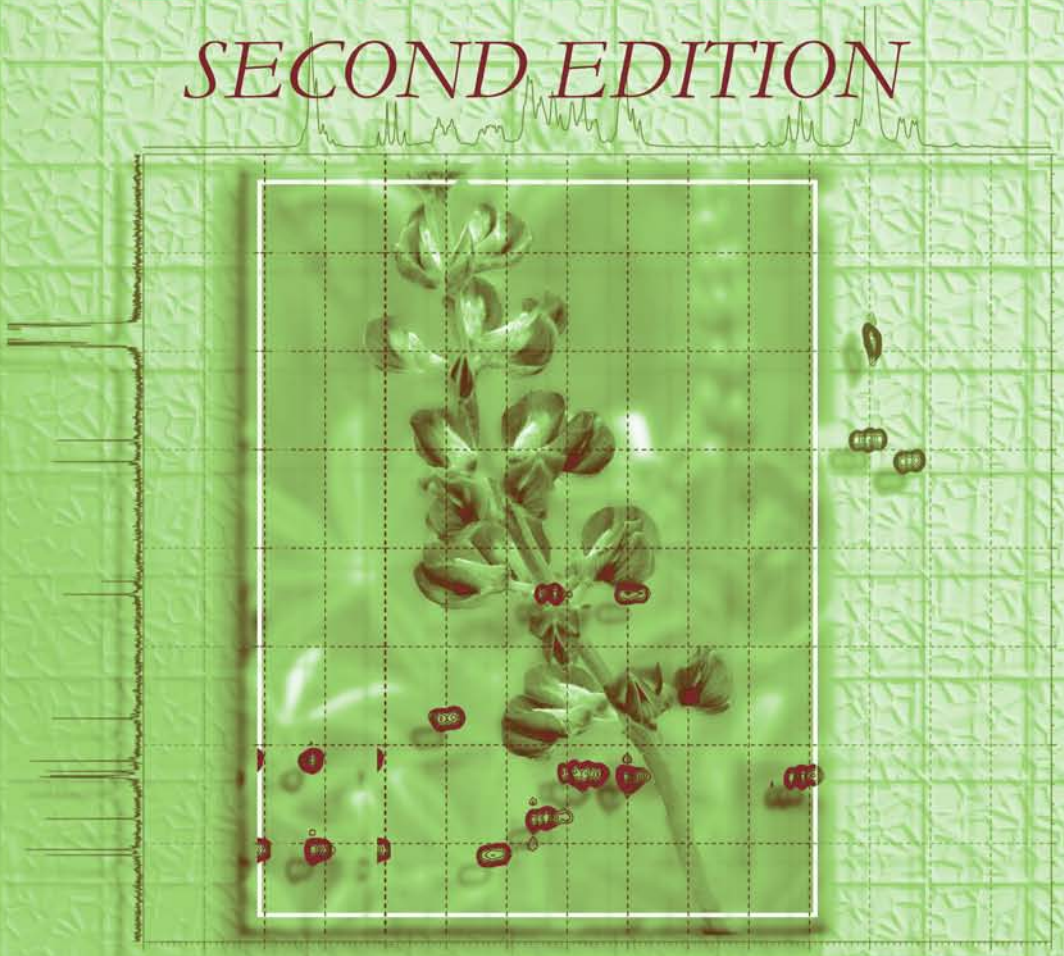
BIOACTIVE NATURAL PRODUCTS

Detection, Isolation, and Structural Determination

SECOND EDITION



Edited by
Steven M. Colegate
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Preface

This edition is a complementary extension of the first edition rather than simply a revised and updated version. Thus, the majority of contributors are new, and the present aspects of bioactive natural product research were not necessarily covered in the first edition. However, following in the same vein as the first edition of this book, the reviewed and edited chapters of this second edition were written by international researchers representing a variety of scientific disciplines. Each contributor consequently approaches bioactive natural product research from a different perspective, highlighting the multidisciplinary and collaborative nature of this type of research.

Again following the ethos of the first edition, contributors to this book were carefully selected to provide an integrated and cohesive treatise of bioactive natural product research, bringing their extensive experience into focus for the benefit of the reader. The contributors to this book have critically reviewed the literature and presented research from their own laboratories that emphasize both the philosophy and rationale that has successfully directed detection and isolation of bioactive natural products. They also review and present the type and quality of structural information that can be acquired and assessed in an integrated way to provide structure assignments for bioactive natural products.

The editors worked closely with all authors to ensure that the individual chapters were amalgamated into a cohesive book that maintained its basic ethos throughout, that is, the imparting of the expertise and experience of successful bioactive natural product researchers to others already researching or planning to research the detection, isolation, and structure determination of bioactive natural products.

Steven M. Colegate
Russell J. Molyneux

Editors

Steven M. Colegate, PhD (University of Western Australia, 1978: organic chemistry) has directed his research mainly, but not exclusively, toward the detection, isolation, and structure determination of those plant-associated bioactive natural products that are toxic to grazing livestock and with the potential to contaminate the human food supply. Experience at the School of Veterinary Studies at Murdoch University, Western Australia, and latterly at the Australian Animal Health Laboratory in Victoria, as leader of the Plant Toxin Research Group within the Division of Livestock Industries of the Commonwealth Scientific and Industrial Research Organisation has firmly entrenched the collaborative, multidisciplinary research ideals that are integral to this book.

Russell J. Molyneux, PhD (University of Nottingham, 1963: organic chemistry) has focused his research, at the Western Regional Research Center in Albany, California, on the chemistry of toxic-range plants. This research has involved close, interdisciplinary collaboration primarily not only with scientists of the Poisonous Plant Research Laboratory in Logan, Utah, but also with many scientists in other parts of the world. The recipient of several awards, the most recent being the 2006 American Chemical Society Spencer Award, Dr. Molyneux is an associate editor of the *Journal of Agriculture and Food Chemistry.*

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1 An Introduction and Overview

Steven M. Colegate and Russell J. Molyneux

CONTENTS

1.1 INTRODUCTION

In the context of this book, and its predecessor, bioactive natural products are those chemical compounds produced by living organisms that exert a biological effect on other organisms. This includes therapeutic activity for diseases of humans and animals, toxic activity responsible for causing human and animal disease, and selective, biodegradable toxicity to help combat pests that may adversely affect our endeavors to feed and otherwise service (e.g., protect cotton crops or plantations of timber used for construction, etc.) the human population. In some cases, these bioactive natural products are secondary metabolites produced by the organism to help protect itself within its own environmental niche. In other cases, the compounds may be integral to the everyday existence of the organism but have serendipitous activity in unrelated biological systems. This latter scenario is exemplified by the search for novel bioactive applications for components of our food, for example, milk and egg-derived proteins.

Several of the contributors to this book have commented on the vast potential offered by natural resources for discovery and development of new therapeutics. They have also commented on the dwindling of such resources in response to the expanding human population and its subsequent demands for food and increasing areas of land on which to live. The extinction of plant and animal species, as mankind encroaches on natural habitats, represents lost and irreplaceable resources, the full potential of which is unpredictable. In the same way, the influence of mankind on aquatic environments parallels that on the terrestrial habitats, with consequently similar concerns for loss of species yet to be investigated or even discovered. The loss of indigenous cultures, as other cultures become influential in an almost cancerous manner, is resulting in the loss of a fount of empirical ethnobotanical knowledge that has been acquired over the course of thousands of years. Indeed, several chapters in this book describe a heavy dependence on ethnobotanical and ethnopharmacological information.

The chapters in this book refer to the detection, isolation, and structural determination of bioactive compounds from terrestrial and aquatic sources, from higher organisms to bacteria and

endophytes. Microbial sources of useful bioactive compounds in conjunction with the capabilities of genetic engineering are extremely important, especially in large-scale production of such compounds.

1.2 THE MULTIDISCIPLINARY APPROACH

Nature recognizes no artificial barriers such as is represented by the "academic disciplines", and thus it is no surprise to find investigators with quite different academic training studying various aspects of bioactive natural products. It is when such diversely trained investigators come together as a team or, at the very least, collaborate very closely with each other, that most benefit will arise from such studies since the investigators approach the subject from differing perspectives, which will, with a little planning, complement and stimulate one another. This multidisciplinary approach enables the solution of specific problems, such as in plant toxicology. For example, in the case of plant-associated intoxication of livestock, the natural products chemist needs to work closely with veterinarians and toxicologists; first to establish the etiology of the disease (e.g., which plant is responsible? What *in vivo* effects are observed?) and then to isolate and identify those specific bioactive compounds that can duplicate the pathogenesis of the intoxication that occurs naturally. The multidisciplinary approach may also enhance the diversity and consequent value of bioactive natural product research. For example, steroidal glycosides isolated on the basis of their mammalian toxicity may also have useful ecdysteroid activity for insect control (see Chapter 21), or the polyhydroxylated alkaloid glycosidase inhibitors again isolated on the basis of mammalian toxicity may have useful anticancer or antiviral activities (see Chapter 15).

Successful multidisciplinary collaboration requires that each individual has an understanding and appreciation of the intellectual and technical contributions to the project of the other team members. The sophistication of modern scientific instrumentation is such that the human aspects of using such equipment are frequently overlooked. Although almost any research problem can presently be solved by the application of the most powerful equipment on the market, it requires imagination and resourcefulness to achieve results when such techniques are unavailable or too expensive to employ. It is then essential for the collaborators to ask themselves appropriate questions with regard to the most expeditious approach to be adopted, for example:

1. Can a single bioassay be used, or must more than one be employed to cover the bioactivity of interest?
2. Is a "value-added" approach warranted, using multiple bioassays to investigate other potential bioactivities?
3. What is the most useful technique for separation of the active compound for both structural determination and possibly large-scale biological testing? Obviously it is inefficient to develop a separation method that yields the milligram quantities necessary for structure elucidation and then to have to develop an entirely different method for preparation of gram quantities for *in vitro* and, ultimately, *in vivo* testing.
4. What are the essential requirements for unambiguous determination of the structure? Can deduced structural features be confirmed using another strategy or analytical technique?
5. Can the information regarding separation and structure of the bioactive compound(s) of concern be integrated to give a useful method for detection and analysis? If so, the time required for such method development may be significantly reduced.
6. What is the role of the compound(s) in the organism from which it is derived? Frequently, this is not known but such knowledge could point the way to useful modes of action and application to other organisms.

Such questions require that the collaborators have a basic understanding of both the potential and the limitations of the techniques and disciplines that each can bring to bear on a problem. This can

only lead to good experimental design, which will further the goals of the project. It is the intention of this book to provide some measure of perception in this regard.

1.3 WHY ISOLATE BIOLOGICALLY ACTIVE NATURAL PRODUCTS?

The use of herbal and other natural medicines has a long history. However, the utilization of whole plant or other crude preparations for therapeutic or experimental reasons can have several drawbacks including:

1. Variation in the amount of the active constituent with geographic areas, from one season to another, with different plant parts and morphology, and with climatic and ecological conditions.
2. Cooccurrence of undesirable compounds causing synergistic, antagonistic, or other undesirable, and possibly unpredictable, modulations of the bioactivity.
3. Changes or losses of bioactivity due to variability in collection, storage, and preparation of the raw material.

Thus, the isolation of natural products that have biological activity toward organisms other than the source has several advantages including the following:

1. Pure bioactive compound can be administered in reproducible, accurate doses with obvious benefits from an experimental or therapeutic aspect.
2. It can lead to the development of analytical assays for particular compounds or for classes of compounds. This is necessary, for example, in the screening of plants for potential toxicity and for quality control of therapeutic formulations or food for human or animal consumption.
3. It permits the structural determination of bioactive compounds, which may enable the production of synthetic material, incorporation of structural modifications, and rationalization of mechanisms of action. This in turn will lead to reduced dependency on plants, for example, as sources of bioactive compounds and will enable investigations of structure/activity relationships, facilitating the development of new compounds with similar or more desirable bioactivities.

1.4 DETECTION AND ISOLATION

To search for a compound, which elicits a particular bioactive response, an appropriate assay is required to screen the source material, and to monitor extracts therefrom and subsequent purification steps. The assays described in this book are varied and include those for antimalarial, anticancer, seed germination, and mammalian toxicity activities, for example. Ideally, the assay should be as simple, specific, and rapid as possible. An *in vitro* test is more desirable than a bioassay using small laboratory animals, which, in turn, is more desirable than feeding large amounts of valuable and hard to obtain extract to larger domestic or laboratory animals. In addition, *in vivo* tests in mammals are often variable and are highly constrained by ethical considerations of animal welfare.

Extraction from the plant is an empirical exercise in which different solvents are utilized under a variety of conditions such as time and temperature of extraction. The success or failure of the extraction process is monitored by the most appropriate assay.

Once extracted from the plant, the bioactive component then has to be separated from the coextractives. With luck, this may involve simple crystallization of the compound from the crude extract, requiring only minor manipulation to yield pure compound. More usually, however, it will involve further solvent partition of the coextractives and extensive chromatography, taking advantage of particular properties of the desired compound such as acidity, polarity, and molecular size.

In some cases, the isolation can be assisted by prior derivatization, imparting more easily manageable properties to the desired compound. As an example of improved separation techniques, the application of high-speed countercurrent chromatography (HSCCC) to the isolation of usefully large amounts of specific bioactive compounds is described in Chapter 10.

Several chapters of this book describe or provide examples of analytical technologies that allow for the structure determination of compounds without the need for rigorous purification. However, in some cases the data are insufficient to allow an unambiguous confirmation of a tentatively assigned structure, and more rigorous structural determination is required with purified compound. Final purification, to provide compounds of suitable purity for such structural analysis, may be accomplished by appropriate techniques such as recrystallization, sublimation, or distillation. It is extremely important that such purification be done if for no other reason than to establish reference standards for sharing with other scientists and for dereplication purposes (e.g., Chapters 5, 8, and 9). The extensive use of HPLC techniques for separation of complex mixtures has led to the perception that symmetrical peaks represent pure compounds (i.e., "the peak is the compound"), which is often not the case when compounds of similar polarities coelute or when mixtures of enantiomers are present (Chapter 7). Furthermore, eluted material is often described as, for example, an "amorphous white solid", but whenever sufficient product is available it should be recrystallized to constant melting point, thus providing a simple physical measure of purity and a benchmark for comparison by other researchers. Similarly, optical rotation should be recorded to establish enantiomeric purity.

1.5 STRUCTURE DETERMINATION

The process of structural determination involves accumulating data from numerous sources, each of which gives some structural information, and the assimilation of this data into a chemical structure that rigorously and uniquely fits all the available structural information. A wide range of spectroscopic instrumentation, such as ultraviolet (UV)/visible (Vis) and infrared (IR) absorption spectroscopies, nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry (MS), currently forms the backbone of modern structural analysis. The development and application of the so-called "hyphenated" approaches (HPLC-MS, HPLC-NMR, HPLC-DAD-MS-NMR, etc.) has increased the speed and sensitivity of structure determinations. This has been particularly valuable in the rapid dereplication of components in extracts, that is, where the structures are already known and where the available data, that might otherwise only provide tentative structure determinations for undescribed compounds, are confirmed by literature reports or comparison with standards.

Prior to the availability of such aids to structural determination and in cases where, despite these aids, ambiguity existed, chemical modification or degradation of the unknown compound was necessary. These latter processes involve the treatment of the unknown compound with functional group-specific reagents or degradation of the compound in a predictable manner until a compound of known structure is obtained. Backtracking should then provide a structure for the unknown compound. Apart from being a time-consuming and exacting art, this method can be fraught with difficulty and ambiguity.

When analysis of the spectroscopic data for an unknown compound is inconclusive, then, if the compound or one of its derivatives is suitably crystalline, a single crystal X-ray diffraction study should be considered. Simplistically, this involves the computer-aided analysis of the diffraction pattern obtained when a single crystal is irradiated with X-rays. Correct interpretation of the data will result in a three dimensional picture of the molecule, including the relative stereochemistry if the molecule is optically active. In some cases, the absolute stereochemistry can also be determined. X-ray diffraction studies can give valuable information on the three dimensional shape of the molecule, bond lengths and angles, and possible intra and intermolecular interactions, but it is important to realize that this is solid-state data and may not be particularly informative as to the behavior of a compound in solution where properties such as hydrogen bonding may have significant effects. The importance of determining the absolute stereochemical nature of the structure is

becoming more self-evident, since enantiomeric (mirror image) structures may have entirely different biological properties. It is not acceptable to evaluate the bioactivity of mixtures of such optically active compounds.

If structural analysis data are ambiguous and the compound is not amenable to an X-ray diffraction study, then chemical synthesis, from precursors of known structure and stereochemistry, is usually sufficient to prove or disprove a proposed structure, including the specific stereochemistry if applicable.

There are traps for the inexperienced and experienced researchers alike. Deciding when a structure has been described unambiguously, or at least sufficiently for the investigator, is sometimes governed by the availability (or lack thereof) of more probing technology. It is sometimes governed by the "need for speed", or other time constraints that may be imposed upon the research. Consequently, the chemical literature is replete with examples of how unambiguous synthesis has been vital in finally establishing a structure for an unknown compound. Conversely, the literature abounds with examples where complete synthesis has disproved a previously published structure that was assigned based on spectroscopic and spectrometric analysis alone.

The power of sophisticated techniques such as NMR spectroscopy and MS for structural elucidation is extremely seductive in their appeal. It is tempting to believe that structures determined by their application are unequivocal, but their misuse can lead to egregious errors. A common mistake is to use a ^{13}C NMR carbon count, in combination with a low-resolution mass spectrum, to determine the molecular formula of a compound. Although the elemental composition can be presumed by this procedure, it cannot be established. For example, carbon atoms with aberrant relaxation times may not be seen in the NMR, and the mass spectrum may not show the molecular ion. Without careful consideration, even high-resolution MS may not be infallible in providing the molecular formula, as the spectrum observed may be that of an impurity or an artifact. For example, alkaloid *N*-oxides often undergo thermal deoxygenation at probe temperatures, giving the mass spectrum of the parent base rather than the *N*-oxide. Combustion analysis is the most reliable method for elemental analysis if sufficient material is available because it establishes both purity and the presence of atoms such as nitrogen or halogens.

It is also important to recognize that a great deal of fundamental structural information can be accumulated by simple chemical or spectroscopic methods prior to resorting to the more expensive and complicated techniques, thus limiting the load on instrument time. For example, a plethora of color tests are available that can be used (either in test tube or as spray reagents) to establish functional groups or compound classes. Simple derivatization reactions, for example, acetylation of hydroxyl groups, can provide the number and type of reactive groups. A technique that has recently become somewhat overlooked is IR spectroscopy. However, it is excellent for demonstrating particular functionalities or for "fingerprinting" a compound for comparison with subsequent isolations. Similarly, UV spectroscopy can be used to determine the degree of conjugation in a molecule and may be quite specific when used in combination with shift reagents. All of these methods should be applied to the fullest extent not only to limit absolute dependence on NMR and MS, but also to confirm the structures derived by such techniques.

1.6 OVERVIEW

Following in the same vein as the first edition of this book, the chapters are written by researchers from a variety of scientific disciplines, each of who consequently approaches bioactive natural product research from a different perspective. This approach serves to highlight the multidisciplinary nature of this type of research. Two of the chapters (Chapters 2 and 3) are significant updates on those presented by the same authors in the first edition of this book. However, to further enhance awareness of the diversity of bioactive natural products research and to derive maximum benefit from personal insights of researchers, the contributor network has been extended with the publication of this edition. Therefore, apart from Chapter 21, the remaining chapters

have been prepared by authors who did not contribute to the first edition. Six of these remaining chapters (Chapters 6, 7, 9, 12, 15, and 21) are different but closely related to chapters presented in the first edition whereas twelve (Chapters 4, 5, 8, 10, 11, 13, 14, and 16–20) are new contributions presenting aspects of bioactive natural product research different from those presented in the first edition.

The contributors to this book have critically reviewed the literature and presented research from their own laboratories that emphasize both the philosophy and rationale that has successfully directed detection and isolation of bioactive natural products. They also review and present the type and quality of structural information that can be acquired and assessed in an integrated way to provide structure assignments.

Several of the authors of this book have reviewed the natural occurrence of compounds with specific activities and describe the rationale guiding the search for such compounds. It must be recognized that the book is not intended to provide a catalog of bioassays but rather to illustrate the extreme diversity of methods and to emphasize the importance of selecting a bioassay appropriate to the activity being sought.

1.6.1 Methods of Detection, Isolation, and Structural Determination

1.6.1.1 Detection and Isolation

Ghisalberti (Chapter 2) has updated his contribution to the previous edition of this book by adding new sections and expanding original sections consistent with the most recent literature, including 282 references. Interweaving his own extensive experience as a natural products chemist into this chapter, Ghisalberti has presented a review of approaches to detecting and isolating bioactive natural products and has illustrated advantages, disadvantages, successes, and failures with examples from the literature. Insights into the practical realities of natural products chemistry abound within this chapter. In addition to briefly discussing the many forms of chromatography available for isolation and purification, a section on extraction-derived and separation-derived artifacts is particularly enlightening. Some of the bioactivities briefly addressed by Ghisalberti are covered in more detail in the following chapters.

Li and Chen (Chapter 10) have provided an overview of HSCCC and have illustrated its many application modes (both analytical and preparative) to a wide variety of natural product structures. The chapter focuses on the HSCCC applications and encourages the reader to examine the original references for more detail on bioactivities and the source of extracts. However, the authors themselves state that "A comprehensive review of the literature on the use of HSCCC in isolation of natural products is not intended; only selected examples are presented and discussed to illustrate the capabilities of this technique and to serve as an entry point for further applications of the various HSCCC methods".

An intriguing review of the application of specifically designed biosensors to the rapid, online detection of bioactive compounds has been described in Chapter 11 by Minunni and Bilia. Among the biosensors described are included those that rely upon catalysis for the detection of specific functional moieties, those that evaluate antioxidant properties, immunosensors, and DNA-based sensors. In the authors' words, "Biosensors represent new analytical devices which appear to be an analyst's dream". Importantly, the authors have demonstrated the wide scope of developmental opportunities that reside within this technology.

1.6.1.2 Structure Determination

Byrne (Chapter 3) has updated his previous chapter that described a strategy for using NMR spectroscopy in the structural elucidation of compounds. A brief description of NMR fundamentals and techniques is accompanied by extensive referencing (208 references) to assist those readers who require more detailed information. For simplicity, and so as not to detract from the NMR essence of

the chapter, Byrne demonstrates this strategy, in the main, by the progressive structural elucidation of one compound. The resulting rational and cumulative acquisition of 1D and 2D NMR information gradually but surely defines the structure of the compound. This strategy, and variations of it, can be detected in various chapters of this book, which concentrate on the structural determination aspects of bioactive natural product research.

Combining detection and quantitation of bioactive natural products with the acquisition of structural NMR data, Pauli, Jaki, Lankin, Walter, and Burton (Chapter 4) describe their experience with the development, optimization, and application of quantitative NMR, especially quantitative ^{1}H NMR. The authors introduce their chapter by outlining the benefits that can accrue from the quantitation of NMR data. They then take the reader step by step through the process of acquiring and optimizing the data, sharing with the reader the benefit of their combined experience.

Again combining detection and quantitation of bioactive natural products with the acquisition of structural NMR data, the coupling of HPLC with NMR is described in Chapter 5 by Wolfender, Queiroz, and Hostettmann. In this chapter the authors initially discuss the development and principle of operation of LC-NMR. They then describe the integration of this technique into dereplication strategies for examining natural product extracts, and the acquisition of appropriate NMR data for *de novo* structure determination. Modes of operation, the advantages and disadvantages, and the application to various case studies are all illustrated with informative figures.

Emphasizing the need for the rapid and reliable dereplication of natural product extracts, Larsen and Hansen (Chapter 8) have addressed the utilization of UV/Vis absorption spectroscopy. An often-neglected technique by comparison with MS and NMR, either alone or hyphenated with chromatographic stages, UV/Vis absorption spectra lack the significant fine structure required for definitive structure determination. However, Larsen and Hansen have clearly shown the potential benefits of utilizing HPLC-DAD UV/Vis databases to automatically screen samples to dereplicate known components and to indicate new potential lead compounds that may be related to the dereplicated bioactive compounds. The general approach presented by Larsen and Hansen can conceivably apply to other datasets that could be compared to assist dereplication of sample extracts.

An important, potentially critical, and often overlooked or underrated aspect of the structure determination of natural products is the absolute stereochemistry. In Chapter 6, with 75 references, Humpf has reviewed the application of the exciton chirality circular dichroism method (including fluorescence-detected exciton-coupled circular dichroism) in deriving the absolute configuration of chiral centers within molecules. In addition to describing the principles behind the circular dichroism method for determining absolute stereochemistry, and the development of new chromophores, Humpf has examined several exemplifying applications of the technique. Taking a different approach to the stereochemical perspective, Lee, Molyneux, and Panter (Chapter 7) describe the observations that led them to suspect enantiomeric contamination of the piperidine alkaloids anabasine and ammodendrine extracted from *Nicotiana* spp. and *Lupinus* spp., respectively. They proceed then to isolate the enantiomers via separation of diastereoisomers and chemical modification back to the enantiomers. Subsequently, a difference in the biological activity of the enantiomers was also demonstrated.

The application of MS was covered in a broad sense in the first edition of this book. In this second edition, Cheng, Chen, and Wang (Chapter 9) have focussed on LC-MS, exemplifying its various modes of application by a heavy reference to bioactives in herbal preparations. Aspects that the authors discuss include "fingerprinting" and thereby quality control of herbal preparations, *de novo* structure analysis, and pharmacokinetic and metabolic analysis. Other applications of LC-MS and the hyphenation of LC-MS with UV/Vis, circular dichroism and NMR can be found throughout this book.

1.6.2 Specific Case Studies

Within this book, the most specific searches for bioactive compounds are those described by Flematti, Ghisalberti, Dixon, and Trengove in Chapter 20 and by Colegate in Chapter 21. Flematti et al. reveal

the reality behind the convoluted path that can sometimes constitute a "final" isolation procedure. The hunt for, the isolation of, and the structure determination of a seed germination stimulant in smoke was a trial of persistence and resourceful dedication that eventually led to the discovery of a butenolide compound that was subsequently synthesized and shown to possess potent seed germination activity. Among other topics, Colegate (Chapter 21) describes the collaborative efforts to isolate and identify the steroidal compounds associated with the ovine toxicity of *Stemodia kingii*. The process involved close collaboration with veterinary toxicologists to define the toxicity and develop suitable bioassays. The structure determination of the bioactivity-guided isolates was based mainly on detailed 1D and 2D NMR analysis.

Su, Zou, Lei, Kong, and Hu (Chapter 18) describe biological fingerprinting analysis with an emphasis on traditional Chinese medicines. This type of analysis is aimed at high-throughput detection of specific activities, particularly at the molecular level where it is based upon the affinity interaction of natural products with biomolecular targets. Despite the emphasis on application in traditional Chinese medicines within the chapter, the approaches described in Chapter 18 are applicable to the detection of bioactive natural products from all sources.

Following on from the U.S. National Cancer Institute's acquisition and screening of natural products as potential anticancer agents described in the first edition of this book, Cragg and Newman (Chapter 12) present a prodigious account (with 320 references) of the number and structural variety of natural products that have been assessed for anticancer activity. The sources of the compounds include terrestrial plants, microbes, and the marine environment. The chapter describes agents in clinical use, clinical development or trials, and in preclinical development. There is some discussion on the mechanism of anticancer activity and the targeted delivery of natural product-based anticancer agents. The authors, experienced as they are in dealing with large-scale screening of bioactive compounds, state that, "Once more, we strongly advocate expanding, not decreasing, the exploration of nature as a source of novel active agents that may serve as the leads and scaffolds for elaboration into desperately needed efficacious drugs for a multitude of disease indications".

Chapters 15, 16, and 19 continue the theme of screening bioactive natural products for specific activity. Nash (Chapter 15) describes the research into the search for alkaloidal natural products that can inhibit glycosidases and therefore have potential value in therapeutic applications. Basically, Nash describes his laboratory's extensive experience in the detection, isolation, and structural identification of these polyhydroxylated alkaloids that mimic the carbohydrate substrates for enzymes and receptor sites. Working from the observation that aflatoxin biosynthesis by *Aspergillus* spp. seems to be substantially inhibited by some tree nut species and cultivars, Molyneux, Mahoney, Kim, and Campbell (Chapter 16) apply many of the techniques also described in other chapters of this book to search for antiaflatoxigenic factors in resistant nuts. After introducing the problem and research challenge, the authors take the reader through the steps of developing the bioassay and using the bioassay to isolate aflatoxin biosynthesis inhibitors. This latter goal required an efficient analytical method to quantitate the production of aflatoxins. Finally, the authors describe the structure determination of an aflatoxigenesis inhibitor and comment upon the mechanism of action. From a generic aspect, the authors state: "The approach described in this chapter for defining the localization of aflatoxin resistance factors by progressively identifying the specific localization within plant species, varieties, organ and finally specific tissue by bioactivity-guided assay can be applied to many other such problems, particularly in the plant world". Mambu and Grellier (Chapter 19) review the ongoing and crucial search for antimalarial compounds. The authors cover rational development of bioassays based on the cycle of malarial infection and application of these bioassays to screening natural products. They discuss the criteria used to select plants for investigation, and the methods used to isolate, purify, and structurally identify active compounds. After describing the activity of various classes of natural product from terrestrial and marine sources, the authors offer comment on the postgenomic possibilities that now exist for aiding the search for new, specifically targeted antimalarials.

Another approach to novel bioactive natural products, exemplified in Chapters 13, 14, and 17, is to "prospect" a specific source for useful activities. Mine and D'Silva (Chapter 17) focus on discovering bioactive peptides from chicken egg (i.e., the shell, shell membrane, albumen, and yolk) proteins. They first rationalize the effort that is going into the discovery of bioactive peptides and then describe the isolation of peptides with various activities including antimicrobial, immunomodulatory, anticancer, and antioxidant, among others. With current interest in the discovery and utilization of novel bioactives, the approaches outlined in this chapter can be applied to other sources of proteins and their constituent peptides. Ojika (Chapter 14) and his research team have been studying bioactive secondary metabolites from myxobacterial species. In particular, Ojika describes the application of a *Phytophthora*-based assay to discover electron transport inhibitors produced by myxobacteria. Ojika has structured his chapter according to the classes of compounds that have been isolated. Within each of these broader classes, the author then discusses the production, isolation, and bioactivity of the secondary metabolites. Casting the net wider, Strobel and Castillo (Chapter 13) describe the detection, isolation, and structure determination of bioactive compounds from endophytic microbes. In this chapter, the authors discuss the need for an increase in the search for bioactive natural products that may have therapeutic benefit although warning of the loss of such resources. Importantly, the rationale for plant selection is a serious consideration described by the authors. Once plants have been collected, the search for endophytes and their consequent isolation and storage are all dealt with in this chapter. The authors then describe various bioactive compounds that have been isolated from endophytic fungi and bacteria. Significantly, these authors also mention the need to work with indigenous people, involving them in the potential benefits accruing from modern researchers tapping into their cultural knowledge.

The final chapter of the book centers around the toxicity of bioactive natural products. Thus, Colegate (Chapter 21) briefly describes the development and utilization of *in vivo* and *in vitro* bioassays designed to aid the search for specific toxins. NMR and MS, especially HPLC-MS, are then utilized for the structure determination of the toxins. Since the toxic natural products can also enter the human food supply, the second part of the chapter describes the development and application of immunochemical methods of detection and the application of HPLC-MS to derive tentative structures for related compounds.

1.7 CONCLUSIONS

Foremost, it is the authors' personal enthusiasm for their bioactive natural product-related research that is evident in the chapters that compose this book. Generally, the authors have given the reader a broad exposure to the scope of bioactive natural product research. This not only includes the "hard science" of detection, isolation, and structure determination of bioactive natural products, but also those aspects that can only come from experience. The latter encompasses selection of source material, consideration for the ethnobotanical knowledge and proprietary rights of indigenous cultures, personal commitment to the search for useful bioactivities and a conviction that nature continues to offer an abundant resource that can provide new bioactive compounds, or lead compounds that can be further modified, to help mankind battle disease and survive the less benign aspects of our environment.

2 Detection and Isolation of Bioactive Natural Products

Emilio L. Ghisalberti

CONTENTS

2.1 INTRODUCTION

Humans have always relied on natural products and have continually explored their application to improving various aspects of our lives. Thus, we have sought to use natural products such as spices, flavoring agents, perfumes, cosmetics, and dyes to control and overcome diseases. Some were recognized as poisons and others were used as insecticides and pesticides. This interest in, and fascination with, natural products continues today and, despite the fact that periodic claims pronounce that the era of natural products chemistry is over, this area of research is still attracting considerable interest and offering significant promise. A recent review on the topic begins with the adage, "There's life in the old dog yet!" and, according to the authors, this also holds true for natural product research.[1]

Natural products research remains one of the main means of discovering bioactive compounds. Since little is known about the etiology of many human, animal, and plant diseases, it is difficult to design potentially active compounds for their treatment and leads from natural sources will continue to be sought.[2] Natural products offer complementary features to synthetic compounds in terms of composition, weight, size, functional groups, and architectural and stereochemical complexity. Importantly, they have evolved to serve a function in specific biological systems such as inhibiting or activating functions of the proteins to which they bind.[3,4] It is interesting to note that of the 877 small organic compounds introduced worldwide as drugs in the period 1981–2002, 61% were natural products, derivatives, or mimics of natural products.[5]

For many years, most natural products chemists were more concerned with the isolation and structural elucidation of 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. Armed with these tools, natural products chemists have ventured into bioassay-guided isolation of metabolites, and they are now turning their attention to the origins of bioactivity. As recently as 2003, Meinwald, a pioneer in the field of chemical ecology commented, "Chemists need to talk to biologists, who could offer valuable guidance … Good field biologists are likely to notice interactions that might provide clues to interesting chemistry".[6] The significance of this statement is more obvious when considered from the perspective of global biodiversity. For flowering plants, about half of the predicted total of 500,000 species have been described. However, only 10% have been studied chemically and many of these only in a cursory manner. The earth's fungal population has been estimated to consist of 1.5 million species of which only 100,000 have been described.[7] Soil microorganisms have been rich sources of natural products, providing pharmacologically important antibiotics and biocatalysts. The heterogeneous soil environment contains a wide variety of microbial niches harboring a high diversity of microorganisms. One gram of soil can contain 10 billion microorganisms of possibly thousands of species. It has been estimated that only 0.1–1.0% of microorganisms can be cultured using current techniques.[8] This need not mean that the metabolites of other species will be unrecoverable. Strategies to access the metagenome from an environmental sample, such as seawater, soil, or insect gut, by the construction and screening of DNA libraries derived from these samples appear to be promising tools for the discovery of bioactive compounds from these otherwise inaccessible species.[9,10]

Myriads of other terrestrial and marine microorganisms should also prove to be as bountiful. New habitats that are rich sources of fungal biodiversity continue to be discovered. As an example, 200 new species of yeasts were recovered from a total of 650 isolates from the guts of beetles.[7] About a million insect species have been described, but this is only a small percentage of the predicted total of 30 million.[11] Spiders are only second to insects as the most diverse group of animals on land.[12] There are roughly 40,000 described species of spiders, but less than 1% of their venoms have been investigated. It seems likely that a study of these venoms will reveal interesting neurochemical agents.

All of these organisms coexist in ecosystems and interact with one another in various ways in which chemistry plays a major role.[13] Clarifying such interactions, and applying the knowledge gained, necessitates a broad multidisciplinary understanding at the individual organism's level as well as interdisciplinary collaboration among natural products chemists, analytical chemists, molecular and cellular biologists, biochemists, and pharmacologists.[14]

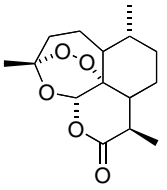
The aim of this chapter is to provide an overview of the range of methods used in the detection of bioactive compounds and to outline the approaches to their isolation and purification. The intention is to provide a newcomer to the field with some working knowledge of the chemical and biological techniques available. Given the breadth of the field, the choice of the topics is, of necessity, selective. Emphasis is given to those strategies and techniques that have been well established and can conveniently be applied, and those that, although still in a state of development, appear to have potential.

2.2 DETECTION OF BIOLOGICALLY ACTIVE METABOLITES

The detection of bioactive metabolites is the starting point for a strategic approach in the search for potentially useful compounds. Clues to the existence of bioactive compounds can arise from disparate sources. Traditional medicine has selected groups of plants that time and experience have shown to be beneficial. Other clues come from observations by scientists in the field who are in the best position to recognize interactions among organisms. Yet others come from screening a large number of organisms for a particular effect, from searches for a particular type of compound, from old metabolites being tested in new screens or, not the least important, from chance discovery of new metabolites displaying pharmacological properties (serendipity). Some examples are given later by way of illustration.

The examples of traditional medicines providing leads to bioactive natural products are legion. It is sufficient to point to some confirmations of the wealth of this source. Artemisinin (qinghaosou) (**1**) is the antimalarial sesquiterpene from a Chinese medicinal herb that has featured in herbal remedies since ancient times.[15] Forskolin (**2**) is the antihypertensive agent from *Coleus forskohlii* Briq. (Labiatae), a plant whose use was described in ancient Hindu Ayurvedic texts.[16] The ginkgo tree, mentioned in Chinese medicinal books from 2800 BC and used in antiasthmatic and antitussive preparations, produces the ginkgolides (**3**), unusual diterpene trilactones containing a tertiary butyl group.[17] Representatives of this group of compounds were first isolated in 1932, their structures were determined in 1967, and their total synthesis was described in 1988. Their involvement in the clinical efficacy of ginkgo tree extracts was reported in 1985 when they were shown to be antagonists of platelet aggregation factor (PAF). Galanthamine (**4**), an alkaloid first isolated in 1951 from *Galanthus woronoii* (Amaryllidaceae), provides an effective symptomatic treatment for patients with Alzheimer's disease and delays the progression of the disease.[18] It appears that the plant came to the notice of scientists in the 1950s when a Russian pharmacologist discovered that the villagers living at the foot of the Caucasian mountains used it to treat poliomyelitis in children. Little is known about the use of this genus in Europe. It has been hypothesized that in Homer's *Odyssey* the plant used by Odysseus to prepare an antidote to Circe's poisonous potion may have been a *Galanthus* species. If so, then the ethnobotanical history of this plant spans 28 centuries. The identification of swainsonine (**5**), a potent α-mannosidase inhibitor, came from the realization that the clinical signs

and pathogenesis of *Swainsona* intoxication in cattle resembled those of an hereditary condition in man and other animals known as α-mannosidosis.[19,20] This stimulated a search for other alkaloids with glycosidase-inhibiting activity and resulted in the discovery of, *inter alia*, castanospermine (**6**), a tetrahydroxyindolizidine alkaloid that inhibits replication of the human immunodeficiency virus (HIV).[21]



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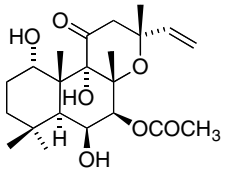

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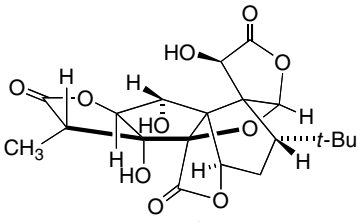

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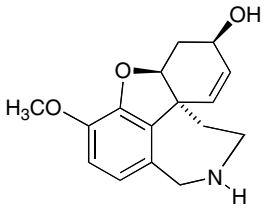

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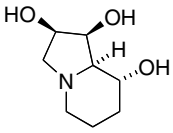

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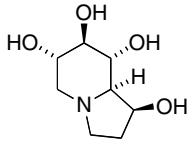

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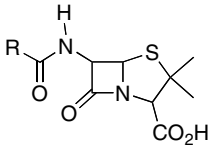

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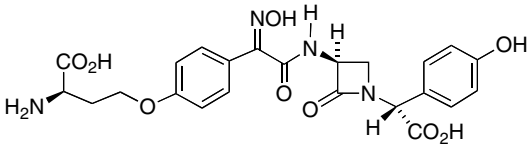

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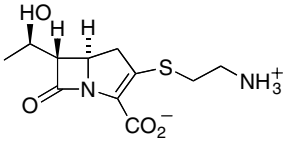

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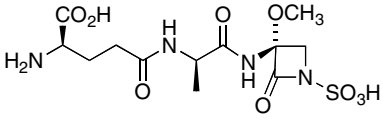

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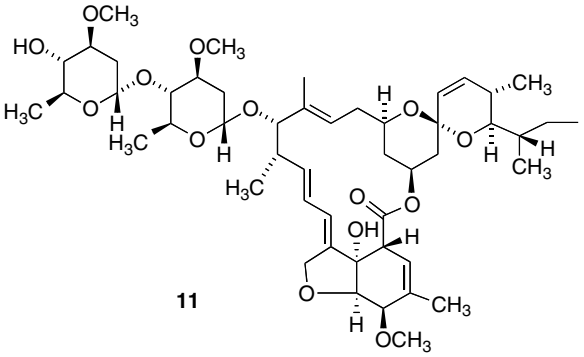
Following the publication of the first positive clinical data on penicillin (**7**) between 1942 and 1944, much effort was concentrated on discovering further antibiotics. Up to 1968, the same methods to target β-lactams were still being used, and one could have concluded that all natural β-lactams had been discovered. The introduction in the 1970s of new screening methods, which, (a) used bacterial strains

supersensitive to β-lactams; (b) tested for inhibition of β-lactamases; or, (c) specifically searched for sulfur-containing metabolites, resulted in the discovery of new antibiotics, that is, the norcardicins (**8**) and carbapenems (**9**) in 1976 and the monobactams (**10**) in the 1980s.[22]

A screen designed to find metabolites from microorganisms active against parasitic infections resulted in the detection and isolation of the avermectins, for example, avermectin 1a (**11**). The producer organism, subsequently named *Streptomyces avermitilis* MA-4680, came from a selection of nearly 2000 cultures from soil samples collected from different environments. Screening was carried out with an *in vivo* mouse assay using mice infected with larvae of the parasitic helminth, *Nematospiroides dubius*. The broth from different bacterial cultures was mixed with the feed given to the mice for 6 days, and the feces and intestinal contents were examined for eggs and worms. The assay required 2 weeks,[23,24] but it is interesting to note that, although such an *in vivo* test was both lengthy and expensive, it simultaneously tested for antiparasitic activity and toxicity to the host. Significantly, a deliberate choice was made to select microorganisms with unusual morphology and nutritional requirements.

The search for enzyme inhibitors of proteases related to various diseases led to the discovery of more than 100 low molecular weight compounds produced in microbial cultures. These compounds have both pharmacological and immunopharmacological activities.[25] Of particular value are mevinolin (**12**) and compactin (**13**), two potent inhibitors of HMG-CoA reductase, an enzyme that controls cholesterol biosynthesis in humans.[26]

Cyclosporin A (**14**), a nonpolar cyclic undecapeptide, was present in an extract from the soil-borne fungus *Tolypocladium inflatum* (formerly *Trichoderma polysporum*). Although it showed



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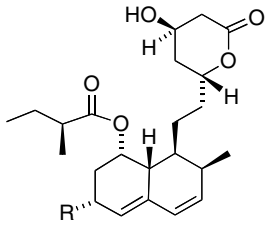

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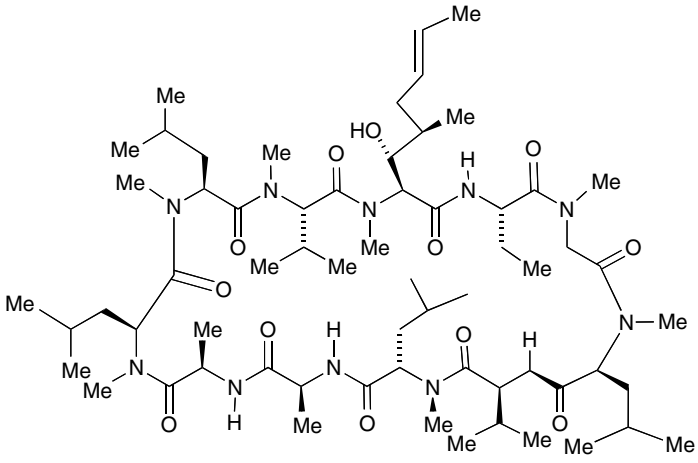
13 R = H



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weak antifungal activity, the low toxicity of this extract prompted its testing in a pharmacological screening program.[27] On *in vitro* screening and in a mouse model, it was shown to inhibit antibody formation, to suppress delayed-type hypersensitivity, and to interfere with the release of inflammatory mediators. The potent immunosuppressive activity was not linked to general cytotoxicity. Cyclosporin A was isolated from the metabolite mixture in 1973 and has since become the prototype of a new generation of immunosuppressants.[27,28]

Another case of serendipity is illustrated by the discovery of the now commercial drugs vincristine (**15**) and vinblastine (**16**) in *Catharanthus roseus*. A random screening program, conducted at Eli Lily and Company, of plants with antineoplastic activity found these anticancer agents in the 40th of 200 plants examined. Ethnomedical information attributed an anorexigenic effect to an infusion from the plant.[29,30]

Many bioactive compounds have been found in the past but, for varying reasons, their activity has not been pursued. On passing through newer screening procedures, a number of these compounds, with previously unsuspected modes of action, are rediscovered (e.g., the ginkgolides), and other activities become apparent.[31] An interesting finding relates to the activity associated with betulinic acid (**17**). This triterpene occurs in many plants, and its structure has been known since 1932. Betulinic acid was found by bioassay-guided fractionation of the ethyl acetate-soluble extract of *Ziziphus mauritiana*, which displayed selective cytotoxicity against human melanoma cell (MEL-2).[32] Biological studies indicate that betulinic acid works by induction of apoptosis. The acid and its derivatives seem to hold some promise as inhibitors of HIV replication.[33]

Azidothymidine (**18**), when first synthesized and tested for anticancer activity, gave negative results. It was later found to be a reverse transcriptase inhibitor effective against the HIV-1 virus. When azidothymidine was first evaluated, the enzyme was not known.[2] The same metabolite can sometimes be detected by different screening procedures. Compactin (**13**) was first discovered[34] as an antifungal compound in 1976 and found shortly after in a screen for cholesterol-lowering agents.[35]

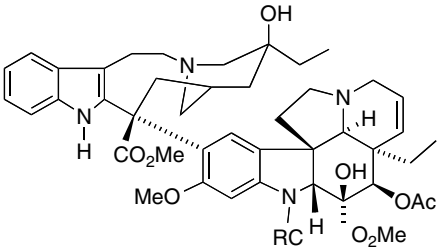
2.3 SCREENING FOR BIOACTIVE METABOLITES

Testing large numbers of extracts or compounds to determine if they produce a biochemical or cellular effect is usually one of the first steps in the discovery of bioactive compounds. In principle, a bioassay is any *in vitro* or *in vivo* system used to detect the biological activity of an extract or a pure substance from a living organism. This may involve testing for antibiotic activity, *in vitro* inhibition tests, pharmacological, agricultural, or veterinary screens, which require diverse *in vitro* assays or *in vivo* animal models. The application of these assays to monitor the presence of a bioactive compound(s) during the isolation process is called bioassay-guided fractionation (isolation). Thus, all fractions generated are tested for biological activity, and those giving a positive test are further processed until the bioactive agent is obtained in a pure form.

As explained later, there are now a large number of bioassays available that differ in degree of sophistication. Simple and inexpensive assays suitable for the rapid screening of extracts in the typical laboratory have been developed. Methods to make screening faster and more efficient are continually being developed. The process by which large numbers of samples are tested in an automated fashion is referred to as high throughput screening (HTS) (Section 2.5.1).

Many factors can complicate matters when using bioassays or bioassay-guided fractionation. The most obvious is that the solubility of the extracts or fractions is limited, and the aliquot of the test sample does not represent the original extract. Most plant extracts can be solubilized by formation of a soluble complex with polyvinylpyrrolidone.[36] In some cases, use of a powerful solvent such as dimethylsulfoxide is possible.[37]

Crude extracts often contain various compounds that may interfere with a bioassay. Phenolic compounds, such as tannins, may hamper receptor binding and enzyme assays through nonselective binding to proteins. Treatment with adsorbents (e.g., XAD-2 resin) that bind aromatic compounds



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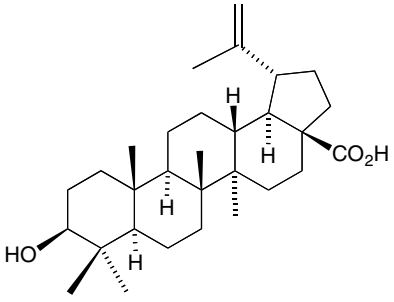

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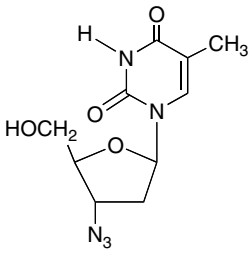

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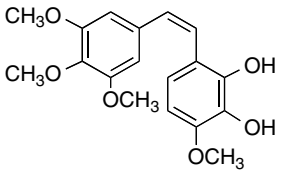

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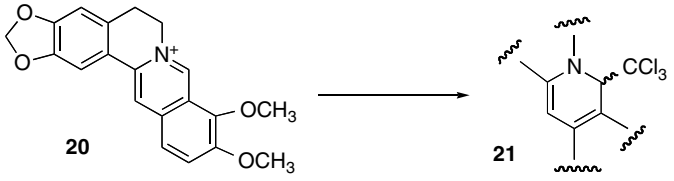


20 **21**

is used to minimize the effect. Linoleic acid, a common fatty acid, binds in the adenosine receptor assay and gives rise to false positives. In cases like these, a preseparation step after extraction is necessary.[38]

Other factors are not so obvious and can be due to one or a combination of effects, including chemical changes during the extraction and manipulation of extracts, antagonistic or synergistic effects. For example, in the isolation of leurosine, a dimeric indole alkaloid related to vinblastine (**16**), the crude alkaloid fraction showed no *in vitro* activity against the P1534 leukemia system, but the pure alkaloid showed pronounced cytotoxicity in the same test.[39] However, fractionation of the extracts from *Combretum caffrum* was complicated by loss of *in vivo* activity. Modification of the extraction of a new batch of the plant eventually led to the isolation of the antineoplastic combretastatin A-1 (**19**).[40] Berberine (**20**) has a weak but quite broad spectrum of activity against a number of pathogenic microorganisms. The artifact (**21**) obtained on contact of ammoniacal solutions of berberine with chloroform has greater *in vitro* antimicrobial activity.[41]

With bioassay-guided fractionation, it is not unusual for the initial extract to show greater activity than any of the fractions obtained. For example, testing extracts of the African plant

Kigelia pinnata for cytotoxicity toward melanoma cancer cell lines gave IC_{50} values that were lower than those of any of the fractions obtained after fractionation through silica gel.[42] In cases like this, the possibility of synergy should be considered and, if possible, checked by testing the bioactivity of the recombined fractions. Evidence to support the occurrence of synergy is increasing, and a number of cases have been discussed in a review.[43]

2.3.1 Primary Screening Assays

There are a number of criteria to be met for a useful front line screening bioassay (basic or primary screen). It must be rapid, convenient, reliable, inexpensive, sensitive, require little material, and be able to identify a broad spectrum of activities. It should also be able to be applied in-house by chemists, botanists, and others who lack the resources or expertise to carry out more elaborate bioassays.

2.3.1.1 Brine Shrimp Lethality Test

A general bioassay that appears capable of detecting a broad spectrum of bioactivity present in crude extracts is the brine shrimp lethality test (BSLT).[44,45] The technique is rapid (24 h), simple (e.g., no aseptic techniques are required), easily mastered, inexpensive, and uses small amounts of test material (2–20 mg or less). The aim of the method is to provide a frontline screen that can be backed up by more specific and more sophisticated bioassays once the active compounds have been isolated. It has been found that brine shrimp toxicity is predictive of cytotoxicity and pesticidal activity. In particular, a positive correlation was found between BSLT toxicity and cytotoxicity toward the 9 KB cell line (human nasopharyngeal carcinoma) and other solid tumors, as well as for the P388 cell line (*in vivo* murine leukemia).

For this test, the tiny crustacean *Artemia salina* is used. The eggs are readily available commercially and kept viable for years if refrigerated. When placed in a brine solution, the eggs hatch within 48 h to provide large numbers of larvae (nauplii). Compounds or extracts are tested at initial concentrations of 10, 100, and 1000 ppm in vials containing 5 mL of brine and 10 brine shrimps in 3 replicates. Survivors are counted after 24 h, with the aid of a stereoscopic microscope, and LC_{50} values at the 95% confidence limit are calculated. Since this test was first introduced in 1982, it has been used in the isolation of *in vivo* active antitumor agents and pesticides produced by plants. Protocols for testing and for bioassay-guided fractionation have been described.[44–46] As an example, the fractionation of the bark of the pawpaw plant (*Asiminia triloba*) is illustrated in Figure 2.1.[46] A simple extraction of the bark with ethanol gave an extract with significant activity in the BSLT. A separation into lipophilic and water-soluble metabolites showed the activity to be associated with the lipophilic fraction. Liquid–liquid partitioning localized the activity in the fraction of medium polarity. The active constituent (asimicin) was subsequently isolated by a combination of chromatographic techniques using the bioassay to identify the most potent fractions in each separation. This plant and other species of the Annonaceae family have been shown to contain potent bioactive acetogenins, which exhibit a broad range of activities such as cytotoxic, antitumor, antimalarial, antimicrobial, immunosuppressive, antifeedant, and pesticidal effects.[47–49]

This assay is more widely used in laboratories with limited resources and provides a particularly useful entry to the detection of bioactive compounds. It has been employed, *inter alia*, to evaluate plants for potential pharmacological activity,[50] to guide the isolation of a new bioactive colchinoid from Jordanian saffron,[51] bioactive neolignans from the leaves of *Magnolia virginiana*,[52] acetylenic acids from *Ximenia americana*,[53] diterpenes from *Haplopappus rigidus*,[54] and triterpenes from *Celaenodendron mexicanum*.[55]

Minor modifications have been made to suit particular cases. The problem of solubility of extracts can be overcome by using DMSO (1%) as a solvent. When this is not sufficient, the solubilizing agents Tween 80 (2% aq.) or polyvinylpyrrolidone, which is not toxic to brine shrimp at concentrations of up to 400 μg/mL in water, can be used.[56] The test can also be carried out in 96-well

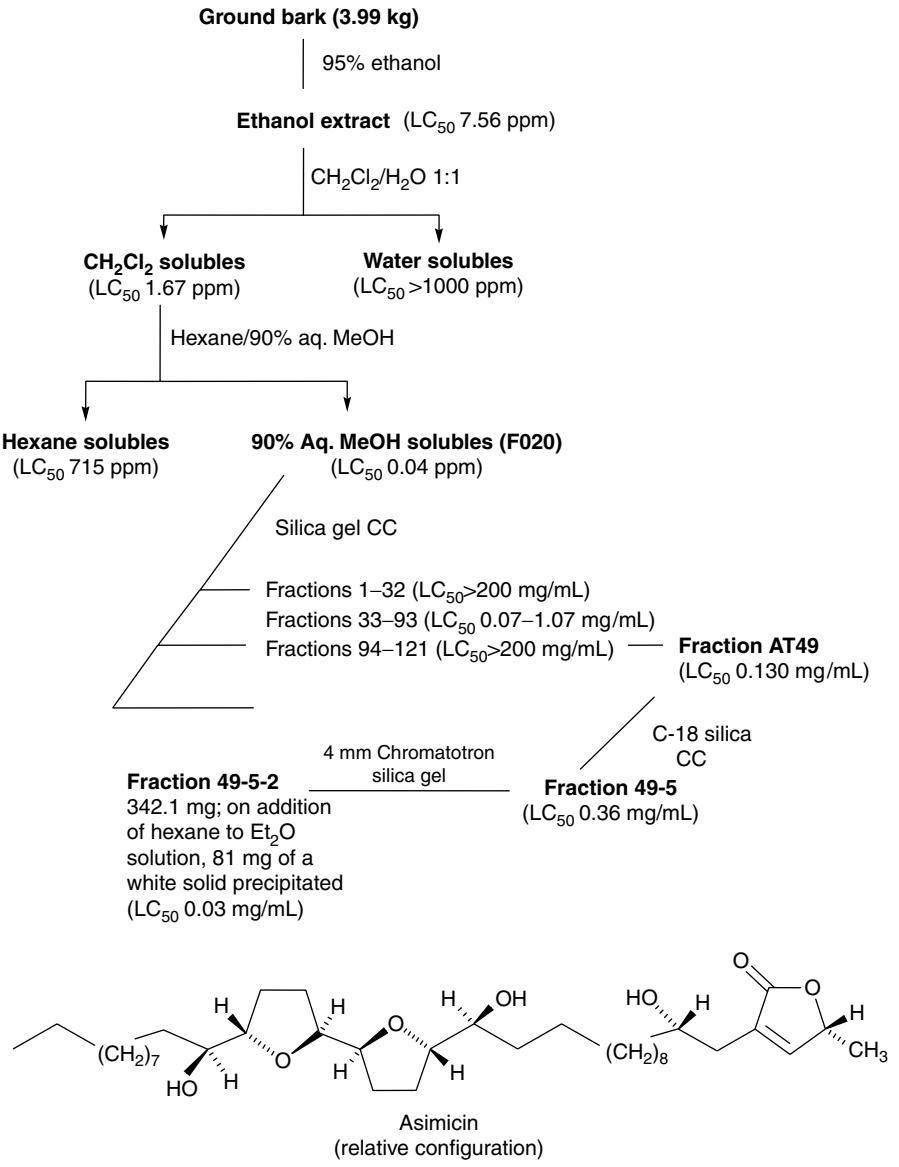


FIGURE 2.1 Bioassay-guided (BSLT) fractionation of an extract from *Asimina trilol*.

microtiter plates. Seawater (50 µL) containing 10–40 larvae is introduced into each spot, and 300 µL of test solution are added, the final concentration of the sample being 8.5, 5.0, and 1.25 mg/mL. The plates are incubated under a light source at 25–30°C, and after 24 h, the number of dead larvae are counted using a microscope. The living larvae are then killed by the addition of 50 µL of phosphate buffer (pH 1), and the total number of larvae is counted.

Recently, a complementary assay based on inhibition of hatching of the cyst in a medium containing different concentrations of organic extract has been described.[57] This bioassay and the BSLT were tested with extracts from 14 species of marine invertebrates and 6 species of microalgae. As a comparison, the extracts were also tested against two human cell lines, lung carcinoma A-549 and colon carcinoma H T-29. Each bioassay detected activity against the human cell lines in 50% of the species, but this increased to 75% if the results of both bioassays were combined. The BSLT

identifies strong anticancer activity but is limited in its predictive capacity to distinguish between strong to moderate and weak potency anticancer compounds. Therefore, the BSLT represents a quick initial screen for potent cytotoxins, but a finer level of discrimination for anticancer activity requires the human cancer cell panel (Section 2.3.2.2).

2.3.1.2 Crown Gall Tumor Bioassay

Another bench-top test that has proven useful monitors the inhibition of crown gall tumor on potato discs.[45] This bioassay is fairly accurate in predicting cytotoxicity to the P388 cell line, giving some false-positives, but few false-negatives. The assay is not meant to replace the P388 assay, but it is particularly convenient for rapid screening of extracts or fractions and does not require expensive equipment or highly trained personnel.

Crown gall is a neoplastic disease induced by the gram-negative bacterium *Agrobacterium tumefaciens*. During infection of the plant material with the bacterium, a large tumor-inducing (Ti) plasmid, found in the bacterial DNA, is incorporated into the plant's chromosomal DNA. The phenols released when the plant is wounded activate the Ti plasmid of the bacterium, which induces cell proliferation without the cells going through apoptosis, thus transforming normal, wounded cells into autonomous tumor cells. The induced tumor is similar in nucleic acid content and histology to human and animal cancers.

In a study aimed at verifying the detection of antineoplastic activity in the potato tumor bioassay, the chemotherapeutic drugs camptothecin (**22**), paclixatel (**23**), podophyllum resin (containing podophyllotoxin, **24**), vincristine (**15**), and vinblastine (**16**) were tested. Each of these drugs exhibited different modes of action on the cell cycle, and all inhibited tumor induction without affecting the viability of the bacterium.[58]

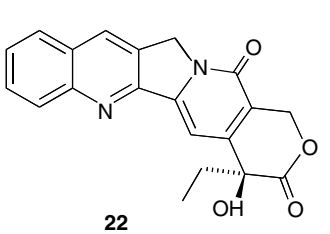
For the assay, potato tubers are surface sterilized, and a core of the tissue is extracted from each tuber. A 2-cm piece is cut from each end, and the remainder is sectioned into 0.5-cm thick discs, which are then placed onto Petri dishes containing 1.5% water agar. A solution (5 mL) of the extract or compound, dissolved in a suitable solvent, is spread over a disk, and the solvent is allowed to evaporate. The discs are then inoculated with 0.1 mL of the bacterial suspension, and the plates are incubated at 27°C. The assay measures the inhibition of tumors induced by the bacterium on potato discs by various extracts.[45] A prerequisite for this test is that the extract or substance being tested does not show antibacterial activity toward *A. tumefaciens*. This can be established by the standard agar plate-assay disk method.[59,60] The common problem of solubilizing lipophilic extracts also surfaces in this assay, although it appears that it will tolerate water:methanol mixtures up to a ratio of 1:1.[60] Importantly, DMSO as a solvent did not interfere with the activity of the bacterium or induce any tumors.[58] Some other examples of the application of this bioassay to determine the bioactivity of extracts of common mullein (*Verbascum thapsus*)[61] and *Ginkgo* are noted.[62]

2.3.1.3 Starfish or Sea Urchin Assay

The eggs of the starfish *Asterina pectinifera* have cell membranes permeable to a variety of substances. Maturation of oocytes, mediated by 1-methyladenine, occurs after 40 min and after 60 min the first polar body is released. A second polar body is released after 100 min, while meiosis is occurring. Insemination can be done at any time of the maturation process and cell division occurs after that. Exposure of the fertilized egg to different compounds will lead to different outcomes. If aphidicolin (**25**), a diterpene that selectively inhibits DNA polymerase A, is added (ca. 10 mg/mL) then the cells die after eight or nine divisions, although this compound does not affect the maturation process. Vinblastine (**16**), an inhibitor of microtubule assembly, stops the formation of the first polar body and the first cell division. When cycloheximide (**26**), a protein synthesis inhibitor, is added, the breakdown of the germinal vesicle (meiotic division) and the first mitotic division are affected.

Inhibitors of RNA synthesis show no effect on the maturation stage and allow the embryo to develop to the 64–128 cell stage. Cytolytic agents affect both types of division. This assay appears to be useful to determine which test samples should be investigated for *in vivo* antineoplastic activity.[63]

The fertilized sea urchin egg assay is a similar technique that can detect inhibitors of DNA and RNA synthesis, microtubule assembly, and protein synthesis. It is less selective than the starfish egg assay and is relatively insensitive to a series of antineoplastic agents.[64] A number of new metabolites isolated from Red Sea corals were tested for their activity in the BSLT, crown gall, and sea urchin assays. All gave positive tests and, for example, the oxylipin (**27**) showed 74% inhibition in the crown gall test.[65]



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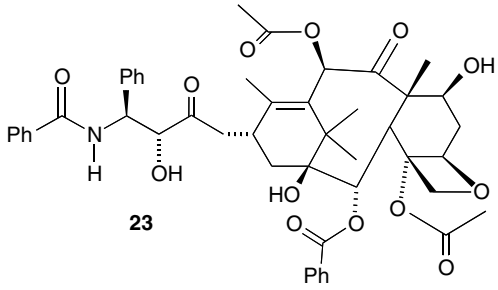

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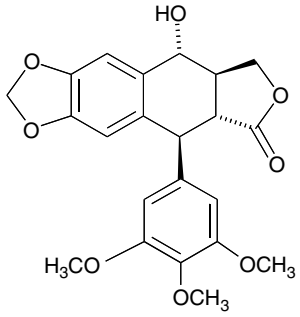

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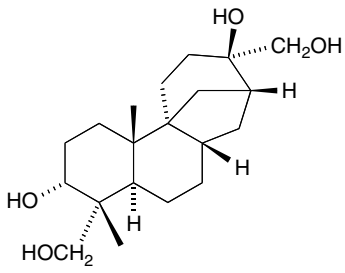

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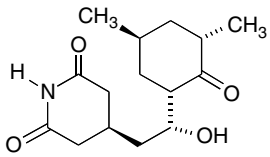

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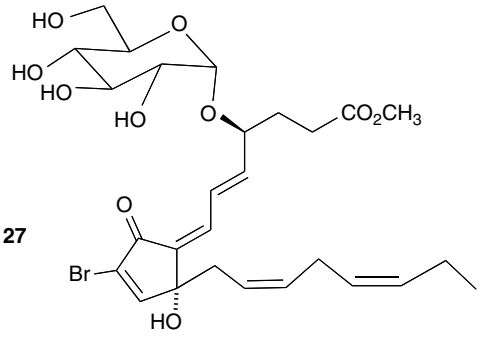

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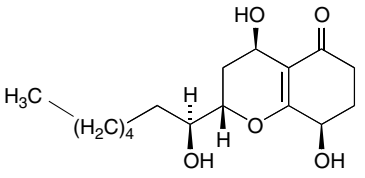
2.3.1.4 Bioassays for Antibiotic Activity

The classical agar diffusion methods have been used to isolate and identify antibiotic-producing microorganisms. These screening methods helped to discover the principal antibiotics against gram-positive bacteria and partly against gram-negative pathogens and pathogenic fungi. Several authors have discussed the history and development of classical and modern approaches to screening for antibiotic metabolites.[66–68]

There are a number of simple tests for antibiotic activity that can be carried out with simple equipment and a minimum of microbiological expertise. The following example illustrates the assay used for the detection of an antibiotic (**28**) produced by a strain of *Trichoderma koningii* antagonistic to the "take-all" pathogen of wheat and other cereals.[69,70] A plug (7-mm diameter) taken from the growing edge of a colony of the "take-all" fungus (grown on 1/5 potato dextrose agar, PDA) was placed at the center of a Petri dish containing 1/5 PDA. Samples of the ethyl acetate extract of the broth from a liquid culture of *T. koningii*, or from subsequent chromatographic fractions of the extract, were dissolved in ethanol (2 mg/mL). Ten milliliter of this solution was dispensed directly on top of the plug, and the solvent was allowed to evaporate in a laminar flow cabinet. The dish was incubated at 20°C, and the growth of the pathogen measured as area of the colony after 2, 3, 4, and 5 days compared to a control treated only with ethanol. Inhibition of growth was obvious after 2 days with the controls showing a colony area of 110 mm^2 and the test samples an area of 32 mm^2.



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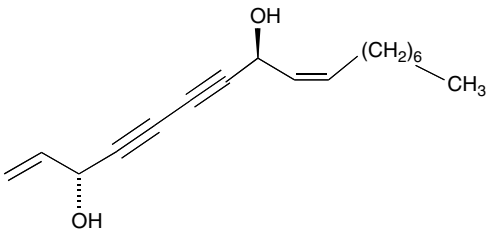

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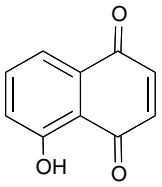

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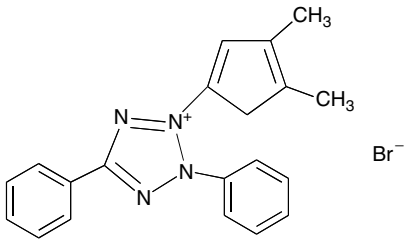

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In screening for antibiotics, the primary screen can be used not only to detect bioactivity, but also for fermentation control aimed at production of larger amounts of material. In agar-dilution streak assays, up to seven different organisms can be screened simultaneously on a Petri dish at a fixed concentration of extract. Weak antimicrobial agents present in low concentrations (<1%) can be detected. Representative microorganisms responsible for human infections of significance are normally chosen for screening. Typical examples are *Staphylococcus aureus* (representing the gram-positives), *Escherichia coli*, *Salmonella gallinarum*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (gram-negatives), *Mycobacterium smegmatis* (acid-fasts), and *Candida albicans* (yeasts and fungi).[71] Qualitative and quantitative assays, in combination with a positive control (e.g., streptomycin sulfate), can be carried out.[72]

Cole[73] has published a particularly detailed review on key antifungal and antibacterial assays that can easily be performed in a typical laboratory. The interpretation of data from such assays is critically discussed, and recommendations for the applications of the assays are made. The topic of comparability of results in antifungal testing of filamentous fungi has been addressed. Two known antifungal agents, the polyacetylene falcarindiol (**29**) and the naphthoquinone juglone (**30**), were tested in various dilution and diffusion assays against three microfungi, *Botrytis cinerea*, *Cladosporium herbarum*, and *Fusarium avenaceum*. Scoring by direct observation with image

analysis and the fluorescein diacetate method showed comparability of results. Although thin-layer bioautography and the radial growth rate method are deviated, they were considered to have some merit. The authors conclude that microdilution offers the potential to become the general standard methodology.[74] Although these techniques are useful as frontline screens, selective search strategies have been devised and these have identified a variety of new antibiotics.[75–79]

2.3.1.5 Bioautography

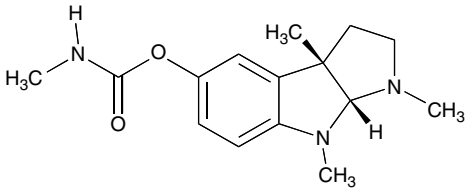
Bioautography is a very useful and relatively simple laboratory technique for the rapid detection of compounds that affect the growth rate of test organisms. The method tests the bioactivity of the analyte, which could have antibacterial, antifungal, or antiprotozoan activity, etc. There are three variants of the method: agar diffusion (contact), immersion, and direct bioautography. The most commonly used method is direct bioautography.[80] In this case, the sample is separated by thin layer chromatography (TLC), the plate is dried, and then dipped in a suspension of the test organism growing in a suitable broth, or the suspension is sprayed onto the plate. After an incubation period, zones of inhibition are made visible by spraying the plate with a reagent (e.g., the colorless *p*-iodonitrotetrazolium chloride or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT; **31**]) that is specific for dehydrogenase activity. The tetrazolium salt is converted into an intensely pink-colored formazan over a period of 4 h after which antibacterial compounds appear as clear spots against a colored background. This is a very sensitive assay that gives accurate localization of the bioactive compounds. It is also very useful in tracking bioactivity through different fractionation processes.

A number of test organisms have been used to monitor bioactivity by bioautography. The fungal plant pathogen *Cladosporium cucumerinum* is nonpathogenic to humans, readily forms spores, and grows on TLC plates with the correct medium.[81,82] The presence of antibacterial compounds is indicated by a white spot against a grey background. An active control such as amphotericin B should be used. The sensitivity of the assay is increased by addition of detergent (e.g., Triton X-100) to the dye.[80] Another suitable plant pathogen is *Valsa ceratosperma*, which can be used with TLC plates, such as silica gel G60 F_{254}, aluminium- and glass-backed plates, RP-8, RP-18 F254S, and RP-18 WF254S plates (Merck).[83]

Direct bioautography using yeasts such as *C. albicans* can be problematic in which case the agar overlay method is used instead.[80,84] In this method, the developed TLC plate is covered with an agar layer containing the test organism. The compounds diffuse into the agar during the incubation period after which the plate is sprayed with MTT to reveal areas of inhibition observed as clear spots against a purple background.[85] *Bacillus subtilis*, *E. coli*, and *S. aureus* are often used as representative bacteria. In these cases, an active control such as ampicillin or chloramphenicol should also be included. Other workers have been able to use the direct method with five human pathogens, including *C. albicans*.[85]

Bioautography also accommodates tests involving enzymes and receptors. Particular interest has been shown in detecting inhibitors of acetylcholinesterase since they form the basis of the newest drugs for the management of Alzheimer's disease.[86] Galanthamine (**4**), an alkaloid from some members of the Amaryllidaceae family, has recently been approved in some countries for the treatment of Alzheimer's disease. It slows down the progress of neurological degeneration by inhibiting acetylcholinesterase as well as binding to and modulating the nicotinic acetylcholine receptor. Because of the importance of this, a simple and rapid bioautographic enzyme assay on TLC plates was developed to screen acetyl and butyrylcholinesterase inhibition by plant extracts.[87] The test relies on the deacetylation of 1-naphthyl acetate to 1-naphthol by acetylcholinesterase, which in turn is allowed to react with Fast Blue B to give a purple-colored diazonium dye. Regions of the TLC plate that contain an inhibitor show up as white spots against the purple background. Detection limits were determined using the standards, physostigmine (**32**) (1 ng), galanthamine (**4**) (10 ng), boldine (**33**) (0.1 μg), and caffeine (10 μg). This assay was used to flag plant species that contain potential inhibitors of the

cholinesterases. Coincidentally, only a short time previously, a different group reported on similar work.[88] The two methods were essentially the same, except that the Ellman reagent (5,5′-dithiobis-(2-nitrobenzoic acid)) was used as the enzyme detector in the second assay. It appears that the first assay has lower detection limits (by a factor of 10 for physostigmine; **32**).



