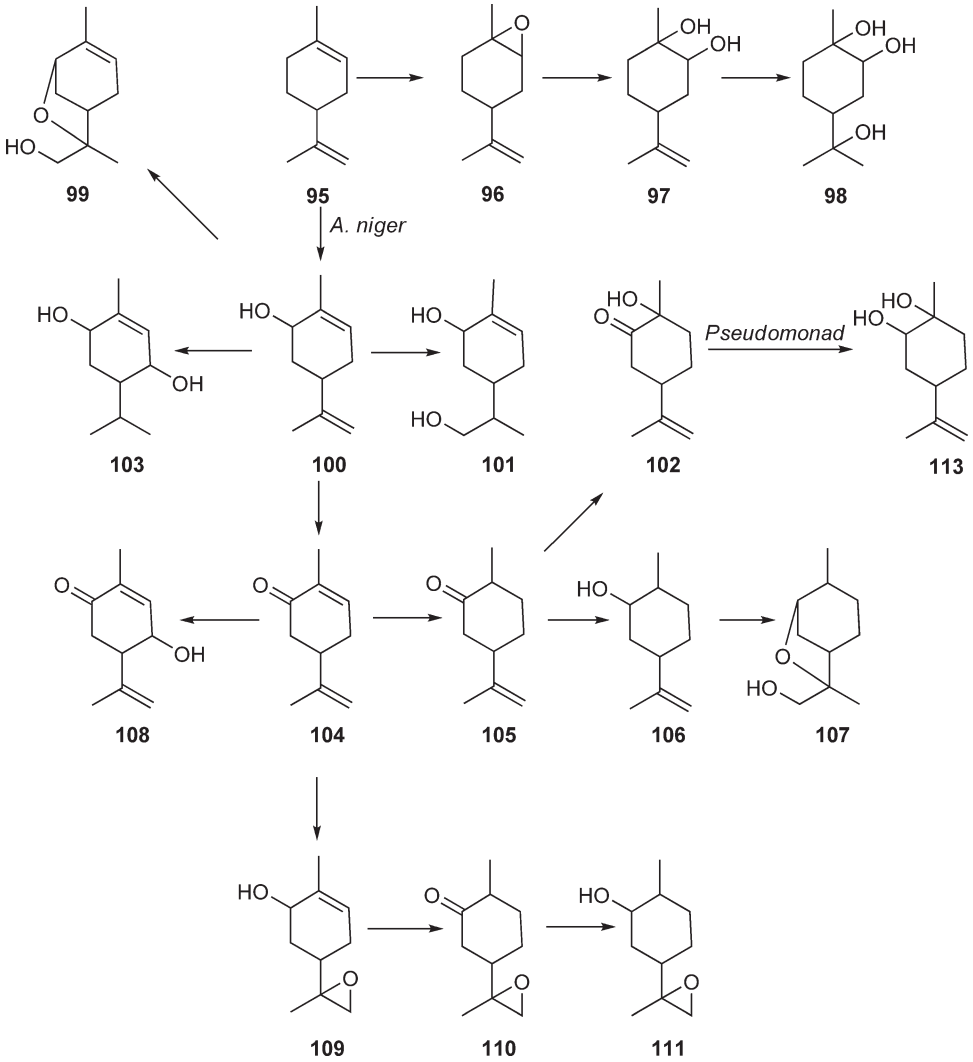
The first data on fungal bioconversion of limonene (**95**) date back to the late 1960s.[60,61] Three soil microorganisms that grew rapidly in a mineral salt medium containing appropriate terpene substrates as the sole carbon sources were isolated. The microorganisms belonged to the class Fungi Imperfecti, and they had been tentatively identified as *Cladosporium* species. One of these strains, designated *Cladosporium* sp. T_7, was isolated on (+)-limonene (**95**). The growth medium of this strain contained 1.5 g l^{-1} *trans*-limonene-1,2-diol (**97a**). Minor quantities of the corresponding *cis*-1,2-diol (**97b**) were also isolated. The same group isolated a fourth microorganism from a terpene-soaked soil on a mineral salt medium containing (+)-limonene as the sole carbon source.[60] The strain, *Cladosporium*, designated T_{12} was capable of converting (+)-limonene (**95**) into an optically active isomer of α-terpineol (**80**) in yields of ~1 g l^{-1}.



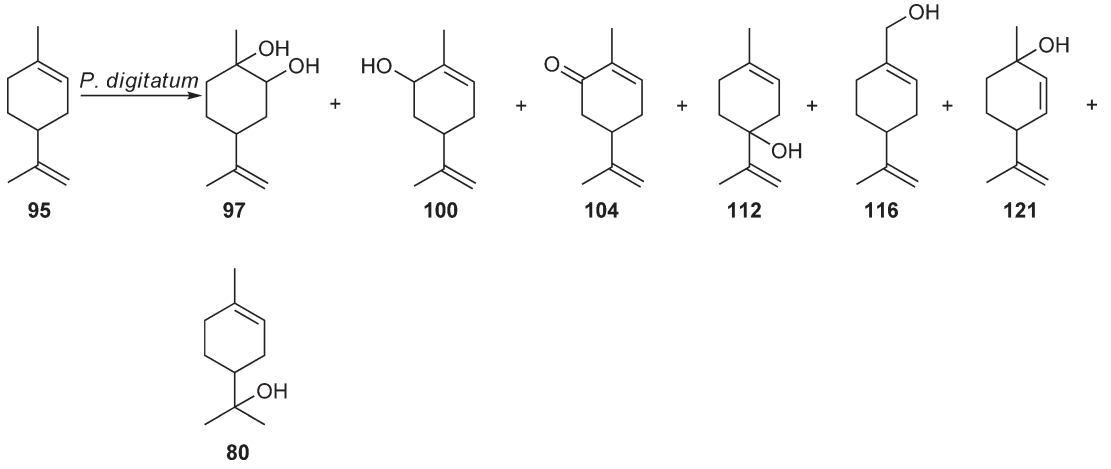
a: Pathway 1
b: Pathway 2
c: Pathway 3 (Main pathway)

Scheme 27 Structures of (*R*)-limonene (**95**) and its enantiomer (**95′**), and degradation pathways of limonene (**95**) by a soil Pseudomonad species strain (L).

α-Terpineol (**122**) was obtained from (+)-limonene (**95**) by biotransformation in fungi such as *P. digitatum*, *P. italicum*, and *Cladosporium* and several bacteria (**Scheme 29**). (+)-*cis*-Carveol (**100**), (4*S*)-(+)-carvone (**104′**) (an important constituent of caraway seed and dill-seed oils[55,62]), and 1-*p*-menthene-6,9-diol (**113**) were also obtained from *P. digitatum* and *P. italicum*. (+)-Carvone (**104′**) is a natural potato sprout-inhibiting, fungistatic,



Scheme 28 Formation of 8-*p*-menthene-1,2-*trans*-diol (**97**) from the biotransformation of (+)-limonene (**95**) and (+)-carvone (**104′**) by *Aspergillus niger* TBUYN-2.



Scheme 29 Biotransformation products of limonene (**95**) by *Penicillium digitatum* and *Penicillium italicum*.

and bacteriostataic compound.[63,64] It is important to note that the microbial transformation of (−)-carvone (**104**, the 'spearmint flavor') has not yet been described.[52] However, the biotransformation of limonene to (−)-carvone (**104**) was patented by a Japanese group:[65] a *Corynebacterium* species grown on limonene was able to produce about 10 mg l^{-1} of 99% pure (−)-carvone (**104**) in 24–48 h.

Mattison *et al.*[66] isolated *Penicillium* sp. from rotting orange rind that utilized limonene (**95**) and converted it rapidly to α-terpineol (**80**). Bowen[67] isolated two common *Citrus* molds, *P. italicum* and *P. digitatum*, responsible for the post harvest diseases of *Citrus* fruits. Fermentation of *P. italicum* on limonene (**95**) yielded *cis*- (**100b**) and *trans*-carveol (**100a**) (26%) as the major products, together with *cis*- and *trans*-*p*-mentha-2,8-dien-1-ol (**121**) (18%), (+)-carvone (**104**) (6%), *p*-mentha-1,8-dien-4-ol (**112**) (4%), perillyl alcohol (**116**) (3%), and 8-*p*-menthene-1,2-diol (**97**) (3%). Conversion of **95** by *P. digitatum* yielded the same products in lower yields (**Scheme 29**).

The biotransformation of limonene (**95**) by *A. niger* is a very important example of fungal bioconversion because limonene is one of the most abundant monocyclic monoterpenes in the plant kingdom and both (+)- and (−)-enantiomers are commercially available. Screening for fungi capable of metabolizing the bicyclic monoterpene hydrocarbon α-pinene (**130**) yielded a strain of *A. niger* NCIM 612 that was also able to transform limonene (**95**). This fungus was able to carry out three types of oxygenative rearrangements α-terpineol (**80**), carveol (**100**), and *p*-mentha-2,8-dien-1-ol (**121, 122**) (**Scheme 30**).[68] In 1985, Abraham *et al.*[2] investigated the biotransformation of (*R*)-(+)-limonene (**95**) by the fungus *P. digitatum*. A complete transformation of the substrate to α-terpineol (**80**) by *P. digitatum* DSM 62840 was obtained with 46% yield of pure product.

The production of glycols from limonene (**95**) and other terpenes with a 1-menthene skeleton was reported in *Corynespora cassiicola* DSM 62475 and *D. gossypina* ATCC 10936.[69] Accumulation of glycols during fermentation was observed. An extensive overview of the microbial transformations of terpenoids with a 1-*p*-menthene skeleton was published by Abraham *et al.*[70]

A list of limonene (**95**) and related compounds obtained as metabolites is given in **Scheme 31**.

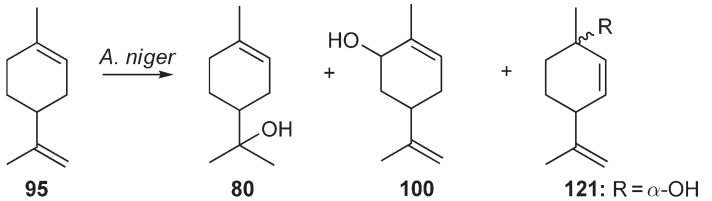
The biotransformation of (+)-limonene (**95**) was carried out by using *Aspergillus cellulosae* M-77.[71] It is important to note that (+)-limonene (**95**) was mainly converted to a new metabolite (+)-isopiperitenone (**123**) (19%) together with (1*S*,2*S*,4*R*)-(+)-limonene-1,2-*trans*-diol (**97a**) (21%), (+)-*cis*-carveol (**100b**) (5%), and (+)-perillyl alcohol (**116a**) (12%) (**Scheme 32**).

(+)-Limonene (**95**) was biotransformed by a kind of *Citrus* pathogenic fungus, *P. digitatum* (Pers.; Fr.) Sacc. KCPYN, to isopiperitenone (**123**, 7% GC ratio), 2α-hydroxy-1,8-cineole (**124b**, 7%), (+)-limonene-1,2-*trans*-diol (**97a**, 6%), and (+)-*p*-menthane-1β,2α,8-triol (**98a**, 45%) as the major products and (+)-*trans*-sobrerol (**125a**, 2%), (+)-*trans*-carveol (**100a**), (+)-carvone (**104′**), (−)-isodihydrocarvone (**105a**), and (+)-*trans*-isopiperitenol (**126a**) as the minor products (**Scheme 33**).[72]

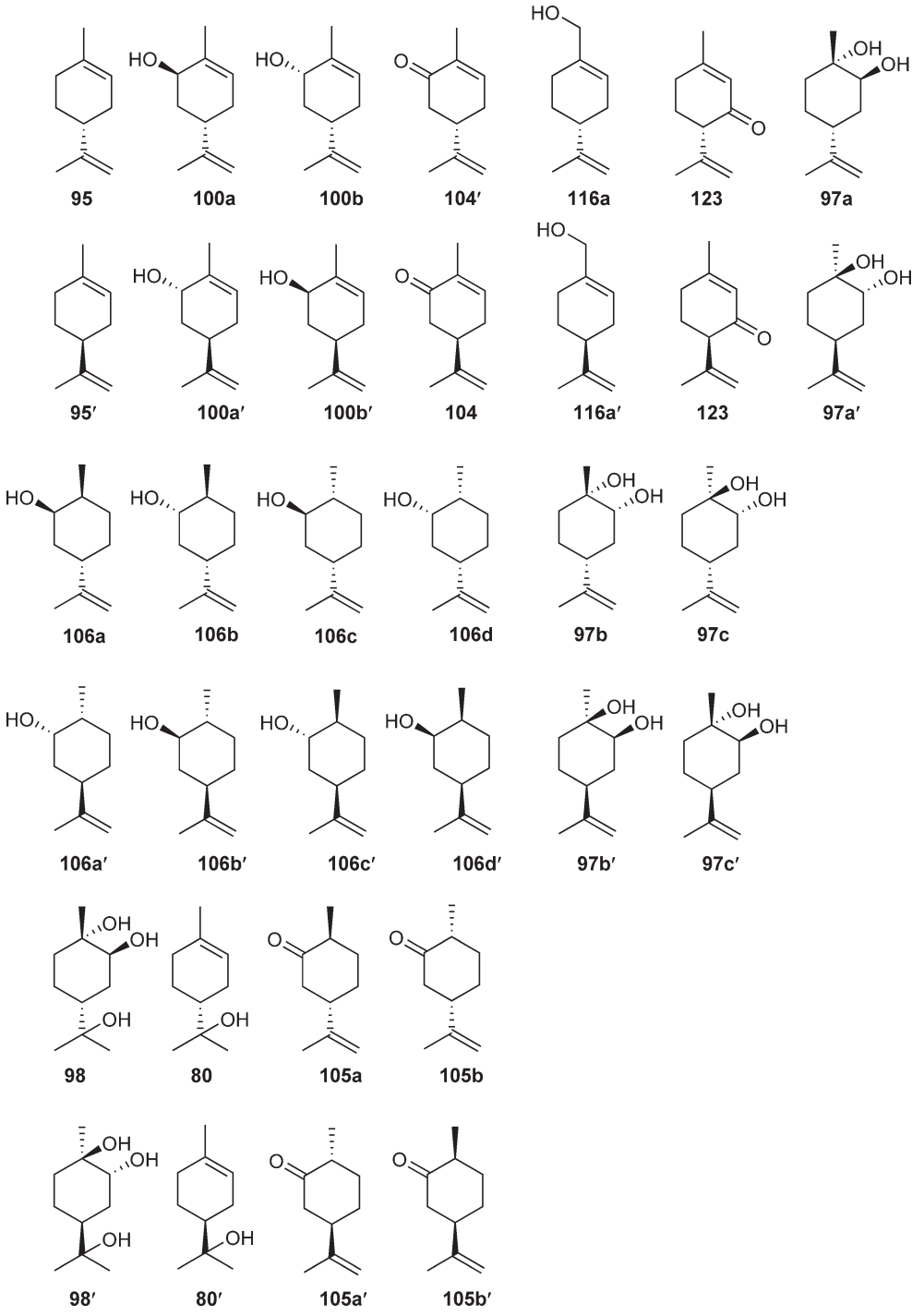
The metabolic pathways of (+)-limonene by *P. digitatum* are shown in **Scheme 34**.

On the other hand, (−)-limonene (**95′**) was also biotransformed by the *Citrus* pathogenic fungus *P. digitatum* (Pers.; Fr.) Sacc. KCPYN to isopiperitenone (**123′**), 2α-hydroxy-1,8-cineole (**124b′**), (−)-limonene-1,2-*trans*-diol (**97a′**), *p*-menthane-1,2,8-diol (**125a′**), and terpineol (**80′**) as the major products together with (+)-*trans*-sobrerol (**125′**), (+)-*trans*-carveol (**100a′**), (−)-carvone (**104**), (−)-dihydrocarvone (**105′**), and (+)-isopiperitenol (**126a′**) as the minor products (**Scheme 35**).[72,73]

A newly isolated unidentified red yeast converted (+)-limonene (**95**) mainly to (+)-limonene-1,2-*trans*-diol (**97a**), (+)-*trans*-carveol (**100a**), (+)-*cis*-carveol (**100b**), and (+)-carvone (**104′**) together with (+)-limonene-1,2-*cis*-diol (**97b**) as the minor product (**Scheme 36**).[74]

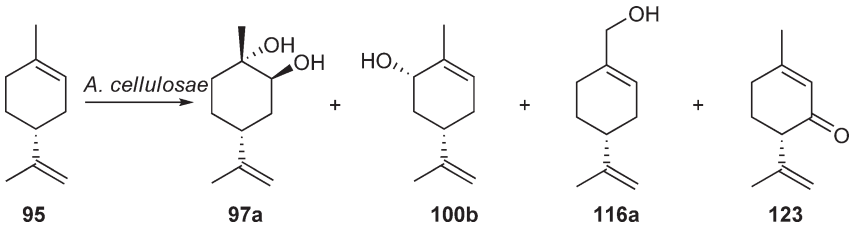


Scheme 30 Biotransformation of limonene (**95**) by *Aspergillus niger* NCIM 612.

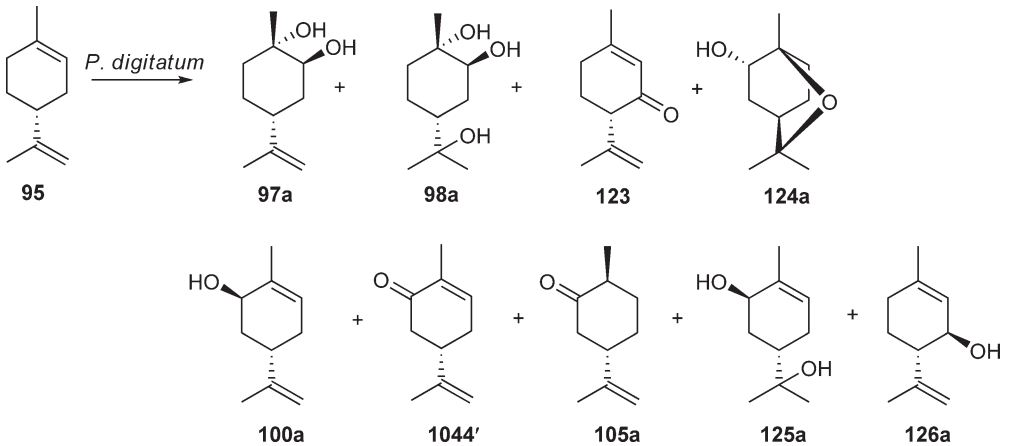


Scheme 31 List of limonene and related compounds as substrates for the biotransformation.

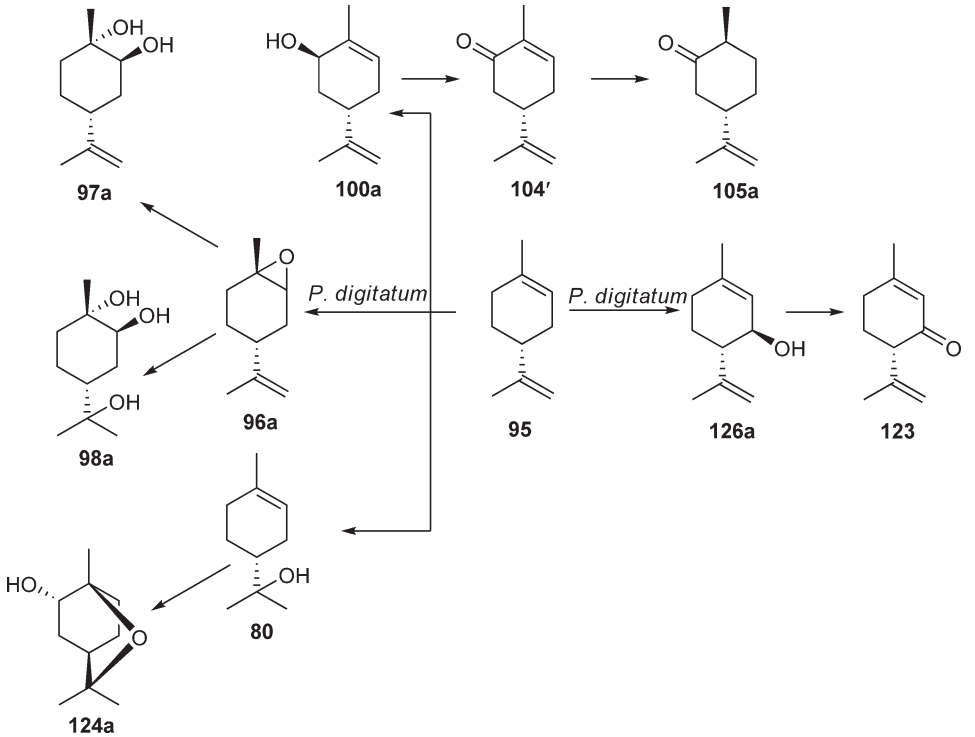
Cladosporium sp. T_7 was cultivated with (+)-limonene (**95**) as the sole carbon source, and it converted **95** to *trans-p*-menthane-1,2-diol (**97a**) (**Scheme 36**).[75] On the other hand, the same red yeast converted (−)-limonene (**95′**) mainly to (−)-limonene-1,2-*trans*-diol (**97a′**), (−)-*trans*-carveol (**100a′**), (−)-*cis*-carveol (**100b′**), and (−)-carvone (**104**) together with (−)-limonene-1,2-*cis*-diol (**97b′**) as the minor product (**Scheme 37**).[74]



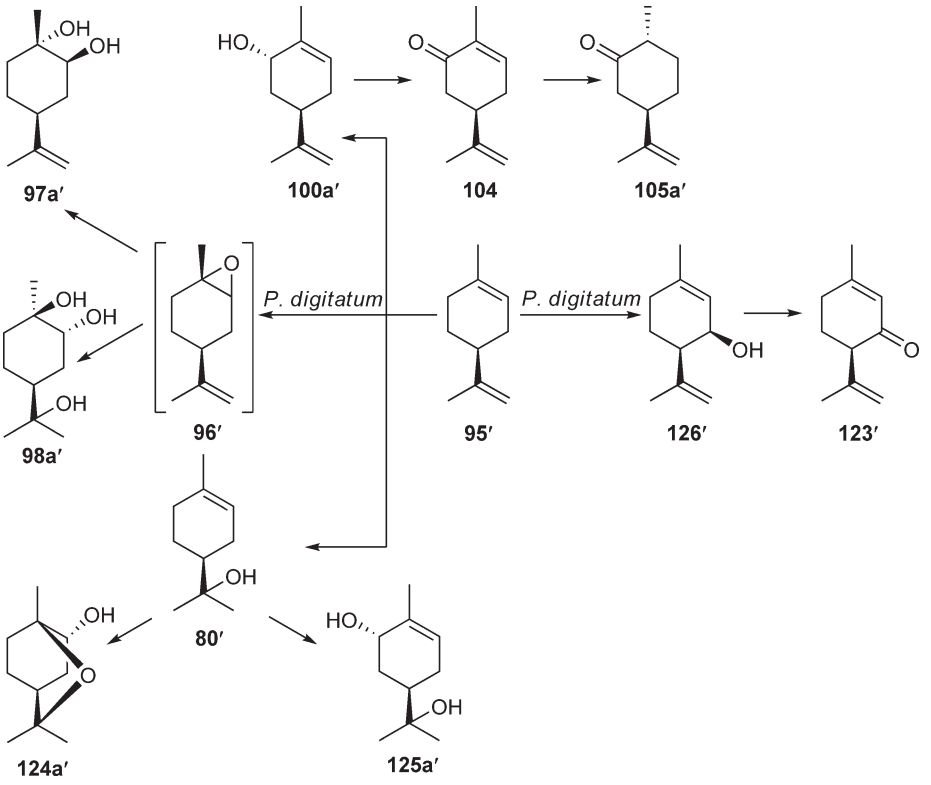
Scheme 32 Biotransformation of (+)-limonene (**95**) by *Aspergillus cellulosae* IFO4040.



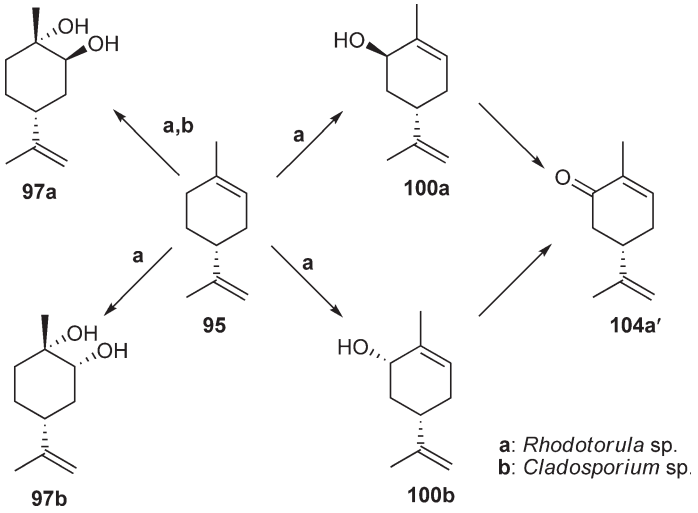
Scheme 33 Metabolites of (+)-limonene (**95**) by the *Citrus* pathogenic fungus *Penicillium digitatum* (Pers.; Fr.) Sacc. KCPYN.



Scheme 34 Biotransformation of (+)-limonene (**95**) by the *Citrus* pathogenic fungus *Penicillium digitatum* (Pers.; Fr.) Sacc. KCPYN.

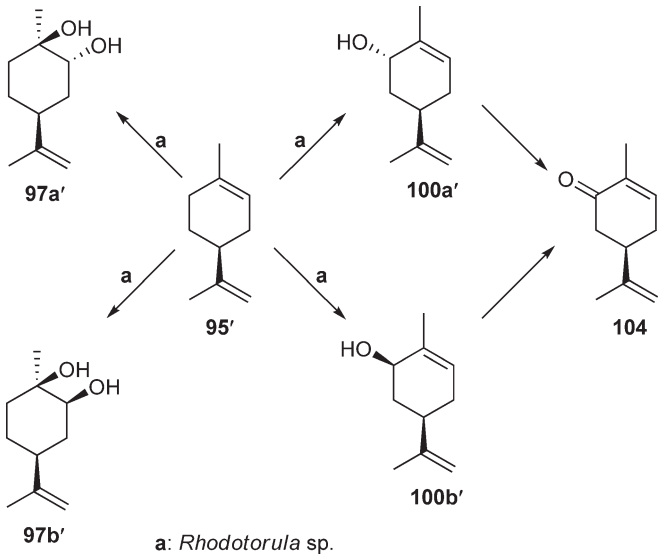


Scheme 35 Biotransformation of (−)-limonene (**95′**) by the *Citrus* pathogenic fungus *Penicillium digitatum* (Pers.; Fr.) Sacc. KCPYN.



Scheme 36 Biotransformation of (+)-limonene (**95**) by the red yeast *Rhodotorula* and *Cladosporium* species T_7.

3.19.2.2.1(ii) Limonene-1,2-epoxide (96a and 96b) The biotransformation of (+)- and (−)-limonene (**95** and **95′**), (+)- and (−)-α-terpineol (**80** and **80′**), (+)- and (−)-limonene-1,2-epoxide (**96a** and **96b**), and caraway oil was carried out by the *Citrus* pathogenic fungus *P. digitatum* (Pers.; Fr.) Sacc. KCPYN and a newly isolated red yeast, a kind of *Rhodotorula* sp. *Penicillium digitatum* KCPYN converted limonenes (**95** and **95′**) to the corresponding isopiperitone (**123** and **123′**), 1α-hydroxy-1,8-cineol (**124b** and **124a′**), limonene-1,2-*trans*-diol



Scheme 37 Biotransformation of (−)-limonene (**95′**) by *Rhodotorula* species.

(**97a** and **97a′**), *p*-menthane-1,2,8-triol (**98** and **98′**), and *trans*-sobrerol (**125a′**) as the main products. (−)-α-Terpineol (**80** and **80′**) was the precursor of 2α-hydroxy-1,8-cineol (**124a** and **124a′**) and *p*-menthane-1,2,8-triol (**98** and **98′**). (+)- and (−)-Limonene-1,2-epoxide (**96b** and **96a**) were also the precursors of limonene-1,2-*trans*-diol (**97a**). *Rhodotorula* sp. also biotransformed (+)- and (−)-limonene (**95** and **95′**) to the corresponding *trans*- and *cis*-carveol (**100a′** and **100b′**) as the major products. This microbe also converted caraway oil, which is an equal mixture of (+)-limonene (**95**) and (+)-carvone (**104**). (+)-Limonene (**95**) disappeared and (+)-carvone (**104**) was produced and accumulated in the culture broth.[74]

(4*S*)-(−)- (**95′**) and (4*R*)-(+)-Limonene (**95**) and their epoxides (**96** and **96′**) were incubated with *Cyanobacterium*. It was found that the transformation was enantio- and regioselective. *Cyanobacterium* biotransformed only (4*S*)-limonene (**95′**) to (−)-*cis*- (**100b′**, 11.1%) and (−)-*trans*-carveol (**100a′**, 5%) in a low yield. On the other hand, (4*R*)-limonene oxide (**96b**) was converted to limonene-1,2-*trans*-diol (**97a′**) and 1-hydroxy-(+)-dihydrocarvone (**102a**). However, (4*R*)-(+)-limonene (**95**) and (4*S*)-limonene oxide (**96a**) were not converted at all (**Scheme 38**).[76]

(+)-Limonene (**95**) was transformed mainly to (+)-*p*-menth-1-ene-8,9-diol (uroterpenol, **127**) and (+)-*p*-meththa-1,8-diene-7-oic acid (perillic acid, **118**) by *S. litula* larvae. Similarly, (−)-limonene (**95′**) was converted by the same insect larvae as mentioned above mainly to (−)-*p*-menth-1-ene-8,9-diol (uroterpenol, **127′**) and (−)-*p*-mentha-1,8-dien-7-oic acid (perillic acid, **118′**) (**Scheme 39**).[77]

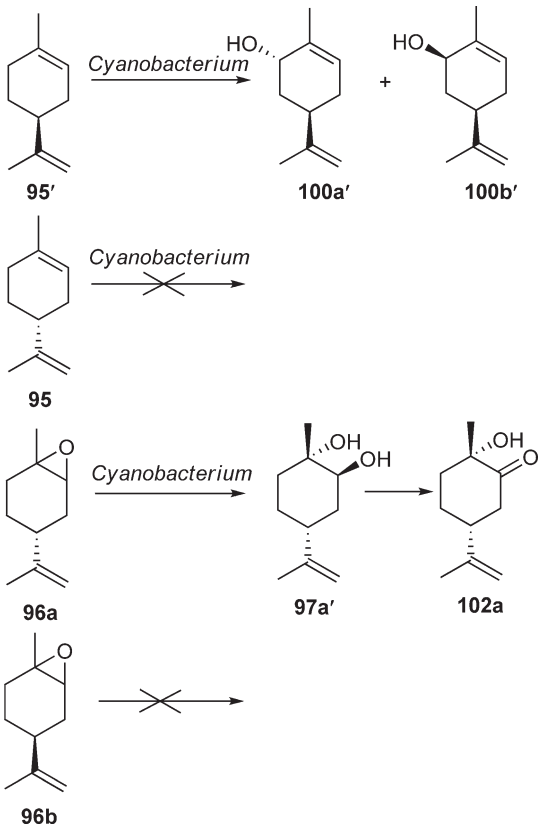
Kieslich *et al.*[78] found a nearly complete microbial resolution of a racemate in the biotransformation of (±)-limonene by *P. digitatum* DSM 62840. The (*R*)-(+)-limonene (**95**) is converted to the optically active (+)-α-terpineol (**80**), $[\alpha]_D = +99°$, while the (S)-(−)-limonene (**95′**) is presumably adsorbed onto the mycelium or degraded via unknown pathways (**Scheme 40**).[78]

(+)-Limonene (**95**) is metabolized by liver microsomes to (+)-*trans*-carveol (**100a**) and (+)-perillyl alcohol (**116a**) (**Scheme 41**).[79]

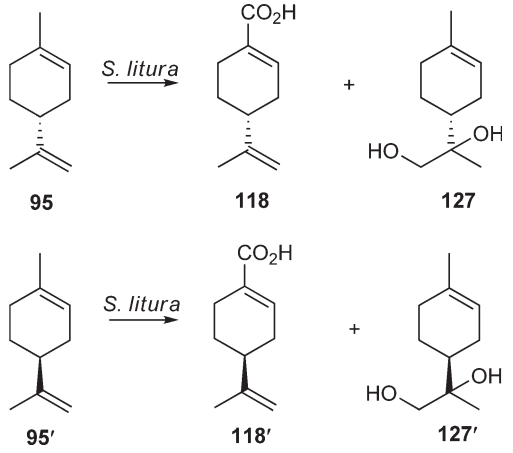
(4*S*)- and (4*R*)-Limonene epoxides (**96a′** and **96a**) were biotransformed by *Cyanobacterium* to 8-*p*-menthene-1α,2β-ol (**97a**, 68.4%) and 1α-hydroxy-8-*p*-menthen-2-one (**102b**, 31.6%) (**Scheme 42**).[76]

The mixture of (+)-*trans*- (**96a**) and *cis*- (**96b**) and the mixture of (−)-*trans*- (**96a′**) and *cis*-limonene-1,2-epoxide (**96b′**) were biotransformed by the *Citrus* pathogenic fungus *P. digitatum* (Pers.; Fr.) Sacc. KCPYN to (1*R*,2*R*,4*R*)-(−)-*trans*- (**97c′**) and (1*S*,2*S*,4*S*)-(+)-8-*p*-menthene-1,2-*trans*-diol (**97c**) and (−)-*p*-menthane-1,2,8-triols (**98b** and **98b′**) (**Scheme 43**).[73]

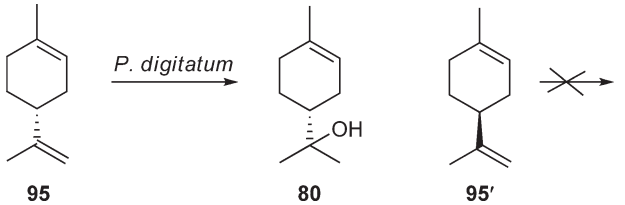
Biotransformation of 1,8-cineole (**128**) by *A. niger* gave racemic 2α-hydroxy-1,8-cineole (**124a** and **124a′**).[22] When racemic 2α-hydroxy-1,8-cineole (**124a** and **124a′**) was biotransformed by *G. cingulata*, only



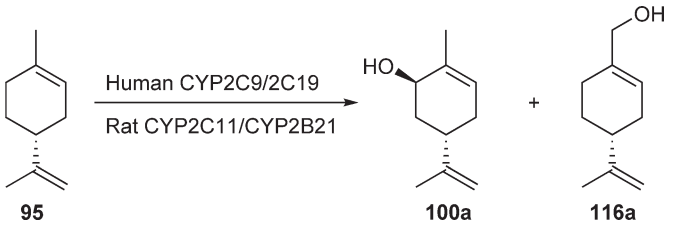
Scheme 38 Biotransformation of (+)- (**95**) and (−)-limonene (**95′**) and limonene epoxide (**96a** and **96b**) by *Cyanobacterium*.



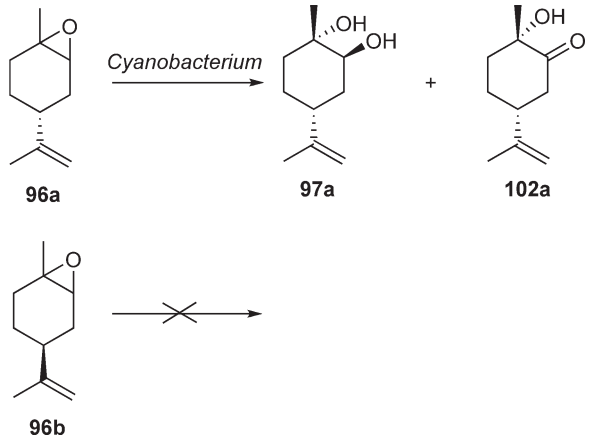
Scheme 39 Biotransformation of (+)- (**95**) and (−)-limonenes (**95′**) by *Spodoptera litura*.



Scheme 40 Microbial resolution of racemic limonenes (**95** and **95′**) and formation of optically active α-terpineol (**80**) by *Penicillium digitatum*.



Scheme 41 Metabolism of (+)-limonene (**95**) by human and rat P-450 enzymes.



Scheme 42 Enantioselective biotransformation of (4*R*)- (**96a**) and (4*S*)-limonene epoxides (**96b**) by *Cyanobacterium*.

(−)-2α-hydroxy-1,8-cineole (**124a′**) was selectively esterified with malonic acid to form its malonate (**129a′**). The malonate was hydrolyzed to form optically pure **124a**.[80] On the other hand, the *Citrus* pathogenic fungus *P. digitatum* biotransformed limonene (**95**) to optically pure **124a** (**Scheme 44**).[74]

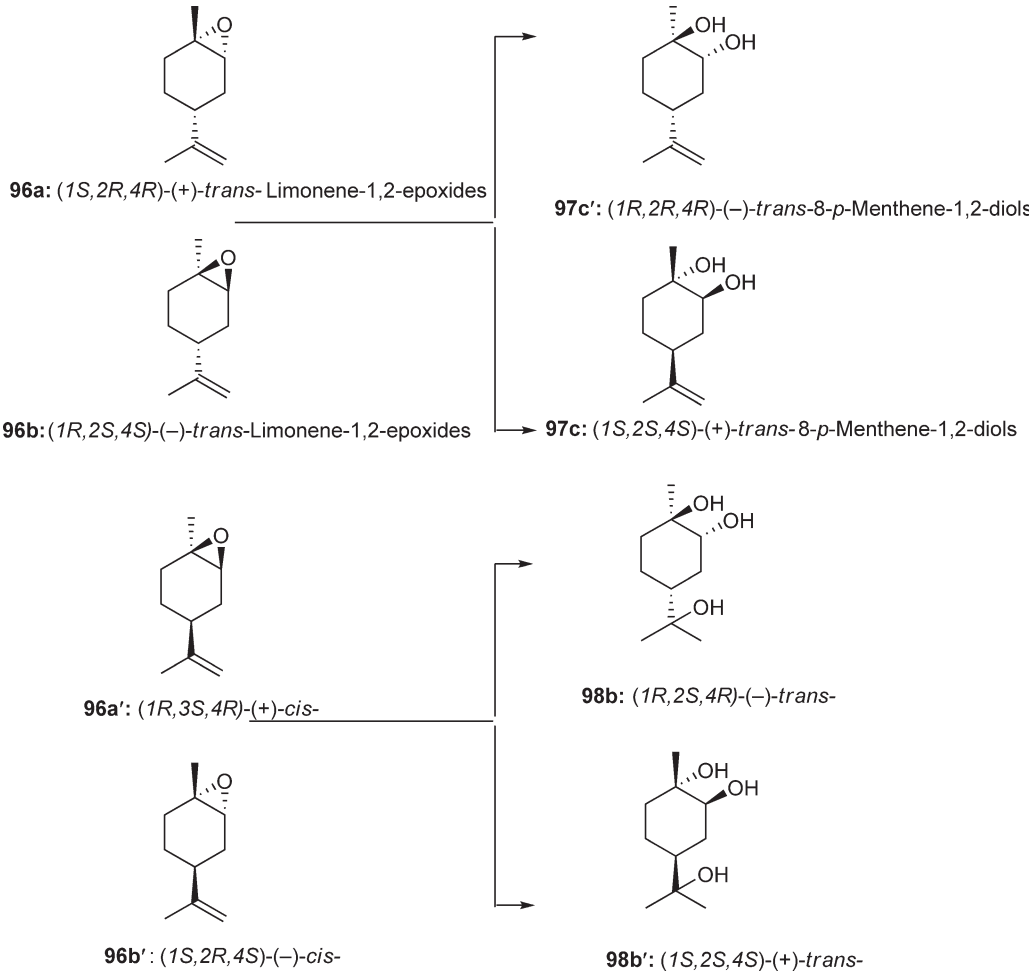
When (*R*)-limonene (**95**), (*S*)-limonene (**95′**), (1*S*,5*R*)-α-pinene (**130**), (1*R*,5*R*)-α-pinene (**130′**), and (1*R*,6*R*)-3-carene (**132**) were administered to the cultured cells of *Nicotiana tabacum*, they were enantio- and stereoselectively converted to the corresponding epoxides (**96a**, **96b**, and **133**) in the presence of hydrogen peroxide and *p*-cresol by a radical mechanism (**Scheme 45**).[81]

The enzyme (p38) concerned with the epoxidation reaction was purified from the cultured cells by cation-exchange chromatography. The enzyme not only had epoxidation activity as shown in **Scheme 46**,[81] but also peroxidase activity. The amino acid sequence of p38 showed 89% homology in its nine amino acid overlap with horseradish peroxidase.

3.19.2.2.1(iii) Isolimonene (134) *Spodoptera litura* converted (1*R*)-*trans*-isolimonene (**134**) to (1*R*,4*R*)-*p*-menth-2-ene-8,9-diol (**135**) (**Scheme 47**).[82]

3.19.2.2.1(iv) p-Menthane (136a and 136b) Hydroxylation of *trans*- and *cis*-*p*-menthane (**136a** and **136b**) by microorganisms is also very interesting from the viewpoint of the formation of important perfumes such as (−)-menthol (**33b**) and (−)-carvomenthol (**247b′**), plant growth regulators, and mosquito repellents such as *p*-menthane-*trans*-3,8-diol (**191b**), *p*-menthane-*cis*-3,8-diol (**191a′**),[23] and *p*-menthane-2,8-diol (**142a**).[61] *Pseudomonas mendocina* strain SF biotransformed **136b** stereoselectively to *p*-*cis*-menthan-1-ol (**137**) (**Scheme 48**).[83]

On the other hand, the biotransformation of the mixture of *p*-*trans*- (**136a**) and *cis*-menthane (**136b**) (45:55, peak area in GC) by *A. niger* gave *p*-*cis*-menthane-1,9-diol (**138**) via *p*-*cis*-menthan-1-ol (**137**). No metabolite was obtained from **136a** (**Scheme 48**).[84]



Scheme 43 Biotransformation of (+)-*trans*- (**96a**), (+)-*cis*- (**96b**), (–)-*trans*- (**96a′**), and (–)-*cis*-limonene-1,2-epoxide (**96b′**) by the *Citrus* pathogenic fungus *Penicillium digitatum* (Pers.; Fr.) Sacc. KCPYN.

3.19.2.2.1(v) 1-p-Menthene (139) Concentrated cell suspension of *Pseudomonas* sp. strain (PL) was inoculated on medium containing 1-*p*-menthene (**139**) as the sole carbon source.[85] Compound **139** was degraded to β-isopropyl pimelic acid (**140**) and methylisopropyl ketone (**141**) (**Scheme 49**).[85]

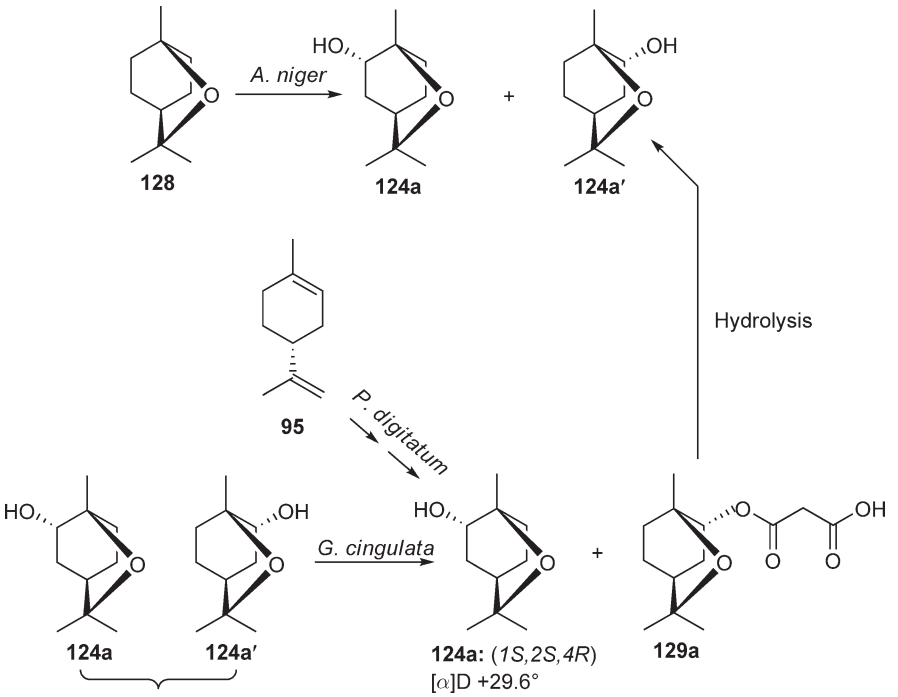
As shown in **Scheme 50**, *S. litura* converted (4*R*)-*p*-menth-1-ene (**139**) at C-7 position to (4*R*)-phellandric acid (**142**).[82] On the other hand, when *Cladosporium* sp. T_1 was cultivated with (+)-limonene (**95**) as the sole carbon source, it converted **139′** to *trans*-*p*-menthane-1,2-diol (**143**).[75]

3.19.2.2.1(vi) 3-p-Menthene (144) When *Cladosporium* sp. T_8 was cultivated with 3-*p*-menthene (**144**) as the sole carbon source, it was converted to *trans*-*p*-menthane-3,4-diol (**145**) as shown in **Scheme 51**.[75]

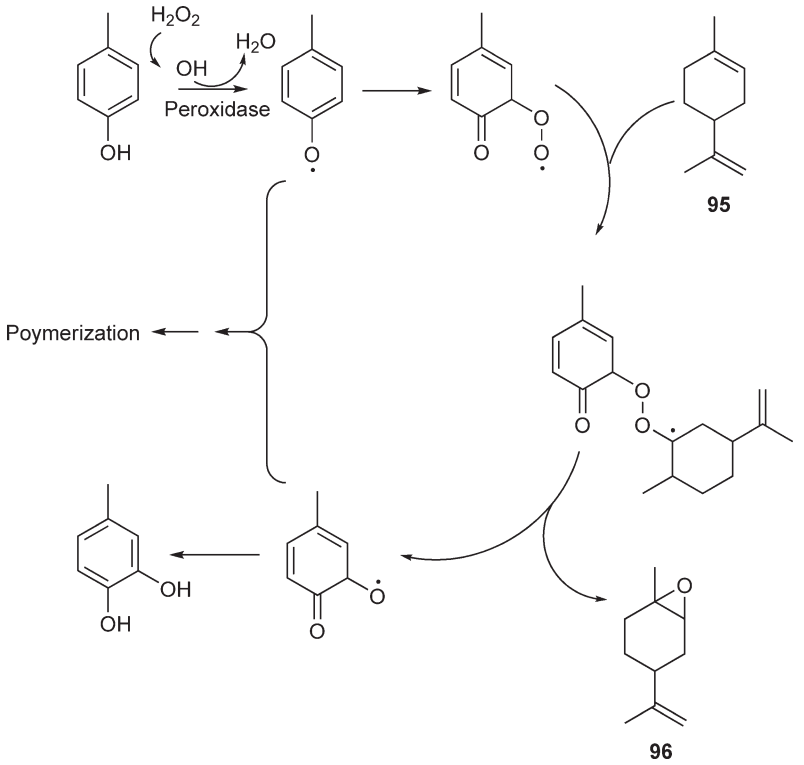
3.19.2.2.1(vii) α-Terpinene (146) α-Terpinene (**146**) was converted by *S. litura* to α-terpinene-7-oic acid (**149**) and *p*-cymene-7-oic acid (**151**, cuminic acid) (**Scheme 52**).[86]

A soil Pseudomonad has been found to grow with *p*-mentha-1,3-dien-7-al (**148**) as the sole carbon source and produce α-terpinene-7-oic acid (**149**) in a mineral salt medium.[87,88]

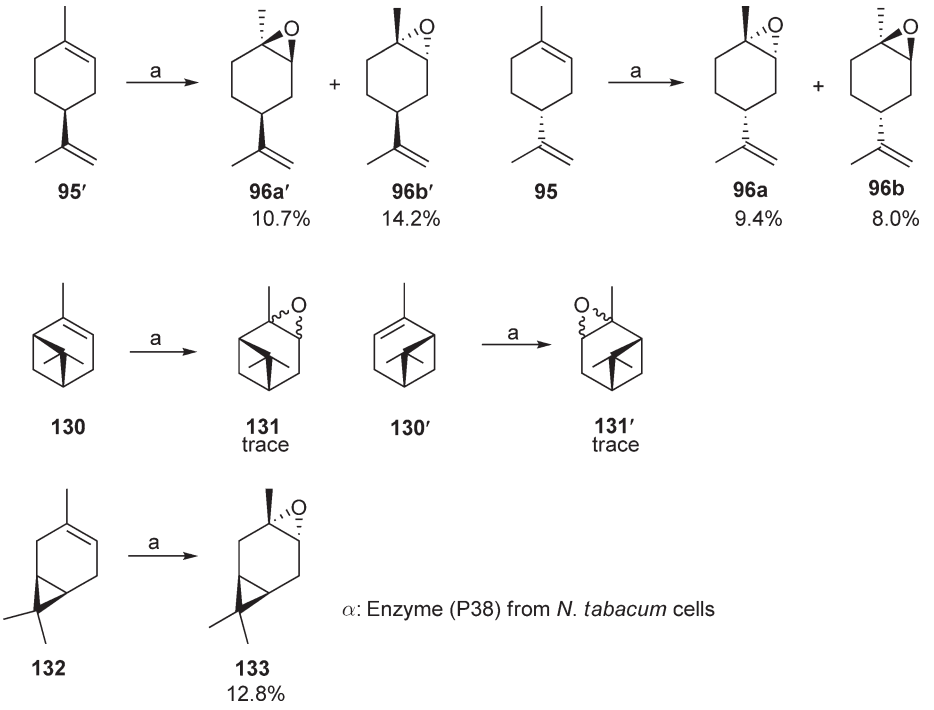
3.19.2.2.1(viii) γ-Terpinene (153) γ-Terpinene (**153**) was converted by *S. litura* to *p*-mentha-1,4-diene-7-oic acid (**154**, 46%) and *p*-cymen-7-oic acid (**152**, cuminic acid, 48%) (**Scheme 53**).[89]



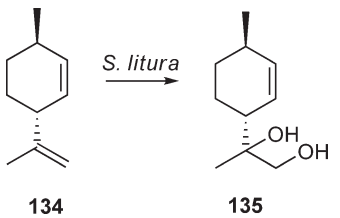
Scheme 44 Formation of optically pure (+)- (**124b**) and (−)-2α-hydroxy-1,8-cineole (**124b′**) from the biotransformation of 1,8-cineole (**128**) and (+)-limonene (**95**) by the *Citrus* pathogenic fungus *Penicillium digitatum* (Pers.; Fr.) Sacc. KCPYN and *Aspergillus niger* TBUYN-2.



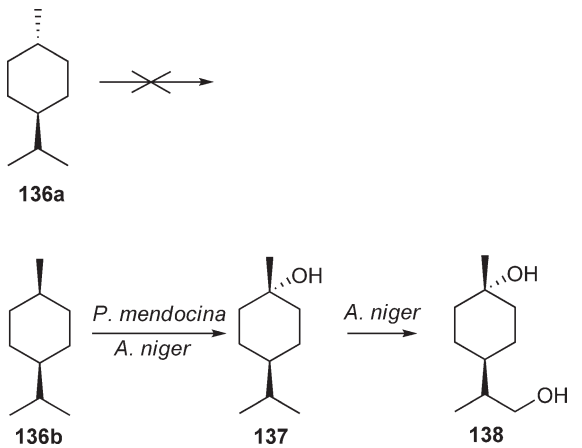
Scheme 45 Proposed mechanism for the epoxidation of (+)-limonene (**95**) with p38 from the cultured cells of *Nicotiana tabacum*.



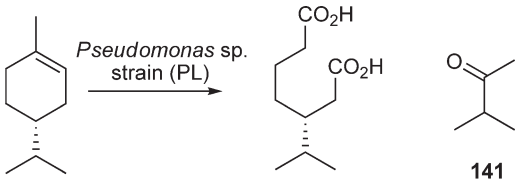
Scheme 46 Epoxidation of limonene (**95**), α-pinene (**130**), and 3-carene (**132**) with p38 from the cultured cells of *Nicotiana tabacum*.



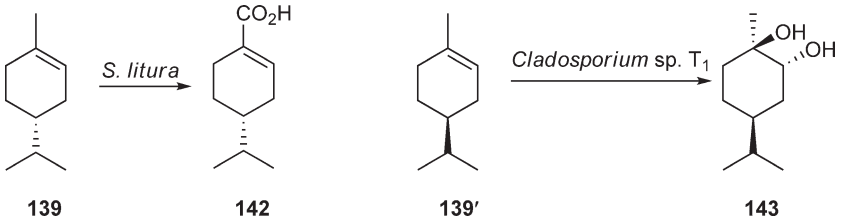
Scheme 47 Biotransformation of (1*R*)-*trans*-isolimonene (**134**) by *Spodoptera litura*.



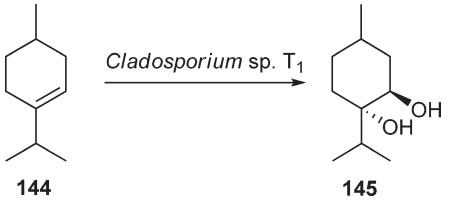
Scheme 48 Biotransformation of the mixture of *trans*- (**136a**) and *cis*-*p*-menthane (**136b**) by *Pseudomonas mendocina* SF and *Aspergillus niger* TBUYN-2.



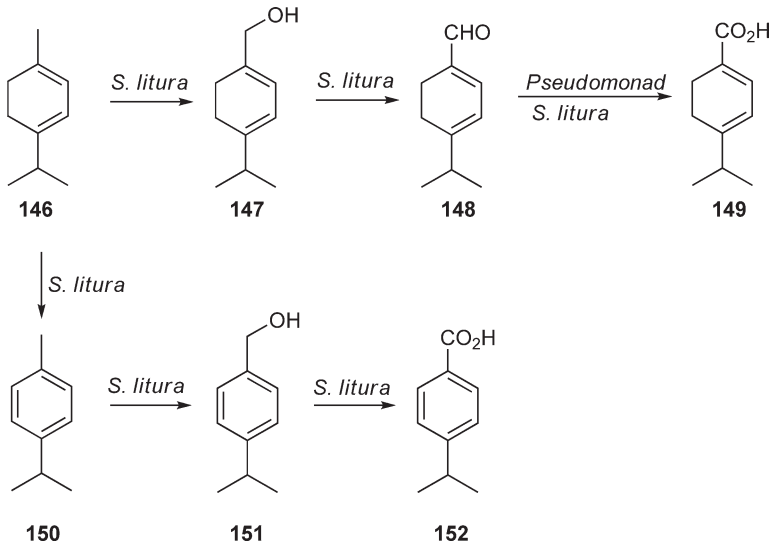
Scheme 49 Biodegradation of (4*R*)-1-*p*-menthene (**139**) by *Pseudomonas* species strain (PL).



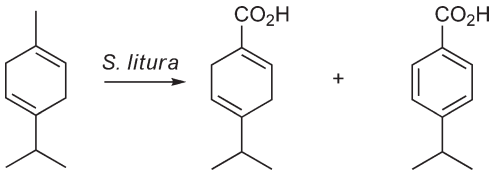
Scheme 50 Biotransformation of (4*R*)-*p*-menth-1-ene (**139**) by *Spodoptera litura* and *Cladosporium* species T_1.



Scheme 51 Biotransformation of *p*-menth-3-ene (**144**) by *Cladosporium* species T_8.



Scheme 52 Biotransformation of α-terpinene (**146**) by *Spodoptera litura* and *p*-mentha-1,3-dien-7-al (**148**) by a soil Pseudomonad.

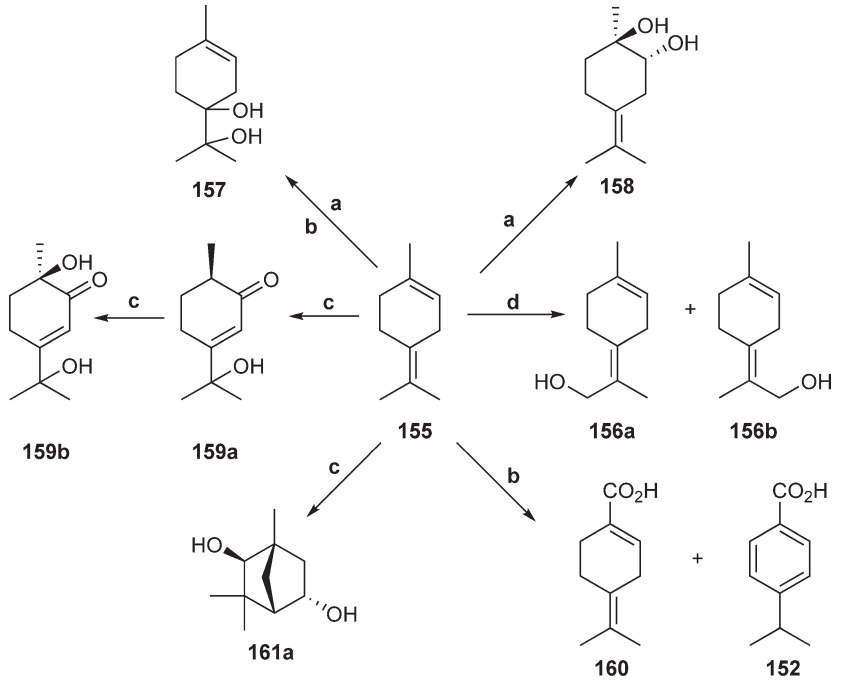


Scheme 53 Biotransformation of γ-terpinene (**153**) by *Spodoptera litura*.

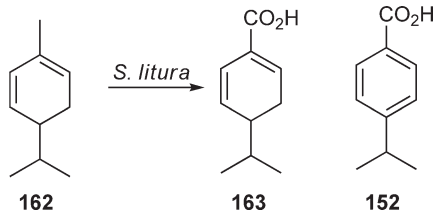
3.19.2.2.1(ix) Terpinolene (155) Terpinolene (**155**) was converted by *A. niger* to (1*R*)-8-hydroxy-3-*p*-menthen-2-one (**159a**), (1*R*)-1,8-dihydroxy-3-*p*-menthen-2-one (**159b**), and 5β-hydroxyfenchol (**161a**).[90] In the case of *C. cassiicola*, it was converted to terpinolene-1,2-*trans*-diol (**158**) and terpinolene-4,8-diol (**157**).[2] Furthermore, in the case of rabbits, terpinolene-9-ol (**156a**) and terpinolene-10-ol (**156b**) were formed from **155**.[91] *Spodoptera litura* also converted **155** to 1-*p*-menthene-4,8-diol (**157**), cuminic acid (**152**, 29%, a major product), and terpinolene-7-oic acid (**160**) (**Scheme 54**).[82]

3.19.2.2.1(x) α-Phellandrene (162) α-Phellandrene (**162**) was converted by *S. litura* to (4*R*)-*p*-mentha-1,5-dien-7-oic acid (**163**, 41%) and *p*-cymen-7-oic acid (**152**, cuminic acid, 55%) (**Scheme 55**).[89]

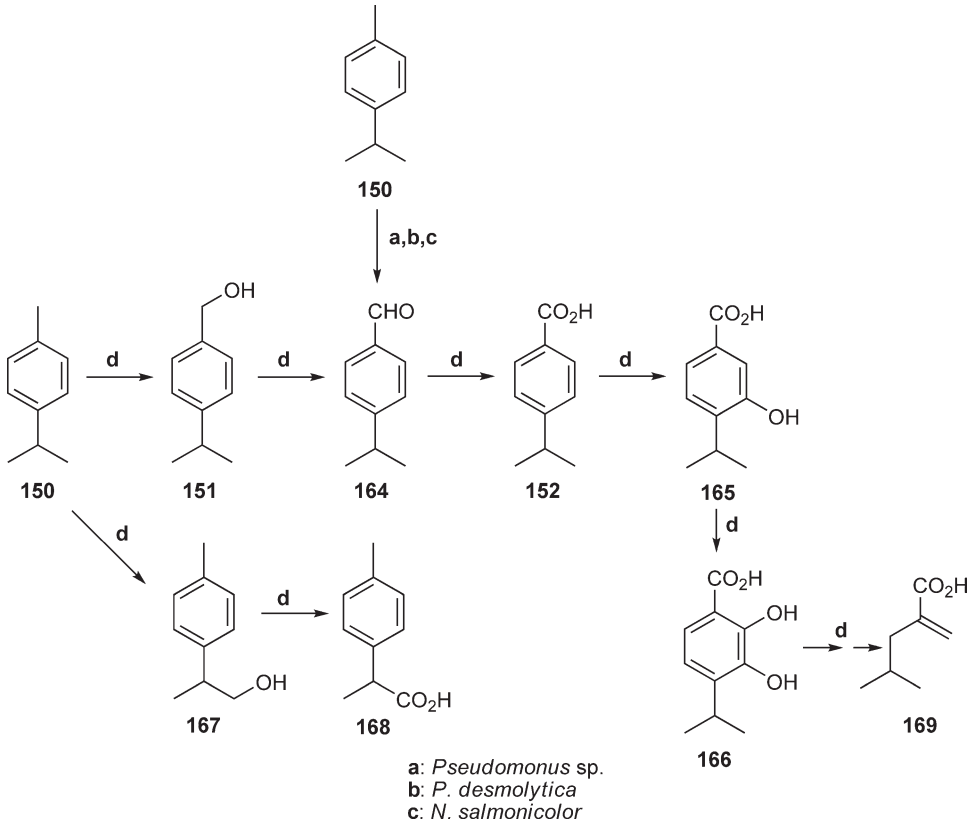
3.19.2.2.1(xi) p-Cymene (150) *Pseudomonas* sp. strain (PL) was cultivated with *p*-cymene (**150**) as the sole carbon source and it gave cumyl alcohol (**151**), cumic acid (**152**), 3-hydroxycumic acid (**165**), 2,3-dihydroxycumic acid (**166**), 2-oxo-4-methylpentanoic acid (**169**), 9-hydroxy-*p*-cymene (**167**), and *p*-cymene-9-oic acid (**168**) as shown in **Scheme 56**.[92] On the other hand, *p*-cymene (**150**) was converted regiospecifically to cumic acid (**152**) by *Pseudomonas* sp., *P. desmolytica*, and *Nocardia salmonicolor* (**Scheme 56**).[92–94]



Scheme 54 Biotransformation of terpinolene (**155**) by *Aspergillus niger*, *Corynespora cassiicola*, *Spodoptera litura*, and rabbits.



Scheme 55 Biotransformation of α-phellandrene (**162**) by *Spodoptera litura*.



Scheme 56 Biotransformation of *p*-cymene (**150**) to cumic acid (**152**) by *Pseudomonas* species, *Pseudomonas desmolytica*, and *Nocardia salmonicolor*.

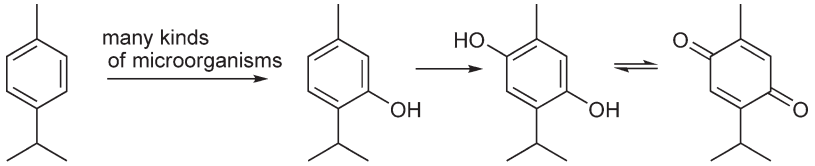
p-Cymene (**150**) is converted to thymoquinone (**172**) and its analogues, **170** and **171**, by various kinds of microorganisms (**Scheme 57**).[95]

3.19.2.2.2 Monocyclic monoterpene aldehydes

3.19.2.2.2(i) Perillaldehydes (117 and 117′) and their related compounds (116a′; 173a, 173b, 174a, 174b) In **Scheme 58**, the structures of perillaldehydes (**117** and **117′**) and their related compounds are shown.

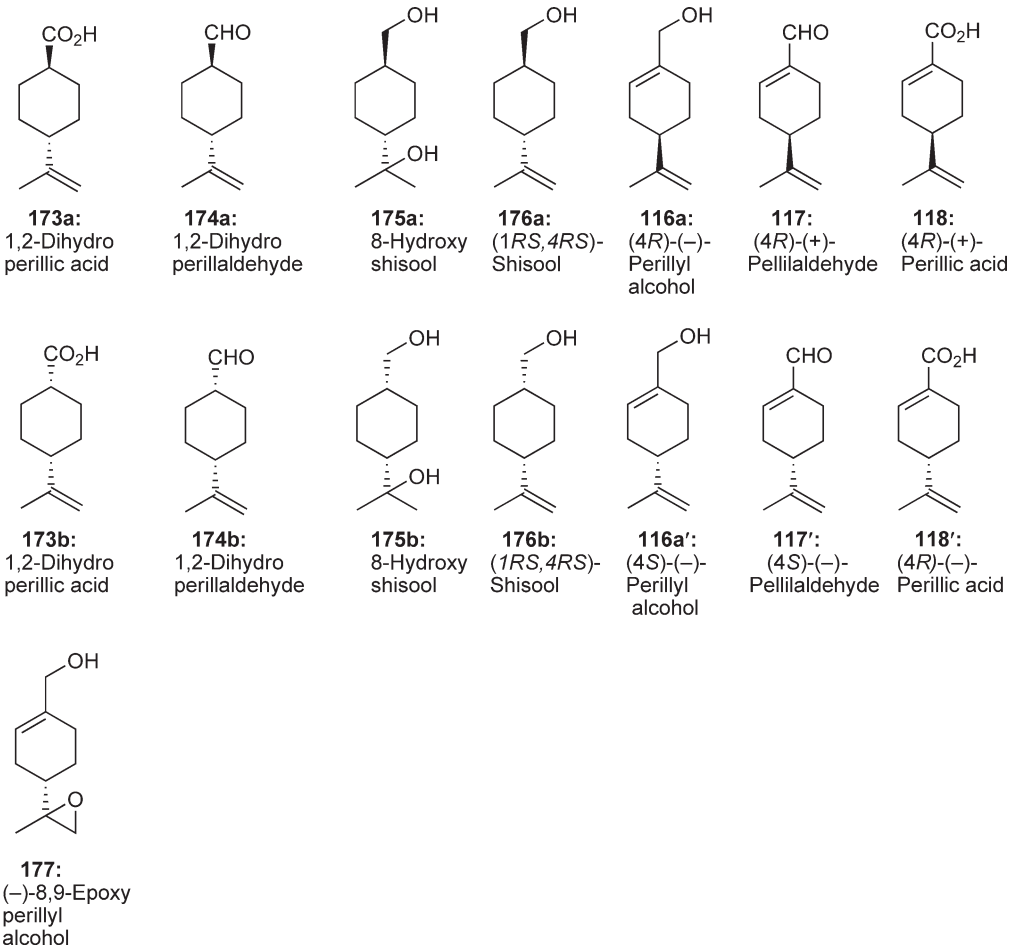
Biotransformation of (−)-perillaldehyde (**117**), (+)-perillaldehyde (**117′**), (−)-perillyl alcohol (**116a′**), *trans*-1,2-dihydroperillaldehyde (**174a**), and *cis*-1,2-dihydroperillaldehyde (**174b**) was carried out by *E. gracilis* Z,[19] *D. tertiolecta*,[20,21] *Chlorella ellipsoidea* IAMC-27,[96] *S. ikutamanensis* Ya-2-1,[24] and other microorganisms (**Scheme 59**).[87]

(−)-Perillaldehyde (**117**) is readily transformed to (−)-perillyl alcohol (**116a′**) and *trans*-shisool (**176a**), which is well known for its characteristic fragrance, as the major products and (−)-perillic acid (**118′**) as the minor product. (−)-Perillyl alcohol (**116a′**) is also transformed to *trans*-shisool (**176a**) as the major product



150 **170** **171** **172**

Scheme 57 Biotransformation of *p*-cymene (**150**) to thymoquinone (**172**) and analogues by microorganisms.

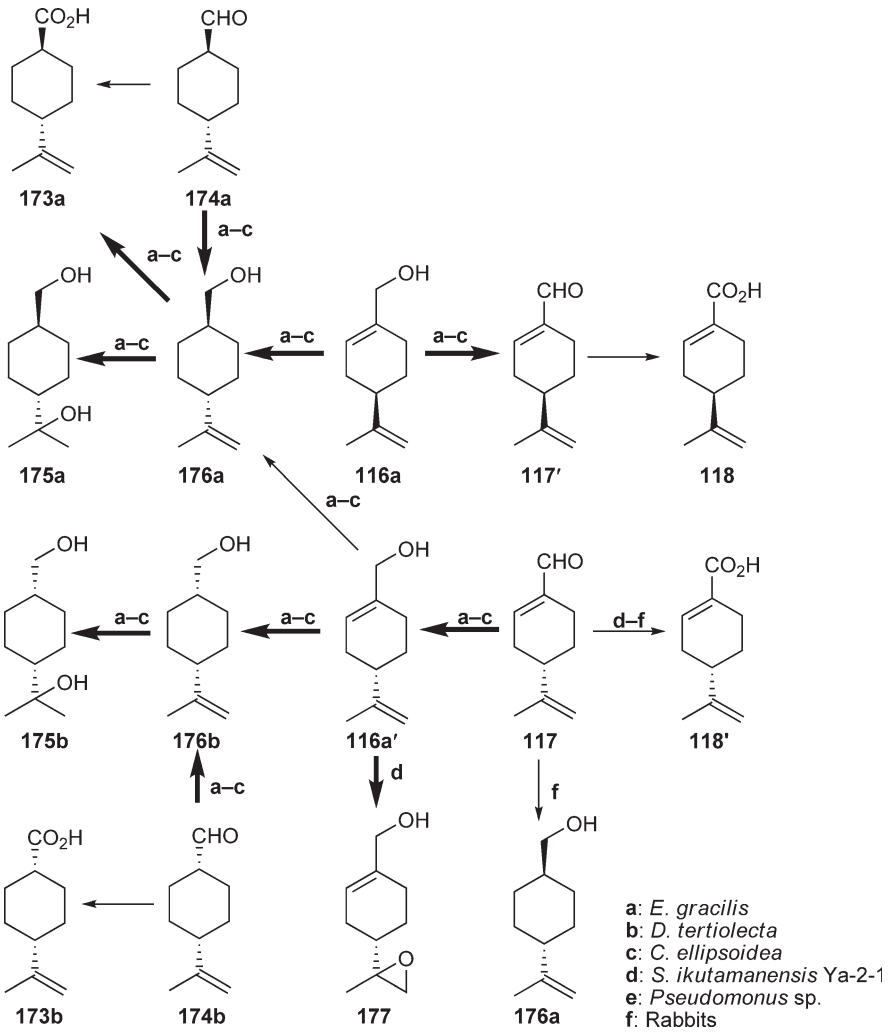


Scheme 58 Structures of perillaldehydes (**117** and **117′**) and their related compounds.

together with *cis*-shisool (**176b**) and 8-hydroxy-*cis*-shisool (**175b**). Furthermore, *trans*-shisool (**176a**) and *cis*-shisool (**176b**) are hydroxylated to 8-hydroxy-*trans*-shisool (**175a**) and 8-hydroxy-*cis*-shisool (**175b**), respectively. *trans*-1,2-Dihydroperillaldehyde (**174a**) and *cis*-1,2-dihydroperillaldehyde (**174b**) are also transformed to **176a** and **176b** as the major products and *trans*-shisoic acid (**173a**) and *cis*-shisoic acid (**173b**) as the minor products, respectively. Compound **173a** was also formed from **176a**. In the biotransformation of (±)-perillaldehyde (**117** and **117′**), the same results as described in the case of **117** were obtained. In the case of *S. ikutamanensis* Ya-2-1, (−)-perillaldehyde (**117**) was converted to (−)-perillic acid (**118′**), (−)-perillyl alcohol (**116a′**), and (−)-perillyl alcohol-8,9-epoxide (**177**), the last compound being the major product.[24,97]

A soil Pseudomonad has been found to grow with (−)-perillaldehyde (**117**) as the sole carbon source and to produce (−)-perillic acid (**118′**) in a mineral salt medium.[87]

On the other hand, rabbits metabolized (−)-perillaldehyde (**117**) to (−)-perillic acid (**118′**) along with shisool (**176a**) as the minor product.[98]



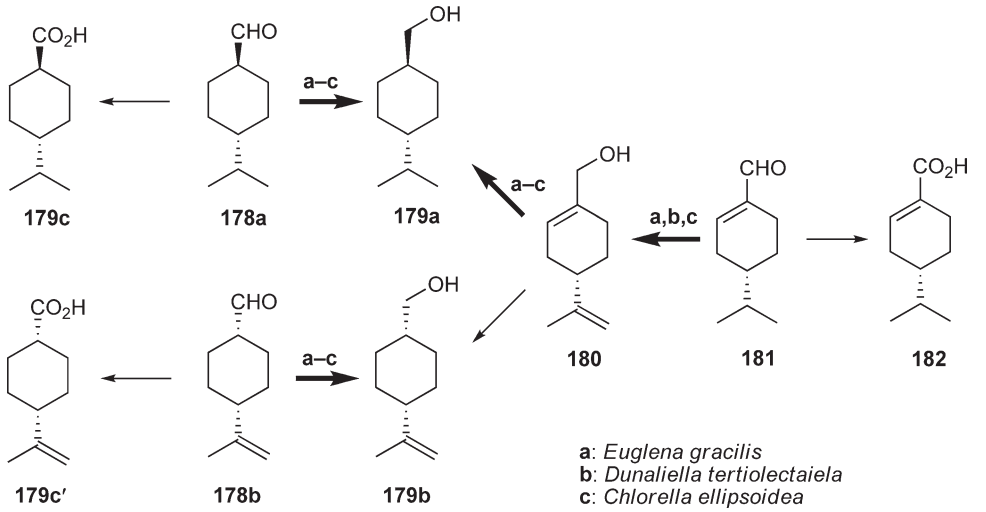
Scheme 59 Metabolic pathways of perillaldehyde (**117** and **117′**) by *Euglena gracilis Z*, *Dunaliella tertiolecta*, *Chlorella ellipsoidea* IAMC-27, *Streptomyces ikutamanensis* Ya-2-1, a soil Pseudomonad, and rabbits.

3.19.2.2.2(ii) Phellandral (181) and tetrahydroperillaldehyde (178a and 178b) Biotransformation of (−)-phellandral (**181**), *trans*-tetrahydroperillaldehyde (**178a**), and *cis*-tetrahydroperillaldehyde (**178b**) was carried out by microorganisms.[19,20,24,96] (−)-Phellandral (**181**) was metabolized mainly via (−)-phellandrol (**180**) to *trans*-tetrahydroperillyl alcohol (**179a**). *trans*-Tetrahydroperillaldehyde (**178a**) and *cis*-tetrahydroperillaldehyde (**178b**) were also transformed to *trans*-tetrahydroperillyl alcohol (**179a**) and *cis*-tetrahydroperillyl alcohol (**179b**) as the major products and *trans*-tetrahydroperillic acid (**179a**) and *cis*-tetrahydroperillic acid (**179b**) as the minor products, respectively (**Scheme 60**).

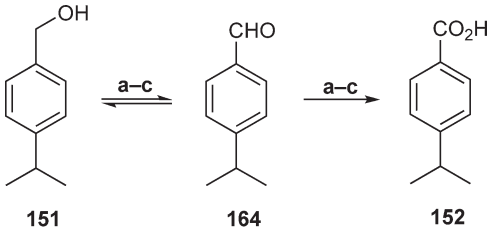
3.19.2.2.2(iii) Cuminaldehyde (164) Cuminaldehyde (**164**) is transformed by *Euglena*,[19] Dunaliella,[21] and *S. ikutamanensis*[24] to cumin alcohol (**151**) as the major product and cuminic acid (**152**) as the minor product (**Scheme 61**).

3.19.2.2.3 Monocyclic monoterpene alcohols

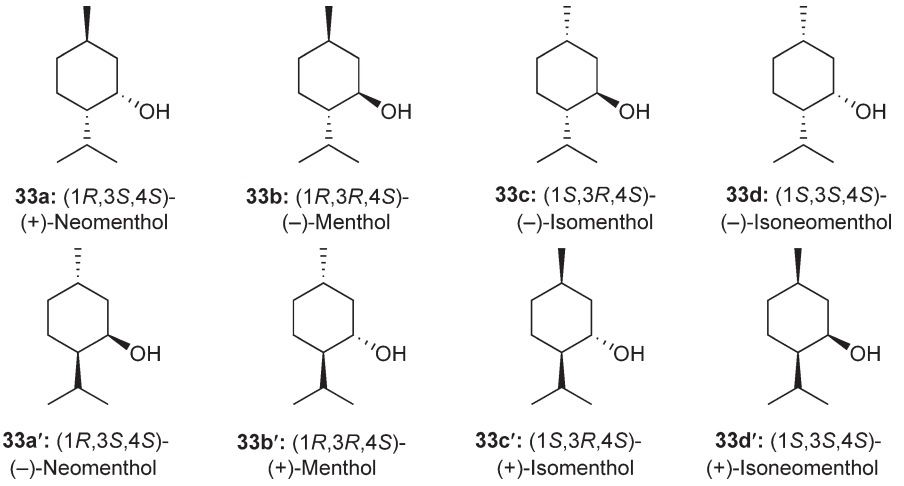
3.19.2.2.3(i) Menthol (33b and 33b′) In **Scheme 62**, (−)-menthol (**33b**) and its isomers are presented. Menthol (**33**) is one of the rare naturally occurring monocyclic monoterpene alcohols that have not only various physiological properties, such as sedative, anesthetic, antiseptic, gastric, and antipruritic properties, but



Scheme 60 Metabolic pathways of (−)-phellandral (**181**) by microorganisms.



Scheme 61 Metabolic pathway of cuminaldehyde (**184**) by microorganisms.



Scheme 62 Structures of (−)-menthol (**33b**) and its isomers.

also characteristic fragrance.[41] There are in fact eight isomers with a menthol (*p*-menthan-3-ol) skeleton, (−)-menthol (**33b**) being the most important one, because of its cooling and refreshing effect. It is the major component of peppermint and corn mint oils obtained from the *Mentha piperita* and *M. arvensis* species.

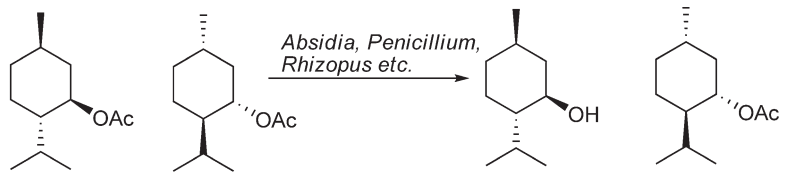
Many attempts have been made to produce (−)-menthol (**33b**) from inexpensive terpenoid sources, but these sources also unavoidably yielded the (±)-isomers (**33b** and **33b′**): isomenthol (**33c**), neomenthol (**33a**), and neoisomenthol (**33d**).[52] Especially Japanese researchers have been active in this field, maybe because of the large demand for (−)-menthol (**33b**) in Japan itself, that is 500 t year^{-1}.[99] Indeed, most literature deals with the enantiomeric hydrolysis of (±)-menthol (**33b** and **33b′**) esters to optically pure (−)-menthol (**33b**). The asymmetric hydrolysis of (±)-menthyl chloroacetate by an esterase of *Alginomonas nonfermentans* FERM-P-1924 has been patented by the Japanese Nippon Terpene Chemical Co.[100,101] Investigators from the Takasago Perfumery Co. Ltd. claim that certain selected species of *Absidia*, *Penicillium*, *Rhizopus*, *Trichoderma*, *Bacillus*, *Pseudomonas*, and others asymmetrically hydrolyze esters of (±)-menthol isomers such as formates, acetates, propanoates, caproates, and esters of higher fatty acids (**Scheme 63**).[102–104]

Numerous investigations on the resolution of the enantiomers by selective hydrolysis with microorganisms or enzymes were carried out. Satisfactory results were obtained by Yamaguchi *et al.*[103] with the asymmetric hydrolysis of (±)-methyl acetate (**33b-Ac** and **33b′-Ac**) by a mutant of *Rhodotorula mucilaginosa*, yielding 44 g of (−)-menthol (**33b**) from a 30% (±)-menthyl acetate mixture per liter of culture medium in 24 h. The latest development is the use of immobilized cells of *R. minuta* in aqueous saturated organic solvents for menthyl succinate (**33b-succinate**) (**Scheme 64**).[105]

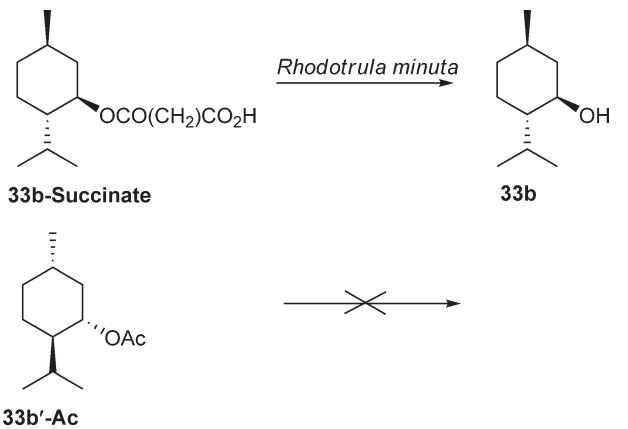
Besides the hydrolysis of menthyl esters, the biotransformation of menthol and its enantiomers has also been published.[90,106]

Incubation of (−)-menthol (**33b**) with *Cephalosporium aphidicola* for 12 days yielded 10-acetoxymenthol (**183b-Ac**), 1α-hydroxymenthol (**186b**), 6α-hydroxymenthol (**185b**), 7-hydroxymenthol (**184b**), 9-hydroxymenthol (**187b**), and 10-hydroxymenthol (**183b**) (**Scheme 65**).[107]

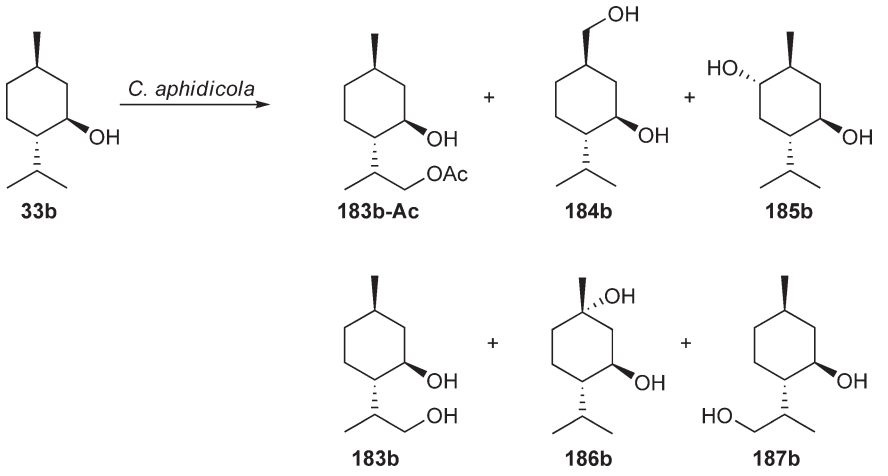
The fungal biotransformation of (−)- (**33b**) and (+)-menthols (**33b′**) by *A. niger* and *A. cellulosae* was described.[90] *Aspergillus niger* THBYN-2 converted (−)-menthol (**33b**) to 1- (**186b**), 2- (**189b′**), 6- (**185b**),



Scheme 63 Asymmetric hydrolysis of racemic menthyl acetate (**33b-Ac** and **33b′-Ac**) to obtain pure (−)-menthol (**33b**).



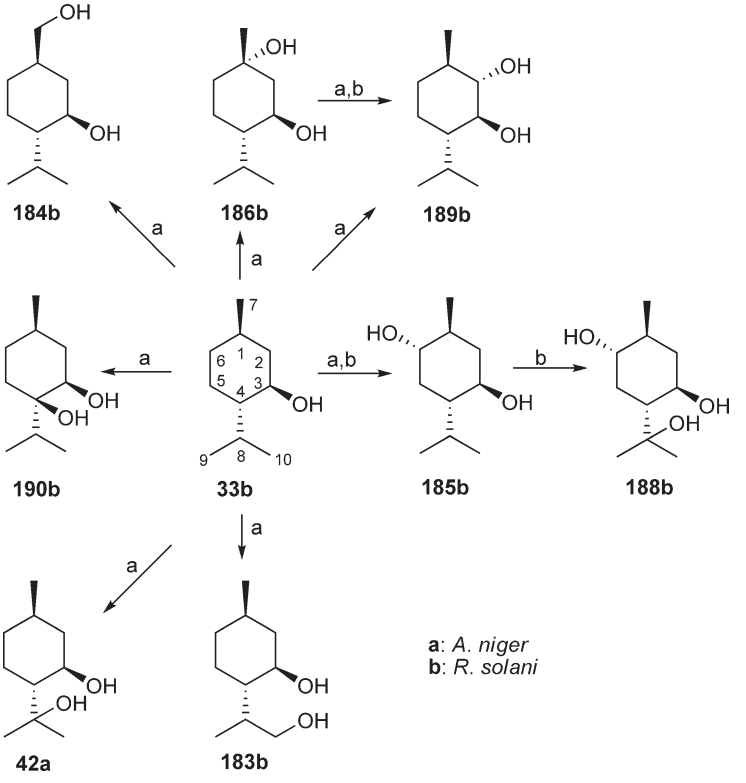
Scheme 64 Asymmetric hydrolysis of racemic menthyl succinate (**33b-** and **33b′-**succinates) to obtain pure (−)-menthol (**33b**).



Scheme 65 Biotransformation of (−)-menthol (**33b**) by *Cephalosporium aphidicola*.

7-(**184b**), and 9-hydroxymenthols (**183b**) and the mosquito repellent active 8-hydroxymenthol (**42a**) (**Scheme 66**). The bioconversion of (+)-menthol (**33b′**), (+)-neomenthol (**33a**) and its enantiomer (**33a′**), and (+)-isomenthol (**33c′**) by *A. niger* was studied later by Noma and Asakawa,[108] mainly giving hydroxylated products (see later). Takahashi *et al.*[109] reported that (+)-neomenthol (**33a**) and (−)-neomenthol (**33a′**) and (+)-isomenthol (**33c′**) were biotransformed by *A. niger* to afford six and seven hydroxylated derivatives from **33a** and **33a′**, respectively. From (+)-isomenthol (**33c′**), only 1α-hydroxy (**186c′**) and 6β-hydroxy derivatives (**185c′**) were obtained by the same fungus as described above.

Aspergillus cellulosae M-77 biotransformed (−)-menthol (**33b**) to 4β-hydroxymenthol (**190b**) predominantly. The formation of **190b** is also observed in *A. cellulosae* IFO4040 and *A. terreus* IFO6123, but its yield is much less than that obtained from **33b** by *A. cellulosae* M-77 (**Table 1**).[90]



Scheme 66 Metabolic pathways of (−)-menthol (**33b**) by *Aspergillus niger* and *Rhizoctonia solani*.

Table 1 Metabolites of (−)-menthol (**33b**) by various *Aspergillus* species (static culture)

Microorganisms	*186b*	*42b*	*189b*	*184b*	*185b*	*183b*	*190b*
Aspergillus awamori IFO4033	+	++	−	+	++	+++	−
Aspergillus fumigatus IFO4400	−	+	−	+	+	+	−
Aspergillus sojae IFO4389	++	+	+	−	−	++++	−
Aspergillus usami IFO4338	−	−	−	+	−	+++	−
Aspergillus cellulosae M-77	+	−	−	+	−	++	++++
Aspergillus cellulosae IFO4040	−	+	−	−	−	++	++
Aspergillus terreus IFO6123	+	+	+	−	+	+	−
Aspergillus niger IFO4049	−	+	−	+	−	+++	−
Aspergillus niger IFO4040	−	+	−	+++	−	+++	−
Aspergillus niger TBUYN-2	+	++	+	+	++	++	−

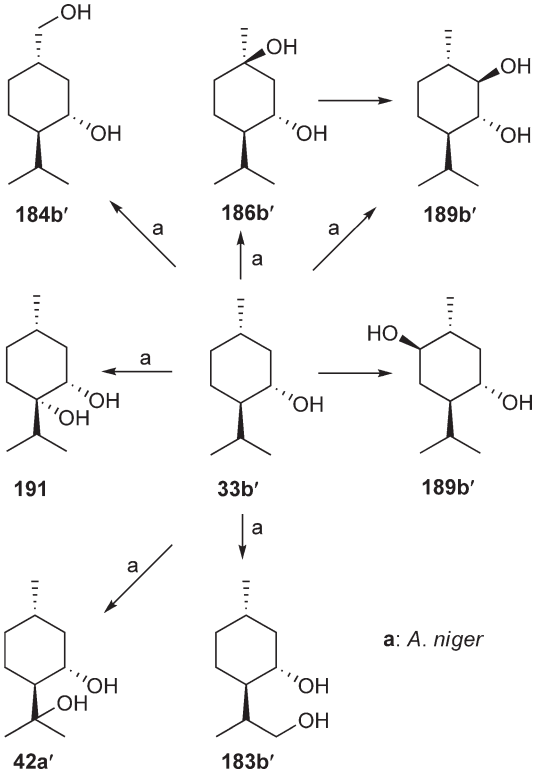
Symbols +, ++, +++, etc. are relative concentrations estimated by GC–MS.

On the other hand, the soil-borne plant pathogenic fungus *Rhizoctonia solani* converted (−)-menthol (**33b**) to (−)-1α- (**186b**) and (−)-6α-hydroxymenthols (**185b**) and (+)-6,8-dihydroxymenthol (**188b**) (**Scheme 66**).[110]

Furthermore, (+)-menthol (**33b′**) was smoothly biotransformed by *A. niger* to 1β-hydroxymenthol (**186b′**), 6β-hydroxymenthol (**185b′**), 2β-hydroxymenthol (**189b′**), 4α-hydroxymenthol (**190b′**), 7-hydroxymenthol (**184b′**), 8-hydroxymenthol (**191b′**), and 9-hydroxymenthol (**183b′**) (**Scheme 67**) (**Table 2**).[90,111]

Spodoptera litura converted (−)- and (+)-menthols (**33b** and **33b′**) to the corresponding 10-hydroxy products (**184b** and **184b′**) (**Scheme 68**).[112]

(−)-Menthol (**33b**) was glycosylated by *Eucalyptus perriniana* suspension cells to (−)-menthol diglucoside (**192**, 26.6%) and another menthol glycoside. On the other hand, (+)-menthol (**33b′**) was glycosylated by the same suspension cells to (+)-menthol di- (**192′**, 44.0%) and triglucosides (**193**, 6.8%) (**Scheme 69**).[113]

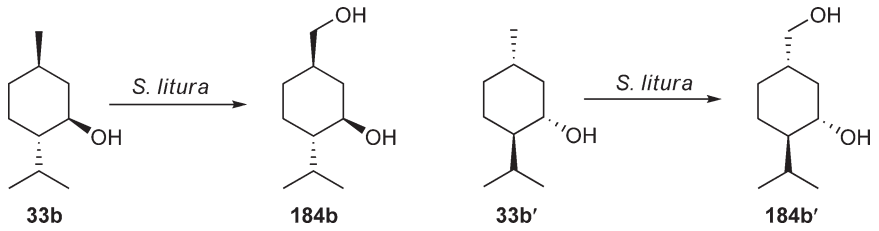


Scheme 67 Metabolic pathways of (+)-menthol (**33b′**) by *Aspergillus niger*.

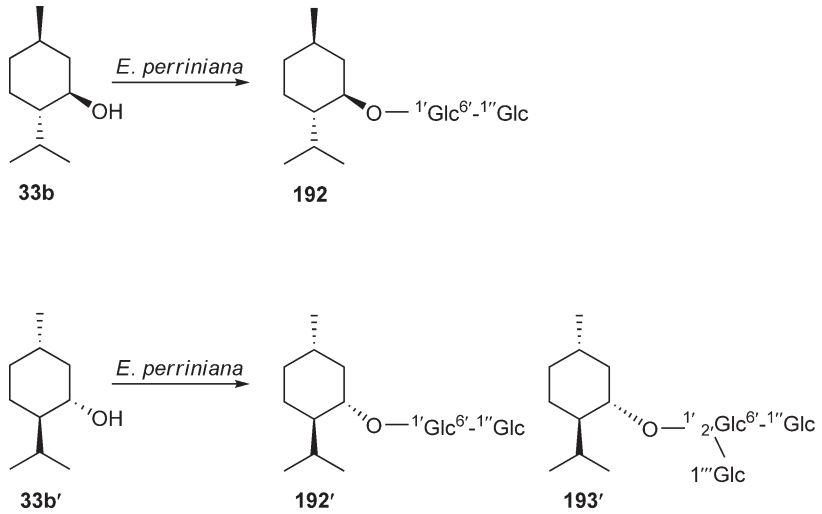
Table 2 Metabolites of (+)-menthol (**33b′**) by various *Aspergillus* species (static culture)

Microorganisms	*186b′*	*42a′*	*189b′*	*184b′*	*185b′*	*183b′*	*190*
Aspergillus awamori IFO4033	+	++	−	+++	−	+++	−
Aspergillus fumigatus IFO4400	+	++	−	+	−	++	−
Aspergillus sojae IFO4389	+	++	−	−	−	+++	−
Aspergillus usami IFO4338	+	−	−	+	−	+++	−
Aspergillus cellulosae M-77	−	+	−	−	−	++	++++
Aspergillus cellulosae IFO4040	+	+	−	−	++	+	+
Aspergillus terreus IFO6123	+	+++	+	+	+	++	−
Aspergillus niger IFO4049	+	−	−	−	+	+++	−
Aspergillus niger IFO4040	+	++	−	+	−	++	−
Aspergillus niger TBUYN-2	++	+	−	+++++	+	+	−

Symbols +, ++, +++, etc. are relative concentrations estimated by GC–MS.

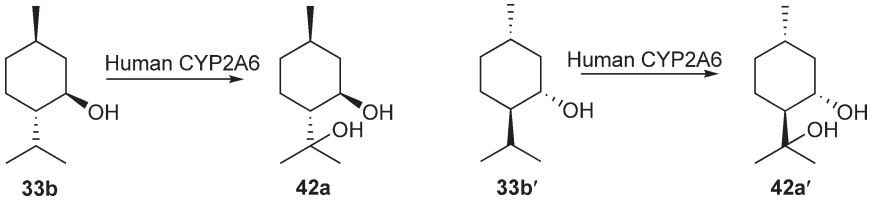


Scheme 68 Biotransformation of (−)- (**33b**) and (+)-menthol (**33b′**) by *Spodoptera litura.*



Scheme 69 Biotransformation of (−)- (**33b**) and (+)-menthol (**33b′**) by suspension cells of *Eucalyptus perriniana.*

(−)-Menthol (**33b**) and its enantiomer (**33b′**) were converted to their corresponding 8-hydroxy derivatives (**42a** and **42a′**) by human CYP2A6 (**Scheme 70**).[114] By various assays, cytochrome P-450 molecular species responsible for the metabolism of (−)- (**33b**) and (+)-menthol (**33b′**) were determined to be CYP2A6 and CYP2B1 in human and rat. Kinetic analysis using Lineweaver-Burk Plot showed that K and V_{max} values for the oxidation of (−)- (**33b**) and (+)-menthol (**33b′**) by recombinant CYP2A6 and CYP2B1 were 28 μmol l^{-1} and 10.33 nmol per min per nmol P-450 and 27 μmol l^{-1} and 5.29 nmol per min per nmol P-450, 28 μmol l^{-1} and 3.58 nmol per min per nmol P-450 and 33 μmol l^{-1} and 5.3 nmol per min per nmol P-450, respectively (**Scheme 70**).[114]



Scheme 70 Biotransformation of (−)-menthol (**33b**) and its enantiomer (**33b′**) by human CYP2A6.

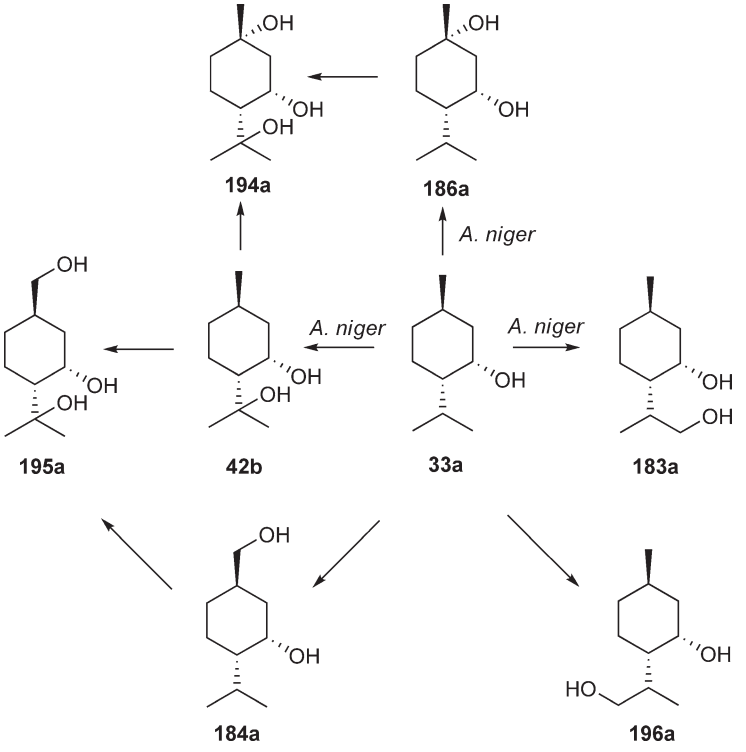
3.19.2.2.3(ii) Neomenthol (33a and 33a′) (+)-Neomenthol (**33a**) is biotransformed by *A. niger* TBUYN-2 to five kinds of diols (**42b**, **183a**, **184a**, **186a**, and **196a**) and two kinds of triols (**194a** and **195a**) as shown in (**Scheme 71**).[108]

(−)-Neomenthol (**33a′**) is biotransformed by *A. niger* to six kinds of diols (**42b′**, **184a′**, **185a′**, **189a′**, **191a′**, **196a′**, and **197a′**) and a triol (**194a′**) as shown in **Scheme 72**.[108]

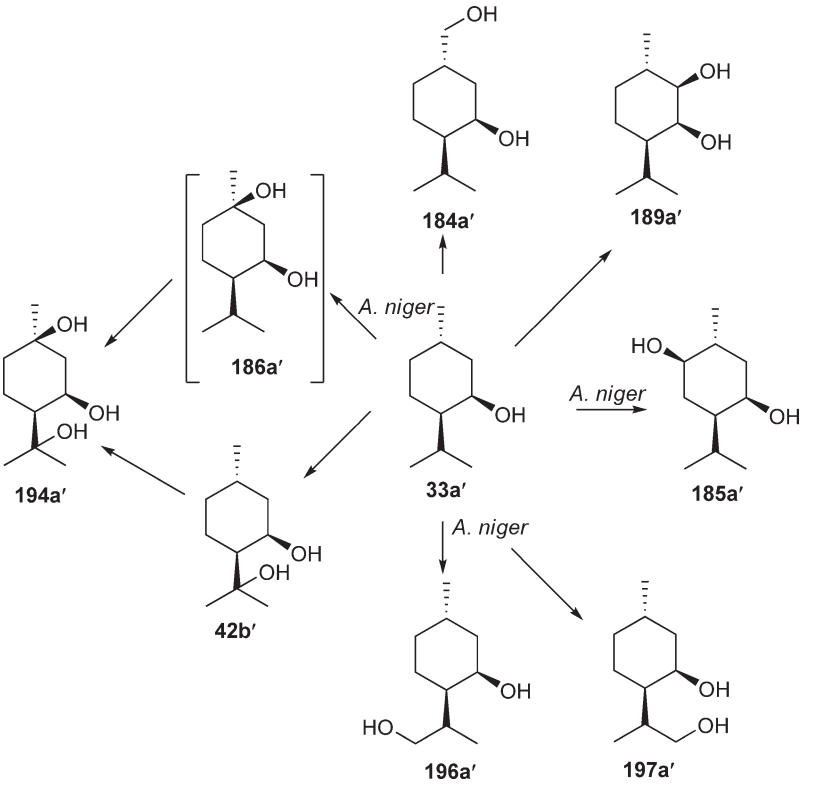
3.19.2.2.3(iii) Isomenthol (33c′) (+)-Isomenthol (**33c′**) was biotransformed to two kinds of diols (6β-hydroxy- (**185c′**) and 1β-hydroxyisomenthol (**186c′**)) by *A. niger* (**Scheme 73**).[108]

(±)-Isomenthyl acetate (**33c-Ac** and **33c′-Ac**) was asymmetrically hydrolyzed to (−)-isomenthol (**33c**) together with (+)-isomenthol acetate (**33c′-Ac**) by many microorganisms and esterases (**Scheme 74**).[104]

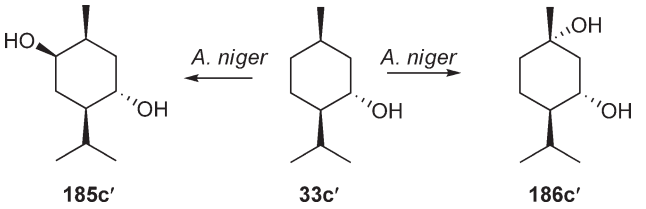
3.19.2.2.3(iv) Isopulegol (38 and 38′) (−)-Isopulegol (**38**) was biotransformed by *S. litura* larvae to 9-hydroxy-(−)-menthol (**183b**), 7-hydroxy-(−)-isopulegol (**198**), and 10-hydroxy-(−)-isopulegol (**199**). On the other hand, (+)-isopulegol (**38′**) was biotransformed by the same larvae in the same manner to 9-hydroxy-(+)-menthol (**183b′**), 7-hydroxy-(+)-isopulegol (**198′**), and 10-hydroxy-(+)-isopulegol (**199′**) (**Scheme 75**).[115]



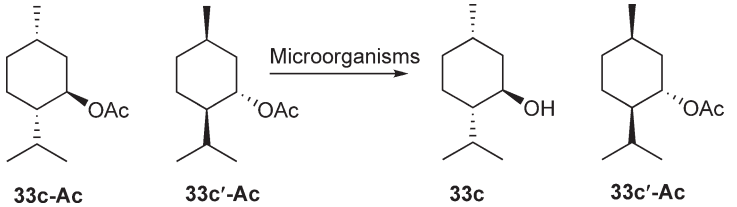
Scheme 71 Metabolic pathways of (+)-neomenthol (**33a**) by *Aspergillus niger*.



Scheme 72 Metabolic pathways of (−)-neomenthol (**33a′**) by *Aspergillus niger*.

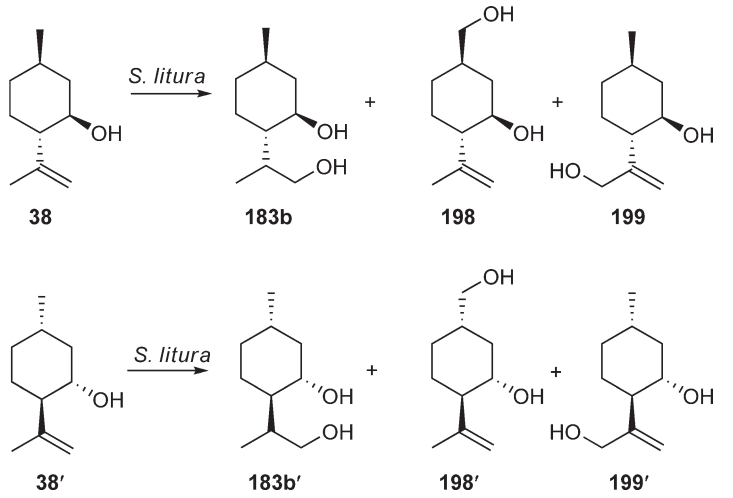


Scheme 73 Metabolic pathways of (+)-isomenthol (**33c**) by *Aspergillus niger*.

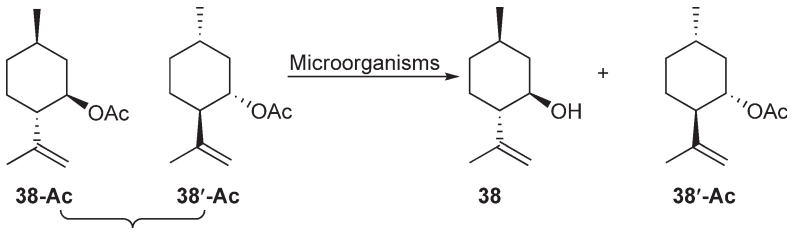


Scheme 74 Microbial resolution of (±)-isomenthyl acetate (**33c-Ac** and **33c′-Ac**) by microbial esterase.

Microbial resolution of (±)-isopulegyl acetate (**38-Ac** and **38′-Ac**) was studied in microorganisms. The substrates (**38-Ac** and **38′-Ac**) were hydrolyzed asymmetrically to a mixture of (−)-isopulegol (**38**) and (+)-isopulegyl acetate (**38′-Ac**) (**Scheme 76**).[116]



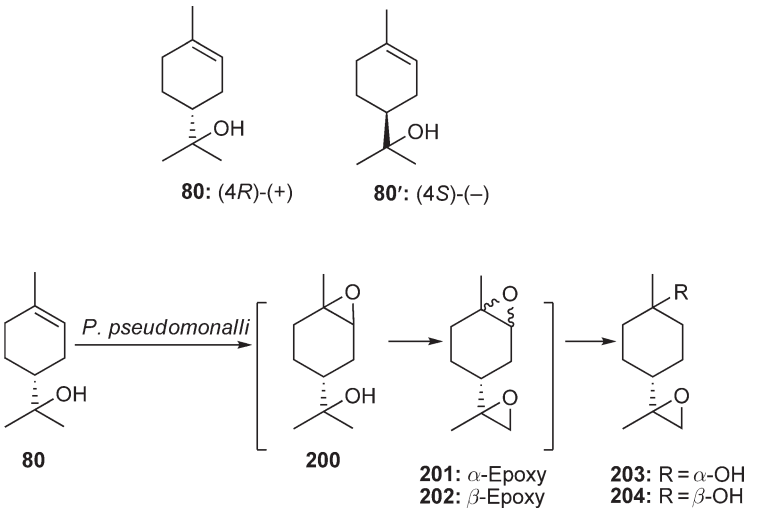
Scheme 75 Biotransformation of (−)- (**38**) and (+)-isopulegol (**38′**) by *Spodoptera litura*.



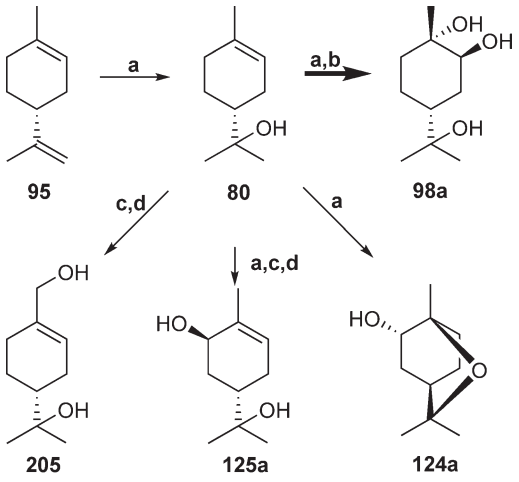
Scheme 76 Microbial resolution of (±)-isopulegyl acetate (**38-Ac** and **38′-Ac**) by microorganisms.

3.19.2.2.3(v) α-Terpineol (80 and 80′) *Pseudomonas pseudomonallei* strain T was cultivated with α-terpineol (**80**) as the sole carbon source and it gave 8,9-epoxy-*p*-menthan-1-ol (**203** and **204**) via monoepoxide (**200**) and diepoxide (**201** and **202**) as intermediates (**Scheme 77**).[117]

(+)-α-Terpineol (**80**) was formed from (+)-limonene (**95**) by the *Citrus* pathogenic fungus *P. digitatum* (Pers.; Fr.) Sacc. KCPYN, which was further biotransformed to *p*-menthane-1α,2β,8-triol (**98a**),



Scheme 77 Structures of (+)- (**80**) and (−)-terpineols (**80′**), and biotransformation of (+)-α-terpineol (**80**) to 8,9-epoxy-*p*-menthan-1-ol (**202**) by *Pseudomonas pseudomonalli* strain T.



a: *P. digitatum*
b: *Penicillium* species
c: *A. niger*
d: *Catharanthus roseus*

Scheme 78 Biotransformation of (+)-α-terpineol (**80**) by the *Citrus* pathogenic fungus *Penicillium digitatum* (Pers.; Fr.) Sacc. KCPYN, *Penicillium* sp.YuzuYN, and *Aspergillus niger* Tiegh (CBAYN).

2α-hydroxy-1,8-cineole (**124a**), and (+)-*trans*-sobrerol (**125a**) (**Scheme 78**).[72,74] *Penicillium* sp. YuzuYN also biotransformed **80** to **98a**. Furthermore, *A. niger* Tiegh (CBAYN) and *C. roseus* biotransformed **80** to **125a** and (+)-oleuropeyl alcohol (**205**), respectively (**Scheme 78**).[72,74,118]

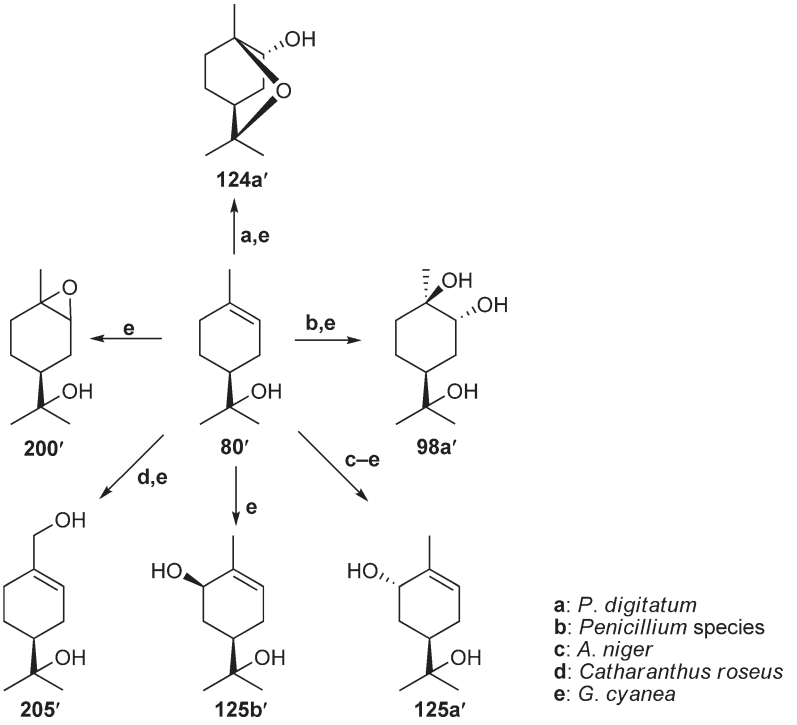
Gibberella cyanea DSM 62719 biotransformed (−)-α-terpineol (**80′**) to *p*-menthane-1β,2α,8-triol (**98a′**), 2α-hydroxy-1,8-cineole (**124a′**), 1,2-epoxy-α-terpineol (**200′**), (−)-oleuropeyl alcohol (**205′**), (−)-*trans*-sobrerol (**125a′**), and *cis*-sobrerol (**125b′**) (**Scheme 79**).[70] In the case of *P. digitatum* (Pers.; Fr.) Sacc. KCPYN, *Penicillium* sp.YuzuYN, and *A. niger* Tiegh (CBAYN), **80′** was biotransformed to **98′**, **125a′**, and **200′**, respectively (**Scheme 79**).[72,74] *Catharanthus roseus* biotransformed **80′** to **125a′** and **205′** (**Scheme 79**).[118]

3.19.2.2.3(vi) Terpinen-4-ol (122 and 122′) *Gibberella cyanea* DSM 62719 biotransformed (*S*)-(+)-terpinen-4-ol (**122**, 1-*p*-menthen-4-ol) to *p*-menthane-1β,2α,4α-triol (**206**), 1-*p*-menthene-4α,6-diol (**207**), and 2α-hydroxy-1,4-cineole (**208b**).[70] *Aspergillus niger* TBUYN-2 also biotransformed (+)-terpinen-4-ol (**122**) to 2α-hydroxy-1,4-cineole (**208b**) and (+)-*p*-menthane-1β,2α,4α-triol (**206**) (**Scheme 80**).[74] On the other hand, *S. litura* biotransformed (*S*)- (**122**) and (*R*)-terpinen-4-ol (**122′**) to (*S*)- (**209**) and (*R*)-*p*-menth-1-en-4,7-diol (**209′**), respectively (**Scheme 80**).[119]

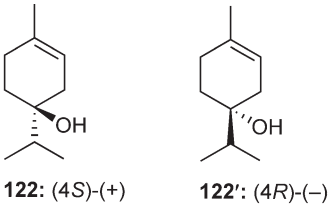
3.19.2.2.3(vii) Thymol (170) and thymol methyl ether (217) Thymol (**170**) was converted at a concentration of 14 mg% by *Streptomyces humidus*, Tu-1, to (1*R*,2*S*)- (**210**) and (1*R*,2*R*)-2-hydroxy-3-*p*-menthen-5-one (**211**) as the major products (**Scheme 81**).[120] On the other hand, in a *Pseudomonas* species, thymol (**170**) was biotransformed to 6-hydroxythymol (**171**), thymol-7-oic acid (**212**), 7-hydroxythymol (**213**), 7,9-dihydroxythymol (**214**), 9-hydroxythymol (**215**), and thymol-9-oic acid (**216**) (**Scheme 81**).[121]

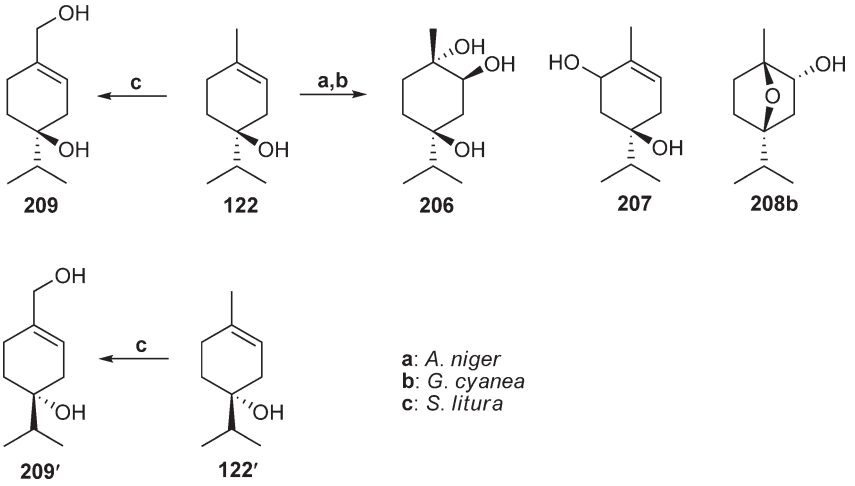
Thymol methyl ether (**217**) was converted by fungi *A. niger*, *Mucor ramannianus*, *Rhizopus arrhizus*, and *Trichothecium roseum* to 7-hydroxy- (**218**) and 9-hydroxythymol methyl ether (**219**) (**Scheme 81**).[122]

3.19.2.2.3(viii) Carvacrol (220) and carvacrol methyl ether (229-Me) When cultivated in a liquid medium with carvacrol (**220**) as the sole carbon source, bacteria isolted from savory and pine consumed carvacrol in the range of 19–22% within 5 days of cultivation. The fungi isolated grew much slower and after 13 days of cultivation consumed 7.1–11.4% carvacrol (**220**). Pure strains belonging to the bacterial genera *Bacterium*, *Bacillus*, and *Pseudomonas*, as well as fungal strains from *Aspergillus*, *Botrytis*, and *Geotrichum* genera, were also

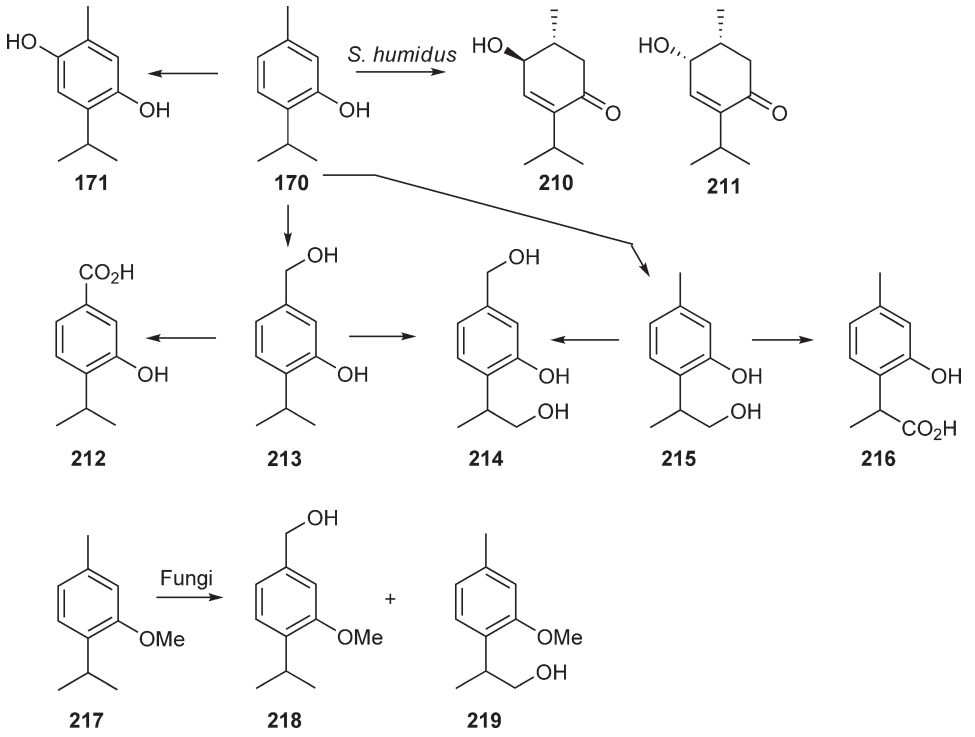


Scheme 79 Biotransformation of (−)-α-terpineol (**80′**) by *Gibberella cyanea* DSM 62719, *Penicillium digitatum* (Pers.; Fr.) Sacc. KCPYN, *Penicillium* species YuzuYN, and *Aspergillus niger* Tiegh (CBAYN).





Scheme 80 Structures of (+)- (**122**) and (−)-terpinene-4-ol (**122′**), and biotransformation of (−)-terpinen-4-ol (**122′**) by *Gibberella cyanea* DSM 62719, *Aspergillus niger* TBUYN-2, and *Spodoptera litura*.



Scheme 81 Biotransformation of thymol (**170**) and thymol methyl ether (**217**) by the actinomycete strain *Streptomyces humidus*, Tu-1, and fungi *Aspergillus niger*, *Mucor ramannianus*, *Rhizopus arrhizus*, and *Trichothecium roseum*.

tested for their ability to grow in a medium containing carvacrol (**220**). Among them, only in *Bacterium* sp. and *Pseudomonas* sp. carvacrol (**220**) uptake was monitored. Both *Pseudomonas* sp. 104 and 107 consumed 19% carvacrol. These two strains also exhibited the highest cell mass yield and the highest productivity (1.1 and 1.2 $g\,l^{-1}$ per day).[123]

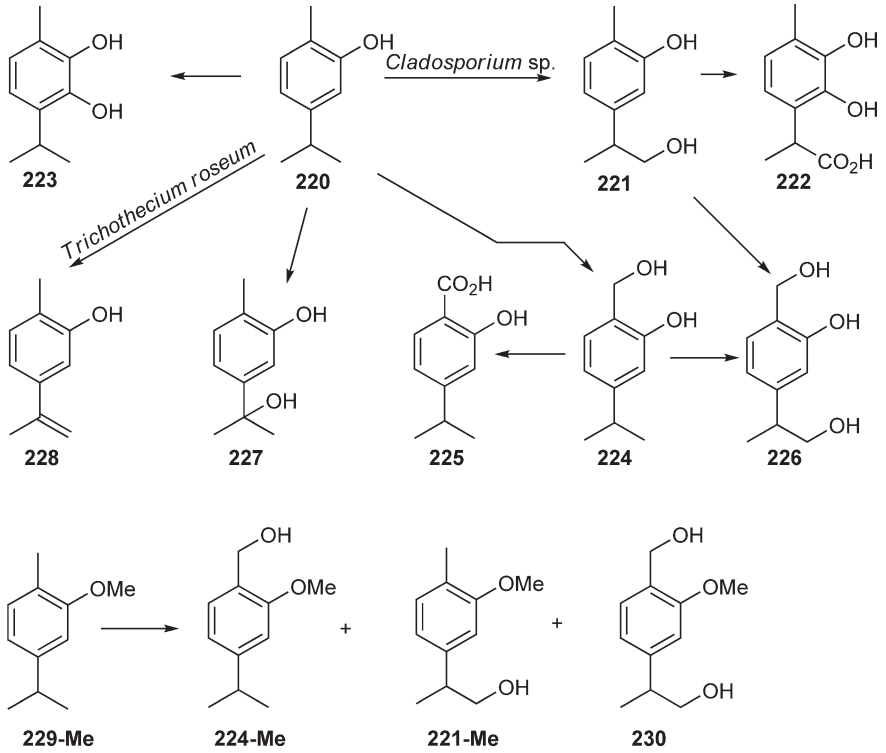
Carvacrol (**220**) was biotransformed to 9-hydroxy- (**221**), carvacrol-9-oic acid (**222**), 3-hydroxy- (**223**), 7-hydroxy- (**224**) and carvacrol-7-oic acid (**225**), 8,9-dihydroxycarvacrol (**226**), 8-hydroxycarvacrol (**227**), and 8,9-dehydrocarvacrol (**228**) by rats[124] and microorganisms[125] including *T. roseum* and *Cladosporium* sp. Furthermore, carvacrol methyl ether (**229-Me**) was converted by the same fungi to 7-hydroxy- (**224-Me**) and 9-hydroxycarvacrol methyl ether (**221-Me**) and 7,9-dihydroxycarvacrol methyl ether (**230**) (**Scheme 82**).[125]

3.19.2.2.3(ix) **cis-** ***(100b and 100b′) and*** **trans-*Carveol (100a and 100a′)*** First, soil Pseudomonad biotransformed (+)-limonene (**95**) to (+)-carvone (**104′**) and (+)-1-*p*-menthene-6,9-diol (**113b**) via(+)-*cis*-carveol (**100b**) as shown in **Scheme 83**.[53,54]

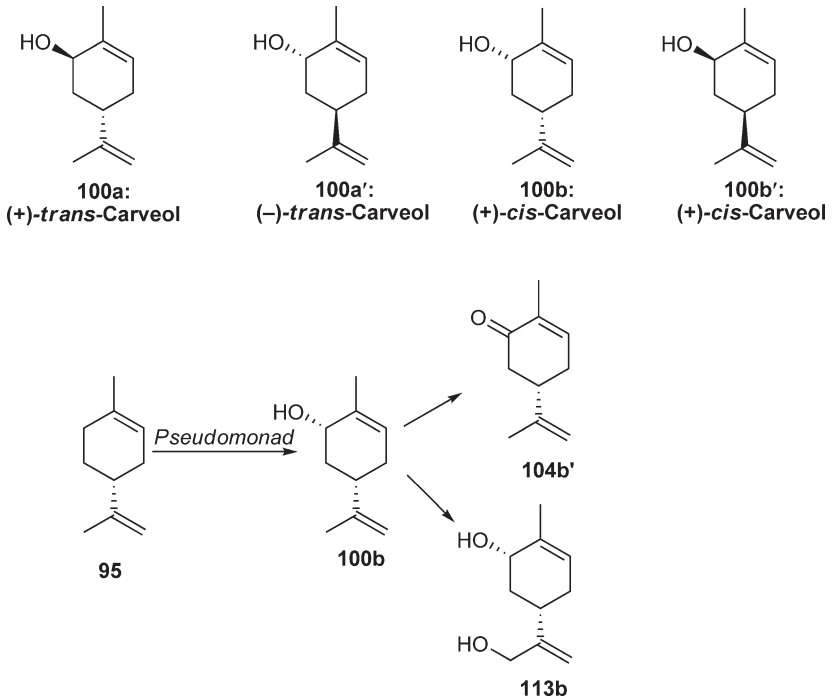
Second, *Pseudomonas ovalis*, strain 6-1, biotransformed the mixture of (−)-*cis*-carveol (**100b′**) and (−)-*trans*-carveol (**100a′**) (94:6, GC ratio) to (−)-carvone (**104**),[126] which was further metabolized reductively to (+)-dihydrocarvone (**105a′**), (+)-isodihydrocarvone (**105b′**), (+)-neodihydrocarveol (**106a′**), and (−)-dihydrocarveol (**106b′**).[97] Hydrogenation at C-1,2 position did not occur, but dehydrogenation at C-6 position occurred forming (−)-carvone (**104**) (**Scheme 84**).

On the other hand, in *Streptomyces* A-5-1 and *Nocardia* 1-3-11, which were isolated from soil, (−)-carvone (**104**) was mainly reduced to (−)-*trans*-carveol (**100a′**) and (−)-*cis*-carveol (**100b′**), respectively. On the other hand, (−)-*trans*-carveol (**100a′**) and (−)-*cis*-carveol (**100b′**) were dehydrogenated to **104** by strain 1-3-11 and other microorganisms.[24] The reaction between *trans*- and *cis*-carveol (**100a′** and **100b′**) and (−)-carvone (**104**) is reversible (**Scheme 84**).[127]

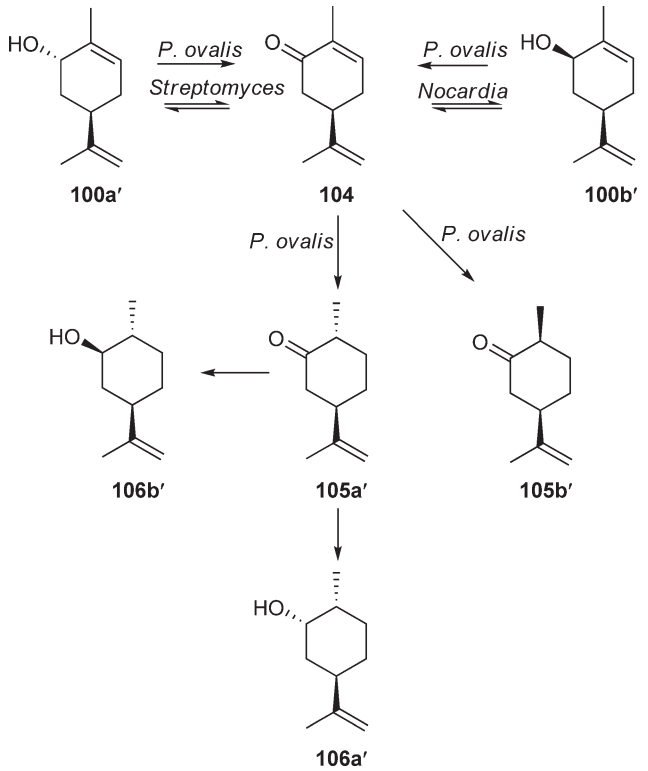
Third, an investigation of the biotransformation of the mixture of (−)-*trans*- (**100a′**) and (−)-*cis*-carveol (**100b′**) (60:40, GC ratio) was carried out by using 81 strains of soil actinomycetes. All actinomycetes produced



Scheme 82 Biotransformation of carvacrol (**220**) and carvacrol methyl ether (**229**) by rats and microorganisms.

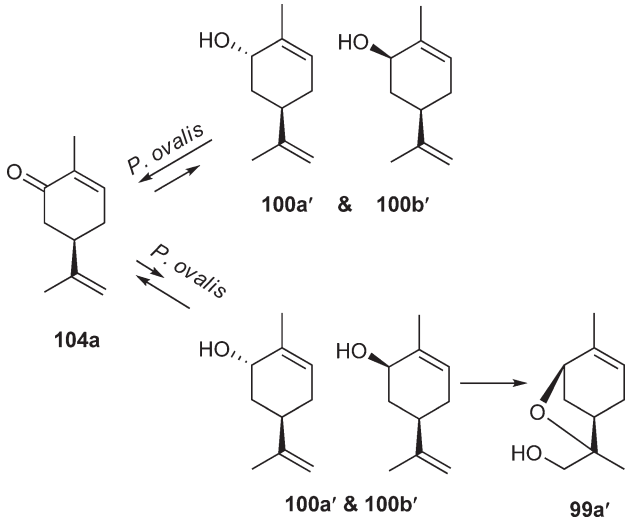


Scheme 83 Structures of (+)-*trans*-carveol (**100a**) and its isomers, and proposed metabolic pathway of (+)-limonene (**95**) and (+)-*cis*-carveol (**100b**) by soil Pseudomonad.

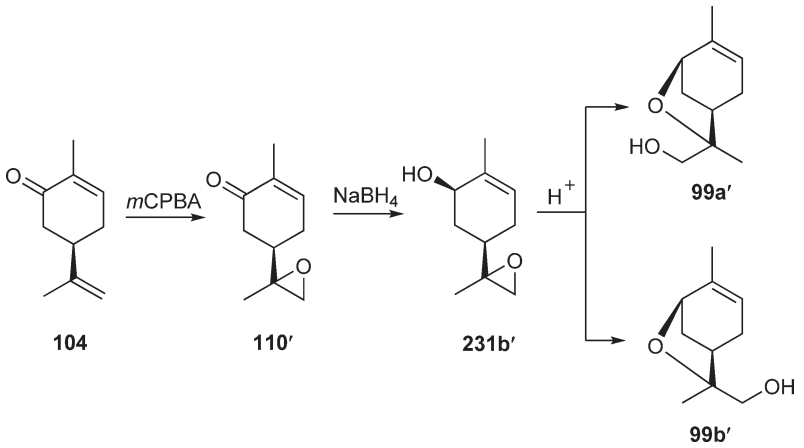


Scheme 84 Biotransformation of (−)-*trans*- (**100a′**) and (−)-*cis*-carveol (**100b′**) (6:94, GC ratio) by *Pseudomonas ovalis* strain 6-1, *Streptomyces* A-5-1, and *Nocardia* 1-3-11.

(−)-carvone (**104**) from the mixture of (−)-*trans*- (**100a′**) and (−)-*cis*-carveol (**100b′**) (60:40, GC ratio). However, 41 strains of actinomycetes converted (−)-*cis*-carveol (**100b′**) to (4*R*,6*R*)-(+)-6,8-oxidomenth-1-en-9-ol (**99a′**), which is named as bottrospicatol after the name of the microorganism *Streptomyces bottropensis* (bottro) and (−)-*cis*-carveol (**100b′**) containing *Mentha spicata* (spicat) and alcohol (ol) (**Scheme 85**).[128,129]



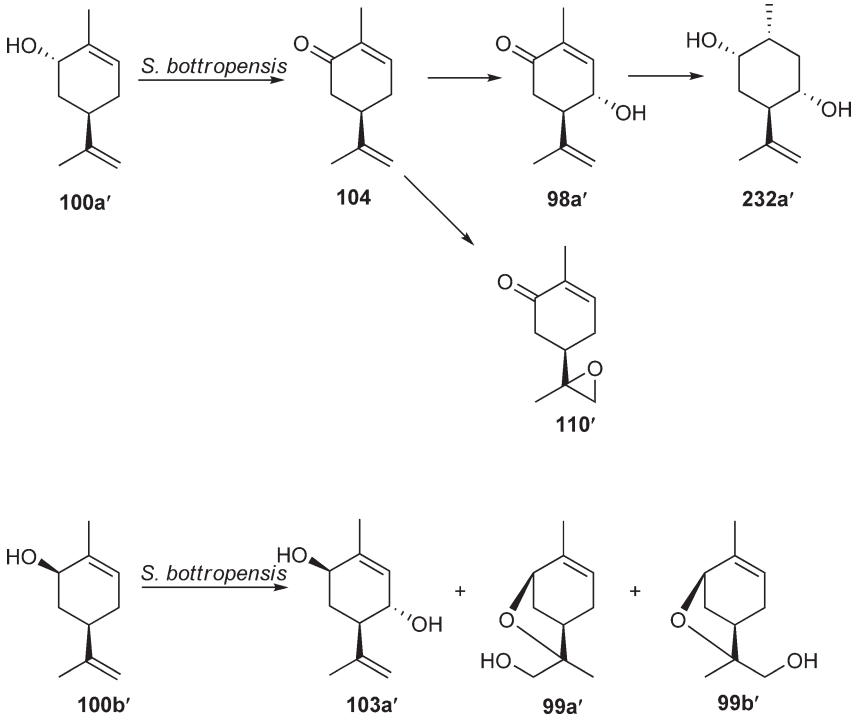
Scheme 85 Metabolic pathways of *cis*-carveol (**100b′**) by *Pseudomonas ovalis* strain 6-1, *Streptomyces bottropensis* SY-2-1, and other microorganisms.



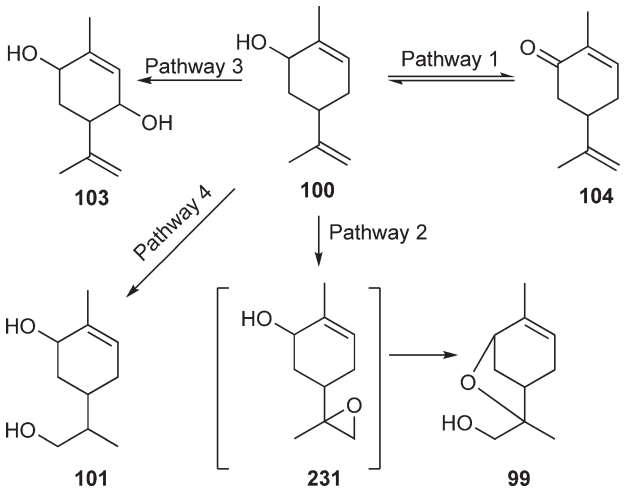
Scheme 86 Preparation of (+)-bottrospicatol (**99a′**) and (+)-isobottrospicatol (**99b′**) from (−)-carvone (**104**) with *m*-chloroperbenzoic acid (CPBA).

(+)-Bottrospicatol (**99a′**) was prepared by epoxidation of (−)-carvone (**104**) with *m*CPBA to (−)-carvone-8,9-epoxide (**110′**), followed by stereoselective reduction with $NaBH_4$ to alcohol, which was immediately cyclized with 0.1 N H_2SO_4 to give a diastereo mixture of bottrospicatol (**99a′** and **99b′**) (**Scheme 86**).[128]

Further investigation showed that *S. bottropensis* SY-2-1[130] follows different metabolic pathways for (−)-*trans*-carveol (**100a′**) and (−)-*cis*-carveol (**100b′**): it converted (−)-*trans*-carveol (**100a′**) to (−)-carvone (**104**), (−)-carvone-8,9-epoxide (**110′**), (−)-5β-hydroxycarvone (**98**), and (+)-5β-hydroxyneodihydrocarveol (**232a′**) (**Scheme 87**), whereas (−)-*cis*-carveol (**100b′**) was converted to (+)-bottrospicatol (**99a′**) and (−)-5β-hydroxy-*cis*-carveol (**103a′**) as the major products together with (+)-isobottrospicatol (**99b′**) as the minor product as shown in **Scheme 87**.[23,129,131,132]



Scheme 87 Biotransformation of (−)-*trans*- (**100a′**) and (−)-*cis*-carveol (**100b′**) by *Streptomyces bottropensis* SY-2-1 and *Streptomyces ikutamanensis* Ya-2-1.



Scheme 88 General metabolic pathways of carveol (**100**) by microorganisms.

In the metabolism of *cis*-carveol (**100**) by microorganisms, there are four pathways (pathways 1–4) as shown in **Scheme 88**. In pathway 1, *cis*-carveol (**100**) is metabolized to carvone (**104**) by dehydrogenation at C-2 position.[126,127]. In pathway 2, *cis*-carveol (**100**) is metabolized via epoxide as an intermediate to bottrospicatol (**99**) by rearrangement at C-2 and C-8[128,129,132] positions. In pathway 3, *cis*-carveol (**100**) is hydroxylated at C-5 position to give 5-hydroxy-*cis*-carveol (**103**). In pathway 4, *cis*-carveol (**100**) is metabolized to 1-*p*-menthene-2,9-diol (**101**) by hydroxylation at C-9 position.[53,54]

The effect of (−)-*cis*- (**100b′**) and (−)-*trans*-carveol (**100a′**) conversion products of *S. bottropensis* SY-2-1 on the germination of lettuce seeds was examined and the result is shown in **Table 3**. (+)-Bottrospicatol (**99′**) and (−)-carvone-8,9-epoxide (**110′**) showed strong inhibitory effect on the germination of lettuce seeds.

Streptomyces bottropensis SY-2-1[130] also has different metabolic pathways for (+)-*trans*-carveol (**100a**) and (+)-*cis*-carveol (**100b**): it converted (+)-*trans*-carveol (**100a**) to (+)-carvone (**104′**), (+)-carvone-8,9-epoxide (**110**), and (+)-5β-hydroxycarvone (**108a**) (**Scheme 89**),[131,133] whereas (+)-*cis*-carveol (**100b**) was converted to (−)-isobottrospicatol (**99c′**) and (+)-5-hydroxy-*cis*-carveol (**103a**) as the major products and (−)-bottrospicatol (**99c**) as the minor product as shown in **Scheme 90**.[23,132,134]

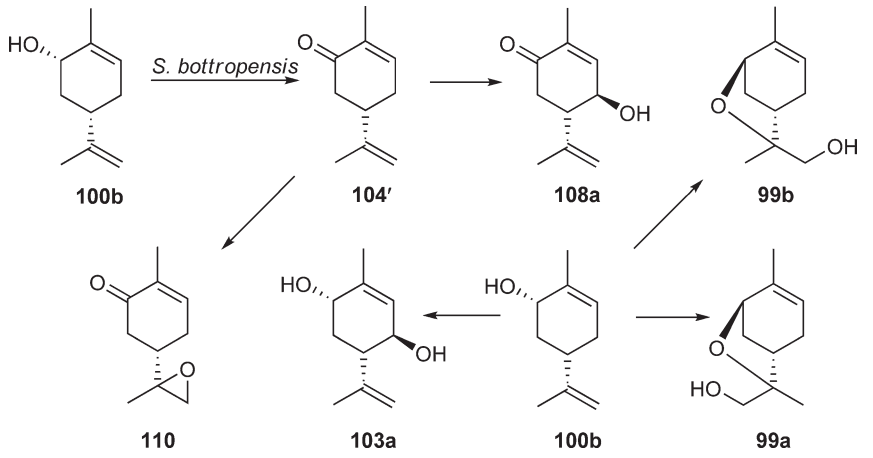
The role of (+)-bottrospicatol (**99a′**) and related compounds in seed germination and root elongation of plants was examined with reference to barnyard grass, wheat, garden cress, radish, green foxtail, and lettuce.[23]

Isomers and derivatives of bottrospicatol were prepared by the procedure shown in **Scheme 91**.[23] The chemical structure of each compound was confirmed by interpretation of spectral data. The effects of all isomers and derivatives on the germination of lettuce seeds were compared. (+)-Bottrospicatol (**99a′**) showed the highest germination inhibitory activity among the different isomers. Interestingly, (−)-isobottrospicatol

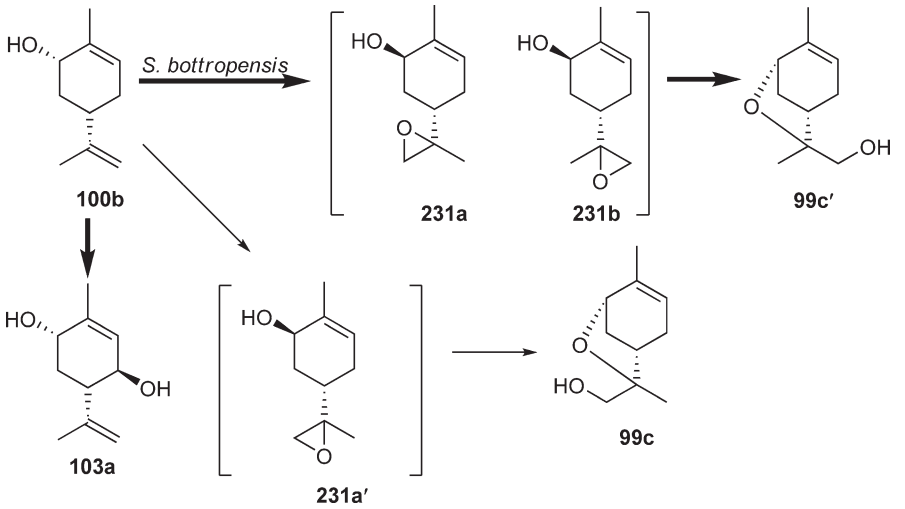
Table 3 Effects of (−)-*cis*- (**100b′**) and (−)-*trans*-carveol (**100a′**) conversion products by *Streptomyces bottropensis* SY-2-1 on the germination of lettuce seeds

	Germination rate (%)	
Compounds	*24 h*	*48 h*
(−)-Carvone (**104**)	47	89
(+)-Bottrospicatol (**99a′**)	3	48
(−)-Carvone-8, 9-epoxide (**110′**)	2	77
5β-Hydroxyneodihydrocarveol (**232a′**)	86	96
5β-Hydroxycarvone (**98a′**)	91	96
Control (water)	95	96

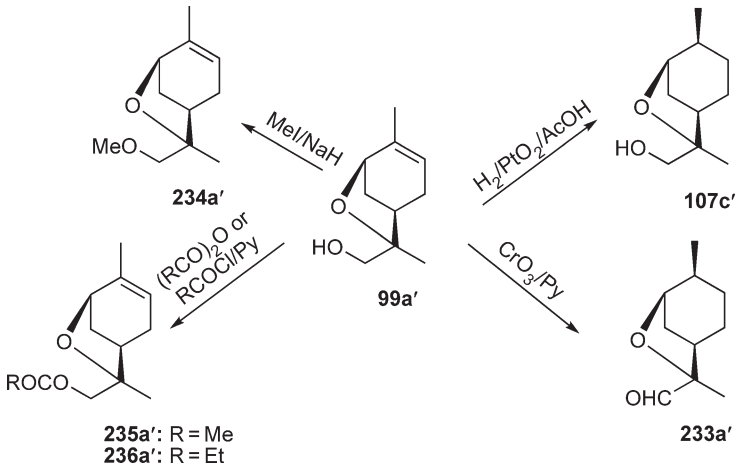
Concentration of each compound was adjusted at 200 ppm.



Scheme 89 Metabolic pathways of (+)-*trans*- (**100a**) and (+)-*cis*-carveol (**100b**) by *Streptomyces bottropensis* SY-2-1.



Scheme 90 Metabolic pathways of (+)-*cis*-carveol (**100b**) by *Streptomyces bottropensis* SY-2-1 and *Streptomyces ikutamanensis* Ya-2-1.



Scheme 91 Preparation of (+)-bottrospicatol (**99a′**) derivatives.

(**99b**) was not effective even at a concentration of 500 ppm. (+)-Bottrospicatol methyl ether (**234a′**) and esters (**235a′** and **236a′**) exhibited weak inhibitory activities. The inhibitory activity of (−)-isodihydrobottrospicatol (**107c′**) was as high as that of (+)-bottrospicatol (**99a′**). Furthermore, an oxidized compound, (+)-bottrospicatal (**233a′**), exhibited higher activity than (+)-bottrospicatol (**99a′**). So, the germination inhibitory activity of (+)-bottrospicatal (**233a′**) against several plant seeds such as lettuce, green foxtail, radish, garden cress, wheat, and barnyard grass was examined. The results indicate that (+)-bottrospicatal (**233a′**) is a selective germination inhibitor with activity as follows: lettuce > green foxtail > radish > garden cress > wheat > barnyard grass.

Enantio- and diastereoselective biotransformation of *trans*-carveols (**100a** and **100a′**) by *E. gracilis* Z[135] and *Chlorella pyrenoidosa* IAM C-28 was studied.[96]

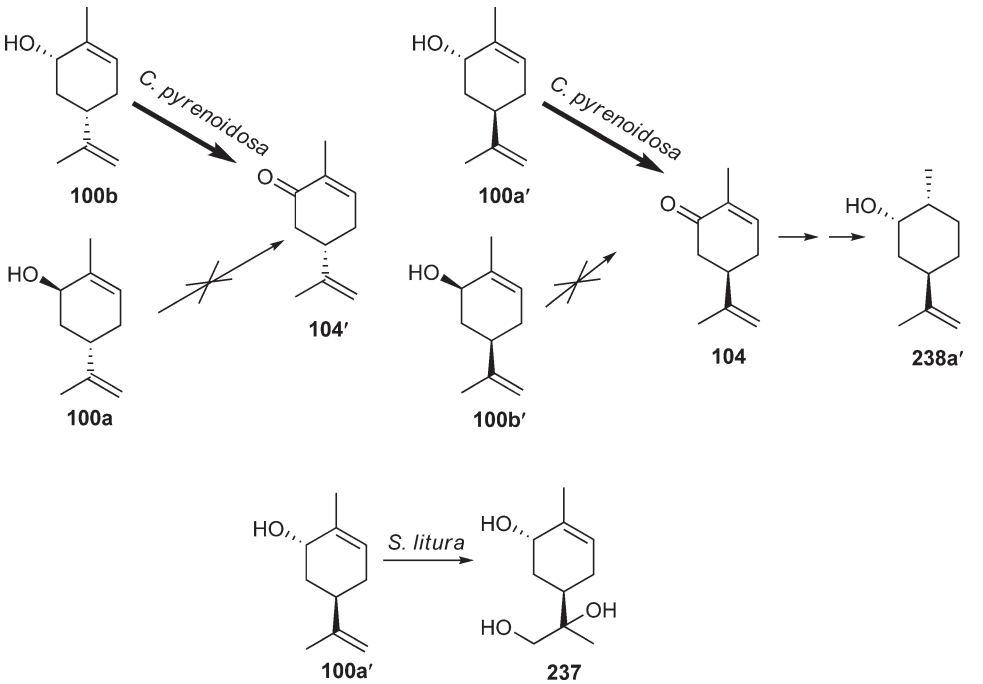
In the biotransformation of racemic *trans*-carveol (**100a** and **100a′**), *C. pyrenoidosa* IAM C-28 showed high enantioselectivity for (−)-*trans*-carveol (**100a′**) to give (−)-carvone (**104**), while (+)-*trans*-carveol (**100a**) was not converted at all. The same *C. pyrenoidosa* IAM C-28 showed high enantioselectivity for (+)-*cis*-carveol (**100b**) to give (+)-carvone (**104**) in the biotransformation of racemic *cis*-carveol (**100b** and **100b′**). (−)-*cis*-Carveol (**100b′**) was not converted at all. The same phenomenon was observed in the biotransformation of the mixture of (−)-*trans*- and (−)-*cis*-carveol (**100a′** and **100b′**) and the mixture of (+)-*trans*- and (+)-*cis*-carveol (**100a** and **100b**) as shown in **Scheme 92**. The high enantioselectivity and high diastereoselectivity for the dehydrogenation of (−)-*trans*- and (+)-*cis*-carveol (**100a** and **100b′**) were shown in *E. gracilis* Z,[135] *C. pyrenoidosa* IAM C-28,[96] *N. tabacum*, and other *Chlorella* species (**Scheme 92**).

On the other hand, the high enantioselectivity for **100a′** was observed in the biotransformation of racemic (+)-*trans*-carveol (**100a**) and (−)-*trans*-carveol (**100a′**) by *C. sorokiniana* SAG to give (−)-carvone (**104**).

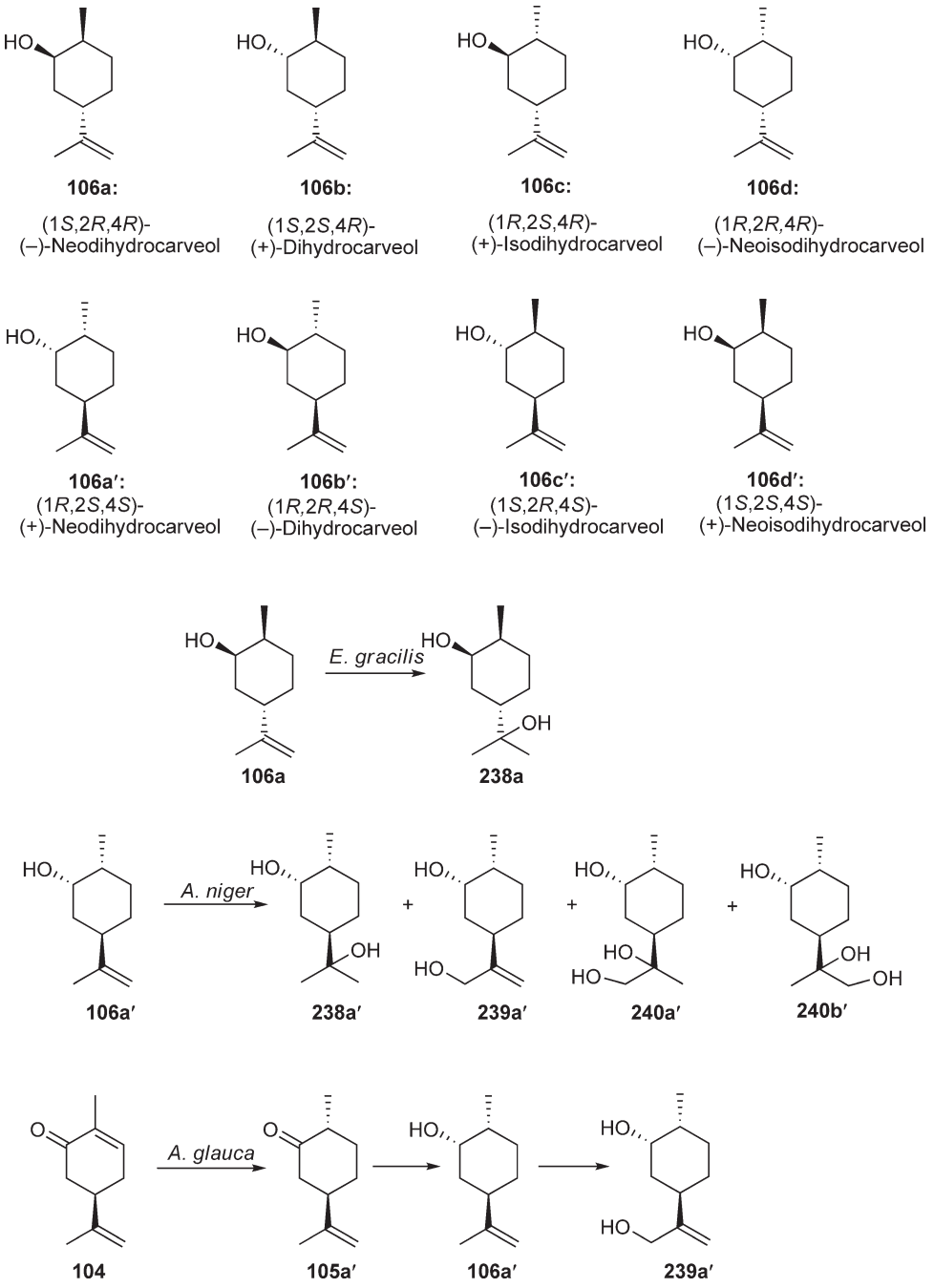
It was considered that the formation of (−)-carvone (**104**) from (−)-*trans*-carveol (**100a′**) by diastereo- and enantioselective dehydrogenation is a very interesting phenomenon in order to produce mosquitocidal (+)-*p*-menthane-2,8-diol (**238a′**) (**Scheme 92**).[61]

(4*R*)-*trans*-Carveol (**100a′**) was converted by *S. litura* to 1-*p*-menthene-6,8,9-triol (**237**) (**Scheme 92**).[82]

In **Scheme 93**, the chemical structures of eight kinds of dihydrocarveols are demonstrated.



Scheme 92 Enantio- and diastereoselective biotransformation of *trans*- (**100a** and **100a′**) and *cis*-carveols (**100b** and **100b′**) by *Euglena gracilis* Z and *Chlorella pyrenoidosa* IAM C-28 and biotransformation of (4*R*)-*trans*-carveol (**100a′**) by *Spodoptera litura*.



Scheme 93 Structures of dihydrocarveols (**106a–106d′**), and biotransformation of (–)- and (+)-neodihydrocarveol (**106a** and **106a′**) by *Euglena gracilis* Z, *Aspergillus niger* TBUYN-2, and *Absidia glauca*.

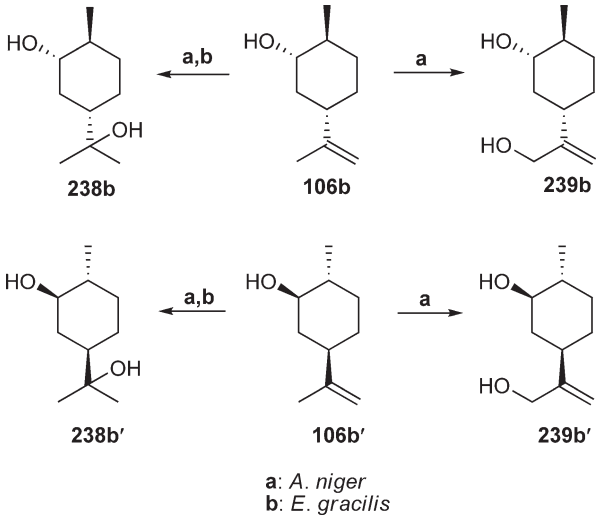
3.19.2.2.3(x) Neodihydrocarveol (106a and 106a′) (+)-Neodihydrocarveol (**106a′**) was converted to *p*-menthane-2,8-diol (**238a′**), 8-*p*-menthene-2,8-diol (**239a′**), and *p*-menthane-2,8,9-triols (**240a′** and **240b′**) by *A. niger* TBUYN-2 (**Scheme 93**).[56,57,109] In the case of *E. gracilis* Z, mosquitocidal (+)-*p*-menthane-2,8-diol (**238a′**) was formed stereospecifically from (–)-carvone (**104**) via (+)-dihydrocarvone (**105a′**) and (+)-neodihydrocarveol(**106a′**).[61,136] (–)-Neodihydrocarveol (**106a**) was also readily and stereospecifically converted by *E. gracilis* Z to (–)-*p*-menthane-2,8-diol (**238a**) (**Scheme 93**).[136]

On the other hand, *A. glauca* converted (−)-carvone (**104**) stereospecifically to (+)-8-*p*-menthene-2,8-diol (**239a′**) via (+)-dihydrocarvone (**105a′**) and (+)-neodihydrocarveol (**106a′**) (**Scheme 93**).[137]

3.19.2.2.3(xi) Dihydrocarveol (106b and 106b′) (+)- (**106b**) and (−)-Dihydrocarveol (**106b′**) were converted by 10 kinds of *Aspergillus* spp. to mainly (+)- (**239b′**) and (−)-10-hydroxydihydrocarveol (**239b**, 8-*p*-menthene-2,10-diol) and (+)- (**238b′**) and (−)-8-hydroxydihydrocarveol (**238b**, *p*-menthane-2,8-diol), respectively (**Scheme 94**).[136,138] The metabolic pattern of dihydrocarveols is shown in **Table 4**.

In the case of biotransformation by *S. bottropensis* SY-2-1, (+)-dihydrocarveol (**106b**) was converted to (+)-dihydrobottrospicatol (**107b**) and (+)-dihydroisobottrospicatol (**107b′**), whereas (−)-dihydrocarveol (**106b′**) was metabolized to (−)-dihydrobottrospicatol (**107a**) and (−)-dihydroisobottrospicatol (**107a′**). (+)-Dihydroisobottrospicatol (**107b′**), and (−)-dihydrobottrospicatol (**107a′**) are the major products (**Scheme 95**).[139]

3.19.2.2.3(xii) Isodihydrocarveol (106c and 106c′) *Euglena gracilis* Z converted (+)-isodihydrocarveol (**106c**) and (−)-isodihydrocarveol (**106c′**) to the corresponding 8-hydroxyisodihydrocarveols **238c** and **238c′**, respectively (**Scheme 96**).[136]

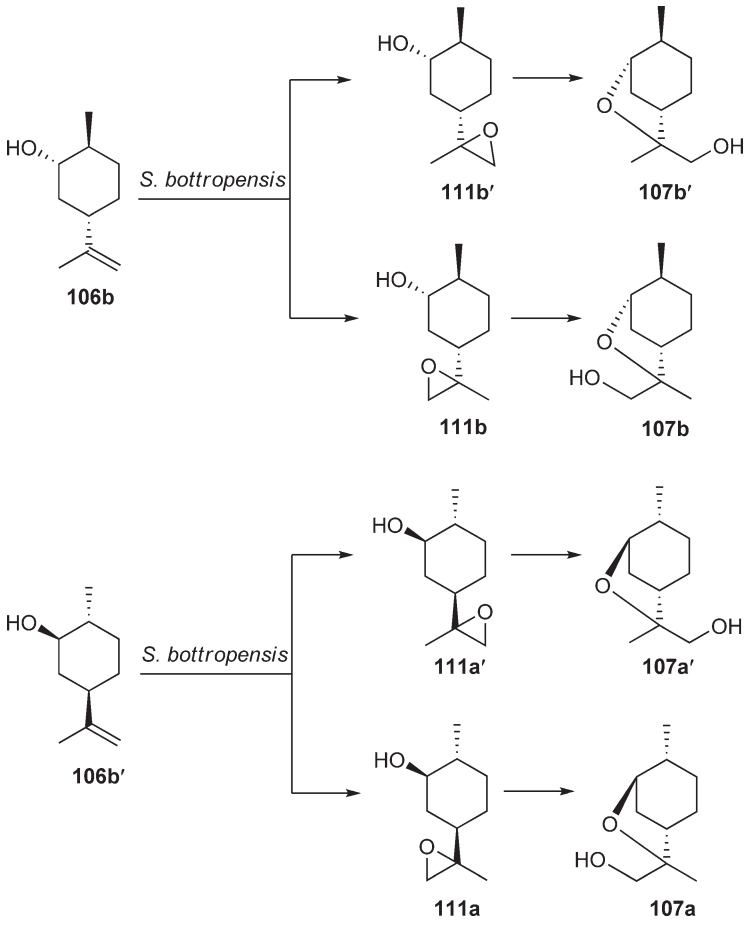


Scheme 94 Biotransformation of (+)- (**106b**) and (−)-dihydrocarveol (**106b′**) by 10 kinds of *Aspergillus* species and *Euglena gracilis* Z.

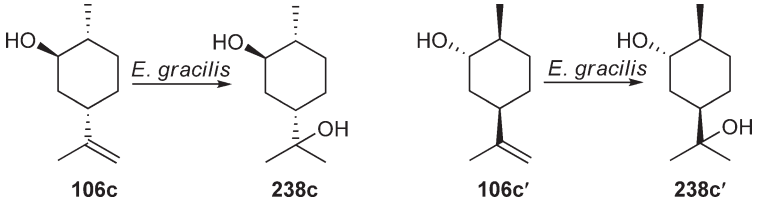
Table 4 Metabolic pattern of dihydrocarveols (**106b** and **106b′**) by 10 kinds of *Aspergillus* species

	Compounds			*Compounds*		
Microorganisms	*106b′*	*238b′*	*c.r. (%)*	*106b*	*238b*	*c.r. (%)*
Aspergillus awamori IFO4033	0	98	99	3	81	94
Aspergillus fumigatus IFO4400	0	14	34	+	6	14
Aspergillus sojae IFO4389	0	47	59	1	50	85
Aspergillus usami IFO4338	0	32	52	+	5	7
Aspergillus cellulosae M-77	0	27	52	+	7	14
Aspergillus cellulosae IFO4040	0	30	55	1	5	8
Aspergillus terreus IFO6123	0	79	92	+	18	46
Aspergillus niger IFO4034	0	29	49	+	8	12
Aspergillus niger IFO4049	4	50	67	9	34	59
Aspergillus niger TBUYN-2	29	68	100	30	53	100

c.r., conversion ratio.



Scheme 95 Biotransformation of (+)- (**106b**) and (−)-dihydrocarveol (**106b′**) by *Streptomyces bottropensis* SY-2-1.

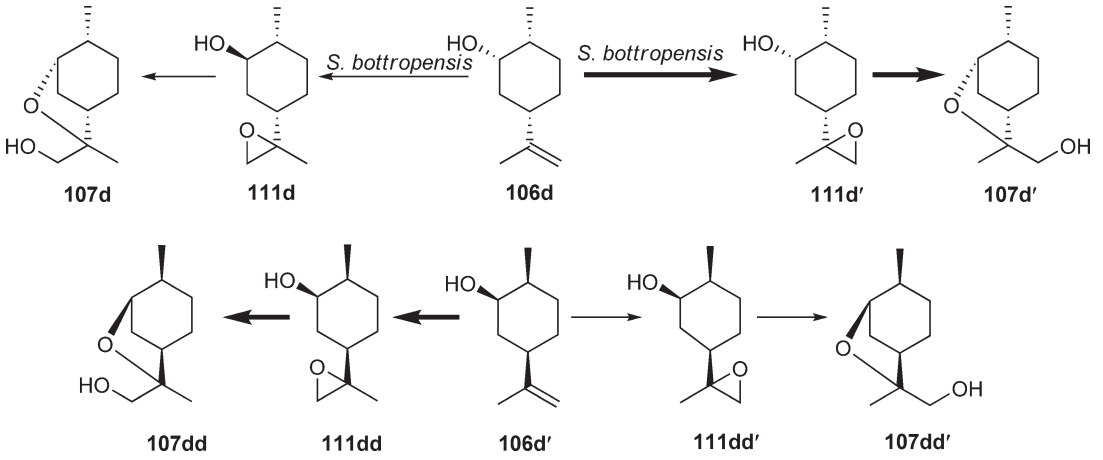


Scheme 96 Biotransformation of (+)- (**106c**) and (−)-isodihydrocarveol (**106c′**) by *Euglena gracilis* Z.

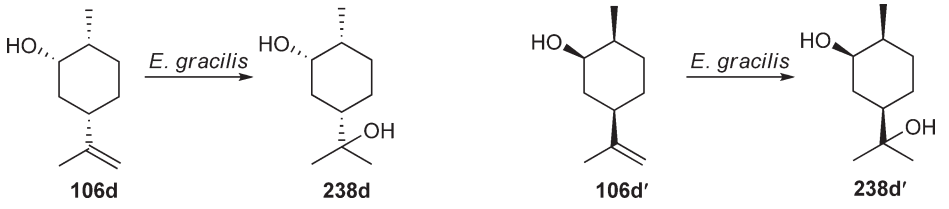
3.19.2.2.3(xiii) Neoisodihydrocarveol (106d and 106d′) In the case of biotransformation by *S. bottropensis* SY-2-1, (−)-neoisodihydrocarveol (**106d**) was converted to (+)-isodihydrobottrospicatol (**107d**) and (+)-isodihydroisobottrospicatol (**107d′**), whereas (+)-neoisodihydrocarveol (**106d′**) was metabolized to (−)-isodihydrobottrospicatol (**107dd**) and (−)-isodihydroisobottrospicatol (**107dd′**). (+)-Isodihydroisobottrospicatol (**107d′**) and (−)-isodihydrobottrospicatol (**107dd**) are the major products (**Scheme 97**).[139]

Euglena gracilis Z converted (−)- (**106d**) and (+)-neoisodihydrocarveol (**106d′**) to the corresponding 8-hydroxyneoisomenthols **238a** and **238d′**, respectively (**Scheme 98**).[136]

Eight kinds of 8-hydroxydihydrocarveols (**238a** and **238d′**, 8-*p*-menthane-2,8-diols) were obtained from carvone (**104** and **104′**), dihydrocarvones (**105a** and **105b**; **105a′** and **105b′**), and dihydrocarveols (**106a–106d** and **106a′–d′**) by *E. gracilis* Z[136] as shown in **Scheme 99**.



Scheme 97 Formation of dihydroisobottrospicatols (**107**) from neoisodihydrocarveol (**106d** and **106d′**) by *Streptomyces bottropensis* SY-2-1.



Scheme 98 Biotransformation of (+)- (**106d**) and (−)-neoisodihydrocarveol (**106d′**) by *Euglena gracilis* Z.

3.19.2.2.3(xiv) Perillyl alcohol (116 and 116′) (−)-Perillyl alcohol (**116′**) was epoxidized by *S. ikutamanensis* Ya-2-1 to give 8,9-epoxy-(−)-perillyl alcohol (**177′**) (**Scheme 100**).[24] (−)-Perillyl alcohol (**116′**) was glycosylated by *E. perriniana* suspension cells to (−)-perillyl alcohol monoglucoside (**241**) and diglucoside (**242**) (**Scheme 100**).[113,140]

Furthermore, 1-perillyl-β-glucopyranoside (**241′**) was converted into the corresponding oligosaccharides (**243–246**) by cyclodextrin glucanotransferase (**Scheme 100**).[140]

3.19.2.2.3(xv) Carvomenthols (247a–d′) In **Scheme 101**, (+)-carvomenthol (**247b**) and its stereoisomers (**247a,c,d, 247b′-d′**) are demonstrated.

(+)-Iso- (**247c**) and (+)-neoisocarvomenthol (**247d**) were formed from (+)-carvotanacetone (**248a**) via (−)-isocarvomenthone (**264a**) by *P. ovalis*, strain 6-1, whereas (+)-neocarvomenthol (**247a′**) and (−)-carvomenthol (**247b′**) were formed from (−)-carvotanacetone (**248a′**) via (+)-carvomenthone (**264a′**) by the same bacterium; **264a**, **264a′**, and **247d** were the major products (**Scheme 101**).[141]

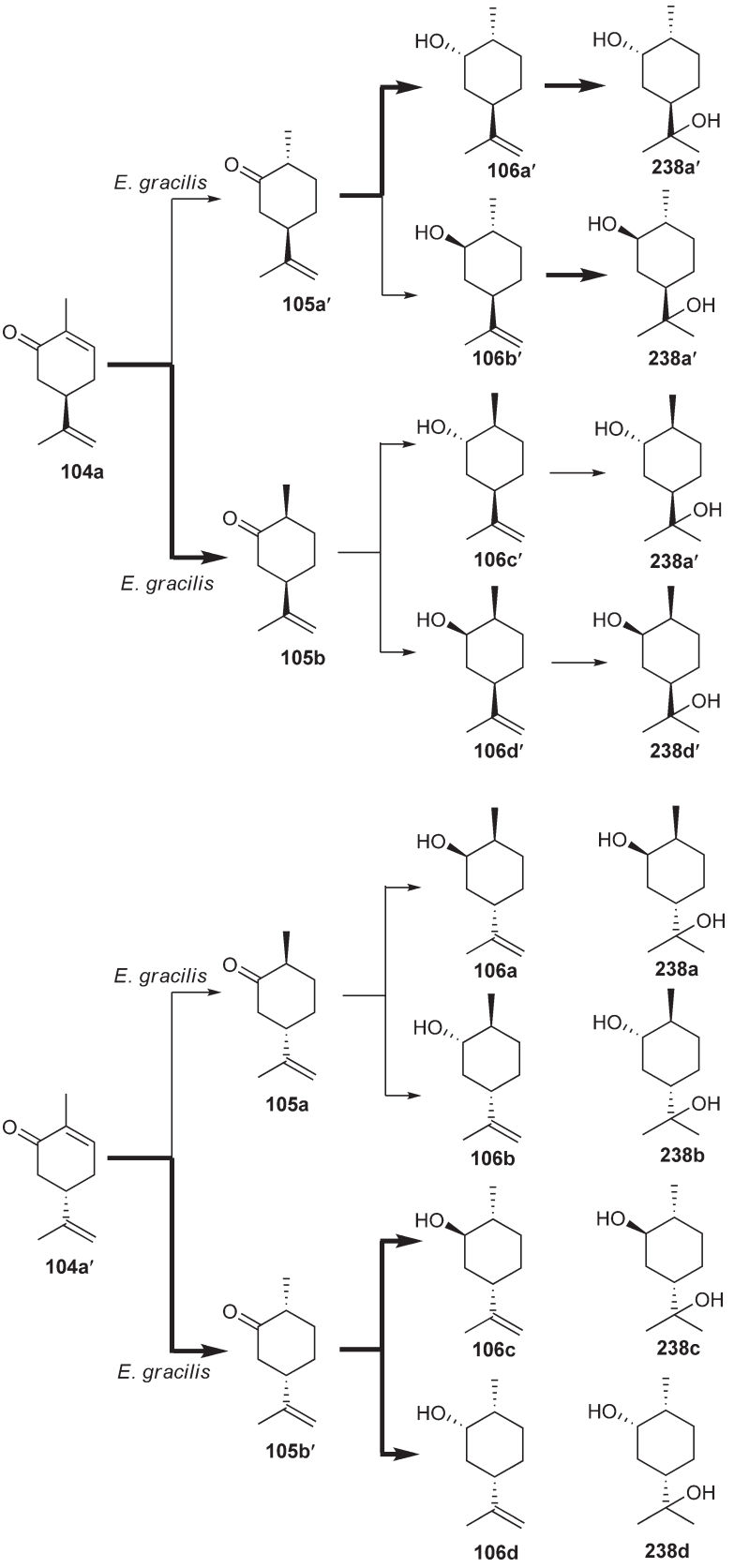
Microbial resolution of carvomenthols was carried out by using selected microorganisms such as *Trichoderma S* and *B. subtilis* var. *niger*.[142] Racemic carvomenthyl acetate, racemic isocarvomenthyl acetate, and racemic neoisocarvomenthyl acetate were asymmetrically hydrolyzed to (−)-carvomenthol (**247b′**), (−)-isocarvomenthol (**247c**), and (+)-neoisocarvomenthol (**247d′**), respectively, together with each non-reacted substrate, (+)-carvomenthyl acetate, (+)-isocarvomenthyl acetate, and (−)-neoisocarvomenthyl acetate. Racemic neocarvomenthyl acetate was not hydrolyzed (**Scheme 102**).[142]

3.19.2.2.4 Monocyclic monoterpene ketone

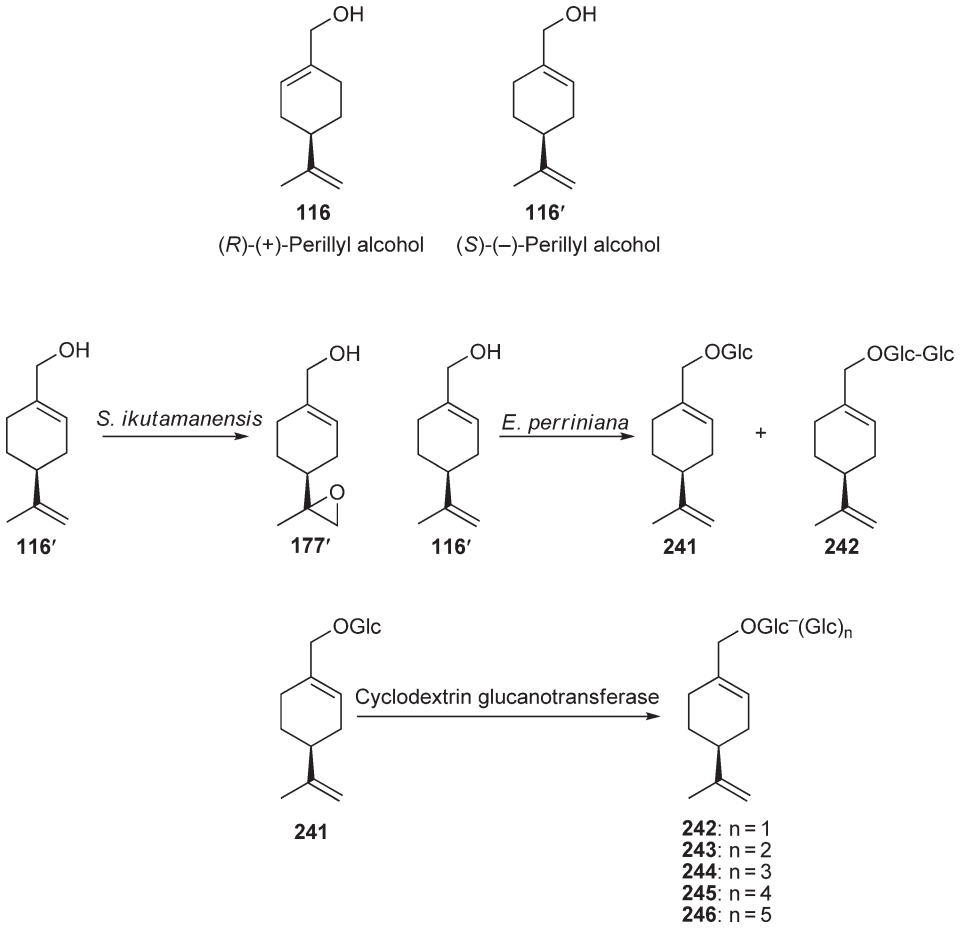
3.19.2.2.4(i) α,β-Unsaturated ketone

3.19.2.2.4(i)(a) Carvone (104 and 104′) In **Scheme 103**, the stereostructures of (−)-carvone (**104′**) and (+)-carvone (**104**) are demonstrated.

Carvone occurs as (+)-carvone (**104**), (−)-carvone (**104′**) (**Scheme 103**), or racemic carvone. (*S*)-(+)-Carvone (**104**) is the major component of caraway oil (~60%) and dill oil and has a herbaceous odor



Scheme 99 Formation of eight kinds of 8-hydroxydihydrocarveols (**238a–238d** and **238a′–238d′**) from (+)- (**104**) and (−)-carvone (**104′**), dihydrocarvones (**105a** and **105b**, and **105a′** and **105b′**), and dihydrocarveols (**106a–106d** and **106a′–106d′**) by *Euglena gracilis* Z.



Scheme 100 Biotransformation of (–)-perillyl alcohol (**116**) by *Streptomyces ikutamanensis* Ya-2-1 and suspension cells of *Eucalyptus perriniana* and (–)-perillyl alcohol monoglucoside (**242**) by cyclodextrin glucanotransferase.

reminiscent of caraway and dill seeds. (*R*)-(−)-Carvone (**104′**) is present in spearmint oil at a concentration of 70–80% and has a herbaceous odor similar to spearmint.[41]

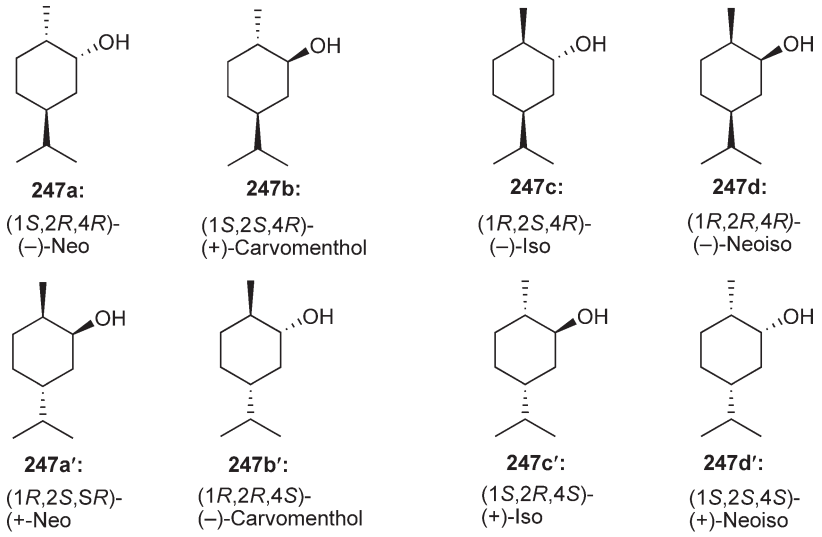
The distribution of carvone convertible microorganisms is summarized in **Table 5**. When ethanol was used as a carbon source, 40% of bacteria converted (+)- (**104′**) and (−)-carvone (**104**). On the other hand, when glucose was used, 65% of bacteria converted carvone. In the case of yeasts, 75% converted (+)- (**104′**) and (−)-carvone (**104**). In the case of fungi, 90 and 85% converted **104** and **104′**, respectively. In actinomycetes, 56 and 90% converted **104** and **104′**, respectively.

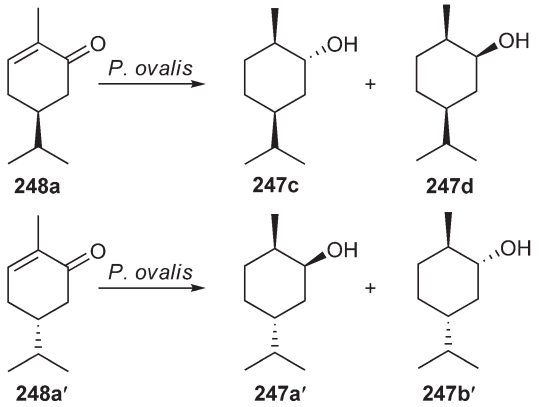
Many microorganisms except for some strains of actinomycetes were capable of hydrogenating the C═C double bond at C-1,2 position of (+)- (**104′**) and (−)-carvone (**104**) to give mainly (−)-isodihydrocarvone (**105b′**) and (+)-dihydrocarvone (**105a′**), respectively (**Scheme 104**, **Table 6**).[126,143–146]

Furthermore, it was found that (−)-carvone (**104**) was converted via (+)-isodihydrocarvone (**105a′**) to (+)-isodihydrocarveol (**106b′**) and (+)-neoisodihydrocarveol (**106d′**) by some strains of actinomycetes.[147,148] (−)-Isodihydrocarvone (**106b**) was epimerized to (−)-dihydrocarvone (**105a**) after the formation of (−)-isodihydrocarvone (**105b**) from (+)-carvone (**104′**) by the growing cells, resting cells, and cell-free extracts of *Pseudomonas fragi* IFO3458.[149]

Consequently, the metabolic pathways of carvone by microorganisms are summarized in the following eight groups (**Scheme 104**):

Group 1: (−)-carvone (**104**)–(+)-dihydrocarvone (**105a′**)–(+)-neodihydrocarveol (**106a′**)
Group 2: **104**–**105a**–(−)-dihydrocarveol (**106b′**)

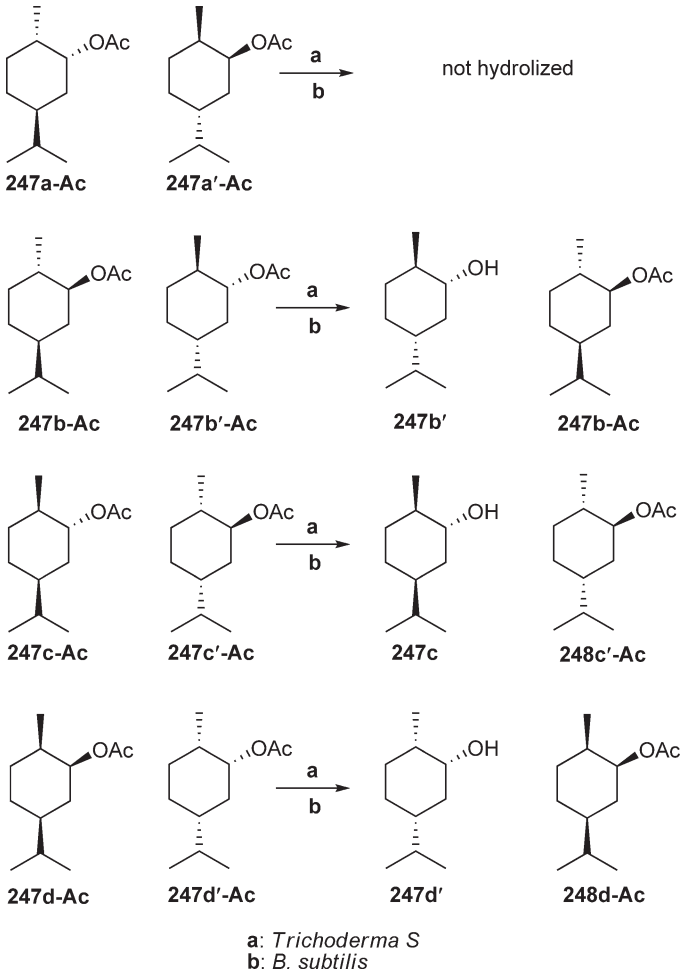




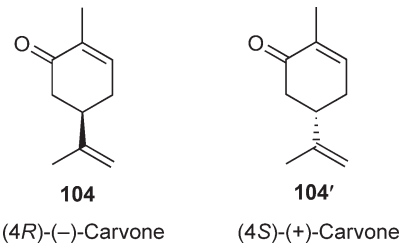
Scheme 101 Structures of carvomenthol (**247b**) and its stereoisomers (**247a, 247c, 247d, 247b′–d**′) and formation of (−)-iso- (**247c**), (−)-neoiso- (**247d**), (+)-neo- (**247c**′), and (−)-carvomenthols (**247d**′) from (+)- (**248a**) and (−)-carvotanacetones (**248a**′) by *Pseudomonas ovalis* strain 6-1.

Group 3: **104**–**105a**′–**106a**′ and **106b**′
Group 4: **104**–(+)-isodihydrocarvone (**105b**′)–**106c**′ and **106d**′
Group 5: (+)-carvone (**104**′)–(−)-isodihydrocarvone (**105b**′)–(−)-neoisodihydrocarveol (**106d**)
Group 6: **104**′–**105b**′–**106c**
Group 7: **104**′–**105b**′–**106c** and **106d**
Group 8: **104**–**105a**–**106a** and **106b**

The result of the mode of action of both the hydrogenation of carvone and the reduction of dihydrocarvone by microorganisms is as follows. In bacteria, only two strains were able to convert (−)-carvone (**104**) via (+)-dihydrocarvone (**105a**′) to (−)-dihydrocarveol (**106b**′) as the major product (group 3); when ethanol was used as a carbon source, 12.5% of (−)-carvone (**104**) convertible microorganisms belonged to this group and, when glucose was used, 8% belonged to this group;[143,149] when (+)-carvone (**104**′) was converted, one strain converted it into a mixture of (−)-isodihydrocarveol (**106c**′) and (−)-neoisodihydrocarveol (**106d**) (group 7, 6 and 4% of **104**′ convertible bacteria belonged to this group when ethanol and glucose were used, respectively) and four strains converted it via (−)-isodihydrocarvone (**105b**) to (−)-dihydrocarvone (**105a**) (group 8, 6 and 15% of (+)-carvone (**104**′) convertible bacteria belonged to this group when ethanol and



Scheme 102 Microbial resolution of carvomenthols by *Trichoderma S* and *Bacillus subtilis* var. *niger*.



Scheme 103 Structures of (4*S*)-(+)-carvone (**104′**) and (4*R*)-(−)-carvone (**104**).

glucose were used, respectively).[149] In yeasts, 43% of carvone convertible yeasts belong to group 1, 14% to group 2, and 33% to group 3 (of this group, three strains are close to group 1) and 12% to group 5, 5% to group 6, and 27% to group 7 (of this group, three strains are close to group 5 and one strain is close to group 6). In fungi, 51% of fungi metabolized (−)-carvone (**104**) by way of group 1 and 3% via group 3, but there was no strain capable of metabolizing (−)-carvone (**104**) via group 2, whereas 20% of fungi metabolized (+)-carvone (**104′**) via group 5 and 29% via group 7, but there was no strain capable of metabolizing (+)-carvone (**104′**) via group 6. In actinomycetes, (−)-carvone (**104**) was converted to dihydrocarveols via group 1 (49%), group 2 (0%), group 3 (9%), and group 4 (28%), whereas (+)-carvone (**104′**) was converted to dihydrocarveols via group 5 (7%), group 6 (0%), group 7 (19%), and group 8 (0%).

Table 5 Distribution of (+)- (**104′**) and (−)-carvone (**104**) convertible microorganisms[136]

Microorganisms	*Number of microorganisms used*	*Number of carvone convertible microorganisms*	*Ratio (%)*
Bacteria	40	16 (ethanol, **104′**)	40
		16 (ethanol, **104**)	40
		26 (glucose, **104′**)	65
		26 (glucose, **104**)	65
Yeasts	68	51 (**104′**)	75
		51 (**104**)	75
Fungi	40	34 (**104′**)	85
		36 (**104**)	90
Actinomycetes	48	27 (**104′**)	56
		43 (**104**)	90

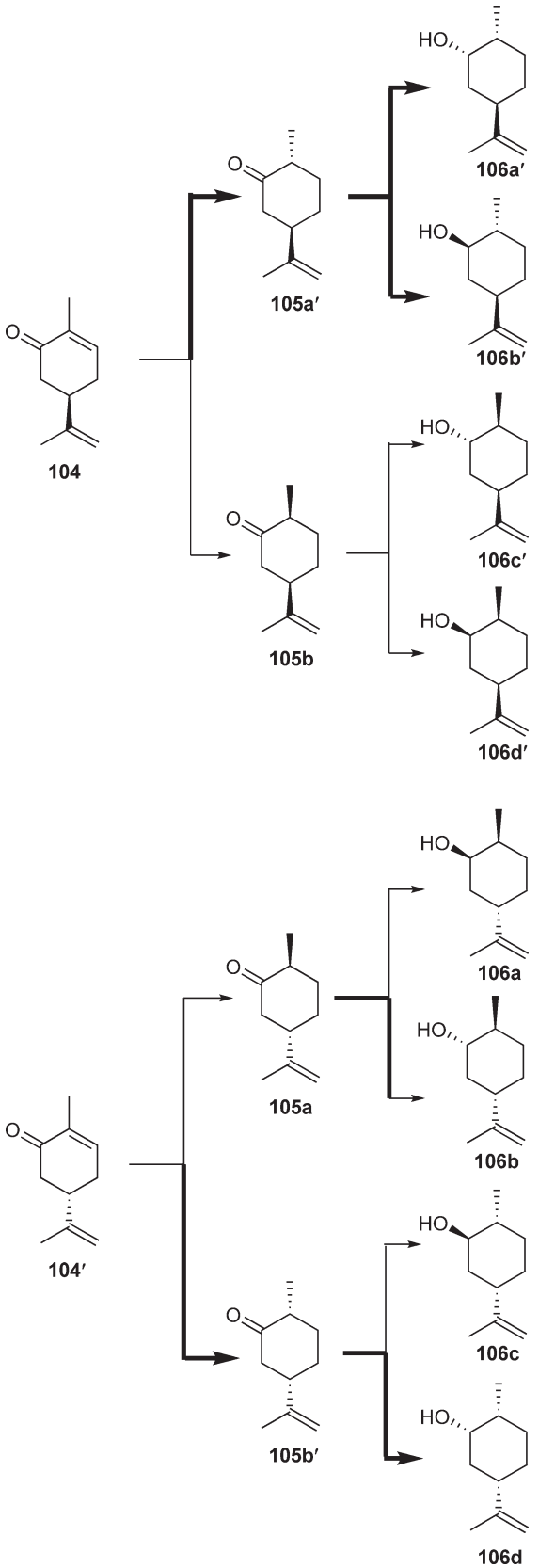
Furthermore, (+)-neodihydrocarveol (**106a′**) stereospecifically formed from (−)-carvone (**104**) by *A. niger* TBUYN-2 was further biotransformed to mosquitocidal (1*R*,2*S*,4*R*)-(+)-*p*-menthane-2,8-diol (**238a′**), (1*R*,2*S*,4*R*)-(+)-8-*p*-menthene-2,10-diol (**239a′**), and the mixture of (1*R*,2*S*,4*R*,8*S*/*R*)-(+)-*p*-menthane-2,8,9-triols (**240a** and **240a′**), while *A. glauca* ATCC 22752 gave **239a′** stereoselectively from **106a′** (**Scheme 105**).[122]

On the other hand, (−)-carvone (**104**) was biotransformed stereoselectively to (+)-neodihydrocarveol (**106a′**) via (+)-dihydrocarvone (**105a′**) by a strain of *A. niger*,[145] *E. gracilis* Z,[136] and *Chlorella miniata*.[150] Furthermore, in *E. gracilis* Z, mosquitocidal (1*R*,2*S*,4*R*)-(+)-*p*-menthane-2,8-diol (**238a′**) was obtained stereospecifically from (−)-carvone (**104**) via **105a′** and **106a′** (**Scheme 105**).

As the microbial method for the formation of mosquitocidal **238a′** was established, the production of (+)-dihydrocarveol (**106b**) and (+)-neodihydrocarveol (**106a′**) as precursors of mosquitocidal **238a′** was investigated by using 40 strains of bacteria belonging to *Escherichia*, *Aerobacter*, *Serratia*, *Proteus*, *Alcaligenes*, *Bacillus*, *Agrobacterium*, *Micrococcus*, *Staphylococcus*, *Corynebacterium*, *Sarcina*, *Arthrobacter*, *Brevibacterium*, *Pseudomonas*, and *Xanthomonas* spp.; 68 strains of yeasts belonging to *Schzosaccharomyces*, *Endomycopsis*, *Saccharomyces*, *Schwanniomyces*, *Debaryomyces*, *Pichia*, *Hansenula*, *Lipomyces*, *Torulopsis*, *Saccharomycodes*, *Cryptococcus*, *Kloeckera*, *Trigonopsis*, *Rhodotrula*, *Candida*, and *Trichosporon* spp.; 40 strains of fungi belonging to *Mucor*, *Absidia*, *Penicillium*, *Rhizopus*, *Aspergillus*, *Monascus*, *Fusarium*, *Pullularia*, *Keratinomyces*, *Oospora*, *Neurospora*, *Ustilago*, *Sporotrium*, *Trichoderma*, *Gliocladium*, and *Phytophythora* spp.; and 48 strains of actinomycetes belonging to *Streptomyces*, *Actinoplanes*, *Nocardia*, *Micromonospora*, *Microbispora*, *Micropolyspora*, *Amorphosporangium*, *Thermopolyspora*, *Planomonospora*, and *Streptosporangium* spp.

The results showed that 65% of bacteria, 75% of yeasts, 90% of fungi, and 90% of actinomycetes converted (−)-carvone (**104**) to (+)-dihydrocarvone (**105a′**) or (+)-neodihydrocarveol (**106a′**). Many microorganisms are capable of converting (−)-carvone (**104**) to (+)-neodihydrocarveol (**106a′**) stereospecifically. Some of the useful microorganisms are listed in **Tables 7** and **8**. There is no good chemical method to obtain (+)-neodihydrocarveol (**106a′**) in large quantities. It was considered that the method utilizing microorganisms is a very useful means and better than the chemical synthesis for the production of mosquitocidal precursor (+)-neodihydrocarveol (**106a′**).

(−)-Carvone (**104**) was biotransformed by *P. ovalis* to (−)-isodihydrocarvone (**105b**), (−)-isodihydrocarveol (**106c′**), and (−)-neoisodihydrocarveol (**106d**) as the major products (**Scheme 106**).[144] *Aspergillus niger* TBUYN-2 also converted the same substrate (**104**) to mainly (+)-8-hydroxyneodihydrocarveol (**238a′**), (+)-8,9-epoxyneodihydrocarveol (**111e**), and (+)-10-hydroxyneodihydrocarveol (**239a′**) via (+)-dihydrocarvone (**105a′**) and (+)-neodihydrocarveol (**106a′**). *Aspergillus niger* TBUYN-2 dehydrogenated (+)-*cis*-carveol (**100b**) to give (+)-carvone (**104′**), which was further converted to (−)-isodihydrocarvone (**105b**). Compound **105b** was further metabolized by four pathways to 10-hydroxy-(−)-isodihydrocarvone (**249b**), (1*S*,2*S*,4*S*)-*p*-menthane-1,2-diol (**97d**) via 1α-hydroxy-(−)-isodihydrocarvone (**102b**) as an intermediate, (−)-isodihydrocarveol (**105c**), and (−)-neoisodihydrocarveol (**106d**), which was further converted to isodihydroisobottrospicatol (**107d**) via 8,9-epoxy-(−)-neoisodihydrocarveol (**111c**). Compound **107d** was the major product (**Scheme 107**).[57]

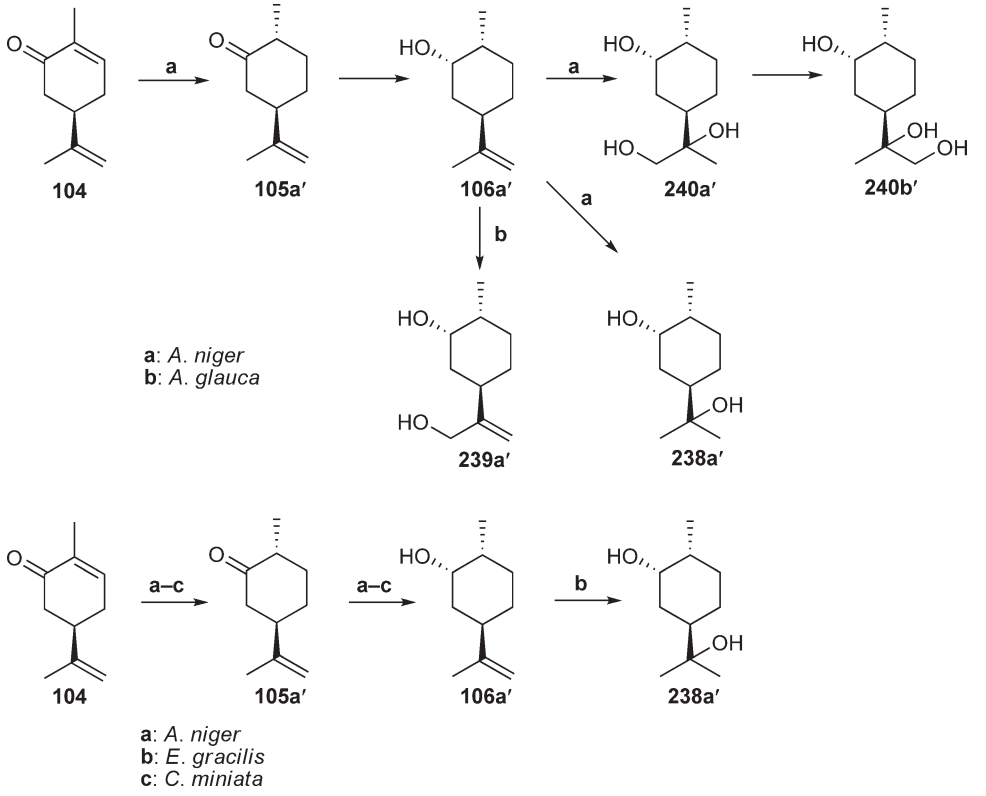


Scheme 104 Biotransformation of (+)- (**104′**) and (−)-carvone (**104**) by various kinds of microorganisms.

Table 6 The ratio of microorganisms that carried out the hydrogenation of C═C double bond of carvone by *si* plane attack and microorganisms that converted carvone

Microorganisms	*Ratio (%)*
Bacteria	100[a] 96[b]
Yeasts	74
Fungi	80
Actinomycetes	39

[a] When ethanol was used.
[b] When glucose was used.



Scheme 105 Metabolic pathways of (−)-carvone (**104**) by *Aspergillus niger* TBUYN-2, *Absidia glauca* ATCC 22752, *Euglena gracilis* Z, and *Chlorella miniata*.

In the case of the plant pathogenic fungus *A. glauca*, (−)-carvone (**104**) was metabolized to the diol 8-*p*-menthene-2,8-diol (**239a′**).[128]

(+)-Carvone (**104′**) was converted by five bacteria and one fungus[151] to (−)-dihydrocarvone (**105a**), (−)-isodihydrocarvone (**105b**), and (−)-neoisodihydrocarveol (**106d**). Sensitivity of the microorganisms to (+)-carvone (**104′**) and some of the products prevented yields exceeding 0.35 $g\,l^{-1}$ in batch cultures. The fungus *Trychoderma pseudokoningii* gave the highest yield of (−)-neoisodihydrocarveol (**106d**) (**Scheme 108**). (+)-Carvone (**104′**) is known to inhibit the growth of the fungus *Fusarium sulphureum* when it is administered via the gas phase.[64] Under the same conditions, the related fungus *Fusarium solani* var. *coeruleum* was not inhibited. In liquid medium, both fungi were found to convert (+)-carvone (**104′**), with the same rate, mainly to (−)-isodihydrocarvone=(**105b**), (−)-isodihydrocarveol (**106c′**), and (−)-neoisodihydrocarveol (**106d**).

Table 7 Summary of microbial and chemical hydrogenation of (−)-carvone (**104**) for the formation of (+)-dihdyrocarvone (**105a′**) and (+)-isodihydrocarvone (**105b**)[146]

	Compounds	
Microorganisms	*105a′*	*105b*
Amorphosporangium auranticolor	100	0
Microbiospora rosea IFO3559	86	0
Bacillus subtilis var. *niger*	85	13
Bacillus subtilis IFO3007	67	11
Pseudomonas polycolor IFO3918	75	15
Pseudomonas graveolens IFO3460	74	17
Arthrobacter pascens IFO121139	73	12
Picha membranaefaciens IFO0128	70	16
Saccharomyces ludwigii IFO1043	69	18
Alcalygenes faecalis IAM B-141-1	70	13
Zn-25% KOH-EtOH	73	27
Raney-10% NaOH	71	19

Table 8 Summary of microbial and chemical reduction of (−)-carvone (**104**) for the formation of (+)-neodihydrocarveol (**106a′**)[146]

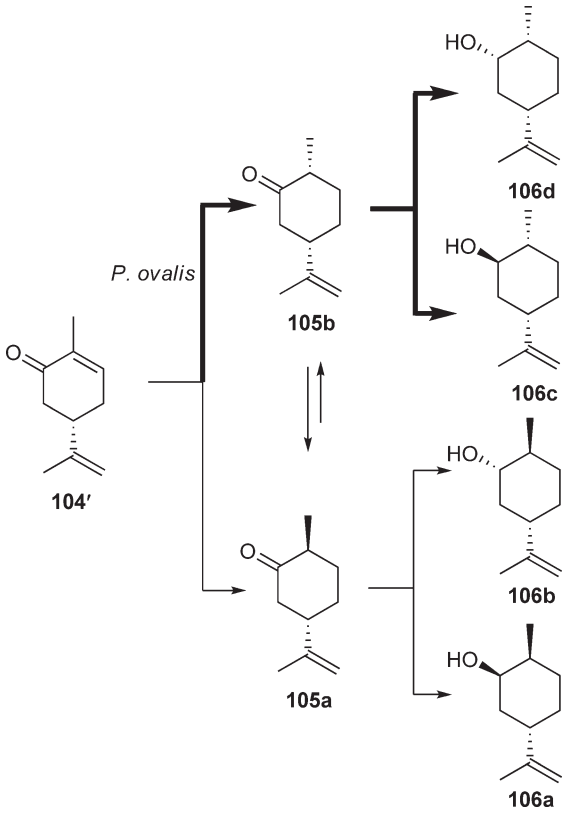
	Compounds					
Microorganisms	*105a′*	*105b*	*106a′*	*106b′*	*106c′*	*106d′*
Torulopsis xylinus IFO454	0	0	100	0	0	0
Monascus anka var. *rubellus* IFO5965	0	0	100	0	0	0
Fusarium anguioides Sherbakoff IFO4467	0	0	100	0	0	0
Phytophthora infestans IFO4872	0	0	100	0	0	0
Kloeckera magna IFO0868	0	0	98	2	0	0
Kloeckera antillarum IFO0669	19	4	72	0	0	0
Streptomyces rimosus	+	0	98	0	0	0
Penicillium notatum Westling IFO464	6	2	92	0	0	0
Candida pseudotropicalis IFO0882	17	4	79	0	0	0
Candida parapsilosis IFO0585	16	4	80	0	0	0
$LiAlH_4$	0	0	17	67	2	13
Meerwein–Ponndorf–Verley reduction	0	0	29	55	9	5

Biotransformation of carvone to carveols by actinomycetes. The distribution of actinomycetes that are capable of reducing the carbonyl group of carvone containing α,β-unsaturated ketone to (−)-*trans*- (**100a′**) and (−)-*cis*-carveol (**100b′**) was investigated.

Of 93 strains of actinomycetes, 63 strains were capable of converting (−)-carvone (**104**) to carveols. The percentage of microorganisms that produced carveols from (−)-carvone (**104**) to total microorganisms was about 71%. Microorganisms that produced carveols were classified into three groups according to the formation of (−)-*trans*-carveol (**100a′**) and (−)-*cis*-carveol (**100b′**): group 1, (−)-carvone – **100b′** only; group 2, (−)-carvone – **100a′** only; and group 3, (−)-carvone – mixture of **100a′** and **100b′**. Three strains belonged to group 1 (4.5%), 34 strains belonged to group 2 (51.1%), and 29 strains belonged to group 3 (44%); in group 3, 2 strains were close to group 1 and 14 strains were close to group 2.

Streptomyces A-5-1 isolated from soil converted (−)-carvone (**104**) to **105a′–105d′** and (−)-*trans*-carveol (**100a′**), whereas *Nocardia* 1-3-11 converted (−)-carvone (**104**) to (−)-*cis*-carveol (**100b′**) together with **100a′** and **105a′**.[127] In the case of *Nocardia*, the reaction between **104** and **100b′** was reversible and the predominant direction of the reaction was from **100b′** to **104** (**Scheme 109**).[127,147]

Biotransformation of carvone by actinomycetes. (−)-Carvone (**104**) was metabolized by actinomycetes to (−)-*trans*- (**100a′**) and (−)-*cis*-carveol (**100b′**) and (+)-dihydrocarvone (**105a′**) as reduced metabolites. Compound **100b′** was further metabolized to (+)-bottrospicatol (**107c′**). Furthermore, **104** was hydroxylated



Scheme 106 Metabolic pathways of (+)-carvone (**104′**) by *Pseudomonas ovalis* strain 6-1 and many other microorganisms.

at C-5 position and C-8,9 position to give 5β-hydroxy-(−)-carvone (**108a′**) and (−)-carvone-8,9-epoxide (**110′**), respectively. Compound **108a′** was further metabolized to 5β-hydroxyneodihydrocarveol (**232a′**) via5α-hydroxydihydrocarvone (**250a′**) (**Scheme 109**).

Metabolic pattern of (+)-carvone (**104**) is similar to that of (−)-carvone (**104**) in *S. bottropensis*. (+)-Carvone (**104′**) was converted by *S. bottropensis* to (+)-carvone-8,9-epoxide (**110**) and (+)-5β-hydroxycarvone (**108**) (**Schemes 110** and **111**). (+)-Carvone-8,9-epoxide (**110**) has light sweet aroma and a strong inhibitory effect on the germination of lettuce seeds.[133,139,152,153]

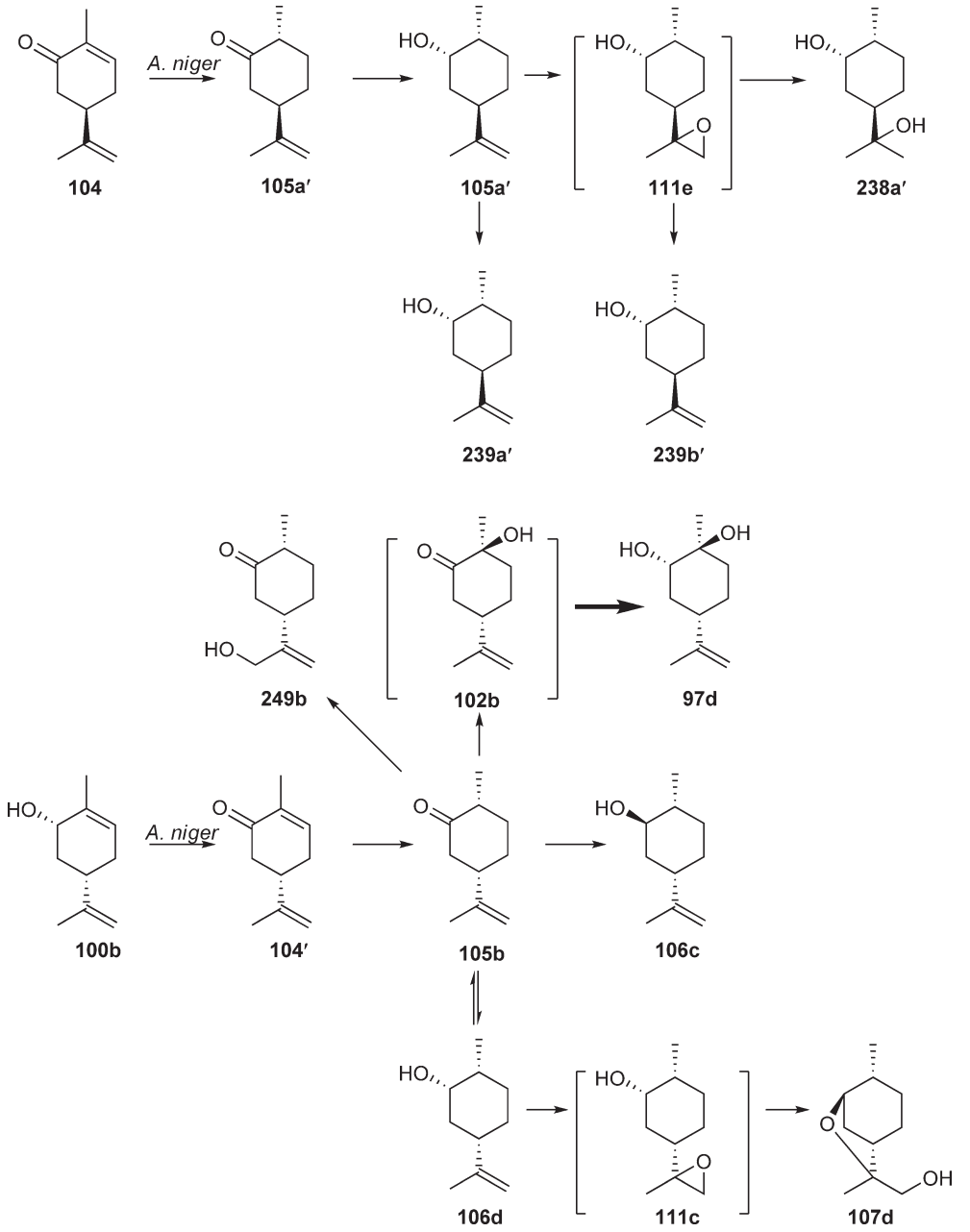
An investigation of (−)-carvone (**104**) and (+)-carvone (**104′**) conversion pattern was carried out by using rare actinomycetes. The conversion pattern was classified as follows:

Group 1: carvone (**104**)–dihydrocarvones (**105**)–dihydrocarveol (**106**)–dihydrocarveol-8,9-epoxide (**248**)–dihydrobottrospicatols (**107**)–5-hydroxydihydrocarveols (**232**)
Group 2: carvone (**104**)–carveols (**100**)–bottrospicatols (**99**)–5-hydroxy-*cis*-carveols (**103**)
Group 3: carvone (**104**)–5-hydroxycarvone (**108**)–5-hydroxyneodihydrocarveols **240**)
Group 4: carvone (**104**)–carvone-8,9-epoxides (**110**)

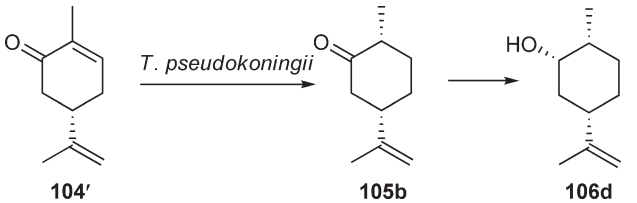
Of 50 rare actinomycetes, 22 strains (44%) were capable of converting (−)-carvone (**104**) to (−)-carvone-8,9-epoxide (**110′**) via pathway 4 and (+)-5β-hydroxycarvone (**108a′**), (+)-5α-hydroxycarvone (**108b′**), and (+)-5β-hydroxyneodihydrocarveol (**232a′**) via pathway 3.[154]

On the other hand, in the case of (+)-carvone (**104′**) conversion, 44% of rare actinomycetes were capable of converting (+)-carvone (**104′**) to (+)-carvone-8,9-epoxide (**110**) via pathway 4 and (−)-5α-hydroxycarvone (**108a**), (−)-5β-hydroxycarvone (**108b**), and (−)-5α-hydroxyneodihydrocarveol (**232a**) via pathway 3.[154]

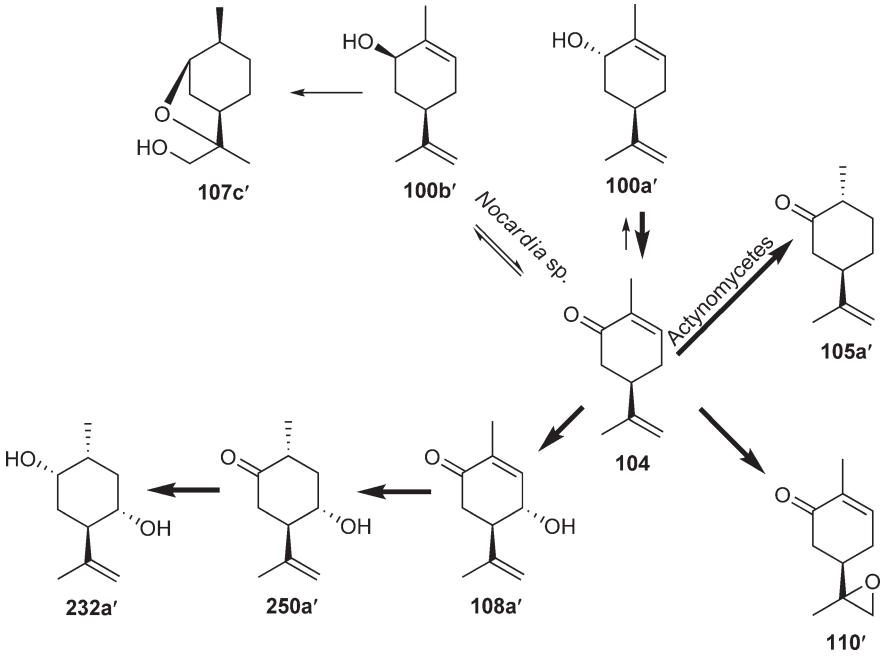
Citrus pathogenic fungi *A. niger* Tiegh (CBAYN) and *A. niger* TBUYN-2 hydrogenated C═C double bond at C-1,2 position of (+)-carvone (**104′**) to give (−)-isodihydrocarvone (**105b′**) as the major product



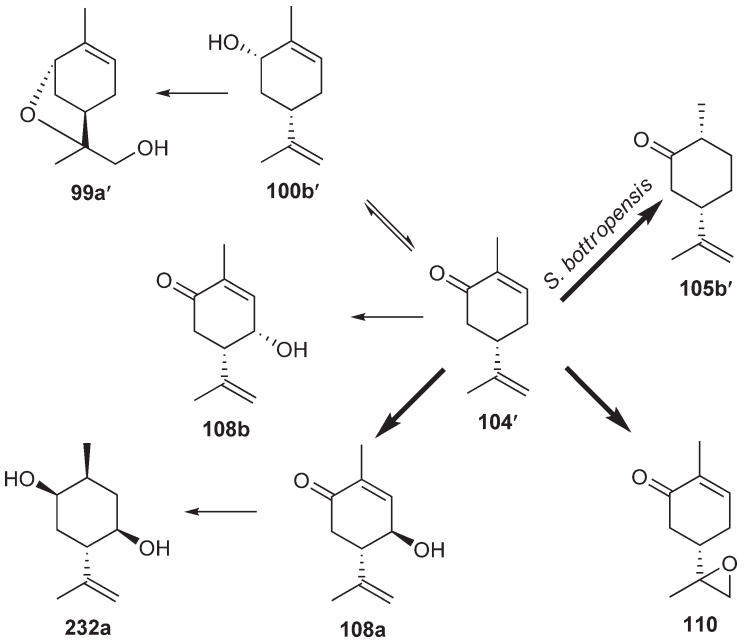
Scheme 107 Possible major metabolic pathways of (+)-carvone (**104′**) and (−)-carvone (**104′**) by *Aspergillus niger* TBUYN-2.



Scheme 108 Biotransformation of (+)-carvone (**104′**) by *Trychoderma pseudokoningii*.

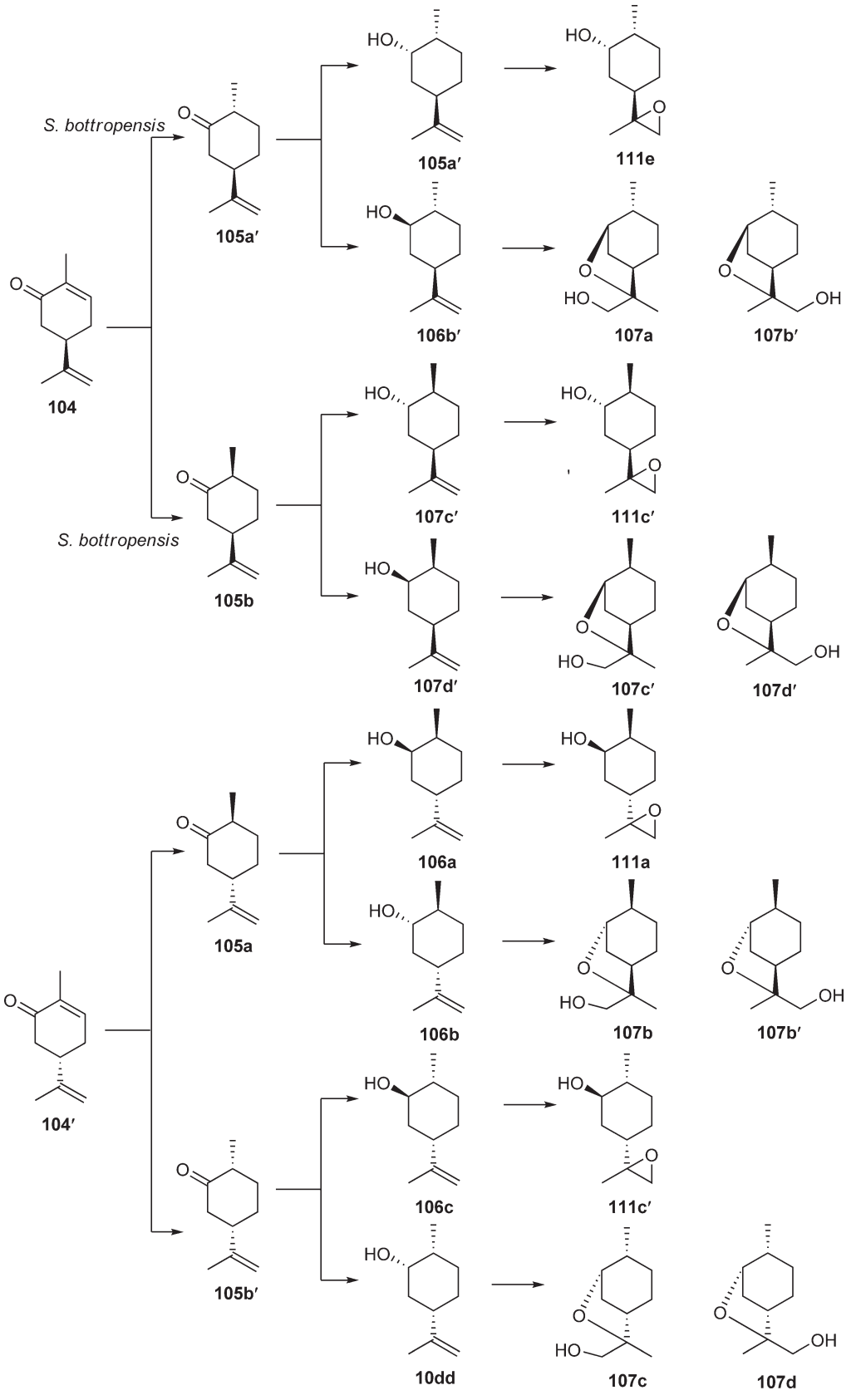


Scheme 109 Metabolic pathways of (−)-carvone (**104**) by *Streptomyces bottropensis* SY-2-1, *Streptomyces ikutamanensis* Ya-2-1, *Streptomyces* A-5-1, and *Nocardia* 1-3-11.

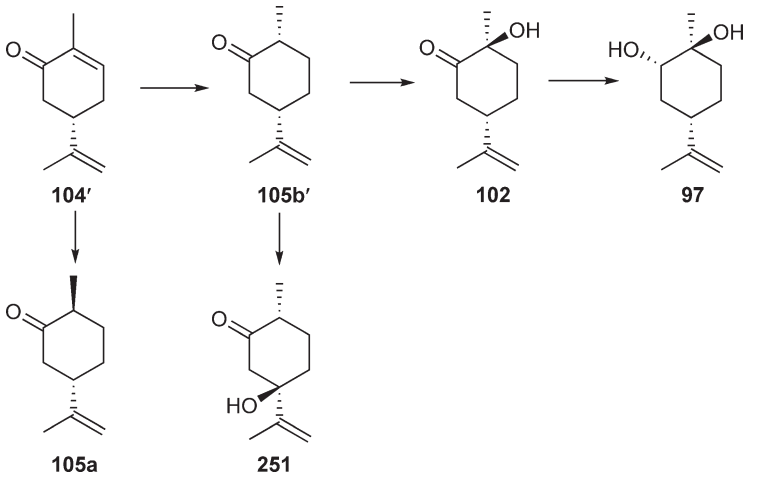


Scheme 110 Metabolic pathways of (+)-carvone (**104′**) by *Streptomyces bottropensis* SY-2-1 and *Streptomyces ikutamanensis* Ya-2-1.

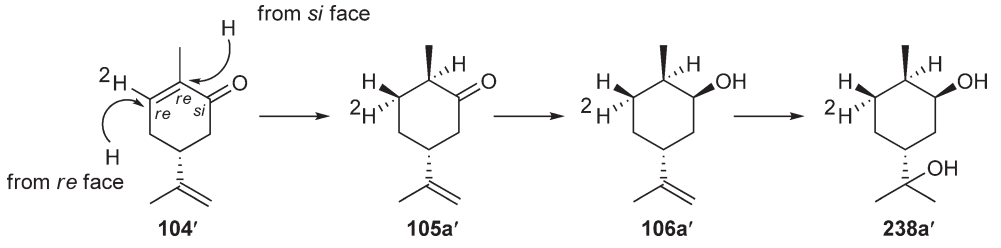
together with a small amount of (−)-dihydrocarvone (**105a**). Compound **105b′** was further metabolized via two pathways: one pathway led to the formation of (+)-1β-hydroxyneoisodihydrocarveol (**97**) via (+)-1β-hydroxyisodihydrocarvone (**102**) and the other pathway gave (+)-4α-hydroxyisodihydrocarvone (**251**) (**Scheme 112**).[155]



Scheme 111 Metabolic pathways of (+)- (**104′**) and (−)-carvone (**104**) and dihydrocarveols (**106a–d** and **106a′–d′**) by *Streptomyces bottropensis* SY-2-1 and *Streptomyces ikutamanensis* Ya-2-1.



Scheme 112 Metabolic pathways of (+)-carvone (**104′**) by *Citrus* pathogenic fungi *Aspergillus niger* Tiegh (CBAYN) and *Aspergillus niger* TBUYN-2.



Scheme 113 The stereospecific hydrogenation of the C═C double bond of α,β-unsaturated ketones, the reduction of saturated ketone, and the hydroxylation by *Euglena gracilis* Z.

The biotransformation of enones such as (−)-carvone (**104**) by the cultured cells of *C. miniata* was examined. It was found that the cells reduced stereoselectively the enones from *si* face at α-position of the carbonyl group and then the carbonyl group from *re* face (**Scheme 113**).[156,157]

The stereospecific hydrogenation occurs independent of the configuration and the kind of the substituent at C-4 position, so that the methyl group at C-1 position is fixed mainly at *R* configuration. [2-^{2}H]-(−)-Carvone ([2-^{2}H]-**104′**) was synthesized in order to clear up the hydrogenation mechanism at C-2 by microorganisms. Compound [2-^{2}H]-**104′** was also readily biotransformed to [2-^{2}H]-(8-hydroxy-(+)-neodihydrocarveol) (**238a′**) via [2-^{2}H]-(+)-neodihydrocarveol (**106a′**). On the basis of ^{1}H-NMR spectral data of compounds **106a′** and **238a′**, hydrogenation of the carbon–carbon double bond at the C-1 and C-2 position by *A. niger* TBUYN-2, *E. gracilis* Z, and *D. tertiolecta* occurs from the *si* face and *re* face, respectively, namely, antiaddition (**Scheme 113**, **Table 9**).[156]

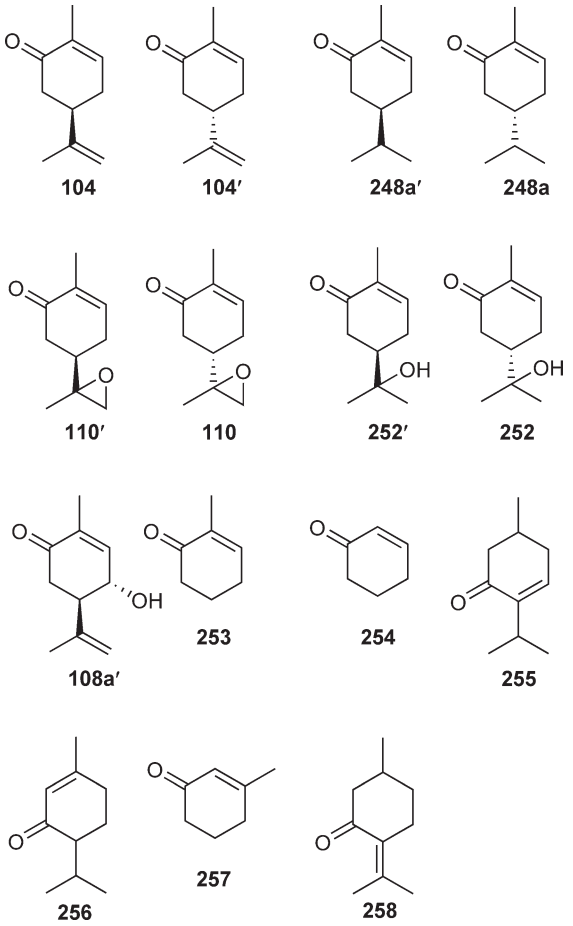
In order to understand the mechanism of the hydrogenation of α,β-unsaturated ketone of (−)-carvone (**104**) and the reduction of carbonyl group of dihydrocarvone (**105a′**), (−)-carvone (**104**), (+)-dihydrocarvone (**105a′**), and the analogues of (−)-carvone (**104**) were chosen and the conversion of the analogues was carried out by using *P. ovalis*, strain 6-1. As the analogues of carvone (**104** and **104′**), (−)- (**248a′**) and (+)-carvotanacetone (**248a**), 2-methyl-2-cyclohexenone (**253**), the mixture of (−)-*cis*- (**100b′**) and (−)-*trans*-carveol (**100a′**), 2-cyclohexenone, racemic menthenone (**275**), (−)-piperitone (**256**), (+)-pulegone (**258**), and 3-methyl-2-cyclohexenone (**257**) were chosen. Of these analogues, (−)- (**248a′**) and (+)-carvotanacetone (**248a**) were reduced to (+)-carvomenthone (**264a′**) and (−)-isocarvomenthone (**264a**), respectively. 2-Methyl-2-cyclohexenone (**253**) was mainly reduced to (−)-2-methylcyclohexanone, but other compounds were not reduced.

Table 9 Summary of the stereospecificity of the reduction of the C═C double bond of [2-^{2}H]-(−)-carvone ([2-^{2}H]-**104′**) by various kinds of microorganisms

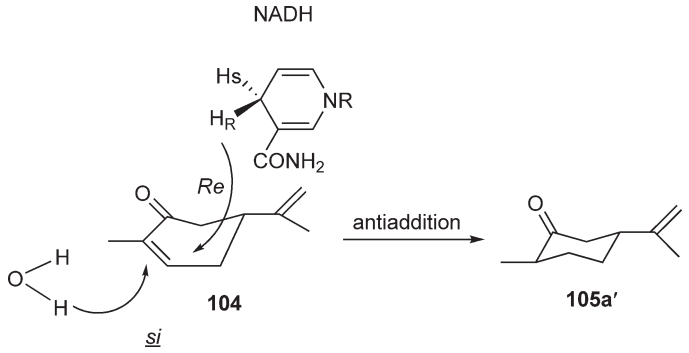
	Stereochemistry at C-2H of compounds	
Microorganisms	*106a′*	*238a′*
Aspergillus niger TBUYN-2	*β*	
Euglena gracilis Z	*β*	*β*
Dunaliella tertiolecta	*β*	
Cultured cells of *Nicotiana tabacum*[158]		

The efficient formation of (+)-dihydrocarvone (**105a**), (−)-isodihydrocarvone (**105b′**), (+)-carvomenthone (**264a′**), (−)-isocarvomenthone (**264b**), and (−)-2-methylcyclohexanone from (−)-carvone (**104′**), (+)-carvone (**104**), (−)-carvotanacetone (**248a**), (+)-carvotanacetone (**248a′**), and 2-methyl-2-cyclohexenone (**253**) suggested at least that C═C double bond conjugated with carbonyl group may be hydrogenated from behind (*si* plane).[126,159] In **Scheme 114**, several substrates used for the hydrogenation of C═C double bond with some microorganisms are shown.

Carvone reductase prepared from *E. gracilis* Z, which catalyzes the NADH-dependent reduction of the C═C bond adjacent to the carbonyl group, was characterized with regard to the stereochemistry of the



Scheme 114 Substrates used for the hydrogenation of C═C double bond with *Pseudomonas ovalis* strain 6-1, *Streptomyces bottropensis* SY-2-1, *S. ikutamanensis* Ya-2-1, and *Euglena gracilis* Z.



Scheme 115 Stereochemistry in the reduction of (−)-carvone (**104′**) by the reductase from *Euglena gracilis* Z.

hydrogen transfer to the substrate. The reductase was isolated from *E. gracilis* Z and was found to reduce stereospecifically the C═C double bond of carvone by *anti* addition of hydrogen from the *si* face at α-position to the carbonyl group and the *re* face at β-position. The hydrogen atoms participating in the enzymatic reduction at α- and β-position to the carbonyl group originate from the medium and the *pro*−4*R* hydrogen of NADH, respectively (**Scheme 115**, **Table 10**).[160]

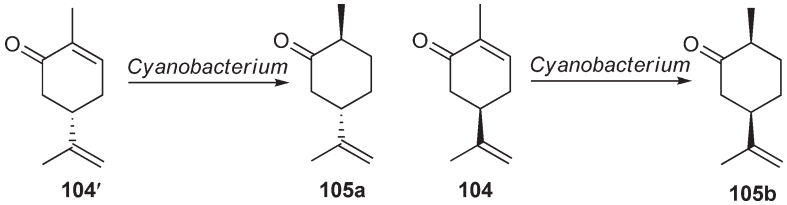
In the case of biotransformation using *Cyanobacterium*, (+)- (**104′**) and (−)-carvone (**104**) were converted by a different type of pattern to give (+)-isodihydrocarvone (**105b′**, 76.6%) and (−)-dihydrocarvone (**105a**, 62.2%), respectively (**Scheme 116**).[38]

On the other hand, the cultured cells of *Cataranthus rosea* biotransformed (−)-carvone (**104**) to 5α-hydroxy-(+)-neodihydrocarveol (**232a′**, 57.5%), 5β-hydroxy-(+)-neodihydrocarveol (**232b′**, 18.4%), 5β-hydroxy-(−)-carvone (**108b′**), 4α-hydroxy-(−)-carvone (**259a′**, 6.3%), 10-hydroxycarvone (**260′**), 5β-hydroxycarvone (**108a′**), and 5β-hydroxydihydrocarvone (**250a**) as the metabolites as shown in **Scheme 117**,[37,38,161] whereas (+)-carvone (**104**) gave 5β-hydroxy-(+)-carvone (**108a**, 65.4%) and 4β-hydroxy-(+)-carvone (**259a**, 34.6%) (**Scheme 117**, **Table 11**).[37,38,161]

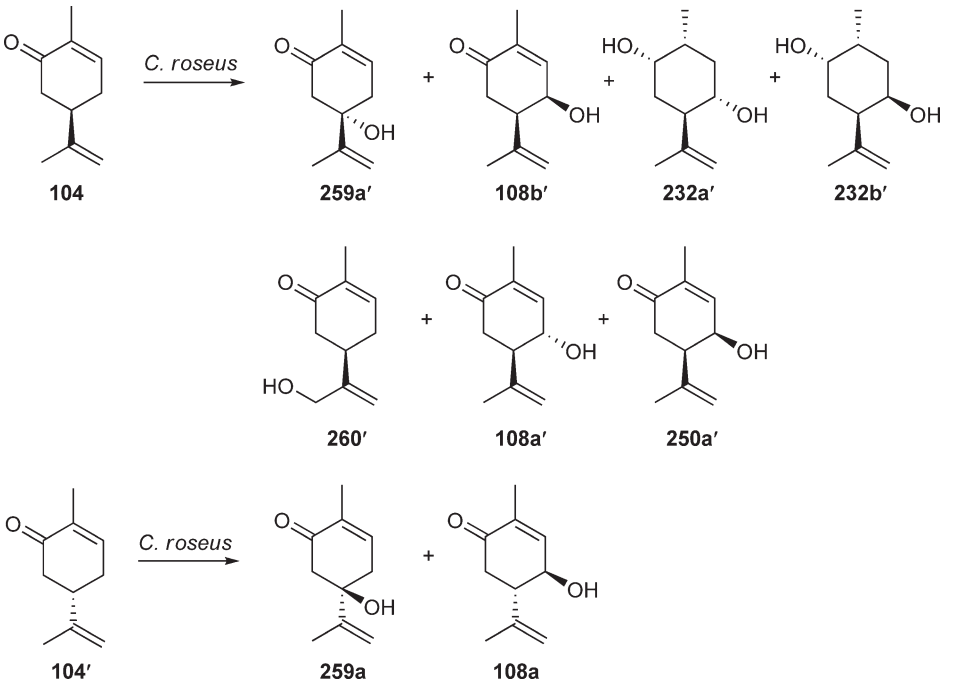
(−)-Carvone (**104**) was incubated with *Cyanobacterium*, enone reductase (43 kDa) isolated from the bacterium, and microsomal enzyme to give (+)-isodihydrocarvone (**105b′**) and (+)-dihydrocarvone (**105a′**). Cyclohexenone derivatives (**253** and **262**) were incubated with the same enone reductase and microsomal enzyme to give the dihydroderivatives (**261a**, **263a**) with *R* configuration in excellent ee (over 99%) and the metabolites (**261b**, **263b**) with *S* configuration in relatively high ee (85 and 80%).[162]

Table 10 Purification of the reductase from *Euglena gracilis* Z

	Total protein (mg)	*Total activity (unit × 10^4)*	*Sp. act. (units per g protein)*	*Fold*
Crude extract	125	2.2	1.7	1
DEAE Toyopearl	7	1.5	21	12
AF-Blue Toyopearl	0.1	0.03	30	18



Scheme 116 Biotransformation of (+)- and (−)-carvone (**104′** and **104**) by *Cyanobacterium*.



Scheme 117 Biotransformation of (+)- and (−)-carvone (**104′** and **104**) by *Catharanthus roseus*.

Table 11 Enantioselectivity in the reduction of enones (**253** and **262**) by enone reductase

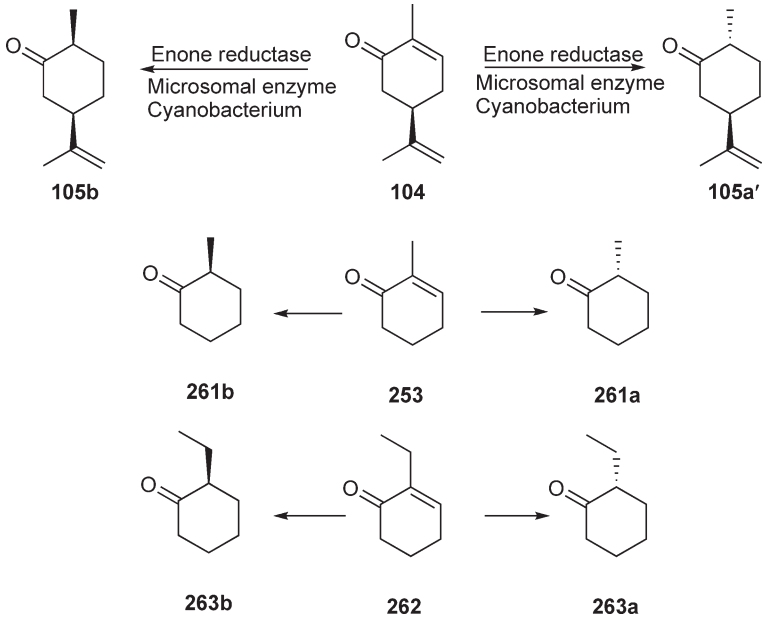
Microsomal enzyme	*Substrate*	*Product*	*ee*	*Configuration*[a]
−	**253**	**261a**	>99	*R*
−	**262**	**263a**	>99	*R*
+	**253**	**261b**	85	*S*
+	**262**	**263b**	80	*S*

[a] Preferred configuration at α-position to the carbonyl group of the products.

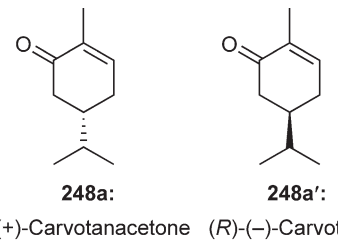
In contrast, almost all the yeasts tested showed reduction of carvone, although the enzyme activity varied. Reduction of (−)-carvone (**104**) was often much faster than reduction of (+)-carvone (**104′**). Some yeasts only reduced the carbon–carbon double bond to yield the dihydrocarvone isomers (**105a′** and **105b′**, and **105a** and **105b**) with the stereochemistry at C-1 with *R* configuration, while others also reduced the ketone to give the dihydrocarveols with the stereochemistry at C-2 always with *S* configuration for (−)-carvone (**104**), but sometimes *S* configuration and sometimes *R* configuration for (+)-carvone (**104′**). In the case of (−)-carvone (**104**), yields increased up to 90% within 2 h (**Scheme 118**).[163]

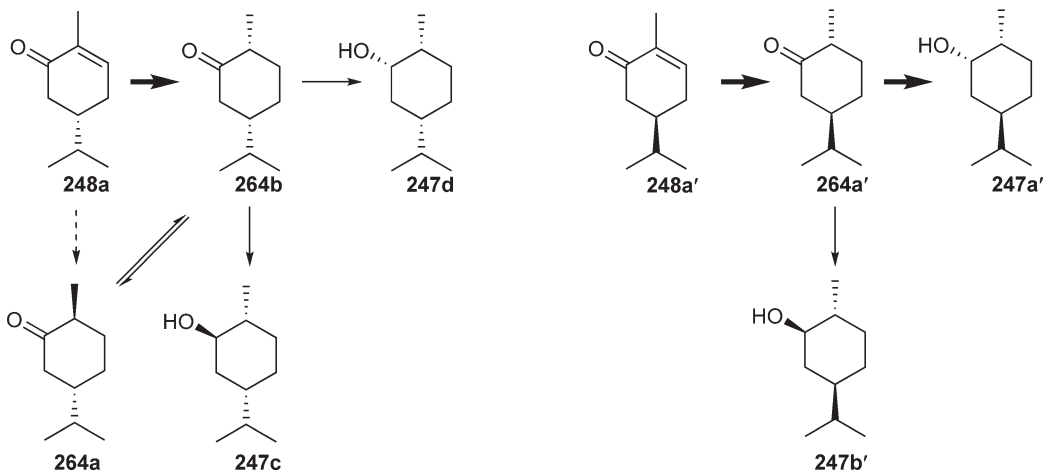
3.19.2.2.4(i)(b) Carvotanacetone (248 and 248′) In the conversion of (+)- (**248**) and (−)-carvotanacetone (**248′**) by *P. ovalis*, strain 6-1, (−)-carvotanacetone (**248′**) was converted stereospecifically to (+)-carvomenthone (**264a′**) and the latter compound was further converted to (+)-neocarvomenthol (**247a′**) and (−)-carvomenthol (**247b′**) in small amounts, whereas (+)-carvotanacetone (**248**) was converted mainly to (−)-isocarvomenthone (**264b**) and (−)-neoisocarvomenthol (**247d**), forming (−)-carvomenthone (**264a**) and (−)-isocarvomenthol (**247c**) in small amounts as shown in **Scheme 119**.[141]

Biotransformation of (−)-carvotanacetone (**248a′**) and (+)-carvotanacetone (**248a**) by *S. bottropensis* SY-2-1 was carried out.[164] As shown in **Scheme 120**, (+)-carvotanacetone (**248a**) was converted by *S. bottropensis* SY-2-

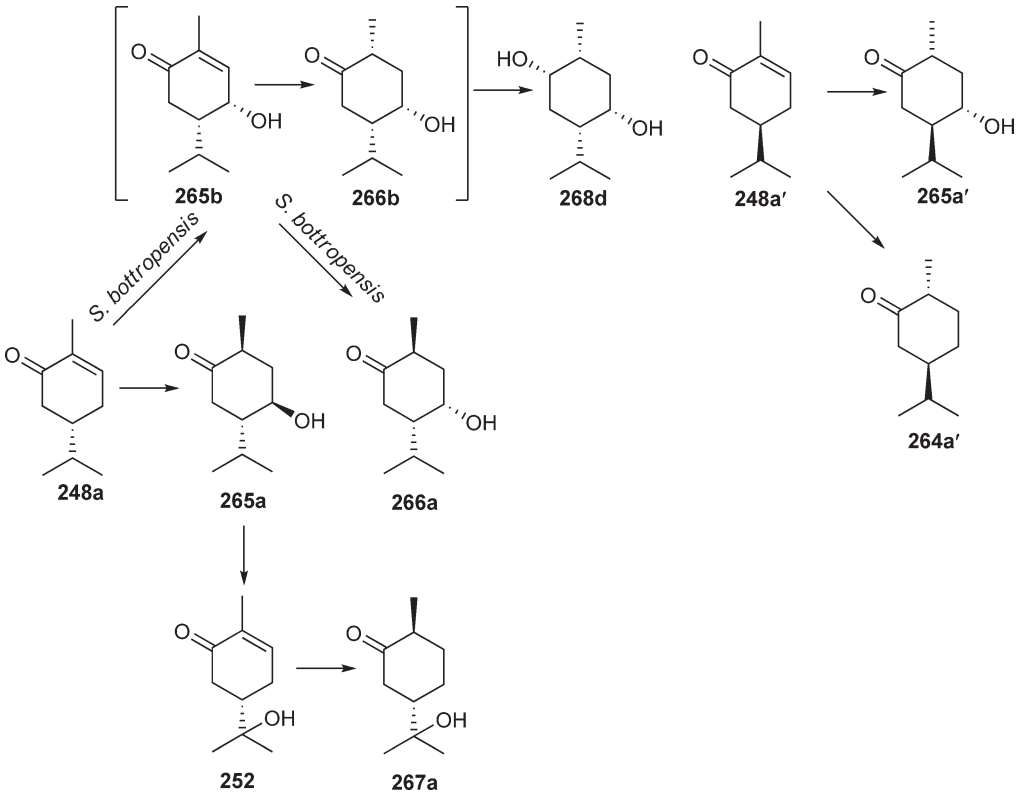


Scheme 118 Biotransformation of 2-methyl-2-cyclohexenone (**253**) and 2-ethyl-2-cyclohexenone (**262**) by enone reductase.





Scheme 119 Structures of (+)- (**248a**) and (–)-carvotanacetone (**248′**), and their metabolic pathways by *Pseudomonas ovalis* strain 6-1.



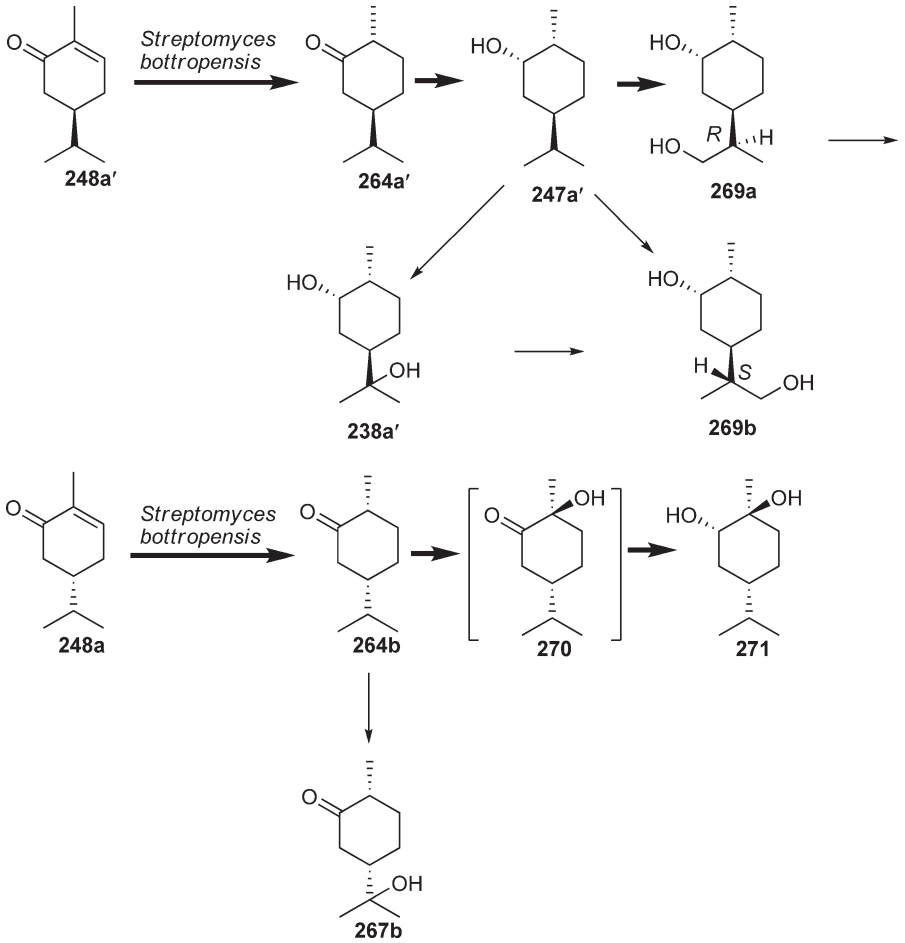
Scheme 120 Proposed metabolic pathways of (+)-(**248a**) and (−)-carvotanacetone (**248a′**) by *Streptomyces bottropensis* SY-2-1.

1 to 5α-hydroxy-(+)-neoisocarvomenthol (**268d**), 5β-hydroxy-(+)-carvotanacetone (**265a**), 5α-hydroxy-(−)-carvomenthone (**266a**), 8-hydroxy-(+)-carvotanacetone (**252**), and 8-hydroxy-(−)-carvomenthone (**267a**), whereas (−)-carvotanacetone (**248a′**) was converted to 5α-hydroxy-(−)-carvotanacetone (**265a′**) and 8-hydroxy-(−)-carvotanacetone (**264a′**).

Aspergillus niger TBUYN-2 converted (−)-carvotanacetone (**248a′**) to (+)-carvomenthone (**264a′**), (+)-neocarvomenthol (**247a′**), diastereoisomeric *p*-menthane-2,9-diols (**269a** (8*R*) and **269b** (8*S*) in the ratio of 3:1), and 8-hydroxy-(+)-neocarvomenthol (**238a′**). On the other hand, the same fungus converted (+)-carvotanacetone (**248a**) to (−)-isocarvomenthone (**264b**), 1α-hydroxy-(+)-neoisocarvomenthol (**271a**) via 1α-hydroxy-(+)-isocarvomenthone (**270**), and 8-hydroxy-(−)-isocarvomenthone (**267b**) as shown in **Scheme 121**.[165]

3.19.2.2.4(i)(c) Piperitone (256) A large number of yeasts were screened for the biotransformation of (−)-piperitone (**256**). A relatively small number of yeasts gave hydroxylation products of (−)-piperitone (**256**). Products obtained from (−)-piperitone (**256**) were 7-hydroxypiperitone (**274**), *cis*-6-hydroxypiperitone (**272b**), *trans*-6-hydroxypiperitone (**273a**), and 2-isopropyl-5-methylhydroquinone (**171**). Yields for the hydroxylation reactions varied between 8 and 60%, corresponding to the product concentrations of 0.04–0.3 g l^{-1}. None of the yeasts tested reduced (−)-piperitone (**256**).[163] During the initial screen with (−)-piperitone (**256**), only hydroxylation products were obtained. The hydroxylation products (**273a** and **273b**, **274**) obtained with nonconventional yeasts belonging to the genera *Arxula*, *Candida*, *Yarrowia*, and *Trichosporon* have recently been described (**Scheme 122**).[163]

3.19.2.2.4(i)(d) Pulegone (258) (*R*)-(+)-Pulegone (**258**), a monoterpene ketone with a mint-like odor, is the major component (up to 80–90%) of the essential oil (Pennyroyal oil) of *Mentha pulegiium*, which is sometimes used in beverages and as food additive for human consumption and occasionally in herbal medicine as an



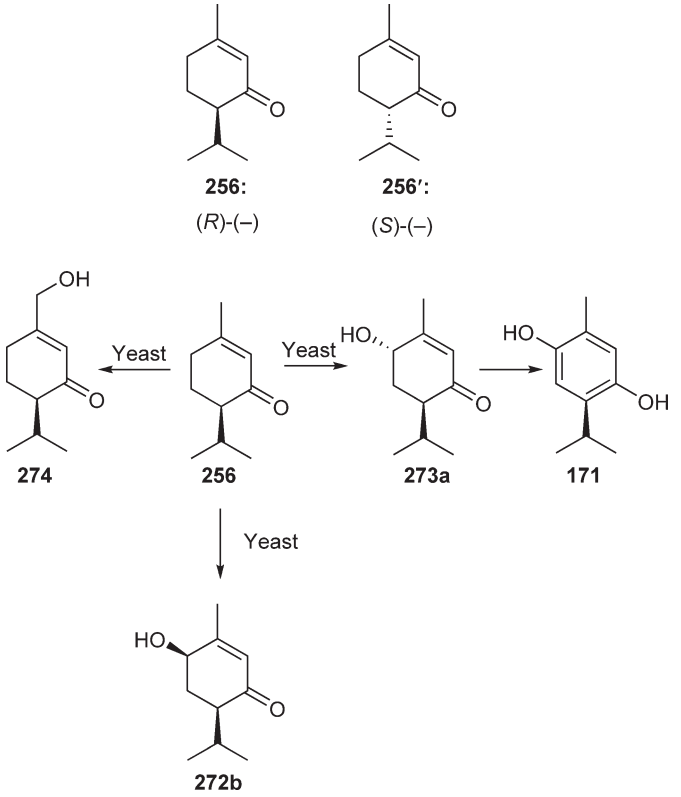
Scheme 121 Proposed metabolic pathways of (+)-(**248a**) and (−)-carvotanacetone (**248a′**) by *Aspergillus niger* TBUYN-2.

abortifacient drug. The biotransformation of (+)-pulegone (**258**) by fungi was investigated.[166] Most fungal strains grown in a usual liquid culture medium were able to metabolize (+)-pulegone (**258**) to some extent in the concentration range of 0.1–0.5 g l^{-1}; higher concentrations were generally toxic, except for a strain of *Aspergillus* sp. isolated from mint leaves infusion, which was able to survive in concentrations of up to 1.5 g l^{-1}. The predominant product was generally l-hydroxy-(+)-pulegone (**276**) (20–30% yield). Other metabolites were present in lower amounts (5% or less). The formation of 1-hydroxy-(+)-pulegone (**276**) was explained by hydroxylation at a tertiary position. Its dehydration to piperitenone (**277**), even under the incubation conditions, during isolation or derivative reactions precluded any tentative determination of its optical purity and absolute configuration.

Botrytis allii converted (+)-pulegone (**258**) to (−)-(1*R*)-8-hydroxy-4-*p*-menthen-3-one (**279**), piperitenone (**277**), and 8-hydroxymenthone (**280**).[167,168] *Hormonema isolate* (UOFS Y-0067) quantitatively reduced (+)-pulegone (**258**) and (−)-menthone (**275a**) to (+)-neomenthol (**33a**) (**Scheme 123**).[163]

The biotransformation by the recombinant reductase and the transformed *Escherichia coli* cells was examined with pulegone (**258** and **258′**), carvone (**104** and **104′**), and verbenone (**281**) as substrates. The recombinant reductase catalyzed the hydrogenation of the exocyclic C═C double bond of pulegone (**258**) to give menthone derivatives (**Tables 12** and **13**, **Scheme 124**).[169]

3.19.2.2.4(i)(e) Piperitenone (277) and isopiperitenone (285) Piperitenone (**277**) is metabolized to 5-hydroxypiperitenone (**284**), 7-hydroxypiperitenone (**287**), and 7,8-dihydroxypiperitone (**282**). Isopiperitenol (**286**) is reduced to isopiperitenone (**285**), which is further metabolized to piperitenone (**277**) and



Scheme 122 Structures of (–)-piperitone (**256**) and its enantiomer (**256′**), and hydroxylation products of (–)-piperitone (**256**) by yeast.

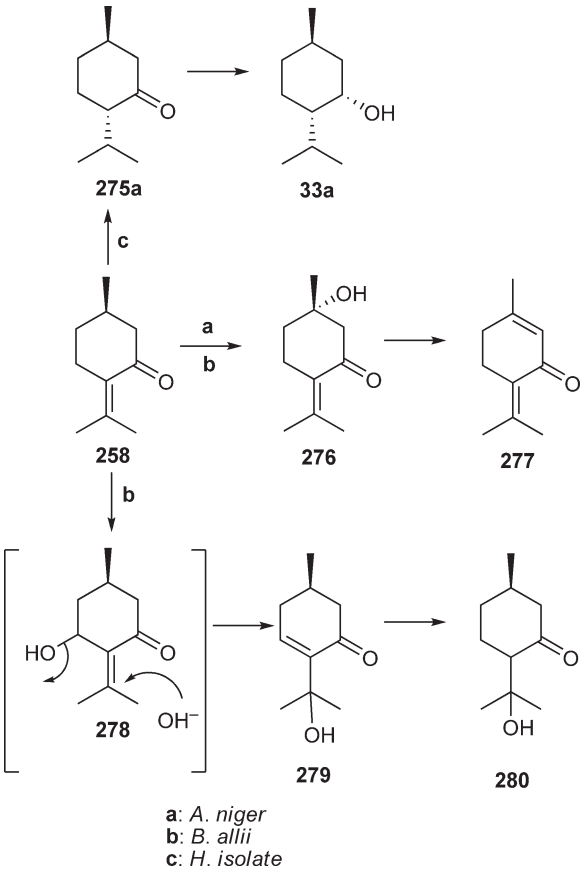
7-hydroxy- (**283**), 10-hydroxy- (**291**), 4-hydroxy- (**290**), and 5-hydroxyisopiperitenone (**289**). Compounds **285** and **277** are isomerized to each other. Pulegone (**258**) is metabolized to **277**, 8,9-dehydromenthenone (**288**), and 8-hydroxymenthenone (**279**) as shown in the biotransformation of the same substrate using *B. allii* (**Scheme 125**).[168,170]

Hormonema isolate (UOFS Y-0067) reduced (4*S*)-isopiperitenone (**285**) to (3*R*,4*S*)-isopiperitenol (**286a**), a precursor of (−)-menthol (**33b**) (**Scheme 126**).[163]

3.19.2.2.4(ii) Saturated ketone

3.19.2.2.4(ii)(a) Dihydrocarvone (105a and 105a′) and isodihydrocarvone (105b and 105b′) In **Scheme 127**, the structures of dihydrocarvones (**105a** and **105a′**) and isodihydrocarvones (**105b** and **105b′**) are presented. In the reduction of saturated carbonyl group of dihydrocarvone by microorganisms, (+)-dihydrocarvone (**105a′**) is converted stereospecifically to either (+)-neodihydrocarveol (**106a′**) or (−)-dihydrocarveol (**106b′**) or nonstereospecifically to a mixture of **106a′** and **106b′**, whereas (−)-isodihydrocarvone (**105b′**) is converted stereospecifically to either (−)-neoisodihydrocarveol (**106d**) or (−)-isodihydrocarveol (**106c**) or nonstereospecifically to a mixture of **106c** and **106d**.[126,143–146]

(+)-Dihydrocarvone (**105a′**) and (+)-isodihydrocarvone (**105b′**) are easily isomerized chemically to each other. In the microbial transformation of (−)-carvone (**104**), the formation of (+)-dihydrocarvone (**105a′**) is predominant. (+)-Dihydrocarvone (**105a′**) is reduced to (+)-neodihydrocarveol (**106a′**) and (−)-dihydrocarveol (**106b′**) or either of the two, whereas in the biotransformation of (+)-carvone (**104′**), (+)-isodihydrocarvone (**105b**) is formed predominantly. (+)-Isodihydrocarvone (**105b′**) is reduced to (+)-isodihydrocarveol (**106c**) and (+)-neoisodihydrocarveol (**106d**) (**Scheme 127**).



Scheme 123 Biotransformation of (+)-pulegone (**258**) by *Aspergillus* species, *Botrytis allii*, and *Hormonema isolate* UOFS Y-0067.

Table 12 Substrate specificity in the reduction of enones by recombinant pulegone reductase

Entry number (reaction time (h))	*Substrates*	*Products*	*Conversions (%)*
1 (3)	(*R*)-(+)-Pulegone (**258**)	(1*R*,4*R*)-Isomenthone (**275b**)	4.4
2 (12)	(*R*)-Pulegone (**258**)	(1*S*,4*R*)-Menthone (**275a′**)	6.8
3 (3)	(*S*)-(−)-Pulegone (**258′**)	(1*R*,4*R*)-Isomenthone (**275b**)	14.3
4 (12)	(*S*)-Pulegone (**258′**)	(1*S*,4*R*)-Menthone (**275a′**)	15.7
5 (12)	(*R*)-(−)-Carvone (**104**)	(1*S*,4*S*)-Isomenthone (**275b′**)	0.3
6 (12)	(*S*)-(+)-Carvone (**104′**)	(1*R*,4*S*)-Menthone (**275a**)	0.5
7 (12)	(1*S*,5*S*)-Verbenone (**281′**)	(1*S*,4*S*)-Isomenthone (**275b′**)	1.6
8 (12)	(1*R*,5*R*)-Verbenone (**281**)	(1*R*,4*S*)-Menthone (**275a**)	2.1
		–	n.d.
		–	n.d.
		–	n.d.
		–	n.d.

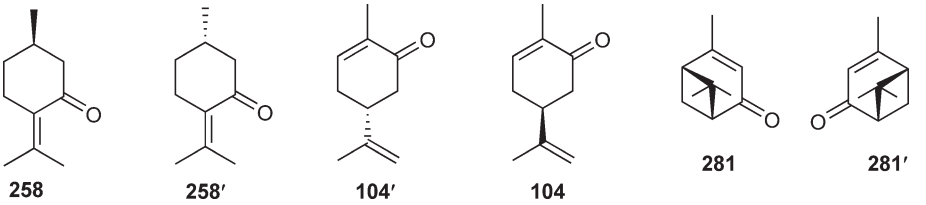
n.d., not detected.

However, *P. fragi* IFO3458, *P. fluorescens* IFO3081, and *Aerobacter aerogemes* IFO3319 and IFO12059 formed (−)-dihydrocarvone (**105a**) predominantly from (+)-carvone (**104′**). In the time-course study of the biotransformation of (+)-carvone (**104′**), it appeared that the predominant formation of (−)-dihydrocarvone (**105a**) is due to the epimerization of (−)-isodihydrocarvone (**105b′**) by epimerase of *P. fragi* IFO3458.[149]

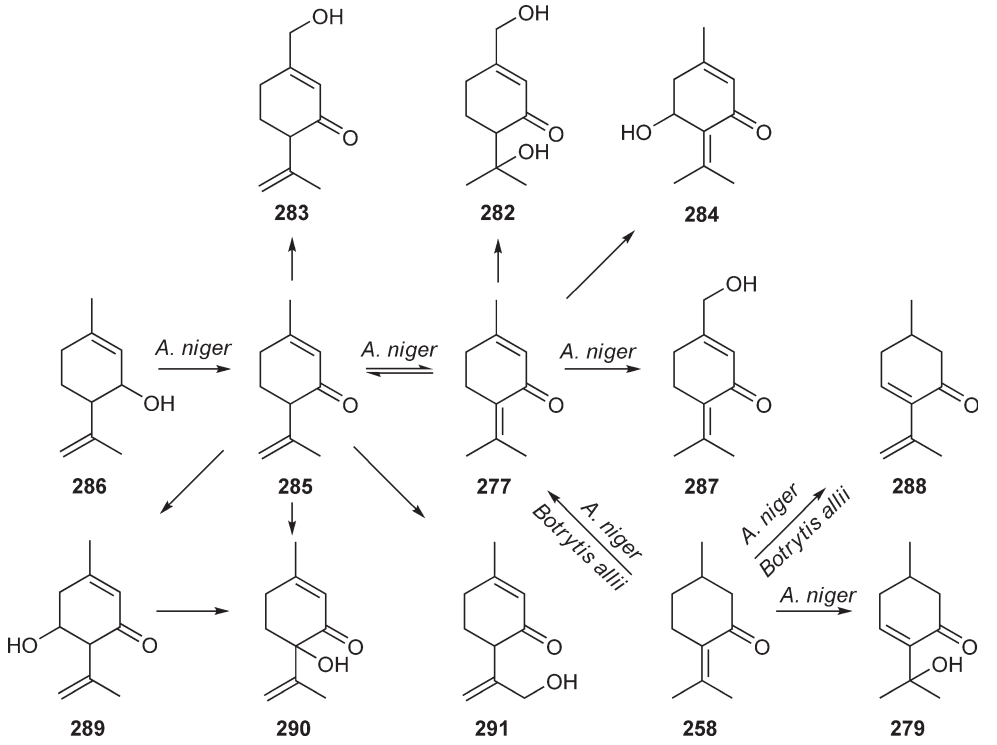
Table 13 Biotransformation of pulegone (**258** and **258′**) by transformed *Escherichia coli* cells

Substrates	*Products*	*Conversion (%)*
(*R*)-(+)-Pulegone (**258**)	(1*R*,4*R*)-Isomenthone (**275b**)	26.8
(*S*)-(−)-Pulegone (**258′**)	(1*S*,4*R*)-Menthone (**275a′**)	30
	(1*S*,4*S*)-Isomenthone (**275b′**)	32.3
	(1*R*,4*S*)-Menthone (**275a**)	7.1

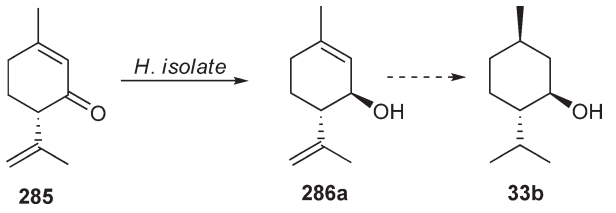
Reaction time of the transformation reaction is 12 h.



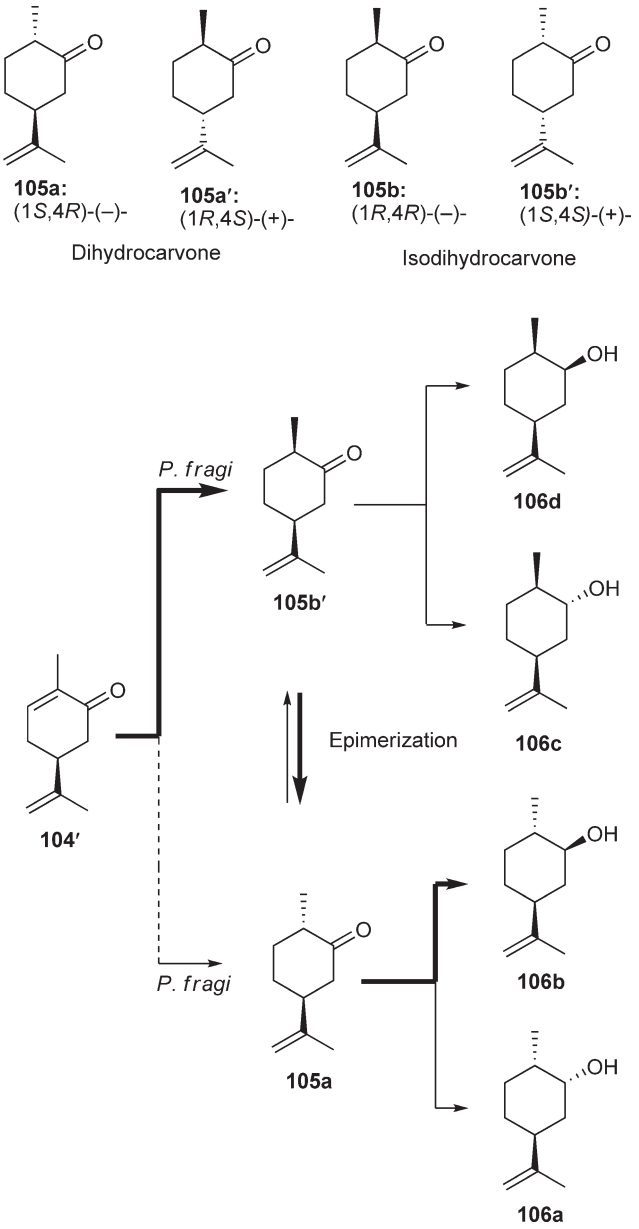
Scheme 124 Structures of substrates (**104**, **104′**, **258**, **258′**, **281**, and **281′**) for reduction by recombinant pulegone reductase.



Scheme 125 Biotransformation of isopiperitenone (**285**) and piperitenone (**277**) by *Aspergillus niger* TBUYN-2.



Scheme 126 Biotransformation of isopiperitenone (**285**) by *Hormonema isolate* UOFS Y-0067.



Scheme 127 Structures of dihydrocarvone (**105a, 105a′**), isodihydrocarvone (**105b, 105b′**), and proposed metabolic pathways of (+)-carvone (**104′**) and (–)-isodihydrocarvone (**105b′**) by *Pseudomonas fragi* IFO3458.

Isodihydrocarvone epimerase. Preparation of isodihydrocarvone epimerase: The cells of *P. fragi* IFO3458 were harvested by centrifugation and washed 5 times with 0.01 mol l^{-1} KH_2PO_4–Na_2HPO_4 buffer (pH 7.2). Bacterial extracts were prepared from the washed cells (20 g from 3 l medium) by sonic lysis (Kaijo Denki Co., Ltd., 20Kc., 15 min, 5–7 °C) in 100 ml of the same buffer. Sonic extracts were centrifuged at 25 500*g* for 30 min at –2 °C. The opalescent yellow supernatant fluid had the ability to convert (–)-isodihydrocarvone (**105b**) to (–)-dihydrocarvone (**105a**). On the other hand, the broken cell preparation was incapable of converting (–)-isodihydrocarvone (**105b′**) to (–)-dihydrocarvone (**105a**). The enzyme was partially purified from this supernatant fluid about 56-fold with heat treatment (95–97 °C for 10 min), ammonium sulfate precipitation (0.4–0.7 saturation), and DEAE-Sephadex A-50 column chromatography.

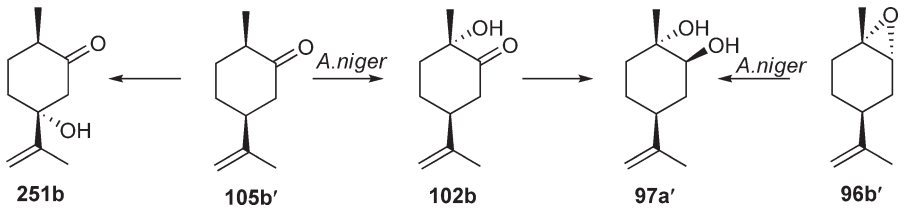
The reaction mixture consisted of a mixture of (−)-isodihydrocarvone (**105b′**) and (−)-dihydrocarvone (**105a′**) (60:40 or 90:10), 1/30 mol l^{-1} KH_2PO_4–Na_2HPO_4 buffer (pH 7.2), and the crude or partially purified enzyme solution. The reaction was started by the addition of the enzyme solution and stopped by the addition of ether. The ether extract was applied to analytical gas layer chromatography (GLC) (Shimadzu Gas Chromatograph GC-4A, 10% PEG-20M, 3 m × 3 mm, temperature 140–170 °C at the rate of 1 °C min^{-1}, N_2 35 ml min^{-1}), and the epimerization was assayed by measuring the peak areas of (−)-isodihydrocarvone (**105b′**) and (−)-dihydrocarvone (**105a**) in GLC before and after the reaction.

The crude extract and the partially purified preparation were found to be very stable to heat treatment; 66 and 36% of the epimerase activity remained after treatment at 97 °C for 60 and 120 min, respectively.[149]

A strain of *A. niger* TBUYN-2 hydroxylated (−)-isodihydrocarvone (**105b′**) at C-1 position to give 1α-hydroxyisodihydrocarvone (**102b**), which was readily and smoothly reduced to (1*S*,2*S*,4*S*)-(−)-8-*p*-menthene-1,2-*trans*-diol (**97a′**), which was also obtained from the biotransformation of (−)-*cis*-limonene-1,2-epoxide (**96b′**) by microorganisms and decomposition by 20% HCl (**Scheme 128**).[57] Furthermore, *A. niger* TBUYN-2 and *A. niger* Tiegh (CBAYN) biotransformed (−)-isodihydrocarvone (**105b**) to (−)-4α-hydroxyisodihydrocarvone (**251b**) and (−)-8-*p*-menthene-1,2-*trans*-diol (**97a′**) as the major products together with a small amount of 1α-hydroxyisodihydrocarvone (**102b**) (**Scheme 128**).[155]

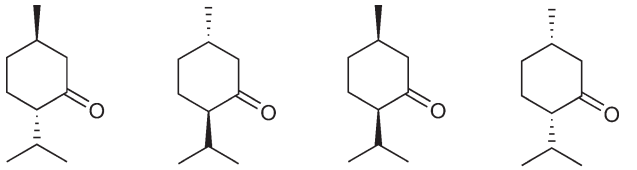
3.19.2.2.4(ii)(b) Menthone (275a and 275a′) and isomenthone (275b and 275b′) The growing cells of *P. fragi* IFO3458 epimerized 17% of racemic isomenthone (**275b** and **275b′**) to menthone (**275a** and **275a′**) (**Scheme 129**).[149] (−)-Menthone (**275a**) was converted by *Pseudomonas fluorescens* M-2 to (−)-3-oxo-4-isopropyl-1-cyclohexane carboxylic acid (**292a**), (+)-3-oxo-4-isopropyl-1-cyclohexane carboxylic acid (**292b**), and (+)-3-hydroxy-4-isopropyl-1-cyclohexane carboxylic acid (**293a**). On the other hand, (+)-menthone (**275a′**) was converted to (+)-7-hydroxymenthone (**294a**), (+)-3-oxo-4-isopropyl-1-cyclohexane carboxylic acid (**292a′**), and (−)-3-oxo-4-isopropyl-1-cyclohexane carboxylic acid (**292b′**). Racemic isomenthone (**275b** and **275b′**) was converted to racemic 1-hydroxy-1-methyl-4-isopropylcyclohexane-3-one (**295**), racemic piperitone (**256**), racemic 3-oxo-4-isopropyl-1-cyclohexene-1-carboxylic acid (**296**), (+)-3-oxo-4-isopropyl-1-cyclohexane carboxylic acid (**292b**), (−)-3-oxo-4-isopropyl-1-cyclohexane carboxylic acid (**292a**), and (+)-3-hydroxy-4-isopropyl-1-cyclohexane carboxylic acid (**293a**) (**Scheme 130**).[171]

The soil plant pathogenic fungus *R. solani* 189 converted (−)-menthone (**275a**) to 4β-hydroxy-(−)-menthone (**297**, 29%) and 1α,4β-dihydroxy-(−)-menthone (**278**, 71%) (**Scheme 131**).[172] (−)-Menthone (**275a**) was transformed by *S. litura* to 7-hydroxymenthone (**294a′**), 7-hydroxyneomenthol (**294c**), and 7-



251b **105b′** **102b** **97a′** **96b′**

Scheme 128 Biotransformation of (−)-isodihydrocarvone (**105b′**) and (−)-*cis*-limonene-1,2-epoxide (**96b′**) by *Aspergillus niger* TBUYN-2 and *Aspergillus niger* Tiegh (CBAYN).



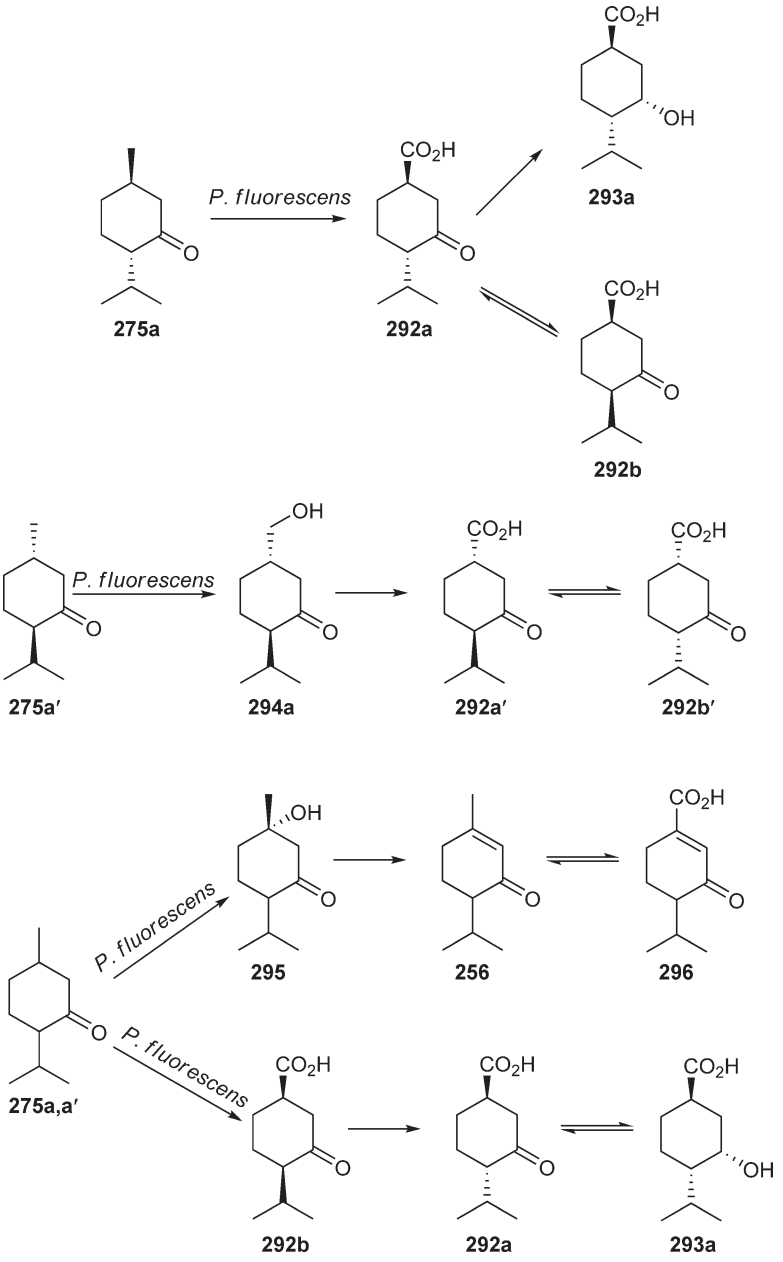
275a: (1*R*,4*S*)-(−)-Menthone

275a′: (1*S*,4*R*)-(+)-Menthone

275b: (1*R*,4*R*)-(+)-Isomenthone

275b′: (1*S*,4*S*)-(−)-Isomenthone

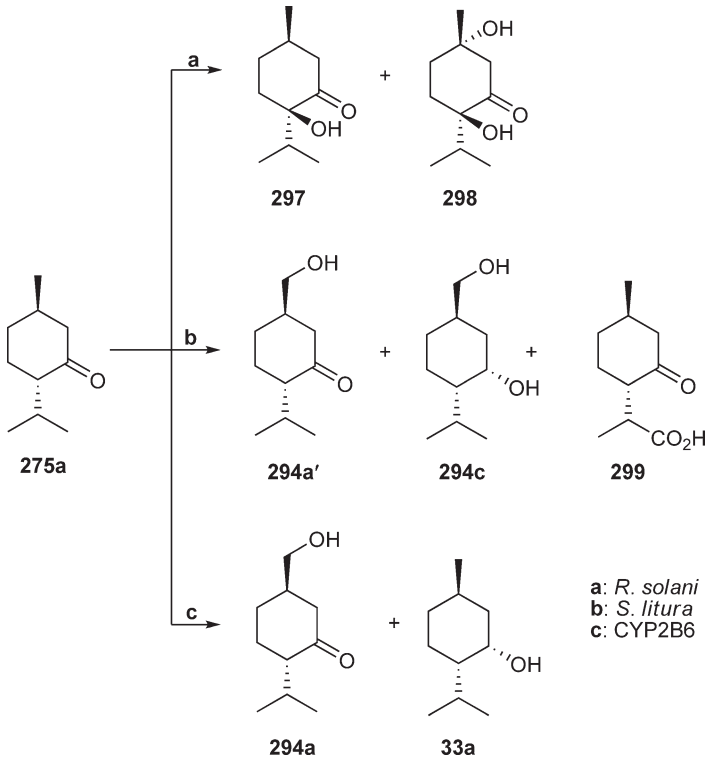
Scheme 129 Structures of racemic menthones (**275a** and **275a′**) and isomenthones (**275b** and **275b′**).



Scheme 130 Biotransformation of (–)- (**275a**) and (+)-menthone (**275a′**) and racemic isomenthones (**275b** and **275b′**) by *Pseudomonas fluorescens* M-2.

hydroxy-9-carboxymenthone (**299**) (**Scheme 131**).[173] (−)-Menthone (**275a**) was metabolized to 7-hydroxymenthone (**294a′**) and (+)-neomenthol (**33a**) by human liver microsomes (CYP2B6). Of 11 recombinant human P-450 enzymes (expressed in *Trichoplusia ni* cells) tested, CYP2B6 catalyzed the oxidation of (−)-menthone (**275a**) to 7-hydroxymenthone (**294a′**) (**Scheme 131**).[174]

3.19.2.2.4(ii)(c) 3-Thujone (300a and 300a′) and 3-isothujone (300b and 300b′) In **Scheme 132**, 3-thujones (**300a** and **300a′**) and 3-isothujones (**300b** and **300b′**) are demonstrated. β-Pinene (**337**) is metabolized to 3-thujone (**300a**) via α-pinene (**130**).[175] α-Pinene (**130**) is metabolized to thujone (**300a**). Thujone (**300a**) is



Scheme 131 Metabolic pathway of (−)-menthone (**275a**) by *Rhizoctonia solani* 189, *Spodoptera litura*, and human liver microsome (CYP2B6).

biotransformed to thujoyl alcohol (**301a**) by *A. niger* TBUYN-2.[94] Furthermore, (−)-3-isothujone (**300b**) prepared from *Armois* oil is biotransformed by the plant pathogenic fungus *B. allii* IFO9430 to 4-hydroxythujone (**302b**) and 4,6-dihydroxythujone (**303b**) (**Scheme 132**).[176]

3.19.2.2.5 Cyclic monoterpene epoxide

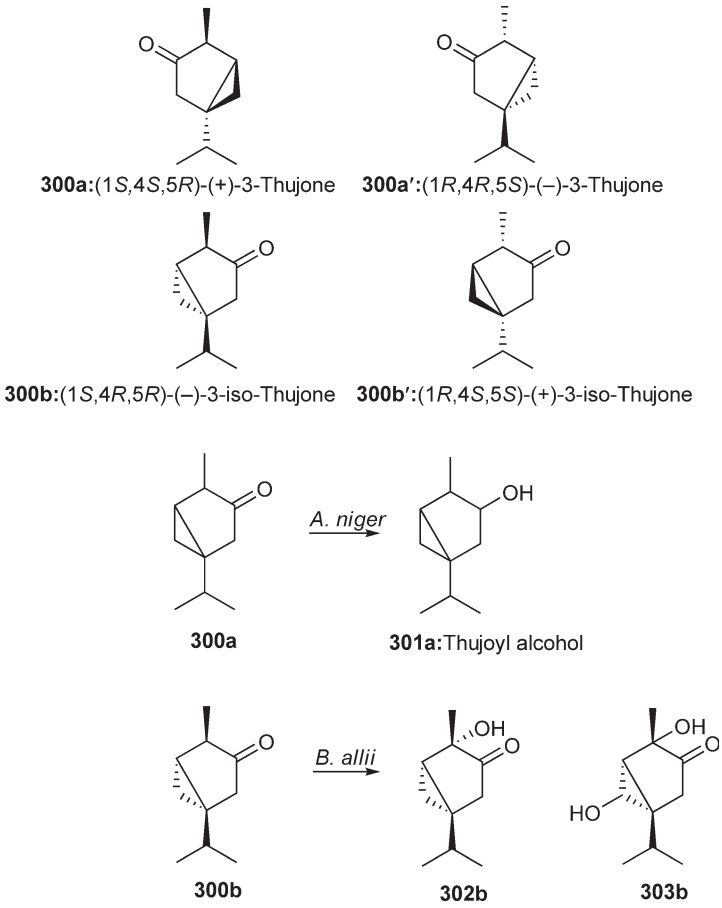
3.19.2.2.5(i) 1,8-Cineole (128) 1,8-Cineole (**128**) is the major component of the essential oil of *Eucalyptus adiata* var. *australiana* leaves, and its concentration in the oil is ~75%, which corresponds to 31 mg g^{-1} fr. wt. leaves.[177]

The most effective utilization of **128** is very important in terms of renewable biomass production. It would be of interest, for example, to produce more valuable substances, such as plant growth regulators, by the microbial transformation of **128**. The first reported utilization of **128** was presented by in 1979 MacRae *et al.*,[178] who showed that it was a carbon source for *Pseudomonas flava* growing on *Eucalyptus* leaves. Growth of the bacterium in a mineral salt medium containing **128** resulted in oxidation at C-2 position of **128** to give the metabolites (1*S*,4*R*,6*S*)-(+)-2α-hydroxy-1,8-cineole (**124b**), (1*S*,4*R*,6*R*)-(−)-2β-hydroxy-1,8-cineole (**124a**), (1*S*,4*R*)-(+)-2-oxo-1,8-cineole (**304**), and (−)-(*R*)-5,5,-dimethyl-4-(3′-oxobutyl)-4,5-dihydrofuran-2(3H)-one (**305**) (**Scheme 133**).

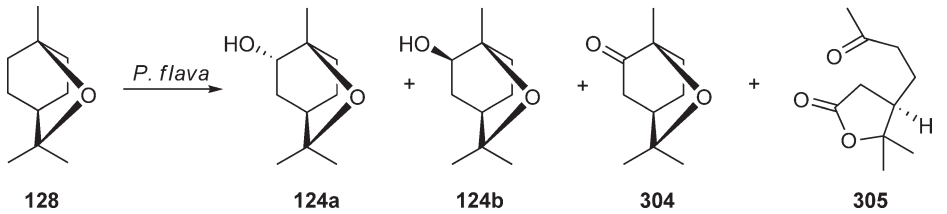
Streptomyces bottropensis SY-2-1 biotransformed 1,8-cineole (**128**) stereochemically to (+)-2α-hydroxy-1,8-cineole (**124a**) as the major product and (+)-3α-hydroxy-1,8-cineole (**306b**) as the minor product.[179] The recovery ratio of 1,8-cineole metabolites as ether extract was ~30% in *S. bottropensis* SY-2-1.

In the case of *S. ikutamanensis* Ya-2-1, 1,8-cineole (**128**) was biotransformed regioselectively to (+)-3α-hydroxy-1,8-cineole (**306b**, 46%) and (+)-3β-hydroxy-1,8-cineole (**306b**, 29%) as the major products.[180] The recovery ratio as ether extract was ~8.5% in *S. ikutamanensis* Ya-2-1 (**Scheme 134**).

When (+)-3α-hydroxy-1,8-cineole (**306b**) was used as a substrate in the culture medium of *S. ikutamanensis* Ya-2-1, (+)-3β-hydroxy-1,8-cineole (**306a**, 32%) was formed as the major product together with a small



Scheme 132 Structures of (+)-3-thujone (**300**) and its isomers, and biotransformation of (–)-3-isothujone (**300b**) by *Aspergillus niger* TBUYN-2 and *Botrytis allii* IFO9430.

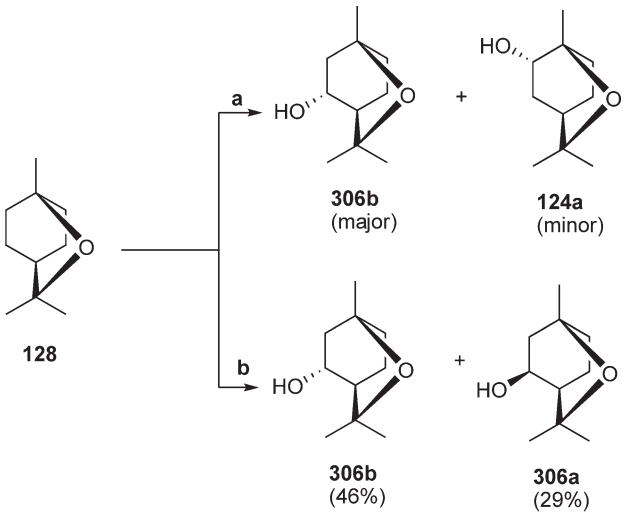


Scheme 133 Biotransformation of 1,8-cineole (**128**) by *Pseudomonas flava*.

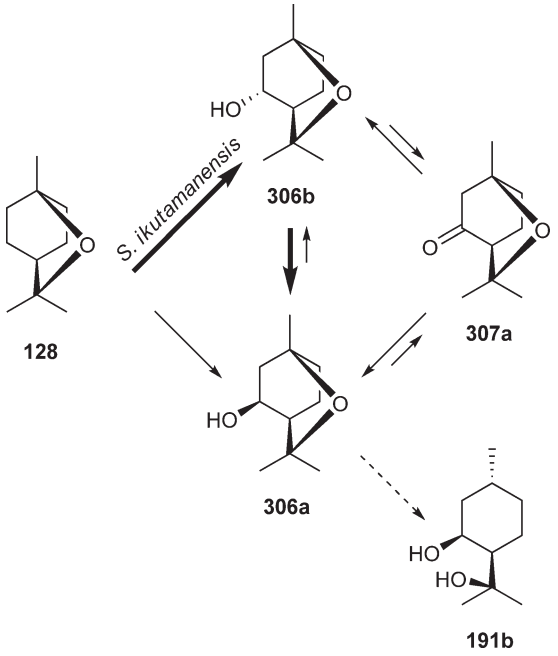
amount of (+)-3-oxo-1,8-cineole (**307a**, 1.6%). When (+)-3β-hydroxy-1,8-cineole (**306a**) was used, (+)-3-oxo-1,8-cineole (**307a**, 9.6%) and (+)-3α-hydroxy-1,8-cineole (**306b**, 2%) were formed. When (+)-3-oxo-1,8-cineole (**307a**) was used, (+)-3α-hydroxy- (**306b**, 19%) and (+)-3β-hydroxy-1,8-cineole (**306a**, 16%) were formed.

Based on the above results, it is obvious that (+)-3β-hydroxy-1,8-cineole (**306b**) is formed mainly in the biotransformation of 1,8-cineole (**128**), (+)-3α-hydroxy-1,8-cineole (**306a**), and (+)-3-oxo-1,8-cineole (**307a**) by *S. ikutamanensis* Ya-2-1. The production of (+)-3β-hydroxy-1,8-cineole (**306b**) is interesting, because it is a precursor of the mosquito repellent *p*-menthane-3,8-diol (**191b**) (**Scheme 135**).[180]

When *A. niger* TBUYN-2 was cultured in the presence of 1,8-cineole (**128**) for 7 days, it was transformed to three alcohols (racemic 2α-hydroxy-1,8-cineoles (**124a** and **124a′**), racemic 3α-hydroxy-1,8-cineoles (**306b** and **306b′**),

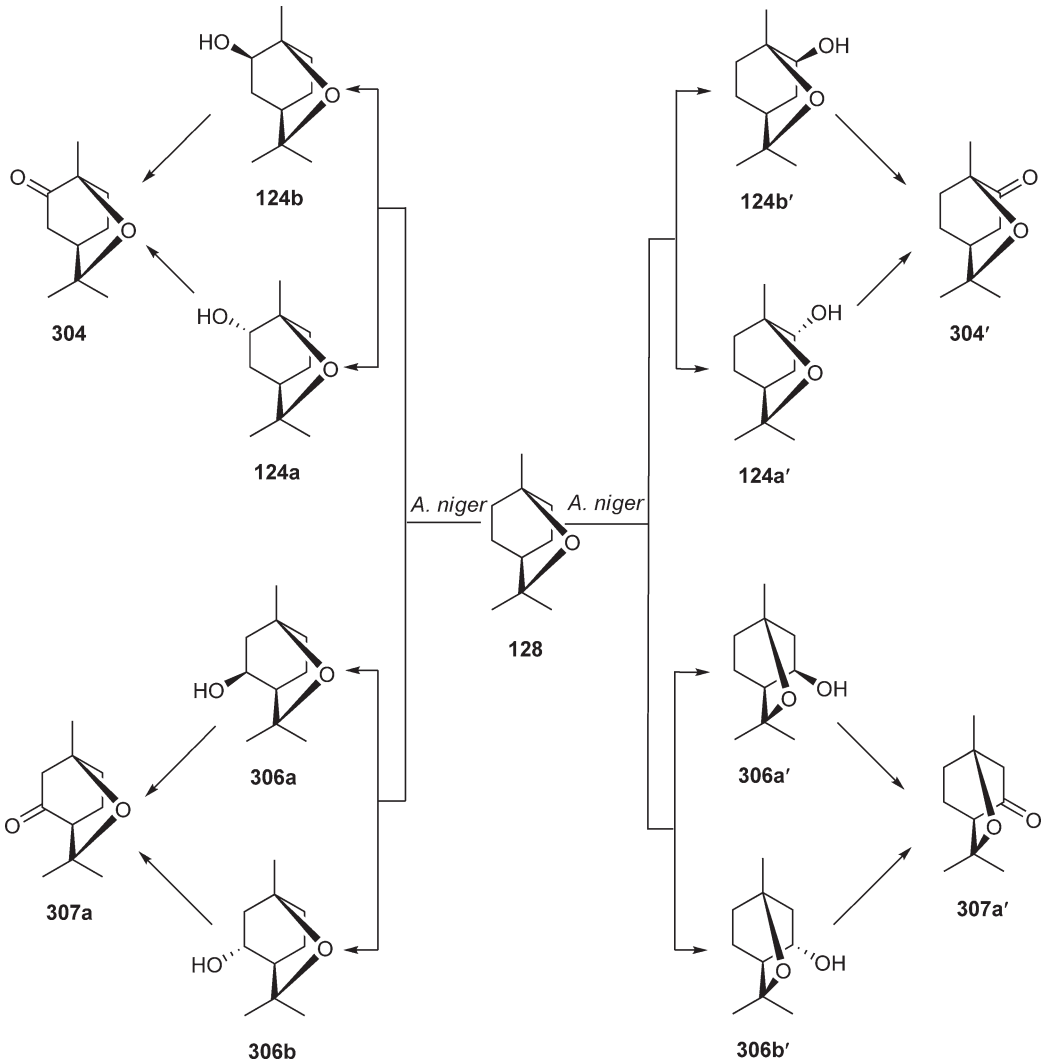


Scheme 134 Biotransformation of 1,8-cineole (**128**) by *Streptomyces bottropensis* SY-2-1 and *Streptomyces ikutamanensis* Ya-2-1.



Scheme 135 Biotransformation of 1,8-cineole (**128**), (+)-3α-hydroxy-1,8-cineole (**124b**), (+)-3β-hydroxy-1,8-cineole (**124a**), and (+)-3-oxo-1,8-cineole (**304**) by *Streptomyces ikutamanensis* Ya-2-1.

and racemic 3β-hydroxy-1,8-cineoles (**306a** and **306a′**)) and two ketones (racemic 2-oxo- (**304** and **304′**) and racemic 3-oxo-1,8-cineoles (**307a** and **307a′**)) (**Scheme 136**). The formation of 3α-hydroxy- (**306b** and **306b′**) and 3β-hydroxy-1,8-cineoles (**306a** and **306a′**) is of great interest not only due to the possibility of the formation of *p*-menthane-3,8-diol (**42a** and **42a′**), the mosquito repellents, and plant growth regulators that are synthesized chemically from 3α-hydroxy- (**306b** and **306b′**) and 3β-hydroxy-1,8-cineoles (**306a** and **306a′**), but also from the viewpoint of the utilization of *E. adiata* var. *austgraliana* leaves oil as biomass. An Et_2O extract of the culture broth (products and **128** as substrate) was recovered in 57% of substrate (w/w) (**Scheme 136**).[22,181]

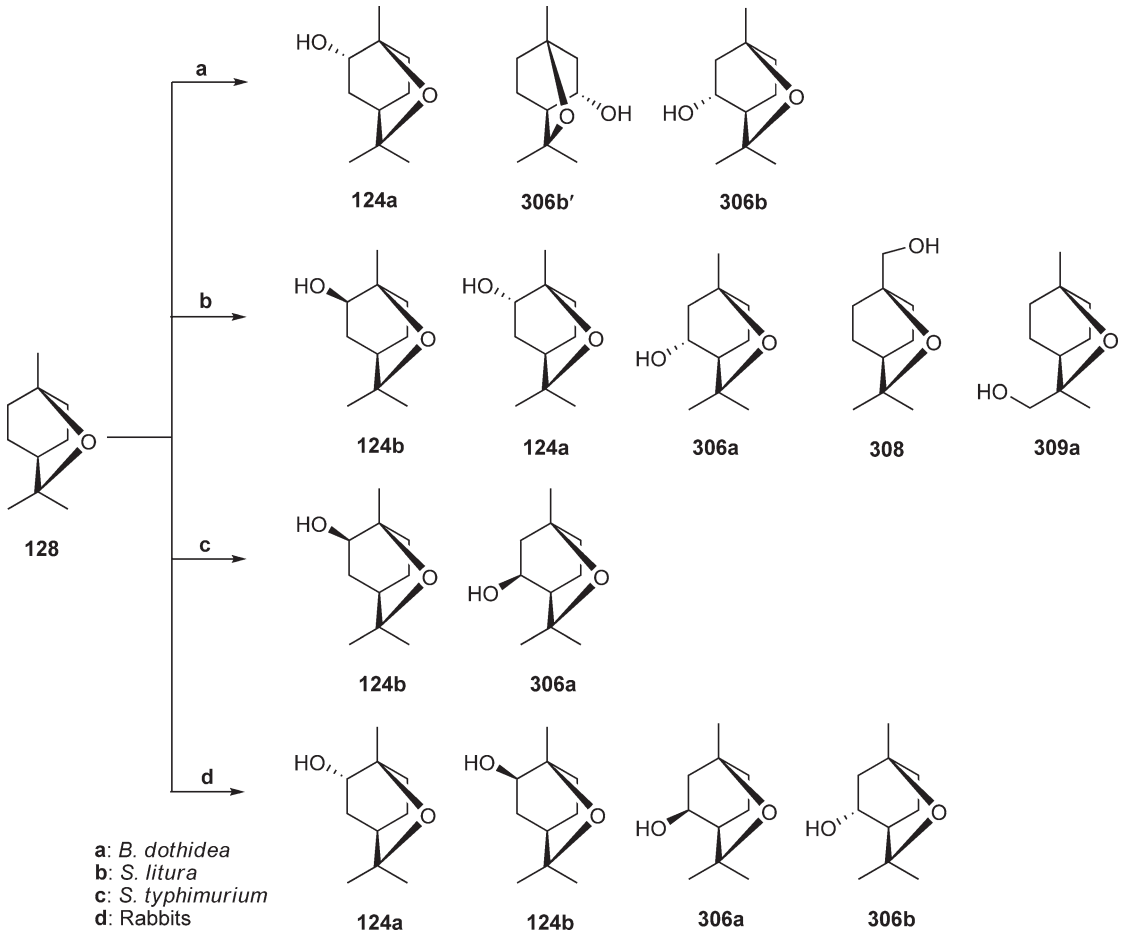


Scheme 136 Biotransformation of 1,8-cineole (**128**) by *Aspergillus niger* TBUYN-2.

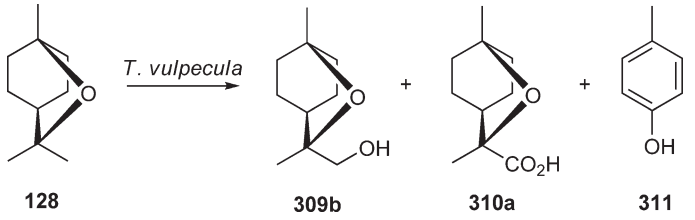
The plant pathogenic fungus *Botryosphaeria dothidea* converted 1,8-cineole (**128**) to optically pure (+)-2α-hydroxy-1,8-cineole (**124b**) and racemic 3α-hydroxy-1,8-cineole (**306b** and **306b′**), which were oxidized to optically active 2-oxo- (**304**) (100% ee) and racemic 3-oxo-1,8-cineole (**307a** and **307a′**), respectively. Cytochrome P-450 inhibitor 1-aminobenzotriazole inhibited the hydroxylation of the substrate (**Scheme 137**).[181] *Spodoptera litura* also converted 1,8-cineole (**128**) to give three secondary alcohols (**306b**, **124a**, and **124b**) and two primary alcohols (**308** and **309**).[182] *Salmonella typhimurium* OY1001/3A4 and NADPH-P-450 reductase hydroxylated 1,8-cineole (**128**) to 2β-hydroxy-1,8-cineole (**124a**, $[\alpha]_D = +9.3$, 65.3% ee) and 3β-hydroxy-1,8-cineole (**306a**, $[\alpha]_D = -27.8$, 24.7% ee).[183] In rabbits, 1,8-cineole (**128**) was metabolized to 2α-hydroxy-1,8-cineole (**124a**), 2β-hydroxy-1,8-cineole (**124b**), 3β-hydroxy-1,8-cineole (**306a**), and 3α-hydroxy-1,8-cineole (**306b**).[184]

Extraction of the urinary metabolites from brush tail possums (*Trichosurus vulpecula*) maintained on a diet of fruit impregnated with 1,8-cineole (**128**) yielded *p*-cresol (**311**) and the novel C-9 oxidized products 9-hydroxy-1,8-cineole (**309b**) and 1,8-cineole-9-oic acid (**310a**) (**Scheme 138**).[185,186]

1,8-Cineole (**128**) was converted into 2α-hydroxy-1,8-cineole (**124a′**) by CYP450 in human and rat liver microsomes. Cytochrome P-450 molecular species responsible for the metabolism of 1,8-cineole (**128**) were



Scheme 137 Biotransformation of 1,8-cineole (**128**) by *Botryosphaeria dothidea*, *Spodoptera litura*, and *Salmonella typhimurium*.

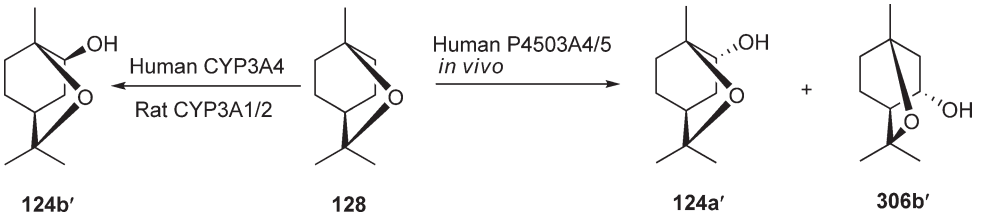


Scheme 138 Metabolism of 1,8-cineole (**128**) in brush tail possums (*Trichosurus vulpecula*).

determined to be CYP3A4 and CYP3A1/2 in human and rat, respectively.[187] Kinetic analysis showed that K_m and V_{max} values for the oxidation of 1,8-cineole (**128**) by pregnenolone-16α-carbonitrile (PCN)-treated human and rat liver microsomes and by recombinant CYP3A4 were 50 µmol l^{-1} and 90.9 nmol per min per nmol P-450, 20 µmol l^{-1} and 11.5 nmol per min per nmol P-450, and 90 µmol l^{-1} and 47.6 nmol per min per nmol P-450, respectively (**Scheme 139**).[188]

The above results were confirmed by the investigation of human urine after the oral administration of cold medication containing 1,8-cineole (**128**). In human urine, 2β-hydroxy-1,8-cineole (**124b′**) and 3α-hydroxy-1,8-cineole (**306b′**) were identified by GC–MS.

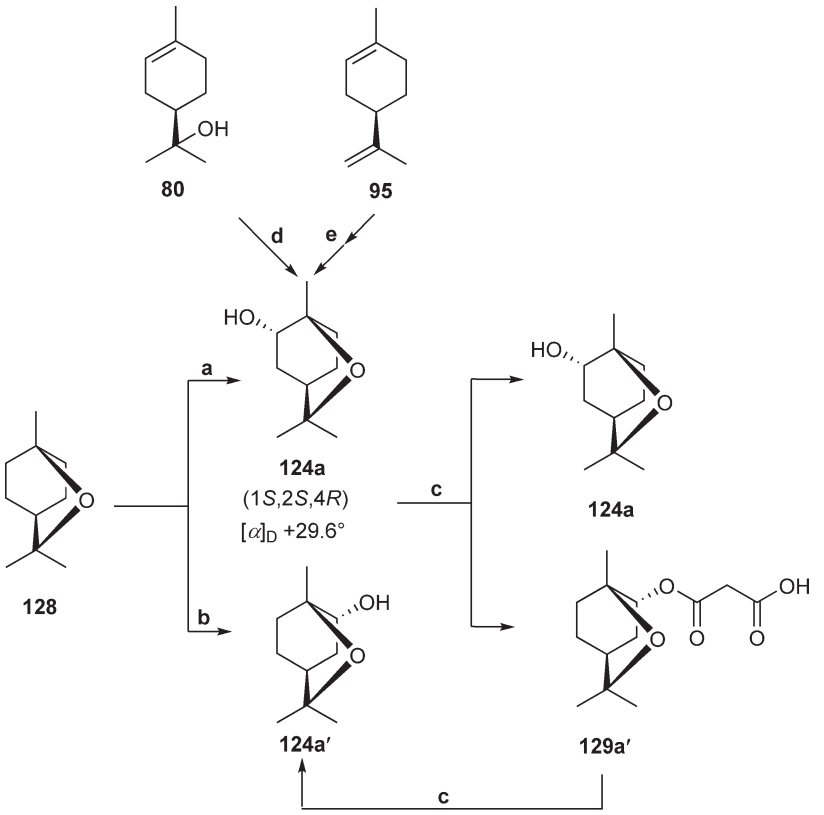
Microbial resolution of racemic 2α-hydroxy-1,8-cineoles (**124a** and **124a′**) was carried out by using *G. cingulata*. The mixture of **124a** and **124a′** was added to a culture of *G. cingulata* and esterified to give after



Scheme 139 Proposed metabolism of 1,8-cineole (**128**) by human CYP-450, rat liver microsome, human CYP3A4/CYP3A5, and *in vivo*.

24 h (1*R*,2*R*,4*S*)-1,8-cineole-2-yl-malonate (**129a′**) in 45% yield (100% ee). The recovered alcohol showed 100% ee of the (1*S*,2*S*,4*R*)-enantiomer (**124b**).[80] On the other hand, optically active (+)-2α-hydroxy-1,8-cineole (**124a**) was also formed from (+)-limonene (**95**) by a strain of *Citrus* pathogenic fungus *P. digitatum* (**Scheme 140**).[73,183]

Aspergillus niger biotransformed 1,8-cineole (**128**) to 2α- (**124a′** and **124b**) and 3α-hydroxy-1,8-cineol (**306a′** and **306a**) as the major components, which were oxidized by PCC–Al_2O_3 to racemic 2- (**304′** and **304**) and 3-oxo-1,8-cineoles (**307a′** and **307a**),[181] while **128** was converted by *B. dothidea* into the same alcohols,



a: *A. niger*
b: *A. niger, R. dothidea, P. flava*
c: *G. cingulata*
d: *G. cyanea*
e: *P. digitatum*

Scheme 140 Formation of 2α-hydroxy-1,8-cineoles (**124b** and **124b′**) from 1,8-cineole (**128**) and optical resolution by *Glomerella cingulata* and *Aspergillus niger* TBUYN-2 and **124b′** from (+)-limonene (**95**) by *Penicillium digitatum*.

Table 14 Stereoselectivity in the biotransformation of 1,8-cineole (**128**) by *Aspergillus niger*, *Botryosphaeria dothidea*, and *Pseudomonas flava*[181]

	Products							
Microorganisms	*124b and 124b′*		*124a and 124a′*		*306b and 306b′*		*306a and 306a′*	
Aspergillus niger TBUYN-2	2	:	43	:	49	:	6	
304 and **304′**, **307** and **307′**			50:50		41:59			
Botryosphaeria dothidea	4	:	59	:	34	:	3	
304 and **304′**, **307** and **307′**			100:0		53:47			
Pseudomonas flava[178]	29	:	71	:	0	:	0	
304 and **304′**, **307** and **307′**			100:0					

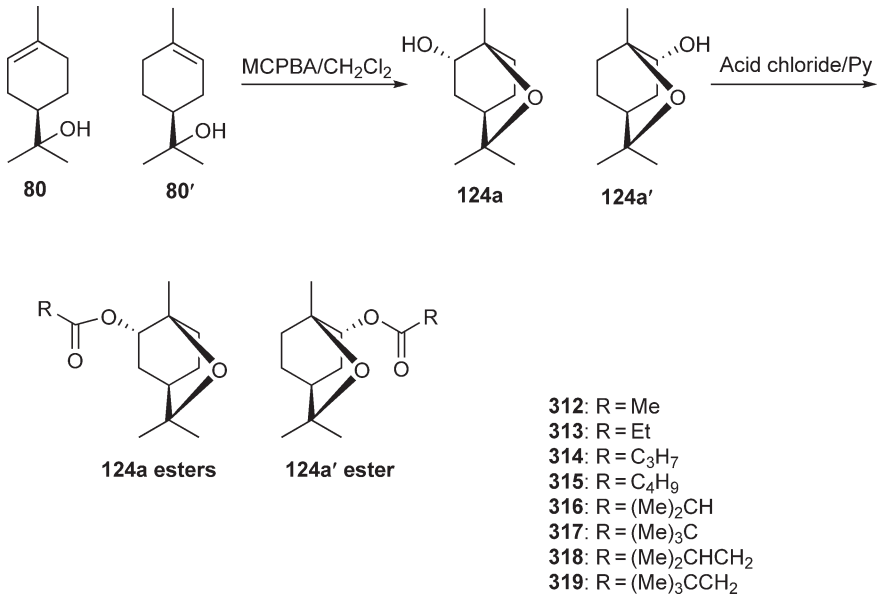
which were also oxidized to optically active 2-oxo- (**304**) (100% ee) and racemic 3-oxo-1,8-cineoles (**307a′** and **307a**) as shown in **Table 14**. The same phenomenon was observed in the biotransformation of **128** by *P. flava*.[178] Each optical isomer was analyzed by CDX-B capillary GC column. Cytochrome P-450 inhibitor 1-aminobenzotriazole inhibited the hydroxylation of 1,8-cineole (**128**).[181]

Esters of racemic 2α-hydroxy-1,8-cineole (**124a** and **124a′**) were prepared by a convenient method (**Scheme 141**) and their odors were characteristic. Then the products were tested against antimicrobial activity and their microbial resolution was studied (**Table 15**).[189]

Resolution of racemic 2α-acetoxy-1,8-cineole (**312** and **312′**) by *G. cingulata* was carried out. Both (+)-(**312**) and (−)-2α-acetoxy-1,8-cineole (**312′**) could be quantitatively obtained in an enantiomerically pure form (50% yield, 100% ee) (**Scheme 142**). In addition, odor differences between the enantiomers are also described. In both compounds (acetoxy and hydroxyl), the (+)-enantiomers tended to have more bright, light, and sweet odors than their (−)-antipodes (**Table 16**).[190]

1,8-Cineole (**128**) was glucosylated by *E. perriniana* suspension cells to 2α-hydroxy-1,8-cineole monoglucoside (**320**, 16.0% and **320′**, 16.0%) and diglucoside (**321**, 1.4%) (**Scheme 143**).[113]

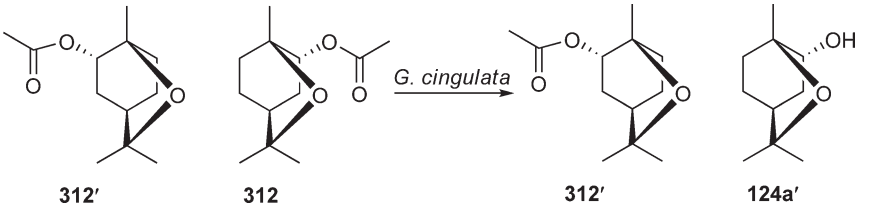
3.19.2.2.5(ii) 1,4-Cineole (322) Regarding the biotransformation of 1,4-cineole (**322**), *S. griseus* transformed **322** to 2β- (**208b′**, 6%), 2α- (**208a′**, 3%), and 8-hydroxy-1,4-cineole (18%, **324**), whereas *Bacillus cereus*



Scheme 141 Chemical synthesis of esters (**312–319**) of racemic 2α-hydroxy-1,8-cineoles (**124b** and **124b′**).

Table 15 Yield and enantiomeric excess (ee) of esters of racemic 2α-hydroxy-1,8-cineole (**124a** and **124a′**) on the microbial resolution by *Glomerella cingulata*[189]

	0 h	*24 h*		*48 h*	
Compounds	*% ee*	*% ee*	*Yield (%)*	*% ee*	*Yield (%)*
312	(−)36.3	(+)85.0	24.0	(+)100	14.1
313	(−)36.9	(+)73.8	18.6	(+)100	8.6
314	(−)35.6	(+)33.2	13.7	(+)75.4	3.5
315	(−)36.8	(+)45.4	14.4	(+)100	2.3
316	(−)35.4	(−)21.4	25.2	(+)20.6	8.0
317	(−)36.7	(−)37.8	31.5	(−)40.6	15.2
318	(−)36.1	(−)29.8	46.8	(−)15.0	24.0
319	(−)36.3	(−)37.6	72.2	(−)39.0	36.9

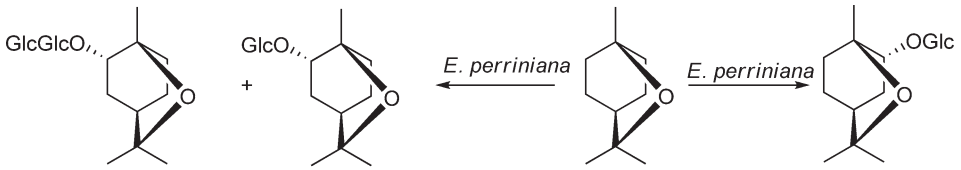


312′ **312** **312′** **124a′**

Scheme 142 Microbial resolution of racemic 2α-acetoxy-1,8-cineolse (**312** and **312′**) by *Glomerella cingulata*.

Table 16 Odor description of enantiomers **124b** and **124b′** and **312** and **312′**[190]

Compound	*Odor description*
(−)-(1*R*,2*R*,4*S*)-**312**	Camphorous, dry odor
(+)-(1*S*,2*S*,4*R*)-**312′**	Sharp, fruity, sweet odor
(−)-(1*R*,2*R*,4*S*)-**124a**	Camphorous odor
(+)-(1*S*,2*S*,4*R*)-**124a′**	Cineole-like, sweet odor

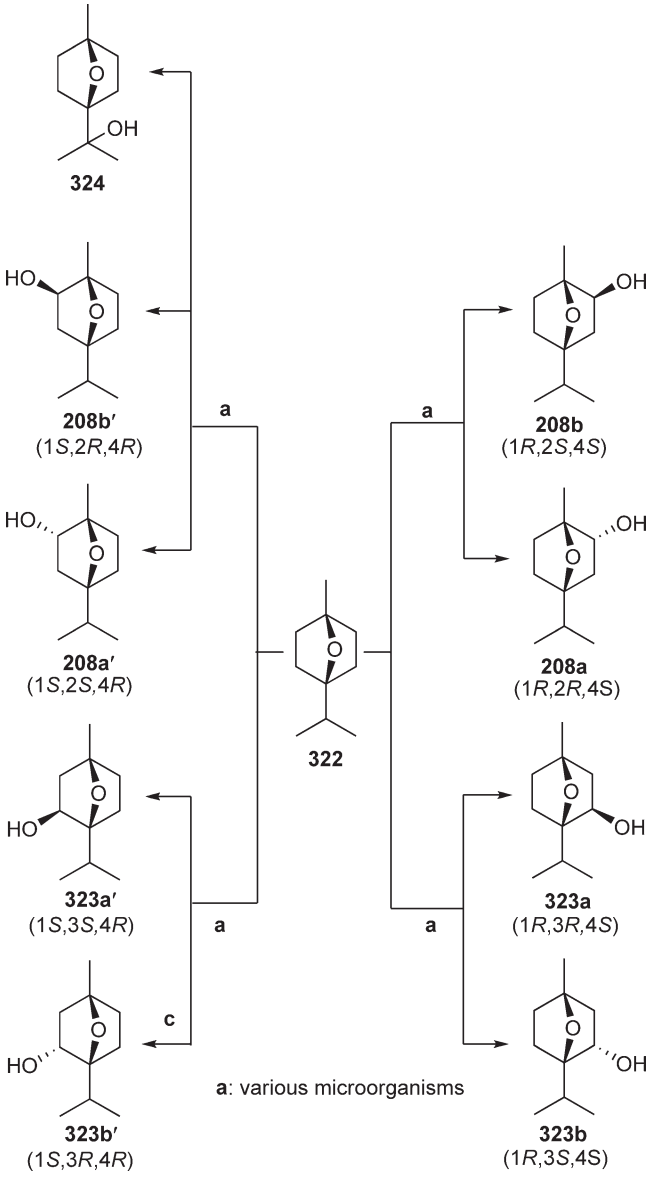


321 **320** **128** **320′**

Scheme 143 Biotransformation of 1,8-cineole (**128**) by suspension culture of *Eucalyptus perriniana*.

transformed 1,4-cineole (**322**) to 2α-hydroxy-1,4-cineole (**208a**, 3.8%) and 2β-hydroxy-1,4-cineole (**208b′**, 21.3%).[191] On the other hand, a strain of *A. niger* biotransformed 1,4-cineole (**322**) regiospecifically to 2α-hydroxy-1,4-cineole (**208a′**)[192] and (+)-3α-hydroxy-1,4-cineole (**323b′**)[193] along with the formation of 8-hydroxy-1,4-cineole (**324**) and 9-hydroxy-1,4-cineole (**325**)[194] (**Schemes 144** and **145**).

Microbial optical resolution of racemic 2α-hydroxy-1,4-cineoles (**208a′** and **208b′**) was carried out by using *G. cingulata*.[80] The mixture of 2α-hydroxy-1,4-cineoles (**208a** and **208a′**) was added to a culture of *G. cingulata* and esterified to give after 24 h (1*R*,2*R*,4*S*)-1,4-cineole-2-yl-malonate (**326**) in 45% yield (100% ee). The recovered alcohol showed an ee of 100% of the (1*S*,2*S*,4*R*)-enantiomer (**208a**). On the other hand, optically active (+)-2α-hydroxy-1,4-cineole (**208a**) was also formed from (−)-terpinen-4-ol (**122**) by *Gibberella cyanea* DSM[70] and *A. niger* TBUYN-2 (**Scheme 146**).[74]

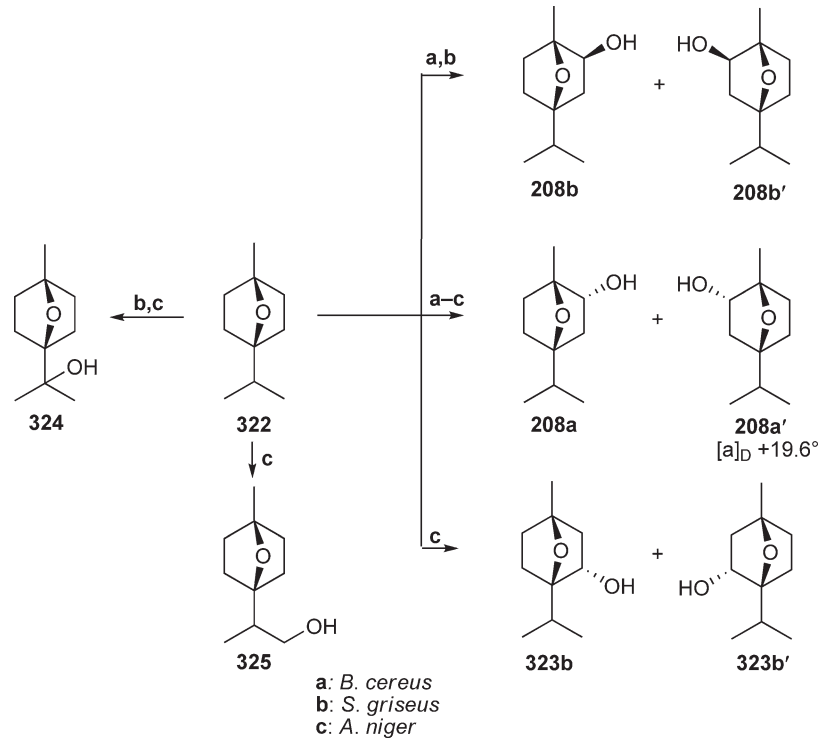


Scheme 144 Metabolic pathways of 1,4-cineole (**322**) by microorganisms.

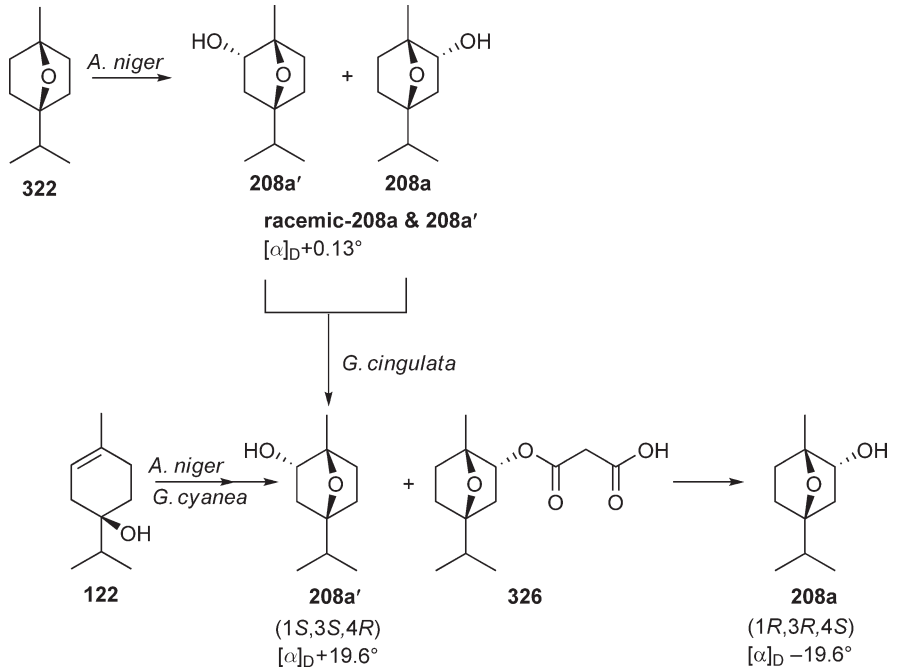
The metabolism of 1,4-cineole (**322**) was studied in rabbits. Four neutral and one acidic metabolites were isolated from the urine and were shown to be 9-hydroxy-1,4-cineole (**325**), 3,8-dihydroxy-1,4-cineole (**337**), 8,9-dihydroxy-1,4-cineole (**328**), 1,4-cineole-8-en-9-ol (**329**), and 1,4-cineole-9-carboxylic acid (**330**) as shown in **Scheme 147**.[195] 1,4-Cineole (**322**) was oxidized at C-2 position by CYP3A4 in humans and by CYP3A1/2 in rats, to give 2β-hydroxy-1,4-cineole (**208b**) (**Scheme 147**).[196]

3.19.2.2.6 Bicyclic monoterpene hydrocarbons

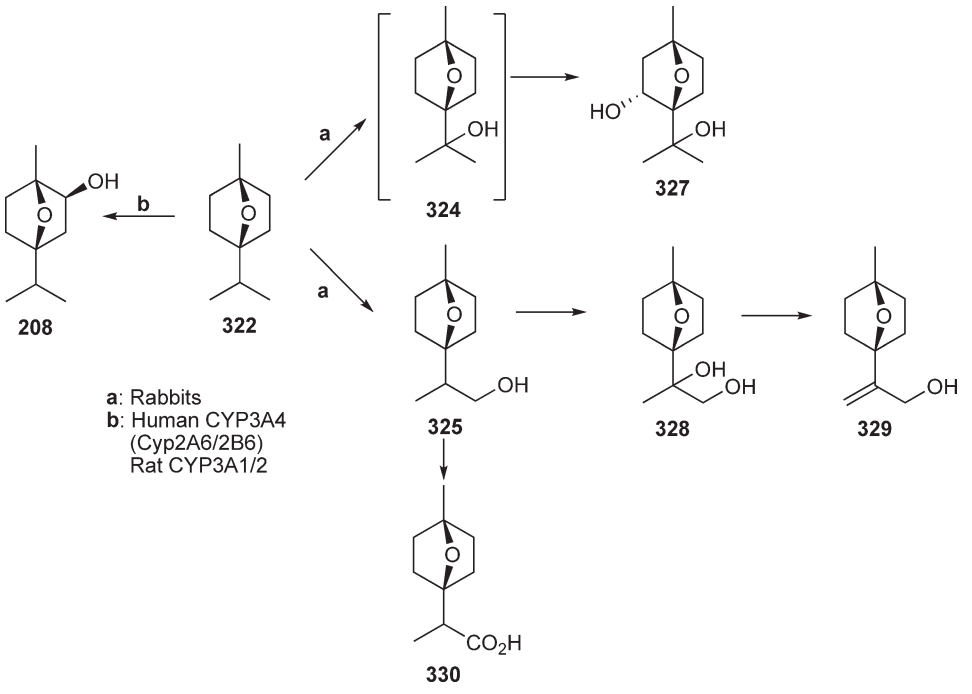
3.19.2.2.6(i) α-Pinene (130 and 130′) α-Pinene (**130** and **130′**) is the most abundant terpene in nature and is obtained industrially by fractional distillation of turpentine.[52] (+)-α-Pinene (**130**) occurs in the oil of *Pinus palustris* Mill. at concentrations of up to 65% and in the oil of *Pinus caribaea* at concentrations of 70%.[41] On the other hand, *P, caribaea* contains (−)-α-pinene (**130′**) at concentrations of 70–80%.[41]



Scheme 145 Metabolic pathways of 1,4-cineole (**322**) by *Aspergillus niger* TBUYN-2, *Bacillus cereus*, and *Streptomyces griseus*.



Scheme 146 Formation of optically active 2α-hydroxycineol (**208a** and **208a′**) from 1,4-cineole (**322**) and terpinene-4-ol (**122′**) by *Aspergillus niger* TBUYN-2, *Gibberella cyanea*, and *Glomerella cingulata*.



Scheme 147 Proposed pathways of metabolism of 1,4-cineole (**322**) by rabbit, human, and rat P-450 enzymes.

The biotransformation of (+)-α-pinene (**130**) by *A. niger* NCIM 612 was investigated.[1,197] A 24-h shake culture of this strain metabolized 0.5% (+)-α-pinene (**130**) in 4–8 h. After fermentation, the culture broth contained (+)-verbenone (**281′**) (2–3%), (+)-*cis*-verbenol (**331b**) (20–25%), (+)-*trans*-sobrerol (**125a**) (2–3%), and (+)-8-hydroxycarvotanacetone (**252**) (**Scheme 148**).[1,197]

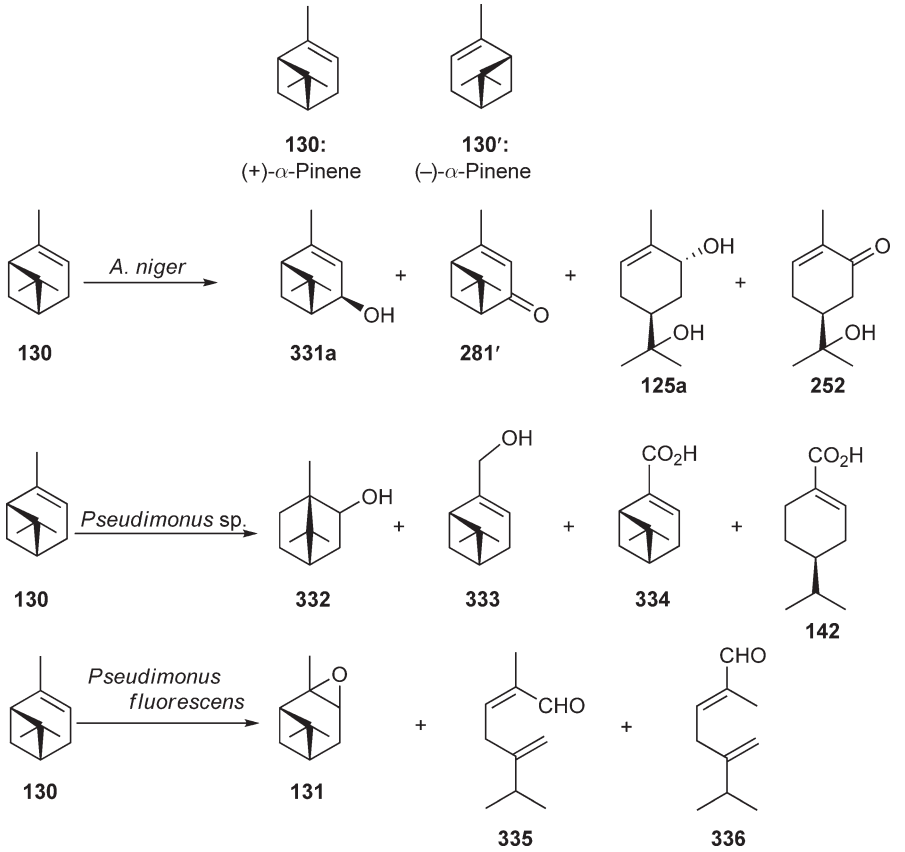
The degradation of (+)-α-pinene (**130**) by a soil *Pseudomonas* sp. (PL strain) was investigated by Hungund *et al.*[85] A terminal oxidation pattern was proposed, leading to the formation of organic acids through ring cleavage. The fermentation of (+)-α-pinene (**130**) was carried out in shake cultures using a soil *Pseudomonas* sp. (PL strain) that is able to grow on (+)-α-pinene (**130**) as the sole carbon source and the products obtained were borneol (**332**), myrtenol (**333**), myrtenic acid (**334**), and α-phellandric acid (**142**) (**Scheme 148**).[198]

The degradation of (+)-α-pinene (**130**) by *P. fluorescens* NCIMB 11671 was studied and a pathway for the microbial breakdown of (+)-α-pinene (**130**) was proposed as shown in **Scheme 148**.[199,200] The attack of oxygen is initiated by enzymatic oxygenation of the 1,2-double bond to form α-pinene epoxide (**131**), which then undergoes a rapid rearrangement to produce a unsaturated aldehyde, occurring as two isomeric forms. The primary product of the reaction (*Z*)-2-methyl-5-isopropylhexa-2,5-dien-1-al (**335**, isonovalal) can undergo chemical isomerization to the *E*-form (novalal, **336**). Isonovalal (**335**), the native form of the aldehyde, possesses citrus, woody, and spicy notes, whereas novalal (**336**) has woody, aldehydic, and cyclone notes. The same biotransformation reaction was carried out using *Nocardia* sp. strain P18.3.[201,202]

Pseudomonas PL strain and PIN 18 degraded α-pinene (**130**) by the pathway proposed in **Scheme 149** to give two hydrocarbons, limonene (**95**) and terpinolene (**155**), and a neutral metabolite, borneol (**332**). A probable pathway has been proposed for the terminal oxidation of β-isopropylpimelic acid (**140**) in the PL strain and PIN 18.[198]

Pseudomonas PX 1 biotransformed (+)-α-pinene (**130**) to (+)-*cis*-thujone (**310**) and (+)-*trans*-carveol (**100a**) as the major compounds. Compounds **100a**, **341**, **343**, and **348** have been identified as fermentation products (**Scheme 150**).[175,203]

Aspergillus niger TBUYN-2 biotransformed (−)-α-pinene (**130′**) to (−)-α-terpineol (**80′**) and (−)-*trans*-sobrerol (**125a′**).[204] The mosquitocidal (+)-(1*R*,2*S*,4*R*)-1-*p*-menthane-2,8-diol (**106a′**) was also obtained as a crystal in the biotransformation of (−)-α-pinene (**130′**) by *A. niger* TBUYN-2 (**Scheme 150**).[61,204]



Scheme 148 Structures of (+)- (**130**) and (−)-α-pinene (**131′**), and biotransformation of (+)-α-pinene (**130**) by *Aspergillus niger* NCIM 612, *Pseudomonas* species (PL strain), and *Pseudomonas fluorescens* NCIMB 11671.

(1*R*)-(+)-α-Pinene (**130**) and its enantiomer (**130′**) were biotransformed by *S. litura* to the corresponding (+)- and (−)-verbenones (**281** and **281′**) and (+)- and (−)-myrtenols (**333** and **333′**) (**Scheme 151**).[205]

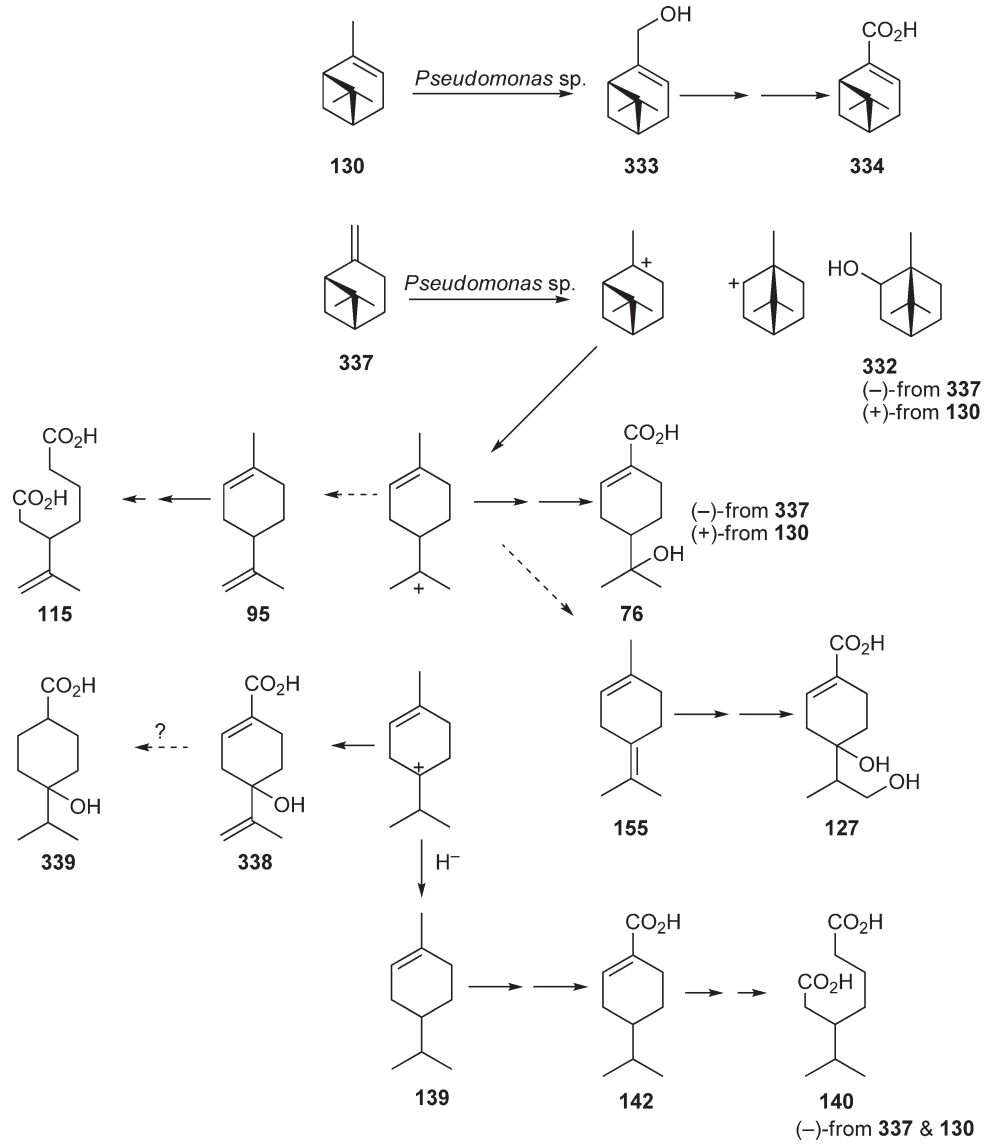
(−)-α-Pinene (**130′**) was converted by human liver microsomes (CYP2B6) to (−)-*trans*-verbenol (**331b′**) and (−)-myrtenol (**333′**) (**Scheme 151**).[206]

In rabbits, (+)-α-pinene (**130**) was metabolized to (−)-*trans*-verbenols (**331b**) as the major metabolites together with myrtenol (**333**) and myrtenic acid (**334**). The purities of (−)-*trans*-verbenol (**331b**) formed from (−)- (**130′**), (+)- (**130**), and (±)-α-pinene (**130** and **131′**) were 99, 67, and 68%, respectively. This means that the biotransformation of (−)-**131′** in rabbits is remarkably efficient in forming (−)-*trans*-verbenol (**331b**) (**Scheme 151**).[207]

(−)-α-Pinene (**131′**) was biotransformed by the plant pathogenic fungus *B. cinerea* to 3α-hydroxy-(−)-β-pinene (**349a′**, 10%), 8-hydroxy-(−)-α-pinene (**350**, 12%), 4α-hydroxy-(−)-pinene-6-one (**351**, 16%), and (−)-verbenone (**281′**) (**Scheme 151**).[208]

3.19.2.2.6(ii) β-Pinene (337 and 337′) (+)-β-Pinene (**337**) is found in many essential oils. Optically active and racemic β-pinene are present in turpentine oils, although in smaller quantities than (+)-α-pinene (**130**).[41]

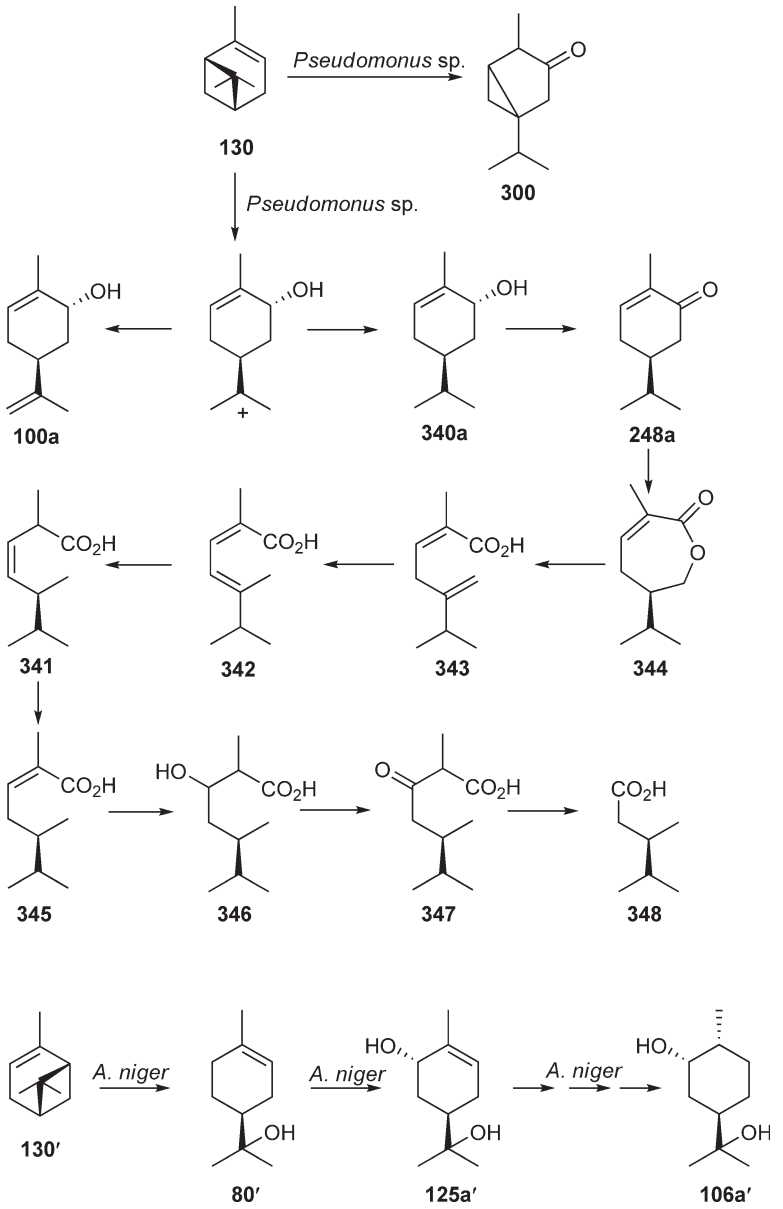
Shukla *et al.*[209] obtained a similarly complex mixture of transformation products from (−)-β-pinene (**337′**) through degradation by a *Pseudomonas* sp. (PL strain). On the other hand, Bhattacharyya and Ganapathy[210] indicated that *A. niger* NCIM 612 acts differently and more specifically on the pinenes by preferably oxidizing (−)-β-pinene (**337′**) at the allylic position to form the interesting products pinocarveol (**349a′**) and pinocarvone (**352**), besides myrtenol (**333′**) (see **Scheme 152**). Furthermore, the conversion of (−)-β-pinene (**337′**) by *P. putida-arvilla* (PL strain) gave borneol (**332**) (**Scheme 152**).[68]



Scheme 149 Metabolic pathways of degradation of α- (**130**) and β-pinene (**337**) by a soil Pseudomonad (PL strain) and *Pseudomonas* PIN 18.