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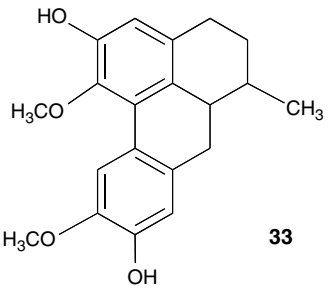

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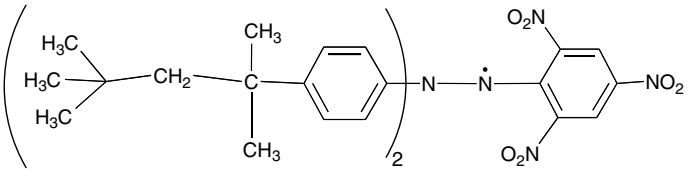

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Due to the growing interest in natural antioxidants, a bioautographic assay using stable radicals such as DPPH (2,2-diphenyl-1-picrylhydrazyl; **34**) has been introduced to screen for radical scavenging activity.[89,90] Radical scavengers reduce the radical producing white spots on a purple background.

Another interesting variant of bioautography is an assay for visualizing the binding properties of secondary metabolites to biomacromolecules, for example, DNA-binding molecules can be detected via the differential chromatographic mobility of a compound with and without the presence of DNA.[91] The limitations of bioautography are mainly the restricted number of relevant biological targets, and the fact that bioautography essentially only provides qualitative results.[92]

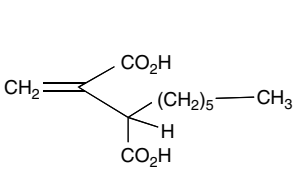
A 2D-TLC direct bioautographic assay for the detection of antifungal agents has been described.[93] In this method, the TLC plate is developed once with a polar solvent, turned 90°, and developed again with a nonpolar solvent system. The plates are sprayed with a nutrient broth/spore suspension, and the culture is allowed to grow directly on the surface of the silica gel. Two dimensional chromatography is well suited to resolving extracts containing lipophilic compounds. Considerable variation was observed among extracts (<1 mg/plate) from different origins (plants, algae, microorganisms, and invertebrates) tested against the important plant pathogens *Colletotrichum acutatum, C. fragariae, C. gloeosporioides*, and *Phomopsis* spp. Inhibition of fungal growth was assessed 3–4 days after treatment. This method has been coupled to a microbioassay in which conidia of *Botrytis cinerea*, *Colletotrichum* spp., *Fusarium oxysporum*, *Phomopsis obscurans*, and *P. viticola* in a 96-well microtiter format is challenged with pure compounds or fractions at concentrations of 0.3, 3.0, and 30.0 µM. Fungal growth is evaluated by measuring absorbance of each well at 620 nm for up to 120 h.[93,94]

2.3.1.6 Allelopathy

While the major plant-growth regulators, that is, ethylene, auxins, cytokinins, gibberellins, and abscisic acid, are intimately involved in the differentiation and development of plants and the

etiology of plant diseases, substantial evidence now suggests that there are a number of natural products that can regulate the growth of plants. These compounds are called allelochemicals and they exhibit allelopathy, which is defined as any direct or indirect effect (stimulatory or inhibitory) by one plant (also algae, bacteria, and fungi) on another through production of compounds released into the environment. Strategies for allelochemical discovery are similar to those used in drug discovery and involve screening of crude extracts and pure compounds for bioactivity. The most widely used bioassays for allelochemicals are seed germination and seedling growth studies. In developing a bioassay, the major consideration is the selection of the target species.[95] *Lemna minor* (duckweed) is often used for plant–plant interactions in aquatic environments, whereas barnyard grass, gooseweed, and ducksalad are more relevant for study of allelopathy in rice. Test samples of mono and dicotyledons should be included to determine potential selectivity. Macias[96] has suggested standardizing allelopathy bioassays by determining appropriate standard target species and using mixtures of commercial herbicides as an internal standard.

A simple, quick bioassay to test crude extracts or fractions utilizes lettuce seeds. A quantity of a solution of the sample (5 mL, concentration of 0.5–5 mg/mL) is introduced into a Petri dish. A Teflon ring supporting a small screen is placed in the dish. Twenty lettuce seeds are placed on the screen and allowed to germinate under white light for 72 h. The germination rate at each concentration is recorded as a percentage of controls and individual root and coleoptile length are determined.[97] Interestingly, lettuce seedlings are insensitive to auxin (indoleacetic acid) but respond to gibberellin-type activity. Selective plant-growth regulating effects of marine algal constituents on lettuce and rice seedlings have been noted using a similar assay.[98]



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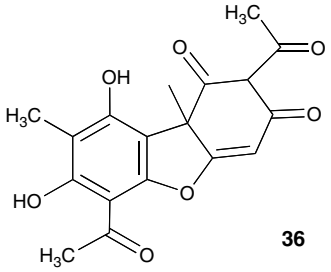

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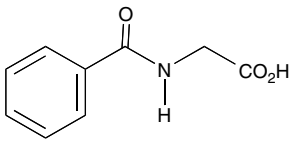

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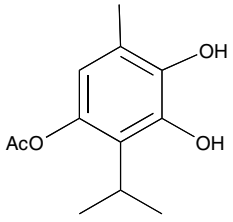

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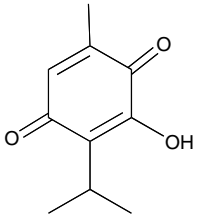

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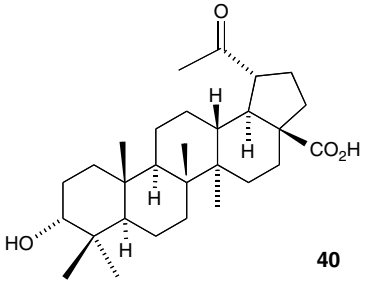

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Another assay frequently used to detect plant-growth regulating substances is the etiolated wheat coleoptile bioassay.[99,100] This assay has also detected compounds that have antimicrobial, antiamoebic, and immunosuppressive activity.[99] It is important to be aware of some problems with bioassays that monitor the inhibitory effects of metabolites on seed germination. Lower phytotoxin concentrations can produce equivalent or greater inhibitory effects than higher concentrations.[101] It is also well known that some compounds that are inhibitory at high concentrations can be growth promoting at lower concentrations and vice versa. For instance, (+)-hexylitaconic acid (**35**) at 20 ppm promoted lettuce root growth by 250%, but at 100, 200, and 500 ppm the growth of shoots and roots was inhibited.[102]

McLaughlin and Rogers[45] have described a bioassay for plant-growth stimulants and inhibitors. In this assay, *L. minor* is the test organism. Single *Lemna* plants, consisting of three fronds (one mother and two daughter fronds), are placed in two vials containing an inorganic salt and EDTA medium (2 mL) and known concentrations of the test sample to obtain 5, 50, and 500 ppm. The vials are placed in translucent, glass-covered dishes and incubated at 27–29°C under fluorescent and incandescent light. After 7 days, the number of fronds is counted and FI_{50} (inhibition of 50% frond proliferation) and FP_{50} (50% frond proliferation) are calculated. Using this assay, it was found that usnic acid (**36**) has potent herbicidal activity (FI_{50} 0.91 ppm) and that hippuric acid (**37**) is a plant-growth stimulant (FP_{50} 125 ppm). Bioassay-guided fractionation of a leaf extract of *Laggera decumbens* (Asteraceae) with the *Lemna* bioassay led to the isolation of two phytotoxic metabolites, 5-acetoxy-2-hydroxythymol (**38**) and 3-hydroxythymoquinone (**39**).[103] The triterpene 3α-hydroxy-20-oxo-30-norlupane (**40**), isolated from *Salvia nubicola*, was found to have significant growth promoting activity.[104]

A primary herbicide screen to test for microbial production of herbicides utilizes surface-sterilized seeds of garden cress (*Lepidium sativum*), barnyard grass (*Echinochloa crusgalli*), and cucumber (*Cucumus sativum*).[105,106] As an example, seeds of garden cress and barnyard grass are placed on filter paper in Petri dishes and moistened with culture broth diluted with distilled water (1:4; v/v). The dishes are incubated in darkness at 25–28°C for 72 h. Germination and radicle growth of the seeds are then evaluated compared to controls. This assay has been used to screen 1500 soil microbial isolates, mostly actinomycetes, for herbicidal activity. Of the 4–12% that showed strong inhibition of seed germination or seedling growth, only 1–2% was strongly herbicidal in a secondary screen, which involved pot trials with a greater range of test samples and observation over a 14-day period.

Another screen monitoring for phytotoxicity involves placing a few microliters of the test solution over a small puncture on a detached leaf of the target plant. The leaf is then placed in a Petri dish containing a filter paper saturated with water. The cover of the dish is sealed with Parafilm, and the dish is incubated under controlled temperature and light conditions. Activity is normally observed by the appearance of chlorotic, necrotic, or colored spots spreading radially from the point of application compared to the control. The effect can be seen in as short a period as 24 h.[107,108] The assay can be complicated by a number of factors. First, there is the usual problem of solubilizing an extract. Some weeds can tolerate a 1:1 mixture of methanol:water without any apparent damage to the control. Alternatively, some solubilizing agent, for example, Tween, can be used. The more interesting problem is one of host selectivity. Some toxins affect all plant species used in the bioassay, others affect only certain species and, at reduced concentrations, even select among different cultivars. This assay was used in the isolation of maculosin (**41**), the first host-specific toxin isolated from a weed pathogen.[109] Other assays involving CO_2 fixation, effects on organelles, and tests on whole plants, plant parts, organelles, or tissue cultures have been described to monitor herbicidal activity.[110]

A two-volume compilation entitled *Biologically Active Natural Products: Agrochemicals and Biologically Active Natural Products: Pharmaceuticals*, published in 1999, emphasized the point that natural products are a common interest in agrochemical and pharmaceutical studies. The first of these books includes details on a number of bioassays for plant-growth regulating activity.[111]

Laboratory bioassays are useful in establishing allelopathic potential of a compound or extract, but they should be followed up by greenhouse and field studies to determine if activity is maintained in the natural environment. This topic is the subject of a recent review.[112]

2.3.1.7 Insecticidal Activity

A simple bench-top assay to determine the pesticidal activity of extracts, fractions, or compounds uses eggs of the yellow fever mosquitoes, *Aedes aegypti*. The assay takes 4 days and requires small amounts of material (5–25 mg). Rotenone (**42**), a natural insecticide, can be used as a positive control in the assay. Pure substances with LC_{50} values of $\leq$1 mg/mL are worthy of further consideration.[45]

This assay was used in a study of the pesticidal activity of the annonaceous acetogenins isolated from, *inter alia*, *Asimina triloba* and *Annona muricata*. Both of these species provide extracts that are complex mixtures of compounds and display potent pesticidal effects. The authors make an interesting case for the use of crude plant extracts as pesticides rather than pure individual compounds. They refer to the work of Feng and Isman,[113] who showed that green peach aphids, which were repeatedly treated with pure azadirachtin (**43**) or a refined neem seed extract, after 40 generations developed resistance to azadirachtin, but not to the neem extract that also contains azadirachtin.

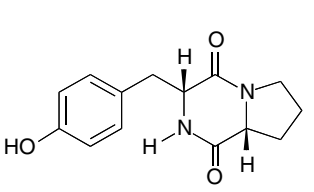
In a major screening program, Alkofahi et al.[46] submitted many species of higher plants for testing against seven indicator organisms that are agronomic pests, that is, mosquito larvae (*A. aegypti*), blowfly larvae, corn rootworm, two-spotted spider mite (*Tetranychus urticae*), southern armyworm (*Spodoptera eridania*), and melon aphid (*Aphis gossypii*). As an example, an aqueous solution, containing 5000 ppm of crude or fractionated extract, was applied to the leaves of a squash plant, and the leaves were allowed to dry. The leaves were removed and placed in Petri dishes along with armyworm larvae. After 3 days, the percent mortality was assessed. The tests with other organisms generally took between 1 and 3 days. The active fractions from the pawpaw fractionation scheme (F020, AT49, and asimicin; Figure 2.1) were compared to the commercial pesticides pyrethrum and rotenone and proved to be of comparable activity. A review on botanical insecticides, deterrents, and repellants has been published recently.[114]

2.3.2 Specialized Screening Assays

All of the "frontline" assays described in the previous section have proven useful in bioassay-guided fractionation leading to the isolation and identification of bioactive metabolites. There are a great number of other assays being used, and newer ones are constantly being developed. However, they are of increasing sophistication and require the skills and expertise of biological scientists. Some of these specialized assays are described in this section, but the emphasis is now on the type of information that can be obtained rather than providing a protocol of the assays. This is also a convenient point to consider the topic of synergy.[43,115,116]

The fact that a whole or partially purified extract has more or less bioactivity than any single isolated constituent is not new but, until recently, has not been systematically investigated. Evidence for the occurrence of synergistic effects is accumulating, and although this phenomenon is being discussed in the context of phytomedicines, it is of significance for any mixture of bioactive compounds. If two bioactive substances of a mixture have the same pharmacological target, an additive effect is expected. If, in contrast, two or more substances of a mixture have different targets, a synergistic effect may result, provided that none of the substances have antagonistic effects.[115]

A particularly telling example of this was observed with the ginkgolides, the PAF antagonists from *Ginkgo biloba*. Mixtures of ginkgolides A (**3**) and B (**44**) showed a lower IC_{50} than that obtained for either of the two metabolites individually. The presence of other ginkgolides and ginkgoflavones also appears to have an effect. A PAF-antagonizing effect in humans is produced by a mixture of ginkgolides A, B, and C at doses between 100 and 240 mg. An equivalent effect



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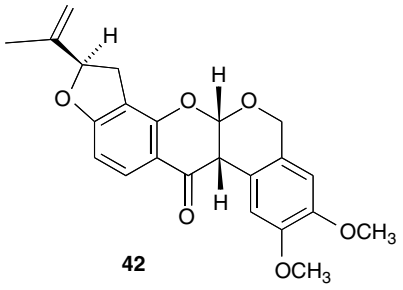

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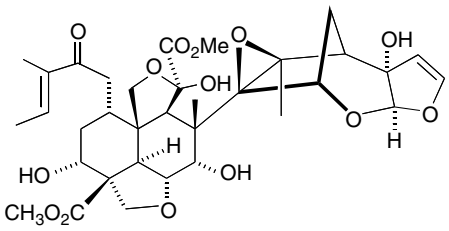

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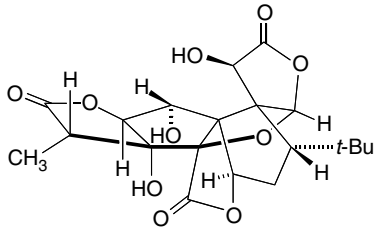

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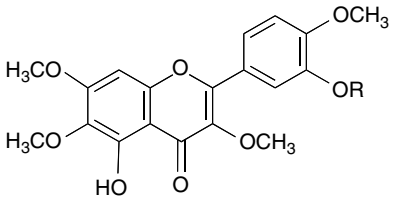

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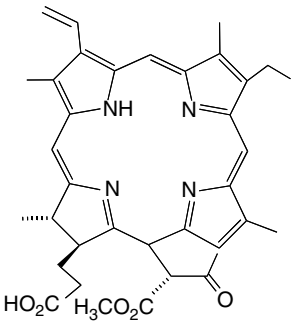
46 R = H



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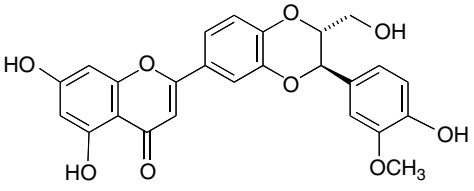

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is observed with a dose of 120 mg of a standardized *Ginkgo* extract containing only 6–7 mg of the ginkgolides.[43,115]

In an *in vitro* antimalarial bioassay, the activity of artemisinin from *Artemisia annua* was enhanced by the presence of the flavonoids artemetin (**45**) and casticin (**46**).[115] As another example, the antibacterial activity of berberine (**20**) from *Berberis freemontii* against a resistant strain of *S. aureus* was potentiated by the addition of two other constituents of the plant, the flavolignan 5′-methoxyhydrocarpin (**47**) and the porphyrin, pheophorbide a (**48**). Neither possessed antibiotic activity when tested alone.[117]

2.3.2.1 Antiviral Activity

Viral diseases, both chronic and emerging, pose the greatest health risk to humans and are an increasing worldwide health concern. Considerable activity has been observed in the last 20 years in

this field with the National Cancer Institute (NCI) implementing anti-acquired immunodeficiency syndrome (AIDS) screening and antiviral development programs in parallel with its long-standing anticancer drug program.[118] An *in vitro* antiviral assay was used to test extracts from 61 higher plants.[119] Antiviral activity was observed in approximately 20% of the plants, which was higher than expected. It was suggested that the antiviral activity detected was due to phytoalexins produced by the plant as a protection against plant viruses. These assays simultaneously allow an estimation of cytotoxicity (loss of the cell monolayer in which the plaques are normally formed). Active extracts or compounds then become candidates for testing against tumor cell lines, for example, P388 or L1210.

Next to malaria, the HIV, resulting in AIDS, is the leading infectious cause of death in the world, and the search for new, effective, and safe as well as affordable anti-HIV drugs is a matter of some urgency. There is also a continuing requirement for new antiviral drugs since an increasing number of patients infected with HIV show degrees of resistance to the currently approved drugs. Much effort has been invested in the search for natural products with anti-HIV activity, but none has so far been included in the list of recommended antiretroviral agents. It is anticipated that this situation will change soon. Recent listings of anti-HIV agents that show promise are impressive, and contain compounds form all the major classes of secondary metabolites. As examples, two compounds of particular interest are the coumarin (+)-calanolide A (**49**) and the triterpene betulinic acid (**17**).[120] A number of coumarins were isolated using an anti-HIV bioassay-guided fractionation of an extract of *Calophyllum lanigerum* var. *austrocoriacenum*. Of these, (+)-calanolide A (**49**) exhibited potent activity against HIV-1 reverse transcriptase, showed an unique sensitivity profile to nonnucleoside reverse transcriptase inhibitor (NNRTI)-sensitive viruses and had a synergistic effect with other anti-HIV drugs. This compound and congeners represent a novel and distinct subgroup of the NNRTI family, and they appear to be useful in combination therapy. Synergy with AZT was also demonstrated in the hollow fiber mouse model (Section 2.3.2.2). Currently, (+)-calanolide A (**49**) is undergoing clinical trials to evaluate its safety in both healthy and HIV-infected volunteers.

Betulinic acid (**17**), together with the biosynthetically related oleanolic and ursolic acids, is a widespread plant metabolite well known to most natural products chemists. All three triterpenes inhibit HIV-1 protease activity *in vitro*. Betulinic acid was found to be active *in vivo* using athymic mice carrying human melanomas. Further biological studies suggest that betulinic acid works by induction of apoptosis.[121,122]

In another study, 156 natural products and 100 plant extracts were examined for inhibition of HIV-1 reverse transcriptase, using an assay developed for the detection of the enzyme in virions. Of the natural products, benzophenanthridine alkaloids showed potent activity. In examining plant extracts, polyphenolic substances such as tannins, inhibitors of the enzyme themselves, complicated matters. The method favored for removal of these was polyamide chromatography, carried out on 3 mg of plant extract. Solubilization of the extracts was achieved using DMSO.[123]

A bioautographic assay to screen for antiviral agents from insects and plants involves overlaying a TLC plate on HSV (*Herpes simplex* virus)-infected CV-1 (monkey kidney) cells and, after incubation, looking for areas of viral growth inhibition.[124] Antiviral testing of 40 plant species by this method suggested that terrestrial plants and marine species contain about the same proportion of antiviral compounds. Extracts from 30 species of insects were tested and some were found to be active. In addition to antiviral information, this assay also gives an indication of cytoxicity by showing inhibition of the CV-1 cells.

A number of reviews detailing natural products with antiviral activity have been published and serve to illustrate the wide range of compounds that may have potential application.[125–127] Historical precedents suggest that diverse screening strategies should be used in the discovery of new HIV-1 agents. To this end, a recent review and report detail a number of cell-based and biochemical methods that can be utilized in antiviral screens, whereas others can be formatted for HTS or utilized as secondary screenings.[128,129]

2.3.2.2 Cytotoxicity, Antitumor, and Antineoplastic Activity

Most of the assays to test for these activities have been developed by the NCI and implemented in extensive programs, for example, The Natural Product Discovery Group, for the discovery of natural product cancer therapeutic agents. Since 1990, more than 100,000 compounds have been screened for anticancer activity in the Developmental Therapeutics Program. A large number of bioassays have been used to detect antitumor substances in extracts and to monitor chromatographic fractionation.

More recently, the P388 murine leukemia model (for *in vitro* and *in vivo* testing) has been favored in terms of sensitivity and predictivity, although advanced *in vivo* testing included additional mouse tumors, that is, Lewis lung carcinoma, colon 38, and CD8f1 mammary. To test the sensitivity of the P388 assay, 18 cancer chemotherapeutic drugs and natural products were assayed. All were detected by toxicity at 0.01 μg or 1.0 μg with P388 cells. Using human HT-29 colon carcinoma cells, only 11 of the 18 were detected at 10 μg.[130]

In recent years, several interesting papers have detailed the collaborative programs funded by the NCI that involve research of multi-institutional groups from both academic and corporate laboratories.[131] Kinghorn et al.[116,132,133] are investigating plants collected mainly from the tropical rainforests as potential sources of cancer chemotherapeutic agents. Another group is adopting a molecular approach to discover anticancer leads in marine natural products.[134]

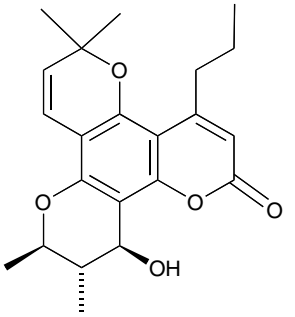
As an example, the steps involved in the evaluation of plant samples for potential anticancer agents are given.[132] Chloroform-soluble extracts from each plant sample are washed with sodium chloride solution to remove tannins that would otherwise interfere with protein-based bioassays. The organic solvent–soluble portion of the extract is screened against a panel of about 25 *in vitro* bioassays including human tumor cell lines, as well as diverse mechanistic and cell-based assays. Plant extracts with selective and potent cytotoxicity are tested against the P388 *in vivo* murine leukemia model. The active extracts are subjected to LC with mass spectrometry (LC-MS) dereplication (Section 2.5.2) to determine if the extracts contain previously isolated cytotoxic compounds. This involves subjecting the extract to high-performance liquid chromatography (HPLC), directing the output through the UV detector (280 nm), and splitting the stream into two uneven portions. The smaller portion is passed into a mass spectrometer, whereas the larger portion is fractionated in a 96-well plate, and each well is reassayed. If activity is associated with unknown compounds, bioassay-guided fractionation is used to aid the isolation of the active agents.[133] Once they are obtained in pure form, the active compounds are evaluated in all the *in vitro* assays available to the program.

Differential solubility can be a problem, particularly when the extract is not water soluble, in which case methanol or dimethylsulfoxide may need to be used. The presence of large amounts of substances that can interfere with the assays, either dominating spectrometric measurement (e.g., pigments) or masking the biological effects (e.g., tannins, fatty acids) of smaller amounts of an active principle, must be considered.[135]

Another *in vivo* assay used as a secondary screen to select compounds for additional biological testing is the hollow fiber assay.[136] This assay relies on the fact that the majority of human tumor cell lines can be grown inside hollow fibers to form a heterogeneous solid tumor model. These semipermeable fibres (polyvinylidene fluoride) containing tumor cells are implanted (intraperitoneal or subcutaneous) in host mice, and the mice are then treated with the test sample and sacrificed on day 7 and the fibers retrieved. The effect of treatment is determined by the net growth percentage of cell relative to changes in body weight.[132] Compounds that give positive responses at the subcutaneous site seem to be more important for follow-up testing than those showing activity only at the intraperitoneal site.

By way of illustration, the results obtained for two natural products are compared.[136] The nor-diterpene, 13-hydroxy-15-oxozoapatlin (**50**), isolated from the root bark of *Parinari curatellifolia* (Zimbabwe) and *P. sprucei* (Venezuela) was shown to be cytotoxic to cultured KB (nasopharyngeal), LNCaP (human hormone-dependent prostate), and LuI (human lung) cells

with ED_{50} values of 1.2, 1.5, and 5.1 μg/mL, respectively. In the hollow fiber assay, it showed inhibition with KB (68.7%) and LNCaP cells (87.7%) but did not inhibit growth of LuI cells at the intraperitoneal site. No indication of bodyweight loss was observed, indicating that further testing was warranted. In the event, **50** was found to be a G2DNA damage check-point inhibitor and to exhibit antimitotic activity.



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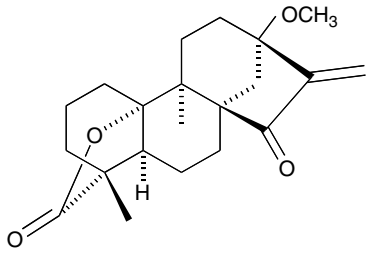

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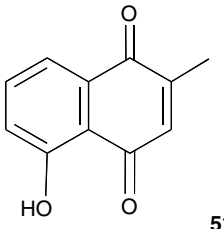

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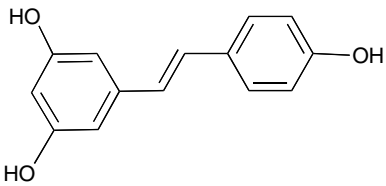

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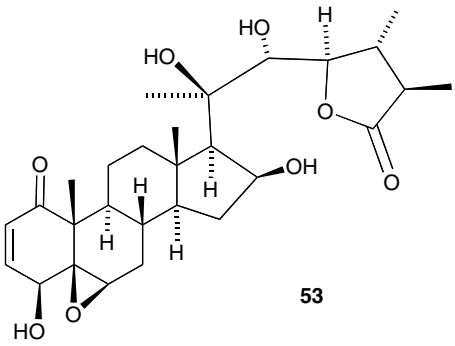

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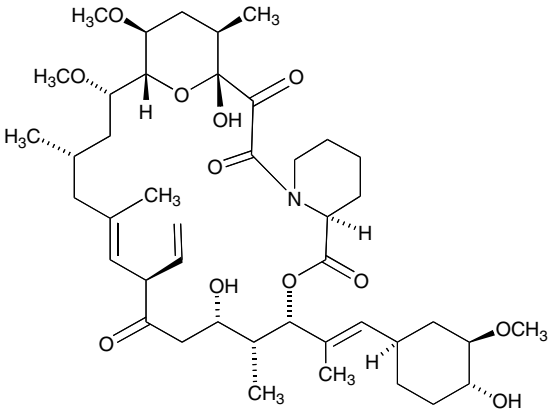
As a comparison, plumbagin (**51**), a well-known naphthoquinone, was also tested. It showed cytotoxicity toward KB (ED_{50}, 0.1 μg/mL), LNCaP (0.8 mg/mL), and LuI (0.3 μg/mL) cells. In the hollow fiber assay, concentrations of 7.5 and 10 mg/kg were lethal to one or more mice, and significant bodyweight loss was observed at 5 mg/kg. No tumor growth inhibition was noted at either of the two sites, and thus plumbagin was not selected for further investigation.

Another strategy is to detect cancer chemopreventive agents by using mechanism-based assays.[132,133] The search for compounds that inhibit carcinogenesis by (a) preventing the formation of carcinogens; (b) preventing binding of the carcinogen to target (blocking); or (c) preventing the development of the tumor, has led to the deployment of a number of bioassays. Application of these assays in guiding the fractionation of selected plant extracts have led to the identification of the isoflavones biochanin A and genistein, and the flavones apigenin and chrysin, as chemopreventive agents.[135] Several other promising compounds have been isolated, including resveratrol (**52**) and the steroid ixocarpalactone (**53**).[133]

More recently, approaches have expanded to include antimutagenicity, antioxidant, HL-60 cell differentiation, quinine reductase induction, aromatase inhibition, cyclooxygenase 1- and 2-inhibition, protein kinase C inhibition, ornithine decarboxylase inhibition, and estrogen receptor antagonist/agonist bioassays.

The NCI has tested over 90,000 extracts of terrestrial plants, marine plants, and invertebrates in its human cancer/60 cell-line prescreen that includes the HL-60 and K-562 leukemia cell lines. These data have been sifted so as to identify genera and families (e.g., Myrsinaceae, Sapindaceae) that appear to contain antileukemic agents.[137]

A multiauthored volume *Anticancer Agents from Natural Products* provides a historical, biochemical, and chemical rationale to the search for plant, marine, or microbial sources of anticancer agents. The authors discuss approaches to drug discovery and development and ways of to obtain viable amounts of the drugs.[138]



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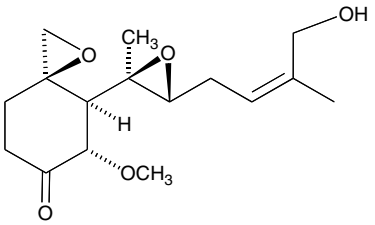

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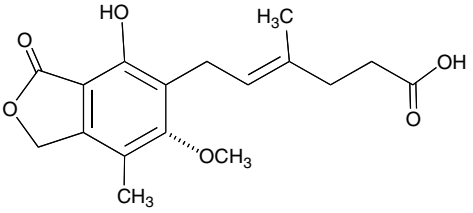

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2.3.2.3 Immunosuppressive Activity

Both terrestrial and marine organisms provide metabolites with immunosuppressive activity. A review of natural products as immunosuppressive agents also contains a very readable introduction to the role of T cells in the immune response.[139]

The discovery of cyclosporin A (**14**) and its remarkable immunosuppressive activity have contributed greatly to successful transplants of heart, liver, kidney, and bone marrow. Cyclosporin A is a fungal metabolite that exhibits a high degree of selectivity for T-cell lymphocytes but without cytotoxicity, in contrast to many other immunosuppressive agents. It is interesting to note that more than 750 analogues of cyclosporin A have been prepared, but none has had superior activity. In a systematic screening program aimed at finding metabolites with similar activity to cyclosporin A (**14**), a soil *Streptomyces* was found to produce the polyketide FK506 (**54**). The activity of this metabolite is 100 times greater than cyclosporin A (**14**).[139]

The sesquiterpene 15-hydroxyovalicin (**55**) has been reported to have an IC_{50} of 1 nM in the murine mixed lymphocyte reaction. This compares favorably with an IC_{50} of 13 nM observed for FK506. Mycophenolic acid (**56**), a metabolite of *Penicillium brevicompactum*, has been the subject

of chemical and biological studies for over a hundred years.[140] Apart from antitumor and antiviral activity, **56** also exhibits immunosuppressive activity. Specifically, it inhibits the inosine monophosphate dehydrogenase and guanylate synthetase, enzymes that are responsible for the production of GDP, GTP, and dGTP. Lymphocytes do not have a salvage pathway, and thus mycophenolic acid leads to inhibition of DNA synthesis. The prodrug has been successfully used to prevent renal graft rejection.[140]

2.3.2.4 Antimalarial Activity

Malaria is directly responsible for the deaths of 1–2 million people each year and a number of other deaths due to malaria-related anemia. Human malaria is caused by four species of *Plasmodium* that are transmitted by female mosquitoes (*Anopheles* spp.). Infection in humans leads to cerebral malaria, and other complications and the majority of cases of malaria and deaths are caused by *P. falciparum*.[141] In the last 30 years, malaria has become more prevalent, largely due to the development of resistance to chloroquine and other antimalarial agents. In 1972, the sesquiterpene artemisinin (**1**) was isolated from *A. annua* (Asteraceae) and was shown to be a rapid and effective agent against resistant parasites, to have a higher chemotherapeutic index than chloroquine, and is as good as quinine for the treatment of cerebral malaria.[15]

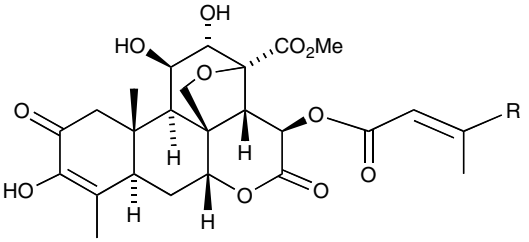
In 1976, the asexual cycle of *P. falciparum* was successfully cultured in human erythrocytes and since then *P. falciparum* has been used in *in vitro* screens. Before then, antimalarial screening relied on *in vivo* avian and rodent models. A semiautomated assay in which the viability of the parasite and the activity of the test sample are related to the uptake of [^{3}H]-hypoxanthine, a nucleic acid precursor, has been widely used. This technique was applied to evaluate the activity of crude, ethanol extracts of *A. annua* and *A. vulgaris*. This *in vitro* test discriminated between the two extracts with IC_{50} values of 3.9 and 250 μg/mL, respectively. *A. annua* contains artemisinin (**1**), which is absent in *A. vulgaris*.

Protocols for determination of *in vitro* anti-*P. falciparum* activity and *in vivo* anti-*P. berghei* activity in mice have been described.[142,143] It is worthwhile noting that *in vitro* antimalarial activity does not parallel cytotoxicity against KB cells (human epidermoid carcinoma of the mouth). Thus, bruceantin (**57**) is three times as active as brusatol (**58**) in the antimalarial test but 10 times more toxic to KB cells.

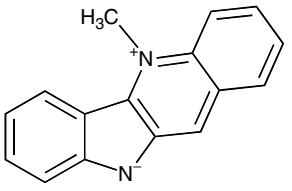
Malarial lactate dehydrogenase, which requires 3-acetylpyridine adenine dinucleotide as a coenzyme in the oxidation of L-lactate to pyruvate, has been used to measure the susceptibility of *P. falciparum* to a drug *in vitro*. Several methods to determine the activity of this enzyme are available. Colorimetric tests provide an alternative to the radiolabelled hypoxanthine method. Since parasite growth is shown by the production of a dark blue color, tests can even be carried out without the need for a spectrophotometer.[144,145]

Apart from being able to detect activity against erythrocytic parasites, methods have been developed to assess activity against liver stage parasites and gametocytes. It is now possible to examine plant extracts and compounds for their abilities to interfere with the pathological processes involved in cerebral malaria such as the adhesion of infected erythrocytes to cerebral microvessels and the action of cytokines.[146]

Many plant extracts have been evaluated for *in vitro* antiplasmodial activities, and some have been tested *in vivo*, usually in mice infected with *P. berghei* or *P. yoelii*.[147–149] Although the development of artemisinin and its derivatives was a major advance in the chemotherapy of malaria, it does have limitations, particularly in terms of availability and cost. This means that there still is an urgent need for effective and affordable antimalarial therapies. In searching for a lead compound, Wright[150] has made a case for the alkaloid cryptolepine (**59**) that occurs in relatively large amounts (>1%) in the roots of the west African plant *Cryptolepis sanguinolenta*. Other possibilities include the alkaloid nitidine (**60**)[151] and the euglobals, the acylphloroglucinol-terpene adducts found in *Eucalyptus* species.[152]



57 R = $CH(CH_3)_2$
58 R = CH_3



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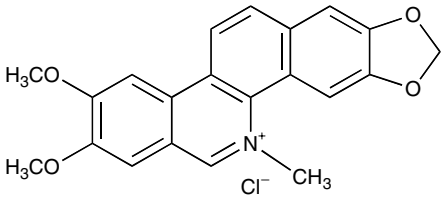

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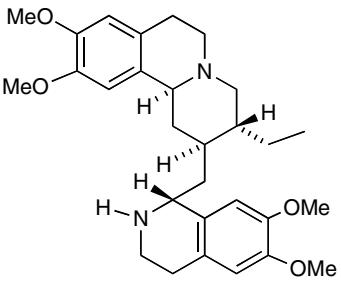

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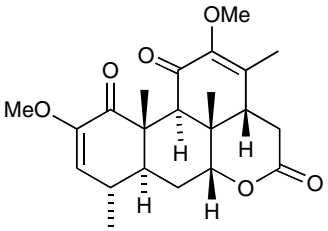

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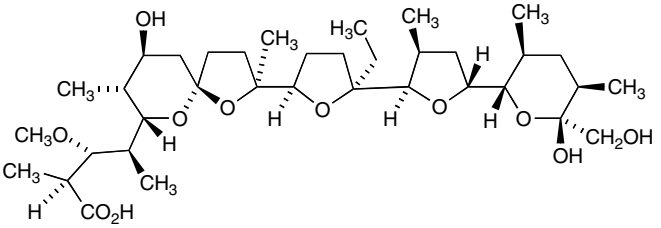

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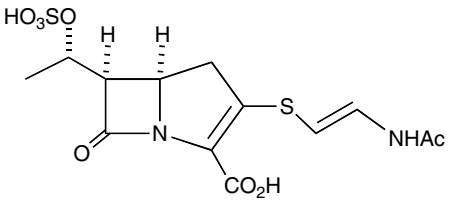

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2.3.2.5 Amoebicidal Activity

In vitro screening methods have been developed to evaluate plant extracts and natural products with activity against *Entamoeba histolytica*, the causative agent of amoebic dysentery.[153] Emetine (**61**) and 2,3-dehydroemetine were found to have IC_{50} values of 0.07 and 0.16 μg/ML, respectively, whereas metronidazole, a well-tolerated drug for this infection but known to cause tumors in experimental animals, had a value of 0.22 μg/mL. However, emetine has high cytotoxicity against guinea pig ear keratinocytes, and the cytotoxicity/amoebicidal ratio is <1, whereas quassin (**62**) has a more

favorable ratio of 132. A number of extracts from simaroubaceous plants and their alkaloid and quassinoid components have been tested.[141–143] A suggestion that anticancer drugs should also be tested for antiparasitic activity has recently been made.[154]

2.3.2.6 Antimycobacterial Activity

It has been estimated that one third of the world's population is infected with the tubercle bacillus (TB; *Mycobacterium tuberculosis*). Although only a small percentage of infected individuals develop clinical TB, about 8 million new cases and 2 million deaths occur every year. The problem is compounded by the fact that the HIV pandemic has provided a large number of susceptible individuals, thus increasing the global burden of TB. In the 1940s and 1950s, a number of efficacious anti-TB agents became available with the last, rifampin, being introduced in the 1960s. However, as seen with most anti-infective agents, the use of these drugs has resulted in the generation of resistant strains.[155]

One of the problems in applying bioassay-guided fractionation to biological extracts concerns the choice of the test organism. *M. tuberculosis* needs to be handled in appropriate biosafety facilities (BL-3). Alternative, avirulent strains are available (*M. bovis* BCG, *M. tuberculosis* $H_{37}Ra$), and require class-2 conditions. A number of bioassays have been used to test extracts, fractions, or compounds for anti-TB activity.[155] Macro and microagar dilution methods require up to 18 days to detect growth of colonies. In radiorespirometry, the growth or inhibition of the mycobacteria is measured from the extent of oxidation of [1-^{14}C] palmitic acid to $^{14}CO_2$ over a period of 1 week. Other methods, which lend themselves to adoption in HTSs (Section 2.5.1), are the microbroth dilution method in which growth is measured colorimetrically, fluorometrically, or by use of an indicator dye, and by a reporter gene assay using firefly luciferase.

The carbazole alkaloids are a class of metabolites that show moderate but consistent activity. The well-known antimicrobial alkaloid berberine (**20**) has been reported to exhibit antimycobacterial activity in a number of studies. The alkaloid inhibited the growth of *M. intracellulare* with an MIC of 0.78–1.56 μg/mL, and *M. smegmatis* and *M. tuberculosis* with an MIC of 25 μg/mL. Reviews on antimycobacterial natural products are available.[156,157]

2.4 ISOLATION AND SEPARATION

In the following sections, the various techniques available for the isolation, separation, and purification of biologically active metabolites are considered. At this stage of the investigation, the chemist is working in close collaboration with the biological scientists. The task of the chemist is to isolate and identify the substance(s) responsible for the bioactivity observed, although often no information is available regarding the nature and class of compounds to which the metabolite belongs. The identification of the metabolite as being biologically active is not more important than ensuring that the compound is pure and properly characterized. The natural products chemist has to ensure that adequate amounts of metabolites are isolated in as pure a form as possible to allow proper characterization, both chemical and biological.

It is useful to consider the following incident described by Nakanishi.[158] The structure of azadirachtin, a potent insect antifeedant, was proposed in 1975 by Nakanishi and coworkers and was based largely on results obtained by applying a new ^{13}C-NMR technique. The evidence was not convincing. For many years, the sample of azadirachtin became a test sample for newly described NMR pulse sequences in attempts to obtain more rigorous evidence for the structure, but progress was hampered by a lack of pure material. This continued until a postdoctoral research fellow devised an isolation scheme[159] that provided 10 g of the compound and, with this, the possibility of studying its chemistry. By the time that Nakanishi's group had arrived at a revised structure (**43**) in 1986, it was too late; two other groups had established the correct structure.

The temptation to bypass careful isolation and purification processes is compounded by the fact that what takes weeks to achieve in the laboratory may have to be described in one sentence in the experimental section of the paper. While structure determination has in many ways become quite routine, isolation and purification have not. This is not apparent from most papers dealing with the isolation, purification, and structural determination of natural products. Contributing to this is the attitude that purification is a trial and error process that, in any event, is not transposable from one problem to the next. In many papers long and involved separation sequences are described, but the rationale for the methods chosen is often not disclosed, even *ex post facto*.

In this context, an example illustrates the confusion that can be introduced by ignoring the importance of purity. Two papers, which appeared almost simultaneously, have described the isolation of *ent*-kauran-3-oxo-16α,17-diol from terrestrial plants. In one, the compound is said to have a melting point of 173–174°C and optical activity of $[\alpha]_D$ −39.2° ($CHCl_3$), and in the other, the melting point is not given and an optical rotation of $[\alpha]_D$ −73.1° ($CHCl_3$) is quoted. The latter sample is reported to be cytotoxic. Either the two compounds are different, in which case there has been an overreliance on interpretation of spectroscopic data, or, if they are the same, the samples differ in homogeneity. A paper published as recently as 2003 describes the preparation of this compound by semisynthesis. In the experiment, it is described as a "white foam", and although IR, MS, and NMR parameters are presented, the entry for this compound concludes with the phrase "further physical data" citing the two references mentioned earlier.

A commentary entitled "Quantity over Quality"[160] laments the declining standards in organic chemistry. The writer is concerned with the shortcuts taken by authors as evidenced from their papers. Some examples are the cavalier attitude toward ensuring purity of compounds, obtaining elemental analysis, quoting optical rotation values, reporting the NMR data for new substances without nuclei-signal assignments, and publication in a format that does not require the full characteristics of new compounds to be presented. Coincidentally, a short time after, a review by Nicolaou and Snyder[161] presented a number of cases in which the structures of natural products have been misassigned. The authors conclude: "Although the past half century has witnessed a remarkable improvement in our ability to isolate and characterize complex natural products, mistakes are still a relative common occurrence".

There are now several books available on the topic of natural products isolation. Some of the earlier compilations are still quite useful,[162–164] but the more recent ones are broader in scope.[165–168]

2.4.1 Extraction

In choosing a solvent for extraction, its ability to extract components of a solute has to be considered. For instance, ionic solutes can be extracted from aqueous solutions with nonpolar solvents if neutral complexes can be generated in the aqueous phase before extraction. The more efficient the extraction step the greater is the range of compounds present in an extract. The need to use pure solvents for extractions is obvious. Less obvious is the need to carry out the extraction under mild conditions utilizing, whenever possible, solvents of low reactivity. The possibility of generating artifacts should never be discounted (Section 2.6).

There are many basic extraction procedures described in the literature that can be tried and refined if necessary, although a trial and error approach is often required. Solvent partition schemes have been outlined for screening plants for antitumor agents[169] or screening organisms for antitumor metabolites.[170,171] The isolation of bioactive compounds is almost "always fraught with difficulties and every step requires judgment, improvisation, and discovery".[171]

2.4.1.1 Dry Biological Material

Air or freeze-dried samples are normally extracted with a variety of solvents, and sometimes sequentially from low to high polarity, if a crude fractionation of metabolites is sought. Generally,

however, a polar solvent such as ethyl acetate or methanol is used, and the separation of compounds is left until a later chromatographic step. It is useful to consider the possibility that the choice of solvent used can determine to some extent whether exo or endocellular metabolites, or a mixture of both, will be extracted. With dried material, ethyl acetate or low-polarity solvents will only rinse or leach the sample, whereas alcoholic solvents presumably rupture cell membranes and extract a greater amount of endocellular materials. For instance, washing a plant sample with ether afforded a sesquiterpene alcohol and di and trihydroxyflavones, whereas extraction with methanol provided a different sesquiterpene alcohol and a diterpene diol, both of which are also soluble in ether.[172] In the extraction of dried algal samples, more lipophilic material can be extracted with dichloromethane after soaking the sample in methanol than from an initial dichloromethane or methanol extraction. A good system with high extraction potential is a 1:1 mixture of the two solvents.

Of the extraction methods used to provide fractions suitable for bioassays, the following[46] is representative. Dried, powdered material is extracted by cold percolation or Soxhlet extractor with 95% ethanol (fraction 1). The ethanol extract is partitioned between chloroform and water (1:1), which normally generates three fractions: the water solubles (fraction 2), chloroform solubles (fraction 3), and a fraction comprising any insoluble material at the solvent interface (fraction 4). The chloroform solubles are then partitioned between hexane and methanol with enough water (normally 10%) added so as to generate two phases. This gives rise to two other fractions, the 90% methanol solubles (fraction 5) and the hexane solubles (fraction 6). If seeds or leaves coated with waxes or fatty materials are used, washing with light petroleum prior to solvent extraction is recommended. The individual fractions are submitted for bioassay, and a qualitative and sometimes quantitative measure of their activity can be obtained. This protocol has been widely used particularly in the detection and isolation of the biologically active acetogenins from the Annonaceae[173] and in the assaying for cytotoxicity and antitumor activity of bryophytes.[174,175]

In crude plant extracts, various compounds might be present that interfere with bioassays or are common constituents of plant extracts. Phenolic compounds may bind to and inactivate proteins and thus may affect receptor binding and enzyme assays. Tannins can be present in high concentration in some plant extracts, and they have been shown to inhibit HIV replication and infection of cells. Passage through polyamide XAD-2, PVPP, or other adsorbents that bind aromatic compounds can be used to remove them. Polysaccharides and their sulfonated derivatives reduce the HIV titer in cells. They can be precipitated from the aqueous extract by addition of ethanol.

2.4.1.2 Fresh Material

The water content in samples of fresh material make the dichloromethane–methanol solvent mixture ideal for extracting purposes. Once the extract has been partitioned and the methanol removed from the aqueous fraction, this can be back-extracted with ethyl acetate and then with butanol to separate the lipophilic material from the water-soluble fraction.

In the case of fermentation metabolites, the mycelial mat should be separated from the culture broth by filtration and extracted separately since some metabolites, although they may be partially exuded into the broth, adhere to the mycelium. This is the case for the avermectins (**11**), which are lipophilic and are always found to adhere to the mycelial cake after broth filtration.[24] In fermentations producing the polyether antibiotics, for example, monensin (**63**), most of the metabolites are contained in the filter cake if the fermentation broth is allowed to stand for an hour at room temperature before filtration.[176] With the *Actinoplanes* strain that produces the ramoplanin complex of glycolipodepsipeptide antibiotics, 85% of the activity is found in the mycelial mat. This can be extracted with water–methanol or water–acetone.[177] In any event, the mycelium may very well contain different endocellular metabolites.

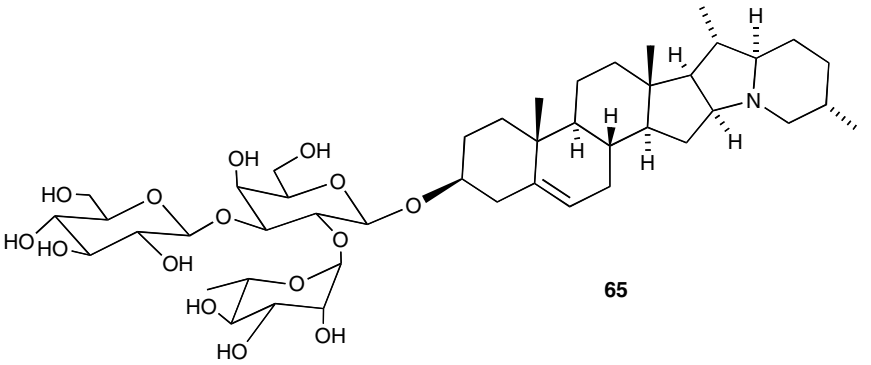
For small quantities of fresh biological material, freezing with liquid nitrogen is useful. The frozen material can then be ground to a powder and extracted in the normal way. With this technique endocellular material is also extracted.

2.4.1.3 Liquid Culture Broth and Other Biological Fluids

These can be extracted sequentially with solvents of increasing polarity from petroleum ether to butanol. The pH of the medium should be checked before extraction. In some cases, extraction of the medium at varying pH ensures maximum recovery of extract and may lead to partial fractionation.

In a number of cases, the organic material can be concentrated by passage of the broth through Amberlite XAD-2 or XAD-4 (polystyrene divinylbenzene) resin columns. These are neutral resins with no ion exchange groups, the adsorption process occurring through hydrophobic forces. To maximize adsorption, addition of 1–5% sodium chloride to the solution is recommended. For some compounds, for example, the carbapenems (**9**), elution with water is sufficient, more lipophilic substances are desorbed with organic solvents (e.g., aqueous acetone or methanol).

Ion-pair extraction can be very useful in extracting sulfated carbapenems at neutral pH.[178] The antibiotic (**64**) and related sulfates are produced in small amounts (μg/mL) in liquid cultures of *Streptomyces olivaceus*. Direct extraction of these sulfates with organic solvents is unsatisfactory because of the acid labile carbapenem nucleus. Neutral extraction can be achieved with dichloromethane containing 10 mM of a quaternary ammonium salt, for example, benzyldimethyl-*n*-hexadecylammonium chloride (BDHA). The organic layer is then back-extracted with 3% aqueous sodium iodide to remove the ammonium species. Another advantage of this method is that it leaves nonsulfated analogues in the aqueous solution. The zwitterionic thienamycin (**9**) can similarly be extracted, however, a more polar solvent, methylisobutyl ketone, and lower pH (~3), necessary for



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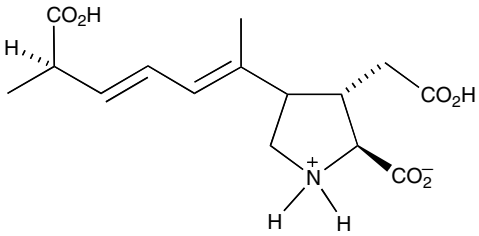

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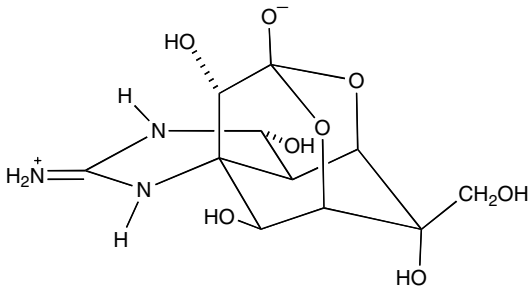

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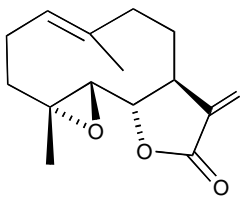

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ion-pair formation, are required. Contact times for the extraction and back-extraction need to be kept to a minimum to avoid decomposition of the metabolite.[178]

Centrifugal partition chromatography is a technique that potentially could be useful for the extraction of compounds from solutions (Section 2.4.2.1). The solution is employed as the mobile phase and a solvent or mixture of solvents with a high affinity for the compound of interest is chosen as the stationary phase.[179]

2.4.1.4 Extraction of Water-Soluble Metabolites

Surprisingly, water is often not the best solvent for the extraction of water-soluble compounds. This is a consequence of many of these compounds being intimately associated with lipophilic structures such as membranes. In fact, the best method of extraction is to use methanol or ethanol, preferably after powdering the biomass following treatment with liquid nitrogen. Treatment with liquid nitrogen, and subsequent storage at low temperatures, should be carried out immediately after collection of the biomass to minimize the possibility of peptidases, glycosidases, sulfatases, and oxidases reacting with the compounds of interest. For example, steroidal glycoalkaloids (**65**) are known to undergo partial hydrolysis by glycosidases on extraction of the metabolites from plant material.[180]

In cases where the source organism comes from a saline environment, removal of the salt is necessary. Desalting can be achieved by use of columns or cartridges containing reverse-phase solid supports (C8 or C18) or neutral polymers such as XAD-2, -4, or -7. In these cases, one is relying on the retention of the organic compound by the absorbent, whereas inorganic ions will elute with the water. This method cannot be used if the organic compound is strongly polar or in an ionized form, in which case size-exclusion chromatography using Sephadex G-10 or Bio-Gel P-2 can be used. These resins in general do not retard large molecules, whereas elution of the smaller inorganic compounds will be delayed.[181]

For isolation and purification purposes, there are a number of chromatographic supports available that operate through different mechanisms. These include anion and cation exchange, ligand exchange, reverse phase, and size exclusion. Silica gel–based supports frequently can irreversibly retain some compounds, possibly due to a combination of hydrogen bonding and pore structures. For example, the linear polycyclic ethers, brevetoxins, and some cyclic peptides can be trapped in silica gel and cannot be desorbed even with polar solvents. Covalent binding between the silica oxygens and electrophilic sites on the bioactive compound can contribute to the loss of material.

A number of alternative supports such as C18 reverse phase, polystyrene divinylbenzene, and hydroxyethylmethacrylate and dimethylmethacrylate copolymers modified with different functional groups (e.g., amino, diol, cyano) are available. These supports give better recovery and are more tolerant of high or low pH and high buffer concentrations. Some of the more commonly used supports are those in the Sephadex series, that is, Sephadex G-10, G-15, G-25, G-50, and G-100 where the number represents the amount of water adsorbed by the beads on swelling. Sephadex G-10 and G-15 are useful for the separation of natural products. The beads can also be swollen in DMSO, DMF, ethylene glycol, and aqueous methanol to give supports that are used to fractionate water-soluble metabolites. Another popular support is Sephadex LH-20, which is a modified G-25 with increased lipophilicity and is well suited for the separation of organic extracts.[181]

Brief descriptions of the isolation of two water-soluble metabolites are presented as examples. The first involves the isolation of the potent marine neurotoxin, tetrodotoxin (**66**).[182] Cultures of bacterial colonies isolated from toxic marine puffer fishes were centrifuged to remove bacterial cells. The supernatant was treated with activated charcoal and filtered through a Buchner funnel. The metabolite was desorbed from the charcoal with 1% acetic acid in 20% aqueous ethanol, and the solution was lyophilized. The residue was dissolved in 10 mL of 0.03 M acetic acid and subjected to gel filtration through a column (2 × 50 cm) of Bio-Gel P-2 equilibrated with 0.03 M acetic acid. The toxic fractions, determined using a mouse bioassay, were lyophilized. The residue was dissolved in 10 mL of acetic acid and was subjected to cation exchange chromatography through a column of

Bio-Rex 70 (H^+). The toxin was eluted by a linear gradient from 0 to 0.03 M acetic acid. Toxic fractions were combined and subjected to chemical analysis.

The second example concerns the isolation and purification of domoic acid (**67**), a glutamate agonist and neuroexcitant from a marine diatom.[181] A 50-day-old culture of the diatom was centrifuged to remove algal cells. The supernatant was adjusted to pH 3 with dilute HCl and passed through C-18 reverse-phase silica gel in a Buchner funnel. The silica bed was washed with water, and the adsorbed material was desorbed with methanol. After removal of the solvent, the residue was loaded onto a Sephadex LH-20 column and eluted with methanol. The elution was monitored by UV absorption at 250 nm. Fractions containing domoic acid were pooled and further purified by rechromatography on a C-18 column, eluting with a gradient from 0.1% acetic acid in 10% methanol to 0.1% acetic acid in 50% methanol. Domoic acid was mostly eluted with 30% methanol.

2.4.1.5 Removal of Fatty Material

Many extracts contain significant amounts of fatty material that can seriously interfere with subsequent chromatographic separation. A number of methods for partitioning such extracts are used. The extract can be dissolved in a mixture (1:1) of methanol–petroleum ether, and enough water (~10%) is added so as to give two phases, which are separated.[183] The aqueous methanol layer can be concentrated, and the organic material is recovered by back-extraction with ethyl acetate. Toluene–methanol, heptane–acetonitrile, or heptane–ethylene glycol can also be used. In the last case, the ethylene glycol layer is diluted with ether, and the ethylene glycol is removed by washing with water.[24]

2.4.1.6 Supercritical Fluid and Accelerated Solvent Extraction

In this process, the material to be extracted is placed in a pressure vessel, and a liquefied gas at a specific temperature and pressure is pumped through it. The extracting solvent is pumped into a separation chamber, the pressure is reduced, and the solvent (now a gas) is recovered for reuse, whereas the extract remains in the chamber. This essentially eliminates the need for concentration. Typical gases used are carbon dioxide, ethanol, and propane; these can be modified by addition of small amounts of a cosolvent, for example, methanol, acetonitrile, acetone, or water. Supercritical CO_2 extraction requires only moderate temperature (31°C), making it attractive for heat-sensitive compounds.

Supercritical fluid extraction (SFE) offers some advantages over conventional extraction methods, particularly when the class of compound(s) to be extracted is known. The advantages include reduced extraction time, reduced volume of solvent used, and more selective extraction. Supercritical fluids are not supersolvents, but they have a relatively high liquidlike density, low viscosity, and high diffusivity.[184]

The main disadvantages of the technique are that it requires pressure equipment, and the extraction efficiency is highly dependent on the particle size and amount of water contained in the material to be extracted. Extraction yields have been shown to depend on the type of matrix involved. The extraction of the immunosuppressant cyclosporin A (**14**) from the mycelial mat of *Beauvaria nivea* that had been subjected to varying degrees of air and oven drying showed such a dependence. The best yield of the metabolite was obtained from partly air-dried biomass, whereas oven-dried material gave the lowest yields. Scanning electron microscopy showed that the oven-dried sample had formed a solid surface in contrast to the porous surface of the air-dried sample.[185]

Another study compared the extractability of the metabolites from a number of microorganisms, that is, *Penicillium, Aspergillus*, and *Streptomyces* species. All the components of interest that were extractable with dichloromethane and methanol were also extractable by SFE with methanol-modified carbon dioxide.[186] The active sesquiterpene parthenolide (**68**) from the feverfew plant (*Tanacetum parthensis*) could be efficiently and selectively extracted by SFE without degradation.[187] SFE induced less racemization of the tropane alkaloid atropine than classical liquid–solid extraction procedures.[188]

A number of reviews detail the application of SFE methods for the extraction of natural products,[189,190] including a review of the important antimalarial artemisinin (**1**).[191] It appears that SFE can be a useful complementary technique to other methods of extraction. Although it seems that it does not recover more extract than that obtained by conventional extraction methods, it does so in a shorter time, more cleanly and with much less solvent. Because the density of the fluid can easily be varied, there is the potential for fractionation of the extract and for selective extraction.

Accelerated solvent extraction (ASE) is a related technique that normally operates at higher temperatures (50–200°C) and can be used for the extraction of more polar compounds. Reviews on these and other extraction methods (ultrasound- and microwave-assisted) are available.[192,193] An inaugural review on "Modern Extraction Techniques" is part of the biannual series of reviews in *Analytical Chemistry*.[194]

2.4.2 Chromatography

The separation of one or more substances from a crude extract or fractions of an extract can be a long and expensive process. Obtaining a pure compound often requires several separation steps involving different chromatographic techniques (multistep and multidimensional). This is particularly the case when dealing with bioactive metabolites, where the target compound(s) may be present only in trace quantities in a matrix of dozens of other constituents. There are many cases in which the isolation of the bioactive metabolite is a relatively straightforward process, especially if the fractionation is guided by a bioassay. The antibiotic agent (**28**) produced by a strain of *Trichoderma koningii*, which suppressed the "take-all" fungus of wheat, was isolated from a liquid culture in the following way.[70] Extraction of the culture broth with ethyl acetate gave a yellow oil, which, from TLC, appeared to contain one major and several minor components. The bioactivity was associated with this fraction, and this was subjected to flash chromatography (FC) on silica gel. Gradient elution (light petroleum to ethyl acetate) gave six fractions of which only the most polar exhibited bioactivity. This fraction, while giving rise to interpretable spectroscopic data, was shown by TLC to be contaminated with another compound of similar R_f value. Fortunately, this impurity could be removed by chromatography on alumina (activity I) without loss of bioactivity.

Most separation procedures, however, require diverse chromatographic methods, and a selection of the more widely used is given in the following sections. In multistep chromatography, the results from one chromatographic step have to be evaluated before a decision can be made on the next step. This is where expertise, intuition, trial and error, and serendipity can all play a part. The availability of high-field NMR instruments, with their greater dispersion power, can usefully be harnessed to guide the selection of separation methods. Even with mixtures of several compounds, it is often possible to infer the class to which the major components belong. An early indication of the nature of, and functionalities associated with, the compound(s) of interest can greatly facilitate rational design of the separation process.

2.4.2.1 Liquid–Liquid Chromatography

2.4.2.1.1 Countercurrent Chromatography

This technique relies on the partitioning of compounds between two immiscible liquid phases. Separation is determined by the different partition coefficients of the solutes. Although countercurrent distribution (CCD) techniques, developed in the 1950s by Craig, provided high-resolution capabilities for separation, the method never achieved widespread use. Some reasons for this might be that the instrumentation was cumbersome and fragile, the process required a lot of solvent, and the multitube glass apparatus demanded significant time for cleaning and setting up. Nevertheless the technique was found useful in separations in the actinomycin, peptide antibiotics, lincomycin, erythromycin, and streptomycin classes of compounds.[164]

Following the developmental work of Itoh in the mid-1960s, a number of other countercurrent chromatographic (CCC) instruments became available, but in the meantime, developments in solid

phase support chromatography, especially HPLC, had attracted the natural products chemists, and CCC was largely overlooked.

2.4.2.1.2 Modern Countercurrent Chromatography

Modern CCC presents a number of advantages compared to adsorption and exclusion chromatography. As a form of liquid–liquid partition chromatography, it provides a complementary technique to other forms of chromatography. Importantly, there is no irreversible adsorption, as can occur with solid supports, and thus there is complete recovery of the sample loaded. A range of solvents can be employed, from nonaqueous to buffered acidic and alkaline solvents. The composition of the two immiscible phases can be fine-tuned to achieve the desired resolution. In addition, there is little risk of degradation of the solutes, solvent consumption is low, and there is no requirement for expensive columns.

Crude extracts can easily be fractionated in a single step, preliminary to further separation. An additional advantage is the ability to adopt either a normal or reverse-phase elution mode with the same two-phase solvent system. Although the efficiency in terms of theoretical plates cannot match that of HPLC, it has other advantages as a consequence of the high-stationary to mobile-phase ratio. In HPLC, about 20% of the volume of the column is stationary phase available for interaction with the solute. For CCC, the stationary-phase content can be as high as 80%. CCC can also be adapted to a continuous extraction or enrichment mode. CCC has the disadvantage of requiring an initial large capital cost, but it has low operating costs.

Marston and Hostettmann[195] have listed the commercially available instruments and described the components of typical rotating coil, cartridge, and disk instruments. Analytical instruments are of the rotating coil type with a capacity varying from 10 to 50 mL. They can be used for screening for bioactive compounds in crude extracts and for microscale isolation. In bioassay-guided fractionation, the collected fractions should be submitted for bioassay. Continuous monitoring systems, such as UV absorption or evaporative light scattering detection, are most convenient. TLC analysis of each fraction remains the surest method of monitoring separation, but the order of elution will not necessarily be reflected by R_f values since, in TLC, adsorption processes are additionally involved.

The key feature for a successful CCC separation is the choice of solvent system. Some of the more general ones that can be used as a starting point are chloroform–methanol–water (10:3:7) or the less polar, hexane–ethyl acetate–methanol–water (1:1:1:1). Many others are available, and these have been listed in terms of the solvent to be used according to the (biosynthetic) class of compounds to be separated. However, this is of limited use because often there is no information as to the class of compound involved. Also, the metabolites of interest may be present in small amounts and masked by more abundant compounds. In these cases, the choice of solvent system is best determined by trial separations using analytical CCC.

It is also possible to use TLC as a guide to solvent selection. A small amount of the sample is thoroughly mixed with an equal amount of the upper or lower phase of the solvent system. The same volume of the two resulting phase-based mixtures is applied to a TLC plate that is then allowed to develop in the organic (or less polar) phase. A suitable system is one in which there is equal distribution between the two phases and with R_f values between 0.2 and 0.5. A similar preliminary test on the solvent system can be carried out by HPLC. The crude sample is dissolved in a volume of the upper-phase solvent, and a small amount is analyzed by HPLC. Then, an equal volume of the lower-phase solvent is shaken with the sample solution and the upper phase is reanalyzed and the peak areas are compared to those of the first chromatogram. The partition coefficient (K) for each component is calculated from the following equation: $K = (A_1 - A_2)/A_2$, where A_1 and A_2 are the peak areas measured from the two chromatograms.

The choice of the solvent systems for samples that are mixtures of potentially charged compounds, such as alkaloids, is best approached by trial and error using analytical CCC. As an example, separation of a mixture of alkaloids isolated from *Coptis chinensis*, a Chinese medicinal herb, was attempted using a chloroform–methanol–aqueous hydrochloric acid system on a 30 mL multilayer coil system, with a separation time of 2 h.[196] Suitable separation of four alkaloids, including

the antibacterial berberine (**20**), was achieved with chloroform–methanol–0.2 M hydrochloric acid (8:3:4) after scaling up to a 236 mL system. By way of comparison, good separation was obtained on TLC with benzene–ethyl acetate–methanol–isopropanol–aqueous ammonia (12:6:3:3:1). A number of other examples of successful separation of bioactive compounds are discussed by Marston and Hostettman.[195]

A way of reducing run times or to separate components of widely different polarity is to use a solvent gradient. Although it may appear counterintuitive, gradient elution can be used in CCC. Moreover, it is possible in some cases to vary the composition of one phase whereas the other remains nearly constant. Another recent improvement is the combination of CCC with MS, and this provides a complementary method to LC–MS for the analysis of nonvolatile or thermally unstable compounds.

Several books on this technique have been published in the last decade.[196–201] More detailed descriptions of the instrumentation, techniques, and factors influencing resolution, together with illustrative examples have been discussed. Guides to solvent systems useful for the separation or purification of different classes of compounds have been compiled.[195,202,203]

2.4.2.2 Planar Chromatography

Planar chromatography (PC) is a collective term for all analytical, micropreparative, and preparative separation methods in which the mobile phase moves through the stationary phase in a planar (flat bed) arrangement. These methods are essentially of two types: TLC and forced-flow planar chromatography (FFPC). In TLC (and the largely superseded paper chromatography), the movement of the compounds to be separated is the result of the driving force of the mobile phase and the retarding action of the stationary phase. FFPC includes centrifugal thin layer chromatography (CTLC), overpressure layer chromatography (OPLC), and electroplanar chromatography (EPC). With these methods, the mobile phase migrates through the stationary phase under the influence of forced flow in addition to capillary action. All methods can provide high resolution, reproducibility, and high throughput. Some, such as EPC, are still largely in a state of development and the instrumentation is both complex and expensive. There is also a move toward mechanization of the individual steps in PC. An applicator that allows sample application in linear, circular, or anticircular PC has been developed.[204]

The considerable activity in the development of these techniques is apparent from the several books, reviews, and papers on the topic.[204–209] The sixth (and last) edition of the classical textbook *Chromatography* edited by Heftmann was published in 2004.[205] The practical aspects of these techniques have been extensively covered by Gibbons and Gray.[206]

2.4.2.2.1 Preparative Thin Layer Chromatography

Traditional preparative thin layer chromatography (PTLC) remains the most basic and most economical of separation techniques since, in its simplest form, it only requires a supply of precoated or homemade plates, a micropipette, developing chamber, sprayer, and an oven. Its usefulness as an analytical method ensures its survival and presence in every chemistry laboratory. Silica gel is the most used layer material. The availability of fine particle silica, bonded phase, and cellulose layers, as well as plates with bonded chiral phases, extends the applicability of the technique.

In the preparative mode, it can be adapted for the separation of milligram to gram quantities. Although a number of TLC plates with different adsorbents are commercially available, the advantage of homemade plates is that any thickness (up to 5 mm) and many compositions (addition of silver nitrate or buffers) can be accommodated. PTLC also has a number of disadvantages. The amount of sample per plate is low (maximum of 100 mg on a 2-mm thick, 20 × 20 cm plate), and when loading is performed manually it can be haphazard. Furthermore, recovery of the purified compound from the plate can be mechanically difficult and potentially hazardous, as containment of fine adsorbent particles requires special precautions.

2.4.2.2.2 Centrifugal Thin Layer Chromatography

A technique that has become popular is CTLC.[210] In this method, the circular preparative plate is rotated at 800 rpm whereas the sample is introduced in the center of the plate, followed by eluent (3–6 mL/s), under a nitrogen atmosphere. In the more popular version, the plate is rotated inclined to the horizontal. The concentric bands of compounds migrate to the periphery of the circular plate are collected. The separation of UV-active substances can be monitored with the aid of a UV lamp, but, in any event, the fractions collected are analyzed by analytical TLC to decide which can be pooled. This method is relatively simple, fast, does not require scraping of bands, the coated plate can be washed and regenerated, step gradient elution is possible, and the contact time between compound and stationary phase is reduced. In terms of loading capacity, a circular plate can tolerate the same amount of material as three ordinary TLC plates. The drawbacks are that coated plates are not commercially available, the maximum thickness of the layer is 4 mm, and there is a restricted choice of stationary phases. CTLC has been useful for the separation of a wide range of substrates from polyacetylenes to saponins and nucleotides.[210] A knowledge of good laboratory practices, an understanding of the principles involved, and a "feel" for the substances to be separated, which comes with experience, goes a long way in making PTLC a powerful separation and purification method.

Recently, the coupling of CTLC online with MS was described.[211] Using low-pressure fittings and a self-aspirating atmospheric pressure, chemical ionization (APCI) probe proved a viable means to couple online CTLC with APCI-MS.

2.4.2.2.3 Overpressure Layer Chromatography and Automated Multiple Development

OPLC can be used for analytical or preparative separations. In OPLC, the vapor phase of PTLC is replaced by an elastic membrane under external pressure. The mobile phase is driven by a pump through the sorbent layer, and thus the planar plate becomes a planar column. Depending on the desired mobile-phase velocity, pressures from 2 to 100 bar can be used. The compounds are eluted from the plate (20 or 40 cm), and fractions are individually collected. Sample sizes may vary from 50 to 500 mg. OPLC can handle increased solute loading, has higher efficiencies than PTLC, has reduced separation times, and provides resolutions equivalent to those obtained with HPLC.[212,213]

The usefulness of OPLC for the separation and characterization of natural products, such as coumarins, flavonoids, anthocyanins, alkaloids, and essential oils, has been illustrated. Quaternary ammonium salts were readily separated by using silica gel plates and a mixture of ethyl acetate–tetrahydrofuran–acetic acid (60:20:20) as eluent. The automated multiple development (AMD) system is a complementary technique that is characterized by the progressive development of a chromatographic plate, with a drying step between each development. Elution is realized in 20–25 steps that corresponds to approximately 40 m of adsorbent.[214]

2.4.2.2.4 Multidimensional Planar Chromatography

Resolution of complex mixtures often requires sequential and multiple fractionation using different chromatographic media. For these cases, it is useful to harness forced-flow methods because they allow optimal mobile phase-flow velocity. Optimum performance laminar chromatography is an example of forced-flow TLC using a planar 2D column. The multidimensional capacity is not limited to the separation technique alone, but also to the multitude of sample application and detection methods available.[215] It can be used as an analytical or semipreparative method (20 × 20 cm, 500 μm thick) and can be adapted for bioautography and 2D development techniques.

Online and off-line multidimensional chromatography has long been used in the separation of selected metabolites from poorly resolved mixtures. In recent years, comprehensive 2D gas chromatography (GC × GC) has proven useful in handling difficult volatile samples. The LC equivalent (LC × LC) is more complex to perform, but its potential is attracting more attention.[216]

The developments in this field can be followed by referring to the fundamental reviews section of *Analytical Chemistry*, a biannual review (latest issue 2006)[217] and to various other journals, that is, *Analytical Chemistry, Chromatographia, Journal of Chromatography* (parts A and B and the

bibliographic issues), *Journal of Chromatographic Science, Journal of Liquid Chromatography & Related Technologies*, *Acta Chromatographica*, and a recent journal devoted to the topic, *Journal of Planar Chromatography—Modern TLC*.

2.4.2.3 Column Chromatography

2.4.2.3.1 Gel Filtration

Size exclusion chromatography, or gel filtration, is an important technique in the area of biopolymers. An extension of the molecular sieves exclusion principle, it also obeys the basic principles of chromatography. The support, or gel, is a neutral porous material that allows molecules of different sizes to penetrate into the gel to different extents. This is a reversible process so that small molecules passing into the interior of the gel can be eluted out. For any given gel, there is an exclusion limit, a molecular weight above which no penetration into the gel will occur. In general, molecules will be eluted in the reverse order of their molecular weight, although for smaller molecules other factors such as polarity will play a role.