Pseudomonas pseudomallai isolated from local sewage sludge by an enrichment culture technique utilized caryophyllene as the sole carbon source.[211] Fermentation of (−)-β-pinene (**337′**) by *P. pseudomallai* in a mineral salt medium (Seubert's medium) at 30 °C with agitation and aeration for 4 days yielded camphor (**353′**), isoborneol (**332b′**), borneol (**332a′**), α-terpineol (**80′**), and β-isopropyl pimelic acid (**140′**) (**Scheme 152**). Using a modified Czapek–Dox medium and keeping the other conditions the same, the pattern of the metabolic products was dramatically changed. The metabolites were *trans*-pinocarveol (**349a′**), myrtenol (**333′**), α-fenchol (**354a′**), α-terpineol (**80′**), myrtenic acid (**334′**), and two unidentified products (**Scheme 152**).[211]

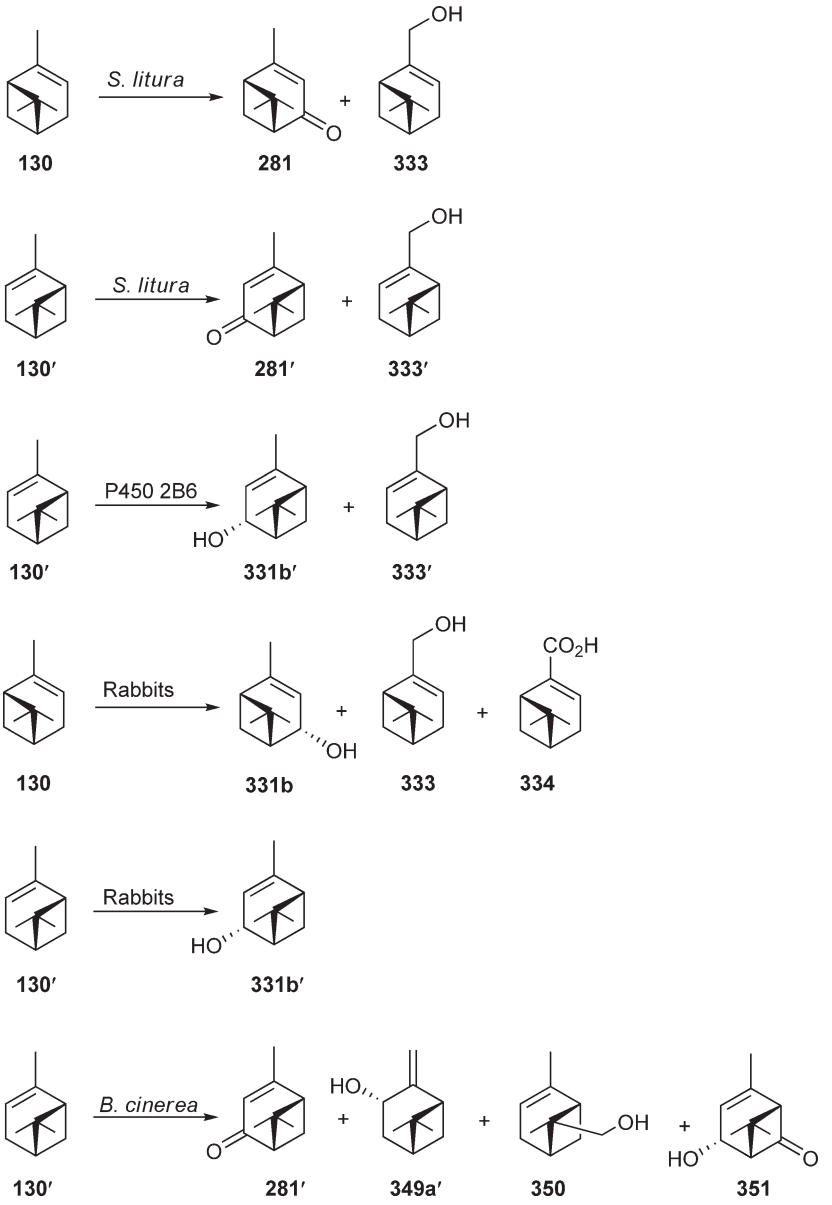
(−)-β-Pinene (**337′**) was converted by the plant pathogenic fungus *B. cinerea* to four new compounds, namely (−)-pinane-2α,3α-diol (**355′**), (−)-6β-hydroxypinene (**356**), (−)-4α,5-dihydroxypinene (**357**), and (−)-4α-hydroxypinen-6-one (**358**) (**Scheme 153**).[212] (−)-Pinane-2α,3α-diol (**355′**) and related compounds were further biotransformed by microorganisms as shown in **Scheme 153**.



Scheme 150 Proposed metabolic pathways for (+)-α-pinene (**130**) degradation by *Pseudomonas* PX 1 and biotransformation of (−)-α-pinene (**130′**) by *Aspergillus niger* TBUYN-2.

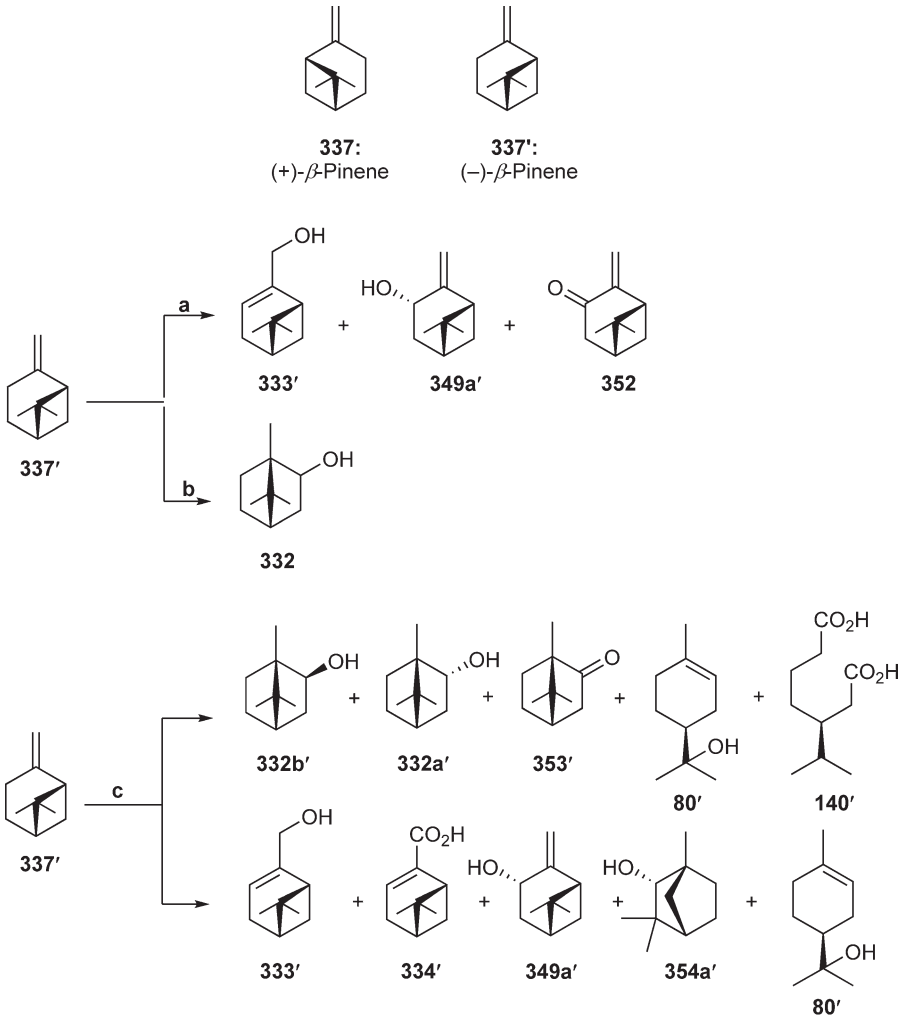
As shown in **Scheme 153**, (+)- (**337**) and (−)-β-pinenes (**337′**) were biotransformed by *A. niger* TBUYN-2 to (+)-α-terpineol (**80**) and (+)-oleuropeyl alcohol (**210**) and their antipodes (**80′** and **210′**), respectively. The hydroxylation process of α-terpineol (**80′**) to oleuropeyl alcohol (**210**) was completely inhibited by 1-aminobenzotriazole, a cytochrome P-450 inhibitor.[204]

(−)-β-Pinene (**337′**) was at first biotransformed by *A. niger* TBUYN-2 to (+)-*trans*-pinocarveol (**349a′**).[213] (+)-*trans*-Pinocarveol (**349a′**) was further transformed by three pathways: in the first pathway, (+)-*trans*-pinocarveol (**349a′**) was metabolized to (+)-pinocarvone (**352a′**), (−)-3-isopinanone (**359**), (+)-2α-hydroxy-3-pinanone (**360b′**), and (+)-2α,5-dihydroxy-3-pinanone (**361b′**); in the second pathway, (+)-*trans*-pinocarveol (**349a′**) was metabolized to (+)-6β-hydroxyfenchol (**362a′**); and in the third pathway, (+)-*trans*-pinocarveol (**349a′**) was metabolized to (−)-6β,7-dihydroxyfenchol (**363a′**) via epoxide and diol as intermediates (**Scheme 153**).[213]



Scheme 151 Biotransformation of (+)- (**130**) and (−)-α-pinene (**130′**) by *Spodoptera litura* and rabbits, and (−)-α-pinene (**131′**) by human liver microsomes (CYP2B6) and *Botrytis cinerea.*

(−)-β-Pinene (**337′**) was metabolized by *A. niger* TBUYN-2 via three pathways as shown in **Scheme 154** to give (−)-α-pinene (**130′**), (−)-α-terpineol (**80′**), and (+)-*trans*-pinocarveol (**349a′**). (−)-α-Pinene (**130′**) was further metabolized by three pathways. At first, (−)-α-pinene (**130′**) was metabolized via (−)-α-pinene epoxide (**131′**), *trans*-sobrerol (**125a′**), (−)-8-hydroxycarvotanacetone (**262**), and (+)-8-hydroxycarvomenthone (**276**) to (+)-*p*-menthane-2,8-diol (**100a′**), which is also formed from (−)-carvone (**104**) metabolism. Second, (−)-α-pinene (**130′**) was metabolized to myrtenol (**333′**), which is metabolized by rearrangement reaction to give (−)-oleuropeyl alcohol (**210′**). (−)-α-Terpineol (**80′**), which is formed from β-pinene (**337′**), was also metabolized to (−)-oleuropeyl alcohol (**210′**), and (+)-*trans*-pinocarveol (**349a′**) formed from (−)-β-pinene (**337′**) was metabolized to pinocarvone (**352′**), 3-pinanone (**359**), 2α-hydroxy-3-pinanone (**360b′**), 2α,5-dihydroxy-3-pinanone (**361b′**), and 2α,9-dihydroxy-3-pinanone (**366a′**).



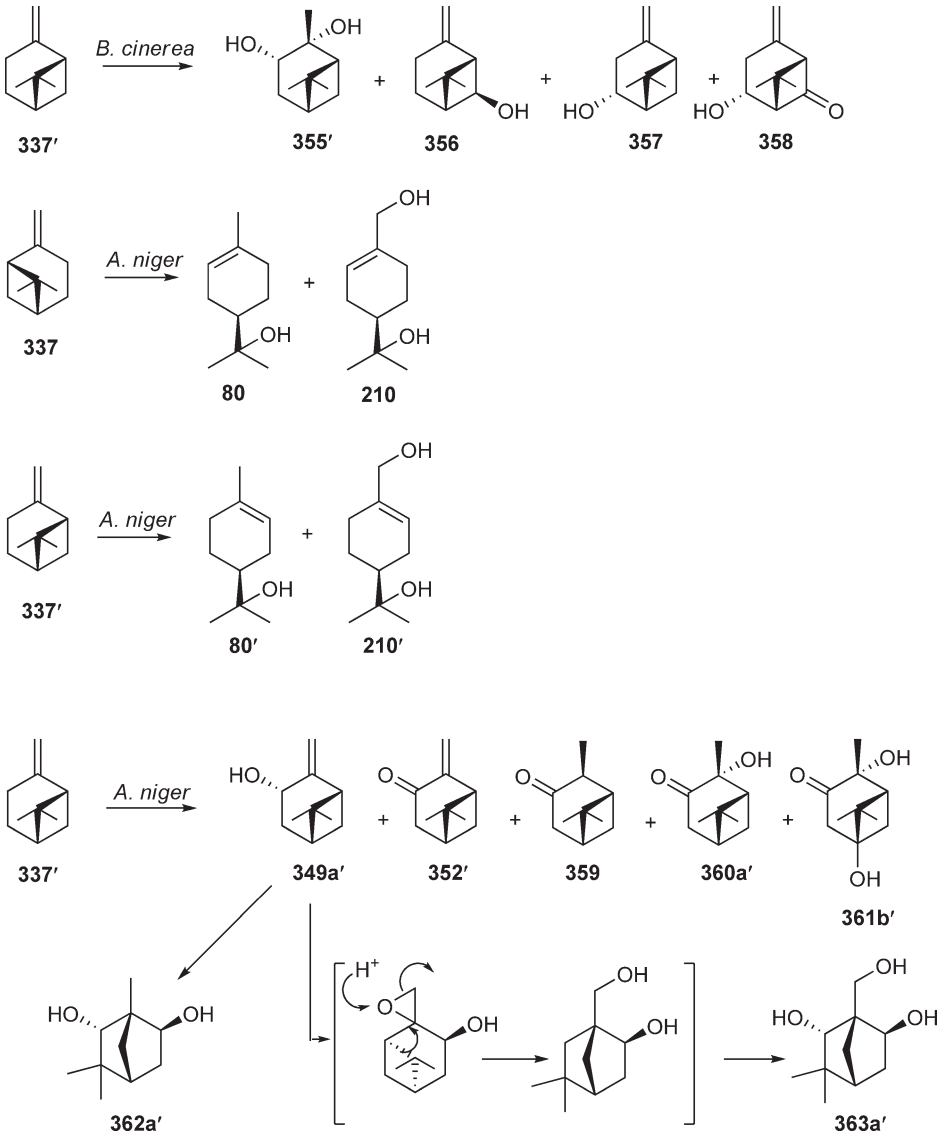
a: *A. niger*
b: *P. putida-arvilla*
c: *P. pesudomallalai*

Scheme 152 Structures of (+)- (**337**) and (–)-β-pinene (**337′**), and biotransformation of (–)-β-pinene (**337′**) by *Aspergillus niger* NCIM 612, *Pseudomonas putida-arvilla* (PL strain), and *Pseudomonas pseudomallai*.

Furthermore, (+)-*trans*-pinocarveol (**349a′**) was metabolized by rearrangement reaction to 6β-hydroxyfenchol (**362a′**) and 6β,7-dihydroxyfenchol (**363a′**) (**Scheme 154**).[213]

(–)-β-Pinene (**337′**) was metabolized by *A. niger* TBUYN-2 to (+)-*trans*-pinocarveol (**362a′**), which was further metabolized to 6β-hydroxyfenchol (**362a′**) and 6β,7-dihydroxyfenchol (**363a′**) by a rearrangement reaction (**Scheme 155**).[213] 6β-Hydroxyfenchol (**362a′**) was also obtained from (–)-fenchol (**354a′**). (–)-Fenchone (**368′**) was hydroxylated by the same fungus to give 6β- (**367b′**) and 6α-hydroxy-(–)-fenchone (**362b**). There is a close relationship between the metabolism of (–)-β-pinene (**337′**) and those of (–)-fenchol (**354a′**) and (–)-fenchone (**368′**).

(–)-β-Pinene (**337′**) and (–)-α-pinene (**130′**) were isomerized to each other. Both were metabolized via(–)-α-terpineol (**80′**) to (–)-oleuropeyl alcohol (**210′**) and (–)-oleuropeic acid (**368′**). (–)-Myrtenol (**333′**) formed from (–)-α-pinene (**130′**) was further metabolized via cation to (–)-oleuropeyl alcohol (**210′**) and (–)-oleuropeic acid (**76**). (–)-α-Pinene (**130′**) was further metabolized by *A. niger* TBUYN-2 via

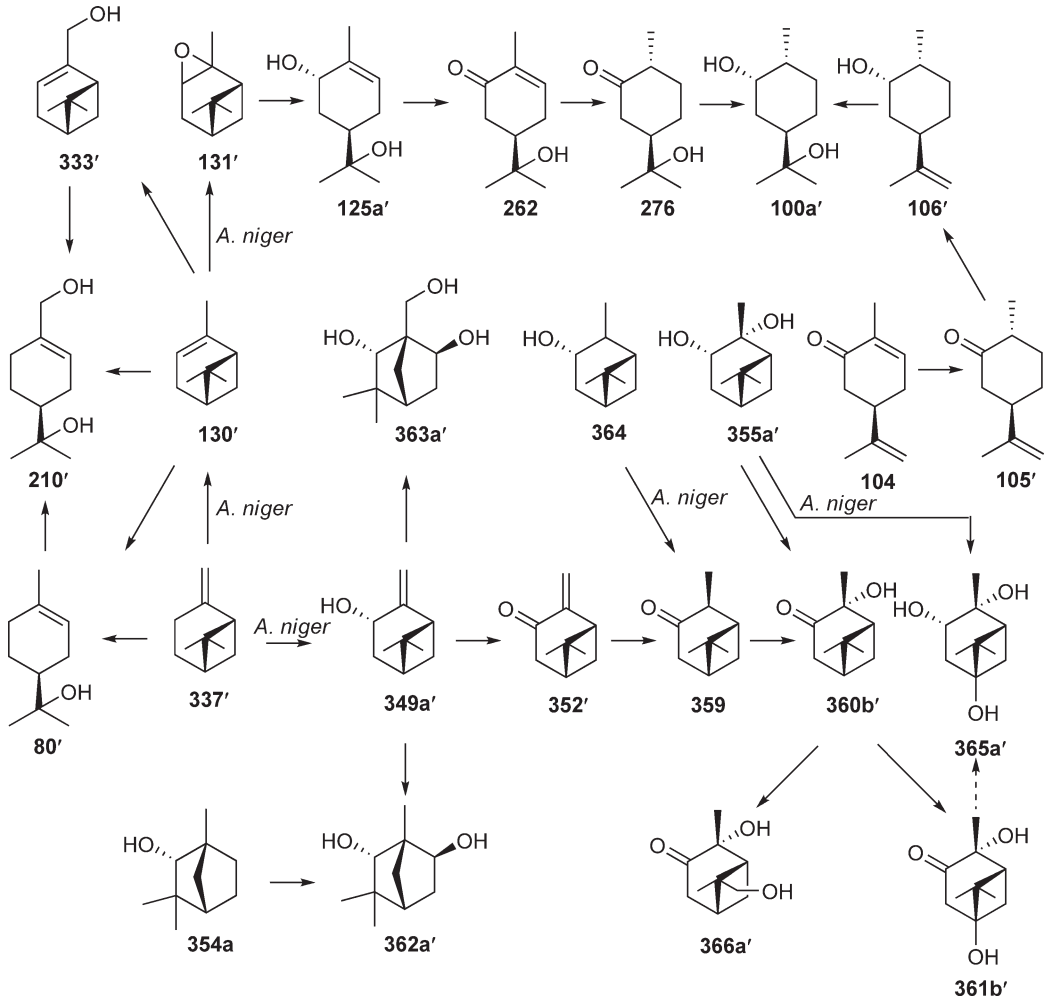


Scheme 153 Biotransformation of (−)-β-pinene (**337′**) by *Botrytis cinerea*, (+)- (**337**) and (−)-β-pinene (**337′**) by *Aspergillus niger* TBUYN-2, and (−)-β-pinene (**377′**) and (+)-*trans*-pinocarveol (**349a′**) by *Aspergillus niger* TBUYN-2.

(−)-α-pinene epoxide (**131′**) to *trans*-sobrerol (**125a′**), (−)-8-hydroxycarvotanacetone (**252′**), (+)-8-hydroxycarvomenthone (**276a′**), and mosquitocidal (+)-*p*-menthane-2,8-diol (**100a′**) (**Scheme 156**).[1,204,214,215]

The major metabolites of (−)-β-pinene (**337′**) were *trans*-10-pinanol (myrtanol) (**364**) (39%) and (−)-1-*p*-menthene-7,8-diol (oleuropeyl alcohol) (**210′**) (30%). In addition, (+)-*trans*-pinocarveol (**349a′**) (11%) and (−)-α-terpineol (**80′**) (5%) were also formed. Verbenol (**331a′** and **331b′**) and pinocarveol (**349a′**) were oxidation products of α- (**130′**) and β-pinene (**337′**), respectively, in the bark beetle *Dendroctonus frontalis*. (−)-*cis*- (**331a′**) and (+)-*trans*-Verbenols (**331b′**) have pheromonal activity in *Ips paraconfussus* and *Dendroctonus brevicomis*, respectively (**Scheme 157**).[207]

3.19.2.2.6(iii) Camphene (368) Racemate camphene (**368**) is a bicyclic monoterpene hydrocarbon found in *Liquidamar* species, *Chrysanthemum*, *Zingiber officinale*, *Rosmarinus offinialis*, and other plants. Rabbits converted it into camphene-2,10-glycols (**370** and **370′**) as the major metabolites, along with 7-hydroxycamphene (**369b**)

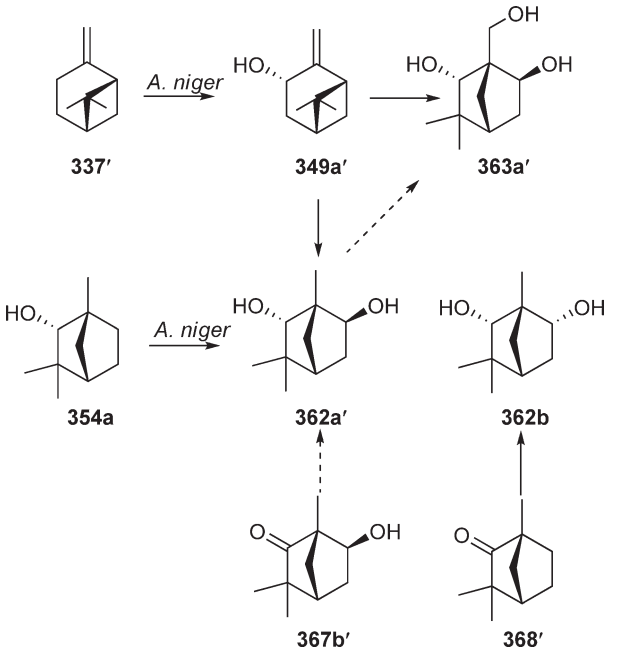


Scheme 154 Biotransformation of (−)-α-pinene (**130′**), (−)-β-pinene (**337′**), and related compounds by *Aspergillus niger* TBUYN-2.

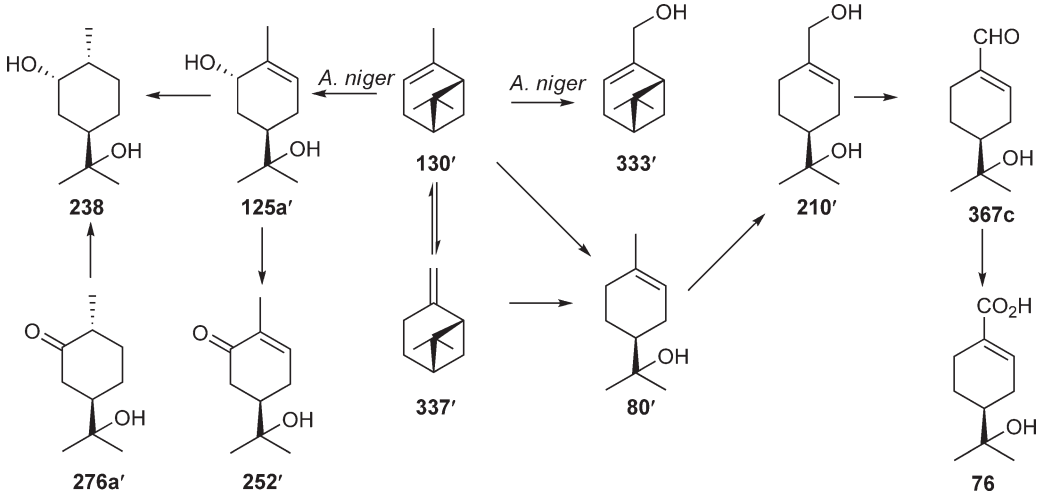
and 6-exo-hydroxycamphene (**359**).[216] The structures of **370** and **370′** were confirmed by the preparation of the same ketone (**371**) by periodate oxidation reaction as shown in **Scheme 158**. On the basis of the production of the glycols (**370** and **370′**) in good yield, these alcohols might be formed through their epoxides as shown in **Scheme 158**.

3.19.2.2.6(iv) 3-Carene (132) and carane (372a) (+)-3-Carene (**132**) was biotransformed by rabbits to afford *m*-mentha-4,6-dien-8-ol (**373**) (71.6%) as the major metabolite together with its aromatized *m*-cymen-8-ol (**374**). Position C-5 in the substrate is thought to be more easily hydroxylated than C-2 by enzymatic systems in the rabbit liver. In addition to the ring opening compound, 3-carene-9-ol (**375**), 3-carene-9-carboxylic acid (**376**), 3-caren-10-ol-9-carboxylic acid (**377**), 3-carene-10-ol (**378**), chamic acid (**379**), and 3-carene-8,10-dicarboxylic acid (**380**) were formed. The formation of such compounds is explained by stereoselective hydroxylation and carboxylation of *gem*-dimethyl group (**Scheme 159**).[207] In the case of (−)-*cis*-carane (**372a**), C-9 or C-10 methyl group was stereoselectively oxidized to monool (**381**), monocarboxylic acid (**382**), and dicarboxylic acid (**383**) as shown in **Scheme 159**.[207]

(+)-3-Carene (**132**) was converted by *A. niger* NC 1M612 to hydroxylated compounds (**384** and **385**) of 3-carene-2-one or 3-carene-5-one, which were not fully identified (**Scheme 160**).[214]



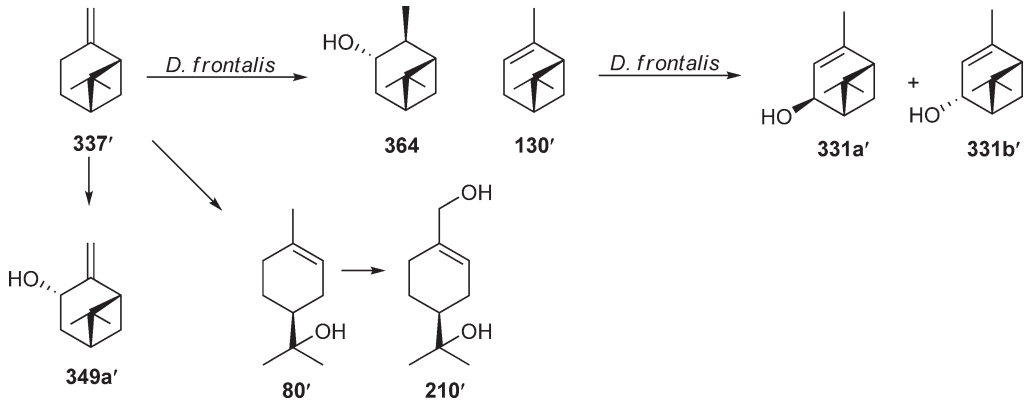
Scheme 155 Relationship between the metabolism of (−)-β-pinene (**337′**), (+)-fenchol (**354′**), and (−)-fenchone (**368′**) by *Aspergillus niger* TBUYN-2.



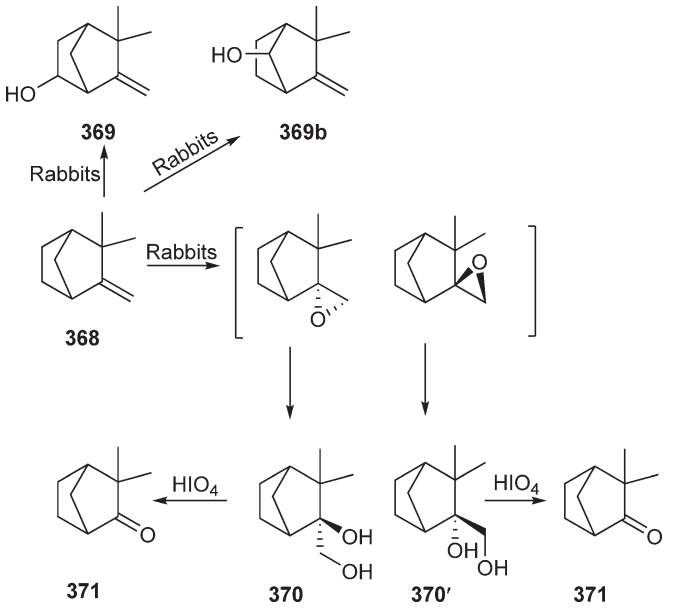
Scheme 156 Metabolic pathways of (−)-β-pinene (**337′**) and related compounds by *Aspergillus niger* TBUYN-2.

3.19.2.2.7 Bicyclic monoterpene aldehydes

3.19.2.2.7(i) Myrtenal (386′) and myrtanal (387a′ and 387b′) In **Scheme 161**, the structures of (+)-myrtenal (**386′**) and (+)-myrtanal (**387a′**) and their isomers are shown. *Euglena gracilis* Z biotransformed (−)-myrtenal (**386′**) to (−)-myrtenol (**333′**) as themajor product and (−)-myrtenoic acid (**334′**) as the minor product. However, further hydrogenation of (−)-myrtenol (**333′**) to *trans*- and *cis*-myrtanol (**388a′** and **388b′**) did not occur even at a concentration less than ~50 mg l^{-1}. (*S*)-*trans* and (*R*)-*cis*-Myrtanal (**387a′** and **387b′**) were also transformed to *trans*- and *cis*-myrtanol (**388a′** and **388b′**) as the major products and (*S*)-*trans*- (**389a′**) and (*R*)-*cis*-myrtanoic acid (**389b′**) as the minor products, respectively (**Scheme 161**).[19]



Scheme 157 Metabolism of (−)-β-pinene (**337′**) by bark beetle, *Dendroctonus frontalis*.



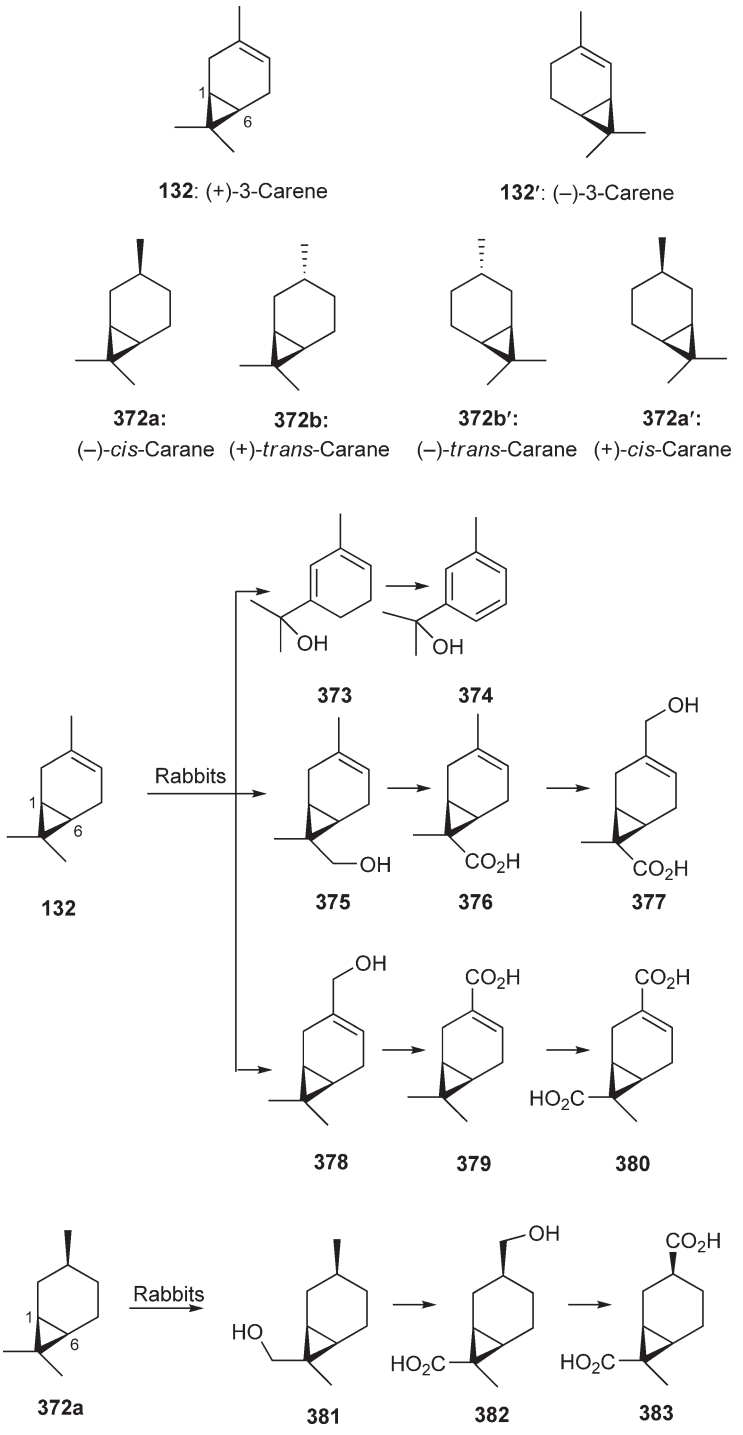
Scheme 158 Biotransformation of racemic camphene (**368**) by rabbits.

In the case of *A. niger* TBUYN-2, *A. sojae*, and *A. usami*, (−)-myrtenol (**333′**) was further metabolized to 7-hydroxyverbenone (**390′**) as the minor product together with (−)-oleuropeyl alcohol (**210′**) as the major product.[217,218] (−)-Oleuropeyl alcohol (**210′**) is also formed from (−)-α-terpineol (**80′**) by *A. niger* TBUYN-2 (**Scheme 161**).[204]

Rabbits metabolized myrtenal (**386′**) to myrtenic acid (**334′**) as the major metabolite and myrtanol (**388a′** or **388b′**) as the minor metabolite (**Scheme 161**).[98]

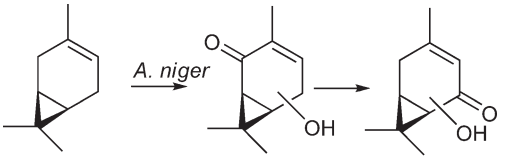
3.19.2.2.8 Bicyclic monoterpene alcohols

3.19.2.2.8(i) Myrtenol (333 and 333′) (−)-Myrtenol (**333′**) was biotransformed mainly to (−)-oleuropeyl alcohol (**210′**), which was formed from (−)-α-terpineol (**80′**) as the major product by *A. niger* TBUYN-2. In the case of *A. sojae* IFO4389 and *A. usami* IFO4338, (−)-myrtenol (**333′**) was metabolized to 7-hydroxyverbenone (**390′**) as the minor product together with (−)-oleuropeyl alcohol (**210′**) as the major product (**Scheme 161**).[217]



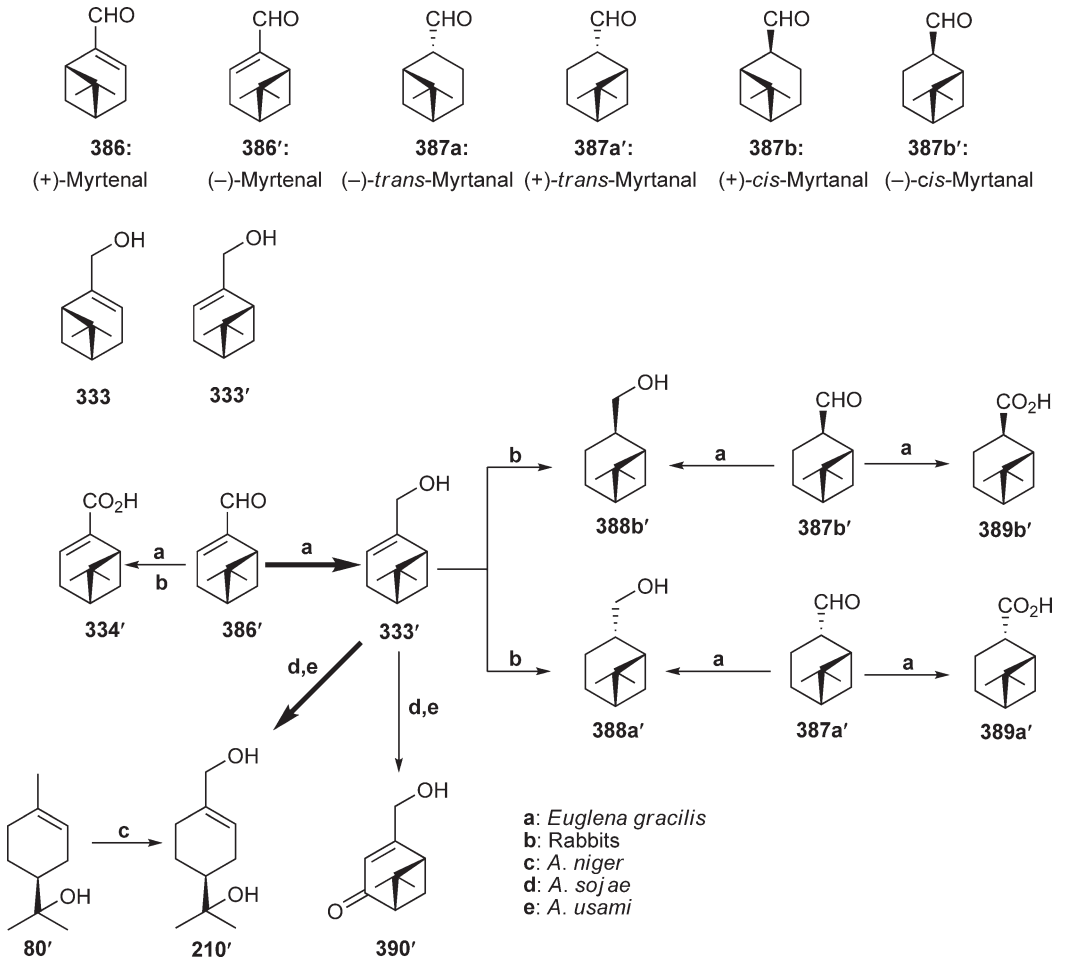
Scheme 159 Structures of (+)- (**132**) and (–)-3-carenes (**132′**) and stereoisomers (**372a, 372a′, 372b, 372b′**) of carane, and metabolic pathways of (+)-3-carene (**132**) and (–)-*cis*-carane (**372a**) by rabbits.

3.19.2.2.8(ii) **cis-** *(388b and 388b′) and* **trans-***Myrtanol (388a and 388a′)* *Spodoptera litura* converted (–)-*trans*-myrtanol (**388a**) and its enantiomer (**388a′**) to the corresponding myrtanic acid (**389a** and **389a′**) (**Scheme 162**).[219]



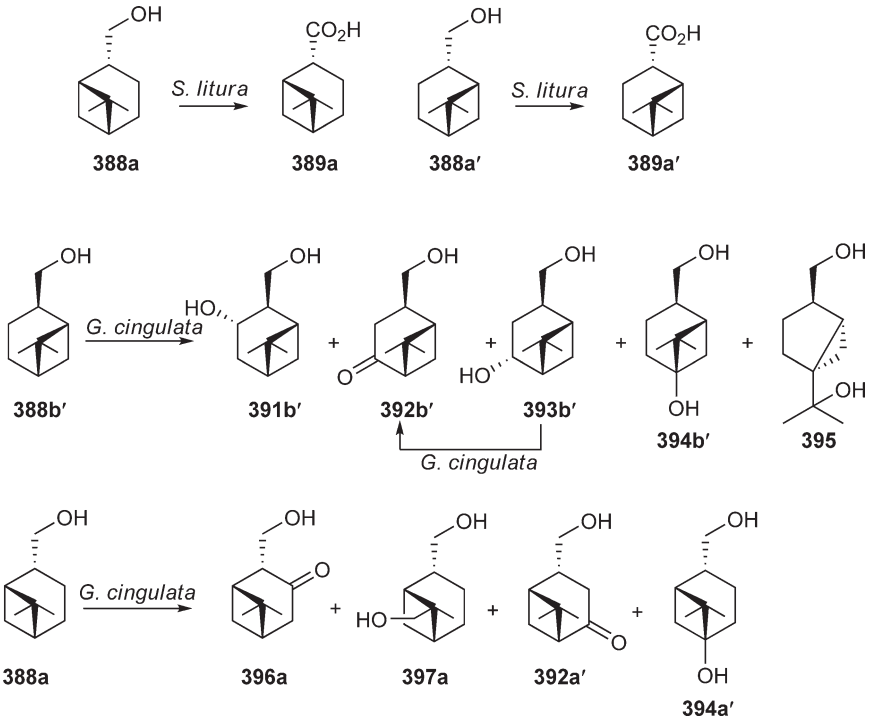
132 **384** **385**

Scheme 160 Metabolic pathways of (+)-3-carene (**132**) by *Aspergillus niger* NC 1M612.



Scheme 161 Structures of myrtenals (**386** and **386′**) and myrtanals (**387a, 387a′, 387b, 387b′**), and biotransformation of (–)-myrtenal (**386′**) and (+)-*trans*- (**387a′**) and (–)-*cis*-myrtanal (**387b′**) by microorganisms and (–)-myrtenol (**333′**) and (–)-α-terpineol (**80′**) by *Aspergillus niger* TBUYN-2.

Glomerella cingulata biotransformed (–)-*cis*-myrtanol (**388b′**) to (3*S*)-3-hydroxy-*cis*-myrtanol (**391b′**), 4-oxo-*cis*-myretanol (**392b′**), (4*R*)-4-hydroxy-*cis*-myrtanol (**393b′**), 5-hydroxy-*cis*-myrtanol (**394b′**), and (1*R*,4*R*,5*S*)-thujane-7,10-diol (**395**). (4*R*)-4-Hydroxy-*cis*-myrtanol (**393b′**) was further converted to 4-oxo-*cis*-myrtanol (**392b′**). In contrast, (+)-*trans*-myrtanol (**388a**) was converted to 3-oxo-*trans*-myrtanol (**396a**), 9-hydroxy-*trans*-myrtanol (**397a**), 4-oxo-*trans*-myrtanic acid (**392a′**), and 5-hydroxy-*trans*-myrtanic acid (**394a′**) (**Scheme 162**).[220]



Scheme 162 Biotransformation of (−)-*trans*-myrtanol (**388a**) and its enantiomer (**388a′**) by *Spodoptera litura* and (−)-*cis*-myrtanol (**388b′**) and (+)-*trans*-myrtanol (**388a**) by *Glomerella cingulata*.

3.19.2.2.8(iii) Pinocarveols (349) **Scheme 163** shows the structures of pinocarveols (**349**). (+)-*trans*-Pinocarveol (**349a′**) was biotransformed by *A. niger* TBUYN-2 via two pathways: in the first pathway, (+)-*trans*-pinocarveol (**349a′**) was metabolized via (+)-pinocarvone (**352′**), (−)-3-isopinanone (**359**), and (+)-2α-hydroxy-3-pinanone (**360b′**) to (+)-2α,5-dihydroxy-3-pinanone (**361b′**). In the second pathway, (+)-*trans*-pinocarveol (**349a′**) was metabolized to epoxide by rearrangement reaction to give 6β-hydroxyfenchol (**362a′**) and 6β,7-dihydroxyfenchol (**363a**).[213] *Spodoptera litura* converted (+)-*trans*-pinocarveol (**349a′**) to (+)-pinocarvone (**352′**) as the major product (**Scheme 163**).[221]

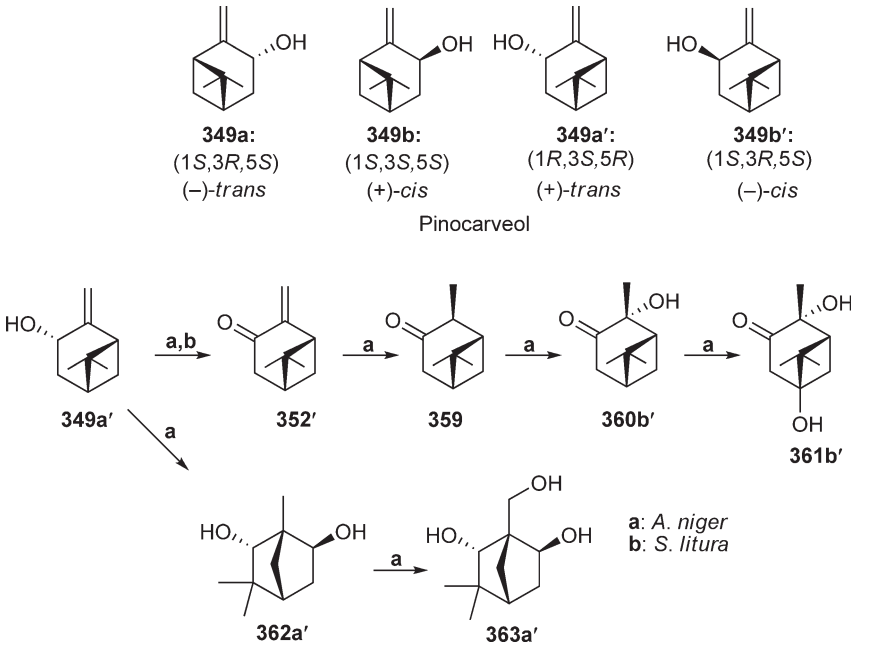
3.19.2.2.8(iv) Pinane-2,3-diol (355a, 355a′, 355b, 355b′) The enantiomers of *trans*- and *cis*-isomers of pinane 2,3-diols (**355a–355b′**) are shown in **Scheme 164**. (−)-Pinane-2α,3α-diol (**355b**) was obtained as one of the metabolites of (−)-β-pinene (**337′**) by *B. cinerea*.[212]

This result led us to study the biotransformation of (−)-pinane-2,3-diol (**355a′**) and (+)-pinane-2,3-diol (**355b**) by *A. niger* TBUYN-2. (−)-Pinane-2,3-diol (**355a′**) was readily biotransformed to (−)-pinane-2,3,5-triol (**365b′**) and (+)-2,5-dihydroxy-3-pinanone (**361b′**) as the major products and (+)-2-hydroxy-3-pinanone (**360b′**) as the minor product.

On the other hand, (+)-pinane-2,3-diol (**355b**) was also readily biotransformed to (+)-pinane-2,3,5-triol (**365a**) and (−)-2,5-dihydroxy-3-pinanone (**361a**) as the major products and (−)-2-hydroxy-3-pinanone (**360a**) as the minor product (**Scheme 164**).[215] *Glomerella cingulata* transformed (−)-pinane-2,3-diol (**355a′**) to a small amount of (+)-2α-hydroxy-3-pinanone (**360b′**, 5%),[222] whereas (+)-pinane-2,3-diol (**355b**) was transformed to a small amount of (−)-2α-hydroxy-3-pinanone (**360a**, 10%) and (−)-3-acetoxy-2α-pinanol (**398b**, 30%) (**Scheme 164**).[223]

3.19.2.2.8(v) Isopinocampheol (364a and 364a′) In **Scheme 165**, the chemical structures of (−)-isopinocampheol (**364a**) and (+)-isopinocampheol (**364a′**) and their related compounds are presented.

Biotransformation of isopinocampheol (3-pinanol) (**364a′**) in 100 bacterial and fungal strains yielded 1-, 2-, 4-, 5-, 7-, 8-, and 9-hydroxyisopinocampheol besides three rearranged monoterpenes, one of them bearing the



Scheme 163 Structures of pinocarveols (**349a, 349a′, 349b, 349b′**), and biotransformation of (+)-*trans*-pinocarveol (**349a′**) by *Aspergillus niger* TBUYN-2 and *Spodoptera litura*.

novel isocarene skeleton. A pronounced enantioselectivity between (−)- (**364a**) and (+)-isopinocampheol (**364a′**) was observed. The phylogenetic position of the individual strains could be seen in their ability to form the products from (+)-isopinocampheol (**364a′**). The formation of 1,3-dihydroxypinane (**401a′**) is a domain of bacteria, while 3,5- (**406a′**) and 3,6-dihydroxypinane (**404a′**) were mainly formed by fungi, especially those of the phylum Zygomycotina. The activity of *Basidiomycotina* toward oxidation of isopinocampheol was rather low. Such information can be used in a more effective selection of strains for screening (**Scheme 165**).[224]

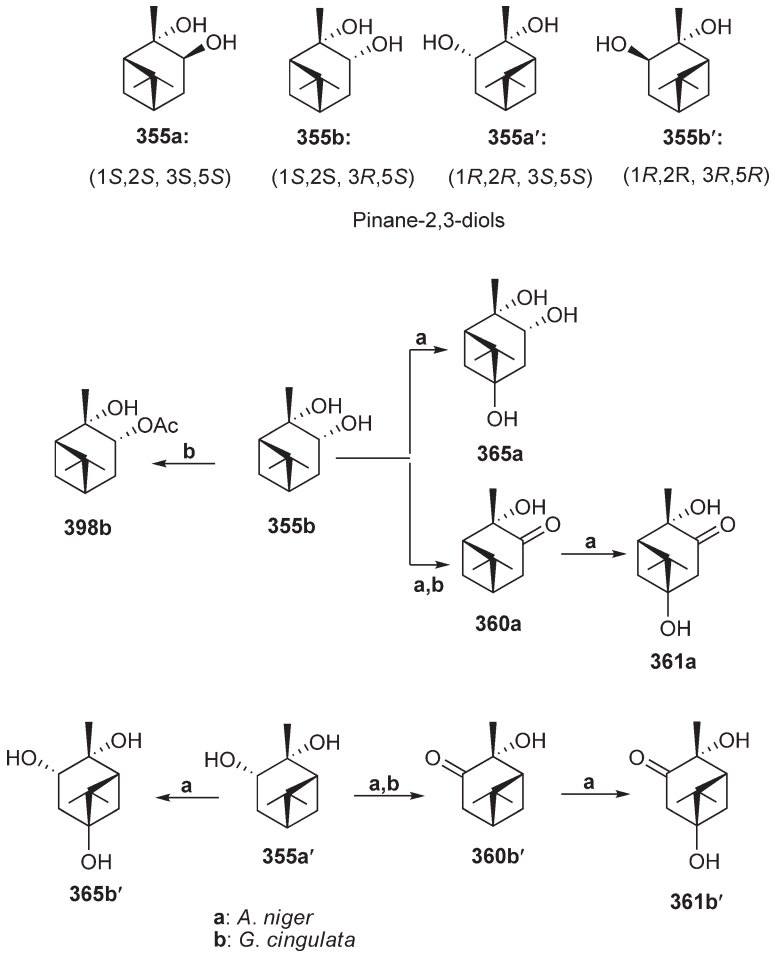
(+)-Isopinocampheol (**364a′**) was metabolized to 4β-hydroxy-(+)-isopinocampheol (**410a′**), 2β-hydroxy-(+)-isopinocampherol acetate (**408a′**), and 2α-methyl,3-(2-methyl-2-hydroxypropyl)-cyclopenta-1β-ol (**409**) (**Scheme 166**).[224]

(−)-Isopinocampheol (**364a**) was converted by *S. litura* to (1*R*,2*S*,3*R*,5*S*)-pinane-2,3-diol (**355a**) and (−)-pinane-3,9-diol (**399a**), whereas (+)-isopinocampheol (**364a′**) was converted to (+)-pinane-3,9-diol (**399a′**) (**Scheme 166**).[225]

(−)-Isopinocampheol (**364a**) was biotransformed by *A. niger* TBUYN-2 to (+)-(1*S*,2*S*,3*S*,5*R*)-pinane-3,5-diol (**404a**, 6.6%), (−)-(1*R*,2*R*,3*R*,5*S*)-pinane-1,3-diol (**401a**, 11.8%), and pinane-2,3-diol (**355a**, 6.6%), whereas (+)-isopinocampheol (**364a′**) was biotransformed by *A. niger* TBUYN-2 to (+)-(1*S*,2*S*,3*S*,5*R*)-pinane-3,5-diol (**405a′**, 6.3%) and (−)-(1*R*,2*R*,3*R*,5*S*)-pinane-1,3-diol (**401a′**, 8.6%) (**Scheme 167**).[226] On the other hand, *G. cingulata* converted (−)- (**364a**) and (+)-isopinocampheol (**364a′**) mainly to (1*R*,2*R*,3*S*,4*S*,5*R*)-3,4-pinanediol (**410a**) and (1*S*,2*S*,3*S*,5*R*,6*R*)-3,6-pinanediol (**404a′**), respectively, together with (**355a**), (**404a**), (**405a′**), and (**399a′**) as the minor products.[225] Some similarities exist between the major metabolites of *G. cingulata* and *R. solani* (**Scheme 167**).[225]

3.19.2.2.8(vi) Borneol (332a and 332a′) and isoborneol (332b and 332b′) (−)-Borneol (**332a′**) was biotransformed by *P. pseudomonalli* strain H to (−)-camphor (**353′**), 6-hydroxycamphor (**412a′**), and 2,6-diketocamphor (**411**) (**Scheme 168**).[227]

Euglena gracilis Z showed enantio- and diastereoselectivity in the biotransformation of (+)- (**332a**), (−)- (**332a′**), and (±)-racemic borneols (an equal mixture of **332a** and **332a′**) and (+)- (**332b**), (−)- (**332b′**), and (±)-isoborneols (an equal mixture of **332b** and **332b′**). The enantio- and diastereoselective dehydrogenation of (−)-borneol (**332a′**) was carried out to give (−)-camphor (**353′**) in ~50% yield.[157,228] The conversion ratio



Scheme 164 Structures of pinane-2,3-diols (**355a, 355a′, 355b, 355b′**), and biotransformation of (+)-pinane-2,3-diol (**355a′**) and (−)-pinane-2,3-diol (**355b**) by *Aspergillus niger* TBUYN-2 and *Glomerella cingulata*.

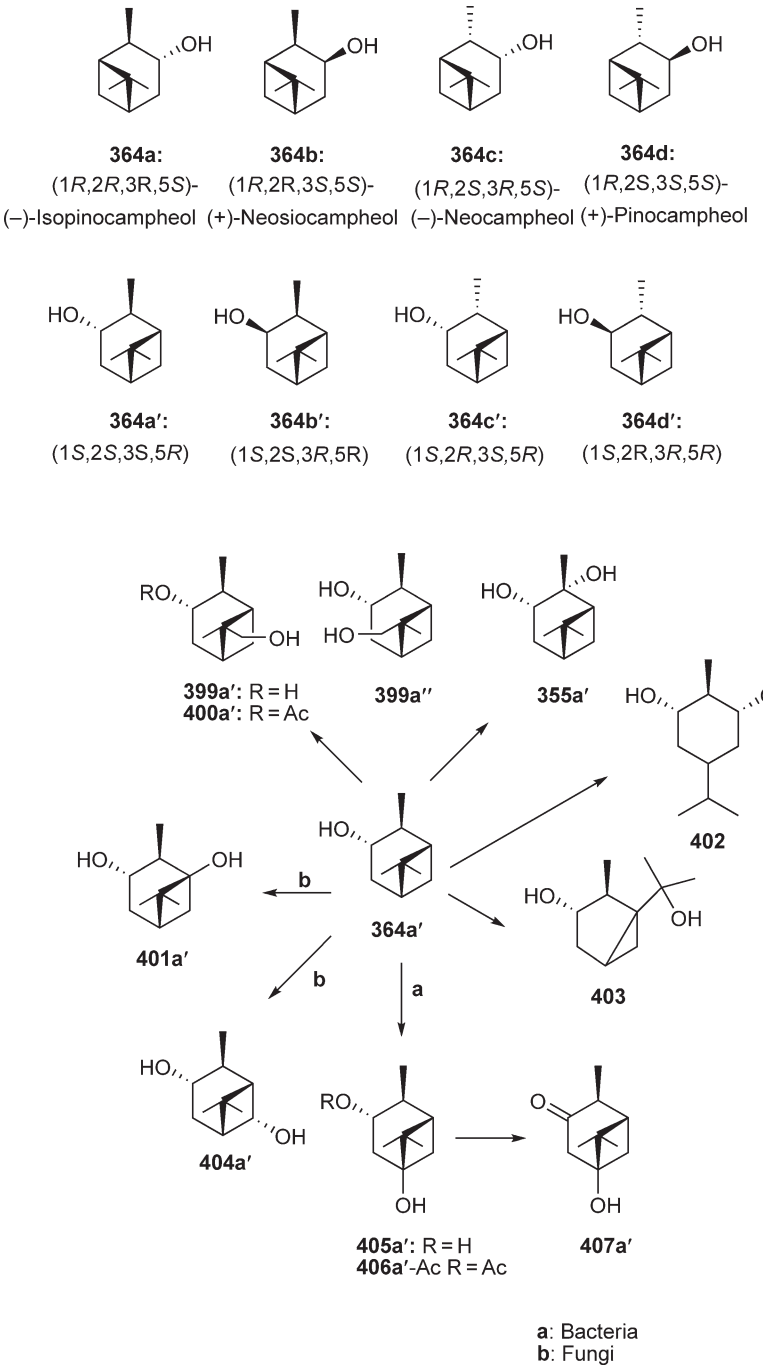
was always ~50% even at different concentrations of (−)-borneol (**332a′**). When (−)-camphor (**353′**) was used as a substrate, it was also converted to (−)-borneol (**332a′**) in 22% yield for 14 days. Furthermore, (+)-camphor (**353**) was also reduced to (+)-borneol (**332a**) in 4 and 18% yield for 7 and 14 days, respectively (**Scheme 169**).[157,228]

(+)- (**332a**) and (−)-Borneols (**332a′**) were biotransformed by *S. litura* to (+)- (**332aa**) and (−)-bornane-2,8-diols (**332a′a′**), respectively (**Scheme 169**).[229]

Glomerella cingulata biotransformed (+)- (**413a**) and (−)-bornyl acetate (**413a′**) to (+)- (**414a**) and (−)-5-β-hydroxybornyl acetate (**414a′**), (+)- (**415a**) and (−)-5-oxo-bornyl acetate (**415a′**), and (+)- (**332a**) and (−)-borneol (**332a′**), respectively (**Scheme 170**).[230]

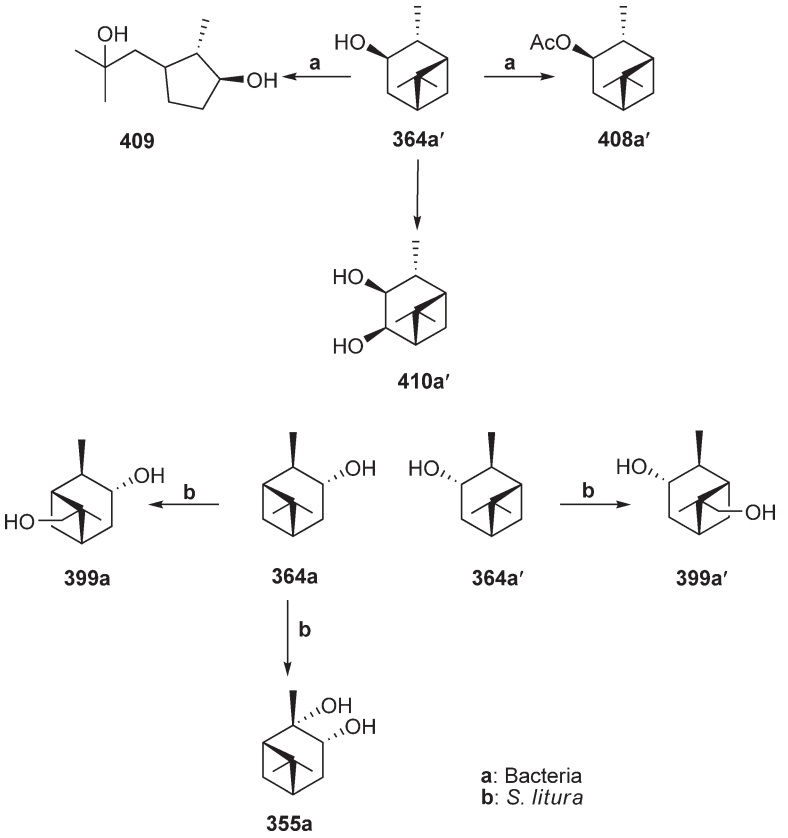
3.19.2.2.8(vii) Fenchol (354a and 354a′) and fenchyl acetate (419a and 419a′) (1*R*,2*R*,4*S*)-(+)-Fenchol (**354a**) was converted by *A. niger* TBUYN-2 to (−)-fenchone (**368′**), (+)-6β-hydroxyfenchol (**362a′**), (+)-5β-hydroxyfenchol (**161b**), and 5α-hydroxyfenchol (**161c**) (**Scheme 171**),[213] while *A. cellulosae* IFO4040 yielded only **368′** from the same substrate. The larvae of common cutworm, *S. litura*, converted (+)-fenchol (**354a**) to (+)-10-hydroxyfenchol (**416a**), (+)-8-hydroxyfenchol (**417a**), (+)-6β-hydroxyfenchol (**362a′**), and (−)-9-hydroxyfenchol (**418a**) (**Scheme 171**).[231]

(+)-*trans*-Pinocarveol (**349a′**), which was formed from (−)-β-pinene (**337′**), was metabolized by *A. niger* TBUYN-2 to 6β-hydroxy-(+)-fenchol (**362a′**) and 6β,7-dihydroxy-(+)-fenchol (**365a′**). (−)-Fenchone (**368′**)

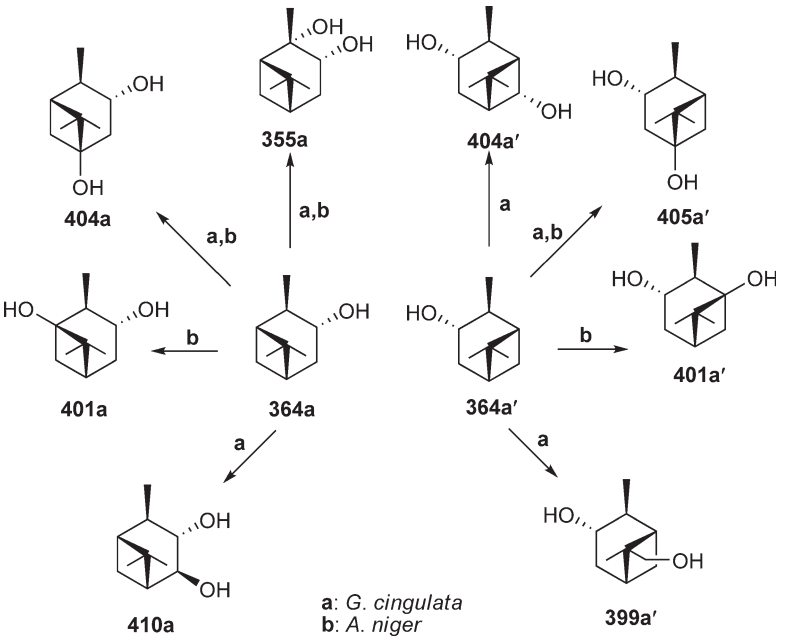


Scheme 165 Structures of (–)-isopinocampheol (**364a**) and its isomers, and metabolic pathways of (+)-isopinocampheol (**364a′**) by microorganisms.

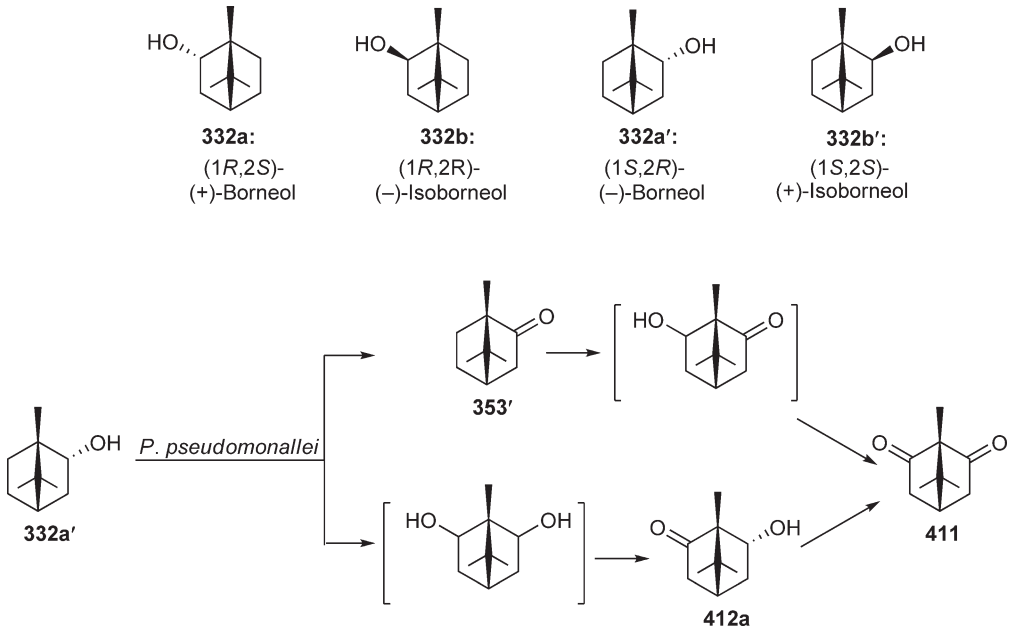
was also metabolized to 6α-hydroxy- (**367b′**) and 6β-hydroxy-(−)-fenchone (**367a′**). (+)-Fenchol (**354a**) was metabolized to 6β-hydroxy-(+)-fenchol (**362a′**) by *A. niger* TBUYN-2. The relationship between the metabolisms of (+)-*trans*-pinocarveol (**349a′**), (−)-fenchone (**368′**), and (+)-fenchol (**354a**) by *A. niger* TBUYN-2 is shown in **Scheme 172**.[213]



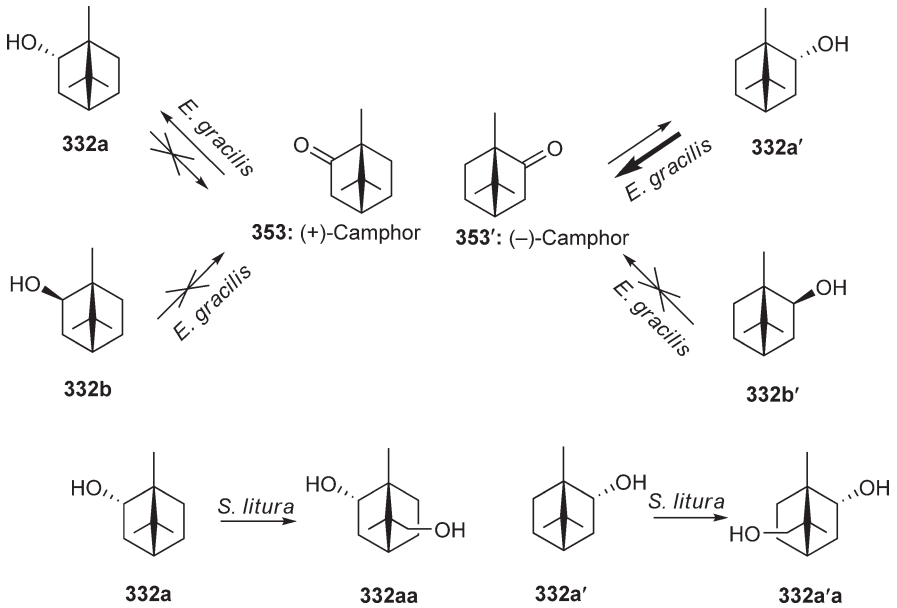
Scheme 166 Metabolic pathways of (−)-pinocampheol (**364d′**) by microorganisms and biotransformation of (−)- (**364a**) and (+)-isopinocampheol (**364a′**) by *Spodoptera litura*.



Scheme 167 Biotransformation of (−)- (**364a**) and (+)-isopinocampheol (**364a′**) by *Aspergillus niger* TBUYN-2 and *Glomerella cingulata*.

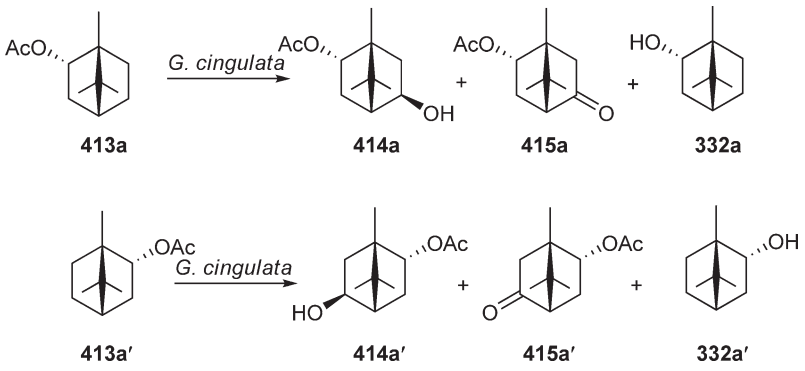


Scheme 168 Structures of (+)-borneol and its isomers, and biotransformation of (–)-borneol (**332a′**) by *Pseudomonas pseudomonallei* strain H.

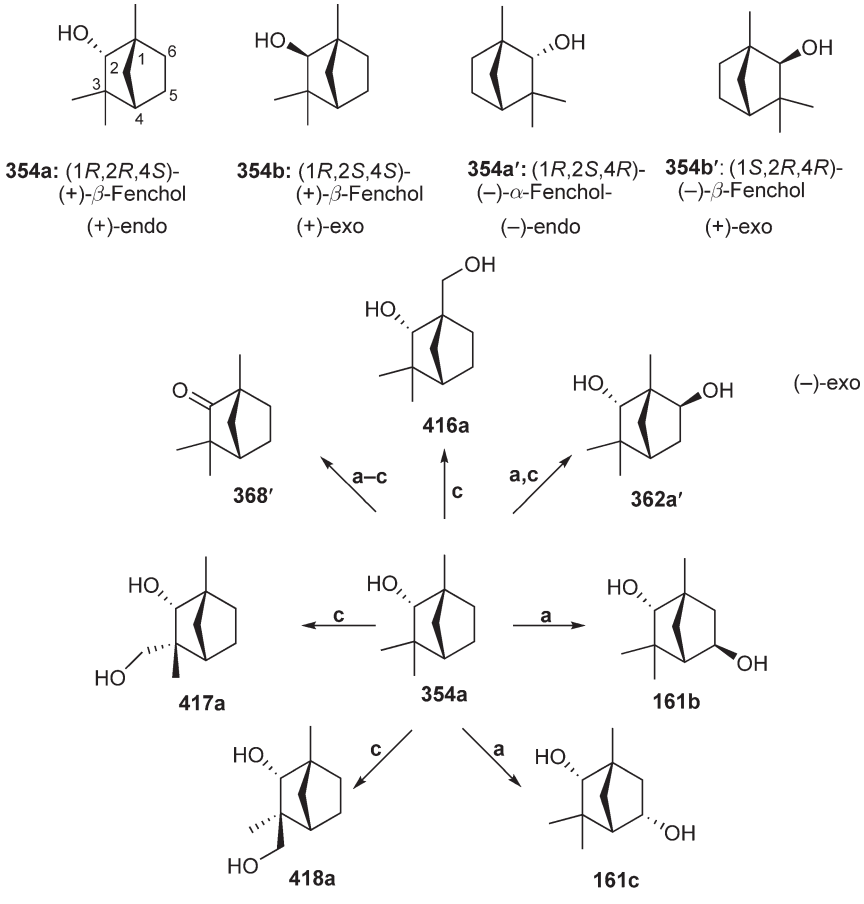


Scheme 169 Enantio- and diastereoselectivity in the biotransformation of (+)- (**332a**) and (–)-borneols (**332a′**) by *Euglena gracilis* Z and *Spodoptera litura.*

(+)-α-Fenchyl acetate (**419a**) was metabolized by *G. cingulata* to (+)-5-β-hydroxy-α-fencyl acetate (**420a**, 50%) as the major metabolite and (+)-fenchol (**354a**, 20%) as the minor metabolite.[232] On the other hand, (–)-α-fenchyl acetate (**419a′**) was metabolized to (–)-5-β-hydroxy-α-fencyl acetate (**420a′**, 70%) as the major metabolite and (–)-fenchol (**354a′**, 10%) as the minor metabolite by *G. cingulata* (**Scheme 172**).[232]

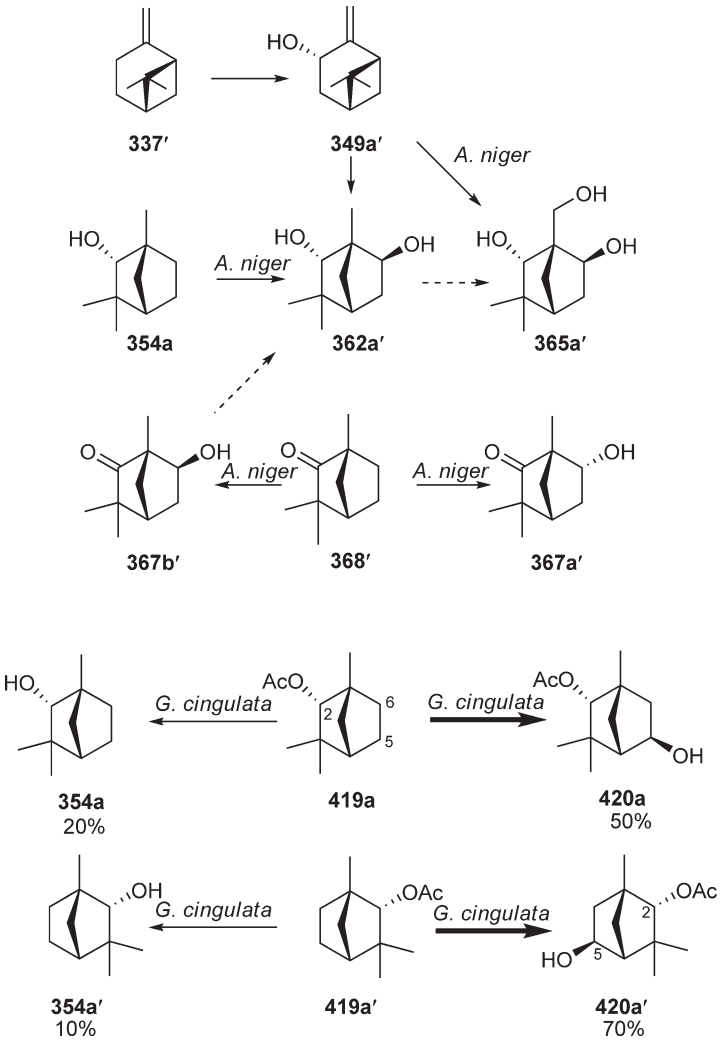


Scheme 170 Biotransformation of (+)- (**413a**) and (−)-bornyl acetate (**413a′**) by *Glomerella cingulata*.

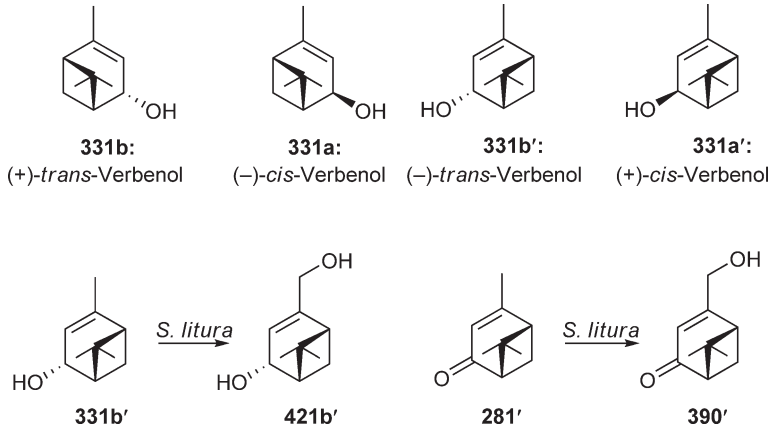


Scheme 171 Structures of (+)-β-fenchol (**354a**) and its isomers, and biotransformation of (+)-β-fenchol (**354a**) by *Aspergillus niger* TBUYN-2, *A. cellulosae* IFO4040, and *Spodoptera litura.*

3.19.2.2.8(viii) **cis-*Verbenol (331a and 331a′) and* trans-*verbenol (331b and 331b′)*** (−)-*trans*-Verbenol (**331b′**) was biotransformed by *S. litura* to 10-hydroxyverbenol (**421b′**). Furthermore, (−)-verbenone (**281′**) was also biotransformed in the same manner to 10-hydroxyverbenone (**390′**) (**Scheme 173**).[233]



Scheme 172 Metabolism of (+)-*trans*-pinocarveol (**349a′**), (−)-fenchone (**368′**), and (+)-fenchol (**354a**) by *Aspergillus niger* TBUYN-2 and (+)- (**419a**) and (−)-α-fencyl acetate (**419a′**) by *Glomerella cingulata*.



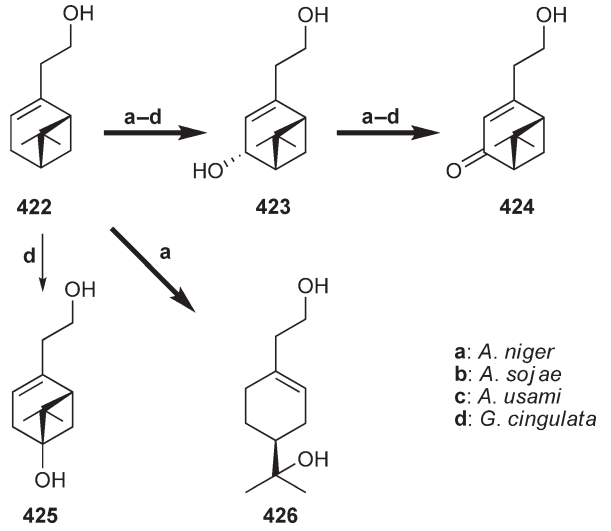
Scheme 173 Structures of (+)-*trans*-verbenol (**331b**) and its isomer, and metabolism of (−)-*trans*-verbenol (**331b′**) and (−)-verbenone (**281′**) by *Spodoptera litura.*

Urine from sawmill workers exposed to α-pinene, β-pinene, and Δ-3-carene was collected and hydrolyzed with β-glucuronidase at pH 5.0 for 24 h at 37 °C. *trans*-Verbenol was detected as a major peak in GC–MS and *cis*-verbenol was also detected.[234]

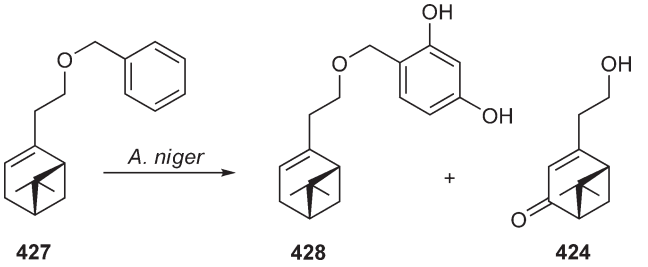
3.19.2.2.8(ix) Nopol (422) and nopol benzyl ether (427) Biotransformation of (−)-nopol (**422**) was carried out at 30 °C for 7 days at the concentration of 100 mg per 200 ml medium by *A. niger* TBUYN-2, *A. sojae* IFO4389, and *A. usami* IFO4338. Incubation of (−)-Nopol (**422**) with *A. niger* TBUYN-2 gave 7-hydroxymethyl-1-*p*-menthen-8-ol (**426**). In the case of *A. sojae* IFO4389 and *A. usami* IFO4338, (−)-nopol (**422**) was metabolized to 4-oxonopol (**424**) as the minor product together with 7-hydroxymethyl-1-*p*-menthen-8-ol (**426**) as the major product. On the other hand, *G. cingulata* biotransformed (−)-nopol (**422**) to (4*R*)-(−)-4-hydroxynopol (**423**), 4-oxonopol (**424**), and 5-hydroxynopol (**425**) (**Scheme 174**).[218,235]

Biotransformation of (−)-nopol benzyl ether (**427**) was carried out at 30 °C for 8–13 days at the concentration of 277 mg per 200 ml medium by *A. niger* TBUYN-2, *A. sojae* IFO4389, and *A. usami* IFO4338. (−)-Nopol benzyl ether (**427**) was biotransformed by *A. niger* TBUYN-2 to 4-oxonopl-2′,4′-dihydroxy benzyl ether (**428**) and (−)-oxonopol (**424**) (**Scheme 175**). 4-Oxonopol-2′,4′-dihydroxybenzyl ether (**428**) shows a strong antioxidative activity (IC_{50} 30.23 µmol l^{-1}). The antioxidative activity of 4-oxonopol-2′,4′-dihydroxybenzyl ether (**428**) is the same as that of butyl hydroxyl anisol (BHA).[218,235]

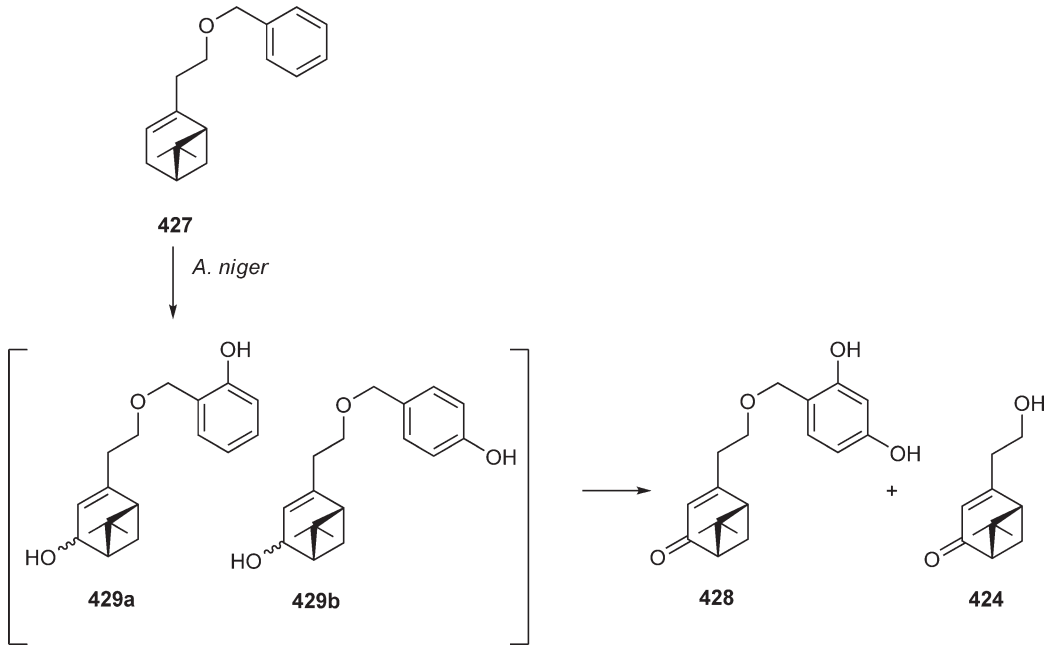
The *Citrus* pathogenic fungus *A. niger* Tiegh (CBAYN) also transformed (−)-nopol (**422**) to (−)-oxonopol (**424**) (**Scheme 174**) and 4-oxonopol-2′,4′-dihydroxybenzyl ether (**428**) through two intermediates (**429a** and **429b**) (**Scheme 176**).[218,235]



Scheme 174 Biotransformation of (−)-nopol (**422**) by *Aspergillus niger* TBUYN-2, *A. sojae* IFO4389, *A. usami* IFO4338, and *Glomerella cingulata*.



Scheme 175 Biotransformation of (−)-nopol benzyl ether (**427**) by *Aspergillus niger* TBUYN-2.



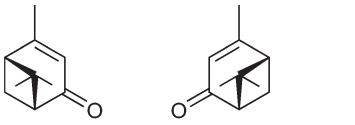
Scheme 176 Proposed metabolic pathways of (−)-nopol benzyl ether (**455′**) by microorganisms.

3.19.2.2.9 Bicyclic monoterpene ketones

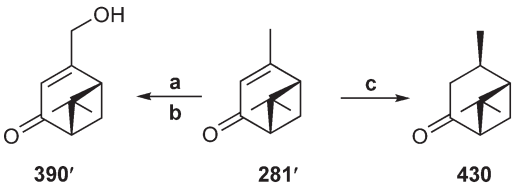
3.19.2.2.9(i) α,β-Unsaturated ketone

3.19.2.2.9(i)(a) Verbenone (281 and 281′) (−)-Verbenone (**281′**) is a component of the essential oil from rosemary species such as *R. officinalis* L., *Verbena triphylla*, and *Eucalyptus globules*, and is used as a spice, a perfume, and to make herbal tea.

(−)-Verbenone (**281′**) was hydrogenated by the reductase of *N. tabacum* to give (−)-isoverbanone (**430**) (**Scheme 177**).[160,236–239] On the other hand, rat and human liver microsomal cytochrome P-450 enzymes converted (−)-verbenone (**281′**) to 10-hydroxyverbenone (**390′**).[240]



281: (+)-Verbenone **281′:** (–)-Verbenone



a: Human CYP2A6, CYP2B6
b: Rat CYP2C11, CYP2B1
c: *N. tabacum* (Verbenone reductase)

Scheme 177 Structures of (+)- (**281**) and (−)-verbenones (**281′**), and hydrogenation of (−)-verbenone (**281′**) to (−)-isoverbanone (**430**) by verbenone reductase of *Nicotiana tabacum* and to (−)-10-hydroxyverbenone (**390′**) by human CYP2A6 (CYP2B6) and rat CYP2C11 (CYP2B1).

3.19.2.2.9(i)(b) Pinocarvone (352 and 352′) *Aspergillus niger* TBUYN-2 transformed (+)-pinocarvone (**352′**) to (−)-isopinocamphone (**359**), 2α-hydroxy-3-pinanone (**360b′**), and 2α,5-dihydroxy-3-pinanone (**361b′**) together with a small amount of 2α,10-dihydroxy-3-pinanone (**366a′**) (**Scheme 178**).[213]

3.19.2.2.9(ii) Saturated ketone

3.19.2.2.9(ii)(a) Camphor (353 and 353′) (+)- (**353**) and (−)-Camphor (**353′**) are found widely in nature, (+)-camphor (**353**) being more abundant. It is the major component of oils obtained from the camphor tree *C. camphora*.[41] The hydroxylation of (+)-camphor (**353**) by *P. putida* C_1 was described.[241] The substrate was hydroxylated exclusively in its 5-exo- (**435a**) and 6-exo- (**412a**) positions.

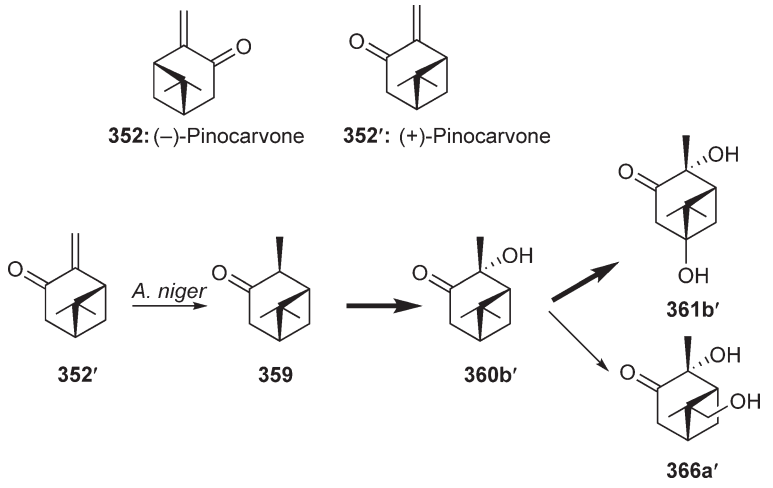
Although only limited success was achieved in understanding the catabolic pathways of (+)-camphor (**353**), the key roles for methylene group hydroxylation and biological Baeyer–Villiger monooxygenases in ring cleavage strategies were established.[242] A degradation pathway of (+)-camphor (**353**) by *P. putida* ATCC 17453 and a soil diphtheroid strain T_1 was proposed.[242]

Metabolic pathway of (+)-camphor (**353**) by microorganisms is shown in **Scheme 179**. (+)-Camphor (**353**) is metabolized to 3-hydroxy- (**432**), 5-hydroxy- (**435a**), 6-hydroxy- (**412a**), and 9-hydroxycamphor (**431a**) and 1,2-campholide (**436**). 6-Hydroxycamphor (**412a**) is degradatively metabolized to 6-oxocamphor (**411**), 4-carboxymethyl-2,3,3-trimethylcyclopentanone (**441**), 4-carboxymethyl-3,5,5-trimethyl-tetrahydro-2--pyrone (**442**), isohydroxycamphoric acid (**446**), isoketocamphoric acid (**447**), and 3,4,4-trimethyl-5-oxo-*trans*-2-hexenoic acid (**448**), whereas 1,2-campholide (**436**) is degradatively metabolized to 6-hydroxy-1,2-campholide (**439**), 6-oxo-1,2-campholide (**440**), 5-carboxymethyl-3,4,4-trimethyl-2-cyclopentenone (**443**), 6-carboxymethyl-4,5,5-trimethyl-5,6-dihydro-2-pyrone (**444**), and 5-carboxymethyl-3,4,4-trimethyl-2-heptene-1,7-dioic acid (**445**). 5-Hydroxycamphor (**435a**) is metabolized to 6-hydroxy-1,2-campholide (**439**), 5-oxocamphor (**438**), and 6-oxo-1,2-campholide (**440**). 3-Hydroxycamphor (**432**) is metabolized to camphorquinone (**433**) and 2-hydroxyepicamphor (**434**) (**Scheme 179**).[243–250]

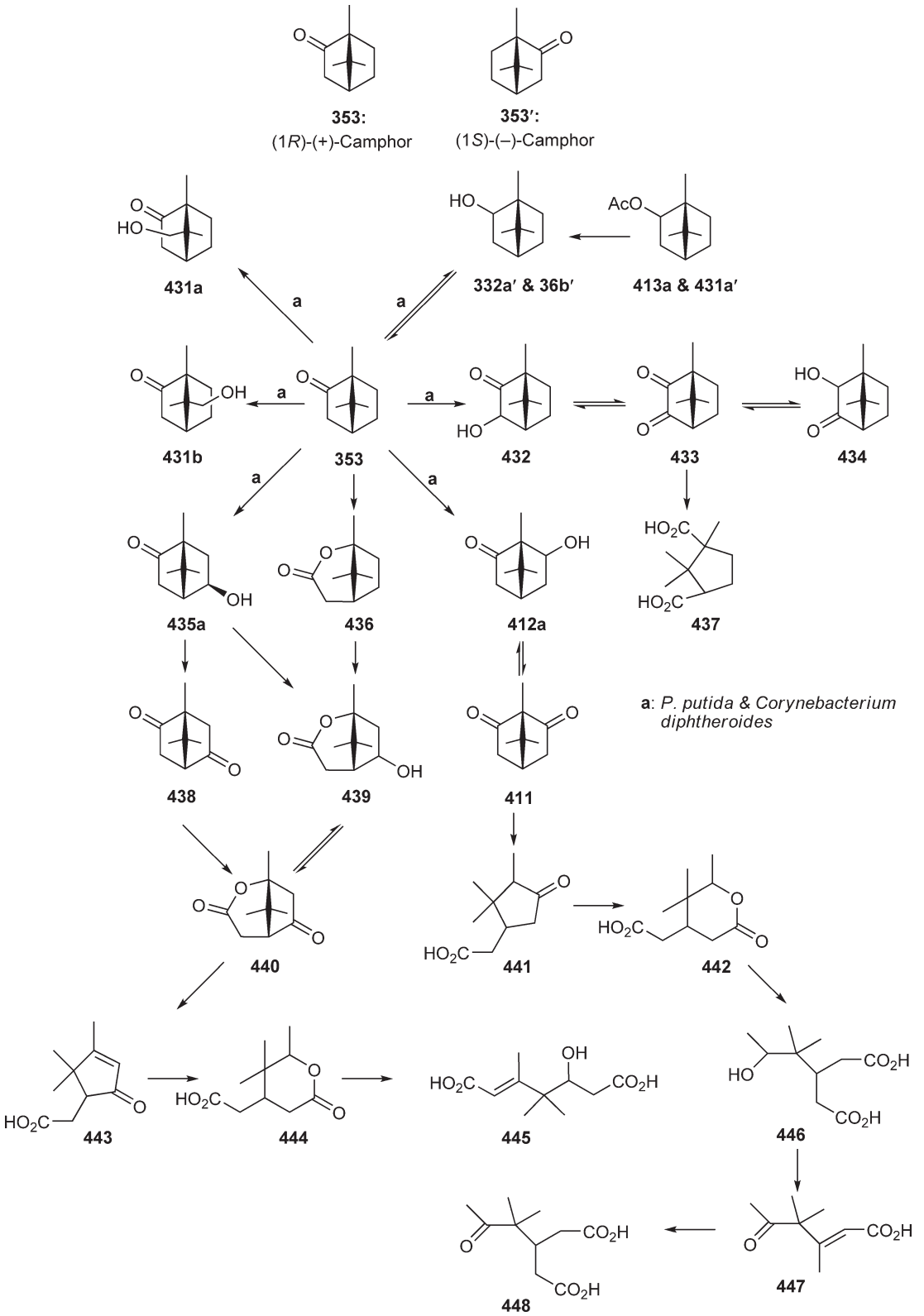
Human CYP2A6 converted (+)-camphor (**353**) and (−)-camphor (**353′**) to 6-*endo*-hydroxycamphor (**412a**) and 5-*exo*-hydroxycamphor (**371b**), while rat CYP2B1 converted (+)-camphor (**353**) to 5-*endo*- (**435a**), 5-*exo*- (**371b**), and 6-*endo*-hydroxycamphor (**412a**) and 8-hydroxycamphor (**431b**) (**Scheme 180**).[251,252]

(+)-Camphor (**353**) was glycosylated by *E. perriniana* suspension cells to 6-*endo*-hydroxycamphor (**412a**) and (+)-camphor monoglycoside (**449**) (**Scheme 180**).[113]

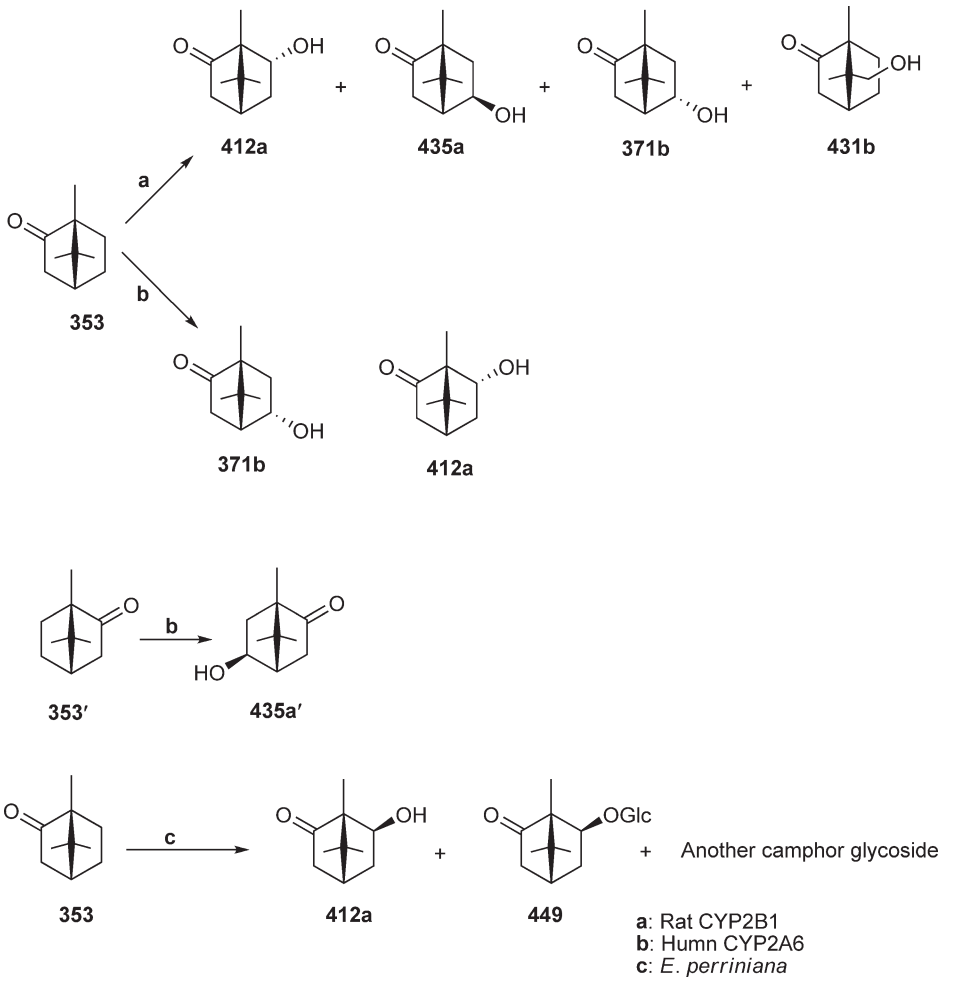
Spodoptera litura larvae hydroxylated (+)-camphor (**353**) to (+)-5β-hydroxycamphor (**435a**), (+)-5α-hydroxycamphor (**435b**), and (+)-8-hydroxycamphor (**431a**), whereas (−)-camphor (**353′**) was converted to (−)-5β-hydroxycamphor (**435a′**), (−)-5α-hydroxycamphor (**435b′**), and (−)-8-hydroxycamphor (**431a′**) (**Scheme 181**).[253]



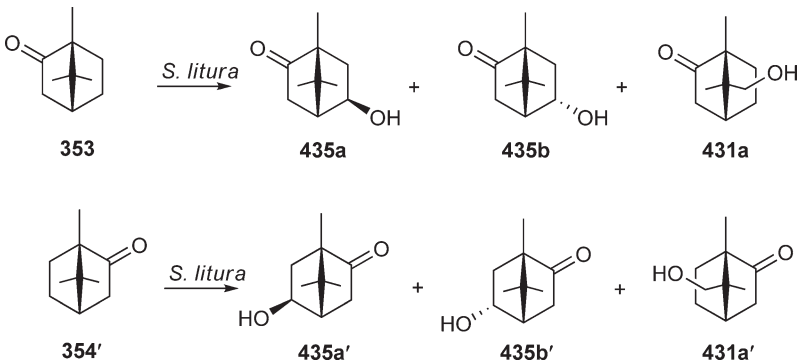
Scheme 178 Structures of (−)- (**352**) and (+)-pinocarvone (**352′**), and biotransformation of (+)-pinocarvone (**352′**) by *Aspergillus niger* TBUYN-2.



Scheme 179 Structures of (+)- (**353**) and (−)-camphor (**353′**), and metabolic pathways of (+)-camphor (**353**) by *Pseudomonas putida* and a soil diphtheroid strain T_1.

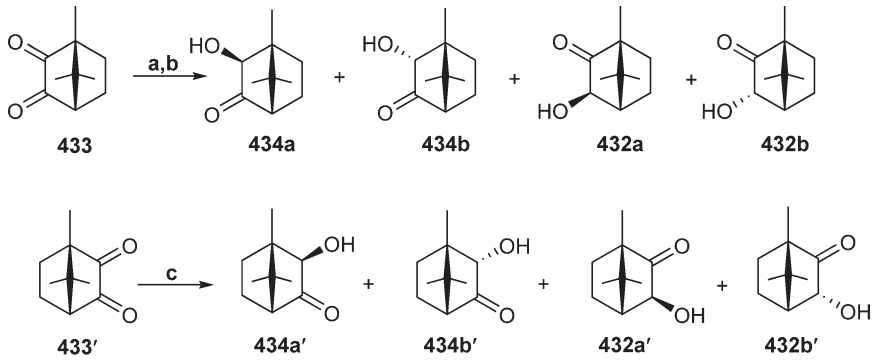


Scheme 180 Biotransformation of (+)-camphor (**353**) by rat P-450 enzyme and suspension cells of *Eucalyptus perriniana* and (+)- (**353**) and (−)-camphors (**353′**) by human P-450 enzymes.



Scheme 181 Biotransformation of (+)- (**353**) and (−)-camphors (**353′**) by *Spodoptera litura*.

3.19.2.2.9(ii)(b) Camphorquinone (433 and 433′) (+)- (**433**) and (−)-Camphorquinones (**433′**) were readily reduced by various fungi. The reduction of (+)-camphorquinone (**433**) by *G. cingulata* and *Mucor mucedo* afforded (−)-(3*S*)-*β*-hydroxycamphor (**434a**) with stereoselectivity, together with its isomers (**432a**, **432b**, and



a: *G. cingulata*
b: *M. mucedo*
c: *A. niger*

Scheme 182 Biotransformation of (+)- (**433′**) and (−)-camphorquinone (**433**) by *Glomerella cingulata, Mucor mucedo*, and *Aspergillus niger.*

Table 17 Reduction of (+)- and (−)-camphorquinones (**433** and **433′**) by various microorganisms

				Product ratio (%)			
Substrates	*Microorganisms*	*Incubation time (h)*	*Yield of products (wt%)*	*434a*	*434b*	*432a*	*432b*
433	*Aspergillus niger*	24	97	51	0	13	33
	Fusarium solani	72	97	28	0	35	34
	Glomerella cingulata	9	99	7	0	70	22
	Mucor mucedo	24	99	14	0	71	14
	Rhizoctoina solani	24	98	8	0	37	53
				434a′	**434b′**	**432a′**	**432b′**
433′	*Aspergillus niger*	24	98	3	80	12	3
	Fusarium solani	72	98	8	26	57	7
	Glomerella cingulata	9	98	17	24	51	6
	Mucor mucedo	24	97	2	30	22	43
	Rhizoctonia solani	24	99	2	33	40	24

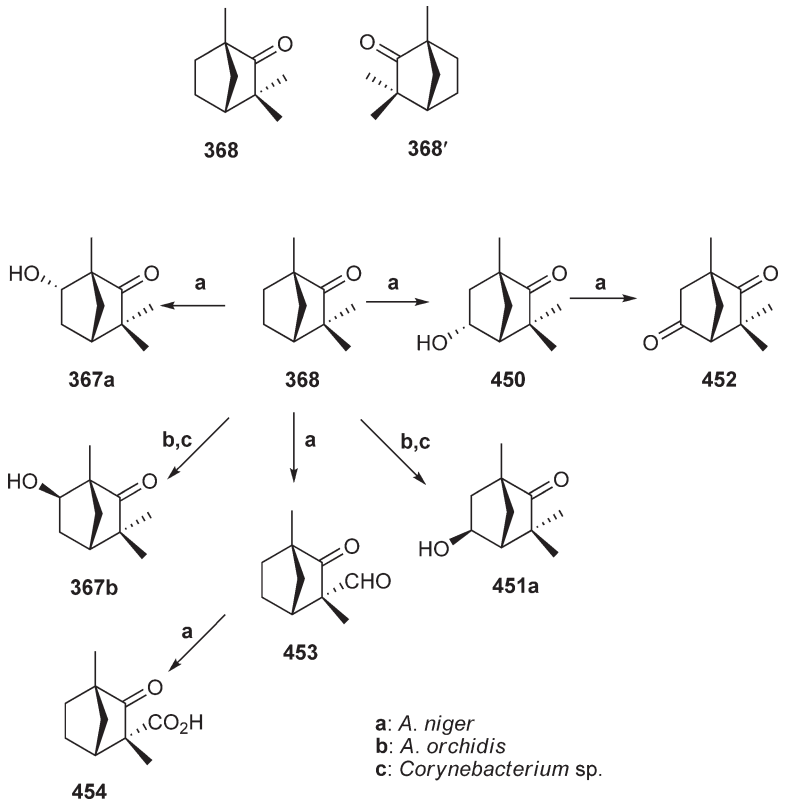
434b) whereas the reduction of (−)-camphorquinone (**433′**) by *A. niger* produced mainly (+)-(2*R*)-β-hydroxyepicamphor (**434a′**) with high stereoselectivity and its related isomers (**432a′**, **432b′**, and **434b′**) (**Scheme 182**, **Table 17**).[254]

3.19.2.2.9(ii)(c) Fenchone (368 and 368′) Incubation of (+)-fenchone (**368**) with *Corynebacterium* sp.[255] and *Absidia orchidis*[256] gave 6β-hydroxy- (**367b**) and 5β-hydroxyfenchones (**451**) (**Scheme 183**). On the other hand, *A. niger* biotransformed (+)-fenchone (**368**) to (+)-6α- (**367a**) and (+)-5α-hydroxyfenchones (**450**),[257,258] 5-oxofenchone (**452**), 9-formylfenchone (**453**), and 9-carboxyfenchone (**454**) (**Scheme 183**).[257,258]

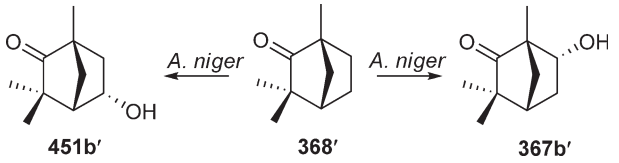
Furthermore, *A. niger* biotransformed (−)-fenchone (**368′**) to 5α- (**451b′**) and 6α-hydroxyfenchones (**367b′**) (**Scheme 184**).[259]

(+)- and (−)-Fenchones (**368** and **368′**) were converted to 6β-hydroxyfenchone (**367b** and **367a′**), 6α-hydroxyfenchone (**367a** and **367b′**), and 10-hydroxyfenchone (**455** and **455′**) by P-450. Of the 11 recombinant human P-450 enzymes tested, CYP2A6 and CYP2B6 catalyzed the oxidation of (+)- (**368**) and (−)-fenchone (**368′**) (**Scheme 185**).[260,261]

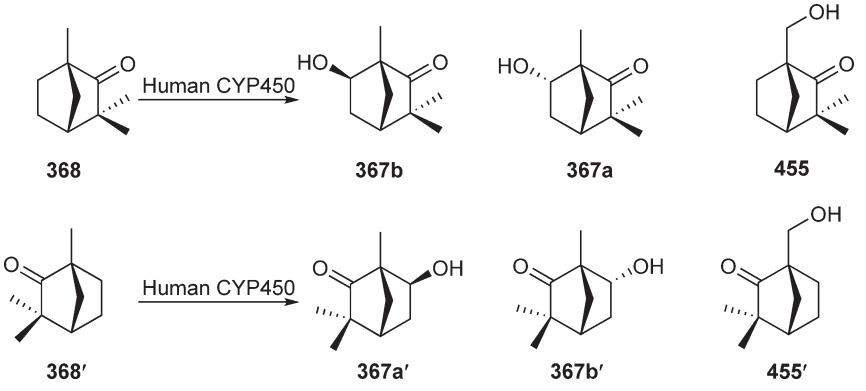
The larvae of *S. litura* biotransformed (+)-fenchone (**368**) to mainly (+)-6β-hydroxyfenchone (**367b**), (+)-6α-hydroxyfenchone (**367a**), (+)-10-hydroxyfenchone (**455**), and (+)-3-oxo-2,2,4-trimethylcyclopentylacetic acid (**456**), together with (+)-5β-hydroxyfenchone (**451a**) as the minor compound. On the other hand, the



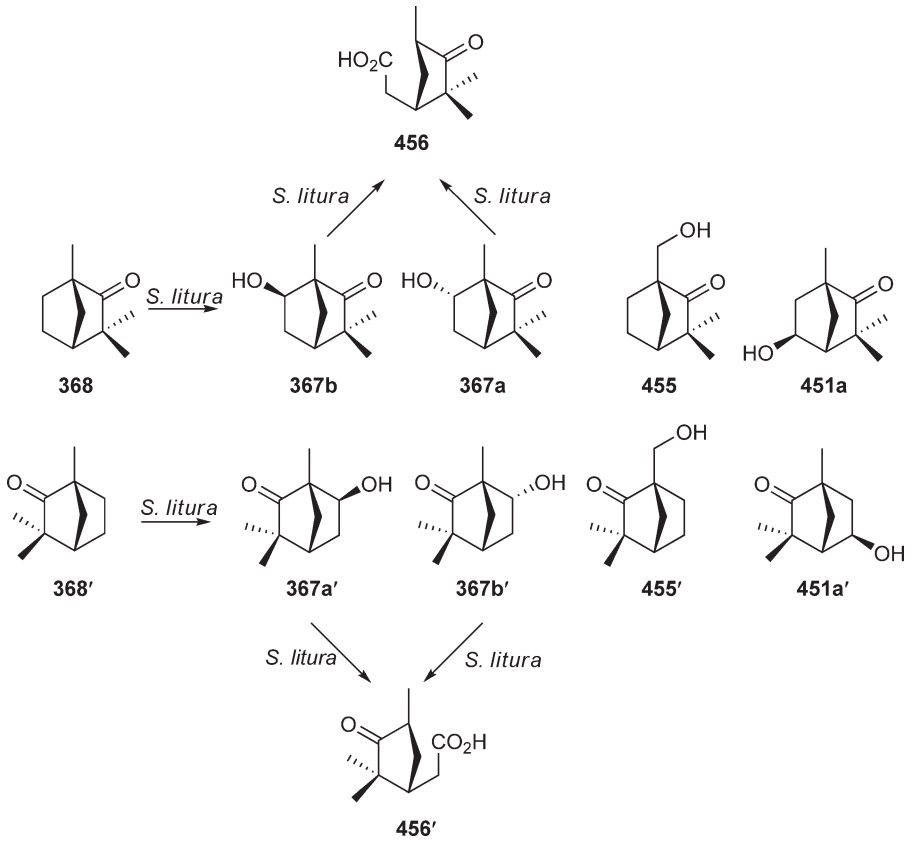
Scheme 183 Structures of (+)- (**368**) and (−)-fenchone (**368′**), and metabolic pathways of (+)-fenchone (**368**) by *Corynebacterium* species, *Absidia orchidis*, and *Aspergillus niger* TBUYN-2.



Scheme 184 Metabolic pathway of (−)-fenchone (**368′**) by *Aspergillus niger* TBUYN-2.



Scheme 185 Biotransformation of (+)- (**368**) and (−)-fenchones (**368′**) by P-450 enzymes.



Scheme 186 Metabolic pathway of (+)- (**368**) and (−)-fenchones (**368′**) by *Spodoptera litura*.

larvae transformed (−)-fenchone (**368′**) to mainly (−)-10-hydroxyfenchone (**455′**), (−)-6β-hydroxyfenchone (**367b′**), and (−)-5 β-hydroxyfenchone (**451a′**), together with (−)-6α-hydroxyfenchone (**367a′**) and (−)-3-oxo-2,2,4-trimethylcyclopentylacetic acid (**456′**) as the minor compounds (**Scheme 186**).[262]

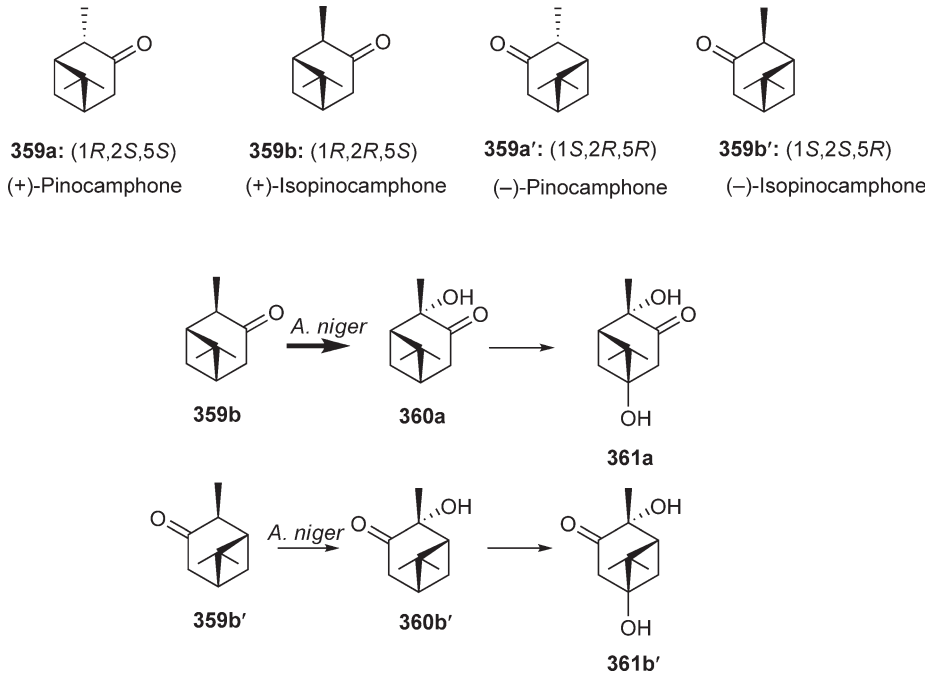
3.19.2.2.9(ii)(d) Pinocamphone (359a and 359a′) and isopinocamphone (359b and 359b′) (+)- (**359b**) and (−)-Isopinocamphone (**359b′**) were biotransformed by *A. niger* to (−)- (**360a**) and (+)-2-hydroxy-3-pinanone (**360b′**) as the major products, respectively, which strongly inhibit the germination of lettuce seeds, and (−)- (**361a**) and (+)-2,5-dihydroxy-3-pinanone (**361b′**) as the minor products, respectively (**Scheme 187**).[215,263]

3.19.2.2.9(ii)(e) 2-Hydroxy-3-pinanone Incubation of (−)-2α-hydroxy-3-pinanone (**360a**) with *A. niger* TBUYN-2 afforded (−)-2α,5-dihydroxy-3-pinanone (**361b**) predominantly, whereas the fungus converted (+)-2α-hydroxy-3-pinanone (**360b′**) mainly to 2α,5-dihydroxy-3-pinanone (**361b′**), 2α,9-dihydroxy-3-pinanone (**366a′**), and (−)-pinane-2 α,3α,5-triol (**365b′**) (**Scheme 188**).[215,263]

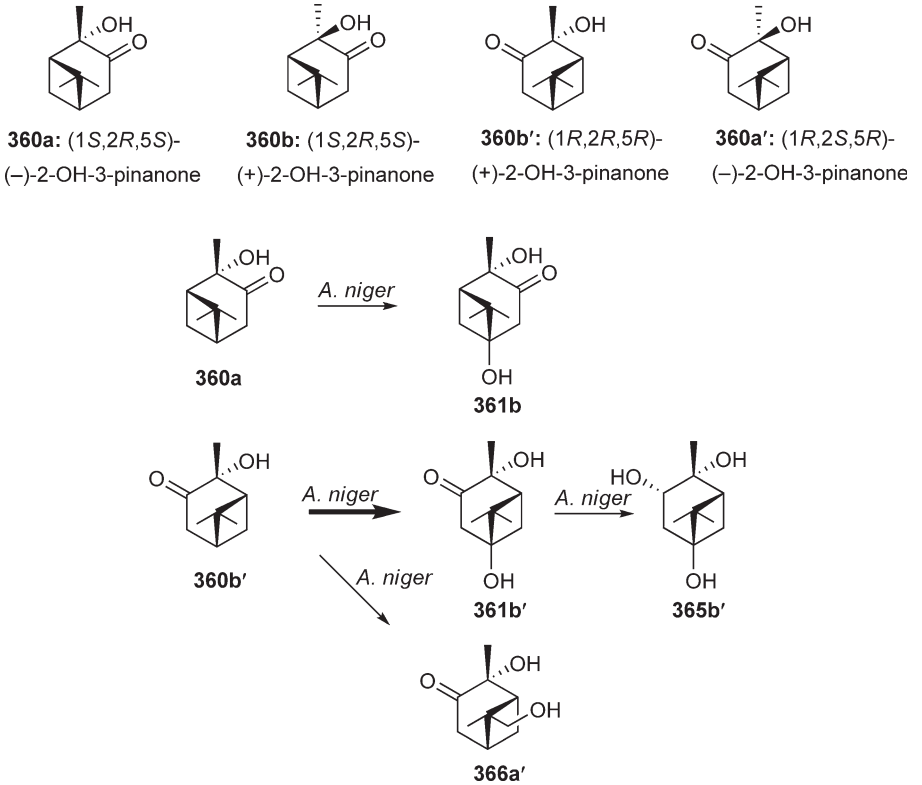
The relationship between the metabolism of β-pinene (**337** and **337′**), isopinocamphone (**359a** and **359b′**), 2α-hydroxy-3-pinanone (**360b** and **360b′**), and pinane-2,3-diol (**355a** and **355a′**) in *A. niger* TBUYN-2 and *B. cinerea* is shown in **Schemes 189** and **190**.

Aspergillus niger TBUYN-2 metabolized (+)-β-pinene (**377**) to pinane-2,3-diol (**355a**), isopinocamphone (**359a**), and 2α-hydroxy-3-pinanone (**360a**), which was further converted to 2α,5-dihydroxy-3-pinanone (**361a**) and 2a,10-dihydroxy-3-pinanone (**366a**) as shown in **Scheme 189**.

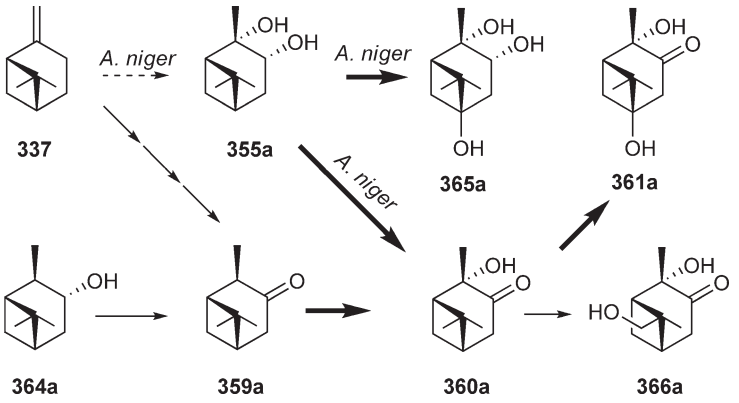
The same metabolic pathyway as mentioned above was observed with (−)-β-pinene (**337′**) using *A. niger* TBUYN-2 and *B. cinerea* to give enantiomers pinane-2,3-diol (**355a′**), isopinocamphone (**359b′**), 2α-hydroxy-3-pinanone (**360a′**), 2α,5-dihydroxy-3-pinanone (**361b′**), and 2α,10-dihydroxy-3-pinanone (**366a′**) as shown in **Scheme 190**.[215,263] Thus, in this case, enantioselectivity of both substrates by the treated fungi has not been observed.



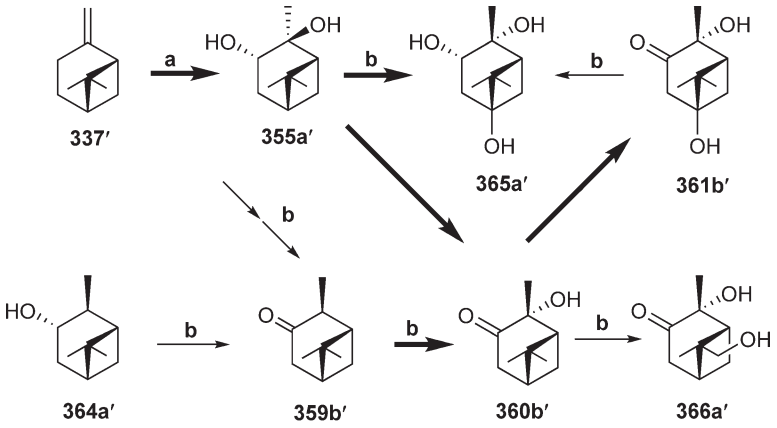
Scheme 187 Structure of (+)-pinocamphone (**359a**) and its isomers, and biotransformation of (+)-isopinocamphone (**359b**) and its enantiomer (**359b′**) by *Aspergillus niger* TBUYN-2.



Scheme 188 Structures of (–)-2-hydroxy-3-pinanone (**360a**) and its isomers, and biotransformation of (–)- (**360b**) and (+)-2-hydroxy-3-pinanone (**360b′**) by *Aspergillus niger* TBUYN-2.



Scheme 189 Relationship between the metabolism of β-pinene (**337**), isopinocamphone (**359a**), 2α-hydroxy-3-pinanone (**360a**), and pinane-2,3-diol (**355a**) by *Aspergillus niger* TBUYN-2.



a: *B. cinerea*
b: *A. niger*

Scheme 190 Relationship between the metabolism of β-pinene (**337′**), isopinocamphone (**359b′**), 2α-hydroxy-3-pinanone (**360b′**), and pinane-2,3-diol (**355a′**) by *Aspergillus niger* TBUYN-2 and *Botrytis cinerea.*

3.19.3 Mosquitocidal and Knockdown Activity

Knockdown and mortality activity toward the mosquito *Culex quinequefasciatus* was carried out for the metabolites of (+)- (**355a**) and (−)-pinane-2,3-diols (**355a′**) and (+)- and (−)-2-hydroxy-3-pinanones (**360b** and **360b′**) by Dr. Radhika Samarasekera, Industrial Technology Institute, Sri Lanka. (−)-2-Hydroxy-3-pinanone (**360b′**) showed mosquito knockdown activity and mosquitocidal activity at the concentration of 1 and 2% (**Table 18**).

3.19.4 Antimicrobial Activity

The microorganisms were refreshed in Mueller Hilton Broth (Merch) at 35–37 °C, and inoculated on Mueller Hinton Agar (Mast Diagnostics, Merseyside, UK) media for the preparation of inoculum. *Escherichia coli* (NRRL B-3008), *P. aeruginosa* (ATCC 27853), *Enterobacter aerogenes* (NRRL 3567), *S. typhimurium* (NRRL B-4420), *Staphylococcus epidermidis* (ATCC 12228), methicillin-resistant *Staphylococcus aureus* (MRSA) (Clinical Isolate,

Table 18 Knockdown and mortality activity toward mosquito

Compounds	*Knockdown (%)*	*Mortality (%)*
(+)-2,5-Dihydroxy-3-pinanone (**361a**, 2%)	27	20
(−)-2,5-Dihydroxy-3-pinanone (**361a′**, 2%)	NT	7
(+)-2-Hydroxy-3-pinanone (**360a**, 2%)	40	33
(−)-2-Hydroxy-3-pinanone (**360a′**, 2%)	100	40
(−)-2-Hydroxy-3-pinanone (**360a′**, 1%)	53	7
(+)-Pinane-2,3,5-triol (**365a**, 2%)	NT	NT
(−)-Pinane-2,3,5-triol (**365a′**, 2%)	13	NT
(+)-Pinane-2,3-diol (**355a**, 2%)	NT	NT
(−)-Pinane-2,3-diol (**355a′**, 2%)	NT	NT

The results are against *Culex quinequefasciatus*. NT, not tested.

Table 19 Biological activity of pinane-2,3,5-triol (**365a** and **365a′**), 2,5-dihydroxy-3-pinanone (**361a** and **361a′**), and 7-hydroxymethyl-1-*p*-menthene-8-ol (**426**) toward MRSA[266]

	MIC (mg ml^{-1})							
	Compounds					*Control*		
Microorganisms	*365a*	*361a′*	*361a*	*365a′*	*426*	*ST1*	*ST2*	*ST3*
Escherichia coli	0.5	0.5	0.25	0.5	0.25	0.007	0.0039	NT
Pseudomonas aeruginosa	0.5	0.125	0.125	0.25	0.25	0.002	0.0078	NT
Enterobacter aerogenes	0.5	0.5	0.25	0.5	1.00	0.007	0.0019	NT
Salmonella typhimurium	0.25	0.125	0.125	0.25	0.25	0.01	0.0019	NT
Candida albicans	0.5	0.125	0.125	0.25	1.00	NT	NT	0.0625
Staphylococcus epidermidis	0.5	0.5	0.25	0.5	1.00	0.002	0.0009	NT
MRSA	0.25	0.125	0.125	0.25	0.125	0.5	0.031	NT

MRSA, methicillin-resistant *Staphylococcus aureus*; NT, not tested; ST1, Ampicillin-Na (Sigma); ST2, chloramphenicol (Sigma); ST3, ketoconazole (Sigma).

Faculty of Medicine, Osmangazi University, Eskisehir, Turkey), and *Candida albicans* (Clinical Isolate, Faculty of Medicine, Osmangazi University, Eskisehir, Turkey) were used as pathogen test microorganisms. Microdilution broth susceptibility assay (R1,R2) was used for the evaluation antimicrobial activity of the samples. Stock solutions were prepared in dimethyl sulfoxide. Dilution series were prepared from 2 mg ml^{-1} in sterile distilled water in micro test tubes from where they were transferred to 96-well microtitre plates. Overnight-grown bacterial and candial suspensions in double-strength Mueller–Hilton broth (Merck) were standardized to ~10^8 CFU ml^{-1} using McFarland No. 0.5 (10^6 CFU ml^{-1} for *C. albicans*). Later, 100 µl of each bacterial suspension was added to each well. The last row containing only the serial dilutions of samples without microorganisms was used as negative control. Sterile distilled water, medium, and microorganisms served as a positive growth control. After incubation at 37 °C for 24 h, the first well without turbidity was determined as the minimal inhibition concentration (MIC); chloramphenicol (Sigma), ampicillin (Sigma), and ketoconazole (Sigma) were used as standard antimicrobial agents (**Table 19**).[264,265]

3.19.5 Microbial Transformation of Terpenoids as Unit Reaction

Microbiological oxidation and reduction patterns of terpenoids and related compounds by fungi belonging to *Aspergillus* spp. containing *A. niger* TBUYN-2 are summarized in **Tables 20** and **21**. Dehydrogenation of secondary alcohols to ketones, hydroxylation of both nonallylic and allylic carbons, oxidation of olefins to form diols and triols via epoxides, reduction of both saturated and α,β-unsaturated ketones, and hydrogenation

Table 20 Microbiological oxidation and reduction patterns of monoterpenoids by *Aspergillus niger* TBUYN-2

Microbiological oxidation		
Oxidation of alcohols	Oxidation of primary alcohols to aldehydes and acids	
	Oxidation of secondary alcohols to ketones	(−)-*trans*-Carveol (**100a′**), (+)-*trans*-carveol (**100a**), (−)-*cis*-carveol (**100b′**), (+)-*cis*-carveol (**100b**), 2α-hydroxy-1,8-cineole (**124b**), 3α-hydroxy-1,8-cineole (**306b**), 3β-hydroxy-1,8-cineole (**306a**)
Oxidation of aldehydes to acids		
Hydroxylation	Hydroxylation of nonallylic carbon	(−)-Isodihydrocarvone (**106b**), (−)-carvotanacetone (**248′**), (+)-carvotanacetone (**248**), *cis*-*p*-menthane (**136**), 1α-hydroxy-*p*-menthane (**137**), 1,8-cineole (**128**), 1,4-cineole (**322**), (+)-fenchone (**368**), (−)-fenchone (**368′**), (−)-menthol (**33b**), (+)-menthol (**33b′**), (−)-neomenthol (**33a′**), (+)-neomenthol (**33a**), (+)-isomenthol (**33c′**)
	Hydroxylation of allylic carbon	(+)-Neodihydrocarveol (**106a′**), (−)-dihydrocarveol (**106b′**), (+)-dihydrocarveol (**106b**), (+)-limonene (**95**), (−)-limonene (**95′**)
Oxidation of olefins	Formation of epoxides and oxides	
	Formation of diols	(+)-Neodihydrocarveol (**106a′**), (+)-dihydrocarveol (**106b**), (−)-dihydrocarveol (**106b′**), (+)-limonene (**98**), (−)-limonene (**98′**)
	Formation of triols	(+)-Neodihydrocarveol (**106a′**)
Lactonization		
Microbiological reduction		
Reduction of aldehydes to alcohols		
Reduction of ketones to alcohols	Reduction of saturated ketones	(+)-Dihydrocarvone (**106a′**), (−)-isodihydrocarvone (**106b**), (+)-carvomenthone (**268a′**), (−)-isocarvomenthone (**264b**)
	Reduction of α,β-unsaturated ketones	
Hydrogenation of olefins	Hydrogenation of olefin conjugated with carbonyl group	(−)-Carvone (**104′**), (+)-carvone (**104**), (−)-carvotanacetone (**248a′**), (+)-carvotanacetone (**248a**)
	Hydrogenation of olefin not conjugated with a carbonyl group	

Table 21 Microbiological oxidation, reduction, and other reaction patterns of monoterpenoids by *Euglena gracilis* Z

Microbiological oxidation		
Oxidation of alcohols	Oxidation of primary alcohols to aldehydes and acids	
	Oxidation of secondary alcohols to ketones	(−)-*trans*-Carveol (**100a′**), (+)-*cis*-carveol (**100b**), (+)-isoborneol (**332b′**)[a]
Oxidation of aldehydes to acids		Myrtenal (**386**), myrtanal (**435**), (−)-perillaldehyde (**117**), *trans*- and *cis*-1,2-dihydroperillaldehydes (**174a** and **174b**), (−)-phellandral (**181**), *trans*- and *cis*-tetrahydroperillaldehydes (**178**), cuminaldehyde (**164**), (+)- and (−)-citronellal (**20** and **20′**)[b]
Hydroxylation	Hydroxylation of nonallylic carbon	
	Hydroxylation of allylic carbon	(+)-Limonene (**95**), (−)-limonene (**95′**)
Oxidation of olefins	Formation of epoxides and oxides	
	Formation of diols	
	Formation of triols	(4*R*)-*trans*-Carveol (**100a′**), (+)- and (−)-neodihydrocarveol (**106a′** and **106a**), (−)-β-pinene (**337′**)
Lactonization		Camphor (**353**)
Microbiological reduction		
Reduction of aldehydes to alcohols	Reduction of terpene aldehydes to terpene alcohols	Myrtenal (**386**), myrtanal (**435**), (−)-perillaldehyde (**117**), *trans*- and *cis*-1,2-dihydroperillaldehydes (**174a** and **174b**), phellandral (**181**), *trans*- and *cis*-1,2-dihydroperillaldehydes (**20** and **20′**), *trans*- and *cis*-tetrahydroperillaldehydes (**178**), cuminaldehyde (**164**), citral (**275** and **276**), (+)-(**261**) and (−)-citronellal (**261′**)
	Reduction of aromatic and related aldehydes to alcohols	
	Reduction of aliphatic aldehydes to alcohols	
Reduction of ketones to alcohols	Reduction of saturated ketones	(+)-Dihydrocarvone (**105a′**), (−)-isodihydrocarvone (**105b**), (+)-carvomenthone (**264a′**), (−)-isocarvomenthone (**264b**), (+)-dihydrocarvone-8,9-epoxides (**110a′**), (+)-isodihydrocarvone-8,9-epoxides (**110b′**), (−)-dihydrocarvone-8,9-epoxides (**110a**)
	Reduction of α,β-unsaturated ketones	
Hydrogenation of olefins	Hydrogenation of olefin conjugated with carbonyl group	(−)-Carvone (**104**), (+)-carvone (**104′**), (−)-carvotanacetone (**248′**), (+)-carvotanacetone (**248**), (−)-carvone-8,9-epoxides (**109′**), (+)-carvone-8,9-epoxides (**109**)
	Hydrogenation of olefin not conjugated with a carbonyl group	
Hydrolysis		
Hydrolysis	Hydrolysis of ester	Geranyl acetate (**22-Ac**), racemic menthyl acetates (**33b-Ac** and **33b′-Ac**), racemic 2α-acetoxy-1,8-cineole (**312** and **312′**), racemic carvomenthol acetates (**248-Ac**)
Hydration		
Hydration	Hydration of C=C bond in isopropenyl group to tertiary alcohol	(+)-Neodihydrocarveol (**106a**), (−)-dihydrocarveol (**106b′**), (+)-isodihydrocarveol (**106c**), (+)-neoisodihydrocarveol (**106d**), (−)-neodihydrocarveol (**106a′**), (+)-dihydrocarveol (**106b**), (−)-Isodihydrocarveol (**106c′**), (−)-neoisodihydrocarveol (**106d′**), *trans*- and *cis*-shisools (**176a** and **176b**)
Isomerization		
Isomerization		Geraniol (**22**), nerol (**25**)

[a] Diastereo- and enantioselective dehydrogenation is observed in carveol, borneol, and isoborneol.
[b] Acids were obtained as minor products.

of olefin conjugated with the carbonyl group were the characteristic features in the biotransformation of terpenoids and related compounds by *Aspergillus* spp.

In conclusion, acyclic, monocyclic, and bicyclic monoterpenoids were biotransformed by using various bacteria, fungi, insect larvae, mammals, and human and rat enzymes to indroduce oxygen atom at their allylic position to afford secondary alcohols and ketones. These reactions proceeded stereo- and regiospecifically. Even at nonactivated carbon atom, oxidation reaction occurs to give secondary, tertiary, or even primary alcohols. These reactions proceed stereo- and regiospecifically. Cultured cells of *Eucalyptus* and *Catharanthus* species led to the production of glycosides of secondary alcohol. Some microalgae such as *Chlorella*, *Dunaliella*, and *E. gracilis*, fungus *Geotrichum candidum*, and bacterial species of the genus *Streptomyces* are also very useful bioreactors for introducing oxygen function to nonactivated carbons and for reducing double bond, ketone, and aldehyde group. Some *Cyanobacterium* species oxidize primary alcohol to aldehyde. Optical resolution of racemic esters and secondary alcohols was observed in *Absidia*, *Bacillus*, *Glomerella*, *Penicillium*, and *Rhizopus* species. *Aspergillus niger, Streptomyces fumidus*, and *Cladosporium* species directly introduced hydroxyl group on benzene ring. Epoxidation in dihydrocarveols was also seen in biotransformation using *S. bottropensis*.

Several metabolites from monoterpenoids showed antimicrobial, mosquitocidal, mosquito repellent, antioxidant, and germination inhibitory activity of plant seeds as well as insect pheromone activity. The present methods are cheap, rapid, and one-step reactions in water and nonhazardous for directly introducing oxygen function on allylic and nonactive carbon atoms and for reducing double bond stereospecifically. They are very useful methods for the production of fragrant components including certain insect pheromones from commercially available cheap natural and unnatural monoterpenoids and related compounds and monoterpenoids from essential oils.

References

1. P. K. Bhattacharyya; B. R. Prema; B. D. Kulkarni; S. K. Pradhan, *Nature* **1960**, *187*, 689–690.
2. W.-R. Abraham; H. M. R. Hoffmann; K. Kieslich; G. Reng; B. Stumpf, In *Enzymes in Organic Synthesis*; R. Porter, S. Clark, Eds.; Ciba Foundation Symposium 111; Pitman Press: London, 1985; pp 146–160.
3. D. Busmann; R. G. Berger, *J. Biotechnol.* **1994**, *37*, 39–43.
4. M. Miyazawa; T. Murata; H. Kameoka, In *Biotransformation of β-Myrcene by Common Cutworm Larvae*, Spodoptera litura *as a Biocatalyst*, Proceedings of the 42nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Masaki, Ed.; Gifu: Japan, 1998; pp 123–125.
5. H. Narushima; T. Omori; Y. Minoda, *6th Proc. Int. Ferment. Symp.* **1980**, *3*, 525 –351.
6. H. Takeuchi; M. Miyazawa, In *Biotransformation of (–)- and (+)-Citronellene by the Larvae of Common Cutworm (*Spodoptera litura*) as Biocatalyst*, Proceedings of the 49th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; M. Hatanaka, Ed.; Fukui: Japan, 2005; pp 426–427.
7. W. Seubert; U. Remberger, *Biochem. Z.* **1963**, *338*, 245–246.
8. W. Seubert; E. Fass; U. Remberger, *Biochem. Z.* **1963**, *338*, 265–275.
9. W. Seubert; E. Fass, *Biochem. Z.* **1964**, *341*, 23–34.
10. W. Seubert; E. Fass, *Biochem. Z.* **1964**, *341*, 35–44.
11. K. M. Madyastha, *Proc. Ind. Acad. Sci. (Chem. Sci.)* **1984**, *93*, 677–686.
12. S. S. Joglekar; R. S. Dhavlikar, *Appl. Microbiol.* **1969**, *18*, 1084–1087.
13. T. Hayashi; H. Takashiba; H. Ueda; C. Tsutsumi, Nippon Nogei Kagaku Kaishi **1967**, 41.254. source: CA 67, no. 79878g.
14. S. G. Cantwell; E. P. Lau; D. S. Watt; R. R. Fall, *J. Bacteriol.* **1978**, *135*, 324–333.
15. J. Babcka; J. Volf; J. Lebeda; P. Czchec, Patent 56-9686b, 1956.
16. K. M. Madyastha; P. K. Bhattacharyya; C. S. Vaidyanathan, *Can. J. Microbiol.* **1977**, *23*, 230–239.
17. J. Rama Devi; P. K. Bhattacharyya, *Indian J. Biochem. Biophys.* **1977**, *14*, 359–363.
18. K. M. Madyastha; V. Renganathan, *Indian J. Biochem. Biophys.* **1983**, *20*, 136–140.
19. Y. Noma; H. Takahashi; Y. Asakawa, *Phytochemistry* **1991**, *30*, 1147–1151.
20. Y. Noma; N. Miki; E. Akehi; E. Manabe; Y. Asakawa, In *Biotransformation of Monoterpenes by Photosynthetic Marine Algae,* Dunaliella tertiolecta, Proceedings of the 35th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Bustugan, Ed.; Nagoya: Japan, 1991; pp 112–114.
21. Y. Noma; E. Akehi; N. Miki; Y. Asakawa, *Phytochemistry* **1992**, *31*, 515–517.
22. H. Nishimura; Y. Noma; J. Mizutani, *Agric. Biol. Chem.* **1982**, *46*, 2601–2604.
23. H. Nishimura; Y. Noma, In *Biotechnology for Improved Foods and Flavors*; G. R. Takeoka, R. Teranishi, P. J. Williams, A. Kobayashi, Eds.; ACS Symposium Series 637, American Chemical Society: Washington, DC, 1996; pp 173–187.
24. Y. Noma; M. Toyota; Y. Asakawa, In *Reduction of Terpene Aldehydes and Epoxidation of Terpene Alcohols by* S. ikutamanensis, *Ya-2-1*, Proceedings of the 30th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Suga, Ed.; Hiroshima: Japan, 1986; pp 204–206.
25. K. M. Madyastha; N. S. R. Krishna Murthy, *Appl. Microbiol. Biotechnol.* **1988**, *28*, 324–329.
26. K. M. Madyastha; N. S. R. Krishna Murthy, *Tetrahedron Lett.* **1988**, *29*, 579–580.

27. A. Rapp; H. Mandery, In *Bioflavour'87. Analysis – Biochemistry – Biotechnology*; P. Schreier, Ed.; Walter de Gruyter and Co.: Berlin, 1988; pp 445–452.
28. P. Brunerie; I. Benda; G. Bock; P. Schreier, In *Bioflavour' 87. Analysis – Biochemistry – Biotechnology*; P. Schreier, Ed.; Walter de Gruyter and Co.: Berlin, 1988; pp 435–444.
29. P. Brunerie; I. Benda; G. Bock; P. Schreier, *Appl. Microbiol. Biotechnol.* **1987**, *27*, 6–10.
30. P. Brunerie; I. Benda; G. Bock; P. Schreier, *Z. Naturforsch.* **1987**, *42c*, 1097–1100.
31. G. Bock; I. Benda; P. Schreier, *Appl. Microbiol. Biotechnol.* **1988**, *27*, 351–357.
32. M. Miyazawa; H. Nankai; H. Kameoka, *Nat. Prod. Lett.* **1996**, *8*, 303–305.
33. J. C. R. Demyttenaere; I. E. I. Koninckx; A. Meersman, In *Flavour Science. Recent Developments*; A. J. Taylor, D. S. Mottram, Eds.; The Royal Society of Chemistry: Cambridge, UK, 1996; pp 105–110.
34. J. C. R. Demyttenaere; M. del Carmen Herrera; N. De Kimpe, *Phytochemistry* **2000**, *55*, 363–373.
35. J. C. R. Demyttenaere; H. L. De Pooter, *Phytochemistry* **1996**, *41*, 1079–1082.
36. J. C. R. Demyttenaere; H. L. De Pooter, *Flavour Fragr. J.* **1998**, *13*, 173–176.
37. H. Hamada; H. Yasumune, In *The Hydroxylation of Monoterpenoids by Plant Cell Biotransformation*, Proceedings of the 39th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Uyehara, Ed.; Utsunomiya: Japan, 1995; pp 375–377.
38. M. Kaji; H. Hamada; T. Furuya, In *Biotransformation of Monoterpenes by* Cyanobacterium *and Plant Suspension Cells*, Proceedings of the 46th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Asakawa, Ed.; Tokushima: Japan, 2002; pp 323–325.
39. H. Hamada; A. Matsumoto; J. Takimura, In *Biotransformation of Acyclic Monoterpenes by Biocatalysts of Plant Cultured Cells and* Cyanobacterium, Proceedings of the 48th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Kajiwara, Ed.; Yamaguchi: Japan, 2004; pp 393–395.
40. H. Takeuchi; M. Miyazawa, In *Biotransformation of Nerol by the Larvae of Common Cutworm (*Spodoptera litura*) as a Biocatalyst*, Proceedings of the 48th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Kajiwara, Ed.; Yamaguchi: Japan, 2004; pp 399–400.
41. K. Bauer; D. Garbe; H. Surburg, Eds., In *Common Fragrance and Flavor Materials: Preparation, Properties and Uses*, 2nd revised ed.; VCH Publishers: New York, **1990**, p 218.
42. T. Murakami; I. Ichimoto; C. Tstsumom, *Nippon Nogei Kagaku Kaishi* **1973**, *47*, 699–703.
43. L. David; H. Veschambre, *Tetrahedron Lett.* **1984**, *25*, 543–546.
44. G. Bock; I. Benda; P. Schreier, *J. Food Sci.* **1986**, *51*, 659–662.
45. W.-R. Abraham; B. Stumpf; H.-A. Arfmann, *J. Essent. Oil Res.* **1990**, *2*, 251–257.
46. J. C. R. Demyttenaere; H. M. Willemen, *Phytochemistry* **1998**, *47*, 1029–1036.
47. M. Miyazawa; K. Yokote; H. Kameoka, In *Biotransformation of Linalool Oxide by Plant Pathogenic Microorganisms,* Glomerella cingulata, Proceedings of the 38th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; M. Ando, Ed.; Niigata: Japan, 1994; pp 101–102.
48. H. Hamada; T. Furuya, In *Hydroxylation of Monoterpenes by Plant Suspension Cells*, Proceedings of the 44th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; H. Nishimura, Ed.; Sapporo: Japan, 2000; pp 167–168.
49. T. Oritani; K. Yamashita, *Agric. Biol. Chem.* **1973**, *37*, 1923–1928.
50. T. Murata; M. Miyazawa, In *Biotransformation of Dihydromyrcenol by Common Cutworm Larvae,* Spodoptera litura *as a Biocatalyst*, Proceedings of the 43rd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Ohga, Ed.; Oita: Japan, 1999; pp 393–394.
51. M. Miyazawa; T. Murata, *J. Oleo Sci.* **2001**, *50*, 921–925.
52. V. Krasnobajew, Terpenoid. In *Biotechnology*; K. Kieslich, Ed.; Verlag Chemie: Weinheim, 1999; Vol. 6a, pp 97–125.
53. R. S. Dhavalikar; P. K. Bhattacharyya, *Indian J. Biochem.* **1966**, *3*, 144–157.
54. R. S. Dhavalikar; P. N. Rangachari; P. K. Bhattacharyya, *Indian J. Biochem.* **1966**, *3*, 158–164.
55. G. Fenaroli, In *Fenaroli's Handbook of Flavor Ingredients*; T. E. Furia, N. Bellanca, Eds.; CRC Press: Cleveland, OH, 1975; Vol. 2, pp 6–563.
56. Y. Noma; M. Toyota; Y. Asakawa, In *Biotransformation of (−)-Carvone and (+)-Carvone by Aspergillus* spp, Annual Meeting of Agricultural and Biological Chemistry, Sapporo: Japan, 1985; p 68.
57. Y. Noma; M. Toyota; Y. Asakawa, In *Biotransformation of Carvone. 6. Biotransformation of (−)-Carvone and (+)-Carvone by a Strain of Aspergillus niger*, Proceedings of the 29th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Fujisawa, Ed.; Mie: Japan, 1985; pp 235–237.
58. K. R. Cadwallander; R. J. Braddock; M. E. Parish; D. P. Higgins, *J. Food Sci.* **1989**, *54*, 1241–1245.
59. K. R. Cadwallader; R. J. Braddock, *Dev. Food Sci.* **1992**, *29*, 571–584.
60. G. Kraidman; B. B. Mukherjee; I. D. Hill, *Bacteriol. Proc.* **1969**, *69*, 63.
61. Y. Noma, In *Aromatic Plants from Asia, Their Chemistry and Application in Food and Therapy*; L. Jiarovetz, N. X. Dung, V. K. Varshney, Eds.; Har Krishan Bhalla & Sons: Dehradun, 2007; pp 169–186.
62. H. J. Bouwmester; J. A. R. Davies; H. Toxopeus, *J. Agric. Food Chem.* **1995**, *43*, 3057–3064.
63. K. Oosterhaven; K. J. Hartmans; J. J. C. Scheffer, *Potato Res.* **1995**, *38*, 219–230.
64. K. Oosterhaven; B. Poolman; E. J. Smid, *Ind. Crops Prod.* **1995**, *4*, 23–31.
65. K. Takagi; Y. Mikami; Y. Minato; I. Yajima; K. Hayashi, Japanese Patent 72-38998, 1972.
66. J. E. Mattison; L. L. McDowell; R. H. Baum, *Bacteriol. Proc.* **1971**, 141.
67. E. R. Bowen, *Proc. Fla. State Hort. Soc.* **1975**, *88*, 304–308.
68. J. Rama Devi; P. K. Bhattacharyya, *J. Indian Chem. Soc.* **1978**, *55*, 1131–1137.
69. W.-R. Abraham; K. Kieslich; H. Reng; B. Stumpf, *3rd European Congress on Biotechnology*; Verlag Chemie: Weinheim, 1984; Vol. 1, pp 245–248.
70. W.-R. Abraham; B. Stumpf; K. Kieslich, *Appl. Microbiol. Biotechnol.* **1986**, *24*, 24–30.
71. Y. Noma; S. Yamasaki; Y. Asakawa, *Phytochemistry* **1992**, *31*, 2725–2727.

72. Y. Noma; Y. Asakawa, In *Biotransformation of (+)-Limonene and Related Compounds by* Citrus *Pathogenic Fungi*, Proceedings of the 50th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Inoue, Ed.; Yokohama: Japan, 2006; pp 431–433.
73. Y. Noma; Y. Asakawa, In *Biotransformation of Limonene and Related Compounds by Newly Isolated Low Temperature Grown* Citrus *Pathogenic Fungi and Red Yeast*, Book of Abstracts of the 38th International Symposium on Essential Oils; J. Novak, C. Franz, Eds.; Graz: Japan, 2007; p 7.
74. Y. Noma; Y. Asakawa, In *Microbial Transformation of Limonene and Related Compounds*, Proceedings of the 51st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Ohta, Ed.; Nagahama: Japan, 2007; pp 299–301.
75. B. B. Mukherjee; G. Kraidman; I. D. Hill, *Appl. Microbiol.* **1973**, *25*, 447–453.
76. H. Hamada; M. Kaji; T. Hirata; T. Furuya, In *Enantioselective Biotransformation of Monoterpenes by* Cyanobacterium, Proceedings of the 47th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Kurata, Ed.; Tokyo: Japan, 2003; pp 162–163.
77. M. Miyazawa; T. Wada; H. Kameoka, *J. Agric. Food Chem.* **1998**, *48*, 300–303.
78. K. Kieslich; W.-R. Abraham; P. Washausen, In *Topics in Flavor Research*; R. G. Berger, S. Nitz, P. Schreier, Eds.; Eichborn: Marzling Hangenham, 1985; pp 405–427.
79. M. Miyazawa; A. Sugie; T. Shimoda, *Drug Metab. Dispos.* **2002**, *30*, 602–607.
80. M. Miyazawa; K. Yokote; H. Kameoka, In *Biotransformation of 2*-Endo-*Hydroxy-1,4-Cineole by Plant Pathogenic Microorganism,* Glomerella cingulata, Proceedings of the 39th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Uyehara, Ed.; Utsunomiya: Japan, 1995; pp 352–353.
81. T. Yawata; M. Ogura; K. Shimoda; S. Izumi; T. Hirata, In *Epoxidation of Monoterpenes by the Peroxidase from the Cultured Cells of* Nicotiana tabacum, Proceedings of the 42nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Masaki, Ed.; Gifu: Japan, 1998; pp 142–144.
82. M. Miyazawa; T. Wada; H. Kameoka, In *Biotransformation of* p-*Menthanes Using Common Cutworm Larvae,* Spodoptera litura *as a Biocatalyst*, Proceedings of the 40th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Kurokawa, Ed.; Saga: Japan, 1996, pp 80–81.
83. Y. Tsukamoto; S. Nonomura; H. Sakai, *Agric. Biol. Chem.* **1975**, *39*, 617–620.
84. Y. Noma; H. Takahashi; T. Asakawa, In *Microbiological Conversion of* p-*Menthane 1. Formation of* p-*Menthane-1,9-Diol from* p-*Menthane by a Strain of* Aspergillus niger, Proceedings of the 34th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Kawamoto, Ed.; Takamatsu: Japan, 1990; pp 253–255.
85. B. L. Hungund; P. K. Bhattacharyya; P. N. Rangachari, *Indian J. Biochem.* **1970**, *7*, 80–81.
86. M. Miyazawa; T. Wada; H. Kameoka, In *Biotransformation of Terpinene, Limonene and* α*-Phellandrene in Common Cutworm Larvae,* Spodoptera litura *Fabricius*, Proceedings of the 39th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Uyehara, Ed.; Utsunomiya: Japan, 1995; pp 362–363.
87. H. Kayahara; T. Hayashi; C. Tatsumi, *J. Ferment. Technol.* **1973**, *51*, 254–259.
88. M. Miyazawa; T. Wada; H. Kameoka, *J. Agric. Food Chem.* **1996**, *44*, 2889–2893.
89. M. Miyazawa; T. Wada, *J. Agric. Food Chem.* **2000**, *48*, 2893–2895.
90. Y. Asakawa; H. Takahashi; M. Toyota; Y. Noma, *Phytochemistry* **1991**, *30*, 3981–3987.
91. Y. Asakawa; M. Toyota; T. Ishida; T. Takemoto, In *Metabolites in Rabbit Urine after Terpenoid Administration*, Proceedings of the 27th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; H. Irie, Ed.; Nagasaki: Japan, 1983; pp 254–256.
92. K. M. Madyastha; P. K. Bhattacharyya, *Indian J. Biochem.* **1968**, *5*, 161–167.
93. K. Yamada; S. Horiguchi; J. Tatahashi, *Agric. Biol. Chem.* **1965**, *29*, 943–948.
94. Y. Noma, unpublished data, 2000.
95. F. Demirci; H. Berber; K. H. C. Baser, In *Biotransformation of p-Cymene to Thymoquinone*, Book of Abstracts of the 38th International Symposium on Essential Oils; J. Novak, C. Franz, Eds.; Graz: Austria, 2007; p 6.
96. Y. Noma; K. Matsueda; I. Maruyama; Y. Asakawa, In *Biotransformation of Terpenoids and Related Compounds by* Chlorella *Species*, Proceedings of the 41st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Ito, Ed.; Morioka: Japan, 1997; pp 227–229.
97. Y. Noma; M. Miyazawa; K. Yamamoto; H. Kameoka; T. Inagaki; H. Sakai, In *Microbiological Conversion of Perillaldehyde. Biotransformation of* l- *and* dl-*Perillaldehyde by* Streptomyces ikutamanensis*, Ya-2-1*, Proceedings of the 28th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; H. Kotake, Ed.; Kanagawa: Japan, 1984; pp 174–176.
98. T. Ishida; Y. Asakawa; T. Takemoto, *Res. Bull. Hiroshima Inst. Technol.* **1981**, *15*, 79–91.
99. L. Janssens; H. L. De Pooter; N. M. Schamp; E. J. Vandamme, *Process Biochem.* **1992**, *27*, 195–215.
100. Y. Watanabe; T. Inagaki, Japanese Patent 77.12.989. No. 187696x, 1977.
101. Y. Watanabe; T. Inagaki, Japanese Patent 77.122.690. No. 87656g, 1977.
102. T. Moroe; S. Hattori; A. Komatsu; Y. Yamaguchi, Japanese Patent 2.036.875. No. 98195t, 1971.
103. Y. Yamaguchi; A. Komatsu; T. Moroe, *J. Agric. Chem. Soc. Jpn.* **1977**, *51*, 411–416.
104. T. Oritani; K. Yamashita, *Agric. Biol. Chem.* **1973**, *37*, 1695–1700.
105. T. Omata; N. Iwamoto; T. Kimura; A. Tanaka; S. Fukui, *Appl. Microbiol. Biotechnol.* **1981**, *11*, 119–204.
106. O. P. Shukla; R. C. Bartholomeus; I. C. Gunsalus, *Can. J. Microbiol.* **1987**, *33*, 489–497.
107. Au. Atta-ur-Rahman; M. Yaqoob; A. Farooq; S. Anjum; F. Asif; M. I. Choudhary, *J. Nat. Prod.* **1998**, *61*, 1340–1342.
108. Y. Noma; Y. Asakawa, In *Aspergillus spp.: Biotransformation of Terpenoids and Related Compounds*, Biotechnology in Agriculture and Forestry, Medicinal and Aromatic Plants VIII; Y. P. S. Bajaj, Ed.; Springer: Berlin, 1995; pp 62–96.
109. H. Takahashi; Y. Noma; M. Toyota; Y. Asakawa, *Phytochemistry* **1994**, *35*, 1465–1467.
110. M. Miyazawa; H. Kawazoe; M. Hyakumachi, *J. Chem. Technol. Biotechnol.* **2003**, *78*, 620–625.
111. Y. Noma; H. Takahashi; Y. Asakawa, In *Microbiological Conversion of Menthol. Biotransformation of (+)-Menthol by a Strain of* Aspergillus niger, Proceedings of the 33rd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; A. Yoshikoshi, Ed.; Sendai: Japan, 1989; pp 124–126.
112. M. Miyazawa; S. Kumagae; H. Kameoka, *J. Agric. Food Chem.* **1999**, *47*, 3938–3940.

113. H. Hamada; Y. Kondo; M. Kaji; T. Furuta, In *Glycosylation of Monoterpenes by Plant Suspension Cells*, Proceedings of the 46th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Asakawa, Ed.; Tokushima: Japan, 2002; pp 321–322.
114. K. Nakanishi; M. Miyazawa, In *Biotransformation of (+)- and (–)- Menthol by liver Microsomal Humans and Rats*, Proceedings of the 49th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; M. Hatanaka, Ed.; Fukui: Japan, 2005; pp 423–425.
115. M. Ohsawa; M. Miyazawa, In *Biotransformation of (+)- and (–)-Isopulegol by the Larvae of Common Cutworm (*Spodoptera litura*) as a Biocatalyst*, Proceedings of the 45th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Hirai, Ed.; Toyama: Japan, 2001; pp 375–376.
116. T. Oritani; K. Yamashita, *Agric. Biol. Chem.* **1973**, *37*, 1687–1689.
117. T. Hayashi; S. Uedono; C. Tatsumi, *Agric. Biol. Chem.* **1972**, *36*, 690–691.
118. H. Hamada; T. Harada; T. Furuya, In *Hydroxylation of Monoterpenes by Algae and Plant Suspension Cells*, Proceedings of the 45th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Hirai, Ed.; Toyama: Japan, 2001; pp 366–368.
119. M. Miyazawa; S. Kumagae, *J. Agric. Food Chem.* **2001**, *49*, 4312–4314.
120. Y. Noma; M. Toyota; Y. Asakawa, In *Microbial Transformation of Thymol Formation of 2-Hydroxy-3-p-Menthen-5-One by* Streptomyces humidus*, Tu-1*, Proceedings of the 28th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; H. Kotake, Ed.; Kanagawa: Japan, 1984; pp 177–179.
121. E. M. Chamberlain; S. Dagley, *Biochem. J.* **1968**, *110*, 755–763.
122. F. Demirci; N. Kirimer; B. Demirci; Y. Noma; K. H. C. Baser, *The Biotransformation of Thymol Methyl Ether by Different Fungi*, XII Biotechnology Congress, Book of Abstracts; **2001**, p 47.
123. B. Schwammle; E. Winkelhausen; S. Kuzmanova; W. Steiner, *Food Technol. Biotechnol.* **2001**, *39*, 341–345.
124. L. T. Ausgulen; E. Solheim; R. R. Scheline, *Pharmacol. Toxicol.* **1987**, *61*, 98–102.
125. F. Demirci, Microbial Transformation of Bioactive Monoterpenes. Ph.D. Thesis, Anadolu University, Eskisehir, Turkey, **2000**, pp 1–137.
126. Y. Noma, *Nippon Nogeikagaku Kaishi* **1977**, *51*, 463–470.
127. Y. Noma, *Agric. Biol. Chem.* **1980**, *44*, 807–812.
128. H. Nishimura; S. Hiramoto; J. Mizutani, In *Biological Activity of Bottrospicatol and Related Compounds Produced by Microbial Transformation of (–)-*cis*-Carveol Towards Plants*, Proceedings of the 27th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; H. Irie, Ed.; Nagasaki: Japan, 1983; pp 107–109.
129. Y. Noma; H. Nishimura; S. Hiramoto; M. Iwami; C. Tstsumi, *Agric. Biol. Chem.* **1982**, *46*, 2871–2872.
130. Y. Noma; M. Iwami, *Bull. Tokushima Bunri Univ.* **1994**, *47*, 99–110.
131. Y. Noma; H. Nishimura, In *Microbiological Conversion of Carveol. Biotransformation of (–)-*Cis*-Carveol and (+)-*Cis*-Carveol by* S. bottropensis*, Sy-2-1*, Proceedings of the 28th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; H. Kotake, Ed.; Kanagawa: Japan, 1984; pp 171–173.
132. Y. Noma; H. Nishimura, *Agric. Biol. Chem.* **1987**, *51*, 1845–1849.
133. Y. Noma; H. Nishimura, In *Biotransformation of Carvone. 4. Biotransformation of (+)-Carvone by* Streptomyces bottropensis*, SY-2-1*, Proceedings of the 26th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; M. Funamizu, Ed.; Yamagata: Japan, 1982; pp 156–159.
134. Y. Noma; H. Nishimura; C. Tatsumi, In *Biotransformation of Carveol by Actinomycetes. 1. Biotransformation of (–)-Cis-Carveol and (–)-*Trans*-Carveol by* Streptomyces bottropensis*, SY-2-1*, Proceedings of the 24th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; M. Kikuchi, Ed.; Koriyama: Japan, 1980; pp 67–70.
135. Y. Noma; Y. Asakawa, *Phytochemistry* **1992**, *31*, 2009–2011.
136. Y. Noma; H. Takahashi; Y. Asakawa, In *Formation of 8 Kinds of* p*-Menthane-2,8-Diols from Carvone and Related Compounds by* Euglena gracilis *Z. Biotransformation of Monoterpenes by Photosynthetic Microorganisms. Part VIII*, Proceedings of the 37th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Yogi, Ed.; Okinawa: Japan, 1993; pp 23–25.
137. F. Demirci; Y. Noma; N. Kirimer; K. H. C. Baser, *Z. Naturforsch.* **2004**, *59c*, 389–392.
138. Y. Noma, In *Formation of p-menthane-2,8-diols from (–)-Dihydrocarveol and (+)-dihydrocarveol by* Aspergillus *spp*, The Meeting of Kansai Division of The Agricultural and Chemical Society of Japan, Kagawa, Japan, 1988; p. 28.
139. Y. Noma, *Kagaku to Seibutsu* **1984**, *22*, 742–746.
140. N. Yonemoto; S. Sakamoto; T. Furuya; H. Hamada, In *Preparation of (–)-Perillyl Alcohol Oligosaccharides*, Proceedings of the 49th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; M. Hatanaka, Ed.; Fukui: Japan, 2005; pp 108–110.
141. Y. Noma; S. Nonomura; H. Sakai, *Agric. Biol. Chem.* **1974**, *38*, 1637–1642.
142. T. Oritani; K. Yamashita, *Agric. Biol. Chem.* **1973**, *37*, 1691–1694.
143. Y. Noma; C. Tatsumi, *Nippon Nogeikagaku Kaishi* **1973**, *47*, 705–711.
144. Y. Noma; S. Nonomura; H. Ueda; C. Tatsumi, *Agric. Biol. Chem.* **1974**, *38*, 735–740.
145. Y. Noma; S. Nonomura, *Agric. Biol. Chem.* **1974**, *38*, 741–744.
146. Y. Noma, *Ann. Res. Stud. Osaka Joshigakuen Jr. Coll.* **1976**, *20*, 33–47.
147. Y. Noma, *Nippon Nogeikagaku Kaishi* **1979**, *53*, 35–39.
148. Y. Noma, *Ann. Res. Stud. Osaka Joshigakuen Jr. Coll.* **1979**, *23*, 27–31.
149. Y. Noma; S. Nonomura; H. Sakai, *Agric. Biol. Chem.* **1975**, *39*, 437–441.
150. T. Gondai; M. Shimoda; T. Hirata, In *Asymmetric Reduction of Enone Compounds by* Chlorella miniata, Proceedings of the 43rd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Ohga, Ed.; Nagasaki: Japan, 1999; pp 217–219.
151. A. A. Verstegen-Haaksma; H. J. Swarts; B. J. M. Jansen; A. de Groot; N. Bottema-MacGillavry; B. Witholt, *Ind. Crops Prod.* **1995**, *4*, 15–21.
152. Y. Noma; H. Nishimura, In *Biotransformation of (–)-Carvone and (+)-Carvone by* S. ikutamanensis *Ya-2-1*, Annual Meeting of Agricultural and Biological Chemical Society, Book of Abstracts; 1983; p 390.

153. Y. Noma; H. Nishimura, In *Biotransformation of Carvone. 5. Microbiological Transformation of Dihydrocarvones and Dihydrocarveols*, Proceedings of the 27th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; H. Irie, Ed.; Nagasaki: Japan, 1983; pp 302–305.
154. Y. Noma; H. Sakai, *Ann. Res. Stud. Osaka Joshigakuen Jr. Coll.* **1984**, *28*, 7–18.
155. Y. Noma; Y. Asakawa, In *New Metabolic Pathways of (+)-Carvone by* Citrus *Pathogenic* Aspergillus niger *Tiegh CBAYN and* A. niger *TBUYN-2*, Proceedings of the 52nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; W. Okazaki, Ed.; Gunma: Japan, 2008; pp 206–208.
156. Y. Noma; T. Higata; T. Hirata; Y. Tanaka; T. Hashimoto; Y. Asakawa, In *Biotransformation of [6-^{2}H]-(−)-Carvone by* Aspergillus niger, Euglena gracilis *Z and* Dunaliella tertiolecta, Proceedings of the 39th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Uyehara, Ed.; Utsunomiya: Japan, 1995; pp 367–368.
157. Y. Noma; Y. Asakawa, In Euglena gracilis *Z. Biotransformation of Terpenoids and Related Compounds*, Proceedings of the Biotechnology in Agriculture and Forestry, Medicinal and Aromatic Plants X; Y. P. S. Bajaj, Ed.; Springer: Berlin, 1998; pp 194–237.
158. T. Suga; T. Hirata; H. Hamada, *Bull. Chem. Soc. Jpn.* **1986**, *59*, 2865–2867.
159. Y. Noma; S. Nonomura; H. Ueda; H. Sakai; C. Tsutsumi, In *Microbial Transformation of Carvone*, Proceedings of the 18th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Suga, Ed.; Chiba: Japan, 1974; pp 20–23.
160. K. Shimoda; T. Hirata; Y. Noma, *Phytochemistry* **1998**, *49*, 49–53.
161. H. Hamada; T. Furuya; N. Nakajima, In *The Hydroxylation and Glycosylation by Plant Catalysts*, Proceedings of the 40th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Kurokawa, Ed.; Saga: Japan, 1996; pp 111–112.
162. K. Shimoda; N. Kubota; H. Hamada; M. Kaji, In *Cyanobacterium Catalyzed Asymmetric Reduction of Enones*, Proceedings of the 47th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Kurata, Ed.; Tokyo: Japan, 2003; pp 164–166.
163. M. S. van Dyk; E. van Rensburg; I. P. B. Rensburg; N. Moleleki, *J. Mol. Catal. B: Enzym.* **1998**, *5*, 149–154.
164. Y. Noma; M. Toyota; Y. Asakawa, In *Microbiological conversion of (−)-carvotanacetone and (+)-carvotanacetone by* S. bottropensis *SY-2-1*, Proceedings of the 29th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Fujisawa, Ed.; Mie: Japan, 1985; pp 238–240.
165. Y. Noma; H. Takahashi; M. Toyota; Y. Asakawa, In *Microbiological Conversion of (−)-Carvotanacetone and (+)-Carvotanacetone by a Strain of* Aspergillus niger, Proceedings of the 32nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Shima, Ed.; Mie: Japan, 1988; pp 146–148.
166. M. Ismaili-Alaoui; B. Benjulali; D. Buisson; R. Azerad, *Tetrahedron Lett.* **1992**, *33*, 2349–2352.
167. M. Miyazawa; H. Huruno; H. Kameoka, *Chem. Express* **1991**, *6*, 479–482.
168. M. Miyazawa; H. Huruno; H. Kameoka, *Chem. Express* **1991**, *6*, 873.
169. T. Watanabe; H. Nomura; T. Iwasaki; A. Matsushima; T. Hirata, In *Cloning of Pulegone Reductase and Reduction of Enones with the Recombinant Reductase*, Proceedings of the 51st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Ohta, Ed.; Nagahama: Japan, 2007; pp 323–325.
170. Y. Noma; H. Takahashi; T. Hashimoto; Y. Asakawa, In *Biotransformation of isopiperitenone, 6-gingerol, 6-shogaol and neomenthol by a strain of* Aspergillus niger, Proceedings of the 37th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Katsumura, Ed.; Nishinomiya: Japan, 1992; pp 26–28.
171. Y. Sawamura; S. Shima; H. Sakai; C. Tatsumi, In *Microbiological Conversion of Menthone*, Proceedings of the 18th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Suga, Ed.; Chiba: Japan, 1974; pp 27–29.
172. H. Nonoyama; H. Matsui; M. Hyakumachi; M. Miyazawa, In *Biotransformation of (−)-Menthone Using Plant Parasitic Fungi,* Rhizoctonia solani *as a Biocatalyst*, Proceedings of the 43rd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Ohga, Ed.; Oita: Japan, 1999; pp 387–388.
173. Y. Hagiwara; H. Takeuchi; M. Miyazawa, In *Biotransformation of (+)-and (−)-Menthone by the Larvae of Common Cutworm (*Spodoptera litura*) as a Biocatalyst*, Proceedings of the 50th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Inoue, Ed.; Yokohama: Japan, 2006; pp 279–280.
174. M. Miyazawa; K. Nakanishi, *Biosci. Biotechnol. Biochem.* **2006**, *70*, 1259–1261.
175. G. H. Gibbon; S. J. Pirt, *FEBS Lett.* **1971**, *18*, 103–105.
176. M. Miyazawa; H. Furuno; H. Kameoka, In *Biotransformation of Thujone by Plant Pathogenic Microorganism,* Botrytis allii *IFO 9430*, Proceedings of the 36th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Katsumura, Ed.; Nishinomiya: Japan, 1992; pp 197–198.
177. H. Nishimura; D. M. Paton; M. Calvin, *Agric. Biol. Chem.* **1980**, *44*, 2495–2496.
178. I. C. MacRae; V. Alberts; R. M. Carman; I. M. Shaw, *Aust. J. Chem.* **1979**, *32*, 917–922.
179. Y. Noma; H. Nishimura, In *Microbiological Transformation of 1,8-Cineole. Oxidative Products from 1,8-Cineole by* S. bottropensis*, SY-2-1*, Annual Meeting of Agricultural and Biological Chemical Society, Book of Abstracts; 1980; p 28.
180. Y. Noma; H. Nishimura, In *Microbiological Transformation of 1,8-Cineole. Production of 3β-Hydroxy-1,8-Cineole from 1,8-Cineole by* S. ikutamanensis*, Ya-2-1*, Annual Meeting of Agricultural and Biological Chemical Society, Book of Abstracts; 1981; p 196.
181. Y. Noma; K. Hirata; Y. Asakawa, In *Biotransformation of 1,8-Cineole. Why do the Biotransformed 2α- and 3α-Hydroxy-1,8-Cineole by* Aspergillus niger *Have No Optical Activity?* Proceedings of the 40th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Kurokawa, Ed.; Saga: Japan, 1996; pp 89–91.
182. Y. Hagiwara; M. Miyazawa, In *Biotransformation of Cineole by the Larvae of Common Cutworm (*Spodoptera litura*) as a Biocatalyst*, Proceedings of the 51st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Ohta, Ed.; Nagahama: Japan, 2007; pp 304–305.
183. H. Saito; M. Miyazawa, In *Biotransformation of 1,8-Cineole by* Salmonella typhimurium *OY1001/3A4*, Proceedings of the 50th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Inoue, Ed.; Yokohama: Japan, 2006; pp 275–276.
184. M. Miyazawa; H. Kameoka; K. Morinaga; K. Negoro; N. Mura, *J. Agric. Food Chem.* **1989**, *37*, 222–226.
185. T. M. Flynn; I. A. Southwell, *Aust. J. Chem.* **1979**, *32*, 2093–2095.
186. I. A. Southwell; T. M. Flynn, *Xenobiotica* **1980**, *10*, 17–23.

187. M. Duisken; F. Sander; B. Blomeke; J. Hollender, *Biochim. Biophys. Acta* **2005**, *1722*, 304–311.
188. M. Shindo; T. Shimada; M. Miyazawa, In *Metabolism of 1,8-Cineole by Cytochrome P450 Enzymes in Human and Rat Liver Microsomes*, Proceedings of the 44th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; H. Nishimura, Ed.; Sapporo: Japan, 2000; pp 141–143.
189. Y. Hashimoto; M. Miyazawa, In *Microbial Resolution of Esters of Racemic 2*-Endo-*Hydroxy-1,8-Cineole by* Glomerella cingulata, Proceedings of the 45th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Hirai, Ed.; Toyama: Japan, 2001; pp 363–365.
190. M. Miyazawa; Y. Hashimoto, *Tetrahedron: Asym.* **2001**, *12*, 3185–3187.
191. W. Liu; A. Goswami; R. P. Steffek; R. L. Chemman; F. S. Sariaslani; J. J. Steffens; J. P. N. Rosazza, *J. Org. Chem.* **1988**, *53*, 5700–5704.
192. M. Miyazawa; Y. Noma; K. Yamamoto; H. Kameoka, *Chem. Express* **1991**, *6*, 771–774.
193. M. Miyazawa; Y. Noma; K. Yamamoto; H. Kameoka, *Chem. Express* **1992**, *7*, 125–128.
194. M. Miyazawa; Y. Noma; K. Yamamoto; H. Kameoka, *Chem. Express* **1992**, *7*, 305–308.
195. Y. Asakawa; M. Toyota; T. Ishida, *Xenobiotica* **1988**, *18*, 1129–1134.
196. M. Miyazawa; M. Shindo; T. Shimada, *Xenobiotica* **2001**, *31*, 713–723.
197. B. R. Prema; P. K. Bhattacharyya, *Appl. Microbiol.* **1962**, *10*, 524–528.
198. O. P. Shukla; P. K. Bhattacharyya, *Indian J. Biochem.* **1968**, *5*, 92–101.
199. D. J. Best; N. C. Floyd; A. Magalhaes; A. Burfield; P. M. Rhodes, *Biocatal. Biotransform.* **1987**, *1*, 147–159.
200. D. J. Best; K. J. Davis, *Soap Perfum. Cosmet.* **1988**, *4*, 47.
201. E. T. Griffiths; P. C. Harries; R. Jeffcoat; P. W. Trudgill, *J. Bacteriol.* **1987**, *169*, 4980–4983.
202. E. T. Griffiths; S. M. Bociek; P. C. Harries; R. Jeffcoat; D. J. Sissons; P. W. Trudgill, *J. Bacteriol.* **1987**, *169*, 4972–4979.
203. G. H. Gibbon; N. F. Millis; S. J. Pirt, In *Degradation of* α*-Pinene by Bacteria*, Proceeding of IV IFS, Fermentation Technology Today; Osaka, Japan, 1972; pp 609–612.
204. Y. Noma; J. Watanabe; T. Hashimoto; Y. Asakawa, In *Microbiological Transformation of* β*-Pinene*, Proceedings of the 45th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Hirai, Ed.; Toyama: Japan, 2001; pp 88–90.
205. M. Miyazawa; H. Yanagihara; H. Kameoka, In *Biotransformation of Pinanes by Common Cutworm Larvae,* Spodoptera litura *as a Biocatalyst*, Proceedings of the 40th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Kurokawa, Ed.; Saga: Japan, 1996; pp 84–85.
206. A. Sugie; M. Miyazawa, In *Biotransformation of (*$-$*)-*α*-Pinene by Human Liver Microsomes*, Proceedings of the 47th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Kurata, Ed.; Tokyo: Japan, 2003; pp 159–161.
207. T. Ishida; Y. Asakawa; T. Takemoto; T. Aratani, *J. Pharm. Sci.* **1981**, *70*, 406–415.
208. A. Farooq; S. Tahara; Choudhary; M. I. Atta-ur-Rahman; K. H. C. Baser; F. Demirci, *Z. Naturforsch.* **2002**, *57c*, 303–306.
209. O. P. Shukla; M. N. Moholay; P. K. Bhattacharyya, *Indian J. Biochem.* **1968**, *5*, 79–91.
210. P. K. Bhattacharyya; K. Ganapathy, *Indian J. Biochem.* **1965**, *2*, 137–145.
211. R. S. Dhavlikar; A. Ehbrecht; G. Albroscheit, *Dragoco Rep.* **1974**, *3*, 47–49.
212. A. Farooq; M. I. Choudhary; Tahara; S. Atta-ur-Rahman; K. H. C. Baser; F. Demirci, *Z. Naturforsch.* **2002**, *57c*, 686–690.
213. Y. Noma; Y. Asakawa, In *New Metabolic Pathways of* β*-Pinene and Related Compounds by* Aspergillus niger, Book of Abstracts of the 36th International Symposium on Essential Oils; J. Bernath, E. Nemeth, A. Kozak, Eds.; Budapest: Hungary, 2005; p 32.
214. Y. Noma; M. Furusawa; T. Hashimoto; Y. Asakawa, In *Stereoselective Formation of (1*R*, 2*S*, 4*R*)-(*$+$*)-*p*-Menthane-2,8-Diol from* α*-Pinene*, Book of Abstracts of the 33rd International Symposium on Essential Oils; A. C. Figueiredo, J. G. Barroso, L. G. Pedro, Eds.; Lisbon: Portugal, 2002; p 142.
215. Y. Noma; F. Kamino; T. Hashimoto; Y. Asakawa, In *Biotransformation of (*$+$*)- and (*$-$*)-Pinane-2,3-Diol and Related Compounds by* Aspergillus niger, Proceedings of the 47th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Kurata, Ed.; Tokyo: Japan, 2003; pp 91–93.
216. T. Ishida; Y. Asakawa; T. Takemoto; T. Aratani, *J. Pharm. Sci.* **1979**, *68*, 928–930.
217. Y. Noma; Y. Asakawa, In *Microbial Transformation of (*$-$*)-Myrtenol and (*$-$*)-Nopol*, Proceedings of the 49th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; M. Hatanaka, Ed.; Fukui: Japan, 2005; pp 78–80.
218. Y. Noma; Y. Asakawa, In *Biotransformation of* β*-Pinene, Myrtenol, Nopol and Nopol Benzyl Ether by* Aspergillus niger *TBUYN-2*, Book of Abstracts of the 37th International Symposium on Essential Oils; D. Joulain, Ed.; Grasse: France, 2006; p 144.
219. M. Miyazawa; S. Kumagae; H. Kameoka, In *Biotransformation of (*$+$*)*-Trans-*Myrtanol and (*$-$*)*-Trans-*Myrtanol by Common Cutworm Larvae,* Spodoptera litura *as a Biocatalyst*, Proceedings of the 41st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Ito, Ed.; Morioka: Japan, 1997; pp 389–390.
220. M. Miyazawa; Y. Suzuki; H. Kameoka, *Phytochemistry* **1997**, *45*, 935–945.
221. M. Miyazawa; H. Yanahara; H. Kameoka, In *Biotransformation of* Trans-*Pinocarveol by Plant Pathogenic Microorganism,* Glomerella cingulata, *and by the Larvae of Common Cutworm*, Spodoptera litura *Fabricius*, Proceedings of the 39th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Uyehara, Ed.; Utsunomiya: Japan, 1995; pp 360–361.
222. F. Kamino; M. Miyazawa, In *Biotransformation of (*$+$*)-and (*$-$*)-Pinane-2,3-Diol Using Plant Pathogenic Fungus,* Glomerella cingulata *as a Biocatalyst*, Proceedings of the 49th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; M. Hatanaka, Ed.; Fukui: Japan, 2005; pp 395–396.
223. F. Kamino; Y. Noma; Y. Asakawa; M. Miyazawa, In *Biotransformation of (1S,2S,3R,5S)-(*$+$*)-Pinane-2,3-Diol Using Plant Pathogenic Fungus,* Glomerella cingulata *as a Biocatalyst*, Proceedings of the 48th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Kajiwara, Ed.; Yamaguchi: Japan, 2004; pp 383–384.
224. A. Wolf-Rainer, *Z. Naturforsch.* **1994**, *49c*, 553–560.
225. M. Miyazawa; Y. Suzuki; H. Kameoka, *Phytochemistry* **1997**, *45*, 945–950.
226. Y. Noma; T. Hashimoto; S. Uehara; Y. Asakawa, unpublished data, 2009.
227. T. Hayashi; T. Kakimoto; H. Ueda; C. Tatsumi, *J. Agric. Chem. Soc. Jpn.* **1969**, *43*, 583–587.

228. Y. Noma; A. Sogo; S. Fujii; N. Miki; T. Hashimoto; Y. Asakawa, In *Biotransformation of Terpenoids and Related Compounds*, Proceedings of the 36th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Katsumura, Ed.; Nishinomiya: Japan, 1992; pp 199–201.
229. Y. Miyamoto; M. Miyazawa, In *Biotransformation of (+)- and (−)-Borneol by the Larvae of Common Cutworm (*Spodoptera litura*) as a Biocatalyst*, Proceedings of the 45th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Hirai, Toyama, Ed.; Yamaguchi: Japan, 2001; pp 377–378.
230. M. Miyazawa; Y. Miyasato, *J. Chem. Technol. Biotechnol.* **2001**, *76*, 220–224.
231. M. Miyazawa; Y. Miyamoto, *Tetrahadron* **2004**, *60*, 3091–3096.
232. Y. Miyazato; M. Miyazawa, In *Biotransformation of (+)- and (−)-α-Fenchyl Acetated Using Plant Parasitic Fungus,* Glomerella cingulata *as a Biocatalyst*, Proceedings of the 43rd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Ohga, Ed.; Oita: Japan, 1999; pp 213–214.
233. T. Yamanaka; M. Miyazawa, In *Biotransformation of (−)-*Trnas-*Verbenol by Common Cutworm Larvae,* Spodoptera litura *as a Biocatalyst*, Proceedings of the 43rd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Ohga, Ed.; Oita: Japan, 1999; pp 391–392.
234. K. Eriksson; J. O. Levin, *Int. Arch. Occup. Environ. Health* **1990**, *62*, 379–383.
235. Y. Noma; Y. Asakawa, In *Microbial Transformation of (−)-Nopol Benzyl Ether*, Proceedings of the 50th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Inoue, Ed.; Yokohama: Japan, 2006; pp 434–436.
236. T. Suga; T. Hirata, *Phytochemistry* **1990**, *29*, 2393–2406.
237. K. Shimoda; D. I. Ito; S. Izumi; T. Hirata, *J. Chem. Soc. Perkin Trans.* **1996**, *1*, 355–358.
238. K. Shimoda; S. Izumi; T. Hirata, *Bull. Chem. Soc. Jpn.* **2002**, *75*, 813–816.
239. T. Hirata; K. Shimoda; T. Gondai, *Chem. Lett.* **2000**, *29*, 850–851.
240. M. Miyazawa; A. Sugie; T. Shimada, *Drug Metab. Dispos.* **2003**, *31*, 1049–1053.
241. W.-R. Abraham; H.-A. Arfmann; B. Stumpf; P. Washausen; K. Kieslich, In *Bioflavour'87. Analysis – Biochemistry – Biotechnology*; P. Schreier, Ed.; Walter de Gruyter and Co.: Berlin, 1988; pp 399–414.
242. P. W. Trudgill, *Biodegradation* **1990**, *1*, 93–105.
243. W. H. Bradshaw; H. E. Conrad; E. J. Corey; I. C. Gunsalus; D. Lednicer, *J. Am. Chem. Soc.* **1959**, *81*, 5507.
244. H. E. Conrad; R. DuBus; I. C. Gunsalus, *Biochem. Biophys. Res. Commun.* **1961**, *6*, 293–297.
245. H. E. Conrad; R. DuBus; M. J. Mamtredt; I. C. Gunsalus, *J. Biol. Chem.* **1965**, *240*, 495–503.
246. H. E. Conrad; K. Lieb; I. C. Gunsalus, *J. Biol. Chem.* **1965**, *240*, 4029–4037.
247. I. C. Gunsalus; P. J. Chapman; J.-F. Kuo, *Biochem. Biophys. Res. Commun.* **1965**, *18*, 924–931.
248. P. J. Chapman; G. Meerman; I. C. Gunsalus; R. Srinivasan; K. L. Rinehart, Jr., *J. Am. Chem. Soc.* **1966**, *88*, 618–619.
249. R. A. Hartline; I. C. Gunsalus, *J. Bacteriol.* **1971**, *106*, 468–478.
250. T. Oritani; K. Yamashita, *Agric. Biol. Chem.* **1974**, *38*, 1961–1964.
251. K. Gyoubu; M. Miyazawa, In *Biotransformation of (+)- and (−)-Camphor by Liver Microsome*, Proceedings of the 50th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Inoue, Ed.; Yokohama: Japan, 2006; pp 253–255.
252. K. Gyoubu; M. Miyazawa, *Biol. Pharm. Bull.* **2007**, *30*, 230–233.
253. M. Miyazawa; Y. Miyamoto, *J. Mol. Catal. B: Enzym.* **2004**, *27*, 83–89.
254. M. Miyazawa; M. Nobata; M. Hyakumachi; H. Kameoka, *Phytochemistry* **1995**, *39*, 569–573.
255. P. J. Chapman; G. Meerman; I. C. Gunsalus, *Biochem. Biophys. Res. Commun.* **1965**, *20*, 104–108.
256. B. Pfrunder; C. Tamm, *Helv. Chim. Acta* **1969**, *52*, 1643–1654.
257. M. Miyazawa; K. Yamamoto; Y. Noma; H. Kameoka, *Chem. Express* **1990**, *5*, 237–240.
258. M. Miyazawa; K. Yamamoto; Y. Noma; H. Kameoka, *Chem. Express* **1990**, *5*, 407–410.
259. K. Yamamoto; M. Miyazawa; H. Kameoka; Y. Noma, In *Biotransformation of* d- *and* l-*Fenchone by a Strain of* Aspergillus niger, Proceedings of the 28th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; H. Kotake, Ed.; Yokohama: Japan, 1984; pp 168–170.
260. K. Gyoubu; M. Miyazawa, In *Biotransformation of (+)- and (−)-Fenchone by Liver Microsomes*, Proceedings of the 49th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; M. Hatanaka, Ed.; Fukui: Japan, 2005; pp 420–422.
261. M. Miyazawa; K. Gyoubu, *Biol. Pharm. Bull.* **2006**, *29*, 2354–2358.
262. M. Miyazawa; Y. Miyamoto, *J. Mol. Catal. B: Enzym.* **2005**, *32*, 123–130.
263. Y. Noma; M. Furusawa; T. Hashimoto; Y. Asakawa, In *Biotransformation of (+)- and (−)-3-Pinanone by* Aspergillus niger, Proceedings of the 48th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Kajiwara, Ed.; Yamaguchi: Japan, 2004; pp 390–392.
264. E. W. Koneman; S. D. Allen; W. M. Janda; P. C. Schreckenberger; W. C. Winn, *Color Atlas and Textbook of Diagnostic Microbiology*; Lippincott-Raven Publishers: Philadelphia, PA, 1997.
265. D. Amsterdam, Susceptibility Testing of Antimicrobials in Liquid Media. In *Antibiotics in Laboratory Medicine*, 4th ed.; V. Lorian, Ed.; Williams Wilkins, Maple Press: Baltimore, MD, 1997.
266. G. Iscan, unpublished data, 2005.

Biographical Sketches

Professor Yoshiaki Noma was born in 1947 in Hyogo Prefecture. He graduated from the Faculty of Agriculture, Okayama University, and then joined the Graduated School of Agricultural and Chemical Sciences of Okayama University and Osaka Prefectural University and obtained a Ph.D. degree from Osaka Prefectural University in 1975. Professor Noma was an associate professor at Osaka Joshi-Gakuen Junior College, and in 1988 he moved to the Department of Human Life Sciences at Tokushima Bunri University as a full professor. Professor Noma is a fellow of the Japan Society for Bioscience, Biotechnology, and Agrochemistry. He is also a fellow of the Chemical Society of Japan and Japanese Society of Nutrition and Food Sciences.

Professor Noma has published over 55 research papers and reviews on several topics connected with the microbiological biotransformation of monoterpenes and sesquiterpenoids, the metabolic pathways of monoterpenoids, and the chemistry and biological activity of metabolites. At present, his research on biotransformation includes substances from liverworts and higher plants of potential use in cancer, antioxidants, and mosquitocidal compounds. He is also interested in the study of biosynthesis of biological active compounds.

Professor Asakawa studied organic chemistry at the graduate school of Hiroshima University. He was appointed as a research assistant there in 1969, obtained his Ph.D. degree in 1972, and then joined Universite Louis Pasteur, France as a postdoctoral fellow, where he worked for 2 years with Professor Guy Ourisson. In 1976, he moved to the Faculty of Pharmaceutical Sciences, Tokushima Bunri University as an associate professor, became full professor in 1981, served twice as Dean, and is currently the Director of the Institute of Pharmacognosy (1986–present) and the president of Phytochemical Society of Asia (2007–till date). He is the coeditor of *Phytomedicine* and serves on the editorial boards of *Phytochemisty*, *Phytochemistry Letters*, *Planta Medica*, *Fitoterapia*, *Flavour and Fragrance Journal*, *Natural Product Communication*, *Natural Product Research*, *Spectroscopy*, *Arkivoc*, *Current Chemical Biology*, and *Malaysian Journal of*

Sciences, among others. He has published 540 original papers, 20 reviews, and 27 books and monographs. For his outstanding research he was awarded the first Hedwig medal (1983), the Pergamon Phytochemistry Prize and Certificate (1997), The Tokushima Newspaper prize (1997), and the ISEO prize (2004). Over the years, he has welcomed 37 postdoctoral researchers from various countries into his laboratory.

3.20 Biotransformation of Sesquiterpenoids

Yoshinori Asakawa and Yoshiaki Noma, Tokushima Bunri University, Yamashiro-cho, Tokushima, Japan

3.20.1 Introduction

Recently, environment friendly green or clean chemistry is emphasized in the field of organic and natural product chemistry. Noyori's team showed that high efficient production of (−)-menthol using (S)-BINAP-Rh catalyst is one of the most important green chemistries[1,2] and that 1000 tons of (−)-menthol has been produced by this method in 1 year. On the other hand, enzymes of microorganisms and mammals are able to transform a huge variety of organic compounds, such as terpenoids, alkaloids, steroids, antibiotics, and amino acids from crude drugs and spore-forming green plants to produce pharmacologically and medicinally valuable substances.

Since Meyer and Neuberg[3] studied the microbial transformation of citronellal, there are a great number of reports concerning biotransformation of essential oils, terpenoids, steroids, alkaloids, and acetogenins. In 1988, Mikami[4] reported the review article of biotransformation of terpenoids entitled 'Microbial Conversion of Terpenoids'. Lamare and Furstoss[5] reviewed biotransformation of more than 25 sesquiterpenoids by microorganisms. In this chapter, the recent advances in the biotransformation of natural and synthetic compounds using microorganisms including algae and mammals are described.

3.20.2 Biotransformation of Sesquiterpenoids by Microorganisms

3.20.2.1 Highly Efficient Production of Nootkatone (2) from Valencene (1)

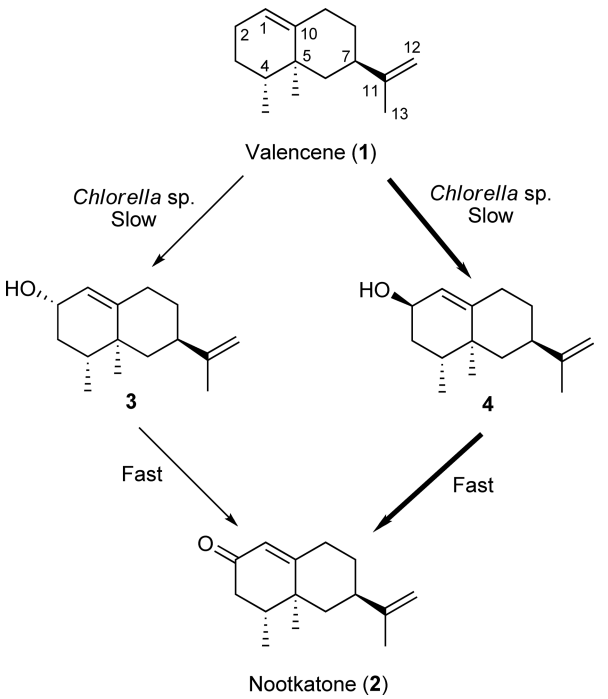
The most important and expensive grapefruit aroma, nootkatone (2), decreases the somatic fat ratio,[6] and therefore its highly efficient production has been requested by the cosmetic and fiber industrial sectors. Previously, valencene (1) from the essential oil of Valencia orange was converted into nootkatone (2) by biotransformation using *Enterobacter* sp. only in 12% yield,[7] *Rodococcus* KSM-5706 in 0.5% yield with a complex mixture,[8] and using cytochrome P-450 (CYP450) in 20% yield with other complex products.[9] Nootkatone (2) was chemically synthesized from valencene (1) with $AcOOCMe_3$ in three steps and chromic acid in low yield[10] and using surface-functionalized silica supported by metal catalysts such as Co^{2+} and Mn^{2+} with *tert*-butyl hydroperoxide in 75% yield.[11] However, these synthetic methods are not safe because they involve toxic heavy

metals. An environment-friendly method for the synthesis of nootkatone which does not use any heavy metals such as chromium and manganese must be designed. The commercially available and cheap sesquiterpene hydrocarbon (+)-valencene (**1**) obtained from Valencia orange oil was efficiently converted into nootkatone (**2**) by biotransformations using *Chlorella*,[12] *Mucor* species,[13] *Botryosphaeria dothidea*, and *Botryodiplodia theobromae*.[14–16]

Chlorella fusca var. *vacuolata* IAMC-28 was inoculated and cultivated stationary under illumination in Noro medium. Czapek-peptone medium was used for the biotransformation of the substrate by *Aspergillus cellulosae*, *A. niger*, *B. dothidea*, *B. theobromae*, *Fusarium culmorum*, and *Mucor* species. *Aspergillus niger* was isolated in our laboratories from the soil of Osaka prefecture, and was identified according to its physiological and morphological characters.

(+)-Valencene (**1**) (20 mg 50 ml^{-1}) isolated from the essential oil of Valencia orange was added to the medium and biotransformed by *C. fusca* for a further 18 days to yield nootkatone (**2**) (GC–MS peak area: 89%; isolated yield: 63%).[14–16] The reduction of **2** with $NaBH_4$ and $CeCl_3$ gave 2α-hydroxyvalencene (**3**) in 87% yield, followed by Mitsunobu reaction with *p*-nitrobenzoic acid, triphenylphosphine, and diethyl azodicarboxylate to give nootkatol (2β-hydroxyvalencene) (**4**), possessing calcium-antagonistic activity isolated from *Alpinia oxyphylla*[17] in 42% yield. Compounds **3** and **4** thus obtained were easily biotransformed by *C. fusca* and *C. pyrenoidosa* for only 1 day to give a high yield (80–90%) of nootkatone (**2**). The biotransformation of compound **1** was further carried out by *C. pyrenoidosa* and *C. vulgaris*[14,15] and soil bacteria[18] to give a good yield of nootkatone. In the time course of the biotransformation of **1** by *C. pyrenoidosa*, the yield of nootkatone (**2**) and nootkatol (**4**) in the absence of 2α-hydroxyvalencene (**3**) had increased with a decrease in the yield of compound **1**, and subsequently the yield of **2** increased with a decrease in the yield of **3**. In the metabolic pathway of valencene (**1**), **1** was slowly converted into nootkatol (**4**), and subsequently **4** was rapidly converted into **2**, as shown in **Scheme 1**.

A fungus strain *Mucor* sp. isolated from the soil found adhering to the thalloid liverwort *Pallavicinia subcilita*, was inoculated and cultivated statically in Czapek-peptone medium (pH 7.0) at 30 °C for 7 days. Compound **1** (20 mg 50 ml^{-1}) was added to the medium and incubated for a further 7 days. This resulted in a high yield (82%) of nootkatone (**2**).[15,16]



Scheme 1 Biotransformation of valencene (**1**) by *Chlorella* species.

The biotransformation from **1** to **2** was also examined using the plant pathogenic fungi *B. dothidea* and *B. theobromae* (a total of 31 strains) separated from fungi infecting various types of fruit, and so forth. The same size of substrate **1** was incubated with *B. dothidea* and *B. theobromae* to obtain nootkatone (42–84%).[15]

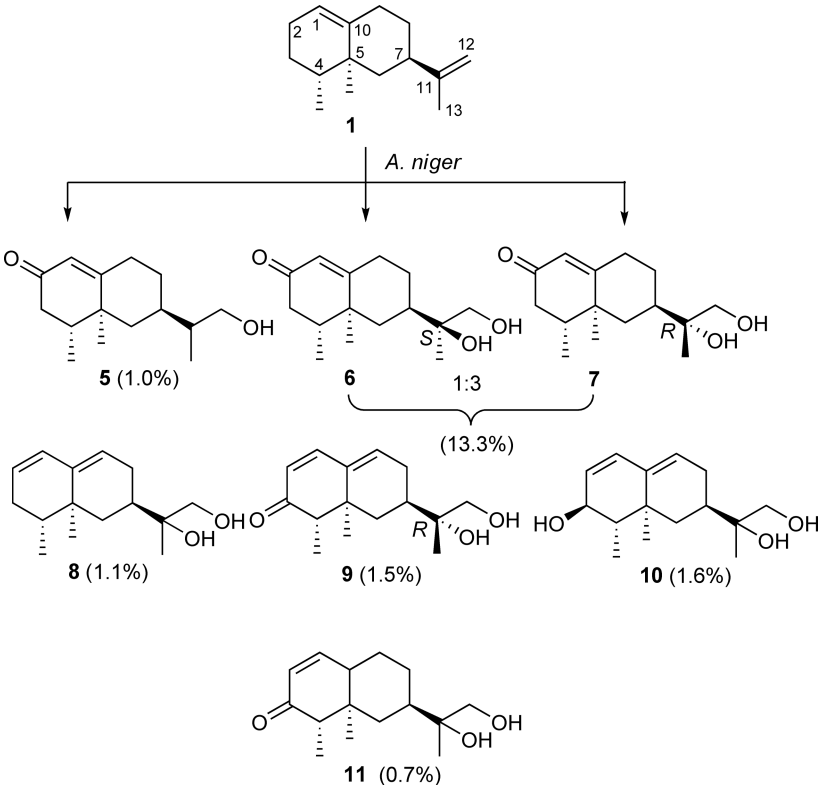
The expensive grapefruit aromatic, nootkatone (**2**) used in the cosmetic and fiber industries, was obtained in large quantities by the biotransformation of (+)-valencene (**1**), which can be cheaply obtained from Valencia orange by *Chlorella* species, fungi such as *Mucor* species, *B. dothidea*, and *B. theobromae*. This is a very inexpensive and clean oxidation reaction which does not use any heavy metals, and thus this method is expected to find applications in the industrial production of nootkatone.

3.20.2.2 Biotransformation of Valencene (1) by *Aspergillus niger* and *A. wentii*

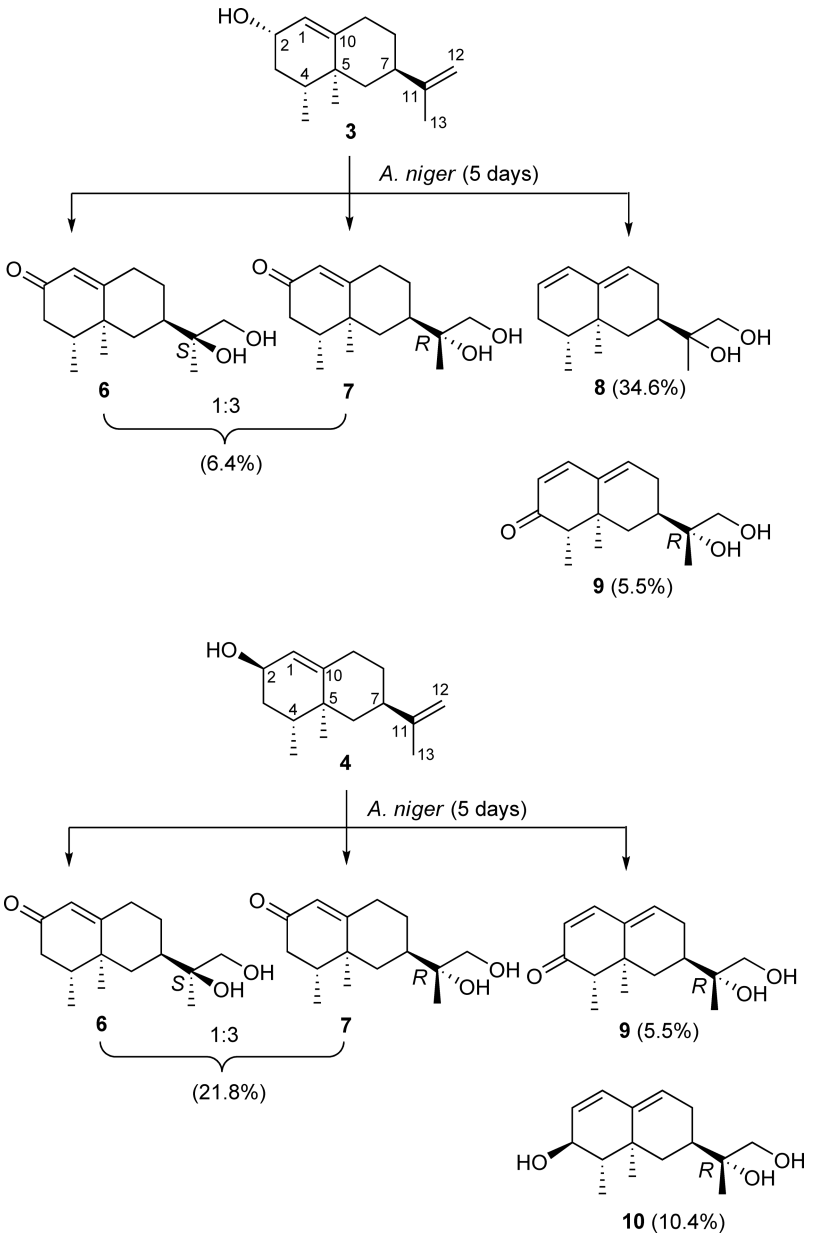
Valencene (**1**) from Valencia orange oil was cultivated by *A. niger* in Czapek-peptone medium, for 5 days to yield six metabolites **5** (1.0%), **6** and **7** (13.5%), **8** (1.1%), **9** (1.5%), **10** (2.0%), and **11** (0.7%). Ratio of compounds **6** (11*S*) to **7** (11*R*) was determined as 1:3 by HPLC analysis of their thiocarbonates (**12** and **13**) (**Scheme 2**).[16]

Compounds **8–11** could be biosynthesized by the elimination of a hydroxy group of 2-hydroxyvalencenes (**3**, **4**). Compound **3** was biotransformed for 5 days by *A. niger* to give three metabolites **6** and **7** (6.4%), **8** (34.6%), and **9** (5.5%). Compound **4** was biotransformed for 5 days by *A. niger* to give three metabolites **6** and **7** (21.8%), **9** (5.5%), and **10** (10.4%) (**Scheme 3**).

Both ratios of **6** (11*S*) to **7** (11*R*) obtained from **3** and **4** were 1:3, respectively. From the above results, plausible metabolic pathways of valencene (**1**) and 2-hydroxyvalencene (**3**, **4**) by *A. niger* are shown in **Scheme 4**.[16]



Scheme 2 Biotransformation of valencene (**1**) by *Aspergillus niger*.

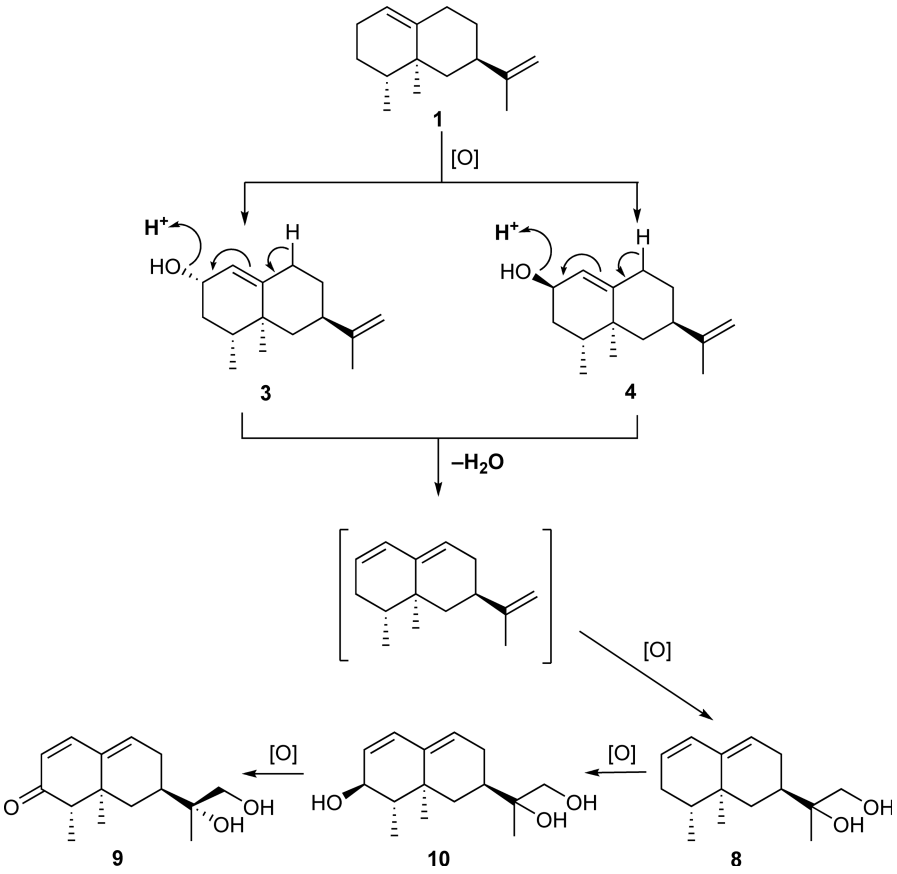


Scheme 3 Biotransformation of 2α-hydroxyvalencene (**3**) and 2β-hydroxyvalencene (**4**) by *Aspergillus niger.*

Aspergillus wentii and *Eurotrium purpurasens* converted valencene (**1**) into 11,12-epoxide (**14a**) and the same diol (**6**, **7**)[19] as well as nootkatone (**2**) and 2α-hydroxyvalencene (**3**).[20]

Kaspera *et al.*[21] reported that valencene (**1**) was incubated in submerged cultures of the ascomycete *Chaetomium globosum*, to give nootkatone (**2**), 2α-hydroxyvalencene (**3**), valencene 11,12-epoxide (**14a**), together with a valencene ketodiol, valencenediols, a valencene ketodiol, a valencene triol, or valencene epoxydiol which were detected by LC–MS spectra and GC–MS of trimethyl silyl derivatives. These metabolites are accumulated preferably inside the fungal cells (**Scheme 5**).

The metabolites of valencene, nootkatone (**2**), (**3**), and (**14a**), indicated grapefruit, with sour and citrus with bitter odors, respectively. Nootkatone 11,12-epoxide (**14**) showed no volatile fragrant properties.



Scheme 4 Possible pathway of biotransformation of valencene (**1**) by *Aspergillus niger*.

3.20.2.3 Biotransformation of Nootkatone (2) by *Aspergillus niger*

Aspergillus niger was inoculated and cultivated under rotation (100 rpm) in Czapek-peptone medium at 30 °C for 7 days. (+)-Nootkatone (**2**) (80 mg per 200 ml), which was isolated from the essential oil of grapefruit was added to the medium and further cultivated for 7 days to obtain two metabolites, 12-hydroxy-11,12-dihydronootkatone (**5**) (10.6%) and C-11 stereo-mixtures (51.5%) of nootkatone-11*S*,12-diol (**6**) and its 11*R* isomer (**7**) (11*R*:11*S* = 1:1) (**Scheme 6**).[16,22,23]

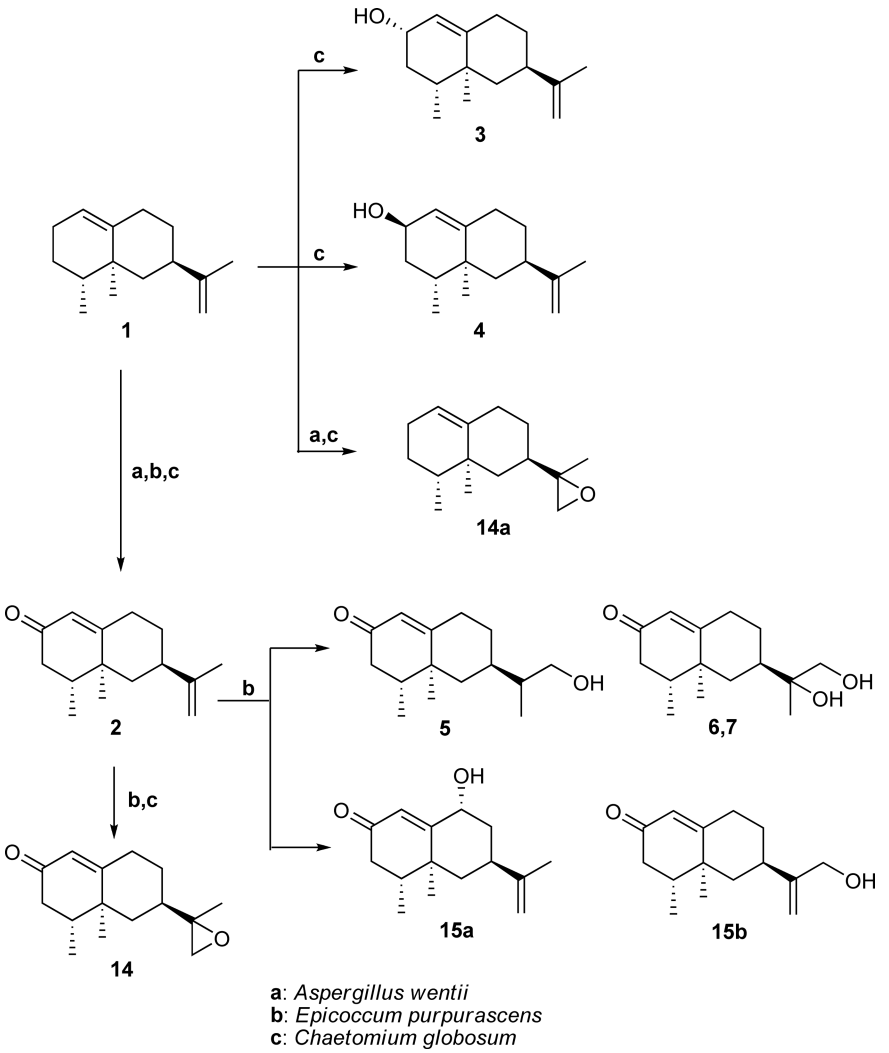
11,12-Epoxide (**14**) obtained by the epoxidation of nootkatone (**2**) with *m*CPBA was biotransformed by *A. niger* for 1 day to yield **6** and **7** (11*R*:11*S* = 1:1) in good yield (81.4%). 1-Aminobenzotriazole, an inhibitor of CYP450, inhibited the oxidation process of **1** into compounds **5**–**7**.[16] From the above results, possible metabolic pathways of nootkatone (**2**) by *A. niger* might be considered as shown in **Scheme 7**.

The same substrate was incubated with *A. wentii* to produce diol (**6**, **7**) and 11,12-epoxide (**14**).[19]

3.20.2.4 Biotransformation of Nootkatone (2) by *Fusarium culmorum* and *Botryosphaeria dothidea*

(+)-Nootkatone (**2**) was added to the same medium as mentioned above including *F. culmorum* to yield nootkatone-11*R*,12-diol (**7**) (47.2%) and 9β-hydroxynootkatone (**15**) (14.9%).[16]

Compound **7** was stereospecifically obtained at C-11 by biotransformation of **1**. Purity of compound **7** was determined as approximately 95% by HPLC analysis of the thiocarbonate (**13**).



Scheme 5 Biotransformation of valencene (**1**) and nootkatone (**2**) by *Aspergillus wentii*, *Epicoccum purpurescens*, and *Chaetomium globosum.*

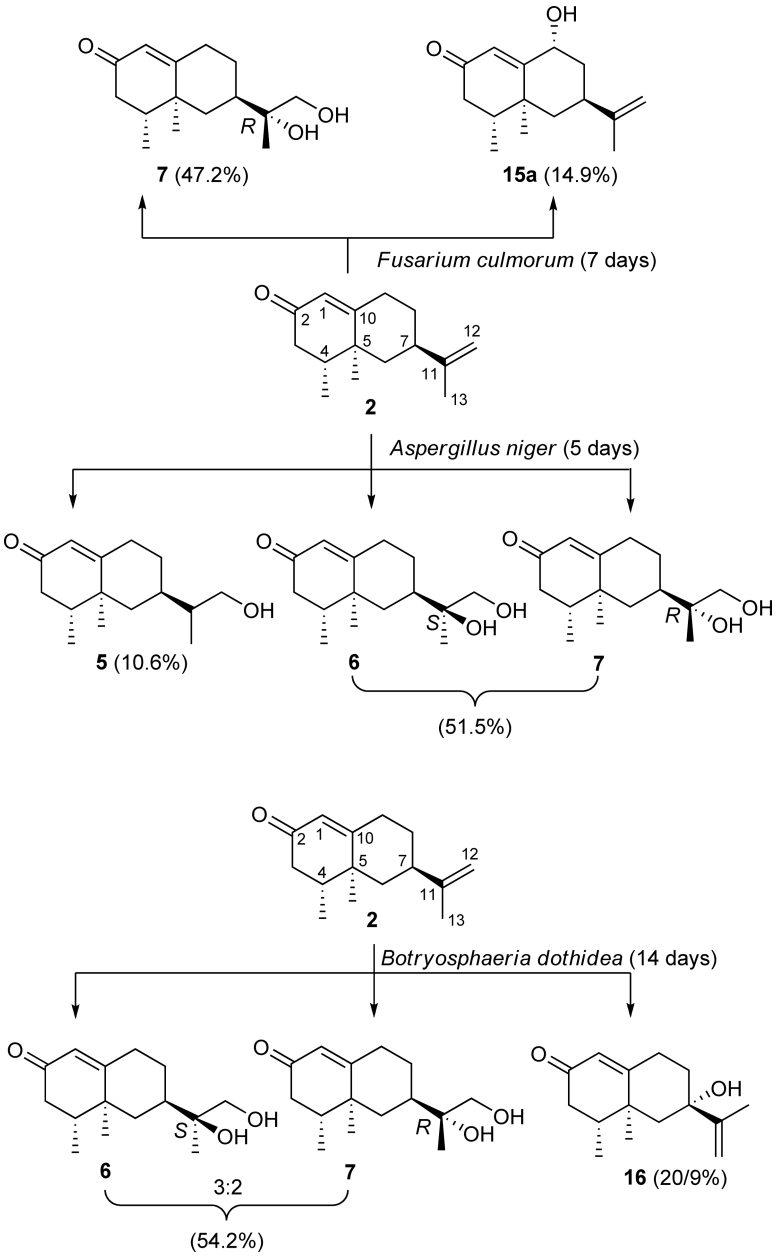
The biotransformation of nootkatone (**2**) was observed in the plant pathogenic fungus, *B. dothidea*, which infected peach. *Botryosphaeria dothidea* (Peach PP8402) was cultivated for 14 days along with (+)-nootkatone (**2**) to yield nootkatone diols (**6** and **7**) (54.2%) and 7α-hydroxynootkatone (**16**) (20.9%). Ratio of compounds **6** to **7** was determined as 3:2 by HPLC analysis of the thiocarbonates (**12**, **13**).[16] Nootkatone (**2**) was administered into rabbits to give the same diols (**6**, **7**).[24,25]

Epicoccum purpurascens also biotransformed nootkatone (**2**) to **5**–**7**, **14**, and **15a**.[20]

The biotransformation of **2** by *A. niger* and *B. dothidea* resembled oral administration given to rabbits since the ratio of major metabolites 11*S*- (**6**) and 11*R*-nootkatone-11,12-diol (**7**) was similar. It is noteworthy that the biotransformation of **2** by *F. culmorum* yields stereospecific nootkatone-11*R*, 12-diol (**7**) (**Scheme 8**). [16]

Metabolites **3**–**5**, **12**, and **13** from (+)-nootkatone (**2**) and **14**–**17** from (+)-valencene (**1**) did not exhibit an effective odor.

Dihydronootkatone (**17**), which exhibits citrus odor, possesses antitermite activity and was also treated with *A. niger* to obtain 11*S*-mono- (**18**) and 11*R*-dihydroxylated products (**19**) (the ratio 11*S* and 11*R* = 3:2). On the other hand, *A. cellulosae* reduced the ketone group at C-2 of **17** to give 2α- (**20**) (75.7%) and 2β-hydroxynootkatone (**21**) (0.7%) (**Scheme 9**).[22]

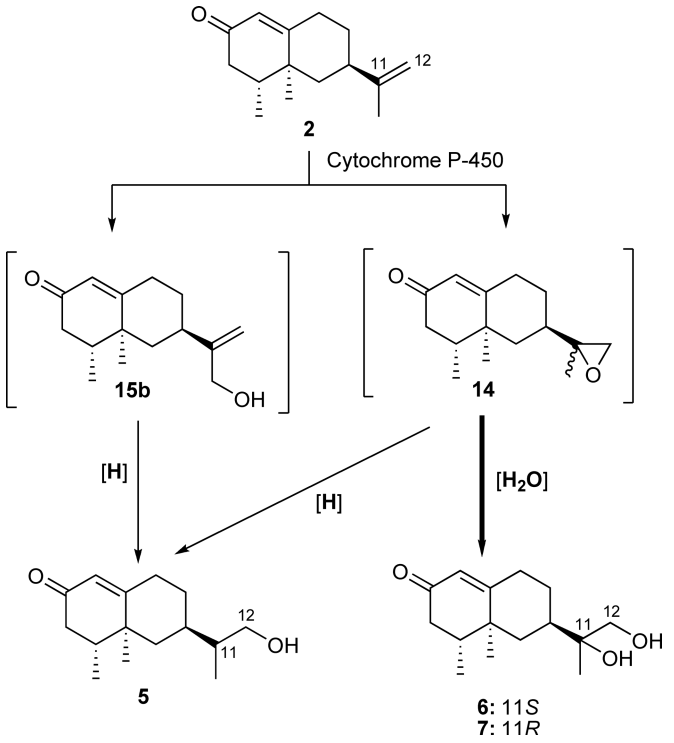


Scheme 6 Biotransformation of nootkatone (**2**) by *Fusarium culmorum*, *Aspergillus niger*, and *Botryosphaeria dothidea.*

Tetrahydronootkatone (**22**) also shows antitermite and mosquito-repellent activity. It was incubated with *A. niger* to give two hydroxylated compounds (**23**, 13.6% and **24**, 9.9%) similar to those obtained from **17** (**Scheme 10**).[26]

8,9-Dehydronootkatone (**25**) was incubated with *A. niger* to give four metabolites: a unique acetonide (**26**, 15.6%), monohydroxylated (**27**, 0.2%), dihydroxylated (**28**, 69%), and a carboxyl derivative (**29**, 0.8%) (**Scheme 11**).

When the same substrate was treated with *Aspergillus sojae* IFO 4389, compound **25** was converted into a monohydroxylated product (**30**, 15.8%) different from that mentioned above. *Aspergillus cellulosae* is an interesting fungus since it did not yield the same products as mentioned above but it produced trinorsesquiterpene ketone (**31**, 6%) and nitrogen-containing aromatic compound (**32**) (**Scheme 12**).[22]



Scheme 7 Possible pathway of biotransformation of valencene (**1**) by cytochrome P-450.

Mucor species also oxidized compound **25** to give three metabolites: 13-hydroxy-8,9-dehydronootkatone (**33**, 13.2%), an epoxide (**34**, 5.1%), and diol (**35**, 19.9%).[22] The same substrate was incubated with cultured suspension cells of the liverwort, *Marchantia polymorpha* to yield **33** (**Scheme 13**).[27]

Although *Mucor* species could give nootkatone (**21**) from valencene (**1**), this fungus biotransformed the same substrate (**25**) to the same alcohol (**30**, 13.2%) obtained from the same starting compound (**25**) in *A. sojae*, a new epoxide (**34**, 5.1%) and diol (**35**, 9.9%).

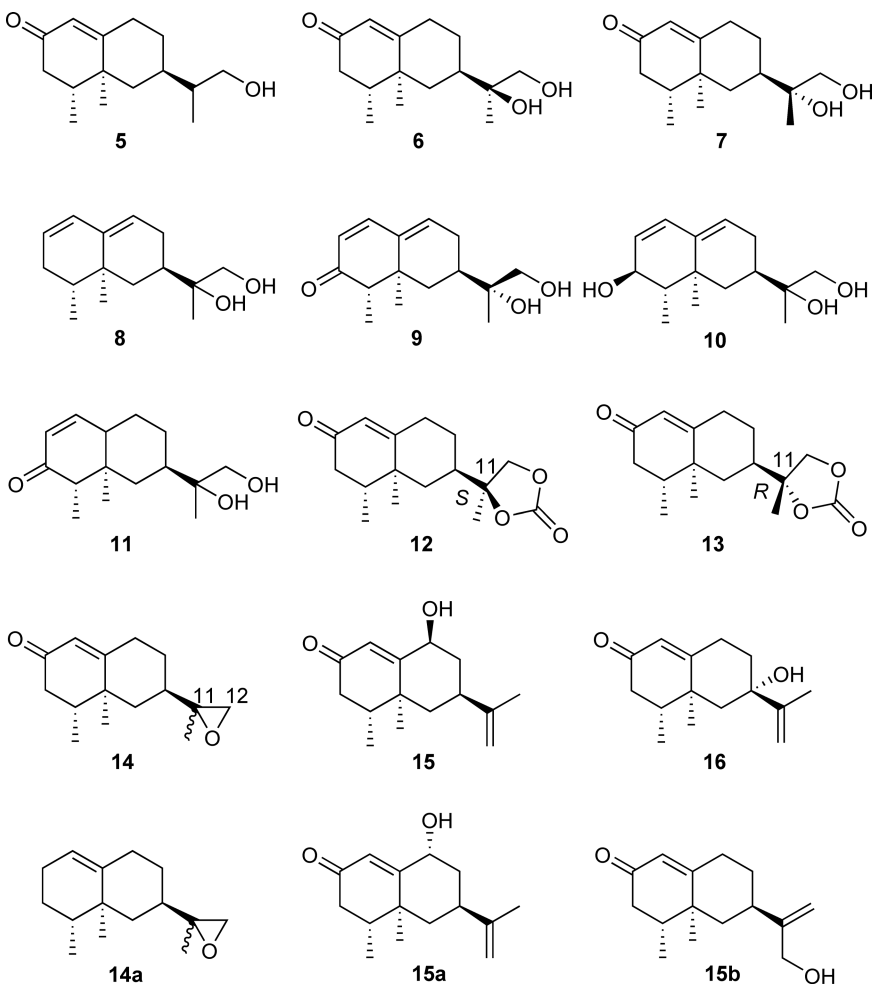
The metabolites (**3**, **4**, **20**, and **21**) inhibited growth of lettuce stem, and **3** and **4** inhibited germination of the same plant.[28]

Valerianol (**35a**), from *Valeriana officinalis* whose dried rhizome is traditionally used for its carminative and sedative properties, was biotransformed by *Mucor plumbeus* to produce three metabolites: a bridged ether (**35b**), a triol (**35c**), which might be formed through C1-10 epoxide, and **35d** which arises from double dehydration.[29] In this case, allylic oxidative compounds have not been found (**Scheme 14**).

3.20.2.5 Biotransformation of (+)-1(10)-Aristolene (36) from the Crude Drug *Nardostachys chinensis* by *Chlorella fusca*, *Mucor* Species, and *Aspergillus niger*

The structure of sesquiterpenoid, (+)-1(10)-aristolene (=calarene) (**36**) from the crude drug *Nardostachys chinensis* was similar to that of nootkatone. 2-Oxo-1(10)-aristolene (**38**) shows antimelanin-inducing activity and excellent citrus fragrance. On the other hand, the enantiomer (**37**) of **36** and (+)-aristolone (**41**) were also found in the liverworts as the natural products. To obtain compound **38** and its analogues, compound **36** was incubated with *C. fusca* var. *vacuolata* IAMC-28, *Mucor* species, and *A. niger* (**Scheme 15**).[30]

Chlorella fusca var. *vacuolata* was inoculated and cultivated stationary in Noro medium (pH 8.0) at 25 °C for 7 days and (+)-1(10)-aristolene (**36**) (20 mg per 50 ml) was added to the medium and further incubated for 10–14 days and cultivated stationary under illumination (pH 8.0) at 25 °C for 7 days to



Scheme 8 Metabolites **5–11**, **14–15b** from valencene (**1**) and nootkatone (**2**) by various microorganisms.

yield 1(10)-aristolen-2-one (**38**, 18.7%), (−)-aristolone (**39**, 7.1%), and 9-hydroxy-1(10)-aristolen-2-one (**40**). Compounds **38** and **40** were found in *Aristolochia* species (**Scheme 16**).

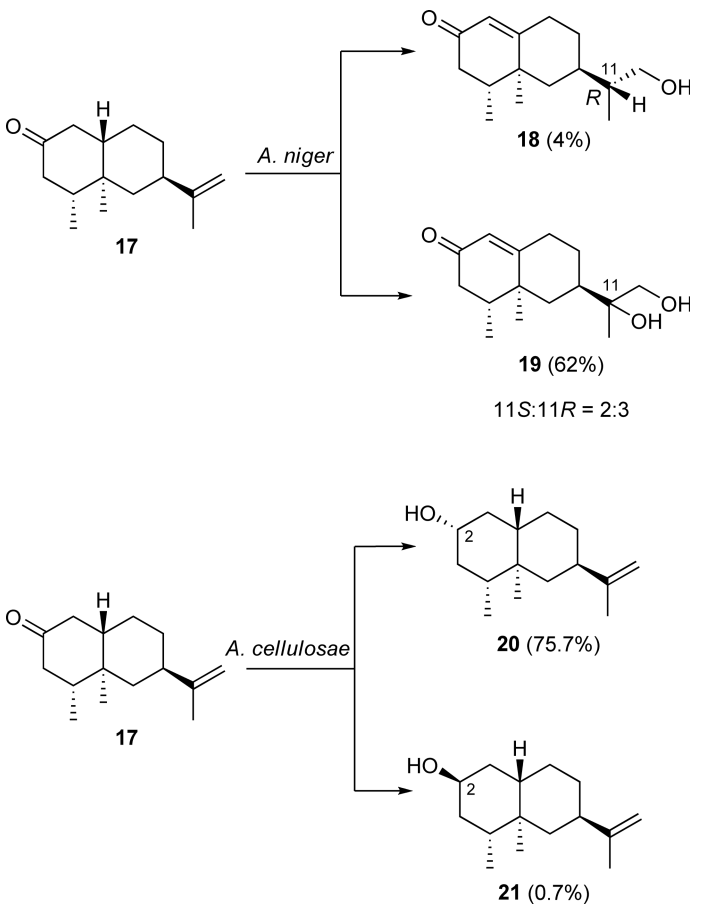
Mucor species was inoculated and cultivated under rotation (100 rpm) in Czapek-peptone medium (pH 7.0) to which (+)-1(10)-aristolene (**36**) (100 mg per 200 ml) was added. The crude metabolites contained **38** (0.9%) and **39** (0.7%) as very minor products (**Scheme 17**).

Although *Mucor* species produced a large amount of nootkatone (**2**) from valencene (**1**), however, only poor yield of similar products as those from valencene (**1**) was seen in the biotransformation of tricyclic substrate (**36**). Possible biogenetic pathway of (+)-1(10)-aristolene (**36**) is shown in **Scheme 18**.

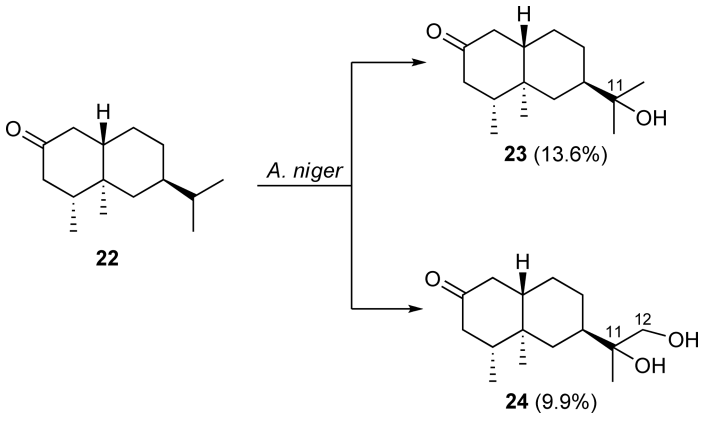
Aspergillus niger was inoculated and cultivated under rotation (100 rpm) at 30 °C for 3 days. (+)-1(10)-Aristolene (**36**) (100 mg 200 ml^{-1}) was added to the medium and further maintained for 7 days. From the crude metabolites, four new metabolic products (**42**, 1.3%), (**43**, 3.2%), (**44**, 0.98%), and (**45**, 2.8%) were obtained in very poor yield (**Scheme 19**). Possible metabolic pathways of **36** by *A. niger* are shown in **Scheme 20**.

Commercially available (+)-1(10)-aristolene (**36**) was treated with *Diplodia gossypina* and *Bacillus megaterium*. Both microorganisms converted **36** into four (**46–49**; 0.8, 1.1, 0.16, 0.38%) and six metabolites (**40, 50–55**; 0.75, 1.0, 1.0, 2.0, 1.1, 0.5, 0.87%), together with **40** (0.75%), respectively (**Scheme 21**).[31]

It is noteworthy that *Chlorella* and *Mucor* species introduce hydroxyl group at C-2 of the substrate (**36**) as seen in biotransformation of valencene (**1**), while *D. gossypina* and *B. megaterium* oxidize C-2, C-8, C-9, and/or

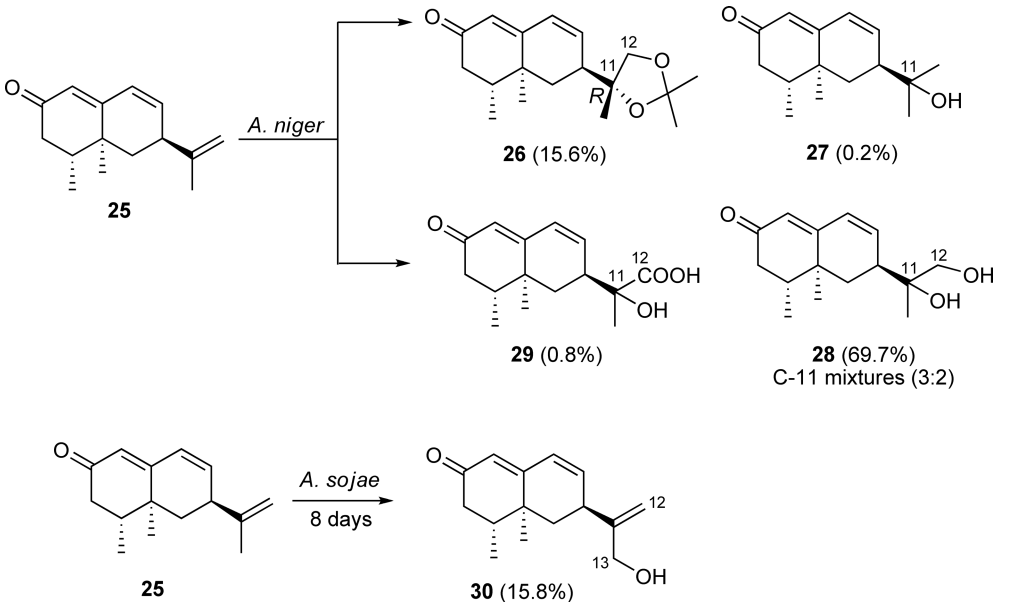


Scheme 9 Biotransformation of dihydronootkatone (**17**) by *Aspergillus niger* and *A. cellulosae.*

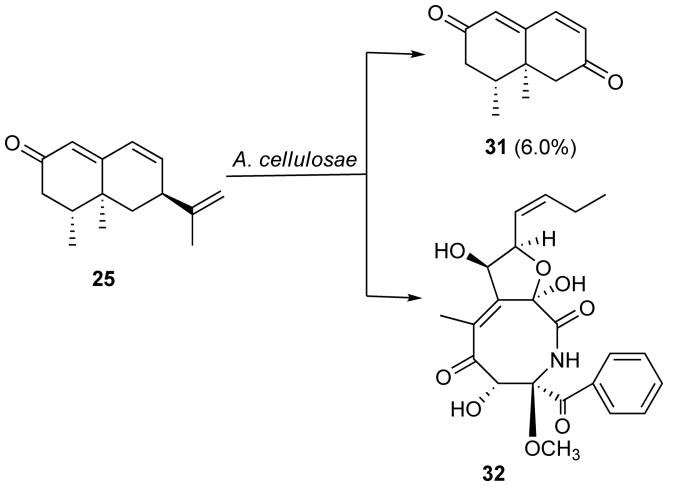


Scheme 10 Biotransformation of tetrahydronootkatone (**22**) by *Aspergillus niger*.

1,1-dimethyl group on a cyclopropane ring. *Aspergillus niger* oxidizes not only C-2, but also stereoselectively oxidize one of the gem-dimethyl groups on cyclopropane ring. Stereoselective oxidation of one of the gem-dimethyl of cyclopropane and cyclobutane derivatives is observed in biotransformation using mammals (see Section 3.20.3).



Scheme 11 Biotransformation of 8,9-dehydronootkatone (**25**) by *Aspergillus sojae*.

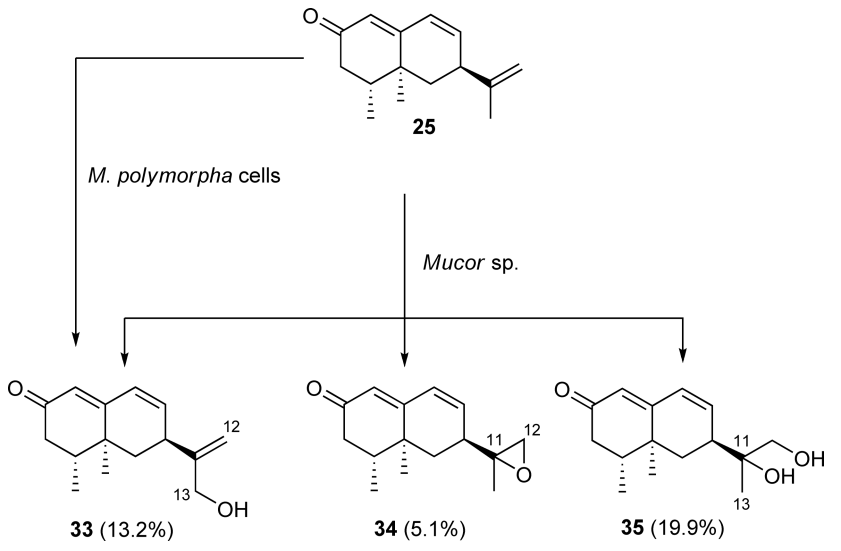


Scheme 12 Biotransformation of 8,9-dehydronootkatone (**25**) by *Aspergillus cellulosae*.

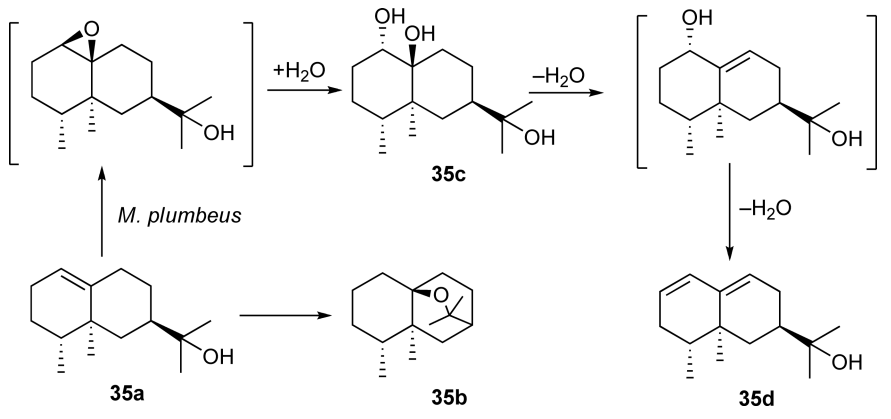
3.20.2.6 Biotransformation of Various Sesquiterpenoids by Microorganisms

Aromadendrane-type sesquiterpenoids have been found not only in higher plants but also in liverworts and marine sources. Three aromadendrenes (**56**, **57**, **58**) were biotransformed by *D. gossypina*, *B. megaterium*, and *Mycobacterium smegmatis*.[31] Aromadendrene (**56**) (800 mg) was converted by *B. megaterium* to yield a diol (**59**) and a triol (**60**) of which **59** (7 mg) was a major product. The triol (**60**) was also obtained from the metabolite of (+)-(1*R*)-aromadendrene (**56**) by the plant pathogen *Glomerella cingulata*.[32] Allo-aromadendrene (**57**) (1.2 g) was also treated with *M. smegmatis* to yield **61** (10 mg) (**Scheme 22**).[31]

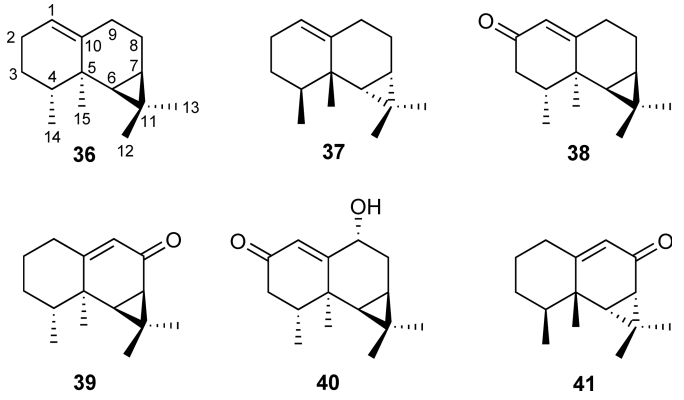
The same substrate was also incubated with *G. cingulata* to yield C-10 epimeric triol (**62**).[32] Globulol (**58**) (400 mg) was treated with *M. smegmatis* to give only a carboxylic acid (**63**) (210 mg). The same substrate (**58**) (1 g) was treated with *D. gossypina* and *B. megaterium* to yield two diols, **64** (182 mg), **65**, and a triol (**66**) from the former and **67**–**69** from the latter organism among which **64** (60 mg) was predominant.[31] *G. cingulata* and *Botrytis cinerea* also bioconverted globulol (**58**) into diol (**64**) regio- and stereoselectively (**Schemes 23** and **24**).[33]



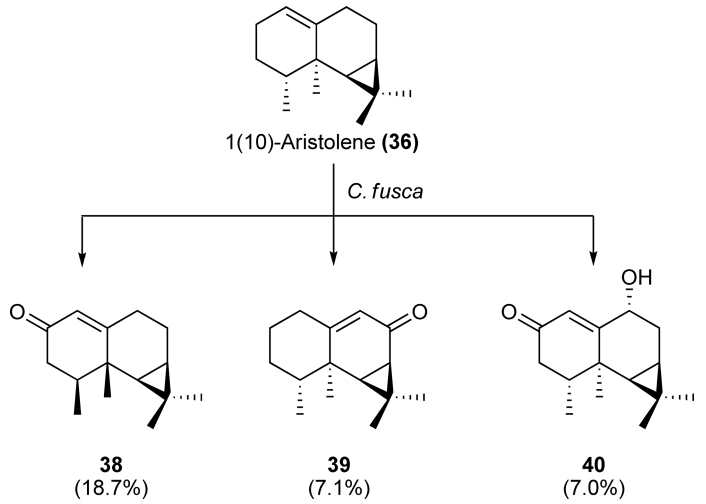
Scheme 13 Biotransformation of 8,9-dehydronootkatone (**25**) by *Marchantia polymorpha* and *Mucor* species.



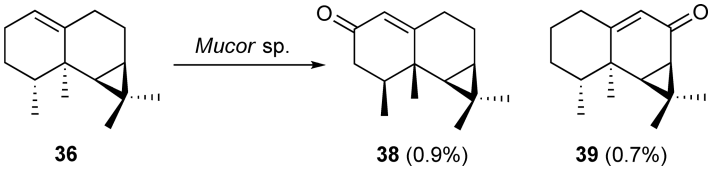
Scheme 14 Biotransformation of valerianol (**35a**) by *Mucor plumbeus.*



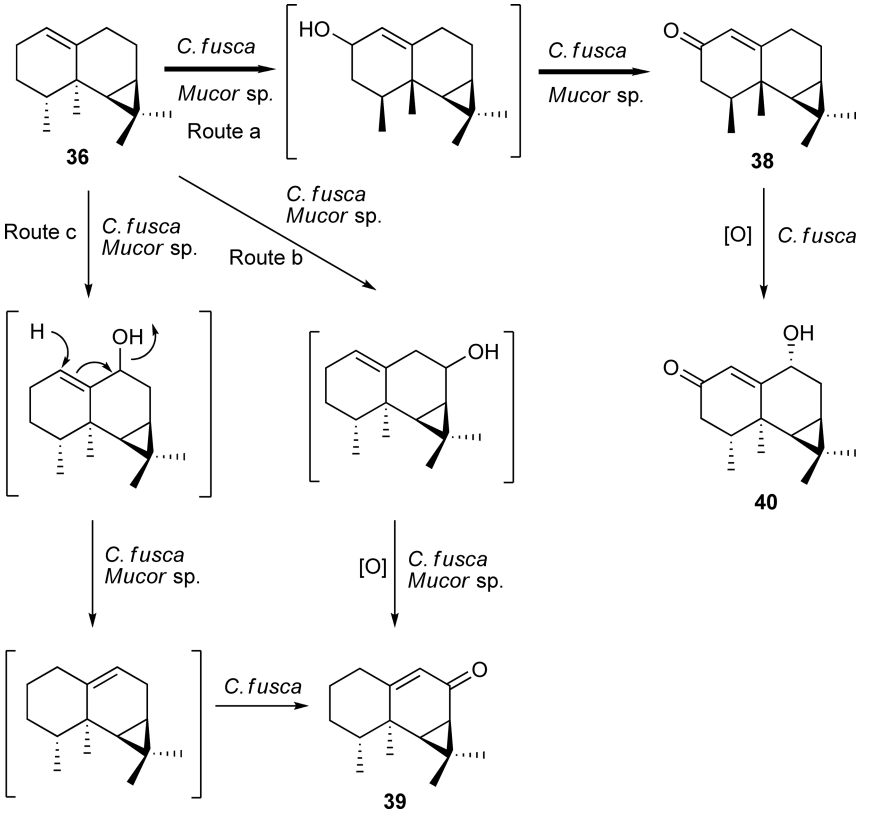
Scheme 15 Naturally occurring aristolane sesquiterpenoids.



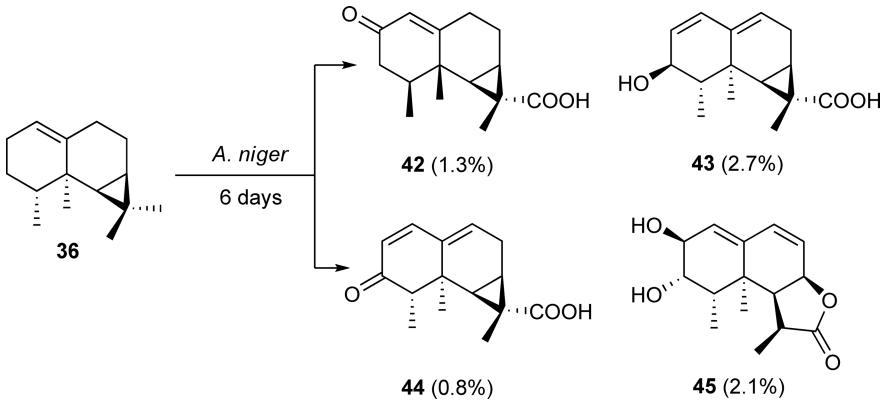
Scheme 16 Biotransformation of 1(10)-aristolene (**36**) by *Chlorella fusca*.



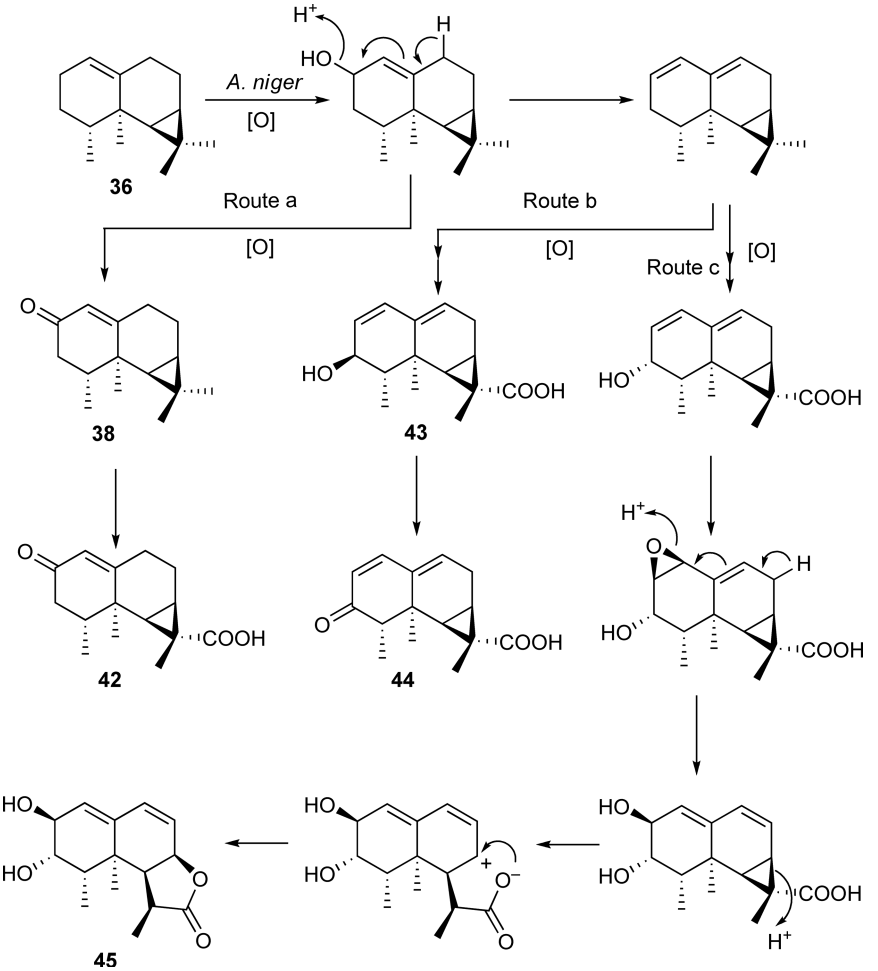
Scheme 17 Biotransformation of 1(10)-aristolene (**36**) by *Mucor* species.



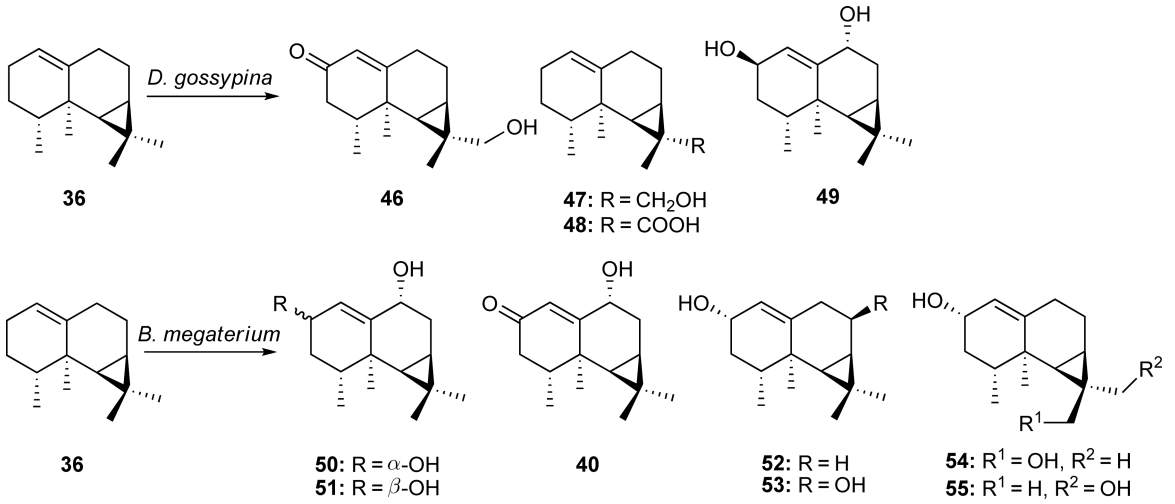
Scheme 18 Possible pathway of biotransformation of 1(10)-aristolene (**36**) by *Chlorella fusca* and *Mucor* species.



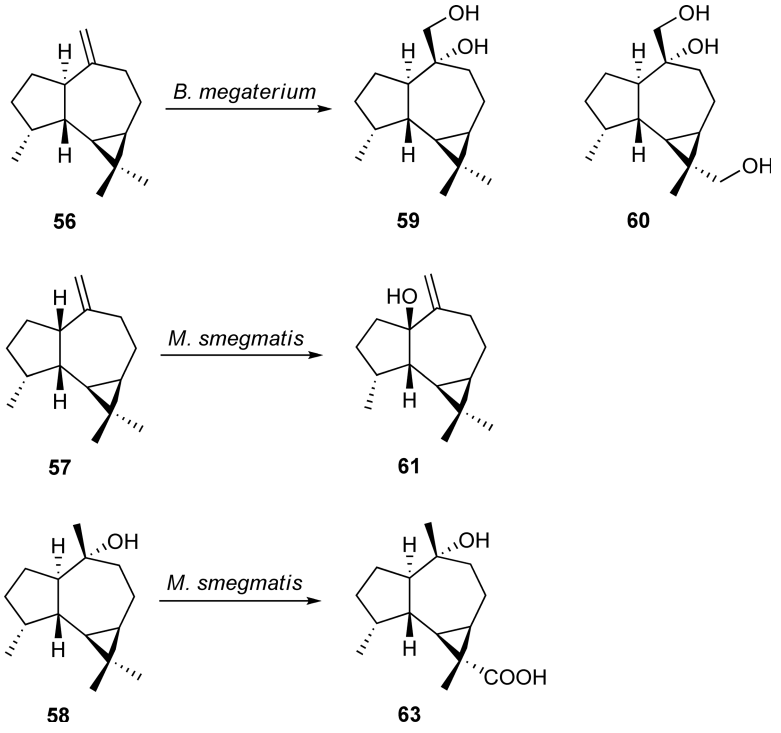
Scheme 19 Biotransformation of 1(10)-aristolene (**36**) by *Aspergillus niger.*



Scheme 20 Possible pathway of biotransformation of 1(10)-aristolene (**36**) by *Aspergillus niger.*

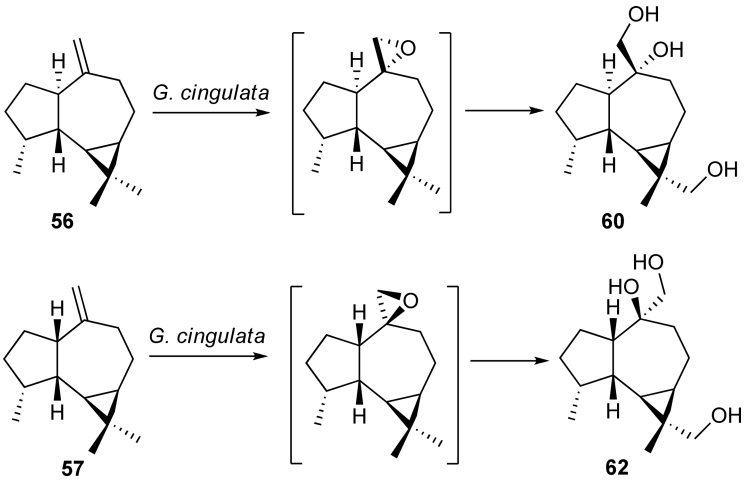


Scheme 21 Biotransformation of 1(10)-aristolene (**36**) by *Diplodia gossypina* and *Bacillus megaterium.*

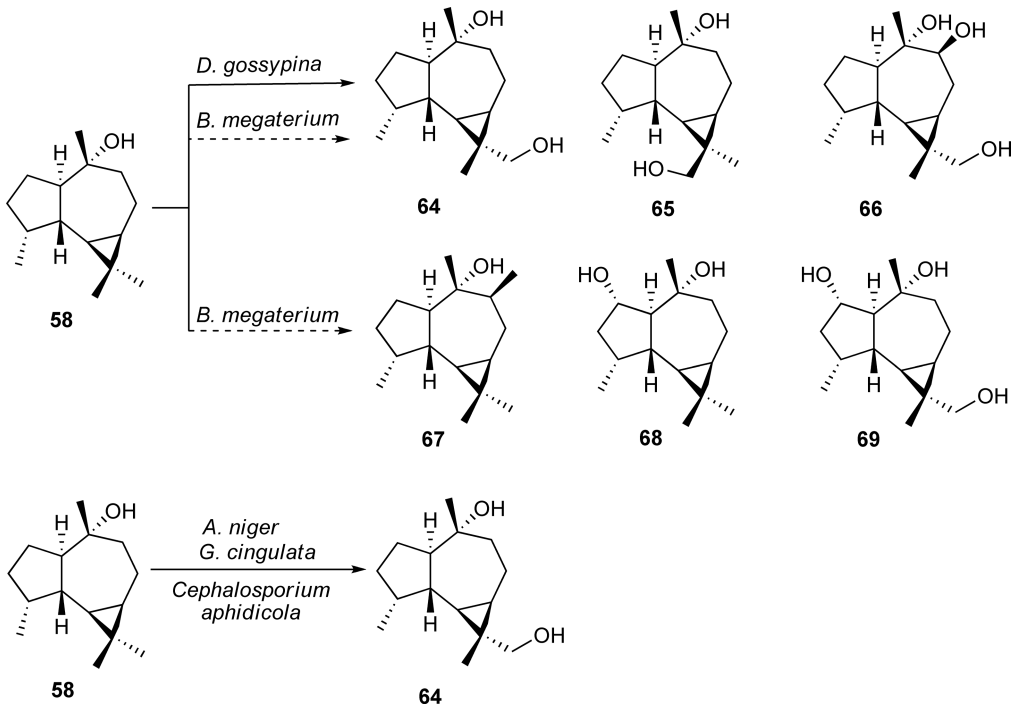


Scheme 22 Biotransformation of aromadendrene (**56**), alloaromadendrene (**57**), and globulol (**58**) by *Bacillus megaterium* and *Mycobacterium smegmatis*.

Globulol (**58**) (1.5 g) and 10-epiglobulol (**70**) (1.2 ml) were separately incubated with *Cephalosporium aphidicola* in shake culture for 6 days to give the same diol **64** (780 mg) as obtained from the same substrate by *B. megaterium* mentioned above and **71** (720 mg), respectively.[34] *Aspergillus niger* also converted globulol (**58**) and epiglobulol (**70**) into a diol (**64**), three 13-hydroxylated globulol (**71, 72, 74**), and 4α-hydroxylated product (**73**). The epimerization at C-4 is a very rare example.[35]



Scheme 23 Biotransformation of aromadendrene (**56**) and alloaromadendrene (**57**) by *Glomerella cingulata.*

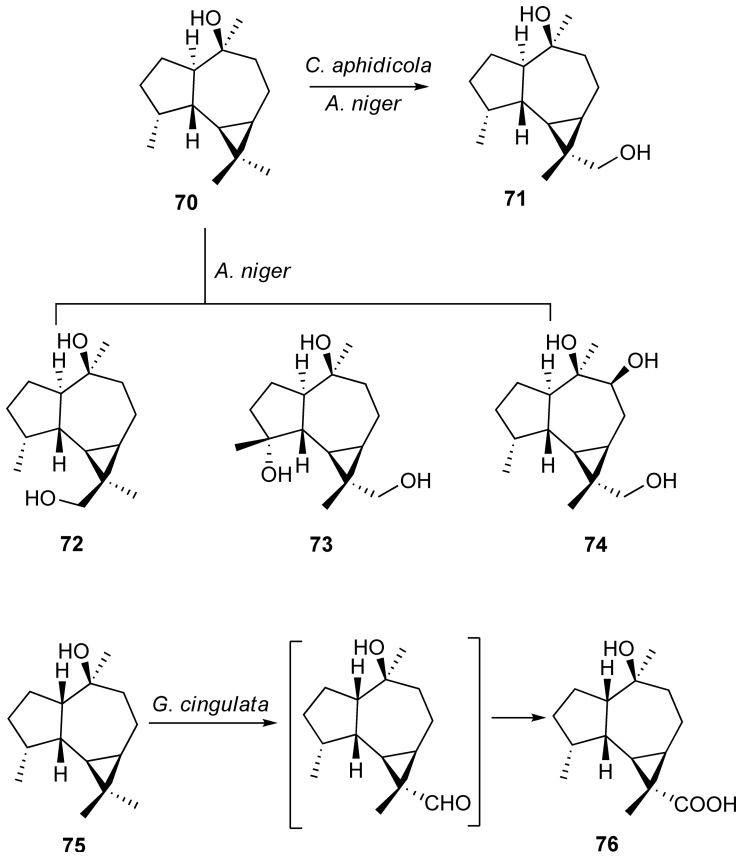


Scheme 24 Biotransformation of globulol (**58**) by various microorganisms.

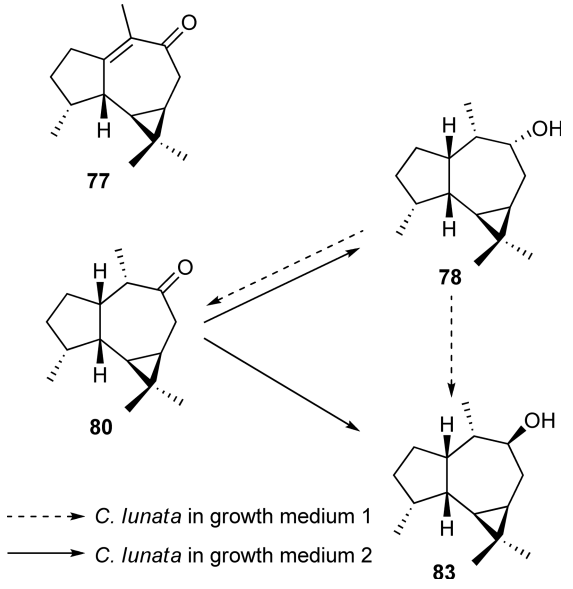
Ledol (**75**), an epimer at C-1 of globulol, was incubated with *G. cingulata* to yield C-13 carboxylic acid (**76**) (**Scheme 25**).[33]

Squamulosone (**77**), aromadendr-1(10)-en-9-one isolated from *Hyptis verticillata* (Labiatae), was reduced chemically to give **78–82** which were incubated with the fungus *Curvularia lunata* in two different growth media (**Scheme 26**).

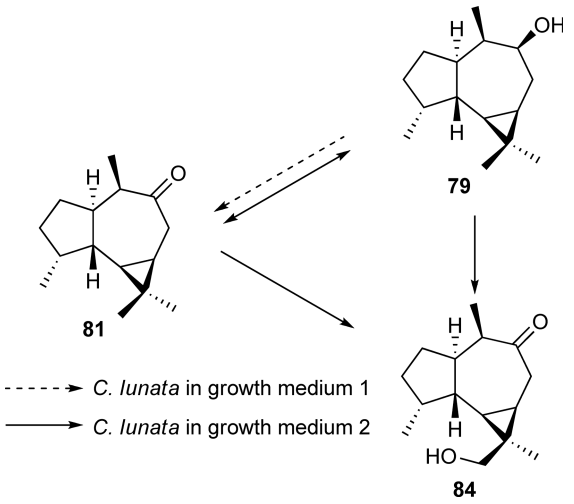
From tetrahydro derivative (**78**) of **77**, two metabolites **80** and **83** were obtained by the same fungus as described above. Compounds **79** and **80** were metabolized to give ketone **81** as the sole product and **78** and **83**, respectively. From compound **81**, two metabolites, **79** and **84** were obtained (**Scheme 27**). From the metabolite of the substrate (**82**), five products (**84–88**) were isolated (**Scheme 28**).[36]



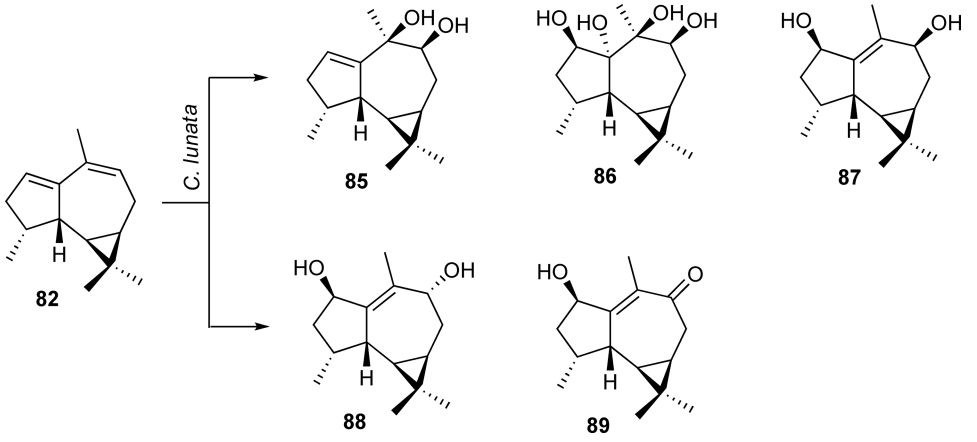
Scheme 25 Biotransformation of 10-epi-globulol (**70**) and ledol (**75**) by *Cephalosporium aphidicola*, *Aspergillus niger*, and *Glomerella cingulata.*



Scheme 26 Biotransformation of aromadendra-9-one (**80**) by *Curvularia lunata.*



Scheme 27 Biotransformation of 10-epi-aromadendra-9-one (**81**) by *Curvularia lunata*.

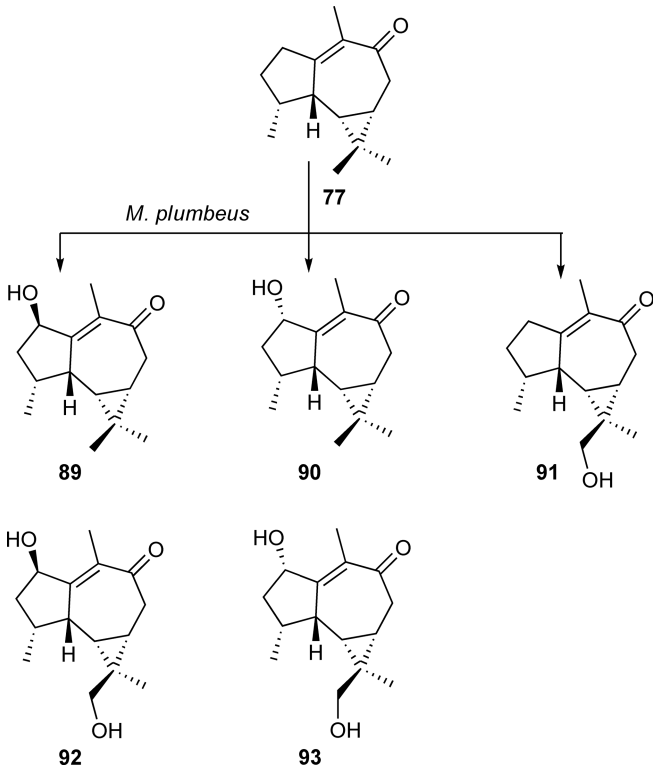


Scheme 28 Biotransformation of aromadendra-1(10),9-diene (**82**) by *Curvularia lunata*.

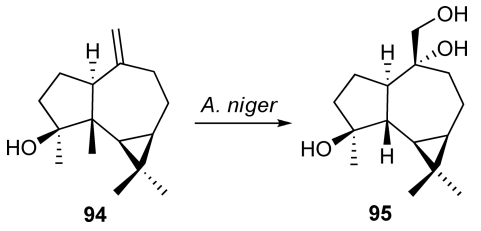
Squamulosone (**77**) was treated with the fungus *M. plumbeus* ATCC 4740 to give not only cyclopentanol derivatives (**89**, **90**) but also C-12 hydroxylated products (**91–93**) (**Scheme 29**).

Spathulenol (**94**), which is found in many essential oils, was fed by *A. niger* to give a diol (**95**).[37] *ent*-10β-Hydroxycyclocolorenone (**96**) and myli-4(15)-en-9-one (**96a**) isolated from the liverwort *Mylia taylorii* were incubated with *A. niger* IFO 4407 to give C-10 epimeric product (**97**)[38] and 12-hydroxylated product (**96b**), respectively (**Schemes 30** and **31**).[39]

(+)-*ent*-Cyclocolorenone (**98**), one of the major compounds isolated from the liverwort *Plagiochila sciophila*,[40,41] was treated with *A. niger* to yield three metabolites: 9-hydroxycyclocolorenone (**99**, 15.9%), 12-hydroxy-(+)-cyclocolorenone (**100**, 8.9%), and a unique cyclopropane-cleaved metabolite, 6β-hydroxy-4,11-guaiadien-3-one (**101**, 35.9%) and 6β,7β-dihydroxy-4,11-guaiadien-3-one (**102**, trace) of which **101** was a major component. The enantiomer (**103**) of **98** isolated from *Solidago altissima* was biotransformed by the same organism to give 13-hydroxycyclocolorenone (**103a**, 65.5%), the enantiomer of **100**, 1β,13-dihydroxycyclocolorenone (**103b**, 5.0%) and its C11-epimer (**103c**).[30] It is noteworthy that no cyclopropane-cleaved compounds from **103** have been detected in the crude metabolites even in GC–MS analysis (**Scheme 32**).



Scheme 29 Biotransformation of squamulosone (**77**) by *Mucor plumbeus*.

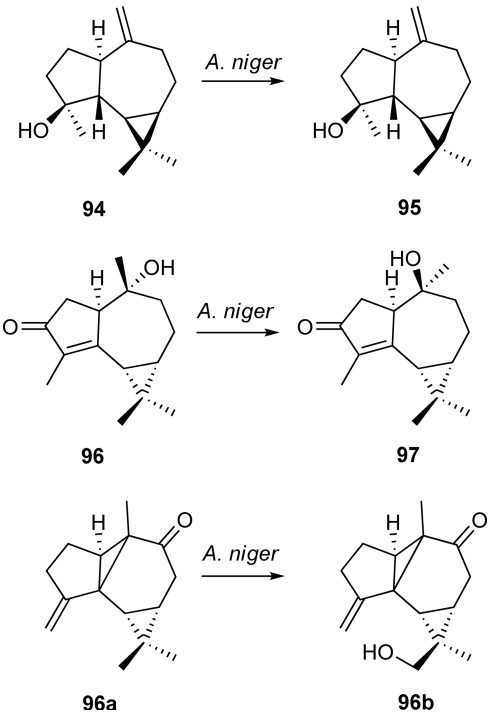


Scheme 30 Biotransformation of spathulenol (**94**) by *Aspergillus niger*.

Plagiochiline A (**104**) which are potent insect antifeedants showing cytotoxicity and piscidal activity are very pungent 2,3-secoaromadendrane sesquiterpenoids having 1,1-dimethyl cyclopropane ring, isolated from the liverwort *Plagiochila fruticosa*. Plagiochilide (**105**) is the major component of this liverwort. In order to get a more pungent component, the lactone (**105**, 101 mg) was incubated with *A. niger* to give two metabolites **106** (32.5%) and **107** (9.7%). Compound **105** was incubated in *A. niger* including 1-aminobenzotriazole, the inhibitor of CYP450, to produce only **106**, since this enzyme plays an important role in the formation of carboxylic acid (**107**) from primary alcohol (**106**). Unfortunately, the two metabolites were not pungent (**Scheme 33**).[30,42]

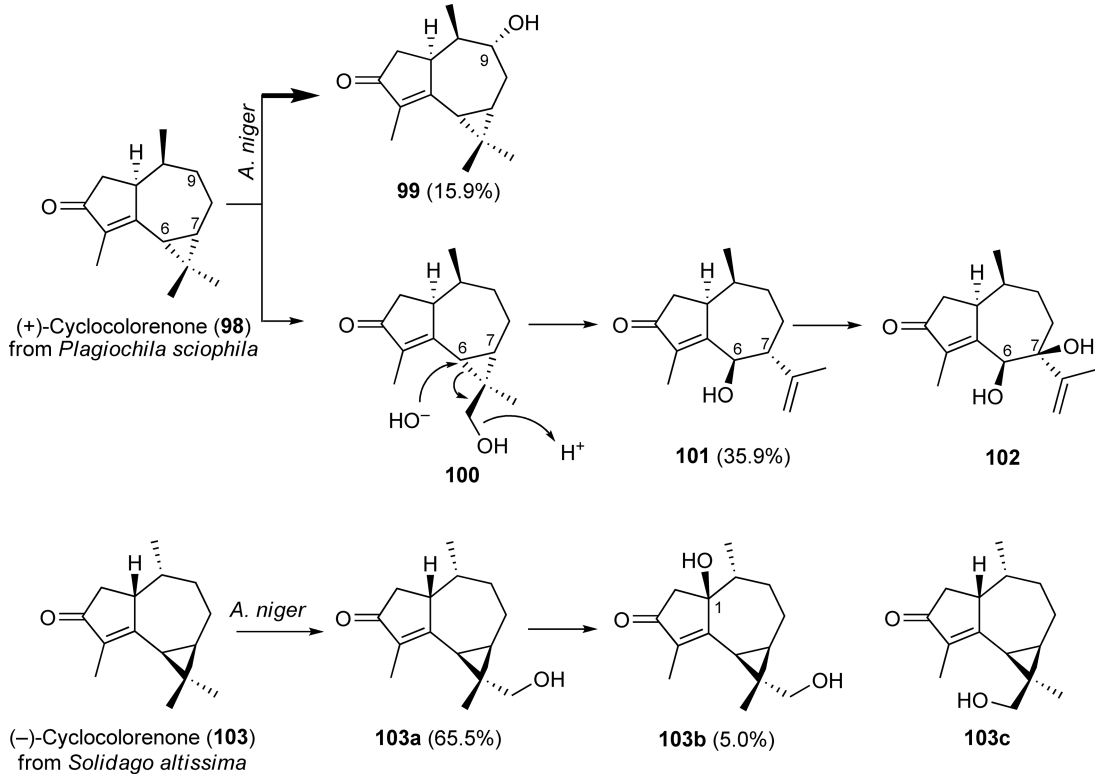
Partheniol, 8α-hydroxybicyclogermacrene (**108**) isolated from *Parthenium argentatum* × *P. tomentosa* was cultured in the media of *Mucor circinelloides* ATCC 15242 to yield six metabolites, a humulane (**109**), three maaliane- (**110, 112, 113**), an aromadendrane- (**111**), and a tricyclohumulane triol (**114**), the isomer of compound (**111**). Compounds **110, 111,** and **114** were isolated as their acetates (**Scheme 34**).

Compound **110** might originate from the substrate by acidic *trans*-annular cyclization since the broth had a pH of 6.4 just before extraction.[43]

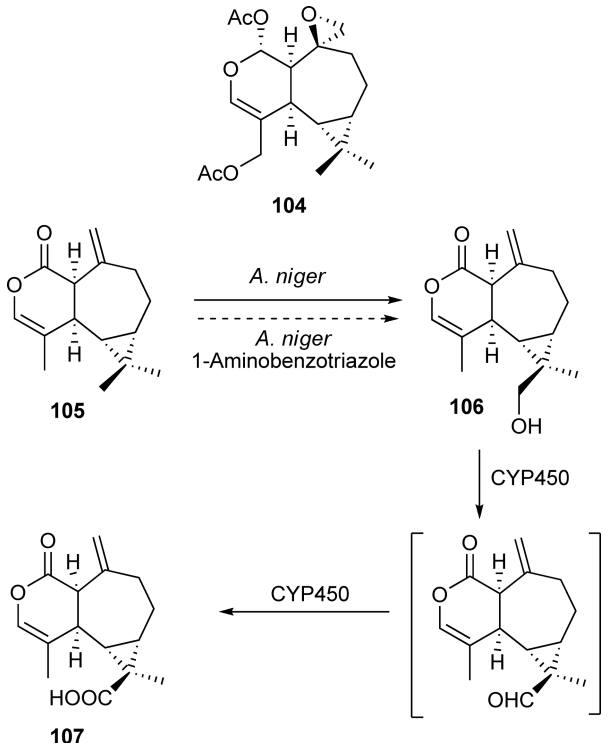


94 **95** **96** **97** **96a** **96b**

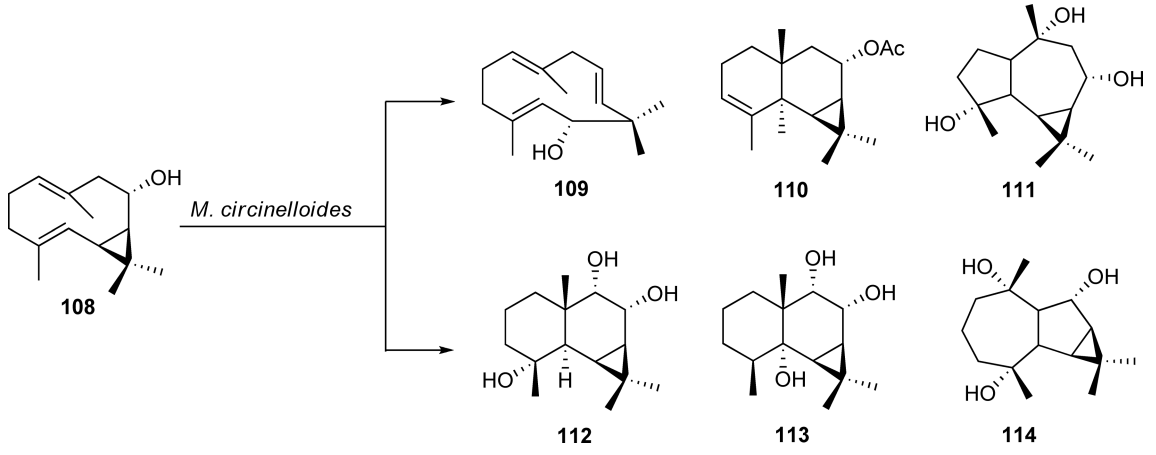
Scheme 31 Biotransformation of spathulenol (**94**), *ent*-10β-hydroxycyclocolorenone (**96**), and myli-4(15)-en-9-one (**96a**) by *Aspergillus niger.*



Scheme 32 Biotransformation of (+)-cyclocolorenone (**98**) and (–)-cyclocolorenone (**103**) by *Aspergillus niger.*



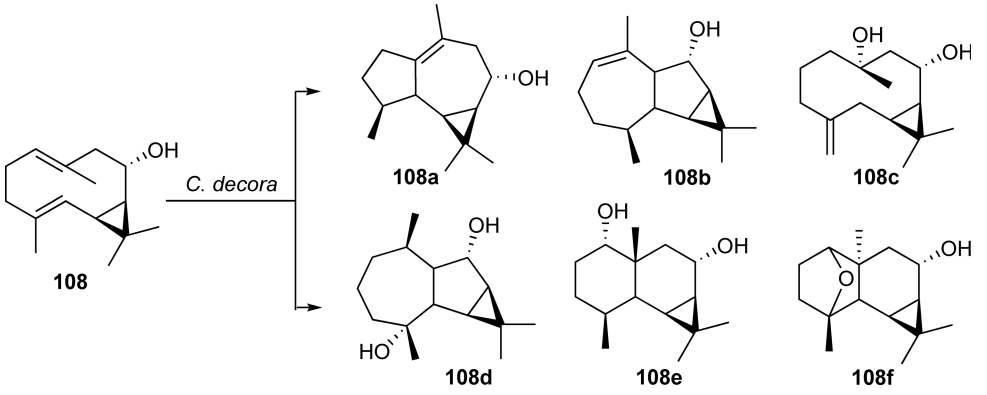
Scheme 33 Biotransformation of plagiochiline C (**104**) by *Aspergillus niger*.



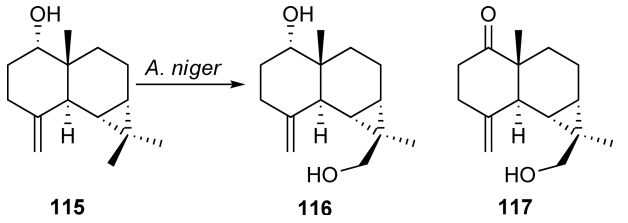
Scheme 34 Biotransformation of 8α-hydroxybicyclogermacrene (**108**) by *Mucor circinelloides*.

The same substrate (**108**) was incubated with the fungus *Calonectria decora* to yield six new metabolites (**108a–108f**). In these reactions hydroxylation, epoxidation, and *trans*-annular cyclization were evidenced (**Scheme 35**).[44]

ent-Maaliane-type sesquiterpene alcohol, 1α-hydroxymaaliene (**115**), isolated from the liverwort *M. taylorii*, was treated with *A. niger* to yield two primary alcohols (**116, 117**).[45] Such an oxidation pattern of 1,1-dimethyl group on cyclopropane ring has been found in aromadendrane series as described above and mammalian biotransformation of a monoterpene hydrocarbon, Δ^3-carene (**Scheme 36**).[46]



Scheme 35 Biotransformation of 8α-hydroxybicyclogermacrene (**108**) by *Calonectria decora.*



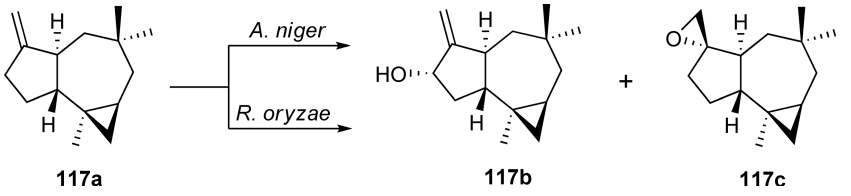
Scheme 36 Biotransformation of 1α-hyroxymaaliene (**115**) by *Aspergillus niger.*

9(15)-Africanene (**117a**), a tricyclic sesquiterpene hydrocarbon isolated from marine soft corals of *Simularia* species, was biotransformed by *A. niger* and *Rhizopus oryzae* for 8 days to give 10α-hydroxy- (**117b**) and 9α,15-epoxy derivative (**117c**) (**Scheme 37**).[47]

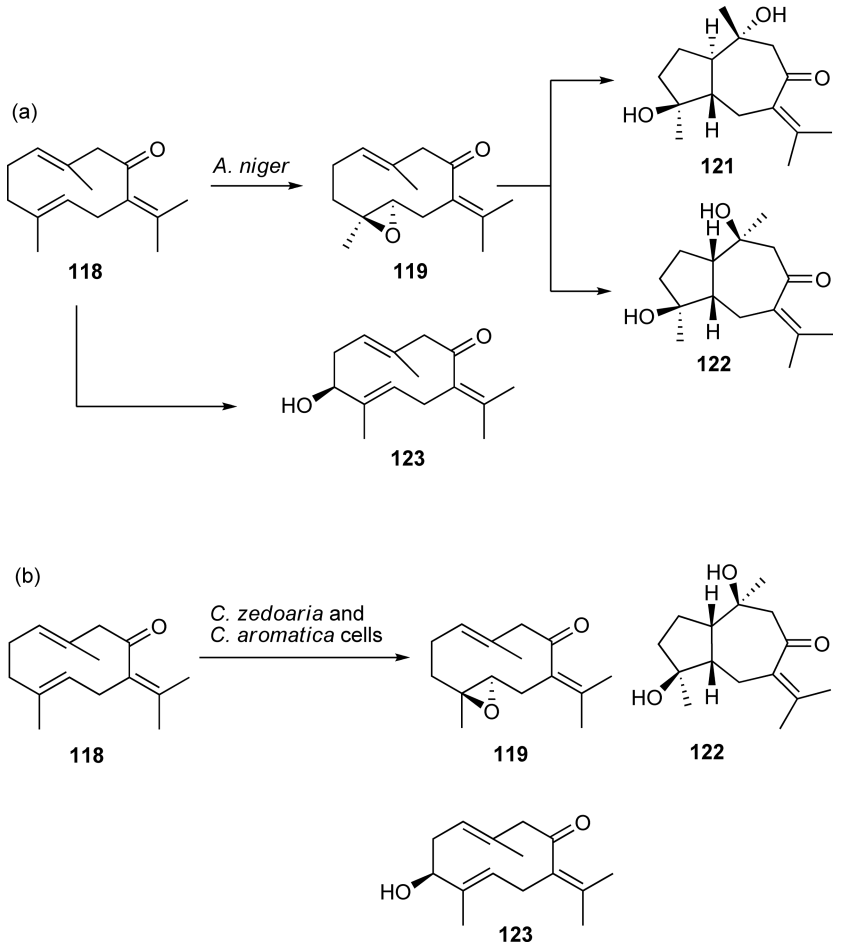
Germacrone (**118**), (+)-germacrone-4,5-epoxide (**119**) and curdione (**120**) isolated from *Curcuma aromatica* which has been used as crude drug was incubated with *A. niger*. From compound **119** (700 mg), two naturally occurring metabolites, zedoarondiol (**121**) and isozedoarondiol (**122**) were obtained.[48] Compound **119** was cultured in callus of *C. zedoaria* and *C. aromatica* to give the same secondary metabolites **121**, **122**, and **124** (**Schemes 38**(**a**) and **38**(**b**)).[49]

Aspergillus niger biotransformed germacrone (**118**, 3 g) to very unstable 3β-hydroxygermacrone (**123**), and 4,5-epoxygermacrone (**119**) which was further converted into two guaiane sesquiterpenoids (**121**) and **122** through *trans*-annular type reaction (**Scheme 38a**).[48] The same substrate was incubated in the microorganism, *Cunninghamella blakesleeana* to yield germacrone-4,5-epoxide (**119**),[50] while treatment of **118** in the callus of *C. zedoaria* gave four metabolites **121**, **122**, **125**, and **126** (**Scheme 39**).[51]

The same substrate (**118**) was treated with plant cell cultures of *Solidago altissima* (Asteraceae) for 10 days to give various hydroxylated products (**121**, **127**, **125**, **128–132**).[51] Guaiane (**121**) underwent further rearrangement by C4–C5 cleavage and C5–C10 *trans*-annular cyclization to the bicyclic hydroxyketone (**128**) and diketone (**129**) (**Scheme 40**).[51]



Scheme 37 Biotransformation of 9(15)-africanene (**117a**) by *Aspergillus niger* and *Rhizopus oryzae.*



Scheme 38 Biotransformation of germacrone (**118**) by *Aspergillus niger*.

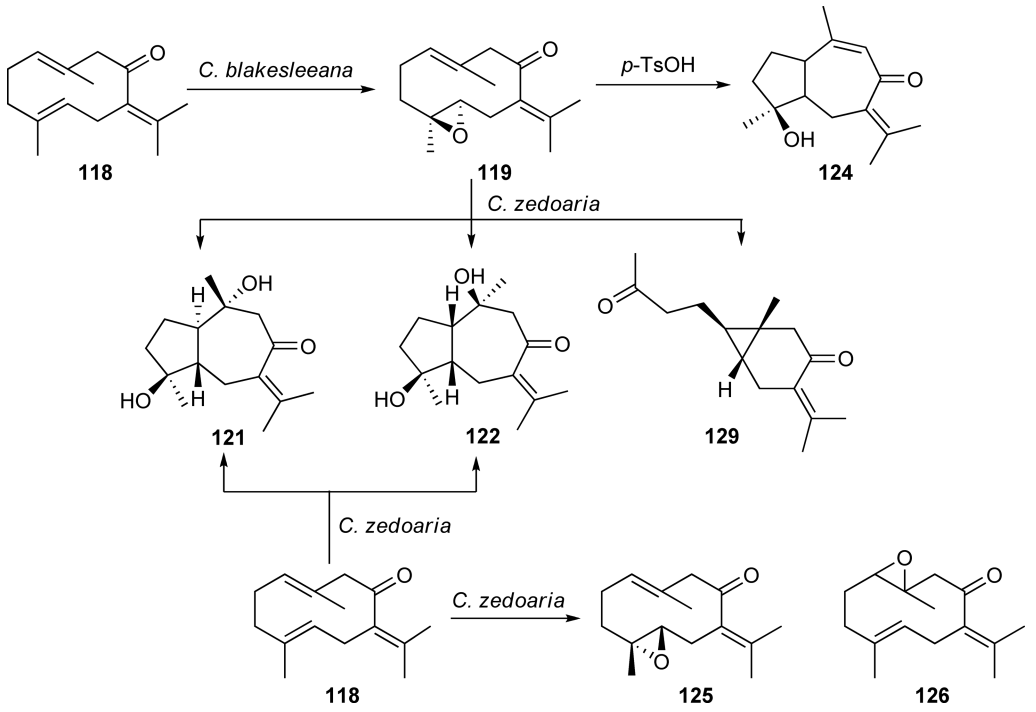
Curdione (**120**) was also treated with *A. niger* to yield two allylic alcohols (**133, 134**) and a spirolactone (**135**). *Curcuma aromatica* and *C. wenyujin* produce spirolactone (**135**) which might be formed from curdione through *trans*-annular reaction *in vivo* and which was biotransformed to spirolactone diol (**135**) (**Scheme 41**).[52,53]

Aspergillus niger also converted shiromodiol diacetate (**136**) isolated from *Neolitsea sericea* into 2β-hydroxy derivative (**137**) (**Scheme 42**).[39]

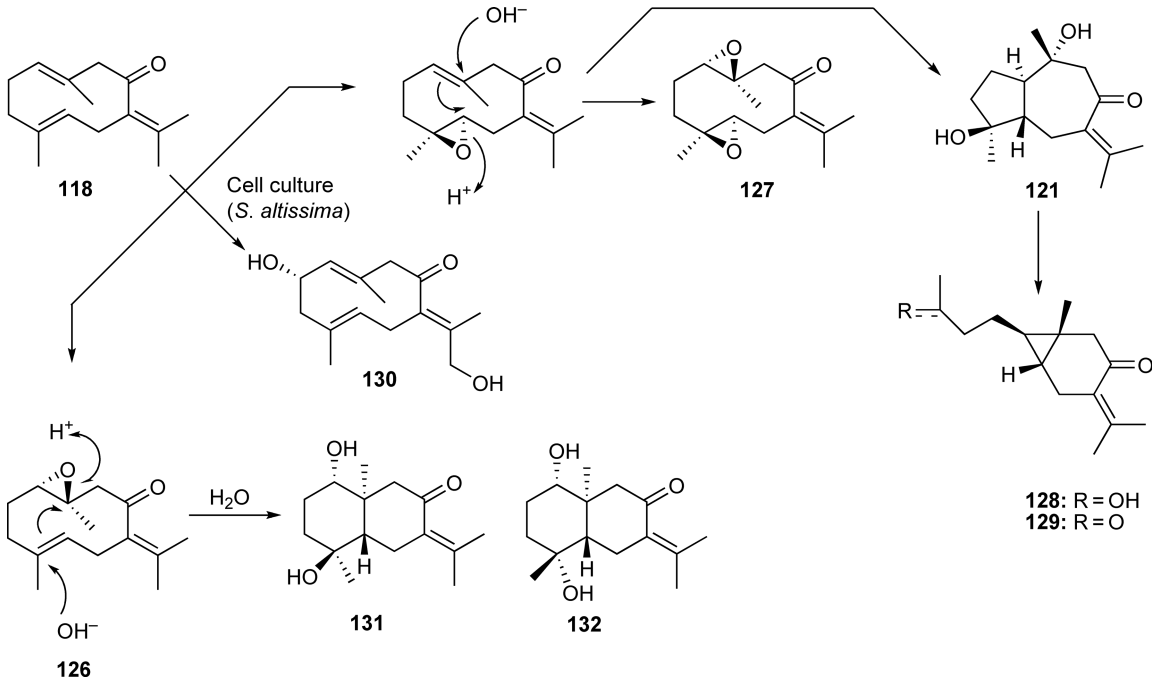
Twenty strains of filamentous fungi and four species of bacteria were screened initially by thin-layer chromatography for their biotransformation capacity of curdione (**120**). *Mucor spinosus*, *M. polymorphosporus*, *Cunninghamella elegans*, and *Penicillium janthinellum* were found to be able to biotransform curdione (**120**) to more polar metabolites. Incubation of curdione with *M. spinosus*, which was a most potent strain to produce metabolites for 4 days using potato medium gave five metabolites (**134, 134a–134d**) among which compounds **134c** and **134d** are new products (**Scheme 43**).[54]

Many eudesmane-type sesquiterpenoids have been biotransformed by several fungi and various oxygenated metabolites obtained.

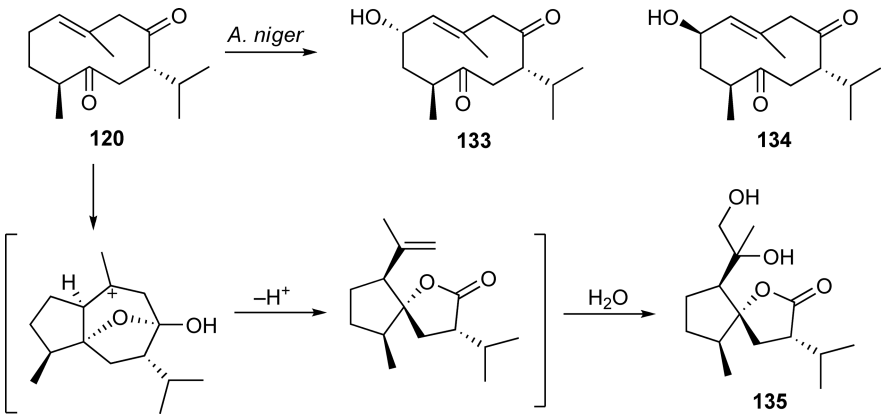
β-Selinene (**138**) is an ubiquitous sesquiterpene hydrocarbon of seed oil from many species of Apiaceae family, for example, *Cryptotenia canadensis* var. *japonica* which is widely used as a vegetable in Japanese soups. β-Selinene was biotransformed by plant pathogenic fungus *G. cingulata* to give an epimeric mixture (1:1) of 1β,11,12-trihydroxy product (**139**).[55] The same substrate was treated with *A. wentii* to give 2α,11,12-trihydroxy derivative (**140**).[56]



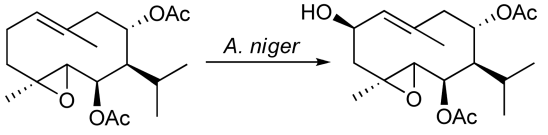
Scheme 39 Biotransformation of germacrone (**118**) by *Cunninghamella blakeleeana* and *Curcuma zedoaria* cells.



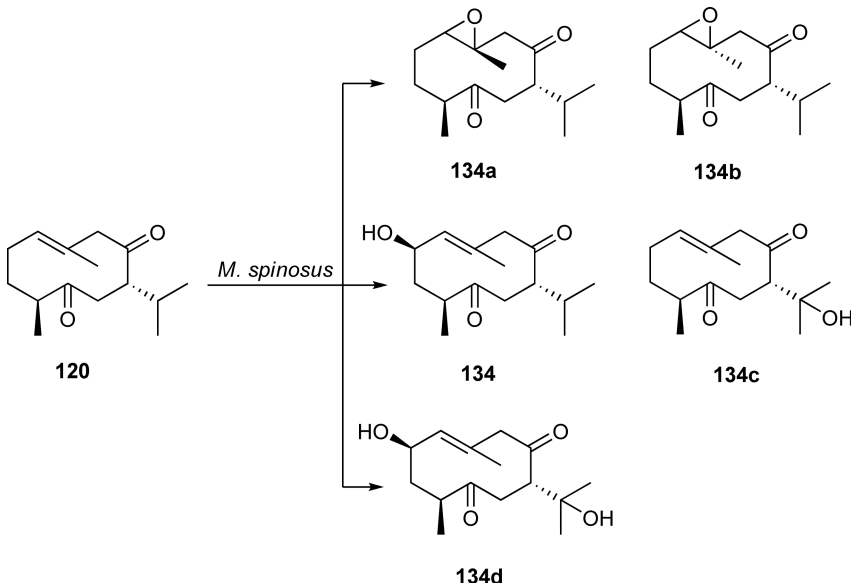
Scheme 40 Biotransformation of germacrone (**118**) by *Solidago altissima* cells.



Scheme 41 Biotransformation of curdione (**120**) by *Aspergillus niger.*



Scheme 42 Biotransformation of shiromodiol diacetate (**136**) by *Aspergillus niger.*



Scheme 43 Biotransformation of curdione (**120**) by *Mucor spinosus.*

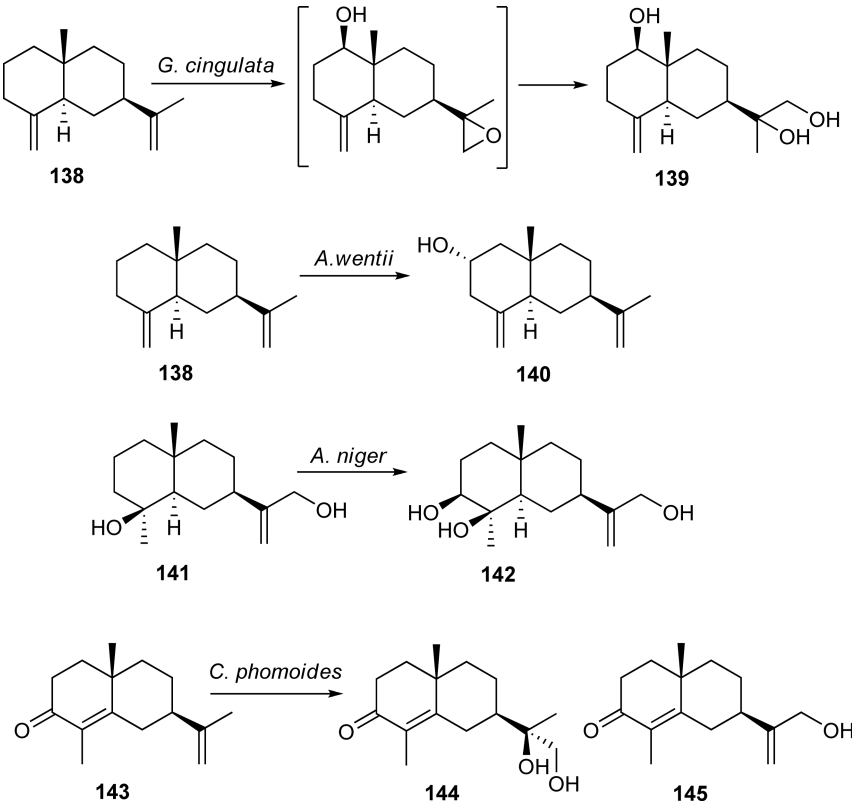
Eudesm-11(13)-en-4,12-diol (**141**) was biotransformed by *A. niger* to give 3β-hydroxy derivative (**142**).[57]

α-Cyperone (**143**) was fed by *Collectotrichum phomoides*[5] to yield 11,12-diol (**144**) and 12-monool (**145**) (**Scheme 44**).[37]

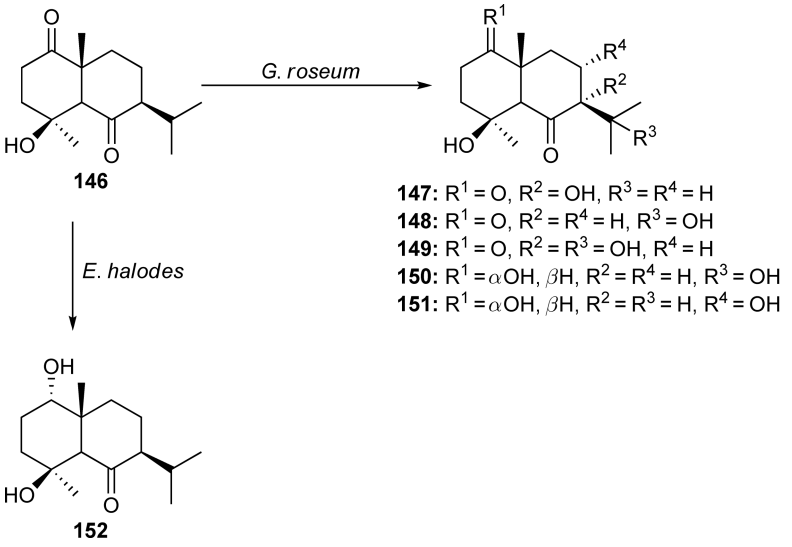
The filamentous fungi *Gliocladium roseum* and *Exserohilum halodes* were used as the bioreactors for 4β-hydroxyeudesmane-1,6-dione (**146**) isolated from *Sideritis varoi* subsp. *cuatrecasasii*. The former fungus transformed **146** to 7α-hydroxyl- (**147**), 11-hydroxy- (**148**), 7α,11-dihydroxy- (**149**), 1α,11-dihydroxy- (**150**), and 1α,8α-dihydroxy derivatives (**151**), while *E. halodes* gave only 1α-hydroxy product (**152**) (**Scheme 45**).[58]

Orabi[59] reported that *Beauvaria bassiana* is the most efficient microorganism to metabolize plectanthone (**152a**) among 20 microorganisms, such as *Absidia glauca*, *Aspergillus flavipes*, *Beauvaria bassiana*, *Cladosporium resinae*, and *Penicillium frequentans*. The substrate **152a** was incubated with *B. bassiana* to give metabolites **152b** (2.1%), **152c** (21.2%), **152d** (2.5%), **152e** (no data), and **152f** (1%) (**Scheme 46**).

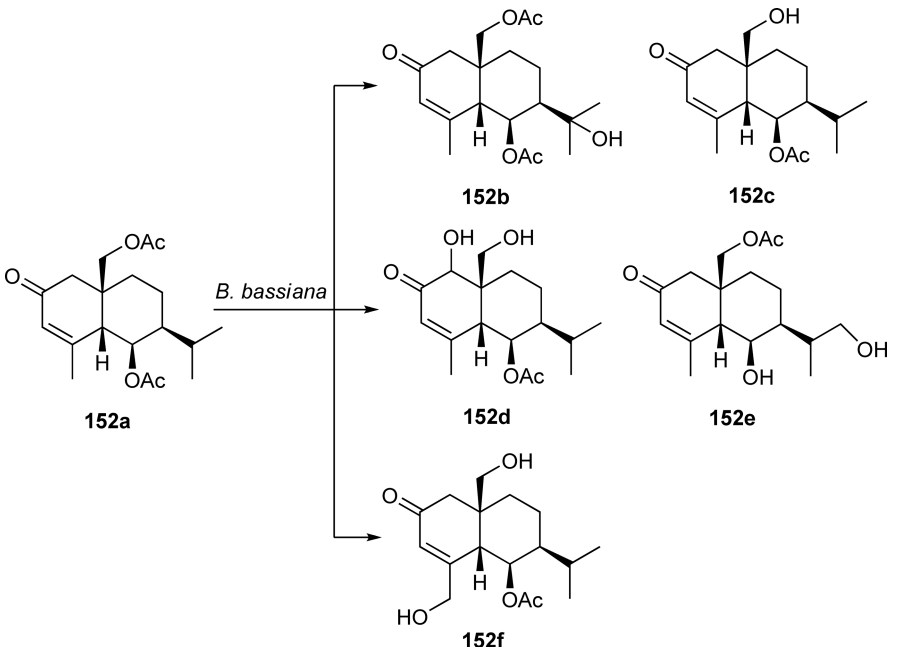
(−)-α-Eudesmol (**153**) isolated from the liverwort, *Porella stephaniana* was treated with *A. cellulosae* and *A. niger* to give 2-hydroxy (**154**) and 2-oxo derivatives (**155**) among which the latter product was predominantly obtained. This bioconversion was completely blocked by 1-aminobenzotriazole, CYP450 inhibitor. Compound **155** has been known as a natural product, isolated from *Pterocarpus santalinus*.[60] Biotransformation of α-eudesmol (**153**) isolated from the dried *Atractylodes lancea* was reinvestigated by *A. niger* to give 2-oxo-11,12-dihydro-α-eudesmol (**156**) together with 2-hydroxy- (**154**), 2-oxo-α-eudesmol (**155**). β-Eudesmol (**157**) was treated with *A. niger*, with the same culture medium to yield 2α- (**158**) and 2β-hydroxy-α-eudesmol (**159**) and 2α,11,12-trihydroxy-β-eudesmol (**160**) and 2-oxo derivative (**161**) which was further isomerized to compound **162** (**Scheme 47**).[60,61]



Scheme 44 Biotransformation of eudesmenes (**138**, **141**, **143**) by *Aspergillus wentii*, *Glomerella cingulata*, and *Collectotrichum phomoides*.

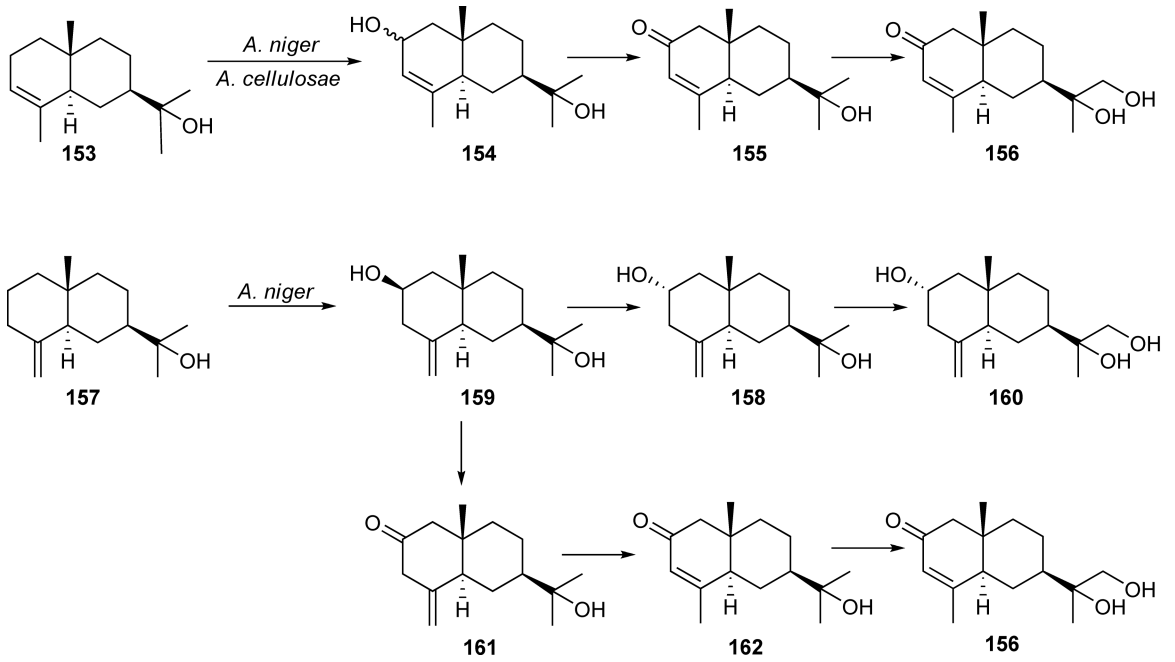


Scheme 45 Biotransformation of 4β-hydroxy-eudesmane-1,6-dione (**146**) by *Gliocladium roseum* and *Exserohilum halodes.*

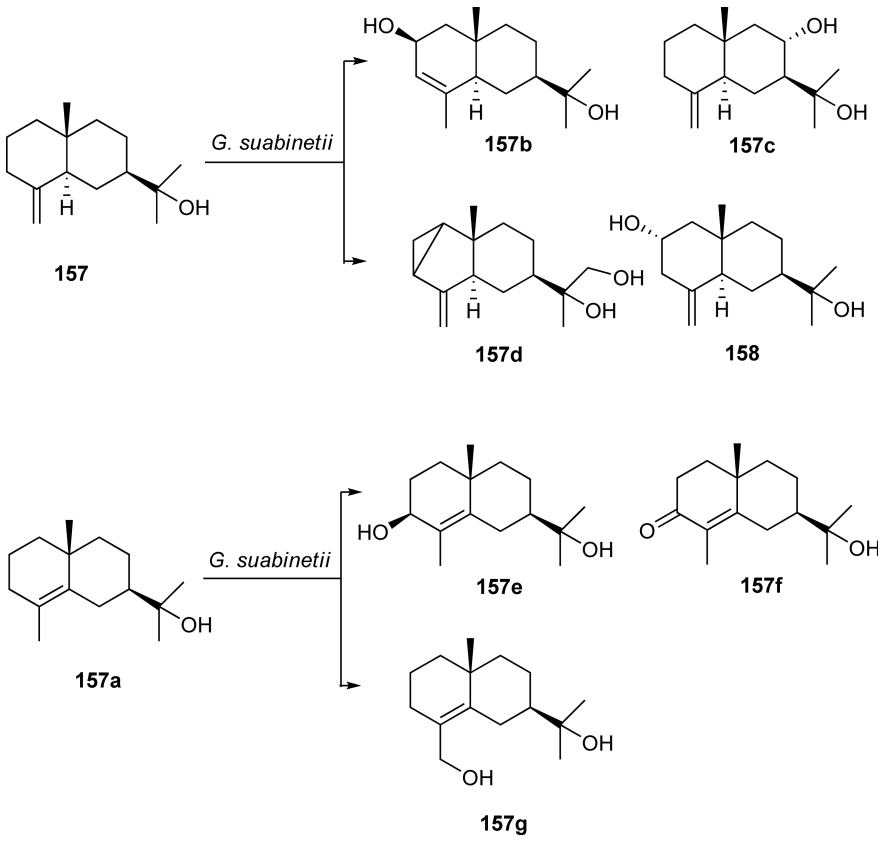


Scheme 46 Biotransformation of eudesmenone (**152a**) by *Beauvaria bassiana.*

Three new hydroxylated metabolites (**157b–157d**) along with a known **158** and **157e–157g** were isolated from the biotransformation reaction of a mixture of β-eudesmol (**157**) and γ-eudesmol (**157a**) by *Gibberella suabinetii.* The metabolites proved a super activity of the hydroxylase, dehydrogenase, and isomerase enzymes. The hydroxylation is a common feature; on contrary, cyclopropyl ring formation-like compound (**158d**) is very rare (**Scheme 48**).[62]



Scheme 47 Biotransformation of α-eudesmol (**153**) and β-eudesmol (**157**) by *Aspergillus niger* and *A. cellulosae.*



Scheme 48 Biotransformation of β-eudesmol (**157**) and γ-eudesmol (**157a**) by *Gibberella suabinetii.*

A furanosesquiterpene, atractylon (**163**) obtained from *Atractylodis* rhizomes was treated with the same fungus to yield atractylenolide III (**164**) possessing inhibition of increased vascular permeability in mice induced by acetic acid.[63]

The biotransformation of sesquiterpene lactones have been carried out by using different microorganisms.

Costunolide (**165**), a very unstable sesquiterpene γ-lactone, from *Saussurea radix*, was treated with *A. niger* to produce three dihydrocostunolides (**166–168**).[64] Costunolide is easily converted into eudesmanolides (**169–172**) in diluted acid, and thus **166–168** might be biotransformed after being cyclized in the medium including the microorganisms. If the crude drug including costunolide (**165**) is orally administered, **165** will be easily converted into **169–172** by the stomach juice (**Scheme 49**).

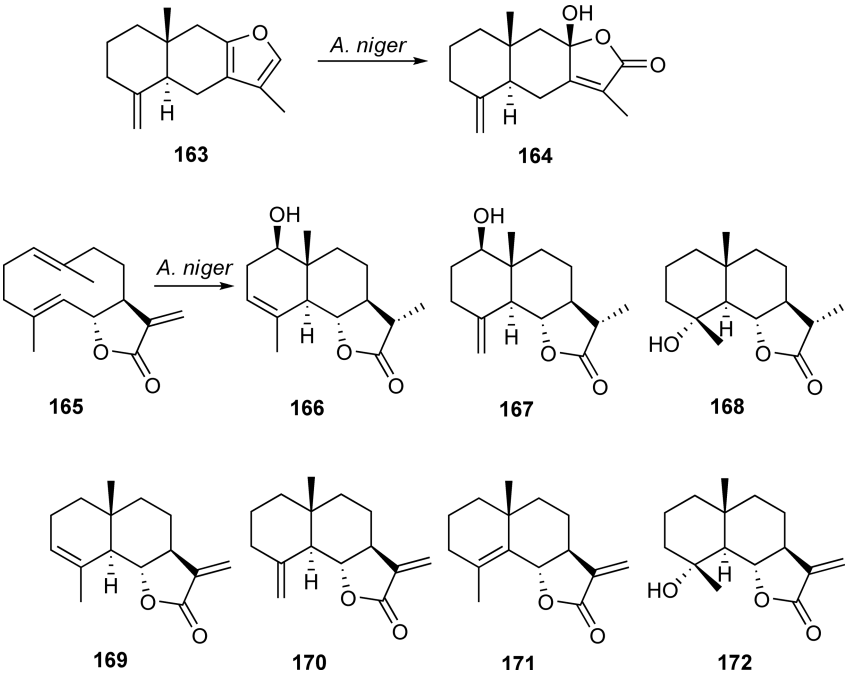
(+)-Costunolide (**165**), (+)-cnicin (**172a**), and (+)-salonitgenolide (**172b**) were incubated with *Cunninghamella echinulata* and *Rhizopus oryzae*.

The former fungus converted compound **165** into four metabolites: (+)-11β,13-dihydrocostunolide (**165a**), 1β-hydroxyeudesmanolide, (+)-santamarine (**166a**), (+)-reynosin (**166b**), and (+)-1β-hydroxyarbusculin A (**168a**), which might be formed from 1β,10α-epoxide (**166c**). Treatment of **172a** with *C. echinulata* gave (+)-salonitenolide (**172b**) (**Scheme 50**).[65]

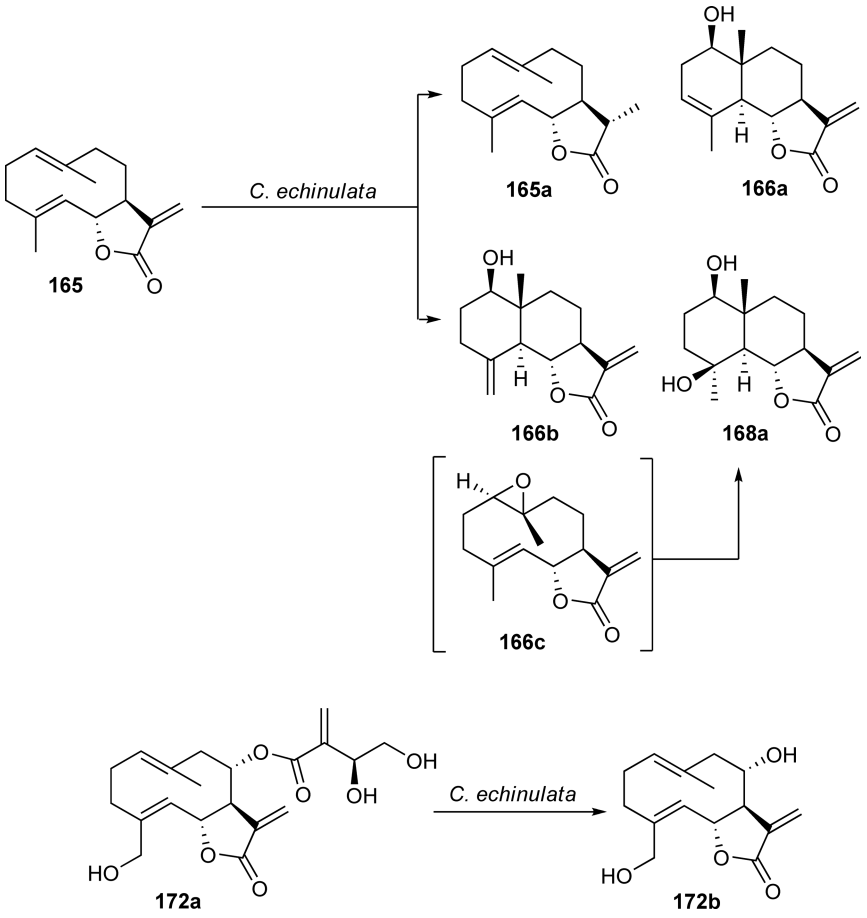
α-Cyclocostunolide (**169**), β-cyclocostunolide (**170**), and γ-cyclocostunolide (**171**) prepared from costunolide were cultivated in *A. niger*. From the metabolite of **169**, four dihydro lactones (**173–176**) were obtained, among which sulfur-containing compound (**176**) was predominant (**Scheme 51**).

The same substrate (**169**) was cultivated for 3 days by *A. cellulosae* to yield a sole metabolite, 11β,13-dihydro-α-cyclocostunolide (**177**). Possible metabolic pathways of **169** by both microorganisms were shown in **Scheme 52**.

A double bond at C11–C13 of **169** was first reduced stereoselectively to yield **177**, followed by oxidation at C-2 to give **173**, and then further oxidation occurred to furnish two hydroxyl derivatives (**174**, **175**) in *A. niger*. The sulfide compound (**176**) might be formed from **175** or by Michael condensation of ethyl 2-hydroxy-3-mercaptopropanate, which might originate from Czapek-peptone medium into exomethylene group of α-cyclocostunolide.[63,66]



Scheme 49 Biotransformation of atractylon (**163**) and costunolide (**165**) by *Aspergillus niger*.



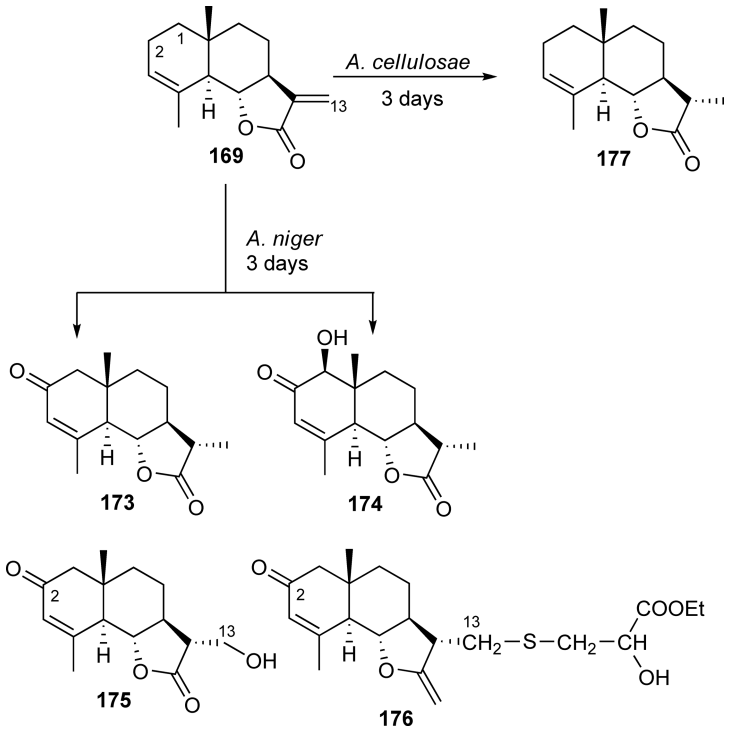
Scheme 50 Biotransformation of costunolide (**165**) and its derivative (**172a**) by *Cunninghamella echinulata* and *Rhizopus oryzae*.

Aspergillus niger converted β-cyclocostunolide (**170**) into 2-oxygenated metabolites (**173**, **174**, **178–181**) of which **173** was predominant. It is suggested that compounds **173** and **174** might be formed during biotransformation period since metabolite media after 7 days was acidic (pH 2.7). Surprisingly, *A. cellulosae* gave a sole product 11β,13-dihydro-β-cyclocostunolide (**182**) which was abnormally folded in mycelium of *A. cellulosae* as a crystal form after biotransformation of **170**. On the other hand, the metabolites were normally liberated in medium outside of the mycelium of *A. niger* and *B. dothidea* (**Scheme 53**).[63,66]

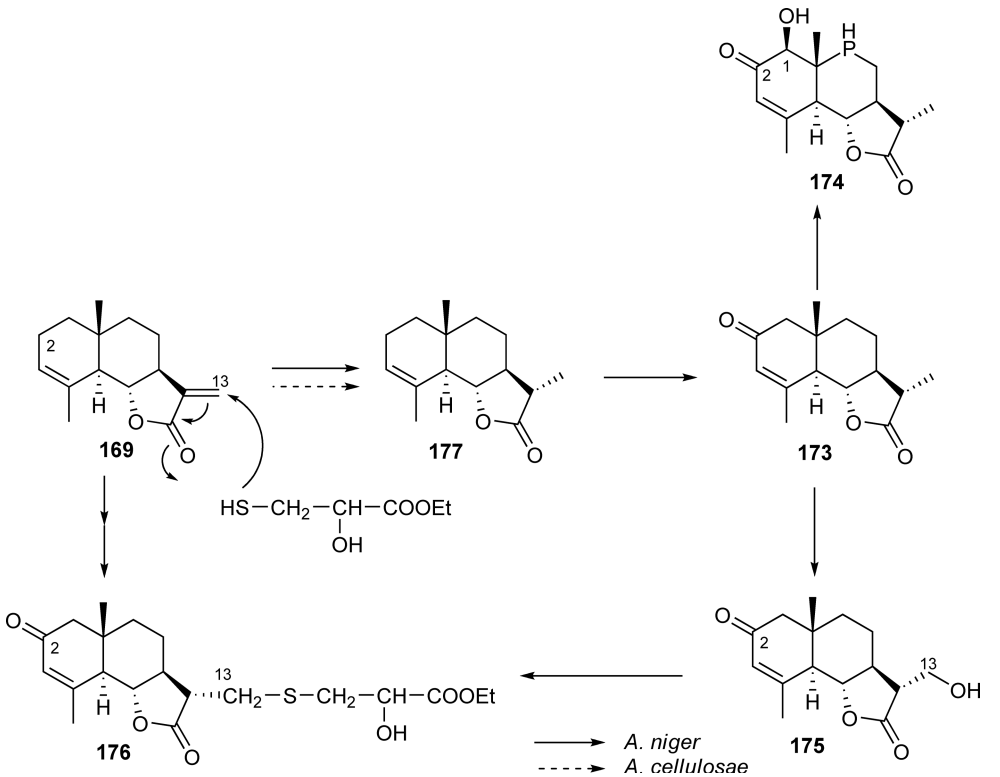
Botryosphaeria dothidea has no stereoselectivity to reduce C11–C13 double bond of β-cyclocostunolide (**170**) since this organism gave two dihydro derivatives **182** (16.7%) and **183** (37.8%), as shown in **Scheme 54**.

It is noteworthy that both α- and β-cyclocostunolides were biotransformed by *A. niger* to give the sulfur-containing metabolites (**176**, **181**). Possible biogenetic pathway of **170** is shown in **Scheme 55**.

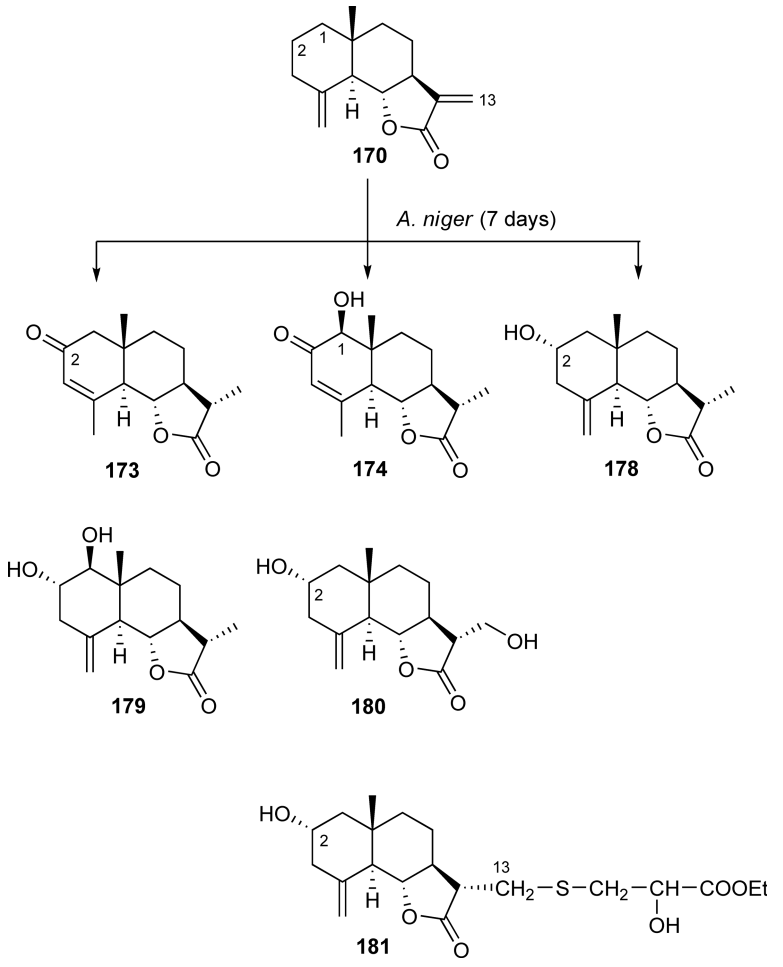
When γ-cyclocostunolide (**171**) was cultivated in *A. niger*, dihydro-α-santonin (**187**, 25%) and its related C-11,13 dihydro derivatives **184–186**, **188**, **189** were obtained in small amounts. Compound **186** was recultivated for 2 days in the same organism as mentioned above to yield **187** (25%) and 5β-hydroxy-α-cyclocostunolide (**189**, 54%). Recultivation of **185** for 2 days in *A. niger* yielded compound **187** as a sole metabolite. During the biotransformation of **171**, no sulfur-containing product was obtained. Both *A. cellulosae* and *B. dothidea* produced only dihydro-γ-cyclocostunolide (**184**) from the substrate (**171**) (**Scheme 56**).[63,66]



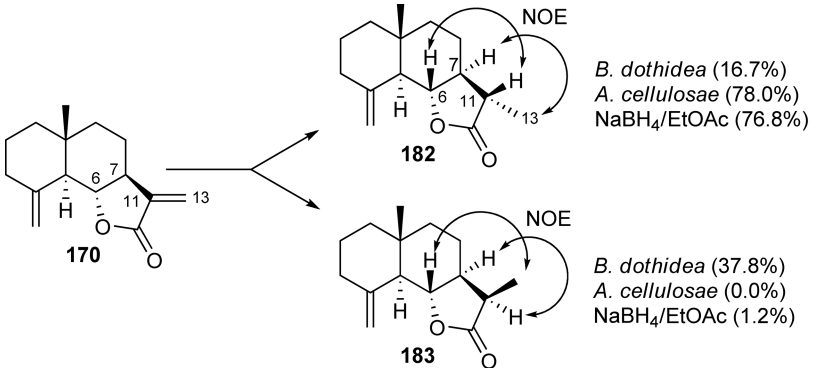
Scheme 51 Biotransformation of α-cyclocostunolide (**169**) by *Aspergillus niger* and *A. cellulosae.*



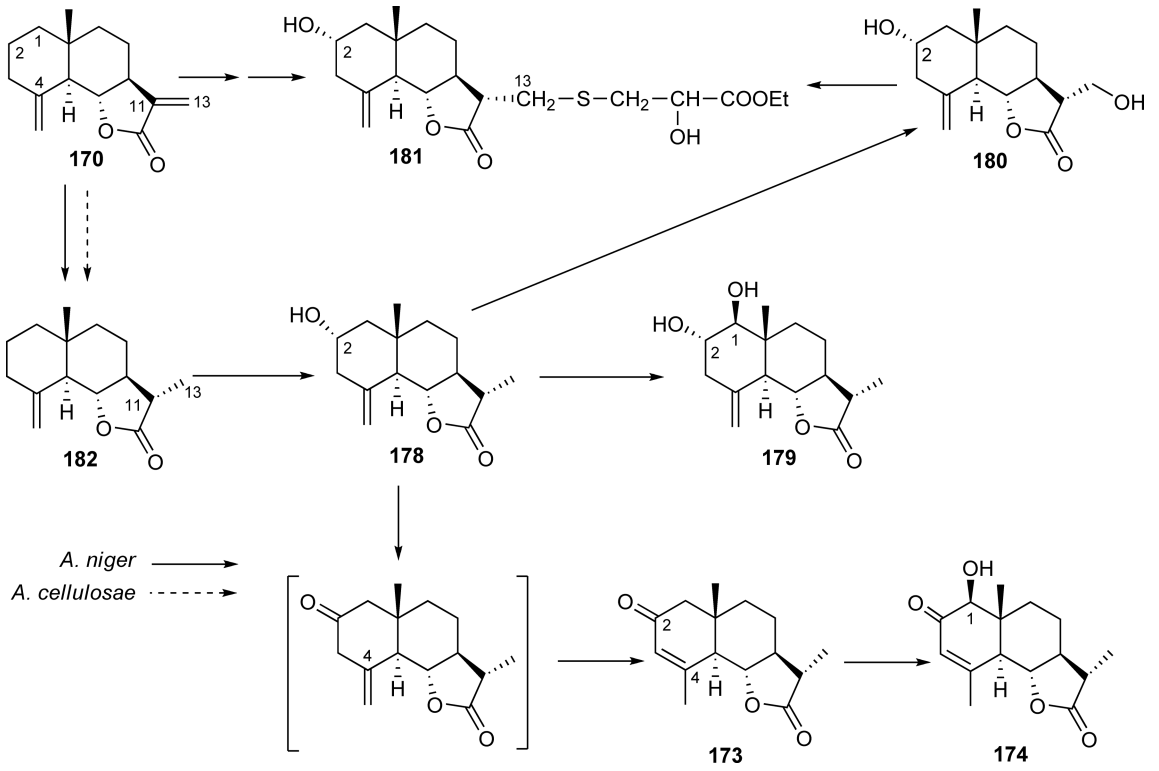
Scheme 52 Possible pathway of biotransformation of α-cyclocostunolide (**169**) by *Aspergillus niger* and *A. cellulosae.*



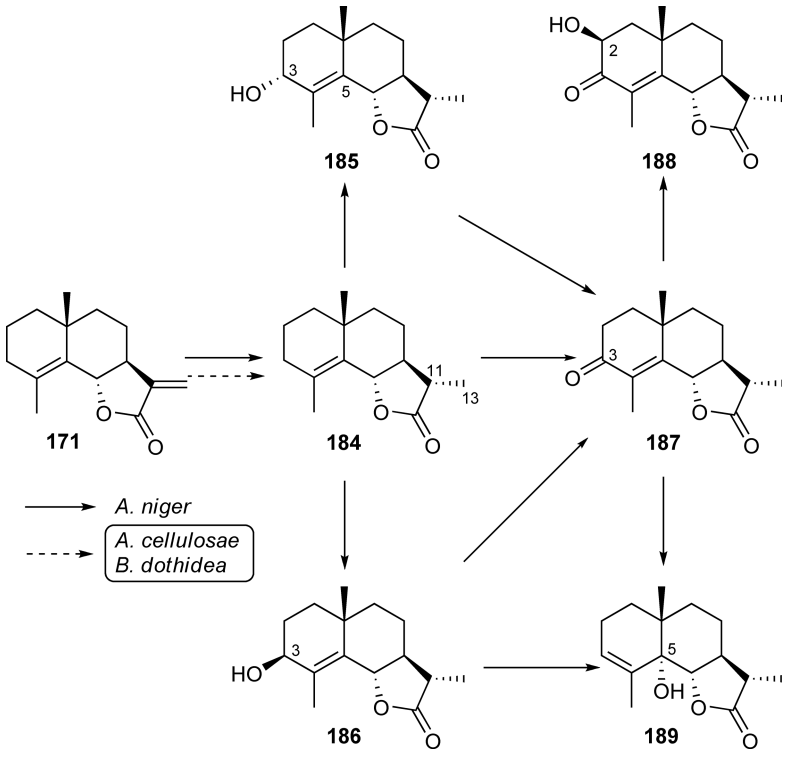
Scheme 53 Biotransformation of β-cyclocostunolide (**170**) by *Aspergillus niger*.



Scheme 54 Biotransformation of β-cyclocostunolide (**170**) by *Aspergillus cellulosae* and *Botryosphaeria dothidea.*



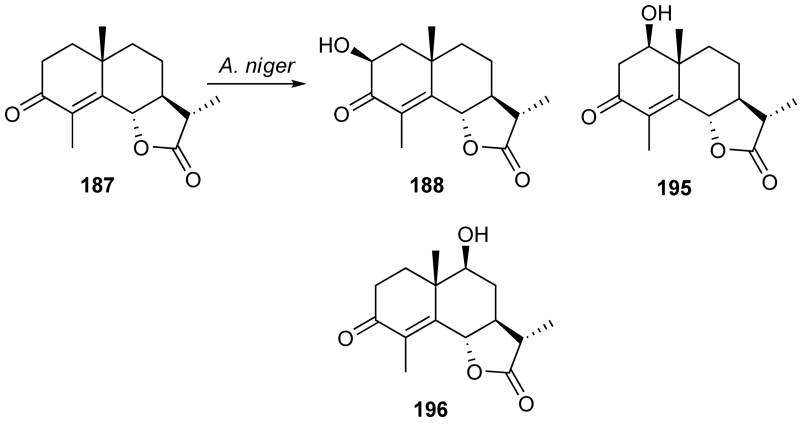
Scheme 55 Possible pathway of biotransformation of β-cyclocostunolide (**170**) by *Aspergillus niger* and *A. cellulosae.*



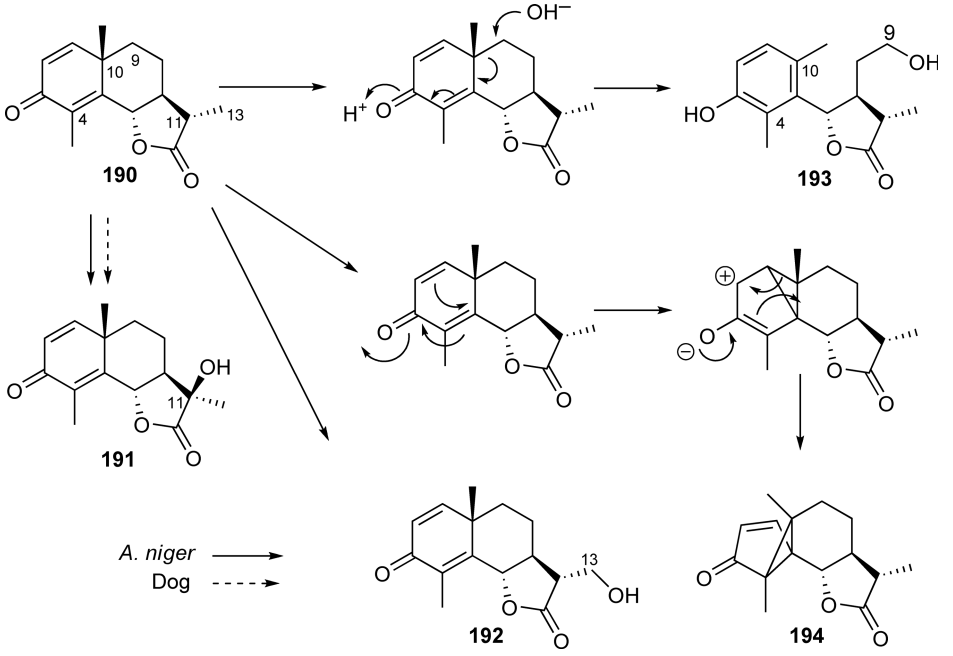
Scheme 56 Biotransformation of γ-cyclocostunolide (**171**) by *Aspergillus niger*, *A. cellulosae*, and *Botryosphaeria dothidea.*

Santonin (**190**) has been used as a vermicide against roundworm. *Cunninghamella blakesleeana* and *A. niger* converted **190** into **187**.[67] When **187** was fed to *A. niger* for 1 week it yielded 2β-hydroxy-1,2-dihydro-α-santonin (**188**, 39%) as well as 1β-hydroxy-1,2-dihydro-α-santonin (**195**, 6.5%), 9β-hydroxy-1,2-dihydro-α-santonin (**196**, 6.9%), and α-santonin (**190**, 5.4%), which might be obtained from dehydroxylation of **188**, as a minor component.[63] Compound **188** was isolated from the crude metabolite of γ-cyclocostunolide (**171**) by *A. niger* as mentioned above (**Scheme 57**).

It was treated with *A. niger* for 7 days to give **191** (18.3%), **192** (2.3%), **193** (19.3%), and **194** (3.5%) of which **193** was the major metabolite. Compound **191** was isolated from dog's urine after oral administration of **190**. The structure of compound **194** was established as lumisantonin obtained by the photoreaction of **190**. α-Santonin **190** was not converted into 1,2-dihydro derivative by *A. niger*, whereas the other strain of *A. niger* gave a single product, 1,2-dihydro-α-santonin (**187**) (**Scheme 58**).[63]



Scheme 57 Biotransformation of dihydro-α-santonin (**187**) by *Aspergillus niger*.



Scheme 58 Biotransformation of α-santonin (**190**) by *Aspergillus niger* and dog.

Ata and Nachtigall[68] reported that α-santonin (**190**) was incubated with *Rhizopus stolonifera* to give **187a** and **183b**, while with *Cunninghamella bainieri*, *C. echinulata*, and *M. plumbeus* to yield the known 1,2-dihydro-α-santonin (**187**) (**Scheme 59**).