Sephadex LH-20, obtained by alkylation of most of the hydroxyl groups of Sephadex G-25 (exclusion limit ca. 5000 Da), has become the most popular of the hydrophilic gels in the isolation of natural products. The derivatization adds lipophilicity to the gel that still retains its hydrophilicity. The gel swells in polar solvents such as water, methanol, and tetrahydrofuran. In the gel filtration mode, using a single solvent, compounds are separated according to their size and those with molecular weight greater than 4000 Da are not retained. If a solvent mixture is used, the more polar solvent will be taken up by the gel, thus generating a two-phase system with the stationary and mobile phases of different composition. In this case a partition mechanism can operate, leading to separation of compounds. Sephadex LH-20 is particularly useful in the removal of high molecular weight and polymeric material from a sample. Since these often cause problems in later chromatographic steps, gel filtration is often a prerequisite step to CCC and pressure LC.

A remarkable example is illustrated by the method used for the isolation of bryostatin 1, an antineoplastic compound from the bryozoan *Bugula neritina*.[218] The isolation scheme is shown in Figure 2.2. The dichloromethane-soluble portion of an extract was subjected to solvent partitioning, and the portion soluble in carbon tetrachloride was collected. The extract (214 g) was divided among five large columns of Sephadex LH-20, prepared in dichloromethane–methanol. The separation was bioassay-guided and yielded ca. 122 g of material showing activity. The separation process was repeated using a less polar solvent combination to furnish fractions from which bryostatin 1 could be crystallized. Bryostatin 2 (deacetyl-bryostatin 1) could also be isolated in this separation.

Partition chromatography on Sephadex LH-20 has also been used for the separation of various avermectins[24] and is the most common method for the preparative isolation of condensed and hydrolysable tannins.[219,220] It is worthwhile noting that the separation of different tannins is induced by their different adsorptivities on the gel. Because of this, higher molecular weight tannins are not easily recovered from the column with organic solvents. In these cases, the use of a more stable vinyl polymer gel, for example, Diaion HP-20 is indicated.[220]

2.4.2.3.2 Preparative Column Chromatography

The conventional gravity-driven, open-column chromatography method is still widely used in both rapid filtration and true separation modes. Sample to support ratios of 1:10 to 1:300 can be used depending on the difficulty of separation of the components of the mixture. Various stationary phases of different particle size (10–200 μm) and porosity (50 nm) are available. Silica is the most widely used phase, but several bonded silica phases (cyano, amino, hydroxyl, nitro) are also used, although they are more expensive. Polyacrylamide beads (45–180 μm) and the cross-linked dextrans (Sephadex) are useful as inert packing in the chromatography of labile compounds. Both phases swell in water, which is also used as the mobile phase. Styrene-divinylbenzene polymers (XAD, HP, SP resins) are useful for reverse-phase chromatography, although their relatively large

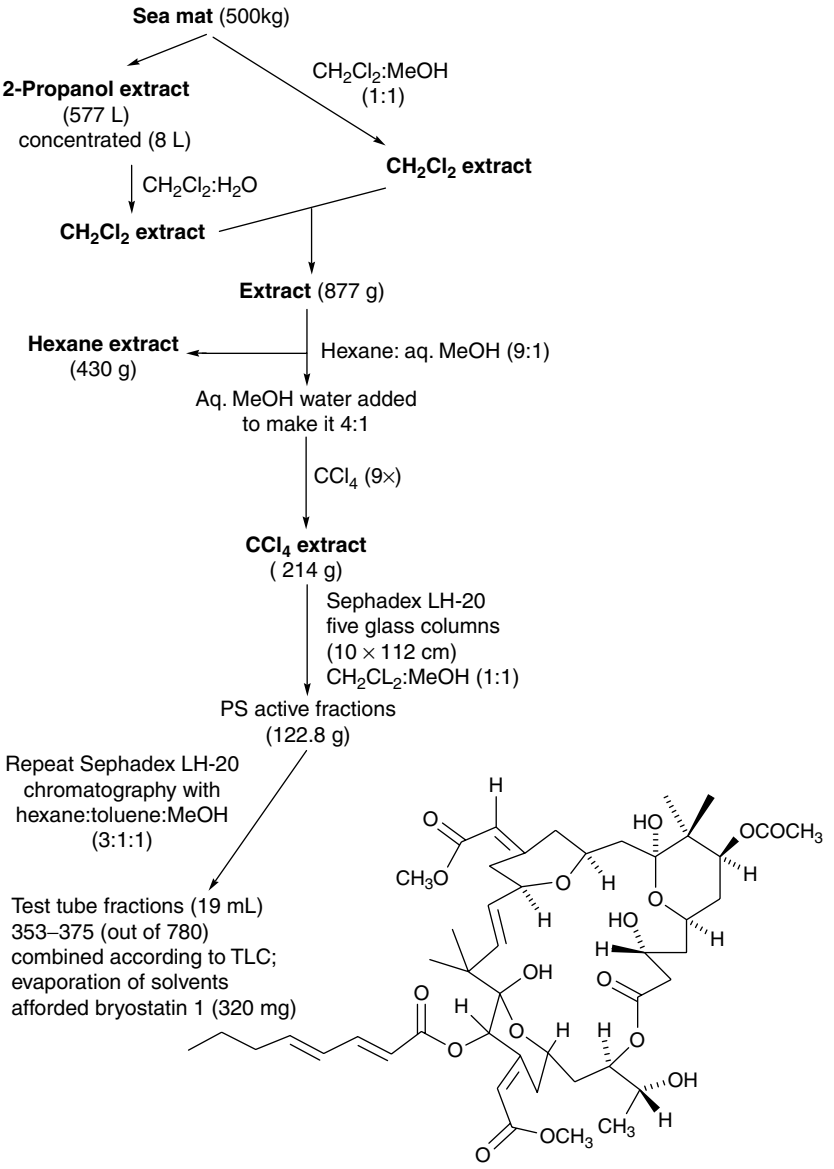


FIGURE 2.2 Isolation of bryostatin 1 from *Bugula neritina*.

beads (250–600 μm) preclude high-resolution separation. They can be used to advantage in desalting and extraction of organic compounds from aqueous media. Alumina (Al_2O_3), initially much used in open-column chromatography, should be used only with highly stable compounds. In both the acidic or basic forms, it can become an excellent catalyst for undesired reactions.

Open-column chromatography is a slow method with the consequence that material is lost by irreversible adsorption, particularly with silica and alumina, has low reproducibility (the ease with which columns can be packed often leads to carelessness) and requires large amounts of solvents, which, in step gradient solvent elution, are not easily recoverable. Two more recent techniques, FC and vacuum liquid chromatography (VLC), have been widely adopted in natural products laboratories and, in terms of convenience, are superior to their forerunner, dry-column chromatography.

2.4.2.3.2.1 Flash Chromatography

FC is a very convenient and simple technique that finds application in both synthetic and natural products chemistry.[221] Briefly, a column is preferably dry-filled with adsorbent, the sample is introduced, and the solvent is forced through the column under pressure from compressed air or nitrogen (ca. 1 bar above atmospheric pressure). Silica gel (25–200 μm) is most frequently used, the sample loading depending on the ΔR_f of the compounds to be separated. With ΔR_f of ca. 0.1, the suggested column size is 50 mm (diameter) for a sample loading of 1 g.

Bonded phases, including chiral stationary phases, can also be used. Reverse-phase FC has been shown to be particularly useful in the partitioning of polar bioactive metabolites.[222] The method involves coating the crude extract onto a reverse-phase support. This is loaded as an aqueous slurry or a powder onto a column packed with the same support (100 g for 20 g of extract), and normal elution, for example, step gradient from water, methanol to dichloromethane, can be carried out. Ten to twelve fractions are collected, which can be tested for bioactivity and can be further processed by semipreparative RPLC. The solid support can be recycled many times.

For samples on the milligram scale, a convenient variant of this technique utilizes cartridges that function as short columns. Developed originally for off-line cleanup of samples prior to HPLC (Section 2.4.2.4.3), these cartridges are available with both normal and reverse-phase packing and are made to fit on the end of a syringe. A solution of the sample is deposited on the column normally, and elution with different solvents is achieved by pressure applied with a syringe containing the solvent. This method is useful not only for sample preparation,[223] but also for separation[224] and purification of compounds.

2.4.2.3.2.2 Vacuum Liquid Chromatography

VLC rivals the foregoing FC method for simplicity, but the flow of the solvent is maintained by vacuum. The column is prepared in a sintered glass funnel using TLC grade packing (aluminium oxide, silica gel, or reverse-phase supports). Uniform packing is achieved by initially tapping the funnel on the bench and then by application of a vacuum from below the funnel. The sample is applied uniformly at the top of the support. Step gradient elution is used and the column can be allowed to run dry after collection of each fraction, approximating multiple-development PTLC. All the usual stationary-phase adsorbents can be used, and the technique is applicable to large-scale separations. Sample sizes from a few milligrams to 50 g can be accommodated by choosing the appropriately sized funnel. The advantages over PTLC (e.g., reduced cost, time saving, and resolution) have been listed,[225] and detailed description of the simple apparatus has been given.[225–227]

Additionally, in a similar way to the use of cartridges and small columns with FC, many different types of cartridge or column (e.g., normal and reverse phase, and ion exchange) can be used with vacuum manifolds to isolate compounds. Larger samples can be distributed over several columns in the manifold and the relevant fractions from each pooled prior to evaporation of the mobile phase to recover the compound(s).

2.4.2.4 Preparative Pressure Liquid Chromatography

This term covers those techniques of column chromatography in which pressure is applied by a pump operating above 2 bar pressure. Preparative, in this context, refers to amounts ranging from micrograms to kilograms. The division between low- (up to 5 bar), medium- (5–20 bar), and high-pressure (>20 bar) liquid chromatography is not simply arbitrary but reflects the use of different columns with different size packing material and size of sample that can be fractionated.

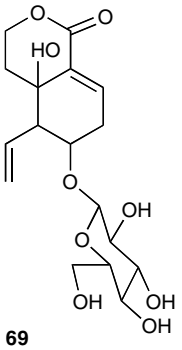
2.4.2.4.1 Low-Pressure LC

This can be conducted with homemade glass or stainless steel columns. Ready-filled glass columns (240 × 10 mm to 440 × 37 mm) packed with silica or RP-8 support (40–60 μm) are commercially available. The system requires a pump capable of reaching 6 bar pressures and an injection system. Smaller columns are suitable for sample loads up to 200 mg, whereas the larger size will tolerate up

to 3 gm. The selection of solvent can be extrapolated from TLC, and normally a single solvent combination (isocratic) is used. Some representative examples have been discussed.[210] This technique is most useful for processing crude extracts into discreet smaller fractions and, while it may not necessarily provide pure compounds, the individual fractions can be submitted to chromatography with higher resolution potential.

2.4.2.4.2 Medium-Pressure LC

This mode uses larger columns and higher pressures delivered by a reciprocating pump. It is a useful substitute for open column chromatography or FC in terms of sample load with the advantage of higher resolution and shorter separation times. Compared to these other two techniques, packings with smaller particle size (25–40 μm) are used. A flow rate of 100 mL/min is usual and high loading capacity (1:25) can be achieved. The power of the technique can be illustrated with reference to the separation of the four major secoiridoid glycosides from *Gentiana lactea*.[228] Crude extract (1.5 g) was applied to a reverse-phase column (46 cm × 26 mm i.d.; LiChroprep RP-8, 15–25 μm)



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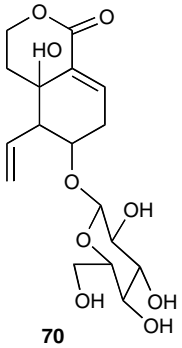

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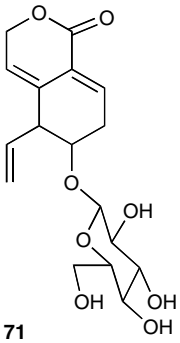

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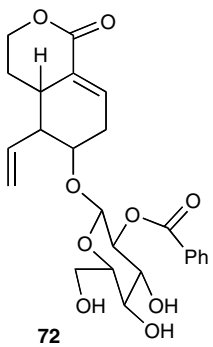

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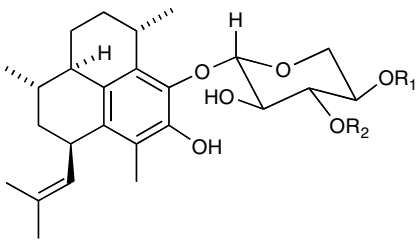
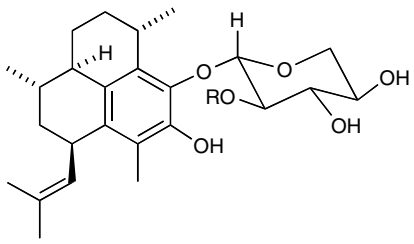


	R_1	R_2
73	**Ac**	**H**
75	**H**	**Ac**



74 R = Ac
76 R = H

under a maximum pressure of 36 bar, equipped with an UV detector (254 nm) and eluted with 20 and 30% aqueous methanol (flow rate 18 mL/min). The four compounds appeared between 50 and 180 min and were collected to yield (**69**) (8 mg), (**70**) (43 mg), (**71**) (37 mg), and (**72**) (14 mg), in order of elution. The resolution approached that obtainable by HPLC (Section 2.4.2.4.3), which was used to establish optimum conditions for separation.

2.4.2.4.3 High-Performance (Pressure) LC

In the 20 or so years since commercial instruments became available, HPLC has had tremendous impact on separation methodology.[229] The development in HPLC was spurred on by the discovery of DNA and the need for the separation of nanogram to microgram levels of nucleotides and nucleosides generated from hydrolysis of DNA and RNA. Commercial instruments were first referred to as "nucleic acid analyzers" or "amino acid analyzers". Developed initially for the biochemical market, HPLC has been adopted by the natural products chemist as both a preparative and an analytical technique. It has spawned the medium- and low-pressure LC methods. The development of microparticulate, chemically bonded supports added a new dimension. Reverse-phase supports were used to achieve separations not easily obtained by ion exchange, or normal adsorption, or partition chromatography. HPLC has expanded to include ion exchange, size exclusion, affinity, immunoaffinity, ion, and chiral chromatography. Besides the normally used silica, supports include other oxides, carbon, polymeric resins, hydroxyapatite beads, agarose, and chiral phases. Packings of various particle and pore sizes are available, and columns (stainless steel, glass-lined stainless steel, plastic cartridges) come in various sizes. Detection is still not as reliable and sensitive as one might wish. Photodiode array (uninterrupted acquisition of UV-visible spectra) is an extension and improvement on the early UV-visible detector systems. The refractive index (RI) detection method is not very sensitive and is subject to variations when using gradient elution.

The so-called universal HPLC detection method, able to detect a wide range of different compounds, was developed in the late 1980s. This method, ELSD, involves transforming the eluent from the HPLC column into a fine spray, evaporating the spray droplets, and then detecting the resultant aerosol particles by light scattering. This allows detection of compounds that do not possess chromophores and, unlike many other detection methods, is compatible with gradient HPLC. However, ELSD often generates very different responses for compounds of the same molecular weight and makes quantitation difficult.

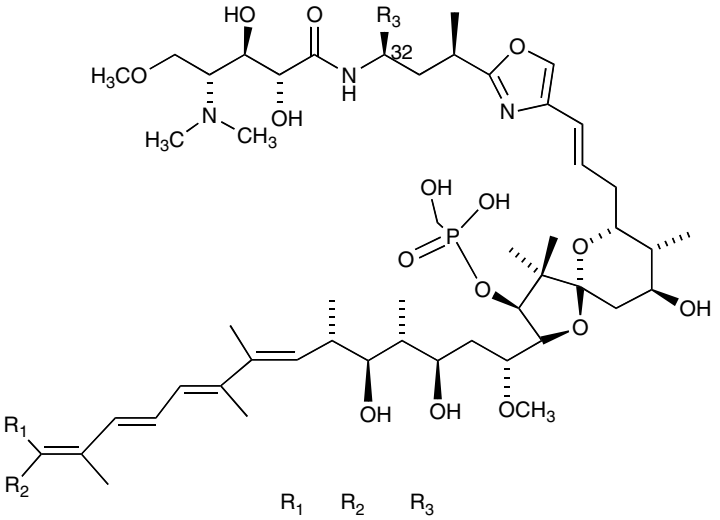
A more sensitive version of ELSD, called aerosol charge detection (ACD), has been developed.[230] In the ACD method, the aerosol particles are given an electrical charge by passing them close to a stream of charged nitrogen. The charged aerosol particles are then detected by an electrometer, which generates a signal in direct proportion to the quantity of each particle.[230]

The advantages of HPLC include ruggedness, versatility, and separating power; particularly for hydrophilic, thermolabile compounds. The major disadvantage is the capital and maintenance costs. The excellent contributions from the *Journal of Chromatography Library*, volume 43[164] and 69B,[205] contain several chapters illustrating the application of HPLC techniques.

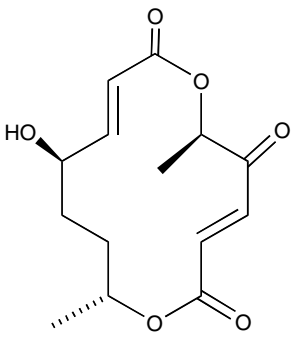
2.4.3 Separation of Similar Compounds

In the field of bioactive metabolites, HPLC almost always represents the final separation and purity determination step. A simple example is illustrated by the isolation and purification of four pseudopterosins, the anti-inflammatory, and analgesic diterpene glycosides from the sea whip *Pseudopterogorgia elisabethae*.[231,232] The ethyl acetate and chloroform extracts of homogenized frozen animals were combined and reextracted with chloroform. Initial separation of the extract by VLC (40 g on 300 g silica) yielded three fractions (each 0.5 L) containing the diterpenes. The first obtained by elution with ethyl acetate–dichloromethane (1:9–3:7) was a mixture of **73** and **74**, the less polar monoacetate analogues. These were separated by HPLC (μ-Porasil; isooctane–ethyl acetate; 1:1). The other fractions contained **75** and **76** and were similarly separated and purified

with a more polar mixture of isooctane and ethyl acetate (15:85). Interestingly, the monoacetate (**75**) is more polar than **73** and **74**, presumably because one possible hydrogen bonding arrangement between the glycosidic oxygen and the C-2 oxygen in the D-xylose unit is not possible in **75**.



	R_1	R_2	R_3
77	CN	H	H
78	H	CN	H
79	CN	H	CH_3
80	H	CN	CH_3



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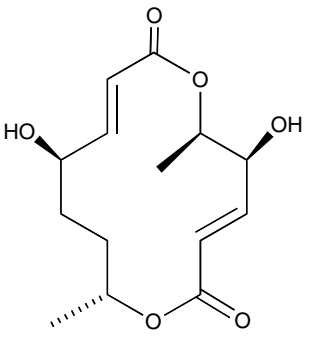


82

Another example involves the isolation of the calyculins, potent antitumor metabolites from the sponge *Discodermia calyx*.[233] The sponge extract showed strong activity in the starfish egg assay,[63,234] and so the fractionation was guided by the use of this assay. The frozen sponge (1 kg) was homogenized and extracted with ethanol (3 × 5 L). The extract was partitioned between dichloromethane and water, the activity being associated with the organic fraction. This fraction (2.2 g) was subjected to low-pressure LC on silica gel using dichloromethane–methanol as the eluent. The active fractions thus obtained were purified by reverse-phase HPLC (octadecylsilane) eluting with methanol–water, 8:2. Four active substances were obtained (ca. 20–150 mg) and designated as calyculins A–D. The major compound, calyculin A, had potent antitumor activity[170] although it was highly toxic to mice also. The separation of these four compounds illustrates the resolution achievable by HPLC. Calyculin A (**77**) and B (**78**) differ only in the geometry of the terminal double bond, as do C (**79**) and D (**80**). Each pair is distinguished from the other by the presence or absence of a methyl group at C-32. These differences in the lipophilic portions of the molecules are sufficient to affect the degree of partitioning of each molecule into the lipophilic stationary phase.

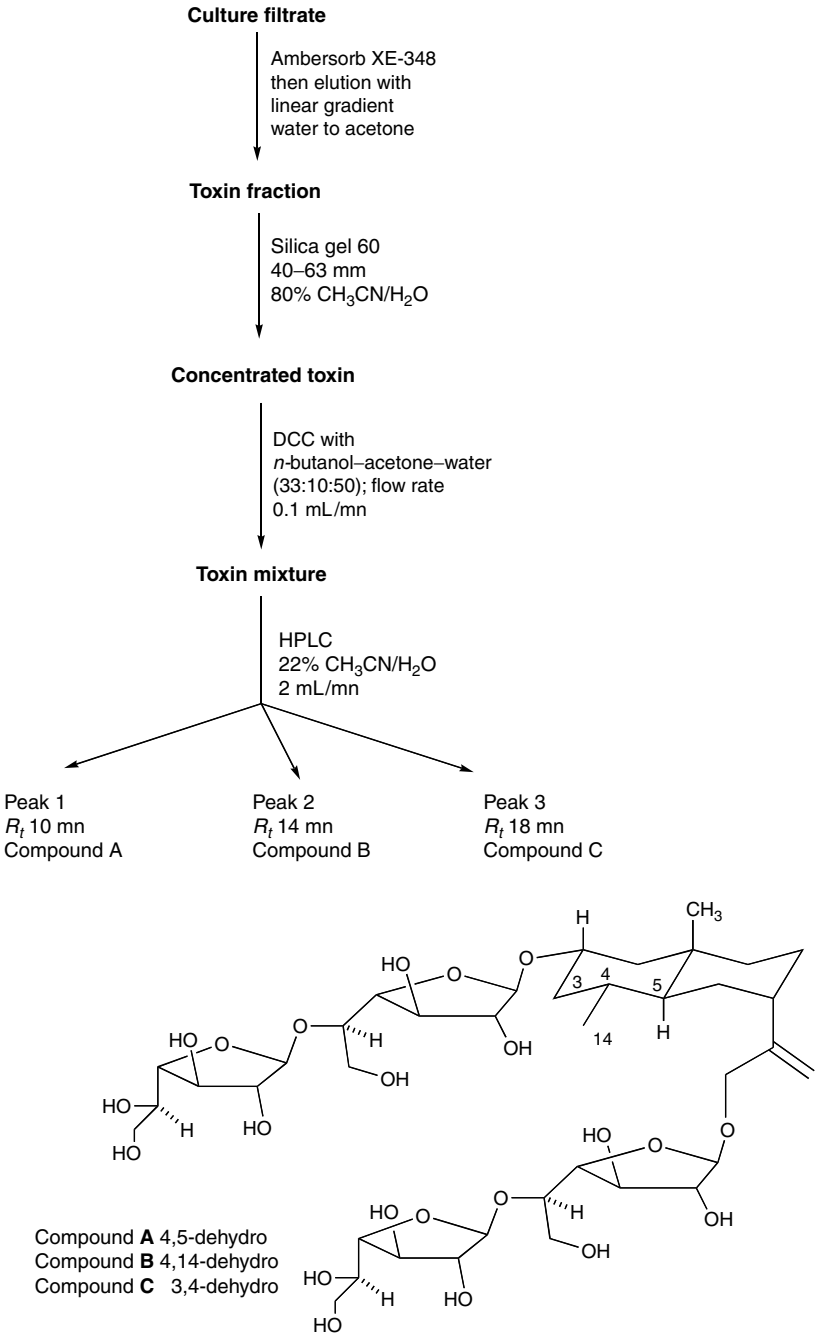


FIGURE 2.3 Isolation and separation of the phytotoxic metabolite from *Helminthosporium sacchari*.

The separation of the three phytotoxins produced by *Helminthosporium sacchari* also relied on reverse-phase HPLC (Figure 2.3).[235,236] The toxin mixture was concentrated from the liquid broth on Ambersorb, then by CC on silica gel and DCCC. The final separation by HPLC (μ-Bondapak C18) took into account the fact that the compounds differ only in the lipophilic portion of the molecule. Thus using a reverse-phase support ensures that this portion will be "recognized" by the lipophilic

C-18 ends of the support. Normal phase support would interact mainly with the hydrophilic portion of the molecule, which is identical in all three compounds.

2.4.4 Separation of Different Classes of Compounds

In most of the examples given so far, the focus has been on the isolation of one type of compound responsible for the bioactivity. Two cases are now considered where the fractionation of a biologically active extract led to the separation of different classes of compounds.

An extract of the microalga *Hymenomonas* sp. showed strong Ca-releasing activity in sarcoplasmic reticulum. The isolation and separation of the active component, hymenosulfate, was carried out as shown in Figure 2.4.[237] There are several features of interest in this separation scheme. Somewhat

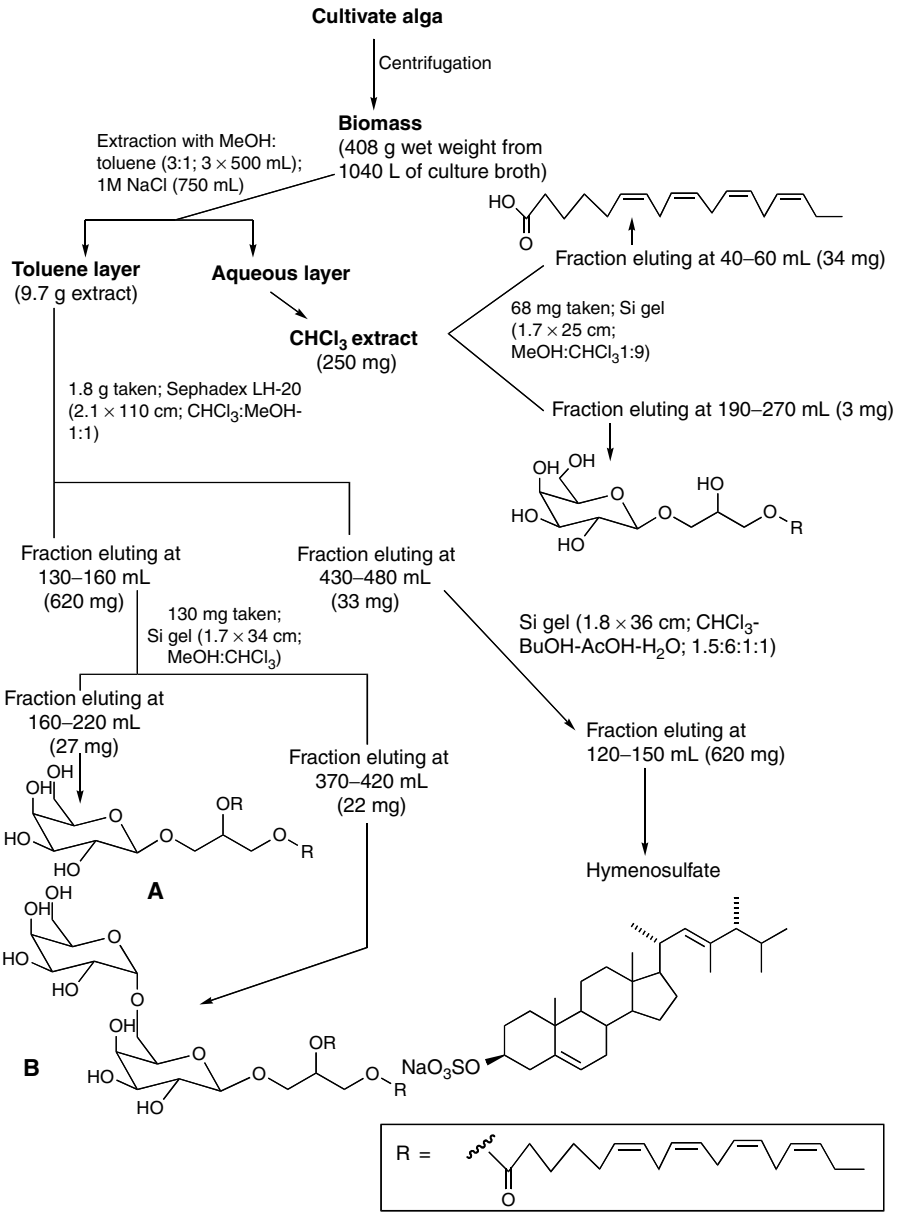


FIGURE 2.4 Isolation of metabolites from *Hymenomonas* sp. (haptophyte).

surprisingly, the sterol sulfate is initially extracted into toluene. Given the cooccurrence of the glycolipids A, B, and C, this might be a reflection of the detergent nature of these compounds, which may enhance the solubility of the sterol sulfate in toluene. Second, the fractionation step on Sephadex LH-20 neatly separates the glycolipid components from the sterol that is of lower molecular weight and polarity. The glycolipids A and B were separated by FC on silica gel using a polar solvent system. Also interesting is the isolation of the fatty acid D from what was initially the aqueous methanol layer. This may be due to its detergent-like character, but since it does not appear in the toluene extract, the more likely explanation is that it is, together with C, an artifact of the extraction and arises from hydrolysis of A.

The second example concerns the separation of the bioactive metabolites from *Annona bullata*, a tree native to Cuba, extracts from which showed cytotoxic and pesticidal activities. The bioassay-guided separation scheme is illustrated in Figure 2.5.[238] The ethanol extract of the bark was partitioned between chloroform and water, and highest activity was found to be located in the

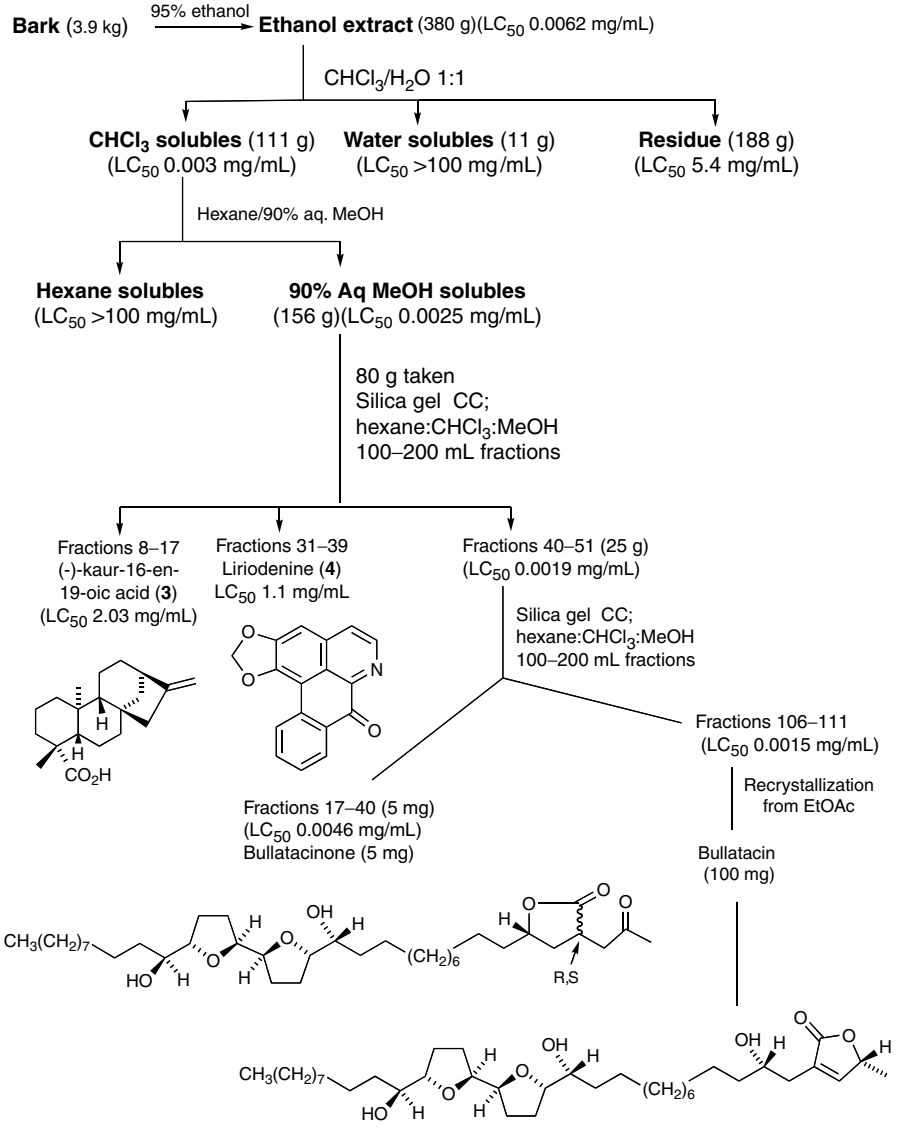


FIGURE 2.5 Isolation of bioactive metabolites from *Annona bullata*.

chloroform-soluble portion. This fraction was defatted by another partitioning process using hexane and aqueous methanol, and the bioactivity was found associated with the polar phase. Open CC of this fraction, monitored by TLC, separated the extract into three major fractions. The first yielded kaurenoic acid, a tetracyclic diterpene, and the second, liriodenine, a tertiary alkaloid. The less polar nature of the diterpene can be rationalized if the hindered nature of the axial carboxylic acid is recognized. The third fraction contained a mixture of the principal active acetogenins, which could be separated by further CC; the order of elution was as expected considering the fact that bullatacinone is a dihydroxy keto lactone whereas bullatacin is a trihydroxy lactone. All four metabolites isolated showed activity in the BSLT bioassay, but only the acetogenins had pronounced cytotoxic effects.

2.4.5 Other Chromatographic Techniques

There are many other techniques that cannot be covered because of space limitations. Ion-pair chromatography is a combination of ion exchange and adsorption chromatography employing HPLC. Although its major applications have been in analytical and bioanalytical chemistry, the adaptation of this technique to the separation of charged as well as neutral molecules has proven valuable in natural products chemistry.[239] Chiral separation methods are also attracting increasing attention.[239,240] These methods are important in phytochemical studies where conclusive information regarding the optical purity of particular metabolites is required. Affinity chromatography[241] and capillary zone electrophoresis[242] are techniques whose role may increase in the future.

2.5 MODERN STRATEGIES

2.5.1 High Throughput Screening

One of the major developments in the discovery of bioactive compounds has been the introduction of small-scale *in vitro* bioassays whereby many samples are evaluated in the same biological test for their effect on a protein or cellular process. This bioassay technique is called high throughput screening (HTS), by virtue of the fact that many samples can be tested in a short period of time. The move to increase throughput is also associated with a reduction in size of test volumes, from milliliters to microliters (even nanoliters with miniaturization), and high-density microwell plates (1536-well) with a 1–2 μL capacity are used. Processes are automated to make them less labor-intensive, more reproducible, and less expensive; and many routine tasks, such as pipetting, assay reading, sample storage, and dispensing are performed by robots. In essence, this method operates on detecting a specialized effect of a test compound or extract on receptors or enzymes (single-target specific bioassay) or on intact cells, isolated organs, whole animals (multitarget functional bioassay). High throughput bioassays are used to test large numbers of extracts obtained from collections of living organisms and to monitor the fractions generated from chromatographic separation steps deployed in the isolation of bioactive components. High throughput technology needs a large number of samples, and pharmaceutical companies make use of chemical libraries generated by combinatorial chemistry, combinatorial biosynthesis, engineering of biosynthetic pathways, biotransformation, elicitation of plant cultures, and induction of microbial secondary metabolism.[243]

The following example is given to illustrate the logic of the HTS technique, albeit in a simplified and shortened form.[244] A marine algae-derived fungus, identified as a *Gliocladium* sp., gave an extract that showed strong cytotoxic and antibacterial activity. A portion (100 μg) of the extract was separated by HPLC with photododiode array detection (DAD). The eluent from the DAD was split into a 1:10 ratio between the evaporative light scattering detector (ELSD) and the fraction collector, with the eluent being collected over 22 min into a 96-well microtitre "master" plate (88 × 0.25 mL fractions; 2.5–24.5 min). A daughter plate was prepared (5 μL dispensed from each well of the master plate; equivalent to a total 2 μg of extract), the solvent was evaporated, each well was inoculated

with P388 cells (murine leukemia), and the plate was incubated for 3 days. The bioactivity across the plate was revealed by addition of yellow MTT solution to each well. Living cells convert this to the purple formazan. The "bioactivity chromatogram" generated was compared to the DAD and ELSD chromatograms. Bioactivity was confined to fractions that eluted between 11 and 13.5 min, whereas the DAD and ELSD chromatograms showed essentially three peaks at 10.8 (A), 12.5 (B), and 15.7 min (C).

An "antibacterial chromatogram" using *B. subtilis* was produced in a similar way, with the exception that 500 μg of extract was required to generate the master plate so that an appropriate response was obtained.[244] This chromatogram clearly showed that antibacterial activity was associated with cytotoxicity and that the bioactive compound eluted at 12.5 min. The three compounds were separated by semipreparative HPLC and the structures deduced by NMR and MS analysis. The bioactive metabolite (B) was assigned structure **81**. Compound A was the dihydro analogue (**82**) whereas C was a new cyclodepsipeptide. Many other biological chromatograms can be obtained using different assays. A more sophisticated approach that involves the production and analysis of natural products libraries has been described, for example, preparation of a library from the bark of *Taxus brevifolia*.[245]

In the past, one of the difficulties in the detection of bioactive compounds was the lack of bioassays to screen for the activity of interest. Recombinant DNA technologies have facilitated the development of cell-based bioassays. Eukaryotic cells can be engineered to produce a specific gene product in response to a stimulus. If the gene product itself has activity that can be monitored this, in essence, is reporting the presence of the stimulus. Reporter genes are frequently used as indicators of transcriptional activity or activation of particular signaling pathways within the cell. In a recent review, the different types of inter and extracellular reporter gene products and their application in bioassays of natural products have been discussed.[246]

Validation of preliminary hits by mammalian animal models is slow and expensive. In recent years, the use of the zebrafish as a vertebrate model organism has been found to overcome these problems.[247–249] Zebrafish-based assays combine the advantage of HTS assays, compared to mammalian models, and greater relevance to humans. They can be performed in the same way as HTS cell-based assays. Zebrafish (*Danio rerio*) is a small freshwater teleost (fish with bony skeleton) that is easy to maintain and breed. The embryo develops externally, is transparent, and small. Testing for bioactivity is simple. Embryos are raised for 5 days in individual wells of a 96-well plate in 100 μL of "fish" water and test compounds, dissolved in "fish" water, diffuse into the embryo. Zebrafish have been used to study the toxic effects of environmental pollutants, toxicity of drug candidates, effects of xenobiotics, toxicity to organs, detection of compounds with antiangiogenic activity, and compounds that modulate apoptosis. Apoptosis can easily be detected in zebrafish embryos using fluorescent labeling techniques with acridine orange and fluorescence-conjugated caspase substrate.

2.5.2 Dereplication

When searching for bioactive metabolites and new lead compounds, the usual procedure involves biological screening followed by bioassay-guided isolation. However, this often leads to the isolation of known or undesirable metabolites. The process of identifying known compounds responsible for the activity of an extract prior to bioassay-guided isolation is referred to as *dereplication*. This can mean either full identification of a compound after only partial purification, or partial identification to the level of a class of compounds. Full identification in these cases relies on comparison with a characterized standard. Partial identification serves to (a) identify undesirable compounds, such as tannins, polyphenols, and fatty acids, (b) to prioritize samples for extraction, and (c) to gather information on the type of compound to facilitate subsequent isolation. Dereplication strategies generally involve a combination of bioassay, separation science, spectroscopic methods, and database searching and can be regarded as chemical or biological screening processes.

2.5.2.1 Biological Screening

In bioassay-guided fractionation, fractions from an HPLC separation of an active extract are collected in microtitre plates, the HPLC solvent is removed and replaced by a compatible solvent (water, buffer, DMSO), and then each fraction is assayed. This is time- and labor-intensive and not cost effective. To overcome this potential bottleneck, a system in which the bioactivity is measured in the HPLC effluent stream and the bioactive compounds are chemically characterized online would be more efficient. Such systems have recently been described for a range of targets, for example, the human estrogen receptor, the urokinase receptor, acetylcholinesterase, and phosphodiesterase. In essence, the extract is injected into the HPLC, and the compounds, on elution, are passed into a closed continuous flow reaction detection system where the bioactivity of individual compounds is measured.

As an example, the online bioassay to screen extracts for binding to the estrogen receptor (ER), α and β, is considered.[250] The extract is injected into the HPLC, the ER is added to the effluent, and the mixture is allowed to interact (30 s). In the second step, the remaining free sites on the ER are saturated with a fluorescent ligand, for example, coumestrol. The phytoestrogen coumestrol has a high affinity for the ER and exhibits fluorescence with a maximum at 438 nm when excited at 340 nm. The emission fluorescence of the bound coumestrol is shifted to 410 nm and has an intensity about four times higher than that of coumestrol. Thus, the presence of estrogenic compounds is detected by the reduced intensity at 410 nm for the ER–coumestrol complex. This approach is applicable to a great number of enzyme targets, for example, kinases, phosphatases, phosphodiesterases, angiotensin-converting enzymes, and caspases.

This method can be extended to include chemical screening by introducing a splitter after the analytical column and directing part of the effluent from the LC to a mass spectrometer. In this way, biochemical responses are rapidly correlated to MS and MS/MS data.

2.5.2.2 Chemical Screening

In chemical screening, a technique as simple as TLC can be used, but these days the preference is for HPLC linked to different detectors (hyphenated techniques) to be employed. Instruments with HPLC coupled to a UV diode array (LC-UV) have been available for some time and have been employed in dereplication. The high-resolution separation achievable in LC combined with fast UV diode array detectors allows the UV spectra of practically every compound in a mixture to be acquired. Although most organic chemists make limited use of UV parameters in structural elucidation, some classes of compounds such as polyketides and alkaloids have characteristic spectra. For example, an initial UV-based library was compiled for dereplication of 187 mycotoxins. This has been extended to 474 fungal metabolites and has been enhanced by the inclusion of mass spectral parameters.[13]

The combination of LC-MS was the next major advance in chemical screening. Interfacing HPLC with MS provides one of the most sensitive methods of molecular analysis. It allows the molecular weight of a compound to be determined, which, if measured at high resolution, can lead to a unique molecular formula. Importantly, it can also provide structural information for the compounds being analyzed. There are a number of different LC-MS interfaces available: thermospray (TSP), electrospray ionization (ESI), continuous flow fast atom bombardment (CF-FAB), and atmospheric pressure chemical ionization (APCI). TSP and APCI allow ionization of moderately polar compounds up to a mass of 800 Da. Larger polar compounds such as glycosides are best analyzed using CF-FAB or ESI. In particular the deployment of ESIMS provided an excellent tool for the study of natural products. ESI is a soft ionization technique that can be optimized to produce, in the positive mode, protonated or sodiated ions from a broad range of metabolites. Although all these methods lead to soft ionization of the constituents of an extract, some fragmentation occurs. The origin and fate of the ions produced can be tracked by tandem MS/MS or by multiple stage MS/MS in ion trap systems. This can provide information that is of significance in determining the structures of the constituents.

Although nuclear magnetic resonance spectroscopy is less sensitive than MS, it provides a powerful complementary technique for identification and quantitative analysis of metabolites in an extract. Metabolite fingerprinting using LC-NMR is a fast, convenient, and effective tool for distinguishing among groups of related compounds. Thus, using the array of hyphenated techniques, it is possible to dereplicate single metabolites or a mixture of metabolites. It is now possible to determine, with high probability, the structure of a compound without the need for isolation.

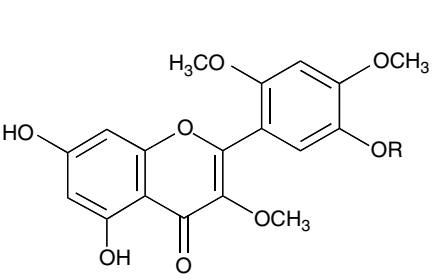
An example that serves to illustrate many of the techniques mentioned is presented.[251] It has been selected because the compounds involved are structurally simple and not too distracting. *Blumea gariepina* is an African plant that was suspected of containing neurotoxins. Closer examination failed to reveal such toxicity in the extracts, but the dichloromethane extract of the aerial parts of the plant showed marked toxicity against the pathogenic fungus *Cladosporium cucumerinum* and significant acetylcholinesterase inhibition. This extract was chosen to evaluate a strategy for chemical and biological screening. For chemical screening, LC was coupled with UV/DAD, MS, and NMR independently. The LC-UV/DAD system had postcolumn addition of UV shift reagents. The LC-MS coupling included an APCIMS^n arrangement. The extract was submitted to two standard TLC bioautography tests for a rapid assessment of the biological activity. The antifungal test showed two spots of fungal growth inhibition against *C. cucumerinum*, and the acetylcholinesterase test showed three spots of activity.

A combined LC-UV/DAD and positive ion APCIMS^n analysis was carried out. Eight major peaks were observed whose UV spectra indicated two chromophoric groups. Compounds A–C showed absorption maxima at 210, 260, and 353 nm and compounds E–H had maxima at 210 and 275 nm, suggesting the presence of flavonols and other phenolics, respectively. The LC-MS data showed that group A–C displayed protonated ions between 330 and 388 Da and the second group at 150–222 Da. Fragment ions arising from the loss of 15, 31, and 42 Da in the LC-APCIMS^n indicated the presence of methyl, methoxyl, and acetate groups.

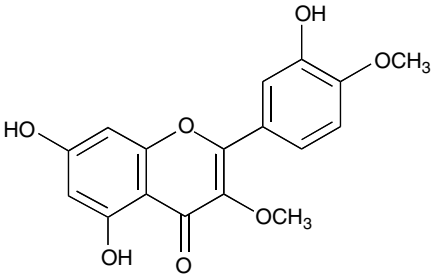
The next step was to obtain the ^{1}H NMR spectrum of each compound after which the sample would be collected for biochemical screening. To facilitate this, a high loading for the LC separation was required, and so a C18 radial compression column was selected instead of an analytical column. A loading of 10 mg of extract was injected onto the LC, and satisfactory resolution was obtained at 1 mL/min flow rate. During this analysis, fractions (1 mL) were collected every minute for 70 min, and these were used for the bioautography. The fractions eluting between 18 and 40 min displayed notable activity. Analysis of the data obtained by LC-APCIMS^n and LC-NMR measurement indicated that compound A was 5,7,2′,5′-tetrahydroxy-3,4′-dimethoxyflavone (**83**). Assignment of the structure of B required isolation of this material. Subsequent MS and NMR data showed it to be 5′-acetoxy-5,7,2′-trihydroxy-3,4′-dimethoxyflavone (**84**). Compound C was 3′,5,7-trihydroxy-3,4′-dimethoxyflavone (**85**). Compound D could not be identified from online data and attempts to isolate it failed. A tentative structure (**86**) was suggested. Compound E was 2-isopropyl-4-hydroxy-5-methylphenylacetate (**87**), F was 2-isopropyl-3-hydroxy-5-methylphenylacetate (**88**), G was thymol (**89**), and H was acetyl thymol (**90**).

2.5.2.3 New Approach to Natural Products Discovery

Extraction of a biological sample is normally the first step in the search for bioactive natural products. Natural products chemists are usually aware of the potential problems that this can create. The processes of sampling, homogenization, lyophilization, and extraction with organic solvents can all affect the nature and relative amounts of metabolites present *in vivo*. Moreover, extraction and chromatography will discriminate against some classes of compounds and favor others. Because of these considerations, Meinwald and coworkers have adopted the approach of direct NMR spectroscopic acquisition and analysis before subjecting the extract to chromatography.[252] Apart from providing a "high-fidelity" snapshot of the constituents in the extract, the information acquired from 1D- and 2D-NMR spectra may be sufficient to identify all of the compounds of interest.



83 R = H
84 R = Ac



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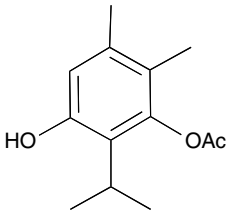

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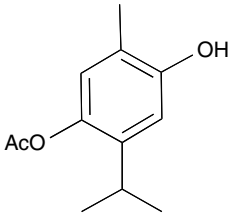

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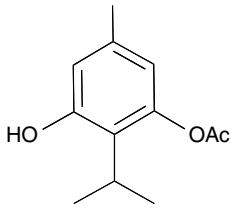

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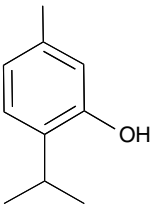

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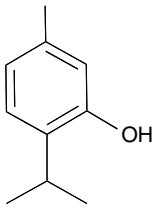

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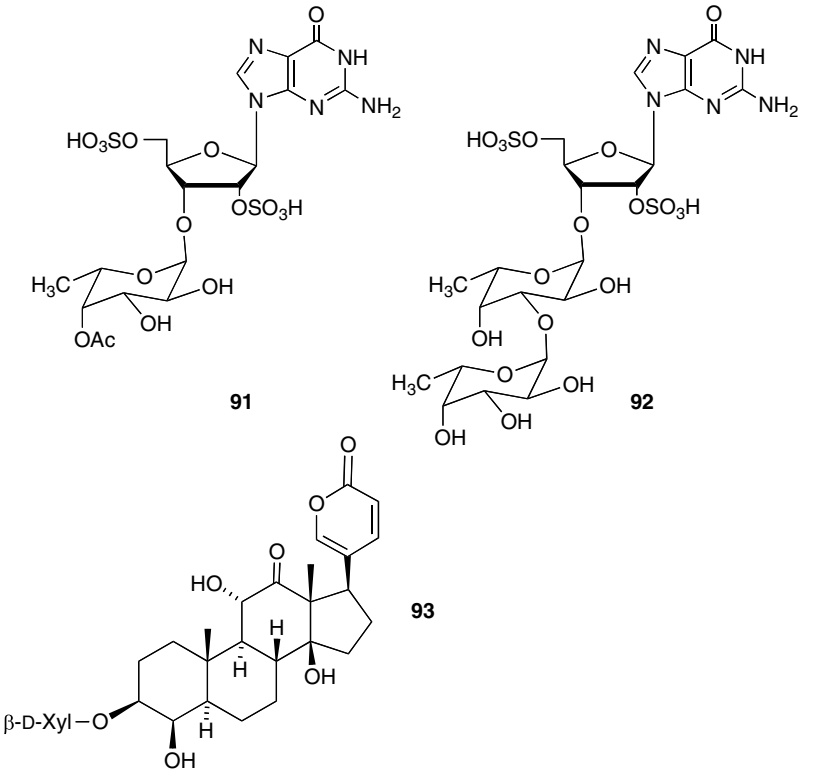
At least, it will provide information to allow rational decisions to be made about the best method of fractionation. The NMR data of the metabolites subsequently isolated can be checked against that in the original "snapshot" to determine if any component has been lost or altered.

This approach has been illustrated in a project aimed at the characterization of the components in the venom of the hobo spider, *Tegenaria agrestis*.[252] Spider venoms show diverse bioactivity including blocking the neuronal nicotinic acetylcholine receptor, increasing the secretion of parathyroid hormone, and inhibiting atrial fibrillation. Previous work had resulted in the discovery of the unique, disulfated glyconucleoside (**91**). Surprisingly, no other example of this type of compound had been found in other spider venoms. Venom from *T. agrestis* was obtained through electrostimulation of the venom gland. Acquisition of NMR spectra of the crude venom and analysis of the results allowed the identification of four compounds, including **92**, and tentative assignment of structure to three other compounds. In retrospect, it appears that compounds such as **91** are relatively unstable and, moreover, they are not very efficiently ionized under electrospray conditions.

In an extension of this approach, the applicability of a capillary NMR probe (CapNMR) for the analysis of mass-limited biological samples was investigated.[253] The CapNMR probe features a very small flow cell (5 μL) with an active volume of 3 μL (1.5 μL is also possible). Essentially, this means that a low molecular weight metabolite can be measured at below the microgram level.[254,255]

Using this technique, Gronquist et al.[253] investigated the metabolites present in the blood of the firefly, *Lucidota agra*. Certain nocturnal fireflies are known to produce steroidal pyrones as a defense against predators. *L. agra* is a diurnal firefly, and it was of some interest to investigate the nature of its defensive arsenal. To this end, the hemolymph of five fireflies was obtained, and ^{1}H-NMR spectroscopy revealed the presence of approximately 10 steroidal pyrones. The NMR spectra showed considerable overlap of signals, thus necessitating partial HPLC fractionation.



The whole-body extract (dichloromethane:methanol; 1:1) of 50 fireflies was partly separated by HPLC into 11 fractions. Attempts to take advantage of the enhanced sensitivity of a cryoprobe were not encouraging, largely due to the poor line shape obtained in the dfqCOSY spectra and the poor sensitivity in the HMBC experiment. Use of the CapNMR probe overcame these obstacles and analysis of the 1D and 2D (dfqCOSY, NOESY, HMBC, and HMQC) spectra allowed the structures of 13 new steroid pyrones (e.g., **93**) to be proposed.

These applications amply demonstrate the power of the NMR techniques and the interpretative skills of experienced natural products chemists. However, in all cases mentioned, the structures proposed should be regarded as working hypotheses. Confirmation of the structures must await chemical studies or syntheses.

2.6 ARTIFACTS

2.6.1 Artifacts from Extraction

In the isolation of bioactive molecules, it is essential to extract under the mildest possible conditions. In certain cases, this might involve carrying out the extraction under an inert atmosphere, as for the isolation of tunichrome B-1, the reducing blood pigment from the tunicate *Ascidia nigra* (Figure 2.6). Tunichromes are extremely sensitive to air and water, and consequently the blood from the animals was collected in test tubes under a current of dry, oxygen-free argon in the presence of *tert*-butylhydroxyphenyl sulfide or *tert*-butyl sulfide as an antioxidant.[256,257]

Some algal metabolites particularly those containing *bis*-enol acetate (e.g., **94**) moieties and polyhalogenated metabolites (e.g., **95**) from green algae are very unstable and difficult to isolate. The widely different melting points and optical rotations quoted for samples of what is apparently the same compound leaves room for suspicion. Freshly collected material either frozen or stored in solvent for long periods of time may decompose.[258]

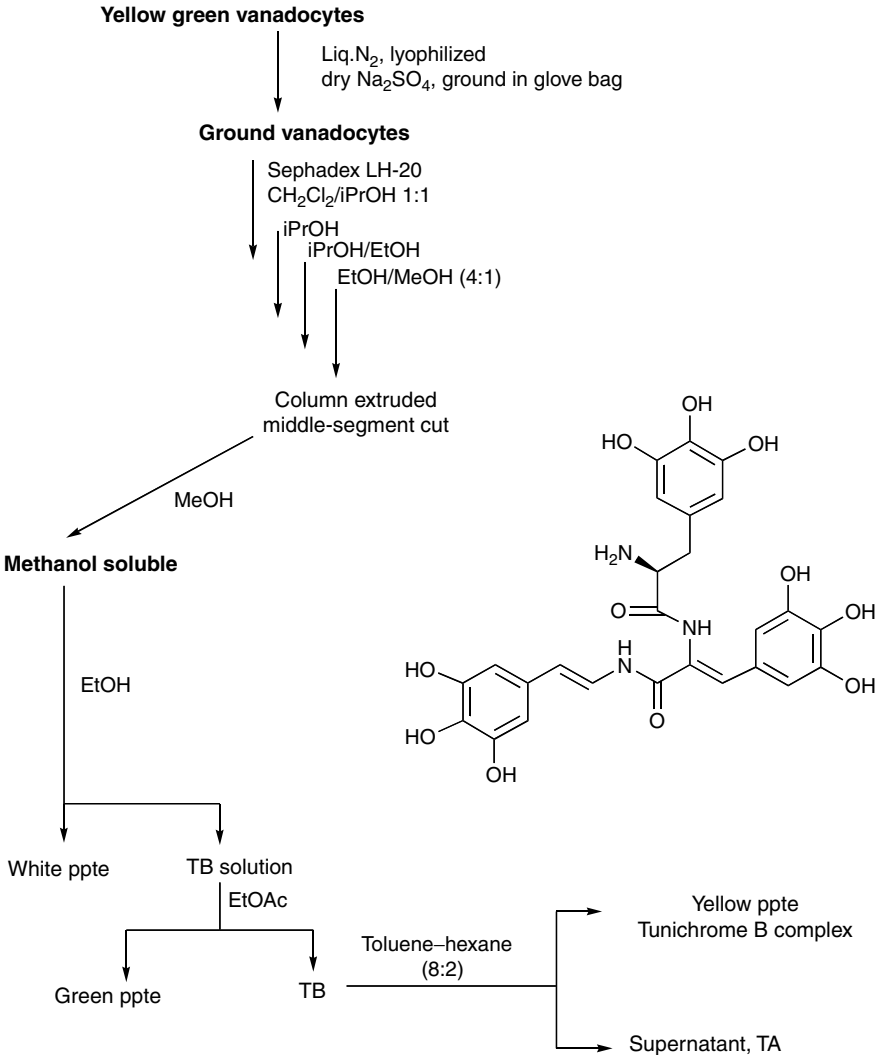
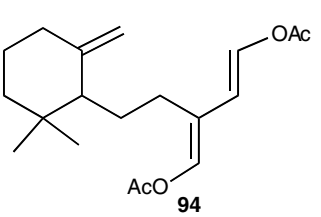


FIGURE 2.6 Isolation of tunichrome B complex from *Ascidia nigra* (the structure of one of the components is shown).

Solutions of organic solvents are normally evaporated under vacuum. Aqueous solutions of an extract are better concentrated by adsorption (Section 2.4.2) for large volumes or with the cartridge method[259] for small volumes. Freeze drying (lyophilization) is a mild concentration technique and is useful in those cases where extracts contain inorganic salts and acids, which could catalyze reactions if the temperature is allowed to increase. As an example, weak bases such as sodium acetate and ammonia initiate epimerization at C-8′ in the strained enolizable molecules of the podophyllotoxin group (**96**) forming mixtures, which contain as little as 1% of the original bioactive compound.[260] It has been suggested that this might occur even in saline preparations.[261] Weak acids can also induce formation of artifacts. The dehydration of **97–98** occurs on wetting a chloroform solution of **97** with dilute hydrochloric acid, or on contact with neutral alumina or silica gel.[262,263]

Solvents often appear as the cause of problem in the formation of artifacts. Eleutherobin (**99**) is a metabolite of a soft coral, *Eleutherobia* spp., collected off the coast of Western Australia. It shows taxol-like activity as a microtubule-stabilizing antimitotic agent, but is 50 times more potent than taxol against a range of *in vitro* cell lines.[264]



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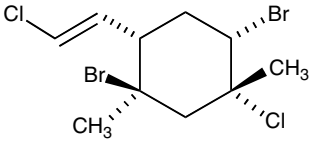

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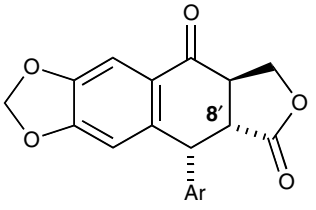

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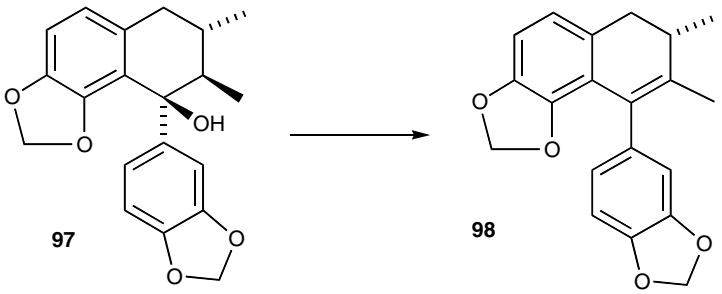
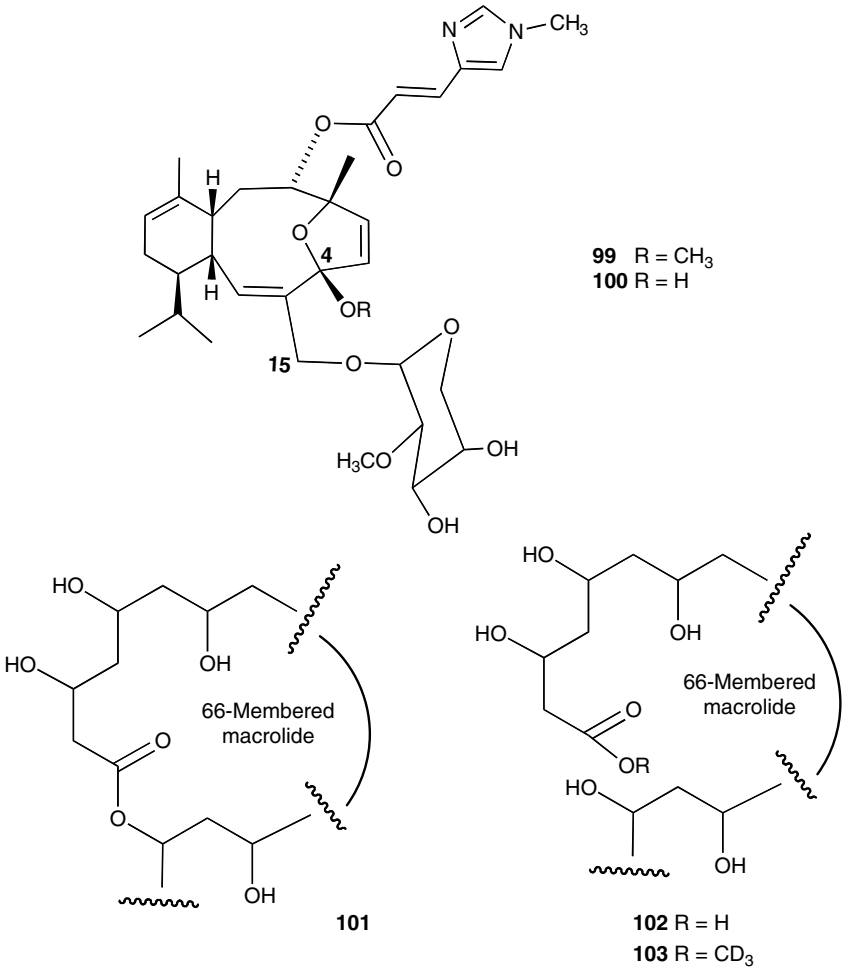


It has been shown that eleutherobin (**99**) is an artifact arising from the C-4 hemiketal (**100**) on exposure to methanol. If methanol is excluded from the extraction, the hemiketal is obtained, whereas use of ethanol leads to the C-4 ethylketal. Interestingly, it appears that formation of the methyl ketal occurs when a methyl, hydroxymethyl, or a glycosidic functionality is present at C-15, but not if an ester is present at this position.

Zooxanthellamide Cs are vasoconstrictive, polyhydroxylated macrolides from a marine dinoflagellate, *Symbiodinium* sp.[265] Three of these metabolites display lactones containing 63-, 64-, and 66-membered rings (partial structure **101**), the largest found so far among the natural macrolides. Although they can be extracted and isolated by HPLC, they are unstable in D_2O or CD_3OH and collapse to the carboxylic acid (**102**) and the trideuterated methyl ester (**103**), respectively.

The isolation of the bisbenzylisoquinoline alkaloids from *Cyclea peltata* by normal methods, involving partitioning of extracts with citric acid solution, basifying with ammonia followed by ion exchange, alumina, and silica gel chromatography, led to the formation of artifacts.[266] These arose from involvement of ethylene glycol and dichloromethane used in extraction steps (**104–106**). Fractionation of extracts rich in berberine (**20**) by partitioning ammoniacal solutions with chloroform leads to the formation of the adduct (**21**). On contact with acetone, (**21**) the adduct (**107**) is produced.[41] The formation of acetonides, on extraction of glycols with acetone, and of acetals, on extraction of ketones with methanol or ethanol, can occur. The β-addition of alcoholic solvents to conjugated systems (**108–109**) is also possible.[267] Condensation of aryl aldehydes with acetone, the extracting solvent, has been noted.[268] The dienedioic acid (**110**) was obtained as the diethyl ester after extraction of the bark of *Phebalium nudum* with ethanol.[269] The possibility of exchanging methoxyl groups of an acetal or carboxylic ester with ethoxyl group should not be discounted.[270,271] The exchange of a carboxylic methyl ester to form the amide (**111–112**) on contact with aqueous ammonia has been reported.[272] Tuliposide A (**113**), isolated from *Tulipa gesneriana* L., is unstable even at −20°C and

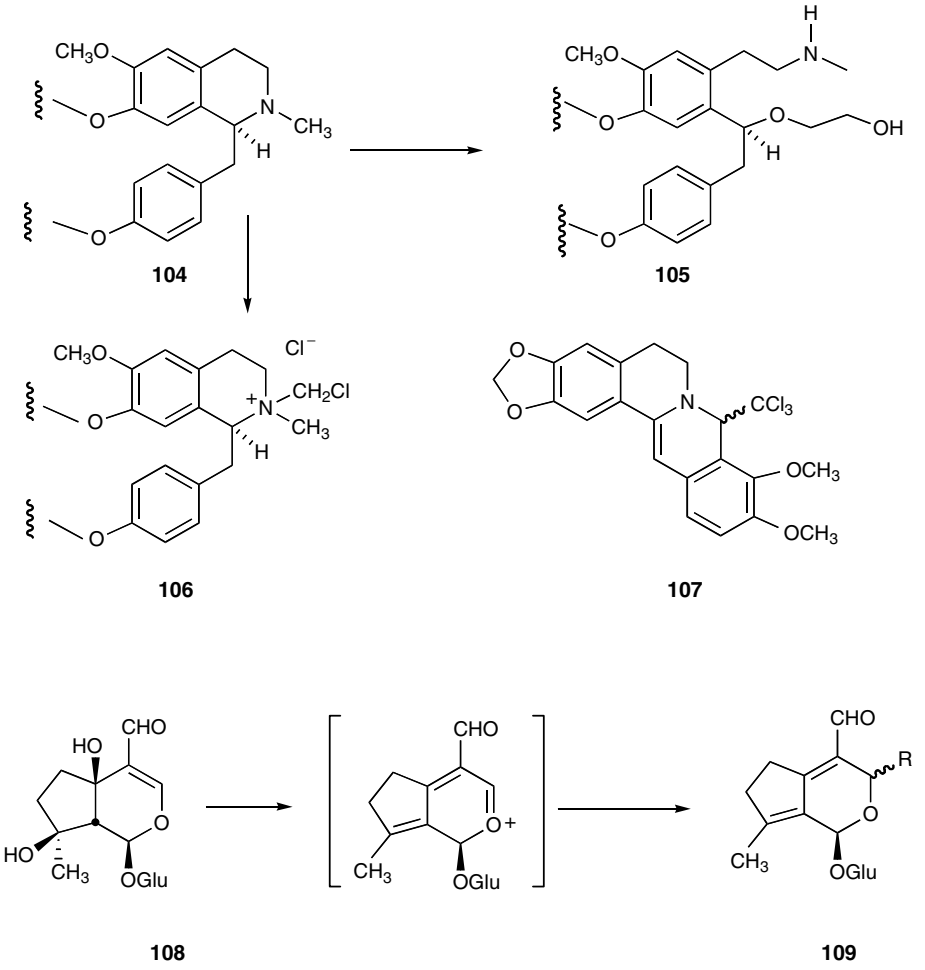


converts to the positional isomer (**112**). On contact with dilute acid, **113** is hydrolyzed to give the α-methylene lactone (**115**).[41] The observation that polygodial (**116**) is generated from the diacetate (**117**) on exposure to wet ether or silica gel[273] suggests that polygodial may be an artifact. The presence of free polygodial in the organisms would be unlikely, given the reactivity of the dialdehyde group.

2.6.2 Artifacts from Separations

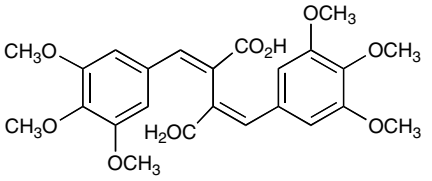
In an investigation of the metabolites produced by the salt water culture of a *Penicillium chrysogenum* strain, an unusual [3.2.1]bicyclooctadione (**118**) was obtained.[274] The presence of an ethoxy group, very rare in secondary metabolites, raised the possibility that **118** may be an artifact. Given the structure of the cooccurring metabolites, the most likely explanation was to assume that in the extraction step, in which dichloromethane, methanol, ethyl acetate, and petroleum ether were used, or in the HPLC separation step when acetonitrile was used as the eluent, a suitable precursor reacted with ethyl vinyl ether (see **119**). The origin of this contaminant remains a mystery.

Problems may also arise at the separation steps, particularly when active alumina is used.[275] A range of reactions such as aldolization, dehydration, hydration, hydride transfer, and skeletal rearrangements can occur.[276] The occurrence of *N*- and *O*-demethylation, oxidation, Hoffmann



β-elimination, rearrangements, and ring expansion when isoindolobenzazepine alkaloids are chromatographed on silica gel has been noted.[277] Crude extracts can survive relatively unchanged over a period of years, but, on separation, the individual components are often unstable to air, solvents, nonneutralized glass surfaces, and light. An example of the instability of a pure compound to light involves the retrochalcone tepanone. Exposure of solutions of tepanone, or its methoxy derivative, to ordinary laboratory light was found to lead to an equilibrium mixture of *E*- and *Z*-isomers.[278]

Any natural product chemist who has had to work on the milligram scale knows the frustration of having to exclude or remove plasticizers. They have become the most widely distributed isolatable "unnatural products". They appear to be present in solvents and chemicals, gases (from plastic tubing), plants (stored in plastic bags), and even in microorganisms (nutrients stored in plastic bags). Indeed, up to 1973, there had been 21 reports of the natural occurrence of phthalic acid and dialkyl phthalates,[279] and the presence of these and dimethyl terephthalates in algae has been reported.[280] Less obvious are a number of artifacts and contaminants that plague the analytical chemist and could fool the natural products chemist. For instance, in an investigation of the metabolites thought to be responsible for the plant-growth promotion activity of the coprophilous fungus, *Sordaria fimicola*, two major metabolites were isolated from this source.[281] The first was indole carboxaldehyde, a catabolic product of the auxin indoleacetic acid, the formation of which would explain the



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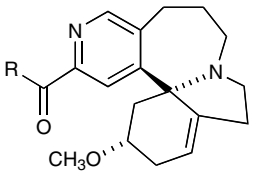
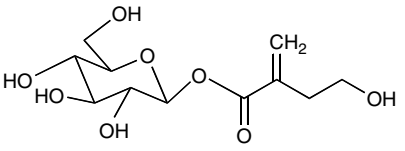


111 R = OCH_3
112 R = NH_2



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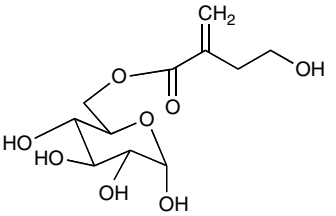

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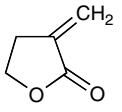

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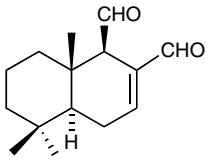

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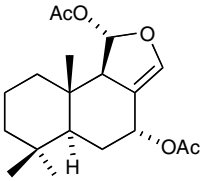

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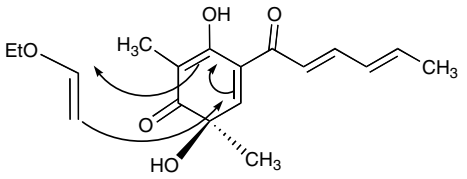

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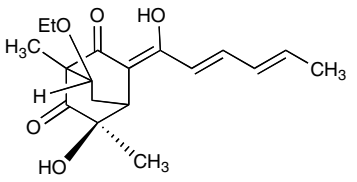

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activity observed. The second was triacontanol, a "metabolite", which most natural products chemists would prefer to ignore. However, triacontanol is a potent plant–growth stimulant and, to add a measure of uncertainty, it is a known contaminant of some filter papers. A useful compilation of such "nuisance" substances together with indications of their likely origin has been published.[282]

2.7 CONCLUDING REMARKS

In this review, an attempt has been made to present some of the more widely used methods in the detection and isolation of bioactive natural products. Examples of both simple and specialized bioassays, often deployed in tandem, have been considered. Much effort is being invested in developing rapid bioassays that allow monitoring single and multitarget effects. At the same time, analytical methods have been refined to achieve previously unimagined levels of resolution and sensitivity. Hyphenated techniques, for example, LC-DAD-MS, LC-DAD-NMR, can be arranged in series or in parallel for chemical screening and can be interfaced with continuous-flow biochemical detection. The structures and bioactivity profiles of many metabolites can be obtained without the need of their isolation. Herein lies the conundrum. It is important to remember that a structure deduced

from an analysis of its spectroscopic properties is useful as a working hypothesis. More evidence for the structure (or elimination of alternatives) is required and may involve comparison to standard compounds, chemical correlations, and synthesis. In this context, the words of Chesterton's *Father Brown*, "No machine can lie, nor can it tell the truth", are a sobering reminder. The isolation of sufficient quantities of a bioactive metabolite, so that its structure can be rigorously established and its range of activities adequately defined, remains a demanding and important task.

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3 Nuclear Magnetic Resonance Spectroscopy: Strategies for Structural Determination

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CONTENTS

3.1 INTRODUCTION

A glance at any recent chemistry journal immediately illustrates the reliance that modern chemistry places on nuclear magnetic resonance (NMR) spectroscopy as a quick and reliable method for the elucidation of molecular structures. NMR spectroscopy has been the single most important physical method for the determination of molecular structures for more than 40 years. The power of the technique lies not only in defining the numbers and types of nuclei present in an organic molecule, but also in describing their individual chemical environments and, more importantly, the way they are interconnected. Driven by its potential to determine the structures of organic compounds, NMR spectroscopy has seen substantial development in the six decades since the first experiments. In particular, the implementation of the pulsed Fourier transform (FT) method[1] and, subsequently, the concept of multidimensional experiments[2] provided the seeds for vibrant growth. There are currently hundreds of multipulse experiments available to the NMR spectroscopist. However, only a small proportion of these procedures are regularly employed for the solution of molecular structures. The most useful experiments have been the subject of numerous reviews.[3–15]

This chapter aims at introducing NMR spectroscopy to the reader who is unfamiliar with the technique. Within the context of the multidisciplinary nature of bioactive natural product research, the chapter will briefly review the most utilized NMR experiments with the aim of highlighting the types of information that each can provide. Theoretical and experimental details of each procedure will be kept to a minimum with references to the literature for readers who require further information. Section 3.2, introduces the essential concepts. Subsequently, the most useful NMR techniques will be discussed approximately in the order that they would be applied to solve the structure of an unknown compound. Sample spectra have been chosen with the aim of clearly demonstrating each technique without the need for detailed argument.

3.2 BASIC PRINCIPLES OF NMR SPECTROSCOPY

Descriptions of the fundamental aspects of NMR spectroscopy can be found, in varying degrees of detail, in most of the numerous NMR books available. However, those published before 1980 may not describe all of the concepts necessary for the understanding of modern pulsed techniques. These principles have been covered in concise,[16,17] more detailed,[18–22] and comprehensive form.[23–27] A book, directed at the organic chemist, which describes modern spectroscopic methods is recommended.[5]

3.2.1 The Conditions Necessary for Resonance

When placed in a magnetic field, nuclei that have a nonzero spin quantum number (I) are able to absorb energy from the radio frequency range of the electromagnetic spectrum. The frequency (v_0) at which a particular nucleus absorbs energy is dependent on the type of nucleus concerned (characterized by its gyromagnetic ratio, γ) and the strength of the magnetic field (B_0) into which it is placed. This relationship is described mathematically by the Lamor equation (Equation 3.1).

$$v_0 = \frac{\gamma B_0}{2\pi} \tag{3.1}$$

In the study of natural compounds using NMR spectroscopy, the nuclei of prime concern are ^{1}H and ^{13}C, both of which have a spin quantum number of ½. It should be noted that both of the isotopes ^{12}C and ^{16}O have $I = 0$, and therefore do not give an NMR signal. The magnetic properties of some nuclei that have a spin quantum number of ½ are shown in Table 3.1. In a magnetic field, the

TABLE 3.1
Properties of Some Nuclei with $I = 1/2$

Isotope	Natural Abundance (%)	NMR Frequency (MHz)		Relative Sensitivity[a,b]
		At 4.70 T	At 11.74 T	
^{1}H	99.98	200.00	500.00	1.0
^{13}C	1.11	50.29	125.72	1.59×10^{-2}
^{15}N	0.37	20.26	50.66	1.04×10^{-3}
^{31}P	100.00	80.96	202.40	6.63×10^{-2}

[a] For an equal number of nuclei at constant field.

[b] The expected relative strength of an NMR signal can be obtained from the product of the relative sensitivity and the natural abundance.

nuclei of an isotope with $I = ½$ can occupy either of two energy states. Nuclei in the lower energy state precess about the direction of the magnetic field whereas those in the higher energy state have the opposite orientation. The relative populations of the two states are described by the Boltzmann distribution, which results in only an extremely small population excess in the lower energy state. This small population excess gives rise to the net absorption of energy when the nuclei are irradiated with the correct radio frequency. The absorbed energy is subsequently lost to the surroundings (the lattice) over a period of time. This process is termed relaxation and is characterized by the spin–lattice relaxation time (T_1). The spin–lattice relaxation times of protons (^{1}H nuclei) are usually of the order of seconds whereas those of ^{13}C nuclei may be tens of seconds. A second relaxation mechanism (spin–spin relaxation) involves the exchange of energy between nuclear spins and has an associated spin–spin relaxation time (T_2). The magnitudes of the relaxation times of the nuclei in a particular sample must be considered in the preparation for each NMR experiment.

3.2.2 The Chemical Shift

Soon after the first observation of nuclear magnetic resonance, it became clear that the specific environment of a nucleus slightly modifies the magnetic field it experiences.[28] The nuclei are shielded from the applied magnetic field to differing extents depending on the electron density about each nucleus. Thus the frequency at which a nucleus is able to absorb energy is characteristic of the environment of the particular nucleus and it is this effect, that is, the chemical shift, together with the phenomenon of spin–spin coupling (Section 3.2.3) that makes nuclear magnetic resonance spectroscopy so useful for structural determinations. The chemical shift (δ), expressed in parts per million (ppm), is defined as[29,30]

$$\delta\ (\text{ppm}) = \frac{10^6(\upsilon_{\text{sample}} - \upsilon_{\text{reference}})}{\upsilon_{\text{reference}}} \tag{3.2}$$

The chemical shift is thus independent of the magnetic field strength of the spectrometer used to record a spectrum. Tetramethylsilane (TMS) is used as the reference for both ^{1}H and ^{13}C spectra and is assigned the chemical shift of 0.00 ppm. In the NMR spectra of natural products, most signals for the ^{1}H nuclei occur within a range of 10 ppm whereas the ^{13}C nuclei resonate over a range of 250 ppm.

3.2.3 Spin–Spin Coupling

On examination of a typical ^{1}H spectrum, it is immediately obvious that most of the resonances do not appear as single lines. Many of the signals are split into multiplets due to the effect of neighbouring nuclei. This phenomenon, which is known as spin–spin coupling, occurs via the bonding

electrons and thus provides information about the interconnection of coupled nuclei. In the simplest case, spin–spin coupling between two chemically different nuclei (each with spin ½) results in the resonance of each being split into two lines, that is, a doublet (d). The separation between the two lines is the same for each resonance and is known as the coupling constant (J), which is expressed in hertz. The magnitude of J depends on both the nature and number of bonds involved and the angular relationship of the coupled nuclei, but is independent of the magnetic field strength at which the spectrum is recorded. Each signal may show coupling to several nuclei. In documentation, J may be preceded by a superscripted number, indicating the number of bonds through which the nuclei are coupled, and followed by a subscript indicating the nuclei involved, for example, $^{2}J_{CH}$. The magnitude of three-bond coupling constants is dependant on the dihedral angle between the coupled nuclei and thus provides valuable stereochemical information.[31,32] Coupling constants over four or more bonds are often too small to be observed. However, the appearance of such long-range couplings is significant as it is usually indicative of particular stereochemical arrangements of atoms. There are many simple descriptions of the mechanism of spin–spin coupling and the use of the magnitude of the coupling constant to define the stereochemical relationship of coupled nuclei.[22,33] If the chemical shift separation between the coupled nuclei is very small compared with their mutual coupling constant, the coupling pattern of each signal will be distorted. The separation between lines in such "second order" signals is no longer directly related to coupling constants and more detailed analysis is required.[20,21,33,34] Experiments that facilitate the interpretation of spin–spin coupling networks are described in Section 3.5.

3.2.4 The Nuclear Overhauser Effect

The nuclear Overhauser effect (nOe) provides a means for establishing the proximity of nuclei in space and thus supplies valuable information about molecular geometry.[35,36] The effect manifests itself as a change in the intensity of the NMR signal of a nucleus when the resonance of a second nucleus is irradiated by an additional radio frequency field. For an intensity change to be apparent, the observed nucleus must relax through the irradiated nucleus via a dipole–dipole mechanism. The magnitude of the effect falls away rapidly as the separation between the irradiated and the observed nuclei increases. In the case of small molecules, the maximum possible intensity change is defined as follows:

$$\text{Enhancement factor}_{\max} = \frac{\gamma_{\text{saturated}}}{2\,\gamma_{\text{observed}}} \tag{3.3}$$

Thus in homonuclear experiments, signals may be enhanced by up to 50% whereas ^{13}C signals, measured with ^{1}H decoupling, may be increased by 200%. In the case of moderately sized molecules (in the range 1000–2000 Da) the maximum enhancement factor can fall away to zero, while large molecules have a maximum enhancement factor of −1.[5,35]

3.2.5 Pulsed Fourier Transform NMR Spectroscopy

Prior to the introduction of pulsed FT NMR spectroscopy,[37–41] nearly three decades after the first NMR experiments,[42,43] the continuous wave (CW) method was used to record spectra. Although some early experiments employed pulses,[44] the routine use of pulsed FT NMR spectroscopy was delayed by the limits of computer technology and the absence of a suitable FT algorithm[45] until the early 1970s. Pulsed FT NMR offered much greater sensitivity than the CW method and thereby, routine access to insensitive nuclei such as ^{13}C. In CW spectrometers, each nucleus in the sample is brought into resonance by slowly sweeping the radio frequency (or magnetic field) through the chemical shift range of the isotope concerned. The sweep normally requires hundreds of seconds. Pulsed spectrometers use a short duration radio frequency pulse to excite all of the nuclei of a particular isotope simultaneously. The resulting signal is recorded over a few seconds. The effect of the pulse is best explained by considering a group of identical nuclei in a rotating frame of reference.

In this frame of reference, depicted in Figure 3.1, the *x*- and *y*-axes are spinning about the direction of the magnetic field (the *z*-axis) at the natural frequency of the nuclei. Thus the individual nuclei appear stationary and the excess of nuclei oriented in the direction of the field gives rise to a net magnetization in this direction (M_z). A short pulse of radio frequency applied along the *x*-axis (solid arrow, Figure 3.1A) results in the magnetization being tipped toward the –*y* axis.[10,23,25] The receiver coils detect the magnetization in the *x*–*y* plane and the resulting signal (known as a Free Induction Decay [FID]) (Figure 3.2A) is digitized and stored in computer memory. The pulse is described by the angle through which it tips the magnetization. Thus, after a 90° pulse, the magnetization is aligned along the –*y* axis giving maximum signal (Figure 3.1B). A 180° pulse

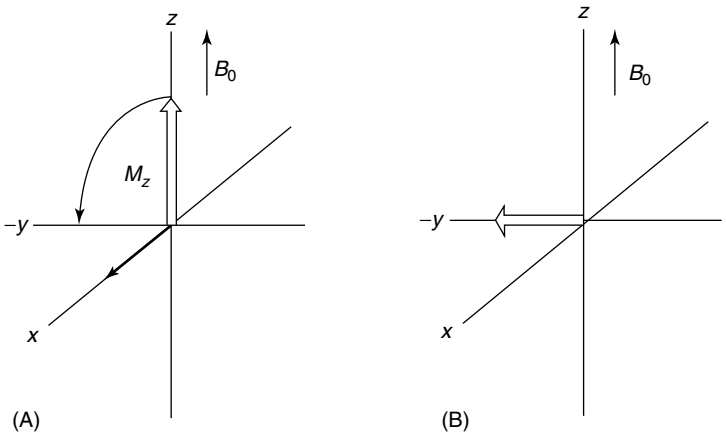


FIGURE 3.1 (A) The effect of a 90° pulse on the net magnetization (Mz). (B) The magnetization immediately after the pulse.

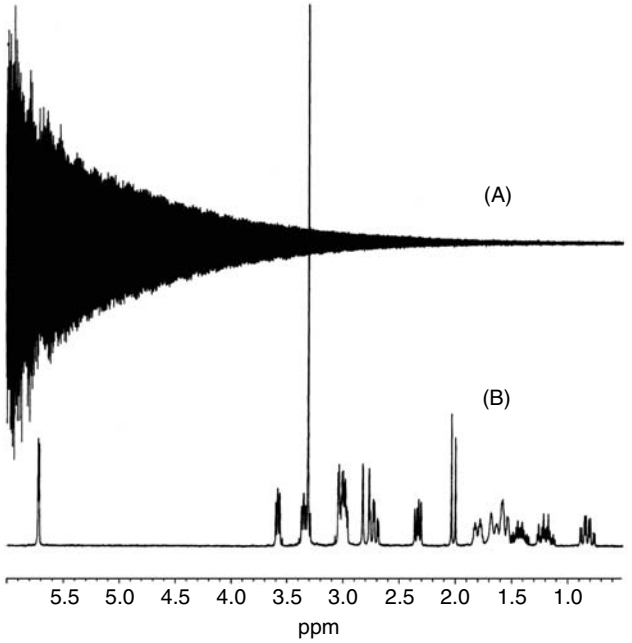


FIGURE 3.2 (A) A typical Free Induction Decay (FID). (B) The spectrum arising from Fourier transformation of the FID.

tips the magnetization into the $-z$ direction. The basic pulsed experiment consists of a single pulse (usually 30–90°) followed by acquisition of the FID. The process is repeated, with the insertion of a suitable delay before each pulse to allow for complete relaxation, until sufficient signal has been added into computer memory. Fourier transformation of the FID (a function of time) produces the normal spectrum (see Figure 3.2B).

3.2.6 Pulse Sequences and Two Dimensional NMR Experiments

The ability to manipulate the nuclear magnetization, by combining pulses of varying lengths into sequences, has led to the design of numerous experiments, each providing specific structural information. As an illustration of a pulse sequence, the COSY (**CO**rrelation **S**pectroscop**Y**) experiment will be described. Since this procedure is a two dimensional (2D) experiment, the basic concepts of 2D NMR spectroscopy[46] will be introduced simultaneously.

The COSY pulse sequence, in its simplest and original form,[47] is illustrated as follows:

$$\begin{array}{l} \text{Relaxation delay} - 90^\circ - t_1 - 90^\circ - \text{FID}(t_2) \\ \text{Preparation} \quad : \text{Evolution} \quad : \text{Acquisition} \end{array}$$

The pulse sequence begins with a delay, to allow for relaxation, followed by two 90° pulses separated by a short delay (t_1). After the second 90° pulse, the FID is acquired to complete the sequence. The pulse sequence of this 2D experiment is described as having three periods: the "preparation" period to allow the nuclear spins to reach equilibrium before each cycle, the "evolution" period during which the nuclear spins are allowed to evolve under the influence of the pulses and the delay, and finally the "acquisition" period. During the COSY experiment, the sequence is repeated for a particular value of t_1 until the computer has acquired sufficient signal. The process is repeated for a number of different t_1 values (typically 256 or 512) with an FID being stored for each value. Each FID is then subjected to Fourier transformation to yield a spectrum. If these spectra are viewed, the appearance of each signal differs from spectrum to spectrum depending on the particular value of t_1 used. Fourier transformation, with respect to t_1, gives the COSY spectrum, which is a surface in 3D space. Two of the axes are frequencies (giving rise to the term 2D NMR spectroscopy), whereas the third is intensity. The spectrum is best plotted as a contour map (e.g., see Figure 3.8) similar to its geographical equivalent. The COSY spectrum shows which nuclei are involved in spin-spin coupling interactions and its interpretation is described in Section 3.5. The preparation, evolution, and acquisition periods are common to all 2D experiments. Some 2D experiments, for example, the NOESY (**N**uclear **O**verhauser **E**ffect **S**pectroscop**Y**) experiment (which indicates nOe interactions between nuclei) has a mixing period between the evolution and acquisition periods. The choice and use of 2D experiments for elucidating molecular structures together with their individual attributes have been described.[3,7,9,10,12]

3.2.7 The NMR Spectrometer

Modern pulsed NMR spectrometers are extremely sophisticated and expensive scientific instruments. The spectrometer features a strong, stable, and homogeneous magnetic field supplied by a superconducting magnet. Modern research spectrometers have superconducting magnets with fields ranging from 4.70 T to the current technological limit of 21.14 T. These magnetic field strengths correspond to ^{1}H resonance frequencies of 200–900 MHz (spectrometers are denoted in terms of their ^{1}H resonance frequency). The spectrometer console houses the radio frequency transmitters and receivers, the digitizer, the pulse field gradient amplifiers, and the temperature control unit. A computer workstation is responsible for overall spectrometer control, the mathematical transformation of the data, and the operation of peripheral devices.

A continuing demand for spectrometers with stronger magnetic fields arises for three reasons. First, the sensitivity of the spectrometer increases significantly with the magnetic field strength ($\alpha B_0^{3/2}$). Second, at higher magnetic fields, there is greater separation between signals, which results in significantly less overlap of multiplets, and therefore spectra are more easily interpreted. Finally, the increased separation of signals also results in the simplification of those "second order" multiplets, which, at lower fields, are complicated due to their close proximity. Thus higher magnetic field strengths not only give greater access to more dilute solutions of the less sensitive nuclei, but the accompanying simplification of spectra allows the study of larger and more complex molecules.

Since the publication of the first edition of this book, there have been significant improvements in instrumentation, which have greatly enhanced spectrometer sensitivity and dramatically reduced experiment times. The maximum field strength available on commercial spectrometers has increased from 14.09 (600 MHz) to 21.14 T (900 MHz), providing a significant gain in sensitivity. Cryogenically cooled probes[48,49] offer a three- to fourfold improvement in the signal to noise ratios (S/N) compared to conventional probes of the same diameter. Thus by adding a cryogenically cooled probe to a 600 MHz spectrometer, one can approximate the sensitivity of a 900 MHz spectrometer. Small volume probes[48] also offer large sensitivity gains, with a 1 mm probe giving similar performance to a 5 mm cryoprobe.

The most notable of the improvements has been the utilization of pulsed field gradients.[50,51] They are now employed in almost all 2D experiments and many one dimensional (1D) experiments, resulting in significantly improved performance and superior quality spectra. Total acquisition times have been greatly reduced (some 2D spectra can be recorded with a single scan per t_1 increment). Pulsed field gradients also allow more efficient suppression of undesired signals and easier data processing. Experiments using pulsed field gradients may be referred to in the literature as gradient-enhanced (ge), gradient-selected (gs), or gradient-accelerated to differentiate them from traditional experiments.

The integration of high-performance liquid chromatography (HPLC) equipment with an NMR spectrometer (LC–NMR) in fully automated systems, sometimes also incorporating a mass spectrometer, has revolutionized the screening process for bioactive compounds.

3.3 STRATEGY FOR SOLVING STRUCTURES

Although the exact strategy used to determine a structure is dictated by the individual demands of each sample, a typical procedure is as follows. First, the routine ^{1}H and ^{13}C spectra are recorded and examined to identify the numbers of each type of nucleus present. This information is integrated with that obtained from other physical methods. The chemical shift of each signal describes the likely environment of the nucleus producing it. Proton-coupling networks are then established and the magnitudes of the coupling constants are used to define the angular relationship and the number of bonds between the coupled nuclei. Subsequently, the proton resonances are correlated with those of the carbon to which they are directly attached. Subunits consisting of contiguous, protonated carbon atoms terminated by heteroatoms or nonprotonated carbon atoms will now be apparent. The subunits may be linked by looking for long-range proton–proton or proton–carbon couplings between the nuclei at the ends of adjoining units. Nuclear Overhauser experiments may also indicate the proximity of nuclei on separate subunits. Usually, some logical connections between the subunits can be made due to the obvious presence of well-known skeletal units (e.g., an indole residue). If sufficient sample is available, experiments using carbon–carbon coupling may be employed to assemble units connected by quaternary carbon atoms.

Heavy demand on instrument time usually necessitates a careful choice of experiments to obtain the required information in the shortest possible time. In the following sections, many of the techniques, which can be used to implement this strategy, are briefly described. More detailed case studies[7,12,52–61] as well as concise examples of the individual experiments[62,63] are available.

3.4 OBTAINING THE BASIC INFORMATION

To obtain high-resolution spectra, careful preparation of the NMR sample is required. Optimum sample concentrations should be used since halving the sample concentration increases the time required to acquire a suitable spectrum by a factor of four. The practical aspects of NMR spectroscopy have been described.[5,62,64]

3.4.1 The ^{1}H Spectrum

Almost all structural studies begin with the recording of a ^{1}H spectrum, the ^{1}H nucleus being the most easily observed and consequently the most studied. A typical ^{1}H spectrum of a natural product, recorded at moderate field strength (7.05 T, 300 MHz), is shown in Figure 3.3. The number of protons giving rise to each signal (and subsequently the number of protons in the molecule) can be determined by comparing the areas under each signal, that is, from the integrals. The integrals in this spectrum (Figure 3.3) are displayed in both digital form and the conventional form in which measurement of the vertical displacement of the integral line over a multiplet indicates its relative area. Ideally, we would like to see each multiplet completely separated from the others to allow the spectrum to be easily interpreted. In the absence of spectrometers with higher field strengths, overlap may be reduced by employing a different solvent or by using lanthanide shift reagents[65,66] that form a complex with the molecule in question, resulting in a substantial increase in the chemical shift of nuclei close to the point of complexation.

The chemical shift of each signal characterizes the environment of the proton from which it arises. Thus, in Figure 3.3, for example, the signal at 5.71 ppm is due to an olefinic proton, and the three-proton singlet at 3.30 ppm indicates the presence of a methoxyl group in the molecule.

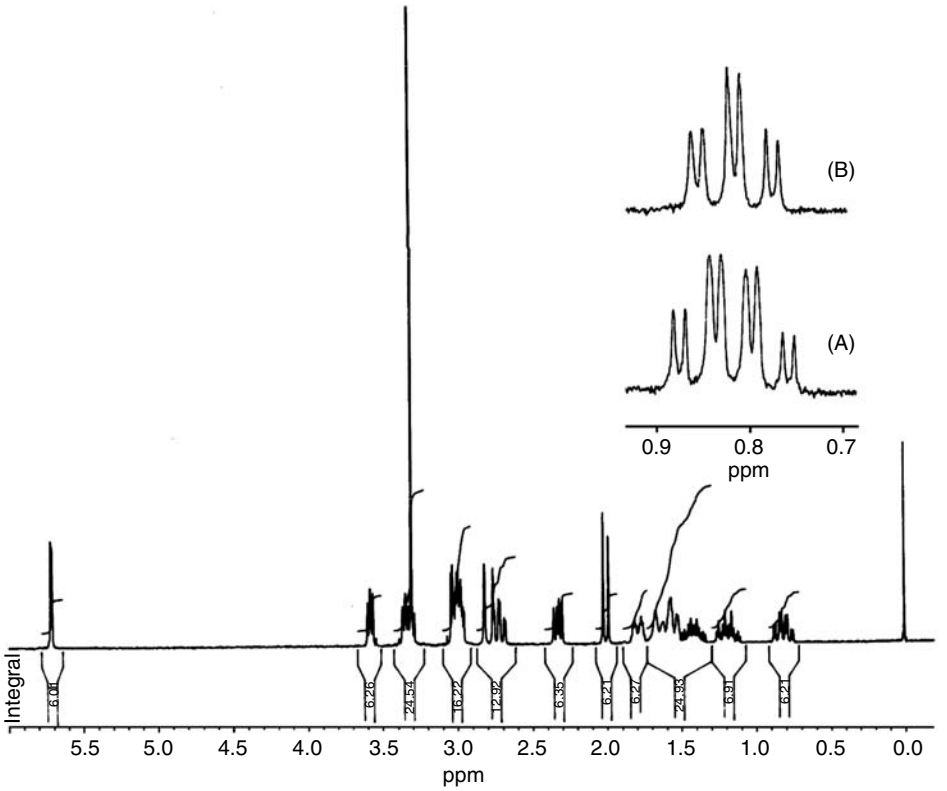


FIGURE 3.3 The ^{1}H spectrum of an alkaloid from *Margaritaria indica*. (A) Expansion of the signal at 0.82 ppm. (B) The same signal while irradiating at 3.00 ppm.

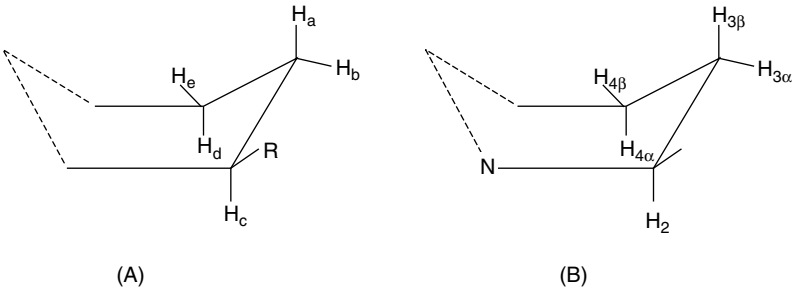
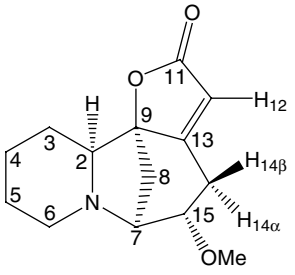


FIGURE 3.4 (A) The expected arrangement of protons coupled to H_a. (B) The actual arrangement in 15α-methoxy-14,15-dihydrophyllochrysine (**1**) (i.e., $H_a = H_{3\beta}$).

Where the coupling pattern of a multiplet is easily interpretable, the numbers and stereochemical orientation of adjacent protons can be determined. For example, the appearance of the multiplet at 0.82 ppm (Figure 3.3, inset A) provides significant information about its surroundings. The coupling pattern is indicative of an axial proton on a six-membered ring (such as H_a in Figure 3.4A), which has two large vicinal diaxial couplings ($^3J_{HH}$ to H_c and H_d), which are of equal magnitude to its geminal coupling constant ($^2J_{HH}$ to H_b) and a smaller axial–equatorial coupling ($^3J_{HH}$ to H_e). The absence of a second axial–equatorial coupling indicates the likely presence of an adjacent equatorial substituent (R). Similarly, the magnitude of the sole coupling constant of the signal arising from the lone olefinic proton in the spectrum defines its immediate environment. It is typical of allylic coupling (i.e., $^4J_{HH}$ via the double bond), and therefore the carbon to which it is attached must be flanked on both sides by fully substituted carbon atoms. Furthermore, its coupling partner will be oriented at approximately 90° to the plane of the double bond as it is in this orientation that allylic couplings are maximized.[33] The spectrum shown in Figure 3.3 is that of 15α-methoxy-14,15-dihydrophyllochrysine (**1**)[67,68] and the signals, just described, arise from $H_{3\beta}$ (see Figure 3.4B) and H_{12} (which is allylically coupled to $H_{14\beta}$), respectively.

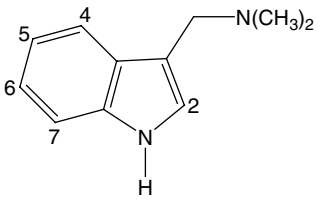


1

It is seldom possible to completely analyze the coupling networks from the ^1H spectrum alone. Experiments, which aid the analysis of the proton spin systems, are described in Sections 3.5 and 3.6. Resonances suspected of being due to exchangeable protons, for example OH, NH can usually be identified by their disappearance after shaking the NMR sample with D_2O.

The FID may be manipulated using one of the various window functions available on most NMR spectrometers, resulting in either increased sensitivity (useful in the case of dilute solutions) or enhanced resolution. Resolution enhancement techniques aid interpretation of spectra by allowing the individual lines of a multiplet to be seen more clearly. Often small, but informative long-range couplings may be revealed using this procedure. Figure 3.5 shows a part of the aromatic pattern

from the spectrum of gramine (**2**). The advantages of the resolution enhanced (Figure 3.5B) over the normal spectrum (Figure 3.5A) are obvious.



2

Comprehensive tabulated descriptions of proton chemical shifts and coupling constants are available.[33,69,70]

3.4.2 The ^{13}C Spectrum

The ^{13}C spectrum[71–74] provides important structural information since it arises from the nuclei which form the framework of the molecule, in contrast to the ^{1}H nuclei that are at the periphery. It also confirms the presence of carbonyl groups and other non-protonated carbon atoms whose

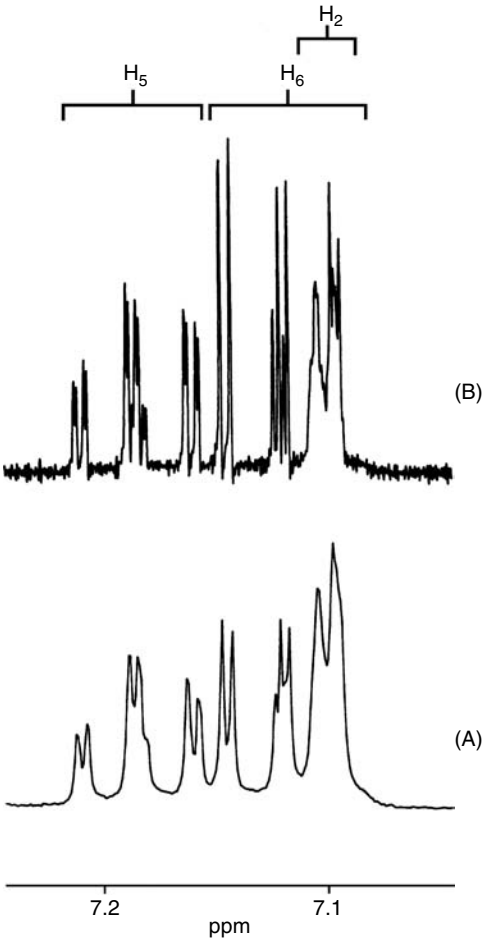


FIGURE 3.5 A section of the aromatic pattern of the ^{1}H spectrum of gramine (**2**). (A) Normal. (B) resolution enhanced.

presence in a molecule can only be inferred from the ^{1}H spectrum. Despite the problem of low relative sensitivity and low natural abundance, modern spectrometers can obtain a ^{13}C spectrum from a few milligrams of material in a relatively short time.

3.4.2.1 The ^{1}H Decoupled ^{13}C Spectrum

The normal ^{13}C spectrum is acquired with full proton decoupling.[75,76] In the absence of coupling to ^{1}H nuclei, all of the ^{13}C signals in the spectrum appear as single lines allowing the number of carbons in the molecule to be readily determined. By employing proton decoupling, a great improvement in the sensitivity of the ^{13}C experiment is achieved due, in part, to the collapse of multiplets into singlets and, in part, from the nOe. The latter effect results in the protonated carbons being enhanced up to nearly three times their intensity without nOe. Nonprotonated carbons can therefore usually be identified by their lower relative intensity in the ^{1}H decoupled ^{13}C spectrum. Figure 3.6A shows the composite pulse decoupled[75,76] spectrum of 15α-methoxy-14,15-dihydrophyllochrysine (**1**). All 14 carbon signals can be clearly seen. The nonproton-bearing carbons, that is, C_9, C_{11}, and C_{13}, are readily identified, having only half to one-third of the intensity of the protonated nuclei. The likely environment of each carbon can be deduced from its chemical shift. The signal at 112.99 ppm (C12) falls within the range for olefinic carbons (100–170 ppm) and its double-bond partner must be one of the two signals that occur at approximately 170 ppm, which is indicative of an olefinic carbon that is β to a carbonyl group. The other signal in this region can be assigned to the carbonyl carbon,

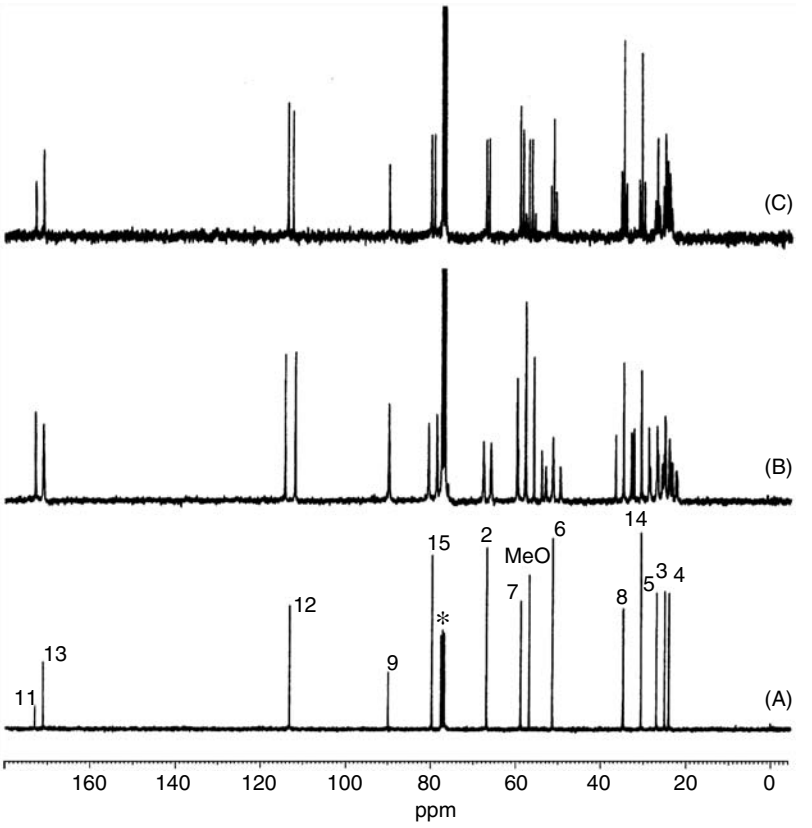


FIGURE 3.6 ^{13}C spectra of 15α-methoxy-14,15-dihydrophyllochrysine (**1**). (A) Composite pulse decoupled. (B) Fully coupled. (C) Single frequency off-resonance decoupled (*$CDCl_3$).

its chemical shift being characteristic of an ester (lactone) carbonyl group. Similarly, assignments of the other resonances can be made.

Knowledge of the number of ^{1}H nuclei attached to each carbon (see Section 3.4.2.2) is necessary for precise assignment based on chemical shift. In addition to general compilations of ^{13}C data[71–73] and specific information on natural products,[77,78] alkaloids,[79–81] steroids,[82,83] saccharides,[84–86] aromatic compounds,[87,88] and amino acids, peptides, and proteins[89–91] is available.

3.4.2.2 Determining the Number of Directly Attached Hydrogen Atoms

3.4.2.2.1 Fully Coupled ^{13}C Spectra

Signals in a fully coupled ^{13}C spectrum[21] are split by large $^{1}J_{CH}$ (typically 125–170 Hz, but may be as large as 250 Hz) and smaller, but significant, $^{2}J_{CH}$ (typically 0–8 Hz) and $^{3}J_{CH}$ (typically 3–13 Hz). The number of large (one-bond) couplings to a particular carbon signal indicates the number of directly attached hydrogen atoms, whereas the smaller couplings provide information about protons on neighbouring carbons.[71,73,74,87,92,93] In the coupled spectrum of **1**, as displayed in Figure 3.6B, the smaller two- and three-bond couplings are not resolved. The signals due to quaternary carbons, that is, C_9, C_{11}, and C_{13}, appear as singlets; tertiary carbons, that is, C_2, C_7, C_{12}, and C_{15}, as doublets; secondary carbons, that is, C_3, C_4, C_5, C_6, C_8, and C_{14}, as triplets; and the primary carbon, that is, OCH_3, as a quartet.

Even in this relatively simple spectrum, the multiplicity of some signals is not immediately obvious due to overlap with other resonances. In more complex molecules, extensive overlap of signals can severely limit the extraction of useful information from the coupled ^{13}C spectrum. For this reason, and because the time taken to acquire the data is usually prohibitive, proton-coupled ^{13}C spectra are rarely recorded.

3.4.2.2.2 Single-Frequency Off-Resonance Decoupled Spectra

In this procedure, the decoupler is set to irradiate at a single frequency, 1000–2000 Hz, outside the ^{1}H spectral range. In the resulting spectrum (Figure 3.6C), one-bond couplings are reduced to 40–60 Hz, thus simplifying the interpretation. For example, the quartet at 56.72 ppm, which is due to the methoxyl group, is now clearly separated from the adjacent doublet arising from C_7. This method offers a significant sensitivity advantage over fully coupled ^{13}C spectra and was the procedure of choice prior to the introduction of spin echo (Section 3.4.2.2.3) and polarization transfer (Section 3.4.2.2.4) techniques. However, carbons attached to strongly coupled protons may be distorted in the single-frequency off-resonance (SFOR) decoupled spectrum and overlap is still a problem in crowded regions.

3.4.2.2.3 J-Modulated Spin Echo Procedures

Experiments that employ a spin echo,[44] modulated by $^{1}J_{CH}$, have been described as *J*-modulated spin echo procedures,[5,13] but are also known by several acronyms, that is, SEFT (**S**pin **E**cho **F**ourier **T**ransform),[14] GASPE (**G**ated **S**pin **E**cho),[94] and APT (**A**ttached **P**roton **T**est).[95] They are superior to the SFOR decoupling method because signals appear as singlets, thus simplifying spectra and providing a further large gain in sensitivity. The *J*-modulated spin echo procedure is represented schematically as follows:

^{13}C	$90°–\tau–180°–\tau–$FID
^{1}H decoupler	OFF ON

The delay between pulses (τ) is set to $1/J_{CH}$, which, in the ensuing spectrum, results in signals due to CH and CH_3 carbons being of opposite phase to those of CH_2 and quaternary carbons. The phase variation arises as the spins evolve under the influence of spin–spin coupling during the period when the decoupler is off. In the *J*-modulated spin echo spectrum (Figure 3.7E) of 15α-methoxy-14,

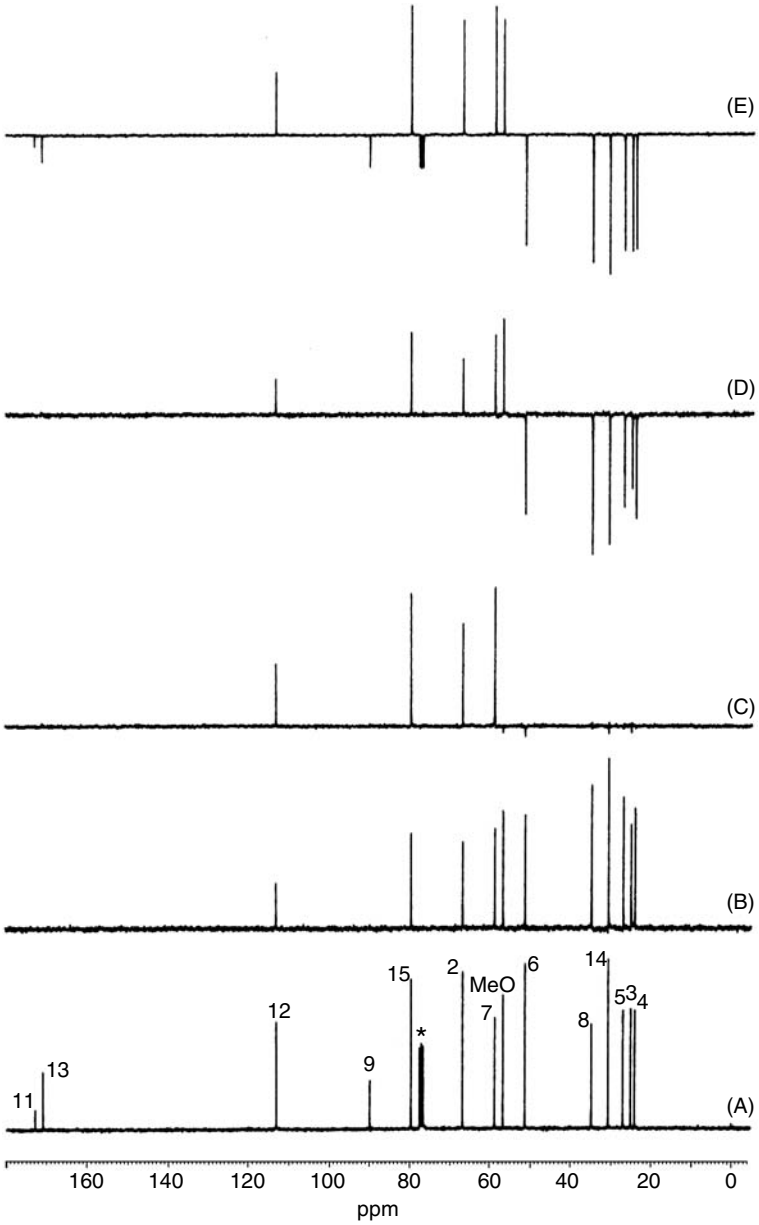


FIGURE 3.7 ^{13}C spectra of 15α-methoxy-l4,15-dihydrophyllochrysine (**1**). (A) Composite pulse decoupled spectrum. (B) DEPT-45°. (C) DEPT-90°. (D) DEPT-135°. (E) *J*-modulated spin echo spectrum (*$CDCl_3$).

15-dihydrophyllochrysine (**1**), the quaternary and CH_2 signals are negative and those of CH and CH_3 carbons are positive. The same sequence, using a delay, where $\tau = 1/2J_{CH}$, produces a spectrum that shows only quaternary carbon resonances.

3.4.2.2.4 Methods Involving Polarization Transfer: DEPT, INEPT

Currently, the most commonly used method for determining the number of hydrogens bonded to each carbon atom is the DEPT (**D**istortionless **E**nhancement by **P**olarization **T**ransfer) experiment.[96,97] This method, together with its precursor, the INEPT (**I**nsensitive **N**uclei **E**nhancement

by **P**olarization **T**ransfer) experiment,[98] involves transfer of magnetization from protons to their directly attached carbons. Thus fully substituted carbons do not give signals in DEPT or INEPT spectra. These experiments offer a further sensitivity gain over the *J*-modulated spin echo procedure. The DEPT pulse sequence can be represented schematically as

^{1}H $\quad 90°–\tau–180°–\tau–\Theta°–\tau–$decoupling

^{13}C $\quad 90°–\tau–180°–\tau–$FID

The delay (τ) is set to $1/2J_{CH}$. The spectra of **1** shown in Figures 3.7B, 3.7C, and 3.7D, were recorded with the DEPT sequence using a final ^{1}H pulse angle (θ) of 45°, 90°, and 135°, respectively. In the DEPT-45° spectrum (Figure 3.7B) all protonated carbons appear as positive singlets, whereas in the DEPT-90° spectrum (Figure 3.7C) only those resonances of carbons bearing one hydrogen can be seen. The DEPT-135° spectrum (Figure 3.7D) again shows all protonated carbon signals with CH_3 and CH resonances being positive, whereas CH_2 signals are negative. Thus, the number of hydrogens attached to each carbon in the molecule can readily be determined by comparing the ^{1}H decoupled (Figure 3.7A), DEPT-90°, and DEPT-135° spectra. The CH and CH_2 signals are immediately obvious from the DEPT-90° and DEPT-135° spectra, respectively. Additionally, positive signals in the DEPT-135° spectrum, which do not appear in the DEPT-90° spectrum, must arise from CH_3 groups. Resonances of quaternary carbons are those appearing in the ^{1}H-decoupled spectrum but not in the DEPT-135° spectrum. The DEPT-45° spectrum therefore would appear to be redundant if signal multiplicity is to be determined by visual inspection alone but must be recorded if editing of spectra is required. The three DEPT spectra may be edited (added and subtracted) to produce a further three spectra, each showing only CH, CH_2, or CH_3 resonances. Modern spectrometers are capable of interpreting the DEPT spectra automatically.

The INEPT sequence produces analogous spectra to those shown for the DEPT experiment, however, a delay between pulses (rather than a pulse angle) is altered to produce the individual spectra. The sequence is more sensitive to the accuracy of setting delays than the DEPT experiment.

Variations of the mentioned sequences and other methods employing polarization transfer to differentiate the number of protons on each carbon atom have been reviewed.[8]

3.5 ESTABLISHING THE ^{1}H COUPLING NETWORKS

3.5.1 Spin–Spin Decoupling Experiments

Homonuclear spin–spin decoupling (double resonance) experiments are the oldest methods for determining proton-coupling networks, but are now rarely used. A typical experiment involves the irradiation of an individual resonance with a second radio frequency field of suitable power while the FID is acquired. In the resulting spectrum, the multiplets, which were coupled to the irradiated nucleus, are simplified since the splitting arising from the irradiated nucleus has been removed. For example, the signal due to $H_{3\beta}$, in 15α-methoxy-14,15-dihydrophyllochrysine (**1**), is simplified when H_2 is irradiated (Figure 3.3, inset B). The disappearance of a large diaxial coupling compared with the normal spectrum (Figure 3.3, inset A) confirms the predicted relationship between H_2 and $H_{3\beta}$.

Spectrometers allow a number of decoupling experiments to be performed automatically, with each multiplet in the spectrum being irradiated in turn. Analysis of the resulting spectra allows most, if not all, of the coupling interactions in the spectrum to be defined. Problems arise when signals are very close or overlap with each other. Selective irradiations are then no longer possible and the results in these cases may be ambiguous. The detection of changes to signals obscured in overlapping multiplets may be aided by using the spin decoupling difference technique.[99]

3.5.2 Two Dimensional Homonuclear Correlation Experiments

The COSY experiment (Section 3.2.6) is an extremely powerful method for tracing spin–spin coupling within an organic molecule. A simple COSY spectrum of **1** is displayed in Figure 3.8. In this contour plot, signals corresponding to the 1D spectrum can be found along the diagonal (lower left to upper right). Cross peaks (those not on the diagonal) indicate spin–spin coupling between two nuclei, the chemical shifts of which are described by the horizontal and vertical coordinates of the cross peak. Thus the cross peak marked **a** in Figure 3.8 arises from coupling between H_{12} and $H_{14\beta}$. It should be noted that the cross peaks are symmetrically disposed about the diagonal and therefore the cross peak **b** shows the same correlation.

The analysis of a COSY spectrum begins at one or more readily identified resonances, for example, H_{12} or $H_{3\beta}$, from which the coupling networks may be traced. The spectrum arising from the COSY sequence incorporating a double-quantum filter (DQF)[100–102] is illustrated in Figure 3.9 in which the normal 1D spectrum is shown immediately above the COSY–DQF spectrum. In this experiment, the intense resonances due to singlets, for example, the methoxyl group in **1**, are removed, which results in significantly improved clarity. A large amount of information can be extracted from the spectrum. Starting from H_{15}, for example, cross peaks indicate coupling to H_7 and $H_{14\beta}$. In turn, H_7 is clearly coupled to $H_{8\alpha}$, which is additionally coupled to $H_{8\beta}$. Similarly, cross

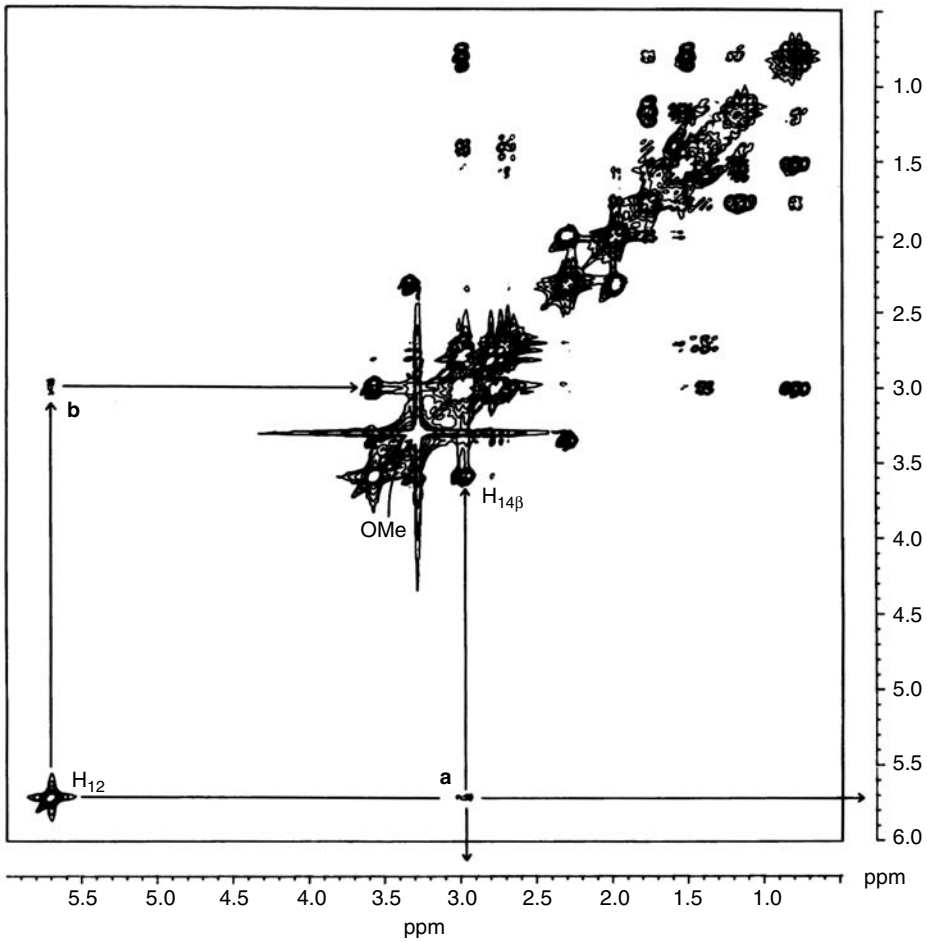


FIGURE 3.8 The COSY spectrum of 15α-methoxy-14,15-dihydrophyllochrysine (**1**).

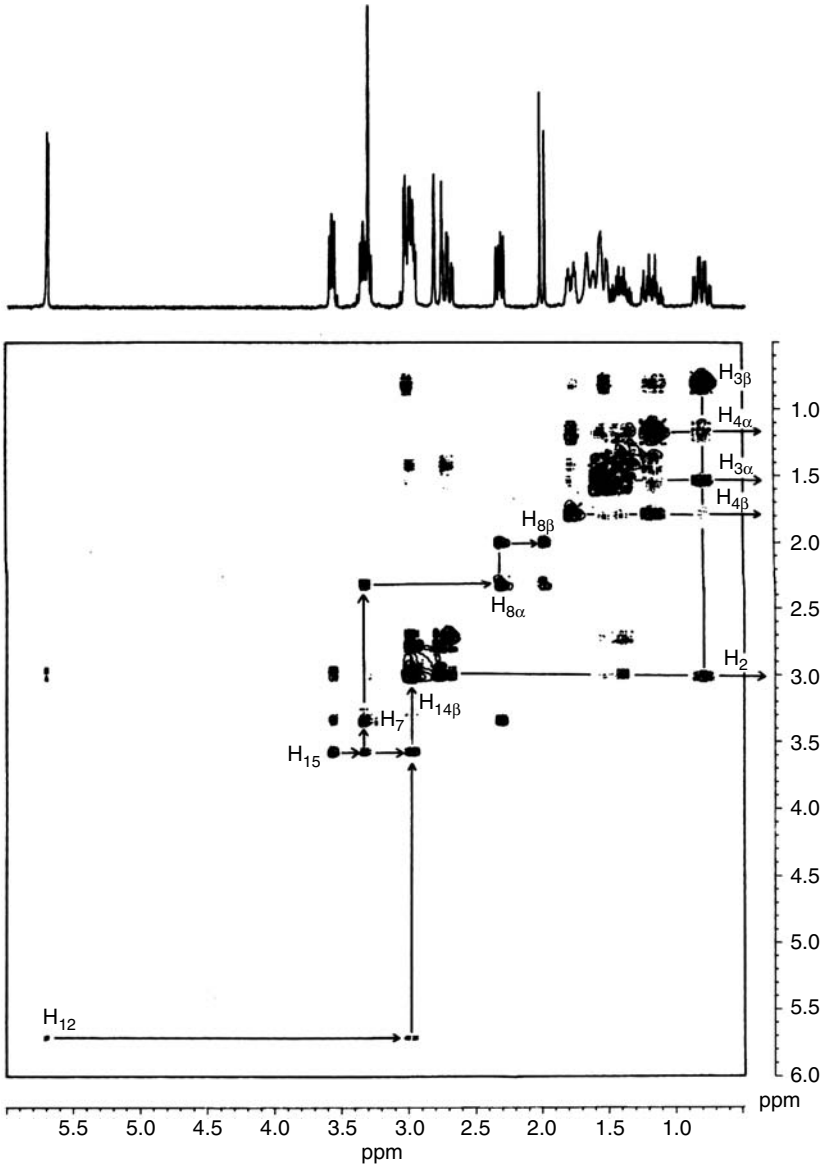


FIGURE 3.9 The double-quantum filtered (DQF) COSY of 15α-methoxy-14,15-dihydrophyllochrysine (**1**).

peaks arising from $H_{3\beta}$ allow the location of all of its coupling partners, namely $H_{4\alpha}$, $H_{3\alpha}$, $H_{4\beta}$, and H_2. It should be noted that experiments described in Section 3.6 can be used at this stage to readily identify the protons attached to the same carbon atom.

Routine COSY experiments now incorporate pulsed field gradients.[103–110] The 500 MHz ge-COSY spectrum of **1** is shown in Figure 3.10. All of the correlations described are also marked on this spectrum. The superior quality of the spectrum incorporating pulsed field gradients is clearly evident. The spectrum was acquired in less than 1/8th of the time required for a similar spectrum without gradients.

There are many other variations of the COSY experiment.[7,10] COSY experiments acquired in the phase-sensitive mode[111,112] can not only define which nuclei are coupled, but analysis of the cross

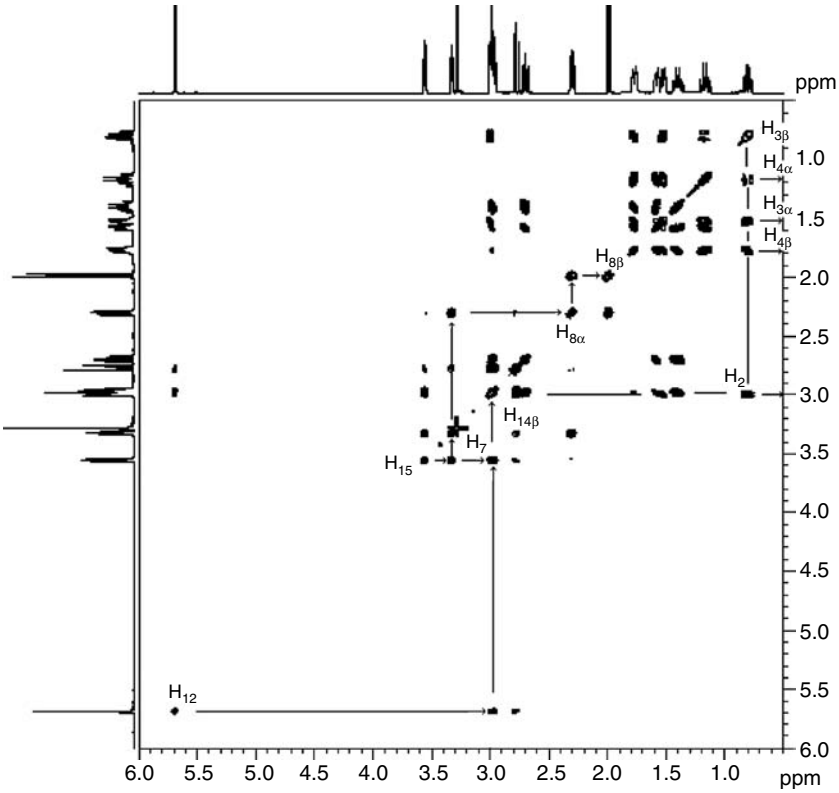


FIGURE 3.10 The ge-COSY spectrum of 15α-methoxy-14,15-dihydrophyllochrysine (**1**) incorporating pulsed field gradients.

peaks also shows which coupling constants arise from which nuclei, that is, which are the active coupling constants. Insertion of a delay into the COSY sequence emphasizes cross peaks arising from small, long-range couplings.[113]

The TOCSY (**TO**tal **C**orrelation **S**pectroscop**Y**)[103,114–116] or HOHAHA (**HO**monuclear **HA**rtmann–**HA**hn) experiments[117,118] can be employed to define all of the protons within a coupling network even though some of them may not be directly coupled. The choice of experimental mixing time, in these experiments, allows the selection of direct-, single-, double-, or multiple-relayed connectivities. Relay coherence transfer experiments may also be useful for identifying all of the hydrogen nuclei within a subunit.[5,10] However, these experiments are less sensitive than the TOCSY sequence.

Despite the obvious advantages of the mentioned 2D spectra, analysis of congested regions of the spectrum may occasionally prove to be difficult. In such cases, double-quantum 2D experiments[119,120] may aid interpretation since these experiments provide an alternative presentation of the data.

3.5.3 One Dimensional Versions of the Cosy Experiment

When only a small number of correlations need to be determined, 1D versions of the COSY experiments (with[121–124] or without pulsed field gradients[125–127]) can be used. In such an experiment the first 90° pulse of the COSY sequence is usually a "soft" pulse, that is, a low power pulse that covers a narrow frequency range, which selectively excites a particular proton resonance. The 1D spectrum, resulting from the 1D COSY pulse sequence, shows only signals for the nuclei that are coupled to the

nucleus that was selectively irradiated. Soft pulses are generated on modern spectrometers by pulse-shaping units. The use of selective pulses in NMR spectroscopy has been reviewed.[128] However, the efficiency of the 2D ge-COSY experiments has resulted in little need for the 1D experiments.

3.6 CORRELATING RESONANCES DUE TO DIRECTLY BONDED ^{1}H AND ^{13}C NUCLEI

Although experiments in Section 3.4 were able to describe the number of hydrogens attached to each carbon atom, they were unable to show which ^{1}H resonances are associated with a particular carbon. Such information is a useful aid in the assignment of resonances in the individual ^{1}H and ^{13}C spectra and, together with knowledge of the proton-coupling networks, allows the complete definition of subunits of adjacent protonated carbons.

The first method used to correlate carbon resonances with those of their directly attached protons involved the acquisition of a series of ^{13}C spectra. Each spectrum was acquired with decoupling at a single ^{1}H frequency.[129] To produce the series, the frequency of the decoupler was stepped through the proton chemical shift range. By locating the carbon resonances that sharpened for a particular ^{1}H decoupling frequency, one could relate the resonances due to directly bonded ^{1}H and ^{13}C nuclei. This procedure was obviously very time-consuming. Modern heteronuclear correlation techniques are considerably more efficient.

3.6.1 Two Dimensional Heteronuclear Chemical Shift Correlation Experiments

The first generation of heteronuclear correlation experiments, for example, HETCOR and COLOC (**CO**rrelation via **LO**ng-range **C**ouplings), involved the excitation or decoupling of protons with the detection of carbon signals. In contrast, inverse methods, for example, HMQC[130] and HSQC[131,132] detect the proton magnetization and offer significantly greater sensitivity over the carbon-detected experiments. The HMQC (**H**eteronuclear **M**ultiple-**Q**uantum **C**orrelation)[130] experiment is eight times more sensitive than its conventional equivalent.[10] Some of the techniques described earlier have an inverse equivalent, for example, the inverse DEPT experiment.[133]

The ge-HSQC (**H**eteronuclear **S**ingle-**Q**uantum **C**oherence)[134,135] spectrum of **1** is displayed in Figure 3.11. The spectrum is readily analyzed with the horizontal and vertical coordinates of each peak defining the chemical shifts of a proton and its directly attached carbon, respectively. Thus peak (**a**) in Figure 3.11 indicates that the proton giving a resonance at 3.58 ppm (H_{15}) is directly bonded to the carbon resonating at 79.6 ppm (C_{15}). In the case of the methylene carbon atoms (i.e., C_3, C_4, C_5, C_6, C_8, and C_{14}), two peaks can be seen for each carbon chemical shift, thereby allowing the ready location of each of the geminal proton signals. This information can be extremely useful as in the case of the protons on C_3. The multiplet due to $H_{3\beta}$ (0.82 ppm) has three large coupling constants of equal magnitude (see Section 3.4.1), that is, two from protons on adjacent carbons and one from its geminal partner ($H_{3\alpha}$). Although decoupling experiments show the locations of the resonances of the three protons coupled to $H_{3\beta}$, they do not directly indicate which one arises from $H_{3\alpha}$ due to the equal magnitude of the coupling constants. However, the location of the $H_{3\alpha}$ signal is immediately obvious from the HSQC spectrum (Figure 3.11). Its chemical shift is given by the second peak at the same ^{13}C chemical shift as $H_{3\beta}$. In the normal 1D spectrum, the ^{1}H signal of $H_{3\alpha}$ is hidden within overlapping multiplets. In a similar manner, the ^{1}H chemical shifts of each of the other geminal pairs can also be determined.

The second experiment regularly employed for one-bond heteronuclear chemical shift correlation, using inverse detection, is the HMQC experiment.[130,136–140] The application of this experiment, and some of its variants, in natural products chemistry has been reviewed.[12] The HMQC and HSQC spectra of the same compound can appear almost identical with the HSQC spectrum offering better resolution in the carbon dimension. The HSQC experiment is the preferred procedure.

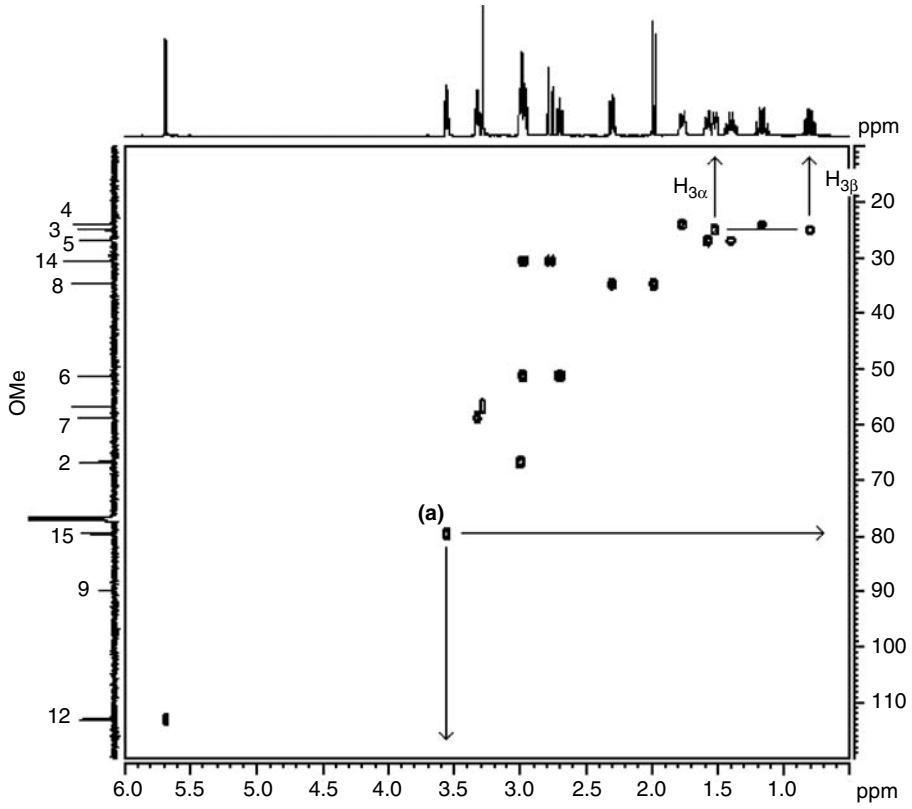


FIGURE 3.11 The ge-HSQC (**H**eteronuclear **S**ingle-**Q**uantum **C**oherence) spectrum of 15α-methoxy-14,15-dihydrophyllochrysine (**1**).

Inverse-detected 2D heteronuclear correlation experiments have also been termed C,H-COSY experiments.[10]

A spectrum arising from the carbon-detected 2D experiment employed to correlate the chemical shifts of directly bonded ^{1}H and ^{13}C nuclei (HETCOR[141–145] or H,C-COSY) is illustrated in Figure 3.12. The analysis of a HETCOR spectrum is analogous to that of a HSQC or HMQC, except that the axes of the spectrum are normally reversed. The HETCOR experiment can offer better resolution in the ^{13}C dimension.

3.6.2 One Dimensional Heteronuclear Chemical Shift Correlation Experiments

The 1D analog of the HMQC sequence is the SELINCOR experiment.[12,146,147] The pulse sequence uses a selective carbon pulse, applied at the chemical shift of the carbon atom of interest, and detects the responses of the attached protons.

The selective ge-1D HSQC experiment[148,149] incorporates pulsed field gradients to produce excellent 1D HSQC spectra, which show only the signals of protons directly attached to a specific ^{13}C atom. The ge-1D HMQC experiment[149,150] provides analogous information.

There are a number of carbon-detected 1D heteronuclear shift correlation experiments,[150–157] which may be useful when only a small number of correlations are of interest or if the limited amount of the sample available precludes a 2D experiment. Some of these experiments such as SHECOR[151,152] (**S**elective **HE**teronuclear **COR**relation), SEPT[153] (**S**elective IN**EPT**) and SDEPT[153,154] (**S**elective **DEPT**) employ selective proton pulses whereas others, including SINEPT[156] and CHORTLE,[157] use nonselective proton pulses.

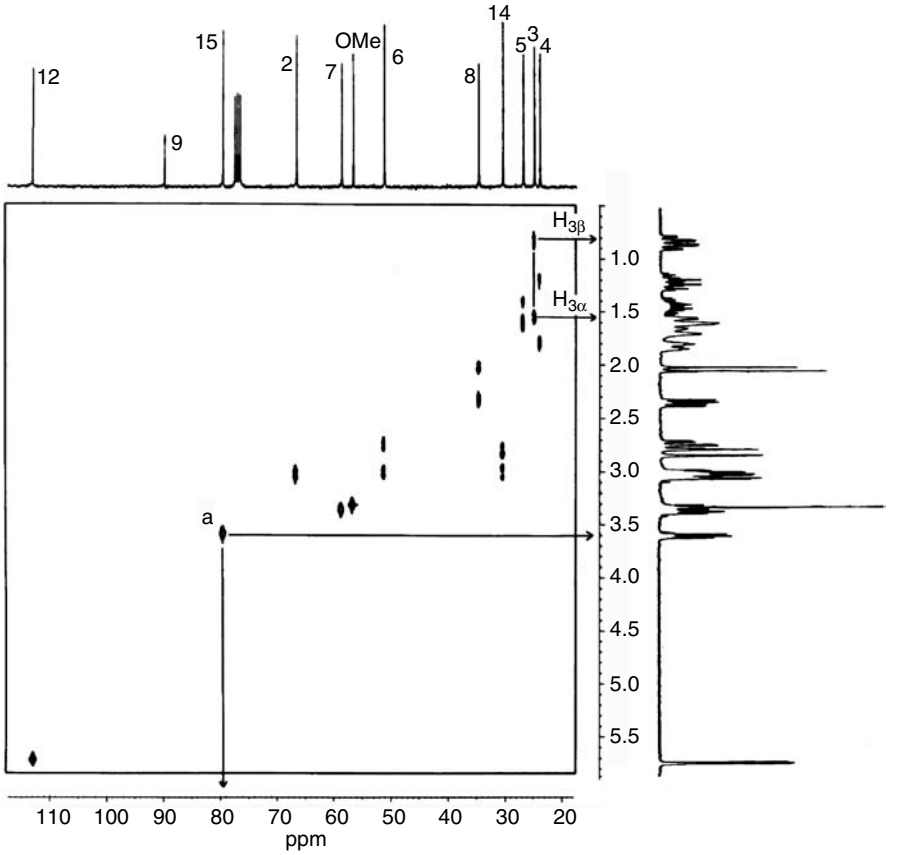


FIGURE 3.12 The heteronuclear chemical shift correlation spectrum (H,C-COSY) of 15α-methoxy-14,15-dihydrophyllochrysine (**1**).

The selective ge-1D HSQC experiment has been used as a building block for more complex experiments, for example, the selective ge-1D HSQC–COSY,[149] and the selective ge-1D HSQC–NOESY.[149,158] A similar series of experiments exist for the ge-1D HMQC experiment.[149,150]

3.7 LONG-RANGE HETERONUCLEAR CHEMICAL SHIFT CORRELATION

Knowledge of the correlation between protons and their directly bonded carbon atoms is a prerequisite for experiments involving long-range correlations.

The experiments described in the previous sections allow the determination of all of the subunits of contiguous, protonated carbon atoms within a molecule. Thus in the case of **1**, the fragments consisting of C_2–C_3–C_4–C_5–C_6 and C_{14}–C_{15}–C_7–C_8; C_{12} and their attached protons have now been defined. The problem of linking these units to form the correct structure now exists. Some logical connections can usually be made. For example, the chemical shifts of C_2, C_6, and C_7 indicate that they are likely to be attached to the lone nitrogen in the molecule. Evidence of long-range proton–proton coupling may show the proximity of units as in the case of the allylic coupling between H_{12} and $H_{14\beta}$, which defines the interconnection of C_{14}, C_{13}, and C_{12}.

An extremely useful method for determining the connectivity of subunits in the molecule is by long-range heteronuclear chemical shift correlation techniques. These methods relate protons and carbons, separated by two or three bonds, via their coupling constant. Two-bond carbon–hydrogen

couplings ($^2J_{CH}$—typically 0–8 Hz) are able to show the relationship between quaternary carbons and the protons on adjacent carbon atoms whereas three-bond couplings ($^3J_{CH}$—typically 3–13 Hz) are able to correlate resonances across a quaternary carbon or heteroatom or correlate a quaternary carbon atom with protons on the β carbon. Long-range correlations can also confirm interconnections, deduced from previous experiments, within a subunit.

3.7.1 Long-Range Heteronuclear Correlation Techniques

3.7.1.1 Two Dimensional Methods

Two dimensional long-range heteronuclear chemical shift correlation methods, together with examples of their application, have been reviewed.[159] The standard H,C-COSY[144] experiment may be optimized for long-range correlations[160,161] but relaxation, during the long delays required by the small coupling constants, results in a significant loss of sensitivity. Also, modulation by one-bond couplings may result in the loss of some correlation peaks. Constant evolution time experiments,[162–165] including the COLOC[162,163] and XCORFE[165] experiments, were designed to circumvent the relaxation problem but they are still affected by one-bond modulations.

The significant gain in sensitivity, provided by inverse detection, makes the HMBC (**H**eteronuclear **M**ultiple-**B**ond **C**orrelation) experiment[166,167] the preferred experiment for long-range heteronuclear correlation. The ge-HMBC[136,139,140] spectrum of **1**, optimized for long-range carbon–hydrogen couplings of 10 Hz, is shown in Figure 3.13. The experiment is useful in defining the location of the nonprotonated carbon atoms, that is, C_9, C_{11}, and C_{13}, within the molecule, as well

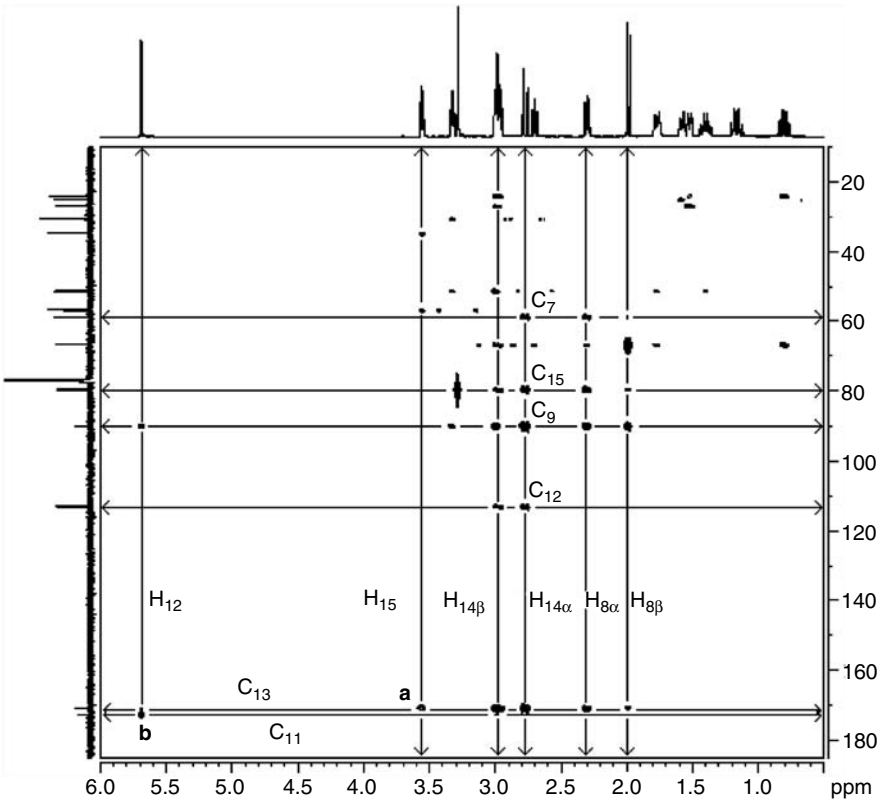


FIGURE 3.13 The ge-HMBC (**H**eteronuclear **M**ultiple-**B**ond **C**orrelation) spectrum of 15α-methoxy-14,15-dihydrophyllochrysine (**1**) optimized for $J_{CH} = 10$ Hz.

as confirming the interconnections deduced from other experiments. Peaks in the spectrum indicate long-range coupling between the nuclei at the corresponding ^{13}C (vertical axis) and ^{1}H (horizontal axis) chemical shifts. Hence, the peak (**a**) indicates a long-range interaction between H_{15} (3.58 ppm) and the nonprotonated carbon at 170.8 ppm. Some ambiguity may arise when it is unclear whether a peak arises from a two- or a three-bond coupling. In such cases, logical argument based on other long-range interactions and on information from procedures described in earlier sections will normally remove the uncertainty. Thus, as most of the fragments containing H_{15} have already been defined, the peak (**a**) must arise via a three-bond interaction between H_{15} and the olefinic carbon attached to C_{14}, that is, C_{13}. This carbon also shows long-range correlations with $H_{14\alpha}$, $H_{14\beta}$, H_{12}, $H_{8\alpha}$, and $H_{8\beta}$. The extended unit C_8–C_7–C_{15}–C_{14}–$C_{13} = C_{12}$ is now defined. In addition to the preceding correlation, $H_{14\alpha}$ shows interactions with C_7, C_{12}, C_{15}, and the quaternary carbon at 89.8 ppm. Since H_{12} also has a long-range interaction with this quaternary carbon, it (C_9) must be attached to C_{13}. Extension of this subunit to include C_{11} is indicated by peak (**b**) together with the chemical shifts of C_{12} and C_{13}, which indicate they are α and β to a carbonyl group, respectively. In some cases, it may be necessary to record a second spectrum, optimized for a different long-range proton–carbon coupling constant to see all of the required correlations.