α-Santonin (**190**) and 6-epi-α-santonin (**198**) were cultivated in *Absidia coerulea* for 2 days to give 11β-hydroxy- (**191**, 71.4%) and 8α-hydroxysantonin (**197**, 2.0%), while 6-epi-santonin (**198**) yielded four major products (**199–201, 206**) and four minor analogues (**202, 203–205**). *Asparagus officinalis* also biotransformed α-santonin (**190**) to three eudesmanolides (**187**, **207, 208**) and a guaianolide (**209**) as a small amount. 6-Epi-santonin (**198**) was also treated in the same bioreactor as mentioned above to give **199** and **206**, the latter of which was obtained as a major metabolite (44.7%) (**Scheme 60**).[69]

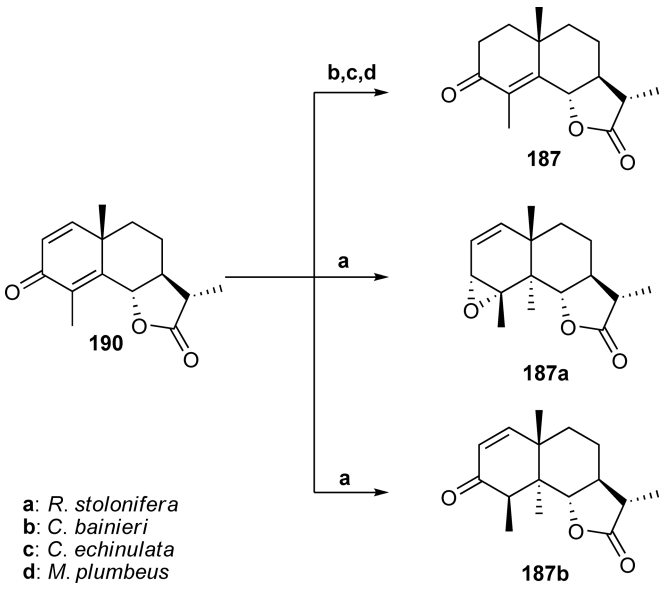
α-Santonin (**190**) was incubated in the cultured cells of *Nicotiana tabacum* and the liverwort *M. polymorpha*. *Nicotiana tabacum* cells gave 1,2-dihydro-α-santonin (**187**) (50%) for 6 days. The latter cells also converted α-santonin to 1,2-dihydro-α-santonin, but conversion ratio was only 28% (**Scheme 61**).[70]

6-Epi-α-santonin (**198**) and its tetrahydro analogue (**210**) were also incubated with fungus *Rhizopus nigricans* to give 2α-hydroxydihydro-α-santonin (**211**),[71] the epimer of **188** obtained from the biotransformation of dihydro-α-santonin (**187**) by *A. niger*.[63] The product **211** might be formed through 1,2-epoxide of **198**. Compound **210** was converted through carbonyl reduction to furnish **212** and **213** under epimerization at C-4 (**Scheme 62**).[71]

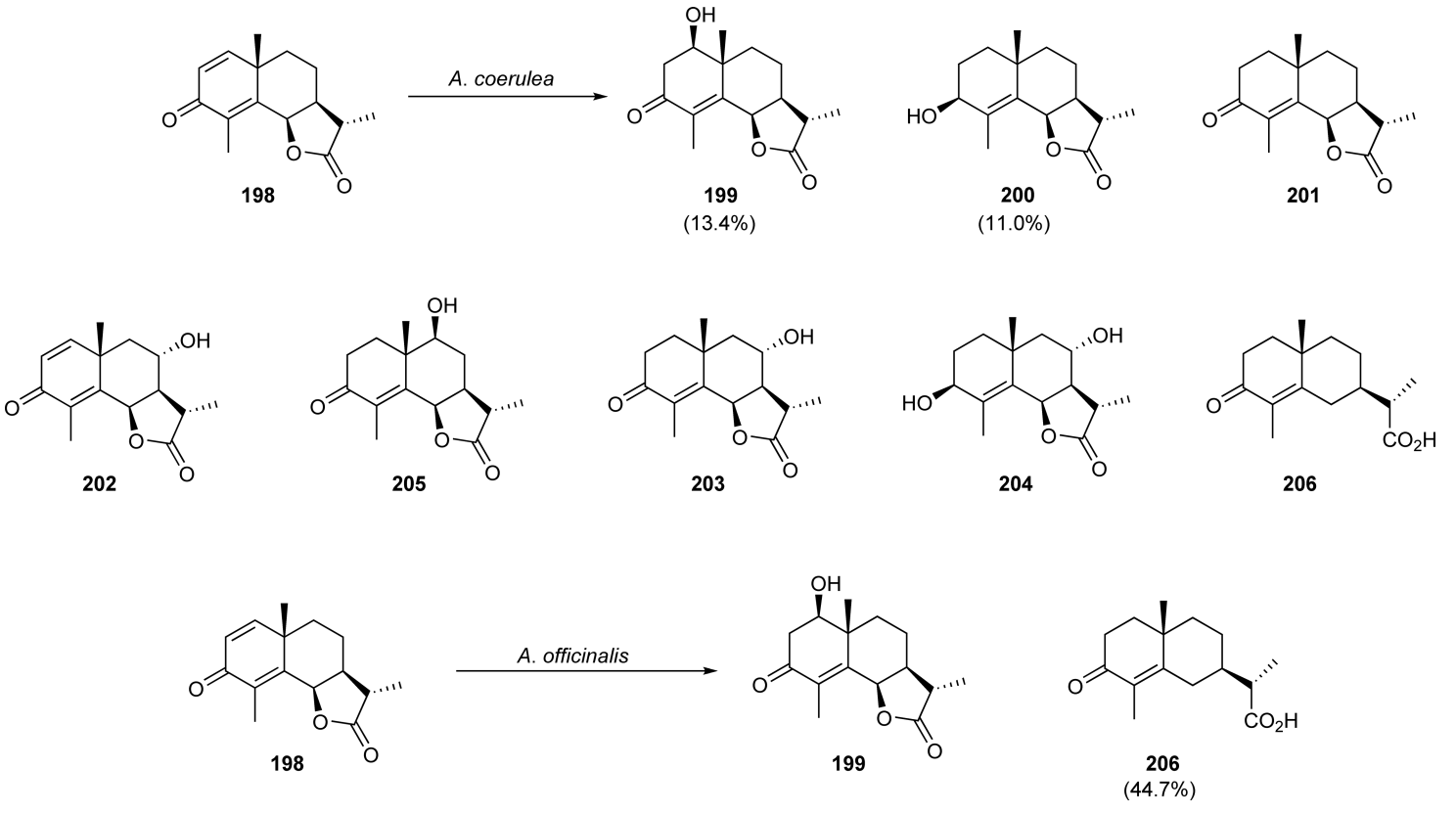
1,2,4β,5α-Tetrahydro-α-santonin (**214**) prepared from α-santonin (**190**) was treated with *A. niger* to yield six metabolites (**215–220**) of which **219** was the major product (21%). When the substrate (**214**) was treated with CYP450 inhibitor, 1-aminobenzotriazole, only **215** was obtained without its homologues, **216–220**, whereas the C-4 epimer (**221**) of **214** was converted by the same microorganism to yield a single metabolite (**222**) (73%). Further oxidation of **222** did not occur. This reason might be considered by the steric hindrance of β (axial) methyl group at C-4 (**Scheme 63**).[63]

7α-Hydroxyfrullanolide (**223**) possessing cytotoxicity and antitumor activity, isolated from *Sphaeranthus indicus* (Compositae), was bioconverted by *A. niger* to yield 13*R*-dihydro derivative (**224**). The same substrate was also treated with *A. quardilatus* (wild type) to give 13-acetyl product (**225**) (**Scheme 64**).[72]

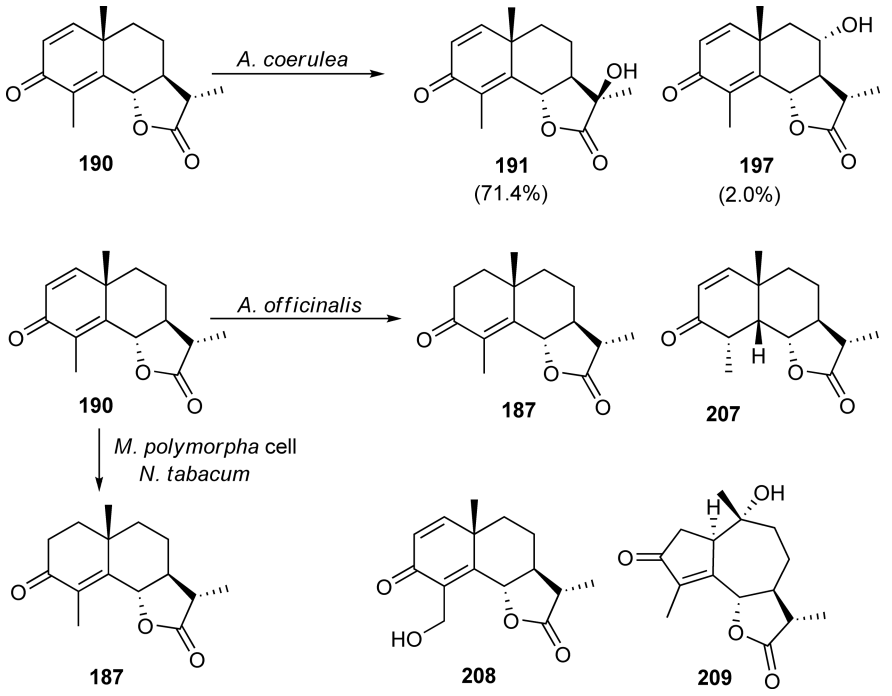
Incubation of (−)-frullanolide (**226**), obtained from the European liverwort, *Frullania tamarisci* subsp. *tamarisci*, causes a potent allergenic contact dermatitis, was incubated with *A. niger* to give dihydrofrullanolide (**227**), nonallergenic compound in 31.8% yield. In this case, C11–C13 dihydro derivative was not obtained.[73]



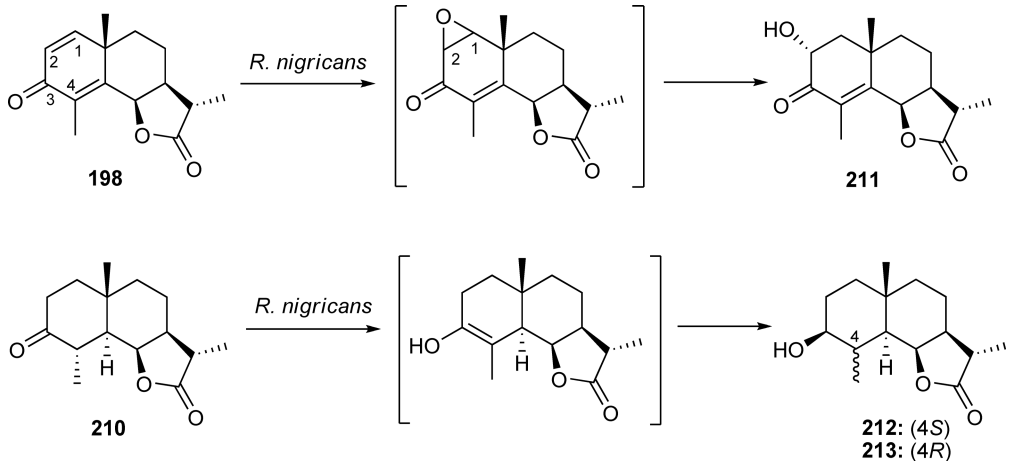
Scheme 59 Biotransformation of α-santonin (**190**) by *Rhizopus stolonifera*, *Cunninghamella bainieri*, *C. echinulata*, and *Mucor plumbeus*.



Scheme 60 Biotransformation of α-epi-santonin (**198**) by *Absidia coerulea* and *Asparagus officinalis.*



Scheme 61 Biotransformation of 6-epi-α-santonin (**190**) by *Absidia coerulea* and *Asparagus officinalis*, *Marchantia polymorpha*, and *Nicotiana tabacum.*

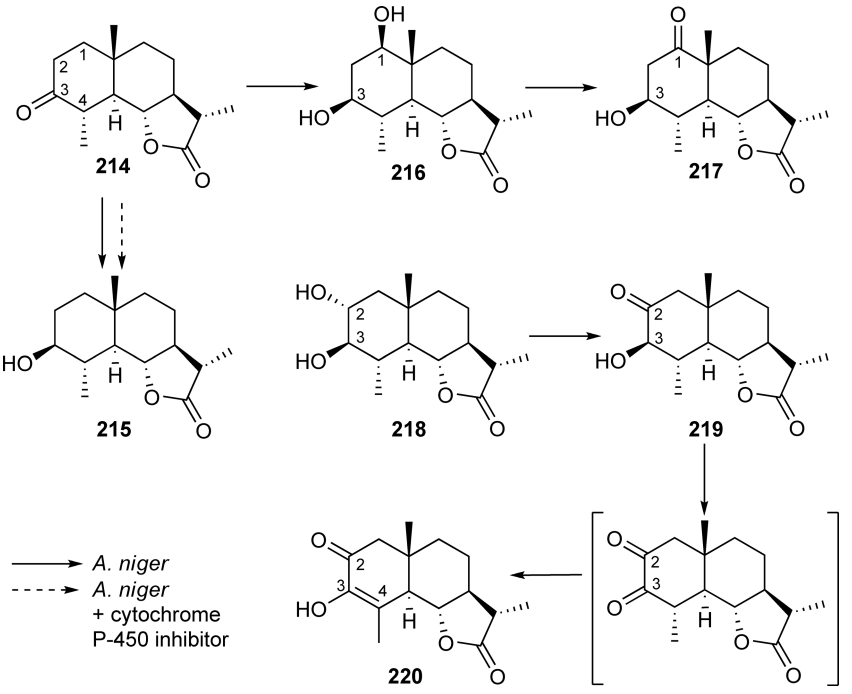


Scheme 62 Biotransformation of epi-α-santonin (**198**) and tetrahydrosantonin (**210**) by *Rhizopus nigricans.*

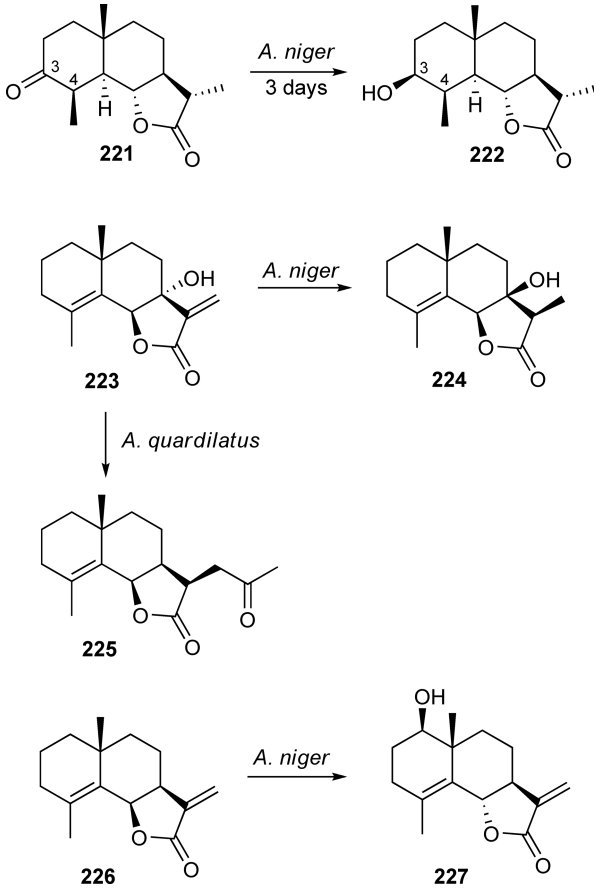
Guaiane-type sesquiterpene hydrocarbon, (+)-γ-gurjunene (**228**), was treated with plant pathogenic fungus *G. cingulata* to give two diols: (1*S*,4*S*,7*R*,10*R*)-5-guaien-11,13-diol (**229**) and (1*S*,4*S*,7*R*,10*S*)-5-guaien-10,11,13-triol (**230**) (**Scheme 65**).[74,75]

Glomerella cingulata converted guaiol (**231**) and bulnesol (**232**) into 5,10-dihydroxy (**233**) and 15-hydroxy derivative (**234**), respectively (**Scheme 66**).[76]

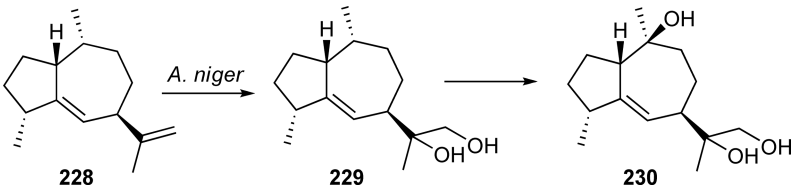
When *Eurotium rubrum* was used as a bioreactor of guaiene (**235**), rotunodone (**236**) was obtained.[77] Guaiol (**231**) was also transformed by *A. niger* to give a cyclopentane derivative, pancherione (**237**), and two dihydroxy guaiols (**238**, **239**)[45] of which **237** was obtained from the same substrate using *Eurotium rubrum* for 10 days (**Scheme 67**).[77,78]



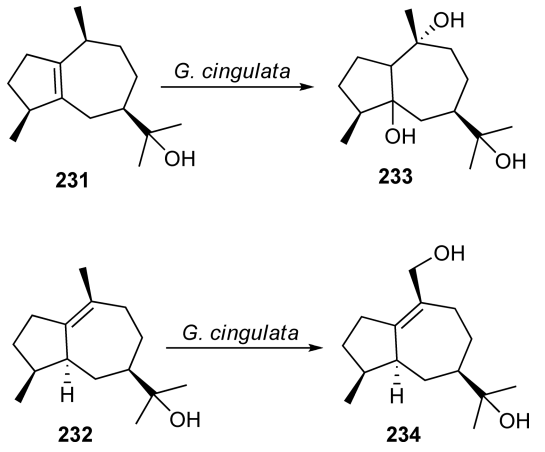
Scheme 63 Biotransformation of 1,2,4β,5α-tetrahydro-α-santonin (**214**) by *Aspergillus niger.*



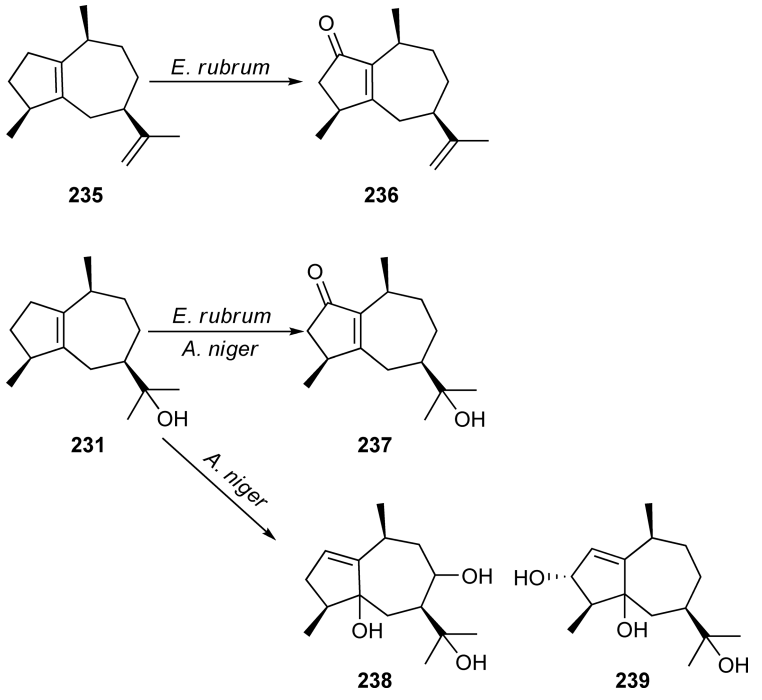
Scheme 64 Biotransformation of C4-epimer (**221**) of **214**, 7α-hydroxyfrullanolide (**223**) and frullanolide (**226**) by *Aspergillus niger* and *A. quardilatus.*



Scheme 65 Biotransformation of (+)-γ-gurjunene (**228**) by *Glomerella cingulata.*



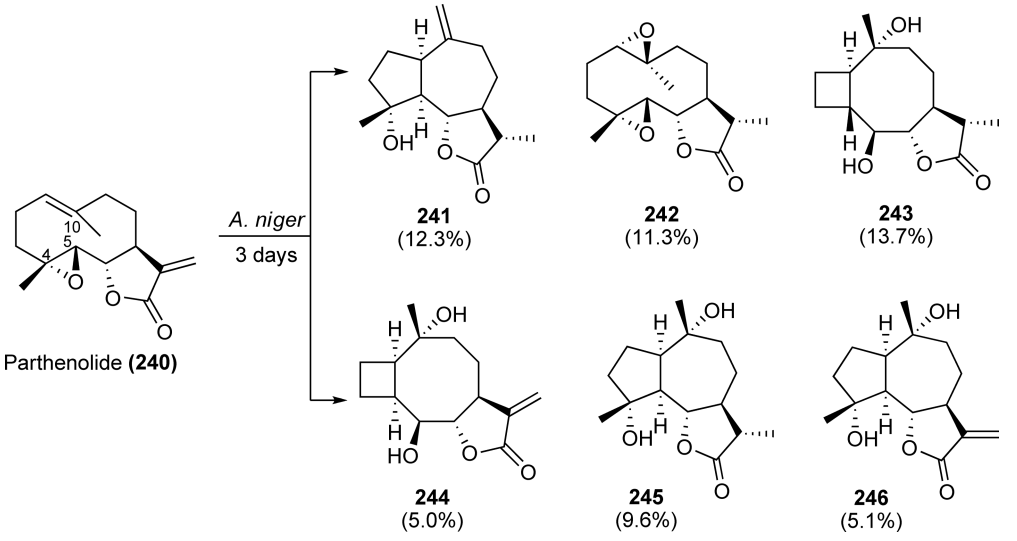
Scheme 66 Biotransformation of guaiol (**221**) and bulnesol (**232**) by *Glomerella cingulata.*



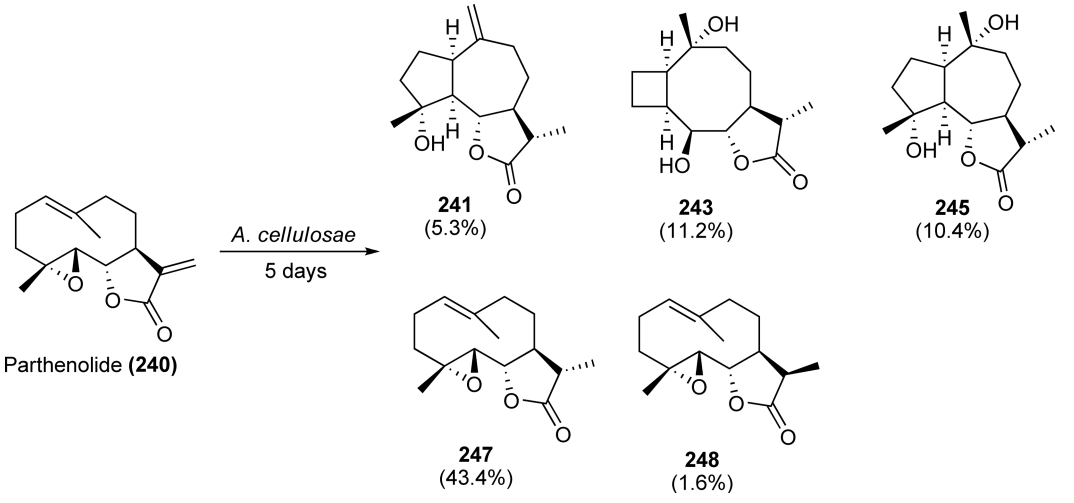
Scheme 67 Biotransformation of guaiene (**235**) by *Eurotium rubrum* and guaiol (**231**) by *Aspergillus niger* and *Eurotium rubrum*.

Parthenolide (**240**), a germacrane-type lactone, isolated from the European feverfew (*Tanacetum parthenium*) as a major constituent shows cytotoxic, antimicrobial and antifungal, anti-inflammatory, antirheumatic activity, apoptosis inducing, and NF-κB and DNA-binding inhibitory activity. This substrate was incubated with *A. niger* in Czapek-peptone medium for 2 days to give six metabolites (**241**, 12.3%, **242**, 11.3%, **243**, 13.7%, **244**, 5.0%, **245**, 9.6%, and **246**, 5.1%).[73] Compound **244** was a naturally occurring lactone from *Michelia champaca*.[79] The stereostructure of compound **243** was established by X-ray crystallographic analysis (**Scheme 68**).

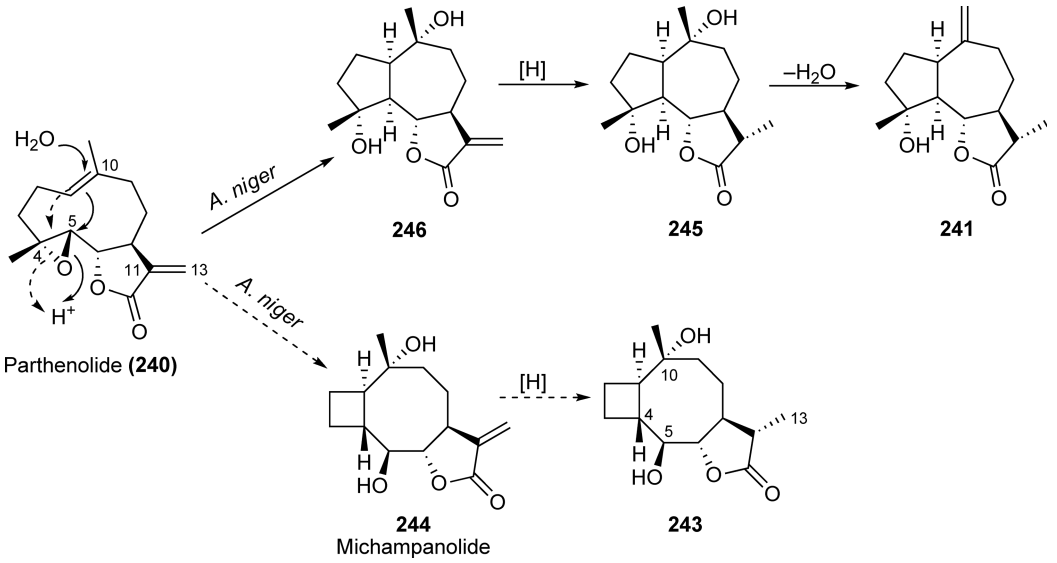
When parthenolide (**240**) was treated with *A. cellulosae* for 5 days, two new metabolites, 11β,13-dihydro- (**247**, 43.5%) and 11α,13-dihydroparthenolides (**248**, 1.6%), were obtained together with the same metabolites (**241**, 5.3%, **243**, 11.2%, **245**, 10.4%) as described above (**Scheme 69**). Possible metabolic root of **240** has been shown in **Scheme 70**.[73]



Scheme 68 Biotransformation of parthenolide (**240**) by *Aspergillus niger*.



Scheme 69 Biotransformation of parthenolide (**240**) by *Aspergillus cellulosae*.



Scheme 70 Possible pathway of biotransformation of parthenolide (**240**).

Galal *et al.*[80] reported that *Streptomyces fulvissimus* or *Rhizopus nigricans* converted parthenolide (**240**) into 11α-methylparthenolide (**247**) in 20–30% yield, while the metabolite 11β-hydroxyparthenolide (**248**) was obtained by incubation of **240** with *R. nigricans* and *Rhodotorula rubra*. In addition to the metabolite **247**, *S. fulvissimus* gave minor polar metabolite, 9β-hydroxy derivative (**248a**) in low yield (3%). The same metabolite (**248a**) was obtained from **247** by fermentation of *S. fulvissimus* as a minor constituent. Furthermore, 14-hydroxyparthenolide (**248b**) was obtained from **240** and **247** as a minor component (4%) by *R. nigricans* (**Scheme 71**).

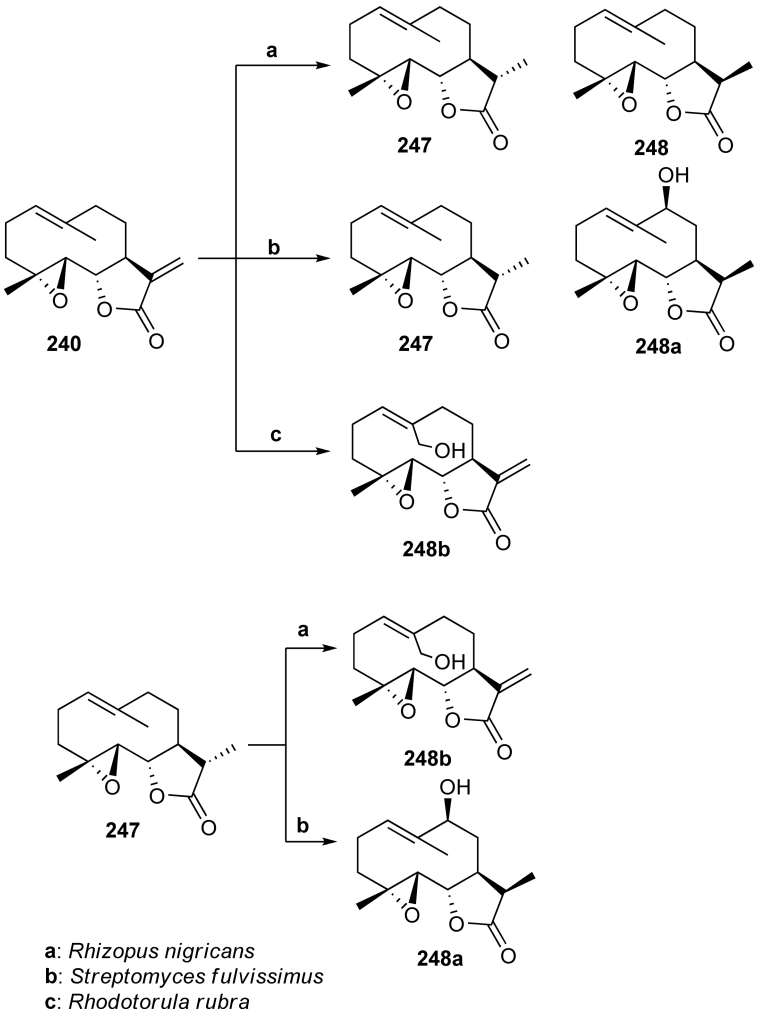
Pyrethrosin (**248c**), a germacranolide, was treated with the fungus *Rhizopus nigricans* to yield five metabolites (**248d–248h**). Pyrethrosin itself and metabolite **248e** displayed cytotoxic activity against human malignant melanoma with IC_{50} 4.20 and 7.5 $\mu g\,ml^{-1}$, respectively. Metabolite **248h** showed significant *in vitro* cytotoxic activity against human epidermoid carcinoma (KB cells) and against human ovary carcinoma with IC_{50} <1.1 and 8.0 $\mu g\,ml^{-1}$, respectively. Compounds **248f** and **248i** were active against *Cryptococcus neoformans* with IC_{50} 35.0 and 25 $\mu g\,ml^{-1}$, respectively, while **248a** and **248g** showed antifungal activity against *Candida albicans* with IC_{50} 30 and 10 $\mu g\,ml^{-1}$. Metabolites **248g** and its acetate (**248i**) derived from **248g** showed antiprotozoal activity against *Plasmodium falciparum* with IC_{50} 0.88 and 0.32 $\mu g\,ml^{-1}$, respectively, without significant toxicity. Compound **248i** also exhibited pronounced activity against the chloroquine-resistant strain of *P. falciparum* with IC_{50} 0.38 $\mu g\,ml^{-1}$ (**Scheme 72**).[81]

(−)-Dehydrocostuslactone (**249**), inhibitors of nitric oxide synthases and TNF-α, isolated from *Saussurea* radix, was incubated with *C. echinulata* to yield (+)-11α,13-dihydrodehydrocostuslactone (**250a**). The epoxide **251** and a C-11 reduced compound (**250**) were obtained by the above microorganisms.[81]

Cunninghamella echinulata and *R. oryzae* bioconverted **249** into C-11/C-13-dihydrogenated **250** and C-10/C-14 epoxidated product (**251**). Treatment of **252a** in *C. echinulata* and *R. oryzae* gave (−)-16-(1-methyl-1-propenyl)eremantholide (**252b**) (**Scheme 73**).[81]

The same substrate (**249**) was fed to *A. niger* for 7 days to yield four metabolites: costuslactone (**250**), and their derivatives (**251–253**) of which **251** was the major product (28%), while the same substrate was cultivated with *A. niger* for 10 days, two minor metabolites (**254, 255**) were newly obtained in addition to **252** and **253** of which the latter lactone was predominant (20.7%) (**Scheme 74**).[63]

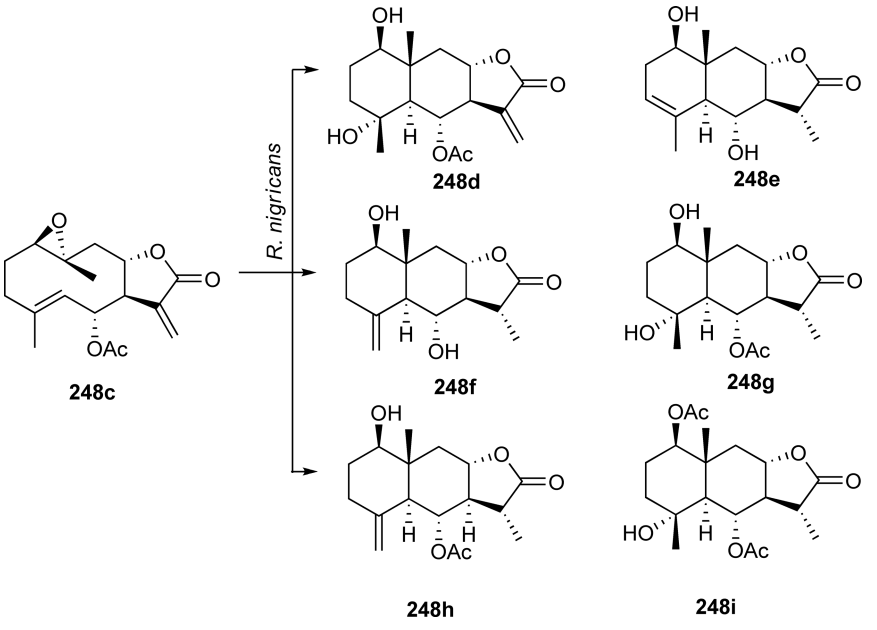
When compound (**249**) was treated with *A. niger* in the presence of 1-aminobenzotriazole, **249** was completely converted into 11β,13-dihydro derivative (**250**) for 3 days; however, further biodegradation did not occur for 10 days.[63,82] The same substrate (**249**) was cultivated with *A. cellulosae* IFO to furnish 11,13-dihydro- (**250**) (82%) for only one day and then the product (**250**) slowly oxidized into 11,13-dihydro-8β-hydroxycostuslactone (**256**) (1.6%) from 8 days (**Scheme 75**).[63,82]



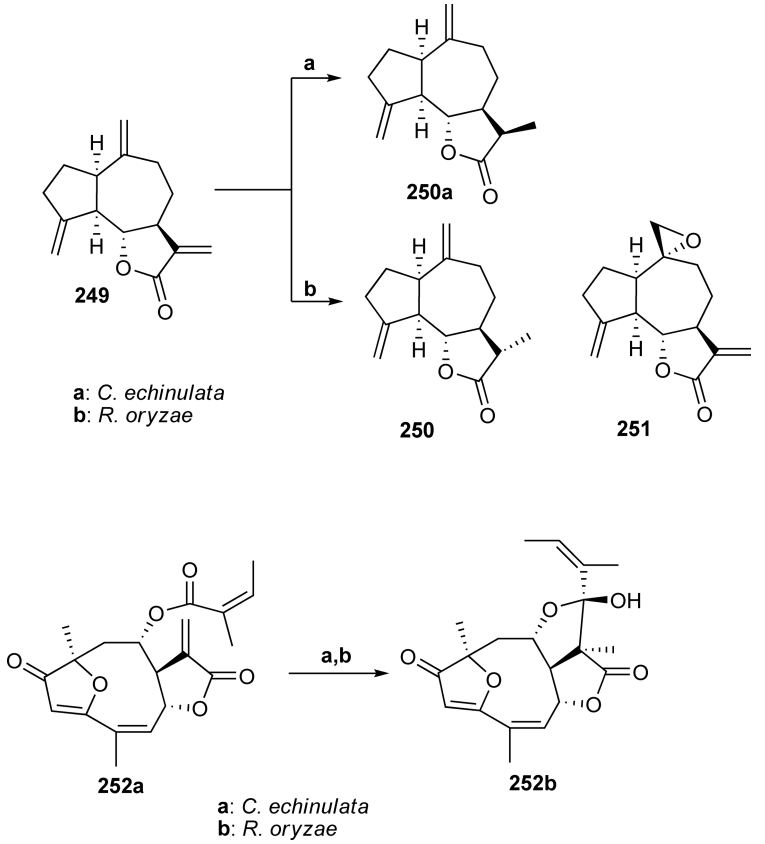
Scheme 71 Biotransformation of parthenolide (**240**) and its dihydro derivative (**247**) by *Rhizopus nigricans*, *Streptomyces fulvissimus*, and *Rhodotorula rubra.*

The lactone (**249**) was biodegraded by the plant pathogen *B. dothidea* for 4 days to give the metabolites **250** (37.8%) and **257** (8.6%), while *A. niger* IFO-04049 (4 days) and *A. cellulosae* for one day gave only **250**. Thus *B. dothidea* demonstrated low stereoselectivity to reduce C11–C13 double bond.[63] Furthermore, three *Aspergillus* species, *A. niger* IFO 4034, *A. awamori* IFO 4033, and *A. terreus* IFO6123, were used as bioreactors for compounds **249**. *Aspergillus niger* IFO 4034 gave three products (**250, 251, 252**) of which **252** was predominant (56% in GC–MS). *Aspergillus awamori* IFO 4033 and *A. terreus* IFO 6123 converted **249** into **250** (56% from *A. awamori*, 43% from *A. terreus*) and **252** (43% from *A. awamori*, 57% from *A. terreus*), respectively (**Scheme 76**).[63]

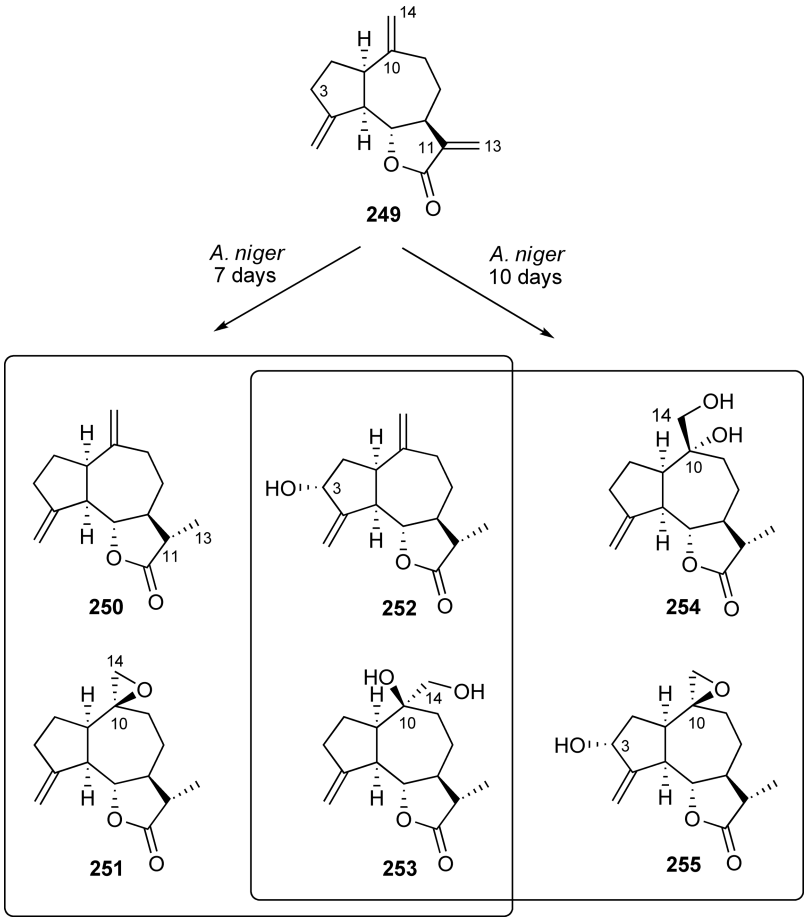
Vernonia arborea (Asteraceae) contains zaluzanin D (**258**) in high content. Ten microorganisms were used for the biotransformation of compound **258**. *Botrytis cinerea* converted **258** into **259** and **260** (85:15%) and *Fusarium equiseti* gave **259** and **260** (33:66%). Compound **258** was incubated with *C. lunata*, *Colletotrichum lindemuthianum*, *Alternaria alternata*, and *Phyllosticta capsici* to produce **259** as the sole metabolite in good yield. *Sclerotinia sclerotiorum* gave only deacetyl product (**261**), while *Rhizoctonia solani* gave **260** and **262–264** among which **263** and **264** are major products. Stereospecific reduction of exocyclic double bond of α-methylene γ-butyrolactone is generally seen during biotransformation of sesquiterpenes with this functional group (**Scheme 77**).[83]



Scheme 72 Biotransformation of pyrethrosin (**248c**) by *Rhizopus nigricans.*



Scheme 73 Biotransformation of (−)-dehydrocostuslactone (**249**) and rearranged guaianolide (**252a**) by *Cunninghamella echinulata and Rhizopus oryzae.*



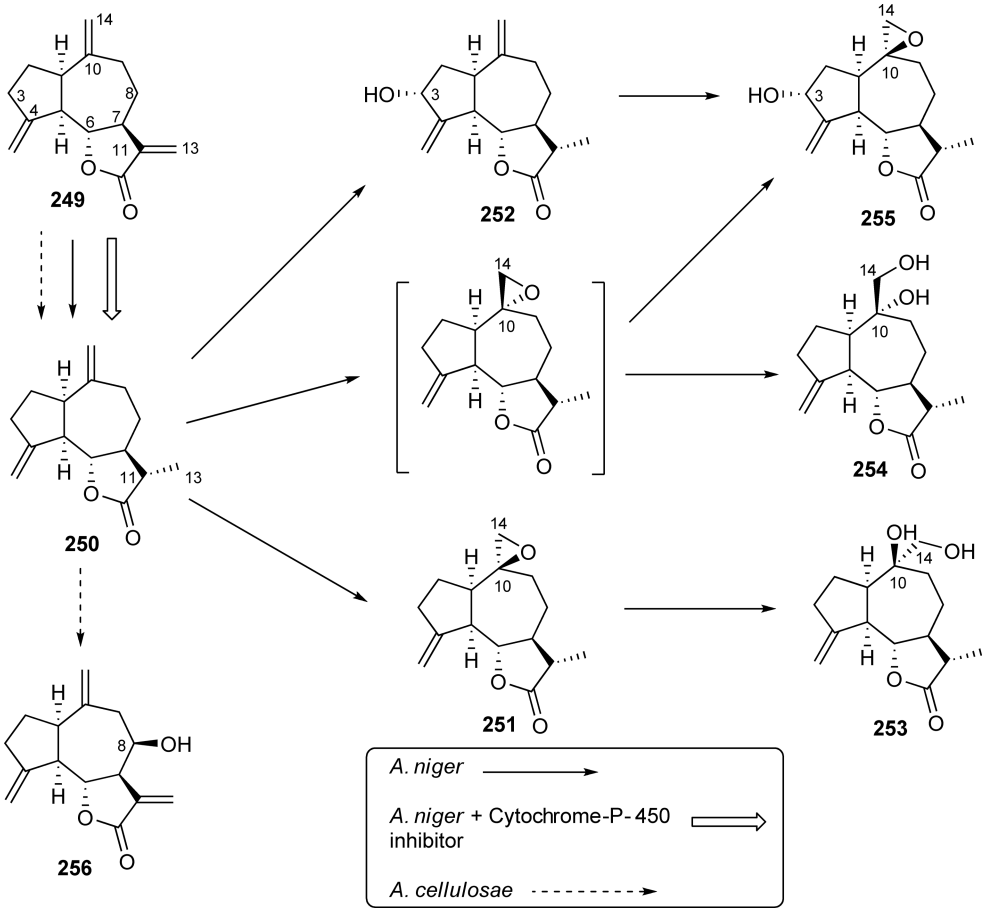
Scheme 74 Biotransformation of (–)-dehydrocostuslactone (**249**) by *Aspergillus niger*.

Incubation of parthenin (**264a**) with the fungus *Beauveria bassiana* in modified Richard's medium gave C11–C13 reduced product (**264b**) in 37% yield, while C-11 α-hydroxylated product (**264c**) was obtained in 32% yield from the broth of the fungus *Sporotrichum pulverulentum* using the same medium (**Scheme 78**).[84]

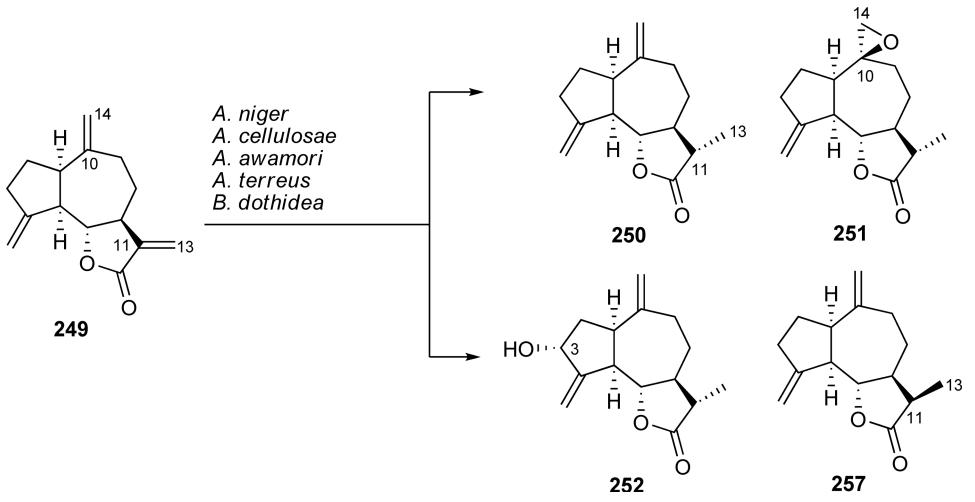
Cadina-4,10(15)-dien-3-one (**265**) possessing insecticidal and ascaricidal activity, from Jamaican medicinal plant *Hyptis verticillata*, was metabolized by *C. lunata* ATCC 12017 in potato dextrose to give 12hydroxy-dihydrocadinene (**266**), 3α-hydroxydihydrocadinene (**267**), and 3α-hydroxy-4,5-dihydrocadinene (**268**), while **265** was incubated by the same fungus in peptone, yeast and beef extracts, and glucose medium, only **267** and **268** were obtained. Compound **267** derived synthetically was treated with the same fungus *C. lunata* to yield three metabolites (**269–271**) (**Scheme 79**).[85]

The incubation of the same substrate (**265**) in *M. plumbeus* ATCC 4740 in high iron-rich medium gave **270**, which was obtained from *C. lunata* mentioned above (**268, 272, 273, 277–279**). In low iron-rich medium, this fungus converted the same substrate **265** into three epoxides (**274–276**), a tetraol (**280**) with common metabolites (**268, 273, 277, 278**), and **271** which was the same metabolite used by *C. lunata*.[86] It is interesting to note that only epoxides were obtained from the substrate (**265**) by *Mucor* fungus in low iron medium (**Scheme 80**).

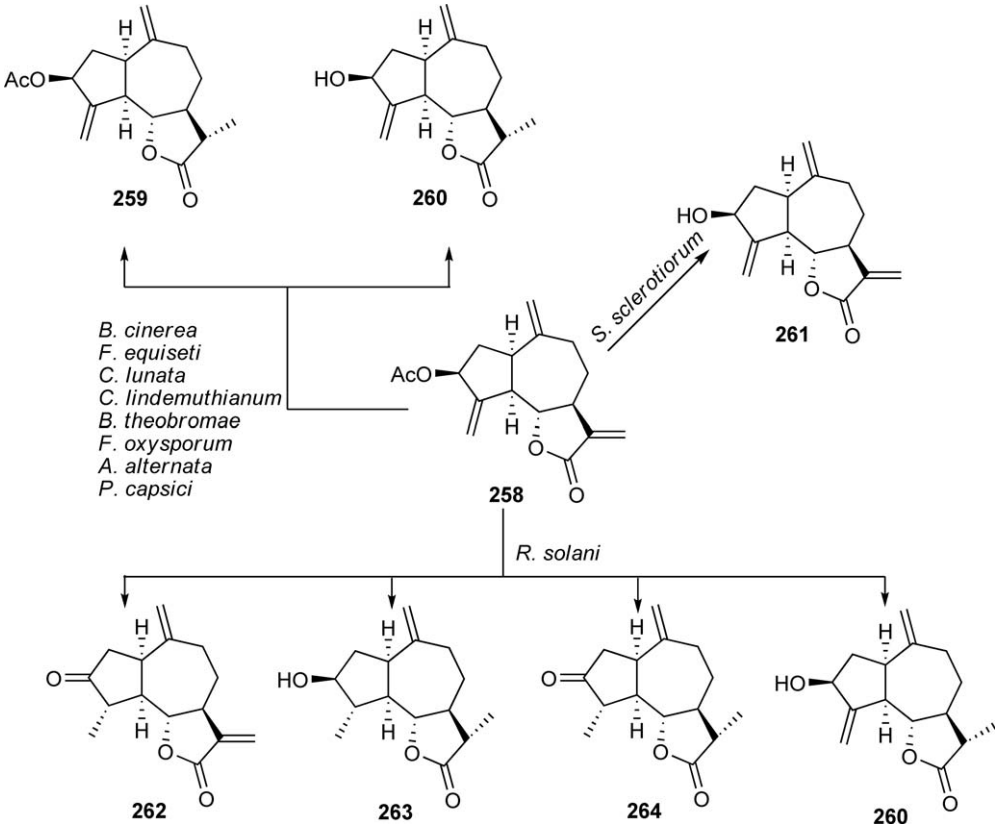
The same substrate (**265**) was incubated with the deuteromycete fungus, *Beauveria bassiana*, which is responsible for the muscaridine disease in insects, in order to obtain new functionalized analogues with improved biological activity. From compound **265**, nine metabolites were obtained. The insecticidal potential of the metabolites (**267, 268, 268a–268f**) were evaluated against *Cylas formicarius*. The metabolites **268, 268d, 273** showed enhanced activity compared with the substrate (**265**). The plant growth regulatory activity of the



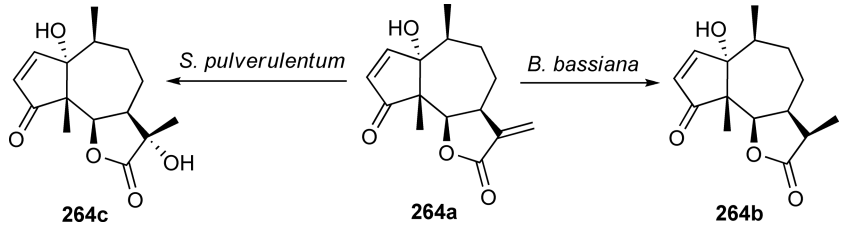
Scheme 75 Possible pathway of biotransformation of (−)-dehydrocostuslactone (**249**) by *Aspergillus niger* and *A. cellulosae.*



Scheme 76 Biotransformation of (−)-dehydrocostuslactone (**249**) by *Aspergillus* species and *Botryosphaeria dothidea.*



Scheme 77 Biotransformation of zaluzanin D (**258**) by various fungi.



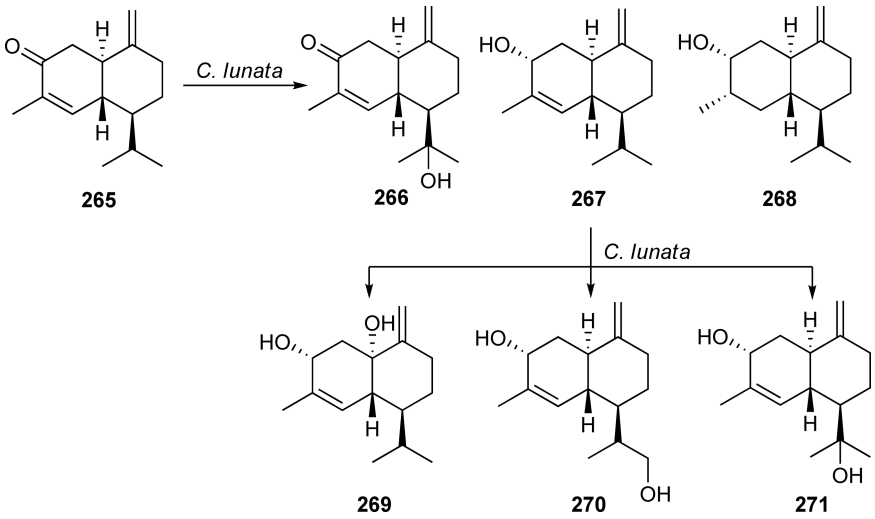
Scheme 78 Biotransformation of parthenin (**264a**) by *Sporotrichum pulverulentum* and *Beauveria bassiana.*

metabolites against radish seeds was tested. All the compounds showed inhibitory activity; however, their activity was less than that of colchicines (**Scheme 81**).[87]

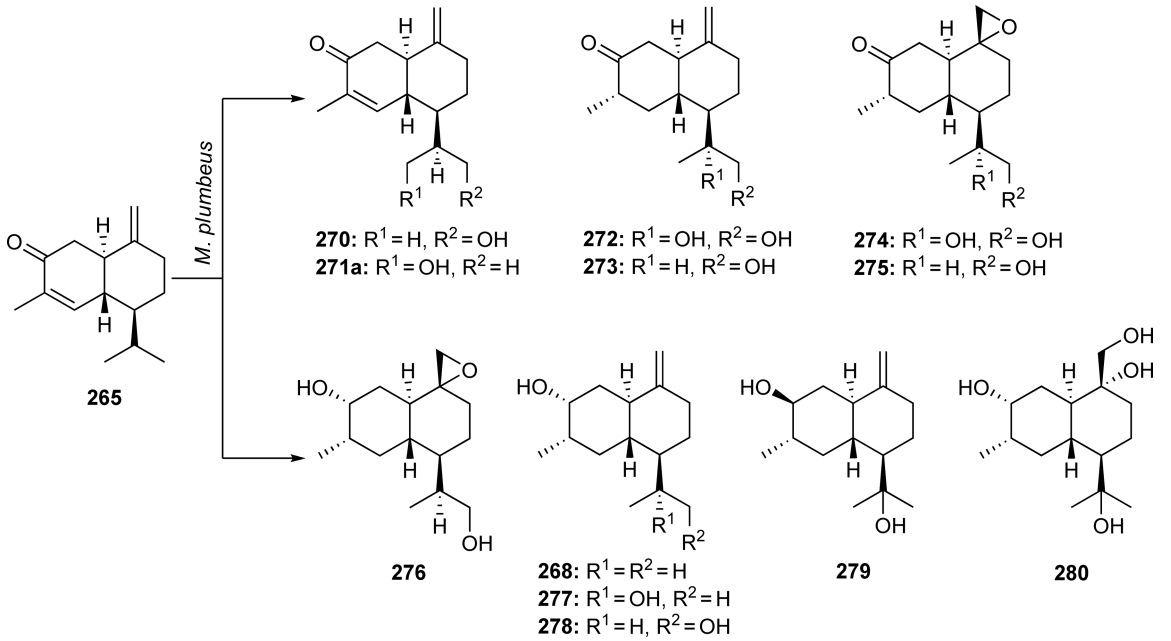
Cadinane-type sesquiterpene alcohol (**281**) isolated from the liverwort *M. taylorii* gave a primary alcohol (**282**) by *A. niger* treatment (**Scheme 82**).[45]

Fermentation of (−)-α-bisabolol (**282a**) possessing anti-inflammatory activity with plant pathogenic fungus *G. cingulata* for 7 days yielded oxygenated products (**282b–282e**) of which compound **282e** was predominant. 3,4-Dihydroxy products (**282b–282d**) could be formed by hydrolysis of the 3,4-epoxide from **282a** and **282c** (**Scheme 83**).[88]

El Sayed *et al.*[89] reported microbial and chemical transformation of (*S*)-(+)-curcuphenol (**282g**) and curcudiol (**282n**), isolated from the marine sponges, *Didiscus axeata.* Incubation of compound **282g** with *Kluyveromyces marxianus* var. *lactis* resulted in the isolation of six metabolites (**282h–282j**). The same substrate was incubated with *Aspergillus alliaceus* to give the metabolites **282p**, **282q**, and **282s** (**Scheme 84**).

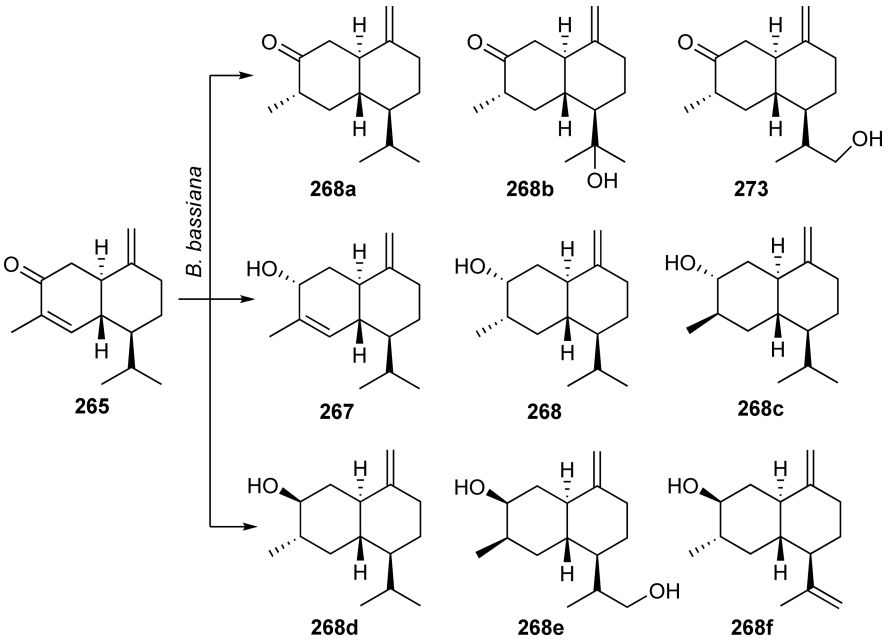


Scheme 79 Biotransformation of cadina-4,10(15)-dien-3-one (**265**) by *Curvularia lunata*.

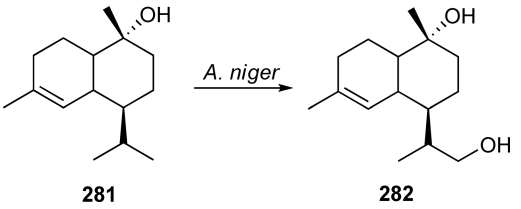


Scheme 80 Biotransformation of cadina-4,10(15)-dien-3-one (**265**) by *Mucor plumbeus*.

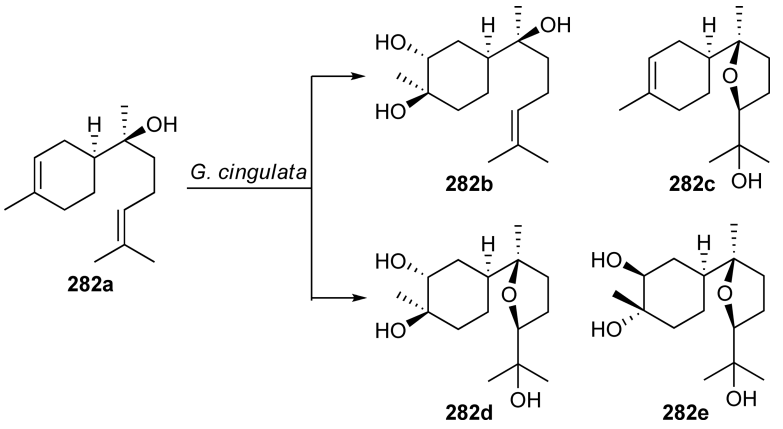
Compounds **282g** and **282n** were treated with *Rhizopus arrhizus* and *Rhodotorula glutinus* for 6 and 8 days to yield glucosylated metabolites, 1α-D-glucosides (**282o**) and **282r**, respectively. The substrate itself showed antimicrobial activity against *C. albicans*, *C. neoformans*, and MRSA-resistant *Staphylococcus aureus* and *S. aureus* with MIC and MFC/MBC ranges of 7.5–25 and 12.5–50 $\mu g\,ml^{-1}$ respectively. Compounds **282g** and **282h** also exhibited *in vitro* antimalarial activity against *Plasmodium falciparum* (D6 clone) and *P. falciparum* (W2 clone) of 3600 and 3800 $ng\,ml^{-1}$ (selective index (SI) >1.3), and 1800 (SI>2.6) and 2900 $ng\,ml^{-1}$(SI>1.6), respectively (**Scheme 84**).[89]



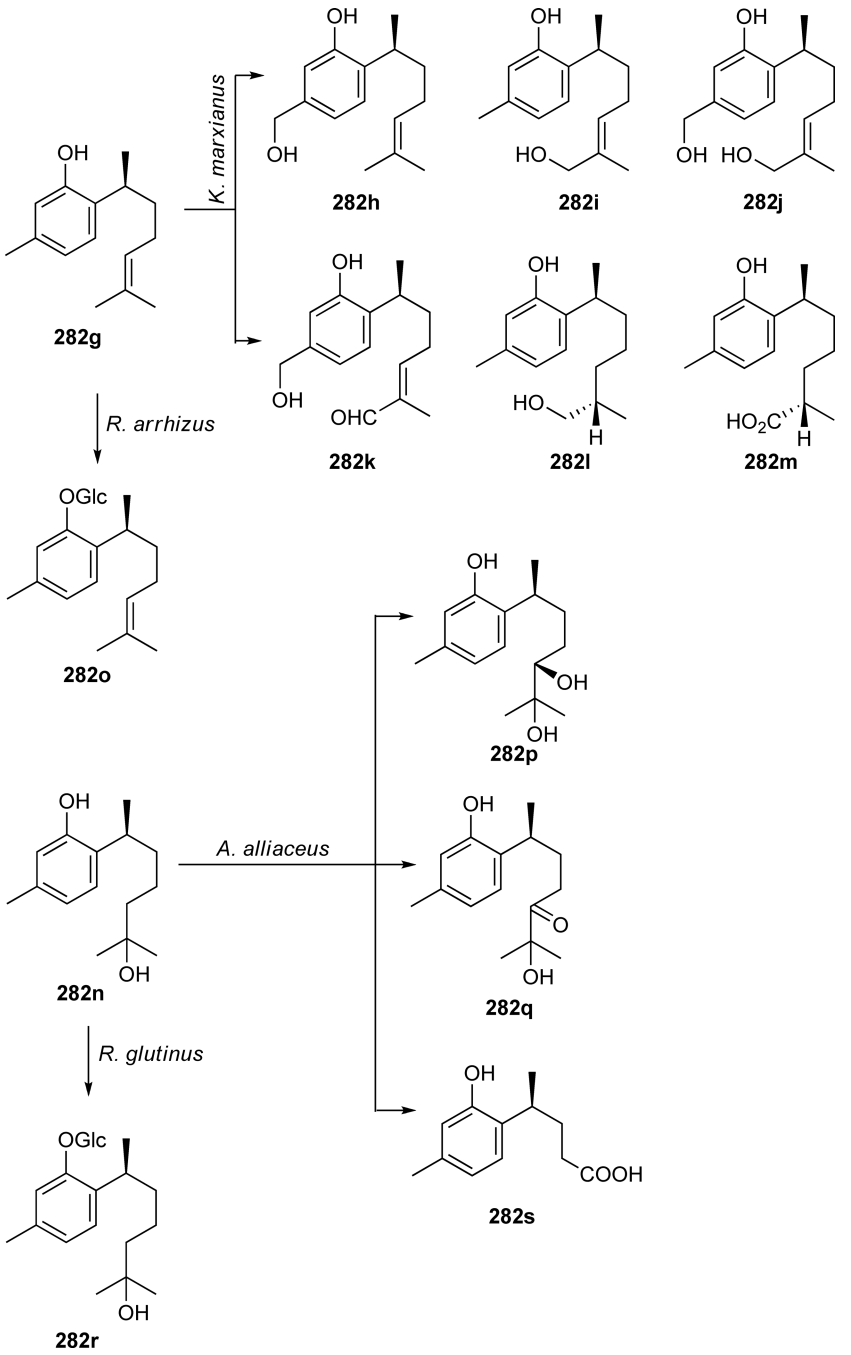
Scheme 81 Biotransformation of cadina-4,10(15)-dien-3-one (**265**) by *Beauveria bassiana.*



Scheme 82 Biotransformation of cadinol (**281**) by *Aspergillus niger*.



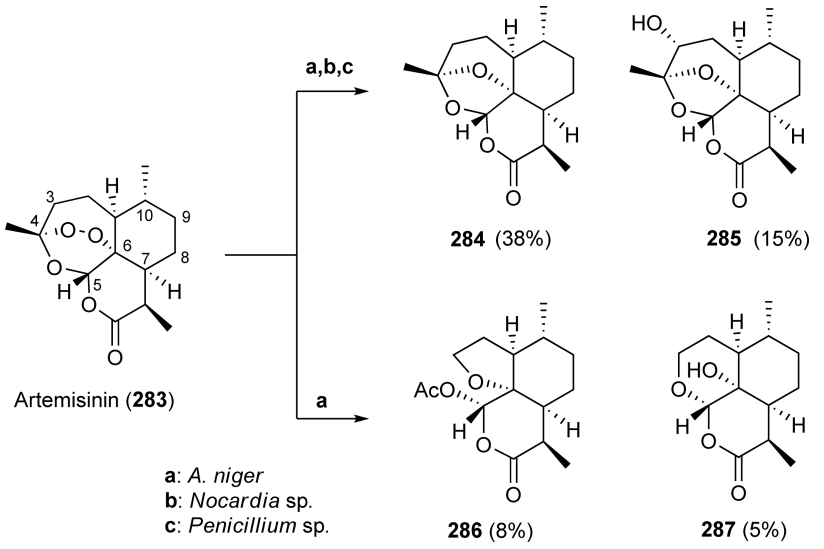
Scheme 83 Biotransformation of β-bisabolol (**282a**) by *Glomerella cingulata.*



Scheme 84 Biotransformation of (*S*)-(+)-curcuphenol (**282g**) by *Kluyveromyces marxianus* and *Rhizopus arrhizus* and curcudiol (**282n**) by *Aspergillus alliaceus* and *Rhodotorula glutinus*.

Artemisia annua is one of the most important Asteraceae species used as an antimalarial plant. There are many reports of microbial biotransformation of artemisinin (**283**), which is an active antimalarial rearranged cadinane sesquiterpene endoperoxide, and its derivatives to give novel antimalarials with increased activities or differing pharmacological characteristics.

Lee *et al.*[90] reported that deoxoartemisinin (**284**) and its 3α-hydroxy derivative (**285**) were obtained from the metabolites of artemisinin (**283**) incubated with *Nocardia corallina* and *Penicillium chrysogenum* (**Scheme 85**).



Scheme 85 Biotransformation of artemisinin (**283**) by *Aspergillus niger*, *Nocardia corallina*, and *Penicillium chrysogenum*.

Zhan *et al.*[91] reported that incubation of artemisinin (**283**) with *C. echinulata* and *A. niger* for 4 days at 28 °C resulted in the isolation of two metabolites, 10β-hydroxyartemisinin (**287a**) and 3α-hydroxydeoxyartemisinin (**285**), respectively.

Compound **283** was also biotransformed by *A. niger* to give four metabolites deoxyartemisinin (**284**, 38%), 3α-hydroxydeoxyartemisinin (**285**, 15%), and two minor products (**286**, 8% and **287**, 5%).[92]

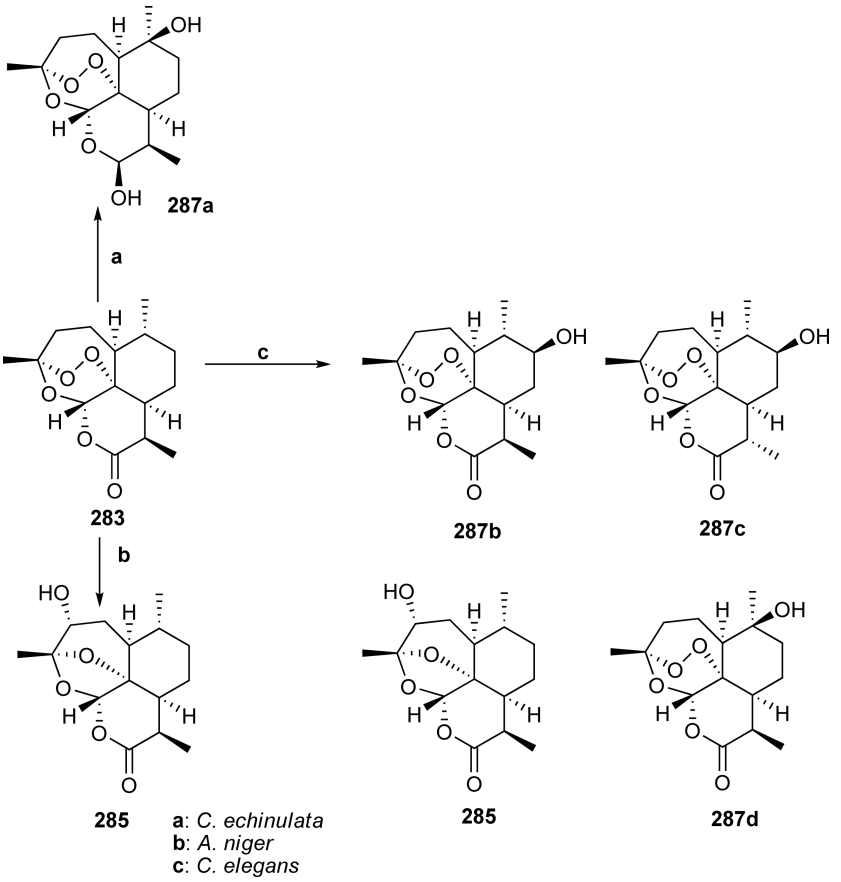
Artemisinin (**283**) was also bioconverted by *C. elegans*. During this process, 9β-hydroxyartemisinin (**287b**, 78.6%), 9β-hydroxy-8α-artemisinin (**287c**, 6.0%), 3α-hydroxydeoxoartemisinin (**285**, 5.4%), and 10β-hydroxyartemisinin (**287d**, 6.5%) have been formed. On the basis of QSAR and molecular modeling investigations, 9β-hydroxy derivatization of artemisinin skeleton may yield improvement in antimalarial activity and may potentially serve as an efficient means of increasing water solubility (**Scheme 86**).[93]

Albicanal (**288**) and (−)-drimenol (**289**) are simple drimane sesquiterpenoids isolated from the liverwort, *Diplophyllum serrulatum*, and many other liverworts, and higher plants. The latter compound was incubated with *M. plumbeus* and *Rhizopus arrhizus*. The former microorganism converted **289** into 6,7α-epoxy- (**290**), 3β-hydroxy-(**291**), and 6α-drimenol (**292**) yielding 2, 7, and 50%, respectively. On the other hand, the latter species produced only 3β-hydroxy derivative (**291**) in 60% yield (**Scheme 87**).[94]

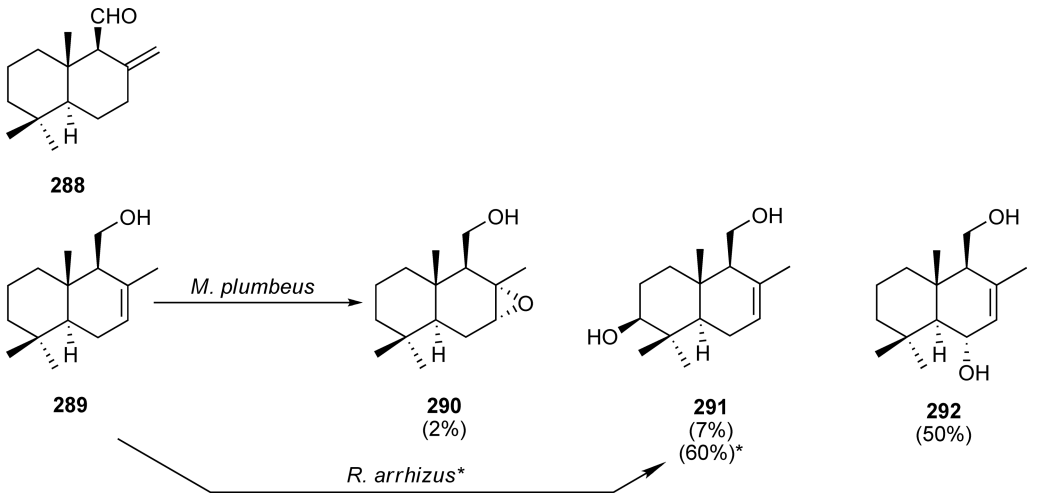
(−)-Polygodial (**293**) possessing piscicidal, antimicrobial, and mosquito repellant activity is the major pungent sesquiterpene diol isolated from *Polygonum hydropiper* and the liverwort, *Porella vernicosa* complex. Polygodial was incubated with *A. niger*, however, because of its antimicrobial activity, no metabolite was obtained.[95] The diol (**295**) prepared from polygodial (**293**) was also treated in the same manner as described above to yield 3β-hydroxy- (**297**) which was isolated from *Marasmius oreades* as antimicrobial activity[96] and 6α-hydroxypolygodiol (**298**) in 66–70% and 5–10% yield, respectively.[94] The same metabolite (**297**) was also obtained from polygodiol (**295**) as the only metabolite from the culture broth of *A. niger* in Czapek-peptone medium for 3 days in 70.5% yield[95], while the C-9 epimeric product (**296**) from isopolygodial (**294**) was incubated with *Mucor plumbeus* to yield 3β-hydroxy- (**299**) and 6α-hydroxy derivative (**300**) in low yield of 7 and 13%.[94] Drim-9α-hydroxy-11β,12-diacetoxy-7-ene (**301**) derived from polygodiol (**295**) was treated in the same manner as described above to yield its 3β-hydroxy derivative (**302**, 42%) (**Schemes 88** and **89**).[95]

Cinnamodial (**303**) from the Malagasy medicinal plant, *Cinnamosma fragrans*, was also treated in the same medium including *A. niger* to furnish three metabolites in very low yield (**304**, 2.2%, **305**, 0.05%, and **306**, 0.62%). Compounds **305** and **306** are naturally occurring cinnamosmolide, possessing cytotoxicity and antimicrobial activity, and fragrolide. In this case, the introduction of 3β-hydroxy group was not observed (**Scheme 90**).[97]

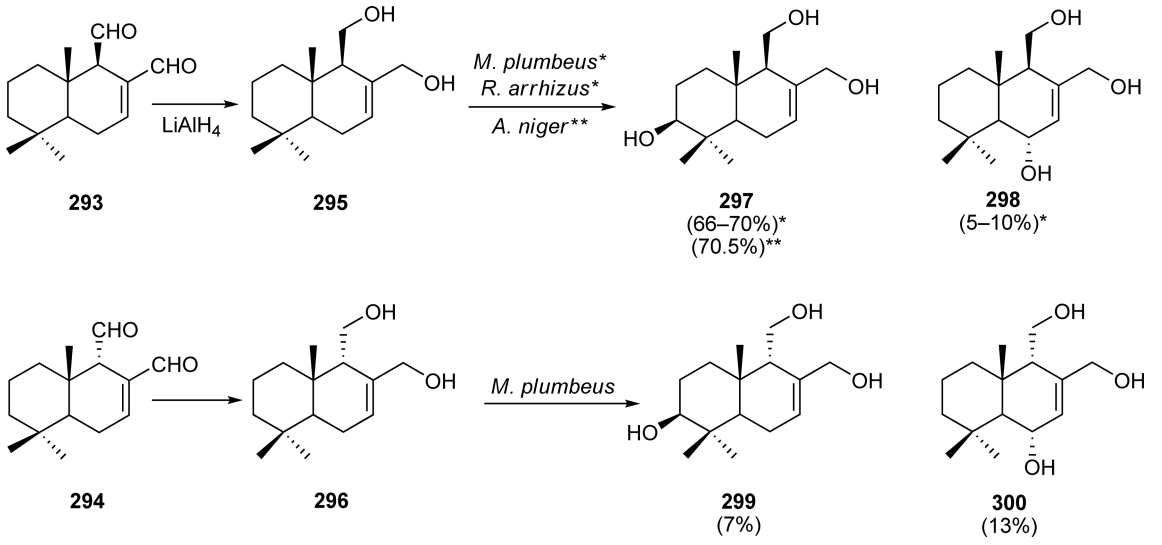
Naturally occurring rare drimane sesquiterpenoids with isocitric acid (**307–314**) were biosynthesized by the fungus *Cryptoporus volvatus*. Among these compounds, in particular, cryptoporic acid E (**312**) possesses antitumor



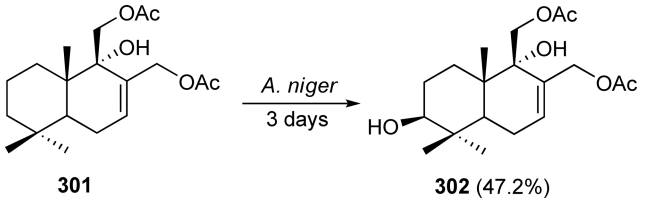
Scheme 86 Biotransformation of artemisinin (**283**) by *Cunninghamella echinulata*, *C. elegans*, and *Aspergillus niger*.



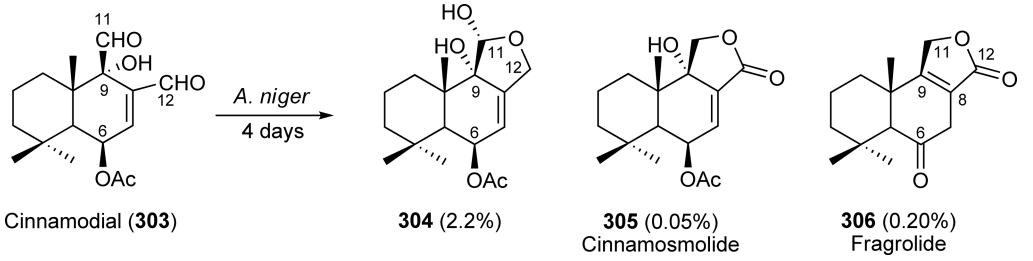
Scheme 87 Biotransformation of drimenol (**289**) by *Mucor plumbeus* and *Rhizopus arrhizus*.



Scheme 88 Biotransformation of polygodiol (**295**) by *Mucor plumbeus*, *Rhizopus arrhizus*, and *Aspergillus niger*.



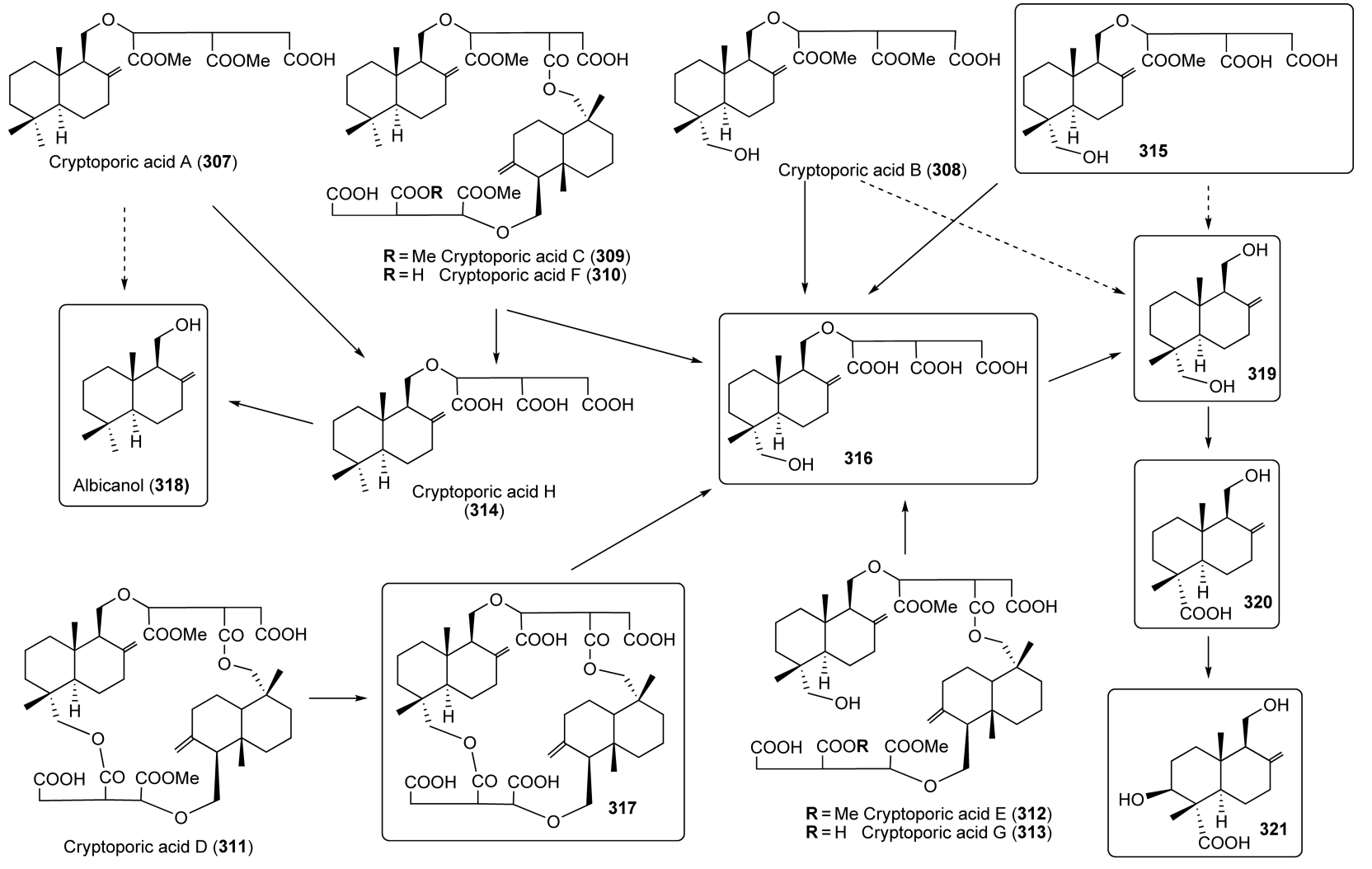
Scheme 89 Biotransformation of drim-9α-hydroxy-11,12-diacetoxy-7-ene (**301**) by *Aspergillus niger*.



Scheme 90 Biotransformation of cinnamodial (**303**) by *Aspergillus niger*.

promoter, anticolon cancer, and very strong super oxide anion radical scavenging activities.[98] When the fresh fungus is allowed to stand under moisture conditions, an olive-colored fungus *Paecilomyces varioti* grows on the surface of the fruiting body of this fungus. Two kilograms of the fresh fungus was infected with *C. volvatus* for 1 month, followed by the extraction of methanol to give the crude extract, followed by purification using silica gel and Sephadex LH-20 to give five metabolites (**316**, **318–321**), which were not found in the fresh fungus.[99] Compound **318** was also isolated from the liverworts, *Bazzania* and *Diplophyllum* species (**Scheme 91**).[40,41]

Liverworts produce a large number of enantiomeric mono-, sesqui-, and diterpenoids to those found in higher plants and lipophilic aromatic compounds. It is also noteworthy that some liverworts produce metabolites of enantiomeric terpenoids. The more interesting phenomenon in the chemistry of liverworts is that the different species in the same genus, for example, *Frullania tamarisci* subsp. *tamarisci* and *F. dilatata* produce totally enantiomeric terpenoids. Various sesqui- and diterpenoids, bibenzyls, and bisbibenzyls isolated from several liverworts show characteristic fragrant odor, intensely hot and bitter taste, muscle relaxing, antimicrobial,



Scheme 91 Biotransformation of cryptoporic acids (**307–317**, **316**) by *Paecilomyces varioti*.

antifungal, allergenic contact dermatitis, antitumor, insect antifeedant, superoxide anion release inhibitory, piscicidal, and neurotrophic activity.[40,41,100–104] In order to obtain the different kinds of biologically active products and to compare the metabolites of both normal and enantiomers of terpenoids, several secondary metabolites of specific liverworts were biotransformed by *Penicillium sclerotiorum*, *A. niger*, and *A. cellulosae*.

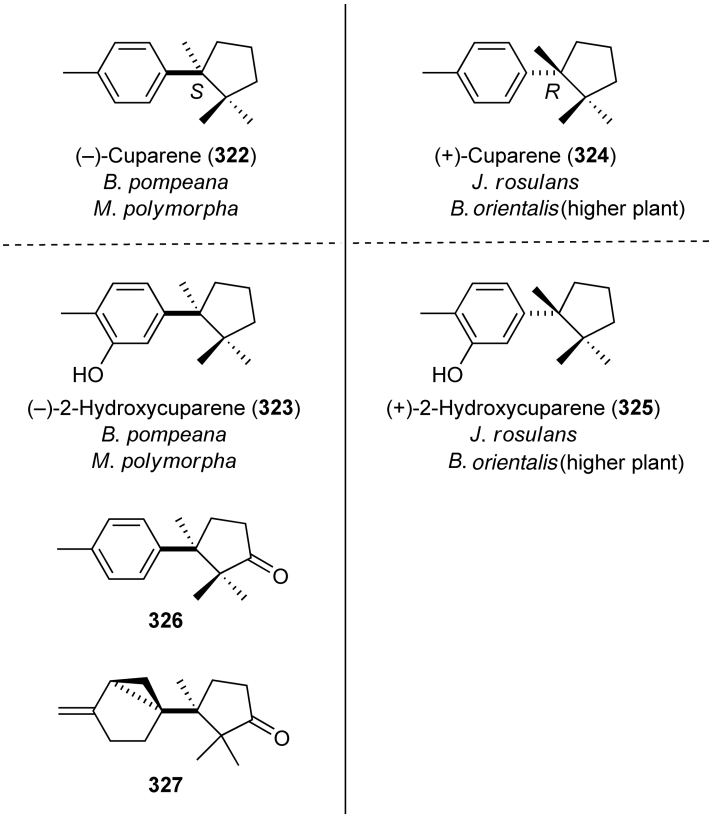
(−)-Cuparene (**322**) and (−)-2-hydroxycuparene (**323**) have been isolated from the liverworts, *Bazzania pompeana* and *M. polymorpha*, while its enantiomer (+)-cuparene (**324**) and (+)-2-hydroxycuparene (**325**) from the higher plants, *Biota orientalis*, and the liverwort *Jungermannia rosulans*. (*R*)-(−)-α-Cuparenone (**326**) and grimaldone (**327**) demonstrate intense flagrance. In order to obtain such compounds from both cuparene and its hydroxy compounds, both enantiomers mentioned above were cultivated with *A. niger* (**Scheme 92**).[105]

From (−)-cuparene (**322**), five metabolites (**328–332**) all of which contained cyclopentanediols or hydroxycyclopentanones were obtained. An aryl methyl group was also oxidized to give primary alcohol, which was further oxidized to yield carboxylic acids (**329–331**) (**Scheme 93**).[105]

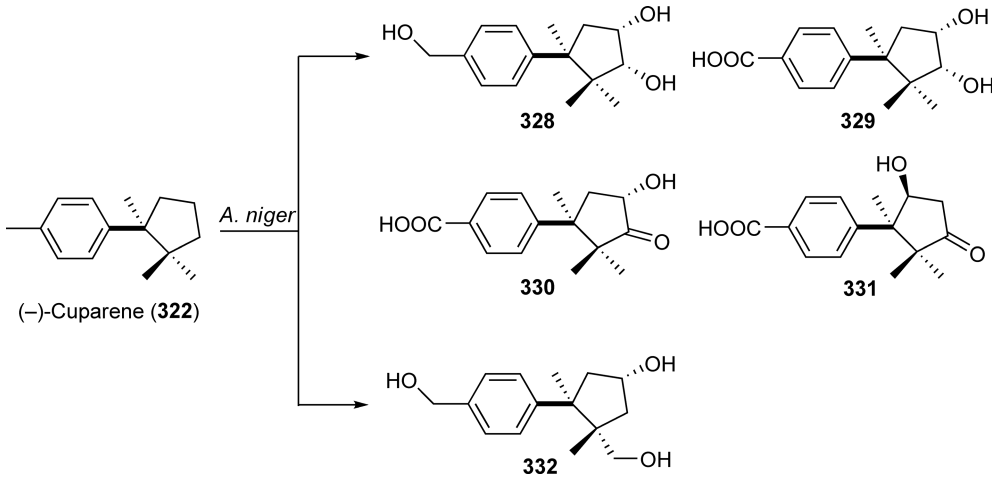
From (+)-cuparene, six metabolites (**333–338**) were obtained. These are structurally very similar to those found in the metabolites of (−)-cuparene, except for the presence of an acetonide (**336**), but are not identical. All metabolites possess benzoic acid moiety (**Scheme 94**).

The possible biogenetic pathways of (+)-cuparene (**324**) has been proposed in **Scheme 95**. Unfortunately, none of the metabolites shows strong mossy odor.[106] The presence of an acetonide in the metabolites has also been seen in those of dehydronootkatone (**25**).[22]

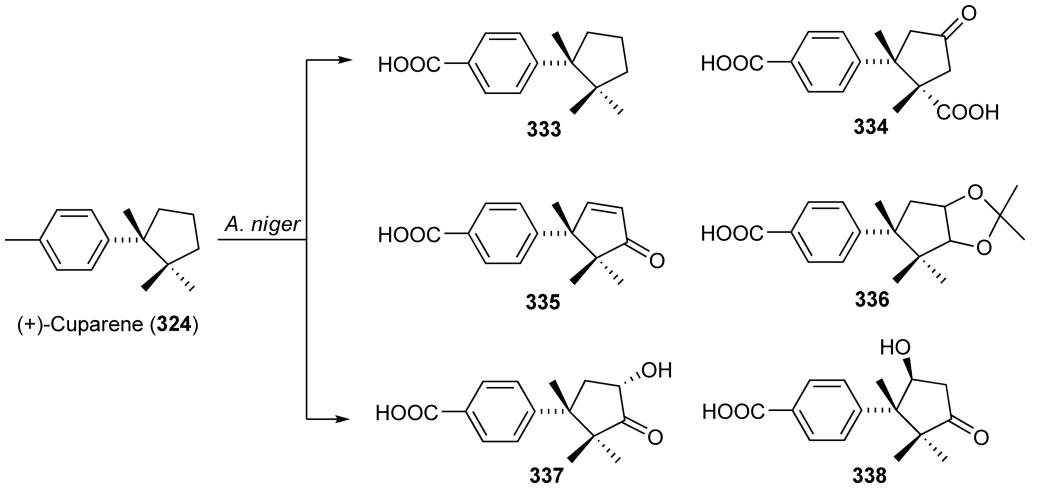
The liverwort *Herbertus adancus*, *H. sakuraii*, and *Mastigophora diclados* produce (−)-herbertene, the C-3 methyl isomer of cuparene, with its hydroxy derivatives, for example, herbertanediol (**339**) that shows NO production inhibitory activity[107] and herbertenol (**342**). Treatment of compound **339** in *Penicillium sclerotiorum* in Czapek-polypeptone medium gave two dimeric products, mastigophorene A (**340**) and mastigophorene B (**341**), which showed neurotrophic activity (**Scheme 96**).[108]



Scheme 92 Naturally occurring cuparene sesquiterpenoids (**322–327**).



Scheme 93 Biotransformation of (–)-cuparene (**322**) by *Aspergillus niger*.

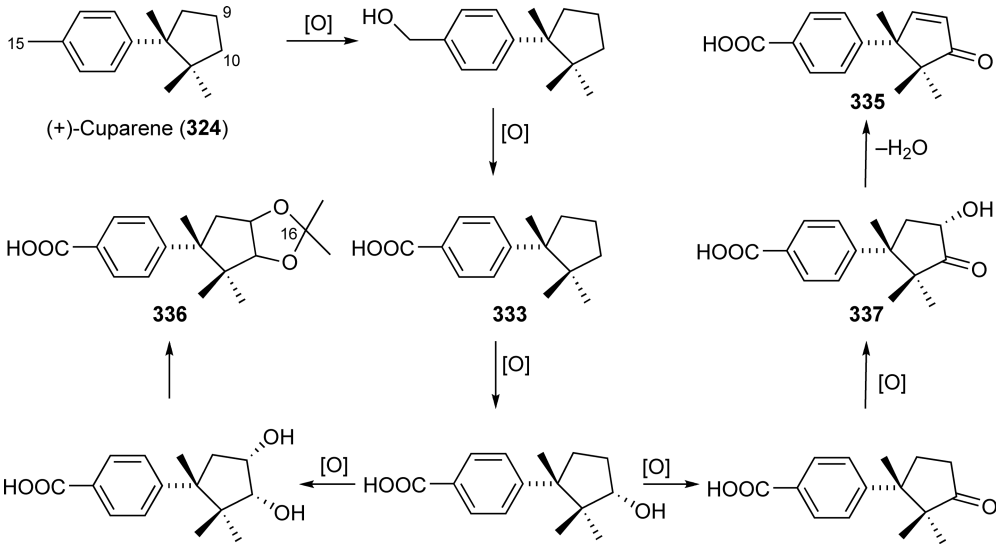


Scheme 94 Biotransformation of (+)-cuparene (**324**) by *Aspergillus niger*.

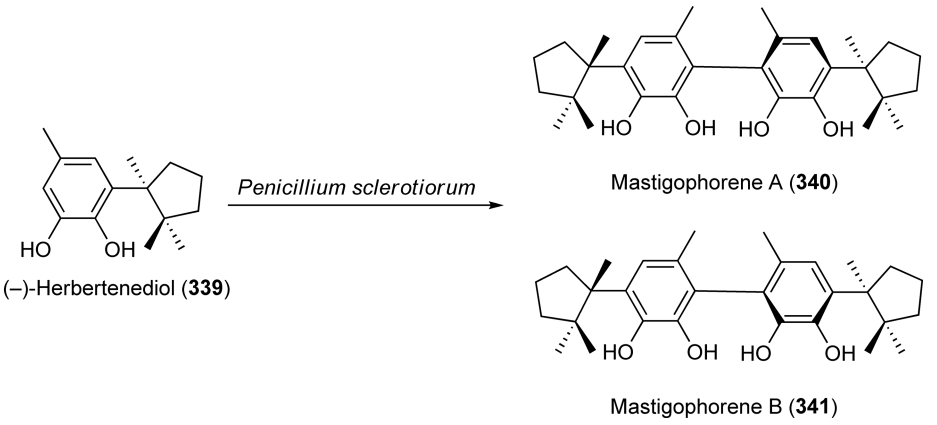
When (−)-herbertenol (**342**) was biotransformed for 1 week by the same fungus, no metabolic product was obtained, however, five oxygenated metabolites (**344–348**) were obtained from its methyl ether (**343**). The possible metabolic pathway is shown in **Schemes 97 and 98**. Except for the presence of the ether (**348**), the metabolites from **342** resemble those found in (−)- and (+)-cuparene.[106]

(−)-Maalioxide (**349**) obtained from the liverwort, *P. sciophila* was incubated with *A. niger* to yield three metabolites, 1β-hydroxy-(**350**), 1β,9β-dihydroxy-(**351**), and 1β,12-dihydroxymaalioxides (**352**), of which **351** was predominant (53.6%). When the same substrate was cultured with *A. cellulosae*, 7β-hydroxymaalioxide (**353**) was obtained as a sole product in 30% yield.[109] The same substrate (**349**) was also incubated with the fungus *M. plumbeus* to obtain a new metabolite, 9β-hydroxymaalioxide (**354**) together with two known hydroxylated products (**350**, **353**).[110]

(−)-Maalioxide (**349**) was oxidized by *m*-chloroperbenzoic acid to give a very small amount of **353** (1.2%), together with 2α-hydroxy- (**355**, 2%) and 8α-hydroxymaalioxide (**356**, 1.5%) which have not been obtained in the metabolite of **349** in *A. niger* and *A. cellulosae* (**Scheme 99**).[111]



Scheme 95 Possible pathway of biotransformation of (+)-cuparene (**324**) by *Aspergillus niger*.

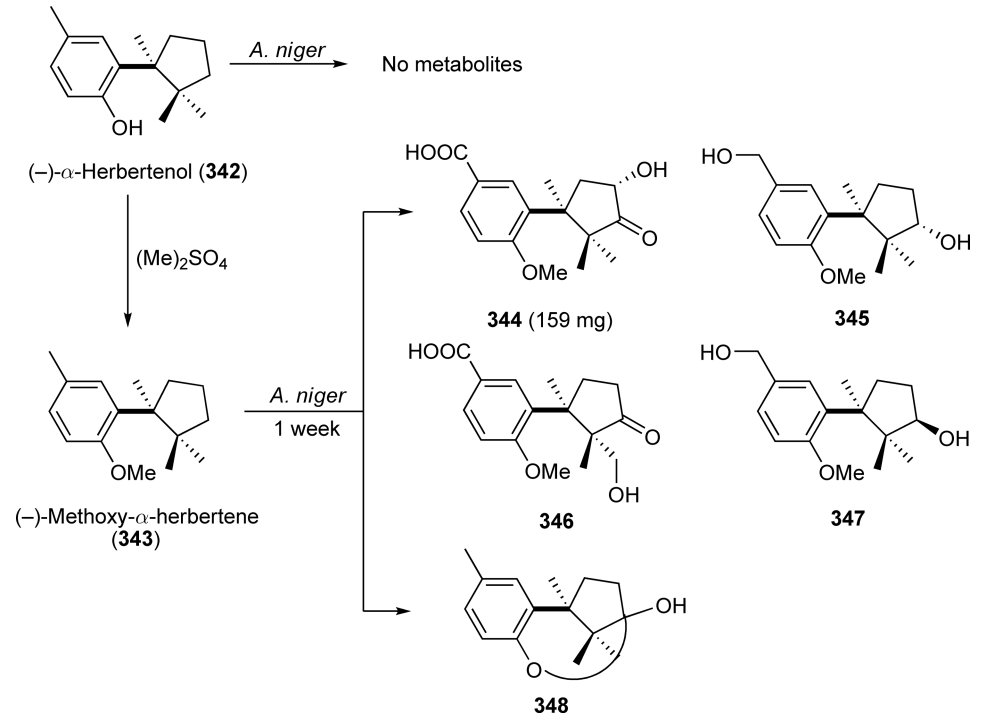


Scheme 96 Biotransformation of (–)-herbertenediol (**339**) by *Penicillium sclerotiorum*.

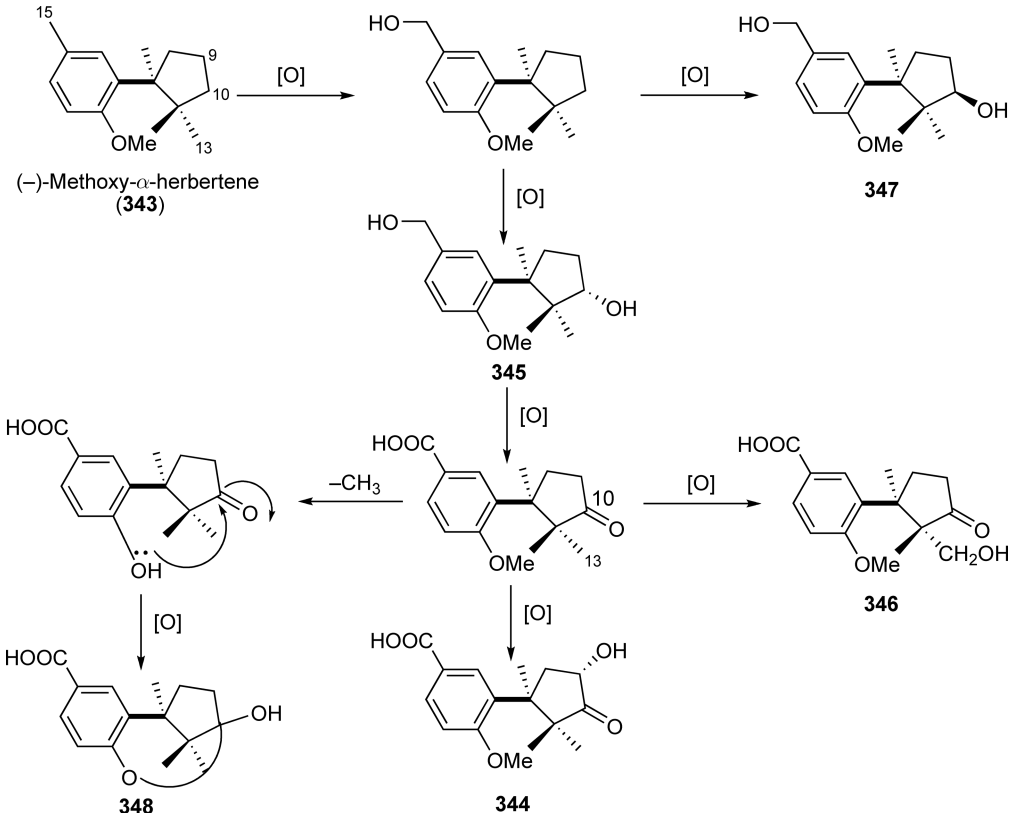
Plagiochila sciophila is one of the most important liverworts, since it produces bicyclohumulenone (**357**) that possesses strong mossy odor and is expected to manufacture a compounding perfume. In order to obtain a better perfume, **357** was treated with *A. niger* for 4 days to give 4α,10β-dihydroxybicyclohumulenone (**358**, 27.4%) and bicyclohumurenone-12-oic acid (**359**). An epoxide (**360**) prepared by *m*-chloroperbenzoic acid was further treated with the same fungus as described above to give the 10β-hydroxy derivative (**361**, 23.4%). Unfortunately, these metabolites possess only faint mossy odor (**Scheme 100**).[42]

The liverwort *Reboulia hemisphaerica* biosynthesizes cyclomyltaylanoids such as **362** and *ent*-1α-hydroxy-β-chamigrene (**367**). Biotransformation of cyclomyltaylan-5-ol (**362**) by *A. niger* gave four metabolites, 9β-hydroxy- (**363**, 27%), 9β,15-dihydroxy- (**364**, 1.7%), 10β-hydroxy- (**365**, 10.3%), and 9β,15-dihydroxy derivative (**366**, 12.6%). In this case, the stereospecific introduction of hydroxyl group was observed but its regiospecificity was not seen in this substrate (**Scheme 101**).[112]

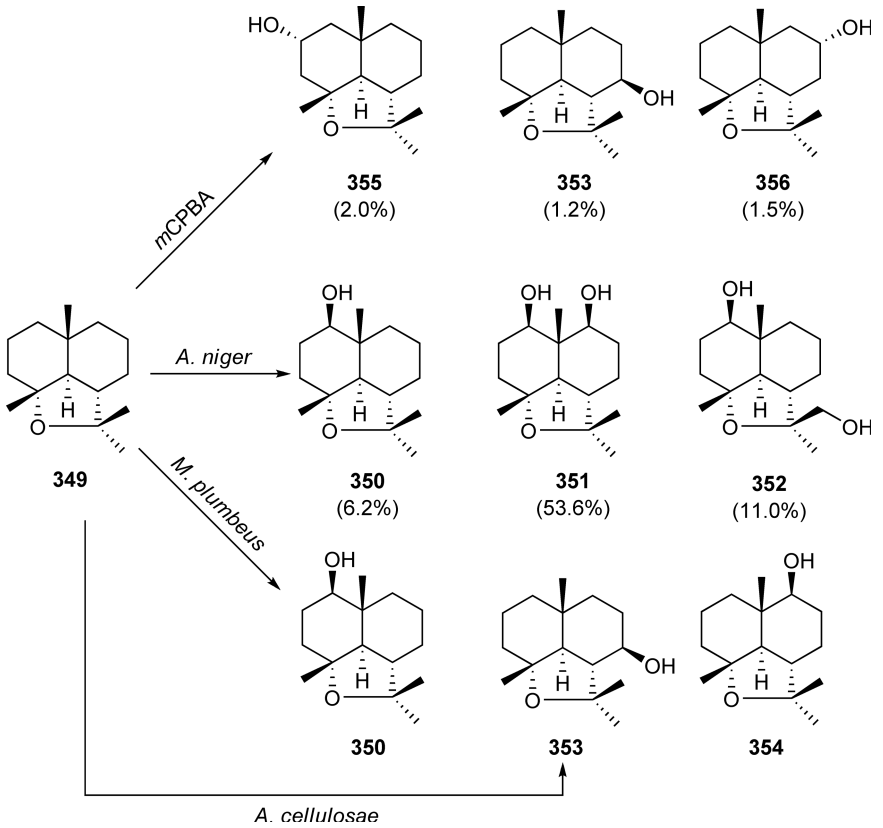
ent-1α-Hydroxy-β-chamigrene (**367**) was inoculated in the same manner as described above to give three new metabolites (**368–370**) of which **370** was the major product (46.2% in isolated yield). The hydroxylation of



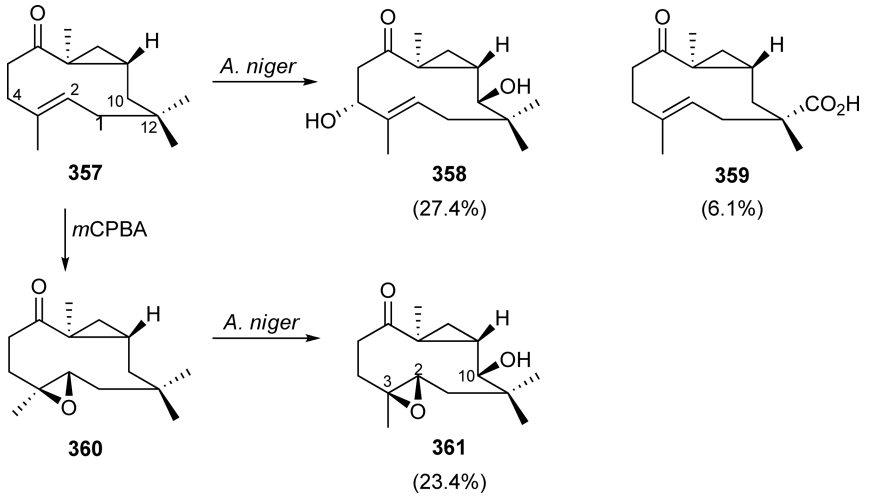
Scheme 97 Biotransformation of (–)-methoxy-α-herbertene (**343**) by *Aspergillus niger.*



Scheme 98 Possible pathway of biotransformation of (–)-methoxy-α-herbertene (**343**) by *Aspergillus niger.*



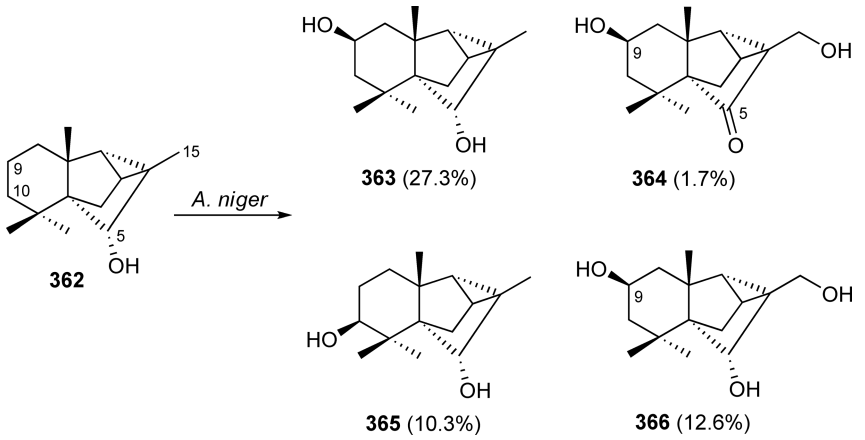
Scheme 99 Biotransformation of maalioxide (**349**) by *Aspergillus niger*, *A. cellulosae*, and *Mucor plumbeus.*



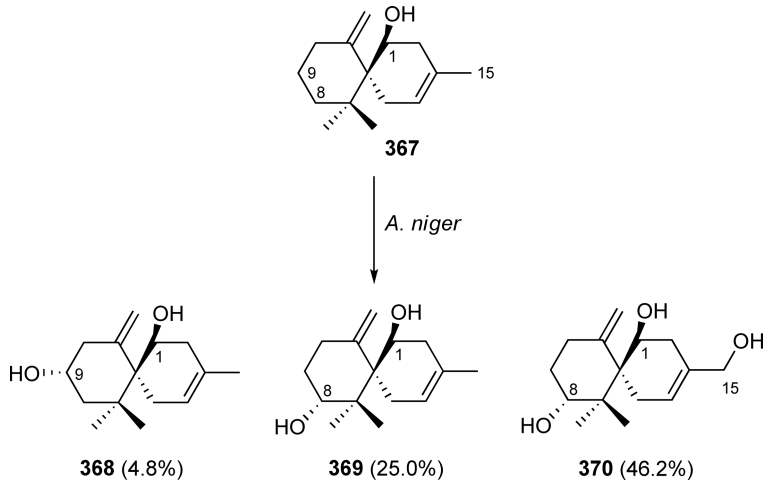
Scheme 100 Biotransformation of bicyclohumulenone (**357**) by *Aspergillus niger.*

vinyl methyl group has been known to be very common in case of microbial and mammalian biotransformation (**Scheme 102**).[112,113]

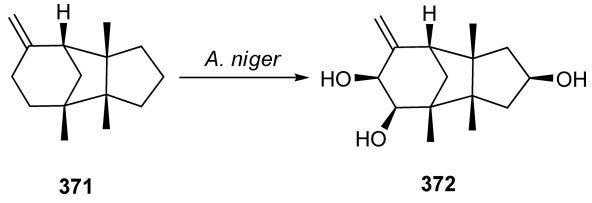
β-Barbatene (=gymnomitrene) (**4**), a ubiquitous sesquiterpene hydrocarbon, from liverwort such as *Plagiochila sciophila* and many other Jungermanniales liverworts were treated in the same manner using *A. niger* for 1 day, which gave a triol, 4β,9β,10β-trihydroxy-β-barbatene (**27**, 8%) (**Scheme 103**).[42]



Scheme 101 Biotransformation of cyclomyltaylan-5-ol (**362**) by *Aspergillus niger*.



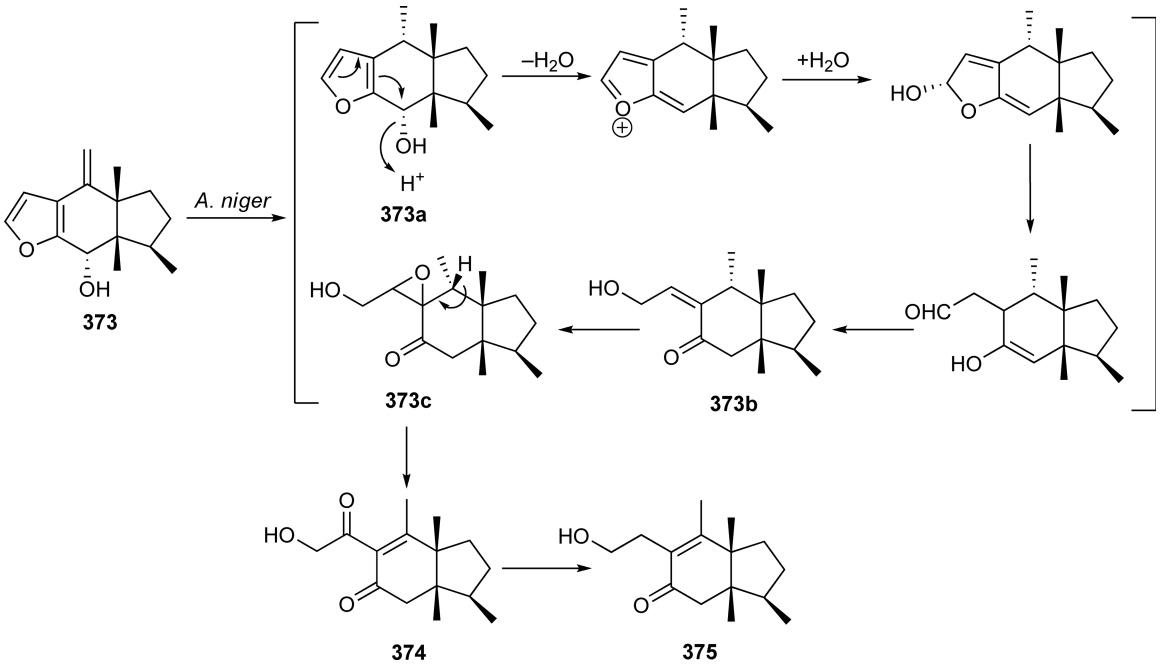
Scheme 102 Biotransformation of *ent*-1α-hydroxy-β-chamigrene (**367**) by *Aspergillus niger*.



Scheme 103 Biotransformation of β-barbatene (**371**) by *Aspergillus niger*.

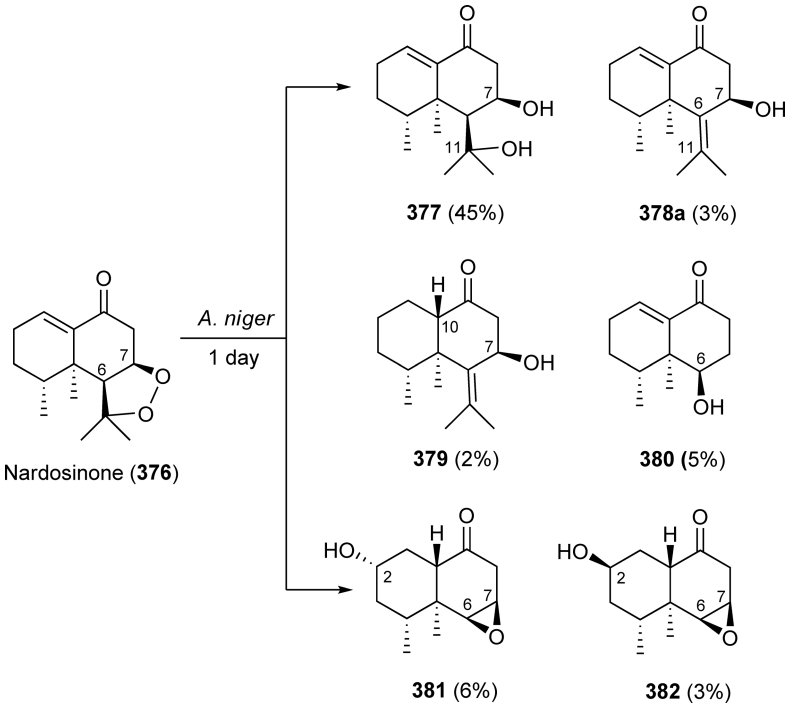
Pinguisane sesquiterpenoids have been isolated from the Jungermanniales, Metzgeriales, and Marchantiales. In particular, the Lejeuneaceae and Porellaceae are rich sources of this unique type of sesquiterpenoids. One of the major furanosesquiterpene (**373**) was biodegraded by *A. niger* to yield primary alcohol (**375**) which might be formed from **374** as shown in **Scheme 104**.[114]

In order to obtain more pharmacologically active compounds, the secondary metabolites from crude drugs and animals were biotransformed by some fungi. Nardosinone (**376**) isolated from *N. chinensis* which has been used for headache, stomachache, diuresis, and antimalarial drug, hinesol (**384**), possessing spasmolytic activity, obtained from *A. lanceae* rhizomes and (−)-ambrox (**391**) from ambergris were biotransformed by *A. niger*, *A. cellulosae*, and *B. dothidea*.

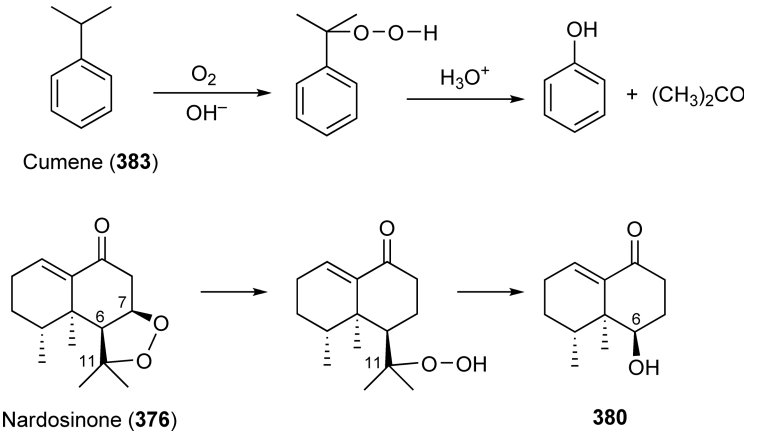


Scheme 104 Biotransformation of pinguisanol (**373**) by *Aspergillus niger*.

Nardosinone (**376**) was incubated with *A. niger* as described above for 1 day to give six metabolites (**377**, 45%, **378**, 3%, **379**, 2%, **380**, 5%, **381**, 6%, and **382**, 3%). Compounds **380–382** are unique trinorsesquiterpenoids although their yield is very poor. Compound **380** might be formed by a similar manner to that of phenol from cumene (**383**) (**Schemes 105** and **106**).[92]

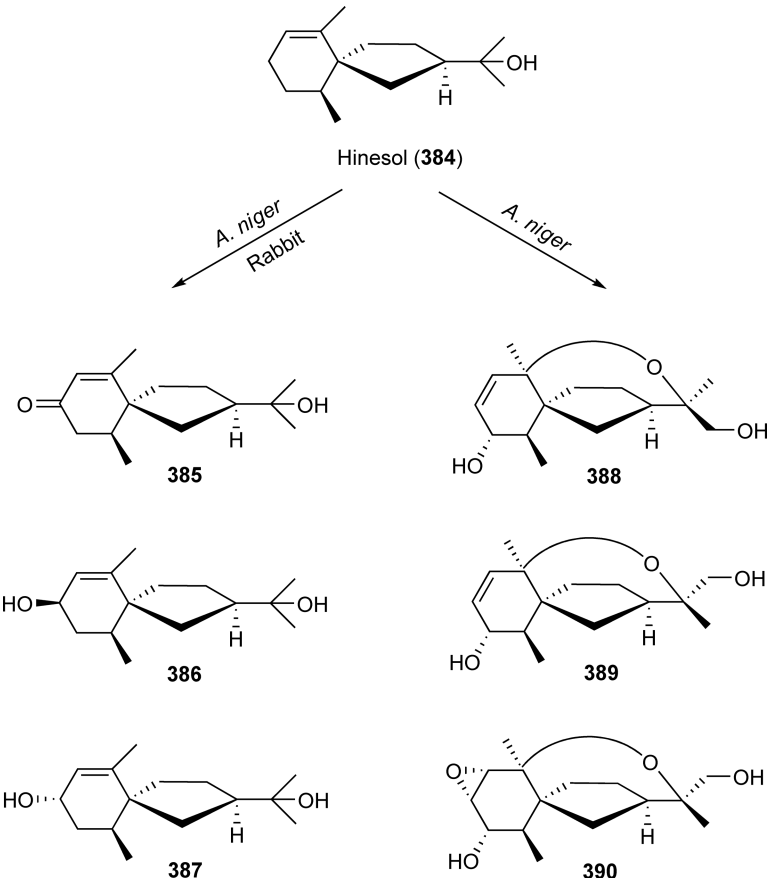


Scheme 105 Biotransformation of nardosinone (**376**) by *Aspergillus niger*.



Scheme 106 Possible pathway of biotransformation of nardosinone (**376**) to trinornardosinone (**380**) by *Aspergillus niger*.

From hinesol (**384**), two allylic alcohols (**386, 387**) and their oxygenated derivative (**385**), and three unique metabolites (**388–390**) having oxirane ring were obtained. The metabolic pathway is very similar to that of oral administration of hinesol since the same metabolites (**395–387**) were obtained from the urine of rabbits (**Scheme 107**).[63,115,116]



Scheme 107 Biotransformation of hinesol (**384**) by *Aspergillus niger*.

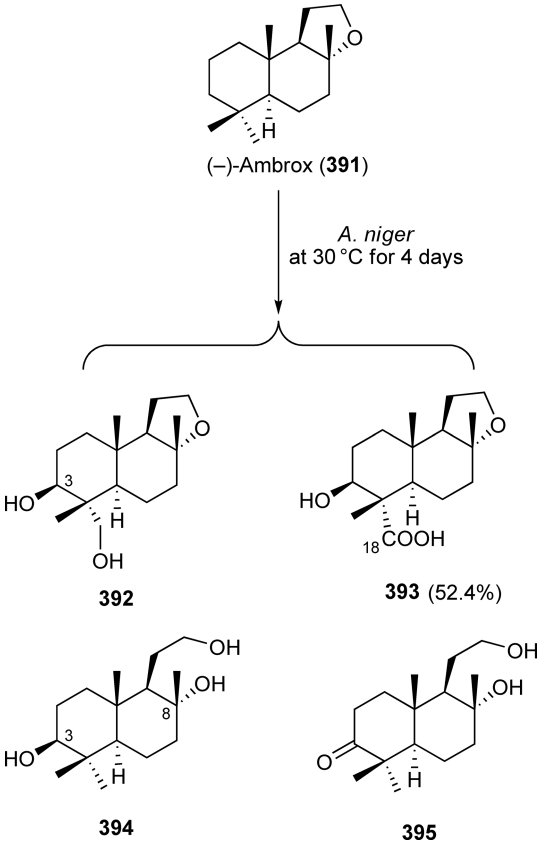
To obtain a large amount of ambrox (**391**), labda-12,14-dien-7α,8-diol obtained from the liverwort, *Porella pettottetiana* as a major component, was chemically converted into (−)-ambrox through six steps in relatively high yield.[117] Ambrox was added to Czapek-peptone medium including *A. niger* for four days, followed by chromatography of the crude extract to yield four oxygenated products (**392–395**) among which the carboxylic acid (**393**, 52.4%) is the major product (**Scheme 108**).[63]

When ambrox (**391**) was biotransformed by *A. niger* for 9 days in the presence of 1-aminobenzotriazole, an inhibitor of CYP450, compounds **396** and **397** were obtained instead of the metabolites **392–395** which were obtained by incubation of ambrox in the absence of the inhibitor. Ambrox was cultivated with *A. cellulosae* for 4 days in the same medium to yield C-1 oxygenated products (**398** and **399**), the former of which was a major product (41.3%) (**Scheme 109**).[63]

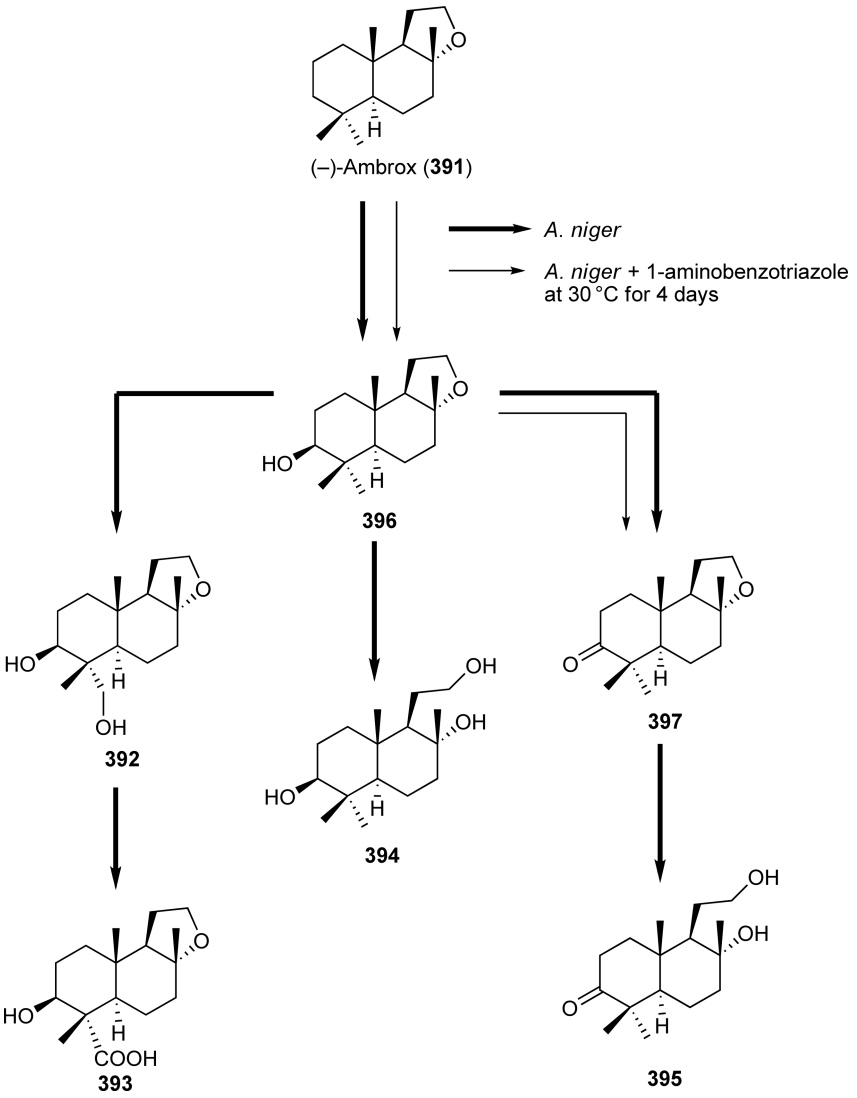
The metabolite pathways of ambrox are quite different between *A. niger* and *A. cellulosae*. Oxidation at C-1 occurred in *A. cellulosae* to yield **398** and **399** which was also provided by John's oxidation of **398**, while oxidation at C-3 and C-18 and ether cleavage between C-8 and C-12 occurred in *A. niger* to give **392–395**. Ether cleavage seen in *A. niger* is very rare.

Fragrance of the metabolites **392–395** and 7α-hydroxy-(−)-ambrox (**400**) and 7-oxo-(−)-ambrox (**401**) obtained from labdane diterpene diol were estimated. Only **399** demonstrated a similar odor to ambrox (**391**) (**Scheme 110**).[63]

(−)-Ambrox (**391**) was also microbiotransformed with *Fusarium lini* to give mono-, di-, and trihydroxylated metabolites (**401a–401d**), while incubation of the same substrate with *Rhizopus stolonifera* yielded two metabolites (**394**, **396**), which were obtained from **391** by *A. niger* as mentioned above, together with **397** and **401e** (**Scheme 111**).[118]



Scheme 108 Biotransformation of (−)-ambrox (**391**) by *Aspergillus niger*.



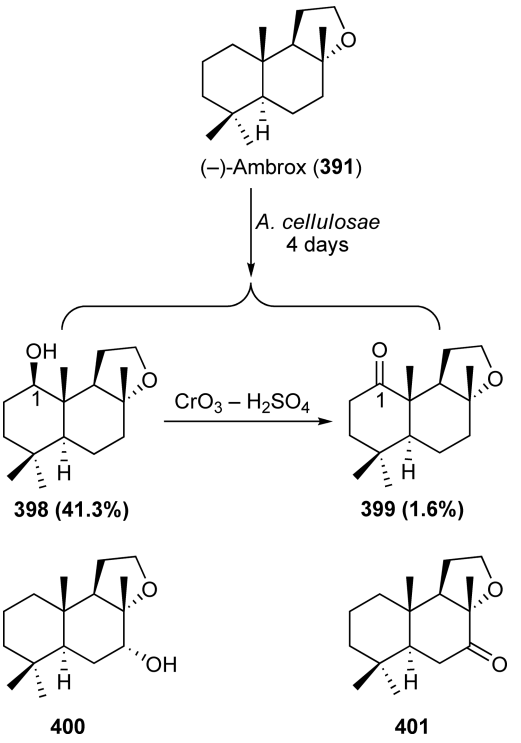
Scheme 109 Possible pathway of biotransformation of (–)-ambrox (**391**) by *Aspergillus niger.*

The sclareolide (**402**) which is a C-12 oxo derivative of ambrox was incubated with *M. plumbeus* to yield three metabolites: 3β-hydroxy- (**403**, 7.9%), 1β-hydroxy- (**404**, 2.5%), and 3-ketosclareolide (**405**, 7.9%) (**Scheme 112**).[119]

Aspergillus niger in the same medium as mentioned above converted sclareolide (**402**) into two new metabolites (**406, 407**), together with known compounds (**403**, **405**) of which 3β-hydroxysclareolide (**403**) is preferentially obtained (**Scheme 113**).[120]

From the metabolites of sclareolide (**402**) incubated with *C. lunata* and *A. niger*, five oxidized compounds (**403–405, 405a, 405b**) were obtained. Fermentation of **402** with *Gibberella fujikuroii* yielded **403–405, 405a**. The metabolites **403** and **405a** were formed from the same substrate by the incubation of *F. lini*. No microbial transformation of **402** was observed with *Pleurotus ostreatus* (**Scheme 114**).[121]

Compound **391** was treated with *C. lunata*, which gave metabolites **396** and **401e**, while *Cunninghamella elegans* yielded compounds **401e** and **396** and (+)-sclareolide (**402**) (**Scheme 114**). The metabolites **396, 401a–401e** from **391** do not release any effective aroma when compared to **391**. Compound **394** showed a strong sweet odor quite different from the amber-like odor.[118]



Scheme 110 Biotransformation of (–)-ambrox (**391**) by *Aspergillus cellulosae*.

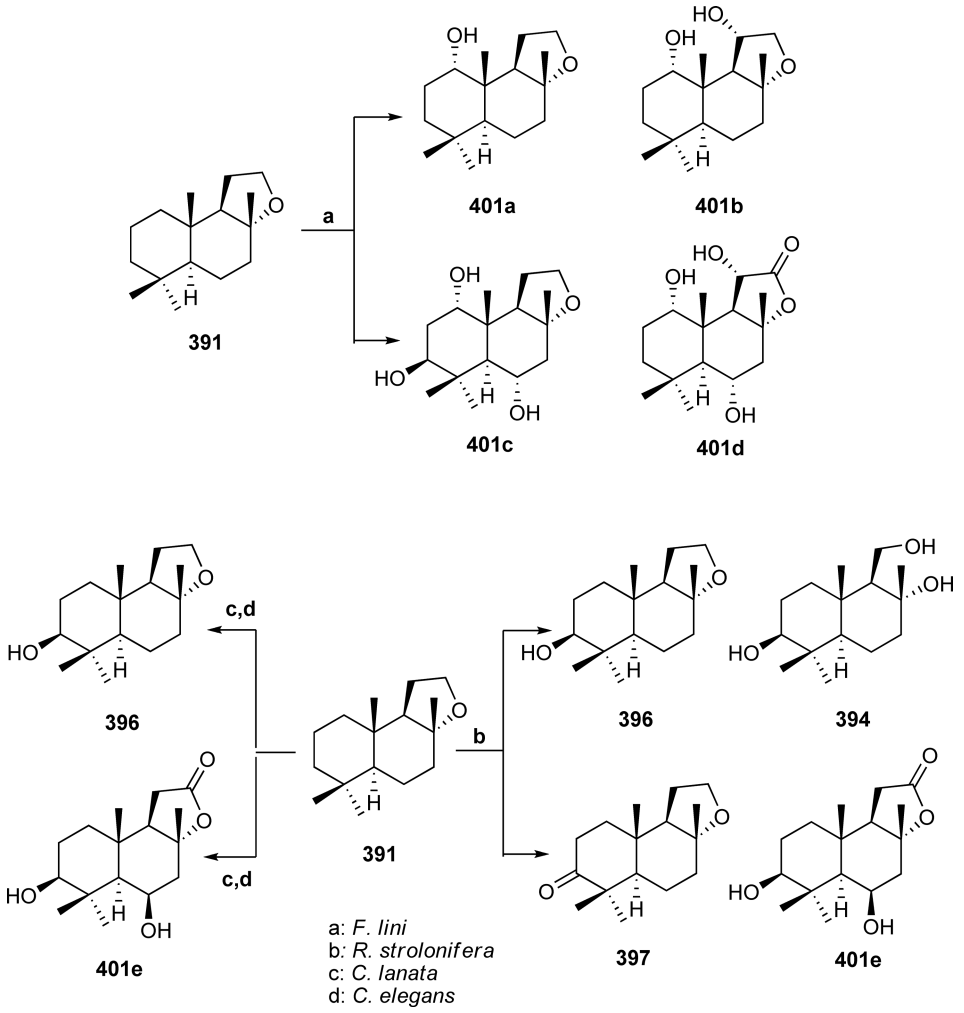
Sclareolide (**402**) exhibited phytotoxic and cytotoxic activity against several human cancer cell lines. *Cunninghamella elegans* produced new oxidized metabolites (**403**, **404**, **405a–405e**), resulting from the enantioselective hydroxylation. Metabolites (**403**, **404**, and **405a**) have been known as early as biotransformed products of **402** by many different fungi and have shown cytotoxicity against various human cancer cell lines. The metabolites **403**, **404**, and **405a** indicated significant phytotoxicity at higher dose against *Lemna minor* L. (**Scheme 114**).[118]

Ambrox (**391**) and sclareolide (**402**) were incubated with the fungus *C. aphidicola* for 10 days in shake culture to give 3β-hydroxy- (**396**), 3β,6β-dihydroxy- (**401g**), 3β,12-dihydroxyambrox (**401h**) and sclareolide 3β,6β-diol (**401f**), and 3β-hydroxy- (**403**), 3-keto- (**405**), and sclareolide 3β,6β-diol (**401f**), respectively (**Scheme 115**).[122]

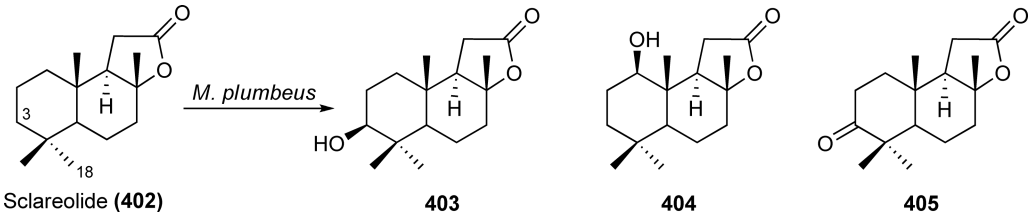
Zerumbone (**408**), which is easily isolated from the wild ginger, *Zingiber zerumbet*, and its epoxide (**409**) were incubated with *F. culmorum* and *A. niger* in Czapek-peptone medium, respectively. The former fungus gave (1*R*,2*R*)-(+)-2,3-dihydrozerumbol (**410**) stereospecifically through either 2,3-dihydrozerumbone (**408a**) or zerumbol (**408b**) or both and accumulated **410** in the mycelium. The facile production of optically active **410** will lead to a useful material of woody fragrance, namely 2,3-dihydrozerumbone. *Aspergillus niger* biotransformed **408** through epoxide (**409**) to several metabolites containing zerumbone-6,7-diol as a main product. The same fungus converted the epoxide (**409**) into three major metabolites containing (2*R*,6*S*,7*S*,10*R*,11*S*)-1-oxo7,9-dihydroxyisodaucane (**413**) through dihydro derivatives (**411**, **412**). However, *A. niger* biotransformed **409** only into **412** in the presence of CYP450 inhibitor, 1-aminobenzotriazole.[123]

The same substrate was incubated in the *A. niger*, *A. oryzae*, *Candida rugosa*, *C. tropicalis*, *Mucor mucedo*, *Bacillus subtilis*, and *Schizosaccharomyces pombe*, however, no metabolites have been obtained. All microbes except for *S. pombe*, bioconverted zerumbone epoxide (**409**) prepared by oxidation using mCPBA into two diastereoisomers, 2*R*,6*S*,7S-dihydro-derivative (**411**) and 2*R*,6*R*,7*R*-derivative (**412**) with over 99% ee (**Scheme 116**).[124]

Several microorganisms and a few mammals (see Section 3.20.3) were used for the biotransformation of (+)-cedrol (**414**) that is widely distributed in the cedar essential oils. Plant pathogenic fungus *G. cingulata* converted cedrol (**414**) into three diols (**415–417**) and 2α-hydroxycedrene (**418**).[125] The same substrate (**414**) was



Scheme 111 Biotransformation of (−)-ambrox (**391**) by *Fusarium lini* and *Rhizopus stolonifera*.

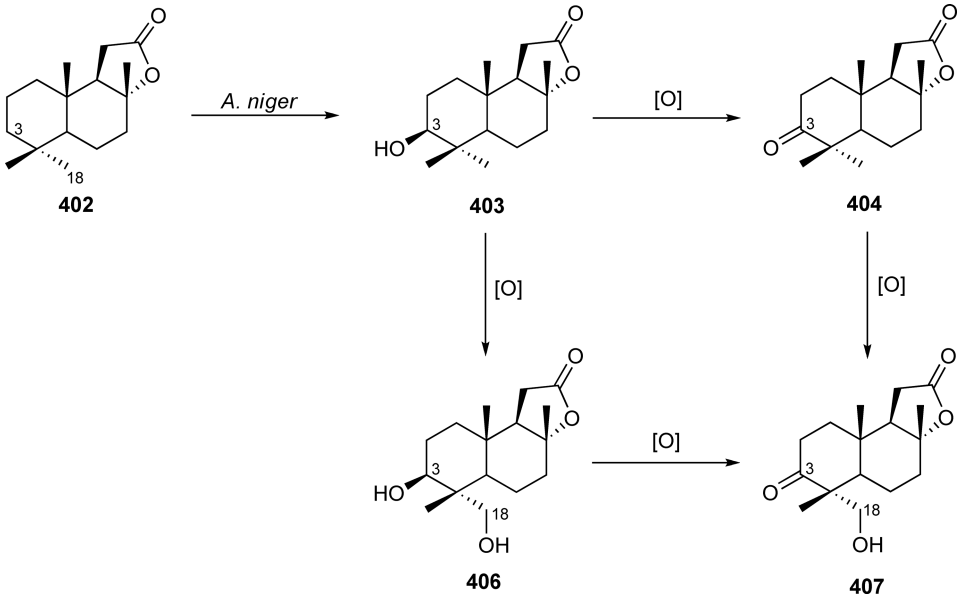


Scheme 112 Biotransformation of (+)-sclareolide (**402**) by *Mucor plumbeus*.

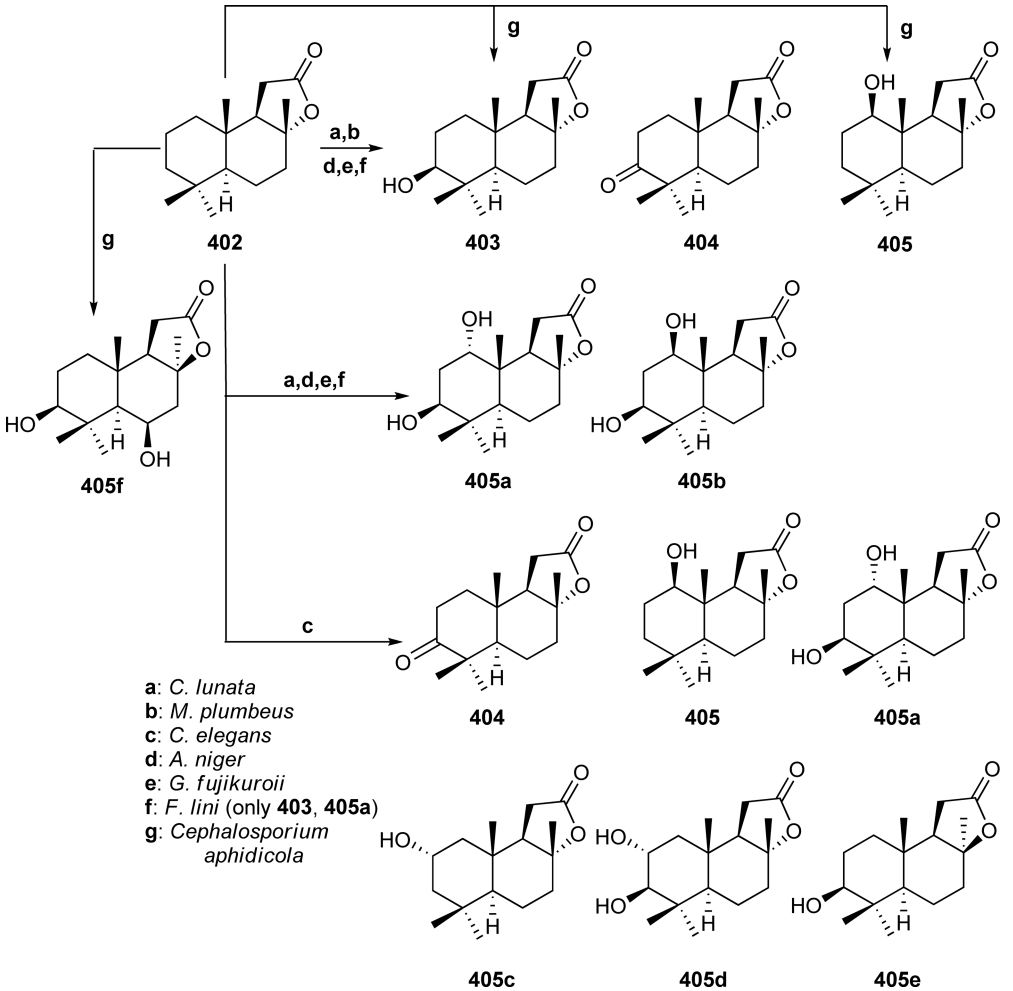
incubated with *A. niger* to give **416** and **417** together with a cyclopentanone derivative (**419**).[37] Human skin microbial flora, *Staphylococcus epidermidis*, also converted (+)-cedrol into 2α-hydroxycedrol (**415**) (**Scheme 117**).[126]

Cephalosporium aphidicola bioconverted cedrol (**414**) into **417**.[127] On the other hand, *Corynespora cassiicola* produced **419** in addition to **417**.[128] It is noteworthy that *B. cinerea* which damages many flowers, fruits, and vegetables biotransformed cedrol to different metabolites (**420–422**) from these mentioned above.[129]

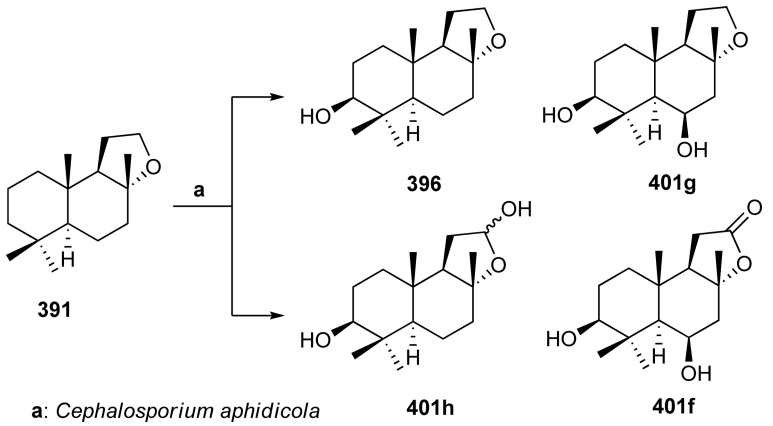
4α-Hydroxycedrol (**424**) was obtained from the metabolite of cedrol acetate (**423**) by using *G. cingulata* (**Scheme 118**).[130]



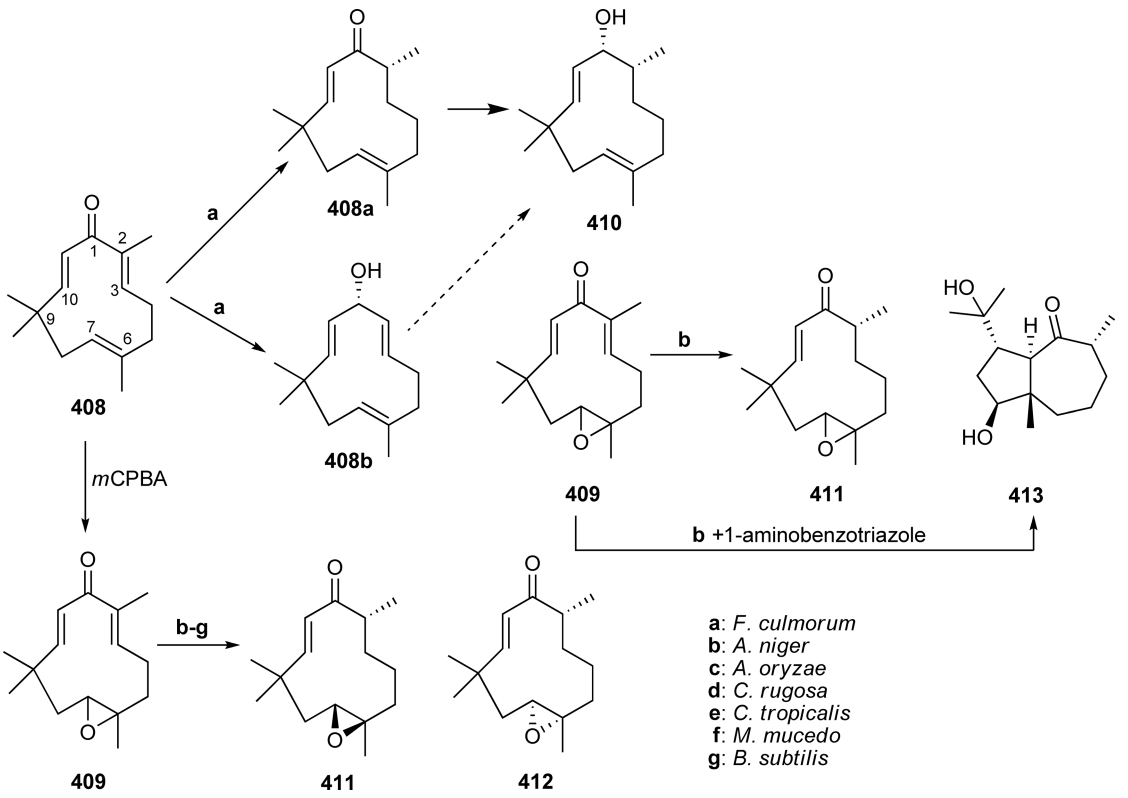
Scheme 113 Biotransformation of (+)-sclareolide (**402**) by *Aspergillus niger*.



Scheme 114 Biotransformation of (+)-sclareolide (**402**) by various fungi.



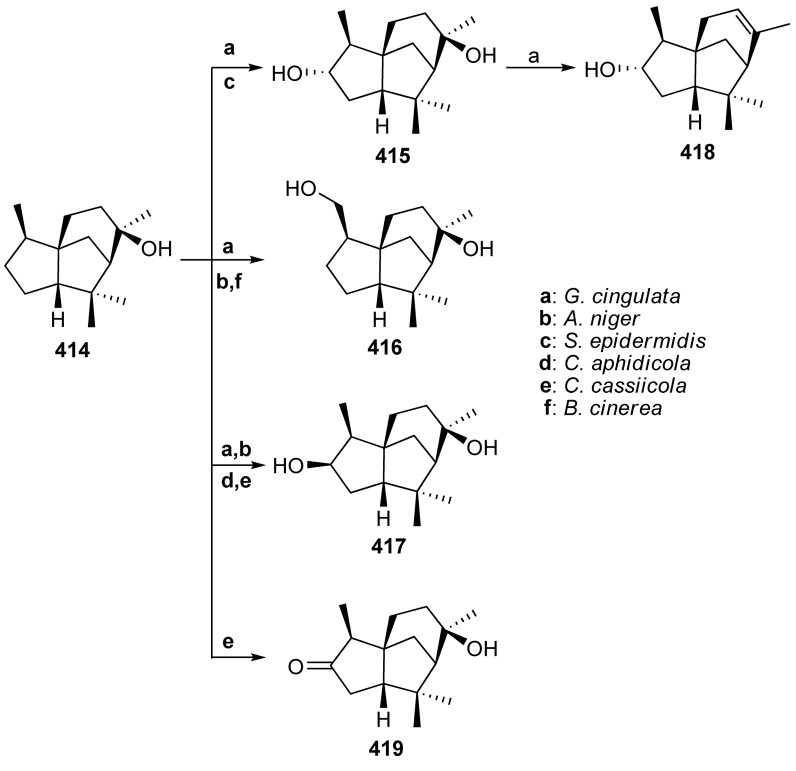
Scheme 115 Biotransformation of (−)-ambrox (**391**) by *Cephalosporium aphidicola*.



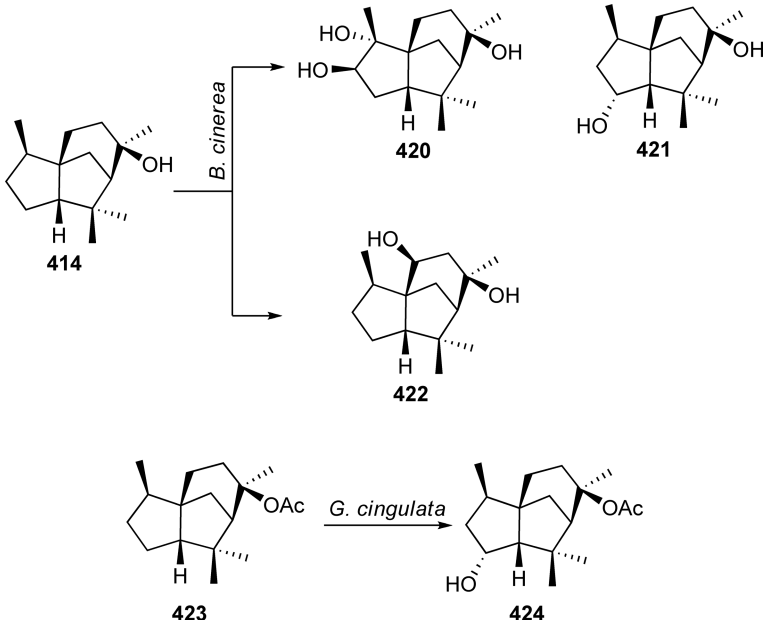
Scheme 116 Biotransformation of zerumbone (**408**) by various fungi.

Patchouli alcohol (**425**) was treated with *B. cinerea* to give three metabolites two tertiary alcohols (**426**, **427**), four secondary alcohols (**428**, **430**, **430a**), and two primary alcohols (**430b**, **430c**) of which compounds **425**, **427**, and **428** are the major metabolites,[129] while plant pathogenic fungus *G. cingulata* converted the same substrate into 5-hydroxy- (**426**) and 5,8-dihydroxy derivative (**429**) (**Scheme 119**).

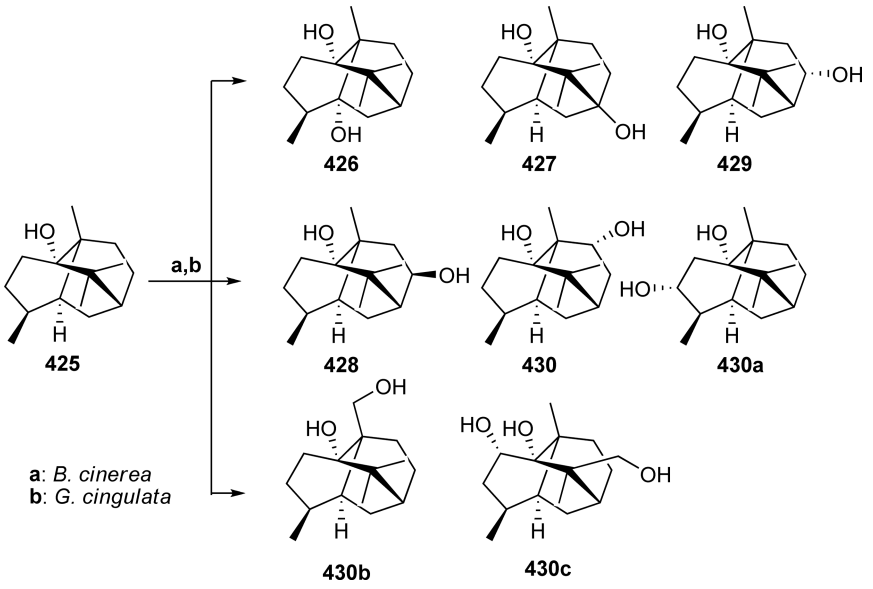
In order to confirm the formation of **429** from **426**, the latter product was reincubated in the same medium including *G. cingulata* to yield **429** (**Scheme 120**).[131]



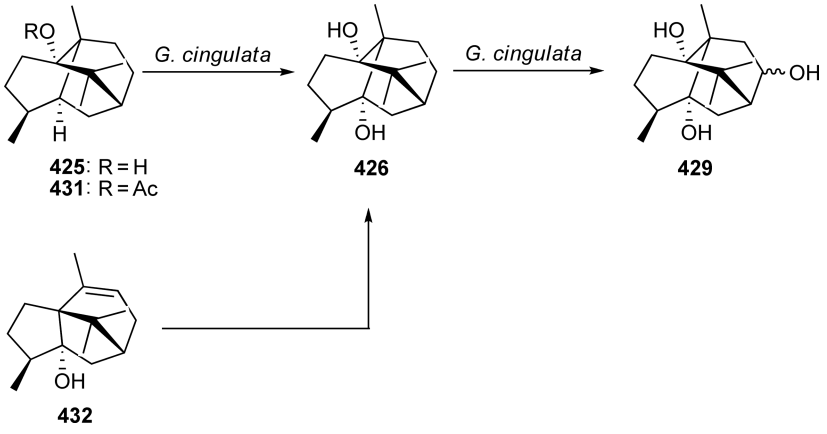
Scheme 117 Biotransformation of cedrol (**414**) by various fungi.



Scheme 118 Biotransformation of cedrol (**414**) by *Botrytis cinerea Glomerella cingulata.*



Scheme 119 Biotransformation of patchoulol (**425**) by *Botrytis cinerea*.



Scheme 120 Biotransformation of patchoulol (**425**) by *Glomerella cingulata*.

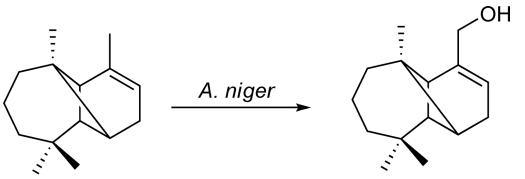
Patchouli acetate (**431**) was also treated in the same medium to give **426** and **429**.[132] 5-Hydroxy-α-patchoulene (**432**) was incubated with *G. cingulata* to yield 1α-hydroxy derivative (**426**).[133]

(−)-α-Longipinene (**433**) was treated with *A. niger* to yield 12-hydroxylated product (**434**).[134]

Ginsenol (**435**) that was obtained from the essential oil of *Panax ginseng* was incubated with *B. cinerea* to yield four secondary alcohols (**436–439**) and two cyclohexanone derivatives (**440**) from **437** and **441** from **438** or **439**. Some of the oxygenated products were considered as potential antifungal agents to control *B. cinerea* (**Schemes 121** and **122**).[135]

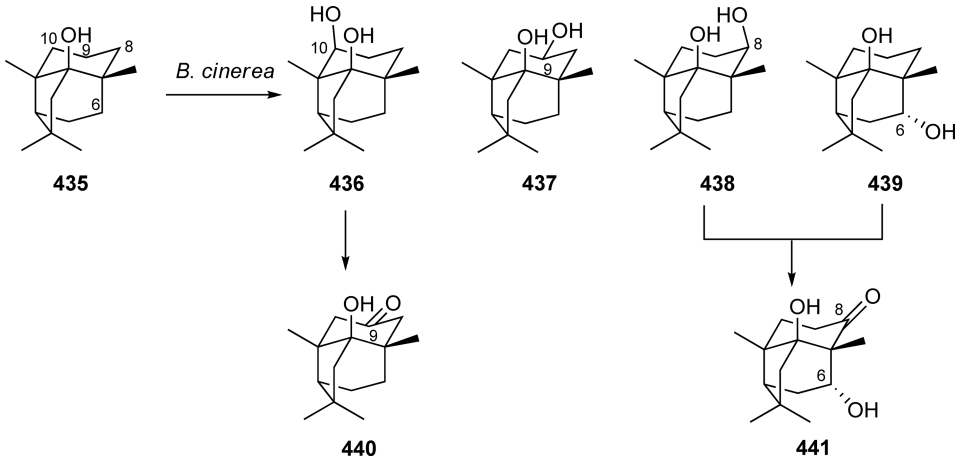
(+)-Isolongifolene-9-one (**442**) that was isolated from some cedar trees was treated with *G. cingulata* for 15 days to yield two primary alcohols (**443**, **444**) and a secondary alcohol (**445**) (**Scheme 123**).[136]

Choudhary *et al.*[137] reported that fermentation of (−)-isolongifolol (**445a**) with *F. lini* resulted in the isolation of three metabolites, 10-oxo- (**445b**), 10α-hydroxy- (**445c**), and 9α-hydroxyisolongifolol (**445d**). When the same substrate was incubated with *A. niger* to yield the products **445c** and **445d**, both products showed inhibitory activity against butylcholinesterase enzyme in a concentration-dependent manner with IC_{50} 13.6 and 299.5 $\mu mol\,l^{-1}$, respectively (**Scheme 124**).



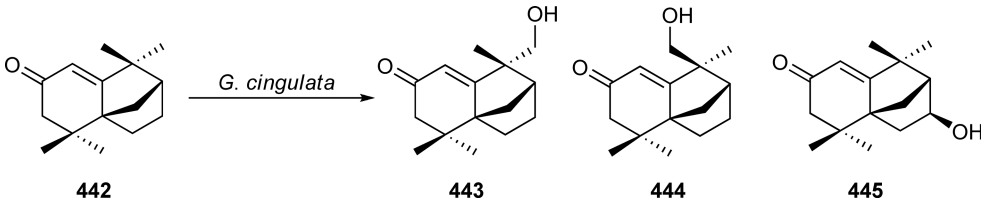
433 **434**

Scheme 121 Biotransformation of α-longipinene (**433**) by *Aspergillus niger.*



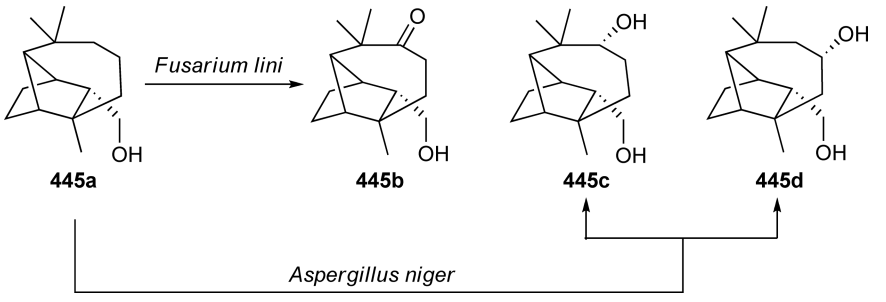
435 **436** **437** **438** **439** **440** **441**

Scheme 122 Biotransformation of ginsenol (**435**) by *Botrytis cinerea.*



442 **443** **444** **445**

Scheme 123 Biotransformation of (+)-isolongifolene-9-one (**442**) by *Glomerella cingulata.*



445a **445b** **445c** **445d**

Scheme 124 Biotransformation of (−)-isolongifolol (**445a**) by *Aspergillus niger* and *Fusarium lini.*

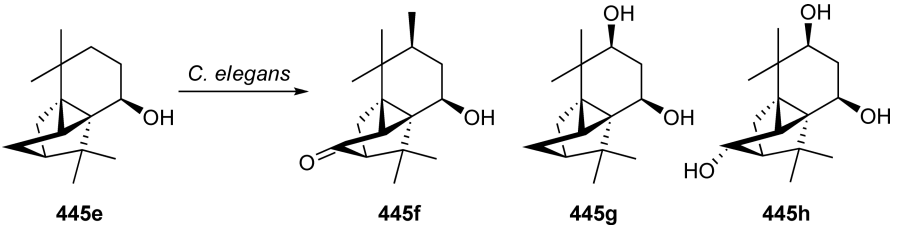
(+)-Cycloisolongifol-5β-ol (**445e**) was fermented with *C. elegans* to yield three oxygenated metabolites, 11-oxo- (**445f**), 3β-hydroxy- (**445g**), and 3β,11α-dihydroxy derivative (**445h**) (**Scheme 125**).[138]

A daucane-type sesquiterpene derivative, lancerroldiol *p*-hydroxybenzoate (**446**), was hydrogenated with cultured suspension cells of the liverwort, *M. polymorpha*, to give 3,4-dihydrolancerodiol (**447**) (**Scheme 126**).[27]

Widdrane sesquiterpene alcohol (**448**) was incubated with *A. niger* to give oxo and oxy derivatives (**449**, **450**) (**Scheme 127**).[57]

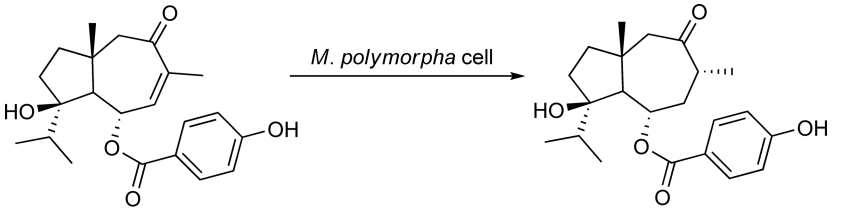
(−)-β-Caryophyllene (**451**), one of the ubiquitous sesquiterpene hydrocarbons found not only in higher plants but also in liverworts, was biotransformed by *Pseudomonas cruciviae*, *Diplodia gossypina*, and *Chaetomium cochlioides*.[5] *Pseudomonas cruciviae* gave a ketoalcohol (**452**),[139] while the latter two species produced the 14-hydroxy-5,6-epoxide (**454**), its carboxylic (**455**) and 3α-hydroxy- (**456**), and norcaryophyllene alcohol (**457**) all of which might be formed from caryophyllene C-5, C-6-epoxide (**453**). Oxidation pattern of (−)-β-caryophyllene by the fungi is very similar to that by mammals (see Section 3.20.3) (**Scheme 128**).

Fermentation of (−)-β-caryophyllene (**451**) with *D. gossypina* yielded 14 different metabolites (**453**–**457j**) among which 14-hydroxy-5,6-epoxide (**454**) and the corresponding acid (**455**) were the major metabolites. Compound **457j** is structurally very rare and is found in *Poronia punctata*. The main reaction path is epoxidation at C-5, C-6 as mentioned above and selective hydroxylation at C-4 (**Scheme 129**).[140]



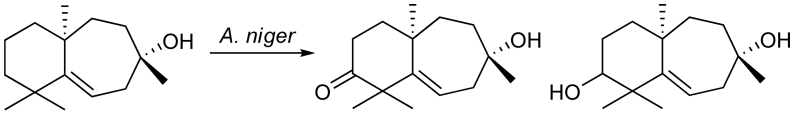
445e **445f** **445g** **445h**

Scheme 125 Biotransformation of (+)-cycloisolongifol-5β-ol (**445e**) by *Cunninghamella elegans*.



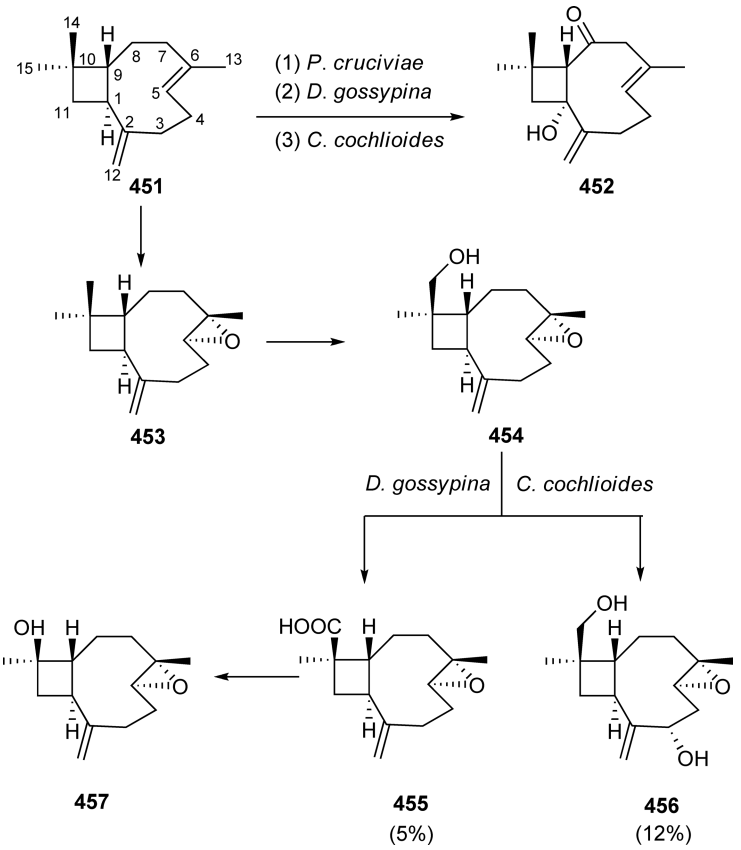
446 **447**

Scheme 126 Biotransformation of lancerroldiol *p*-hydroxybenzoate (**446**) by *Marchantia polymorpha* cells.



448 **449** **450**

Scheme 127 Biotransformation of widdrol (**448**) by *Aspergillus niger*.



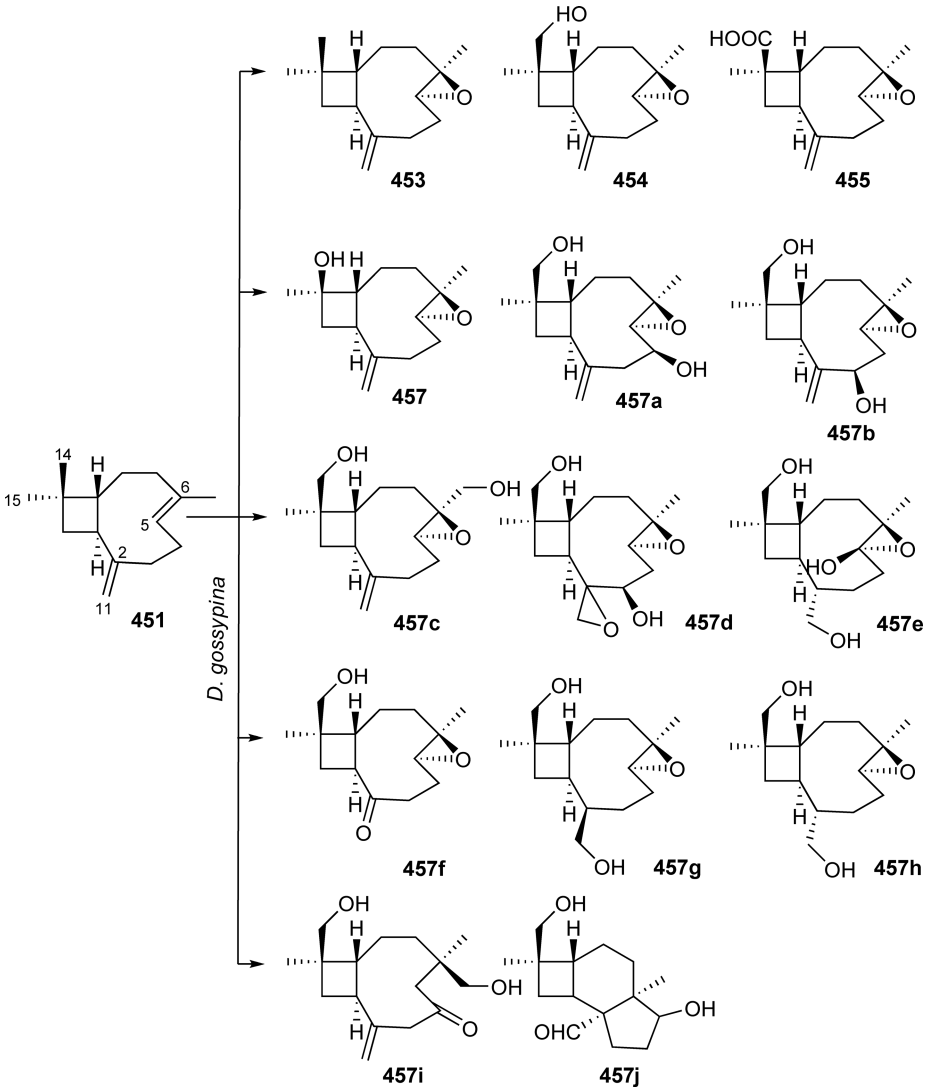
Scheme 128 Biotransformation of (−)-β-caryophyllene (**451**) by *Pseudomonas cruciviae*, *Diplodia gossypina*, and *Chaetomium cochlioides*.

(−)-β-Caryophyllene epoxide (**453**) was incubated with *C. aphidicola* for 6 days to yield two metabolites (**457l, 457m**), while *Macrophomina phaseolina* biotransformed the same substrate to 14-hydroxy (**454**) and 15-hydroxy derivatives (**457k**). The same substrate was treated with *A. niger*, *G. fujikuroii*, and *R. stolonifera* for 8 days and *F. lini* for 10 days to yield the metabolites **457n–457r**. All metabolites were estimated for butyrylcholine esterase inhibitory activity and compound **457k** was found to show potent activity to galanthamine HBr (IC_{50} 10.9 vs. 8.5 μmol l^{-1}) (**Scheme 130**).[141]

The fermentation of (−)-β-caryophyllene oxide (**453**) using *B. cinerea* and isolation of the metabolites was carried out by Duran *et al.*[142] Kobuson (**457w**) was obtained with 14 products (**457s–457u, 457x**). Diepoxides **457t** and **457u** could be the precursors of epimeric alcohols **457q** and **457y** obtained through reductive opening of the C-2, C-11-epoxide. The major reaction paths are stereoselective epoxidation and introduction of hydroxyl group at C-3. Compound **457ae** has a caryolane skeleton (**Scheme 131**).

When isoprobotryan-9α-ol (**458**) produced from isocaryophyllene was incubated with *B. cinerea*, it was hydroxylated at tertiary methyl groups to give three primary alcohols (**459–461**) (**Scheme 132**).[143]

Acyclic sesquiterpenoids, racemic *cis*-nerolidol (**462**), and nerylacetone (**463**) were treated with the plant pathogenic fungus, *G. cingulata*.[144] From the former substrate, a triol (**464**) was obtained as the major product. The latter was bioconverted to give the two methyl ketones (**465, 467**) and triol (**468**) among which **465** was the predominant. The C-10, C-11 diols (**464, 465**) might be formed from both epoxides of the substrates, followed by the hydration although no C-10, C-11-epoxides were detected (**Scheme 133**).

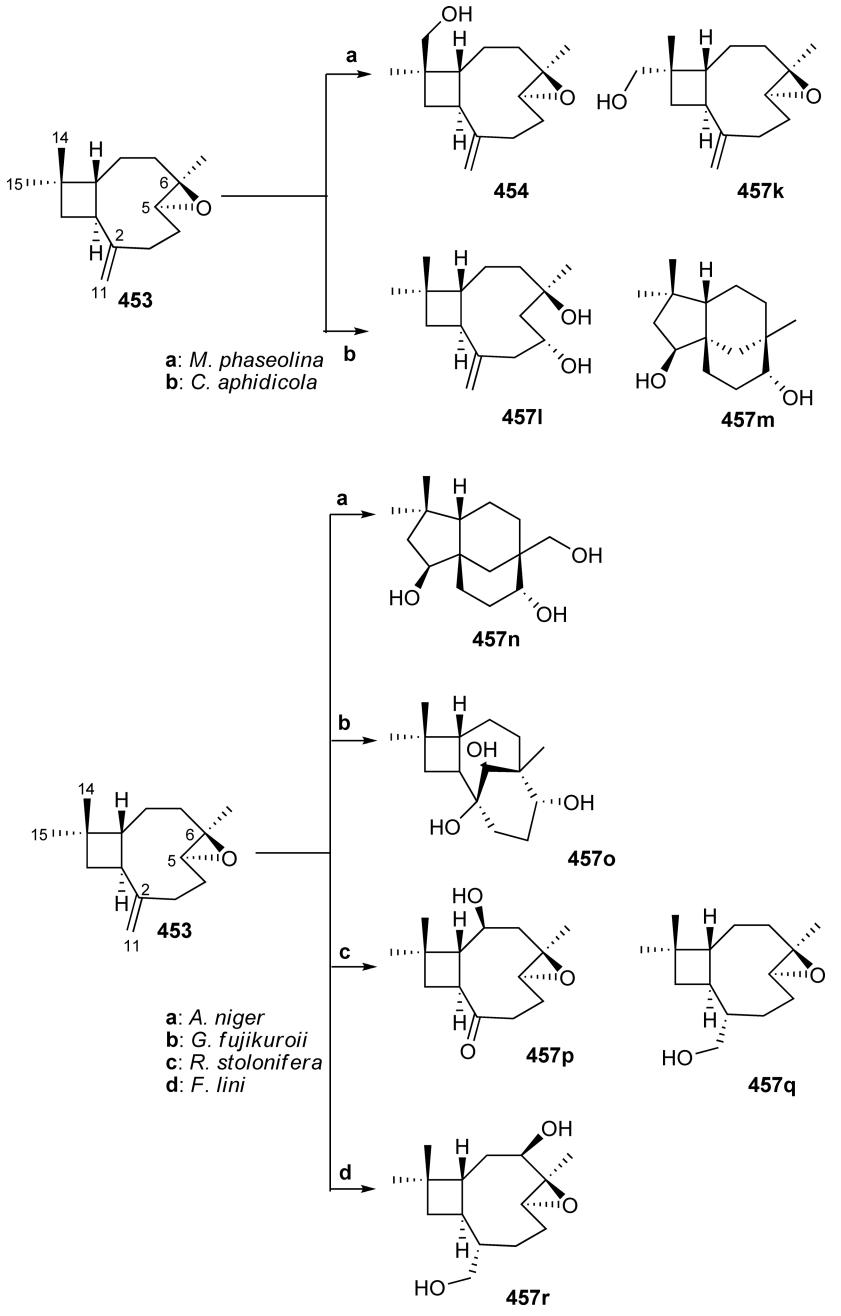


Scheme 129 Biotransformation of (–)-β-caryophyllene (**451**) by *Diplodia gossypina*.

Racemic *trans*-nerolidol (**469**) was also treated with the same fungus to yield ω-2 hydroxylated product (**471**) and C-10, C-11-hydroxylated compounds (**472**) as seen in racemic *cis*-nerolidol (**462**) (**Scheme 134**).[145]

12-Hydroxy-*trans*-nerolidol (**472a**) is an important precursor in the synthesis of interesting flavor of α-sinensal. Hrdlicka *et al.*[146] reported the biotransformation of *trans*- (**469**) and *cis*-nerolidol (**462**) and *cis*/*trans*-mixture of nerolidol using repeated batch culture of *A. niger* grown in computer-controlled bioreactors. *Trans*-nerolidol (**469**) gave **472a** and **472** and *cis*-isomer (**462**) yielded **464a** and **464**, respectively. From a mixture of *cis*- and *trans*-nerolidol, 12-hydroxy-*trans*-nerolidol **472a** (8%) was obtained in postexponential phase at high dissolved oxygen. At low dissolved oxygen condition, the mixture yielded **472a** (7%) and **464a** (6%), respectively (**Scheme 135**).

From geranyl acetone (**470**) incubated with *G. cingulata*, four products (**473–477**) were formed. It is noteworthy that the major compounds from both substrates (**469, 470**) were ω-2 hydroxylated products, but not C-10, C-11 dihydroxylated products as seen in *cis*-nerolidol (**462**) and nerylacetone (**463**) (**Scheme 134**).[147]

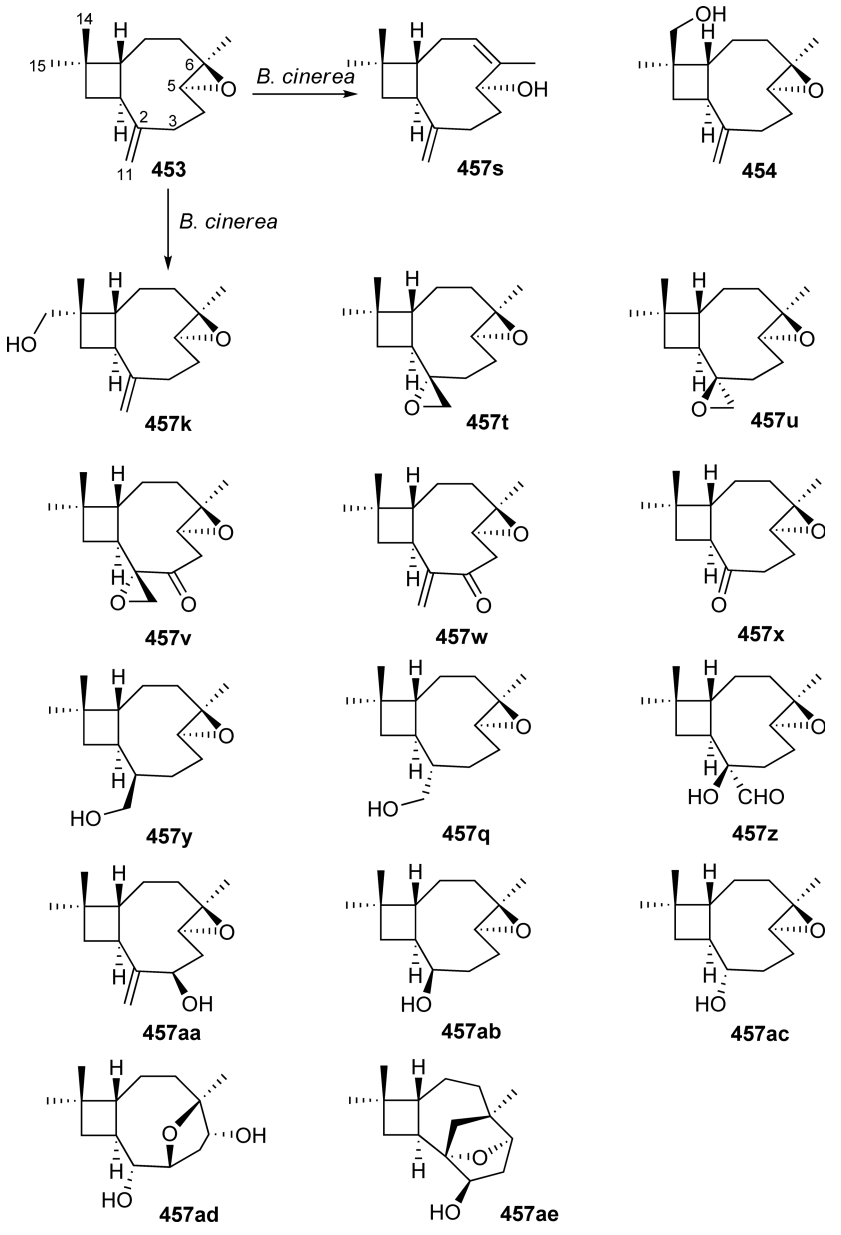


Scheme 130 Biotransformation of (–)-β-caryophyllene epoxide (**453**) by various fungi.

The same fungus bioconverted (2*E*,6*E*)-farnesol (**478**) to four products, ω-2 hydroxylated product (**479**) that was further oxidized to give C-10, C-11 dihydroxylated compound (**480**) and 5-hydroxy derivative (**481**), followed by isomerization at C-2,3 double bond to yield a triol (**482**) (**Scheme 136**).[147]

The same substrate was bioconverted by *A. niger* to yield two metabolites, 10,11-dihydroxy- (**480**) and 5,13-hydroxy derivative (**480a**) (**Scheme 137**).[148]

The same fungus also converted (2*Z*,6*Z*)-farnesol (**483**) into three hydroxylated products: 10,11-dihydroxy-(2*Z*,6*Z*)-farnesol (**484**), 10,11-dihydroxy (2*E*,6*Z*)-farnesol (**485**), and (5*Z*)-9,10-dihydroxy-6,10-dimethyl-5-undecen-2-one (**486**) (**Scheme 138**).[149]



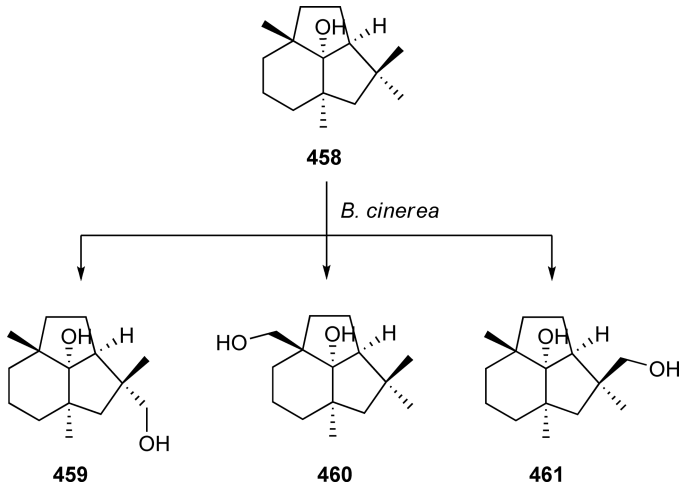
Scheme 131 Biotransformation of (−)-β-caryophyllene epoxide (**453**) by *Botrytis cinerea*.

A linear sesquiterpene 9-oxonerolidol (**487**) was treated with *A. niger* to give ω-1 hydroxylated product (**488**) (**Scheme 139**).[37]

Racemic diisophorone (**488a**) dissolved in ethanol was incubated with the Czapeck–Dox medium of *A. niger* to yield 8α- (**488b**), 10β- (**488c**), and 17-hydroxydiisophorone (**488d**).[150]

On the other hand, the same substrate was fed with *Nicotiana crassa* and *C. aphidicola* to yield only 8β-hydroxydiisophorone (**488e**) in 20 and 10% yield, respectively (**Scheme 140**).[151]

From the metabolites of 5β,6β-dihydroxypresilphiperfolane 2β-angelate (**488f**) using the fungus *Mucor ramannianus*, 2,3-epoxyangeloyloxy derivative (**488g**) was obtained (**Scheme 141**).[152]



Scheme 132 Biotransformation of isoprobotryan-9α-ol (**458**) by *Botrytis cinerea.*

3.20.3 Biotransformation of Sesquiterpenoids by Mammals, Insects, and Cytochrome P-450

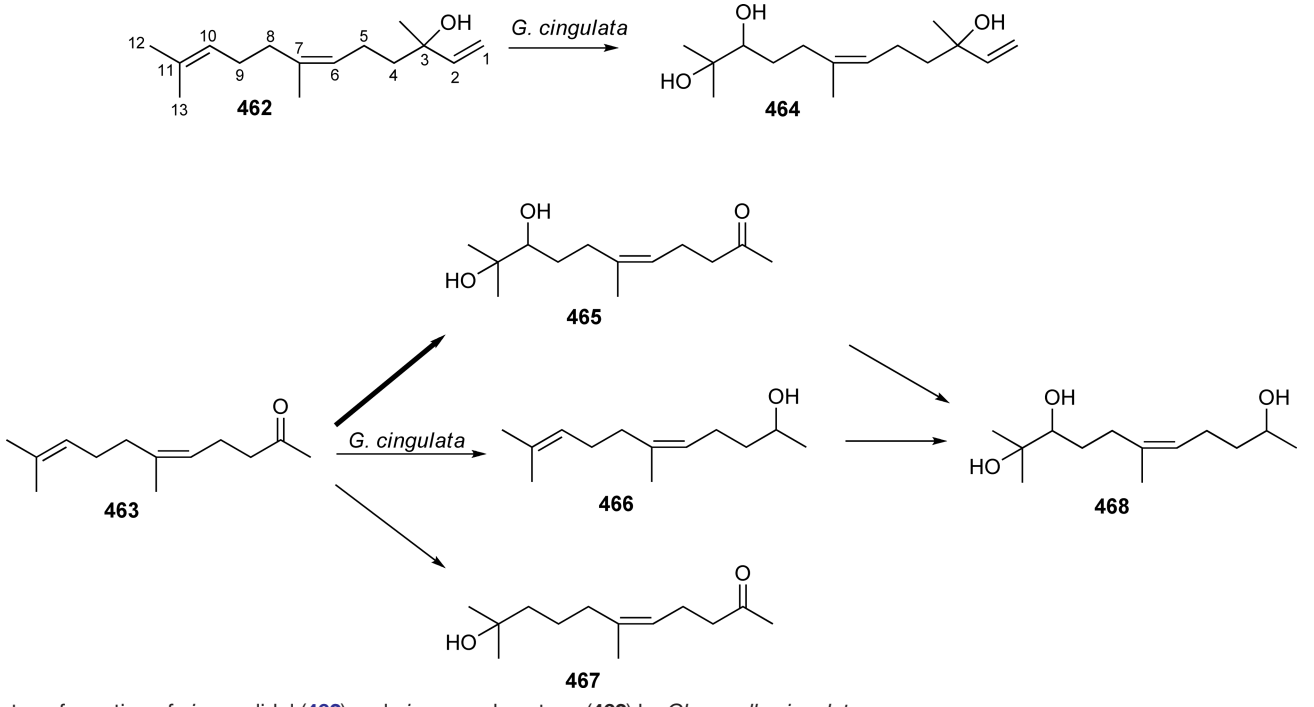
3.20.3.1 Animals (Rabbits) and Dosing

Six male albino rabbits (2–3 kg) were starved for 2 days before the experiment. Monoterpenes were suspended in water (100 ml) containing polysorbate 80 (0.1 g) and were uniformly homogenized. This solution (20 ml) was administered to each rabbit through a stomach tube followed by water (20 ml). This dose of sesquiterpenoids corresponds to 400–700 mg kg^{-1}. Rabbits were housed in stainless-steel cages and were allowed rabbit food and water *ad libitum.* The urine was collected daily for 3 days after drug administration and stored at 0–5 °C until the time of analysis. The urine was centrifuged to remove feces and hair at 0 °C and the supernatant was used for the experiments. The urine was adjusted to pH 4.6 with acetate buffer and incubated with β-glucuronidase-arylsulfatase (3 ml per 100 ml of fresh urine) at 37 °C for 48 h, followed by continuous ether extraction for 48 h to obtain the metabolite. The ether extract was washed with 5% $NaHCO_3$ and 5% NaOH to remove the acidic and phenolic components, respectively.[46]

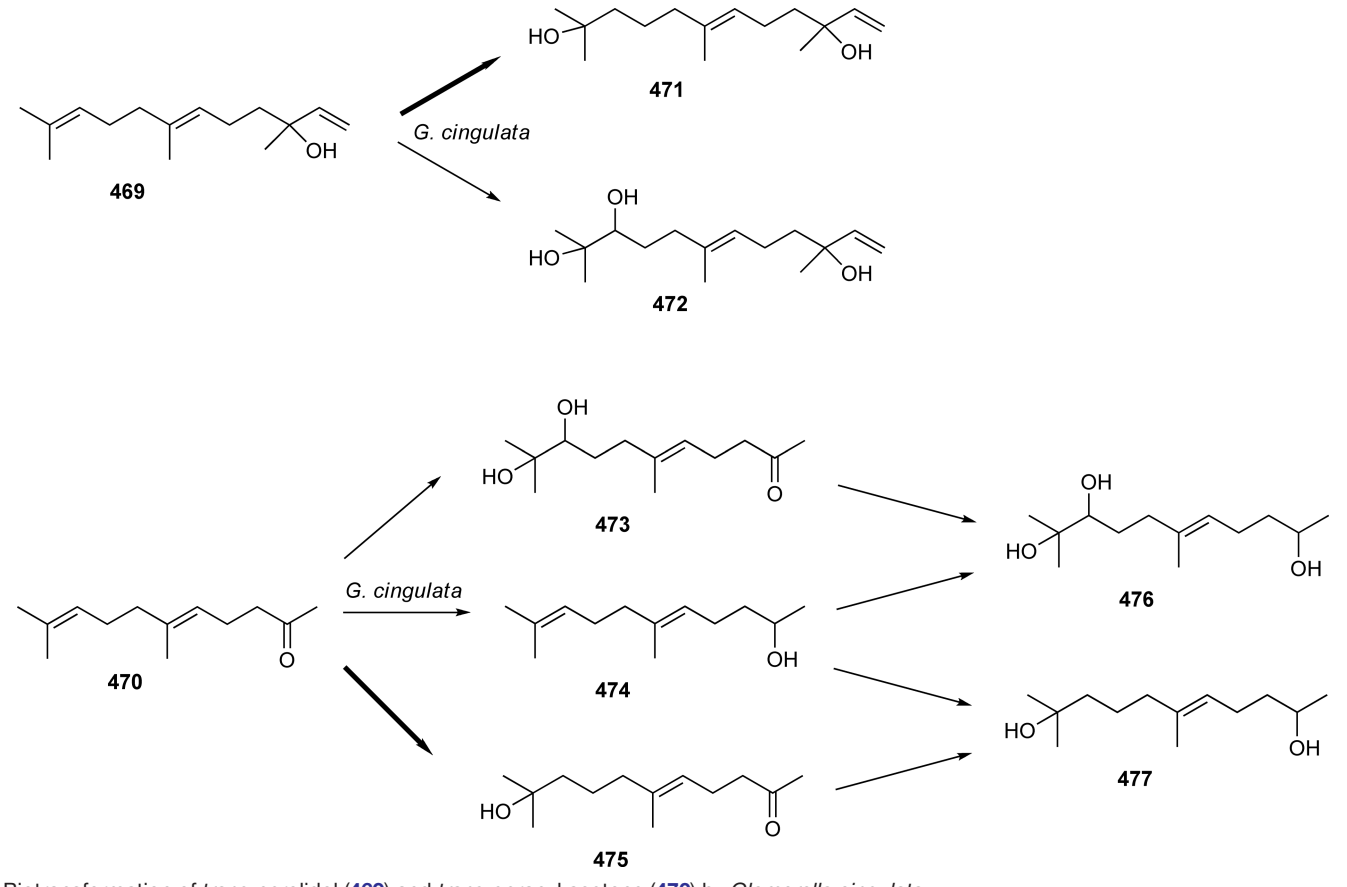
3.20.3.2 Sesquiterpenoids

Wild rabbits (hair) and deer damage the young leaves of *Chamaecyparis obtusa*, one of the most important tree used in furniture and house constructions in Japan. The essential oil of the leaves contains a large amount of (−)-longifolene (**489**). Longifolene (36 g) was administered to 18 of the rabbits to obtain the metabolites (3.7 g) from which an aldehyde (**490**) (35.5%) was isolated in pure state. In the metabolism of terpenoids having an exomethylene group, glycol formation was often found, but in the case of longifolene, a diol was not formed. Introduction of an aldehyde group in biotransformation is very remarkable. Stereoselective hydroxylation of the gem dimethyl group on a seven-membered ring is reported for the first time (**Scheme 142**).[153]

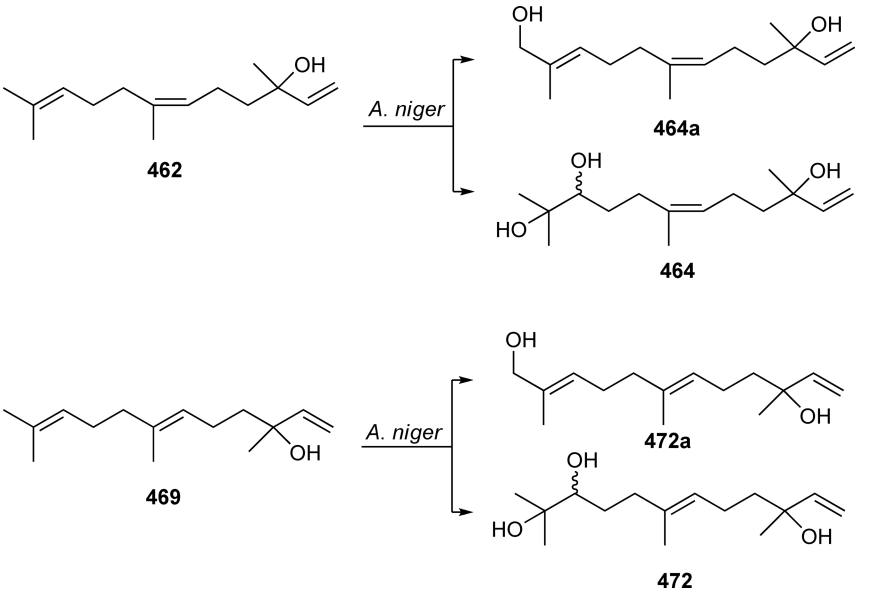
(−)-β-Caryophyllene (**451**) is one of the ubiquitous sesquiterpene hydrocarbons in the plant kingdom and the main component of beer hops and clove oil, and is being used as a culinary ingredient and as a cosmetic in soaps and fragrances. (−)-β-Caryophyllene is cytotoxic against breast carcinoma cells and its epoxide is toxic to *Planaria* worms. It contains a unique 1,1-dimethylcyclobutane skeleton. (−)-β-Caryophyllene (3 g) was treated in the same manner as described above to yield the crude metabolite (2.27 g) from which (10*S*)-14-hydroxycaryophyllene-5,6-oxide (**491**) (80%) and a diol (**492**) were



Scheme 133 Biotransformation of *cis*-nerolidol (**462**) and *cis*-geranyl acetone (**463**) by *Glomerella cingulata*.



Scheme 134 Biotransformation of *trans*-nerolidol (**469**) and *trans*-geranyl acetone (**470**) by *Glomerella cingulata.*



Scheme 135 Biotransformation of *cis*- (**462**) and *trans*-nerolidol (**469**) by *Aspergillus niger*.

obtained.[154] Later, compound (**491**) was isolated from the Polish mushroom, *Lactarius camphorates* (Basidiomycetes) as a natural product.[155] 14-Hydroxy-β-callyophyllene and 1-hydroxy-8-keto-β-caryophyllene have been found in Asteraceae and *Pseudomonas* species, respectively. In order to confirm that caryophyllene epoxide (**453**) is the intermediate of both metabolites, it was treated in the same manner as described above to give the same metabolites **491** and **492** of which **491** was predominant (**Scheme 143**).[24,154]

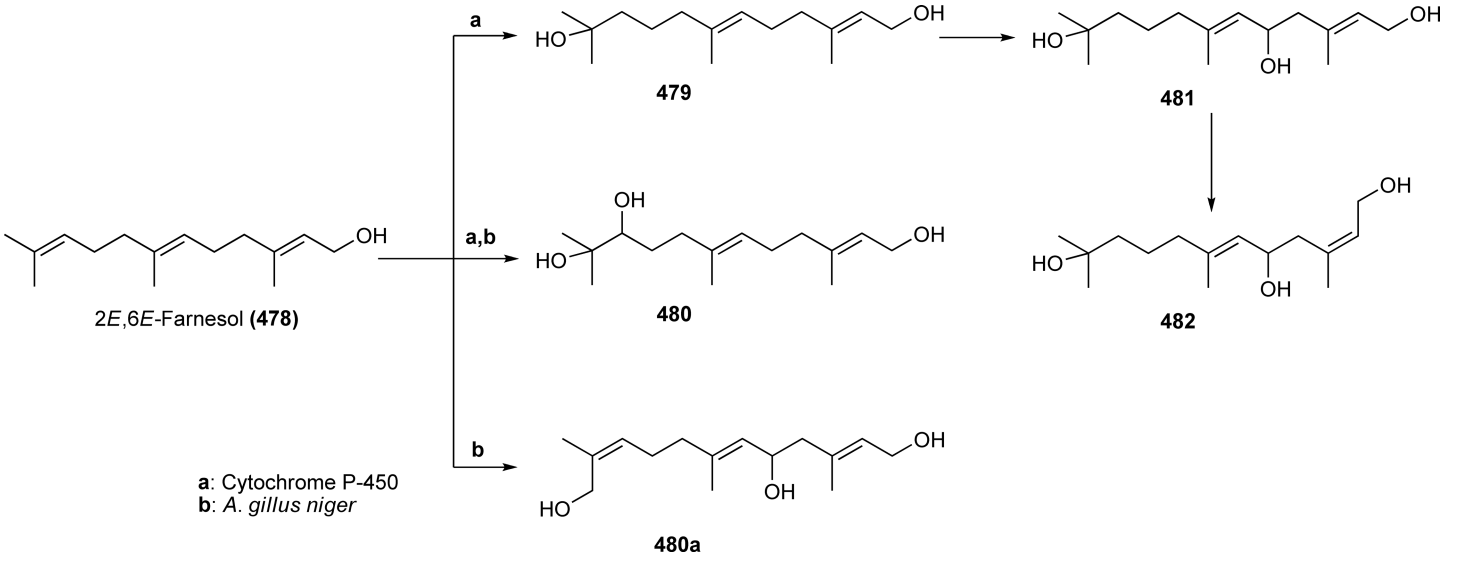
The grape fruit aroma, (+)-nootkatone (**2**) was administered into rabbits to give 11,12-diol (**6, 7**). The same metabolism has been found in the biotransformation of nootkatone by microorganisms as mentioned in the previous paragraph. Compounds (**6, 7**) were isolated from the urine of hypertensive subjects and named urodiolenone. The production of **6, 7** seems to occur intermittently from the nootkatone or grape fruit. Synthetic racemic nootkatone epoxide (**14**) was incubated with rabbit-liver microsomes to give 11,12-diol (**6, 7**).[25] Thus, the role of the epoxide was clearly confirmed as an intermediate of nootkatone (**2**).

(+)-*ent*-Cyclocolorenone (**98**) and its enantiomer (**103**) were biotransformed by *Aspergillus* species to give cyclopropane-cleaved metabolites as described in the previous paragraph.

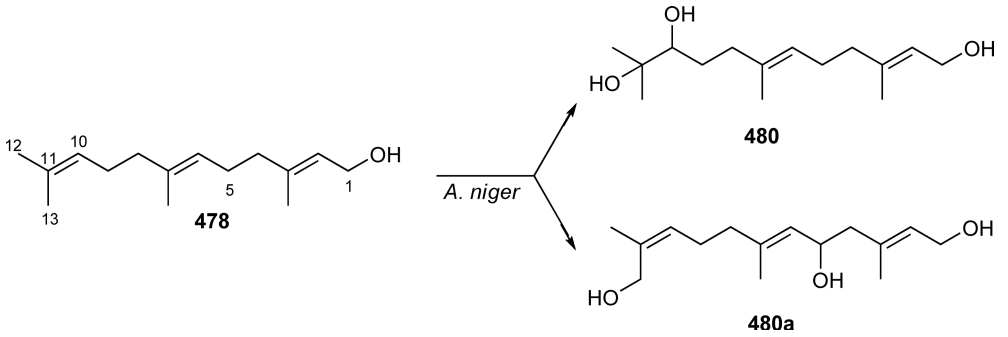
In order to compare the metabolites between mammals and microorganisms, the essential oil (2 g per rabbit) containing (−)-cyclocolorenone (**103**) obtained from *Solidago altissima* was administered in rabbits to obtain two metabolites, 9β-hydroxycyclocolorenone (**493**) and 10-hydroxycyclocolorenone (**494**).[24] 10-Hydroxyaromadendrane-type compounds are well known as the natural products. No oxygenated compound of cyclopropane ring was found in the metabolites of cyclocolorenone in rabbit (**Scheme 144**).

From the metabolites of elemol (**495**) possessing the same partial structures of monoterpene hydrocarbon, myrcene, and nootkatone, one primary alcohol (**496**) was obtained from rabbit urine after administration of **495** (**Scheme 145**).[24]

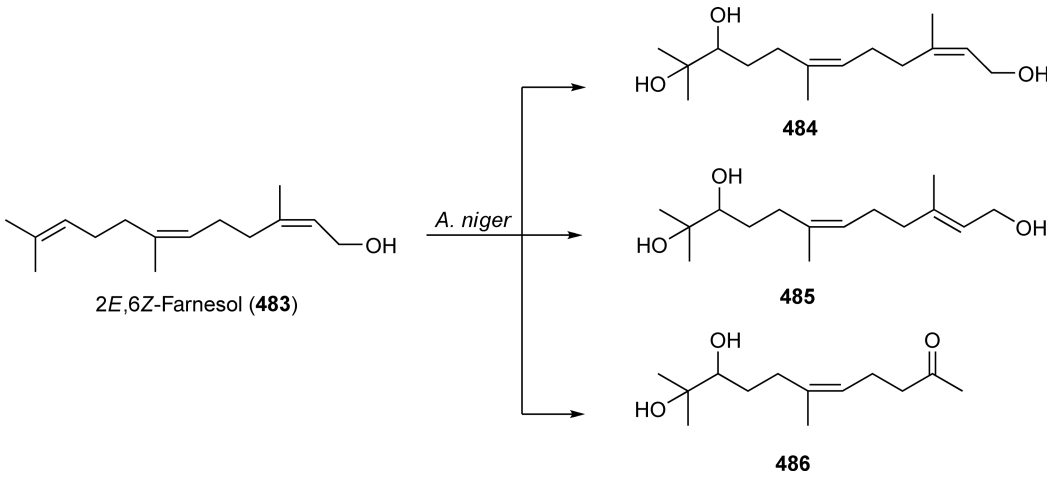
Components of cedar wood such as cedrol (**414**) and cedrene shorten the sleeping time of mice. In order to search for a relationship between scent, olfaction, and detoxifying enzyme induction, (+)-cedrol (**414**) was administered to rabbits and dogs. From the metabolites from rabbits, two C-3 hydroxylated products (**418** and **497**) and diol (**415** or **416**) may be formed after hydrogenation of the double bond. Dogs converted cedrol (**414**) into the different metabolite products, C-2 (**498**), C-2/C-14 hydroxylated products (**499**), together with the same C-3 (**415**) and C-15 hydroxylated products (**416**) as those found in the metabolites of microorganisms and rabbits. The above species-specific metabolism is very remarkable.[156]



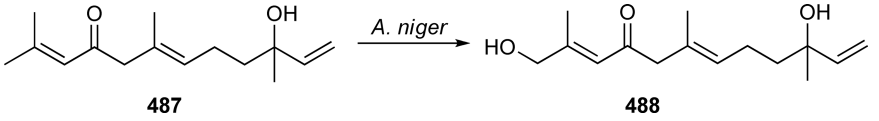
Scheme 136 Biotransformation of 2*E*,6*E*-farnesol (**478**) by cytochrome P-450 and *Aspergillus niger*.



Scheme 137 Biotransformation of 2*E*,6*E*-farnesol (**478**) by *Aspergillus niger*.



Scheme 138 Biotransformation of 2*Z*,6*Z*-farnesol (**478**) by *Aspergillus niger*.

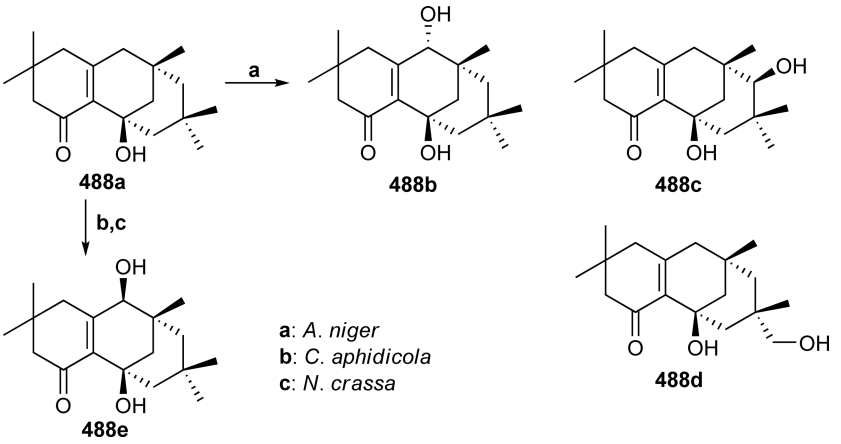


Scheme 139 Biotransformation of 9-oxo-*trans*-nerolidol (**487**) by *Aspergillus niger*.

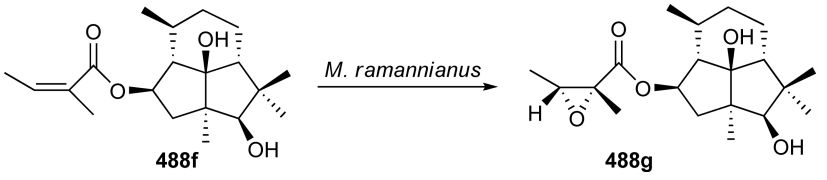
The microorganisms *C. aphidicoda*, *C. cassiicola*, *B. cinerea*, and *G. cingulata* also biotransformed cedrol to various C-2, C-3, C-4, C-6, and C-15 hydroxylated products as shown in the previous paragraph. The microbial metabolism of cedrol resembles that of mammals (**Scheme 146**).

Patchouli alcohol (**425**) with fungi static properties is one of the important essential oils in perfumery industry. Rabbits and dogs gave two oxidative products (**500, 501**) and one norpatchoulen-1-one (**502**) possessing a characteristic odor. This pathogen gave totally different five metabolites (**426–430**) from those found in the urine metabolites of mammals as described above (**Scheme 147**).[157]

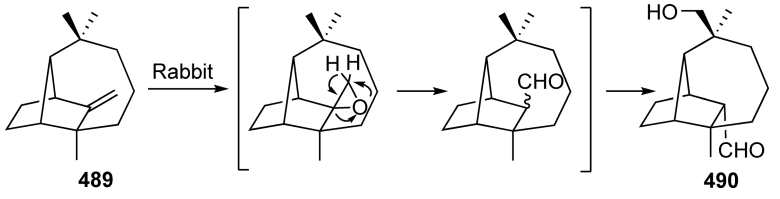
Sandalwood oil contains mainly α-santalool (**503**) and β-santalool. Rabbits converted α-santalool into three diastereomeric primary alcohols (**504–506**) and dogs converted α-santalool into carboxylic acid (**507**) (**Scheme 148**).[158]



Scheme 140 Biotransformation of diisophorone (**488a**) by *Aspergillus niger*, *Cephalosporium aphidicola*, and *Neurospora crassa*.



Scheme 141 Biotransformation of 5β,6β-dihydroxypresilphiperfolane 2β-angelate (**488f**) by *Mucor ramannianus.*



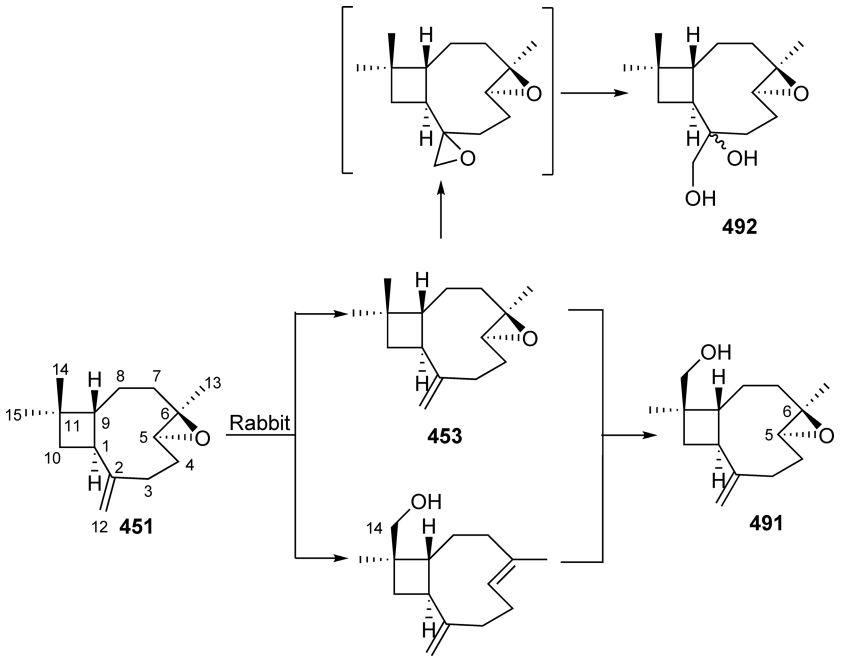
Scheme 142 Biotransformation of longifolene (**489**) by rabbit.

(2*E*,6*E*)-Farnesol (**478**) was treated with cockroach cytochrome P-450 (CYP4C7) to form region- and diastereospecifically ω-hydroxylated at the C-12 methyl group to the corresponding diol (**508**) with 10*E*-configuration (**Scheme 149**).[159]

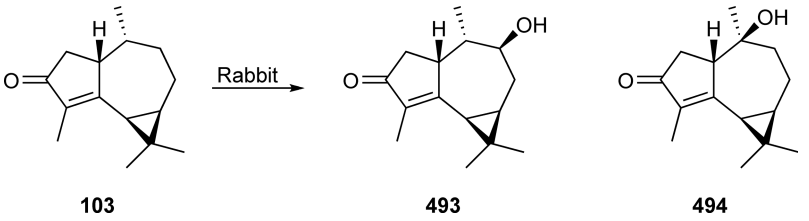
Juvenile hormone III (**509**) was also treated with cockroach CYP4C7 to the corresponding 12-hydroxylated product (**510**).[159]

The African locust converted the same substrate (**509**) to 7-hydroxy product (**511**) and 13-hydroxylated product (**512**). It is noteworthy that the African locust and the cockroach showed clear species specificity for introduction of oxygen function.[160]

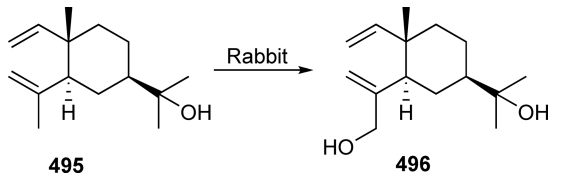
In conclusion, a number of sesquiterpenoids were biotransformed by various fungi and mammals to yield many metabolites, several of which showed antimicrobial and antifungal, antiobesity, cytotoxic, and neurotrophic activity. Microorganisms introduce oxygen atoms at allylic positions to give secondary hydroxyl and keto groups. Double bond is also oxidized to give epoxide, followed by hydrolysis to yield diol. These reactions proceed stereo- and regiospecifically. Even at nonactivated carbon atom, oxidation reaction occurs to give primary alcohol. Some fungi such as *A. niger* cleave cyclopropane ring with a



Scheme 143 Biotransformation of (−)-β-caryophyllene (**451**) by rabbit.



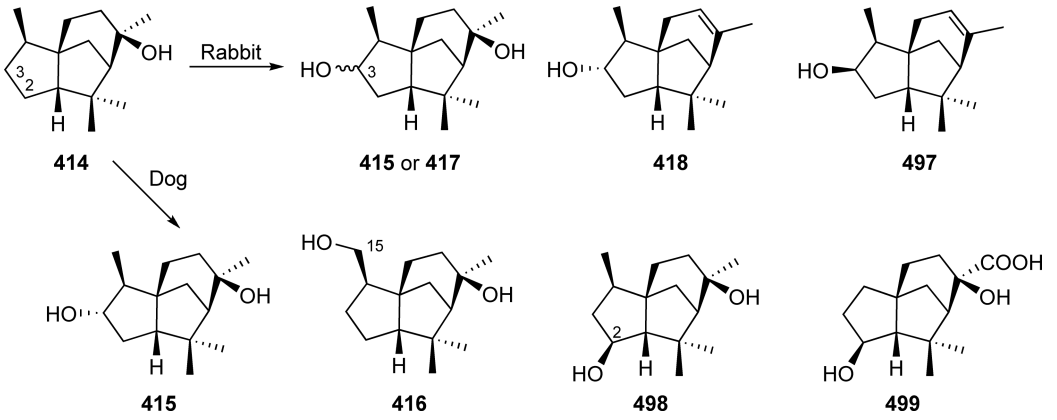
Scheme 144 Biotransformation of (+)-*ent*-cyclocolorenone (**101**) by rabbit.



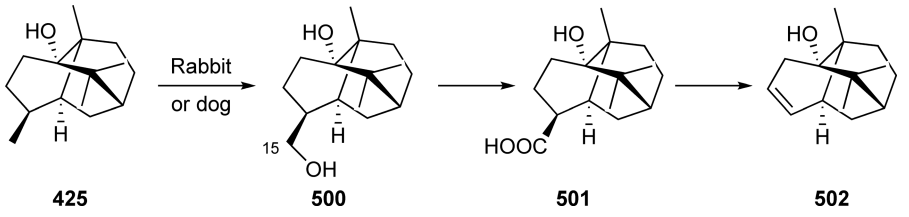
Scheme 145 Biotransformation of elemol (**495**) by rabbit.

1,1-dimethyl group. It is noteworthy that *A. niger* and *A. cellulosae* produce the totally different metabolites from the same substrates. Some fungi bring about reduction of carbonyl group, oxidation of aryl methyl group, phenyl coupling, and cyclization of 10-membered ring sesquiterpenoids to give C6/C6- and C5/C7-cyclic or spiro compounds. Cytochrome P-450 is responsible for the introduction of oxygen function into the substrates.

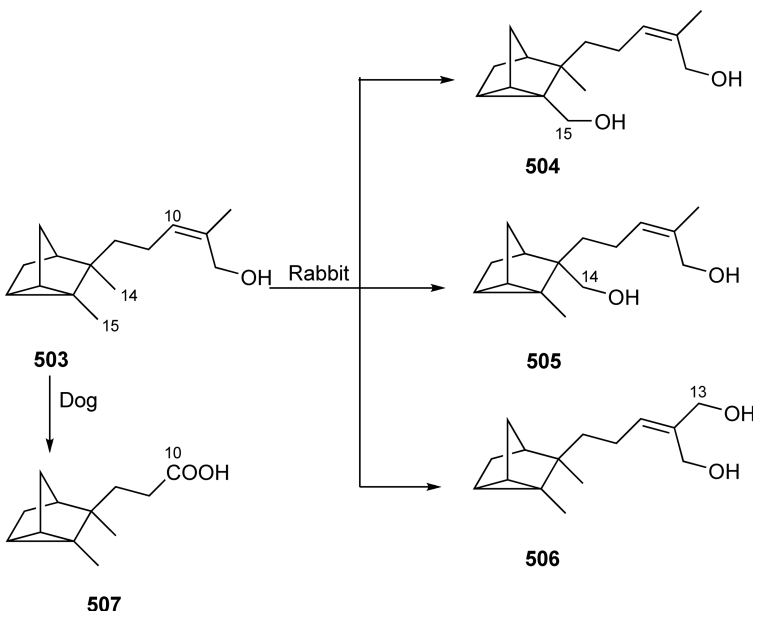
The present methods are very useful for the production of medicinal and agricultural drugs as well as fragrant components from commercially available cheap natural, unnatural terpenoids or a large amount of terpenoids from higher medicinal plants and spore-forming plants such as liverworts and fungi.



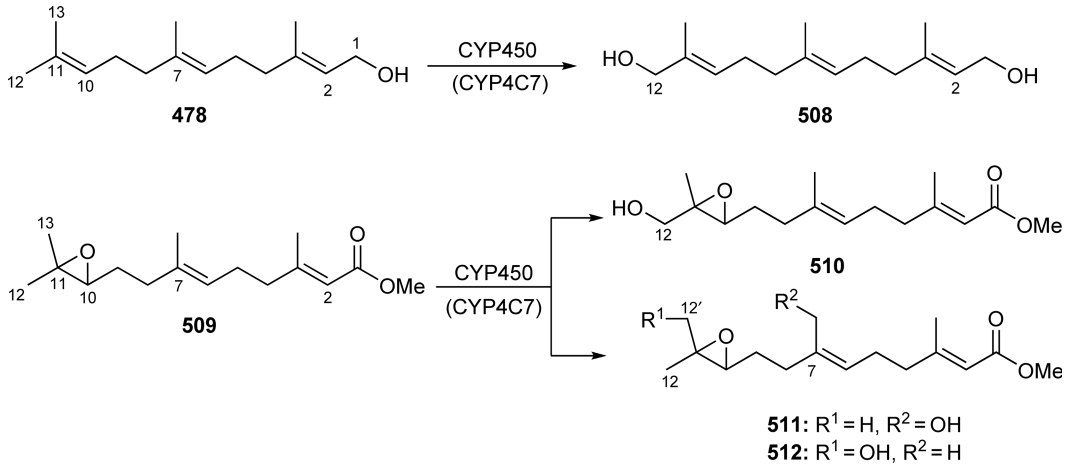
Scheme 146 Biotransformation of cedrol (**414**) by rabbit or dog.



Scheme 147 Biotransformation of patchouli alcohol (**425**) by rabbit or dog.



Scheme 148 Biotransformation of santalool (**503**) by rabbit or dog.



Scheme 149 Biotransformation of 2*E*,6*E*-farnesol (**478**) by cockroach cytochrome P-450 and 10,11-epoxyfarnesic acid methyl ester (**509**) by African locust cytochrome P-450.

The methodology discussed in this chapter is very simple one-step reaction in water, nonhazard, and very cheap and it gives many valuable metabolites possessing different properties from those of the substrates.

References

1. K. Tani; T. Yamagata; S. Otsuka; S. Akutagawa; H. Kumobayashi; T. Taketomi; H. Takaya; A. Miyashita; R. Noyori, *J. Chem. Soc. Chem. Commun.* **1982**, 600–601.
2. S. Otsuka; K. Tani, *Synthesis* **1991**, *9*, 665–680.
3. P. Meyer; C. Neuberg, *Biochem. Z.* **1915**, *71*, 174–179.
4. Y. Mikami, *Microbial Conversion of Terpenoids. Biotechnology and Genetic Engineering Reviews*. Intercept Ltd.: Wimborne, UK, 1988; Vol. 6, pp 271–320.
5. V. Lamare; R. Furstoss, *Tetrahedron* **1990**, *46*, 4109–4132.
6. S. Haze; K. Sakai; Y. Gozu, *Jpn. J. Pharmacol.* **2002**, *90*, 247–253.
7. R. S. Dhavalikar; G. Albroscheit, *Dragoco Rep.* **1979**, *20*, 251–258.
8. M. Okuda; K. Sonohara; H. Takikawa, *Jpn. Kokai Tokkyo Koho* **1994**, *30*, 39–67.
9. R. J. Sowden; S. Yasmin; N. H. Rees; S. G. Bell; L.-L. Wong, *Org. Biomol. Chem.* **2005**, *3*, 57–64.
10. C. W. Wilson III; P. E. Saw, *J. Agric. Food Chem.* **1978**, *26*, 1430–1432.
11. J. A. R. Salvador; J. H. Clark, *Green Chem.* **2002**, *4*, 352–356.
12. T. Hashimoto; Y. Asakawa; Y. Noma; C. Murakami; M. Tanaka; T. Kanisawa; M. Emura, *Jpn. Kokai Tokkyo Koho* 70492A, 2003.
13. T. Hashimoto; Y. Asakawa; Y. Noma; C. Murakami; M. Furusawa; T. Kanisawa; M. Emura; M. Mitsuhashi, *Jpn. Kokai Tokkyo Koho* 250591A, 2003.
14. M. Furusawa; T. Hashimoto; Y. Noma; Y. Asakawa, *Chem. Pharm. Bull.* **2005**, *53*, 1513–1514.
15. M. Furusawa; T. Hashimoto; Y. Noma; Y. Asakawa, *Chem. Pharm. Bull.* **2005**, *53*, 1423–1429.
16. T. Hashimoto; Y. Noma; C. Murakami; N. Nishimatsu; M. Tanaka; Y. Asakawa, In *Biotransformation of Valencene and Aristolene*, Proceeding of the 45th Symposium on Chemistry of Terpenes, Essential Oils and Aromatics, Toyama, Japan, 2001; Y.Hirai, Ed.; pp 345–347.
17. N. Shoji; A. Umeyama; Y. Asakawa; T. Takeout; K. Nocoton; Y. Ohizumi, *J. Pharm. Sci.* **1984**, *73*, 843–844.
18. Y. Noma; M. Furusawa; C. Murakami; T. Hashimoto; Y. Asakawa, In *Formation of Nootkatol and Nootkatone from Valencene by Soil Microorganisms*, Proceedings of the 45th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Toyama, Japan, 2001; Y.Hirai, Ed.; pp 91–92.
19. T. Takahashi; M. Miyazawa, In *Biotransformation of (+)-Nootkatone by* Aspergillus wentii, *as Biocatalyst*, Proceedings of the 49th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics Fukui, Japan, 2005; M.Hatanaka, Ed.; pp 393–394.
20. T. Takahashi; M. Miyazawa, In *Biotransformation of Sesquiterpenes Which Possess an Eudesmane Skeleton by Microorganisms*, Proceedings of the 50th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Yokohama, Japan, 2006; S.Inoue, Ed.; pp 256–257.
21. R. Kaspera; U. Krings; T. Nanzad; R. G. Berger, *Appl. Microbiol. Biotechnol.* **2005**, *67*, 477–583.
22. M. Furusawa; Y. Noma; T. Hashimoto; Y. Asakawa, In *Biotransformation of* Citrus *Oil Nootkatone, Dihydronootkatone and Dehydronootkatone*, Proceedings of the 47th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Tokyo, Japan, 2003; T. Kurata, Ed.; pp 142–144.

23. T. Hashimoto; Y. Noma; C. Murakami; M. Tanaka; Y. Asakawa, In *Microbial Transformation of* α*-Santonin Derivatives and Nootkatone*, Proceedings of the 44th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Sapporo, Japan, 2000; H. Nishimura, Ed.; pp 157–159.
24. Y. Asakawa; T. Ishida; M. Toyota; T. Takemoto, *Xenobiotica* **1986**, *6*, 753–767.
25. T. Ishida; T. Matsumoto; S. Masumoto, *Bull. Hiroshima Inst. Technol. Rec.* **2005**, *39*, 59.
26. M. Furusawa, Biotransformation of Sesquiterpenoids Obtained from Crude Drugs and Liverworts: Production of Functionalized Substances. Ph.D. Thesis, Tokushima Bunri University, Tokushima, Japan, 2006; pp 1–156.
27. M.-E. F. Hegazy; C. Kuwata; Y. Sato; M. Otsuka; T. Iwasaki; A. Matsushima; T. Hirata, In *Research and Development of Asymmetric Reaction Using Biocatalysts-Biotransformation of Enones by Cultured Cells of* Marchantia polymorpha, Proceedings of the 49th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Fukui, Japan, 2005; M. Hatanaka, Ed.; pp 402–404.
28. T. Hashimoto; Y. Asakawa, Biological Activity of Fragrant Substances from Citrus and Herbs, and Production of Functional Substances Using Microbial Biotransformation. In *Development of Medicinal Foods*; M. Yoshikawa, Ed.; CMC Publisher: Tokyo, 2007; pp 168–184.
29. S. F. Arantes; J. R. Hanson; P. B. Hitchcok, *Phytochemistry* **1999**, *52*, 1063–1067.
30. M. Furusawa; T. Hashimoto; Y. Noma; Y. Asakawa, *Chem. Pharm. Bull.* **2006**, *54*, 861–868.
31. W.-R. Abraham; K. Kieslich; B. Stumpf; L. Ernst, *Phytochemistry* **1992**, *31*, 3749–3755.
32. M. Miyazawa; T. Uemura; H. Kameoka, *Phytochemistry* **1995**, *40*, 793–796.
33. M. Miyazawa; T. Uemura; H. Kameoka, *Phytochemistry* **1994**, *37*, 1027–1030.
34. R. L. Hanson; J. M. Wasylyk; V. B. Nanduri; D. L. Cazzulino; R. N. Patel; L. J. Szarka, *J. Biol. Chem.* **1994**, *269*, 22145–22149.
35. K. Hayashi; H. Morikawa; H. Nozaki; D. Takaoka, In *Biotransformation of Globulol and Epiglubulol by* Aspergillus niger *IFO 4407*, Proceedings of the 42nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Gifu, Japan, 1998; Y.Masaki, Ed.; pp 136–138.
36. D. O. Collins; W. F. Reynold; P. B. Reese, *Phytochemistry* **2002**, *60*, 475–481.
37. H. Higuchi; R. Tsuji; K. Hayashi; D. Takaoka; A. Matsuo; H. Nozaki, In *Biotransformation of Sesquiterpenoids by* Aspergillus niger, Proceedings of the 45th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Toyama, Japan, 2001; Y.Hirai, Ed.; pp 354–355.
38. K. Hayashi; H. Morikawa; A. Matsuo; D. Takaoka; H. Nozaki, In *Biotransformation of Sesquiterpenoids by* Aspergillus niger *IFO 4407*, Proceedings of the 43rd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Oita, Japan, 1999; K.Ohota, Ed.; pp 208–210.
39. H. Nozaki; K. Asano; K. Hayashi; M. Tanaka; A. Masuo; D. Takaoka, In *Biotransformation of Shiromodiol Diacetate and Myli-4(15)-en-9-one by* Aspergillus niger *IFO 4407*, Proceedings of the 40th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Saga, Japan, 1996; S. Kurokawa, Ed.; pp 108–110.
40. Y. Asakawa, Chemical Constituents of the Hepaticae. In *Progress in the Chemistry of Organic Natural Products*; W. Herz, H. Grisebach, G. W. Kirby, Eds.; Springer: Vienna, 1982; Vol. 42, pp 1–285.
41. Y. Asakawa, Chemical Constituents of the Bryophytes. In *Progress in the Chemistry of Organic Natural Products*; W. Herz, G. W. Kirby, R. E. Moore, W. Steglich, Ch. Tamm, Eds.; Springer: Vienna, 1995; Vol. 65, pp 1–618.
42. T. Hashimoto; Y. Noma; Y. Goto; S. Takaoka; M. Tanaka; Y. Asakawa, In *Biotransformation of Sesquiterpenoids from the Liverwort* Plagiochila *species*, Proceedings of the 47th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Tokyo, Japan, 2003; K. Kurata, Ed.; pp 139–141.
43. G. T. Maartooq, *Phytochemistry* **2002**, *59*, 39–44.
44. G. A. Maatooq, *Z. Naturforsch.* **2002**, *57C*, 680–685.
45. H. Morikawa; K. Hayashi; K. Wakamatsu; D. Takaoka; H. Haraguchi; A. Matsuo; H. Nozaki, In *Biotransformation of Sesquiterpenoids by* Aspergillus niger *IFO 4407*, Proceedings of the 44th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Sapporo, Japan, 2000; H. Nishimura, Ed.; pp 151–153.
46. T. Ishida; Y. Asakawa; T. Takemoto, *J. Pharm. Sci.* **1982**, *71*, 965–966.
47. Y. Venkateswarlu; P. Ramesh; P. S. Reddy; K. Jamil, *Phytochemistry* **1999**, *52*, 1275–1277.
48. H. Takahashi, Transformation of Terpenoids and Aromatic Compounds by Selected Microorganisms. Ph.D. Thesis, Tokushima Bunri University, Tokushima, Japan, 1994; pp 1–115.
49. N. Sakui; M. Kuroyanagi; M. Sato; A. Ueno, In *Transformation of Ten-membered Sesquiterpenes by Callus of* Curcuma, Proceedings of the 32nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Myazaki, Japan, 1988; K. Shima, Ed.; pp 322–324.
50. H. Hikino; T. Konno; T. Nagashima; T. Kohama; T. Takemoto, *Tetrahedron Lett.* **1971**, *12*, 337–340.
51. S. Sakamoto; N. Tsuchiya; M. Kuroyanagi; A. Ueno, *Phytochemistry* **1994**, *35*, 1215–1219.
52. Y. Asakawa; H. Takahashi; M. Toyota, *Phytochemistry* **1991**, *30*, 3993–3997.
53. N. Sakui; M. Kuroyamagi; Y. Ishitobi; M. Sato; A. Ueno, *Phytochemistry* **1992**, *31*, 143–147.
54. X. C. Ma; M. Ye; L. J. Wu; D. A. Guo, *Enzyme Microb. Technol.* **2006**, *38*, 367–371.
55. M. Miyazawa; Y. Honjo; H. Kameoka, *Phytochemistry* **1997**, *44*, 433–436.
56. T. Takahashi; I. Horibe; M. Miyazawa, In *Biotransformation of* β*-Selinene by* Aspergillus wentii, Proceedings of the 51st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Nagahama, Japan, 2007; S. Ohta, Ed.; pp 319–320.
57. K. Hayashi; H. Morikawa; A. Matsuo; D. Takaoka; H. Nozaki, In *Biotransformation of Sesquiterpenoids by* Aspergillus niger *IFO 4407*, Proceedings of the 43rd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Oita, Japan, 1999; K. Ohga, Ed.; pp 208–210.
58. A. Garcia-Granados; M. C. Gutierrez; F. Rivas; J. M. Arias, *Phytochemistry* **2001**, *58*, 891–895.
59. K. Y. Orabi, *J. Nat. Prod.* **2000**, *63*, 1709–1711.
60. Y. Noma; T. Hashimoto; A. Kikkawa; Y. Asakawa, In *Biotransformation of* (−)-α*-Eudesmol by* Asp. niger *and* Asp. cellulosae *M-77*, Proceedings of the 40th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Saga, Japan, 1996; S. Kurokawa, Ed.; pp 95–97.

61. Y. Noma; T. Hashimoto; S. Kato; Y. Asakawa, In *Biotransformation of* (+)-β-*Eudesmol by* Aspergillus niger, Proceedings of the 41st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Morioka, Japan, 1997; T. Ito, Ed.; pp 224–226.
62. G. A. Maatooq, *Z. Naturforsch.* **2002**, *57C*, 654–659.
63. T. Hashimoto; Y. Noma; Y. Asakawa, *Heterocycles* **2001**, *54*, 529–559.
64. A. M. Clark; C. D. Hufford, *J. Chem. Soc. Perkin Trans.* **1979**, *1,* 3022–3028.
65. A. F. Barrero; J. E. Oltra; D. S. Raslan; D. A. Saude, *J. Nat. Prod.* **1999**, *62*, 726–729.
66. T. Hashimoto; Y. Noma; Y. Matsumoto; Y. Akamatsu; M. Tanaka; Y. Asakawa, In *Biotransformation of Sesquiterpenoids by Microorganisms. (5): Biotransformation of Dehydrocostuslactone*, Proceedings of the 43rd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Oita, Japan, 1999; K. Ohga, Ed.; pp 202–204.
67. Atta-ur Rahman; M. I. Choudhary; F. Shaheen; A. Rauf; A. Farooq, *Nat. Prod. Lett.* **1998**, *12*, 215–222.
68. J. R. Hanson; A. Truneh, *Phytochemistry* **1996**, *42*, 1021–1023.
69. L. Yang; K. Fujii; J. Dai; J. Sakai; M. Anodo, In *Biotransformation of* α-*Santonin and its C-6 Epimer by Fungus and Plant Cell Cultures*, Proceedings of the 47th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Tokyo, Japan, 2003; K. Kurata, Ed.; pp 148–150.
70. A. Matsushima; M.-E. F. Hegazy; C. Kuwata; Y. Sato; M. Otsuka; T. Hirata, In *Biotransformation of Enones Using Plant Cultured Cells-the Reduction of* α-*Santonin*, Proceedings of the 48th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Yamaguchi, Japan, 2004; T. Kajiwara, Ed.; pp 396–398.
71. A. Amate; A. Garcia-Granados; A. Martinez; A. S. de Buryage; J. L. Breton; M. E. Onorato; J. M. Arias, *Tetrahedron* **1991**, *47*, 5811–5818.
72. Atta-ur Rahman; M. I. Choudhary; A. Ata; M. Alam; A. Farooq; S. Perveen; M. S. Shekhani, *J. Nat. Prod.* **1994**, *57*, 1251–1255.
73. T. Hashimoto; M. Sekita; M. Furusawa; Y. Noma; Y. Asakawa, In *Biotransformation of Sesquiterpene Lactones, (-)-Parthenolide and (-)-Frullanolide by Microorganisms*, Proceedings of the 49th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Fukui, Japan, 2005; M. Hatanaka, Ed.; pp 387–389.
74. M. Miyazawa; S. Akazawa; H. Sakai; H. Kameoka, In *Biotransformation of* (+)-γ-*Gurjunene Using Plant Pathogenic Fungus,* Glomerella cingulata *as a Biocatalyst*, Proceedings of the 41st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Morioka, Japan, 1997; T. Ito, Ed.; pp 218–219.
75. M. Miyazawa; Y. Honjo; H. Kameoka, *Phytochemistry* **1998**, *49*, 1283–1285.
76. M. Miyazawa; Y. Honjo; H. Kameoka, In *Biotransformation of Gguaiol and Bulnesol Using Plant Pathogenic Fungus* Glomerella cingulata *as a Biocatalyst*, Proceedings of the 40th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Saga, Japan, 1996; S. Kurokawa, Ed.; pp 82–83.
77. A. Sugawara; M. Miyazawa, In *Biotransformation of Guaiene Using Plant Pathogenic Fungus,* Eurotium rubrum *as a Biocatalyst*, Proceedings of the 48th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Yamaguchi, Japan, 2004; T. Kajiwara, Ed.; pp 385–386.
78. M. Miyazawa; A. Sugawara, *Nat. Prod. Res.* **2006**, *20*, 731–734.
79. U. Jacobsson; V. Kumar; S. Saminathan, *Phytochemistry* **1995**, *39*, 839–843.
80. A. M. Galal; A. S. Ibrahim; J. S. Mossa; F. S. El-Feraly, *Phytochemistry* **1999**, *51*, 761–765.
81. A. M. Galal, *J. Nat. Prod.* **2001**, *64*, 1098–1099.
82. T. Hashimoto; Y. Noma; Y. Akamatsu; M. Tanaka; Y. Asakawa, In *Biotransformation of Sesquiterpenoids by Microorganisms. (5): Biotransformation of Dehydrocostuslactone*, Proceedings of the 43rd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Oita, Japan, 1999; K. Ohga, Ed.; pp 202–204.
83. G. N. K. Kumari; S. Masilamani; R. Ganesh; S. Aravind, *Phytochemistry* **2003**, *62*, 1101–1104.
84. K. K. Bhutani; R. N. Thakur, *Phytochemistry* **1991**, *30*, 3599–3600.
85. D. O. Collins; P. B. Reese, *Phytochemistry* **2002**, *59*, 489–492.
86. D. O. Collins; P. L. D. Ruddock; J. Chiverton; C. de Grasse; W. F. Reynolds; P. B. Reese, *Phytochemistry* **2002**, *59*, 479–488.
87. G. O. Buchanan; L. A. D. Williams; P. B. Reese, *Phytochemistry* **2000**, *54*, 39–45.
88. M. Miyazawa; H. Nankai; H. Kameoka, *Phytochemistry* **1995**, *39*, 1077–1080.
89. K. A. El Sayed; M. Yousaf; M. T. Hamann; M. A. Avery; M. Kelly; P. Wipf, *J. Nat. Prod.* **2002**, *65*, 1547–1553.
90. I.-S. Lee; H. N. ElSohly; E. M. Coroom; C. D. Hufford, *J. Nat. Prod.* **1989**, *52*, 337–341.
91. J. Zhan; H. Guo; J. Dai; Y. Zhang; D. Guo, *Tetrahedron Lett.* **2002**, *43*, 4519–4521.
92. T. Hashimoto; Y. Noma; N. Nishimatsu; M. Sekita; M. Tanaka; Y. Asakawa, In *Biotransformation of Antimalarial Sesquiterpenoids by Microorganisms*, Proceedings of the 47th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Tokyo, Japan, 2003; K. Kurata, Ed.; pp 136–138.
93. I. A. Parshikov; K. M. Muraleedharan; M. A. Avery, *Appl. Microbiol. Biotechnol.* **2004**, *64*, 782–786.
94. G. Aranda; I. Facon; J.-Y. Lallemand; M. Leclaire, *Tetrahedron Lett.* **1992**, *33*, 7845–7848.
95. M. Sekita; M. Furusawa; T. Hashimoto; Y. Noma; Y. Asakawa, In *Biotransformation of Pungent Tasting Polygodial from* Polygonum hydropiper *and Related Compounds by Microorganisms*, Proceedings of the 49th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Fukui, Japan, 2005; M. Hatanaka, Ed.; pp 380–381.
96. W. A. Ayer; P. A. Craw, *Can. J. Chem.* **1989**, *67*, 1371–1380.
97. M. Sekita; T. Hashimoto; Y. Noma; Y. Asakawa, In *Biotransformation of Biologically Active Terpenoids, Sacculatal and Cinnamodial by Microorganisms*; Proceedings of the 50th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Yokohama, Japan, 2006; S. Unoue, Ed., pp 406–408.
98. Y. Asakawa; T. Hashimoto; Y. Mizuno; M. Tori; Y. Fukuzawa, *Phytochemistry* **1992**, *31*, 579–592.
99. H. Takahashi; M. Toyota; Y. Asakawa, *Phytochemistry* **1993**, *33*, 1055–1059.
100. Y. Asakawa, Terpenoids and Aromatic Compounds with Pharmaceutical Activity from Bryophytes. In *Bryophytes: Their Chemistry and Chemical Taxonomy*; D. H. Zinsmeister, R. Mues, Eds.; Clarendon Press: Oxford, 1990; pp 369–410.
101. Y. Asakawa, Phytochemistry of Bryophytes. In *Phytochemicals in Human Health Protection, Nutrition, and Plant Defense*; J. Romeo, Ed.; Kluwer Academic/Plenum Publishers: New York, 1999; Vol. 33, pp 319–342.
102. Y. Asakawa, *Pure Appl. Chem.* **2007**, *75*, 557–580.
103. Y. Asakawa, *Nat. Prod. Commun.* **2008**, *3*, 77–92.

104. Y. Asakawa; A. Ludwiczuk, *Med. Plants Poland World* **2008**, *14*, 33–53.
105. T. Hashimoto; Y. Noma; S. Takaoka; M. Tanaka; Y. Asakawa, In *Biotransformation of Valencene and Aristolene*, Proceedings of the 45th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Toyama, Japan, 2001; H. Hirai, Ed.; pp 348–350.
106. T. Hashimoto; Y. Noma; Y. Asakawa, In *Biotransformation of Cuparane- and Herbertane-type Sesquiterpenoids*, Proceedings of the 50th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Yokohama, Japan, 2006; S. Inoue, Ed.; pp 263–265.
107. L. Harinantenaina; D. N. Quang; T. Nishizawa; T. Hashimoto; C. Kohchi; G.-I. Soma; Y. Asakawa, *Phytomedicine* **2007**, *14*, 486–491.
108. L. Harinantenaina; Y. Noma; Y. Asakawa, *Chem. Pharm. Bull.* **2005**, *53*, 256–257.
109. T. Hashimoto; Y. Noma; Y. Goto; M. Tanaka; S. Takaoka; Y. Asakawa, *Heterocycles* **2004**, *62*, 655–666.
110. Y. Wang; T.-K. Tan; G. K. Tan; J. D. Connolly; L. J. Harrison, *Phytochemistry* **2006**, *67*, 58–61.
111. M. Tori; M. Sono; Y. Asakawa, *Bull. Chem. Soc. Jpn.* **1990**, *63*, 1770–1776.
112. M. Furusawa; T. Hashimoto; Y. Noma; Y. Asakawa, In *The structure of New Sesquiterpenoids from the Liverwort* Reboulia hemisphaerica *and Their Biotransformation*, Proceedings of the 49th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Fukui, Japan, 2005; M. Hatanaka, Ed.; pp 235–237.
113. M. Furusawa; T. Hashimoto; Y. Noma; Y. Asakawa, *Chem. Pharm. Bull.* **2006**, *54*, 996–1003.
114. E. L. Lahlou; Y. Noma; T. Hashimoto; Y. Asakawa, *Phytochemistry* **2000**, *54*, 455–460.
115. T. Hashimoto; S. Kato; M. Tanaka; S. Takaoka; Y. Asakawa, In *Biotransformation of Sesquiterpenoids by Microorganisms (4): Biotransformation of Hinesol by* Aspergillus niger, Proceedings of the 42nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Gifu, Japan, 1998; M. Masaki, Ed.; pp 127–129.
116. T. Hashimoto; Y. Noma; S. Kato; M. Tanaka; S. Takaoka; Y. Asakawa, *Chem. Pharm. Bull.* **1999**, *47*, 716–717.
117. T. Hashimoto; K. Shiki; M. Tanaka; S. Takaoka, *Heterocycles* **1998**, *49*, 315–325.
118. M. I. Choudhary; S. G. Musharraf; A. Sami; Atta-ur-Rahman, *Helv. Chim. Acta* **2004**, *87*, 2685–2694.
119. G. Aranda; M. S. Kortbi; J.-Y. Lallemand; A. Neuman; A. Hammoumi; I. Facon; R. Azerad, *Tetrahedron* **1991**, *47*, 8339–8350.
120. T. Hashimoto; M. Fujiwara; K. Yoshikawa; A. Umeyama; M. Tanaka; Y. Noma, In *Biotransformation of Sclareolide and Sclareol by Microorganisms*, Proceedings of the 51st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Nagahama, Japan, 2007; S. Ohta, Ed.; pp 316–318.
121. Atta-ur-Rahman; A. Farooq; M. I. Choudhary, *J. Nat. Prod.* **1997**, *60*, 1038–1040.
122. J. R. Hanson; A. Truneh, *Phytochemistry* **1996**, *42*, 1021–1023.
123. Y. Noma; T. Hashimoto; S. Sawada; T. Kitayama; Y. Asakawa, In *Microbial Transformation of Zerumbone*, Proceedings of the 46th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics 2002; pp 313–315.
124. E. Nishida; Y. Kawai, In *Bioconversion of Zerumbone and Its Derivatives*, Proceedings of the 51st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Tokushima, Japan, 2007; Y. Asakawa, Ed.; pp 387–389.
125. M. Miyazawa; H. Nakai; H. Kameoka, *Phytochemistry* **1995**, *40*, 69–72.
126. Y. Itsuzaki; K. Ishisaka; M. Miyazawa, In *Biotransformation of (+)-Cedrol by Using Human Skin Microbial Flora* Staphylococcus epidermidis, Proceedings of the 46th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Tokushima, Japan, 2002; Y. Asakawa, Ed.; pp 101–102.
127. J. R. Hanson; H. Nasir, *Phytochemistry* **1993**, *33*, 835–837.
128. W.-R. Abraham; P. Washausen; K. Kieslich, *Z. Naturforch. C* **1987**, *42*, 414–419.
129. J. Aleu; J. R. Hanson; R. Hernandez-Galan; I. G. Collad, *J. Nat. Prod.* **1999**, *62*, 437–440.
130. H. Matsui; Y. Minamino; M. Miyazawa, In *Biotransformation of (+)-Cedryl Acetate by* Glomerella cingulata, *Parasitic Fungus*, Proceedings of the 43rd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Oita, Japan, 1999; K. Ohga, Ed.; pp 215–216.
131. M. Miyazawa; H. Matsui; H. Kameoka, In *Biotransformation of Patchouli Alcohol Using Plant Parasitic Fungus* Glomerella cingulata *as a Biocatalyst*, Proceedings of the 41st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Morioka, Japan, 1997; T. Ito, Ed.; pp 220–221.
132. H. Matsui; M. Miyazawa, In *Biotransformation of Pathouli Acetate Suing parasitic Fungus* Glomerella cingulata *as a Biocatalyst*, Proceedings of the 44th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Morioka, Japan, 2000; T. Ito, Ed.; pp 149–150.
133. M. Miyazawa; H. Matsui; H. Kameoka, In *Biotransformation of Unsaturated Sesquiterpene Alcohol Using Plant Parasitic Fungus*, Glomerella cingulata *as a Biocatalyst*, Proceedings of the 42nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Gifu, Japan, 1998; Y. Masaki, Ed.; pp 121–122.
134. K. Sakata; I. Horibe; M. Miyazawa, In *Biotransformation of (+)-α-Longipinene by Microorganisms as a Biocatalyst*, Proceedings of the 51st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Nagahama, Japan, 2007; S. Ohta, Ed.; pp 321–322.
135. J. Aleu; R. Hermandez-Galan; J. R. Hanson; P. B. Hitchcock; I. G. Collado, *J. Chem. Soc. Perkin Trans. 1* **1999**, 727–730.
136. K. Sakata; M. Miyazawa, In *Biotransformation of (+)-Isolongifolen-9-one by* Glomerella cingulata *as a Biocatalyst*, Proceedings of the 50th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Yokohama, Japan, 2006; S. Inoue, Ed.; pp 258–260.
137. M. I. Choudhary; S. G. Musharraf; S. A. Nawaz; S. Anjum; M. Pervez; H. K. Fun; Atta-ur-Rahman, *Bioorg. Med. Chem.* **2005**, *13*, 1939–1944.
138. M. I. Choudhary; W. Kausar; Z. A. Siddiqui; Atta-ur-Rahman, *Z. Naturforsch.* **2006**, *61B*, 1035–1038.
139. J. R. Devi, *Indian J. Biochem. Biophys.* **1979**, *16*, 76–79.
140. W. R. Abraham; L. Ernst; B. Stumpf, *Phytochemistry* **1990**, *29*, 115–120.
141. M. I. Choudhary; Z. A. Siddiqui; S. A. Nawaz; Atta-ur-Rahman, *J. Nat. Prod.* **2006**, *69*, 1429–1434.
142. R. Duran; E. Corrales; R. Hernandez-Galan; G. Collado, *J. Nat. Prod.* **1999**, *62*, 41–44.
143. J. Aleu; R. Hernandez-Galan; I. G. Collad, *J. Mol. Catal. B* **2002**, *16*, 249–253.
144. M. Miyazawa; H. Nakai; H. Kameoka, *Phytochemistry* **1995**, *40*, 1133–1137.

145. M. Miyazawa; H. Nakai; H. Kameoka, *J. Agric. Food. Chem.* **1996**, *44*, 1543–1547.
146. P. J. Hrdlicka; A. B. Sorensen; B. R. Poulsen; G. J. G. Ruijter; J. Visser; J. J. L. Iversen, *Biotechnol. Progr.* **2004**, *20*, 368–376.
147. M. Miyazawa; H. Nakai; H. Kameoka, *Phytochemistry* **1996**, *43*, 105–109.
148. K. M. Madyastha; T. L. Gururaja, *Indian J. Chem.* **1993**, *32B*, 609–614.
149. H. Nankai; M. Miyazawa; H. Kameoka, In *Biotransformation of (Z,Z)-Farnesol Using Plant Pathogenic Fungus,* Glomerella cingulata *as a Biocatalyst*, Proceedings of the 40th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics, Saga, Japan, 1996; S. Kurokawa, Ed.; pp 78–79.
150. I. Kiran; H. N. Yildirim; J. R. Hanson; P. B. Hitchcock, *J. Chem. Technol. Biotechnol.* **2004**, *79*, 1366–1370.
151. I. Kiran; T. Akar; A. Gorgulu; C. Kazaz, *Biotechnol. Lett.* **2005**, *27*, 1007–1010.
152. K. Y. Orabi, *Z. Naturforsch.* **2001**, *56C*, 223–227.
153. T. Ishida; Y. Asakawa; T. Takemoto, *J. Pharm. Sci.* **1982**, *71*, 965–966.
154. Y. Asakawa; Z. Taira; T. Takemoto; T. Ishida; M. Kido; Y. Ichikawa, *J. Pharm. Sci.* **1981**, *70*, 710–711.
155. W. M. Daniewski; P. A. Grieco; J. Huffman; A. Rymkiewicz; A. Wawrzun, *Phytochemistry* **1981**, *20*, 2733–2734.
156. L. Bang; G. Ourisson, *Tetrahedron Lett.* **1975**, *16*, 1881–1884.
157. L. Bang; G. Ourisson; P. Teisseire, *Tetrahedron Lett.* **1975**, *16*, 2211–2214.
158. J.-L. Zundel, Etude Chimique et Biochimique de l'Essence de Santal. Ph.D. Thesis, Universite Louis Pasteur, Strasbourg, France, 1976; pp 57–70.
159. T. D. Sutherland; G. C. Unnithan; J. F. Andersen; P. H. Evans; M. B. Muratakiev; L. Z. Szabo; E. A. Mash; W. S. Bowers; R. Feyereisen, *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12884–12889.
160. E. Darrouzet; B. Mauchamp; G. D. Prestwich; L. Kerhoas; I. Ujvary; F. Couillaud, *Biochem. Biophys. Res. Commun.* **1997**, *240*, 752–758.

Biographical Sketches

Professor Yoshinori Asakawa studied organic chemistry at the graduate school of the Hiroshima University. Here he was appointed as a research assistant in 1969, obtained his Ph.D. degree in 1972, and later did his postdoctoral research at the Universite Louis Pasteur, France where he worked for 2 years with Professor Guy Ourisson. In 1976, he moved to the Faculty of Pharmaceutical Sciences, Tokushima Bunri University, as an associate professor, full professor in 1981, served twice as Dean, and is currently Director of the Institute of Pharmacognosy (1986–present) and the president of the Phytochemical Society of Asia (2007–present). He is the coeditor of *Phytomedicine* and serves on editorial boards of *Phytochemistry*, *Phytochemistry Letters*, *Planta Medica*, *Fitoterapia*, *Flavour and Fragrance Journal*, *Natural Product Communication*, *Natural Product Research*, *Spectroscopy*, *Arkivoc*, *Current Chemical Biology*, and *Malaysian Journal of Sciences*, among others. He has published 540 original papers, 20 reviews, and 27 books and monographs. For his outstanding research he was awarded the first Hedwig medal (1983), the Pergamon Phytochemistry Prize and Certificate (1997), The Tokushima News Paper prize (1997), and the ISEO prize (2004). Over the years, he has mentored 37 postdoctoral researchers from various countries.

Professor Yoshiaki Noma was born in 1947 in Hyogo Prefecture and graduated from the Faculty of Agriculture, Okayama University, and then entered into the Graduate School of Agricultural and Chemical Sciences of Okayama University and Osaka Prefectural University and obtained a Ph.D. from the Osaka Prefectural University in 1975. Professor Noma was an associate professor at Osaka Joshi-Gakuen Junior College, later in 1988 he moved to the Department of Human Life Sciences at Tokushima Bunri University as a full professor. Professor Noma is a Fellow of the Japan Society for Bioscience, Biotechnology, and Agrochemistry. He is also a Fellow of the Chemical Society of Japan and Japanese Society of Nutrition and Food Sciences.

Professor Noma has published over 55 research papers and reviews on several topics connected with the microbiological biotransformation of monoterpenes and sesquiterpenoids, the metabolic pathways of monoterpenoids, and the chemistry and biological activity of metabolites. At present, his research on biotransformation includes substances from liverworts and higher plants of potential use in cancer, antioxidant, and mosquitocidal compounds. He is also interested in the study of the biosynthesis of biologically active compounds.

3.21 Biotransformation of Di- and Triterpenoids, Steroids, and Miscellaneous Synthetic Substrates

Yoshinori Asakawa and Yoshiaki Noma, Tokushima Bunri University, Yamashiro-cho, Tokushima, Japan

3.21.1 Biotransformation of Di- and Triterpenoids and Steroids

Many naturally occurring diterpenoids such as labdanes, kaurene, stemodane, sacculatane, taxanes, and abietanes are used as substrates of microbial biotransformation.

Sclareol (**1**), one of the major components of *Salvia sclarea*, was incubated with *Cephalosporium aphidocola*,[1] *Bacillus sphaericus*, *Cunninghamella elegans*, and *Diplodia gossypina*.[2] *C. aphidocola* converted **1** to three hydroxylated compounds, 3β-hydroxy- (**3**), 18-hydroxy- (**4**), and 18-acetoxysclareol (**6**), and one very unstable 14,15-epoxide (**7**), whereas *B. sphaericus* converted **1** to **3** and **4**, *C. elegans* to 2α-hydroxysclareol (**2**), together with **3** and **4**, and *D. gossypina* to **2**–**4** and 19-hydroxysclareol (**5**) (**Scheme 1**). The ability of fungi to biotransform sclareol is higher than that of bacteria. 2-Hydroxysclareol (**2**) is formed by zygomycotina and deuteromycotina.[2] Hashimoto *et al.*[3] reported that *Aspergillus niger* converted **1** to 3β-dihydroxy derivative (**3**).

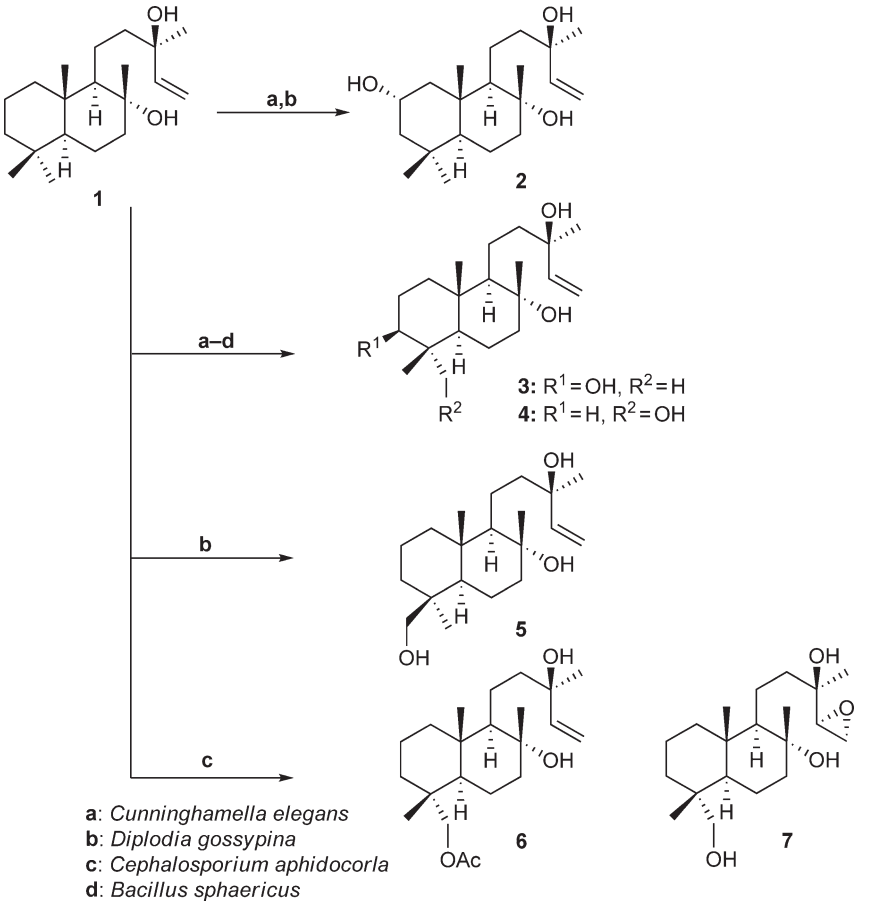
Sclareol (**1**), manool (**8**), and 7-hydroxymanool (**9**) were incubated with *Mucor plumbeus*. In each substrate, 3-hydroxylated compounds (**3**, **12**, **13**) have been ubiquitously found along with 2α- (**11**), 6α- (**10**, **15**), 7α- (**13**), 18-hydroxy (**4**), and 7α,19-dihydroxy products (**16**). 3-Oxomanool (**14**), which might be formed from **12** by further oxidation, was obtained in 80% yield[4] (**Scheme 2**).

13,14,15-Trihydroxylabd-7-ene (**17**) and 13,14,15-trihydroxylabd-8(17)-ene (**18**) obtained from *Madia* species were incubated with the fungus *Debaryomyces hansenii* to afford 6-oxo-8-ene (**19**) and 7α-hydroxy derivatives (**20**), and 3-hydroxy (**21**) and 3-oxo derivatives (**22**), respectively. The same substrates (**17**, **18**) were fermented with *A. niger* to furnish 7α-hydroxy (**20**), 3β-hydroxy (**21**), and 3-oxo derivatives (**22**)[5] (**Scheme 3**). Antimicrobial activity of the metabolites **17**–**22** was tested against three bacteria, *Bacillus cereus*, *Staphylococcus aureus*, and *Escherichia coli*. Compound **21** showed the highest activity against all tested bacteria.[5]

Two *ent*-18-acetoxy-6-oxo-13-epi-manoyl oxides (**23**) and their 13-isomer (**24**) were biotransformed by *Fusarium moniliforme* and *Neurospora crassa*, respectively (**Schemes 4,5**).

Biotransformation of **23** by *F. moniliforme* yielded deacetylated metabolites **25** (17%), **26**, (16%), and **27** (6%). The same substrate (**23**) was incubated with *N. crassa* to afford the same metabolites **25** (18%), **26** (22%), and **27** (18%) as well as **28** (3%), **29** (10%), **30** (2%), and **31** (2%), whereas incubation of **24** with *F. moniliforme* afforded the metabolites **32** (21%), **33** (10%), and **34** (9%). The structure of the metabolite **33** was confirmed by the analysis of ^{1}H NMR data of diacetate (**35**) obtained from **33** by acetylation. *N. crassa* converted the same substrate to **32** (17%), **33** (9%), **34** (27%), and *ent*-11β-hydroxy derivatives **37** (12%) and **38** (2%), together with two new metabolites, *ent*-11α-hydroxy (**36**) (6%) and 11-oxo derivative (**39**) (3%). *F. moniliforme* caused hydroxylation at C-11 with different stereoselectivity and by the *ent*-α-face. *N. crassa* showed less stereoselectivity to yield both isomers together with an 11-oxo product.[6]

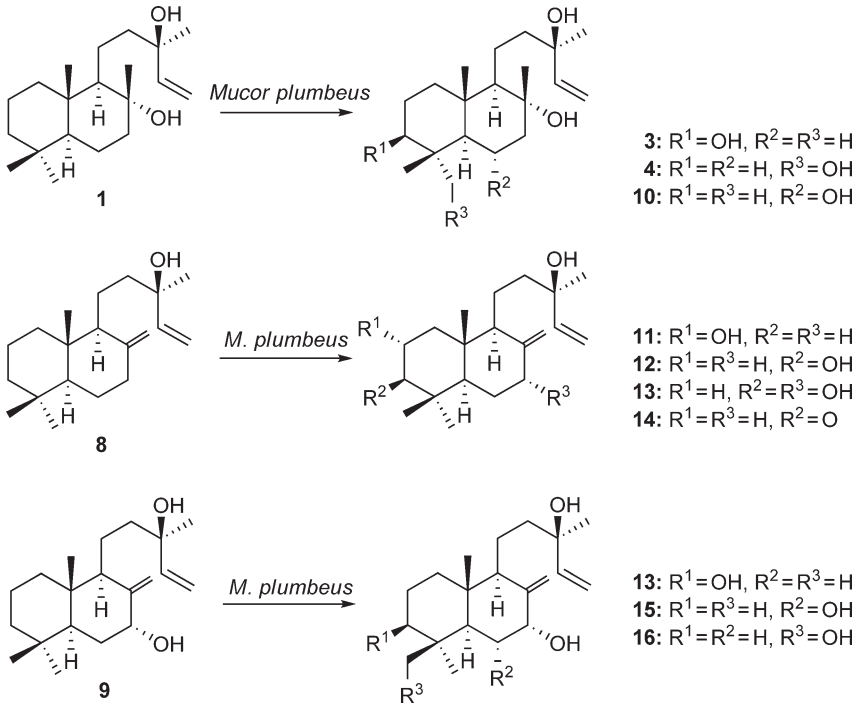
The substrate isolated from *Sideritis perfoliata*, 2β-hydroxy-*ent*-13-*epi*-manoyl oxide (**40**), was incubated with *Gibberella fujikuroi* to yield the metabolites 12β- (**41**), 6β- (**42**), and 20-hydroxy derivatives (**43**) among which **41** was predominantly obtained[7] (**Scheme 6**).



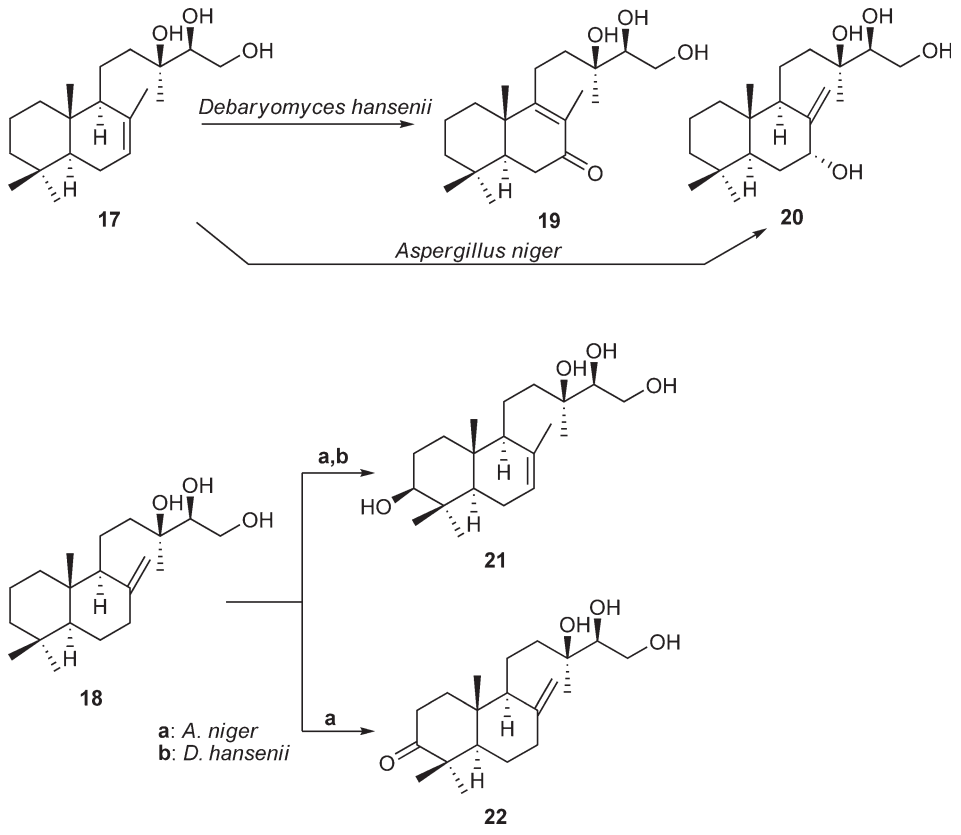
Scheme 1 Biotransformation of sclareol (**1**) by *C. aphidocola*, *B. sphaericus*, *C. elegans*, and *D. gossypina*.

The structure of the diterpenoids stemodin (**44**) and stemodinone (**45**) found in the shrub *Stemodia maritima* is similar to that of aphidicolin, which shows a potent cytotoxic and antiviral activity.[8] In order to obtain their derivatives with potential biological activity, Buchanan and Reese[8] and Lamm *et al.*[9] carried out biotransformation of **44**, **45**, and stemarin (**46**) and related compounds, stemodin 2α-(*N*,*N*-dimethylcarbamate)- (**47**) and stemarin 19-(*N*,*N*-dimethylcarbamate) (**48**) by *Beauveria bassiana* and *Cunninghamella echinulata* var. *elegans* (**Scheme 7**). The former microbe converted **44** to 19-hydroxystemodin (**49**), **45** to 19-hydroxystemodinone (**50**), and **46** to 1β-hydroxystemarin (**51**) and 19-carboxystemarin (**52**). The latter organism converted **44** to 7α- (**53**), 7β- (**54**), 11β- (**55**), and 3β-hydroxystemodin (**56**) (**Scheme 8**). The products **54** and **56** were also obtained from **44** by incubation with *Phanerochaete chrysosporium*. *C. echinulata* converted stemodinone (**45**) to stemodin (**44**), the same products (**53**, **54**) as described above, and 14-hydroxy (**57**) and 7β-hydroxy derivative (**58**) (**Scheme 9**). *P. chrysosporium* also gave 19-hydroxystemodinone (**50**) from stemodinone (**45**) (**Scheme 9**). 2α-(*N*,*N*-dimethylcarbamate (**47**) was incubated with *C. echinulata* to afford 6α- (**59**) and 7α-hydroxy derivatives (**60**) (**Scheme 10**). The same organism degraded stemarin (**46**) and its related compound stemarin-19-(*N*,*N*-dimethylcarbamate) (**48**) to 7α-hydroxy-19-carboxylic acid (**61**), and stemarin-2α-hydroxy-19-(*N*, *N*-dimethylcarbamate) (**62**), stemarin-2α,8β-dihydroxy-19-(*N*, *N*-dimethylcarbamate) (**63**) and decarbamate product (**52**), which was also obtained from stemarin (**46**) (**Scheme 10**). The substrate **46** and two carbamates (**47**, **48**) were not changed by *P. chrysosporium*.

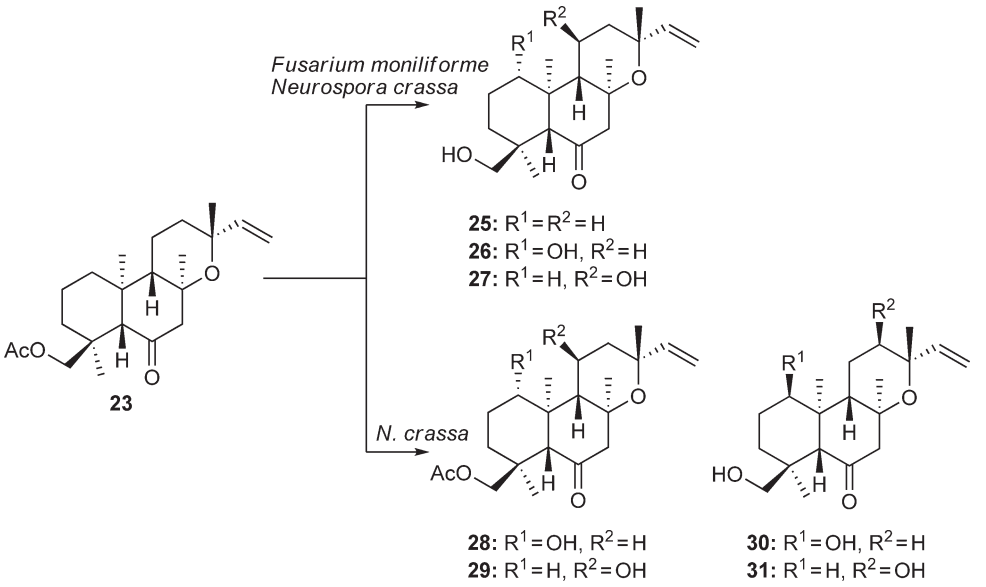
Biotransformation of (−)-kaur-16-en-19-oic acid (**64**), the major constituent of *Cacalia bulbifera*, was carried out to obtain 7-hydroxylated products, which are important intermediates of gibberellin biosynthesis. Incubation of the substrate with *Cunninghamella blakesleeana* gave four metabolites, 7β-hydroxy (**65**), 16-hydroxy (**66**), 16α,17-dihydroxy (**67**), and 7β,16α-dihydroxy derivatives (**68**)[10] (**Scheme 11**).



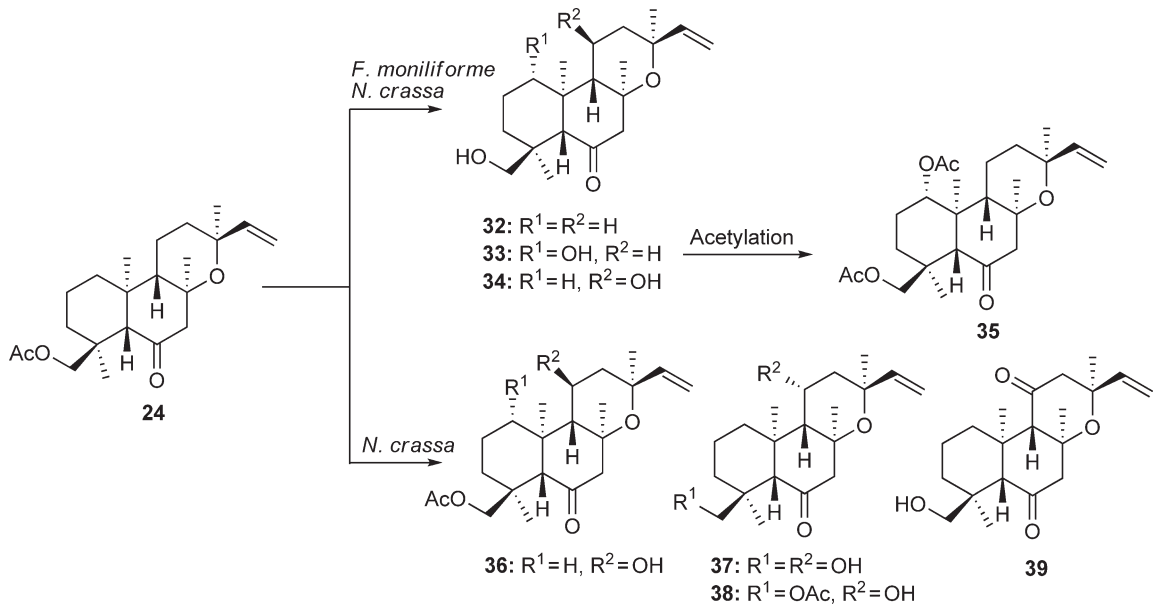
Scheme 2 Biotransformation of sclareol (**1**), manool (**8**), and 7-hydroxymanool (**9**) by *M. plumbeus.*



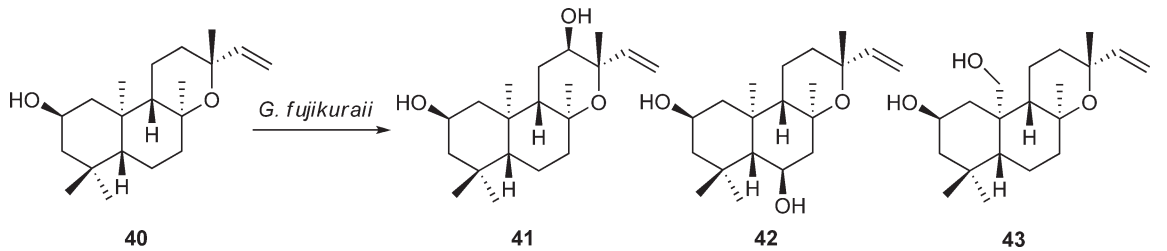
Scheme 3 Biotransformation of 13,14,15-trihydroxylabd-7-ene (**17**) and 13,14,15-trihydroxylabd-8(17)-ene (**18**) by *D. hansenii.*



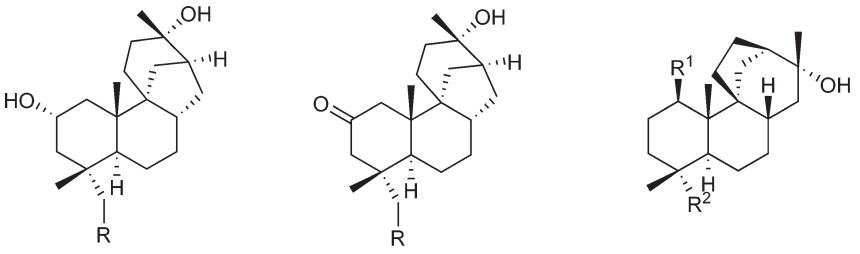
Scheme 4 Biotransformation of *ent*-18-acetoxy-6-oxo-13-epi-manoyl oxide (**23**) by *F. moniliforme* and *N. crassa.*



Scheme 5 Biotransformation of *ent*-18-acetoxy-6-oxomanoyl oxide (**24**) by *F. moniliforme* and *N. crassa.*



Scheme 6 Biotransformation of 2β-hydroxy-*ent*-13-*epi*-manoyl oxide (**40**) by *Gibberella fujikuroi.*

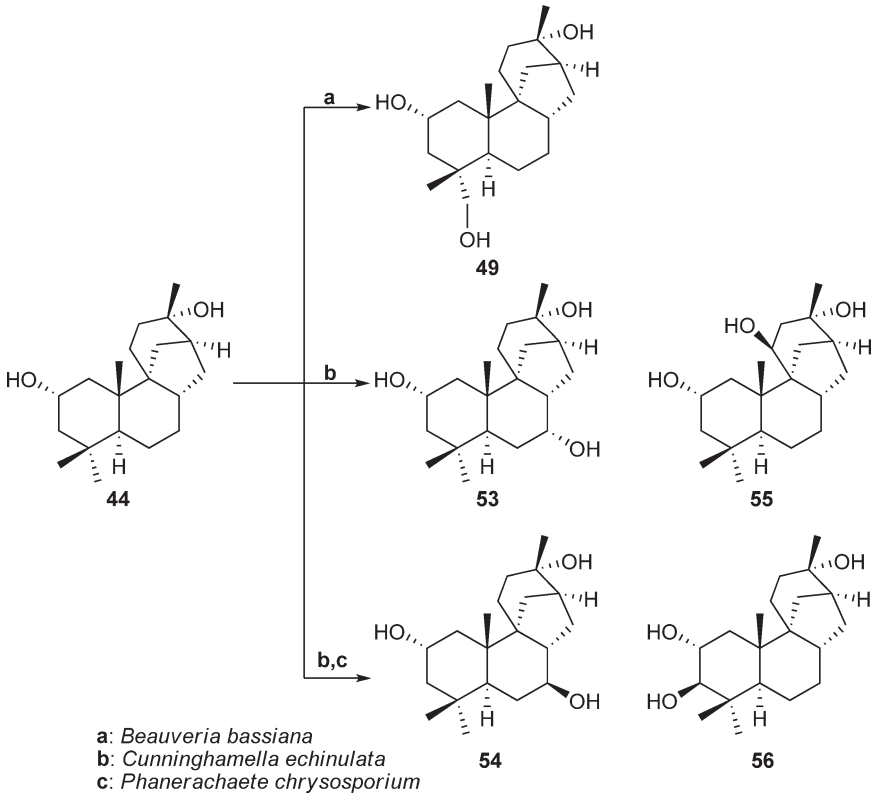


Stemodin (**44**): R = H
49: R = OH

Stemodinone (**45**): R = H
50: R = OH

Stemarin (**46**): R^1 = H, R^2 = CH_2OH
51: R^1 = OH, R^2 = CH_2OH
52: R^1 = H, R^2 = CO_2H

Scheme 7 Stemodin (**44**), stemodinone (**45**), and stemarin (**46**), substrates for biotransformation.

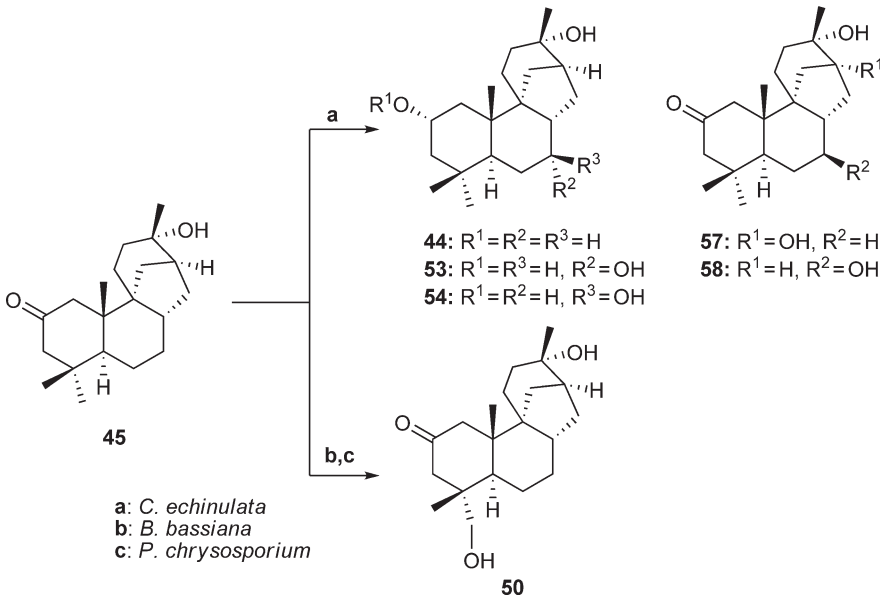


a: *Beauveria bassiana*
b: *Cunninghamella echinulata*
c: *Phanerachaete chrysosporium*

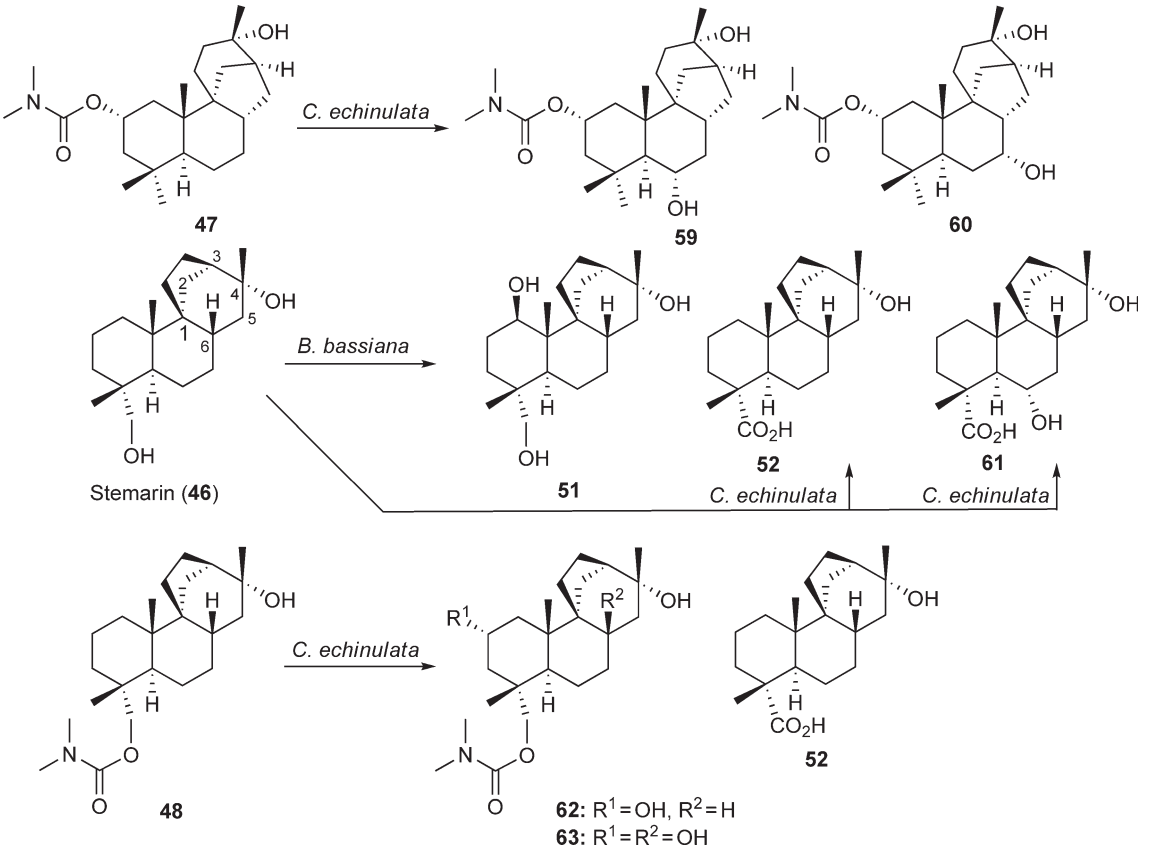
Scheme 8 Biotransformation of stemodin (**44**) by *B. bassiana*, *C. echinulata* var. *elegans*, and *P. chrysosporium.*

A number of *ent*-kaurane derivatives, *epi*-candicandiol (=*ent*-7α,18-dihydroxykaur-16-ene),[11] *ent*-kaur-16-ene 7-, 15-, and 18-alcohols,[12] *ent*-3β-hydroxy-kaur-16-ene,[13] candidiol (=*ent*-15β,18-dihydroxykaur-16-ene),[14] *ent*-15β-hydroxykaurene,[15] *ent*-7α,15β-hydroxykaurene,[16] *ent*-15β-hydroxykaur-16-ene,[17] and 7-oxo-*ent*-kaurene derivatives[18] were biotransformed by *G. fujikuroi* in conjunction with gibberellin biosynthesis. The biotransformation of *epi*-candicandiol and candicandiol (**70**) by *M. plumbeus* was also reported by the same authors.[19]

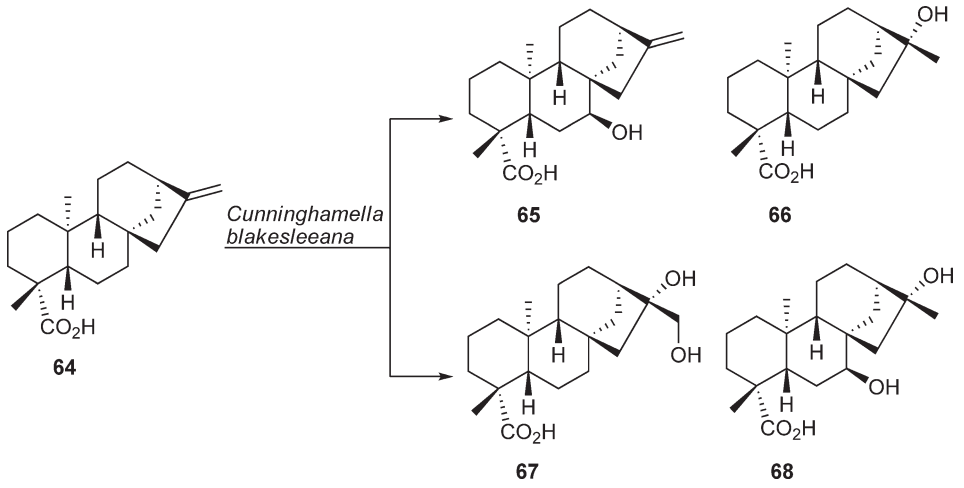
Furthermore, the biotransformation of 7α-hydroxy-*ent*-kaur-16-ene (epicandiol A) (**69**) was carried out by the fungus *G. fujikuroi* to afford 7α,16α,17-trihydroxy-*ent*-kaurane (**72**) and fujenoic acid (**73**), a B-ring opened anhydride derivative (**Scheme 12**). Incubation of candicandiol (**70**) and canditriol (**71**) with the same fungus gave 19-hydroxylated (**74**) and 11β-dihydroxy derivative (**75**), respectively[20] (**Scheme 12**).



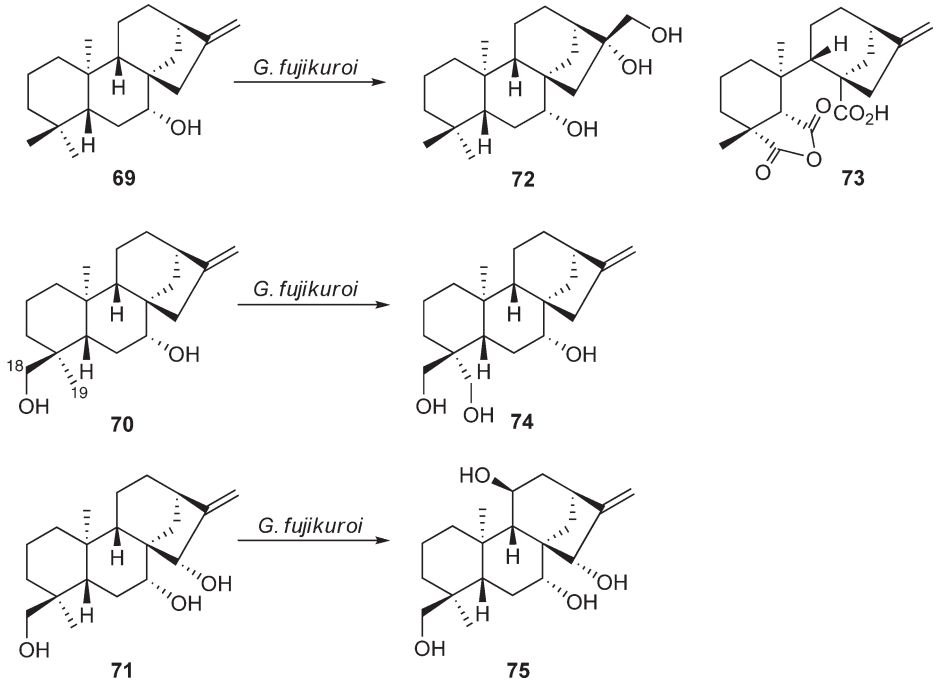
Scheme 9 Biotransformation of stemodinone (**45**) by *B. bassiana*, *C. echinulata* var. *elegans*, and *P. chrysosporium.*



Scheme 10 Biotransformation of stemarin (**46**), stemodinone-2α-(*N*,*N*-dimethylcarbamate) (**47**), and stemarin 19-(*N*,*N*-dimethylcarbamate) (**48**) by *B. bassiana* and *C. echinulata* var. *elegans.*



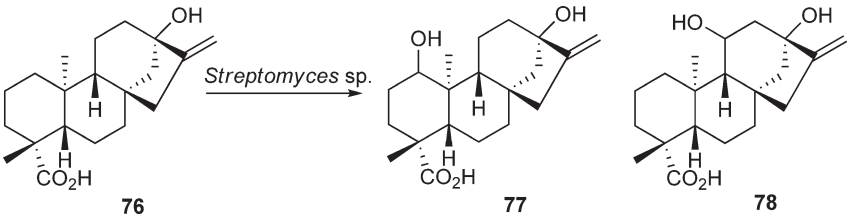
Scheme 11 Biotransformation of (−)-kaur-16-en-19-oic acid (**64**) by *C. blakesleeana*.



Scheme 12 Biotransformation of 7α-hydroxy-*ent*-kaur-16-ene (epicandiol A) (**69**), candicandiol (**70**), and canditriol (**71**) by *Gibberella fujikuroi*.

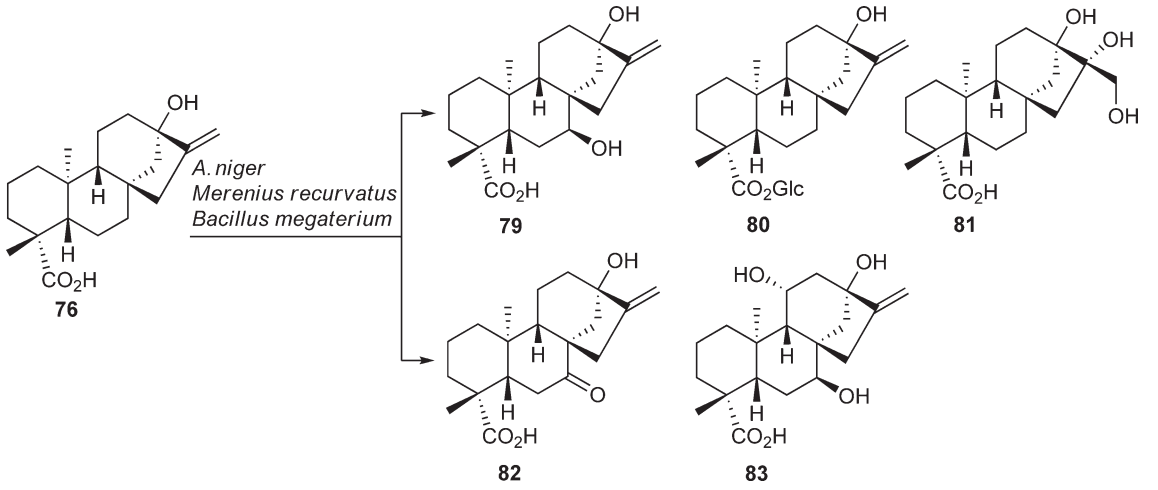
Steviol (**76**) prepared from the natural sweetener stevioside possesses interesting pharmacological activities, such as antiglycemic activity and inhibition of oxygen uptake. **Steviol** (**76**) was biotransformed by *Streptomyces* species to 1-hydroxy- (**77**) and 11-hydroxysteviol (**78**)[21] (**Scheme 13**).

Yang *et al.*[22] reported that three microbes *Bacillus megaterium*, *Mucor recurvatus*, and *A. niger* converted steviol (**76**) into 7β- (**79**), glucosylated steviol (**80**), and 16,17-dihydroxy- (**81**), 7-oxo- (**82**), and 7β,11α-dihydroxy derivatives (**83**) (**Scheme 14**). A triol (**81**) might be formed by the hydrolysis of epoxide derived from the substrate. This assumption was confirmed by the formation of the same product from 16α,17-epoxide (**84**) using *M. recurvatus*. This fungus also biohydrogenated **84** into two nor-kaurane derivatives (**87**, **89**) and four oxygenated kaurenes



76 77 78

Scheme 13 Biotransformation of steviol (**76**) by *Streptomyces* species.



76 79 80 81 82 83

Scheme 14 Biotransformation of steviol (**76**) by *B. megaterium*, *M. recurvatus*, and *Aspergillus niger*.

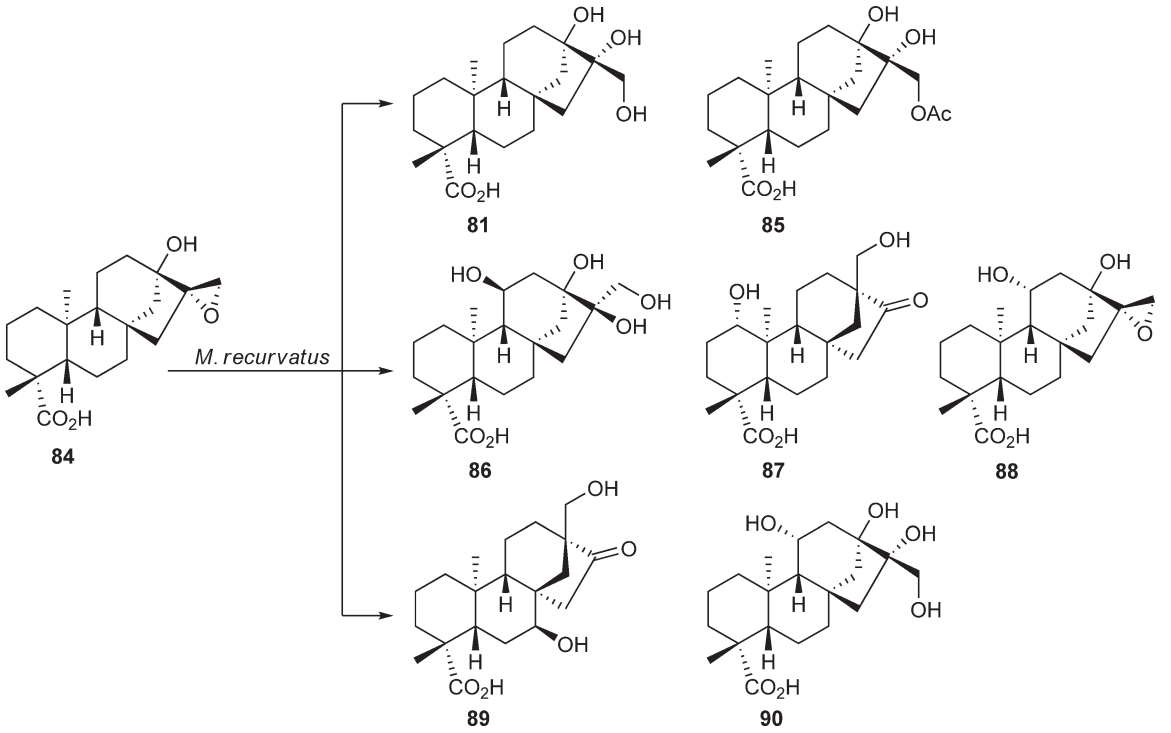
(**85**, **86**, **88**, **90**), which were formed by hydrolysis of the epoxide (**84**) as mentioned above and 11α-hydroxy derivative of the substrate (**84**) (**Scheme 15**). Compound **81** had more potent antihyperglycemic activity than the substrate (**76**). Compounds **80**, **81**, and **83** showed glucocorticoid agonist effects; however, the activity was weaker than the reference compounds methylprednisolone and dexamethasone.[22]

The strong pungent diterpene dialdehyde, sacculatal (**91**), from the liverwort, *Trichocoleopsis sacculata* and *Pellia endiviifolia*, possessing cytotoxicity against cancer cell lines, antimicrobial and anti-HIV1 activity did not give any metabolites with *A. niger*. *A. niger* biotransformed sacculatane diol (**92**) obtained from **91** by reaction with $LiAlH_4$ to give a small amount of the metabolites **93** (6.1%), **94** (6.7%), **95** (4.5%), **96** (1.6%), and 3β-hydroxy sacculatane diol (**97**)[23] (**Scheme 16**). *A. niger* converted **92** to secondary alcohols stereo- and regiospecifically. This phenomenon is generally observed in 4,4-dimethyl-type sesquiterpenoids as shown in the previous chapter. Possible biogenetic pathway of sacculatane diol (**92**) by *A. niger* is shown in **Scheme 17**.

Tobacco cembranoids show insecticidal activity, prostaglandin biosynthesis, plant growth, and fungal spore germination inhibitory activity, and antitumor-promoting activity, block the expression of the behavioral sensitization to nicotine, and inhibit neuronal acetylcholine receptors in rats. In order to explore antiproliferative activity of various derivatives of 2,7,11-cembratriene-4,6-diol (**98**), the semisynthesis and biotransformation of **98** were carried out.[24]

Mucor ramannianus ATCC 9628 and *C. elegans* ATCC 7929 biotransformed compound **98** to 10*S*,11*S*-epoxy analogue (**99**) as the major metabolite. 6-*O*-Acetyl analogue (**100**) was converted by the marine symbiotic *B. megaterium* MO31 to 2,7,11-cembratriene-4,6,10-triol (**101**) (**Scheme 18**). However, compound **101** showed no effect on mammary tumor cell growth.[24]

The microbiotransformation of dehydroabieta-18-ol (**102**) and 1α,18-dihydroxydehydroabietane (**110**) was carried out by the fungus *M. plumbeus*, which shows a broad substrate specificity.[25] From the former compound,



Scheme 15 Biotransformation of steviol-16α,17-epoxide (**84**) by *Merenius recurvatus.*

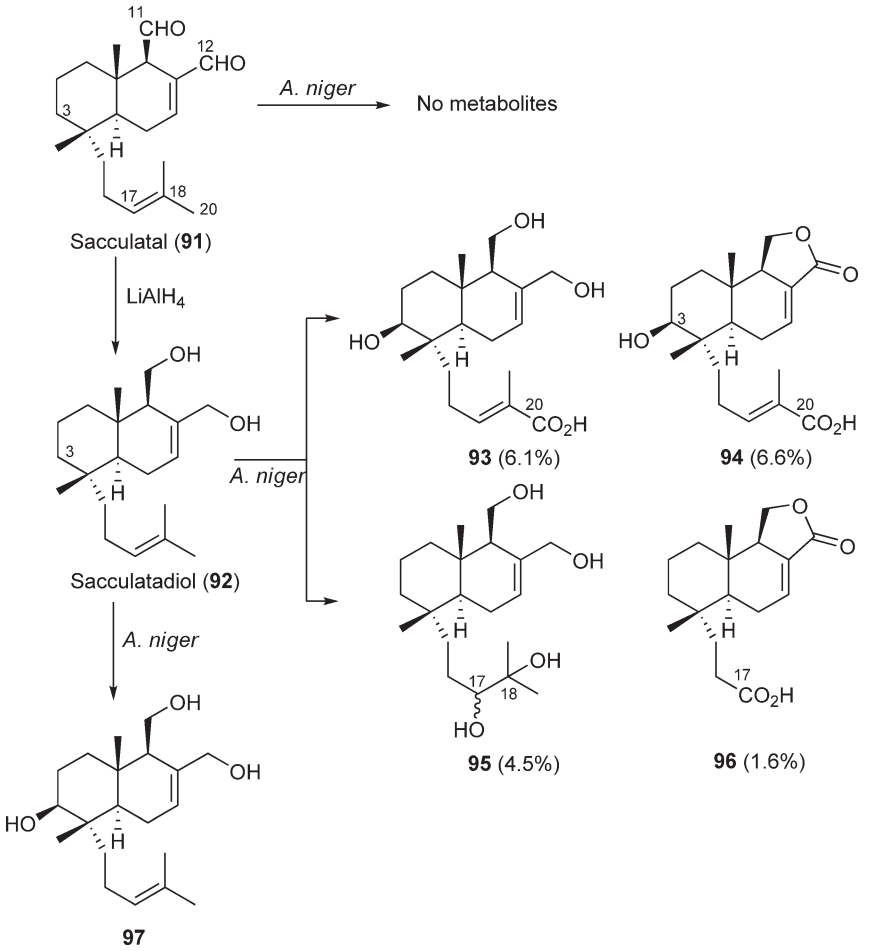
7α,15-dihydroxy (**103**), 7α,15-methoxy (**104**), 2-oxo-7β-hydroxy (**105**), 2-oxo-15-hydroxy derivatives (**106**), 2-oxo-15,16-dihydroxy (**107**), and 2α-hydroxy-15,16-methylene derivatives (**108**), along with a tentatively assigned 2α-hydroxy derivative (**109**), were obtained (**Scheme 19**).

Compound **107** might be formed through a dehydrated product (**108**) from **103**, followed by epoxidation and hydrolysis. 1α,18-Dihydroxydehydroabietane (**110**) was also treated in the same manner as described above to afford 2α-hydroxy (**111**), 7α-hydroxy (**112**), and 7β-hydroxy derivatives (**113**) (**Scheme 20**). It is interesting to note that the presence of 1α-hydroxy group on A-ring of **110** inhibits 15-hydroxylation.

A number of mono- and sesquiterpenoids were administered to rabbits and the structures of their metabolites were investigated as discussed in the previous chapters. However, the study of the metabolism of di- and triterpenoids in mammals is rare. Asakawa *et al.*[26] reported the metabolism of (−)-abietic acid (**102a**) and dehydroabietic acid (**102d**) from Chinese pine rosin in rabbits. Each substrate was separately administered to rabbits as their sodium salts (each 20 g). After 1 day, urine samples were collected and adjusted to pH 6.0 with phosphate butter (disodium hydrogen phosphate-citric acid) and incubated with β-glucuronidase/arylsulfatase at 37 °C for 48 h and ether extracts were separated into neutral and acidic fractions as usual. The acidic fraction was further methylated with dimethyl sulfate in acetone or methyl iodide in the presence of potassium carbonate in acetone.

After column chromatography, (−)-dimethyl abieta-7,13-dien-16,18-dioate (**102c**) was isolated from the metabolite of **102a**, whereas five metabolites, (+)-methyl 15-hydroxyabieta-8,11,13-trien-18-oate (**102f**), (+)-methyl abieta-8,11,13,15-tetraen-18-oate (**102h**), (+)-methyl 16-hydroxyabieta-8,11,13-trien-18-oate (**102j**), (+)-methyl 16-acetoxyabieta-8,11,13-trien-18-oate (**102l**), and methyl 7-oxo-abieta-8,11,13-trien-18-oate (**102n**), were identified from the metabolites of **102d** (**Scheme 21**). Thus one free acid (**102b**) and five free acids (**102e, 102g, 102i, 102k, 102n**) were suggested to be the true metabolites from each substrate.

In order to obtain a large amount of taxoids that show an exciting therapeutic profile, many hemisyntheses as well as microbial transformation and cell cultures have been carried out since they are of limited availability.

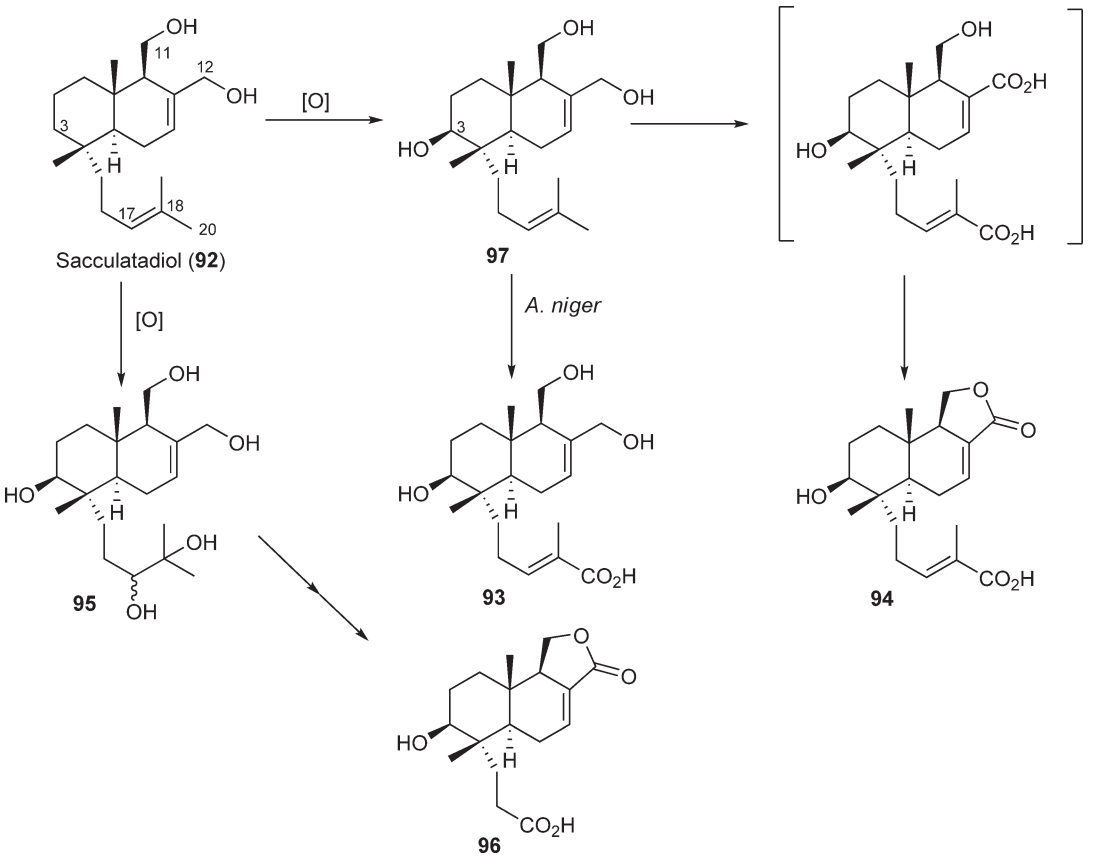


Scheme 16 Biotransformation of sacculatadiol (**92**) by *Aspergillus niger*.

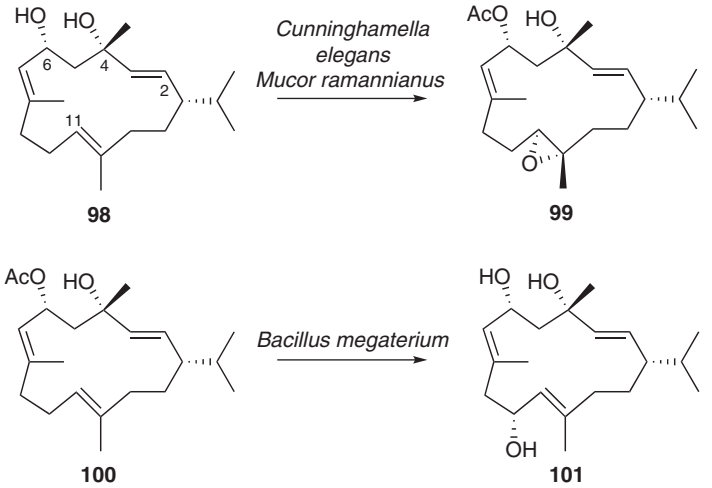
A strain of *Nocardioides albus* isolated from soil produced an extracellular enzyme that specifically removed the C-13 side chain from paclitaxel (=taxol) (**114**), an anticancer drug, and cephalomannine (**115**) and other related compounds. A strain of *Nocardioides luteus* from soil produced an intracellular 10-deacetylase that removed the 10-acetate from baccatin (**116**) and paclitaxel (**Scheme 22**) (**114**).[27]

The enhancement of 10-deacetylbaccatin (10-DAB) III (**117**) by enzymatic treatment of crude extracts from yew tree is very valuable for increasing the amount and purification of this key precursor for the synthesis of paclitaxel and its related compounds since an estimated 20 000 pounds of yew bark from 2000 to 3000 trees is needed to obtain 1 kg of paclitaxel.[27]

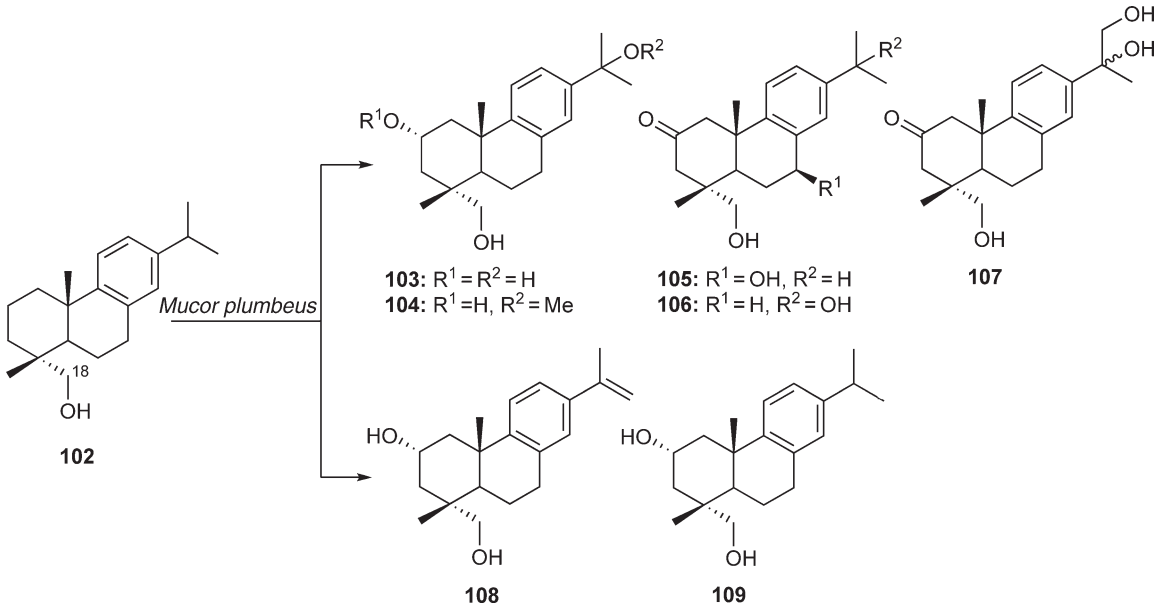
Selective deacetylation and hydroxylation of 2α,5α,10β,14β-tetraacetoxy-4(20),11-taxadine (**118**) were carried out by microbial biotransformation using *C. echinulata* AS 3.[28] The results indicated that the substrate (**118**) was stereospecifically hydroxylated at C-6 position to give **119** and **120**, together with **121** and 4(20)-epoxide (**122**), which were obtained by deacetylation at C-10 (**Scheme 23**). The main product (**119**), which showed poor antitumor activity, was obtained in relatively good amount (33%). The hydroxylation of taxol might be catalyzed by cytochrome P-450 in *C. echinulata* since in human liver also taxol is stereospecifically catalyzed by this enzyme. It is noteworthy that the production of epoxide (**122**) was found in this experiment since the C-4(20) oxirane ring was considered to be derived biogenetically from exomethylene at C-4 by epoxidation. It is suggested that the epoxidase in this fungus could epoxidize the exomethylene group in taxane derivatives and provides some proof of the biosynthetic pathway of taxoids possessing epoxide at C-4(20) position.



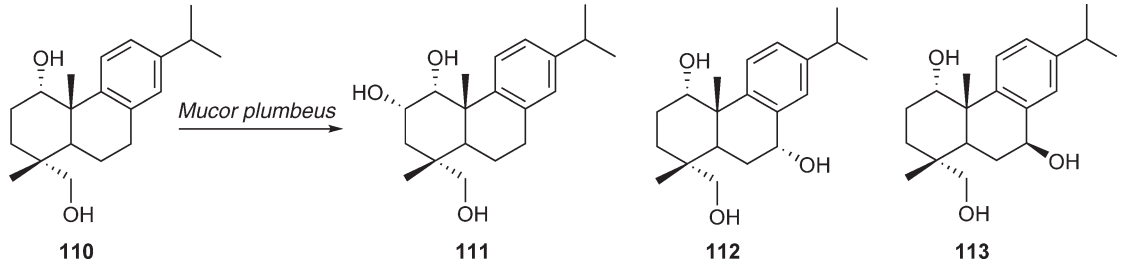
Scheme 17 Biogenetic pathway of sacculatane diol (**92**) by *A. niger*.



Scheme 18 Biotransformation of 2,7,11-cembratriene-4,6-diol (**98**) and 6-*O*-acetyl analogue (**100**) by *Mucor ramannianus*, *Cunninghamella elegan*s, and *Bacillus megaterium.*



Scheme 19 Biotransformation of dehydroabieta-18-ol (**102**) by *Mucor plumbeus*.

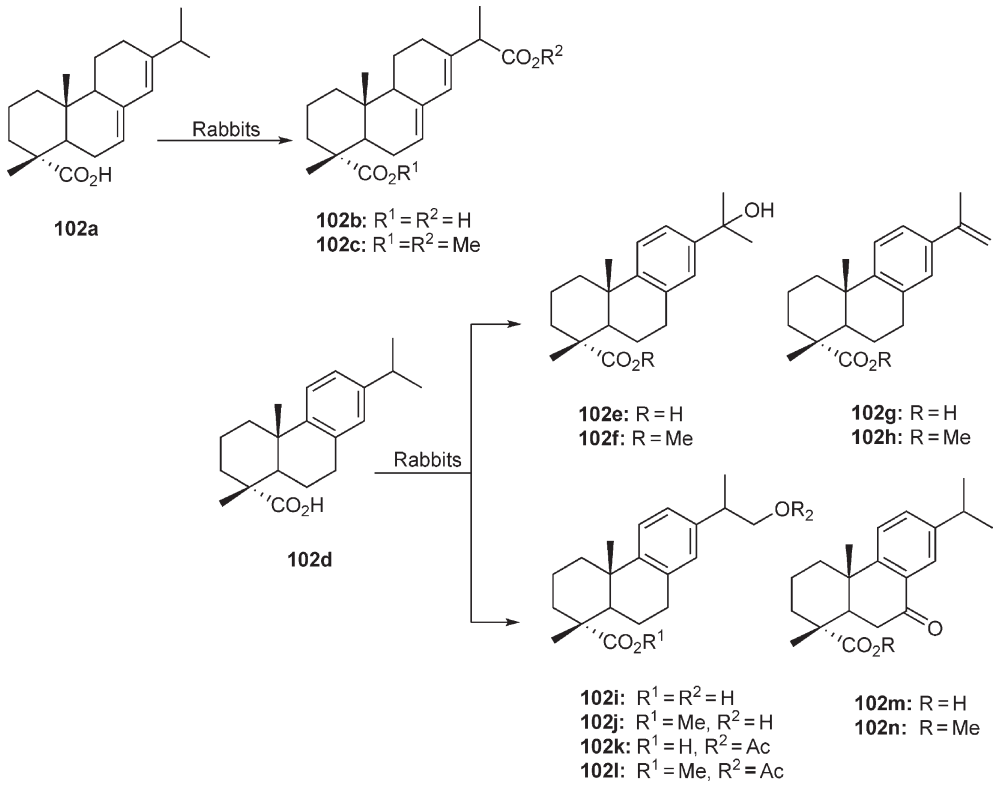


Scheme 20 Biotransformation of 1α,18-dihydroxydehydroabietane (**110**) by *Mucor plumbeus*.

About 80 strains of fungi from the inner bark of *Taxus yunnanensis* were screened by Zhang *et al.*[29] for their ability to biotransform 10-deacetyl-7-epitaxol (**123**) and 1β-hydroxybaccatin I (**126**) and it was found that *Microsphaeropsis onychiuri*, *Mucor* species, and *Alternaria alternata* were able to selectively hydrolyze and epimerize **123** and **124**. *M. onychiuri* converted **123** to 10-DAB III (**116**), 10-DAB V (**124**), and 10-deacetyltaxol (**125**). *Mucor* species biodegraded **123** to **124** and **125** (**Scheme 24**). The substrate **126** was converted by *A. alternata* to 5-deacetyl-1β-hydroxybaccatin I (**127**), 13-deacetyl-1β-hydroxybaccatin I (**128**), and 5,13-dideacetyl-1β-hydroxybaccatin I (**Scheme 25**) (**129**).

Biotransformation of sinenxan (=taxuyunnanine C), 2α,5α,10β,14β-tetraacetoxy-4(10),11-taxadien (**118**), isolated from callus tissue cultures of *Taxus* species was carried out by *Ginkgo biloba* cell suspension cultures.[30] 9α-Hydroxy (**130**) and C-10 deacetyl derivatives (**131**) were obtained together with six minor products (**119, 132–136**) among which compounds **130** and **131** are the major metabolites (**Scheme 26**). These results show that regio- and stereoselective hydroxylation at C-6 and C-9, and selective deacetylation at C-10 and C-14 positions were displayed by employing the same *G. biloba* cell line, exhibiting the reaction diversity of sinenxan A catalyzed by *Ginkgo* cells.

Incubation of taxuyunnanine C (**118**) and its analogues (**140, 141**) with the fungus *Absidia coerulea* IFO 4011 produced metabolites that were regio- and stereoselectively hydroxylated at the 7β position. From compound **118**, five derivatives (**121, 130, 137–139**) were obtained from *A. coerulea* (**Scheme 27**). In this case, compound **121** was obtained as the major product (16%); on the contrary, the yield of 9α-hydroxylated product (**130**) was very poor (1%). When compound **140** was incubated with *A. coerulea* for 7 days, six metabolites (**142–147**) were formed, of which metabolite **142** was predominant (**Scheme 28**). Compound **141** was administered to 2-day-old cell cultures of the same fungus to give seven metabolites (**148–154**) of which **150** was the major product (**Scheme 29**).



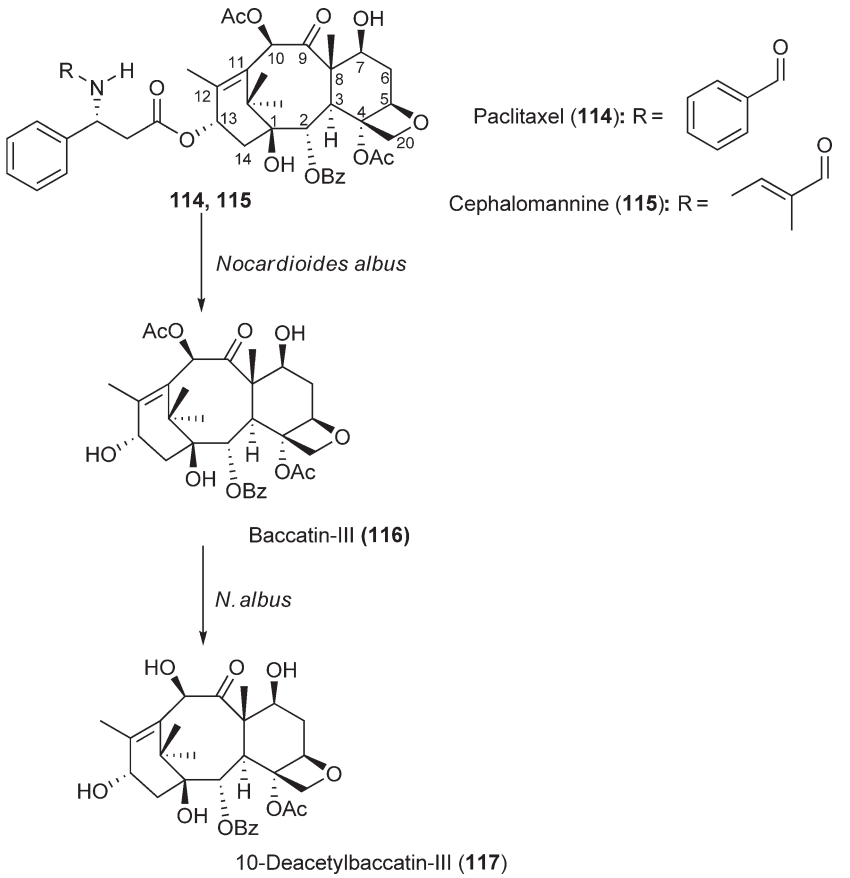
Scheme 21 Biotransformation of (−)-abietic acid (**102a**) by rabbits.

Ginkgo callus also bioconverted **118** to **130** (70%), which was further incubated with *A. coerulea* to afford compound **131** (2%), whereas **118** was converted to **137** (5%) by *A. coerulea*, followed by *G. biloba* to furnish compound **155** in very poor yield. The metabolites **130** and **137** were acetylated giving rise to acetate (**156**, **159**). Compound **156** was further converted into **157** (2%) and **158** (2%), and compound **159** into **160**. The desired hydroxylation took place in both cases, and their corresponding 7β- and 9α-hydroxylated products (**157**, **160**) were obtained (**Scheme 30**). Such a simple modification of C-14 oxygenated taxanes with different substitution groups supplies very useful intermediates for the semisynthesis of paclitaxel and other pharmacologically active taxanes from easily obtained natural products such as taxuyunnanine C.[30,31]

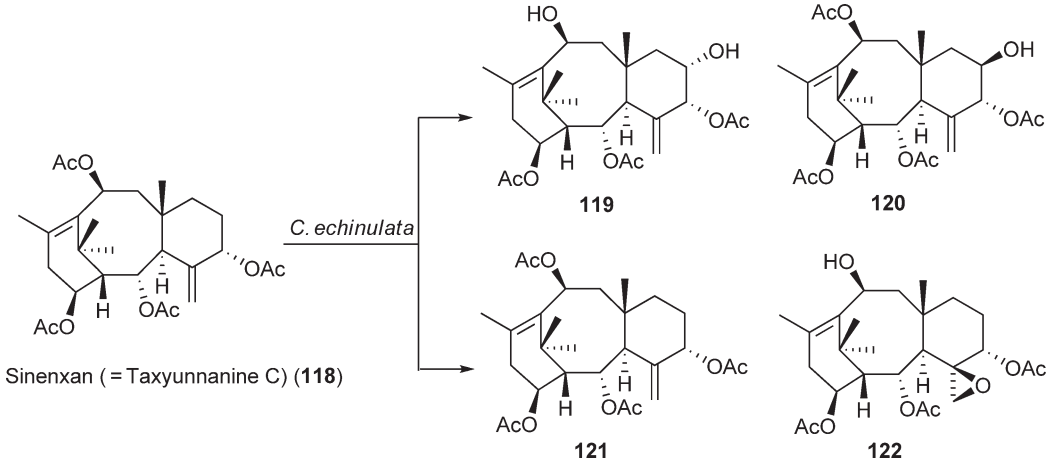
Taxuyunnanine C (**118**) was coincubated with cyclodextrin to increase the yield of the metabolites; however, the desired product (**130**) was not increased. In this case, three more metabolites (**161**, **162**, and **163**) were obtained in a very small amount, in addition to compound **130**[30] (**Scheme 31**).

10-DAB III (**117**) was incubated with *Curvularia lunata* and *Trametes hirsuta* to give compounds **124**, **164**, and **165**. Metabolites **124** and **164** were also produced by cultures of *B. bassiana*, *Pseudomonas fluorescens*, *Epicoccum* sp., *A. alternata*, *C. echinulata* var. *echinulata*, and *Ophiobolus herpotrichus*. *T. hirsuta* belonging to the Basidiomycetes transforms 10-DAB into metabolite **165** in good yield (44%) (**Scheme 32**). The substrate 13-DeBAC (**166**) was converted by *Epicoccum* sp., *Streptomyces griseus*, *Streptomyces catenulae*, *A. alternata*, *B. bassiana*, *M. plumbeus*, *O. herpotrichus*, and *D. celatrina* to give C-7 isomerized product (**167**) only, whereas *A. alternata* and *G. fujikuroi* gave C-10 deacetyl product (**165**) (**Scheme 32**). Twenty-nine microorganisms were tested for the biotransformation of 10-DAB; however, the new introduction of the functional group into the core of the taxane molecules is difficult.[32]

Cephalomannine (**115**) was biotransformed by *Luteibacter* species isolated from the soil around *Taxus cuspidata* to give eight metabolites, baccatin III (**116**), baccatin V (**168**), 10-DAB III (**117**), 10-deacetyl-10-oxobaccatin V (**169**), 7-epicephalomannine (**170**), which was the major product (~13%), 10-acetylcephalomannine (**171**), 10-deacetyl-7-epicephalomannine (**172**), and 3′-*N*-debenzoyl-3′-*N*-(2-methylbutyryl)-7-epitaxol (**173**) among which **173** was a new compound (**Scheme 33**). 7β-D-Xylosyl-10-deacetyltaxol (**174**) was treated in the same manner as mentioned above to give a C-13 side-chain eliminated metabolite (**175**) (**Scheme 34**). Paclitaxel (**114**), a

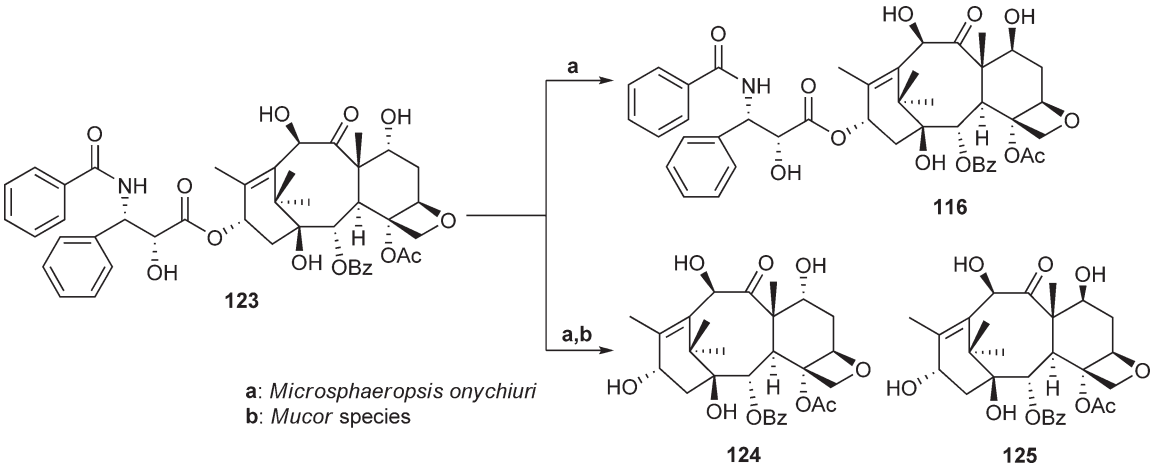


Scheme 22 Biotransformation of paclitaxel (=taxol) (**114**), cephalomannine (**115**), and baccatin III (**116**) by *N. albus* and *N. luteus*.



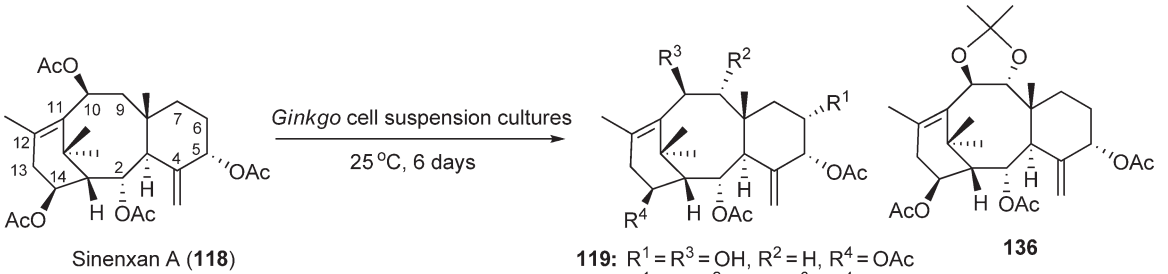
Scheme 23 Biotransformation of 2α,5α,10β,14β-tetraacetoxy-4(20),11-taxadine (**118**) by *C. echinulata*.

clinically important chemotherapeutic drug, and its related compounds were tested for their inhibitory activities against five human cancer cell lines such as HCT-8 and Bel-7402. The metabolites tested demonstrated less potent activities than paclitaxel.[33]



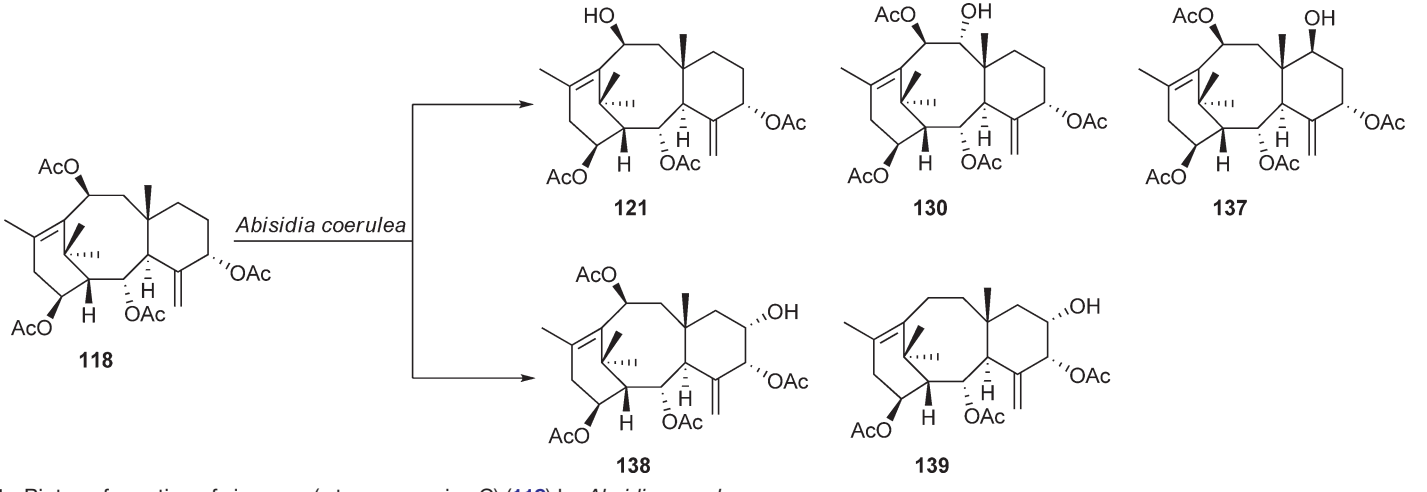
Scheme 24 Biotransformation of 10-deacetyl-7-epitaxol (**123**) by *M. onychiuri* and *Mucor* species.

Scheme 25 Biotransformation of 1β-hydroxybaccatin I (**126**) by *A. alternata.*

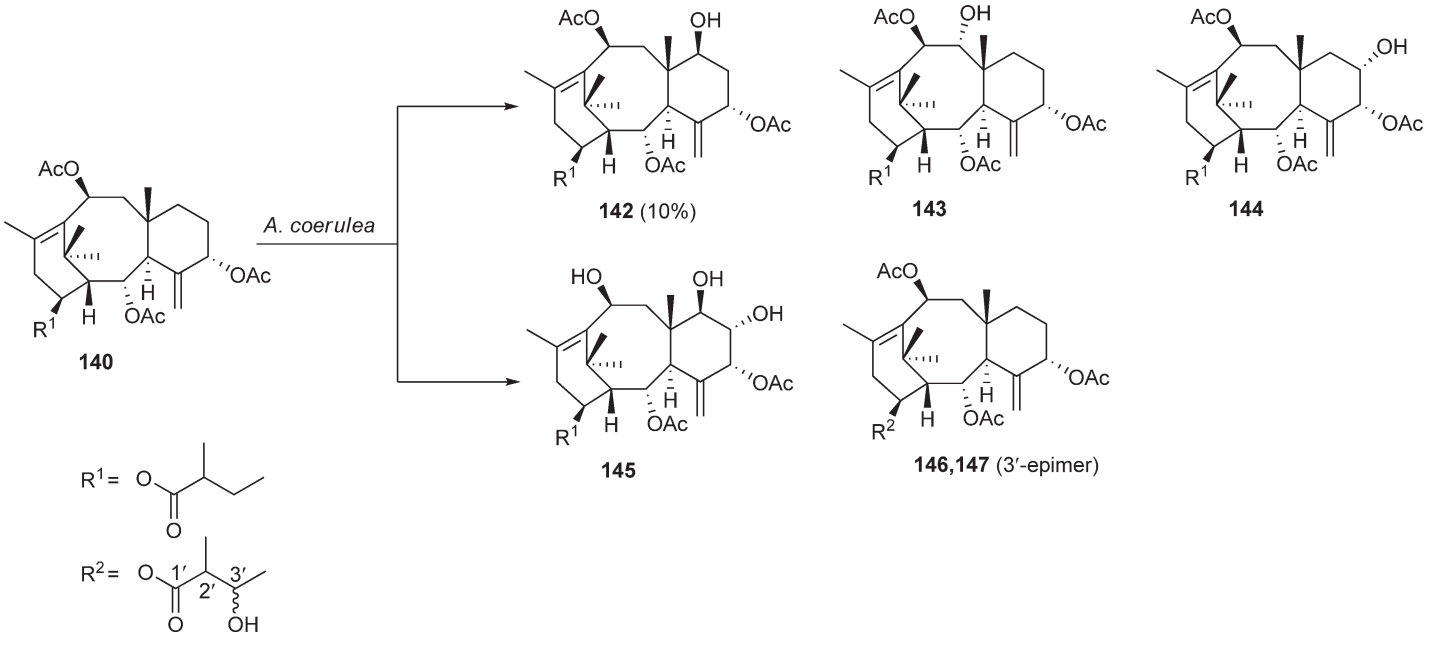


Scheme 26 Biotransformation of sinenxan (=taxuyunnanine C), 2α,5α,10β,14β-tetraacetoxy-4(10),11-taxadien (**118**), by cultured cells of *Ginkgo biloba.*

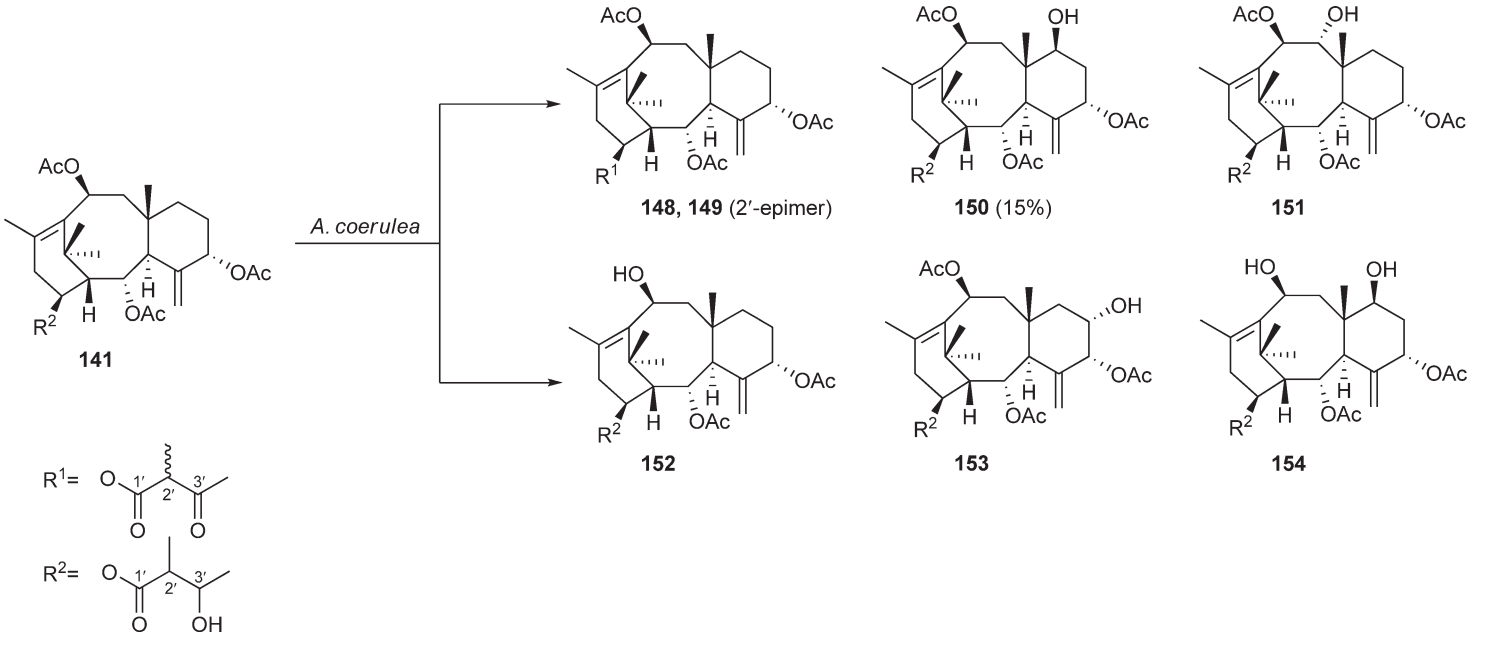
Protopanaxatriol is one of the well-known aglycones of ginsenosides and it has been shown that it reduces proliferation of human leukemia THP cells and other cancer cell lines. The incubation of 20(*S*)-protopanaxatriol (**176**) with the fungus *Mucor spinosus* gave 10 metabolites (**177–185**) (**Scheme 35**) and these were tested against three human cancer cell lines, BGC-823, HeLa, and HL-60 cells. Among the metabolites, compounds **178, 179,** and **184** showed more potent inhibitory activity against HL-60 cell line than the substrate. Thus 12-carboxylation or hydroxylation at C-28 or C-29 increases the cytotoxic activities.[34]



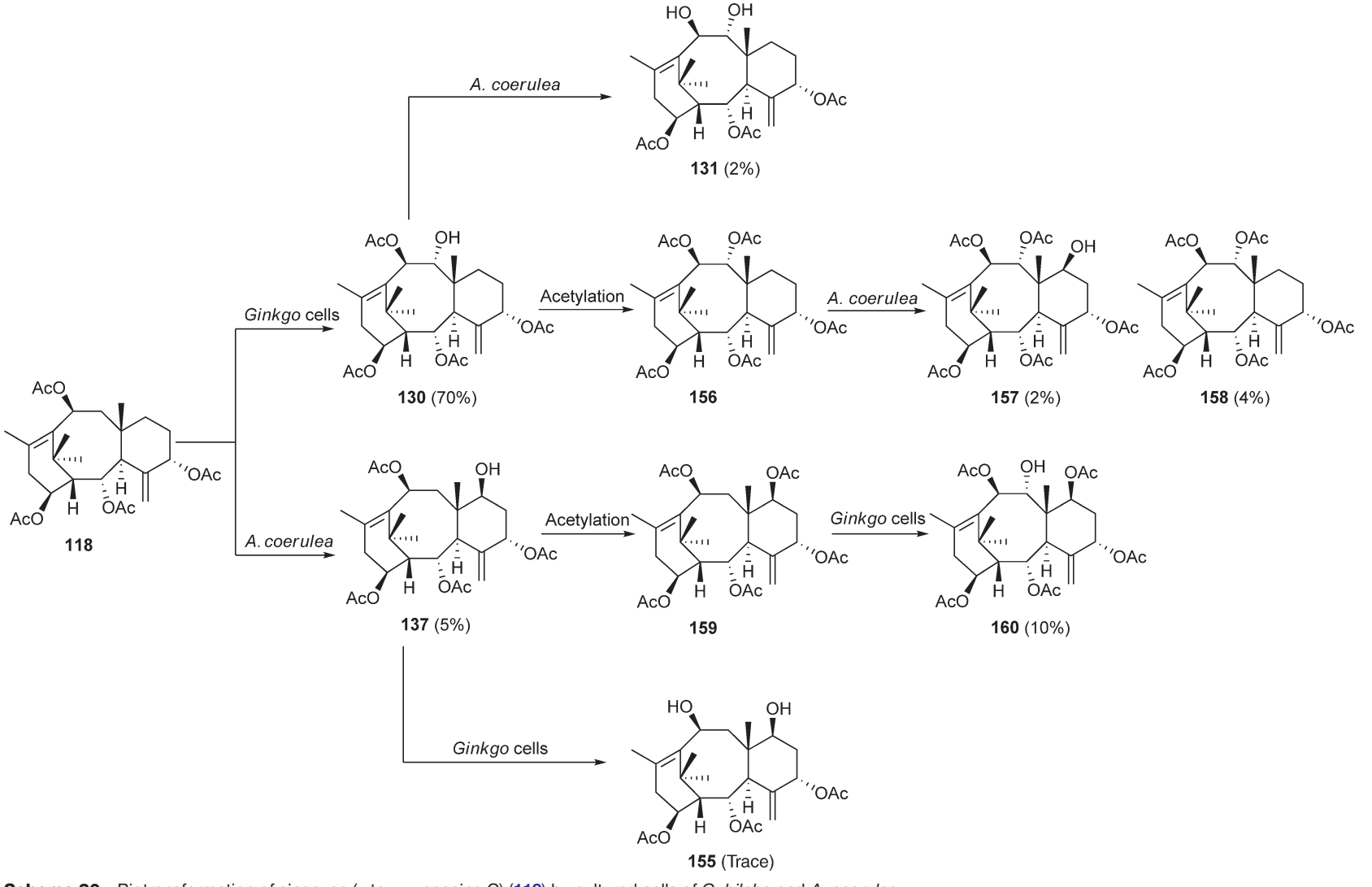
Scheme 27 Biotransformation of sinenxan (=taxuyunnanine C) (**118**) by *Absidia coerulea*.



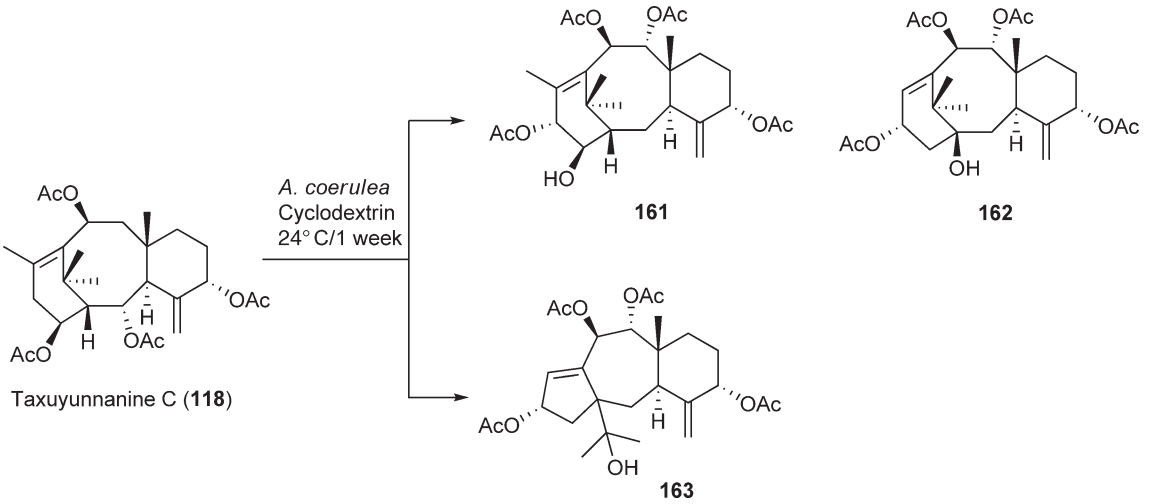
Scheme 28 Biotransformation of taxane compound (**140**) by *A. coerulea.*



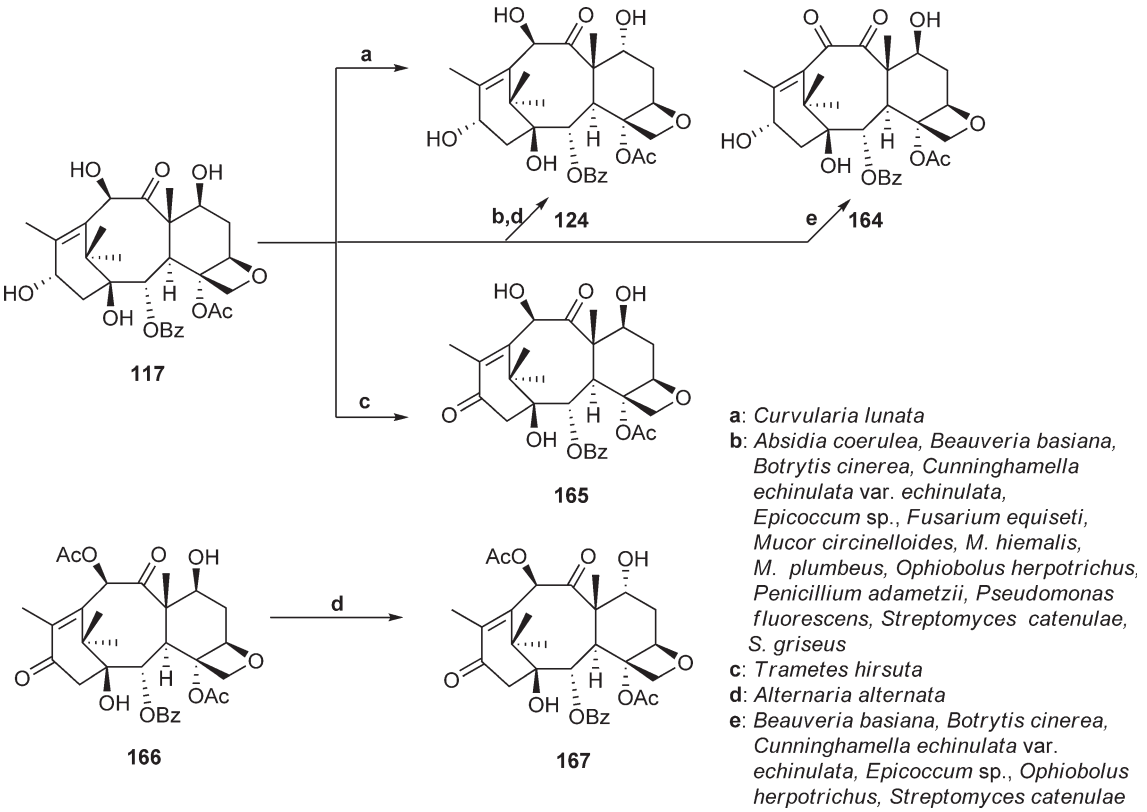
Scheme 29 Biotransformation of taxane compound (**141**) by *A. coerulea.*



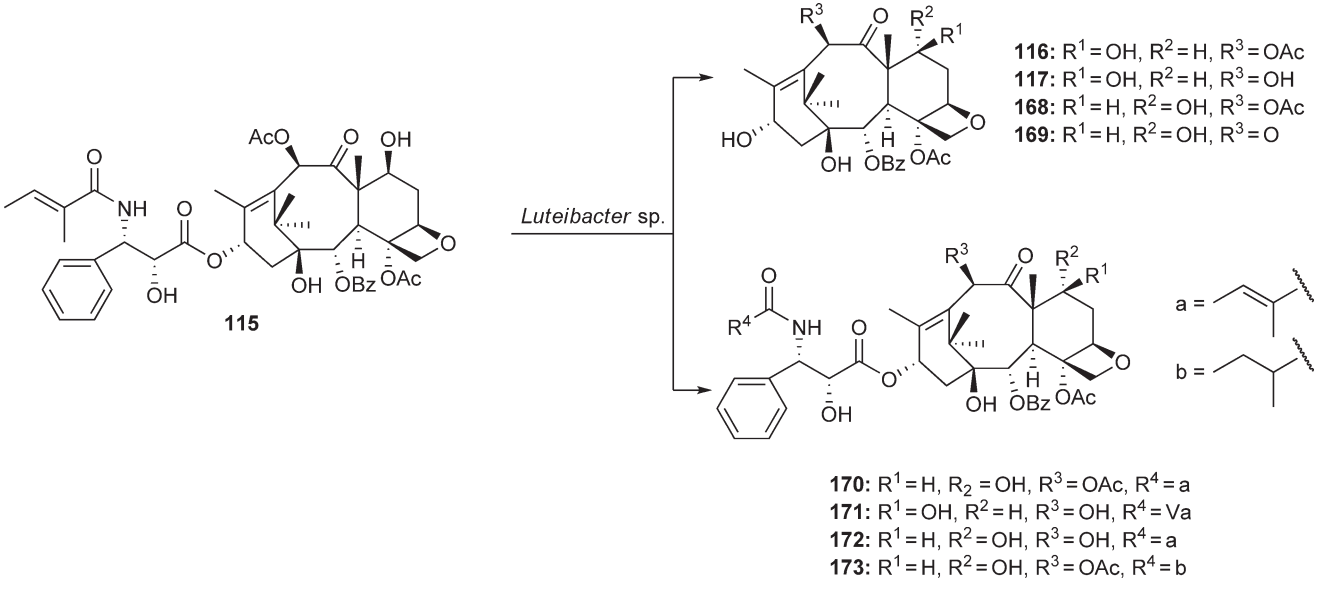
Scheme 30 Biotransformation of sinenxan (=taxuyunnanine C) (**118**) by cultured cells of *G. biloba* and *A. coerulea.*



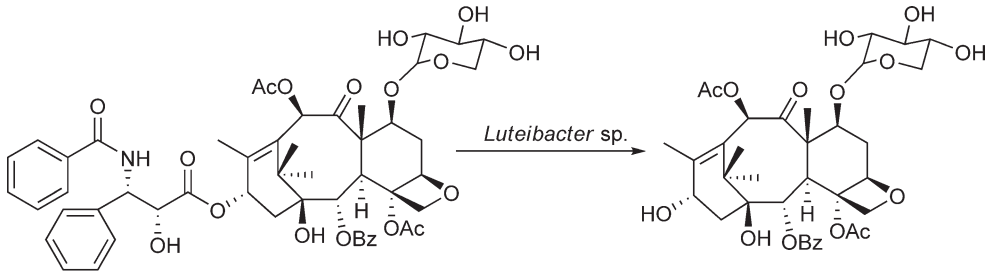
Scheme 31 Biotransformation of sinenxan (=taxuyunnanine C) (**118**) by *Absidia* with cyclodextrin.



Scheme 32 Biotransformation of 10-DAB III (**117**) and 13-DeBAC (**166**) by *C. lunata*, *Trametes hirsute*, *B. bassiana*, *P. fluorescens*, *Epicoccum* sp., *A. alternata*, *C. echinulata* var. *echinulata*, *O. herpotrichus*, and *T. hirsuta.*

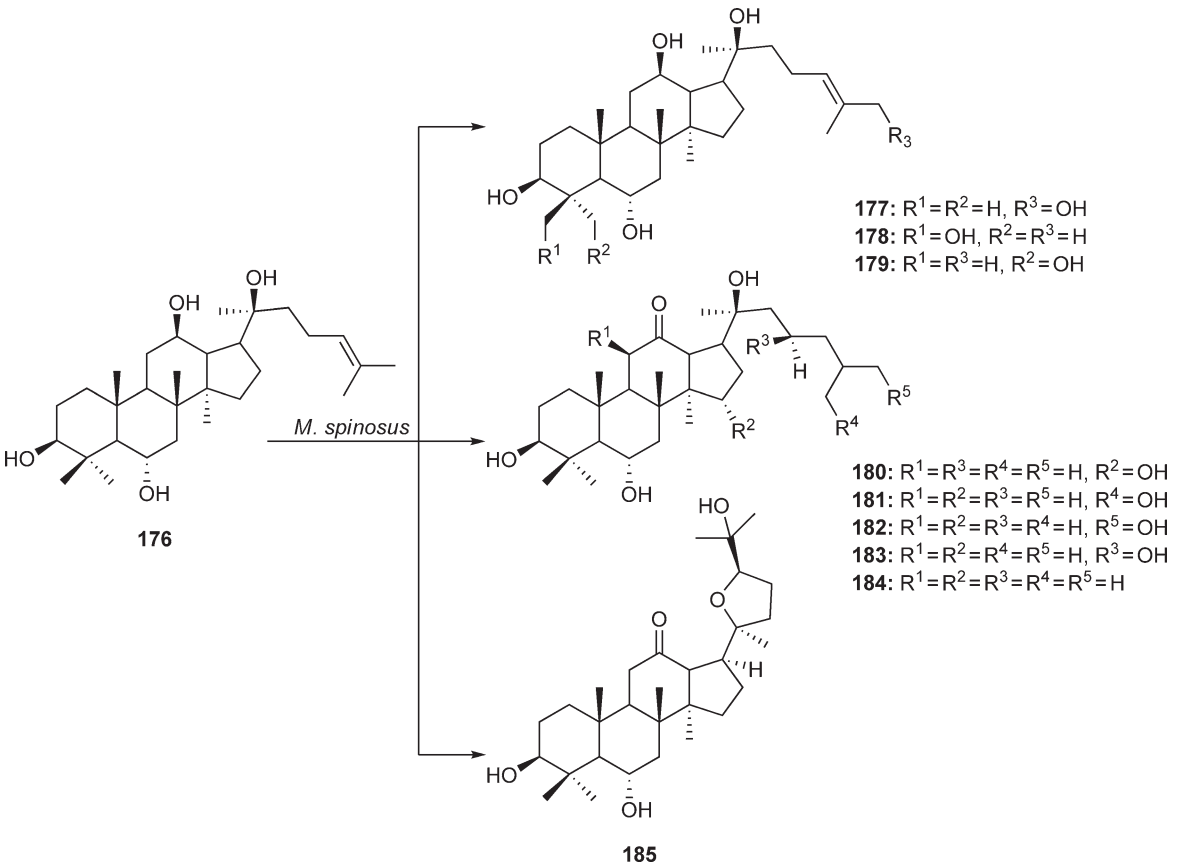


Scheme 33 Biotransformation of cephalomannine (**115**) by *Luteibacter* species.



174 **175**

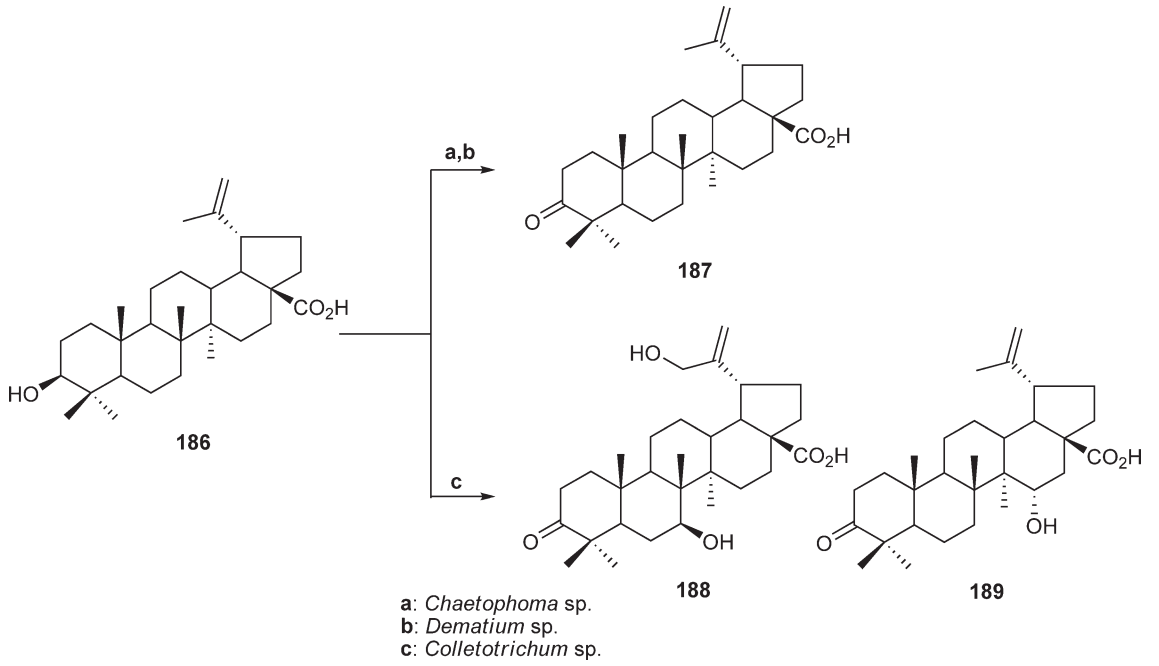
Scheme 34 Biotransformation of 7β-D-xylosyl-10-deacetyltaxol (**174**) by *Luteibacter* species.



176 **185**

Scheme 35 Biotransformation of 20(*S*)-protopanaxatriol (**176**) by *Mucor spinosus*.

Betulinic acid (**186**) possessing antimelanoma, antileukemia, antimalaria, and anti-HIV activities was fed to *Cunninghamella*[35] and *B. megaterium*.[36] The glycosylation was seen in the metabolites of **186** with the former fungus. The latter organism converted **186** into betulonic acid (**187**) with anti-inflammatory, antimelanoma, and antiviral activities and C-1, C-7, C-11, and C-15 hydroxylated products. *B. megaterium*, *C. elegans*, and *Mucor mucedo* resulted in the production of C-1, C-3, C-6, C-7, C-11, and C-15 oxidized compounds.[37] *Nocardia* species esterified **186** to afford methyl betulinate.[38] The substrate (**187**) was also incubated with *Chaetomium longirostre* to produce C-7 and C-15 hydroxylated compounds and A-ring cleaved derivative at C-3.[39]



Scheme 36 Biotransformation of betulinic acid (**186**) by *B. megaterium*, *Chaetophoma*, *Dematrium,* and *Colletotrichum* species.

Bastos *et al.*[40] have reported the biotransformation of both substrates (**186, 187**) by the fungi *Arthrobotrys*, *Chaetophoma*, *Dematrium*, and *Colletotrichum* isolated from *Platanus orientalis*. *Chaetophoma* and *Dematrium* species converted **186** into betulonic acid (**187**). When the same substrate was incubated with *Colletotrichum* species, 3-oxo-7β,30-dihydroxylup-20(29)-en-28-oic acid (**188**) and 3-oxo-15α-hydroxylup-20(29)-en-28-oic acid (**189**) were formed (**Scheme 36**). *Arthrobotrys* converted **187** into 3-oxo-7β,30-dihydrozylup-20(29)-en-28-oic acid (**188**), 3-oxo-7β-hydrolup-20(29)-en-28-oic acid (**190**), and 3-oxo-7β,15α-dihydrolup-20(29)-en-28-oic acid (**191**) (**Scheme 37**). *Colletotrichum* converted betulonic acid (**187**) into **188** and **189**, whereas *Chaetophoma* gave 3-oxo-25-hydroxylup-20(29)-en-28-oic acid (**192**) (**Scheme 37**). Such experiments might be valuable in the study of metabolism of other cyclic triterpenoids in mammals.

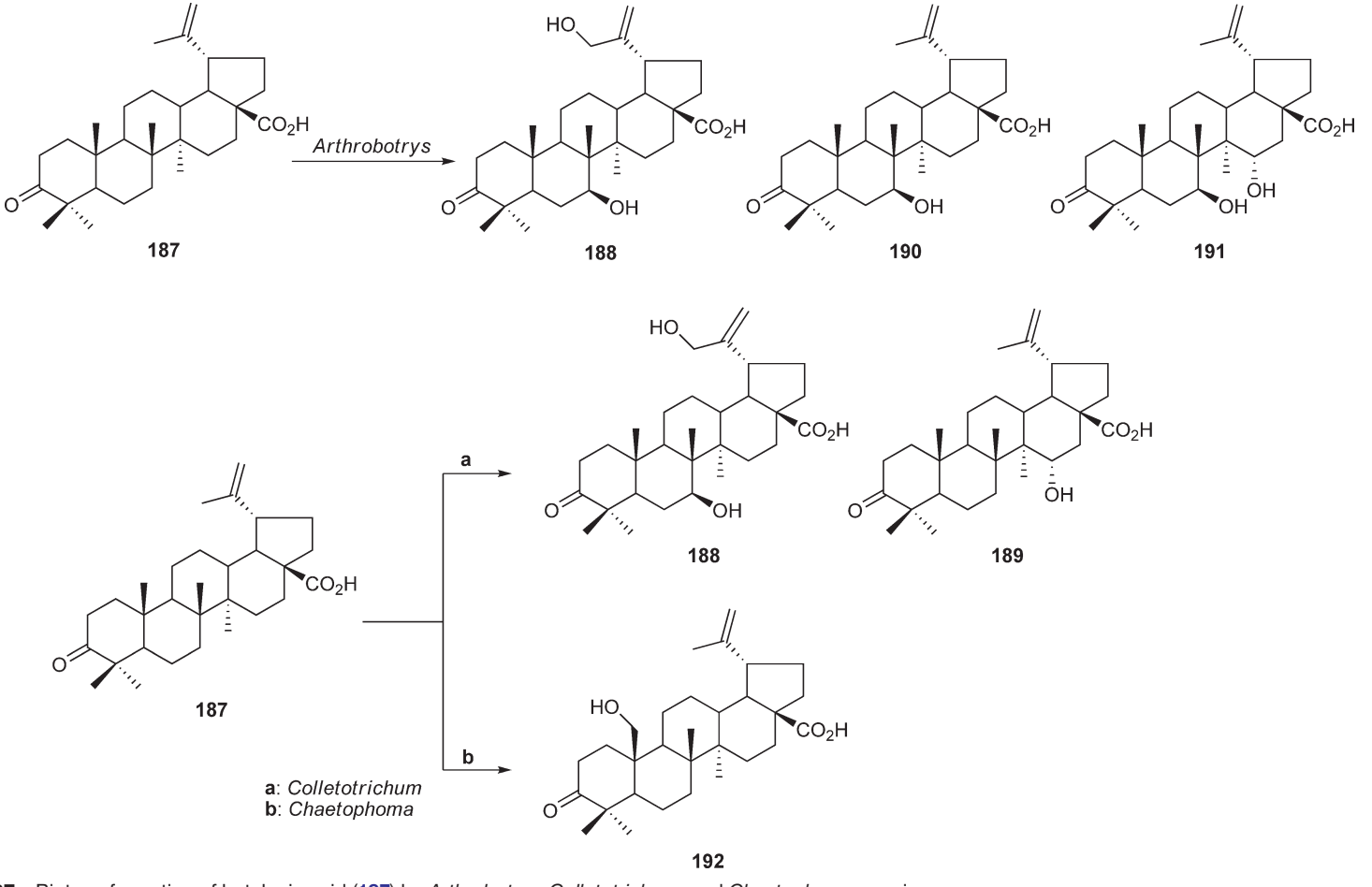
A green cell suspension culture of the liverwort, *Marchantia polymorpha*, was used as a bioreactor that converted testosterone (**193**) and epitestosterone (**195**) into 6β-hydroxytestosterone (**194**) and androst-4-ene-3,17-diene (**198**) in yields of 18 and 40%, respectively (**Scheme 38**). The results showed that the cultures of *M. polymorpha* regio- and stereoselectively hydrated C-6 position of **193** and oxidized a 17α-hydroxyl group to the corresponding ketone (**196**) without hydroxylation at any other carbon atom in **195**.[41]

Progesterone (**197**) was reduced by the cultured cells of the same liverwort as mentioned above to give α-pregnan-3,20-dione (**198**) but conversion ratio was low (15%). In this case, the reduction of a double bond was observed without any oxidation[42] (**Scheme 39**).

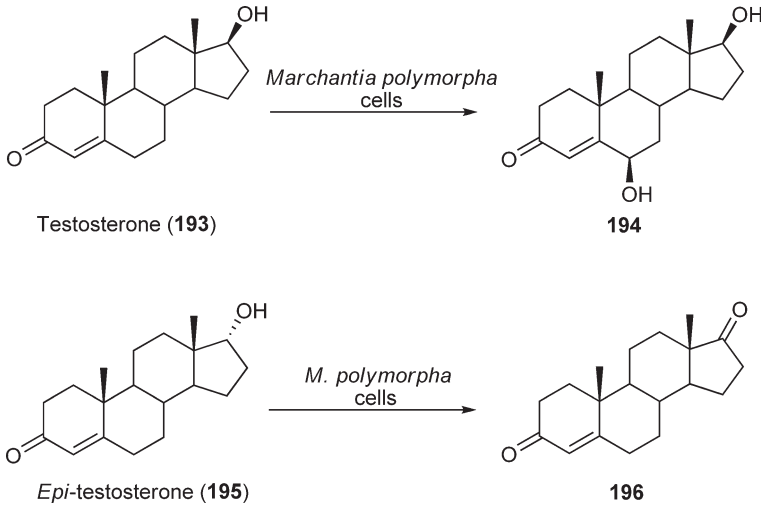
The biotransformation of many steroids has been carried out using microalgae, fungi, and bacteria. *Nostoc muscorum*, a freshwater blue-green alga that has various important enzymes such as peroxidase, hydrogenase, and alkaline phosphatase, was used for the biotransformation of hydrocortisone (**199**) as an exogenous substrate. Compound **99** was converted into 17-keto- (**200**), 17β- (**201**), and 20β-hydroxy derivatives (**202**)[43] (**Scheme 40**).

3.21.2 Biotransformation of Ionones, Damascones, and Adamantanes

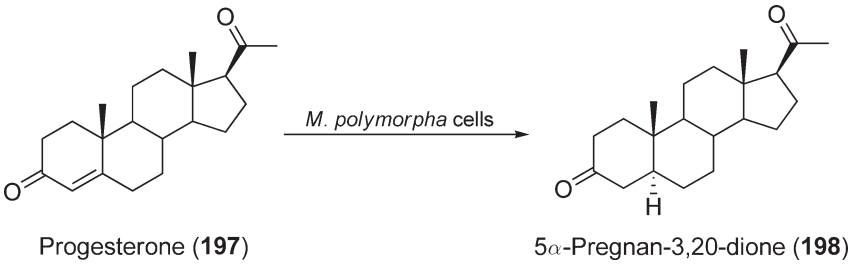
Racemic α-ionone (**203**) was converted to 4-hydroxy-α-ionone (**204**), which was further dehydrogenated to 4-oxo-α-ionone (**205**) by *Chlorella ellipsoidea* IAM C-27 and *Calluna vulgaris* IAM C-209. α-Ionone (**203**) was reduced preferentially to α-ionol (**206**) by *Chlorella sorokiniana* and *Chlorella salina*.[44] α-Ionol (**206**) was oxidized



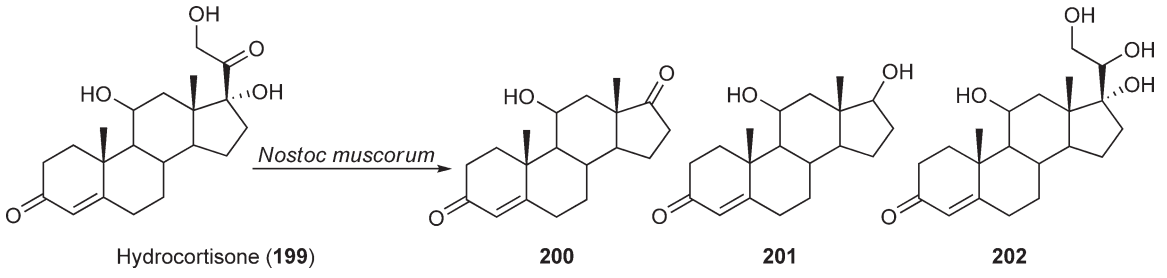
Scheme 37 Biotransformation of betulonic acid (**187**) by *Arthrobotrys*, *Colletotrichum*, and *Chaetophoma* species.



Scheme 38 Biotransformation of testosterone (**193**) and epitestosterone (**195**) by cultured cells of the liverwort, *M. polymorpha*.



Scheme 39 Biotransformation of progesterone (**197**) by cultured cells of the liverwort, *M. polymorpha*.



Scheme 40 Biotransformation of hydrocortisone (**199**) by *N. muscorum*.

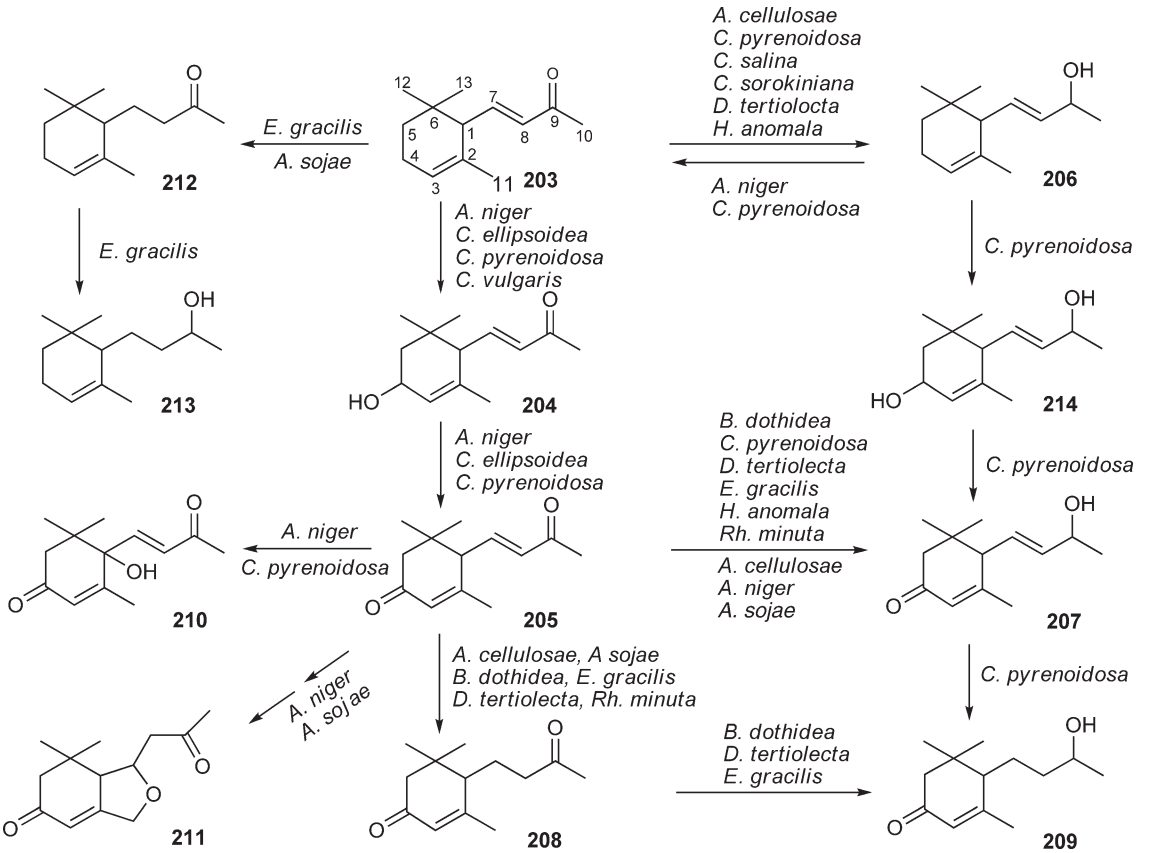
by *Chlorella pyrenoidosa* to afford 4-hydroxy-α-ionol (**214**). The same substrate was fed to the same microorganism and *A. niger* to furnish α-ionone (**203**).[45] 4-Oxo-α-ionone (**205**), which is one of the major products of biotransformation of α-ionone (**203**) by *A. niger*, was transformed reductively by *Hansenula anomala*, *Rhodotorula minuta*, *Dunaliella tertiolecta*, *Euglena gracilis*, *C. pyrenoidosa* C-28, and other eight kinds of *Chlorella* species, *Botryosphaeria dothidea*, *Aspergillus cellulosae* IFO 4040, and *Aspergillus sojae* IFO 4389 to give 4-oxo-α-ionol (**207**), 4-oxo-7,8-dihydro-α-ionone (**208**), and 4-oxo-7,8-dihydro-α-ionol (**209**). Compound **205** was also

oxidized by *A. niger* and *A. sojae* to give 1-hydroxy-4-oxo-α-ionone (**210**) and 7,11-oxido-4-oxo-7,8-dihydro-α-ionone (**211**). The C_7–C_8 double bond of α-ionone (**203**), 4-oxo-α-ionone (**205**), and 4-oxo-α-ionol (**207**) was easily reduced to the corresponding dihydro products **212**, **208**, and **209**, respectively, by *Euglena*, *Aspergillus*, *Botryosphaeria*, and *Chlorella* species. The metabolite **212** was further reduced to **213** by *E. gracilis*[46–48] (**Scheme 41**).

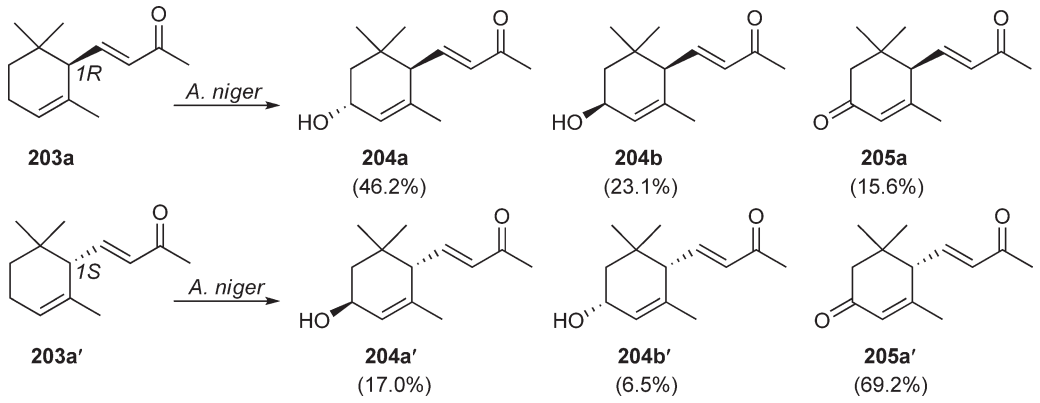
(+)-(*1R*)-α-Ionone (**203a**), $[\alpha]_D$ +386.5°, 99% enantiomeric excess (ee), and (−)-(1*S*)-α-ionone (**203a′**), $[\alpha]_D$ −361.6°, 98% ee, which were obtained by optical resolution of racemic α-ionone (**203**), were fed to *A. niger* for 4 days in Czapek-peptone medium. From **203a**, 4α-hydroxy-α-ionone (**204a**), 4β-hydroxy-α-ionone (**204b**), and 4-oxo-α-ionone (**205a**) were obtained, whereas from compound **203b**, the enantiomers (**204a′**, **204b′**, **205a′**) of the metabolites of **203a** were obtained; however, there were differences in their yields. In the case of **203a**, 4α-hydroxy-α-ionone (**204a**) was obtained as the major product, while **205a′** was predominantly obtained from **203a′** (**Scheme 42**). This oxidation was inhibited by 1-aminobenzotriazole; thus, CYP-450 contributes to this oxidation process.[49]

β-Ionone (**214a**) was bioconverted to 5*S*- (**214e**) and 3*R*-hydroxy-β-ionone (**214g**) and the corresponding ketones (**214f**, **214h**) by *A. niger*.[50] Compound **214a** was incubated with *Gongronella butleri* to afford **214g** predominantly,[51] whereas *Lasiodiplodia theobromae* reduced the C_7–C_8 double bond of **214a** to give dihydro (**214c**) and oxidative products (**214m**)[52] (**Scheme 43**).

Biotransformation of β-ionone (**214a**) was also studied by using 10 kinds of *Aspergillus* species, *A. awamori*, *A. fumigatus*, *A. sojae*, *A. usami*, *A. cellulosae* M-77, *A. cellulosae* IFO 4040, *A. terreus*, *A. niger* IFO 4034, *A. niger* IFO 4049, and a strain of *A. niger*, *D. tertiolecta* (algae), *E. gracilis* (protozoa), *H. anomala* (yeast), *Saccharomyces cerevisiae* (yeast), and *Streptomyces ikutamanensis* (Actinomycetes).[46] Two strains of *A. cellulosae*, *H. anomala*, and *S. cerevisiae*



Scheme 41 Biotransformation of α-ionone (**203**) and the related compounds (**204–208**, **212**, **214**) by *Aspergillus cellulosae*, *A. niger*, *A. sojae*, *Botryosphaeria dothidea*, *Chlorella ellipsoidea*, *C. pyrenoidosa*, *C. salina*, *C. sorokiniana*, *C. vulgaris*, *Dunaliella tertiolecta*, *Euglena gracilis*, *Hansenula anomala*, and *Rhodotorula minuta*.



Scheme 42 Biotransformation of (+)-(*1R*)-α-ionone (**203a**) and (−)-(1*S*)-α-ionone (**203a′**) by *A. niger*.

converted **214a** to β-ionols (**214b, 214b′**) predominantly. *D. tertiolecta* and *E. gracilis* produced the same alcohols (**214b, 214b′**) and their dihydro derivatives (**214d, 214d′**) together with C_7–C_8 dihydro-β-ionone (**214c**) (**Scheme 43**).

Except *A. cellulosae*, the other *Aspergillus* species transformed β-ionone to 3α-hydroxy-β-ionone (**214g**) as the major product, followed by oxidation to afford 3-oxo-β-ionone (**214h**). β-Ionone on incubation with the three strains of *A. niger* gave 5α-hydroxy-β-ionone (**214e**), 5-oxo-β-ionone (**214f**), and **214g**. *Aspergillus niger* and *S. ikutamanensis* converted 3-oxo-β-ionone (**214h**) to 5-hydroxy-β-ionone (**214i**), 7,8-dihydro derivative (**214j**), and their dihydro products (**214l, 214m**). *A. sojae* produced 3-oxo-9-hydroxy-β-ionone (**241k**) in small amounts. When compounds **214j** and **214c** were incubated with the same microorganisms, 4-oxo-β-cyclohomogeraniol (**214n**) and β-cyclohomogeraniol (**214o**) were obtained, respectively, through Baeyer–Villiger-type oxidation reaction. The dehydrogenation of **214b** and **214b′** occurred in *A. niger* to give β-ionone (**214a**) (**Scheme 43**).

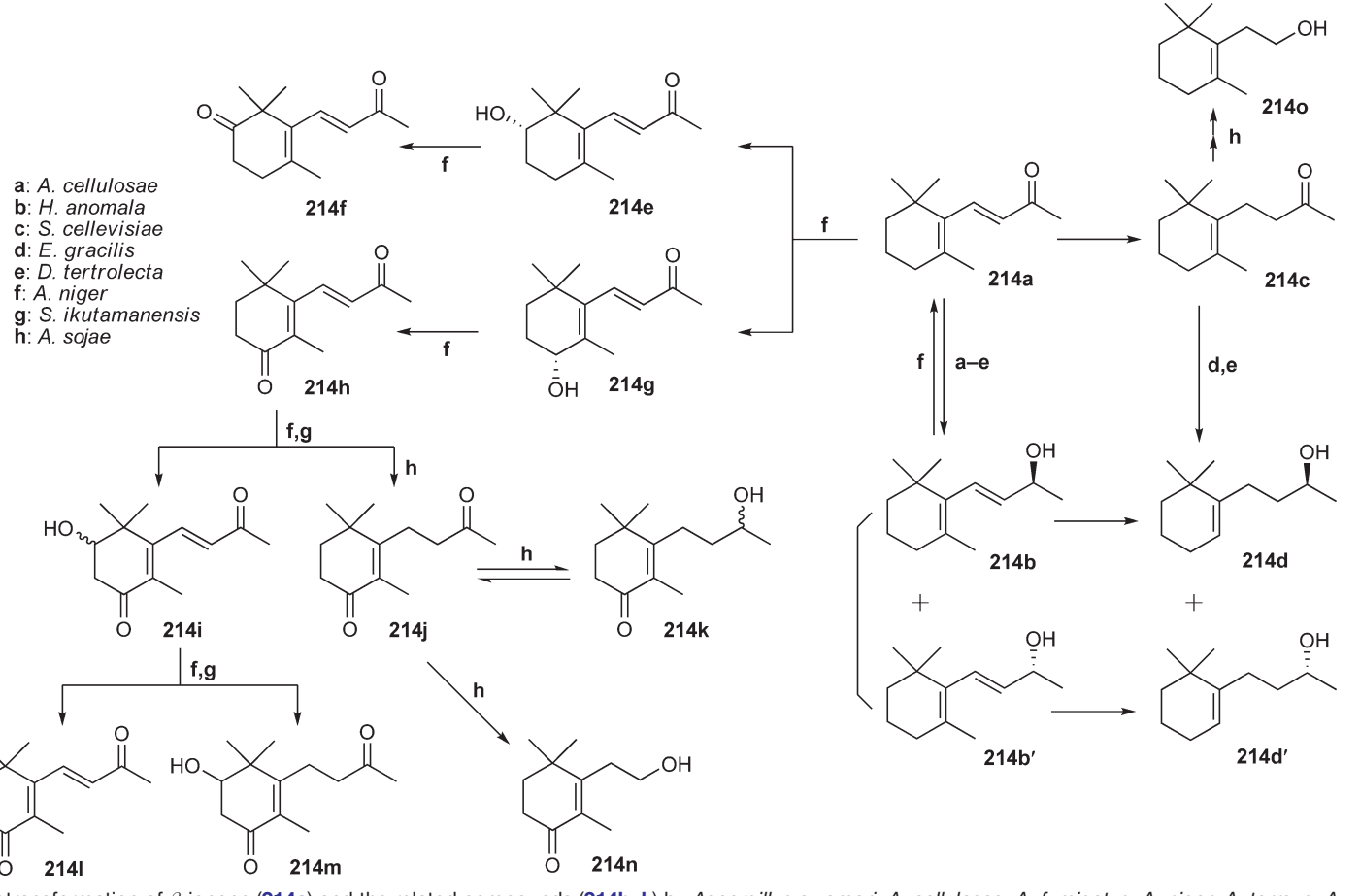
α-Damascone (**215**) on incubation with *A. niger* in Czapek-peptone medium give *cis*- (**216**) and *trans*-3-hydroxy-α-damascones (**217**) and 3-oxo-α-damascone (**218**), and with *Aspergillus* afforded 3-oxo-8,9-dihydro-α-damascone (**219**). The hydroxylation process of **215** to **216** and **217** was inhibited by CYP-450 inhibitor. *H. anomala* reduced α-damascone (**215**) to α-damascol (**220**). *Cis*- (**216**) and *trans*-4-hydroxy-α-damascone (**217**) were fed to *C. pyrenoidosa* in Noro medium to give 4-oxodamascone (**218**)[53] (**Scheme 44**).

A. niger converted β-damascone (**221**) to 5-hydroxy-β-damascone (**222**), 3-hydroxy-β-damascone (**223**), 5-oxo-β-damscone (**224**), 3-oxo-β-damscone (**225**), and 3-oxo-1,9-dihydroxy-1,2-dihydro-β-damascone (**226**) as the minor components. In the case of *A. terreus*, 3-hydroxy-8,9-dihydro-β-damascone (**227**) was also obtained[53] (**Scheme 45**).

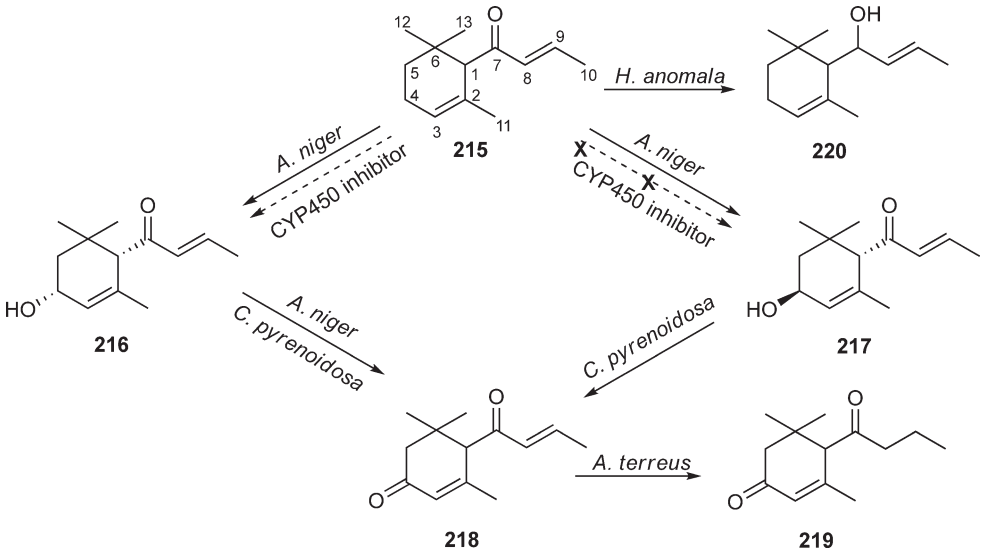
Adamantane derivatives have been used as medicinal drugs. In order to obtain the drugs, adamantanes were incubated with many microorganisms, such as *A. niger*, *A. awamori*, *A. cellulosae*, *A. fumigatus*, *A. sojae*, *A. terreus*, *B. dothidea*, *C. pyrenoidosa* IMC C-28, *Chlorella sorokiriana*, *Fusarium culmurorum*, *E. gracilis*, and *H. anomala*.

Adamantane (**228**) was incubated with *A. niger*, *Aspergillus cellulosea*, and *B. dothidea* in Czapek-peptone medium. The same substrate was also treated in *C. pyrenoidosa* in Noro medium. Compound **228** was converted to both 1-hydroxyadamantane (**229**) and 9α-hydroxyadamantane (**230**) by all four microorganisms, followed by oxidation to give 1,9α-dihydroxyadamantanol (**231**) by *A. niger*, which was further oxidized to 1-hydroxyadamantane-9-one (**232**), followed by reduction to afford 1,9β-hydroxyadamantane (**234**). *A. niger* gave the metabolite **231** as the major product in 80% yield. *A. cellulosae* converted **228** to **229** and **230** in the ratio of 81:19. *Chlorella pyrenoidosa* gave **229**, **230**, and adamantane-9-one (**233**) in the ratio of 74:16:10. 9α-hyeroxyadamantane (**230**) was directly converted by *C. pyrenoidosa*, *A. niger*, and *A. cellulosae* to afford **233**, which was also reduced to 9α-adamantanol (**230**) by *A. niger* (**Scheme 46**). The biotransformation of adamantane, however, did not occur by the microorganisms *H. anomala*, *C. sorokiriana*, *D. tertiolecta*, and *E. gracilis*.[54]

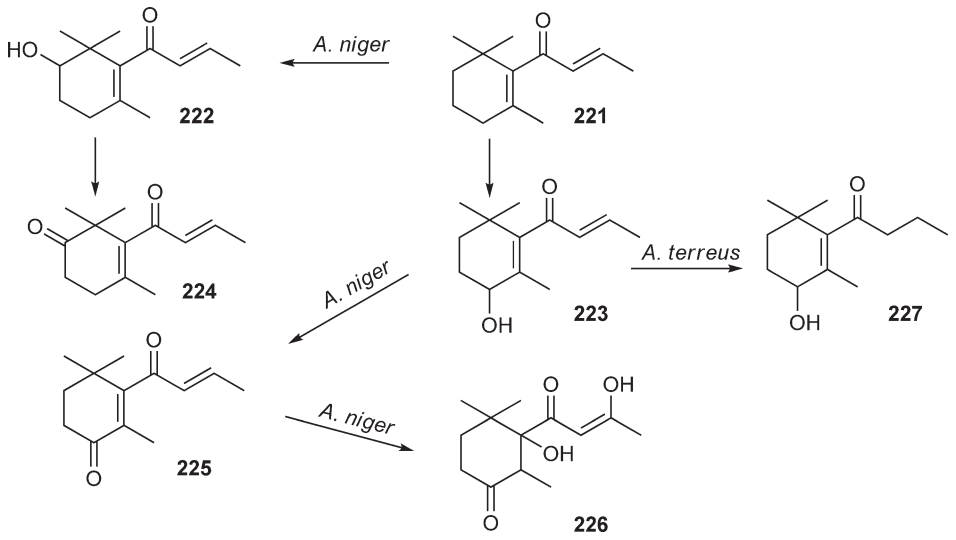
Adamantanes (**228–233**) were also incubated with various fungi including *F. culmurorum*. 1-Hydroxyadamantane-9-one (**232**) was reduced stereoselectively to **231** by *A. niger*, *A. cellulosae*, *B. dothidea*, and *F. culmurorum*. On the contrary, *F. culmurorum* reduced **231** to **232**. *A. cellulosae* and *B. dothidea* bioconverted



Scheme 43 Biotransformation of β-ionone (**214a**) and the related compounds (**214b–k**) by *Aspergillus awamori*, *A. cellulosae*, *A. fumigatus*, *A. niger*, *A. terreus*, *A. usami*, *Dunaliella tertiolecta*, *Euglena gracilis*, *Hansenula anomala*, and *Streptomyces ikutamanensis.*



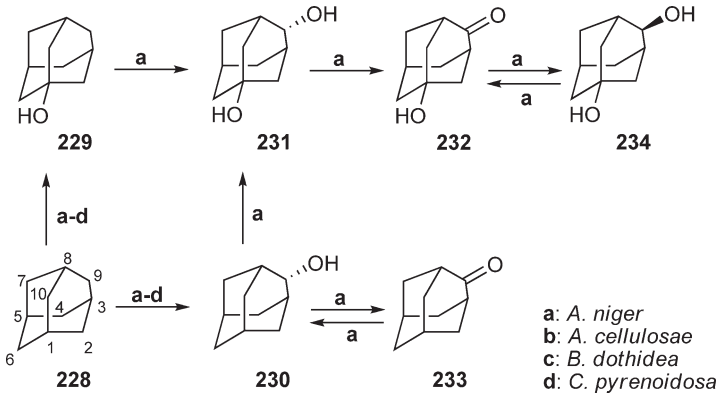
Scheme 44 Biotransformation of α-damascone (**215**) by *A. niger*, *A. terreus*, *C. pyrenoidosa*, and *H. anomala*.



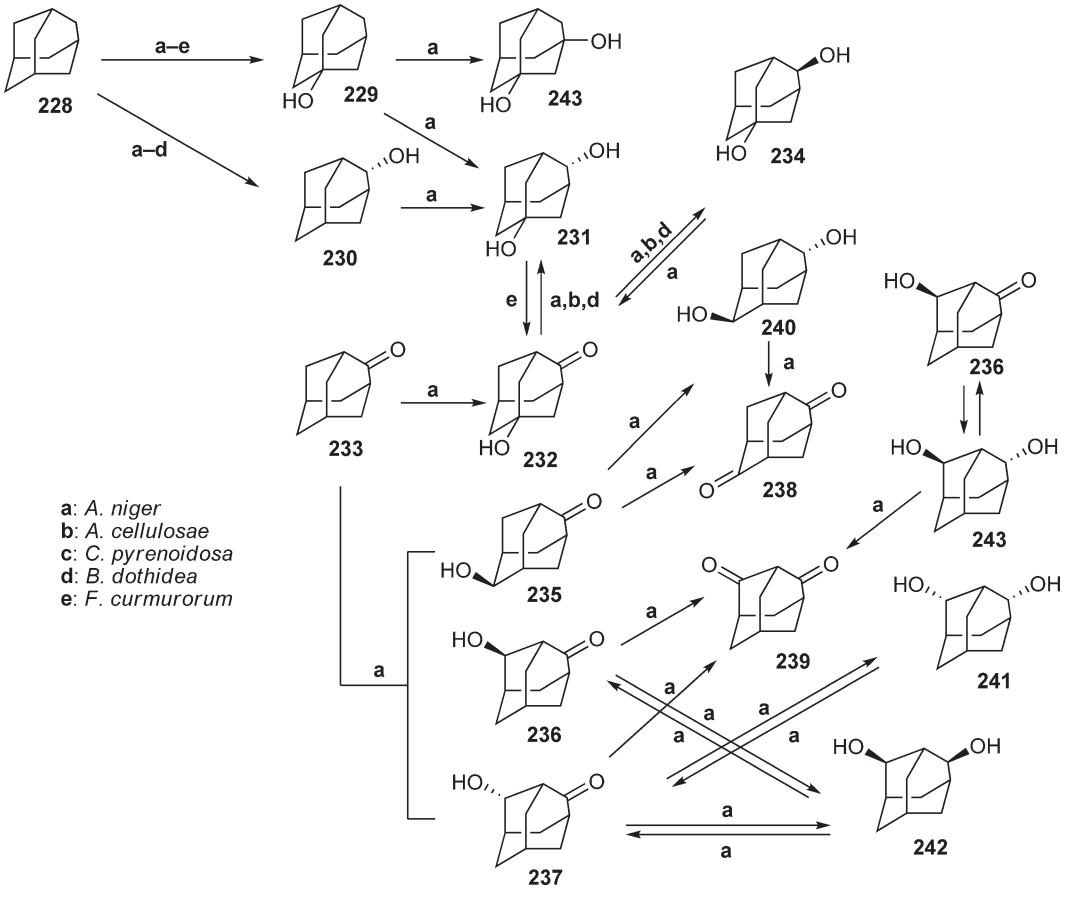
Scheme 45 Biotransformation of β-damascone (**221**) by *A. niger* and *A. terreus*.

232 to 1,9β-hydroxyadamantane (**234**) stereoselectively. *A. niger* nonstereoselectively converted **233** to **235** and **236**, which were further converted to diketone (**238, 239**) and diol (**240**). It is noteworthy that oxidation and reduction reactions were observed between ketoalcohol (**237**) and diols (**241, 242**). The same phenomenon was also seen between **236** and **243**. The latter diol was also oxidized by *A. niger* to furnish diketone (**239**).[55,56] Direct hydroxylation at C-3 of 1-hydroxyadamantane (**229**) was seen in the incubation of **229** with *A. niger* to afford 1,3-dihydroxyadamantane (**243**) (**Scheme 47**).

4-Adamantanone (**233**) showed promotion effect on cell division of the fungus, while 1-adamantanol (**229**) and adamantane-9-one (**234**) inhibited germination of lettuce seed. 1-Hydroxyadamantane-9-one (**232**) inhibited elongation of root of lettuce, while adamantane-1,4-diol (**234**) and adamantane itself (**228**) promoted root elongation.[54,55]

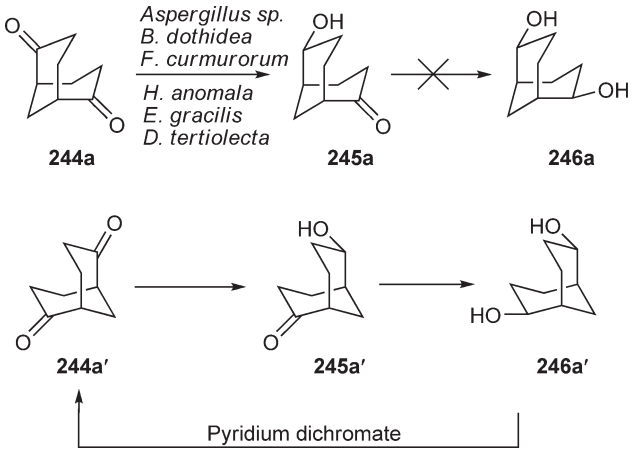


Scheme 46 Biotransformation of adamantane (**228**) and the related compounds (**229–234**) by *A. cellulosae*, *A. niger*, *B. dothidea*, and *C. pyrenoidosa.*



Scheme 47 Biotransformation of adamantane (**228**) and the related compounds (**229–237**, **239–243**) by *A. niger*, *B. dothidea*, *F. culmorum*, *E. gracilis*, *D. tertiolecta*, and *H. anomala.*

Stereoselective reduction of racemic bicycle[3.3.1]nonane-2,6-dione (**244a**, **244a′**) was carried out by *A. awamori*, *A. fumigatus*, *A. cellulosae*, *A. sojae*, *A. terreus*, *A. niger*, *B. dothidea*, *Fusarium culmorum* in Czapek-peptone, *H. anomala* in yeast, *E. gracilis in* Hunter, and *D. tertiolecta* in Noro medium. All microorganisms reduced **244a**



Scheme 48 Biotransformation of bicycle[3.3.1]nonane-2,6-dione (**244a**, **244a′**) by *A. awamori*, *A. cellulosae*, *A. fumigatus*, *A. niger*, *A. sojae*, *A. terreus*, *B. dothidea*, *F. culmorum*, *E. gracilis*, *D. tertiolecta*, and *H. anomala*.

and **244a′** to give corresponding monoalcohols (**245a, 245a′**) and optically active (−)-diol (**246a′**) ($[\alpha]_D$ −71.8° in the case of *A. terreus*), which was formed by enantioselective reduction of racemic monoalcohol, namely **245a** and **245a′**. The enantiomer (**246a**) of **246a′** was not obtained from the racemic diones (**244a, 244a′**)[56] (**Scheme 48**).

3.21.3 Biotransformation of Aromatic Compounds

Essential oils contain aromatic compounds, like *p*-cymene, carvacrol, thymol, vanillin, cinnamaldehyde, eugenol, chavicol, safrole, and asarone (**247**) among others.

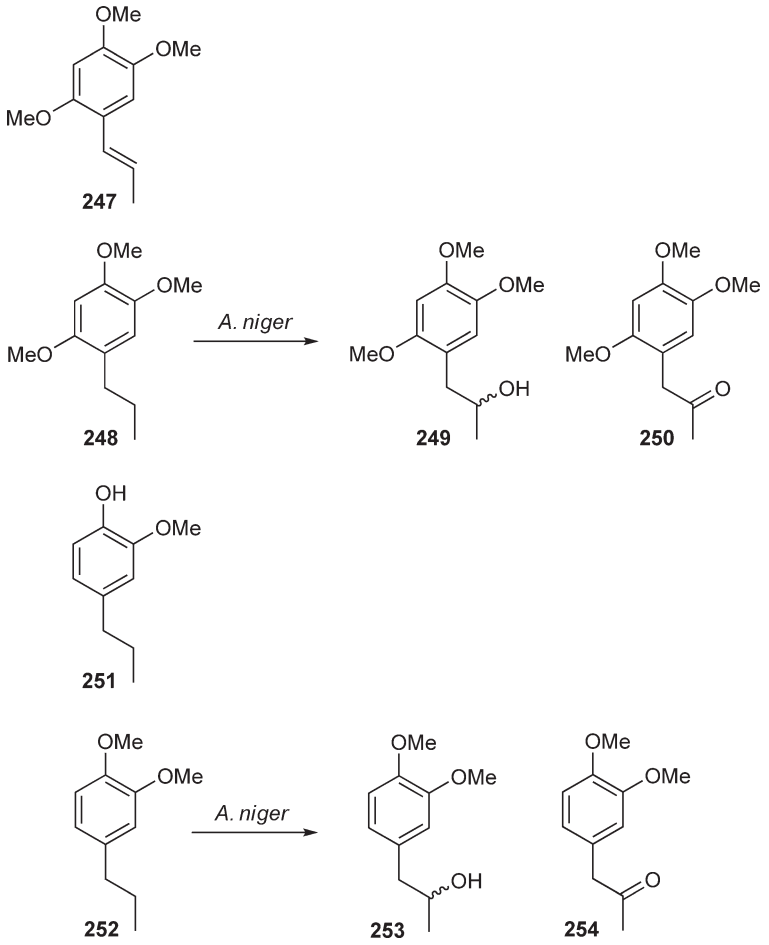
Takahashi[57] reported that simple aromatic compounds, such as propylbenzene, hexylbenzene, decylbenzene, *o*- and *p*-hydroxypropiophenones, *p*-methoxypropiophenone, 4-hexylresorcinol, and methyl 4-hexylresorcionol, were incubated with *A. niger*. From hexylbenzene and decylbenzene, ω1-hydroxylated products were obtained, whereas from propylbenzene, ω2-hydroxylated metabolites were obtained. However, the free phenolic compounds were not biodegraded.[57]

Asarone (**247**) and dihydroeugenol (**251**) were not biotransformed by *A. niger*. However, dihydroasarone (**248**) and methyldihydroeugenol (**252**) were biotransformed by the same fungus to produce a small amount of 2-hydroxy (**249, 250**) and 2-oxo derivatives (**253, 254**). The chirality at C-2 was determined to be *R* and *S* mixtures (1:2) by modified Mosher's method[57] (**Scheme 49**).

Microalgae *Chlorella* species are excellent oxidation bioreactors as mentioned earlier. Monoterpene aldehydes and related aldehydes were reduced to the corresponding primary alcohols, indicating that these green algae also possess reductase activity.

The following aromatic aldehydes were incubated with *C. ellipsoidea*. Benzaldehyde, *o*-, *m*-, and *p*-hydroxybenzaldehydes, *o*-, *m*-, and *p*-chlorobenzaldehydes, *o*-, *m*-, and *p*-cyanobenzaldehydes, *o*-, *m*-, and *p*-nitrobenzaldehydes, *o*-, *m*-, and *p*-anisaldehydes, *o*-, *m*-, and *p*-tolualdehydes, *o*-, *iso*-, and terephthalaldehydes, *o*-vanillin, vanillin, isovanillin, ethylvanillin, veratraldehyde, 2,4-dimethylbenzaldehyde, 2,4-, 2,5-, and 3,4-dihydroxybenzaldehydes, 2,3-, 2,4-, 2,5-, and 3,5-dimethoxybenzaldehydes, 2,3-, 2,4-, 2,6-, 3,4-, and 3,5-dichlorobenzaldehydes, 2,3,4- and 3,4,5-trimethoxybenzaldehydes, phenylacetaldehyde, 2-, 3-phenylpropionaldehyde, cinnamaldehyde, and α-methylcinnamaldehyde were reduced to their corresponding primary alcohol in good yield. Cinnamaldehyde and α-methylcinnamaldehyde gave a small amount of 3-phenylpropanol and α-methyl-3-phenylpropanol.[44]

The microalgae *E. gracilis* and *D. tertiolecta* also contain reductase. 2-Cyanobenzaldehyde, *o*-, *m*-, and *p*-anisaldehydes, salicylaldehyde, *o*-, *m*-, and *p*-tolualdehydes, *o*-chlorobenzaldehyde, *p*-hydroxybenzaldehyde, *o*- *m*-, and *p*-nitrobenzaldehyde, 3-cyanobenzaldehyde, vanillin, isovanillin, *o*-vanillin, nicotine aldehyde,

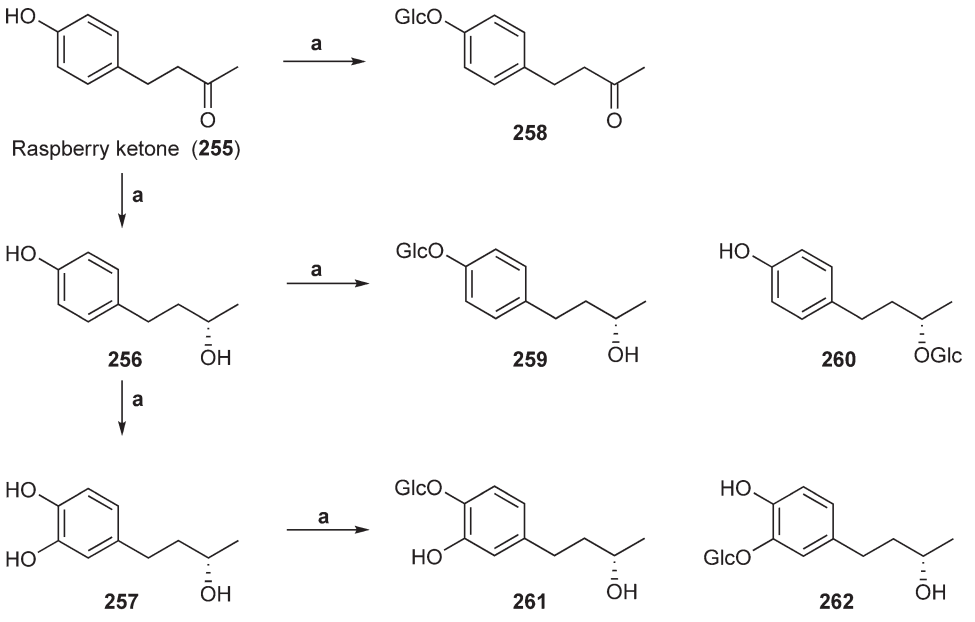


Scheme 49 Biotransformation of dihydroasarone (**248**) and methyldihydroeugenol (**252**) by *A. niger*.

3-phenylpropionaldehyde, ethyl vanillin, veratraldehyde, 3-nitrosalicylaldehyde, phenylacetaldehyde, and 2-phenylpropanaldehyde were incubated with *E. gracilis* or *D. tertiolecta* to afford the corresponding primary alcohols. 2-Cyanobenzaldehyde gave its corresponding alcohol with phthalate. *m*- and *p*-Chlorobenzaldehyde gave their corresponding alcohols and *m*- and *p*-chlorobenzoic acids. *o*-Phthaldehyde and *p*-phthalate, and *iso*- and terephthaldehydes gave their corresponding monoalcohols and dialcohols. When cinnamaldehyde and α-methyl cinnamaldehyde were incubated in *E. gracilis* or *D. tertiolecta*, cinnamyl alcohol and 3-phenylpropanol, and 2-methylcinnamyl alcohol and 2-methyl-3-phenylpropanol were obtained in good yield.[48,57–59]

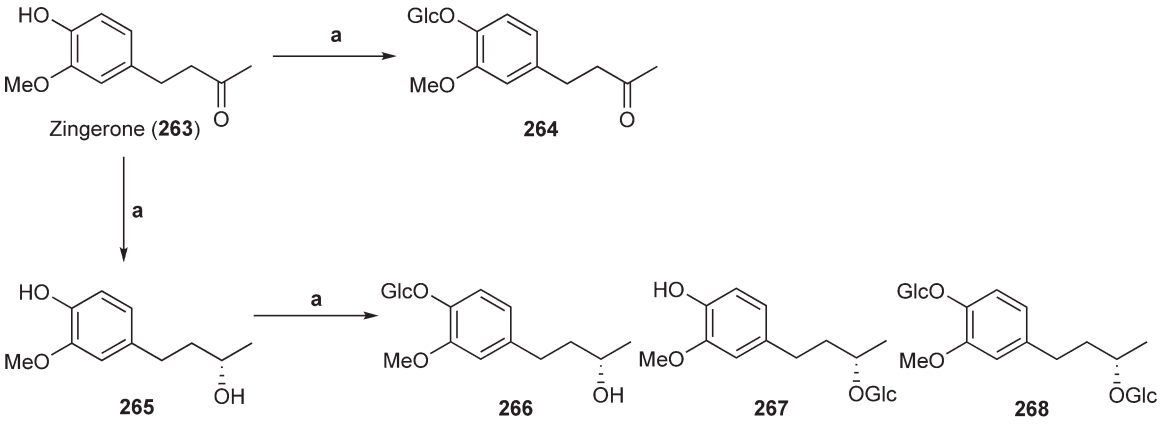
Raspberry ketone (**255**) and zingerone (**263**) are the major components of raspberry (*Rubus idaeus*) and ginger (*Zingeber officinale*) and these are used as food additives and spices. When substrates **255** and **263** were incubated with the *Phytolacca americana* cultured cells for 3 days, two secondary alcohols (**256, 257**) as well as five glucosides (**258–262**) were produced from **255**, and a secondary alcohol (**265**) and four glycoside products (**264, 266–268**) were produced from **263**. In the case of raspberry ketone, the phenolic hydroxyl group was preferably glycosylated after reduction of the carbonyl group of the substrate occurred. It is interesting to note that one more hydroxyl group was introduced into the benzene ring to give **257**, which was further glycosylated to one of the phenolic hydroxyl groups, and no glycoside of the secondary alcohol at C-2 was obtained (**Scheme 50**).

On the contrary, zingerone (**263**) was converted into **265**, followed by glycosylation to give glucosides (**266, 267**) of both phenolic and secondary hydroxyl groups and a diglucoside (**268**) of both phenolic and secondary hydroxyl group in the molecule (**Scheme 51**). It is the first report on the introduction of individual glucose residues onto both phenolic and secondary hydroxyl groups by cultured plant cells.[60]



a: *Phytolacca americana* cells

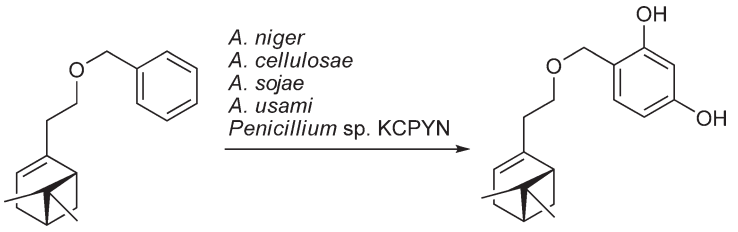
Scheme 50 Biotransformation of raspberry ketone (**255**) by cultured cells of *Phytolacca americana*.



a: *Phytolacca americana* cells

Scheme 51 Biotransformation of zingerone (**263**) by cultured cells of *P. americana*.

(−)-Nopol benzyl ether (**269**) was smoothly biotransformed by *A. niger*, *A. cellulosae*, *A. sojae*, *A. usami*, and *Penicillium* species in Czapek-peptone medium to give (−)-4-oxonopol-2′,4′-dihydroxybenzyl ether (**270**, 23% in the case of *A. niger*), which demonstrated antioxidant activity (ID_{50} 30.23 μmol l^{-1}), together with a small amount of nopol (6.3% in *A. niger*) (**Scheme 52**). The antioxidant activity of **270** is same as that of butylhydroxyanisol (BHA). The direct introduction of oxygen function on the phenyl ring by microorganisms is very rare.[61]

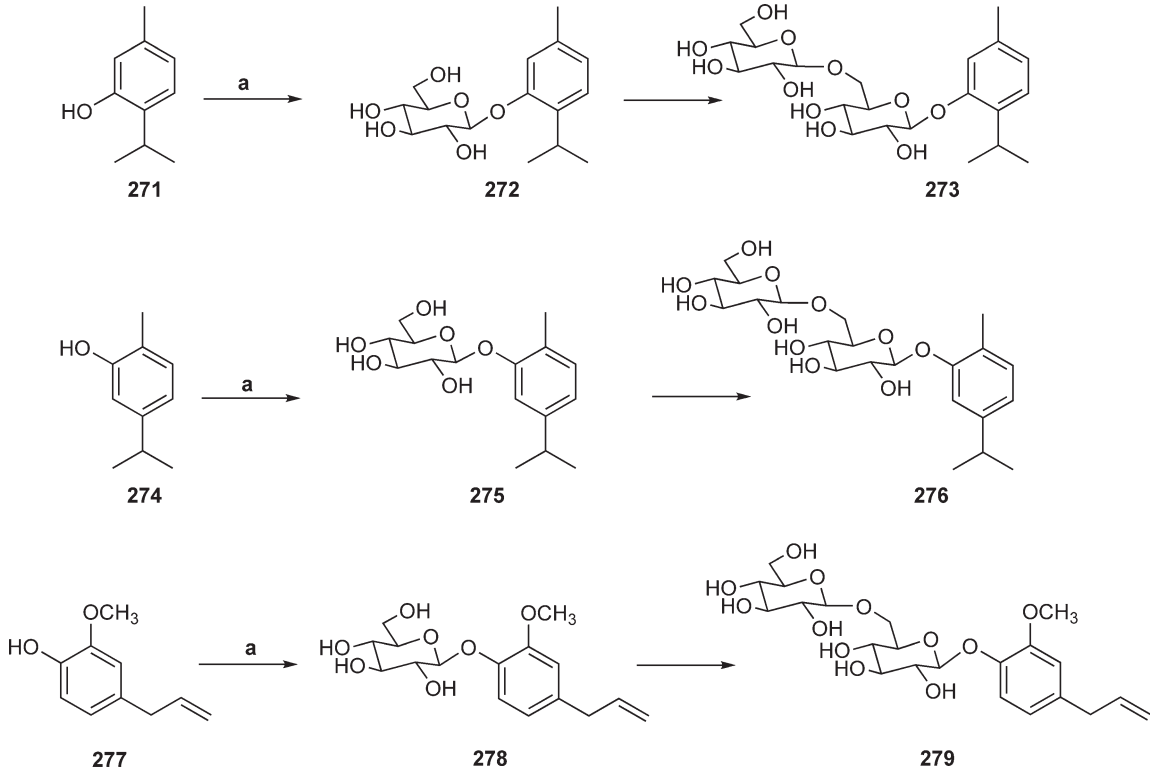


(–)-Nopol benzylether (**269**) **270** (23%)

Scheme 52 Biotransformation of (–)-nopol benzyl ether (**269**) by *A. niger*, *A. cellulosae*, *A. sojae*, *A. usami*, and *Penicillium* species.

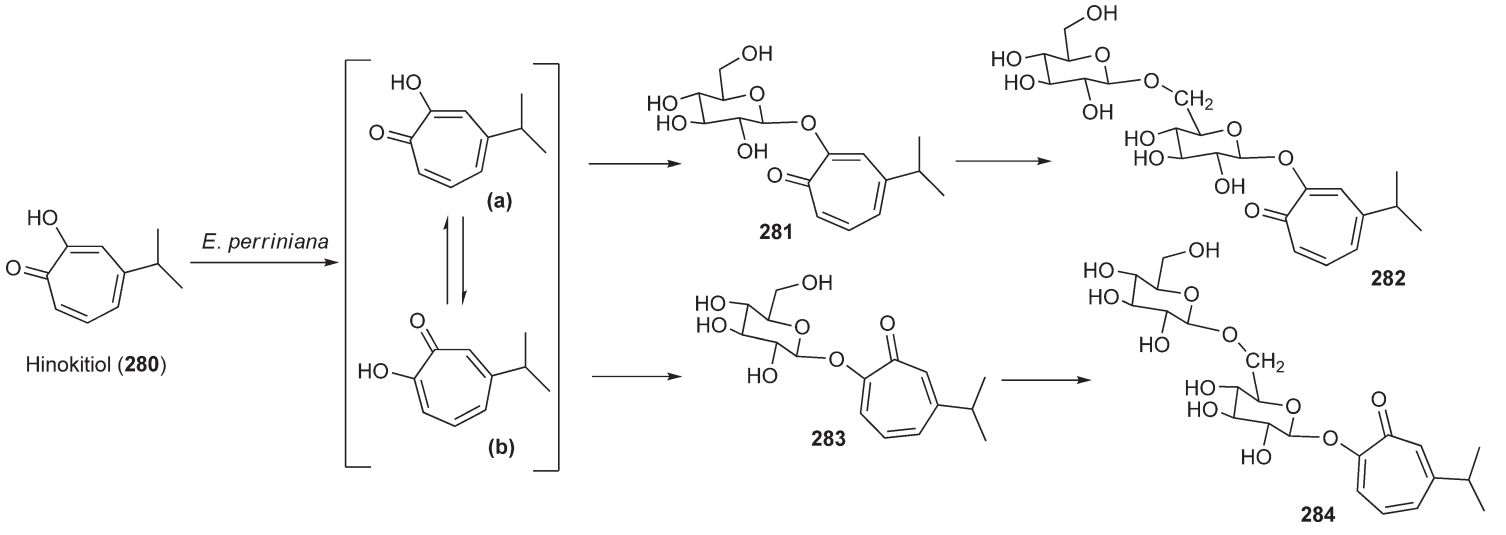
Thymol (**271**), carvacrol (**274**), and eugenol (**277**) were glucosylated by glycosyl transferase of *Eucalyptus perriniana* cultured cells to glucoside (**272**, 3%; **275**, 5%; **278**, 7%) and gentiobioside (**273**, 87%; **276**, 56%; **279**, 58%) (**Scheme 53**). The yield of thymol glycosides was 1.5 times higher than that of carvacrol and 4 times higher than that of eugenol. Such glycosylation is useful to obtain higher water-soluble products from natural and commercially available secondary metabolites for food additives and cosmetic fields.[62]

Hinokitiol (**280**), which is easily obtained from cell suspension cultures of *Thujopsis dolabrata* and possesses potent antimicrobial activity, was incubated with cultured cells of *E. perriniana* for 7 days to give its monoglucosides (**281**, **283**, 32%) and gentiobiosides (**282**, **284**)[63,64] (**Scheme 54**).



a: *Eucalyptus perriniana* cells

Scheme 53 Biotransformation of thymol (**271**), carvacrol (**274**), and eugenol (**277**) by cultured cells of *Eucalyptus perriniana*.



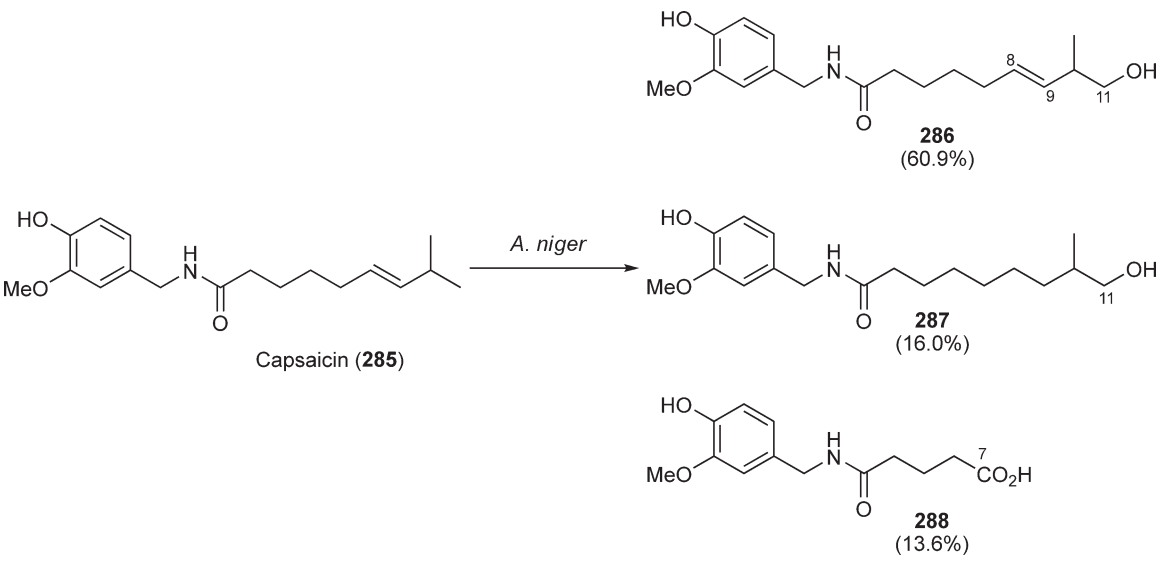
Scheme 54 Biotransformation of hinokitiol (280) by cultured cells of *E. perriniana*.

Capsicum annuum contains capsaicin (**285**). Capsaicin and its homologues having an alkylvanillylamide possess various interesting biological properties such as anti-inflammatory and antioxidant effects, saliva- and stomach juice-inducing activity, analgesic, antigenotoxic, antimutagenic, anticarcinogenic, and antirheumatoid arthritis effects, and are used in the treatment of diabetic neuropathy and as food additives. On the contrary, because of potent pungency and irritation on skin and mucous membrane, capsaicin has not yet been permitted as a medicinal drug. In order to reduce the typical pungency and application of nonpungent capsaicin metabolites to the crude drug, capsaicin (**285**) (600 g) including 30% of dihydrocapsaicin (**289**) was incubated in Czapek-peptone medium including *A. niger* for 7 days to give three metabolites, ω1-hydroxylated capsaicin (**286**, 60.9%), 8,9-dihydro-ω1-hydroxycapsaicin (**287**, 16%), and a carboxylic acid (**288**, 13.6%) (**Scheme 55**). All the metabolites do not show pungency.

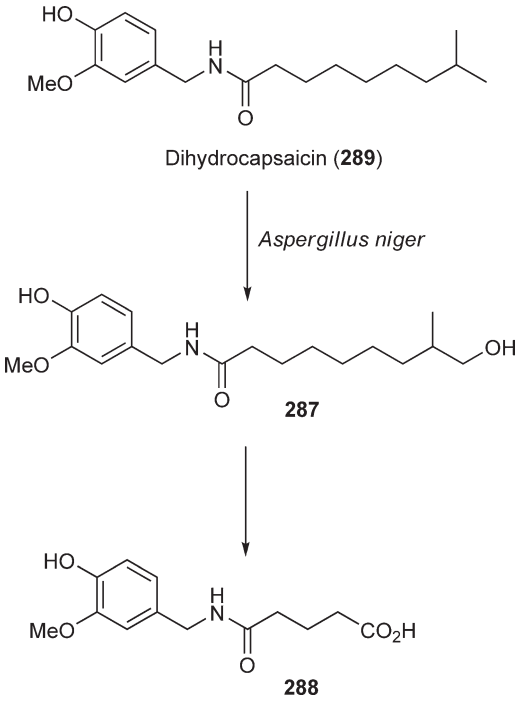
Dihydrocapsaicin (**289**) was also treated in the same manner as described above to afford ω1-hydroxydihydrocapsaicin (**287**, 80.9%) in high yield and carboxylic acid (**288**, 5.0%) (**Scheme 56**). Capsaicin itself showed carbachol-induced contraction in bronchus of 60% at the concentration of 1 μmol l^{-1}. 11-Hydroxycapsaicin (**286**) retained this activity of 60% at the concentration of 30 μmol l^{-1}. Dihydrocapsaicin (**289**) showed the same activity of contraction in bronchus at the same concentration as that of capsaicin. However, the activity of contraction in bronchus of 11-hydroxy derivative (**287**) was weaker (50% at 30 μmol l^{-1}) than that of the substrate. Since both metabolites (**286**, **287**) are tasteless, these products might be of value for the crude drug although the contraction in bronchus is weak. DPPH radical-scavenging activity test of capsaicin and dihydrocapsaicin derivatives was carried out. 11-Hydroxycapsaicin (**286**), 11-dihydrocapsaicin (**287**), and capsaicin showed higher activity than *dl*-α-tocopherol, and 11-dihydroxycapsaicin displayed strong scavenging activity (IC_{50} 50 μmol l^{-1}) (Hashimoto and Asakawa, unpublished results).

Shimoda *et al.*[65] reported the bioconversion of capsaicin (**285**) and 8-nordihydrocapsaicin (**293**) by cultured cells of *Catharanthus roseus* to give more water-soluble capsaicin derivatives. From capsaicin, three glycosides, capsaicin 4-*O*-β-D-glucopyranoside (**290**), one of the capsaicinoids in the fruit of *Capsicum* with 1/100 weaker pungency than capsaicin, 4-*O*-(6-*O*-β-D-xylopyranosyl)-β-D-glucoside (**291**), and 4-*O*-(6-*O*-α-L-arabinosyl)-β-D-glucopyranoside (**292**) were obtained. 8-Nordihydrocapsaicin (**293**) was also incubated with the same cultured cells to afford products (**294**–**296**) with reduced pungency and enhanced water solubility (**Scheme 57**). Since many synthetic capsaicin glycosides possess remarkable pharmacological activity, such as decreasing liver and serum lipids, the present products will be valuable as prodrugs.

Z. officinale contains various sesquiterpenoids and pungent aromatic compounds such as 6-shogaol (**297**) and 6-gingerol (**302**) and their pungent compounds, which possess cardio tonic and sedative activity. 6-Shogaol



Scheme 55 Biotransformation of capsaicin (**285**) by *A. niger*.



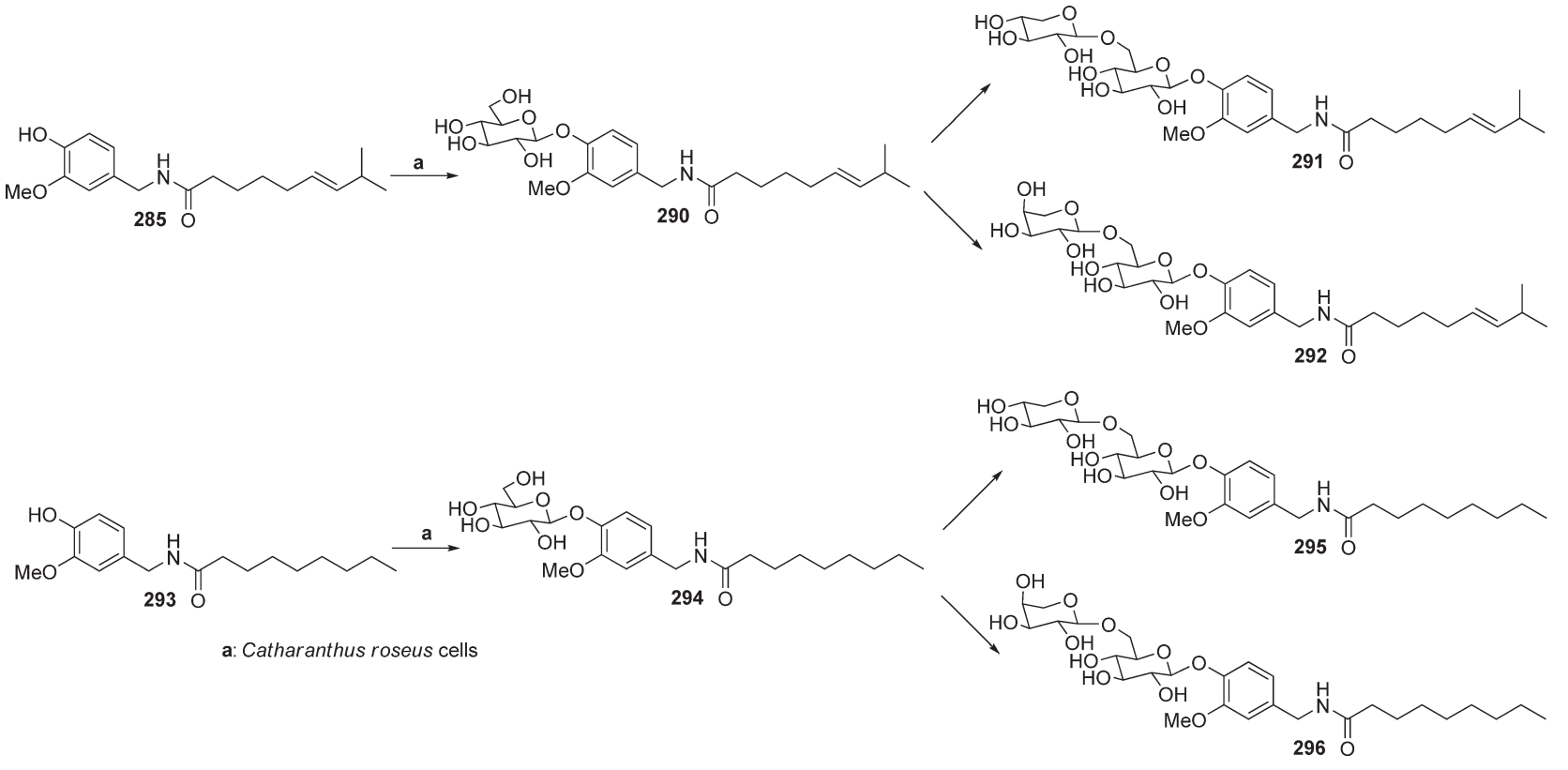
Scheme 56 Biotransformation of dihydrocapsaicin (**289**) by *A. niger.*

(**297**) was incubated with *A. niger* in Czapek-peptone medium for 2 days to afford ω1-hydroxy-6-shagaol (**298**, 9.9%), which was further converted to 8-hydroxy derivative (**299**, 16.1%), a γ-lactone (**300**, 22.4%), and 3-methoxy-4-hydroxyphenylacetic acid (**301**, 48.5%)[46,66] (**Scheme 58**).

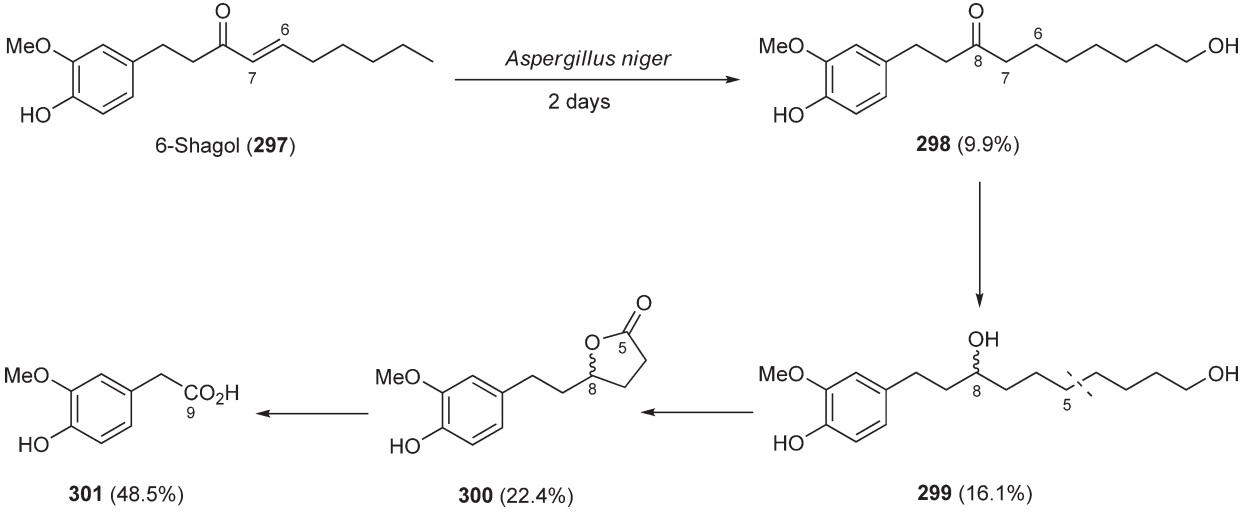
6-Gingerol (**302**) (1 g) was treated in the same condition as mentioned above to yield six metabolites, ω1-hydroxy-6-gingerol (**303**, 39.8%), its carboxylic derivative (**305**, 14.5%), a γ-lactone (**307**) (16.9%), which might be formed from **305**, its 8-hydroxy-γ-lactone (**308**, 12.1%), ω2-hydroxy-6-gingerol (**304**, 19.9%), and 6-deoxy-gingerol (**306**, 14.5%)[46,66] (**Scheme 59**). The metabolic pathway of 6-gingerol (**302**) resembles that of 6-shagaol (**297**). The metabolic pathway of 6-shogaol and dihydrocapsaicin (**289**) is also similar since both substrates gave carboxylic acids as the final metabolites.

Isopropyl naphthalene (**309**) is used as jet-printing media or pressure-sensitive copying paper in the industry and is found even in drinking water. In order to understand the fate of compound **309** and compare the metabolites of *p*-cymene and dehydroabietic acid (**102d**), **309** (2.2 g per rabbit) was administered orally to rabbit. Eight urinary metabolites, 2-(1-naphthyl)-2-propanol (**310**), 2-(1-naphthyl)-1-propanol (**311**), 2-(1-naphthyl)-1,2-propanediol (**312**), 4-isopropyl-1,2-naphthoquinone (**313**), 4-isopropyl-1-naphthol (**314**), 4-isopropyl-2-naphthol (**315**), 5-isopropyl-2-naphthol (**316**), and 2-(1-naphthyl)-propanoic acid (**317**), which was identified by its methyl ester, were isolated after β-glucuronidase and arylsulfatase treatment and extraction with chloroform, followed by chromatography on silica gel. The enzymatic oxidation of **309** might occur by three different metabolic pathways as shown in **Scheme 60**. ω-2 and ω-1 oxidation reactions of **309** were observed in routes (a) and (b) to give **310** and **311**, followed by further oxidation to afford the corresponding primary alcohol (**312**) and carboxylic acid (**317**). In order to confirm these pathways, **310** and **311** were administered to rabbits to give the same metabolites, **312** and **317**, respectively.

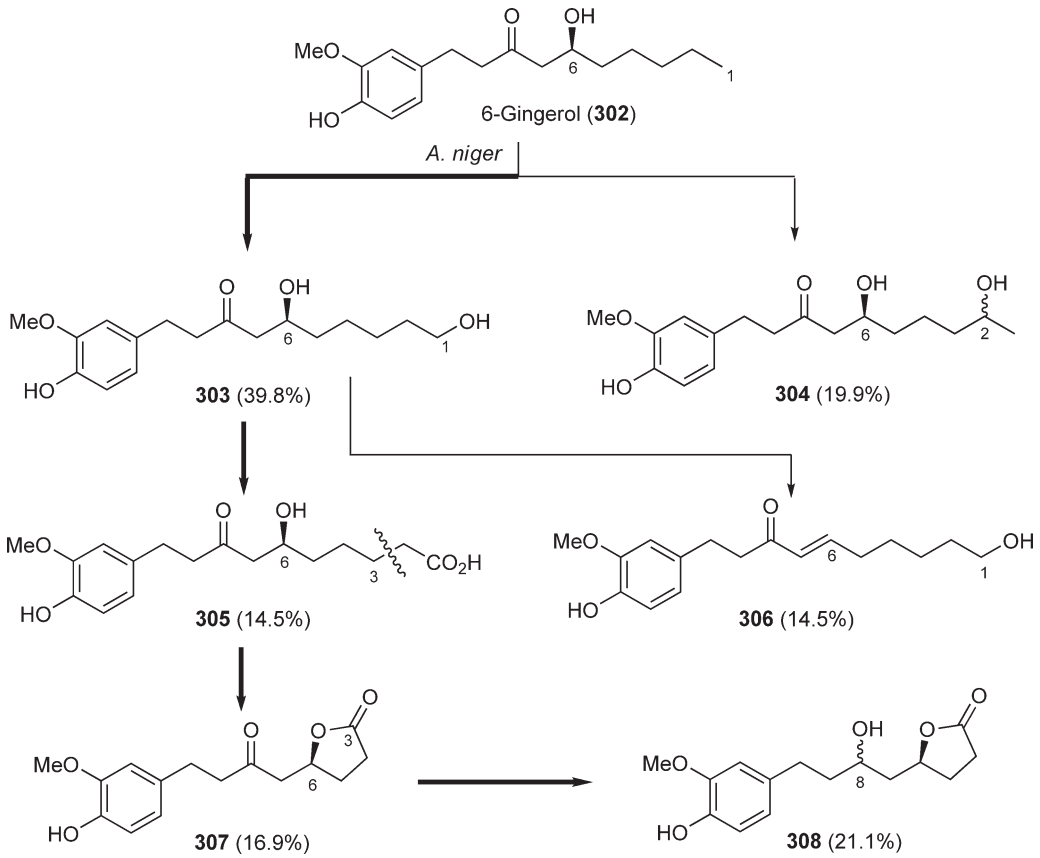
Although no phenolic metabolites have been found in the metabolites of *p*-cymene, cumene, or 4-isopropenyltoluene, the regiospecific introduction of a phenolic hydroxyl group in an aromatic ring was observed in 1-isopropylnaphthalene (**309**) and the formation of *o*-quinone (**313**) is very characteristic.[67] The metabolites **311**, **312**, and **317** have an asymmetric carbon. The ratio of (2*R*)-(1-naphthyl)-propanoic acid and its enantiomer is 52:48.



Scheme 57 Biotransformation of capsaicin (**285**) and 8-nordihydrocapsaicin (**293**) by cultured cells of *C. roseus.*



Scheme 58 Biotransformation of 6-shogaol (297) by *A. niger.*



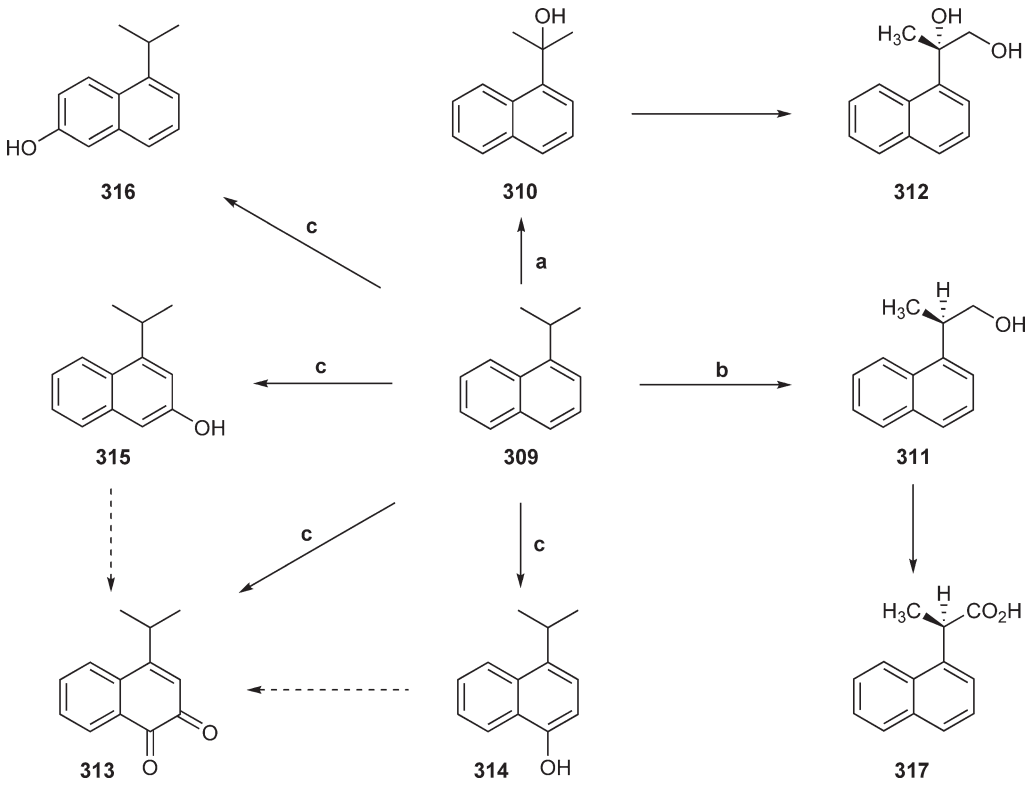
Scheme 59 Biotransformation of 6-gingerol (**302**) by *A. niger*.

The isoflavone derivative 2,3-dehydrokievitone (**318**) was metabolized by two fungi, *Botrytis cinerea* or *Aspergillus flavus*, to give benzofurano- (**322**), pyrano-isoflavones (**323**), and a glycol (**324**). Monomethoxy derivative (**319**) was also incubated with *B. cinerea* to afford an epoxide (**320**) and a glycol (**321**) having *S*-absolute stereochemistry of epoxy and secondary alcohol[68] (**Scheme 61**).

The formation of water-soluble vitamin derivatives from lipophilic vitamins was achieved by glycosylation in cultured plant cells of *P. americana* and *C. roseus*. Two chromanols (**325, 326**) and vitamin E (**327**) were incubated with *P. americana* to afford monoglucosides (**329**, 63%; **330**, 35%; **331**, 7%), respectively (**Scheme 62**). The longer side chain dramatically decreases the yield of the metabolites. On the contrary, *C. roseus* converted the same substrate (**325**) into β-glucoside (**329**, 56%) and gentiobioside (**332**, 14%) (**Scheme 63**). From compound **326**, β-glucoside (**330**, 32%) and β-gentiobioside (**333**, 5%) were obtained. When compound **327** was used as the substrate, only β-glucoside (**331**, 8%) was produced. Retinol (vitamin A) (**328**) was fermented with *P. americana* to afford β-glucoside (**334**, 31%)[69] (**Scheme 62**).

α-Tocopherol (vitamin E) (**327**) and δ-tocopherol (**335**) were also incubated with cultured plant cells of *E. perriniana* to give water-soluble vitamin E series, β-glucoside (**331, 336**), β-gentiobioside (**337, 339**), and β-rutinoside (**338, 400**), which are more water-soluble compounds (**Scheme 63**). The relative incorporation of δ-tocopherol was about 1.7 times higher than that of α-tocopherol at 2 days.[70]

The suppressive action of the glucosides **329–334** on IgE antibody formation was examined. Compound **330** exerted the strongest action among the glycosides tested, whereas no actions were observed in the case of **332–334**, indicating that vitamin E and its homologue glycosides may be useful antiallergic and anti-inflammatory prodrugs since vitamin E glycosides reduce their toxicity and enhance their oral absorption.[69]



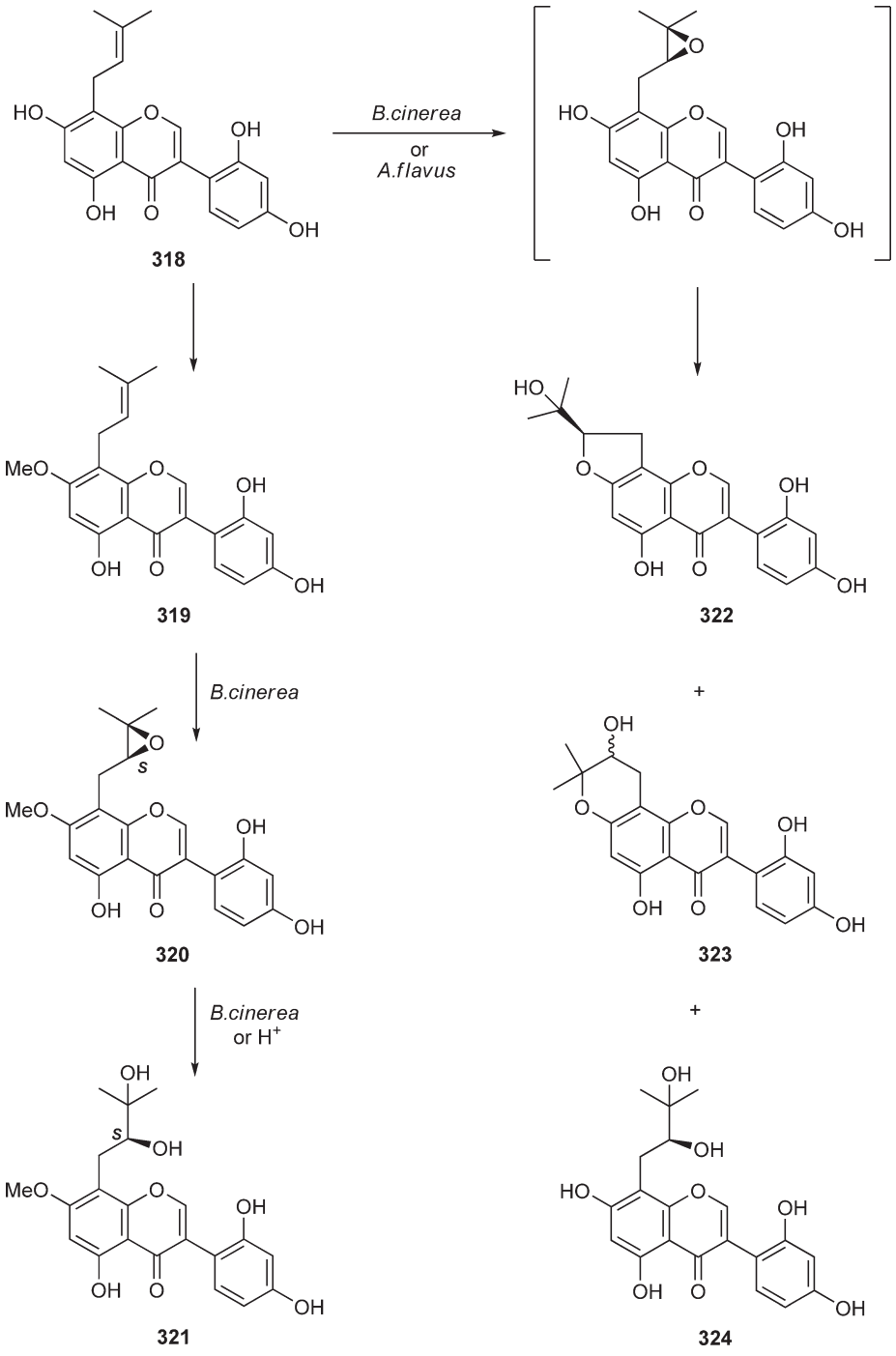
Scheme 60 Biotransformation of isopropyl naphthalene (**309**) by rabbits.

Bisphenol A (**401**), which is suspected of being an endocrine disturbing chemical, particularly for female, is widely used as starting material for manufacturing polymers. Hamada *et al.*[71] studied biotransformation of **401** using suspension cultured cells of *E. perriniana* and found that it is capable of regioselective hydroxylation and glycosylation to give the metabolites **402** (41.7%), **403** (18.7%), and **406** (6.3%). The product **404** might be formed by hydroxylation of the substrate **401** at C-6 of A-ring and C-12 of B-ring to give **407** and **408**, followed by glycosylation of each phenolic hydroxyl group. Furthermore, bisphenol (**401**) was treated in the same manner as described above to give the same metabolites (**402, 403, 406**) in almost the same yield as described above and two new glucosides (**404**, 6%; **405**, 13%), which might be formed from **407**[72] (**Scheme 64**). The formation of such glycosides from bisphenol A (**401**) will decrease endocrine disturbance.

Benzophenone (**409**) was also incubated with *E. perriniana* to produce 4-*O*-β-D-gluco-pyranosylbenzophenone (**412**, 23%), 4-*O*-[6-*O*-(α-L-rhamnopyranosyl benzophenone (**414**, 16%), diphenylmethyl β-D-glucopyranoside (**413**, 29%), and 6-*O*-(β-D-glucopyranosyl)-α-D-glucopyranoside (**415**, 24%), which might be formed through their corresponding intermediates (**410, 411**)[72] (**Scheme 65**).

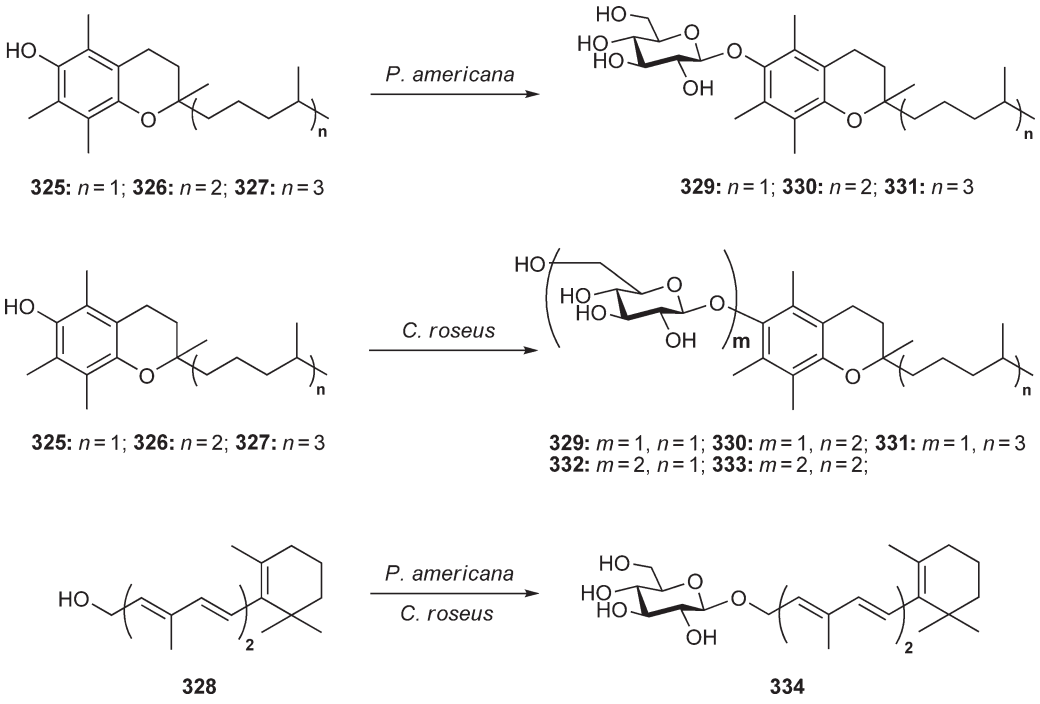
The cultured cells of *E. perriniana* converted phenol (**416**) and monofluorophenols (**417–419**) into their glucosides (**420**, 17.2%; **421**, 49.2%; **422**, 19.6%; and **423**, 28.0%), respectively (**Scheme 66**). Especially, *o*-fluorophenol was quantitatively bioconverted into its phenyl glucoside after 1 h incubation of the cultured cells.[73]

Phenylpropanoids having allyl group, methyleugenol, safrole, elemicin, and asarones series cause genotoxicity by forming adducts with DNA. The seed of nutmeg (*Myristica fragrans*) contains various phenylpropanoids and has been found to show toxicological side effects in clinical application. Kasahara *et al.*[74] reported the microbiological transformation of myrisligan (**424**) in rats and by fecal intestinal bacteria *in vivo* to give one metabolite (**425**). The same compound was biotransformed by rat liver microsomes *in vitro* to give eight metabolites (**426–432**) (**Scheme 67**) and it was suggested that toxicity of nutmeg is not solely caused by phenylpropane with allyl group, but some of the neolignans with the same allylic group.[75]



Scheme 61 Biotransformation of 2,3-dehydrokievitone (**318**) by *Botrytis cinerea* and *Aspergillus flavus*.

Grifolin (**433**) isolated from the inedible mushroom, *Albatrellus confluens*, was treated in *A. niger* in Czapek-peptone medium to give seven metabolites **434–440** of which **436** was the major product (**Scheme 68**). Neogrifolin (**441**) was also treated in the same manner as described above to afford the metabolites **442–444** of which compound **444** was predominant (**Scheme 69**). The metabolites obtained and the substrates were tested against methicillin-resistant



Scheme 62 Biotransformation of chromanols (**325**, **326**) and vitamin E (**327**) by cultured cells of *P. americana* and *C. roseus*.

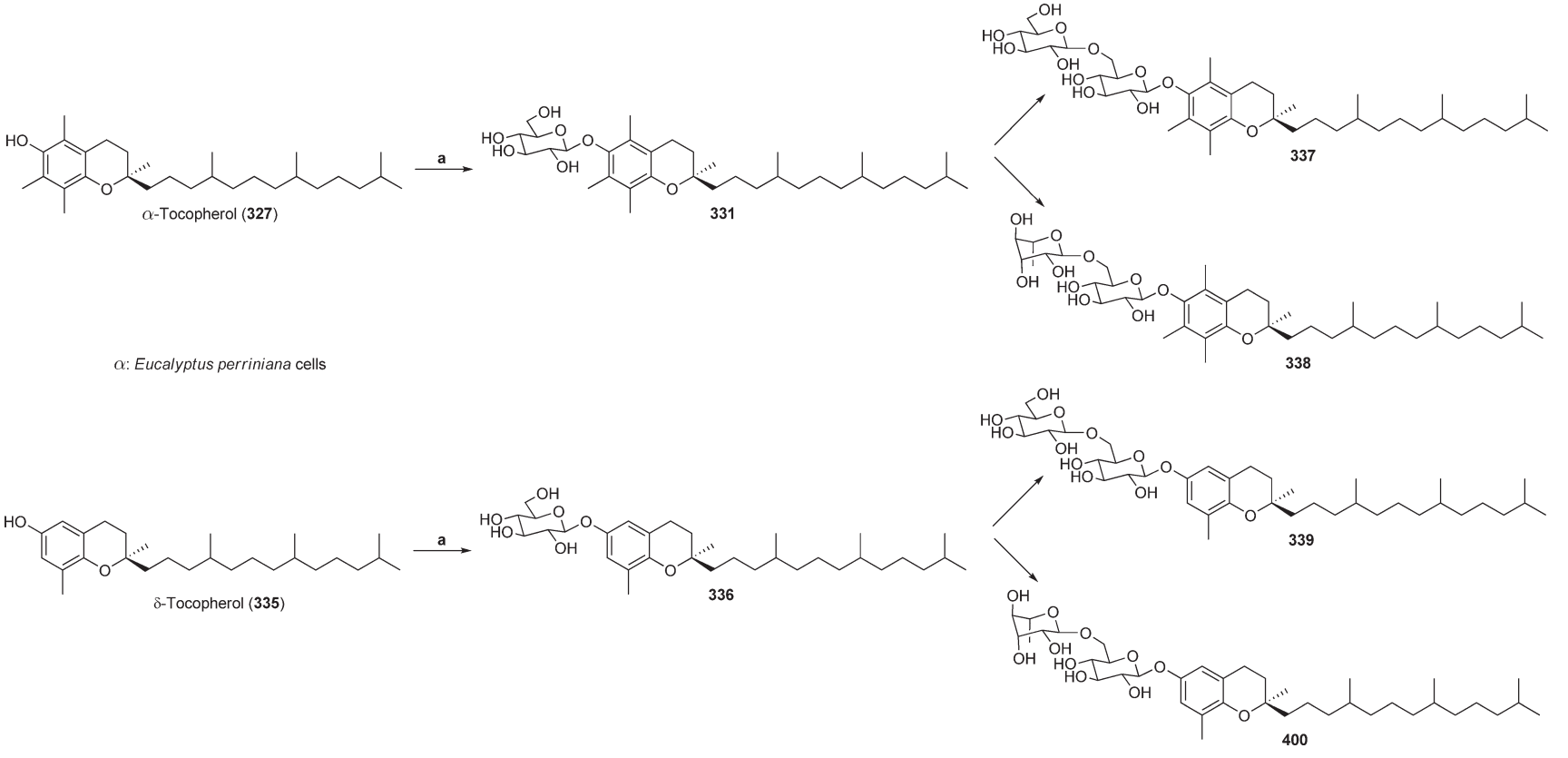
S. aureus (MRSA). Grifolic acid (**433**) showed strong antibacterial activity and compound **433** and neogrifolin (**441**) showed strong cytotoxic activity against HL-60.[76]

Immobilized cells of *Daucus carota* transformed acetophenone (**445**) into (1*S*)-phenethyl alcohol (**446a**) stereoselectively (**Scheme 70**). The same substrate was also bioconverted into (1*S*)-alcohol by immobilized cells of *Nicotiana tabacum*; however, neither stereoselective reduction nor oxidation was observed. High enantiomerically pure (1*S*)- (**446a**) and (1*R*)-alcohol (**446b**) (>99% ee) were produced by the biotransformation of acetophenone using *D. carota* and *Gardenia jasminoides*.[77]

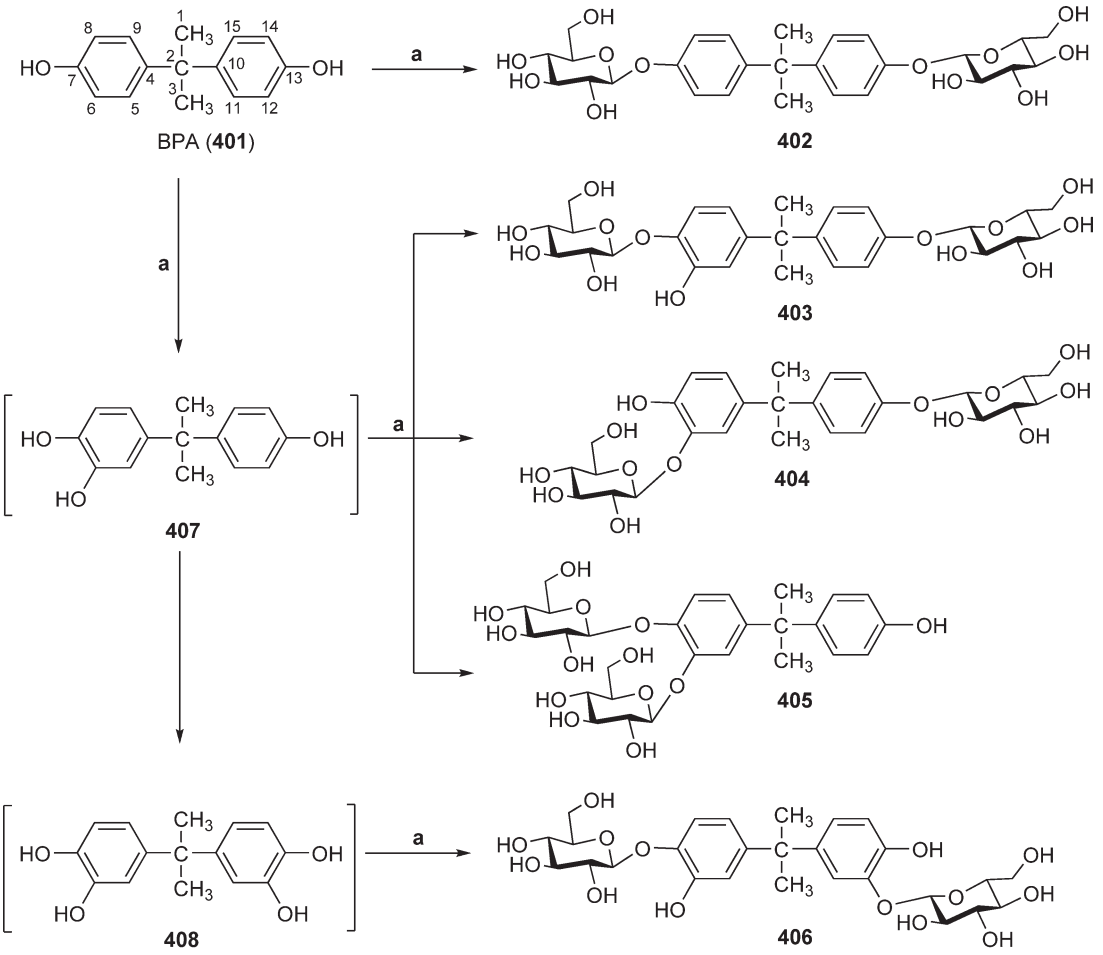
The effect of high hydrostatic pressure and high-pressure homogenization on the microbial reduction of acetophenone (**445**) was investigated. The yeast strain *S. cerevisiae* and *Yarrowia lipolytica* were used as microorganisms. In the case of the former fungus, acetophenone (**445**) was converted to (1*S*)-alcohol (**446a**), whereas the same substrate was converted to (*1R*)-alcohol (**446b**) by the latter fungus. In high-pressure condition, enantioselectivity is higher than statistic condition. But in both conditions, the yield of product from **445** was poor.[78]

Acetophenone (**445**) was incubated with a microalga, *E. gracilis*, to afford 1-phenylethanol (**446**); however, its ee is very poor (10%).[57] The same substrate (**445**) and propiophenone (**455**) were fed to *C. ellipsoidea* C-27(K) to furnish stereospecifically *S*-form-rich alcohols (**446a**, **447a**) in 84% ee, respectively[44] (**Scheme 71**).

H. anomala is a useful microorganism to produce chiral compounds. When racemate of 1-phenylethyl alcohol (**446**) was added into the medium of *H. anomala*, only 1*S*-form (**446a**) was stereoselectively converted to acetophenone (**445**), which was further reduced selectively to 1*R*-form (**446b**) and only **446b** was accumulated in the medium. The same phenomenon was observed in racemic 1-phenylpropanol (**447**), 1-phenylbutanol (**449**), 1-phenylpentanol (**450**), 1-phenylhexanol (**451**), 1-phenylheptanol (**452**), and 1-phenyloctanol (**453**) to give their corresponding ketones (**455–462**), followed by dehydrogenation to give each alcohol (**447b–467**) with *R*-configuration. In the case of 1-phenylnonanol (**453**) and 1-phenyldecanol (**454**), only *S*-form was selectively dehydrogenated to give the corresponding ketones (**461**, **462**), which were not reduced to each alcohol with *S*-configuration at all (**Scheme 71**).



Scheme 63 Biotransformation of α-tocopherol (vitamin E) (**327**) and δ-tocopherol (**335**) by *E. perriniana.*



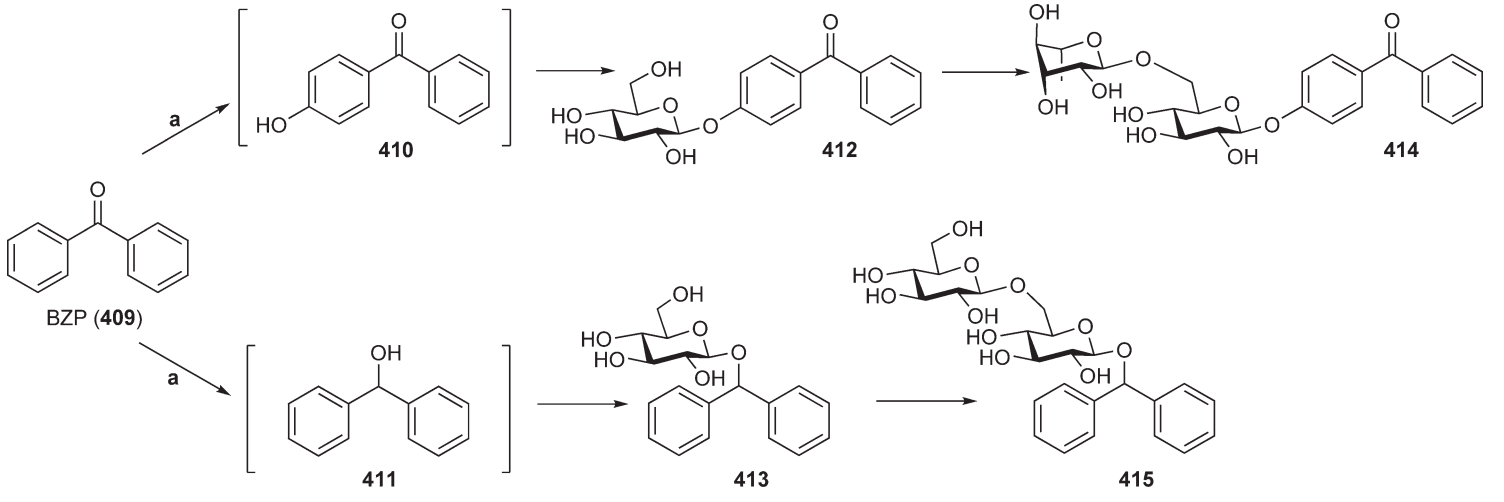
Scheme 64 Biotransformation of bisphenol A (**401**) by cultured cells of *E. perriniana*.

H. anomala also exhibited a remarkable enantioselectivity on the reduction of isobutyrophenone (**468**) to give the corresponding secondary alcohol (**469**) with *S*-configuration in 100% ee[79] (**Scheme 71**).

One of the environmentally friendly microorganisms, *G. candidum*, immobilized with a water-absorbent polymer in hexane and cyclohexane improved reactivity in enantioselective oxidation of 1-arylethanols (**446a, 446b, 470a, 470b**). Cyclohexanone as an additive improves the rate of oxidation as well as ee of the remaining alcohol. Racemic 1-phenylethanol (**446a, 446b**) and 1-(2-furyl) ethanol (**470a, 470b**) gave 99% ee of corresponding 1-arylethanols (**446b**, 50%; **471b**, 50%) and the corresponding ketones (**445**, 50%; **472**, 50%)[80] (**Scheme 72**).

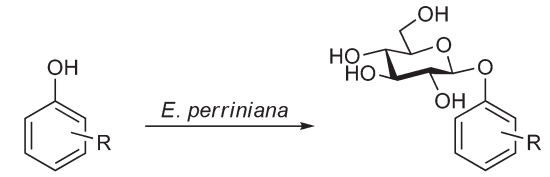
Incubation of aryl ketones (**445, 472–478**) with immobilized *G. candidum* in 2-hexanol and hexane system gave the corresponding secondary alcohols (**446a, 471, 479–484**) with *S*-configuration in 99% ee[81] (**Scheme 72**).

Reduction of alkylphenones, such as acetophenone (**445**), propiophenone (**455**), butyrophenone (**456**), 1-phenyl-2-propanone, benzylacetone, *m*-chloroacetophenone (**474**), and *p*-chloroacetophenone (**475**), proceeded enantioselectively to give pure *S*-arylalkanols in excellent yields when they were reduced by incubation on relatively dilute media under the condition of shorter reaction time, large amount of *G. candidum*, and the use of an argon atmosphere[82] (**Scheme 72**).



a: *Eucalyptus perriniana* cells

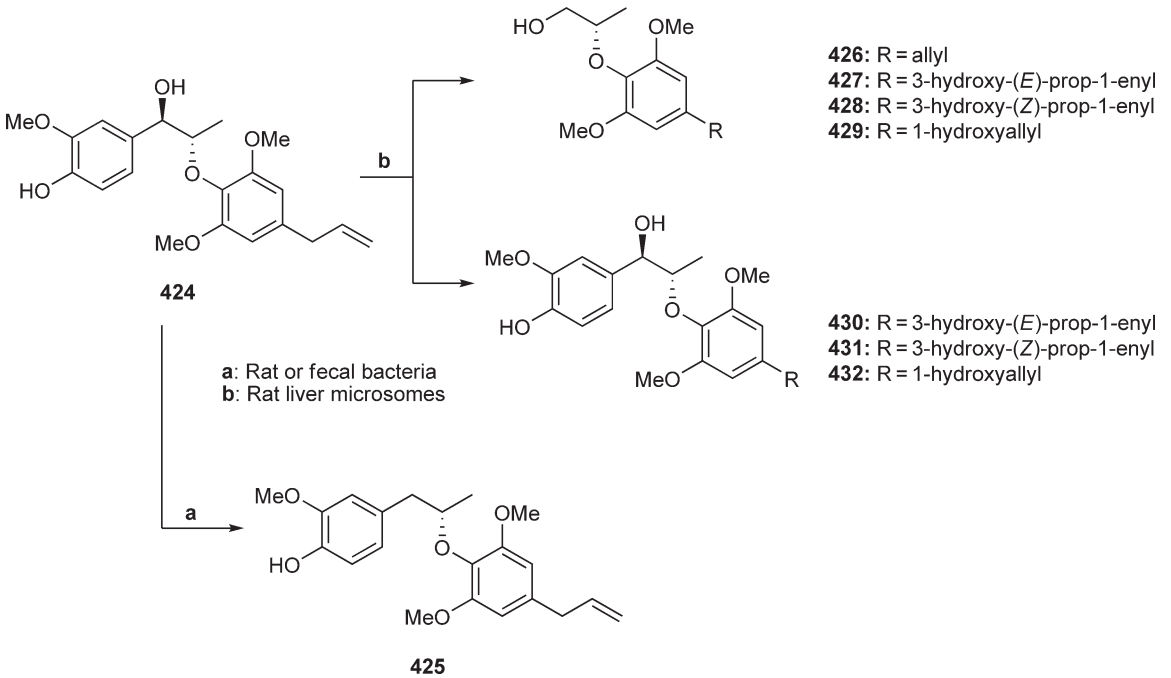
Scheme 65 Biotransformation of benzophenone (**409**) by cultured cells of *E. perriniana.*



416: R = H
417: R = *o*-F
418: R = *m*-F
419: R = *p*-F

420: R = H
421: R = *o*-F
422: R = *m*-F
423: R = *p*-F

Scheme 66 Biotransformation of phenol (**416**) and monofluorophenols (**417–419**) by cultured cells of *E. perriniana.*

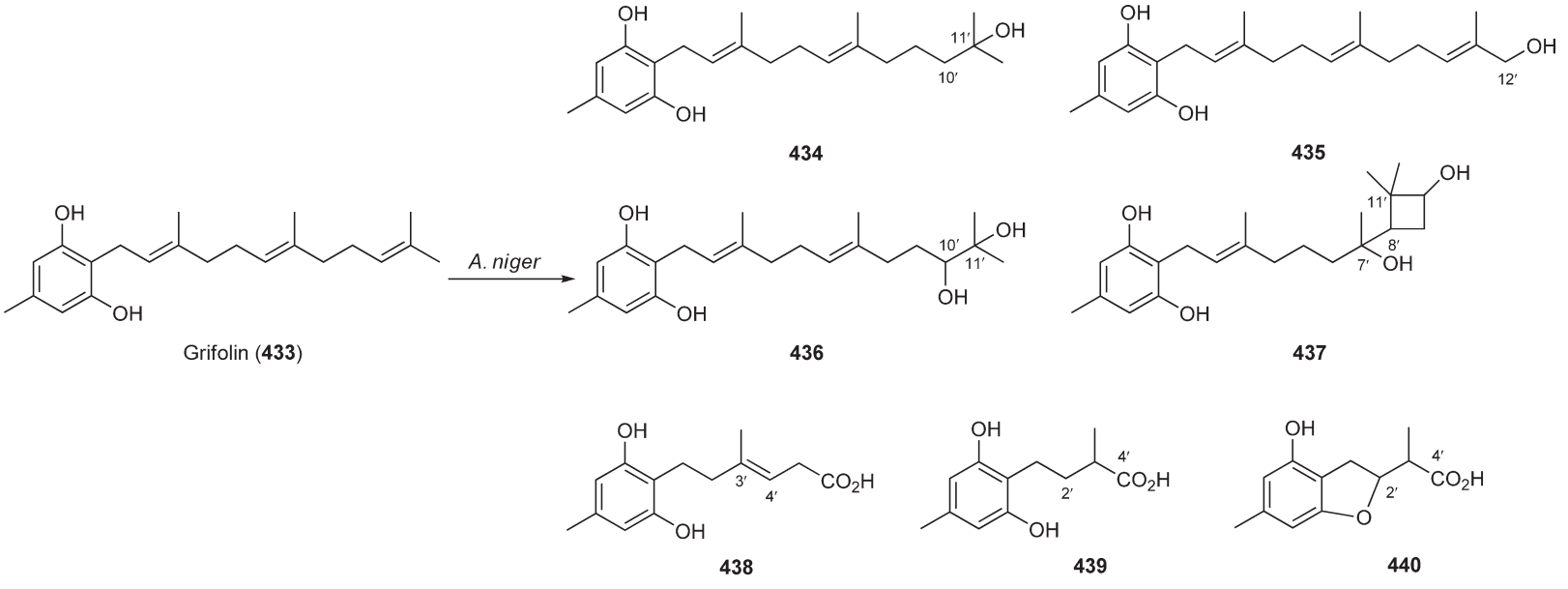


426: R = allyl
427: R = 3-hydroxy-(*E*)-prop-1-enyl
428: R = 3-hydroxy-(*Z*)-prop-1-enyl
429: R = 1-hydroxyallyl

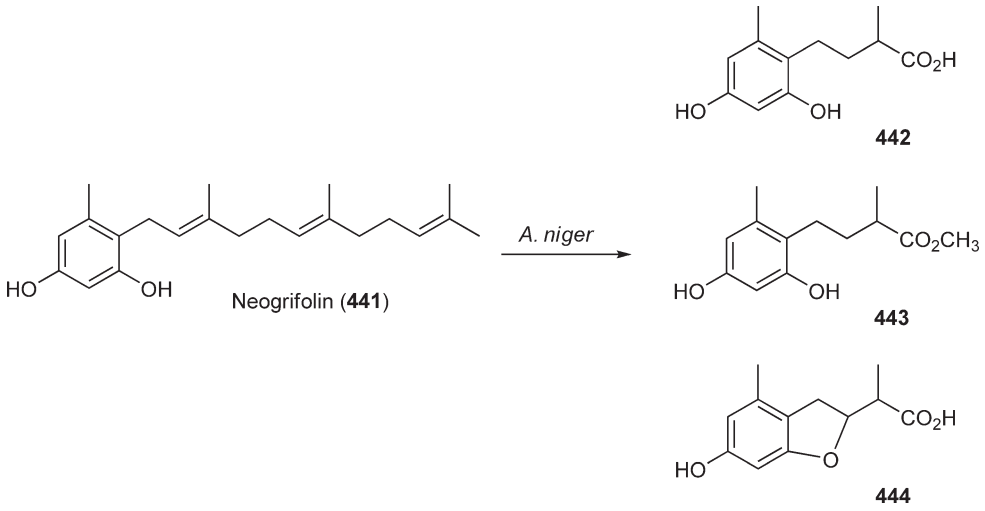
430: R = 3-hydroxy-(*E*)-prop-1-enyl
431: R = 3-hydroxy-(*Z*)-prop-1-enyl
432: R = 1-hydroxyallyl

Scheme 67 Biotransformation of myrisligan (**424**) by rats, fecal intestinal bacteria, and rat liver microsomes.

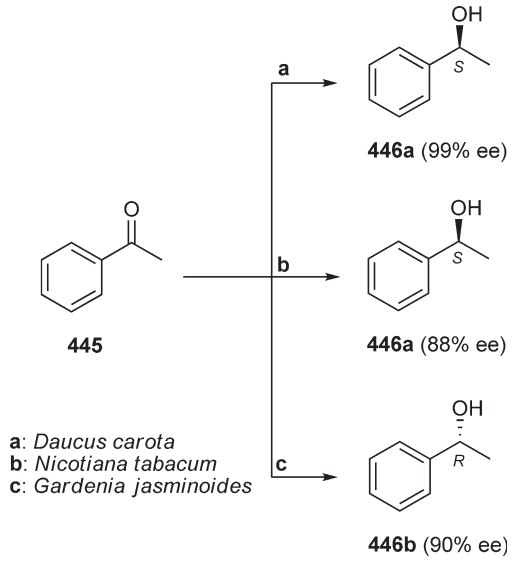
The acetone powder from *G. candidum* IFO4597 (APG4) catalyzes the reduction of ketones giving outstanding results in the presence of 2-hexanol or cyclopentanol and a small amount of a coenzyme, NAD^+. For example, acetophenone (**445**) and its chloro derivatives (**473, 474, 475**) are reduced by this method to give the corresponding (1*S*)-alkanols (**446a, 480, 481**) in good yield (89–99%) with high ee (99%). *o*-, *m*-, and *p*-Bromofluoroacetophenones, *o*-tolylacetophenone (**476**), 2-phenylethylacetophenone, and methoxyacetophenone were also treated in the same manner as described above to afford the corresponding secondary alcohols with *S*-configuration, whereas monofluoroacetophenone was converted to the corresponding secondary alcohol with *R*-configuration (**Scheme 72**). The reaction mechanism is as follows. As acetophenone is reduced to 1-alkanol, $NAD(P)^+$ is formed, which in turn is reduced to NAD(P)H by the coupled oxidation of 1-alkanol. Racemic 1-alkanol is used for the reaction; however, (1*S*)-alkanol is oxidized selectively to reduce the substrate to (*S*)-alcohol. Storage of the powder in a freezer reserves the enzyme activity for more than 1 year, whereas the resting cell of *G. candidum* is active for only a few days.[83,84]



Scheme 68 Biotransformation of grifolin (**433**) by *A. niger*.



Scheme 69 Biotransformation of neogrifolin (**441**) by *A. niger*.

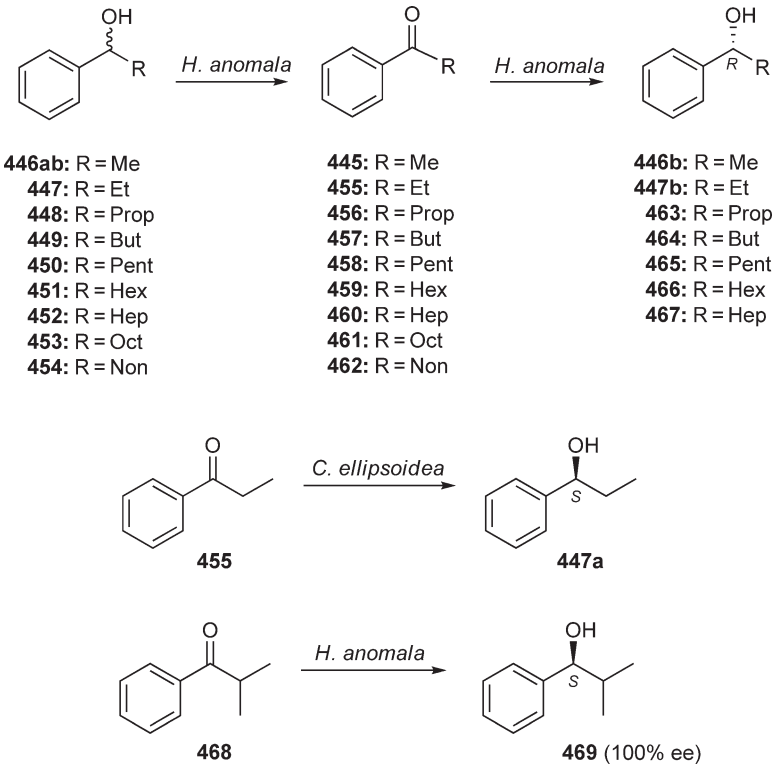


Scheme 70 Biotransformation of acetophenone (**445**) by immobilized cells of *D. carota*, *N. tabacum*, and *G. jasminoides*.

Reduction of acetophenone (**445**), *o*-tolylacetophenone (**476**), and *o*-, *m*-, and *p*-fluoroacetophenones and their related compounds were biotransformed by immobilized *G. candidum* cells in the presence of supercritical carbon dioxide to afford the corresponding (1*S*)-secondary alcohols (**446a, 482**) in 11–96% yield with 96–99% ee. In the case of monofluoroacetophenone, (*1R*)-secondary alcohol was obtained[85] (**Scheme 72**).

Saika and Naoshima[86] reported that *m*- (**474**) and *p*-chloroacetophenone (**475**), and *o*- (**470c**), *m*- (**470f**), and *p*-acetylpyridines (**470g**) were incubated with immobilized carrot cells to afford the corresponding (1*S*)-secondary alcohols (**471c, 471f, 471g**). *p*-Nitro-, *p*-bromo-, and *p*-methoxyacetophenones were also treated in the same manner as described above to give (1*S*)-secondary alcohols.

m- (**474**) and *p*-Chloroacetophenones (**475**), *o*- (**470c**), *m*- (**470f**), and *p*-acetylpyridines (**470g**), trifluoroacetylpyridine (**470d**), and 2-trifluoroacetyl-5-bromopyridine (**470e**) were also biotransformed by immobilized *G. candidum* cells to give the corresponding secondary alcohols (**480, 481**) and (**471c–g**) with (1*S*)-configuration (99–100% ee) in 4.4–90% yield[87] (**Scheme 72**).



Scheme 71 Biotransformation of acetophenone (**445**) and the related compounds (**455–462**), and 1-phenethyl alcohol (**446a**, **446b**) by *E. gracilis*, *C. ellipsoidea*, and *H. anomala.*

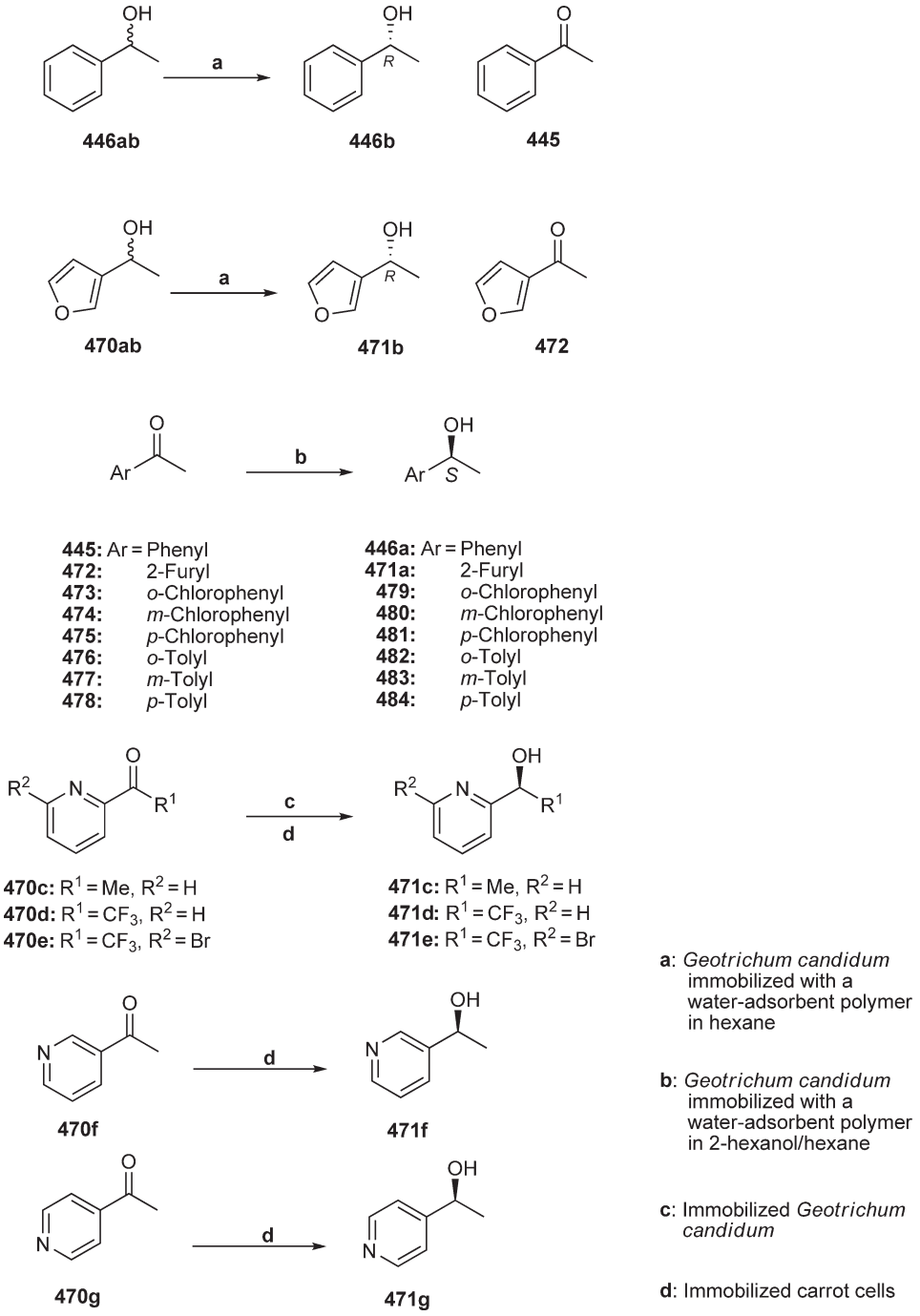
Nakamura *et al.*[88] summarized stereoselective oxidation and reduction of 1-arylethanols and alkylphenones by immobilized *G. candidum* in an organic solvent.

Butyrophenone (**456**), pentanophenone (**457**), hexanophenone (**458**), heptanophenone (**459**), octanophenone (**460**), isobutyrophenone (**468**), *o*-, *m*-, and *p*-tolylacetophenones (**476–478**), *m*-hydroxyacetophenone, and *o*-, *m*-, and *p*-methoxyacetophenones were also incubated with *C. ellipsoidea* in Noro medium to afford the corresponding secondary alcohols, whereas *C. pyrenoidosa* bioconverted racemic 1-phenylbutanol (**448**) into butyrophenone (**456**) in 20% yield. *C. pyrenoidosa* bioconverted acetophenone (**445**), hexanophenone (**458**), and heptanophenone (**459**) into the corresponding secondary alcohols with *R*-configuration. In addition, 1-phenyl-2-butanone, isobutyrophenone (**468**), *trans*-4-phenyl-3-buten-2-one, and benzoylacetone were incubated with the same algae to afford 1-phenyl-2-butanol (57%), 1-phenyl-2-methyl-1-propanol (**469**), *trans*-4-phenyl-2-oxo-4-butanol (*R*:*S* = 50:50), and 4-phenyl-4-oxo-2-butanol, respectively[44] (**Scheme 71**).

Immobilized acetone powdered *Glomerella candidum* was approached to trifluoromethyl ketones containing a sulfur functionality. 1,1,1-Trifluoro-3-(phenylthio)propan-2-one (**485**) was fed to immobilized *G. candidum*, except for the presence of cyclopentanol in the place of 2-hexanol as reducing agent, to afford the corresponding 2-alkanol (**486**) with *R*-configuration in 98% ee. In the case of 1,1,1-trifluoro-5-(phenylthio)propan-2-one (**487**) and trifluoroacetyl-α-thiophene (**489**), the corresponding 2-alkanols (**488**, **490**) were obtained with *S*- and *R*-configuration, respectively, in 99% ee[89] (**Scheme 73**).

The tissue cultured cells of the thalloid liverwort, *M. polymorpha*, in Murashige and Skoog (MS) medium could reduce trifluoroacetophenone (**491**), trifluorobenzylacetone (**492**), perfluoroacetophenone (**493**), and trifluoroacetyl-α-thiophene (**489**) to the corresponding secondary alcohols (**494–496**) with *S*-configuration and **490** with *R*-configuration[90] (**Scheme 74**).

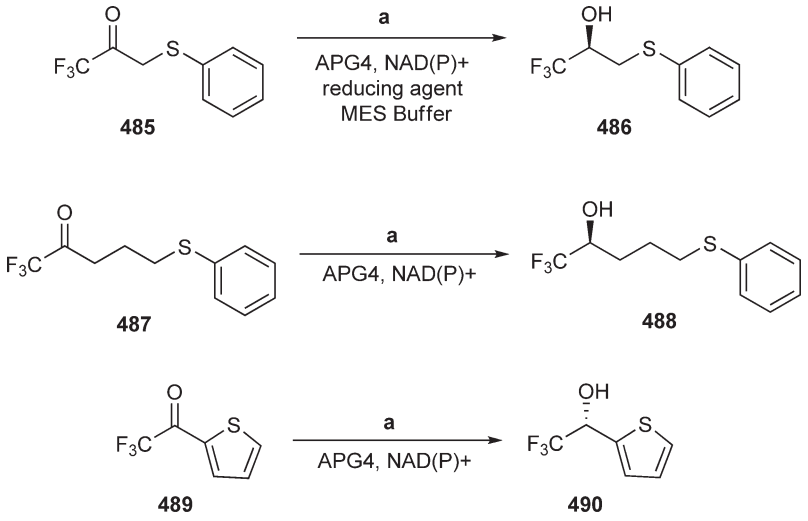
Cyanobacterium (*Synechococcus* sp. PCC 7942) also converted under illumination (1000 lux) perfluoroacetophenone (**493**) to (2*S*)-alcohol (**496**) in 90% yield and its ee was 99%.



Scheme 72 Biotransformation of aryl ketones (**445**, **472–478**) by *G. candidum*.

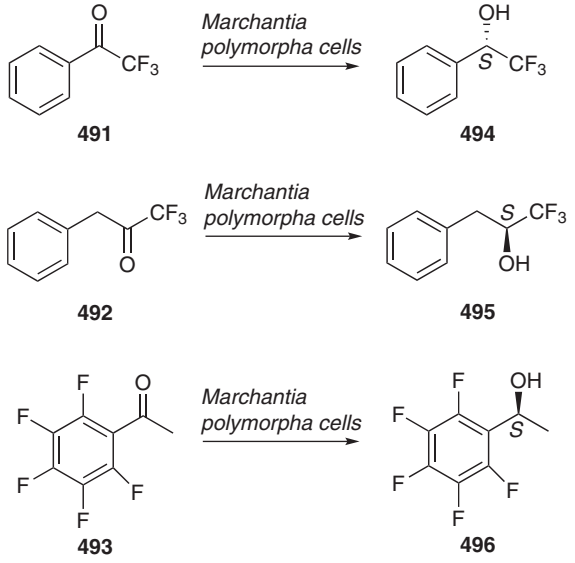
o-, *m*-, and *p*-Chloroacetophenones (**473–475**), *o*-, *m*-, and *p*-tolylacetophenones (**476–478**), *o*-, *m*-, and *p*-methoxyacetophenones, and *o*-, *m*-, and *p*-fluoroacetophenones were also treated in the same manner as described above to give the corresponding (2*S*)-alcohols in 96–100% ee.[91]

In the biotransformation of benzalacetone (**496a**), benzalacetophenone (**496b**), and benzoyl acetone (**496i**), reduction of double bond in either the side chain or carbonyl group was observed. Compound **496a** was incubated with *Aspergillus* species and *E. gracilis* to give only 4-phenyl-2-butanone (**496c**) and a mixture



a: Immobilized *Geotrichum candidum*

Scheme 73 Biotransformation of 1,1,1-trifluoro-3-(phenylthio)propan-2-one (**485**) and the related compounds (**487**, **489**) by *M. polymorpha* cells.

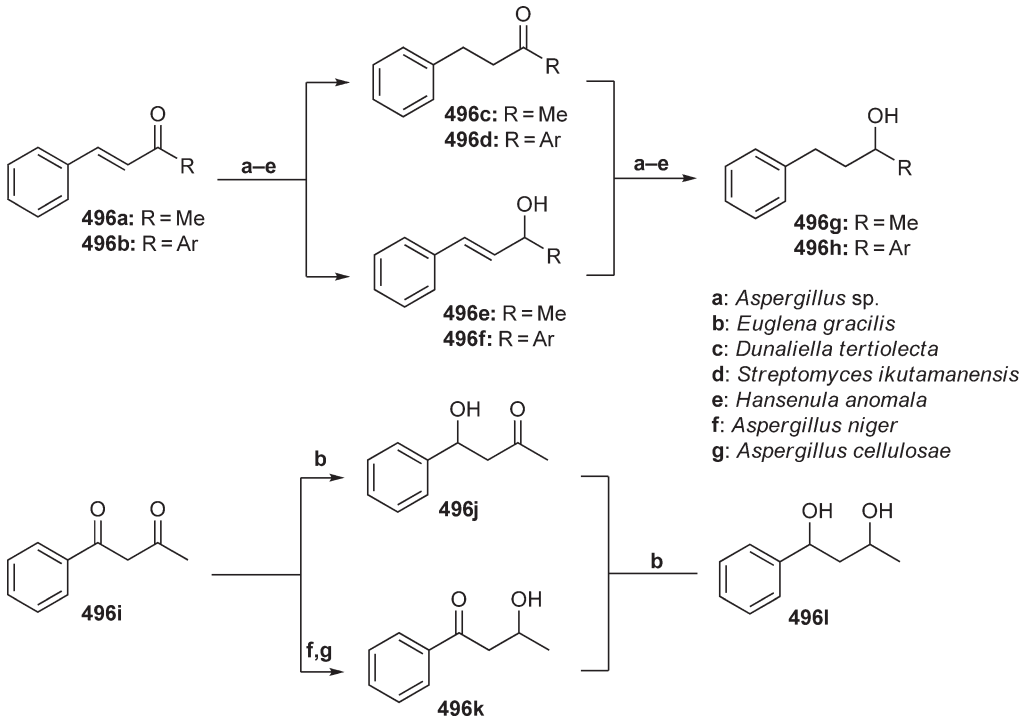


Scheme 74 Biotransformation of trifluoroacetophenone (**491**) and the related compounds (**492**, **493**) by cultured cells of *M. polymorpha.*

of **496c** and 4-phenyl-2-butanol (**496g**). *D. tertiolecta*, *S. ikutamanensis*, and *H. anomala* gave the same mixture (**496c**, **496g**) and 4-phenyl-2-buten-3-ol (**496e**) (**Scheme 75**).

All microorganisms described above converted **496b** to **496d**; however, **496d** was not reduced to **496h** except in *E. gracilis*. There are no microorganisms to reduce **496f**. In the case of benzoylacetone (**496i**), *A. niger* and *A. cellulose* biotransformed it to only **496k**, whereas *E. gracilis* produced **496j–496l**[46,47] (**Scheme 75**).

Biotransformation of 1-(4-methoxybenzo)cyclobutenecarbonitrile (**497**) with suspension cultured cells of cotton in MS medium gave benzoketone (**498**, 23.1%), which is a key intermediate in the synthesis of



Scheme 75 Biotransformation of benzalacetone (**496a**), benzalacetophenone (**496b**), and benzoyl acetone (**496i**) by *A. cellulosae*, *A. niger*, *D. tertiolecta*, *E. gracilis*, *H. anomala*, and *S. ikutamanensis*.

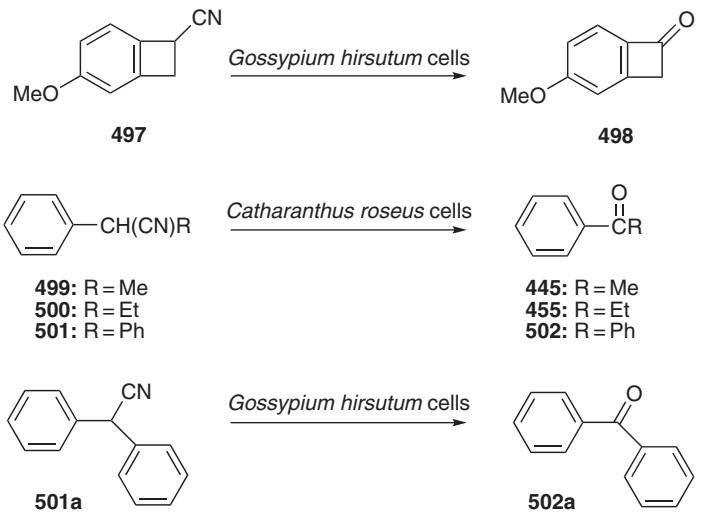
14α-hydroxyestrone (**Scheme 76**). Alkyl cyanides (**499–501a**) were treated in the same manner described above to give the corresponding alkylphenone (**445, 455, 502a**) in yields of 37.5, 9.1, and 58.8%.[92]

Racemic styrene oxides (**503a, 503b**) were incubated with buffered resting-cell suspension of *A. niger* for 7 h to give (2*R*)-diol (**504**) in 54% yield with low ee (51%) and (1*S*)-styrene oxide (**503a**) was recovered with a very high ee (28% yield, 96%). The substrates **503a** and **503b** were incubated with *A. niger* and *Beauveria sulfurescens* to afford only (2*R*)-diol (**504**) in 92% yield and 89% ee, whereas **503a** and **503b** were fed to *B. sulfurescens* only to afford (2*R*)-diol (**504**) with 83% ee in 47% yield and (*1R*)-styrene oxide was recovered showing high ee (34% yield, 98% ee) after only 2 h (**Scheme 77**). These results show that the two different microorganisms can achieve an enantioselective hydrolysis of racemic styrene oxide (**503a, 503b**) and present an opposite enantioselectivity for the substrate enantiomers.[93]

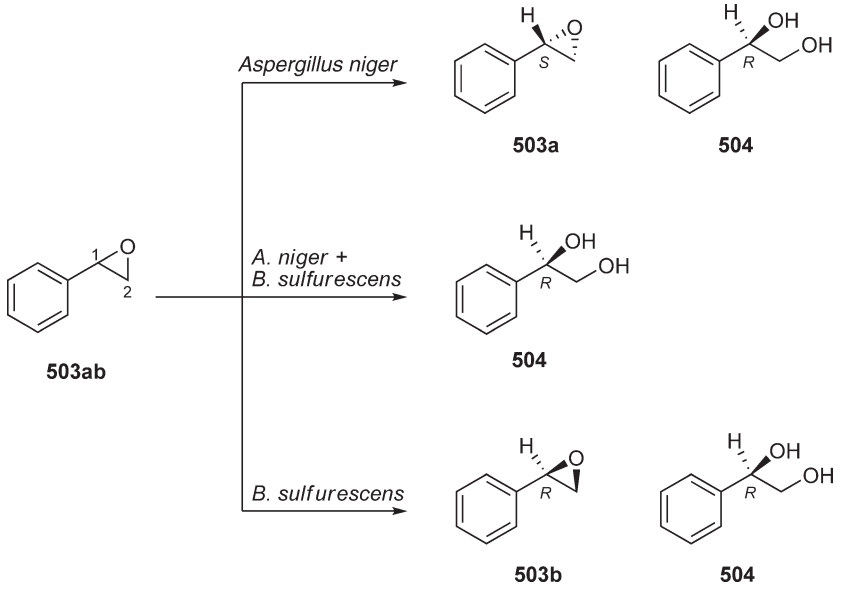
Reduction of 1-carbomethoxy-2-tetralone (**505**) and 3-carbomethoxy-2-tetralone (**507**) was carried out by yeast and fungal strains. *S. cerevisiae*, *Rhizopus arrhizus*, *Mucor racemosus*, and *Sporotrichum exile* converted **505** into **506a** having (1*R*,2*S*)-configuration, whereas *Saccharomyces montanus* and *Aspergillus ochraceus* produced **506a** and **506b** possessing (1*R*,2*S*)- and (1*R*,2*R*)-configuration and only **506b** (*1R*,2*R*), respectively (**Scheme 78**).

When 3-carbomethoxy-2-tetralone (**507**) was incubated with *S. cerevisiae* and *S. montanus*, product **508a** with (2*S*,3*R*)-configuration was obtained in good yield (99–97%). *R. arrhizus*, *M. racemosus*, and *S. exile* gave both **508a** (2*S*,3*R*) and **508b** (2*S*,3*S*) products whose ee is excellent (89–94%), whereas *A. ochraceus* gave (2*R*,3*S*)-carbomethoxyalcohol (**508c**) in 89% ee and *Rhodotorula glutinis* only (2*S*,3*S*)-metabolite (**508b**) in 95% ee (**Scheme 78**). These results afford valuable asymmetric hydroxy ester synthons for organic synthesis.[94]

Sixty thousand microorganisms from Saskatchewan soil were screened for growth on the cytokinin N^6-benzyladenine (**509**) as carbon source. *Serratia proteamaculans* biotransformed **509** into 8-hydroxy-N^6-benzyladenine (**512**). Adenine (**510**) and isopentenyladenine (**511**) were also incubated with the same microorganism to afford 8-hydroxyadenine (**513**) and 8-hydroxyisopentenyladenine (**514**), respectively (**Scheme 79**). This is the first report on the metabolites of cytokinins resulting from biotransformation of *S. proteamaculans* and first hydroxylated cytokinin metabolites reported to be natural products.[95]



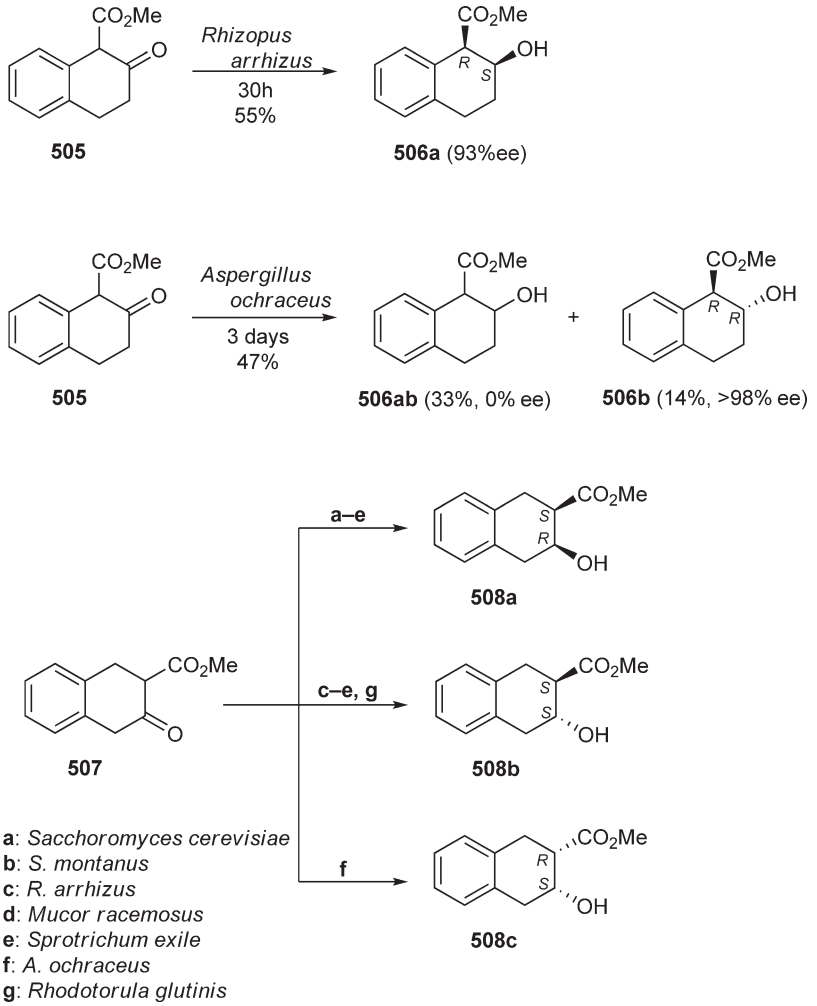
Scheme 76 Biotransformation of 1-(4-methoxybenzo)cyclobutenecarbonitrile (**497**) and alkyl cyanides (**499–501a**) by cultured cells of *Gossypium hirsutum*.



Scheme 77 Biotransformation of racemic styrene oxide (**503a**, **503b**) by *Aspergillus niger* and *Beauveria sulfurescens.*

3.21.4 Biotransformation of Cyclohexane Derivatives and Other Selected Synthetic Compounds by Microorganisms

Various α,β-unsaturated ketones (**515**, **518**, **521**, **524**) were biotransformed by *B. sulfurescens*. 4-Methyl-2-cyclohexenone (**515**) gave saturated ketone (**516a**, **516b**) (65%) and saturated alcohol (**517**) (35%). Racemic 5-methyl- (**518**) and 6-methyl-2-cyclohexenone (**521**) gave saturated ketones (**519**, **522a**, **522b**, **523a**, **523b**) (70% from **518** and 30% from **521**) and saturated alcohol (**520**) (30% from **518** and 70% from **521**). Both products **522a**, **522b** and **523a**, **523b** showed optical activity, resulting from preferential attack on one

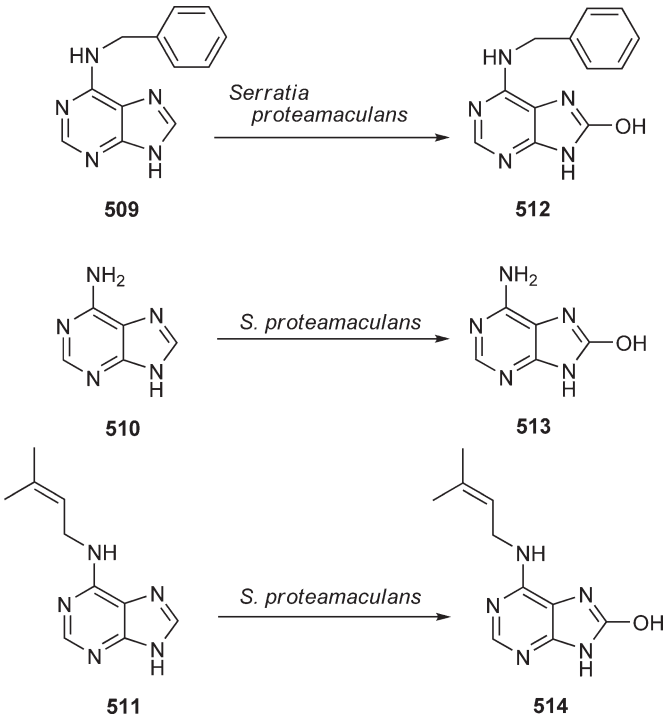


Scheme 78 Biotransformation of 1-carbomethoxy-2-tetralone (**505**) and 3-carbomethoxy-2-tetralone (**507**) by *Rhizopus arrhizus*, *Aspergillus ochraceus*, *Saccharomyces cerevisiae*, *S. montanus*, *Mucor racemosus*, *Sporotrichum exile*, and *Rhodotorula glutinis.*

enantiomer of the saturated ketones **522a** and **522b** (**Scheme 80**). Reduction of the carbonyl group was slower than that of the double bond of the unsaturated ketone and the mixture of ketones **522a** and **522b** was dextrorotatory, showing a proportion of 80% of the *S*-isomer (**522a**). On the contrary, 2,6-dimethyl- (**536**) and 2,6,6-trimethylcyclohexene-1-one did not give any metabolites by this microbe. 3,5,5-Trimethylcyclohex-2-en-1-one (**524**) was reduced by the same microorganism during 5 days incubation though much more slowly than lesser substituted homologues, to give a saturated ketone **525** in 27%, having *R*-configuration at C-2 and a secondary alcohol (**526**) in 8% yield having 1*S*, 2*R*-configuration[96] (**Scheme 80**).

A. niger also converted 4-methyl-2-cyclohexenone (**515**) and 3-methyl-2-cyclohexenone (**518**) to 4-methylcyclohexanone (**516a, 516b**) and 4-methylcyclohexanol (**517**), and 3-methylcyclohexanone (**519a, 519b**) and 3-methylcyclohexanol (**520**)[46]

D. tertiolecta reduced cycloalkenones, cycloalkanones, and linear alkanones to the corresponding secondary saturated ketones and/or saturated alcohols. 2-Methyl-2-cyclohexene-1-one (**528**) gave (−)-2-methylcyclohexanone (**522b**) and (+)-2-methylcyclohexanol (**523b**) (**Scheme 81**). 2-Cyclohexanone was also easily converted to cyclohexanone and cyclohexanol. 4-Methylcyclohexanone (**516a, 516b**), 3-methylcyclohexanone (**519a, 519b**), and 2-methylcyclohexanone (**522a, 522b**) were incubated with *D. tertiolecta* to afford *cis*- and *trans*-4-methyl hexanols (**517a, 517b**, 71%:29%; **520a, 520b**, 73%:16%; **523a, 523b**, 55%:28%), respectively (**Scheme 80**).



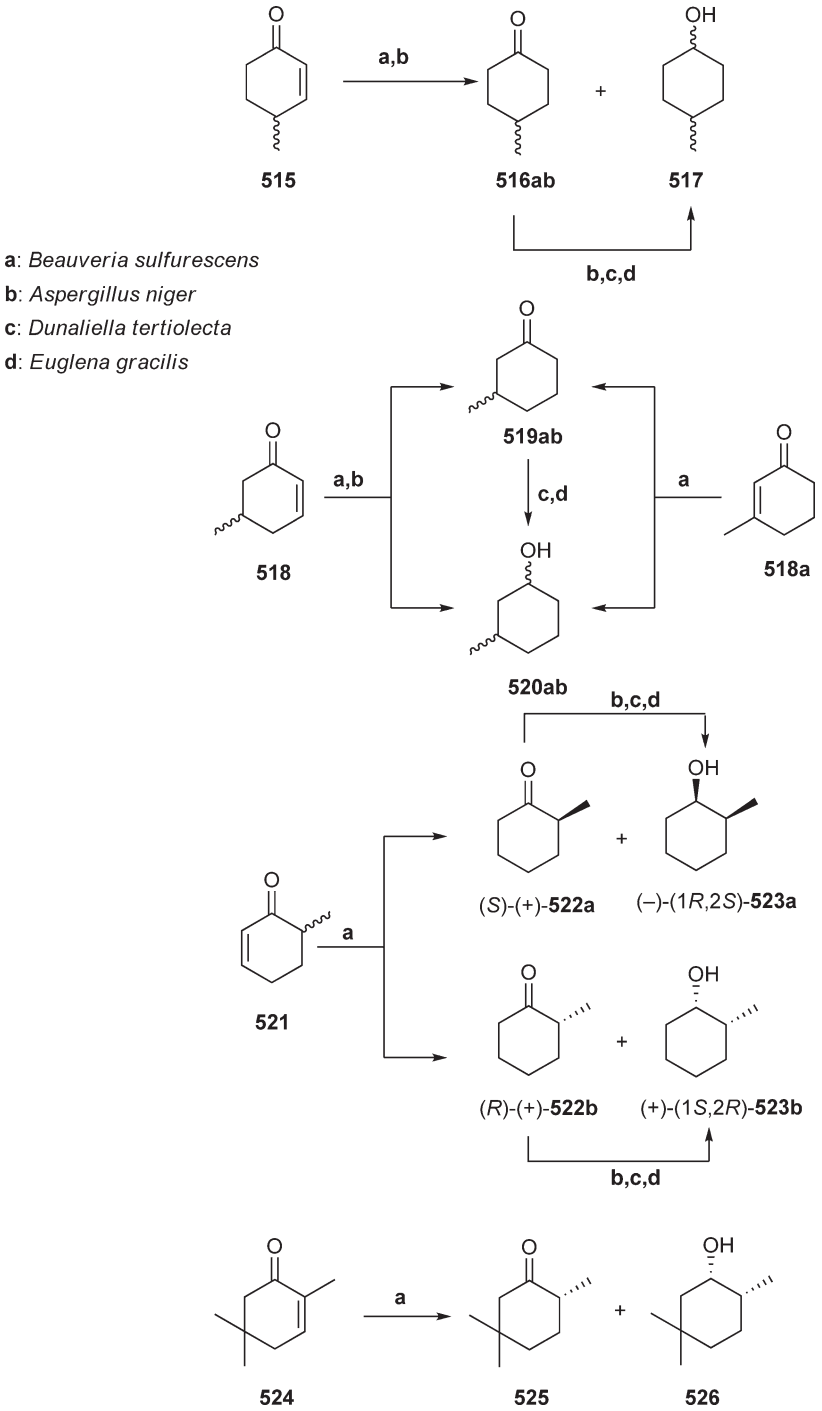
Scheme 79 Biotransformation of N^6-benzyladenine (**509**), adenine (**510**), and isopentenyladenine (**511**) by *Serratia proteamaculans*.

Cyclobutanone, cyclopentanone, cyclohexanone, cycloheptanone, cyclooctanone, cyclodecanone, and cyclododecanone were converted into the corresponding secondary alcohols in 20–100% yield for 2–8 days. 2-Butanone, 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone, and 2-decanone and related liner ketone were fed to *D. tertiolecta* to give the corresponding secondary alcohols.[48]

Racemic 4-methyl-2-cyclohexanone (**516a, 516b**), 3-methyl-2-cyclohexanone (**519a, 519b**), and 2-methyl-2-cyclohexanone (**522a, 522b**) were converted by *A. niger* to the corresponding secondary alcohols (**517, 520, 523a, 523b**). 2-Methyl-2-hexenone (**528**) and cyclohexanone were also treated in the same manner as described above to afford the saturated ketone (**522a, 522b**), secondary alcohol (**523a, 523b**), and cyclohexanol[46] (**Scheme 80**).

When 1-methylcyclohexene was incubated with *A. cellulosae*, 3α- and 3β-hydroxy-1-methylcyclohexenes were obtained. Treatment of these two products with the same fungus gave 3-methyl-2-cyclohexanone (**519**). Cyclohexene was also treated in the same manner as described above to afford 3-hydroxycyclohexene and 2-cyclohexenone.[46] On the basis of the above results, microbial biotransformation of cycloalkanones and cycloalkanols (C-5, C-6, C-10, and C-12) was carried out. Equal mixtures of cycloalkanones and cycloalkanols (C-5, C-8, C-10, and C-12) were fed to 10 kinds of *Aspergillus* species. In the case of *A. awamori* and two strains of *A. cellulosae*, cycloalkanones of C-5, C-7, C-8, and C-10 and cycloalkanols of C-6 and C-12 were formed preferentially. In the case of *A. niger* IFO4034 and *A. fumigatus*, all cycloalkanones were predominant. *Aspergillus niger* IFO4049, a strain of *A. niger*, *A. sojae*, and *A. terreus* accumulated cycloalkanones of C-7, C-8, C-10, and C-12 predominantly. Cycloalkanones of C-5, C-7, C-8, C-10, and C-12 were accumulated in the case of *A. usami*. In *A. awamori*, *A. niger* IFO4049, and a strain of *A. niger*, C-8, C-10, and C-12 were further bioconverted to the corresponding hydroxylated products.[46]

E. gracilis also contains reductase as seen in *Aspergillus* and *Dunaliella*.[47] 4-Methylcyclohexanone (**516a, 516b**), 3-methylcyclohexanone (**519a, 519b**), and 2-methylcyclohexanone (**522a, 522b**) gave the corresponding *cis*/*trans* secondary alcohols (**517**) (*cis*:*trans* 25%:75%), **520a, 520b** (80%:20%), and 2-methylcyclohexanols (**523a, 523b**) (46%:39%). 2-Methylcyclohexenone (**518**) gave (−)-2-methylcyclohexanone (**522b**) and (+)-2-



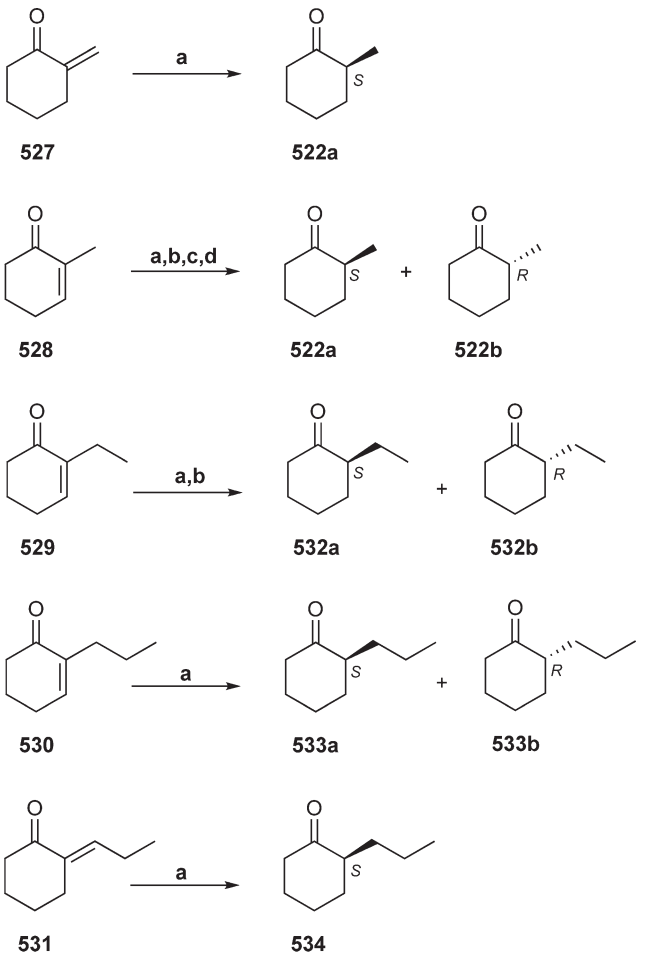
Scheme 80 Biotransformation of cyclohexene derivatives (**515**, **518**, **521**, **524**) and the related compounds (**516a**, **516b**,**519a**, **519b**, **522a**, **522b**) by *A. niger*, *B. sulfurescens*, *D. tertiolecta*, *E. gracilis*, and *H. anomala.*

methylcyclohexanol (**Scheme 80**) (**523b**). The same phenomenon occurred in the case of 2-cyclohexanone to give cyclohexanone, which was further reduced to cyclohexanol. Cycloalkanones (C-4 to C-10) were fed to *E. gracilis* to afford the corresponding secondary cycloalcohols in 16–100% yield for 6–11 days.

In the case of cyclobutanone, in the beginning, it was easily converted to cyclobutanol in 98% yield, which in turn was gradually dehydrogenated to cyclobutanone in 93% yield. The preferential order for the reduction of cycloalkanones was C-4 > C-7 > C-6 > C-12 > C-8 > C-10, whereas the preferential order for the dehydrogenation of the cycloalkanols was C-5 > C-4 > C-10 > C-8 > C-7 > C-12. It is noteworthy that cyclopentanone was not reduced and cyclohexanol was not dehydrogenated at all. In the case of the biotransformation of an equal mixture of cycloalkanones and cycloalkanols, the same phenomenon as described above was observed. A number of linear alkyl ketones were also treated in the same manner as described above to give the corresponding saturated secondary alcohols.[47]

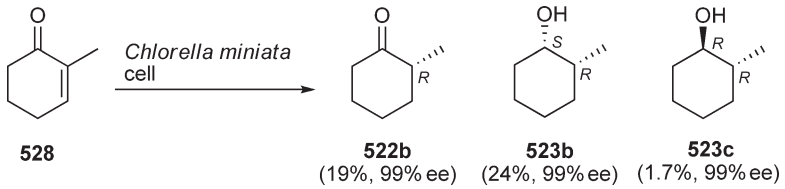
Cyclohexenone derivatives (**528, 529**) were treated with the enone reductase isolated from Cyanobacterium only and the same enzyme in complex with microsomal enzyme to give the dihydro derivatives (**522a, 532a**) with *S*-configuration in excellent ee (over 99%) and the metabolites **522b** and **532b** with *R*-configuration in relatively high ee (85 and 80%)[97] (**Scheme 81**).

2-Methyl-2-cyclohexene-1-one (**528**) was incubated with *Chlorella miniata* to give (*R*)-2-methylcyclohexanone (**522b**, 19% 99% ee) and then (1*R* 2*R*)-2-methylcyclohexanol (**532a**, 1.7%, 99% ee) and (1*S*,2*R*)-2-



a: *Nicotiana tabacum* cells
b: *Cyanobacterium*
c: *Aspergillus niger*
d: *Dunaliella tertiolecta*

Scheme 81 Biotransformation of alkyl cyclohexanones (**527–530**) and alkenyl cyclohexane (**531**) by *Aspergillus niger*, *Dunaliella tertiolecta*, *Cyanobacterium*, and cultured cells of *Nicotiana tabacum*.



Scheme 82 Biotransformation of 2-methyl-2-cyclohexene-1-one (**528**) by cultured cells of *Chlorella miniata.*

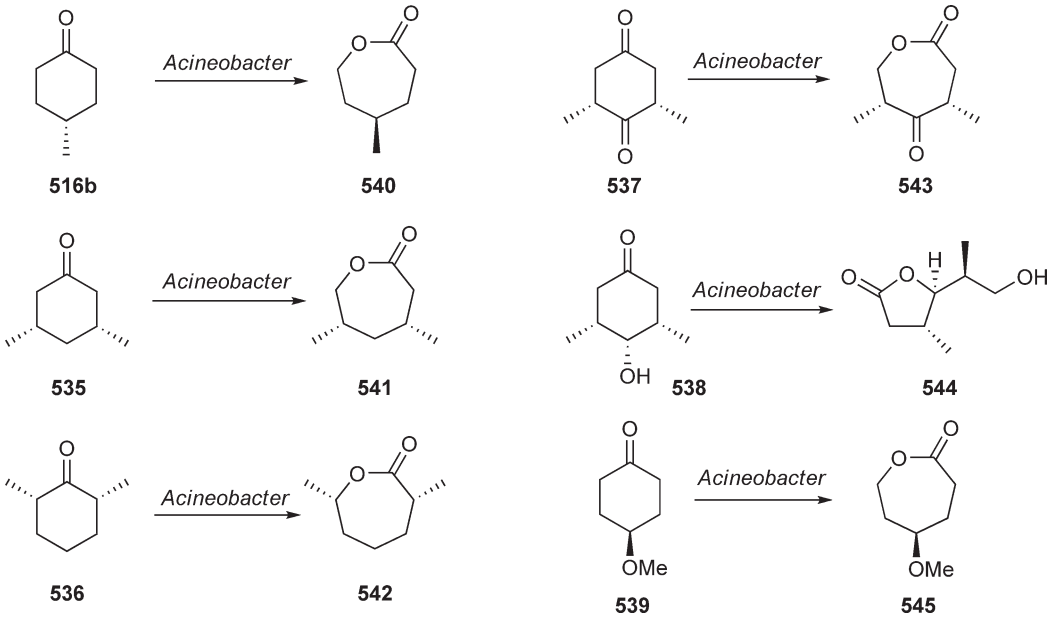
methylcyclohexanol (**532b**, 24%, 99% ee) (**Scheme 82**). However, 2-ethyl-, 2-propyl-, 2-pentyl-2-cyclohexene-1-one, and 3-methyl-2-cyclohexen-1-one were not biotransformed by this alga.[98]

Asymmetric hydrogenation of the C–C double bond of enones (**527–531**) was carried out by reductases from *Nicotiana tubacum* using MS medium for 3 weeks. The enone reductase reduced enantiotropically the C–C double bond of enones to give optically active 2-alkylated ketones (**522a–534**)[99,100] (**Scheme 82**).

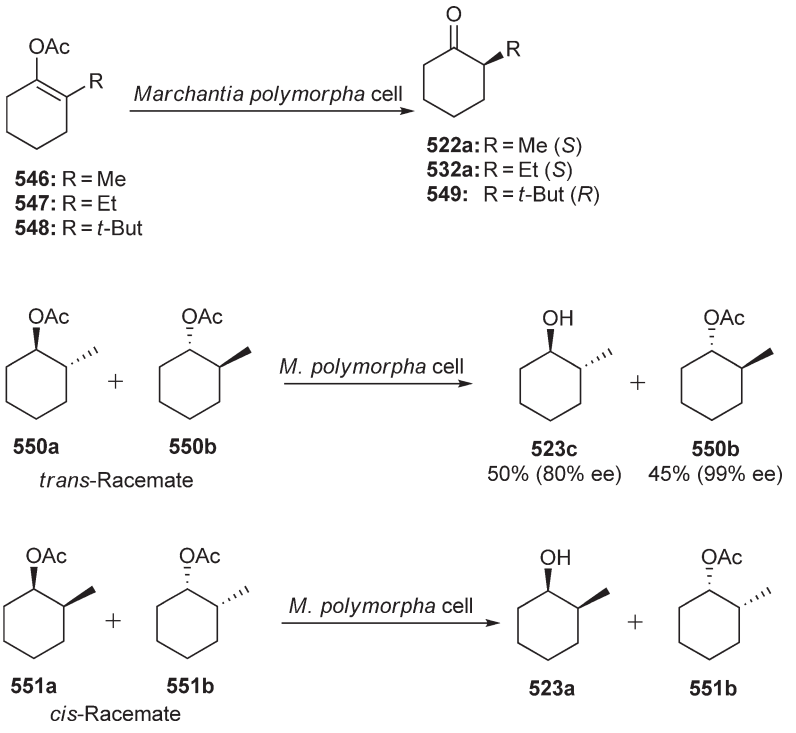
Enzymatic Baeyer–Villiger oxidation with the enzyme isolated from *Acinetobacter* NCIB 9871, cyclohexanone oxygenase, in the enantioselective preparation of lactones from a number of mesomeric cyclohexanones (**516b, 535–539**) was reported by Taschner and Black.[101] The enantiomeric purity of absolute configuration was determined by conversion of the alcohol to (−)-α-methyl-α-(trifluoromethyl)phenylacetic acid ester. Except lactone **545**, all the other lactones (**540–544**) showed negative optical rotation with more than 98% ee (**Scheme 83**).

The cultured cells obtained from the liverwort, *M. polymorpha*, transformed several enol acetates, such as 1-acetoxy-2-alkylcyclohexenes (**546–548**), to the corresponding ketones (**522a, 532a, 549**) enantioselectively (**Scheme 84**). The enzyme that hydrolyzed enol acetates was purified from the suspension cultured cells by hydrophobic chromatography on butyl-TOYOPEARL. One of two enzymes played a role in the hydrolysis of enol acetates.[102]

Hirata *et al.*[103] reported enantioselectivity in the hydrolysis of racemic *trans*- (**550a, 550b**) and *cis*-2-methylcyclohexyl acetates (**551a, 551b**) with cultured cells of the liverwort, *M. polymorpha.* The cultured cells hydrolyzed preferentially those acetates possessing the *R*-configuration, (*1R,2R*)-(−)-*trans*-2-methylcyclohexanol (**523c**) in 50% yield with 80% ee. The acetate was recovered unchanged in a 45% yield and identified as (1*S*,2*S*)-(+)-*trans*-2-methylcyclohexanyl acetate (**550b**) with 99% ee. From *cis*-isomers, *cis*-alcohol (**523a**) and *cis*-acetate (**551b**) were obtained (**Scheme 84**).



Scheme 83 Biotransformation of mesomeric cyclohexanones (**516b,535–539**) by *Acinetobacter.*



Scheme 84 Biotransformation of 1-acetoxy-2-alkylcyclohexenes (**546–548**) and racemic *trans*- (**550a, 550b**) and *cis*-2-methylcyclohexyl acetates (**551a, 551b**) by cultured cells of *M. polymorpha*.

Reduction of acyclic α,β-unsaturated ketone (**552–554**) by *B. sulfurescens* gave mainly the corresponding saturated ketone **555–557**, having *S*-configuration at C-3, and a small amount of saturated alcohols **558** and **559** with *R*-configuration. 3-Methyl-hexa-2-en-4-one (**560**) and 4-methylhepta-3-en-5-one (**561**) treated in the same manner as mentioned above afforded only saturated ketones (**562, 563**) with *R*-configuration[96] (**Scheme 85**). The enone reductase of *N. tabacum* cells reduced the C–C double bond of 1-octen-3-one (**564**) to give 3-octanone (**565**)[99,100] (**Scheme 85**).

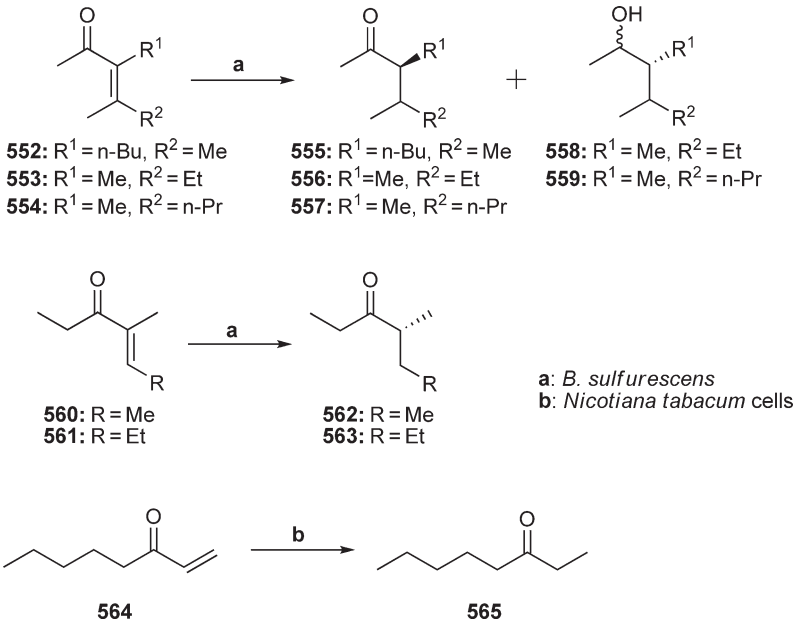
Methanol yeast *Candida boidinii* KK912 biotransformed polyols, such as 1,2,4-butanetriol (**566**), to a keto derivative (**567**) in 78.1% yield. The secondary hydroxyl groups of 2-butano, 2,3-butanediol, and 1,2,3-butanetriol were also oxidized by the same fungus to yield their corresponding carbonyl derivatives[104] (**Scheme 86**).

G. candidum and *Galactomyces reesii* in acetone powder converted ethyl 2-methyl-3-oxobutanoate (**568**) to give only one isomer, 2*R*,3*S*-hydroxy ester (**569**) with high selectivity and excellent ee and high yield (99%)[105] (**Scheme 86**).

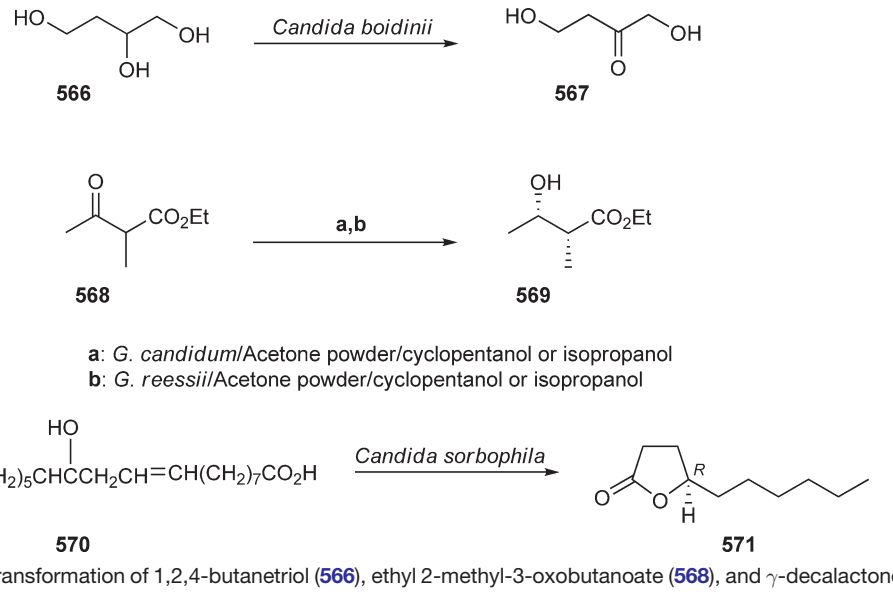
γ-decalactone (**571**) possesses characteristic odor of peach, strawberry, and apricot. Thus, this compound is one of the essential materials in the flavor and fragrance industry. Ricinoleic acid (=12(*R*)-hydroxy-9*Z*-octadecenoic acid) (**570**) was biotransformed by *Candida sorbophila* strain FC 58 in yeast and malt extract, polypeptone, trace amounts of $CuSO_4$, and L-carnitine to produce γ-decalactone (**571**) (in high yield 44.2 g l^{-1}) after 20 days incubation. Chiral analysis indicated that (*R*)-form was dominant and was over 99.9% ee[106] (**Scheme 86**).

Optically active 2-methylbutyric acid (**572a, 572b**) is a useful scent. *Pseudomonas* predominantly produces 2*R*-methylbutyric acid (**572a**) (5.2 g from 1 l of the medium) from racemic 2-methylbutyric acid (**572a, 572b**). (*R*)- (**573a**) and (*S*)-filberone (**573b**), which are the characteristic scent of hazelnut, were synthesized by both optical isomers (**572a, 572b**)[107] (**Scheme 87**).

Alkyl methyl ketones, such as 2-heptanone, 2-octanone, 2-nonanone, 3-octanone, and alkyltrifluoromethylketones like 1-trifluoromethyl-2-octanone and trifluoromethyl-2-nonanone were cultured with immobilized cells of carrot (*D. carota*) to give the corresponding secondary alcohols (*S*-configuration for alkyl alcohols and *R*-configuration for alkyltrifluoromethyl alcohols) with 30–93% ee in 30–45% yield.[86]



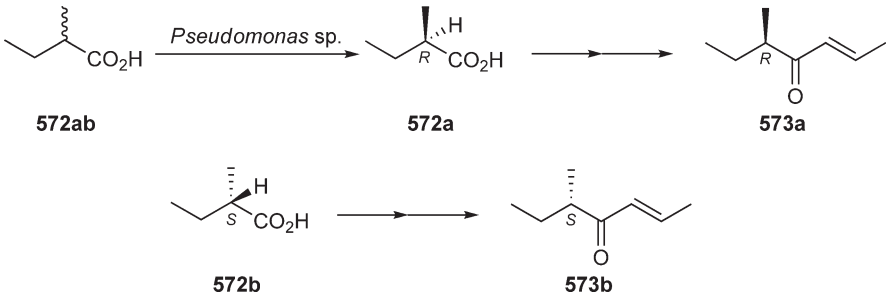
Scheme 85 Biotransformation of acyclic α,β-unsaturated ketones (**552–554**), 3-methyl-hexa-2-en-4-one (**560**), 4-methylhepta-3-en-5-one (**561**), and 1-octen-3-one (**564**) by *B. sulfurescens* and cultured cells of *N. tabacum*.



Scheme 86 Biotransformation of 1,2,4-butanetriol (**566**), ethyl 2-methyl-3-oxobutanoate (**568**), and γ-decalactone (**570**) by *C. boidinii*, *G. candidum*, *G. reesii*, and *C. sorbophila*.

2-Alkanones, 2-octanone, 2-nonanone, 2-decanone, and 2-undecanone as well as β-keto esters, such as methyl-, ethyl-, and neopentyl 3-oxo-butanoates, were also treated in immobilized acetone powder of *G. candidum* in the presence of 2-alkanol or cyclopentanol with coenzyme NAD^+ or $NADP^+$ to afford the corresponding secondary alcohols having *S*-configuration with 99% ee.[84]

The effect of high hydrostatic pressure and high-pressure homogenization on the microbial reduction of 2-methyl-1,5-hexadiene (**574**) and acetyl γ-lactone (**575**) was investigated. The yeast strain *S. cerevisiae* and



Scheme 87 Optical resolution of racemic 2-methylbutyric acid (**572a**, **572b**) by *Pseudomonas* species.

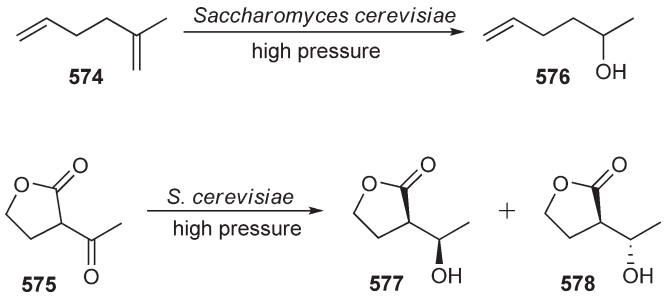
Yarrowia lipolytica were used as microorganisms. In high-pressure condition, enantioselectivity of the product **576** is higher than statistic condition. But in both conditions, the yield of the metabolite **576** was poor. In the case of the γ-lactone (**575**), both yield and ee of **577** (3*R*,1*R* and 3*S*,1*S*) and **578** (3*R*,1*S*) are excellent[78] (**Scheme 88**).

When 2-undecylcyclopentanone (**579**) was incubated with the strain *Acinetobacter calcoaceticus*, which was grown on cyclohexanol as the only carbon source, the metabolite 5-hexadecanolide (**580**) in 25–40% yield with an optical purity of 32–74% ee was obtained (**Scheme 89**). This procedure allows the three-step synthesis of (*S*)-(−)-5-hexadecanolide (**580**) as well as direct access to (*R*)-(+)-2-undecylcyclopentanone, a precursor for chemical synthesis of (*R*)-(+)-5-hexadecanolide, a pheromone isolated from the oriental hornet *Vespa orientalis* in good optical purity.[108]

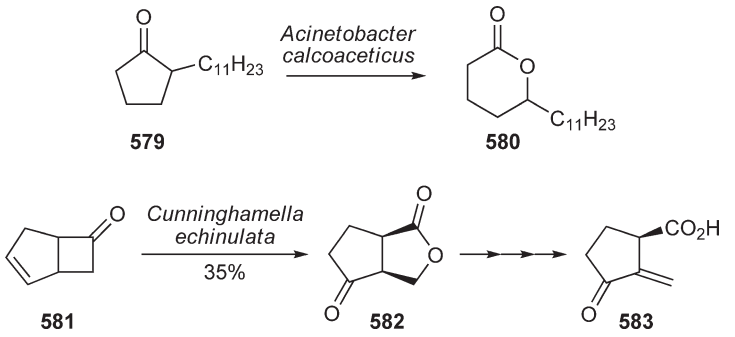
The antibiotic (−)-(*R*)-sarkimysin (**583**), which exhibited a potent antitumor activity, was synthesized through the formation of (−)-(1*R*,5*S*)-γ-lactone (**582**), which was obtained from racemic cyclobutanone (**581**) using *C. echinulata*[109] (**Scheme 89**).

Geotrichum candidum IFO 5767 and 4597 converted 2,2-disubstituted 1,3-ketone (**584**) to the corresponding 3*S*-hydroxyketones (**585a** (2*S*,3*S*) and **586a** (2*R*,3*S*)) enantioselectively. 3-Meso (*syn* diol form) (**587**) was treated in the same fungus to give (2*R*,3*R*) and (2*S*,3*R*) products (**585b**, **586b**). *Mucor hiemalis* IAM 6095 converted meso (*anti* diol form) (**588**) to give the same products as mentioned above[110] (**Scheme 90**).

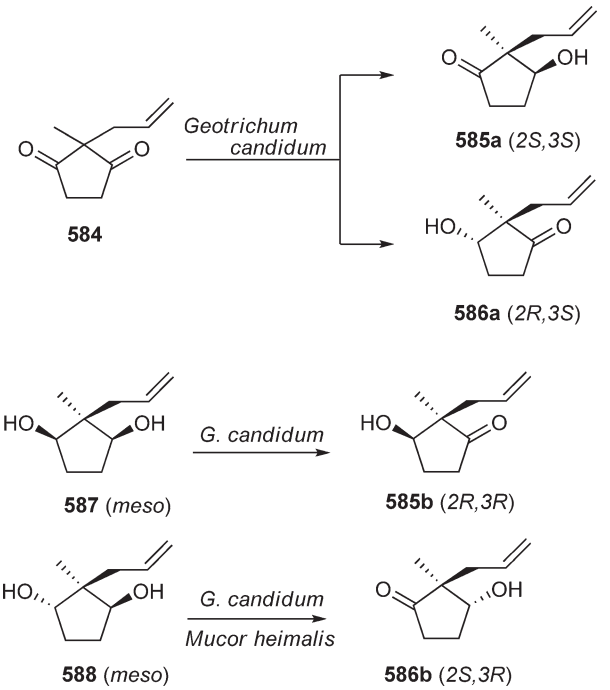
N-Substituted maleimides (**589–591**) and *N*-phenyl-2-methylmaleimide (**595**) in dimethylsulfoxide were administered to suspension cultured cells of plants and Cyanobacterium, such as *N. tabacum*, *C. roseus*, *M. polymorpha*, *Parthenocissus tricuspidata*, *Gossypium hirsutum*, and *Cynechococcus* species in MS medium. The C–C double bond of the substrates **589–591** was reduced to afford succinimide derivatives **592–594**. Compound **591** was smoothly reduced by incubation for 5 days with cultured cells and for 12 h with *Cyanobacterium* species to furnish *N*-phenylsuccinimide (**594**) in over 99% conversion yield. *N*-Phenyl-2-methylmaleimide (**595**) was fed to suspension cultured cells of *N. tabacum*, *M. polymorpha*, and *Cynechococcu*s species to obtain (*R*)-*N*-phenyl-2-methylsuccinimide (**596**) in 61–86% yield (**Scheme 91**). Enantiomeric purity of the metabolite was 98–99% ee. Hydrogenation of maleimides is realized with discrimination of the enantiotopic face of the double bond of 2-methylmaleimide derivative to afford optically pure (*R*)-2-methylsuccinimide derivative.[111]



Scheme 88 Biotransformation of 2-methyl-1,5-hexadiene (**574**) and acetyl γ-lactone (**575**) by *S. cerevisiae*.



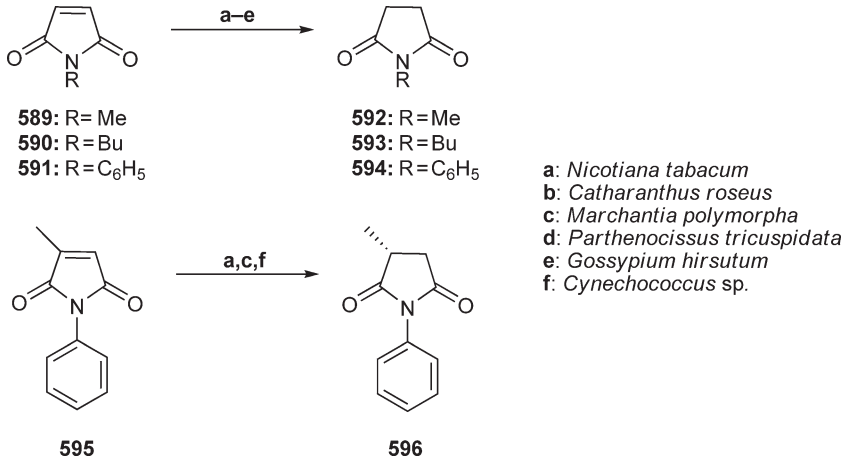
Scheme 89 Biotransformation of 2-undecylcyclopentanone (**579**) and cyclobutanone (**581**) by *A. calcoaceticus* and *C. echinulata.*



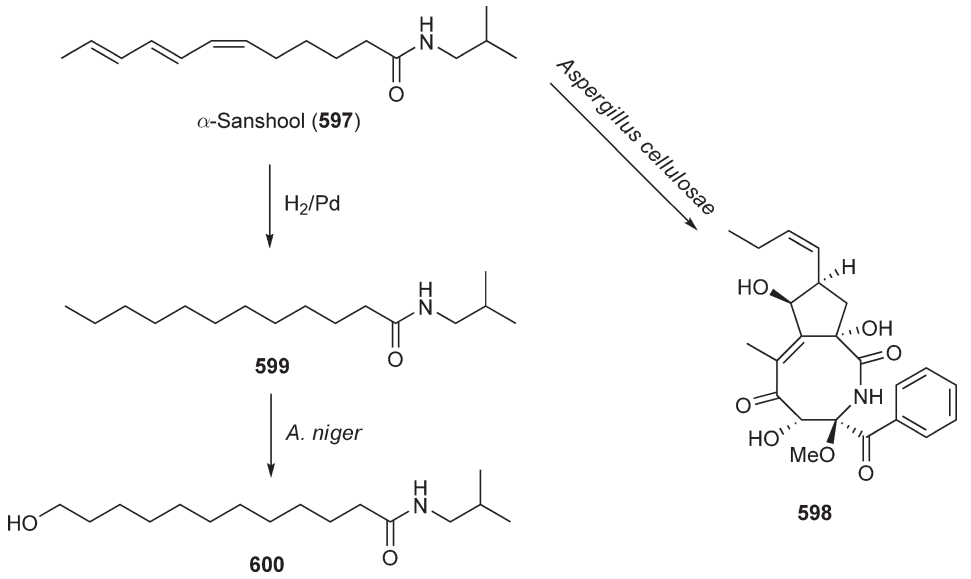
Scheme 90 Biotransformation of 2,2-disubstituted 1,3-ketone (**584**), 3-meso (*syn* diol form) (**587**), and meso (*anti* diol form) (**588**) by *G. candidum* and *M. hiemalis.*

α-Sanshool (**597**) obtained from *Zanthoxyli fructus* shows insecticide, diuretic, stomachic, and irritant activity. Biotransformation of α-sanshool did not proceed by *A. niger*, while *A. cellulosae* produced a complex nitrogen-containing substance (**598**). Perhydro-α-sanshool (**599**), however, gave ω1-hydroxylated product (**600**) (Asakawa and Hashimoto, unpublished results) (**Scheme 92**).

The regio- and stereoselective hydroxylation of substituted octalones by several fungal strains was estimated. *Mucor plumbeus* bioconverted **601a** into **603a** and **604a** (in the ratio 66%:22%), whereas compound **602b** gave only *ent*-3 (**603b**) in 77% yield. *Beauveria bassiana* biotransformed **602b** to *ent*-3 (**603b**) and *ent*-4 (**604b**) in 15% yield each. Incubation of (4*aS*,8*S*)-**602c** with *M. plumbeus* afforded **605** and **606** in 40 and 27% yield, respectively. This method gave new and valuable regio- and stereoselective functionalized synthons for organic synthesis[112] (**Scheme 93**).



Scheme 91 Biotransformation of N-substituted maleimides (**589–591**) and *N*-phenyl-2-methylmaleimide (**595**) by cultured cells of *Nicotiana tabacum*, *C. roseus*, *M. polymorpha*, *P. tricuspidata*, *G. hirsutum*, and *Cynechococcus* species.

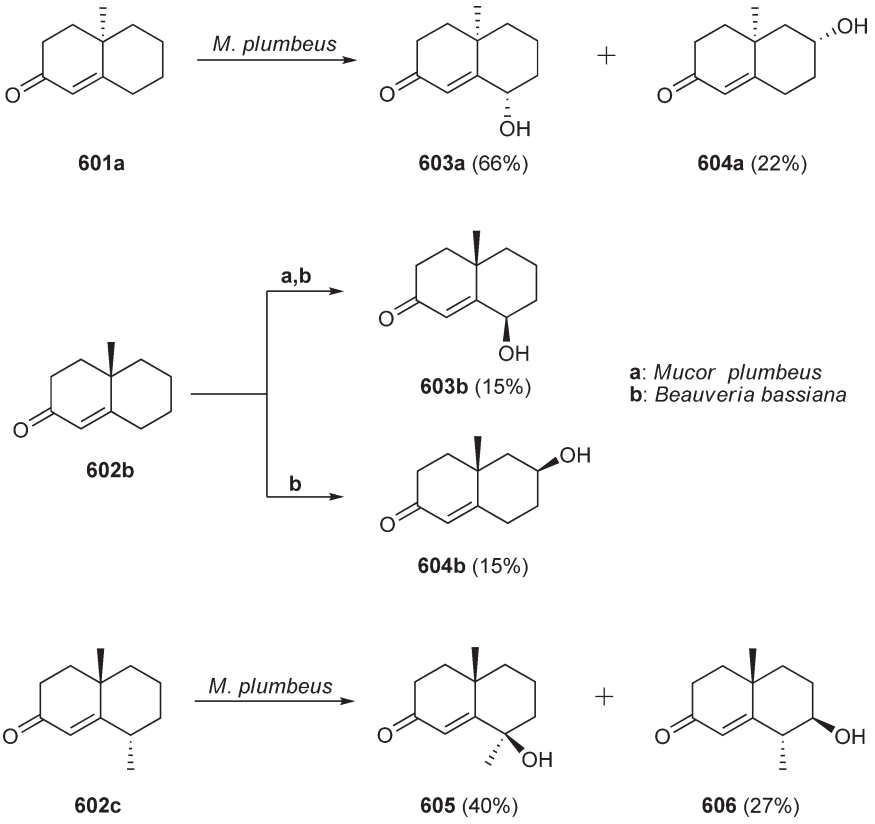


Scheme 92 Biotransformation of α-sanshool (**597**) and perhydro-α-sanshool (**599**) by *A. cellulosae* and *A. niger.*

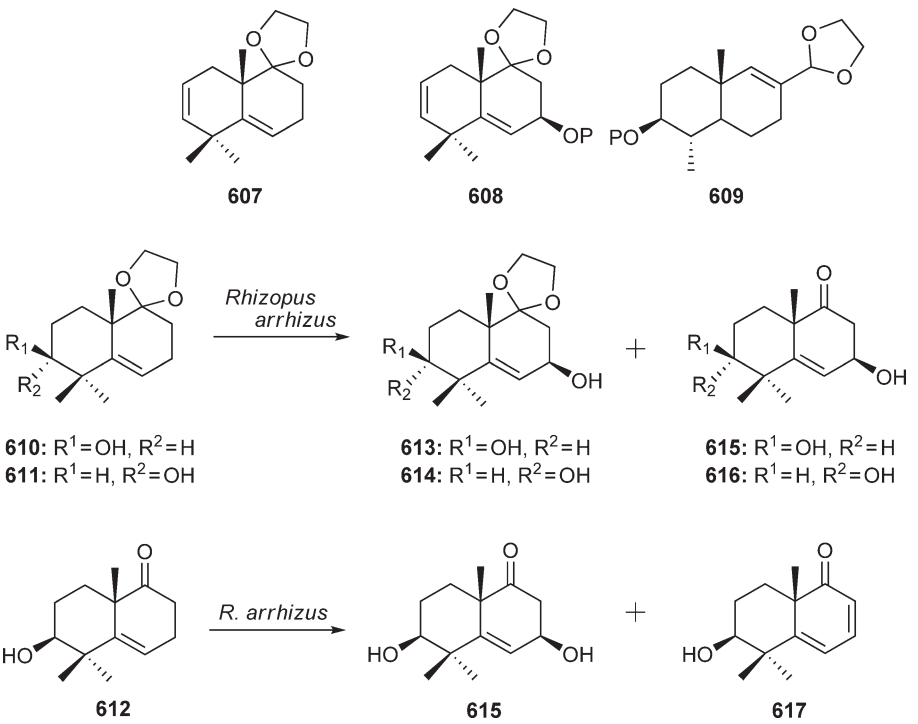
Chiral fragments like **607–609** are potentially very useful building blocks in stereoselective total synthesis of highly oxygenated bioactive terpenoids such as forskolin and bruceantin. Compounds **610** and **611**, and **612** obtained from Wieland-Miescher ketone were incubated with *R. arrhizus* to afford 7β-hydroxy octalone derivatives **613**, **614** and **615**, **616**, and **615** and **617**, respectively[113] (**Scheme 94**).

The same fungus biohydroxylated the similar substrate **618** to **619–621** (**Scheme 95**), **622** to **625** and **626**, **623** to **627**, **624** to **628** and **629**, **630** to **607** and **631** (**Scheme 96**), and furthermore, **607** to **632–635** (**Scheme 97**). Even in the presence of acetate, phosphate, free ketone, or dioxolane substituents, this reaction occurred, but not a benzyl group.[114]

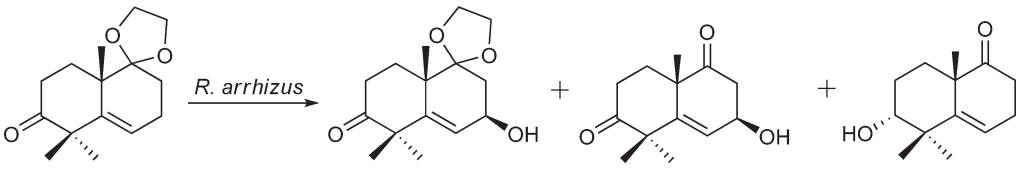
The similar substrate **636a** and its enantiomer **636b**, and related substrates **637–641** were treated in the same manner as described above to afford the corresponding 7α- and 7β-hydroxylated products (**642–652**) in good yield[115] (**Schemes 98, 99**).



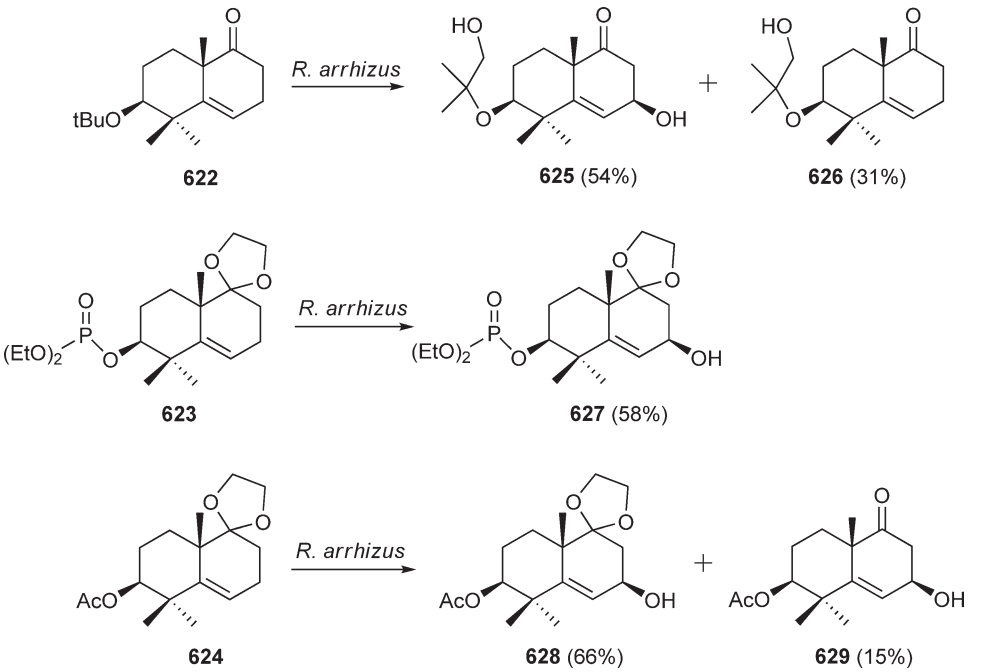
Scheme 93 Biotransformation of octalone derivatives (**601a, 602b, 602c**) by *Beauveria bassiana* and *Mucor plumbeus*.



Scheme 94 Useful building block of chiral fragments (**607–609**) and biotransformation of 4,4-dimethyl octalone derivatives (**610–612**) by *R. arrhizus*.



Scheme 95 Biotransformation of octalone derivative (**618**) by *Rhizopus arrhizus*.



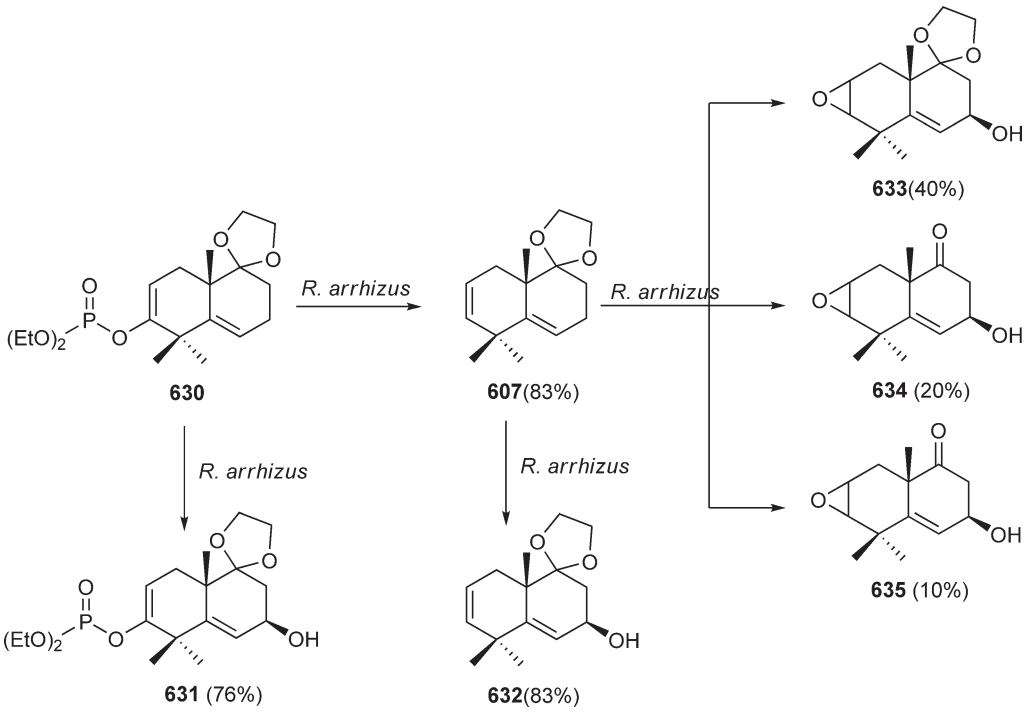
Scheme 96 Biotransformation of octalone derivatives (**622–624**) by *Rhizopus arrhizus*.

A review of the biotransformation of terpenoids, acetogenins, and aromatic compounds by suspension cultured cells published till 1995 was reported by Suga and Hirata.[116]

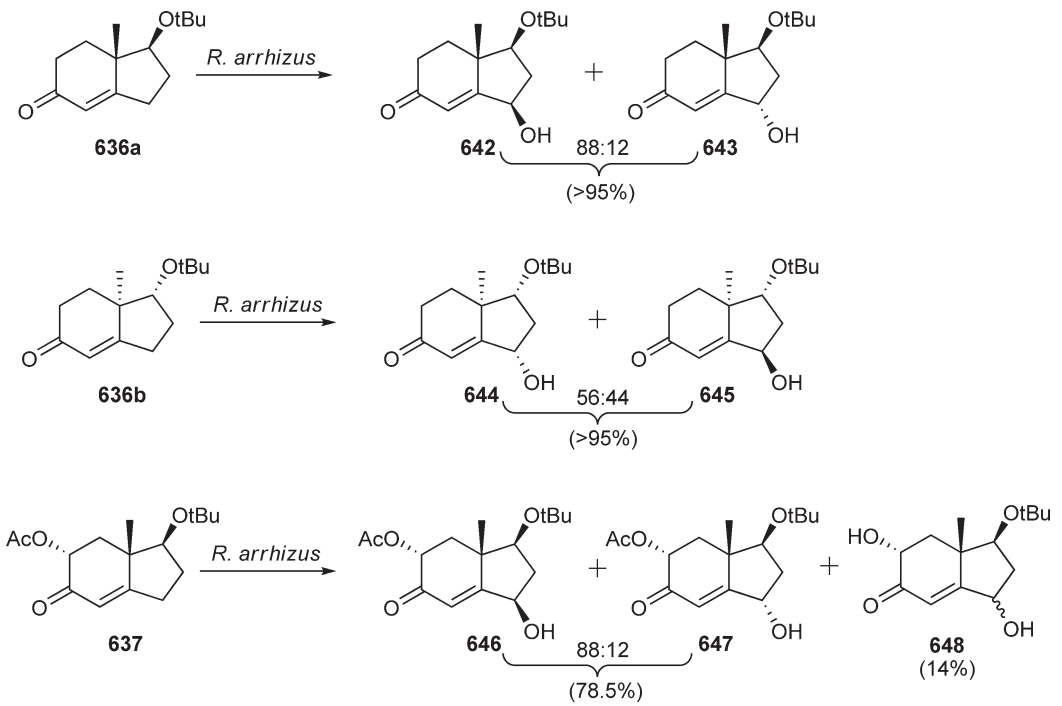
In conclusion, many oxygenated diterpenoids were microbiologically transformed by various fungi. Generally, nonactivated methylene, methine, and methyl group of almost all the substrates were oxidized to give secondary, tertiary, and primary alcohols. Hydroxylation occurred regio- and stereoselectively and the resulting secondary alcohols were further oxidized to afford ketones.

The ketone group of cyclohexane and its derivatives and diterpenoids was reduced to the corresponding secondary alcohol stereoselectively. Such a phenomenon has been observed in sesquiterpenoid biotransformation. In diterpenoids with 4,4-dimethyl group at A-ring and other cyclohexane with 1,1-dimethyl group, introduction of oxygen function at C-3 was observed as seen in drimane sesquiterpenoids. 3-Hydroxy group at A-ring of some triterpenoids and 17-hydroxy steroids, and a dienone at A-ring of steroids were oxidized and reduced to give ketone and cyclohexanone derivatives, respectively.

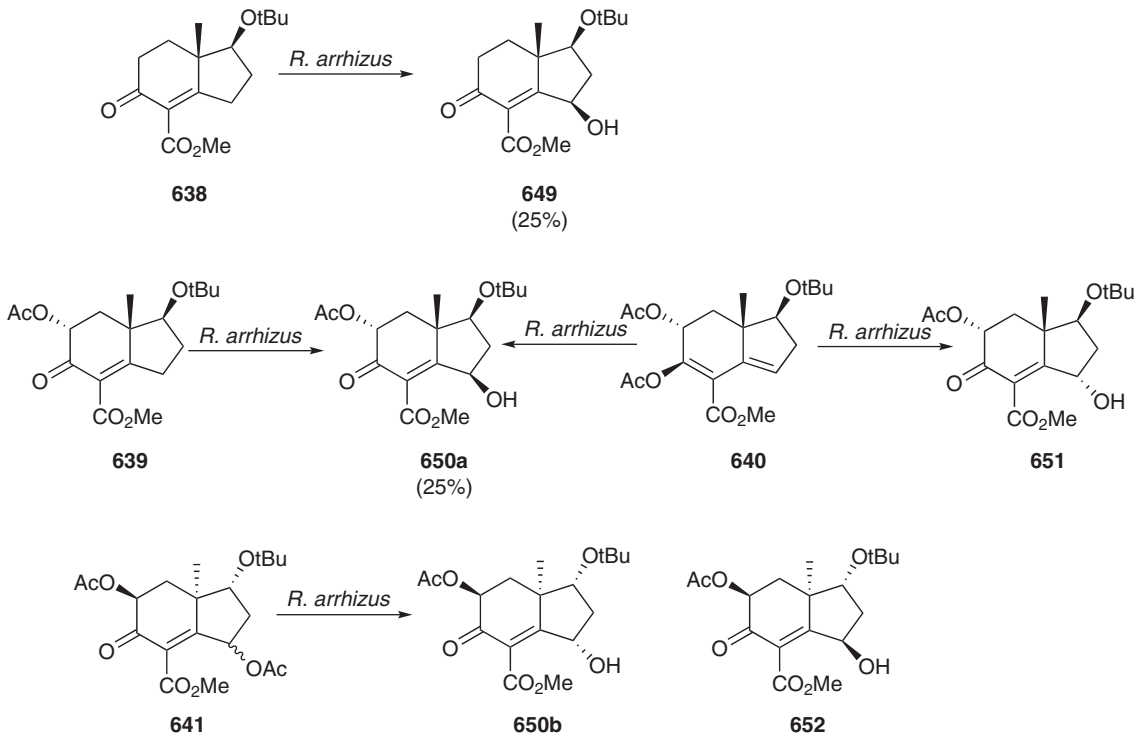
Study on the biotransformation of nitrogen-containing substances is rare except for the taxane diterpenoids. Hydrolysis of ester group was observed in taxoids (**114–116, 118, 123**, etc.) and the resulting metabolites do not show potent anticancer activity. Such reactions were also observed in labdane and cembrane diterpenoids. Acetylation also occurred in cembranes and taxoids. Microorganisms



Scheme 97 Biotransformation of octalone derivatives (**607**, **630**) by *Rhizopus arrhizus.*



Scheme 98 Biotransformation of octalone derivatives (**636a, 636b, 637**) by *Rhizopus arrhizus.*



Scheme 99 Biotransformation of octalone derivatives (**638, 639, 641**) by *R. arrhizus.*

introduce oxygen atom at allylic position to give secondary hydroxyl and keto groups. Allylic hydroxylation and further oxidation of secondary alcohol as well as reduction of double bond were seen in α-(**203**) and β-ionones (**214a**), and α-damscones (**215**). Introduction of a hydroxyl group at benzylic and homobenzylic positions was observed in abietanes (**102**, **102a**) and isopropyl naphthalene (**309**) by microorganisms and rabbits. The direct introduction of hydroxyl group on benzene ring was confirmed in nopol benzyl ether (**269**).

Glycosylation of secondary alcohol and/or phenolic hydroxyl group in raspberry ketone (**255**), zingerone (**263**), phenolic monoterpenes (**271**, **274**), eugenol (**277**), tocopherol, hinokitiol (**280**), capsaicin (**285**), 8-nordihydrocapsaicin (**293**), vitamin A (**328**), and vitamin E (**327**) by using suspension cultured cells of higher plants such as *Eucalyptus*, *Phytolacca*, and *Catharanthus* species is noteworthy since these glycosides are soluble in water and the resulting materials will be useful for prodrugs. Bisphenol (**401**) and phenol derivatives were also glycosylated to give di- and monoglycosides, respectively.

Aspergillus niger cleaved the side chains of capsaicin (**285**), shogaol (**297**), and ginerol (**302**), to give γ-lactone and/or carboxylic acid. An isoprene moiety of glifolin (**433**) and neoglifolin (**441**) was degraded to afford carboxylic acids and dihydrofurane derivatives.

Plant suspension cultured cells like *D. carota*, *N. tabacum*, and *G. jasminoides*, microalgae *E. gracilis* and *Chlorella* species, fungus *G. candidum*, and yeast *H. anomala* are useful microorganisms to obtain both enantiomers of 1-phenylalkanol from alkylphenones.

In pungent aromatic components, like capsaicins (**285**, **289**), gingerol (**302**), and shogaol (**297**), ω-hydroxylated metabolites were obtained. The hot tasting of the original substrates disappeared in the resulting compounds. A double bond was also oxidized to give an epoxide, which was further stereospecifically hydrolyzed to afford diols as seen in stylene oxide (**503a**, **503b**).

The present methods are very simple one-step reaction in water, nonhazard, and very cheap and very useful for the production of many valuable functionalized compounds in high yield from fungi and plants secondary metabolites or synthetic compounds.

Acknowledgments

We thank Dr. Agnieszka Ludwiczuk for drawing the structures and valuable discussion.

References

1. J. B. Hanson; P. B. Hitchcock; H. Nashir; A. Truneh, *Phytochemistry* **1994**, *36*, 903–906.
2. W.-R. Abraham, *Phytochemistry* **1994**, *36*, 1421–1424.
3. T. Hashimoto; M. Fujiwara; K. Yoshikawa; A. Umeyama; M. Tanaka; Y. Noma, In *Biotransfromation of Sclareolide and Sclareol by Microorganisms*. Proceedings of the 51 st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Ohta, Ed.; Nagahama, Japan, 2007; pp 316–318.
4. G. Aranda; M. S. E. Kortbi; J.-Y. Lallemand; A. Neuman; A. Hammoumi; I. Facon; R. Azerad, *Tetrahedron* **1991**, *47*, 8339–8350.
5. M. S. A. Haridy; A. A. Ahmed; M. Doe, *Phytochemistry* **2006**, *67*, 1455–1459.
6. H. Ghoumari; M.-H. Benajiba; A. Garcia-Granados; A. Fernandez; A. Martinez; F. Rivas; J. M. Arias, *Phytochemistry* **2006**, *67*, 2294–2302.
7. B. M. Fraga; P. Gonzalez; M. G. Hernandez; S. Suarez, *Phytochemistry* **2003**, *62*, 67–70.
8. G. O. Buchanan; P. B. Reese, *Phytochemistry* **2001**, *56*, 141–151.
9. A. Lamm; W. Reynold; P. B. Reese, *Phytochemistry* **2006**, *67*, 1088–1093.
10. N. A. El-Emary; G. Kusano; T. Takemoto, *Chem. Pharm. Bull.* **1976**, *24*, 1664 1657.
11. B. M. Fraga; J. R. Hanson; M. G. Hernandez, *Phytochemistry* **1978**, *17*, 812–814.
12. B. M. Fraga; J. R. Hanson; M. G. Hernandez; F. Y. Sarah, *Phytochemistry* **1980**, *17*, 1087–1091.
13. B. M. Fraga; A. G. Gonzalez; J. R. Hanson; M. G. Hernandez, *Phytochemistry* **1981**, *20*, 57–61.
14. B. M. Fraga; M. G. Hernandez; F. G. Tellado; A. Perales, *Phytochemistry* **1986**, *25*, 1235–1237.
15. B. M. Fraga; M. G. Hernandez; C. E. Diaz; P. Gonzalez; R. Guillermo, *Phytochemistry* **1988**, *27*, 3131–3136.
16. B. M. Fraga; M. G. Hernandez; P. Gonzalez, *Phytochemistry* **1991**, *30*, 2567–2571.
17. B. M. Fraga; M. G. Hernandez; P. Gonzalez, *Phytochemistry.* **1992**, *31*, 3845–3849.
18. B. M. Fraga; P. Gonzalez; M. G. Hernandez; S. Suarez, *Tetrahedron* **2005**, *61*, 5623–5632.
19. B. M. Fraga; L. Alvarez; S. Suarez, *J. Nat. Prod.* **2003**, *66*, 327–331.
20. B. M. Fraga; C. Bressa; P. Gonzalez; R. Guillermo; M. G. Hernandez; S. Suarez, *Phytochemistry* **2007**, *68*, 1557–1563.
21. K. Moriwake; K. Hashimoto; T. Terai; A. Nishinaga, In *Interconversion of Steviol (4) Biotransformation of Steviol Using Soil Microbes*, Streptomyces *sp., a Biocatalyst*. Proceedings of the 42nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Masaki, Ed.; Gifu, Japan, 1998; pp 148–150.
22. L.-M. Yang; F.-L. Hsu; S.-F. Chang; J.-T. Cheng; J.-Y. Hsu; C.-Y. Hsu; P.-C. Liu; S.-J. Lin, *Phytochemistry* **2007**, *68*, 562–570.
23. M. Sekita; T. Hashimoto; Y. Noma; Y. Asakawa, In *Biotransformation of Biologically Active Terpenoids, Sacculatal and Cinnamodial by Microorganisms*. Proceedings of the 50th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Inoue, Ed.; Yokohama, Japan, 2006; pp 406–408.
24. K. A. El Sayed; S. Laphookhieo; M. Yousaf; J. A. Prestridge; A. B. Sguride; V. B. Wali; P. W. Sylvester, *J. Nat. Prod.* **2008**, *71*, 117–122.
25. B. M. Fraga; M. G. Hernandez; J. M. Artega; S. Suarez, *Phytochemistry* **2003**, *63*, 663–668.
26. Y. Asakawa; T. Ishida; M. Toyota; T. Takemoto, *Xenobiotica* **1986**, *16*, 753–767.
27. R. L. Hanson; J. M. Wasylyk; V. B. Nanduri; D. L. Cazzulino; R. N. Patel; L. J. Szarka, *J. Biol. Chem.* **1994**, *269*, 22145–22149.
28. S. Hu; X. Tian; W. Zhu; Q. Fang, *Tetrahedron* **1996**, *52*, 8739–8746.
29. J. Zhang; L. Zhang; Z. Wang; D. Qiu; D. Sun; J. Guo; Q. Fang, *J. Nat. Prod.* **1998**, *61*, 497–500.
30. J. Dai; M. Ando; S. Zhang; J. Sakai; J. Bai, In *Biotransformation of C-14 Oxygenated 4(20),11-Taxadienes by Fungus* Absidia coerulea *IFO 4011*. Proceedings of the 46th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Asakawa, Ed.; Tokushima, Japan, 2002; pp 326–327.
31. J. Dai; L. Yan; J.-I. Sakai; M. Ando, *Tetrahedron* **2005**, *61*, 5507–5517.
32. A. Arnone; A. Bava; A. Alemani; G. Nasini; E. Bombardeli; G. Fontana, *J. Mol. Catal. B : Enzym.* **2006**, *42*, 95–98.
33. J. Li; J. Dai; X. Chen; P. J. Zhu, *J. Nat. Prod.* **2007**, *70*, 1846–1849.
34. J. Zang; H. Guo; Y. Tian; P. Liu; N. Li; J. Zhou; T. Guo, *Phytochemistry* **2007**, *68*, 2523–2530.
35. P. Chatterjee; J. M. Pezzuto; S. A. Kouzi, *J. Nat. Prod.* **1999**, *62*, 761–763.
36. P. Chatterjee; S. A. Kouzi; J. M. Pezzuto; M. T. Hamann, *Appl. Environ. Microbiol.* **2000**, *66*, 3850–3855.
37. S. A. Kouzi; P. Chatterjee; J. M. Pezzuto; M. T. Hamann, *J. Nat. Prod.* **2000**, *63*, 1653–1657.
38. J. Zhang; Z.-H. Cheng; B.-H. Yu; G. A. Cordell; S. Z. Qiuc, *Tetrahedron Lett.* **2005**, *46*, 2337–2340.
39. T. Akihisa; Y. Takamine; K. Yoshizumi; H. Okuda; Y. Kimura; M. Ukiya; T. Nakahara; T. Yokochi; E. Ichiishi; H. Nishino, *J. Nat. Prod.* **2002**, *65*, 278–282.

40. D. Z. L. Bastos; I. D. Pimentel; D. A. de Jesus; B. H. Oliveira, *Phytochemistry* **2007**, *68*, 834–839.
41. H. Hamada; H. Konishi; H. J. Williams; A. I. Scott, *Phytochemistry* **1991**, *30*, 2269–2270.
42. A. Matsushima; M.-E. F. Hegazy; C. Kuwata; Y. Sato; M. Otsuka; T. Hirata, In *Biotransformation of Enones Using Plant Cells-the Reduction of* α*-Santonine*. Proceedings of the 48th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Kajiwara, Ed.; Yamaguchi, Japan, 2004; pp 396–398.
43. M. T. Yazdi; H. Arabi; M. A. Faramarzi; Y. Ghasemi; M. Amini; S. Shokravi; F. A. Mohseni, *Phytochemistry* **2004**, *65*, 2205–2209.
44. Y. Noma; K. Matsueda; I. Maruyama; Y. Asakawa, In *Biotransformation of Terpenoids and Related Compounds by* Chlorella *Species*. Proceedings of the 41st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Ito, Ed.; Morioka, Japan, 1997; pp 227–229.
45. Y. Noma; Y. Asakawa, In *Microbiological Transformation of 3-Oxo-*α*-Ionone of Optically Active (+)-*α *-Ionone*. Proceedings of the 42nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; H. Nishimura, Ed.; Sapporo, Japan, 1998; pp 133–135.
46. Y. Noma; Y. Asakawa, *Aspergillus* spp: biotransformation of terpenoids and related compounds. In *Biotechnology in Agriculture and Forestry 33. Medicinal and Aromatic Plants VIII*; Y. P. S. Bajaj, Ed.; Springer, Berlin, 1995; Vol. 33, pp 65–96.
47. Y. Noma; Y. Asakawa, *Euglena gracilis* Z: biotransformation of terpenoids and related compounds. In *Biotechnology in Agriculture and Forestry 41. Medicinal and Aromatic Plants X*; Y. P. S. Bajaj, Ed.; Springer: Berlin, 1998; Vol. 41, pp 195–237.
48. Y. Noma; Y. Asakawa, *Dunaliella tertiolecta* (green microalga): culture and biotransformation of terpenoids and related compounds. In *Biotechnology in Agriculture and Forestry 28. Medicinal and Aromatic Plants VII*; Y. P. S. Bajaj, Ed.; Springer: Berlin, 1994; Vol. 28, pp 195–202.
49. T. Hashimoto; Y. Noma; H. Matsumoto; H. Tomita; M. Tanaka; Y. Asakawa, In *Microbial Biotransformation of Optically Active (+)-Ionone and (*$-$*)-Ionone*. Proceedings of the 44th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; H. Nishimura, Ed.; Sapporo, Japan, 2000; pp 154–156.
50. Y. Mikami, *Biotechnol. Genet. Eng. Rev.* **1988**, *6*, 271–320.
51. K. Kieslich; W. R. Abraham; B. Stumpf; P. Washausen, *Top. Flavor Res.* **1985**, 405.
52. V. Krasmobajew; D. Heimlinger, *Helv. Chim. Acta* **1982**, *65*, 1590–1601.
53. Y. Noma, T. Hashimoto, Y. Asakawa, In *Microbiological Transformation of Damascone (1)*. Proceedings of the 45th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Hirai, Ed.; Toyama, Japan, 2001; pp 93–95.
54. Y. Noma; T. Hashimoto; Y. Akamatsu; S. Takaoka; Y. Asakawa, In *Microbiological Transformation of Adamantane (Part 1)*. Proceedings of the 43rd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Ohga, Ed.; Oita, Japan, 1999; pp 199–201.
55. Y. Noma; T. Hashimoto; Y. Asakawa, In *Microbiological Transformation of Adamantanes (2)*. Proceedings of the 45th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Hirai, Ed.; Toyama, Japan, 2001; pp 96–98.
56. Y. Noma; Y. Takahashi; Y. Asakawa, In *Stereoselective Reduction of Racemic Bicyclo[3.3.1]Monane-2,6-Dione and 5-Hydroxy-2-Adamantanone by Microorganisms*. Proceedings of the 47th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Kurata, Ed.; Tokyo, Japan, 2003; pp 118–120.
57. H. Takahashi, Transformation of Terpenoids and Aromatic Compounds by Selected Microorganisms. Ph.D. Thesis, Tokushima Bunri University, Tokushima, Japan, 1994; pp 1–115.
58. Y. Noma; Y. Okajima; T. Takahashi; Y. Asakawa, *Phytochemistry* **1991**, *30*, 2969–2971.
59. Y. Noma; E. Akehi, N. Miki; Y. Asakawa, *Phytochemistry* **1992**, *31*, 515–517.
60. K. Shimoda; T. Harada; H. Hamada; N. Nakajima; H. Hamda, *Phytochemistry* **2007**, *68*, 487–492.
61. Y. Noma; Y. Asakawa, In *Microbial Transformation of (*$-$*)-Nopol Benzyl Ether*. Proceedings of the 50th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Inoue, Ed.; Yokohama, Japan, 2006; pp 434–436.
62. K. Shimoda; Y. Kondo; T. Nishida; H. Hamada; N. Nakajima; H. Hamada, *Phytochemistry* **2006**, *67*, 2256–2261.
63. H. Hamada; Y. Miyamoto; T. Furuya; N. Nakajima, In *The Biotransformation of Terpenes by Plant Suspension Cells*. Proceedings of the 41st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Ito, Ed.; Morioka, Japan, 1997; pp 222–223.
64. H. Hamada; F. Murakami; T. Furuya, In *Production of Hinokitiol Glycoside*. Proceedings of the 42nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Masaki, Ed.; Gifu, Japan, 1998; pp 145–147.
65. K. Shimoda; S. Kwon; A. Utsuki; S. Ohiwa; H. Katsuragi; K. Yonemoto; H. Hamada; H. Hamada, *Phytochemistry* **2007**, *68*, 1391–1396.
66. H. Takahashi; T. Hashimoto; Y. Noma; Y. Asakawa, *Phytochemistry* **1993**, *34*, 1497.
67. T. Matsumoto; T. Ishida; Y. Takeda; K. Soh; I. Kubo; M. Sakamoto, *Chem. Pharm. Bull.* **1995**, *43*, 216–222.
68. S. Tahara; J. L. Ingham; J. Mizutani, *Phytochemistry* **1989**, *28*, 2079–2084.
69. K. Shimoda; Y. Kondo; K. Abe; H. Hamada; H. Hamada, *Tetrahedron Lett.* **2006**, *47*, 2695–2698.
70. K. Shimoda; Y. Kondo; M. Akagi; K. Abe; H. Hamada; H. Hamada, *Phytochemistry* **2007**, *68*, 2678–2683.
71. H. Hamada; R. Tomi; Y. Asada; T. Furuya, *Tetrahedron Lett.* **2002**, *43*, 4087–4089.
72. Y. Kondo; K. Shimoda; K. Miyahara; H. Hamada; H. Hamada, *Plant Biotechnol.* **2006**, *23*, 291–296.
73. Y. Kondo; K. Shimoda; N. Kubta; H. Hamada; H. Hamada, *Plant Biotechnol.* **2006**, *23*, 329–331.
74. H. Kasahara; M. Miyazawa; H. Kameoka, *Phytochemistry* **1995**, *40*, 1515–1517.
75. F. Li; X.-W. Yang, *Phytochemistry* **2008**, *69*, 765–771.
76. T. Hashimoto; Y. Ohara; K. Yoshikawa; A. Umeyama; Y. Noma; Y. Asakawa, In *Biotransformation of Grifolin Derivatives from Mushroom Belonging to Scutigeraceae by* Aspergillus niger. Proceedings of the 51st Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Ohta, Ed.; Nagahama, Japan, 2007; pp 313–315.
77. Y. Akakabe; Y. Naoshima, *Phytochemistry* **1994**, *35*, 661–664.
78. G. Fantin; M. Fogagnolo; M. E. Guerzoni; R. Lanciotti; A. Medici; P. Pedrini; D. Russi, *Tetrahedron Asymmet.* **1996**, *7*, 2879–2887.
79. Y. Noma; M. Kataoka; Y. Asakawa, In *Enantioselectivity in the Biotransformation of Racemic 1-Phenyl-1-Alkanols and Related Compounds by* Hansenula anomala. Proceedings of the 40th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Kurokawa, Ed.; Saga, Japan, 1996; pp 92–94.
80. K. Nakamura; Y. Inoue; A. Ohno, *Tetrahedron Lett.* **1994**, *35*, 4375–4376.
81. K. Nakamura; Y. Inoue; A. Ohno, *Tetrahedron Lett.* **1995**, *36*, 265–266.
82. K. Nakamura; T. Matsuda; A. Ohno, *Tetrahedron Asymmet.* **1996**, *7*, 3021–3024.

83. K. Nakamura; K. Kitano; T. Masuda; A. Ohno, *Tetrahedron Lett.* **1996**, *37*, 1629–1632.
84. K. Nakamura; T. Matsuda; N. Nakajima, In *Stereochemical Control of Asymmetric Reduction of Ketones by* Geotrichum candidum. Proceedings of the 42nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Masaki, Ed.; Gifu, Japan, 1998; pp 159–160.
85. T. Matsuda; Y. Inoue; T. Harada; K. Nakamura, In Proceedings of the 44th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; H. Nishimura, Ed.; Sapporo, Japan, 2000; pp 160–163.
86. T. Saika; Y. Naoshima, In *Enantioselective Bioreduction of Prochiral Ketones with Carrot Cells*. Proceedings of the 40th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Kurokawa, Ed.; Saga, Japan, 1996; pp 105–107.
87. K. Nakamura; I. Misawa; T. Matsuda; T. Yokoi; Y. Mikata; A. Ohno, In *Asymmetric Reduction of Aromatic Ketones Containing a Nitrogen Functional by* Geotrichum candidum. Proceedings of the 42nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Masaki, Ed.; Gifu, Japan, 1998; pp 161–162.
88. K. Nakamura; Y. Inoue; T. Matsuda; I. Misawa, *J. Chem. Soc. Perkin Trans. 1* **1999**, 2397–2402.
89. K. Nakamura; T. Matsuda; M. Shimizu; T. Fujisawa, *Tetrahedron* **1998**, *54*, 8393–8402.
90. K. Nakamura; R. Yamanaka; A. Ohno; H. Hamada, In *Asymmetric Reduction of Ketones with the Cultured Suspension Cells of* Marchantia polymorpha. Proceedings of the 42nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Masaki, Ed.; Gifu, Japan, 1998; pp 157–158.
91. R. Yamanaka; K. Nakamura, In *Asymmetric Syntheses of Optically Active Alcohols by Phototroph*. Proceedings of the 45th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Hirai, Ed.; Toyama, Japan, 2001; pp 369–371.
92. H. Hamada; T. Tanaka; T. Furuya; H. Takahata; H. Nemoto, *Tetrahedron Lett.* **2001**, *42*, 909–911.
93. S. Pedragoss-Moreau; A. Archelas; R. Furstoss, *J. Org. Chem.* **1993**, *58*, 5533–5536.
94. C. Abalain; D. Buisson; B. Azerad, *Tetrahedron Asymmet.* **1996**, *7*, 2983–2996.
95. J. L. Taylor; L. I. Haharia; H. Chen; E. Anderson; S. Abrams, *Phytochemistry* **2006**, *67*, 1887–1894.
96. A. Kergomard; M. F. Renard; H. Veschambre, *J. Org. Chem.* **1982**, *47*, 792–798.
97. K. Shimoda; N. Kubota; H. Hamada; M. Kaji, In *Cyanobacterium-Catalyzed asymmetric Reduction of Enones*. Proceedings of the 47th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Kurata, Ed.; Tokyo, Japan, 2003; pp 164–166.
98. T. Gondai; K. Shimoda; T. Hirata, In *Asymmetric Reduction of Enone Compounds by* Chlorella miniata. Proceedings of the 43rd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Ohga, Ed.; Oita, Japan, 1999; pp 217–219.
99. T. Hirata; K. Shimoda; T. Gondai, *Chem. Lett.* **2000**, 850–851.
100. K. Shimoda; S. Izumi; T. Hirata, *Bull. Chem. Soc. Jpn.* **2002**, *75*, 813–816.
101. M. J. Taschner; D. J. Black, *J. Am. Chem. Soc.* **1988**, *110*, 6892–6893.
102. D. Ohba; N. Furuya; M. Aoki; K. Shimoda; R. Utsumi; S. Izumi; T. Hirata, In *Asymmetric Induction in the Hydrolysis of Enol Acetates by the Cultured Cells of* Marchantia polymorpha. Proceedings of the 40th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Kurokawa, Ed.; Saga, Japan, 1996; pp 113–115.
103. T. Hirata; S. Izumi; K. Akita; H. Yoshida; S. Gotoh, *Tetrahedron Asymmet.* **1993**, *4*, 1465–1466.
104. S. Matsumura; T. Kawamori; S. Yoshikawa, *Chem. Lett.* **1991**, 729–730.
105. K. Nakamura; Y. Yuki; T. Matsuda; A. Ohno; T. Kitayama; T. Okamoto, In *Diastereoselective Reduction of Ethyl 2-Methyl-3-Oxobutanoate by Acetone Powder of Microbe*. Proceedings of the 40th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; S. Kurokawa, Ed.; Saga, Japan, 1996; pp 98–99.
106. M. Iimori; K. Mitsuhashi; A. Shinohara; K. Tokoro, In *Production of Optical Active γ-Decalactone in Biotransformation by* Candida sorbophila. Proceedings of the 47th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; K. Kurata, Ed.; Tokyo, Japan, 2003; pp 94–96.
107. T. Tachihara; H. Hashimoto; T. Komai; S. Tamogami; M. Ogino, In *Microbial Resolution of 2-Methylbutyric Acid and Its Application*. Proceedings of the 48th Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; T. Kajiwara, Ed.; Yamaguchi, Japan, 2004; pp 387–389.
108. V. Alphand; A. Archelaas; R. Furstoss, *J. Org. Chem.* **1990**, *55*, 347–350.
109. L. Andrau; J. Lebreton; P. Viazzo; V. Alphand; R. Furstoss. *Tetrahedron Lett.* **1997**, *38*, 825–826.
110. K. Nakamura; M. Takeuchi; A. Ohno, In *Construction of a Chiral Quaternary Carbon Center by Microbial Asymmetric Reduction of a Prochiral 1,3-Diketone*. Proceedings of the 42nd Symposium on the Chemistry of Terpenes, Essential Oils and Aromatics; Y. Masaki, Ed.; Gifu, Japan, 1998; pp 163–164.
111. T. Hirata; A. Takarada; M.-E. F. Hegazy; Y. Sato; A. Matsushita; Y. Kondo; A. Matsuki; H. Hamada, *J. Mol. Catal. B: Enzym.* **2005**, *37*, 131–134.
112. A. Hammoumi; G. Revial; J. D'Angelo; J. P. Girault; R. Azerad, *Tetrahedron Lett.* **1991**, *32*, 651–654.
113. Z. Ouazzani; S. Arseniyadis; R. Alvarez-Manzaneda; E. Cabrera; G. Ourisson, *Tetrahedron Lett.* **1991**, *32*, 647–650.
114. Z. Ouazzani; S. Arseniyadis; R. Alvarez-Manzaneda; A. Rumbero; G. Ourisson, *Tetrahedron Lett.* **1991**, *32*, 1983–1986.
115. S. Arseniyadis; Z. Ouazzani; R. Rodriguez; A. Rumbero; G. Ourisson, *Tetrahedron Lett.* **1991**, *32*, 3573–3576.
116. T. Suga; T. Hirata, *Phytochemistry* **1990**, *29*, 2393.

Biographical Sketches

Professor Asakawa studied organic chemistry at the graduate school of Hiroshima University. He was appointed as a research assistant there in 1969, obtained his Ph.D. degree in 1972, and then went as a postdoctoral fellow to the Université Louis Pasteur, France, where he worked for 2 years with Professor Guy Ourisson. In 1976, he moved to the Faculty of Pharmaceutical Sciences, Tokushima Bunri University as an associate professor, became full professor in 1981, served twice as Dean, and is currently the Director of the Institute of Pharmacognosy (1986–present) and the president of Phytochemical Society of Asia (2007–). He is the coeditor of Phytomedicine and serves on the editorial boards of *Phytochemisty*, *Phytochemistry Letters*, *Planta Medica*, *Fitoterapia*, *Flavour and Fragrance Journal*, *Natural Product Communication*, *Natural Product Research*, *Spectroscopy*, *Arkivoc*, *Current Chemical Biology*, and *Malaysian Journal of Sciences*, among others. He has published 540 original papers, 20 reviews, and 27 books and monographs. For his outstanding research, he was awarded the first Hedwig medal (1983), the Pergamon Phytochemistry Prize and Certificate (1997), The Tokushima Newspaper prize (1997), and the ISEO prize (2004). Over the years, he has welcomed 37 postdoctoral researchers from various countries into his laboratory.

Professor Yoshiaki Noma was born in 1947 in Hyogo Prefecture. He graduated from the Faculty of Agriculture, Okayama University, and then entered into the Graduated School of Agricultural and Chemical Sciences of Okayama University and Osaka Prefectural University and obtained Ph.D. from Osaka Prefectural University in 1975. Professor Noma was an associate professor at Osaka Joshi-Gakuen Junior College, and in 1988 he moved to the Department of Human Life Sciences at Tokushima Bunri University as a full professor. Professor Noma is a fellow of the Japan Society for Bioscience, Biotechnology, and Agrochemistry. He is also a fellow of the Chemical Society of Japan and Japanese Society of Nutrition and Food Sciences.

Professor Noma has published over 55 research papers and reviews on several topics connected with the microbiological biotransformation of monoterpenes and sesquiterpenoids, the metabolic pathways of monoterpenoids, and the chemistry and biological activity of metabolites. At present, his research on biotransformation includes substances from liverworts and higher plants of potential use in cancer, those with antioxidant properties, and mosquitocidal compounds. He is also interested in the study of biosynthesis of biological active compounds.

3.22 Beer Flavor

Leen C. Verhagen, University of Leiden, Leiden, The Netherlands

3.22.1 Introduction

Beer is one of the most widespread and largely consumed alcoholic drinks in the world. The total world's beer production amounts to about 1.7 billion liters. The major beer producing countries are Germany, USA, and China. The brewing industry is dominated by some very big groups, of which the top five together produces some 45% of the world's total beer production (see **Table 1**).

Beer is an alcoholic beverage made from the ingredients – water, malted barley, hops, and yeast.

The main constituents of beer are water (~92%), ethanol (~4%), carbohydrates (~2.8%), proteins (~0.5%), and carbon dioxide (~0.5%). However, besides these main constituents, there are many minor components, which originate from the raw materials, or are formed *de novo* during the brewing process or storage of the beer. Although many of these components have been identified and their origin elucidated, numerous minor components today are still unknown. On the whole, beer is an extremely complex beverage and its flavor depends on the delicate balance between its numerous components.

As for many food products, the flavor of beer changes during storage. Temperature, light, and oxygen initiate chemical reactions changing the flavor balance, because flavor compounds increase or decrease in concentration or are newly formed. Beer aging is a major quality issue, because the product may after a period of time no longer meet the consumer's expectation. The chemistry of the phenomenon of beer flavor aging has been studied for more than 40 years and found to be extremely complex. Brewers use the results to develop technological process improvements to control the shelf-life of their products in a better way. Despite these efforts, aging is still one of the main concerns for global brewers. The type of flavor changes during storage is only partly controllable and consumers experience this in the market place.

This chapter deals with the chemistry of beer flavor. The origin and formation of the dominant flavors and off-flavors in beer is described, with emphasis on the bittering compounds originating from hop, which is a minor ingredient but with a huge impact on the sensory and physical quality of beer.[1] In addition, some of the chemical changes that occur during storage of beer are highlighted.

3.22.1.1 Brewing Process

Brewing is a multistage process. It starts with the mixing of barley malt and brewing water (so-called mashing) and heating of the slurry. Enzymes in the malt degrade starch and proteins and a mixture of sugars, peptides, and amino acids are formed.

Malt contains a range of carbohydrates, composed of insoluble cellulose and soluble hemicellulose, dextrin, starch, and sugars. Starch, which accounts for about 50–60% of the weight of malt, is composed of amylose, which decomposes during mashing into maltose and maltotriose and amylopectins which decomposes into glucose molecules (**1**).

Table 1 World's beer production in 2006

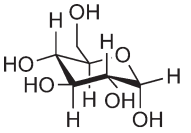
Region of production	*2006 (1000 hl)*
European Union	386 169
Rest of Europe	182 550
Europe total	*568 719*
North America	255 458
Central America/Caribbean	94 343
South America	172 279
America total	*522 080*
Asia	*508 037*
Africa	*78 807*
Australia/Oceania	*21 295*
World Total	*1 698 938*

Source: Barth Report 2006/2007, (http://www.barthhaasgroup.com).

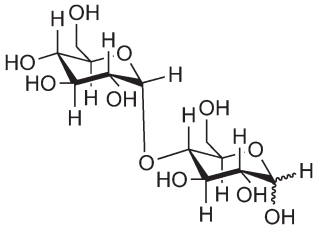
The most important reaction during mashing is the conversion of starch into low-molecular weight fermentable sugars and unfermentable higher molecular weight dextrin. Maltose (**2**), the most common carbohydrate associated with brewing consists of two glucose units and maltotriose (**3**) of three glucose units (**Figure 1**). Maltotriose is still fermentable by most brewing yeast strains while higher dextrins are not.[2] Sucrose, another disaccharide, is also present in malt though in low concentration. The cellulose components in the malt do not give fermentable extract or flavor.

Time, temperature, and pH are important factors influencing the enzymatic breakdown of the starch molecules. The principal enzymes, alpha- and beta-amylase, have a different temperature and pH operating range. Alpha-amylase is more temperature resistant and has an optimum between 72 and 75 °C, but is destroyed at 80 °C. It has an optimum pH between 5.6 and 5.8. For beta-amylase, the optimum temperature is between 60 and 65 °C and the pH between 5.4 and 5.5. The difference in temperature optimum is used by the brewer to control the composition of the mash and the ratio of fermentable and nonfermentable sugars. The higher the temperature used for the mashing process, the greater the proportion of unfermentable dextrins in the liquor. The latter contribute to the body and the mouthfeel of the final beer. Mashing at lower temperatures results in more fermentable sugars and subsequently a higher alcohol production during fermentation.

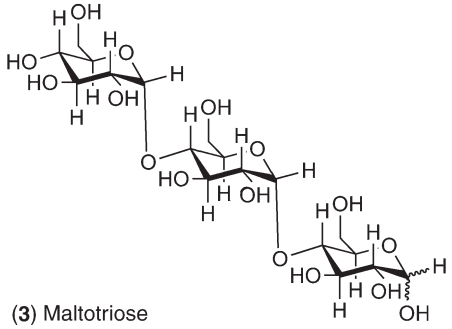
Malted barley contains polyunsaturated fatty acids, such as linoleic and linolenic acid, which readily form oxidation products, which can be the precursors for aging compounds formed in the final beer.[3–6] During mashing enzymatic and nonenzymatic oxidation of the unsaturated fatty acids takes place. Reduction of oxygen contact during mashing has a positive effect on the flavor stability of the final beer.[7] Brewing with barley-malt lacking the enzyme lipoxygenase-1 also results in better flavor stability of the final beer.[8,9]



(**1**) α-Glucose



(**2**) Maltose



(**3**) Maltotriose

Figure 1 Fermentable sugars.

After the mashing is completed, filtration is carried out to obtain a solution containing about 12–14% (w/w) sugar, which is called sweet wort. With the filtration of the mash (called lautering or mash filtration) solid materials such as spent grains are removed. Together with the solids and the turbidity much of the unwanted fatty acid materials are also removed. The effects of the clarity of the wort after lautering on the fermentation performance and later on the flavor stability of the final beer has been a subject of many studies.[10,11]

After the lautering, the sweet wort is boiled for at least 1 h together with hops, the flowers (so-called cones) of the female hop plant which provide flavor to beer. The boiling serves several purposes: sterilization, deactivation of enzymes, protein precipitation, color formation, removal of unwanted volatile components and, very important, the conversion (isomerization) of the main constituents of the hops, the α-acids, into the iso-α-acids, the main bittering compounds found in beer. During boiling of the wort the following changes occur.

1. Proteins and phenolic compounds from the malt form insoluble complexes and precipitate. This is important to increase the colloidal stability of the final product.
2. The wort becomes darker because of the formation of melanoidins, as a result of reactions of sugars with amino acids, oxidation of polyphenols, and caramelization of sugars.
3. Many volatile compounds, which are present in the malt and hops, such as volatile sulfur components, aldehydes, and hydrocarbons, are evaporated. This is important for the quality of the final beer, as many of these volatile compounds are considered negative for beer flavor.

Dimethyl sulfide (DMS) is a particularly important malt component, which is rapidly lost during the boiling of the wort. To decompose its precursor, *S*-methylmethionine (SMM), adequate boiling time is required. If the boiling is stopped too soon the remaining SMM can still decompose during the cooling of the wort, but without evaporation of the DMS formed. Consequently, a very high concentration of DMS can carry through in the final beer where it is considered an off-flavor.

Boiling concentrates the wort to its desired strength for fermentation. On average, the volume decreases by 8–10% per hour of boiling. Finally, boiling also sterilizes the wort, which is important to avoid microbiological spoilage during the next steps in the process, fermentation and maturation. After the boiling, the wort is cooled and solid materials, precipitated proteins, spent grain, and spent hops, are removed and the clear liquid (hopped wort) is ready for fermentation. Yeast is added and the solution is aerated to facilitate the yeast growth. During the main fermentation phase, yeast converts the fermentable carbohydrates in the wort into ethanol and carbon dioxide. During fermentation numerous other flavor-active volatile components, such as esters, aldehydes, and higher alcohols, are being formed as by-products, which have an important contribution to the flavor of the final beer. The composition of these flavors depends on the yeast strain and the fermentation conditions, enabling the brewers to create unique flavors in different beer types.

After the main fermentation the liquid, called green beer or young beer, is not yet ready for consumption. It contains too many undesirable flavor components, also formed during the main fermentation. It requires a period of maturation or conditioning of several weeks at low temperature during which off-flavor compounds are either transformed (reduced) into less flavor-active compounds by the remaining yeast cells or are purged by the carbon dioxide which is still formed in this phase of the process.

The most dominant compounds, which need monitoring during the maturation phase, are diacetyl and 2,3-pentanedione. These compounds are particularly unwanted in lager-type beers because of their very low flavor threshold value. Only when the content of these flavor-active compounds has decreased to below their critical concentration the beer is ready for filtration and can eventually be packaged in kegs, bottles, or cans.

In order to avoid problems with microbiological contamination in the packaged beer, the bottled or canned beer may be pasteurized. Alternatively, cold sterile filtration can be used before bottling of the beer. A simplified scheme with the steps in the brewing process is depicted in **Figure 2**.

Malting and brewing technology have remained very traditional over the years, but the efficiency of the process has increased through understanding of the technology and the underpinning science. Innovation in the brewing industry is driven by cost reduction, for example, by more efficient use of the raw materials and lower energy consumption, and the need for improved quality, safety, and wholesomeness of the final product.[12]

Extensive state-of-the-art knowledge of brewing science and practice is described in a standard work by Briggs *et al.*[13] Research and innovation in brewing process and technology and their effects on beer flavor have been reviewed by Bamforth[14] and by Meilgaard.[15]

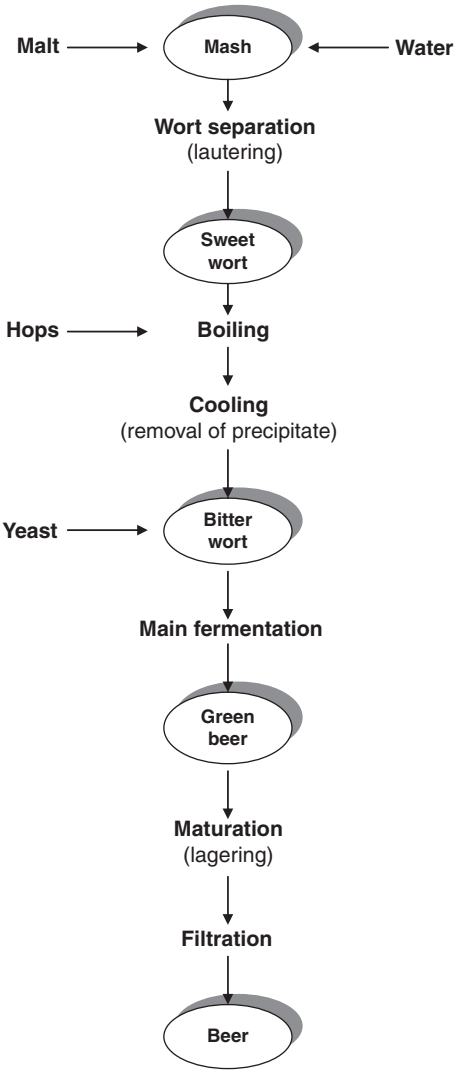


Figure 2 Main steps in the brewing process.

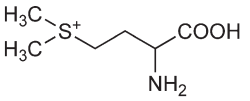
3.22.1.2 Ingredients in Beer Production

3.22.1.2.1 Barley malt

Barley is the major raw material for beer brewing. It is so well suited for brewing because it has a high content of enzymes for the conversion of starch into sugars and it contains proteins necessary for yeast nutrition. Per liter of beer about 160 g of barley is used.

Barley is grown for food, for brewing, and for feed. The world barley production amounts to about 130–150 million tons, but only about 20% of it is suitable for beer production. The rest is used for food and feed purposes. There are two types of barley: six-row and two-row barley that differ in enzyme, protein, and starch content. In general, two-row barley has a lower content in enzymes and proteins, but contains more starch, although this can differ between cultivars. Barley husks contain a relative high content of polyphenols, which impart astringent flavors to beer.

Prior to brewing, barley has to be subjected to a treatment called malting. It serves the purpose to convert insoluble starch into soluble starch and sugars, the breakdown of complex proteins to generate nutrients for the yeast and to develop enzymes.



(**4**)
S-Methylmethionine (SMM)

(**5**)
Dimethyl sulfide (DMS)

Figure 3 *S*-Methylmethionine and dimethyl sulfide.

The malting process consists of three steps: steeping, germination, and kilning. Steeping begins with the mixing of barley grains with water to raise the moisture content and to start the metabolic reactions in the barley kernel. The wet barley is stored for a period of 3–4 days at an elevated temperature of about 60 °C and high humidity to start the germination process in which small amounts of sugars, soluble starch, and enzymes are being formed and complex proteins are degraded into soluble peptides.

During germination the synthesis of the compound SMM (**4**) occurs, which is the main precursor of DMS (**5**), an important beer flavor component (**Figure 3**). The SMM content is variety dependent. Germination is stopped by drying (kilning) of the malt. The higher the kilning temperature the more SMM is decomposed and the resulting DMS is evaporated from the malt.

During the kilning phase color is formed due to Maillard-type reactions and caramelization of sugars. The higher the kilning temperature, the more color compounds are formed. By controlling time and temperature pale colored or darker colored malts are made. Most of the color of beer is due to the malt or malt-mixtures used for it. Malt also has a distinct contribution to the flavor of beer, which is more pronounced in darker colored beers, and on the flavor stability of beer due to the presence of antioxidants.[16–19] Higher kilning temperatures also reduce the residual activity of the enzyme lipoxygenase,[20] which plays an important role in lipid oxidation and beer flavor stability.

After the kilning, the rootlets of the germinated kernels are being removed. This is important as the rootlets contain relatively high levels of carbonyl compounds, for example, (*E*)-2-nonenal. Many short-chain carbonyls have low flavor threshold values and give beer a stale flavor when present above their threshold level.

In addition to carbohydrates and proteins, malt also contains vitamins, polyphenols, lipids, and fatty acids. The latter can affect the foam quality while oxidation of unsaturated fatty acids can induce stale flavors in beer. Malted barley can partly be replaced by other starch or sugar containing raw materials such as corn, rise, or malt syrup (the so-called adjuncts).

3.22.1.2.2 Yeast

The metabolizing of carbohydrates by brewing yeast leads to the production of carbon dioxide and ethanol. At the same time a whole range of other by-products are being formed which are of major importance for the final beer flavor. There are two types of brewing yeast: the ale top-fermenting type *Saccharomyces cerevisiae* and the bottom-fermenting type lager yeast *Saccharomyces uvarum*, also known as *Saccharomyces carlsbergensis*. Today, both types are classified as members of *Saccharomyces cerevisiae*.

Bottom-fermenting and top-fermenting yeasts are used at different temperatures. After the main fermentation, when the available sugars have been used, the yeast cells flocculate together and, depending on yeast strain and temperature, will drop to the bottom of the tank or rise to the top and float on the liquid. Typically, lager-beer strains are bottom fermenting at temperatures 5–8 °C, while ales and wheat beers are top fermented at 10–25 °C.

To save tank capacity brewers increasingly ferment highly concentrated wort and dilute the final product back to the desired alcohol strength. However, brewing yeast can behave different when the sugar concentration of the wort is getting very high.[21]

Traditional beer fermentation and lagering takes place in batch processes in cylinder-conical tanks. Continuous fermentation, using immobilized cells, is increasingly used in the bio-industry for economic reasons and is also recognized to have potential for the brewing industry. It is successfully used by some breweries, though not widely. Improved technological control of the flavor profile, in the production of flavor-active compounds such as higher alcohols, esters, and the vicinal diketones diacetyl and 2,3-pentanedione still needs further investigation.[22–26]

3.22.1.2.3 Water

Beer is composed of more than 90% (w/w) water. The mineral content of the brewing water is of importance in the various steps in the brewing process but also has an important contribution to the flavor of beer.

Historically, breweries were located at sites with water supplies having a consistent and characteristic composition. In modern breweries, the mineral composition of brewing water is often adjusted to the required composition. Water treatment typically involves pH reduction, mineral salt adjustment, dechlorination, removal of particles, and microbiological control.

3.22.1.2.4 Hop

An essential raw material for beer production is hops.[27,28] Until the 1920s brewers used large quantities of hops mainly to prevent microbiological infections because of the aseptic activity of hop bitter acids. In modern brewing practice with high standards of hygiene this is no longer an issue.

Today the main reason for the use of hop is to impart a bitter taste to beer, but it also enhances the aroma and helps to stabilize beer foam. Compared to barley, hop is a minor ingredient but with an extremely important contribution to beer flavor. The quantity of hops varies per beer style, but on average only about 0.5–2 g of hop is used per liter of beer depending on variety, bitter acid content, and beer style. Typically, US beers are very low in bitterness, while traditional English ales can be very bitter and German and Czech beers can be very bitter and heavily flavored with aroma hop varieties.

Hop is an agricultural crop commercially grown in the moderate temperature zones between the 35th and 55th latitude, both in the northern and southern hemisphere. Although hops are grown in many countries, the commercially most important production areas are found in the Pacific North West of the United States (in the states Oregon, Washington, and Idaho), in Europe (mainly Germany and Czech Republic) and in China. Hop is a perennial plant with male and female plants, of which only the latter has brewing value. Hop growing takes places between April and August in the northern hemisphere and between October and February in the southern hemisphere.

The size of the hop industry is relatively small because hops are almost exclusively used for the production of beer. The total world hop production amounted in 2006 to approximately 86 000 tons. In the past 10 years, the total area under cultivation dramatically decreased from approximately 77 000 ha in 1996 to approximately 49 500 ha in 2006. Hop production fell in this period from approximately 124 000 tons in 1996 to the current 86 000 tons in 2006. In the same period world beer production increased from 1268 million hl to about 1698 million hl.

The reasons for the dramatic decline in the hop production area are that globally brewers have gradually reduced the amount of hop in their beer in response to consumer preferences for less bitter beer. Increasingly they are also using more sophisticated hop products, which have higher utilization rates compared to the traditional hop use. At the same time successful hop breeding programs and improved hop cultivation practices have elevated both the yield of tons of hops per hectare as well as the amount of the most important compounds in hops, the bitter acids.

The flowers of the female hop plant, called cones, contain a sticky yellow material, the so-called lupulin glands, which contain the hop bitter acids and essential oils (**Photos 1** and **2**). Male plants do not produce cones and therefore do not have value for the brewers.

Raw hop cones contain up to 20% or more of their dry weight as bitter acids. The relative amount of the different compounds depends on the variety. Many different varieties or cultivars of hop exist. Commercial varieties are classified into groups according to their use in brewing[29] and are generally divided into categories of bitter hops and aroma hops. More recently, with the advent of new varieties, hops are being grouped as fine aroma, aroma, bitter hops (often called dual purpose hops) and high-α-acid varieties. Both aroma hops and bitter hops contain bitter compounds and essential oil, but in different quantities and ratios in composition. Brewers select hop varieties depending on the beer style they produce. Beers with a very distinct hop aroma are usually produced with (mixtures of) aroma hop varieties, which are added in steps to the boiling wort at different time intervals and in part just before the end of the boiling with the aim to preserve the aroma as much as possible. As a consequence the α-acids in these late hop dosages are not or only partly isomerized. Contrary to this, beers with a more neutral aroma are mostly produced with bitter hops which are added at the start of the boiling in order to obtain maximum isomerization of the α-acids.

Photo 1 Hop flowers (cones).

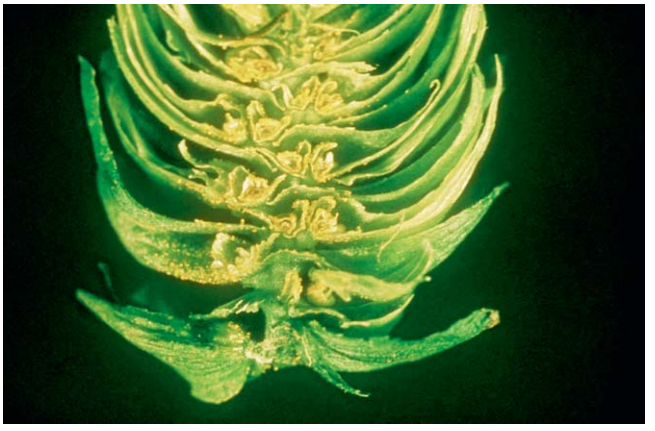


Photo 2 Lupulin glands inside of the hop cone.

Hop plants grow fast and vigorously and require large amounts of fertilizer. As a consequence hop can contain a relatively high concentration of nitrate and can significantly contribute to the total nitrate content of beer. Hops are sensitive to a range of diseases and insects and chemical or biological protection is required for commercial production of the crop. The use of agrochemicals is of concern for both environmental and economical reasons. Brewers are also concerned about the possibility of residues of the chemicals remaining on the hops, which potentially could end up in the final beer. Hop breeding programs are aiming to increase the natural resistance of the hops in order to reduce or eliminate the use of agrochemicals.

At present hops are almost exclusively used in beer brewing, but research efforts are made to find other applications. Hops also are gaining interest because of their antioxidant activity and potential bio-activity of their constituents,[30,31] which may open new applications, outside the brewing industry.

3.22.2 Hop Chemistry

Hop is a natural product with a very complex composition.[1,32–34] Hop cones contain different groups of organic compounds. The major classes of compounds are shown in **Table 2**.

Table 2 Composition of dried hop cones

Major compound class	*Quantity (% w/w)*
α-Acids	2–17
β-Acids	2–10
Essential oil	0.5–3.0
Polyphenols and tannins	3–6
Monosaccharides	2
Amino acids	0.1
Proteins	15
Lipids and fatty acids	1–5
Pectins	2
Ash and salts	10
Cellulose and lignins	40–50
Water	8–12

Source: Reproduced from J. L. Benitez; A. Forster; D. De Keukeleire; M. Moir; F. R. Sharpe; L. C. Verhagen; K. T. Westwood, *Hops and Hop Products – Manual of Good Practice*; EBC Technology and Engineering Forum, Hans Carl Verlag: Nurnberg, 1997; ISBN 3-418-00758-9.

For the brewers three groups of hop compounds are of particular importance: the bitter acids, essential oil, and polyphenols. The bitter acids are unique to hops and no other plant species in the world is known to contain such compounds, but constituents in hop essential oil and hop polyphenols are not unique and similar compounds are found in other plants as well.

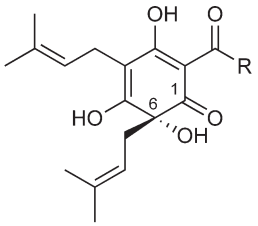
Historically, the group of the hop bitter compounds were collectively called resin, a mixture of compounds, some of which were known, some of which were not. The hop resin was classified on solubility: the so-called soft resin is soluble in hexane, while hard resin is not. Both the soft resin and the hard resin fractions are soluble in cold methanol and diethyl ether. The provision that total resin should be soluble in cold methanol was included to distinguish between hop resin and wax. Hop wax consists of mixtures of long-chain alcohols, acids, esters, and hydrocarbons, all of which are very poorly soluble in cold methanol.

The soft resin fraction contains two related series of compounds, called α-acids and β-acids. The hard resin fraction consists of a mainly undefined mixture of oxidation products of the soft resins. The hard resin fraction of the hops increases when the hop gets oxidized due to improper post-harvest treatment or with poor storage condition. For high-quality hop the hard resin fraction should be as low as possible and is typically for fresh hops 1.0–2.0% (w/w).[35]

In the past a less specific method, the Lead Conductometric Value (LCV), was used for the α-acid analysis.[36] The method is based on a conductometric titration of a toluene extract of hops with lead(II)acetate and does not only determines the total amount of α-acids but also includes other undefined bitter compounds. At the present time specific analysis of the hop α- and β-acids can be made using HPLC. For fresh hops the HPLC α-acid content and the LCV are not very different, typically the ratio of LCV/α-HPLC is 1.1–1.2. When hop deteriorates because of oxidation the ratio increases and can serve as an indicator for deterioration.

3.22.2.1 α-Acids

The most important group of compounds within the resin fraction are the α-acids. This group consists of up to five homologues differing in the acyl side chain (**Figure 4**). The main components are called cohumulone (**6**), humulone (**7**), and adhumulone (**8**), while pre- and posthumulone are only present as minor components. Humulone is sometimes called *n*-humulone or 'normal' humulone, to distinguish from the other members of the group of α-acids. This notation can however be confusing, since the IUPAC nomenclature has reserved the term '*n*' for straight chain carbon groups.[1]



α-Acids

R = –$CH(CH_3)_2$ Cohumulone (**6**)
–$CH_2CH(CH_3)_2$ Humulone (**7**)
–$CH(CH_3)CH_2CH_3$ Adhumulone (**8**)

Figure 4 α-Acids.

All hop cultivars contain the five different α-acids, but the ratio of the individual compounds varies, in particular the ratio of humulone to cohumulone is variety dependent and cohumulone is found in the range of 20–35% of the total α-acids content of the hop.[37] The relative amount of adhumulone within the α-acids group is more or less constant in the different varieties. Typically, aroma hops are relatively low in α-acids (3–6%) while bitter hops have a high content of α-acids (up to 18%). For the brewers the α-acids are of prime importance because they are the precursors for the bitter compounds in beer, the so-called iso-α-acids (see Section 3.22.2.3). The conversion of α-acids into iso-α-acids depends on temperature, time, and pH. The α-acids are weak acids, with pK_a of 5.1 for humulone[38] and are poorly soluble in water. In the brewing process the initial pH is about 5.6 and decreases to about pH 4.3 in the final beer, therefore, the main portion of the α-acids is lost due to precipitation and by adsorption to solids which are removed from the process. Prolonged heating also results in losses of the iso-α-acids due to degradation to uncharacterized products. The overall yield of iso-α-acids is at best only 30–35% of the amount of α-acids dosed at the start of the wort boiling. This poor utilization and the difficulty of controlling the isomerization are of concern to brewers because it contributes to inconsistency in the taste. Also, for economic reasons, an elevated yield would be more preferable.

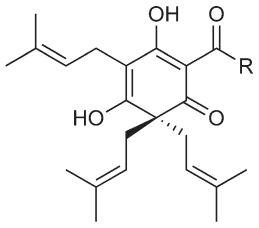
Factors affecting the isomerization rate and the overall utilization have been studied extensively. A study on the effect of glucose, maltose, calcium, and pH in the range 4.8–6.0, across a temperature range of 90–130 °C, revealed that none of these affect the rate of production of the iso-α-acids, but pH was found to have a marked effect on the measured α-acids concentration which is attributed to solubility.[39]

The isomerization reaction is found to be first order, with reaction rate varying as a function of temperature.[40] A small portion of the α-acids survives the brewing process unchanged. In beer unisomerized α-acids can be detected in concentrations of 0.1–4 mg l^{-1}, but they do not contribute to the sensory bitterness of beer.[41]

Of the three main α-acids cohumulone is the most polar and is found to have the highest utilization throughout the brewing process. The isomerization product of cohumulone is believed to give poorer bitterness quality in beer and to contribute to harshness and astringency,[42] but data are conflicting. Despite disagreement on the effect of cohumulone on beer bitterness quality some brewers select hop varieties low in cohumulone content for their beers and in breeding programs the cohumulone content is one of the selection criteria and targets for new varieties.

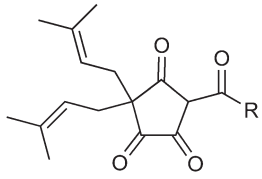
3.22.2.2 β-Acids

The second group of bitter acids in hop resin are the β-acids. They also consist of five different homologues: colupulone (**9**), lupulone (**10**), adlupulone (**11**), and minor quantities of prelupulone and postlupulone (**Figure 5**). Similar to the α-acids, the various β-acids differ in the acyl side chain. Compared to the α-acids, the solubility of β-acids in water is even lower. β-Acids are weak acids with pK_a of approximately 6.1.[37] All hops contain both α- and β-acids. The ratio of α/β-acids can be lower than 1.0 for some aroma hops, but for bitter hops the ratio is in the range 1.5–4.0.



β-Acids

R = $-CH(CH_3)_2$ Colupulone (**9**)
$-CH_2CH(CH_3)_2$ Lupulone (**10**)
$-CH(CH_3)CH_2CH_3$ Adlupulone (**11**)



(**12**) Hulupone

Figure 5 β-Acids and hulupone.

The transformation of β-acids in the brewing process is quite different from the α-acids. Most importantly, they do not undergo isomerization because β-acids lack the tertiary alcohol function on carbon atom 6. Instead, β-acids undergo a variety of oxidation reactions, mainly involving the double bonds of the prenyl side chains in the molecule. A series of oxidation products has been identified by Verzele and De Keukeleire.[33] Auto-oxidation of β-acids results in hulupones (**12**), which can be present in aged, oxidized hops. Hulupones are bitter in taste, survive the brewing process, and contribute to the sensory bitterness intensity and quality. This is one of the explanations of the observation that beer brewed with oxidized, deteriorated hops still exhibits sensory bitterness despite low or even absence of iso-α-acids in the beer. For consistent bitterness quality, it is desirable to use fresh hops and to avoid as much as possible oxidation and degradation products of α- and β-acids in beer.

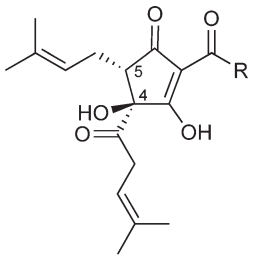
Although for the brewer the β-acids have little value, traditional hop products intrinsically contain both α- and β-acids. Because of their low solubility in water most of the β-acids in the hop precipitate in the wort and is removed from the process together with other solids and in beer only traces of β-acids can be detected, typically in the range of 10–100 $\mu g\,l^{-1}$. β-Acids are however commercially used as a starting material for the production of modified hop products with specific properties, such as the reduced iso-α-acids (see Section 3.22.2.4).

In the sugar industry β-acids are used as preservative in the processing of sugar beets.[43–45]

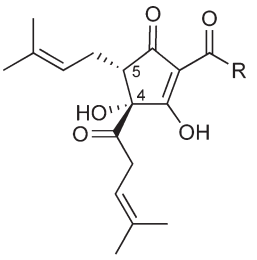
3.22.2.3 Iso-α-Acids

Iso-α-acids are a main quality factor in beer.[46] Traditionally they are formed during the boiling of wort with hop where the hop α-acids thermally isomerize into the intense bitter tasting iso-α-acids. The iso-α-acids also stabilize beer foam[47,48] and are antibacterial agents.[49,50]

When isomerized, each α-acid gives rise to two iso-α-acids, distinguished as *trans*-iso-α-acid (**13**) and *cis*-iso-α-acid (**14**), due to the spatial arrangement of the tertiary alcohol function at carbon atom 4 and the prenyl side chain at carbon atom 5. Because hop contains three main α-acids, at least six major iso-α-acids are present in beer (*cis*-isohumulone and *trans*-isohumulone, *cis*-isocohumulone and *trans*-isocohumulone and



(**13**) *trans*-iso-α-acids



(**14**) *cis*-iso-α-acids

R = $-CH(CH_3)_2$	Isocohumulone
$-CH_2CH(CH_3)_2$	Isohumulone
$-CH(CH_3)CH_2CH_3$	Isoadhumulone

Figure 6 Iso-α-acids.

cis-isoadhumulone and *trans*-isoadhumulone) (**Figure 6**). The ratio of the *cis*- and *trans*-iso-α-acids is determined by the isomerization conditions. In boiling wort, with a pH of about 5.6, the *cis*/*trans* ratio is typically around 2/1. Instead of isomerizing the α-acids in the brewing kettle, α-acids can also be isolated from hop or hop extract, purified and chemically isomerized using alkaline or magnesium catalyst. In these media, the *cis*/*trans* ratio differs from the ratio found under brewing conditions. Elevated pH of the isomerization medium gives *cis*/*trans* ratios of around 75/25, while magnesium catalyzed isomerization results in about 50% of the *cis* isomer.[51]

α-Acids can also be isomerized by light at a wavelength in the region 350–366 nm. This results in formation of 100% of the *trans* isomer.[33] However this process is not applied commercially.

The importance of the ratio of the *cis* and *trans* stereoisomeric bittering compounds for beer quality aspects, such as bitter intensity and bitterness quality and for foam stability and quality, are not yet clear. The sensory bitterness intensity of iso-α-acids is not linear with concentration and the perceived intensity depends on the beer type. Small differences in concentration are more difficult to detect at higher concentrations and in complex matrixes.

Studies with individual *cis*- and *trans*-iso-α-acids in model systems have indicated that *cis*-isohumulone is the most bitter iso-α-acid and *trans*-iso-cohumulone the least. Similar differences were demonstrated when the pure compounds were added to unhopped beer although the differences were partially masked by the background flavor of the unhopped beer.[52] The results demonstrate the difficulty with sensory testing of bitterness because many factors can affect the perceived bitterness. This has led to contradictory and confusing data on the sensory bitterness of iso-α-acids and in particular of the so-called reduced or modified iso-α-acids which are mainly used for downstream addition to beer (see Section 3.22.2.4).

Iso-α-acids in beer are not stable to visible light. When beer is exposed to day light photochemical degradation of the iso-α-acids results in the formation of a highly flavor-active compound in beer,[53] often called sunstruck flavor (see Section 3.22.5.2). To avoid this problem iso-α-acids can be modified (reduced) to various hydrogenated compounds.

3.22.2.4 Reduced Iso-α-Acids

Hydrogenation of iso-α-acids results in three different classes of the so-called modified or reduced iso-α-acids, called dihydro-, tetrahydro-, and hexahydroiso-α-acids, referring to the number of hydrogen atoms added into the molecule. Reduced iso-α-acids are stable to visible light, but also exhibit differences in bitterness and foam-enhancing properties. The chemistry of the reduced iso-α-acids is complex and has been described in detail by Verzele and De Keukeleire.[32,33]

3.22.2.4.1 ρ-Iso-α-acids

Treatment of iso-α-acids with sodium borohydride reduces the carbonyl group in the side chain at C4 to a tertiary alcohol group to form dihydroiso-α-acid (**15**), also known as ρ-iso-α-acid (**Figure 7**). Formation of the secondary alcohol group results in a new chiral center and thus two epimeric dihydroiso-α-acids are formed. This occurs for each of the individual iso-α-acids. Therefore, ρ-iso-α-acid theoretically contains in total 12 dihydroiso-α-acids, two for each iso-α-acid. In practice this is however not always found to occur. *trans*-ρ-Iso-α-acids are almost absent in ρ-iso-α-acid mixtures produced under caustic conditions. Goldstein and Ting[54–56] separated and identified stereoisomers of isomerized α-acid derivatives and found that reduction under neutral conditions resulted in the expected *cis* and *trans* diastereoisomers while reduction of *cis*/*trans*-iso-α-acids under caustic conditions only produces *cis*-isomers.

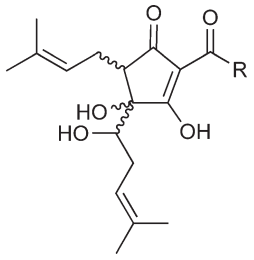
The reason for this is thought to be steric hindrance to the borohydride group when the iso-α-acids are in the *trans*-configuration. Also retro-isomerization to α-acids and subsequent re-isomerization to the more borohydride-reactive *cis*-orientation has been speculated.[54] ρ-Iso-α-acids are completely stable toward light. Their solubility in water is greater than that of the regular iso-α-acids.

3.22.2.4.2 Tetrahydroiso-α-acids

Hydrogenation of the double bonds in each of the two alkyl side chains of regular iso-α-acids with hydrogen and palladium as catalyst results in six tetrahydroiso-α-acids (**16**): *cis*/*trans*-tetrahydroisocohumulone, *cis*/*trans*-tetrahydroisohumulone, and *cis*/*trans*-tetrahydroisoadhumulone (**Figure 8**).

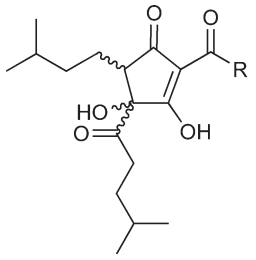
Tetrahydro-iso-α-acids can be derived from α-acids or from β-acids. The relative composition of the resulting mixture is different, because β-acids naturally contain a higher proportion of their co-homologues than α-acids. However, also the stereochemistry of the tetrahydroiso-α-acids prepared from either α-acids or from β-acids is different. When derived from the α-acids, there are six major tetrahydroiso-α-acids. Using β-acids as the source, there are 12 different tetrahydroiso-α-acids in the mixture.

Using chiral phase HPLC the enantiomeric compounds (+)*cis*- and (−)*trans*-tetrahydroiso-α-acids were found for α-acid-derived compounds and racemic (±)-*cis*- and (±)-*trans*-tetrahydroiso-α-acids were found when derived from β-acids.[56]



(**15**) ρ-iso-α-acids

Figure 7 ρ-Iso-α-acids.



(**16**) Tetrahydroiso-α-acids

Figure 8 Tetrahydroiso-α-acids.

Tetrahydroiso-α-acids are less soluble in water than regular iso-α-acids and exhibit a strong foam-enhancing capacity. Brewers use this property and add small quantities, typically 3–5 mg l^{-1}, of tetrahydroiso-α-acids to beer which otherwise contains regular hop bitter acids, to increase the foam stability.

Tetrahydroiso-α-acids are also used for the production of light stable beer to prevent formation of sunstruck flavor when the beer is exposed to light. Recently it has been demonstrated that tetrahydroiso-α-acids are not completely stable to light but in beer do not lead to the typical sunstruck flavor compound.[57,58]

3.22.2.4.3 Hexahydroiso-α-acids

The third class of chemically modified iso-α-acids is the hexahydroiso-α-acids (**17**). These compounds can be produced either by hydrogenation of ρ-iso-α-acids or sodium borohydride reduction of tetrahydroiso-α-acids. Similar as for the production of ρ-iso-α-acids from regular iso-α-acids, in borohydride reduction under neutral conditions the *cis*/*trans* configurations is retained and in total 12 hexahydroiso-α-acids can be derived from tetrahydroiso-α-acid when α-acids had been the source of the tetrahydroiso-α-acids.[56]

Hexahydroiso-α-acids prepared by borohydride reduction of tetrahydroiso-α-acids with β-acids as the source, theoretically give rise to eight hexahydroiso-α-acids for each co-, *n*-, and ad-variant, and so in total 24 components could be in the mixture. Hexahydroiso-α-acids show low solubility in water or in beer and exhibit potent foam-stabilizing properties, which makes them difficult to use in brewing (**Figure 9**).

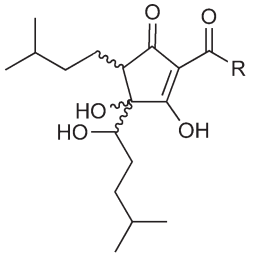
Reduced hop products are commercially available with various trade names and are used by the brewers to enhance the foaming capacity or the foam stability of their beers, to create a specific sensory bitterness or to make a beer resistant to light-induced formation of sunstruck off-flavor.

The sensory bitterness of ρ-, tetra-, and hexahydroiso-α-acids relative to regular iso-α-acids is difficult to assess and conflicting results can be obtained. Originally reported as 0.6×, 1.5–2×, and 1.2× the bitterness of regular iso-α-acids respectively,[59] these values have ever since been quoted in literature and in commercial brochures. The data however were obtained for the compounds dissolved in water. Studies of the sensory bitterness of the compounds in different beer styles at various concentrations confirmed the bitterness of ρ-iso-α-acids indeed to be significantly lower than regular iso-α-acids, but tetrahydro- and hexahydroiso-α-acids were found to be approximately equal in bitterness to regular iso-α-acids.[60,61]

The sensory characteristics of reduced iso-α-acids deviate from that of regular iso-α-acids and can be dependent on the beer style. In general ρ-iso-α-acids are found to be less bitter and mellower in taste, while tetra- and hexahydroiso-α-acids are more lingering and sometimes described as metallic when present above a certain concentration. Finally, overdosing of tetrahydro and hexahydroiso-α-acids in beer can result in artificial, coarse looking foam.

3.22.2.5 Preparation, Isolation, and Purification of Hop α- and β-Acids

Hop α- and β-acids can be synthesized *de novo*, but the methods are laborious and with low yield.[62,63] On laboratory scale as well as on commercial scale, separation and purification of α- and β-acids is more easily achieved by making use of their acidity and solubility.



(**17**) Hexahydroiso-α-acids

Figure 9 Hexahydroiso-α-acids.

Starting from a (commercial) CO_2 extract, typically containing about 50% α-acid and 25% β-acid, the α-acids are selectively extracted into an aqueous solution of about pH 8–9 (carbonate solution) leaving β-acids, oil, and other materials behind. By using an aqueous solution of pH 11–12 for extraction, the β-acids can be isolated.[64]

Pure α-acids can be obtained by complexation with *o*-phenylenediamine. Repeated recrystallization of the crystalline complex reveals pure complexes of the α-acids, which can be liberated by acid treatment.

The pure β-acids colupulone and lupulone can be obtained by direct recrystallization of the compound isolated from the CO_2 hop extract. CO_2 as extraction solvent already enables substantial separation between the α-acids and β-acids as groups, which can then be used as starting materials to prepare the pure analogues.

Preparative HPLC methods for the isolation of the pure analogues of the bitter acids can also be applied, but usually require large quantities of the mobile phase. Centrifugal Partition Chromatography has also successfully been used to separate and isolate larger quantities of the individual analogues of the α-acids and the β-acids.[65–67]

3.22.2.6 Preparation and Purification of Iso-α-Acids

For isolation and purification of iso-α-acids the pure α-acids can be isomerized in alkaline solution to yield a mixture of the *cis*- and *trans*-iso-α-acids. The diastereomers can be separated by preparative liquid chromatography. More efficient is to separate the mixture by formation of the dicyclohexylamine (DCHA) complex of the iso-α-acids of which the *trans*-isomer selectively precipitates, leaving behind the *cis*-isomer in solution.[68] Pure *trans*-iso-α-acids are obtained by treating the salt with an inorganic acid. The DCHA salts of the *trans*-iso-α-acids are stable at room temperature for an extended period of time and have been adopted as standards for quantitative HPLC analysis by international brewery organizations.

Pure *trans*-iso-α-acids can also stereospecifically be prepared by photo-isomerization of pure α-acids.[69,70] Almost complete separation of *cis*- and *trans*-isomers is also possible using cyclodextrins.[71] This method offers an elegant opportunity to prepare pure compounds suitable for study of the sensory properties of the individual isomers, because no harmful solvents or chemicals are needed in this separation process.

Instead of making use of hop extract and purified α-acid analogues, for larger scale preparation of pure iso-α-acids the most efficient way is to start with a commercial pre-isomerized extract which typically contains 30% iso-α-acids in alkaline water of pH 10. This solution is easily available in large quantities and can be used to apply the various methods for separation and isolation of the *cis*- and *trans*-isomers.

3.22.2.7 Essential Oil

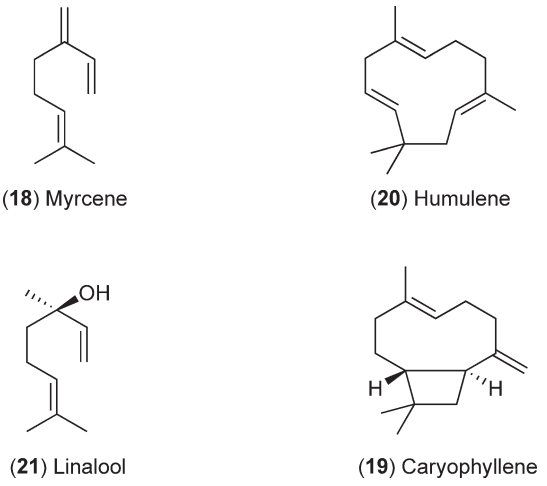
Essential oil represents a small volatile fraction of the hops and is responsible for the unique aroma of hop and beer. Typically, the essential oil content ranges from 0.5 to 1.5 ml $100\,g^{-1}$ for aroma hop varieties and 1.0–2.5 ml $100\,g^{-1}$ for the bitter hop varieties.

The essential oil fraction of hops is very complex with many different components. Moir[72] reported that well over 300 compounds had been identified, divided over various chemical classes (**Table 3**).

Table 3 Classes and number of compounds in hop essential oil

Group	*Approximate number of compounds identified*
Hydrocarbons	60
Aldehydes/ketones	60
Esters	70
Acids	10
Alcohols	60
Oxygen heterocyclics	30
Sulfur compounds	30

Source: Reproduced from M. Moir, *EBC Symposium on Hops, Monograph XXII*, *The Netherlands*; Hans Carl Verlag: Nurnberg, 1994; pp 165–180, ISBN 3-418-00746-5.



(**18**) Myrcene (**20**) Humulene

(**21**) Linalool (**19**) Caryophyllene

Figure 10 Myrcene, humulene, caryophyllene, and linalool.

Sophisticated multidimensional capillary gas chromatography indicates the possible presence of many more compounds in hop oil[73–75] of which only a relative small number has been identified. The relevance of many of these compounds for the flavor of beer is also still unknown.

Hydrocarbons are the most dominant compounds and constitute 40–80% of the total oil content. Within this class, the monoterpene myrcene (**18**) and the sesquiterpenes caryophyllene (**19**), and humulene (**20**) are the most dominant (**Figure 10**). As an example, the hop oil in the variety Hallertauer Mittelfrue consists of approximately 17% of myrcene, approximately 55% of humulene, and approximately 15% of caryophyllene. Humulene is believed to be partly responsible for the pleasant aroma of whole hop cones, while myrcene is regarded as negative in this respect. High-quality aroma hops are usually low in myrcene and high in humulene. Myrcene and the sesquiterpene hydrocarbons are very sensitive to oxidation, which leads to many derivatives that can also be found in hops.

Oxygenated compounds represent 20–50% of the total essential oil in hops. The fraction is very complex of composition including about 70 esters, many derived from straight and branched chain acids and alcohols. It is difficult to prove if these compounds are transferred into beer, since identical compounds are also formed as fermentation by-products.

Oxidation of the carbon–carbon double bond in compounds such as humulene and caryophyllene lead to epoxides, which have been detected in beer and believed to play a role in the flavor of beer.[76] Owing to their high volatility most of the aroma components present in the hop or hop products are lost in the traditional brewing process,[77] but modern analytical methods have revealed the presence of several hop oil-derived compounds in beer, in particular compounds derived as a result of oxidative degradation of hop oil components.[78–80]

Despite several research studies it is still difficult to explain hop flavor in beer by the presence of specific compounds. Linalool (**21**), an oxidation product of myrcene, is believed to be either responsible for hop flavor of beer or at least is a good marker compound for hop flavor.[81] In hops around 94% exists as R-linalool. In the brewing process racemization takes place leading to a considerable lower amount of R-linalool in beer, which further decreases by continued racemization to S-linalool during beer aging, resulting in flavor loss.[82]

Esters such as 2-methylpropyl isobutyrate and 2-methylbutyl isobutyrate contribute to fruity notes of hops. Short-chain fatty acids, such as 2-methylbutyric acid, cause the cheesy aroma, which develops when hop deteriorates.

Hop flavor compounds occur in hop in free and in glycosidically bound form. The amount differs per variety and type of hop product. CO_2 extracts of hop contain very little glycosidic bound flavor compounds, but in ethanol extract they are present. The glycosidic compounds also have been found in beer and slow liberation of the flavor-active aglycone may explain the increase in concentration of certain hop aroma compounds in beer over time.[83]

3.22.2.8 Polyphenols

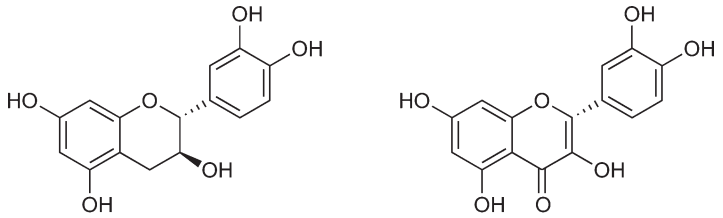
Hop contains many phenolic compounds. Low-molecular weight (poly-) phenols are known to have antioxidant activity and help to protect beer against oxidation and to improve the taste stability, but also are thought to contribute to harsh and astringent taste characteristics. High-molecular weight polyphenols partly form insoluble complexes with proteins and precipitate during the brewing process. When present in excess, high-molecular weight polyphenols contribute to the color of beer and may give haze formation when the beer is stored or cooled. The amount of polyphenols in beer derived from hops is much lower than the amount derived from malt and depends on the form of hop product the brewer uses.[84]

Polyphenol classes and compounds found in hop are procyanidins (e.g., catechin (**22**)), chalcones (e.g., xanthohumol), and flavonols (e.g., quercetin (**23**))[31] (**Figure 11**). Phenolic compounds from hops are only slightly soluble, but some survive the brewing process to end up in beer where they can form polymers.

In recent years, polyphenols in hops have gained renewed attention because of their bioactivity and in search for new applications for hops. The compounds 8-prenylnaringenin (**24a**) and xanthohumol (**24b**) in particular has become subject to research because of their supposed medicinal properties.[30,85] A variety of potential positive activities has been claimed, such as against osteoporosis and diabetes and to prevent breast cancer.

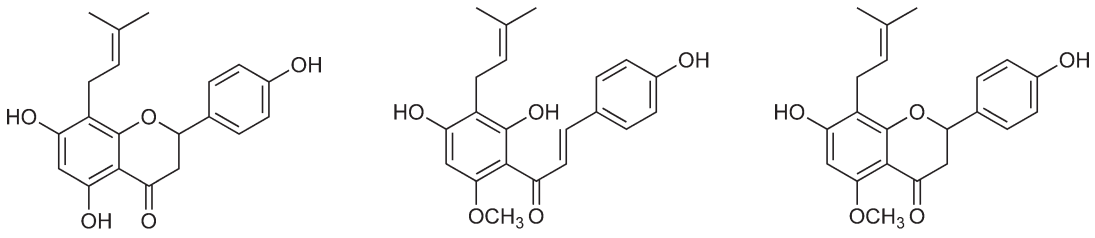
Xanthohumol is present in hops in small quantities. Typical concentrations found are 0.2–1.0%, depending on the hop variety. Xanthohumol is not present in CO_2 hop extracts and only by using whole hop cones, hop pellets, or ethanol hop extract xanthohumol can be introduced into beer. In the brewing process xanthohumol is converted (isomerized) into isoxanthohumol (**25**), in a similar reaction as for the α-acids (**Figure 12**). In most beers the concentration of xanthohumol is quite low, approximately 0.1 mg l^{-1}, but concentrations >1 mg l^{-1} were found in special dark beers and stout beer.[86] The concentration of isoxanthohumol can be as high as 3 mg l^{-1}, depending on the hop variety and quantity of hops used in the brew.

Compared to xanthohumol the possible medicinal activity of isoxanthohumol is much lower and at the concentration present in beer there is no health beneficial effect expected of iso-xanthohumol from beer consumption.



(**22**) (+)-Catechin (**23**) Quercetin

Figure 11 Catechin and quercetin.



(**24a**) 8-Prenylnaringenin (**24b**) Xanthohumol (**25**) Isoxanthohumol

Figure 12 8-Prenylnaringenin, xanthohumol, and iso-xanthohumol.

3.22.2.9 Biosynthesis of the Hop Bitter Acids

The biosynthetic pathway of the formation of hop bitter acids has been studied quite extensively.[87–91] For the main hop bitter acids, the acyl side chains of the phloroglucinol ring are derived from three amino acids, valine (for cohumulone), leucine (for humulone), and isoleucine (for adhumulone). The derivatives are formed in a reaction catalyzed by the enzyme chalcone synthase and in two subsequent prenylation steps converted into deoxy-α-acids (**26**), which are subsequently further oxidized to yield the α-acids (**Figure 13**).

The α- and β-acids and the prenylated chalcones desmethylxanthohumol and xanthohumol have been found to be present in low concentration from the onset of flowering, not only in the female hop cones but also in male inflorescences. During the development of the female cones the levels gradually increases. Although the highest concentrations of these compounds are present in the hop cones, they are also found in the leaves of fully grown hop.[92]

3.22.3 Hop Processing

Hop bitter acids are not very stable and are quickly oxidized. Therefore, hop is processed directly after harvest into a variety of products to stabilize the bitter acids and hop oil components.

3.22.3.1 Drying

The drying process is directly done after the harvest to reduce the water content in the hop cones to about 10%. This process is critical for the quality of the hops. Drying at very high temperature or for extensive periods of

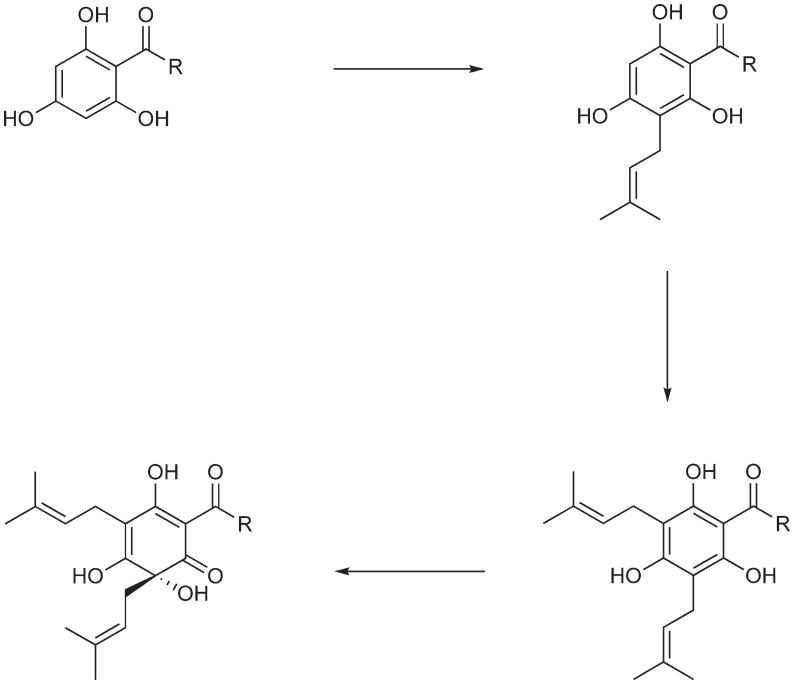


Figure 13 Main steps in the biosynthesis of hop bitter acids.

time can harm the hop bitter acids and hop oil components. The dry hops are subsequently pressed into bales to reduce the volume and to exclude oxygen from the hop cones as much as possible. The hop bitter acids in the baled hop is still quite sensitive to oxidation and the bales have to be stored below 5 °C to preserve the bitter acids and the hop oil components as much as possible.

3.22.3.2 Hop Pellets

Hops can be further converted into pellets. In this process dried hops are milled and the powder pressed into granules (pellets) of approximately 4×10 mm. The pellets are packaged in aluminum foil packs either flushed with an inert gas or as vacuum packs. Nevertheless, even after such packaging, the bitter acids and oil components are subject to oxidation and deterioration, so that cold storage is needed as well to slow down these reactions.

Hop pellets have a chemical composition close to the original composition in the hop cones. A special process concerns the production of concentrated or enriched hop pellets. In this process, the lupulin glands, containing the hop acids and the hop oil, are separated from the cellulose material. For this separation the hop cones are first frozen at -30 to -40 °C in order to harden the lupulin glands, which are then separated from the hop cones by special crushing and sieving machines. The enriched hop powder is subsequently transformed into hop pellets. These 'enriched' pellets contain about twice the content of hop bitter acids and hop oil compared to the regular hop pellets, but only half the amount of polyphenols, nitrate, heavy metals and, if present, pesticide residues.

3.22.3.3 Hop Extract

A third processing option is extraction of the hops with solvents. A major advantage is that the volume of the extract is much smaller than the volume of whole hops and of hop pellets. In the past a variety of organic solvents have been used, such as methanol, dichloromethane, and hexane. Today most common is the use of liquid or supercritical CO_2 or ethanol as extraction solvents. Depending on the α-acid content of the starting hop material, hop extracts can contain up to 50% α-acids.

3.22.3.3.1 CO_2 extraction

Extraction with CO_2 is made at elevated pressure and temperature. When compressed, CO_2 gas converts into a liquid with extraction properties and at a pressure above 73 bar and at a temperature above 31 °C the so-called super critical CO_2 is formed. The solvent power is highest for nonpolar or slightly polar compounds. By choosing specific combinations of pressure and temperature, the extraction selectivity can be influenced, which enables selective extraction of fractions of the hop, for example, the bitter acids or the essential oil. In this way extracts enriched with specific hop components can be produced.[93–95]

Removal of the extraction solvent is made by simply reducing the pressure, so that CO_2 extracts are not exposed to high temperatures during solvent evaporation and protected against oxidation by the CO_2 atmosphere.

Because of the nonpolar nature of CO_2 the extract does not contain polyphenols such as xanthohumol and other polar compounds present in the hops, although supercritical CO_2 is less selective as a solvent than liquid CO_2 and may contain small quantities of tannins and polyphenols. The CO_2 extract is very stable, and storage in closed tins or containers for several years at room temperature does not affect the α-acid content.

3.22.3.3.2 Ethanol extraction

Extraction of hops with ethanol makes the extracts more complex in composition because ethanol co-extracts some of the more polar components such as polyphenols. The extraction is made with 90% ethanol (obtained from fermentation) and 10% water. Ethanol is removed by vacuum and the remaining extract naturally separates in an ethanol phase containing almost all the hop bitter acids and hop oil and an aqueous phase, or tannin extract phase, containing most of the water-soluble substances. Owing to the elevated temperature required to evaporate the ethanol, a small portion of the α-acids is converted into iso-α-acids during solvent removal.

Table 4 Composition of hops and processed products (cultivar Taurus, crop 2005)

Compound class	*Raw hops*	*scCO$_2$ extract*	*Ethanol extract*
Essential oil	1.5%	5%	4%
α-Acids	15%	55%	45%
β-Acids	6%	21%	18%
Xanthohumol	1%	–	3%
Iso-α-acids	–	–	1%

Source: M. Biendl; C. Pinzl, *Arzneipflanze-Hopfen*; Deutsches Hopfen Museum: Wolnzach, 2007; ISBN 3-929749-05-X.

Although most of the polyphenols are found in the aqueous phase, prenylflavonoids are extracted into the ethanol phase. Xanthohumol for instance is only present in ethanol extract but not in CO_2 extracts.

As an example, **Table 4** shows the composition of the main classes of compounds in raw hop, CO_2 extract, and ethanol extract.

Today most brewers use either hop pellets or hop extract, depending on the type of beer they want to produce. Small pub or microbrewers still use whole hop cones. They often use very special blends of varieties to get highly flavored beers and consistency of their beer is often a lower priority than it is for the major brewers.

3.22.3.4 Isomerized Hop Products

Since the yield of the conversion of α-acids in hops into iso-α-acids in beer is only 30–35%, the hop industry has developed products which are already isomerized and can be added at various points in the brewing process and preferably as late as possible to get the highest utilization.

These so-called pre-isomerized products can be made under much more favorable conditions for the isomerization to give almost quantitative yields, as opposed to the relative poor yield of the isomerization in the brewing process. Isomerized extracts exist in various forms. The starting materials for isomerized products are conventional hop pellets or extracts. Isomerized pellets are produced by blending magnesium oxide in the hop powder before pelletization and subsequent storage of the packaged pellets at temperature of 50 °C for a certain period of days.

Isomerized extracts can be added to the brewing process after the wort boiling or after the fermentation, resulting in substantial higher utilization of the bitter acids. Isomerized hop extracts exist in various forms and composition. In its purest form isomerized extract only contains 20–30% (w/w) pure iso-α-acids as their potassium salt in an alkaline water solution. This solution is diluted before use and can be injected into the beer stream. The main advantage of the use of the latter product is improved control and consistency of the final beer bitterness.

Complete absence of the hops in the wort boiling and downstream addition of isomerized extract however also affects the sensory flavor profile of the beer and is therefore not directly applicable for existing beer brands. This observation indicates that during the wort boiling hop or hop extract contributes also many minor compounds to the typical final beer flavor. These compounds are either present in the hop or hop product, retained in the wort to pass the brewing process or are formed during the wort boiling or fermentation from precursors present in hop. To compensate for the lack of hop flavor in beer only made with isomerized hop products, special hop aroma products may be added. For instance, the volatile compounds in hop can be fractionated into floral fractions and spicy fractions by combined CO_2 extraction and column chromatographic separation. Brewers may use these hop aroma fractions to enhance the hop flavor of specific beer styles. These aroma fractions are almost always dosed to beer as late as possible, in the cold phase of the process.

3.22.4 Beer Flavors and Off-Flavors

The appreciation of any food product or beverage depends on its taste, aroma, and visual appearance. Flavor is arguably the most important aspect of the quality of food and beverages.[96] Beer is an extremely complex product and a mixture of volatile and nonvolatile components which originate from the raw materials, are

formed during the brewing process or caused by aging of the beer and deterioration of certain flavor compounds. Understanding the contributions of the raw materials and the processing conditions to the flavor of beer is essential for the brewers to control and optimize existing products and helps in the development of new products. Terminology to describe and compare the sensory properties of beer is essential for studies of beer flavor.[97]

The concentration of the various flavor compounds in beer varies over a wide range, from $g\,l^{-1}$ to $ng\,l^{-1}$. The importance of a compound for the flavor of beer is not its concentration but the impact that the particular compound has on the taste or aroma.

The complex composition of beer makes it difficult to identify and analyze the compounds responsible for the perceived flavor. Application of modern analytical methods such as gas chromatography–olfactometry (GC–O), a technique for evaluation of aroma characteristics of volatiles separated by GC, and a technique called flavor dilution chromatography is often used for this purpose and enables identification of the so-called character-impact compounds. The use of GC–O methods for analysis and quality assessment of alcoholic beverages has recently been reviewed by Plutowska and Wardencki.[98]

The flavor of beer is a dynamic system, which is changing over time. Many factors play a role in these changes and it is a challenge for the brewer to maintain the original quality as long as possible.

3.22.4.1 Carbon Dioxide, Ethanol, and Sugars

CO_2 and ethanol in beer are formed during the metabolism of sugars in the wort under the anaerobic conditions found in brewery fermentation. The CO_2 concentration of beer is typically around $5\,g\,l^{-1}$ for lager beers, but can be as high as $10\,g\,l^{-1}$ for certain specific beer styles. The ethanol content of a beer depends on the amount of fermentable sugars present in the wort. For most beer styles it is typically in the range of 3–5% by volume, but some special beers can have up to 8% alcohol.

Glucose (**1**), maltose (**2**), maltotriose (**3**), and in low concentration also fructose and saccharose can be present in beer and contribute to its sweetness. Higher molecular weight dextrins contribute to the fullness and mouthfeel of beer. They usually arise from the malt and have survived the fermentation process either because the fermentation was stopped at an early stage or because the sugars were unfermentable.

Many attempts have been made to produce beers low in alcohol content or without alcohol. Obvious ways to do so are to stop fermentation at an early stage or to remove the alcohol from the partial or fully fermented beverages. Alcohol diffusion through membranes and evaporation of alcohol under vacuum have become established methods.[99] The flavor of the low and nonalcohol products is quite different from the flavor of regular beer. Many of these products have a malt or wort flavor, which makes them less attractive to the consumer. Limited fermentation helps to improve the flavor. Low-alcohol beers (0.5–2% v/v) are therefore more appreciated than nonalcohol beers and are gaining interest.

3.22.4.2 Organic Acids

Beer pH is usually in the range of 3.9–4.4 although some exceptions are found. The pH is caused by the presence of organic acids (**Table 5**). These acids come from the yeast but their concentration in the final beer depends on the fermentation conditions. Vigorous fermentations will result in more acid release and higher concentration in the final product. Except for the effect on the pH, several of the acids also have impact on the sensory properties of beer in their undissociated forms.

pH is a key factor influencing beer aging and stability[100] and has a significant effect on astringency: the higher the pH the lower the astringency perceived.[101]

3.22.4.3 Inorganic Anions and Cations (Salts)

Inorganic ions are of importance for the flavor of beer (**Table 6**). They mainly come from the water used for brewing, although some also originate from raw materials. Based on experience, various brewers use a wide range of water compositions for their beer production. The principal ions present in brewing water are calcium, sodium, magnesium, potassium, sulfate, chloride, carbonate, and nitrate. Minor concentrations of iron, copper,

Table 5 Major organic acids found in beer

Organic acid	*Typical concentration range in beer (mg l^{-1})*
Acetic acid	30–200
Propanoic acid	1–5
Butanoic acid	0.5–1.5
2-Methylpropanoic acid	0.1–2
Pentanoic acid	0.03–0.1
2-Methylbutanoic acid	0.1–0.5
3-Methylbutanoic acid	0.1–2
Lactic acid	20–80
Pyruvic acid	15–150
Succinic acid	16–140

Source: Reproduced from P. S. Hughes; E. D. Baxter, *Beer Quality, Safety and Nutritional Aspects*; The Royal Society of Chemistry: Cambridge, UK, 2001; ISBN 0-85404-588-0.

Table 6 Main inorganic ions in beer

Ion	*Typical concentration range in beer (mg l^{-1})*	*Source*
Potassium	200–450	Malt
Sodium	20–350	Brewing materials, water
Calcium	25–120	Brewing materials, water
Magnesium	50–90	Brewing materials, water
Chloride	120–500	Water
Sulfate	100–430	Water
Oxalate	5–30	Malt
Phosphate	170–600	Malt
Nitrate	0.5–2.0	Water, hops

Source: Reproduced from P. S. Hughes; E. D. Baxter, *Beer Quality, Safety and Nutritional Aspects*; The Royal Society of Chemistry: Cambridge, UK, 2001; ISBN 0-85404-588-0.

and zinc are also found. Divalent cations in the brewing water, such as Mg, can have an effect on the conversion of the hop bitter acids during boiling of wort.

For the flavor of beer, sulfate and chloride are of particular importance. While sulfate is found to contribute to dry, harsh salty taste, chloride is thought to give body and a soft sweet taste. When present in excess, Fe^{3+} ions can lead to a metallic taste of beer.

Metal ions, in particular copper and iron, are believed to play a role in the oxidation of beer leading to stale flavor.

3.22.4.4 Bitterness

Bitterness is a major quality factor for beer and a typical taste characteristic. The bitterness of beer is almost completely caused by the iso-α-acids (**13,14**). Typical concentration for the iso-α-acids in beer is in the range of 5–50 mg l^{-1} and varies per beer style.

The perceived bitterness of the iso-α-acids depends on the type of beer and the concentration. In beers low in flavor the perceived bitterness is different than for beers rich in flavors. Today brewers are not only using traditional hopping but also increasingly use (modified) pre-isomerized products. Tetrahydroiso-α-acids can be present in small quantities when used to increase the foam stability of beer, while complete replacement of the regular hopping by mixtures of reduced pre-isomerized iso-α-acids is being used to produce light stable beers.[102] This practice complicates the analysis of the bitterness of beer. Traditionally, brewers use a spectrophotometric method for the determination of the analytical bitterness. The absorbance of an iso-octane extract of acidified beer is measured at 270 nm and the value multiplied by a factor of 50. The result is expressed as Bitterness Units (BU). The method has been devised long ago and the factor of 50 was empirically established

for beers made with fresh hops, such that 1 mg l^{-1} of iso-α-acid present in beer gives 1 BU as measured by the spectrophotometric method. However, when (part of) the bitterness is derived from the modern hop products the multiplication factor is no longer 50, but higher. In particular, the reduced iso-α-acids have lower molar absorptivity at the wavelength used in the method and therefore underestimate the amount of reduced iso-α-acids present. For example, 1 mg of tetrahydroiso-α-acid when measured in the spectrophotometric method using the multiplication factor 50 gives a value of about 0.6 BU.

The spectrophotometric method also does not allow discriminating between regular iso-α-acids and the various modified iso-α-acids. This is especially a drawback when mixtures of hop bittering products have been used and consequently the result of the method no longer reflects the sensory bitterness. Using HPLC the various bitter compounds can be resolved and quantified and is recommended for analysis of these more complex products.[103,104]

Iso-α-acids are rather labile compounds and gradually degrade when exposed to oxygen or light. Owing to oxidation many derivatives are already formed during the brewing process. Light exposure of beer quickly results in the formation of a typical off-flavor, which is caused by the photochemical degradation of the hop bitter acids (see Section 3.22.5.2).

The analytical and sensory bitterness of beer decreases during storage. HPLC analysis shows *trans*-iso-α-acids to degrade more quickly than the *cis* isomers,[105,106] so that the *cis*/*trans* ratio changes in time. This phenomenon is of interest as an indicator for beer aging.

It is still unclear what the fate of the bitter acids is and no degradation products have been identified yet, but degradation of the iso-α-acids in beer decreases the sensory bitterness and results in the formation of products which are believed to contribute to aging flavors. The sensory bitterness decreases due to the decrease in concentration of the iso-α-acids, but the perceived intensity can also be suppressed by the stale flavors which develop simultaneously. Differences in stability between the *cis*- and *trans*-isomers, suggest that for optimal consistency of beer bitterness, the highest possible *cis* content of the bitter acids in beer may be preferable.

3.22.4.5 Esters

Volatile esters are important for beer flavor, in particular ethyl acetate and 2- and 3-methylbutyl acetate (often called iso-amyl acetate) because these esters are present in concentration well above their flavor threshold value. Esters give beer a fruity character. Normal concentrations in beer are 20–40 mg l^{-1} for ethyl acetate and 2–5 mg l^{-1} for iso-amyl acetate. Esters are produced by yeast and their concentration is amongst others dependent on the density of the fermentation medium (wort) and the amount of oxygen present. The yeast strain itself also affects the amount of ester formation. Other esters commonly found in beer are ethyl hexanoate, ethyl octanoate, and 2-phenylethyl acetate.

When beer flavor deteriorates several new esters are synthesized in reactions between ethanol and organic acids, while the concentration of some flavor-positive esters decreases.

3.22.4.6 Aldehydes

Beer contains many flavor-active aldehydes which have been formed during the various stages in the process. They are produced by oxidation of the corresponding alcohols or are derived from fatty acids and lipids present in the malt.

The most dominant aldehyde present in beer is acetaldehyde. It is commonly found in concentrations between 2 and 10 mg l^{-1}. Its flavor threshold value depends on the type of beer, but is in the range of 5–50 mg l^{-1}. Above its threshold level, acetaldehyde can give beer a typical green apple flavor.

Aldehydes play an important role in the flavor changes occurring during aging of beer (see Section 3.22.5).

3.22.4.7 Higher Alcohols

Besides ethanol, beer also contains a range of other alcohols but at a much lower concentration and often below their flavor threshold values. The formation of these so-called higher alcohols, also called fusel alcohols, is dependent on the yeast strain. Important alcohols are 2-methylpropanol, 2-methylbutanol and

3-methylbutanol, and 2-phenylethanol. They have strong flavors and can have a warming effect on the taste of beer. Higher alcohols are the direct precursors of esters found in beer and contribute to the positive beer flavor.

3.22.4.8 4-Vinylguaiacol

Some beers contain relatively high concentrations of 4-vinylguaiacol (**27**) (4-VG). It is formed by thermal or enzymatic decarboxylation of ferulic acid (**28**) (**Figure 14**). It gives beer a phenolic or clove-like flavor which is typical for beer made from wheat or wheat malt with top-fermentation yeast strains, but is considered an off-flavor in Pilsners beers. In beer made with barley malt the ferulic acid is released from the malt during mashing and small quantities of 4-VG are formed during the wort boiling phase. When wheat or wheat-malt is used ferulic acid is found to be mainly formed by decarboxylation of ferulic acid during fermentation, causing higher levels of 4-VG in beer.[107]

The presence of 4-VG in regular Pilsner-type beer at concentrations above 10 μl^{-1} is an indication that the beer may have been contaminated with wild yeast strains.[108]

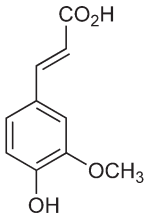
3.22.4.9 Malt Flavors

Since barley malt is the main raw material for beer, it obviously contributes to the flavor of beer. Malt contains a number of aldehydes, but during the kilning process the majority of these compounds disappear. Many flavor-active components are also formed due to chemical reactions taking place in this process, such as degradation of phenolic acids and of fatty acid-derived products, leading to a range of volatile compounds. Many of these compounds however are lost during the boiling and fermentation steps in the brewing process.

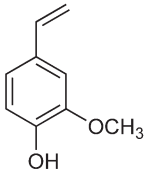
Lipids in malt are only sparingly soluble in water and are lost to a large extent due to adsorption to solids. This is desirable, since lipids and lipid oxidation products are believed to be major precursors for typical staling flavors formed when beer ages. On the other hand, malt polyphenols are thought to contribute to the flavor stability of the final beer.

3.22.4.10 Vicinal Diketones

During fermentation considerable amounts of the vicinal diketones 2,3-butanedione (**29**) (diacetyl) and 2,3-pentanedione (**30**) is formed (**Figure 15**). In beer, the former in particular gives a buttery or butterscotch aroma. The precursor of diacetyl is alpha-acetolactate which is excreted by yeast cells and decomposes in the wort to yield diacetyl. Yeast has a great capacity to reduce the diacetyl content in the wort. In contact with healthy yeast, diacetyl is reduced to form acetoin and subsequently 2,3-butane-diol. For pentanedione a similar reaction occurs. For the reduction of diacetyl and pentanedione a long enough maturation period is required and usually decrease of diacetyl below a certain level is used as an indicator to monitor if maturation is completed.



(**28**) Ferulic acid



(**27**) 4-Vinylguaiacol

Figure 14 Ferulic acid and 4-vinylguaiacol.

(**29**) Diacetyl (**30**) 2,3-Pentanedione

Figure 15 Diacetyl and 2,3-pentanedione.

Diacetyl is detectable in almost all beers. In lager beers, having less body and flavor, diacetyl is regarded a defect, but it can be a desirable flavor in certain heavily hopped beer styles, such as British ales. The taste threshold value of diacetyl in beer depends on the beer type and is for Pilsner style beers as low as $0.03\,mg\,l^{-1}$. Despite being present at low concentration, both diacetyl and pentanedione are considered to make a significant contribution to the overall sensory quality and flavor balance of a beer.

3.22.4.11 Sulfur Components

Sulfur components can be very potent in flavor and in beer several volatile sulfur components are present. The most abundant is sulfur dioxide which can be as high as $10–15\,mg\,l^{-1}$. SO_2 is naturally formed during fermentation but can also be added. The compound has a particular effect on beer flavor because of its capacity to chemically bind carbonyl compounds and helps to suppress oxidation flavors.[109,110] When SO_2 is added, mostly in the form of sodium or potassium metabisulfite, to increase the shelf life, legal limits have been set to the amount that may be added.

When present in low concentrations, sulfur compounds may contribute positively to the overall beer flavor, but when present above threshold level, they are considered very negative for the quality of the flavor. The main sources for sulfur compounds in beer are malt, hops, and yeast. Hydrogen sulfide is in particular a compound to be avoided in beer, because of its very unpleasant rotten eggs flavor. H_2S can be formed from the breakdown of sulfur containing amino acids. However, because H_2S has a high volatility most of it will disappear in the fermentation process together with CO_2.

DMS (**5**), is another important sulfur compound responsible for the sulfury note of beer. It is a desirable component in low concentration, but above its threshold value it is considered an off-flavor. During malting DMS is formed because of the breakdown of SMM (**4**) as the precursor, which is formed during the kilning of malt. Most of the DMS is evaporated during the wort-boiling step in the brewing process (see Section 3.22.1.1).

3.22.5 Beer Flavor Deterioration

Consistency of flavor is important for brand recognition and image. Beer flavor is not static and changes over time. The sensory properties are the result of both the formation of flavor-active compounds in concentrations above their flavor threshold value and the degradation of flavor-active compounds to below their flavor threshold value. Positive flavors such as fruity, floral, and estery notes tend to decrease when beer ages. For the overall impression of the flavor the decrease of positive flavors may be as important as the development of stale flavors.[111] The perceived flavor is further complicated due to interactions between compounds, either enhancing or suppressing.[112]

As beer is a mixture of many different compounds, many chemical reactions can occur. However, these reactions depend on the actual conditions used for storage of the beer. Also, there are differences in the aging characteristics between various beer styles. Strong flavors in dark beers, for instance, mask the development of aging flavors and result in better sensory flavor stability[113] although analytically the concentration of the staling compounds increases.

3.22.5.1 Aging

Aging of beer is one of the main concerns for major brewers who produce large volumes of beer which is consumed all over the world. The complex chemistry of beer aging has recently been critically reviewed by Vanderhaegen *et al.*[114]

Over the past several years aging of beer was believed to be caused by carbonyl compounds. For the first time in 1966 the significant increase in volatile carbonyls during beer storage parallel to the development of stale flavors was reported. In particular, the so-called cardboard flavor was found typical for beer aging. (*E*)-2-Nonenal (**31**) (**Figure 16**) was found to give beer a cardboard flavor when added and in 1970 (*E*)-2-nonenal was indeed detected in beer which had been acidified and heated.[115] For a long period of time, (*E*)-2-nonenal was considered the main indicator for beer staling and responsible for the initial flavor changes in beer during storage. Other flavor changes such as harsh and astringent taste[116] and wine and whiskey-like flavors[117] were found to develop in strongly aged beer. It was reported that in beer stored at 40 °C (*E*)-2-nonenal increased to concentrations above its threshold level within days, but when stored at 20 °C this was not found even after several months of storage.[118]

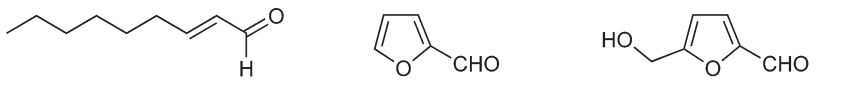
Extreme storage conditions, acidification, and elevated temperature, were initially needed to form analytically detectable quantities of (*E*)-2-nonenal in beer. Detection of the compound in beer under normal storage conditions was not possible due to the extreme low flavor threshold concentration of $\leq 0.1\ \mathrm{ng\,l^{-1}}$. Many attempts have been made to develop quantitative analysis methods to measure the concentration of (*E*)-2-nonenal in beer at this level. Direct gas chromatographic analysis was not possible due to the overwhelming number of compounds in head space or solvent extracts of beer. Laborious methods based on concentration and derivatization of aldehydes with specific reagents, followed by analysis with GC–mass spectrometry, GC-electron capture detection or HPLC with UV or fluorescence detection eventually led to methods enabling the detection of (*E*)-2-nonenal and other carbonyl compounds formed under normal beer storage conditions.[119,120]

Much attention has been devoted to the mechanism of beer aging, which has been attributed to the oxidation of unsaturated fatty acids. These compounds occur in malted barley. Oxidation intermediates of these fatty acids were believed to be precursors for carbonyls and in particular (*E*)-2-nonenal. However it was found that only under acidic conditions, at pH 2, the conversion of these oxidation products could happen and therefore had to be excluded as possible precursors under normal beer conditions.

Drost *et al.*[121] defined that during wort production enzymatic and nonenzymatic oxidation of fatty acids results in a compound source which potentially could lead to the formation of (*E*)-2-nonenal and released during storage of the final product. This so-called 'nonenal potential' is determined as the amount of nonenal that is released after heating of wort, at pH 4, in a closed tube in an inert argon atmosphere for 2 h. The chemical nature of the 'potential' is still largely unknown, but the increase of nonenal in aging beer is most probably the result of oxidation processes early in the beer production chain, particularly during the mashing stage.

Evidence shows that nonenal is already present in wort as Schiff bases with amino acids or proteins and that the bound nonenal in this form passes through the brewing process into the final beer, where hydrolyzes over time results in the release of nonenal to concentrations above the sensory threshold level.[122,123] Free nonenal present in wort is found to be quickly reduced into nonenol by yeast during fermentation and therefore it is believed not to be an important source for nonenal found in beer.[124]

Aldehydes readily form adducts with SO_2, which is formed in relative high concentration during fermentation and the adduct could potentially pass into beer where it is hydrolyzed to release the carbonyls again. Nonenal can form an adduct with SO_2 at the carbonyl function as well as on the double bond. Although adducts with the carbonyl group are reversible, the adduct on the double bond in nonenal is irreversible and therefore the SO_2 adducts of nonenal in beer cannot release nonenal of any significance. Increase of nonenal in beer with



(**31**) (*E*)-2-Nonenal (**32**) Furfural (**33**) 5-Hydroxymethylfurfural

Figure 16 Nonenal, furfural, and 5HMF.

aging is therefore thought to be mainly the result of oxidation reactions in the first part of the brewing process and it is believed that both chemical oxidation, by oxygen, and enzymatic oxidation, by lipoxygenases, play a role. Studies showed that 70% of the (*E*)-2-nonenal formed during beer staling originates from the wort boiling phase and the other 30% from the mashing phase of the brewing process.[125,126]

Many heterocyclic compounds in beer are products of the Maillard reaction. The best known Maillard products in aged beer are furfural (**32**) and 5-hydroxymethylfurfural (**33**) (**Figure 16**). Although the compounds remain well below their flavor threshold value, they are considered as useful markers for beer staling and heat load during the brewing process.[127]

3.22.5.2 Sunstruck Off-Flavor

An important off-flavor sulfur component in beer is 3-methyl-2-butene-1-thiol (MBT). This compound has an extreme low flavor threshold value, typically in lager beer around 5–10 ng l^{-1} and causes the so-called sunstruck or skunk flavor to beer.

MBT is formed by photochemical reactions taking place when beer is exposed to light in the wavelength range of 350–550 nm. It is formed because of the degradation of iso-α-acids in the presence of a photosensitizer, for example, riboflavin, and other sulfur sources present in beer.[128]

The commonly postulated mechanism for the formation of MBT (**34**) in beer is shown in **Figure 17**. The light-sensitive part in the iso-α-acid molecule is the tertiary alcohol function at the C4 carbon atom and the

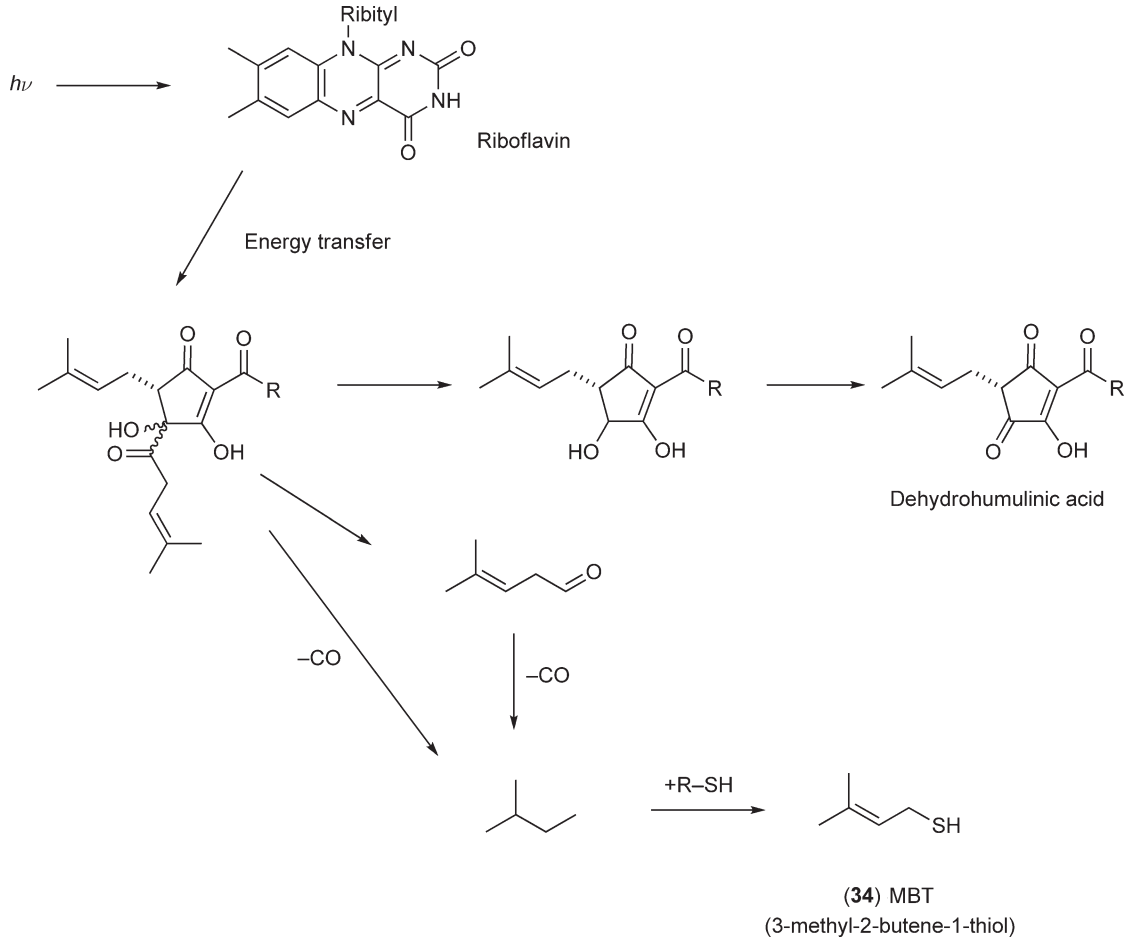


Figure 17 Mechanism for the formation of sunstruck off-flavor in beer. Reproduced from P. S. Hughes; E. D. Baxter, *Beer Quality, Safety and Nutritional Aspects*; The Royal Society of Chemistry: Cambridge, UK, 2001; ISBN 0-85404-588-0.

adjacent carbonyl function in the side chain. Photosensitized cleavage of the C—C bond by light is thought to be followed by subsequent loss of carbon monoxide of the acyl radical and recombination with sulfur radicals from a sulfur source in beer to form MBT.