The COLOC spectrum of **1**, optimized for long-range carbon–hydrogen couplings of 9 Hz, is illustrated in Figure 3.14. Analysis of the spectrum resulting from the COLOC sequence is analogous to that described for the HMBC experiment, except that the axes are normally reversed.

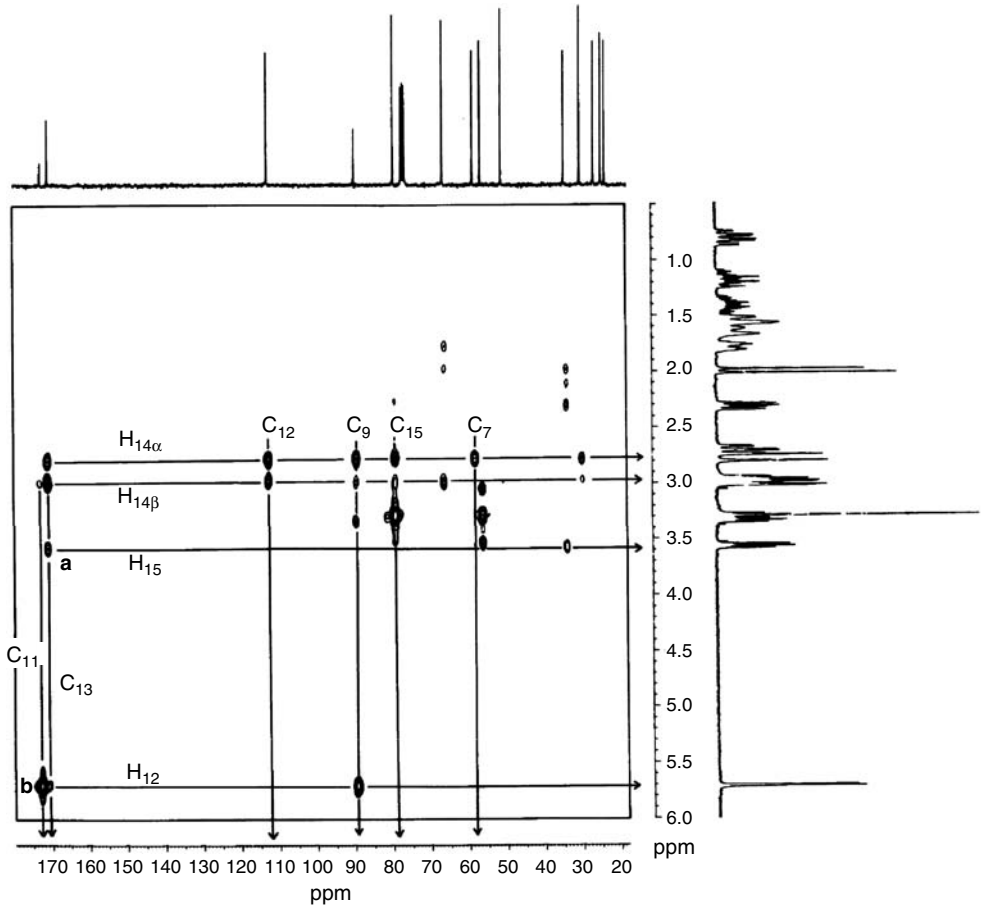


FIGURE 3.14 The COLOC spectrum of 15α-methoxy-14,15-dihydrophyllochrysine (**1**) optimized for $J_{CH} = 9$ Hz.

Experiments[168,169] have been designed to remove the ambiguity that arises when it is difficult to differentiate two-bond from three-bond correlations. The ^2J,^3J-HMBC experiment[168] differentiates ^2J from ^3J correlations to protonated carbons, whereas the H2BC[169] experiment aims to enhance two-bond correlations while suppressing correlations through three or more bonds.

The FUCOUP experiment[170] (correlation with **FU**ll **COUP**ling) and the DEPT-based sequences[171–173] provide other alternatives for long-range heteronuclear correlation.

3.7.1.2 One Dimensional Methods

One dimensional versions of the HMBC experiment,[174–176] which employ frequency-selective carbon pulses, may be employed when the quantity of material available or low solubility prevents the recording of a full HMBC spectrum.[175] The selective ge-1D HMBC[149,150,177] has been employed in our laboratories to determine the structure of the smoke-derived germination stimulant (see Chapter 20, Section 20.4.1).[178] This butenolide compound was initially available in an extremely small quantity. Several ge-1D HMBC experiments were used, each determining which protons were located within two or three bonds of a particular carbon.

The INAPT (**I**nsensitive **N**uclei **A**ssigned by **P**olarization **T**ransfer) experiment,[179,180] a version of the INEPT sequence, which uses selective proton pulses, has been used for the structural determination of natural products.[181]

3.7.2 Relayed Coherence Transfer Experiments

Protons may also be correlated with neighbouring carbon atoms via H−H−C relay experiments.[182–187] In these experiments, magnetization is passed from one proton to a neighbouring proton and subsequently to the carbon directly attached to the second proton. Such experiments, however, are less sensitive than the standard heteronuclear chemical shift correlation experiment.

3.8 ESTABLISHING MOLECULAR STRUCTURE VIA ^{13}C–^{13}C COUPLING

The 2D INADEQUATE (**I**ncredible **N**atural **A**bundance **D**ouble-**QU**A**n**tum **T**ransfer **E**xperiment) sequence[188–194] offers an unambiguous method for tracing the interconnection of carbon atoms in a molecule. However, the technique suffers from extremely low sensitivity as the experiment detects only those molecules with adjacent ^{13}C nuclei, that is, one molecule in 10,000. Thus, a typical experiment requires hundreds of milligrams of material. In spite of this problem there are a number of examples of its use in the literature.[15,195]

Selective 1D versions[196,197] of the 2D INADEQUATE procedure are only recommended when one key piece of information is required to determine a structure due to low sensitivity.

3.9 ESTABLISHING THE PROXIMITY OF NUCLEI THROUGH SPACE

The nuclear Overhauser effect allows the identification of those nuclei within a molecule that are close in space, irrespective of whether the nuclei are spin–spin coupled or not. There are two experiments commonly used to detect nOe interactions between nuclei—the nOe difference experiment and the 2D NOESY technique. The latter procedure is most useful when studying large molecules, such as proteins, whereas the former is able to detect small nuclear Overhauser enhancements and is usually employed in the study of small- to medium-sized molecules. The information from such experiments is valuable in defining the relative orientation of substituents and can aid the assembly of subunits into a molecular structure.

3.9.1 The NOE Difference Experiment

The early method that was used to show nOe interactions involved measurement of the integral of a signal while another resonance was being irradiated. This integral was then compared to the value

of the integral of the same signal, obtained when the decoupler was offset from any resonance. Enhancements were noted as the percentage variation in the integration measurements (Section 3.2.4). The nOe difference experiment[99] is an extension of this method.

Prior to an experiment, oxygen is removed from the sample solution[64] thereby excluding an alternative relaxation pathway and optimizing nOe enhancements. The experiment involves irradiating a resonance for several seconds with a second radio frequency field. The irradiating field is then turned off and a FID is acquired. The sequence is repeated for a number of scans and the resulting FID is stored. Each resonance of interest is irradiated in turn and a final spectrum is acquired with the decoupler offset from any resonance. To minimize the effects caused by spectrometer variations during the experiment, a small number of scans are used for each irradiation frequency and the cycle of irradiations is repeated many times. Difference spectra, which are obtained by subtracting the final standard spectrum from each of the spectra in which a resonance was irradiated, show only those resonances that have nOe interactions with the irradiated signal together with a negative signal for the irradiated nucleus itself. For example, Figure 3.15A shows the nOe difference spectrum of **1** obtained from the irradiation of $H_{8\alpha}$. Enhancements can be seen for its geminal partner $H_{8\beta}$ (27%) and also for H_7 (6%). In addition, and more importantly, an enhancement of H_2 (8%) is observed. H_2 belongs to a separate proton-coupling network and therefore the nOe indicates the spatial proximity of $H_{8\alpha}$ and H_2 on the underside of the molecule. The nOe difference spectrum also allows the signal due to H_2 to be seen clearly, in contrast to the normal ^1H spectrum in which this resonance is partially obscured due to overlap with other resonances.

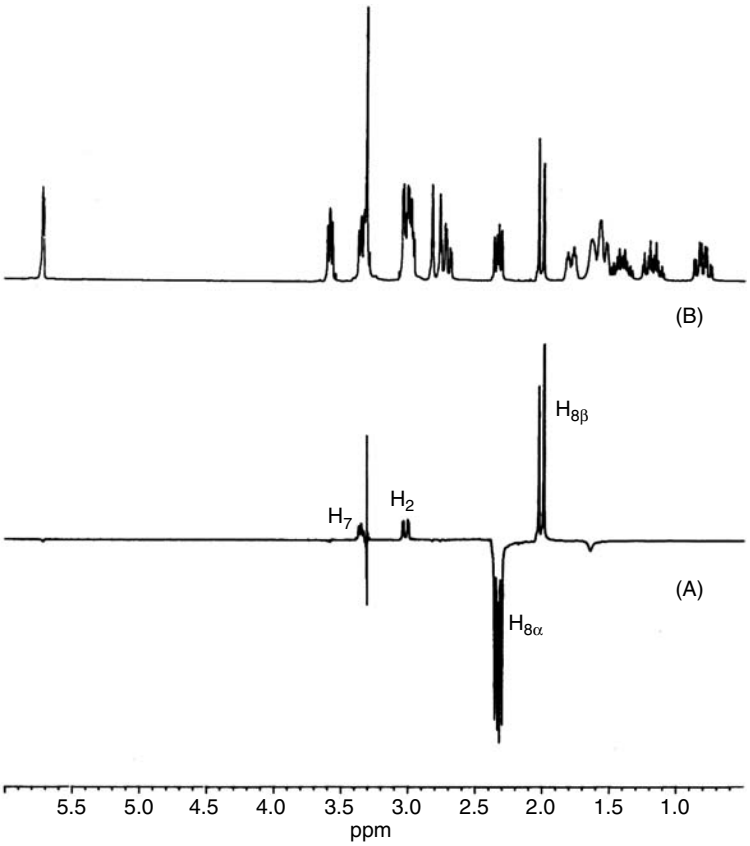
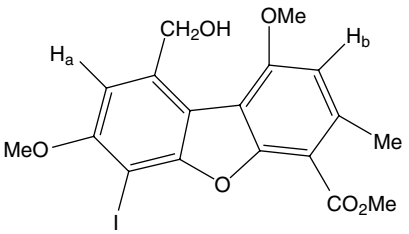


FIGURE 3.15 (A) The nOe difference spectrum arising from irradiation of $H_{8\alpha}$ of 15α-methoxy-14,15-dihydrophyllochrysine (**1**). (B) The normal ^1H spectrum.

Another extremely useful interaction, defined by the nOe difference experiment, is that between H_{12} and $H_{14\alpha}$. Each shows an enhancement when the other is irradiated thereby confirming their proximity. Thus the angular relationships between H_{12} and both $H_{14\alpha}$ and $H_{14\beta}$ have been defined by NMR techniques (the latter relationship by allylic coupling—see Section 3.4.1). The nOe difference experiment is only suitable for small molecules.

3.9.2 The Two Dimensional NOESY Experiment

The NOESY spectrum[35,198–200] is very similar in appearance to the COSY spectrum described in Section 3.5.2 with peaks corresponding to the 1D spectrum lying along the diagonal. In the case of the NOESY spectrum, off-diagonal peaks indicate nOe interactions (exchange of magnetization) rather than spin–spin coupling interactions. A simple example of a NOESY spectrum can be seen in Figure 3.16. This spectrum was produced from a dilute solution of the iodo compound (**3**), a by-product in the synthesis of a naturally occurring dibenzofuran.[201] The location of the iodine substituent, which had just been introduced, was in question. The solution to this problem was immediately obvious from the NOESY spectrum. The off-diagonal peaks corresponding to the aromatic hydrogen H_a indicate nOe interactions with both the $-CH_2O-$ protons and those of a methoxyl group. Thus H_a is located between these two substituents. The other aromatic proton resonance (H_b) shows interactions with a methoxyl group and the aromatic methyl group, thereby defining its location and consequently the location of the iodine substituent. The off-diagonal peaks marked (**a**) in this spectrum (Figure 3.16) arise from chemical exchange between the hydroxyl proton and those of the water in the dilute solution.



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The ge-NOESY spectrum[198,202] of **1** is shown in Figure 3.17. In this particular spectrum, only the nOe cross peaks are displayed, with a dotted line indicating the diagonal. A large amount of stereochemical information can be obtained from the spectrum. The nOe interactions among $H_{8\beta}$, H_7, H_2, and $H_{8\alpha}$, as described in Section 3.9.1, are illustrated. In addition, peak (**a**) shows the strong nOe interaction between H_{12} and $H_{14\alpha}$. An adjacent, less intense cross peak shows a weaker interaction between H_{12} and $H_{14\beta}$.

3.9.3 The ge-1D NOESY Experiment

The selective 1D NOESY spectrum[196,203,204] is almost identical in appearance to that of the nOe difference experiment (see Figure 3.15). However, it is of superior quality and can be obtained in a shorter timeframe and therefore is the preferred experiment. The selectively excited peak appears as a negative signal. The only other signals visible in the spectrum are those that arise due to nOe interactions with the selectively excited proton.

3.9.4 The ROESY Experiment

The NOESY experiment can fail for moderately sized molecules for which the nOe falls to zero. In this case the ROESY (**R**otating Frame **O**verhauser **E**nhancement **S**pectroscop**Y**) experiment[205–207]

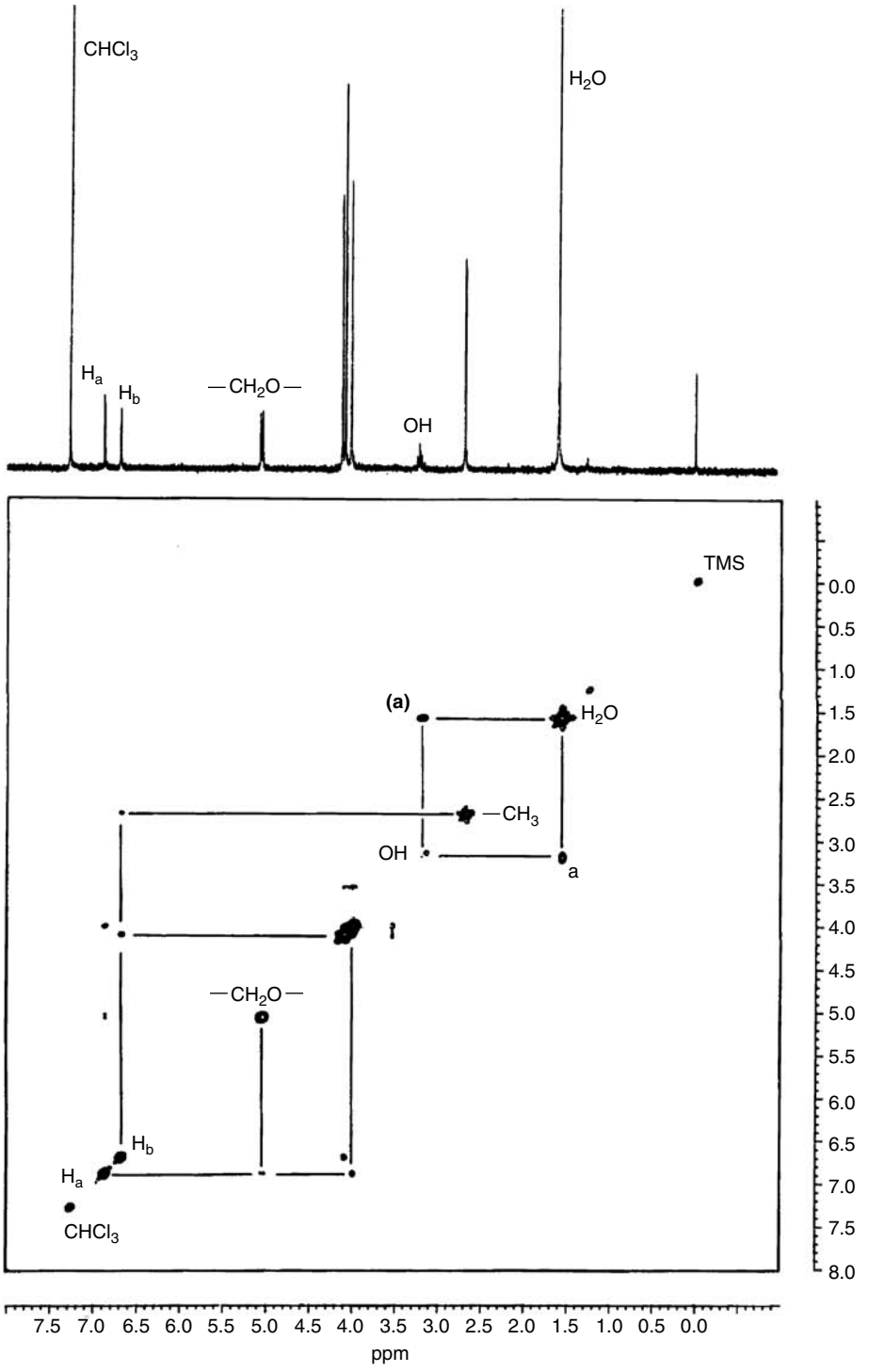

FIGURE 3.16 The NOESY spectrum of the iodo compound 3.

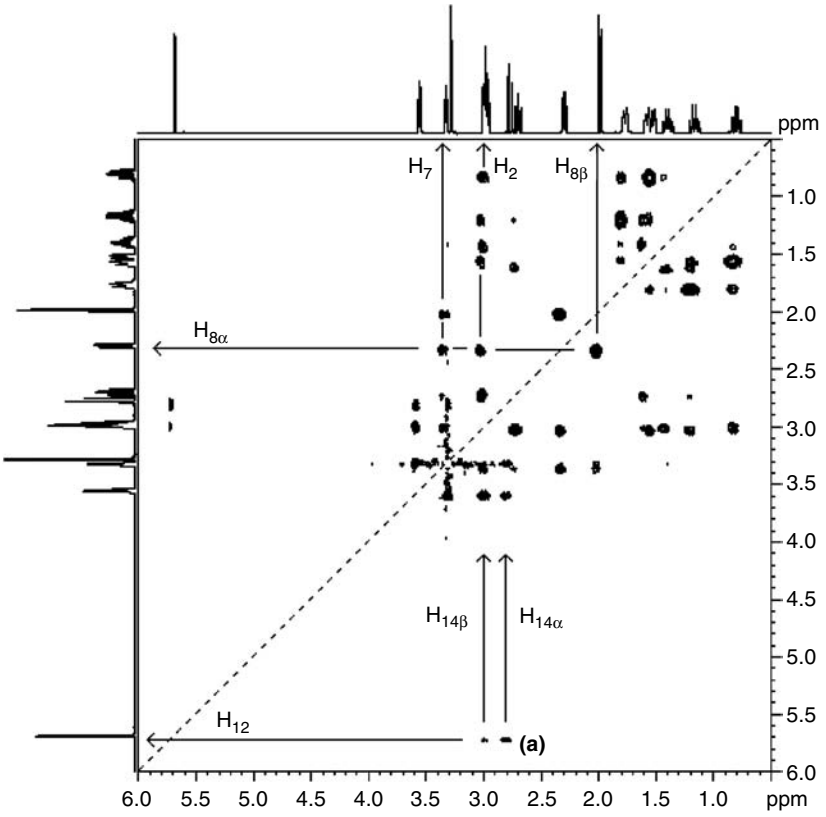

FIGURE 3.17 The ge-NOESY spectrum of 15α-methoxy-14,15-dihydrophyllochrysine (**1**).

can be used. In the rotating frame of reference, nOe enhancements are positive irrespective of molecular size. The 2D-ROESY spectrum is identical in appearance to the NOESY spectrum, however, the analysis of the spectrum can be complicated by the appearance of COSY and TOCSY cross peaks. One dimensional versions of the ROESY experiment are also available.[196,203,204,206,207]

3.10 THREE DIMENSIONAL EXPERIMENTS

Modern NMR spectroscopy offers a comprehensive range of efficient 1D and 2D experiments that are usually sufficient for determining the structures of bioactive natural products. In cases where extensive overlap of peaks in 2D experiments hides key information, analysis may be aided by spreading the data into a third frequency dimension.[6,62,208] Such experiments, which produce large data sets, are not routine and will only be described briefly.

Three dimensional (3D) experiments are usually derived by the effective combination of 2D experiments, and incorporate three time periods. Peaks in the 3D spectrum, which result from Fourier transformation with respect to the three time periods, are points in 3D space. The spectra are analyzed by selecting planes from the 3D spectrum. For example, a plane selected at the chemical shift of a particular carbon atom in a 3D HMQC–COSY[208] experiment will show a COSY spectrum displaying only the interactions associated with the protons attached to the particular carbon atom. Similarly, selections of the planes associated with the other carbon atoms will allow the analysis of the related COSY interactions.

3.11 CONCLUDING REMARKS

In the preceding sections, only selected information arising from each spectrum has been discussed, with the aim of indicating how the analysis of each spectrum is approached. Thorough analysis of each spectrum and integration of all of the available information is required to arrive at the correct structure. Demands on instrument time will often necessitate the selection of those experiments that are most likely to give the required information in the shortest time. The choice between a 2D technique and its 1D analog is usually based on the number of interactions to be determined, the quantity of sample, and the amount of instrument time available.

Although X-ray crystallography has been nominated as "the ultimate arbiter of chemical structures",[57] there are many constraints on this technique. NMR is still the first technique turned to by research workers in the quest to determine the structures of biologically active organic compounds.

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4 Quantitative NMR of Bioactive Natural Products

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CONTENTS

4.1 INTRODUCTION

4.1.1 The New Kid on the Block?

4.1.1.1 A New Potential Role for NMR

As natural products research evolves from a basic chemistry-oriented science into an interdisciplinary effort located on the chemistry–biology ("bioactivity") interface, the requisite technology is advancing rapidly. Today, the concerted acquisition of a standard set of one dimensional (1D) $^{1}H/^{13}C$ and two dimensional (2D) COSY, HSQC, NOESY/ROESY, and HMBC NMR spectra, together with other spectroscopic and spectrometric information, in particular MS/MS^{n}, allows for almost routine structural identification of known, or elucidation of unknown, "small" organic molecules with varying degrees of structural complexity. From the perspective of interdisciplinary research groups with a keen interest in the overarching theme of bioactive natural products, the wealth of structural NMR methodology already seems sufficiently complex. Consequently, the addition of yet another implement to the NMR toolbox can appear to be excessive at first sight.

However, this assessment is unbalanced when considering the biological and chemical implications of the insights that quantitative structural information can provide, including, for example, the relative composition of mixtures or the purity of compounds that result from a long isolation/purification process. It is reasonable to assert that the benefits and potential long-term implications of quantitative NMR (qNMR) far outweigh the investment in effort that is required when qNMR is incorporated into routine characterization protocols for biologically active natural products.[1] Informally, qNMR can be viewed as "the new kid on the block" in a neighborhood inhabited by a myriad of routine 1D and 2D NMR experiments,[2] beside other major spectroscopic and spectrometric methods, for example, MS, CD/ORD, and IR. The objective of this chapter, which will deal mainly with quantitative ^{1}H NMR (qHNMR), is to explain qNMR concepts and outline qNMR technology, provide examples of applications, and develop a perspective of how the new "fellow citizen" can be advantageously integrated into the neighborhood of advanced analytical tools for the characterization of bioactive natural products.

4.1.1.2 Definition of qNMR Terms

Although qHNMR can already be considered to be as routine as "standard" ^{1}H NMR (HNMR), it is still reasonable to emphasize the quantitative aspect of the qNMR experiments. Therefore, due to the lack of official nomenclature, in keeping with the majority of previous literature, and for the sake of consistency in nomenclature and a previously proposed working definition,[1] the term "quantitative NMR" will herein be abbreviated as "qNMR". Accordingly, the small preceding character "q" for "quantitative" is used in the same way as, for example, "g" is commonly used to designed "gradient-enhanced" or "gradient-selected" NMR experiments, such as in "gHSQC". Designation of the observed nuclei, for example, protons (^{1}H), is added in analogy to accepted nomenclature, and

leads to "qHNMR" to describe the basic quantitative 1D proton NMR experiment, which will be the main focus for the rest of this chapter.

4.1.2 The Biological Perspective of Quantitative NMR

4.1.2.1 Biological Activity and qNMR Correlation

To elicit biological activity, an agent must have a specific chemical structure or contain essential structural features (the "pharmacophore" model). Thus, variation of chemical structure produces variation in biological activity, either in the form of the change of potency, for example, the radical-scavenging polyphenolic antioxidants in which reactive potential correlates with biological effect, or by completely changing the type of biological response such as androgenic, gestagenic, and estrogenic steroids.

Developing analogs or derivatives of a natural or synthetic lead compound ultimately represents a semiempirical, targeted variation of its structure and subsequent generation of structure–activity relationships, with the ultimate goal of finding the most potent (pharmacodynamics, activity) and most suitable (pharmacokinetics, toxicity, formulation) candidate.

Since biological activity and chemical structure are directly connected (Figure 4.1), all structural information that can be gleaned from a biologically active compound or material is relevant. NMR methodology can contribute in multiple ways to this biology–chemistry relationship by virtue of both its comprehensive qualitative (measuring δ, J, nuclear Overhauser enhancements [NOEs], T_1, T_2) and quantitative nature (qNMR). It is this combination of NMR and qNMR that forms a synergistic information hub, which enhances the supply of valuable structural data about the "small" (low molecular weight) natural molecules that are involved in biological activity. Accordingly, the NMR/qNMR combination can be utilized to perform the following tasks:

- Determine or confirm a chemical structure
- Establish structure–activity relationships and provide insight into structural equilibria (e.g., tautomerism or zwitterionic formation)
- Determine the purity of the bioactive material
- Reveal the presence of impurities that are known to interfere with the bioassay
- Probe for the presence of closely related compounds, often found in natural products analysis, that is, the "biosynthetic cocktail"
- Explore the composition of a mixture, that is, the metabolomic approach.

It should be emphasized that, especially when compared to their synthetic counterparts, natural products are unique in that they have to be purified from complex matrices, the "biosynthetic cocktails". This places additional demands on the processes (mostly chromatographic) involved in their purification and complicates the structural analysis. In this context, NMR itself has intrinsic differentiation capability, that is, the chemical shift (δ) 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 via spin–spin (J) or dipolar (D) coupling, heteronuclear chemical shift (δ_X), or diffusion (D), which adds significantly to the differentiation power of NMR.[2] By adding a combined quantitative–qualitative dimension to the structural NMR portfolio of the analyte, qNMR in general, and qHNMR in particular, can make valuable contributions to the overall picture that correlates biological activity with chemical structure (Figure 4.1).

4.1.2.2 Metabolomics and Biomarkers

At any given time, biological systems from single cells to mammals exhibit distinct chemical profiles comprising primary and secondary metabolites (a metabolome) indicative of their overall biological

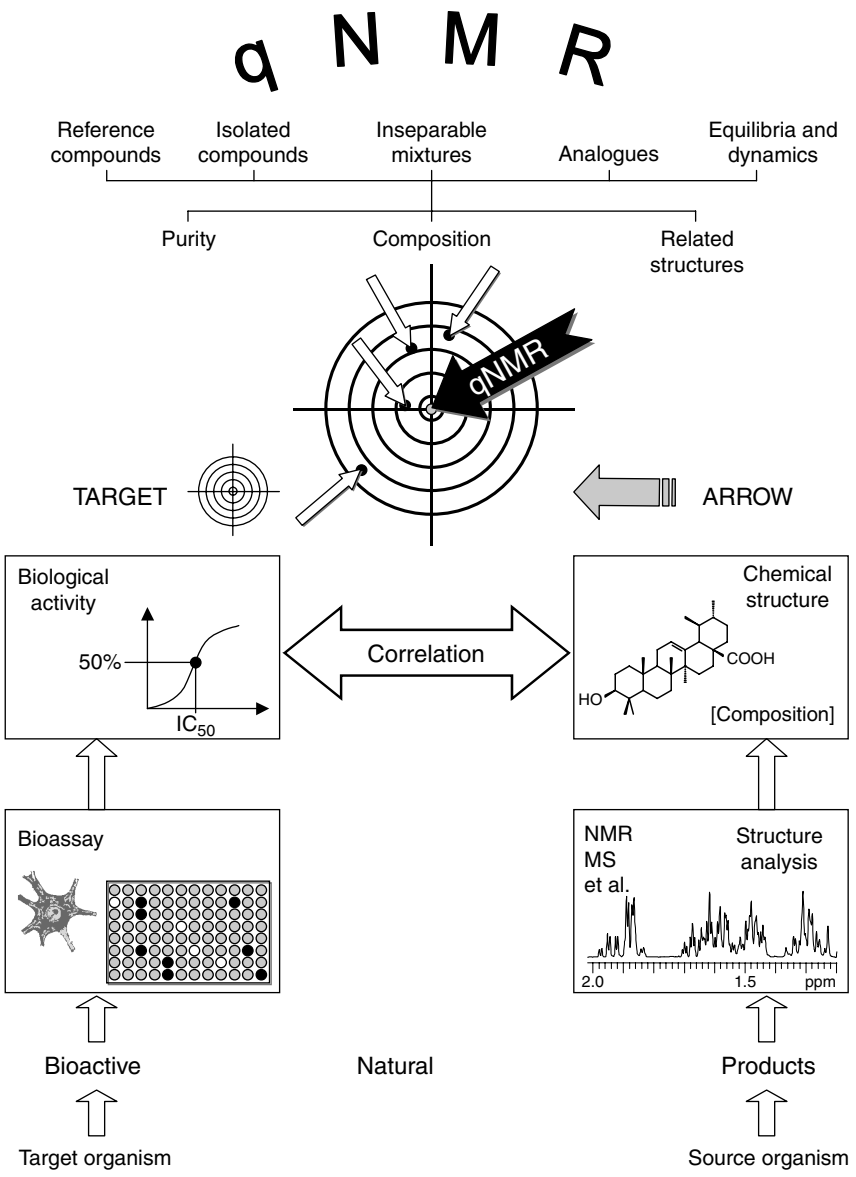

FIGURE 4.1 The overarching role and potential impact of qNMR in the study of bioactive natural products. Toward the principal goal of correlating biological activity and chemical structure, qNMR contributes valuable information beyond qualitative aspects of structure by adding the quantitative dimension. Implementing qHNMR analysis in the routine spectroscopic workflow provides regular access to information regarding purity, composition of mixtures, and related structures with no significant additional effort.

status. Metabolomics seeks to define this status by analyzing profiles of metabolites ("metabolomic fingerprints") of cells, tissues, organs, biofluids, or in some cases whole organisms, either *in vivo* or from extracts. The complexity of the fingerprint and, indeed, the whole undertaking is obvious. Depending on the method used, comprehensive analysis of biofluids or other representative samples is capable of generating hundreds or thousands of metabolite signals.

Aside from their identification and assignment, one main challenge is the actual selection of biomarkers, which are specific analytes that allow pinpointing of a certain biological status.

In health-related research, a frequent objective is to identify metabolic biomarkers that are related to major public health challenges. One exemplifying application of HNMR-based metabolomics involves environmental toxicology, where studies using the Japanese medaka (*Oryzias latipes*) embryo assay showed that metabolic endpoints provide more sensitive, specific, and unbiased biomarkers of toxicity than nonspecific indicators such as hatching success.[3]

In the context of applying automated NMR for metabolomic analysis ("metabonomics"), the INTERMAP study has shown that HNMR represents a very robust analytical tool.[4] Not only was HNMR found to be highly reproducible, it can also be used with quantitative accuracy. This exemplifies and underlines the potential of qHNMR for NMR-based metabolomic applications and in the search for biomarkers.

4.1.3 The Benefits of Quantitative NMR

4.1.3.1 The Dual Character of qNMR

Although the number of publications employing qNMR has been steadily increasing, it has not kept pace with the almost exponential increase in the total amount of published scientific literature.[1] In this context, another observation is that qNMR assays have been widely used in the pharmaceutical and chemical industries but, typically, have remained unpublished and, thus, inaccessible and invisible to the scientific community, due to their proprietary nature. In the recent literature, however, it has become customary to designate qNMR as a separate entity of NMR analysis. Because NMR spectra in general, and 1D NMR spectra in particular, contain some degree of quantitative information by default, it could be argued whether a separate designation of qNMR versus NMR is indeed justified. One factor to be taken into account, however, is that the quantitative nature of the workhorse NMR experiment, that is, the "standard" 1D HNMR spectrum, has been living in the shadows in terms of further development for most of the half century since NMR was established, regardless of whether acquisition was performed in a continuous wave (CW) or FT mode. Thus, whereas the qualitative, that is, structural, information in HNMR spectra has been widely exploited, the quantitative information has mostly been neglected. The process of stimulating this valuable feature of HNMR spectra has begun only recently as evident from several publications dealing with fundamental aspects of qNMR.[5–9]

4.1.3.2 The No-Cost Extension Aspect of qHNMR

Possibly one of the most attractive features of qNMR analysis of natural products is that it essentially comes at no extra cost. Adding qNMR to the standard identification portfolio of an isolated pure compound, which typically consists of physicochemical, spectrometric, and spectroscopic data, for example, UV, IR, ORD/CD, MS/HR-MS, ^{1}H NMR, ^{13}C NMR, and 2D NMR, does not create significant extra experimental expense in the form of spectrometer time or supplies. The only additional investment is the time needed to evaluate the quantitative information in the 1D qHNMR spectra, provided such data are available. The proposal to add qNMR evaluation to the standard portfolio of bioactive substances does not, therefore, add much effort to the already demanding requirements for compound dereplication or structural elucidation, and does not necessarily raise the bar for publication. More likely, qNMR provides valuable information concerning purity, relative composition and constitution of the active agent, and has great potential to enhance consistency in the literature of bioactive natural products. As detailed below, on today's high-field FT-NMR spectrometers only minimal efforts are required to migrate from what is widely considered a "standard" 1D HNMR experiment to the implementation of a qHNMR experiment for routine HNMR spectroscopy. Thus, in terms of costs and efforts, qHNMR can be considered a "no-cost extension" of current day-to-day, workhorse HNMR analysis.

4.1.4 General Quantitative NMR Considerations

4.1.4.1 General Advantages of qNMR

From a practical point of view, one of the most striking advantages of qNMR is its nondestructive nature. This is particularly important for the precious, mass-limited samples representative of many natural products, and allows for qNMR analyses to be performed prior to, or in parallel with, their evaluation in a bioassay using the very same sample, that is, "bioassay ex-NMR tube". A second major advantage of qNMR is that, compared to all other spectroscopic/spectrometric methods, it has the nearest to constant proportionality to the concentration of the analyte. This applies, in particular, across all ^{1}H-containing stoichiometric compounds, provided "quantitative conditions" are met when conducting the qHNMR. Third, qNMR is unique in that it permits "weighing the unweighable": As long as concentrations and volumes are known, masses can be determined directly by comparison with precisely weighed external and internal standards, in turn directly providing the limits to purity on a weight/weight basis. Owing to the excellent linearity of qNMR over a wide dynamic range, this approach can be scaled down to "practically unweighable" amounts of natural products, such as microgram amounts of minor isolates that were obtained from a bioactivity-guided isolation procedure.

4.1.4.2 Nuclei Suitable for qNMR

Although, in general, qNMR can be applied to all NMR-observable nuclei, the proton-detected variant, that is, qHNMR, will remain the qNMR workhorse for natural products analysis due to sensitivity considerations, until further major enhancements in NMR sensitivity can be achieved. The recently increasing availability, in both industrial and academic settings, of ultra-high-field spectrometers (750–900 MHz) fitted with cryoprobes offers unique opportunities for qHNMR analysis, since they increase signal dispersion and reduce data collection time by factors of up to 4–8 times when compared to routine 400–500 MHz instruments.

Given the dominance of C, H, O, and N in most natural products, ^{13}C (qCNMR)[10–12] is the next best alternative to protons, but is limited to only 1.6% of ^{1}H sensitivity for equal numbers of nuclei at constant field, and has only 1.1% natural abundance.[13] Very little qNMR work has been done on nitrogen (^{14}N/^{15}N, qNNMR[14,15]) or oxygen (^{17}O, qONMR[16]), both of which suffer from severe limitations in sensitivity and cannot be considered workable target nuclei for qNMR analysis of bioactive natural products at this point of technology development. For phosphorylated natural products, however, ^{31}P qPNMR can be a very attractive method that, similar to qCNMR, has the advantage of a wide chemical shift dispersion (hundreds of ppm) and signal resolution. Similarly, ^{19}F qFNMR has a high sensitivity (83% of ^{1}H) and chemical shift range but, at present, it is a specialty method for a small group of fluorinated natural products.[17,18]

4.2 THE QUANTITATIVE NMR EXPERIMENT

4.2.1 General Considerations

Performance of qNMR experiments requires the establishment of quantitative conditions, which relate to both NMR hardware and software settings, as well as to considerations associated with the sample itself. To provide guidance for the establishment of quantitative conditions, five main points are addressed in the following sections: (i) preparation of the sample (Section 4.2.2); (ii) and (iii) achievement of signal proportionality and calibration (Section 4.2.3); and (iv) organization of a unified qNMR workflow (Section 4.2.4), including optimization of acquisition parameters (Section 4.2.4.1) and postacquisition processing (Section 4.2.4.2).

4.2.2 Sample Preparation

In solution NMR, sample preparation starts with the choice of the solvent. There are more things to consider than just "sample solubility" when it comes to the choice of an appropriate solvent,

especially when doing qHNMR: (i) the solvent signal(s), typically the signal(s) of the residual protons in deuterated solvents, as well as the resonance of dissolved water in organic solvents, might overlap with compound signals; (ii) the influence of solvent viscosity on the spectrum resolution; and (iii) exchangeable deuterium contained in (residual) water or protic solvents such as CD_3OD, but not CD_3OH, might affect the detection of exchangeable protons in the molecule. As exchangeable protons can lead to broad signals (depending on the exchange rate), appearing as "bumps" or other imperfections in the baseline that cannot be reliably integrated, they are not eligible for quantification purposes. This, however, does not necessarily mean that the last point can be neglected for qNMR purposes.

Unless solubility or polarity dictates the choice, the nonviscous solvents (e.g., acetone, acetonitrile, chloroform, dichloromethane, and methanol) are preferable over the more viscous solvents (e.g., benzene, dimethylformamide [DMF], dimethylsulfoxide [DMSO], pyridine, toluene, and water). Certain applications for quantified solutions, such as the preparation of reference materials for subsequent use with mass spectrometric techniques, may preclude the use of fully deuterated solvents.[5] Chloroform is the solvent of choice for quantitative assessments due to the good locking of the $CDCl_3$ signal and the good shimming performance of the sharp singlet of its residual solvent signal, and the low costs. However, the instability and pH reactivity of many compounds in the presence of residual acid in chloroform should be considered. For more polar compounds, the authors prefer the use of methanol over DMSO, due to improved chemical shift dispersion leading to better differentiation of intramolecular and intermolecular signals.[19] With regard to potential overlap between the analyte and the residual solvent and water signals, reference should be made to textbook literature[20,21] and charts supplied by NMR solvent manufacturers.

As any extraneous signal is a potential detriment for quantitation, particular care should be taken in sample preparation as well as solvent handling and storage. Many of the organic solvents, including deuterated water (D_2O), are hygroscopic, requiring a desiccator for storage of bottles and avoidance of exposure to air. Commercially available NMR solvents range in deuteration grades from 99.5 to 99.995%. However, these specifications only refer to the degree of deuteration and not to other impurities, which means that the "highest quality" solvent is not necessarily the purest. Prescreening of batches of NMR solvents can be a good investment of time since quality can vary greatly from batch to batch and also among manufacturers.

Solutions for qHNMR need to be clear and free from dust and fibers. Samples are best filtered directly into the NMR tube keeping in mind the inertness and leachability of the filter material. The sample as well as all tools, namely NMR tubes, pipettes, filters, etc., are best dried in a desiccator prior to use in order to minimize water contamination and the consequent, large analytically disturbing water signals. If internal standards are used, or absolute quantitation is required, it is crucial to obtain the precise weight of the analyte, measured using a microanalytical balance operating in the precision range of 0.1 mg. In this latter case, good solubility of the sample material is especially important since losses may occur during filtration. Weighing the sample directly into the NMR tube is complicated, requiring special practical skills, but has the obvious advantage of producing the most reliable absolute weight since no sample transfer is required. Internal standards can be added directly or as normalized solutions. Whenever volumetric steps are involved, the total solvent volume needs to be determined to allow for subsequent quantitative calculations such as percentage purity. In a series of qHNMR experiments, it is helpful to standardize the filling height of the NMR tube in order to simplify calculations and to facilitate manual or automatic shimming.

To ensure suitability for qNMR experiments, most readily assessed by the general ability to perform good shimming on the sample and to achieve high resolution (e.g., the *o*-dichlorobenzene [ODCB] resolution test), NMR tubes should be of high precision and quality, and free from dust and scratches. Standard 3 or 5 mm outer diameter tubes with 7 or 8 in. length are suitable. Having used quality NMR tubes from different manufacturers, the authors are unaware of significant differences in performance or other factors that affect suitability for qNMR. Constancy of cross-sectional area of tubes can be monitored by "absolute" NMR measurements which include all contributions to

signal intensity.[5] Provided that the aforementioned sample preparation guidelines are followed, the constituents of any given mixture can routinely be assessed down to a level of 1–2%. Occasionally, much lower levels of quantitation (0.1% or even ppm range) can be achieved, especially for low molecular–weight compounds such as solvents.[11]

4.2.3 Signal Proportionality

4.2.3.1 Radio Frequency Excitation and NMR Observables

When considering NMR experiments in general, but specifically the quantitative aspects of qHNMR experiments, excitation of all of the proton spins in the sample is achieved by irradiation of the sample with a high-power radio frequency (RF) pulse tuned to the proton frequency. All of the ^{1}H spins in the sample are excited at once. The excitation bandwidth, that is, the region of the proton spectrum being excited, is inversely proportional to the length of the pulse,[22] which typically lasts several microseconds. Therefore, to achieve uniform excitation over the ^{1}H frequency range of interest, the pulse must be short enough to adequately cover this range. This is critically important for good qNMR and is more difficult to achieve with nuclei having a large chemical shift range. The resultant signal response, which is known as a free-induction decay (FID), or time domain spectrum, is subjected to a digital Fourier transformation (FT). The result of the FT is conversion of the time domain spectrum (FID) into the more familiar frequency domain spectrum, from which structural information pertaining to the sample may be extracted in the form of NMR observables; that is, chemical shift (δ), spin–spin coupling (J-coupling [Hz]), and quantitative integrated intensity information.

In qHNMR, the integrated intensities of the signals in a sample are directly proportional to the number of spins giving rise to the signal. The ratios of these integrated intensities are used to derive the number of protons present within a molecule. For example, if a sample represents a single chemical species, information about its structure can be obtained from (i) the NMR chemical shifts (δ), which depend on the chemical environments within the molecule; (ii) the J-coupling information, which reveals how the protons are chemically bonded and thus how structural components are assembled within the molecule; and (iii) the quantitative measure of integrated signal intensities, which affords ratios of the number of protons at different sites within a molecule. This important fundamental aspect of the NMR technique has served as the foundation of structural NMR spectroscopy that has been widely used and routinely applied for decades.

4.2.3.2 External Quantitative Analysis

The fact that protons within one molecule can be related quantitatively for structural purposes (internal or intramolecular quantitative analysis) suggests that this technique can also be employed for external quantitative analysis, that is, at an intermolecular level. Thus, if a known chemical entity has an impurity, the "ideal" situation for qHNMR is met when the NMR observables (i.e., chemical shifts, J-couplings) of the impurity are also known and when the signals from the impurity are "out in the open", that is, with little or no overlap of signals arising from the major chemical entity with signals arising from the minor impurity. Consequently, selection, integration, and comparison of signals arising from the major and minor components, and correction for the number of protons that each of the signals represents, will provide a ratio of the impurity to the major chemical entity. Normalizing the result to 100% provides a quantitative measure of the impurity level. The essence of this very simple illustration is representative of the most currently practiced application of qNMR techniques.

4.2.3.3 Spectrometer Hardware Configuration for Removal of Sidebands, Artifacts, and Satellites

The success of "internal" qNMR, for structural purposes, combined with "external" quantitative analysis, for the purposes of assessing amount and purity, depends on the presence of necessary

spectrometer hardware, on proper hardware configuration, and on the software-controlled acquisition parameters that control the hardware operation. There are key acquisition parameters that require optimization to create accurate and precise qNMR data, irrespective of the observation nucleus. However, discussions here will be restricted to the specific details of qHNMR experiments.

Although internal quantitative qHNMR analysis to obtain the internal ratios of protons within a single molecular system is routinely practiced, there are two critically important experimental conditions concerning the spectrometer hardware configuration that are necessary for successful external qHNMR analysis, that is, sample spinning (or lack thereof) and removal of ^{13}C satellites.

4.2.3.3.1 Sample Spinning

qHNMR data should be acquired on a static sample, that is, the NMR tube should not be spinning during the data collection. This serves to eliminate residual spinning sidebands, albeit small but frequently comparable in size to low-level sample impurities, from the resultant spectrum and, thus, reduces the effects of overlap. The sidebands can reduce the accuracy of quantitation by distributing the total area of each peak between the central resonance and the equally spaced sidebands, which may overlap with other peaks. Spinning also modulates the probe *Q* factor, which can adversely affect the steadiness of probe RF tuning and matching, and RF pulsing conditions, thereby degrading the accuracy of comparisons with external standards in separate tubes. Spectrometers produced within the past 15 years allow ready shimming of static samples to <0.5 Hz line width (where solvent viscosity permits), with excellent lineshape.

4.2.3.3.2 Removal of ^{13}C Satellites

Simplification of qHNMR spectra may be achieved with broadband decoupling of the ^{13}C region to remove the ^{13}C satellites from the ^{1}H spectrum. When HNMR spectra are acquired from a compound at natural ^{13}C-abundance (1.1%), 98.9% of the ^{1}H nuclei are bound to ^{12}C, a nonmagnetic "NMR-silent" nucleus, whereas the remaining 1.1% are bound to ^{13}C, which, with a spin quantum number = 1/2, is an NMR-active nucleus. The signals arising from ^{1}H attached to ^{13}C are split into a doublet with a separation equal to $^{1}J_{C,H}$ (125–250 Hz). On the one hand, each component of the doublet contains 0.55% of the total ^{1}H resonance intensity (half of the ^{13}C natural abundance of 1.1%), which can complicate the assessment of the impurity level in a sample. On the other hand, because the natural abundance of ^{13}C shows little variation across natural products of differing origin, the ^{13}C satellites can serve as a reasonably precise internal standard for impurity levels.[23]

Decoupling of ^{13}C from ^{1}H can be achieved by applying strong RF irradiation at the appropriate ^{13}C frequency (^{13}C frequency = ^{1}H frequency/3.9767). Since ^{13}C resonances are usually dispersed over a large frequency range, broadband decoupling of ^{13}C requires higher power than for decoupling of ^{1}H, and the consequent heat generation can be deleterious to qNMR. Composite-pulse decoupling schemes require less power for a given bandwidth of decoupling, and the authors have successfully used globally optimized alternating-phase rectangular pulses (GARP) decoupling,[24] in which the decoupler is turned on only during the acquisition time (an "inverse-gated" decoupling technique), thereby reducing heating effects to the sample and concurrently providing good decoupling over a wide ^{13}C spectral region of about 220 ppm, while avoiding the induction of {^{13}C}-^{1}H NOEs. Initial reports of the use of this decoupling approach to ^{1}H qNMR utilized "Waltz-16" decoupling.[25,26] The pulse sequence profile for the qHNMR experiment in conjunction with GARP composite-pulse decoupling is illustrated in Figure 4.2.

4.2.3.4 Other Considerations

Acquiring a qHNMR spectrum in a nonspinning mode and with broadband (GARP) ^{13}C decoupling of the region produces an HNMR spectrum that only consists of signals arising from the major component(s) and the minor impurities. Removal of the spinning sidebands and ^{13}C satellites serves to eliminate ambiguity regarding which signals arise from impurities and which signals arise from sideband artifacts.

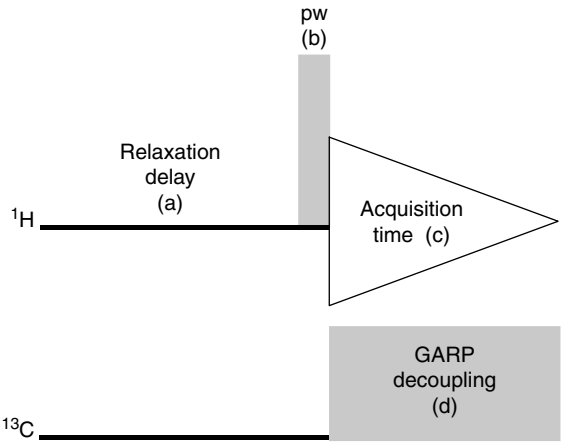

FIGURE 4.2 A standard pulse profile for qHNMR experiments showing (a) the relaxation delay, (b) the excitation pw, (c) acquisition time of the ^{1}H FID, and (d) GARP decoupling of ^{13}C during the acquisition time (inverse-gated decoupling).

At this point it should be noted that this assumes that all other basic spectrometer functions must be properly working and correctly set (e.g., absence of quadrature phase images, proper RF in-line filters, accurate frequency tuning and impedance matching, calibration of pulse width and power levels of observed and decoupler pulses, etc.). All contemporary NMR spectrometers are equipped with the necessary hardware to perform the ^{13}C heterodecoupling function since this capability is also a requisite for executing various other types of NMR experiments such as multidimensional NMR. This enables the ^{13}C-decoupled qHNMR experiment to be performed in a routine manner.

4.2.4 Common NMR Workflow

4.2.4.1 Optimization of qHNMR Acquisition Parameters

In addition to the aforementioned specific spectrometer hardware requirements necessary for performing the qHNMR experiment, there are a number of acquisition parameters that are under software control. These enable further manipulation of the hardware to ensure a favorable outcome of the qHNMR experiment and they need to be properly adjusted or experimentally optimized. The following discussion addresses, in a practical context, the key acquisition parameter settings and their effect on the qHNMR experiment and hopefully serves as a set of guidelines, or "cookbook recipe", for acquiring qHNMR data on virtually any contemporary pulsed NMR spectrometer.

4.2.4.1.1 Spectral Window Selection

The spectral window, or the region of RF excitation and observation that is used in a qHNMR experiment, partly depends on where the signals of interest reside. In general, spectral windows of width W (Hz) can be preselected as a component of a qHNMR standardized acquisition parameter set. Initially, a rather wide qHNMR spectral window can be predetermined empirically. This can then be optimized for each solvent, and adjusted to the specific conditions of a sample. The final spectral window for qHNMR should, however, always include ca. 2 ppm added on to both the high-field and low-field ends of the desired region of interest. This is recommended regardless of instrumentation but particularly in older instruments that do not have oversampling and digital filtering (also known as digital signal processing). The analog audio frequency filters (hardware), used to restrict or eliminate aliasing (folding) of unwanted signals from outside of the desired spectral window, multiply the intensity of the resonances by a function that is uniform over the central region (possibly with

some "ripple") and tailing off toward zero at each end. As a result, signal intensity appearing at the ends of the spectral window (out of the uniform region of the analog filter) is severely attenuated (>70%) and also phase-distorted. This will lead to significant errors in the integrated intensities of the spectrum when attempting any kind of quantitative application. On most contemporary NMR spectrometers, the use of oversampling and digital filtering provides a highly uniform filter function with very sharp cut-offs at the edges of the pass band, freedom from phase distortion, and improved baseline response; however in practice it is still recommended to use a somewhat wider spectral window than the region containing NMR signals. It is also important to ensure that the region being excited by the RF pulse is wider than that is necessary from the sheer perspective of the observed signal range, by using an excitation pulse with duration $\ll 1/W$,[22] necessitating high-power RF pulses to obtain uniform 90° excitation with high-field (>600 MHz ^{1}H frequency) instruments. Taken together, the above precautions should lead to a flat baseline with undistorted integrated intensities, uniform phase, and elimination of signal attenuation at the edges of the spectrum.

4.2.4.1.2 Transmitter Offset Selection

In NMR, the transmitter RF used for the excitation of the desired spectral region is offset from the basic RF of the spectrometer and is typically set in the center of the region of interest. Accordingly, the transmitter offset is a software parameter that controls the frequency of the transmitter pulse and, therefore, the location in the HNMR spectrum, where the transmitter output (hardware) will be set. On many spectrometers, the position of the transmitter is automatically adjusted depending on the spectral window selected, or it can again be predefined as a parameter value in a standardized qHNMR parameter set. By keeping a wide predefined spectral window fixed, the position of the transmitter offset should be manually adjusted, depending on the solvent used, to cover the specific range of signals in the sample of interest.

4.2.4.1.3 Pulse Width Selection

Spin lattice relaxation time (T_1) is an important factor in NMR quantitation. Repeated pulses are usually required to achieve adequate signal-to-noise ratios (S/N) in the proton NMR spectra of natural products and, therefore, sufficient time between pulses must be allowed for full recovery of sample magnetization. The pulse width (pw) represents the length of pulse duration (in microseconds) that converts equilibrium magnetization (z) into transverse magnetization (x, y) at a specified transmitter power level. If all equilibrium magnetization is converted into transverse magnetization by the application of an RF pulse, this is defined as a "90° pulse". A single 90° pulse will produce maximum signal intensity from fully relaxed nuclei. However, if a 90° excitation pulse is followed by successive 90° pulses spaced by intervals that are not large compared to the spin lattice relaxation time T_1, the signal following each pulse will be partially saturated. When it is important to obtain the maximum S/N in a given time from a particular signal, the optimum choice of flip angle (α) for a relaxation delay (d_1) is the Ernst angle (α_E),[27] defined as

$$\cos \alpha_E = e^{-d_1/T_1}$$

Although this relation is a useful guide to the choice of conditions for obtaining, for example, ^{13}C spectra from small amounts of natural products, it is not appropriate for precise qNMR since the relative intensities of peaks from analytes and standards will vary to different extents depending on their individual T_1 values. Although T_1 values of individual nuclei may be determined by the inversion recovery experiment,[28] this is a slow process particularly with limited amounts of sample, since spurious results will be obtained unless relaxation delays $>5T_{1max}$ are employed during the pulse sequence. Where the T_1 relaxation times of the sample are unknown, or in the case that individual T_1 determinations are impractical, it is a reasonable approach to assume that the majority of protons will have $T_1 < 2$ s in most solvents. However, it has to be kept in mind that this assumption may severely limit achievable precision and, therefore, should not be made when doing precise qNMR work.

Consideration of the interdependence of α, T_1 for a particular nucleus, and the pulse repetition rate (T), shows that for qHNMR 90° pulses combined with long T (such that $T/T_1 > 4.61$) will lead to a better S/N than a smaller flip angle with a shorter delay, where the criterion in both cases is completeness of relaxation to better than 1% between successive accumulations.[29] Usually some compromise is necessary, as not all peaks of a given analyte will have the same T_1. Although measurement of the T_1 values of all protons can be a time-consuming process with mass-limited samples, it only needs to be performed once per analyte and will provide important information for the subsequent qHNMR analysis. Depending on the degree of quantitative accuracy desired, however, it is usually more time-efficient to use "long" relaxation delays (d_1, 15–60 s), assuming that most T_1 values will be less than one-fifth of this value in most solvents, with 90º pulses, which will also reduce the error resulting from an erroneous setting of the pulse length and minimize heating effects in ^{13}C-decoupled qHNMR.

4.2.4.1.4 Acquisition Time Selection

As noted earlier, following RF excitation, creation and measurement of the FID represents the basic acquisition of a 1D NMR spectrum. The length of time that is spent to acquire (or digitize) the FID is defined as the acquisition time. The acquisition time selected is related, in part, to the spectral window and to the desired level of digitization of the resultant NMR spectrum upon FT, and conforms to the Nyquist[20,30] relationship. In general, fine digitization (low Hertz/point values; typically <0.2 Hz/point) is preferable for quantitative work. For example, at a proton frequency of 400 MHz, it can be generally recommended to select 64 K real data points for a 20 ppm spectral window to yield 0.125 Hz/point digital resolution, which results overall in an acquisition time of 4 s. Reducing the acquisition time to 2 s results in the sampling of 32 K real data points, and acceptance of reduced digital resolution at 0.25 Hz/point, but it will reduce sample heating if ^{13}C decoupling is used. Some improvement in digitization, or restoration of the lost digitization due to a reduction in the acquisition time, can be achieved by postacquisition zero filling of the FID by a factor of two. Zero filling beyond a factor of two will serve to improve the overall smoothness of the spectral information, but will not replace actual resolution in an otherwise truncated FID.[31]

4.2.4.1.5 Relaxation Delay Selection

The delay, in seconds, following the pulse in a qHNMR experiment is referred to as the relaxation delay (d_1). This delay is inserted to allow the excited nuclei to reestablish their equilibrium z-magnetization before application of the next pulse occurs. If the pulse excitation is a 90° pulse, that is, all equilibrium magnetization is converted into transverse (x, y) magnetization, the relaxation delay should be set to five times the longest relaxation time (T_1) of any proton in the sample. If the pulse excitation uses a <90° pulse, for example, a 30° or Ernst angle pulse, a shorter relaxation delay can in principle be employed. The optimum relaxation delay can be empirically determined by setting the excitation pulse width to a fixed value, while varying the value of the relaxation delay and monitoring the variation of the integrated intensity per pulse of the resultant spectrum. For a qHNMR experiment, this is the recommended approach to optimizing the relaxation delay for the given NMR hardware, keeping in mind the sample dependence of T_1.

Another consideration that requires examination is the time that the FID takes to decay to essentially zero intensity, below the noise level. If the acquisition time is set to 4 s, but >95% of the FID signal intensity has decayed within 2 s, then a shorter relaxation delay can be used. The sum of the acquisition time and the relaxation delay is known as the pulse repetition time.

A not so obvious aspect of the recycle delay that needs to be mentioned here relates to the ^{13}C GARP decoupling previously discussed and the heat generation, associated with the broadband decoupling, which is produced during the acquisition time. On the one hand, the recycle delay can be reduced in length while still avoiding relaxation time effects and obtaining good quantitative results. On the other hand, because decoupling of the carbon frequency is achieved during the acquisition, it is generally necessary to lengthen the recycle delay to bring about a reduction of the duty cycle of the GARP decoupling in order to reduce unfavorable heating effects. For the previously introduced

example with an acquisition time of 4 s (400 MHz, 64 K real data points, 20 ppm spectral window, 0.125 Hz/point digital resolution), a recycle delay of 20 s is necessary to create a 20% decoupling duty cycle. For a 2-s acquisition time, the minimum required recycle delay becomes 10 s accordingly. As resonances are integrated, the extra resolution obtained by increasing the acquisition time above 2 s has no further advantage for quantitation. In general, it is important to reduce the heating effects as they can lead to line broadening and, if the sample is heat sensitive, to degradation of the sample during the NMR experiment. Heating should also be avoided when using separate external standards (Sections 4.2.3.2 and 4.3.3.2).[5]

4.2.4.1.6 Setting the Number of Scans or Transients

Selection of the number of scans or transients will depend on the level of desired sensitivity and the molecular weight and molar concentration of the analyte(s). Sensitivity is generally defined as the achieved S/N of the spectrum with respect to a particular signal in the sample. For a qHNMR spectrum, sensitivity will not only depend on the amount of sample but also on the sample complexity and the level of impurities present. It is important to note that the overall S/N of the resultant spectrum will increase only with the square root of the number of scans or transients. For the qHNMR experiment, the better the S/N of the spectrum, especially with respect to the lowest-level component/impurity in the sample, the better the quantitative accuracy that can be achieved. Thus, selection of the number of scans or transients for a qHNMR experiment will be variable and sample dependent. For example, in natural product applications of the qHNMR experiment involving isolated compounds or fractions, the number of scans could be typically 128–1024 (1 K), depending on the level of impurity, desired S/N, and the desired accuracy of the final analysis. Additional scans may be necessary depending on the sample size.

4.2.4.1.7 Receiver Gain Setting

The receiver gain, which is the amount the analog signal is amplified prior to digitization, is generally set automatically on most NMR spectrometers but it is a critical parameter to set correctly. It is set prior to initiation of any data collection on the NMR spectrometer, including qHNMR experiments. If the receiver gain is set too high, saturation of the analog to digital converter can result in "FID clipping" and signal attenuation, or in some cases elimination, as well as severe baseline distortions and spurious peaks. Setting the gain too low can lead to a spectrum with reduced S/N, failure to detect small peaks in the presence of large, and limitation of the S/N ultimately attainable, resulting in artificial, time-consuming data acquisition.[31,32] The receiver gain is a parameter that can be manually overridden by the spectroscopist. Normally, for 1D ^{1}H experiments, including qHNMR, an automatic determination of "correct" receiver gain is available on most commercial NMR spectrometers. The spectroscopist, however, should routinely check the value set by the spectrometer and be wary of abnormally high receiver gain settings prior to initiating any kind of qHNMR experiment. A suggested procedure is to set the receiver gain at least a factor of 2–4 lower than the setting arrived at automatically, particularly when a number of samples are being compared using a solvent suppression method or external standards (Section 4.3.3.2). It might be useful to calibrate the receiver gain setting versus typical sample concentrations in order to evaluate the range of receiver gain settings that can be realistically expected.

4.2.4.1.8 Steady-State ("Dummy") Pulses

The use of repetitions of the pulse sequence without sampling the FID, that is, "dummy pulses", prior to any NMR experiment, establishes a steady quasi-state prior to actually collecting and digitizing the FID information. All NMR spectrometers have this capability and, depending on the nature of the experiment, the number of steady-state pulses used will vary. The function of these pulses is very simple and tends to improve the reproducibility of the data being collected. Once the experiment is in a steady-state condition, collection of the FID information will begin. For 1D experiments, in general, the number of steady-state pulses should be at least the number of steps in the phase cycle (typically four), whereas in the case of some 2D experiments the number could be a

much larger power of 2. Depending on the desired accuracy and reproducibility for qHNMR, an optimized number of steady-state pulses can be determined empirically.

4.2.4.1.9 Carbon Decoupling

With regard to ^{13}C decoupling, the ^{13}C spectral window and the ^{13}C decoupler offset need to be set correctly. The width of the ^{13}C window to be decoupled can typically cover only the region concerned with protonated carbons (0–180 ppm). However, to ensure that all protonated carbons are decoupled, and to standardize the acquisition parameter set, a spectral region of 0–220 ppm is recommended. In this fashion, aldehyde carbon resonances will be included in the decoupling process. The carbon transmitter position, that is, ^{13}C decoupler offset, would then be centered in this region, in this case at 110 ppm.

4.2.4.2 Postacquisition Processing

For consistent and reproducible qHNMR analysis, the establishment of a spectral processing concept is essential. Factors affecting digitization and data processing have been comprehensively reviewed[31] and, although subsequent improvements in computer hardware and instrumentation have reduced the likelihood of many of the artifacts discussed, the underlying principles still apply. The first step is the determination of the optimal window function and parameters (apodization function). When applying a window function, it should be borne in mind that the integrated intensities of all resonances in the spectrum obtained by FT of the apodized FID will be multiplied by the value of the first point of the window function. Usually for quantitative work, a window function having a value of 1.0 at zero time is applied, in which case the starting intensities are unaltered from those of the original FID. Such a window function is the negative exponential $\exp[-\alpha(t/T_{FID})]$, where α is positive, t is the time after the first point of spectral acquisition, and T_{FID} represents the time constant of the original FID. However, window functions applying negative exponentials may have disadvantages in some circumstances as the area of the resulting Lorentzian lineshape contains a higher proportion of area in the wings than do Gaussian lineshapes, which result from Gaussian apodization, that is, $\exp(-\beta[t/T_{FID}]^2)$. This function, where β is positive, also has its maximum 1.0 at $t = 0$ (the first point), and the intensities of the resulting spectrum are again unaltered.

Lorentzian–Gaussian (LG) multiplication,[31] by a function $F = \varepsilon \exp(\alpha t/T_{FID} - \beta[t/T_{FID}]^2)$, is frequently employed with complex spectra of natural products as a means of obtaining a balance between resolution enhancement and S/N. The variables in the function are usually determined individually to optimize each spectrum. This function will multiply the first point of the FID by the factor ε, which may be difficult to determine when using software supplied by instrument manufacturers. For instance, ε is in some cases scaled automatically such that the maximum value of F is 1.0, in which case $\varepsilon < 1.0$ and the spectral integrals will be less than those obtained with a negative exponential window function. LG multiplication is compatible with the use of an internal standard for quantitation since the contribution of each resonance to the first point of the FID is reduced to the same extent. Nevertheless, it is advisable to use values of α and β that avoid negative artifacts on either side of peaks, as these would interfere with integration. The authors have experienced that, when using internal standards, a mild LG multiplication with Gaussian factors around 0.05 (=5% of the total acquisition time of the FID) and line broadening factors around 0.3 Hz (sometimes entered as negative value) provides lineshape improvement with minimal S/N loss. LG multiplication is not advisable when comparing samples and standards in separate tubes (external standard) due to the need to ensure constant proportionality at all stages of signal processing. If attempts were made to optimize resolution of each individual spectrum, this would risk alteration of ε and would bias the relative intensities between spectra.

Overall, the processing steps of a raw qHNMR FID are: (i) correction of the baseline of the FID (removal of DC offset); (ii) application of optimized window function; (iii) zero filling as needed; (iv) FT; (v) phase correction, typically performed in manual mode; (vi) subtraction of disturbing broad resonances such as those from water or other –OH/NH/XH exchangeable protons by repeated

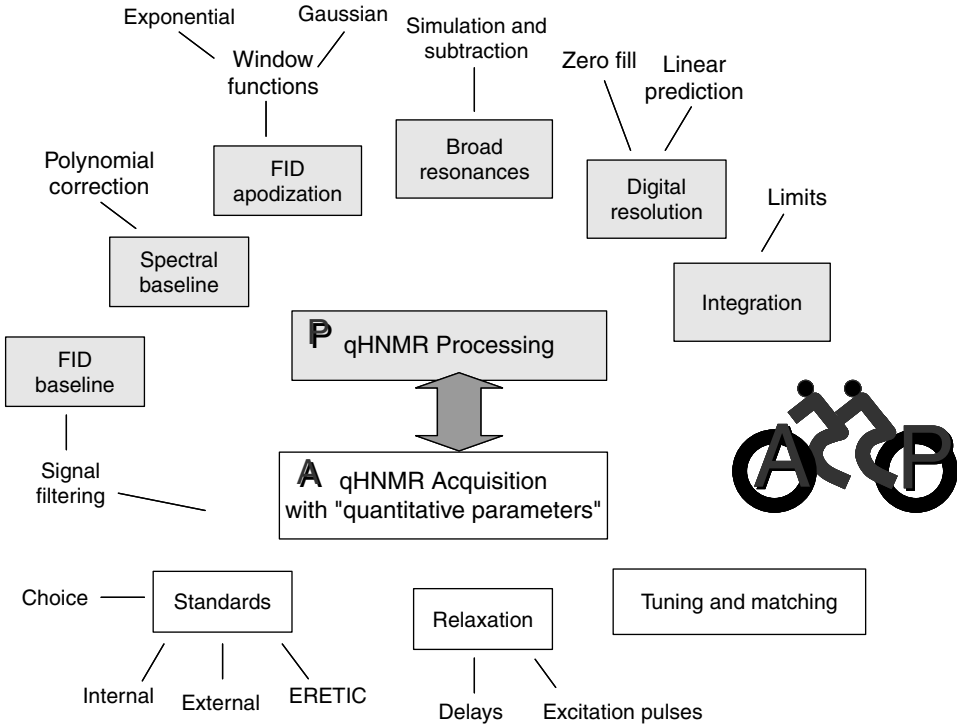

FIGURE 4.3 The tandem role of acquisition (A) and processing (P) parameters in qHNMR. In any qNMR assay, the tandem of acquisition and processing has to work cooperatively. Although in acquisition "quantitative conditions" (see text) ensure the yield of valid raw qHNMR data (FIDs), spectral processing has to also consider several factors that affect quantitation. Thus, a well-founded qHNMR processing concept ultimately has to define and document a number of processing parameters that were used in the assay ("quantitative procedures"). Although some of them are sample dependent and require individual optimization for each analytical problem, this figure provides a topical overview of the processing components/steps that are likely to have a major impact on integration and, thus, quantitation of the signals. Because acquisition and processing work in tandem, the definition of reproducible experimental procedures in qHNMR requires the proper choice, specification, and documentation of both "quantitative conditions" (A) and "quantitative procedures" (P).

simulation and subtraction; and finally (vii) baseline flattening by application of nth ($n < 10$) order polynomial correction (Figure 4.3).

Regarding the achievement of a flat baseline, there are numerous tools available in NMR processing software packages. The tools typically follow two basic approaches, fitting of the experimental baseline with an "artificial" curve (usually an nth order polynomial), or manual fitting of single, rather well-defined resonances (e.g., residual water peak). Both approaches then involve subtraction of the fitted curves from the qHNMR spectrum. The extent to which baseline correction is required for successful qHNMR quantitation largely depends on the nature of the sample (there are also influences from the spectrometer hardware, which are beyond the scope of this chapter). In the authors' experience, adequate ("flat") "quantitative baselines" routinely require at least a single-step polynomial correction, but sometimes, in particular with more complex or less pure natural products, multiple manual steps of line fitting and subtraction. An example is provided in Figure 4.4.

As a general guideline, when integrating the signals of the main component, the set of resonances with the lowest integrals per proton should be averaged to provide the integral reference since these would have the lowest likelihood of overlap with signals from other components and, hence, the highest selectivity. One of these signals could be assigned an arbitrary integral value of

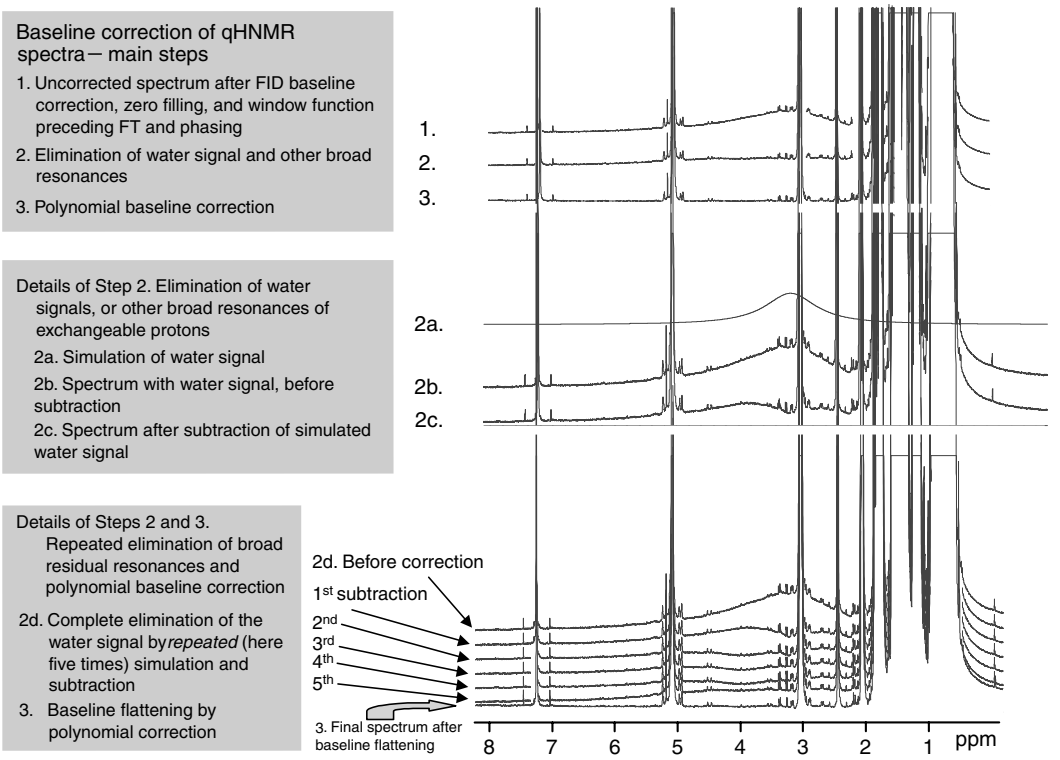

FIGURE 4.4 Influence of different processing steps on the spectral baseline. Achievement of a flat baseline is one of the key prerequisites for reliable qNMR quantification. Depending on the sample, several steps may be required to eliminate imperfections in the baseline, which are typically due to broad resonances of exchangeables such as water. This is illustrated for a qHNMR spectrum of ursolic acid (see Figure 4.8) in $CDCl_3$. To focus on the impurities and the imperfections of the baseline, the main sample peaks are drawn off-scale. In practice, a combination of multiple subtractions of simulated broad resonances and polynomial baseline correction was found to yield good results, while still requiring manual optimization of simulation and polynomial curve parameters. As observed with manual versus automatic phasing, the human eye serves as an excellent tool in the judgment of symmetry and planarity.