The formation of MBT occurs very rapidly when beer is exposed to daylight, either in the bottle or in the drinking glass. Brewers therefore try to protect beer by packaging in amber glass bottles or green colored bottles. The latter protect the beer however only to a certain extent. Obviously, beer packaged in steel or aluminum cans are fully protected against light.

Alternatively, brewers can replace their regular hopping products with the reduced hop products, ρ-, tetra-, or hexahydroiso-α-acids or mixtures thereof, as described above, but for their regular products replacement will almost invariably lead to a difference in flavor, because of the decrease in complexity of the flavor. The photochemical degradation of hop bittering principles is only partly understood but has recently regained renewed interest.[57,58,129]

3.22.6 Microbiological Deterioration

Beer is a very stable microbiological medium and only a few microorganisms are capable of growing in it. Growth of mycotoxin-producing fungi during the malting process, wild yeasts producing off-flavors, development of turbidity in the packaged beer due to growth and metabolic activity of wild yeasts, certain lactic acid bacteria, and anaerobic Gram-negative bacteria can have a negative impact on beer quality. There is an increasing development of novel approaches to exploit inhibitory components present in raw materials to enhance microbiological stability of beer.[130,131]

3.22.7 Prospects

The chemistry of beer flavor is extremely complex. Despite the fact that current data are overwhelming it is only partly possible to predict and control beer flavor. The great number of compounds occurring at widely varying concentrations makes complete analytical characterization of beer or its raw materials currently impossible. Sensory information of individual compounds is still lacking and the interactions between flavor compounds leading to flavor masking, suppression, or enhancement is not well understood yet.

Developments in analytical methods, enabling study of metabolic processes and analysis of the complex flavor composition of beer and its raw materials, in combination with modern sensory evaluation methods, will in the future enable brewers to optimize and control the flavor of existing products and to develop new products. Ideally this information should also be coupled to consumer research data and consumer preferences.

For mainstream brewers consistency in production is of utmost importance as their consumers have expectations about the taste and flavor of the brand. Aging is one of the problems requiring in-depth scientific knowledge about the mechanisms behind the various staling characteristics and the possibility to translate the knowledge into practical measures and control. Increasingly, brewers exploit natural inhibitory compounds to protect beer from deterioration during shelf life. Malt and hops are rich sources of antioxidants which can potentially be used for this purpose.[132]

Acknowledgments

Professor Paul Hughes, International Centre for Brewing and Distilling, Heriot-Watt University, Edinburgh, is gratefully acknowledged for drawing the chemical structures and Mr. Willy Mitter, Technical Director of Simon H. Steiner, Hopfen, GmbH, Mainburg, Germany for providing the hop photographs.

References

1. D. De Keukeleire, *Quim. Nova* **2000**, *23* (1), 108–112.
2. C. R. Zastrow; M. A. Mattos; C. Hollatz; B. U. Stambuk, *Biotechnol. Lett.* **2000**, *22* (6), 455–459.
3. K. Wackerbauer; S. Meyna; M. Westphal, *Monatsschrift Brauwiss.* **2003**, *56* (1–2), 27–33.

4. K. Wackerbauer; S. Meyna; S. Marre, *Monatsschrift Brauwiss.* **2003**, *56* (9–10), 174–178.
5. K. Wackerbauer; S. Meyna, *Monatsschrift Brauwiss.* **2002**, *55* (3–4), 52–57.
6. K. Wackerbauer; S. Meyna, *Monatsschrift Brauwiss.* **2002**, *55* (5–6), 110–114.
7. M. J. T. J. Arts; C. Grun; R. L. de Jong; H. P. Voss; A. Bast; M. J. Mueller; G. R. M. M. Haenen, *J. Agric. Food. Chem.* **2007**, *55* (17), 7010–7014.
8. N. Hiroto; H. Kuroda; K. Takoi; T. Kaneko; T. Kaneda; H. Yoshida; M. Takashio; M. Ito; K. Takeda, *Cereal Chem.* **2006**, *83* (3), 250–254.
9. L. F. Guido; P. Boivin; N. Benismail; C. R. Goncalves; A. A. Barros, *Eur. Food Res. Technol.* **2005**, *220* (2), 200–206.
10. F. Kuhbeck; W. Back; M. Krottenthaler, *J. Inst. Brew.* **2006**, *112* (3), 215–221.
11. F. Kuhbeck; W. Back; M. Krottenthaler, *J. Inst. Brew.* **2006**, *112* (3), 222–231.
12. P. S. Hughes; E. D. Baxter, *Beer Quality, Safety and Nutritional Aspects*; The Royal Society of Chemistry: Cambridge, UK, 2001; ISBN 0-85404-588-0.
13. D. E. Briggs; C. A. Boulton; P. A. Brookes; R. Stevens, *Brewing: Science and Practice*; Woodhead Publishing: Cambridge, UK, 2004; ISBN 1855734907.
14. C. W. Bamforth, *J. Sci. Food Agric.* **2000**, *80* (9), 1371–1378.
15. M. Meilgaard, *J. Inst. Brew.* **2001**, *107* (5), 271–286.
16. S. Coghe; E. Martens; H. D'Hollander; P. J. Dirinck; F. R. Delvaux, *J. Inst. Brew.* **2004**, *110* (2), 94–103.
17. E. L. Inns; L. A. Buggey; C. Booer; H. E. Nursten; J. M. Ames, *J. Agric. Food. Chem.* **2007**, *55* (16), 6539–6546.
18. S. Coghe; B. Gheeraert; A. Michiels; F. R. Delvaux, *J. Inst. Brew.* **2006**, *112* (2), 148–156.
19. L. F. Guido; A. F. Curto; P. Boivin; N. Benismail; C. R. Goncalves; A. A. Barros, *J. Agric. Food. Chem.* **2007**, *55* (3), 728–733.
20. S. Sovrano; S. Buiatti; M. Anese, *Food Chem.* **2006**, *99* (4), 711–717.
21. O. S. Younis; G. G. Stewart, *J. Am. Soc. Brew. Chem.* **1999**, *57* (2), 39–45.
22. R. Willaert; V. A. Nedovic, *J. Chem. Technol. Biotechnol.* **2006**, *81* (8), 1353–1367.
23. T. Branyik; A. A. Vicente; P. Dostalek; J. A. Teixeira, *Biotechnol. Progr.* **2005**, *21* (3), 653–663.
24. I. Virkajarvi; N. Pohjala, *J. Inst. Brew.* **2000**, *106* (5), 311–318.
25. M. Tata; P. Bower; S. Bromberg; D. Duncombe; J. Fehring; V. Lau; D. Ryder; P. Stassi, *Biotechnol. Prog.* **1999**, *15* (1), 105–113.
26. P. H. Pilkington; A. Margaritis; N. A. Mensour; I. Russell, *J. Inst. Brew.* **1998**, *104* (1), 19–31.
27. J. L. Benitez; A. Forster; D. De Keukeleire; M. Moir; F. R. Sharpe; L. C. Verhagen; K. T. Westwood, *Hops and Hop Products Manual of Good Practice*; EBC Technology and Engineering Forum, Hans Carl Verlag: Nurnberg, 1997; ISBN 3-418-00758-9.
28. H. J. Barth; C. Klinke; C. Schmidt, *The Hop Atlas*; Joh. Barth & Sohn: Nurnberg, 1994; ISBN 3-418-00745-7.
29. A. Forster; R. Schmidt, *Brauwelt* **1993**, *40*, 2036–2057.
30. M. Biendl; C. Pinzl, *Arzneipflanze-Hopfen*; Deutsches Hopfen Museum: Wolnzach, 2007; ISBN 3-929749-05-X.
31. J. F. Stevens; C. L. Miranda; D. R. Buhler; M. L. Deinzer, *J. Am. Soc. Brew. Chem.* **1998**, *56* (4), 136–145.
32. M. Verzele, *J. Inst. Brew.* **1986**, *92* (1), 32–48.
33. M. Verzele; D. De Keukeleire, *Chemistry and Analysis of Hop and Beer Bitter Acids*; Elsevier: Amsterdam, 1991; ISBN 0-444-88165-4.
34. M. Moir, *J. Am. Soc. Brew. Chem.* **2000**, *58* (4), 131–146.
35. K. Krofta, *Plant Soil Environ.* **2003**, *49* (6), 261–268.
36. L. C. Verhagen, Hop Analysis. In *Modern Methods of Plant Analysis*; H. F. Linskens, J. F. Jackson, Eds.; Springer Verlag: Berlin, 1988; Vol. 7; ISBN 3-540-18308-6, pp 67–84.
37. G. B. Nickerson; P. A. Williams, *J. Am. Soc. Brew. Chem.* **1986**, *44*, 91–94.
38. W. J. Simpson, *J. Inst. Brew.* **1993**, *99*, 317–326.
39. M. G. Malowicki; T. H. Shellhammer, *J. Agric. Food Chem.* **2005**, *53* (11), 4434–4439.
40. M. G. Malowicki; T. H. Shellhammer, *J. Am. Soc. Brew. Chem.* **2006**, *64* (1), 29–32.
41. A. Fritsch; T. H. Shellhammer, *J. Am. Soc. Brew. Chem.* **2007**, *65* (1), 26–28.
42. F. L. Rigby, *Proc. Am. Soc. Brew. Chem.* **1972**, *30*, 46–50.
43. A. Juste; M. S. Krause; B. Lievens; M. Klingeberg; C. W. Michiels; K. A. Willems, *J. Appl. Microbiol.* **2008**, *104* (1), 51–59.
44. G. Pollach; W. Hein; D. Beddie, *Zuckerindustrie* **2002**, *127* (12), 921–930.
45. W. Hein; G. Pollach; F. Emerstorfer, *Zuckerindustrie* **2006**, *131* (7), 477–491.
46. P. S. Hughes, *J. Inst. Brew.* **2000**, *106* (5), 271–276.
47. C. W. Bamforth, *J. Inst. Brew.* **1985**, *91*, 370–383.
48. C. W. Bamforth, *J. Inst. Brew.* **2004**, *110* (4), 259–266.
49. W. J. Simpson; A. R. W. Smith, *J. Appl. Bacteriol.* **1992**, *72*, 325–334.
50. C. A. Blanco; A. Rojas; D. Nimubona, *Trends Food Sci.Technol.* **2007**, *18* (3), 144–149.
51. H. Koller, *J. Inst. Brew.* **1969**, *75*, 175–179.
52. P. S. Hughes; W. J. Simpson, *J. Am. Soc. Brew. Chem.* **1996**, *54*, 234–237.
53. J. Templar; K. Arrigan; W. J. Simpson, *Brew. Dig.* **1995**, *70* (5), 18–25.
54. H. Goldstein; P. L. Ting, *EBC Symposium on Hops*, *Monograph XXII*, *The Netherlands*; Hans Carl Verlag: Nurnberg, 1994; pp 141–153, 154–162, ISBN 3-418-00746-5.
55. P. L. Ting; H. Goldstein, *J. Am. Soc. Brew. Chem.* **1996**, *54* (2), 103–109.
56. P. L. Ting; S. Kay; D. Ryder, *J. Am. Soc. Brew. Chem.* **2007**, *65* (1), 9–14.
57. K. Huvaere; B. Sinnaeve; J. Van Bocxlaer; D. DeKeukeleire, *Photochem. Photobiol. Sci.* **2004**, *3* (9), 854–858.
58. K. Huvaere; M. L. Andersen; L. H. Skibsted; A. Heyerick; D. De Keukeleire, *J. Agric. Food Chem.* **2005**, *53* (5), 1489–1494.
59. P. H. Todd; P. A. Johnson; L. R. Worden, *Tech. Q. Master Brew. Assoc. Am.* **1972**, *9*, 31–35.
60. A. Weiss; C. Schonberger; W. Mitter; M. Biendl; W. Back; M. Krottenthaler, *J. Inst. Brew.* **2002**, *108* (2), 236–242.
61. A. Fritsch; T. H. Shellhammer, *J. Am. Soc. Brew. Chem.* **2008**, *66* (2), 88–93.
62. L. Givaudan, Process for the Manufacture of Humulones. Canadian Patent 1,052,821, 17 April 1979.
63. L. Givaudan, Cie Societe Anonyme. British Patent 1,381,443, January 1975.

64. J. Cowles; H. Goldstein; E. Chicoye; P. L. Ting, Process for the Separation of Beta-Acids from Extract Containing Alpha Acids and Beta Acids. U.S. Patent 4,590,296, 20 May 1986.
65. A. C. J. Hermans-Lokkerbol; A. C. Hoek; R. Verpoorte, *J. Chromatogr. A* **1997**, *771*, 71–79.
66. A. C. J. Hermans-Lokkerbol; R. Verpoorte, *J. Chromatogr. A* **1994**, *664*, 45–53.
67. A. C. J. Hermans-Lokkerbol; R. Verpoorte, *Euro. Brew. Conv. Mongr.* **1994**, *22*, 273–274.
68. H. A. Thornton; J. Kulandai; M. Bond; M. P. Jontef; D. B. Hawthorne; T. E. Kavanagh, *J. Inst. Brew.* **1993**, *99*, 473–477.
69. B. J. Clarke; R. P. Hildebrand, *J. Inst. Brew.* **1965**, *71*, 26–36.
70. M. L. Viriot; J. C. Andre; M. Niclause; R. Flayeux; M. Moll, *J. Inst. Brew*. **1980**, *86*, 21–24.
71. A. Khatib, Studies of Iso-α-acids: Analysis, Purification, and Stability. Ph.D. Thesis, Leiden University: Leiden, 2006; ISBN 90-9020979-4.
72. M. Moir, *EBC Symposium on Hops, Monograph XXII, The Netherlands*; Hans Carl Verlag: Nurnberg, 1994; pp 165–180, ISBN 3-418-00746-5.
73. M. T. Roberts; J. P. Dufour; A. C. Lewis, *J. Sep. Sci.* **2004**, *27*, 473–478.
74. G. T. Eyres; P. J. Marriott; J. P. Dufour, *J. Agric. Food Chem.* **2007**, *55* (15), 6252–6261.
75. M. Steinhaus; W. Wilhelm; P. Schieberle, *Eur. Food Res. Technol.* **2007**, *226*, 45–55.
76. M. Deinzer; X. Yang, *EBC Symposium on Hops, Monograph XXII*, *The Netherlands*; Hans Carl Verlag: Nurnberg, 1994; pp 181–197, ISBN 3-418-00746-5.
77. A. J. Irwin, *J. Inst. Brew.* **1989**, *95*, 185–194.
78. T. Kishimoto; A. Wanikawa; K. Kono; K. Shibata, *J. Agric. Food. Chem.* **2006**, *54* (23), 8851–8861.
79. T. Kishimoto; A. Wanikowa; N. Kagami; K. Kawatsura, *J. Agric. Food. Chem.* **2005**, *53* (2), 4701–4707.
80. H. T. Fritsch; P. Schieberle, *J. Agric. Food. Chem.* **2005**, *53* (19), 7544–7551.
81. G. Lermusieau; M. Bulens; S. Collin, *J. Agric. Food. Chem.* **2001**, *49* (8), 3867–3874.
82. D. Kaltner; M. Steinhaus; W. Mitter; M. Biendl; P. Schieberle, *Monatsschrift Brauwiss.* **2003**, *56* (11–12), 192–196.
83. H. Kollmannsberger; M. Biendl; S. Nitz, *Monatsschrift Brauwiss.* **2006**, *59* (5/6), 83–89.
84. A. Mikyska; M. Hrabak; D. Haskova; J. Scrogl, *J. Inst. Brew.* **2002**, *108* (1), 78–85.
85. J. F. Stevens; J. E. Page, *Phytochemistry* **2004**, *65* (10), 1317–1330.
86. C. J. Walker, *Brauwelt* **2003**, *143*, 1709–1712.
87. F. Drawert; J. Beier, *Phytochemistry* **1976**, *15*, 1695–1696.
88. K. W. M. Zuurbier; S. Y. Fung; J. J. C. Scheffer; R. Verpoorte, *Phytochemistry* **1995**, *38* (1), 77–82.
89. S. Y. Fung; K. W. M. Zuurbier; N. B. Paniego; J. J. C. Scheffer; R. Verpoorte, *Phytochemistry* **1997**, *44* (6), 1047–1053.
90. K. W. M. Zuurbier; S. Y. Fung; J. J. C. Scheffer; R. Verpoorte, *Phytochemistry* **1998**, *49* (8), 2315–2322.
91. N. B. Paniego; K. W. M. Zuurbier; S. Y. Fung; R. van der Heijden; J. J. C. Scheffer; R. Verpoorte, *Eur. J. Biochem.* **1999**, *262* (2), 612–616.
92. J. De Keukeleire; G. Ooms; A. Heyerick; I. Roldan-Ruiz; E. Van Bockstaele; D. De Keukeleire, *J. Agric. Food. Chem.* **2003**, *51*, 4436–4441.
93. C. R. Langezaal; A. Chabdra; S. T. Katsiotis; J. J. C. Scheffer; A. B. de Haan, *J. Sci. Food Agric.* **1990**, *53* (4), 455–463.
94. Z. Zekovic; I. Pfaf-Sovljanski; O. Grujic, *J. Serb. Chem. Soc.* **2007**, *72* (1), 81–87.
95. G. Brunner, *J. Food Eng.* **2005**, *67* (1–2), 21–33.
96. M. Moir, *J. Inst. Brew.* **1992**, *98* (3), 215–220.
97. S. A. Langstaff; M. J. Lewis, *J. Inst. Bre*w. **1993**, *99* (1), 31–37.
98. B. Plutowska; W. Wardencki, *Food Chem.* **2008**, *107* (1), 449–463.
99. C. Zufall; K. Wackerbauer, *Monatsschrift Brauwiss.* **2000**, *53* (9–10), 164–179.
100. C. W. Bamforth, *MBAA Tech. Q.* **2001**, *38* (1), 1–9.
101. N. Francois; C. Guyt-Declerck; B. Hug; D. Callemien; B. Govaerts; S. Collin, *Food Qual. Prefer.* **2006**, *17* (6), 445–452.
102. B. Jaskula; E. Syryn; K. Goiris; G. De Rouck; F. Van Opstaele; J. De Clippeleer; G. Aerts; L. De Cooman, *J. Am. Soc. Brew. Chem.* **2007**, *65* (1), 38–46.
103. G. Vanhoenacker; D. De Keukeleire; P. Sandra, *J. Chromatogr. A* **2004**, *1035* (1), 53–61.
104. D. Harms; F. Nitzsche, *J. Am. Soc. Brew. Chem.* **2001**, *59* (1), 28–31.
105. P. S. Hughes; I. D. Menneer; M. T. Walters; G. Marinova, *Proc. Congr. Eur. Brew. Conv.* **1997**, *28*, 231–238.
106. L. De Cooman; G. Aerts; H. Overmeire; D. De Keukeleire, *J. Inst. Brew.* **2000**, *106*, 169–178.
107. S. Coghe; K. Benoot; F. Delvaux; B. Vanderhaegen; F. R. Delvaux, *J. Agr. Food. Chem.* **2004**, *52* (3), 602–608.
108. I. McMurrough; D. Madigan; D. Donelly; J. Hurley; A. M. Doyle; G. Hennigan; N. McNulty; M. R. Smyth, *J. Inst. Brew.* **1996**, *102* (5), 327–332.
109. J.-P. Dufour; M. Leus; A. J. Baxter; A. R. Hayman, *J. Am. Soc. Brew. Chem.* **1999**, *57* (4), 138–144.
110. M. Nyborg; H. Outtrup; T. Dreyer, *J. Am. Soc. Brew. Chem.* **1999**, *57* (1), 24–28.
111. C. W. Bamforth, *MBAA Tech. Q.* **2000**, *37* (2), 165–171.
112. M. C. Meilgaard, *MBAA Tech. Q.* **1975**, *12* 107–117, 151–168.
113. B. Vanderhaegen; F. Delvaux; L. Daenen; H. Verachtert; F. R. Delvaux, *Food Chem.* **2007**, *103* (2), 404–412.
114. B. Vanderhaegen; H. Neven; H. Verachtert; G. Derdelincks, *Food Chem.* **2006**, *95*, 357–381.
115. A. M. Jamieson; J. E. A. Van Gheluwe, *Proc. Am. Soc. Brew. Chem.* **1970**, *28*, 192–197.
116. R. M. Lewis; R. M. Pangborn; L. A. S. Tanno, *MBAA Tech. Q.* **1974**, *11*, 83–86.
117. B. W. Drost; P. van Eerde; S. F. Hoekstra; J. Strating, Proceedings of the 13th Congress of the European Brewery Convention, 1971; pp 451–458.
118. P. van Eerde; J. Strating, *European Brewery Convention*; Flavor Symposium, Monograph VII, 1981; pp 117–121.
119. J. Strating; W. M. Westra; L. C. Verhagen; F. Ph. Slotema, *MBAA Tech. Q.* **1979**, *16* (4), 176–181.
120. L. C. Verhagen; J. Strating; U. R. Tjaden, *J. Chromatogr.* **1987**, *393*, 85–96.
121. B. W. Drost; R. van der Berg; F. J. M. Freijee; E. G. van der Velde; M. Hollemans, *J. Am. Soc. Brew. Chem.* **1990**, *48*, 124–131.
122. S. Noel; S. Collin, Proceedings of the 25th Congress of the European Brewery Convention, 1995; pp 483–490.
123. G. Lermusieau; S. Noel; C. Liegeois; S. Collin, *J. Am. Soc. Brew. Chem.* **1999**, *57*, 29–33.

124. A. J. Irwin; R. L. Barker; P. Pipast, *J. Am. Soc. Brew. Chem.* **1991**, *49*, 140–148.
125. S. Noel; C. Liegeois; G. Lermusieau; E. Bodart; C. Badot; S. Collin, *J. Agric. Food. Chem.* **1999**, *47* (10), 4323–4326.
126. C. Liegeois; N. Meurens; C. Badot; S. Collin, *J. Agric. Food. Chem.* **2002**, *50* (26), 7634–7638.
127. C. Shimizu; Y. Nakamura; K. Miyai; S. Araki; M. Takashio; K. Shinotsuka, *J. Am. Soc. Brew. Chem.* **2001**, *59* (2), 51–58.
128. Y. Kuroiwa; N. Hashimoto; H. Hashimoto; E. Kokubo; K. Nakagawa, *J. Am. Soc. Brew. Chem. Proc.* **1961**, *19*, 181–193.
129. A. Heijerick; Y. N. Zhao; P. Sandra; K. Huvuaere; F. Roelens; D. De Keukeleire, *Phytochem. Photobiol. Sci.* **2003**, *2* (3), 306–314.
130. A. Vaughan; T. O'Sullivan; D. van Sinderen, *J. Inst. Brew.* **2005**, *111* (4), 355–371.
131. D. P. Lowe; E. K. Arendt, *J. Inst. Brew.* **2004**, *110* (3), 163–180.
132. P. L. Ting; L. Lusk; J. Refling; S. Kay; D. Ryder, *J. Am. Soc. Brew. Chem.* **2008**, *66* (2), 116–126.

Biographical Sketch

Leen C. Verhagen obtained his M.Sc. degree in analytical chemistry from Leiden University. He worked for 32 years in the corporate R&D department of the Heineken Company and retired in the year 2007.

He started the development of HPLC for brewery applications and became the manager of the Process & Product Analysis section and of the Brewing Science & Technology section within the R&D department. His research interests are beer flavor, hop chemistry and development, and the application of instrumental analysis methods for flavor research.

He has been a member of the European Brewery Convention Analysis Committee and the American Society of Brewing Chemists. For more than ten years he represented Heineken in the US Hop Research Council and served the Council for four years as President.

He is co-author of the European Brewery Convention Manual of Good Practice 'Hop and Hop Products'.

3.23 Chemistry of Tea

Ulrich H. Engelhardt, Institut für Lebensmittelchemie, Braunschweig, Germany

3.23.1 Introduction

Tea is one of the most popular beverages in the world and it is the most consumed beverage next to water. There are different types of tea, for example, white, green, oolong, black, and pu-erh tea. All types of tea are made from *Camellia sinensis*, the major varieties being *C. sinensis* var. *sinensis* and var. *assamica*. As a rule of thumb, the Assam variety has higher contents of phenolics and also a higher enzyme activity than the sinensis variety. There are also a number of subtypes, for example, *C. sinensis* var. *khenghe bai hao* and var. *fudin bai hao* grown only in Fujian province in China and traditionally used for white tea manufacture.

Tea is grown in the tropical and subtropical regions of the world. The major tea-producing countries are China, India, Sri Lanka, Kenya, Indonesia, Vietnam, Turkey, and Japan, but it is also grown in Argentina, Georgia, and other countries. The world tea production in 2007 was 3 871 339 tons, the major contributors being China (1 186 500 tons), India (949 220 tons), Sri Lanka (304 600 tons), Vietnam (153 000 tons), Kenya (315 000 tons), and Indonesia (192 000 tons).[1] India and China have high domestic consumption and consequently the export is only around 20% of the total production, while Sri Lanka and Kenya have an export of more than 90% of the total production.

Historically, tea has been used as a traditional medicine in China for more than 1000 years. Today, tea is used as a beverage and as an ingredient in other beverages, such as ready-to-drink (rtd) beverages. Tea is also used in cosmetics because of its antiaging properties. Traditionally, in some countries like China and Japan, far more green tea is consumed compared to black tea. In India and Sri Lanka, black tea is predominantly consumed. Tea consumption in Europe and the United States has changed over the past 10–15 years. Green tea consumption was not common in Europe earlier but nowadays – due to possible health benefits – the consumption has reached figures up to 20% of the total tea consumption in some countries.

3.23.2 Manufacture of Tea

Information on botanical classification, breeding, appropriate weather conditions, soils, and other premanufacture steps can be found in Willson and Clifford.[2] The first step in tea manufacture is plucking. Tea is usually plucked manually ('two leaves and a bud'); however, in some areas, mechanical plucking is employed.[3] Plucking standards do affect the composition and quality of tea, as stalks have a different composition compared to leaves. Tea bushes can reach a height of several meters but for the ease of plucking they are regularly pruned to a height of 1–1.5 m.[4,5] The principle of the manufacture of tea is described in **Figure 1**. The scheme in **Figure 1** is only an overview, as the details of the manufacturing process are often not available and it is not possible to cover the manufacture of every special tea.

Shading of tea has become a common practice in tea-growing areas worldwide. Shaded teas have a higher amount of amino acids and a lower amount of polyphenols.[6] The reports on the physiological activity of theanine (see Section 3.23.6.7) have led to an increase in the amount of tea produced in the shade. In Japan, green tea is traditionally made from shaded and unshaded leaves, for example, Gyokuro and Matcha are made from 90% shaded leaves, while Sencha, Bancha, and Kamairicha are made from unshaded leaves.[6] In most Japanese teas, the enzyme deactivation is accomplished by steaming, which means the tea is treated at 95–105 °C for 30–45 s. However, there are also Japanese pan-fired teas, for example, Kamairi-cha.[7] In China, pan firing (dry heat treatment) is more common. For some green teas, withering is also employed. A special type of tea is roasted green tea called Hoji-cha or Houjicha. It is made from crude green tea – usually not the best quality – by a thermal treatment.[7] There is also – as no agreed definition exists – some debate regarding green and white teas. It is common knowledge that green tea is nonfermented while black tea is fermented. For white teas, this is not true: there are publications that state that white tea is (slightly) fermented,[8] while other papers state that white tea is nonfermented.[9,10] A detailed overview of the different types of tea can be found in Hara *et al.*[11]

In China, a number of special kinds of tea are prepared traditionally and the manufacture of these special teas is often not known in detail. In some cases, even trade professionals are not familiar with the products, for example, with yellow tea. Oolong teas are the so-called semifermented teas. But what semifermented means is

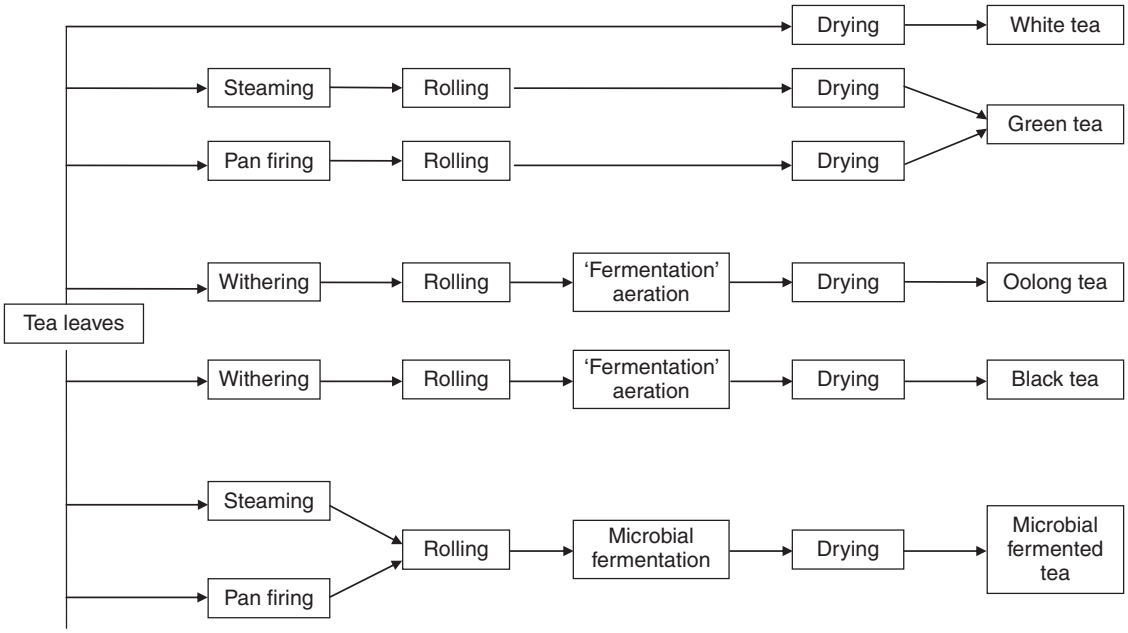


Figure 1 The principle of tea manufacture.[3–7,11]

not clear. There is a difference between different types of oolongs with regard to the degree of the so-called fermentation. In the case of black tea production, the withering step is crucial for subsequent processing. Changes during withering are loss of moisture and increase in the permeability of cell walls. Enzyme reactions take place leading to the formation of aroma precursors.[3,5]

The so-called fermentation is in fact an enzyme conversion of leaf constituents, mainly the catechins (flavanols) by leaf enzymes. The following are the most important enzymes. Polyphenoloxidase (PPO), EC 1.14.18.1, also referred to as tyrosinase (monophenol monooxygenase), is an enzyme containing copper at the active site. Peroxidase (POD), EC 1.11.1.7, is an enzyme responsible for the decomposition of H_2O_2 to water, among other reactions.[5,12,13] Catalase is also present in fresh tea leaf.[12] From a manufacturer's point of view, it is essential in case of oolong and black tea that the enzymes (usually present in the epidermal cells) and the substrates come in contact with each other and also that oxygen (which is used by PPO as an electron acceptor) is available. This is accomplished by a mechanical treatment of the leaves, the so-called rolling.[3,5,14] There are different ways to do this: (1) using a rolling machine to produce larger particles and (2) using a crushing–tearing–curling (CTC) machine to make smaller leaf grades. There are other possibilities, for example, the Lawrie Tea Processor (LTP), the Rotorvane, the Boruah rolling machine, and the Legg cutting machine.[14] As a rule of thumb, the smaller the particles, the more rapid and exhaustive the fermentation will be. As will be shown later, the degradation (oxidation/polymerization) of leaf flavonoids is more intensive.

The final drying step in tea manufacture is often referred to as firing. This step is crucial to reduce the moisture so that the tea is stable. There is also a step in the production called grading. This is essentially a sieving procedure. Some tea grades are Broken Orange Pekoe (BOP), Broken Orange Pekoe Fannings (BOPF), fannings, and dust, among others.[15]

On the regulatory or standardization site, there is some more work to be done as definitions for some types of tea do not exist (e.g., for white tea).[16] The International Organization for Standardization (ISO) working group on tea has set up definitions of black tea (currently modified) and is working on the definition of green tea. For black tea, the following definition was agreed to: "Tea derived solely and exclusively, and produced by acceptable processes, notably withering, leaf maceration, aeration and drying, from the tender shoots of varieties of the species *Camellia sinensis* (L.) Kuntze, known to be suitable for making tea for consumption as a beverage."[17]

The basic requirements of green and black teas are currently discussed on an international base and a proposal has been made: a minimum of 9% total phenolics for black teas and a minimum of 11% total phenolics and a minimum of 7% catechins for green teas.[18] The water extractables do have a limit at 32% for both.

3.23.3 Constituents of Tea and Chemical Reactions during Manufacture

Phenolic compounds are currently the most important constituents of tea and there are innumerable reports on the potential health benefits of these compounds. It is known that all the flavonoids do have antioxidative properties, which will be discussed briefly in Section 3.23.6.

The principle of the biosynthesis of the most important flavonoids is shown in **Figure 2**, compiled from different sources.[19,20] The precursors are carbohydrates, which are converted to shikimic acid and *p*-coumaric acid, which combine with three molecules of malonyl-CoA to form 4,2′,4′,6′-tetrahydroxychalcone, which already has the C6–C3–C6 backbone of the flavonoids. In **Figure 2**, only compounds with one hydroxyl group are shown; further hydroxylation occurs subsequently. Also, derivatization such as glycosylation is believed to occur at a later stage. It is known that flavonoid biosynthesis is light dependent via phenylalanine ammonia lyase (PAL), which is one of the key enzymes. More recently, it was shown that the high concentrations of flavanols are due to the strong activities of anthocyanidin reductase and dihydroflavonol 4-reductase/leucoanthocyanidin 4-reductase.[21]

In the literature, sometimes, all phenolic compounds in tea are called polyphenols; however, this is not true for compounds like gallic acid or mono-caffeoylquinic acids, which have to be referred to as simple phenols.[22] Tea usually contains 10–25% total phenolics depending on the source of tea and also the method of determination.[23] The chemistry of tea flavonoids has been reviewed based on the available literature in 1997.[24] In the following sections, the most important groups of phenolic compounds are presented.

3.23.3.1 Flavanols (Catechins)

Flavanols or catechins are the major phenolic constituents of fresh tea leaves. The older research and the identification are summarized in a few papers.[5,20] Commonly, the term catechins refers to (+)-catechin (+C), epicatechin (EC), epigallocatechin (EGC), epigallocatechin-3-*O*-gallate (EGCG), and epicatechin-3-*O*-gallate (ECG), with EGCG and ECG/EGC being the most abundant. The structures are shown in **Figure 3**. Other catechins have been identified, such as 3″- and 4″-methyl-epigallocatechin gallate.[25] Other minor catechins are epicatechin-3-(3-*O*-methylgallate) and epigallocatechin-(3-*O*-methylgallate) obtained from fresh leaves;[26] epiafzelechin-3-*O*-gallate, epicatechin-3-*O*(4-*O*-methylgallate), epicatechin-3-*O*-*p*-hydroxybenzoate, and epigallocatechin-3-*O*-cinnamate from oolong tea;[27] epigallocatechin-3,3′-di-*O*-gallate, epigallocatechin-3,4′-di-*O*-gallate, and epigallocatechin-3-*O*-*p*-coumarate from green leaves;[28] and epigallocatechin-3-*O*-caffeoate and epiafzelechin-3-*O*-gallate from fresh leaves.[29]

The amount of catechins in tea samples varies considerably. In some black teas, there are only traces of catechins present, while the amounts in Darjeeling teas can be as high as 10%. The most important reaction during the manufacture of black or oolong teas is the conversion of flavanols into theaflavins (TFs) and thearubigins (TRs) (see Sections 3.23.3.6 and 3.23.3.7). This leads to a dramatical decrease in catechin concentration in most black teas. Sometimes, only traces of the major catechin EGCG can be detected; however, in Darjeeling black teas, the amounts of catechins are as high as in green teas.[23]

Compositional data for catechins in green and black teas can be found in the literature. However, the results are often not comparable as different extraction conditions have been used. For the detection of geographic origin, there is often the problem of authenticity of the samples. Since 3 years, data collection by an ISO working group is ongoing using origin teas and also standardized procedures.[30,31] More than 300 green and black teas have been analyzed and the participating laboratories worldwide took part in several ring trials to ensure the quality of the results. These data on origin teas will become available in the near future.[32]

Table 1 is an overview and does not cover all data available from the literature. A more comprehensive overview – although not containing every publication – can be found in the USDA database on flavonoids.[38,39] The pattern of this group of flavonoids in tea might be dependent on geographic origin. In Darjeeling black tea

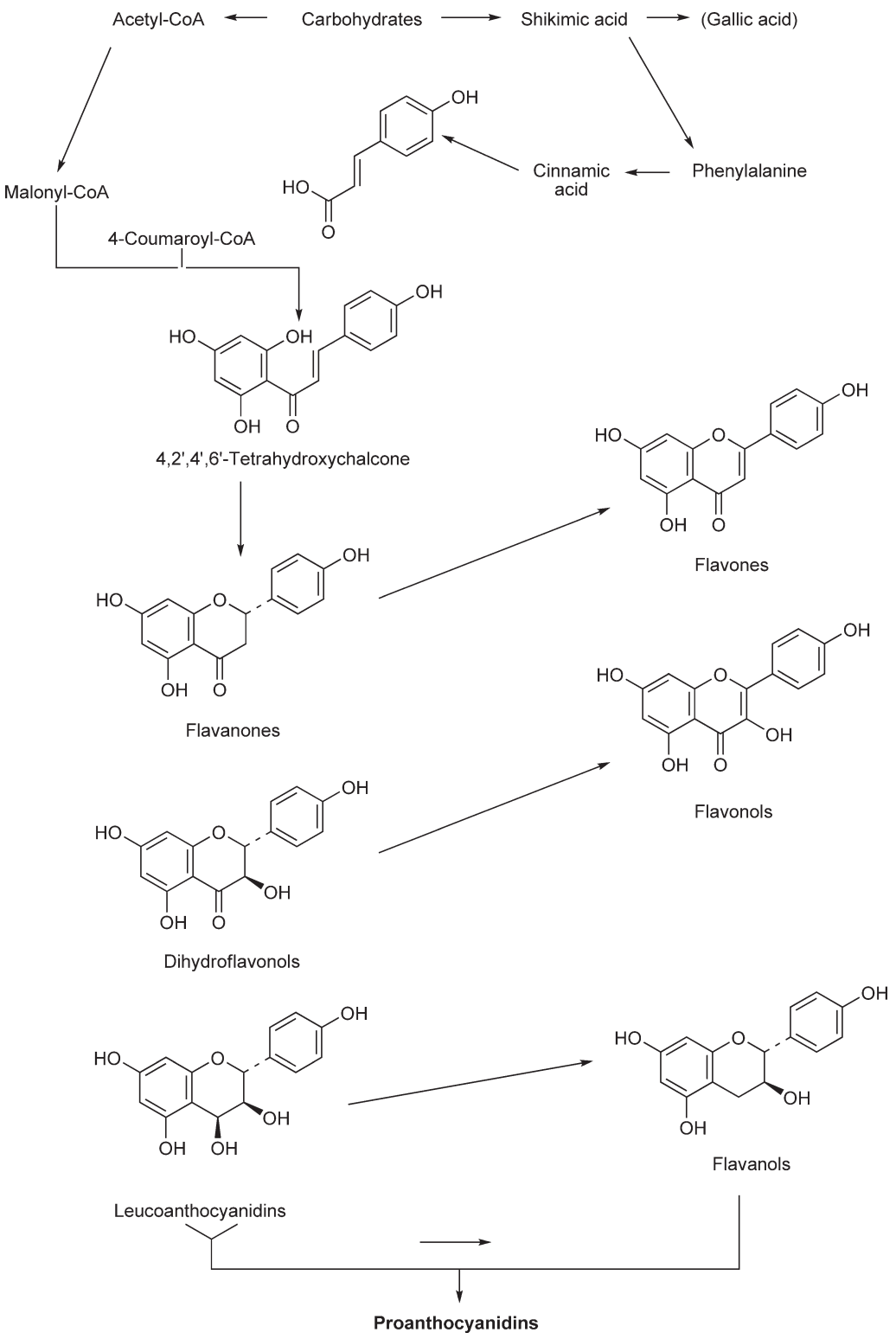


Figure 2 Biosynthesis of tea flavonoids.

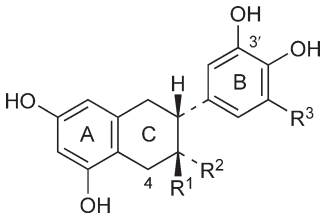


Figure 3 Structures of catechins (flavan-3-ols) from tea. (−)epigallocatechin gallate: $R^1 = H$, $R^2 =$ gallate, $R^3 = OH$; (−)epigallocatechin: $R^1 = H$, $R^2 = OH$, $R^3 = OH$; (−)epicatechin gallate: $R^1 = R^3 = H$, $R^2 =$ gallate; (+)gallocatechin: $R^1 = R^3 = OH$, $R^2 = H$; (−)epicatechin: $R^1 = R^3 = H$, $R^2 = OH$; (+)catechin: $R^1 = OH$, $R^2 = R^3 = H$.

Table 1 Major catechins in tea samples

Tea sample	*Epigallocatechin*	*Epicatechin*	*Epigallocatechin gallate*	*Epicatechin gallate*	*Total content*	*Reference(s)*
Darjeeling	0.11–0.86	0.00–0.40	2.78–7.07	0.90–2.23	4.40–10.00	33
Assam	0.00–0.14	0.00–0.24	0.58–1.61	0.31–0.94	0.89–2.81	33
Sri Lanka	0.05–1.35	0.28–1.00	1.24–4.55	0.68–1.66	2.25–8.42	33
Kenya	0.05–0.31	0.11–0.58	0.47–1.49	0.33–0.69	1.68–2.99	33,34
Commercial blends	0.00–1.03	0.04–0.63	0.05–2.84	0.17–26.80	0.54–6.95	35
Oolongs	0.78–11.15	0.08–0.60	0.28–3.07	0.06–0.92	1.50–15.60	36
China, green	2.02–4.65	0.61–3.79	6.10–11.61	1.10–5.47	11.29–19.54	33
Japan, green	2.47–4.76	0.61–1.68	4.28–8.18	0.62–1.45	8.46–15.89	33,34
Assam, green	4.31–4.57	0.82–0.88	12.57–12.82	2.61–2.61	20.62–20.56	33
Green teas	0.99–9.47	0.126–0.73	1.75–4.82	0.46–1.40	3.50–14.46	36
Fresh leaves	1.27–2.73	1.21–2.17	9.51–13.86	0.88–2.09	13.83–20.39	37
Commercial blends, green	0.00–1.42	0.01–0.26	0.002–5.36	0.12–2.71	0.44–10.00	35

Data are given in g per 100 g dry matter.

samples, the EGCG content was always higher than ECG and in most cases EGC was higher than ECG, while this was different in Assam samples (EGC > EGCG > ECG).[40,41] Sri Lankan samples had the same pattern in principle as Darjeeling samples; however, this was based on a small number of samples (six each).[41] Data on the epimers (gallocatechin (GC), gallocatechin gallate (GCG), catechin gallate (CG)) are quite scarce. These epimers do play a more important role in canned green tea beverages. Data for GC were between 0.07 and 0.45%, for CG between 0 and 0.01%, and for GCG between 0.20 and 0.17% in normal green teas.[34] The amount of GCG was of the same order of magnitude in another study: 0.09–0.18% in green teas ($n = 5$), 0.08–0.11% ($n = 3$) in oolongs, and traces in black teas. In some canned and bottled green tea beverages, the amount of GCG was more than EGCG.[42]

3.23.3.2 Flavonol Glycosides

As early as 1953, Japanese researchers detected more than 20 flavonol glycosides (FOGs) and they could identify nearly half of them (see **Table 2**). Examples of the structures of tea flavonols and flavones are given in **Figure 4**. Their role in the composition of black teas was underestimated in earlier studies, as no quantitative determinations were carried out and the content was referred to as 'traces'.[43] In the 1990s, isolation and NMR work were carried out leading to the identification of 14 FOG and an HPLC method following a polyamide clean-up was developed for the quantification.[44] NMR data are necessary to confirm aglycone, the position of the bonds between aglycone and the sugar moieties, and the anomeric configuration of the sugars. NMR data for FOG can also be found in a more recent paper.[53] **Table 2** gives an overview of the compounds identified.

As can be seen from the table, the flavonols in tea are mainly present in the form of mono-, di-, and triglycosides. The free aglycones – kaempferol, myricetin, and quercetin – have been detected in

Table 2 Flavonol glycosides identified in tea samples

Compound	*Abbreviation*	*Isolated from/ identified in*	*Reference(s)*
Kaempferol-3-*O*-β-D-glucopyranoside (astragalin)	K-glu	Fresh leaves, green tea, black tea	44–58
Kaempferol-3-*O*-β-D-galactopyranoside	K-gal	Fresh leaves, green tea, black tea	44,53,56,57
Quercetin-3-*O*-β-D-glucopyranoside (isoquercitrin)	Q-glu	Fresh leaves, green tea, black tea	44–53
Quercetin-3-*O*-β-D-galactopyranoside	Q-gal	Green tea, black tea	44–45,56,57
Quercetin-7-*O*-glucoside	Q-7-glu	Fresh leaves	48
Myricetin-3-*O*-β-D-glucopyranoside	M-glu	Fresh leaves, green tea, black tea	44,45–51, 53,55–57,59
Myricetin-3-*O*-β-D-galactopyranoside	M-gal	Fresh leaves, green tea, black tea	44,53,56,57
Myricetin-3-*O*-β-D-rhamnopyranoside (myricitrin)	M-rha	Fresh leaves	47
Quercetin-3-*O*-α-L-rhamnoside (quercitrin)	Q-rha	Fresh leaves, green tea, black tea	47,55
Kaempferol-3-*O*-[-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside]	K-rut	Fresh leaves, green tea, black tea	44–60
Quercetin-3-*O*-[-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (rutin)	Q-rut	Fresh leaves, green tea, black tea	44–58,60,61
Myricetin-3-*O*-[-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside]	M-rut	Fresh leaves, green tea, black tea	44–46,53,55–57
Kaempferol-3-*O*-[β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside]	K-rdg	Fresh leaves, green tea, black tea	44–48,50,51,53, 55–57,62
Quercetin-3-*O*-[β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside]	Q-rdg	Fresh leaves, green tea, black tea	44–51,53–57,62
Kaempferol-3-*O*-[β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→6)β-D-galactopyranoside]	K-grg	Green tea, black tea	9,10,12–14,17,20,44, 53,55–57,60,63
Quercetin-3-*O*-[β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→6)β-D-galactopyranoside]	Q-grg	Green tea, black tea	44,53,55–57,60,63

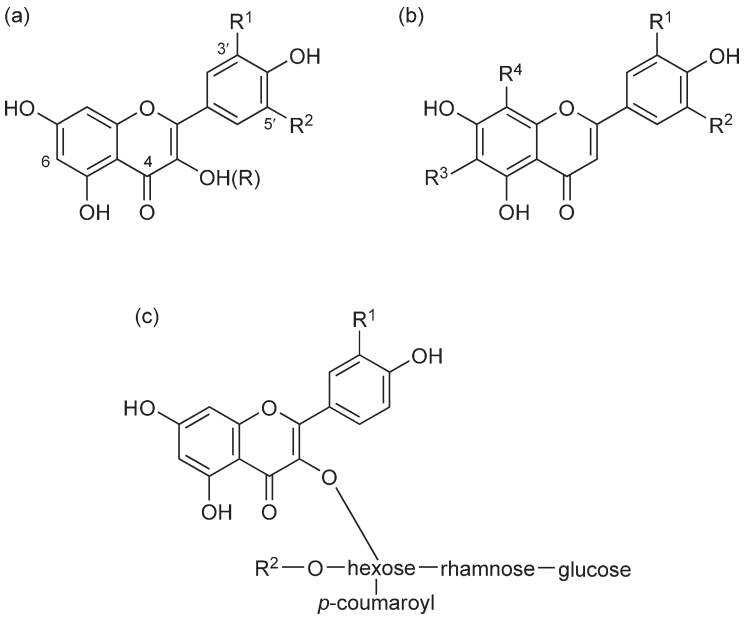


Figure 4 (a) Flavonol aglycones. Kaempferol: $R^1 = R^2 = H$; quercetin: $R^1 = OH$, $R^2 = H$; myricetin: $R^1 = R^2 = OH$. The sugars are attached at position 3 via a glycosidic bond. (b) Flavone aglycones. Apigenin: $R^1 = R^2 = H$; luteolin: $R^1 = OH$, $R^2 = H$. The sugars are attached at C-6 and/or C-8. (c) Flavonol tetrasaccharide (see text).

somestudies;[50,64,65] however, these are in most cases not detectable. In tea flowers, 3,5,8,4′-tetrahydroxy-7-methoxyflavone was identified along with its 3-*O*-glucoside, 3-*O*-rutinoside, and another glycoside.[66] In some Chinese tea samples, kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-β-D-galactopyranoside], kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside], and kaempferol-3-*O*-α-[-L-rhamnopyranosyl-(1 → 3)-(4‴-*O*-acetyl)-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] have been detected.[67]

Recently, eight acylated tri- and tetraglycosides have been identified in oolong teas; however, no data on their levels are available yet.[60] The structures of the acylated sugar moieties are shown in **Figure 4**. The tetraglycosides of quercetin and kaempferol with a hexose as R^1 were present in most samples analyzed, with the quercetin compounds being more abundant. The derivatives of quercetin and kaempferol having a rhamnose in R^2 position and those having an arabinose in R^1 position were not detected in most of the samples.

According to data available from the literature, the figures for FOG change only slightly during fermentation.[47] Quantification of FOG shows that their proportion in some black teas is higher than that of catechins, as FOG are not substantially changed during the manufacturing process. Depending on whether FOG are calculated as glycosides or aglycones, the total content is between 1 and 2%. In **Table 3** the data for the glycosides are given.

The pattern of FOG has been used in an approach to detect the geographic origin of the samples.[68] Using a data set of 45 black teas, the application of multivariate data analysis led to the conclusion that the differentiation of Darjeeling samples from other samples was undoubtedly possible based on the criteria Q-rut and Q-rdg.[68] A more recent work gave rise to the statement that FOG are responsible for the astringent taste of tea.[53,69] In an activity-guided fractionation, it was shown that neither the catechins nor the TFs are responsible for the astringent taste of black tea brews. The flavon-3-ol glycosides, above all Q-rut, were shown to give a velvety and also a mouth-coating sensation, while the catechins were described as astringent and the TFs as mouth-drying and rough. The threshold data were very much lower compared to catechins and TFs. **Table 4** gives an overview. The tea brews were analyzed more comprehensively; data are given for catechins, TFs,

Table 3 Flavonol glycosides in tea

Compound	*Content (mg kg^{-1})*	*Reference(s)*
Myricetin-3-*O*-rutinoside	n.d.–3236	34,44,57
Myricetin-3-*O*-galactoside	n.d.–1541	44,57
Myricetin-3-*O*-glucoside	n.d.–2512	44,57
Quercetin-glucorhamnogalactoside	n.d.–3598	44,57
Quercetin-rhamnodigalactoside	n.d.–7637	44,57
Quercetin-rhamnogalactoside	n.d.–1304	57
Quercetin-3-*O*-rutinoside	407–5344	44,57
Quercetin-3-*O*-galactoside	n.d.–1332	44,57
Quercetin-3-*O*-glucoside	195–2734	44,57
Kaempferol-glucorhamnogalactoside	n.d.–3365	44,57
Kaempferol-rhamnodigalactoside	101–5779	44,57
Kaempferol-3-*O*-galactoside	73–829	44,57
Kaempferol-3-*O*-rutinoside	n.d.–3039	34,44,57
Kaempferol-3-*O*-glucoside	41–2590	34,44,57

Data are given for the glycosides.
n.d., not detected.

Table 4 Threshold data for tea flavonoids

Class of flavonoids	*Threshold (compound) data in μmol l^{-1}*	*Reference*
Catechins	190 (epigallocatechin gallate) to 930 (epicatechin)	53
Theaflavins	13 (theaflavin-3,3′-digallate) to 26 (theaflavic acid)	53
Flavonoid glycosides	0.001 (quercetin-3-*O*-rutinoside) to 19.8 (kaempferol-3-*O*-glucorhamnogalactoside)	53

FOG, caffeine, amino acids, organic acids, and sugars.[69] It was also stated that FOG also contribute to bitterness by amplifying the bitter taste of caffeine.[69] The threshold data for astringency and bitterness can also be found in other papers.[43,70]

3.23.3.3 Flavone Glycosides

In tea, a number of flavone glycosides have been detected, the majority being flavone 6- and/or 8-C-glycosides (FCG). Apigenin-8-C-glycoside (vitexin),[45,50,51,56,71–73] apigenin-6-C-glycoside (isovitexin),[56,74] apigenin-6,8-C-diglucoside (vicenin 2),[50,51] apigenin-6-C-[1 → 2 glycosyl-glucoside],[75] apigenin-6-C-pentosyl-8-C-glucoside,[56] apigenin-6-C-glucosyl-8-C-pentoside,[56] apigenin-6-C-glucosyl-7-*O*-glucoside,[76] and apigenin-8-C-glucosyl-7-*O*-glucoside have been identified.[51] More recently, apigenin-8-C-[α-L-rhamnopyranosyl-(1 → 2)-*O*-β-D-glucopyranoside] was identified in a study on astringent taste.[53] In the literature, a few more compounds are described; however, in most cases, the nature of the sugar moiety was not elucidated.

Seven FCG were determined by HPLC, five apigenin compounds (apigenin-6,8-C-diglucoside, apigenin-6-C-glucoside-8-C-arabinoside, apigenin-6-C-arabinoside-8-C-glucoside, apigenin-8-C-glucoside, and apigenin-6-C-glucoside) as well as two luteolin compounds (luteolin-6-C-glucoside and luteolin-8-C-glucoside).[41] In this study, the amounts (total content of the FCG) were between 0.48 and 2.69 g kg^{-1}, which is well below the amounts of FOG. No correlation between the amounts or pattern of the FCG and the geographic origin was observed. However, this was based on a limited set of samples (16 black, 2 green, and 1 oolong sample). In another study, the amounts of the same compounds were determined in 50 green and black teas each. In black tea, the range was from 0.2 to 1.2 g kg^{-1} (calculated as aglycones, average 0.51 g kg^{-1}, calculated as glycosides 0.9 g kg^{-1}), and in green teas from 0.05 to 1.4 g kg^{-1} (average 0.86 g kg^{-1}, as glycosides 1.6 g kg^{-1});[23] individual data can be found in Lakenbrink.[57] FCG are very stable compounds, so their determination could be useful for the determination of tea-based rtd beverages if the instant was treated with alkali hydroxide.[77]

3.23.3.4 Proanthocyanidins and Bisflavanols

The occurrence of proanthocyanidins and bisflavanols in tea (**Figure 5**) has been confirmed in a number of papers. **Table 5** gives an overview of the proanthocyanidins identified. Besides proanthocyanidins, a number of bisflavanols (see the structure in **Figure 5**) have been identified. Roberts[81] detected three colorless compounds (bisflavanols A–C) in black tea. Later, bisflavanols A and B were also found in fresh leaves.[28,82] Four more compounds were identified along with the three already mentioned and were named theasinensins A–G.[82] The theasinensins D and E (*S* configuration of the biphenyl unit) are stereoisomers of A and C (*R* configuration).

The structural elucidation of both proanthocyanidins and bisflavanols requires sophisticated NMR work along with chemical analysis. NMR data can be found in the literature.[28,29,57,76,78,79,82] The total content of

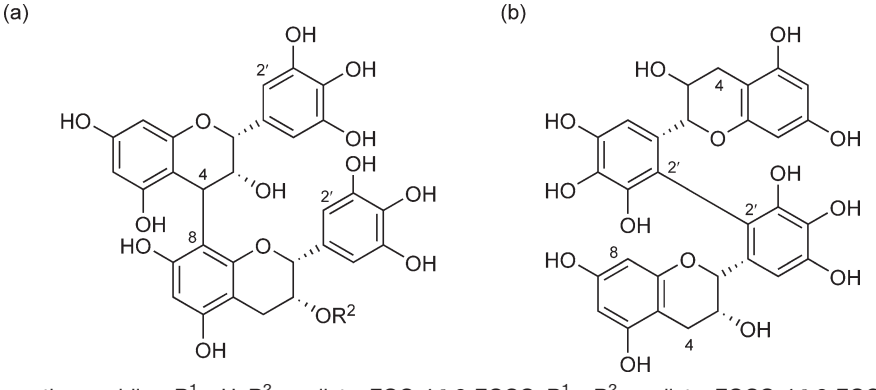


Figure 5 (a) Proanthocyanidins: $R^1 = H$, $R^2 =$ gallate: EGC-4β-8-EGCG; $R^1 = R^2 =$ gallate: EGCG-4β-8-EGCG; (b) Bisflavanols: $R^1 = R^2 = H$: bisflavanol C; $R^1 = H$, $R^2 =$ gallate: bisflavanol B; $R^1 = R^2 =$ gallate: bisflavanol A.

Table 5 Proanthocyanidins identified in tea

	Isolated from	*Reference(s)*
Dimers		
Epicatechin-(4β→8)-epicatechin (procyanidin B2)	FL, GT, BT	28,29,57,76–80
Catechin-(4α→8)-catechin (procyanidin B3)	FL, OT	29,78
Catechin-(4α→8)-epicatechin (procyanidin B4)	GT, FL, OT	29,76,78
Catechin-(4α→8)-epigallocatechin	FL, OT	29,78
Gallocatechin-(4α→8)-epicatechin	FL, OT	29,78
Gallocatechin-(4α→8)-epigallocatechin (prodelphinidin B4)	FL, OT	29,60,78
Trimers		
[Epicatechin-(4β→8)]$_2$-epicatechin (procyanidin C1)	GT, FL, BT	29,57,76,80
Galloylated dimers		
Epiafzelechin gallate-(4β→6)-epigallocatechin gallate	GT, OT, BT	57,78,80
Epicatechin-(4β→8)-epicatechin gallate	FL, GT, BT	28,57,60,80
Catechin-(4α→8)-epicatechin gallate	FL	28
Epicatechin-(4β→8)-epigallocatechin gallate	GT, OT, BT	57,60,78,80
Catechin-(4α→8)-epigallocatechin gallate	OT	79
Epigallocatechin-(4β→8)-epicatechin gallate	GT, OT, BT	57,76,78,80
Epigallocatechin-(4β→8)-epigallocatechin gallate	GT, OT, BT	28,57,78,80
Epicatechin gallate-(4β→8)-epicatechin gallate	FL, GT, OT, BT	28,57,78,80
Epicatechin gallate-(4β→6)-epicatechin gallate	GT, OT, BT	57,78,80
Epicatechin gallate-(4β→8)-epigallocatechin gallate	GT, OT, BT	57,78,80
Gallocatechin-(4α→8)-epigallocatechin gallate	GT, OT, BT	57,72,74
Epicatechin gallate-(4β→6)-epigallocatechin gallate	GT, OT, BT	57,78,80
Epigallocatechin gallate-(4β→8)-epicatechin gallate	GT, OT, BT	57,78,80
Epigallocatechin gallate-(4β→6)-epicatechin gallate	GT, OT, BT	57,78,80
Epigallocatechin gallate-(4β→8)-epigallocatechin gallate	GT, OT, BT	57,78,80
Epigallocatechin gallate-(4β→6)-epigallocatechin gallate	GT, OT, BT	57,78,80
Epigallocatechin-(4β→8, 2β→7)-epigallocatechin gallate (prodelphinidin A2-gallate)	GT, OT, BT	57,60,78,80
Gallocatechin-(4α→8)-epicatechin gallate	GT, BT	57,80
Epiafzelechin gallate-(4β→8)-epigallocatechin gallate	GT, BT	57,80
Epiafzelechin gallate-(4β→8)-epicatechin gallate	GT, BT	57,60,79,80
Epiafzelechin gallate-(4β→6)-epicatechin gallate	GT, BT	57,60,79,80

FL, fresh leaves; GT, green tea; OT, oolong tea; BT, black tea.

proanthocyanidins (determined by HPLC) in green tea was 0.13–1.89% (average 0.84%, $n = 29$) while it was lower in black tea (0.10–0.98%, average 0.50%, $n = 9$). The amount of bisflavanols was lower in green tea 0.01–0.11% (average 0.05%) and higher in black tea (0.33–0.81%, average 0.65%).[57,80] First, this gives rise to the assumption that there is a decrease in proanthocyanidins during the enzyme oxidation while bisflavanols are formed. This is in tune with a fermentation study in which the behavior of four different proanthocyanidins and two bisflavanols during fermentation was studied.[83] It was stated that the galloylated proanthocyanidins rapidly decreased while the nongalloylated remained nearly constant. The bisflavanols went through a maximum after 2h of fermentation and were higher after 12 h of fermentation compared to the fresh leaves. According to the literature, it appears to be very likely that the same precursors as for the TFs also form the bisflavanols.[12,24,84,85]

Second, it can be stated that the amounts of bisflavanols and proanthocyanidins are of the same order of magnitude as the FOG and, consequently, will contribute to sensory and health attributes. The extraction kinetics of consumer brews has been studied using green tea. It was shown that a 3 min aqueous extraction gave recoveries from 0 to 80% compared to an exhaustive extraction, with the compounds having a 4→6 interflavonoid bond being lower.[80]

3.23.3.5 Theasinensins

In this chapter, theasinensins are referred to as bisflavanols and are discussed in Section 3.23.3.4.

3.23.3.6 Theaflavins

TFs are the main oxidation products of catechins with a known structure. Consequently, these are found only in fermented teas; however, small amounts may also be present in green tea. Historically, the oxidation products obtained during the manufacture of black tea were named by Roberts *et al.*[86,87] He separated the colored oxidation products into two groups, TFs and TRs. Ongoing research led to the structural elucidation of TFs, which are reddish-orange pigments with a benzotropolone moiety. The real structural elucidation was conducted by Collier *et al.*[88] using modern spectroscopic methodology. The pathway/mechanism of TF formation was established before this, but it is still valid.[89] Basically, two flavanols, one with two and the other with three hydroxyl groups at the B-ring, combine by an oxidative coupling via *o*-quinones. This conversion is shown in principle in **Figure 6**. More detailed schemes are available in the literature.[84,85,90]

It is known that TFs go through a maximum as a prolonged enzymatic oxidation leads to a decrease and the TFs might be at least in part converted into TRs.[91,92] In **Table 6**, the precursors and the resulting TFs and related compounds are listed. Besides the major TFs, minor compounds like isotheaflavin and neotheaflavins have been identified.[88] Theaflavic acids were shown to be benzotropolone compounds as well as theaflagallines.[93,94] Other minor TFs identified were theaflavinat B,[95] isotheaflavin-3′-gallate, and neotheaflavin-3-gallate.[96] More recently, three new benzotropolone derivatives have been detected in a study on the mechanism of catechin oxidation. Theadibenzotropolone A and B were the first benzotropolone-type trimers of catechins found in black tea extract.[97,98] Methylated TFs such as theaflavin-3-*O*-(3-*O*-methyl)gallate and theaflavin-3-*O*-(3-*O*-methyl)gallate-3′-gallate have been detected.[99] Another type of phenolic pigment not containing a benzotropolone system was named theacitrins.[100] These are dimeric flavanols but they do have a C6–C5–C5 system instead of the benzotropolone moiety. ECG trimers and tetramers were produced from model reactions along with theaflavate A.[101] **Figure 7** gives an overview of the structures of selected TFs and related compounds.

TFs contribute to the bright orange-red color of tea brews, which might influence tea taster evaluations. Earlier, there was an effort to find a correlation between TF content and the tea taster's assessment but no real satisfying concept was internationally agreed upon. It has been claimed that TFs do have an important influence on the astringency of black teas and therefore determine tea taster evaluations.[102–105] It was shown in the 1990s that there was no correlation between TF concentration and the astringent taste but a correlation was shown between the concentration of catechins and the astringent taste. More recently, in a bioactivity-guided fractionation, it was concluded that FOG are responsible for astringency (see Section 3.23.3.2).

Compositional data on TF content are relatively scarce. Some of the literature data have been determined by the flavognost method, which is not capable of differentiating between the individual TF compounds. One of the problems is due to the fact that no calibration standards are commercially available. In black teas, the total content of the four major TFs will hardly exceed 2.5%. Selected data from the literature are compiled in **Table 7**.

The TF content from HPLC data for Assam samples produced under different conditions was between 0.76 and 2.12% (total content).[107] According to Friedman *et al.*,[35] the concentration of TFs (sum of the contents of four major ones) in commercial black teas was 0–0.88% (from aqueous extracts, calculated for dry leaves). In the paper, the individual data for the four major TFs can be found. Additional data for TFs in dry tea leaves or tea brews can also be found in the literature[108–111] or in the USDA databases.[38,39] These data are in most cases of the same order of magnitude as those from earlier, non-HPLC work.[3,5]

Quantitative data for other TF-related compounds are even more scarce. The amounts of theaflavin-3-*O*-(3-*O*-methyl) gallate and theaflavin-3-*O*-(3-*O*-methyl)gallate-3′-gallate in four samples were in the range of 0.005–0.017 and 0.013–0.038 g per 100 g.[99] Other minor TF-related compounds (theaflavic and epitheaflavic acids, epitheaflavic acid-3-gallate, neotheaflavin, epitheaflagalline, epitheaflagalline-3-gallate) were shown to contribute only 5–10% of the total content of all TFs detected.[23] The content of individual minor TFs has also been calculated.[33] It was shown that the results of TF determination by HPLC and the photometric flavognost method are different; in most cases, the photometric method gave higher results.[106]

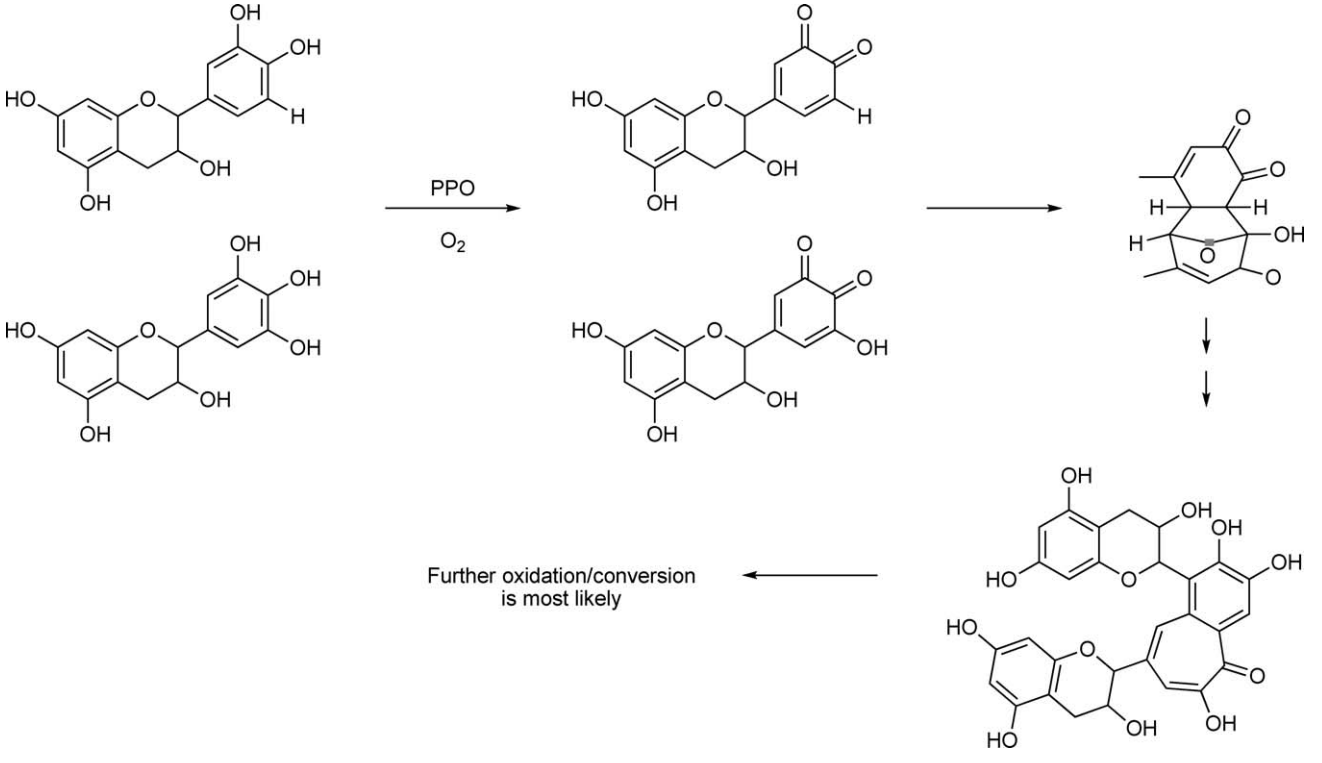


Figure 6 Enzymatic conversion in black tea manufacture.

Table 6 Formation of theaflavins and related compounds

Precursor 1	*Precursor 2*	*Product*
Epicatechin	Epigallocatechin	Theaflavin
Epicatechin	Epigallocatechin gallate	Theaflavin-3-gallate
Epicatechin gallate	Epigallocatechin	Theaflavin-3′-gallate
Epicatechin gallate	Epigallocatechin gallate	Theaflavin-3,3′-digallate
Epicatechin	Gallocatechin	Isotheaflavin
Catechin	Epigallocatechin	Neotheaflavin
Epicatechin	Gallic acid	Epitheaflavic acid
Catechin	Gallic acid	Theaflavic acid
Epigallocatechin	Gallic acid	Epitheaflagalline
Gallocatechin	Gallic acid	Theaflagalline
Epigallocatechin gallate	Gallic acid	Epitheaflagalline-3-gallate

3.23.3.7 Thearubigins

The so-called TRs are the major constituents of black teas accounting for 70–80% of the total phenols. This section will provide a brief overview of TRs. The current knowledge of TR has not changed much in the past few years. Earlier findings have been summarized by Haslam.[12] The term TRs refers to a group of colored phenolic oxidation products. The question arose whether or not to stick to this name.[12] Despite the fact that a lot of work has been done, there is no real full picture of the structures of TR yet. What is known is that TRs are formed during the enzymatic transformation of flavanols, they are water soluble, at least some of the compounds are colored, and they are acidic.[12] A chromatographic separation of TR compounds is currently not possible. At least some of them are responsible for a hump in the chromatogram. Consequently, a real determination of TR is currently not possible; however, data can be found in the literature but they have to be treated with caution. One of the concepts was spectrophotometric determination after extraction, which was later shown to suffer from interference of FOG.[112]

Roberts and Williams[86] named all brown acidic pigments in black tea as TRs and defined subgroups (called SI, SIa, SII), which were obtained by extraction with ethyl acetate (SI). Later, HPLC was employed to separate the TR and at least a part of the fraction was found to elute as an unresolved hump.[113–116] Based on this, an alternative classification of TR was proposed: group I (excluded from HPLC separation), group II (resolved by HPLC), and group III (not resolvable by HPLC).[113] Using normal-phase HPLC, acetyl and methyl derivatives could be separated; however, it was also observed that the acetyl derivatives tended to change in a time-dependent manner.[117] A different wording was used for one fraction: theafulvins.[118] This stands for a polymeric brown fraction containing no caffeine, protein, or FOG and eluting as a hump from RP-18 columns. The fraction was isolated by means of column chromatography (Solka-Floc cellulose) and also evaluated by NMR. The results showed that the fraction was based on flavanols.[114,115,118] High-speed countercurrent chromatography (HSCCC) was used to isolate a fraction (the hump one) free of resolvable compounds.[119] The fraction contained 35% of phenolics as determined by the Folin–Ciocalteu assay calculated as gallic acid equivalents and an antioxidant activity of 3.6 mmol trolox g^{-1} in the trolox equivalent antioxidant capacity (TEAC) test. The same papers gave rise to the assumption that TR at least in part contains bisflavanol structures in the backbone, which was elucidated by NMR work. There are some structures of TR as condensation products of proanthocyanidin gallates with catechins-derived compounds having a benzotropolone moiety.[120]

In the research on TR, a lot of work has been done employing model fermentation systems using different enzymes.[121–124] However, this did not give rise to a full understanding of the chemistry as no individual structure could be elucidated. Currently, our knowledge of TR is still very limited and structures remain to be elucidated. Maybe in the future, the term will be omitted, as it is nothing but a generic term for all oxidation products.

3.23.3.8 Hydrolyzable Tannins

Not much is known about the hydrolyzable tannins in tea except about strictinin. Strictinin (1-*O*-galloyl-4,6-(−)-hexahydroxydiphenoyl-*β*-D-glucose; **Figure 8**) is a well-known tea constituent. It was first detected in

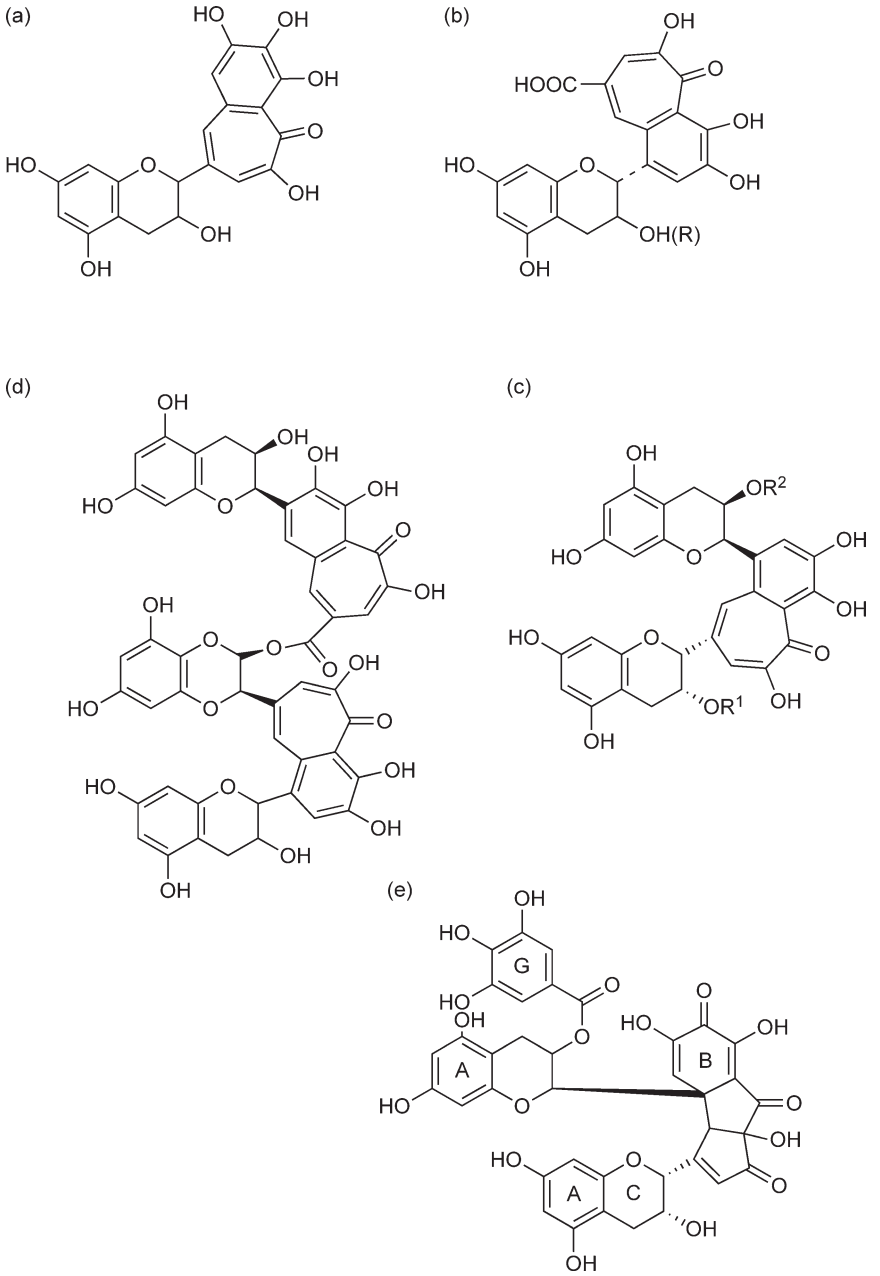


Figure 7 (a) Theaflagallines/epitheaflagallines; (b) theaflavic/epitheaflavic acids; (c) theaflavin ($R^1 = R^2 = H$), theaflavin 3-gallate ($R^1 =$ gallate, $R^2 = H$), theaflavin 3′-gallate ($R^1 = H$, $R^2 =$ gallate), theaflavin 3,3′-digallate ($R^1 = R^2 =$ gallate); (d) benzoditropolone; (e) theacitrin A.

Table 7 Content of the four major theaflavins in tea samples

Sample	*Theaflavin*	*Theaflavin-3-gallate*	*Theaflavin-3′-gallate*	*Theaflavin-3,3′-digallate*	*Total content*	*Reference(s)*
Darjeeling teas	0.10–0.15	0.06–0.13	0.04–0.08	0.07–0.26	0.28–0.56	33,99,102
Assam teas	0.15–0.22	0.22–0.41	0.16–0.29	0.42–1.13	0.96–1.91	33,99,102
Sri Lankan teas	0.18–0.25	0.13–0.34	0.12–0.22	0.13–0.35	0.61–1.15	33,99,106
African teas	0.39–0.50	0.42–0.65	0.36–0.48	0.37–0.66	1.66–2.30	33
Chinese teas	0.03–0.15	0.09–0.27	0.04–0.17	0.23–0.31	0.44–0.89	33

Data are given in g per 100 g dry matter.

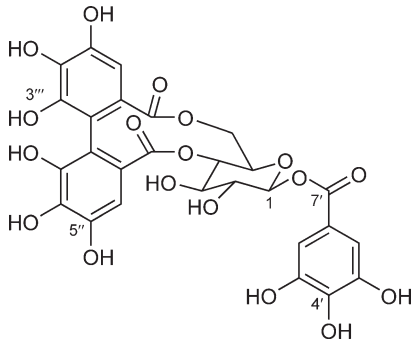


Figure 8 Structures of 1-*O*-galloyl-4,6-(−)-hexahydroxydiphenoyl-β-D-glucose (strictinin) (R = H) and 1-*O*-digalloyl-4,6-(−)-hexahydroxydiphenoyl-β-D-glucose (R = gallate).

green tea[76] together with 1,4,6-tri-*O*-galloyl-β-D-glucose, and later also in black tea.[83] A derivative (1-*O*-digalloyl-4,6-(−)-hexahydroxydiphenoyl-β-D-glucose) was identified later.[57,80] Like other ellagitannins, strictinin seems to have some interesting properties (e.g., an antiallergic effect).[125]

Data on the content of these compounds are scarce. A brewing study estimated the content of strictinin between 1.05 and 7.4 g kg^{-1} (average 4.5 g kg^{-1}, 20 green teas tested).[126] The contents in two cultivars from Japan were between 6.9 and 14.8 g kg^{-1}. The strictinin content varied depending on the plant material analyzed, with buds and first leaves having 34 g kg^{-1}, second leaves 26.4 g kg^{-1}, down to the fifth leaves 7.6 g kg^{-1} and the stems 4.3 g kg^{-1}.[125] The content was between 6.9 and 14.8 g kg^{-1} when the complete material (bud to fifth leaf) was analyzed. In another study, much lower amounts (177–1285 mg kg^{-1}) were detected.[80] In a fermentation series, the amount of strictinin decreased with increasing fermentation time.[80] Strictinin was also determined to be responsible for sediment formation during the storage of green tea beverages.[127] It is hydrolyzed during the heat sterilization of the beverage yielding ellagic acid, which reacts with proteins resulting in sediment formation.

3.23.3.9 Phenolic Acids and Derivatives

The most abundant phenolic acid derivative in fresh tea leaves is theogallin. It was elucidated by Roberts as 3-galloylquinic acid.[128] Owing to the changes in the International Union of Pure and Applied Chemistry (IUPAC) nomenclature, it is now referred to as 5-galloylquinic acid. According to the literature, tea is the only relevant source of theogallin. Gallic acid (**Figure 9**) is also present in tea samples. In fresh leaves, the concentration of gallic acid is much lower compared to theogallin. As gallic acid is released from galloylated

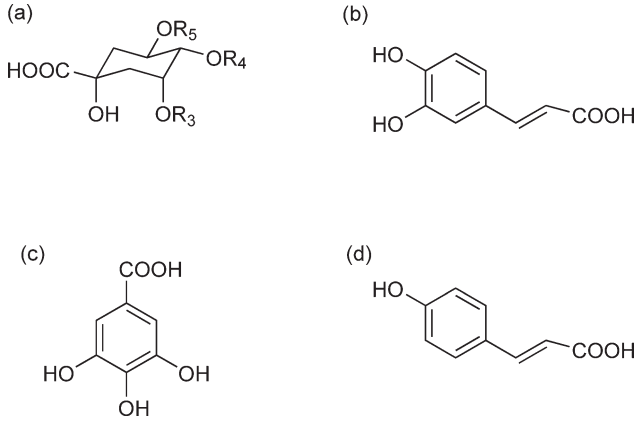


Figure 9 Structures of (a) quinic acid, (b) caffeic acid, (c) gallic acid, and (d) *p*-coumaric acid.

Table 8 Theogallin and gallic acid in green and black teas[23,33]

	Theogallin (% dry matter)	*Gallic acid (% dry matter)*
Green tea	0.08–1.41 Average: 0.64	0.01–0.19 Average: 0.09
Black tea	0.11–1.01 Average: 0.65	0.16–0.60 Average: 0.26

catechin-derived species, such as galloylated catechins, during fermentation, the amount in black and oolong tea is higher; however, in most black teas, the concentration of theogallin is still higher as is the concentration of gallic acids. **Table 8** is an overview of theogallin and gallic acid contents in green and black teas. It is based on around 50 green and black tea samples each.[23,33] Other phenolic acid derivatives have also been detected (**Figure 9**).

The presence of a number of isomers of coumaroylquinic acid (CouQA) and caffeoylquinic acid (CQA) has been established.[43,54,58,60,129–133] Six isomers (3-, 4-, 5-CQA and 3-, 4-, 5-CouQA) have been determined in 12 black teas.[132] The total content of these isomers was between 0.25 and 0.68%. The 4-isomers were most abundant. Both the order of magnitude and the abundance of isomers (for CQA) are in tune with another study.[131] In four teabag products, the content was between 0.3 and 0.45% with the same order with respect to isomer composition.[57,111] More recently, 3- and 4-galloylquinic acid were identified in a commercial green tea by LC–MS^n procedures,[134] while digalloylquinic acids were not detected. Data on the amounts in tea are currently not available.

3.23.3.10 Alkaloids

The presence of alkaloids, caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine), in tea is well established. Other purine alkaloids, such as theacrine, have also been detected. In a special kind of tea (*Camellia assamica* var. *Kucha*), theacrine (1,3,7,9-tetramethyluric acid) was present in higher amounts and it was shown that the biosynthetic pathway led from adenosine to caffeine and then to thecarine.[135] **Figure 10** shows some structures of tea alkaloids.

Caffeine is the most abundant alkaloid in tea. The content is usually between 1.5 and 5%, with the concentration in green and black teas being very similar.[5,16,20,23,33,40] Also present in relevant amounts is theobromine (usually 0.1–0.4%). Data on theophylline in tea are quite scarce. In one study, theophylline was detectable in the bud and the first leaf (0.13–0.18%) but not in the second or third leaf.[136] In a survey including 27 black, 13 green, and 5 pu-erh teas, no theophylline was detected in 19 samples while the other samples contained up to 0.049%.[137]

The biosynthesis of alkaloids in tea plants has been studied frequently.[138–140] According to Mizutani *et al.*,[141] the biosynthesis of caffeine in tea leaves follows the following pathway: *S*-adenosyl-L-methionine (SAM) → *S*-adenosyl-L-homocysteine (SAH) → adenosine → adenine → AMP → IMP → XMP → xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine. Caffeine is known to be bitter and consequently the compound is involved in the bitter taste of tea, which might be amplified by FOG.[69] It is worth noting that the amount of caffeine is high in white tea samples.

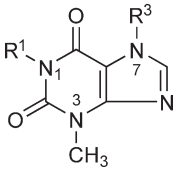


Figure 10 Structures of alkaloids from tea. Caffeine: $R^1 = R^2 = CH_3$; theobromine: $R^1 = H$, $R^2 = CH_3$; theophylline: $R^1 = CH_3$, $R^2 = H$.

3.23.3.11 Enzymes

The key role of PPO, POD, and catalase in tea – especially black and oolong tea chemistry – has already been described in Section 3.23.2. Besides these enzymes, tea contains a number of other enzymes. The basic enzymes are summarized in a review.[5] The enzymes mentioned are 5-dehydroshikimate reductase (EC 1.1.1.25), PAL, tea leaf chorophyllase, pectin methylesterase, tea leaf ribonuclease, malate dehydrogenase, and acid phosphatases, among others. There is not much information on tea enzymes in the more recent literature. It was shown that β-primeverosidase (EC 3.2.1.149) plays an important role in the aroma formation of oolong and black teas by liberating aroma precursors.[141–145]

3.23.3.12 Proteins and Amino Acids

Fresh tea leaves contain up to 30% protein. In black tea, 15–23% of proteins have been determined with less than 2% being water soluble.[146] Not much literature is available on tea proteins as they do not play a significant role in the beverage. The presence of free amino acids in tea has been established; these amino acids influence the aroma properties of green tea. In a recent paper, alanine, arginine, asparagine, aspartic acid, glutamic acid, isoleucine, histidine, leucine, phenylalanine, serine, theanine, threonine, and tyrosine have been determined by HPLC in green, white, black, and pu-erh teas. The concentration of most amino acids was detected between 0 and 0.3%, with the concentration in pu-erh teas being very low.[9] Using principal component analysis (PCA), a differentiation of white, green, black, and oolong teas was possible, with glutamic acid, asparagine, serine, alanine, leucine, and isoleucine contributing to this differentiation the most. In this study, 21 green, 28 black, 11 white, 13 oolong, and 21 pu-erh samples were analyzed.

Among the amino acids, theanine (*N*-ethylglutamic acid) has attracted most interest for a long time as it is occurs (nearly) exclusively in tea and accounts for as much as 50% of the free amino acids in tea and also because of possible health benefits. It was detected by Japanese researchers as an unknown compound but the major one in the amino acid fraction of tea and was named theanine.[5] Later, it was isolated and finally identified to be *N*-ethylglutamic acid.[147] The biosynthesis of theanine occurs in the young rootstocks of tea from glutamic acid and ethylamine (the precursor is L-alanine) by the action of L-theanine synthase. The biosynthesis is shown in **Figure 11**.

Theanine naturally occurs in tea nearly exclusively as L-theanine.[148] The content of theanine in tea can vary widely. In the earlier literature, it was reported that there is a much higher amount of theanine in green compared to black tea.[5] More recently, it was stated that the amount of theanine was reduced during the so-called fermentation.[149] Regardless of whether or not this is the case, no systematic difference can be drawn from the data for green and black teas (see **Table 9**). As can be seen, there is a considerable difference within the

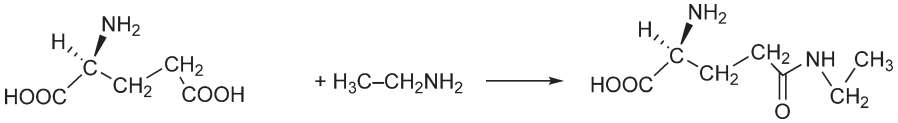


Figure 11 Theanine biosynthesis.

Table 9 Theanine content in tea

Tea variety	*Theanine (%)*	*Number of samples*	*Reference*
White tea	0.53–3.37	11	9
Green tea	0.160–0.337	21	9
Green tea	0.61–1.15	8	150
Black tea	0.049–0.412	28	9
Black tea	0.75–1.37	4	148
Green and black	0.28–0.53	9	151
Green and black	0.15–1.20	40	152
Green and black	0.87–1.81	4	131
Green, oolong, black	0.60–2.38	17	149

data, reflecting not only a variation in tea or the growing conditions (e.g., shading) but also a difference in analysis. It has been shown recently that L-theanine also serves as an enhancer of the umami taste of Japanese green tea together with theogallin and gallic acid, among others.[153]

3.23.3.13 Carotenoids

The occurrence of at least 16 carotenoids in tea with total amounts of the order of magnitude around 25–100 mg per 100 g is known.[154,155] The carotenoids play an important role as aroma precursors in black tea.[156] In earlier papers, up to 14 different carotenoids have been identified,[5] with neoxanthin, violaxanthin, lutein, and β-carotene being the most abundant ones.[155] Using HPLC with photodiode array detection in acetone extracts of different teas (intact fresh leaves, green teas, one black and one pu-erh tea), 6–19 chlorophylls and 5–13 carotenoids were identified. As a rule of thumb, it was stated that with the processing of the leaves the number of chlorophylls increased while the opposite was true for carotenoids.[157] In fresh tea leaves, lutein was determined in an ethanol extract from green tea leaves at a concentration of 0.015%.[158] The fate of the four major carotenoids was followed from fresh leaf through a 3 h fermentation process. There was a decrease during fermentation for neoxanthin (initially 51 mg kg^{-1}) to 46% of the original in the dried and fired tea, violaxanthin (initially 120 mg kg^{-1}) to 36%, lutein (initially 260 mg kg^{-1}) to 59%, and β-carotene (initially 102 mg kg^{-1}) to 60%.[156] As already mentioned, carotenes play an important role in black tea aroma formation. β-Carotene is converted to β-ionone and terpenoid-like aldehydes and ketones in the first oxidation step and dihydroactinidiole, 2,2,6-trimethylcyclohexanone, 5,6-epoxy ionone, and 2,2,6-trimethyl-6-hydroxycyclohexanone are the secondary oxidation products. It was proposed that the other carotenoids are converted in a similar manner.

3.23.3.14 Chlorophylls

Chlorophylls are present in tea in low concentrations. According to Hara *et al.*,[11] the amount of chlorophyll *a* and *b* is around 1.4 mg g^{-1} but this is dependent on climatic variations and the clone.[5] In freshly plucked leaves, the amount of chlorophyll *a* was 1.5–5.4 mg g^{-1} and that of chlorophyll *b* 0.7–2.1 mg g^{-1} depending on the clone analyzed.[159] In this study, it was shown that the degradation of chlorophylls into pheophytin and pheophorbide was higher in orthodox compared to CTC teas from the same source.[159]

3.23.3.15 Carbohydrates

Several carbohydrates along with phosphates have been identified in different parts of the tea plant. Pectin is also present in tea leaves. In tea extract solids, around 4% polysaccharides, 0.15% pectin, and 6.5% sugars (fructose, glucose, sucrose, *m*-inositol along with small amounts of maltose and raffinose) have been determined.[43] An overview of the earlier literature is available.[5,11]

Current research is more focused on tea polysaccharides and glycoproteins, as claims have been made that they might contribute to the health benefits of tea.[125] Immunostimulating activities, blood glucose-reducing and antioxidant effects, and antiadhesive effects (of an acidic polysaccharide fraction) were reported, among others.[125,160–162] After extraction and precipitation, the tea glycoprotein (TPS) was purified by gel chromatography.[160] The molecular mass was determined to be around 110 kDa (by both HPLC and gel chromatography) and the mol% was determined to be 6.49 (arabinose), 2.6 (xylose), 6.53 (fucose), 43.27 (glucose), and 41.11 (galactose). The amino acid composition was determined as well.[160] TPS was isolated using gel filtration and the elution studied by gel permeation chromatography.[162] The content of TGC (tea glycoprotein) was 0.608–1.274% in six different samples.[162] The compositional analysis of tea polysaccharide showed 3.5% protein, 44.2% neutral sugars, and 43.1% uronic acids.[161] In the same study, it was stated that this fraction had a very low toxicity making it safe for use in dietary supplements. Further research is necessary to give a full picture of the structures.

3.23.3.16 Lipids

According to Mahanta,[129] the total amount of lipids in tea is around 4%. During the manufacture of black tea, lipids change and their contribution to black tea aroma is worth noting. There is a loss of linolenic acid and to a smaller extent of linolic and palmitic acids.[163] In the same study, the behavior of more polar lipids, such as phosphatidylcholine, mono- and digalactosidediglyceride, phosphatidylethanolamine, sterol acyl monoglucoside, phosphatidylglycerol, cerebroside, and sterol monoglucoside, was studied. There was a decrease to 27–90% of the contents in fresh leaves except for sterol (acyl) monoglucoside, which increased a bit.[163] Several saponins are present in tea;[5] for example, brassinosteroids have been identified in very small amounts.[129] More recently, several saponins (theasaponins and assamsaponins, TR-saponins) were initially isolated from seeds and roots of the tea plant.[164–169] From tea flowers, bioactive floratheasaponins were isolated,[167] and from tea leaves the isolation and structural elucidation of isotheasaponins by NMR were reported.[168] **Figure 12** shows the structures of isotheasaponins.

3.23.3.17 Minerals

Tea is rich in manganese, fluoride, and aluminum compared to other food sources. **Table 10** gives some data for tea samples compiled from different papers. The metal content has also been employed to differentiate between teas from different geographic origins based on 46 samples. Using PCA, it was possible to discriminate teas from China, Japan, India, Sri Lanka, and Kenya.[170] Tea is known to be protective against dental caries. It has been stated that polyphenols at least in part are responsible for this property, for example, by inhibiting enzymes of *Streptococcus mutans* or *Porphyromonas gingivalis*.[173–175] Also, the fluoride content is known to be high in the tea plant. Fluoride content between 90 and 600 mg kg^{-1} (average 238 mg kg^{-1}), 40–334 mg kg^{-1}(average 118 mg kg^{-1}, $n = 35$) has been determined.[176–179] The concentration in beverages is usually between 1 and 2 mg l^{-1} when water without added fluoride is used.[178,179]

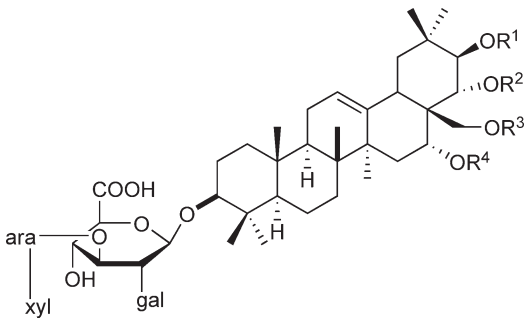


Figure 12 Basic structure of isotheasaponins from tea. On R^1–R^4 are acetyl and cinnamic acid moieties. ara = arabinose, xyl = xylose, gal = galactose.

Table 10 Selected elements in tea samples[146,170–172]

Element	*Content (mg kg^{-1})*	*Element*	*Content (mg kg^{-1})*
N	33 000–73 000	Cu	8–70
P	2000–6500	Al	200–2560
K	19 000–34 000	Zn	19–51
Ca	900–10 000	Ba	1–15
Mg	1100–4272	Na	35–1760
Fe	56–2037	Mn	148–1533

3.23.3.18 Volatiles/Flavor Compounds

More than 600 volatiles have been identified in tea samples till date. It is quite clear that the aroma of the so-called fermented tea has to be quite different from the aroma of green tea. There are some reviews available compiling the compounds identified.[129,180–183] Among the compounds identified, there are more than 70 hydrocarbons (aliphatic, aromatic, and terpenoid), around 90 alcohols (aliphatic, aromatic, and terpenoid), around 70 aldehydes (aliphatic, aromatic, and terpenoid) and ketones each, around 70 acidic compounds (aliphatic, aromatic, and terpenoid), more than 80 esters, 25 lactones, around 20 phenolics, around 40 oxygen-containing compounds (furanoids, aromatic, ionone-related), and around 90 nitrogen-containing compounds (pyrroles, pyridines, pyrazines, etc.) and sulfur-containing compounds.[129,180–183] The overall concentration of volatiles in black teas is around 100 mg kg^{-1}.[183] The methodology has changed over the years; static headspace,[183] dynamic headspace,[184] solvent extractions, distillation/extraction,[185,186] and solvent-assisted flavor evaporation (SAFE)[187] have been used, among others.

While earlier work focused on identification and some correlation related to quality, nowadays, approaches like aroma extract dilution analysis (AEDA) are more common. Initial studies using this approach led to the identification of 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone, (*E*)-β-damascenone, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, and linalool as the compounds with the highest flavor dilution (FD) factors.[188] This was confirmed in part by another study, but also other compounds with high FDs were identified, such as methyl salicylate, geraniol, and phenylacetaldehyde.[189]

One of the reasons for the different results could be that in one study a China sample and in the other study a Darjeeling sample were used. Important progress was made in another study also with a Darjeeling sample.[187] The results indicated that vanillin, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 2-phenylethanol, and (*E*,*E*,*Z*)-2,4,6-nonatrienal had the highest impact on tea aroma due to their high FD factors. Overall, 24 compounds had FD factors above 4. Also, a comparison was made between the relevant aroma compounds in tea infusion and tea leaves. It became obvious that some compounds (e.g., geraniol, among others) were much more important for the aroma of the infusion.[187]

In Sri Lankan Dimbula tea infusions, the sweet and/or juicy note was identified to be due to *cis*- and *trans*-4,5-epoxy-decenals.[190] It was suggested that both compounds are formed from linoleic acid and its hydroperoxide during withering and fermentation by enzyme action (lipoxygenase).[190]

Houjicha (roasted green tea) has a different aroma, as its formation is based on biosynthesis and also roasting. In a recent study, 2-ethyl-3,5-dimethylpyrazine and 2-ethyl-3,6-dimethylpyrazine were identified by AEDA as the most relevant odorants.[191] Synergistic effects were detected in a sensory study with jasmine tea infusion.[192] The addition of subthreshold concentrations of 4-hexanolide to model solutions and to jasmine tea infusion led to significant stronger aroma impressions.[192]

In a study on green tea aroma, it was confirmed that the degradation products of furan fatty acids 3-methyl-2,4-nonanedione and 3-hydroxy-3-methyl-2,4-nonanedione contribute to the flavor of green tea beverages, the latter compound by increasing a sweet creamy note. Similar compounds were recently identified as 1-methyl-2-oxopropyl hexanoate and 1-methyl-2-oxoheptyl acetate contributing to floral juicy notes while 2-butyl-4,5-dimethyl-3(2*H*)-furanone gives a sweet buttery sensation.[193]

Aroma changes in Longjing tea infusions caused by aging have recently been studied with the result that newly formed aroma compounds such as 2-butyl-2-octenal influence the perceived aroma and changes in aroma release are due to interactions with nonvolatiles.[194]

To summarize, currently, the key aroma compounds for some teas have been identified and by far not for all types of tea and origins.

3.23.3.19 Organic Acids

Among the organic acids detected in tea, oxalic acid is of importance as it might contribute to kidney stone formation.[195] Data from the literature show that tea contains 0.375–1.5 g per 100 g of oxalic acid.[20,195–198] The discussion on the relevance for kidney stone formation is ongoing; however, in human studies, it was shown that oxalic acid from tea did not dramatically elevate urine oxalic acid levels and also tea consumed with milk did

not at all.[196] Other studies recognized a protection against urinary stones by tea probably due to its antioxidative properties.[199,200]

Quinic acid was identified to be one of the major acids in green tea.[201] Other acids have also been determined by capillary electrophoresis (CE).[198] The range found in six teas (two green, two black, and two postfermented) was for oxalic acid 12.1–166.3 mg l^{-1} (corresponding to 0.07–1% in dry leaves), citric acid trace to 31.7 mg l^{-1} (corresponding trace to 0.19%), malic acid trace to 40.9 mg l^{-1} (trace to 0.25%), quinic acid 13.5–316.4 mg l^{-1} (0.08–1.90%), and aspartic acid and glutamic acid trace to 64.5 mg l^{-1} each (trace to 0.25%). The concentrations of these acids were quite low in the postfermented samples. The data for dry leaves are not given in the paper, and were calculated using extraction conditions based on the assumption of an exhaustive extraction.

In one of the postfermented samples, a huge peak of lactic acid was found (no data given). The determination of anions by HPLC (ion exchange) in three types of green tea gave the following figures: acetic acid 0.58–1.06%, ascorbic acid 0.95–2.23%, succinic acid 4.9–7.8%, malic acid 0.36–0.55%, and citric acid 0.74–0.85%.[202] The data for succinic acid appear to be very high.

3.23.3.20 Vitamins

Tea is not a relevant source of vitamins; however, some data on the vitamin content can be found in the literature. Ascorbic acid content in green teas is 60–250 mg per 100 g;[6] according to other sources it is n.d.–145 (25 samples of different origins, average 32.7 mg per 100 g).[203] Based on a recommended dietary allowance (RDA) of 100 mg day^{-1}, 1 l of tea will contribute a maximum of 25% based on the assumption of an exhaustive extraction and complete stability. Other water-soluble vitamins have also been detected but their contribution to the RDA is even less, except folic acid.[146] In the lipid fraction, relevant amounts of fat-soluble vitamins have been found; however, the extraction into the beverage is not quite clear.[146]

3.23.3.21 Acrylamide

Acrylamide is a thermogenic compound in thermal-treated foods and has been classified by the International Agency for Research on Cancer (IARC) as probably carcinogenic to humans. Acrylamide has been detected in roasted tea.[191,204] The precursors for acrylamide formation are asparagine and suitable carbonyl compounds such as reducing sugars. Levels of acrylamide in roasted green tea are between 250 and 1880 μg kg^{-1}.[191] The roasting temperature strongly affects both the sensory properties and the acrylamide content. Moreover, the degradation of catechins is also temperature dependent. In a comparison between two roasting temperatures (160 and 180 °C) at different times, it was shown that a roasting temperature of 160 °C is superior as the acrylamide levels were lower (2 compared to 4 μg l^{-1} in tea infusions), the typical aroma compounds are present at adequate concentrations, and the degradation of catechin is lower compared to that at 180 °C.[191]

3.23.4 Extraction and Storage of Tea

There is a small review from 1982 on the storage of black tea.[205] The loss of quality and character was ascribed to a loss of TFs, which is driven by moisture. The extraction of compounds from the leaves into the infusion is dependent on various factors such as particle size, water-to-tea ratio, temperature, and brewing time. In a brewing study with (black) teabags employing short brewing times (25 s up to 2 min), data for total soluble solids, total phenolics, catechins, alkaloids, phenolic acids, TFs, flavonol, and flavone glycosides were determined.[111]

It became evident that the extraction efficiency of catechins and TFs was below 50% in a 2 min brew, while FOG were much better extracted (up to 70%) as was caffeine (55–90%).[111] For comparison purposes, an extraction using 70% aqueous methanol was employed, which was regarded as an exhaustive extraction. The brew solids consisted of 25–31% phenolics, 22–27% flavonoid derivatives, 15–19% TR,

1.2–2.8% TFs, 0.9–6.5% catechins, 1.6–2.2% FOG, 0.21–0.46% FCG, and 8.6–11.2% caffeine. In this study, total flavonoids were calculated using total phenolics (Folin, calculated as gallic acid equivalents) minus sum of the contents of gallic acid, theogallin, and chlorogenic acids. TR was determined by using the total flavonoid content minus the sum of the contents of flavonoids detected (catechins, TF, FOG, FCG).[111]

Surprisingly, one of the findings was that the caffeine/total phenolics ratio was relatively constant over the brewing time, which contradicts the statement that caffeine is released from the leaves immediately while phenolics are extracted later. These results are in principle the same as those of another UK brewing study.[206]

More recently, different steeping methods were used with teabags filled with 3 g ground black, green, oolong, paochong, and pu-erh teas and 150 ml of water at different temperatures for 0.5–4 min.[208] The steeping was repeated eight times using the same bag to mimic the Chinese procedure for tea ceremonies. It was shown that at 70 °C the second infusion contained the highest concentration of caffeine, catechins, and gallic acid while at 85 and 100 °C the first infusions contained the highest concentration. The storage of the brews at 25 °C for 36 h led to a decrease in catechins while gallic acid increased and caffeine remained unchanged. Storage at 4 °C did not give rise to significant changes.[207] In earlier studies, it was stated that storage at 70 or 80 °C for a few hours also led to a decrease in catechins.[41,208] The degradation of catechin in tea drinks was studied by Chen *et al.*[42]

Brewing experiments for theanine showed that after 3 min more than 80% of the total theanine was transferred to the brew.[152] Storage experiments were carried out with dry formulations containing instant green tea, ascorbic acid, sucrose, and citric acid and with instant green tea alone at relative humidities of 0–85% over a period of 3 months. The stability of the catechins was monitored by HPLC. For relative humidity (rh) <43%, the catechins were stable in all samples; however, caking was observed. At higher humidities, degradation occurred, which increased due to the presence of citric acid at rh <58% and ascorbic acid at rh <75%.[209] Storage experiments with tea drinks (canned and bottled) have been performed.[42,210] It was shown that an epimerization occurs due to thermal treatment, which might be affected by the presence of other ingredients.

Tea cream is a precipitate formed when a strong black tea brew cools down. It can remove up to 30% of the total soluble solids. The phenomenon was first published by Roberts.[211] Creaming does not necessarily occur with all strong tea brews and the interaction does not take place in the presence of organic solvents. Tea cream is a problem in the production of instant black teas when the brews are concentrated. The cream consists in principle of TR, TF, and caffeine; however, other flavonoids, nitrogen-containing compounds, and minerals are also present in small amounts.[212,213]

A more recent study on the mechanism of cream formation using X-ray scattering came to the conclusion that TFs are important for the initiation of creaming when small clusters (3 nm diameter) are formed. Caffeine later fills the gaps and is an important constituent of the cream but it is unnecessary for the initiation of creaming.[214] It was also found that calcium plays an important role by neutralizing the charge of the associates. Tea creaming can be reduced by changing the solubility of polyphenols (glucosylation or degallation) or by removing calcium.[214]

The creaming in semifermented teas was also studied. It was found that the leaf/water ratio affected the amount, not the composition of cream. The most important constituents were catechins (30%), caffeine (20%), and protein (16%) but FOG were also involved.[215]

Tea scum is an interaction that sometimes occurs in tea brews. The phenomenon leads to the formation of an oily film. There are only a few papers in the literature on tea scum; however, it is quite clear that calcium and additionally carbonate are involved in scum formation.[216] It can be avoided by using more soft water or, alternatively, by adding citric acid to the tea. This problem is less on the tea side and more on the water side. The scum formation is about the same in decaffeinated and normal black teas.[217] The formation is inhibited by a flow of nitrogen over the surface of the brew while a flow of oxygen enhances it, showing that an oxidation process is involved.[216]

Sediments in green tea beverages are different and probably caused by the conversion of strictinin to ellagic acid and a subsequent formation of precipitates with proteins (see Section 3.23.3.8).

3.23.5 Products

3.23.5.1 Decaffeinated Teas

Tea is decaffeinated by various methods. Briefly, the tea is prewetted and extracted by some organic solvent such as dichloromethane or ethyl acetate.[15] Alternatively, an extraction using supercritical carbon dioxide can be used. The decaffeination technology affects the constituents drastically in case of ethyl acetate because besides caffeine most of the catechins and catechin-related compounds are also removed.[23] For most of the black teas, it is not as important because the catechin levels are low anyway, but in case of green tea the characteristic compounds are removed. A number of patents have been awarded for decaffeination using carbon dioxide.

The influence of CO_2 decaffeination on green tea volatiles has been studied. As expected, some of the nonpolar aroma compounds like hexanal and 2-(*E*)-hexenal were removed or decreased.[217] The use of supercritical CO_2 in combination with a cosolvent (water) was studied to get a more effective retention of catechins while caffeine should be effectively removed.[218] It was suggested to use a partially decaffeinated tea for the production of decaffeinated instant teas. To get the low caffeine figures necessary for the final product, it was necessary to treat the brews with active carbon to remove excess caffeine; however, catechins are also removed in part.[219]

3.23.5.2 Instant Teas and Ready-to-Drink Beverages

Most kinds of teas are used for producing instant powders, which are commonly used for the production of rtd beverages. In the case of black teas, cream formation is one issue that has to be addressed by the production process. Extraction is in most cases performed using water and countercurrent procedures.[220] Depending on the usage of the instant tea, there are a number of treatments, the details of which are often not accessible. Cream processing can be accomplished by maintaining the temperature above 65 °C or by removal of the cream, for example, by cooling and centrifugation. Doing this, the yield is decreased and the overall aroma sensation changes. Cream treatment is especially necessary for cold water-soluble instants, which are commonly used for beverages like ice tea. There is not much information available on technology details; however, one of the procedures employed is the treatment with sodium hydroxide and afterward neutralization using organic acids, for example, citric acid. Another approach to avoid haze formation is the use of tannase, which hydrolyzes the phenolic compounds, which as a result get smaller and more water soluble.[221]

The use of tea in ice tea is often assessed by using the caffeine content but caffeine could be added for adulteration purposes. Chemical treatments like alkali hydroxide give rise to changes in the flavonoid pattern; however, FOG are still detectable in most samples. However, there are treatments that also destroy FOG. To check whether or not tea was used in ice tea beverages and to get an idea how much tea was used, an assessment based on the content of FCG was done with 21 beverages.[57,77] The most traditional rtd product is lemon ice tea, which basically consists of brewed or (mostly) instant tea, citric and ascorbic acids, sucrose/glucose and/or artificial sweetener, and aroma.[220]

3.23.6 Potential Health Effects of Tea, Its Flavonoids, and Theanine

The number of papers published in the past 10 years on the potential health effects of tea is hard to count. Initially, there were papers from Asia focusing on green tea, which in the 1990s had only a small proportion of the tea market outside Asia. Later, black tea was also studied for potential health effects. The phenolic compounds, especially the flavonoids, are responsible for most of the health effects Since long, the health effects of theanine have been discussed and more recent studies have also reestablished their health effects. It is well established that tea flavonoids exhibit a strong antioxidant activity in the established *in vitro* test systems, such as TEAC and oxygen radical absorbance capacity (ORAC), among others.[222,223]

3.23.6.1 Bioavailability and Metabolism

Flavonoids do have a number of positive health effects in *in vitro* studies. The question is whether or not they are bioavailable *in vivo* and most importantly in humans. A number of reviews are available on the bioavailability of flavonoids in general and from tea and the potential health effects of flavonoids in foods and especially as constituents of teas.[224–228] It has been estimated that roughly 5% of the phenolic compounds are absorbed in the duodenum and only 5% of these reach the plasma unchanged.[227] A review on bioavailability concludes that gallic acid and isoflavones are the best-absorbed phenolics, followed by catechins, flavanones, and quercetin glycosides. At the lower end are the galloylated catechins, proanthocyanidins, and anthocyanins.[226] Another review on the bioavailability and bioefficacy of several subgroups of polyphenols based on intervention studies with humans concluded that there is an effect on biomarkers for plasma antioxidants and energy metabolism; however, these results are more limited compared to *in vitro* studies.[224]

The plasma levels of catechins were different, with EGC having the shortest rise and elimination time (half-time of elimination 1.7 h), while ECG had the slowest elimination (half-time 6.9 h) and EGCG had the slowest rise time but an intermediate half-time of elimination (3.9 h).[229] It has been shown that catechins are bioavailable; however, only 1.68% of a high dose of catechins were found in plasma, urine, and feces.[230]

It is quite clear from the literature that the dietary flavonoids are not well absorbed from the small intestine but are transformed by the gut microflora to be absorbed in the colon. These metabolites are hydroxyphenyl-γ-valerolatones and phenolic acids.[231] For catechin, it was proposed that 3′-methoxy-catechin is formed as well as the colon metabolites 3,4-dihydroxyvalerolactone, 3-hydroxyvalerolactone, and phenylpropionic acid, which are transformed into 3-methoxy-4-hydroxyphenylvalerolactone and hippuric acid. 3′-Methoxy-catechin, 3-methoxy-4-hydroxyphenylvalerolactone, 3-hydroxyphenylvalerolactone, and hippuric acid are excreted in the urine.[231] It was deduced from *in vitro* and *in vivo* studies that EGCG might be degallated by the action of an esterase followed by the formation of 4′-methyl-EGC or by glucuronidation. Other metabolites are 4′,4″-dimethyl EGCG, and at high levels (animal study) EGCG-2″-cysteine and EGCG-2′-cysteine.[232] Also, EGCG and EGC sulfates are formed by sulfotransferase.[232] **Figure 13** shows the structures of some metabolites.

Another approach used to obtain information on bioavailability and biotransformation was NMR spectroscopy of human urine.[233] It was confirmed that hippuric acid was the major metabolite formed after ingestion of black tea and as a new metabolite 1,3-dihydroxyphenyl-2-*O*-sulfate was identified by HPLC–MS–^{1}H-NMR.[233] The same group used a high-resolution ^{1}H-NMR-based metabonomics approach to determine metabolic profiles in urine and plasma after green and black tea consumption in a human randomized crossover study with 17 participants.[234] The excretion of hippuric acid and 1,3-dihydroxyphenyl-2-*O*-sulfate was found to be about the same, while after green tea consumption, some citric acid intermediates were higher, suggesting an

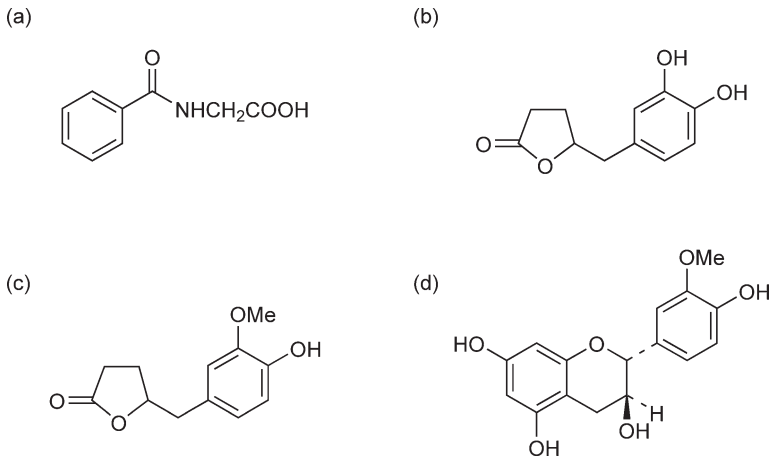


Figure 13 Selected metabolites of tea catechins. (a) Hippuric acid; (b) 3,4-dihydroxyphenylvalerolactone; (c) 3-methoxy-4-hydroxyphenylvalerolactone; (d) 3′-methoxy-catechin.

influence on energy metabolism. Moreover, several unidentified metabolites were found after green tea consumption. Recently, the human urine metabolite profile of tea catechins was determined by LC–MS. More than 20 metabolites have been identified including EGC-4′ and 7-glucuronide, EGC sulfates, methylated EGC glucuronides and sulfates, and sulfated and glucuronidated valerolactones.[235] The effects of green tea catechins in *in vivo* animal models have led to the conclusion that green tea catechins might have protective effects against various types of degenerative diseases.[236]

3.23.6.2 Cardiovascular System/Heart Health

The current status and research needs are summarized from an International Life Sciences Institute (ILSI) workshop on flavonoids and heart health.[237] The molecular targets for tea polyphenols and protective effects on the cardiovascular system have been reviewed recently. It was hypothesized that tea catechins and possibly other constituents act either as inhibitors or activators of kinases depending on whether the cell is activated or resting.[238] The galloyl group of the polyphenols in question seems to be very important.

3.23.6.3 Anticancer Properties of Tea

Recently, a Japanese prospective study concluded that green tea may be associated with a decreased risk of advanced prostate cancer.[239] A review on green and black teas and the risk of colorectal cancer from 2006 came to the conclusion that there is strong evidence for the protective effect of both green and black teas against colorectal cancer from *in vitro* and nonhuman *in vivo* studies.[240]

The molecular mechanism and human relevance of tea and cancer prevention were reviewed and the activity of tea constituents against various types of cancer, including skin, lung, stomach, liver, and pancreas cancer, was established in numerous animal studies; however, the mechanism of action is not fully understood and may vary in different experimental systems.[241] The authors also state data from human studies are currently inconclusive.[241] EGCG acts as an inhibitor of dihydrofolate reductase (a key enzyme in the folate metabolism) at concentrations found in a normal green tea consumer. This might be the mechanism for the observed activity of green teas against some types of cancer.[242,243] The question was brought up whether or not EGCG could also be responsible for the negative effects due to folate depletion in some individuals.[244]

Green tea catechins, especially EGCG, have been reviewed with respect to their molecular targets.[245] The targets are cell cycle and apoptotic proteins, transcription factors, protein kinases growth factors. It was concluded that the results from laboratory studies are most promising and if the effects can be confirmed by human studies it might help in future cancer therapy. The limiting factors are the relatively low bioavailability and the autooxidation of EGCG.[245]

3.23.6.4 Antimicrobial Activities of Tea

The antibacterial, antitoxic, antiviral, and antifungal activities of tea flavonoids have been extensively reviewed in 2007.[246] The bottom line is that there are a number of research needs, for example, whether or not the *in vitro* activities are also valid *in vivo* (in humans), determination of additive/synergistic effects, and checking the effect against antibiotic-resistant (foodborne) pathogens, among others.[247]

3.23.6.5 Tea with or without Milk

The never-ending battle came up again in 2007. There are papers in the literature stating that milk does not change the bioavailability and the protective effects of tea constituents. It was shown in a human study that milk changes neither the absorption of total phenolics and catechins nor the increase in plasma antioxidant activity.[247] The results of other studies were the same in principle.[248–251] A laboratory study came to the conclusion that there are losses for catechin (green tea) and TFs (black tea) but the overall antimutagenic properties remained unchanged.[252]

Another human study came to the conclusion that milk counteracts the positive effects of tea on the vascular system.[253] In response, there were a few comments disagreeing with the number of individuals involved, the study design, and the relevance of the findings, among others.[254–256]

3.23.6.6 Miscellaneous

The photoprotective effects of green tea polyphenols have been reviewed and it was concluded that they have protective effects against damages caused by both UVA and UVB and, consequently, have the potential to be used together with normal sunscreens.[257] It was shown that the mechanism of traditional sunscreens and that of tea phenolics are different.[257] With regard to antiobesity and antidiabetic effects, the results from a number of studies are promising; however, there is a demand for further human studies.[258–260]

To summarize, despite the fact that a lot of progress has been made during the past 10 years, still there is a necessity for further studies with regard to the beneficial effects of polyphenols in humans and their mechanism of action.

3.23.6.7 Theanine

The physiological effects of theanine have regained interest in the past few years. It was concluded from a double-blind randomized human study that theanine counteracts the effects of caffeine in part and reduces raised blood sugar level.[261] A more recent review on theanine and caffeine suggests that theanine supports caffeine action (e.g., enhances mental performance) and also modulates the effects of higher doses of caffeine.[262] It was suggested from a human study that theanine and caffeine in combination do have a different pharmacological effect compared to caffeine alone.[263] In another human study, electroencephalogram (EEG) was recorded after ingestion of decaffeinated green tea enriched with theogallin and theanine. It was concluded that there still was a stimulating effect due to L-theanine and theogallin.[264] It was concluded from an animal study (rats) that theanine might improve learning and memory.[265] Also, in rats, there was a modification (inhibition) of glutamine transport across plasma membranes.[266]

3.23.7 Analytical Section

This section focuses on the determination of phenolic species and theanine.

3.23.7.1 Determination of (Poly)Phenols and Flavonoids

The extraction of (poly)phenols and flavonoids is a crucial step. To gain compositional data, it is essential to use conditions that ensure an exhaustive extraction of the compounds (or at least reproducible recoveries) to be analyzed and the stability of the compounds during the analytical procedure. For brewing studies, exhaustive extraction is not important but ensuring the reproducibility of the extraction and the stability of the compounds is crucial.

The determination of total phenolics according to ISO 14502-1 is as follows: the extraction is carried out using 70% methanol (aqueous) at 70 °C (0.2 g of ground tea, 5 ml extraction solvent, 10 min extraction, centrifugation, repeat extraction with another 5 ml of solvent).[30] The extract is diluted and a Folin–Ciocalteu assay is conducted.[267,268] The calibration is done with gallic acid monohydrate, and consequently the results are expressed as gallic acid equivalents.[30]

Numerous methods have been used for the determination of total phenolics, for example, one similar to the ISO standard, the Folin–Denis method, the Loewenthal method based on oxidation by $KMnO_4$ with and without adsorption of the phenolics by hide powder or gelatin, a methodology using copper precipitation, and a gravimetric method using adsorption to hide powder.[269–271] A more recent approach was the use of FT–NIR for the determination of total phenolics in green tea with the ISO method as reference. The results were quite good when using a proper algorithm.[272]

The chromatography of tea constituents has been reviewed frequently. A review from 1992 compiles especially the HPLC methods for the measurement of catechins, TFs, and FOG.[273] In 2000, a review on

HPLC measurements of flavonoids from plants compiled the literature not only for tea but also for numerous other foods.[274] There are two extensive reviews on the determination of tea polyphenols and more specifically tea catechins, covering the literature up to 1999 and 2000, respectively, including HPLC, CE, GC, TLC, and hyphenated methods.[275,276] One of the most important problems for some of the analytes is the lack of available, affordable, and sufficient pure calibration standards. There is one international standard method for the determination of catechins in tea.[31] The same extraction procedure as described for total phenolics is followed. After that the sample is diluted fivefold with a stabilization solution (10% acetonitrile with 500 $\mu g\,ml^{-1}$ of EDTA and ascorbic acid) and then separated by HPLC on a Phenyl-Hexyl column using a gradient. There are two concepts described for the quantification. One relies on a conventional concept using authentic standards, the other is a relative response factor concept based on a calibration against caffeine and a calculation with conversion factors for the individual catechins set up with very pure (water content determined) catechin in an international ring test. The latter concept enables laboratories worldwide to carry out this determination independent of expensive, unstable, and sometimes not really pure catechin standards. The method is currently set up for the determination of gallic acid, caffeine, catechin, EC, EGC, EGCG, and ECG; however, it is also possible to separate theogallin, theobromine, gallocatechin, GCG, and CG. Moreover, using multiple wavelength detection, it is also possible to determine FOG, some hydroxycinnamic acids, and the major TFs.

Currently, it can be stated that HPLC–DAD using different types of RP-18 columns with gradients consisting of acidified or buffered water and acetonitrile, sometimes methanol, is most frequently used, nowadays often in combination with an additional mass spectrometric detection. For extraction, the use of aqueous alcohols, similar to the ISO procedure, is the method of choice. A recent study compared several extraction techniques for tea polyphenols and catechins using aqueous alcohols and it was concluded that microwave-assisted extraction (MAE) gave the highest yields.[277]

The instrumentation has changed in the past few years, so there is a tendency of using HPLC–DAD often hyphenated to ESI-MS for the detection of as much tea constituents as ever possible, including catechins, FOG, TFs, flavone glycosides, proanthocyanidins, and phenolic acids.[34,131,277] These methods often do not employ any clean-up procedures. Possible clean-up procedures include polyamide column chromatography, which has been successfully employed for the isolation of FOG and FCG prior to the HPLC determination.[44,278] For the determination of catechins, solid-phase extraction (SPE) is sometimes used. The clean-up of TF can be achieved by extraction using ethyl acetate or by column chromatography on Sephadex. Another approach is the use of a rotary perforator.[23,33] SPE has also been used for the clean-up of TFs.[99]

A simultaneous quantitative determination of nine catechins (including the epimers GCG, GC, and CG), gallic acid, alkaloids, and strictinin in tea and bottled tea drink by HPLC–DAD using catechol as internal standard was published.[126]

Recently, analytical HSCCC was used for the separation of polyphenols, including catechins, TFs, phenolic acids, and FOG.[279] NIR spectroscopy has also been used for the determination of alkaloids and major catechins in green tea leaves with HPLC as a reference method.[280] HSCCC was used for the isolation of pure flavonoids from tea, for example, catechins, FOG, proanthocyanidins, strictinin, or TFs as calibration standards.[119,281–284]

The analysis of biological fluids is a special task due to the often low concentration in plasma and urine and also because conjugates have built up. The HPLC systems might be similar to those employed for the determination in tea; however, it is necessary to use MS/MS detection systems or CoulArray detectors. The clean-up often includes an (enzyme) hydrolysis and SPE on different types of columns.[285–288]

3.23.7.2 Determination of L-Theanine

The determination of amino acids requires a derivatization, for example using *o*-phthaldialdehyde (OPA) or other reagents like phenylisothiocyanate (PITC), fluorenylmethoxycarbonylchloride (FMOC), dansylchloride (5-(dimethylamino)-1-naphthalinsulfonylchloride), and 6-amino-quinolyl-*N*-hydroxysuccinimidylcarbamate (AQC) prior to HPLC separation.[9,149,289] Based on the literature, which states that normally 98% of the theanine in tea is L-theanine, a simple method was adopted by an ISO working group. The method relies on an aqueous extraction (10 min, hot water). The sample is either directly injected or after passage through a polyamide column (to get rid of potentially interfering phenolics) to an RP-18 column with water as an eluent

and a detection wavelength of 210 nm. Theanine elutes after around 6 min and is quite well separated from other polar tea constituents.[152]

3.23.7.3 Authenticity and Quality Aspects

Several studies have been conducted to detect the geographic origin of tea samples or the type of tea. The use of minerals, FOG, and amino acids has already been mentioned. Other approaches are the use of ^{1}H-NMR and multivariate analysis with nearly 200 tea samples to get a differentiation between white, green, oolong, and black teas and also between Chinese and Japanese teas.[290] Catechins and alkaloids were successfully used to differentiate between fermented and nonfermented samples and also between black teas from six geographic origins based on 45 samples from the retail.[137] Recently, GC–(TOF)–MS has been used in a metabolomics approach to predict the quality of Japanese green teas as determined by professional tea tasters. The results were based on acids, amino acids, and sugars, among others. The results were quite promising; however, the authors state that there are still problems to overcome.[291] The same authors published a different approach for the same purpose more recently using FT–NIR making use of the 5500–5200 cm^{-1} spectral region.[292]

Abbreviations

aq.	aqueous
bt	black tea
C	(+)-catechin
CE	capillary electrophoresis
CG	catechin gallate
CouQA	*p*-coumaroylquinic acid
CQA	caffeoylquinic acid
d.m.	dry matter
DAD	photo diode-array detector
EA	epiafzelechin
EAG	epiafzelechin gallate
EC	epicatechin
ECG	epicatechin gallate
EGC	epigallocatechin
EGCG	epigallocatechin gallate
ESI	electrospray ionisation
FCG	flavone C-glycoside
FL	fresh leaf
FOG	flavonol O-glycoside
GCG	gallocatechin gallate
gt	green tea
HHDP	hexahydroxydiphenoyl
HSCCC	high speed countercurrent chromatography
i.d.	internal diameter
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
K-	kaempferol-
M-	myricetin-
n.d.	not detected
NIR	near-infrared reflectance spectroscopy
OT	oolong tea
PA	polyamide
PPO	polyphenoloxidase
Q-	quercetin-

RDA	recommended dietary allowances
RP	reversed-phase
rtd	ready to drink
SPE	solid phase extraction
TF	theaflavin
TOF	time of flight
TR	thearubigin

Nomenclature

gal	galactose
glu	glucose
rha	rhamnose
rut	rutinose

References

1. Food and Agricultural Organization of the United Nations. http://faostat.fao.org/ (accessed March 15, 2009).
2. K. C. Willson; M. N. Clifford, Eds., *Tea: Cultivation to Consumption*; Chapman & Hall: London, 1992.
3. Y. Hara; S. J. Luo; R. L. Wickremasinghe; T. Yamanishi, *Food Rev. Int.* **1995**, *11*, 457–471.
4. Y. Hara; S. J. Luo; R. L. Wickremasinghe; T. Yamanishi, *Food Rev. Int.* **1995**, *11*, 371–374.
5. R. L. Wickremasinghe, Tea. In *Advances in Food Research*; C. O. Chichester, E. M. Mrak, G. F. Stewart, Eds.; Academic Press, New York, 1978, Vol. 24, pp 229–286.
6. C.-D. Chu; L.-R. Juneja, General Chemical Composition of Green Tea and its Infusion. In *Chemistry and Applications of Green Tea*; T. Yamamoto, L. R. Juneja, D.-J. Dhu, M. Kim, Eds.; CRC Press: Boca Raton, 1997; pp 13–22.
7. M. Kawakami; A. Kobayashi, Carotenoid-Derived Aroma Compounds in Tea. In *Carotenoid-Derived Aroma Compounds;* P. Winterhalter, R. Rouseff, Eds.; ACS Symposium Series 802; American Chemical Society: Washington, DC, 2002; pp 145–159.
8. T. Hashimoto; M. Goto; H. Sakakibara; N. Oi; M. Okamoto; K. Kanazawa, *Phytother. Res.* **2007**, *21*, 668–670.
9. A. Alcázar; O. Ballesteros; J. M. Jurado; F. Pablos; M. J. Martin; J. L. Vilches; A. Navalón, *J. Agric. Food Chem.* **2007**, *55* (15), 5960–5965.
10. A. K. Sharma, Why Tea. In *Medicinal Properties of Tea*; B. Banerjee, T. C. Chaudhuri, Eds.; Oxford & IBH Publishing Co. Pvt. Ltd: New Delhi, 2005; p XV.
11. Y. Hara; S. J. Luo; R. L. Wickremasinghe; T. Yamanishi, *Food Rev. Int.* **1995**, *11*, 409–434.
12. E. Haslam, *Phytochemistry* **2003**, *64*, 61–73.
13. A. Robertson, The Chemistry and Biochemistry of Black Tea Production – The Non-Volatiles. In *Tea: Cultivation to Consumption*; K. C. Willson, M. N. Clifford, Eds.; Chapman & Hall: London, 1992; pp 555–601.
14. M. G. Hampton, Production of Black Tea. In *Tea: Cultivation to Consumption*; K. C. Willson, M. N. Clifford, Eds.; Chapman & Hall: London, 1992; pp 459–511.
15. M. Gill, Speciality and Herbal Teas. In *Tea: Cultivation to Consumption*; K. C. Willson, M. N. Clifford, Eds.; Chapman & Hall: London, 1992; pp 513–534.
16. Y. Hilal; U. H. Engelhardt, *J. Verbr. Lebensm.* **2007**, *2*, 414–421.
17. International Organization für Standardization (ISO) 3720. *Black Tea – Definition and Basic Requirements;* Corrigenda: ISO 3720:1986/Corr.1: 1992, ISO 3720:1986/Corr.2:2004, 1986.
18. ISO TC 34/SC8. *Resolutions Taken During the 22nd Meeting of ISO/TC 34/SC 8;* Hangzhou, China, 31st March – 2nd April 2008 (document N609).
19. W. Heller; G. Forkmann, Biosynthesis of Flavonoids. In *The Flavonoids: Advances in Research since 1986*; J. B. Harborne, Ed.; Chapman & Hall: London, 1994; pp 499–535.
20. M. A. Bokuchava; N. I. Skobeleva, The Chemistry and Biochemistry of Tea and Tea Manufacture. In *Advances in Food Research*; C. O. Chichester, E. M. Mrak, G. F. Stewart, Eds.; Academic Press: New York, 1969, Vol. 17, pp 215–292.
21. P. A. Punyasiri; I. S. Abeysinghe; V. Kumar; D. Treutter; D. Duy; C. Gosch; S. Martens; G. Forkmann; T. C. Fischer, *Arch. Biochem. Biophys.* **2004**, *431* (1), 22–30.
22. M. N. Clifford, *Crit. Rev. Food Sci. Nutr.* **2001**, *41* (5), 393–397.
23. U. H. Engelhardt; C. Lakenbrink; S. LapczynskiAntioxidative Phenolic Compounds in Green/Black Tea and Other Methylxanthine Containing Beverages. In *Caffeinated Beverages*; T. H. Parliment, C.-T. Ho, P. Schieberle, Eds.; ACS Symposium Series 754; American Chemical Society: Washington, DC, 1999; pp 111–118.
24. D. A. Balentine; S. A. Wiseman; L. C. M. Bouwens, *Crit. Rev. Food Sci. Nutr.* **1997**, *37*, 693–704.
25. F.-L. Chiu; J.-K. Lin, *J. Agric. Food Chem.* **2005**, *53*, 7035–7042.

26. R. Saijo, *Agric. Biol. Chem.* **1982**, *46*, 1969–1970.
27. F. Hashimoto; G. Nonaka; I. Nishioka, *Chem. Pharm. Bull.* **1987**, *35*, 611–616.
28. G. Nonaka; O. Kawahara; I. Nishioka, *Chem. Pharm. Bull.* **1983**, *31*, 3906–3914.
29. F. Hashimoto; G. Nonaka; I. Nishioka, *Chem. Pharm. Bull.* **1989**, *37*, 77–85.
30. International Organization für Standardization (ISO) 14502-1. *Determination of Substances Characteristic of Green and Black Tea – Part 1: Content of Total Polyphenols in Tea – Colorimetric Method Using Folin-Ciocalteu Reagent;* Corrigenda: ISO 14502-1:2005/Corr.1:2006, 2005.
31. International Organization für Standardization (ISO) 14502-2. *Determination of Substances Characteristic of Green and Black Tea – Part 2: Content of Catechins in Green Tea – Method Using High-performance Liquid Chromatography;* Corrigenda: ISO 14502-2:2005/Corr.1:2006, 2005.
32. J. Obuchowicz; K. Donelly; U. H. Engelhardt, Flavanols and total phenolics in green and black origin teas. In preparation.
33. S. Lapczynski, Untersuchungen über Theaflavine und Flavanole in grünen und schwarzen Tees. Thesis, TU Braunschweig, 2000 (in German).
34. H. Sakakibara; Y. Honda; S. Nakagawa; H. Ashida; K. Kanazawa, *J. Agric. Food Chem.* **2003**, *51* (3), 571–581.
35. M. Friedman; S.-Y. Kim; S.-J. Lee; G.-P. Han; J.-S. Han; K.-R. Lee; N. Kozukue, *J. Food Sci.* **2005**, *70* (9), C550–C559.
36. Y.-S. Lin; Y.-J. Tsai; J.-S. Tsay; J.-K. Lin, *J. Agric. Food Chem.* **2003**, *51*, 1864–1873.
37. M. Saravanan; K. M. Maria John; R. Raj Kumar; P. K. Pius; R. Sasikumar, *Phytochemistry* **2005**, *66* (5), 561–565.
38. USDA Database for the Flavonoid Content of Selected Foods, 2003. http://www.nal.usda.gov (accessed March 17, 2009).
39. USDA Database for the Flavonoid Content of Selected Foods, Release 2.1, 2007. http://www.nal.usda.gov (accessed March 17, 2009).
40. S. Kuhr; U. H. Engelhardt, *Z. Lebensm. Unters. Forsch.* **1991**, *192*, 526–529.
41. S. Kuhr, Flavanole, Flavon-C-glykoside und höhermolekulare Polyphenole im Tee. Thesis, TU Braunschweig, 1993 (in German).
42. Z.-Y. Chen; Q. Y. Zhu; D. Tsang; Y. Huang, *J. Agric. Food Chem.* **2001**, *49* (1), 477–482.
43. G. W. Sanderson; A. S. Ranadive; L. S. Eisenberg; F. J. Farrell; R. Simons; C. H. Manley; P. Coggon, Contribution of Polyphenolic Compounds to the Taste of Tea. In *Phenolic, Sulfur, and Nitrogen Compounds in Food Flavors;* G. Charalambous, I. Katz, Eds.; ACS Symposium Series 26; American Chemical Society: Washington, DC, 1976; pp 14–46.
44. U. H. Engelhardt; A. Finger; B. Herzig; S. Kuhr, *Dtsch. Lebensmitt. Rundsch.* **1992**, *88*, 69–73.
45. E. A. H. Roberts; R. A. Cartwright; D. J. Wood, *J. Sci. Food Agric.* **1956**, *7*, 637–646.
46. E. A. H. Roberts; W. Wight; D. J. Wood, *New Phytol.* **1958**, *57*, 211–225.
47. M. A. Bokuchava; M. Ul`yanova, *Fenol`nye Soedin. Ikh Biol. Funkts., Mater Vses. Simp.* **1966**, *1*, 224–228.
48. K. G. Mikaberidze; I. I. Moniava, *Chem. Nat. Compounds (USSR)* **1971**, *7*, 813.
49. I. D. Chkhikvishvili; V. A. Kurkin; M. N. Zaprometov, *Khim. Prir. Soedin.* **1984**, *5*, 661–662.
50. I. D. Chkhikvishvili; V. A. Kurkin; M. N. Zaprometov, *Prikl. Biokhim. Mikrobiol.* **1986**, *22* (3), 410–422.
51. I. D. Chkhikvishvili; M. N. Zaprometov, *Subtrop. Kult.* **1986**, *4*, 73–77.
52. R. G. Bailey; I. McDowell; H. E. Nursten, *J. Sci. Food Agric.* **1990**, *52*, 509–525.
53. S. Scharbert; N. Holzmann; T. Hofmann, *J. Agric. Food Chem.* **2004**, *52* (11), 3498–3508.
54. A. J. Stewart; W. Mullen; A. Crozier, *Mol. Nutr. Food Res.* **2005**, *49* (1), 52–60.
55. K. R. Price; M. J. C. Rhodes; K. A. Barnes, *J. Agric. Food Chem.* **1998**, *46*, 2517–2522.
56. A. Finger, Flavonol- und Flavonglykoside des Tees. Thesis, TU Braunschweig, 1991 (in German).
57. C. Lakenbrink, Strukturaufklärung und Bestimmung von Proanthocyanidinen und anderen flavonoiden Inhaltsstoffen des Tees. Thesis, TU Braunschweig, 2000 (in German).
58. D. Del Rio; A. J. Stewart; W. Mullen; J. Burns; M. E. Lean; F. Brighenti; A. Crozier, *J. Agric. Food Chem.* **2004**, *52* (10), 2807–2815.
59. R. R. Dhindzholiya; M. R. Pruidze; R. G. Dadiani, *Appl. Biochem. Microbiol.* **1979**, *15*, 589–594.
60. J. Dou; V. S. Y. Lee; J. T. C. Tzen; M.-R. Lee, *J. Agric. Food Chem.* **2007**, *55*, 7462–7468.
61. Y. Oshima; T. Nakabayashi, *J. Agric. Chem. Jpn.* **1953**, *27*, 274–276.
62. K. Z. Dzemukhadze; M. N. Nestruk, *Dokl. Akad. Nauk SSSR* **1960**, *133*, 469–471.
63. A. Finger; U. H. Engelhardt; V. Wray, *Phytochemistry* **1991**, *30* (6), 2057–2060.
64. K. G. Mikaberidze; I. I. Moniava, *Khim. Prir. Soedin.* **1974**, *4*, 519–520.
65. Y. R. Liang; Z. S. Liu; Y. R. Xu; Y. L. Hu, *J. Sci. Food Agric.* **1990**, *53*, 541–548.
66. Y. Sakamoto, *Agric. Biol. Chem.* **1969**, *33* (6), 818–825.
67. C. Lakenbrink; T. M. L. Lam; U. H. Engelhardt; V. Wray, *Nat. Prod. Lett.* **2000**, *14* (4), 233–238.
68. M. Winterstein; A. Finger, *Dtsch. Lebensmitt. Rundsch.* **1998**, *94* (8), 262–267.
69. S. Scharbert; T. Hofmann, *J. Agric. Food Chem.* **2005**, *53* (13), 5377–5384.
70. Z. Ding; S. Kuhr; U. H. Engelhardt. *Z. Lebensm. Unters. Forsch.* **1992**, *195*, 108–111.
71. K. M. Dzhemukhadze; M. N. Nestjuk, *Dokl. Akad. Nauk SSSR* **1960**, *131* (1), 203–205.
72. Y. Sakamoto, *Agric. Biol. Chem.* **1967**, *31* (9), 1029–1034.
73. I. D. Chkhikvishvili; V. A. Kurkin; M. N. Zaprometov, *Khim. Priod. Soedin.* **1985**, *1*, 118–119.
74. Y. Sakamoto, *Agric. Biol. Chem.* **1969**, *33* (6), 959–961.
75. A. Chaboud; J. Raynaud; L. Debourcieu, *Pharmazie* **1986**, *41*, 745–746.
76. G. Nonaka; R. Sakai; I. Nishioka, *Phytochemistry* **1984**, *23*, 1753–1755.
77. U. H. Engelhardt, Authenticity of Tea and Tea Products. In *Authentication of Food and Wine*; S. E. Ebeler, G. R. Takeoka, P. Winterhalter, Eds.; ACS Symposium Series 952; American Chemical Society: Washington, DC, 2007; pp 138–146.
78. F. Hashimoto; G. Nonaka; I. Nishioka, *Chem. Pharm. Bull.* **1989**, *37*, 3255–3263.
79. C. Lakenbrink; U. H. Engelhardt; V. Wray, *J. Agric. Food Chem.* **1999**, *47*, 4621–4624.
80. U. H. Engelhardt; C. Lakenbrink; O. Pokorny, Proanthocanidins, Bisflavanols, and Hydrolyzable Tannins in Green and Black Teas. In *Nutraceutical Beverages. Chemistry, Nutrition, and Health Effects*; F. Shahidi, D. K. Weerasinghe, Eds.; ACS Symposium Series 871; American Chemical Society: Washington DC, 2004; pp 254–264.
81. E. A. H. Roberts, *J. Sci. Food Agric.* **1958**, *9*, 381–390.
82. F. Hashimoto; G. Nonaka; I. Nishioka, *Chem. Pharm. Bull.* **1988**, *36*, 1676–1684.

83. F. Hashimoto; G. Nonaka; I. Nishioka, *Chem. Pharm. Bull.* **1992**, *40*, 1383–1389.
84. T. Tanaka; I. Kuono, *Food Sci. Technol. Res.* **2003**, *9* (2), 128–133.
85. T. Tanaka; M. Matsuo; I. Kuono, *J. Agric. Food Chem.* **2005**, *53*, 7571–7578.
86. E. A. H. Roberts; D. M. Williams, *J. Sci. Food Agric.* **1958**, *9*, 212–216.
87. E. A. H. Roberts; A. Cartwright; M. Oldschool, *J. Sci. Food Agric.* **1957**, *8*, 72–80.
88. P. D. Collier; T. Bryce; R. Mallows; P. E. Thomas; D. J. Frost; O. Korver; C. Wilkins, *Tetrahedron* **1973**, *29*, 125–142.
89. Y. Takino; H. Imagawa; H. Horikawa; A. Tanaka, *Agric. Biol. Chem.* **1964**, *28*, 64–71.
90. Y. Li; T. Tanaka; I. Kouno, *Phytochemistry* **2007**, *68* (7), 1081–1088.
91. A. Robertson, *Phytochemistry* **1983**, *22*, 889–896.
92. A. Robertson, *Phytochemistry* **1983**, *22*, 897–903.
93. D. T. Coxon; A. Holmes; W. D. Ollis, *Tetrahedron Lett.* **1970**, *11* (60), 5247–5250.
94. G. Nonaka; F. Hashimoto; I. Nishioka, *Chem. Pharm. Bull.* **1986**, *34*, 61–65.
95. Y. Cai; J. Lewis; A. L. Davis; A. P. Davies; J. P. G. Wilkins, *Polyphenols Commun., Bordeaux (France)* **1996**, *96*, 49–50.
96. J. R. Lewis; A. L. Davis; Y. Cai; A. P. Davies; J. P. G. Wilkins; M. Pennington, *Phytochemistry* **1998**, *49*, 2511–2519.
97. S. Sang; S. Tian; X. Meng; R. E. Stark; R. T. Rosen; C. S. Yang; C.-T. Ho, *Tetrahedron Lett.* **2002**, *43*, 7129–7133.
98. S. Sang; S. Tian; R. E. Stark; C. S. Yang; C.-T. Ho, *Bioorg. Med. Chem.* **2004**, *12*, 3009–3017.
99. M. Nishimura; K. Ishiyama; A. Watanabe; S. Kawano; T. Miyase; M. Sano, *J. Agric. Food Chem.* **2007**, *55* (18), 7252–7257.
100. A. L. Davis; J. R. Lewis; Y. Cai; C. Powell; A. P. Davis; J. P. G. Wilkins; P. Pudney; M. N. Clifford, *Phytochemistry* **1997**, *46* (8), 1397–1402.
101. R. Kusano; T. Tanaka; Y. Matsuo; I. Kouno, *Chem. Pharm. Bull.* **2007**, *55* (12), 1768–1772.
102. P. O. Owuor; S. G. Reeves; K. Wanyoko, *J. Sci. Food Agric.* **1986**, *37*, 507–513.
103. E. A. H. Roberts; R. F. Smith, *Analyst* **1961**, *86*, 94–98.
104. P. J. Hilton; R. T. Ellis, *J. Sci. Food Agric.* **1972**, *23*, 227–232.
105. I. McDowell; S. Taylor; C. Gay, *J. Sci. Food Agric.* **1995**, *69*, 467–474.
106. B. Steinhaus; U. H. Engelhardt, *Z. Lebensm. Unters. Forsch.* **1989**, *188*, 509–511.
107. A. M. Baruah; P. K. Mahanta, *J. Agric. Food Chem.* **2003**, *51* (22), 6578–6588.
108. W. Shao; C. Powell; M. N. Clifford, *J. Sci. Food Agric.* **1995**, *69*, 535–540.
109. A. J. Stewart; W. Mullen; A. Crozier, *Mol. Nutr. Food Res.* **2005**, *49*, 52–60.
110. D. Del Rio; A. J. Stewart; W. Mullen; J. Burns; M. E. Lean; F. Brighenti; A. Crozier, *J. Agric. Food Chem.* **2004**, *52* (10), 2807–2815.
111. C. Lakenbrink; S. Lapczynski; B. Maiwald; U. H. Engelhardt; *J. Agric. Food Chem.* **2000**, *48* (7), 2848–2852.
112. E. A. H. Roberts; R. F. Smith, *J. Sci. Food Agric.* **1963**, *14*, 689–699.
113. R. G. Bailey; H. E. Nursten; I. McDowell, *J. Chromatogr.* **1991**, *542*, 115–128.
114. R. G. Bailey; H. E. Nursten; I. McDowell, *J. Chromatogr. A* **1994**, *662*, 101–112.
115. R. G. Bailey; H. E. Nursten; I. McDowell, *J. Sci. Food Agric.* **1994**, *64*, 231–238.
116. L. Bruschi; E. L. Copeland; M. N. Clifford, *Food Chem.* **1999**, *67*, 143–146.
117. B. L. Wedzicha; T. J. Donovan, *J. Chromatogr.* **1989**, *478*, 217–224.
118. R. G. Bailey; H. E. Nursten; I. McDowell, *J. Sci. Food Agric.* **1992**, *59*, 365–375.
119. A. Degenhardt; U. H. Engelhardt; A. S. Wendt; P. Winterhalter, *J. Agric. Food Chem.* **2000**, *48* (11), 5200–5205.
120. M. C. Menet; S. Sang; C. S. Yang; C. T. Ho; R. T. Rosen, *J. Agric. Food Chem.* **2004**, *52* (9), 2455–2461.
121. A. Finger, *J. Sci. Food Agric.* **1994**, *66*, 293–305.
122. M. A. Dix; C. J. Fairley; D. J. Millin; D. E. Swaine, *J. Sci. Food Agric.* **1981**, *32*, 920–932.
123. S. C. Opie; A. Robertson; M. N. Clifford, *J. Sci. Food Agric.* **1990**, *50*, 547–561.
124. T. Bond; A. P. Davies; A. L. Davis; J. R. Lewis; A. Degenhardt; U. H. Engelhardt, *Lebensmittelchemie* **2002**, *56*, 11.
125. M. Monobe; K. Ema; F. Kato; M. Maeda-Yamamoto, *J. Agric. Food Chem.* **2008**, *56* (4), 1423–1427.
126. Y. Mizukami; Y. Sawai; Y. Yamaguchi, *J. Agric. Food Chem.* **2007**, *55* (13), 4957–4964.
127. H. Niino; I. Sakane; K. Okanoya; S. Kuribayashi; H. Kinugasa, *J. Agric. Food Chem.* **2005**, *53* (10), 3995–3999.
128. E. A. H. Roberts; M. Myers, *J. Sci. Food Agric.* **1958**, *9*, 701–715.
129. P. K. Mahanta, Colour and Flavour Characteristics of Made Tea. In *Modern Methods of Plant Analysis – New Series*; H. F. Linskens, J. F. Jackson, Eds.; Springer Verlag: New York, 1988; Vol. 8, 221–295.
130. A. Kiehne; U. H. Engelhardt, *Z. Lebensm. Unters. Forsch.* **1996**, *202*, 299–302.
131. X. Zhu; B. Chen; M. Ma; X. Luo; F. Zhang; S. Yao; Z. Wan; D. Yang; H. Hang, *J. Pharm. Biomed. Anal.* **2004**, *34* (3), 695–704.
132. U. H. Engelhardt; A. Finger; B. Herzig, *Lebensmittelchem. Gerichtl. Chem.* **1989**, *43*, 58–59.
133. M.-J. Li; Q.-K. Chen; H.-F. Wang, *Tea Sci. Res. J. (Hangzhou)*, **1983**, 112.
134. M. N. Clifford; S. Stoupi; N. Kuhnert, *J. Agric. Food Chem.* **2007**, *55* (8), 2797–2807.
135. X.-Q. Zheng; C.-X. Ye; M. Kato; A. Crozier; H. Ashihara, *Phytochemistry* **2002**, *60* (2), 129–134.
136. C. N. Chen; C. M. Liang; J. R. Lai; Y. J. Tsai; J. S. Tsay; J. K. Lin, *J. Agric. Food Chem.* **2003**, *51* (25), 7495–7503.
137. P. L. Fernández; F. Pablos; M. J. Martín; A. G. González, *J. Agric. Food Chem.* **2002**, *50* (7), 1833–1839.
138. T. Suzuki; G. R. Waller, Metabolism and Analysis of Caffeine and Other Methylxanthines in Coffee, Tea, Cola, Guarana and Cacoa. In *Modern Methods of Plant Analysis – New Series*; H. F. Linskens, J. F. Jackson, Eds.; Springer Verlag: New York, 1988; Vol. 8, 184–220.
139. T. Suzuki; E. Takahashi, *Biochem. J.* **1975**, *146* (1), 87–96.
140. H. Ashihara; T. Suzuki, *Front. Biosci.* **2004**, *9*, 1864–1876.
141. M. Mizutani; H. Nakanishi; J.-I. Ema; S.-J. Ma; E. Noguchi; M. Inohara-Ochiai; M. Fukuchi-Mizutani; M. Nakao; K. Sakata, *Plant Physiol.* **2002**, *130* (4), 2164–2176.
142. D. Wang; E. Kurasawa; Y. Yamaguchi; K. Kubota; A. Kobayashi, *J. Agric. Food Chem.* **2001**, *49* (4), 1900–1903.
143. P. Saikia; P. K. Mahanta, *J. Agric. Food Chem.* **2002**, *50* (26), 7691–7699.
144. S. J. Ma; M. Mizutani; J. Hiratake; K. Hayashi; K. Yagi; N. Watanabe; K. Sakata, *Biosci. Biotechnol. Biochem.* **2001**, *65* (12), 2719–2729.
145. N. K. Rana; P. Mohanpuria; S. K. Yadav, *Appl. Biochem. Biotechnol.* **2008**, *39* (1), 49–56.

146. G. V. Stagg; D. J. Millin, *J. Sci. Food Agric.* **1975**, *26*, 1439–1459.
147. R. Cartwright; E. A. H. Roberts; F. D. Wood, *J. Sci. Food Agric.* **1954**, *5*, 597–599.
148. K. H. Ekborg-Ott; A. Taylor; D. W. Armstrong, *J. Agric. Food Chem.* **1997**, *45*, 353–363.
149. Y. Ying; J. W. Ho; Z.-Y. Chen; J. Wang, *J. Liq. Chromatogr. Relat. Technol.* **2005**, *28* (5), 727–737.
150. M. Friedman; B. E. Mackey; H. J. Kim; I. S. Lee; K. R. Lee; S. U. Lee; E. Kozukue; N. Kozukue, *J. Agric. Food Chem.* **2007**, *55* (2), 243–253.
151. R. Thippeswamy; K. G. Gouda; D. H. Rao; A. Martin; L. R. Gowda, *J. Agric. Food Chem.* **2006**, *54* (19), 7014–7019.
152. U. H. Engelhardt; M. Simonides, *Lebensmittelchemie* **2007**, *61*, 139–140.
153. S. Kaneko; K. Kumazawa; H. Masuda; A. Henze; T. Hofmann, *J. Agric. Food Chem.* **2006**, *54* (7), 2688–2694.
154. Y. Hara; S. J. Luo; R. L. Wickremasinghe; T. Yamanishi, *Food Rev. Int.* **1995**, *11*, 435–456.
155. Y. Hara; S. J. Luo; R. L. Wickremasinghe; T. Yamanishi, *Food Rev. Int.* **1995**, *11*, 477–525.
156. G. W. Sanderson; H. Co; J. G. Gonzalez, *J. Food Sci.* **1971**, *36*, 231–236.
157. Y. Suzuki; Y. Shioi, *J. Agric. Food Chem.* **2003**, *51* (18), 5307–5314.
158. I. Fukuda; I. Sakane; Y. Yabushita; R. Kodoi; S. Nishiumi; T. Kakuda; S. Sawamura; K. Kanazawa; H. Ashida, *J. Agric. Food Chem.* **2004**, *52* (9), 2499–2506.
159. P. K. Mahanta; M. Hazarika, *J. Sci. Food Agric.* **1985**, *36*, 1133–1139.
160. W. Dongfeng; W. Chenghong; L. Jun; Z. Guiwen, *J. Agric. Food Chem.* **2001**, *49*, 507–510.
161. H. Chen; M. Zhang; Z. Qu; B. Xie, *J. Agric. Food Chem.* **2007**, *55* (6), 2256–2260.
162. S. Nie; M. Xie; Y. Wang, *Anal. Bioanal. Chem.* **2005**, *383*, 680–686.
163. A. J. Wright; M. J. Fishwick, *Phytochemistry* **1979**, *18*, 1511–1513.
164. I. Kitagawa; K. Hori; T. Motozawa; T. Murakami; M. Yoshikawa, *Chem. Pharm. Bull. (Tokyo)* **1998**, *46* (12), 1901–1906.
165. T. Murakami; J. Nakamura; H. Matsuda; M. Yoshikawa, *Chem. Pharm. Bull. (Tokyo)*, **1999**, *47* (12), 1759–1764.
166. T. Murakami; J. Nakamura; T. Kageura; H. Matsuda; M. Yoshikawa, *Chem. Pharm. Bull. (Tokyo)* **2000**, *48* (11), 1720–1725.
167. M. Yoshikawa; T. Morikawa; K. Yamamoto; Y. Kato; A. Nagatomo; H. Matsuda, *J. Nat. Prod.* **2005**, *68* (9), 1360–1365.
168. Y. Lu; T. Umeda; A. Yagi; K. Sakata; T. Chaudhuri; D. K. Ganguly; S. Sarma, *Phytochemistry* **2000**, *53* (8), 941–946.
169. M. Yoshikawa; T. Wang; S. Sugimoto; S. Nakamura; A. Nagatomo; H. Matsuda; S. Harima, *Yakugaku Zasshi* **2008**, *128* (1), 141–145.
170. P. L. Fernández-Cáceres; M. J. Martín; F. Pablos; A. G. González, *J. Agric. Food Chem.* **2001**, *49* (10), 4775–4779.
171. S. Natesan; V. Ranganathan, *J. Sci. Food Agric.* **1990**, *51*, 125–139.
172. C. Cabrera; R. Artacho; R. Giménez, *J. Am. Coll. Nutr.* **2006**, *25* (2), 79–99.
173. J. Smullen; G. A. Koutsou; H. A. Foster; A. Zumbé; D. M. Storey, *Caries Res.* **2007**, *41* (5), 342–349.
174. S. Otake; M. Makimura; T. Kuroki; Y. Nishihara; M. Hirasawa, *Caries Res.* **1991**, *25*, 438–442.
175. M. Okamoto; A. Sugimoto; K. P. Leung; K. Nakayama; A. Kamaguchi; N. Maeda, *Oral Microbiol. Immunol.* **2004**, *19*, 118–120.
176. W. Feldheim; S. O. Miehe, *Z. Lebensm. Unters. Forsch.* **1979**, *169*, 453–456.
177. H. Albers; U. H. Engelhardt, *Kaffee Tee Markt* **1988**, *XXXVII/11*, 3–4 (in German).
178. M. Reto; M. E. Figueira; H. M. Filipe; C. M. Almeida, *Plant Foods Hum. Nutr.* **2007**, *62* (4), 139–144.
179. C. B. Walters; J. C. Sherlock; W. H. Evans; J. I. Read, *J. Sci. Food Agric.* **1983**, *34*, 523–528.
180. T. Yamanishi; A. Kobayashi, Progress of Tea Aroma Chemistry. In *Flavor Chemistry: 30 Years of Progress*; R. Teranishi, Ed.; Kluwer: New York, 1999; pp 135–145.
181. J. M. Robinson; P. O. Owuor, Tea Aroma. In *Tea: Cultivation to Consumption*; K. C. Willson, M. N. Clifford, Eds.; Chapman & Hall: London, 1992; pp 603–647.
182. S. M. Constantinides; R. Hoover; P. A. Karakoltsidis; T. C. Kelly; Y. C. Lu; M. Namiki; P. Schreier; P. Winterhalter, *Food Rev. Int.* **1995**, *11*, 371–542.
183. P. Schreier, Analysis of Tea Volatiles. In *Modern Methods of Plant Analysis – New Series*; H. F. Linskens, J. F. Jackson, Eds.; Springer: Berlin, 1988; Vol. 8, 296–320.
184. O. G. Vitzthum; P. Werkhoff; P. Hubert, *J. Agric. Food Chem.* **1975**, *23*, 999–1003.
185. W. Mick; E. Goetz; P. Schreier, *Lebenson. Wiss. Technol.* **1984**, *17*, 104–106.
186. W. Mick; P. Schreier, *J. Agric. Food Chem.* **1984**, *32*, 924–929.
187. C. Schuh; P. Schieberle, *J. Agric. Food Chem.* **2006**, *54* (3), 916–924.
188. H. Guth; W. Grosch, *Flavour Fragrance J.* **1993**, *8*, 173–178.
189. H. Masuda; K. Kumazawa, The Change in the Flavor of Green and Black Tea Drinks by the Retorting Process. In *Caffeinated Beverages*; T. H. Parliment, C.-T. Ho, P. Schieberle, Eds.; ACS Symposium Series 754; 1999; American Chemical Society: Washington DC, pp 337–346.
190. K. Kumuzawa; Y. Wada; H. Masuda, *J. Agric. Food Chem.* **2006**, *54*, 4795–4801.
191. Y. Mizukami; Y. Sawai; Y. Yamaguchi, *J. Agric. Food Chem.* **2008**, *56* (6), 2154–2159.
192. Y. Ito; K. Kubota, *Mol. Nutr. Food Res.* **2005**, *49*, 61–68.
193. R. Naef; A. Jaquier; A. Velluz; B. Maurer, *J. Agric. Food Chem.* **2006**, *54* (24), 9201–9205.
194. Y. Cheng; T. Huynh-Ba; I. Blank; F. Robert, *J. Agric. Food Chem.* **2008**, *56* (6), 2160–2169.
195. P. M. Zarembski; A. Hodgkinson, *Br. J. Nutr.* **1962**, *16*, 627–634.
196. G. P. Savage; M. J. S. Charrier; L. Verhanen, *Eur. J. Clin. Nutr.* **2003**, *57*, 415–419.
197. J. Charrier; G. P. Savage; L. Vanhanen, *Asia Pac. J. Clin. Nutr.* **2002**, *11* (4), 298–301.
198. H. Horie; Y. Yamauchi; K. Kohata, *J. Chromatogr. A* **1998**, *817*, 139–144.
199. B. C. Jeong; B. S. Kim; J. I. Kim; H. H. Kim, *J. Endourol.* **2006**, *20* (5), 356–361.
200. Y. Itoh; T. Yasui; A. Okada; K. Tozawa; Y. Hayashi; K. Kohri, *J. Urol.* **2005**, *173* (1), 271–275.
201. K. Sakata; S. Sakuraba; A. Yagi; K. Ina; T. Hara; T. Takeo, *Agric. Biol. Chem.* **1986**, *50* (7), 1919–1921.
202. M.-Y. Ding; P.-R. Chen; G.-A. Luo, *J. Chromatogr. A* **1997**, *764*, 341–345.
203. H. Hoehne, Methodenentwicklung zur Bestimmung von Flavanolen und Ascorbinsäure in Tee mittels Kapillarelektrophorese. Thesis, TU Braunschweig, 1999 (in German).
204. M. Yoshida; H. Ono; Y. Chuda; H. Yada; M. Ohnishi-Kameyama; H. Kobayashi; A. Ohara-Takada; C. Matsuura-Endo; M. Mori; N. Hayashi; Y. Yamaguchi, *Adv. Exp. Med. Biol.* **2005**, *561*, 405–413.
205. M. Sivaplan, *Tea Q.* **1982**, *51* (4), 185–189.

206. S. Khokhar; S. G. Magnusdottir, *J. Agric. Food Chem.* **2002**, *50* (3), 565–570.
207. D. J. Yang; L. S. Hwang; J. T. Lin, *J. Chromatogr. A* **2007**, *1156* (1–2), 312–320.
208. D. Stach; O. J. Schmitz, *J. Chromatogr. A* **2001**, *924*, 519–522.
209. J. Ortiz; M. G. Ferruzzi; L. S. Taylor; L. J. Mauer, *J. Agric. Food Chem.* **2008**, *56* (11), 4068–4077.
210. L. F. Wang; D. M. Kim; C. Y. Lee, *J. Agric. Food Chem.* **2000**, *48* (9), 4227–4232.
211. E. A. H. Roberts, *J. Sci. Food Agric.* **1963**, *14*, 700–705.
212. R. F. Smith, *J. Sci. Food Agric.* **1968**, *19*, 530–534.
213. P. Rutter; G. Stainsby, *J. Sci. Food Agric.* **1975**, *26*, 455–463.
214. E. Jöbstl; J. P. Fairclough; A. P. Davies; M. P. Williamson, *J. Agric. Food Chem.* **2005**, *53* (20), 7997–8002.
215. Y. C. Chao; B. H. Chiang; *J. Sci. Food Agric.* **1999**, *79*, 1767–1774.
216. M. Spiro; D. Jaganyi, *Food Chem.* **1994**, *49* (4), 359–365.
217. S. Lee; M. K. Park; K. H. Kim; Y. S. Kim, *J. Food Sci.* **2007**, *72* (7) S497–S502.
218. K. J. Huang; J. J. Wu; Y. H. Chiu; C. Y. Lai; C. M. Chang, *J. Agric. Food Chem.* **2007**, *55* (22), 9014–9020.
219. J. H. Ye; Y. R. Liang; J. Jin; H. L. Liang; Y. Y. Du; J. L. Lu; Q. Ye; C. Lin, *J. Agric. Food Chem.* **2007**, *55* (9), 3498–3502.
220. M. Saltmarsh, Instant tea. In *Tea: Cultivation to Consumption*; K. C. Willson, M. N. Clifford, Eds.; Chapman & Hall: London, 1992; pp 535–554.
221. D. K. Boadi; R. J. Neufeld, *Enzyme Microb. Technol.* **2001**, *28*, 590–595.
222. G. Paganga; N. Miller; C. A. Rice-Evans, *Free Radic. Res.* **1999**, *30*, 153–162.
223. S. A. Wiseman; D. A. Balentine; B. Frei, *Crit. Rev. Food Sci. Nutr.* **1997**, *37*, 705–718.
224. P. C. H. Hollman; L. B. M. Tijburg; C. S. Yang, *Crit. Rev. Food Sci. Nutr.* **1997**, *37* (8), 719–738.
225. G. Williamson; C. Manach, *Am. J. Clin. Nutr.* **2005**, *81* (Suppl. 1), 243S–255S.
226. C. Manach; G. Williamson; C. Morand; A. Scalbert; C. Rémésy, *Am. J. Clin. Nutr.* **2005**, *81* (Suppl. 1), 230S–242S.
227. M. N. Clifford, *Planta Med.* **2004**, *70* (12), 1103–1114.
228. P. M. Aron; J. A. Kennedy, *Mol. Nutr. Food Res.* **2008**, *52* (1), 79–104.
229. J. M. van Amelsvoort; K. H. van het Hof; J. N. Mathot; T. P. Mulder; A. Wiersma; L. B. Tijburg, *Xenobiotica* **2001**, *31* (12), 891–901.
230. B. A. Warden; L. S. Smith; G. R. Beecher; D. A. Balentine; B. A. Clevidence, *J. Nutr.* **2001**, *131* (6), 1731–1737.
231. M. R. Olthof; P. C. Hollman; M. N. Buijsman; J. M. van Amelsvoort; M. B. Katan, *J. Nutr.* **2003**, *133* (6), 1806–1814. Erratum in *J. Nutr.* **2003**, *133* (8), 2692.
232. J. D. Lambert; S. Sang; C. S. Yang, *Mol. Pharm.* **2007**, *4* (6), 819–825.
233. C. A. Daykin; J. P. van Duynhoven; A. Groenewegen; M. Dachtler; J. M. van Amelsvoort; T. P. Mulder, *J. Agric. Food Chem.* **2005**, *53* (5), 1428–1434.
234. F. A. van Dorsten; C. A. Daykin; T. P. Mulder; J. P. van Duynhoven, *J. Agric. Food Chem.* **2006**, *54* (18), 6929–6938.
235. S. Sang; M. J. Lee; I. Yang; B. Buckley; C. S. Yang, *Rapid Commun. Mass Spectrom.* **2008**, *22* (10), 1567–1578.
236. V. Crespy; G. Williamson, *J. Nutr.* **2004**, *134* (Suppl. 12), 3431S–3440S.
237. J. W. Erdman, Jr.; D. Balentine; L. Arab; G. Beecher; J. T. Dwyer; J. Folts; J. Harnly; P. Hollman; C. L. Keen; G. Mazza; M. Messina; A. Scalbert; J. Vita; G. Williamson; J. Burrowes, *J. Nutr.* **2007**, *137* (3 Suppl. 1), 718S–737S.
238. V. Stangl; H. Dreger; K. Stangl; M. Lorenz, *Cardiovasc. Res.* **2007**, *73* (2), 348–358.
239. N. Kurahashi; S. Sasazuki; M. Iwasaki; M. Inoue; S. Tsugane, *Am. J. Epidemiol.* **2008**, *167* (1), 71–77.
240. C.-L. Sun; J.-M. Yuan; W.-P. Koh; M. C. Yu, *Carcinogenesis* **2006**, *27* (7), 1301–1309.
241. C. S. Yang; D. J. Lambert; J. Ju; G. Lu; S. Sang, *Toxicol. Appl. Pharmacol.* **2007**, *224* (3), 265–273.
242. E. Navarro-Perán; J. Cabezas-Herrera; L. S. Campo; J. N. Rodríguez-López, *Int. J. Biochem. Cell Biol.* **2007**, *39* (12), 2215–2225.
243. E. Navarro-Perán; J. Cabezas-Herrera; F. García-Cánovas; M. C. Durrant; R. N. Thorneley; J. N. Rodríguez-López, *Cancer Res.* **2005**, *65* (6), 2059–2064.
244. M. D. Lucock; P. D. Roach, *Cancer Res.* **2005**, *65* (18), 8573.
245. S. Shankar; S. Ganapathy; R. K. Srivastava, *Front Biosci.* **2007**, *12*, 4881–4899.
246. M. Friedman, *Mol. Nutr. Food Res.* **2007**, *51*, 116–134.
247. J. A. Kyle; P. C. Morrice; G. McNeill; G. G. Duthie, *J. Agric. Food Chem.* **2007**, *55* (12), 4889–4894.
248. V. C. Reddy; G. V. Vidya Sagar; D. Sreeramulu; L. Venu; M. Raghunath, *Ann. Nutr. Metab.* **2005**, *49* (3), 189–195.
249. P. C. Hollman; K. H. van Het Hof; L. B. Tijburg; M. B. Katan, *Free Radic. Res.* **2001**, *34* (3), 297–300.
250. K. H. van het Hof; G. A. Kivits; J. A. Weststrate; L. B. Tijburg, *Eur. J. Clin. Nutr.* **1998**, *52* (5), 356–359.
251. R. Leenen; A. J. Roodenburg; L. B. Tijburg; S. A. Wiseman, *Eur. J. Clin. Nutr.* **2000**, *54* (1), 87–92.
252. F. Catterall; A. I. Kassimi; M. N. Clifford; C. Ioannides, *Anticancer Res.* **2003**, *23* (5A), 3863–3867.
253. M. Lorenz; N. Jochmann; A. von Krosigk; P. Martus; G. Baumann; K. Stangl; V. Stangl, *Eur. Heart. J.* **2007**, *28* (2), 219–223.
254. V. R. Prabhakar; N. Venkatesan, *Eur. Heart. J.* **2007**, *28* (11), 1397–1398.
255. E. von Elm; G. Antes, *Eur. Heart. J.* **2007**, *28* (11), 1398–1399.
256. M. Pfeuffer; J. Schrezenmeir, *Eur. Heart J.* **2007**, *28*, 1266–1267.
257. N. Yusuf; C. Irby; S. K. Katiyar; C. A. Elmets, *Photodermatol. Photoimmunol. Photomed.* **2007**, *23* (1), 48–56.
258. S. Wolfram; Y. Wang; F. Thielecke, *Mol. Nutr. Food Res.* **2006**, *50* (2), 176–187.
259. J. K. Lin; S. Y. Lin-Shiau, *Mol. Nutr. Food Res.* **2006**, *50* (2), 211–217.
260. Y. H. Kao; H. H. Chang; M. J. Lee; C. L. Chen, *Mol. Nutr. Food Res.* **2006**, *50* (2), 188–210.
261. P. J. Rogers; J. E. Smith; S. V. Heatherley; C. W. Pleydell-Pearce, *Psychopharmacology (Berlin)* **2008**, *195* (4), 569–577.
262. J. Bryan, *Nutr. Rev.* **2008**, *66* (2), 82–90.
263. C. F. Haskell; D. O. Kennedy; A. L. Milne; K. A. Wesnes; A. B. Scholey, *Biol. Psychol.* **2008**, *77* (2), 113–122.
264. W. Dimpfel; A. Kler; E. Kriesl; R. Lehnfeld; I. K. Keplinger-Dimpfel, *Nutr. Neurosci.* **2007**, *10* (3–4), 169–180.
265. T. Yamada; T. Terashima; H. Honma; S. Nagata; T. Okubo; L. R. Juneja; H. Yokogoshi, *Biosci. Biotechnol. Biochem.* **2008**, *72* (5), 1356–1359.
266. T. Kakuda; E. Hinoi; A. Abe; A. Nozawa; M. Ogura; Y. Yoneda, *J. Neurosci. Res.* **2008**, *86* (8), 1846–1856.
267. V. L. Singleton; J. A. Rossi, *Am. J. Enol. Vitic.* **1965**, *16* (3), 144–158.
268. V. L. Singleton; R. Orthofer; R. M. Lamuela-Raventos, *Methods Enzymol.* **1999**, *299*, 152–178.
269. J. Lowenthal, *Z. Anal. Chem.* **1877**, *16*, 33–48 and 201.

270. M. Kapel; R. Karunanithy, *Analyst* **1974**, *99*, 661–665.
271. G. Bonifazi; E. Capt, *Z. Anal. Chem.* **1932**, *88*, 297–298.
272. Q. Chen; J. Zhao; M. Liu; J. Cai; J. Liu, *J. Pharm. Biomed. Anal.* **2008**, *46* (3), 568–573.
273. A. Finger; S. Kuhr; U. H. Engelhardt, *J. Chromatogr.* **1992**, *624*, 293–315.
274. H. M. Merken; G. R. Beecher, *J. Agric. Food Chem.* **2000**, *48* (3), 577–599.
275. G. R. Beecher; B. A. Warden; H. Merken, *Proc. Soc. Exp. Biol. Med.* **1999**, *220* (4), 267–270.
276. J. J. Dalluge; B. C. Nelson, *J. Chromatogr. A* **2000**, *881*, 411–424.
277. T. Sultana; G. Stecher; R. Mayer; L. Trojer; M. N. Qureshi; G. Abel; M. Popp; G. K. Bonn, *J. Agric. Food Chem.* **2008**, *56* (10), 3444–3453.
278. U. H. Engelhardt; A. Finger; S. Kuhr, *Z. Lebensm. Unters. Forsch.* **1993**, *197*, 239–244.
279. A. Yanagida; A. Shoji; Y. Shibusawa; H. Shindo; M. Tagashira; M. Ikeda; Y. Ito, *J. Chromatogr. A* **2006**, *1112* (1–2), 195–201.
280. H. Schulz; U. H. Engelhardt; A. Wegent; H. Drews; S. Lapczynski, *J. Agric. Food Chem.* **1999**, *47* (12), 5064–5067.
281. A. Degenhardt; U. H. Engelhardt; C. Lakenbrink; P. Winterhalter, *J. Agric. Food Chem.* **2000**, *48* (8), 3425–3430.
282. A. Degenhardt; U. H. Engelhardt; P. Winterhalter; Y. Ito, *J. Agric. Food Chem.* **2001**, *49*, 1730–1736.
283. K. Wang; Z. Liu; J. A. Huang; X. Dong; L. Song; Y. Pan; F. Liu, *J. Chromatogr. B* **2008**, *867* (2), 282–286.
284. C. Yang; D. Li; X. Wan, *J. Chromatogr. B* **2008**, *861* (1), 140–144.
285. T. Unno; Y. M. Sagesaka; T. Kakuda, *J. Agric. Food Chem.* **2005**, *53* (26), 9885–9889.
286. M. de L. Mata-Bilbao; C. Andrés-Lacueva; E. Roura; O. Jáuregui; C. Torre; R. M. Lamuela-Raventós, *J. Agric. Food Chem.* **2007**, *55* (22), 8857–8863.
287. M. Masukawa; Y. Matsui; N. Shimizu; N. Kondou; H. Endou; M. Kuzukawa; T. Hase, *J. Chromatogr. B* **2006**, *834* (1–2), 26–34.
288. T. P. Mulder; C. J. van Platerink; P. J. Wijnand Schuyl; J. M. van Amelsvoort, *J. Chromatogr. B* **2001**, *760* (2), 271–279.
289. M. J. Desai; D. W. Armstrong, *Rapid Commun. Mass Spectrom.* **2004**, *18* (3), 251–256.
290. M. Fujiwara; I. Ando;, K. Arifuku, *Anal. Sci.* **2006**, *22* (10), 1307–1314.
291. W. Pongsuwan; E. Fukusaki; T. Bamba; T. Yonetani; T. Yamahara; A. Kobayashi, *J. Agric. Food Chem.* **2007**, *55* (2), 231–236.
292. T. Ikeda; S. Kanaya; T. Yonetani; A. Kobayashi; E. Fukusaki, *J. Agric. Food Chem.* **2007**, *55* (24), 9908 9812.

Biographical Sketch

Ulrich H. Engelhardt was born in 1955. He obtained his Ph.D. in 1984 for his research investigations on nonvolatile acids in coffee. He became an assistant professor in 1984, and from 1999 he is working as an adjunct professor at the Institute of Food Chemistry, TU Braunschweig. He is a scientific advisor of the German tea trade organization (since 1994) and is working in cooperation with the ISO working group on tea (since 1995) and has led the German delegation at the meetings in Columbo (1997), Calcutta (1999), Mombaza (2001), Hangzhou (2003), Hamburg (2005), and Hangzhou (2008). He is also the chairman of the working groups on tea and coffee of the German Institute of Standards (DIN) (since 2004) and a member of the editorial board of Deutsche Lebensmittel-Rundschau (since 2007). Professor Engelhardt's research interests include polyphenols, flavonoids, and phenolic acids in tea, coffee, herbal infusions, and related beverages; bitter and acidic compounds in coffee; and chromatography, hyphenated methods (GC–MS and LC–MS), and capillary electrophoresis. He has handled several research projects on tea, coffee, and herbals sponsored by the German minister of economy via the AIF/FEI, the DFG, and various companies. His teaching interests are food analysis, food toxicology, and chemistry and technology of foods containing polyphenols. He has published around 85 papers mainly on coffee and tea chemistry. Professor Engelhardt is the recipient of the Kurt-Täufel award from the German Chemical Society (1996).

3.24 Chemistry of Cannabis

Arno Hazekamp, Justin T. Fischedick, Mónica Llano Díez, Andrea Lubbe, and Renee L. Ruhaak, Leiden University, Leiden, The Netherlands

3.24.1 An Introduction to the Cannabis Plant

Almost no plant has been studied as much as the Cannabis plant (*Cannabis sativa* L.); more than 10 000 papers have been published describing various aspects of Cannabis as a biologically active plant. Nonetheless, it is hard to think of a medical topic that can so strongly divide the research community as the medicinal use of Cannabis. It may even be stated that Cannabis is the most controversial plant in the history of mankind. But imagine if *C. sativa* were to be discovered today, growing in some remote spot of the world, it would be hailed as a wonder of nature; a new miracle plant with the potential to treat anything ranging from headaches to neurological disorders to cancer. Still, the potential of Cannabis was largely ignored until the discovery of the human endocannabinoid system, about a decade ago. Nowadays, it is known that many of our own body functions are controlled by Cannabis-like substances in our brain, immune system, and other organs.

But Cannabis plants are interesting to human society in more ways. As a fiber plant, Cannabis produces some of the best and most durable fibers of natural origin, historically used to produce ropes and sails for sea ships, paper, banknotes, and even the first Levi's jeans. Modern applications include dashboards for exclusive cars and insulation for houses. The oil of the hempseed was found to be well balanced with regard to the ratio of omega-3- to omega-6 fatty acids for human nutrition, and can be used as a sustainable alternative to fish oil. Furthermore, the oil is ideal as an ingredient for body oils and lipid-enriched creams.

The medicinal use of Cannabis has a very long history. However, the availability of alternative treatments, absence of quality control, and sociopolitical pressure led to a decline in the medical use of Cannabis by the beginning of the twentieth century. As a result, in the past decades its medicinal potential continued to be disputed. But despite its illegality, people have continued to obtain Cannabis on the black market for self-medication.

At least one bioactivity of Cannabis is undisputed: the psychoactive effect of delta-9-tetrahydrocannabinol (THC) is one of the best-studied biological activities in the world. As a result, the attention has shifted from the Cannabis plant as a whole, to its main psychoactive component. Interestingly, THC, a terpenophenolic compound, contains no nitrogen atom and therefore is not an alkaloid, which is rare among the psychotropically active compounds. Furthermore, therapeutically used THC is among the most nonpolar compounds used in medicine today. Chemically, THC belongs to a group of closely related compounds known as cannabinoids, and they are considered the main bioactive components of Cannabis. Up to date, already 70 different cannabinoids have been described, several of which were found to be bioactive in one or more ways.

Cannabis has the potential to evolve into useful and much needed new medicines, but this is seriously obstructed by its classification as a dangerous narcotic. But as shown in the case of the poppy plant (*Papaver somniferum*) and the opiates derived from it (e.g., morphine, codeine), the distinction between a dangerous drug of abuse and a medicine can be made by proper, unbiased, and well-conducted research, combined with a rational approach. Relevant biological activities, as shown by thorough research in the laboratory, and finally confirmed through properly conducted clinical trials, are the best guarantee for the future of Cannabis as a medicine. The information presented in this chapter should help researchers of various disciplines to understand the current scientific status of the Cannabis plant and its constituents.

3.24.1.1 The Different Forms of Cannabis

Together with coffee and tobacco, Cannabis is the most commonly used psychoactive drug worldwide, and it is the single most popular illegal drug. Worldwide over 160 million people are using Cannabis regularly and these numbers are still rising.[1] With such high popular demand, it is not surprising that Cannabis and its products are known under a large variety of names. Some of the most widely used ones are defined here.

The commonly used term 'marijuana' or 'marihuana' traditionally describes the Cannabis plant when used as a recreational drug, and is frequently associated with the negative effects or social impact of the drug. 'Weed' is another name for Cannabis when used as a recreational drug. In contrast, when the term 'hemp' is used, it usually refers to the use of Cannabis as a source of fiber, making the term fiber-hemp therefore somewhat superfluous. Because of the inexact and unscientific nature of these terms, they will not be used in this chapter. Instead, the proper scientific name 'Cannabis' will be consistently used to describe the plant *C. sativa* L. in all its varieties.

When discussing about Cannabis for recreational, medicinal, or scientific use, what is usually referred to are the female flowers (also known under the Latin name *flos*), being the most potent part of the plant. The dried resin obtained from these flowers is generally known as 'hash', or 'hashish', although a large variety of names exist throughout the world. This resin is the source of the most important bioactive components of the Cannabis plant, the cannabinoids, which will be the main focus throughout this chapter.

Finally, 'dronabinol' is another name for the naturally occurring (−)-*trans*-isomer of THC, often used in a medical context in the scientific and political literature, and adopted by the World Health Organization.

3.24.1.2 The Botany of *Cannabis sativa*

The basic material of all Cannabis products is the plant *C. sativa* L. (**Figure 1**). It is an annual, usually dioecious, more rarely monoecious, wind-pollinated herb, with male and female flowers developing on separate plants. It propagates from seed, grows vigorously in open sunny environments with well-drained soils, and has an abundant need for nutrients and water. It can reach up to 5 m (16 ft) in height in a 4–6-month growing season. However, in modern breeding and cultivation of recreational Cannabis, the preferred way to propagate the plants is by cloning, using cuttings of the so-called mother plant. As this term indicates, female plants are used for this purpose, as they produce significantly higher amounts of psychoactive compounds than the male plants.

The sexes of Cannabis are anatomically indistinguishable before they start flowering, but after that, the development of male and female plants varies greatly. Shorter days, or more accurately longer nights, induce the plant to start flowering.[2] The female plant then produces several crowded clusters of individual flowers (flower tops); a large one at the top of the stem and several smaller ones on each branch, whereas the male

Figure 1 *Cannabis sativa*. A female plant in full bloom. Photo courtesy by Bedrocan BV, The Netherlands.

flowers hang in loose clusters along a relatively leafless upright branch. The male plants finish shedding their pollen and die before the seeds in the female plants ripen, that is 4–8 weeks after being fertilized. A large female can produce over 1 kg of seed. If the seed survives, it may germinate the next spring.

According to current botanical classification, Cannabis belongs, only with *Humulus* (hops), to the small family of Cannabinaceae (also Cannabaceae or Cannabidaceae).[3–5] Despite this close relationship, cannabinoids (see Section 3.24.2.1) can only be found in *C. sativa*. In the genus *Humulus*, even in grafting experiments between *Cannabis* and *Humulus*, no cannabinoids have been found,[6,7] but instead a variety of the so-called bitter acids, such as humulone, adhumulone, and cohumulone are produced. The close relationship between both plant species is clearly shown by the fact that both compounds (cannabinoids and bitter acids, respectively) are derived from similar biosynthetic pathways (see Section 3.24.2.2). Furthermore, both are excreted as a resinous mixture by glandular hairs, mainly found on female flowers.

The current systematic classification of Cannabis is:[8]

Division Angiosperms
Class Dicotyledon
Subclass Archichlamydeae
Order Urticales
Family Cannabinaceae
Genus *Cannabis*
Species *sativa* L.

Because of centuries of breeding and selection, a large variation of cultivated varieties (or cultivars) has been developed. Already, more than 700 different cultivars have been described[9] and many more are thought to exist. As a result, there has been extensive discussion about further botanical and chemotaxonomic classification. So

far, several classifications of Cannabis have been proposed. Originally, this was a classification into *C. sativa* L., *Cannabis indica* Lam., and *Cannabis ruderalis* Janisch[10–12] or *C. sativa* L. subsp. *sativa* and *C. sativa* subsp. *indica*.[13–15] However, it is becoming commonly accepted that Cannabis is monotypic and consists only of a single species *C. sativa*, as described by Leonard Fuchs in the sixteenth century.[16–19]

To solve the controversy in a biochemical way, a first chemical classification was done by Grlic,[20] who recognized different ripening stages. Fetterman *et al.*[21] described different phenotypes based on quantitative differences in the content of main cannabinoids and he was the first to distinguish the drug and fiber types. Further extension and perfection of this approach was subsequently done by Small and Beckstead,[22] Turner *et al.*,[23] and Brenneisen and Kessler.[24] However, it was found that a single plant could be classified into different phenotypes, depending on its age. Although these chemotaxonomic classifications do not strictly define the contents of the main cannabinoids for each chemotype, it does provide a practical tool for classification. A final validation of Cannabis classification awaits further chemotaxonomic and genetic research.

For forensic and legislative purposes, the most important classification of Cannabis types is that into the fiber type and the drug type. The main difference between these two is found in the content of the psychotropically active component THC, and its acidic precursor tetrahydrocannabinolic acid (THCA, see Section 3.24.2.2): a high content of THC + THCA classifies as drug-type Cannabis, whereas a low THC + THCA content (below 0.2–0.3% of dry weight) is found in fiber-type Cannabis. All Cannabis varieties presently used for medicinal purposes belong to the drug type, because of their high content of the biologically active THC. But although fiber-type Cannabis is currently not used for medicinal or recreational purpose, it does contain components that have been found to be biologically active, most notably the cannabinoid cannabidiol (CBD). This indicates that the distinction between the two types may have limited relevance for medicinal research into Cannabis.

3.24.1.3 A Short History of Cannabis

Cannabis most likely originates from Central Asia, as archeological evidence indicates that it was already cultivated in China for food and fiber 10 000 years ago. Even in ancient Egyptian mummies, clues have been found for the use of Cannabis as food or medicine.[25] In fact, Cannabis is one of the oldest known medicinal plants and is described in almost every ancient handbook on plant medicine, most commonly in the form of a tincture or a tea.[26,27] Some religions were closely related with the properties of the Cannabis plant. For example, in Hindu legend, Cannabis is believed to be the favorite food of the god Shiva, because of its energizing properties. As Cannabis spread from Asia toward the West, almost every culture came into contact with this miracle plant. Nowadays, varieties of Cannabis can be found in all temperate and tropical zones, except in humid, tropical rain forests.[28]

Despite the fact that Cannabis was grown on a large scale in many countries, the abuse as a narcotic remained uncommon in Western countries until relatively recently. People were largely unaware of the psychoactive properties of Cannabis and it is unlikely that early cultivars, selected mainly for their seed or fiber qualities, contained significant amounts of the psychoactive THC. The medicinal use of Cannabis was introduced in Europe only around 1840, by a young Irish doctor, William O'Shaughnessy, who served for the East India Trading Company in India, where the medicinal use of Cannabis was widespread. Unlike the European fiber Cannabis, these Indian varieties did contain a reasonable amount of bioactive cannabinoids. In the following decades, the medicinal use of Cannabis saw a short period of popularity both in Europe and in the United States. At the top of its popularity, more than 28 different medicinal preparations were available with Cannabis as active ingredient, which were recommended for indications as various as menstrual cramps, asthma, cough, insomnia, support of birth labor, migraine, throat infection, and withdrawal from opium use.[27]

However, because no tools existed for quality control, it was impossible to prepare a standardized medicine, so patients often received a dose that was either too low, having no effect, or too high, resulting in serious side effects. Moreover, Cannabis extract was not water-soluble and therefore could not be injected (in contrast to, e.g., the opiates), whereas oral administration was found to be unreliable because of its slow and erratic absorption. Because of such drawbacks, the medicinal use of Cannabis increasingly disappeared in the beginning of the twentieth century, and in 1937 Cannabis was removed from the US pharmacopoeia, a move that was

followed by most other Western countries.[27] Isolation and structure elucidation of the first pure active substances from Cannabis was not achieved until the 1960s.[29]

Only since the flower-power-time of the 1960s, the smoking of Cannabis as a recreational drug has become a widely known phenomenon in the Western world. From then on, import of stronger varieties from the tropics, combined with a growing expertise in breeding and cultivation, led to a steady increase in psychoactive potency. Contemporary recreational Cannabis has increasingly become a high-tech crop, grown indoors under completely artificial conditions.

An extensive review on the history of Cannabis and its uses by humans has recently been published.[30]

3.24.1.4 Chemical Constituents of Cannabis

With currently 538 known constituents, Cannabis is one of the chemically best-studied plants.[31] But because most of these constituents have not yet been properly characterized for biological activity, the Cannabis plant could be called a 'neglected pharmacological treasure trove'.[32] Extensive reviews of Cannabis constituents are available in the literature.[4,27,33] The most important classes are listed in **Table 1**. The most interesting among these constituents are those found in the secretions of the head cells of glandular hairs (trichomes) distributed across the surface of the Cannabis plant. Although trichomes can be found all over the male and female plants, they are particularly concentrated on the bracts that support the female inflorescence. Solitary resin glands, consisting of one or two dozen cells, most often form at the tips of slender trichome stalks that form as extensions of the plant surface, as shown in **Figure 2**. The resin excreted by the glands contains a variety of constituents, any of which might play a role in the biological activities of the Cannabis plant. Among these are terpenoids, flavonoids, and cannabinoids. Resin collects under a thin waxy membrane surrounding the secretory head cells. In these extracellular resin pockets, the secreted compounds are segregated from the secretory cells, protecting it from both oxidative degradation and enzymatic change. A layer of abscission cells at the base of each secretory head allows the gland to be easily removed.[34]

The adaptational significance of the resin glands remains speculative. Although the resin gives a certain defense against insect and fungal attack, Cannabis crops are still vulnerable to attack by a wide variety of pests, particularly under greenhouse conditions. Certainly, the intoxicating effects of Cannabis resin have increased Cannabis predation by humans, as well as encouraged its domestication, thus dramatically widening its distribution. It has been shown that the cannabinoids, cannabigerolic acid (CBGA) and THCA, induce cell

Table 1 An overview of compounds identified in Cannabis

Compound class	*Compounds identified*
Terpenoids	>120
Cannabinoids	>70
Hydrocarbons	50
Sugars and related compounds	34
Nitrogenous compounds	27
Noncannabinoid phenols	25
Flavonoids	23
Fatty acids	22
Simple acids	21
Amino acids	18
Simple ketones	13
Simple esters and lactones	13
Simple aldehydes	12
Proteins, glycoproteins, and enzymes	11
Steroids	11
Elements	9
Simple alcohols	7
Pigments	2
Vitamin	1 (vitamin K)

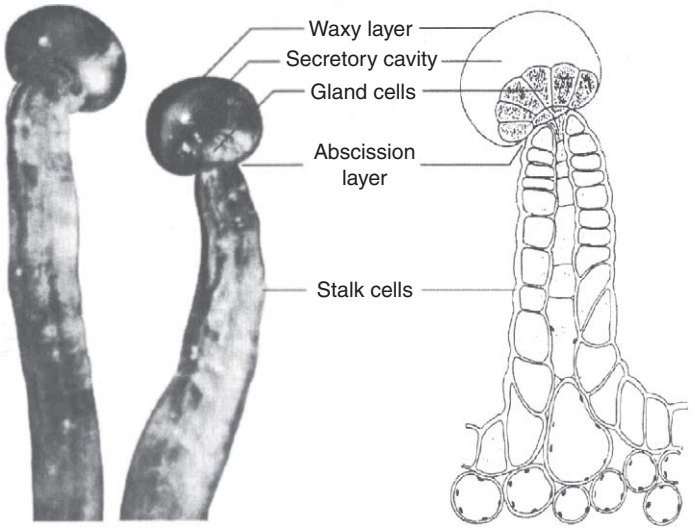


Figure 2 Microscope photograph and drawing of a Cannabis resin gland, with secretory head cells visible underneath the transparent cannabinoid- and terpenoid-rich resin. Photo courtesy by Hashish and R. Clarke, Los Angeles: Red Eye Press, 1998. Reprinted with permission. Drawing from HASHISH!, by R. Clarke.

death through apoptosis in some plant cells as well as insect cells.[35] Furthermore, formation of THCA was found to be linked to hydrogen peroxide formation, which may contribute to the self-defense of the Cannabis plant. These results strongly suggest that cannabinoids act as plant defense compounds, which is a common function of plant secondary metabolites.[36]

The compounds described in **Table 1** have all been identified as a constituent of some preparation of Cannabis: herbal plant material, whole extracts, and chromatographic fractions, or illicit material such as hashish. In many cases, the material used has been obtained from an uncontrolled source (e.g., materials confiscated by authorities) and its quality cannot be guaranteed. It is therefore not certain how many compounds, identified from such materials, should be considered as artifacts, resulting from oxidation, and enzymatic, thermal, or other degradation. In fact, even THC itself is not produced by the metabolism of the Cannabis plant but rather is formed by thermal decarboxylation (loss of CO_2) of THCA. Further degradation of THC results in the formation of cannabinol (CBN) or delta-8-tetrahydrocannabinol (delta-8-THC). Also, many terpenoids are known to be susceptible to degradation upon storage or extraction. As such, the chemical composition of any given Cannabis preparation depends not only on its biosynthetic composition, but also on factors such as age, conditions of storage, and method of extraction. Any biological activity claimed for such preparations should therefore be considered with some care.

Cannabinoids, terpenoids, flavonoids, and fatty acids (hemp oil) comprise the most interesting classes of biologically active compounds from Cannabis. They will be discussed in detail in Sections 3.24.2 and 3.24.5, and constituents in these classes that deserve more scientific attention will be highlighted. Several less significant classes of secondary metabolites found in Cannabis will be discussed shortly in Section 3.24.5.4.

3.24.2 Cannabinoids

Cannabinoids are considered to be the main biologically active constituents of the Cannabis plant, and they can be found nowhere else in nature (for an exception, see Section 3.24.2.2). The majority of biological activities attributed to Cannabis have so far been linked to cannabinoids, and more specifically to THC. The naturally occurring cannabinoids form a complex group of closely related compounds of which currently 70 are known and well described.[4,33,31] New cannabinoids, although present in very minor quantities, have been discovered very recently. These include 11 new cannabinoid esters and a number of other cannabinoid structures.[37,38]

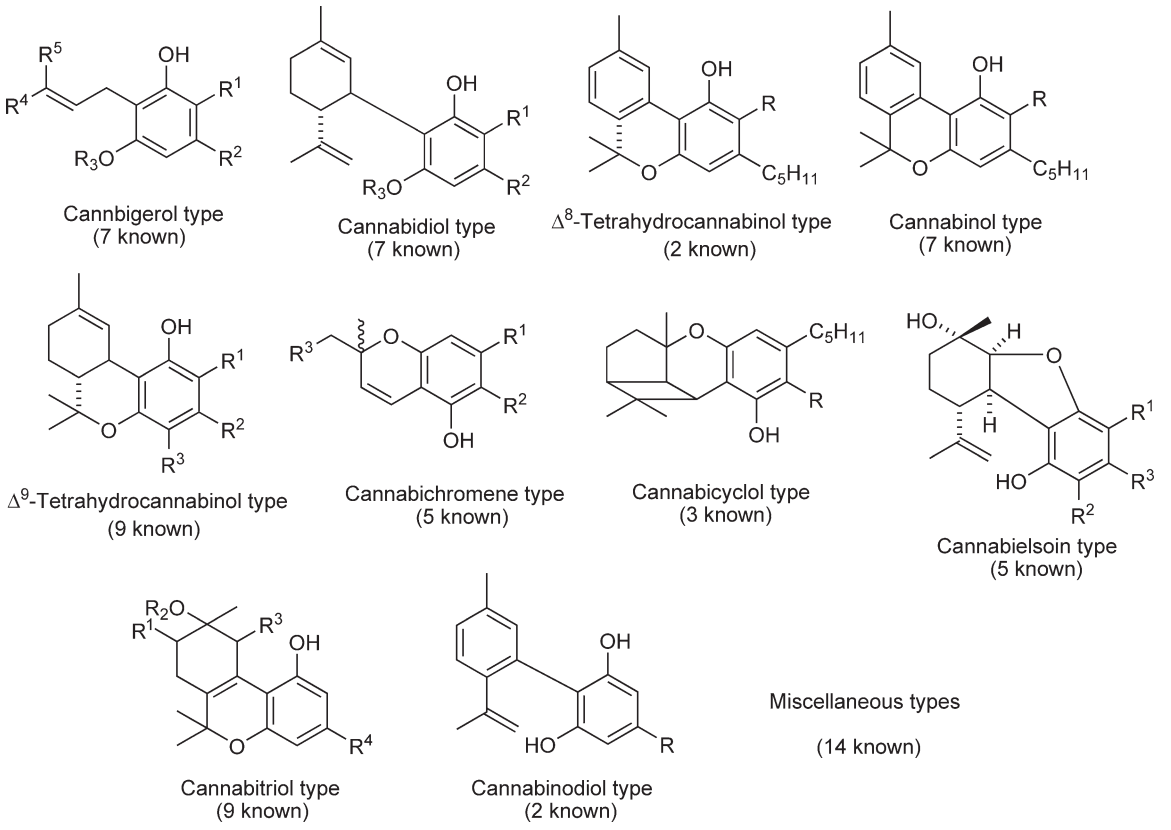


Figure 3 The structural types of cannabinoids found in *Cannabis sativa* L.

There are trace compounds that might have a limited occurrence in certain varieties, but they may add to our increasing understanding of the complexity of cannabinoid biosynthesis.

Cannabinoids can be divided into 10 main structural types (**Figure 3**). All other compounds that do not fit into the main types are grouped as miscellaneous.

3.24.2.1 Cannabinoids Defined

Until the 1980s, the term cannabinoids represented by definition the group of typical terpenophenolic C_{21} compounds present in *C. sativa*, their carboxylic acids, analogues, and transformation products. But from this rather restricted pharmacognostic definition, considerable expansion is now required. A modern definition will put more emphasis on synthetic chemistry and on pharmacology, and would also include related structures or compounds that affect cannabinoid receptors. This, however, creates several chemical subcategories of cannabinoids. The term 'cannabinoids' now represents the whole set of endogenous, natural, and synthetic ligands of the cannabinoid receptors, belonging to a wide variety of chemical families. The plant-derived cannabinoids are now often termed phytocannabinoids. When the word cannabinoids is used in this chapter, the naturally occurring phytocannabinoids are meant, unless indicated otherwise. It should be emphasized that not all phytocannabinoids bind to the cannabinoid receptors.

The first cannabinoid was isolated in 1940.[39] Chemical analysis indicated it to be an alcohol, so it obtained the rather straightforward name CBN ($C_{21}H_{26}O_2$). It was, however, found to be inactive as a psychoactive compound. Chemically, the cannabinoids belong to the terpenophenols, which are common in nature. Cannabinoids are accumulated in the glandular hairs (see Section 3.24.1.4), where they typically make up more than 80% of the subcuticular secretion. In general, all plant parts can contain cannabinoids, except for the seeds. The traces of cannabinoids found in seeds are most likely a result of contamination with Cannabis resin

from the flowers.[17,40] Essentially, there are no qualitative differences in cannabinoid spectrum between plant parts, only quantitative differences.[21,41] The highest cannabinoid concentrations (in percentage of dry weight plant material) can be found in parts of the flowers and fruits. In the foliage leaves the content is lower, and in the stems and, even more so, the roots the content is very low.[42] Cannabis grown outdoors generally has lower levels of cannabinoids when compared to indoor grown plants. When grown under artificial, high-yielding conditions, Cannabis flowering parts can be obtained with a resin content of up to 25–30%, mainly consisting of THCA, the acidic precursor of THC (see Section 3.24.2.2).

3.24.2.2 Biosynthesis of the Cannabinoids

The cannabinoids most commonly detected in herbal Cannabis materials are shown in **Figure 4**. For the chemical numbering of cannabinoids, five different nomenclature systems have been used so far,[43] but the most commonly used system nowadays is the dibenzopyran numbering, which is also adopted by Chemical Abstracts. In Europe, the monoterpenoid system based on *p*-cymene has also been widely used. As a result, the major cannabinoid delta-9-THC is sometimes described as delta-1-THC in older manuscripts. In this chapter, the dibenzopyran numbering is consistently used, therefore THC is fully described as (−)-*trans*-Δ^9-tetrahydrocannabinol (**Figure 5**).

It is commonly thought that cannabinoids are unique compounds only found in Cannabis. However, some exceptions exist in the plant kingdom: In *Helichrysum umbraculigerum* Less., a species from the family Compositae, the presence of CBGA, cannabigerol (CBG), and analogues to CBG was reported.[44] Moreover, in liverworts from *Radula* species the isolation of geranylated bibenzyls analogous to CBG was reported,[45] suggesting the homology of genes from the cannabinoid pathway in at least some other species.

3.24.2.2.1 The acidic cannabinoids

In all biosynthetic pathways for cannabinoids that were postulated until 1964, CBD or cannabidiolic acid (CBDA) was regarded as the key intermediate, which was supposedly built from a monoterpene and olivetol or olivetolic acid (OA), respectively. However, Gaoni and Mechoulam[46] showed that CBG is the common precursor of cannabinoids, biosynthesized through the condensation of geranyldiphosphate and olivetol or OA. Subsequently, they concluded that CBD, THC, and CBN all derive from CBG and differ mainly in the way this precursor is cyclized.[47–50] A further improvement of our understanding of cannabinoid biosynthesis came when Shoyama *et al.*[51,52] concluded that neither the free phenolic (noncarboxylic acid) forms of the cannabinoids nor cannabinolic acid (CBNA) were produced by the living plant. Instead, they postulated a biosynthetic pathway based on geraniol and a polyketoacid, resulting in the production of the acidic cannabinoids. The same conclusion was reached by Turner and Hadley[53] after the study of African Cannabis types.

It is now known that cannabinoids are produced by the metabolism of the plant in the form of carboxylic acids, where the substituent at position 2 is a carboxyl moiety (–COOH).[52] Incorporation studies with ^{13}C-labeled glucose have confirmed that geranyl diphosphate (GPP) and OA are specific intermediates in the biosynthesis of cannabinoids.[54,55] The first specific biosynthetic step is the condensation of GPP with OA into CBGA, catalyzed by the prenylase enzyme geranyldiphosphate:olivetolate-geranyltransferase (GOT).[54] Furthermore, biosynthetic pathways finally became clear by identification and subsequent cloning of the genes responsible for the conversion of CBGA to THCA, CBDA, and cannabichromenic acid (CBCA), respectively.[56–58] Further oxidation of THCA leads to the formation of CBNA, which is still formed after the plant material is harvested and high levels could be due to poor storage conditions (**Figure 6**).

The terpenoid GPP is derived from the deoxyxylulose phosphate/methyl-erythritol phosphate (DOXP/MEP) pathway.[55,59] Not much is known about the biosynthesis of OA yet, but it has been proposed that a polyketide synthase (PKS) could be involved.[59] However, a PKS specifically yielding OA has not been found to date. Interestingly, OA itself has never been isolated from the plant material, possibly indicating it to be a very short-lived intermediate.

Mahlberg and Kim[60] reported that glandular trichomes are exclusively specialized to synthesize high amounts of cannabinoids and that other tissues contain only very low amounts. These authors distinguished three types of glandular trichomes in Cannabis, with different localization, morphology, and cannabinoid content. Cannabinoids are deposited in the noncellular, secretory cavity of glandular trichomes. However, after

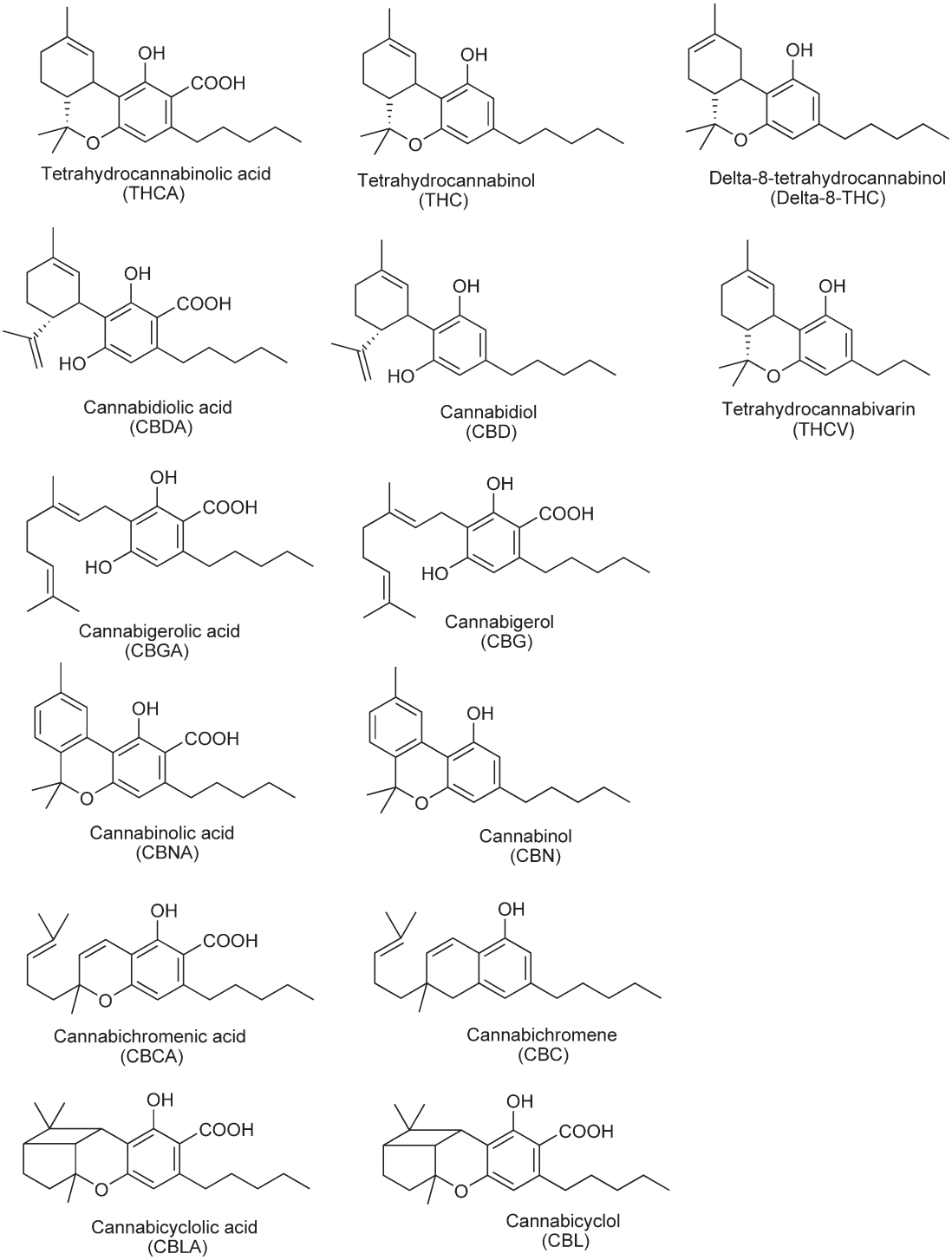
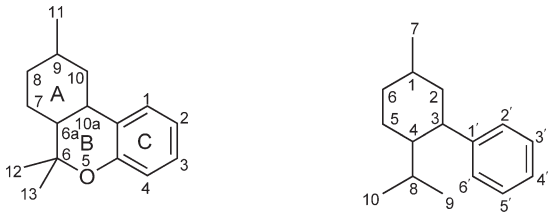


Figure 4 Structures of the cannabinoids most commonly found in Cannabis plant materials. All cannabinoids have the (6a*R*,10a*R*)-orientation, according to the chemical numbering shown in **Figure 5**.

confirming the presence of the central precursor CBGA, as well as THC synthase activity in the secretory cavity, it was suggested that this is not only the site of cannabinoid accumulation, but also the site of cannabinoid biosynthesis.[35]



Dibenzopyran-numbering Monoterpene-numbering based on *p*-cymene

Figure 5 Two most commonly used numbering systems for the cannabinoids. The dibenzopyran system is used in this chapter.

3.24.2.2.2 Occurrence of short-chain cannabinoids and other homologues

Most commonly, the acidic cannabinoids produced by plant metabolism contain a pentyl side chain, derived from the OA moiety. Cannabinoids with propyl side chains result if GPP condenses with divarinic acid instead of OA, into cannabigerovarinic acid (CBGVA). The three known cannabinoid synthase enzymes are not selective for the length of the alkyl side chain, and will convert CBGVA into the propyl homologues of CBDA, THCA, and CBCA.[61] All chain lengths from –methyl to –pentyl have been found in naturally occurring cannabinoids, probably all arising from the incorporation of shorter chain homologues of OA. The side chain is important for the affinity, selectivity, and pharmacological potency for the cannabinoids receptors.

Many other minor acidic cannabinoids have been identified over the years, including monomethyl and other types of esters.[38,62] The biosynthetic pathways explaining this variation have been studied.[59]

3.24.2.3 A Phytochemical Classification of Cannabinoids

Cannabinoids are produced by the metabolism of the plant in the form of carboxylic acids as discussed in Section 3.24.2.2. However, a range of other types of cannabinoids have been detected in Cannabis. For a clear phytochemical discussion of the cannabinoids, they can most conveniently be divided into three groups: acidic cannabinoids; neutral cannabinoids; and 'artifacts'. This practical classification of the cannabinoids is shown in **Figure 7**.

An important distinction that can be made within the group of cannabinoids is between the so-called acidic and neutral cannabinoids. Consequently, in fresh plant material almost no neutral cannabinoids can be found, but theoretically all cannabinoids are present in this acidic form. These can be converted into their decarboxylated analogues under the influence of light, heat, or prolonged storage, by losing the relatively unstable carboxylic group in the form of carbon dioxide.[63] For the purpose of this chapter, the original plant-derived carboxylic acids will be indicated as 'acidic' cannabinoids, whereas their decarboxylated counterparts are indicated as 'neutral' cannabinoids, even though the presence of the phenolic moiety in the neutral cannabinoids may of course classify them as acids as well.

The group of the acidic cannabinoids includes a large number of structures. The most common types of acidic cannabinoids found in a typical drug-type Cannabis plant are THCA, CBDA, CBGA, and CBCA. These acids can be converted to their neutral counterparts by decarboxylation to form THC, CBD, CBG, and cannabichromene (CBC), respectively. An example of this conversion is shown in **Figure 8**.

The group of cannabinoids that occur as a result of degradative conditions deserve some special attention, because their presence is largely the result of variable and unpredictable conditions during all the stages of growing, harvesting, processing, storage, and use. As a result, a well-defined Cannabis preparation may change rapidly into a product with significantly different biological effects. Degradation of THC results in the formation of CBN and delta-8-THC, whereas THCA can further degrade into CBNA.[4] Particularly, in samples that have been stored for an extended period, CBN can be found in relatively large amounts. The isomerization of delta-9-THC to delta-8-THC is well documented and occurs particularly at elevated temperature, where the equilibrium is toward the delta-8 isomer.[49,50,64] The cannabinoids cannabicyclol

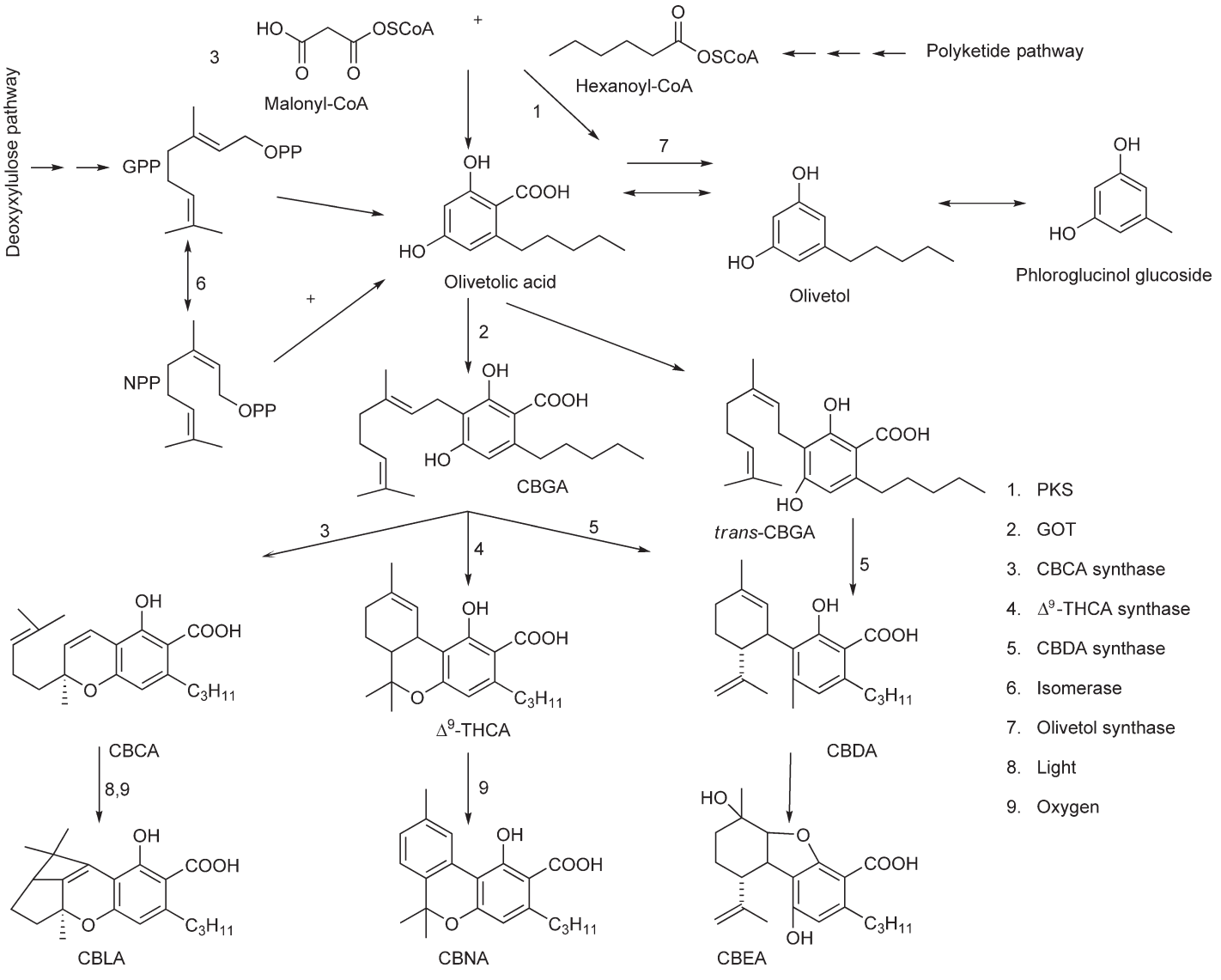


Figure 6 General overview of the biosynthesis of cannabinoids and putative routes. Reproduced with permission from I. J. Flores-Sanchez; R. Verpoorte, *Phytochem. Rev.* **2008**, *7*, 615–639.

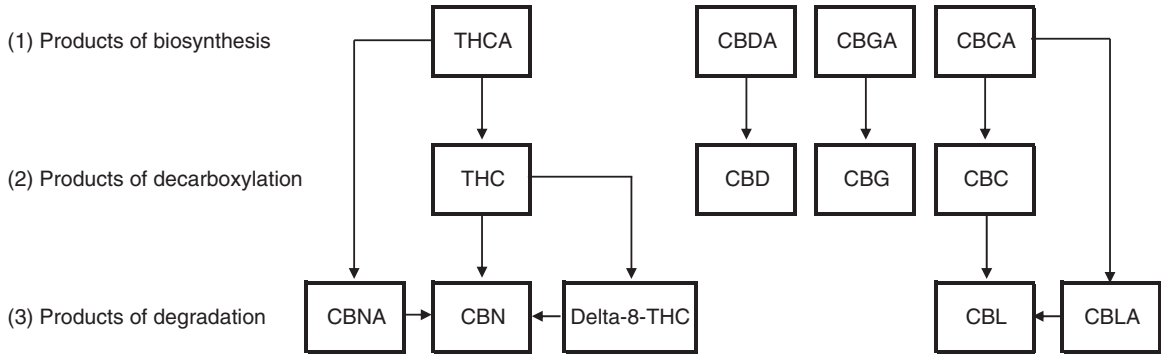


Figure 7 A practical way to categorize the relationships between the major cannabinoids found in herbal Cannabis. Three different groups may be distinguished: (1) cannabinoids produced by biosynthesis of the plant; (2) cannabinoids resulting from natural decarboxylation of acidic cannabinoids; and (3) degradation products resulting from various influences, such as UV-light, oxidation, or isomerization. Arrows indicate the routes of conversion.

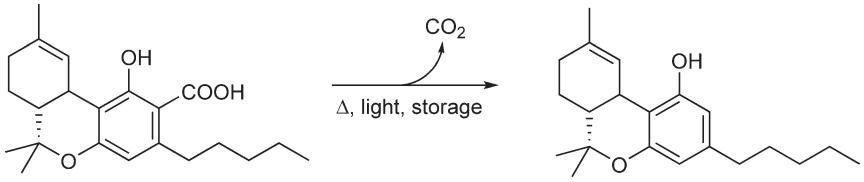


Figure 8 Decarboxylation: the example THCA → THC is shown.

(CBL) and cannabicyclolic acid (CBLA) arise by the exposure of CBC and CBCA, respectively, to UV radiation, leading to cross-linking of two double bonds in the molecule.[65]

Although a large variety of cannabinoids is known, this does not mean that all cannabinoids are detectable in all Cannabis products. They were identified over several decades of Cannabis research, studying many different Cannabis products and different and sometimes rare types of Cannabis plants from a variety of origins and qualities. There can be an enormous variation in their quantitative ratios. The different chemical types of cannabinoids have been well described.[4,66] Understanding how the cannabinoids are (chemically) related to each other is important when studying Cannabis samples, as degradation and changes in the cannabinoid profile might occur as a result of storage or breeding conditions, variations in preparation of medicines, heating, and so on.

3.24.2.4 Chromatographic Analysis of Cannabinoids

For the analysis of highly pure, single cannabinoid preparations, specific analytical procedures can be easily developed. However, most phytochemical and pharmacological studies are far more complex than that. For example, studies on cannabinoid biosynthesis and localization in Cannabis require accurate identification of the whole cannabinoid profile in fresh plant tissues. Also, the identification, classification (e.g., fiber type vs drug type), and individualization (e.g., source tracing) of Cannabis plants and products do not often rely on the analysis of only a single cannabinoid. Furthermore, increasingly *in vitro* and *in vivo* studies are being conducted with whole Cannabis preparations, either as raw plant materials (e.g., powdered flower tops) or as extracts.[67–70] The bioactivities found for such preparations are possibly the result of the acidic cannabinoids,[71] and consequently a method must be available to identify and quantify neutral as well as acidic cannabinoids present in the plant materials used.

For the chemical analysis of cannabinoids, the analytical methods that are available have been extensively reviewed by Raharjo and Verpoorte.[72] Because of the complex chemistry of Cannabis, advanced separation techniques, such as high-performance liquid chromatography (HPLC) and gas chromatography (GC), often coupled with mass spectrometry (MS) detection, are often necessary for the acquisition of the typical chemical profiles of Cannabis constituents. However, especially for screening purposes and on-site field testing, noninstrumental techniques like thin-layer chromatography (TLC) and color reactions may be helpful, too.

3.24.2.4.1 Decarboxylation

Owing to the thermal lability of the acidic cannabinoids, indirect methods have been widely used for their determination. These indirect methods are based on the decarboxylation of the acids and subsequent HPLC determination of the neutral cannabinoids formed. But although decarboxylation occurs naturally over time, during storage of cannabinoids, it is difficult to perform a quantitative decarboxylation under experimental conditions. When performing the thermal decarboxylation of cannabinoid acids in either the presence or absence of organic solvents in an open reactor, an optimum temperature at which the velocity of the decarboxylation would be high enough and simultaneous evaporation of neutral cannabinoids would not occur could not be found.[63] Consequently, it is not possible in this manner to obtain an amount of neutral cannabinoids equivalent to that of the cannabinoid acids from which they were decarboxylated. During the decarboxylation on different sorbent surfaces, the evaporation of cannabinoids was hindered by sorptive effects, but simultaneous side-reactions occurred, causing chemical changes of the neutral cannabinoids.[63]

3.24.2.4.2 Microscopy

Identifying a plant sample as *C. sativa* L. may be done simply by using macroscopic and microscopic evaluation of the intact plant material. The botanical identification of plant specimens consists of physical examination of the intact plant morphology and habit (leaf shape, male and female inflorescences, etc.) followed by the microscopical examination of leaves for the presence of cystolith hairs (as shown in **Figure 2**). The very abundant trichomes, which are present on the surface of the fruiting and flowering tops of Cannabis, are the most characteristic features to be found in the microscopic examination of herbal Cannabis products.[62]

3.24.2.4.3 Color reactions

The most common color spot tests include those developed by Duquenois and its modifications. A study of 270 different plant species and 200 organic compounds has shown that the Duquenois–Levine modification is most specific.[73] The fast blue B salt test is the most common color reaction for the visualization of TLC patterns (see below) but may also be used as spot test on a filter paper.[74]

It must be stressed that positive reactions to color tests are only presumptive indications of the possible presence of Cannabis products or materials containing Cannabis products. A few other materials, often harmless and uncontrolled by national legislation or international treaties, may react with similar colors to the test reagents.[62]

3.24.2.4.4 Thin-layer chromatography

One- and two-dimensional TLC is suited for the acquisition of qualitative cannabinoid profiles from plant material. Both normal-phase and reversed-phase TLC methods have been described.[75] For selective visualization of cannabinoids, the TLC plate can be sprayed with 0.5% fast blue B salt (*o*-dianisidine-bis-(diazotized)-zinc double salt) in water, followed by 0.1 mol l^{-1} NaOH.[74] For quantitation, instrumental TLC coupled to densitometry is necessary. High-pressure TLC and overpressured layer chromatography have been developed for the reproducible and fast determination and isolation of neutral and acidic cannabinoids.[76–78]

3.24.2.4.5 Gas chromatography

The use of GC, commonly coupled to flame ionization detection (FID) or MS detection, permits the analysis of a large variety of cannabinoids with very high resolution. However, derivatization is necessary (e.g., silylation and methylation) when information about cannabinoid acids, the dominating cannabinoids in the plant, is required.[62] Because it is hard to perform a quantitative derivatization for all components in a complex mixture, GC analysis may have only limited value when studying the authentic composition of Cannabis products.

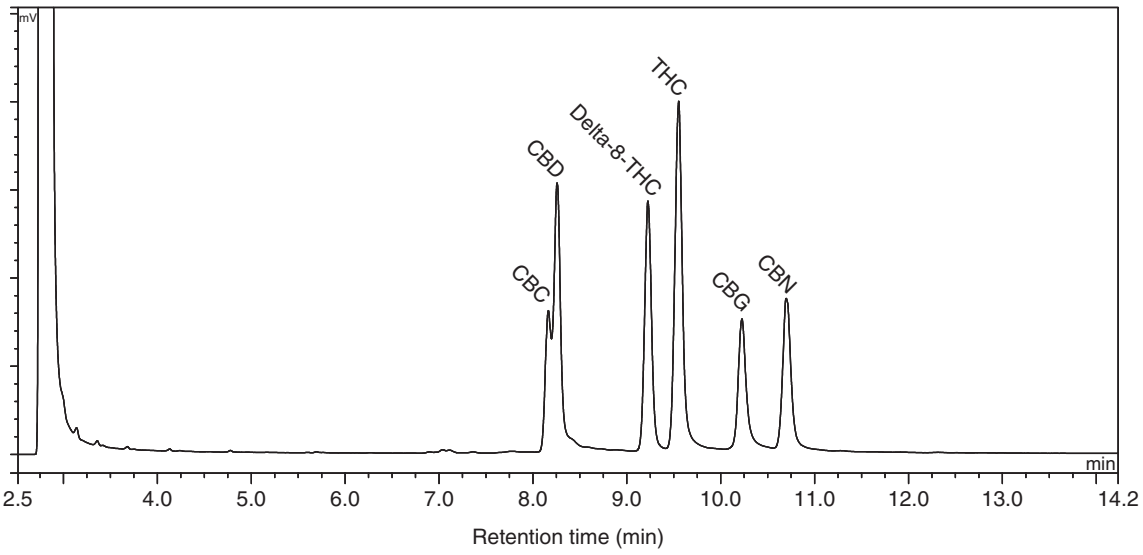


Figure 9a A typical GC chromatogram (FID detection) obtained according to Hazekamp *et al.*[75] Column: Durabond fused silica capillary column (30 m × 0.25 mm inner diameter) coated with DB-1 at a film thickness of 0.1 μm (J&W Scientific Inc., Rancho Cordova, CA). The oven temperature was programmed from 100 to 280 °C at a rate of 10 °C min^{-1}.

GC/MS is the method of choice for creating Cannabis profiles and signatures (chemical fingerprints), a tool for attributing the country of origin, the conditions of cultivation (indoor, outdoor), and so on. A representative GC-FID chromatogram is shown in **Figure 9(a)**.

3.24.2.4.6 High-performance liquid chromatography

HPLC has made it possible to simultaneously detect cannabinoids in both the acid and neutral forms, without the need of derivatization. By making use of an UV- or photodiode array (PDA) detector, cannabinoids can be efficiently analyzed without causing degradation of sample components. Thus, HPLC has become the method of choice for most laboratories. A representative HPLC chromatogram is shown in **Figure 9(b)**. However, the analysis of all major cannabinoids in a typical Cannabis extract is not easily achieved, because of the complex composition resulting in chromatographic overlap of peaks. To overcome this problem, the use of MS detection (LC–MS) to distinguish between overlapping chromatographic peaks is becoming increasingly important.[75,79]

Validated HPLC methods exist for the analysis of cannabinoids according to the American United States Pharmacopoeia (USP) or German the German Drug Codex (DAC) guidelines. However, these were specifically developed for the analysis of impurities in highly pure preparations of THC, derived from either synthetic (USP method) or natural source (DAC method). They were not intended, and hence not validated, for use with whole Cannabis plant materials. More recently, a fully validated pharmacopoeia method was developed for the quality control of Cannabis produced for the Dutch medicinal Cannabis program.[80] This ultra-performance LC (UPLC) method has been validated according to International Conference on Harmonization (ICH) guidelines and is suitable for the analysis of a wide range of authentic cannabinoids in herbal Cannabis. A representative UPLC chromatogram is shown in **Figure 9(c)**.

3.24.2.4.7 Other techniques

Occasionally, new methods are explored for the analysis of cannabinoids. The applicability of capillary electrochromatography with photodiode array UV detection for the analysis of cannabinoids has been demonstrated.[81] Also, supercritical fluid chromatography has been studied,[82] but with limited success. Supercritical fluid chromatography is characterized by shorter analysis times than GC or HPLC and does not require derivatization.

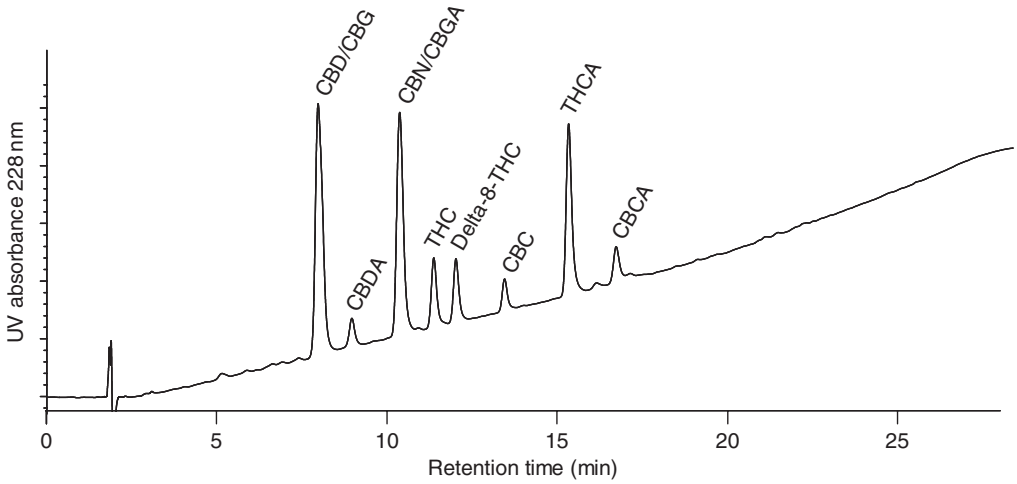


Figure 9b A typical HPLC chromatogram (228 nm) obtained according to Hazekamp *et al*.[115] Column: Waters XTerra MS C_{18} (2.1 × 150 mm, 3.5 μm); eluent: methanol/water gradient with linear increase of methanol from 65 to 100% over 25 min; flow rate: 1.5 ml min^{-1}; detection wavelength: UV at 228 nm. The baseline was not corrected for the influence of the methanol gradient.

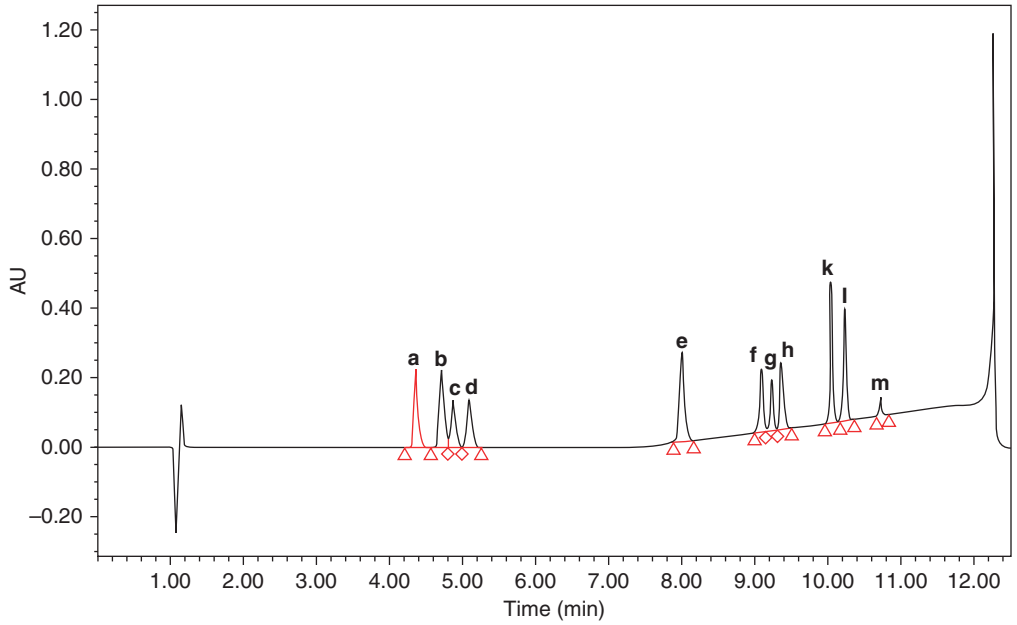


Figure 9c A typical UPLC chromatogram obtained using the following method: Column: Waters C_{18} analytical column (1.7 μm, 2.1 × 150 mm); eluent: acetonitrile/water/0.1% formic acid; 0–6 min isocratic 70% acetonitrile, 6–10.5 min gradient to 100% acetonitrile, 10.5–11 min hold at 100% acetonitrile; flow rate: 0.3 ml min^{-1}; detection wavelength: UV at 228 nm. a, CBDA; b, CBGA; c, CBG; d, CBD; e, CBN; f, THC; g, delta-8-THC; h, CBNA; k, CBC; l, THCA; m, CBCA.

3.24.2.4.8 Spectroscopic and chromatographic data

A final important factor in the effective analysis of the cannabinoids is the availability of reliable spectroscopic and chromatographic data. Although such data have been published for most known cannabinoids during isolation and identification experiments (see Turner *et al*.[4] for an overview), they were scattered over a huge amount of scientific papers. In 2005, Hazekamp *et al*.[75] determined chromatographic and spectroscopic data for 16 different naturally occurring cannabinoids as well as two human metabolites under standardized conditions.

Spectroscopic analyses performed were UV-absorbance, infra-red spectral analysis, (GC–) mass spectrometry, and spectrophotometric analysis. Also, the fluorescent properties of the cannabinoids were presented. Chromatographic data includes relative retention times in HLPC, GC, and TLC. In a similar standardized fashion, the complete ^{1}H- and ^{13}C-NMR assignments of several major cannabinoids have been summarized.[83]

3.24.3 Sites and Mechanisms of Action of Cannabinoids

The majority of studies on the biological effects of Cannabis constituents have been done with cannabinoids. No other class of Cannabis compounds received anything near as much attention. The reason for this is clear: cannabinoids are unique to the Cannabis plant, whereas all the other classes of compounds can also be found elsewhere in nature. Therefore, the majority of this chapter is dedicated particularly to the cannabinoids. And among the cannabinoids, virtually all studies have focused on the effects of THC. So almost by necessity, this section will focus on THC to discuss what is known about the receptor-binding, its metabolism, and other mechanisms involved in understanding the biological effects of cannabinoids. However, other cannabinoid constituents will be discussed where possible, based on the literature available.

In general, biological effects take place through activation of receptors, so it needs no explanation that the psychoactive effects of THC led to the hunt for specific binding sites. Analogues of THC, chemically modified and radiolabeled, served as a tool for the identification of cannabinoid receptor 1 (CB1) in the rat brain, soon followed by the discovery of the CB2 receptor. These findings prompted the search for endogenous ligands, which was guided by the chemical concept that, by homology to the highly lipophilic THC, physiological cannabinoid receptor ligands were to be looked for among endogenous lipids rather than the more polar peptides like the endorphins.

It is now known that cannabinoid receptors can be found in most parts of the brain, as well as in the immune system and a variety of other organs. Their distribution seems to explain many of the observed effects of Cannabis consumption. Such a variety of effects was concisely summarized by Di Marzo *et al.*:[84] endocannabinoids make you 'feel less pain, control your movement, relax, eat, forget (posttraumatic), sleep, and protect your neurons'. The activation of the endogenous cannabinoid system could represent a crucial and important component for each of these functions. The endocannabinoid system that is responsible for our physiological response to Cannabis use is analogous to the morphine–endorphin system. Interestingly, cross talk between the two systems has been shown.[85]

In this section, the sites and mechanisms of action of the cannabinoids are discussed. After describing the discovery of Cannabis receptors, the endocannabinoids will be discussed. The understanding of this endogenous system explains the effects of Cannabis on human physiology, which will be further clarified by looking in more detail at THC. Information on absorption, distribution, metabolism, and elimination will help to explain the possible role of synergy and interaction between Cannabis components. Finally, the effect of administration forms on the observed biological effects of cannabinoids is discussed.

3.24.3.1 The Cannabinoid Receptors: CB1 and CB2

The cannabinoid receptors are a class of receptors under the G-protein-coupled receptor superfamily. Their ligands are known as cannabinoids or endocannabinoids depending on whether they come from external or internal (endogenous) sources. Cannabinoid receptors have a protein structure defined by an array of seven transmembrane-spanning helices with intervening intracellular loops and a C-terminal domain that can associate with G proteins of the $G_{i/o}$ family.[86]

Until the discovery of specific Cannabis receptors, the biochemical mode of action of cannabinoids was much debated. Because of their lipophilic character, cannabinoids can penetrate cellular membranes by simple diffusion. Therefore, possible explanations for cannabinoid activity initially included unspecific membrane binding resulting in fluidity and permeability changes of neural membranes, the inhibition of acetylcholine synthesis, an increase in the synthesis of catecholamines, and an interaction with the synaptosomal uptake of serotonin.[87,88] However, it was established in the mid-1980s that cannabinoid activity is highly stereoselective,[89] indicating the existence of a receptor-mediated mechanism.

The first reliable indications that cannabinoids act through receptors came when it was shown that cannabinoids can act as inhibitors of the adenylate cyclase second messenger pathway in brain tissue and neuroblastoma cell lines. This activity was dose-dependent, stereospecific, and could be modulated by pertussis toxin.[90–94] Finally, a stereospecific G-protein-coupled cannabinoid receptor was found and cloned.[95] It was named 'cannabinoid-binding receptor type 1' or CB1. The CB1 receptor is most clearly localized in the central nervous system (CNS), therefore it is often called the 'central receptor', but it is also found in certain peripheral organs and tissues, such as lungs, liver, and kidneys.[96] CB1 receptors are thought to be the most widely expressed G-protein-coupled receptors in the brain. Activation leads to the inhibition of adenylate cyclase activity.[97] The CB1 receptor also modulates ion channels, inducing, for example, the inhibition of N- and P/Q-type voltage-sensitive Ca^{2+} channels and the activation of G-protein inwardly rectifying K^{+} channels. Besides these well-established cannabinoid receptor-coupled events, cannabinoid receptors have also been shown to modulate several signaling pathways that are more directly involved in the control of cell proliferation and survival, including extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt, focal adhesion kinase (FAK), and the ceramide pathway.[98]

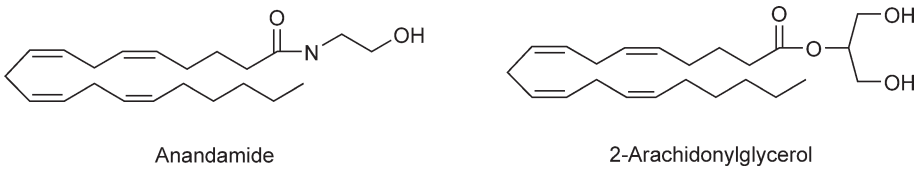
Subsequently, a second cannabinoid receptor (CB2) was found with a possible role in immunological processes.[99] The CB2 receptor was first described as a peripheral G-protein-coupled receptor (GPCR), mainly localized in the immune system, therefore it is often called the 'peripheral receptor'. However, nowadays it appears that the situation is more complex, as CB2 expression was also reported to be present in neurons of the brain.[100] It is primarily expressed by immune tissues such as leukocytes, spleen, and tonsils, and it shows a different selectivity than the centrally acting CB1. So far, the physiological roles of CB2 receptors are proving more difficult to establish, but at least one seems to be the modulation of cytokine release.[101] Recently, it has been recognized that CB2 may play a functionally relevant role in the CNS, mediated through microglial cells.[102]

The cannabinoid signaling system is teleologically millions of years old, as it has been found in mammals, fish, and invertebrates down to very primitive organisms such as the hydra.[103] Surprisingly, the protein sequences of CB1 and CB2 show only about 45% homology. There are indications that CB receptors are evolutionary related to the vanilloid receptors.[104] The transient receptor potential vanilloid receptor 1 (TRVR1) can be activated by the fatty acid amide compound capsaicin. Based on the chemical similarities between capsaicin and endocannabinoids (see Section 3.24.3.2), it was hypothesized that TRVR1 and proteins of the endocannabinoid system share common ligands. This was confirmed when it was demonstrated that the endocannabinoid anandamide activates TRVR1 receptors.[105] Also, it was found in isolated blood vessel preparations that some endocannabinoids can activate vanilloid receptors on sensory neurons,[106] which raises the possibility that endocannabinoids are endogenous agonists for vanilloid receptors.[107] These receptors might therefore be putatively regarded as CB3 receptors.

There is mounting evidence of novel receptors expressed in endothelial cells and in the CNS that have cannabimimetic and therapeutic effects independent of the mechanisms described above.[108] In 2007, the binding of several cannabinoids to a GPCR (GPR55) in the brain was described.[109] These receptors are more likely to be functionally related than structurally, as there is currently no evidence for additional cannabinoid receptors in the human genome. However, not all of the effects of cannabinoids can be explained by receptor-mediated effects, and it is believed that at least some effects are nonspecific and caused through membrane perturbation.[110,111]

3.24.3.2 The Endocannabinoid System

Based on the observation that all natural cannabinoids are highly lipid-soluble, an attempt was made to isolate endogenous ligands for the cannabinoid receptors from fatty tissues of animals. Finally, a single compound could be isolated from the brain tissue of pigs, with a high affinity for the CB1 receptor. It was chemically identified as arachidonic acid ethanolamide, and named anandamide, from the Sanskrit word for 'eternal bliss'.[112] A few years later, a related compound was isolated from canine gut with an affinity for both cannabinoid receptors: 2-arachidonylglycerol (2-AG).[113] Structures of these two compounds are shown in **Figure 10**. In recent years, a variety of compounds with endocannabinoid activity have been isolated or



Anandamide 2-Arachidonylglycerol

Figure 10 Structures of the two major endocannabinoids.

synthesized,[114,115] interestingly, all having an eicosanoid-related structure. Cannabinoid receptors and their endogenous ligands together constitute what is referred to as the endogenous cannabinoid, or endocannabinoid, system.

The endocannabinoid system is now known to be a ubiquitous neuromodulatory system with wide-ranging actions. It consists of cannabinoid receptors, endogenous cannabinoids, and enzymes responsible for their production, transport, and degradation. The endocannabinoid system can be found even in very primitive organisms, indicating it has a fundamental role in basic physiology. There are currently two main families of endocannabinoids that have been extensively characterized. The first are amides of arachidonic acid and ethanolamide; the typical example of this family is anandamide. The second family includes glycerol esters related to 2-AG. The biosynthetic pathways for both families of endocannabinoids are complex, but well reviewed.[116,117]

There are several pathways known for the synthesis and degradation of endocannabinoids, so there appears to be a high redundancy. Basically, endocannabinoids exist intracellularly as precursors in the plasma membrane of neurons as part of certain phospholipids. They are produced on demand by distinct biochemical pathways involving phospholipases C and D, as well as other enzymes. These events are triggered by the enhancement of intracellular calcium concentrations that follow cell depolarization or the mobilization of intracellular calcium stores subsequent to the stimulation of protein-coupled receptors from the G_q/G_{11} family. Accordingly, the enzymes catalyzing anandamide and 2-AG are calcium-sensitive. After formation, endocannabinoids are transported across the cell membrane for interaction with their extracellular binding sites on cannabinoid receptors.[116,117]

Endocannabinoids serve as extracellular retrograde messengers, with characteristics very different from other neurotransmitters such as acetylcholine, γ-aminobutyric acid (GABA), and dopamine. Endocannabinoids are described as retrograde transmitters because they most commonly travel backward against the usual synaptic transmitter flow: they are released from the postsynaptic cell and act on the presynaptic cell, where the target receptors are densely concentrated. Like the endorphins, endocannabinoids exert a homeostatic function. But because of their chemical (nonwater-soluble) nature, they cannot travel unaided for long distances in the aqueous medium surrounding the cells from which they are released. Therefore, endocannabinoids do not typically function like hormones, which can affect cells throughout the body, but instead they act as local (autocrine or paracrine) mediators. Activation of the cannabinoid receptors temporarily reduces the amount of conventional neurotransmitter released, thereby controlling the incoming synaptic signal. The ultimate effect of this process depends on the nature of the transmitter that is controlled, which itself depends on the function of the tissue where the cannabinoid receptors are expressed. Simply said, endocannabinoids produced by a certain neuron are modulators of the flow of other neurotransmitters produced by that same neuron.

Degradation is an important mechanism to regulate endocannabinoid activity, as the duration of endocannabinoid effect is dependent on the localization of the degrading enzymes.[117] The degradation system involves reuptake into the presynaptic cell, followed by rapid hydrolysis of the amide or ester bonds. How endocannabinoids move from the extracellular space to the interior of a cell for degradation remains unclear, but there is indirect evidence for specific proteins facilitating the membrane transport.[118] 2-AG exhibits higher selectivity and efficacy for CB1 and CB2 receptors than anandamide, which also interacts with noncannabinoid receptor targets. Therefore, it is not surprising that the levels of the two compounds are regulated in different ways. However, the main enzyme that inactivates both anandamide and 2-AG (and others) by hydrolysis is fatty acid amide hydrolase (FAAH). It was isolated after the synthesis of inhibitors of endocannabinoid degradation, which were then used for affinity chromatography purification of the degrading enzyme.[119] 2-AG is also inactivated by monoacylglycerol lipase (MAGL).

Interestingly, in *Arabidopsis thaliana*, a functional homologue of the mammalian FAAH has been cloned,[120] and fatty acid ethanolamines with high homology to anandamide have been discovered in several plant species.[121] These findings provide support at the molecular level for a conserved mechanism between plants and animals for the metabolism of *N*-acylethanolamines.

3.24.3.3 Pharmacokinetics of the Cannabinoids (ADME)

Cannabinoid pharmacokinetic research has been especially challenging because of low analyte concentrations in serum, rapid and extensive metabolism, and physicochemical characteristics that hinder the separation of drugs of interest from biological matrices, and from each other. Although many other administration forms have been developed (see Section 3.24.3.5 on administration forms), oral administration, often in the form of synthetic THC (Marinol), has been most extensively studied.

Although the metabolic fate of THC is well known,[27] not much has been reported on the other cannabinoids. After oral administration, THC is almost completely absorbed (90–95%); however, because of the first-pass metabolism by the liver and high lipid solubility, 90% or more of orally administered THC never reaches the sites of activity in the body in its native form.[122,123] THC and its metabolites are extensively protein bound in the blood (~97%) and rapidly distributed to highly vascularized tissues and the brain. Serum concentrations peak at approximately 0.5–4 h after oral dosing, and decline over several days. After oral administration, THC has an onset of action of approximately 1–2 h and duration of psychoactive effects is 4–6 h after administration.

In humans, plasma THC concentration profiles are similar after smoking or intravenous administration, with prompt onset and steady decline. Metabolism occurs mainly in the liver by microsomal hydroxylation, and oxidation catalyzed by enzymes of the P-450 complex. Nearly 100 metabolites have been identified for THC alone. Besides the liver, other tissues like the heart and lungs are also able to metabolize cannabinoids, albeit to a lesser degree. The two major metabolites of THC are 11-hydroxy-THC (11-OH-THC) and 11-nor-9-carboxy-THC (11-COOH-THC). 11-OH-THC is the most important psychotropic metabolite, being about twice as psychoactive as THC, and it has a similar kinetic profile as the parent molecule.[124] In contrast, 11-COOH-THC has no psychotropic activity. Most of the 11-COOH-THC is finally converted into its glucuronide form, with a glucuronic acid moiety linked to the carboxylic group. This is the major form of THC excreted into urine. When THC is inhaled through smoking or vaporizing, it avoids first-pass metabolism, and conversion into 11-OH-THC and further metabolites takes place much slower.[124]

Metabolism is the major route for the elimination of THC from the body. The elimination is biphasic; there is a rapid distribution phase (initial half-life about 4 h), believed to be due to the highly lipophilic nature of the drug and redistribution into lipid-rich tissues, and a terminal half-life of around 25–30 h for THC and 11-OH-THC. Plasma half-life for 11-COOH-THC may be even as long as 25–75 h.[125,126]

Only negligible amounts of THC are excreted in unchanged form; less than 5% of an oral dose is recovered unchanged in the feces. Most of the absorbed dose (65–80%) is excreted as metabolites in the feces and a lesser amount in the urine (20–35%). Among the metabolites, 11-COOH-THC is a major one identified in both urine and feces, both in its native form and in the form of its glucuronide. Because of its large volume of distribution ($\sim 10\,l\,kg^{-1}$), THC and its metabolites may be excreted at low levels for prolonged periods of time. Following single-dose administration, low levels of THC metabolites have been detected for more than 5 weeks in the urine and feces.[126]

For the other cannabinoids, only the metabolism of CBD and CBN has been described to some extent.[127] An important aspect of CBD is that it inactivates certain types of cytochromes, which may be important because serious drug–drug interactions may occur in the case that CBD is coadministered with drugs that are metabolized mainly by the enzyme system containing these P-450 isozymes.[128]

So far, virtually nothing was reported on absorption, distribution, metabolism, and excretion (ADME) of the major cannabinoid THCA. In a case study (unpublished data by the author), it was found that the oral consumption of 30 mg pure THCA did not lead to psychotropic effects and no THC metabolites could be detected in urine by a standardized GC–MS detection method for THC. So, despite their structural similarity, the metabolism of THCA seems to be quite distinct from THC.

3.24.3.4 Structure–Activity Relationships of Cannabinoids

There is a central problem in the discussion about making Cannabis or THC medicinally available: the curative properties of Cannabis/THC are mediated mainly by the same receptors that cause its unwanted psychoactive side effects. So, just as for other psychoactive drugs (e.g., morphine, benzodiazepines), the accepted medicinal applications are limited. As a result, a major goal of cannabinoid research nowadays is to separate beneficial from unwanted effects by means of medicinal chemistry, studying structure–activity relationships (SARs). The same approach is also used to develop agonists or antagonists with a high selectivity for only one of the cannabinoid receptors.

The absolute configuration of naturally occurring THC is *trans*-(6a*R*,10a*R*), resulting in a negative specific rotation. The preparation of the *cis*-(+)-enantiomer of THC, and subsequent pharmacological comparison to its natural counterpart, gave a decisive argument for the stereospecificity of the binding, and thereby reinforcing the cannabinoid–receptor interaction hypothesis. Since this discovery, cannabinoids have been extensively studied to understand the relationships between their structure and affinity for the Cannabis receptors. For this purpose, a very large number of synthetic cannabinomimetics have been made and systematically tested for receptor binding on CB1 and CB2.

Large numbers of compounds have been studied for the SAR of both known CB receptors. But in addition to CB1 and CB2 receptors, pharmacological studies have strongly suggested the existence of other cannabinoid receptor subtypes,[129] the most likely candidate being the vanilloid TRVR1 receptor (see Section 3.24.3.1). This essentially means that all available cannabinoids produced for SAR studies could be tested again for binding affinity to this receptor. This may lead to new clues about the mechanism of action for certain bioactivities of cannabinoids.

Classical cannabinoids and endocannabinoids are both agonists of the CB receptors, but because of the different structural features of CB1 and CB2, their SAR is not entirely similar. The CB1 receptor has been proposed to exists in two different conformational states; one where it is bound to the secondary system (by G-proteins) and one in which it is uncoupled.[130] Agonists of the CB1 receptor bind to the precoupled stage activating the receptor, whereas antagonists bind to the receptor without activating it. Because antagonists prevent endogenous cannabinoids from binding, activation is interrupted. Inverse agonists bind to the uncoupled receptor, blocking it and avoiding precoupling of the receptor.

The SAR of classical cannabinoids and endocannabinoids has been studied intensively, and several reviews have been published.[131–136] The major cannabinoids THC[113] and delta-8-THC[137] bind to the CB1 receptor with moderate affinity and do not show specificity for either. CBN, however, does show a slight specificity for the CB2 receptor.[138] It should be noted that values published for CB-receptor affinity may be strongly dependent on the type of tissue and animal species used in the study. Although rat CB1 is 97% homologous with human CB1, critical differences do exist.[139] Also, the type of agonist to be displaced has an influence on the value of K_i reported. As a result, binding efficiencies reported in the literature often show a range of values. An overview is presented by Pertwee,[140] and a summary is presented in **Table 2**.

By introducing a large variety of chemical modifications, four pharmacophores have so far been identified for the classical cannabinoids prototype. They are listed below. For chemical numbering of carbon positions, see **Figure 5**.

Table 2 Range of reported K_i values for the major phytocannabinoids, according to Pertwee[140]

Phytocannabinoid	*CB1 K_i (nmol l^{-1})*	*CB2 K_i (nmol l^{-1})*
THC	5.05–80.3	3.13–75.3
Delta-8-THC	44–47.6	39.3–44
THCV	46.6–75.4	62.8
CBN	120.2–1130	96.3–301
CBD	4350–27 542	2399–4200
CBG	440	337

3.24.3.4.1 An alkyl substituent at C-3

An alkyl group on the C-3 aromatic position seems necessary for binding affinity to the CB receptors, and in the naturally occurring cannabinoids this side chain ranges from the most commonly observed pentyl ($-C_5H_{11}$), down to methyl ($-CH_3$). Changes in the alkyl group of natural cannabinoids lead to wide variations in affinity and selectivity for the cannabinoid receptors. It is now well established that the introduction of a dimethylalkyl side chain greatly increases affinity,[141] suggesting that the introduction of a branched substituent enhances the affinity of CB receptor ligands. The best substituents found so far are 1,1-dimethylheptyl or 1,2-dimethylheptyl.[137,142] One of the strongest CB1 agonists ever made falls into this class: HU210. It has a binding affinity to CB1 of up to 800 times more potent than THC, but without the psychoactive effects.[143]

Sometimes, varying the side chain may also lead to surprising results: the propyl analogue of THC, naturally occurring in the Cannabis plant, is an antagonist of both CB1 and CB2 receptors with fairly high affinity, instead of a weak agonist, as was expected based on its structural similarity to THC.[144]

3.24.3.4.2 A hydroxyl substituent at C-1

The phenolic hydroxyl group at C-1 has to be freely available for significant CB_1 binding. Removal of this hydroxyl group or conversion into a methoxy group leads to selective CB2-binding affinity.[137] A possible explanation for the inactivity of the acidic cannabinoids in receptor binding may be the occurrence of hydrogen bonding between the hydroxyl group and the adjacent carboxyl group.

3.24.3.4.3 The substituent at C-9/C-11

The methyl group at C-9 is not an absolute requirement for binding affinity. Introduction of a hydroxyl group at C-11 was in fact shown to increase affinity of THC as well as delta-8-THC for both CB1 and CB2 receptors.[145] The 11-hydroxy substituent is present in the primary metabolite of THC, 11-OH-THC, which has a more potent psychoactive effect than THC. Further oxidation (to aldehydes or carboxylic acids) eliminates the psychoactive effect but induces analgesic and anti-inflammatory properties. Ajulemic acid (AJA, a carboxylic acid) and nabilone (an aldehyde) are examples of synthetic analogues of THC developed for clinical use (see Section 3.24.6.2).

3.24.3.4.4 An aliphatic hydroxyl at C-6

The hydroxyl group attached to C-6 should be bound to an optimal chain length of three carbon atoms.[146] In the structure of THC, this pharmacophore is a part of ring B (see **Figure 5**, carbon# 6, 12, and 13). In contrast, in the structure of CBD these aliphatic carbons are spatially separated from the hydroxyl, and consequently CBD does not bind to either cannabinoid receptor.

There are several structural similarities between endocannabinoids and the plant-derived cannabinoids that bind to the CB receptors: both classes have a polar head group and a hydrophobic chain with a terminal *n*-pentyl group. More specifically, the pentyl side chain in cannabinoids is present in the endocannabinoids as the last five carbons of the fatty acid chain, and the OH at the C-3 position might correspond to the polar hydroxyl end of the endocannabinoids. Furthermore, the relative distances between these functional groups are comparable because of the ring system in cannabinoids, which can be mimicked by the four double bonds in the endocannabinoids.[147] The fatty acid acyl chain of endocannabinoids should be 20–22 carbons long, with at least three homoallylic double bonds.[148] It is proposed that the acyl chain can assume more than one conformation and that flexibility is necessary to mimic the tricyclic core of the classical cannabinoids.[149] Recently, evidence was also presented for common binding sites to the CB receptors.[150]

The knowledge obtained from these SAR studies has played a crucial role in the development of some of the cannabinoids in clinical use, as described in Section 3.24.6.2. However, the resemblance between phytocannabinoids and endocannabinoids, as described above, does not often apply to the cannabinomimetics, which may structurally be much unrelated to endocannabinoids.

3.24.3.5 Administration Forms

Studies with Cannabis or Cannabis-based preparations have been performed with a large variety of administration forms, ranging from pulmonary (smoking and vaporizing), to sublingual, topical, and oral preparations

(tea, milk decoctions, and baked products). A common factor of all administration forms of (herbal) Cannabis is a heating step, which is essential for the conversion of the acidic cannabinoids into their pharmacologically more active neutral counterparts. Based on the administration form, many changes to the original profile of compounds may occur by, for example, the extent of heating (decarboxylation of acidic cannabinoids), the type of storage (e.g., tea is stored differently from Cannabis cigarettes), degradation, loss by evaporation (e.g., terpenoids), and metabolism. As a result, a different spectrum of compounds is finally entering the bloodstream, and consequently a different type and duration of effects may be observed.

Smoking and oral administration are the two most commonly studied administration forms in clinical trials. The few studies that have directly compared the two forms of THC delivery show smoking to be more effective than oral administration.[151–153] Inhalation of THC avoids the first-pass effect but causes a rapid peak in blood levels accompanied by a spike in psychoactivity. A dose of 2–5 mg of THC consumed through smoking reliably produces blood concentrations above the effective level within a few minutes.[154,155] As a result, Cannabis smoking is a convenient method of administration, allowing self-titration of the desired effects. However, inhalation of toxic compounds during Cannabis smoking poses a serious hazard. This risk is not thought to be due to cannabinoids, but rather due to noxious pyrolytic by-products.[156,157] Consequently, the shortcomings of smoked Cannabis have been widely viewed as a major obstacle for the approval of crude (herbal) Cannabis as a medicine by public health authorities.[158] Nevertheless, inhaling is about equal to intravenous injection in efficiency, while considerably more practical.[159,160] Smoking Cannabis in the presence of tobacco can almost double the release of THC into the smoke, compared to smoking pure Cannabis. The mechanism for this is however still unclear.[161]

Cannabis vaporization or volatilization is a technique aimed at suppressing irritating respiratory toxins by heating Cannabis to a temperature where active cannabinoid vapors are formed, but below the point of combustion where pyrolytic toxic compounds are released. Vaporization offers patients who use medicinal Cannabis the advantages of the pulmonary route of administration, that is, rapid delivery into the bloodstream, ease of self-titration, and concomitant minimizing the risk of over- and underdosing, while avoiding the respiratory disadvantages of smoking. A few studies have been performed in recent years showing that vaporizing can be considered an efficient way of administration of Cannabis as well as pure cannabinoids,[162] resulting in bioavailability of THC comparable to smoking.[163]

In contrast, despite its convenient use, oral THC is notoriously unreliable in its effects.[164] Drawbacks of this administration route include its large variability in bioavailability, and extensive first-pass metabolism. The onset of effects is slow, precluding effective individual titration. In a study performed with orally administered THC, 2 h after oral administration of 10–15 mg, 84% of the subjects had no measurable level of THC in their blood. After 6 h, 57% still had none.[165]

When Cannabis is consumed in the form of a decoction, it is often referred to as 'tea'. Although Cannabis tea is a relatively popular administration form for self-medication among medicinal users, virtually no standardized studies have been performed with it. However, a recent study[166] showed Cannabis tea to be a robust and reproducible administration form for cannabinoids, with relatively high levels of acidic cannabinoids present.

As a result of the factors described above, the choice of administration form can have a major influence on the observed biological effects. For example, early studies indicated that oral doses of THC were no more effective for pain than codeine, and produced a significant amount of dysphoric effects.[167,168] Thus, it was believed that THC could only produce analgesia at doses that were high enough to cause behavioral side effects, and therefore was dropped as potential analgesic.[169] However, when using the parenteral or systemic route, THC and a range of cannabinomimetics have demonstrated potent analgesic effects up to 10 times that of morphine in animal models of acute and neuropathic pain.[170–173]

3.24.4 Biological Effects of the Cannabinoids

Virtually all studies on the biological activities of cannabinoids have been performed on the neutral cannabinoids. However, it should be stressed that most of the neutral cannabinoids discussed below are not biosynthesized by the Cannabis plant as such. Instead, acidic cannabinoids (carboxylic acids) are formed, that will yield neutral cannabinoids upon heating (e.g., recreational use by smoking) or prolonged storage, as

discussed in more detail in Section 3.24.2.4.1. However, it is now becoming increasingly clear that the acidic cannabinoids may have biological activities of their own, and should not be merely considered as 'precursors' of the active, neutral cannabinoids.

It would be impossible here to give a comprehensive overview of all described bioactivities of the cannabinoids, in particular for THC. However, they have been well reviewed in a number of papers and books.[27,174,175] Only the most important or remarkable biological activities will be discussed here, whereas their clinical implications will be described in more detail in Section 3.24.6.1 of this chapter.

3.24.4.1 Delta-9-Tetrahydrocannabinol

THC is the pharmacologically and toxicologically most relevant constituent found in the Cannabis plant, producing a myriad of effects in animals and humans. A frequently used way to review the biological effects of THC is by distinguishing central from peripheral effects, reflecting the classical physiological distribution of the cannabinoid-binding receptors CB1 (the 'central' receptor) and CB2 (the 'peripheral' receptor), as discussed in Section 3.24.3.1. However, the exact mechanism of action of cannabinoids is not exactly clear, as CB1 receptors are increasingly found outside the CNS,[96] whereas CB2 receptors are now known to be present in the nervous system, for example, in rat microglial cells and other brain-associated cells during inflammation.[176]

In a toxicological sense, the CB1-mediated central effects of THC are most important, because they are directly related to the psychological effects of Cannabis use. The most conspicuous psychological effects of THC in humans have been divided into four groups:[177] affective, sensory, somatic, and cognitive. In fact, most documented cannabinoid effects are mediated by the central cannabinoid receptor, and the behavioral effects caused by Cannabis or THC are generally consistent with the anatomical distribution of the cannabinoid receptors, in particular CB1 in the brain. However, neuroprotective properties in ischemia and hypoxia are examples of some known receptor-independent actions of THC and other cannabinoids. Furthermore, both THC (CB1- and CB2-binding) and CBD (nonbinding) potentiate the extinction of conditioned incentive learning, indicating that screening of receptor binding does not necessarily show the potential of cannabinoids.[178]

The best-established palliative effect of THC is the inhibition of chemotherapy-induced nausea and vomiting, mainly in cancer patients. Today, oral capsules containing dronabinol (Marinol) or its synthetic analogue nabilone (Cesamet) are approved for this purpose (see Section 3.24.6.2). Also, herbal Cannabis has been shown to reduce nausea in the majority of users, when ingested or inhaled. The effect of THC on nausea and vomiting has been confirmed in clinical trials.[179,180] It is however unclear how Cannabis or THC compares to the more recently developed, and very efficient serotonin ($5HT_3$) receptor antagonists for the treatment of nausea. Other potential palliative effects of THC in cancer patients include appetite stimulation and pain inhibition.

THC increases the metabolic rate in the brains of animals and humans,[181] and it decreases body temperature, but only at high doses. The increase in heart rate observed after THC administration is clearly dose-dependent and closely associated with THC plasma concentrations. As a result, cardiovascular problems are generally considered a contraindication for the medicinal use of Cannabis or THC. The results of a well-designed clinical trial using inhaled THC suggest that the increase in heart rate is not mediated by brainstem centers but is established by a direct effect of THC on the heart.[124] In the same study a wide array of CNS and non-CNS parameters were monitored after administration. THC had clear dose-dependent effects on postural stability, and body sway was found to be a very reliable indicator of THC blood levels. The high densities of CB1 receptors found in the basal ganglia, cerebellum, amygdala, and forebrain may explain these observations.

In particular in inexperienced users, THC can induce unpleasant effects including anxiety, panic, and paranoia. There are suggestions that in a small number of cases THC is capable of precipitating psychosis, involving delusions and hallucination.[182] If these disorders exist they seem to be rare, because they most likely require very high doses of THC, the prolonged use of highly potent forms of Cannabis, or a preexisting genetic vulnerability.[183] The causal link between Cannabis use and the development of psychosis has not been definitely proven, because of the large amount of parameters to be considered. However, there is enough reason to be precautious and communicate these 'suspicions' in a fair and balanced way. Although the

psychological effects caused by THC or Cannabis are a major drawback in their medical applications, many physical effects are already achieved below the threshold of psychological effects.

The effect that THC decreases intraocular pressure and improves blood circulation in the eye was found serendipitously as part of a study that tried to find easy physiological markers to screen drivers for driving under the influence of drugs. As a result, a variety of studies have targeted THC and other cannabinoids as potential new drugs in the treatment of glaucoma, the leading cause of irreversible blindness. The neuroprotective properties of THC may also be useful in this respect, leading to a dual effect in the protection of the retina and optic nerve.[184]

Anticonvulsant effects have been described for psychotropic as well as nonpsychotropic cannabinoids, including THC, CBD, CBN, 11-OH-THC, and delta-8-THC. THC relaxes muscles and has hypokinetic and anticonvulsant effects. This is one of the major reasons why THC is studied as a treatment for multiple sclerosis, and it may also have significance in epilepsy.

THC exerts an atropine-like effect on salivary secretion resulting in dry mouth. It also causes bronchodilation, comparable to the standard drug salbutamol.[185] This indicates that there is a potential for THC-like substances to treat asthma. However, no recent studies have been performed in this field.

Receptors of the CB2 type are present on white blood cells and affect the immune system, which may be a reason why Cannabis is often used as self-medication by immunocompromised individuals. THC is now considered an immunomodulator, capable of either enhancing or suppressing the function of a range of immune cells. These effects may be modulated by other constituents present in Cannabis.[186] Many described anti-inflammatory effects of THC and other cannabinoids are probably mediated by complex interactions with the immune system.

In early studies, THC was suggested to be mutagenic or carcinogenic. But in fact, an increasing number of studies are showing its anticancer properties.[187] Currently, there is convincing evidence that THC may play a role in the treatment of several types of cancer. In addition to apoptosis and inhibition of proliferation, THC might exert its antitumor effects by inhibiting tumor angiogenesis and metastasis. However, there is some controversy here: although THC has antiproliferative effect in tumors expressing cannabinoid receptors, those with low or no expression suffer increased growth and metastasis due to THC-induced suppression of the antitumor immune response.[188] More research is needed to clearly identify the therapeutic role of THC in cancer treatment.

A promising recent discovery is that THC relaxes the colon and reduces the colonic motility and tone after a meal.[189] This points out the potential for CB receptors to modulate colonic motor function in intestinal disease such as irritable bowel syndrome or Crohn's disease.

An important aspect of the evaluation of THC effects is that it may have a biphasic effect, causing opposite effects at high versus low concentrations. For example, in hefty doses, THC may protect the brain against various types of damage, whereas in tiny doses, potentially adverse effects would come through.[190] The potentially adverse doses would be much lower than those normally obtained from smoking a joint. However, a large dose inevitably becomes a small one as the body slowly clears it out. This may explain the many conflicting results obtained in clinical studies on THC and Cannabis: many studies use low concentrations of THC to prevent possible psychological effects in test subjects. Inadvertently, these low doses may cause exactly those adverse effects the study tries to prevent.[190]

3.24.4.2 Cannabidiol

CBD is, together with CBG, the major nonpsychotropic cannabinoid found in Cannabis. It is the principal cannabinoid present in fiber-type Cannabis (in the form of its carboxylic acid CBDA), a plant that is easily available to researchers, in contrast to the strictly controlled drug-type Cannabis varieties. Second to THC, the pharmacological effects of CBD have been best studied of all cannabinoids. It has powerful antioxidant properties, more potent than ascorbate and α-tocopherol. Also, it has notable anti-inflammatory and immunomodulatory effects.[191] Furthermore, sedating, hypnotic, antiepileptic, and antidystonic effects have been described. Also, CBD is a modulator of some types of opioid receptors,[192] and can modulate sleep in rats.[193]

CBD was found to have antianxiety effects.[194] In a clinical trial, oral administration of 400 mg of CBD resulted in decreased anxiety and increased mental sedation in test subjects.[195] It was concluded that CBD

possesses anxiolytic properties, possibly mediated by an action on limbic and paralimbic brain areas, where it reduced regional cerebral blood flow. These anxiolytic properties might prove useful in psychiatry. Possibly the most significant conclusion of this study is that a dose as high as 400 mg of CBD had no adverse effects. CBD was furthermore found to have antipsychotic benefits.[196]

A prominent effect of CBD was found in a variety of cancer studies. In a mouse model of metastatic breast cancer, CBD reduced the aggressiveness of breast cancer cells, by inhibiting a crucial protein for cancer development.[197] The study concluded that CBD represents the first nontoxic exogenous agent that can significantly inhibit metastatic breast cancer cells leading to the downregulation of tumor aggressiveness. Currently, there is a limited range of options in treating certain aggressive forms of cancer. CBD offers the hope of a nontoxic therapy that could achieve significant results without any of the painful side effects associated with standard therapy. Both *in vitro* and *in vivo* CBD were able to produce a significant antitumor activity on glioma cells. This antiproliferative effect of CBD was shown to be correlated to induction of apoptosis, which suggests a possible application of CBD as an antineoplastic agent. Effects were partially prevented by a (nonpsychoactive) CB2 receptor antagonist, suggesting a role for CB2 in cancer treatment.[198]

In another study performed on a panel of tumor cell lines with a variety of plant-derived cannabinoids, CBD was the most potent inhibitor of cancer cell growth, with significantly lower potency in noncancer cells. A CBD-rich Cannabis extract was equipotent to CBD, whereas CBG and CBC followed in the rank of potency.[199] It was suggested that the observed effect was due to the capability of CBD to induce apoptosis through cannabinoid receptors, or cannabinoid/vanilloid receptor-independent elevation of intracellular Ca^{2+} and reactive oxygen species. These data support the further testing of CBD and CBD-rich extracts for the potential treatment of cancer.

In many Cannabis varieties CBD is present in significant amounts.[200] However, only since a few years there is serious attention for THC–CBD interaction and this is mostly in studies on multiple sclerosis. Earlier studies focusing on the effect of THC alone have generally shown the use of Cannabis to be ineffective in many disease models, and such negative results unfortunately helped to shape the controversy in the discussion on the moral and ethical sides of Cannabis use in multiple sclerosis and other diseases.[201] It is known that CBD inhibits the metabolism of THC, by blocking its conversion to the more psychoactive 11-OH-THC by cytochrome P-450 (CYP) 3A11.[202] Possibly this is the reason why CBD is known to antagonize the psychotropic effects of THC.[203] Even though higher doses of THC are capable of inducing psychotic problems in some users, CBD seems to have an antipsychotic effect, its presence balancing the negative impact of THC consumption.[26] This property of CBD is exploited in the Cannabis-based medicine Sativex (discussed in Section 3.24.6.2).

3.24.4.3 Delta-8-Tetrahydrocannabinol

Delta-8-tetrahydrocannabinol(Delta-8-THC) is a positional isomer of delta-9-THC with a similar pharmacological profile and slightly lower psychoactive potency. Even though delta-8-THC has been very important for SAR studies on the classical cannabinoids, not many bioactivity studies have been done with the pure compound. It is probably not produced by plant metabolism, but rather it is an artifact caused by the degradation of THC (Section 3.24.2.3). In very low concentrations (0.001 mg kg^{-1} in mice, intraperitoneal (i.p.) injection), it increased food consumption, more than THC, whereas performance and activity of the animals were similar.[204] This low dose is equivalent to about 0.1 mg for an average human, an amount that could easily be formed by degradation of THC during the smoking of Cannabis (or be already present in aged plant material). Consequently, it could, at least partially, be responsible for the 'munchies', a popular name for Cannabis-induced increase in appetite.

In a rat study, it was found that behavioral suppression by delta-8-THC was mediated by activation of the arachidonic acid cascade through the CB1 receptor.[205] This may be a useful model to study the amotivational syndrome in humans.

3.24.4.4 Cannabigerol

CBG is one of the major cannabinoids found in most Cannabis varieties. It has shown relevant antibiotic effects,[206] and could decrease intraocular pressure.[207] CBG has been called 'inactive' when compared to THC, but it has slight affinity for CB1 receptors, approximately equal to that of CBD.[90] Like CBD, it has analgesic and

anti-inflammatory properties, indicating that there is scope for developing cannabinoid drugs that do not have the psychoactive properties of THC.[208] In one study,[209] CBG was evaluated for antitumor efficacy against mouse skin melanoma cells and showed a significant *in vivo* activity using an methylthiazoltetrazolium (MTT)-based cell viability assay.

Of several cannabinoids tested, CBG had the strongest potency to inhibit platelet aggregation.[210] However, in recent years no further studies have been reported on the biological activities of CBG.

3.24.4.5 Cannabinol

In 1940, CBN was the first cannabinoid to be isolated and purified from Cannabis. Although CBN is not produced by the metabolism of the plant, it is easily formed from THC by degradation during drying, storing, and consumption (heating) of Cannabis products. As a result, it may play a significant role in several effects attributed to Cannabis consumption. It is a very weakly psychotropic cannabinoid, whose effect is only measurable after intravenous administration. CBN has significant anticonvulsant, sedative, and other pharmacological activities likely to interact with the effects of THC.[211] It was shown to decrease heart rate without affecting coronary blood flow,[212] to decrease intestinal motility,[213] and to inhibit platelet aggregation.[204] Furthermore, CBN is a downregulator of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), thereby counteracting the effects of THC, which increases NF-κB.[214]

3.24.4.6 Cannabichromene

This cannabinoid has hardly been studied at all. However, in most Cannabis varieties analyzed, CBC (in the form of CBCA) can be detected in significant amounts. CBC was shown to have sedative effects. By itself it has only a low analgesic effect in mice, but it increased the analgesic action of THC when administered together.[215] A Cannabis extract rich in CBC altered behavioral despair on the mouse tail suspension test of depression.[216]

3.24.4.7 Tetrahydrocannabivarin

Tetrahydrocannabivarin (THCV) is structurally similar to THC, except for a shortened side chain; it has a propyl ($-C_3$) side chain instead of a pentyl ($-C_5$), and for a long time it was thought to be a slightly less potent little brother of THC, exhibiting similar properties. However, quite unexpectedly, there is evidence that THCV is a CB1 and CB2 receptor antagonist.[144] Although the mechanism of action is not yet fully understood, a very recent study suggest that THCV, alongside standard CB1 receptor antagonists, has therapeutic potential to combat diseases involving cerebellar dysfunction and hyperexcitability, such as epilepsy.[217]

3.24.4.8 The Acidic Cannabinoids

Isolated in 1955, CBDA was the first discovered cannabinoid acid, whereas CBCA was isolated from Cannabis in 1968.[218] Up to date, only sporadic reports have been made on CBCA or CBDA, and rarely have the pure compounds been used for the evaluation of biological activity. As an exception to this rule, pure CBDA was found to display a potent antimicrobial effect.[219] In a study examining the composition of hemp seed oil and its potential as an important source of nutrition, it was observed that extracts containing higher concentrations of CBDA displayed more pronounced antimicrobial activity.[220] Because it is now known that seeds do not produce cannabinoids, the observed levels of CBDA must have resulted from external contamination of seeds by the hemp flowers surrounding it.

Not much is known about the biological effects or human metabolism of the acidic cannabinoids, but older studies at least indicate that the most common acidic cannabinoid, THCA, is not psychoactive in monkeys.[221] CB-receptor-binding assays indicate that the acidic cannabinoids, as well as their esters, are not binding. In the more potent varieties of Cannabis, THCA may be present in levels up to more than 20% of dry weight. However, a quantified, highly pure standard of THCA, as needed for analytical research as well as studies on biological effects, has not been available until recent years.[222] As a result, the potential value of THCA as an immunomodulating agent has only been discovered very recently.[71] Further studies on the biological activities of THCA, and on clinical formulation of this compound, are currently under way.

The acidic cannabinoids are biosynthesized by specialized trichomes, and stored extracellularly, which may indicate that they are cytotoxic. Therefore, the toxicity of CBGA and THCA, in suspension-cultured Cannabis cells and tobacco BY-2 cells, was compared with that of OA, the phenolic moiety in cannabinoids.[35] In 10-day-old suspension-cultured cells of *C. sativa*, 24-h treatment with CBGA and THCA at 50 $\mu mol\,l^{-1}$ caused 100% cell death as demonstrated by trypan blue staining, whereas OA did not have any effect on the cells. The same study also showed that both CBGA and THCA induced apoptosis not only in plant cells but also in insect (*Spodoptera frugiperda*, Sf9) cells, suggesting that cannabinoids may act as plant defense compounds. Since cannabinoid-producing glandular trichomes are distributed in physically fragile young tissues of the Cannabis plant, THCA and CBGA, and possibly other acidic cannabinoids, would protect these tissues from predators such as insects. This was the first report suggesting the physiological importance of THCA and CBGA as apoptosis-inducing defense compounds.

In a later study, it was observed that besides THCA, CBCA also has the ability to induce necrotic cell death through mitochondrial dysfunction in the leaf cells of the Cannabis plant itself.[223]

3.24.5 Noncannabinoid Constituents of Cannabis

Besides some major cannabinoids, no constituents derived from Cannabis plant material have been developed for medical use. Nevertheless, there are several constituents or even whole classes of compounds that may play a significant role in the observed effect of some Cannabis-based preparations. Many of these preparations are a part of self-medication by patients, but they have not been studied in controlled experiments.

Cannabis contains a large number as well as a significant amount of terpenoids. These compounds can be easily evaporated and are consequently inhaled by smoking. Smoke of Cannabis contains a high level of carcinogens, tar, and obnoxious gases (such as CO). However, smoking of Cannabis (without tobacco) does not seem to be associated with lung disease. In contrast, there are even positive effects reported on asthma. It is thought that the positive properties of the terpenoids are at least partially responsible for this. Indeed, several terpenoids identified in Cannabis have known anti-inflammatory, antiapoptosis, or neuroprotective effects (discussed in more detail in Section 3.24.5.1). Furthermore, Cannabis terpenoids and flavonoids may increase cerebral blood flow, enhance cortical activity, and kill respiratory pathogens.[224]

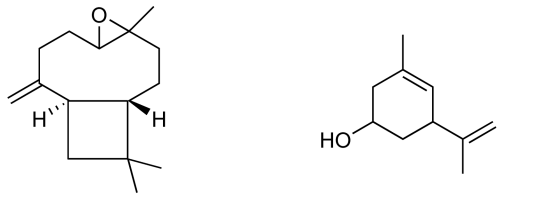
Plants offer a wide range of chemical diversity and have been a growing domain in the search for effective cannabinoid receptor ligands.[225] An increasing number of natural compounds from other species is found to bind to the CB receptors. An exciting discovery was that certain isobutyl analogues of anandamide from *Echinacea* species constitute a new class of cannabinomimetics, which specifically engage and activate CB2.[226,227] More recently, the ubiquitous sesquiterpene β-caryophyllene was found to bind to CB2.[228] Relatively high concentrations of this compound can be found in many plant species, including Cannabis.

The examples mentioned above illustrate the limitations of focusing solely on the cannabinoids. Even though many Cannabis constituents are found ubiquitous in other species, many of them have only been poorly characterized. At the same time, we have strong indications that the biological activities ascribed to Cannabis use cannot be explained by the presence of cannabinoids alone. The following sections will therefore list the most relevant noncannabinoid constituents found in Cannabis. For a complete overview of compounds found in Cannabis materials, the reader is referred to many reviews.[4,33,62]

3.24.5.1 Terpenoids

Terpenoids make up a large percentage of the essential oil of *C. sativa* L. To date, more than 120 terpenoids have been found in Cannabis, including 58 monoterpenoids, 38 sesquiterpenoids, 1 diterpenoid, 2 triterpenoids, and 4 other terpenoids. Two excellent reviews have been published summarizing these compounds and how they were identified.[4,33] Terpenoids display a wide range of biological activities and hence may play a role in some of the pharmacological effects of various Cannabis preparations.

Although cannabinoids are odorless, the volatile monoterpenoids and sesquiterpenoids are the compounds that give Cannabis its distinct smell. The sesquiterpenoid β-caryophyllene-epoxide (**Figure 11**), for example, is the main compound that search dogs are trained to recognize.[229] Only one unusual terpenoid can be found in



β-Caryophyllene-epoxide *m*-Mentha-1,8(9)-dien-5-o1

Figure 11 Two special terpenoids found in Cannabis.

Cannabis: the monoterpenoid *m*-mentha-1,8(9)-dien-5-ol. All others can be found ubiquitously in nature. For this reason the terpenoids of Cannabis did not receive much scientific interest, until it was found that the terpenoid profile of Cannabis products can help in determining the origin of Cannabis in custom seizures.[230]

3.24.5.1.1 Biosynthesis and composition of Cannabis essential oils

The terpenoids in Cannabis are frequently extracted from herbal material by steam distillation or vaporization. Typical yields of the terpene essential oils from fresh plant material range from 0.05 to 0.29% (v/w).[224] The essential oil of Cannabis is mainly composed of monoterpenoids and sesquiterpenoids with monoterpenoids dominating. Since self-administered Cannabis plant material is usually consumed as an (air-)dried product, the change in terpenoid content and concentration in relation to the fresh plant material is important to note. It has been reported that the essential oil content of a Cannabis plant changed from 0.29% essential oil (v/w) in the fresh product to 0.8% (v/w) after 1 week of drying, as a result of water loss. Following storage at room temperature for up to 3 months in a paper bag, the total essential oil was then reduced to 0.57% (v/w). Furthermore, it was observed in the essential oil that the relative percentage of monoterpenoids decreased whereas the relative percentage of sesquiterpenoids increased.[231]

Environmental conditions such as plant density, harvest time, pollination, and climate conditions may all play a role in composition and yield of Cannabis essential oils.[232] The cultivar of the plant also plays a role in the terpenoid composition. A study that analyzed the terpenoids of 157 different strains of Cannabis from various known origins found statistically significant differences in terpenoid composition. Even though these differences were not always indicative of what chemotaxonomic type the Cannabis strains belonged to, it may play a role in differential medicinal effects.[233]

3.24.5.1.2 Biological activities of terpenoids

The observation that whole Cannabis extracts may produce effects greater than expected from THC content alone has led researchers to postulate as to what other components in Cannabis could be responsible for enhancing or modulating the effects of THC. The terpenoids present in Cannabis display a wide range of biological activities that may be involved in regulating the effects of THC as well as producing their own unique pharmacological effects.[224] An overview of some of the known biological activities of terpenoids that have been identified in Cannabis is shown in **Table 3**.

Some undesired side effects of THC may be decreased or modulated in the presence of terpenoid compounds. For example, THC is known to cause acetylcholine deficits in the hippocampus, which may lead to short-term memory loss. This effect can be alleviated in rats by administering tacrine, an alkaloid that inhibits acetylcholine esterase, the primary enzyme involved in the breakdown of acetylcholine in cholinergic receptors.[223] Indeed, tacrine has blocked THC-induced memory loss behavior in rats. Interestingly, many of the terpenoids present in Cannabis display similar acetylcholine esterase inhibition, including pulegone, limonene, limonene oxide, α-terpinene, γ-terpinene, terpinen-4-ol, carvacrol, L- and D-carvone, 1,8-cineole, *p*-cymene, fenchone, and pulegone-1,2-epoxide.[224] For this reason, terpenoids are investigated for the treatment of Alzheimer's disease.

THC has been known to cause negative psychological reactions such as anxiety and depersonalization.[175] Some of these effects may again be alleviated by the terpenoids present in Cannabis, because of their sedative and antidepressive effects.[224] Cannabis terpenoids such as linalool, citronellol, and α-terpinene were shown to

Table 3 Summary of terpenoid biological activity[224,228]

Terpenoid	*Known properties*
β-Myrcene	Analgesic, anti-inflammatory, antibiotic, antimutagenic
β-Caryophyllene	Anti-inflammatory, cytoprotective, antimalarial, CB2 agonist
D-Limonene	Immune potentiator, antidepressant, antimutagenic
Linalool	Sedative, antidepressant, anxiolytic, immune potentiator
Pulegone	Acetylcholinesterase (AChE) inhibitor, sedative, antipyretic
1,8-Cineol	AChE inhibitor, stimulant, antibiotic, antiviral, anti-inflammatory, antinociceptive
α-Pinene	Anti-inflammatory, bronchodilator, stimulant, antibiotic, antineoplastic, AChE inhibitor
α-Terpineol	Sedative, antibiotic, AChE inhibitor, antioxidant, antimalarial
Terpineol-4-ol	AChE inhibitor, antibiotic
p-Cymene	Antibiotic, anticandidal, AChE inhibitor

have significant sedative effects, as indicated by decreased activity in a mice motility model after the inhalation of these compounds.[234] Limonene is a common component of Cannabis essential oil,[231] and it was shown to have a strong antidepressant effect by inhibiting the secretion of hypothalamic–pituitary–adrenal (HPA) stress hormones and normalization of CD4:CD8 ratios.[235] Limonene is also under investigation as an antimutagenic compound because of its multiple anticarcinogensis mechanisms. These effects may reduce some of carcinogenic effects of compounds present in Cannabis smoke.[224]

Cannabis and Cannabis extracts are used in pain relief.[175] Although many of the pain-relieving properties of Cannabis have been attributed to cannabinoids, terpenoids present in Cannabis may also exhibit pain-relieving effects. One of the most abundant terpenoids in Cannabis is β-myrcene,[231] which exhibits a potent analgesic effect as well as anti-inflammatory effect.[236,237] Other terpenoids present in Cannabis, such as carvacrol, exhibit a potent anti-inflammatory effect, even greater than that of THC.[238]

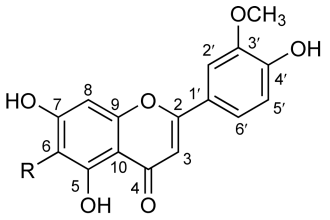
Cannabis extracts are known to effect blood–brain barrier (BBB) permeability,[239] thereby potentially altering the pharmacokinetics of THC and other cannabinoids. Since terpenoids are well known to interact with lipid membranes, they may be responsible for this observed activity. Terpenoids have also been shown to increase cerebral blood flow,[240] which may enhance cognitive brain functions in a way similar to ginkgolides in *Ginkgo biloba*.[224]

Terpenoids known to be present in Cannabis have a variety of effects, including antibacterial, antifungal, antiviral, and antimalarial activity. Besides the general health-promoting effect of these antimicrobial activities, they may also be important in reducing the dangers of recreational smoking of herbal Cannabis contaminated with microbial organisms.[224] A number of studies have investigated the antimicrobial effects of Cannabis essential oil.[241] One conclusion was that terpenoids from hash oil (obtained from drug cultivars of Cannabis, high in THC content) displayed an antimicrobial effect that was greater than essential oil derived from fiber cultivars.[242]

Finally, terpenoids present in Cannabis may play an important role in the chemical ecology of the plant. For example, they have been shown to be involved in the pesticidal properties of the Cannabis plant.[224] Terpenoids have been detected in the pollen of male Cannabis plants, which may play an important role in either attracting organisms involved in pollination or in repelling harmful organisms.[243]

3.24.5.2 Flavonoids

In total, 23 flavonoids have been reported from Cannabis.[31] Some bioactivity studies have been performed on flavonoids from Cannabis, although not nearly as much as on the cannabinoids or terpenoids. Much has been speculated about the role of these compounds in the therapeutic effect of Cannabis. They are often believed to synergistically enhance some beneficial effects, or reduce unwanted side effects of cannabinoids when Cannabis is taken in a crude form. Much remains to be learnt about these flavonoids, related to their effect not only on consumers of Cannabis but also on their role in the plant, and how various factors affect their biosynthesis and distribution in the plant.



R = CH_2-CH=$C(CH_3)_2$, Cannflavin B
R = CH_2-CH=$C(CH_3)CH_2$-CH_2-CH=$C(CH_3)_2$, Cannflavin A

Figure 12 Structures of cannflavins A and B.

Flavonoids have been extracted from the leaves,[244,245] flowers,[83] pollen,[246] and stems[247] of the plant. The aglycones or conjugated *O*-glycosides of kaempferol, quercetin, apigenin, and luteolin have been found, as well as the C-glycosides of vitexin, isovitexin, orientin, and their *O*-glycosides.[4,62] Two flavonoids are so far unique to the Cannabis plant; these are the prenylated flavonoids called cannflavin A and cannflavin B, as shown in Figure 12.[244,248]

In older studies, flavonoid glycosides were hydrolyzed with acid to yield aglycones, before identification. These were characterized with UV spectral properties and behavior in various chromatographic systems.[247,249] The result is that for the glycosides, the exact number and linkage positions of the sugar moieties are mostly not known.[4] In a few later studies, additional spectroscopic techniques were used to determine the structures of these flavonoids more precisely.[246,250]

3.24.5.2.1 Biosynthesis of flavonoids in Cannabis

Although the flavonoid pathway has been extensively studied in several plants, there is no specific data on the biosynthesis of flavonoids in Cannabis. However, the general pathway for flavone and flavonol biosynthesis as it is expected to occur in Cannabis is described by Flores-Sanchez.[251] There is currently no evidence indicating the presence of flavonoids in glandular trichomes.

A few studies have investigated differences in flavonoid content between different strains of Cannabis. Clark and Bohm[252] investigated the flavonoid content of 53 different Cannabis varieties grown from seeds from nine different countries, and found considerable plant to plant variation. The distribution of flavonoids in different varieties followed a pattern based on agronomic use, with clear differences between high-cannabinoid- and low-cannabinoid-producing strains.

Vanhoenacker *et al.*[253] found that cannabinoid-free Cannabis did not produce prenylated flavonoids in leaves or flowers, indicating that the biosynthesis of flavonoids in Cannabis may be linked to that of cannabinoids, and therefore these two polyketide biosynthetic pathways may be competitive. So far, not much is known about how biotic and abiotic factors influence levels and distribution of flavonoids in Cannabis.

3.24.5.2.2 Biological effects of flavonoids

Flavonoids have many roles in the physiology of plants. They provide plant pigmentation and flavor, are involved in plant growth and development, provide resistance to pathogens and predators, as well as protect against harmful effects of UV radiation.[254,255]

Flavonoids also show a large variety of pharmacological and biological activities.[256] As phenolic compounds, they can act as metal chelators and free radical scavengers, and more recently much attention has been given to the role of flavonoids as antioxidants.[257–259] Flavonoids are able to modify the activities of many enzyme systems in the human body, and have been shown to alter the function of various cell types in mammalian cell systems.[260] Many of these properties are related to immune functions, cellular transformation, and tumor growth and metastasis. Other biological activities reported include antibacterial, antifungal, and antiviral effects.[261,262]

Segelman *et al.*[263] isolated orientin and two flavone C-glycosides (orientin-2″-*O*-D-glucopyranoside and vitexin-2″-*O*-*β*-D-glucopyranoside) from a Mexican strain of Cannabis. These compounds were tested in a rat

lens aldose reductase enzyme assay, and found to have inhibitory properties against this enzyme, that is implicated in the pathogenesis of cataracts in humans with diabetes and galactosemia.

In a recent review of the chemistry and pharmacology of marijuana smoke,[264] no mention is made of flavonoids in Cannabis smoke. Nevertheless, it is likely that some flavonoids are present in Cannabis smoke. Most authors cite the work of Sauer *et al.*[265] who assumed that the estrogenic effect of marijuana smoke condensate was due to the presence of apigenin. Lee *et al.*[266] also tested marijuana smoke condensate for estrogenic effects, and went further by fractionating it to find out which components were responsible for the observed effect. They identified phenol and one phenolic derivative in the active fraction, but no flavonoids were found.

3.24.5.2.3 Therapeutic potential

Flavonoids may be important for the overall therapeutic effect of THC and the other cannabinoids by either synergistically enhancing them or reducing their side effects.[224] Flavonoids may counteract some unwanted effects caused by THC, such as the upregulation of tumor necrosis factor-alpha (TNF-α).[267] Flavonoids are particularly adept at inhibiting CYP monooxygenase enzymes, thereby potentially altering the pharmacokinetics of THC, which is converted into 11-OH-THC by the same enzymes. Such CYP-suppressing flavonoids may therefore act as chemoprotective agents by blocking the conversion of procarcinogens such as benzo[α]pyrene and aflatoxin B1, two harmful agents potentially found in Cannabis smoke, as a result of contamination of herbal Cannabis with molds.

Some flavonoids isolated from Cannabis have been tested for pharmacological effects. Cannflavins A and B were found to inhibit prostaglandin E2 in human rheumatoid synovial cells. Cannflavin A did so with 30 times more potency than aspirin.[244] Cannflavin A inhibited cyclooxygenase (COX) and lipoxygenase enzymes and thus had anti-inflammatory properties.[268] It is perhaps not surprising that these compounds show strong biological activities, because substitution with a prenyl group increases lipophilicity of flavonoids and give the molecules strong affinity for biological membranes.[269] Prenylated flavonoids are attracting increasing attention from the scientific community because of their potent antioxidant and anticancer effects, and their potential for treating menopausal problems.[270] Therefore, it is possible that the cannflavins will be shown to possess more biological properties in the future.

Clinical studies on flavonoids have shown that often very little unchanged aglycone is present in human plasma, but that conjugated metabolites such as glucuronic acid conjugates are present at high concentrations.[271] The bioactive forms *in vivo* may thus not be the naturally occurring phytochemical forms but rather their metabolites derived from them after absorption in the body.[272] The uptake of flavonoids and their *in vivo* metabolites are also different for different cell types. Consequently, the concept of oral bioavailability and activity of dietary flavonoids is clearly a complex topic. Nonetheless, it is possible that flavonoids in Cannabis taken orally have potential beneficial effects.

3.24.5.3 Hemp Oil

When Cannabis is cultivated for the production of fiber or seeds, only specially selected varieties with a very low THC content are legally allowed to be used. In that case, it is usual to use the term hemp instead of Cannabis (see Section 3.24.1.1). In recent years, scientific knowledge on the composition and benefits of hemp oil has increased significantly. The oil of Cannabis seeds has been promoted as a good source of the healthy polyunsaturated fatty acids, and may be considered a sustainable alternative to fish oil. It is widely used in body care products, lubricants, paints, and for other industrial uses, while its antimicrobial properties and emollient effect make it a useful ingredient for soaps, shampoos, and detergents.

Hemp oil is obtained from mature hemp seeds, grown outdoors.[273] After harvest, the seed is dried to reduce its moisture content, which also prevents sprouting during storage. Hemp seed contains about 30–35% oil by weight.[273,274] Because hemp oil is considered to be a relatively unstable product, it is not extracted by means of steam or organic solvents, but mainly by cold-pressing methods. Cold-pressed, unrefined hemp oil is light green, with a nutty, grassy flavor, whereas refined hemp oil is clear with little flavor. Chlorophyll and the carotenoid pigments found in mature seeds provide the natural dark green color to the oil.

3.24.5.3.1 Composition of hemp oil

Hemp seed typically contains about 25% high-quality protein and 35% fat in the form of an excellent quality oil. It has a remarkable fatty acid profile, being high in the desirable omega-3 fatty acids and also delivering some γ-linolenic acid (GLA), which is deficient in the average Western diet.[275] Although work by Ross *et al.*[276] showed no significant difference in the fatty acid composition of the oil generated from drug- or fiber-type seeds, the content of such higher fatty acids may vary considerably with variety, climate, and growing conditions.

Hemp oil typically contains 50–70% linoleic acid (LA; C18:2, an omega-6 fatty acid) and 15–25% α-linolenic acid (ALA; C18:3, an omega-3 fatty acid),[273] which is roughly in the 3:1 ratio that matches our nutritional needs (see Section 3.24.5.3.3). Furthermore, hemp oil provides significant amounts of some higher fatty acids such as GLA (C18:3; omega-6) and stearidonic acid (SDA; C18:4; omega-3).[273] Oleic acid (C18:1) and saturated fatty acids (mainly palmitic, stearic acids) both make up about another 10% of the oil.[27] In some hemp varieties, the omega-9 fatty acid eicosenoic acid (EA; C20:1) is present in amounts up to 0.5%;[273,277] however, most varieties typically contain much less.

Because hemp oil contains a high proportion of polyunsaturated fatty acids,[278] the double bonds that provide such unsaturation may be degraded because of oxidation by exposure to air, light, and/or elevated temperatures. At temperatures above 200 °C, undesirable *trans*-fatty acids are gradually formed, which may lead to the formation of aldehydes, causing the oil to become rancid. As a result, it is generally recommended that hemp oil should not be used for frying or baking, but preferably should be consumed cold.[273,275] However, results obtained by Molleken and Theimer,[277] who subjected hemp oil to a series of heat treatments before analyzing the fatty acid composition, showed that the stability of hemp oil is much better than generally assumed: *trans*-fatty acids were not formed under normal cooking conditions, and heated native hemp oils were quite stable under high-temperature conditions (up to 250 °C), presumably because of the presence of significant amounts of the antioxidant γ-tocopherol. In general, extra addition of tocopherols is recommended as preservative for hemp oil.[279]

Besides fatty acids, moderate to high concentrations of the vitamin E are present in hemp oil as well as small amounts of phytosterols, phospholipids, chlorophyll, carotenes, and several minerals.[27]

3.24.5.3.2 Therapeutic potential

Many edible oils (e.g., hemp, sunflower, soybean, pumpkin, and canola) contain significant amounts of the health-promoting omega-6 fatty acid LA. However, only some of these oils simultaneously provide significant amounts of the omega-3 ALA (**Figure 13**). It is important to notice that only hemp oil provides a ratio of LA to ALA close to 3:1, which is suggested as optimal for human nutrition.[273,280,281] Furthermore, hemp oil contains GLA and SDA. No other edible plant oil has these nutritional advantages.

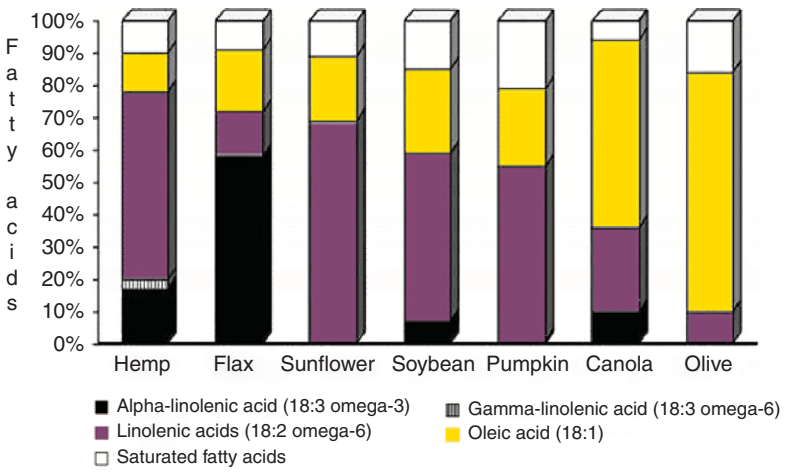


Figure 13 Typical fatty acid composition of vegetable oils. Reproduced with permission from G. Leson; P. Pless; J. Roulac, *Hemp Foods and Oils for Health*; Hemptech: Sebastopol, CA, 1999.

The unbalanced intake of omega-6 and omega-3 fatty acids is associated with many chronic diseases such as cardiovascular disease, diabetes, cancer, obesity, autoimmune diseases, rheumatoid arthritis, asthma, and depression.[282] The average Western diet provides a ratio of omega-6 to omega-3 of about 15:1. An increased intake of omega-3 fatty acids, through their eicosanoid metabolites, has been shown to result in lower blood pressure and blood cholesterol levels, playing an important role in the prevention and treatment of coronary artery disease, cancer, and hypertension. Moreover, it helps normalize fat metabolism and decreases insulin dependence in diabetics. Omega-3 fatty acids also increase overall metabolic rate and membrane fluidity, and exhibit anti-inflammatory properties, specifically with regard to relieving arthritis.[281,283]

Nutritionists suggest that daily requirements should range from 9 to 18 g of LA and 6 to 7 g of ALA, which would be equivalent to the consumption of three to five tablespoons of hemp oil. However, individuals who consume a diet high in saturated fatty acids or *trans*-fatty acids will require more, as well as people who are overweight or under great stress.[220,281]

3.24.5.3.3 Cannabinoid contamination of hemp oil products

Because hemp oil is produced for applications in food, the fear exists that the oil may be contaminated with significant amounts of the psychoactive component THC. Although no cannabinoids are metabolically produced by the hemp seed itself, they may be detected in hemp oil because cannabinoids as well as other components present in the resin may be transferred from the flowers and leaves onto the seeds, and subsequently to the oil during pressing. Thorough cleaning of the seeds, including the removal of the seed coat (dehulling), and the use of varieties with a certified low THC content (or more accurately: THCA content, see Section 3.24.2.3) are ways of preventing such contamination.[27] Certified low-THC hemp seed is currently available from Canada, Europe, and China and is under development in Australia and the United States. Today, hemp is grown throughout the world – except in the United States, where it is illegal to grow the plant but allowed to import, manufacture, and sell products made from it.

In order to ensure the safety of hemp products (oil and other), strict legal limits have been set for the level of THC allowed, ranging from 10 ppm in Canada to 50 ppm in Switzerland.[284] Nowadays, THC quantities observed in hemp oil are usually so small that there is no possibility of intoxication and hence no potential negative effects on human health. Use of cosmetics based on hemp oil typically does not result in positive urine tests for marijuana use. The minimal amounts of THC in hemp oil are probably not absorbed through the skin and/or do not cause any relevant uptake into the bloodstream.

3.24.5.4 Other Components Found in Cannabis

Besides the major classes of compounds described above, some other classes are worth mentioning as well. They will be described shortly in the following sections. An excellent review of the functions, occurrence, and biosynthetic pathways for the production of these minor components of *C. sativa* has been given by Flores-Sanchez and Verpoorte.[60]

3.24.5.4.1 Alkaloids and nitrogenous compounds

In Cannabis, 27 nitrogenous compounds have been detected, of which 10 have been identified as alkaloids.[4,33] Some of the more unusual constituents of Cannabis include an amide formed between *p*-hydroxy-(*trans*)-cinnamic acid and 2-(*p*-hydroxyphenyl)-ethylamine, which was isolated from the roots of Mexican Cannabis, and the spermidine-type alkaloids cannabisativine and anhydrocannabisativine (see **Figure 14**), isolated from the roots and aerial parts of various Cannabis strains.[4]

Other interesting alkaloids include choline, neurine, L-(+)-isoleucine-betaine and muscarine (protoalkaloids), hordenine (a phenethylamine), and trigonelline (a pyridine). The concentration of choline and neurine from dried roots was only 0.01%.[285] The presence of muscarine in Cannabis plants has later been questioned.[286] Methods for the synthesis of cannabisativine[287] as well as the biosynthesis of choline and atropine by hairy root cultures of *C. sativa*[288] have been reported.

Although alkaloids are generally considered to be a most interesting class of compounds for biological activity, there is currently no relevant information on the pharmacological profile of these Cannabis alkaloids.

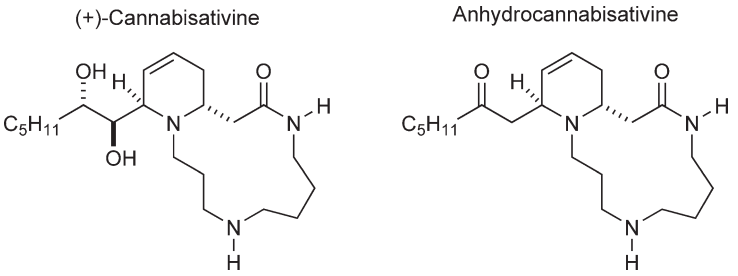


Figure 14 The structures of the Cannabis alkaloids cannabisativine and anhydrocannabisativine.

Some studies suggest pharmacological activities of smoke condensate and aqueous or crude extracts containing Cannabis alkaloids.[289,290]

3.24.5.4.2 Noncannabinoid phenols

Twenty-five noncannabinoid (and nonflavonoid) phenols were identified in Cannabis. These include simple phenols such as eugenol and related phenols, dihydrostilbenes or bibenzyl compounds (e.g., canniprene), and dihydrophenanthrenes and spiro-indans (e.g., Cannabispiran, Cannabispirenone). The dihydrostilbenes and spiroindans are closely related, and they possibly have the same biosynthetic origin. Several spiroindans have only been found in Cannabis.[291]

Only recently, the phenanthraquinone denbinobin (**Figure 15**) was identified in Cannabis extracts. First isolated from *Ephemerantha lonchophylla*, this compound inhibits NF-κB and causes apoptosis in human leukemic cells through reactive oxygen species. Furthermore, in a concentration-dependent manner, it induces apoptosis in human leukemic cells through Akt inactivation, Bad activation, and mitochondrial dysfunction.[292]

3.24.5.4.3 Stilbenoids

Nineteen stilbenoids have been identified in Cannabis.[4,33] They have been isolated from stem, leaves, and resin.[60] Studies have reported antibacterial activity for certain Cannabis stilbenoids.[293] It has been suggested that their biosynthesis could have a common origin, with dihydroresveratrol as a central intermediate.[60] However, no comprehensive reports about the biosynthesis of spirans or about the regulation of the stilbenoid pathway in Cannabis currently exist.

3.24.5.4.4 Lignanamides and phenolic amides

Cannabis fruits and roots have yielded 11 compounds identified as phenolic amides and lignanamides.[60] The phenolic amides include *N-trans*-coumaroyltyramine, *N-trans*-feruloyltyramine, and *N-trans*-caffeoyltyramine; the lignanamide group includes Cannabisins A–G and grossamide. In general, lignanamides belong to the lignan group, and the Cannabis lignanamides are classified as lignans of the arylnaphthalene derivative type.

The phenolic amides have cytotoxic, anti-inflammatory, antineoplastic, cardiovascular, and mild analgesic activity. For the lignanamides grossamide Cannabisin-D and Cannabisin-G, a cytotoxic activity was reported.[60] The presence and accumulation of phenolic amides in response to wounding and UV light suggests a chemical defense against predation in plants.[294] For the lignanamides Cannabisin-B and Cannabisin-D, a potent feeding deterrent activity was reported.[60]

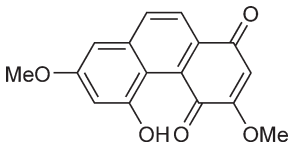


Figure 15 The structures of the Cannabis phenanthraquinone denbinobin.

The structures of the lignanamides and phenolic amides from Cannabis suggest condensation and polymerization reactions in their biosynthesis starting from the precursors tyramine and CoA-esters of coumaric, caffeic, and coniferic acids. However, it has also been suggested that these lignanamides could be isolation artifacts.[295,296] Further biosynthesis studies are necessary to elucidate their origin.

3.24.6 Cannabis as a Medicine

The clinical potential of the cannabinoids is large; some people suggest that Cannabis could be the 'aspirin of the twenty-first century', pointing out the impulse secondary metabolites from Cannabis may give to contemporary medicine.[9] However, much of the evidence for the medicinal use of Cannabis or cannabinoids is anecdotal and it turns out to be very challenging to confirm many of these findings by clinical trials. Also, it is often unknown which constituents are responsible for the effects observed after administration of herbal Cannabis or extracts. The lack of appropriate animal models with the complexity of the human brain hampers the study of the behavioral effects of these compounds. Therefore, experimental studies have concentrated on measurable physiological effects, and, as a result, the understanding of the underlying biology is only slowly improving. But despite these limitations, a number of cannabinoids of natural as well as synthetic origin have been developed for clinical use; most often as agonists or antagonists of CB receptors. These compounds are often the result of extensive studies on SARs performed on the plant-derived cannabinoids, their chemical derivatives, and their metabolites. In contrast, the clinical evaluation or development of the noncannabinoid constituents of Cannabis is minimal.

Although the structure of THC was elucidated by means of NMR spectroscopy already in 1964, relatively little clinical research took place for a long time. Research on the medicinal potential of cannabinoids got a new impulse after the discovery of the cannabinoid receptors in the 1990s. During the extended period between these two events, the cannabinoid character of a large variety of compounds was assessed through a panel of *in vivo* assays, one of the earliest being the dog ataxia test.[297] However, the most widely used set of assays were known as the cannabinoid tetrad,[298] which comprised four different behavioral tests performed mostly in mice: diminution of temperature (hypothermia), immobility in a multiple photoelectric cell chamber (diminution of locomotion), a ring test or bar test (catalepsy), and a hot-plate or tail-flick test (analgesia). A positive response in all four tests was the criterion to consider a compound as a 'classical' cannabinoid. To date, activity in this mouse behavioral battery has been a reliable predictor of psychotomimetic activity in humans. Nowadays, it is understood that the observed effects in the cannabinoid tetrad can in fact be attributed to CB1 activation.

Cannabis preparations have been used in the treatment of numerous diseases, with marked differences in the available supporting data. Clinical studies with single cannabinoids (natural or synthetic) or whole plant preparations (e.g., smoked Cannabis, encapsulated extract) have often been inspired by positive anecdotal experiences of patients using crude Cannabis products for self-treatment. The antiemetic,[299] appetite-enhancing,[300] analgesic,[301] and muscle relaxant effects,[302] and the therapeutic use in Tourette's syndrome,[303] were all discovered or rediscovered in this manner. Incidental observations have also revealed therapeutically useful effects. The discovery of decreased intraocular pressure with THC administration, potentially useful in the treatment of glaucoma, was made serendipitously during a systematic investigation of healthy Cannabis users.[304] However, anecdotes as to the efficacy of Cannabis or THC in indications that have not been confirmed in controlled studies have to be judged with caution. Nevertheless, the therapeutic potential of Cannabis and the cannabinoids is large.

3.24.6.1 Therapeutic Potential of Cannabinoids

The therapeutic potential of cannabinoids can be clarified by pointing out the central physiological importance of the endocannabinoid system, as described in Section 3.24.3. The cannabinoid system is involved in a wide range of physiological functions and might be related to a general stress–recovery system. One yet unproven but intriguing idea is that endocannabinoids may set the 'analgesic tone' of the body, with the level of their production acting as a kind of pain thermostat.[305] It is likely that such a system relies on the combined activities of a range of compounds. Strategies to modulate endocannabinoid activity include inhibition of reuptake into

cells and inhibition of their degradation to increase concentration and duration of action. The effect of cannabinoids or synthetic cannabinomimetics interacting with such an endocannabinoid system could be on multiple levels, other than receptor binding alone. Some of such interactions have already been described.[305]

Cannabinoids make up a significant group of compounds with diverse properties, and even based on the limited data available it may be expected that at least several of them have therapeutic potential. Most known cannabinoids have been tested to describe their relative (psychoactive) potency in comparison to THC, either in receptor-binding assays or in THC-specific assays. However, testing non-THC cannabinoids as serious candidates for new leads can sometimes lead to completely counterintuitive results, as shown in the case of THCV; although its psychoactive potency is roughly similar to THC (about 75%)[4,306], later *in vivo* testing surprisingly showed that THCV should rather be considered an antagonist of THC activity.[144]

An exciting notion is that cannabinoids, and possibly also noncannabinoids present in the plant, may exert their effect independent of cannabinoid receptors altogether. In this respect, most studies are focused on the interaction between the cannabinoid and the opioid system. It is becoming increasingly clear that cross talk between the cannabinoid and the opioid system exists,[85] but our understanding of this field is only just beginning, as discussed in more detail in Section 3.24.6.3.4.

3.24.6.2 Current Status of Cannabinoid Medicines

An increasing number of pharmaceutical companies start to pick up the idea of cannabinoids or their antagonists as therapeutic drugs. At present a number of preparations based on the biological activities of the cannabinoids are available, but not all of these have been fully registered as drugs. Most preparations are pure compounds based on the pharmacological actions of THC. The major ones are shown in **Figure 16**.

The most commonly prescribed cannabinoid-based medicines are Marinol (synthetic THC in sesame oil, Solvay Pharmaceuticals) and Cesamet (nabilone, Valeant Pharmaceuticals International). They are registered for the indication of nausea and vomiting associated with cancer chemotherapy. Marinol is also approved for anorexia and cachexia in HIV/AIDS. The patent on Marinol will expire in 2011, opening the way for the development of generic preparations of synthetic, as well as naturally derived THC.

Although there are clear indications that some pharmacological effects may vary according to the fact if a cannabinoid is taken alone, or in combination with other cannabinoids, not much work has been done on the

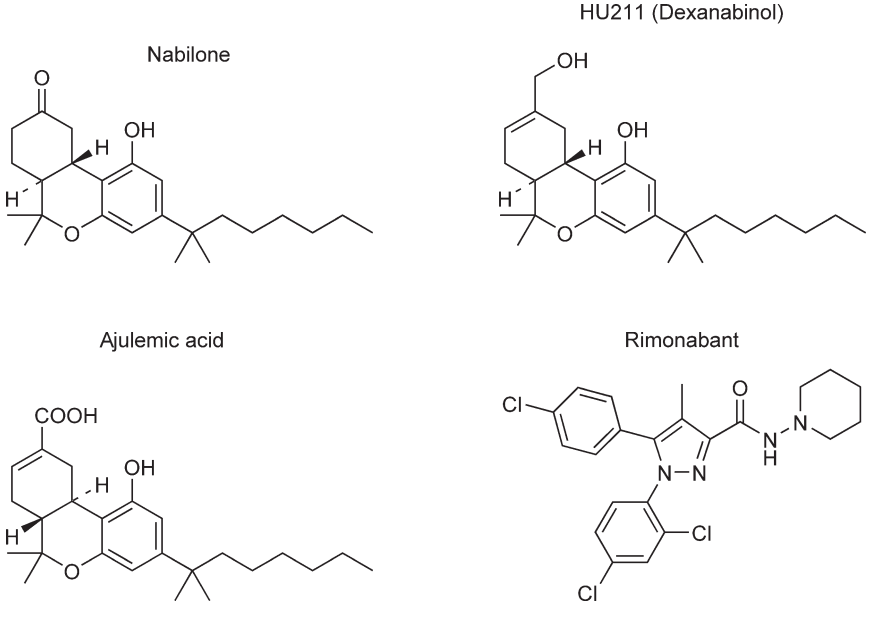


Figure 16 The structures of several cannabinoid receptor agonists currently in clinical use or under development.

activities of combined cannabinoids. However, there is a major exception: Sativex (GW Pharmaceuticals, UK) is a sublingual spray based on a mixture of two distinct standardized Cannabis extracts. The final preparation contains equal amounts of THC and CBD. The presence of CBD is thought to reduce the metabolism of THC by cytochromes in the liver, thereby increasing the half-life of THC in the blood. Because of the use of whole extracts, nonstandardized amounts of ballast components are also present, such as minor cannabinoids and terpenoids.[307] Sativex is currently registered only in Canada, but registration is pending in several European countries.

Cannador (European Institute for Oncological and Immunological Research, Germany) is an oral capsule containing a Cannabis extract, with a ratio of THC:CBD that does not appear to be fully standardized. Although it has been used in several clinical trials, it has not yet been registered as a drug. It has been clinically tested for the reduction of tremor in multiple sclerosis,[308] and postoperative pain management.[70]

Rimonabant (Acomplia, by Sanofi-Aventis) was the first selective CB1 receptor blocker to be approved for use anywhere in the world.[309] It is an inverse agonist for the cannabinoid receptor CB1, intended as a new prescription antiobesity drug. It was released in the form of a tablet under the name Acomplia, but has very recently been pulled off the market out of concern over its depressive side effects. Many other pharmaceutical companies were working on similar CB1 agonists (e.g., Taranabant by Merck & Co, and SR141716 by Eli Lilly), but most have discontinued their work after the withdrawal of Rimonabant.

AJA (also known as CT-3, developed by the University of Massachusetts Medical School, Worcester, Massachusetts, USA)[310] is a synthetic analogue of the human THC metabolite 11-carboxy-THC (see Section 3.24.3.3). Although the mechanism of AJA action remains largely unknown, it has potent analgesic and anti-inflammatory activity,[311] without the psychotropic action of THC. Psychoactive effects may be limited as a result of reduced crossing of the BBB, and greater activity at peripheral rather than central cannabinoid receptors.[312] A major advantage of AJA is that, unlike the nonsteroidal anti-inflammatory drugs (NSAIDs), it is not ulcerogenic at therapeutic doses. It has been studied in clinical trials for chronic neuropathic pain.[313]

Dexanabinol (also known as HU-211, developed at The Hebrew University, School of Pharmacy, Jerusalem, Israel) is a dihydroxylated synthetic cannabinoid resembling THC that is devoid of cannabimimetic effects and does not bind to cannabinoid receptors.[314] The neuroprotective effect of dexanabinol is related to its unique capacity to act as a noncompetitive antagonist of the NMDA receptor, to block COX-2 enzymes, and to prevent inflammation by inhibiting secretion of TNFα and other inflammatory cytokines in the CNS.[315] Dexanabinol may be useful in the treatment of traumatic brain injury.[316]

3.24.6.3 Approaches for Further Development

Extensive information about Cannabis as a medicament goes beyond the possibilities of this chapter and the readers are referred to several comprehensive books[27,317] and recent reviews[69,131,307] on this subject. However, some major developments will be briefly discussed here. The following sections discuss some suggested targets for further development of Cannabis compounds as a drug.

Cannabinoids, as found naturally occurring in the plant, provide the pharmaceutical developer with a variety of challenges, including low water solubility, variable bioavailability, and psychoactive side effects. As a result, current research on synthetic cannabinoids focuses mainly on new chemical entities (NCEs) that can agonize or antagonize one of the CB receptors specifically, or that can affect the endocannabinoid system otherwise (e.g., FAAH inhibitors). Possible fields of application include obesity, anorexia, and neuroprotection. Some of these molecules have proven to be powerful agonists. But although they are extremely useful for nonclinical studies to uncover the functions of the endocannabinoid system, they may be too psychoactive to be used in human subjects.

3.24.6.3.1 Improving the biological availability of cannabinoids

THC is a light yellow resinous oil, sticky at room temperature that hardens upon refrigeration. THC is highly lipophilic, practically insoluble in water,[318] having an octanol:water partition coefficient (at pH 7) of at least 6000:1.[319] As a result, it has been difficult to develop effective formulations for human use.[162] So far, every attempt to make classical cannabinoids more water soluble has yielded only compounds without significant CB

binding. Several examples illustrate the difficulties in handling the high lipophilicity of cannabinoids; the synthetic cannabinoid dexanabinol was evaluated in phase I clinical trial by intravenous (i.v.) infusion in cremophor–ethanol vehicle diluted with saline, whereas Sativex is a sublingual spray containing ethanol and propylene glycol as solubilizers.[320] A possible way to handle this obstacle is the creation of water-soluble prodrugs. Examples are glycinates and salts of amino acid esters containing tertiary and quaternary heterocyclic N-atoms.[321] Also, the use of cyclodextrins as solubilizers seems to be feasible.[322]

3.24.6.3.2 Selective activation of cannabinoid receptors

There is a fundamental problem with using the cannabinoid receptor as a drug target: the main target for most therapeutic activities is CB1 and this is the same receptor that causes most of the adverse effects. Dissociation of the adverse effects from the therapeutic effects of Cannabis may therefore never be truly possible. Furthermore, excessive stimulation of CB1 leads to receptor tolerization and this is a particular problem of strong agonism.[323] Also, there may be risks associated with the long-term use of CB blockers (antagonists): a good example is the increased risk of depression with the prolonged use of Rimonabant.

But although CB1 is generally considered to be centrally active, it is also expressed on nerves outside the CNS, for example, on nerve terminals, dorsal root ganglia, and the vasculature. Therefore, a possible strategy for drug development is to develop compounds that are excluded from the BBB, to selectively activate the peripherally located CB1 receptors. This may limit psychoactivity while producing benefits for disorders such as pain, asthma, and glaucoma.

Cannabinoids inhibit pain in virtually every experimental pain paradigm via either CB1- or CB2-like activity, dependent on the type of nociceptive pathway being studied. This finding is consistent with high concentrations of CB1 receptors on primary afferent nociceptors, particularly in the dorsal spinal cord, whereas peripheral CB2 receptors have been implicated in the control of inflammatory pain.[135] CB2 selective agents, working on the peripherally located CB2 receptors, without activating the CB1 receptors that may induce a psychoactive effect, may have therapeutic value. Guindon and Hohmann[324] reviewed behavioral, neurochemical, and electrophysiological data, which identify cannabinoid CB2 receptors as a therapeutic target for treating pathological pain states with limited, centrally mediated side effects. Cheng and Hitchcock[325] reviewed the present development of cannabinoid agonists with an emphasis on selective CB2 agonists and peripherally restricted CB1 or CB1/CB2 dual agonists for the treatment of inflammatory and neuropathic pain.

3.24.6.3.3 Modulating the endocannabinoid system

Endocannabinoids, through interaction with the CB receptors, have a range of effects on the nervous system. They are weak agonists and these agents naturally stimulate receptors without much potential for inducing psychoactive effects. For this reason, modulation of the endocannabinoid system is an exciting target for cannabinoid therapy. But although endocannabinoids may be interesting as therapeutic agents, their instability and rapid metabolism limit their utility in preclinical and clinical research. To date, no endocannabinoid agents have been administered to humans.

How many and what functions of the endocannabinoids occur tonically under conditions of physiological homeostasis is unclear at present. The fact that CB1 and CB2 receptor knockout, at least in certain genetic backgrounds, does not produce a strong phenotype in unchallenged animals suggests that this system becomes important mostly under pathological conditions. Indeed, endocannabinoid signaling often undergoes dramatic tissue-specific changes in both animal models of disorders and in human diseases.[117]

As discussed in Section 3.24.3.2, endocannabinoids are made on demand, act only locally, and are metabolized immediately after action. As a result, the duration of their action is very limited. Compounds that affect the levels of endocannabinoids, by inhibiting membrane transport or hydrolysis, thereby prolonging their life span, offer promising potential for further research and pharmaceutical development. During a variety of diseases there are changes in endocannabinoid concentrations at the site of pathology. Targeting of endocannabinoid degradation through inhibition of the reuptake mechanism or enzymes that cause degradation could locally target sites of damage while limiting effects in uninvolved areas.

3.24.6.3.4 Interaction with other neurotransmitter pathways

An exciting observation is that THC reduces chronic pain in patients who do not get sufficient pain relief from opioids alone. Therefore, a promising development is the combined use of THC with opioids. Although the brain has more CB1 than opioid receptors, a review by the U.S. Institute of Medicine has commented on how little is known about cannabinoids in comparison with opiates.[326] The obvious analogy between the history of research on opiates and cannabinoids suggests good reason for optimism about the future of cannabinoid drug development.[327,328] Many studies have been performed on Cannabis and pain, but they are hard to compare because of the large variety of pain models used, and the subjective nature of pain. However, a variety of synthetic analogues and derivatives of THC and other cannabinoids have been designed with an improved analgesic effect, but without the psychotropic side effects of THC.

Opioids and cannabinoids share several pharmacological effects, including antinociception, hypothermia, inhibition of locomotor activity, hypotension, and sedation.[169] It is therefore not surprising that cross talk between the two systems has been shown.[85] The coupling of both receptor types, through inhibitory $G_{i/o}$ proteins, to similar intracellular signaling pathways underlies, to some extent, their similarities in actions.[86] Interactions between the pathways probably explain why antagonists of each receptor type sometimes counteract the pharmacological effects induced by the stimulation of the other type.[327] Coadministration of various cannabinoids with morphine produced a greater-than-additive effect with respect to antinociception in mice.[329] Although both cannabinoids and opioids are accompanied by undesirable side effects at high doses, it was found that THC can enhance the potency of opioids such as morphine, thereby dramatically reducing the dose needed for pain control in some clinical indications.[330,331]

Yet another potential target for interaction with (endo)cannabinoids is ceramide, a ubiquitous sphingolipid second messenger that plays an important role in the control of cell fate. Cannabinoid-induced acute ceramide generation might rely on *de novo* synthesis through the induction of sphyngomyelin hydrolysis. As a result of this activity, cannabinoids, like other ceramide-generating agents,[332] might be considered as potential therapeutic drugs for the management of malignant tumors.

3.24.7 Practical Aspects of Cannabis Research

Thanks to the relatively recent discovery of the human endogenous cannabinoid system, it seems that Cannabis-based medicines may have a bright future. But there are a lot of obstacles to be taken first. Some are a direct result of the nature of the plant and the chemical characteristics of its constituents, but others are clearly the result of social, cultural, and as a result, legal bias toward a hazardous plant. Some of the most important aspects are discussed below.

3.24.7.1 Legal Aspects

Starting from 1954, the World Health Organization (WHO) has claimed that Cannabis and its preparations no longer serve any useful medical purpose and are therefore essentially obsolete. Up to that moment, Cannabis legislation had been based on a large number of conventions, causing considerable confusion in the execution of treaties. Under pressure of increasing reports that Cannabis was a drug dangerous to society, it was proposed to combine all in single convention, the draft of which was finally accepted by the United Nations in 1961. In the following years, several complementary treaties were made to strengthen it. Under the 'Single Convention on Narcotic Drugs', Cannabis and its products were defined as dangerous narcotics with a high potential for abuse and no accepted medicinal value. It reflected the belief that Cannabis was a dangerous narcotic with a threat that was equal to the most dangerous opiates, as it was strongly believed that Cannabis use could serve as stepping stone to the use of such drugs. Since the Single Convention, the potential danger of Cannabis abuse by recreational users has been much higher on the political agenda than any of its benefits as a source for fiber, food, or medicines. The distinction between medicinal and recreational use is thereby made only in a few countries.

It can be observed that new scientific insights on Cannabis are only slowly and reluctantly incorporated into new legislation. However, in recent years a large variety of scientific and clinical data has become available,

further showing the physiological effects of cannabinoids and the endocannabinoid system. And in several Western countries important obstacles for a real acceptance of medicinal Cannabis have already been addressed, as serious steps are taken toward decriminalization of Cannabis use or even providing medicinal Cannabis products to patients. These shifts constitute the first steps away from the dominant drug policy paradigm advocated by the United States, which is punishment-based prohibition, and it signals that the Single Convention may start to reach its expiry date. The legislation that follows it will depend for a large part on the quality of the research available. However, good arguments will finally not be enough; what is most needed is a change in mentality; in politics, but also in the way research is conducted.

3.24.7.2 Availability of Plant Materials and Reference Standards

Although a huge number of scientific papers have been published on Cannabis over the past decades, many aspects still remain unclear. The world today is full of Cannabis myth and mystery. A major reason is the unavailability of standardized plant materials. As a result of a prohibition on the breeding of the Cannabis plant, researchers worldwide have virtually no access to fresh plant materials. In fact, most plant material used for Cannabis research comes from customs seizures or governmental agencies. The type of Cannabis (cultivar), the breeding and storage conditions, and age of the plant materials are often unknown to the researcher. Microbiological contamination of such herbal Cannabis has been frequently described. Although it remains a speculation, the production of toxins by these microbes may play a significant role in at least some of the observed adverse effects in medical studies.[80]

For more than half a century, the medicinal research has been driven by the search for the components responsible for the psychoactive effects of Cannabis. As a result, THC has been in the spotlights for decades, but other Cannabis constituents were largely neglected. By doing so, it is often forgotten that more than 700 different varieties of Cannabis have been described. In many research papers, it is assumed that differences between Cannabis cultivars are only defined by the total content of THC (which is defined as THC + THCA). The total fingerprint of other compounds present is usually not reported and very often these compounds were not even analyzed. As a result, such studies can never be repeated, because the (exact) same type of Cannabis cannot be obtained by other researchers trying to duplicate the results. It is obvious that there is a serious need for the availability of standardized herbal Cannabis for research. Such materials are currently only available from a very limited number of sources. The best example is the Netherlands, where pharmaceutical-grade Cannabis is produced as part of the medicinal Cannabis program of the Dutch Health Ministry, and this material can be exported for research worldwide.[80]

Finally, independent of the method used for cannabinoid analysis, reliable standards are needed for the compounds to be studied, to allow high-quality, quantitative research on the pharmacological and medicinal aspects of Cannabis. However, up to very recently, only a few of the major cannabinoids were commercially available (THC, CBD, CBN, and delta-8-THC). Without a doubt, this lack of reference standards has been a great obstacle for the detailed study and understanding of Cannabis. In recent years, however, the number of suppliers of cannabinoid standards has been growing (e.g., Echo Pharmaceuticals, Lipomed, THC Pharm, Sigma-Aldrich).

3.24.7.3 Social Aspects

Although the complex chemical nature of the Cannabis plant and its constituents has complicated its study, a significant role was also played by social and cultural views on Cannabis in recent history. Although Cannabis has a long history, the twentieth century advent of modern purified pharmaceuticals made Cannabis products increasingly less popular with physicians. Only after the biochemical basis of Cannabis activity has been elucidated, which is in fact only in the last 10–15 years, scientific interest has been somewhat revived.

A major argument of health authorities against the medicinal use of herbal Cannabis as currently available has been that it is a highly variable product with respect to composition and (microbiological) contamination. This may be true when comparing different varieties, but the composition of single well-defined varieties of Cannabis can be highly standardized. This fact has been clearly shown by the medicinal Cannabis program currently going on in the Netherlands, where growing under the regime of Good Agricultural Practice (GAP),

in combination with technical and hygienic measures, has shown to produce Cannabis of high, pharmaceutical quality.[80] Furthermore, procedures for standardized prescription botanical products have been formalized by the FDA, providing a further blueprint for regulatory approval of phytochemicals and botanical medicines.[333]

Cannabis studies often have to deal with problems that are largely unknown to other fields of research. These include difficulties to find funding, get results published, or to obtain permission to perform clinical trials. Also, the restrictions on import/export of Cannabis materials and its extracts or pure components can postpone studies for long periods of time. Fortunately, in several Western countries such restrictions are slowly becoming less strict. Still, the continuing fear of potential psychoactive effects of Cannabis frequently interferes with performing, mostly clinical, studies. In fact, the continuous increase in (psychoactive) potency of modern Cannabis varieties is boosting political as well as societal fears of addiction and health problems, and this seems to make the acceptance of Cannabis as a source of potential new medicines even harder.

It is clear that, in time, Cannabis-based medicines should be standardized, efficacious, and safe preparations, as much as any other approved medicine. Therefore, the main challenges for the near future are standardization of Cannabis-based medicines, obtaining clinical proof of its claimed activities, and improving the acceptance among authorities and health professionals. The dominant view is that the proof of the activities of Cannabis must come from statistically significant randomized clinical trials, acceptable to regulatory bodies in various countries and adhering to the modern scientific method. However, times are changing, and other plant-based medicines, such as Chinese traditional medicine, are gaining ground as part of Western medicine. In some cases, traditional use of such herbal medicine by large groups of patients may be accepted by the authorities as sufficient proof of safety and efficacy. It remains to be seen whether Cannabis can benefit from such developments.

3.24.8 Conclusion

Perhaps Cannabis is best known for its use as a psychoactive drug. However, it should also be recognized as provider of the strongest fibers found in the plant kingdom, and source of some of the healthiest and most nutritious edible oils. In fact, the Cannabis plant can probably meet more of human's needs than any other plant can. As a medicinally active plant, Cannabis has been used by cultures all over the world for millennia, making it one of the oldest known medicinal plants.

But despite the great potential of Cannabis, its classification as a narcotic drug and its increasing demonization in most cultures around the world have so far delayed its successful development into modern medicines. Since the United Nations adopted the 'Single Convention on Narcotic Drugs' in 1961, Cannabis and its products have been politically defined as dangerous narcotics with a high potential for abuse and no accepted medicinal value. Following that, a huge number of studies have been published on all aspects of Cannabis use, but there is currently still no scientific consensus on the usefulness of Cannabis as a medicine. New scientific insights on Cannabis are only slowly and reluctantly incorporated into new legislation. The reasons for this are diverse, and they have been discussed in more detail in this chapter. As a result, the world today appears to be full of Cannabis myth and mystery.

At least one bioactivity of Cannabis is undisputed: the psychoactive effect of THC is one of the best-studied biological activities in the world. As a result, scientific attention has largely shifted from the Cannabis plant as a whole, to its main psychoactive component(s). Chemically, THC belongs to a group of closely related compounds known as cannabinoids, and they are commonly considered the main bioactive components of Cannabis. These terpenophenolic compounds are unique to the Cannabis plant and are found nowhere else in nature. Up to date, already 70 different cannabinoids have been described, but only a few of the major ones have been characterized for biological activities, including CBD and CBN. Nevertheless, the activities that have been discovered so far provide enough reasons to find out what else the Cannabis plant has to offer us.

Besides cannabinoids, Cannabis contains over 450 other identified components. Much remains to be learnt about most of these compounds, as the medicinal properties of Cannabis do not seem to be completely understood based on the cannabinoids alone. What we need to learn about these constituents is related not only to their effect on consumers of Cannabis, but also to their role in the plant, and how various factors affect their biosynthesis and distribution in the plant.

Most interesting among the Cannabis constituents are the secretions of the glandular trichomes, found in high density on the female flowers. Besides cannabinoids, terpenoids are present in high amounts, and more that 100 different types have been identified in Cannabis. Although none of them are unique to Cannabis, many of them have well-described biological activities. And because they are easily volatilized, they are present in Cannabis smoke, which is the most commonly used form of Cannabis administration for both recreational and medicinal users. Although it would be very useful to understand the interaction between the cannabinoids and terpenoids in a variety of medical or psychological conditions, such studies are yet to be undertaken.

Hemp oil is obtained from the mature seeds of Cannabis, and may be an upcoming 'superfood'. Hemp seed is very rich in easily digestible protein content, and its oil has one of the healthiest lipid compositions among the edible plant oils. However, the general confusion between hemp and 'marijuana' still stands in the way of accepting hemp as a major new food crop in most countries. Nutritional studies focused on the health benefits of a hemp-oil-enriched diet may help to increase the acceptance of this valuable resource.

Many additional classes of compounds can be found in Cannabis, including flavonoids, alkaloids, and stilbenoids, and all have been covered in this chapter. But because most of these constituents have not yet been properly characterized for biological activity, the Cannabis plant could be called a 'neglected pharmacological treasure trove'.[32] There is still plenty of work to do for the coming generation of plant researchers, to make us truly understand the potential of the Cannabis plant.

The pharmacological effects of the Cannabis plant have intrigued scientists for centuries, and after the elucidation of the structure of THC in 1964, this led to a hunt for specific binding sites. Finally, the discovery of such sites, the CB1 and CB2 receptors, has provided us with an increasingly clear understanding of the effects of Cannabis. It is now known that cannabinoid receptors can be found in most parts of the brain, as well as in the immune system and a variety of other organs. Their distribution seems to explain many of the observed effects of Cannabis consumption.

Cannabinoid receptors are part of the endocannabinoid system, which is now known to be a ubiquitous neuromodulatory system with wide-ranging actions. It comprises cannabinoid receptors, endogenous cannabinoids, and enzymes responsible for their production, transport, and degradation. The endocannabinoid system can be found even in very primitive organisms, indicating that it has a fundamental role in basic physiology. Its activation seems to represent a crucial and important component for the proper functioning of a wide range of physiological functions. The discovery of the endocannabinoid system has opened up a whole new and exciting field of medical and biological research.

The medicinal potential of Cannabis was largely underestimated until the discovery of the human endocannabinoid system. Now that the significance of this system is becoming increasingly clear, Cannabis as a subject for scientific study should have a brighter future. It is now understood that many biological activities of Cannabis are mediated through a real mechanism, involving not only the endocannabinoid system, but potentially also through cross talk with other systems, including the opioid receptors. And there is increasing evidence that the vanilloid receptor may have a double function as a putative CB3 receptor. Obviously, the discovery of new receptors and ligands may only further our interest in this field.

An increasing number of pharmaceutical companies have started to pick up the idea of (synthetic) cannabinoids and their antagonists as therapeutic drugs. At present a number of preparations based on the biological activities of the cannabinoids are already available, as mentioned in more detail in this chapter. A considerable number of cannabinoid-based medicines are expected to enter the market in the coming years, particularly in the field of synthetic cannabinoid receptor agonists and antagonists. A future with Cannabis-based medicines therefore seems very likely, and a further understanding of Cannabis as a medicine through scientific research is warranted.

However, there is a fundamental problem with using the cannabinoid receptor as a drug target: the main target for most therapeutic activities is CB1 and this is the same receptor that causes most of the (psychoactive) adverse effects. Furthermore, their pharmacokinetic properties set the cannabinoids apart from almost any other type of biologically active compounds used in medicine: the cannabinoids are virtually insoluble in water, causing them to bind to adipose and other tissues, and remain in the body for extended periods of time, up to several months. A major goal of SAR studies is therefore to create more water-soluble cannabinoids that are easier to administer and to dose in an effective manner. Other major goals include the development of CB1

agonists that are excluded from the BBB, and specific CB2 agonists that may be used in treating indications related to the immune system and inflammation.

The Cannabis constituents make up a significant group of compounds with diverse properties, and even based on the limited data available it may be expected that at least several of them have therapeutic potential. Unfortunately, much of the evidence for the medicinal use of Cannabis or cannabinoids is anecdotal and it turns out to be very challenging to confirm these findings by clinical trials. Also, it is often unknown which constituents are actually responsible for the effects observed after the administration of herbal Cannabis or extracts. Only a few indications have been more or less confirmed by clinical testing, including multiple sclerosis, cancer- and AIDS-related nausea and vomiting, chronic pain, and Tourette's syndrome. However, many more indications are currently under some form of investigation, one of the most exciting recent findings being that cannabinoids may be very effective in the treatment of some forms of cancer.

But even without considering these pharmaceutical developments, research on the medicinal use of Cannabis is important simply because Cannabis is already used for self-medication by an huge number of chronically ill people worldwide, often risking harsh legal punishments by doing so. They use Cannabis medicinally, with a large array of different administration forms, to ameliorate the symptoms of diseases varying from cancer and multiple sclerosis, to epilepsy, psychological disorders, and irritable bowl syndrome. It is interesting to note that Cannabis in such situations often seems to be used for ailments that cannot sufficiently be treated with conventional medicine, indicating a specific niche for Cannabis medications. The presence of such a large group of experienced users provides the (medical) researcher with an enormous potential reservoir of knowledge, comparable to ethnopharmacological field studies performed in remote places like the Amazon rainforest, or central Africa. It would therefore probably be wise if future studies on the biological activities of Cannabis would consider including these experiences into a more multidisciplinary approach.

Phytochemical analysis of the Cannabis plant, and in particular its cannabinoids, has been complicated in the past, because of overlapping spectroscopic and chromatographic properties, combined with a severe lack of reliable standards. For the analysis of highly pure, single cannabinoid preparations, specific analytical procedures can be easily developed. However, most phytochemical and pharmacological studies are far more complex than that. Because of the complex chemistry of Cannabis, advanced separation techniques are often necessary for the acquisition of the typical chemical profiles of Cannabis constituents.

More recently, the increasing availability of quantified standards has led to new impulses for analytical science to develop reliable, validated methods for the analysis of the many different types of Cannabis preparations that are available today. Unfortunately, most scientific publications still only mention the THC content of the Cannabis material used in the study, refraining from analyzing or publishing the content of other potentially important biologically active ingredients. But it is likely that using only the total THC content to characterize different Cannabis varieties is not sufficient to understand the complex biological effects of this plant. Ideally, a comprehensive overview of the cannabinoid content (i.e., the chemical fingerprint) of Cannabis preparations used in studies should become an integral part of scientific reports on the effects of Cannabis.

It may be concluded that *C. sativa* as a biologically active plant is currently at an exciting crossroads of science, politics, and culture. Advanced modern techniques such as NMR spectroscopy, principal component analysis, high-resolution MS detection, and various chromatographic improvements make it possible to isolate, identify, and study virtually any constituent that is wished. Currently, there seem to be no more major analytical obstacles for a full understanding of the composition, effects, and usefulness of the Cannabis plant. But although numerous laboratories in the world are allowed to work with even the most dangerous and addictive class/schedule I drugs, many of them do not have permission to work with the relatively mild Cannabis plant in any way or form. In this respect, Cannabis as a subject for scientific research is clearly in a league of its own.

So what is needed now is very clear: scientists must be able to take up the challenges that lay ahead, without the restrictions that are currently holding them back. Traditionally, the function of science is to perform unbiased, peer-reviewed, and reproducible research that is open to discussion after the results have been presented. However, in the case of Cannabis-related studies, these basic principles are all too often challenged by public opinion, political barriers, and legal restrictions, even before the studies can take place.

Clearly, this approach does not stimulate a science-based evolution of our perception of Cannabis as a dangerous drug without medicinal value. Access to (research-grade) Cannabis materials, reference standards,

and validated analytical methods are among the basic requirements to set up the types of studies that should tell us if, and when, Cannabis and its biologically active constituents may be useful in modern medicine. Hopefully, this chapter has been able to inform a new generation of Cannabis researchers about the work to be done, so they can help to make the best of the Cannabis plant and its preparations.

Acknowledgements

B. V. Bedrocan (the official grower of medicinal-grade cannabis in the Netherlands) and Ms. Blair van Pelt are acknowledged for providing the funds to the author to perform field studies on the medicinal properties of medicinal cannabis, and hemp, respectively.

References

1. World Drug Monitor, United Nations Office on Drugs and Crime, 2006.
2. R. C. Clarke, *Marijuana Botany*; Ronin Publishing: Berkeley, CA, 1981.
3. D. Frohne; U. Jensen, *Systematik des Pflanzenreichs unter besonderer Berücksichtigung chemischer Merkmale und pflanzlicher Drogen*; Gustav Fischer: Stuttgart, 1973; p 100.
4. C. E. Turner; M. A. Elsohly; E. G. Boeren, *J. Nat. Prod.* **1980**, *43* (2), 169–234.
5. R. E. Schultes; A. Hofmann, *Pflanzen der Götter: Die magischen Kräfte der Rausch- und Giftgewächse*; Hallwag Verlag: Bern, Switzerland, 1980; p 93.
6. L. Crombie; W. M. L. Crombie, *Phytochemistry* **1975**, *14*, 409–412.
7. C. Fenselau; S. Kelly; M. Salmon; S. Billets, *Food Cosmet. Toxicol.* **1976**, *14*, 35–39.
8. T. Lehmann; R. Brenneisen, *J. Liq. Chromatogr.* **1995**, *18*, 689–700.
9. W. Snoeijer, *A Checklist of Some Cannabaceae Cultivars. Part 1: Cannabis*; Div. Pharmacognosy, Leiden/Amsterdam Centre for Drug Research: Leiden, The Netherlands, 2001.
10. R. E. Schultes; W. M. Klein; T. Plowman; T. E. Lockwood, *Bot. Mus. Lealf. Harv. Univ.* **1974**, *23*, 337–367.
11. L. C. Anderson, *Bot. Mus. Lealf. Harv. Univ.* **1974**, *24*, 29–36.
12. W. A. Emboden, *Econ. Bot.* **1974**, *28*, 304–310.
13. E. Small; A. Cronquist, *Taxon* **1976**, *25*, 405–435.
14. E. Small; P. Y. Jui; L. P. Lefkovitch, *Syst. Bot.* **1976**, *1*, 67–84.
15. A. Cronquist, *An Integrated System of Classification of Flowering Plants*; CUP: New York, 1981; p 193.
16. J. A. Beutler; A. H. Der Marderosian, *Econ. Bot.* **1978**, *32*, 378–394.
17. C. Lawi-Berger, *Contribution à l'étude chimiotaxonomique du genre Cannabis (Cannabaceae). Dissertation*, University of Geneva, Switzerland, 1982.
18. C. Lawi-Berger; M. N. Miège; I. Kapétanidis; J. Miège, *C.R. Acad. Sci. Paris* **1982**, *295*, 397–402.
19. R. Brenneisen, *Pharm. Acta Helv.* **1983**, *58*, 314–320.
20. L. Grlic, *Bull. Narc.* **1968**, *20*, 25–29.
21. P. S. Fetterman; E. S. Keith; C. W. Waller; O. Guerrero; N. J. Doorenbos; M. W. Quimby, *J. Pharm. Sci.* **1971**, *60*, 1246–1249.
22. E. Small; H. D. Beckstead, *Lloydia* **1973**, *36*, 144–165.
23. C. E. Turner; M. A. ElSohly; P. C. Cheng; G. Lewis, *J. Nat. Prod.* **1979**, *42*, 317–319.
24. R. Brenneisen; T. Kessler, *Pharm. Acta Helv.* **1987**, *62*, 134–139.
25. S. Balabanova; F. Parsche; W. Pirsig, *Naturwissenschaften* **1992**, *79* (8), 358.
26. A. W. Zuardi, *Rev. Bras. Psiquiatr.* **2006**, *28* (2), 153–157.
27. F. Grotenhermen; E. Russo, Eds., *Cannabis and Cannabinoids: Pharmacology, Toxicology, and Therapeutic Potential*; Haworth Press: Binghamton, NY, 2002.
28. H. J. Conert; E. J. Jäger; J. W. Kadereit; W. Schultze-Motel; G. Wagenitz; H. E. Weber; G. Hegi, *Illustrierte Flora von Mitteleuropa*; Paul Parey: Berlin/Hamburg, 1992; pp 283–295, 473–474 .
29. Y. Gaoni; R. Mechoulam, *J. Am. Chem. Soc.* **1964**, *86*, 646–647.
30. E. B. Russo, *Chem. Biodivers.* **2007**, *4*, 1614–1648.
31. M. A. ElSohly; D. Slade, *Life Sci.* **2005**, *78* (5), 539–548.
32. R. Mechoulam, *Br. J. Pharmacol.* **2005**, *146* (7), 913–915.
33. S. Ross; M. A. ElSohly, *Zagazig J. Pharm. Sci.* **1995**, *4*, 1–10.
34. E. S. Kim; P. G. Mahlberg, *Mol. Cells* **2003**, *15* (3), 387–395.
35. S. Sirikantaramas; F. Taura; Y. Tanaka; Y. Ishikawa; S. Morimoto; Y. Shoyama, *Plant Cell Physiol.* **2005**, *46* (9), 1578–1582.
36. S. Sirikantaramas; F. Taura; S. Morimoto; Y. Shoyama, *Curr. Pharm. Biotechnol.* **2007**, *8* (4), 237.
37. M. M. Radwan; S. A. Ross; D. Slade; S. A. Ahmed; F. Zulfiqar; M. A. Elsohly, *Planta Med.* **2008**, *74* (3), 267–272.
38. S. A. Ahmed; S. A. Ross; D. Slade; M. M. Radwan; F. Zulfiqar; M. A. Elsohly, *J. Nat. Prod.* **2008**, *71* (4), 536–542.
39. R. Adams; B. R. Baker; R. B. Wearn, *J. Am. Chem. Soc.* **1940**, *62* (8), 2204–2207.
40. S. A. Ross; Z. Mehmedic; T. P. Murphy; M. A. Elsohly, *J. Anal. Toxicol.* **2000**, *24* (8), 715–717.
41. B. I. Field; R. R. Arndt, *J. Pharm. Pharmacol.* **1980**, *32*, 21–24.
42. J. K. Hemphill; J. C. Turner; P. G. Mahlberg, *J. Nat. Prod.* **1980**, *43*, 112–122.
43. N. B. Eddy, *The Question of Cannabis*; Bibliography United Nations Commision on Narcotic Drugs, E/CN7/49, 1965.

44. F. Bohlmann; E. Hoffmann, *Phytochemistry* **1979**, *18*, 1371–1374.
45. Y. Asakawa; K. Takikawa; M. Toyota; T. Takemoto, *Phytochemistry* **1982**, *21*, 2481–2490.
46. Y. Gaoni; R. Mechoulam, *Proc. Chem. Soc.* **1964**, 82.
47. R. Mechoulam; Y. Gaoni, *Tetrahedron* **1965**, *21*, 1223–1229.
48. R. Mechoulam; Y. Gaoni, *Fortsch. Chem. Org. Naturst.* **1967**, *25*, 175–213.
49. R. Mechoulam, *Science* **1970**, *168*, 1159–1166.
50. R. Mechoulam, *Marihuana*; Academic Press: New York, 1973.
51. Y. Shoyama; T. Yamauchi; I. Nishioka, *Chem. Pharm. Bull.* **1970**, *18*, 1327–1332.
52. Y. Shoyama; M. Yagi; I. Nishioka; T. Yamauchi, *Phytochemistry* **1975**, *14*, 2189–2192.
53. C. E. Turner; K. Hadley, *J. Pharm. Sci.* **1973**, *62*, 251–258.
54. M. Fellermeier; M. H. Zenk, *FEBS Lett.* **1998**, *427*, 283–285.
55. M. Fellermeier; W. Eisenreich; A. Bacher; M. H. Zenk, *Eur. J. Biochem.* **2001**, *268*, 1596–1604.
56. F. Taura; S. Morimoto; Y. Shoyama; R. Mechoulam, *J. Am. Chem. Soc.* **1995**, *117* (38), 9766–9767.
57. F. Taura; S. Morimoto; Y. Shoyama, *J. Biol. Chem.* **1996**, *271* (29), 17411–17416.
58. S. Morimoto; K. Komatsu; F. Taura; Y. Shoyama, *J. Phytochem.* **1998**, *49* (6), 1525–1529.
59. I. J. Flores-Sanchez; R. Verpoorte, *Phytochem. Rev.* **2008**, *7*, 615–639.
60. P. G. Mahlberg; E. S. Kim, *J. Ind. Hemp* **2004**, *9*, 15–36.
61. E. P. M. de Meijer; K. M. Hammond; M. Micheler, *Euphytica* **2009**, *165*, 293–311.
62. R. Brenneisen, Chemistry and Analysis of Phytocannabinoids and Other Cannabis Constituents. In *Forensic Science and Medicine: Marijuana and the Cannabinoids*; M. A. ElSohly, Ed.; Humana Press Inc.: Totowa, NJ, 2006; pp 17–49.
63. T. Veress; J. I. Szanto; L. Leisztner, *J. Chromatogr.* **1990**, *520*, 339–347.
64. R. K. Razdan, *Progr. Org. Chem.* **1973**, *8*, 78–101.
65. L. Crombie; R. Ponsford; A. Shani; B. Yagnitinsky; R. Mechoulam, *Tetrahedron Lett.* **1968**, *55*, 5771–5772.
66. M. A. ElSohly; H. N. ElSohly; C. E. Turner, Cannabis: New Constituents and Their Pharmacological Action. In *Topics in Pharmaceutical Sciences*; D. D. Breimer, R. Speiser, Eds.; Elsevier Science Publishers: New York, NY, 1985; pp 429–439.
67. C. Perras, *Issues Emerg. Health Technol.* **2005**, *72*, 1–4.
68. T. Nadulski; F. Pragst; G. Weinberg; P. Roser; M. Schnelle; E. M. Fronk; A. M. Stadelmann, *Ther. Drug Monit.* **2005**, *27* (6), 799–810.
69. B. Ben Amar, *J. Ethnopharm.* **2006**, *105*, 1–25.
70. A. Holdcroft; M. Maze; C. Dore; S. Tebbs; S. Thompson, *Anesthesiology* **2006**, *104* (5), 1040–1046.
71. K. C. Verhoeckx; H. A. Korthout; A. P. van Meeteren-Kreikamp; K. A. Ehlert; M. Wang; J. van der Greef; R. J. Rodenburg; R. F. Witkamp, *Int. Immunopharmacol.* **2006**, *6* (4), 656–665.
72. T. J. Raharjo; R. Verpoorte, *Phytochem. Anal.* **2004**, *15* (2), 79–94.
73. K. Bailey, *J. Forensic Sci.* **1979**, *24*, 817–841.
74. D. Corrigan; J. J. Lynch, *Planta Med.* **1980**, *40*, 163–169.
75. A. Hazekamp; C. Giroud; A. Peltenburg; R. Verpoorte, *J. Liq. Chromatogr. Relat. Technol.* **2005**, *28* (15), 2361–2382.
76. D. Debruyne; F. Albessard; M. C. Bigot; M. Moulin, *Bull. Narc.* **1994**, *46*, 109–121.
77. J. Pothier; N. Galand; C. Viel, *J. Toxicol. Clin. Exp.* **1992**, *12*, 495–501.
78. P. Oroszlan; G. Verzar-Petri; E. Mincsovics; T. Szekely, *J. Chromatogr.* **1987**, *388*, 217–224.
79. A. A. Stolker; J. van Schoonhoven; A. J. de Vries; I. Bobeldijk-Pastorova; W. H. Vaes; R. van den Berg, *J. Chromatogr. A* **2004**, *1058* (1–2), 143–151.
80. A. Hazekamp, *Cannabinoids* **2006**, *1* (1), 1–9.
81. I. S. Lurie; R. P. Meyers; T. S. Conver, *Anal. Chem.* **1998**, *70* (15), 3255–3260.
82. B. Backstrom; M. D. Cole; M. J. Carrott; D. C. Jones; G. Davidson; K. Coleman, *Sci. Justice* **1997**, *37* (2), 91–97.
83. Y. H. Choi; A. Hazekamp; A. M. G. Peltenburg-Looman; M. Frederich; C. Erkelens; A. W. M. Lefeber; R. Verpoorte, *Phytochem. Anal.* **2004**, *15* (6), 345–354.
84. V. Di Marzo; D. Melck; T. Bisogno; L. De Petrocellis, *Trends Neurosci.* **1998**, *21*, 521–528.
85. J. Corchero; J. Manzanares; J. A. Fuentes, *Crit. Rev. Neurobiol.* **2004**, *16*, 159–172.
86. A. C. Howlett, *Handb. Exp. Pharmacol.* **2005**, *168*, 53–79.
87. W. L. Dewey, *Pharmacol. Rev.* **1986**, *38*, 151–178.
88. R. G. Pertwee, *Pharmacol. Ther.* **1988**, *36*, 189–261.
89. R. Mechoulam; W. A. Devane; R. Glaser, *Cannabinoid Geometry and Biological Activity*. CRC Press: Boca Raton, FL, 1992; pp 1– 33.
90. W. A. Devane; F. A. Dysarz; M. R. Johnson; L. S. Melvin; A. C. Howlett, *Mol. Pharmacol.* **1988**, *34*, 605–613.
91. A. C. Howlett, *Mol. Pharmacol.* **1985**, *27*, 429–436.
92. A. C. Howlett; J. M. Qualy; L. L. Khachatrian, *Mol. Pharmacol.* **1986**, *29*, 307–313.
93. A. C. Howlett, *Neuropharmacology* **1987**, *26*, 507–512.
94. M. Bidaut-Russell; W. A. Devane; A. C. Howlett, *J. Neurochem.* **1990**, *55*, 21–26.
95. L. A. Matsuda; S. J. Lolait; M. J. Brownstein; A. C. Young; T. I. Bonner, *Nature* **1990**, *346*, 651–654.
96. V. DiMarzo, *Drug Discov. Today* **2008**, *13* (23–24), 1026–1041.
97. K. Mackie; B. Hille, *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3825–3829.
98. G. Velasco; I. Galve-Roperh; C. Sánchez; C. Blázquez; A. Haro; M. Guzmán, *Life Sci.* **2005**, *77*, 1723–1731.
99. S. Munro; K. L. Thomas; M. Abu-Shaar, *Nature* **1993**, *365* (6441), 12–13.
100. M. D. van Sickle; M. Duncan; P. J. Kingsley; A. Mouihate; P. Urbani; K. Mackie; N. Stella; A. Makriyannis; D. Piomelli; J. S. Davison; L. T. Marnett; V. DiMarzo; Q. J. Pittman; K. D. Patel; K. A. Sharkey, *Science* **2005**, *310*, 329–332.
101. F. Molina-Holgado; E. Pinteaux; J. D. Moore; E. Molina-Holgado; C. Guaza; R. M. Gibson; N. J. Rothwell, *J. Neurosci.* **2003**, *23* (16), 6470–6474.
102. G. A. Cabral; E. S. Raborn; L. Griffin; J. Dennis; F. Marciano-Cabral, *Br. J. Pharmacol.* **2008**, *153* (2), 240–251.
103. L. De Petrocellis; D. Melck; T. Bisogno; A. Milone; V. Di Marzo, *Neuroscience* **1999**, *92* (1), 377–387.

104. J. M. McPartland; P. Pruitt, *J. Cannabis Ther.* **2002**, *2* (1), 73–104.
105. D. Smart; M. J. Gunthorpe; J. C. Jerman; S. Nasir; J. Gray; A. I. Muir; J. K. Chambers; A. D. Randall; J. B. Davis, *Br. J. Pharmacol.* **2000**, *129*, 227–230.
106. P. M. Zygmunt; J. Petersson; D. A. Andersson; H. Chuang; M. Sørgård; V. DiMarzo; D. Julius; E. D. Högestätt, *Nature* **1999**, *400* (6743), 452–457.
107. R. G. Pertwee, *Handb. Exp. Pharmacol.* **2005**, *168*, 1–51.
108. M. Begg; P. Pacher; S. Bátkai; D. Osei-Hyiaman; L. Offertáler; F. M. Mo; J. Liu; G. Kunos, *Pharmacol. Ther.* **2005** *106* (2), 133–145.
109. E. Ryberg; N. Larsson; S. Sjögren; S. Hjorth; N. O. Hermansson; J. Leonova; T. Elebring; K. Nilsson; T. Drmota; P. J. Greasley, *Br. J. Pharmacol.* **2007**, *152* (7), 1092–1101.
110. A. Makriyannis, *The Role of Cell Membranes in Cannabinoid Activity in Cannabinoid Receptors*; Academic Press: London, 1995; pp 87–115.
111. S. Oddi; P. Spagnuolo; M. Bari; A. D'Agostino; M. Maccarrone, *Int. Rev. Neurobiol.* **2007**, *82*, 327–337.
112. W. A. Devane; L. Hanus; A. Breuer; R. G. Pertwee; L. A. Stevenson; G. Griffin; D. Gibson; A. Mandelbaum; A. Etinger; R. Mechoulam, *Science* **1992**, *258*, 1946–1949.
113. R. Mechoulam; S. Ben-Shabat; L. Hanus; M. Ligumsky; N. E. Kaminski; A. R. Schatz; A. Gopher; S. Almog; B. R. Martin; D. R. Compton; R. G. Pertwee; G. Griffin; M. Bayewitch; J. Barg; Z. Vogel, *Biochem. Pharmacol.* **1995**, *50*, 83–90.
114. R. Mechoulam; E. Fride; V. Di Marzo, *Eur. J. Pharmacol.* **1998**, *359*, 1–18.
115. R. G. Pertwee, *Int. J. Obes. (Lond.)* **2006**, *30* (Suppl. 1), S13–S18.
116. V. Di Marzo; T. Bisogno; L. De Petrocellis, *Chem. Biol.* **2007**, *14*, 741–756.
117. V. Di Marzo; S. Petrosino, *Curr. Opin. Lipidol.* **2007**, *18*, 129–140.
118. M. J. McFarland; E. L. Barker, *Pharmacol. Ther.* **2004**, *104*, 117–135.
119. B. Koutek; G. D. Prestwich; A. C. Howlett; S. A. Chin; D. Salehani; N. Akhavan; D. G. Deutsch, *J. Biol. Chem.* **1994**, *269*, 22937–22940.
120. R. Shrestha; R. A. Dixon; K. D. Chapman, *J. Biol. Chem.* **2003**, *278*, 34990–34997.
121. K. D. Chapman, *Prog. Lipid. Res.* **2004**, *43*, 302–327.
122. M. A. Peat, *Adv. Anal. Toxicol.* **1989**, *2*, 186–217.
123. M. E. Wall, *Clin. Pharmacol. Ther.* **1983**, *34*, 352–363.
124. L. Zuurman; C. Roy; R. Schoemaker; A. Hazekamp; J. den Hartigh; J. C. M. E. Bender; R. Verpoorte; J. L. Pinquier; A. F. Cohen; J. van M. A. Gerven, *J. Psychopharmacol.* **2008**, *22* (7), 707–716.
125. A. Reiter; J. Hake; C. Meissner; J. Rohwer; H. J. Friedrich; M. Oehmichen, *Forensic Sci. Int.* **2001**, *119*, 248–253.
126. F. Grotenhermen, *Clin. Pharmacokinet.* **2003**, *42*, 327–360.
127. M. A. Huestis, *Handb. Exp. Pharmacol.* **2005**, *168*, 657–690.
128. I. Yamamoto; K. Watanabe; S. Narimatsu; H. Yoshimura, *Int. J. Biochem. Cell Biol.* **1995**, *27* (12), 1365.
129. K. Mackie; N. Stella, *AAPS J.* **2006**, *8*, E298–E306.
130. G. Milligan; R. A. Bond; M. Lee, *Trends Pharmacol. Sci.* **1995**, *16*, 10–13.
131. A. Goutopoulos; A. Makriyannis, *Pharmacol. Ther.* **2002**, *95*, 103–117.
132. S. L. Palmer; G. A. Thakur; A. Makriyannis, *Chem. Phys. Lipids* **2002**, *121*, 3–19.
133. G. A. Thakur; S. P. Nikas; C. Li; A. Makriyannis, *Handb. Exp. Pharmacol.* **2005**, *168*, 209–246.
134. G. A. Thakur; R. I. Duclos, Jr.; A. Makriyannis, *Life Sci.* **2005**, *78*, 454–466.
135. A. C. Howlett; F. Barth; T. I. Bonner; G. Cabral; P. Casellas; W. A. Devane; C. C. Felder; M. Herkenham; K. Mackie; B. R. Martin; R. Mechoulam; R. G. Pertwee, *Pharmacol. Rev.* **2002**, *54*, 161–202.
136. G. A. Thakur; S. P. Nikas; A. Makriyannis, *Mini. Rev. Med. Chem.* **2005**, *5* (7), 631–640.
137. J. W. Huffman; J. Liddle; S. Yu; M. M. Aung; M. E. Abood; J. L. Wiley; B. R. Martin, *Bioorg. Med. Chem.* **1999**, *7* (12), 2905–2914.
138. M. H. Rhee, *J. Med. Chem.* **1997**, *40*, 3228–3233.
139. J. M. McPartland; M. Glass; R. G. Pertwee, *Br. J. Pharmacol.* **2007**, *152*, 583–593.
140. R. G. Pertwee, *Br. J. Pharmacol.* **2008**, *153*, 199–215.
141. W. J. Ryan; W. K. Banner; J. L. Wiley; B. R. Martin; R. K. Razdan, *J. Med. Chem.* **1997**, *40*, 3617–3625.
142. J. W. Huffman; J. R. Miller; J. Liddle; S. Yu; B. F. Thomas; J. L. Wiley; B. R. Martin, *Bioorg. Med. Chem.* **2003**, *11*, 1397–1410.
143. W. A. Devane; A. Breuer; T. Sheskin; T. U. Järbe; M. S. Eisen; R. Mechoulam, *J. Med. Chem.* **1992**, *35* (11), 2065–2069.
144. A. Thomas; L. A. Stevenson; K. N. Wease; M. R. Price; G. Baillie; R. A. Ross; R. G. Pertwee, *Br. J. Pharmacol.* **2005**, *146* (7), 917–926.
145. J. W. Huffman; S. M. Bushell; J. R. Miller; J. L. Wiley; B. R. Martin, *Bioorg. Med. Chem.* **2002**, *10*, 4119–4129.
146. L. S. Melvin; G. M. Milne; M. R. Johnson; B. Subramaniam; G. H. Wilken; A. C. Howlett, *Mol. Pharmacol.* **1993**, *44*, 1008–1015.
147. W. Tong; E. R. Collantes; W. J. Welsh; B. A. Berglund; A. C. Howlett, *J. Med. Chem.* **1998**, *41*, 4207–4215.
148. A. D. Khanolkar; A. Makriyannis, *Life Sci.* **1999**, *65*, 607–616.
149. P. H. Reggio; H. Traore, *Chem. Phys. Lipids* **2000**, *108*, 15–35.
150. R. P. Picone; A. D. Khanolkar; W. Xu; L. A. Ayotte; G. A. Thakur; D. P. Hurst; M. E. Abood; P. H. Reggio; D. J. Fournier; A. Makriyannis, *Mol. Pharmacol.* **2005**, *68*, 1623–1635.
151. V. Vinciguerra; T. Moore; E. Brennan, *N. Y. State J. Med.* **1988**, *85*, 525–527.
152. A. E. Chang; D. J. Shiling; R. C. Stillman; N. H. Goldberg; C. A. Seipp; I. Barofsky; R. M. Simon; S. A. Rosenberg, *Ann, Intern. Med.* **1979**, *91*, 819–824.
153. R. S. Hepler; R. Petrus, *Experiences with Administration of Marijuana to Glaucoma Patients*; Plenum Medical Book Company: New York, 1976; pp 63–76.
154. R. D. Mattes; K. Engelman; L. M. Shaw; M. A. Elsohly, *Pharmacol. Biochem. Behav.* **1994**, *49*, 187–195.
155. M. E. Wall; M. Perez-Reyes, *J. Clin. Pharmacol.* **1981**, *21*, 178S–189S.
156. F. C. Hiller; F. J. J. Wilson; M. K. Mazumder; J. D. Wilson; R. C. Bone, *Fundam. Appl. Toxicol.* **1984**, *4*, 451–454.
157. P. Matthias; D. P. Tashkin; J. A. Marques-Magallanes; J. N. Wilkins; M. S. Simmons, *Pharmacol. Biochem. Behav.* **1997**, *58*, 1145–1150.

158. J. E. Joy, *Marijuana and Medicine: Assessing the Scientific Base*; Institute of Medicine: Washington, DC, 1999.
159. S. Agurell; M. Halldin; J. E. Lindgren; A. Ohlsson; M. Widman; H. Gillespie; L. Hollister, *Pharmacol. Rev.* **1986**, *38*, 21–43.
160. A. Ohlsson; J. E. Lindgren; A. Wahlen; S. Agurell; L. E. Hollister; H. K. Gillespie, *Clin. Pharmacol. Ther.* **1980**, *28*, 409–416.
161. F. Van der Kooy; B. Pomahacova; R. Verpoorte, *Inhal. Toxicol.* **2009**, *21* (2), 87–90.
162. A. Hazekamp; R. Ruhaak; L. Zuurman; J. van Gerven; R. Verpoorte, *J. Pharm. Sci.* **2006**, *95* (6), 1308–1317.
163. D. I. Abrams; H. P. Vizoso; S. B. Shade; C. Jay; M. E. Kelly; N. L. Benowitz, *Clin. Pharmacol. Ther.* **2007**, *82* (5), 572–578.
164. L. Grinspoon; J. B. Bakalar; L. Zimmer; J. P. Morgan, *Science* **1997**, *277* (5327), 749.
165. R. D. Mattes; L. M. Shaw; J. Edling-Owens; K. Engelman; M. A. Elsohly, *Pharmacol. Biochem. Behav.* **1993**, *44*, 745–747.
166. A. Hazekamp; K. Bastola; H. Rashidi; J. Bender; R. Verpoorte, *J. Ethnopharm.* **2007**, *113*, 85–90.
167. R. Noyes; S. F. Brunk; D. H. Avery; A. Canter, *Clin. Pharmacol. Ther.* **1975**, *18*, 84–89.
168. F. A. Campbell; M. R. Tramer; D. Carroll; D. J. Reynolds; R. A. Moore; H. J. McQuay, *BMJ* **2001**, *323*, 13–16.
169. D. L. Cichewicz, *Life Sci.* **2004**, *74*, 1317–1324.
170. A. H. Lichtman; B. R. Martin, *Pharmacol. Biochem. Behav.* **1997**, *57*, 7–12.
171. J. A. Fuentes; M. Ruiz-Gayo; J. Manzanares; G. Vela; I. Reche; J. Corchero, *Life Sci.* **1999**, *65*, 675–685.
172. A. Fox; A. Kesingland; C. Gentry; K. McNair; S. Patel; L. Urban; I. James, *Pain* **2001**, *92*, 91–100.
173. M. R. Johnson; L. S. Melvin; T. H. Althuis; J. S. Brinda; C. A. Harbert; G. M. Milne; A. Weissman, *J. Clin. Pharmacol.* **1981**, *21*, 271s–282s.
174. Di V. Marzo, *Cannabinoids*; Springer: New York, 2004.
175. E. M. Williamson; F. J. Evans, *Drugs* **2000**, *60* (6), 1303–1314.
176. E. S. Onaivi; H. Ishiguro; J. P. Gong; S. Patel; P. A. Meozzi; L. Myers; A. Perchuk; Z. Mora; P. A. Tagliaferro; E. Gardner; A. Brusco; B. E. Akinshola; B. Hope; J. Lujilde; T. Inada; S. Iwasaki; D. Macharia; L. Teasenfitz; T. Arinami; G. R. Uhl, *PLoS ONE* **2008**, *20* (3), e1640.
177. M. Perez-Reyes, *NIDA Res. Monogr.* **1990**, *99*, 42–62.
178. L. A. Parker; P. Burton; R. E. Sorge; C. Yakiwchuk; R. Mechoulam, *Psychopharmacology* **2004**, *175*, 360–366.
179. E. Meiri; H. Jhangiani; J. J. Vredenburgh; L. M. Barbato; F. J. Carter; H. M. Yang; V. Baranowski, *Curr. Med. Res. Opin.* **2007**, *23* (3), 533–543.
180. A. Jatoi; H. E. Windschitl; C. L. Loprinzi; J. A. Sloan; S. R. Dakhil; J. A. Mailliard; S. Pundaleeka; C. G. Kardinal; T. R. Fitch; J. E. Krook; P. J. Novotny; B. Christensen, *J. Clin. Oncol.* **2002**, *20* (2), 567–573.
181. I. B. Adams; B. R. Martin, *Addiction* **1996**, *91* (11), 1585–1614.
182. B. Favrat; A. Ménétrey; M. Augsburger; L. E. Rothuizen; M. Appenzeller; T. Buclin; M. Pin; P. Mangin; C. Giroud, *BMC Psychiatry* **2005**, *5*, 17.
183. C. Henquet; A. Rosa; L. Krabbendam; S. Papiol; L. Fananás; M. Drukker; J. G. Ramaekers; J. van Os, *Neuropsychopharmacology* **2006**, *31* (12), 2748–2757.
184. T. Järvinen; D. W. Pateb; K. Lainea, *Pharmacol. Ther.* **2002**, *95* (2), 203–220.
185. S. J. Williams; J. P. Hartley; J. D. Graham, *Thorax* **1976**, *31* (6), 720–723.
186. J. McPartland, In *Advantages of Polypharmaceutical Herbal Cannabis Compared to Single-Ingredient, Synthetic Tetrahydrocannabinol. Biosource Hemp*: Proceedings of the Third International Symposium, Nova Institut: Wolfsburg, Germany, 2000.
187. B. Costa, *Chem. Biodivers.* **2007**, *4*, 1664–1677.
188. R. J. McKallip; M. Nagarkatti; P. S. Nagarkatti, *J. Immunol.* **2005**, *174*, 3281–3289.
189. T. Esfandyari; M. Camilleri; I. Busciglio; D. Burton; K. Baxter; A. R. Zinsmeister, *Am. J. Physiol. Gastrointest. Liver Physiol.* **2007**, *293* (1), G137–G145.
190. I. Tselnicker; O. Keren; A. Hefetz; C. G. Pick; Y. Sarne, *Neurosci. Lett.* **2007** *411* (2), 108–111.
191. A. M. Malfait; R. Gallily; P. F. Sumariwalla; A. S. Malik; E. Andreakos; R. Mechoulam; M. Feldmann, *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97* (17), 9561–9566.
192. M. Kathmann; K. Flau; A. Redmer; C. Tränkle; E. Schlicker, *Naunyn Schmiedeberg's Arch. Pharmacol.* **2006**, *375* (5), 354–361.
193. E. Murillo-Rodríguez; D. Millán-Aldaco; M. Palomero-Rivero; R. Mechoulam; R. Drucker-Colín, *FEBS Lett.* **2006**, *580* (18), 4337–4345.
194. A. W. Zuardi; I. Shirakawa; E. Finkelfarb; I. G. Karniol, *Psychopharmacology (Berl.)* **1982**, *76* (3), 245–250.
195. de Souza J. A. Crippa; A. W. Zuardi; G. E. J. Garrido; L. Wichert-Ana; R. Guarnieri; L. Ferrari; P. M. Azevedo-Marques; J. E. C. Hallak; P. K. McGuire; G. F. Busatto, *Neuropsychopharmacology* **2004**, *29*, 417–426.
196. A. W. Zuardi; S. L. Morais; F. S. Guimaraes; R. Mechoulam, *J. Clin. Psychiatry* **1995**, *56*, 485–486.
197. S. D. McAllister; R. T. Christian; M. P. Horowitz; A. Garcia; P. Y. Desprez, *Mol. Cancer Ther.* **2007**, *6* (11), 2921–2927.
198. P. Massi; A. Vaccani; S. Ceruti; A. Colombo; M. P. Abbracchio; D. Parolaro, *J. Pharmacol. Exp. Ther.* **2004**, *308* (3), 838–845.
199. A. Ligresti; A. S. Moriello; K. Starowicz; I. Matias; S. Pisanti; L. De Petrocellis; C. Laezza; G. Portella; M. Bifulco; V. Di Marzo, *J. Pharmacol. Exp. Ther.* **2006**, *318* (3), 1375–1387.
200. L. Grlic, *Bull. Narc.* **1976**, *14*, 37–46.
201. J. T. Ungerleider; T. Andrysiak, *Int. J. Addict.* **1985**, *20*, 691–699.
202. L. M. Bornheim; K. Y. Kim; J. Li; B. Y. Perotti; L. Z. Benet, *Drug Metab. Dispos.* **1995**, *23* (8), 825–831.
203. L. M. Bornheim; M. P. Grillo, *Chem. Res. Toxicol.* **1998**, *11* (10), 1209–1216.
204. Y. Avraham; D. Ben-Shushan; A. Breuer; O. Zolotarev; A. Okon; N. Fink; V. Katz; E. M. Berry, *Pharmacol. Biochem. Behav.* **2004**, *77* (4), 675–684.
205. T. Yamaguchi; T. Kubota; S. Watanabe; T. Yamamoto, *J. Neurochem.* **2004**, *88* (1), 148–154.
206. G. Appendino; S. Gibbons; A. Giana; A. Pagani; G. Grassi; M. Stavri; E. Smith; M. M. Rahman, *J. Nat. Prod.* **2008**, *71* (8), 1427–1430.
207. B. K. Colasanti, *J. Ocul. Pharmacol.* **1990**, *6* (4), 259–269.
208. E. A. Formukong; A. T. Evans; F. J. Evans, *Inflammation* **1988**, *12* (4), 361–371.
209. S. H. Baek; D. S. Han; C. N. Yook; Y. C. Kim; J. S. Kwak, *Arch. Pharm. Res.* **1996**, *19* (3), 228–230.
210. E. A. Formukong; A. T. Evans; F. J. Evans, *J. Pharm. Pharmacol.* **1989**, *41* (10), 705–709.

211. B. E. Akinshola; A. Chakrabarti; E. S. Onaivi, *Neurochem. Res.* **1999**, *24* (10), 1233–1240.
212. G. Nahas; R. Trouve, *Proc. Soc. Exp. Biol. Med.* **1985**, *180* (2), 312–316.
213. J. E. Shook; T. F. Burks, *J. Pharmacol. Exp. Ther.* **1989**, *249* (2), 444–449.
214. A. C. Herring; N. E. Kaminski, *J. Pharmacol. Exp. Ther.* **1999**, *291*, 1156–1163.
215. W. M. Davis; N. S. Hatoum, *Gen. Pharmacol.* **1983**, *14* (2), 247–252.
216. R. E. Musty; R. A. Deyo, In *Cannabichromene (CBC) Extract Alters Behavioral Despair on the Mouse Tail Suspension Test of Depression. Proceedings of the International Cannabinoid Research Society, 2003 Symposium on the Cannabinoids*, Burlington, VT, USA, 2003; p 146.
217. Y. L. Ma; S. E. Weston; B. J. Whalley; G. J. Stephens, *Br. J. Pharmacol.* **2008**. *154* (1), 204–215.
218. Y. Shoyama; T. Fujita; T. Yamauchi; I. Nishioka, *Chem. Pharm. Bull. (Tokyo)* **1968**, *16* (6), 1157–1158.
219. G. Petri, In *Biotechnology in Agriculture and Forestry: Medicinal and Aromatic Plants I*; Y. P. S. Bajaj, Ed.; Springer-Verlag: Heidelberg, 1988; Vol. 4, pp 333–349.
220. C. Leizer; D. Ribnicky; A. Poulev; S. Dushenkov; I. Raskin, *J. Nutraceut. Funct. Med. Foods* **2000**, *2* (4), 35–53.
221. H. Edery; Y. Grunfeld; G. Porath; Z. Ben-Zvi; A. Shani; R. Mechoulam, *Arzneimittelforschung* **1972**, *22* (11), 1995–2003.
222. A. Hazekamp; R. Simons; A. Peltenburg-Looman; M. Sengers; R. van Zweden; R. Verpoorte, *J. Liq. Chromatogr. Relat. Technol.* **2004**, *27* (15), 2421–2439.
223. S. Morimoto; Y. Tanaka; K. Sasaki; H. Tanaka; T. Fukamizu; Y. Shoyama; F. Taura, *J. Biol. Chem.* **2007**, *282* (28), 20739–20751.
224. J. M. McPartland; E. B. Russo, *J. Cannabis Ther.* **2001**, *1*, 103–132.
225. K. Woelkart; O. M. Salo-Ahen; R. Bauer, *Curr. Top. Med. Chem.* **2008**, *8* (3), 173–186.
226. S. Raduner; A. Majewska; J. Z. Chen; X. Q. Xie; J. Hamon; B. Faller; K. H. Altmann; J. Gertsch, *J. Biol. Chem.* **2006**, *281*, 14192–14206.
227. J. Gertsch; S. Raduner; K. H. Altmann, *J. Recept. Signal Transduct. Res.* **2006**, *26* (5–6), 709–730.
228. J. Gertsch; M. Leonti; S. Raduner; I. Racz; J. Z. Chen; X. Q. Xie; K. H. Altmann; M. Karsak; A. Zimmer, *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105* (26), 9099–9104.
229. E. Stahl; R. Kunde, *Tetrahedron Lett.* **1973**, *30*, 2841–2844.
230. R. Brenneisen; M. A. ElSohly, *J. Forensic Sci.* **1988**, *33*, 1385–1404.
231. S. A. Ross; M. A. ElSohly, *J. Nat. Prod.* **1996**, *59* (1), 49–51.
232. C. Meier; V. Mediavilla, *J. Int. Hemp Assoc.* **1998**, *5* (1), 16–20.
233. K. W. Hillig, *Biochem. Syst. Ecol.* **2004**, *32* (10), 875–891.
234. G. Buchbauer; L. Jirovetz; W. Jäger; C. Plank; H. Dietrich, *J. Pharm. Sci.* **1993**, *82* (6), 660–664.
235. T. Komori; R. Fujiwara; M. Tanida; J. Nomura, *Eur. Neuropsychopharmacol.* **1995**, *5* (4), 477–480.
236. B. B. Lorenzetti; G. E. Souza; S. J. Sarti; D. Santos Filho; S. H. Ferreira, *J. Ethnopharmacol.* **1991**, *34* (1), 43–48.
237. V. S. Rao; A. M. Menezes; G. S. Viana, *J. Pharm. Pharmacol.* **1990**, *42* (12), 877–878.
238. S. Burstein; C. Varanelli; L. T. Slade, *Biochem. Pharmacol.* **1975**, *24* (9), 1053–1054.
239. A. K. Agrawal; P. Kumar; A. Gulati; P. K. Seth, *Res. Commun. Subst. Abuse* **1989**, *10*, 155–168.
240. C. Nasel; B. Nasel; P. Samec; E. Schindler; G. Buchbauer, *Chem. Senses* **1994**, *19* (4), 359–364.
241. J. Novak; K. Zitterl-Eglseer; S. G. Deans; C. M. Franz, *Flavour Fragrance J.* **2001**, *16* (4), 259–262.
242. G. Fournier; M. R. Paris; M. C. Fourniat; A. M. Quero, *Ann. Pharm. Fr.* **1978**, *36* (11–12), 603–606.
243. M. Rothschild; G. Bergstrom; S. A. Wangberg, *Bot. J. Linn. Soc.* **2005**, *147* (4), 387–397.
244. M. L. Barrett; A. M. Scutt; F. J. Evans, *Experientia* **1986**, *42* (4), 452–453.
245. R. R. Paris; M. R. Paris, *C.R. Hebd. Seances Acad. Sci. D* **1973**, *277* (21), 2369–2371.
246. S. A. Ross; M. A. ElSohly; G. N. N. Sultana; Z. Mehmedic; C. F. Hossain; S. Chandra, *Phytochem. Anal.* **2005**, *16* (1), 45–48.
247. M. Gellert; I. Novak; M. Szell; K. Szendrei, Glycosidic components of *Cannabis sativa* L. I. Flavonoids. UN Document ST/SOA/SER.S/50, 20 September, 1974.
248. M. L. Barrett; D. Gordon; F. J. Evans, *Biochem. Pharmacol.* **1985**, *34* (11), 2019–2024.
249. R. R. Paris; E. Henri; M. Paris, *Plant. Med. Phytother.* **1976**, *10*, 144–154.
250. A. B. Segelman; F. P. Segelman; A. E. Star; H. Wagner; O. Seligmann, *Phytochemistry* **1978**, *17* (4), 824–826.
251. I. J. Flores-Sanchez, *Polyketide Synthases in Cannabis sativa L. Dissertation,* University of Leiden, Leiden, The Netherlands, 2008.
252. M. N. Clark; B. A. Bohm, *Bot. J. Linn. Soc.* **1979**, *79*, 249–257.
253. G. Vanhoenacker; P. Van Rompaey; D. De Keukeleire; P. Sandra, *Nat. Prod. Lett.* **2002**, *16* (1), 57–63.
254. L. Bravo, *Nutr. Rev.* **1998**, *56* (11), 317–333.
255. D. Treutter, *Plant Biol.* **2005**, *7* (6), 581–591.
256. E. Middleton, *Int. J. Pharm.* **1996**, *34* (5), 344–348.
257. A. Braca; G. Fico; I. Morelli; F. De Simone; F. Tome; N. De Tommasi, *J. Ethnopharm.* **2003**, *86* (1), 63–67.
258. C. G. Fraga, *IUBMB Life* **2007**, *59* (4–5), 308–315.
259. P. G. Pietta, *J. Nat. Prod.* **2000**, *63* (7), 1035–1042.
260. E. Middleton; C. Kandaswami; T. C. Theoharides, *Pharmacol. Rev.* **2000**, *52* (4), 673–751.
261. T. P. T. Cushnie; A. J. Lamb, *Int. J. Antimicrob. Agents* **2005**, *26* (5), 343–356.
262. B. Ozcelik; I. Orhan; G. Toker, *Z. Naturforsch. C, J. Biosci.* **2006**, *61* (9–10), 632–638.
263. A. B. Segelman; F. P. Segelman; S. D. Varma; H. Wagner; O. Seligmann, *J. Pharm. Sci.* **1977**, *66* (9), 1358–1359.
264. H. N. Elsohly; M. A. Elsohly, Marijuana Smoke Condensate: Chemistry and Pharmacology. In *Marijuana and the Cannabinoids*; M. A. Elsohly, Ed.; Humana Press: Totowa, NJ, 2007.
265. M. A. Sauer; S. M. Rifka; R. L. Hawks; G. B. Cutler; D. L. Loriaux, *J. Pharmacol. Exp. Ther.* **1983**, *224* (2), 404–407.
266. S. Y. Lee; S. M. Oh; K. H. Chung, *Toxicol. Appl. Pharmacol.* **2006**, *214* (3), 270–278.
267. M. E. Gerritsen; W. W. Carley; G. E. Ranges; C. P. Shen; S. A. Phan; G. F. Ligon; C. A. Perry, *Am. J. Pathol.* **1995**, *147* (2), 251–272.
268. A. T. Evans; E. A. Formukong; F. J. Evans, *Biochem. Pharmacol.* **1987**, *36*, 2035–2037.
269. B. Botta; A. Vitali; P. Menendez; D. Misiti; G. Delle Monache, *Curr. Med. Chem.* **2005**, *12* (6), 713–739.

270. J. F. Stevens; J. E. Page, *Phytochemistry* **2004**, *65* (10), 1317–1330.
271. T. Walle, *Free Radic. Biol. Med.* **2004**, *36* (7), 829–837.
272. J. P. E. Spencer; M. M. A. El Mohsen; C. Rice-Evans, *Arch. Biochem. Biophys.* **2004**, *423* (1), 148–161.
273. J. L. Deferne; D. W. Pate, *J. Int. Hemp Assoc.* **1996**, *3* (1), 4–7.
274. K. Jones, *Nutritional and Medicinal Guide to Hemp Seed*; Rainforest Botanical Laboratory: Gibsons, British Columbia, Canada, 1995.
275. G. Leson; P. Pless; J. Roulac, *Hemp Foods and Oils for Health*; Hemptech: Sebastopol, CA, 1999.
276. S. A. Ross; H. N. ElSohly; E. A. ElKashoury; M. A. ElSohly, *Phytochem. Anal.* **1996**, *7*, 279–283.
277. H. Molleken; R. Theimer, *J. Int. Hemp Assoc.* **1997**, *4* (1), 13–18.
278. D. Wirtshafter, Nutrition of Hemp Seeds and Hemp Seed Oil. In *Bioresource Hemp*, 2nd ed.; Nova-Institute: Cologne, Germany, 1995; pp 546–555.
279. C. McEvoy; M. Edwards; M. Snowden, *Pharm. Technol. Eur.* **1996**, *8* (6), 36–40.
280. J. C. Callaway, *Euphytica* **2004**, *140*, 65–72.
281. U. Erasmus, *Fats That Heal, Fats That Kill*; Alive Books: Burnaby, BC, 1999.
282. A. P. Simopoulos, *Biomed. Pharmacother.* **2006**, *60* (9), 502–507.
283. A. P. Simopoulos, *J. Am. Coll. Nutr.* **2002**, *21* (6), 495–505.
284. F. Grotenhermen; M. Karus; D. Lohmeyer, Derivation of THC Limits for Food, Part II. http://www.naihc.org/hemp_information/content/nova_report/part2.html
285. C. E. Turner; M. L. Mole, *JAMA* **1973**, *225* (6), 639.
286. R. Mechoulam, Alkaloids in *Cannabis sativa* L. In *The Alkaloids: Chemistry and Pharmacology*; A. Brussi, Ed.; Academic Press Inc: USA, 1988, pp 77–93.
287. T. Hamada, *J. Pharm. Soc. Jpn.* **2005**, *125*, 1–16.
288. I. Wahby; D. Arráez-Román; A. Segura-Carretero; F. Ligero; J. M. Caba; A. Fernández-Gutiérrez, *Electrophoresis* **2006**, *27*, 2208–2215.
289. J. M. Johnson; L. Lemberger; M. Novotny; R. B. Forney; W. S. Dalton; M. P. Maskarinec, *Toxicol. Appl. Pharmacol.* **1984**, *72*, 440–448.
290. F. K. Klein; H. Rapoport, *Nature* **1971**, *232*, 258–259.
291. A. Raman; A. Joshi, *The Chemistry of Cannabis*; CRC Press: Boca Raton, FL, USA, 1998.
292. C. T. Kuo; M. J. Hsu; B. C. Chen; C. C. Chen; C. M. Teng; S. L. Pan; C. H. Lin, *Toxicol. Lett.* **2008**, *177* (1), 48–58.
293. J. Molnar; K. Csiszar; I. Nishioka; Y. Shoyama, *Acta Microbiol. Hung.* **1986**, *33*, 221–231.
294. K. Back; S. M. Jang; B. C. Lee; A. Schmidt; D. Strack; K. M. Kim, *Plant Cell Physiol.* **2001**, *42* (5), 475–481.
295. D. C. Ayres; J. D. Loike, Lignans: Chemical, Biological and Clinical Properties. In *Chemistry and Pharmacology of Natural Products*; J. D. Phillipson, D. C. Ayres, H. Baxter, Eds.; Cambridge University Press: Cambridge, 1990; p 402.
296. N. G. Lewis; L. B. Davin, Lignans: Biosynthesis and Function. In *Comprehensive Natural Products Chemistry, Vol. 1: Polyketides and Other Secondary Metabolites Including Fatty Acids and Their Derivatives*; D. H. R. Barton, K. Nakanishi, O. Meth-Cohn, U. Sankawa, Ed.; Elsevier Science Ltd.: Oxford, 1999; pp 639–712.
297. R. P. Walton; L. F. Martin; J. H. Keller, *J. Pharmacol. Exp. Ther.* **1938**, *62*, 239.
298. B. R. Martin, *Pharmacol. Rev.* **1986**, *38*, 45–74.
299. D. A. Dansak, *Cannabis as an Antiemetic and Appetite Stimulant in Cancer Patients*; McFarland & Co.: Jefferson, NC, 1997; pp 69–83.
300. T. F. Plasse; R. W. Gorter; S. H. Krasnow; M. Lane; K. V. Shepard; R. G. Wadleigh, *Pharmacol. Biochem. Behav.* **1991**, *40* (3), 695–700.
301. R. Noye; D. A. Baram, *Compr. Psychiatry* **1974**, *15* (6), 531–535.
302. D. B. Clifford, *Ann. Neurol.* **1983**, *13* (6), 669–671.
303. K. R. Muller-Vahl; H. Kolbe; U. Schneider; H. M. Emrich, *Forsch. Komplementarmed.* **1999**, *6* (Suppl. 3), 23–27.
304. R. S. Hepler; I. R. Frank, *JAMA* **1971**, *217* (10), 1392.
305. G. Watts, *BMJ* **2004**, *329*, 257–258.
306. L. E. Hollister, *Pharmacology* **1974**, *11*, 3–11.
307. E. B. Russo; G. W. Guy, *Med. Hypotheses* **2006**, *66*, 234–246.
308. P. Fox; P. G. Bain; S. Glickman; C. Carroll; J. Zajicek, *Neurology* **2004**, *62* (7), 1105–1109.
309. L. F. Van Gaal; A. M. Rissanen; A. J. Scheen; O. Ziegler; S. Rossner, *Lancet* **2005**, *365*, 1389–1397.
310. S. H. Burstein; M. Karst; U. Schneider; R. B. Zurier, *Life Sci.* **2004**, *75* (12), 1513–1522.
311. R. B. Zurier; Y. P. Sun; K. L. George; J. A. Stebulis; R. G. Rossetti; A. Skulas; E. Judge; C. N. Serhan, *FASEB J.* **2009**, *23* (5), 1503–1509.
312. K. Salim; U. Schneider; S. Burstein; L. Hoy; M. Karst, *Neuropharmacology* **2005**, *48* (8), 1164–1171.
313. M. Karst; K. Salim; S. Burstein; I. Conrad; L. Hoy; U. Schneider, *JAMA* **2003**, *290* (13), 1757–1762.
314. J. J. Feigenbaum; F. Bergmann; S. A. Richmond; R. Mechoulam; V. Nadler; Y. Kloog; M. Sokolovsky, *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86* (23), 9584–9587.
315. C. L. Darlington, *IDrugs* **2003**, *6*, 976–979.
316. A. I. Maas; G. Murray; H. Henney, III; N. Kassem; V. Legrand; M. Mangelus; J. P. Muizelaar; N. Stocchetti; N. Knoller; *Lancet Neurol.* **2006**, *5* (1), 38–45.
317. R. Mechoulam, Ed., *Cannabinoids as Therapeutics*; Birkhaeuser Verlag: Basel, Switzerland, 2005; p 272.
318. E. R. Garrett; C. A. Hunt, *J. Pharm. Sci.* **1974**, *63*, 1056–1064.
319. B. F. Thomas; D. R. Compton; B. A. Martin, *J. Pharmacol. Exp. Ther.* **1990**, *255* (2), 624–630.
320. C. Scully, *Br. Dent. J.* **2007**, *203* (6), E12.
321. E. Stern; D. M. Lambert, *Chem. Biodivers.* **2007**, *4* (8), 1707–1728.
322. A. Hazekamp; R. Verpoorte, *Eur. J. Pharm. Sci.* **2006**, *29* (5), 340–347.
323. R. N. Kumar; W. A. Chambers; R. G. Pertwee, *Anaesthesia* **2001**, *56*, 1059–1068.
324. J. Guindon; A. G. Hohmann, *Br. J. Pharmacol.* **2008**, *153*, 319–334.

325. Y. Cheng; S. A. Hitchcock, *Exp. Opin. Investig. Drugs* **2007**, *16*, 951–965.
326. J. E. Joy; S. J. Watson, Jr.; J. A. Benson, Jr., Eds., *Marijuana and Medicine – Assessing the Science Base*; National Academy Press: Washington, DC, 1999.
327. D. Vigano; T. Rubino; D. Parolaro, *Pharmacol. Biochem. Behav.* **2005**, *81* (2), 360–368.
328. R. G. Pertwee, *Br. J. Pharmacol.* **2006**, *147* (Suppl. 1), S163–S171.
329. P. B. Smith; S. P. Welch; B. R. Martin, *J. Pharmacol. Exp. Ther.* **1994**, *268*, 1382–1387.
330. I. J. Williams; S. Edwards; A. Rubo; V. L. Haller; D. L. Stevens; S. P. Welch, *Eur. J. Pharmacol.* **2006**, *539* (1–2), 57–63.
331. S. Narang; D. Gibson; A. D. Wasan; E. L. Ross; E. Michna; S. S. Nedeljkovic; R. N. Jamison, *J. Pain* **2008**, *9* (3), 254–264.
332. N. S. Radin, *Biochem. J.* **2003**, *371*, 243–256.
333. *Guidance for Industry: Botanical Drug Products*; US Food and Drug Administration, Center for Drug Evaluation and Research: Rockville, MD, 2004; p 48.

Biographical Sketches

Dr. Arno Hazekamp was born on 15 March, 1976 in the Netherlands. He studied at Leiden University, the Netherlands, where he obtained his Bachelor's diploma in the field of molecular biology from the School of Biology. He continued his studies at the same university, School of Pharmaceutical Science, where he worked for several years in the Department of Pharmacognosy, under Professor Rob Verpoorte. In 2000, Arno finished his M.Sc. research on the ethnopharmacology of a Thai medicinal plant, and graduated with honors. Subsequently, he was employed as a technician and laboratory manager at Leiden University.

In November 2001, Arno started as a Ph.D. student under the supervision of Professor Rob Verpoorte. His research project was focused on the medicinal properties of the Cannabis plant, and on the practical obstacles that stand between this plant and its development into a modern medicine. Arno worked closely with the official grower of medicinal Cannabis in the Netherlands, Bedrocan BV, and was involved in numerous projects regarding quality control, product development, and basic research regarding medicinal Cannabis. Arno was actively involved in setting up the medicinal Cannabis program of the Dutch Health Ministry, and was a strong advocate of a more science-based approach on the medicinal use of Cannabis in the Netherlands and abroad. During his Ph.D., Arno spent several periods at the Institut Universitaire de Médecine Légale (IUML) in Lausanne, Switzerland, focusing on the forensic aspects of Cannabis use.

After finishing his Ph.D. in 2007, Arno started a phytochemical contract laboratory, and was the first to make a wide range of highly pure cannabinoid standards commercially available. He was also involved in the early phase of Echo Pharmaceuticals, a Dutch pharmaceutical company, developing a sublingual administration form of THC and other cannabinoids. Arno is currently working as a phytochemist with a larger consortium of biotech companies that work together under the name Product Isolation from Nature (PRISNA), providing phytochemical services and consultancy for the development of plant-based products.

Arno continues to have a strong interest in the medicinal use of Cannabis, with a specific focus on controlled growing, quality control, and safe access for medical patients. In 2009, Arno became a board member of the International Association for Cannabis as Medicine (IACM). Througout his career, Arno has maintained contact with patients, caretakers, and patient-organizations. These connections and experiences serve as an inspiration for evolving research projects. He is an active traveller and lecturer, and is considered a professionally trained medicinal Cannabis advocate. However, most of his time is still spent in the laboratory.

Andrea Lubbe was born in Belville, South Africa. She completed a B.Sc. degree in Molecular and Cellular Biology at the University of Stellenbosch, South Africa, followed by an honours degree in Plant Physiology at the same university. She obtained an M.Sc. degree in Natural Products at the University of Leiden in The Netherlands, and is currently at the same institution pursuing a Ph.D. degree. The focus of her current work is agronomic factors affecting alkaloid production in *Narcissus* species.

Justin T. Fischedick was born in the United States in 1984. He is currently a Ph.D. student at Leiden University studying various aspects of the Cannabis plant. He is specifically interested in the interactions and synergy among the many biologically active components of Cannabis. Mr. Fischedick earned his Masters degree in Biology at Leiden University in 2008 and his B.Sc. degree from the State University of New York College of Environmental Science and Forestry in 2006.

Renee Ruhaak was born in Leiden, The Netherlands. She completed a B.Sc. degree in BioPharmaceutical Sciences at Leiden University, The Netherlands, where she continued her studies for a Master degree and finished in 2005. Her interests are in methods of application for Cannabis and cannabinoids, which was the topic of her master thesis. Currently, she holds a Ph.D. position at the Leiden University Medical Centre on the topic of glycosylation analysis in the study of healthy ageing.

Mónica Llano Díez was born in Oviedo, Spain, and graduated in Biochemistry at the University of Oviedo, Spain. She did an internship training program in the Department of Molecular Structural Biology, University of Greifswald, Germany, and participated as postgraduate student in the project 'Metabolic profiling of *Catharanthus Roseus* cells elicited with *Pythium* and jasmonic acid using ^{1}H-NMR' at the University of Leiden, The Netherlands. Currently, she is doing her Ph.D. in Neurophysiology at the University of Uppsala, Sweden.

3.25 Chemistry of Coffee

S. Oestreich-Janzen, CAFEA GmbH, Hamburg, Germany

3.25.1 Overview

3.25.1.1 Coffee's Origin

The coffee plant most probably originated in Africa and Madagascar. Early cultivation is reported in the Ethiopian Highlands and also on the other side of the Red Sea, in Yemen – no surprise, since climate and geography are similar. According to legends, coffee stepped out of Africa most likely in the first millennium. Other narratives deal with the discovery of roasting, leading to the consumption of coffee as a beverage.

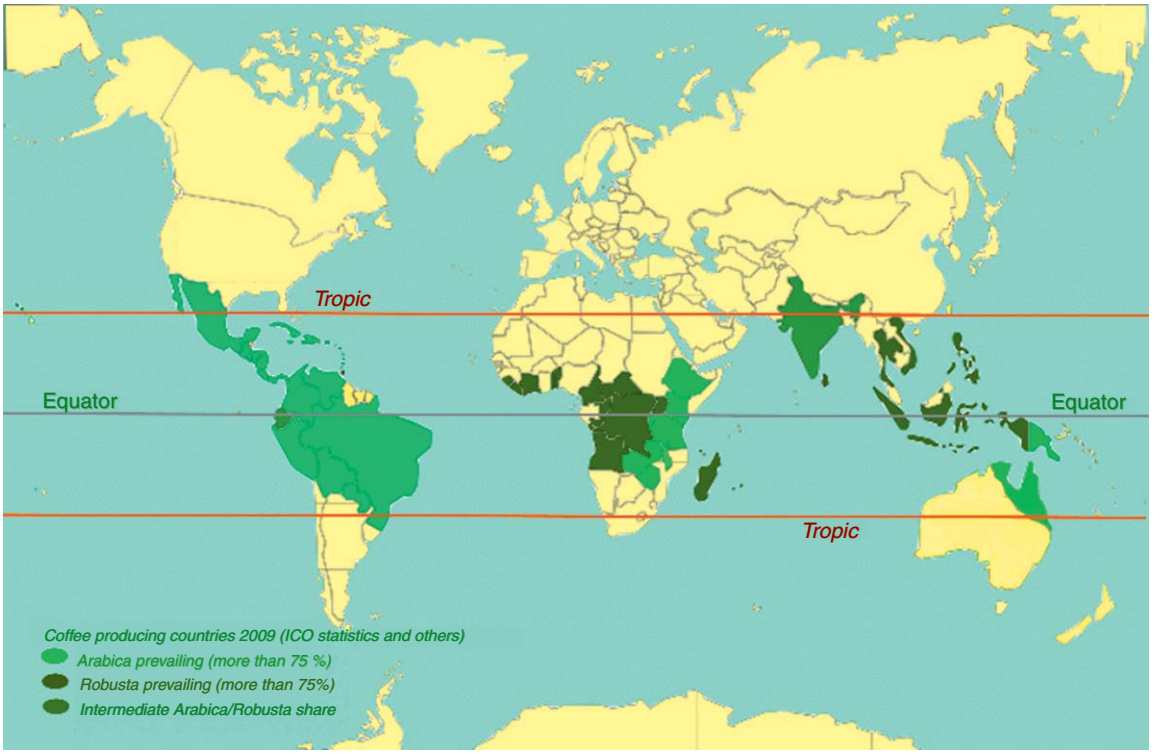


Figure 1 Coffee producing countries around the globe.

Coffee grows best in areas lying between the tropics. Today, it is found in all continents belonging to this geographic belt, **Figure 1**, even in Australia and very recently, not yet shown here, at the Northern tropic in the Yunnan highland of China. There are two main species of coffee, Arabica and Robusta, each with its own ecology.

3.25.1.2 Early Cultivation and Consumption

The first reference to coffee in the literature seems to be from Arab scientists in the ninth and tenth centuries (Rhazes, Persian physician (853–935); Avicenna, Arab physician (980–1037), polymath in the world of letters and sciences). Referenced to as 'bun', it was a stimulant food of Ethiopia and Yemen. It might have been chewed and eaten.

The oldest material source is dated about AD 1200. Carbonized paleobotanic Arabica coffee beans were found in 1997 in an archaeological excavation[1] on the eastern side of the Arabian Peninsula, near Oman, together with the early thirteenth-century pottery from Yemen. Since these beans were evidently not at their growing place, they indicate that coffee was traded at that time. The findings also reveal that the beans came from Yemen and people knew about roasting them.[2]

Until 1600, the cultivation of coffee was restricted to Arabia, and mainly centered in the highlands of Yemen, where a sophisticated system of irrigation was practiced.[3] To retain this monopoly, the export of coffee plants was strictly forbidden, and even the beans for trade were said to have been made infertile.

Coffee was economically important for both producers and traders. The Arab Islamic world was familiar with the beverage, even though there were times of restriction, based on whether or not religion permitted consumption of the beverage.[4] Mecca pilgrims took word of the beverage to their distant homelands.

The use of coffee spread with the Ottoman Empire, over its part of the Mediterranean. Coffee brewers progressed into a specialized guild, as reported by Austrian legation members about a procession of guilds at a celebration in Istanbul in 1582.[5]

Detailed information on coffee reached the Christian part of Europe about the same time, through travel reports of scientists[6,7] and books' prints with drawings of the plant and descriptions of its consumption. The

naked beans had circulated among European scientists some time before, mentioned in the updated 1574 edition of an earlier standard work on tropical plants.[8]

Coffee as a commodity for trade did not enter the non-Islamic world until 1615, when a few bags arrived in Venice,[9] followed by demand and supply. The coffee trade's mainstream ran from Mocha, the port in Yemen involved in export, through ship and caravan to Cairo and Alexandria.[4] From there the coffee was distributed to the Ottoman customers and to the consumers in Europe, where coffee houses started flourishing. As early as 1668, coffee had crossed the Atlantic and arrived in Dutch New York.[10]

3.25.1.3 Ways Out of Arabia

Despite all of the protective restrictions adopted in the Arab world, the expanding interest in the beverage encouraged a search for a similar tropical climate for growing coffee plants and efforts to find ways of transferring plants. Possibly, the first expansion was to the west coast of India, where coffee was brought in by Muslim pilgrims from Mecca. The mystical origins of Indian coffee are traced to the seven beans brought to Karnataka by the legendary Baba Budan in 1600.[11,12]

From India, coffee is reported to have moved to Ceylon (now Sri Lanka) in 1658, to the plantations of the Netherlands East India Company, and to Java after 1696.[13] Java became the world's mass supplier for coffee outside Arabia and remained so for half a century; even today, in the United States, a cup of coffee is commonly called a 'cup of Java'.

From the Dutch colonies, single plants were sent to the botanical garden of Amsterdam in 1706; from there, living plants were transported to the Dutch colony in Surinam for cultivation and a grown-up tree was gifted to King Louis XIV in Paris in 1714.[14] Some plants of the next Parisian generation were transferred to French colonies in the Caribbean, for cultivation in an appropriate climate. There is a well-known textbook story of a single plant surviving on a ship to Martinique, nourished by the drinking water of its officer, in about 1720. This was reported by the officer himself 50 years later[15]. The coffee variety originating from Amsterdam, called Typica, made its way step by step into Latin America.

At the same time, there was another route of Arabica plants out of Yemen: 60 seedlings, an official present by the Yemenite sultan[16] to Louis XIV, were sent to the French Bourbon Island (La Réunion, today) in 1715; again, these were a limited number of plants. From there, the Bourbon variety of coffee entered the French colonies in the Americas and in other places.

Coffee cultivation was also promoted by other colonial powers such as Portugal, Spain, and England (and, for a few decades, by Germany also). These powers also expanded cultivation to their respective spheres of influence.

Bourbon and Typica are the prolific ancestors of most of today's Arabica crops – a genetically narrow route that expanded from a few single plants to millions of tons of Arabica per year, all within 300 years.

Other coffee types, which grew without international demand in the humid tropical areas of their origin, became objects of scientific surveys and explorations from the eighteenth century.

Today, independent coffee producing countries cooperate with the consuming ones in a worldwide research for the conservation of the coffee plant's genetic heritage and diversity. Numerous so-called wild or spontaneous varieties deserve genetic resource management. Since they are imminently threatened by advanced deforestation, an effective preservation strategy is needed.

3.25.1.4 Discovery of the Robusta Variety

The tropical areas of Africa, from Guinea to Angola and to Uganda in the east, host different species of coffee. The first to gain economic importance was the Ethiopian Arabica, with its early cultivation in Arabia.

Reports of other species in Africa and their indigenous cultivation and trade date from the nineteenth century. For the East African countries, expeditions of 1857[17] and 1862[18] informed that coffee was familiar to the people there, grown in homegardens,[19,20] and chewed and eaten as green bean for its stimulating effect.[21,22] Discussions on the identity of these species of coffee, whether they were wild Arabicas (prospector's hope) or sui generis, remained ongoing[23] till 1897. Several non-Arabica species were determined by Froehner,[24] including *Coffea canephora* Pierre,[25] with the later extension ex A. Froehner.

Cultivation of this species for use as roast coffee was initiated in 1898,[26,27] and promoted by a Belgian horticultural company.[28,29] This coffee was called Robusta by the Belgians as it proved more robust against diseases and had less ecological requirements in terms of humidity, temperature, and altitude of plantations. Although different in taste, it soon turned out to be a useful alternative for the vulnerable Arabicas, which were disease prone due to their narrow genetic origin.[30] There was an actual demand for resistant plants as a disastrous epidemic of leaf rust, caused by the devastating *Hemileia vastatrix* fungus, started in 1869.[31] The first to suffer from it were the Ceylon coffee plantations. There, at the abandoned plantations, coffee was replaced by tea. The next coffee countries soon to be attacked were India and, then, Indonesia. Eventually, the search for rust-resistant coffee types led to the substitution of Arabica by Robusta, after an intermezzo with the species *Coffea liberica*. During the last century, Robusta made its way to other coffee growing regions and witnessed an enormous expansion in its cultivation.

Besides pests and diseases, coffee crops can also be reduced by drought and frost,[32] thus influencing the available volume of coffee. This happened regularly after a frost in Brazil, causing declines in world supplies in the 1970s. Soon the cultivation of Robusta increased in West Africa, its original homeland.

Since 1990 there has been a steady increase in Robusta production in Vietnam, which is now the world's of coffee in general.[33]

3.25.1.5 Coffee as a Trade Commodity – World Production

The data of total coffee production since 1620 show the overall increase and the rise of new production areas. Compiled from several sources, these data are represented as a graph (**Figure 2**), with the volume of today's big players marked individually.

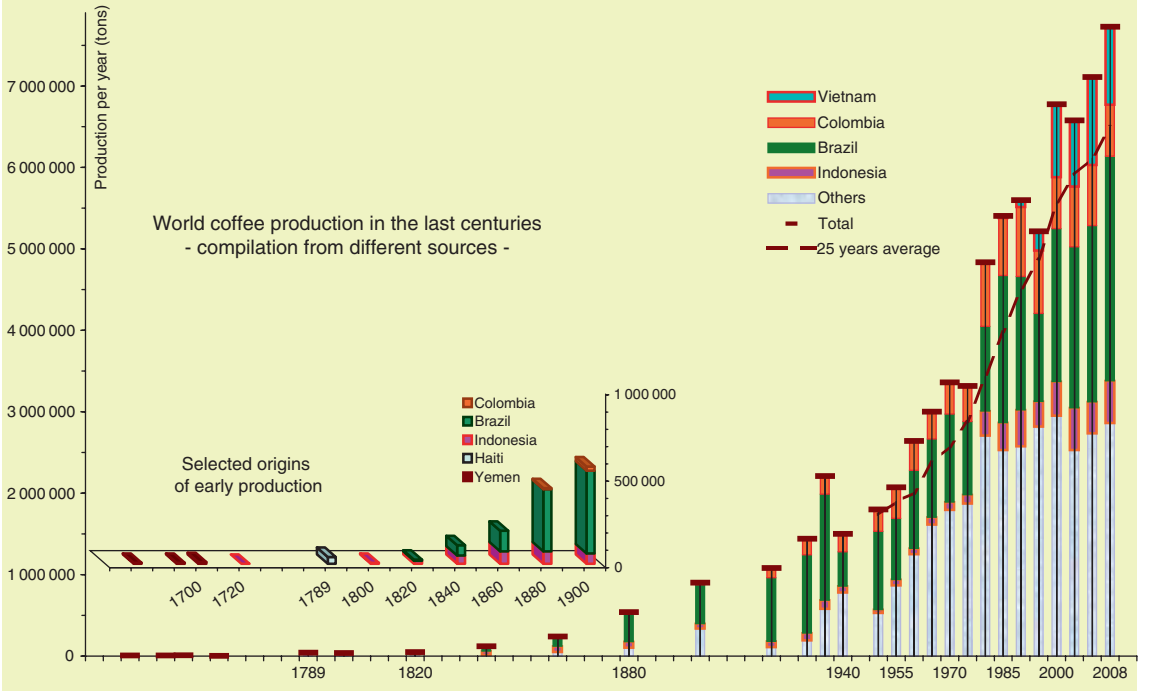


Figure 2 World Coffee production compiled from several sources.

A. 1620, 1700: Becker,[34] p 11.
B. 1670, 1955: Söhn,[35] pp 11, 98.
C. 1720: Eccardi; Sandalj,[9] p 48.
D. 1789, 1936: Dahlgren,[10] pp 22, 41.
E. 1800–1920, 1940, 1950, 1960, 1975: Sivetz,[36] pp 13.
F. 1980–1995: ICO,[33] historical data, total production of member plus non member countries (2009).
G. 2000–2008: ICO,[33] world supply, total production (accessed July 2009).

The known data for world coffee trade started with about 10 000 tons per year in 1700,[34] all from Yemen, followed by supply from Indonesia and from the Caribbean.[35,37] In 2008, a total of about 8 000 000 tons was traded (roughly, Brazil 35%, Vietnam 15%, Colombia 10%, and Indonesia 5%, followed by Ethiopia, Mexico, and India; Arabica constituted 60%). The world production figures are likely higher because there is domestic consumption as well. The volumes of world coffee production and trade before the seventeenth century is not clearly reported; compared with the volumes of today, it was negligible.

3.25.1.6 From Field to Physiology

Coffee passes through several steps to reach its final destination: human consumption. All of these steps – the seed maturation, postharvest processing, storage and transportation on land and ship, roasting, home brewing or industrial extraction, and eventual intermediate treatments – have an impact on its chemical composition.

The economic importance of coffee is evident in agriculture, occupation, and welfare both in producing and consuming countries. Production, international trade, and world wide consumption create normative rules in legislation, administration and standardization in the countries concerned, regarding registration, food surveillance, customs, subsidaries, analytical methods, and many other aspects. Coffee's agriculture has an impact even on the global ecology.

Originally, coffee's stimulating property, its oldest-known physiological effect, was the basis of its use; later on the focus shifted to enjoying aromatic roast coffee beverage as a psychoactive stimulant. Today, due to sophisticated research on the effects and mechanisms of action of coffee, a more balanced view has emerged on the effects of coffee on our well-being and health.

3.25.2 Botany

3.25.2.1 Plant Characteristics, Habit, and Growth

In nonrestricted growth, the coffee plant is a perennial tree or treelet with a single main trunk and horizontal branches, primary, secondary, and tertiary horizontal branches (plagiotropic); pruning can lead to multiple-stem plants.

Coffee's original place is the forest understorey. Coffee plants bear clusters of flowers and cherry-like fruits. A short central taproot fades into axillary and lateral roots and a manifold of feeder bearers and root hairs. The dark green elliptical leaves grow in opposite pairs on the main stem and branches. The wood is dense; the fruits, nectar, and leaves are food resources.

Under tropical conditions, flowering and fruiting happen in parallel; the nearer coffee plants are to the equator, the more pronounced is a bimodal cycle.

Multistem growth is trained by either capping the main stem, giving rise to suckers that develop into new vertical stems with horizontal branching, or bending the principal stem to the horizontal (agobiado technique), with several suckers in a row.

Periodic pruning is done to optimize the plant shape for good fruiting, easy harvesting, and effective disease and pest control, as well as to rejuvenate the plant.

Pests may attack the plants in the field and the beans in store by boring, biting, mining, and sucking, or living as parasites on the root system; fungi, viruses, and bacteria may cause severe coffee diseases.

Further characteristics and figures are given in **Table 1**. For each characteristic, the properties common to all species are written in the first line, followed by the differing ones beneath in separate columns for the species.

The development of flowers from the buds takes several weeks, that of the fruits several months after flowering, with periods of growth and of dormancy. The sequence of steps is enzymatically controlled in-plant and is triggered externally by photoperiodism, relief of water stress, and temperature drop.

In a days-after-flowering (DAF) scale,[43] depicted in **Figure 3**, the tissues of the growing fruit show marked changes in volume ranking of all pericarp, perisperm, and endosperm. Embedded in the start-up pericarp, the perisperm expands to equal the pericarp mass; then, it gets absorbed by the developing endosperm, the final organ for all storage compounds in the fruit.

Propagation to the next generation starts with either seeds from the farmer's own plants, sown directly in the fields, or seedlings from seeds or cuttings delivered by a nursery in plastic bags for planting. Development time in soil from the naked seed to the first leaves is 1–2 months at best. A third method for propagation *in vitro* is

Table 1 Coffee characteristics and requirements for cultivation

Species Character	*Coffea*	*Coffea arabica*	*Coffea canephora*	*Others*
Chromosome status		Amphidiploid ($2n = 4 = 44$ chromosomes)	Diploid ($2n = 22$ chromosomes)	
Ecology	Humid, evergreen tropical forest (cultivation with and without shade trees; intercropping)			
		tropical highlands, (950–) 1200–1950 m	tropical lowlands. (50–)250–1500 m	
		Temperature: moderate, 15–24 °C	Warm, 18–36 °C	
		Rainfall 1200–2200 mm $year^{-1}$	Rainfall 2200–3000 mm $year^{-1}$	
		Exceptional cultivations at sea level (Hawaii)	Sometimes in seasonally dry humid forest, or in gallery forest	
Plant height		4–6 m (pruned 2–3 m)	8–12 m (pruned 2–3 m)	*C.liberica* up to 18 m; also 2 m dwarf species
Canopy diameter		1.2–2 m	1.2–2 m	
Root system	Tap root 0.5–1 m; several axillary roots, vertical depth 1.5–3 m; many lateral roots parallel to the soil, distance 1.5–1.8 m from the trunk; hair roots overall; 90% of the root system in the upper 30 cm of the lateral parts			
		Deeper roots	Shallower roots	
Leaves	Elliptical; margin entire; tip accuminate; dark green to bronze green			
		Length 10–15, width 4–6 cm	Length 22, width 17 cm	
		Development time 7–8 weeks to full expansion and optimum photosynthesis (Arabica); life span 7–10 months (Robusta)		
Inflorescences	Paired, axillary; best flowering at 1-year-old wood; development time 4 months with periods of dormancy			
		4–12 with 16–48 flowers per node	30–100 flowers per node	
Flowers	Hermaphrodite; corollas white or rarely light pink; corolla lobes overlapping; anthers exerted; style long, exerted			
		Autogamous (self-pollinating)	Allogamous (cross-pollinating)	
		Time for pollination 1 day	Receptive to 6 days after flowering	
Fruit	Berry containing two seeds (rarely one or three); each seed with a deep groove, an invagination, on the flat ventral side (‘coffee bean’).			
		Length 12–18 mm	Length 8–16 mm	
		Fruit maturation 7–9 months after flowering	Fruit maturation 9–11 months after flowering	*C.racemosa*: 2 months

The table is compiled from several sources.[24,38–42]

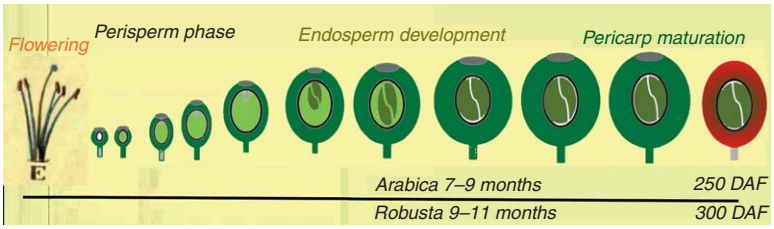


Figure 3 Fruit development in coffee, days-after-flowering scale, compiled from several sources. A. Flower: Linnaeus,[46] p 1. B. Bean development: De Castro; Marraccini,[43] p 177.

used in research: the cultivation of a fragment of tissue from a plant in a suitable substrate, producing a new plant that is genetically identical to the plant from which it originated.[44]

3.25.2.2 Species and Classification

The first attempts at coffee classification were as early as 1623, when Bauhin[45] in his *Pinax theatri botanici* (*Illustrated exposition of plants*) mentioned *Coffea* and set it in relation to Evonymus.

A century later, a comprehensive descriptive systematization was proposed by Linné; he set up plant classes with easily identifiable characters of the floral structure, including the genus *Coffea,* at the very beginning,[46,47] with publications in 1735 and 1737. The final naming of the species as *Coffea arabica* was presented in his '*Species Plantarum*' in 1753.[48] This work was based on direct observations in botanical gardens and herbaria,[49,50] and in discussions and exchanges with colleagues. He explicitly cited Jussieu's description of coffee for the Royal Academy in Paris in 1713/1715.[14]

In **Figure 4** shows the coffee of Linné's original private herbarium, pressed, dried, and fixed on a sheet (N° 2490 of the Linnaean Herbarium at the Linnean society London, Salvage No. 232.1; the mark "india" presumably indicating the origin of the specimen; collection purchased from Linné's widow in 1784).

The official reference for *C. arabica* L. is the type specimen from Clifford's herbarium,[51] which Linné had described in 1737.[49,50] It is designated as a lectotype[52] on the basis of the procedures of the International Botanical Code (the Vienna Code of 2005[53]). 'Lectotype' means that the first publisher had described the specimen prior to the reference to it – the requirement to explicitly design a reference specimen came up later.

Linné's typification became a benchmark for the botany of that time, as underlined by the title 'Order out of Chaos' for an anniversary book of 2007.[54] Although his classification was soon overtaken,[55] the binomial principle of naming remained – the first part indicates the botanic genus; the second is an epithet given by the first author, who is indexed. The overall genus for the coffee species is *Coffea* L., indexed as named by Linnaeus. The principal commercial species used for beverage preparation are *C. arabica* L., and *C. canephora* Pierre ex A.Froehner,[25] commonly called var. Robusta. *Coffea liberica* Bull ex Hiern and some other species are much less important today; there are numerous varieties, including cultivated ones (cultivars).

The botanical classification of coffee has reached a high degree of consensus, presented in international conferences[56,57] and with ongoing publication in the World Checklist of Rubiaceae[58] – coffee is positioned in this family since Jussieu 1789.[55]

Chevalier's system,[59] with four subgenera of *Coffea*, was popular for some decades of the last century. However, it became obsolete the more taxa of the different pools of coffee origin were described and classified.[60–62]

In terms of the Botanical Code, the classification lineage of coffee, starting at the family rank with Rubiaceae, goes down via subfamily Ixoriadeae, tribe Coffeae DC.,[63,64] to the genus *Coffea* L. Some 100 accepted species of the genus, belong to the subgenus *Coffea,* including all beverage coffees; subgenus *Baracoffea* holds about 10 species.[42] That is the actual status.

Nevertheless, there are follow-up refinements.[65]

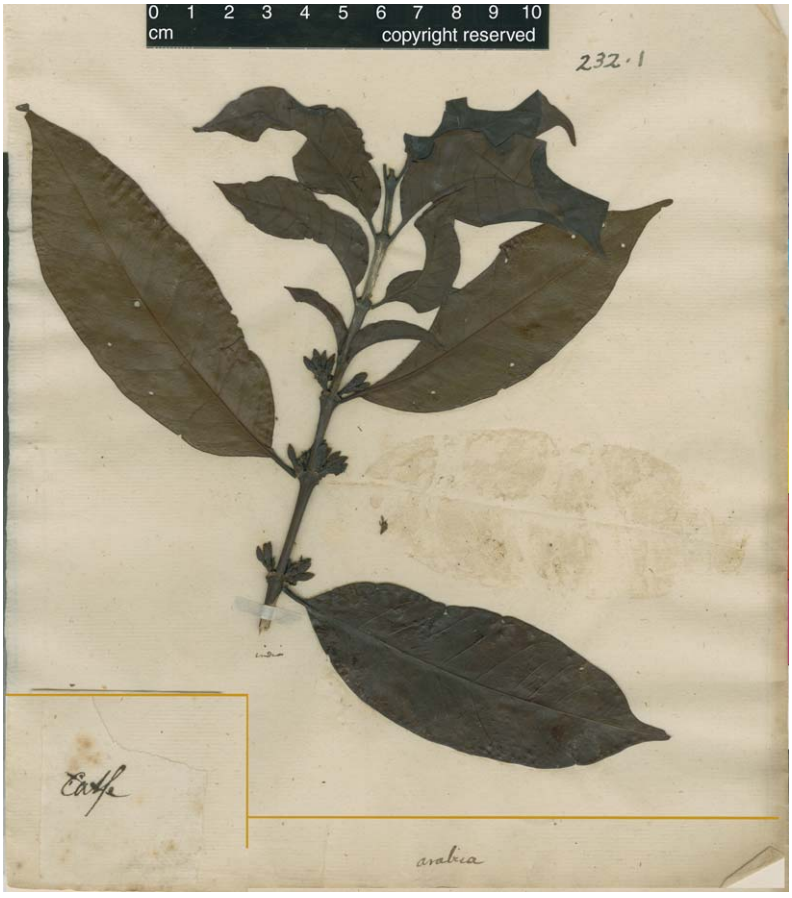


Figure 4 'Caffe' in Linnés herbar, marked 'arabica' by his own hand, courtesy of the Linnean Society, London.

3.25.2.3 Modern Classifications

The changes in classification since Linné's time came along with a change of characterizing properties and of observables, morphological characters against molecular phylogenetics. Involved is a change in the philosophy of systematic biology, "from being considered a plan in the mind of the creator, . . . finally to a phylogenetic mapping of the tree of life".[66] Recently, a 'phylocode' had been proposed, to substitute the Botanical Code's system with its taxa and their hierarchy, at least partially.[67] Discussions are under way.

Modern analytical chemical measurements can now be used in combination with chemometric comparison of secondary plant metabolite levels such as caffeine or chlorogenic acids to classify varieties in phylogenetic trees. Studies of the molecular genetic variation help to identify the relationships of species help to identify their relationships, presented in dendrograms and resulting in genetic clusters of coffees, which can be compared to the biogeographical grouping of coffee clades. Several lineages with geographical or ecological coherence are now recognized,[68] as shown in **Figure 5**.

The phylogenetic approach widens the principles of classification from the morphological similarity of existing plants toward an evolutionary dimension, with genetic clades, where the taxa are traced back to ancestry.

With these instruments, the genetic origin of *C. arabica* could be investigated, with a strongly supported hypothesis: Arabica seems to have been formed by interspecific crossing of diploid progenitors, one species close to *C. eugenioides* as the maternal and another species close to the canephoroid group as the paternal

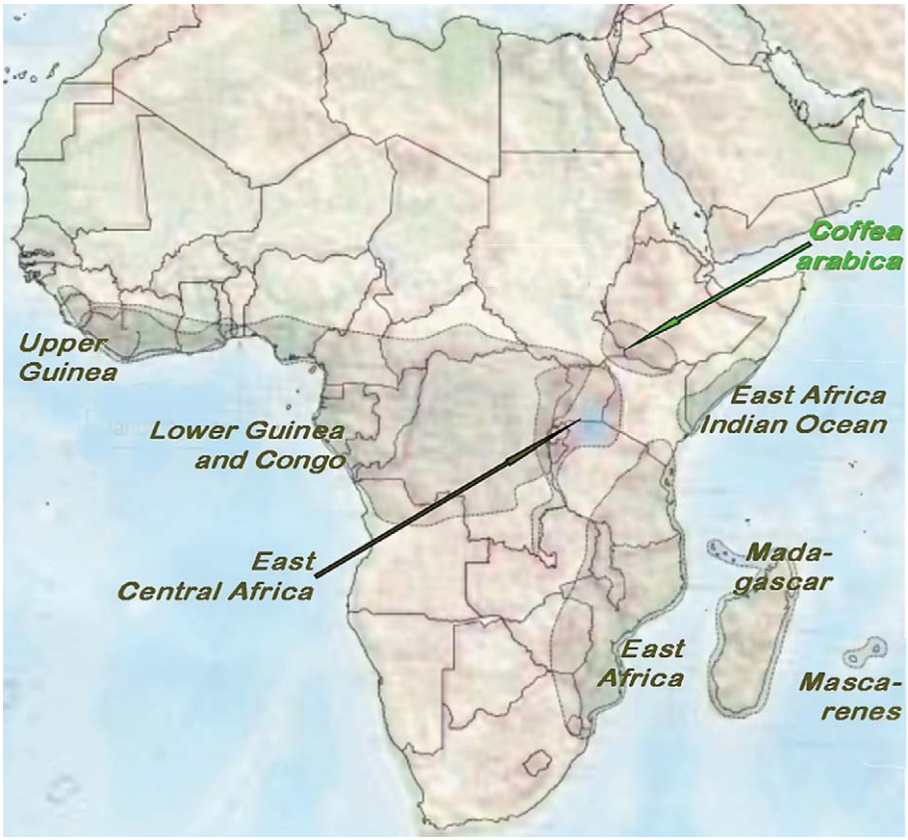


Figure 5 The tropical African origin of coffee, distribution map showing the location of groups of Coffea.

progenitor. This might have taken place in East–Central Africa, very likely in the late Quaternary period.[69] Several mechanisms were discussed – how the new species moved from a tetraploid via progressive diploidization toward the amphidiploid, which it is considered to be now. With the adaptation to the tropical mountain climate of Ethiopia, a low but continued natural selection may have occurred.[70]

In contrast, the high genetic diversity found in the canephora branch of coffee encourages an earlier dating of the origins, as some 500 000 years ago.[71] The clustering of natural *C. canephora* diversity groups, with some 40 species in continental Africa, supports the link to climatic variations in that tropical zone. In the last glacial maximum, an arid period 18 000 years ago, the environment became hostile and few forest refugia[72] remained. There, the differentiation of disseminated subgroups could have occurred,[73] with rare migration of species along the rivers[74] and with unpopulated gaps in-between due to adverse conditions. From the East African group, a dispersal to Madagascar and the neighboring islands might have taken place. They show more than 50 species of the genus *Coffea*, distinct from the continentals: no naturally occurring species is shared between Africa, Madagascar, and the Mascarenes.[42]

Not so far away in history emerged the question about the origin of Bourbon Pointu from Réunion, *C. arabica* 'Laurina' – whether it is a mutation of the Arabica introduced from Yemen or a result of crossing between this Arabica and the indigenous Café marron of the island. A combination of historical and modern analytical research revealed that it is a very young mutant of the Yemenite Arabica that was introduced in Réunion.[75]

The genomic era facilitates the understanding of coffee seed development. Using current knowledge, the metabolic pathways of the major seed storage compounds of coffee were elucidated.[76]

3.25.3 Chemistry Components and Processes

Coffee, as internationally agreed by coffee people, means in the vocabulary ISO, the "fruits and seeds of plants of the genus *Coffea*, usually of the cultivated species, and the products from these fruits and seeds, in different stages of processing and use intended for human consumption" (subclause 1.1. of the ISO 3509 coffee vocabulary),[77] and in the wording of the International Coffee Agreement, the "beans and cherries of the coffee tree, whether parchment, green or roasted, and includes ground, decaffeinated, liquid and soluble coffee" (Article 2 of the said Agreement).[78] Both definitions describe 'coffee' in terms of a series of stages from maturation to consumption.

The composition of coffee varies with the species and with the step in the line. **Table 2** gives an overview of analytical data.

The data are averages from literature reviews,[80] and from investigations executed in other analytical contexts,[82] where the components of constituent groups had been individually determined and summarized.[79]

The in-bean localization of distinct components during the development stages of growing and ripening of the coffee fruit had been observed with electron microscopy supported by tissue coloring. Consecutive papers presented at conferences of association for the science and information on coffee (ASIC) since 1977 featured impressive findings:[83] constituents had been distinguished in their cellular environment and their migrations observed.

An integral view – arrived at through the use of modern instruments – of key biosynthetic pathways of the main coffee seed storage compounds was published recently.[76] Readers may refer to this paper for the plant biochemistry as that will not be further elaborated here.

The following sections deal with the main components, caffeine, carbohydrates, chlorogenic acids, lipids, other nitrogenous compounds, volatiles, and melanoidins, and include the transformation processes.

The first compound covered here is caffeine, associated even by name to our subject, coffee. Caffeine is a nitrogenous compound that is not affected by the central process in coffee chemistry, roasting.

3.25.3.1 Nitrogenous Compounds I: Caffeine

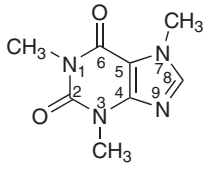
Caffeine, 1,3,7-trimethyl-xanthine, a purine alkaloid, is a secondary metabolite of the coffee plant: the biosynthesis starts from xanthosinemonophosphate.[84] In the metabolic pathway, subsequent methylation steps occur with different N-methyl transferases, methionine being the methyl donor. The purine catabolism of caffeine comprises its degradation via successive demethylation down to carbon dioxide and ammonia.[85]

Table 2 Chemical composition of coffee, mass percent in dry matter, different sources

	Arabica green[a]	*Robusta green*[a]	*Arabica roasted*[b]	*Robusta roasted*[b]	*Arabica instant*[a]	*Robusta instant*[a]
Constituent	*% DW*	*% DW*	*% DW*	*% DW*	*% DW*	*% DW*
Caffeine	1.3	2.3	1.2	2.4	2.5	3.8
Trigonelline	0.8	0.7	0.3[c]	0.3[c]	0.7	0.4
Carbohydrates	53.7	50.7	38	42	46.6	44.7
Chlorogenic acids	8.1	9.9	2.5	3.8	2.6	1.6
Lipids	15.2	9.4	17.0	11.0	0.11	0.26
Amino acids	11.1	11.8	7.5	7.5	6.2	6.0
Organic acids	2.3	1.7	2.4	2.6	8.1	7.9
Melanoidins	–	–	25.4	25.9	25.1	28.6
Volatile aroma	Traces	Traces	0.1	0.1		
Ash (minerals)	3.9	4.4	4.5	4.7	8.0	7.4
Others partly unknown			In melan oidines	Added to melan		

[a] Leloup[79].
[b] Illy and Viani[80].
[c] Macrae[81]

In the coffee plant, caffeine is present in all parts over the ground. The ecological effects of caffeine as an intrinsic chemical defense against herbivory, molluscs, insects, fungi, or bacteria[86] have often been discussed, but experimental results for clear support are difficult to obtain.[87]



Caffeine

Caffeine biosynthesis takes place in the leaves and in the pericarp, the outer part of the fruit. In aged leaves the caffeine content is lower.[88] In the pericarp tissues, light strongly stimulates the methylation step of caffeine synthesis. When the seed inside the fruit starts growing, caffeine is translocated through the membranes and accumulates in the endosperm. There, the final value is reached 8 months after flowering.[89]

The caffeine content of the coffee beans depends on species and variety, from 0.6% in Laurina up to 4% in some extreme Robustas; averages are given in **Table 3**.[90]

Not included here are coffees from the Mascaracoffea group of Madagascar, which do not belong to the 'beverage' coffees.[91] They have very low caffeine contents at the limit of analytical detection (caffeine free)[92] and may serve as genetic resources for further work. These low caffeine species also show low caffeine in their leaves.[93]

The caffeine content in dry matter base is not affected by postharvest processing, neither by the roasting. Although the roasting process occurs well above sublimation temperature, during it only a small percentage of caffeine vanishes, which is overbalanced by the organic weight loss.[91]

Caffeine is a physiologically active compound, and the human exposure to it after a cup of coffee is of interest. Some general calculations can be made: an aqueous extraction at regular brewing conditions transfers the caffeine almost completely into the beverage. A cup of 100 ml with a brew of 55 g l^{-1} of roast and ground coffee, with the world trade ratio of 60% Arabica and an average caffeine content of **Table 2**, supplies about 100 mg caffeine.

Looking beyond the averages, **Table 4** roughly gives the variability (brewing strength 40 g/l)[94,95] of the value, omitting extremes and exotics – a range of plus/minus 100%.

But even the term 'cup' or 'serving' is in motion: the mug has become widespread, with a volume of about 250 ml, and 'jumbos' are on the market, of about 500 ml.

A cup of tea for comparison, has a slightly lower caffeine content: Prepared from a 1.75 g teabag with an average caffeine content for tea of 3%, a cup of 100 ml contains 50 mg, with a natural variability similar to the case of coffee.

The limit for mandatory caffeine labeling in non-coffee and non-tea drinks is set at 150 mg l^{-1}(sets the obligation for caffeine labelling at amounts exceeding 150 mg/l and a sentence intended as warning, "contains high amounts of caffeine," unless (Art.2 2) the beverage is based on coffee or tea);[96] this labeling should help consumers avoid unexpected caffeine intake.

The data given here reflect a standard beverage preparation – others exist. In an espresso-style percolation, for a cup of 30 ml, 6.5 g roast and ground are taken; with the same coffee, about 87 mg caffeine can be expected. The very short time available to extract caffeine from the cellular structure leads to 75–85% extraction yield only.[97]

Table 3 Caffeine content in green beans of different species and varieties

		Leaf	*bean*
Species	*Variety*	*% DW*	*% DW*
C. arabica	Mundo Novo	0.98	1.11
	Typica	0.88	1.05
	Catuai	0.93	1.34
	Laurina	0.72	0.62
C. canephora	Robusta	0.46	>4
	Kouilou/Conillon	0.95	2.36
	Laurentii	1.17	2.45

Table 4 Estimated ranges of caffeine content per cup, standard brewing of different strengths

Species (caffeine range)	*Arabica (0.9–1.6%)*	*Mix 60 Ar/40 Rob (1.7 %)*	*Robusta (1.4–2.9%)*
Brewing strength	*Caffeine per cup mg/100 ml*	*Caffeine per cup mg/100 ml*	*Caffeine per cup mg/100 ml*
40 g l^{-1}[a]	36–64	67	56–116
55 g l^{-1}[b]	50–88	92	77–160
70 g l^{-1}[c]	63–112	118	98–203

Brewing strength according to
[a] NEVO, 1991, Dutch nutritional tables[94]: 40 g l^{-1}.
[b] Mean between a and c: 55 g l^{-1} (German common use).
[c] ISO 6668:2008,[95]: 70 g l^{-1}.

While enjoying his coffee, the consumer may benefit from the stimulating effect of caffeine.

The alerting effects are well known and the mechanisms investigated.[98,99]

After its consumption, caffeine is readily and completely absorbed from the gastrointestinal tract. Within 1 h it is evenly distributed within the body, readily passing the blood–brain barrier. Peak plasma levels occur 30–60 min after ingestion.

Provoked by a cup of regular coffee of the previously-calculated concentration, a caffeine level of 2 mg l^{-1} body fluid is reached (total body fluid taken as 60% of a 70-kg man), just in the range of the stimulatory level of about 1–4 mg l^{-1} body fluid.[100] At blood concentrations such as these, the main mechanism of action in the central nervous system is the antagonism of adenosine receptors, which increases central nervous system activity, with effects on alertness and cognitive control.

During circulation, caffeine is metabolized in the liver via successive demethylation and oxidative degradation to uric acid. The breakdown products are excreted through the kidneys. About 5% of caffeine is excreted unchanged. The half-life ranges from 2.5 to 4.5 h in healthy male adults. For children, women, pregnant women, and people under stress, longer times were reported.

The caffeine content of coffee can be reduced by decaffeination. The process starts with a steam treatment of the green coffee to soften the tissues, followed by solvent extraction. The first patent dates back to 1905.[101] Today, processes run with dichloromethane, ethyl acetate, supercritical or fluid carbon dioxide, or water – each process with its own special technology.[102] In the United States, nondecaffeinated coffee is called 'regular' coffee.

Legal requirements on the caffeine content apply to decaffeinated coffee for the final product for consumption, that is, roast and soluble coffees. In the United States, decaffeination is measured through the degree of decaffeination; common are 97%.[103] The European legislation sets a maximum residual caffeine content of 0.3% for soluble coffee;[104] roast coffee is covered by national legislations, in general 0.1% on dry matter.

The standard analytical methods for caffeine determination employ chromatographic separation and spectrometric detection.[105]

Although caffeine as pure chemical has a clearly bitter taste (it can be used as a "bitter" standard in basic sensory tests), it plays only a minor role in giving a bitter tinge to the coffee beverage.

3.25.3.2 Processes and Reactions

3.25.3.2.1 Postharvest processing: dry and wet methods

The ripe coffee beans, cherry-like, embedded in the pulp of the fruit, need to be dissected soon after harvesting to avoid an uncontrolled fermentation in the wet mucilage, which would cause undesired 'off-flavors' in the cup. The cherries can be processed by either the dry method – sun drying on patios for 3–9 days followed by mechanical removal of the dried outer parts, resulting in 'natural coffee' – or the wet method – pulping, controlled fermentation of the mucilage in an 18–36 h process, then rinsing the residuals and drying to produce

the 'washed coffee'.[106] The metabolism occurring in the beans during the processes differ in their time windows,[107,108] and variations in the composition of aroma precursor can result.[82] This may well explain the observed sensory differentiation of the coffees originating from dry and wet processed beans.[109]

3.25.3.2.2 Roasting

Roasting the coffee beans is an essential transformation, performed at about 200 °C.[110] The coffee beans become dry, expand in volume, become brown and brittle, and develop a characteristic flavor and aroma profile.

During roasting, volatile aromatic compounds and polymeric brown pigments are formed in the beans, while water and carbon dioxide are released. The principal thermally reactive constituents of the raw bean are the monosaccharides and sucrose, free amino acids, chlorogenic acids and trigonelline, and the newly formed precursors of degraded carbohydrates and denaturated proteins. The chemical reactions are complex.

Prevalent is the reaction, as demonstrated by Maillard and coworkers,[111] of free amino acids with reducing sugars, with a cascade of condensations, cleavages, rearrangements, and degradations and oxidative polymerization in parallel.

Another pathway of roasting is the Strecker degradation, leading to pyrazines and oxazoles.

Degradation of trigonelline leads to nicotinic acid, pyrroles, and pyrimidines. Chlorogenic acids form lactones; in Robusta, they may also end up in phenols like guaiacoles.

3.25.3.2.3 Extraction for beverage preparation

Both home brewing of coffee and production of soluble (or instant) coffee at an industrial scale,[36] include the process of aqueous extraction of the solubles from roast and ground coffee.

Home brewing can be done with various extraction techniques, either boiling the coffee, or percolating, or brewing and decanting, or filtering (the usual method), or by single portion pressure extraction in a special apparatus, with partially selective extraction of components. The resulting product is the beverage, ready to drink. In instant coffee production, the extraction is run at higher temperatures; it is followed by concentration of the extract, and drying. The steps are technologically optimized to meet the sensory quality of a brewed coffee.[112] The product obtained is a dry powder, to be reconstituted to the beverage on demand.

The impact on the composition of the product that is finally consumed is summarized in the respective sections.

Other ethnic styles like the infusion of dried coffee husks (qishr) in Yemen or of coffee leaves in Southeast Asia are not covered here, nor a masticatory use (chewing) that might still exist.

3.25.3.3 Carbohydrates

Carbohydrates are products of photosynthesis in plants. During coffee fruit development, they are produced in both the leaves and the pericarp, as reducing sugars (glucose, fructose) and sucrose. Transported to the perisperm and the endosperm in their respective phases of growing,[113] they contribute to sucrose accumulation in the coffee seed. Robusta accumulates about 30% less sucrose than Arabica.[114]

By far, polysaccharides of differing molecular sizes form the largest part of the green coffee carbohydrates. Names like galactomannan and arabinogalactan protein reflect the constitutive units,[115] the 'backbone' chains and the substituting branches; an overview is given in **Table 5**.

Recently, linkage analysis of the moieties revealed a glycoprotein backbone for the arabinogalactan fraction of green coffee, which is now called arabinogalactan protein.[116] The central chain consists of proteins, which account for 0.5–2% of the polymer; they contain between 7 and 12% hydroxyprolin.[117] For arabinogalactan proteins in plant tissues in general, a structure was proposed.[118] The analytical determination of carbohydrates in these structural elements starts with the separation and isolation of the different fractions with chemical and enzymatic reactions.[119] The detailed analytical data in **Table 6**, of 2006, are achieved by this procedure; they confirm and refine those of **Table 5**, of 1987.

Table 5 Main carbohydrate structures in Arabica and Robusta green coffee beans, compiled from different tables[112]

		Arabica	*Robusta*
Fraction	*Structure*	*Wt%*	*Wt%*
Monosaccharides	Fructose, glucose, galactose, and arabinose (traces)	0.2–0.4	0.5–0.70
Oligosaccharides	Mainly sucrose	5.1–8.6	2.2–6.6
Mannan (galactomannan)	Storage carbohydrate, straight chain of β-(1–4)-mannan with low degree of substitution, poorly soluble	22	22
Arabinogalactane-protein (formerly arabinogalactan)	Structural cell wall component; β-(1–3)-galactans substituted with mixed arabinose/galactose branchings, water-soluble, covalently linked to a protein backbone	14–15	16–17
Cellulose (homoglucan)	structural cell-wall components, linear unsubstituted β-(1–4)-glucan, unsoluble	8	8
Hemicellulose	mostly glucan, with some rhamnose (0.3%), xylose (0.2%) from residual parchment	Traces	Traces

Roasting favors the degradation/depolymerization of polysaccharides,[120] and transforms the sugar composition substantially. The resulting oligo- and mono-saccharides can be solubilized during extraction, yielding a characteristic carbohydrate profile.

After roasting, the extractability of mannans in high temperature extraction is enhanced, important for the instant coffee production; a maximum of extraction yield is achieved with medium roast.[121]

Carbohydrates are precursors for flavor generation. They react with proteinaceous components in the well-studied Maillard reaction. The process generates essential contributors to coffee flavor, as either volatile aroma compounds[122] or nonvolatile taste compounds,[123] and, simultaneously, a heterogeneous class of dark brown polymers, the melanoidins with different ranges of molecular-weight.[124]

At darker roasting, pyrolytic degradations take place.

Carbohydrates are major components of both roast and soluble coffees. In home brews prepared from roast and ground coffee, they are present in low quantities.

Analysis of the individual carbohydrates is presented in an internationally accepted standard with high performance anion exchange chromatography (HPAEC),[125] providing profiles of free carbohydrates and of total carbohydrates (the overall carbohydrate composition)

Green coffee and instant coffee as the starting and end points, respectively, of all processing steps are set in parallel in **Table 6**, with the free and total carbohydrate profiles of soluble coffee from medium roast compared with the figures for the corresponding Arabica and Robusta green coffees.

The sucrose of the green coffee disappears totally with roasting, while small amounts of monosaccharides and other disaccharides are released by roasting and extraction. A significant portion of the total carbohydrates is transformed into solubles.

Other investigations show that the mannose/galactose ratio changes during plant development.[126] Several studies on carbohydrate composition give ratios different from the one cited here.

The standard method mentioned previously is also used, when the available carbohydrates and sugars are required for nutritional evaluation – the relevant European legislation[127] says, "any carbohydrate metabolized in man" and "all monosaccharides and disaccharides present in food", respectively. Using the ISO method, the carbohydrates are to be determined individually and summed up – the resulting energetic amounts are negligible for instant coffee,[128] 36 kCal/100 g and 0.7 per cup, respectively; for the roast coffee beverage, probably even lesser.

A 'traditional' procedure for nutritional carbohydrate evaluation, summing up all other components (water, fat, ash, proteins) and taking the difference to 100% for carbohydrates, is not suitable for coffee.

The procedure for specific carbohydrate profiles of soluble coffee may indicate whether or not extraneous material was used for extraction and serves as a criterion for judging soluble coffee's authenticity.[129]

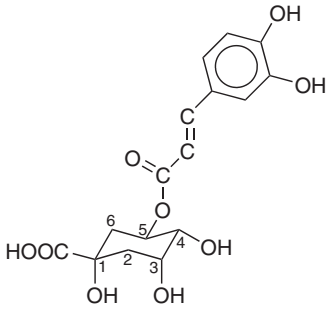
Table 6 Carbohydate composition of Arabica and Robusta green coffees and of the corresponding soluble coffees obtained by industrial manufacturing (roasting, extraction, drying)

Component	*Arabica Green* % DW	*Robusta Green* % DW	*Arabica Instant* % DW	*Robusta Instant* % DW	*Component*	*Arabica Green* % DW	*Robusta Green* % DW	*Arabica Instant* % DW	*Robusta Instant* % DW
					Mannitol	0.43	0.35	0.43	0.28
Free arabinose	0.00	0.00	0.67	0.80	Total arabinose	3.92	4.82	3.07	3.57
Free galactose	0.08	0.03	1.37	1.40	Total galactose	10.37	12.76	12.97	14.13
Free glucose	0.00	0.00	0.34	0.35	Total glucose	9.35	8.93	1.06	1.05
Free fructose	0.09	0.13	0.66	0.67					
Free mannose	0.00	0.00	2.46	2.35	Total mannose	19.85	18.96	18.81	15.34
					Total xylose	0.22	0.27	0.13	0.09
Sum of mono saccharides	**0.17**	**0.16**	**5.50**	**5.57**	**Sum of total carbohydrates**	**49.90***	**48.86***	**37.1**	**35.1**
Sucrose	3.63	1.68	0.00	0.00					
Other disaccharides			3.96	4.03					
Disaccharides	**3.63**	**1.68**	**3.96**	**4.03**					

Compiled from V. Leloup[79], excerpts of **Table 1** therein.

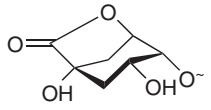
3.25.3.4 Chlorogenic Acids

Chlorogenic acids are widely distributed secondary metabolites in plants, and they are also present in the coffee bean in relatively large quantities.



5-CQA

The parent structure is a conjugate of tetrahydroxy-cyclohexane carboxylic acid (quinic acid) and caffeic acid (3,4-dihydroxy cinamicacid). Due to isomers and epimers in the cylohexane part and substitutions at the aromatic ring, a whole family of chlorogenic acids exists.



1,5-Quinide

The most common chlorogenic acid is 5-*O*-caffeoyl-quinic acid (5-CQA); the formula shows the actual numbering at the caffeic acid moiety.[130] Isomers in the quinic acid part are 3- and 4-CQA, each at an amount of about 10% of 5-CQA. Widespread in the chlorogenic acid family are also substitutions at the aromatic ring, naming the respective cinnamic moiety, with common synomyms ss feruloyl quinic acid (FQA, 4-hydroxy, 3-methoxy-) and p-coumaroylquinic acid (pCoQA, 4-hydroxy-). Their concentration is orders of magnitude lower than for (caffeic) CQA, and again, the 3- and 4-isomers show 10% of the respective 5-*O*-isomer. Several isomeric di-esters of quinic acid exist (e.g. di-caffeoylquinic acid, diCQA), and even ester mixes, like caffeoyl-feruloylquinic acid (CFQA). **Table 7** shows the typical contents of Arabica and Robusta green coffee for these chlorogenic acids, values from Clifford.[131] For analytical determination, HPLC is the method of choice.

Chlorogenic acid is biosynthesized in the perisperm and accumulated in the beans' endosperm;[76] di-CQAs are converted into mono-CQAs during the last phase of bean maturation. The latter is important for harvesting management,[132] as di-CQAs would negatively affect the sensory quality of coffee, and in case of nonuniform ripening and simultaneous harvesting, the immatures might be included in the crop.

Roasting reduces progressively the amount of free chlorogenic acids in coffee, creating a series of transformation products that may be unique to coffee.[133] In the quinic part of CQA, a lactonisation occurs; the chlorogenic lactones (quinides) show a marked bitterness and possible biological effects. Within the series of

Table 7 Typical contents of chlorogenic acid (CGA) and CGA-like components in commercial green coffee beans

	Arabica	*Robusta*
Component	*% d.b.*	*% d.b.*
CQA	5.2–6.5	5.5–8.0
pCoQA	0.03–0.07	0.05–0.06
FQA	0.3–0.5	0.7–1.5
diCQA	0.7–1.0	1.4–2.5
CFQA	n.d.	0.2–0.3

n.d. not detectable.

isomers, the 1,5 quinides are the most common. The general structure is shown here.[134]A great portion of the CQA's of green coffee disappears via Maillard-type reactions into more complex macromolecules, i.e. melanoidins,[135] and partly decomposes into quinic acid and caffeic acid, to form quinides and to be incorporated in the melanoidins. Another transformation leads via decarboxylation and cyclisation to phenylindanes, identified as a strongly bitter component of coffee.[131,149]

Domestic brewing and commercial instantization substantially extract the CQAs from the melanoidines and hydrolyzes the lactones.[131] CQA contents in the brew are about 3% DW, in instant coffee about 5–7%.[133]

In human digestion, CQA is bioavailable; it reacts with the microflora of the gut, and reaches the plasma within one to four hours.[136] The metabolism is under investigation.

Plant-derived phenolics are reported to have wide ranging biological activities and a high potential as antioxidants. Coffee with its chlorogenic acid is one of the richest dietary sources; many studies deal with the fate and effect of chlorogenic acid, in order to elucidate on protective effects against degenerative diseases such as cardiovascular disease, cancers, and also diabetes II: Regular Reviews[137] over the years reveal the accumulating epidemiological evidence and support for the positive health impacts of coffee consumption.

3.25.3.5 Nitrogenous Compounds II

This section deals with those nitrogenous compounds of coffee that are transformed during the processes of roasting and extraction. Proteins, the classic nitrogenous compounds of food, constitute about 12% of green coffee, peptides and free amino acids up to 1.5%, alkaloids 3–4%, of which trigonelline represents about 1%. Most of these compounds are transformed at roasting.

The 'roast-stable' caffeine was covered in the first section of nitrogenous compounds.

3.25.3.5.1 Amino acids and proteins

The free amino acid content of green coffee beans shows a wide range, from 0.001% for methionine in Robusta to 0.1% for glutamic acid in Arabica. For half of the free amino acids, Arabica and Robusta green beans differ significantly. In roast coffee, free amino acids are not detectable.

Amino acids are constituents of peptides and proteins; their individual contribution can be analyzed as 'total amino acids' after appropriate analytical hydrolysis.[137] The sum of total amino acids roughly accounts for the protein content.

Protein content is required for nutritional declarations of foods,[138] which is, in fact, optional.

The traditional determination of protein in food via conversion of total nitrogen into protein content with the legal (!) empirical factor of 6.25 (the Kjeldahl nitrogen method) does not give correct protein values for coffee unless several corrections have been introduced – for the nitrogen of caffeine and trigonelline, for other nonprotein nitrogen, and for those components that in the case of roast coffee do not reach the consumers' beverage. **Table 8** lists the results of free and of total amino acid determination of Arabica and Robusta green coffees, taken from two doctoral theses; data for totals of Arabica roast and brew are added. Trautwein used samples of different origins; great variation was found in each dataset,[139] which is not evident in the overall mean. In total amino acids, the results of Arabica and Robusta overlap widely, as **Table 8** shows.

The free amino acids of green coffees are largely transformed upon roasting. They take part in the Maillard reaction, resulting in components that contribute to flavor and color of the coffee brew. In roasted coffee, only negligible amounts remain.[140]

- Sulfur amino acids, cystine, cysteine, and methionine in green coffee mostly bound in proteins, degrade at roasting, and interact with reducing sugars and Maillard intermediates to form intensely aromatic volatiles, for example, furfurylthiol, an aroma impact compound with a very low aroma threshold value, and thiophenes and thiazoles.
- Hydroxyl-amino acids serine and threonine react with sucrose to give volatile heterocyclic compounds, inter alia the alkylpyrazines.
- Proline and hydroxyproline react with Maillard intermediates to give pyrroles, pyrrolizines, and pyridines and also alkyl-, acyl-, and furfurylpyrroles.
- Tryptophan is transformed into serotonine in the last weeks of grain development.

Table 8 Free and total amino acid content of coffee, green, roasted, brew, from different sources

	Arabica green free AA[a]	*Robusta green free AA*[a]	*Robusta green total AA*[a]	*Arabica green total AA*[b]	*Arabica roast total AA*[b]	*Arabica brew total AA*[b]
Amino acid	*% DW*	*% DW*	*%.DW*	*% DW*	*% DW*	*% DW*
Alanine	0.025	0.034	0.53	0.58	0.61	035
Arginine	0.008	0.018	0.72	0.64	0.00	0.00
Aspartic acid	0.033	0.033	1.03	1.22	1.15	0.73
Cysteine	n.d.	n.d.	0.26	n.d.	n.d.	n.d.
γ-Amino butyric acid	0.028	0.047	0.05			
Glutamic acid	0.102	0.047	2.20	2.43	2.47	2.06
Glycine	0003	0.006	0.69	1.14	103	0.71
Histidine	0.004	0.004	0.35	0.29	0.24	0.15
Ileucine	0.005	0.008	0.45	0.49	0.50	0.26
Leucine	0.006	0.010	0.93	1.09	1.11	0.57
Lysine	0.006	0.011	0.69	0.81	0.11	0.00
Methionine	0.002	0.001		0.14	0.11	0.07
Phenylalanine	0.017	0.021	0.60	0.61	0.63	0.32
Proline				0.64	0.62	0.45
Serine	0.017	0.016	0.57	0.49	0.24	0.15
Threonine	0.003	0.005	0.40	044	0.27	0.12
Tryptophan				n.d.	n.d.	n.d.
Tyrosine	0.005	0.011	0.43	0.34	0.28	0.18
Valine	0.009	0.017	0.58	0.65	0.72	0.28
Sum	0.27	0.29	10.5	12.0	10.1	6.4
Mean	0.02	0.02	0.66	0.75	0.63	0.40

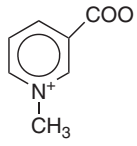
[a] Trautwein[139] samples from different origins.
[b] Beke-dam[137] aspartic and glutamic acid contents, including the amides; sample Colombia Arabica.
n.d. not detectable; AA, amino acids.

The protein content of green coffee is about 10–13%. The protein profile of coffee changes during roasting; the proteins are both fragmented and polymerized, and integrated into melanoidins. Their concentration in the brew is at the end about 6–7%, a figure relevant for nutritional value calculations.[137]

The principal protein of green coffee is a storage protein of 11S type. It is biosynthesized in the endosperm[141] during maturation and accounts for about half of the protein content.[142] The 11S protein has an α- and a β-arm of different length, with a disulfide bridge, and allows covalent bindings of chlorogenic acids at the higher reactive β-branch[143] upon roasting. A 7S- and a 2S-type protein were also reported. The 11S protein itself and the DNAs encoding its expression are subjects of European and US patents.[144]

3.25.3.5.2 Trigonelline

Trigonelline, the *N*-methylpyridinium-3-carboxylate, is, after caffeine, the second most important alkaloid of coffee, with about 1% of the green bean. During leaf development, it is synthesized in the leaves and in the fruits' pericarp and accumulated in the seeds. The direct precursors are nicotinic acid and nicotine amide, deriving from the pyridine nucleotide cycle.[145]

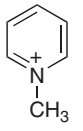


Trigonelline

Trigonelline is rapidly degraded during roasting, strongly depending on temperature and roasting time, with about 60–90% being lost.[146] The products are nicotinic acid via demethylation and methyl-pvridines and pyridines via decarboxylation, with reactive intermediates and further recombination products including pyrrols.

Trigonelline products have an impact on the overall aromatic perception of roast coffee and beverage. Niacin (nicotinic acid), the degradation product of Trigonellin, serves for vitamin supply in human nutrition; it is an accepted vitamin in European legislation.[147]

Physiologically important are the recently identified *N*-methylpyridinium (NMPY) ions;[148] they act *in vivo*, as identified through an activity guided screening procedure in the coffee brew,[149] as key components to turn on the endogeneous antioxidant defense system through induction of the phase II biotransformation enzymes.[149]



N-Methylpyridinium

3.25.3.6 Lipids

Food lipids are substances that are soluble in organic solvents. The category includes structurally different compounds.[150] In green coffee, the biggest part of the lipids is the coffee oil in the beans endosperm, as lipids outside the bean there is a small amount of coffee wax on the outer layers of the bean. The coffee oil consists of triglycerides (the fats), phospholipids, sterols, tocopherols, the coffee characteristic diterpenes, and the respective esters with fatty acids. The coffee wax consists of 5-hydroxytryptamide esters with fatty acids. The fatty acids are unbranched with a chain length of 16–24 carbons.

The overall range of lipid content in green beans is 7–17%, with an average of about 15% for Arabica and 10% for Robusta.

Table 9 shows the relative content of the different components, as averages[151] and ranges,[152] respectively, from literature compilations.

Lipids in coffee serve as carriers for flavors and for fat-soluble vitamins and contribute to texture and mouthfeel in the brew. The diterpenes among them have received attention in recent years due to their different physiological effects.

Table 9 Composition of the lipid fraction of green coffee

	Mass % of total lipids	
Compounds	*Average*[a]	*Range*[b]
Triacylglycerols	75.2	70–80
Esters of diterpene alcohols and fatty acids	18.5	15–18.5
Diterpene alcohols	0.4	01–1.2
Esters of sterols and fatty acids	3.2	1.4–3.2
Sterols	2.2	1.3–2.2
Tocopherols	0.04–0.06	0.3–0.7
Phosphatides	0.1–0.5	0.1
Tryptamine derivatives	0.6–1.0	0.3–1.0

[a] Maier[151].
[b] Viani[152].

3.25.3.6.1 Total lipids

The total lipid content of coffee is most reliably determined via selective solvent extraction with tertiary butyl methyl ether.[153] This separation is the first step of the follow-up fractionating. It is validated as part of a German standard procedure[154]

For further investigations, the total lipids can be fractionated via gel permeation chromatography into free fatty acids, triglycerides, and diterpene fatty acid esters.[155]

3.25.3.6.2 Triacylglycerols

Triacylglycerols accumulate in the fruit endosperm from day 120 onward after flowering. The preceding steps of lipid synthesis[76] are supposed to occur in both the perisperm and the endosperm: prolongation of fatty acids with 2-carbon units, desaturation steps with different enzymes, second desaturation with phosphatidylcholine as the intermediate host of fatty acids, and sequential acylation - to end up with the triacylglycerol.

The final accumulation is visible in the bean by electron microscopy – distinct oil bodies in the coffee material, forming droplets of about 0.5 μ diameter, positioned near the cell wall. **Figure 6** shows this at day 187 after flowering.[156]

The fatty acid distribution of the coffee triglycerides is special in tropical plants, as the polyunsaturated fatty acids (PUFAs) – polyunsaturated fatty acids – exceed the saturated ones: about 50% linoleic acid, C-18, twice doublebonded, versus 30% palmitic acid, C-16; the third in line is oleic acid, C-18 monounsaturated, with about 10%. There are variations between Arabica and Robusta, but in general they are similar.

During roasting, the triacylglyceroles remain unchanged, prone to become the carrier of the emerging flavor volatiles. With very strong roasting, they gather at the outer bean surface, 'sweating'.

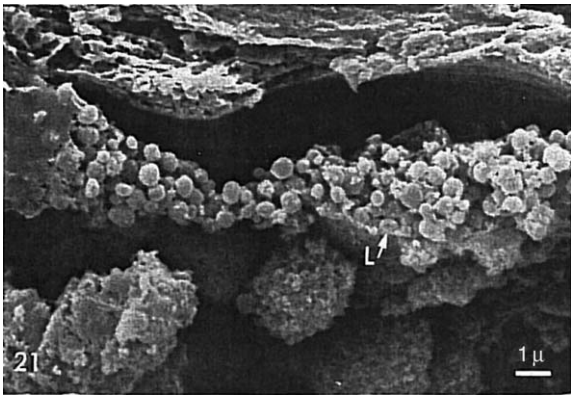


Figure 6 Lipid bodies in the cells of the coffee grain, 187 days after flowering, scanning electron microscopy, Dentan, ASIC 1985.

At the beverage preparation, intended to extract the coffee ingredients for consumption, the lipids in most cases do not reach the brew, as they stick to the spent grounds and are filtered off, in filter home brew as well as in soluble instant coffee production.

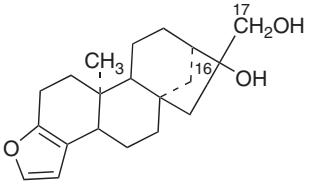
With this in mind, soluble coffee manufacturers often remove the aroma compounds with their carrier coffee oil, before the aqueous extraction and reincorporate them at a final step before packaging – many sophisticated solutions to this challenge exist.[157]

Other brewing methods, like preparation by boiling the roast and ground coffee without filtering separation, leave the lipids in the cup for consumption – an old-fashioned style, which had been used until the late 1970s in Scandinavia.

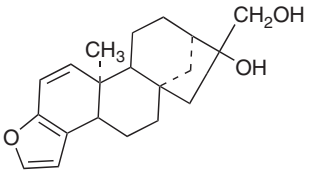
In the true espresso preparation,[158] advancing since half a century, the coffee lipids in the cup play an outstanding role. Because of the quick preparation under pressure, the lipids can reach the beverage to form a stable oil in water emulsion, with high content of aromatic volatiles; the consumer is touched via the retronasal sensation and through enhanced mouthfeel – 'Espresso, a festival for all senses' was the title of a German popular-scientific paper in 2003.[159]

3.25.3.6.3 Diterpenes and diterpene esters

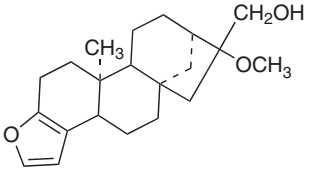
Part of the lipids in coffee are esters of fatty acids with the pentacyclic diterpene alcohols cafestol and kahweol, and the respective methoxylated compounds, esterified at the C-17 position.



Cafestol



Kahweol



16-*O*-methylcafestol

Their content is about 15% of total lipids. The individual esters are present in coffee in different amounts. They range from palmitic, linoleic, oleic, down to stearic acid from 50–10%, and even some saturated C-20- and C-22 fatty acid esters are found, such as arachidic and behenic acids.[160] The odd-numbered fatty acids esters are very minor components.

Kahweol esters are mainly present in Arabica beans, those of cafestol in both Arabica and Robusta, and 16-*O*-methylcafestol esters only in Robusta. The latter is stable on roasting, so an elaborate analytical procedure was proposed for identification of an eventual Robusta content of commercially roasted coffees.[161] According to recent results, an expanded method is claimed to allow the adaptation for instant coffee.[162]

Cafestol, kahweol, and their respective esters undergo decomposition and isomerisation at roasting, to form dehydrocafestol/-kahweol by water elimination, cafestal/kahweal by ester cleavage and oxidation at C-17, and isomerisation and elimination to isokahweol and dehydroisokahweol; paralled by a decrease in the ester contents, strongly depending on roasting conditions.[163]

An adverse association between coffee consumption and serum cholesterol levels, reported in Norwegian study of 1983,[164] was identified as linked to the presence of cafestol (and kahweol) esters in the beverage.[165] Like with the other lipids, their amount is connected to the style of coffee making. Data are shown in **Table 10**, with small pictures of the brewing equipment, a description of the procedures, and with estimates on cholesterol rises according to the literature.[166].

The ester content is high for boiled coffee, French press (Plunger pot), and Middle Eastern style preparations, where there is no separation of grounds, is intermediate in espresso coffee, and negligible in instant and filtered coffees.

Meanwhile, most of the Scandinavians have changed their habit of coffee making from the traditional boiling style to the filtering method; insofar, a cholesterol raising effect of coffee is no longer a problem there. Regarding the other preparation techniques mentioned, the choice is up to the consumer.

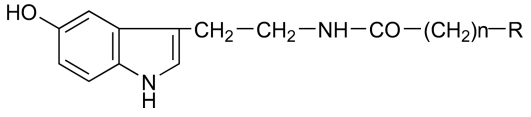
3.25.3.6.4 Sterols, Tocopherols

Coffee contains a number of sterols that are also typical of other seed oils. In addition to 4-desmethylsterols, various 4-methyl- and 4,4-dimethylsterols have been identified, both in free and in esterified form. The distribution of the main desmethylsterols in Robusta and Arabica coffee differs markedly, and with a special statistical evaluation, their use for identification of Robusta in Arabica coffees had been proposed.[167]

Tocopherols in coffee oil hold for about 120 mg kg^{-1}, in Robusta slightly more than in Arabica. They are also found in roast coffee and in the brew and in soluble coffee.

3.25.3.6.5 Coffee wax

Coffee wax, a thin layer on the surface of green coffee beans, is composed of fatty acids of a chain length up to C-22, linked as an amide to the amino-group of serotonine, 5-hydroxytryptophan (C-5-HT, carboxylic-acid-5-hydroxy-tryptamide).



Insoluble in petroleum ether, it is defined and prepared by solubility in chlorinated organic solvents

For some time, its reduction was taken to indicate a 'treatment' of green coffee,[168] executed to reduce possible irritating compounds that might be formed on roasting and hence result in a more digestible coffee brew.

3.25.3.7 Volatiles

The ingredients of this section do not share a common chemical characteristic, but the physical property of being volatile, and contributing to the aroma of coffee.

The estimation of their individual contribution to the overall sensation of coffee smell can be performed with different instruments.

The equipment of choice is the gas chromatograph, with a sniffing port at the detector side (GC-O) for the description and a mass spectrometer (GC-MS) for the identification of the separated components. Precise quantification of the odorants is achieved using stable isotope dilution assays as internal standards.[169]

Based on the minimum identifiable odor concentration, an Odor Value or a Flavor Dilution Factor can be defined, thus ranking the volatiles on the basis of their odor units determined by GC/O. A frequently used visualization is the FD chromatogram: FD factor versus retention index on the chromatographic column used.[170]

The importance of the odor active components on the overall impression of coffee is proved by a recombination experiment. With a synthetic blend of some 20 compounds on the basis of quantitative data, the sensation 'roast coffee' was met. The impact of individual compounds in interaction to the others is evaluated by omission experiments.

The vast majority of coffee volatiles is connected to roast coffee and generated by green coffee roasting.

Table 10 Preparation techniques of coffee brews, resulting levels of cafestol and kahweol in the brew, and predicted effects on serum cholesterol with a habitual consumption of 5 cups per day

		Diterpenes per cup[a]		
Type of coffee	*Preparation technique*[a]	*Cafestol (mg)*	*Kahweol (mg)*	*Predicted rise in serum cholesterol levels with consumption of five cups/day (mmol l^{-1})*[b]
Filtered 	Boiled water is poured over finely ground roasted coffee in a paper filter, by either hand or using an electric coffee maker	0.1	0.1	<0.01
Percolated 	Coarsely ground roasted coffee is extracted by recirculating boiling water until the desired brew strength is reached	0.1	0.1	<0.01
Instant 	2–3 g of soluble coffee granules are dissolved into 150–190 ml of hot water	0.2	0.2	0.01

(*Continued*)

Table 10 (Continued)

Type of coffee	*Preparation technique*[a]	*Diterpenes per cup*[a]		*Predicted rise in serum cholesterol levels with consumption of five cups/day (mmol l^{-1})*[b]
		Cafestol (mg)	*Kahweol (mg)*	
Espresso 	Hot water is forced under high pressure through a bed of finely ground, usually dark roasted, coffee	1.5	1.8	0.10
Mocha 	Just overheated water is forced through a bed of finely ground, usually dark roasted, coffee	1.1	1.4	0.07
Boiled	Coarsely ground coffee is boiled with water for 10 or more min, or infused with hot water('infused' coffee), and the liquid is decanted without the use of a filter	3.0	3.9	0.19

Plunger pot 	Hot water is poured onto coarsely ground coffee, and after 2–5 min the metal screen strainer is pushed down to separate the grounds from the fluid	3.5	4.4	0.23
Turkish/Greek 	Very fine/powdery ground coffee is brought to a boil once or repeatedly, or incubated with hot water (‘mud’ coffee), and the liquid is decanted without the use of a filter	3.9	3.9	0.25

[a] Urgert, and Katan[165]
[b] calculated by Urgert according to Weusten-van der Wouw et al.[166]

3.25.3.7.1 Volatiles in green coffee

In raw coffee, some 200 volatiles have been identified. Using the methods given above, some turned out to be the most important (**Table 11**).

Some of these key odorants allow differentiation between Robusta and Arabica coffees (**Table 12**).[169]

2-Methylisoborneol exhibiting an 'earthy, musty' sensorial impression, is suggested to be the aroma key compound of Robusta coffee.

On thorough investigation, some raw coffee volatiles were identified as characteristic for defects, which are externally caused, maybe by over-fermentation during processing, or by insect attacks (**Table 13**).[80,120]

3.25.3.7.2 Volatiles on roasting

Roast coffee aroma is a complex mixture of more than 1000 volatile compounds. With the instruments mentioned above, their quantity could be condensed to some 20 key aroma compounds.[120]

Of these, the roasty-sulfurous smelling 2-furfurylthiol (FFT) is by far the outstanding odorant. During roasting it is formed by reactions of cysteine with arabinose which is released from the polysaccharides in coffee.[171]

Next in line as revealed by the omission-experiment, are the pyrazines, followed by furanones and further sulfur compounds.

The volatiles can be grouped according to their odor qualities.

Table 11 Key odorants in green coffee and concentration in roast coffee, compiled from Grosch[120]

Odorant	*Concentration* $\mu g\,kg^{-1}$	*FD factor*	*impression*	*Concentration in roast coffee*
3-Isobutyl-2-methoxypyrazine	97	4096	Pea-like	97
2-Methoxy-3,5-dimethylpyrazine	0.5	512	Earthy	1.1
2-Methoxy-3-isopropylpyrazine	2.3	128	Pea-like	2.4
4-Vinylguaiacol	117		Spicy, phenolic	39 000
4-Ethylguaiacol	21			4060
3-Hydroxy-4,5-dimethyl-2-furanone (Sotolon)	0.7	64	Spicy	1870
(E)-β-Damascenone	>0.3		Fruity, honey-like	255

Table 12 Odorants markedly different in Arabica and Robusta green coffees

Odorant	*Robusta green*	*Arabica green*
2-Methylisoborneol	$0.74\,\mu g\,kg^{-1}$	$0.42\,\mu g\,kg^{-1}$
2-Ethyl-3,5-dimethylpyrazine	824	492
2,3-Diethyl-5-methylpyrazine	233	112

Table 13 Green coffee volatiles associated with defects[120]

Odorant	*Impression*	*Defect caused by*
Geosmin	Earthy	Moulds
2,4,6 Trichloroanisol /-phenol	Rioy, corky	Degradation of a fungicide
2-Isopropyl-3-methoxypyrazine	Peasy, potato-taste	Bacterial infection after insect attack
4-Heptenal	Rotten fish	Immature beans
Ethyl-2-methylbutyrate		Uncontrolled fermentation
Ethyl-3methylbutyrate		

The concentrations in solid roast (mg kg^{-1}) and in the brew, FD factors, sensory impressions are given together with the numbering in the chromatographic analysis (**Figure 7**) for medium roasted Arabica coffee (compiled from different sources[120,172,173] – not all entities present). Not all volatiles were grouped.

The **Tables 14–19** contain the positively grouped volatiles with their characteristics, when known – not all compounds identified in the FD-chromatogram are grouped, and not all are completely characterized.

Some of these roast coffee odorants differ markedly between Arabica and Robusta.

3.25.3.7.3 Volatiles in the brew

The data in the tables show the aroma volatiles both in the solid roast and ground coffee and in the brew, prepared as the final step before consumption.

The stability of just these aroma beverage concentrations are of interest, as the consumer appreciates a freshly prepared coffee brew mostly for its pleasing overall aroma – besides the stimulatory effect.

The aroma, however, is not stable, and is rapidly changing after preparation of the coffee brew.

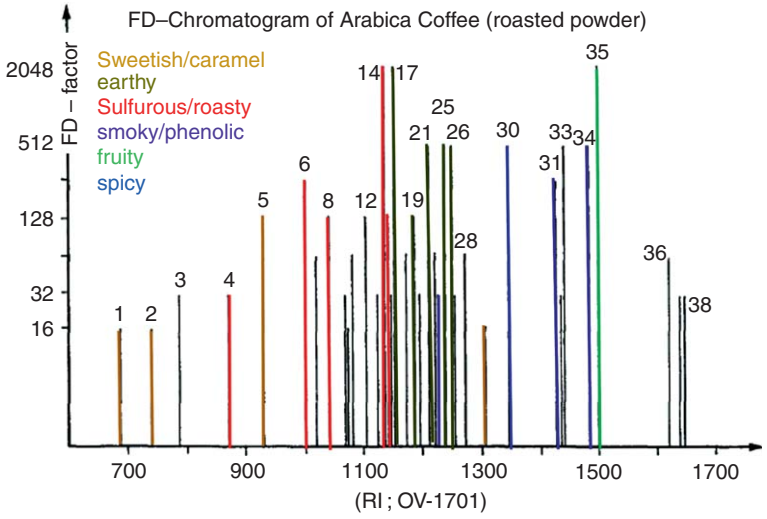


Figure 7 FD-Chromatogram of Arabica roast coffee powder. Reproduced from I. Blank; A. Sen; W,Grosch, Aroma Impact Compounds of Arabica and Robusta Coffee. Qualitative and Quantitative Investigations. *ASIC 14e Colloque*; San Francisco, 1991; pp 117–129.

Table 14 Grouping of key aroma compounds of roast coffee: sweetish/caramel group

	Conc. mg kg^{-1}	*Conc. mg kg^{-1}*			
Sweetish/caramel odorants	*Roast*	*Brew*	*FD factor*	*Impression*	*N° in Figure 7*
4-Hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol)	120	7.2		Caramel-like	24
2,3-Butane-dione (diacetyl)	49.4	3.1		Buttery	1
2,3-Pentane-dione	36.2	1.6		Buttery	5
2-Methylbutanal	23.4	0.9		Malty, pungent, sweety	
2-Ethyl-4-hydroxy-5-methyl-3(2H)-furanone	16.7	0.8		Caramel-like	29
Methylpropanal	28.2	0.8		Malty, pungent, fruity	
3-Methylbutanal	17.8	0.6		Malty, pungent, sweety	2
Vanillin	4.1	0.2	128	Sweet (vanilla)	38

Table 15 Grouping of key aroma compounds of roast coffee: earthy group

	Conc. mg kg^{-1}	Conc. mg kg^{-1}			
Earthy odorants	*roast*	*brew*	*FD factor*	*Impression*	*N° in Figure 7*
2-Ethyl-3,5-dimethylpyrazine	0.326	0.017	2048	Earthy, roasty	17
2,3-Diethyl-5-methylpyrazine	0.090	0.0036	2048	Earthy, roasty	21
2-Ethenyl-3-ethyl-5-methylpyrazine	0.017	0.002		Roasty, earthy	26
3-Isobutyl-2-methoxypyrazine	0.087	0.0015		Earthy, roasty	25
2-Ethenyl-3,5-dimethylpyrazine	0.053	0.001		Roasty, earthy	19

Table 16 Grouping of key aroma compounds of roast coffee: sulfurous/roasty group

	Conc. mg kg^{-1}	Conc. mg kg^{-1}			
Sulfurous/roasty odorants	*Roast*	*Brew*	*FD factor*	*Impression*	*N° in Figure 7*
Methanethiol	455	0.170		Putrid, sulfury	
2-Furfurylthiol	1.7	0.017		Roasty (Coffee)	6
Methional	0.239	0.010		Potato-like, sweet	8
3-Mercapto-3-methyl-butyl formate	0.112	0.005 7	2048	Catty, roasty	14
2-Methyl-3-furan-thiol	0.006 4	0.001 1		Meaty	15
3-Methyl-2-buten-1-thiol	0.009 9	0.000 6		Sulfurous, amine-like	4
Dimethyl trisulfide	0.028			Vegetable (cooked)	

Table 17 Grouping of key aroma compounds of roast coffee: smoky/phenolic group

	Conc. mg kg^{-1}	Conc. mg kg^{-1}			
Smoky/phenolic odorants	*Roast*	*Brew*	*FD factor*	*Impression*	*N° in Figure 7*
4-Vinylguaiacol	55	0.74	512	Spicy, clove-like	34
Guaiacol	3.2	0.12		Phenolic, spicy	23
4-Ethylguaiacol	1.6	0.05		Spicy, clove-like	31

Table 18 Grouping of key aroma compounds of roast coffee: fruity group

	Conc. mg kg^{-1}	Conc. mg kg^{-1}			
Fruity odorants	*Roast*	*Brew*	*FD factor*	*Impression*	*N° in Figure 7*
Acetaldehyde	130	4.7		Fruity, winey	
Propanal	17.4				
β-Damascenone	0.226	0.001 6	2048	Fruity, honey-like	35

Table 19 Grouping of key aroma compounds of roast coffee: spicy group

	Conc. mg kg^{-1}	Conc. mg kg^{-1}			
Spicy odorants	*roast*	*brew*	*FD factor*	*Impression*	*N° in Figure 7*
3-Hydroxy-4,5-dimethyl-2-furanone (Sotolone)	1.6	0.08	2048	Spicy	30
4-Ethyl-3-hydroxy-5-methyl-2(5H)-furanone	0.132		1024	Spicy	

Of the groups of volatile aroma compounds summarized above, the sulfury-roasty components, contribute prevalently to the roasty impression of the beverage which is special for coffee.

Regarding this quality aspect,[174] a marked decrease in the beverage quality can be observed shortly after brewing. It is quantitatively identified through comparative aroma dilution analysis on the aqueous solutions' headspace, which contains the total volatile fraction isolated from coffee brew.

The decrease strongly affects the sulfurous/roasty notes, and the aroma and quality changes observed are connected to losses of the odorous thiols 2-furfurylthiol (FFT), 3-methyl-2-butenthiol (MBT), 3-mercapto-3-methylbutyl formate (MMBF), 2-methyl-3-furanthiol, and methane thiol.

The data further suggest that the thiol-binding activity of pyrazinium derivatives from melanoidins, contributes undoubtedly to the decrease of the sulfury-roasty odor appearing shortly after preparation of the coffee brew, with a pyrazinium radical cation, as the active principle.

3.25.3.8 Melanoidins

During roasting, a marked browning of the coffee beans occurs, together with intrinsic transformation of coffee components, the formation of aromatic volatiles and the formation of a new class of products, the melanoidins.

For a long time, the melanoidins were the dark end of the browning reactions, with unstructured, scarcely defined macromolecular material, which nevertheless enters the brew. The dissolved components of the brew are larger then the molecules of the 'solvent' water, in terms of physicochemistry, we deal with suspensions.

Just these melanoidins were the topics of international scientific networking on 'Melanoidins in Food and Health',[175] of 1999 and on 'Thermally processed foods: possible health implications'[176] of 2004.

The melanoidins are part of the coffee brew, with about 20% of the dry matter, and can be isolated from it. Recent investigations revealed,[141] that they consist of an about 80% fraction with low-molecular-weight compounds (up to 3 kDa),[177] and of a residual high molecular weight fraction (molecular weight up to 12 kDa).[122]

The melanoidins high-molecular-weight fraction was formed with incorporation of arabinogalactan proteins. It is unclear, whether these proteins provide a contribution to human nutrition.

Chlorogenic acids were found to be incorporated as a whole into the melanoidins, contributing to their antioxidant capacity, preferably the higher molecular weight fractions, whilst the free chlorogenic acids drastically decreases during roasting.

Nonenzymatic browning might also be due to caramelization, when pure monosaccharides are heated over a long time; this reaction might not be involved here, as the shorter heating time with amino reactants would prioritize the Maillard reaction. Another possible reaction scheme, lipid oxidation, is not probable, as the lipid content does not decrease on roasting. The Maillard remains the prevalent reaction during heating, which leads to melanoidins.

3.25.4 Conclusions

Coffee contains a wide spectrum of compounds. Many contribute to the flavor of coffee, and some compounds have an effect on our health, such as caffeine with a stimulating effect, chlorogenic acids as antioxidants, nicotinic acid as vitamin and the diterpene esters as cholesterol-increasing compounds. Despite the similar importance of coffee as beverage, the research on health promoting effects has not yet developed to the same extent for coffee as for tea. At least it is clear that the whole processing of coffee beans and the method of coffee brewing play a crucial role for the final mélange of compounds that is consumed when drinking coffee.

References

1. D. Kennet, *Tea Coffee Trade J.* **1998**, *170*, 10–11.
2. D. Kennet, Durham University, Durham. Private communication, February 2009, publication in progress.
3. H. Kopp, Die Landwirtschaft des Yemen: Vom Mokka zum Qat. In *Jemen*; W. Daum, Ed.; Pinguin: Innsbruck, 1987;pp 365–369.
4. A. Huetz de Lemps, Cinq siècles d'aventure. In *Les mondes du Café*; C. Guillard, Ed.. Hors-série of L'amateur de Bordeaux: Paris, 1997; pp 6–15.

5. N. H.v. Presslaw, Aussführlicher Bericht Von Den Unerhörten Wunderbaren Ceremonien. . . Zu Constantinopel Im 1582 Jar. In *Neuwe Chronika Türkischer Nation*; H. Lewenklaw von Amelbeurn, Ed.; C.de Marne, J. Aubrius: Frankfurt, 1590; p 505.
6. L. Rauwolf, *Aigentliche Beschreibung der Raiß inn die Morgenländerin*; Reinmichel: Lauingen, 1582; pp 102–103 (journey in Syria 1573–1576).
7. P. Alpinus, physician and botanist of Venice, Italy, *De medicina Aegyptiorum*; F. de Franceschi: Venice, 1591; Vol. IV, Cap. I–III, p 118–123 (stay in Cairo 1580–1583).
8. C. Clusius, *Aromatum et simplicium aliquot medicamentorum apud Indios nascentium historia*, Latin translation 1567 of G. de Orta, *Colóquios dos simples e drogas*, Ioannes de Endem: Goa, 1563; in the amended 2nd Latin ed. coffee beans are mentioned and depicted on pp 214–215, Plantin: Antwerp, 1574; in the 4 ed., a reference to Rauwolf is added, Plantin: Antwerp, 1593, pp 205–206.
9. F. Eccardi; V. Sandalj, *Coffee. A Celebration of Diversity*, English edition 2002 *of Il Caffè – Territori e Diversità*; Sandalj: Trieste, 2000; p 37.
10. B. E. Dahlgren, *Coffee*; Field Museum: Chicago, 1938; p 7.
11. Coffee Board of India, Central Coffee Research Institute, Leaflet for the coffee Board's Platinum Jubilee, 2000, paragraph 'A little history. . .'.
12. M. Pendergrast, *Uncommon Grounds*; Basic Books: New York, 1999; p 7.
13. J. Thorn, *L'Amateur de Café*, French edition 1996 of *The Coffee Companion. The Connoisseurs Guide to the Worlds Best Brews*; Quintet Publishing: London, 1995; pp 8–16.
14. A. de Jussieu, Histoire du Caffé. In *Mémoires de l'Académie Royale des Sciences*; 1715, pp 291–299; replacing his earlier lecture ''Histoire du Caffé'' 1713. In *Histoire de l'Académie Royale*; Académie Royale des Sciences, Ed.; Pankoucke: Paris, 1757; pp 388–399.
15. B. E. Dahlgren, see Ref. 10, p 22.
16. A. Lécolier; P. Besse; A. Charrier; T-N. Tchakaloff; M. Noirot, *Euphytica* **2009**, *168*, 1–10; citing letters from French archives of 1713.
17. R. F. Burton, *J. Royal Geograph. Soc.* **1859**, *XXIX*, 1–454.
18. J. A. Grant, *A Walk Across Africa*; Blackwood: London and Edinburgh, 1864.
19. R. F. Burton, see Ref. 17, p 290.
20. J. A. Grant, see Ref. 18, p 197.
21. J. A. Grant, see Ref. 18, p 160.
22. R. F. Burton, see Ref. 17, pp 183, 285.
23. O. Warburg, Coffea Arabica L. In *Die Pflanzenwelt Ost-Afrikas und der Nachbargebiete*; A. Engler, Ed.; Reimer: Berlin, 1895; Theil B, Kap. VIII, pp 246–252.
24. A. Froehner, *Engl. Bot. Jahrb.* **1898**, *25*, 233–295 (from Froehners thesis, Rostock 1898).
25. A. Froehner, *Notizbl. Bot. Gart. Berlin-Dahlem,* **1897**, *1*, p 237; with *Coffea canephora* referring to the earlier publication of an 'Illustration with Analysis' by J.-B. L. Pierre, based on a sample by Klaine from Gabon.
26. B. A. Weinberg; B. K. Bealer, *The World of Caffeine*; Routledge Chapman & Hall: London, 2001; p 244.
27. É. Laurent, *C. R. Séances Soc. R. Bot. Belg.* **1898**, *37*, 46–59.
28. P. J. S. Cramer, In *A Review of Literature of Coffee Reseach in Indonesia from about 1602 to 1945*; F. L. Wellman, Ed.; SIC Editorial IICA: Turrialba, Costa Rica, 1957; pp 12–13.
29. H. D. Mac Gillavry, *Der Tropenpflanzer* **1905**, *9* (2), 128–131; describing a delivery from the company L'Horticole Coloniale, Brussels, director Lucien Linden.
30. R. A. Muller; D. Berry; J. Avelino; D. Bieysse, Coffee Diseases. In *Coffee: Growing, Processing, Sustainable Production*; J. N. Wintgens, Ed.;Wiley-VCH: Weinheim, 2004; Part II, Chapter 4, pp 491–545.
31. M. J. Berkeley, *The Gardeners' chronicle and agricultural gazette;* 1869, November; p 1197.
32. F. M. DaMatta; J. D. Cochico Ramalho, *Braz. J. Plant Physiol.* **2006**, *18* (1), 55–81.
33. International Coffee Organization ICO, London, Coffee statistics, published quarterly, 2009.
34. H. Becker, *Kaffee aus Arabien*, Steiner: Wiesbaden 1979, p 11, quoting G. R. Gavin, Aden under British Rule, London 1977, p 18.
35. G. Söhn, *Kleine Kaffeekunde*, 3rd ed.; de Gruyter: Hamburg, 1957; p 18.
36. M. Sivetz; N. W. Desrosier, *Coffee Technology*; AVI: Westport, 1979; p 13.
37. M. Pendergrast, see Ref. 12, p 18.
38. B. Rothfos, *Coffee. The Production*; Gordian: Hamburg, 1980, Engl. ed. of Kaffee. Die Produktion: Hamburg, 1979.
39. E. Dentan, Étude microscopique du développement et de la maturation du grain de café. *ASIC 11e Colloque* Lomé: Togo, 1985; 381–396.
40. M. R. Söndahl; T. W. Baumann, Agronomy II Development and Cell Biology. In *Coffee, Recent Developments*; R. J. Clarke, O. G. Vitzthum, Eds.; Blackwell: Oxford, 2001; p 203.
41. M. R. Söndahl; H. A. M. van der Vossen, The Plant, Origin, Production and Botany. In *Espresso Coffee. The Science of Quality*; A. Illy, R. Viani, Eds.; Elsevier: Amsterdam, 2005; pp 21–29.
42. A. P. Davis; R. Govaerts; D. M. Bridson; P. Stoffelen, *Bot. J. Linn. Soc.* **2006**, *152*, 465–512.
43. R. D. De Castro; P. Marraccini, *Braz. J. Plant Physiol.* **2006**, *18* (1), 175–199.
44. F. Eccardi; V. Sandalj, see Ref. 9, p 69
45. C. Bauhin, *Pinax Theatri Botanici*; L. König: Basel, 1623; p 428 (misprint '498' in *Species Plantarum*, 1st ed.).
46. C. Linnaeus, *Systema naturae*, 1st ed.; Haak: Leiden, 1735; coffea on p 4 (no numerotation).
47. C. Linnaeus, *Genera Plantarum*, 1st ed.; Wishoff: Leiden, 1737; coffea on p 158.
48. C. Linnaeus, *Spedies Plantarum*, 1st ed., Salvius: Stockholm,, 1753; Coffea arabica on p 172.
49. C. Linnaeus, *Hortus Cliffortianus*, printed Leiden, Netherlands, 1737, published Amsterdam 1738; coffea on p 59.
50. C. Linnaeus, *Hortus Upsaliensis*; Salvius: Stockholm, 1748; coffea on p 41.
51. Natural History Museum, The Linnaean Plant Name Typification Project. BM 000558021. http://www.nhm.ac.uk/research-curation/research/projects/linnaean-typification (accessed Nov 2009)
52. D. M. Bridson; B. Verdcourt; Coffea. In *Flora of Tropical East Africa. Rubiaceae (Part 2)*; R. M. Polhill, Ed.; Balkema: Rotterdam 1988; p 713.

53. International Code of Botanical Nomenclature (Vienna Code), *Regnum Vegetabile*; Gantner: Vienna, 2006.
54. C. Jarvis, *Order Out of Chaos*; The Linnean Society: London, 2007.
55. A.-L. de Jussieu, *Genera Plantarum secundum ordines naturales*; Herrissant widow: Paris, 1789; *Coffea* L. on page 204.
56. B. Bremer, *Ann. Mo Bot. Gard.* **2009**, *96*, 4–26.
57. E. Smets; P. De Block; B. Bremer; S. Dessein; S. Huysmans; H. Ochoterena; C. Puff; E. Robbrecht; C. Taylor, Third International Rubiaceae Conference 2006 in Leuven, Belgium; H. Ochoterena; P. De Block; B. Bremer; E. L. Cabral; S. Dessein; M. Endress; S. Razafimandimbison; E. Robbrecht; E. Smets; C. Taylor, Fourth International Rubiaceae Conference 2006, Jalapa, Mexico.
58. R. Govaerts; D. G. Frodin; M. Ruhsam; D. M. Bridson; A. P. Davis, *A World Checklist & Bibliography of Rubiaceae*; Royal Botanic Gardens: Kew, 2007.
59. A. Chevalier, Les caféiers du globe II: Systématique des caféiers et faux caféiers, maladies et insects nuisibles. In *Encyclopédie biologique no. 28*; Le Chevalier: Paris, 1947; p 1–352.
60. J. F. Leroy, *J. Agric. Trop. Bot. Appl.* **1961**, *VIII* (1–3), pp 1–20.
61. D. M. Bridson; B. Verdcourt, Coffea. In *Flora of Tropical East Africa. Rubiaceae (Part 2)*; R. M. Polhill, Ed.; Balkema: Rotterdam, 1988; pp 702–723.
62. P. Stoffelen, Coffea and Psilanthus (Rubiaceae) in Tropical Africa: A Systematic and palynological study, including a revision of the west and central African species. Ph.D. Thesis, Katholieke Universiteit, Leuven, Belgium, 1998.
63. A. P. De Candolle, *Ann. Mus.Hist. Nat.* **1807**, *9*, 216–222 (first description of the Coffeae tribe) and.
64. A. P. De Candolle, *Prodromus systematis naturalis regni vegetabilis*; Treuttel und Würtz: Paris, 1830; Part IV. Saxifragaceae to Dipsacaceae.
65. A. P. Davis; M. Chester; O. Maurin; M. F. Fay, *Am. J. Bot.* **2007**, *94*, 313–329.
66. B. D. Mishler, *Taxon* **2009**, *58*, 61–67.
67. P. D. Cantino; K. de Queiroz, International Code of Phylogenetic Nomenclature, Version 4b, 2007.
68. O. Maurin; A. P. Davis; M. Chester; E. F. Mvungi; Y. Jaufeerally-Fakim; M. F. Fay, *Ann. Bot.* **2007**, *100*, 1565–1583.
69. A. Charrier; A. B. Eskes, Botany and Genetics of Coffee. In *Coffee: Growing, Processing, Sustainable Production*; J. N. Wintgens, Ed.; Wiley-VCH: Weinheim, 2004; p 36.
70. P. Lashermes; M. C. Combes; J. Cros; P. Troustot; F. Anthony; A. Charrier, *Origin and Genetic Diversity of Coffea arabica* L. Based on DNA Molecular Markers. ASIC 16e Colloque; Kyoto, 1995; p 528–536.
71. P. Lashermes, Recent Actualisation of the State Of Knowledge. Private communication, August 2009.
72. J. Maley, *Proc. R. Soc. Edinb.* **1996**, *104B*, 31–73.
73. C. Gomez; S. Dussert; P. Hamon; S. Hamon; A. de Kochko; V. Poncet, *BMC Evol. Biol.* **2009**, *9*, 167ff.
74. M. E. Leal, The African Rain Forest during the Last Glacial Maximum, an Archipelago of Forests in a Sea of Grass. Ph.D. Thesis, Wageningen University, 2004; Chapter 4, 5.
75. A. Lecollier *et al*., see Ref. 16; main text.
76. T. Joët; A. Laffargue; J. Salmona; S. Doulbeau; F. Descroix; B. Bertrand; A. de Kochko; S. Dussert, *New Phytol.* **2009**, *182*, 146–162.
77. ISO 3509:2005 , *Coffee and Coffee Products – Vocabulary*, 4th ed.; ISO: Geneva, 2005.
78. International Coffee Agreement of 2001, between the governments of member states of the International Coffee Organization ICO, London, EU OJ L 326 of 11.12.2001, p 23–39.
79. V. Leloup, Evaluation of Nutritive Value of Soluble Coffee. *ASIC 21e Colloque*; Montpellier, 2006; p 80–87, excerpts of Table 1.
80. A. Illy; R. Viani, *Espresso Coffee, The Chemistry of Quality*; Academic Press: London, 1995; Table 5.2, p 98.
81. R. Macrae, Nitrogenous Components. In *Coffee, Vol. 1: Chemistry*; R. J. Clarke, R. Macrae, Eds.; Elsevier: Amsterdam, 1985; p 119.
82. V. Leloup; C. Gancel; R. Liardon; A. Rytz; A. Pithon, Impact of Dry and Wet Process on Green Coffee Composition and Sensory Characteristics. *ASIC 20e Colloque*; Bangalore, 2004; p 93–101.
83. E. Dentan, Structure fine du grain de café vert. *ASIC 8e Colloque*; Abidjan, 1977; pp 59–64.
84. P. Mazzafera; T. W. Baumann; M. M. Shimizu; M. B. Silvarolla, *Trop. Plant Biol.* **2009**, *2*, 63–76.
85. A. Crozier; T. W. Baumann; H. Ashihara; T. Suzuki, IV; G. R. Waller, *Pathways Involved in the Biosynthesis and Catabolism of Caffeine in Coffea and Camellia*. ASIC 17e Colloque; Nairobi, 1997; pp 106–113.
86. T. W. Baumann, Biochemical Ecology. In *Espresso Coffee: The science of quality*; A. Illy, R. Viani, Eds.; Elsevier: Amsterdam, 2005; Table 2.8, p 58.
87. O. Guerreieo Filho; P. Mazzafera, *Caffeine Does Not Protect Coffee against the Leaf Miner Perileucoptera coffeella*. ASIC 18e Colloque; Helsinki, 1999; pp 520–523.
88. H. Ashihara; A. M. Monteiro; F. M. Gilies; A. Crozier, *Biosynthetic Pathways of Caffeine in Coffea arabica* Leaves. ASIC 16e Colloque; Kyoto, 1995; pp 589–598.
89. T. W. Baumann; H. Wanner, *Planta* **1972**, *108*, 11–20.
90. A. Illy; R. Viani, see Ref. 80, Table 3.6, p 25.
91. A. P. Davis *et al*., see Ref. 42, p 465.
92. J.-J. R. Rakatomalala; T. Kumamoto; T. Aburatani; A. Rabemiafra; C. Nagai; Y. Kawashima; Y. N. Rabenatoandro, *Caffeine Content Distribution among Mascarocoffea* Species in Madagascar. ASIC 20e Colloque; Bangalore, 2004; pp 154–160.
93. H. Ashihara; A. Cozier, *Biosynthesis and Catabolism of Caffeine in Low Caffeine-containing Species of Coffea*. ASIC 19e Colloque; Trieste, 2001; Article PB702.
94. Dutch Nutrient Database, *De Nevo tabel van Stichting Nederlands Voedingsstoffenbestand*, 5th. ed.; Den Haag, 2001; p 644.
95. ISO 6668:2008, *Green Coffee – Preparation of Samples for Use in Sensory Analysis*, 2nd ed.; ISO: Geneva, 2008.
96. Directive 2002/67/EC on the labelling of foodstuffs containing . . . caffeine (OJ L 191, 19.7.2002, p 20–21), art. 2, 1. sets the obligation for caffeine labeling at amounts exceeding 150mg/l and a sentence intended as warning, ''contains high amounts of caffeine,'' unless (Art.2 2) the beverage is based on coffee or tea.
97. M. Petracco, *Physico-Chemical and Structural Characterisation of Espresso Coffee Brew*. ASIC 13e Colloque; Paipa, Colombia, 1989; pp 246–261.
98. J. Snel, *Coffee, Sleep and Wakefulness: Research Trends*. ASIC 20e Colloque; Bangalore, 2004; p 69–76.

99. Z. Tieges, Caffeine and Cognitive Control. Ph.D. Thesis, University of Amsterdam, 2007.
100. J. W. Daly; B. B. Fredholm, Mechanism of Action of Caffein on the Nervous System. In *Coffee, Tea, Chocolate, and the Brain*; A. Nehlig, Ed.; CRC Press: Boca Raton, FL, 2004; p 2.
101. Kaffee-Handels-Aktiengesellschaft, Bremen, Applicant (inventor not named). Verfahren zur Gewinnung kaffeinfreier Kaffeebohnen. Dt. Reichs-Patent 198, 279. October 28, 1905.
102. W. Hölscher, Rohkaffeebehandlung im Verbraucherland. In *Kaffee: Die Zukunft*; J. B. Rothfos, H. Lange, Eds.; Behr's Verlag: Hamburg, 2005; pp 88–109.
103. FDA opinion letter of 1981.
104. Directive 1999/4/EC on coffee extracts etc., (OJ L 66, 13.3.1999, p 26, Art. 2, c).
105. ISO 20481:2008, *Coffee and Coffee Products – Determination of the Caffeine Content Using High Performance Liquid Chromatography (HPLC) – Reference Method*, 1st ed.; ISO: Geneva, 2008.
106. A. Illy; R. Viani, see Ref. 80, pp 40.
107. G. Bytof; S. E. Knopp; P. Schieberle; I. Teutsch; D. Selmar, *Eur. Food Res. Technol.* **2005**, *220*, 245–250.
108. G. Bytof; S.-E. Knopp; P. Schieberle; I. Teutsch; D. Selmar, *Ann. Bot.* **2007**, *100*, 61–66.
109. P. Mazzafera; R. P. Purcino, *Post Harvest Processing methods and Physiological Alterations in the Coffee Fruit*. ASIC 20e Colloque; Bangalore, 2004; pp 811–819.
110. R. Eggers, Röstkaffee. In *Kaffee: Die Zukunft*; J. B. Rothfos, H. Lange, Eds.; Behr's Verlag: Hamburg, 2005; pp 112–137.
111. F. Ledl; E. Schleicher, *Angew. Chem.* **1990**, *102*, 597–626.
112. R. Viani; M. Petracco, Coffee. In *Ullmann's Encyclopedia of Industrial Chemistry*, 7th ed., Electronic; 2007; Section 6.2, p 1–30.
113. C. Geromel; L. P. Ferreira; S. M. C. Guerreiro; A. A. Cavalari; D. Pot; L. F. P. Pereira; T. Leroy; L. G. E. Vieira; P. Mazzafera; P. Marraccini, *J. Exp. Bot.* **2006**, *12, 57*, 3243–3258.
114. I. Privat; S. Foucrier; A. Prins; T. Epalle1; M. Eychenne; L. Kandalaft; V. Caillet; C. Lin; S. Tanksley; C. Foyer; J. McCarthy, *New Phytol.* **2006**, *178* (4), 781–797.
115. R. Viani, see Ref. 112, Table 8; citing A. G. W. Bradbury; D. J. Hallyday, see Ref. 117.
116. A. G. W. Bradbury; D. J. Hallyday, *J. Agric. Food Chem.* **1990**, *38*, 389–392.
117. R. J. Redgwell; D. Curti; M. Fischer; L. B. Fay, *Carbohydr. Res.* **2002**, *337* (3), 239–253.
118. A. M. Showalter, *Cell. Mol. Life Sci.* **2001**, *58*, 1399–1417.
119. A. G. W. Bradbury; D. J. Halliday, *Polysaccharides in Green Coffee Beans*. ASIC 12e Colloque; Montreux, 1987; pp 265–269.
120. V. Leloup; R. Liardon, *Analytical Characterisation of Coffee Carbohydrates*. ASIC 15e Colloque; Montpellier, 1993; pp 861–863.
121. V. Leloup; J. H. deMichieli; R. Liardon, *Characterisation of Oligosaccharides in Coffee Extracts*. ASIC 17e Colloque; Nairobi, 1997; pp 122–127.
122. W. Grosch, Volatile Compounds. In *Coffee: Recent Developments*; R. J. Clarke, O. G. Vitzthum, Eds.; Blackwell Science: Oxford, 2001; pp 68–89.
123. S. Homma, Non-Volatile Compounds Part II. In *Coffee: Recent Developments*; R. J. Clarke, O. G. Vitzthum, Eds.; Blackwell Science: Oxford, 2001; pp 50–67.
124. E. K. Bekedam; H. A. Schols; M. A. J. S. Van Boekel; G. Smit, *J. Agric. Food Chem.* **2006**, *24*, 7658–7666.
125. ISO 11292:1995 , *Instant Coffee – Determination of Free and Total Carbohydrate Contents – Method Using High-Performance Anion-Exchange Chromatography*, 1st ed.; ISO: Geneva, 1995.
126. R. J. Redgwell; D. Curti; J. Rogers; P. Nicolas; M. Fischer, *Planta* **2003**, *217* (2), 316–326.
127. Directive 90/496/EEC on nutrition labelling for foodstuffs (OJ L 276, 6.10.1990, p 40–44, with subsequent amendments); definitions as of Art.1, (4), (d) and (e), unchanged since 1990.
128. V. Leloup, see Ref. 79, Table 3.
129. J. Prodolliet; M. Bruelhart; M. Blanc; V. Leloup; G. Cherix; C. Donnelly; R. Viani, *J. AOAC Int.* **1995**, *78* (3), 761–768.
130. K. Kraehenbuehl; R. Bel-Rhlid; O. Mauroux; N. Page-Zoerkler; K. Gartenmann, *Selective Hydrolysis of Chlorogenic Acid Lactones and Impact on Coffee Taste*, ASIC 22e Colloque: Campinas, 2008, 352–356.
131. M. N. Clifford, *The Nature of Chlorogenic Acids – Are They Advantageous Compounds in Coffee?* ASIC 17e Colloque; Nairobi, 1997; pp 79–91.
132. H. C. de Menezes, *Food chem.* **1994**, *50* (3), 293–296.
133. V. Leloup; A. Louvrier; R. Liardon, *Degradation Mechanisms of Chlorogenic Acids during Roasting*. ASIC 16e Colloque; Kyoto, 1995, pp 192–198.
134. M. Ginz, Bittere Diketopiperazine und Chlorogensäurederivate in Röstkaffee. Ph.D. Thesis, University of Braunschweig 2004.
135. E. K. Bekedam, Coffee Brew Melanoidins. Structural and Functional Properties of Brown-Colored Coffee Compounds. Ph.D. Thesis, University of Wageningen, 2008.
136. M. Monteiro; A. Farah; D. Perrone; L. C. Trugo; C. Donangelo, *J. Nutr.* **2007**, *137*, 2196–2201.
137. M. N. Clifford, *Chlorogenic Acids – Their Characterization, Transformation during Roasting, and Potential Dietary Significance*. ASIC 21e Colloque; Montpellier, 2006; pp 36–49.
138. EU directive 90/496/EEC, see Ref. 127, definition as of Art.1, (4), (c), unchanged since 1990.
139. E. Trautwein, Untersuchungen über den Gehalt von freien und gebundenen Aminosäuren in verschiedenen Kaffeesorten sowie über deren Verhalten während des Röstens. Ph.D. Thesis, University of Kiel, 1987.
140. S. Homma, see Ref. 121.
141. P. Marraccini; C. Allard; M.-L. Andre; C. Courjault; C. Gaborit; N. Lacoste; A. Meunier; S. Michaux; V. Petit; P. Priyono; J. W. Rogers; A. Deshayes, *Update on Coffee Biochemical Compounds, Protein and Gene Expression during Bean Maturation and in other Tissues*. ASIC 19e Colloque; Trieste, 2001, Article B214.
142. W. J. Rogers; G. Bezard; A. Deshayes; I. Meyer; V. Petiard; P. Marraccini, *An 11S-Type Storage Protein from Coffea arabica* Endosperm: Biochemical Characterization, Promoter Function and Expression during Grain Maturation. ASIC 17e Colloque; Nairobi, 1997; pp 161–167.
143. H. M. Rawel; S. Rohn; J. Kroll, *Dtsch. Lebensm. Rundsch.* **2005**, *101*, 148–161.
144. P. Marraccini; J. Rogers, Coffee Storage Proteins. U.S. Patent 6,617,433, 9 September 2003.
145. H. Ashihira; X.-Q. Zheng; C. Nagai, *ASIC 20e Colloque*; Bangalore, 2004; pp 807–810.

146. R. Macrae, see Ref. 81, Fig. 2, p 130.
147. European directive 90/496/EEC, see Ref. 127: Nicotinic acid is mentioned as vitamin Niacin Annex I under vitamins and minerals, which may be labeled, OJ L 276, 6.10.1990, p 40, unchanged since 1990.
148. R. H. Stadler; N. Varga; C. Milo; B. Schilter; F. A. Vera; D. H. Welti, *J. Agric. Food Chem.* **2002**, *50*, 1200–1206.
149. T. W. Hofmann; V. Somoza, *Antioxidant Components in Roast Coffee*. ASIC 20e Colloque; Bangalore, 2004; pp 77–87.
150. K. Speer; I. Kölling-Speer, *Braz. J. Plant Physiol.* **2006**, *18* (1), 201–216.
151. H. G. Maier, *Kaffee*; Parey: Hamburg, 1981; p 21.
152. R. Viani; M. Petracco, see Ref. 112, table 19, originating from Ullmann's 5th edition, 1986.
153. K. Speer, *Z. Lebensm. Unters. Forsch.* **1989**, *189*, 326–330.
154. DIN 10779:1999, *Analysis of Coffee and Coffee Products – Determination of 16-O-Methyl Cafestol Content of Roasted Coffee – HPLC-method*; Beuth: Berlin, 1999.
155. K. Speer; N. Sehat; A. Montag, Fatty Acids in Coffee. *ASIC 15e Colloque*; Montpellier, 1993; pp 583–592.
156. E. Dentan, see Ref. 39, Fig. 21, p 391.
157. M. Sivetz, see Ref. 36, pp 434–483.
158. M. Petracco, Espresso. In *Kaffee: Die Zukunft*; J. B. Rothfos, H. Lange, Eds.; Behr's Verlag: Hamburg, 2005; pp 140–143.
159. K. Roth, *Chem. unserer Zeit* **2003**, *37*, 280–282.
160. K. Folstar; W. Pilnik; J. G. de Heus; H. C. van der Plas, *Lebensm. Wiss. Technol.* **1975**, *8*, 286–288.
161. K. Speer, In *16-O-Methylcafestol – a New Diterpene in Coffee; the Fatty Acid Esters of 16-O-Methylcafestol.* Proceedings of the Euro Food Chemistry VI, Hamburg, Germany; W. Baltes, T. Eklund, R. Fenwick, W. Pfannhauser, A. Ruiter, H.-P. Thier, Eds.; Behr's: Hamburg, 1991; Vol. 1, pp 338–342.
162. K. Speer; T. Kurzrock; I. Kölling-Speer, *16-O-Methylcafestol in the Quality Controlof Instant Coffees*. ASIC 20e Colloque; Bangalore, 2004; pp 880–883.
163. T. Kurzrock; I. Kölling-Speer; K. Speer, In *Identification of Dehydrocafestol Fattyacid Esters in Coffee.* In Proceedings of the 20th International Symposium on Capillary Chromatography; P. Sandra, A. J. Rackstraw, Eds.; Naxos: Schriesheim, Germany, 1998; No 27.
164. D. S. Thelle; E. Arnesen; O. H. Førde, *N. Engl. J. Med.* **1983**; *308* (24), 1454–1457.
165. R. Urgert; M. B. Katan, *J. R. Soc. Med.* **1996**, *89* (11), 618–623.
166. M. P. M. E. Weusten-van der Wouw; M. B. Katan; R. Viani; A. C. Huggett; R. Liardon; P. G. Lund-Larsen; D. S. Thelle; I. Ahola; A. Aro; S. Meyboom; A. C. Beynen, *J. Lipid Res.* **1994**, *35*, 721–733, p 729.
167. A. Duplatre; C. Tisse; J. Estienne, *Ann. Fals. Exp. Chim.* **1984**, *828*, 259–270.
168. O. Culmsee, *Dtsch. Lebensmitt. Rundsch.* **1975**, *71*, 425–427.
169. W. Grosch; P. Semmelroch; C. Masanetz, *Quantification of Potent Odorants in Coffee*. ASIC 15e Colloque; Montpellier, 1993; pp 545–549.
170. I. Blank; A. Sen; W. Grosch, *Aroma Impact Compounds of Arabica and Robusta Coffee. Qualitative and Quantitative Investigations*. ASIC 14e Colloque; San Francisco, 1991; pp 117–129.
171. W. Grosch, *Key Odorants of Roasted Coffee: Evaluation, Release, Formation*. ASIC 18e Colloque; Helsinke, 1999; pp 17–21.
172. H. Steinhardt; W. Hoelscher, *Storage Related Changes of Low Boiling Volatiles in Whole Coffee Beans*. ASIC 14e Colloque; San Francisco, CA, 1991; pp 156–164.
173. K. Cale; N. Imura, *Recovery of Beneficial Coffee Aromas from Thermal Hydrolyzates*. ASIC 15e Colloque; Kyoto, 1995; pp 685–694.
174. T. Hofmann; M. Czerny; P. Schieberle, *Instrumental Analysis and Sensory Studies on the Role of Melanoidins in the Aroma Staling of Coffee Brew*. ASIC 19e Colloque; Trieste, 2001; CD-ROM paper C308.
175. COST action 919 – Melanoidins in Food and Health; 5th Framework Programme of the EU (1999–2004).
176. COST action 927 – Thermally processed foods: possible health implications, within the 6th Framework Programme of the EU 2004 to 2009 – the Wageningen university took part in this programme with the melanoidin research group of the PhD thesis. E. K. Bekedam, see Ref. 137.
177. E. K. Bekedam; E. Ros; H. A. Schols; M. A. J. Boekels; G. Smit, *J. Agric. Food Chem.* **2008**, *56*, 4060–4067.

3.26 Chemistry of Wine

Véronique Cheynier, Rémi Schneider, Jean-Michel Salmon, and Hélène Fulcrand, Montpellier Research Centre, Montpellier, France

3.26.1 Introduction

Wines are alcoholic drinks made by fermenting the juice of fruits or berries, but a narrower definition, restricted to alcoholic beverages obtained from the fermentation of grapes, is most commonly accepted and is relevant to this chapter.

Wine composition varies greatly with the grape variety, the vintage characteristics, the vine growing conditions, the grape ripeness at harvest, and the wine-making practices. In brief, wine-making process involves a series of successive operations, the sequence of which varies considerably and largely determines the wine type. Thus, wine composition partly reflects the composition of the grapes used to make it but also differs from it considerably. This is due to the partial transfer of grape components into the wine and changes occurring during wine-making and aging processes, in particular as a result of fermentation.

The grape berry contains large amounts of sugars (glucose and fructose), representing 20–25% of the berry weight at harvest, and these are transformed into alcohol, representing 10–15% of the wine volume, by alcoholic fermentation. Similarly, malic acid, which is one of the major organic acids present in the berry, is converted to lactic acid through malolactic fermentation. In white wine making, the first step (usually after crushing) is pressing. This separates the solid parts (i.e., skins, seeds, and eventually stems) from the juice, and the juice is then fermented separately. In red wine making, fermentation is achieved on the whole must obtained after crushing, and pressing is performed only after the maceration phase. Maceration enables extraction of constituents present in the skins and seeds into the fermenting must, including not only the red pigments, but also tannins, volatile compounds and aroma precursors, and plant cell wall polysaccharides. Changes taking place during winemaking involve both biochemical and chemical processes. The former result from yeast metabolism and from the action of various enzymes originating from grape, yeast, and other microorganisms, or from the action of exogenous enzymes added as process aids. Biochemical processes occur mostly in the early stages of the process, while chemical reactions continue throughout wine aging.

This chapter is divided into three parts: the first part describes the composition changes due to yeast and bacterial primary metabolism, the second part the chemistry of aroma compounds, and the third part the chemistry of phenolic compounds in wine.

3.26.2 Chemistry Associated with Wine Fermentations

In wine-making conditions, the yeast *Saccharomyces cerevisiae* carries out alcoholic fermentation in almost anaerobic conditions (available O_2 concentration in the must is below $7\,mg\,l^{-1}$ at the start of fermentation). Metabolism in these conditions is thus strictly fermentative.

Grape must has a high fermentable sugar content (from 140 to $260\,g\,l^{-1}$, as a function of maturity), composed of similar amounts of glucose and fructose. It is also highly acidic ($pH \approx 3.0–3.5$). Complete fermentation by *S. cerevisiae* under these conditions produces between 8 and 15% (v/v) ethanol, together with several other fermentation products generated to maintain the balance in yeast oxidoreductive metabolism.

Malolactic fermentations of wine by lactic acid bacteria (*Oenococcus oeni* or other lactobacilli) are particularly sought to increase the microbiological stability of the finished wine. This bacterial fermentation process favors the development of physiochemical and sensory characteristics of wine.

3.26.2.1 Products of Yeast Primary Metabolism (Alcoholic Fermentation)

After a period of latency, lasting from a few hours to several days, the yeast grows until its maximum population size is reached, usually by the point at which the fermentation is 20–30% complete. The maximum rate of fermentation is reached at the end of this phase. The yeast population reached differs greatly between musts, and generally lies between 50 and 250×10^6 cells ml^{-1}. Most of the sugar is fermented during the stationary phase. Yeasts no longer divide during this phase and their activity decreases steadily, although survival rates generally exceed 80%, or even 90%, other than in stopped fermentations.

Ethanol is not immediately produced in grape musts inoculated with *S. cerevisiae*.[1] Many compounds other than ethanol are formed at the start of fermentation: glycerol, pyruvate, succinate, and other organic acids.[2,3] In addition, the biosynthesis of carbon compounds (amino acids and sugars in particular), essential for biomass production, occurs through hexose metabolism and therefore does not lead to the formation of ethanol. In these conditions, a number of fermentative by-products are formed, reestablishing the chemical equilibrium in the cell.

3.26.2.1.1 Ethanol and carbon dioxide

Ethanol is the main product of alcoholic fermentation, and its extracellular concentration may reach 12–14% (v/v) during normal fermentation. It is widely accepted that the synthesis of one degree of alcohol (1% (v/v)) during alcoholic fermentation represents the consumption of 16.5–17 g of reducing sugar (glucose or fructose) per liter. The cell membrane is highly permeable to small hydrophilic molecules, such as ethanol, which thus reaches equilibrium by simple diffusion across the plasma membrane.[4] Counting by weight, carbon dioxide is the second most important product of alcoholic fermentation. Carbon dioxide production is a function of the *S. cerevisiae* strain used, and in enological conditions, the mean yield of carbon dioxide may be considered to be 0.4–0.5 g per gram of sugar consumed.

3.26.2.1.2 Glycerol

The glycerol concentrations reached under enological fermentation conditions are generally between 5 and $11\,g\,l^{-1}$, depending on the yeast strain used. The main purpose of glycerol production is to replenish the pool of NAD^+ cofactors within the yeast.[5] The major determinants of glycerol production during fermentation are the maintenance of redox equilibrium, biomass generation, and the anabolic cellular reactions leading to the generation of an excess of reduced NADH cofactors. Glycerol production also promotes resistance to strong osmotic pressure in the yeast. In such conditions, large amounts of glycerol accumulate in the yeast cytoplasm, and surplus glycerol subsequently leaves the cell by a process of simple diffusion. Glycerol production is generally linked to the production of succinate and acetate, the synthesis of which leads to a marked NADH production.

3.26.2.1.3 Organic acids

More than a hundred organic acids are formed during the course of alcoholic fermentation. Some (acetate, succinate, alpha-ketoglutarate, malate, and citrate) are generated directly from pyruvate by limited activity of the tricarboxylic acid cycle. These organic acids may have a direct effect on the organoleptic nature of the

finished product and the pH of the wine. Of these organic acids, succinate, like glycerol, is one of the major by-products of fermentation. Its concentration at the end of fermentation remains below 1 g l^{-1}, and accounts for between 0.3 and 0.5% of the fermented sugars.[6] Other organic acids (isovaleric and isobutyric acids) are probably derived from the synthetic pathways for amino acids and higher alcohols.

Most of the remaining organic acids are produced during the synthesis of fatty acids from malonyl coenzyme A. These fatty acids are the components of yeast membranes. The main fatty acids produced by yeast during alcoholic fermentation are palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), and oleic acid (C18:1). Fatty acids with shorter carbon chains (caproic acid (C6:0), caprylic acid (C8:0), and capric acid (C10:0)) are also produced by this pathway, although they may also be generated by the oxidation of longer chain fatty acids. These fatty acids are clearly the preferred precursors for the synthesis of esters during fermentation. Most of these fatty acids remain localized within the cell rather than being released into the surrounding medium. Nevertheless, due to their small size and strong hydrophobicity, short-chain fatty acids may be excreted by the yeast, and many of these compounds act as autoinhibitory factors.

3.26.2.1.4 Higher alcohols and keto acids

Most of the higher alcohols produced by yeast are derived directly from the carbon skeleton of the amino acids assimilated during alcoholic fermentation. The amino group is removed by transamination, and the corresponding keto acid is then decarboxylated to form an aldehyde (**Figure 1**). This aldehyde is reduced by the alcohol dehydrogenase enzyme, characteristic of alcoholic fermentation, leading to the formation of a higher

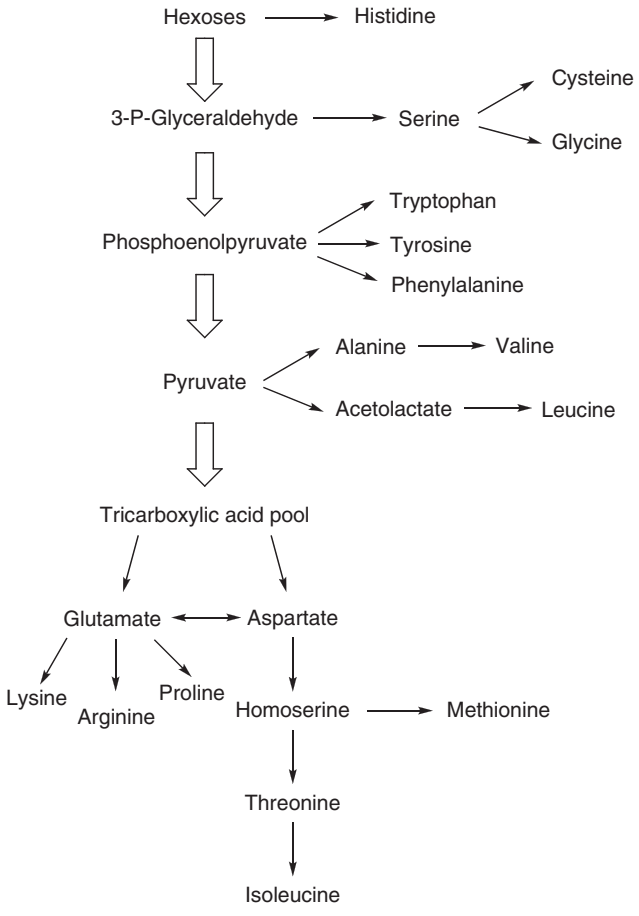


Figure 1 Carbon filiations between amino acids and carbon metabolism intermediates during amino acids synthesis in fermenting yeast.

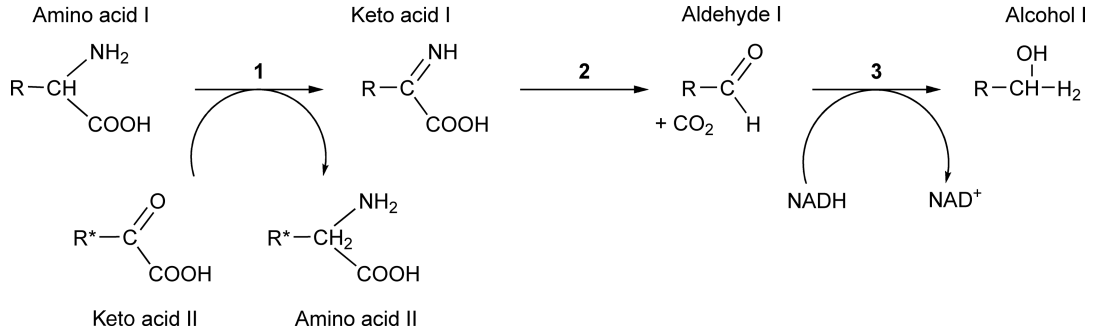


Figure 2 Schematic representation of the biosynthesis of higher alcohols from amino acids and related alpha-keto acids in fermenting yeast. (1): transamination, (2): decarboxylation, (3): dehydrogenase reaction.

alcohol with one carbon atom less than the starting amino acid. However, higher alcohols may also be generated by other pathways, as such alcohols are synthesized even in the absence of amino acid catabolism (during the stationary phase, in particular). In such cases, a keto acid containing one atom more than the corresponding higher alcohol is decarboxylated and reduced during the biosynthesis of an amino acid. Another pathway for the biosynthesis of branched-chain higher alcohols has been demonstrated that involves a direct action of enzymes specific for branched-chain amino acids.[7] The main higher alcohols synthesized during alcoholic fermentation are n-propanol, isobutanol, amylic and isoamylic alcohols, and phenylethanol. The concentrations of these compounds vary between 50 and 300 mg l^{-1} in enological conditions.[8] There is therefore a direct relationship between a higher alcohol and the amino acid from which it is derived (**Figure 2**). It should be noted that clarification or any other treatment of the must aiming to decrease the amount of insoluble material (such as proteins in particular) may influence the final higher alcohol content of wine.[9]

During the synthesis of higher alcohols from amino acids, transamination reactions generate a number of different carbon skeletons, including keto acids (**Figure 3**). These keto acids are then excreted into the external medium, or are decarboxylated and then reduced to form the corresponding higher alcohol. At the beginning of fermentation, when the amino acids required have not yet been taken up by the yeast, the synthesis of keto acids and the corresponding higher alcohols is based on sugar metabolism via pyruvate.[10,11]

3.26.2.1.5 Esters

Esters, produced during alcoholic fermentation, constitute the largest group of compounds affecting the organoleptic quality. They are generated by an enzymatic reaction involving coenzyme A fatty acyl derivatives and free alcohols.[12] The formation of acetate esters during fermentation is directly linked to the level of alcohol

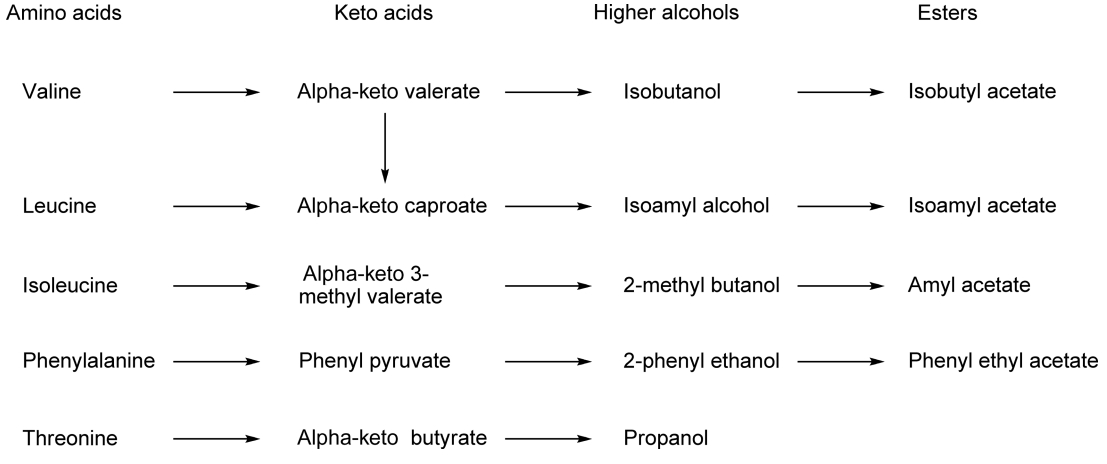


Figure 3 Relationship between amino acids, alpha-keto acids, higher alcohols and esters.

acetyltransferase activity in the cell and to the concentrations of the respective alcohol precursors.[13] In general, the presence of unsaturated fatty acids in the must stimulates cell growth during anaerobiosis and reduces the rate of production of short-chain fatty acids and the corresponding ethyl esters.[14]

3.26.2.1.6 Acetoin, 2,3-butanediol, and diacetyl

Acetoin is produced by yeast during the alcoholic fermentation process. It both affects the bouquet of the wine and, in particular, is a precursor for the biosynthesis of 2,3-butanediol and diacetyl. The formation of 2,3-butanediol can contribute to the overall aromatic balance of the wine,[15] whereas diacetyl is considered to be an organoleptic defect, particularly due to its characteristic odor and low perception threshold.[16] In general, *Saccharomyces* yeasts – unlike apiculate yeasts (*Kloeckera*, *Hanseniaspora*) and yeasts of other genus (*Zygosaccharomyces*) – produce small quantities of acetoin.[17,18] The concentrations of acetoin normally produced by *S. cerevisiae* during alcoholic fermentation vary from levels below the detection threshold to several tens of milligrams per liter, with certain strains able to produce up to 200 mg of acetoin per liter in exceptional conditions. The mechanism by which *S. cerevisiae* generates acetoin during fermentation remains unclear. Three synthetic pathways have been proposed,[18] all based directly on the decarboxylation of pyruvate to form acetaldehyde, an important step in the production of ethanol through glycolysis:

- The first pathway is based on the synthesis of alpha-acetolactate by *S. cerevisiae*. The synthesis of this compound is dependent on very low concentrations of oxygen ($<0.2\ mg\ l^{-1}$) and is linked to catabolism of the amino acids threonine and lysine.[19] Alpha-acetolactate is then decarboxylated to form acetoin.[20]
- The second pathway involves the direct condensation of two molecules of acetaldehyde.[21] This synthetic pathway is therefore independent of the synthesis of other intermediates.
- The third synthetic pathway starts with the condensation of acetaldehyde and acetyl coenzyme A to form diacetyl, which is then reduced to form acetoin.[21] The operation of this pathway is increased by aeration of the medium.[22]

In general, *S. cerevisiae* rapidly reduces diacetyl to generate acetoin and then 2,3-butanediol. This rapid metabolism, in particular, accounts for the small quantities of diacetyl produced during the growth phase of yeast.[19] The formation of acetoin depends principally on the NAD^+/NADH ratio and on the intracellular concentration of pyruvic acid.[23]

3.26.2.1.7 Sulfur compounds

Yeasts do not normally produce large amounts of sulfites (SO_2) in enological conditions (between 10 and $30\ mg\ l^{-1}$),[24] but certain strains can produce over $100\ mg\ l^{-1}$.[25,26] The composition of the medium may affect yeast metabolism, resulting in SO_2 overproduction, particularly in conditions of high sugar concentration,[27] or when the concentration of sulfate (SO_4^{2-}) or pantothenic acid is too high.[28]

Hydrogen sulfide (H_2S) is normally produced in small amounts during fermentation.[29] However, certain strains of *S. cerevisiae* can produce up to $1\ mg\ l^{-1}$.[25] This compound is the final product in the sulfate reduction chain, and is also an intermediate in the synthesis of sulfur-containing amino acids. The production of H_2S is increased by high temperatures or high pH during fermentation.[30] H_2S production may also be stimulated by a strong assimilable nitrogen deficiency.[31] The addition of sulfite (SO_2) as an antimicrobial agent may also provide a source of sulfur for yeast and may contribute to the production of significant amounts of H_2S.[32] Yeast is also able to form H_2S from sulfur itself, which is used as an antifungal treatment by vine growers.[33]

Dimethyl sulfide (DMS; see Section 3.26.3.1.2) is a thioester produced from *S*-methylmethionine (SMM) or dimethyl sulfoxide (DMSO) of grape, but only from SMM at prefermentation stages.[34] During fermentation, *S. cerevisiae* yeast is able to reduce DMSO to DMS,[35] and some yeast and lactic acid bacteria strains can use SMM as a sulfur source. Nevertheless, because of its high volatility, DMS produced by yeasts is mainly removed with the carbon dioxide produced by the alcoholic fermentation. DMS concentrations in bottled wines are generally between 5 and $50\ \mu g\ l^{-1}$.[36]

Other sulfides (including diethylsulfide and dimethyldisulfide in particular) are formed during fermentation, at concentrations of up to 0.5–$2\ \mu g\ l^{-1}$.[36] These low concentrations probably result in the oxidation of certain mercaptans, or the microbiological degradation of certain sulfur-containing pesticides.

Carbon sulfides and disulfides are formed in wine at concentrations of less than 2 $\mu g\,l^{-1}$. They are mostly generated metabolically, but may also be produced by the degradation of certain fungicides by *S. cerevisiae*.[37]

Mercaptans are sulfur-containing molecules principally generated metabolically: methylmercaptan is formed through the metabolism of methionine[38] and ethylmercaptan is formed through hydrogen sulfide metabolism.[39] These molecules are extremely reactive and are readily oxidized to form disulfides.[40]

Thioesters are principally synthesized from mercaptans, but may also be generated by the enzymatic degradation of methionine.[41] Their concentrations do not exceed 5–20 $\mu g\,l^{-1}$.[36]

3.26.2.1.8 Effect of yeast strain on wine organoleptic characteristics

There is increasing evidence to suggest that yeast has an important effect on the production of aromatic compounds, but this effect is difficult to estimate. Indeed, the synthesis of these molecules is influenced not only by the strain, but also by the composition of the must and the fermentation process. Different and complex interaction mechanisms may be involved, generating contradictory observations, depending on the conditions used. Nevertheless, indisputable evidence for the activity of certain strains has been obtained:

- Various cryotolerant strains have been selected for fermentation at very low temperatures (10–12 °C). Under such conditions, these strains produce particularly large quantities of the esters 2-phenyl ethanol and 2-phenyl acetate, affecting the fruity character of wines.
- Different yeasts produce different amounts of volatile varietal thiols, which are strongly implicated, for example, in the aromatic character of Sauvignon wines.[42] However, even in these cases, the factors operating during fermentation (molecular precursors, metabolic pathways implicated, and the effect of operative variables) have not been fully elucidated.[43,44]

3.26.2.1.9 Effect of temperature on wine organoleptic characteristics

Generally, very low temperatures (10–15 °C) during fermentation promote the production of aromatic compounds, such as esters. Such temperatures can therefore be used to increase the fruity character of wine. Recent studies have also demonstrated the importance of temperature in terms of the release of varietal aromatic compounds, including thiols in particular. They have shown that, whatever the strain used, the production of these compounds is greater at high temperatures (20 °C) than at low temperatures (13 °C).

3.26.2.2 Products of Bacterial Primary Metabolism (Malolactic Fermentation)

Malolactic fermentation, whether spontaneous or induced by inoculation with lactic acid bacteria, leads to the conversion of L(−)-malic acid in wine to L(−)- or D(−)-lactic acid, depending on the *Lactobacillus* species involved and the substrates degraded. For each gram of malic acid metabolized, 0.67 g of lactic acid and 0.33 g (165 ml) of CO_2 are produced. In parallel, lactic acid bacteria can also break down 0.3–2 g of residual sugar per liter to form about 100 mg of D(−)-lactic acid per liter.[45] This is accompanied by a whole range of physicochemical and sensory characteristics, representing another tool for modifying the final style of the wine produced.

3.26.2.2.1 Malolactic fermentation process

Spontaneous malolactic fermentation (using indigenous lactic acid bacteria) may begin only after a very long latency period (from a few weeks to several months). This phase is extended by low pH of the wine. *Oenococcus oeni* is the preferred species at low pH values, and *Lactobacillus* and *Pediococcus* are the predominant species at high pH. During malolactic fermentation, the bacterial population often reaches between 10^6 and 10^7 cells ml^{-1}, with only 10^3–10^4 cells ml^{-1} present at the end of alcoholic fermentation. After the exponential growth phase, the bacterial population declines rapidly (due to high temperatures and concentrations of SO_2).

The adaptation phase for the lactic acid bacteria associated with spontaneous malolactic fermentation is thus at high risk of the growth of unwanted microorganisms, particularly in wines with a low active SO_2 content or high levels of volatile activity. The use of a lactic starter has been developed for such cases, as a starter is already adapted to the wine-making environment,[45] whether at the beginning, during the course of, or at the end of alcoholic fermentation. In some cases, spontaneous malolactic fermentation in wines with a high pH value and low SO_2

content can generate unpleasant tastes and odors, characterized by sulfur in the nose, phenol or moldy odors, and a bitter and oily aftertaste. These defects are never observed following inoculation with selected bacteria.

3.26.2.2.2 Effects on wine characteristics

Malolactic fermentation, through deacidification, generally leads to a decrease in titratable acidity of 1.0–4.6 g l^{-1} (equivalent to tartaric acid) and an increase in the pH of wine, between 0.1 and 0.45 units (generally 0.1–0.25 pH units). The higher pH may result in higher microbial instability. However, malolactic fermentation may also be considered advantageous in terms of the microbiological stability of wine. Indeed, several contaminating bacterial species may consume malic acid. In addition, lactic acid bacteria use nutrients during their growth (amino acids, peptides, and vitamins), thus reducing their bioavailability.

Malolactic bacteria also produce by-products that either directly influence sensory perception or interact with bitter or astringent substances already present in the wine. These effects are highly dependent on the strains of lactic acid bacteria used and may, in some cases, have negative effects, masking the fruity or varietal characters, or, alternatively, may result in the greater development of these characters.[45] Diacetyl is a by-product of citric acid metabolism in a number of lactic acid bacteria. It is generally considered to have a negative effect on sensory characters. However, this compound is generally eventually reduced by yeast (even during the aging of wine on lees) or lactic acid bacteria, forming acetoin and 2,3-butanediol, which have virtually no sensory effect on wine at normal concentrations.

3.26.3 Chemistry of Aroma Compounds in Wine

More than a thousand aroma compounds have been identified in grapes and wines. These compounds are formed throughout the production process of grapes and along the elaboration process of wines. For better understanding, and thanks to the works of Drawert,[46] aromas are classified considering the period of their formation. Thus, for simplicity and coherence, this chapter will focus on three groups, in consideration of the previous classification:

- varietal compounds, either free forms or those obtained from precursors present in grapes,
- compounds formed during winemaking that are essentially fermentative compounds, and
- compounds formed during the maturation of wines.

3.26.3.1 Varietal Compounds

Varietal compounds present in grapes are subdivided into two classes: those that are found in a free form, which means volatile and directly perceptible by the olfactory mucosa, and those that are formed during the biotechnological process of winemaking from specific precursors present in grapes after one or two cleavages.

3.26.3.1.1 Aroma compounds in free form

Volatile free compounds of grapes, susceptible of giving a typicity to the wine aroma, are found only in a few varieties and correspond to three groups: monoterpenic compounds, 2-alkyl-3-methoxypyrazines, and rotundone.

3.26.3.1.1(i) Monoterpenic compounds These compounds (**Figure 4**) include a few compounds with cyclic skeletons such as α-terpineol (**1**) and mainly compounds with acyclic skeletons such as linalool (**2**), nerol (**3**), hotrienol (**4**), geraniol (**5**), and citronellol. They are the characteristic of 'muscat-like grape' varieties and of some Alsacian or Iberic varieties, such as Gewürztraminer, Alvarinho, or Loureiro, in which they are responsible for the floral notes.[47] Other monoterpenes, with the same carbon skeletons but with higher degrees of oxidation, polyols, and acids, are also present in these grape varieties, but most of them are odorless. Odorant monoterpenes are mainly found in the solid parts of the grapes; thus, wine-making operations contributing to the contact between juice and berry skin (macerations) or those that destroy the structure of the cell walls (enzymes, carbonic maceration) help to extract them.

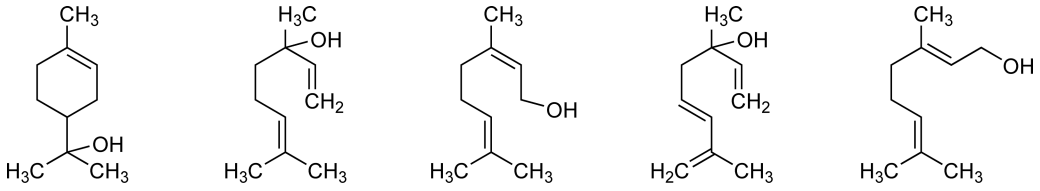


Figure 4 Main terpenols of grapes and wines (**1** = α-terpineol, **2** = linalool, **3** = nerol, **4** = hotrienol, **5** = geraniol).

These compounds along with many dioxygenated terpenes are also present in glycosylated forms (see Section 3.26.3.1.2).

3.26.3.1.1(ii) 2-Alkyl-3-methoxypyrazines 2-Alkyl-3-methoxypyrazines (**Figure 5**), which are disubstituted dinitrogenated aromatic heterocycles, exhibit green pepper, aristolochia, green pea, and asparagus aromas. They were initially identified in Cabernet Sauvignon,[48] and their contents, a few nanograms per liter, are close to the olfactive perception threshold. They can therefore type grape aromas of that variety and of other grape varieties such as Sauvignon, Cabernet Franc, and Merlot in which they are detected. They are considered as unfavorable to wine aromas, and hence wineries try to minimize their content. This can be achieved by some vine management techniques (in particular leaves stripping), by picking at higher maturation degree, and by using wine-making processes limiting their extraction from the berry skins, or maximizing their heat degradation (heated prefermentation maceration, flash pasteurization, etc.).

3.26.3.1.1(iii) Rotundone Another free varietal compound, rotundone (**6**; **Figure 6**), presenting notable pepper aromas was recently identified in Australian Syrah grapes.[49] This sesquiterpene with a guaiane skeleton, also identified in white and black pepper, is present in these Syrah grapes in low amounts, but higher than its olfactive perception threshold in red wine (16 ng l^{-1}). Ongoing research will help define its importance in enology.

3.26.3.1.2 Aroma compounds generated from precursors in grape

3.26.3.1.2(i) C_6 compounds These compounds, with vegetal and freshly cut grass aromas, are formed in the must before fermentation, by the sequential action of grape lipase, lipoxygenase, and alcohol dehydrogenase on the lipidic precursors of linoleic and linolenic acids.[50] At this stage, the aldehydic, hexenal, and hexanal forms, which are the most odorant, are dominant. During fermentation, these forms are mainly reduced by yeasts to alcohol, principally hexanol, which is not very odorant, and hexenol, which is more odorant but found in minor quantities. Their management is achieved by removing leaves from the crop and by limiting trituration during transfer and mixing of the press content.[51]

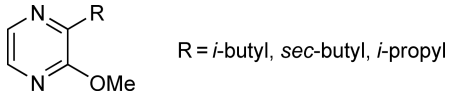


Figure 5 2-Alkyl-3-methoxypyrazines in grapes.

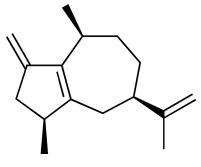


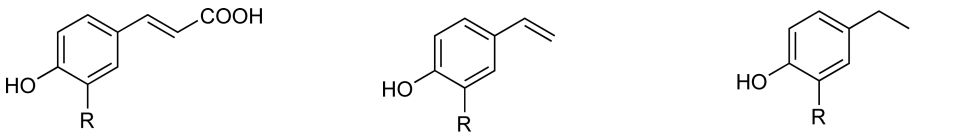
Figure 6 Rotundone (**6**).

These compounds also exist in glycosylated forms in grape berries, at levels that are much lower than those formed during prefermentation operations, but that indicate the existence of an *in vivo* biogenesis way.[47]

3.26.3.1.2(ii) Volatile phenols The major compounds of this class (**Figure 7**) are 4-vinylphenol and 4-ethylphenol, and 4-vinylguaiacol and 4-ethylguaiacol. 4-Vinylphenol (**9**) and 4-vinylguaiacol (**10**) are formed by decarboxylation of hydroxycinnamic acids (see Section 3.26.4.1.1), that is, *p*-coumaric acid (**7**) and ferulic acid (**8**), respectively, by yeasts. The quantities formed in red wines are very low compared to those found in white or rose wines, because decarboxylase is inhibited by catechic tannins.[52] In white and rose wines, these derivatives never reach significant amounts, also because they are rapidly degraded in this medium, mainly with the addition of ethanol to the vinyl group.[53] They are mainly responsible, in case their influence becomes perceptible, for masking the fruity notes or for giving aroma defects known as a phenolic taste.[52] Regarding ethylic derivatives (**11, 12**), they are perceptible only in wines that have been contaminated by yeasts of the *Brettanomyces* genus, which, contrary to *S. cerevisiae* yeasts or wine lactic acid bacteria, contain vinylphenol reductase, necessary for the reduction of vinylic derivatives. These ethylphenols are then responsible for a pronounced phenolic taste.

These vinylic derivatives, along with other phenolic compounds, are also present in glycosylated forms (cresols, methyl salicylate, guaiacol, vanilla and derivatives, eugenol and derivatives, zingerone, frambinone, and syringaldehyde and derivatives), but considering their concentrations, they contribute only rarely to the aromas of young or old wines.

3.26.3.1.2(iii) Volatile compounds coming from glycoconjugates Highlighted through Cordonnier and Bayonove,[54] glycosidic precursors contain a carbohydrate part attached by an oside linkage to a volatile compound, the aglycone, which is odorant or nonodorant. The carbohydrate part of grape glycosides, presenting a small variety, allows their classification into four groups (**Figure 8**): β-D-glucopyranosides (**13a**), monoglucosides that contain only glucose, and three classes of diglycosides (**13b, 13c,** and **13d**).[47]



R = H: *p*-coumaric acid (**7**) R = OCH_3: ferulic acid (**8**)
R = H: 4-vinylphenol (**9**) R = OCH_3: 4-vinylguaiacol (**10**)
R = H: 4-ethylphenol (**11**) R = OCH_3: 4-ethylguaiacol (**12**)

Figure 7 Hydroxycinnamic acid precursors and volatile phenols in wines.

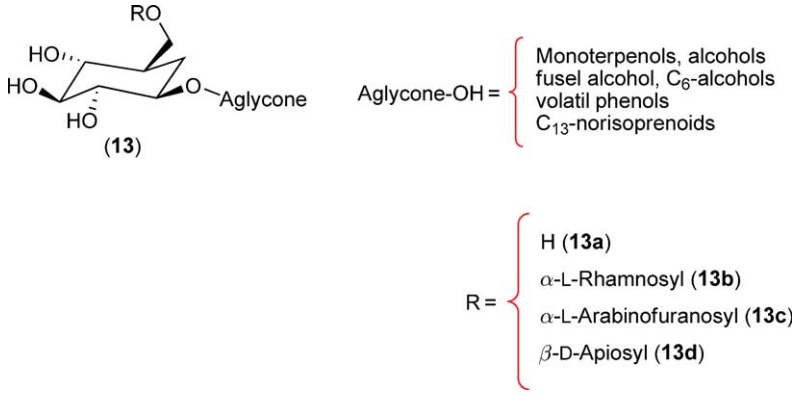


Figure 8 Glycosidic aroma precursors in grapes.

The formation of odorant compounds implies the release of odorant aglycones in wine by acid-catalyzed hydrolysis. The nonodorant aglycones can undergo acid-catalyzed chemical rearrangements to form odorant compounds. These transformations take place mainly during prefermentation steps and fermentation.[55,56] In wines without residual sugars, grape glycosides can also be hydrolyzed more rapidly enzymatically using appropriate exogenous glycosidases, such as glycosidasic enzyme preparations of filamentous fungi.[50]

Recent studies call into question the hypothesis on the negligible role of yeasts and lactic acid bacteria in the formation of aroma compounds from glycoconjugates and in particular on their global sensory influence on wine.[57,58]

Thus, the many volatile compounds coming from glycoconjugates belong to the main grape volatile compound classes: C_6 compounds, fusel alcohols, volatile phenols, monoterpenols, and C_{13}-norisoprenoids. The first three classes have been described above. Thus, only the last two classes will be considered in the following paragraphs.

Glycosylated monoterpenes differ from free monoterpenes described in Section 3.26.3.1.1 only in their proportion in different grape varieties. Glycosylated monoterpenes are generally more abundant than the free form, in particular dioxygenated monoterpenes, which are dominant in nonaromatic grape varieties.

Bayonove[47] recalls that C_{13}-norisoprenoids identified in grapes are mainly di- or trioxygenated and glycoconjugated. They are formed in the berries by oxidative degradation of C_{40}-isoprenoidic precursors, namely carotenoid pigments involved in photosynthetic activity in the berry. The key enzyme of this formation was recently identified and the primary products of the cleavage resulting from its action can later be transformed by oxidases or reductases and finally glycosylated by glycosyl transferases.[59]

These hydrolytic and rearrangement processes lead to many volatile compounds, but only around 10 can reach contents close to or above the olfactive perception thresholds in wines of non-Muscat-like varieties. The best known are linalol, rose oxide, 1,8-cineol, wine lactone, eugenol, guaiacol, zingerone, methyl salicylate, β-damascenone, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), and (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB).

It should be recalled that ionones are not formed from the degradation of glycosidic precursors, but from the degradation, at prefermentative and fermentative stages, of one of the most abundant carotenoids in grapes, β-carotene.[60]

3.26.3.1.2(iv) Furaneol This furanic derivative is a compound with a characteristic smell of caramel or strawberry and is formed by the degradation of sugars in young and old *Vitis vinifera* wines. Its formation, mainly in young wines, results from glycosidic precursors, because the chemical degradation of sugars cannot take place in such conditions. Indeed, this compound was identified among aglycones from grape glycoside extracts of Merlot, Cabernet Sauvignon, and Syrah.[61,62] Nevertheless, no furaneol glycoside structure was found in grapes, whereas the glucoside and its malonate derivative have been identified in several fruits, including strawberry. A homologue of furaneol, homofuranel, was also identified in many wines.[61,63,64] Its contribution to aromas of Grenache rose wine as a fruit flavor enhancer in synergy with furaneol was studied,[64] but its formation mechanisms are not known.

3.26.3.1.2(v) Varietal sulfur compounds Varietal sulfur compounds are formed, during fermentation, from S-conjugates of grape cysteine (**14**; **Figure 9**). *Saccharomyces cerevisiae* yeasts degrade these precursors by their S-lyase activity, giving rise to varietal thiols, which are very odorant compounds. Four varietal thiols are presently known to be detected in wines, 4-methyl-4-mercaptopentan-2-one (4MMP), 4-methyl-4-sulfanyl-pentan-2-ol (4MMPOH), 3-mercaptohexanol (3MH), and 3-mercaptohexyl acetate (Ac3MH). These thiols derive from three cysteine conjugates that have been identified in grape berries.[65,66] Moreover, a conjugate of 3-mercaptohexanol to glutathione (**15**) has been identified in Sauvignon Blanc musts, and may also be a precursor of 3-mercaptohexanol.[67]

Even though the contents of 4MMP, 3MH, and Ac3MH are around tens or hundreds of parts per trillion (ppt) in wines, they give Sauvignon wines characteristic notes of yew, grapefruit, and passion fruit, because their thresholds of perception are very low in wines, that is, 3, 60, and 4 ng l^{-1}, respectively. 3-Sulfanyl-hexanol and its acetate are also present in many grape varieties, which is not the case for 4-methyl-4-sulfanyl-pentan-2-one. The ubiquity of 3MH is in part due to another way of formation of this volatile thiol during fermentation, not

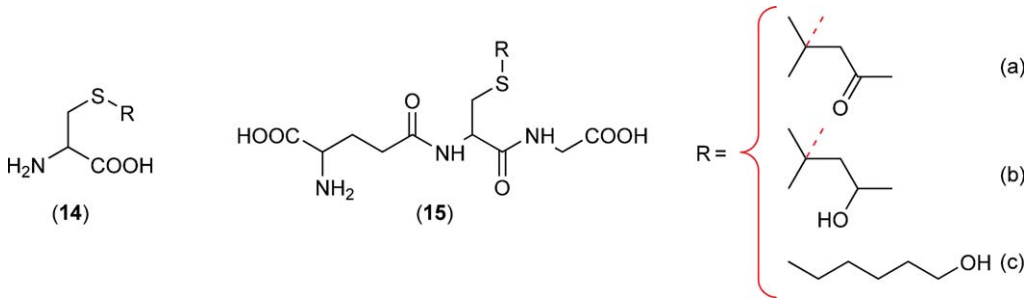


Figure 9 Precursors of volatile thiols present in grapes (**14** = cysteinyl conjugates, **15** = glutathionyl conjugates, a = 4-methylpentan-2-one, b = 4-methylpentan-2-ol, c = hexanol).

from S-conjugates of grape cysteine, but from the addition of sulfur sources to (*E*)-2-hexenal in musts during fermentation.[68] Furthermore, this mode of formation has been demonstrated under the same conditions for 4-methyl-4-sulfanyl-pentan-2-one via mesityl oxide, but this last molecule and its hydrate have never been identified in grapes.

3.26.3.1.2(vi) Dimethylsulfide Dimethylsulfide is a light compound highlighted by Du Plessis and Loubster[69] in wine and has an olfactive perception threshold of around 25 $\mu g\,l^{-1}$.[70] Its contents in young wines are often lower than the olfactive perception threshold, but can reach 900 $\mu g\,l^{-1}$ in evolved wines.[71] Recent data show that this compound is more often perceived positively, but its contribution to wine aroma is complex. At high concentrations in well-evolved wines, mainly those coming from late harvested white grapes, this compound brings truffle notes. At lower concentrations, it contributes to red wine fruity notes, in particular by a potentialization effect.[34,35,72]

Varietal DMS is produced during fermentation from SMM and dimethylsulfoxide of grape, but only from SMM at prefermentation stages.[34,71,73]

During fermentation stages, *S. cerevisiae* yeast is able to reduce DMSO to DMS,[35] and some yeast and lactic acid bacteria strains can use SMM as a sulfur source. Nevertheless, because of its high volatility, varietal DMS produced by yeasts is for the most part removed with the carbon dioxide produced by the alcoholic fermentation, and by this way, DMS concentrations in bottled wines are generally very low.

On the other hand, postfermentation, varietal DMS contents increase with time and heat during maturing in bottles, to reach contents of around 1 $mg\,l^{-1}$, but at this stage, DMS is only produced from SMM by a purely chemical reaction.[34,71]

3.26.3.2 Aroma Compounds Generated during Winemaking

3.26.3.2.1 Fusel alcohols, ramified aldehydes, and ramified acids

Although some of these compounds exist in glycoside form in grapes, the contents of fusel alcohols and the corresponding ramified fatty acids are much lower than those produced by yeast secondary metabolism during fermentation, as described in Sections 3.26.2.1.4 and 3.26.2.1.5 The ratio of ramified fatty acids to fusel alcohols depends on the redox level of yeasts, and in enology this ratio is on the order of a few percent.

Ramified aldehydes, the intermediate products of fusel alcohols or ramified fatty acids, are present in traces in young wines after fermentation.[74]

Generally, conditions that slow down fermentation are unfavorable for the formation of fusel alcohols.[70] Thus, a high levels of ammonium ions, a low fermentation temperature, and a low must pH lead to a decrease in fusel alcohols content. As for the influence of the must amino acid contents, some report a reduction in the formed superior alcohol contents with a reduction in the amino acid contents,[75] while others report the contrary.[76]

3.26.3.2.2 Linear fatty acids

These fermentative compounds, produced by yeasts in far larger quantities than the ramified fatty acids seen above, are associated with the lipid metabolism. The most abundant fatty acids of linear structure and with an even number of carbon atoms are produced by yeasts as described in Section 3.26.2.1.3. Like ramified fatty acids, they have unpleasant odors, but only acetic acid, by far the most abundant, has concentrations in wines that are above the olfactive perception threshold.[70]

3.26.3.2.3 Esters

Fermentative esters are mainly classified into three groups. The first group includes ethylic esters of linear fatty acids, associated with the lipid metabolism. The other two groups are associated with the nitrogen metabolism and include on the one hand fusel alcohol acetates and on the other hand ethylic esters of ramified fatty acids. Biogenetically, they all derive from the action of water, ethanol, and superior alcohols on the corresponding Acyl-*S*-CoEnzyme A, catalyzed by yeast esterases (see Section 3.26.2.1.5).

Fermentation conditions favoring the formation of high contents of linear fatty acid esters are the absence of oxygen, the clarification of musts, and temperatures between 15 and 18 °C. In the same way, anaerobic conditions are favorable for the formation of fusel alcohol acetates by stimulating alcohol acetyltransferases, but the availability of required quantities of alcohols would also lead to an increase in acetates. Nevertheless, as explained above, aerobic conditions are favorable for the formation of ramified fatty acids, and would also be favorable to their ethylic esters. However, the contents of these esters are very low in young wines.

Acetic acid and ethyl acetate are, by far, the most abundant fatty acid and ester, but their perception thresholds are also very high. Despite their unpleasant smell, these two compounds are necessary in the global wine aroma with variable optimal contents according to the wine, but with limit values neighboring 600 mg l^{-1} for the former and 100 mg l^{-1} for the latter. This contribution is modulated by the type of wine, as indicated by Ferreira *et al.*,[77] and the relative proportions of esters give complex effects of synergies and disguises. These synergy effects between esters were highlighted in a recent study on cold prefermentative maceration for red and rose wines with Merlot and Cabernet Sauvignon in order to explain the genesis of fruity notes, enhanced in the corresponding wines.[78]

3.26.3.2.4 Fermentative sulfur compounds

These volatile sulfur compounds are formed in low quantities by yeasts during fermentation, but their concentrations generally progress during maturation on lees. Their formation during fermentation is described in Section 3.26.2.1.7.

3.26.3.3 Reactions Occurring during Wine Aging

3.26.3.3.1 Chemical reactions affecting ester composition

The degradation and the transformation of wine fruity aroma is a major technological notion in the evolution of wine aroma. This mechanism is often linked to reactions affecting major fermentative compounds, namely esters, which constitute the vinous note of wines (see Section 3.26.3.2.3).

Fusel alcohol acetates and ethylic esters are formed in young wines in high concentrations (a few micrograms per liter to a few milligrams per liter), but with esters/acids ratios higher than those of the chemical esterification/hydrolysis balance. Consequently, these esters are gradually hydrolyzed during storage.

For acetates, the reaction velocity depends on the medium, mainly its reactant concentration, pH, and temperature. The velocity is determined by the pH and is almost proportional to the proton concentration in the wine.[79–81]

For fatty acid ethylic esters, the hydrolysis velocity varies proportionally to the molecular mass, which explains the rapid hydrolysis of heavy esters.[79]

Ethylic esters of ramified fatty acids are formed in low amounts by yeasts in young wines (rarely above tens of micrograms per liter), and lower than the concentrations corresponding to the chemical esterification/hydrolysis balance. Thus, as the wine ages, residual ramified fatty acids are chemically esterified by the main alcohol in wine, ethanol, to form the corresponding ethylic esters.[82]

Thus, the first group of esters mainly exert a favorable influence on young wine aroma. They become less abundant during storage, whereas the ethylic esters of ramified fatty acids accumulate, partly compensating for the loss of the first group and leading to modifications in the perceived fruity notes.

3.26.3.3.2 Modification of varietal compounds

Some varietal compounds like 3-alkyl-2-methoxypyrazines are particularly stable during both fermentation and wine maturation.[47] In contrast, other compounds never stop undergoing modifications and rearrangements during wine maturation. This is the case for monoterpenic compounds and C_{13}-norisoprenoids in their free or linked forms. Generation of aglycones by hydrolysis of the corresponding glycosylated forms has been presented above (see Section 3.26.3.1.2). We will develop here the modifications affecting monoterpenols and C_{13}-norisoprenoids in their free or linked forms in wines during maturation.

Monoterpenol modifications are the result of many reactions in acid medium – isomerization, ring closure, hydration, dehydration, and oxidation[83] – and these have been described only in model solutions. They seem to affect only free terpenols and not those that are still in glycosylated form. Globally, wine cyclic terpenol contents progress at the expense of young wine monoterpenols.[53,56,83–87] The modification reactions are accelerated at low pH[88] and can be slowed down by storing wine at low temperature (<10 °C).[83,86]

C_{13}-norisoprenoids undergo acid-catalyzed reactions analogous to those observed with terpenic compounds to form compounds that are not present in grapes or young wines. Within these compounds, we can mention isomers of vitispirane (eucalyptus odor), theaspirane (tea odor), β-damascenone (rose odor and exotic flower odor), and TDN (kerosene odor).[84,85] According to studies in model solutions, each of these odorant compounds could be formed in wines by acid catalysis reactions from several norisoprenoidic precursors that are glycosylated and/or nonglycosylated.[89–92] These reactions can take place on the free aglycone or directly on the glycoside. The proportions of the formed compounds are then different, as demonstrated by Skouroumounis *et al.*[90]

Quantitative differences between grape varieties with respect to norisoprenoidic precursors could explain the origin of the typical aroma of some wines. In particular, this is the case of old Riesling wines with characteristic petrol notes, due to the presence of TDN. TDN precursors are present in musts of this grape variety in particularly high concentrations.[85,87,88]

3.26.3.3.3 Reactions occurring during oxidative storage

While most wines undergo a classic maturation in bottles under reductive conditions, some wines, and mainly those having been added with alcohol (i.e., fortified wines), undergo an oxidative maturation, which confers a particular aroma. This oxidative maturing can involve only chemical reactions due to the presence of oxygen, as for wines from Madera, Porto, and some French fortified wines such as Maury, Banyuls, and Rivesaltes. This maturing can nevertheless also involve biochemical reactions, resulting from the development of veil-forming yeasts called 'oxidative yeasts', as in wines from Xeres, Jura Vin Jaune, and Vernaccia di Oristano from Italy and Tokay from Hungary.

During oxidative storage, aldehydes increase significantly, on the one hand due to oxidation of alcohols by a coupled oxidation mechanism involving air oxygen and wine di- and trihydroxyphenols[93] and, on the other hand, due to their biosynthesis by veil-forming yeast. This is the case for acetaldehyde present in these types of wines at concentrations above its perception threshold, so that it contributes to the typicity of Fino sherries[94,95] and Jura Vin Jaune.[96]

Another major reaction observed in this type of maturing is acetalization between aldehydes and alcohols or polyols such as glycerol. This reaction is privileged because of the high concentrations in reactants. Several acetals were detected in these wines, often at concentrations higher than those in nonoxidized wines. 1,1-Diethoxyethane coming from the reaction between acetaldehyde and ethanol is the major acetal because of the high concentrations in reactants. This compound reaches high concentrations in Xeres wines[97] and Jura Vin Jaune[98] that are above its perception threshold. It is the only acetal known to have an olfactive impact in wines.[70] The other identified acetals, mainly dioxolanes and dioxanes coming from acetaldehyde and glycerol, found in Port wines,[99,100] Xeres wines,[97] Jura Vin Jaune,[101] and Grenache fortified wines[102] do not contribute to wine aroma because they are odorless.[101–103]

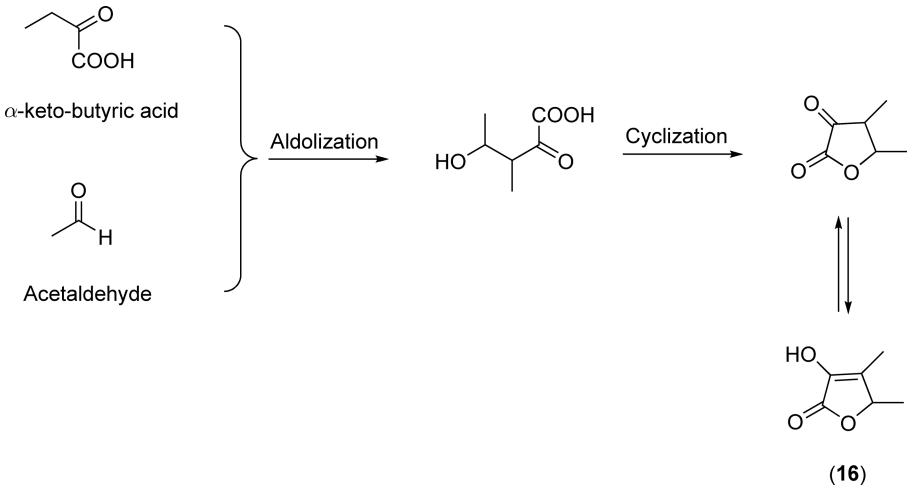


Figure 10 Formation of sotolon (**16**) in wines as described by Guichard *et al.*[104]

Among lactones also formed during this type of maturing, to this day only one is known to have an important impact on aroma: sotolon (3-hydroxy-4,5-dimethyl-2(5*H*) furanone) (**16**; **Figure 10**). Sotolon has an odor close to nut and curry, and has a low perception threshold (10 $\mu g\,l^{-1}$). This compound is present in high amounts in wines matured under a veil of yeasts: Xeres, Tokay from Hungary, and Jura Vin Jaune.[104,105] This compound is formed by aldol condensation of acetaldehyde and α-ketobutyric acid.[106,107] The latter molecule comes from deamination of L-threonine by veil-forming yeasts.

3.26.3.3.4 Reactions occurring during storage in oak barrels

Maturing in oak barrels is considered as essential for the quality of premium wines.

In addition to the reactions due to polyphenols, during maturation in oak barrels, some wood extractable volatile compounds diffuse into wines with different kinetics depending on the compound. The botanical and geographical origin of the oak, the manufacturing conditions (drying and burning), and the use of the barrels can influence the volatile extractable molecules and the wood macromolecules in both quality and quantity.[106–109]

Several oak volatile phenols are transferred to wine during maturation in barrels, such as guaiacol, 4-methylguaiacol, smelling like burnt wood, 4-propylguaiacol, 4-ethyl-2,6-dimethoxyphenol, eugenol, some phenylketones, in particular butyrovanillin, and some aldehydes, mainly vanillin and syringaldehyde.[107,110,111] The extraction of most of these compounds is almost complete after 6 months of storage. The first four compounds are specific wood components that are virtually not found in wine. Others such as vanillin, syringaldehyde, and eugenol are found as traces in wine but their concentrations can significantly increase with the wood contribution.

During maturation in oak barrels, wines are regularly enriched in β-methyl-γ-octalactones,[107] compounds specific to oak wood and with a coconut odor.[112] These molecules give a woody note to wine. Nevertheless, when the concentration of *cis* and *trans* (1:1) forms of β-methyl-γ-octalactones exceeds the 235 $\mu g\,l^{-1}$ threshold in red wine, notes of varnish and coconut can stand out, depreciating the aroma quality.[107]

Burning of wood leads to the formation of aldehydes (furfural, 5-methylfurfural, and 5-hydroxymethylfurfural), rapidly extracted from wine during maturation.[107,111] Their odor is close to bitter almond, which is found in wines matured in new oak barrels. Nevertheless, their concentrations are too low to take part in the olfactive note.[107] In case alcoholic and/or malolactic fermentation takes place in oak barrels, there is the formation of 2-furanmethanethiol (**17**) responsible for grilled coffee notes in wines, as shown by Blanchard.[113] This thiol is formed from the nucleophilic addition of sulfide hydrogen formed during fermentation to the corresponding aldehyde, extracted from the oak wood (**Figure 11**).

It should be recalled that maturation in oak barrels can lead to an increase in the acetic acid content. This is due to the development of acetic bacteria and chemical hydrolysis of acetylated groups of new

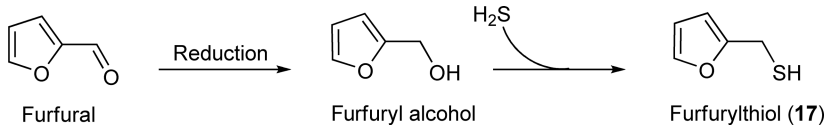


Figure 11 Formation of furfurylthiol as described by Blanchard.[113]

wood hemicelluloses. This hydrolysis takes place with time and leads to only a small increase in the volatile acidity (from 0.1 to 0.2 g l^{-1}).[106,114]

3.26.4 Chemistry of Phenolic Compounds in Wine

Grape and wine phenolic compounds have been extensively studied owing to their quantitative and qualitative importance. Phenolic compounds are ubiquitous plant secondary metabolites that contribute to the yellow, red, or blue pigmentation of various plant organs and play protective roles against biotic (fungi, pests, etc.) and abiotic (UV light exposure) stress. They are involved in the development of color and flavor properties of grape and wine and may play a role in the beneficial health effects attributed to their dietary consumption.

Phenolic compounds show a large diversity of structures, from very simple molecules to complex polymeric species (tannins), hence their variety of properties. Note that the term polyphenols is often used to designate phenolic compounds but should in principle be restricted to molecules containing at least two phenolic rings. Phenolic compounds are classically divided into flavonoids and nonflavonoids. Flavonoids are based on a common C6–C3–C6 skeleton (**Figure 12**) but comprise several groups (e.g., flavones (**18**), flavonols (**19**), dihydroflavonols (**20**), flavan-3-ols (**21**), and anthocyanidins (**22**)) that differ by the oxidation state of their central pyran ring (C ring). Within all groups, substitutions (hydroxylation, methoxylation, glycosylation, acylation, etc.) of the basic structures lead to further complexity and diversity as detailed below. Among flavonoids, anthocyanins, which are the pigments present in red grapes, and flavan-3-ols, which are encountered as oligomers and polymers, called proanthocyanidins or condensed tannins, are particularly important for the quality of red wines. Flavonols and dihydroflavonols are present in small amounts and play very limited role. Nonflavonoids (**Figure 13**) include rather simple molecules such as benzoic acids (**23**), benzylic aldehydes (**24**), and cinnamic acids (**25**), but also polyphenols such as stilbenes (**26**), and complex species such as oligostilbenes and hydrolyzable tannins, all of which can also be substituted. Nonflavonoids are represented in grapes mostly by hydroxycinnamic acids and their tartaric esters, the major factors of the enzymatic

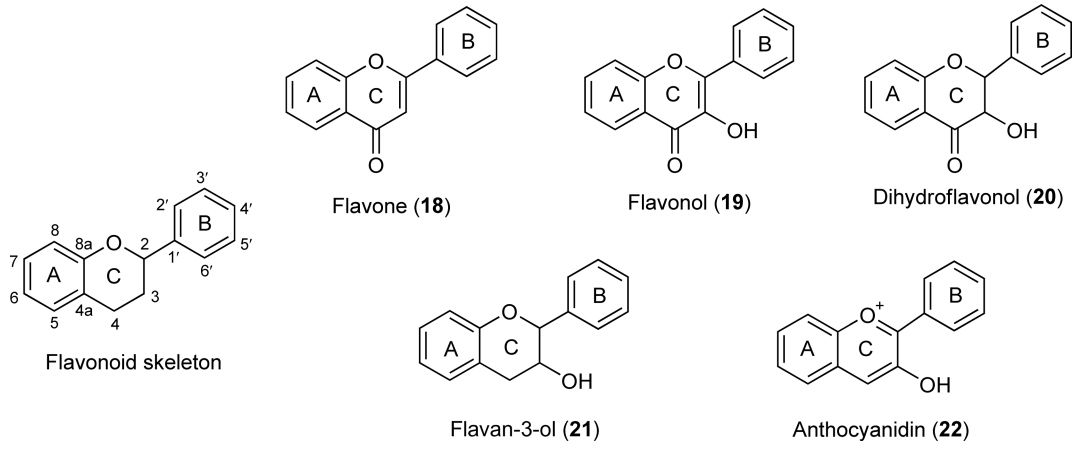
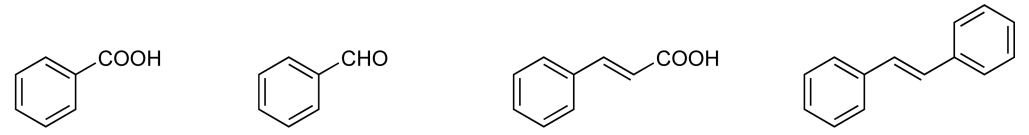


Figure 12 Flavonoid skeleton and general structure of flavonoid families found in grape.



Benzoic acid (**23**) Benzylic aldehyde (**24**) Cinnamic acid (**25**) Stilbene (**26**)

Figure 13 Structures of the main nonflavonoid phenolic families found in grape.

oxidation processes responsible for browning of white musts. Stilbenes are attracting a lot of interest due to their potential health benefits but they are present in rather small amounts in grapes and wines.

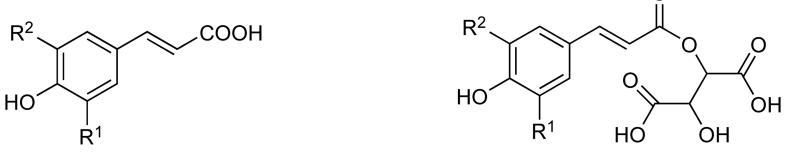
The wine phenolic composition is quite different from that of the grape. First, depending on the wine-making process and especially the extent of pomace contact, only part of the grape components are extracted into the wine. Moreover, all these compounds are highly reactive and are converted to various new species, through biochemical and chemical processes, as the wine is made and aged. Finally, exogenous phenolic compounds may be introduced in the process, for instance extracted from oak in the course of barrel aging or added as enological tannins.

3.26.4.1 Grape Phenolic Composition

Grape phenolic composition has been extensively studied since the pioneer work of Ribéreau-Gayon, who established the nature of major anthocyanins,[115] flavonols,[116] and hydroxycinnamic acids[117] in grape. The presence of proanthocyanidins, then called leucoanthocyanidins, in white wines,[118] red wines,[119] and grapes[120] has also been discovered in the 1950s but further structural determination has been achieved only recently as suitable methods have become available.

3.26.4.1.1 Phenolic acids

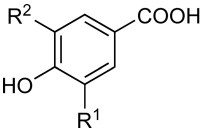
Major phenolic acids in grapes are hydroxycinnamic acids (**Figure 14**), namely *p*-coumaric (**7**), caffeic (**26**), and ferulic (**8**) acids, encountered mostly as tartaric esters (coutaric (**27**), caftaric (**28**), and fertaric (**29**) acids) in the vacuoles of skin and pulp cells of the grape berries.[117] The presence of tartaric esters – instead of the quinic esters more commonly found in fruits – in grape has long been a matter of debate but is now well established. The predominant isomers are in the *trans* (*E*) configuration but the *cis* (*Z*) isomers of the tartaric esters have been reported to be present in small amounts in grape.[121] Other studies have shown evidence of glucosylated derivatives of caftaric and coutaric acids in grape[122] and of coutaric acid in wine,[123] based on the analysis of products released after hydrolysis. Confusion between the *cis* forms and the glycosylated derivatives of tartaric esters has been postulated[121] and there has been some controversy concerning the nature of the small peaks eluting before the major *trans* forms. More recent studies, involving nuclear magnetic resonance (NMR) characterization, identified the *cis* isomers of coutaric acid in Chardonnay grape pomace[124] and of coumaric and coutaric acids in a Riesling wine.[125] The 4-*O*-glucosides of *p*-coumaric and ferulic acid in both the *cis* and the *trans* configuration were also identified in wine,[125] while other hydroxycinnamate derivatives were found



$R^1 = R^2 = H$: *trans*-*p*-coumaric acid (**7**)
$R^1 = OH$; $R^2 = H$: *trans*-caffeic acid (**26**)
$R^1 = OCH_3$; $R^2 = H$: *trans*-ferulic acid (**8**)

$R^1 = R^2 = H$: *trans*-*p*-coutaric acid (**27**)
$R^1 = OH$; $R^2 = H$: *trans*-caftaric acid (**28**)
$R^1 = OCH_3$; $R^2 = H$: *trans*-fertaric acid (**29**)

Figure 14 Structures of the main hydroxycinnamic acids in grape.



$R^1 = R^2 = OH$: gallic acid (**30**)
$R^1 = OH$; $R^2 = H$: protocatechuic acid (**31**)
$R^1 = OCH_3$; $R^2 = H$: vanillic acid (**32**)
$R^1 = R^2 = OCH_3$: syringic acid (**33**)

Figure 15 Structures of the main hydroxybenzoic acids in grape and wine.

only in the *trans* form. The glucose esters of *trans*-*p*-coumaric and *trans*-ferulic acids have been reported in grapes[126] and confirmed in wine.[125] Besides, *p*-coumaric acid and, to a lesser extent, caffeic acid are also encountered in grape as acyl substituents of the anthocyanins (see Section 3.26.4.1.2).

The concentration of hydroxycinnamic esters is higher (2- to 100-fold) in skins than in pulp but the pulp contribution to the whole berry content can exceed that of the skin in some varieties. Large varietal differences in the total amounts[127–129] and in the proportions of each ester[130] have been reported. The predominant hydroxycinnamic ester in grape is always *trans*-caftaric acid (0.06–0.78 mg per gram fresh weight of skin), followed by *trans*-*p*-coutaric acid (0–0.3 mg per gram fresh weight of skin), and *trans*-fertaric acid is present only in low amounts.

Hydroxybenzoic acids (**Figure 15**) are mostly represented in grapes by gallic acid (**30**), which is found in the free form and as an acyl substituent of flavan-3-ols (see Section 3.26.4.1.3). Its 3-*O*-β-glucopyranoside and 4-*O*-β-glucopyranoside have been detected in grape.[124] Other benzoic acids such as protocatechuic (**31**), vanillic (**32**), and syringic (**33**) acids are present in wine but, to our knowledge, have not been isolated from grape. They may result from the degradation of flavonoids.

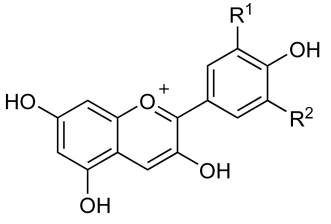
3.26.4.1.2 Anthocyanins

Anthocyanins, which are the red pigments of red and pink grape berries, belong to the flavonoid family. Anthocyanins are glycosylated derivatives, the corresponding aglycones being called anthocyanidins (**22**). Grape anthocyanins are based on five different anthocyanidins, namely cyanidin (**34**), peonidin (**35**), delphinidin (**36**), petunidin (**37**), and malvidin (**38**), that differ by their B-ring substitution pattern (**Figure 16**). In *V. vinifera*, the major anthocyanins are 3-monoglucosides (**39**). Moreover, the glucose can be acylated, at its C-6 position, by acetic acid (**40**), *p*-coumaric acid (**41**), and caffeic acid (**42**).[131,132] The presence in grape of the 3-(6-acetyl)-glucosides and 3-(6-*p*-coumaroyl)-glucosides of all five anthocyanidins as well as that of malvidin-3-(6-caffeoyl)-glucoside has been established in 1978.[131] The use of high-performance liquid chromatography coupled to mass spectrometry detection (HPLC–MS) has recently allowed the detection of additional anthocyanins in grape, namely the 3-caffeoylglucosides of peonidin,[133] cyanidin, petunidin, and delphinidin,[134] as well as the *cis* isomers of the 3-(6-*p*-coumaroyl)-glucoside of malvidin,[135] delphinidin, cyanidin, and petunidin.[136] The presence of the *cis* isomer of malvidin-3-(6-caffeoyl)-glucoside is also suspected. Recently, the existence of anthocyanins acylated with lactic acid has been reported in wine[136] but their identification requires confirmation and they have not so far been detected in grape.

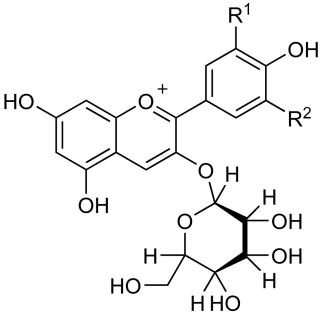
Anthocyanidin 3,5 diglucosides can be encountered in rather large amounts in non-*vinifera* species[115] and are present in trace amounts in *V. vinifera*.[134,137] The 3-(*p*-coumaroylglucoside), 5-glucoside of all five anthocyanidins have been first identified in *V. labrusca* grape juice[138] and in hybrid varieties.[139] More recently, mass signals attributed to these compounds and to the 3-(acetylglucoside), 5-glucoside have been detected in *V. vinifera* (cv. Dornfelder) wines.[137]

The total amounts (from 500 mg kg^{-1} to 3 g kg^{-1}) and the proportions of the various anthocyanins are varietal characteristics.[132,140] As stated above, *V. vinifera* contains only trace amounts of 3,5 diglucosides, which are much more abundant in some non-*vinifera* species.[115] Among the latter, *V. labrusca*, *V. riparia*, and *V. rupestris* can be distinguished from *V. rotundifolia* by the presence of acylated anthocyanins.[132] Within *V. vinifera* cultivars, Pinot noir and Pinot meunier contain no acylated anthocyanins.

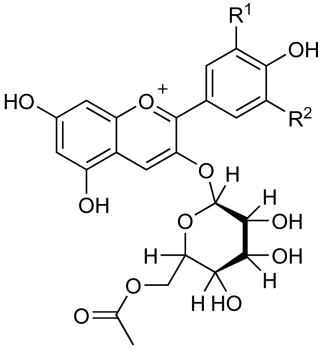
The anthocyanin profile has been proposed for chemotaxonomic classification. In most grape varieties, the major anthocyanins are malvidin derivatives, representing up to 90% in Grenache. Criteria such as the ratio between malvidin and peonidin derivatives[140] or the sum of malvidin + delphinidin + petunidin derivatives to peonidin derivatives[141] reflect the relative activities of hydroxylase enzymes involved in the biosynthesis



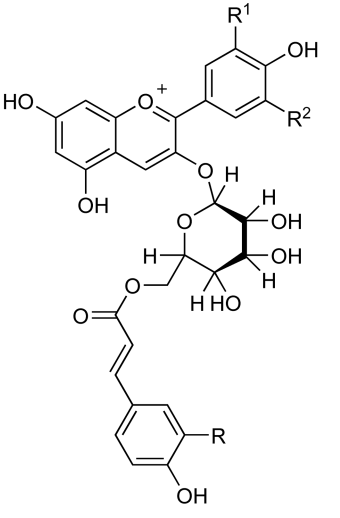
$R^1 = OH$, $R^2 = H$: cyanidin (**34**)
$R^1 = OCH_3$, $R^2 = H$: peonidin (**35**)
$R^1 = R^2 = OH$: delphinidin (**36**)
$R^1 = OCH_3$, $R^2 = OH$: petunidin (**37**)
$R^1 = R^2 = OCH_3$: malvidin (**38**)



Anthocyanidin 3-glucoside (**39**)



Anthocyanidin 3-(6-acetyl)glucoside (**40**)



R = H: anthocyanidin 3-(6-*p*-coumaroyl)glucoside (**41**)
R = OH: anthocyanidin 3-(6-caffeoyl)glucoside (**42**)

Figure 16 Structures of the main grape anthocyanins.

pathways leading to disubstituted and trisubstituted B rings, respectively, which are genetically determined. The malvidin 3-glucoside to delphinidin 3-glucoside ratio reflects the efficiency of the methyl transferase catalyzing methylation of the 3′ and 5′ OH groups while the relative abundance of anthocyanins acylated with acetic or *p*-coumaric acid reflects the efficiency of the relevant acyl transferase activities.

Grape anthocyanin composition also depends on the maturity and vine growing conditions including soil and climate characteristics but also practices such as pruning, fertilization, or watering.[142–145] Anthocyanins start accumulating at veraison and their concentration sometimes drops toward the end of the ripening period, especially in hot climates.[146] However, the amount of total pigments remains constant over the same period, suggesting that anthocyanins are converted to new pigments. Anthocyanin oligomers, possibly linked by either an A-type (carbon–carbon and ether bonds) or B-type (carbon–carbon bonds) linkage, have actually been detected in the skin extracts of Syrah grapes.[147]

3.26.4.1.3 Flavan-3-ols

Flavan-3-ols are encountered in grapes as monomers (**Figure 17**) and as oligomers and polymers (**Figure 18**), and the latter are called condensed tannins or proanthocyanidins. Major grape flavan-3-ol monomers are (+)-catechin (**43**) and (−)-epicatechin (**44**), which differ by the stereochemistry of the hydroxyl group in C3, and

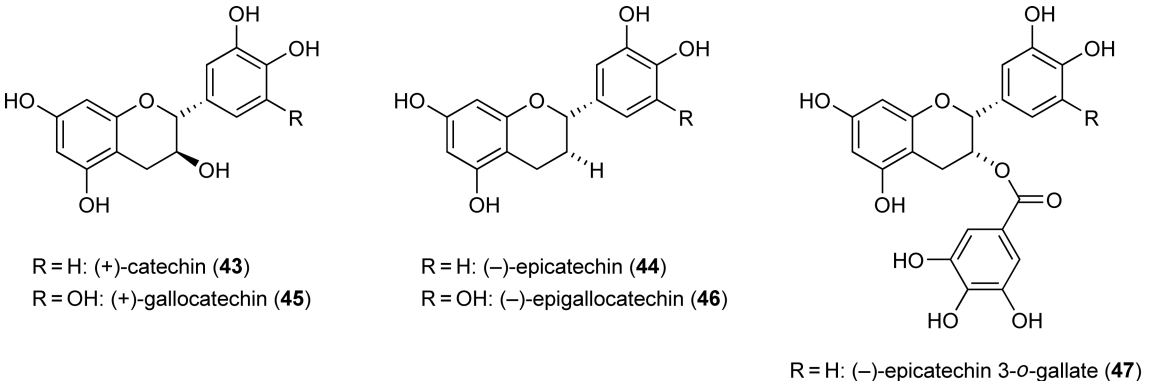


Figure 17 Structures of the main grape flavan-3-ol monomers.

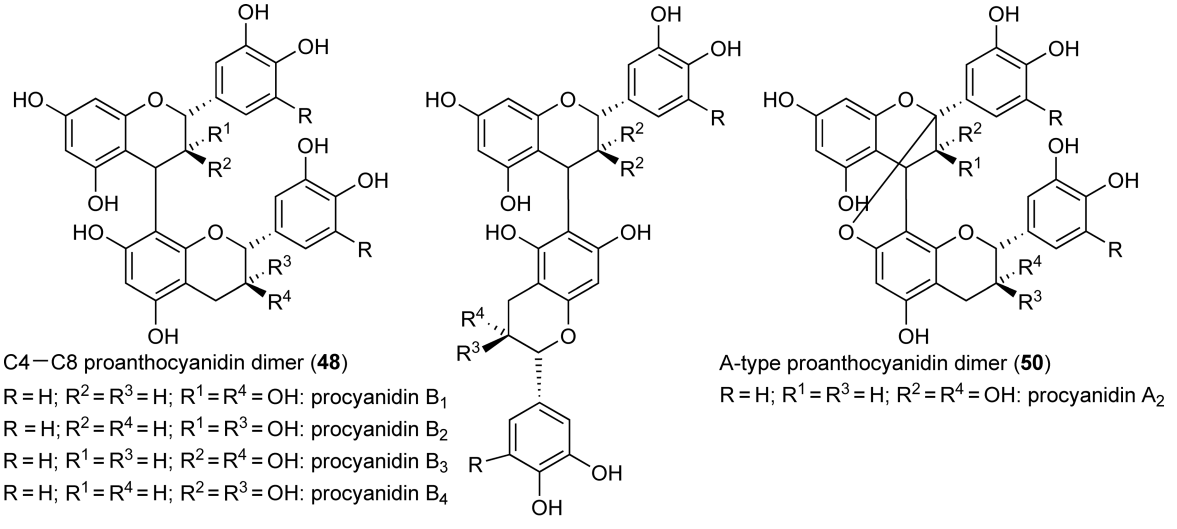


Figure 18 Structures of B-type (C4–C8 and C4–C6) and A-type proanthocyanidin dimers.

(−)-epicatechin 3-gallate (**47**). (+)-Gallocatechin (**45**) and (−)-epigallocatechin (**46**) are present in much lower amounts.[148,149] The 3-gallates of catechin[150] and gallocatechin[151] have also been reported in grapes but not in *V. vinifera*.

Grape proanthocyanidins (**Figure 18**) are based primarily on (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, and (−)-epicatechin 3-gallate units. In addition, (+)-Gallocatechin and (−)-epigallocatechin 3-gallate units have been found in dimers.

In proanthocyanidins, the constitutive flavan-3-ol units are linked by C4—C8 (**48**) or C4—C6 (**49**) linkages, in *trans* from the OH in the third position (i.e., 4α-8 or 4α-6 when the upper unit is catechin or gallocatechin, and 4β-8 or 4β-6 when it is epicatechin or epigallocatechin). These linkages are called B-type linkages and the corresponding proanthocyanidins are called B-type proanthocyanidins. Additional ether linkages (C2—O—C7 (**50**) or C2—O—C5), called A-type linkages, give rise to A-type proanthocyanidins. Acid-catalyzed cleavage of the interflavanic linkages (**Figure 19**) releases an intermediate carbocation (**51**) from each unit involved in the linkage through it C4 position (i.e., extension and upper units). This carbocation oxidizes to the corresponding anthocyanidin, hence the term proanthocyanidin. This reaction has been classically used to detect and measure these compounds.[152,153] The anthocyanidin formed from catechin or epicatechin is cyanidin and that formed from gallocatechin or epigallocatechin is delphinidin, so that the corresponding polymers are designated as procyanidins (**52**) and prodelphinidins (**53**), respectively.

About 20 B-type procyanidin dimers and trimers have been identified in grape seeds[154–156] and grape skins.[157] Those isolated from seeds contain catechin, epicatechin, and epicatechin gallate units, while those from skins, also detected in wine,[158,159] are based mostly on (epi)catechin and (epi)gallocatechin units.

Procyanidin dimer A2 (epicatechin-(4β-8, 2-*O*-7)-epicatechin) has been reported[160] but has not been formally identified in wine. Besides, mass signals detected in wine have been tentatively attributed to methoxylated flavan-3-ol dimers[161] but this requires confirmation.

Higher molecular weight polymers are largely predominant in grape, as in many other plant species.[162] Although this was well established almost 30 years ago, these polymeric compounds, which cannot be easily analyzed and usually appear as unresolved humps in the HPLC profiles, have been neglected for many years. Recent analytical developments have enabled much progress in their characterization. These include depolymerization by acid-catalyzed cleavage in the presence of a nucleophilic agent (e.g., thiolysis, phloroglucinolysis, using toluene-α-thiol (**54**) and phloroglucinol (**55**), respectively, as the nucleophilic agent). Reaction of the intermediate carbocations (**51**) released from the upper and extension units after cleavage of the interflavanic linkages yields a characteristic adduct, while the terminal unit (nonsubstituted in C4) is released as the corresponding monomer. HPLC analysis of the resulting mixture gives access to the amount and proportion of each unit and allows calculation of the average degree of polymerization (DP). It was thus shown that grape seed proanthocyanidins are partly galloylated procyanidins with an average DP around 10,[163] while grape skin proanthocyanidins consist of both prodelphinidins and procyanidins, with average DP values around 30.[164]

Normal phase HPLC analysis, enabling separation of oligomers and polymers as a function of their molecular weight, confirmed that grape seed proanthocyanidins consist of oligomers and lower molecular weight polymers, while skin proanthocyanidins are mostly polymeric material. Mass spectrometry analysis indicated that procyanidin and prodelphinidin units are encountered together in the oligomeric chains. In fact, all constitutive units seem to be randomly distributed in the PA chains. Proanthocyanidins from stems[165] and pulp[166–168] are also mixed procyanidin/prodelphinidin polymers. Values found in literature for the total amount of proanthocyanidins vary from 1.7 to 4.4 g kg^{-1} of berries in skins, from 1.1 to 6.4 g kg^{-1} of berries in seeds, and from 0.2 to 1 g kg^{-1} of berries in pulp.[166,167]

3.26.4.1.4 Other flavonoids

Grape berries also contain flavonols and small amounts of dihydroflavonols (**Figure 20**). The major flavonols in grapes are 3-glycosides of quercetin (**56**) and, to a lesser extent, of myricetin (**57**). Kampferol (**58**), isorhamnetin (**59**), laricitrin (**60**), and syringetin (**61**) derivatives have also been reported. The last three groups, as well as myricetin derivatives, are almost specific to red cultivars,[169] but trace amounts of isorhamnetin[170] and myricetin[171] have been found in some white varieties. Major glycosides in each family are the 3-glucoside (**62**) and the 3-glucuronide (**63**) but other mono- and diglycosides are present in lower amounts.[116,172–175]

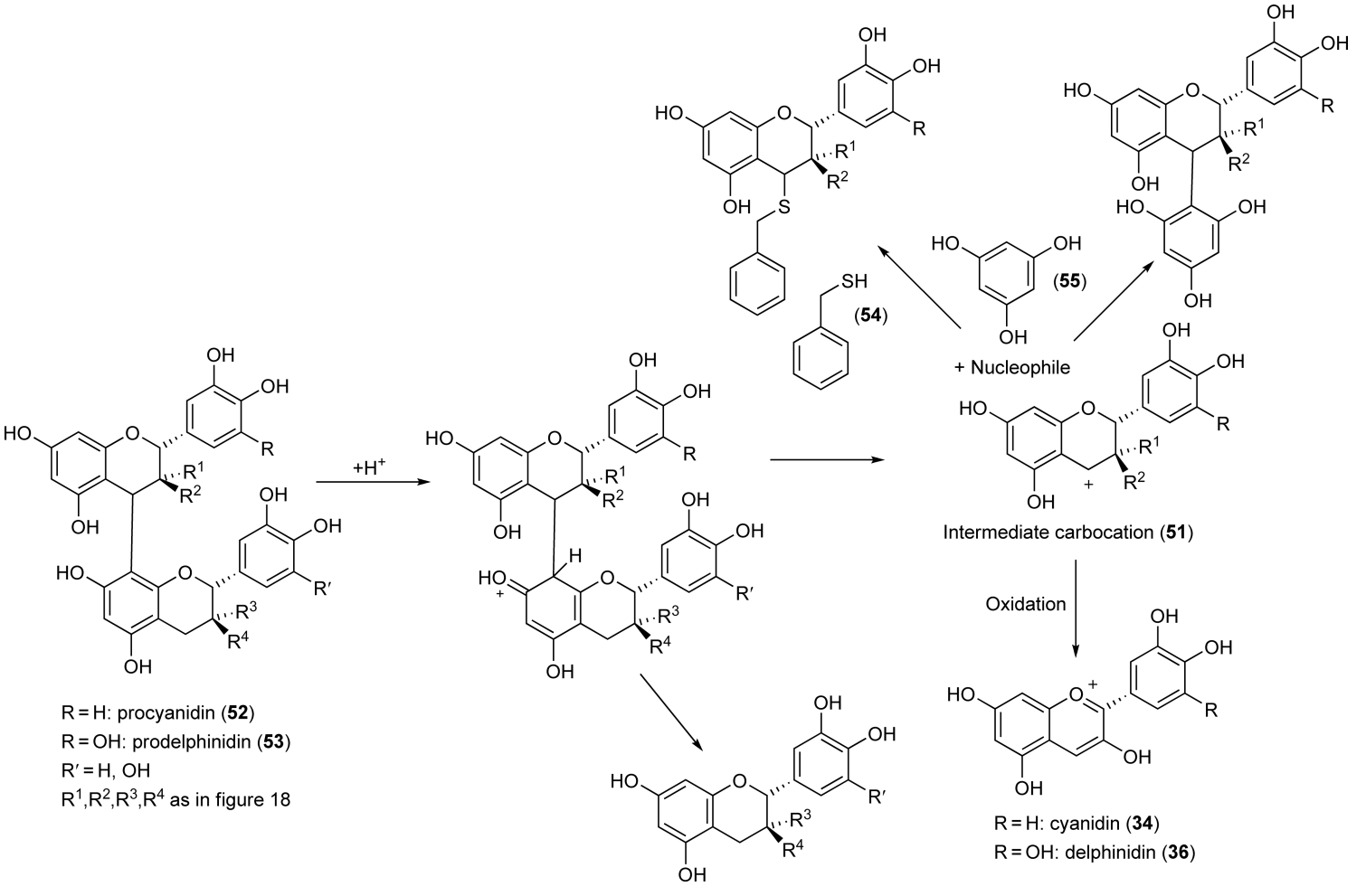
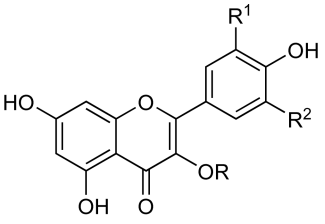
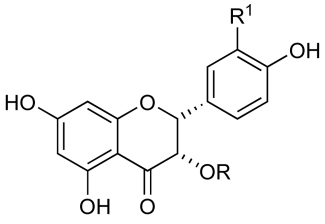


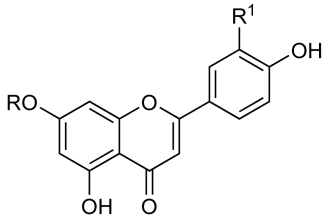
Figure 19 Acid-catalyzed cleavage of proanthocyanidins and subsequent reactions.



R^1 = OH, R^2 = H, R = H: quercetin (**56**);
R = Glc: quercetin 3-glucoside (**62**);
R = GlcA: quercetin 3-glucuronide (**63**)
R^1,R^2 = OH, R = H: myricetin (**57**)
R^1,R^2 = H, R = H: kampferol (**58**)
R^1 = OCH_3, R^2 = H, R = H: isorhamnetin (**59**)
R^1 = OCH_3, R^2 = OH, R = H: laricitrin (**60**)
R^1,R^2 = OCH_3, R = H: syringetin (**61**)



R^1 = OH, R = Rh: astilbin (**64**)
R^1 = H, R = Rh: engeletin (**65**)



R^1 = H, R = Glc: apigenin 7-glucoside (**66**)
R^1 = OH, R = Glc: luteolin 7-glucoside (**67**)

Figure 20 Structures of the main grape flavonols, dihydroflavonols, and flavones.

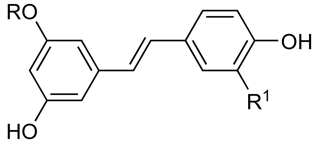
Flavonol contents are generally higher in red cultivars (4–78 mg kg^{-1} of berry) than in white cultivars (2–30 mg kg^{-1} of berry).[169] The amount is greatly increased by UV light exposure.[174–176] This is related to the role of flavonols as UV protectants.[176–178] Values up to 170 mg kg^{-1} (for Viognier berries) or 200 mg kg^{-1} (for Syrah berries) have been reported.[170]

Dihydroflavonols, namely astilbin (dihydroquercetin 3-rhamnoside (**64**)) and engeletin (dihydrokaempferol 3-rhamnoside (**65**)), have also been identified in grape and wine.[179] Engeletin and astilbin concentrations of 9 and 0.6 mg kg^{-1}, respectively, have been reported in grape skins. These compounds are also present in stems,[165] and levels up to 24.22 mg l^{-1} have been reported in wine.[179] Finally, flavones, that is, apigenin 7-glucoside (**66**) and luteolin 7-glucoside (**67**), have been identified in grape leaves.[180]

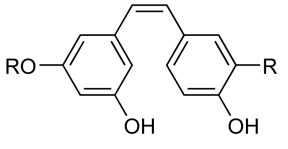
3.26.4.1.5 Stilbenes

Stilbenes, which belong to nonflavonoids, are attracting a lot of interest because of their potential beneficial health effects. They are considered as phytoalexins, which are synthesized in plant tissues in response to attack by fungi such as *Botrytis cinerea*, but also to abiotic stress such as UV irradiation.

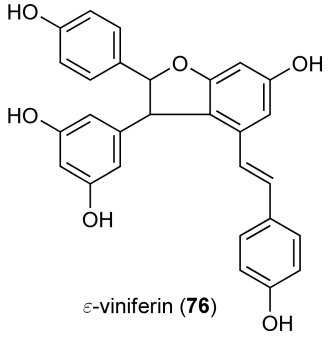
The major stilbenes in grape berries (**Figure 21**) are resveratrol (3,5,4′-trihydroxystilbene, *trans* (**68**) and *cis* (**69**)) and piceatannol (3,5,3′,4′-tetrahydroxystilbene, **70**, **71**) and their respective 3-*O*-β-D-glucosides piceid (**72**, **73**) and astringin (**74**, **75**), all of which can be found in *trans* and *cis* forms. Piceid is usually predominant, with concentrations in the range of 10–60 mg per kg dry skin for both the *trans* and the *cis* form.[180] Resveratrol is found only in the *trans* form in grape, although its *cis* isomer has been identified in wine. Stilbenes are also present in high concentrations in stems and leaves.



R = H, R^1 = H: *trans*-resveratrol (**68**)
R = H, R^1 = OH: *trans*-picetannol (**70**)
R = Glc, R^1 = H: *trans*-piceid (**72**)
R = Glc, R^1 = OH: *trans*-astringin (**74**)



R = H, R^1 = H: *cis*-resveratrol (**69**)
R = H, R^1 = OH: *cis*-picetannol (**71**)
R = Glc, R^1 = H: *cis*-piceid (**73**)
R = Glc, R^1 = OH: *cis*-astringin (**75**)



ε-viniferin (**76**)

Figure 21 Structures of the main grape stilbenes.

Values of 145.52 mg kg^{-1} of *trans*-resveratrol, 61.43 mg kg^{-1} of *trans*-piceid, and 143.85 mg kg^{-1} of *cis*-piceid (on dry stem) have been reported for cv. Castelão.[181] Only *trans*-resveratrol was quantified in grape seeds, with much lower concentration (6.8 mg kg^{-1} of dry seed).

In addition, stilbenes are also found as oligomers, resulting from the oxidation of resveratrol, presumably catalyzed by a peroxidase.[182–184] The major oligomer in grape is ε-viniferin (**76**), a resveratrol cyclic dehydrodimer. Several other resveratrol oligomers including dimers (ampelopsins B, D, and F and palidol) derived from ε-viniferin, trimers (α-viniferin), and tetramers (β-viniferin and hopeaphenols) have been identified in grapevine.[184]

3.26.4.1.6 Distribution in grape

The qualitative profiles and tissue distribution of phenolic compounds in the grape berries are genetically determined. Anthocyanins are located only in the grape skins, with the exception of the teinturier varieties (e.g., *V. vinifera* var. Alicante Bouschet, var. Gamay Fréaux), which also contain anthocyanins in the pulp. Flavonols are also localized in the berry skin, and are present in large amounts in grapevine leaves, in accordance with their role as UV protectants. Flavan-3-ols are found in larger amounts in the seeds than in the skins and only in small amounts in the pulp. The flavan-3-ol composition of seeds is different from that of skin and pulp. Flavan-3-ols are also present in stems[165] and leaves,[185] with intermediate composition between those of seeds and skins. Hydroxycinnamic acids are more abundant in skins but are also present in large amounts in the pulp, the contributions of both compartments to the total berry content being equivalent. Hydroxycinnamic acids are usually considered as the major phenolic compounds in grape pulp.[186] However, recent papers have shown that the pulp of all three Champagne varieties contains similar amounts of proanthocyanidins.[166] Although data on pulp proanthocyanidin content are still scarce, proanthocyanidins have been found in noticeable amounts in all varieties analyzed so far.[166–168] Stilbenes are present in the berry skin but they are also present in low amounts in seeds, and are more abundant in stems and leaves.

3.26.4.2 Phenolic Reactivity

3.26.4.2.1 Reactivity of the phenolic ring

The reactivity of polyphenols is mainly based on the fundamental chemical properties of the simplest phenol composed of a benzene ring with a unique hydroxyl substituent on C1.[187] The conjugation of one of the two unshared pairs of electrons from the oxygen atom of the hydroxyl group with the benzene ring corresponds to the positive electron-donor mesomeric effect (+M). This phenomenon extends the electron delocalization and confers a partial negative charge on the carbons in *ortho* and *para* position with respect to the hydroxyl group. Consequently, phenols are nucleophiles not only through their oxygen atom but also through their carbons in *ortho* and *para* positions with respect to the phenol hydroxyl group. This property is responsible for electrophilic aromatic substitution (EAS) reactions.

Additionally, the presence of substituents displaying a mesomeric effect on the phenol ring greatly affects the homolytic dissociation energy of OH bond (bond dissociation energy (BDE)), which reflects the reducing capacity of the corresponding phenol derivatives.

3.26.4.2.2 Electrophilic aromatic substitution reactions

The nucleophilic property of phenols promotes the electrophilic aromatic substitution reactions that are regioselective of *ortho* and *para* positions with respect to the hydroxyl group of phenol. The presence of additional hydroxyl groups in *meta* position (1,3-dihydroxybenzene, syn. resorcinol and 1,3,5-trihydroxybenzene, syn. phloroglucinol (**55**)) enables the accumulation of electron density at C2, C4, and C6 (*ortho* and *para* positions), thus increasing the nucleophilic character that supports subsequent EAS reactions. This kind of hydroxyl substitution pattern is encountered in the A ring of all grape flavonoids.

3.26.4.2.3 Reductive properties and oxidation

Another important property of the phenol ring is related to its ability to transfer electrons/hydrogen to radicals of high energy such as oxyl (RO·) and peroxyl (ROO·) species. This property is responsible for the antioxidant activity of phenols that mainly corresponds to the conversion of a very reactive radical into a stabilized

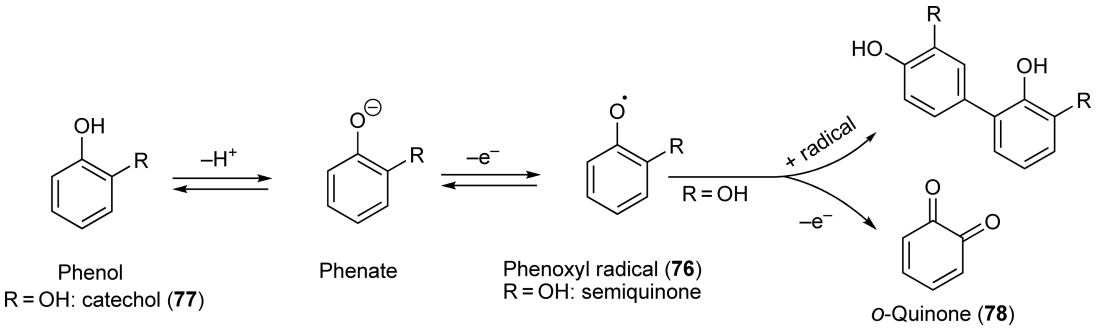


Figure 22 Oxidation of the phenol ring.

phenoxyl radical. The weaker is the BDE, the stronger is the H atom donor character. The phenol undergoes monoelectronic oxidation that gives the corresponding phenoxyl radical. The measure of the reducing capacity of a given substance is generally expressed by the standard redox potential (E°): the more reductive is the substance, the lower is the E° value. Substitution of the phenol with electron-donor substituents at *ortho* (C2 or C6) or *para* (C4) position stabilizes the phenoxyl radical by delocalization of the single electron toward this substituent and thus increases its reducing capacity. The 1,2-dihydroxy (catechol (77)) substitution is particularly favorable, suggesting that the stabilization results not only from electronic effects but also from the formation of an intramolecular hydrogen bond. This kind of hydroxyl substitution pattern is encountered in the structure of phenolic acids and flavonoid B rings.

Oxidation (**Figure 22**) can proceed by monoelectronic transfer that gives phenoxyl radical intermediates (76) or, in the case of *o*-diphenols, by dielectronic process leading to an *o*-quinone (78), depending on the nature of the oxidizing agent. The phenoxyl radicals derived from polyphenols are not very stable and quickly undergo dimerization, disproportionation, or reaction with dioxygen. In the case of a catechol nucleus, the disproportionation pathway is favored, leading to both an *o*-quinone and the restitution of the parent catechol. The *o*-quinones are not stable intermediates either and evolve through various pathways owing to their high electrophilic character:

- dimerization and oligomerization as demonstrated with caffeic acid oxidation,[188–190]
- addition of a nucleophile present in the medium, especially thiol groups contained in amino acids, peptides or proteins, and other phenolic compounds, and
- water or alcohol addition.

Besides, the oxidizing character of *o*-quinones and phenoxyl radicals allows the cooxidation of other substances such as ascorbate ions and other phenols as well. This enables, for instance, indirect oxidation of polyphenols that are not substrates of polyphenoloxidase (see Section 3.26.4.3.2). Whatever the reaction pathway, the oxidation products usually still display phenolic rings that in turn may be oxidized.

3.26.4.2.4 Reactivity of polyphenols

Polyphenols contain several phenolic rings, which can be diversely substituted (e.g., pyrogallol, phloroglucinol, resorcinol, and pyrocatechol), and thus show different reactivities. For instance, the flavonoid backbone corresponds to a 2-phenyl-1,4-benzopyrone, in which two phenolic units, a phloroglucinol (A ring) and a phenol (B ring), can be distinguished. The B ring is diversely substituted, usually according to a pyrocatechol or pyrogallol hydroxylation pattern. In terms of reactivity, this means that flavonoids are prone to react as nucleophiles through their A ring whereas they will be involved in oxidation through their B ring. In addition, flavonoids display a specific reactivity owing to the phenolic subclass to which they belong.

3.26.4.2.5 Specific reactivity of anthocyanins

The anthocyanins are usually represented as their red-colored forms, the flavylium cations. Due to the presence of a positive charge, flavylium cations are unstable and are prone to lose their color. In fact, there

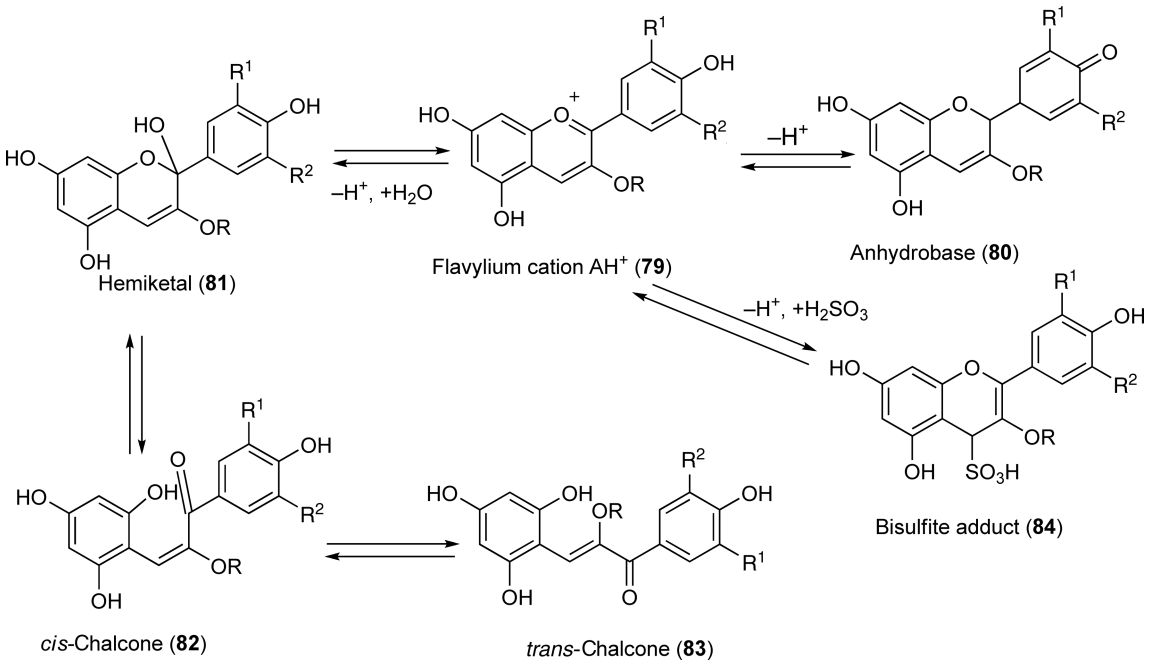


Figure 23 Anthocyanin equilibria in wine (R^1 and R^2 as in **Figure 16**, R = glucoside, 6-acetylglucoside, 6-*p*-coumaroylglucoside, or 6-caffeoylglucoside).

are two ways by which they can yield neutral species: proton transfer corresponding to an acid–base equilibrium and hydration (**Figure 23**).

3.26.4.2.5(i) Acid–base equilibrium In this equilibrium, the cation (AH^+, **79**) is the acidic molecule and the neutral quinoidal base (A, **80**) is the basic species. This equilibrium is instantaneous.[191,192]

$$AH^+ \rightleftharpoons A + H^+, \quad K_a = \frac{[A][H^+]}{[AH^+]}$$

The position of the equilibrium is fixed by the acidity constant ($pK_a = -\log K_a$) characteristic of the anthocyanin molecule. The typical pK_a value for anthocyanin is 4.2, which corresponds to the value measured for the major grape anthocyanin, malvidin 3-glucoside.[193]

3.26.4.2.5(ii) Hydration The second process by which the flavylium cations are converted into neutral species is hydration. This reaction consists of the nucleophilic addition of a water molecule to the pyrylium nucleus, mainly at C2 and, at a lesser extent, at C4. The products of hydration are the colorless hemiketal forms (AOH, **81**), which are partly converted into tautomeric forms, the *cis*- and *trans*-chalcones (**82**, **83**). The hydration reaction is much slower than the proton transfer, and requires several hours to reach the state of equilibrium.

$$AH^+ + H_2O \rightleftharpoons AOH + H^+, \quad K_h = \frac{[AOH][H^+]}{[AH^+]}$$

This equilibrium is characterized by a hydration constant ($pK_h = -\log K_h$) characteristic of the anthocyanin molecule. This hydration equilibrium is also pH dependent. Indeed, the proportion of flavylium and hemiketal forms is determined by the hydration constant to pH ratio. For malvidin 3-glucoside, the pK_h had been estimated at 2.6.[193] This means that at pH > 2.6, malvidin 3-glucoside is mainly in the colorless form. At the pH of the wine (3.6), 90% of the anthocyanins are hydrated. In this form, anthocyanins are able to react as nucleophiles through their A-ring-like flavanols. Therefore, some color-stabilizing mechanisms take place in wine to preserve anthocyanin color. A well-known process occurring originally in the plants is driven by the

aromatic properties of the colored forms of anthocyanins. The planarity and hydrophobicity conferred by the aromatic systems favor vertical stacking of the colored forms of anthocyanins between them (self-association), with other planar structures (copigmentation) or with an aromatic residue connected to the pigment (intramolecular copigmentation).[194–196]

A second way by which anthocyanins preserve their color is by converting their structure into a more stable chromophore through chemical reactions. This can happen only when the cellular integrity of the grape berries is broken and the different constituents come in contact with each other.

Before reviewing these reactions, let us see another major reaction responsible for anthocyanin discoloration in wines: hydrogen sulfite addition.

3.26.4.2.5(iii) Hydrogen sulfite addition Bisulfite is used in food processing, especially in winemaking, to protect wine from microbial and fungal growth as well as from oxidation. However, its disadvantage is the formation of a colorless adduct (**84**) with the red forms of anthocyanins.[197] The structure of this adduct has been recently confirmed.[198]

$$AH^+ + HSO_3^- \rightleftharpoons AHSO_3, \quad K_s = \frac{[AHSO_3]}{[AH^+][HSO_3^-]}$$

Given the value of the dissociation constant of sulfite ($pK_s = 5$), the anthocyanin sulfite adduct is stable in the pH range of wine (3.2–4). Therefore, part of anthocyanins are trapped as noncolored hydrogen sulfite adducts in proportion to the amount of sulfite added to the wine.

3.26.4.2.6 Specific reactivity of proanthocyanidins

In addition to the reactivity imparted by the A and B rings of flavonoid units, proanthocyanidins can undergo cleavage of the bonds (interflavan linkage (IFL)) connecting the flavanol units within the polymeric chain, under acidic conditions (even mild).[199] This actually gives rise to highly reactive carbocations (see Section 3.26.4.1.3; **Figure 19**). They can further oxidize to anthocyanidins (where the proanthocyanidin name comes from). Otherwise they can be trapped by electron-rich components, chemically speaking, nucleophiles.

3.26.4.3 Changes Occurring in Must Preparation

Grape pulp contains hydroxycinnamic acids and flavan-3-ols while anthocyanin pigments are located in the skins (see Section 3.26.4.1.6). The distribution of phenolic compounds in grape, along with their solubility and reactivity, determines their extraction into the must and the wine. Technological processes are also essential to control the extraction of various groups of phenolics from their respective compartments. Thus, white musts and wines are obtained by direct pressing of white or red grapes, while red and rose wines require a skin contact phase in their elaboration to extract the red pigments from the red berry skins. Consequently, the major processes involving phenolic compounds in the early steps of winemaking are extraction of the pigments and tannins, which is essential for the quality of red wines, and enzymatic oxidation, also known as enzymatic browning, which starts as soon as the grapes are crushed or pressed and which is especially important for white wines.

3.26.4.3.1 Extraction of grape phenolic compounds during maceration

Extraction from the grapes starts after crushing and continues throughout maceration, until the skins and seeds are separated from the liquid phase by racking or pressing. Pulp components are readily extracted into the must and make up the major part of phenolic compounds in white musts and wines. In contrast, transfer of flavonoids and stilbenes, which are mostly localized in the solid part of the cluster, is largely determined by the extent of maceration. Diffusion kinetics also depend on the solubility of phenolic compounds in water and hydroalcoholic solutions and on factors such as alcohol content, the temperature, and the extent of must homogenization.[200]

The concentrations of caffeoyltartaric acid and *p*-coumaroyltartaric acid in musts carefully protected from oxidation account for 40 and 20%, respectively, of the amounts available in grape. After crushing and pressing,

caffeoyltartaric acid is the major phenolic compound in grape musts, with concentrations reaching up to 150 mg l^{-1} in some varieties.[201,202] White musts and wines normally contain very little flavonoids and stilbenes. The flavan-3-ol polymers present in pulp are not found in white wines. Whether this is due to oxidation or their adsorption on the plant cell walls, as shown for cider apple,[203] is unknown. Average resveratrol concentrations of 0.48 and 0.67 have been reported in still white wines and Champagne wines, respectively.[204]

Skin contact is sometimes performed to increase the extraction of volatile compounds and aroma precursors, when making white wines from white grapes. This results in an increase in hydroxycinnamic acid content by 20%, and a threefold increase in the concentration of flavan-3-ol monomers and dimers, after 4 h of skin contact.[205] Sulfur dioxide addition at harvest to prevent oxidation as well as thorough pressing also increases the concentrations of flavan-3-ols[206,207] and hydroxycinnamic acids[208] in the resulting musts and wines.

In red wine making, anthocyanins are extracted rather fast. Their concentration reaches a maximum after a few days of fermentation and then decreases,[209,210] as they proceed to other molecular species. As explained above, skin (and pulp) tannins can be distinguished from seed tannins on the basis of their specific constitutive units, namely epigallocatechin and epicatechin gallate, respectively. Consequently, the respective contributions of skins and seeds to wine tannin composition can be determined. Monitoring of proanthocyanidin composition during fermentation showed that extraction of proanthocyanidins from skins roughly parallels that of anthocyanins while that of seed flavan-3-ols is slower.[210,211] Extraction of flavan-3-ols from seeds increases considerably with the ethanol concentration[212] and is thus enhanced by longer maceration.[212] This may be due to the poorer water solubility imparted by higher galloylation rate or due to the particular structure of seed tissues. The average chain length of wine tannins also increases with maceration,[211,212] meaning that lower molecular weight proanthocyanidins diffuse faster and are retained to a lesser extent on plant cell walls than the larger polymers.

The amount of anthocyanins in wine usually represents only 20–40% of the grape potential. Anthocyanins extracted from the pomace do not compensate for this loss, indicating that conversion of grape anthocyanins to other compounds starts very early in the wine-making process. Proanthocyanidins are also only partly extracted into the wine (20–40%) but a larger proportion can be recovered from the pomace.[211] Their rate of conversion to other molecular species appears highly variable.

Several wine-making practices can be used to increase the extraction of phenolic compounds into the wine. Cold soak before fermentation results in higher concentrations of anthocyanins and skin proanthocyanidins, while postfermentation maceration increases the content of seed proanthocyanidins in the final wine.[213] Processes such as thermovinification and flash release, which involves heating the grapes quickly at high temperature (>95 °C) and then placing them under a strong vacuum that causes instant vaporization and cooling, accelerate anthocyanin extraction into the must but also their degradation.[211] The latter reaction is presumably due to increased conversion of the hemiketal to the chalcone form (see Section 3.26.4.2.4), which is known to be favored at higher temperatures,[214] followed by cleavage[215] or rearrangement[216] of the chalcone ring. Flash release also significantly increases the extraction of tannins, especially when associated with maceration.[211] When several enrichment processes, namely flash release, enzyme treatment, runoff, and tannin addition, were tested on Syrah and Grenache wines in the Rhône valley, the tannin content was significantly higher after flash release and tannin addition while runoff increased the anthocyanin concentration. Runoff and flash release also resulted in enhanced color intensity. This can be attributed to increased levels of derived pigments, formed through reactions of anthocyanins with tannins (see Section 3.26.4.4).

Flavonol concentration in wine is usually low. However, it can be higher in wines made from sun-exposed berries[175] or as a result of extraction from leaves.[217] Astilbin and engeletin have also been reported in low amounts in wines.[218]

The transfer rate of stilbenes from grape to wine is extremely low. There was a significant correlation between the concentration of piceid in grape skins and that in the wine but no such relationship was found for resveratrol.[181] In Spanish red wines, resveratrol concentrations varied from 0.1 to 2.5 mg l^{-1} and from 0.03 to 0.16 mg l^{-1} for *trans* and *cis* resveratrol, respectively.[219] Similar values were found in wines from North Africa[220] and Croatia.[221] Piceid is the stilbene present at the highest concentrations, ranging from 4.6 to 45 mg l^{-1} in wines from North Africa[220] and from 0.82 to 38.47 mg l^{-1} in a series of Italian wines.[222] *Trans*-ε-viniferin was found to be present only in red and botrytized sweet white wines with levels between 0.1 and 1.63 mg l^{-1}.[220,223]

Concentrations ranging from 0.2 to 9.2 mg l^{-1} and from 0.3 to 3.8 mg l^{-1} were reported for palidol and (+)-hopeaphenol, respectively, in red wines.[220]

3.26.4.3.2 Enzymatic oxidation

3.26.4.3.2(i) Oxidative enzymes Enzymatic oxidation in must is catalyzed by grape catecholoxidase (*o*-diphenol:oxygen oxidoreductase, (EC 1.10.3.1), usually called polyphenoloxidase (PPO). It catalyzes the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones, using oxygen as a cosubstrate for both reactions (**Figure 24**).

Enzymatic oxidation starts when oxygen, phenolic substrates, and PPO come into contact, as a result of cellular decompartmentation. Other oxidases, including laccase (EC 1.10.3.2), originating from *B. cinerea*, the fungus responsible for both gray rot and noble rot, and peroxidase (EC 1.11.1.7), another oxidase present in grape[224] that uses hydrogen peroxide as a cosubstrate, can also catalyze the conversion of *o*-diphenols to *o*-quinones.

3.26.4.3.2(ii) Phenolic substrates of grape PPO The preferred substrates of grape PPO are hydroxycinnamic derivatives and especially caffeoyltartaric (**28**) and *p*-coumaroyltartaric (**27**) acids,[225,226] which are also the major phenolic constituents of grape juice.[186] From both of these substrates, PPO generates the *o*-quinone of caffeoyltartaric acid (**85**; **Figure 25**).[227] Grape PPO is also active, but to a lesser extent, on flavan-3-ol monomers, catechin, and epicatechin but it cannot oxidize flavonol glycosides, anthocyanins, epicatechin gallate, or proanthocyanidin dimers.[226,228,229] This is presumably due to steric hindrance as flavonol aglycones are good substrates for plant PPOs.

3.26.4.3.2(iii) Reactions of o-quinones as oxidants Quinones are yellow pigments and are highly unstable, being both strong electrophiles and potent oxidants, as described in Section 3.26.4.2.2. In particular, caffeoyltartaric acid *o*-quinone is extremely reactive and plays a major role in oxidative mechanisms leading to must browning.[186,227] Due to the high redox potential of the caffeoyltartaric acid/caffeoyltartaric acid *o*-quinone couple, the *o*-quinone can oxidize numerous other compounds present in the must. These include reductants such as ascorbic acid and sulfites[230,231] and also other *o*-diphenolic compounds, which are then oxidized to secondary *o*-quinones through coupled oxidation processes (**Figure 25, (A)**). Thus, epicatechin gallate,[232] procyanidins,[233] and *o*-diphenolic anthocyanins[234] can be oxidized in musts by caffeoyltartaric acid quinone although they are not substrates for grape PPO.

3.26.4.3.2(iv) Reactions of o-quinones as electrophiles Moreover, primary and secondary quinones can react with nucleophiles (**Figure 25, (B)**). The major example of such reactions in musts is the formation of 2-*S*-glutathionyl caffeoyltartaric acid, called grape reaction product (GRP (**86**), **Figure 25**), which results from the addition of glutathione (**87**) to caffeoyltartaric acid quinone (**Figure 25**).[186,227,235] Reactions of thiols with quinones are well documented,[236,237] but, to our knowledge, GRP is the only known example of such products in plant transformation processes. Moreover, its formation demonstrates the presence of glutathione in rather large amounts in grape (up to 160 mg l^{-1} in musts). GRP is not a substrate for grape PPO but it can be oxidized to GRP *o*-quinone (**88**) by *B. cinerea* laccase[238] and by the caffeoyltartaric acid quinones present in excess after depletion of glutathione.[239] As laccase is known to survive in wines, oxidation of GRP may be responsible for the browning observed in wines made from botrytized grapes.

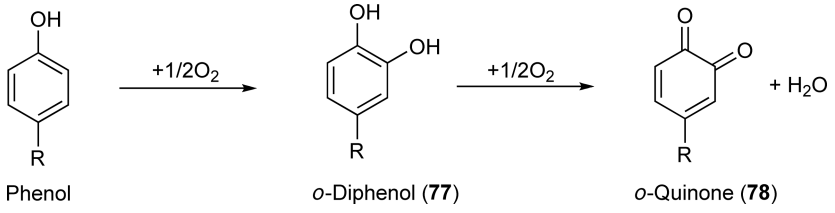


Figure 24 Reactions catalyzed by PPO.

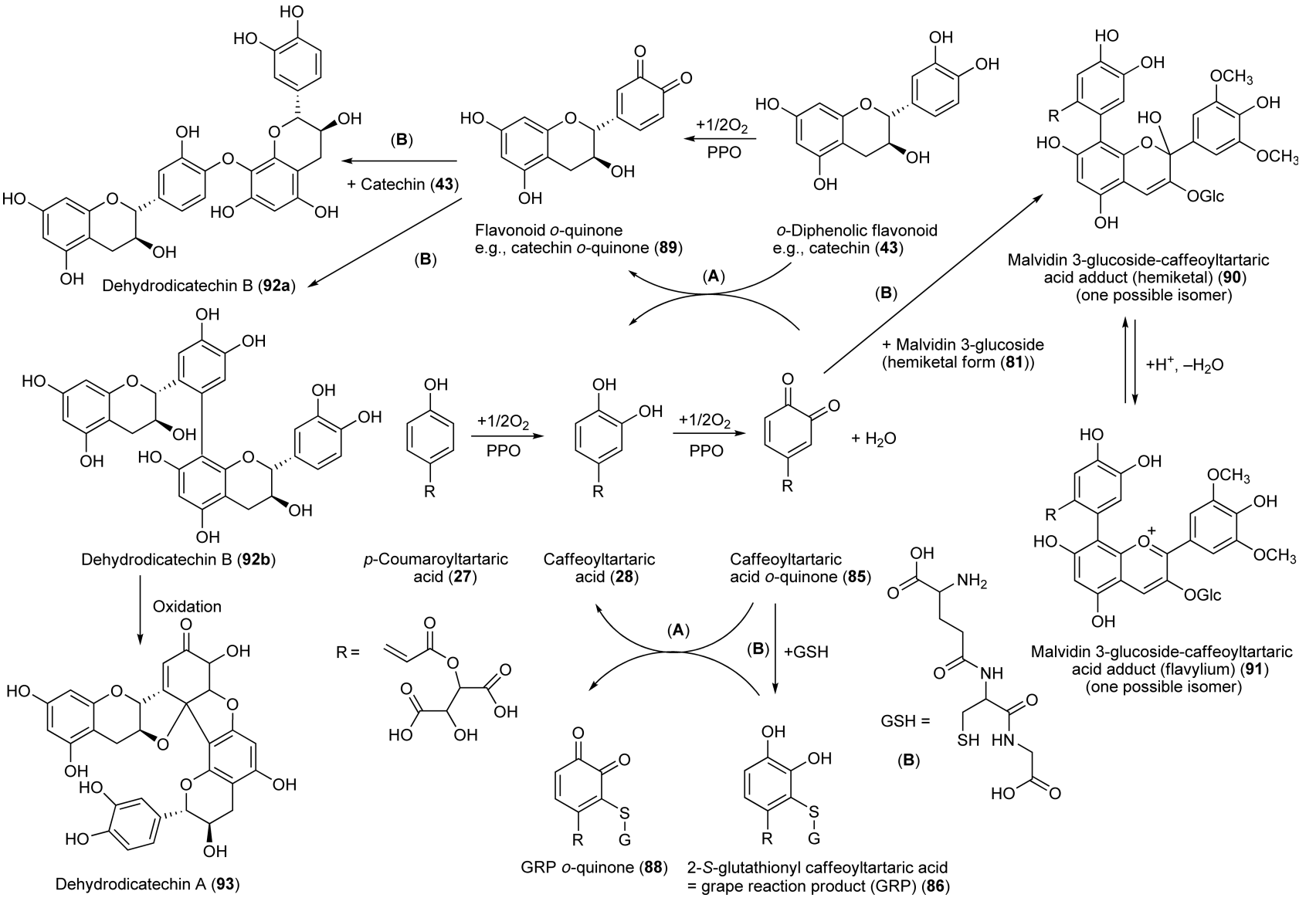


Figure 25 Reactions of *o*-quinones in grape musts.

3.26.4.3.2(v) Enzymatic oxidation processes in grape musts GRP itself is colorless[227] but its oxidation leads to intense browning.[239] The susceptibility of grape musts to enzymatic browning could thus be related to the relative amounts of caffeoyltartaric acid and glutathione, which is a varietal characteristic.[129] Oxygen consumption in musts is related to their caffeoyltartaric (and *p*-coumaroyltartaric) acid content while their tendency to brown depends on the caffeoyltartaric acid to glutathione molar ratio. In musts showing excess glutathione, caffeoyltartaric acid is entirely converted to GRP, which remains stable, with no browning. In musts containing excess caffeoyltartaric acid, two successive phases are observed: first, rapid consumption of caffeoyltartaric acid and formation of GRP take place; then, GRP concentration decreases and caffeoyltartaric acid decay becomes much slower, as some of it is regenerated through coupled oxidation of GRP. This second phase consumes oxygen, as caffeoyltartaric acid is reoxidized after each reduction step. This also results in browning, followed by precipitation of the brown pigments.

Comparison of oxidation kinetics in musts and in model solutions containing identical proportions of caffeoyltartaric acid and glutathione has shown that other must components compete with glutathione for reaction with caffeoyltartaric acid *o*-quinone.[230] A lag phase in which oxygen is consumed while the concentration of caffeoyltartaric acid remains constant could be attributed to instant reduction of the quinone by powerful reductants such as ascorbic acid.[231]

In addition, other nucleophiles can trap the quinone in the same way as glutathione. These include nucleophilic phenolic compounds such as flavonoids, which add to quinones through their A ring C8 or C6 positions. Thus, the addition of malvidin 3-glucoside to caffeoyltartaric acid yields a series of adducts.[240] In fact, the anthocyanin presumably adds to the quinone as the nucleophilic hemiketal (**81**), the resulting hemiketal adduct (**90**) being in equilibrium with the corresponding flavylium (**91**). Similarly, enzymatic oxidation of catechin yields a series of oligomers (called B type dehydrodicatechins) in which the B ring of one unit is linked to the A ring of the other by biphenylether (**92a**) or biphenyl (**92b**) linkages.[241] Several dimers arising from the oxidation of caffeic acid have also been identified.[188–190] Moreover, oxidation kinetics in solutions containing both caffeoyltartaric acid and catechin suggest the formation of mixed reaction products,[242] as shown in apple must.[243,244] The products arising from these coupling reactions are *o*-diphenols and can thus be oxidized further. In particular, B type dehydrodicatechins oxidize intramolecularly to yield dehydrodicatechins A (**93**), which are yellow pigments.

3.26.4.3.2(vi) Influence of the wine-making process The phenolic composition of free run white musts protected from oxidation reflects that of the pulp, which contains hydroxycinnamic esters and flavan-3-ols. However, in white wine making, important oxidation reaction takes place during pressing, unless special care is taken to avoid it. The extent of oxidation depends primarily on the grape composition, but can be modified by technological processes. Thus, hydroxycinnamic acids are almost entirely oxidized during pressing in musts that have high ratios of glutathione to hydroxycinnamic acids. In musts containing larger amounts of hydroxycinnamic acids, oxygen availability appears to be the limiting factor. More important oxidation takes place in a traditional Champagne press, which allows a lot of oxygen circulation in the must,[208] than in a horizontal rotative press.[245] Hyperoxygenation, which involves adding oxygen before fermentation, enhances the oxidation of phenolic compounds. In contrast, early sulfiting maintains higher levels of caffeoyltartaric acid and GRP and limits must browning, due to the reduction of the quinones back to *o*-diphenols and due to PPO inhibition by sulfites.

Skin maceration, whether intentional or accidental, induces enrichment of the must with phenolic compounds and especially flavan-3-ols. Their enzymatic or coupled oxidation induces must browning followed by precipitation of the pigments. However, in musts containing excess glutathione, or with added sulfur dioxide or ascorbic acid, these oxidation mechanisms are limited, so that higher levels of flavan-3-ols are maintained throughout winemaking. This results in higher sensitivity of the wines to nonenzymatic browning, which appears correlated to the flavan-3-ol concentration.[205,246]

3.26.4.3.3 Other biochemical reactions

In addition to polyphenoloxidases, various enzymes originating from yeasts, bacteria, and fungi or added as winemaking aids can modify the phenolic composition of musts and wines.

Pectinases and β-glucanases are the only enzymes allowed for winemaking in Europe. They are used to hydrolyze plant cell wall polysaccharides and facilitate pressing and clarification and also to release aroma

compounds from their nonvolatile glycosidic precursors[50] and to increase extraction of phenolic compounds into the wine.[247,248] Studies on the effect of enzymes on wine phenolic composition have led to conflicting results,[249] some showing an increase in anthocyanin[250–252] and tannin[252] content or color,[252–254] while others reporting a decrease in pigments.[255,256] This may be due to the presence of side activities degrading anthocyanins. Indeed, the commercial enzyme preparations often contain side activities such as cinnamate esterase, β-glucosidase, or tannin acyl hydrolase (tannase).[257] Cinnamate esterase releases caffeic and *p*-coumaric acids from their tartaric esters[225] and the acylated anthocyanins.[53] It is worth pointing out that methods based on anthocyanin profiles, which have been proposed as authentication criteria for varietal wines, are not reliable as the proportion of acylated anthocyanins can be modified during winemaking. β-Glucosidase activities release the flavonol aglycones and the anthocyanidins from the corresponding glucosides, resulting respectively in haze[217] and in color loss. Numerous fungi including *Aspergillus niger* and *B. cinerea* show tannin acyl hydrolase (EC 3.1.1.20) activity, which cleaves the flavan-3-ol galloyl esters.[258] All these hydrolytic reactions also occur spontaneously in wine, under acid catalysis. The hydrolysis products of hydroxycinnamic esters and GRP thus slowly accumulate in wine.[123,235]

Other biochemical processes altering phenolic composition include decarboxylation of hydroxycinnamic acids to volatile phenols (see Section 3.26.3.1.2), catalyzed by yeast cinnamate decarboxylase,[52,259,260] and formation of tyrosol and tryptophol, from tyrosine and tryptophan, respectively.[261] Other yeast enzyme activities have been proposed to catalyze *trans–cis* isomerization, for instance of resveratrol,[262] but it can also take place chemically, in particular following light exposure.[263] Similarly, phenolic acids (gentisic, 3,4-dihydroxybenzoic, *p*-hydroxybenzoic, *m*-hydroxybenzoic, salicylic, syringic, vanillic, protocatechuic) detected in fermenting musts and wine may arise either from the degradation of grape phenolics under the action of yeast enzymes or from spontaneous cleavage of the flavonoid heterocycles.

3.26.4.4 Extraction of Phenolic Compounds from Oak

Higher quality wines are usually barrel aged. Major sensory characteristics contributed by barrel aging are due to extraction from oak of volatiles, including a number of phenolic compounds, as discussed in Section 3.26.3.2.4. Nonvolatile phenolics such as phenolic acids (*p*-hydroxybenzoic, vanillic, syringic) and ellagitannins such as vescalagin (**94**; **Figure 26**) and castalagin (**95**)[264] are also extracted from barrels but at concentrations too low to impact taste properties.[265,266] Maximum concentrations of 21 and 7 mg l^{-1} have been reported for castalagin and vescalagin, respectively. These concentrations are much lower than those extracted with hydroalcoholic solutions, presumably due to the reactions of ellagitannins with other wine constituents. Besides, barrel aging enables slow

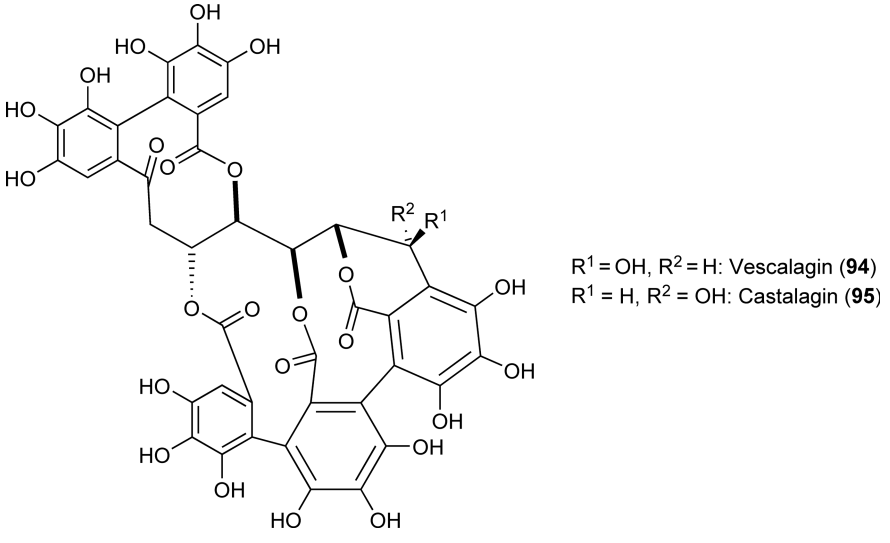


Figure 26 Vescalagin and castalagin.

oxygen transfer into the wine. The disappearance of ellagitannins is directly related to the presence of dissolved oxygen,[267] which enhances their transformation to more stable compounds.[268]

3.26.4.5 Reactions Occurring during Wine Aging

Due to the high reactivity of phenolic compounds, a large number of new molecules are formed in the course of winemaking and, later on, during wine storage and aging. Reactions taking place spontaneously in hydroalcoholic acidic media like wine include hydrolysis of ester and glycosidic linkages as described above (Section 3.26.4.3.3), as well as formation of ethyl esters from carboxylic acids.[123] Moreover, color changes taking place in wine aging, from the purple nuance of young wines toward the red-brown tint of matured wine, have long been ascribed to the conversion of grape anthocyanins to other pigments.[269] Reactions of proanthocyanidins, based on their characteristic C—C bond-breaking and bond-making processes and oxidation mechanisms, are considered responsible for the astringency loss.[199] Reactions between anthocyanins and tannins have been thoroughly studied in the last decade and the main types of products have been characterized. Oxidation, polycondensation, and direct reactions have been shown to generate specific types of linkages, some of which are resistant to acid-catalyzed cleavage.

3.26.4.5.1 Oxidation and subsequent nucleophilic addition, radical coupling

Oxidation can occur by both biochemical and chemical processes. Biochemical processes are prevalent in musts (see Section 3.26.4.3.2) but, as the ethanol content increases during fermentation, enzymatic systems become less effective and chemical oxidation gradually becomes predominant. As discussed in Section 3.26.4.2.3, oxidation of an *o*-diphenol yields an *o*-quinone, which is very reactive, particularly toward other polyphenols. The *o*-quinone can oxidize molecules with lower redox potentials. It is also a powerful electrophile, and it can react with a nucleophile, which can be its nonoxidized form or another phenol. The resulting product is a dimer containing at least one *o*-diphenol function. The dimer, then, is susceptible to further oxidation and can be converted into a trimer. Therefore, oxidation can be a polymerization process.

Chemical oxidation involves metal-catalyzed dioxygen reactions, as the direct reaction between polyphenols and molecular oxygen, commonly referred to as autoxidation, is neither thermodynamically nor kinetically favorable. Autoxidation of polyphenols is primarily governed by their redox potential and it can affect a wider range of molecules than enzymatic oxidation. Therefore, the polyphenol substrates of enzymatic reactions are not necessarily the most reactive molecules toward chemical oxidation. Thus, while grape hydroxycinnamic acids are the major substrates of enzymatic oxidation, the capacity of white wine to brown was shown to be directly related to its flavan-3-ol content.[205,247] Autoxidation products of flavanols exhibit specific linkages, located between the B ring of one unit and the A ring of the other, in their structures,[270,271] as described above for products of enzymatic oxidation.[241] These linkages can be either a biphenyl ether linkage (**92a**) or a biphenyl (**92b**) linkage, which result from radical coupling of two semiquinones and from the nucleophilic addition of the catechin A ring to the *o*-quinone, respectively. Both types of linkages are resistant to acid cleavage and thus much stronger than the interflavanyl linkages of proanthocyanidins. Further oxidation of the biphenyl-linked dimer yields yellow pigments (**93**) by an intramolecular rearrangement of the oxidized dimer, competing with further oxidation-induced polymerization. Furthermore, flavanol autoxidation can be modulated by the presence of other compounds such as phenolic acids. The addition of caffeic acid to a catechin solution enhanced browning of the solution, whereas the addition of protocatechuic acid (3,4-dihydroxybenzoic acid) decreased it. In the latter solution, a colorless product was formed by the addition of a catechin molecule to the *o*-quinone of protocatechuic acid.

Additionally, oxidation is not restricted to phenolic constituents. It can affect other wine constituents, such as ethanol and potassium bitartrate, to produce aldehydes that are reactive toward flavonoids.[272] Furthermore, catalytic species, like radicals and/or metals (Fe, Cu), induce chemical oxidation reactions in the presence of oxygen and polyphenols. In fact, reaction between polyphenols and oxygen, also called autooxidation, is not straightforward and requires that either oxygen or the polyphenol is activated (by radicals and/or metals). The resulting chain reaction can be summarized by the following equation:

$$O_2 + o\text{−diphenol} \rightarrow H_2O_2 + o\text{−quinone}$$

Hydrogen peroxide (H_2O_2) generated from phenol autoxidation can further oxidize ethanol to acetaldehyde,[93] only in the presence of ferrous ions (Fe^{2+}).[273] Oxidation of bitartrate in the presence of Fe^{2+} leads to the formation of various acids and aldehydes,[274,275] including glyoxylic acid, which was shown to be the precursor of yellow pigments through its polycondensation reactions with flavonoids.[272] Polycondensation reactions with aldehydes will be further discussed in the next section.

3.26.4.5.2 Polycondensations with aldehydes

3.26.4.5.2(i) Condensation with acetaldehyde Pigments resulting from condensation reaction of acetaldehyde with one flavan-3-ol and one anthocyanin have been first described in the 1970s.[276] In these products, the two flavonoid units are linked through a methylmethine (—(CH—CH_3)—) bond, often incorrectly called ethyl bridge in enology. Their formation involves an electrophilic aromatic substitution of the protonated form of acetaldehyde on the nucleophilic A ring of a flavonoid, followed by protonation of the resulting adduct (**96**), loss of a water molecule to yield a carbocation (**97**), and nucleophilic addition of the A ring of a second flavonoid molecule to this carbocation (**Figure 27**).[277] Flavan-3-ols, including monomers[276–279] and oligomers,[280] and anthocyanins (in their hydrated nucleophilic form) can take part in these processes, the resulting products being polyflavanols (**98**),[277] polyanthocyanins (**99**),[281] and copolymers of anthocyanins and flavan-3-ols (**100**).[277,279–281] As flavonoids can react in their C6 and C8 positions, several isomers (C6—C6, C8—C8, C6—C8, R and S, as illustrated for flavan-3-ol dimers (**98**)) can be formed and the polymerization reaction can continue. Formal identification of the four (epi)catechin-(CH—CH_3)-malvidin 3-glucoside enantiomers (C8—(CH—CH_3)—C8, R and S for each pair) formed in model solutions has been achieved recently.[282]

The presence of (epi)catechin-(CH—CH_3)-malvidin 3-glucoside, (epi)catechin-(CH—CH_3)-delphinidin 3-glucoside, (epi)catechin-(CH—CH_3)-petunidin 3-glucoside, (epi)catechin-(CH—CH_3)-peonidin 3-glucoside, in the flavylium form,[282] and methylmethine-linked (epi)catechin dimers[283,284] and trimers,[283] and malvidin 3-glucoside-(CH—CH_3)-malvidin 3-glucoside, in the hemiketal-flavylium form,[281] has been established in wine. The proportion of methylmethine bonds between flavan-3-ol units increases with aging but represents only a small percentage of interflavanic linkages.[285]

3.26.4.5.2(ii) Condensation with other aldehydes Other aldehydes can react in the same way as acetaldehyde (**Figure 28**). These include glyoxylic acid (**101**) arising from tartaric acid oxidation,[272] furfural (**102**) and 5-hydroxymethyl furfural (HMF, **103**),[286] which can be formed during toasting of oak barrels, isovaleraldehyde, benzaldehyde, propionaldehyde, isobutyraldehyde, formaldehyde, and 2-methylbutyraldehyde,[287] which are the constituents of spirits added to fortified wines. The products resulting from glyoxylic acid, furfural, and HMF proceed, through dehydration and cyclization, to xanthene derivatives (**104**), which oxidize to orange xanthylium pigments (**105**).[286,287,288,289] This mechanism, which competes with further polymerization, has not been established in the case of methylmethine derivatives although the solutions undergo similar color changes.

3.26.4.5.2(iii) Reactions of condensation products in wine The methylmethine bridges are susceptible to acid-catalyzed cleavage[278] and thus rather unstable in wine. Among them, anthocyanin-flavan-3-ol adducts are more resistant than flavanol oligomers under the very acidic conditions of the thiolysis reaction. However, they cleave spontaneously and release anthocyanins when incubated at higher pH values (2.5 and 5).[290] Cleavage of methylmethine-linked flavanol oligomers leads to rearrangement and polymerization,[278] through random bond-making and bond-breaking processes, and to the formation of vinylflavanols (**106**), which are the precursors of pyranoanthocyanins[291,292] and portisins,[293] as explained below.

Acetaldehyde is produced by yeast metabolism (see Section 3.26.2) but can also be generated by oxidation of ethanol in the presence of oxygen, metal catalysts, and phenolic compounds. The condensation rate is determined by that of aldehyde protonation. Thus, both formation and cleavage of methylmethine derivatives depend on pH. Metal ions also catalyze the oxidation of tartaric acid to glyoxylic acid and the formation of carboxymethine bridges that yield xanthylium pigments,[294] and thus enhance oxidative browning of catechin solutions.[286] Ascorbic acid, acting as a pro-oxidant after an initial lag phase, increases the formation of these pigments.[295] To our knowledge, the only condensation products detected in wine are those formed from acetaldehyde. This is presumably due to the higher concentration of acetaldehyde

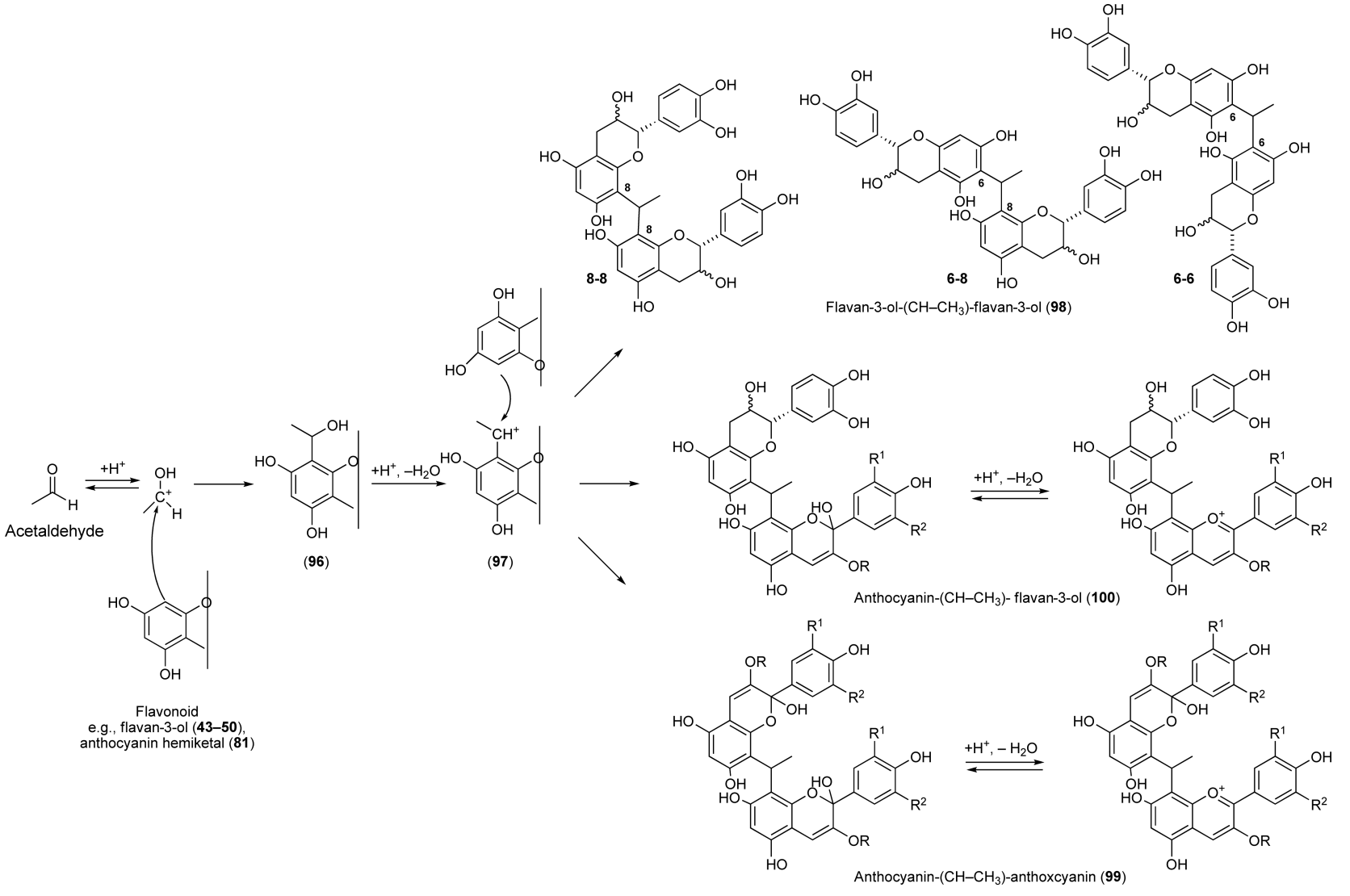


Figure 27 Acetaldehyde polycondensation reactions (R, R^1, and R^2 as in **Figure 23**).

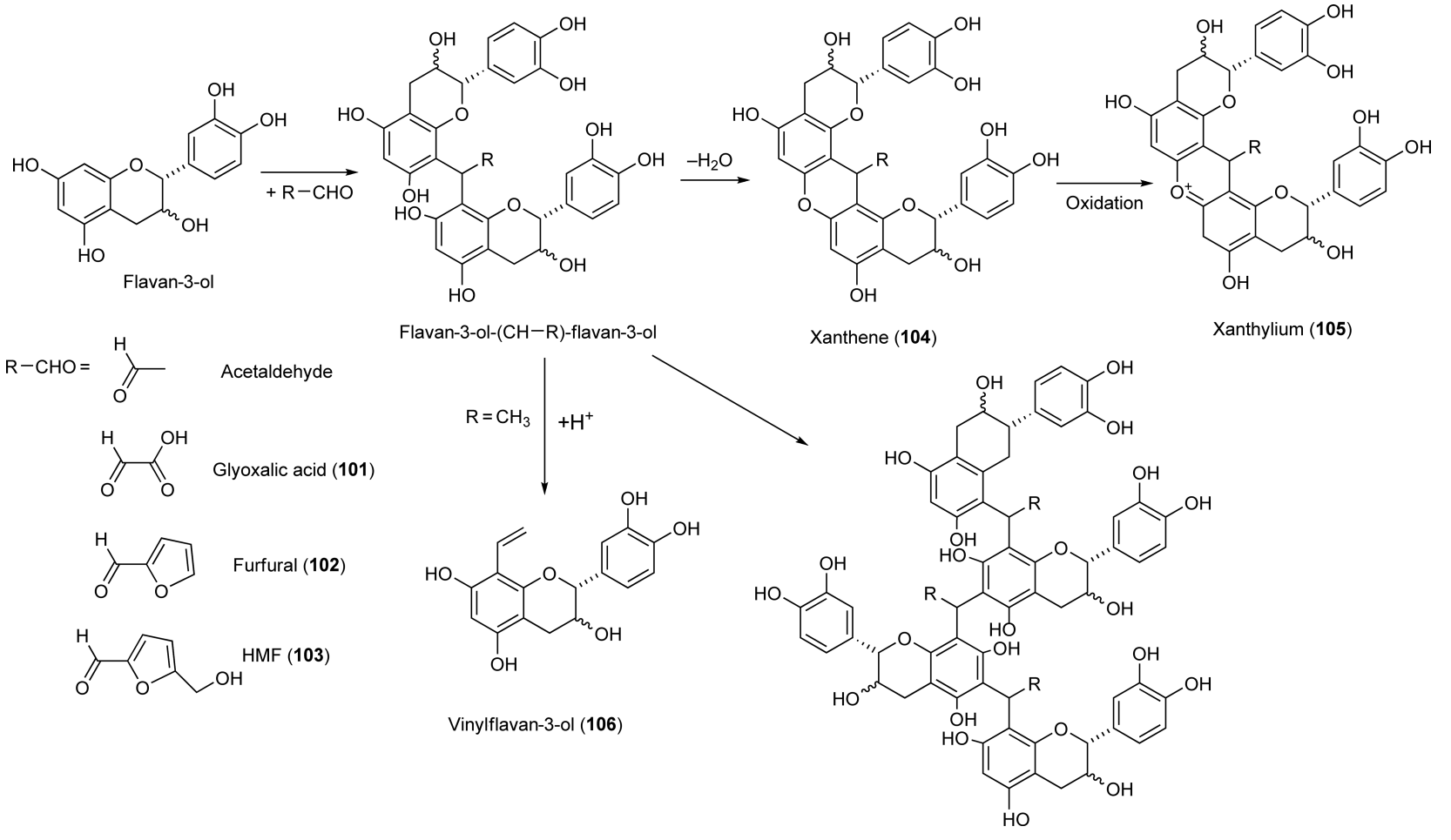


Figure 28 Reactions of flavonoid aldehyde condensation products.

compared to other aldehydes in wine. The rate of glyoxylic acid formation might be limiting, as catechin reacts faster with it than with acetaldehyde.

3.26.4.5.3 Conversion of anthocyanins to pyranoanthocyanins

3.26.4.5.3(i) Phenylpyranoanthocyanins Pyranoanthocyanins (**Figure 29**) result from the reaction of the anthocyanin flavylium cations with compounds showing a doubled bond activated by electron-donating substituents such as hydroxyl or phenyl groups. The first pyranoanthocyanin structure was detected in wine[296] and identified[297] in 1996. Its formation mechanism involves the addition of 4-vinylphenol (**9**), formed by decarboxylation of *p*-coumaric acid (see Section 3.26.3.1.1), to the anthocyanin C4, followed by cyclization with the anthocyanin hydroxyl in C5. Autoxidation of the resulting flavene intermediate (**107**) is thermodynamically favored by rearomatization, generating a new 4-hydroxyphenyl-pyranoanthocyanin chromophore (**108**). Similar derivatives, arising from the reaction of 4-vinylcatechol, 4-vinylguaiacol, and 4-vinylsyringol with malvidin 3-glucoside, have been tentatively identified in wine by mass spectrometry.[298] The catechol derivative (pinotin A) has been isolated from Pinotage wine and its structure confirmed by NMR.[299] Moreover, an alternative pathway has been proposed for the formation of phenyl-pyranoanthocyanins from hydroxycinnamic acids as precursors.[300]

3.26.4.5.3(ii) Pyranoanthocyanins formed from enol precursors Other types of pyranoanthocyanins have since been detected in wines and characterized.[287,301,302] Among them, vitisin A (**109**) and B (**110**)[303] result from the reaction of pyruvic acid and acetaldehyde, respectively, with anthocyanins.[287] Their formation mechanism involves the addition of the double bond of the enol form of the aldehyde or acid to the flavylium C4, followed by dehydration and rearomatization to yield the pyranoanthocyanin structure. This mechanism can be generalized to other enol derivatives, such as ⟨-ketoglutaric acid and 2-hydroxybutan-2-one, which are yeast metabolites, or acetone, which can react with anthocyanins during solvent extraction procedures.[304]

3.26.4.5.3(iii) Flavanyl-pyranoanthocyanins In addition, flavanyl-pyranoanthocyanins (**111**) have been detected in model solutions[305] and in wine.[292,306–308] Several of these products have been isolated from Port wine and their structures confirmed by NMR.[293,309] They probably result from the addition of the 8-vinylflavanol arising from acid-catalyzed cleavage of methylmethine bonds to the anthocyanin.[291] Such intermediates have been detected in the mass spectra of products resulting from the condensation of acetaldehyde with flavan-3-ols.[309]

3.26.4.5.3(iv) Vinylpyranoanthocyanins Finally, a new group of pigments, called portisins (**112**), have been isolated from aged Port wine.[293] Their structure differs from that of flavanyl-pyranoanthocyanins by the presence of a vinyl bridge between the flavanol and the pyranoanthocyanin groups.[310] They are derived from carboxypyranoanthocyanins, formed by the reaction between anthocyanins and pyruvic acid, and 8-vinylflavanols. Their formation mechanism involves decarboxylation of the carboxypyranoanthocyanin followed by addition of the vinyl group to the activated pyran ring. Vinylphenol can react in the same way, yielding phenyl-vinylpyranoanthocyanins.[311]

3.26.4.5.4 Direct flavan-3-ol anthocyanin reactions

This terminology is classically used in enology to designate reactions leading to direct linkages between the flavonoid units, as opposed to indirect linkages such as those arising from condensation with aldehydes. They actually consist of nucleophilic addition mechanisms in which the nucleophile is the A ring of one of the flavonoids and the electrophile the positively charged C4 of the other. The reactivities of flavan-3-ols and anthocyanins give rise to several mechanisms, yielding different products (**Figure 30**), as detailed below.

3.26.4.5.4(i) Anthocyanin-flavan-3-ol adducts In this case, the anthocyanin reacts as the electrophile, in its flavylium cation form, and undergoes nucleophilic addition of a flavan-3-ol. The resulting product, in which the anthocyanin is linked through its C4 (and thus in the upper position) and the flavan-3-ol (lower unit) through one of its C8 and C6 carbons, is called anthocyanin-flavan-3-ol (A-F) or anthocyanin-tannin (A-T) adduct. The nucleophilic addition step gives rise to a flavene (**113**). This intermediate can oxidize to the

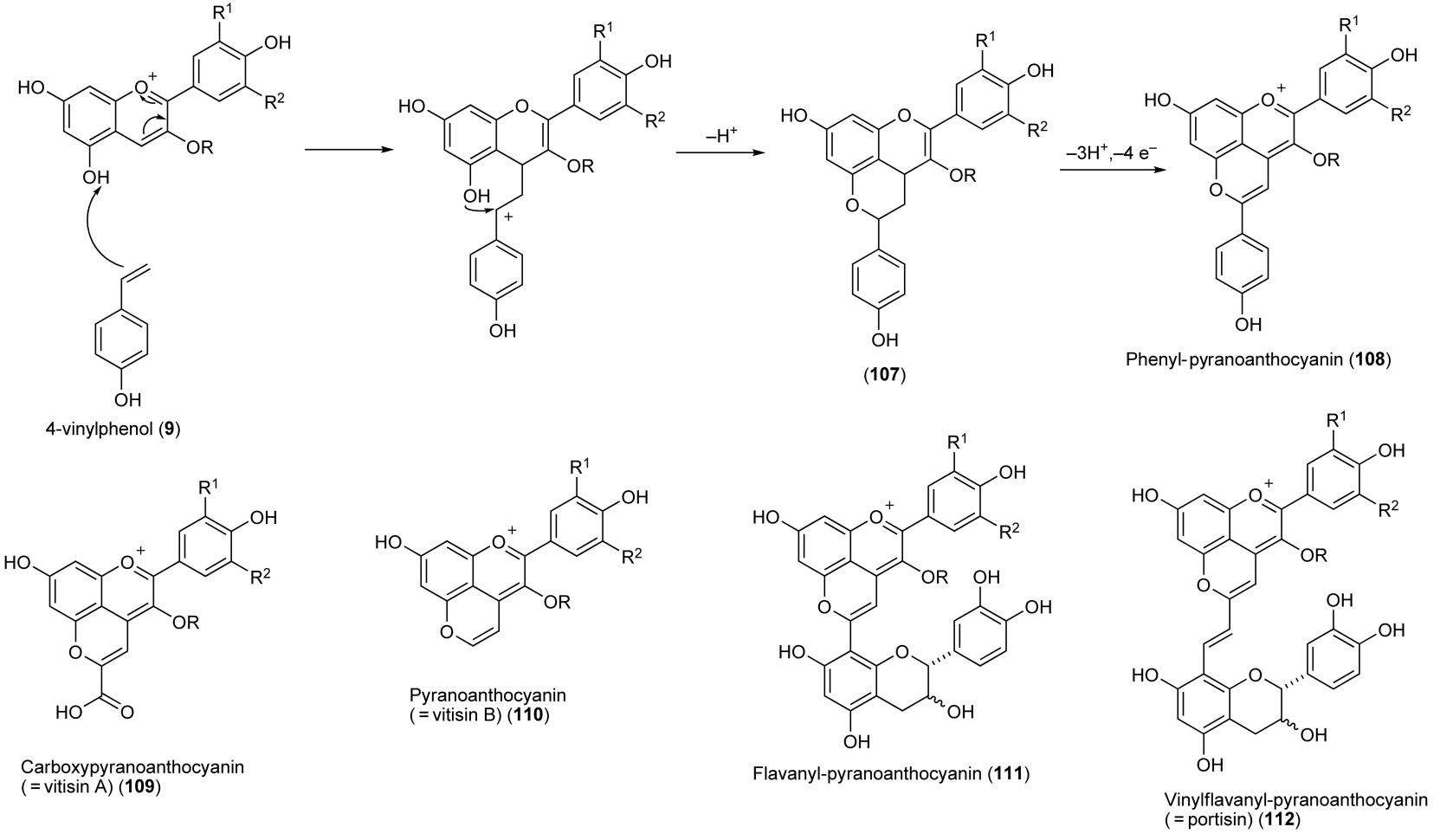


Figure 29 Formation of vinylpyranoanthocyanins and structures of pyranoanthocyanins found in wine (R, R^1, and R^2 as in Figure 23).

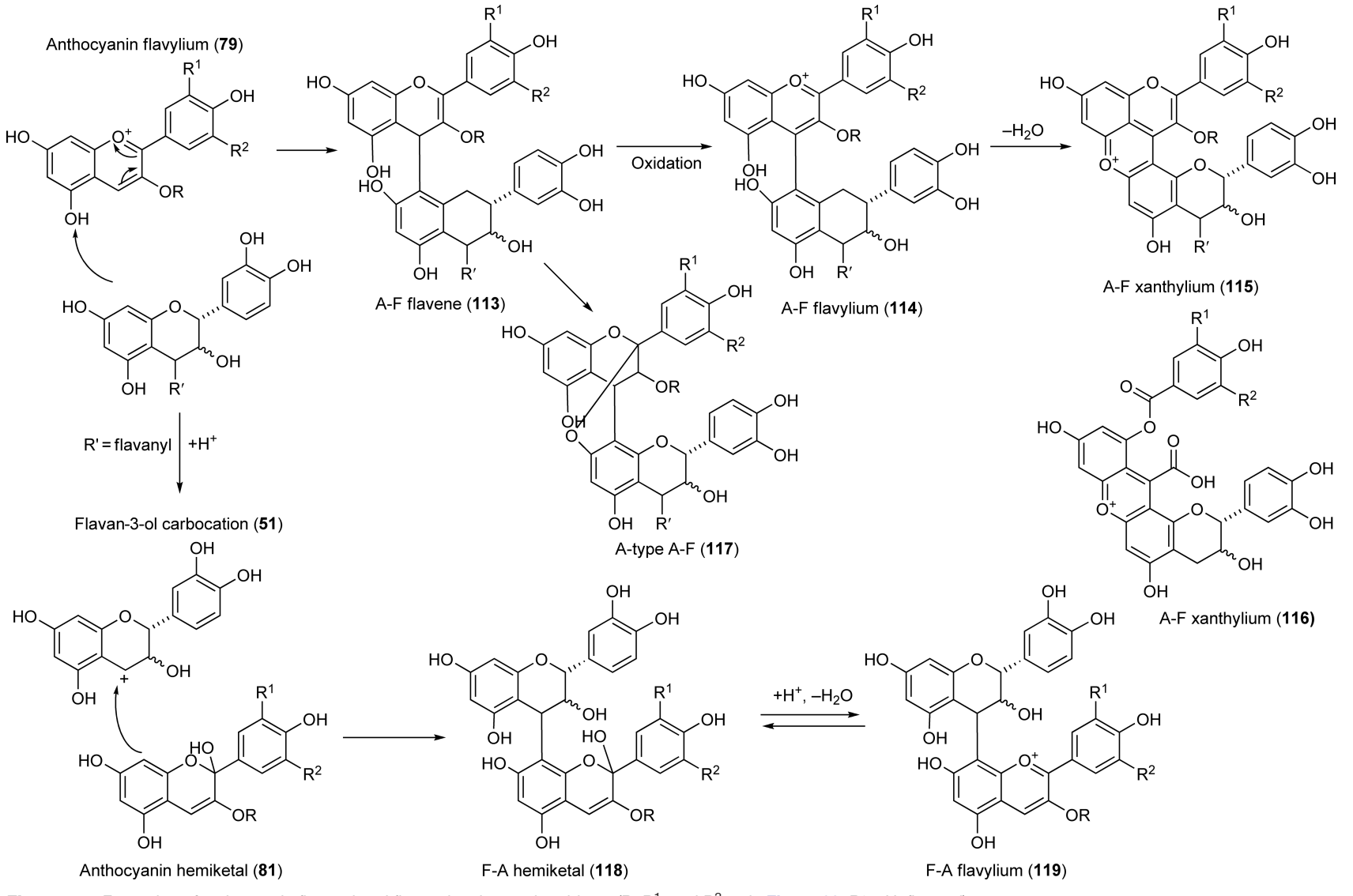


Figure 30 Formation of anthocyanin flavanol and flavanol anthocyanin adducts (R, R^1, and R^2 as in **Figure 23**; R′ = H, flavanyl).

corresponding flavylium (A^+-F (**114**)), possibly through a mechanism in which another anthocyanin flavylium is reduced.[312,313] Model solution studies using malvidin 3-glucoside and a procyanidin dimer, B2 3′-*O*-gallate, have demonstrated the formation of several A^+-F structures, corresponding to the different linkage positions on the flavanol dimer, when the incubation was carried out at pH 3.8.[314] The A^+-F adducts were not observed at pH 2 and, to our knowledge, have never been detected in wine. They have not been detected either in model solution when the flavan-3-ol was a monomer,[215] suggesting that the chain length plays a role in the stabilization of A^+-F species. At pH values above 4, other products in which the flavanol was linked through its B ring to the A ring of malvidin 3-glucoside were formed.[215] The involvement of the B ring indicates that these molecules result from nucleophilic addition of the anthocyanin to the catechin *o*-quinone and thus from oxidative reactions, as described in Section 3.26.4.5.1.