100 units per proton, for example. Statistical considerations as well as other information (see the following paragraph) can be used to decide which resonances should be included in the average. Particular care has to be taken to ensure that the pulse repetition rate is adequate for proper relaxation when using signals of nuclei with long relaxation times as integration references. If in doubt, signals of protons with short relaxation times should be preferred as integration reference.

More specifically, a compound with multiple ^1H resonances requires some systematic process to decide which of these should be used in qHNMR. Where a number of peaks are well defined and clear of overlapping resonances, their areas (per H) should be equal within an error determined by the noise level of the spectrum and should be lower than those of peaks that overlap impurities. The presence of the latter can sometimes be determined by TOCSY spectra indicating the other resonances in the impurity spin systems, which usually will not coincide with other peaks from the analyte. Figure 4.5 shows integral ranges chosen for quantitation of the shellfish toxin, pectenotoxin-11, together with the TOCSY spectrum. In cases where the "impurity" is a compound closely related to the analyte so that most resonances coincide, it may not be possible to quantify them independently. Instead, it may be preferable to measure the total quantity of related compounds and estimate a ratio of the analyte and its derivative from the few separated peaks.

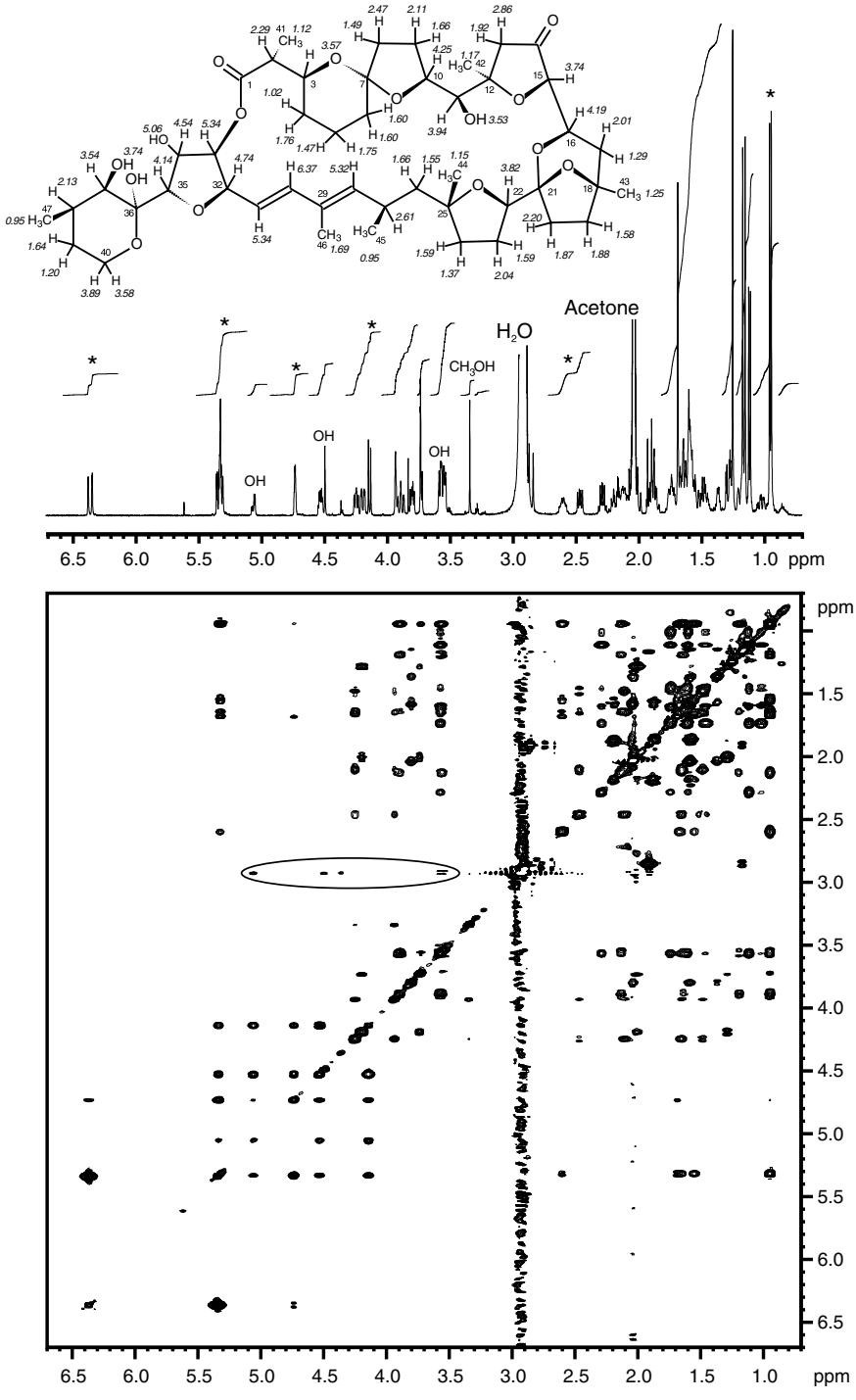

FIGURE 4.5 Integration ranges in the qHNMR analysis of a shellfish toxin. ^{1}H 1D and 2D TOCSY (160 ms mixing time) NMR spectra of pectenotoxin-11 (structure with ^{1}H assignments inserted) in acetone-d_6, illustrating (*) integration ranges suitable for quantitation. Other integral ranges are precluded owing to overlap with impurities (e.g., resonances at δ 0.85, 1.27, and 1.63 ppm), or exchanging OH resonances (encircled) as indicated by 2D TOCSY and COSY, and resolution-enhanced 1D, spectra. Pectenotoxin-11 was provided by the Cawthron Institute, Nelson, New Zealand.

4.3 QUANTITATIVE NMR APPLICATIONS TO BIOACTIVE NATURAL PRODUCTS

4.3.1 Basic Considerations

Many factors influence the detailed choice of qNMR procedures in natural products analysis. These include the desired accuracy and precision, the ease and speed of the operation, sample handling and preparation, toxicity, volatility, instrumental stability, instrumental capability, as well as subsequent uses of samples, and whether it is permissible to mix them with other compounds. The latter point relates to a major choice between the use of internal or external reference standards,[33] the advantages and disadvantages of which will be discussed in the following sections in the light of the above considerations.

4.3.2 Internal Standards

Historically, qNMR has relied on internal standards as a means for calibrating the integrated intensity of signals. Thus, a substance different from the analyte is added to the solution in a known concentration and the relative areas of the analyte and standard signals are measured in the same spectrum. This approach has the advantage that precise tuning and matching of the probe, and pulse calibration, is not necessary except for maximizing S/N. Provided that the pulse sequence contains an adequate relaxation delay to ensure that differential saturation of analyte and standard signals does not occur, the analyte concentration may be calculated from the relative response per ^{1}H nucleus compared to that of the standard. For dilute solutions, a standard may be added to the solvent in bulk before the analyte is added, or resonances of the solvent itself may be used to calculate an analyte/solvent concentration ratio if the solvent is not deuterated and the dynamic range of the spectrometer permits.

4.3.2.1 Some Precautions with Internal Standards

There are many disadvantages to the use of added internal standards. First, a precise amount of standard substance must be added to a measured volume of solution. This requires sufficient quantities of the standard and appropriate equipment for accurate weighing or volumetric measurement. As with any standard prepared by weighing, the exact composition including hydration state, salt form, or both must be known. Adding the standard to bulk solvent before dissolving the analyte simplifies the procedure, but, if concentrated solutions are made up, then the overall volume change may be significant and must be taken into account. The resonances of the standard must not overlap with those of the analyte, and the substances must be mutually unreactive and soluble. The standard must not interfere with subsequent uses of the analyte solution unless it is readily removable.

4.3.2.2 Use of Solvent Signals as Internal Standards

Although the use of solvent signals as internal standards has the obvious advantage of simplicity, their use is restricted by a number of considerations. The solvent must have a precisely known ^{1}H concentration and the overall purity, dryness (if nonaqueous), and absence of chemical exchange effects should be established. Analyte signals must not overlap solvent resonances unless the solution is highly dilute, so that the former have negligible intensity. If these conditions are satisfied, the molar ratio of analyte to solvent may be measured readily in highly concentrated solutions. However, calculation of the molar concentration will require knowledge of the solution density which, in general, will differ from that of the neat solvent.

Highly dilute solutions in protonated solvents present different problems. The analyte signals may be so weak compared to those of the solvent that they cannot be adequately digitized at a gain

setting that is low enough to accommodate the latter. However, the solution density may be assumed equal to the neat solvent density. A possible approach in this case is to record two spectra under the same conditions of probe tuning and excitation pulse: the first at low gain following a single pulse without solvent suppression, and the second with solvent suppression and a higher gain sufficient for adequate digitization of the unsuppressed analyte signals.[5] If the gain ratio is calibrated, for example, by measurement of a sample designed for adequate digitization at both gain settings using the same signal paths within the instrument, there is sufficient information to calculate the analyte concentration knowing the solvent density at the measurement temperature. Multiple scans may be used for the solvent-suppressed spectrum, and each of the spectra must be recorded with adequate relaxation conditions and under the same conditions of probe tuning, although exact tuning and matching is not mandatory.

4.3.3 External Standards

Where it is impractical or undesirable to use internal standards, various methods of external standardization have been devised. These may take the form of (i) a standard solution separated in some way from the analyte solution, (ii) standard and analyte solutions in separate tubes, or (iii) an electronic signal introduced into the instrument at a frequency close to those of the analyte resonances.

4.3.3.1 Concentric Tubes

Tubes containing standard compounds are commonly inserted into tubes containing analyte solutions as a means of calibration.[33] The inverse situation is equally possible but seldom used. The advantages of this approach are that the same standard can be transferred from one analyte sample to another, there is no need for precise tuning and matching of the probe, and the signals from the standard and analyte are obtained in one measurement. Major disadvantages are that the ratio of inner to outer tube diameters needs to be accurately known, or preferably calibrated, and good shimming may not be possible since concentric tube assemblies are less precise than standard tubes. Resonance lineshapes may be seriously compromised, thereby lowering the quality of measurements. Overlapping of analyte and standard resonances must be avoided as for internal standards. The inner tube must be carefully cleaned and dried when transferring from one sample to another to avoid cross-contamination, in which case it is difficult to avoid loss of analyte or alteration of the concentration following measurement. Although the method has been used for hazardous samples, including chemical warfare agents,[34] it is far from ideal for this application since sealing of the toxic analyte cannot be readily maintained.

4.3.3.2 Separate Analyses of Analytes and Standards

The containment of external standards and analytes in separate but identical tubes appears to have received little attention as methodology for qNMR until recently.[5] Likely reasons for the neglect of this approach included doubts about its likely precision and accuracy resulting from anticipated variations in the experimental conditions between samples, such as probe geometry, probe Q factor, instrumental gain, tuning, and tube diameters. The requirements are in some ways more stringent than for other methods, but the advantages are numerous: (i) Tubes may be sealed (especially desirable for toxic analytes); (ii) one set of standards may be compared with numerous analyte solutions; (iii) overlap of standard and analyte resonances is not an issue since separate spectra are recorded for each; and (iv) standards may be made up from a number of different compounds or solvents, sealed and kept indefinitely, and periodically cross-compared to monitor for degradation. Ultimately this approach should permit samples to be run rapidly, provided instrumental stability is good enough for several analyte spectra to be obtained between calibrations.

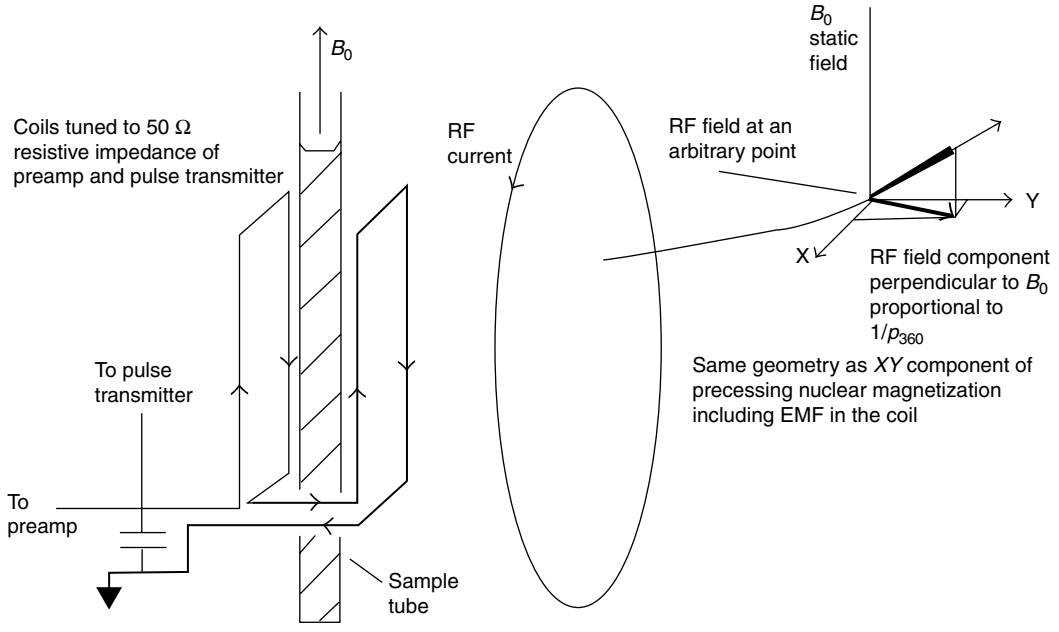

FIGURE 4.6 The use of the 360° pulse length in probe damping compensation in qHNMR. Schematic view of a "single-coil" NMR probe (left) showing the Helmholtz coil pair used for both RF transmission and detection of NMR signals in a static field B_0. The diagram on the right shows how the RF field component in the XY plane perpendicular to B_0, produced at a given position in a sample by RF current in a coil of arbitrary shape, is geometrically equivalent to the XY component of precessing nuclear magnetization at the same position inducing a current in the same coil. For this reason (reciprocity[35]), the 360° pulse length (p_{360}) is inversely proportional to the coil quality factor Q and the product ($p_{360} \times$ integrated intensity) compensates for variable probe damping when comparing samples in separate tubes using a single-coil probe.[5]

The potential disadvantages of the approach, which may have mitigated against its widespread adoption, are several but all are surmountable. The method cannot provide intensity comparisons to a greater accuracy than the reproducibility of the tube diameters. Fortunately, it is possible to demonstrate directly, by comparing integrated spectra of neat liquids such as H_2O, that with high-quality tubes the variation is negligible, that is, $<0.3\%$. Tuning and matching of the probe with each sample is also necessary; however, this operation is highly reproducible with spectrometers produced within the last decade, and is automatic with recent instruments. The remaining hurdle is the large variation in probe Q factor (damping) that occurs upon change of solvent or salt content of aqueous solutions. Compensation for damping is possible for single coil detection in which the excitation and NMR signal detection are performed with the same coil, as the 360° pulse length at constant RF power level is directly proportional to the probe Q factor (Figure 4.6). As the 360° pulse length may be measured precisely, the product of this quantity and integrated signal intensity may be used to compare concentrations of samples in completely different solvents.[5] This is a practical illustration of the principle of reciprocity.[35]

Other precautions are necessary but not difficult to implement: (i) within the instrument detection system, the RF signal path, gain, and absolute processing conditions must be maintained constant between separate standard and analyte samples; (ii) samples must be well-mixed, free from bubbles or suspended particles, and compared at the same temperature; and (iii) standards must be prepared from certified compounds of known composition in pure solvents. Nevertheless, similar precautions are also necessary when using internal standards.

Figure 4.7 illustrates a validation of the use of external standards in separate tubes for samples of various shellfish toxins (structures shown) used in the preparation of certified reference materials, and some other natural products.[5] When the above precautions are kept in mind, it is possible

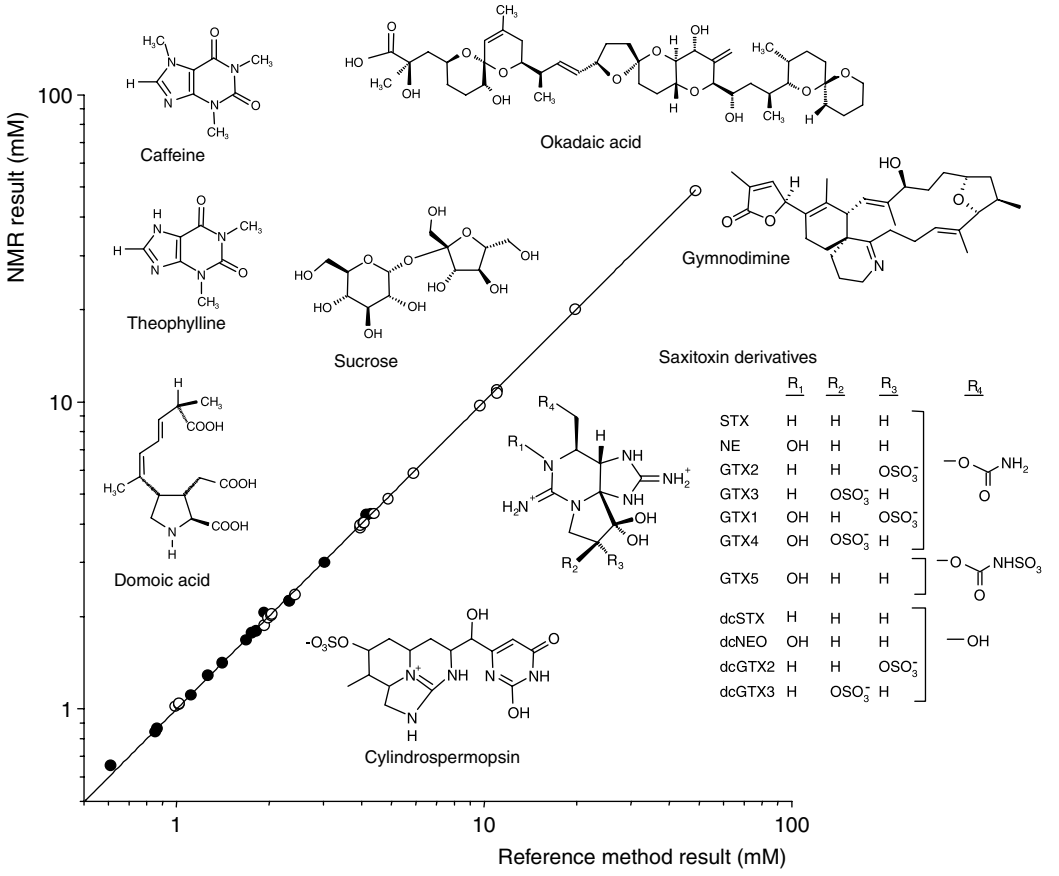

FIGURE 4.7 Comparison of qHNMR with gravimetry and LC analysis. Comparison of solution concentrations of shellfish toxins and USP-grade standard compounds (caffeine, theophylline, and sucrose) determined by NMR, with concentrations of the same solutions determined by gravimetry (open circles) or LC-CLND (chemiluminescence nitrogen detector) (filled circles). NMR measurements were standardized against USP caffeine, sucrose, or theophylline solutions contained in separate but identical precision 5-mm NMR tubes. Linear regressions: for NMR versus gravimetric concentration, slope 1.0019 ± 0.0020, intercept –0.028 ± 0.026, R^2 0.99992; for NMR versus LC-CLND, slope 1.024 ± 0.011, intercept –0.026 ± 0.024, R^2 0.9982. Solvents were H_2O, D_2O, and CD_3OH. Samples and gravimetric or LC-CLND measurements were provided by M.A. Quilliam and the Certified Reference Materials Laboratory at NRC-IMB Halifax. (Adapted from Burton, I.W., Quilliam, M.A., and Walter, J.A., *Anal. Chem.*, 77, 3123, 2005. With permission. Copyright 2005, American Chemical Society.)

to consider this method for efficient automated use with large numbers of samples. The sequence presented by the sample changer could start with a standard, followed by several analyte samples, interspersed with and terminated by one or more other standards. Each sample would be individually shimmed, tuned, and matched automatically, and run at the same receiver gain and probe temperature, preferably the same as the temperature in the sample changer to minimize equilibration times. Adequately long relaxation delays would be necessary, as for all qNMR (Section 4.2.4.1.5). A means for automatic determination of the 360° pulse length time for each sample, and for setting the 90° pulse from this, would need to be devised. For a series of dilute samples/compounds in the same organic solvent, however, the 360° pulse is unlikely to vary significantly, and the initial value could serve for all, provided automatic tuning and matching was maintained. Data processing would be undertaken with exponential ($\exp[-\alpha t/T_{FID}]$) or Gaussian ($\exp(-\beta[t/T_{FID}]^2)$) multiplication and integration at constant absolute scale, but LG multiplication optimized for individual samples would be strictly avoided in this routine qHNMR procedure.

4.3.3.3 Electronic Signals (The ERETIC Method)

The ERETIC method circumvents internal standards by introducing a small exponentially decaying signal from an external RF source to an untuned probe coil or separate antenna mounted within the probe.[36,37] The signal is applied during the acquisition time, at a single frequency within the detection window, but separate from any analyte resonances. The amplitude is chosen to allow convenient integration of this signal alongside the analyte resonances after FT. Implementation of the method on basic instruments requires special RF hardware, but recent multichannel spectrometers may be readily adapted. It is necessary to ensure that the signal amplitude is constant and its integrated intensity calibrated periodically against a certified pure standard. There does not appear to be any physical reason for damping the ERETIC signal since the integrated intensity will depend only on the signal amplitude at the beginning of the acquisition time. Constant signal amplitude would be easier to implement, and line width would be imposed by the window function applied prior to FT.

It has been shown that the intensity of the ERETIC signal varies with probe coil loading to the same extent as NMR signals detected from the sample.[36] The method assumes that the cross-sectional areas of NMR tubes used for both samples and original calibrating solutions are constant, as for other external standards. Changes of flip angle caused by probe *Q* damping or mistuning/mismatching of the transmitter circuitry are not automatically compensated and would need to be taken into account, particularly for samples causing heavy damping.

The main advantage of the method appears to be as a monitor, present in every spectrum, of instrument performance on the detection side. It does not indicate changes on the transmission side, such as pulse power fluctuations, or those resulting from sample tube variations. It is also necessary to assume that the ERETIC signal generator is constant. Total instrument performance may be monitored periodically in other ways with external chemical standards (e.g., by single 90° pulse scans of neat solvents) and, if acceptable stability is demonstrable between calibrations, the use of ERETIC appears superfluous.

4.3.4 Quantitative HNMR Analysis of Ursolic Acid Samples

The ubiquitous pentacyclic plant triterpene, ursolic acid, is a prominent example of a natural product that has been isolated on many occasions. Typically, isolates of ursolic acid are considered "pure" compounds and are used to evaluate the biological activity in various test systems. However, in view of the apparent omnipotent activity of ursolic acid, the potential influence of impurities on the observed bioactivity has not been reported. Toward this end, a qHNMR-based purity evaluation and profiling for nine different reference samples of ursolic acid, originating from commercial sources and with purity certificates being provided, demonstrated the superiority of qHNMR in comparison to results derived from commonly used LC-UV or GC assays.

The solubility problems of precipitated ursolic acid dictated the solvent choice and required the dried sample material, ranging from 4 to 11 mg, to be dissolved in 50 μL of DMSO (99.9% isotopic purity) prior to $CDCl_3$ (99.8% isotopic purity) being added to a final volume of 1000 μL. The samples were prepared according to the previously described guidelines (Section 4.2.2) and in the absence of an internal standard. The NMR spectra were recorded using a Bruker AMX 500 instrument. Chemical shifts (δ in ppm) were referenced to the $CDCl_3$ singlet at δ 7.240 ppm. For all NMR experiments, off-line data analysis was performed using the NUTS software package from Acorn NMR Inc. (USA).

For (im)purity profiling, qHNMR spectra were measured with 512 transients using quantitative conditions (Section 4.2). Acquisition parameters were selected from a reported qNMR method with a precision of detection for minor compounds present at ca. 1% abundance, to be better than 2%.[1,23] The acquisition and processing concepts for optimizing the qHNMR spectra were carried out as described in Section 4.2. The doublet at δ 2.033 ppm of ursolic acid, the main component, served as a reference signal and was set to an arbitrary integral value of 100.

To provide a solid foundation for the subsequent qHNMR analysis, an *ab initio* structure elucidation of ursolic acid was performed. In particular, a full spin-system analysis of the HNMR spectrum of ursolic acid was performed, and the coupling and NOE interactions were fully determined to distinguish between signals (or signal parts) of ursolic acid and those that were due to impurities. In addition, high-sensitivity 2D COSY spectra were recorded to aid in the assignment of impurities. Taking into account all available information about the impurities, including their profiles and their molecular weights, the purity of the ursolic acid reference materials was evaluated using the 100% method.[1,23] This method assumes that all analytes or chemical components are detected (by HNMR), that they can either be identified or assigned, and that the ratios are normalized to a total of 100%. Clearly, when using the 100% method, the certainty or error with which all components can be assigned determines the overall precision or error of the purity assay.

Considering that ursolic acid is nearly ubiquitous in higher plants and is isolated from very different chemotaxonomic matrices, typically by means of chromatography or precipitation, this almost inevitably results in very different profiles of minor impurities contained in the final isolate. Therefore, it was reasonable to hypothesize that the investigated ursolic acid samples were heterogeneous as a group. Indeed, the samples were determined to contain 5–10 different impurities, including oleanolic acid (detected in eight samples) and betulinic acid (detected in six samples). The identities of oleanolic acid and betulinic acid were verified from the following specific marker signals ("fingerprint signals"): a doublet of doublets ($J = 3.4$, 14.3 Hz) at δ 2.760 ppm for oleanolic acid, and a doublet ($J = 2.0$ Hz) at δ 4.615 ppm for betulinic acid. Although other impurities were not assigned to a specific compound, their signals were pieced together with the aid of COSY correlations, combined with logical signal grouping according to integration values (Figure 4.8). Groups of signals with chemical shifts (and multiplicities) that were very similar (or related) to the main compound (ursolic acid) were considered to belong to the same compound class, and the molecular weight of ursolic acid was taken to represent the nearest approximate value. For other known impurities, for example, residual solvents such as MeOH or EtOH, their precise molecular weight was considered in the calculation.

In summary, the qHNMR-derived purity of eight out of the nine ursolic acid reference materials was found to be significantly lower than all of the available declarations, which were based on HPLC-UV methods. In only one case were the qHNMR and LC purities practically identical (0.04% difference). For three out of the nine samples, the qHNMR purity was found to deviate substantially from the LC-based assessment, with differences between 9% and 15% compared to the declared purity. Finally, it has to be noted that, considering the unavoidable signal overlap in the high-field region of the spectra, the "real" purity must be considered to be rather lower than that determined in this study. This example demonstrates the great potential of qHNMR in providing a more universal impurity detection and quantification capability, and in generating meaningful impurity profiles, especially for natural products.

4.3.5 Sample-Specific Considerations

4.3.5.1 Sample Purity

In virtually all applications, qNMR measures the concentration of an analyte in a solution that extends beyond the detecting region within the probe coils, to ensure adequate field homogeneity by approximating an "infinite" cylinder. In principle, it would be possible to measure the total signal from an analyte contained in a spherical sample that is small compared to the detecting region. However, many difficulties intervene to make this procedure impractical for routine use, particularly the loading, positioning, and shimming of the sample containers. When the intensities of NMR signals are used to measure quantities, it is of paramount importance to distinguish the signals that arise from the analyte in question from all other possible contributors to the total NMR spectrum. There is no substitute for a "clean" sample, free of impurities, in which

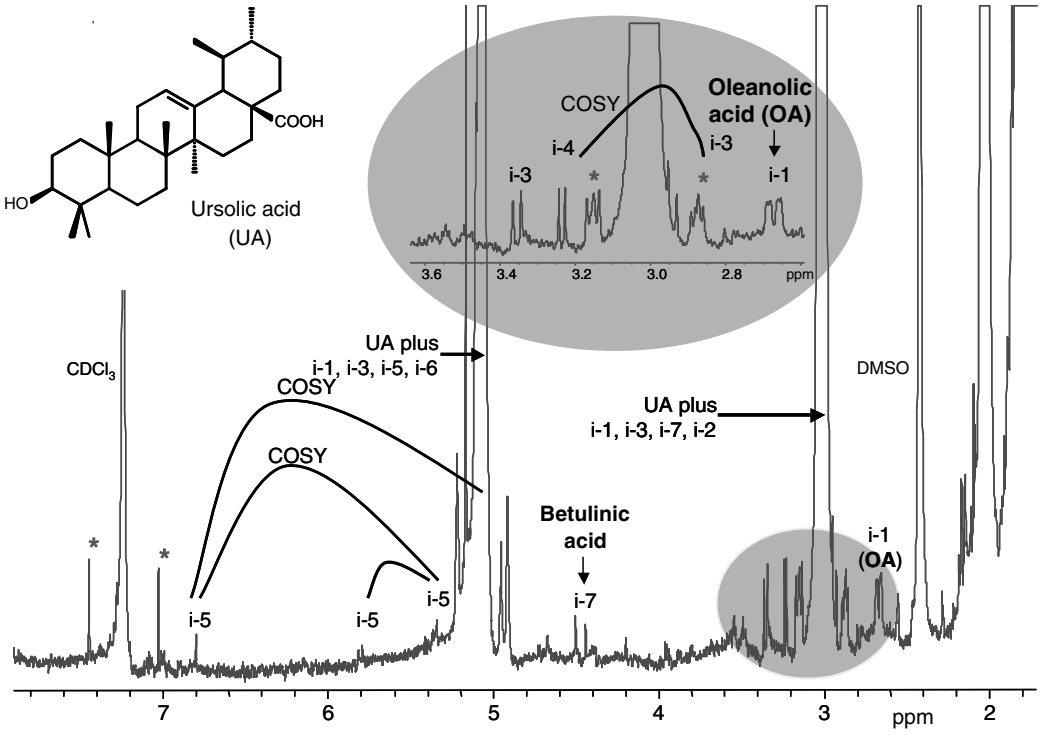

FIGURE 4.8 Impurity profile of an ursolic acid sample. The signals of different impurities, designated as i-1 to i-7, were assigned on the basis of matching integrals and 2D COSY correlations. In addition, reference spectra of oleanolic acid and betulinic acid were used to identify the two impurities on the basis of specific marker signals ("fingerprint signals"). Although oleanolic acid gives rise to a dd at δ 2.67 ppm (J = 3.4, 14.3 Hz), two characteristic AB-type exomethylene multiplets at δ 4.43 and 4.51 ppm are indicative of betulinic acid. This exemplifies how qHNMR can be capable of impurity identification even at very low levels (≤1%), and also demonstrates the diversity of potential impurities found in ursolic acid samples isolated from nature (* denotes ^{13}C satellites; solvent used: $CDCl_3$:DMSO-d_6 [95:5]; 500 MHz).

all nonexchanging resonances are sharp and baselines are flat. In practice, some natural product samples are not purifiable to this extent without unacceptable sample loss, in which case the accuracy of the quantitation will be degraded.

4.3.5.2 Proton Counts

Curve resolution techniques may be employed in some cases to estimate the areas of analyte peaks superposed on those of impurities. To determine concentration, it is also necessary to know enough about the sample and its molecular structure to decide how many ^{1}H nuclei per molecule contribute to a particular resonance, bearing in mind the possible degeneracy of resonances resulting from molecular symmetry. Preferably, the complete structure and HNMR assignment should have been determined, but this is not an absolute requirement provided one is certain of the number of ^{1}H nuclei (per molecule) contributing to at least one readily integrated resonance. The spectra of most samples provide redundant information for quantitation, which represents a unique advantage of NMR.

4.3.5.3 Concentration

To convert concentration to mass of the substance, both the solution volume and the complete composition must be known. Many natural products are not available in sufficient quantity to obtain

crystals, and the purest sample available may contain residual chromatographic support material, salts, and polymeric substances such as silicates that are undetectable by high-resolution HNMR. These may contribute to broadening or loss of NMR signals through adsorption of analyte from free solution. High-molecular weight, ^{1}H-containing impurities may contribute broad underlying peaks that are difficult to integrate independently of analyte peaks, or they may be so broad as to be undetectable and, therefore, are accommodated by baseline correction (Section 4.2.4.2). Sharp peaks from solvents and other low molecular weight–impurities are usually readily recognized (a searchable database based on the study of Jones et al.[11] is available at www.qnmr.org) and discounted for quantitation purposes. Nevertheless, in favorable cases, qNMR can provide an upper limit to the total mass of impurities by comparison with a gravimetrically determined sample mass.

4.3.5.4 Proton Exchange

Peaks from ^{1}H nuclei undergoing intra- or intermolecular exchange, for example, with the solvent, are not usually reliable as a basis for quantitation since their intensity may be distributed between different regions of the spectrum. These may be recognized from ROESY spectra as cross-peaks of opposite sign to those arising from intramolecular dipolar couplings.[2] If at least some ^{1}H resonances from the analyte are clearly distinguishable from solvent, exchanging and impurity peaks, they may be used for quantitation.

4.3.5.5 Paramagnetism

Paramagnetic impurities in low concentrations may have a beneficial effect on quantitation by reducing the relaxation time (T_1) so that shorter relaxation delays (d_1) may be used. However, it is generally preferable to remove them from solution because of their deleterious effects on spectral resolution.

Some natural products form complexes with paramagnetic ions, which may selectively broaden resonances of nuclei close to the paramagnetic center, distort relative intensities of peaks, and make integration of individual resonances difficult or ambiguous.

4.3.5.6 Nuclear Overhauser Enhancements

NOEs may affect integrals of ^{1}H resonances used for quantitation if these resonances are inadvertently irradiated prior to acquisition, for instance, if presaturation is used to suppress signals from nondeuterated solvents.[5] The NOEs may be intermolecular between the solvent and some ^{1}H of the analyte, or intramolecular if an analyte resonance happens to coincide with the solvent resonance. Although the likelihood of intramolecular NOE may be assessed, if the structure and ^{1}H resonance assignments are known for a particular analyte, intermolecular NOEs are not readily predictable as they depend on the degree of solvent exposure of individual analyte protons and the relative contribution of competing intramolecular relaxation pathways. Structural knowledge may indicate likely candidates for this effect, for example, the imino CH of caffeine (see Figure 4.7 for structure). When there is a possibility of NOE, the simplest practice is to disregard any peaks that are significantly enhanced relative to others since most natural products will have protons that vary greatly in their solvent exposure. This strategy also applies to peaks that overlap impurity resonances.

4.4 CONCLUSIONS

Provided that "qNMR conditions" are met, 1D qHNMR has great potential to substitute for "standard" 1D HNMR as a primary analytical tool for the characterization of biologically active natural products.

The key experimental points for working under qNMR conditions consist of

- the proper implementation of a pulse sequence and acquisition parameters;
- the conscientious use of postacquisition methods, in particular window functions and baseline correction tools;
- the precise volumetric or gravimetric preparation of NMR samples; and
- the use of external or internal standards, depending on the application and level of precision required.

Given often the precious nature of the samples, qHNMR evaluation can be performed with excellent precision for absolute quantitation (ca. 1% for main component) by using external rather than internal standards. Alternatively, the approach of subtracting the total impurities from ideal purity (100% method) allows a reliable first quantitative assessment of sample composition. This method takes into account the structural information from fingerprint signals of the main analyte and the impurities, as well as other NMR measurements such as 2D COSY and TOCSY. The use of GARP decoupling in qHNMR minimizes signal overlap by removing ^{13}C satellites and spinning artifacts, and can be implemented on any modern NMR spectrometer.

By virtue of its combined structural and quantitative–analytical characteristics, qHNMR is capable of making a major contribution to any project located at the structure–activity interface of biologically active natural products. A qHNMR portfolio can be easily implemented into the natural products analytical workflow and is likely to provide valuable insights into the composition and purity of natural products, from crude materials to "pure" compounds.

4.5 SUMMARY AND OUTLOOK

The measurement of qHNMR spectra is routine practice in our laboratories. Two main observations led us to believe that qHNMR is not only the "new kid on the block" in the toolkit of the natural products chemist, but also a tool that has potential to proliferate rapidly and become valuable in the hand of other chemical and biomedical sciences.

1. Owing to the S/N requirements for quality qHNMR data, somewhat longer acquisition times are required when compared to the "standard 4–16-scan 1D HNMR experiment" implemented on all modern NMR spectrometers. However, considering the bigger picture, this additional time requirement becomes negligible, especially when taking into account the instrument time that is typically spent to acquire full 2D data sets. More importantly, the relatively high S/N of routine qHNMR spectra offers a major advantage for qualitative NMR work because of their "reserve in S/N". Thus, qHNMR data are much more compatible with apodization functions that significantly enhance spectral resolution. As a result they allow a much-improved interpretation of ^{1}H spin pattern and provide a wealth of structural data that would be inaccessible when using a "standard HNMR spectrum".
2. Although the quantitative nature of NMR has only started to be explored in greater depth, it has already become clear that NMR is more stable (primary analytical method) and sensitive (0.1% range and below; availability of ultra-high-field magnets) than was generally anticipated. At the same time, NMR is a nondestructive (natural products are often priceless) and independent analytical tool, offering a nuclear versus chromatographic (HPLC) view on sample complexity/purity. Taking into account the simultaneous quantitative and qualitative capabilities of qHNMR, the technique combines numerous unique virtues to become a future standard measure in the characterization of all biologically active compounds. It is reasonable to propose inclusion of qHNMR analytical data, such as the results from a basic 100% method quantitation, as part of the standard physical data reported for all newly isolated or synthesized compounds. This only requires

minimal adjustments of established laboratory procedures: beside some simple no-cost modifications in acquisition conditions and parameters for "the routine HNMR experiment", performance of a qualitative qHNMR calculation/evaluation would be the only additional task and time. By and large, qNMR purity data or profiles have all potential to become a standard in the characterization of bioactive molecules.

3. A further area, which is amenable to a qNMR approach, is the measurement of amounts of natural products that are utilized in the form of liquid-soluble complex mixtures without full characterization or purification, such as functional foods, cosmetics, nutraceuticals, and extracts of plant- or animal-derived material used in agricultural, medicinal, and other applications. The properties of these organic materials and the mechanisms of their activities may not be fully understood, but they are "beneficial" in some application, and as such have commercial value. It is also common for the properties of such materials to be highly dependent on the species of source organism, environmental factors, geographic location, time of harvesting, and detailed method of processing. Consequently, there is a need for rapid methods to measure, for example, the consistency of composition of materials produced by a particular manufacturer, as well as the amounts or concentrations of active ingredients, for purposes of trade and monitoring of efficacy or possible adulteration.

Simple qHNMR is well suited in principle for such purposes, and the quantitative information may be obtained with minimal extra effort compared to that needed to record a standard ^{1}H spectrum. The variability of a particular product may be readily assessed from HNMR spectra recorded in a few minutes, either visually or by means of statistical techniques such as principal components analysis based on "binning" of intensity within defined regions of chemical shift. Once the variability is established, the amount of a product may be determined by an analyst from the integrated intensities of selected spectral ranges that are representatives of the product (i.e., not including solvent or contaminant peaks), measured on an absolute scale and calibrated against an internal or external standard. The spectrum of a weighed amount of a dried product, dissolved in a known volume of solvent, could be recorded and calibrated by a manufacturer under defined conditions, and supplied to the customer along with the appropriate experimental protocol. "Binned" spectral regions from calibrated spectra could also be used for this purpose. External standards in separate tubes have advantages for complex mixtures of this type as they circumvent problems of overlap of the spectrum of an internal standard with that of the mixture, or of complexation or chemical reaction of the standard with unknown components of the mixture. The above approaches to quantitation of complex mixtures also apply to biofluids and tissue extracts used in applications ranging from metabolomic studies to therapy.

In summary, future applications of qHNMR are envisioned in three major areas:

1. qHNMR to become a "standard HNMR workhorse" experiment
2. qHNMR purity to become a standard parameter for biologically active compounds
3. qHNMR to become a new analytical method for the characterization of natural materials with complex composition (e.g., [functional] foods, nutraceuticals, botanicals, herbal drugs, cosmetics).

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5 Development and Application of LC-NMR Techniques to the Identification of Bioactive Natural Products

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CONTENTS

5.1 INTRODUCTION

The coupling of high-performance liquid chromatography (HPLC) with nuclear magnetic resonance spectroscopy (LC-NMR) is one of the most powerful methods for the separation and structural elucidation of unknown compounds in mixtures. Recent progress in pulse field gradients and solvent suppression, improvement in probe technology, and construction of high-field magnets have given a new impulse to this technique, which has emerged since the mid-1990s as a very efficient method for the online identification of organic molecules. It took nearly 20 years to establish LC-NMR, and this long period can be mainly attributed to the intrinsically low sensitivity of NMR.

LC-NMR represents an interesting complementary technique to LC-UV-MS for online identification of LC peaks. Many recent applications have demonstrated the usefulness of LC-NMR in the field of natural products chemistry. In this chapter, the principle of operation of LC-NMR, integration of this technique in various dereplication strategies, and its use for the early identification of natural products are discussed. In Section 5.2, direct hyphenation of NMR in online applications is discussed, whereas new trends involving indirect hyphenation of NMR (SPE-NMR or CAP-NMR) for better sensitivity is compared in Section 5.3. The latter will include aspects regarding the possibilities and limitations of the "online" versus "at-line" strategies combined with microfractionation procedures. Finally, a summary of the latest applications is given (Section 5.4).

5.2 DIRECT HYPHENATION OF LC-NMR

5.2.1 Principle of LC-NMR

NMR spectroscopy is the most powerful technique for structural elucidation of natural products. In contrast, HPLC is the most versatile technique for the efficient separation of secondary metabolites directly from a crude mixture without the need for complex sample preparation. The application of NMR as an HPLC detector can thus be regarded as an ideal combination for both separation and structural identification of natural products. Furthermore, the advantage of LC-NMR resides not only in the fact that full structural and stereochemical information can be obtained (by the use of 2D NMR), but also in the fact that it is a highly nonselective detection technique (^{1}H NMR spectroscopy will detect any hydrogen-containing compound present in the HPLC eluate in a sufficient amount regardless of its structure).[1]

From an historical viewpoint, the interest in combining separation methods with ^{1}H NMR spectroscopy arose as early as the end of the 1970s.[2–5] Because of the inherent lack of sensitivity of NMR at that time and the problems related to efficient solvent suppression, it took almost two decades before LC-NMR started to be used practically to solve analytical problems.[1]

5.2.1.1 NMR Flow Cell Design

The heart of an LC-NMR system includes a dedicated continuous-flow probe. In a classical NMR experiment, compounds of interest are dissolved in a deuterated solvent and the sample is rotated in a 5 mm i.d. tube in a Helmholtz coil to eliminate field inhomogeneities (Figure 5.1a). With modern NMR instruments, the tube is no longer rotated since, for highly sophisticated 2D experiments, rotation causes severe distortions. The design of continuous-flow NMR probes for cryomagnets,

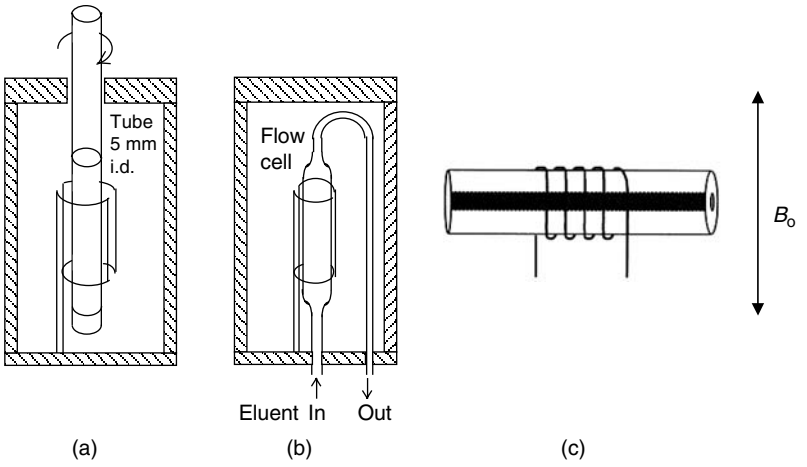

FIGURE 5.1 Schematic of (a) conventional NMR probes, (b) saddle-type continuous-flow LC-NMR probes, and (c) solenoidal continuous-flow LC-NMR probes.

introduced in the early 1980s, was contrary to the classical conventional tube setup. Thus, a U-type glass tube was fixed in the Dewar of the NMR probe body, breaking the central symmetry of the magnetic field. The first application of this design ("saddle"-shaped geometry) (Figure 5.1b) showed an excellent resolution, approaching those achieved with rotation of the NMR tube.[6] Saddle-shaped, continuous-flow NMR probes for routine applications employ detection volumes between 40 and 120 μL, which are much larger than conventional UV detection cells (8 μL) of similar geometry.[7]

The selection of detection volumes depends on a compromise between the needs for chromatography and those required by NMR. Whereas conventional HPLC can accommodate detection volumes of 5–10 μL, NMR needs a larger detection volume for two reasons: lineshape quality and sensitivity.[7]

1. *Lineshape quality*: When operated in the flowing mode (on-flow), there is a distinct residence time (dwell time, τ) of the nuclei in the NMR detection cell, which has to be considered. This factor does not affect the standard NMR experiment since nuclei can remain for an infinite time period within the tube. In LC-NMR, τ is defined by the ratio of the detection volume to the flow rate. If τ is below 5 s, a line broadening of the NMR signal will be observed, which will reduce the NMR spectra resolution. For example, the measured signal half width of chloroform at 400 MHz will be 0.55 Hz if the flow is stopped, 0.75 Hz with a flow of 0.5 mL/min, and 1.05 Hz at 1.0 mL/min. To maintain a tolerable line broadening, the detection volume of the LC-NMR probe was 44 μL in this case, which gives a τ of 5.28 s at 0.5 mL/min.
2. *Sensitivity*: LC-NMR is a volume-sensitive detection technique and requires maximization of NMR-active nuclei by increasing the detection volume. The concentration in later LC-eluting peaks is reduced by a factor of at least three due to the diffusion of the LC chromatographic process, and therefore the NMR detection of these dilute solutions would require very high detection volume in the milliliter range.

The design of the actual flow cells is therefore a compromise between the need for resolution and sensitivity on the NMR side (large volume) and reasonable LC peak separation on the chromatographic side (small volume). As will be discussed later (Section 5.3), less compromise is required with the indirect hyphenation of NMR.

One particularly attractive approach to enhance NMR sensitivity for small-volume, mass-limited samples uses reduced-diameter solenoid NMR coils (Figure 5.1c). As predicted theoretically

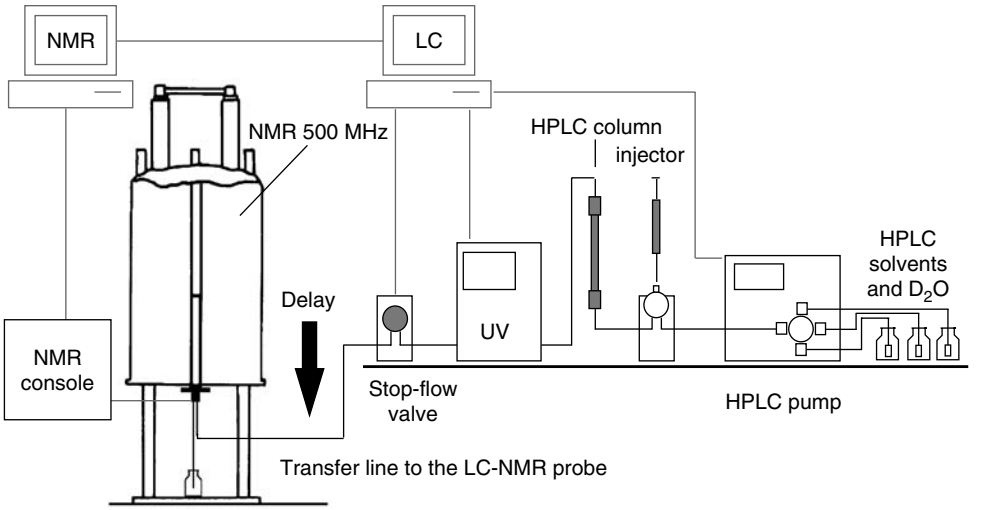

FIGURE 5.2 Typical setup used for on-flow and direct stop-flow LC-NMR experiments. The control of the stop-flow valve is achieved by the computer of the LC-UV system, which triggers both the valve of the HPLC pump and the NMR acquisition computer. A calibrated delay is used for parking the LC peak of interest with precision at the center of the LC-NMR flow probe.

and verified experimentally, the mass sensitivity of an NMR coil, defined as signal-to-noise (S/N) per sample quantity, depends inversely on its diameter.[8] However, for a given coil diameter, solenoidal coils exhibit a several-fold enhancement in NMR sensitivity compared with that of Helmholtz coils. These type of probes, used for capillary LC-NMR (CAP-NMR),[9] are mainly used in indirect hyphenation of NMR (Section 5.3) for the analysis of dried HPLC microfractions redissolved in deuterated solvent and injected by infusion. They can, however, also be directly hyphenated to capillary HPLC for mass-limited applications.[10] Because of the different advantages afforded by the two coil geometries, the specific application determines the best choice for the LC-NMR probe.

5.2.1.2 Experimental Arrangement

From a practical point of view, a classical LC-NMR experiment can be performed on an NMR system with magnetic field strength higher than 9.4 T (^{1}H resonance frequency of 400 MHz) with a dedicated flow probe and a standard HPLC system. In conventional setups, the HPLC will be installed 2–4 m from the magnet, whereas with a shielded magnet, it can be installed close to it. The main constraint will be the installation of the valve before the probe for the recording of continuous- or stop-flow spectra (Figure 5.2). Indeed, though LC-NMR was originally designed for continuous-flow NMR acquisition, the need for full structural assignment of unknown constituents led to major applications in the stop-flow mode, which enables the recording of 2D correlation experiments. In more elaborate setups, multiple loops for postchromatographic analysis of the peaks to be analyzed replace the stop-flow valve; this mode of operation is known as "loop storage" (Section 5.2.2.3).

5.2.1.3 Sensitivity

LC-NMR can be regarded as relatively insensitive due to intrinsic properties of NMR detection, which is a consequence of the small energy gap between ground and excited nuclear spin states,[11] leaving only a small population excess of spins occupying the ground state at thermal equilibrium to give rise to the NMR signal (see Chapter 3, Section 3.2.1). On the other hand, limits of detection will

also depend on the type of magnet used. Ideally, the S/N ratio in NMR is proportional to the 7/4 power of the magnetic field strength. The use of ultra-high field strengths is, however, associated with excessive costs. In contrast, small-volume flow probes (30–120 µL detection volume) in current use offer a mass sensitivity (S/N per mass unit) that is much greater than that of traditional NMR probes.[1]

LC-NMR applications have been reported using magnets of 400–900 MHz. For standard LC-NMR analyses, flow probes with volumes of 30–120 µL have been employed, whereas microflow LC-NMR applications were obtained with microcoils of 20 nL to 5 µL. The use of new cryoflow probes has enhanced the sensitivity by a factor of 3–4 compared to noncryogenated systems.[12] In cryogenically cooled probes, the receiver coils and preamplifiers are cooled to about 20 K. The effect of cooling these electronic components results in reduced electronic noise and thus a better S/N. Thus, use of cryogenic probes enables NMR spectra to be obtained with ca. 3–4 times less material during a given data acquisition period, or to reduce the data acquisition time by a factor of 9–16 with the same sample size.[1] Furthermore, the use of new techniques of NMR data acquisition, such as digital filtering and oversampling, also enhances the S/N.

The mode of operation (Section 5.2.2) strongly affects sensitivity[13] since in continuous-flow measurements, the time of acquisition is limited, whereas in stop-flow or loop storage mode,[14] there is time for the critical number of transients to be recorded. Classically, with a standard LC-NMR flow probe (60 µL on a 500 MHz NMR spectrometer), the detection limits are of about 20 µg on-column for the secoiridoid swertiamarin (**1**, MW 374) in the on-flow mode, whereas a spectrum of good quality is obtained with about 100 µg (Figure 5.3). If the same amount of compound (20 µg) can be injected in a volume corresponding exactly to that of a capillary LC-NMR probe (5 µL on a 500 MHz instrument), a much better S/N is obtained due to the probe design and the effect of the increase in concentration (Figure 5.3). With this latter type of capillary flow probe (CAP-NMR),

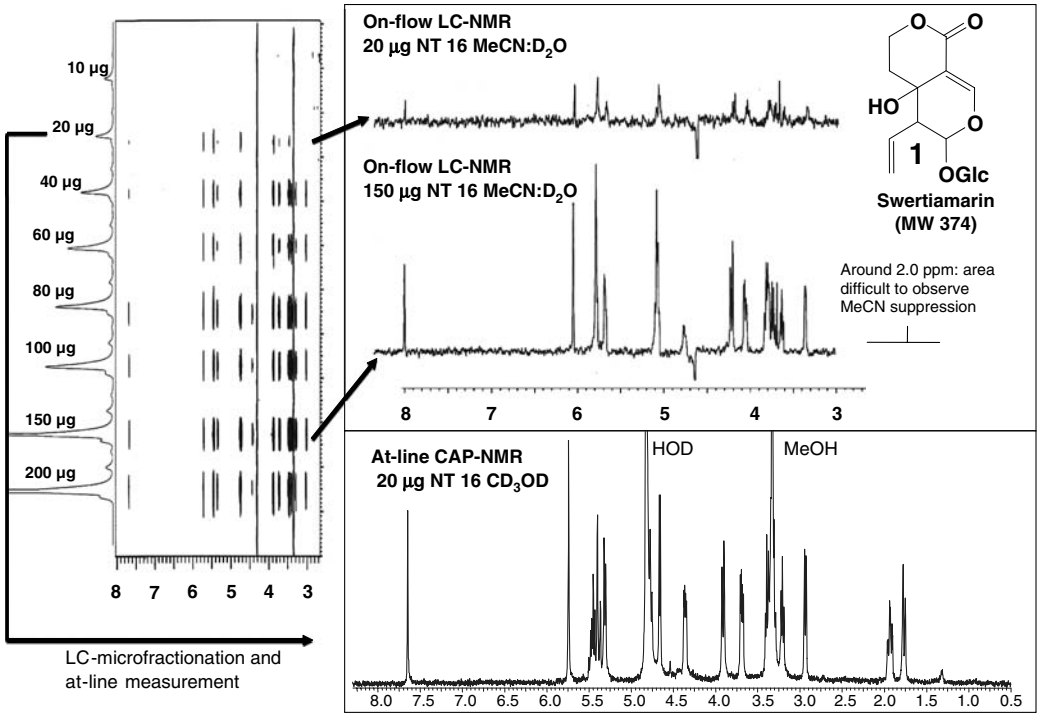

FIGURE 5.3 Estimation of sensitivity and the quality of NMR spectra recorded either by on-flow LC-NMR (500 MHz, flow cell 60-µL active volume) or by at-line CAP-NMR (500 MHz, flow cell 1.5-µL active volume) by injection of different amount of swertiamarin (**1**) always with the same number of scans (NT = 16).

demanding experiments such as inverse ^{1}H–^{13}C correlation (HSQC) can be obtained in about 1 h with 100 μg of this same type of compound, whereas long-range ^{1}H–^{13}C correlations (HMBC) would require 6 h (e.g., see data for sweroside in Figure 5.29).

In the case of very long acquisition times on LC-NMR in the stop-flow mode (overnight experiment), the absolute detection limit of a commercially available LC-NMR probe (100 μL, 500 MHz) was estimated for 3′-deoxy-3′-azidothymidine (AZT) (MW 252). An S/N of 3.0 for the H-1′ proton of the ribose moiety of this molecule was obtained using 85 ng of AZT.[15] More recently, it has been stated that using a 600 MHz magnet, the limit of ^{1}H detection of a compound with MW 500 is roughly 100 ng, but micrograms would be required for routine structure elucidation work.[14]

With the state-of-the-art technology, ^{1}H NMR spectra can thus be acquired in the submicrogram range, whereas 2D correlation data can be acquired on samples in the microgram range.[16] This represents a very important step forward for natural product structural identification work where milligram amounts were previously needed. This renders the work very feasible for the analysis of crude mixtures where several hundred micrograms or milligrams of extract can be loaded onto the column. Furthermore, it is expected that detection limits will decrease further as a result of the continuous technological improvements in this area.

5.2.1.4 Dynamic Range and Solvent Suppression

The main problem of LC-NMR is the difficulty in observing analyte resonances in the presence of the much larger resonances of the mobile phase. This problem is worsened in the case of typical LC reverse-phase operating conditions in which more than one protonated solvent is used, and where the resonances change frequencies during gradient mode analyses. Furthermore, the continuous flow of sample in the detector coil complicates solvent suppression. These problems have now been overcome due to the development of fast, reliable, and powerful solvent suppression techniques. These techniques are mainly presaturation (NOESY presaturation), soft-pulse multiple irradiation, and water suppression enhanced through T_1 effects (WET) presaturation.

All these techniques can be used either in the continuous-flow or the stop-flow mode. The WET approach, in particular, consists of a combination of pulsed field gradients, shaped RF pulses and shifted laminar pulses.[17] By varying the tip angle of the selective RF pulses, the WET sequence can be optimized. This sequence provides a fast and efficient saturation of multiple solvent resonances. It can be used in combination with ^{13}C decoupling for an efficient removal of the ^{13}C satellites of the solvent, which would otherwise strongly reduce the NMR spectrum resolution. Indeed, the ^{13}C satellites are equal to 1.1% of the ^{1}H resonance of CH_3CN. However, ^{13}C decoupling collapses these satellites into the ^{1}H resonance and these satellites are further efficiently suppressed. An example of application of the WET sequence for the suppression of multiple signals from solvent and ion-pair reagent is shown in Figure 5.4 for the analysis of GDP-mannose (**2**).[18] In this example, the signals of CH_3CN and its ^{13}C satellites were efficiently suppressed together with the signal of the residual HOD line. Triethylamine (TEA), a buffer needed for the HPLC separation of this nucleotide, generated three additional ^{1}H signals that were also suppressed during the application of the WET sequence.

5.2.1.5 HPLC Separation of Crude Plant Extracts

HPLC represents the most robust and versatile technique for the separation of natural products since it can allow direct analysis of the metabolites in crude extracts without the need for derivatization. These extracts are usually obtained by maceration of the dried plant material with nonpolar (e.g., CH_2Cl_2) or polar (e.g., MeOH) solvents or by liquid–liquid, acid–base partitioning for the extraction of specific compounds such as the alkaloids. A plant extract is a complex mixture that may contain hundreds of constituents. These products are separated mainly on reverse-phase columns using gradients of MeCN:H_2O or MeOH:H_2O with acidic or alkaline modifiers. The lipophilic extracts can be difficult to dissolve, and therefore, strong solvents such as THF are usually employed for injection in small volume.

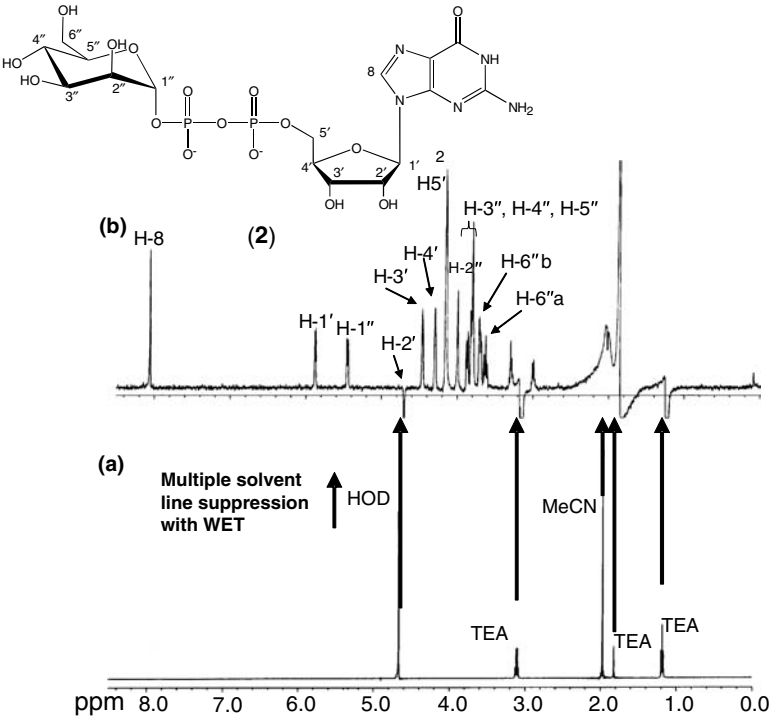

FIGURE 5.4 Multiple suppression of solvent and ion-pair reagents in the LC-NMR spectrum of GDP-mannose with the WET sequence. (a) Stop-flow spectrum recorded without solvent suppression, solvent signals and ion pair reagents hinder the detection of the signals of the analyte GDP-mannose (**2**). (b) Stop-flow spectrum of GDP-mannose (**2**) (200 µg, NT = 1024) with suppression of all signals due to the LC eluent. (Reprinted from Ramm, M., Wolfender, J.-L., Queiroz, E.F., Hostettmann, K., and Hamburger, M., *J. Chromatogr. A*, 1034, 139, 2004. With permission from Elsevier.)

Most of the applications related to crude plant extract profiling have been performed on conventional 4 mm i.d. reverse-phase columns containing 5 µm particles at 1 mL/min flow rate. Compared to these classical HPLC conditions, the recent development of ultra-performance liquid chromatography (UPLC) systems, in combination with columns with very small particle size (1.7 µm), has provided a significant improvement in terms of separation speed and LC resolution.[19] This new separation method has not yet been utilized in LC-NMR, to our knowledge, but might be valuable for a better resolution of otherwise coeluting peaks, especially in the indirect hyphenation of LC-NMR where multiple trapping of peaks difficult to separate is possible. In our experience, the use of UPLC with long gradients and columns coupled in series (300 mm × 2.1 mm i.d., *N*: ca. 80,000) provides a significantly better resolution than conventional HPLC for crude extract profiling. For example, a baseline separation of more than 250 metabolites could be achieved on an enriched extract of *Arabidopsis thaliana* in the frame of a metabolomic study.[20]

When needed, and especially for LC-NMR application, the extracts can be prepurified on solid-phase extraction (SPE) columns for enrichment of specific metabolites according to their polarities. Direct combination of matrix solid-phase dispersion (MSPD) extraction with LC-NMR has also been reported. This technique of sample preparation was reported for the online identification of asterosaponins of the starfish *Asterias rubens*.[21] Of course for a more targeted metabolite enrichment, fractions obtained by preparative chromatography separation of crude extracts can also be analyzed.[22] Prefractionation of crude extracts might represent an important issue according to the complexity of the matrix analyzed because the coelution of different natural products can compromise the

recording of good-quality LC-UV DAD or LC-NMR spectra. The intrinsic selectivity of LC-MS is usually sufficient to resolve problems of coeluting metabolites.

In the case of on-flow LC-NMR applications, and because of the inherent low sensitivity of the method, the amount of sample loaded on the column is much more important than for LC-UV-MS. For crude plant extract analysis, the amount injected on-column can be several milligrams. To cope with these sample loadings, long HPLC columns (250 mm) or columns with large inner diameter (8 mm) are used for standard LC-NMR measurements.[16] Because of the poor solubility of some extracts, dissolution of the sample in deuterated DMSO is often required for injection of such quantities on-column. As a consequence, specific chromatographic conditions have to be developed for LC-NMR to separate large amounts of sample with satisfactory LC resolution and maintain flow conditions compatible with the detection cell used.

The type of loading needed for the on-flow LC-NMR analysis of the antioxidant fraction of the Indonesian plant *Orophea enneandra* (Annonaceae) is illustrated in Figure 5.5.[23] A comparison of the LC-UV traces of the active fraction obtained by injecting 20 µg and 2 mg of sample, as well as

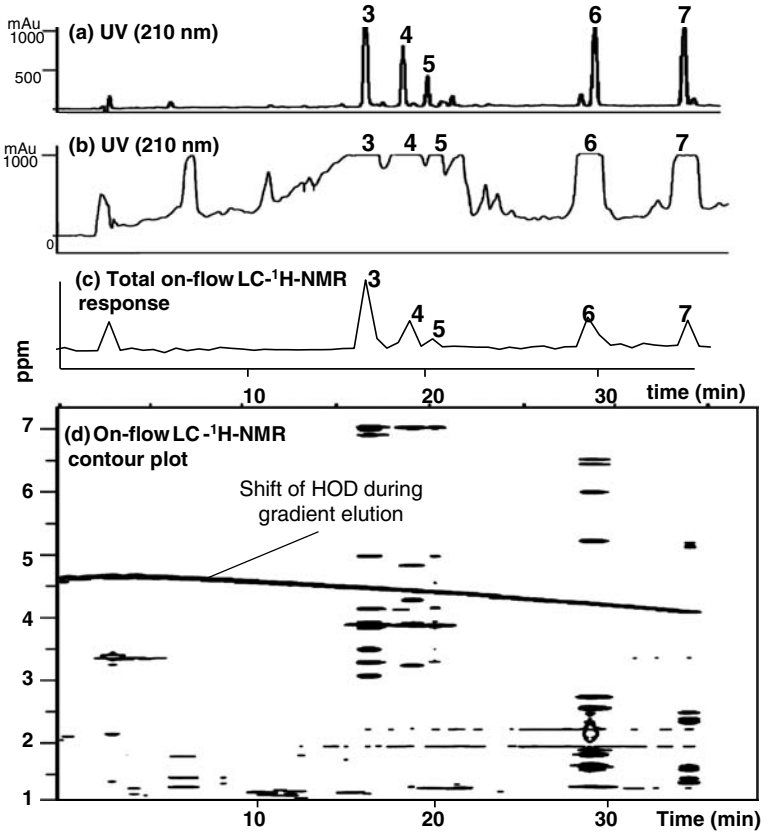

FIGURE 5.5 Comparison of the LC-UV traces and the reconstructed total on-flow LC-^{1}H NMR trace of the enriched fraction of *Orophea enneandra* (Annonaceae). As shown, the on-flow LC-^{1}H NMR of compounds **3**–**7** required a significant overloading of the column. (a) 20 µg injected, (b) 2 mg injected, and (c) 2 mg injected. (d) On-flow LC-^{1}H NMR contour plot. The signal of HOD is negative and continually shifted during the LC gradient. HPLC conditions: column, Nova-Pak C18 (150 mm × 3.9 mm i.d., 4 µm); MeCN:D_2O gradient (20:80 to 95:5 in 50 min); 0.05% TFA; 1 mL/min. LC-NMR conditions: 24 scans/increment, flow cell (60 µL, 3 mm i.d.), 500 MHz. (Reproduced from Wolfender, J.-L., Ndjoko, K., and Hostettmann, K., *Phytochem. Anal.*, 12, 2, 2001. With permission. Copyright John Wiley & Sons Limited.)

the LC-NMR trace obtained by summing the LC-^{1}H NMR responses of all the constituents (total ^{1}H NMR response), is shown. The corresponding 2D on-flow LC-NMR contour plot obtained is shown below these traces (Figure 5.5). The 2 mg injection of the active fraction allowed the recording of five main constituents, that is, lignans (**3**–**5**), tocopherol (**6**), and polyacetylene derivatives (**7**), in the total on-flow LC-NMR trace (Figure 5.5c), but, as indicated by the UV trace recorded in the same conditions (Figure 5.5b), a significant overloading of the column was necessary. The NMR spectra for minor constituents could not be recorded in this mode. The on-flow LC-NMR spectrum recorded for the lignan derivative phylligenin (**3**) is discussed in Section 5.2.5.2 and shown in Figure 5.26.

One way to improve the detection limits of the on-flow mode is to carry out the analysis at low flow (0.1 mL/min) (e.g., the on-flow analysis of *Erythrina vogelii* (Figure 5.10) in Section 5.2.4.1.1) or to run time-slice experiments over a whole chromatogram. Both modes of operation enable a higher number of transients per increment to be recorded and thus a significant improvement in S/N is obtained. In the latter approach, the flow is stopped at defined time intervals and a critical number of scans can be recorded for each increment.[24] An application of this time-slice technique has demonstrated that it was possible to record successfully the LC-NMR spectra of more than 20 compounds in the crude extract of *Gnidia involucrata* (Thymelaceae), a plant widely used in African traditional medicine. A comparison of the 2D contour plot of the on-flow LC-NMR analysis and the time-slice stop-flow analysis clearly demonstrates the gain in sensitivity that can be obtained in the latter mode (Figure 5.6). In the on-flow mode (Figure 5.6a), only the four main constituents of *G. involucrata* were detected, but in the time-slice LC-NMR analysis, several minor constituents were revealed (see compounds eluting between 7 and 12 h in Figure 5.6b). These different constituents correspond to various benzophenone, xanthone, and flavone *O*- and *C*-glycosides.[25]

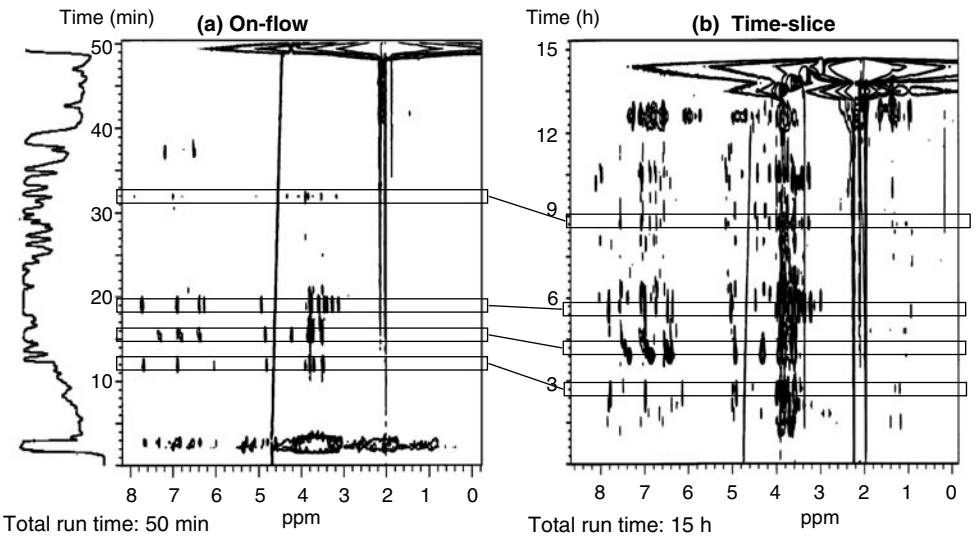

FIGURE 5.6 Comparison of the on-flow and "time-slice" stop-flow LC-NMR contour plots of the MeOH extract of the aerial parts of *Gnidia involucrata* (Thymelaceae). As shown, the on-flow experiment (a) allows the detection of four major compounds, whereas the "time-slice" procedure (b) reveals about 20 components. Amount injected: 20 mg. HPLC conditions: C18 column with radial compression, Waters RCM 8 × 10 (100 mm × 8 mm i.d.); MeCN:D_2O gradient (5:95–20:80 in 50 min); 0.9 mL/min. LC-NMR conditions: 24 scans/increment (on-flow); 1024 scans/increment (time slice); 60 µL flow cell (3 mm i.d.); 500 MHz. (Reproduced from Wolfender, J.-L., Ndjoko, K., and Hostettmann, K., *Phytochem. Anal.*, 12, 2, 2001. With permission. Copyright John Wiley & Sons Limited.)

The on-flow LC-NMR technique requires a compromise to be made on the LC separation side. One way to improve both the sensitivity of NMR detection and the LC resolution is to work in the indirect mode of NMR hyphenation, either by SPE-NMR or CAP-NMR (Section 5.3.1).

5.2.2 Modes of Operation of LC-NMR in Direct Hyphenation

5.2.2.1 On-Flow

In the on-flow mode, the LC-NMR spectra are acquired continuously during the HPLC separation and are stored as a set of scans in discrete increments. This method does not need any complex automation for the control of the HPLC. The on-flow data are processed as a 2D NMR experiment. One dimension of the contour plot represents the NMR ppm scale and the other the time scale (for typical 2D, on-flow LC-NMR plots, see Figures 5.5, 5.10, and 5.13). When the separation is performed in the gradient elution mode, the NMR signals are shifted according to the change in solvent composition. The NMR keeps the main solvent signal at a fixed frequency while the other signals are shifted. This influence of the gradient can be observed by virtue of the shift of the residual HOD line during elution (Figures 5.5 and 5.10). Depending on the analysis, a compromise between the number of scans per increment and the LC resolution has to be made to ensure both a satisfactory sensitivity and reasonable separation of the signals of closely eluting LC peaks. As previously discussed (Section 5.2.1.3), the main drawback of on-flow LC-NMR is its inherent low sensitivity. Practically, therefore, on-flow LC-NMR measurements will be mainly restricted to the direct measurement of the main constituents of a crude extract and usually under "overloaded" LC conditions. Typically, 1–5 mg of crude plant extract will have to be injected on-column.

5.2.2.2 Stop-Flow

Operation in the stop-flow mode requires that the retention times of the analytes of interest are known or that a sensitive method of prior detection, such as LC-UV or LC-MS, is used to trigger LC-NMR data acquisition. In practice, one of these detectors is connected online before the NMR instrument, and the signal of the analyte of interest passing through this detector is used to trigger a valve, which will stop the LC flow exactly when the peak reaches the NMR cell after a calibrated delay (Figure 5.2). The stop-flow mode allows the acquisition of a larger number of transients for any given LC peak, thereby providing satisfactory LC-^{1}H NMR spectra of compounds present in the low-microgram range. The solvent suppression is of a better quality than in the on-flow mode. Recording of various 2D correlation experiments, such as COSY and NOESY, is also possible provided that the concentration of the metabolite is high enough (generally more than 100 µg of sample is required). Inverse ^{1}H–^{13}C correlation experiments can be measured, but, in practice, very high loadings, not always compatible with a good LC separation, are necessary and much better results can be obtained with indirect hyphenation of NMR (Section 5.3).

An example of the improvement in S/N obtained between the on-flow and stop-flow modes is shown in Figure 5.7 for the secoiridoid sweroside (**8**). The LC-NMR spectra of this compound were obtained directly from the analysis of crude extract of *Swertia calycina*, a Gentianaceae species of Africa. In the stop-flow mode, it was possible to obtain a ^{1}H–^{1}H 2D correlation (COSY) spectrum directly from the peak in the crude extract. Despite this, it is important to note that the quality of the spectra obtained in the stop-flow mode is worse than those obtainable in the indirect mode of hyphenated NMR, for example, the CAP-NMR spectra of a similar compound shown in Figure 5.3.

5.2.2.3 Loop Storage

A more practical way to perform stop-flow LC-NMR is to use a loop collector, triggered by a sensitive LC detector, for example, LC-UV or LC-MS, which will automatically collect the peaks of interest without stopping the LC flow. Off-line, postchromatographic analysis of the content of the loops is then automatically performed in LC-NMR.[14]

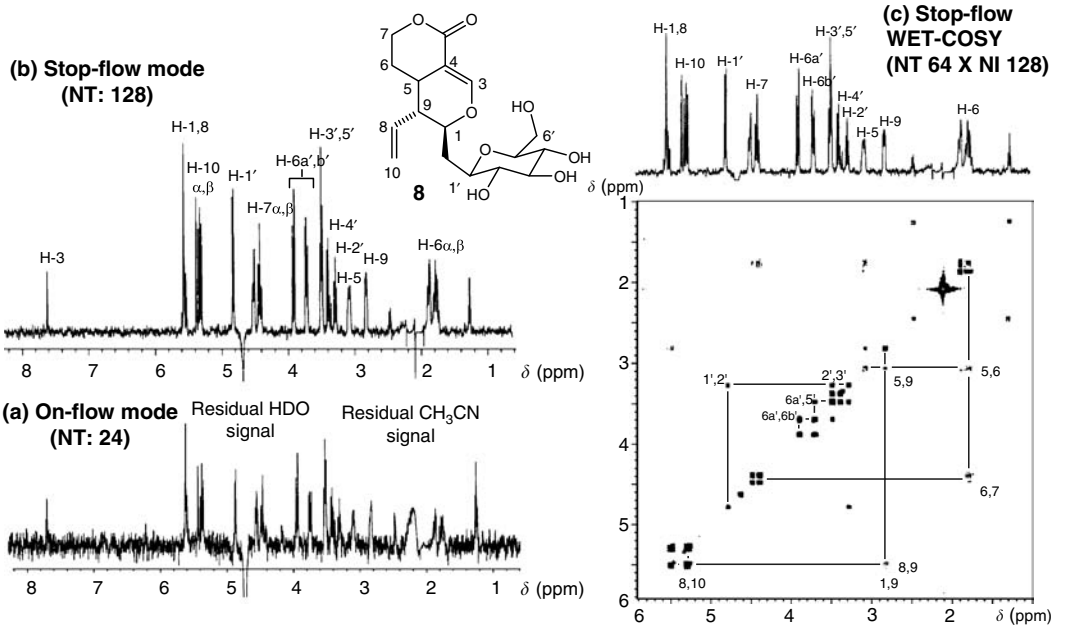

FIGURE 5.7 Comparison of the on-flow (a) and stop-flow (b) LC-^{1}H NMR of sweroside (**8**) in a dichloromethane extract of *Swertia calycina* (Gentianaceae), with direct acquisition of a WET-COSY ^{1}H–^{1}H stop-flow spectrum (c). LC-NMR: 60 µL flow cell (3 mm i.d.); 500 MHz. (Reproduced from Wolfender, J.-L., Ndjoko, K., and Hostettmann, K., *Phytochem. Anal*., 12, 2, 2001. With permission. Copyright John Wiley & Sons Limited.)

5.2.2.4 Time Slicing

When peaks are difficult to separate or are hardly detectable, it is possible to stop the flow at short intervals during the passage of the eluting peak through the NMR flow cell. This method is known as time slicing. It allows chromatographic peak purity to be estimated. This method can also be used for analyzing a complex extract when stop-flow experiments are difficult to trigger based on UV peak detection (e.g., the analysis of *G. involucrata* in Figure 5.6).

5.2.3 "Hypernation": Integration of LC-NMR in Multiple Hyphenation

Even if NMR is arguably the most versatile analytical platform for complex mixture analysis,[14] it is seldom possible to solve the structure of a novel compound by this technique alone. Indeed, common functional groups such as carboxylic acids, phenols, and amino groups are NMR-silent in many solvents because of proton–deuterium exchange. The presence of these groups, however, can be deduced from mass spectrometry (MS), which will provide molecular weight, molecular formula, and molecular fragment information.[14] In natural product chemistry, UV spectra may also provide very valuable information especially for polyphenols, which exhibit characteristic chromophores.[26]

When compared to the classical use of UV spectroscopy, MS, and NMR spectroscopy applied to pure natural products, the integration of all these techniques in their hyphenated forms (LC-UV, LC-MS, and LC-NMR) in a single setup, with centralized acquisition of the spectroscopic data, should ideally permit the complete spectroscopic characterization of different metabolites in a mixture in a single analysis. Furthermore, other existing hyphenated techniques such as LC-IR[27] or LC-circular dichroism (CD)[28] may also bring valuable complementary information. It has been demonstrated that it is possible from a practical point of view to couple HPLC with up to four spectrometers in an extensive hypernated system (LC-UV-FTIR-NMR-MS). This has permitted, for example, the characterization of ecdysteroids in the silene plant *Lychnis flos-coculi*, a member

of the family Caryopyllaceae.[29] In other applications, LC-CD-NMR-MS has shown its potential for the determination of the full absolute stereostructure of new metabolites in plant extracts.[28] These types of extended hypernations did not pose overwhelming technical problems. Rather, practical difficulties have been reported, centered around finding eluents compatible with all the spectroscopic techniques involved, coping with large differences in analyte detectability, and data handling.[30] The main limitation of such systems is of course that they will be only as sensitive as the least sensitive spectrometer used.

The more robust hypernated systems that have evolved into completely automated systems are those involving LC-NMR-MS.[14] The main problems in such systems arise from sample overloading into the MS systems and shift of the molecular weights due to proton–deuterium exchange. These types of problems can, however, be overcome by the use of an efficient postcolumn splitter, which diverts a small portion of the flow into the mass spectrometer and simultaneously enables proton–deuterium back exchange by dilution with an appropriate make-up flow as discussed in Section 5.3.1 and shown in Figure 5.27. However, a deuterium exchange experiment on the MS side might also reveal valuable additional structural information on the number of exchangeable protons in a given molecule, for example, the analysis of alkaloids from *Erythroxylum vacciniifolium* (catuaba) (Section 5.2.4.1.2).[31] The great advantage of LC-NMR-MS is that MS data can be directly correlated to the NMR spectra acquired. LC-MS can also be used to precisely trigger stop-flow or loop storage LC-NMR experiments. Furthermore, the MS detection can be used to deconvolute coeluting components with a limitation for isobaric or isomeric metabolites. Latest developments of this approach involve automated MS-directed LC-SPE-NMR-MS, which provides higher sensitivities.[32] This aspect will be discussed in the indirect hyphenation of NMR (Section 5.3).

In the authors' laboratory, the direct hyphenated LC-NMR analyses are performed independently from the LC-UV-MS runs. This is mainly due to practical reasons (e.g., magnet not shielded), and to minimize the compromises that have to be made between UV-MS and NMR detection. Thus, LC-UV-MS is used as a first dereplication step for the chemical profiling of crude plants extracts. Compounds are tentatively identified on the basis of molecular weight and fragment information, integrated with manual searches in natural product libraries as well as in-house UV spectra libraries. LC-NMR is mainly used in a second step for a more detailed structural investigation of compounds presenting original structural features or displaying interesting activities after LC bioassays (Figure 5.8).[33]

It is noteworthy that additional structural information on the localization of the hydroxyl groups in polyphenols can be obtained online with LC-DAD-UV following postcolumn addition of UV shift reagents. These reagents are classically used for the structural characterization of these products in a pure form.[34] Multiple injections of a given crude extract, each time with another reagent, for example, a strong base, a weak base, or a complexing agent such as $AlCl_3$, provide useful information for localization of the hydroxyl groups on the basis of a comparison of the shifts recorded for the absorption band of the polyphenols. Such techniques have been successfully applied to the characterization of various polyphenols.[35,36] Examples of the type of UV-shifted spectra that can be recorded online are discussed and shown in Section 5.2.4.1.1 and Figure 5.11 for the antifungal isoflavones identified from *E. vogelii* (Leguminosae) or in Section 5.3.3 and Figure 5.32a for a flavonoid glycoside from *Eriophorum scheuchzeri* (Cyperaceae).

The combination of UV, MS, MS/MS, and NMR data has been found to be mandatory for the full or partial structure elucidation of the metabolites of interest in crude plant extract. This will be illustrated in the Section 5.2.4 using different examples from our own research.

5.2.4 Application to the Analysis of Crude Plant Extracts

In the fields of pharmacognosy, phytochemistry, and, more generally, natural product research, LC-hyphenated techniques represent strategic tools for speeding up the drug discovery process. Indeed, to discover new bioactive compounds, which could become new leads or new drugs, extracts from plants or other natural sources should be submitted at the same time to a chemical screening

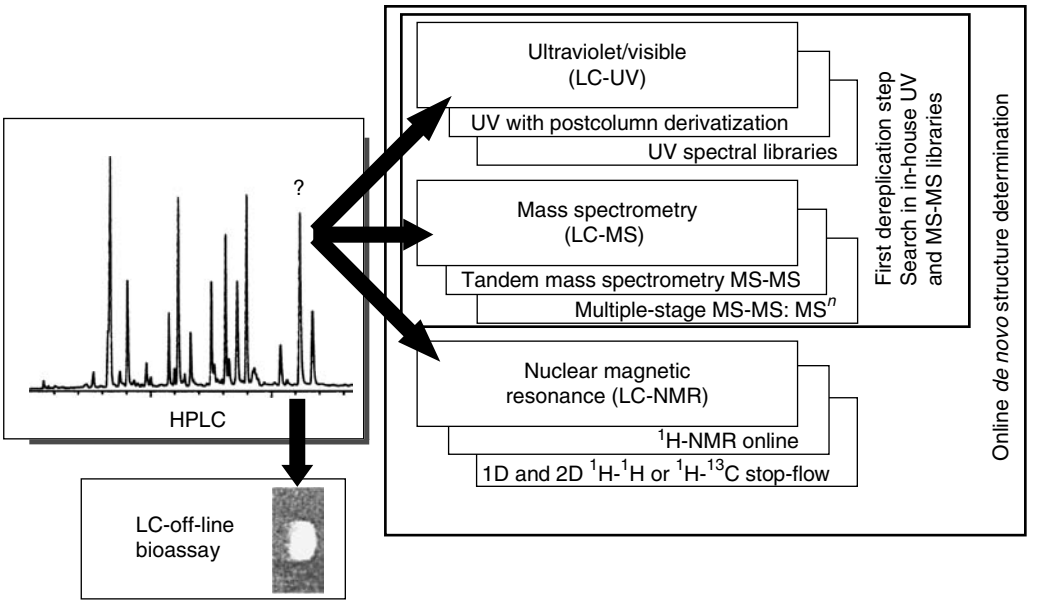

FIGURE 5.8 Type of information that can be obtained for a given LC peak with the different LC-hyphenated techniques. In this approach LC-NMR can be regarded as an efficient addition to LC-UV-MS for *de novo* structure identification of natural products online. (Reprinted from Wolfender, J.-L., Ndjoko, K., and Hostettmann, K., *J. Chromatogr. A*, 1000, 437, 2003. With permission from Elsevier.)

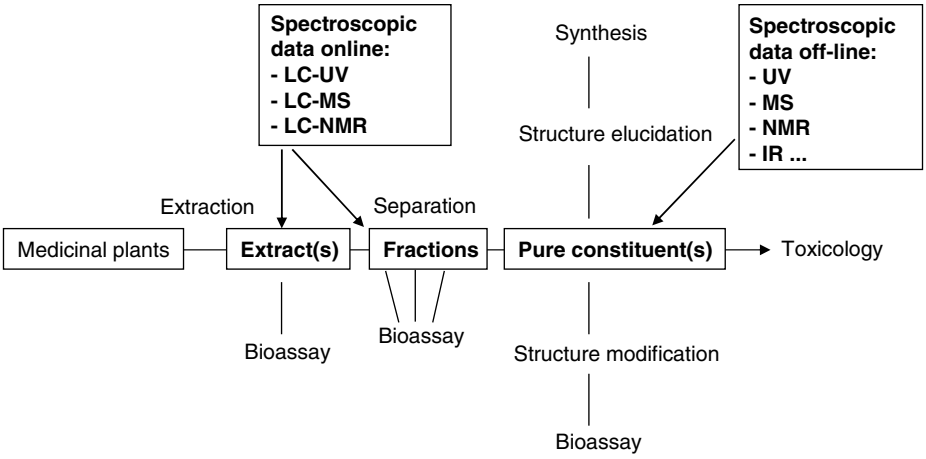

FIGURE 5.9 A procedure for obtaining the active principles from plants and use of LC-hyphenated techniques as strategic analytical screening tools to enable dereplication and rapid online identification in the isolation process.

and to various biological or pharmacological targets. The chemical screening or metabolite profiling based on LC-UV-MS and LC-NMR is aimed at distinguishing between known compounds (dereplication) and new molecules in crude extracts. Thus, the unproductive isolation of known compounds can be avoided, and a targeted isolation of novel constituents or constituents presenting original bioactivities can be undertaken (Figure 5.9).[37]

Metabolite profiling in crude plant extracts is not an easy task to perform due to the structural diversity of natural products.[38] In contrast to the sequencing of genes or proteins, the order of the

atoms and stereochemical orientations have to be elucidated *de novo* for each compound. This is a complex process in which LC-NMR is often mandatory (Figure 5.8). Consequently, and unlike genomics and proteomics, a single analytical technique does not exist that is capable of profiling all secondary metabolites in a crude extract.[39] The complete profiling of all metabolites in an organism as function of its metabolism is often referred to as "metabolomics".[38]

The rich structural complexity of natural products presents a good chance to find new effective drugs. Indeed, this potential of natural products has prompted synthetic chemists to produce them in the laboratory, often with therapeutic applications in mind, and many drugs used today are natural products or derived from natural compounds.[40] Research in pharmacognosy has demonstrated that potent bioactive products can be obtained from plants. In the present drug discovery programs, natural products or compounds derived from natural products account for more than 40% of the new registered drugs.[41,42] A statistical investigation into the structural complementarity of natural and synthetic compounds also proved that the potential for new natural products is not exhausted and they still represent an important source for the lead finding process.[43,44]

5.2.4.1 Chemical Screening by On-Flow LC-NMR

5.2.4.1.1 Antifungal Isoflavones from E. vogelii

In our search for new antifungal agents from plant origin, we have screened numerous plant extracts for activity against the plant pathogenic fungus *Cladosporium cucumerinum* by TLC bioautography.[45] This type of biological assay can be also performed on HPLC fractions for a precise localization of the bioactive compounds in the chromatogram since the amount loaded for LC-NMR (several micrograms in each LC peak) is compatible with the bioassay. This combined LC-NMR and LC bioassay approach is illustrated here by the investigation of an African plant *E. vogelii* (Leguminosae).[46,47]

The crude dichloromethane extract of the roots of *E. vogelii* was first analyzed using high-resolution LC-Q TOF MS/MS together with LC-UV DAD with postcolumn addition of UV shift reagents. The LC-UV trace revealed the presence of a dozen major peaks. These different constituents shared rather similar types of UV spectra, with two main absorption bands of decreasing intensity characteristic for isoflavones. For one group of peaks (**9**–**11**) three maxima were recorded at ca. 210, 260, and 290 (sh) nm; for the other group (**12**–**15**), these bands were present at ca. 210, 290, and 333 (sh) nm. The UV spectra for most of the minor peaks in the chromatogram also indicated one of these two types of chromophores. Using postcolumn addition of a reference compound for mass lock, the high-resolution LC-Q TOF MS and LC-Q TOF MS/MS data provided the molecular formulae. Molecular weights were obtained in all cases with a precision of less than 5 ppm.[48] Presence of fragments due to losses of 56 and 69 Da in the LC-MS/MS spectra of all constituents revealed the possible presence of prenyl chains. From these preliminary LC-UV-MS results, it was concluded that the dichloromethane extract of *E. vogelii* most probably consisted of a combination of various prenylated isoflavanones or isoflavones.

The on-flow LC-^{1}H NMR analysis of the extract, followed by HPLC microfractionation, was carried out on a C18 radial compression column having a large inner diameter (8 mm). This allowed 10 mg of extract to be injected on column, and separation was achieved at a low flow rate (0.1 mL/min) that enabled sensitive detection (256 scans/increment). During the LC-NMR analysis, an LC-microfractionation of the different peaks was performed every 10 min (1 mL fractions) for the antifungal bioautography assays against *C. cucumerinum*. The postchromatographic antifungal assay revealed activity in the fractions associated with the LC peaks eluting at 8.4, 10.8, 11.3, 12.2, and 14.6 h (Figure 5.10). In the on-flow contour plot of the crude extract, the signals of more than ten LC peaks were efficiently recorded. *De novo* structure determination based on the complementary sets of online data obtained was performed for each LC peak of interest.

The ^{1}H NMR data extracted from the low-flow LC-NMR analyses confirmed that all the major constituents of the crude extract of *E. vogelii* were prenylated isoflavones and isoflavanones

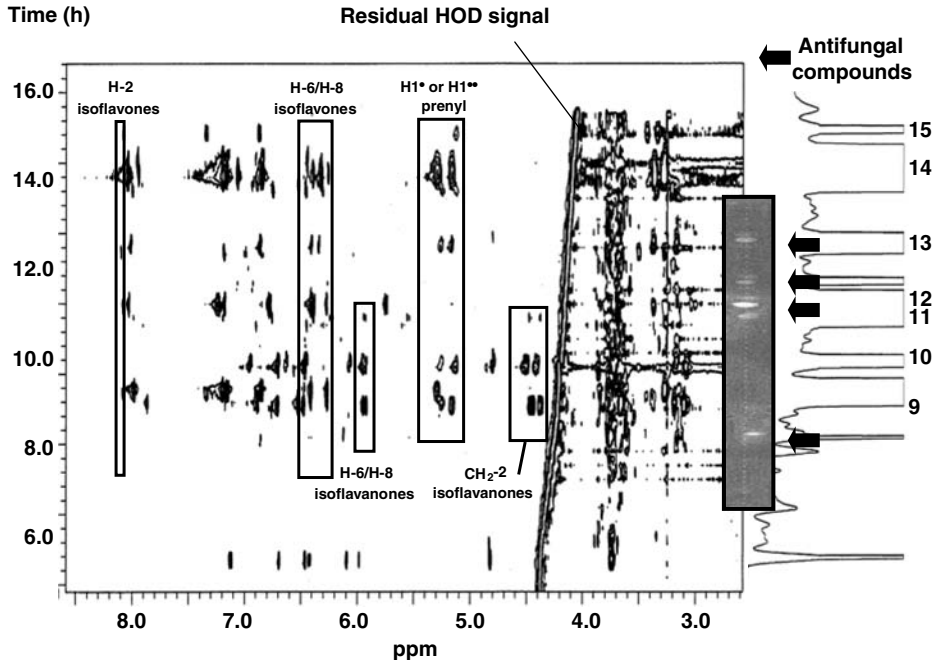

FIGURE 5.10 On-flow LC-NMR contour plot of the crude dichloromethane extract of *E. vogelii* (Leguminosae). On this contour plot, all ^{1}H NMR resonances of the analytes are clearly visible. A sensitive detection was obtained due to the low flow rate used. Several characteristic regions for the resonances of isoflavanones and isoflavones are highlighted. The signals at ca. 4.5 ppm clearly indicate that the isoflavanones are eluting first on the chromatogram. The inset represents the antifungal TLC bioautography assay performed at-line on all HPLC microfractions. Four constituents highlighted with arrows were found to exhibit antifungal activity. HPLC conditions: C18 column with radial compression (Waters RCM 100 mm × 8 mm i.d.); MeCN:D_2O gradient (5:95–100:0; 19 h); 0.05% TFA; 0.1 mL/min. LC-NMR conditions: 265 scans/increment (on-flow); 60 µL flow cell (3 mm i.d.); 500 MHz. (Reprinted from Wolfender, J.-L., Ndjoko, K., and Hostettmann, K., *J. Chromatogr. A*, 1000, 437, 2003. With permission from Elsevier.)

(see **11** and **12** in Figure 5.11). A first evaluation of the on-flow LC-NMR contour plot revealed that in all peaks, aromatic signals between 5.9 and 6.4 ppm were indicative of an A-ring oxygenated in positions 5 and 7. This was in good agreement with chemotaxonomical data, which indicated a 5,7,4′ oxygenation for biosynthetic reasons. For compounds **9**–**11**, methylene protons at 4.39–4.57 ppm were characteristic of an isoflavanone nucleus, whereas for peaks **12**–**15**, the presence of an aromatic singlet at ca. 8 ppm was indicative of isoflavones. This was also confirmed by the presence of two distinct types of chromophore for each group of constituents in the UV-DAD spectra recorded (see **11** and **12** in Figure 5.11). Signals of prenylated groups were detected between 3.0 and 3.3 ppm (H-1″), 5.1 and 5.3 ppm (H-2″), and 1.5 and 1.6 ppm (2 CH_3) for all compounds. These diagnostic signals were readily observable on the contour plot of the on-flow LC-^{1}H NMR experiment, and most of the characteristic resonances are highlighted in Figure 5.10. Isoflavone **12** presented the strongest zone of inhibition in the postchromatographic antifungal bioautography assay. Its molecular formula of $C_{20}H_{19}O_5$ was deduced from the protonated molecular ion (MH^+) peak at *m/z* 339.1208, whereas losses of C_4H_7 or C_5H_9 were characteristic of a prenyl moiety. The LC-^{1}H NMR spectrum showed two singlets at δ 6.26 and 6.40 attributable to two aromatic protons (H-6 and H-8). Three signals corresponding to an ABX system at δ 6.85 (J = 8.0 Hz), 7.14 (J = 8.0 Hz), and 7.19 (br s) were attributed to the B-ring aromatic protons H-5′, H-6′, and H-2′, respectively. Finally, a singlet at δ 7.9 was assigned to H-2, a proton characteristic of an isoflavone nucleus. The positions of the three

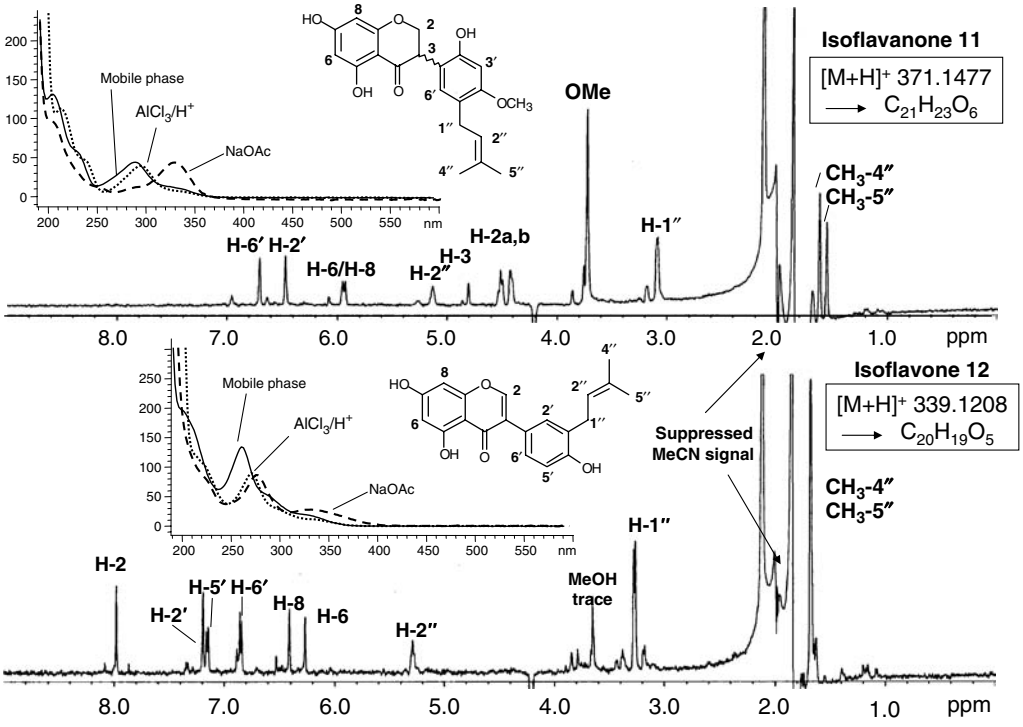

FIGURE 5.11 On-flow LC-NMR spectra of an isoflavanone **11** and the antifungal isoflavone **12**. Complementary UV-DAD and shifted UV-DAD spectra recorded online, as well as exact molecular formula assignments based on LC-APCI QTOFMS data, are also displayed. Same LC-NMR conditions as in Figure 5.10. (Reprinted from Wolfender, J.-L., Ndjoko, K., and Hostettmann, K., *J. Chromatogr. A*, 1000, 437, 2003. With permission from Elsevier.)

hydroxyl groups and the prenyl unit were deduced from the complementary UV-shifted spectra and the MS/MS experiments. The shifted UV spectra (see UV spectra of **11** and **12** in Figure 5.11) were obtained by LC-UV DAD analyses of the extract with postcolumn addition of UV shift reagents.[49] The shifts observed were interpreted according to the rules previously established for the analysis of pure polyphenols.[34] In the case of **12**, the 5,7-dihydroxylation of the A-ring was deduced from the UV shifts observed upon addition of $AlCl_3$ and NaOAc. The CID MS/MS spectra obtained at a collision energy of 40 eV confirmed the presence of two hydroxyl groups on the A-ring (retro Diels–Alder cleavage *m/z* 153.0450 $[A1]^+$). All these data suggested that **12** was isowighteone, a known prenylated isoflavone that was dereplicated by this means.

Compound **11** (Figure 5.11) showed a UV spectrum and a three-proton NMR spin system (δ 4.39 [H-2a], 4.47 [H-2b], and 4.80 [H-3]) typical of an isoflavanone nucleus. The MH^+ ion at *m/z* 371.1477 indicated a molecular formula of $C_{21}H_{22}O_6$ for **11**, and consequently, a substitution by three hydroxyl, one methoxyl, and one prenyl groups was deduced. The presence of the methoxyl group was confirmed by a singlet at δ 3.66 (3H) in the LC-NMR spectrum. The two broad singlets corresponding to H-6 and H-8 (δ 5.93 and 5.95 ppm) were slightly shifted to higher field compared to **12**. In the LC-^{1}H NMR spectrum, two broad singlets at δ 6.47 and 6.70 were attributed to the aromatic protons of the B-ring (Figure 5.11). The position of the prenyl unit and that of the methoxyl group could not, however, be precisely assigned on the basis of ^{1}H NMR data only since the resolution of the aromatic signals did not allow distinction between *meta*- or *para*-coupled protons. These two substituents could be either at 2′ and 5′ or at 3′ and 6′, respectively. A computer simulation indicated that the best match for H-3′ (δ calc. 6.40) and H-6′ (δ calc. 6.64) was found for

a B-ring substitution by a prenyl group at 5′ and a methoxyl at 4′. It was not possible to fully ascertain the structure on these indications alone. However, this information suggested that **11** was probably a new natural product and its targeted isolation was undertaken. Its structure was confirmed to be 5,7,2′-trihydroxy-4′-methoxy-5′-(3-methylbut-2-enyl)-isoflavanone.[47] By following the same approach, integrating LC-^{1}H NMR with UV and MS data, eight polyphenols were thus identified partially or totally online in the crude extract of *E. vogelii*.[48]

5.2.4.1.2 *Tropane Alkaloids from* E. vacciniifolium

Another example of online identification of natural products by on-flow LC-NMR is illustrated by the analysis of the alkaloids from *E. vacciniifolium*, a Brazilian Erythroxylaceae known as "catuaba".[31] Catuaba is popular in Brazil and is used in traditional medicine for its aphrodisiac and tonic properties, even though precise identification of the plant seems to be uncertain.[50] In this case, and in contrast to the example described in Section 5.2.4.1.1, 3 mg of the alkaloid extract was analyzed directly at 1 mL/min with on-flow LC-NMR, with good sensitivity for the main constituents in a 1 h LC run.

To obtain a preliminary idea of the alkaloids present in *E. vacciniifolium*, a combined LC-UV-DAD and positive-ion LC-APCI MS analysis of the alkaloid extract was performed using an alkaline MeCN–H_2O gradient on a C18 column. This preliminary analysis revealed that most of the constituents shared similar UV, MS^2, and MS^3 spectra suggesting a common core structure in all compounds (see, for example, the dependent scan MS^n spectra for **39** in Figure 5.12).[51]

The on-flow LC-NMR analysis revealed similarities between the compounds recorded (**22** and **36**–**39**) (Figure 5.13). The spectra of **22** and **28** were obtained in the stop-flow mode because of their lower abundance (see spectrum of **22** in Figure 5.14). The data suggested that the main constituents were tropane alkaloids esterified with pyrrolic acid moieties.

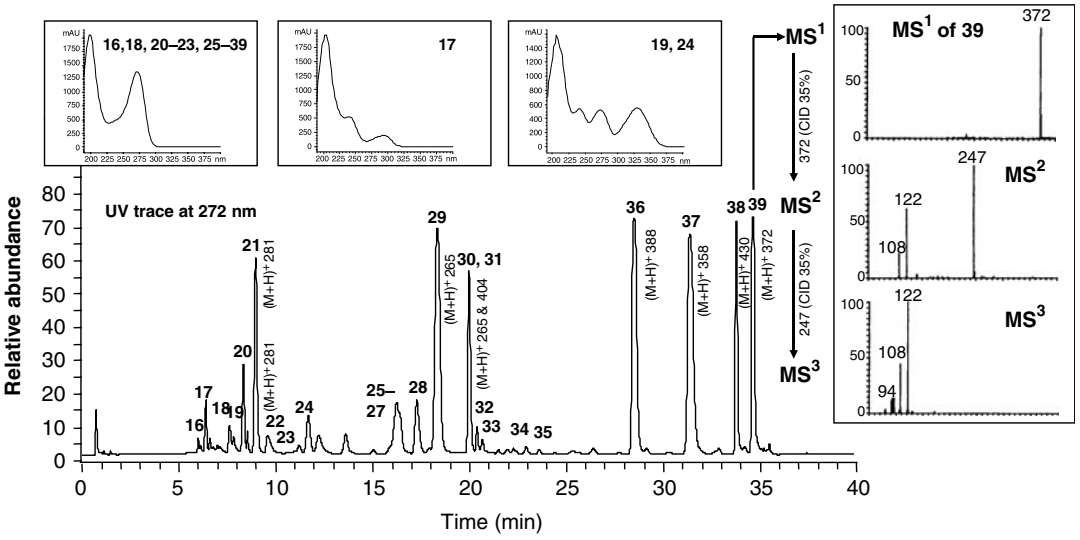

FIGURE 5.12 LC-UV-APCI MS analysis of the crude alkaloid extract of *E. vacciniifolium* (Erythroxylaceae). The UV spectra were recorded between 200 and 500 nm. HPLC conditions: C18 column, Macherey–Nagel CC Nucleodur 100-5 (125 mm × 4.6 mm i.d.); MeCN (+2 mM NH_3):water (+2 mM TEA) gradient from 5:95 to 100:0 in 34 min, including five isocratic elution steps each of 5 min at 5:95, 18:82, 23:77, 35:65, and 40:60; 1.0 mL/min. The MH^+ ions of the main products recorded by LC-APCI MS^n are given. In the inset, the MS^n spectra recorded for compound **39** are displayed. (Reproduced from Wolfender, J.-L., Queiroz, E.F., and Hostettmann, K., *Magn. Reson. Chem.*, 43, 697, 2005. With permission. Copyright John Wiley & Sons Limited.)

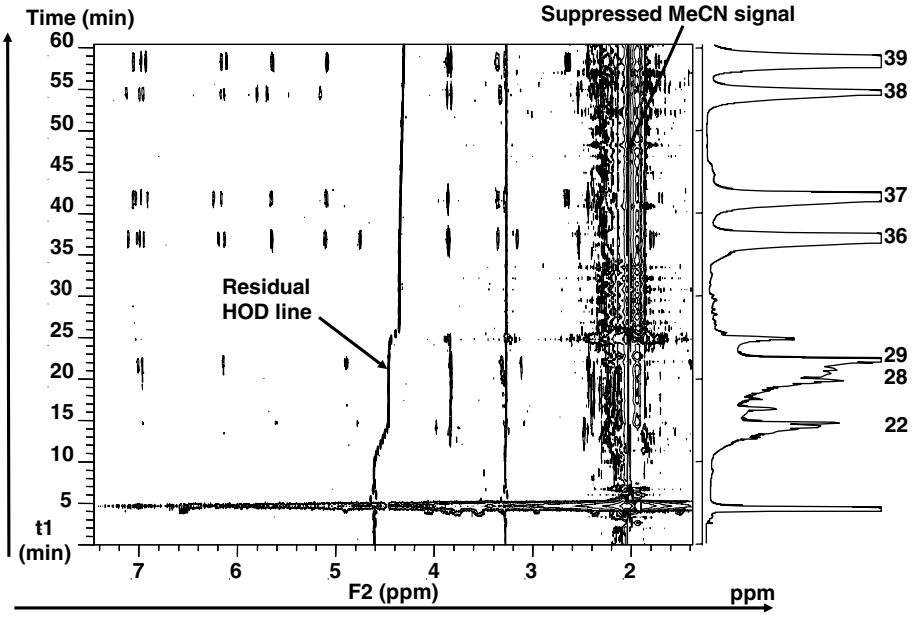

FIGURE 5.13 On-flow LC-NMR contour plot of the alkaloid extract of *E. vacciniifolium* (Erythroxylaceae). As shown, the on-flow experiment allows the detection of seven major compounds. Amount injected: 3 mg. HPLC conditions: C18 column, Macherey–Nagel CC Nucleodur 100-5 (125 mm × 8.0 mm i.d.); MeCN (2 mM NH_3):D_2O (2 mM NH_3) (5:95–100:0; 80 min) gradient including five isocratic elution steps; 1.2 mL/min. LC-NMR conditions: 32 scans/increment (on-flow); 60 μL flow cell (3 mm i.d.); 500 MHz (Reprinted from Zanolari, B., Wolfender, J.-L., Guilet, D., Marston, A., Queiroz, E.F., Paulo, M.Q., and Hostettmann, K., *J. Chromatogr. A*, 1020, 75, 2003. With permission from Elsevier.)

The strategy followed for their identification involved isolation of the main constituent (**37**) in a single step of medium-pressure liquid chromatography and structural elucidation following extensive 2D NMR analyses. It was identified as 3α-(1-methyl-1*H*-pyrrol-2-ylcarbonyloxy)-6β-(1*H*-pyrrol-2-ylcarbonyloxy) tropane (catuabine D).[52] The structure assignments of all the other analogues were performed by comparisons of the on-flow LC-^{1}H NMR and LC-MS^n spectra recorded. The ^{1}H NMR resonances of **37** obtained by on-flow LC-^{1}H NMR (Rt 42 min in Figure 5.14b) differed by less than 0.2 ppm from those of the isolated compound measured in $CDCl_3$ (Figure 5.14a). Typical resonances for a methylpyrrole or a nonmethylated pyrrole substructure were revealed (see H-3″ to H-5″ and H-3′ to H-6′, Figure 5.14b). The structure of the disubstituted tropane core skeleton was shown by three saturated methylenes at δ 1.79 (H_{endo}-2), 2.30 (3H, H_{exo}-2, H_{exo}-4, and H_{exo}-7) and 2.75 (H_{endo}-7). As shown in Figure 5.14, the solvent suppression around 2.10 ppm affected the signal detection of H_{endo}-4 protons. Two methine and one methyl groups linked to the nitrogen atom were also recorded at δ 3.38 (H-5), 3.46 (H-1), and 2.54 (N−CH_3), respectively. The last two oxygenated methine moieties showed resonances at δ 5.19 (H-3) and 5.75 (H-6). The multiplicity (triplet) of the H-3 signal with the coupling constant (J = 4.4 Hz) indicated the α-orientation (i.e., *endo*) of the substituent at C-3. The arrangement of the substituent at C-6 was established by the analysis of the coupling constants of H-6, H-7, and H-5 protons. H-6 of the disubstituted tropane alkaloid showed two couplings (J = 7.7 and 2.8 Hz) with the two H-7 protons, and it did not present any coupling with the vicinal H-5. This observation implied β-orientation (i.e., *exo*) of the substituent and a dihedral angle close to 90° between H-5 and Hα-6.[53]

On the basis of the on-flow LC-^{1}H NMR contour plot of the extract (Figure 5.13), a direct comparison of the chemical shifts of the other compounds indicated that they were analogues of catuabine D (Figure 5.14). For example, the LC-^{1}H NMR spectrum recorded for **39** at 58 min

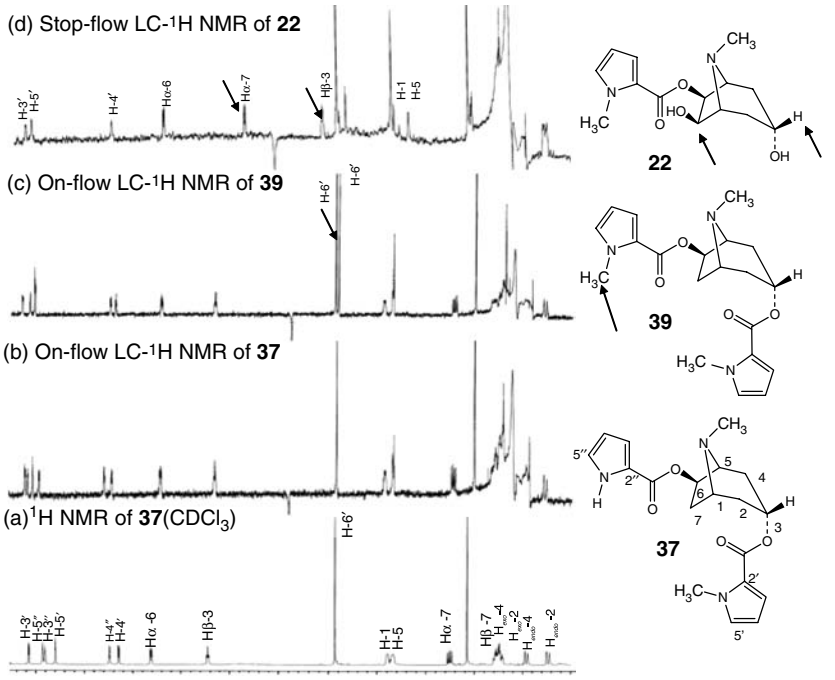

FIGURE 5.14 (a) ^{1}H NMR spectrum of the tropane alkaloid **37** ($CDCl_3$) isolated after a single step of medium-pressure liquid chromatography. On-flow LC-^{1}H NMR spectra of (b) compound **37**, (c) compound **39**, and (d) stop-flow LC-^{1}H NMR spectrum of the minor alkaloid **22**. A direct comparison of the different spectra together with computer simulations enabled the identification of all major constituents of the alkaloid extract of *E. vacciniifolium* (Erythroxylaceae). The main differences are indicated by arrows. For LC-NMR conditions see Figure 5.13. (Reproduced from Wolfender, J.-L., Queiroz, E.F., and Hostettmann, K., *Magn. Reson. Chem*., 43, 697, 2005. With permission. Copyright John Wiley & Sons Limited.)

(Figure 5.14c) exhibited similar chemical shifts to those of **37**, supporting the presence in the molecule of a tropane moiety dioxygenated at C-3 and C-6. The difference between the two compounds was in their ester substituents. In fact, two methyl groups were recorded at δ 3.93 (3H, s, N−CH_3) and 3.94 (3H, s, N−CH_3) in the LC-^{1}H NMR spectrum of **39**, indicating two methylpyrrole acid moieties. The presence of an additional methyl group in the structure of compound **39** was corroborated by the LC-MS data. On the basis of the above evidence, the structure of **39** was elucidated online as 3α,6β-di(1-methyl-1*H*-pyrrol-2-yl-carbonyloxy) tropane. The spectrum of the minor alkaloid **22** (Rt 14 min) was recorded in the stop-flow mode (Figure 5.14d). Its protonated molecular ion MH^+ *m/z* 281) suggested the presence of only one methylpyrrole ester. This compound displayed characteristic NMR differences compared to the trisubstituted tropane alkaloids **37** and **39**. These differences consisted mainly of a downfield shift of Hα-7 due to hydroxylation of this position and an upfield shift of Hβ-3 due to the lack of a methylpyrrolic acid in this position (Figure 5.14d). This compound was thus elucidated as 3α,7β-dihydroxy-6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane.

In a similar fashion, the online investigations indicated the structure of all the main alkaloids (**28**, **36**–**39**).[31] The structures of the minor constituents, not detectable by LC-NMR even in the stop-flow mode, were deduced from the LC-TOF MS (molecular weight) and LC–MS^n (fragmentation) data.[51] Most of these alkaloids could be grouped in sets of isomeric structures. In many cases, a difference of 16 Da between these sets was recorded, indicating the presence of an additional oxygen. To confirm that this oxygen corresponded to an additional hydroxyl group in the molecules, the number of exchangeable protons was calculated on the basis of a comparison of the LC-MS spectra recorded under LC-NMR conditions with deuterated solvents (MeCN:D_2O) and in standard

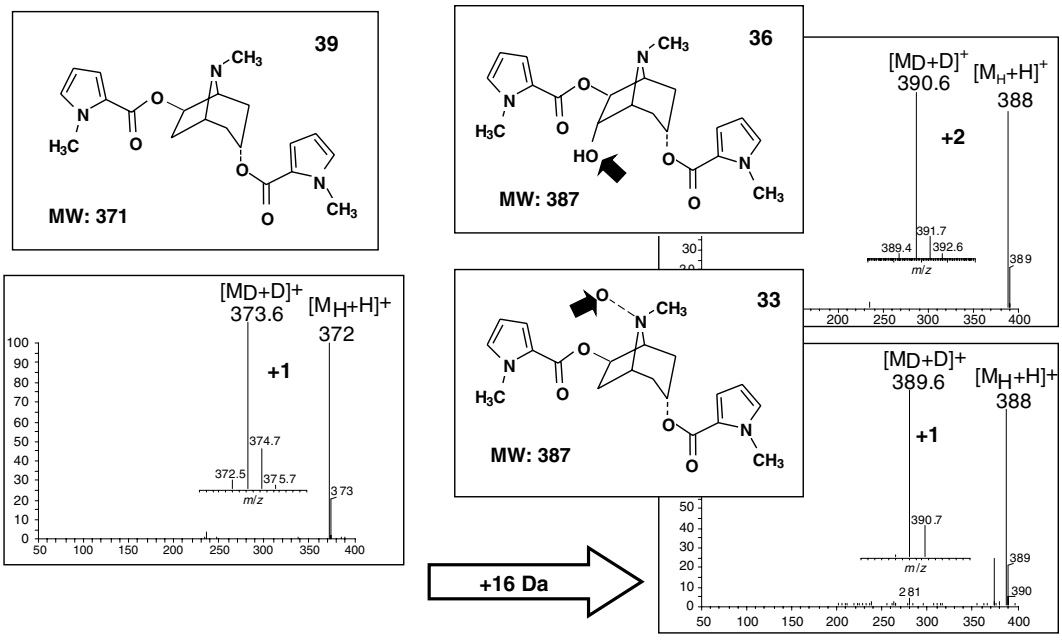

FIGURE 5.15 LC-MS spectra recorded for **39** and two analogues **33** and **36**, with protonated molecules shifted 16 Da, in the extract of *Erythroxylum vacciniifolium* (Erythroxylaceae). The inset in all spectra represent the MD^+ ions obtained when recording the spectra under LC-NMR (i.e., deuterated) conditions. A shift of 1 Da indicated no exchangeable protons; a shift of 2 Da indicated the presence of one hydroxyl. On the basis of these results, the presence of *N*-oxide or hydroxyl groups could be shown for a series of minor constituents of the extract. (Reproduced from Wolfender, J.-L., Queiroz, E.F., and Hostettmann, K., *Magn. Reson. Chem.*, 43, 697, 2005. With permission. Copyright John Wiley & Sons Limited.)

LC-MS experiments with nondeuterated solvents (MeCN:H_2O). As shown in Figure 5.15, for example, **33** and **36** exhibited the same protonated molecule (16 Da more than **39**), but they had different numbers of exchangeable protons (1 for **33** and 2 for **36**). This observation suggested an additional hydroxyl group for **36** and probably the presence of an *N*-oxide in compound **33**. In the same way, five other tropane alkaloids (**16**, **18**, **20**, **25**, and **33**) were deduced to contain an *N*-oxide in their structure in comparison with **22**, **23**, **28**, **35**, and **36**, respectively. Thus, as shown here, the LC-MS spectra recorded in typical LC-NMR conditions may also provide important structural information.[31,51]

On the basis of the above interpretation of LC-NMR and LC-UV–MS^n data, complete or partial structure elucidations of 24 alkaloids bearing the same tropane skeleton were achieved. Most of these compounds were found to be new natural products and these analyses permitted their final targeted isolation.[52]

5.2.4.2 Detailed Structural Investigation by Stop-Flow LC-NMR

5.2.4.2.1 Prenylated Flavanones from Monotes englerii

The combination of LC-UV, LC-MS, and LC-^{1}H NMR data in many cases permits the structural elucidation of various known compounds. These data, however, are not always sufficient for a full online identification of new constituents. If a complete structural identification has to be carried out, different 2D correlation experiments have to be performed. These experiments can be run by LC-NMR in the stop-flow mode, provided that the sample concentration in the LC-NMR cell is high enough (typically hundreds of micrograms are needed). In this mode, ^{1}H–^{1}H correlations such as COSY or NOESY can be measured. Information on ^{13}C can be extracted from ^{1}H–^{13}C correlation

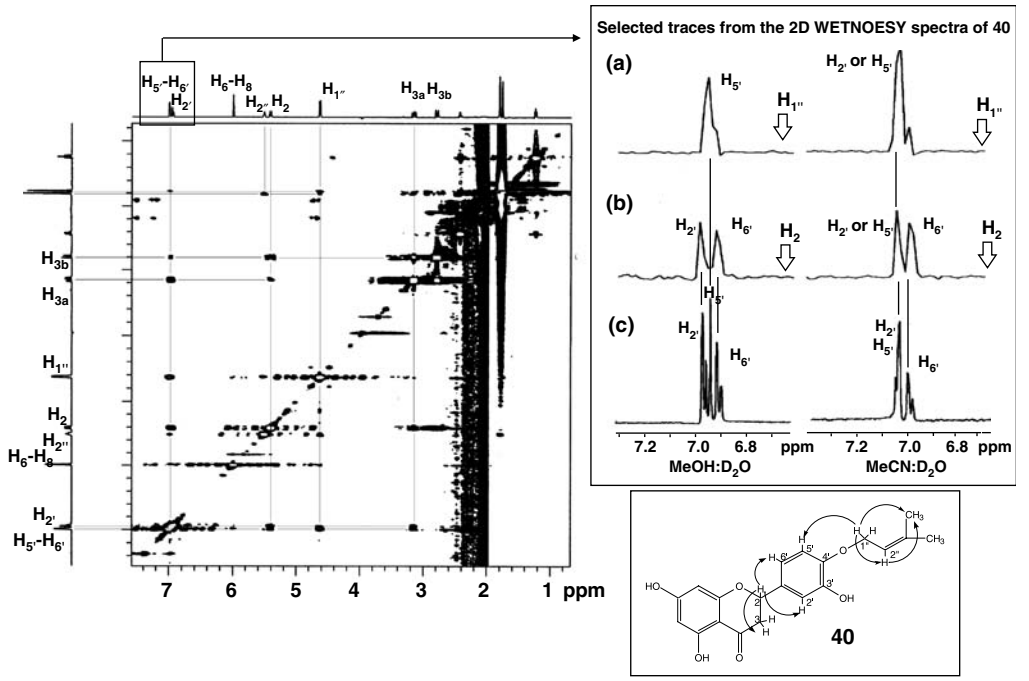

FIGURE 5.16 Stop-flow LC-WET NOESY spectrum of the flavanone **40**. One milligram of the enriched fraction injected; solvent, MeOH:D_2O. Inset: selected traces showing the main NOE correlations in both MeCN:D_2O and MeOH:D_2O solvent systems. LC-WET NOESY: transients 8, increments 256, time: 2.5 h. (Reprinted from Wolfender, J.-L., Ndjoko, K., and Hostettmann, K., *J. Chromatogr. A*, 1000, 437, 2003. With permission from Elsevier.)

experiments such as HSQC or HMBC. These latter types of experiments are, however, very difficult to obtain in the direct hyphenation of LC-NMR and are much more efficiently measured at-line with SPE-NMR or CAP-NMR (Section 5.3.1).

For example, in the study of a Dipterocarpaceae plant *M. englerii*, LC-UV-MS and LC-NMR data enabled the partial characterization of its main antifungal agent to be an *O*-prenylated flavanone (**40**),[54] but the position of the prenylated chain could not be ascertained on the basis of this information only. Stop-flow LC-2D NMR correlation experiments were thus performed on an enriched fraction containing 1 mg of **40**. To determine the exact position of the prenyl unit on the flavanone skeleton of **40**, a 2D LC-WET NOESY experiment was undertaken. The sensitivity was good enough for the observation of clear NOE effects. However, the weak resolution of the H-pattern of the B-ring in the MeCN:D_2O solvent system did not permit the differentiation of H-2′ and H-5′, preventing the full structural assignment of **40** (Figure 5.16). A second stop-flow LC-NMR analysis of the active fraction was then performed in MeOH:D_2O. In this latter solvent system, the three aromatic protons of the B-ring were resolved. Two doublets ($J = 7.8$ Hz) at δ 6.90 and 6.95 were attributable to the *ortho*-coupled protons H-6′ and H-5′, and one broad singlet at δ 6.97 was due to H-2′. A second WET NOESY was performed with this latter solvent system (Figure 5.16). First, an irradiation of H-2 gave enhancement of the H-2′ and H-6′ aromatic protons at δ 6.97 and 6.90 ppm. A correlation with the H-2 and the two protons on C-3 was also shown (Figure 5.16). Second, irradiation of H-1″ gave enhancement of the *ortho*-coupled proton at δ 6.95 ppm (H-5′). Other NOE effects were also measured on the prenyl unit, particularly between H-1″ and H-2″ and between H-1″ and H-5″, as well as between H-2″ and H-4″ (Figure 5.16). According to these observations, the attachment of the *O*-prenyl substituent at position C-4′ was confirmed and **40** could thus be identified online as 2,3-dihydro-5,7-dihydroxy-2-[3-hydroxy-4(3-methyl-2-butenyl)oxyphenyl]-4*H*-1-benzopyran-4-one.

It was found to be a new natural product, and its complete isolation was finally performed for the measurement of its physical data, as well as the determination of its absolute configuration.[54]

It should be noted, however, that absolute configuration determination is possible online using hyphenated CD, that is, HPLC-CD. For example, the online 2D LC-ROESY and LC-TOCSY investigation of an isoquinoline and a naphthylisoquinoline alkaloid from the crude extract of a Dioncophyllaceae species *Habropetalum dawei* allowed the determination of their relative configuration, whereas the absolute configuration was determined online by subsequent stop-flow HPLC-CD experiments and empirical analysis of their CD data.[55]

5.2.4.2.2 *Oxidation Products of Hyperforin from* Hypericum perforatum

LC-NMR in the stop-flow mode was found to be very useful for the structure elucidation of closely related isomers. This was the case for the study of oxidation products of hyperforin, a phloroglucinol derivative that is claimed to be responsible in part for the antidepressive effect of *H. perforatum*.[56] Hyperforin is unstable and its lability to oxidative degradation poses serious problems for standardization and may also dramatically affect the pharmacological activity of the extracts. These oxidation products are difficult to isolate on a preparative scale, and therefore the characterization using hyphenated methods was mandatory.[57]

Hyperforin was treated with *n*-hexane to yield various oxidized forms. The crude hexane solution was analyzed using HPLC-DAD-ESI MS and MS^n. Four main LC peaks were detected upon this treatment (Figure 5.17). Their HPLC separation was difficult to achieve and only a partial resolution was obtained after elution on two phenyl columns connected in series. Furohyperforin (**41**) was identified as the slower eluting peak on the basis of the online data obtained and with the help

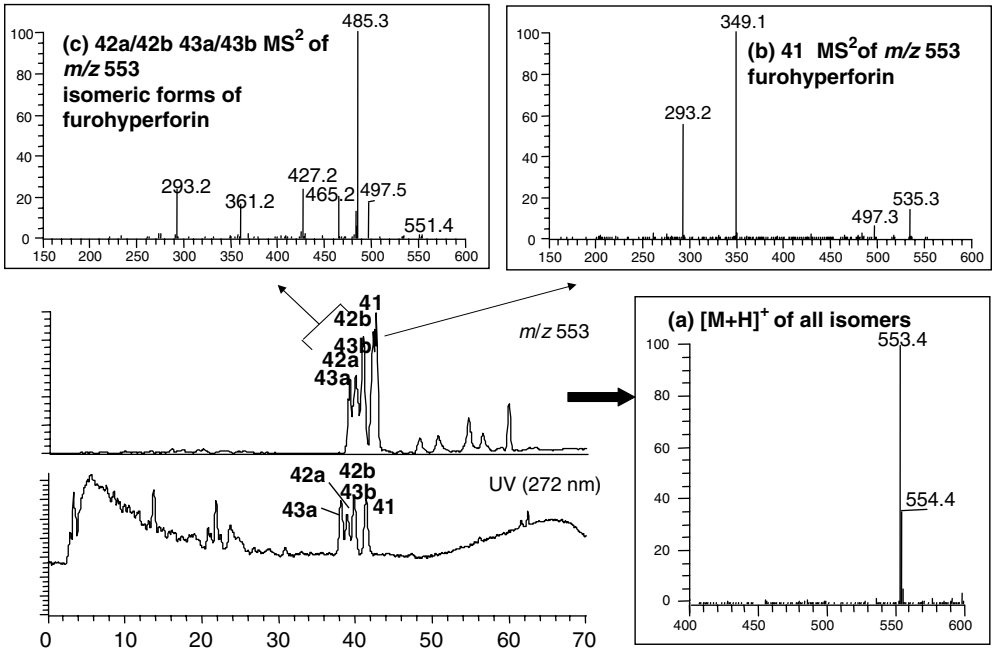

FIGURE 5.17 LC-UV chromatogram (272 nm) and LC-ESIMS single-ion trace of the protonated molecules of **41**, **42a**, **42b**, **43a**, and **43b**. The insets show (a) a typical HPLC-ESIMS spectrum, (b) LC-ESIMS/MS spectrum of furohyperforin (**41**), and (c) typical LC-ESIMS/MS of the isomers **42a/42b // 43a/43b**. The clear difference between the LC-ESIMS/MS of **41** and **42a/42b // 43a/43b** indicates a modification in the position of the oxidized prenyl chain between the isomers.[58] (Reproduced from Wolfender, J.-L., Verotta, L., Fuzzati, N., Strepponi, I., and Hostettmann, K., *Phytochem. Anal.*, 14, 290, 2003. With permission. Copyright John Wiley & Sons Limited.)

of an authenticated standard. The three other peaks (**43a**, **42a**, **42b/43b**) were found to be isomers of furohyperforin with closely related structures. All these products were isomers displaying intense protonated molecules at *m/z* 553 (ESI positive-ion mode) and *m/z* 551 (ESI negative-ion mode) but differed, however, in their LC-MS/MS fragmentation pattern, indicating a modification in the position of the oxidized prenyl chain between the isomers.[58]

To confirm these observations and implications, a stop-flow LC-NMR analysis of the crude hexane solution was carried out. The separation was achieved under isocratic HPLC conditions with an MeCN:D_2O solvent system. Isocratic conditions were preferred to the gradient elution mode to avoid changes in solvent composition. Indeed, solvent composition modifications might significantly affect the spectrum of a given compound. Under the conditions used, a precise comparison of the NMR chemical shifts for the different isomers was made possible (Figure 5.18). A careful analysis of the stop-flow LC-^{1}H NMR data clearly indicated that most of the characteristic resonances of furohyperforin could be assigned for the slower running LC peak **41** as expected. The most characteristic signals were those of the α-hydroxypropyl group formed by oxidation of the prenyl chain of hyperforin attached at C-6. These signals comprised the oxymethine furan proton H-32 (dd, δ 4.63) as well as the methylene H-31 (H-31a, dd, δ 2.48 and H-31b, dd, δ 1.85) characteristic of the ABX system of a $-CH_2CHOR$ moiety, and two shielded sharp singlets of the methyl groups H-34 and H-35 (δ 1.27 and 1.15). In the low-field region of the spectrum, two multiplets integrating for three protons were assigned to H-27 (δ 5.10) and H-17/22 (δ 5.02). The deshielded methylene protons (H-26)

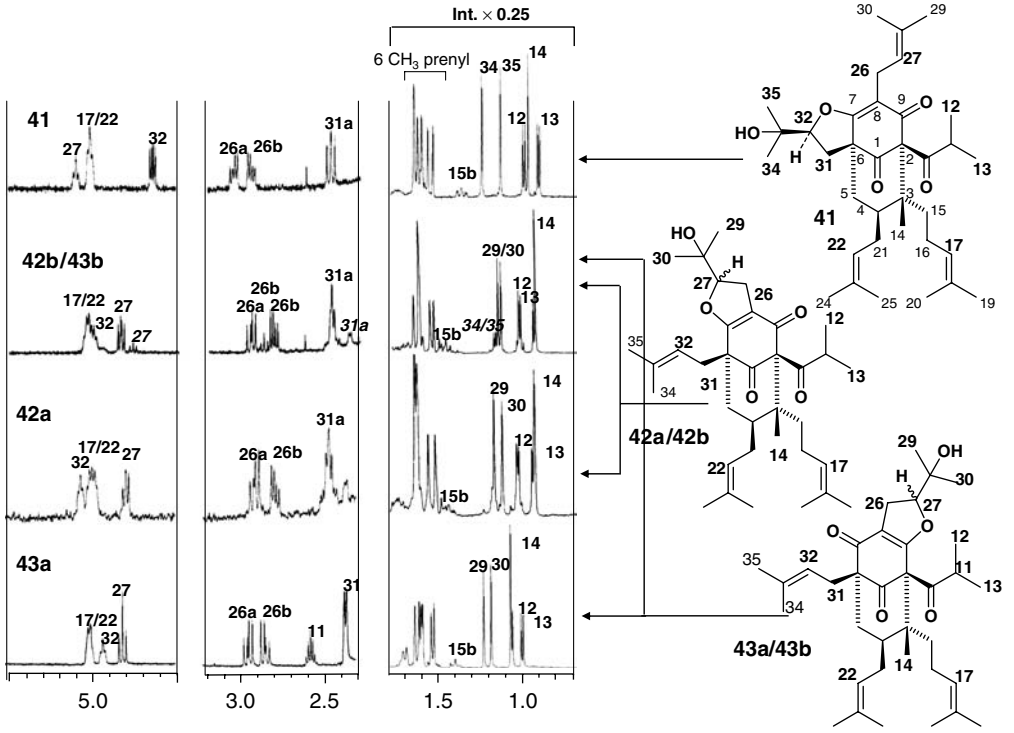

FIGURE 5.18 Stop-flow LC-^{1}H NMR spectra of the isomers **41**, **42a**, **42b**, **43a**, and **43b** with expansion of the most characteristic region. The labels in italics in the stop-flow LC-^{1}H NMR spectrum of **42b/43b** are those corresponding to the coeluting minor peak **43b**. Amount injected: 3 mg. HPLC conditions: Zorbax Eclipse XDB-phenyl column (2 mm × 250 mm × 84.6 mm i.d.); MeCN:D_2O (66:34) isocratic; 1.2 mL/min. LC-NMR conditions: 32 scans/increment (on-flow); 60-μL flow cell (3 mm i.d.); 500 MHz. (Reproduced from Wolfender, J.-L., Verotta, L., Fuzzati, N., Strepponi, I., and Hostettmann, K., *Phytochem. Anal.*, 14, 290, 2003. With permission. Copyright John Wiley & Sons Limited.)

of the prenyl chain linked in C-8 were also clearly detected (dd, δ 3.05, H-26a and δ 2.95 for H-26b). Finally, in the high-field region, five singlets (one of which consisted of two superimposed signals) (6 × 3H, δ 1.55–1.68) accounting for the six methyl groups of the three prenyl chains and two methyl doublets (2 × 3H, δ 1.01, H-12 and δ 0.92, H-13) were also readily detected (Figure 5.18).

Because of the need for solvent suppression (MeCN δ 2.00 and residual HOD δ 4.92), most of the resonances located near to the solvent signals were not detected in the stop-flow LC-^{1}H NMR spectra. Consequently, the methylene signals of the prenyl chains at positions C-4 and C-3 could not be observed with the LC-NMR conditions used.

To complement the online information, LC-WET gCOSY experiments were performed on each of the three LC peaks recorded in the stop-flow mode. For **41**, this experiment confirmed the correlations between H-32 and H-31a/H-31b in the oxidized prenyl chain at C-6, as well as between H-27 and H-26a/H-26b in the prenyl chain linked at position 8 (Figure 5.19).

The assignment of the most characteristic signals of furohyperforin **41** enabled a precise comparison with the unknown isomers **42a**, **43a**, and **42b/43b**. The most striking differences between the LC-NMR stop-flow spectra of these isomers were found between the low-field protons occurring between 4.6 and 5.2 ppm (Figure 5.18). For furohyperforin (**41**), the oxymethine H-32 appeared as a distinct doublet of doublets (dd, δ 4.63), whereas for **42a** and **43a**, this signal was replaced by another dd (H-27) each with a slightly different pattern, that is, δ 4.78 for **42a** and δ 4.80 for **43a**. In contrast to **41**, this oxymethine proton was no longer coupled to the methylene H-31 but to H-26a and H-26b for both **42a** and **43a** as shown by the LC-WET gCOSY recorded (see **42** in Figure 5.19). This indicated that, for these two isomers, the oxymethine function was located in position 27 and thus the prenyl chain in position 8 was oxidized. This prenyl chain modification was also supported by the slight shift of the methyl protons H-34 and H-35 and the methylene H-26. Symmetrically, the proton H-32 of the prenyl chain linked in C-6 was deshielded for **42a** (m, δ 5.06) and **43a** (m, δ 4.95) compared to **41** (dd, δ 4.63). In the LC-WET gCOSY spectrum, H-32 showed a clear

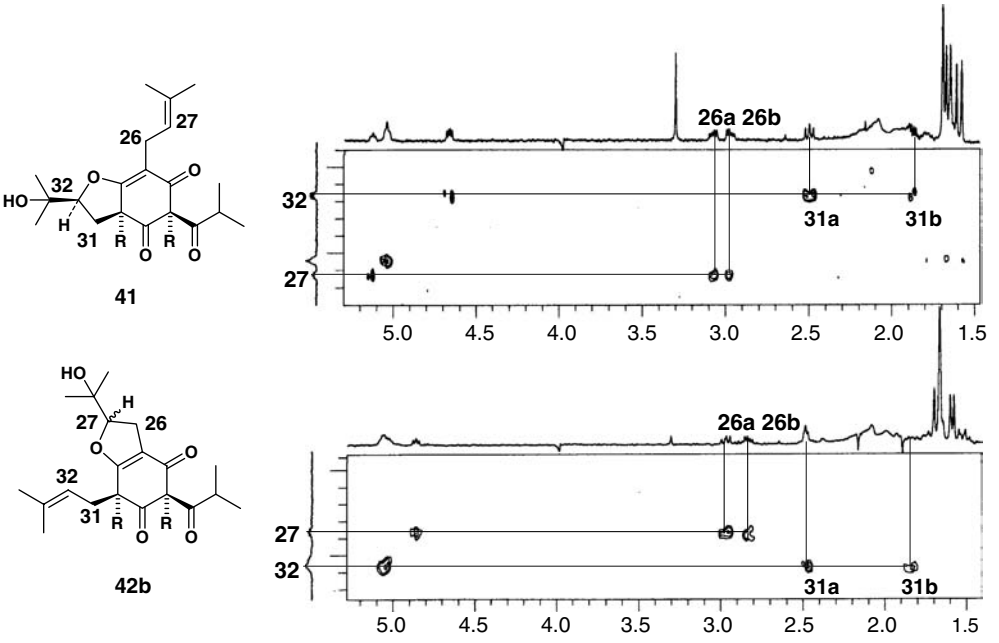

FIGURE 5.19 Stop-flow WET gCOSY spectra of the isomers **41** and **42b** with expansion of the region where the main differences were observed. Same LC-NMR conditions as in Figure 5.18. (Reproduced from Wolfender, J.-L., Verotta, L., Fuzzati, N., Strepponi, I., and Hostettmann, K., *Phytochem. Anal.*, 14, 290, 2003. With permission. Copyright John Wiley & Sons Limited.)

cross-peak with H-31, which, in **42a** and **43a**, was barely shifted but appeared as broad multiplets (see **42** in Figure 5.19). These observations clearly indicated that **42a** and **43a** both had an oxidized prenyl chain at C-8 and differences between hydroxy-dihydrofuran ring closure positions were noticed between each isomer. This was supported by the deshielding of H-11 (δ 2.57) in the case of **43a** compared to **42a** (δ 2.15) and **41** (δ 1.99), which could be explained by a lack of the carbonyl function in C-9. In hyperforin (**41**), a keto–enol equilibrium between the C-7 and C-9 positions is indeed known to occur, and it could be concluded from these LC-NMR measurements that **42a** and **43a** result from the cyclization of two different tautomeric forms. The structures of the epimers **42b** and **43b** were deduced by similar comparison. The proposed structures were further confirmed by HMBC and HSQC experiments on mixtures where the resonances of the different isomers were extracted on the basis of their stop-flow LC-NMR spectra.[57] These oxidation products were found to be new, and an evaluation of their pharmacological profile is underway.

5.2.4.3 Study of Unstable Compounds: Iridoids from *Jamesbrittenia fodina*

Besides its use as a tool for dereplication of natural products in crude plant extracts, LC-NMR can also be very useful for the structural investigation of labile products in simple fractions in combination with standard in-mixture NMR experiments. This is illustrated by the investigation of the methanolic extract of an African Scrophulariaceae species, *J. fodina*.[59]

A preliminary metabolite profiling study performed with LC-UV DAD and LC-ESI MS revealed the presence various common cinnamic ester derivatives. Attention was focused on a pair of putative new isomeric compounds (**44a/44b**). These constituents displayed UV spectra characteristic for cinnamic esters and shared the same molecular weight (MH^+ at *m/z* 669) (Figure 5.20).

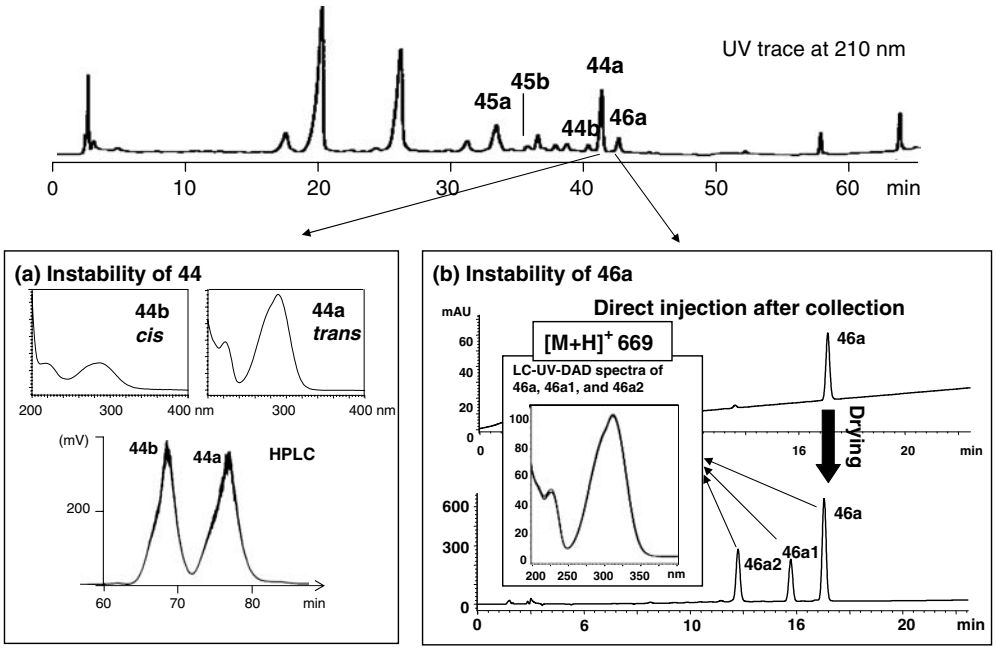

FIGURE 5.20 LC-UV DAD analysis of the methanol extract of *Jamesbrittenia fodina* (Scrophulariaceae). Chromatographic conditions: C18 column, Waters Symmetry (250 mm × 3.9 mm i.d.), MeOH:H_2O gradient (28:72 to 46:54 in 30 min, 46:54 to 62:38 in 16 min, 62:38 to 100:0 in 14 min); injection 50 µg of crude extract; flow rate 1 mL/min. Insets showing (a) the HPLC of the fraction containing **44a** and **44b** with their respective UV-DAD spectra (see Figure 5.21 for LC-NMR conditions) and (b) HPLC chromatogram of **46a** directly after collection and after drying. In this case, all the compounds, **46a**, **46a1**, and **46a2**, had identical UV and MS spectra.

They could not be dereplicated on the basis of the LC-UV-MS analysis, and their isolation was undertaken. These isomers were well separated by semipreparative HPLC, but it was noticed that the resulting fraction still contained the two isomeric peaks in the same proportions. These results suggested that these constituents were probably unstable upon drying of the fraction.

Stop-flow LC-^{1}H NMR analyses of this mixture revealed that the isomers **44a/44b** had common cinnamoyl, rhamnose, and aglycone moieties. The striking difference between the spectra of **44a** and **44b** concerned the signals attributed to the cinnamoyl double bond. Doublets at δ 7.72 (1H, J = 15.9 Hz) and δ 6.42 (1H, J = 15.9 Hz) were characteristic of a *trans* double bond in the case of **44a**, whereas in **44b**, the two corresponding doublets at δ 7.04 (1H, J = 12.6 Hz) and δ 5.84 (1H, J = 12.6 Hz) were typical of a *cis* double bond (Figure 5.21). The amount loaded in the flow cell of the LC-NMR did not permit gHMBC experiments directly on the LC peaks. The mixture of **44a/44b** was thus submitted to classical 1D and 2D NMR analysis, recorded in CD_3OD, that revealed these molecules to be catalpol derivatives. The HMBC correlations proved the attachment positions of the sugars and cinnamoyl moieties. On the basis of these stop-flow LC-^{1}H NMR and in-mixture NMR experiments, the structures of **44a** and **44b** were established to be *trans* and *cis* forms of 6-*O*-[4-*O*-(4-methoxycinnamoyl)-α-L-rhamnopyranoside]catalpol. The *trans* form was described in other members of the Scrophulariaceae family, but there was no mention of the *cis* forms. Other pairs of interconverting *cis*/*trans* isomers (**45a/45b**, Figure 5.20) were analyzed in the same way in the extract.[59]

In the same extract, another cinnamic ester derivative (**46a**) revealed a different type of instability. Indeed, **46a** was found to be transformed after isolation and lyophilization into two additional compounds **46a1** and **46a2** sharing the same UV and same molecular weight as their parent molecule **46a** (Figure 5.20). The absence of chromophore modifications within this series indicated that the instability was not related to a *cis*/*trans* isomerization, as previously observed for **44a/44b**

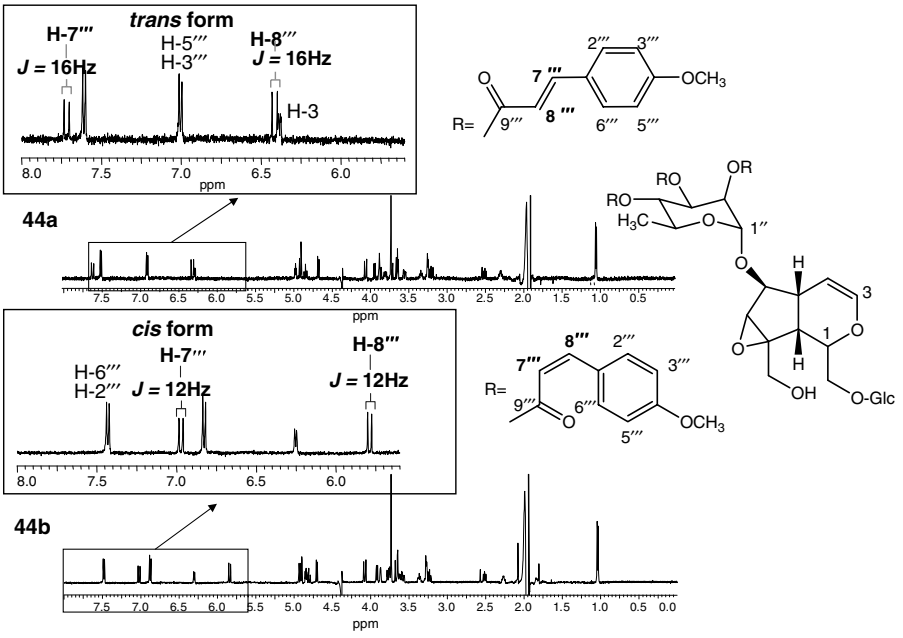

FIGURE 5.21 Stop-flow LC-^{1}H NMR spectra of the *cis* and *trans* isomers **44a** and **44b** isolated from the methanol extract of *Jamesbrittenia fodina* (Scrophulariaceae). HPLC conditions: C18 column with radial compression, Waters RCM 8 × 10 (100 mm × 8 mm i.d.); MeCN:D_2O gradient (25:75 to 30:70; 30 min); 1 mL/min, injection 240 µg. LC-NMR stop-flow 128 transients; 60-µL flow cell (3 mm i.d.); 500 MHz. The insets highlight the main differences in the stop-flow LC-^{1}H NMR spectra.