The flavylium A^+-F adducts are also proposed to yield yellow xanthylium salts (**115**), through dehydration and rearomatization steps.[269,315–317] However, the formation of these pigments has never been confirmed. Structural identification of the yellow pigments formed from the reaction of catechin with malvidin 3-glucoside has been recently achieved.[215] They are actually xanthylium salts derived from the A-F flavene but their structure (**116**) is somewhat different from that originally postulated. Their formation process involves opening and cleavage of the anthocyanin C ring.

Alternatively, the A-F flavene can rearrange to a structure in which both units are linked by an additional ether bond between the C2 of the anthocyanins and the C7 of the flavan-3-ol (A-type bond (**117**)).[318,319] The products obtained in model solutions containing catechin and malvidin 3,5 diglucoside[318] or malvidin 3-glucoside[319] have been formally identified. The malvidin 3-glucoside flavan-3-ol derivatives have been detected in wine, both as dimers (A-F) and as larger oligomers (A-F_n, with $n > 1$).[320–322] In contrast, the formation of flavylium and xanthylium forms in wine remains to be established.

3.26.4.5.4(ii) Flavan-3-ol-anthocyanin adducts Flavan-3-ol-anthocyanin (F-A) adducts, also called tannin-anthocyanin (T-A) adducts, are formed by the inverse reaction in which the anthocyanin (as the nucleophilic hemiketal, AOH (**81**)) adds through its C8 (or C6) position onto the carbocation (**51**) arising from cleavage of the interflavanic linkage of a proanthocyanidin. Unlike the previous one, this reaction, proposed by Ribéreau-Gayon in 1982,[142] is thus restricted to flavan-3-ol oligomers. The F-AOH hemiketal (**118**) can then undergo dehydration and protonation to yield the corresponding flavylium (F-A^+ (**119**)).

Random breaking and making of interflavanic linkages were proposed in 1980 to explain the changes in proanthocyanidin chain length occurring during wine aging[199] and confirmed more recently.[323] Each cleavage produces a carbocation from the upper part of the molecule and a flavan-3-ol from its lower part. Nucleophilic addition of another flavan-3-ol to the carbocation results in a new flavan-3-ol polymer. If the solution contains an excess of monomers, the average chain length gradually decreases. Similarly, formation of F-A adducts actually includes replacing the lower part of a given proanthocyanidin polymer by an anthocyanin unit, thus decreasing or maintaining the number of units in the polymeric chain.

F-A^+ adducts have been observed in model solution at pH 2 but not at pH 3.8, consistent with their acid-catalyzed formation mechanism.[314] However, their presence in red wine, as dimers and larger oligomers, is well established.[211,320–322,324,325]

3.26.4.5.4(iii) Anthocyanin polymers Anthocyanin dimers arising from nucleophilic addition of one anthocyanin (in the hemiketal form) to another (in the flavylium form) have been detected in wine fractions obtained by countercurrent chromatography.[321] The mass spectrometry data obtained for these molecules suggest that the upper unit is in the flavene form, as expected from the addition mechanism, while the lower unit is a flavylium cation. However, their resistance toward thiolysis suggested that they may also be A-type anthocyanin dimers obtained by rearrangement of the flavene. A series of anthocyanin dimers and trimers based on the same structure were also detected in grape skin extracts, suggesting that they may form *in vivo*.[147]

3.26.4.5.5 Other nucleophilic addition reactions

Flavan-3-ol and anthocyanins (in their hemiketal form) can similarly add to other electrophiles. Nucleophilic addition of flavan-3-ols[241] and of anthocyanins[216] to catechin quinone arising from autoxidation has been observed in model solutions (see Sections 3.26.4.5.1 and 3.26.4.5.4). However, the resulting products have not

been detected in wine, presumably due to competition with other reactions such as oxidation of ethanol to acetaldehyde and condensation reactions. In contrast, anthocyanin caffeoyltartaric acid adducts have been found in Gamay wines.[326] These adducts are formed by nucleophilic addition of anthocyanins to the caffeoyltartaric acid quinone generated by enzymatic oxidation during must preparation (see Section 3.26.4.3.2). The carbocation formed by protonation and dehydration of vescalagin, in mildly acidic condition, has also been shown to react with flavan-3-ols, to form complex tannin structures called acutissimin and epiacutissimin.[327] Anthocyanins can replace flavan-3-ols in this reaction. However, no evidence of any of these ellagitannin derivatives has been provided in wine.

3.26.4.5.6 Influence of the wine-making process on polyphenol reactions

The relative importance of the various mechanisms and the proportions of the different products described above depend on many factors. They are primarily determined by the concentration of the precursors, and in particular the concentration of anthocyanins and proanthocyanidins that participate in most reactions and compete in some of the reactions. The tannin to anthocyanin ratio depends on the grape variety, on grape maturity at harvest, and on the extraction rate of tannins and anthocyanins, which is greatly influenced by the winemaking process as detailed above (see Section 3.26.4.3.1). The relative proportion of anthocyanins and flavan-3-ols affects the nature and size of products. Anthocyanins alone appear to be very unstable.[314] Some of them proceed to more stable anthocyanin polymers but a rather large part are lost through cleavage and rearrangement reactions. In the presence of flavan-3-ols, they are converted to different types of anthocyanin-flavan-3-ol derivatives. In the absence of anthocyanins, reactions of proanthocyanidins lead to polymerization and browning while excess amounts of anthocyanins increase the formation of lower molecular weight flavanol anthocyanin adducts. Finally, additional precursors, including various yeast metabolites, are required for some of the reactions. The concentrations of volatile phenols, pyruvic acid, and acetaldehyde depend on the yeast strain and the fermentation conditions.

Among other factors, pH and the extent of oxygen exposure (together with the presence of oxidation catalysts such as metal ions) are particularly important. pH controls the rate of several reactions such as aldehyde protonation and acid-catalyzed cleavage of interflavanic and methylmethine bonds. Moreover, it determines the ratio of the flavylium and hemiketal forms of anthocyanins that serve as precursors in different reaction pathways. Thus, pyranoanthocyanins arising from flavylium cations and acetaldehyde condensation products are more efficiently formed at lower pH values. Formation of flavan-3-ol-anthocyanin adducts is also favored in acidic solutions, meaning that it is limited by the rate of proanthocyanidin cleavage rather than by the availability of anthocyanins in the hemiketal form. However, their concentration was highest in wines containing higher amounts of anthocyanins and, for a given variety, it increased, like the ratio of flavanols to anthocyanins, with the extent of extraction.[211]

Enzymatic oxidation is very important in white wine making and usually results in a marked decrease in polyphenol content (see Section 3.26.4.3.2). In contrast, it is rather limited in red wine making as the oxygen introduced in the pumping over operations is mainly consumed by the fermenting yeasts. Chemical oxidation is promoted by oxygen exposure throughout the wine aging. Microoxygenation is becoming a common practice. This involves adding oxygen continuously in quantities low enough to ensure that the rate of consumption exceeds that of dissolution, to mimic the situation encountered in barrel aging. Some more oxygen can be introduced in transfers and at bottling if no care is taken to avoid it. Finally, variable amounts of oxygen can be allowed through the closures.[328] Monitoring of wine phenolic composition over a 7-month storage period showed that microoxygenation induced higher levels of methylmethine-linked pigments while pyranoanthocyanins appeared to be associated with aging.[307] As mentioned above, methylmethine-linked pigments result from condensation with acetaldehyde, which is an oxidation product of ethanol. These products are rather unstable and proceed to pyranoanthocyanins, which can serve as markers of oxidation. Similarly, carboxypyranoanthocyanins result from the reaction of anthocyanins with pyruvic acid, a yeast metabolite that can also be formed by the oxidation of lactic acid.

It is worth emphasizing that, although much knowledge has been acquired on the reactivity of phenolic compounds, all products detected account only for a small proportion of those actually formed in wine, and that a great majority cannot actually be analyzed by the available methods. It is generally accepted that the lower molecular weight compounds that can be detected and assayed are markers of large families of compounds

formed from the whole series of anthocyanins and/or of proanthocyanidins by the same reactions. Nevertheless, determination of the wine phenolic composition remains a major challenge.

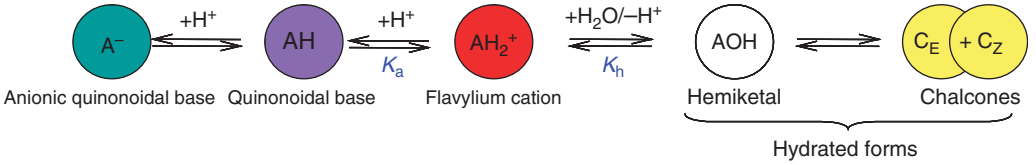
3.26.4.6 Influence of Structures and Reactions on Wine Quality

Phenolic compounds are responsible for the color of red and rose wines and play a role in the oxidative browning of white musts and wines. They also contribute to wine flavor and mouthfeel properties. Some phenolic compounds are volatile odorants or react with other volatile compounds while others are bitter. Moreover, tannins are involved in astringency perception, which is attributed to their interaction with salivary proteins.

3.26.4.6.1 Color properties

The color properties of a molecule depend on the extent of electron delocalization within its structure. Thus, phenolic compounds comprise colorless molecules such as flavan-3-ols, benzoic acids (λ_{max} 280 nm), and hydroxycinnamic acids (λ_{max} 310–325 nm) but also yellow pigments (e.g., flavonols, chalcones, and xanthylium salts, λ_{max} 340–360 nm) and red to blue anthocyanins (λ_{max} 510–560 nm), due to increasing conjugation of the phenolic rings with the adjacent parts of the structure.

As described in Section 3.26.4.2.4, anthocyanins are usually represented as their red flavylium forms but they exist in different forms showing different colors. For a given anthocyanin, the proportion of these different forms is driven by pH.



The quinonoidal bases are the kinetic products formed from the flavylium at pH 3.5 and above. However, they are thermodynamically unstable and can be converted, through the flavylium form, to the hemiketal and chalcone, which are the thermodynamic products. Thus, determination of the hydration constant is the best estimation of anthocyanin color stability in water. K_h and K_a can be determined by UV–visible spectrophotometry, using two series of physicochemical experiments, corresponding to thermodynamic and kinetic measurements.[329] Based on these constants, at pH values of wine (3.2–4.0), grape anthocyanins should be largely colorless (>70%). The color of red and rose wines is ensured through two stabilization mechanisms. On the one hand, a process referred to as copigmentation displaces the hydration equilibrium, as explained above (see Section 3.26.4.2.4).[194,195] Selective trapping of the colored species in copigmentation complexes formed by vertical stacking with other planar molecules (copigments) results in enhanced intensity (hyperchromic effect), due to higher concentrations of the flavylium ions.[330,331] Besides, a slight bathochromic effect is usually observed. On the other hand, derived pigments resulting from reactions of anthocyanins (see Section 3.26.4.4) may show different chemical and color properties. In particular, substitutions of the C ring impede addition of water or sulfite to the flavylium ions. Thus, pyranoanthocyanins have been shown to be resistant to sulfite bleaching.[303,332] Their spectra in the visible range also show a hypsochromic shift compared to those of anthocyanins, and they are thus pink orange rather than red or purple.[296,302–304] In contrast, portisins, which absorb at 575 nm, appear much bluer than genuine anthocyanins.[293]

The hydration and protonation constants of some anthocyanin-derived pigments have been determined.[333] The pK_h of a dimeric F-A adduct (2.6) is similar to that of its anthocyanin precursor (2.8), meaning that it is mostly colorless at the pH of wine. In contrast, pigments resulting from condensation of anthocyanins with catechin and acetaldehyde show higher pK_h (4.2) and lower pK_a (3.3 and 3.5) values than the anthocyanin. Consequently, they remain colored in a wide pH range and show a blue tint, reflecting the larger proportion of quinonoidal bases. The ethyl-linked anthocyanin dimer also appears blue at wine pH values. Its hydration constants were determined at 1.8 and 4.6, meaning that, under mildly acidic conditions, one of the anthocyanin moieties is hydrated while the second one is not.[334]

Thus, F-A adducts probably show little contribution to wine color, unless they are involved in copigmentation, while the formation of acetaldehyde condensation products enhances and stabilizes color. The particular behavior of ethyl-linked anthocyanins is attributed to self-association, which protects the molecule from hydration and facilitates deprotonation.[333,335] Copigmentation experiments showed that the color of catechin-malvidin 3-glucoside is much enhanced in the presence of chlorogenic acid while that of catechin-ethyl-malvidin 3-glucoside remains unchanged.[333]

3.26.4.6.2 Colloidal properties and haze

Phenolic compounds are prone to interact among themselves and with other wine constituents such as metals, proteins, and polysaccharides. These interactions are driven by hydrophobic effect and hydrogen bonding. Depending on the molecules involved and the composition of the medium, these phenomena may lead to the formation of colloidal or insoluble particles.

3.26.4.6.2(i) Self-association of phenolic compounds Self-association of anthocyanins, as well as their association with various phenolic copigments, including flavonols, phenolic acids, and flavan-3-ols, is well documented and plays a major role in color properties, as described above. However, to our knowledge, it has not been reported to lead to colloidal instability.

Self-association has also been observed with all flavan-3-ols tested, from monomers to polymers,[336–339] but the formation of aggregates appeared to be restricted to molecules containing at least three phenolic rings (or two *o*-diphenolic rings).[338] This structural feature is required for the establishment of bridges between two other phenolic molecules.[336] Self-association of galloylated monomers,[340] procyanidins,[339] and oligomers formed by condensation of catechin with acetaldehyde,[341] but not of catechin or epicatechin,[340] resulted in the formation of metastable colloidal particles in wine-like hydroalcoholic medium. Aggregation of proanthocyanidins increased with their average DP (up to 5 and 10, for nongalloylated proanthocyanidins and galloylated procyanidins, respectively). It then decreased for larger polymers, which appear more water soluble. Size and polydispersity of the aggregates increased with flavanol concentration and ionic strength and decreased as the ethanol content increased.[340] Self-association constants recorded for epicatechin were 5 times weaker in 10% ethanol[337] than in water,[336] and no aggregation of proanthocyanidins up to 5 g l^{-1} was observed in 20% ethanol. Finally, the formation of haze and precipitates in white wines has been attributed to aggregation of flavonol aglycones.[217]

3.26.4.6.2(ii) Interactions with proteins, polysaccharides, and glycoproteins Interactions between phenolic compounds and proteins have been extensively studied.[342–345] They rely upon both van der Waals–London interactions and hydrogen bonding.[346–349] Proline-rich protein sequences, encountered for instance in collagen, gelatin, or casein, which are commonly used as protein fining agents in wine, or in some salivary proline-rich proteins, which are reported to be involved in astringency perception, are particularly prone to interact with phenolic compounds. All phenolic compounds can theoretically precipitate proteins, if present in sufficient concentration.[350] Lower molecular weight compounds (e.g., phenolic acids, flavonols, and nongalloylated flavan-3-ol monomers) display moderate affinity for proteins. In contrast, the definition of tannins refers to the ability of these molecules to precipitate proteins.[344] Interaction and precipitation increase with the number of phenolic rings and thus with the DP and the presence of galloyl substituents.[338,342,350] The binding capacity of galloyl glucose esters with a proline-rich peptide increased with the number of galloyl rings.[349] Similarly, no interaction could be measured by isothermal titration calorimetry between catechin or epicatechin and poly-L-proline while association constants in the range of 10^4–10^5 mol l^{-1}, indicating relatively high affinity, were determined for galloylated monomers and procyanidin oligomers.[351] Oxidation of flavan-3-ol monomers, generating oligomeric species, also enhanced interactions with proteins.[352,353] Interaction does not necessarily lead to precipitation. A three-stage mechanism has been proposed for aggregation of flavan-3-ols with proteins, as the polyphenol concentration is increased: ligand binding and folding of the protein, formation of rather small metastable protein–polyphenol aggregates, and bridging of these aggregates leading to haze and precipitation.[354,355]

Protein concentrations in wines are usually below 100 mg l^{-1}[1356,357] but can reach 300 mg l^{-1} in unfined wines.[358] Wine proteins originate mostly from grape but yeast proteins have also been detected. Fining treatments, which involve adding an exogenous protein, such as gelatin, casein, or ovalbumin, are commonly applied to precipitate out tannins, reduce astringency, and improve stability. These treatments result in selective precipitation of higher molecular weight tannins.[359–361] In addition, part of the tannin–protein complexes are soluble[362] so that part of the fining proteins actually remain in the finished wine.

Interactions of phenolic compounds with polysaccharides have also been reported. Polysaccharides and glycoproteins, released from the cell walls of both grapes and yeast, are found in wines in concentrations ranging from 0.2 to 1 g l^{-1}.[363] Arabinogalactans (AGs) and arabinogalactan proteins (AGPs), originating from grapes, are the major polysaccharides in wines.[364,365] Rhamnogalacturonan II (RGII), another grape cell wall constituent containing rare sugars, is also very abundant in wines as it resists enzymatic degradation.[366] Its concentration increases during maceration and is thus much higher in red wines than in white wines. Small amounts of rhamnogalacturonan I and arabinans have also been reported.[365] Yeast polysaccharides consist of mannans and mannoproteins, which are excreted during fermentation or released after autolysis. Among wine polysaccharides, mannoproteins and some arabinogalactan proteins form small and stable particles with procyanidins[339] by a steric stabilization mechanism.[367] In contrast, RGII enhances their aggregation.[341] Polysaccharides also limit the precipitation of tannin protein complexes,[213,346,349] and mannoproteins contribute to protein haze stabilization.[368,369] Gum arabic (a mixture of arabinogalactans and arabinogalactan proteins) is commonly added to prevent aggregation, flocculation, and precipitation of tannins and tannin–protein complexes.[370] However, haze development can be observed with higher doses.[371,372]

3.26.4.6.3 *Flavor and mouthfeel properties*

The contribution of phenolic compounds to wine aroma has been described in Section 3.26.2. In addition, reactions of phenolic compounds with volatile compounds can alter the aroma composition of wines. A particular example of such processes is trapping of odorant vinylphenol through formation of nonvolatile phenyl-pyranoanthocyanins. Reactions of aldehydes with phenolic compounds or addition of sulfur compounds to quinones is also potentially important although their role in the evolution of wine flavor has not yet been established.

Phenolic compounds contribute bitterness and astringency.[373] The former is a taste involving interaction of the molecule with a taste receptor, while the latter is a tactile sensation, resulting from precipitation of salivary proteins and/or adsorption on the mouth epithelium. Bitterness is restricted to rather small molecules such as phenolic acids and their ethyl esters, flavonols, and flavan-3-ol monomers and oligomers that can enter the receptor.[374–377] In contrast, astringency is imparted by higher molecular weight flavan-3-ols that show higher affinity for proteins.

In the flavan-3-ol series, bitterness decreases and astringency increases as the molecular weight increases from monomers to trimers.[375,378] Larger molecular weight proanthocyanidins (above DP 10) were classically considered insoluble and nonastringent[375,379] but recent studies have established that they are actually soluble in wine-like hydroalcoholic solutions[380] and are present in wine.[361] Astringency of proanthocyanidin fractions increases with their chain length and with the extent of galloylation.[380] Selective precipitation of higher molecular weight tannins may explain the reduction of astringency induced by protein fining treatments. However, this may also be due to the inclusion of tannins in soluble protein complexes, which would impede their interaction with salivary proteins. Similar results were obtained in commercial wines, with astringency perception increasing with proanthocyanidin concentration, but also with their average degree of polymerization and galloylation.[381] Longer maceration, resulting in higher levels of proanthocyanidins, and especially of galloylated procyanidins from seeds, is thus expected to enhance wine astringency.

The loss of astringency observed during wine aging is usually ascribed to reactions of tannins but the role of various types of reactions in taste remains to be determined. Oligomers formed from catechin by oxidation or condensation with acetaldehyde interact with proteins and were perceived astringent[382] in the same way as proanthocyanidins. In contrast, wine fractions containing derived pigments did not exhibit astringency.[383] Detailed analysis of a similar fraction allowed detection of lower molecular weight pyranoanthocyanins, anthocyanin oligomers, and A-type anthocyanin-flavanol adducts.[321] No information is available on the taste of other types of flavanol-derived pigments. Whether the loss of astringency is due to a decrease in the average chain length of proanthocyanidins or due to the incorporation of anthocyanin units in the proanthocyanidin structures is still unknown.

Moreover, perception of taste, and perception of astringency in particular, is influenced by the presence of other components of wine. In particular, astringency intensity increases with acidity and ionic strength while bitterness intensity increases with ethanol level.[383,384] Anthocyanins and mannoproteins did not modify the perception of a grape seed tannin fraction but RGII decreased its astringency.[366]

3.26.5 Conclusion and Future Prospects

Much progress has been made in the understanding of wine composition and the chemical and biochemical processes involved in its evolution during winemaking and aging. In some cases, a given compound or reaction can be related to a particular organoleptic feature. For instance, the odor characteristics of a number of volatile constituents as well as the color properties of various pigments originating from grape or formed in wine have been determined. However, there are major gaps in our knowledge of the relationships existing between wine composition and its quality.

Analysis of wine composition remains a major challenge. Analytical tools need to be developed, on the one hand, to characterize and assay compounds that are present in trace amounts but may have important organoleptic impact, and, on the other hand, to determine the large variety of wine polyphenols. Indeed, while phenolic monomers and oligomers can be analyzed by HPLC, the heterogeneity of polymeric compounds, which increases with their chain length and as they react further, leads to poor resolution of components in all separation techniques. The reaction products obtained in model solutions and/or detected in wine are rather simple molecules that can serve as markers of specific reactions and give access to qualitative information on the 'history of wine', but do not reflect the overall composition of wine. A particular objective is to characterize the distribution of molecular species (different molecular weights, units or sequences, branched or linear, etc.) within a given polymer fraction and relate it to its organoleptic properties. Efforts are also aimed at developing high-throughput methods enabling fingerprinting of a large number of samples for quality control or wine-making process management.

Finally, it should be emphasized that such a complex system cannot be approached as the sum of its individual components as interactions between them often result in increasing, decreasing, or modifying the effect that they have separately.

Abbreviations

AG	arabinogalactan
AGP	arabinogalactan protein
AOH	anthocyanin hemiketal
DMS	dimethyl sulfide
DMSO	dimethyl sulfoxide
DP	degree of polymerization
EAS	electrophilic aromatic substitution
GRP	grape reaction product
HMF	hydroxymethyl furfural
HPLC	high-performance liquid chromatography
IFL	interflavanic linkage
NMR	nuclear magnetic resonance
PPO	polyphenoloxidase
RGII	rhamnogalacturonan II
RO·	oxyl radical
ROO·	peroxyl radical
SMM	*S*-methylmethionine

Nomenclature

3MH	3-mercaptohexanol
4MMP	4-methyl-4-mercaptopentan-2-one
4MMPOH	4-methyl-4-sulfanylpentan-2-ol
A^+-F	anthocyanin flavylium-flavan-3-ol
Ac3MH	3-mercaptohexyl acetate
A-F	anthocyanin-flavan-3-ol
A-T	anthocyanin-tannin
C10:0	capric acid
C16:0	palmitic acid
C16:1	palmitoleic acid
C18:0	stearic acid
C18:1	oleic acid
C6:0	caproic acid
C8:0	caprylic acid
E°	redox potential
F-A	flavan-3-ol-anthocyanin
F-AOH	flavan-3-ol-anthocyanin hemiketal
Fe^{2+}	ferrous anion
H_2O_2	hydrogen peroxide
H_2S	hydrogen sulfide
SO_2	sulfide
SO_4^{2-}	sulfate
TDN	1,1,6-trimethyl-1,2-dihydronaphthalene
TPB	(*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene

References

1. A. Pena; G. Cinco; A. Gomez-Puyon; M. Tuena, *Arch. Biochem. Biophys.* **1972**, *153*, 413–425.
2. J. Ribéreau-Gayon; E. Peynaud; M. Lafon, *Am. J. Enol. Vitic.* **1956**, *7*, 53–61.
3. J. Ribéreau-Gayon; E. Peynaud; M. Lafon, *Am. J. Enol. Vitic.* **1956**, *7*, 112–118.
4. R. P. Jones, *FEMS Microbiol. Rev.* **1988**, *54*, 239–258.
5. H. Holzer; W. Bernhardt; S. Scheide, *Biochem. Z.* **1963**, *336*, 495–509.
6. E. Oura, *Process Biochem.* **1977**, *12*, 19–21.
7. S. Derrick; P. J. Large, *J. Gen. Microbiol.* **1993**, *139*, 2783–2792.
8. A. Rapp; G. Versini, In *Influence of Nitrogen Compounds in Grapes on Aroma Compounds of Wine*, Proceedings of the International Symposium on Nitrogen in Grapes and Wines, Seattle, WA, USA, 1991; J. Rantz, Ed.; American Society for Enology and Viticulture: Davis, CA, 1991; pp 156–164.
9. C. Ancin; B. Ayestaran; M. Corroza; J. Garrido; A. Gonzalez, *Food Chem.* **1996**, *55*, 241–249.
10. E. C.-H. Chen, *J. Am. Soc. Brew. Chem.* **1978**, *36*, 39–43.
11. L. Nykänen, *Am. J. Enol. Vitic.* **1986**, *37*, 84–96.
12. K. Nordström, *Svensk Kem. Tidskr.* **1964**, *76*, 510–543.
13. J. R. M. Hammond; J. M. Pye, *Yeast Newslett.* **1996**, *45*, 12–12.
14. P. A. Thurston; P. E. Quain; R. S. Tubb, *J. Inst. Brew.* **1982**, *88*, 90–94.
15. T. Shinoara, *Bull. O.I.V.* **1984**, *641–642*, 606–618.
16. P. Dubois, *Rev. Fr. Oenol.* **1994**, *145*, 27–40.
17. P. Romano; G. Suzzi, *FEMS Microbiol. Lett.* **1993**, *108*, 23–26.
18. P. Romano; G. Suzzi; V. Brandolini; E. Menziani; P. Domizio, *Lett. Appl. Microbiol.* **1996**, *22*, 299–302.
19. E. Geiger; A. Piendl, *Brew. Dig.* **1975**, *67*, 50–63.
20. A. D. Haukeli; S. Lie, *J. Inst. Brew.* **1972**, *78*, 229–232.
21. L. F. Chuang; E. B. Collins, *J. Bacteriol.* **1968**, *95*, 2083–2089.
22. E. B. Collins, *J. Dairy Sci.* **1972**, *55*, 1022–1028.
23. H. H. Dittrich; R. Eschenbruch, *Arch. Mikrobiol.* **1968**, *63*, 63–69.
24. W. Dott; M. Heinzel; H. G. Trüper, *Arch. Microbiol.* **1976**, *107*, 283–285.
25. R. Eschenbruch; P. Bonish; B. M. Fisher, *Vitis* **1978**, *17*, 67–74.

26. M. A. Heinzel; W. Dott; H. G. Trüper, *Wein-Wissenschaft* **1979**, *34*, 192–211.
27. H. Gyllang; M. Winge; C. Korch, In *Regulation of Hydrogen Sulfide Formation During Fermentation*, Proceedings of the 22nd European Brewery Convention (EBC), Zürich, Switzerland, 1989; pp 347–354.
28. P. C. Hartnell; D. J. Spedding, *Vitis* **1979**, *18*, 307–315.
29. P. R. Monk, *Wine Ind. J.* **1986**, *November,* 10–16.
30. B. C. Rankine, *Vitis* **1968**, *7*, 22–49.
31. P. A. Henscke; V. Jiranek, In *Hydrogen Sulfide Formation During Fermentation: Effect of Nitrogen Composition in Model Grape Must*, Proceedings of the International Symposium on Nitrogen in Grapes and Wines, Seattle, WA, USA 18–19 June 1991; American Society of Enology and Viticulture.
32. M. Stratford; A. H. Rose, *J. Gen. Microbiol.* **1985**, *131*, 1417–1424.
33. K. Wenzel; H. H. Dittrich; H. P. Seyffardt; J. Bohnert, *Wein-Wissenschaft* **1980**, *35*, 414–420.
34. M. A. Ségurel; A. Razungles; C. Riou; M. G. Trigueiro; R. L. Baumes, *J. Agric. Food Chem.* **2004**, *53*, 2637–2645.
35. A. Anocibar Beloqui; Y. Kotseridis; A. Bertrand, *J. Int. Sci. Vigne Vin* **1998**, *30*, 167–170.
36. O. A. Leppänen; J. Denslow; P. P. Ronkainen, *J. Inst. Brew.* **1979**, *85*, 350–353.
37. P. Cabras; M. Meloni; F. M. Pirisi, *Rev. Environ. Contam. Toxicol.* **1987**, *99*, 83–117.
38. T. Wainwright; J. F. Mc Mahon; J. Mc Dowell, *J. Sci. Food Agric.* **1972**, *23*, 911–914.
39. O. J. Goniak; A. C. Noble, *Am. J. Enol. Vitic.* **1987**, *38*, 223–227.
40. R. A. Bobet; A. C. Noble; R. B. Boulton, *J. Agric. Food Chem.* **1990**, *38*, 449–452.
41. S. Matsuhi; M. Amaha, *Agric. Biol. Chem.* **1981**, *45*, 1341–1349.
42. D. Dubourdieu; T. Tominaga; I. Masneuf; C. Peyrot des Gachons; M. L. Murat, *Am. J. Enol. Vitic.* **2006**, *57*, 81–88.
43. J. H. Swiegers; E. J. Bartowsky; P. A. Henschke; S. Pretorius, *Aust. J. Grape Wine Res.* **2005**, *11* (2), 139–173.
44. M. Subileau; R. Schneider; J. M. Salmon; E. Degryse, *FEMS Yeast Res.* **2008**, *8* (5), 771–780.
45. S. Krieger; G. Trioli; L. Dulau, Bacteria and Wine Quality – State of the Art, 2000. http://www.lallemandwine.us/library/tech_articles.php.
46. F. Drawert, Formation Des Arômes À Différents Stades De L'èvolution Du Fruit. Enzymes Intervenant Dans Cette Formation. In *Facteurs et régulation de la maturation des fruits*; Colloques Internationaux CNRS: Paris, 1975; pp 309–319.
47. C. Bayonove, L'arome Variètal: Le Potentiel Aromatique Du Raisin. In *Œnologie, Fondements Scientifiques et Technologiques*; C. Flanzy Ed.; Tec & Doc Lavoisier: Paris, 1998; pp 165–181.
48. C. Bayonove; R. Cordonnier; P. Bubois, *C. R. Acad. Sci. Paris* **1975**, *D-281*, 75–78.
49. C. Wood; T. E. Siebert; M. Parker; D. L. Capone; G. M. Elsey; A. P. Pollnitz; M. Eggers; M. Meier; T. Vössing; S. Widder; G. Krammer; M. A. Sefton; M. J. Herderich, *J. Agric. Food Chem.* **2008**, *56* (10), 3738–3744.
50. J. Crouzet; C. Flanzy, Les Enzymes En Oenologie. In *Œnologie, Fondements Scientifiques et Technologiques*; C. Flanzy, Ed.; Tec & Doc Lavoisier: Paris, 1998; pp 362–413.
51. R. Cordonnier; C. Bayonove, *Connaiss. Vigne Vin* **1981**, *15* (4), 269–286.
52. P. Chatonnet; D. Dubourdieu; J. N. Boidron; V. Lavigne, *J. Sci. Food Agric.* **1993**, *62*, 191–202.
53. I. Dugelay; Z. Günata; J. C. Sapis; R. Baumes; C. Bayonove, *J. Agric. Food Chem.* **1993**, *41* (11), 2092–2096.
54. R. Cordonnier; C. Bayonove, *C. R. Acad. Sci. Paris* **1974**, *D-278* (26), 3387–3390.
55. A. Delcroix; Z. Günata; J. C. Sapis; J. C. Sapis; J. M. Salmon; C. Bayonove, *Am. J. Enol. Vitic.* **1994**, *45*, 291–296.
56. Z. Günata; C. Bayonove; R. Baumes; R. E. Cordonnier, *Am. J. Enol. Vitic.* **1986**, *37*, 112–114.
57. N. Loscos; P. Hernandez-Orte; J. Cacho; V. Ferreira, *J. Agric. Food Chem.* **2007**, *55* (16), 6674–6684.
58. P. Hernandez-Orte; M. Cersosimo; N. Loscos; J. Cacho; E. Garcia Moruno; V. Ferreira, *Food Chem.* **2008**, *107* (3), 1064–1077.
59. S. Mathieu; N. Terrier; J. Procureur; F. Bigey; Z. Günata, *J. Exp. Bot.* **2005**, *56* (420), 2721–2731.
60. R. Baumes; J. Wirth; S. Bureau; Y. Gunata; A. Razungles, *Anal. Chim. Acta* **2002**, *458* (1), 3–14.
61. Y. Kotseridis, Etude De L'arôme Des Vins De Merlot Et Carbernet-Sauvignon De La Région Bordelaise. Ph.D. Thesis, University of Bordeaux II, 1999; 268pp.
62. M. Ségurel; A. Razungles; C. Riou; M. G. Trigueiro; R. L. Baumes, *J. Agric. Food Chem.* **2005**, *53* (7), 2637–2645.
63. R. Schneider; A. Razungles; F. Charrier; R. Baumes, *J. Chromatogr. A* **2001**, *936* (1–2), 145–157.
64. V. Ferreira; N. Ortín; A. Escudero; R. López; J. Cacho, *J. Agric. Food Chem.* **2002**, *50* (14), 4048–4054.
65. T. Tominaga; I. Masneuf; D. Dubourdieu, *J. Int. Sci. Vigne Vin* **1995**, *29* (4), 227–232.
66. T. Tominaga; C. Peyrot des Gachons; D. Dubourdieu, *J. Agric. Food Chem.* **1998**, *46*, 5215–5219.
67. C. Peyrot des Gachons; T. Tominaga; D. Dubourdieu, *J. Agric. Food Chem.* **2002**, *50* (14), 4076–4079.
68. R. Schneider; F. Charrier; A. Razungles; R. Baumes, *Anal. Chim. Acta* **2006**, *536*, 58–64.
69. C. S. Du Plessis; G. J. Loubster, *Agrochemophysica* **1974**, *6*, 49–52.
70. P. X. Etiévant, Wine. In *Volatile Compounds in Food and Beverages*; H. Maarse, Ed.; Marcel Dekker: New York, Basel, Honk-Hong, 1991; pp 19–33.
71. L. Dagan, Potentiel Aromatique Des Raisins De Vitis Vinifera L. Cv. Petit Manseng et Gros Manseng. Contribution À L'arôme Des Vins De pays Côtes De Gascogne. Ph.D. Thesis, Ecole Nationale Supérieure d'Agronomie de Montpellier, 2006; 225pp.
72. A. Escudero; E. Campo; L. Fariña; J. Cacho; V. Ferreira, *J. Agric. Food Chem.* **2007**, *55* (11), 4501–4510.
73. N. Loscos; M. Ségurel; L. Dagan; N. Sommerer; T. Marlin; R. Baumes, *Anal. Chim. Acta* **2007**, *621* (1), 24–29.
74. L. Culleré; A. Escudero; J. P. Pérez-Trujillo; J. Cacho; V. Ferreira, *J. Food Composit. Anal.* **2008**, *21* (8), 708–715.
75. L. Nykanen, *Am. J. Enol. Vitic.* **1986**, *37*, 84–86.
76. D. Schulthess; L. Ettlinger, *J. Inst. Brew.* **1978**, *84*, 240–243.
77. B. Ferreira; C. Hory; M. H. Bard; C. Taissant; A. Olsson; Y. Le Fur, *Food Qual. Prefer.* **1995**, *6*, 35–41.
78. B. Pineau; J. C. Barbe; C. Van Leeuwen; D. Dubourdieu, *J. Agric. Food Chem.* **2007**, *55* (10), 4103–4108.
79. D. D. Ramey; C. S. Ough, *J. Agric. Food Chem.* **1980**, *28*, 923–934.
80. J. Marais, *Vitis* **1978**, *17*, 394–403.
81. A. Garofolo; A. Piracci, *Bull. O.I.V.* **1994**, *757–758*, 225–245.
82. M. C. Díaz-Maroto; R. Schneider; R. Baumes, *J. Agric. Food Chem.* **2005**, *53*, 3503–3509.
83. A. Rapp; M. Guntert; M. Ulmeyer, *Z. Lebensm. Unters. Forsch.* **1985**, *180*, 109–116.

84. R. F. Simpson, *Vitis* **1979**, *18*, 148–154.
85. R. F. Simpson; G. C. Miller, *Vitis* **1983**, *22*, 51–63.
86. R. Di Stefano; M. Castino, *Riv. Vitic. Enol.* **1983**, 245–259.
87. J. Marais; C. J. Van Wyk; A. Rapp, *S. Afr. J. Enol. Vitic.* **1992**, *13* (1), 33–44.
88. P. Winterhalter, The Generation of C13-Norisoprenoid Volatils. In *Connaissance aromatique des cépages et qualité des vins*; C. Bayonove, J. Crouzet, C. Flanzy, J. C. Martin, J. C. Sapis, Eds.; Revue Française d'œnologie: France, 1993; pp 65–73.
89. R. Di Stefano, *Riv. Vitic. Enol.* **1989**, 11–23.
90. G. Skouroumounis; A. Massy-Westropp; M. A. Sefton; P. Williams, *Tetrahedron Lett.* **1992**, *33*, 3533–3536.
91. M. A. Sefton; I. L. Francis; P. J. Williams, *Am. J. Enol. Vitic.* **1993**, *44*, 359–370.
92. G. Versini; A. Rapp; J. Marais; F. Mattivi; M. Spraul, *Vitis* **1996**, *35*, 15–21.
93. H. L. Wildenradt; V. L. Singleton, *Am. J. Enol. Vitic.* **1974**, *25*, 119–126.
94. A. Baro; J. Carrasco, *Bull. O.I.V.* **1977**, *554*, 253–267.
95. K. Otsuka; I. Iki; S. I. Nozu; Y. Limura; A. Totsuka, *J. Ferment. Technol.* **1980**, *58*, 353–361.
96. C. Charpentier; T. T. Pham; E. Guichard, *et al.*, Production Du Sotolon Par Les Levures Isolées Des Vins Jaunes Du Jura. In Proceedings of the 5e Symposium International d'Oenologie, Bordeaux, France, 1995; L.-F. Aline, Ed.; Tec & Doc Lavoisier: Paris, 1995; pp 179–182.
97. M. L. Brock; R. E. Kepner; A. D. Webb, *Am. J. Enol. Vitic.* **1984**, *35*, 151–155.
98. P. Dubois, *Rev. Fr. Oenol.* **1984**, *146*, 39–50.
99. A. A. Williams; J. L. Mervyn; H. V. May, *J. Sci. Food Agric.* **1983**, *34*, 311–319.
100. G. De Revel; C. Mendes Moreira; T. Hogg, *et al.*, Etude Des Composés Dicarbonylés Au Cours Des Fermentations Alcoolique Et Malolactique. In Proceedings of the 5e Symposium International d'Oenologie; Bordeaux, France, 1995; L.-F. Aline Tec & Doc Lavoisier: Paris, 1995; pp 419–423.
101. P. Etiévant, *Lebensm. Wiss. Technol.* **1979**, *12*, 115–120.
102. R. Schneider; R. Baumes; C. Bayonove; A. Razungles, *J. Agric. Food Chem.* **1998**, *46*, 3230–3237.
103. P. J. Williams; C. R. Strauss, *J. Inst. Brew.* **1978**, *84*, 144–147.
104. E. Guichard; T. T. Pham; C. Charpentier, *Rev. Oenol.* **1997**, *82*, 32–34.
105. T. T. Pham; E. Guichard; C. Charpentier, Conditions Optimales De Formation Du Sotolon À Partir De L'acide a-Cétobutyrique Dans Le Vin Jaune Du Jura. In Proceedings of the 5e Symposium International d'Oenologie, Bordeaux, France, 1995; L.-F. Aline, Ed.; Tec & Doc Lavoisier: Paris, 1995; pp 613–616.
106. J. V. Aiken; A. C. Noble, *Vitis* **1984**, *23*, 27–36.
107. P. Chatonnet; J. N. Boidron; M. Pons, *Sci. Aliments* **1990**, *10*, 565–587.
108. I. L. Francis; M. A. Sefton; P. J. Williams, *Am. J. Enol. Vitic.* **1992**, *43*, 23–30.
109. G. Masson; J. L. Puech; M. Moutounnet, *Bull. O.I.V.* **1986**, *785–786*, 635–657.
110. P. Dubois, *Rev. Fr. Oenol.* **1989**, *120*, 19–24.
111. J. N. Boidron; P. Chatonnet; M. Pons, *Connaiss. Vigne Vin* **1988**, *22*, 275–294.
112. M. Masuda; K. Nishimura, *Phytochemistry* **1971**, *10*, 1401–1402.
113. L. Blanchard, Recherche Sur La Contribution De Certains Thiols Volatils À L'arôme Des Vins Rouges. Etude De Leur Genèse Et De Leur Stabilitè. Ph.D. Thesis, University of Bordeaux II, 2001; 150pp.
114. P. Chatonnet; J. N. Boidron; D. Dubourdieu, *J. Int. Sci. Vigne Vin* **1993**, 27, 277–298.
115. P. Ribéreau-Gayon, Recherches Sur Les Anthocyanes Des Végétaux. Application Au Genre Vitis. Ph.D. Thesis, Faculté des Sciences de Bordeaux, 1959.
116. P. Ribéreau-Gayon, *C. R. Acad. Sci. Paris* **1964**, *258*, 1335–1337.
117. P. Ribéreau-Gayon, *C. R. Acad. Sci. Paris* **1965**, *260*, 341.
118. J. Masquelier, *Bull. Soc. Chim. Biol.* **1956**, *38*, 65.
119. J. Ribéreau-Gayon, *C. R. Acad. Agric.* **1957**, *43*, 197.
120. J. Ribéreau-Gayon; P. Ribéreau-Gayon, *Am. J. Enol. Vitic.* **1958**, *9*, 1–9.
121. J. D. Baranowski; C. W. Nagel, *J. Agric. Food Chem.* **1981**, *29*, 63–64.
122. B. Y. Ong; C. W. Nagel, *J. Chromatogr.* **1978**, *157*, 345–355.
123. T. C. Somers; E. Verette; K. F. Pocock, *J. Sci. Food Agric.* **1987**, *40*, 67–78.
124. Y. R. Lu; L. Y. Foo, *Food Chem.* **1999**, *65* (1), 1–8.
125. B. Baderschneider; P. Winterhalter, *J. Agric. Food Chem.* **2001**, *49*, 2788–2798.
126. A. Reschke; K. Herrmann, *Z. Lebensm. Unters. Forsch.* **1986**, *182*, 200–204.
127. S. Okamura; M. Watanabe, *Agric. Biol. Chem.* **1981**, *45*, 2063–2070.
128. F. Romeyer; J. C. Sapis; J. J. Macheix, *J. Sci. Food Agric.* **1985**, *36*, 728–732.
129. V. Cheynier; J. M. Souquet; M. Moutounet, *Am. J. Enol. Vitic.* **1989**, *40*, 320–324.
130. J. M. Boursiquot; J. C. Sapis; J. J. Macheix, *C. R. Acad. Sci. III* **1986**, *302*, 177.
131. L. W. Wulf; C. W. Nagel, *Am. J. Enol. Vitic.* **1978**, *29*, 42–49.
132. G. Mazza; E. Miniati, Grapes. In *Anthocyanins in Fruits, Vegetables and Grains*; G. Mazza, E. Miniati, Eds.; CRC Press: Boca Raton, FL, Ann Arbor, MI, London, Tokyo, 1993; pp 149–199.
133. A. A. Baldi; A. Romani; N. Mulinacci; F. Vincieri; B. Caseta, *J. Agric. Food Chem.* **1995**, *43*, 2104–2109.
134. S. Vidal; Y. Hayasaka; E. Meudec; V. Cheynier; G. Skouroumounis, *J. Agric. Food Chem.* **2004**, *52*, 713–719.
135. M. Monagas; C. Gómez-Cordovés; B. Bartolomé; O. Laureano; J. M. Ricardo da Silva, *J. Agric. Food Chem.* **2003**, *51*, 6475–6481.
136. C. Alcalde-Eon; M. Escribano-Bailon; C. Santos-Buelga; J. C. Rivas-Gonzalo, *Anal. Chim. Acta* **2006**, *563*, 238–254.
137. A. Heier; W. Blaas; A. Dross; R. Wittkowski, *Am. J. Enol. Vitic.* **2002**, *53*, 78–86.
138. M. M. Giusti; L. E. Rodriguez-Saona; D. Griffin; R. E. Wrolstad, *J. Agric. Food Chem.* **1999**, *47*, 4657–4664.
139. D. Favretto; R. Flamini, *Am. J. Enol. Vitic.* **2000**, *51*, 55–64.
140. J. P. Roggero; S. Coen; B. Ragonet, *Am. J. Enol. Vitic.* **1986**, *37*, 77–83.
141. J. P. Roggero; J. L. Larice; C. Rocheville-Divore; P. Archier; S. Coen, *Rev. Fr. Oenol.* **1988**, *112*, 41–48.

142. P. Ribéreau-Gayon, The Anthocyanins of Grapes and Wines. In *Anthocyanins as Food Colors*; P. Markakis, Ed.; Academic Press: New York, 1982; pp 209–244.
143. J. L. Larice; P. Archier; C. Rocheville-Divorne; S. Coen; J.-P. Roggero, *Rev. Fr. Oenol.* **1989**, *121*, 7–12.
144. M. González-San José; G. Santa María; C. Diez, *J. Food Compost. Anal.* **1990**, *3*, 54–66.
145. G. Gonzalez-Neves; G. Gil; M. Ferrer, *Food. Sci. Technol. Int.* **2002**, *8*, 315–321.
146. D. Fournand; A. Vicens; L. Sidhoum; J. M. Souquet; M. Moutounet; V. Cheynier, *J. Agric. Food Chem.* **2006**, *54*, 7331–7338.
147. S. Vidal; E. Meudec; V. Cheynier; G. Skouroumounis; Y. Hayasaka, *J. Agric. Food Chem.* **2004**, *52*, 7144–7151.
148. M. V. Piretti; M. Gherdini; G. Serrazanetti, *Ann. Chim.* **1976**, *66*, 429–437.
149. Z. Czochanska; L. Y. Foo; R. H. Newman; L. J. Porter; W. A. Thomas; W. T. Jones, *J. Chem. Soc. Chem. Commun.* **1979**, 375–377.
150. C. Y. Lee; A. Jaworski, *Am. J. Enol. Vitic.* **1987**, *38*, 277–281.
151. C. Y. Lee; A. Jaworski, *Am. J. Enol. Vitic.* **1990**, *41*, 87–89.
152. E. C. Bate-Smith, *Food* **1954**, *23*, 124.
153. L. J. Porter; L. N. Hrstich; B. G. Chan, *Phytochemistry* **1986**, *25*, 223.
154. Z. Czochanska; L. Foo; L. Porter, *Phytochemistry* **1979**, *18*, 1819–1822.
155. M. Boukharta; M. Girardin; M. Metche, *J. Chromatogr.* **1988**, *455*, 406–409.
156. J. M. Ricardo da Silva; J. Rigaud; V. Cheynier; A. Cheminat; M. Moutounet, *Phytochemistry* **1991**, *30*, 1259–1264.
157. M. T. Escribano-Bailon; M. T. Guerra; J. C. Rivas-Gonzalo; C. Santos-Buelga, In *Procyanidin Composition in Skin and Seed from Grapes,* Proceedings 17th International Conference on Polyphenols, Palma de Mallorca, Spain, 1994; R. Brouillard, M. Jay, A. Scalbert, Eds.; pp 225–227.
158. H. Fulcrand; S. Remy; J. M. Souquet; V. Cheynier; M. Moutounet, *J. Agric. Food Chem.* **1999**, *47*, 1023–1028.
159. S. De Pascual-Teresa; J. C. Rivas-Gonzalo; C. Santos-Buelga, *Int. J. Food Sci. Technol.* **2000**, *35*, 33–40.
160. M. H. Salagoity-Auguste; A. Bertrand, *J. Sci. Food Agric.* **1984**, *35*, 1241–1247.
161. J. J. Cooper; A. G. Marshall, *J. Agric. Food Chem.* **2001**, *49*, 5710–5718.
162. Z. Czochanska; L. Y. Foo; R. H. Newman; J. L. Porter, *J. Chem. Soc. Perkin Trans. I* **1980**, 2278–2286.
163. C. Prieur; J. Rigaud; V. Cheynier; M. Moutounet, *Phytochemistry* **1994**, *36*, 781–784.
164. J. M. Souquet; V. Cheynier; F. Brossaud; M. Moutounet, *Phytochemistry* **1996**, *43*, 509–512.
165. J. M. Souquet; B. Labarbe; C. LeGuernevé; V. Cheynier; M. Moutounet, *J. Agric. Food Chem* **2000**, *48*, 1076–1080.
166. C. Mané; J. M. Souquet; D. Olle; C. Verriès; F. Veran; G. Mazerolles; V. Cheynier; H. Fulcrand, *J. Agric. Food Chem.* **2007**, *55*, 7224–7233.
167. J. M. Souquet; F. Veran; C. Mané; V. Cheynier. In *Optimization of Extraction Conditions on Phenolic Yields from the Different Parts of Grape Clusters - Quantitative Distribution of Their Proanthocyanidins.* In *Proceedings of the XXIII International Conference on Polyphenols, Winnipeg, MB, Canada, 2006*; F. Daayf, A. El Hadramy, L. Adam, G. M. Lallance, Eds.; Groupe Polyphénols: Bordeaux, 2006; pp 245–246.
168. C. Verries; J.-L. Guiraud; J.-M. Souquet; S. Vialet; N. Terrier; D. Ollé, *J. Agric. Food Chem.* **2008**, *56*, 5896–8904.
169. F. Mattivi; R. Guzzon; U. Vrhovsek; M. Stefanini; R. Velasco, *J. Agric. Food Chem.* **2006**, *54*, 7692–7702.
170. R. Rodriguez Montealegre; R. Romero Peces; J. L. Chacon Vozmediano; J. Martinez Gascuena; E. Garcia Romero, *J. Food Compost. Anal.* **2006**, *19*, 687–693.
171. S. Talcott; J. Lee, *J. Agric. Food Chem.* **2002**, *50*, 3186–3192.
172. L. W. Wulf; C. W. Nagel, *J. Chromatogr.* **1976**, *116*, 271–279.
173. V. Cheynier; J. Rigaud, *Am. J. Enol. Vitic.* **1986**, *37*, 248–252.
174. M. Downey; J. Harvey; S. Robinson, *Aust. J. Grape Wine Res.* **2003**, *9*, 110–121.
175. S. F. Price; P. J. Breen; M. Vallado; B. T. Watson, *Am. J. Enol. Vitic.* **1995**, *46*, 187–194.
176. S. Spayd; J. Tarara; D. Mee; J. Ferguson, *J. Am. J. Enol. Vitic.* **2002**, *53*, 171–182.
177. M. O. Downey; J. S. Harvey; S. P. Robinson, *Aust. J. Grape Wine Res.* **2004**, *10*, 55–73.
178. J. Cortell; J. A. Kennedy, *J. Agric. Food Chem.* **2006**, *54*, 8510–8520.
179. H. Amira-Guebailia; K. Chira; T. Richard; T. Mabrouk; A. Furiga; X. Vitrac; J. P. Monti; J. C. Delaunay; J. M. Mérillon, *J. Agric. Food Chem.* **2006**, *54*, 9559–9564.
180. M. Hmamouchi; N. Es-Safi; M. Lahrichi; A. Fruchier; E. M. Essassi, *J. Agric. Food Chem.* **1996**, *47* (2), 186–192.
181. B. Sun; A. M. Ribes; M. C. Leandro; A. P. Belchior; M. I. Spanger, *Anal. Chim. Acta* **2006**, *563*, 382–390.
182. M. A. Pedreno; M. Morales; A. A. Calderon; J. M. Zapata; A. A. Ros Barcelo, In *Plant Peroxidases; Biochemistry and Physiology*; C. Obinger, U. Burner, R. Eberman, C. Penel, H. Greppin, Eds.; University of Geneva: Geneva, 1996; pp 338–344.
183. P. Jeandet; A. C. Douillet-Breuil; R. Bessis; S. Debord; M. Sbaghi; M. Adrian, *J. Agric. Food Chem.* **2002**, *50*, 2731–2741.
184. Y. Takaya; K. X. Yan; K. Terashima; Y. H. He; M. Niwa, *Tetrahedron* **2002**, *58*, 9265–9271.
185. C. Tesnière; L. Torregrosa; M. Pradal; J. M. Souquet; C. Gilles; K. Dos Santos; P. Chatelet; Z. Günata, *J. Exp. Bot.* **2006**, *57*, 91–99.
186. V. L. Singleton; J. Zaya; E. Trousdale; M. Salgues, *Vitis* **1984**, *23*, 114–120.
187. O. Dangles, Propriétés Chimiques Des Polyphénols. In *Les polyphénols en agroalimentaire*; P. Sarni-Manchado, V. Cheynier, Eds.; Tec & Doc Lavoisier: Londres, Paris, New York, 2006; pp 29–54.
188. J. J. L. Cilliers; V. L. Singleton, *J. Agric. Food Chem.* **1991**, *39*, 1298–1303.
189. H. Fulcrand; A. Cheminat; R. Brouillard; V. Cheynier, *Phytochemistry* **1994**, *35*, 499–505.
190. P. Hapiot; A. Neudeck; J. Pinson; H. Fulcrand; P. Neta; C. Rolando, *J. Electroanal. Chem.* **1996**, *405*, 169–176.
191. R. Brouillard; J.-E. Dubois, *J. Am. Chem. Soc.* **1977**, *99*, 1359–1364.
192. R. Brouillard; B. Delaporte, *J. Am. Chem. Soc.* **1977**, *99*, 8461–8468.
193. R. Brouillard; G. Mazza; Z. Saad; A. M. Albrecht-Gary; A. Cheminat, *J. Am. Chem. Soc.* **1989**, *111*, 2604–2610.
194. T. Goto; T. Kondo, *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 17–33.
195. R. Brouillard; O. Dangles, Flavonoids and Flower Colour. In *The Flavonoids. Advances in Research Since 1986*; J. B. Harborne, Ed.; Chapman and Hall: London, 1993; pp 565–588.
196. O. Dangles; R. Brouillard, *J. Chem. Soc. Perkin Trans. II* **1992**, 247–257.

197. L. Jurd, *Food Chem.* **1963**, *33*, 225–237.
198. B. Berke; C. Chèze; J. Vercauteren; G. Deffieux, *Tetrahedron Lett.* **1998**, *39*, 5771–5774.
199. E. Haslam, *Phytochemistry* **1980**, *19*, 2577–2582.
200. J. Ribéreau-Gayon; E. Peynaud; P. Ribéreau-Gayon; P. Sudraud, *Traité d'oenologie, Sciences et Techniques du vin, Tome 3: Vinifications, Transformation du vin*; Ed. Dunod: Paris, 1976.
201. C. W. Nagel; J. D. Baranowski; L. W. Wulf; J. R. Powers, *Am. J. Enol. Vitic.* **1979**, *30*, 198–201.
202. V. Cheynier; J. Rigaud; J. M. Souquet; F. Duprat; M. Moutounet, *Am. J. Enol. Vitic.* **1990**, *41*, 346–349.
203. C. M. G. C. Renard; A. Baron; S. Guyot; J. Drilleau, *Int. J. Biol. Macromol.* **2001**, *29*, 115–125.
204. A. I. Romero-Perez; R. M. Lamuela-Raventos; C. Andres-Lacueva; M. Carmen de la Torre-Boronat, *J. Agric. Food Chem.* **2001**, *49*, 210–215.
205. V. Cheynier; J. Rigaud; J. M. Souquet; J. M. Barrillère; M. Moutounet, *Am. J. Enol. Vitic.* **1989**, *40*, 36–42.
206. K. Yokotsuka, *J. Ferment. Bioeng.* **1990**, *70*, 15–21.
207. T. C. Somers; K. F. Pocock, *Vitis* **1991**, *30*, 189–201.
208. V. Cheynier; G. Masson; J. Rigaud; M. Moutounet, *Am. J. Enol. Vitic.* **1993**, *44*, 393–399.
209. C. W. Nagel; L. W. Wulf, *Am. J. Enol. Vitic.* **1979**, *30*, 111.
210. V. Cheynier; C. Prieur; S. Guyot; J. Rigaud; M. Moutounet, *ACS Symp. Ser.* **1997**, *661*, 81.
211. C. Morel-Salmi; J. M. Souquet; M. Bes; V. Cheynier, *J. Agric. Food Chem.* **2006**, *54*, 4270–4276.
212. R. Canals; M. Llaudy; J. Valls; J. M. Canals; F. Zamora, *J. Agric. Food Chem.* **2005**, *53*, 4019–4025.
213. V. Cheynier; M. Dueñas-Paton; E. Salas; C. Maury; J. M. Souquet; P. Sarni-Manchado; H. Fulcrand, *Am. J. Enol. Vitic.* **2006**, *57*, 298–305.
214. R. Brouillard; J. Lang, *Can. J. Chem.* **1990**, *68*, 755–761.
215. M. Dueñas; H. Fulcrand; V. Cheynier, *Anal. Chim. Acta* **2006**, *563*, 15–25.
216. N. E. Es-Safi; E. Meudec; C. Bouchut; H. Fulcrand; P. H. Ducrot; G. Herbette; V. Cheynier, *J. Agric. Food Chem.* **2008**, *56*, 4584–4591.
217. T. C. Somers; G. Ziemelis, *Vitis* **1985**, *24*, 43–50.
218. E. Trousdale; V. L. Singleton, *Phytochemistry* **1983**, *22*, 619–620.
219. P. Vinas; N. Campillo; M. Hernandez-Perez; M. Hernandez-Cordoba, *Anal. Chim. Acta* **2008**, *611*, 119–125.
220. H. Amira-Guebailia; K. Chira; T. Richard; T. Mabrouk; A. Furiga; X. Vitrac; J. P. Monti; J. C. Delaunay; J. M. Merillon, *J. Agric. Food Chem.* **2006**, *54*, 9559–9564.
221. V. Katalinic; I. Ljubenkov; I. Pezo; I. Generalic; O. Stricevic; M. Milos; D. Modun; M. Boban, *Period. Biol.* **2008**, *110*, 77–83.
222. F. Buiarelli; F. Coccioli; R. Jasionowska; M. Merolle; A. Terracciano, *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2955–2964.
223. N. Landrault; F. Larronde; J. C. Delaunay; C. Castagnino; J. Vercauteren; J. M. Merillon; F. Gasc; G. Cros; P. L. Teissedre, *J. Agric. Food Chem.* **2002**, *50*, 2046–2052.
224. C. Poux; A. Ournac, *Ann. Technol. Agric.* **1972**, *21*, 47–67.
225. Y. Z. Gunata; J. C. Sapis; M. Moutounet, *Phytochemistry* **1986**, *26*, 1–3.
226. Y. Z. Gunata; M. Moutounet, *Rev. Fr. Oenol.* **1988**, *113*, 42–47.
227. V. L. Singleton; M. Salgues; J. Zaya; E. Trousdale, *Am. J. Enol. Vitic.* **1985**, *36*, 50–55.
228. E. Harel; A. M. Mayer, *Phytochemistry* **1971**, *10*, 17–22.
229. M. Dubernet; P. Ribéreau-Gayon, *C. R. Acad. Sci. Paris* **1973**, *277* (D), 975–978.
230. V. Cheynier; J. Rigaud; M. Moutounet, *Phytochemistry* **1990**, *29*, 1751–1753.
231. J. Rigaud; V. Cheynier; J. M. Souquet; M. Moutounet, *J. Sci. Food Agric.* **1991**, *57*, 55–63.
232. V. Cheynier; C. Osse; J. Rigaud, *J. Food Sci.* **1988**, *53*, 1729–1732.
233. V. Cheynier; J. M. Ricardo Da Silva, *J. Agric. Food Chem.* **1991**, *39*, 1047–1049.
234. P. Sarni; H. Fulcrand; V. Souillol; J. M. Souquet; V. Cheynier, *J. Sci. Food Agric.* **1995**, *69*, 385–391.
235. V. Cheynier; E. Trousdale; V. L. Singleton; M. Salgues; R. Wylde, *J. Agric. Food Chem.* **1986**, *34*, 217–221.
236. W. S. Pierpoint, *Biochem. J.* **1969**, *112*, 609–617.
237. G. Prota, *J. Invest. Dermatol.* **1980**, *75*, 122–127.
238. M. Salgues; V. Cheynier; Z. Gunata; R. Wylde, *J. Food Sci.* **1986**, *51*, 1191–1194.
239. V. Cheynier; M. W. Van Hulst, *J. Agric. Food Chem.* **1988**, *36*, 10–15.
240. P. Sarni-Manchado; V. Cheynier; M. Moutounet, *Phytochemistry* **1997**, *45*, 1365–1369.
241. S. Guyot; J. Vercauteren; V. Cheynier, *Phytochemistry* **1996**, *42*, 1279–1288.
242. V. Cheynier; N. Basire; J. Rigaud, *J. Agric. Food Chem.* **1989**, *37*, 1069–1071.
243. J. Oszmianski; C. Y. Lee, *J. Agric. Food Chem.* **1990**, *38*, 1892–1895.
244. F. Richard-Forget; M. J. Amiot; P. Goupy; J. Nicolas, *A.C.S. Symp. Ser.* **1995**, *594*, 144–158.
245. V. Cheynier; J. M. Souquet; A. Samson; M. Moutounet, *Vitis* **1991**, *30*, 107–115.
246. R. F. Simpson, *Vitis* **1982**, *21*, 233–239.
247. P. Fernandez-Zurbano; V. Ferreira; C. Pena; A. Escudero; J. Cacho, *J. Food Sci. Technol. Int.* **1999**, *5*, 319–325.
248. O. Munoz; M. Sepulveda; M. Schwartz, *Food Chem.* **2004**, *87*, 487–490.
249. K. L. Sacchi; L. F. Bisson; D. O. Adams, *Am. J. Enol. Vitic.* **2005**, *56*, 197–206.
250. H. Kelebek; A. Canbas; T. Cabaroglu; S. Selli, *Food Chem.* **2007**, *105*, 334–339.
251. I. Revilla; M. L. Gonzalez-SanJose, *Food Chem.* **2003**, *80*, 205–214.
252. M. A. Ducasse; R. M. Canal-Llauberes; M. De Lumley; P. Williams; J. M. Souquet; H. Fulcrand; J. M. Souquet; T. Doco; V. Cheynier, *Food Chem.* **2009**, in press.
253. J. Ducruet; D. An; R. M. Canal-Llauberes; Y. Glories, *Rev. Fr. Oenol.* **1997**, *166*, 16–19.
254. J. Bakker; S. J. Bellworthy; H. P. Reader; S. J. Watkins, *Am. J. Enol. Vitic.* **1999**, *50*, 271–276.
255. J. D. Wightman; S. F. Price; B. T. Watson; R. E. Wrolstad, *Am. J. Enol. Vitic.* **1997**, *48*, 39–48.
256. A. B. Bautista-Ortin; A. Martinez-Cutillas; J. M. Ros-Garcia; J. M. Lopez-Roca; E. Gomez-Plaza, *Int. J. Food Sci. Technol.* **2005**, *40*, 867–878.
257. D. Dubourdieu, *Vitis* **1994**, *187*, 29–30.

258. S. Okamura; M. Watanabe, *Agric. Biol. Chem.* **1982**, *46*, 1839–1848.
259. P. X. Etiévant, *J. Agric. Food Chem.* **1981**, *29*, 65–67.
260. P. Dubois, Volatile Phenols in Wines. In *Flavour of Distilled Beverages, Origin and Developments*; J. R. Pifgott, Ed.; Ellis Horwood: Chichester, 1983; pp 110–119.
261. J.-C. Sapis; P. Ribéreau-Gayon, *Ann. Technol.* **1969**, *18* (3), 221–229.
262. G. J. Soleas; D. M. Goldberg; E. P. Diamandis; A. Karumanchiria; J. Yan, *Am. J. Enol. Vitic.* **1995**, *46*, 346–352.
263. V. L. Singleton; C. F. Timberlake; A. G. H. Lea, *J. Sci. Food Agric.* **1978**, *29*, 403–410.
264. M. Moutounet; P. Rabier; J. L. Puech; E. Verette; J. M. Barillère, *Sci. Aliments* **1989**, *9*, 35–51.
265. T. C. Somers; K. F. Pocock, *Vitis* **1990**, *29*, 109–121.
266. K. F. Pocock; M. A. Sefton; P. J. Williams, *Am. J. Enol. Vitic.* **1994**, *45*, 429–434.
267. M. Moutounet; P. Rabier; F. Sarni; A. Scalbert, *J. Int. Sci. Vigne Vin* **1992**, (Hors série), 75–79.
268. N. Vivas; Y. Glories, *Am. J. Enol. Vitic.* **1996**, *47*, 103–107.
269. T. C. Somers, *Phytochemistry* **1971**, *10*, 2175–2186.
270. W. Sun; J. M. Miller, *J. Mass Spectrom.* **2003**, *38*, 438–446.
271. K. Weinges; W. Ebert; D. Huthwelker; H. Mattauch; J. Perner, *Liebigs Ann. Chem.* **1969**, *726*, 114–124.
272. H. Fulcrand; V. Cheynier; J. Oszmianski; M. Moutounet, *Phytochemistry* **1997**, *46*, 223–227.
273. T. Inui; K. Nakahara; M. Uchida, *Bull. Chem. Soc. Jpn.* **2004**, *77*, 1201–1207.
274. L. Genevoix; J. Ribéreau-Gayon, Eds., Oxydations Et Réductions Dans Les vins. In *Le vin*; Actualités Scientifiques et Industrielles – 1017-Nutrition; Hermann & Cie: Paris, 1947; Chapter 4, pp 71–81.
275. J. Mourgues; L. Deibner, *Ind. Aliment Agric.* **1967**, *84*, 1483–1491.
276. C. F. Timberlake; P. Bridle, *Am. J. Enol. Vitic.* **1976**, *27*, 97–105.
277. H. Fulcrand; T. Doco; N. E. Es Safi; V. Cheynier, *J. Chromatogr.* **1996**, *752*, 85–91.
278. N. E. Es-Safi; H. Fulcrand; V. Cheynier; M. Moutounet, *J. Agric. Food Chem.* **1999**, *47*, 2088–2095.
279. N. E. Es-Safi; H. Fulcrand; V. Cheynier; M. Moutounet, *J. Agric. Food Chem.* **1999**, *47*, 2096–2102.
280. C. Dallas; J. M. Ricardo-da-Silva; O. Laureano, *J. Sci. Food Agric.* **1996**, *70*, 493–500.
281. V. Atanasova; H. Fulcrand; C. Le Guernevé; V. Cheynier; M. Moutounet, *Tetrahedron Lett.* **2002**, *43*, 6151–6153.
282. D. F. Lee; E. E. Swinny; G. P. Jones, *Tetrahedron Lett.* **2004**, *45*, 1671–1674.
283. V. Cheynier; H. Fulcrand; P. Sarni; M. Moutounet, *Analusis* **1997**, *25*, M14–M21.
284. C. Saucier; D. Little; Y. Glories, *Am. J. Enol. Vitic.* **1997**, *48*, 370–373.
285. J. Drinkine; P. Lopes; J. Kennedy; P. L. Teissedre; C. Saucier, *J. Agric. Food Chem.* **2007**, *55*, 6292–6299.
286. N. E. ES-Safi; V. Cheynier; M. Moutounet, *J. Agric. Food Chem.* **2000**, *48*, 5946–5954.
287. J. Pissara; N. Mateus; J. C. Rivas-Gonzalo; C. Santos-Buelga; V. De Freitas, *J. Food Sci.* **2003**, *68*, 476–481.
288. N. E. Es-Safi; C. Le Guerneve; B. Labarbe; H. Fulcrand; V. Cheynier; M. Moutounet, *Tetrahedron Lett.* **1999**, *40*, 5869–5872.
289. N. E. Es-Safi; C. Le Guerneve; H. Fulcrand; V. Cheynier; M. Moutounet, *J. Agric. Food Chem.* **1999**, *47*, 5211–5217.
290. T. Escribano-Bailon; M. Alvarez-Garcia; J. C. Rivas-Gonzalo; F. J. Heredia; C. Santos-Buelga, *J. Agric. Food Chem.* **2001**, *49*, 1213–1217.
291. V. Cheynier; N. E. Es-Safi; H. Fulcrand, *Proceedings of the International Congress on Pigments in Food and Technology,* Sevilla, Spain, 1999; pp 23–35.
292. N. Mateus; A. M. S. Silva; C. Santos-Buelga; J. C. Rivas-Gonzalo; V. de Freitas, *J. Agric. Food Chem.* **2002**, *50*, 2110–2116.
293. N. Mateus; A. M. S. Silva; J. C. Rivas-Gonzalo; C. Santos-Buelga; V. De Freitas, *J. Agric. Food Chem.* **2003**, *51*, 1919–1923.
294. A. C. Clark; P. D. Prenzler; G. R. Scollary, *J. Agric. Food Chem.* **2003**, *51*, 6204–6210.
295. M. P. Bradshaw; V. Cheynier; G. R. Scollary; P. D. Prenzler, *J. Agric. Food Chem.* **2003**, *51*, 4126–4132.
296. J. P. Cameira dos Santos; J.-M. Brillouet; V. Cheynier; M. Moutounet, *J. Agric. Food Chem.* **1996**, *70*, 204–208.
297. H. Fulcrand; P. J. Cameira dos Santos; P. Sarni-Manchado; V. Cheynier; J. Favre-Bonvin, *J. Chem. Soc. Perkin Trans. 1* **1996**, *7*, 735–739.
298. Y. Hayasaka; R. E. Asenstorfer, *J. Agric. Food Chem.* **2002**, *50*, 756–761.
299. M. Schwarz; G. Jerz; P. Winterhalter, *Vitis* **2003**, *42*, 105–106.
300. M. Schwarz; T. C. Wabnitz; P. Winterhalter, *J. Agric. Food Chem.* **2003**, *51*, 3682–3687.
301. J. Bakker; P. Bridle; T. Honda; H. Kuwano; N. Saito; N. Terahara; C. F. Timberlake, *Phytochemistry* **1997**, *44*, 1375–1382.
302. H. Fulcrand; C. Benabdeljalil; J. Rigaud; V. Cheynier; M. Moutounet, *Phytochemistry* **1998**, *47*, 1401–1407.
303. J. Bakker; C. F. Timberlake, *J. Agric. Food Chem.* **1997**, *45*, 35–43.
304. C. BenAbdeljalil; V. Cheynier; H. Fulcrand; A. Hakiki; M. Mosaddak; M. Moutounet, *Sci. Aliments* **2000**, *20*, 203–220.
305. E. M. Francia-Aricha; M. T. Guerra; J. C. Rivas-Gonzalo; C. Santos-Buelga, *J. Agric. Food Chem.* **1997**, *45*, 2262–2266.
306. R. E. Asenstorfer; Y. Hayasaka; G. P. Jones, *J. Agric. Food Chem.* **2001**, *49*, 5957–5963.
307. V. Atanasova; H. Fulcrand; V. Cheynier; M. Moutounet, *Anal. Chim. Acta* **2002**, *458*, 15–27.
308. N. Mateus; E. Carvalho; A. R. F. Carvalho; A. Melo; A. M. Gonzalez-Paramas; C. Santos-Buelga; A. M. S. Silva; V. de Freitas, *J. Agric. Food Chem.* **2003**, *51*, 277–282.
309. C. Saucier; C. Guerra; I. Pianet; M. Laguerre; Y. Glories, *Phytochemistry* **1997**, *46*, 229–234.
310. N. Mateus; J. Oliveira; C. Santos-Buelga; A. M. S. Silva; V. de Freitas, *Tetrahedron Lett.* **2004**, *45*, 3455–3457.
311. N. Mateus; J. Oliveira; J. Pisarra; A. M. González-Paramás; J. C. Rivas-Gonzalo; C. Santos-Buelga; A. M. S. Silva; V. De Freitas, *Food Chem.* **2006**, *97*, 689–695.
312. L. Jurd; A. C. Waiss, *Tetrahedron* **1965**, *21*, 1471–1483.
313. L. Jurd, *Tetrahedron* **1967**, *23*, 1057–1064.
314. E. Salas; F. Fulcrand; E. Meudec; V. Cheynier, *J. Agric. Food Chem.* **2003**, *51*, 7951–7961.
315. L. Jurd; T. C. Somers, *Phytochemistry* **1970**, *9*, 419–427.
316. H. Liao; Y. Cai; E. Haslam, *J. Sci. Food Agric.* **1992**, *59*, 299–305.
317. C. Santos-Buelga; S. Bravo-Haro; J. C. Rivas-Gonzalo, *Z. Lebensm. Unters. Forsch.* **1995**, *201*, 269–274.
318. P. D. Bishop; C. W. Nagel, *J. Agric. Food Chem.* **1994**, *32*, 1022–1026.
319. S. Remy-Tanneau; C. LeGuernevé; E. Meudec; V. Cheynier, *J. Agric. Food Chem.* **2003**, *51*, 3592–3597.

320. S. Remy; H. Fulcrand; B. Labarbe; V. Cheynier; M. Moutounet, *J. Sci. Food Agric.* **2000**, *80*, 745–751.
321. E. Salas; M. Dueñas; M. Schwarz; P. Winterhalter; V. Cheynier; H. Fulcrand, *J. Agric. Food Chem.* **2005**, *53*, 4536–4546.
322. Y. Hayasaka; J. A. Kennedy, *Aust. J. Grape Wine Res.* **2003**, *9*, 210–220.
323. S. Vidal; D. Cartalade; J. M. Souquet; H. Fulcrand; V. Cheynier, *J. Agric. Food Chem.* **2002**, *50*, 2261–2266.
324. E. Salas; V. Atanasova; C. Poncet-Legrand; E. Meudec; J. P. Mazauric; V. Cheynier, *Anal. Chim. Acta* **2004**, *513*, 325–332.
325. C. Alcalde-Eon; M. T. Escribano-Bailón; C. Santos-Buelga; J. C. Rivas-Gonzalo, *Anal. Chim. Acta* **2006**, *563*, 238–254.
326. B. Labarbe, Le potentiel phénolique de la grappe de *Vitis vinifera* var. Gamay noir et son devenir en vinification beaujolaise. Ph.D. Thesis, ENSA-M, UMI, UMII, Montpellier, France, 2000.
327. S. Quideau; M. Jourdes; C. Saucier; Y. Glories; P. Pardon; C. Baudry, *Angew. Chem. Int. Ed.* **2003**, *42*, 6012–6014.
328. G. Skouroumounis; M. Kwiatkowski; L. Francis; H. Oakey; D. L. Capone; B. Duncan; M. A. Sefton; E. Waters, *Aust. J. Grape Wine Res.* **2005**, *11*, 369–377.
329. O. Dangles; N. Saito; R. Brouillard, *J. Am. Chem. Soc.* **1993**, *115*, 3125–3132.
330. G. Mazza; R. Brouillard, *Phytochemistry* **1990**, *29*, 1097–1102.
331. O. Dangles; R. Brouillard, *Can. J. Chem.* **1992**, *70*, 2174–2189.
332. P. Sarni-Manchado; H. Fulcrand; J.-M. Souquet; V. Cheynier; M. Moutounet, *J. Food Sci.* **1996**, *61*, 938–941.
333. M. Dueñas; E. Salas; V. Cheynier; O. Dangles; H. Fulcrand, *J. Agric. Food Chem.* **2006**, *54*, 189–196.
334. V. Atanasova; H. Fulcrand; C. Le Guernevé; O. Dangles; V. Cheynier; M. Moutounet, In *First Evidence of Acetaldehyde-Induced Anthocyanin Polymerization,* In Proceedings of the XXI International Conference on Polyphenols, Marrackech, Morocco; I. El Hadrami, Ed.; 2002; pp 417–418.
335. M. Escribano-Bailon; O. Dangles; R. Brouillard, *Phytochemistry* **1996**, *41*, 1583–1592.
336. N. J. Baxter; T. H. Lilley; E. Haslam; M. P. Williamson, *Biochemistry* **1997**, *36*, 5566–5577.
337. C. Dufour; C. Bayonove, *J. Agric. Food Chem.* **1999**, *47*, 678–684.
338. P. Sarni-Manchado; V. Cheynier, *J. Mass Spectrom.* **2002**, *37*, 609–616.
339. V. Riou; A. Vernhet; T. Doco; M. Moutounet, *Food Hydrocoll.* **2002**, *16*, 17–23.
340. C. Poncet-Legrand; D. Cartalade; J. L. Putaux; V. Cheynier; A. Vernhet, *Langmuir* **2003**, *19*, 10563–10572.
341. C. Saucier; G. Bourgeois; C. Vitry; D. Roux; Y. Glories, *J. Agric. Food Chem.* **1997**, *45*, 1045–1049.
342. E. Haslam; T. H. Lilley, *Crit. Rev. Food Sci. Nutr.* **1988**, *27*, 1–40.
343. A. E. Hagerman, Chemistry of Trannin-Protein Complexation. In *Chemistry and Significance of Condensed Tannins*; R. W. Hemingway, J. J. Karchesy, Eds.; Plenum Press: New York, 1989; pp 323–331.
344. E. Haslam; T. H. Lilley; E. Warminski; H. Liao; Y. Cai; R. Martin; S. H. Gaffney; P. N. Goulding; G. Luck, In *Phenolic Compounds in Food and Their Effects on Health*; C. T. Ho, C. Y. Lee, M. T. Huang, Eds.; American Chemical Society: Washington, DC, 1992; pp 8–50.
345. O. Dangles, Flavonoid-Protein Interactions. In *Flavonoids: Chemistry*, *Biochemistry and Applications*; O. Andersen, K. Markham, Eds.; CRC Press: Boca Raton, FL, 2005; pp 443–470.
346. H. I. Oh; J. E. Hoff; G. S. Armstrong; L. A. Haff, *J. Agric. Food Chem.* **1980**, *28*, 394–398.
347. G. Luck; H. Liao; N. J. Murray; H. R. Grimmer; E. E. Warminski; M. P. Willamson; T. H. Lilley; E. Haslam, *Phytochemistry* **1994**, *37*, 357–371.
348. N. J. Murray; M. P. Williamson; T. H. Lilley; E. Haslam, *Eur. J. Biochem.* **1994**, *219*, 923–935.
349. A. J. Charlton; N. J. Baxter; T. H. Lilley; E. Haslam; C. J. McDonald; M. P. Williamson, *FEBS Lett.* **1996**, *382*, 289–292.
350. J. P. McManus; K. G. Davis; J. E. Beart; S. H. Galffney; T. H. Lilley; E. Haslam, *J. Chem. Soc. Perkin Trans. II* **1985**, 1429–1438.
351. C. Poncet-Legrand; C. Gautier; V. Cheynier; A. Imberty, *J. Agric. Food Chem.* **2007**, *55*, 9235–9240.
352. S. Guyot; P. Pellerin; J. M. Brillouet; M. Moutounet; V. Cheynier, *Biosci. Biotechnol. Biochem.* **1996**, *60*, 1131–1135.
353. P. Sausse; V. Aguie-Beghin; R. Douillard, *Langmuir* **2003**, *19*, 737–743.
354. E. Jobstl; J. R. Howse; J. P. A. Fairclough; M. P. Williamson, *J. Agric. Food Chem.* **2006**, *54*, 4077–4081.
355. C. Pascal; C. Poncet-Legrand; A. Imberty; C. Gautier; P. Sarni-Manchado; V. Cheynier; A. Vernhet, *J. Agric. Food Chem.* **2007**, *55*, 4895–4901.
356. M. Fukui; K. Yokotsuka, *Am. J. Enol. Vitic.* **2003**, *54*, 178–188.
357. P. Jones; R. Gawel; L. Francis; E. Waters, *Food Qual. Prefer.* **2008**, *19*, 596–607.
358. E. J. Waters; G. Alexander; R. Muhlack; K. F. Pocock; C. Colby; B. K. O'Neill; P. B. Hoj; P. Jones, *Aust. J. Grape Wine Res.* **2005**, *11*, 215–225.
359. J. M. Ricardo Da Silva; V. Cheynier; J. M. Souquet; M. Moutounet; C. Cabanis; M. Bourzeix, *J. Sci. Food Agric.* **1991**, *57*, 111–125.
360. P. Sarni-Manchado; A. Deleris; S. Avalonne; V. Cheynier; M. Moutounet, *Am. J. Enol. Vitic.* **1999**, *50*, 81–86.
361. C. Maury; P. Sarni-Manchado; S. Lefèbvre; V. Cheynier; M. Moutounet, *Am. J. Enol. Vitic.* **2001**, *52*, 140–145.
362. C. Maury; P. Sarni-Manchado; S. Lefebvre; V. Cheynier; M. Moutounet, *Am. J. Enol. Vitic.* **2003**, *54*, 105–111.
363. T. Doco; N. Quellec; M. Moutounet; P. Pellerin, *Am. J. Enol. Vitic.* **1999**, *47*, 108–110.
364. J. M. Brillouet; C. Bosso; M. Moutounet, *Am. J. Enol. Vitic.* **1990**, *41*, 29–36.
365. P. Pellerin; S. Vidal; P. Williams; J. M. Brillouet, *Carbohydr. Res.* **1995**, *277*, 135–143.
366. T. Doco; J. M. Brillouet, *Carbohydr. Res.* **1993**, *243*, 333–343.
367. C. Poncet-Legrand; T. Doco; P. Williams; A. Vernhet, *Am. J. Enol. Vitic.* **2007**, *58*, 87–91.
368. E. Waters; P. Pellerin; J. M. Brillouet, *Carbohydr. Polym.* **1994**, *23*, 185–191.
369. I. V. S. Dupin; B. M. McKinnon; C. Ryan; M. Boulay; A. J. Markides; G. P. Jones; P. J. Williams; E. J. Waters, *J. Agric. Food Chem.* **2000**, *48*, 3098–3105.
370. P. Pellerin; J. C. Cabanis, In *Oenologie – fondements scientifiques et technologiques*; C. Flanzy, Ed.; Tec & Doc Lavoisier, 1998; pp 40–92.
371. C. Saucier; D. Roux; Y. Glories, In *Oenologie95*; A. Lonvaud-Funel, Ed.; Tec & Doc Lavoisier: Paris, 1996; pp 395–400.
372. K. J. Siebert; A. Carrasco; P. Y. Lynn, *J. Agric. Food Chem.* **1996**, *44*, 1997–2005.
373. A. C. Noble, In *Bitterness in Foods and Beverages*; Developments in Food Science 25; R. L. Roussef, Ed.; Elsevier: Amsterdam, Oxford, New York, Tokyo, 1990; pp 145–158.
374. A. G. H. Lea, In *Bitterness in Foods and Beverages*; Developments in Food Science 25; R. L. Rouseff, Ed.; Elsevier: Amsterdam, 1990; pp 123–143.

375. A. G. H. Lea; G. M. Arnold, *J. Sci. Food Agric.* **1978**, *29*, 478–483.
376. K. Gacon; H. Peleg; A. C. Noble, *Food Qual. Prefer.* **1996**, *7*, 343–344.
377. J. Hufnagel; T. Hofmann, *J. Agric. Food Chem.* **2008**, *56*, 1376–1386.
378. J. L. Robichaud; A. C. Noble, *J. Sci. Food Agric.* **1990**, *53*, 343–353.
379. J. L. Goldstein; T. Swain, *Phytochemistry* **1965**, *4*, 185–192.
380. S. Vidal; L. Francis; S. Guyot; N. Marnet; M. Kwiatkowski; R. Gawel; V. Cheynier; E. Waters, *J. Sci. Food Agric.* **2003**, *83*, 564–573.
381. S. Preys; G. Mazerolles; P. Courcoux; A. Samson; U. Fischer; M. Hanafi; D. Bertrand; V. Cheynier, *Anal. Chim. Acta* **2006**, *563*, 126–136.
382. S. Vidal; L. Francis; A. C. Noble; M. Kwiatkowski; V. Cheynier; E. Waters, *Anal. Chim. Acta* **2004**, *513*, 57–65.
383. S. Vidal; P. Courcoux; L. Francis; M. Kwiatkowski; R. Gawel; P. Williams; E. Waters; V. Cheynier, *Food Qual. Prefer.* **2004**, *15*, 209–217.
384. U. Fischer; A. C. Noble, *Am. J. Enol. Vitic.* **1994**, *45*, 6–10.

Biographical Sketches

Véronique Cheynier graduated from Institut National Agronomique Paris-Grignon (INAP-G). After obtaining a Ph.D. in Analytical Chemistry at INAP-G, she joined the INRA Institute of Vine Produce in Montpellier in 1983. She then spent a year as a postdoctoral researcher in the Department of Enology and Viticulture at University of California, Davis. Her research group focuses on the structure and analysis of plant phenolic compounds, their biosynthetic pathways, the physicochemical processes involving these compounds, and their influence on the quality of wine and other plant-derived products.

Agricultural engineer and enologist, Rémi Schneider received his Ph.D. in Food Science from the University of Montpellier (France). He is now working in the French Wine Technical Institute (IFV) in Montpellier, and coordinates a Joint Technical Unit between INRA (National Institute for Agricultural Research) and IFV, dedicated to developing new methods for the characterization of grapes and wines quality. His research is focused on the analysis of aromas and aroma precursors.

Jean-Michel Salmon obtained a permanent position of scientist at INRA in 1981. He received his Ph.D. in Microbiology in 1986 from Montpellier University. He is now research director at the 'Enological Sciences' Joint Research Unit at Montpellier within the 'Microbiology and Fermentation Technology' team. His research focuses on the adaptation of fermenting yeasts to the peculiar composition of grape musts, and more particularly on their interaction with oxygen in relation with their lipidic status.

Hélène Fulcrand received her A.B. in Chemistry and Ph.D. in Organic and Analytical Chemistry from Louis Pasteur University (Strasbourg, France). She joined the French National Institute for Agricultural Research in 1993 at Montpellier. She spent a sabbatical year at the University of New Orleans (Louisiana, USA) in 2002 to gain skills in mass spectrometry. Her research focuses on the structural evolution of polyphenols in the course of winemaking and wine aging and on how it affects wine sensory quality, especially wine color.

3.27 Trees: A Remarkable Biochemical Bounty

Ann M. Patten, Daniel G. Vassão, Michael P. Wolcott, Laurence B. Davin, and Norman G. Lewis, Washington State University, Pullman, WA, USA

3.27.1 Trees: Human Reliance on Arborescent Life

The importance of trees and their varied wood/bark constituents cannot be overemphasized, given humanity's critical reliance upon them for almost every aspect of life. Not only do they represent the sources of all of our commercial wood products, but also that of innumerable and often truly remarkable secondary products. Interestingly, while wood and bark usage worldwide is generally regional and application-dependent, many of their so-called secondary products have been traded over huge distances throughout history, for example, spices, fragrances, and so on. In other words, over millennia and across the globe, humans have sought and utilized – for a host of different purposes – a tremendous array of basic raw materials and substances from our diverse tree species. It is thus worth reflecting on the fantastic bounty that trees provide humanity, particularly since it is seldom fully appreciated that tree uses and applications depend upon their chemistries – whether as their diverse phytochemical constituents or as their structural/polymeric (material) components.

Many of our most familiar items are derived from wood. These range from basic writing equipment, such as pencils and paper, to the bulk of our pulp, paper, and fiber-based products that are used in many different applications. They are also the major sources of lumber, wood, and associated wood composites for construction of buildings and their interiors, and being extensively utilized for boats, utility poles, cooking and eating utensils, and so on (**Figure 1**). As a raw material, wood is also often used for the sophisticated design and crafting of musical instruments; for creation of the striking works of art, such as carvings, sculptures, totem poles, jewelry and the like; and for furniture/furnishings, either of rudimentary design and/or of truly creative/artistic expression (**Figure 2**). Such applications largely reflect the physical/mechanical properties of the wood structural biopolymers in their roles as materials. Wood is also used for a plethora of other sundry items and applications.

Trees are sources of other valuable commodities and products, such as rubber (**Figure 3**), lacquers (**Figure 4**), gums (**Figure 5**), syrups (**Figure 6**), resins, oils, varnishes, fossils (amber, **Figure 7**), and miscellaneous intermediate chemicals used in various products. However, they can additionally serve as important providers of specialty chemicals, flavor, and fragrance chemicals, and highly valued medicinals. Examples include common spices, such as cinnamon, cloves, nutmeg, and bay leaves (**Figure 8**); various flavors/fragrances including those from sandalwood, incense (e.g., *Boswellia* spp., *Commiphora* spp.), pine (*Pinus* spp.), and citrus (*Citrus* spp.), and so on; medicinals, such as taxol (**1**), camptothecin (**2**), quinine (**3**), acetylsalicylic acid (**4**), and camphor (**5**) (**Figure 9**); insecticides, such as azadirachtin (**6**) or poisons, such as strychnine (**7**) and cyanogenic compounds (e.g., (*R*)-amygdalin (**8**), (*R*)-prunasin (**9**)) (**Figure 9**). They are also sources of a fantastic array of foodstuffs, including fruits and nuts (**Figure 10**), and various products derived from specific types of foliage. In addition, there are many diverse bark products, ranging from the familiar suberized cork

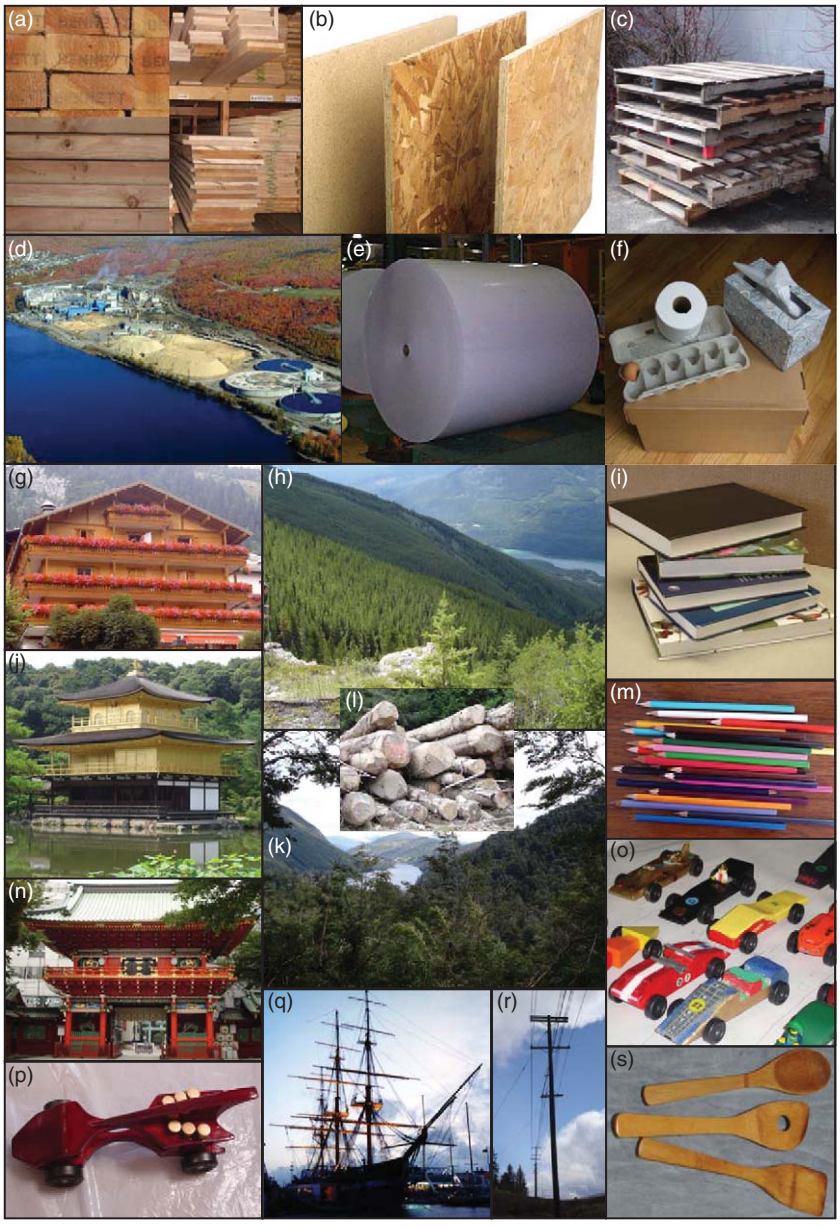


Figure 1 Some familiar uses of wood as a material and as a source of other cellulose-derived products: Wood for lumber (a); Wood composites such as waferboard (b); Wood pallets (c); Aerial photograph of pulp and paper mill (Tembec, Temiscaming, Québec, Canada), with the light-colored wood chip piles in the center of the image used as raw material (d); Paper is wound into large rolls (which can weigh up to ~25 tons), with this resulting from processing wood chips to make pulp, compressing the same to remove water and drying pulp (e); Selected pulp and paper products (toiletries/paper towels, paper tissues, packaging (e.g., egg and cardboard boxes, paper tissues) (f); books (i); newsprint (not shown), and so on. Selected examples of wood products in building construction, such as a typical 'chalet' in the Alps, France (g); the Golden Temple in Kyoto, Japan (j); and the main gate of the Kanda Shrine in Tokyo, Japan (n); Pencils (m); toys (o, p); boats (q); utility poles (r); and cooking/eating utensils (s); A forested hillside in British Columbia, Canada (h); and in the Lake District of Chile (k); with harvested timber temporarily piled (l); Images from L. B. Davin, Washington State University (a, c, f–s); http://www.plywoodnews.com (b); M. G. Paice, Pulp and Paper Institute of Canada, Pointe Claire, Québec, Canada (d); and ForestWorks, North Melbourne, Victoria, Australia (http://www.forestworks.com.au), with permission (e).

Figure 2 Selected miscellaneous uses of woods for artistic and/or functional applications, reflecting various material (wood biopolymer) properties: Mandolin (a); many musical instruments employ wood from specific species in order to produce particular sounds/tones. Hokkaido bear carving (b) (provenance: near Lake Toya, Japan); Wood-carved fruit assortment (c); An elephant carving of red cedar (*Toona ciliata*) wood, with a king and carriage (Visakhapatnam, Andhra Pradesh, India) (d); Decorative carvings on choir stall seating in the Mosteiro dos Jeronimos (Lisbon, Portugal) (e and f); Lion carving in the same monastery (g); Close-up of an oak choir stall (fifteenth century) by Jörg Syrlin the Elder, in the Ulm Cathedral, Germany (h); Totem pole in the Canadian Museum of Civilization (Gatineau, Québec, Canada) (i); Wood sculpture in the Chateau de Vizille garden (Vizille, France) (j); Artificial flowers made from wood shavings (k); and a wooden ox-cart drawing logs (l) (Pucón, Chile). Black-faced ibis carvings (m) (Villaríca, Chile); Wooden bananas decoration (n) (Brotas, Brazil); Fine furniture examples, such as a Louis XIV commode (o) and a Louis XVI bureau (p). Images from L. B. Davin, Washington State University (a, c, e–n); H. Moore, Washington State University (b and d); LG Antique Restoration, Los Angeles, CA, USA (http://ifixantiques.com with permission) (o); and French Accents Antiques (http://www.faccents.com, with permission) (p).

Figure 3 Rubber trees (*Hevea brasiliensis*) (a) are tapped for latex (b) that is used for rubber production. Selected rubber products include tires (c), pencil erasers (d), boots (e), latex gloves (f), and rubber bands (g). Images from D. Nandris; H. Chrestin, Institut de Recherche pour le Development (IRD), Mahidol University, Bangkok, Thailand (a and b), and L. B. Davin, Washington State University (c–g).

products (**Figure 11**)[1,2] to mulch to other highly valued spices, for example, cinnamon (**Figure 8**). Woody plants also globally serve as a principal source of renewable energy, directly as either fuel, such as wood, or as wood-derived by-products, such as lignins, that are solubilized during wood-pulping operations. In short, much of our very existence and human expression depends on the woody growth habit.

Our trees and forests contribute extensively to the quality of our diverse environments, from their aesthetic (visual) contributions to our various landscapes (**Figure 12**), to the quality of the air we breathe, to the many blossoms and fragrances that we so greatly treasure. Furthermore, woody plants serve as important repositories of organic carbon through biological CO_2 sequestration and photosynthesis. Indeed, wood-forming plants can be thought of as nature's most abundant reservoir of organic carbonaceous substances on land and thus are of pivotal importance for global climate chemistry. Yet, humanity's escalating overuse of these resources may lead to long-term environmental crisis. For example, deforestation in the 1990s occurred at a rate of 9.4 million hectares per year, resulting in up to 10% of all trees being listed as either threatened or endangered, with 1002 tree species declared critically endangered (each estimated to be <50 individuals/species) worldwide.[3] There is thus now the challenge of sustainably preserving/utilizing these remarkable resources globally and, of course, the corresponding biochemical diversity encapsulated within. Yet history has repeatedly recorded that failure to sustainably husband forest resources can result in collapse of the civilizations in the regions so impacted.[4] Indeed, we often take for granted humanity's critical reliance on the sustainability of the diverse woody habitats that extend over the very small thin line(s) of topsoil worldwide, and that of their individual growth habits.

In this contribution, we first consider how the woody habit presumably emerged and, by inference, how their quite distinct phytochemical factories may have evolved. Regardless of how this occurred, these developments have ultimately resulted in humanity's massive use of tree resources from both material and phytochemical perspectives. Today, the plant sciences are directed mainly to establishing how such diverse processes occur at the molecular levels, that is, from wood formation to the generation of specialized phytochemicals, including how they ultimately became compartmentalized in different tissues and organs. As the reader will hopefully appreciate from the accompanying text, there has never been more a need to understand the biochemistries of wood formation than now.

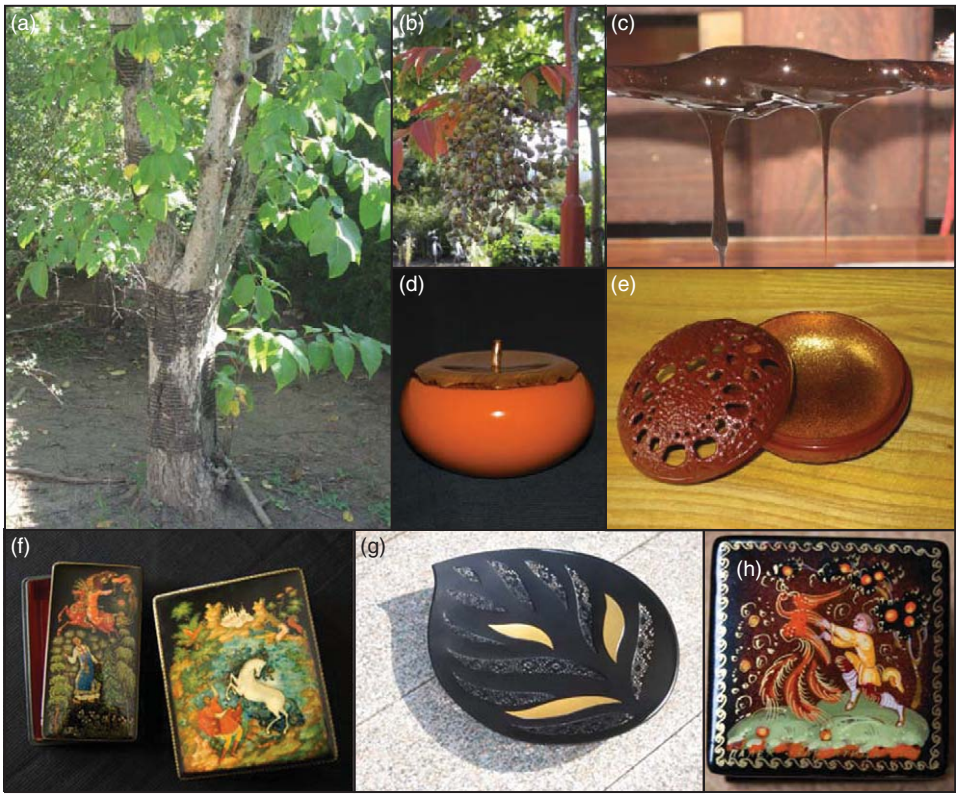


Figure 4 Lacquer tree, objects of art and Russian lacquer boxes. The lacquer tree (*Rhus vernicifera*) (a and b) is the source of urushi (c), used to make objects of art such as in d, e, and g. The most sought after Russian lacquer boxes (f and h) originate from four villages – Palekh, Fedoskino, Kholui, and Mstera. Items in (f) are from Fedoskino, whereas that in (h) is from Palekh. They are made of 'papier mâché' painted with several coats of black lacquer on the outside and red lacquer on the inside. Extremely fine brushes are used to create the fine lines and details in each painting. Finally, when the painting is finished a layer of transparent lacquer is applied. Altogether, this can take several months to complete. Images from S. Ross, Urushi Artist, Japan (a–c, e, and g), and L. B. Davin, Washington State University (d, f, and h).

3.27.2 Evolution of the Woody Growth Habit: Land Colonization and Adaptation

Present-day trees or tree forms are classified under ferns, gymnosperms, and angiosperms, although only the latter two groups produce wood from a cambium. The total number of extant arborescent species is currently difficult to precisely establish because of variations in definitions used.[3] Nevertheless, ~100 000 are thought to be in existence globally, this representing up to 25% of all plant species.[3,5] Of the 100 000 or so tree species, however, there are less than 1000 that are gymnosperms; the bulk are the angiosperms.[6] (Named from the ancient Greek, gymnosperm, meaning naked seed, refers to plants with seeds that are borne externally, as on a scale or similar structure, whereas angiosperm refers to those with vessel seed, indicating the carpel in which the seed develops.[7]) The enormous breadth of the topic of tree resources alone limits any substantial discussion of shrubs, bushes, and lianas (woody vines), even though they can have a woody growth habit.

Plants apparently first emerged on land from their forerunner algal ancestors during the mid-Ordovician period, some 460 Mya.[8,9] Over this lengthy evolutionary period, numerous truly remarkable innovations and adaptations occurred that eventually led to our familiar tree forms. This ultimately afforded the 350 000 or so distinct plant species in existence today, and thus the fantastic diversity in terms of plant shapes, sizes, habitats, and associated phytochemical constituents. In achieving this colonization of land, some of the remarkable evolutionary changes manifested over the passage of time included, among others: generation of specialized cell types, such as lignified (reinforced) secondary cell walls and vascular tissues; formation of protective wood and bark tissues, and other specialized cell types within; the ability to continuously orient/reorient massive photosynthetic canopies;

Figure 5 *Acacia senegal* (a, b) is the source of gum arabic (c). Some selected uses of gum Arabic: as an ingredient in soft drinks, candies, weight loss compositions (e.g., Slim Fast), and so on. Gum arabic is also used as a binder (e) for watercolor paint (f) as it dissolves readily in water. It is also used in shoe polish (g), and as a wettable adhesive, such as in postage stamps (h). Images from D. Lesueur, CIRAD, Tropical Soil Biology and Fertility Institute of CIAT, World Agroforestry Centre, Nairobi, Kenya (a–c), L. B. Davin (d and h), and H. Moore (e–g) Washington State University.

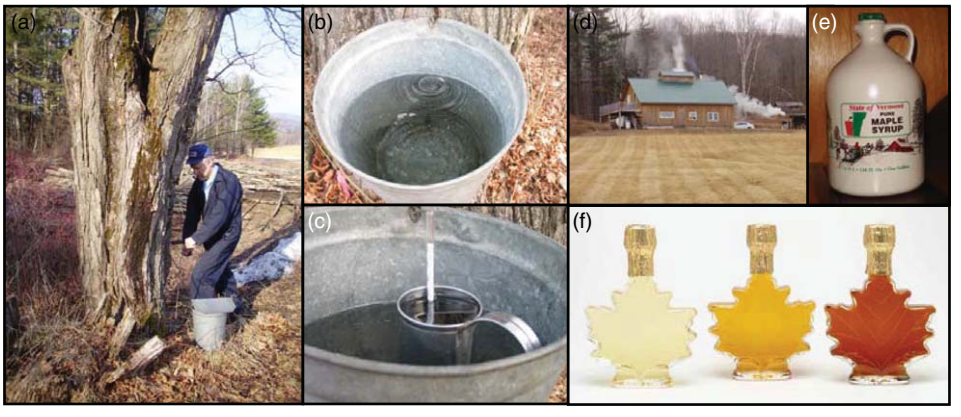


Figure 6 Maple trees (*Acer saccharum*) and maple syrup. Trees are tapped annually for their sap in early spring before buds emerge (a), with sap collection (b and c) and large amounts of sap then processed (d) to produce syrup of different grades (e, f). Most trees are tapped once annually (some twice), with each tap yielding ~80 l of sap which is then concentrated to ~1.5 l. Images from B. Putnam, Putnam Family Farm, Cambridge, Vermont, USA (a–d, f), and L. B. Davin, Washington State University (e).

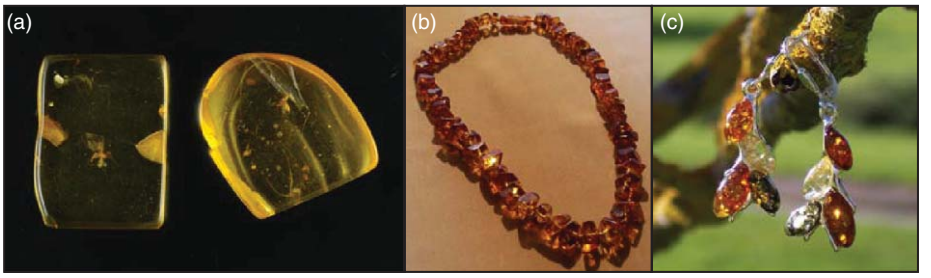


Figure 7 Tree-derived amber (fossilized resin) and jewelry. Amber is found in extensive deposits in different parts of the world: Fly (family *Chironomidae*) in amber (a). Amber is used in the making of jewelry, for example, necklace (b) and earrings (c). Amber specimen from Professor R. S. Zack, Washington State University. Images from H. Moore, Washington State University (a) and Amber Goods – Amber Jewelry (http://www.ambergoods.ie), with permission (b, c).

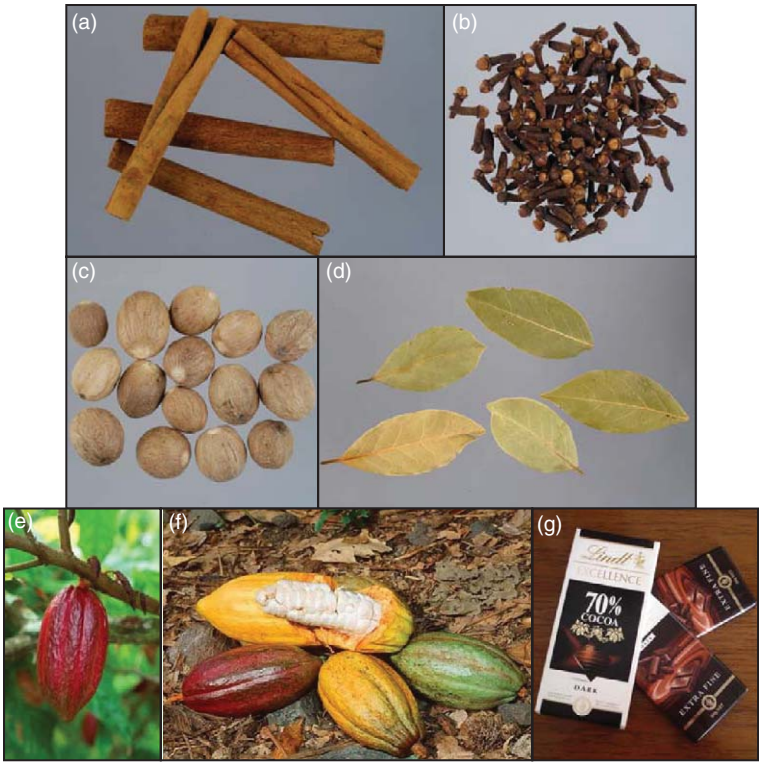


Figure 8 Selected examples of common spices from tree species: Cinnamon (*Cinnamomum verum*; a), cloves (*Syzygium aromaticum*; b), nutmeg (*Myristica fragrans*; c), and bay leaves (*Laurus nobilis*; d). Cocoa beans (*Theobroma cacao*; e–f) can be processed into chocolate (g) and other products. Images from H. Moore, Washington State University (a–d), Consulat de São Tomé & Principe, Marseille, France (e, f) and L. B. Davin, Washington State University (g).

elaboration of a plethora of often species-specific distinct biochemical pathways leading to, for example, chemical defense systems; evolution of distinct plant pollination/reproductive strategies and adaptations, and a myriad of related regulatory processes at the genomic/proteomic and metabolic levels.

3.27.2.1 Land Colonization, the Early Phases: Turgor-Based Stem Support Systems

The earliest terrestrial plants had features similar to today's bryophytes (mosses, liverworts, and hornworts), and were thus nonwoody. They were small in stature, transporting water by hydroid cell types (having primary

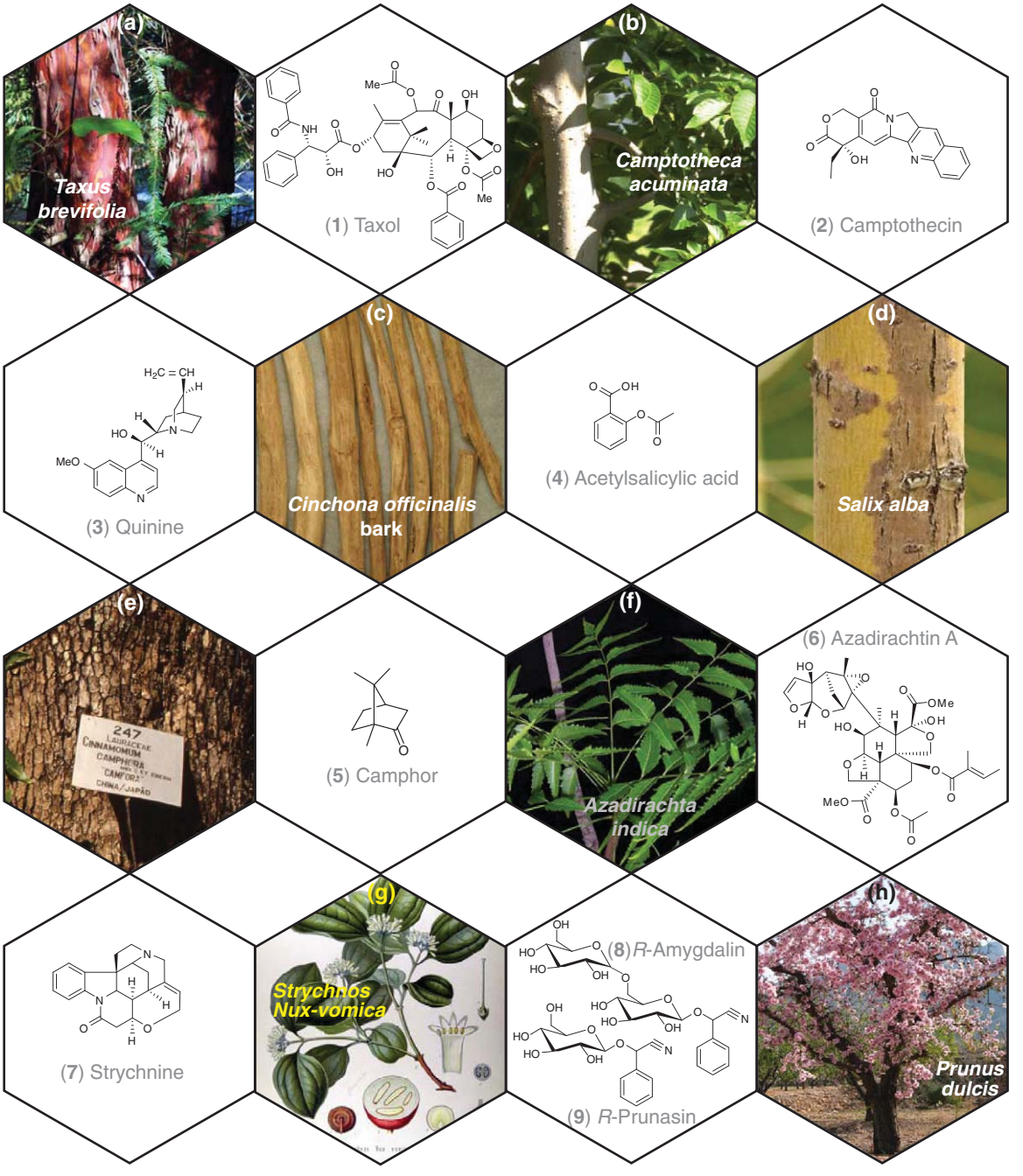


Figure 9 Phytochemical treasures: *T. brevifolia* (a) and *C. acuminata* (b) accumulate the cancer chemotherapeutics, taxol (**1**) and camptothecin (**2**), respectively. *Cinchona* spp. (e.g., *Cinchona calisaya*) (c) and *S. alba* (d) are sources of the medicinals quinine (**3**) and acetylsalicylic acid (**4**). The medicinal compound, camphor (**5**), used as a cough suppressant, is derived from *Cinnamomum camphora* (e). The neem tree (*A. indica*; f) harbors the insecticide, azadirachtin A (**6**). Poisons, such as strychnine (**7**) and cyanogenic compounds, (*R*)-amygdalin (**8**), (*R*)-prunasin (**9**), are from *S. Nux-vomica* (f) and the almond tree *P. dulcis* (g), respectively. Images from R. E. B. Ketchum, Washington State University (a), J. Manhart, Department of Biology, Texas A&M University, College Station, TX (b), The Garden of Medicinal Plants, Kyoto Pharmaceutical University, Kyoto, Japan (c), A. M. Patten, Washington State University (d), L. B. Davin, Washington State University (e), Cal Lemke, University of Oklahoma, Norman, OK (f), Köhler's Medizinal Pflanzen, 1887 (g), R. Sanchez-Perez; F. Dicenta, CSIC-CEBAS, Murcia, Spain (h).

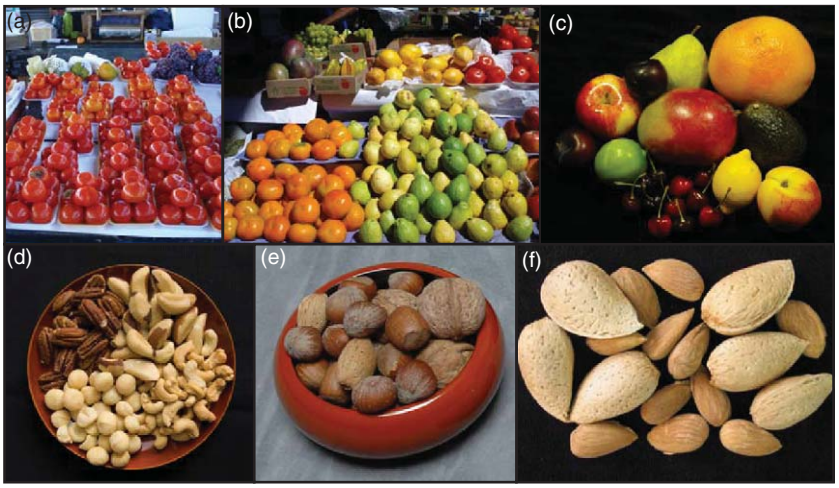


Figure 10 Trees provide humanity with an enormous range of fruit and nut products. Selected examples from temperate and tropical regions. Persimmon (*Diospyros* sp.), guava (*Psidium* sp.), mangoes (*Mangifera* spp.), starfruits (*Averrhoa carambola*) (a and b). Pear (*Pyrus* spp.), avocado (*Persea americana*), peach (*Prunus persica*, various cultivars), lemon (*Citrus limon*), grapefruit (*Citrus* × *paradisi*), cherries (*Prunus avium* and other spp./hybrids), lime (*Citrus limetta* and other spp./hybrids), apple (*Malus domestica* hybrid), plum (*Prunus domestica* and other species/varieties), and mango (*Mangifera* spp.) (c). Brazil nuts (*Bertholletia excelsa*), cashews (*Anacardium occidentale*), macademia nuts (*Macadamia integrifolia* and *M. tetraphylla*) and pecans (*Carya illinoinensis*) (d). Walnuts (*Juglans* spp.), almonds (*Prunus dulcis*), and hazelnuts (*Corylus* spp.) (e). Almonds (*P. dulcis*) with and without the shell (f). Images from J. V. Marques, Instituto de Quimica, Universidade de São Paulo, São Paulo, Brazil (a and b), A. M. Patten, Washington State University (c), L. B. Davin, Washington State University (d) H. Moore, Washington State University (e) and R. Sanchez-Perez; F. Dicenta, CSIC-CEBAS, Murcia, Spain (f).

walls only) and were covered by cuticle layers to help protect the plant body from microbes, UV irradiation, temperature fluctuations, desiccation, and/or other external insults.[9,10] Significant changes in initial cell wall properties and assemblies of these early plant forms then followed, including that of secondary wall formation/thickening in the late Silurian period ~415 Mya. This resulted in the emergence of the first tracheophyte plants, such as the now extinct *Cooksonia*,[11] so-called for their distinctive water-conducting cells, called tracheids (**Figure 13(a)**). Following *Cooksonia* emergence, however, the early plant tracheophytes subsequently diversified, resulting in numerous independent early lineages, as evidenced by the fossil records of *Rhynia* (**Figure 13(c)**),[10] *Sennicaulis*, and *Gossilingia*.[12] Within these plant groups, the tracheid secondary cell walls thus essentially became thicker with control of lateral water movement being improved throughout via defined lateral cell wall pits.[12] The tracheid was a structural advance,[12,13] however, which still exists in quite similar form in present-day plants. For example, Douglas fir (*Pseudotsuga menziesii*) has helical-shaped secondary thickenings in its tracheids (**Figure 13(b)**; ht) partially covering the internal surfaces of its primary cell walls.[14,15] Such modifications, in turn, resulted in changes in plant cell wall biopolymer composition and organization, thereby facilitating their functional properties, for example, in terms of being better able to stand upright and for water conduction, and so forth.

Examination of these early fossils also led to various suggestions of the presence of phenolic-based components, including the structural cell-wall polymers, the lignins, to account for the apparent decay-resistant nature of their secondary wall thickenings.[9,16–20] However, it is yet unclear as to when lignins proper arose.[19,20] Nevertheless, these early tracheid-containing organisms had enhanced resistance to implosion (from the negative pressure of transpiration) that helped maintain turgor pressure,[8,21] and a means of improved water delivery. It is unlikely that they provided either much stem support ($\leq$1% contribution with turgor pressure providing the rest)[10] or vertical height (i.e., $\leq$13–22 cm)[8,10] (**Figure 13(c)**). In any event, evolution of the lignified secondary cell wall reinforcement (whenever it occurred) was a major innovation for vascularization by improving water transport and overall hydration of the plant body.[22]

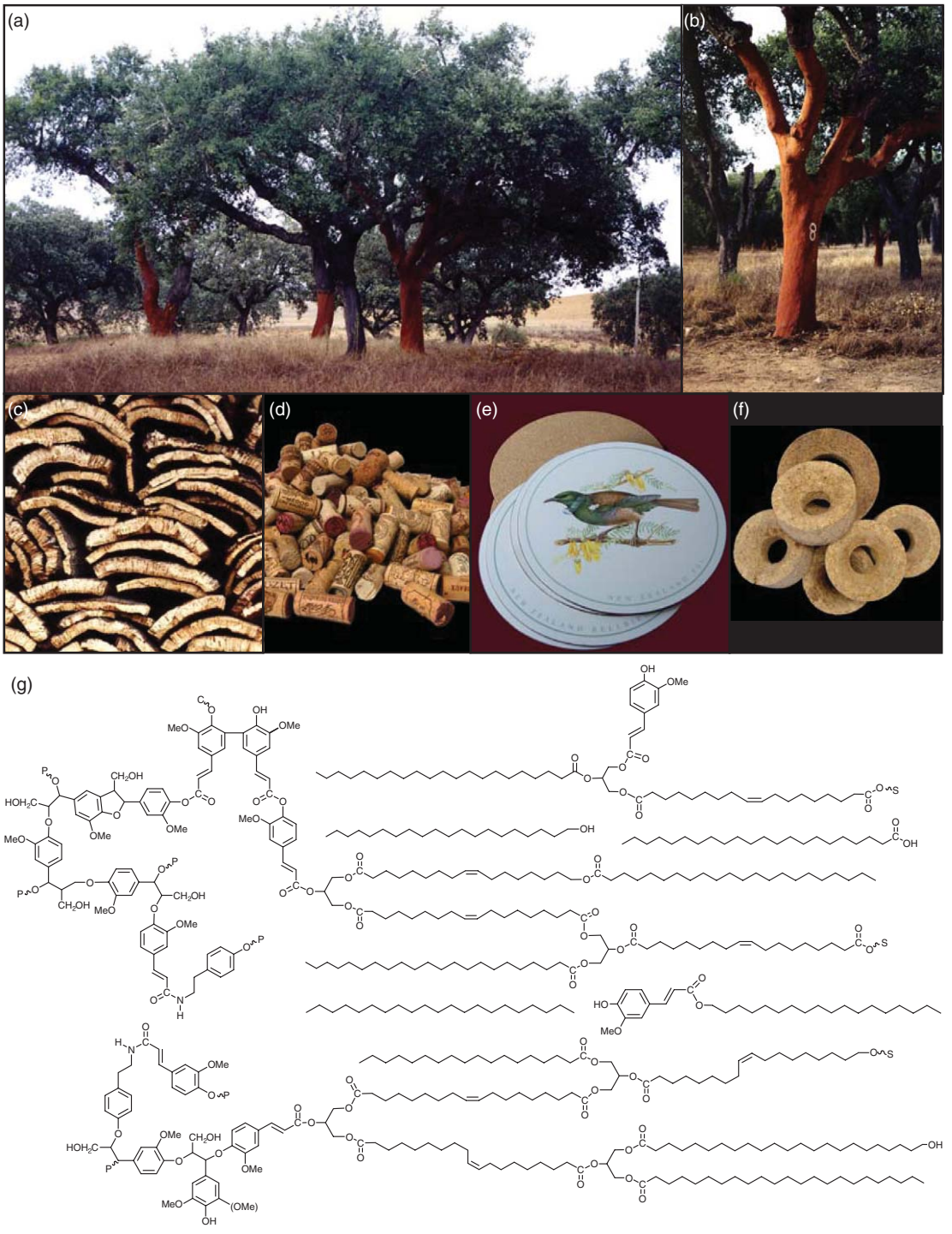


Figure 11 Cork and cork products. Harvesting (a–c) and selected uses (d–f) of oak (*Quercus suber*) bark (cork). Trees stripped of bark (i.e., red-colored stems; a and b) to afford raw cork (c). Cork is used in many products including bottling, place mats, and laboratory materials (d–f). An artistic rendition of a putative model of suberin from potato[1] that is also currently ascribed to in cork[2] (g). Images from L. B. Davin, Washington State University (a–f).

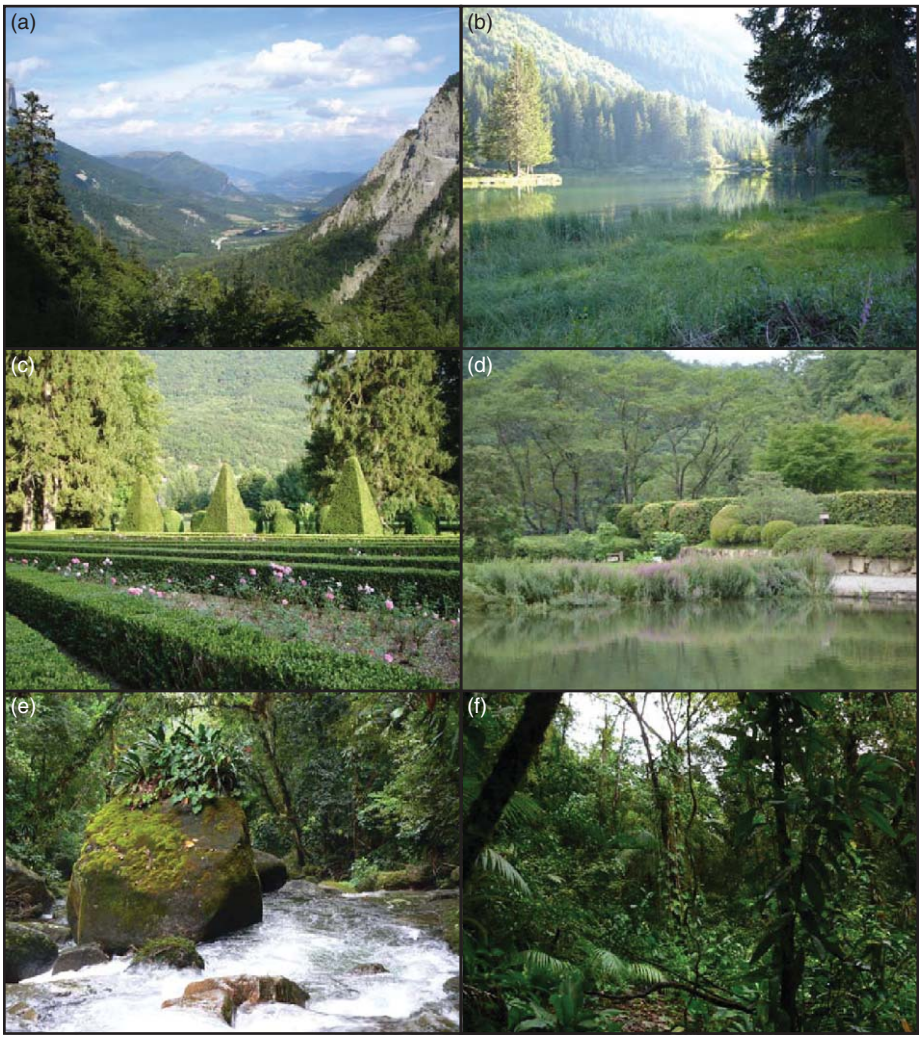


Figure 12 The realm of photosynthesis, carbon sequestration, and plant life diversity. Not only do trees contribute greatly to production of our natural resources, but photosynthesis also modulates atmospheric CO_2 levels. Pictures taken in the French Alps (a–c), Kyoto, Japan (d), and Ubatuba, São Paulo State, Brazil (e, f). Images from L. B. Davin (a–d) and D. G. Vassao (e and f), Washington State University.

3.27.2.2 Stem-Thickening Systems: Further Adaptations

To diversify into drier environments and compete for sunlight and gamete (reproductive cells) dispersal, some plant forms shifted from mainly having a turgor-based stem strength to a more self-supporting system.[8,22] Thus, by the mid-Devonian period, ~390 Mya, several independent lineages had begun to achieve 'self-support' by development of a hypodermal sterome at the periphery of the stem.[10] This consisted of rows of cells with very thick, decay-resistant, secondary cell walls called sclerenchyma. Indeed, one extinct plant form, *Psilophyton dawsonii*,[10] apparently attained ~96% of its stem flexural stiffness from its hypodermal sclerenchyma tissue[10] (sterome(s), **Figure 13(d)**), thereby providing the anatomical and structural means for its fiber-like cells to function specifically in self-support. Continued evolution and diversification of secondary cell walls within water-conducting and structurally reinforcing sclerenchyma cells later improved water transport and stem support, respectively. Additionally, changes in organization at the tissue, cell wall (and biopolymer) levels allowed for tall stems with broad photosynthetic canopies and transport of large amounts of water for photosynthesis.

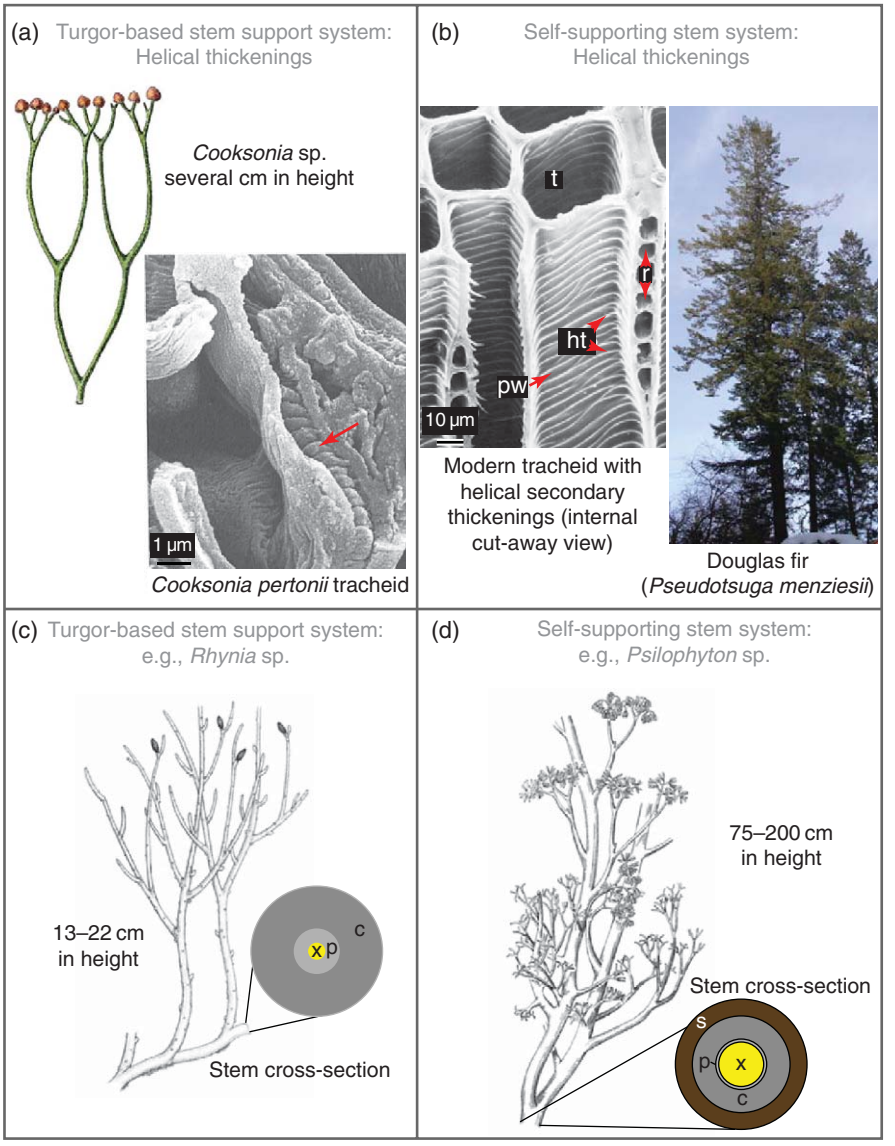


Figure 13 Early and late tracheophyte developments that ultimately lead to woody tissue. These progressions also reflect the biophysical/mechanical properties of the polymeric constituents conferred within the tracheophyte cell wall composite(s). *Cooksonia* spp. were among the earliest tracheophytes, whose tracheids had decay-resistant helical secondary cell wall thickenings (red arrow) with anatomical structures that allowed for improved overall water transport (a). Extant tracheids, such as in Douglas fir (*P. menziesii*) wood, are variations on the basic theme of a primary wall (pw) (i.e., dark background) with internal helical secondary cell wall thickenings (ht) for structural reinforcement (lighter raised surfaces) (b). Mechanical stem strength of early tracheophytes diversified from a system dependent on turgor pressure as in *Rhynia* (c) to a system complemented by a hypodermal sterome fortified by sclerenchyma cells (s) with thick secondary cell walls as in *Psilophyton* (d). Circle diagrams indicate stem structure in cross-section. Abbreviations: c, cortical parenchyma; ht, helical thickening; p, phloem; pw, primary wall; r, ray parenchyma; s, sterome; t, tracheid lumen; x, xylem/tracheids. Reproduced from *Le Monde des Végétaux*, copyright 1997 (http://www.creaweb,fr/bv/banque/cormo-cooksonia.html) and with permission of Wiley Inter Science from D. Edwards, *New Phytol.* **1993**, *125*, 225–247, copyright 1993 (a). Reproduced with permission of Syracuse University Press from H. A. Core; W. A. Cote; A. C. Day, *Wood Structure and Identification*, 2nd ed.; Syracuse University Press: Syracuse, 1979, copyright 1979, with whole tree photograph from L. B. Davin (b). Reproduced with permission of Elsevier from N. Rowe; T. Speck, Hydraulics and Mechanics of Plants: Novelty, Innovation and Evolution. In *The Evolution of Plant Physiology: From Whole Plants to Ecosystems*; A. R. Hemsley; I. Poole, Eds.; Elsevier: Amsterdam, 2004; pp 297–325, copyright 2004; (c, d).

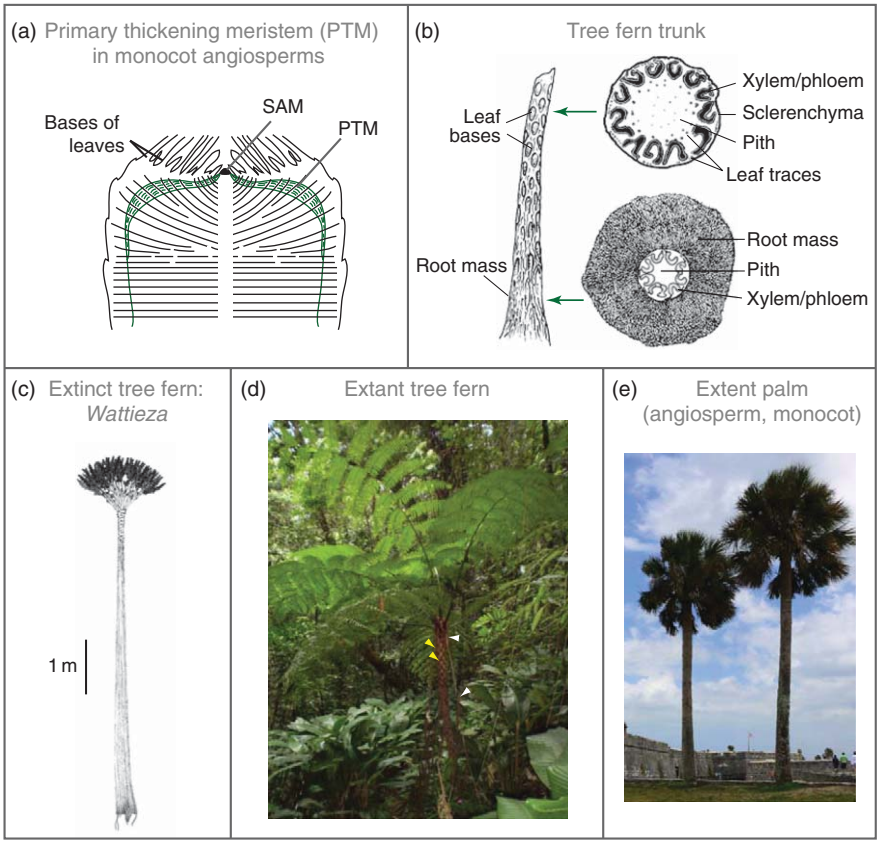


Figure 14 Primary stem-thickening systems. Stem thickening by primary tissues evolved independently in several taxa, including some extinct lineages, ferns, and monocot angiosperms. The primary thickening meristem (PTM) is derived from the shoot apical meristem (SAM), where the PTM produces leaves that add to stem thickness (a). Mechanical strength in tree ferns is derived from placement of xylem and sclerenchyma to the stem periphery, and accumulation of leaf bases along the entire stem and root growth (sometimes from the leaf bases) to form a root mass along the lower portion of the stem (b). The cladoxylopsid, *Wattiezia*, preceded both ferns and horsetails and reached at least 8 m in height (c). In extant tree ferns, old leaves abscise (white arrows) leaving behind prominent leaf scars (yellow arrowheads), with the remnant leaf bases contributing to primary thickening. Some present-day tree ferns (d) can reach ~20 m in height (not shown). Tall extant monocots, such as palms, have similar primary thickening systems (e). Reproduced with permission of Botanical Society of America from D. A. DeMason, *Am. J. Bot.* **1983**, *70*, 955–962, copyright 1983 (a). Reproduced with permission of M. F. Large from M. F. Large; J. E. Braggins, Eds.; *Tree Ferns*; Timber Press: Portland, 2004, copyright 2004 (b). Reproduced with permission of *Nature Publishing Group* (a division of Macmillan Publishers Ltd.) from W. E. Stein; F. Mannolini; L. V. Hernick; E. Landing; C. M. Berry, *Nature* **2007**, *446*, 904–907, copyright 2007 (c). Photos from M. J. Kato, Instituto de Quimica, Universidade de São Paulo, São Paulo, Brazil (d) and D. G. Vassão, Washington State University (e).

Several other stem-thickening strategies subsequently arose, including the development of specialized primary thickening (**Figure 14(a)**) as in the extant tree ferns, numerous monocot angiosperms, and cycads.[23,24] This was also achieved through independent emergence of unifacial (**Figure 15(a)**) and bifacial (**Figure 16(a)**) secondary growth in several very different taxa.[25] Of these adaptations resulting in tree forms, however, only bifacial secondary growth succeeded to the extent of giving rise to the diversity of woody species in existence that we so greatly value today.

The earliest tree form with a perennial trunk emerged during the mid-Devonian period, 397–385 Mya. This was the now extinct *Wattieza* (**Figure 14(c)**), a giant ~8–16 m cladoxylopsid, which represents a lineage that was ancestral to major lineages, such as Pteridophyta (i.e., the ferns) and Sphenophyta (the horsetails).[26] Unlike present-day trees, *Wattieza* apparently lacked a taproot, permanent branches, and secondary growth. Nevertheless, using a primary thickening strategy (see below), it successfully achieved arborescence and comprised the earliest known forest.[26]

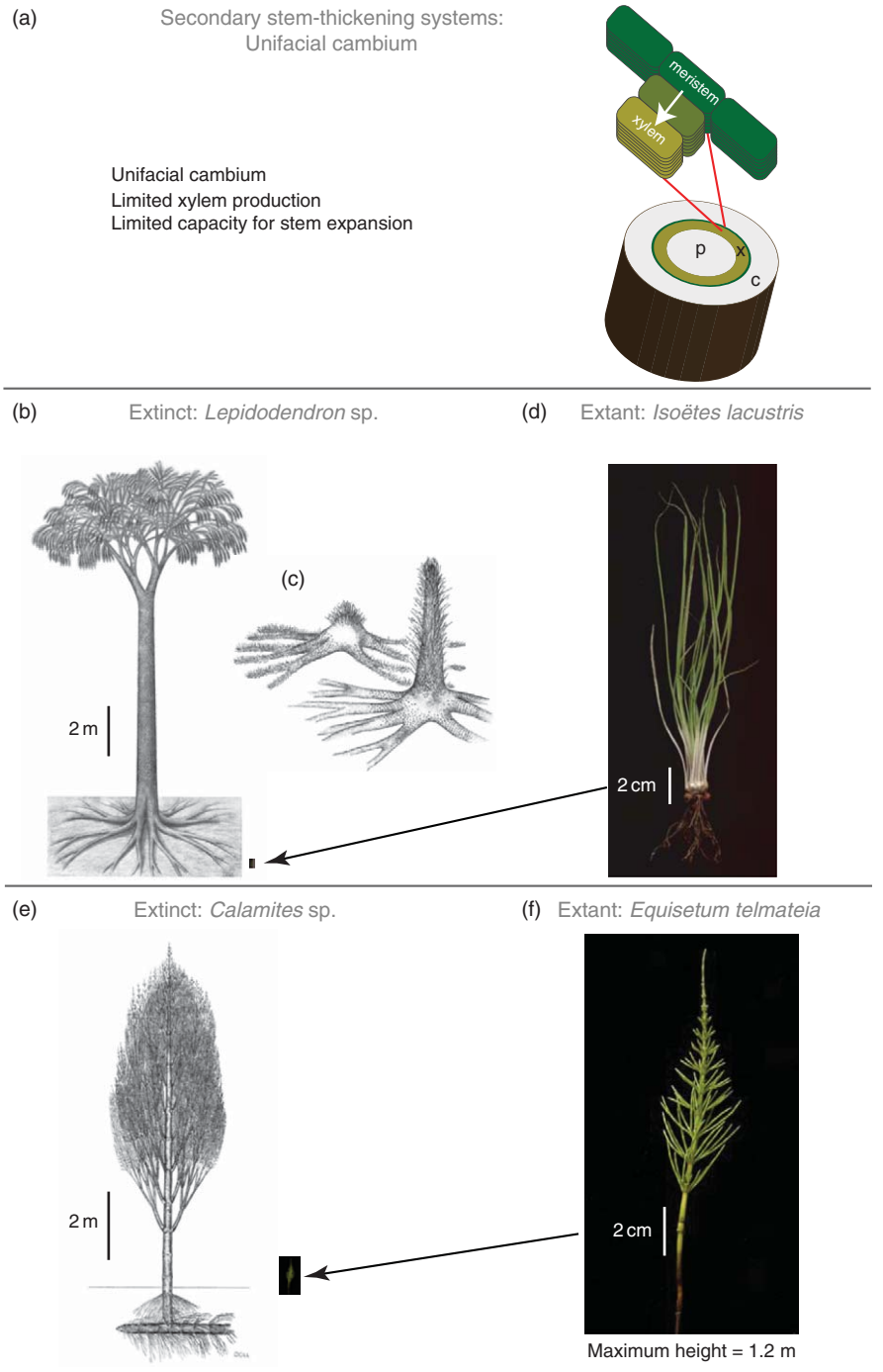


Figure 15 Evolution of the first stem secondary thickening system: the unifacial cambium. This meristem type produced only a limited amount of wood (secondary xylem) because the meristem could not regenerate itself. No secondary phloem was produced (a). Lycophytes emerged ~400 Mya which later developed an unifacial cambium that allowed for tree forms known as early as ~300 Mya, such as in the genus *Lepidodendron* (b). However, these plants spent much of their lives in a stump-like form (c), only generating an active unifacial cambium for short-term stem generation during the reproduction stage (b). Lycophyte tree forms became extinct long ago. Diminutive species exist today, for example, *Isoëtes lacustris* (d). Ancient horsetails also produced substantial tree-like forms by means of an unifacial cambium in the genus *Calamites*, for example (e). These were ultimately unsuccessful as tree forms. Modern horsetails reach no more than ~1.2 m in height (f). Abbreviations: c, cortex; p, pith; x, xylem. Redrawn with permission of Paleontological Society from M. J. Donoghue, *Paleobiology* **2005**, *31*, 77–93, copyright 2005 (a). Reproduced from M. Hirmer, *Handbuch der Paläobotanik*; Druck und Verlag von R. Oldenbourg: München und Berlin, 1927, Vol. 1 (b, e). Reproduced with permission of Elsevier from W. A. Dimichele; T. L. Phillips, *Rev. Palaeobot. Palynol.*, **1985**, *44*, 1–26, copyright 1985 (c). Photos from A. M. Patten, Washington State University (d, f).

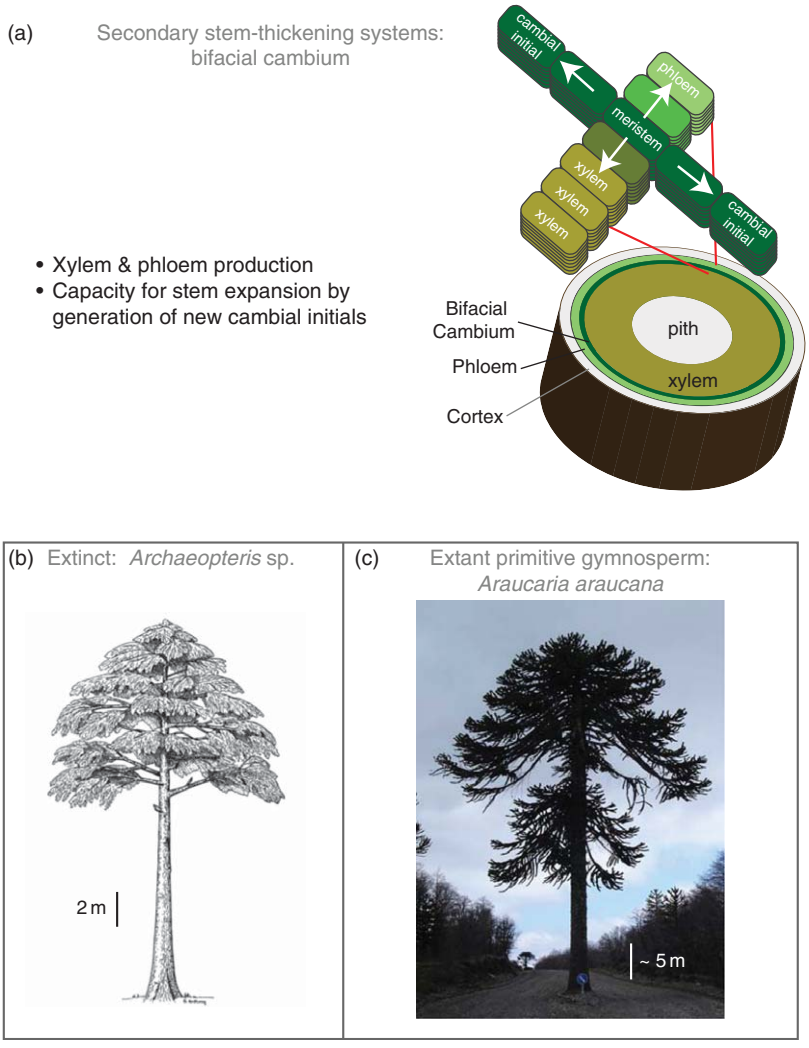


Figure 16 The most prevalent extant stem secondary thickening system: the bifacial cambium. This meristem not only produces secondary phloem and xylem, but also regenerates its own cambial initial cells to allow for stem expansion (a). The earliest tree form considered to have bifacial cambium activity was from the genus *Archaeopteris* (b). This belonged to the progymnosperms, which in turn gave rise to gymnosperms and later angiosperms. *Araucaria araucana* (c) is an example of a primitive extant gymnosperm. Redrawn with permission of Paleontological Society from M. J. Donoghue, *Paleobiology* **2005**, *31*, 77–93, copyright 2005 (a). Reproduced with permission of Botanical Society of America from C. B. Beck, *Am. J. Bot.* **1962**, *49*, 373–382, copyright 1962 (b). Photo from L. B. Davin, Washington State University (c).

Among the Pteridophyta, ferns with tree forms began appearing *ca.* 340 Mya. These were quite abundant by 245 Mya and were represented by several independent lineages.[24] Ancient tree ferns were, however, similar to the extant tree ferns of the Dicksoniaceace and Cyatheaceae that can reach maximum heights of ~20 m (**Figure 14(d)**).[24] Tree ferns lack secondary growth though, instead deriving their stem strength from localization of xylem and sclerenchyma to the stem periphery, and from the accumulation of leaf bases and/or roots around the periphery (**Figure 14(b)**).[7,24,27] This latter stem-strengthening strategy thickens the stem, allowing for an arborescent form. This is considered a primary thickening strategy because the leaves (and leaf bases) are produced from a primary thickening meristem (PTM) (**Figure 14(a)**). This particular strategy resulted in arborescent forms arising independently in several lineages, including monocot angiosperms,[23] such as the present-day palms (**Figure 14(e)**), which can reach as much as 58 m in height.[28] These again reflected changes in cell wall biopolymer content, and tissue and cell wall organization.

In contrast to primary stem-thickening systems, secondary thickening strategies arose producing secondary xylem. These included unifacial (as in the Lycophytina and Sphenophytina) and bifacial cambia. Although the Lycophytina evolved more than 400 Mya, their arborescent forms appeared in the Upper Carboniferous terrestrial landscape ~307–299 Mya[7] (Figures **15(b)** and **15(e)**) but were unlike trees as we know today. These early adaptations ultimately became the ancestors to the present-day, small herbaceous, so-called 'primitive' plants. Within the lycopsid lineage, the Carboniferous *Lepidodendron* spp.[7,29] towered at ~30–35 m (**Figure 15(b)**). However, their distant descendants, such as extant *Isoëtes* spp., lack wood-producing cambia and are comparatively diminutive (**Figure 15(d)**). Similarly, early members of the Sphenophytina, such as *Calamites*,[7,29] stood at ~10–20 m in height (**Figure 15(e)**). By contrast, present-day sphenopsids, the horsetails, reach only a fraction of that maximum height (**Figure 15(f)**).

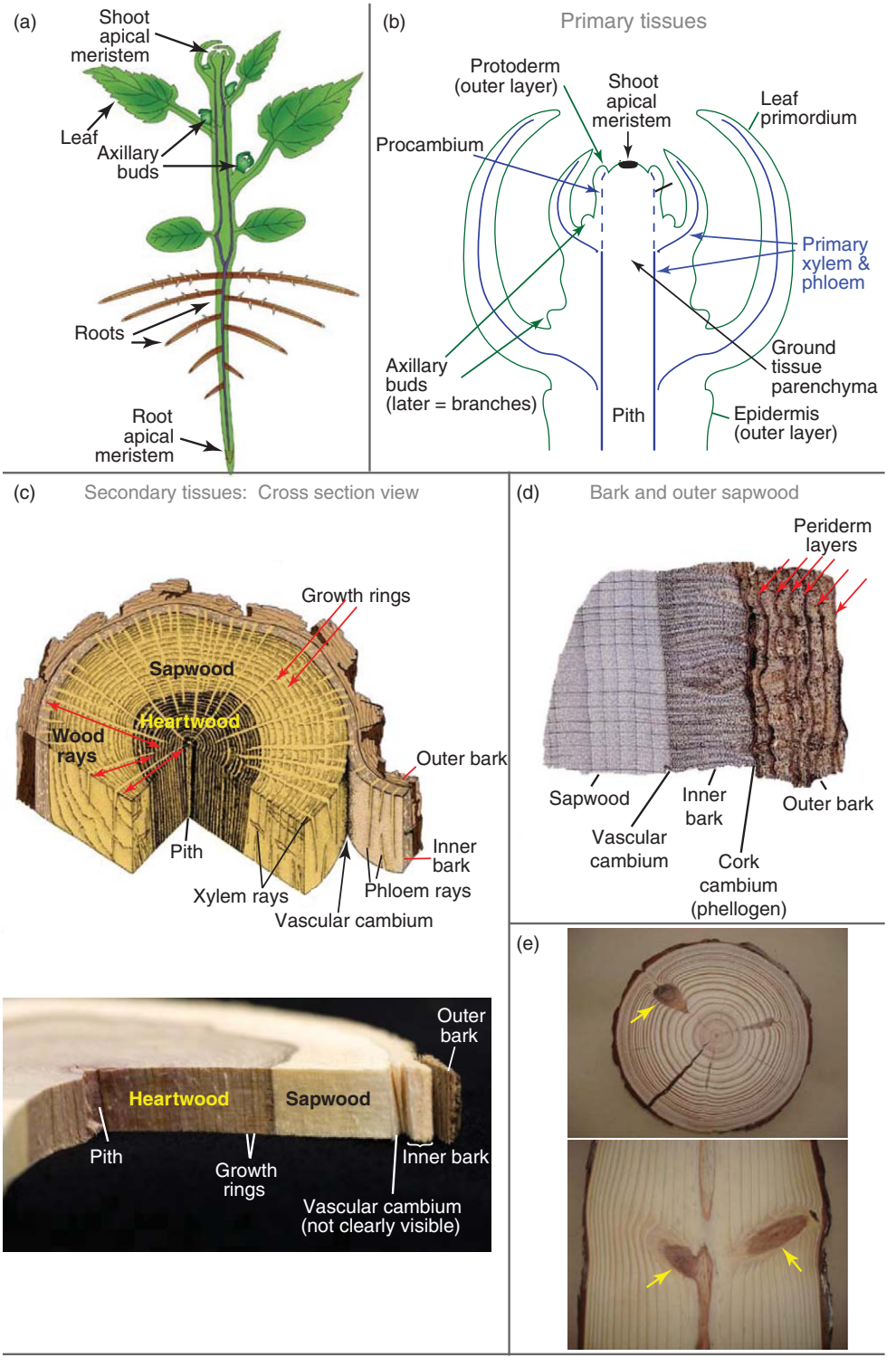
Despite the impressive sizes of *Lepidodendron* and *Calamites* spp. and others, these 'giants' were limited by having an unifacial vascular cambium (**Figure 15(a)**) that was unable to either generate secondary phloem (see Section 3.27.3.1) or regenerate its own cambial initial (stem) cells. As such, their plant bodies were reliant on primary phloem for nutrient transport[7] and in some cases, photosynthetic activity by leaves along most of the trunk.[27] However, they produced very little wood. In fact, their 1–2 m diameter trunks were largely composed of secondary cortex,[7] an unique outer layer with similarities closer to bark.[27] Interestingly, *Lepidodendron* apparently also spent much of its life in a stump-like form (**Figure 15(c)**), only producing a tall trunk to disperse spores for a brief time – a possible consequence of the limited nutrient transport system.[27,30]

By contrast, wood evolution proper was ultimately dependent on the emergence of vascular and cork (phellogen) cambia. In this regard, the bifacial vascular cambium provided the means to produce (1) both sapwood (SW) xylem and phloem within the inner bark, with greater cell division and overall biochemical activity in cell wall generation toward the xylem side (**Figures 16(a)** and **17(c)**) and (2) new vascular cambial initials, which facilitated circumferential expansion (**Figure 16(a)**). Interestingly, the earliest fossil evidence of bifacial cambia dates back to the mid-Devonian period ~360 Mya in the first-known Lignophytes, a group including the progymnosperms. These were the spore-bearing immediate forerunners of seed plants.[8,25] Among them were the first perennial trees[31] with secondary growth, such as the extinct progymnosperm, *Archaeopteris*[32] (**Figure 16(b)**). However, limited to a determinate growth pattern,[8,25] this development did not add much to mechanical strength, although it apparently increased water supply to support enlarged canopy growth. As a result, the early Lignophytes essentially relied on a large outer ring of cortex for most of their stem strength[8,25] that facilitated growth to ~18 m in height for *Archaeopteris*.[32]

It was not until the later development of a periderm (bark) that Lignophytes were able to increase secondary growth, thus producing a secondary body that became a major portion of the trunk. This shifted mechanical strength from the outer portion of the stem to the inner, thereby giving these early trees a means for a more indeterminate growth pattern.[8,33] Additionally, wood rays (see Section 3.27.3.3.1) consistently appeared in all early Lignophytes, suggesting their functional and/or structural necessity for wood.[12,31]

From these early forerunners, the gymnosperms subsequently arose (e.g., *Araucaria araucana*, **Figure 16(c)**), followed by angiosperms, and from these lineages our extant trees/woods were derived (**Figure 18**). Of these, the earliest conifers classified within the gymnosperms emerged during the Carboniferous period ~308 Mya.[31] However, the first conifer wood similar to that observed in extant species did not appear until the late Triassic period ~228–200 Mya.[31,34] Subsequently, angiosperms arose from basal seed plants ~130 Mya,[35] although there remains some controversy as to whether the earliest representatives were either herbaceous or woody.[36] Nevertheless, the earliest angiosperm wood fossils date from the Lower Cretaceous period ~145–100 Mya.[37]

The woody gymnosperms (sometimes called evergreens) are also often referred to as softwoods, whereas angiosperms (flowering, broadleaf, and/or deciduous trees) are frequently described as hardwoods. These softwood and hardwood descriptions generally refer to their lumber/timber properties. Such terms are loosely applied and do not, however, reflect absolute physical ranges of density or other properties.[38–40] Moreover, they are also overgeneralizations: some gymnosperms are deciduous (i.e., with seasonal leaf loss, such as larch (*Larix)* or ginkgo), while some angiosperms are considered evergreen, such as *Illicium* sp. (anise) and *Quercus ilex* (evergreen oak). Nevertheless, the 100 000 or so different wood species extant today provide


(a)
Shoot apical meristem
Leaf
Axillary buds
Roots
Root apical meristem
(b)
Primary tissues
Protoderm (outer layer)
Shoot apical meristem
Leaf primordium
Procambium
Primary xylem & phloem
Axillary buds (later = branches)
Ground tissue parenchyma
Pith
Epidermis (outer layer)
(c)
Secondary tissues: Cross section view
Growth rings
Sapwood
Heartwood
Wood rays
Outer bark
Pith
Inner bark
Xylem rays
Phloem rays
Vascular cambium
Outer bark
Heartwood
Sapwood
Pith
Growth rings
Inner bark
Vascular cambium (not clearly visible)
(d)
Bark and outer sapwood
Periderm layers
Sapwood
Inner bark
Outer bark
Vascular cambium
Cork cambium (phellogen)
(e)

humanity with an enormous richness, which, in turn, has resulted in our crucial dependence on these quite remarkable life forms. Such developments, however, have all resulted from innumerable changes/modifications as to how the vascular plants (chemically) produce their various cell wall types, cellular contents/phytochemicals, tissues, and organs.

3.27.3 Wood Anatomy and Cellular/Tissue Function: The Living and the Dead

The different tissues and cell types found in wood and bark are next considered in order to provide a foundation for the interested reader in terms of both anatomy and physiological function. This is summarized so that the various material/phytochemical properties being subsequently discussed are placed in useful context with tree form, function, and ultimate use. Of course, wood and bark tissues are heterogeneous in terms of their cell types and chemistries, and this variability can often result in specific phytochemical/material properties as discussed later.

3.27.3.1 Tree Growth/Development and Wood/Bark Generation

Growth and development of both gymnosperm and angiosperm trees follow a general sequence to produce highly modular organisms. This modularity allows for redundancy in function, such as multiple branches, wide circumferential distribution of xylem, phloem, and bark. This redundancy, in turn, enables trees to respond to changing environmental conditions and to isolate (compartmentalize) injured tissues.[41,42] Some of this redundancy is also important for various forms of storage tissues, including that for specific phytochemical accumulation.

As in all plants, the initial embryonic/hypocotyl growth of trees involves elongation from their shoot and root apical meristems[43] (SAM and RAM, respectively; **Figure 17(a)**), with the shoot-generating tissues that will eventually form the woody trunk. Shoot primary growth thus consists of the ground tissue or parenchyma, the epidermis or outer layer, and the primary vascular tissues, the primary xylem and phloem[15] (**Figure 17(b)**). As the hypocotyl develop into a young sapling, the secondary meristems then quickly develop to increase lateral growth and, in young tree trunks, the centrally located ground tissue forms the pith. Activation of the vascular cambium next begins generation of secondary tissues, including SW and bark formation. The latter begins to form through secondary phloem development (**Figure 17(c)**) with the appearance of an active cork cambium also called phellogen (**Figure 17(d)**).[44] As the trunk elongates, branches additionally form at intervals as both trunk and branches expand by secondary growth of the wood and bark. The complex junctions between the trunk and the branches are also fortified by knotwood (**Figure 17(e)**, yellow arrows), which is often 'extractives-rich.' Once the base of the trunk matures, heartwood (HW) can then form beginning within the oldest SW closest to the pith (**Figure 17(c)**); however, this phase generally occurs at indeterminate times. HW ultimately becomes the most valuable of the woody resource(s).

During tree growth and development, other external influences may cause numerous variations/irregularities in woody tissue, including responses to mechanical stressors (i.e., reaction wood formation, discussed below) or injury (e.g., leading to further development of secretory structures). The next sections thus discuss the

Figure 17 General tissue systems of trees. Seedling growth occurs through the shoot apical meristem (SAM), giving rise to the shoot system, and the root apical meristem (RAM) leading to the root system. Axillary buds are derived from the SAM (a). The SAM affords all primary tissues, including ground tissue parenchyma, epidermis (from protoderm), primary xylem and phloem, and axillary buds leading to either branches, flowers, or leaves (b). Secondary tissues (cross-section view) of the tree trunk include wood (heartwood (HW) and sapwood (SW)), the ray system, the inner and outer bark, and the vascular cambium that produces both SW and phloem of the inner bark (c). The lower image shows a fresh wood section from lilac (*Syringa vulgaris*) with HW and SW clearly visible (c). Bark and outer SW (longitudinal view) (d), with the cork cambium producing the outer bark (layers of periderm). Cross-section and longitudinal views of knotwood from loblolly pine (*Pinus taeda*) and shortleaf pine (*Pinus echinata*), respectively (e). Reproduced with permission of *Nature Publishing Group* from M. Tsiantis; A. Hay, *Nat. Rev. Genet.* **2003**, *4*, 169–180, copyright 2003 (a). Reproduced with permission of Freeman and Company Publishers from P. H. Raven; R. F. Evert, S. E. Eichhorn, *Biology of Plants*, 7th ed.; Freeman and Company Publishers: New York, 2005, copyright 2005 (c, d). Photo of lilac stem section from A. M. Patten, Washington State University (c). Photos from T. L. Eberhardt, USDA Forest Service, Southern Research Station, Pineville, LA, USA (d).

Figure 18 Gymnosperm and angiosperm trees: growth habits of typical temperate zone examples. Gymnosperms generally have evergreen needle-like leaves and an overall triangular growth form due in part to a regular branching pattern; *Picea* sp. (a). Angiosperms are generally deciduous with a rounded canopy growth form related to its complex branching system; *Salix* sp. (b). Photos from L. B. Davin, Washington State University.

various forms of tissues that are generated during wood and bark development which, in turn, reflect the changes in cell wall chemistries and so forth.

3.27.3.1.1 Juvenile wood

Wood formed during the first three or so years of life of a tree is generally dominated by the so-called juvenile wood[45,46] (**Figure 19(a)**). Although formed by the vascular cambium, juvenile wood differs from SW because its formation is relatively close to the SAM, which influences the vascular cambium activity in a poorly understood manner.[47] In general, juvenile wood tends to have high variability in physical and anatomical

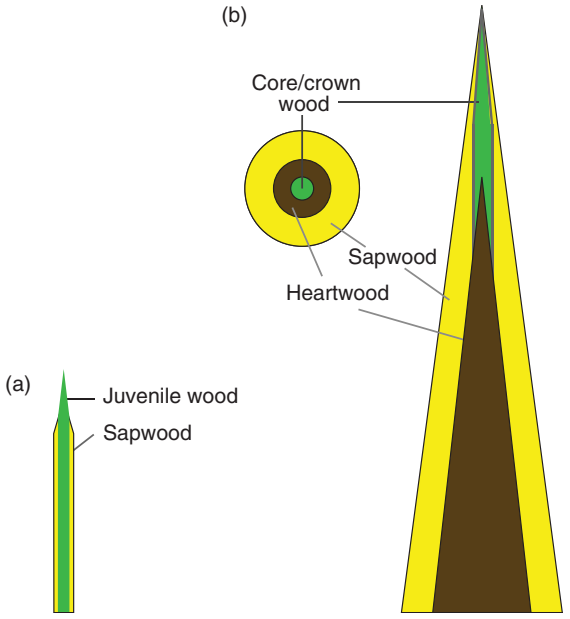


Figure 19 Stylized trunks of the sapling and mature tree. Juvenile wood is the first-formed wood that dominates the sapling trunk (a). It ultimately becomes confined to the core of the trunk with the development of mature SW, and eventually becomes part of the HW (b). Redrawn from P. Saranpää, Wood Density and Growth. In *Wood Quality and Its Biological Basis*; J. R. Barnett, G. Jeronimidis, Eds.; Blackwell Publishing Ltd.: Oxford, UK, 2003; pp 87–117 (b).

properties,[45] with characteristics similar to early wood[48] (see Section 3.27.3.1.2). Gymnosperm juvenile wood has also been reported to have some compression wood (CW)-like characteristics (see Larson *et al.*[45] and Timmel,[49] and references therein). Although it is not yet fully clear what the specific advantages of juvenile wood are, the low stiffness it confers to saplings apparently gives the young stem the mechanical flexibility needed to contend with wind and other bending stresses.[48]

As the tree grows and matures, the SAM continues to influence the vascular cambium nearest the apex, in turn influencing the youngest seasonal wood growth. While retaining some juvenile wood characteristics, this so-called core wood forms the central rings of the trunk. With further maturity, this earliest apical wood has additional variability and is also referred to as crown wood. The differences between juvenile, core, and crown wood are currently considered relatively minor with these three wood types forming a continuum throughout the trunk.[45] Together, they contribute to water transport and overall stem strength until eventually being converted into a part of the HW,[46] and part of the structural core of the tree stem (**Figure 19(b)**).

Juvenile, core, and crown woods are also generally considered to be low-quality resources, because of their high anatomical variability, low-specific gravity, low density and strength, and greater longitudinal shrinkage.[48,50] Such negative characteristics are more pronounced in gymnosperm woods than in angiosperm woods; hence, they have been more widely studied in conifers (see Larson *et al.*,[45] and references therein, and Bao[51]). Of little consequence in the days of old-growth logging, this has become an increasingly important research area because of current and projected uses for fast-growing, short-rotation species.[50]

3.27.3.1.2 Sapwood

The next phase or phases of wood development involve(s) generation of SW, which physiologically is the water-/sap-conducting tissue of the tree. SW retains this functional capacity until its conversion into HW, which can take 5–20 years or so depending on the species. The volume of SW at tree maturity may only account for about 5% of the wood, depending on when – and how extensively HW – is later formed. Nevertheless, SW contains numerous structural (anatomical) elements, and the major vertical/lateral transport networks via its various cell types, and thus the means for both mechanical support and essential water/sap conduction (**Figures 20(a)**[52] and **20(b)**[14]). As discussed later, many of the living components in woody tissues are also present in SW. Indeed, various cell types in the annually generated SW of any given annual ring can remain active for 5–10 years or more.[39,53,54]

In temperate zone species, SW formation varies according to season. That is, the SW first formed annually (the so-called early or springwood) is characterized by high water conductivity and low stiffness to facilitate rapid growth. Late in the growing season, late or summer wood forms, which has greater strength, but lower water conduction, thereby lending additional structural support to the stem (**Figure 20(a)**, see late vs. early wood tracheids). One round of each type of early and late wood growth comprises a single annular ring in the SW, although this can also vary with environmental disturbances. During the first 3 years or so, however, saplings produce annual rings that are dominated by 'springwood,' this being the juvenile wood discussed above.[45] In mature trees, only the outer rings of SW provide effective water conduction, albeit where the radial and axial parenchyma can remain living and active in transport and storage functions.[55]

3.27.3.1.3 Reaction wood

Trees have quite remarkable biochemical mechanisms for responding to mechanical, and perhaps photosynthetic, stressors as well. These responses can be as basic as facilitating the upright orientation of the trunk, and controlling/modulating lateral branching, in order to continually orient the photosynthetic canopy. However, reaction wood formation can also occur in response to external influences, such as either growing on a slope or being battered by wind, snow, and so on. In order to achieve such control over both stem and branch orientations, two very distinct reaction wood systems have evolved to achieve essentially the same effect within the woody gymnosperms and angiosperms. This involves formation of compression wood (CW, **Figure 21(a)**) in gymnosperms with increased lignin content, and tension wood (TW, **Figure 21(d)**) in angiosperms with increased (gelatinous fiber) cellulose levels. Note that while reaction wood can be found in

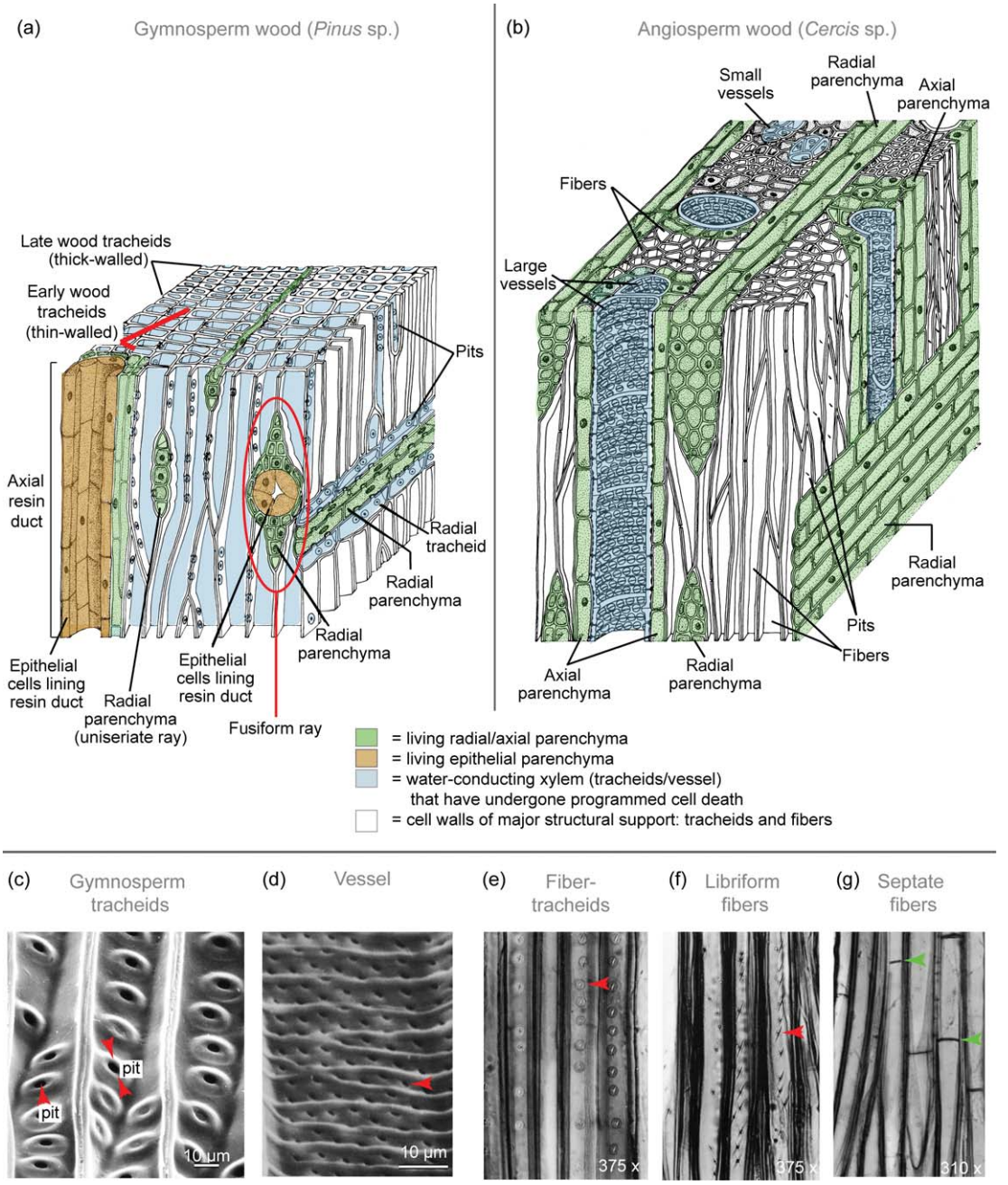


Figure 20 Idealized comparative cross-sectional anatomy between gymnosperm (a) and angiosperm (b) woods. Gymnosperm wood (both early and late wood) is characterized by a majority of tracheids that can function for both structural support and water conduction. Gymnosperm wood rays are composed of radial parenchyma for nutrient/metabolite lateral transport throughout the trunk. In *Pinus* spp. radial tracheids may also occur for lateral water transport (not found in non-*Pinus*). Resin ducts (axial or radial) may be present and function in defense (a). Tracheids are characterized by simple pits that allow for cell-to-cell water transport (c; red arrows). Angiosperm wood has large vessels for water conduction (b), again with pits for lateral water transport (d; red arrow). Adjacent axial parenchyma also function in water/nutrient storage. A complex ray system (radial parenchyma) functions in nutrient/metabolite lateral transport throughout the wood and phloem (phloem not shown). Angiosperm fibers also provide structural support (b). Fiber types in angiosperms vary from fiber tracheids (e) to libriform fibers (f) to septate fibers (g) (pits, red arrows and septa, green arrows). Reproduced with permission of Elsevier from A. Fahn, *Plant Anatomy*, 3rd ed.; Pergamon Press: Oxford, 1982, copyright 1982 (a, b). Reproduced with permission of Syracuse University Press from H. A. Core; W. A. Cote; A. C. Day, *Wood Structure and Identification*, 2nd ed.; Syracuse University Press: Syracuse, 1979, copyright 1979 (c–g).

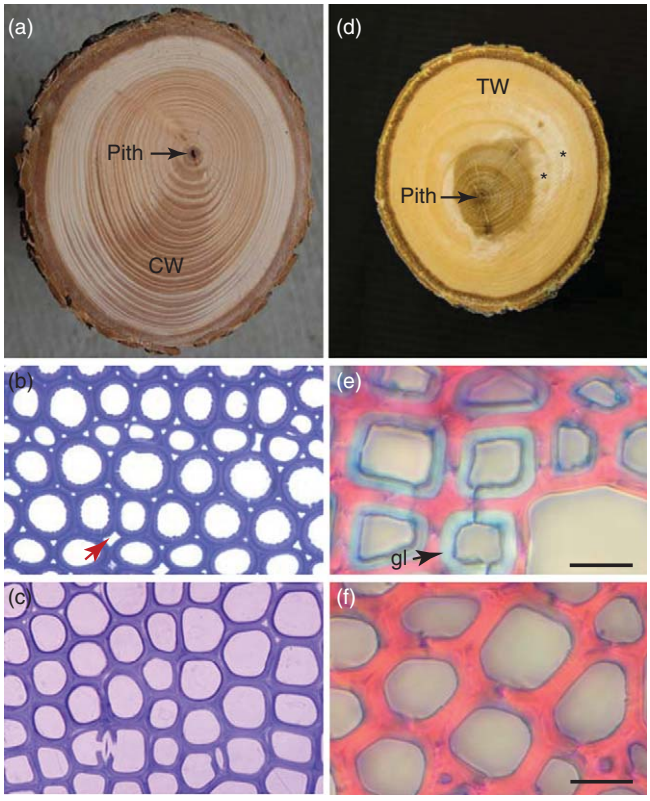


Figure 21 Reaction wood types in gymnosperms and angiosperms. Reaction wood formation often results in eccentric growth relative to the center (pith). Compression (reaction) wood (CW) is formed on the under sides of the gymnosperm branches (shown here for Douglas fir (*Pseudotsuga menziesii*; a) or on the leaning side of the gymnosperm tree trunks. Compression wood tracheid lumens are smaller and rounder (b) than those of normal wood tracheids (c) in Douglas fir. Intercellular spaces occur in severe compression wood (arrow; b). Tension (reaction) wood (TW) is formed on the upper side of the angiosperm tree branches (shown here for *Populus* sp., d) and leaning stems. In *Populus* spp. and a few other species, rough-sawn wood takes on a whitish appearance (*) where concentrated areas of cellulose-rich gelatinous fibers occur (d). In fast-growing species, such as *Populus*, gelatinous fibers may occur in spiral pattern (*, d). Gelatinous fibers (*Populus trichocarpa*; blue stain; e) frequently occur within TW of temperate zone angiosperm trees; normal wood fibers (f). Scale = 50 μm (e, f). Photos from L. B. Davin (a, d). Reproduced with permission of American Society of Plant Biologists from R. Croteau; T. M. Kutchan; N. G. Lewis, Natural Products (Secondary Metabolites). In *Biochemistry and Molecular Biology of Plants*; B. B. Buchanan, W. Gruissem, R. L. Jones, Eds.; American Society of Plant Physiologists: Rockville, MD, 2000; pp 1250–1318, copyright 2000 (b and c). Reproduced with permission of Botanical Society of America from A. M. Patten; M. Jourdes; E. E. Brown; M. P. Laborie; L. B. Davin; N. G. Lewis, *Am. J. Bot.* **2007**, *94*, 912–925, copyright 2007 (e and f).

both SW and HW, it is only formed by cells which were once in a living stage, regardless of the tissue/wood type it ultimately results in.

The gymnosperm compression wood thus provides high compressive strength at the base and undersides of branches (**Figure 21(a)**), and on the undersides of leaning trunks.[49,56] Less commonly, it may also form spiral patterns in rapidly growing saplings, due to the mechanical stress of circumnutational (i.e., bending in many directions) movements.[57,58]

On the other hand, angiosperms form tension wood in response to bending stresses, usually on the upper sides of trunks and branches (**Figure 21(d)**), although spirally formed tension wood can also occur in fast-growing species. Tension wood properties are much more variable than those of compression wood, in part due to the high genotypic/phenotypic diversity among the angiosperm taxa (discussed later).

Nevertheless, despite the structural benefits to the tree, the unique mechanical and chemical properties of reaction wood tissues pose significant complications to commercial processing and are thus a quite undesirable trait. Moreover, these reflect the influences of altering the compositions of the polymeric constituents in the cell

walls on their material properties. For example, the mechanical differences of compression tracheids translate into decreased pulp yield and paper quality.[59–61] Tension wood lumber also differs in fracture and cutting quality and has tendencies to split and crack, among other traits.[62,63]

3.27.3.1.4 Heartwood

Mature trees can also contain large amounts of centrally located HW (**Figure 22**), which at maturity may represent as much as 95% of the merchantable wood.[64] In the most general terms, HW generation is initiated at an indeterminate stage, for example, after about 5 years growth/development or even decades later depending upon the species. HW initiation occurs within the innermost mature SW of the lower trunk, proceeding upward ultimately to comprise most of the trunk. Relative to SW, the HW tissues are generally characterized by both higher density and higher 'extractives' content, whose chemical constituents often contribute positively to the commercial value of many wood species. Extractives often impart a darker coloration to the wood: for example, the red-brownish color of western red cedar (*Thuja plicata*, **Figure 22(a)**), the blackish colored HW of ebony (*Diospyros* spp., **Figure 22(b)**), and the purplish coloration of lilac (*Syringa vulgaris*, **Figure 22(c)**).

The formation of such extractive deposits begins with metabolism in SW ray parenchyma.[65] At later stages, it also occurs in the so-called transition zones (i.e., between SW and HW). Transition zones are only readily observed, however, in some species such as *Syringa* spp. where it appears lighter in color than either SW or HW (**Figure 22(c)**). Our understanding of HW-forming processes is, nevertheless, at a very incomplete level, in large part due to its recalcitrant and varied nature. Technical obstacles include: (1) the presence of copious resinous/phenolic deposits (so-called 'extractives') in HW, some of which are not readily extractable;[64] (2) the quite remarkable variability in amounts and types of major extractives/metabolites present, which can differ markedly according to species, for example, plicatic acid (**10**) derivatives (lignans) in western red cedar,[66–68] the so-called 'hydrolyzable' tannins (e.g., vescalagin (**11**) and castalagin (**12**)) in oak,[69–71] and the so-called 'phlobaphenes' (considered to be acid-catalyzed rearrangement products of 'condensed' tannins) in redwood (*Sequoia sempervirens*)[72,73] and in ebony wood;[74] (3) their variability in appearance, where many HWs are dark, while others are light-colored, such as in the so-called 'ripewoods,' for example, in Norway spruce (*Picea abies*[75]) and *Populus* spp. Note that some species of maples (*Acer* spp.) lack HW,[75] and some other species may accumulate quite low levels of resinous/phenolic deposits, such as spruce (*P. abies*). As discussed later, these differences in chemistries have pivotal effects on their utilization.

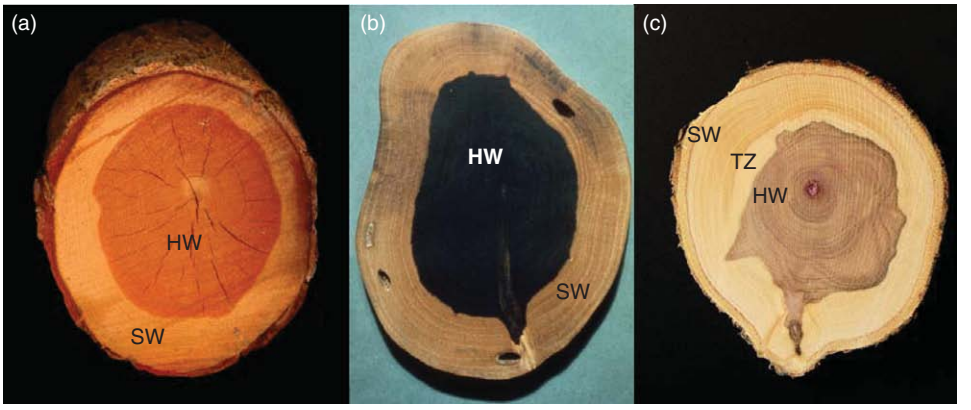
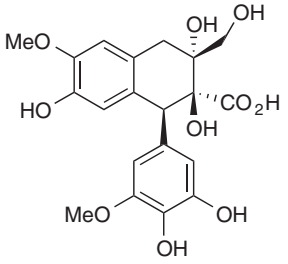
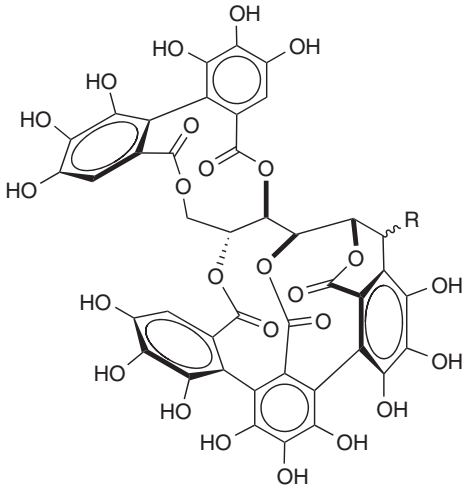


Figure 22 Cross-sections of selected HW-forming trees. Both gymnosperms (e.g., western red cedar, *Thuja plicata*; a) and angiosperms (e.g., ebony, *Diospyros* sp.; b and lilac, *Syringa vulgaris*; c) can often form HW that is darker than the surrounding SW because of copious deposits of 'extractives.' In some species, a transition zone (TZ) is involved in the late stages of HW formation, as is evident between SW and HW in *Syringa vulgaris* (c). The irregular border and extended portions of dark HW (c) are due to branch traces and possible stress factors perceived within the xylem. Photos from A. M. Patten, Washington State University (a, c). Photo from the late W. E. (Ted) Hillis, CSIRO, Australia (b).



(**10**) Plicatic acid



(**11**) R = H, β-OH, Vescalagin
(**12**) R = H, α-OH, Castalagin

3.27.3.1.5 Bark

Bark is the outer tissue that envelopes wood and visually defines the trunk to the human eye. Cork (*Quercus suber)* (**Figure 23(a)**) holds special significance in the field of biology as the tissue that Robert Hooke examined microscopically in 1665, and from which he first coined the term, cell.[76] Bark encompasses a very diverse set of tissues, divided into inner (living) and outer (nonliving) bark regions, that are derived from the vascular cambium and cork cambium, respectively (**Figure 17(d)**). Inner bark provides important metabolic/transport/storage functions and the outer bark facilitates these providing protection against external factors.

Inner bark is derived from the vascular cambium, with the exception of the previously formed primary tissues, the primary phloem and cortex (i.e., from primary meristematic tissues). It thus generally includes all tissues between the vascular cambium and the outer bark including primary and secondary phloem, phloem fiber types (including commercially important bast fibers and lignified fibers, such as sclerenchyma or stone cells), radial and axial parenchyma, cortex, and specialized cell types[47] as discussed later (Section 3.27.3.3). Perhaps the most important function of the inner bark is for long distance transport of nutrients (especially sucrose), metabolites, and systemic signals. Sieve tubes and companion cells of the phloem (not shown) also play central roles in this process.[77–79] Additional functions of inner bark include: storage of starch and lipids,[80] some metabolic functions of ray parenchyma, including respiration, and an inner defense zone that functions as a continuum with the outer bark (see below).

Given the rich contents of the inner bark and the vital functions of the vascular cambium, rather sophisticated physical and chemical defense systems have evolved in the various bark forms. The outermost line of defense rests mostly in the nonliving outer bark. This is generated by the cork cambium (phellogen, **Figure 23(b)**, *) which produces several rows of parenchymatous cells inward, called phelloderm (which may be absent in some species) and numerous rows of suberized and/or lignified phellem outward (**Figure 23(b)**, PM, and inset).[15,81,82] Together, the three tissues of the outer bark, phellem, phellogen, and phelloderm are collectively known as the periderm. Of those trees producing phelloderm, young trees may have limited photosynthesis occurring in either the phelloderm or the cortex of the inner bark[47] (chlorenchyma (Cl), **Figure 23(b)**). Numerous layers of periderm may also form over the years, but not necessarily every year (**Figures 17(d)** and **23(a)**).

The outer bark (chemistries) can provide several centimeters of physical protection, as in cork oak (**Figure 23(a)**) and pines (e.g., *Pinus ponderosa*, *Pinus palustris*, and most other *Pinus* spp.) (**Figure 23(c)**), or be quite thin and subject to peeling, as in birch[39] (e.g., *Betula papyrifera*, *Betula nigra*, and all other *Betula* spp.) (**Figure 23(d)**). Indeed, the variety of bark phenotypes that is found reflect the often species-specific strategies that have evolved for protection and defense. The most obvious defense components of the outer

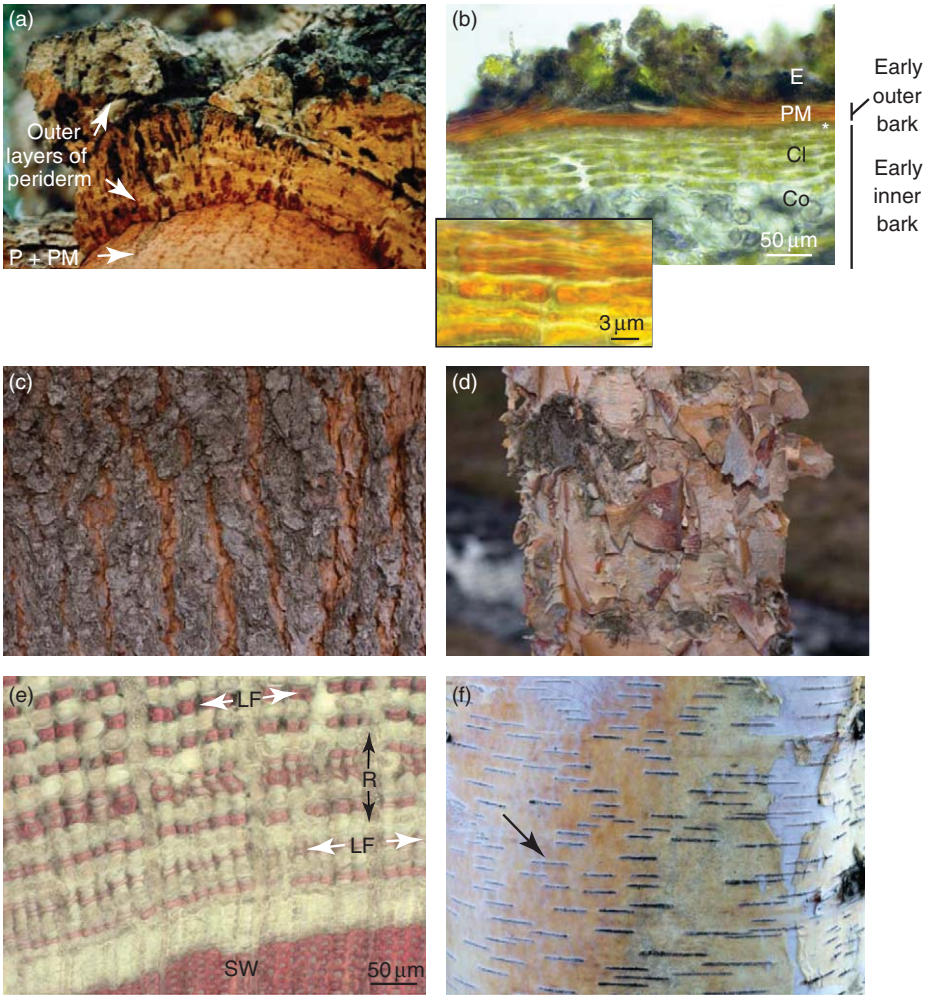
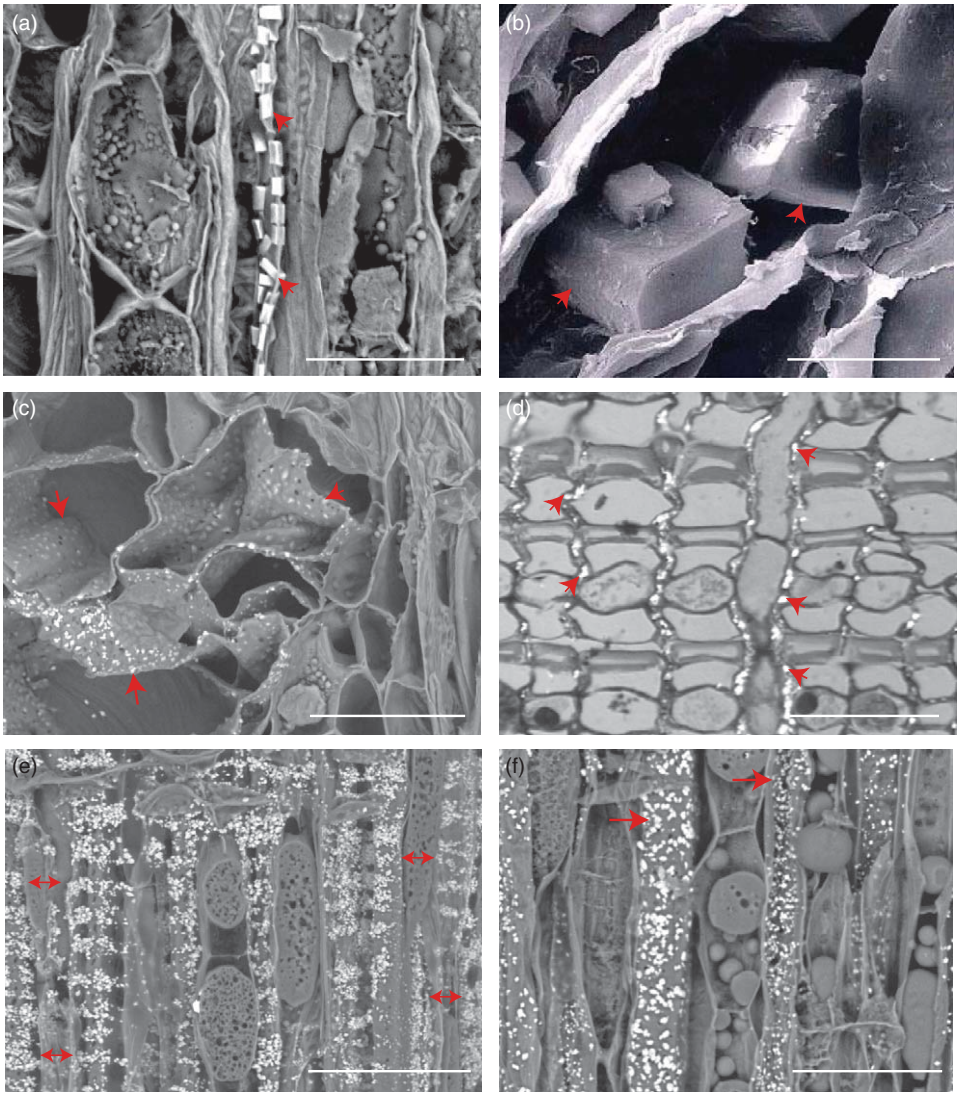
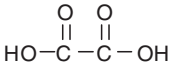


Figure 23 Selected physical anatomical structures of bark. Partially harvested cork from cork oak (*Quercus suber*). The outer layers of the periderm are manually harvested to assure that the phellogen (protected by a layer of red-orange phellem) is left intact to regenerate cork (a). Thin orange-colored phellem is the suberized outer bark layer of a *Tilia* sp. branch. (*) indicates the location of the phellogen between the phellem and the early inner bark (b). Inset (b): Mature phellem cells compress, forming a tight physical barrier. Ponderosa pine (*Pinus ponderosa*): thick bark protects the inner trunk tissues from fire, pests, and other external factors (c). Other species, such as river birch (*Betula nigra*), have thin peeling bark that allows for fast growth and expulsion (by shedding) of insects, microbes, and so on (d). Phloroglucinol–HCl-treated secondary phloem of *Thuja plicata* showing lignification of lignified fiber (LF) cells and SW (pink coloration; e). Lenticels (arrow) allow for gas exchange between the stem and the environment and are visually prominent in birch bark (*Betula platyphylla*) (f). Abbreviations: Cl, chlorenchyma (photosynthetic parenchyma); Co, cortex; E, epidermis; P, phellogen; PM, phellem; R, ray parenchyma. Photos from L. B. Davin (a) and A. M. Patten (b–f), Washington State University.

bark are constitutive and structural in nature, with gymnosperm and angiosperm trees sharing many of the same features. Suberization (cork layers) and wax production in the phellem also imparts sealant properties to bark that includes prevention of water loss.[1,82,83] Suberin is a polymer comprised of both polyaromatic and polyaliphatic domains (**Figure 11(g)**)[1,2,84] and is the major constituent of cork. It acts as barrier to water loss and therefore was essential to land adaptation; it also imparts pathogen resistance.[1,84] Additionally, many phellem cells may become air-filled and provide thermal insulation against fire,[47] with thicker bark improving protection[85,86] as in cork oak (*Q. suber*) (**Figure 23(a)**) and ponderosa pine (*P. ponderosa*) (**Figure 23(c)**).

In some species, inner bark supplements these physical defenses, such as with lignified cells (as in fibers, sclerenchyma, sclerids, stone cells) (**Figure 23(e)**) and/or deposition of calcium oxalate (**13**) crystals (**Figure 24**) either within or among these cells, and in rows of crushed sieve elements.[2,47,87–89] Interestingly, in many non-Pinaceae gymnosperms there is a direct correlation between the extent of calcium oxalate (**13**) crystal formation and the resistance to insect attack, with crystals being deposited within or between phloem cells, such as ray parenchyma, fibers, or stone cells.[90] Shedding outer layers of phellem is another physical defensive strategy





(**13**) Oxalic acid

Figure 24 Calcium oxalate crystal formation provides a formidable physical barrier against herbivores within the secondary phloem of many conifers. Western larch (*Larix occidentalis*) radial section showing a continuous file of crystals (arrows; a). Close-up of crystals (arrows) within a phloem cell of the grand fir (*Abies grandis*) (b). *Araucaria araucana* stone cells with dense distribution of crystals (appearing as whitish specks) (arrows; c). *Thuja plicata* cross-section with intercellular crystal deposition (arrows) between adjacent phloem cells, such as rays, lignified fibers, and sieve elements (d). *Thuja plicata* radial section showing dense crystal distribution throughout the phloem (arrows; e). *Taxus brevifolia* radial section showing heavy calcium oxalate deposits in phloem fibers (arrows; f). Scanning electron (a–c, e, f) and light microscopy (d) used. Scales = 50 μm (a, c, e, f), 10 μm (b), 40 μm (d). Reproduced with permission of Wiley InterScience from J. W. Hudgins; T. Krekling; V. R. Franceschi, *New Phytol.* **2003**, *159*, 677–690, copyright 2003.

with the effect of expelling pathogens, pests, and/or epiphytes (**Figure 23(d)**).[39] Chemical defense strategies are also common in bark tissues, being either constitutive or induced by secretory systems (see Section 3.27.3.3). Overall, such strategies thus help create physical and chemical barriers to invasive microbes and herbivores.

Although defense functions are the most obvious properties, outer bark also facilitates internal metabolic processes, such as of cambial, phloem, and ray/axial parenchyma activities, by providing exchange of limited amounts of CO_2, O_2, and water.[82] Indeed, respiring cells of SW and inner bark produce substantial amounts of CO_2, resulting in a CO_2/O_2 balance that is much higher in tree stems than in the external environment.[91,92] Some CO_2, however, is removed by the transpiration stream. Nevertheless, it is estimated that between 15 and 55% of CO_2 may be retained within the stem and requires an alternate egress.[92] Lateral gas exchange can occur through specialized cell types on the outer surface of bark, called lenticels. These form narrow spaces in the bark that are only very noticeable where bark is thin, such as in saplings, or in the distinct horizontal marks such as those of mature birch bark (**Figure 23(f)**).

3.27.3.2 Water Conduction and Cell Wall Reinforcement in Wood and Bark

The main tissue types present in wood (pith, juvenile wood, SW, and HW), and bark, include both living and nonliving cell types that broadly function in mechanical support, transport, storage, and/or defense. These cell types, in turn, provide the often species-specific material and phytochemical constituent properties of both wood and bark tissues, and a framework for contemplated biotechnological manipulations.

Of the various physiological functions that evolved, hydration and water/nutrient transport were perhaps among some of the earliest, thereby leading to successful colonization of the desiccating terrestrial environments. As such, three factors presumably influenced the anatomical adaptations and chemistries of cell wall containing tracheids and later vessels, all of which represent functional trade-offs:[93] (1) Conductive efficiency of water through the various conduits that evolved, including tracheids and vessels, (2) the need for increased mechanical strength and (3) improved resistance to embolism (**Figure 25**). The first of these factors, conductive efficiency, refers to water delivery in relation to maximum photosynthetic rate, and thus involves maximizing the size of the water conduit. Wide channels result in thinner cell walls, thereby compromising mechanical strength and increasing the opportunity for air introduction and embolism,[93] a common hazard to trees during either water stress or freeze–thaw cycles.[94]

In gymnosperms up to 90% of the SW/HW consists of tracheids.[95,96] For this reason, gymnosperm wood is sometimes considered to have a relatively homogenous structure, given the somewhat regular arrangement and sheer number of tracheids overall (**Figures 20(a)** and **20(c)**).[52,96] The tracheids, in turn, function in both water transport and mechanical support in the SW, whereas those in the HW contribute only to stem support and in protection as a barrier against opportunistic pathogens. By contrast, angiosperm SW tissue is more complex, characterized by vessels, various fibers, and larger ray systems[52,96] (**Figures 20(b), 20(d)–20(g)**). Moreover,

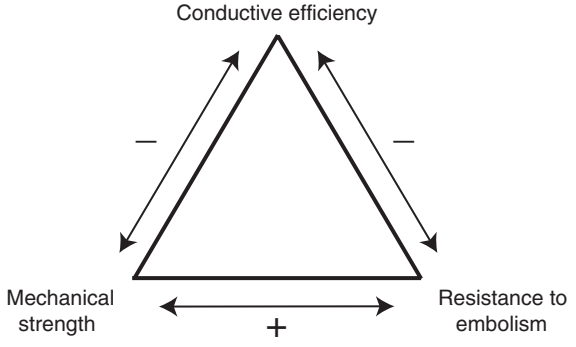


Figure 25 Trade-off triangle wherein xylem evolution has been driven by needs for conductive efficiency, resistance to embolism, and mechanical strength; the latter two have inverse correlations to conductive efficiency. Redrawn with permission of Elsevier from P. Baas; F. W. Ewers; S. D. Davis; E. A. Wheeler, Evolution of Xylem Physiology; A. R. Hemsley, I. Poole, Eds.; *The Evolution of Plant Physiology*; Elsevier Academic Press: London, 2003; pp 273–295, copyright 2003.

angiosperm wood cells divide transport and structural functions: in the SW, the vessels transport water, while the fiber cells provide stem mechanical support.[15]

Taken together, the success of these hydraulic systems in present-day gymnosperm/angiosperm SWs is also due in part to their integrative, compartmentalized, and redundant characteristics, and the chemistries of the cell wall polymeric constituents that help confer such properties.[97] Water transport is thus integrated both vertically, in an end-to-end element arrangement, and laterally through cellular pits. In this way, water drawn up by any root may potentially reach any leaf and laterally connected cell. However, the relatively small diameters of individual water-conducting elements (15–65 μm in tracheids, 40–360 μm in vessels)[95] also help function as a safeguard to limit embolism (air incorporation). Additionally, this redundancy of elements provides a mechanism for compartmentalization of either injury or disease to allow continuous water transport in neighboring functional cells.[97] Measurement of water conductance is difficult to assess because it is influenced by variances in hydraulic functionality (as from injury, described above), sap flux, SW/HW ratio, amounts of stored water (capacitance), and the environment in which an individual tree lives (see Meinzer *et al.*,[54] and references therein). Nevertheless, estimates of daily water transport reach a maximum of ~200 kg day^{-1} in gymnosperms and up to ~800 kg day^{-1} in angiosperms.[54]

3.27.3.2.1 Tracheids: normal and reaction wood

Gymnosperm tracheids have a relatively safe structural design compared to angiosperm vessels, as they are thick walled for good structural support and have a large number of pits with wide apertures ('fenestriform') that allow for rapid refilling via replacement of air by water (**Figures 20(a), 20(c)**, and **26(a)**).[94] However, they also have comparatively narrow water conduits, limiting their conductive efficiency, which can adversely affect growth rate.[98,99] The large percentage of tracheids (~90%)[95] and overall high wood density also reduces storage capacity, for example, of photosynthates, metabolites, and so forth, in the SW of gymnosperm trunks.[98] However, because gymnosperm woods are mainly composed of tracheids, this functional redundancy can often limit deleterious effects of either injury or disease.[97] Furthermore, gymnosperm tracheid structure and wood density may actually give them an advantage under water stress conditions, such as either in drought-stricken or arid environments, or with very tall trunks, in which water stress can result under conditions of very long distance transport.[99]

Tracheids (**Figure 26(a)**) also tend to be long and narrow compared to angiosperm vessels (**Figure 26(b)**) with some reaching as much as a 100:1 length to diameter ratio (not shown).[96] As for most other lignified cell types, such as the angiosperm vessels and fibers, the tracheid structure anatomically consists of a primary cell wall, and an internal secondary cell wall composed of three sublayers (S_1, S_2, S_3, **Figure 27(e)**), and cell wall pits and end plates to facilitate water flow (**Figure 26(a)**).[38,100] Briefly, the three secondary cell walls differ not only in thickness but also in the helical orientation of their cellulose microfibrils (i.e., cellulose molecules (**Figure 27(a)**) assembled in long fibrils that are bundled in a group forming a microfibril[15] (**Figure 27(c)**). The S_1 and S_3 sublayers are very thin with the cellulose microfibrils having an overall S helical configuration, while the S_2 sublayer forms the most substantial layer with a much thicker wall and a Z helical microfibril configuration. This alternating S–Z–S helical structure facilitates resistance to lateral stresses partially through the S_1 and S_3 sublayers,[100] while the S_2 sublayer bears most of the axial loading and longitudinal stiffness of the tracheid.[38,100] When cell thickness varies, however, it is usually from variations in the S_2 sublayer. Cell walls are also strengthened by the deposition of various polymeric hemicelluloses/pectins (**Figures 27(c) and 28**)[101,102] and lignins[103,104] (**Figure 29(a)**[105]). The latter consists mostly of guaiacyl (G-) lignin, with small amounts of *p*-hydroxyphenyl (H-) lignin,[106–108] derived from coniferyl (**15**) and *p*-coumaryl (**14**) alcohols (**Figure 29(b)**), respectively. Full maturation of the tracheid finally involves programmed cell death (PCD) to form the lumen of the tracheid.[109–112]

In compression wood, a greater number of tracheids are found causing more expanded annual rings within the compression wood zone that results in a wider radius below the center (i.e., pith, **Figure 21(a)**). Compression wood tracheids differ from normal tracheids in cell wall structure (thicker S_2, absent S_3, altered microfibril angle (MFA), and decreased overall length)[57] and chemical content (including lower cellulose contents, increased H lignin amounts, and overall lignin contents). This altered chemical content thus gives compression wood greater density[113] and compressive strength, but also causes marked brittleness.[57] Additionally, water conduction in compression wood is hampered by the altered anatomy of its tracheids,[114] which are visibly rounder and have distinctive intercellular spaces between themselves (**Figure 21(b)**), relative to normal tracheids (**Figure 21(c)**).

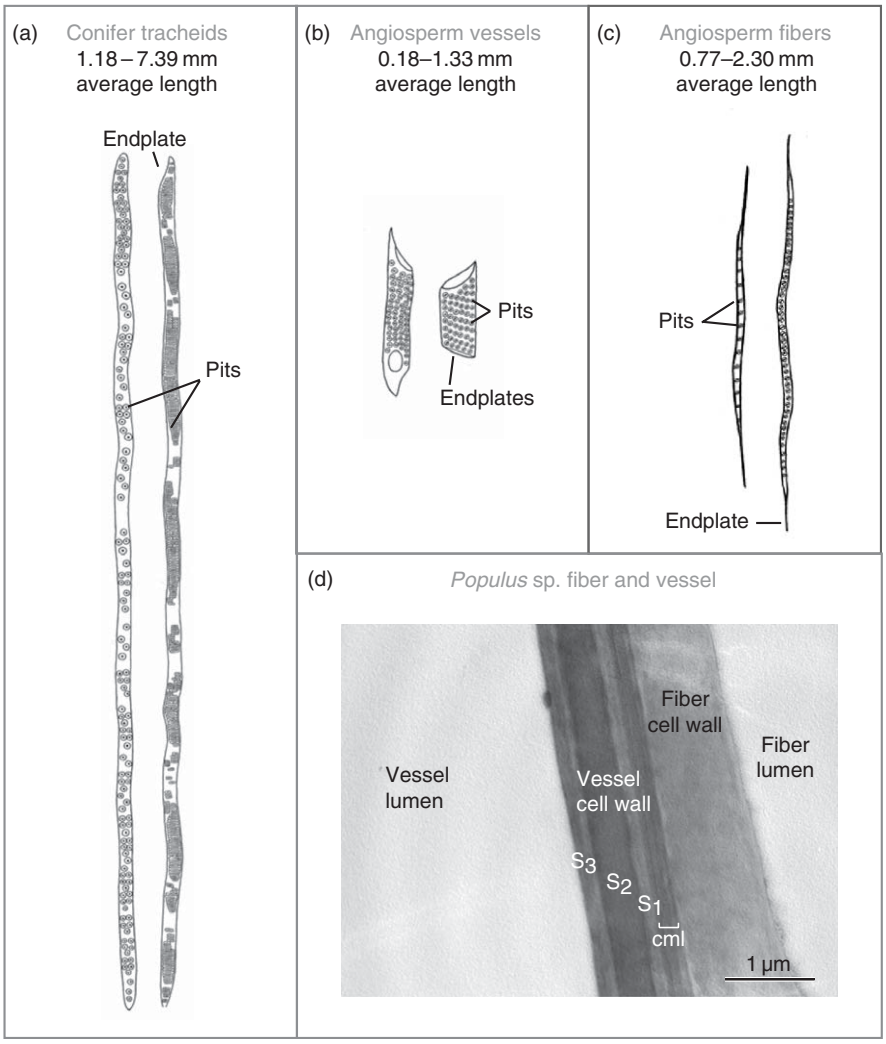


Figure 26 Wood cell walls. Tracheids are the water-conducting and mechanical supporting cells of gymnosperms; water is transported longitudinally through endplates and laterally through pits (a). Angiosperm vessels function primarily to transport water and are individually shorter than tracheids (b). Angiosperm fibers provide the main mechanical structure in angiosperm wood (c). Angiosperm vessels and fibers (d) have the same basic cell wall structure (S_1–S_3) as do gymnosperms (see **Figure 27(e)**). Reproduced from I. W. Bailey; W. W. Tupper, *Proc. Am. Acad. Arts Sci.* **1918**, *54*, 149–204 (a–c). Reproduced with permission of Botanical Society of America from A. M. Patten; M. Jourdes; E. E. Brown; M. P. Laborie; L. B. Davin, N. G. Lewis, *Am. J. Bot.* **2007**, *94*, 912–925, copyright 2007 (d).

3.27.3.2.2 Vessels: normal and reaction wood

Angiosperm wood is readily distinguished by the presence of vessels (**Figures 20(b)**, **20**(**d**), and **26**(**b**)), these being another specialized form of water-conducting elements. Vessels are more efficient water-conducting elements than gymnosperm tracheids due, in part, to shorter cell length, greater width, non-membranous wide perforation end structures, and end-to-end cell alignments. These changes may, in turn, help resist increased internal water tension, due to the wider diameter of vessels.[38]

Vessels also generally have the same overall subcellular (S_1, S_2, and S_3) architectural design of the tracheid cell wall (**Figure 26(d)**). There are, however, important component differences including a steeper cellulose MFA in the S_2 layer[38] and reinforcement with G lignin and small amounts of H- and syringyl (S-) lignin,[115,116] with the latter being sinapyl alcohol (**16**, **Figure 29**(**b**)) derived. However, because vessels are relatively short, vertical water transport requires a higher degree of lateral movement. Hence they have larger numbers of cell wall pits (**Figure 20(d)**), with highly porous membranes to facilitate water flow.[38] Efficiency of water conduction thus

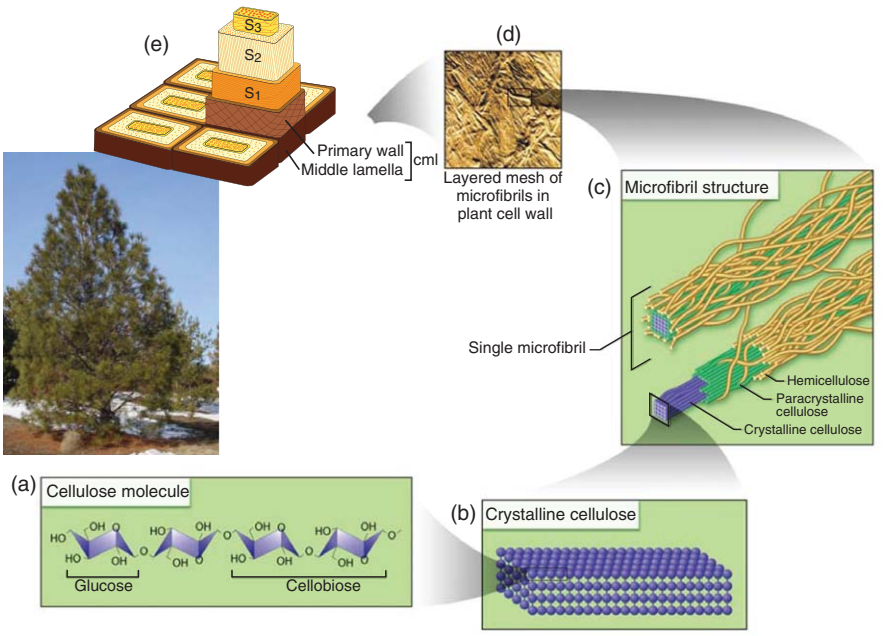


Figure 27 Cellulose and the woody plant cell wall. The cellulose molecule is a linear polymer consisting of thousands of 'anhydroglucoside' residues with cellobiose as the repeating unit in the polymer (a). The hydroxyl groups on the glucose residues from one chain form hydrogen bonds either inter- or intramolecularly within the same or neighboring chains (b) and can ultimately form microfibrils (10–20 nm in diameter and up to 40 or so cellulose chains) (c). Orientation (random) of microfibrils in a primary wall (d). Idealized telescopic representation of a lignified conifer tracheid: three secondary cell wall sublayers (S_1-S_3) with differing cellulose microfibril orientations (indicated by red lines in each layer) form sequentially within the primary wall. Tracheids are adjoined by the middle lamella, which combines with the primary wall to form the compound middle lamella (cml) (e). Reproduced in part from the Genomics: GTL Roadmap, U.S. Department of Energy, Office of Science, August 2005, http://genomicsgtl.energy.gov (a-d). Redrawn with permission of American Society of Plant Biologists from R. Croteau; T. M. Kutchan; N. G. Lewis, Natural Products (Secondary Metabolites). In *Biochemistry and Molecular Biology of Plants*; B. B. Buchanan, W. Gruissem, R. L. Jones, Eds.; American Society of Plant Physiologists: Rockville, MD, 2000; pp 1250–1318, copyright 2000 (e). Photo from L. B. Davin, Washington State University.

translates into a lower investment in vessel numbers. On average, only about 30% of temperate zone angiosperm woody tissues have vessels, in marked contrast to gymnosperm woods that have threefold more tracheids.[95] As discussed earlier, the expanded diameter of the vessels also increases the risk of embolism[117] in angiosperm wood. Interestingly in tension wood, the overall number and size of vessels are reduced (not shown).[118]

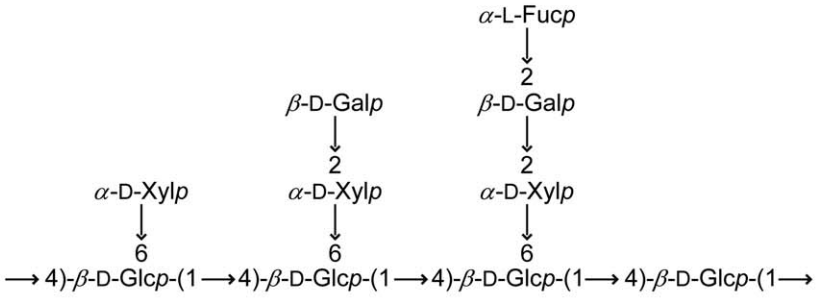
3.27.3.2.3 Fibers: normal and reaction wood

Angiosperm wood fibers (**Figure 26(c)**) are also largely specialized for mechanical/structural support, such as the fiber-tracheids and the libriform fibers (**Figures 20(e)** and **20(f)**). A small portion serve to store metabolites, such as septate fibers (**Figure 20(g)**).[14] As for the water-conducting lignified cells discussed above, fibers also have the familiar primary and secondary cell wall architectures with the three sublayers previously described (**Figure 26(d)**), although they are sometimes difficult to see. In contrast to vessels or tracheids, fibers are enriched in syringyl (S-) lignin, together with smaller amounts of H- and G-lignins.[107,116,119,120] Fibers may also occur in the bark, which in some taxa (e.g., *Tilia* spp.) can include large groups of bast fibers that are either septate or libriform-like.[47]

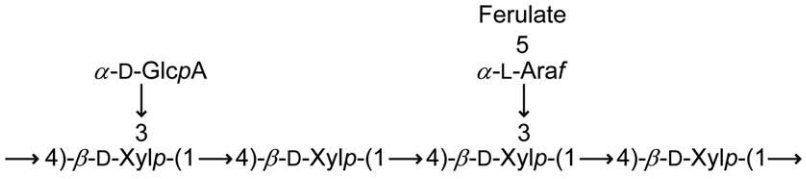
Taken together, the gymnosperm tracheids, angiosperm fiber-tracheids, and libriform fibers can be considered to represent an ascending evolutionary continuum, albeit with variations in the chemistries of the polymeric constituents present.[15,38,121] Indeed, the fiber-tracheids more closely resemble tracheids in terms of length, cell wall thickness, and pit type. However, their pit size is reduced since the functions are modified, and they are often found clustered around vessels, thereby serving in direct mechanical support to the vessels with only minor roles in water transport. The libriform fibers, by contrast, are shorter and thicker than fiber-tracheids. Yet they form the

(a) **Hemicelluloses.** Examples include:

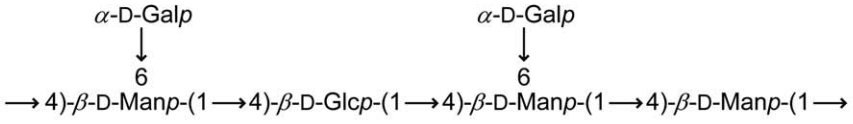
Heteroglucan (fucogalactoxyloglucan)



Heteroxylan (glucuronoarabinoxylan)



Heteromannan (galactoglucomannan)



(b) **Pectins**

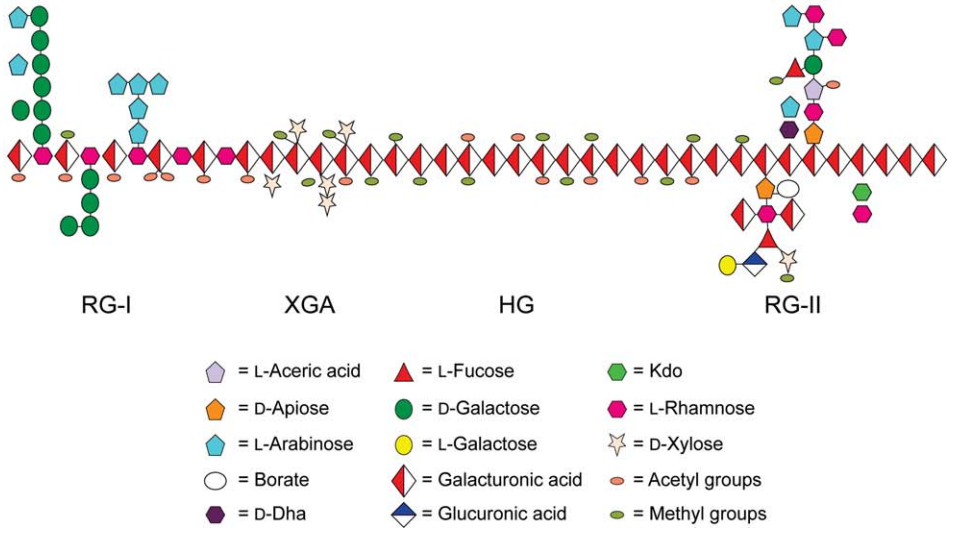


Figure 28 Examples of hemicelluloses (a): Proposed structures of an heteroglucan, an heteroxylan, and an heteromannan.[101] Schematic structure of pectin (b) showing the three main pectic polysaccharides homogalacturonan, (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) linked to each other. Reproduced with permission of Blackwell Publishing from D. Mohnen; M. Bar-Peled; C. Somerville, Cell Wall Polysaccharide Synthesis. In *Biomass Recalcitrance. Deconstructing the Plant Cell Wall for Bioenergy*; M. E. Himmel, Ed.; Blackwell Publishing: Oxford, UK, 2008; pp 94–187, copyright 2008 (b).

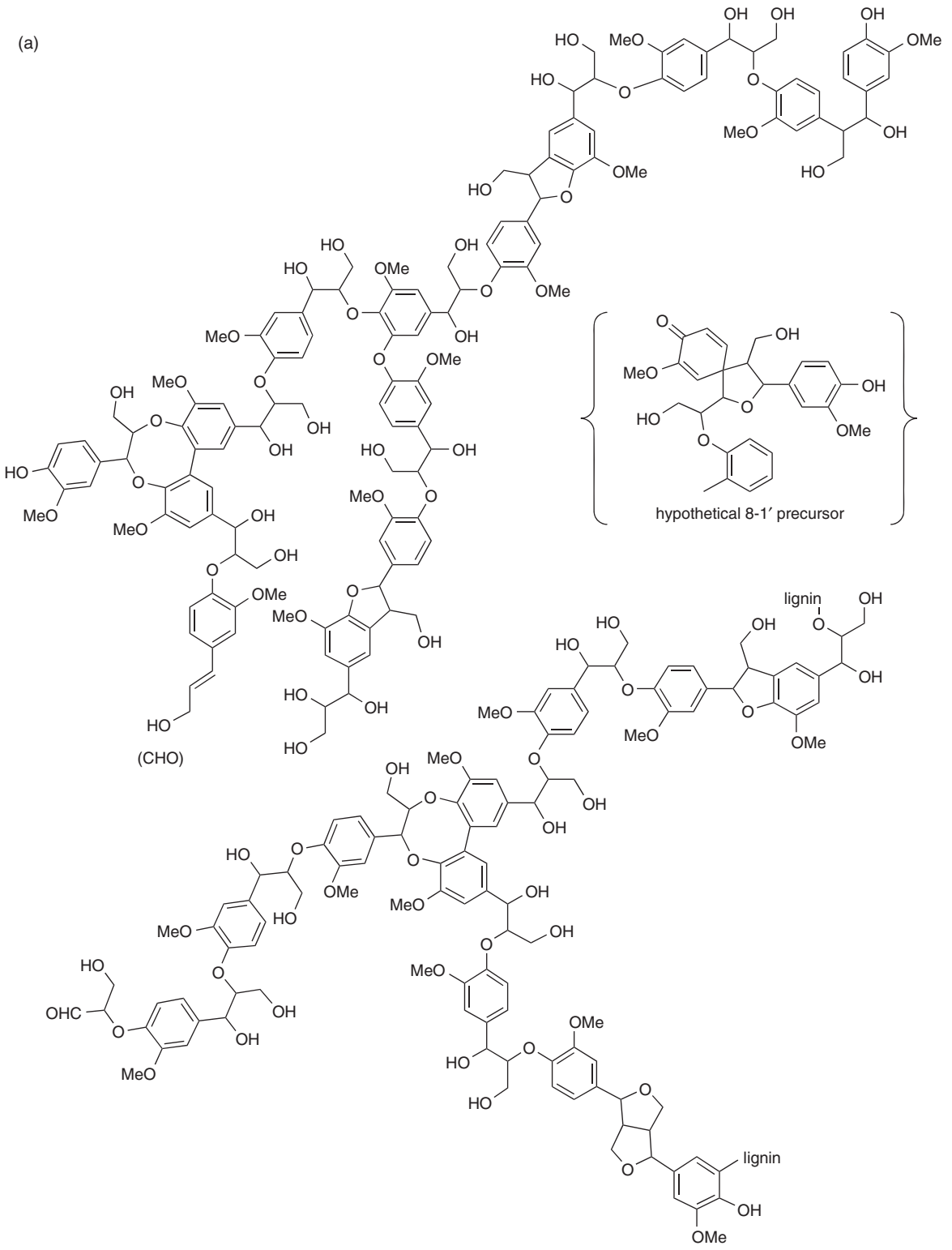


Figure 29 (Continued)

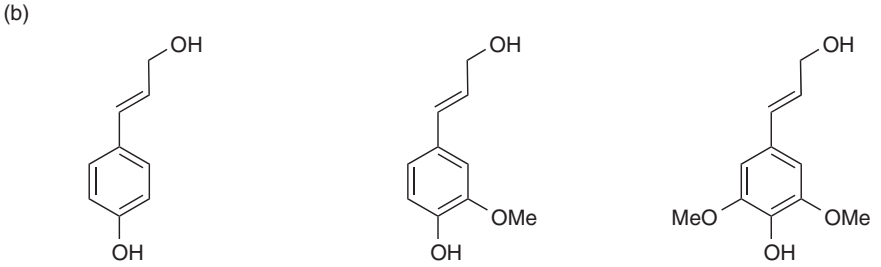


Figure 29 Artistic depictions of (a) lignin structure as envisaged by Brunow *et al*.[105] and (b) monolignols **14–16**.

majority of angiosperm fiber populations, and also serve as the main structural support of the trunk.[15] Wood density associated with hardwoods is thus generally attributed to this fiber type.[39]

The cell wall composition of either libriform fibers or fiber-tracheids may, however, be adaptively altered as in the formation of gelatinous fibers in tension wood of temperate angiosperm species[14] (**Figure 21(e)**). Gelatinous fibers have a thick inner layer that generally replaces one or two of the inner two cell wall layers and are high in cellulose content but lack lignin, appearing to have a gel-like appearance, relative to normal wood[122] (**Figure 21(f)**). In addition to gelatinous fibers (that may not be present in all species), tension wood generally also exhibits eccentric growth, with extended growth to the upper side of the branch (**Figure 21(d)**) or leaning stem. The tree benefits from localized higher tensile strength and greater resistance to impact by tension wood formation.[57] Interestingly, tension wood can be formed in the green herbaceous growth of alfalfa (and in its basal woody tissues).[123]

3.27.3.3 Nature's Diverse Phytochemical Factories in Trees

Curiously, all woody tissues are often viewed as being dead. This is frequently surmised because PCD results in formation of the aforementioned water-conducting elements, including the tracheid and vessel conduits. In addition to these cell types, trees also contain a variety of living cells of specialized metabolic function in their woody (and nonwoody) tissues. These remarkable cell types, or perhaps more accurately, 'phytochemical factories,' can produce a plethora of often species-specific metabolites, such as the so-called extractives that are deposited within wood cells as in HW, or the various exudates/secretions that help defend living wood, phloem, and meristematic tissues.

The overall metabolic compositions found in many of these cell types in any species are, however, generally quite characteristic for a particular taxa, as are their anatomical structures. Accordingly, both of these morphological and chemical features are highly useful for the taxonomic classification of plants.[47,124,125] More importantly, many of the species-specific phytochemicals generated are greatly valued by humanity for a diverse range of uses (see Section 3.27.4).

3.27.3.3.1 Radial, axial, and epithelial parenchyma

There are many living parenchyma cells within SW tissues and provisionally a smaller population in the HW itself.[126,127] For primary growth, the ground parenchyma are initially generated from a primary meristem, these supporting the young stem in a turgor-dependent manner, and which are competent to divide/differentiate as needed.[15,47] Secondary parenchyma are then generated by the vascular cambium and involve three types of parenchyma within SW and HW: radial (ray), axial, and epithelial parenchyma[95] (**Figures 20(a)** and **20(b)**). Of these, the radial and axial populations are linked together, forming a three-dimensional transport network throughout the tree.[128] In some species, functional differences between axial and ray parenchyma are pronounced, with the former primarily involved in starch storage, and the latter in primary and secondary metabolism and transport. However, in other trees, both axial and ray parenchyma may contain starch and other primary or secondary metabolites (see Hillis,[129] and references therein).

Within woody tissues, the ray parenchyma are the most abundant of these cell types (**Figures 20(a)** and **20(b)**), where they reportedly represent between 7–16% of the wood volume in gymnosperms, and 8–40% in angiosperms.[95,130] They are essential for lateral transport of photosynthate, various metabolites and, to a lesser extent, water needed by the incipient and mature xylem and phloem.[40,131,132] Viewed in transverse section, ray

parenchyma (radial wood rays) originate at the vascular cambium and traverse the trunk radially in all directions to the pith, much like the spokes of a wheel (**Figure 17(c)**). This configuration also helps contribute to stabilizing wood structure under bending stresses, by adding resistance to slipping and shearing due to differential stiffness between annual early and late wood tissues.[133,134] For example, beech wood (*Fagus sylvatica*) rays provide substantially high tensile radial strength exceeding that of the axial elements.[135] Ray parenchyma may also play a role in providing resistance to freezing damage.[136,137]

By contrast, axial parenchyma are rare in gymnosperms, but can comprise from ~0.1 to 23% of angiosperm woods depending on the species.[95] This is in part due to their close association with vessels, where they can also function in water and nutrient storage. Indeed, stored water in axial parenchyma may aid rapid vessel refilling in response to air entering the vessel.[39] A specialized form of axial parenchyma cell (i.e., idioblast), the epithelial parenchyma, also functions in secretion and excretion.[47] These are found in both gymnosperms and angiosperms, although the frequency of their presence has generally only been described in the context of associated duct systems: for example, they apparently account for ~1% of the wood in resinous pines.[95] Nevertheless, despite their apparent low frequency, the epithelial parenchyma can serve in vital defensive functions.

Bark and wood tissues may include all three cell types, and other specialized parenchyma for bark photosynthesis (chlorenchyma, **Figure 23(b)**), various chemical defenses (e.g., polyphenolic cells, polyphenolic parenchyma (PP), **Figure 30**), and physical barrier/defenses (e.g., suberized cork (**Figure 23(b)**, inset), stone

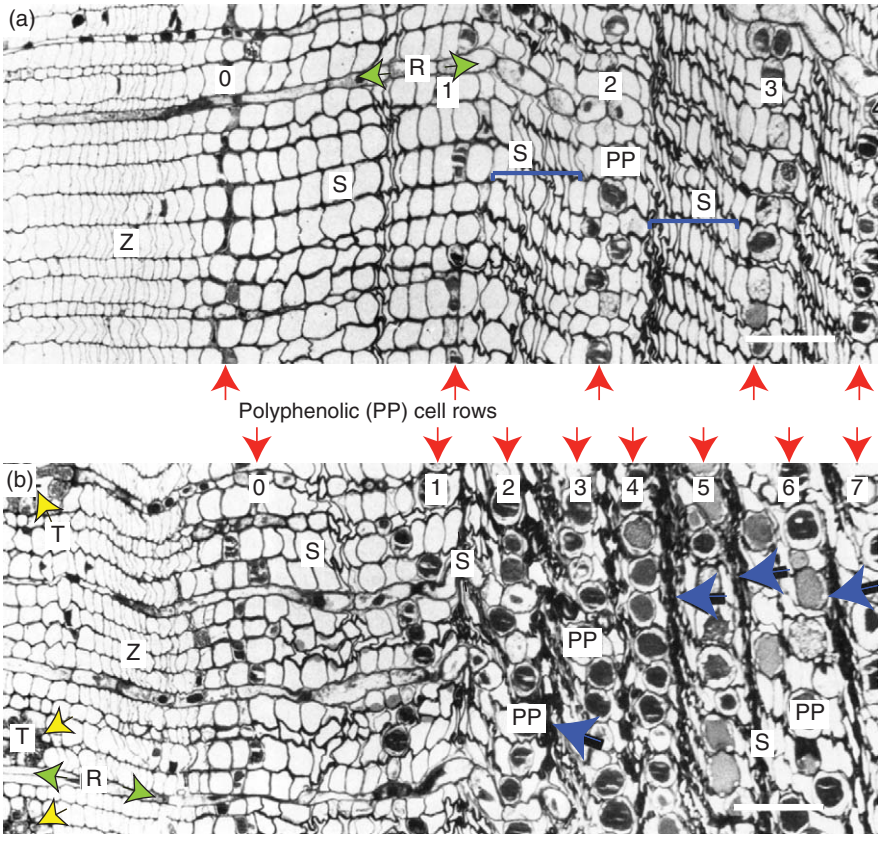


Figure 30 Polyphenolic (PP) cells aligned in rows (red arrows) within secondary phloem. When Norway spruce (*Picea abies*) is surface inoculated (i.e., control) with *Ceratocystis polonica*, the fungus does not penetrate the outer periderm (not shown). Secondary phloem appears similar to normal (data not shown) with PP cell rows widely separated (rows 0–3), sieve cells (S) partially compressed, and an absence of resin ducts (a). Deep inoculation of secondary phloem with *C. polonica* causes enlargement of PP cells (rows 0–7) in combination with collapsed sieve cells (S) (blue arrows) forming a tightly packed physical/chemical barrier system, with traumatic resin ducts (T) (yellow arrows) present (b). Abbreviations: PP, polyphenolic cells; R, ray parenchyma; S, sieve cells; T, traumatic resin duct; Z, cambial zone. Scales = 100 μm. Reproduced with permission of American Botanical Society from V. R. Franceschi; P. Krokene; T. Krekling, E. Christiansen, *Am. J. Bot.* **2000**, *87*, 314–326, copyright 2000.

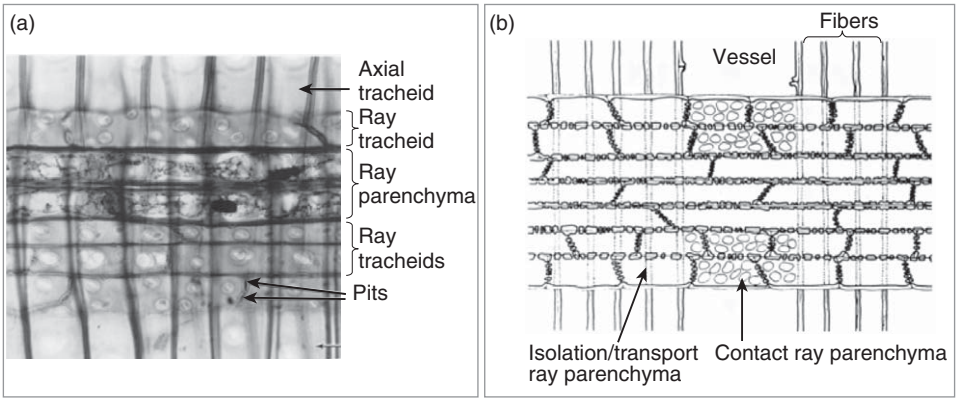


Figure 31 Radial parenchyma structure in longitudinal section in a gymnosperm (*Pinus* sp.) and an angiosperm. Normal (i.e., without resin ducts) *Pinus* wood rays may include both ray parenchyma and ray tracheids for nutrient/metabolite or water transport, respectively (a). Non-*Pinus* species do not form ray tracheids. Angiosperm ray parenchyma include two types: isolation/transport parenchyma for lateral transport or storage and contact parenchyma that have numerous pits connecting to neighboring vessels (b). Reproduced with permission of John Wiley and Sons from R. F. Evert, *Esau's Plant Anatomy. Meristems, Cells, and Tissues of the Plant Body: Their Structure, Function and Development*; John Wiley and Sons: Hoboken, 2006, copyright 2006 (a). Reproduced with permission of Springer Verlag from J. J. Sauter and S. Kloth, *Protoplasma* **1987**, *137*, 45–55, copyright 1987 (b).

cells (**Figure 24(c)**). As a result, many of these cell types in different tissues are involved in various specialized biosynthetic pathways to the so-called 'secondary' metabolites.

Overall, the wood ray structure differs substantially between gymnosperms and angiosperms, being relatively simple in the former and more complex in the latter. In gymnosperms, wood ray columns consist of several cells, while their rows vary between either one (i.e., uniseriate) (**Figure 20(a)**) or two (i.e., biseriate; not shown) rows wide. Less frequently, a horizontal resin duct is also formed within a ray, with this combined structure being known as a fusiform ray (**Figure 20(a)**).[96] In *Pinus* spp. (but not in non-*Pinus* taxa), ray parenchyma can often be combined with ray tracheids, which transport water (**Figure 31(a)**). Similar to axial tracheids of all gymnosperms, the pine ray tracheids have undergone lignification and rapid PCD, and hence can transport water in the lateral direction.[121]

On the other hand, angiosperm wood rays have much more species-specific variability which include variations in width (2–30 cell rows wide) or height (a few microns to 5 cm) (**Figure 20(b)**); orientation of individual cells (upright vs. procumbent); composition (single vs. multiple cell types); and spacing of rays, for example, closely spaced, the so-called 'aggregate rays.'[96] Within rays, there are also apparently two functionally distinct angiosperm ray parenchyma populations, namely contact and isolation/transport cells (**Figure 31(b)**), based on presence/absence of pit connections to vessels, respectively. Of these, the former appear to be well suited to exchange functions, while the latter may specialize in lateral transport of primary metabolites and signaling/transcriptional factors across the trunk or storage.[132]

3.27.3.3.2 Heartwood formation and exudates ('extractives')

In spite of its critical importance to woody plant survival and utilization, the precise order of HW-forming events is not yet well understood. Nevertheless, the 'secondary' metabolites that accumulate often help dictate the properties of a particular (HW) species (see **Figure 22**). As regards deposition of HW 'extractives,' various correlations with HW formation have been noted in ray parenchyma cells within adjacent SW, including starch granule consumption, carbohydrate conversion into secondary metabolites, reduced moisture content, ray parenchyma PCD, vessel occlusion, and extractives deposition, in part, via exudation.[65,129,138–145] For example, in both gymnosperm and angiosperm trees, the sucrose contents are highest in the ray parenchyma of young SW [126] with levels decreasing inward until absent in the HW itself.[75] Sucrose in these cell types is also transported centripetally (inward) following which it is converted into secondary metabolic products in the mature SW in an often species-specific manner (i.e., in terms of the so-called 'extractives' phytochemicals).[146]

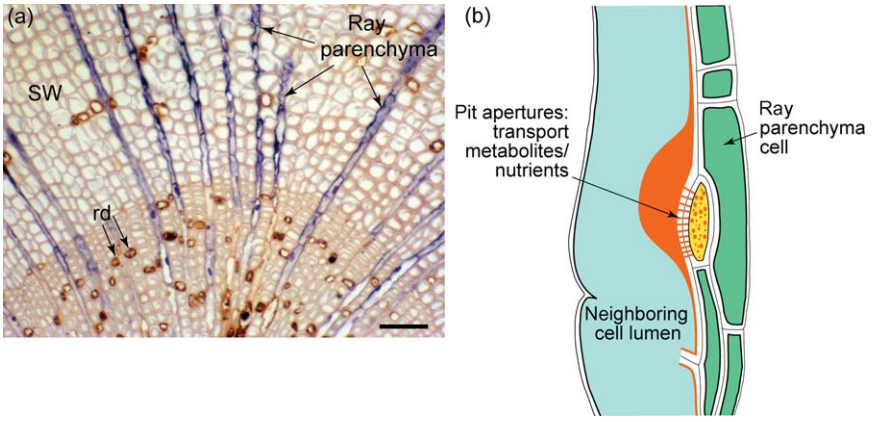


Figure 32 HW-forming processes. Radial parenchyma transport and synthesize a wide variety of nutrients, proteins, and the so-called 'secondary' metabolites (a). Here, the mRNA for dirigent protein, involved in an essential early metabolic step in lignan synthesis for HW formation, has been localized (blue coloration) to the radial parenchyma of a young *Thuja plicata* stem. Lignan-containing resinous deposits (rd) also appear in HW-forming tissue. Illustration of a proposed idealized HW metabolic deposition process: ray parenchyma transport various metabolites into neighboring cells through pit apertures (b). Photo from A. M. Patten, Washington State University (a) and redrawn from M. Chattaway, *Aust. For.* **1952**, *16*, 25–34 (b).

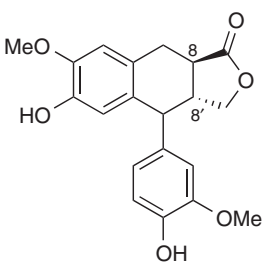
Conversion of SW into HW generally begins mid-summer, within the so-called transition zone,[65] once cambial activity and annual growth decline or cease.[130] Sucrose metabolism occurs by action of either sucrose synthase (SuSy) in SW and/or by cell wall-bound acid invertases within the transition zone and outer HW regions.[75] All of these processes are now known, however, to be mediated by involvement of ray parenchyma (**Figures 32(a) and 32(b)**) that ultimately facilitate secondary metabolite transport between the ray parenchyma and neighboring cells through cell wall pit apertures (**Figure 32(b)**).[64,65,130,147–149]

Concomitant to the so-called extractives formation and deposition, cellular changes occur as the growth season progresses. Parenchyma PCD, related to HW formation, begins in the transition zone and progressively radiates outwards (generally summer into early winter in temperate zone species).[53,75,150] This is evidenced by changes in ray parenchyma nuclei indicating onset of PCD in mature SW parenchyma.[130] Variations in PCD localization[151] and phenolic deposition[149] are noted, however, between tree species, where either a clearly defined transition zone (e.g., as in **Figure 22(c)**), or a less distinct and more diffuse zone exist between SW and HW.[53,129,149] Additionally, closure of pits between tracheids/vessels by the pit membrane (see Kwon *et al.*[148] and Taylor *et al.*,[152] and references therein) or the formation of tyloses (protrusions from the ray parenchyma through the pits into the vessel) may occur, thereby blocking water channels and possibly aiding in extractives accumulation.[39,130,153,154] It has also been shown that a few ray parenchyma remain transcriptionally active within outer HW zones during the HW-forming processes of summerwood.[126,127]

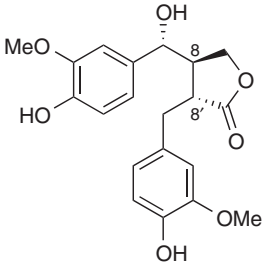
One early theory of HW function considered it as being a metabolic waste storage tissue[147] rather than deployment of a constantly evolving arsenal of distinctive, often species-specific, phytochemicals for this purpose. Others have apparently confused HW metabolite formation with tissues harboring 'abnormal lignins'[155] (see critique by Gang *et al.*[64]). Newer hypotheses and technical approaches now, however, offer more logical and incisive insights into this field of study as discussed herein.

Deposition of HW metabolites, such as the 8′–8′-linked lignans, appears to be concentrated in the transition zone and in the HW itself. Interestingly, Krahmer *et al.*[156] reported higher amounts of HW lignans, (−)-α-conidendrin (**17**), (−)-hydroxymatairesinol (**18**), and (−)-matairesinol (**19**) in the transition zone and HW metabolic regions, relative to the SW of western hemlock (*Tsuga heterophylla*). These chemical components were differentially distributed, with an enrichment of (−)-α-conidendrin (**17**) in tracheids with white floccosoid deposits[156,157] in contrast to enrichment in (−)-hydroxymatairesinol (**18**) in tracheids with clear deposits.[156] Moreover, similar proportions of (−)-α-conidendrin (**17**), (−)-hydroxymatairesinol (**18**), and (−)-matairesinol (**19**) occurred in crossing walls (i.e., with pit contacts allowing translocation) of ray parenchyma that were in

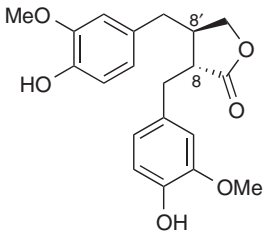
contact with fully occluded tracheids, but not in distal regions within the same ray parenchyma. These findings indicated that final steps of lignan synthesis, for example, occur either adjacent to, or within, tracheid ray parenchyma crossing walls.[156]



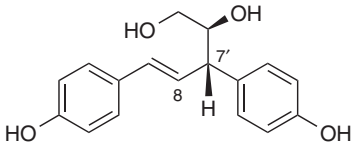
(**17**) (–)-α-Conidendrin



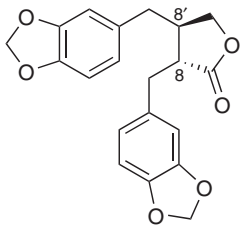
(**18**) (–)-Hydroxymatairesinol



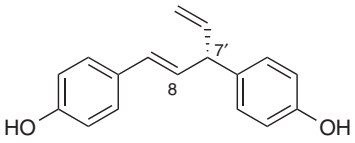
(**19**) (–)-Matairesinol



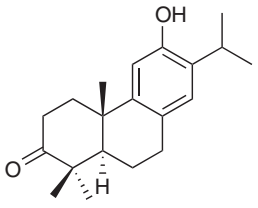
(**20**) Agatharesinol



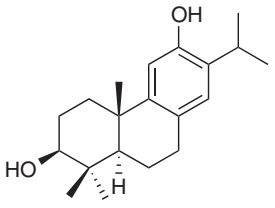
(**21**) (–)-Hinokinin



(**22**) (*E*)-Hinokiresinol



(**23**) Hinokione



(**24**) Hinokiol

Other studies have localized lignans to ray parenchyma and cell walls of adjacent tracheids. For instance, the *Cryptomeria japonica* (Sugi) norlignan, agatharesinol (**20**), was localized to HW ray parenchyma and to neighboring HW tracheids, but not SW.[158] Molecular mapping of (−)-hinokinin (**21**), *E*-hinokiresinol (**22**), and the diterpene phenols, hinokione (**23**) and hinokiol (**24**), in hinoki cypress (*Chamaecyparis obtusa*) indicates their widespread distribution in the cell walls of tracheids and parenchyma throughout the HW–SW transition zone. In contrast, (−)-hinokinin (**21**) is specifically localized to the ray parenchyma in this same region.[159]

3.27.3.3.3 Secretory and other resin-/phenolic-producing structures of wood and bark

Woody gymnosperms and angiosperms biosynthesize a rather enormous range of often highly valued secretory substances. This includes oleoresins (from conifers/cedars and tropical angiosperms), balsams (such as storax, styrex, elemis, frankincense, myrrh, and others), and varnishes and lacquers (such as dammars, mastics, and hard copals).[160] While physiologically these represent various forms of chemical defenses, their viscous/sticky natures also help provide physical barriers to opportunistic organisms. Moreover, their individual phytochemical constituents are generally quite characteristic for a particular plant species. This, in turn, has resulted in their differential usage by humanity over millennia for a myriad of applications, including that of the fossils known as amber (**Figure 7(a)**).[160]

Prior to discussing the phytochemicals present in specific plant species, a brief description of the tissues/cell types and the anatomical structures involved in resin formation is also warranted. It should be noted that the term 'resins' is loosely applied to a wide variety of secondary metabolites, sometimes without specific chemical meaning,[129] although it generally refers to metabolic mixtures containing terpenoids and/or

phenolics.[160] Additionally, the secretory resins may also be formed by a variety of anatomical structures, including the epithelial cells of resin ducts and polyphenolic cells in wood and bark tissues, and colleters, nectaries, and leaf tooth glands in photosynthetic tissues of the canopy.

3.27.3.3.3(i) Secretory resin ducts Resin ducts may form either constitutively ('normal' ducts) or in response to injury ('traumatic' ducts), such as in bark beetle attacks (arrow, **Figure 33(a)**). Normal resin

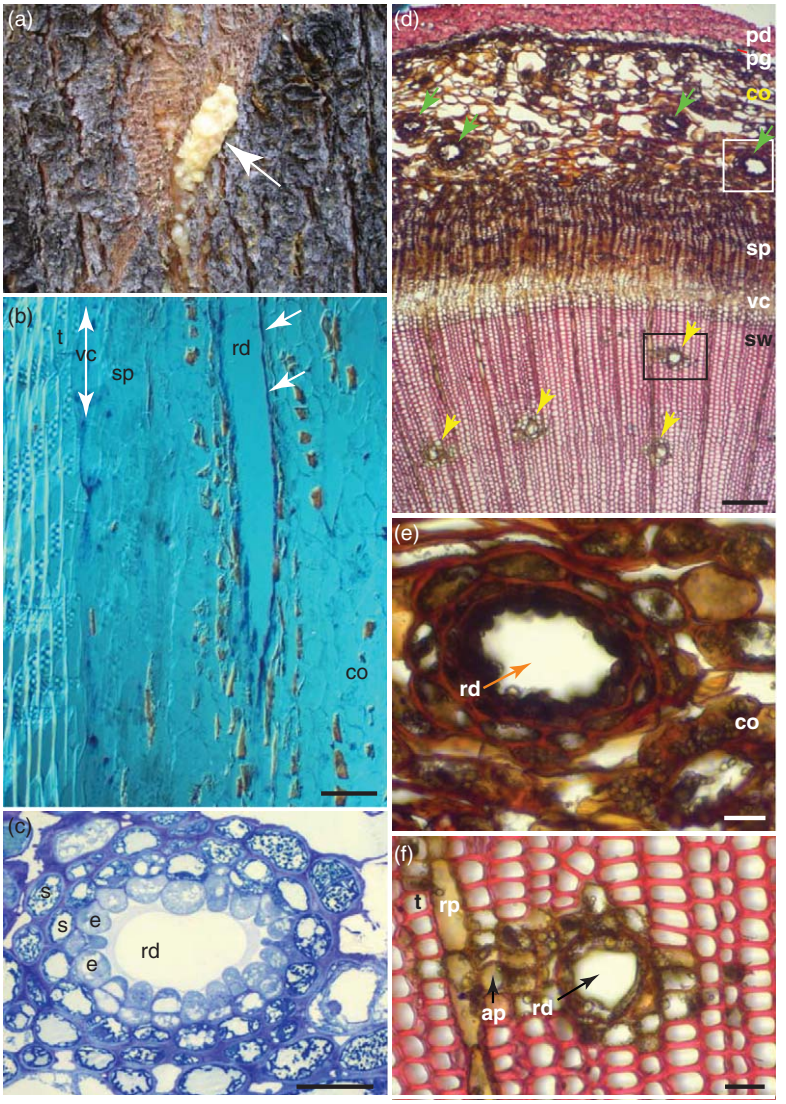


Figure 33 Resin duct structures in bark and wood. A bark beetle attack induced this strong level of resin production in *Pinus* sp. which has sealed off the injured site (arrow; a). Longitudinal section of a resin duct (rd) in *Pinus taeda* SW; *in situ* hybridization detection of mRNA (dark blue colorations, arrows; b) for the phenylpropenal double-bond reductase, an enzyme involved in lignan biosynthesis important to HW formation and defense.[161] Detailed cross-section view of a resin duct located within the secondary phloem of *Abies grandis* (c). Epithelial cells secrete resin into a large central resin duct, where the resin is stored until injury occurs. One or more layers of axial parenchyma (sheath) cells enclose the resin duct structure. Cross-section of loblolly pine (*P. taeda*) branch; resin ducts are present in the cortex and sapwood ducts (yellow and green arrows, respectively; d). Cortical resin duct (e) as marked in (d) white box. Sapwood resin duct (f) as marked in (d) black box. Abbreviations: ap, axial parenchyma; co, cortex; e, epithelial cell; pd, periderm; pg, phellogen; rd, resin duct; s, sheath cell; sp, secondary phloem; sw, sapwood; t, tracheid; vc, vascular cambium. Scale: 50 μm (b), 100 μm (c), 200 μm (d), 30 μm (e, f). Photos from L. B. Davin (a), A. M. Patten (b, d–f) and G. W. Turner (c), Washington State University.

ducts occur singly, and are relatively longer compared to the traumatic resin ducts that tend to be shorter and to occur in groups.[15] Resin ducts (**Figures 20(a)**, **32**(**a**), and **33**(**b**)–**33**(**f**)) are also given various names depending on general appearance: 'canals' if elongated (e.g., dammar canals or even some pine ducts, as in **Figure 33(b)**), 'pockets/cysts/blisters' if globular in appearance (e.g., copal resin pockets), or 'cavities' if distended and irregular in shape.[160] They may also occur in either the radial or the axial plane, with the frequency of the specific form of orientation apparently influenced by age, growth rate, and/or environmental conditions.[162–164] The overall effect appears to be that a higher density of resin canals results in increased resistance to infection.[165,166]

The resin ducts consist of hollow tube-like structures lined with adjacent resin-producing epithelial parenchyma cells (i.e., the aforementioned specialized axial parenchyma or idioblasts) that secrete their often bioactive phytochemical contents into the duct. The latter are, in turn, frequently surrounded by additional axial parenchyma/sheath cells[47] (**Figure 33(c)**) that presumably transport the needed metabolites. However, in some species, the surrounding sheath cells may also contain suberin, thereby providing sealant properties to protect tissue outside of the duct.[167] The most-widely reported process of duct formation (i.e., any type of resin, gum, oil, etc.) is that of schizogenous formation, which occurs where a gap develops between initial (nascent epithelial) cells, following dissolution of the middle lamella bonding cells to one another.[168] As an example, schizogenous development of an oil gland is shown in **Figure 34**.

In gymnosperms, the Pinaceae, Araucariaceae, Cupressaceae, and Podocarpaceae families are the true resin producers with scattered reports from the Cephalotaxaceae, Taxaceae, and Sciadopityaceae.[160] The most important commercial resins from gymnosperms include: the turpentines (i.e., often distillation products of

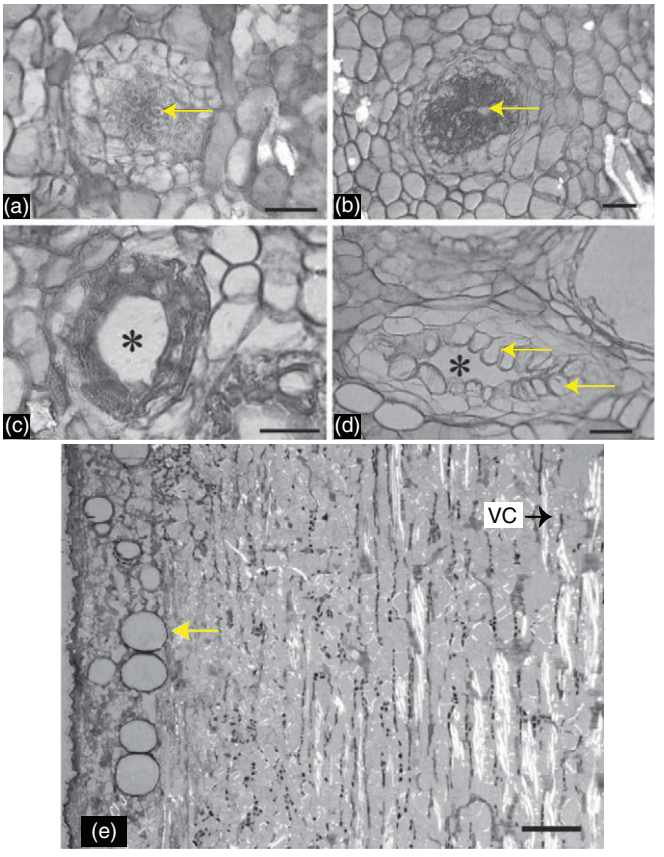


Figure 34 Development of bark traumatic oil glands following injury in *Eucalyptus globulus*. Parenchyma within the trauma zone divide (arrow; a). Early schizogeny between traumatic parenchyma cells (b). Late schizogeny forming a cavity (*; c). Mature oil gland with cavity (*) surrounded by oil-producing epithelial cells (arrows; d). Oil glands typically occur in the outer cortex, far from the vascular cambium (VC) (e). Scale = 10 μm (a–d); 300 μm (e). Reproduced with permission from Springer from A. Eyles; N. W. Davies; C. Mohammed, *Trees – Struct. & Funct.* **2004**, *18*, 204–210, copyright 2004.

resinous mixtures), derived from Pinaceae of North America and Europe, and *Larix* species in Europe; the Canada and Oregon balsams that are harvested from *Abies balsamea* (**Figure 35**)[169,170] and *P. menziesii*, respectively; Sandarac, harvested from *Tetraclinis articulata*[171](Africa, **Figure 35**) and *Callitris* spp. (Australia).[160]

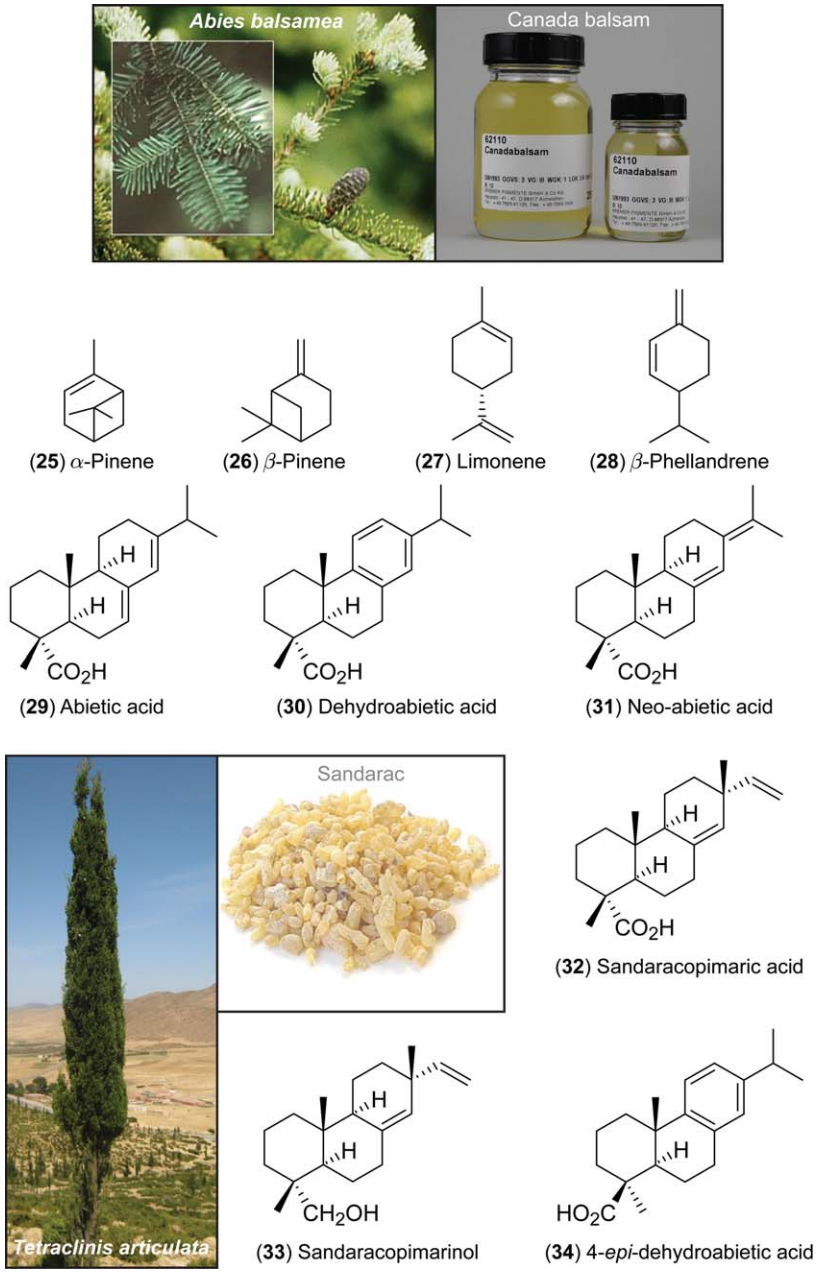


Figure 35 Canada balsam is secreted by cells in the bark of balsam fir (*A. balsamea*) and accumulates in blisters. α- and β-Pinene (**25**, **26**), limonene (**27**), and β-phellandrene (**28**) are the main constituents of the oil obtained after distillation of Canada balsam, whereas abietic (**29**), dehydroabietic (**30**), and neo-abietic (**31**) acids are the major components of the residue.[170] Sandarac is from the sandarac tree (*Tetraclinis articulata*) which is native to Morocco. It is used as a resin for artwork. The main constituents of sandarac are sandaracopimaric acid (**32**), sandaracopimarinol (**33**), and 4-*epi* dehydroabietic acid (**34**).[171] Photos from U. T. Runesson, Lakehead University, Thunder Bay, Ontario, Canada (borealforest.org), from Monica Titelus, Kremer Pigmente (http://www.kremerpigments.com), from Professor Moha Berraho, Université Cadi Ayyad, Marrakech, Morocco and from Cleansing Incense (http://www.cleansingincense.co.uk), with permission.

There are important differences, however, between the resin duct anatomies of the Pinaceae and non-Pinaceae gymnospermous species. The former produce copious amounts of resin by both constitutive and defensive response systems. This is possible because the internal secretory parenchyma that line the resin ducts of the Pinaceae remain alive and active for years, thereby allowing resin accumulation. In contrast, non-*Pinus* gymnosperms produce little constitutive resin reserves, these being stored in resin pockets or blisters. Moreover, genera such as *Abies* and *Tsuga* employ rapid responses to invading organisms by forming traumatic ducts to increase resin production. Given the short-term function of such traumatic structures, their epithelial cells become lignified and die within a short time, thereby limiting the amount of resin produced over the lifetime of non-*Pinus* trees.[47,129,172]

Many angiosperm genera also produce resins, again either constitutively or as a response to invasive organisms. Resin-producing trees occur in numerous angiosperm families worldwide;[173] however, it is the subtropical to tropical members of the Fabaceae and Dipterocarpaceae that produce the greatest quantities.[174,175] Unfortunately, however, the secretory structures of many angiosperm trees have not been studied to the same extent as those of resin-producing gymnosperm trees.[160] It is known that angiosperm resins are formed by secretory epithelial cells lining canals, cavities, pockets, or blisters. Moreover, if their resin structures are numerous and in close proximity within angiosperms, they may anatosmose (i.e., form connections that generate a resinous network).[160] The most well-known resin products from angiosperm species include: copals (**Figure 36**) from *Hymenaea*, *Copaifera*,[176] *Trachylobium*, and *Daniellia* spp. (Fabaceae, West Africa and the Americas); elemi resins (**Figure 37**) from *Canarium*[177,178] and *Dacryodes* spp. (Burseraceae, Pacific islands), and *Protium* spp. (South America); aromatic (in terms of fragrance, not chemical structure) resins (**Figure 38**) from *Boswellia*[179] and *Commiphora* spp.[180,181] (Burseraceae, North Africa, Middle East, Central Asia); mastic/varnishes (**Figure 39**) from *Pistacia* spp.[182,183] and the dammars from *Shorea*, *Pentacme*, *Hopea*, and *Balanocarpus* spp. (Dipterocarpaceae, Asia).[160] In addition, the Tolu and Peru balsams (**Figure 40**) are harvested from members of the genus *Myroxylon*[184](Fabaceae, South America); benzoin and other styrax resins (**Figure 40**) from *Styrax* spp.[185,186] (Styracaceae, Asia and South America, respectively);[160,173] storax from *Liquidambar* spp. (Hamamelidaceae, Asia minor and the Middle East, and North and Central Americas);[160,187] and the anacard lacquer from *Rhus vernicifera* (Anacardiaceae)[160] (**Figure 4** and **Structures 71–74**).[188–192]

3.27.3.3.3(ii) Other polyphenolic and resin-producing idioblasts in bark and some woods Although not common in all genera, some axial idioblasts produce and store phenolic and/or resinous metabolites for defense purposes. These occur as clusters of individual cells and not as part of a larger structure, such as a duct.

In the secondary phloem of all conifer families, the so-called PP cells are the most important of these individual cell types in the constitutive defense systems of bark[81,87] (**Figure 30**). The PP cells are axially oriented and develop in rows within the secondary phloem. As their name implies, their contents are normally polyphenolic in nature, serving as a constitutive antifeedant. Franceschi *et al.*[87] have shown that PP cell size and apparent phenolic content increases in response to injury (**Figure 30(b)**). They suggest that such anatomical changes in PP cells may correlate with overall phloem chemical content changes in response to injury, as noted by other researchers. These include, for example, apparent increases in ferulic acid glucoside (**75**) in ponderosa pine (*P. ponderosa*),[193] and tannin,[194] stilbene, and terpene increases in Norway spruce (*P. abies*).[195] Changes in PP cell size/contents may also co-occur with traumatic resin duct formation, and/or may trigger induction of wound phellogen to produce wound periderm. Hence, overall, this is a coordinated response that is both of a chemical and of a structural defense in nature.[81] Such responses represent yet another wonderful example of the benefits of modularity and compartmentalization of tree growth.[41,42]

In some angiosperms, certain idioblasts also serve in defensive functions at the single cell level. For instance, some *Betula* spp. have SW parenchyma containing various terpenoids, such as **76**, **77**, and lupeol (**78**) as constitutive chemical defenses.[196]

3.27.3.3.4 Laticifers of bark and leaves

Laticifers are latex-producing structures of many plants and which can occur in the bark (and sometimes leaves) of tree species. Latex is an aqueous colloidal solution that varies by species, but usually appears whitish in color (in tree species) and generally includes terpenoids/isoprenoids and various enzymes.[197,198] Smaller quantities of fats, carbohydrates, and/or other phytochemicals may also be present.[47]

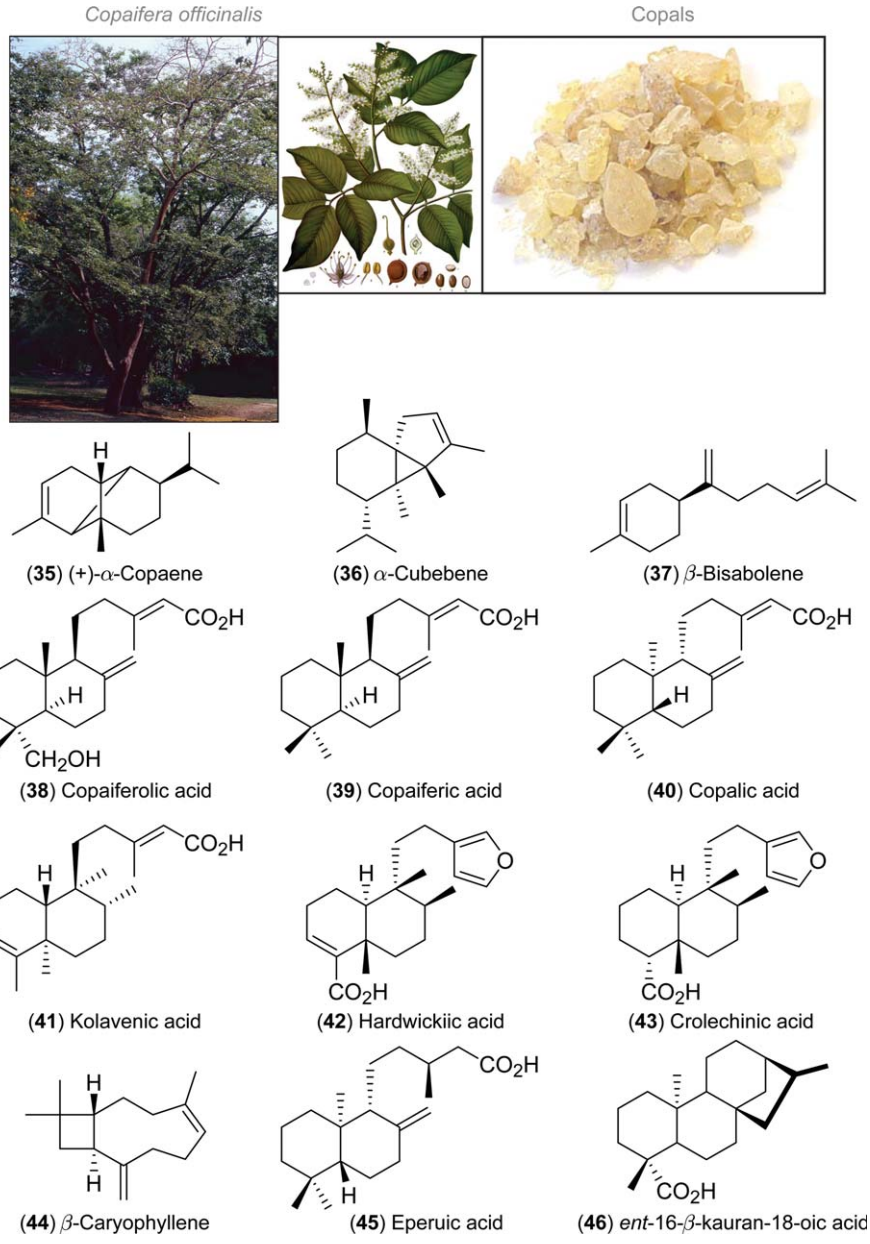


Figure 36 Copals from *Copaifera* spp., with representative diterpenes and sesquiterpenes (**35–46**).[176] Artistic rendition from Köhler's Medizinal – Pflanzen (1887). Photos from Professor David Webb, Spokane, Washington, USA and from Cleansing Incense (http://www.cleansingincense.co.uk), with permission.

Laticifers have been found in over 20 000 herbaceous and woody angiosperm species,[199] and are much more abundant in tropical angiosperms.[160] They are also present in several *Gnetum* species (i.e., gymnosperms) and one fern species (*Regnellidium diphyllum*).[200] Latex compositions can vary by taxon and geographic location. Among latex-producing trees, various well-known latex/rubber products have been developed including: Parà or Hevea rubber (*Hevea brasiliensis*), Ceara or Manicoba rubber (*Manihot glaziovii*), India or Assam rubber (*Ficus elastica*), Lagos silk or African rubber (*Funtumia elastica*), Gutta-Percha (*Palaquium gutta*), Balata (*Manilkara bidentata*), Jelutong (*Dyera costulata*), and Chicle (*Achras zapota*).

Laticifers (**Figure 41(a)**) are classified into two major groups by development and morphology. The simplest form, the nonarticulated type, originates from a single cell, which elongates extensively to form tube-like structures

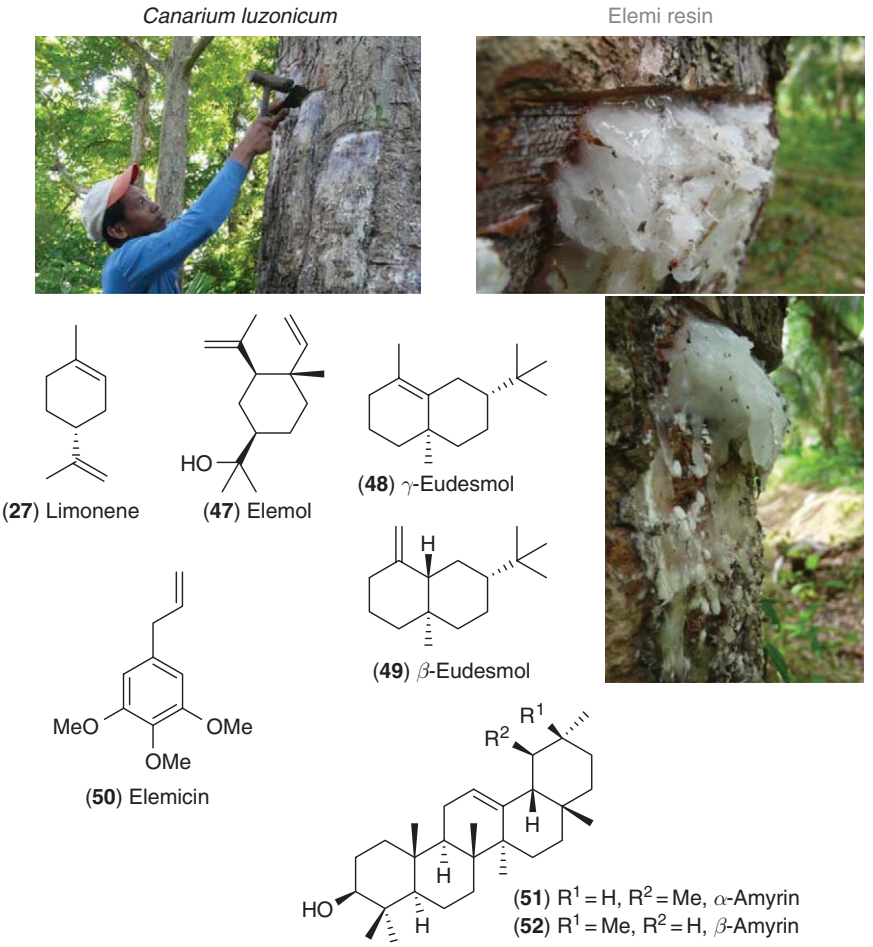


Figure 37 Elemi resin is from *Canarium luzonicum* that grows in the Philippines. From July to November cuts are made in the bark with gum harvested thereafter weekly. The main constituents of the resin are the phenylpropanoid, elemicin (**50**), the monoterpene, limonene (**27**), the sesquiterpenes, elemol (**47**), γ-eudesmol (**48**), β-eudesmol (**49**), α-amyrin (**51**) and β-amyrin (**52**).[177,178] Photos from Biolandes (http://www.biolandes.com), with permission.

that may be either branched or unbranched. In contrast, the articulated type is derived from multiple cells forming compound structures. In both cases, neighboring laticifers may join to form networks (i.e., anastomosis).[15] Little is yet known about laticifers, with most studies focused on the commercially important species, the rubber tree (*H. brasiliensis*) (see Pickard,[201] and references therein) (**Figures 3(a) and 3(b)**). This species develops laticifers in concentric rows (**Figure 41(b)**) with anastomoses forming a network between these in both the primary and secondary phloem (**Figure 41(c)**). Primary and secondary laticifer systems produce compositionally variant latexes, with commercial latex harvested from the secondary laticifers.[202] The quantity of latex produced is dependent on the number of laticifer rows within the secondary phloem,[203] with faster laticifer differentiation (as in some cultivars) improving yield.[202] Repeated tapping of *H. brasiliensis* is known to induce laticifer differentiation.[204,205]

Additionally, latex in *H. brasiliensis* and other species is under high hydrostatic pressure (10–15 atm), with greatest pressure being in the lower parts of the trunk.[206,207] Upon puncture (as in tapping), laticifers release latex, the flow of which appears to be attenuated by the involvement of actin microfilaments, which decrease in content upon repeated tapping as in commercial harvesting.[208]

3.27.3.3.5 Gum-producing structures of wood and bark

Plant gums are generally complex carbohydrate polymers that may also include hydroxyproline-rich proteins, resins, or other components and can be either soluble, partly soluble, or insoluble.[209,210] Because of their

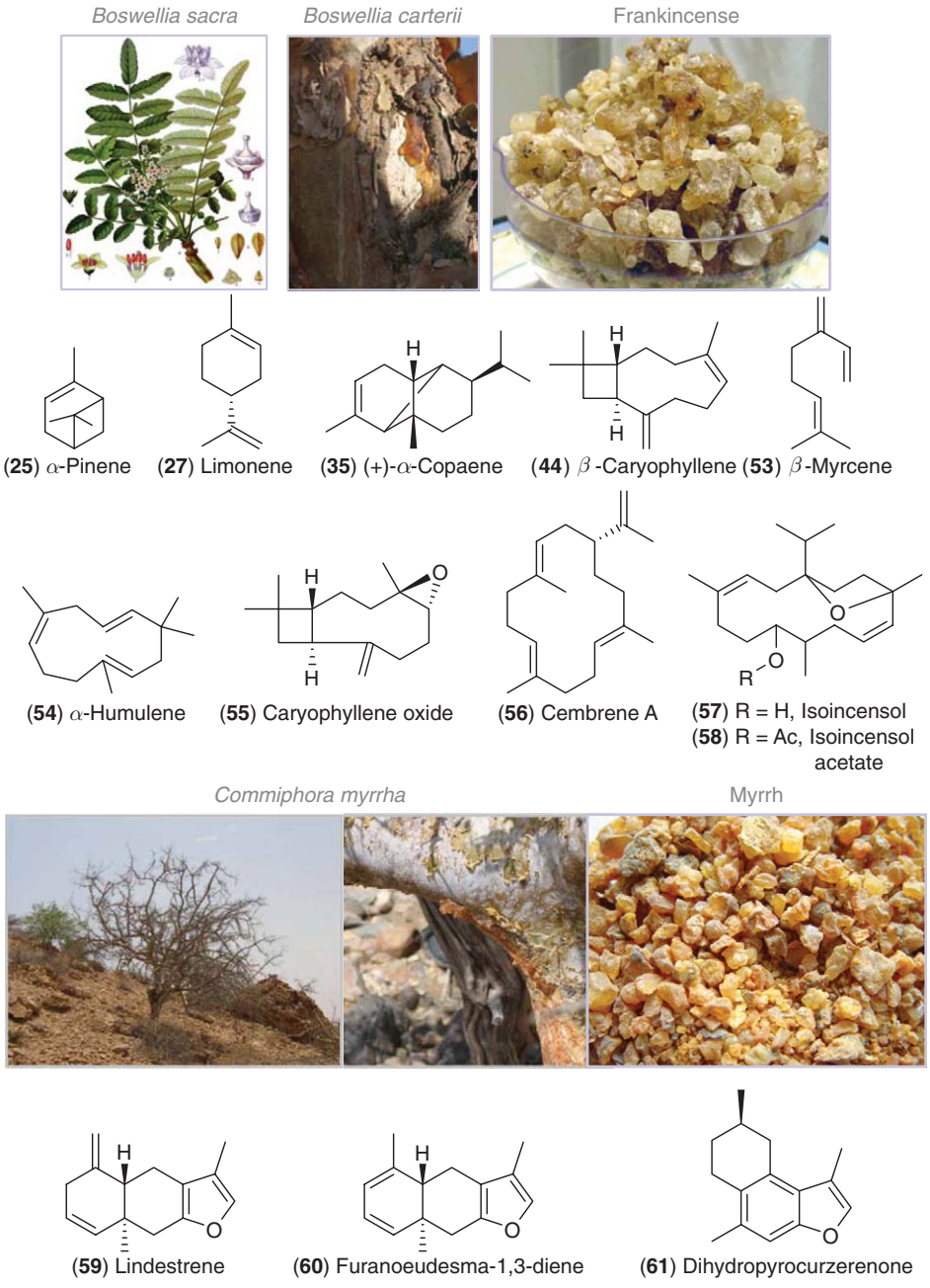


Figure 38 Frankincense and myrrh have been used since the dawn of civilization. Frankincense is collected from *Boswellia* spp. (i.e., *B. sacra*, *B. papyrifera*, *B. frereana*, and *B. carterii*). During the dry season (summer), the tree is wounded, and the gum is collected and sorted. Myrrh is from *Commiphora myrrha*. The main constituents of frankincense are α-pinene (**25**), limonene (**27**), α-copaene (**35**), β-caryophyllene (**44**), β-myrcene (**53**), α-humulene (**54**), caryophyllene oxide (**55**), cembrene A (**56**), isoincensol (**57**), and isoincensol acetate (**58**),[179] whereas lindestrene (**59**), furanoeudesma-1,3-diene (**60**), and dihydropyrocurzerenone (**61**) are largely responsible for the fragrance of myrrh.[180,181] Photos from Biolandes (http://www.biolandes.com), with permission and artistic rendition from Köhler's Medizinal – Pflanzen (1887).

co-occurrence with other secretion types and material properties, gums have been at times confused with resins (especially from leguminous spp.) or latexes.[160] Gums and gum resins of wood and bark are produced by duct structures that have close morphological similarities to resin-producing ducts. Gum-secreting epithelial cells

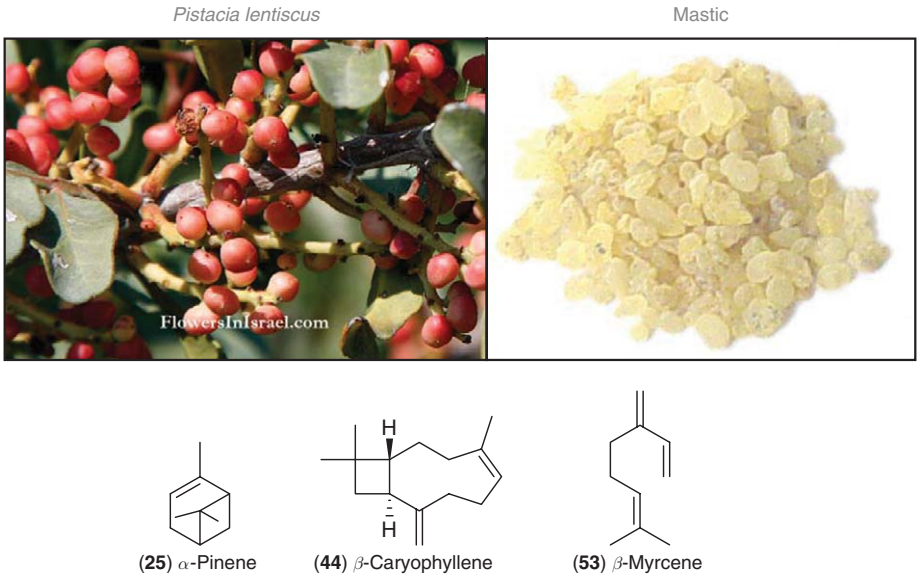


Figure 39 Mastic resin is obtained from branches and trunks upon injury of *Pistacia lentiscus*. *Pistacia* trees are distributed along the Aegean–Mediterranean coast. The main constituents of mastic essential oil are α-pinene (**25**), β-caryophyllene (**44**), and β-myrcene (**53**).[182,183] Photos from Flowers in Israel (http://www.flowersinisrael.com), from Cleansing Incense (http://www.cleansingincense.co.uk), with permission.

also line the gum ducts, with their Golgi bodies being the site of (carbohydrate) synthesis, transport, and secretion.[168] Gum ducts may be formed either constitutively or in response to injury, the latter being referred to as 'gummosis.'[96,209] The gum secretions are, however, complex and variable between species, but often also contain terpenoids and significant levels of other carbohydrate components (especially starch).[15]

While many herbaceous and woody plants produce gums, only a few tree species produce commercial quality and quantities of gums, these currently being limited to families of the Fabaceae, Sterculiaceae, and Combretaceae, with the Fabaceae being the most important.[168,209] Gums produced commercially mainly include: gum Arabic (*Acacia senegal*, **Figure 3**), gum ghatti, (*Anogeissus latifolia*), neem gum (*Azadirachta indica*), gum karaya (*Sterculia urens; Cochlospermum gossypium*), Joel or Jingan gum (*Lannea coromandelica*), and Mesquite gum (*Prosopis juliflora*).[209]

Gummosis or gumming syndrome is the release of gum in response to injury and poses a serious problem in either fruit and/or wood of commercially important fruit tree species, such as citrus crops and *Prunus* spp. (e.g., cherry, plum, peach, and apricots) (**Figure 42**). This can include loss of fruit, loss of high-quality wood (i.e., black cherry, *Prunus serotina*), and even tree death.[211–213] Infected citrus saplings, for example, can also form gum ducts within the cambium zone, depositing gum into young vessels and hampering the water conduit system of the tree.[47]

3.27.3.3.6 Kino

Kinos are exudates of *Eucalyptus* spp. that historically were referred to as 'gums,' most likely due to their viscous and tacky nature; however, they are apparently not enriched in carbohydrates.[214] Instead, kinos are thought to be largely composed of proanthocyanidins as well as monomeric polyphenolic components in some species.[129,215] Kino composition has also been reported to vary by species (see Tippett,[214] and references therein). For instance, *Eucalyptus calophylla*, *Eucalyptus sieberi*, and *Eucalyptus sideroxylon*, among others, are envisaged to consist primarily of polymerized leucocyanidin, while polymerized flavans apparently form the major component of the kinos from *E. platypus*, *E. astringens*, and *E. lehmannii* (see Hillis and Yazaki,[216] and references therein). Distinctive polyphenols (including tannins), and other components have been reported for individual species.[216–218] For the most part, nevertheless, these structures are still very provisional.

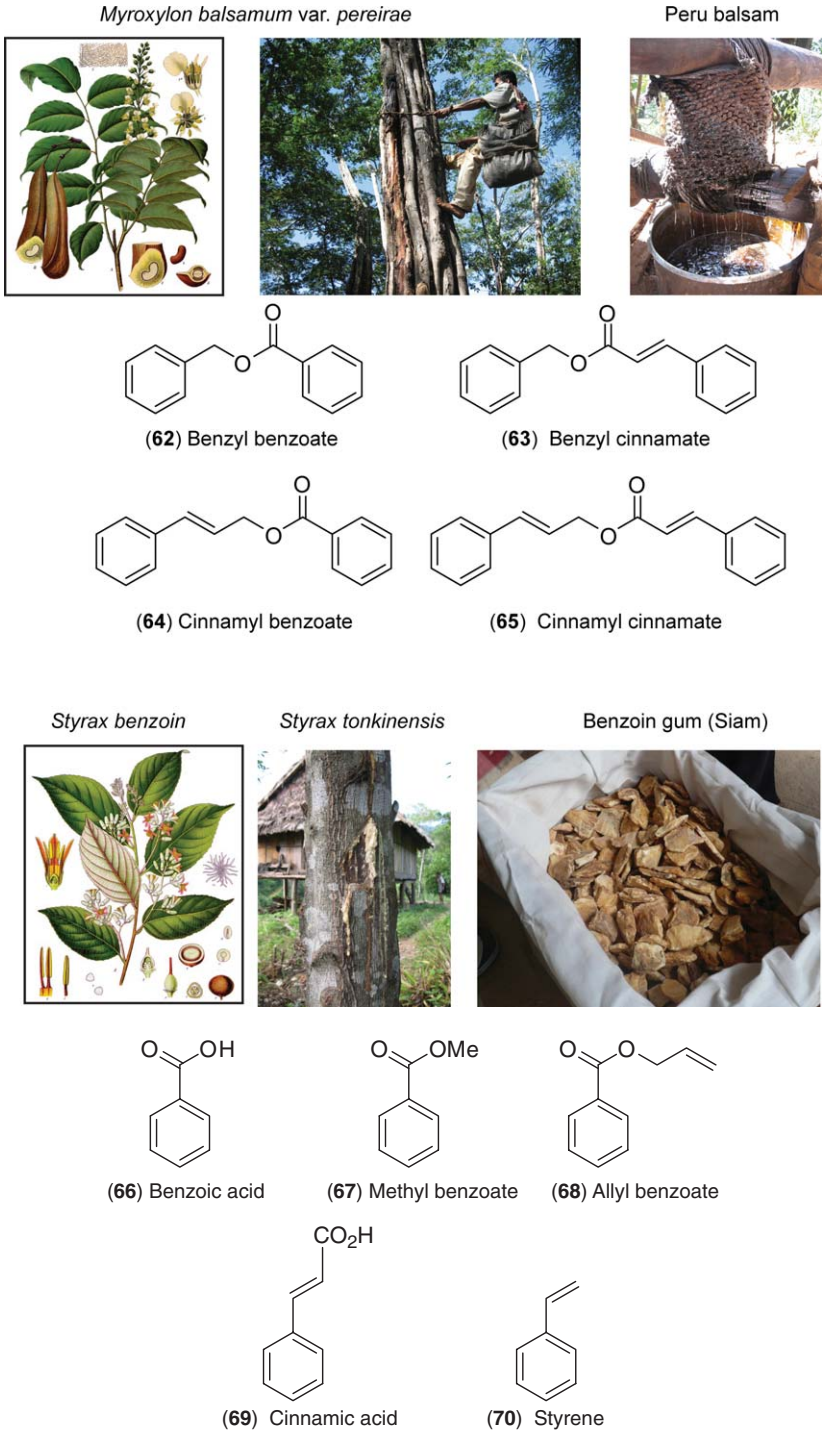
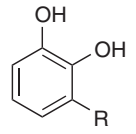
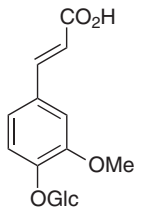


Figure 40 Peru balsam is obtained from *Myroxylon balsamum* var. *pereirae*. The main constituents of the oil obtained by vacuum distillation are benzyl benzoate (**62**), benzyl cinnamate (**63**), cinnamyl benzoate (**64**), and cinnamyl cinnamate (**65**).[184] Sumatra and Siam benzoin gums are from *Styrax benzoin* and *Styrax tonkinensis*, respectively. The gum is produced only after deep incisions are made in the bark. The main volatile constituents are benzyl benzoate (**62**), benzoic acid (**66**), methyl benzoate (**67**), allyl benzoate (**68**) in Siam benzoin, whereas Sumatra benzoin is rich in styrene (**70**), cinnamic acid (**69**), and benzyl cinnamate (**63**).[185,186] Photos from Biolandes (http://www.biolandes.com), with permission and artistic rendition from Köhler's Medizinal – Pflanzen (1887).

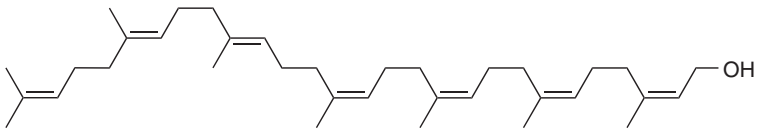
Kino is formed within the so-called 'kino veins' that are duct-like at 1.5–5 mm in diameter and as much as 2 m in length.[129] The veins are associated with injury to the vascular cambium, derived from cambial initial cells, and form to either the xylem side or the phloem side of the cambial zone.[214,219] Kino veins have distinctive morphology with individual structures composed of secretory idioblasts and separated by parenchyma bands; these together form a continuous circumferential zone (**Figure 43(a)**). Occasionally, some parenchyma partitions breakdown, allowing the formation of a kino pocket (not shown). Kino structures are thought to provide a physical/chemical barrier against herbivores and pathogens.[129]



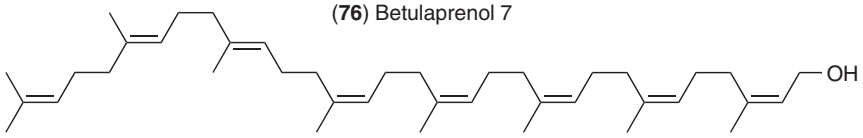
Urushiol
(**71**) R = 8′*Z*, 11′*E*, 13′*Z*-pentadecatrienyl
(**72**) R = 8′*Z*-pentadecenyl
(**73**) R = 8′*Z*, 11′*Z*-pentadecadienyl
(**74**) R = pentadecyl



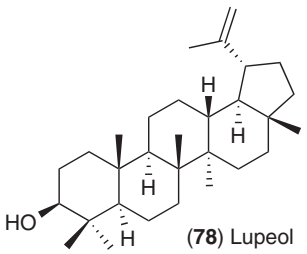
(**75**) Ferulic acid glucoside



(**76**) Betulaprenol 7



(**77**) Betulaprenol 8



(**78**) Lupeol

Besides cambial-associated kino veins, *Eucalpytus* spp. also form other extractive types when injured outside the cambial zone and in association with wound tissue. This wound tissue is noted to form from traumatic parenchyma that is derived from preexisting parenchyma and produces exudates that differ in composition from kinos (**Figure 43(b)**) (see Eyles and Mohammed,[219] and references therein). As in kinos, proanthocyanidins and

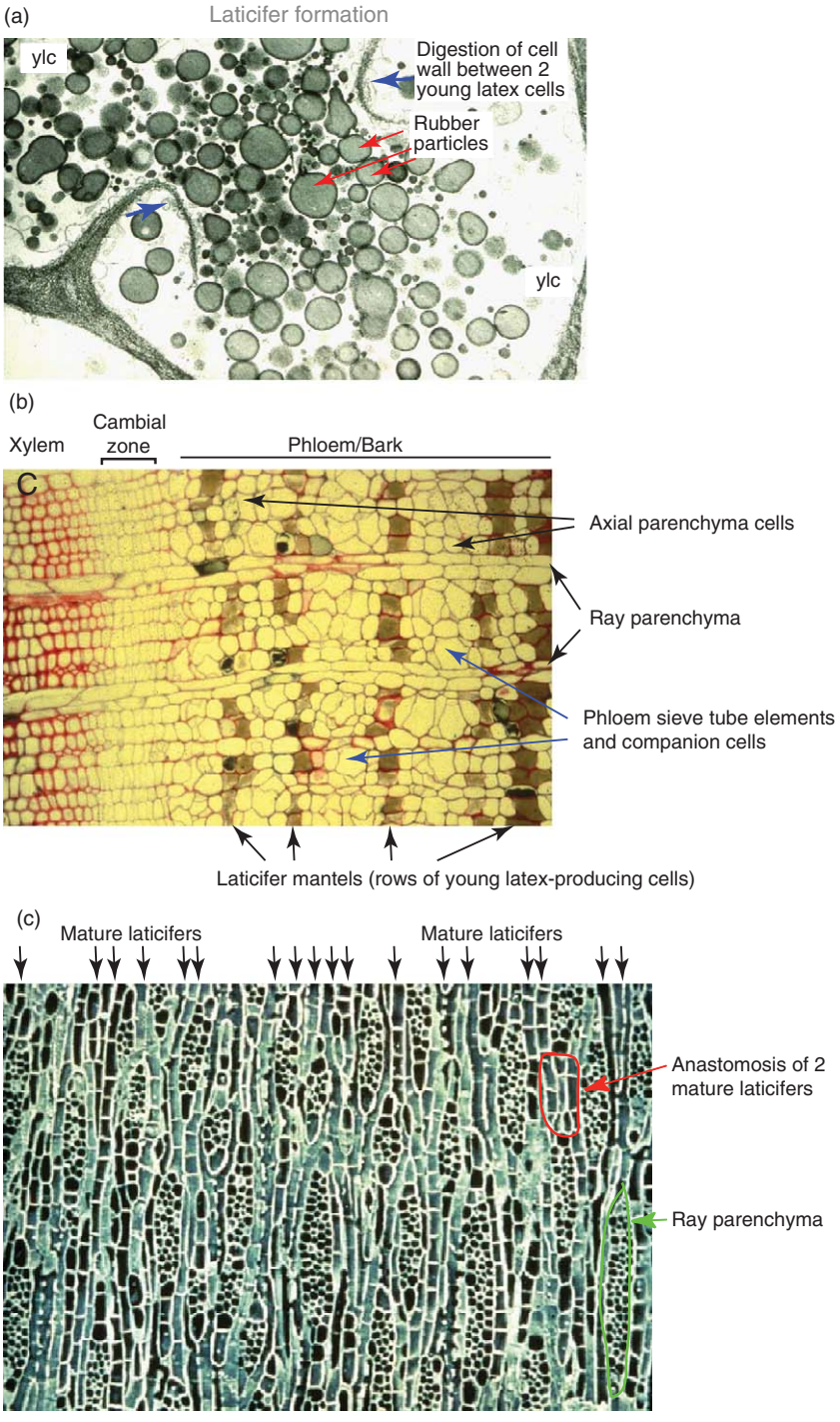


Figure 41 Laticifer anatomy in *Hevea brasiliensis*. Young latex cells (ylc) produce intracellular latex-containing natural rubber particles. A laticifer is formed by the digestion of adjacent cell walls of the young latex cells (a) allowing for the formation of the larger structure. Individual laticifers form rows or 'mantels' in the bark as observed in cross-section (b). Mature laticifers extend throughout the bark (tangential section) and often connect to form complex networks; a laticifer connection is referred to as an 'anastomosis' (c). Images from D. Nandris; H. Chrestin, Institut de Recherche pour le Development (IRD), Mahidol University, Bangkok, Thailand.

Figure 42 Gum exudates. Gum-producing trees, such as the cherry tree (*Prunus* sp.) form viscous sticky exudates in response to injury. Reproduced with permission of Wiley-VCH Verlag GmbHCo. KGaA from J. B. Lambert; J. A. Santiago-Blay; K. B. Anderson, *Angew. Chem. Int. Ed.* **2008**, *47*, 9608–9616, copyright 2008.

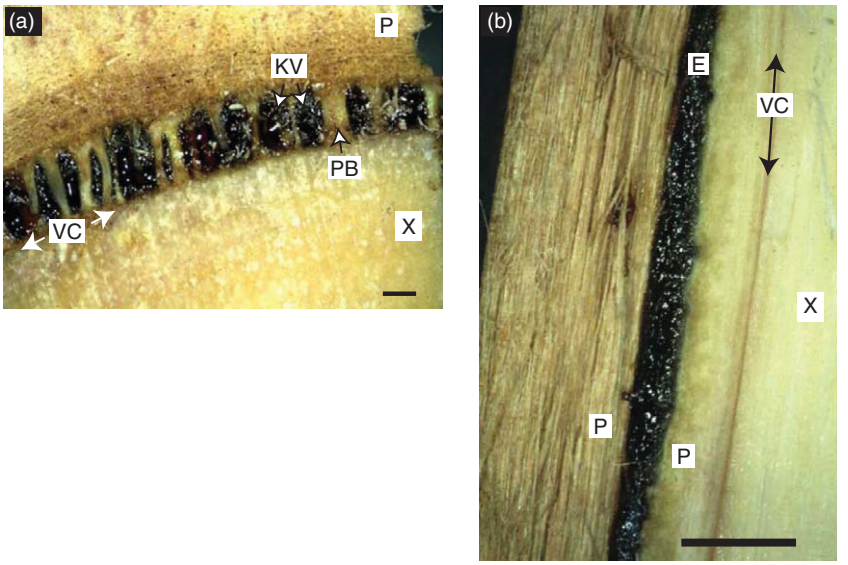


Figure 43 Differential 'extractives' formation in *Eucalyptus* spp. Kino veins (KV) (a) are specialized structures that produce kino extractives. The veins originate from vascular cambium (VC) initials and are discrete structures that typically occur concentrically outside the VC and are separated by bands of parenchyma (PB). They are typically associated with wounding of the VC. Extractives (E) that are chemically distinct from kino can also form in *Eucalyptus* spp. and appear to be involved in wound responses not involving the VC (b). The extractives are produced from traumatic parenchyma derived from pre-existing phloem parenchyma and forming a continuous layer at the site of wounding. Scale = 0.5 mm (a); ~1.5–2 cm (b). Images from A. Eyles, Co-operative Research Centre for Sustainable Production Forestry, Hobart, Tasmania, Australia (a). Reproduced with permission of the Institute of Foresters of Australia from A. Eyles; C. Mohammed, *Aust. For.* **2003**, *66*, 206–212, copyright 2003.

tannins provisionally comprise a portion of extracambial exudates; however, other compounds such as flavanone glycosides, stilbene glycosides, and formylated phloroglucinol compounds occur as unique components.[220,221]

The apparent variety and complexity of chemical defenses produced by *Eucalyptus* spp. underscore the biochemical diversity within trees that we are only beginning to understand, and the expansive resources available to humans.

3.27.3.3.7 Oil-producing structures in wood and bark

In many tree species, the most notable groups including the conifers, *Cinnamomum* spp., *Citrus* spp., and *Eucalyptus* spp., oil may be produced in nearly all tissues.[15,222–224] Some of the so-called 'wood oils' are not true oils (i.e., a liquid volatiles-containing component), but are derived from oleoresins released by distillation from HW, such as cedarwood oil, sandalwood oil, and rosewood oil.[160] That is, these are 'extractive' oil components of stem, and sometimes root, HW.[225] On the other hand, cinnamon, camphor, and other true oils are derived from oil idioblasts of the wood/bark axial and/or ray parenchyma systems,[222,226] although additionally, oil cavities or glands may be present. Similar to gum cavities, these have structural similarities to resin ducts with secretory epithelial cells lining the cavity. Although not well studied in angiosperms, at least one genus has been reported to produce both 'normal' and 'traumatic' oil glands,[227] in the phloem (inner bark) of *Eucalyptus* spp. Traumatic oil glands do not form in the cambial zone, as in kino formation, but form schizogenously within parenchyma of wood or bark in a manner similar to resin duct formation (**Figure 34**).[227]

In general, development of an oil cell begins with deposition of a suberized layer inside the primary cell wall of an incipient oil idioblast. Another cell wall layer is then added inside this and together the three layers form a tightly sealed boundary that permits retention of future oil contents.[228] Oil accumulates within the young cells until the protoplast dies and the oil with cellular remnants occupies the entire cell cavity. Oil is then released when the cell membrane is lysed.[47]

3.27.3.3.8 Mucilage-producing structures of wood and bark

Mucilages are similar to gums in that they are complex polysaccharides, but differ in the variety of different functions they serve and the secretory structures producing them. Germination and dispersal, water retention, and freezing tolerance are just some of the functions that mucilages are considered to participate in.[160] Mucilages may be formed by trichomes, idioblasts (i.e., specialized parenchyma), ducts, or epidermal cells, each of which often vary in morphology in a species-specific manner.[160,229]

In wood and bark, mucilages play important roles in maintaining water status. While trees of optimal size in optimal environments rely on water tension to draw water to the canopy, trees under high saline or drought conditions and trees of extreme height are challenged to maintain sufficient water tension. Mangrove trees apparently secrete mucopolysaccharides to line xylem cells, which assist with water uptake that is high in saline content.[230] ('mangrove' refers to plants adapted to high-saline aqueous environments and includes tree families such as *Rhizophora*, *Bulnesia*, and *Astronium*). Interestingly, at least three genera of giant trees, the *Metasequoia*, *Sequoia*, and *Sequoiadendron* also appear to offset the challenge of long distance water transport by absorbing atmospheric water (i.e., fog) through mucilage of bark, and offsetting the extremely negative water potentials needed to lift water over 200 ft vertically.[231,232]

Mucilage-secretory cells are considered to have a common developmental origin, despite their differing contents, function, and appearance at maturity. This is based on observations within extant 'primitive' or basal angiosperms (i.e., the Laurales and Magnoliales), wherein both oil and mucilage cells appear to have a suberized layer (described above).[229,233] Members of these so-called 'primitive' taxa include the genus *Cinnamomum* wherein both mucilage and oil cells occur in abundance and contribute to the secretory component of cinnamon.

Mucilaginous cells produce polysaccharides that are secreted by the Golgi apparatus.[47] Cells that retain mucilage (**Figures 44(a)** and **44(b)**), however, often develop raphid crystals (long bundled calcium oxalate (**13**) crystals; not shown) that also serve defensive functions.[234–236]

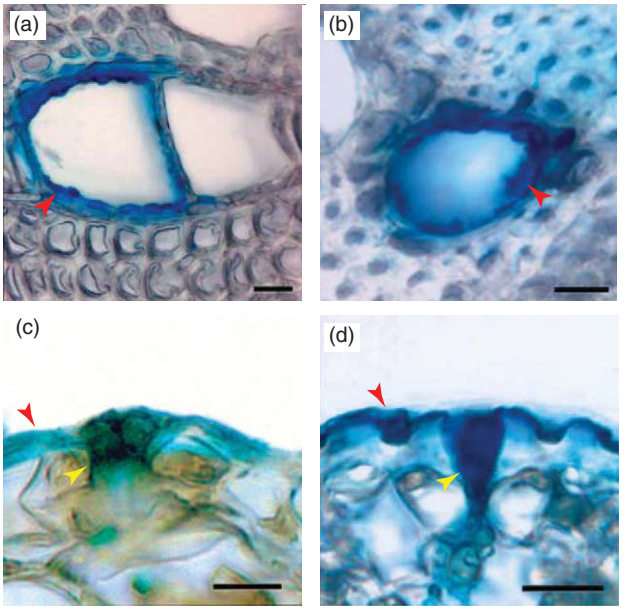


Figure 44 Mucilage as a supplemental water uptake mechanism. In *Populus nigra* (a, c) and *Eucalyptus pilularis* (b, d), for example, mucilage (blue coloration) allows for facilitated stem water transport in vessels (arrowheads; a and b). Mucilage (blue coloration) in leaves allows for capture of atmospheric moisture, especially in leaf epidermis (red arrowheads; c and d) in leaves near the stem apex. Mucilage may also plug the stomata of leaves when the whole organism is under drought stress (yellow arrows; c, d). Reproduced with permission of Springer from D. Zimmermann; M. Westhoff; G. Zimmermann; P. Gessner; A. Gessner; L. H. Wegner; M. Rokitta; P. Ache; H. Schneider; J. A. Vásquez; W. Kruck; S. Shirley; P. Jakob; R. Hedrich, F.-W. Bentrup; E. Bamberg; U. Zimmermann, *Protoplasma* **2007**, *232*, 11–34, copyright 2007.

3.27.3.3.9 Secretory structures of foliage and reproductive tissues

3.27.3.3.9(i) Resin-producing structures of leaf and flower tissues Resin-producing structures of leaves and flowers include colleters, glandular trichomes, marginal leaf glands, and floral and extrafloral nectaries. While secreting resin in some species, these anatomical structures may generate and/or accumulate phytochemical mixtures in other species, including gums or mucilages, or they may produce nectar or release water (guttation). Each structure type also thus has species-specific anatomical variations that in some cases have strong similarities to others because of their similar functions. This has led to somewhat confusing terminology in the literature. For instance, bud colleters of *Populus*, *Betula*, *Alnus*, and *Aesculus* spp. have also been variously referred to as 'glandular trichomes.'[160,237]

Colleters are hair-like structures that, in families such as Salicaceae and Rubiaceae, occur on the adaxial side of young buds, leaves, and/or stipules (i.e., the often leafy appendages at the base of young buds and leaves (**Figure 45(a)**) and secrete resins to coat and presumably protect young tissues.[237–240] Bud scale (i.e., modified stipules) colleters produce resins and/or other secretions to fill gaps and seal the bud, thereby protecting meristematic and primordial leaf tissues. This is an important part of bud set in temperate zone species to allow for successful overwintering. Various names have been given to the colleters, including 'glandular hairs,' 'glandular trichomes,' 'extrafloral nectaries,' 'resin glands,' among others (see Thomas,[237] and references therein). Morphology of these structures can vary between taxa, although the most common is the 'standard type' (after Lersten[238,239]) that appears finger-like (**Figures 45(b)** and **45(c)**). Developmental sequences of the various forms are, nevertheless, very similar. Cells of the protoderm divide to increase the surface area and form an outer layer. Concomitantly, underlying cells divide and elongate upward, pushing the overall form to extend from the tissue surface[240] (**Figures 46(a)–46(d)**). With maturation, the protodermal cells also elongate and differentiate into epidermal secretory cells with an outer cuticular layer. Secretion occurs with breakdown of the cuticle[15,240] (**Figures 45(b)** and **46(e)–46(g)**). Colleters are ephemeral structures, drying and sloughing off once the bud breaks and a given young leaf has expanded.[47]

Trichomes (Greek for 'a growth of hair') are specialized epidermal structures.[47] Although there are many anatomical variations in nature, overall they have a 'hair-like' (as their name implies) usually because of a stalk-like base (a difference between trichomes and colleters). Many of the trichomes found on tree leaves are of a

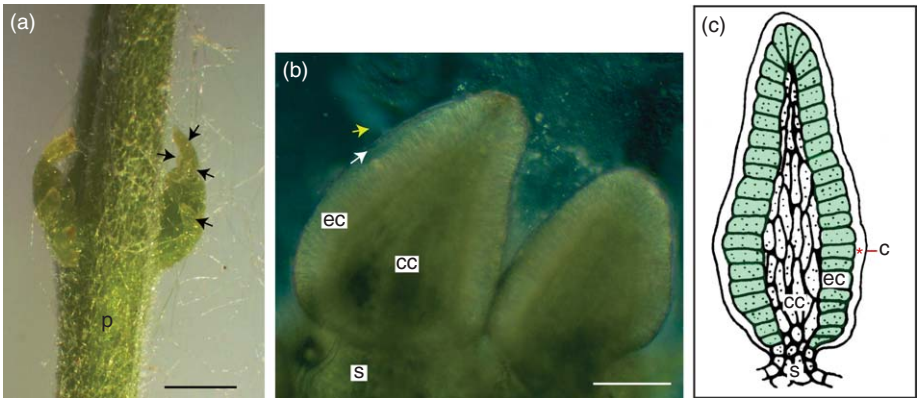


Figure 45 An example of glandular leaf stipules in *Salix* sp.; numerous individual glands comprise the stipule (arrows); structural hairs along the petiole (p) also provide physical protection to young tissues (a). Excised resin glands from *Salix* sp. stipules showing the colleter form mounted as a whole organ (not sectioned). Resin is released where the cuticle (white arrow) is separated from the gland; this sample is mounted in water, which causes the resin to appear cloudy (yellow arrow). Light microscopy with differential interference contrast (b). The most common colleter morphology found in plant tissues is that of the 'standard' colleter, (c). The structure is supported by a short stalk (s) and central columnal cells (cc). The epidermal cells (ec) secrete resin which is retained by the cuticle (c) until it is broken and releases the resin. Scale = 10 mm (a); 100 μm (b). Images from A. M. Patten, Washington State University (a, b). Reproduced with permission of Oxford University Press from V. Thomas, *Ann. Bot.* **1991**, *68*, 287–305, copyright 1991 (c).

structural nature, forming a physical barrier to herbivores and environmental challenges (**Figure 47(c)**, red arrow), and are able to respond to various challenges by increasing their density.[241] On the other hand, there are some families among the trees that have glandular trichomes that secrete resins and other substances. In *Betula* species, peltate glandular trichomes (stalk with rounded top secretory cell) produce resins, with the greatest amounts measured for the surfaces of new growing shoots and leaves.[242] As the tree grows, bark replaces the protective function of some of the trichomes, with the remaining becoming spread out, effectively reducing the overall amount of resin on the leaf or shoot surface.[243]

Marginal leaf glands are resin-secreting structures located in the teeth of maturing leaves in some species such as in Salicaceae and Flacoutiaceae[244] (**Figures 47(a)–47(c)**). In *Populus*, the youngest leaves lack these glands, but can have resin-producing basal glands that function in the same way, albeit at the leaf base[245] (not shown). As the growing season progresses, the bulk of the resin production shifts from the basal glands to the margin leaf (teeth) glands. The latter are most active while the leaf is unrolling and the edges overhang the leaf surface, thereby releasing resin to cover the adaxial (upper) side.[245] Often other leaf marginal structures co-occur with resin glands in the leaf teeth releasing either water (i.e., hydathode structures) or nectar (i.e., nectary) that mixes with the resinous secretions of the leaf tooth gland. Once the leaf is unfurled, the leaf teeth dry and may brown.[245]

Leaf nectaries, as their name implies, secrete sugar-containing nectar to attract insects and other creatures. They may be 'floral' if located on flower tissues (including flowers of trees) or 'extrafloral' if found on non-flower parts, such as leaves.[15,246] Nectar presentation by floral structures is a well-known reward system for pollination, while nectar from leaves (or other nonfloral tissues) is considered to reward predators of herbivores.[247] One example is shown (**Figure 48**) where *Pseudomyrmex* ants feed on the nectar from bullhorn acacia (*Acacia cornigera*). In rare cases, the latter structures secrete resin, although as mentioned previously, nomenclature for the diverse epidermal secretory structures has often been somewhat confused. Overall, nectary structures vary greatly, but resin-producing nectaries have close similarities to other epidermal resin-secreting structures, such as in *Prockia crucis*, a relative of *Populus*. The extrafloral nectaries of this species provisionally produce both sugars (fructose, glucose, and sucrose) and terpenes, phenolics and alkaloids as indicated by histochemical analyses.[248]

3.27.3.3.9(ii) Oil-producing structures of leaves, fruits, and flowers Oil cavities or glands, also have structural similarities to resin ducts with secretory epithelial cells again lining the cavity, with these being common in *Eucalyptus* and citrus leaves, and in citrus rind[47,249] (**Figures 49(a)** and **49(b)**). Oil glands originate from epidermal cells that divide and differentiate to form a series of secretory epithelial cells that line an

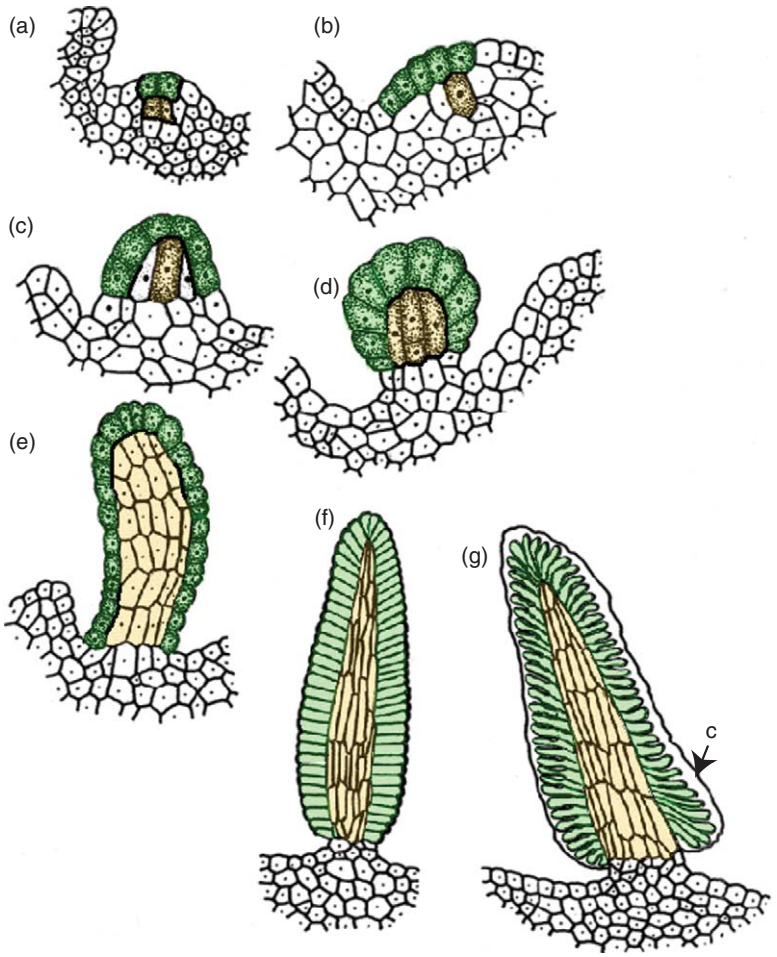


Figure 46 Development of a standard colleter. Protodermal cells (green) differentiate into nascent secretory epidermal cells, with cells below the protoderm differentiating into nascent columnar cells (yellow) (a–d). Further development (e) produces fully mature secretory epidermal cells arranged in a palisade form (green) with a cuticle (black border) and supported by mature columnar cells (yellow) (f). Resin secretion occurs with breakage of the cuticle (c), loosening the palisade structure of the secretory epidermal cells (g). Reproduced with permission of Oxford University Press from S. Mangalan; K. P. Kurien; P. John; G. M. Nair, *Ann. Bot.* **1990**, *66*, 123–132, copyright 1990.

intercellular cavity. The cavity then grows and increases oil content as the leaf matures.[249,250] In *Eucalyptus* leaves, the oil glands are large enough and visible to the naked eye and their species-specific localization in relation to veination is often taxonomically diagnostic.[249] Located on the petals of some tree genera (e.g., jasmine (*Jasminun* spp.)), osmophores emit fragrances to attract pollinators. These structures include a layer of secretory epidermal cells that store essential oils and release volatiles (often terpenes) through stomatal openings.[47,250] Behind the epidermal layer rests 2–5 subepidermal layers that presumably synthesize the oils to be volatized (see Evert,[47] and references therein). Flower petals contain very little essential oil at any given time (e.g., ~0.04–0.08% by weight), instead producing small amounts continuously (under optimal temperature conditions) that amount to several fold higher quantities of airborne molecules.[250]

3.27.3.3.9(iii) Mucilage-producing structures of buds and leaves Mucilage is also produced in the buds and leaves of many tree species.[229] For instance, mucilage cavities within the bud scales of *Tilia cordata* produce polyamines that facilitate freezing tolerance in buds.[251] Similarly, leaf mucilages are produced by idioblasts in *Araucaria* spp., to aid leaf/needle freezing tolerance.[252]

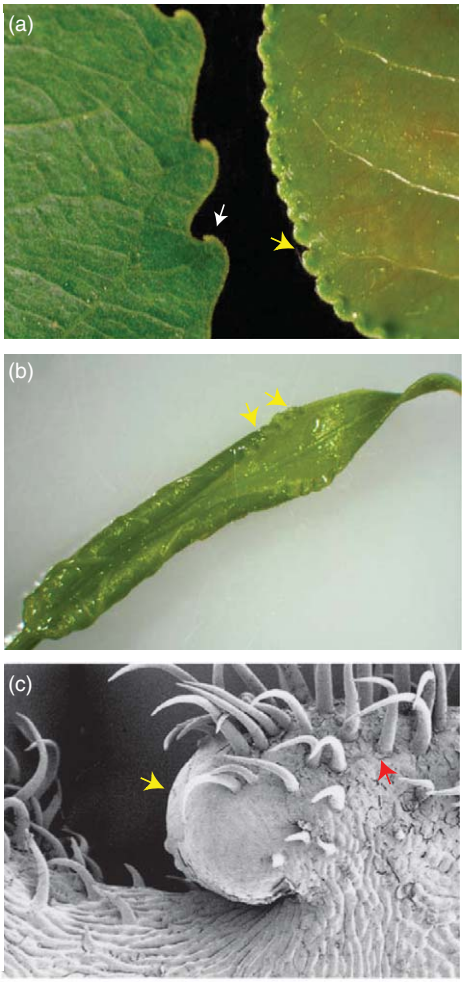


Figure 47 Leaf teeth, leaf tooth gland, and trichomes in *Populus* spp. Leaf teeth of mature leaves of *Populus trichocarpa* (white arrow) occur at regular intervals (a). In young leaves (a), especially as they unfurl (b), the teeth are closely spaced (yellow arrow) and their glands secrete copious amounts of resin as indicated by the shiny leaf surface (a, b). Scanning electron micrograph of resin-secreting leaf tooth gland (yellow arrow) on a young leaf of *Populus cilita* (c). Non-glandular trichomes (red arrow) provide additional protection to the young leaf by structurally and chemically deterring some herbivores. Images from A. M. Patten, Washington State University (a, b). Reproduced with permission of Linnean Society of London from H. P. Wilkinson, *Bot. J. Linn. Soc.* **2007**, *155*, 241–256, copyright 2007 (c).

As mentioned above, the giant trees, *Metasequoia*, *Sequoia*, and *Sequoiadendron* produce mucilage to offset water stress due to their extreme heights by absorbing water from the atmosphere.[232] The phenomena also occur in tall angiosperms, such as *Populus* or *Eucalyptus* spp., of which the apical leaves incorporate epidermal mucilage for water absorption. Additionally, stomatal mucilage plugs aid water conservation, especially under drought conditions[231] (**Figures 44(c)** and **44(d)**).

3.27.4 Nature's Phytochemical Bounty and Tree Biochemical Diversity

The previous sections described, in somewhat general terms, our current – yet very incomplete – understanding of the biological systems leading to the remarkable tissues, cells, and phytochemical constituents that characterize our highly diverse tree species, whether in wood, bark, photosynthetic canopy, reproductive organs, and so forth. These sections though gave only a brief glimpse into the fantastic chemical

Figure 48 Extrafloral nectaries (arrow) in bullhorn acacia (*Acacia cornigera*) attract *Pseudomyrmex* ants which feed on its nectar. This is a well-known mutualism in which the ants vigorously defend the tree from herbivores and competing plants. Image with courtesy of D. L. Perlman/EcoLibrary.org.

diversity which exists, with little in the way of gaining a sense of the true significance at the material (nanochemical) and phytochemical levels. It is, however, those aspects that we now focus upon in the subsequent sections which illustrate the truly novel biochemistries and compartmentation strategies that have emerged over time. These, in turn, reflect the enormous and fascinating differences encountered in the distinct 100 000 or so tree species, including intra-species variability. Such differences in tree species encompass: growth and development, life spans, ecological niches, distinct nanochemistries, tissue material properties and architectures, and the all-important phytochemical diversity, for example, in HW, resins, and so on. These differences also ultimately led to distinct uses of specific tree species for a host of purposes worldwide, and which often helped dictate the course of history and commerce through the ages.

However, it may come as somewhat of a surprise to some readers of just how little true understanding we have of many of these biochemical processes. Indeed, if the only product of this contribution is to bring an appreciation to the urgency of the research needed, both now and in the future, then a useful contribution will have been made.

To attempt to do justice to the incredible biochemical diversity encapsulated in trees, the sections that follow focus upon our current understanding of this biochemical bounty, whether as sources of structural materials in solid wood, or wood composite forms, or as (bioactive) phytochemicals. Indeed, nature has provided us with fantastic opportunities as regards the enormous range of tree genetic blueprints. To emphasize their importance, however, only selected examples can be given due to space constraints. Many of the examples chosen focus upon the tree species which we have so heavily depended historically for our continued existence, whereas other more recent examples are drawn from advances in nanomaterials, medicinal uses, and so on. Indeed, we encourage the interested reader to consider our current state of knowledge and to help gain further insight into the wonderful, if not compelling, opportunities that beckon science and technology, both now and in the near future. To do this, though, requires consideration of our current understanding of how plant cell wall formation occurs, including the factors affecting wood properties and how they are measured/evaluated. This is essential in order to consider, for example, how tree biotechnology of our wood resources might ultimately achieve its stated objectives, namely for improved pulp and paper production, and a source of

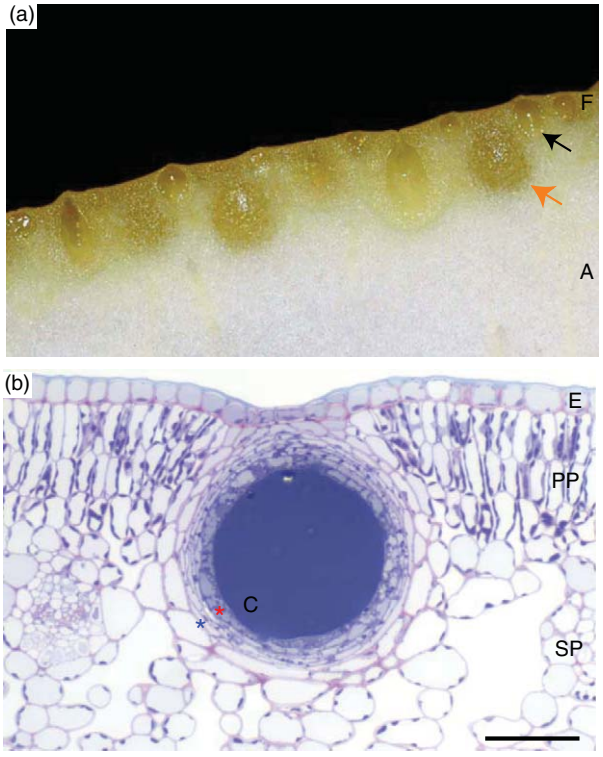


Figure 49 Oil glands in citrus rind and leaves. Oil glands of various sizes occur in the flavedo (F) of the fruit rind just below the epidermis in all citrus fruits (grapefruit, *Citrus* × *paradisi*, shown here) (a). Oil glands sectioned through the center reveal the oil-containing cavity (black arrow), while those sectioned at their outer boundaries appear opaque (orange arrow) (a). The citrus leaf oil gland (*Citrus limon* shown) consists of a central cavity (C) that is lined by oil-secreting epithelial cells (red star), these being surrounded by protective sheath cells (blue star). Anatomy of the fruit and leaf oil glands are very similar. Abbreviations: A, albedo; C, oil gland cavity; E, epidermis; F, flavedo; PP, palisade parenchyma; SP, spongy parenchyma. Scale = 50 μm (b). Images from A. M. Patten (a) and G. W. Turner (b), Washington State University.

new bioenergy/biofuels and bioproducts. Never has there been such an exciting potential to link the scientific enterprise with the needs of humanity as now.

3.27.4.1 The Emergence of Wood as a Structural Nanomaterial: Biomechanical/Biophysical Properties of Various Woods and Wood-Derived Products

Over the millennia, humanity has preferentially selected specific tree species for a multitude of structural applications such as for dwellings/housing (exterior and interior), furnishings, utensils, tool-making (instruments and weapons), musical instruments, water transport, art forms, and so forth (**Figures 1** and **2**). Other than for highly specialized uses, selection of a particular wood (tree) species generally reflects both needed material properties and regional availability. The specific properties that influence species choice include: grain appearance, color, structural performance, and durability as measured by enhanced resistance to a variety of environmental (water, light, and temperature) and biological (including fungi, insect, and bacteria) challenges. Such differences, in turn, result from the distinct species-specific chemistries and material properties of different woods.

It should be noted that historical preferences for structural uses of wood (from the Stone Age onwards) have been difficult to document, since the vast majority of wood materials either have degraded or were disposed of over time. Nevertheless, a few examples of historical usage provide some insight into how our critical reliance upon nature's diverse woods/nanomaterials evolved.

3.27.4.1.1 Ancient usage of wood in building construction

As early as the Neolithic period (10 000 BC), humans used wooden branches, along with other plant materials, to construct nest-like protective dwellings.[253] Later, with the advent of sophisticated building technologies, woods were identified strong enough to bear structural weight and durable enough to last (at least for the lifetime of the builder). As a prime example, the ancient Phoenicians (~3000 BC or so) recognized the strength, beauty, and durability of the Cedar of Lebanon (*Cedrus libani*), also known as atlas cedar, and used it in great amounts to build temples, palaces, and sailing fleets. They also exported large quantities of this timber (by water) to Egypt and Assyria, although the tree resource was ultimately decimated in the process due to overuse. Nevertheless, *C. libani* became so prized that it was used to construct Solomon's Temple in Jerusalem (~950 BC), which was burned and reconstructed twice with *C. libani* heartwood until the temple's final destruction.[254]

Fortunately, some truly remarkable early achievements in timber construction remain today, including what is believed to be the oldest timber building, the Horyu-ji temple in Ikaruga (Nara Prefecture), Japan (5-stories, ~1300 years old, **Figure 50**) and the tallest wooden structure, the Sakyamuni Pagoda in Shanxi Province, China (67.31 m, AD ~1056).[253] The strength and longevity of such stately structures is again largely due to the inherent strength and decay resistance of the HW that they were built from (Japanese cypress and other gymnosperm species).[253] Such striking technological developments at that time thus served as the early forerunners for the manifold uses of wood and wood products that are in architecture/construction today.

Figure 50 The Horyu-ji temple in Ikaruga (Nara Prefecture), Japan. On the right is the Gojunoto (five-story pagoda) and on the left, Kondo (main hall). Most of the temple buildings are built with Hinoki cypress (*Chamaecyparis obtusa*). Through dendrochronological analyses it was estimated that the trees used for the construction of the pagoda and the main hall were felled in 594 and between 667 and 669, respectively. Photograph from Shogakukan Inc., Tokyo, Japan.

3.27.4.1.2 Trade and naval power usage of wood

From the Phoenicians to the nineteenth century shipping industry, wooden vessels were also of central strategic importance in maintaining naval and merchant fleets, and even today many local fishermen and sailors around the world use wooden boats to fish, trade goods, and so forth.

Additionally, during the seventeenth and the eighteenth centuries, British settlers in North America discovered the versatility and value of eastern white pine (*Pinus strobus*) to the extent that it became known as 'the tree that built America.'[255] Indeed, white pine wood became the choice timber for British naval ship masts and planks until the late eighteenth century.[255,256] This was because it was strong and light, with a straight grain and trunk, and clear of branches for as much as 100 ft. Similarly, on the west coast of North America, the native people harvested western red cedar (*T. plicata*) for many uses, including for dugout canoes. Lewis and

Clark were aided by the Nez Percé Indians in finding, felling, and carving cedar canoes at Orofino, Idaho, for their travel from the Clearwater river into the Columbia river before reaching the Pacific Ocean.[257]

Many parts of trees have also long been employed for different hull applications. As one example, birch species (*Betula*) produces bark (**Figure 23(f)**) that is water-resistant, strong, and can be cut from the trunk in long continuous sheets. As a result, it was widely used by North American Indians to sheath canoe frames and protect housing exteriors. Scandinavians also employ birch bark for protecting purposes, such as for roofing.[257,258] Again, these species selections simply reflect the distinctive chemistries and material properties that various wood species have, and which fulfill a particular purpose.

3.27.4.1.3 Musical instruments and sounds from wood

Wood has historically also been an important material for musical instruments. This is due to the orthotropic nature of wood which results from the distinct mechanical properties of the radial, tangential, and longitudinal planes of its anatomy/structure.[259] In this regard, the most desirable woods for musical instruments are straight-grained, wherein the early and late woods have similar densities and smooth transitions,[121] with these being selected based on acoustic properties and ease of handling. As a few examples, some dense tropical wood species are highly sought after for wind instruments (e.g., rosewood (*Dalbergia* spp.) and ebony (*Diospyros* spp.)), since they can be precisely worked and retain overall shape when exposed to humidity. Additionally, rosewood and padauk (*Pterocarpus* spp.) have good side hardness, sound and low damping, and are sought after for xylophones.[259] For pianos, temperate woods with high durability (e.g., hornbeam, maple, beech, and birch) are used for the piano actions, while birch wood is specifically used for hammershanks because its density and moduli produce high sound quality.[259] Indeed, during the early nineteenth century, the German piano maker, J. C. Schleip was reported to have used up to 10 different wood species in a single piano.[121] By contrast, low-density softwoods with good resonance (e.g., spruce, pine, and fir) are used for string instrument soundboards, wherein the wood grain is set parallel to the strings.[121,257,259] The additional quality of sound radiation also makes maple a preferred wood for the sides and back of violins.[259] Sound quality is complex, however, and since the sublime tones of the Cremonese violins (i.e., of Stradivari, Del Gesu, etc.) have yet to be reproduced, their origin remains controversial. Recently, the Cremonese sound has been in part attributed to the unique wood density properties of the spruce tops and maple backs crafted by the seventeenth century masters.[260] Others have postulated that the tori (i.e., tracheid pit membranes that control water flow resistance) of softwood tracheid-bordered pits are ruptured and mineralized with sea salt, thus altering air and/or wood vibrations and thus the overall sound. However, this has been disputed following subsequent examinations.[121] In any case, it has been widely reported for many musical instruments that their frequent use over time increases the wood elasticity, thus improving the sound.[261] Nevertheless, the selection of these woods illustrates the often unique material properties of specific wood species. This, in turn, translates to generation of the sounds that they can produce which result from their cell wall chemical compositions and anatomies.

3.27.4.1.4 Furnishings from wood

Some of the earliest known surviving furnishings made from wood date back to ancient Egypt as those found in King Tutankhamen's tomb, which were placed there nearly 4000 years ago (**Figure 51**). That particular discovery, and those from written records and drawings (papyrus writings, wall paintings, etc.), further emphasized how prized certain wood species, such as ebony, were even millennia ago. Indeed, from then until the present, the artistic expression of furnishings and uses of wood has constantly evolved, as evidenced by written records describing Roman and Greek furnishings, and furniture, and so forth from other periods (such as Tudor, Elizabethan, Georgian, Louis XIV, and Louis XVI; see **Figures 2(o)** and **2(p)**). More recent furnishings have since then embraced Victorian and colonial styles, and those of more modern design. Once again, the different wood species being utilized were chosen based upon both their cell wall structural properties and their aesthetic appearance.

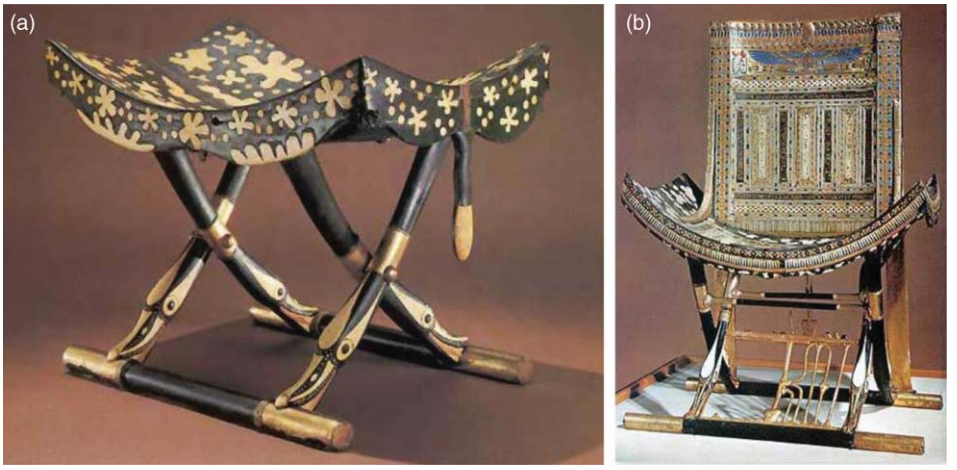


Figure 51 Furniture from King Tutankhamen's tomb. (a) A stool imitating leopard skin, with the seat and the legs made of ebony. (b) Inlaid chair, with the border of the seat made of ebony. Images from Tut Exhibit at http://www.egyptianexhibits.com.

3.27.4.2 Factors Influencing Wood Selection and Usage: Important Physical and Mechanical Properties

Initially, the true physical and (bio)chemical factors influencing choice of various wood species for buildings, shipping, musical instruments, furnishing, and so on were unknown to the artisans at that time. However, scientific enquiry has recently gained much appreciation of the various factors that affect wood performance for specific applications. These studies have ultimately led to our present understanding of specific wood–property relations and how they are affected by, for example, different mechanical properties of the structural cell wall biopolymers. For all wood forms, however, the main factors to be considered are those of durability (factors affecting resistance to opportunistic pathogens, etc.), dimensional stability (factors affected by temperature ranges and moisture levels), and cell anatomy influencing both mechanical and physical performance.

It is important to note that humanity has relentlessly utilized a relatively small number of highly valued wood species (e.g., ebony, mahogany (*Swietenia* spp.), oak (*Quercus* spp.), walnut (*Juglans* spp.), pine (*Pinus* spp.), spruce (*Picea* spp.), etc.), many of which continue to be employed to the present day. However, their utilization has greatly reduced old growth specimens[262] and has driven some tropical species to near extinction.[263] Other negative impacts on wood availability have also been through forest conversion into farmland, and (in tropical areas) through unsustainable usage of fuel wood. As a result, with diminishing old growth resources and an increasing human population, smaller/younger trees of 'poorer' wood quality have been increasingly used. To meet the growing demands of humanity this has necessitated development of innovative wood products, such as the so-called lower quality 'core wood' being placed under wood veneers, or in generation of various glued composite materials, including laminated veneer lumber (LVL), glulam, oriented strand board (OSB), medium density fiberboard (MDF), particle board, plywood, and so forth.[264]

For wood proper, there are three rather disparate features common to all woody plants that generally dictate structural properties: The first are the physicochemical interactions between the structural polymeric components (celluloses, hemicelluloses, and lignins) at the nanoscale level within the cell walls, and the second are those engendered by species-specific anatomical variations in their tissues, with the latter leading to distinct cell wall-type properties (e.g., fibers, vessels, etc.). A third factor affecting wood properties, such as relative durability/resistance to biodegradation, is largely a consequence of the species-specific phytochemical 'extractives' present in HW. The latter can function by either providing physical barriers to water penetration (i.e., encrusted bordered pits in Douglas-fir and tyloses in white oaks) or having bioactivity properties, such as natural biocides, repellents, and so on (**Figure 52**).

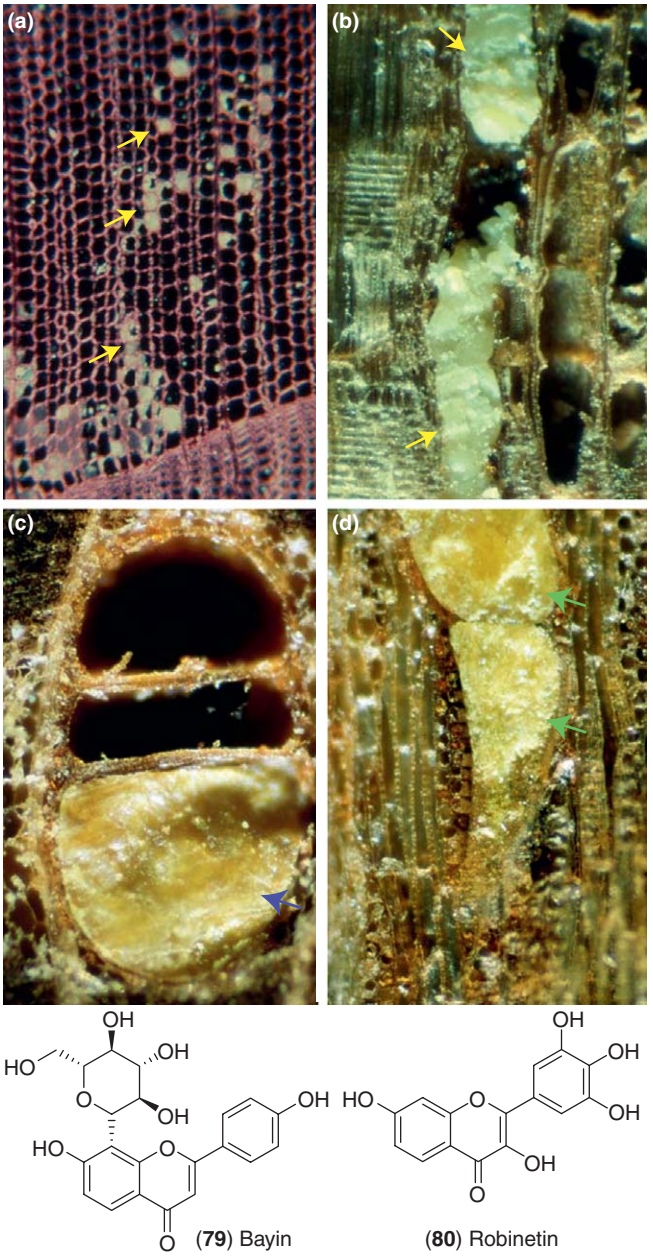


Figure 52 Species-specific extractives deposition. (−)-Hydroxymatairesinol (**18**) and (−)-α-conidendrin (**17**) deposits (arrows) occluding some of the tracheids in western hemlock (*Tsuga heterophylla*) (a). Bayin (**79**) deposition (arrows) within two vessels of *Castanospermum australe* (Moreton Bay chestnut or blackbean) (b). Robinetin (**80**) deposit (arrow) within a vessel of *Intsia bijuga* (aka ipil, merbau, kwila), cross-section (c) and radial section (d). Images from the late W. E. Hillis, CSIRO, Australia.

As far as the first two features are concerned, our understanding of the complex interactions that form structure–property relationships in woody biomaterials[265] has progressed rapidly in recent years due to developments in the science of nanomaterials. In this respect, wood structural properties are largely determined by their behavior to external factors, which can be of a physical, mechanical, or even biological (biodegradation) nature. Such properties of woods vary enormously not only with tree species, but also with tissue origin (e.g., juvenile wood, HW, SW, etc.).

3.27.4.2.1 *Wood properties at the molecular (biopolymer) level* in vivo *and* in vitro

In the 1970s, the work of Cousins[266,267] and Cave[268] began to spur interest into approaching the study of wood as a polymeric composite material. This work was pivotal in beginning to consider wood in this way, and still stands as the only reliable mechanical testing data for evaluating properties of individual amorphous wood polymers.[269] However, these data were obtained using isolated cell-wall polymers (e.g., cellulose, hemicellulose, and/or lignin-derived) rather than with the *in situ* wood polymer composites within the various cell wall types. Despite such shortcomings, the insights gained and trends noted by Cousins and Cave are now used to apply composite mechanics theory to that of mechanical property dependence of wood on the cellulose microfibril angle (MFA). A major finding of this work is that the hemicelluloses, and not celluloses or lignins, are the major components which can decrease mechanical properties with increasing moisture content. It is currently unclear whether the isolated polymers *in vitro* display the same behavior as their native constituents *in vivo*, particularly since the processes needed for disassociation/separation from other wood composite biopolymers/phytochemicals must alter their structures and/or compositions to some extent, as well as their anatomical relationships. Nevertheless, it is envisaged that properties of *in situ* polymers are significantly influenced through their inherent interactions with each other as well as with hydration, the latter being a major natural and vital constituent of native plant material.

One approach to study *in situ* behavior of wood polymers in their native state is to use thermal analytical techniques to discern 'phase changes,' as all materials are capable of changing phase such as solid, liquid, or gaseous. For polymers, changes in mechanical and thermodynamic properties of the material can be linked to their polymeric structures through physical chemistry-based mathematical relationships.[270] Therefore, various techniques now present the opportunity to relate physical and chemical properties together, although this is still complicated by difficulties arising from uncertainties surrounding the morphology of the wood polymers *in situ*.

For amorphous polymers, such as lignins and hemicelluloses, the most important phase change is that from a glassy to rubbery phase (i.e., glass transition), whereas some crystalline polymers can undergo melting as crystals disassociate. For wood polymers in their native state, it is believed that the hemicelluloses and lignins take on amorphous forms in the cell walls, despite the fact that some hemicelluloses can crystallize *in vitro*.[271] By contrast, the crystalline cellulose polymers never display a melt temperature when unmodified, but rather undergo thermal degradation prior to melting.[272] From an analytical perspective, the behavior displayed for melt behavior is more easily discerned and analyzed but, unfortunately, this is not yet applicable for analyzing wood species differences. Instead, only the rather discrete glass transitions that are influenced by factors such as hydration can currently be gauged. It is to be hoped, however, that new techniques and equipment advances may soon improve the usefulness of thermal analysis in order to evaluate cell wall biopolymer properties in the native state.

Attempts to apply thermal analysis techniques to whole wood with the express aim at discerning *in situ* polymer properties were previously conducted by Salmén.[273] This approach used dynamic mechanical analysis (DMA) to discern glass transitions within whole wood and these were assigned to the amorphous lignin component. Later, both differential scanning calorimetry (DSC) and DMA were employed to understand how this glass transition behavior changed as a function of hydration below the fiber saturation point.[274] Critical to these techniques was verification of the viscoelastic phase change using superposition techniques and subsequent computation of activation energies for the event. Kelley *et al.*[274] then further confirmed the amorphous nature of *in situ* lignin, while also corroborating the importance of hemicelluloses with dependence of mechanical properties on hydration. The validity of such approaches was also later established by computing similar activation energy values using an analogous technique from the static mechanical testing of wood in the stress relaxation mode at varying temperature and hydration levels.[275] In other words, the thermodynamic behavior of *in situ* lignin was independently corroborated for a third time using an extension of the original theories to totally different loading scenarios. In aggregate, these studies demonstrated (1) the value of studying wood polymer behavior *in situ* using mechanical techniques and (2) the integral role that moisture plays in the mechanical behavior of wood polymers (see below).

Collectively, these findings have led to a new era in applying polymer and composite techniques to study wood cell walls and their constituent polymers. The fact that natural polymers – lying within complex composite structures (cell walls) which are not well defined – obey general polymer science principles, lends further confidence to the potential of addressing natural materials using these techniques. Indeed, the application of composite and polymer theories has now yielded a better understanding of how the natural polymer morphology and its arrangement in the cell wall can lead to the physical properties displayed by wood species. This is particularly the case for the contribution of MFA in defining directional properties (see below), and for the role of the amorphous wood polymers (i.e., hemicelluloses and lignins) in control of viscoelastic properties over polymer phase changes. Thus, tools are now available for discerning material behaviors *in situ*, these being approaches that can be anticipated to have important contributions to endeavors aimed at manipulating wood properties through genetic manipulations.

3.27.4.2.2 Effects of hydration and temperature on wood chemistries/properties

3.27.4.2.2(i) Influence of hydration and hydrogels The dual roles of hydration (moisture) and temperature in the physico-chemical behavior of all woods cannot be overemphasized as wood properties are significantly influenced by both environmental factors. Wood, like most biological materials, can equilibrate to its environment by taking up more moisture when it is present and this can exist in two forms: (1) *bound water*, physically associated with cell wall chemical constituents through non-covalent interactions and (2) *free water*, present in liquid form, primarily in the cell lumen. As a rule of thumb, above ~30% moisture content (the fiber saturation point) in woody tissues, *free water* begins to accumulate.

In the living tree, the moisture contents of wood are essentially always above the fiber saturation point, even in HW. Despite this fact, the moisture content in trees can vary widely depending on season, species, and site. In living trees, the wood moisture contents (defined as the mass fraction of the total moisture divided by the combined wood and moisture) can be as much as twice the weight of the (dry) wood itself.[276] While this 'free water' has no significant effect on either dimensional stability or mechanical performance, it gives an appreciation of the massive amounts of water involved in supporting the photosynthetic apparatus.

For industrial applications of wood, lower moisture contents are needed, especially those below the fiber saturation point, with these being achieved by either natural or artificial drying. Natural drying methods include air drying where the wood slowly comes into equilibrium with the temperature and relative humidity of the surrounding environment, whereas artificial drying methods utilize kilns with control over both humidity and temperature. It is the bound water, however, that primarily influences wood behavior because of its interaction with the property-defining structural biopolymers (i.e., mainly the celluloses, hemicelluloses, and lignins). Accordingly, any increases in the *bound water moisture content* after drying leads to swelling and decreased mechanical performance. Indeed, it is for this reason that many uses of wood requires some form of protective treatment and/or controlled humidity environment so that these properties can be maintained/stabilized. Specific treatments for stabilizing wood dimensional performances include acetylation of the accessible hydroxylation groups[277] or thermal treatment. While these treatments can impart changes to many wood properties, retarding moisture absorption is the most pronounced and typically the aim.

The inherent interactions of wood polymers with each other and with water have been further studied in the last decade. These, in turn, have resulted in a much better understanding of woody systems particularly with respect to the role of hydration in mechanical behavior. Moisture relationships are of particular interest in wood products because in the living system, wood is inevitably saturated with water when performing the mechanical task of supporting the tree crown. However, in commercial use, wood is typically dried because green wood will eventually dry to reach equilibrium with the prevailing temperature and relative humidity.

In this context, Burgert[278] deduced the relationship of hydration level with that of the hemicellulose components. Here, cyclical compression is shown (**Figure 53**) with a proposed fiber-matrix model to produce the unique mechanical behavior observed that is largely dependent on the water–hemicellulose interaction. With initial compression, a relatively steep slope in the stress–strain curve results indicative of a material of significant integrity but, after passing the yield point and stress release, a permanent strain results. Upon subsequent reloading, the stress–stain slope recovers to its original slope regardless of the

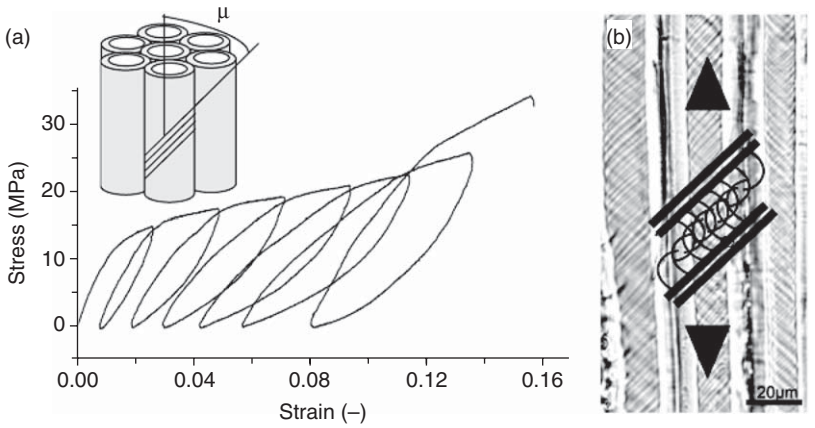


Figure 53 Proposed model for the interaction of cellulose fibers with the hemicellulose and lignin matrix to produce the unique 'slip-stick' mechanical behavior of wood in cyclical compression. Reproduced with permission of the Botanical Society of America from I. Burgert, *Am. J.Bot.* **2006**, *93*, 1391–1401, copyright 2006.

yielding effect. Normally, in such a fatigue experiment, the slope would decay drastically from the initial damage imposed by plastic deformation. However, in wet wood specimens, the labile hydrogen bonding of the wood polymers in the matrix allows for chain slippage with subsequent reptation. Such behavior is thus indicative of a hydrogel, a material with a continuous hydration phase.[279] It is thus becoming increasingly understood that nanoscale structures are being formed in the wood polymers from the interactions of celluloses, hemicelluloses, and lignins.[269] Indeed, such structures have been detected using atomic force microscopy (AFM) (**Figure 54**) and these are believed to accommodate the water absorbed below the fiber saturation point.[280]

At the molecular level, the celluloses, hemicelluloses, and lignins have different levels of hydroxyl group functionalities and these differentially interact with water through hydrogen bonding and other noncovalent interactions[266,267] In addition, the amounts of low-molecular weight extractives can have a large influence on the amount of absorbed moisture as they can be either hydrophilic or hydrophobic.[281]

The hydroxyl group in water also serves to produce interactions among the wood biopolymers which enhance mechanical performance. However, moisture levels below the fiber saturation point can

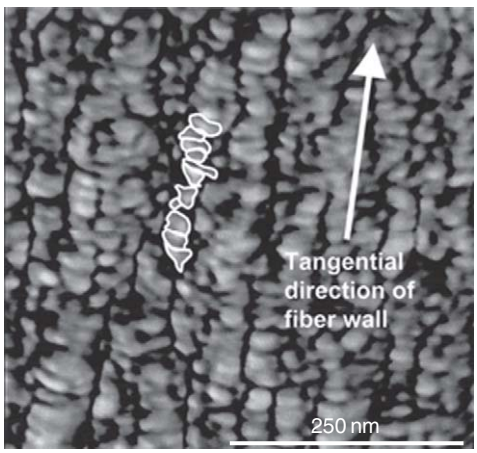


Figure 54 An AFM micrograph depicting nanoscaled structures of cellulose aggregates (light regions) connected by amorphous hemicellulose and lignin matrix (dark regions) in a spruce (*Picea* spp.) cell wall. Reproduced with permission of Springer from J. Fahlén; L. Salmén, *J. Mater. Sci.* **2003**, *38*, 119–126, copyright 2003.

also interrupt intermolecular hydrogen bonding and decrease overall mechanical performance.[282] The interpretation of such interactions was originally solely attributed to the extent of hydrogen bonding (**Figure 27(b)**); however, a better understanding of nanomaterials[264] is providing a more comprehensive and definitive understanding of the property–structure relationships involved. In this regard, current views of wood–water interactions include other effects, such as those influencing the relative degree of crystallinity and paracrystallinity regions in the cellulose, and the nature of the interactions with the various hemicelluloses.[269] Indeed, some imposed thermal histories of wood in service (i.e., under drying, storage, or use conditions) can promote reordering of paracrystalline to crystalline regions. This increased long-range order in crystalline cellulose thus prevents water molecules from entering into the tight microfibril structure, thereby decreasing the ability for moisture absorption and swelling.

An additional mechanism for altering moisture absorption of processed wood can also be achieved by modifying the content of hemicelluloses. Here, a group of wood products known collectively as 'thermally modified wood' are produced through controlled exposure of wood to elevated temperatures. Several mechanisms contribute to this process but the dominant reaction is that of acid hydrolysis of carbohydrates, mainly hemicelluloses, to produce formaldehyde, furfural, and other aldehydes.[283] This chemical degradation process thus results in a profound and lasting decrease for wood products to absorb moisture, which then also reduces the ability to undergo degradation by biological pathogens such as fungi.

3.27.4.2.2(ii) Temperature and wood biopolymer behavior Temperature changes also influence properties of wood biopolymeric materials, and these are generally of both short-term reversible and long-term irreversible effects.[276] Reversible effects occur when increased temperatures soften wood material, whereas when temperatures are lowered the wood increases in stiffness. Indeed, such effects are readily observable in wood still containing moisture heated to temperatures above water's boiling point, and this behavior explains the phenomenon historically exploited in steam bending techniques used to produce curved forms for furniture and sporting goods (i.e. snowshoes, tennis rackets, skis, etc.).

More specifically, under certain conditions, the amorphous wood polymers (i.e., hemicelluloses and lignins) can go through a *phase change* from a glassy (hard and brittle) to a rubbery soft and ductile phase.[273,274] The temperature of this phase change, known as the glass transition temperature (T_g), is dependent on both the polymer type and the amount of absorbed moisture. For moisture saturated wood, the T_g of the hemicelluloses is subambient, whereas the T_g of the lignin is ~60 °C. By contrast, T_g values of both polymers in the dry state are near 200 °C, a temperature approaching that of thermal degradation. This phenomenon of reversible softening of wood polymers can thus be explained as due to an increase in volume through accelerated Brownian motions within the cell wall polymer chains, thereby increasing relative inter- and intramolecular distances. The increased rate of molecular motion also explains the increased rate of deformation that occurs under load at higher temperatures and which can be linked directly to polymeric chain slippage and reptation.[270]

Irreversible thermal effects in wood, by contrast, typically result in both structural and chemical changes via thermal decomposition.[276] The most extreme case, with an organic material such as wood, is that of combustion itself. However, at temperatures lower than that of combustion, or in an anoxic environment, other structural changes can still occur albeit at a more controlled rate. Such conditions are relevant for wood in both processing and end use. For instance, wood can be exposed to temperatures as high as 250 °C for short periods of time during hot pressing of some wood composites. However, wood in attic regions of houses can also experience degradative effects even when exposed to temperatures as low as 70–80 °C for years.[284]

Such thermal exposures can also engender permanent changes in material performance by altering the ability for moisture absorption and tissue mechanical properties.[276] For example, **Figure 55** depicts a thermogravimetric analysis (TGA) curve for four different wood species as shown by fractional mass loss (**Figure 55(a)**) and rate of fractional mass loss (**Figure 55 (b)**) as wood is heated in a controlled atmosphere and at a controlled rate.[285] Although the behavior of all woody species has certain similarities, the small differences that exist in the derivative curves in the range of 150–400 °C illustrate effects that originate from the 'extractives' component of the wood. The latter typically include low-molecular weight carbohydrates, lipids, aromatic compounds, and so on, with their influence being especially evident in **Figure 56**, where the

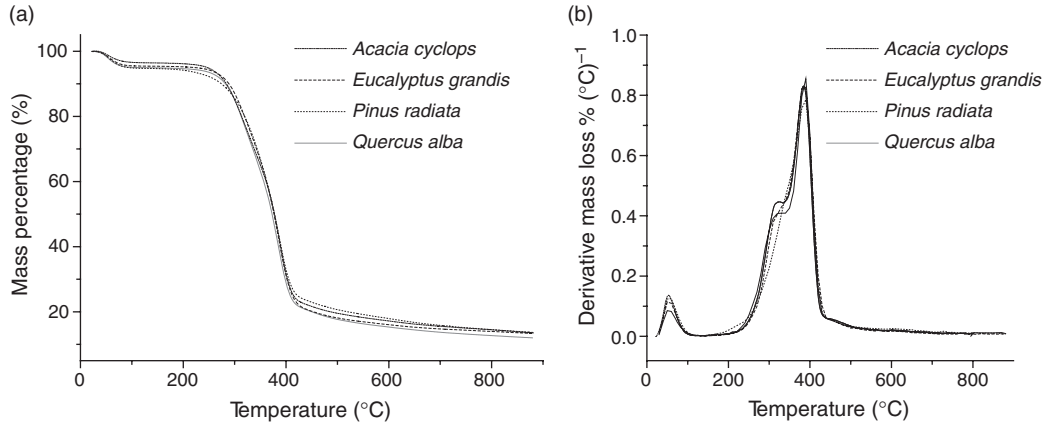


Figure 55 Thermogravimetric analysis (TGA) results of four wood species. Reproduced with permission of Elsevier from A. N. Shebani; A. J. van Reenen; M. Meincken, *Thermochim. Acta* **2008**, *471*, 43–50, copyright 2008.

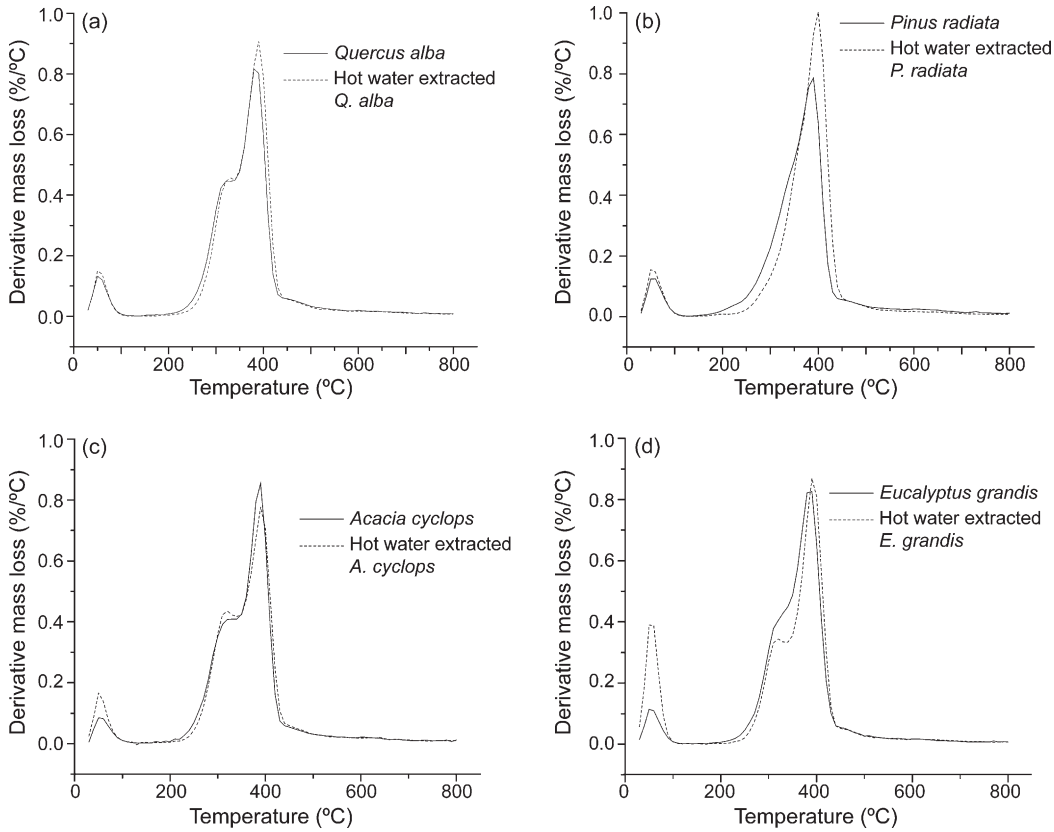


Figure 56 Derivative mass loss curves of four wood species before and after hot water extractions. Reproduced with permission of Elsevier from A. N. Shebani; A. J. van Reenen; M. Meincken, *Thermochim. Acta* **2008**, *471*, 43–50, copyright 2008.

influence of hot water extraction is demonstrated on the derivative mass loss curves of four wood species. The peaks in the derivative mass loss curves indicate the temperature where thermal degradation is the most rapid due to loss of specific material components. In contrast, the peak below 100 °C represents tightly bound moisture on the wood biopolymers. The subsequent changes in these curves from extraction thus reflect the

influence of various low-molecular weight components, and when removed, an increased thermal stability of different woods is evident. In such instances, both onset of thermal degradation around 200 °C and peak temperatures are delayed with extraction. Thus, the main contributors to lowering of thermal stability are considered to be the extractives and hemicelluloses. Such behavior can also be further demonstrated by thermal analysis of isolated wood polymers,[286] with one explanation for such differences being the type and amount of hemicellulose present in a particular wood species.[287] For instance, hemicelluloses degrade at a lower temperature than do lignins, although galactans display enhanced thermal stability when compared to, for example, xylan and glucomannans.

3.27.4.2.2(iii) Additional influences of wood anatomical features

3.27.4.2.2(iii)(a) Cellulose microfibril angle Of the anatomical features present in the cell wall, the cellulose microfibril orientation (MFA) in the S_2 layer of the cell wall (**Figure 27(e)**) is of great importance in determining the longitudinal mechanical properties of solid woods.[269] This can be both experimentally measured and theoretically linked (**Figure 57**) to stiffness changes that result both among distinct species and among different wood forms within the same species.[288] Indeed, this measurement has become important for assessment of wood quality for genetic selection purposes.[289] The theoretical basis of the importance of MFA is that of *anisotropic elasticity*, this being based on fundamental mechanics that evaluates load–deformation behavior of materials with properties that differ by direction. Being semi-crystalline, the cellulose molecules can also aggregate to form microfibrils (**Figure 27(c)**) which, in turn, possess the highest strength and modulus (**Figure 57**). In short, when they are aligned closely with the longitudinal direction of the S2 layer in the cell walls, this imparts high mechanical properties to the wood as a whole.

3.27.4.2.2(iii)(b) Pit structure As a further illustration of differences in wood properties, the effects of variability in pit structure is quite striking. This is because, from a physiological perspective, some species have pit structures particularly well suited for more facile fluid transport, such as in the genus *Pinus* where fenestriform (i.e., window-like) pit structures dominate (**Figure 58**). From a practical (application) perspective, however, this variable property is the reason why pines are preferred for manufacturing preservative-treated wood products, such as exterior deckboards and railings. Indeed, to protect further against possible fungal and insect attack for such applications, the wood is readily infiltrated with preservative treatments such as 'CCA' (the so-called 'chromated copper arsenic'), creosote, and other copper-based systems.[290] On the other hand, in some species such as Douglas-fir (*P. menziesii*), the pit structures are so resistant to fluid transport that normal wood preservation treatment methods are difficult. In such cases, a process called *incising* is used instead, where sharp metal spikes perforate the wood structure thereby enhancing the liquid transport into the wood.

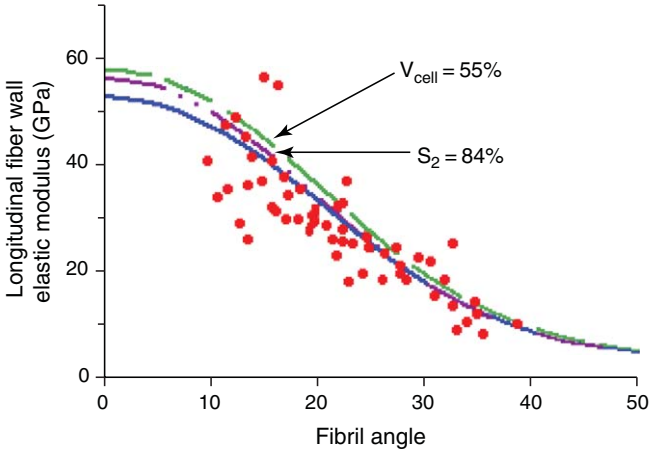


Figure 57 Cell wall modulus predictions fit to data measured by Cave.[268] Reproduced with permission of Elsevier from L. Salmén, *C. R. Biol.* **2004**, *327*, 873–880, copyright 2004.

Figure 58 Micrograph of wood radial face depicting classic fenestriform pits in *Pinus silvestris*. Image from W. Schoch, I. Heller, F. H. Schweingruber, F. Kienast (2004) Wood Anatomy of Central European Species (Online version: www.woodanatomy.ch).

The pit structures are also very important for other processes in wood products that require transport of liquids, such as for coatings, adhesives, and polymer matrix composites. Such processes require several means to facilitate proper interaction of the synthetic polymer (i.e., coating or adhesive) with the wood structure. A very important mechanism in wood is thus that of penetration of liquid polymers into porous wood structures during processing. Once solidified, this penetration provides physical anchorage of the polymer into the wood substrate, while also increasing surface area for chemical interactions. An example of this mechanism is shown in **Figure 59**, with high-density polyethylene (HDPE) penetration through pit structures. This behavior allows for successful production of composites of wood with recycled plastics that have little opportunity for

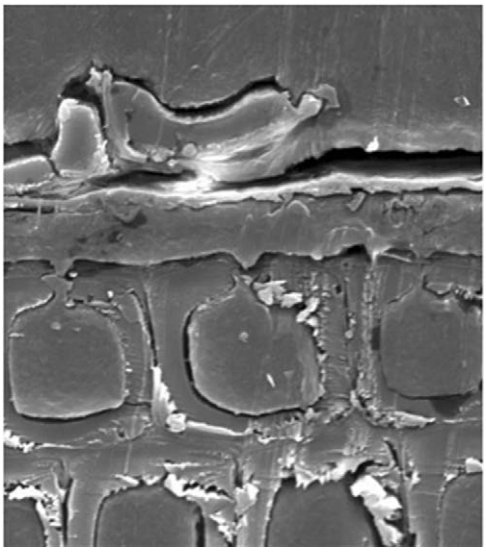


Figure 59 Scanning electron micrograph of polyethylene penetration into wood structure through pit structures. Image from M. P. Wolcott, Washington State University.

chemical interaction. Instead, the strong physical interaction afforded by the mingling of polymer with the wood structure anchors the plastic into the wood to transfer physical stresses.

3.27.4.2.3 Other factors affecting wood properties and performance: loading mode, directionality, and density

3.27.4.2.3(i) Loading mode Measurements of wood mechanical properties are crucial in assessing overall relative strengths (i.e., resistance to fracture) of different wood products, and in their propensity to deform under load. Such measurements also give good predictability into how well a particular wood (tree species) might perform its mechanical functions within the living plant, a topic of increasing significance as biotechnological manipulations of wood formation continue to be explored. The woody plant stem must, of course, also be able to meet mechanical requirements of support, while supporting its major purpose, the photosynthetic canopy that collects solar radiation for photosynthesis.

Mechanical properties are directly linked to both wood anatomy and types of wood cell wall polymers present. Thus, the measurement of such properties provides very useful information with regard to performance differences within and among different species, and as an indication of how wood materials respond to, and are affected by, various physical forces. The primary mechanical properties of interest are those of both strength and stiffness, which reflect load-bearing capacity at fracture and deformability under load, respectively. These, in turn, reflect the properties of the wood cell wall biopolymers, their morphological arrangements, and their general cell wall types. Loads are thus typically placed on wood materials in tension, compression, shear, or flexure modes, with the properties of each varying with loading mode. In pure tension and compression loading, the stiffness is gauged in terms of Young's modulus (E), whereas in flexure mode the property measured is the modulus of elasticity (MOE). To evaluate/compare strength properties, however, stress must be taken into consideration, this being the load per unit area of a material sample. While strength values simply represent the maximum stress supported by a material in either tension or compression, the modulus of rupture (MOR) represents the maximum stress value that a material can support under, for example, a bending load. As the stress varies throughout a wooden beam in the flexure mode, the MOR has maximum values at the outer surfaces.

Definitions/explanations of common mechanical testing terms for woody structural materials are summarized in **Table 1**, as defined by Burgert,[278] with the corresponding standards commonly used for measurement of wood properties described elsewhere.[276]

Table 1 Mechanical terms used with respect to uniaxial tensile tests[278]

Mechanical term	*Definition*
Stress	When a load is applied to a material, stress σ is a measure of the force per unit area. Taking a cube of material, tensile stress is defined as the force perpendicular to the surface divided by its area, $\sigma = F/A$. Shear stress is the force parallel to the surface per unit area, $\tau = F/A$.
Strain	Strain ε is the displacement of a material related to its original length.
Stress–strain diagram	In a stress–strain experiment, the stress σ is measured as a function of strain ε.
Elastic/viscoelastic/plastic	An elastic material returns immediately to its original shape when the load is removed after deformation; a plastic material keeps the deformed shape forever, and a viscoelastic material returns slowly to its original shape. Materials have often a combination of these properties: an elastoplastic material relaxes partially and retains only part of the deformation.
Stiffness	Stiffness is defined as the resistance of an elastic material to its displacement.
Modulus of elasticity	The modulus of elasticity E is a measure of the stiffness and is defined as the slope of the stress–strain curve in the linear-elastic region close to the origin (for small strains).
Strength	Tensile strength is the ultimate tensile stress that results in tensile failure.
Toughness	Toughness is defined as the resistance to fracture of a material or as the amount of energy that is absorbed before rupture. Toughness can be measured by calculating the area below the stress–strain curve.

3.27.4.2.3(ii) Directionality The mechanical behavior of wood products (including in trees) depends not only on the loading mode (i.e., tension, compression, shear, etc.), but also on the direction where the load is placed. This directional dependence of wood structural properties again reflects its *anisotropy* and can be readily observed by simply examining the ease of breaking a piece of wood across the grain. As indicated earlier, wood is an *orthotropic* material because there are three planes of symmetry that run normal to the three principal directions in the material, longitudinal, radial, and tangential (**Figure 60**), with the origin of these directions directly attributed to the orientation of various cell types within the wood (see **Figures 20(a)** and **20(b)**). Tracheids and fibers, being the primary load-bearing cells in wood, are longitudinally oriented and thus enhance strength and stiffness properties in that direction. The ray parenchyma also provide additional strengthening capacity in the radial direction (as compared to the tangential which is the weakest) despite their relatively weak anatomical structures. An approximate ratio for longitudinal (L), radial (R), and tangential (T) stiffness properties was described by Holmberg *et al.*:[291]

$$E_L : E_R : E_T \approx 20 : 1.6 : 1$$

As to be expected from the above, there are substantial differences in strength properties between the different loading modes. For example, in general, the stress–strain relationship in compression loses its proportionality at far lower loads than it does in tension (**Figure 61**).[291,292] This behavior is especially pronounced in the compression mode perpendicular to the grain, where thin cell walls readily succumb to the applied load by

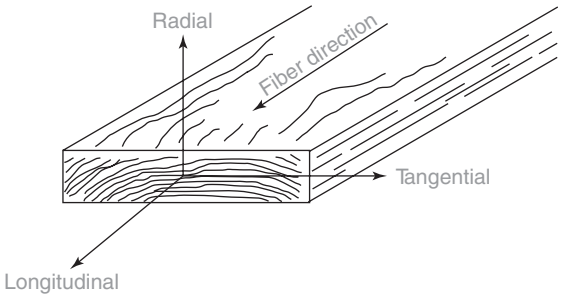


Figure 60 Primary orthogonal material directions for wood.

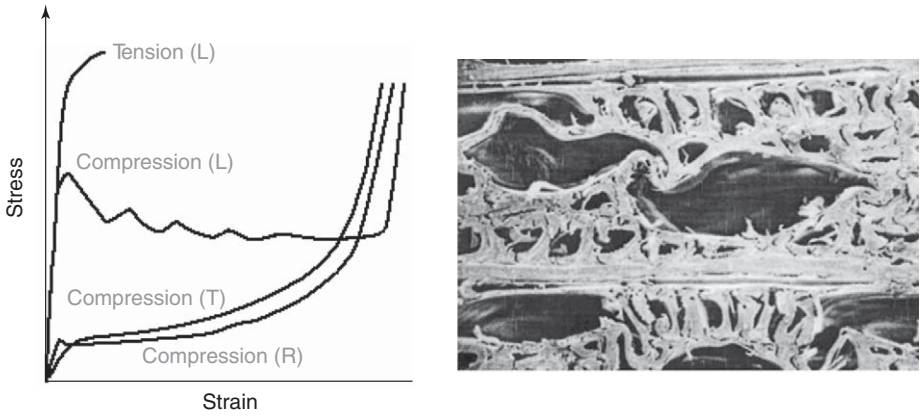


Figure 61 Representative stress–strain relation of tension and compression loading for different directions of wood (a). Micrograph depicting cell collapse resulting from compression loading (b). Reproduced with permission of Elsevier from S. Holmberg; K. Persson; H. Petersson, *Comput. Struct.* **1999**, *72*, 459–480, copyright 1999 (a). Reproduced with permission of the Society of Wood Science & Technology from M. P. Wolcott; E. L. Shutler, Jr. *Wood Fiber Sci.* **2003**, *35*, 540–551, copyright 2003 (b).

buckling.[293] Buckling behavior, in turn, leads to cell collapse which significantly limits load-bearing capacity of wood. Understanding this phenomenon is important because many times only a simple flexural test is used to evaluate the influence of different treatments on mechanical properties. However, in some use or processing scenarios, wood is loaded specifically in the compression mode which results in an unique behavior.

Thus, once again, the differences observed in the above properties emphasize why one woody plant species being preferentially utilized over another.

3.27.4.2.3(iii) Density One of the single most important physical properties influencing wood mechanical behavior and properties is that of its 'density.' This property has a large role in many materials as it represents the amount of mass per unit volume of material able to resist external forces. Simply speaking, higher wood densities result in larger amounts of material mass to resist any given force. However, the actual 'cell wall' densities of woody tissues across all species are apparently relatively invariant, as the wood cell wall itself generally has a density of $\sim 1.45\,g\,cm^{-3}$ regardless of species, age, or growing conditions.[294]

By contrast, the *bulk* density of wood can vary greatly among different species ranging from $0.16\,g\,cm^{-3}$ for balsa (*Ochroma pyramidale*) to $1.33\,g\,cm^{-3}$ for guaiacum wood (Lignum vitae, *Guaiacum officinale*).[295] Such differences are due to variations in anatomical features such as lumen diameter, parenchyma content, level of extractives present, and so on. For example, *G. officinale* has ~18–25% resin by weight, containing various lignans.[188] Consequently, an extremely hard and dense wood, such as guaiacum wood, has traditionally been used as bearings for lubrication in hydroelectric dams, because it can support high compression stresses and perform well even when submerged in water.[296]

The density of most commercial wood species in North America, for example, ranges from 0.35 to $0.75\,g\,cm^{-3}$, this including low-density species such as spruce (*Picea* spp.), fir (*Abies* spp.), and pines (*Pinus* spp.) to other species such as oak (*Quercus* spp.) and hickory (*Carya* spp.), which are toward the higher end.[276] For this reason, medium density oak and hickory perform particularly well in railroad ties, because the energy imparted by repetitive loading of the rail bearing on the tie can be dissipated by cell wall deformations.[297] Conversely, because mechanical properties are generally proportional to density, light-weight species often lack the ability to support enough load in a given application. However, some low-density commercial species such as Sitka spruce (*Picea sitchensis*) are prized for their high strength-to-weight ratio and have historically been used in weight-sensitive applications, such as in aircraft and sporting goods. In addition, balsa today is still used as a preferred core material for many carbon-fiber panel structures. Indeed, one example of this application is the Advanced Enclosed Mast/Sensor (AEM/S) program for the US Navy, which is used to house sensitive electronic components and is the largest composite structure on US Navy vessels.[298] For such applications, the balsa core provides light weight, with appropriately high compression strength to maintain separation of the carbon fiber composite skins. As yet another example, the excessive deformations that occur when cell walls are buckled under load are crucial to properly forming strand-based wood composites, such as the commonly used orientated strand board (OSB).[275] Indeed, this property enables the use of low-density species, for example, aspen (*Populus* spp.) for such applications, since this behavior is crucial to properly form an adhesive area between the different wood strands.

3.27.4.2.4 Predicting wood qualities/performance for genetic selection/manipulation through noninvasive techniques

Developments in modern analytical and microscopy techniques have recently contributed significantly to the understanding of structure–property relations in wood and other plant materials.[269] However, most of these techniques are impractical for rapid assessment necessary for screening, for example, in genetic selection. Therefore, techniques have been recently sought to provide a rapid assessment tool for predicting mechanical properties of wood as a function of microstructural measurements. Such tools could also be applied to either genetic selection programs or genetic manipulation research aimed at altering the chemical compositions or structures, and evaluating their influence on the mechanical and physiological functions of the plant.

One tool, termed SilviScan, has been developed by CSIRO laboratories with the aim of exploiting the strong control over density and MFA on mechanical performance.[289] This tool uses a variety of X-ray diffraction and

densitometry techniques to measure microstructural parameters for a wood sample.[299] These parameters can then be used to predict a number of mechanical properties in wood and provide distinctly different data than other slower analytical techniques.[300] For instance, Wu *et al.*[300] studied 10 different hardwood species using both SilviScan and nanoindentation (embedding of a pyramid-shaped nanoscale probe) techniques and found that the SilviScan measurements were significantly different among the species and highly correlated to the solid wood properties. In contrast, less species differences were found with nanoindentation tests and the measurements were more closely correlated to hardness measurements. It appears that the nanoscale mechanical properties measured within the cell wall may elucidate specific polymer interaction but microscaled factors such as MFA have more influence over the solid wood properties. This finding would be consistent with the theoretical modeling assessed by Salmén and Burgert.[269]

Another technique of growing interest takes a different approach. Near-infrared (NIR) spectroscopy utilizes the multivariate analysis of the spectral signature in the NIR range (500–2400 nm) to develop strong correlations to multiple chemical, physical, and mechanical properties.[301] Ultimately, the spectroscopic signals of interest originate from chemical functionalities within the wood materials. However, these signatures also appear to be highly correlated with other microstructural parameters such as MFA. The theoretical basis for this empirical observation is not immediately clear because the NIR spectroscopy measures chemical functionality, whereas other studies have shown that cell wall and polymer morphologies are closely tied to physical performance. It is possible that the measurements provided by the NIR are actually a secondary effect whereby certain functional groups are either correlated to or control structural features in the wood. Whatever the basis, the usefulness of this technique is predicated on the development of a trained database correlating the property of interest to spectral properties. But the strong success in developing these correlations leads to interesting questions about how chemical compositions in plant tissues give rise to both anatomical structures and physical properties.

3.27.4.3 Lignins, Celluloses, Hemicelluloses, and Plant Cell Wall Formation/Deconstruction via Genetic Engineering: A New Era Beckons

The previous sections have provided some insights into the remarkable anatomical architectures that make up gymnosperm and angiosperm woody tissues. These, in turn, reflect the differential deposition of celluloses, hemicelluloses, lignins and, to a much lesser extent, cell wall proteins in a cell-specific manner. These differences result in the distinctive biophysical/mechanical properties of the polymers in each cell type.

As a result, wood tissues are mainly a complex blend of these polymeric entities arranged very specifically within cell walls. This arrangement spans, however, many levels at the nanoscale and proceeds to macroscopic features discernible to the naked eye. As a result, these physicochemical and anatomical features dictate physical performance.

It is not, however, the purpose of this contribution to describe the chemistry and biochemistry of these cell wall biopolymers in detail as they are comprehensively discussed elsewhere,[101–104,302] and in Chapters 6.16 and 6.17. The interested reader is thus strongly encouraged to review these comprehensive contributions on lignins, celluloses, and hemicelluloses.

It does need to be emphasized that very little is still yet known about how these distinct macromolecular assemblies occur to afford vessels, fibers, tracheids – in both 'normal' and 'reaction' wood tissues, and how such modifications are achieved to generate pits, end plates, and the like. However, the recent explosion of genomics and proteomics in the study of woody plant formation[303–306] is beginning to shed needed light on the overall hierarchal control over these systems.

In a related manner, our understanding of many of the factors involved in individual polymer formation is still poorly understood in terms of the factors controlling the molecular weight distribution (MWD) for cellulose, in terms of initiation, propagation, and termination reactions. Nor is it well understood yet as to what factors control cellulose MFA (with cellulose chains being laid down initially in a 'random' manner in the primary walls, and then having a more carefully defined deposition process in the secondary walls). In an analogous manner, the various hemicelluloses in wood (from pectins in the primary wall/middle lamella) to that of how the various polymers (mainly galactoglucomannans in softwoods and arabinoxylans in

hardwood)[102] are biosynthesized still remain a considerable challenge to fully define, not only in terms of initiation, propagation, and termination reactions of the polymeric backbones, but also as regards side-chain/branching processes. In terms of lignification, there also remain a whole series of poorly understood processes – including how monomer transport is controlled, and regulated, and how lignin primary structure is achieved. Together with all of the above, the roles of the various structural cell wall proteins (e.g., proline-rich glycoproteins, extensins, etc.) are also very poorly understood in terms of the cell-specific processes that they are involved in.

Currently, however, there is a renaissance – or really a beginning – in attempting to define cell wall assembly processes leading to what is collectively known as wood (architectures). This is being driven, in large part, by the desire to use plants as a source of renewable energy, which, in turn, requires solution to the so-called 'lignocellulose' recalcitrance problem that prevents ready deconstruction and fermentation of our woody resources. In this context, the interested reader is strongly encouraged to review the monograph entitled 'Biomass Recalcitrance; Deconstructing the Plant Cell Wall for Bioenergy.'[307]

In this regard, trees present a greater challenge though to genetic manipulation and commercial production than do herbaceous crop species. Some of the biological reasons for this include: long generation times, mode of pollination (e.g., by wind in some species or unknown in many tropical species), their obligate outbreeding systems (i.e., they cannot produce viable inbred lines),[308] their large genome sizes, and the sizes of their stem, canopy, and root systems that consequently require large tracks of land. Work has, however, progressed on understanding tree genetics with a growing number of wood-related databases and the complete sequencing of the genome of *Populus trichocarpa*.[309] Wood biotechnology is still in its earliest stages as only a very small portion of the possible genomes, proteomes, transcriptomes, and so on has been studied. Moreover, little confirmation exists for the identity of putative genes, their gene product functionality or regulation. Most of the present work on the molecular biology of trees is thus geared toward information gathering (i.e., genomics, proteomics, etc.) and we are only at the earliest stages of this process.

Accordingly, the biotechnological potential of wood and related products might not be fully realized without perhaps a systems approach to help define regulation and molecular interactions at all physiological levels. For instance, increasing wood or bark volume or altering stem branching will require a clear understanding of the respective meristems giving rise to each tissue type, as well as the interacting signals between them. Theoretically, increasing lignocellulose resources could also be potentially achieved by controlling cell identity, differentiation, or cell wall synthesis processes. However, overcoming the challenges of, or utilizing the unique properties presented by, juvenile, reaction, or heartwoods will presumably require a comprehensive view of inter-related molecular mechanisms.

Another complicating factor is that the various attempts to biotechnologically modify various woody tissues, in terms of their polymeric cell wall constituents and their specific cell wall types (fibers, vessels, tracheids, etc.), have generally lacked any comprehensive testing of the resulting modified wood material properties. Moreover, such biotechnologically modified wood forms are typically only examined at a juvenile wood developmental stage. Indeed, even the effects of biotechnological manipulations on water and nutrient (fluid) conduction properties are also sadly lacking in essentially all studies to date. Such omissions in testing have thus frequently led to wood properties being gauged simply in terms of 'visible to the naked eye' phenotypes, and thus to a fairly simplistic view of structural (material) properties that result. Comprehensive testing will be an absolute minimum requirement in understanding any wood material in terms of its biophysical and mechanical properties.

3.27.4.4 Heartwood Diversity and Formation

In spite of the critical dependence humanity places on HW formation, this has been one of the most greatly overlooked and under-studied areas in plant sciences. In this context, some discussion is provided to place research findings made thus far in useful context, as well as to summarize the urgent needs that remain.

3.27.4.4.1 A phytochemical extravaganza: from heartwood color to biological function

As alluded above, there is enormous variability/complexity in wood cell wall architecture and cross-sectional patterns in different tree species. This complexity is perhaps only superseded by the fantastic diversity of HW

metabolites that accumulate in the central core of trees. However, some of the most attractive and durable HWs are those generally used for furniture, carvings, housing, and so forth, because of their appealing colors, structural integrity, and biodegradation resistance. Moreover, some of the most brilliantly colored HW constituents are relatively readily removable and color stable, with several having found applications worldwide for centuries as sources of dyes.[310–314]

There are at least two types of HW formation envisaged to occur. For Type 1 HW phenotype, the deposition of 'extractives' starts at the SW–HW transition zone (e.g., as for *Robinia pseudoacacia*), whereas for Type 2, the 'extractive' precursors accumulate in the older SW, with these then undergoing further transformations (e.g., oxidation, hydrolysis) at the SW–HW transition zone (e.g., *Juglans*).[149] Moreover, for HW-forming temperate species, the HW metabolites are mainly laid down constitutively (e.g., *Quercus*, *Pinus*, *Juglans*, *Cryptomeria*, *Robinia*, etc.) toward the fall (autumn) of each year.[315] Over periods spanning many years, however, this deposition process progresses radially outwards from the pith to ultimately more or less encompass most of the woody tissue. In such cases, the HW metabolites also infiltrate the cell wall, the effects of which overall help engender dimensional stability (limiting shrinkage and swelling), and enhancing durability. Additionally, although not forming HW proper, some other species such as European ash (*Fraxinus excelsior*) and European beech (*F. sylvatica*) produce discolored wood in response to external factors (e.g., to either injury or pathological death). Such effects occur independently of season, with the resulting tissue being formed discontinuously and where the polyphenols apparently do not infiltrate the cell walls.[315]

In extractives-rich species, the HW metabolites are generally present as complex mixtures, the levels of which in such tissues can often ultimately represent a major repository of (sequestered) organic carbon. These 'extractives' mixtures also frequently encompass several classes of secondary metabolites which, depending upon the species, include aromatics/phenolics, terpenoids, alkaloids, and so on, although typically they are of the former category. Of the aromatics/phenolics, the most commonly found are the proanthocyanidins/condensed tannins and related metabolites (see Chapter 6.18), hydrolyzable tannins (see Chapters 3.21 and 4.16), flavonoids, (oligo)lignans (see Chapter 1.23), napthoquinones, xanthones, and stilbenes. Some HW metabolites may also result from mixed biosynthetic pathway origins, for example, via mixed phenolic and terpenoid metabolism, such as prenylated flavonoids, xanthones, and so forth. Interestingly, HW 'extractives' can also accumulate in a broad range of molecular sizes/MWDs. For example, western red cedar HW accumulates various metabolites ranging from plicatic acid (**10**) to oligomeric lignans of MW ~13 000.[316] Surprisingly, MWDs are generally not reported in studies of the so-called HW extractives.

The term HW 'extractives' is arguably a misnomer, since not all metabolites present are readily extractable, unless deposited in pure form in the lumen of tracheids and vessels (**Figure 52**). More often than not, however, being impregnated into lignified cell wall tissues, they are difficult to remove under normal solvent extraction procedures. Indeed, extractive amounts in HWs can range from being quite low (<2–3%), such as in black spruce (*Picea mariana*),[317,318] to levels of up to 18–25%, such as in western red cedar (*T. plicata*)[316] and *G. officinale*.[188] Indeed, those with low HW metabolite contents (such as the gymnosperm spruce above) are often preferred feedstocks for the pulp and paper industry (with another advantage in gymnosperms being their longer tracheid lengths). On the other hand, lower extractive contents generally reflect a greater susceptibility to attack by opportunistic pathogens.

In many cases, the HW extractives have some level of cytotoxic, antifungal, antibacterial, and antioxidant properties and/or other biocidal/biological modes of action. They can thus help provide chemical defenses in response to (potential) attack by opportunistic pathogens, and thereby protect the central core of the nonliving part of the tree.

Table 2 depicts a fairly limited selection of phytochemical HW pigments (and putative pro-pigments) **81–166** isolated from mixtures of metabolites present in specific HWs, including their structures, compound classes, and range of colors (**Figure 62**).[310–314,316,319–375] It must be emphasized though that the pigments and colors in woods can be influenced not only by individual phytochemical components, but also from all of the constituents present in the overall mixtures. pH, metal content, and 'aging' of tissues can affect coloration as well.

For the examples chosen, color generation appears to generally result from oxidation of a di-phenol of some type, to generate the corresponding *ortho*- or *para*-quinones (**Table 2** and **Figure 62**). Striking examples of important dye pigments are the brilliant red dyes that have been used for centuries, including: brazilin (**82**) and

Table 2 Selected examples of some important HW pigments/putative propigments

Latin name (family)	*Common name*	*Heartwood appearance*	*Examples of pigments*	*Examples of other metabolites in heartwood, including putative propigments*	*Examples of uses*
Berchemia zeyheri (Rhamnaceae)	Red ivory or pink ivory	Pink	Unknown	Maesopsin (**81**); oligomeric benzofuranoids and others;[319] isoflavanone-benzofuranone;[320,321] bibenzoflavonoids[322]	Billiard cues
Caesalpinia echinata (Fabaceae)	Brazilwood (Pau-Brasil, Pernambuco)	Bright orange when fresh, dark red to red-brown when exposed to air[310]	Brazilin (**82**),[311] Brazilein (**83**)[312]		Heartwood used to make violin bows Dyewood for cotton, woolen cloth, red ink
Caesalpinia sappan (Fabaceae)	Sappanwood	Dark red to brown with fine striping	Brazilin (**82**), brazilein (**83**)[312]	Chalcones,[323] dibenzoxocins,[324] homoisoflavonoids[325]	Dyewood, red
Calophyllum lankaensis, *C. wightianum* (Clusiaceae)		Deep red to red or pink-brown, or orange-red	Zeyloxanthonone (**84**) with palmitic acid[326]	Triterpenes, flavonoids	
Chlorophora excelsa, *C. regia* (Moraceae)	Iroko	Variable from pale yellow-brown to dark brown with light markings		Flavonoids, stilbenes, and geranylstilbenes, e.g., excelsaoctaphenol (**85**)[327] and chlorophorin (**86**) [328]	Exterior applications, e.g., boat building (general – decking – framing – planking), boxes and crates, bridge construction, building materials, canoes. Also, carving, chairs, chests, counter, and table tops, etc.
Cordia alliodora (Boraginaceae)	Spanish elm, Ecuador laurel, satinwood	Light olive-brown fresh with black streaks; golden-brown to brown[310]		Alliodorin (**87**)[329]	Boat decking, cabinetry, guitar/bass building[329]
Cordia elaeagnoides (Boraginaceae)	Bocote	Red-brown with irregular brown or black streaks		Hydroquinone terpenoids **88–90**[330]	Furniture, inlays, cabinets, veneers, tool handles, etc.

(*Continued*)

Table 2 (Continued)

Latin name (family)	*Common name*	*Heartwood appearance*	*Examples of pigments*	*Examples of other metabolites in heartwood, including putative propigments*	*Examples of uses*
Cryptomeria japonica (Cupressaceae)	Sugi	Reddish color to black	Dehydrohydroxy-sugiresinol (**91**)[331,332] and products of oxidative coupling products of hydroxysugiresinol (**92**)[332]	Hydroxysugiresinol (**92**), sugiresinol (**93**), sequirin C (**94**)[333]	Construction, decoration
Dalbergia candenatensis (Fabaceae)		Deep red	Candenatone (**95**)[313]		Red dyestuff [313]
Dalbergia congestiflora (Fabaceae)	Granadillo	Purple[334]	Neocandenatone (**96**)[334]		Musical instruments; dye for fabrics, paints, etc.; food colorant (proposed)
Dalbergia nigra (Fabaceae)	Brazilian rosewood, Jacarandá-da-Bahia	Violet brown to purple-black with black streaks[310]	Dalbergiones, e.g., (*R*)-4-methoxydalbergione (**97**), (*S*)-4,4′-dimethoxydalbergione (**98**), (*S*)-4-hydroxy,4′-methoxydalbergione (**99**) [335–337]	Isoflavones, e.g., caviunin (**100**)[338]	High-end furniture and paneling, cabinetry, musical instruments
Dalbergia retusa (Fabaceae)	Cocobolo	Deep red to red-brown with black mottling[310]	Retusapurpurin A (**101**)[339], obtusaquinone (**102**)[340]		Musical instruments (but not those touching lips), mirror frame, clock cases, furniture, bowls, etc.
Diospyros celebica (Ebenaceae)	Macassar ebony	Black with pink and/or gray stripes	Naphthoquinones and binaphthoquinones, e.g., diosindigo B, B_1 and B_2 (**103–105**), celebaquinone (**106**)[341]	Macassar II (**107**) and III (**108**)[341,342]	Musical instruments, cabinetry, luxury furniture, knife handles, walking sticks
Diospyros melanoxylon (Ebenaceae)	Coromandel ebony persimmon	Dark brown[343]	Naphthoquinones and binaphthoquinones, e.g., diosindigo A (**109**) and B (**103**), violet quinone (**110**), biramentacéone (**111**)[344]		Boxes, combs, beams

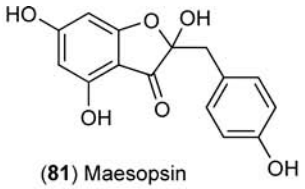
Diospyros morrisiana (Ebenaceae)	Morris persimmon		Isodiospyrin (**112**)[345]		
Garcinia morella (Clusiaceae)	Batuan		Flavonoids/ biflavonoids, e.g., morelloflavone (**113**), and chromenoxanthones, e.g., morellin (**114**) [346]		Artist's yellow dyes; Gamboge/used to dye silk robes of Buddhist monks
Gonystylus bancanus Thymelaeaceae	Ramin	White to yellow	5-Hydroxy-7,4′-dimethoxyflavone (**115**)[347]		Furniture, window blinds
Guaiacum officinale (Zygophyllaceae)	Lignum vitae, ironwood	Greenish yellow		Guaiaretic acid (**116**)[188,348] and related lignans	Submarine propeller shaft, bearings, chopping blocks, casters, etc.
Haematoxylum campechianum (Fabaceae)	Logwood tree Palo del Brasil	Brownish when dry Reddish-brown	Haematoxylin (**117**), Haematein (**118**)[349]		Dyes for cotton, woolen goods, leather, furs, silks, histological stains
Larix kaempferi, syn. *L. leptolepis* (Pinaceae)	Japanese larch	Yellow/brown		Flavonoids, e.g., distylin (=dihydroquercetin, **119**)[350]	Construction work, fences
Libocedrus decurrens (Cupressaceae)	Incense cedar	Light to medium brown	Thymoquinones, e.g., 3-libocedroxy-thymoquinone (**120**)[351]		Pencils
Liriodendron tulipifera (Magnoliaceae)	Yellow poplar	Tan, but variable	Noraporphine alkaloids, e.g., liriodenine (**121**)[352]	Lignans, e.g., syringaresinol (**122**)[353]	Interior finish of houses, panels, coffins, wooden ware, organs
Maclura pomifera (Moraceae)	Osage orange	Orange to yellow tinted	Flavonoids, e.g., morin (**123**), stilbenes, e.g., 2,3′,4,5′-tetrahydroxystilbene (**124**), and xanthones, e.g., 1,3,6,7-tetrahydroxyxanthone (**125**)[354]		Fence posts (lasting more than a century), cutting boards
Mansonia altissima (Malvaceae)	Mansonia	Yellow brown or dark gray brown, frequently with a purplish cast, often shows light and dark bands	Sesquiterpene quinones, e.g., mansonones A, C, and G (**126**–**128**)[355]		

(*Continued*)

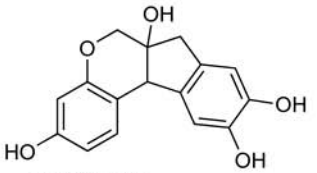
Table 2 (Continued)

Latin name (family)	*Common name*	*Heartwood appearance*	*Examples of pigments*	*Examples of other metabolites in heartwood, including putative propigments*	*Examples of uses*
Melanorrhea spp. (Anacardiaceae)			Rengasine (**129**)[356]		
Mesua ferrea (Clusiaceae)	Gangaw	Dark red to red-brown	Xanthones, e.g., euxanthone (**130**), mesuaxanthone A (**131**), mesuaxanthone B (**132**)[357]		Structural timber, railroad ties
Millettia laurentii (Fabaceae)	Wenge	Dark brown to black with alternating light and dark streaks	Flavonoids, e.g., laurentiquinone (**133**), laurentinol (**134**)[358]	Flavonoids, guanidine alkaloids (e.g., millaurine (**135**))[359]	Musical instruments, flooring, sculpting, paneling, furniture
Morinda citrifolia (Rubiaceae)	Noni	Roots	Anthraquinones, e.g., morindin (**136**) and **137–141**[360,361]		Dyeing clothes
Peltogyne spp. (Fabaceae)	Purpleheart, Guarabu, (numerous common names)	Purple		Peltogynol (**142**)[362]	Industrial construction, flooring, furniture, tool handles, etc.
Pseudotsuga menziesii (Pinaceae)	Douglas fir	Light brown	5,5′-Bisdihydroquercetin (**143**) and higher proanthocyanidin oligomers/ polymers[363,364]	Flavonoids, e.g., distylin (=dihydroquercetin) **119**[364,365]	Timbers, railroad ties, poles, flooring, furniture
Pterocarpus marsupium (Fabaceae)	Indian kino tree	Dark tan	6,4′ dihydroxy-7-methylaurone 6-*O*-rhamnopyranoside (**144**), 4,6,3′,4′-tetrahydroxy-aurone 6-rhamnopyranoside (**145**)[366]	Flavonoids	
Pterocarpus santalinus (Fabaceae)	Red sandalwood	Reddish brown	Santalin A (**146**) and santalin B (**147**)[314]		Source of brilliant red dye, classical Chinese furniture
Rhus vernicifera (Anacardiaceae)	Japanese lacquer tree			Fustin (**148**), fisetin (**149**)[367]	Source of urushiol (e.g., **71–74**) for lacquer (see **Figure 4**)
Robinia pseudoacacia (Fabaceae)	Black locust	Greenish yellow, dark yellow, golden brown		Robinetin (**150**), dihydrorobinetin (**151**)[368]	Fence posts, small watercraft

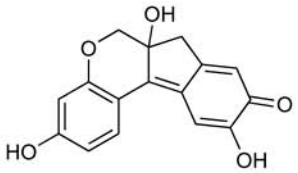
Tabebuia chrysantha (Bignoniaceae)	Yellow ipê or Araguaney	Light brown to golden	Naphthoquinones, naphthoquinols and dimers thereof, e.g., dehydrotectol (**152**), tetrahydrotectol (**153**), β-lapachone (**154**), dehydro-α-lapachone, (**155**), dehydro-β-lapachone, (**156**), 1-hydroxy-anthraquinone (**157**)[369]		Flooring, posts
Tecomella undulata (Bignoniaceae)	Desert teak, Marwar teak		Tecomaquinone I (**158**), α-lapachone (**159**)[370,371]		Timber, firewood
Tectona grandis (Lamiaceae)	Teak	Dark golden-brown when fresh, near-black when exposed to air[310]	Anthraquinones, e.g., 9,10 dimethoxy-2-methyl-anthra-1,4 quinone (**160**), tectoquinone (**161**), barleriaquinone-I (**162**), and naphthoquinones, e.g., dehydro-α-lapachone (**155**), 5-hydroxylapachol (**163**), deoxylapachol (**164**), dehydro-α-isodunnione (**165**)[372–374]		Sailboat decks, shipbuilding, planking, furniture, paneling (durable, remarkable termite resistance)
Thuja plicata (Cupressaceae)	Western red cedar	Orange-red	Plicatic acid (**10**)-derived polymer ($\sim$13 000 MW)[316]	Plicatic acid (**10**)[68] plus related metabolites from **10**	Outdoor construction, posts, siding, closets and chests
Zanthoxylum flavum (Rutaceae)	West Indian satinwood	Golden-yellow to brown		Suberosin (**166**)[375]	High-end furniture



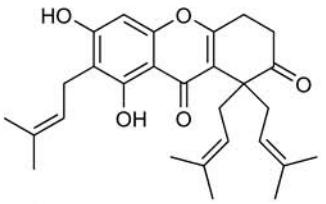
(**81**) Maesopsin
(*Berchemia zeyheri*)
[PROPIGMENT?]



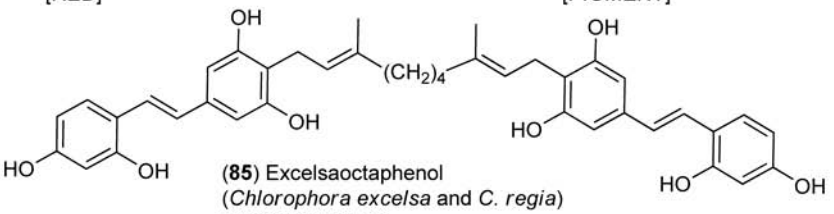
(**82**) Brazilin
(*Caesalpinia echinata*)
[RED]



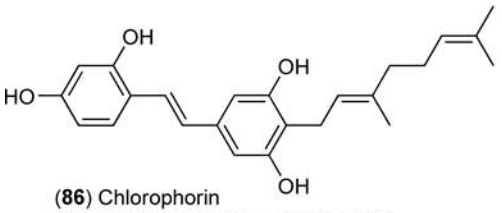
(**83**) Brazilein
(*Caesalpinia echinata*)
[RED]



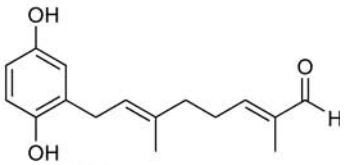
(**84**) Zeyloxanthonone
(*Calophyllum lankaensis*)
[PIGMENT]



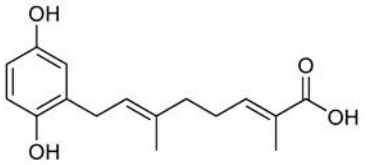
(**85**) Excelsaoctaphenol
(*Chlorophora excelsa* and *C. regia*)
[PROPIGMENT?]



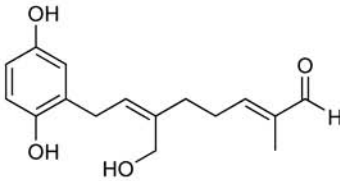
(**86**) Chlorophorin
(*Chlorophora excelsa* and *C. regia*)
[PROPIGMENT?]



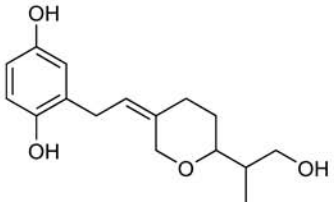
(**87**) Alliodorin
(*Cordia alliodora*)
[PROPIGMENT?]



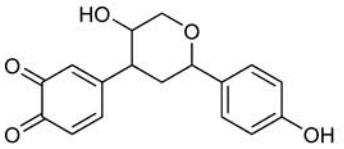
(**88**) (*Cordia elaeagnoides*)
[PROPIGMENT?]



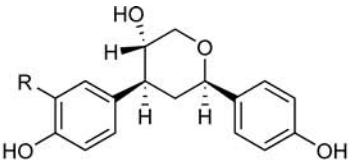
(**89**) (*Cordia elaeagnoides*)
[PROPIGMENT?]



(**90**) (*Cordia elaeagnoides*)
[PROPIGMENT?]

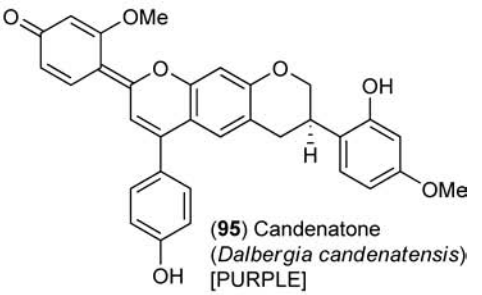


(**91**) Dehydrohydroxysugiresinol
(*Cryptomeria japonica*)
[REDDISH-ORANGE]

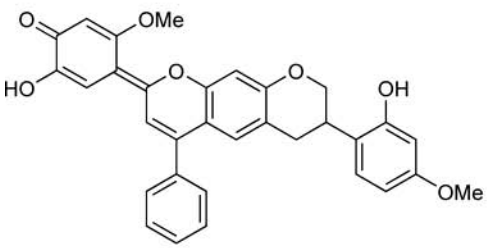


(**92**) R = OH, Hydroxysugiresinol
(**93**) R = H, Sugiresinol
(*Cryptomeria japonica*)
[PROPIGMENT?]

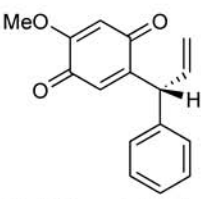
(**94**) Sequirin C
(*Cryptomeria japonica*)
[PROPIGMENT?]



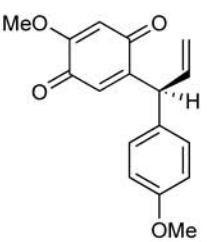
(**95**) Candenatone
(*Dalbergia candenatensis*)
[PURPLE]



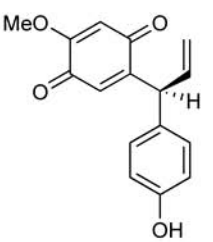
(**96**) Neocandenatone
(*Dalbergia congestiflora*)
[PURPLE]



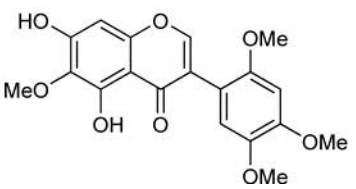
(**97**) (*R*)-4-Methoxydalbergione
(*Dalbergia nigra*)
[YELLOW]



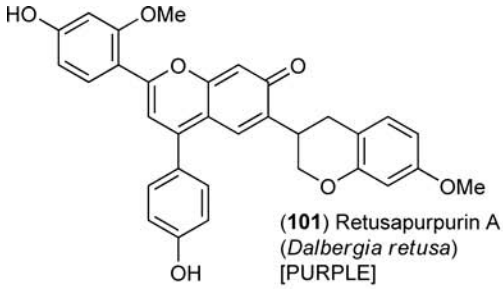
(**98**) (*S*)-4,4′-Dimethoxydalbergione
(*Dalbergia nigra*)
[ORANGE]



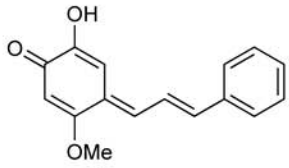
(**99**) (*S*)-4-Hydroxy,4′-
methoxydalbergione (*Dalbergia nigra*)
[ORANGE]



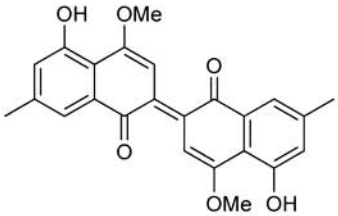
(**100**) Caviunin
(*Dalbergia nigra*)
[PROPIGMENT?]



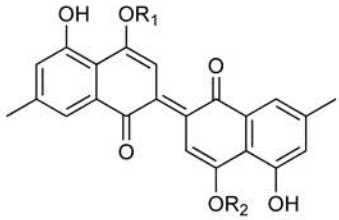
(**101**) Retusapurpurin A
(*Dalbergia retusa*)
[PURPLE]



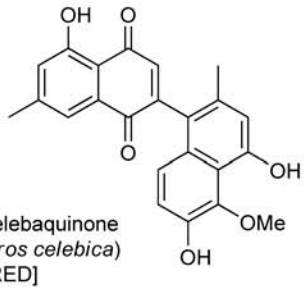
(**102**) Obtusaquinone
(*Dalbergia retusa*)
[RED]



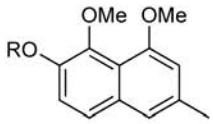
(**103**) Diosindigo B
(*Diospyros melanoxylon*)
[BLUE]



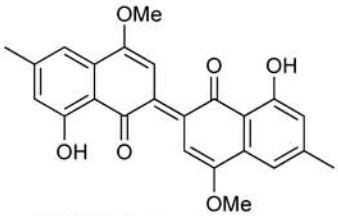
(**104**) R_1 = Me, R_2 = Et, Diosindigo B_1
(**105**) $R_1 = R_2$ = Et, Diosindigo B_2
(*Diospyros celebica*)
[BLUE, isolation artefacts]



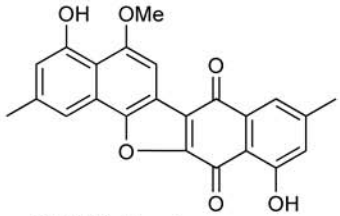
(**106**) Celebaquinone
(*Diospyros celebica*)
[COLORED]



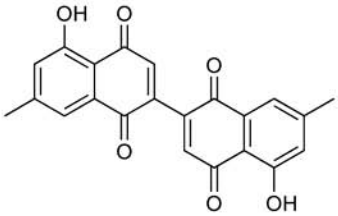
(**107**) R = H, Macassar II
(**108**) R = Me, Macassar III
(*Diospyros celebica*)
[PROPIGMENT?]



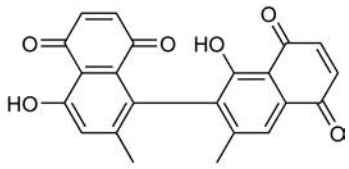
(**109**) Diosindigo A
(*Diospyros melanoxylon*)
[BLUE]



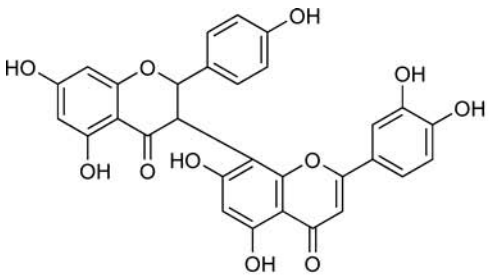
(**110**) Violet quinone
(*Diospyros melanoxylon*)
[VIOLET]



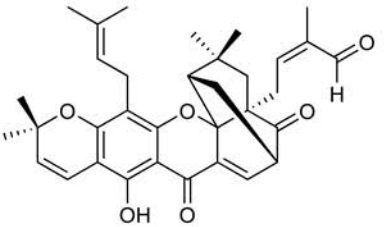
(**111**) Biramentacéone
(*Diospyros melanoxylon*)
[ORANGE]



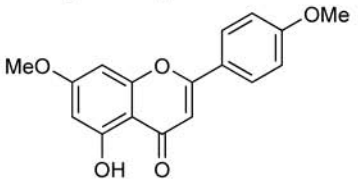
(**112**) Isodiospyrin
(*Diospyros morrisiana*)
[ORANGE]



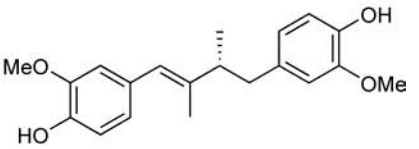
(**113**) Morelloflavone
(*Garcinia morella*)
[YELLOW]



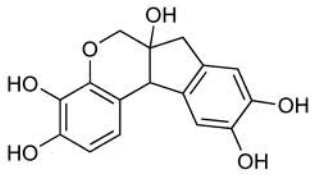
(**114**) Morellin
(*Garcinia morella*)
[YELLOW]



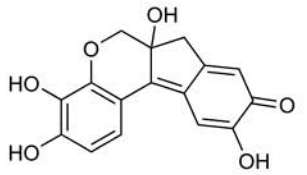
(**115**) 5-hydroxy-7,4′-dimethoxyflavone
(*Gonystylus bancanus*)
[YELLOW]



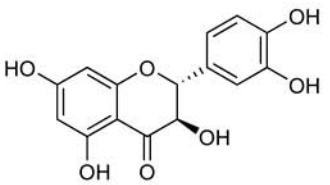
(**116**) Guaiaretic acid
(*Guaiacum officinale*)



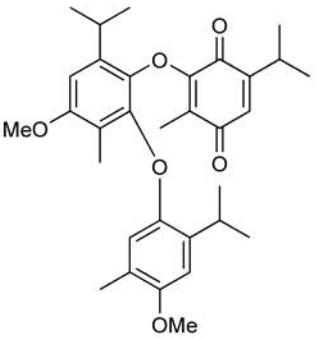
(**117**) Haematoxylin
(*Haematoxylum campechianum*)
[RED]



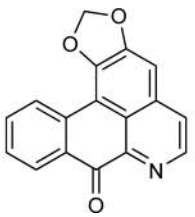
(**118**) Haematein
(*Haematoxylon campechianum*)
[RED]



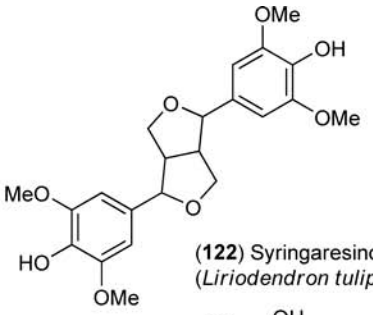
(**119**) Distylin (=dihydroquercetin)
(*Larix kaempferi* and
(*Pseudotsuga menziesii*)
[PROPIGMENT?]



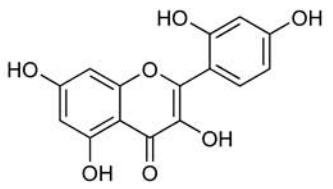
(**120**) 3-Libocedroxythymoquinone,
(*Libocedrus decurrens*)
[DARK RED]



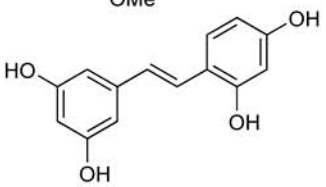
(**121**) Liriodenine, yellow
(*Liriodendron tulipifera*)
[YELLOW]



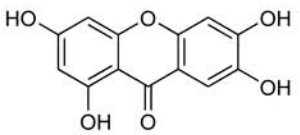
(**122**) Syringaresinol
(*Liriodendron tulipifera*)



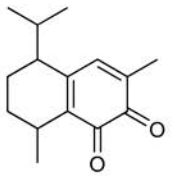
(**123**) Morin
(*Maclura pomifera*)
[YELLOW]



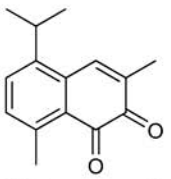
(**124**) 2,3′,4,5′-Tetrahydroxystilbene
(*Maclura pomifera*)
[YELLOW]



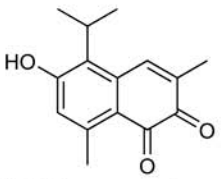
(**125**) 1,3,6,7-Tetrahydroxy-xanthone
(*Maclura pomifera*)
[YELLOW]



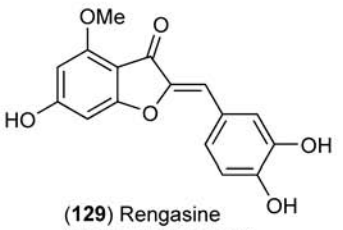
(**126**) Mansonone A
(*Mansonia altissima*)
[RED]



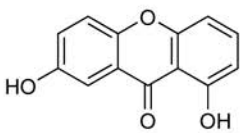
(**127**) Mansonone C
(*Mansonia altissima*)
[ORANGE]



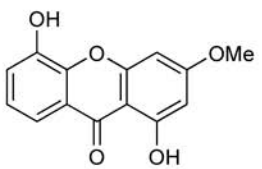
(**128**) Mansonone G
(*Mansonia altissima*)
[ORANGE]



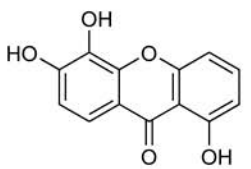
(**129**) Rengasine
(*Melanorrhea* spp.)
[YELLOW]



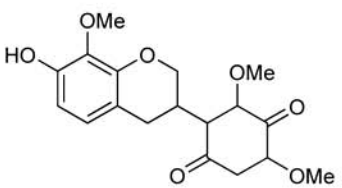
(**130**) Euxanthone
(*Mesua ferrea*)
[YELLOW]



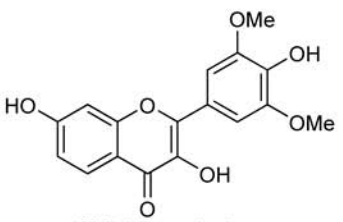
(**131**) Mesuaxanthone A
(*Mesua ferrea*)
[YELLOW]



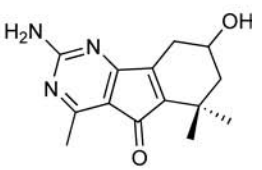
(**132**) Mesuaxanthone B
(*Mesua ferrea*)
[YELLOW]



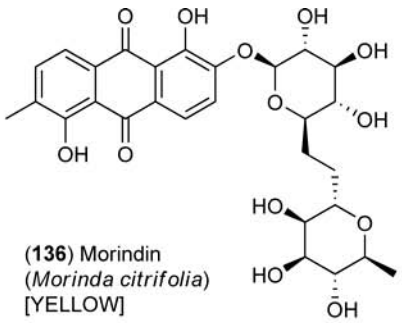
(**133**) Laurentiquinone
(*Millettia laurentii*)
[ORANGE]



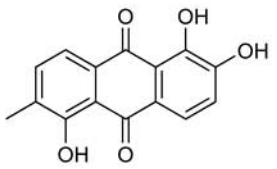
(**134**) Laurentinol
(*Millettia laurentii*)
[GREEN]



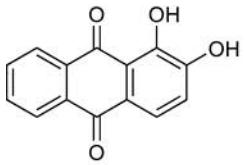
(**135**) Millaurine
(*Millettia laurentii*)
[YELLOW]



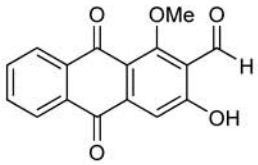
(**136**) Morindin
(*Morinda citrifolia*)
[YELLOW]



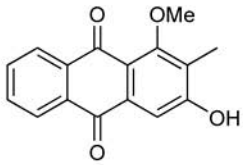
(**137**) Morindone
(*Morinda citrifolia*)
[YELLOW?]



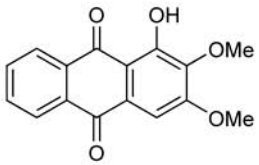
(**138**) Alizarin
(*Morinda citrifolia*)
[RED]



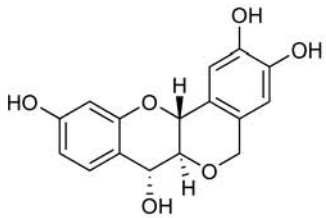
(**139**) Damnacanthal
(*Morinda citrifolia*)
[ORANGE-YELLOW]



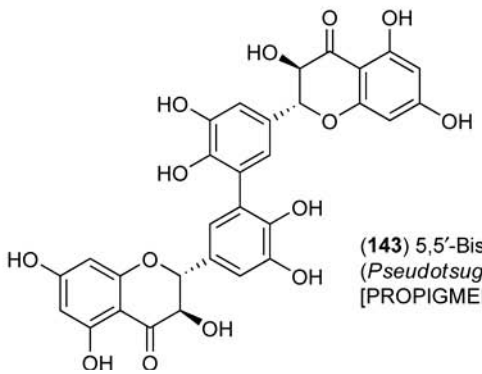
(**140**) Rubiadin 1-methyl ether
(*Morinda citrifolia*)
[YELLOW]



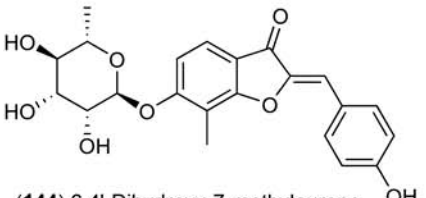
(**141**) Anthragallol 2,3-dimethyl ether
(*Morinda citrifolia*)



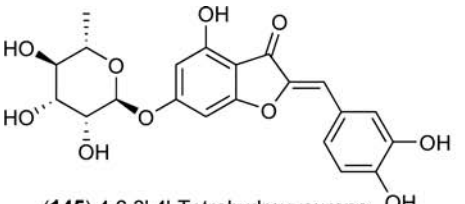
(**142**) Peltogynol
(*Peltogyne* spp.)
[PROPIGMENT?]



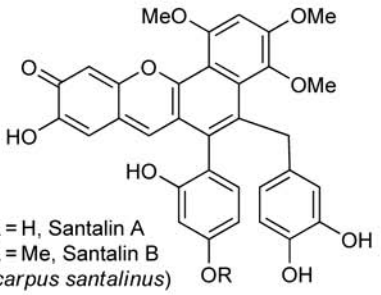
(**143**) 5,5′-Bisdihydroquercetin
(*Pseudotsuga menziesii*)
[PROPIGMENT?]



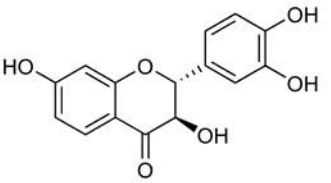
(**144**) 6,4'-Dihydroxy-7-methylaurone 6-*O*-rhamnopyranoside (*Pterocarpus marsupium*) [YELLOW]



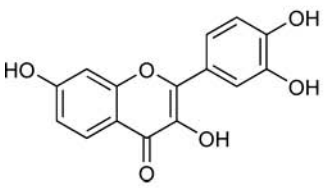
(**145**) 4,6,3',4'-Tetrahydroxyaurone 6-*O*-rhamnopyranoside (*Pterocarpus marsupium*) [YELLOW]



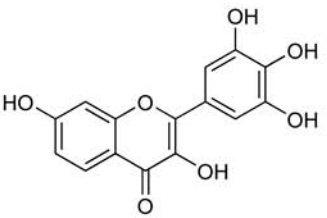
(**146**) R = H, Santalin A
(**147**) R = Me, Santalin B
(*Pterocarpus santalinus*) [RED]



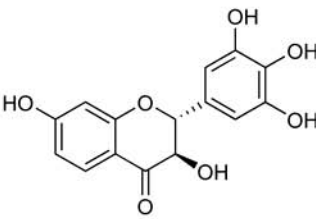
(**148**) Fustin (*Rhus vernicifera*) [PROPIGMENT?]



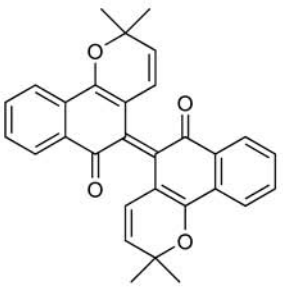
(**149**) Fisetin (*Rhus vernicifera*) [PROPIGMENT?]



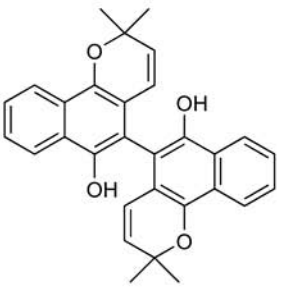
(**150**) Robinetin (*Robinia pseudoacacia*) [PROPIGMENT?]



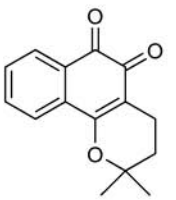
(**151**) Dihydrorobinetin (*Robinia pseudoacacia*) [PROPIGMENT?]



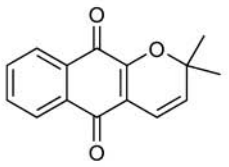
(**152**) Dehydrotectol (*Tabebuia chrysantha*) [BLUE-GREEN]



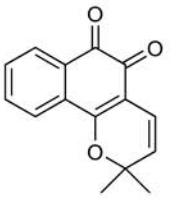
(**153**) Tetrahydrotectol (*Tabebuia chrysantha*) [YELLOW]



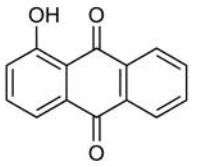
(**154**) β-Lapachone (*Tabebuia chrysantha*) [ORANGE]



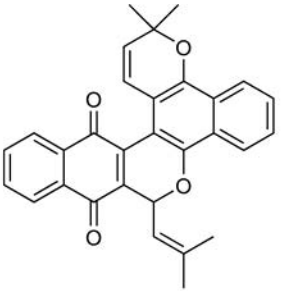
(**155**) Dehydro-α-lapachone (*Tabebuia chrysantha* and *Tectona grandis*) [ORANGE]



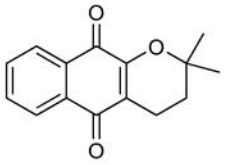
(**156**) Dehydro-β-lapachone (*Tabebuia chrysantha*) [YELLOW]



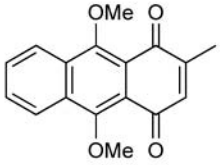
(**157**) 1-hydroxyanthraquinone (*Tabebuia chrysantha*) [YELLOW]



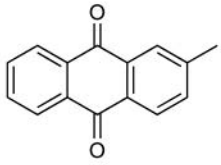
(**158**) Tecomaquinone I
(*Tecomella undulata*)
[DARK-BLUE/GREEN]



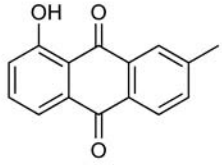
(**159**) α-Lapachone
(*Tecomella undulata*)
[YELLOW]



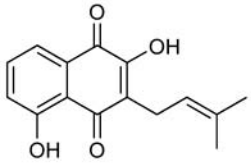
(**160**) 9,10-Dimethoxy-2-methyl-anthra-1,4 quinone
(*Tectona grandis*)
[YELLOW]



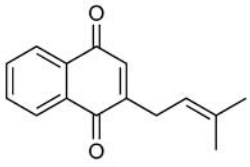
(**161**) Tectoquinone
(*Tectona grandis*)
[GOLDEN YELLOW]



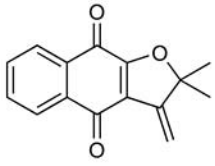
(**162**) Barleriaquinone-I
(*Tectona grandis*)
[ORANGE-YELLOW]



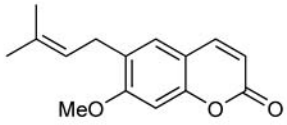
(**163**) 5-Hydroxylapachol
(*Tectona grandis*)
[BROWN]



(**164**) Deoxylapachol
(*Tectona grandis*)
[GOLDEN YELLOW]



(**165**) Dehydro-α-isodunnione
(*Tectona grandis*)
[YELLOW]



(**166**) Suberosin
(*Zanthoxylum flavum*)

brazilein (**83**) from *Caesalpinia echinata* and *C. sappan*;[310,311] hematoxylin (**117**) from *Haematoxylum campechianum*,[349] and santalin A (**146**) and B (**147**) from *Pterocarpus santalinus*;[314] the yellow dyes containing morindin (**136**) (and related pigments) from roots of Noni (*Morinda citrifolia*),[360,361] and gamboge from *Garcinia morella*,[346] which accumulates morelloflavone (**113**), morellin (**114**), and so forth. Indeed, such pigments have been utilized extensively over centuries for the dyeing of clothes, leading to the endangerment/near-extinction of some of these species. While many of these dyes have since been replaced by synthetics, a few are in use today, for example, as dyes for staining of microscopy samples (e.g., haematoxylin (**117**)),[376] or that of gamboge used for dyeing robes of Buddhist monks.

Other examples of pigments that strikingly affect HW colors, or are presumed so to do, include: the reddish-orange of dehydrohydroxysugiresinol (**91**) from sugi (*C. japonica*);[331,332] plicatic acid (**10**) from western red cedar (*T. plicata*)[68] and its (oxidized) derivatives thereof; the purple pigments, candenatone (**95**),[313] neo-candenatone (**96**),[334] and retusapurpurin A (**101**)[339] from *Dalbergia* species; the dark blue pigments from *Diosypros*, such as diosindigo A (**109**) and B (**103**);[341,344] the yellow pigments liriodenine (**121**) in *Liriodendron*

Figure 62 Selected examples of some important heartwood. Images from http:hobbithouseinc.com/personal/woodpics/, with permission.

tulipifera;[352] the orange coloration of laurentiquinone (**133**) in *Millettia* species;[358] the presumed pro-pigment peltogynol (**142**) for the striking purple color of purpleheart (*Peltogyne* spp.);[362] and the blue-greens of dehydrotectol (**152**) in *Tabebuia* spp.[369]

These examples, however, give just a tiny glimpse of the truly remarkable structural diversity in HWs and from which their fantastic colors and, in many cases, bioactivities derive. It should be emphasized that only a very few biosynthetic pathways to these metabolites have been studied at this point.

3.27.4.4.2 Phytochemical factories in heartwood metabolite deposition: parenchyma cells

The most important cell types apparently involved in HW metabolite generation are the parenchyma which can transport sucrose (from the cambial regions) and convert it into species-specific/characteristic HW metabolites such as those above. At the HW–SW transition zone, many of these (phenolic) metabolites then – at least in part – frequently undergo some form of oxidative conversion, often partially involving coupling/polymerization, to afford a range of molecular entities including the precursor monomeric, oligomeric, and/or polymeric constituents. These are then exudated/secreted through ray parenchyma (**Figures 20(a), 20(b)**, and **32(a)**), and ultimately, the molecular entities so produced typically result in some type of coloration (**Figure 62**) of, and protection to, the HW.

To date, the study of HW formation has focused upon several distinct but somewhat complementary approaches. These include: (1) delineation of overall metabolic events and processes occurring between the cambial and HW–SW transition zones; (2) identification of proteins, enzymes, and genes involved in specific biochemical pathways (e.g., to western red cedar and loblolly pine lignans); (3) studies of the biochemistry(ies) of the HW–sapwood transition zone; and (4) application of sequencing technologies to better establish the patterns/profiles of gene expression (the so-called wood 'omics') at different developmental stages in HW-generating tissues.

3.27.4.4.3 Metabolic cross-talk: primary and secondary metabolic events in parenchyma cells between the cambial and heartwood–sapwood transition zones leading to heartwood metabolites

Very little work has been carried out to fully delineate the relationship between primary (sucrose synthesis) and secondary metabolism, including how these processes are compartmentalized and organized/differentiated along the parenchyma cells between the cambium and the SW–HW transition zone. Preliminary studies began to examine this question, however, using black locust (*R. pseudoacacia*) as an experimental model, a species that accumulates robinetin (**150**) and dihydrorobinetin (**151**) in its HW.[146] By autumn, the activities of sucrose synthase (SuSy) and a neutral invertase, which are required for sucrose cleavage, were found to increase at the SW–HW transition zone, these being correlated in the case of SuSy with increased protein amounts (as determined by Western blotting) and transcripts (established by semiquantitative RT-PCR analyses).[377] Concomitantly, increased enzymatic activities of phenylalanine ammonia lyase (PAL, the first enzyme in the phenylpropanoid pathway) and chalcone synthase (CHS, the first enzyme in the flavonoid pathway),[146,149,150,378] were also noted, together with increases in levels of the HW products, robinetin (**150**) and dihydrorobinetin (**151**).[146] These results suggest a close correlation between sucrose turnover and flavonoid production, but also demonstrate how little is yet known of these metabolic processes.

Accordingly, much more work is needed to fully delineate the overall biochemical processes (and regulatory/transcriptional controls) operative in parenchyma cells between the cambial and SW–HW transition zone(s) which lead to HW metabolite generation.

3.27.4.4.4 Selected biochemical pathways: proteins, enzymes, and genes involved in heartwood lignan and flavonoid formation

Very few biochemical pathways leading to HW metabolites have thus far been established. Most work has focused upon lignan (e.g., in western red cedar[127,379–381] and loblolly pine[148,161,382]) and flavonoid (e.g., in black locust[146,149,150,315,377,378] and black walnut[153]) pathways.

The early steps in plicatic acid (**10**) biosynthesis in western red cedar have been investigated in this laboratory, with a detailed description given in Chapter 1.23. In summary, the first step involves stereoselective coupling of two *E*-coniferyl alcohol (**15**) moieties to afford (+)-pinoresinol (**167**), a reaction facilitated by

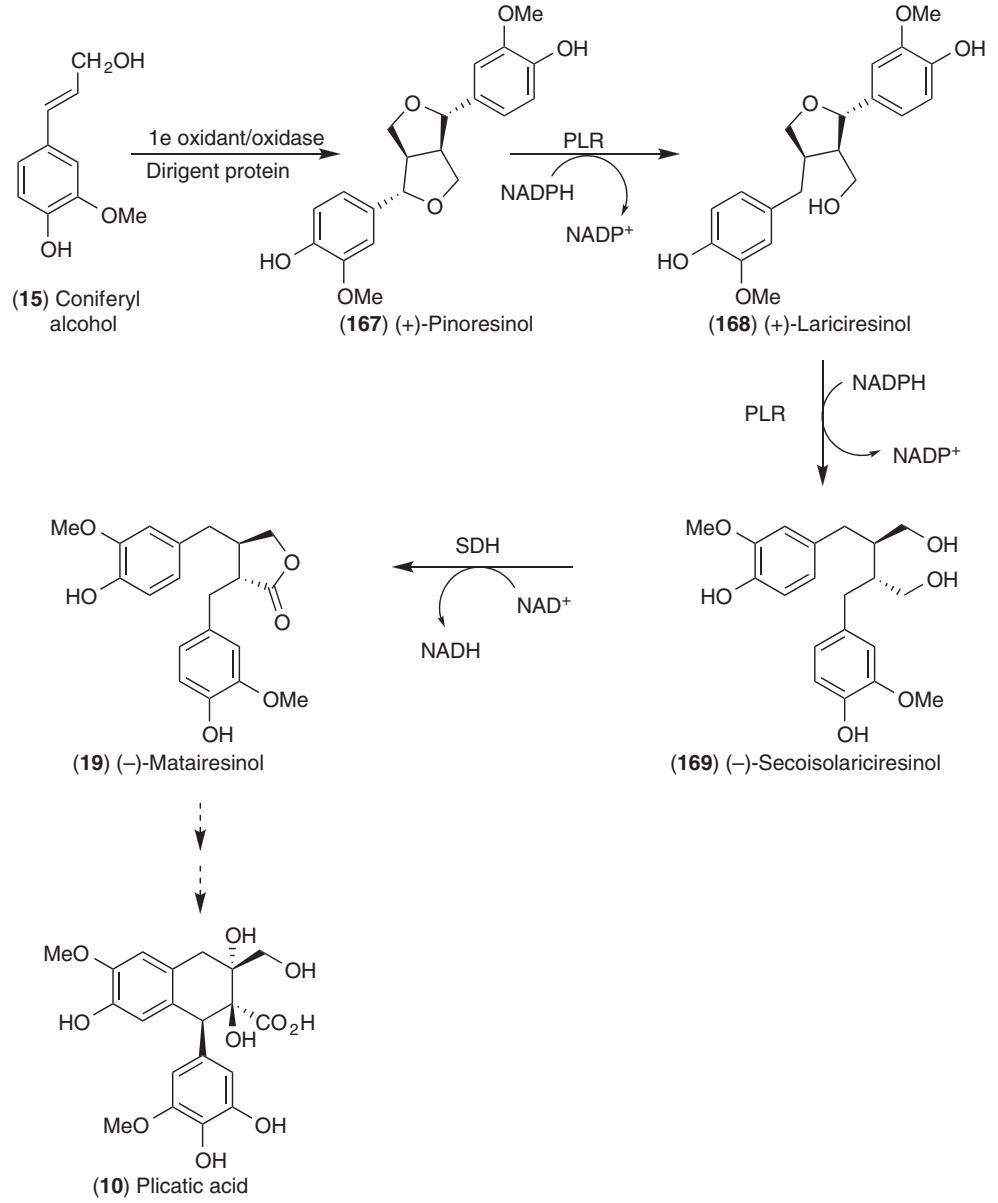


Figure 63 Proposed biosynthetic pathway to plicatic acid (**10**) in western red cedar (*Thuja plicata*).

dirigent proteins.[380,381,383] (+)-Pinoresinol (**167**) then undergoes two sequential reductions by pinoresinol/lariciresinol reductase to afford (+)-lariciresinol (**168**) and (−)-secoisolariciresinol (**169**),[379,384] respectively, with the latter converted into matairesinol (**19**)[385] (**Figure 63**). Further modifications ultimately afford plicatic acid (**10**) and its congeners.

As regards the first transformation, nine dirigent protein genes were cloned from western red cedar,[380,381] with the localization of their corresponding transcripts investigated using an RNA probe common to all isoforms. Dirigent protein transcripts were found in SW ray parenchyma (**Figure 32(a)**) suggesting transport of the lignans, at least in part, from SW to HW, and in the vascular cambium (not shown).[127] Interestingly, dirigent transcripts were also detected in the cork cambium, indicating a defense response in these tissues as well.

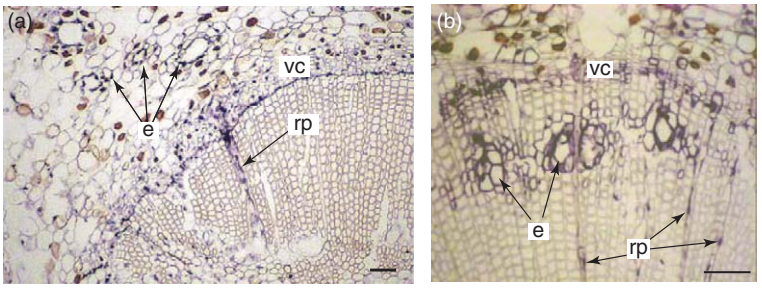


Figure 64 Phenylpropenal double bond reductase (PPDBR) and phenylcoumaran benzylic ether reductase (PCBER) localization in loblolly pine (*Pinus taeda*). PPDBR gene expression in young stems (a). PCBER protein localization in *P. taeda* (1-year old) seedlings (b). Bars: 50 μm (a) and 400 μm (b). Reproduced with permission of Elsevier from H. Kasahara; Y. Jiao; D. L. Bedgar; S.-J. Kim; A. M. Patten; Z.-Q. Xia; L. B. Davin; N. G. Lewis, *Phytochemistry* **2006**, *67*, 1765–1780, copyright 2006 (a). Reproduced with permission of Elsevier from M. Kwon; L. B. Davin; N. G. Lewis, *Phytochemistry* **2001**, *57*, 899–914, copyright 2001 (b).

In another example, biosynthesis of the 8–5′-linked lignans, dehydrodiconiferyl alcohol (**170**) and its derivatives **171**–**173**, was investigated in *Pinus taeda* (see Chapter 1.23). Transcripts of phenylpropenal double bond reductase (PPDBR)[161] and phenylcoumaran benzylic ether reductase (PCBER) protein[148] were localized to the SW ray parenchyma and vascular cambium, with PPDBR and PCBER also being detected in the axial parenchyma of bark resin ducts and SW resin ducts, respectively (**Figures 64(a)** and **64(b)**). PCBER can reduce – albeit very inefficiently – (±)-dehydrodiconiferyl alcohols (**170**) and (±)-dihydrodehydrodiconiferyl alcohols (**172**) into the 7-*O*-4′-reduced products, (±)-isodihydrodehydrodiconiferyl alcohols (IDDC, **171**) and (±)-tetrahydrodehydrodiconiferyl alcohols (TDDC, **173**), respectively (**Figure 65(a)**),[386] whereas PPDBR is considered to convert (±)-dehydrodiconiferyl aldehydes (**174**) and coniferyl aldehyde (**176**) into (±)-dihydrodehydrodiconiferyl aldehydes (**175**) and dihydroconiferyl aldehyde (**177**), respectively (**Figure 65(b)**).[161]

3.27.4.4.5 Transformations at the sapwood–heartwood transition zone

Several studies have been directed toward establishing the nature of biochemical conversions at the SW–HW transition zone in black walnut (*Juglans nigra*). The brown color of black walnut HW (**Figure 62**) is proposed to be due to the oxidation/polymerization of flavanols (e.g., by putative peroxidases) at the SW–HW transition zone.[387] As for black locust,[146,149,150,378] transcripts of genes involved in flavonoid biosynthesis (i.e., CHS, flavanone 3-hydroxylase (F3H) and dihydroflavonol 4-reductase (DFR)) have been shown to correlate well with flavanol accumulation in the SW–HW transition zone during the winter months.[153]

Finally, understanding transcriptional regulation of HW formation in terms of extractives production/secondary metabolism will also depend in part on deciphering PCD of ray parenchyma within the transition zone and HW. In contrast to earlier assumptions of complete cell death throughout HW tissues, two recent studies have shown that a few parenchyma cells remain living in the outer HW during the transition process.[126,127] Moreover, there may be distinct populations of ray parenchyma[388] that are responsible for 'extractives' formation, as it has been reported that parenchyma that undergo PCD relatively early in the growing season do not form extractives.[389] Single cell analyses of various types will presumably be extremely informative in helping elucidate HW-forming processes from transcripts through extractives accumulation.

3.27.4.4.6 'Omics' and heartwood formation

Although the technological advances in sequencing continue to rapidly develop, few studies have yet focused upon HW formation. The most extensive of these thus far are that of *C. japonica* and *R. pseudoacacia*. Over a thousand ESTs were sequenced from *C. japonica* HW-forming tissues (Forestry and Forest Products

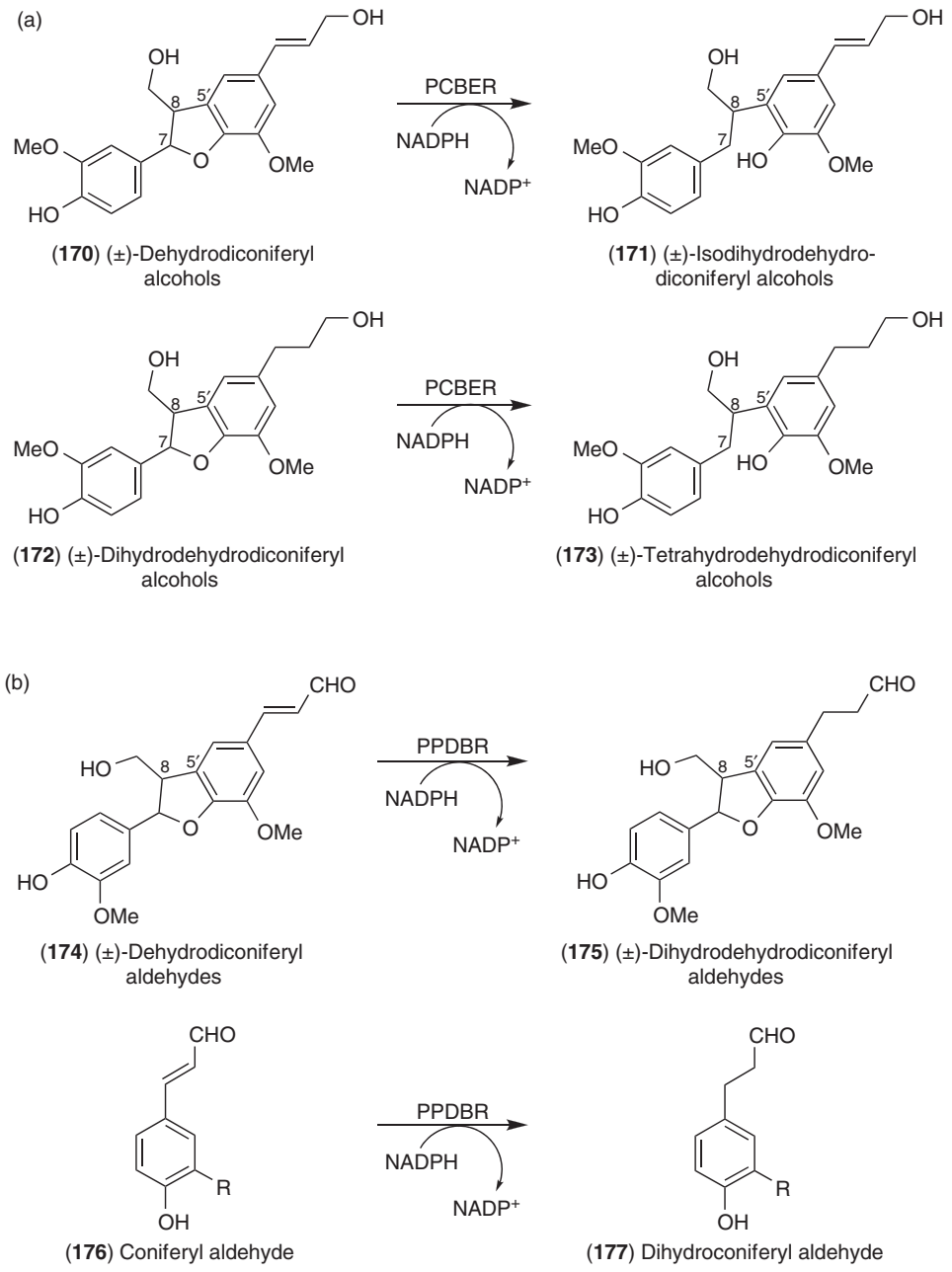


Figure 65 Reactions catalyzed by phenylcoumaran benzylic ether reductase (PCBER; a) and phenylpropenal double bond reductase (PPDBR; b) from loblolly pine (*Pinus taeda*).

Research Institute; GenBank accession numbers: DC882454-DC883482); however, no further work has been published so far. Additionally, mRNA was isolated from the bark/cambial regions, SW regions, and transition zones of mature black locust trees, with over 2900 expressed sequence tags (ESTs) being obtained.[390] Of these ~55% had no homology to any known sequences. From the ~2300 unigenes identified, microarrays were constructed and used in hybridization analyses to determine their expression profiles within the trunk of the tree. Specifically, genes involved in sucrose metabolism were highly

expressed in the SW, whereas those involved in flavonoid biosynthesis were at the SW–HW transition zone.[390] Such data are thus in accordance with the earlier findings of Magel *et al.*[146,149,150,377,378] At least 570 putative genes were also differentially expressed in the transition zone between summer and autumn by microarray analyses,[391] with >50% and <70% of the summer and fall transition zone ESTs unidentified. These data, when taken together, underscore the current incompleteness of our understanding of HW-forming processes at the molecular level(s).

3.27.4.4.7 Challenges for the future

Unraveling the complexity of HW formation, in its fullest sense, will require much more knowledge than that currently available. As indicated above, there is much needed to be established. This includes how HW metabolite deposition constitutively occurs, as well as whether their formation is inducible; how transcriptional control over deposition is effectuated; establishing both the commonalities and distinctions in biochemical pathways between the various (often) species-specific HW extractives; what initiates and controls axial/ray parenchyma cell formation, and metabolite transport from the cambial regions sapwood–HW transition zone; what controls differential carbon allocation to the various HW-forming biochemical pathways both within and between different plant species; how exudation and/or cell wall impregnation is initiated, regulated, and terminated; what oxidative and/or other biochemical processes are operational at the SW–HW transition zone, and how PCD of the specific cell types is engendered. When these are much better understood, these will provide much better insight into this remarkable diversity and how these processes truly occur.

3.27.4.5 Medicinals and Their Phytochemical Factories/Compartments

Trees are often considered throughout the world as a source of innumerable remedies and medicines, particularly as regards traditional/folk medicinal usage. However, at least from a western viewpoint, there are currently only a relatively small number of rigorously established pharmaceuticals from tree resources. It must be emphasized though that only a relatively small proportion of tree phytochemical constituents have been comprehensively evaluated for their effectiveness against specific diseases – this being further complicated by the fact there is no single battery of tests that can be universally applied to each and every disease condition. Regardless of this, tree phytochemicals or, in many cases, their crude phytochemical extracts have a long history in medicinal and health-protecting applications worldwide, as well as in their usage by numerous cultures as folk/traditional medicines. As a result, many tree extracts (or their phytochemical components) are used in human health applications worldwide such as in cough/flu remedies, and so forth.

Tables 3[222,392–406] and **4**[407–412] (and accompanying structures) summarize some of the most potent tree medicinals (or derivatives thereof) and/or extracts used medicinally including those in clinical trial status. Once again, however, the breadth of this topic results in only a sampling of medicinal treatment uses for illustrative purposes.

3.27.4.5.1 Alkaloids and their phytochemical factories

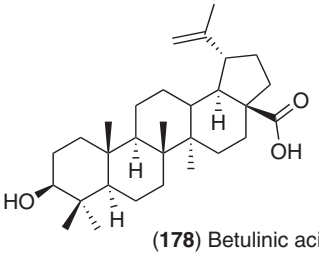
Of the tree species providing humanity with key pharmaceuticals, perhaps the alkaloids are the most notable. Striking examples include the structurally complex pharmaceuticals such as Taxol® (**1**), 10-deacetylbaccatin III (**189**), camptothecin (**2**), quinine (**3**), theobromine (**181**), theophylline (**182**), caffeine (**183**), pilocarpine (**185**), yohimbine (**186**), reserpine (**187**), and ajmaline (**188**). Of these, Taxol (**1**) and camptothecin (**2**) (and derivatives/homologs thereof) are some of nature's most efficacious cancer treatments.

Table 3 Selected proven medicinals/pharmaceuticals, and others in clinical trials, from tree species

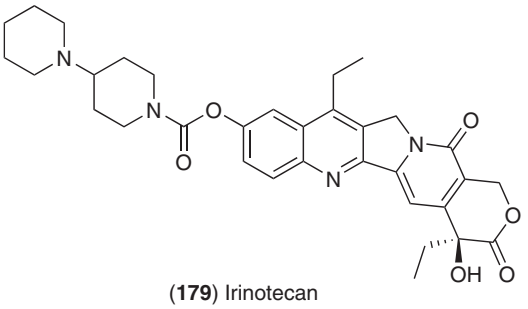
Latin name	*Common name*	*Compound(s) and semisynthetic derivative*	*Tissue occurrence*	*(Putative*) cell type/compartment*	*Medicinal use*	*Product*
Betula alba (Betulaceae)	White birch	Betulinic acid (**178**)	Outer bark[392]	NR[a]	Neoplasia (abnormal cell proliferation), inflammation, anti-HIV	Bevirimat
Camptotheca acuminata (Nyssaceae)	Happy tree	Camptothecin (**2**), irinotecan (**179**), and topotecan (**180**)	Stem wood[393], root bark,[394] leaves,[395,396] endosperm, and embryo[395–397]	Glandular trichomes* on leaves[395,413]	Ovarian cancer, coleo-rectal cancer	Hycamtin, camptosar
Cinchona officinalis (Rubiaceae)	Red cinchona	Quinine (**3**)	Bark[398]	Parenchyma cells*[398]	Malaria, fever, cardiac arrhythmia (abnormal heart rhythm)	Qualaquin
Cinnamomum camphora (Lauraceae)	Camphor tree	Camphor (**5**)	Leaves, bark, wood, roots, seeds[222,399]	NR	Pain, spasms, viral infection, exterior contamination (i.e., antiseptic), arthritis, itching, coughing, excess mucus, flatulence, and other digestive conditions, warts, cold sores, hemmorhoids	Vicks VapoRub®
Ilex paraguariensis (Aquifoliaceae)	Maté	Theobromine (**181**), theophylline (**182**), caffeine (**183**)	Leaves	Epicuticular wax (possibly other tissues)[400,401]	Weight reduction	Theobromine is included in supplements claiming to reduce weight, e.g., Dexatrim®
Camellia sinensis (Theaceae)	Tea	Caffeine (**183**)	Leaves			
Coffea spp. (Rubiaceae)	Coffee	Caffeine (**183**)	Fruits			
Theobroma cacao (Malvaceae)	Cocoa	Theobromine (**181**), caffeine (**183**)	Fruits			

Illicium verum (Schisandraceae)	Chinese star anise	(−)-Shikimic acid (**184**)	Seeds[402,403]	NR	Influenza	Oseltamivir (formerly: Tamiflu)
Pilocarpus pennatifolius (Rutaceae)	Jaborandi (shrub to small tree)	Pilocarpine (**185**)	Leaves[404]	NR	Ophthalmology (e.g., glaucoma, to reduce pupil size), to treat dry mouth (xerostomia)	Numerous brand names including: Ocusert Pilo-20 and Pilo-40, Pilopine HS, Salagen
Pausinystalia johimbe (Rutaceae)		Yohimbine (**186**)	Bark	NR	Stimulant	
Rauvolfia serpentina (Apocynaceae)	Snakeroot/ serpentwood	Reserpine (**187**) Ajmaline (**188**)	Root, leaves[405]	NR	Sedative, hypertensive Antiarrhythmic	Serpalan, Reserpine
Salix spp. (Salicaceae)	Willow	Acetylsalicyclic acid (**4**)	Bark		Analgesic, antipyretic, and anti-inflammatory	Aspirin
Taxus brevifolia (Taxaceae)	Pacific yew	Taxol® (**1**)	Bark[406]	Storage occurs in cell walls of phloem, vascular cambium, and wood		Abraxane, paclitaxel, taxol
		10-deacetylbaccatin III (**189**)	Leaves			

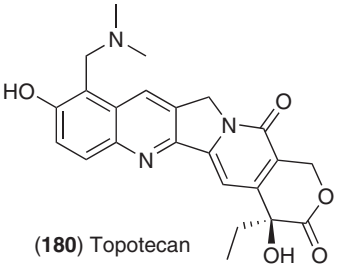
[a] NR = not reported.



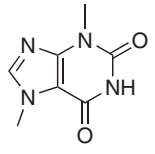
(**178**) Betulinic acid



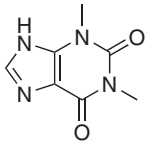
(**179**) Irinotecan



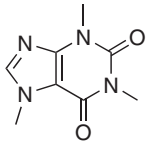
(**180**) Topotecan



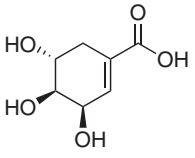
(**181**) Theobromine



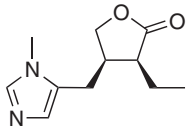
(**182**) Theophylline



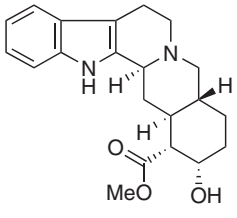
(**183**) Caffeine



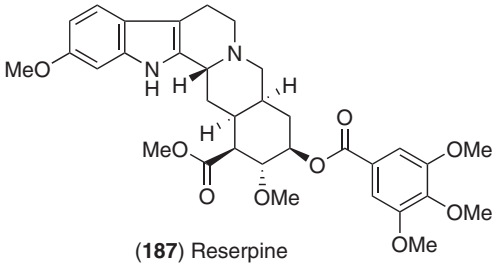
(**184**) (–)-Shikimic acid



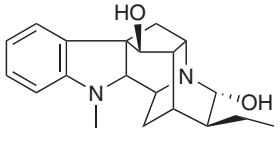
(**185**) Pilocarpine



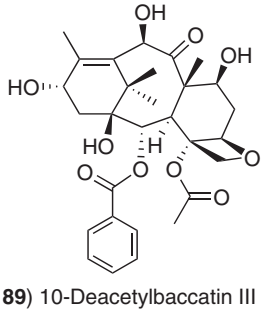
(**186**) Yohimbine



(**187**) Reserpine



(**188**) Ajmaline



(**189**) 10-Deacetylbaccatin III

3.27.4.5.1(i) Camptothecin Camptothecin (**2**), a quinoline alkaloid, is commercially obtained from stem[393] tissues of the 'Happy Tree' (*Camptotheca acuminata*), which is now mainly grown in plantations for this purpose. However, camptothecin (**2**) also accumulates in leaves,[395,396] seeds (endosperm and embryo),[395–397] and root bark,[394] with very preliminary evidence suggesting accumulation in glandular trichomes, at least in the leaves.[395,413] Its semisynthetic derivatives, irinotecan (**179**) and topotecan (**180**),

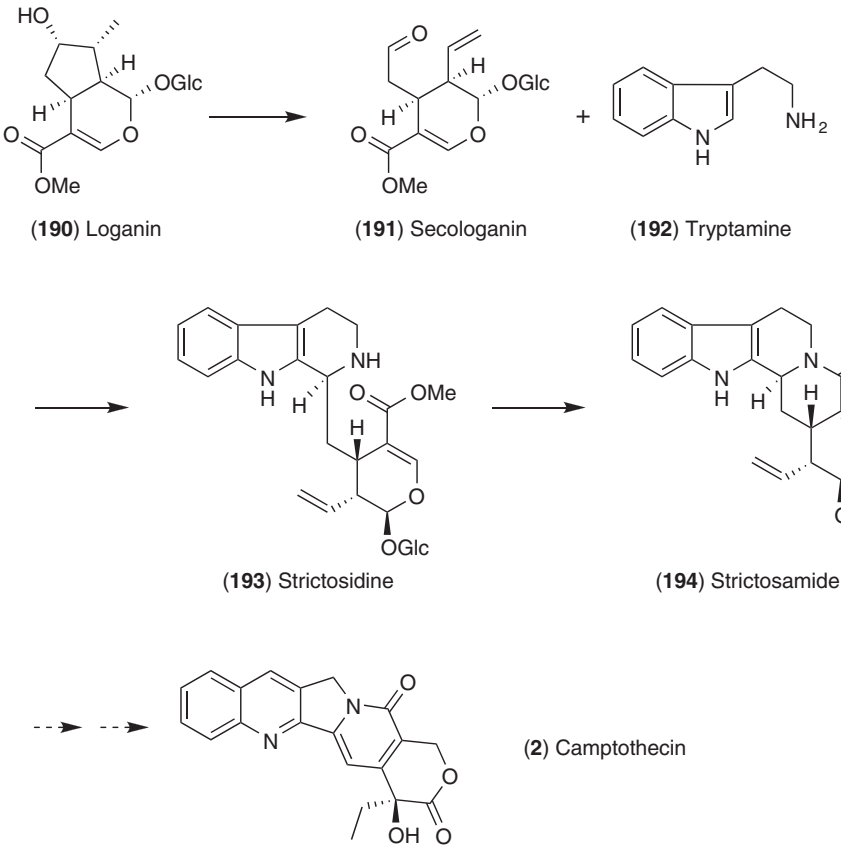
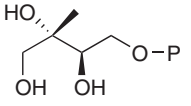


Figure 66 Proposed pathway to camptothecin (**2**) in *Camptotheca acuminata.*

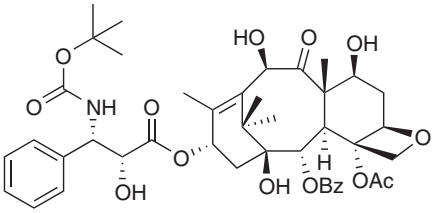
are currently extensively used for coleorectal and ovarian cancer treatments, as well as being evaluated in numerous other clinical trials. In terms of discovery, camptothecin (**2**) was originally isolated by Wall *et al.*[393] in pioneering studies in 1966 as a novel alkaloidal leukemia and tumor inhibitor. Its mode of action is that of binding to topoisomerase 1 and DNA complex, which thus prevents DNA re-ligation resulting in apoptosis via DNA damage.[414,415]

Very little is yet known definitively about camptothecin (**2**) biosynthesis. Early studies in the late 1970s[416,417] established, however, that it was derived from strictosidine (**193**) and strictosamide (**194**) (**Figure 66**). More recently, stable isotope labeling by administering (1-^{13}C glucose) to hairy root cultures of another camptothecin-producing plant species, *Ophiorrhiza pumila*, demonstrated that the secologanin (**191**)-derived segment of the molecule was of non-mevalonate origin, being instead from 2*C*-methyl-D-erythritol 4-phosphate (MEP, **195**, see Chapter 1.12).[418] None of the genes, pathway intermediate enzymatic steps, and transcription factors are, however, as yet known, nor is how transport and compartmentation effectuated. Interestingly, while there is one report that an endophytic fungus from *C. acuminata* produces camptothecin (**2**) and analogs,[419] repeated culturing of the fungus resulted in a strain no longer producing the alkaloid, thus leaving in doubt whether this fungus truly produces camptothecin (**2**).

3.27.4.5.1(ii) Taxol and baccatin III Taxol (**1**), a diterpene alkaloid, is present in near trace amounts (<0.001%) in tissues of various *Taxus* species and was also discovered by the same pioneers, Wall and Wani,[406] in 1971 from *Taxus brevifolia*. It functions medicinally by stabilizing cytoskeletal microtubules from depolymerization, thus suppressing mitosis during cell division. It is successfully used in the treatment of numerous aggressive carcinoma types considered difficult to treat, including breast, lung, ovarian, squamous cell carcinomas of the head and neck, and other cancers.[420] Challenges in obtaining this drug in sustainable and



(**195**) 2C-methyl-D-erythritol 4-phosphate (MEP)



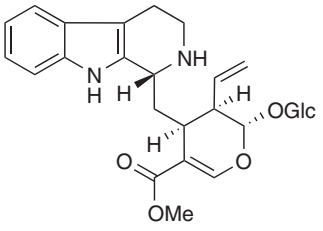
(**196**) Taxotere

sufficient amounts spurred considerable research activities, leading to semisynthesis of Taxol® and Taxotere®. In this regard, Holton *et al.*[421] chemically converted the much more abundant 10-deacetylbaccatin III (**189**) into Taxol® (**1**), whereas Potier's group[422,423] produced a drug named Taxotere® (**196**) by a series of reactions from 10-deacetylbaccatin III (**189**). Taxol® (**1**) can also be commercially produced in cell culture.[424–428] All together, these represent the main sources of *Taxus*-derived drugs used for cancer treatment.

Taxol® (**1**) is provisionally considered to accumulate in the parenchyma cells of stem and bark tissues.[429] Its biosynthesis has been extensively studied with several enzymatic steps now known, and genes encoding the corresponding proteins identified. Our current understanding of the Taxol® (**1**) biosynthetic pathway is summarized in **Figure 67**, and the reader is encouraged to review the substantial literature on this subject.[430,431] Of particular interest is the entry point to the pathway catalyzed by taxadiene synthase, which converts geranylgeranyl pyrophosphate (**199**) into taxa-4(5),11(12)-diene (**200**).[432–434] The remaining steps, whose genes and enzymes remain to be established, are also highlighted in **Figure 67**. Interestingly, formation of the oxetane ring has no biochemical precedence; however, this step has not yet been biochemically established.

3.27.4.5.1(iii) Quinine Quinine (**2**) is a quinoline-type alkaloid present in *Cinchona* tree bark, which together with related alkaloids can accumulate up to 16% of the bark weight.[435] Commonly used as an antimalarial, it is also a mild antipyretic/analgesic in common cold preparations. Additionally, it is useful in treating certain muscular disorders, especially nocturnal leg cramps, as well as being tested in numerous clinical trials for treating babesiosis (a serious or life-threatening illness). Although the precise site(s) of biosynthesis and accumulation are unknown, recent UV Raman spectroscopy of bark samples appear to provide visualization data that may be consistent with an axial parenchyma localization.[398]

As regard its biosynthesis, radiolabeling tracer studies in the 1970s[436–438] suggested it was derived from vincoside (**208**), and, more recently, a strictosidine synthase was purified from a *C. robusta* suspension culture.[439] Nothing is, however, known about any other pathway enzyme/gene or how pathway regulation is effectuated.



(**208**) Vincoside

3.27.4.5.1(iv) Theobromine, theophylline, and caffeine Xanthine alkaloids, theobromine (**181**), theophylline (**182**), and caffeine (**183**) are present in tissues of various woody species. All three are present in maté (*Ilex paraguarensis*) leaves,[400,401] and theobromine (**181**)/caffeine (**183**) (and ~300 other natural

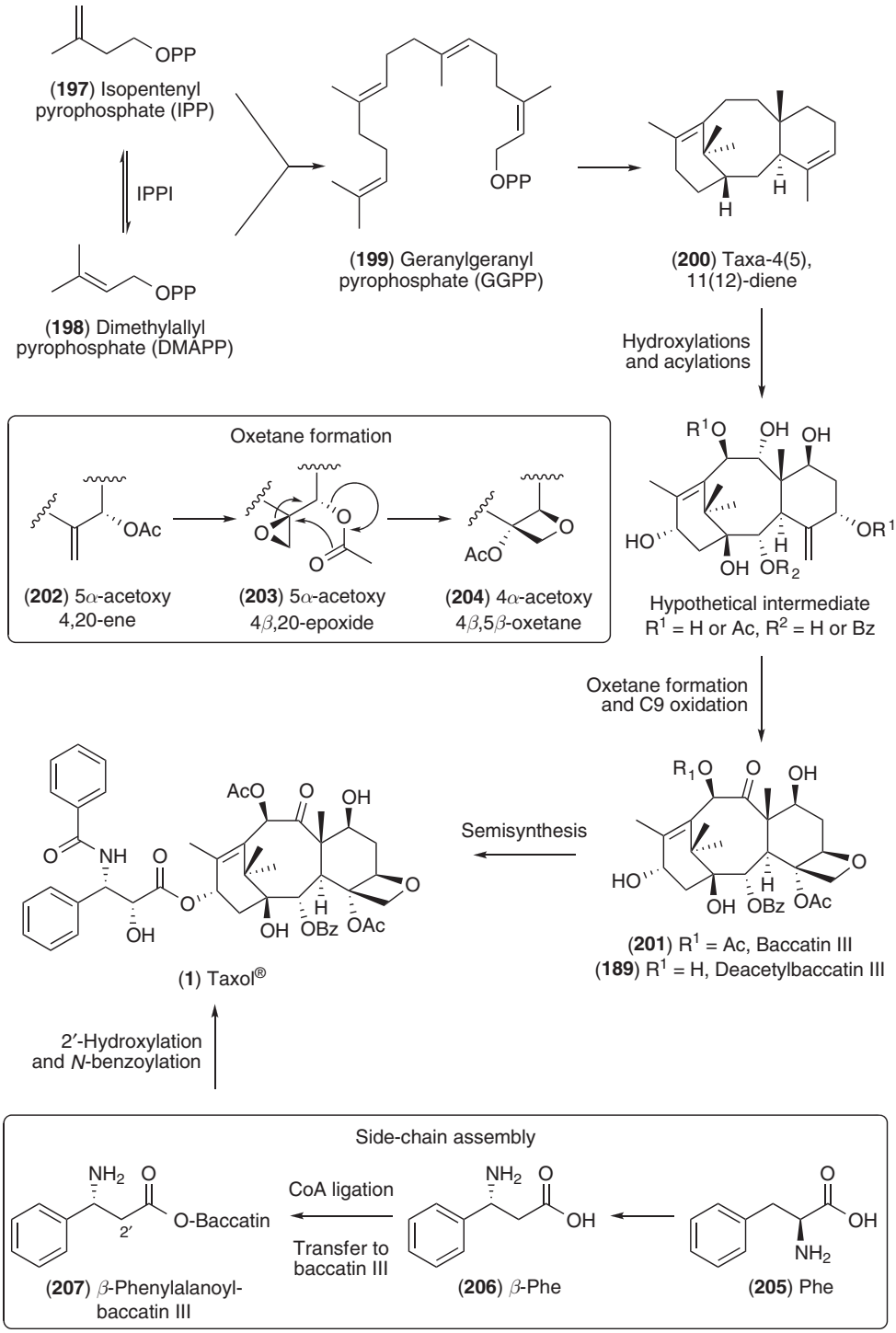


Figure 67 Current understanding of the Taxol® (**1**) biosynthetic pathway. Reproduced with permission of Springer from R. Croteau, R. E. B. Ketchum, R. M. Long, R. Kaspera, M. R. Wildung, *Phytochem. Rev*. **2006**, *5*, 75–97, copyright 2006.

products) accumulate in cocoa beans from cacao (*Theobroma cacao*).[440] Caffeine (**183**) is also part of the extracts from tea (*Camellia sinensis*) leaves and coffee (*Coffea arabica*) beans (see Chapters 3.21 and 3.22).

In general, these alkaloids find medicinal utility as stimulants and vasodilators. They are formed from xanthosine (**209**, **Figure 68**) and their biosynthetic pathway is described in detail in Chapters 3.21 and 3.22.

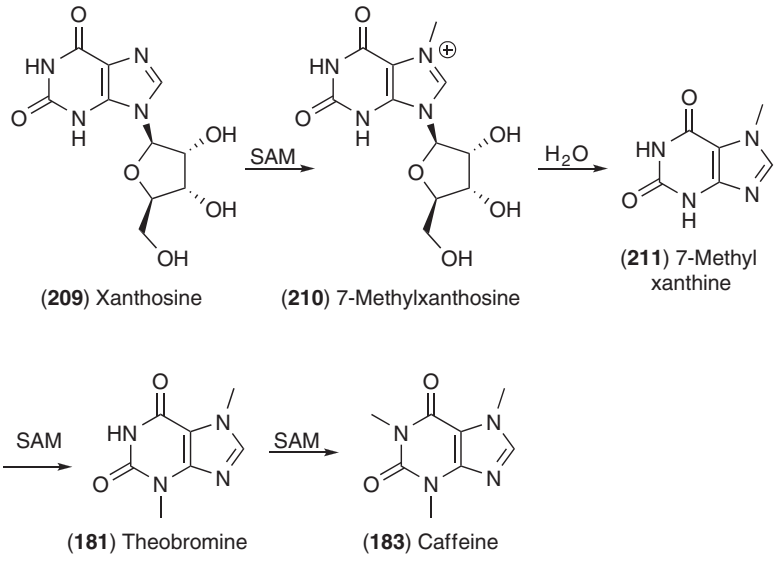


Figure 68 Biosynthetic pathway to the xanthine alkaloids, theobromine (**181**) and caffeine (**183**).

3.27.4.5.1(v) Yohimbine, reserpine, and ajmaline Yohimbine (**186**), reserpine (**187**), and ajmaline (**188**) are monoterpenoid indole alkaloids of the yohimbine and Coryanthe type. Reserpine (**187**) and ajmaline (**188**) accumulate in the root tissue of *Rauvolfia serpentina*,[405] and this plant species has been used medicinally for more than 3000 years. Clinically, reserpine (**187**) is used as an antihypertensive/sedative, whereas ajmaline (**188**) is employed for cardiac arrhythmias. Yohimbine (**186**), by contrast, is found in the bark of *Pausinystalia johimbe* (synonymous with *Coryanthe johimbe*). It has been used in traditional medicines in the treatment of angina and hypertension, but is more commonly employed in organic/psychogenic impotence, and used as an aphrodisiac.

Yohimbine (**186**), reserpine (**187**), and ajmaline (**188**) biosynthetic pathways have mainly been studied by the research groups of Meinhart Zenk[441–443] and Joachim Stöckigt[444–446] While the cell specificity and regulation have not been established, a significant amount of progress (intermediates, enzymes, and genes) has been established using cell cultures from *R. serpentina*. Their biosynthetic pathways go through either strictosidine (**193**) or vincoside (**208**) (see **Figures 69–71**), following which there are a number of intriguing structural rearrangements to afford the corresponding alkaloid types.

3.27.4.5.1(vi) Pilocarpine Pilocarpine (**185**) is an imidazole-type alkaloid present in jaborandi (*Pilocarpus pennatifolius*) leaves, which contain about 1% alkaloids with pilocarpine (**185**) amounting to about half.[404] It has been used for over 100 years for the treatment of chronic open-angle glaucoma and acute angle-closure glaucoma. It is also used as an antidote for scopolamine (**226**), atropine (**227**), and hyoscyamine (**228**) poisoning, in

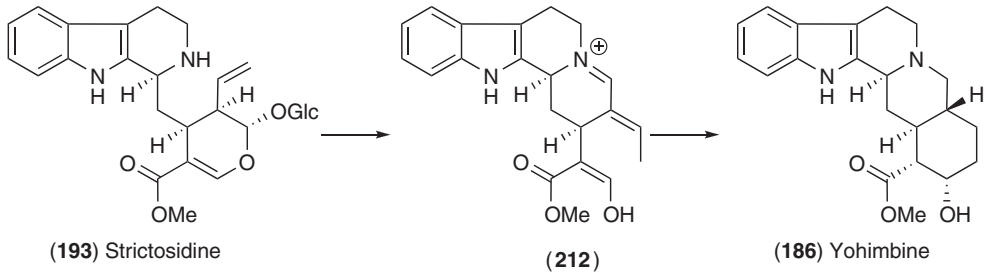


Figure 69 Biosynthetic pathway to yohimbine (**186**) in *Pausinystalia johimbe*.

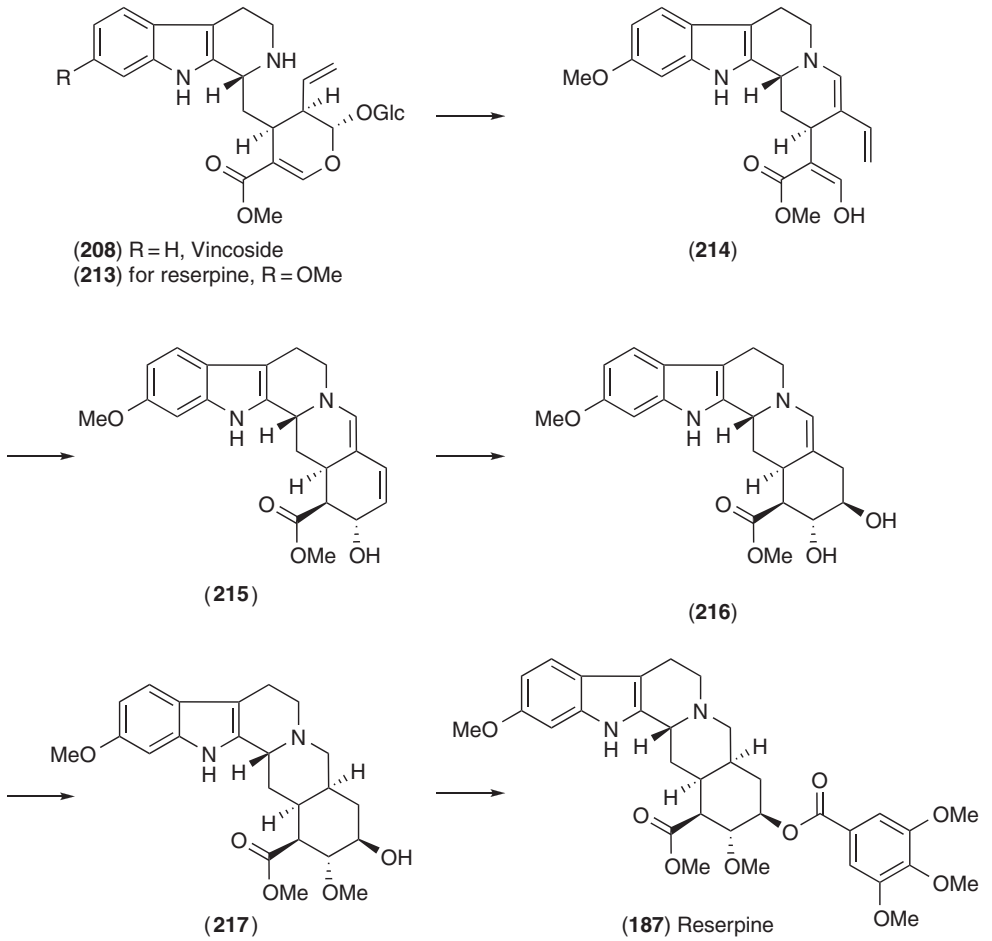


Figure 70 Biosynthetic pathway to reserpine (**187**) in *Rauvolfia serpentina*.

ophthalmology to reduce pupil size, and to treat dry mouth (xerostomia). The latter condition can occur, for example, as a side effect of radiation therapy for head/neck cancers. It also stimulates secretion of large amounts of saliva and sweat, and is used to diagnose cystic fibrosis. There are nine pharmaceuticals using pilocarpine (**185**), and it is currently in numerous clinical trials.

In terms of compartmentation and biosynthesis, nothing is yet known about intermediates, enzymes, genes, pathway regulation, and/or cellular location.

3.27.4.5.2 Terpenoids

Several woody plant species serve as sources of other important medicinals, either proven or in clinical trial, such as camphor (**5**) from the camphor tree (*Cinnamomum camphora*) where it accumulates in the bark. Camphor (**5**) is used medicinally as an ingredient in various medications to treat a number of conditions, such as coughing, itching, arthritis, excess mucus, flatulence, and other digestive conditions. Other triterpenoids include betulinic acid (**178**) derivatives from white birch (*Betula alba*), aescin (**229**), and related triterpene glycosides from horse chestnut (*Aesculus hippocastanum*), α- and β-boswellic acids (**230/231**) and derivatives from frankincense (*Boswellia* spp.), and the terpenic trilactone, ginkgolide A (**233**), ginkgolide B (**234**), and bilobalide (**235**) from Ginkgo (*Ginkgo biloba*). Finally, oleuropein (**236**), an oleoside-type secoiridoid is isolated from olives (*Olea europaea*). While these represent only a tiny fraction of the terpenoids with (potential) medicinal properties, it must be emphasized that many woody plant species continue to be investigated for bioactive terpenoids.

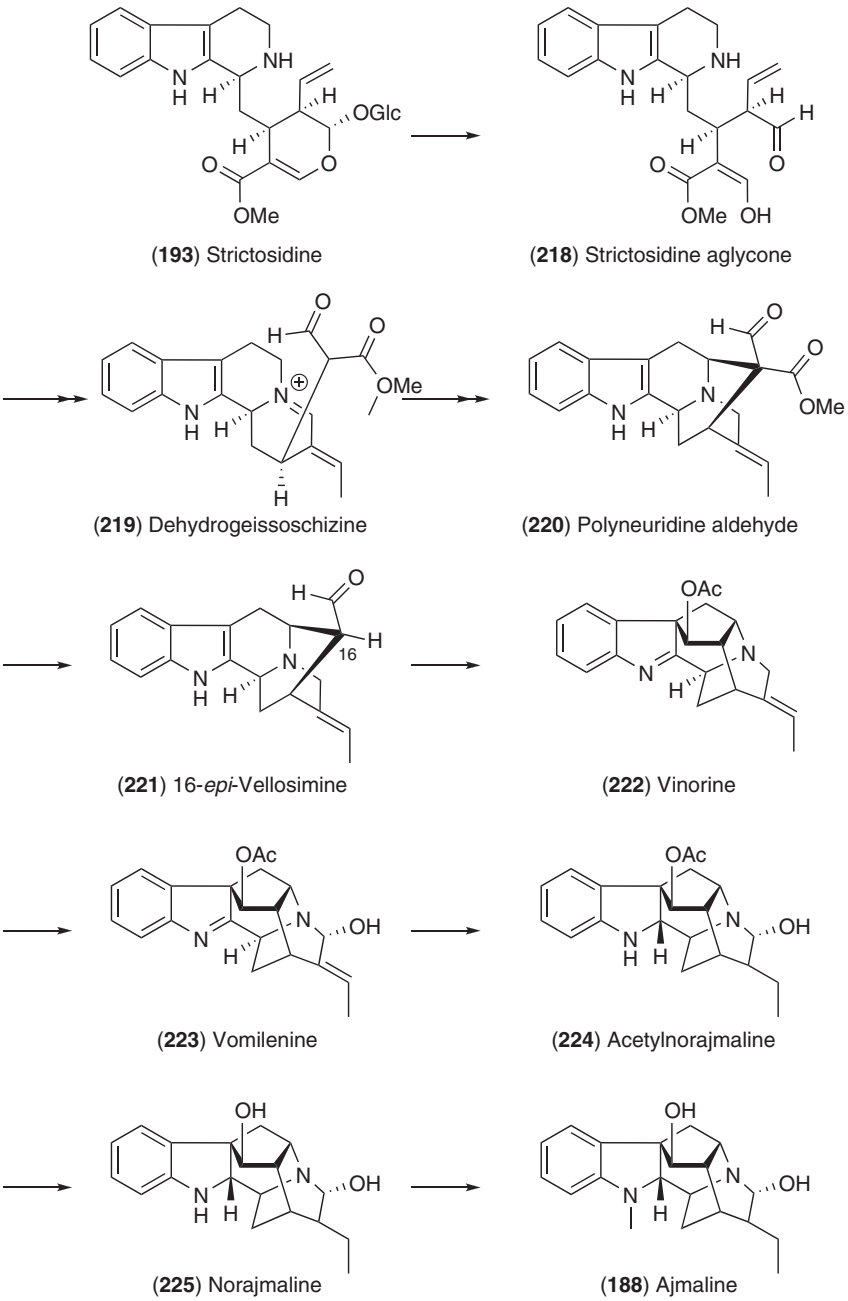
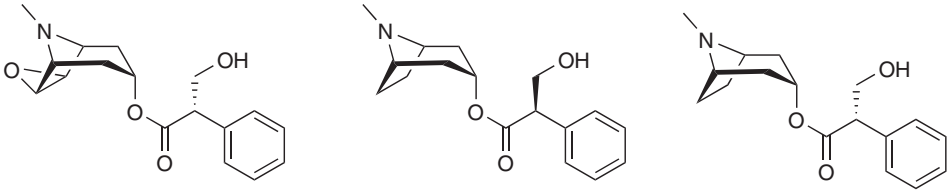


Figure 71 Biosynthetic pathway to ajmaline (**188**) in *Rauvolfia serpentina*.

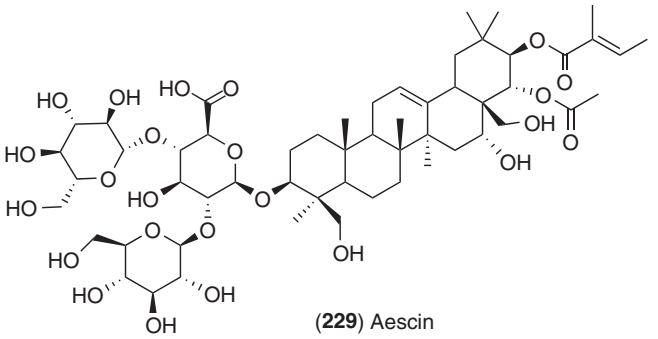


The general biosynthetic pathways to these terpene and triterpene derivatives are described in Chapters 1.15–1.18.

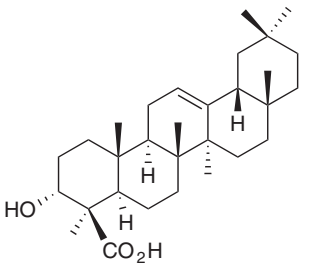
Table 4 Selected clinical trial of extracts from tree species

Latin name	*Common name*	*Compound(s)*	*Tissue occurrence*	*(Putative) cell type/ compartment*	*Medicinal properties*	*Clinical trials*
Aesculus hippocastanum (Sapindaceae)	Horse chestnut	Aescin (**229**) (triterpene glycosides)	Nut bark of young branches[407]	NR[a]	Antihypertensive	
Boswellia sacra, B. serrata (Burseraceae)	Frankincense	α- and β-boswellic acids (**230/231**) and derivatives	Bark	Resin duct: epithelial cells[408]	Inflammatory disease reaction including asthma, some arthritic conditions, some colon disorders[409]	Anticancer, osteoarthritis, and asthma effects
Cinnamonum verum (Lauraceae)	Cinnamon	Cinnamaldehyde (**232**)	Bark	Phloem oil cells, mucilage cells[222]	Diabetes treatment, antispasmodic, antifungal, antibacterial, carminative	Diabetes treatment and cardiac protection potential
Ginkgo biloba (Ginkgoaceae)	Ginkgo	Ginkgolide A (**233**) Ginkgolide B (**234**) Bilobalide (**235**)	Roots and leaves[410]	NR	Preventing/managing memory loss, improving circulation to brain and central nervous system, anti-inflammatory, antihistamines	Numerous
Olea europaea (Oleaceae)	Olive tree	Oleuropein (**236**)	Fruit: mesocarp,[411] leaves[412]	NR	Lowers blood pressure, antispasmodic, antioxidant, bone density	Bone density

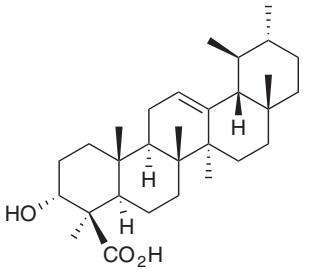
[a] NR = Not reported.



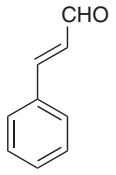
(**229**) Aescin



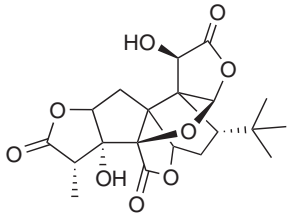
(**230**) α-Boswellic acid



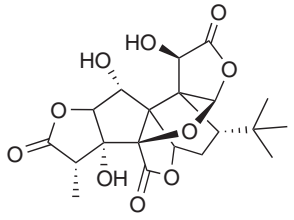
(**231**) β-Boswellic acid



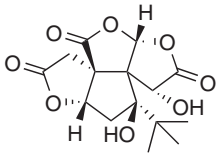
(**232**) Cinnamaldehyde



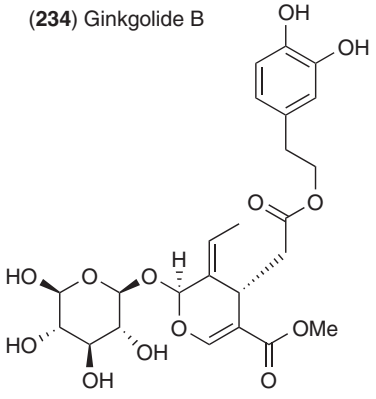
(**233**) Ginkgolide A



(**234**) Ginkgolide B



(**235**) Bilobalide



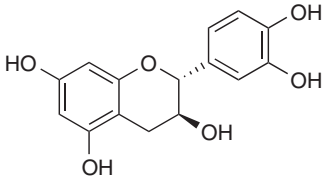
(**236**) Oleuropein

3.27.4.5.3 Aromatics/aromatic pathway plant medicinals

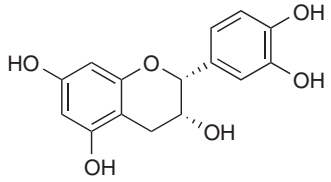
Plant products such as (–)-shikimic acid (184) from the Chinese star anise tree (*Illicium verum*)[402] are used to treat influenza, in the form of Oseltamivir (formerly Tamiflu), whereas methyl salicylate (**4**) from willow (*Salix*

spp.) is employed as an analgesic, antipyretic, and anti-inflammatory. The bulk of the other aromatics found in a large number of trees serve as antibacterials, such as cinnamaldehyde (**232**); antioxidants, astringents, and possible anticancer substances, such as catechin (**237**), *epi*-catechin (**238**), and derivatives; in helping improve bone density, such as hesperidin (**239**), or are being tested as possible anticancer/anti-HIV agents, such as rutin (**240**).

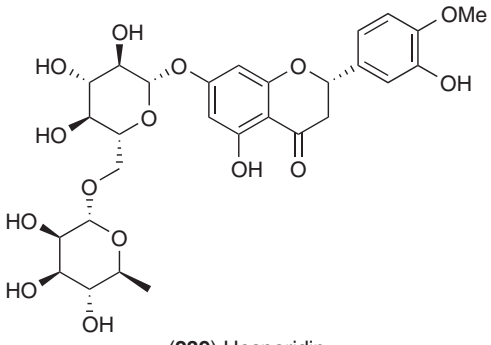
The biosynthetic pathways to these aromatic pathway compounds are described in Chapter 1.12.



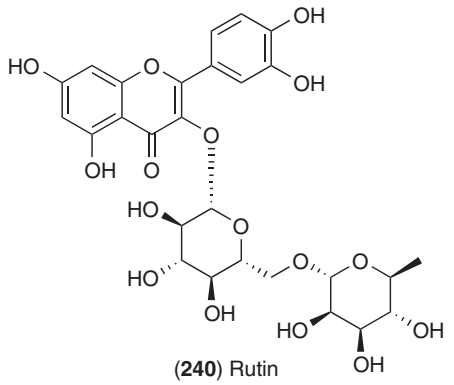
(**237**) Catechin



(**238**) *epi*-Catechin



(**239**) Hesperidin



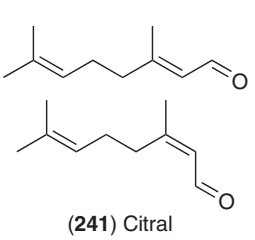
(**240**) Rutin

3.27.4.6 Spices and Food Additives

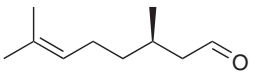
In addition to the extensive usage of tree-derived phytochemicals in medicinal/health-related applications, numerous plant constituents – again often present as complex mixtures – serve as either spices, food colorants, and/or food additives.

3.27.4.6.1 Spices

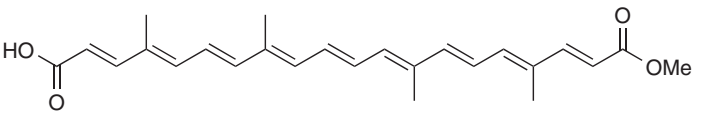
The history of many spice applications can be traced back to the dawn of civilization. These plant species have been selected for taste, odor, and the ability to help preserve foodstuffs. As can be gleaned from **Table 5**[447–495] and accompanying structures, many of the plant-derived spices are now very familiar household items, although their actual phytochemical constituents may not be. Most are of either terpene or phenolic (phenylpropanoid) origin, with only a few exceptions.



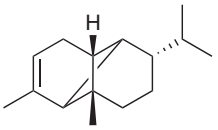
(**241**) Citral



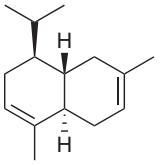
(**242**) Citronellal



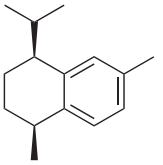
(**243**) Bixin



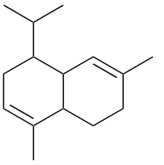
(**244**) Ylangene



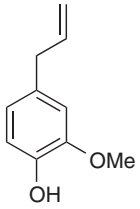
(**245**) β-Cadinene



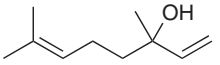
(**246**) Calamenene



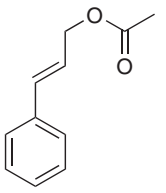
(**247**) 4,9-Cadinadiene



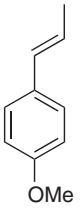
(**248**) Eugenol



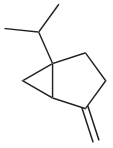
(**249**) Linalool



(**250**) Cinnamyl acetate

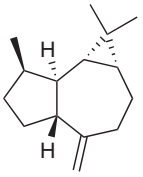


(**251**) Anethole

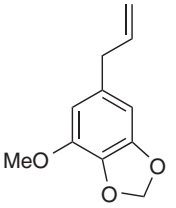


(**252**) Sabinene

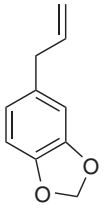
(**253**) 1,8-Cineole



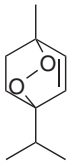
(**254**) Aromadendrene



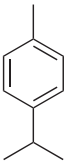
(**255**) Myristicin



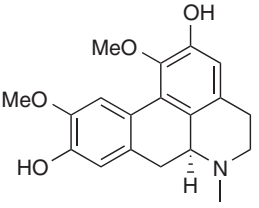
(**256**) Safrole



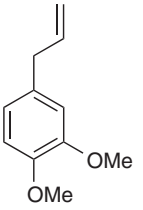
(**257**) Ascaridol



(**258**) *p*-Cymene



(**259**) Boldine



(**260**) Methyleugenol

Table 5 Selected spices and their characteristic chemical constituents from tree species

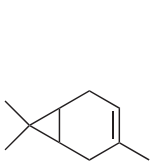
Latin name	*Common name*	*Part used*	*Characteristic metabolites*	*Native*	*Uses*
Backhousia citriodora (Myrtaceae)	Lemon myrtle	Leaves fresh or dried	Two chemotypes: 1. Citral (**241**) rich (80–95%) 2. L-citronellal (**242**) rich (85–89%)[447–450]	Subtropical rainforest of Queensland, Australia	Spice (cooking) Flavoring (beverage) Essential oil used for medicinal, cosmetic, and aromatherapy purposes[448]
Bixa orellana (Bixaceae)	Annatto, Roucou	Reddish pulp that surrounds the seeds	Bixin (**243**)[451–454]	Tropical regions of Americas	Flavoring and red food coloring, e.g., in cheeses (Cheddar, Red Leicester), margarine
Cinnamomum burmannii (Lauraceae)	Indonesian Cinnamon Java cassia, Fagot cassia, Padang cinnamon	Stem bark	Bark oil contains: Cinnamaldehyde (**232**, 92–98%), β-caryophyllene (**44**)[455,456]	Western Sumatra	Spice
Cinnamomum cassia (Lauraceae)	Chinese cassia	Stem bark	Bark oil contains: Cinnamaldehyde (**232**, 92–98%), β-caryophyllene (**44**), and ylangene (**244**)[457,458]	Southern China, Burma, Laos, Vietnam	Spice
Cinnamomum loureiroi (Lauraceae)	Vietnamese cinnamon	Stem bark	Cinnamyl aldehyde (**232**), copaene (**35**), β-cadinene (**245**), calamenene (**246**), and cadinadiene-4,9 (**247**)[459]	Northern Vietnam	Spice
Cinnamomum tamala (Lauraceae)	Indian Bay-leaf Indian cassia	Leaves	Several chemotypes identified: 1. Eugenol (**248**; ~74%)[460] 2. Cinnamaldehyde (**232**) and linalool (**249**)[461] 3. Linalool (**249**)[462,463] 4. β-Caryophyllene (**44**), linalool (**249**), and caryophyllene oxide (**55**)[464]	South slopes of the Himalayas	Spice
Cinnamomum zeylanicum (Lauraceae)	Cinnamon (true cinnamon)	Inner bark	Bark oil contains: Cinnamaldehyde (**232**, 85–93%), cinnamyl acetate (**250**), β-caryophyllene (**44**), linalool (**249**), and eugenol (**248**)[458,465]	Sri Lanka and South India	Spice

(*Continued*)

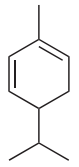
Table 5 (Continued)

Latin name	*Common name*	*Part used*	*Characteristic metabolites*	*Native*	*Uses*
Illicium verum (Illiciaceae)	Star anise Badiane	Pericarp	Anethole (**251**, up to 95% of the oil)[466]	Southwest China	Major component of garam masala in Indian cuisine. Used in Chinese and Malay Indonesian cuisine
Juniper communis (Cupressaceae)	Juniper	Berries	Essential oil is mainly composed of monoterpenes: α-pinene (**25**), limonene (**27**), myrcene (**53**), sabinene (**252**)[467–469]	Northern hemisphere	Flavoring of gin Sauerkraut Venison seasoning
Laurus nobilis (Lauraceae)	European bay leaves	Leaves	Cineole (**253**), α-pinene (**25**), β-pinene (**26**), linalool (**249**), eugenol (**248**), sabinene (**252**)[470]	Europe	Spice
Litsea cubeba (Lauraceae)	May chang	Fruit processed for its oil	Citral (**241**; 70–85% of the oil)[471]	China, Indonesia, Taiwan, and other parts of Southeast Asia	
Murraya koenigii (Rutaceae)	Curry tree Kadipatta	Leaves	Monoterpenes: β-phellandrene (**28**), α-pinene (**25**), and β-pinene (**26**) are more abundant in North Indian plants. Sesquiterpenes: β-caryophyllene (**44**) and aromadendrene (**254**) in South Indian plants[472]	India	Curry leaves should be used fresh or they lose their flavor. Curry powder is not made from curry leaves
Myristica fragrans (Myrtaceae)	Nutmeg	Seed endosperm	Contents depend whether nutmeg is from West Indian, Indian, or Sri-Lankan origin. Sabinene (**252**), α-pinene (**25**), β-pinene (**26**), myrcene (**53**), 1,8-cineole (**253**), myristicin (**255**), limonene (**27**), safrole (**256**)[473]	Banda Islands	Spice
Myristica fragrans (Myrtaceae)	Mace	Aril surrounding endosperm	Sabinene (**252**), α-pinene (**25**), β-pinene (**26**), myrcene (**53**), 1,8-cineole (**253**), myristicin (**255**), limonene (**27**), safrole (**256**)[473]	Banda Islands	Spice

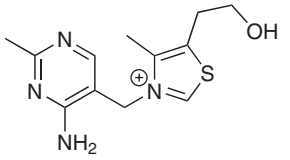
Myrtus communis (Myrtaceae)	Myrtle	Dried leaves Dried berries	Linalool (**249**), limonene (**27**), α-pinene (**25**), 1,8-cineole (**253**) are the main constituents of the oil[474–476]	North Western to Eastern Mediterranean	Flavor and fragrance industries
Peumus boldus (Monimiaceae)	Boldo leaves	Leaves	Ascaridol (**257**),[477] Limonene (**27**), *p*-cymene (**258**),1,8 cineole (**253**), β-phellandrene (**28**),[478] boldine (**259**)[479–482]	Chile (shrubby evergreen)	Spices
Pimenta dioica (Myrtaceae)	Allspice Jamaica pepper Kurundu Myrtle pepper Pimento Newspice	Dry berries (berries are picked when green and unripe)	Eugenol (**248**), methyleugenol (**260**), several flavonoids. Jamaica (**248** = 65–90%),[483,484] Mexico[485] (**260** = 50–60%)	West Indies, Southern Mexico, Central America	Spices
Sassafras albidum (Lauraceae)	Sassafras	Oil obtained from distillation of root bark	Safrole (**256**; up to 85% of the essential oil)[486]	Deciduous forest of Eastern USA	Flavoring agents (originally in root beer)
Schinus terebinthifolius (Anacardiaceae)	Pepper Rosé	Peppercorn	Δ3-Carene (**261**), α-pinene (**25**), α-phellandrene (**262**), limonene (**27**)[487]		Spice
Syzygium aromaticum (Myrtaceae)	Clove	Dried unopened flower buds	Eugenol (**248**; 80–95%)[488–491]	Banda Island	Spice
Tamarindus indica (Fabaceae)	Tamarind	Pulp from pods	Rich in thiamine (**263**)[492] 2-Acetylfuran (**264**) coupled with furfural (**265**) and 5-methylfurfural (**266**) contribute to the overall aroma of tamarind. The citrus component is attributed to limonene (**27**), terpinen-4-ol (**267**), α-terpineol (**268**), and geraniol (**269**).[493]	India	To flavor drinks, candies, sauces (Worcestershire), curries, chutney
Umbellularia californica (Lauraceae)	California bay leaves	Leaves	Umbellulone (**270**), 1,8-cineole (**253**), sabinene (**252**)[494]	Southern USA	Spice
Xylopia aethiopica (Annonaceae)	Negro pepper	Fruits	α-Pinene (**25**), sabinene (**252**), 1,8-cineole (**253**), germacrene D (**271**)[495]	Tropical Asia	Spice



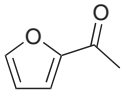
(**261**) Carene



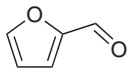
(**262**) α-Phellandrene



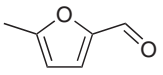
(**263**) Thiamine



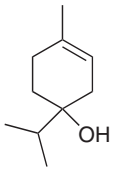
(**264**) 2-Acetylfuran



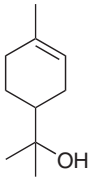
(**265**) Furfural



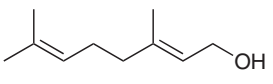
(**266**) Methylfurfural



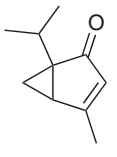
(**267**) Terpinen-4-ol



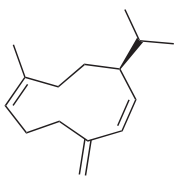
(**268**) α-Terpineol



(**269**) Geraniol



(**270**) Umbellulone

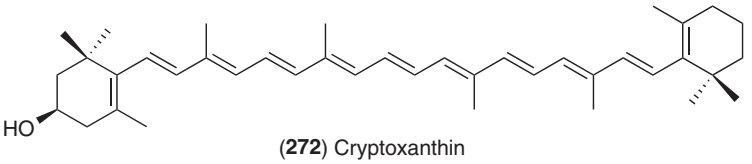


(**271**) Germacrene D

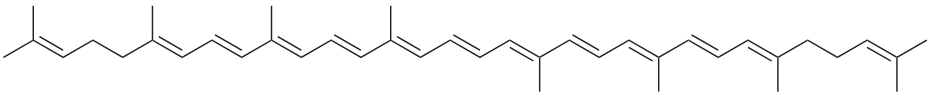
The biochemical pathways to the allyl/propenyl phenols, such as eugenol, have been comprehensively described in Chapter, as have the general pathways to the monoterpenes and sesquiterpenes (see Chapters 1.15 and 1.16). Little is yet known about the cell-types harboring many of these substances, and much needs to be done to place this on a sound scientific footing.

3.27.4.6.2 Food additives

Many tree-derived substances are used as food additives (**Table 6**).[496–507,509–511] These range from colorants, such as the bixin (**243**) used as a red colorant in cherries and margarine, to lycopene (**272**), cryptoxanthin (**273**), or tannins as other food colorants. Food additives can serve as thickening and/or gelling agents, including gum arabic, carob gum, gum karanya, and sassafras, these being of carbohydrate origins. The vast majority of these additives are, however, in flavoring applications.



(**272**) Cryptoxanthin



(**273**) Lycopene

3.27.5 Concluding Remarks

The putative evolutionary origins and adaptations of the woody growth habit, which facilitated colonization of land were first discussed, with this necessitating a brief consideration of the changes in woody plant anatomies,

Table 6 Selected food additives from tree species

Latin name	*Common name*	*Family*	*Compound(s)*	*Tissue*	*Food additive attributes*
Acacia catechu	Catechu, Cachou	Fabaceae	Catechu extract	Heartwood[496]	Flavoring
Acacia senegal	Gum Arabic	Fabaceae	Gum Arabic	Cambium (no duct structures)[497]	Thickener, gum, stabilizer, emulsifier
Bixa orellana	Annatto	Bixaceae	Bixin (**243**)	Seeds[498]	Coloring
Ceratonia siliqua	Carob	Fabaceae	Galactomannan	Seeds[499]	Carob gum: Thickener, gelling agent
Citrus spp.	E.g., lemon, orange, grapefruit trees	Rutaceae	Citric acid	Fruit (juice)[500]	Acidifier, flavoring
e.g., *Citrus* spp., *Carica papaya*, *Malus domestica*	Citrus spp., papaya, and apple trees (and many subtropical fruits)	Rutaceae, Caricaceae Rosaceae	Cryptoxanthin (**272**)	Fruit (juice) vesicle plastids[501]	Coloring
Citrus × *paradisi*	Grapefruit	Rutaceae	Lycopene (**273**)	Fruit pulp[502]	Coloring
Juniperus communis	Common Juniper	Cupressaceae	Biflavones, diterpenes	Berry tubercules, leaf oleoresin ducts[503]	Berry extract: Flavoring
Mangifera spp.	Mango	Anacardiaceae	Amchur/Mango powder	Dried raw unripe fruit[504]	Flavoring
Myroxylon spp.	Quina, Tolu	Fabaceae	Peru/Tolu balsam	Resin ducts[160]	Flavoring
Prunus dulcis	Almond	Rosaceae	Linoleic and oleic acids, triglycerides	Almond (nut) kernel[505,506]	Almond oil: Cooking oil, flavoring
Quercus spp.	Oak	Fagaceae	Tannins	Heartwood[507]	Coloring, flavoring
Sassafras spp.	Sassafras	Lauraceae	Citral (**241**)[508]	Leaf oil cells[509]	Filé powder/gumbo filé: Thickener, flavoring
Sterculia spp.	Tropical chestnut	Malvaceae	Gum Karaya	Gum duct[510,511]	Thickener, gum, stabilizer, emulsifier

architectures, and cellular/tissue functions that were manifested over hundreds of millions of years. Consideration was also given to the emergence of some of the truly fascinating biochemical processes that occurred during woody plant evolution, in terms of their growth, development, and reproductive processes (including plant–pollinator/seed dispersal systems), and to their defense functions (such as plant–pathogen, plant–insect, plant–plant, and plant–herbivore interactions). As regards the latter, particular emphasis was placed upon the oft species-specific phytochemical-derived bioactivities that evolved, as additional biochemical pathways (both constitutive and inducible) emerged over the passage of time. Among others, this included various secretory processes in trees, such as those involved in HW formation and their associated specialized metabolic biochemistries, which often afford distinctive bioactive, species-specific, constituents. Bark formation was briefly considered as well, including what is known about some of the biochemical pathways (and the cell types involved) that lead to its bioactive constituents.

In terms of the exciting progress made and promise for the future, this contribution would not have been complete without consideration of current and potential biotechnological manipulations of woody plant cell wall formation/structure (composition, content, and architecture) as well. This is of topical interest given the rapidly emerging needs for useful fast-growing woody species as a source of 'both' sustainable renewable (bio)energy and other bioproduct streams, including that for future petrochemical substitutes. In this context, HW and bark manipulations also potentially represent important biotechnological targets for tree improvement and/or downstream use by humanity, and progress made therein is described and discussed.

This contribution additionally addresses some of the historical uses of woods, which often reflect their biological (bioactivity) properties, and the underlying reasons for the same. These ranged from some of the myriad reasons for human utilization of wood, to a summation of selected examples of their distinctive biological roles in plant defense(s) against pathogens/pests, and so on. Indeed, such properties were often central in determining suitability and uses of a particular wood species for a specific application, whether for lumber, furniture, and so forth, or for applications as specialty intermediate chemicals, medicinals, and/or in various foodstuffs.

While not discussed in any detail, in terms of postharvest chemistries and material properties of wood resources, humanity has also developed various chemically harsh processes for wood-pulping operations, and others for producing specific wood product composites (plywood, particleboard, waferboard, etc.). Of these, however, the wood-pulping processes are largely derived from the nineteenth/twentieth century technologies. Accordingly, there is much interest, if not an urgent need, to now develop new transformative technologies to identify more facile means to produce such products and/or improved versions thereof. In an analogous manner, our wood resources increasingly need to be considered in terms of suitability for applications in high-performance composites and other specialty applications, including nanochemistry.

Acknowledgments

The authors thank the U.S. Department of Energy (DE FG03-97ER20259), the U.S. National Science Foundation (MCB-0417291), the United States Department of Agriculture (Agricultural Plant Biochemistry #2006-03339), the National Institute of General Medical Sciences (1RC2MØ92561-Ø1) the BioEnergy Science Center, the U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science, McIntire Stennis, and the G. Thomas and Anita Hargrove Center for Plant Genomic Research for generous financial support.

References

1. M. A. Bernards, *Can. J. Bot.* **2002**, *80*, 227–240.
2. S. P. Silva; M. A. Sabino; E. M. Fernandes; V. M. Correlo; L. F. Boesel; R. L. Reis, *Int. Mater. Rev.* **2005**, *50*, 345–365.
3. S. Oldfield; C. Lusty; A. MacKinven, *The World List of Threatened Trees*; World Conservation Press: Cambridge, UK, 1998; p 650.
4. M. Bologna; J. C. Flores, *Europhys. Lett.* [Online] **2008**, *81*, e48006.
5. R. W. Scotland; A. H. Wortley, *Taxon* **2003**, *52*, 101–104.

6. J. D. Palmer; D. E. Soltis; M. W. Chase, *Am. J. Bot.* **2004**, *91*, 1437–1445.
7. E. M. Gifford; A. S. Foster, *Morphology and Evolution of Vascular Plants*, 3rd ed.; W.H. Freeman and Company: New York, 1989; p 626.
8. R. M. Bateman; P. R. Crane; W. A. DiMichele; P. R. Kenrick; N. P. Rowe; T. Speck; W. E. Stein, *Annu. Rev. Ecol. Syst.* **1998**, *29*, 263–292.
9. L. E. Graham, *Origin of Land Plants*; John Wiley and Sons, Inc.: New York, 1993; p 287.
10. N. Rowe; T. Speck, Hydraulics and Mechanics of Plants: Novelty, Innovation and Evolution. In *The Evolution of Plant Physiology: From Whole Plants to Ecosystems*; A. R. Hemsley, I. Poole, Eds.; Elsevier Academic Press: London, 2004; pp 297–325.
11. D. Edwards, *New Phytol.* **1993**, *125*, 225–247.
12. P. Kenrick; P. R. Crane, *The Origin and Early Diversification of Land Plants. A Cladistic Study*; Smithsonian Institution Press: Washington, DC, 1997; p 441.
13. D. Edwards; K. L. Davies; L. Axe, *Nature* **1992**, *357*, 683–685.
14. H. A. Core; W. A. Côté; A. C. Day, *Wood Structure and Identification*, 2nd ed.; Syracuse University Press: Syracuse, NY, 1979; Vol. 6, p 182.
15. K. Esau, *Plant Anatomy*, 2nd ed.; John Wiley and Sons, Inc.: New York, 1965; p 767.
16. C. K. Boyce; G. D. Cody; M. L. Fogel; R. M. Hazen; C. M. O. D. Alexander; A. H. Knoll, *Int. J. Plant Sci.* **2003**, *164*, 691–702.
17. M. E. Cook; W. E. Friedman, *Int. J. Plant Sci.* **1998**, *159*, 881–890.
18. W. E. Friedman; M. E. Cook, *Philos. Trans. R Soc. Lond. B – Biol. Sci.* **2000**, *355*, 857–868.
19. G. Ewbank; D. Edwards; G. D. Abbott, *Org. Geochem.* **1996**, *25*, 461–473.
20. N. G. Lewis; E. Yamamoto, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1990**, *41*, 455–496.
21. J. A. Raven, *Adv. Bot. Res.* **1977**, *5*, 153–219.
22. K. J. Niklas, *Plant Biomechanics: An Engineering Approach to Plant Form and Function*; The University of Chicago Press: Chicago, IL, 1992; p 607.
23. D. A. DeMason, *Am. J. Bot.* **1983**, *70*, 955–962.
24. M. F. Large; J. E. Braggins, *Tree Ferns*; Timber Press: Portland, OR, 2004; p 359.
25. N. Rowe; T. Speck, *New Phytol.* **2005**, *166*, 61–72.
26. W. E. Stein; F. Mannolini; L. V. Hernick; E. Landing; C. M. Berry, *Nature* **2007**, *446*, 904–907.
27. M. J. Donoghue, *Paleobiology* **2005**, *31*, 77–93.
28. M. Bernal; A. Navas; S. Madriñan, In *Developmental Morphology and Growth Patterns of the Wax Palm Ceroxylon quindiuense.* Botany & Plant Biology Joint Congress, Chicago, IL, 2007; abstract 2333.
29. M. Hirmer, *Handbuch der Paläobotanik*; Druck und Verlag von R. Oldenbourg: München und Berlin, 1927.
30. W. A. Dimichele; T. L. Phillips, *Rev. Palaeobot. Palynol.* **1985**, *44*, 1–26.
31. R. A. Savidge, *Forest Chron.* **2008**, *84*, 498–503.
32. C. B. Beck, *Am. J. Bot.* **1962**, *49*, 373–382.
33. T. Speck; N. P. Rowe, *J. Plant Res.* **1994**, *107*, 443–460.
34. R. A. Savidge, *Bull. Geosci.* **2007**, *82*, 301–328.
35. P. S. Soltis; D. E. Soltis, *Am. J. Bot.* **2004**, *91*, 1614–1626.
36. T. S. Feild; N. C. Arens; J. A. Doyle; T. E. Dawson; M. J. Donoghue, *Paleobiology* **2004**, *30*, 82–107.
37. K. Takahashi; M. Suzuki, *IAWA J.* **2003**, *24*, 269–309.
38. B. G. Butterfield, Wood Anatomy in Relation to Wood Quality. In *Wood Quality and Its Biological Basis*; J. R. Barnett, G. Jeronimidis, Eds.; Blackwell Publishing, Ltd.: Oxford, 2003; pp 30–52.
39. J. D. Mauseth, *Plant Anatomy*; The Benjamin/Cummings Publishing Company, Inc.: Menlo Park, CA, 1988; p 560.
40. A. C. Wiedenhoeft; R. B. Miller, Structure and Function of Wood. In *Handbook of Wood Chemistry and Wood Composites*; R. M. Rowell, Ed.; CRC Press: Boca Raton, FL, 2005; pp 9–33.
41. T. M. Hinckley; P. J. Schulte, Stems in the Biology of the Tissue, Organism, Stand, and Ecosystem. In *Plant Stems: Physiology and Functional Morphology*; B. L. Gartner, Ed.; Academic Press, Inc.: San Diego, CA, 1995; pp 409–428.
42. A. L. Shigo, *Annu. Rev. Phytopathol.* **1984**, *22*, 189–214.
43. M. Tsiantis; A. Hay, *Nat. Rev. Genet.* **2003**, *4*, 169–180.
44. P. H. Raven; R. F. Evert; S. E. Eichhorn, *Biology of Plants*, 7th ed.; W.H. Freeman and Company Publishers: New York, 2005; p 686.
45. P. R. Larson; D. E. Kretschmann; A. Clark, III; J. G. Isebrands, Formation and Properties of Juvenile Wood in Southern Pines: A Synopsis. In *U.S.D.A., F. S.*, Forest Products Laboratory, Madison, WI, 2001; p 42.
46. P. Saranpää, Wood Density and Growth. In *Wood Quality and Its Biological Basis*; J. R. Barnett, G. Jeronimidis, Eds.; Blackwell Publishing Ltd.: Oxford, UK, 2003; pp 87–117.
47. R. F. Evert, *Esau's Plant Anatomy. Meristems, Cells, and Tissues of the Plant Body: Their Structure, Function and Development;* 3rd Ed. John Wiley and Sons, Inc.: Hoboken, NJ, 2006; p 601.
48. J. R. Barnett; V. A. Bonham, *Biol. Rev.* **2004**, *79*, 461–472.
49. T. E. Timell, *Compression Wood in Gymnosperms*; Springer-Verlag: Berlin, 1986; Vol. 1: *Bibliography, Historical Background, Determination, Structure, Chemistry, Topochemistry, Physical Properties, Origin, and Formation of Compression Wood*, p 2150.
50. D. E. Kretschmann, *Properties of Juvenile Wood, Techline document: VI-7.* USDA Forest Service, Forest Products Laboratory: Madison, WI, 1998. http://www.fpl.fs.fed.us
51. F. C. Bao; Z. H. Jiang; X. M. Jiang; X. X. Lu; X. Q. Luo; S. Y. Zhang, *Wood Sci. Technol.* **2001**, *35*, 363–375.
52. A. Fahn, *Plant Anatomy*, 3rd ed.; Pergamon Press: Oxford, 1982.
53. R. Spicer, Senescence in Secondary Xylem: Heartwood Formation as an Active Developmental Program. In *Vascular Transport in Plants*; N. M. Holbrook, M. A. Zwieniecki, Eds.; Elsevier Academic Press: San Diego, CA, 2005; pp 457–475.
54. F. C. Meinzer; B. J. Bond; J. M. Warren; D. R. Woodruff, *Funct. Ecol.* **2005**, *19*, 558–565.
55. H. Ziegler, *Holz Roh Werkst.* **1968**, *26*, 61–68.
56. M. Kwon; D. L. Bedgar; W. Piastuch; L. B. Davin; N. G. Lewis, *Phytochemistry* **2001**, *57*, 847–857.

57. J. R. Barnett; G. Jeronimidis, Reaction Wood. In *Wood Quality and Its Biological Basis*; J. R. Barnett, G. Jeronimidis, Eds.; CRC Press LLC: Boca Raton, FL, 2003; pp 118–136.
58. F. W. Telewski, *IAWA Bull.* **1988**, *9*, 269–274.
59. P. Karenlampi, *Paperi ja Puu – Pap. Timber* **1992**, *74*, 650–664.
60. H. Lohrasebi; W. E. Mabee; D. N. Roy, *J. Wood Chem. Technol.* **1999**, *19*, 13–25.
61. N. Terziev; G. Daniel; A. Marklund, *Holzforschung* **2008**, *62*, 149–153.
62. C. Coutand; G. Jeronimidis; B. Chanson; C. Loup, *Wood Sci. Technol.* **2004**, *38*, 11–24.
63. C. Plomion; G. Leprovost; A. Stokes, *Plant Physiol.* **2001**, *127*, 1513–1523.
64. D. R. Gang; M. Fujita; L. B. Davin; N. G. Lewis, The 'Abnormal Lignins': Mapping Heartwood Formation Through the Lignan Biosynthetic Pathway. In *Lignin and Lignan Biosynthesis*; N. G. Lewis, S. Sarkanen, Eds.; ACS Symposium Series; American Chemical Society. Washington, DC, 1998; Vol. 697, pp 389–421.
65. M. Chattaway, *Aust. For.* **1952**, *16*, 25–34.
66. J. A. F. Gardner; G. M. Barton; H. MacLean, *Can. J. Chem.* **1959**, *37*, 1703–1709.
67. J. A. F. Gardner; B. F. MacDonald; H. MacLean, *Can. J. Chem.* **1960**, *38*, 2387–2394.
68. J. A. F. Gardner; E. P. Swan; S. A. Sutherland; H. MacLean, *Can. J. Chem.* **1966**, *44*, 52–58.
69. W. Mayer; W. Gabler; A. Riester; H. Korger, *Justus Liebigs Ann. Chem.* **1967**, *707*, 177–181.
70. W. Mayer; H. Seitz; J. C. Jochims; K. Schauerte; G. Schilling, *Justus Liebigs Ann. Chem.* **1971**, *751*, 60–68.
71. B. Fernández de Simón; E. Cadahía; E. Conde; M. C. García-Vallejo, *Holzforschung* **1999**, *53*, 147–150.
72. E. Zavarin; K. Snajberk, *Tappi J.* **1963**, *46*, 320–323.
73. E. Zavarin; K. Snajberk; R. M. Smith, *Tappi J.* **1965**, *48*, 574–577.
74. W. E. Hillis; P. Soenardi, *IAWA J.* **1994**, *15*, 425–437.
75. E. Magel; A. Abdel-Latif; R. Hampp, *Holzforschung* **2001**, *55*, 135–145.
76. W. J. Croft, *Under the Microscope: A Brief History of Microscopy*; World Scientific Publishing Co.: Hackensack, NJ, 2006; p 138.
77. S. Dinant, *C. R. Biol.* **2008**, *331*, 334–346.
78. R. Turgeon; S. Wolf, *Annu. Rev. Plant Biol.* **2009**, *60*, 207–221.
79. A. J. E. Van Bel, *Plant Cell Environ.* **2003**, *26*, 125–149.
80. T. Krekling; V. R. Franceschi; A. A. Berryman; E. Christiansen, *Flora (Jena)* **2000**, *195*, 354–369.
81. V. R. Franceschi; P. Krokene; E. Christiansen; T. Krekling, *New Phytol.* **2005**, *167*, 353–375.
82. K. J. Lendzian, *J. Exp. Bot.* **2006**, *57*, 2535–2546.
83. B. Groh; C. Hübner; K. J. Lendzian, *Planta* **2002**, *215*, 794–801.
84. M. A. Bernards; N. G. Lewis, *Phytochemistry* **1998**, *47*, 915–933.
85. P. M. Fernandes; J. A. Vega; E. Jiménez; E. Rigolot, *For. Ecol. Manage.* **2008**, *256*, 246–255.
86. T. E. Kolb; J. K. Agee; P. Z. Fulé; N. G. McDowell; K. Pearson; A. Sala; R. H. Waring, *For. Ecol. Manage.* **2007**, *249*, 141–157.
87. V. R. Franceschi; P. Krokene; T. Krekling; E. Christiansen, *Am. J. Bot.* **2000**, *87*, 314–326.
88. J. W. Hudgins; E. Christiansen; V. R. Franceschi, *Tree Physiol.* **2004**, *24*, 251–264.
89. M. Trockenbrodt, *Ann. Bot.* **1995**, *75*, 281–284.
90. J. W. Hudgins; T. Krekling; V. R. Franceschi, *New Phytol.* **2003**, *159*, 677–690.
91. R. Spicer; N. M. Holbrook, *J. Exp. Bot.* **2007**, *58*, 1313–1320.
92. R. O. Teskey; A. Saveyn; K. Steppe; M. A. McGuire, *New Phytol.* **2008**, *177*, 17–32.
93. P. Baas; F. W. Ewers; S. D. Davis; E. A. Wheeler, Evolution of Xylem Physiology. In *The Evolution of Plant Physiology*; A. R. Hemsley, I. Poole, Eds.; Elsevier Academic Press: London, 2004; pp 273–295.
94. M. J. Clearwater; G. Goldstein, Embolism Repair and Long Distance Water Transport. In *Vascular Transport in Plants*; N. M. Holbrook, M. A. Zwieniecki, Eds.; Elsevier, Inc.: San Diego, CA, 2005; pp 375–399.
95. A. J. Panshin; C. de Zeeuw, *Textbook of Wood Technology. Structure, Identification, Properties, and Uses of the Commercial Woods of the United States and Canada*, 4th ed.; McGraw-Hill Book Company: New York, 1980; p 722.
96. S. G. Pallardy, *Physiology of Woody Plants*, 3rd ed.; Elsevier: San Diego, CA, 2008; p 454.
97. P. Cruiziat; H. Cochard; T. Améglio, *Ann. Sci. For.* **2002**, *59*, 723–752.
98. B. J. Enquist; G. B. West; E. L. Charnov; J. H. Brown, *Nature* **1999**, *401*, 907–911.
99. U. G. Hacke; J. S. Sperry; W. T. Pockman; S. D. Davis; K. A. McCulloch, *Oecologia* **2001**, *126*, 457–461.
100. L. Donaldson, *IAWA J.* **2008**, *29*, 345–386.
101. P. J. Harris; B. A. Stone, Chemistry and Molecular Organization of Plant Cell Walls. In *Biomass Recalcitrance. Deconstructing the Plant Cell Wall for Bioenergy*; M. E. Himmel, Ed.; Blackwell Publishing: Oxford, UK, 2008; pp 61–93.
102. D. Mohnen; M. Bar-Peled; C. Somerville, Cell Wall Polysaccharide Synthesis. In *Biomass Recalcitrance. Deconstructing the Plant Cell Wall for Bioenergy*; M. E. Himmel, Ed.; Blackwell Publishing: Oxford, UK, 2008; pp 94–187.
103. L. B. Davin; A. M. Patten; M. Jourdes; N. G. Lewis, Lignins: A Twenty-First Century Challenge. In *Biomass Recalcitrance. Deconstructing the Plant Cell Wall for Bioenergy*; M. E. Himmel, Ed.; Blackwell Publishing: Oxford, UK, 2008; pp 213–305.
104. L. B. Davin; M. Jourdes; A. M. Patten; K. W. Kim; D. G. Vassao; N. G. Lewis, *Nat. Prod. Rep.* **2008**, *25*, 1015–1090.
105. G. Brunow; I. Kilpeläinen; J. Sipilä; K. Syrjänen; P. Karhunen; H. Setälä; P. Rummakko, Oxidative Coupling of Phenols and the Biosynthesis of Lignin. In *Lignin and Lignan Biosynthesis*; N. G. Lewis, S. Sarkanen, Eds.; ACS Symposium Series; American Chemical Society. Washington, DC, 1998; Vol. 697, pp 131–147.
106. B. J. Fergus; A. R. Proctor; J. A. N. Scott; D. A. I. Goring, *Wood Sci. Technol.* **1969**, *3*, 117–138.
107. S. Saka; D. A. I. Goring, Localization of Lignins in Wood Cell Walls. In *Biosynthesis and Biodegradation of Wood Components*; T. Higuchi, Ed.; Academic Press: Orlando, FL, 1985; pp 51–62.
108. P. Whiting; D. A. I. Goring, *Wood Sci. Technol.* **1982**, *16*, 261–267.
109. A. L. Samuels; M. Kaneda; K. H. Rensing, *Can. J. Bot.-Rev. Can. Bot.* **2006**, *84*, 631–639.
110. H. Fukuda, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1996**, *47*, 299–325.
111. H. Fukuda, *Plant Mol. Biol.* **2000**, *44*, 245–253.
112. A. H. L. A. N. Gunawardena, *J. Exp. Bot.* **2008**, *59*, 445–451.

113. B. L. Gartner, Patterns of Xylem Variation within a Tree and their Hydraulic and Mechanical Consequences. In *Plant Stems: Physiology and Functional Morphology*; B. L. Gartner, Ed.; Academic Press: San Diego, CA, 1995; pp 125–149.
114. R. Spicer; B. L. Gartner, *Tree Physiol.* **1998**, *18*, 777–784.
115. A. M. Anterola; N. G. Lewis, *Phytochemistry* **2002**, *61*, 221–294.
116. Y. Watanabe; Y. Kojima; T. Ona; T. Asada; Y. Sano; K. Fukazawa; R. Funada, *IAWA J.* **2004**, *25*, 283–295.
117. U. G. Hacke; J. S. Sperry; J. Pitterman, Efficiency Versus Safety Tradeoffs for Water Conduction in Angiosperm Vessels Versus Gymnosperm Tracheids. In *Vascular Transport in Plants*; N. M. Holbrook, M. A. Zwieniecki, Eds.; Elsevier, Inc.: San Diego, CA, 2005; pp 333–353.
118. B. Jourez; A. Riboux; A. Leclercq, *IAWA J.* **2001**, *22*, 133–157.
119. B. J. Fergus; D. A. I. Goring, *Holzforschung* **1970**, *24*, 118–124.
120. B. J. Fergus; D. A. I. Goring, *Holzforschung* **1970**, *24*, 113–117.
121. W. C. Dickison, *Integrative Plant Anatomy*; Academic Press: San Diego, CA, 2000; p 533.
122. A. B. Wardrop, In *The Reaction Anatomy of Arborescent Angiosperms.* The Formation of Wood in Forest Trees. The Second Symposium Held under the Auspices of the Maria Moors Cabot Foundation for Botanical Research, Harvard Forest, April, 1963; M. H. Zimmermann, Ed.; Academic Press: New York, 1964; pp 405–456.
123. A. M. Patten; M. Jourdes; E. E. Brown; M.-P. Laborie; L. B. Davin; N. G. Lewis, *Am. J. Bot.* **2007**, *94*, 912–925.
124. N. T. Mirov, *Annu. Rev. Biochem.* **1948**, *17*, 521–540.
125. T. Reynolds, *Phytochemistry* **2007**, *68*, 2887–2895.
126. E. Magel; S. Hauch; L. De Filippis, Random Amplification of Polymorphic DNA and Reverse Transcription Polymerase Chain Reaction of RNA in Studies of Sapwood and Heartwood. In *Wood Formation in Trees: Cell and Molecular Biology Techniques*; N. Chaffey, Ed.; Taylor and Francis: London, 2002; pp 319–337.
127. A. M. Patten; L. B. Davin; N. G. Lewis, *Phytochemistry* **2008**, *69*, 3032–3037.
128. N. Chaffey; P. Barlow, *Planta* **2001**, *213*, 811–823.
129. W. E. Hillis, *Heartwood and Tree Exudates*; Springer-Verlag: Berlin, 1987.
130. W. E. Hillis, The Formation of Heartwood and Its Extractives. An Overview. In *Phytochemicals in Human Health Protection, Nutrition, and Plant Defense*; J. T. Romeo, Ed.; Kluwer Academic/Plenum Publishers: New York, 1999; pp 215–253.
131. Y. Murakami; R. Funada; Y. Sano; J. Ohtani, *Ann. Bot.* **1999**, *84*, 429–435.
132. J. J. Sauter; W. Witt, Structure and Function of Rays: Storage, Mobilization, Transport. In *Trees – Contributions to Modern Tree Physiology*; H. Rennenberg, W. Eschrich, H. Ziegler, Eds.; Backhuys Publishers: Leiden, The Netherlands, 1997; pp 177–195.
133. W. Albrecht; K. Bethge; C. Mattheck, *J. Arboricult.* **1995**, *21*, 83–87.
134. A. Reiterer; I. Burgert; G. Sinn; S. Tschegg, *J. Mater. Sci.* **2002**, *37*, 935–940.
135. I. Burgert; D. Eckstein, *Trees – Struct. Funct.* **2001**, *15*, 168–170.
136. S. Fujikawa; K. Kuroda, *Micron* **2000**, *31*, 669–686.
137. Z. Ristic; E. N. Ashworth, *Plant Physiol.* **1994**, *104*, 737–746.
138. J. J. Balatinecz; R. V. Kennedy, *For. Prod. J.* **1967**, *17*, 57–64.
139. R. K. Bamber, *J. Inst. Wood Sci.* **1972**, *6*, 32–35.
140. R. K. Bamber; G. W. Davies, *Holzforschung* **1969**, *23*, 83–84.
141. W. G. Craib, *Notes from the Royal Botanic Garden, Edinburgh. LXVI* 1923; pp 1–8.
142. A. Frey-Wyssling; H. H. Bosshard, *Holzforschung* **1959**, *13*, 129–137.
143. J. Bauch; W. Schweers; H. Berndt, *Holzforschung* **1974**, *28*, 86–91.
144. W. E. Hillis; F. R. Humphreys; R. K. Bamber; A. Carle, *Holzforschung* **1962**, *16*, 114–121.
145. K. Yamamoto, *Res. Bull. Hokkaido Univ. For.* **1982**, *39*, 245–296.
146. E. Magel; C. Jay-Allemand; H. Ziegler, *Trees – Struct. Funct.* **1994**, *8*, 165–171.
147. C. M. Stewart, *Science* **1966**, *153*, 1068–1074.
148. M. Kwon; L. B. Davin; N. G. Lewis, *Phytochemistry* **2001**, *57*, 899–914.
149. E. A. Magel, Biochemistry and Physiology of Heartwood Formation. In *Cell and Molecular Biology of Wood Formation*; R. A. Savidge, J. R. Barnett, N. Napier, Eds.; BIOS Scientific Publishers, Ltd., Oxford, UK, 2000; pp 363–376.
150. E. A. Magel; A. Drouet; A. C. Claudot; H. Ziegler, *Trees* **1991**, *5*, 203–207.
151. T. Nobuchi; S. Takahara; H. Harada, *Bull. Kyoto Univ. For.* **1979**, *51*, 239–246.
152. A. M. Taylor; B. L. Gartner; J. J. Morrell, *Wood Fiber Sci.* **2002**, *34*, 587–611.
153. I. Beritognolo; E. Magel; A. Abdel-Latif; J.-P. Charpentier; C. Jay-Allemand; C. Breton, *Tree Physiol.* **2002**, *22*, 291–300.
154. E. Magel; W. Einig; R. Hampp, Carbohydrates in Trees. In *Carbohydrate Reserves in Plants – Synthesis and Regulation*; A. K. Gupta, N. Kaur, Eds.; Elsevier: Amsterdam, 2000; pp 317–336.
155. J. Ralph; J. J. MacKay; R. D. Hatfield; D. M. O'Malley; R. W. Whetten; R. R. Sederoff, *Science* **1997**, *277*, 235–239.
156. R. L. Krahmer; R. W. Hemingway; W. E. Hillis, *Wood Sci. Technol.* **1970**, *4*, 122–139.
157. G. M. Barton, *For. Prod. J.* **1963**, *13*, 304.
158. T. Nagasaki; S. Yasuda; T. Imai, *Phytochemistry* **2002**, *60*, 461–466.
159. K. Saito; T. Mitsutani; T. Imai; Y. Matsushita; K. Fukushima, *Anal. Chem.* **2008**, *80*, 1552–1557.
160. J. H. Langenheim, *Plant Resins: Chemistry, Evolution, Ecology, and Ethnobotany*; Timber Press: Portland, OR, USA, 2003; p 586.
161. H. Kasahara; Y. Jiao; D. L. Bedgar; S. J. Kim; A. M. Patten; Z. Q. Xia; L. B. Davin; N. G. Lewis, *Phytochemistry* **2006**, *67*, 1765–1780.
162. C. A. Blanche; P. L. Lorio; R. A. Sommers; J. D. Hodges; T. E. Nebeker, *For. Ecol. Manage.* **1992**, *49*, 151–165.
163. J. D. DeAngelis; T. E. Nebeker; J. D. Hodges, *Can. J. Bot. – Rev. Can. Bot.* **1986**, *64*, 1046–1049.
164. X. Moreira; L. Sampedro; R. Zas; A. Solla, *Trees – Struct. Funct.* **2008**, *22*, 771–777.
165. R. I. Alfaro; F. He; E. Tomlin; G. Kiss, *Can. J. Bot. – Rev. Can. Bot.* **1997**, *75*, 568–573.
166. D. Boucher; Y. Mauffette; R. Lavallée, *Can. J. For. Res. – Rev. Can. Rech. For.* **2001**, *31*, 2026–2034.
167. C. A. LaPasha; E. A. Wheeler, *IAWA Bull.* **1990**, *11*, 227–238.
168. M. N. B. Nair, Some Notes on Gum and Resin Ducts and Cavities in Angiosperms. In *The Cambial Derivatives*; M. Iqbal, Ed.; Gebrüder Borntraeger: Berlin, 1995; Vol. IX, pp 317–340.

169. F. Bender, *Dept. For. Rural Develop. Can.* **1967**, *1182F*, 1–17.
170. A. Mills, *Ann. Sci.* **1991**, *48*, 173–185.
171. N. Sugimoto; M. Kuroyanagi; T. Kato; K. Sato; A. Tada; T. Yamazaki; K. Tanamoto, *Shokuhin Eiseigaku Zasshi* **2006**, *47*, 76–79.
172. R. Buvat, *Ontogeny, Cell Differentiation, and Structure of Vascular Plants*; Springer-Verlag: Berlin, 1989; p 581.
173. C. L. Mantell, *Econ. Bot.* **1950**, *4*, 203–241.
174. J. H. Langenheim, *Science* **1969**, *163*, 1157–1169.
175. J. H. Langenheim, *Am. Sci.* **1990**, *70*, 16–24.
176. V. F. Veiga Junior; A. C. Pinto, *Quim. Nova* **2002**, *25*, 273–286.
177. R. Clery. Fragant Adventures in Madagascar. In *Advances in Flavours and Fragrances, from the Sensation to the Synthesis*; K. A. D. Swift, Ed. Royal Society of Chemistry, Cambridge, UK, 2002; pp 92–98.
178. J. De la Cruz-Canizares; M.-T. Domenech-Carbo; J.-V. Gimeno-Adelantado; R. Mateo-Castro; F. Bosch-Reig, *J. Chromatogr. A* **2005**, *1093*, 177–194.
179. S. Hamm; J. Bleton; J. Connan; A. Tchapla, *Phytochemistry* **2005**, *66*, 1499–1514.
180. G. Ohloff, *Scent and Fragrances. The Fascination of Odors and their Chemical Perspectives*; Springer-Verlag: Berlin, 1994.
181. A. Dekebo; E. Dagne; O. Sterner, *Fitoterapia* **2002**, *73*, 48–55.
182. D. Papanicolaou; M. Melanitou; K. Katsaboxakis, Changes in Chemical Composition of the Essential Oil of Chios ''mastic resin'' from *Pistacia lentiscus* var. *Chia* Tree during Solidification and Storage. In *Food Flavors: Generation, Analysis and Process Influence*; G. Charalambous, Ed.; Elsevier: Oxford, 1995; pp 303–310.
183. S. Boztok; B. Cokuysal, *Asian J. Chem.* **2007**, *19*, 593–599.
184. A. M. Api, *Contact Derm.* **2006**, *54*, 179.
185. C. Castel; X. Fernandez; L. Lizzani-Cuvelier; A.-M. Loiseau; C. Perichet; C. Delbecque; J.-F. Arnaudo, *Flavour Fragr. J.* **2006**, *21*, 59–67.
186. X. Fernandez; L. Lizzani-Cuvelier; A. -M. Loiseau; C. Périchet; C. Delbecque, *Flavour Fragr. J.* **2003**, *18*, 328–333.
187. S. Jordan, *Ind. Eng. Chem.* **1917**, *9*, 770–771.
188. F. E. King; J. G. Wilson, *J. Chem. Soc.* **1964**, 4011–4024.
189. Y. Yamauchi; R. Oshima; J. Kumanotani, *J. Chromatogr.* **1980**, *198*, 49–56.
190. Y. Yamauchi; T. Murakami; J. Kumanotani, *J. Chromatogr.* **1981**, *214*, 343–348.
191. Y. Yamauchi; R. Oshima; J. Kumanotani, *J. Chromatogr.* **1982**, *243*, 71–84.
192. Y. Du; R. Oshima; J. Kumanotani, *J. Chromatogr.* **1984**, *284*, 463–473.
193. P. Bonello; A. J. Storer; T. R. Gordon; D. L. Wood; W. Heller, *J. Chem. Ecol.* **2003**, *29*, 1167–1182.
194. F. Brignolas; B. Lacroix; F. Lieutier; D. Sauvard; A. Drouet; A. -C. Claudot; A. Yart; A. A. Berryman; E. Christiansen, *Plant Physiol.* **1995**, *109*, 821–827.
195. H. Viiri; E. Annila; V. Kitunen; P. Niemelä, *Trees – Struct. Funct.* **2001**, *15*, 112–122.
196. E. L. Back, *J. Wood Sci.* **2002**, *48*, 167–170.
197. J. D'Auzac; H. Crétin; B. Marin; C. Lioret, *Physiol. Veg.* **1982**, *20*, 311–331.
198. J. R. Hunter, *Trees – Struct. Funct.* **1994**, *9*, 1–5.
199. T. M. Lewinsohn, *Chemoecology* **1991**, *2*, 64–68.
200. H. -D. Behnke; S. Herrmann, *Protoplasma* **1978**, *95*, 371–384.
201. W. F. Pickard, *New Phytol.* **2008**, *177*, 877–888.
202. J.-H. Yu; X. Zeng; S.-G. Yang; H.-S. Huang; W. M. Tian, *J. Rubber Res.* **2008**, *11*, 43–51.
203. J. B. Gomez, *Anatomy of Hevea and Its Influence on Latex Production*; Malaysia Rubber Research and Development Board: Kuala Lumpur, Monograph No. 7, 1982.
204. B.-Z. Hao; J.-L. Wu, *Acta Bot. Sin.* **1982**, *24*, 388–391.
205. B.-Z. Hao; J.-L. Wu, *Chin. J. Trop. Crops* **1984**, *5*, 19–23.
206. B. R. Buttery; S. G. Boatman, *Science* **1964**, *145*, 285–286.
207. B.-Z. Hao; J.-L. Wu, *Ann. Bot.* **2000**, *85*, 37–43.
208. Z. Q. Gao; C. X. Meng; N. H. Ye, *J. Plant Biochem. Biotechnol.* **2008**, *17*, 189–192.
209. B. Nair. In *Sustainable Utilization of Gum and Resin by Improved Tapping Technique in Some Species.* Seminar on Harvesting of Non-wood Forest Products, Menemen-Izmir, Turkey, 2000; Joint FAO/ECE/ILO Committee on Forest Technology, Management and Training.
210. R. L. Davidson, *Handbook of Water-Soluble Gums and Resins*; McGraw-Hill Book Company: New York, 1980; p 700.
211. B. D. Barnd; M. D. Ginzel, In *Causes of Gummosis in Black Cherry (Prunus serotina); Planting and Care of Fine Hardwood Seedlings: FNR-229-W.* Hardwood Tree Improvement and Regeneration Center, Purdue University Cooperative Extension Service, USDA Forest Service: West Lafayette, IN, 2008.
212. K. Matsumoto; J.-P. Chun; N. Nakata; F. Tamura, *J. Food Qual.* **2008**, *31*, 205–215.
213. K. S. Derrick; L. W. Timmer, *Annu. Rev. Phytopathol.* **2000**, *38*, 181–205.
214. J. T. Tippett, *IAWA Bull.* **1986**, *7*, 137–143.
215. D. S. Skene, *Aust. J. Bot.* **1965**, *13*, 367–378.
216. W. E. Hillis; Y. Yazaki, *Phytochemistry* **1974**, *13*, 495–498.
217. W. E. Hillis, *Biochem. J.* **1964**, *92*, 516–521.
218. M. O. Freitas; M. A. S. Lima; E. R. Silveira, *Magn. Reson. Chem.* **2007**, *45*, 262–264.
219. A. Eyles; C. Mohammed, *Aust. For.* **2003**, *66*, 206–212.
220. A. Eyles; N. W. Davies; C. Mohammed, *Can. J. For. Res.* **2003**, *33*, 2331–2339.
221. A. Eyles; N. W. Davies; C. Mohammed, *J. Chem. Ecol.* **2003**, *29*, 881–898.
222. P. N. Ravindran; M. Shylaja; K. Nirmal Babu; B. Krishnamoorthy, Botany and Crop Improvement of Cinnamon and Cassia. In *Cinnamon and Cassia: The Genus Cinnamomum*; P. N. Ravindran, K. Nirmal Babu, M. Shylaja, Eds.; CRC Press: Boca Raton, FL, 2004; Vol. 36, pp 14–79.
223. O. Boussaada; M. Skoula; E. Kokkalou; R. Chemli, *J. Essent. Oil Bear. Plants* **2007**, *10*, 453–464.
224. A. Eyles; N. W. Davies; T. Mitsunaga; R. Mihara; C. Mohammed, *For. Pathol.* **2004**, *34*, 225–232.

225. J. J. W. Coppen, *Flavours and Fragrances of Plant Origin*; Food and Agriculture Organization of the United Nations: Rome, 1995; Vol. 1, p 101.
226. C. R. Metcalfe; L. Chalk, *Anatomy of the Dicotyledons. Leaves, Stem, and Wood in Relation to Taxonomy with Notes on Economic Uses*. Clarendon Press: Oxford, 1950; Vol. 2.
227. A. Eyles; N. W. Davies; C. M. Mohammed, *Trees – Struct. Funct.* **2004**, *18*, 204–210.
228. M. E. Bakker; P. Baas, *Acta Bot. Neerl.* **1993**, *42*, 133–139.
229. M. Gregory; P. Baas, *Israel J. Bot.* **1989**, *38*, 125–174.
230. U. Zimmermann; H.-J. Wagner; M. Heidecker; S. Mimietz; H. Schneider; M. Szimtenings; A. Haase; R. Mitlöhner; W. Kruck; R. Hoffmann; W. König, *Trees – Struct. & Funct.* **2002**, *16*, 100–111.
231. D. Zimmermann; M. Westhoff; G. Zimmermann; P. Geßner; A. Gessner; L. H. Wegner; M. Rokitta; P. Ache; H. Schneider; J. A. Vásquez; W. Kruck; S. Shirley; P. Jakob; R. Hedrich; F.-W. Bentrup; E. Bamberg; U. Zimmermann, *Protoplasma* **2007**, *232*, 11–34.
232. U. Zimmermann; H. Schneider; L. H. Wegner; A. Haase, *New Phytol.* **2004**, *162*, 575–615.
233. P. Baas; M. Gregory, *Israel J. Bot.* **1985**, *34*, 167–186.
234. A. P. Kausch; H. T. Horner, *Am. J. Bot.* **1983**, *70*, 691–705.
235. A. P. Kausch; H. T. Horner, *Can. J. Bot.-Rev. Can. Bot.* **1984**, *62*, 1474–1484.
236. Z.-Y. Wang; K. S. Gould; K. J. Patterson, *Int. J. Plant Sci.* **1994**, *155*, 342–349.
237. V. Thomas, *Ann. Bot.* **1991**, *68*, 287–305.
238. N. R. Lersten, *Am. J. Bot.* **1974**, *61*, 973–981.
239. N. R. Lersten, *Bot. J. Linn. Soc.* **1974**, *69*, 125–136.
240. S. Mangalan; K. P. Kurien; P. John; G. M. Nair, *Ann. Bot.* **1990**, *66*, 123–132.
241. P. Dalin; J. Ågren; C. Björkman; P. Huttunen; K. Kärkkäinen, Leaf Trichome Formation and Plant Resistance to Herbivory. In *Induced Plant Resistance to Herbivory*; A. Schaller, Ed.; Springer: Berlin, 2008; pp 89–105.
242. E. Valkama; J.-P. Salminen; J. Koricheva; K. Pihlaja, *Ann. Bot.* **2003**, *91*, 643–655.
243. S. P. Lapinjoki; H. A. Elo; H. T. Taipale, *New Phytol.* **1991**, *117*, 219–223.
244. H. P. Wilkinson, *Bot. J. Linn. Soc.* **2007**, *155*, 241–256.
245. J. D. Curtis; N. R. Lersten, *Am. J. Bot.* **1974**, *61*, 835–845.
246. R. Schmid, *Bot. Rev.* **1988**, *54*, 179–232.
247. E. Pacini; S. W. Nicolson, Introduction. In *Nectaries and Nectar*; S. W. Nicholson, M. Nepi, E. Pacini, Eds.; Springer: Dordrecht, 2007; pp 1–18.
248. M. Thadeo; M. F. Cassino; N. C. Vitarelli; A. A. Azevedo; J. M. Araújo; V. M. M. Valente; R. M. S. A. Meira, *Am. J. Bot.* **2008**, *95*, 1515–1522.
249. I. Brooker, Botany of the Eucalypts. In *Eucalyptus. The Genus Eucalyptus*; J. J. W. Coppen, Ed.; Taylor and Francis: London, 2002; Vol. 22, pp 3–35.
250. A. Fahn, *Secretory Tissues in Plants*; Academic Press: London, 1979; p 302.
251. A. Santanen; L. K. Simola, *Trees – Struct. Funct.* **2007**, *21*, 337–344.
252. A. A. Mastroberti; J. E. de Araujo Mariath, *Protoplasma* **2008**, *232*, 233–245.
253. I. Smith; M. A. Snow, *For. Chron.* **2008**, *84*, 504–510.
254. W. R. Chaney; M. Basbous, *Econ. Bot.* **1978**, *32*, 118–123.
255. M. D. Abrams, *Bioscience* **2001**, *51*, 967–979.
256. D. C. Peattie, *A Natural History of Trees of Eastern and Central North America*; Houghton Mifflin: Boston, MA, 1991; p 606.
257. D. C. Peattie, *A Natural History of Western Trees*; Houghton Mifflin: Boston, MA, 1991.
258. H. Petroski, *The Toothpick: Technology and Culture*; Random House: Toronto, ON, 2007; p 443.
259. U. G. K. Wegst, *Am. J. Bot.* **2006**, *93*, 1439–1448.
260. B. C. Stoel; T. M. Borman, *PLoS ONE* [Online] **2008**, *3*, e2554.
261. U. G. K. Wegst, *Ann. Rev. Mater. Res.* **2008**, *38*, 323–349.
262. J. R. Stritthoit; D. A. DellaSala; H. Jiang, *Conserv. Biol.* **2006**, *20*, 363–374.
263. S. Oldfield, *Oryx* **2008**, *42*, 159–160.
264. J. G. Haygreen; J. L. Bowyer, *Forest Products and Wood Science. An Introduction*, 2nd ed.; Iowa State University Press: Ames, IO, 1989.
265. C. D. W. Wilkinson; M. Riehle; M. Wood; J. Gallagher; A. S. G. Curtis, *Mater. Sci. Eng., C* **2002**, *19*, 263–269.
266. W. J. Cousins, *Wood Sci. Technol.* **1978**, *12*, 161–167.
267. W. J. Cousins, *Wood Sci. Technol.* **1976**, *10*, 9–17.
268. I. D. Cave, *Wood Sci. Technol.* **1978**, *12*, 75–86.
269. L. Salmén; I. Burgert, *Holzforschung* **2009**, *63*, 121–129.
270. J. D. Ferry, *Viscoelastic Properties of Polymers*, 3rd ed.; John Wiley & Sons: New York, 1980; p 641.
271. I. Gabrielii; P. Gatenholm; W. G. Glasser; R. K. Jain; L. Kenne, *Carbohydr. Polym.* **2000**, *43*, 367–374.
272. A. R. Sanadi; D. F. Caulfield; R. E. Jacobson; R. M. Rowell, *Ind. Eng. Chem. Res.* **1995**, *34*, 1889–1896.
273. L. Salmén, *J. Mater. Sci.* **1984**, *19*, 3090–3096.
274. S. S. Kelley; T. G. Rials; W. G. Glasser, *J. Mater. Sci.* **1987**, *22*, 617–624.
275. M. P. Wolcott; F. A. Kamke; D. A. Dillard, *Wood Fiber Sci.* **1994**, *26*, 496–511.
276. Forest Products Laboratory. *Wood Handbook: Wood as an Engineering Material*; University Press of the Pacific: Honolulu, Hawaii, 2000.
277. R. M. Rowell, Chemical Modification of Wood. In *Handbook of Engineering Biopolymers, Homopolymers, Blends and Composites*; D. Fakirov, D. Bhattacharyya, Eds.; Hanser Gardner: Cincinnati, OH, 2007, pp 673–691.
278. I. Burgert, *Am. J. Bot.* **2006**, *93*, 1391–1401.
279. D. J. Cosgrove, *Annu. Rev. Cell Dev. Biol.* **1997**, *13*, 171–201.
280. J. Fahlén; L. Salmén, *J. Mater. Sci.* **2003**, *38*, 119–126.
281. E. T. Choong; S. S. Achmadi, *Wood Fiber Sci.* **1991**, *23*, 185–196.

282. I. D. Cave, *Wood Sci. Technol.* **1972**, *6*, 157–161.
283. B. Tjeerdsma; M. Boonstra; A. Pizzi; P. Tekely; H. Militz, *Holz als Roh- Werkstoff* **1998**, *56*, 149–153.
284. D. W. Green; J. W. Evans; B. A. Craig, *Wood Fiber Sci.* **2003**, *35*, 499–523.
285. A. N. Shebani; A. J. van Reenen; M. Meincken, *Thermochim. Acta* **2008**, *471*, 43–50.
286. M. V. Ramiah, *J. Appl. Polym. Sci.* **1970**, *14*, 1323–1337.
287. F. Beall, *Wood Fiber Sci.* **1969**, *1*, 215–226.
288. L. Salmén, *C. R. Biol.* **2004**, *327*, 873–880.
289. R. Evans; J. Ilic, *For. Prod. J.* **2001**, *51*, 53–57.
290. M. H. Freeman; C. R. McIntyre, *For. Prod. J.* **2008**, *58*, 6–27.
291. S. Holmberg; K. Persson; H. Petersson, *Comput. Struct.* **1999**, *72*, 459–480.
292. M. P. Wolcott; E. L. Shutler, Jr., *Wood Fiber Sci.* **2003**, *35*, 540–551.
293. K. E. Easterling; R. Harrysson; L. J. Gibson; M. F. Ashby, *Proc. R. Soc. Lond. A – Math. Phys. Eng. Sci.* **1982**, *383*, 31–41.
294. F. F. Wangaard, *The Mechanical Properties of Wood*; John Wiley & Sons: New York, 1950; p 377.
295. B.-E. van Wyk; M. Wink, *Medicinal Plants of the World*; Timber Press: Portland, OR, 2004.
296. W. C. Unwin, *The Elements of Machine Design*; Longmans, Green, and Co.: London, 1882.
297. J. Bodig; B. A. Jayne, *Mechanics of Wood and Wood Composites*; Krieger Publishing Company: Malabar, FL, 1993.
298. A. P. Mouritz; E. Gellert; P. Burchill; K. Challis, *Compos. Struct.* **2001**, *53*, 21–41.
299. C. Buksnowitz; U. Müller; R. Evans; A. Teischinger; M. Grabner, *Wood Sci. Technol.* **2008**, *42*, 95–102.
300. Y. Wu; S. Wang; D. Zhou; C. Xing; Y. Zhang, *Wood Fiber Sci.* **2009**, *41*, 64–73.
301. S. S. Kelley; T. G. Rials; R. Snell; L. H. Groom; A. Sluiter, *Wood Sci. Technol.* **2004**, *38*, 257–276.
302. R. H. Atalla; J. W. Brady; J. F. Matthews; S.-Y. Ding; M. E. Himmel, *Biomass Recalcitrance*; M. E. Himmel, Eds.; Blackwell Publishing: Oxford, UK, 2008; pp 188–212.
303. D. B. Neale; P. K. Ingvarsson, *Curr. Opin. Plant Biol.* **2008**, *11*, 149–155.
304. J. L. Wegrzyn; J. M. Lee; B. R. Tearse; D. B. Neale, *Int. J. Plant Genomics* [Online] **2008**, e412875.
305. C. Plomion; N. Bahrman; P. Costa; C. Dubos; J.-M. Frigerio; J.-M. Gion; C. Lalanne; D. Madur; C. Pionneau; S. Gerber, Proteomics for Genetic and Physiological Studies in Forest Trees: Application in Maritime Pine. In *Molecular Genetics and Breeding of Forest Trees;* S. Kumar, M, Fladung, Eds. Crop Science Series, Food Products Press: Binghamton, NY, **2004**, 53–79.
306. S. G. Ralph; H. J. E. Chun; N. Kolosova; D. Cooper; C. Oddy; C. E. Ritland; R. Kirkpatrick; R. Moore; S. Barber; R. A. Holt; S. J. M. Jones; M. A. Marra; C. J. Douglas; K. Ritland; J. Bohlmann, *BMC, Genomics* [Online] **2008**, *9,* e484.
307. M. Himmel, Ed., *Biomass Recalcitrance; Deconstructing the Plant Cell Wall for Bioenergy*; Blackwell Publishing: Oxford, UK, 2008; p 505.
308. A. T. Groover, *Trends Plant Sci.* **2007**, *12*, 234–238.
309. G. A. Tuskan; S. DiFazio; S. Jansson; J. Bohlmann; I. Grigoriev; U. Hellsten; N. Putnam; S. Ralph; S. Rombauts; A. Salamov; J. Schein; L. Sterck; A. Aerts; R. R. Bhalerao; R. P. Bhalerao; D. Blaudez; W. Boerjan; A. Brun; A. Brunner; V. Busov; M. Campbell; J. Carlson; M. Chalot; J. Chapman; G.-L. Chen; D. Cooper; P. M. Coutinho; J. Couturier; S. Covert; Q. Cronk; R. Cunningham; J. Davis; S. Degroeve; A. Déjardin; C. dePamphilis; J. Detter; B. Dirks; I. Dubchak; S. Duplessis; J. Ehlting; B. Ellis; K. Gendler; D. Goodstein; M. Gribskov; J. Grimwood; A. Groover; L. Gunter; B. Hamberger; B. Heinze; Y. Helariutta; B. Henrissat; D. Holligan; R. Holt; W. Huang; N. Islam-Faridi; S. Jones; M. Jones-Rhoades; R. Jorgensen; C. Joshi; J. Kangasjärvi; J. Karlsson; C. Kelleher; R. Kirkpatrick; M. Kirst; A. Kohler; U. Kalluri; F. Larimer; J. Leebens-Mack; J. C. Leplé; P. Locascio; Y. Lou; S. Lucas; F. Martin; B. Montanini; C. Napoli; D. R. Nelson; C. Nelson; K. Nieminen; O. Nilsson; V. Pereda; G. Peter; R. Philippe; G. Pilate; A. Poliakov; J. Razumovskaya; P. Richardson; C. Rinaldi; K. Ritland; P. Rouzé; D. Ryaboy; J. Schmutz; J. Schrader; B. Segerman; H. Shin; A. Siddiqui; F. Sterky; A. Terry; C.-J. Tsai; E. Uberbacher; P. Unneberg; J. Vahala; K. Wall; S. Wessler; G. Yang; T. Yin; C. Douglas; M. Marra; G. Sandberg; Y. Van de Peer; D. Rokhsar, *Science* **2006**, *313*, 1596–1604.
310. C. A. Berni; E. Bolza; F. J. Christensen, In *South American Timbers – The Characteristics, Properties and Uses of 190 Species.* Commonwealth Scientific and Industrial Research Organization (CSIRO), Division of Building Research, 1979.
311. A. W. Gilbody; W. H. Perkin; J. Yates, *J. Chem. Soc., Trans.* **1901**, *79*, 1396–1411.
312. D. S. Kim; N.-I. Baek; S. R. Oh; K. Y. Jung; I. S. Lee; H.-K. Lee, *Phytochemistry* **1997**, *46*, 177–178.
313. M. O. Hamburger; G. A. Cordell; N. Ruangrungsi; P. Tantivatana, *J. Org. Chem.* **1988**, *53*, 4161–4165.
314. K. N. Gurudutt; T. R. Seshadri, *Phytochemistry* **1974**, *13*, 2845–2847.
315. E. A. Magel; C. Hillinger; W. Höll; H. Ziegler, Biochemistry and Physiology of Heartwood Formation: Role of Reserve Substances. In *Trees – Contribution to Modern Tree Physiology*; H. Rennenberg, W. Eschrich, H. Ziegler, Eds.; Backhuys Publishers: Leiden, 1997; pp 477–506.
316. C. I. Johansson; J. N. Saddler; R. P. Beatson, *Holzforschung* **2000**, *54*, 246–254.
317. U. P. Agarwal; S. A. Ralph, *Appl. Spectrosc.* **1997**, *51*, 1648–1655.
318. B. L. Browning, *The Chemistry of Wood*; Interscience Publishers: New York, 1963.
319. R. Bekker; R. S. Smit; E. V. Brandt; D. Ferreira, *Phytochemistry* **1996**, *43*, 673–679.
320. R. Bekker; E. V. Brandt; D. Ferreira, *Tetrahedron Lett.* **1998**, *39*, 6407–6410.
321. R. Bekker; E. V. Brandt; D. Ferreira, *Tetrahedron* **1999**, *55*, 10005–10012.
322. R. Bekker; D. Ferreira; K. J. Swart; E. V. Brandt, *Tetrahedron* **2000**, *56*, 5297–5302.
323. M. Nagai; S. Nagumo; I. Eguchi; S.-M. Lee; T. Suzuki, *Yakugaku Zasshi* **1984**, *104*, 935–938.
324. M. Nagai; S. Nagumo, *Heterocycles* **1986**, *24*, 601–605.
325. M. Namikoshi; H. Nakata; T. Saitoh, *Phytochemistry* **1987**, *26*, 1831–1833.
326. F. M. Dean; F. H. Herbstein; M. Kapon; G. M. Reisner, *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.* **1991**, *C47*, 137–141.
327. L. P. Christensen; J. Lam, *Phytochemistry* **1989**, *28*, 917–918.
328. J. Minn; W. H. Daly; I. I. Negulescu; K. D. McMurtrey; T. P. Schultz, *J. Agric. Food Chem.* **1996**, *44*, 2946–2947.
329. K. L. Stevens; L. Jurd, *Tetrahedron* **1976**, *32*, 665–668.
330. G. D. Manners, *J. Chem. Soc., Perkin Trans. 1: Org. Bio-Org. Chem. (1972–1999)* **1983**, 39–43.
331. Y. Kai; H. Kuroda; F. Teratani, *Mokuzai Gakkaishi* **1972**, *18*, 315–321.

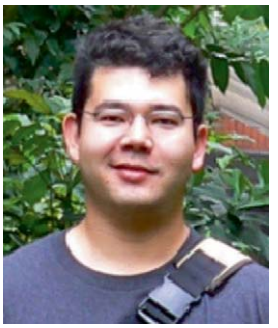
332. Y. Kai; F. Teratani, *Mokuzai Gakkaishi* **1977**, *23*, 499–503.
333. K. Takahashi, *Mokuzai Gakkaishi* **1981**, *27*, 654–657.
334. B. E. Barragán-Huerta; J. Peralta-Cruz; R. F. Gonzalez-Laredo; J. Karchesy, *Phytochemistry* **2004**, *65*, 925–928.
335. W. B. Eyton; W. D. Ollis; I. O. Sutherland; O. R. Gottlieb; M. Taveira Magalhães; L. M. Jackman, *Tetrahedron* **1965**, *21*, 2683–2696.
336. W. B. Eyton; W. D. Ollis; I. O. Sutherland, L. M. Jackman; O. R. Gettlieb; M. Taveira Magalhães*, Proc. Chem. Soc.* **1962**, 301–302.
337. M. Gregson; W. D. Ollis; B. T. Redman; I. O. Sutherland; H. H. Dietrichs; O. R. Gottlieb, *Phytochemistry (Elsevier)* **1978**, *17*, 1395–1400.
338. O. R. Gottlieb; M. Taveira. Magalhães, *J. Org. Chem.* **1961**, *26*, 2449–2453.
339. M. Czakó; L. Marton, *Phytochemistry* **2001**, *57*, 1013–1022.
340. L. Jurd; K. Stevens; G. Manners, *Phytochemistry* **1972**, *11*, 3287–3292.
341. B. C. Maiti; O. C. Musgrave, *J. Chem. Soc., Perkins Trans. 1* **1986**, 675–681.
342. A. G. Brown; J. C. Lovie; R. H. Thomson, *J. Chem. Soc.* **1965**, 2355–2361.
343. G. S. Sidhu; A. V. B. Sankaram, *Justus Liebigs Ann. Chem.* **1966**, *691*, 172–176.
344. A. V. B. Sankaram; V. V. Narayana Reddy; G. S. Sidhu, *Phytochemistry (Elsevier)* **1981**, *20*, 1093–1096.
345. T. J. Lee; Y. M. Lin; T. S. Shih; F. C. Chen, *International conference on chemistry and Biotechnology of Biologically Active Natural Products*. Federation of European chemical societies: Varna, Bulgaria, 1981; vol. 3, pp 290–302.
346. C. G. Karanjgaokar; P. V. Radhakrishnan; K. Venkataraman, *Tetrahedron Lett.* **1967**, 3195–3198.
347. S. Ahmad, *Planta Med.* **1983**, *48*, 62–63.
348. R. D. Haworth; C. R. Mavin; G. Sheldrick, *J. Chem. Soc.* **1934**, 1423–1429.
349. H. G. M. Edwards; L. F. C. de Oliveira; M. Nesbitt, *Analyst (Cambridge, U.K.)* **2003**, *128*, 82–87.
350. K. Nishida; H. Ito; T. Kordo, *J. Japan Tech. Assoc. Pulp Paper Ind*. **1952,** *6*, 261–263, 322
351. E. Zavarin, *J. Org. Chem.* **1958**, *23*, 1198–1204.
352. M. A. Buchanan; E. E. Dickey, *J. Org. Chem.* **1960**, *25*, 1389–1391.
353. C.-L. Chen; H.-M. Chang; E. B. Cowling, *Phytochemistry* **1976**, *15*, 547–550.
354. M. L. Wolfrom; H. B. Bhat, *Phytochemistry* **1965**, *4*, 765–768.
355. N. Tanaka; M. Yasue; H. Imamura, *Tetrahedron Lett.* **1966**, 2767–2773.
356. L. Farkas; M. Nogradi; L. Pallas, *Magy. Kem. Foly.* **1964**, *70*, 301–303.
357. T. R. Govindachari; B. R. Pai; P. S. Subramaniam; U. R. Rao; N. Muthukumaraswamy, *Tetrahedron* **1967**, *23*, 243–248.
358. P. Kamnaing; S. N. Y. F. Free; A. E. Nkengfack; G. Folefoc; Z. T. Fomum, *Phytochemistry* **1999**, *51*, 829–832.
359. D. Ngamga; S. N. Y. F. Free; Z. T. Fomum; A. Chiaroni; C. Riche; M. T. Martin; B. Bodo, *J. Nat. Prod.* **1993**, *56*, 2126–2132.
360. C. D. Mell, *Text. Color.* **1928**, *50*, 531–532.
361. A. D. Pawlus; D. A. Kinghorn, *J. Pharm. Pharmacol.* **2007**, *59*, 1587–1609.
362. G. M. Robinson; R. Robinson, *J. Chem. Soc.* **1935**, 744–752.
363. V. Dellus; A. Scalbert; G. Janin, *Holzforschung* **1997**, *51*, 291–295.
364. V. Dellus; I. Mila; A. Scalbert; C. Menard; V. Michon; C. L. M. du Penhoat, *Phytochemistry* **1997**, *45*, 1573–1578.
365. W. H. Hoge, *Tappi* **1954**, *37*, 369–376.
366. P. Mohan; T. Joshi, *Phytochemistry* **1989**, *28*, 2529–2530.
367. M. Hasegawa; T. Shirato, *Nippon Kagaku Zasshi* **1951**, *72*, 223–224.
368. D. G. Roux; E. Paulus, *Biochem. J.* **1962**, *82*, 324–330.
369. A. R. Burnett; R. H. Thomson, *J. Chem. Soc. Sect. C Org. Chem.* **1968**, 850–853.
370. P. Singh; P. Khandelwal; N. Hara; T. Asai; Y. Fujimoto, *Indian J. Chem., Sect. B: Org. Chem. Incl. Med. Chem.* **2008**, *47B*, 1865–1870.
371. K. C. Joshi; A. K. Sharma; P. Singh, *Planta Med.* **1986**, *52*, 71–72.
372. P. Singh; S. Jain; S. Bhargava, *Phytochemistry* **1989**, *28*, 1258–1259.
373. K. C. Joshi; P. Singh; R. T. Pardasani, *Planta Med.* **1977**, *32*, 71–75.
374. R. M. Khan; S. M. Mlungwana, *Phytochemistry* **1999**, *50*, 439–442.
375. F. E. King; J. R. Housley; T. J. King, *J. Chem. Soc.* **1954**, 1392–1399.
376. M. Titford, *Biotech. Histochem.* **2005**, *80*, 73–78.
377. S. Hauch; E. Magel, *Planta* **1998**, *207*, 266–274.
378. E. Magel; B. Hübner, *Bot. Acta* **1997**, *110*, 314–322.
379. M. Fujita; D. R. Gang; L. B. Davin; N. G. Lewis, *J. Biol. Chem.* **1999**, *274*, 618–627.
380. M. K. Kim; J.-H. Jeon; L. B. Davin; N. G. Lewis, *Phytochemistry* **2002**, *61*, 311–322.
381. M. K. Kim; J.-H. Jeon; M. Fujita; L. B. Davin; N. G. Lewis, *Plant Mol. Biol.* **2002**, *49*, 199–214.
382. D. R. Gang; M. A. Costa; M. Fujita; A. T. Dinkova-Kostova; H.-B. Wang; V. Burlat; W. Martin; S. Sarkanen; L. B. Davin; N. G. Lewis, *Chem. Biol.* **1999**, *6*, 143–151.
383. L. B. Davin; H.-B. Wang; A. L. Crowell; D. L. Bedgar; D. M. Martin; S. Sarkanen; N. G. Lewis, *Science* **1997**, *275*, 362–366.
384. A. T. Dinkova-Kostova; D. R. Gang; L. B. Davin; D. L. Bedgar; A. Chu; N. G. Lewis, *J. Biol. Chem.* **1996**, *271*, 29473–29482.
385. Z.-Q. Xia; M. A. Costa; H. C. Pélissier; L. B. Davin; N. G. Lewis, *J. Biol. Chem.* **2001**, *276*, 12614–12623.
386. D. R. Gang; H. Kasahara; Z.-Q. Xia; K. Vander Mijnsbrugge; G. Bauw; W. Boerjan; M. Van Montagu; L. B. Davin; N. G. Lewis, *J. Biol. Chem.* **1999**, *274*, 7516–7527.
387. L. Dehon; J. J. Macheix; M. Durand, *J. Exp. Bot.* **2002**, *53*, 303–311.
388. S. Nakaba; T. Kubo; R. Funada, *Trees – Struct. Funct.* **2008**, *22*, 623–630.
389. T. Nobuchi; K. Kuroda; R. Iwata; H. Harada, *Mokuzai Gakkaishi* **1982**, *28*, 669–676.
390. J. M. Yang; S. Park; D. P. Kamdem; D. E. Keathley; E. Retzel; C. Paule; V. Kapur; K.-H. Han, *Plant Mol. Biol.* **2003**, *52*, 935–956.
391. J. M. Yang; D. P. Kamdem; D. E. Keathley; K.-H. Han, *Tree Physiol.* **2004**, *24*, 461–474.
392. P. A. Krasutsky, *Nat. Prod. Rep.* **2006**, *23*, 919–942.
393. M. E. Wall; M. C. Wani; C. E. Cook; K. H. Palmer; A. T. McPhail; G. A. Sim, *J. Am. Chem. Soc.* **1966**, *88*, 3888–3890.
394. Z. Zhang; S. Li; S. Zhang; C. Liang; D. Gorenstein; R. S. Beasley, *Planta Med.* **2004**, *70*, 1216–1221.

395. S. Y. Li; Y. J. Yi; Y. J. Wang; Z. Z. Zhang; R. S. Beasley, *Planta Med.* **2002**, *68*, 1010–1016.
396. A. Valletta; A. R. Santamaria; G. Pasqua, *Nat. Prod. Res.* **2007**, *21*, 1248–1255.
397. J. Zhang; Y. Yu; D. Liu; Z. Liu, *Phytomedicine* **2007**, *14*, 50–56.
398. T. Frosch; M. Schmitt; J. Popp, *J. Phys. Chem. B* **2007**, *111*, 4171–4177.
399. P. N. Ravindran; K. Nirmal Babu, Introduction. In *Cinnamon and Cassia: The Genus Cinnamomum*; P. N. Ravindran, K. Nirmal Babu, M. Shylaja, Eds.; CRC Press: Boca Raton, FL, 2004; pp 1–13.
400. M. L. Athayde; G. C. Coelho; E. P. Schenkel, *Phytochemistry* **2000**, *55*, 853–857.
401. P. Mazzafera, *Rev. Bras. Fisiol. Veg.* **1994**, *6*, 149–151.
402. H. Liu; Q. Li; Y. Zhang; Z. Zhou, *Chromatographia* **2009**, *69*, 339–344.
403. M. Enserink, *Science* **2006**, *312*, 382–383.
404. C. U. B. Pinheiro, *Econ. Bot.* **1997**, *51*, 49–58.
405. J. H. Trapold; A. J. Plummer; F. F. Yonkman, *J. Pharmacol. Exp. Ther.* **1954**, *110*, 205–214.
406. M. C. Wani; H. L. Taylor; M. E. Wall; P. Coggon; A. T. McPhail, *J. Am. Chem. Soc.* **1971**, *93*, 2325–2327.
407. C. R. Sirtori, *Pharmacol. Res.* **2001**, *44*, 183–193.
408. S. S. Ghosh; S. K. Purkayastha, *Indian Forester* **1960**, *86*, 684–695.
409. E. Ernst, *BMJ* [Online] **2008**, *337*, a2813.
410. A. Cartayrade; E. Neau; C. Sohier; J.-P. Balz; J.-P. Carde; J. Walter, *Plant Physiol. Biochem.* **1997**, *35*, 859–868.
411. S. Mazzuca; A. Spadafora; A. M. Innocenti, *Plant Sci.* **2006**, *171*, 726–733.
412. N. S. A. Malik; J. M. Bradford, *J. Food Agric. Environ.* **2008**, *6*, 8–13.
413. M. López-Meyer; C. L. Nessler; T. D. McKnight, *Planta Med.* **1994**, *60*, 558–560.
414. Y.-H. Hsiang; R. Hertzberg; S. Hecht; L. F. Liu, *J. Biol. Chem.* **1985**, *260*, 14873–14878.
415. R. P. Verma; C. Hansch, *Chem. Rev.* **2009**, *109*, 213–235.
416. A. H. Heckendorf; C. R. Hutchinson, *Tetrahedron Lett.* **1977**, *48*, 4153–4154.
417. C. R. Hutchinson; A. H. Heckendorf; J. L. Straughn; P. E. Daddona; D. E. Cane, *J. Am. Chem. Soc.* **1979**, *101*, 3358–3369.
418. Y. Yamazaki; M. Kitajima; M. Arita; H. Takayama; H. Sudo; M. Yamazaki; N. Aimi; K. Saito, *Plant Physiol.* **2004**, *134*, 161–170.
419. S. Kusari; S. Zühlke; M. Spiteller, *J. Nat. Prod.* **2009**, *72*, 2–7.
420. E. K. Rowinsky; R. C. Donehower, *N. Engl. J. Med.* **1995**, *332*, 1004–1014.
421. R. A. Holton; C. Somoza; H. B. Kim; F. Liang; R. J. Biediger; P. D. Boatman; M. Shindo; C. C. Smith; S. Kim; H. Nadizadeh; Y. Suzuki; C. L. Tao; P. Vu; S. H. Tang; P. S. Zhang; K. K. Murthi; L. N. Gentile; J. H. Liu, *The Total Synthesis of Paclitaxel Starting from Camphor.* In *Taxane Anticancer Agents;* G. I. Georg, T. T. Chen, I, Ojima, D. M. Vyas, Eds: ACS Symposium Series; Washington, DC, **1995**, Vol. 583, pp 288–301.
422. D. Guénard; F. Guéritte-Voegelein; P. Potier, *Acc. Chem. Res.* **1993**, *26*, 160–167.
423. F. Guéritte-Voegelein; D. Guénard; F. Lavelle; M. T. Le Goff; L. Mangatal; P. Potier, *J. Med. Chem.* **1991**, *34*, 992–998.
424. M. Hezari; R. E. B. Ketchum; D. M. Gibson; R. Croteau, *Arch. Biochem. Biophys.* **1997**, *337*, 185–190.
425. R. E. B. Ketchum; R. B. Croteau, The *Taxus* Metabolome and the Elucidation of the Taxol® Biosynthetic Pathway in Cell Suspension Cultures. In *Biotechnology in Agriculture and Forestry;* K. Saito, R. A. Dixon and L. Willmitzer, Eds. Springer, Berlin, 2006; Vol. 57 Plant Metabolomics, pp 291–309.
426. R. E. B. Ketchum; L. Wherland; R. B. Croteau, *Plant Cell Rep.* **2007**, *26*, 1025–1033.
427. R. E. B. Ketchum; T. Horiguchi; D. Qiu; R. M. Williams; R. B. Croteau, *Phytochemistry* **2007**, *68*, 335–341.
428. D. Frense, *Appl. Microbiol. Biotechnol.* **2007**, *73*, 1233–1240.
429. W. A. Russin; D. D. Ellis; J. R. Gottwald; E. L. Zeldin; M. Brodhagen; R. F. Evert, *Int. J. Plant Sci.* **1995**, *156*, 668–678.
430. R. Croteau; R. E. B. Ketchum; R. M. Long; R. Kaspera; M. R. Wildung, *Phytochem. Rev.* **2006**, *5*, 75–97.
431. R. Kaspera; R. Croteau, *Phytochem. Rev.* **2006**, *5*, 433–444.
432. R. Croteau; J. Hefner; M. Hezari; N. G. Lewis, Taxol Biosynthesis: Cyclization and Early Hydroxylation Steps of the Pathway. In *Current Topics in Plant Physiology;* D. L. Gustine, H. E. Flores, Eds. American Society of Plant Physiologists, Rockville, MD, 1995; Vol. 15, Phytochemical and Health, pp. 94–104.
433. M. Hezari; N. G. Lewis; R. Croteau, *Arch. Biochem. Biophys.* **1995**, *322*, 437–444.
434. M. R. Wildung; R. Croteau, *J. Biol. Chem.* **1996**, *271*, 9201–9204.
435. D. Tsimachidis; P. Česla; T. Hájek; G. Theodoridis; P. Jandera, *J. Sep. Sci.* **2008**, *31*, 1130–1136.
436. A. R. Battersby; E. S. Hall, *J. Chem. Soc. D* **1970**, 194–195.
437. A. R. Battersby; R. J. Parry, *J. Chem. Soc. D* **1971**, 31–32.
438. A. R. Battersby; R. J. Parry, *J. Chem. Soc. D* **1971**, 30–31.
439. L. H. Stevens; C. Giroud; E. J. M. Pennings; R. Verpoorte, *Phytochemistry* **1993**, *33*, 99–106.
440. E. O. Afoakwa; A. Paterson; M. Fowler; A. Ryan, *Crit. Rev. Food Sci. Nutr.* **2008**, *48*, 840–857.
441. D. Eichinger; A. Bacher; M. H. Zenk; W. Eisenreich, *Phytochemistry* **1999**, *51*, 223–236.
442. N. Hampp; M. H. Zenk, *Phytochemistry* **1988**, *27*, 3811–3815.
443. T. M. Kutchan; N. Hampp; F. Lottspeich; K. Beyreuther; M. H. Zenk, *FEBS Lett.* **1988**, *237*, 40–44.
444. X. Ma; J. Koepke; G. Fritzsch; R. Diem; T. M. Kutchan; H. Michel; J. Stoeckigt, *Biochim. Biophys. Acta, Proteins Proteomics* **2004**, *1702*, 121–124.
445. M. Ruppert; X. Ma; J. Stoeckigt, *Curr. Org. Chem.* **2005**, *9*, 1431–1444.
446. J. Stöckigt; S. Panjikar; M. Ruppert; L. Barleben; X. Ma; E. Loris; M. Hill, *Phytochem. Rev.* **2007**, *6*, 15–34.
447. J. J. Brophy; R. J. Goldsack; C. J. R. Fookes; P. I. Forster, *J. Essent. Oil Res.* **1995**, *7*, 237–254.
448. J. C. Doran; J. J. Brophy; E. V. Lassak; A. P. N. House, *Flavour Fragr. J.* **2001**, *16*, 325–328.
449. A. R. Penfold; F. R. Morrison; H. H. G. McKern; J. L. Willis; M. C. Spies, *Aust. J. Sci.* **1950**, *13*, 27.
450. A. R. Penfold; F. R. Morrison; J. L. Willis; H. H. G. McKern; M. C. Spies, *J. Proc. R. Soc. N. S. W.* **1952**, *85*, 123–126.
451. W. Diemair; H. Janecke; D. Heusser, *Naturwissenschaften* **1952**, *39*, 211.
452. A. Z. Mercadante; A. Steck; H. Pfander, *J. Agric. Food Chem.* **1997**, *45*, 1050–1054.
453. K. Venkataraman, *J. Indian Inst. Sci.* **1924**, *7*, 225–231.
454. L. Zechmeister; R. B. Escue, *Science* **1942**, *96*, 229–230.

455. X.-d Ji; Q.-l. Pu; H. M. Garraffo; L. K. Pannell, *J. Essent. Oil Res.* **1991**, *3*, 373–375.
456. B. Shan; Y.-Z. Cai; J. D. Brooks; H. Corke, *J. Agric. Food Chem.* **2007**, *55*, 5484–5490.
457. A. Jayatilaka; S. K. Poole; C. F. Poole; T. M. P. Chichila, *Anal. Chim. Acta* **1995**, *302*, 147–162.
458. N. K. Leela, Cinnamon and Cassia. In *Chemistry of Spices*; V. A. Parthasarathy, B. Chempakam, T. J. Zachariah, Eds.; CAB International: Wallingford, UK, 2008; pp 124–145.
459. Z.-T. Jiang; R. Li; Y. Wang, *J. Essent. Oil-Bear. Plants* **2008**, *11*, 267–270.
460. A. Baruah; S. C. Nath; A. K. Hazarika, *Indian Perfum.* **2004**, *48*, 437–438.
461. S. C. Joshi; D. S. Bisht; R. C. Padalia; K. K. Singh; C. S. Mathela, *J. Essent. Oil-Bear. Plants* **2008**, *11*, 278–283.
462. S. C. Nath; A. K. Hazarika; R. S. Singh, *J. Spices Aromat. Crops* **1994**, *3*, 33–35.
463. P. Yadav; N. K. Dubey, *Indian J. Pharm. Sci.* **1994**, *56*, 227–230.
464. A. Ahmed; M. I. Choudhary; A. Farooq; B. Demirci; F. Demirci; K. H. C. Baser, *Flavour Fragr. J.* **2000**, *15*, 388–390.
465. U. M. Senanayake; T. H. Lee; R. B. H. Wills, *J. Agric. Food Chem.* **1978**, *26*, 822–824.
466. G. Singh; S. Maurya; M. P. de Lampasona; C. Catalan, *J. Sci. Food Agric.* **2005**, *86*, 111–121.
467. P. S. Chatzopoulou; S. T. Katsiotis, *Flavour Fragr. J.* **2006**, *21*, 492–496.
468. B. M. Damjanovic; D. Skala; D. Petrovic-Djakov; J. Baras, *J. Essent. Oil Res.* **2003**, *15*, 90–92.
469. P. K. Koukos; K. I. Papadopoulou, *J. Essent. Oil Res.* **1997**, *9*, 35–39.
470. A. Kilic; H. Hafizoglu; H. Kollmannsberger; S. Nitz, *J. Agric. Food Chem.* **2004**, *52*, 1601–1606.
471. K. Kahuku; R. Kato, *Nippon Kagaku Kaishi (1921–47)* **1938**, *59*, 1096–1098.
472. V. K. Raina; R. K. Lal; S. Tripathi; M. Khan; K. V. Syamasundar; S. K. Srivastava, *Flavour Fragr. J.* **2002**, *17*, 144–146.
473. N. K. Leela, Nutmeg and Mace. In *Chemistry of Spices*; V. A. Parthasarathy, B. Chempakam, T. J. Zachariah, Eds.; CAB International: Wallingford, UK, 2008; pp 165–189.
474. A. Fernandes Costa; J. Cardoso do Vale, *Not. Farm.* **1952**, *18*, 194–200.
475. C. I. G. Tuberoso; A. Barra; A. Angioni; E. Sarritzu; F. M. Pirisi, *J. Agric. Food Chem.* **2006**, *54*, 1420–1426.
476. M. Vanhaelen; R. Vanhaelen-Fastré, *Planta Med.* **1980**, *39*, 164–167.
477. E. Miraldi; S. Ferri; G. G. Franchi; G. Giorgi, *Fitoterapia* **1996**, *67*, 227–230.
478. R. Vila; L. Valenzuela; H. Bello; S. Cañigueral; M. Montes; T. Adzet, *Planta Med.* **1999**, *65*, 178–179.
479. M. Bachstez; G. Cavallini, *Chim. Ind. (Milan, Italy)* **1937**, *19*, 126–128.
480. P. Pietta; P. Mauri; E. Manera; P. Ceva, *J. Chromatogr.* **1988**, *457*, 442–445.
481. V. Quercia; B. Bucci; G. Iela; M. Terracciano; N. Pierini, *Boll. Chim. Farm.* **1978**, *117*, 545–548.
482. H. Vogel; I. Razmilic; M. Muñoz; U. Doll; J. San Martin, *Planta Med.* **1999**, *65*, 90–91.
483. A. O. Tucker; M. J. Maciarello; L. R. Landrum, *J. Essent. Oil Res.* **1991**, *3*, 195–196.
484. L. Jirovetz; G. Buchbauer; I. Stoilova; A. Krastanov; A. Stoyanova; E. Schmidt, *Ernaehrung (Vienna, Austria)* **2007**, *31*, 55–62.
485. J. García-Fajardo; M. Martínez-Sosa; M. Estarrón-Espinosa; G. Vilarem; A. Gaset; J. M. de Santos, *J. Essent. Oil Res.* **1997**, *9*, 181–185.
486. D. Kamdem; D. A. Gage, *Planta Med.* **1995**, *61*, 574–575.
487. L. C. A. Barbosa; A. J. Demuner; A. D. Clemente; V. Fonseca de Paula; F. M. D. Ismail, *Quim. Nova* **2007**, *30*, 1959–1965.
488. W. Lenz, *Z. Anal. Chem.* **1894**, *33*, 193–200.
489. H. Thoms, *Arch. Pharm.* **1903**, *241*, 592–603.
490. A. K. Srivastava; S. K. Srivastava; K. V. Syamsundar, *Flavour Fragr. J.* **2005**, *20*, 51–53.
491. K. Chaieb; H. Hajlaoui; T. Zmantar; A. B. Kahla-Nakbi; M. Rouabhia; K. Mahdouani; A. Bakhrouf, *Phytother. Res.* **2007**, *21*, 501–506.
492. C. D. Miller; K. Bazore, *Fruits of Hawaii. Description, Nutritive Value, and Use.* University of Hawaii Agricultural Experiment Station, Honolulu, HI, Vol. 96; 1945.
493. P. L. Lee; G. Swords; G. L. K. Hunter, *J. Agric. Food Chem.* **1975**, *23*, 1195–1199.
494. R. G. Kelsey; O. McCuistion; J. Karchesy, *Nat. Prod. Commun.* **2007**, *2*, 779–780.
495. K. Koba; K. Sanda; C. Raynaud; C. Guyon; J.-P. Chaumont; L. Nicod, *J. Essent. Oil Res.* **2008**, *20*, 354–357.
496. M. A. Hye; M. A. Taher; M. Y. Ali; M. U. Ali; S. Zaman, *J. Sci. Res.* **2009**, *1*, 300–305.
497. J.-P. Joseleau; G. Ullmann, *Phytochemistry* **1990**, *29*, 3401–3405.
498. K. M. Davies, An Introduction to Plant Pigments in Biology and Commerce. In *Plant Pigments and Their Manipulation*; K. M. Davies, Ed. Blackwell Publishing Ltd.: Oxford, 2004; pp 1–22.
499. M. Srivastava; V. P. Kapoor, *Chem. Biodivers.* **2005**, *2*, 295–317.
500. T. N. S. Varma; C. V. Ramakrishnan, *Nature* **1956**, *178*, 1358–1359.
501. J. F. Fisher; R. L. Rouseff, *J. Agric. Food Chem.* **1986**, *34*, 985–989.
502. M.-U.-D. Khan; G. Mackinney, *Plant Physiol.* **1953**, *28*, 550–552.
503. F. Shahmir; L. Ahmadi; M. Mirza; S. A. A. Korori, *Flavour Fragr. J.* **2003**, *18*, 425–428.
504. R. K. Goyal; A. R. P. Kingsly; M. R. Manikantan; S. M. Ilyas, *Biosys. Eng.* **2006**, *95*, 43–49.
505. L. R. Beuchat; R. E. Worthington, *J. Food Technol.* **1978**, *13*, 355–358.
506. A. R. Nassar; B. S. El-Tahawi; S. A. S. El-Deen, *J. Am. Oil Chem. Soc.* **1977**, *54*, 553–556.
507. M. Moutounet; P. Rabier; J.-L. Puech; E. Verette; J.-M. Barillère, *Sci. Aliments* **1989**, *9*, 35–51.
508. K. M. Kaler; W. N. Setzer, *Nat. Prod. Commun.* **2008**, *3*, 829–832.
509. W. C. West, *Bull. Torrey Bot. Club* **1969**, *96*, 329–344.
510. A. R. S. Menon; A. M. Babu, *Phyton (Austria)* **1989**, *29*, 41–47.
511. R. C. Setia; J. J. Shah, *Indian J. Exp. Biol.* **1977**, *15*, 297–301.

Biographical Sketches

Ann M. Patten received her B.Sc. in microbiology (Pennsylvania State University, 1988), an M.S. in forest resources (University of Idaho, 1999), and a Ph.D. in molecular plant sciences (Washington State University, 2007) under the supervision of Professor Norman G. Lewis. Her Ph.D. studies were supported by Helen/Loyal H. Davis and John/Edith McDougall Fellowships. Currently, Dr. Patten is carrying out postdoctoral studies in the Lewis' group; her research focuses on plant cell wall formation/lignification using various types of light and electron microscopic approaches.



Daniel G. Vassão obtained his B.S. in chemistry from the University of São Paulo, Brazil, in 2002 and then completed his Ph.D. requirements in biochemistry under the supervision of Professor Lewis in 2008. His Ph.D. thesis involved establishing the biosynthetic routes leading to allyl/propenyl phenols, such as chavicol/eugenol in herbs and spices, and also to structurally related (neo)lignans. He has received several awards including the SMB Excellence in Graduate Research (2007), an Arthur M. and Kate Eisig-Tode Research Scholarship, the Charles Glen King Prize (2008), the John and Maggie McDougall Fellowship (2006/2007), and the Helen and Loyal H. Davis (2004/2005) Fellowship.

Professor Michael P. Wolcott received both B.Sc. (wood science, 1982) and M.Sc. (forestry, 1985) degrees from the University of Maine at Orono, and a Ph.D. degree in materials engineering science from the Virginia Polytechnic Institute and State University in 1989. Before joining Washington State University, Dr. Wolcott was an associate professor of Wood Science at West Virginia University. His current research interests emphasize natural fiber and wood composites design, thermoplastic composites, fiber–polymer interaction, viscoelasticity, and adhesion. For more than a decade, Dr. Wolcott has led research teams in developing novel building materials for the US Navy, aiming to reduce toxic chemical exposure from docks, pier, and other shoreline structures.

Laurence B. Davin received her B.Sc. (1983) and Ph.D. (1987) degrees in plant biochemistry/physiology from the Université Paul Sabatier (Toulouse, France). Later, Dr. Davin studied glucosinolate biogenesis with Ted Underhill in the National Research Council of Canada's Plant Biotechnology Institute as a postdoctoral fellow. Since 1989, she has turned her attention to lignan, lignin, and allyl/propenyl phenol biosynthesis at both the Virginia Polytechnic Institute and State University (Blacksburg, VA) and at the Institute of Biological Chemistry (Washington State University). Dr. Davin is a member of the Editorial Board for *Recent Advances in Phytochemistry*.

Professor Norman G. Lewis completed his B.Sc. at the University of Strathclyde (Honors, chemistry, 1973) and Ph.D. at the University of British Columbia (chemistry, 1977). Next, he pursued postdoctoral studies at Cambridge University (chemistry, 1978–80) with Professor Sir Alan R. Battersby. Before joining Washington State University in 1990, he held various research positions at ICI, the Pulp and Paper Research Institute of Canada, the National Research Council of Canada, and Virginia Tech. His research interests mainly involve lignan, lignin, and allyl/propenyl phenol biosynthesis. He serves on numerous scientific advisory committees, grant panels, and editorial boards, including as Regional Editor (*Phytochemistry*), Monitoring Editor (*Plant Physiology*), and as Executive Editor of *Advances in Plant Biochemistry and Molecular Biology.*

3.28 The Chemistry of *Arabidopsis thaliana*

M. Soledade C. Pedras and Qingan Zheng, University of Saskatchewan, Saskatoon, SK, Canada

3.28.1 Introduction

Plants from the wild species *A. thaliana* (L.) Heynh. (thale cress) have a rosette of small leaves and a main stem (up to 30–40 cm at the adult stage) terminating on an inflorescence. The inflorescence contains a series of flowers consisting of four white petals surrounded by four sepals, with the youngest flowers at the top. The flowers normally self-fertilize causing the ovary to elongate and develop into a fruit called a silique, containing 30–60 seeds at maturity. Mature seeds soaked in water germinate within a few days, and in optimal growth conditions form a rosette, bolts, and flowers within 4 weeks. Under continuous light, at 25 °C, with good nutrition, the commonly used ecotypes Columbia and Landsberg have a generation time of around 6 weeks.[1]

Arabidopsis thaliana, many times referred to as 'Arabidopsis', has a long history of use as a model system. An article by Alexander Braun in 1873 describing a mutant plant found near Berlin appears to be the first nontaxonomic reference of Arabidopsis. The widespread acceptance of *A. thaliana* as a model plant occurred in the 1980s and was followed by a remarkable revolution in plant genetics, physiology, and molecular genetics.[2] This outcome was partly due to the relatively small genome (115 409 949 base pairs of DNA, 25 498 genes distributed in five chromosomes ($2n = 10$)) of *A. thaliana*, and because mutational analysis and informative mutations in any gene could be carried out in populations of a feasible size.[2] Subsequent combination of multiple research efforts concentrated on this model plant accelerated dramatically the understanding of plant molecular processes related for instances, with cell morphogenesis, root development, disease resistance, cold and freezing resistance, and hormone effects. The sequence of the genome of *A. thaliana*, the third multicellular organism to have its genome sequenced, was published at the end of 2000.[3] Unexpectedly at that time, the Arabidopsis genome revealed substantial gene duplication and segmental duplications, with ~60% of the genome thought to have derived from a single duplication event. Achievement of the complete genome sequencing has generated a series of large-scale projects aimed at discovering the functions of the ~25 000 genes identified in *A. thaliana* (http://www.tigr.org/tdb/e2k1/ath1/; http://www.nsf.gov/pubs/2002/bio0202/start.htm). In addition, the availability of

large populations of gene disruption lines has increased enormously the impact of Arabidopsis genomics on various research areas of plant science.[4] Importantly, comparison of the rice and *A. thaliana* proteomes shows that 71% of predicted rice proteins are reasonably similar to *A. thaliana* proteins. This similarity indicates that determination of orthologous relationships between genes of crop plants and *A. thaliana* is a reasonable strategy to obtain information on gene function and thus many laboratories are considering this approach.

3.28.1.1 The Crucifer Family

The species *A. thaliana* is a member of the family Brassicaceae that contains about 340 genera and 3700 species distributed worldwide. The enormous agricultural importance of the Brassicaceae family, commonly known as crucifer, is mainly due to the genus *Brassica*, which includes oilseeds as well as many vegetables, condiments, and fodder crops. Several *Brassica* species are widely cultivated (*B. carinata*, *B. juncea*, *B. napus*, *B. nigra*, *B. oleracea*, and *B. rapa*) and contain important subspecies as well. For example, *B. rapa* comprises a number of morphologically diverse crops, including vegetables like Chinese cabbage, Pak choi and turnip, and oilseed types that encompass rapeseed, canola, and yellow and brown Sarson. The haploid genome equivalent of *B. rapa* is about 529 Mb and is closely related to the genome of *A. thaliana* (125 Mb haploid genome equivalent).[5] *A. thaliana* and *Brassica* species evolved from a common ancestor around 14.5–20.4 Ma.[6,7] The economic significance of several cultivated *Brassica* species and amenability to genetic analysis, as well as facile acclimatization, explain the enormous amount of research on various species of this family.

3.28.1.2 Secondary Metabolites of Plants and Metabolomics

The chemistry of plants and other nonmammalian living organisms is generally equated with secondary metabolism. Secondary metabolites are naturally occurring low molecular weight organic compounds (also known as natural products) characteristic of a particular group of organisms (CONAP II, vol. 9). Secondary metabolites are produced by living organisms and have specific roles that contribute to fitness of the species, contrary to primary metabolites that are common to all living organisms, for example, amino acids, proteins, lipids, carbohydrates, polysaccharides, nucleotides, and nucleic acids. Primary metabolites are therefore essential for life, whereas in general, secondary metabolites being significant within particular groups will contribute to fitness and development within such groups.[8,9] Because structurally related secondary metabolites are often restricted to a set of species within a phylogenetic group, some taxonomic issues can be clarified using metabolite profiles. Molecular phylogeny uses chemical structures to obtain information on an organism's evolutionary relationships.[10] The result of a molecular phylogenetic analysis is expressed in a so-called phylogenetic tree.

The dramatic improvements in the sensitivity of analytical methods have contributed to the development and fast advancement of the so-called 'omics' technologies. The primary aim of 'omics' is the identification of all gene products present in a certain organism.[11] Basically, metabolomics is the qualitative and quantitative nonbiased analysis of the complete set of metabolites produced by a living organism at a given time of its life cycle. Thus, metabolomics is an important approach to investigate metabolic networks and to understand biosynthetic pathways.[12] In an early example of the comprehensive analysis of plant metabolites, gas chromatography–mass spectrometry (GC–MS) analysis was used to analyze the metabolites of *A. thaliana* leaves; over 300 compounds were detected,[13] of which half could be assigned a chemical structure. Clearly, the very substantial quantitative and structural differences among the various metabolites produced by any living organism indicate that there is no known single analytical method that can provide a nonbiased evaluation of a complete metabolome.[14] Although GC–MS and liquid chromatography–mass spectrometry (LC−MS) are perhaps the most widely used methods, a combination of various analytical methods, including chromatography–mass spectrometry (LC−MS), are required before the complete metabolome of any species can be determined. In addition, the metabolite profile obtained for an organism at any given stage of its life cycle is highly dependent on sampling methods, particularly in the case of phytohormones,[15,16] that must be clearly defined for an assessment of the potential detection range and significance.

3.28.2 Secondary Metabolites of *Arabidopsis thaliana*

Considering the enormous impact of *A. thaliana* on the advancement of current scientific knowledge in a number of areas, it is not surprising to find various reviews dealing with its secondary metabolites, some of which are focused on specific metabolites or groups, for example, flavonoids,[17] ascorbic acid,[18] camalexin,[19] or indolyl-3-acetonitriles,[20] whereas others are quite general.[21] In 2005, a review on secondary metabolites of *A. thaliana* by D'Auria and Gershenzon[21] reported about 170 compounds grouped in seven major classes (indole and indole-sulfur compounds (10), glucosinolates (35), phenylpropanoids (20), benzenoids (25), flavonoids (15), terpenes (50), and fatty acids and derivatives (15)) and suggested that many additional metabolites would be discovered. In this comprehensive review, the secondary metabolites of *A. thaliana* (total number over 270) are grouped and reported according to their biosynthetic building blocks. Plant hormones or metabolites with signaling roles (e.g., γ-aminobutyric acid),[22,23] cofactors, carbohydrates, oxylipins, and metabolites speculated to be produced are not included.

3.28.2.1 Metabolites Derived from Acetate

Metabolites derived from acetate comprise those derived from condensation of the basic building blocks, acetate and malonate, whose biosynthesis is carried out by fatty acid synthases in the case of fatty acids, and polyketide synthases in the case of polyketides (CONAP II, vol. 1) or related enzymes in the case of derivatives. Polyketides isolated from *A. thaliana* do not appear to have been reported. A substantial number of metabolites having a polyketide component and another basic building block such as shikimate are, strictly speaking, considered to have mixed biosynthetic origin. In the case of *A. thaliana*, these metabolites are the flavonoids covered in Section 3.28.2.2.2 'Metabolites of mixed origin: Shikimate and malonate – flavonoids and their glycosides'.

3.28.2.1.1 Fatty acid derivatives

Most fatty acids are esterified with glycerol and stored in seeds as acyl glycerols, which are lipids and considered primary metabolites. Long-chain acyl-CoA synthetases activate free fatty acids to acyl-CoA thioesters that are then used in fatty acid-derived metabolic pathways,[24] in which plant species produce characteristic mixtures of fatty acids. Plants use fatty acid derivatives, the jasmonates (CONAP II, vol. 4), as biological regulators with roles in the regulation of developmental and defense-related gene expression.[25,26]

Headspace analysis of herbivore-infested and artificially damaged *A. thaliana* plants contains metabolites that are not detected in undamaged plants. Caterpillar-infested plants showed induced emissions of certain metabolites including alcohols and ketones (**Figure 1**). The presence of tentatively identified cycloheptadienes **a1** and **a2** in the volatile blend is unusual as these compounds are not common among higher plants.[27] Protection of above ground organs of plants includes a cuticle consisting of intracuticular waxes, embedded in a polymer matrix of cutin, and epicuticular waxes deposited on the outer surface of the cutin layer. The cuticular waxes are formed in epidermal cells in biosynthetic pathways that first generate the very long-chain carbon skeletons, and then modify them into diverse aliphatic lipid classes. Wax biosynthesis utilizes 16:0 and 18:0 acyl precursors supplied by a plastid-localized elongation pathway. *Arabidopsis thaliana* contains alkyl esters with chain lengths between C_{38} and C_{52}, with C_{42}, C_{44}, and C_{46} favored. The esters of wild types are dominated by 16:0 acyl moieties, and the chain lengths of esterified alcohols are between C_{20} and C_{32} (**Figure 1**).[28]

3.28.2.2 Metabolites Derived from Shikimate

The shikimate pathway is found in plants and microorganisms but not in animals. This pathway involves several metabolic steps initiated with the coupling of phosphoenolpyruvate and erythrose-4-phosphate to yield chorismate, the precursor of the aromatic amino acids such as phenylalanine, tyrosine, and tryptophan, and of many secondary metabolites that may serve as substrates for other metabolic pathways as well (CONAP II, vol. 1). The penultimate enzyme of the shikimate pathway is the target for the herbicide glyphosate.[29]

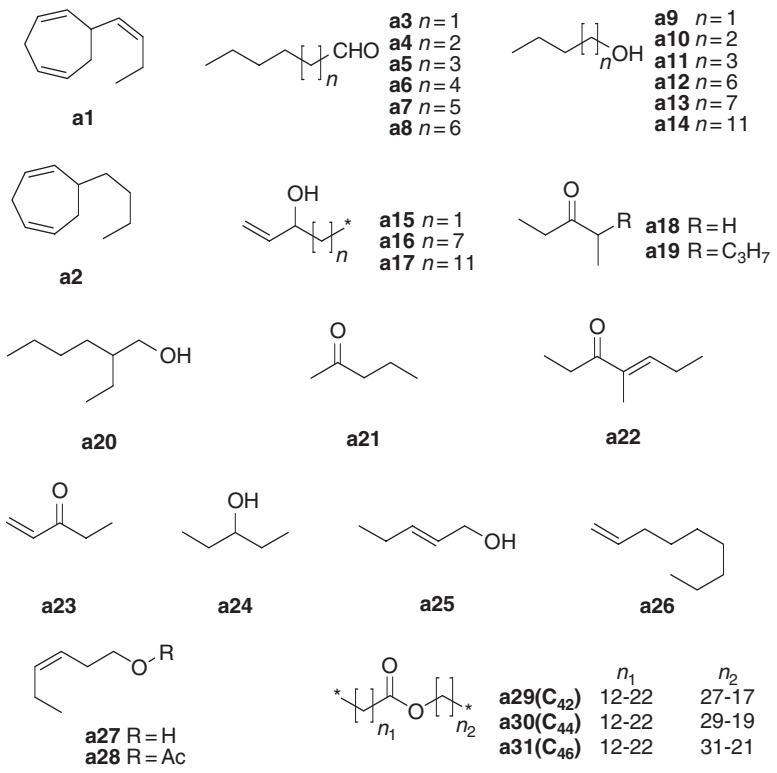


Figure 1 Fatty acid derivatives (**a1–a28**)[27] and epicuticular waxes (**a29–a31**)[28] from *A. thaliana*.

3.28.2.2.1 Phenylpropanoids and derivatives

Plant phenylpropanoids are a vast and structurally diverse group of phenylalanine-derived metabolites (C_6–C_3) that play crucial roles in the interaction of plants with other living organisms. The deamination of phenylalanine by phenylalanine ammonia lyase (PAL) produces cinnamic acid (**b1**), the precursor of the phenylpropanoids. A hydroxylase catalyzes the introduction of the hydroxyl group at the para position of the phenyl ring of cinnamic acid (**b1**), producing 4-coumaric acid (**b2**). The structural diversity of phenylpropanoids (**Figure 2**) is due to a variety of further enzymatic transformations, including acylation, condensation, cyclization, glycosylation, hydroxylation, methylation, and prenylation.[30] Phenylpropanoids are precursors of most benzenoid metabolites (C_6–C_1) after loss of two carbons of the side chain, as for example in vanillin (**c5**) biosynthesis, although some benzenoid metabolites may derive directly from intermediates early in the shikimate pathway.[31] Many of the phenylpropanoids (**Figure 2**) and benzenoids (**Figure 3**) from *A. thaliana* identified thus far are also produced in other plant families.

3.28.2.2.2 Metabolites of mixed origin: Shikimate and malonate – flavonoids and their glycosides

The wide variety of flavonoids produced by plants are biosynthesized from the phenylpropanoid 4-coumaric acid (**b2**) and three malonyl-CoA units. The carboxyl group of 4-coumaric acid is first activated by the formation of a thioester bond with CoA, which then condenses sequentially with the malonyl-CoA units. Much of the current understanding between plant differentiation and secondary metabolism is due to work on flavonoid metabolism in *A. thaliana*, which has addressed the relationship between secondary metabolism and epidermal and seed differentiation. It is clear that a common set of transcription factors control both the flavonoid pathway and the specific aspects of cellular differentiation.[9] For example, proanthocyanidins (or

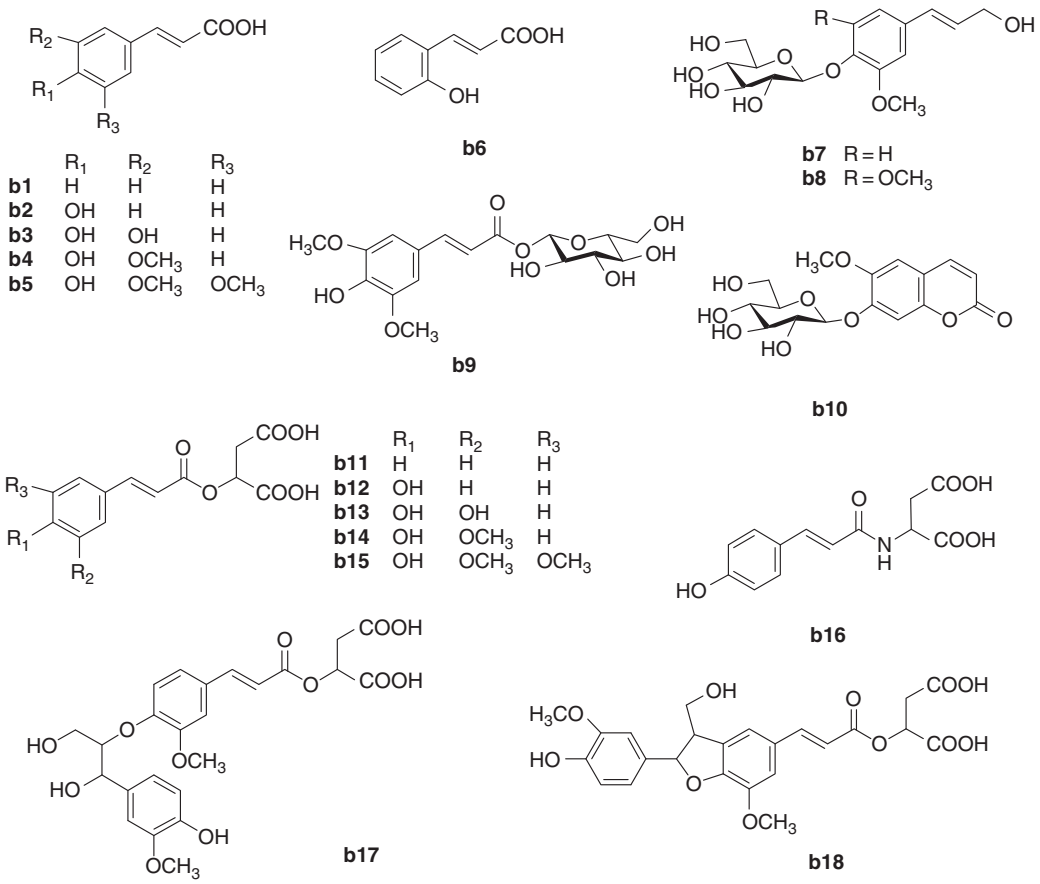


Figure 2 Phenylpropanoids (C6–C3) from *A. thaliana*: cinnamic acid (**b1**), 4-coumaric acid (**b2**), caffeic acid (**b3**), ferulic acid (**b4**), sinapic acid (**b5**), 2-coumaric acid (**b6**), coniferin (**b7**), syringin (**b8**), sinapoyl ®-D-glucose (**b9**), scopolin (**b10**), malates (**b11–b16**), and lignans (**b17, b18**).[32–36]

condensed tannins) accumulate in the innermost layer of the developing seed coat after fertilization and until full seed maturity. Many flavonoids such as anthocyanidins and flavonols are glucosylated, for example, **d4** and **d8**, which were first reported from *A. thaliana* (**Figure 4**).

3.28.2.3 Isoprenoids

Like all other plant species, *A. thaliana* produces a great variety of isoprenoids (CONAP II, vol. 1), which represent the largest group of secondary metabolites reported from this species.

3.28.2.3.1 Terpenes

Monoterpenes (C_{10}) and sesquiterpenes (C_{15}) are known to be produced by *A. thaliana* (**Figures 5** and **6**).[21,42,43] Many of these terpenoids are volatiles and were obtained by solid-phase microextraction coupled with GC–MS; 24 monoterpenes, 26 sesquiterpenes, and 12 aromatic structures, predominantly observed in inflorescences were reported from *A. thaliana*.[37]

Genome data mining in *A. thaliana* indicated that this plant could synthesize a number of triterpenes, although traditional isolation and/or analytical detection methods showed only the triterpenes lupeol (**g8**), α-amyrin (**g11**), and β-amyrin (**g12**).[44] Indeed, likely because of the genome mining methodology, the number

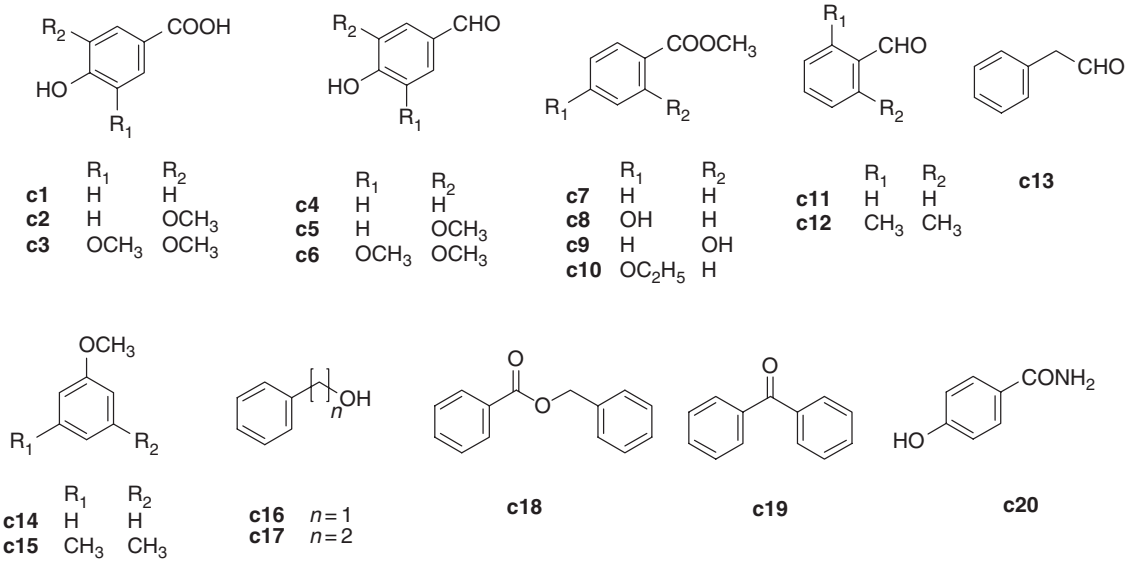


Figure 3 Benzenoids (C_6–C_1) from *A. thaliana*: benzoic acid derivatives (**c1–c3**, **c7–c10**, **c18**, **c20**), benzaldehyde derivatives (**c4–c6**, **c11–c12**), phenylacetaldehyde (**c13**), phenols (**c14**, **c15**), phenyl alcohols (**c16**, **c17**), and benzophenone (**c19**).[35,37]

of triterpenes from *A. thaliana* is higher than that ever reported for other plants (**Figure 7**). Similarly, a comprehensive analysis of genes coding for enzymes involved in the metabolism of isoprenoids in *A. thaliana* allowed the generation of a knowledge-based metabolic map of isoprenoid metabolic pathways.[45] Similar to many other plants, *A. thaliana* produces diterpenes (CONAP II, vol. 1) that include the plant hormones gibberellins and tetraterpenes such as phytoene (**g14**), β-carotene (**g15**), α-carotene (**g16**), zeaxanthin (**g17**), violaxanthin (**g18**), and lutein (**g19**) (**Figure 7**).

3.28.2.3.2 Steroids

Most of the higher plant sterols are found as free sterols used as membrane components. A minute fraction of sterols, predominantly campesterol and its epimer 22(23)-dihydrobrassicasterol, form the pool of brassinosteroid precursors that function as plant growth hormones (**Figure 8**). In general, for a given plant species, the composition of the sterol mixture is genetically determined.[49]

3.28.2.4 Alkaloids and Sulfur Metabolites

Similar to most species within the Brassicaceae family, *A. thaliana* biosynthesizes only relatively simple alkaloids, although a number of gene homologues encoding enzymes involved in the synthesis of more complex alkaloids have been annotated.[52] However, 18 genes annotated as 'putative tropinone reductase' in the Arabidopsis genome, which shared about 50% identity to tropinone reductases of Solanaceae, were questioned due to the absence of tropane alkaloids and presence of nortropane alkaloids in many Brassicaceae species.[53]

3.28.2.4.1 Indole alkaloids

The wide variety of simple indole alkaloids characteristic of Brassicaceae is also found in *A. thaliana* (**Figure 9**). Most of these indole alkaloids appear to be derived from tryptophan, including the phytohormone indolyl-3-acetic acid (**i15**)[54] and its conjugates **i17–i22** (**Figure 9**). Like all Brassicaceae species investigated thus far, the phytoalexins found in *A. thaliana*, camalexin (**i23**) and rapalexin A (**i25**) are indole-containing metabolites. Camalexin (**i23**) was the only phytoalexin[55] known in *A. thaliana*[19] until 2008 when rapalexin A (**i25**), first

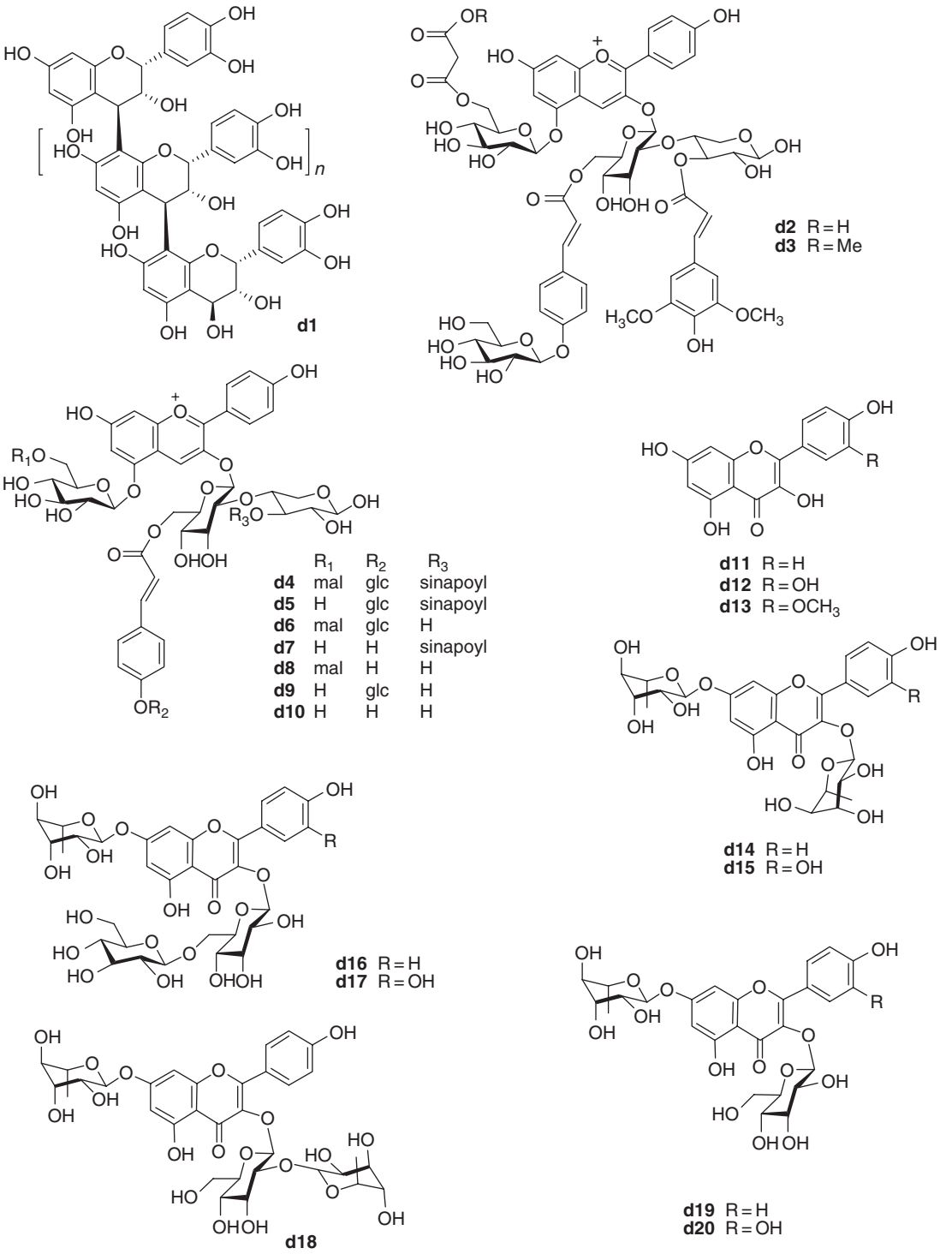


Figure 4 Flavonoids from *A. thaliana*: proanthocyanidin (**d1**), cyanidins (**d2–d10**), kampferol and glycosides (**d11, d14, d16, d18, d19**), rhamnetin (**d13**), and quercetin and glycosides (**d12, d15, d17, d20**).[38–41]

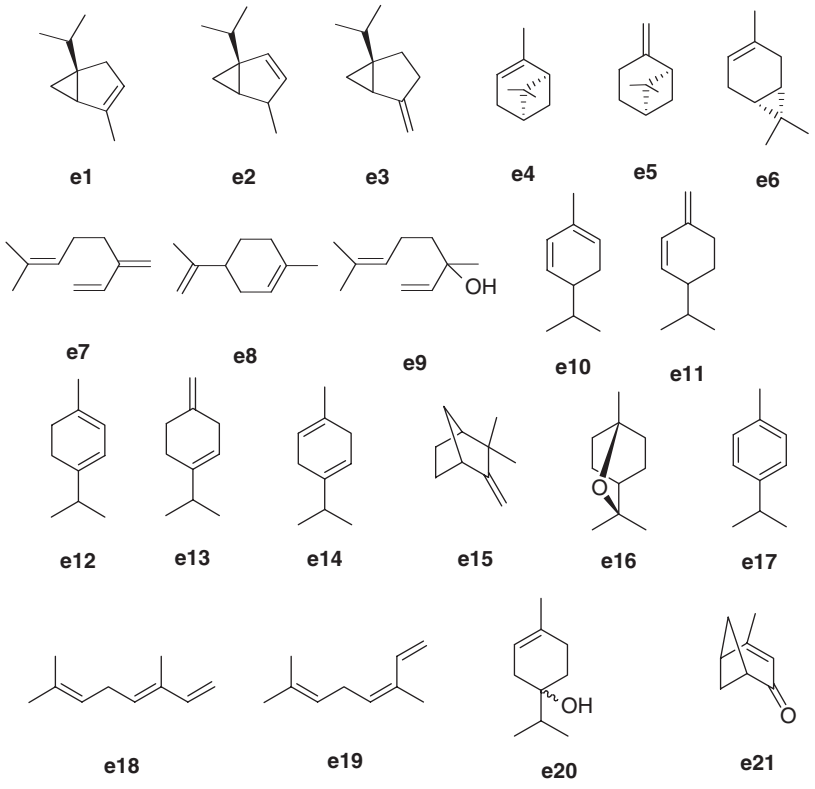


Figure 5 Monoterpenes from *A. thaliana*: α-thujene (**e1**), β-thujene (**e2**), sabinene (**e3**), α-pinene (**e4**), β-pinene (**e5**), 3-carene (**e6**), β-myrcene (**e7**), limonene (**e8**), linalool (**e9**), α-phellandrene (**e10**), β-phellandrene (**e11**), α-terpinene (**e12**), β-terpinene (**e13**), γ-terpinene (**e14**), camphene (**e15**), 1,8-cineole (**e16**), *p*-cymene (**e17**), (*Z*)-β-ocimene (**e18**), (*E*)-β-ocimene (**e19**), α-terpineol (**e20**), and verbenone (**e21**).[42,43]

isolated from *B. rapa*,[56] was reported from stressed *A. thaliana* leaves.[57] The biosynthetic pathways of a few of these indole alkaloids are under intense investigation and some of their genes have been cloned and a few enzymes have been purified.[19,20,55,58,59]

3.28.2.4.2 Glucosinolates

Glucosinolates are found in the order Capparales, which includes the economically important Brassicaceae. Glucosinolates are secondary metabolites derived from a variety of amino acids, several of which are not part of protein ribosomal biosynthetic pathways. They occur naturally as salts of sulfate and are therefore very polar compounds classified as aliphatic, aromatic, and indolic, depending on the amino acid from which they are derived (**Figure 10**). A protein amino acid can undergo a series of chain elongations prior to assembly into a glucosinolate that in turn can be subjected to additional metabolic transformations such as hydroxylations, methylations, and oxidations.[67] The glucosinolates found in *A. thaliana* ecotype Columbia are derived from tryptophan, several chain-elongated methionine homologues, chain-elongated phenylalanine, and phenylalanine. The bishomomethionine-derived glucosinolates 4-methylthiobutylglucosinolate (**j9**) and 4--methylsulfinylbutylglucosinolate (**j3**) account for more than 50% of the total glucosinolate content in the rosette leaves of *A. thaliana*.[68]

The biosynthetic pathways of the various groups of glucosinolates have been intensely investigated for decades but great advances have been made in the last several years specifically due to the availability of multiple *A. thaliana* ecotypes and mutants.[69,70] Because most of the biosynthetic genes of the glucosinolate

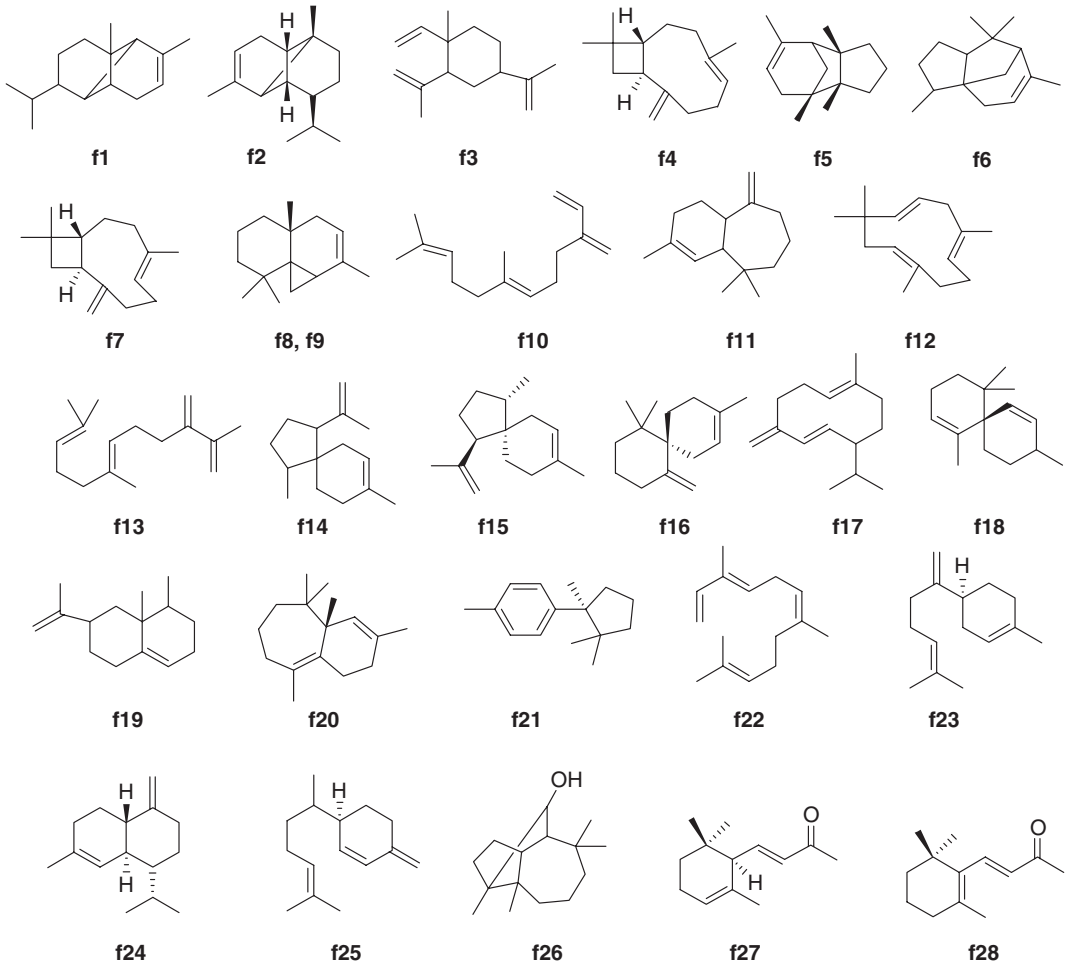


Figure 6 Sesquiterpenes from *A. thaliana*: α-ylangene (**f1**), α-copaene (**f2**), β-elemene (**f3**), (*Z*)-β-caryophyllene (**f4**), α-barbatene (**f5**), α-cedrene (**f6**), (*E*)-β-caryophyllene (**f7**), (*Z*)-thujopsene (**f8**), (*E*)-thujopsene (**f9**), (*Z*)-β-farnesene, (**f10**), α-himachalene (**f11**), α-humulene (**f12**), (*E*)-β-farnesene (**f13**), α-acoradiene (**f14**), β-acoradiene (**f15**), β-chamigrene (**f16**), germacrene D (**f17**), α-chamigrene (**f18**), valencene (**f19**), β-himachalene (**f20**), cuparene (**f21**), (*E*,*E*)-α-farnesene (**f22**), β-bisabolene (**f23**), δ-cadinene (**f24**), β-sesquiphellandrene (**f25**), longiborneol (**f26**), α-ionone (**f27**), and β-ionone (**f28**).[42,43]

pathways have been cloned and some enzymes isolated, additional important key questions focus on their biosynthetic regulation and pathway engineering.[67,71,72]

3.28.2.4.3 Metabolites derived from glucosinolates and other sulfur-containing metabolites

It is well known that under certain conditions, for example, tissue damage, glucosinolates can be enzymatically hydrolyzed by endogenous myrosinases (thioglucohydrolases) to yield metabolic products that include isothiocyanates, thiocyanates, and nitriles (**Figure 11**).[79] However, not all potential isothiocyanates (**Figure 11**) derived from glucosinolates (**Figure 10**) produced in *A. thaliana* plants can be detected. Certain glucosinolates, namely, indolyl containing **j35–j38**, yield rather unstable intermediates prone to further reactions. The stable glucosinolate degradation metabolites have a variety of biological activities, including antifungal and anticarcinogenic, as discussed in the Section 3.28.3 'Ecological Roles of Secondary Metabolites of *Arabidopsis thaliana*'. In addition to these metabolites, a few sulfur-containing volatiles, including sulfur, were detected in *A. thaliana* as well (**Figure 12**).

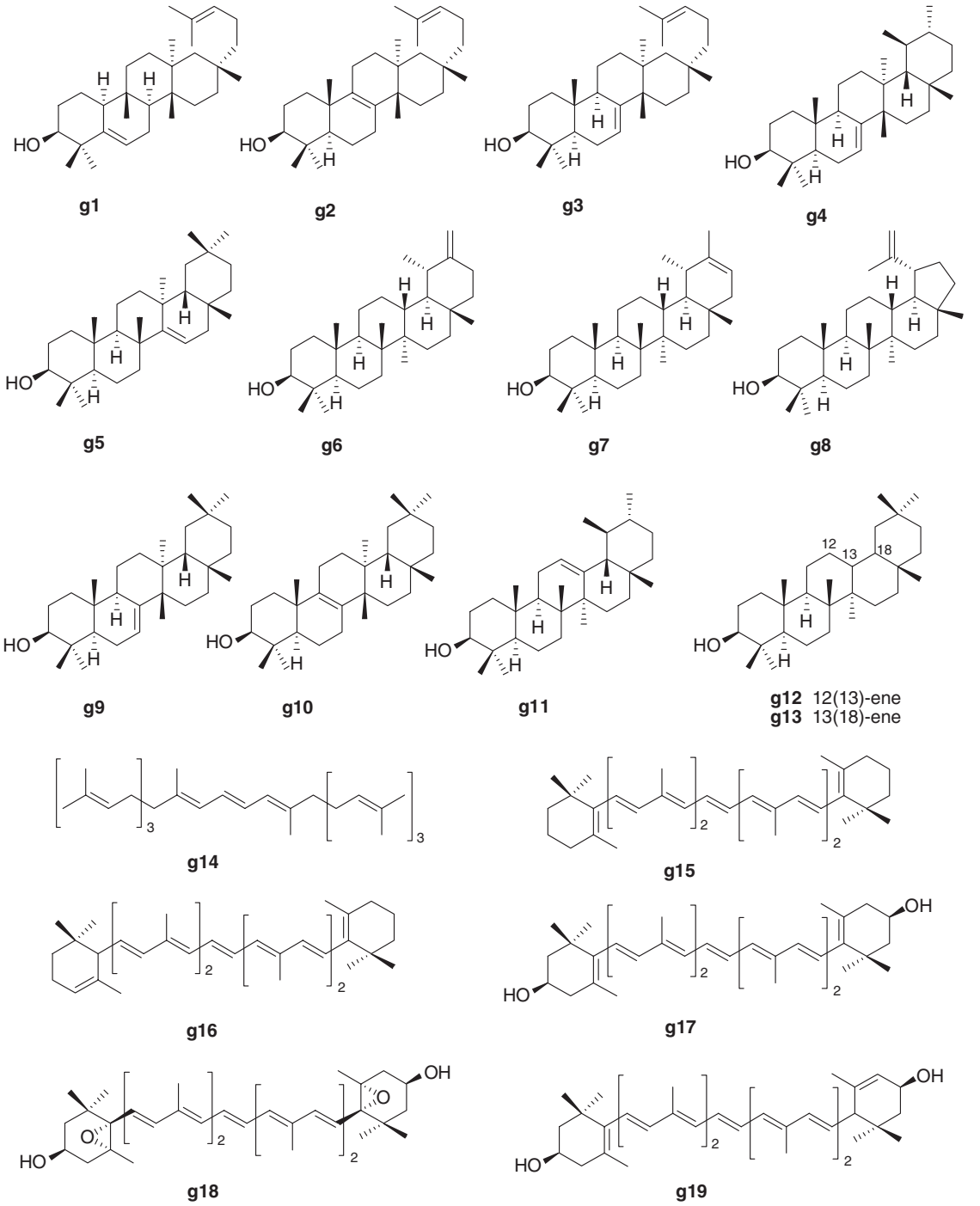


Figure 7 Triterpenes and tetraterpenes from *A. thaliana*: baruol (**g1**), columbiol (**g2**), lemmaphylladienol (**g3**), baurenol (**g4**), taraxerol (**g5**), taraxasterol (**g6**), ψ-taraxasterol (**g7**), lupeol (**g8**); multiflorenol (**g9**), isomultiflorenol (**g10**), α-amyrin (**g11**), β-amyrin (**g12**), δ-amyrin (**g13**), phytoene (**g14**), β-carotene (**g15**), α-carotene (**g16**), zeaxanthin (**g17**), violaxanthin (**g18**), and lutein (**g19**).[44–48]

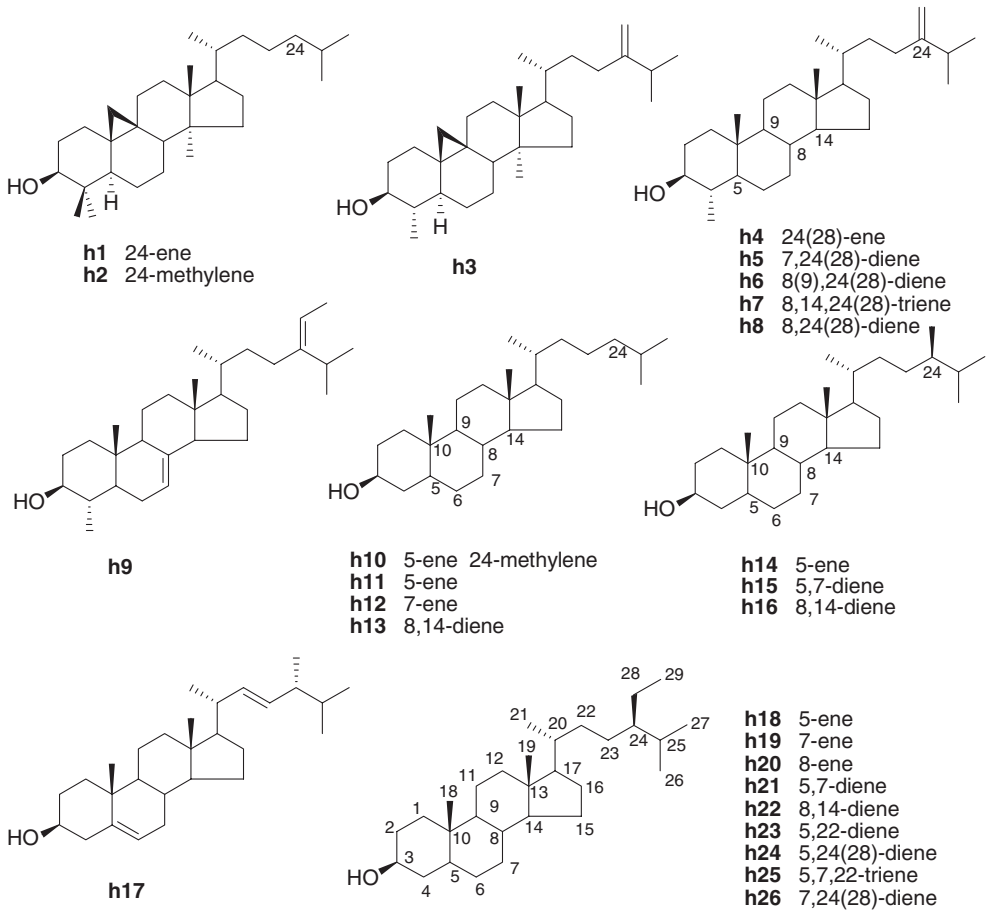


Figure 8 Steroids from *A. thaliana*: cycloartenol (**h1**), 24-methylene cycloartanol (**h2**), cycloeucalenol (**h3**), 24-methylene pollinastanol (**h4**), 24-methylene lophenol (**h5**), obtusifoliol (**h6**), 4α-methyl-ergosta-8, 14, 24(28)-trien-3β-ol (**h7**), 4α-methyl-ergosta-8, 24(28)-dien-3β-ol (**h8**), 24-ethylidene lophenol (**h9**), 24-methylene cholesterol (**h10**), cholesterol (**h11**), Δ^7-cholesterol (**h12**), $\Delta^{8,14}$-cholesterol (**h13**), campesterol (**h14**), $\Delta^{5,7}$-campesterol (**h15**), $\Delta^{8,14}$-campsterol (**h16**), brassicasterol (**h17**), sitosterol (**h18**), Δ^7-sitosterol (**h19**), Δ^8-sitosterol (**h20**), $\Delta^{5,7}$-sitosterol (**h21**), $\Delta^{8,14}$-sitosterol (**h22**), stigmasterol (**h23**), isofucosterol (**h24**), $\Delta^{5,7}$-stigmasterol (**h25**), and Δ^7-avensterol (**h26**).[49–51]

3.28.3 Ecological Roles of Secondary Metabolites of *Arabidopsis thaliana*

Multiple and integrated approaches including 'omics' and genetic engineering technologies are essential to understand and establish the ecological roles of secondary metabolites, including their function in disease and pest resistance. The availability of diverse populations and mutants of *A. thaliana* makes it a unique model to investigate the ecological roles of secondary metabolites. Among the biogenetic classes of secondary metabolites biosynthesized in *A. thaliana*, the glucosinolate–myrosinase system and the phytoalexins camalexin and rapalexin A (**Figure 9**, **i23**, and **i25**, respectively) are characteristic of crucifers, and it is therefore pertinent to discuss the current understanding of their ecological roles in this review. For many decades, the glucosinolate–myrosinase system (**Figure 11**) of Brassicaceae is known to be involved in plant defense programs, and isothiocyanates (**Figure 12**) are known to play an important defensive role against certain pathogenic bacteria, fungi, and pests. Natural variation of certain loci in *A. thaliana* has been related to the differential control of herbivory, depending on whether the insect is a crucifer generalist or specialist.[80] Two species of flea beetles, the cruciferous feeding specialists *Phyllotreta nemorum* and *P. cruciferae*, do not discriminate between *A. thaliana* lines that express high levels of 4-hydroxybenzyl glucosinolate and wild-type plants,[81] indicating that a

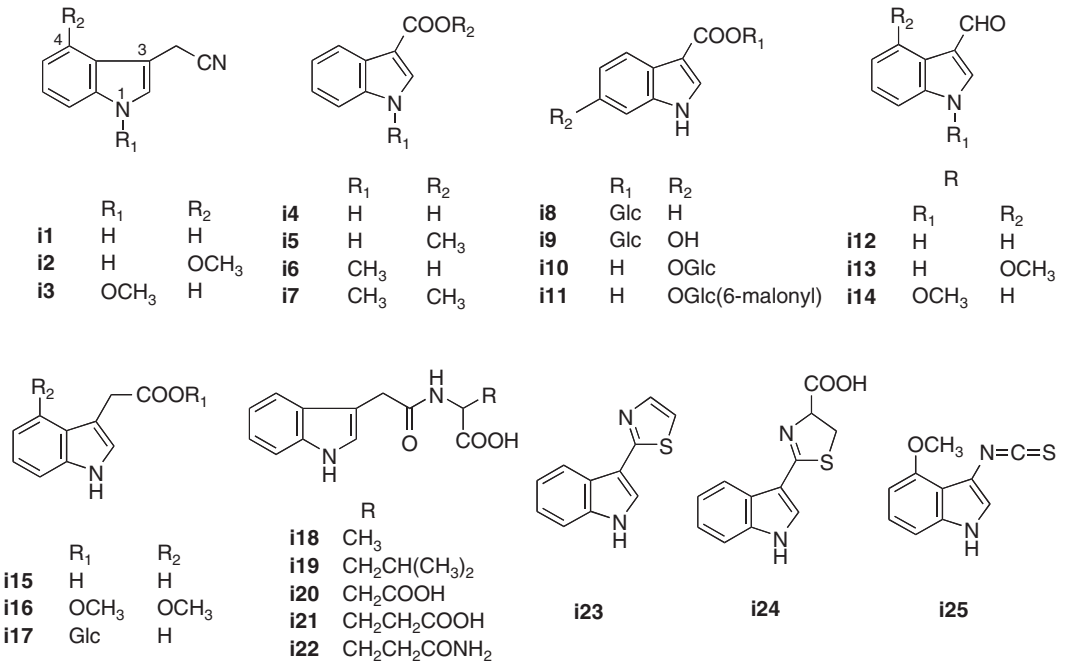


Figure 9 Indolyl-3-acetonitriles (**i1–i3**), indole-3-carboxylic acid (**i4**) and derivatives **i5–i11**, indole-3-carboxaldehydes **i12–i14**, indolyl-3-acetic acid (**i15**) and derivatives **i16–i22**, phytoalexins camalexin (**i23**) and rapalexin A (**i25**), and the camalexin precursor camalexic acid (**i24**) from *A. thaliana*.[20,57,60–66]

significant change in the glucosinolate profile does not affect the ability of flea beetles to recognize and feed on *A. thaliana*. On the contrary, *P. nemorum* can discriminate strongly between transgenic *A. thaliana* expressing high levels of dhurrin,[82] a cyanogenic glucoside not usually produced in crucifers, and wild-type plants. The flea beetles consume up to 80% less of the transgenic *A. thaliana* leaf material as compared with wild-type leaves, and the majority of flea beetle larvae die when fed the dhurrin containing lines. The ability to make these dhurrin-producing transgenic plants, thus introducing a new metabolic pathway in *A. thaliana*, is an important milestone in plant metabolic engineering of secondary metabolites. Importantly, the production of dhurrin had little impact on the visual plant phenotype, metabolome, and transcriptome of *A. thaliana*.[83] Glucosinolates can act as feeding deterrants as well, as high glucosinolate amount can deter the generalist herbivors *Trichoplusia ni* and *Spodoptera exigua*. Glucosinolates can have feeding stimulant activity as found for the specialist *Plutella xylostella*.[84] When *Myzus persicae* (green peach aphid) feeds on *A. thaliana* leaves, aliphatic glucosinolates pass through the aphid gut intact, but indole glucosinolates are mostly degraded, causing a decrease in glucosinolate content but an induction in production of 4-methoxyindolyl-3-methylglucosinolate. This glucosinolate profile is limited to the area in which aphids are feeding. 4-Methoxyindolyl-3-methylglucosinolate (**j36**) is a significantly greater aphid deterrent in the absence of myrosinase than indolyl-3-methylglucosinolate (**j35**). Addition of purified indolyl-3-methylglucosinolate (**j35**) to the petioles of mutant leaves of *A. thaliana* not producing indole glucosinolates indicates that indolyl-3-methylglucosinolate is a precursor of 4-methoxyindolyl-3-methylglucosinolate.[85] However, feeding experiments with deuterated glucosinolate precursors indicate that oxidation and methylation at positions 1 or 4 of the indole ring occur at earlier stages.[55,86] Glucosinolate accumulation and expression of glucosinolate biosynthetic genes in response to four herbivores in *A. thaliana* wild-type and mutant lines lead to increased aliphatic glucosinolate content for three of four herbivores tested: the aphid generalist *M. persicae*, the aphid specialist *Brevicoryne brassicae*, and the lepidopteran generalist *Spodoptera exigua*. The lepidopteran specialist *Pieris rapae* does not affect aliphatic glucosinolate content in the wild-type line, but indole glucosinolates increase slightly. However, gene expression associated with aliphatic glucosinolate biosynthesis increases after feeding by all species. *Arabidopsis thaliana* lines with

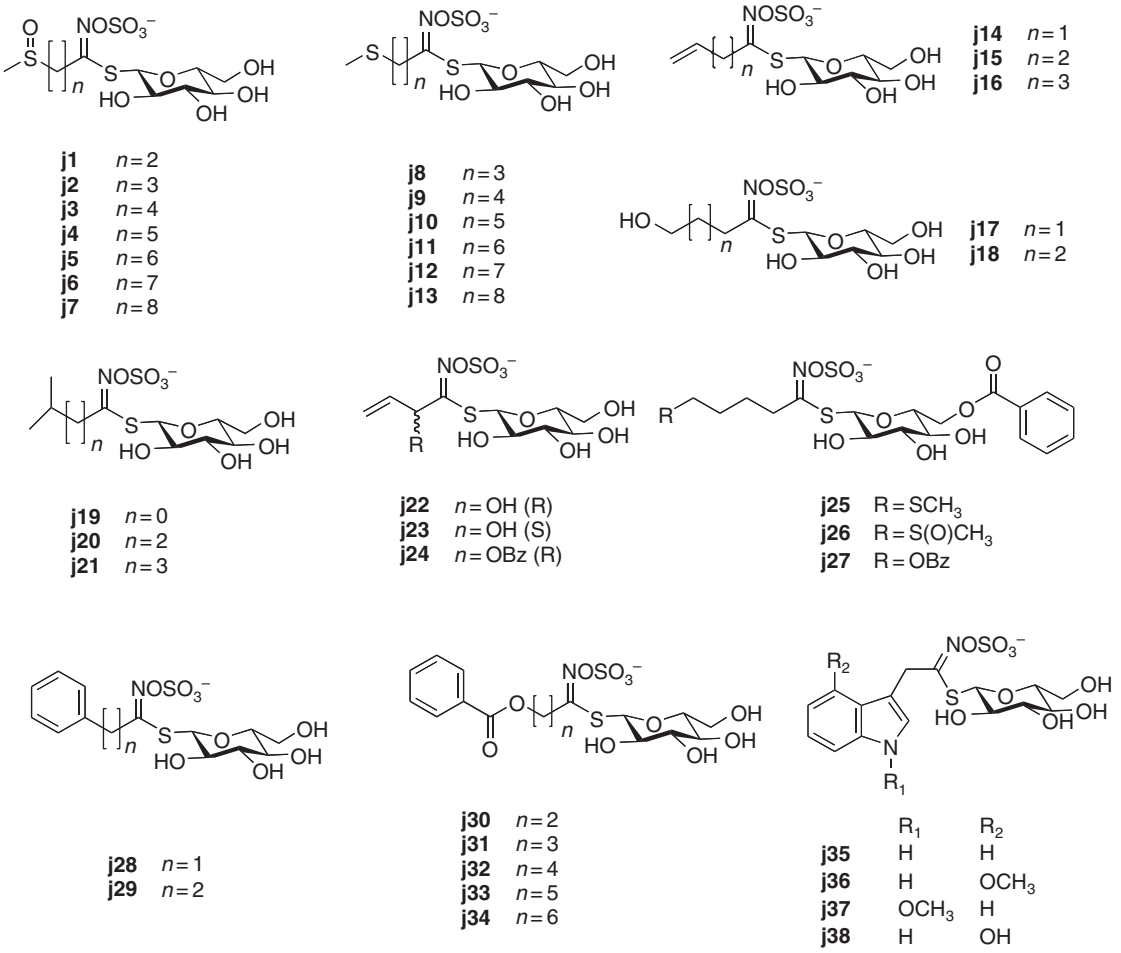


Figure 10 Aliphatic (**j1–j27**), aromatic (**j28–j34**), and indole (**j35–j38**) glucosinolates from *A. thaliana*.[73–78]

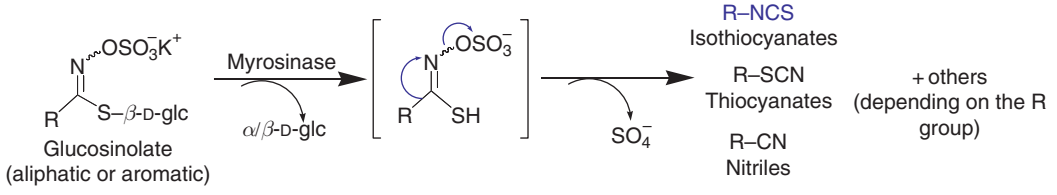


Figure 11 Myrosinase (thioglucohydrolase; E.C. 3.2.1.147) mediated transformation of glucosinolates.[79]

mutations in jasmonate, salicylate, and ethylene signaling diverge in gene expression, glucosinolate content, and insect performance compared with wild type. In contrast to the wild-type response, gene transcripts of aliphatic glucosinolate biosynthesis do not generally increase in the mutants. Both glucosinolate content and gene expression data indicate that salicylate and ethylene signaling repress some jasmonate-mediated responses to herbivory.[87] It is believed that myrosinase-independent degradation of indole glucosinolates during aphid feeding is an alternate plant defense strategy for deterring these insects, which are able to avoid or prevent myrosinase-catalyzed glucosinolate activation.[88]

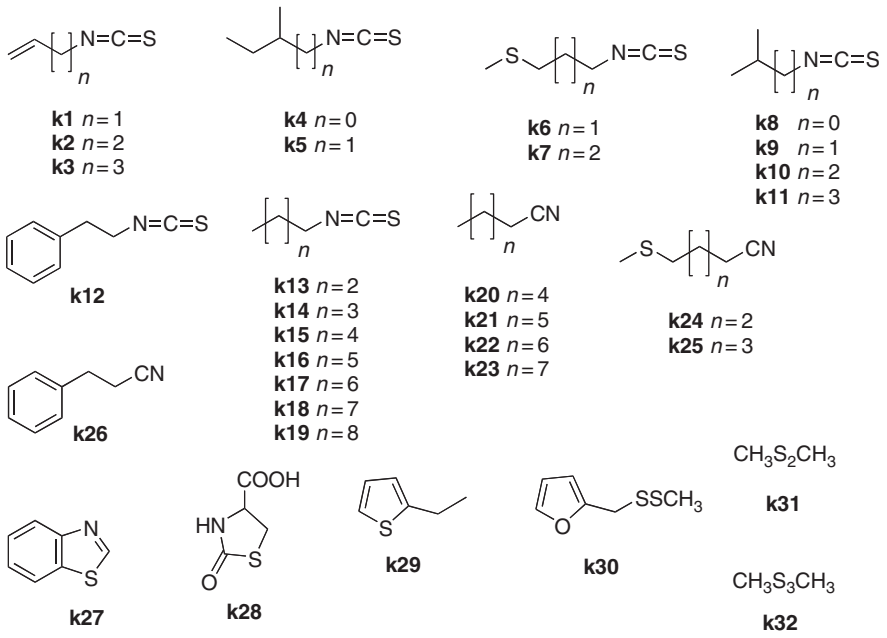


Figure 12 Metabolites derived from glucosinolates isothiocyanates (**k1–k19**), nitriles (**k20–k26**), and sulfur containing metabolites (**k27–k32**) from *A. thaliana*.[37,68,74,75]

The disease resistance of genetically engineered *A. thaliana* producing increased quantities of specific glucosinolates revealed that these metabolites have important functions. Isopropyl and methylpropyl glucosinolates enhanced resistance of transgenic *A. thaliana* against the bacterial soft-rot pathogen *Erwinia carotovora*, whereas 4-hydroxybenzyl or benzyl glucosinolates increases resistance toward the bacterial pathogen *Pseudomonas syringae*. However, disease resistance of engineered *A. thaliana* to the necrotrophic fungus *Alternaria brassicicola* is not increased by altering glucosinolates; indeed, introduction of aromatic glucosinolates can lead to increased susceptibility.[89] Overall, it is clear that, like other plant species, *A. thaliana* uses different pathogen-inducible defense programs that contribute to resistance against particular pathogens.[90] Quantitative and qualitative metabolite analyses of extracts of *A. thaliana* wild type and mutant root cultures infected with the root-pathogenic oomycete *Pythium sylvaticum* indicate that with respect to phenylpropanoid metabolites, roots differ greatly from leaves, whereas indole metabolite profiles are similar in roots and leaves. Moreover, pathogen defense in both leaves and roots appears to be associated with a decrease in soluble phenylpropanoids and an increase in soluble indole metabolites.[32] Sulfur-related genes, namely, sulfur-rich defense proteins and enzymes involved in glucosinolate metabolism in *A. thaliana*, are also affected strongly by the phytohormone methyl jasmonate, altogether indicating an important connection between the regulation of sulfur-related genes and the plant defense programs.[91] In general, sulfur deficiency in plants causes retarded and chlorotic growth and can affect substantially crop yields negatively. Considering the number of sulfur-containing metabolites produced in *A. thaliana*, sulfur starvation is expected to have a larger impact. In fact, more than 2700 genes were affected by sulfur starvation. For example, a thioglucosidase is strongly induced in the roots after 24 h of sulfur deficiency, whereas phenylalanine ammonia lyase, involved in the phenylpropanoid pathway, is strongly reduced after 6 and 10 days of sulfur deficiency. Metabolome analysis shows that ~10% of the 6000 metabolites detected were affected significantly, as for example tryptophan, whose concentration increased up to 28-fold after 13 days of sulfur starvation.[92] Because of all these important aspects, there is a great interest in controlling the level of specific glucosinolates in crops to improve nutritional value and pest and disease resistance.

The phytoalexins camalexin (**i23**) and rapalexin A (**i25**) (**Figure 9**) are produced in *A. thaliana* as well as in a few other crucifer species[19,55–57] in response to biotic and abiotic stress. Phytoalexins are plant secondary metabolites produced *de novo* in response to various forms of stress, including pathogen attack. Because plants in

general produce phytoalexin blends with different antifungal activity,[55] it is likely that phytoalexins other than camalexin and rapalexin A are produced in *A. thaliana*. Although numerous studies address the biological role of camalexin (**i23**), because rapalexin A (**i25**) is recently isolated from *B. rapa*, only its activity against the biotrophic plant pathogenic oomycete *Albugo candida* is known to be high.[56] Like in many other species, leaves of *A. thaliana* display efficient resistance responses to *B. cinerea* using basic defense mechanisms such as cell wall modification, papilla formation, synthesis of antimicrobial compounds, H_2O_2 and nitric oxide production, retraction of water from primary infections, and autophagy-like survival responses. Cell wall modification and papilla formation are among the first visible responses, whereas camalexin synthesis appears to occur at a later stage and is of critical importance for restriction of colonization.[93] It appears that camalexin does not affect the resistance of *A. thaliana* to the biotrophic oomycete *Hyaloperonospora parasitica*, the biotrophic ascomycete *Erysiphe orontii*, or the hemibiotrophic bacterium *Pseudomonas syringae* but is important for resistance to *A. brassicicola*, *B. cinerea*, and *Leptosphaeria maculans*.[19] Because a few fungal pathogens (*Sclerotinia sclerotiorum*[94] and *Rhizoctonia solani*[95]) are able to detoxify camalexin, its quantification in infected plant tissues might not provide the amounts actually produced by infected plants. In addition to phytoalexins, plants produce constitutive defenses known as phytoanticipins,[55] in which indolyl-3-acetonitriles (**Figure 9**) and isothiocyanates (**Figure 12**) are included due to their inhibitory activity against microbial pathogens.

Contrary to alkaloids such as glucosinolates, flavonoids occur in a much wider range of plant families. Accordingly, their roles in the defense of plants against pathogens, herbivores, and environmental stress have been widely investigated. The induction of defense-related flavonoids is affected by diverse factors including competition between growth and secondary metabolism.[84] For example, flavonoids affect auxin transport and development in *A. thaliana*, although the flavonoids that are specifically responsible for these effects are not yet clear.[96] Similarly, terpenoids and steroids are widely occurring in plants and thus aspects related to their ecological roles are well studied. For example, transgenic lines of *A. thaliana* plants expressing high levels of free, hydroxylated, and glycosylated linalool derivative showed growth retardation and repelled the aphid *Myzus persicae*.[97]

Finally, it is pertinent to mention that glucosinolates, via the corresponding isothiocyanates, have positive and negative dietary effects on mammalian systems. Positive effects may include anticarcinogenic and enhanced antioxidant activity, whereas negative roles include poor palatability, decreased food efficiency, hypertrophy and hyperplasia of the thyroid, goiter, liver lesions, and necrosis.[98,99] The negative effects of glucosinolates, for example, poor palatability and decreased food efficiency, may protect the plant as it can deter mammals from feeding on crucifers.

3.28.4 Conclusions

Wild-type populations and mutants of *A. thaliana* with modified metabolite contents will continue to provide means to investigate secondary metabolic biosynthetic pathways, including those of phytoalexins and phytoanticipins, assess the biological roles of individual metabolites, and test the feasibility of producing plants with tailor-made disease resistance traits. Specifically, considering that the molecular mechanisms of regulation of the sulfate assimilation pathways are not understood, the role of Arabidopsis as a model plant will continue to have impact on many research programs across the world.[92] Integration and analysis of metabolomics and transcriptomics data of *A. thaliana* grown under sulfur deficiency show that a group of metabolites/genes regulated by the same mechanism cluster together and that the metabolism of glucosinolates is regulated coordinately. Therefore, it is likely that this approach will continue be used to provide further information on the regulation of metabolite pathways of *A. thaliana*.[100] Extensive use of 'omics' should provide detailed understanding of defense programs in *A. thaliana*, leading to the identification of gene functions and potential extrapolation to genetically engineer naturally occurring pathways in important crop species. In a decade from now, perhaps most metabolic secondary pathways of *A. thaliana* will have been mapped and expressed in other species/organisms and their ecological roles understood, an important achievement to facilitate environmentally sustainable worldwide production of staple crops.

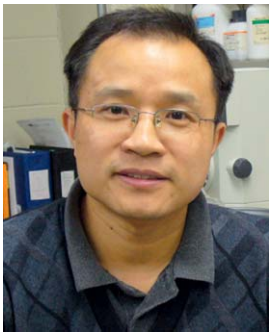
References

1. E. M. Meyerowitz, *Ann. Rev. Genet.* **1987**, *21*, 93–111.
2. E. M. Meyerowitz, *Plant Physiol.* **2001**, *125*, 15–19.
3. The Arabidopsis Initiative, *Nature* **2000**, *408*, 796–815.
4. M. Bevan; S. Walsh, *Genome Res.* **2005**, *15*, 1632–1642.
5. J. S. Johnston; A. E. Pepper; A. E. Hall; Z. J. Chen; G. Hodnett; J. Drabek; R. Lopez; H. J. Price, *Ann. Bot.* **2005**, *95*, 229–235.
6. J. E. Bowers; B. A. Chapman; J. Rong; A. H. Paterson, *Nature* **2003**, *422*, 433–438.
7. M. A. Lysak; C. Lexer, *Plant Syst. Evol.* **2006**, *259*, 175–198.
8. T. Hartmann, *Phytochemistry* **2007**, *68*, 2831–2846.
9. P. Broun, *Curr. Opin. Plant Biol.* **2005**, *8*, 272–279.
10. M. Wink, *Phytochemistry* **2003**, *64*, 3–19.
11. K. Kai; J. Horita; K. Wakasa; H. Miyagawa, *Phytochemistry* **2007**, *68*, 1651–1663.
12. E. Fridman; E. Pichersky, *Curr. Opin. Plant Biol.* **2005**, *8*, 242–248.
13. O. Fiehn; J. Kopka; P. Dormann; T. Altmann; R. N. Trethewey; L. Willmitzer, *Nature Biotechnol.* **2000**, *18*, 1157–1161.
14. W. B. Dunn; D. I. Ellis, *Trends Anal. Chem.* **2005**, *24*, 285–294.
15. E. A. Schmelz; J. Engelberth; J. H. Tumlinson; A. Block; H. T. Alborn, *Plant J.* **2004**, *39*, 790–808.
16. L. S. Barkawi; Y.-Y. Tam; J. A. Tillman; B. Pederson; J. Calio; H. Al-Amier; M. Emerick; J. Normanly; J. D. Cohen, *Anal. Biochem.* **2008**, *372*, 177–188.
17. L. Lepiniec; I. Debeaujon; J. M. Routaboul; A. Baudry; L. Pourcel; N. Nesi; M. Caboche, *Annu. Rev. Plant Biol.* **2006**, *57*, 405–430.
18. C. Barth; M. De Tullio; P. L. Conklin, *J. Exp. Bot.* **2006**, *57*, 1657–1665.
19. E. Glawischnig, *Phytochemistry* **2007**, *68*, 401–406.
20. K. Ljung; A. K. Hull; M. Kowalczyk; A. Marchant; J. Celenza; J. D. Cohen; G. Sandberg, *Plant Mol. Biol.* **2002**, *49*, 249–272.
21. J. C. D'Auria; J. Gershenzon, *Curr. Opin. Plant Biol.* **2005**, *8*, 308–316.
22. Y. Miyashita; A. G. Good, *Plant Cell Physiol.* **2008**, *49*, 92–102.
23. N. Bouché; H. Fromm, *Trends Plant Sci.* **2004**, *9*, 1360–1385.
24. E. E. Farmer; H. Weber; S. Vollenweider, *Planta* **1998**, *206*, 167–174.
25. S. Goepfert; Y. Poirier, *Curr. Opin. Plant Biol.* **2007**, *10*, 245–251.
26. J. M. Shockey; M. S. Fulda; J. A. Browse, *Plant Physiol.* **2002**, *129*, 1710–1722.
27. C. Lai; L. Kunst; R. Jetter, *Plant J.* **2007**, *50*, 189–196.
28. R. M. P. Van Poecke; A. P. Maarten; D. Marcel, *J. Chem. Ecol.* **2001**, *27*, 1911–1928.
29. K. M. Herrmann; L. M. Weaver, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1999**, *50*, 473–503.
30. J. P. Noel; M. B. Austin; E. K. Bomati, *Curr. Opin. Plant Biol.* **2005**, *8*, 249–253.
31. A. R. Knaggs, *Nat. Prod. Rep.* **2003**, *20*, 119–136.
32. P. Bednarek; B. Schneider; A. Svatos; N. J. Oldham; K. Hahlbrock, *Plant Physiol.* **2005**, *138*, 1058–1070.
33. H. P. Mock; V. Wray; W. Beck; J. W. Metzger; D. Strack, *Phytochemistry* **1993**, *34*, 157–159.
34. A. Rohde; K. Morreel; J. Ralph; G. Goeminne; V. Hostyn; R. De Rycke; S. Kushnir; J. van Doorsselaere; J.-P. Joweleau; M. Vuylsteke; G. van Driessche; J. van Beeumen; E. Messens; W. Boerjan, *Plant Cell* **2004**, *16*, 2749–2771.
35. J. Tan; P. Bednarek; J. Liu; B. Schneider; A. Svatos; K. Hahlbrock, *Phytochemistry* **2004**, *65*, 691–699.
36. T. S. Walker; H. P. Bais; K. M. Halligan; F. R. Stermitz; J. M. Vivanco, *J. Agric. Food Chem.* **2003**, *51*, 2548–2554.
37. J. Rohloff; A. M. Bones, *Phytochemistry* **2005**, *66*, 1941–1955.
38. S. J. Bloor; S. Abraham, *Phytochemistry* **2002**, *59*, 343–346.
39. I. Debeaujon; N. Nesi; P. Perez; M. Devic; O. Grandjean; M. Caaboche; L. Lepiniec, *Plant Cell* **2003**, *15*, 2514–2531.
40. T. L. Graham, *Plant Physiol. Biochem.* **1998**, *36*, 135–144.
41. M. Viet; G. F. Pauli, *J. Nat. Prod.* **1999**, *62*, 1301–1303.
42. F. Chen; D. Tholl; J. C. D'Auria; A. Farooq; E. Pichersky; J. Gershenzon, *Plant Cell* **2003**, *15*, 481–494.
43. F. Chen; D. K. Ro; J. Petri; J. Gershenzon; J. Bohlmann; E. Pichersky; D. Tholl, *Plant Physiol.* **2004**, *135*, 1956–1966.
44. S. Lodeiro; Q. Xiong; W. K. Wilson; M. D. Kolesnikova; C. S. Onak; S. P. T. Matsuda, *J. Am. Chem. Soc.* **2007**, *129*, 11213–11222.
45. B. M. Lange; M. Ghassemian, *Plant Mol. Biol.* **2003**, *51*, 925–948.
46. T. Husselstein-Muller; H. Schaller; P. Benveniste, *Plant Mol. Biol.* **2001**, *45*, 75–92.
47. M. Suzuki; T. Xiang; K. Ohyama; H. Seki; K. Saito; T. Muranaka; H. Hayashi; Y. Katsube; T. Kushiro; M. Shibuya; Y. Ebizuka, *Plant Cell Physiol.* **2006**, *47*, 565–571.
48. M. A. Jenks; H. A. Tuttle; S. D. Eigenbrode; K. A. Feldmann, *Plant Physiol.* **1995**, *108*, 359–377.
49. H. Schaller, *Prog. Lipid Res.* **2003**, *42*, 163–175.
50. S. Fujioka; T. Yokota, *Ann. Rev. Plant Biol.* **2003**, *54*, 137–164.
51. A. Schaeffer; R. Bronner; P. Benveniste; H. Schaller, *Plant J.* **2001**, *25*, 605–615.
52. P. J. Facchini; D. A. Bird; B. St-Pierre, *Trends Plant Sci.* **2004**, *9*, 116–122.
53. A. Brock; T. Herzfeld; R. Paschke; M. Koch; B. Draeger, *Phytochemistry* **2006**, *67*, 2050–2057.
54. S. Abel, *ACS Chem. Biol.* **2007**, *2*, 380–384.
55. M. S. C. Pedras; Q. A. Zheng; V. K. Sarma-Mamillapalle, *Nat. Prod. Commun.* **2007**, *2*, 319–330.
56. M. S. C. Pedras; Q. A. Zheng; G. S. Ravi, *Chem. Commun*. **2007**, 368–370.
57. M. S. C. Pedras; A. M. Adio, *Phytochemistry* **2008**, *69*, 889–893.
58. M. Burow; Z.-Y. Zhang; J. A. Ober; V. M. Lambrix; U. Wittstock; J. Gershenzon; D. J. Kliebenstein, *Phytochemistry* **2008**, *69*, 663–671.
59. B. G. Hansen; B. A. Halkier, *Planta* **2005**, *221*, 603–606.

60. I. Barlier; M. Kowalczyk; A. Marchant; K. Ljung; R. Bhalerao; M. Bennett; G. Sandberg; C. Bellini, *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 14819–14824.
61. N. M. Barratt; W. Dong; D. A. Gage; V. Magnus; C. D. Town, *Physiol. Plant* **1999**, *105*, 207–217.
62. J. Hagemeier; B. Schneider; N. J. Oldham; K. Hahlbrock, *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 753–758.
63. M. Kowalczyk; G. Sandberg, *Plant Physiol.* **2001**, *127*, 1845–1853.
64. A. Östin; M. Kowalczyk; R. P. Bhalerao; G. Sandberg, *Plant Physiol.* **1998**, *118*, 285–296.
65. Y. Y. Tam; E. Epstein; J. Normanly, *Plant Physiol.* **2000**, *123*, 589–595.
66. J. Tsuji; E. P. Jackson; D. A. Gage; R. Hammerschmidt; S. C. Somerville, *Plant Physiol.* **1992**, *98*, 1304–1309.
67. B. A. Halkier; J. Gershenzon, *Annu. Rev. Plant Biol.* **2006**, *57*, 303–333.
68. G. W. Haughn; L. Davin; M. Giblin; E. W. Underhill, *Plant Physiol.* **1991**, *97*, 217–226.
69. M. Nafisi; I. E. Sønderby; B. G. Hansen; F. Geu-Flores; H. H. Nour-Eldin; M. H. H. Nørholm; N. B. Jensen; J. Li; B. A. Halkier, *Phytochem. Rev.* **2006**, *5*, 331–346.
70. C. D. Grubb; S. Abel, *Trends Plant Sci.* **2006**, *11*, 89–100.
71. D. J. Kliebenstein; J. Kroymann; P. Brown; A. Figuth; D. Pedersen; J. Gershenzon; T. Mitchell-Olds, *Plant Physiol.* **2001**, *126*, 811–825.
72. T. Gigolashvili; B. Berger; H.-P. Mock; C. Muller; B. Weisshaar; U.-I. Flugge, *Plant J.* **2007**, *50*, 886–901.
73. N. Agerbirk; B. L. Petersen; C. E. Olsen; B. A. Halkier; J. K. Nielsen, *J. Agric. Food Chem.* **2001**, *49*, 1502–1507.
74. R. A. Cole, *Phytochemistry* **1976**, *15*, 759–762.
75. M. E. Daxenbichler; G. F. Spencer; D. G. Carlson; G. B. Rose; A. M. Brinker; R. G. Powell, *Phytochemistry* **1991**, *30*, 2623–2638.
76. L. R. Hogge; D. W. Reed; E. W. Underhill; G. W. Haughn, *J. Chromatogr. Sci.* **1988**, *26*, 551–556.
77. J. Ludwig-Muller; K. Pieper; M. Ruppel; J. D. Cohen; E. Epstein; G. Kiddle; R. Bennett, *Planta* **1999**, *208*, 409–419.
78. M. Reichelt; P. D. Brown; B. Schneider; N. J. Oldham; E. Stauber; J. Tokuhisa; D. J. Kliebenstein; T. Mitchell-Olds; J. Gershenzon, *Phytochemistry* **2002**, *59*, 663–671.
79. A. M. Bones; J. T. Rossiter, *Phytochemistry* **2006**, *67*, 1053–1067.
80. D. J. Kliebenstein; J. Kroymann; T. Mitchell-Olds, *Curr. Opin. Plant Biol.* **2005**, *8*, 264–271.
81. J. K. Nielsen; M. L. Hansen; N. Agerbirk; B. L. Petersen; B. A. Halkier, *Chemoecology* **2001**, *11*, 75–83.
82. K. A. Nielsen; D. B. Tattersall; P. R. Jones; B. L. Moller, *Phytochemistry* **2008**, *69*, 88–98.
83. A. V. Morant; K. Jorgensen; B. Jorgensen; W. Dam; C. E. Olsen; B. L. Moller; S. Bak, *Metabolomics* **2007**, *3*, 383–398.
84. D. J. Kliebenstein, *Plant Cell Environ.* **2004**, *27*, 675–684.
85. J. H. Kim; G. Jander, *Plant J.* **2007**, *49*, 1008–1019.
86. M. S. C. Pedras; D. P. O. Okinyo, *Org. Biomol. Chem.* **2008**, *6*, 51–54.
87. I. Mewis; J. G. Tokuhisa; J. C. Schultz; H. M. Appel; C. Ulrichs; J. Gershenzon, *Phytochemistry* **2006**, *67*, 2450–2462.
88. M. De Vos; J. H. Kim; G. Jander, *BioEssays* **2007**, *29*, 871–883.
89. G. Brader; M. D. Mikkelsen; B. A. Halkier; P. E. Tapio, *Plant J.* **2006**, *45*, 758–767.
90. K. F. M.-J. Tierens; B. P. H. J. Thomma; M. Brouwer; J. Schmidt; K. Kistner; A. Porzel; B. Mauch-Mani; B. P. A. Cammue; W. F. Broekaert, *Plant Physiol.* **2001**, *125*, 1688–1699.
91. R. Jost; L. Altschmied; E. Bloem; J. Bogs; J. Gershenzon; U. Haehnel; R. Haensch; T. Hartmann; S. Kopriva; C. Kruse; R. Mendel; J. Papenbrock; M. Reichelt; H. Rennenberg; E. Schnug; A. Schmidt; S. Textor; J. Tokuhisa; A. Wachter; M. Wirtz; T. Rausch; R. Hell, *Photosynth. Res.* **2005**, *86*, 491–508.
92. S. Kopriva, *Ann. Bot.* **2006**, *97*, 479–495.
93. P. Van Baarlen; E. J. Woltering; M. Staats; J. A. L. Van Kan, *Mol. Plant Pathol.* **2007**, *8*, 41–54.
94. M. S. C. Pedras; P. W. K. Ahiahonu, *Bioorg. Med. Chem.* **2002**, *10*, 3307–3312.
95. M. S. C. Pedras; A. Q. Khan, *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2255–2260.
96. D. Treutter, *Environ. Chem. Lett.* **2006**, *4*, 147–157.
97. A. Aharoni; A. P. Giri; S. Deuerlein; F. Griepink; W.-J. de Kogel; F. W. A. Verstappen; H. A. Verhoeven; M. A. Jongsma; W. Schwab; H. J. Bouwmeester, *Plant Cell* **2003**, *15*, 2866–2884.
98. K. R. Anilakumar; F. Khanum; A. S. Bawa, *J. Food Sci. Technol.* **2006**, *43*, 8–17.
99. O. Vang, Chemopreventive Potential of Compounds in Cruciferous Vegetables. In *Carcinogenic and Anticarcinogenic Food Components*; W. Baer Dubowska, A. Bartoszek, D. Malejka-Giganti, Eds.; Taylor & Francis Group: Florida, USA, 2006; pp 303–328.
100. M. Y. Hirai; M. Klein; Y. Fujikawa; M. Yano; D. B. Goodenowe; Y. Yamazaki; S. Kanaya; Y. Nakamura; M. Kitayama; H. Suzuki; N. Sakurai; D. Shibata; J. Tokuhisa; M. Reichelt; J. Gershenzon; J. Papenbrock; K. Saito, *J. Biol. Chem.* **2005**, *280*, 25590–25595.

Biographical Sketches

M. Soledade C. Pedras was born in Portugal where she obtained a B.Sc. in Chemistry and a Lic. degree in organic chemistry from the University of Porto. She came to Canada with a NATO fellowship to study organic chemistry in the Department of Chemistry, University of Alberta, from where she graduated with a Ph.D. degree in 1986. Dr. Pedras accepted a postdoctoral Canadian Government Laboratory Visiting Fellowship at the Plant Biotechnology Institute – National Research Council of Canada in Saskatoon where she stayed as a researcher until June 1994. She then moved to the Department of Chemistry at the University of Saskatchewan, where she is a full professor since July 1998. She is currently a Tier 1 Canada Research Chair in Bioorganic and Agricultural Chemistry. Her studies involve the determination of the chemical mediators of the interaction between plants (crucifers) and pathogens, and application of this information to understand and predict the disease resistance of plants and control virulence of plant pathogens. Her projects include biosynthetic pathways of phytoalexins, phytoanticipins and phytotoxins, isolation of detoxifying enzymes from plant pathogens and design and synthesis of paldoxins, and other inhibitors of metabolic processes specific to fungi.



Qingan Zheng obtained a Bachelor degree in Medicine from Hubei College of Traditional Chinese Medicine. After that, he worked in Wuhan Institute of Botany, the Chinese Academy of Science, as a research associate, later on as an assistant professor in research and development of new medicines from natural sources. He obtained his Master degree in Science, in Wuhan Institute of Botany in 1998. From September 2000 to June 2003, he studied natural products chemistry in State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, the Chinese Academy of Sciences, as a Ph.D. candidate. His research topic was the chemical constituents and chemical ecological variation of a traditional Chinese medicine Dragon's blood (resin from *Dracaena cochinchinensis*). Since September 2004, he worked as a postdoctoral fellow and later on as research

associate in Professor Pedras' laboratory, in the Department of Chemistry, University of Saskatchewan, Canada. His research interest is to use integrated methodology to understand the interaction of plants and pathogens, including analysis and synthesis of secondary metabolites related to the defensive responses.