(Figure 5.20). As in the case of **44a/44b**, to determine the origin of this degradation, in-mixture NMR with extensive 2D correlation experiments was performed in the first step. It revealed that all three isomers (**46a**, **46a1**, and **46a2**) were aucubin derivatives and shared similar sugar and aglycone residues. In the second step, a stop-flow LC-NMR analysis enabled the recording of the individual spectra of compounds **46a**, **46a1**, and **46a2**. These spectra displayed many similarities (Figure 5.22) but several significant differences. A downfield shift was observed for the peak corresponding to the methyl of the rhamnose (H-6″) in **46a1** and **46a2** compared to **46a** and two signals at about δ 3.5 and 4.0 were present in **46a1** and **46a2** but absent in **46a**. The LC-NMR spectra of **46a1** and **46a2** were very similar; however, presence of two almost superposed doublets for both H-7‴ and H-8‴ and almost superposed singlet for H-7 in different ratios in **46a1** and in **46a2** suggested a transformation already occurring in the LC-NMR flow probe cell.

A recording of stop-flow spectra on the peak corresponding to **46a2** at different time intervals over 24 h revealed that this compound was first transformed to **46a1** and finally back to **46a**. These experiments clearly indicated that the differences between **46a** and **46a1/46a2** are due to transformations that took place on the rhamnose moiety of this aucubin derivative. Complementary LC-NMR WET TOCSY experiments were carried out by selective irradiation of the methyl H-6″ of the rhamnose unit of the different isomers and confirmed that the differences between the isomers were related to the position of the cinnamoyl moiety on one of three hydroxyls of the rhamnose unit at C-2″ (**46a2**), C-3″ (**46a1**), and C-4″ (**46a**).[60] The instability observed in this case was related to transacylation reactions of the cinnamoyl moiety on the rhamnose units of the aucubin derivatives.

5.2.4.4 Study of Epimerization Reactions

In our on-going search for antifungal compounds from higher plants, the most promising lead compound has been discovered in the root bark of a tree from Zimbabwe, *Bobgunnia madagascariensis* (Fabaceae), previously known as *Swartzia madagascariensis*.[61] Its root bark contained pale yellow metabolites that strongly inhibited growth of both *Candida albicans* and *C. cucumerinum*

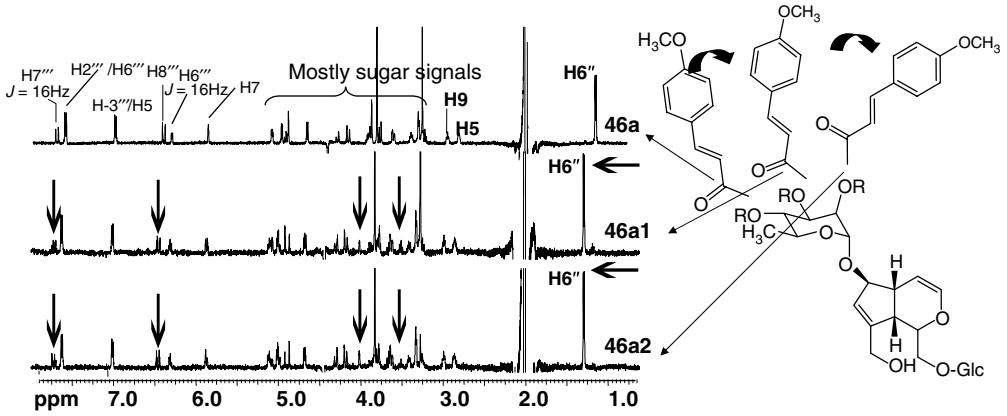

FIGURE 5.22 Stop-flow LC-^{1}H NMR spectra of **46a** and two different isomers (**46a1** and **46a2**) formed during the drying of **46a** isolated from the methanol extract of *Jamesbrittenia fodina* (Scrophulariaceae). Although MS and UV spectra were identical (see Figure 5.20), characteristic differences (highlighted by arrows) in the stop-flow LC-^{1}H NMR spectra proved a transacylation of the rhamnose moiety of **46a** by the cinnamoyl unit. HPLC conditions: C18 column with radial compression, Waters RCM 8 × 10 (100 mm × 8 mm i.d.); MeCN: D_2O gradient (20:80 to 36:44 in 20 min.); 1 mL/min, injection 200 µg. LC-NMR stop-flow 128 transients; 60-µL flow cell (3 mm i.d.); 500 MHz. (Reproduced from Wolfender, J.-L., Queiroz, E.F., and Hostettmann, K., *Magn. Reson. Chem.*, 43, 697, 2005. With permission. Copyright John Wiley & Sons Limited.)

in TLC bioautography assays. The main antifungal compound from this extract was identified as a new type of natural product, a "quinone methide" diterpene with a cassane skeleton.[61] Two other antifungal compounds in this extract (**47** and **48**) could not be isolated on the preparative scale. These compounds were probably two isomers that were interconverting in solution. To determine the structures of these compounds, on-flow and stop-flow LC-NMR experiments were performed on the enriched fraction containing both isomers.[62] The on-flow LC-NMR analysis demonstrated very close similarity between the signals of **47** and **48**. Indeed, all the signals in the high-field region of the 2D NMR spectrum (δ 1.0–4.5 ppm, Figure 5.23) were common for both compounds. However, the signals detected in the δ 5.5–7.0 ppm region differed significantly between these two compounds. Thus, two singlets appeared at δ 5.9 and 6.5 ppm for **47**, whereas they were shifted to δ 5.8 and 6.7 ppm in the case of **48**.

Repeated stop-flow LC-NMR analysis of **48** demonstrated the conversion to **47** in solution. The LC separation was stopped exactly when peak **48** was eluting in the LC-NMR cell. The first stop-flow LC-NMR spectrum of this peak obtained after 15 min of stabilization showed that **48** was indeed the major constituent in this peak, but already small signals of **47** were observable (Figure 5.23). A comparison of the integration of the signals of **47** and **48** indicated that **48** was present at more than 93% in the LC-NMR flow cell after 15 min. This stop-flow experiment was maintained for 2 h, and spectra were recorded every 15 min. As displayed on the expansion of the stop-flow spectra in Figure 5.23, the signals of compound **47** were increasing, whereas those of **48** were decreasing with time. The ratio of **47** reached one-third that of **48** after 105 min proving that indeed **48** was converted into **47** in solution. Furthermore, the sum of the integrals of H-7 and H-17 of **47** and **48** remained constant during the entire stop-flow experiment demonstrating that no other compound was formed.

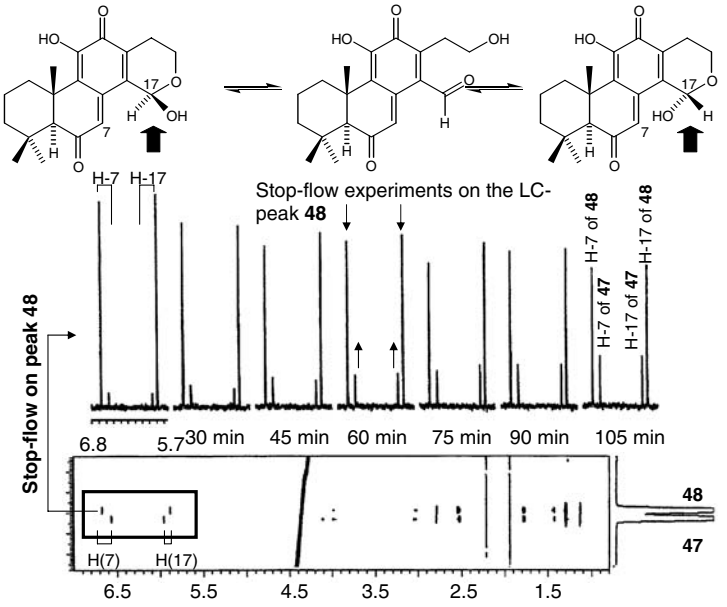

FIGURE 5.23 On-flow LC-2D NMR contour plot of the fraction containing **47/48** from *Bobgunnia madagascariensis* (Leguminosae). The signals of both products were identical except for those found in the δ 5.7–6.8 ppm region. Also shown are stop-flow LC-NMR spectra (expansion of the 5.7–6.8 ppm region) performed on peak **48** after different times. HPLC conditions: injected 300 µg; C18 column NovaPak (250 × 4.6 mm i.d., 4 µm); MeCN:D_2O gradient (25:75 to 55:45 in 45 min); 1 mL/min. LC-NMR conditions: 24 scans/increment, flow cell (60 µL, 3 mm i.d.), 500 MHz. (Reprinted from Wolfender, J.-L., Ndjoko, K., and Hostettmann, K., *J. Chromatogr. A*, 1000, 437, 2003. With permission from Elsevier.)

As in the case of the unstable iridoids of *J. fodina*, the structures of **47** and **48** were finally determined on the basis of standard 1D and 2D NMR correlation experiments performed in-mixture taking into account the assignment of individual signals of **47** and **48** determined from the LC-^{1}H NMR spectra. Thus, a careful study of the HMBC, DQCOSY, and NOE correlations allowed the mapping of a hemiacetal functionality (−CH_2−CH_2−O−C*HOH−) between C-13 and C-14. Epimerization of the chiral center (*) in this hemiacetal group, through ring opening and closing (Figure 5.23), results in the observed equilibrium between **47** and **48**. The diastereoisomers **47** and **48** were found to be new natural products, and both isomers had important antifungal properties.[62] As shown in these last examples, LC-NMR is not only applicable as a complement for metabolite profiling but also a powerful tool for the study of compounds not isolatable, or not stable in a pure form.

5.2.4.5 Online Absolute Configuration Determination

Determination of the absolute configuration remains a challenging task in the structure elucidation of natural products. For online determination, LC-CD has proven to be useful as exemplified by its application to isoquinoline alkaloids.[28]

Classically, chemical methods, such as Mosher's ester synthesis, have been frequently used for the characterization of various isolated natural products bearing secondary alcohol functions.[63] In this case, the H^1-NMR spectra of (*R*)- and (*S*)-2-methoxy-2-phenyl-(trifluoromethyl) acetic acid (MTPA) ester derivatives of the analytes are compared. The difference of the chemical shifts of the (*S*)-MTPA and (*R*)-MTPA diastereoisomers ($\Delta\delta_H = \delta_S - \delta_R$) indicates whether the alcohol function is (*R*) or (*S*) on the basis of established conformational models.[64]

Until recently, Mosher's ester synthesis was only used in combination with standard NMR. The recent study of crude reaction mixtures using LC-UV-MS and LC-NMR has proved to be a rapid, sensitive method for absolute configuration determination. This online method can be applied to restricted amounts of samples, or fractions, difficult to obtain in a pure form, and the absolute configuration can be determined on few micrograms of natural products.

This technique was applied to the determination of the absolute configuration in two α-pyrones (**49** and **50**) isolated from the root bark of *Ravensara crassifolia* (Lauraceae).[65] Two aliquots (50 µg each) of α-pyrone **49** were esterified with (*R*)- and (*S*)-MTPA. Five percent of the residual reaction mixture (i.e., 10 µL) was analyzed by LC-ESI MS under isocratic LC conditions to verify the completion of the esterification reaction. The remaining reaction mixture (95%) was then submitted to stop-flow LC-NMR analysis using the same isocratic LC conditions. A fast cleanup of the sample was thus obtained, and the LC-^{1}H NMR spectra of the Mosher's esters were recorded in the stop-flow mode. A preliminary on-flow run demonstrated the extent of cleanup, which can be obtained using LC-NMR (Figure 5.24). Pyridine, excess MTPA, and traces of starting material eluted rapidly, whereas the ester derivatives eluted between 8 and 12 min. The use of isocratic conditions was found to be essential since precise comparison of the chemical shifts has to be made and changes in solvent composition would hinder such an online approach.

Comparison of LC-^{1}H NMR spectra of the (*R*)-MTPA **49a** and (*S*)-MTPA **49b** derivatives showed significant chemical shift differences for the protons near the MTPA ester (Figure 5.25). These diagnostic shifts were used for determination of the absolute configuration. Suppression of the MeCN solvent resonance (δ 2.0 ppm) hampered a clear assignment of the signals recorded at δ 1.60–2.20 ppm. For an unambiguous chemical shift assignment of each proton for both Mosher's esters (**49a** and **49b**), ^{1}H–^{1}H WET-COSY spectra were recorded. With these 2D correlation experiments, precise chemical shift assignments for H-1′, H-3′, and H-4′ were made possible. Differences for all chemical shifts of (*S*)-MTPA **49a** and of (*R*)-MTPA **49b** were calculated using the formula ($\Delta\delta_H = \delta_S - \delta_R$). Positive δ_H values were recorded for the α-pyrone moiety (C-1′), which was placed on the right side of the Mosher model. Negative δ_H values of C-3′/C-5′ indicated that the alkyl chain had to be placed on the left side of the model (Figure 5.25). Finally, the proton geminal to the esterified

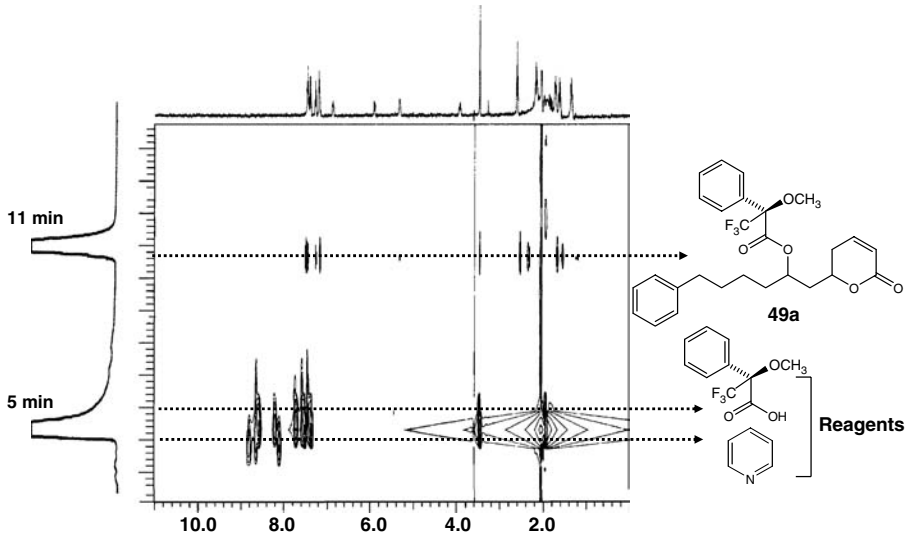

FIGURE 5.24 On-flow LC-NMR contour plot obtained after injection of the crude reaction mixture of the (*R*)-MTPA Mosher's ester **49a**. HPLC conditions: Injected 250 µg; µ-BondaPak C18 column (100 mm × 8 mm i.d., 10 µm); MeCN:D_2O (80:20, 35 min); 0.8 mL/min. LC-NMR conditions: 32 scans/increment, flow cell (60 µL, 3 mm i.d.), 500 MHz. (Reprinted from Wolfender, J.-L., Ndjoko, K., and Hostettmann, K., *J. Chromatogr. A*, 1000, 437, 2003. With permission from Elsevier.)

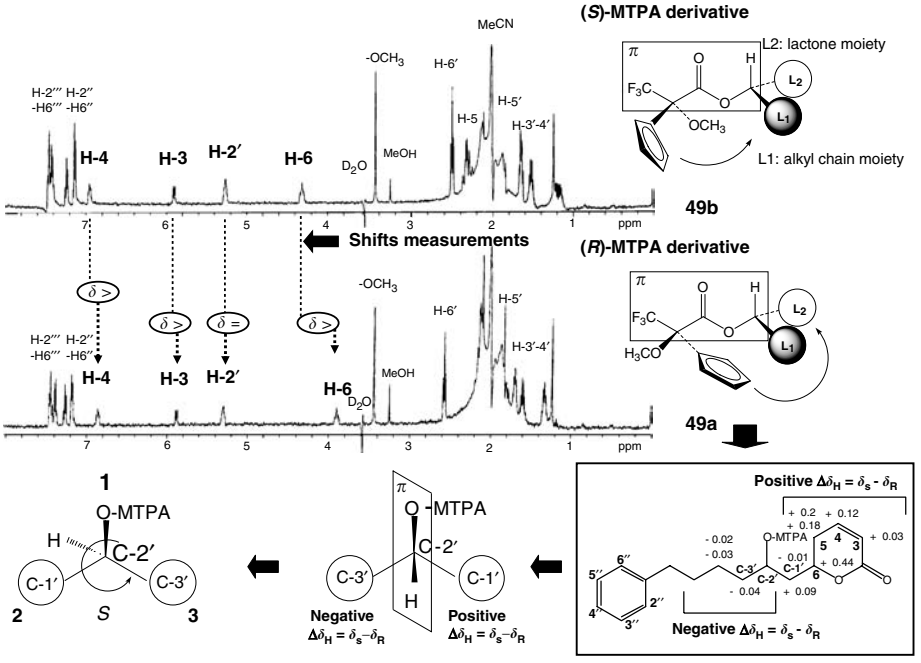

FIGURE 5.25 Stop-flow LC-NMR spectra of the Mosher's esters derivatives **49a** and **49b** (50 µg injected; NT = 128 acquisitions each) and determination of the absolute configuration with the $\Delta\delta_H$ values ($\Delta\delta_H = \delta_S - \delta_R$) of compound **49** using the Mosher method. Same LC-NMR conditions as in Figure 5.24. (Reprinted from Wolfender, J.-L., Ndjoko, K., and Hostettmann, K., *J. Chromatogr. A*, 1000, 437, 2003. With permission from Elsevier.)

hydroxyl group was placed in the opposite side of the plane, and the absolute configuration at C-2′ was determined using the Cahn–Ingold–Prelog priority rule. This arrangement indicated a sinistrorse rotation and confirmed the *S* absolute configuration for the asymmetric center C-2′ (Figure 5.25).

The same type of absolute configuration determinations based on LC-NMR analyses were made for other α-pyrones isolated from the same plant.[65] The same approach also enabled the absolute configuration determination of a tetrahydrophenanthrene from *Heliotropium ovalifolium*.[66]

5.2.5 Limitations of the Direct Hyphenation of LC-NMR

As shown in the different examples discussed, direct hyphenation of LC-NMR enables a lot of structural problems to be solved. This approach provides the possibility to rapidly obtain NMR structural information, complementary to LC-UV and LC-MS spectra, on the main constituent(s) of a crude plant extract. Furthermore, it is invaluable for the structure determination of closely related isomers or unstable compounds. It has an additional advantage that a direct connection to the eluting LC peaks can be made in conjunction with other spectroscopic data when used in a hypernated system. The direct hyphenation of NMR requires, however, compromises to be made that alter the quality of the data in comparison with conventional NMR experiments.

5.2.5.1 Restricted Observable NMR

One problem linked to the use of solvent suppression is that the signals of the analytes of interest, which reside under the solvent peak, will be suppressed together with the solvent signal. An example of this problem is demonstrated by the LC-NMR analysis of the lignan derivative phylligenin (**3**) from an enriched fraction of *O. enneandra* (Annonaceae).[23] The on-flow spectrum recorded for phylligenin (**3**, Figure 5.5 and Figure 5.26a) in MeCN:D_2O presented all the characteristic ^1H signals of this compound with the exception of H-7, which was suppressed together with the HOD line. This can be a major drawback when dealing with unknown constituents. To detect all analyte signals, one alternative is to carry out the solvent suppression in two independent solvent systems such as MeCN:D_2O and MeOH:D_2O.[33] In this case, the solvent signal to be suppressed will be shifted at different frequencies and all the analyte signals can be observed by comparison of the spectra obtained. This approach, however, necessitates optimizing the separation of the constituent of interest in two independent solvent systems.

In direct hyphenation of NMR, another way to observe all analyte signals is to perform the separation in fully deuterated solvents (CD_3CN:D_2O). This is relatively expensive on 4 mm i.d. columns but has been used practically in the industry. This approach can, however, be used on microcolumns where the solvent consumption is limited.[67]

One interesting way to circumvent the problems related to this solvent suppression issue might be the use of high-temperature chromatography with superheated D_2O, which is now emerging as a separation technique. The use of high-temperature LC in combination with temperature gradient provides an alternative to conventional LC with the advantage of using only D_2O as a solvent, compatible with UV, MS, and NMR in the case of hypernation. However, issues related to natural product sample degradation need to be considered.

5.2.5.2 Chemical Shift Differences

Another problem occurring in LC-NMR is that the chemical shifts recorded in a typical reverse-phase solvent, for example, MeCN:D_2O or MeOH:D_2O, will differ from those reported in standard deuterated NMR solvents. This can be a drawback if precise comparisons with literature data have to be performed. In the example of phylligenin (**3**) (Figure 5.26), the chemical shifts of the aromatic protons of this compound were significantly different depending on whether the spectrum was recorded using on-flow conditions in MeCN:D_2O (Figure 5.26a) or in standard conditions on the pure constituent, that is, $CDCl_3$ (Figure 5.26b) or CD_3OD (Figure 5.26c). This example demonstrates

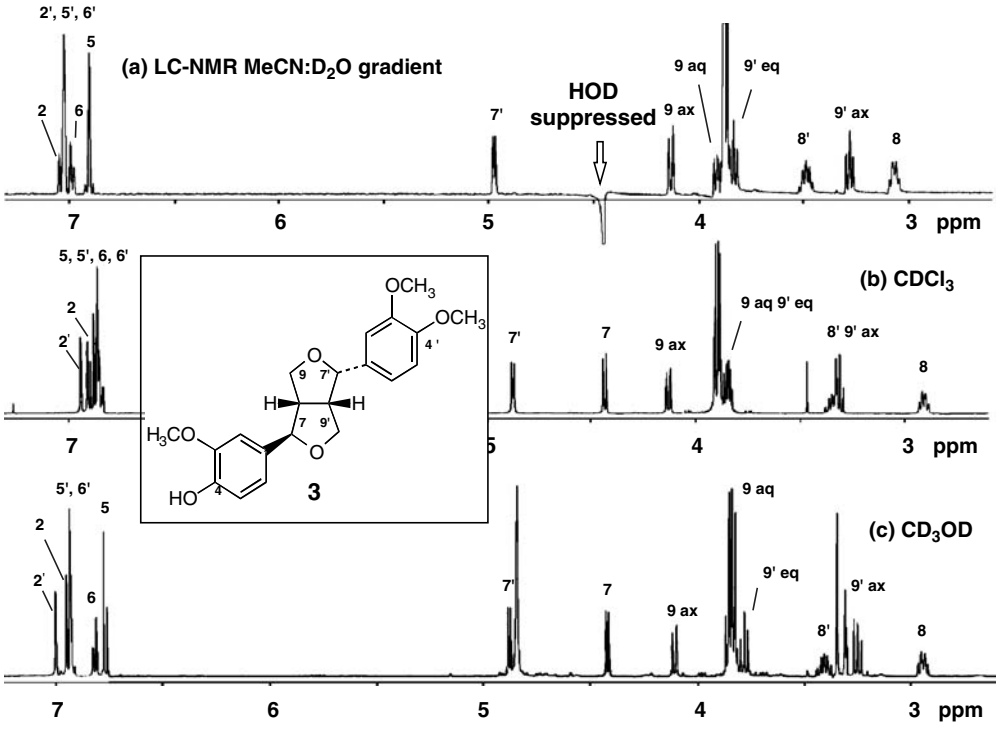

FIGURE 5.26 Comparison of the on-flow LC-^{1}H NMR of the lignan pylligenin (**3**) found in the enriched fraction of *O. enneandra* (Annonaceae) (for LC-NMR conditions see Figure 5.5) and the same spectrum recorded in conventional deuterated NMR solvent $CDCl_3$ and CD_3OD. As shown, water suppression hindered the detection of H-7 in LC-NMR, and aromatic patterns are difficult to compare. (Reproduced from Wolfender, J.-L., Ndjoko, K., and Hostettmann, K., *Phytochem. Anal.*, 12, 2, 2001. With permission. Copyright John Wiley & Sons Limited.)

that a simple comparison of chemical shifts between reference and LC-NMR spectra cannot be performed and often other stop-flow 2D correlation experiments are needed to complete the signal assignments made in LC-NMR. Furthermore, these differences will hinder the creation of LC-^{1}H NMR databases for automatic assignment of known constituents.

5.2.5.3 Lack of Sensitivity

Another limitation of LC-NMR is that the elution volumes of the peaks are often bigger than the volume of flow cell. This dilution phenomenon causes loss of sensitivity; however, this can be addressed using at-line techniques that enable preconcentration of sample or dilution of dried LC peaks in volumes that match the volume of the flow cell.

5.3 INDIRECT HYPHENATION OF NMR: AT-LINE VERSUS ONLINE APPROACHES

To cope with the limitations related to the direct hyphenation of NMR, analytical strategies based on the indirect hyphenation of this technique have been devised.[68] These at-line strategies represent new trends in NMR hyphenation mainly directed toward a better sensitivity of detection and better data quality but require more sample handling.

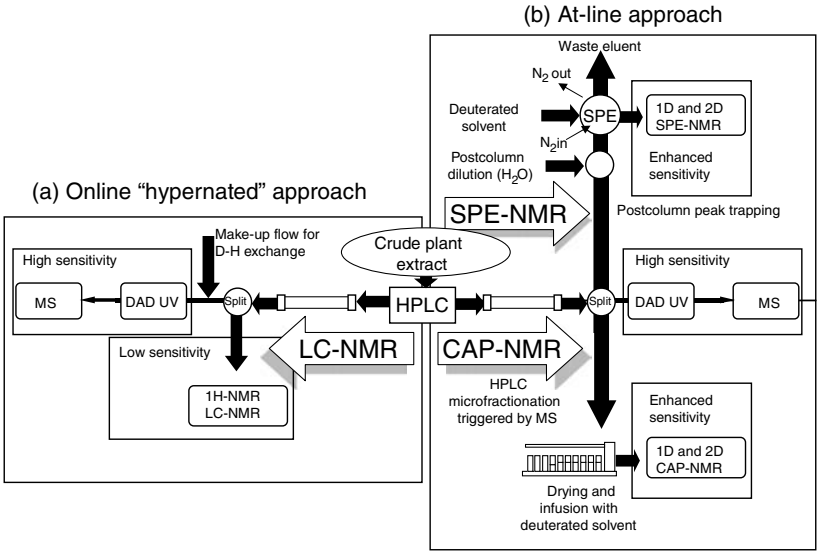

FIGURE 5.27 Two ways of obtaining UV, MS, and NMR information on a crude plant extract. (a) Online approach: in this mode, all detection methods can be coupled together in what is called a "hypernated" system and the LC-NMR is operated on-flow. (b) At-line approach: the less sensitive detection method LC-NMR can be operated at-line by either SPE-NMR or CAP-NMR for an optimum detection of sample, whereas UV and MS data are acquired during LC acquisition.

As shown in Figure 5.27, one possibility to overcome the limitations of the direct hyphenation of LC-NMR is to work at-line, either trapping the LC peaks on SPE columns, that is, LC-SPE-NMR,[12,68] or with HPLC microfractionation followed by drying and reinjection of the concentrated LC peak in a deuterated solvent in a microflow capillary LC-NMR probe, that is, CAP-NMR.[9,69]

5.3.1 SPE-NMR

SPE offers reproducible, rapid, and selective preparation of samples. The LC-separated peaks are diluted postcolumn with water and trapped automatically on SPE columns.[14] After drying the SPE column with nitrogen to remove all solvents used for chromatographic separation, the trapped analyte is eluted from the SPE column to the NMR flow cell probe with a deuterated solvent of choice (Figure 5.27).[32] This technique presents the advantage that the separation can be carried out with normal protonated solvents and, moreover, allows multiple trapping of the same analyte from repeated LC injections to circumvent problems of low concentration. The operation can be fully automated on state-of-the-art systems, but optimization of the trapping conditions is required for optimum performances.[70] In this respect, the use of either semipreparative or analytical columns, with or without multiple trapping, has been investigated, and it was found that the choice of the optimum separation strategy is mainly related to the polarity of the analyte of interest.[71]

The first LC-SPE-NMR analysis on natural products was carried out on an extract of Greek oregano[12] and led to the successful identification of various flavonoids, rosmarinic acid, and the monoterpene, carvacrol, on the basis of a combination of UV, MS, and NMR spectra. Another interesting study has demonstrated LC-SPE-NMR for the identification of antioxidants in a commercial rosemary extract.[72] Several recent applications are summarized in Table 5.1.

TABLE 5.1
Recent Applications (2005–2006) of LC-NMR for Online or At-Line Natural Products Identification

Products	Origin	Mode	Experiment	Instrumentation	Reference
Flavonoids granaticinic acid derivatives	*Streptomyces violaceoruber* Tü 22	LC-NMR, on-flow	^{1}H NMR	600 MHz, 120-μL flow cell	86
Phenolics	*Blumea gariepina*	LC-NMR, on-flow	^{1}H NMR	500 MHz, 60-μL flow cell	87
Phenolics	*Mangifera indica* (Mango juice)	LC-NMR, on-flow	^{1}H NMR	500 MHz, 120-μL flow cell	88
Carotenoids	Thermally processed vegetables	LC-NMR, stop-flow	^{1}H NMR, WETg-COSY	500 MHz, 65-μL flow cell	89
Phenolics	Cell culture of *Macleaya* and *Corydalis* species	LC-NMR, stop-flow	^{1}H NMR, WET-NOESY	500 MHz, 60-μL flow cell	90
Roquefortine (toxins)	*Penicillium roqueforti*, *P. paneum*, and *P. crustosum*	LC-NMR, stop-flow	^{1}H NMR	500 MHz, 60-μL flow cell	91
Iridoid glycosides	*Jamesbrittenia fodina*	LC-NMR, stop-flow	^{1}H NMR	500 MHz, 60-μL flow cell	60
Flavonoids	*Citrus sinensis*	LC-NMR, stop-flow	^{1}H NMR	400 MHz, 60-μL flow cell	92
Isoflavones	*Radix astragali*	CAP-LC-NMR, stop-flow	^{1}H NMR	600 MHz, 1.5-μL solenoidal flow cell	10
N.A.	Cell culture of *Arabidopsis thaliana*	LC-NMR	N.A.	N.A.	93
Jacaranone glycosides	*Senecio scandens*	LC-NMR	N.A.	N.A.	94
Flavonoids and cardenolides	*Kanahia laniflora*	LC-SPE-NMR 10 mm × 2 mm Hysphere C18 HD SPE trap cartridges	^{1}H NMR, COSY NOESY, HMBC, HSQC	600 MHz, 30-μL flow cell	95
Phenolics	Olive oil	LC-SPE-NMR 10 mm × 2 mm Hysphere trap cartridges	^{1}H NMR, 1D NOESY, TOCSY	600 MHz, 30-μL flow cell	96
Flavonoids, chorogenic acids	*Rhaponticum carthamoides*	LC-SPE-NMR 10 mm × 2 mm Hysphere Resin SH trap cartridge	^{1}H NMR, HMBC	400 MHz, 120-μL flow cell	97
Iridoid glycosides	*Harpagophyton procumbens*	LC-SPE-NMR 10 mm × 2 mm Hysphere Resin GP or Hysphere C18 trap cartridges	^{1}H NMR	600 MHz, 30-μL flow cell	98

Isoflavonoids	*Smirnowia iranica*	LC-SPE-NMR C18 HD SPE trap cartridge	^{1}H NMR, ^{13}C DEPT, COSY, NOESY, HMBC, HSQC	600 MHz, 30-μL flow cell	81
Phenolics	*Tilia europea*, *Urtica dioica*, *Lonicera periclymenum*, and *Hypericum perforatum*	LC-SPE-NMR 10 mm × 2 mm Hysphere GP trap cartridges	^{1}H NMR, 1D NOESY	400 MHz, 120-μL flow cell	99
Isomeric tropane alkaloids	*Schizanthus grahamii*	LC-SPE-NMR 10 mm × 2 mm HySphere GP resin trap cartridges LC-NMR, stop-flow, loop storage	^{1}H NMR, COSY	600 MHz, 60-μL flow cell	82
Chinane-type tricyclic diterpenes	*Harpagophytum procumbens*	LC-SPE-NMR 10 mm × 2 mm HySphere GP resin trap cartridges	^{1}H NMR, COSY, NOESY, HMBC, HSQC	600 MHz, 30-μL flow cell	100
Cardenolides and steroids	*Lucidota atra*	CAP-NMR	^{1}H NMR, COSY, NOESY, HMBC, HSQC	600 MHz, 1.5-μL solenoidal flow cell	85
Acetylated oligorhamnosides	*Cleistopholis patens*	CAP-NMR	^{1}H NMR, ^{13}C NMR, COSY, NOESY, HMBC, HSQC	600 MHz, 1.5-μL solenoidal flow cell	74
Ursene triterpenes	*Diospyros dendo*	CAP-NMR	^{1}H NMR, COSY	600 MHz, 1.5-μL solenoidal flow cell	101
Cyclolignans	*Scyphocephalium ochocoa*	CAP-NMR	^{1}H NMR, COSY, NOESY, HMBC, HSQC	600 MHz, 1.5-μL solenoidal flow cell	102
Acylated caprylic alcohol glycosides	*Arctostaphylos pumila*	CAP-NMR	N.A.	600 MHz, 1.5-μL solenoidal flow cell	103
Indolosesquiterpene	*Greenwayodendron suaveolens*	CAP-NMR	^{1}H NMR, COSY, NOESY, HMBC, HSQC	600 MHz, 1.5-μL solenoidal flow cell	104
Iridoid glycosides	*Penstemon centranthifolius*	CAP-NMR	^{1}H NMR, COSY, NOESY, HMBC, HSQC	600 MHz, 1.5-μL solenoidal flow cell	73

Note: N.A.: Not available; flow cell active volumes are indicated.

5.3.2 CAP-NMR

In the application of CAP-NMR, the same LC microfractionation procedure described for LC-SPE-NMR can be used except that the samples are not trapped on SPE. The microfractions are collected and dried before reconstituting in the appropriate solvent (Figure 5.27). Some recent applications of CAP-NMR have demonstrated the potential for this technique in high-throughput natural product chemistry methods for the structural characterization of iridoid glycosides from *Penstemon centranthifolius*[73] or for the determination of acetylated oligorhamnoside derivatives from *Cleistopholis patens*.[74] Automation for the direct infusion of 10 µL samples from a low volume, 384-well plate has recently been demonstrated to be feasible.[75] Some examples are described in the following sections and other examples can be found in Table 5.1.

5.3.2.1 Rutin

Figure 5.28 shows an example of the improvement in spectrum quality that can be obtained with CAP-NMR analysis of 50 µg of rutin (**51**) (MW 610) after HPLC microfractionation, compared to that obtained with stop-flow LC-NMR. The flow cell used for LC-NMR had an active volume of 60 µL, whereas that of the CAP-NMR was 1.5 µL. For CAP-NMR, 50 µg of rutin was collected after separation on an analytical column and dried. The sample was redissolved in 5 µL of CD_3OD and pushed into the CAP-NMR flow probe with a calibrated push volume of CD_3OD. As shown in Figure 5.28, the spectrum obtained with CAP-NMR had a good lineshape (<1 Hz) and no interference due to solvent suppression.

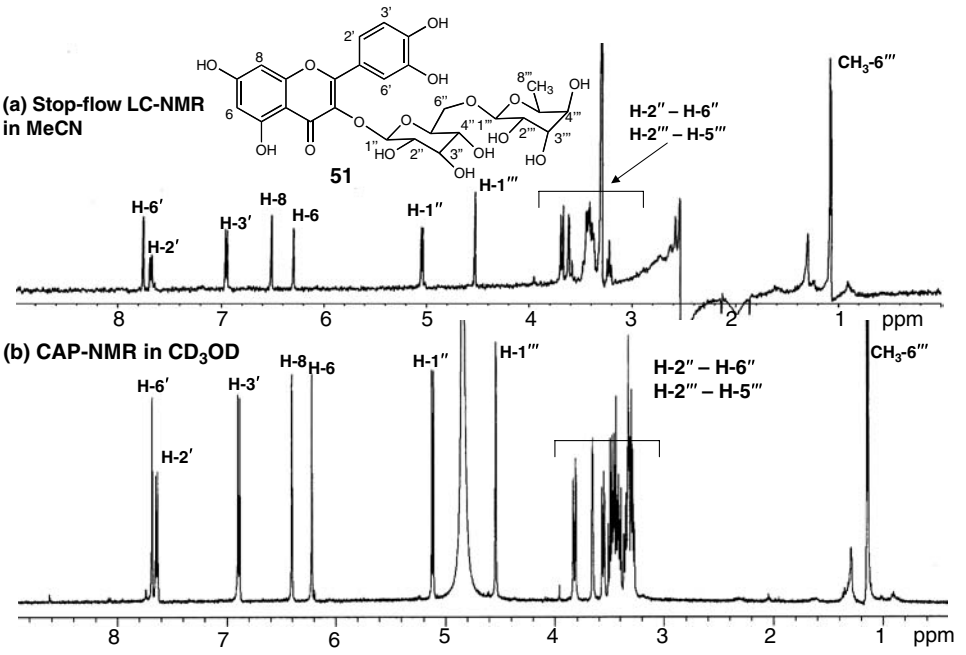

FIGURE 5.28 Comparison between stop-flow LC-^{1}H NMR and CAP-NMR spectra of rutin (20 µg). (a) Stop-flow LC-^{1}H NMR in MeCN (NT = 256), 60-µL "observe volume" flow cell (Varian, Palo Alto, CA); 500 MHz. (b) CAP-NMR spectra in CD_3OD (NT = 16). The sample was dissolved in 6.5 µL of CD_3OD and parked in the probe with a push volume of 5 µL. NMR: 1.5-µL "observe volume" flow cell (Protasis/MRM Corp., Savoy, IL); 500 MHz. (Reproduced from Wolfender, J.-L., Queiroz, E.F., and Hostettmann, K., *Magn. Reson. Chem.*, 43, 697, 2005. With permission. Copyright John Wiley & Sons Limited.)

5.3.2.2 Swertiamarin and Sweroside

The improvement in spectra possible with CAP-NMR has already been illustrated in Figure 5.3 for 20 µg of the secoiridoid swertiamarin (**1**) (Section 5.2.1.3). The indirect ^{1}H–^{13}C NMR experiments that can be recorded on 100 µg of a similar secoriridoid, sweroside (**52**) are illustrated in Figure 5.29. In this case, all 2D NMR experiments for a complete structure elucidation (COSY, HSQC, and HMBC) were obtained in about 8 h.

For single ^{1}H NMR spectra measurement with CAP-NMR, the quantities can be significantly lowered and it has been shown that 340 ng of sucrose yields a good 1D spectrum in less than 10 min.[9] However, at the low microgram level, residual solvent signals begin to interfere with the analyte signals of interest. In this case, the application of the WET solvent suppression sequence enables the recording of good-quality data. This is illustrated in Figure 5.30 with the ^{1}H NMR spectrum recorded on 2 µg of swertiamarin (**1**) in CD_3OD. A recent study demonstrated the capability of this probe for the structure determination of various iridoids from the crude extract of *P. centranthifolius* (Scrophulariaceae).[73] Other recent examples can be found in Table 5.1.

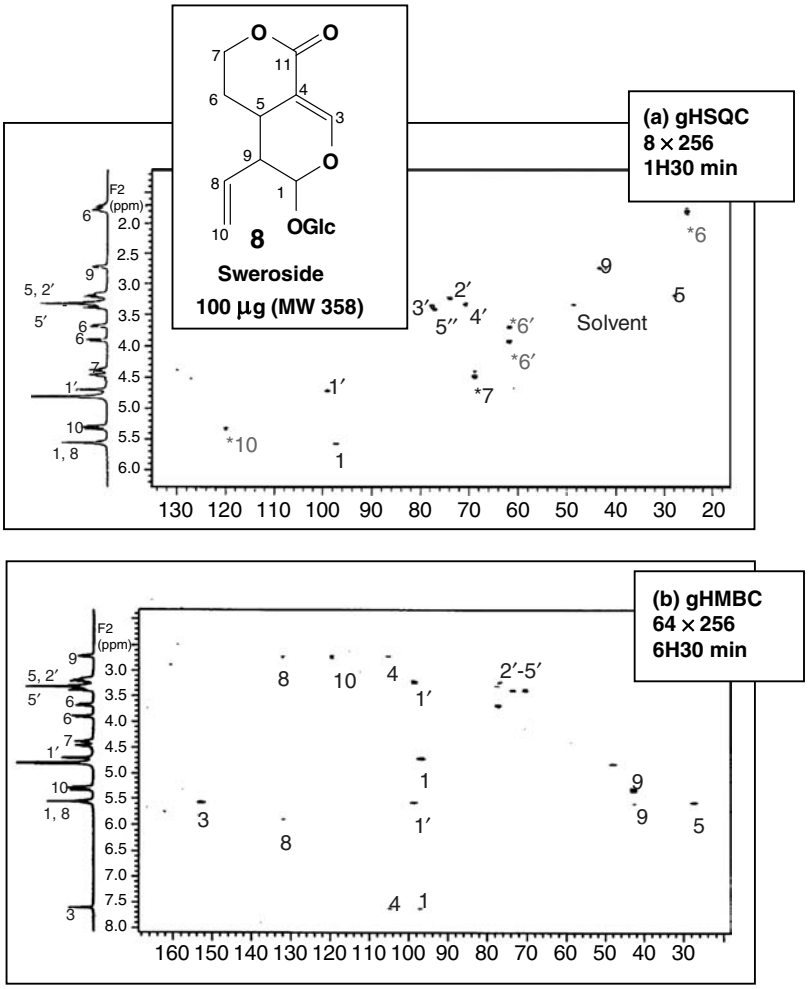

FIGURE 5.29 (a) gHSQC and (b) gHMBC CAP-NMR spectra in CD_3OD obtained on 100 µg of sweroside (**8**) showing that all ^{13}C information can be obtained in about 8 h for a compound of MW 358 (For LC-^{1}H NMR and COSY, see also Figure 5.7). The sample was dissolved in 6.5 µL of CD_3OD and parked in the probe with a push volume of 5 µL. NMR: 1.5 µL "observe volume" flow cell (Protasis/MRM Corp., Savoy, IL); 500 MHz.

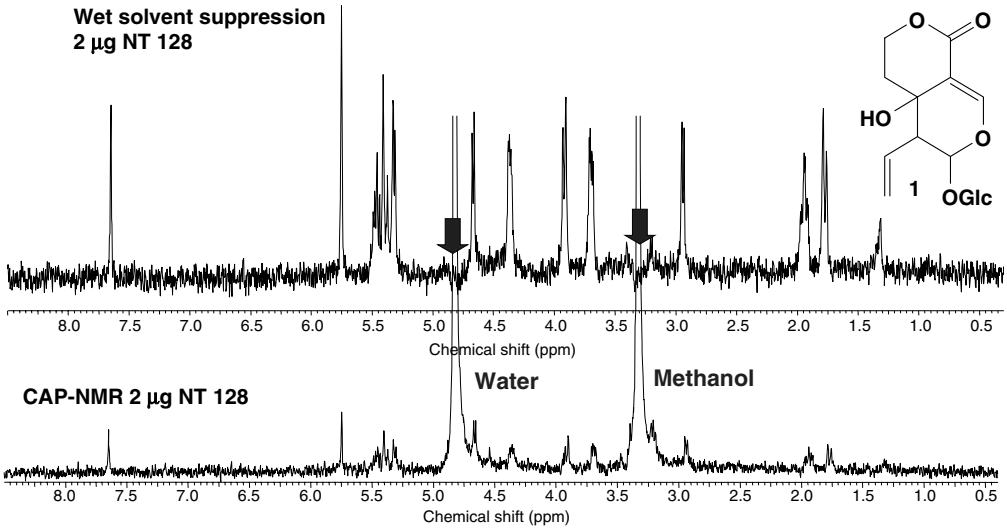

FIGURE 5.30 Sensitivity of CAP-NMR detection. ^{1}H-CAP-NMR spectra in CD_3OD obtained on 2 µg of swertiamarin (**1**). Application of the WET solvent suppression technique to CAP-NMR provides a good-quality spectrum with only 128 transients. (For LC-^{1}H NMR and CAP-NMR on larger amounts, see Figure 5.3.) The sample was dissolved in 6.5 µL of CD_3OD and parked in the probe with a push volume of 5 µL. NMR: 1.5-µL "observe volume" flow cell (Protasis/MRM Corp., Savoy, IL); 500 MHz.

5.3.3 *De Novo* Structural Determination: Antioxidant Flavonoids from *E. scheuchzeri*

At-line measurements have the considerable advantage of providing spectra recorded in standard deuterated solvents with the highest possible concentration for a given amount of compound. Furthermore, access to ^{13}C-NMR information deduced indirectly from inverse detection experiments (HSQC and HMBC) is feasible, providing numerous invaluable structural information for *de novo* structural determination of natural products.

To determine the structure of major and minor constituents from a Swiss alpine Cyperaceae, *E. scheuchzeri*, both on-flow LC-NMR and at-line CAP-NMR were used. The study of this plant was motivated by the fact that the extreme habitat of this alpine plant suggested a strong adaptation to UV-B irradiation, which may partly consist of an increase in the amount of UV-absorbing phenolic compounds. Therefore, the antioxidant potential of the extract was evaluated by TLC autography using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical as spray reagent[76] and by online methods.[77] The combination of both online, postcolumn addition of DPPH and online LC-UV-MS and LC-NMR enabled a rapid dereplication of the main antioxidant, which was found to be rutin (**51**), a widespread flavonoid.[78]

The other main flavonoids present in the crude extract were characterized using LC-UV-MS and on-flow LC-^{1}H NMR. To obtain good-quality spectra for all the main constituents of the crude methanolic extract, a relatively large amount (80 mg) was injected on-column and the separation was achieved in 160 min at 1 mL/min flow rate. The on-flow LC-NMR contour plot (Figure 5.31) confirmed that all the major constituents of the crude extract were flavonoids, with characteristic signals in the aromatic proton region between δ 6 and 8 ppm. The aromatic signals between δ 6 and 6.9 ppm were indicative of an A-ring oxygenated in positions 5 and 7, whereas chemical shifts appearing between δ 6.9 and 8 ppm were characteristic of the B-ring protons. In addition, signals between δ 3.0 and 4.2 ppm were indicative for sugar moieties, with respective anomeric protons between δ 4.4 and 5.3 ppm. The ^{1}H NMR and UV and MS spectra of the most polar compound (**52**)

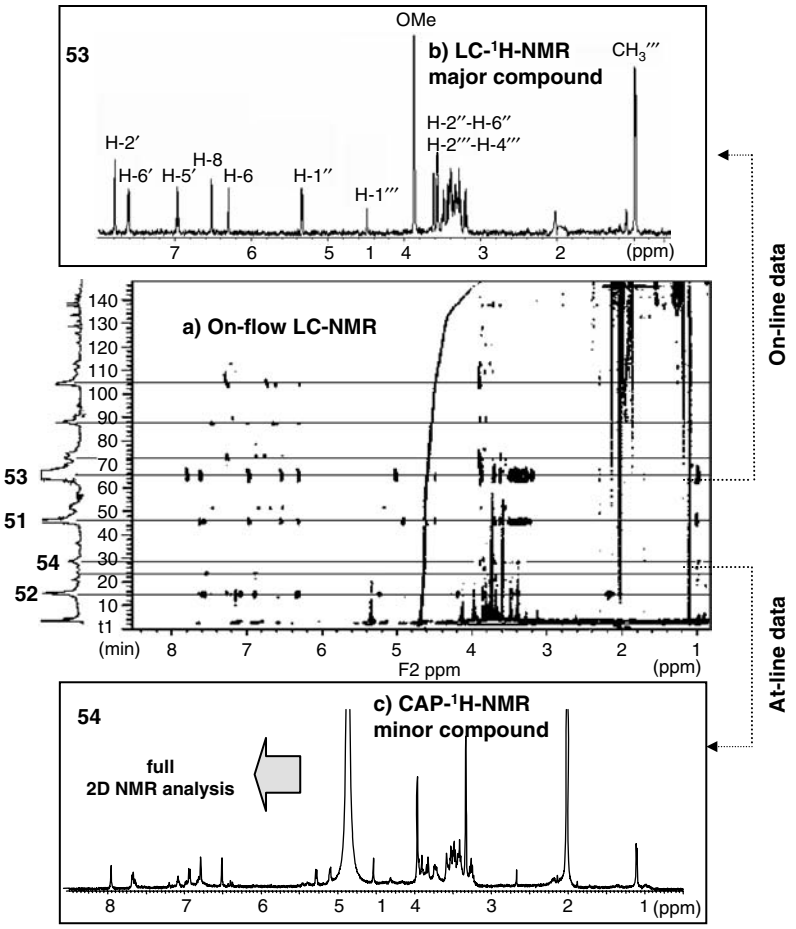

FIGURE 5.31 (a) On-flow LC-NMR contour plot of the crude MeOH extract of *E. scheuchzerii* (Cyperaceae) showing the ^{1}H NMR resonances of all the main constituents. (b) On-flow spectrum of the main flavonoid, isorhamnetin 3-*O*-rutinoside (**53**) (NT = 32). (c) CAP-NMR spectrum of minor flavonoid isorhamnetin 3-*O*-rutinoside-7-*O*-β-D-glucopyranoside (**54**) recorded after microfractionation and elution in CD_3OD (NT = 32). HPLC conditions: C18 column with radial compression, Waters RCM 8 × 10 (100 mm × 8 mm i.d.); MeCN:D_2O (0.05% TFA) gradient (5:95 to 65:35 in 160 min); 1 mL/min, injection 80 mg. LC-NMR conditions: 32 scans/increment, flow cell (60 μL, 3 mm i.d.), 500 MHz.

differ from these flavonoids. It could be identified as chlorogenic acid on the basis of comparison with literature data.

The main flavonoid glycoside of the extract, compound **53**, presented a very clear ^{1}H NMR spectrum in the on-flow conditions with a good S/N. The presence of an isorhamnetin aglycone was shown by a methoxyl group at δ 3.88 ppm and a characteristic aromatic pattern, that is, two *meta*-coupled doublets at δ 6.50 (H-8, d, J = 1.95 Hz) and 6.30 ppm (H-6, d, J = 1.95 Hz), two doublets at δ 7.79 (H-2′, d, J = 1.95 Hz) and 6.97 (H-5′, d, J = 8.3 Hz), and a double doublet at δ 7.62 (H-6′, d, J = 1.95 and 8.3 Hz). The presence of two anomeric protons at δ 5.35 and 4.50 ppm confirmed the presence of two sugars. The proton signal at δ 5.35 (1H, d, J = 6.82 Hz) was attributed to a 3-*O*-β-D-glucoside, and the position of attachment of this sugar on the aglycone was ascertained by the interpretation of the UV-shifted spectra recorded online. The second anomeric proton at δ 4.50 confirmed that the second sugar was attached to the glucose. In addition, the methyl group at δ 0.9 ppm indicated that the second sugar was a rhamnosyl moiety. Thus, **53** was identified as

isorhamnetin 3-*O*-rutinoside. On the basis of the same type of interpretation, the structures of other major flavonoids of the extract were ascertained.[78,79]

Even if significant amounts of the extract were injected on-column, the on-flow LC-NMR analysis failed to give good-quality data on the minor constituents. In this case, the minor constituents were investigated at-line by microfractionation of the LC peaks of interest using the same chromatographic conditions used for on-flow LC-NMR followed by CAP-NMR analysis. As an example, 1D and 2D CAP-NMR spectra recorded for the minor flavonoid **54** of *E. scheuchzeri* are illustrated in Figures 5.31 and 5.32. The ^{1}H NMR signals of **54** were hardly discernable in the on-flow contour plot of the crude extract. Thus, the LC peak corresponding to **54** (MH^+ 787) was collected on the basis of a microfractionation procedure triggered by MS. The sample was dried and redissolved in 5 µL of CD_3OD (exact volume of the flow cell) and pushed into the CAP-NMR flow probe with a calibrated push volume of CD_3OD.

The CAP-^{1}H NMR spectrum recorded for **54** (Figure 5.32c) presented a good lineshape (<1 Hz), no inference due to solvent suppression, and a good S/N even though this compound was difficult to detect in the on-flow approach. All signals recorded were characteristic for a flavonol glycoside with an aglycone bearing a methoxy group. The presence of three anomeric signals at δ 5.34, 5.09, and 4.5 ppm indicated the presence of three sugars, one of them being rhamnose since methyl group protons were observed at δ 0.9 ppm. The presence of the sugars was also confirmed by the LC-APCI MS spectrum, which showed consecutive losses of 308 (162 + 146) and 162 Da to yield the aglycone ion at *m/z* 317. Further to the ^{1}H NMR data, a complete series of COSY, TOCSY, NOESY, HSQC, and HMBC spectra were obtained in 48 h (the amount of compound was estimated to be about 50 µg). In a 1D NOE experiment (Figure 5.32d), it was possible, for example, to ascertain the position of the methoxy group in 3′. A very demanding long-range indirect ^{1}H–^{13}C HMBC experiment (NT 256 × NI 256; 24 h) (Figure 5.32e) was very helpful in providing information on the sugar

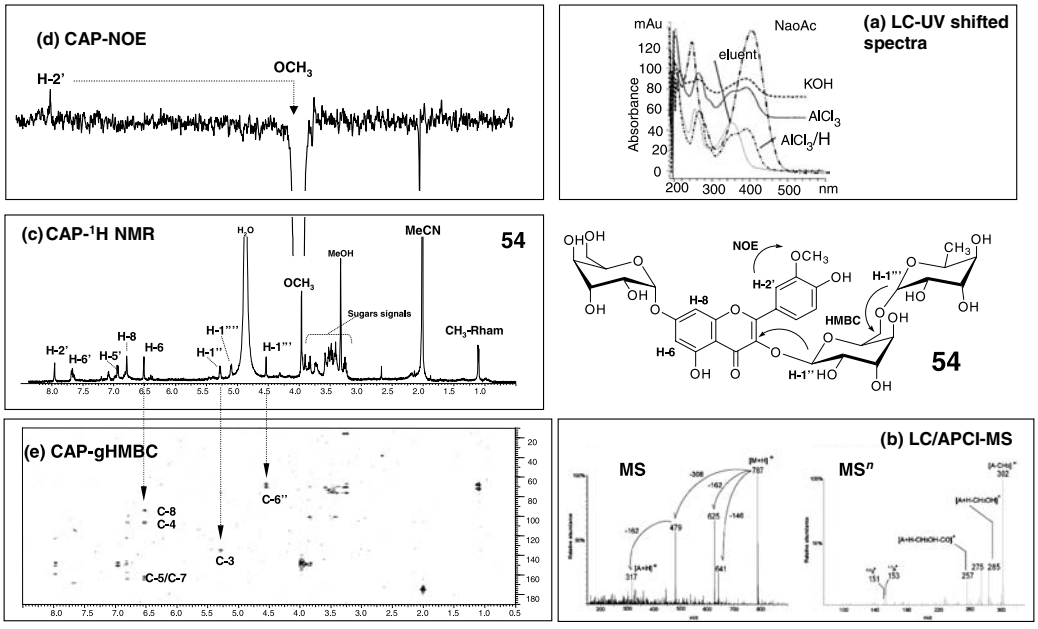

FIGURE 5.32 Summary of all at-line and online information obtained for the minor flavonoid, isorhamnetin 3-*O*-rutinoside-7-*O*-β-D-glucopyranoside (**54**) from the crude MeOH extract of *E. scheuchzeri* (Cyperaceae). For the at-line CAP-NMR spectra, **54** (ca. 50 µg) was dissolved in 6.5 µL of CD_3OD and parked in the probe with a push volume of 5 µL. (a) LC-UV shifted spectra, (b) LC-APCI MS data, (c) CAP-^{1}H NMR (NT = 32), (d) CAP-NOE (NT = 512), and (e) CAP-gHMBC (NT 256 × NI 256; 24 h); (1.5-µL active volume), 500 MHz.

position. For example, the clear correlations between H-1‴ and C-6″ and between H-1″ and C-3 indicate the presence of rutinoside moiety attached at C-3. Furthermore in this case the spectra were recorded in CD_3OD and a good matching with literature data was feasible. Thus, compound **54** was finally identified as isorhamnetin 3-*O*-rutinoside-7-*O*-β-D-glucopyranoside. On the basis of this approach, another 13 polyphenols were identified in the methanolic extract of *E. scheuchzeri*.[79]

5.4 REVIEW OF THE LATEST APPLICATIONS OF DIRECT AND INDIRECT HYPHENATION OF NMR

On the basis of some applications realized in our laboratory, we have illustrated various facets of the use of hyphenated methods for rapid structural determination. In this field of research, the number of applications of online identification of natural products is growing rapidly. In Table 5.1, a summary of the most recent applications (2005–2006) is presented including the type of experiments and the probe used. To complement this table, recent reviews also cover the latest developments.[1,32,68,80]

As shown in Table 5.1, about one-third of the recent applications in the field of natural products are still performed by using classical LC-NMR in both on-flow and stop-flow modes. Generally, in these modes, ^1H NMR spectra were recorded. In direct NMR hyphenation, it is noteworthy that microflow capillary probes can be employed for the direct analysis of crude plant extract in the stop-flow mode. For example, only 33 μg of extract *Radix astragali* (Fabaceae) injected on-column enabled the recording of the stop-flow spectra of the four main isoflavones of the extract that were fully characterized by this means.[10]

A significant number of recent applications (Table 5.1) are performed using LC-SPE-NMR providing ^1H–^1H as well as ^1H–^{13}C data. As an example, the analysis of the roots of *Smirnowia iranica* (Fabaceae) using LC-SPE-NMR with multiple trapping allowed the identification of ten new isoflavonoids and seven previously described constituents.[81]

To improve sensitivity, LC-SPE-NMR spectra were also acquired using cryogenic flow probes. For example, the identification of four isomeric tropane alkaloids of an endemic Chilean plant, *Schizanthus grahamii* (Solanaceae), has been reported.[82] Thus, only 260 μg of an enriched fraction, containing mainly four isomers, was sufficient to obtain high-quality ^1H and ^1H–^1H spectra. Some authors combined LC-UV-MS-^1H NMR and LC-SPE-NMR to completely assign the structure of new natural products. This was the case for the structure determination of new isomeric divanilloylquinic acids from *Fagara zanthoxyloides* (Rutaceae) that may play a useful role in sickle cell disease.[83]

At present, CAP-NMR is probably less widely used than SPE-NMR, but the technique has important potential and presents an interesting alternative.[84] Different recent applications, mainly from Hu et al., have demonstrated their capabilities. This is exemplified by the structure determination of three new and five known partially acetylated antibacterial oligorhamnoside derivatives that were obtained from *C. patens* (Annonaceae) via high-throughput natural products chemistry procedures. The structure determinations were based on complete HSQC, HMBC, and NOESY experiments in combination with high-resolution ESI MS.[74]

Another impressive example, which shows the efficiency of CAP-NMR for mass-limited samples, is the identification of 13 steroids from a small collection of only 50 fireflies (*Lucidota atra*).[85] In this case, 11 HPLC fractions were obtained from a whole insect body extract and the structures were determined on the basis of a complete set of 2D ^1H–^1H and ^1H–^{13}C spectra.

5.5 CONCLUSION

The complementary application of LC-NMR with other LC-hyphenated techniques is playing an increasingly important role in phytochemical investigations designed to search for new bioactive lead compounds. In the drug discovery process, natural products still represent an important source of new pharmacophores, and development of efficient dereplication protocols is essential for a rational approach.

The combination of LC-UV DAD and LC-MS information is very helpful in the first step of dereplication, especially when this information is combined with botanical and chemotaxonomical considerations for cross-search in natural product databases. This approach is, however, limited because of the lack of commercially available LC-UV DAD, LC-MS, or LC-MS/MS databases, and thus, often, complementary information from LC-NMR is necessary for the dereplication process or for full or partial characterization of unknowns. Indeed, for a deeper online structural investigation of natural products, very often the order of the atoms and their stereochemical orientations have to be elucidated, and for this, the use of NMR is mandatory. In direct hyphenation, NMR can be operated in the on-flow mode in fully hypernated systems or as complementary analytical method to LC-UV-MS dereplication in separated analyses. LC-NMR plays a key role and allows the recording of critical complementary online structure information when LC-UV-MS data are insufficient for unambiguous peak identification. The operation of NMR online necessitates, however, that some compromises be made because of the low sensitivity inherent to this spectroscopic method. The need for solvent suppression and the problems of chemical shift differences observed in typical reverse-phase solvent mixtures represent some major limitations.

Despite these limitations, on-flow LC-NMR provides important ^{1}H NMR information on the main constituents of an extract, and the on-flow contour plot readily reveals the similarities found in groups of constituents. These aspects allow a rapid chemical screening of the main compound(s) of an extract; furthermore, a direct estimation of the relative quantities of the main products can be obtained since NMR provides an absolute quantification of protonated species. On-flow NMR represents an invaluable tool for the investigation of unstable compounds since sample handling is very limited. This direct hyphenation of NMR, however, did not provide high-quality NMR data; for in-depth structural investigation, indirect hyphenation of NMR offers numerous advantages, and alternative approaches such as LC-SPE-NMR or CAP-NMR should be considered. These at-line techniques are becoming more and more frequently used in the field of natural products.

Indeed, the complete *de novo* structure determination of natural products requires access to ^{13}C information as well as to different sets of 2D NMR spectra. These types of spectra can be provided to some extent by direct hyphenation of LC-NMR in the stop-flow mode. However, acquisition in this mode still suffers from the compromises that have to be made for the solvent suppression issues. Furthermore, because of LC peak broadening, the elution volumes are not optimized for the best sensitivity. SPE-NMR and CAP-NMR represent robust methods for the rapid at-line identification of natural products. These methods require more sample handling and automation than for the direct hyphenation of NMR, but this still represents only a small percentage of the time required for the time-consuming acquisition of 2D NMR experiments. With such at-line methods, a whole range of 2D NMR experiments can be recorded with a few tenths of micrograms of compounds with only minute amounts of deuterated solvent. The SPE-NMR approach requires a good optimization of the trapping parameter, but it has the advantage that it can be fully automated in commercially available systems.

The CAP-NMR approach presents the advantage that a complete choice of deuterated solvent is possible, since the samples are microfractionated, dried, and reconstituted in the minimum of appropriate solvent to enhance sensitivity. Furthermore, because of its small volume, this type of probe has low residual solvent background signals. In this case, however, solubility problems might present an issue for some types of natural products especially when 2D NMR experiments need to be recorded. Other at-line approaches involving the use of sensitive cryogenic probes for limited-sample application are also very valuable and provide the possibility of working with more dilute solutions if solubility problems are encountered, but the cost of this type of technology is considerably higher. When working with crude extracts, one important task is to develop high-resolution LC methods to avoid problems of coelution that might hamper a correct structure determination. In this respect, the use of very long columns with high efficiency at high pressure and high temperature

has to be investigated in more depth. The latest development in UPLC may bring some efficient solutions to this issue.

There is no unique strategy for the online identification of natural products. Many ways to tackle the issues related to dereplication and rapid characterization are possible. The impressive technological developments made in the field of LC-hyphenated techniques and particularly LC-NMR open new fields of investigation in natural product chemistry where a rapid chemical screening with minute amounts of extracts becomes practically feasible. Phytochemical investigations can now be performed at the microgram level for preliminary chemical investigation. In addition to the progress in analytical methods, it is also essential to establish a direct link between the dereplication process and biological screening via sensitive bioassays that have to be performed online or at-line.

It is safe to argue that the integration of these sensitive spectroscopic methods, hyphenated with HPLC separation, with highly sensitive bioassays that can be coupled online or used at-line after microfractionation will considerably speed up the drug discovery process and thus contribute to the discovery of new useful drugs from natural sources. The combination of chemoanalytical and biological data, and automated dereplication will become essential. This probably will represent the greatest challenge for a few years to come.

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6 Determination of the Absolute Configuration of Bioactive Natural Products Using Exciton Chirality Circular Dichroism

Hans-Ulrich Humpf

CONTENTS

6.1 INTRODUCTION

The term "chirality" is derived from the Greek word *kheir*, meaning "handedness". Any organic molecule that lacks reflection symmetry exists in more than one form. These forms are like the left- and right-hand nonsuperimposable mirror images of each other and are known as chiral objects (enantiomers). Enantiomers, which differ only in their absolute configuration, possess identical physical and chemical properties except in two important points: they rotate the plane of polarized light in opposite directions and they react or interact at different rates with other chiral compounds. For example, the interaction between biologically active compounds, receptor proteins, or enzymes often shows a high or complete stereoselectivity. Basically all natural products, such as proteins, nucleic acids, sugars, lipids, amino acids, hormones, vitamins, antibiotics, plant/animal/marine natural products, etc., are optically active or chiral.

Classical examples of biologic stereoselectivity are the taste of amino acids. Whereas D-amino acids generally exhibit a sweet taste, the L-enantiomers are tasteless or bitter. Many other examples of chiral chemicals with enantioselective physiological, pharmacological, or toxicological effects can be found in the literature, for example, sodium glutamate (*S*-(+): flavor enhancing), penicillamine

(*R*-(+): extremely toxic; *S*-(−): antiarthritic), or omeprazole, a proton pump inhibitor, which is more pharmacologically active in humans in its *S*-form.[1,2] Another example is ibuprofen; however, in this case the inactive *R*-isomer is converted into the active *S*-isomer in the human body.[3]

Because of the increasing interest in enantiomers, the development of methods to synthesize single isomers has become one of the most important fields in synthetic organic chemistry over the last decades.[4] For their basic work creating catalysts that could produce only one stereoisomer, the Nobel Prize for Chemistry in 2001 was awarded to William S. Knowles, K. Barry Sharpless, and Ryoji Noyori. Besides synthetic approaches to enantioselective syntheses, biotransformations, for example, using enzymes, have become key technologies.[5]

On the basis of the seminal experiment of Pasteur in 1848 on the manual separation of the two enantiomorphs of sodium ammonium tartrate tetrahydrate, van't Hoff[6] and Le Bel[7] developed the concept of asymmetry in 1874. Pasteur recognized that the two isomers polarized light differently, one to the left and the other to the right, and that this must be due to an asymmetric arrangement of the atoms in the optically active molecules.[8] Following Kekule's recognition in 1858 that carbon has a valence of 4,[9] van't Hoff and Le Bel independently recognized that two different forms exist, when four different groups are attached to a carbon atom.[6,7] However, it took over a century from Pasteur's experiments until the first assignment of the absolute configuration using X-ray by Bijvoet et al. in 1951.[10] In addition to X-ray, chiroptical data were acquired as early as the mid-nineteenth century, and chiroptical methods such as polarimetry, optical rotatory dispersion (ORD), or circular dichroism (CD) were extensively used to differentiate between stereoisomers.[1,11] However, the interpretation of chiroptical data and the assignment of the absolute configuration of molecules was limited and only based on the comparison with compounds of known stereochemistry and on empirical rules. For this reason, Harada and Nakanishi[12,13] developed the CD exciton chirality method, a nonempirical method that enables the determination of the absolute configuration of organic molecules without reference to authentic examples. The method has already been described in detail in several books, chapters, and review articles.[11,13–16] The basic theories underlying the exciton chirality phenomenon were already provided in the 1930s by Kuhn's "coupled oscillator" theory[17] and Kirkwood's "group polarizability" theory.[18]

6.2 EXCITON CHIRALITY METHOD

6.2.1 Basic Principle of the Exciton Chirality Method

The exciton chirality CD method is a nonempirical microscale procedure to determine the absolute configuration and conformation of organic molecules and has been widely used in the field of organic chemistry and natural product analysis.[13–16] The CD exciton chirality method is based on the "through space" coupling of two or more chromophores in chiral substrates giving rise to a bisignate CD curve. Initially, this method correlated the chirality of 1,2-glycols with signs of the intense π–π* Cotton effects (CEs) of dibenzoates. The signs of these split CEs (couplets) establish the absolute sense of twist of the electric transition moments in a nonempirical manner. The coupling of two identical chromophores is shown by the *p*-substituted bisbenzoates of a vicinal cyclohexanediol (Figure 6.1). If the spatial orientation of the transition dipoles of two chromophores is clockwise (looking from the chromophore in front to the chromophore in the rear as shown in Figure 6.1A or 6.2A), defined as positive chirality (Figure 6.1B), the CD shows a positive first CE at higher wavelength and a negative CE at lower wavelength (Figure 6.1E) and *vice versa*.[13,14]

When the electric transition moments (μ, bold lines in Figures 6.1A and 6.2) of two (or more) chromophores interact, the energy levels of the excited state split and a stabilized dipole–dipole interaction at a lower energy level (α-state) and a destabilized dipole–dipole interaction at a higher energy level (β-state) are observed (Figure 6.1C). In the UV/Vis spectra the excited states are observed as a red-shifted α-state (stabilized dipole–dipole interaction) and a blue-shifted β-state

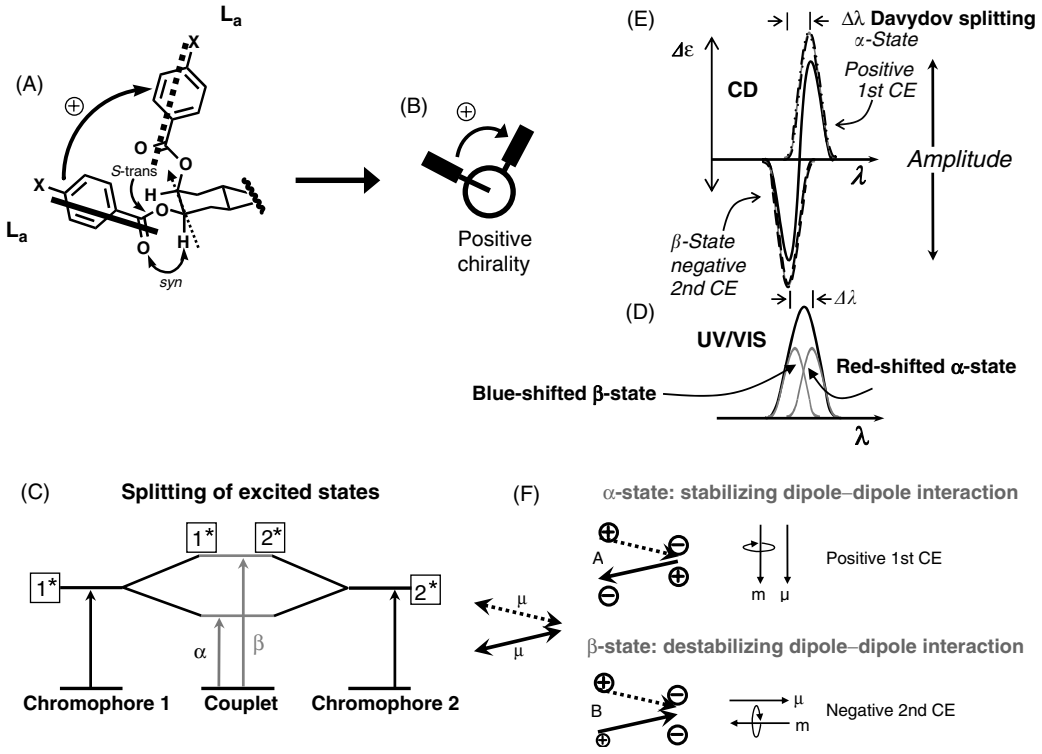

FIGURE 6.1 Exciton coupling of two identical chromophores.

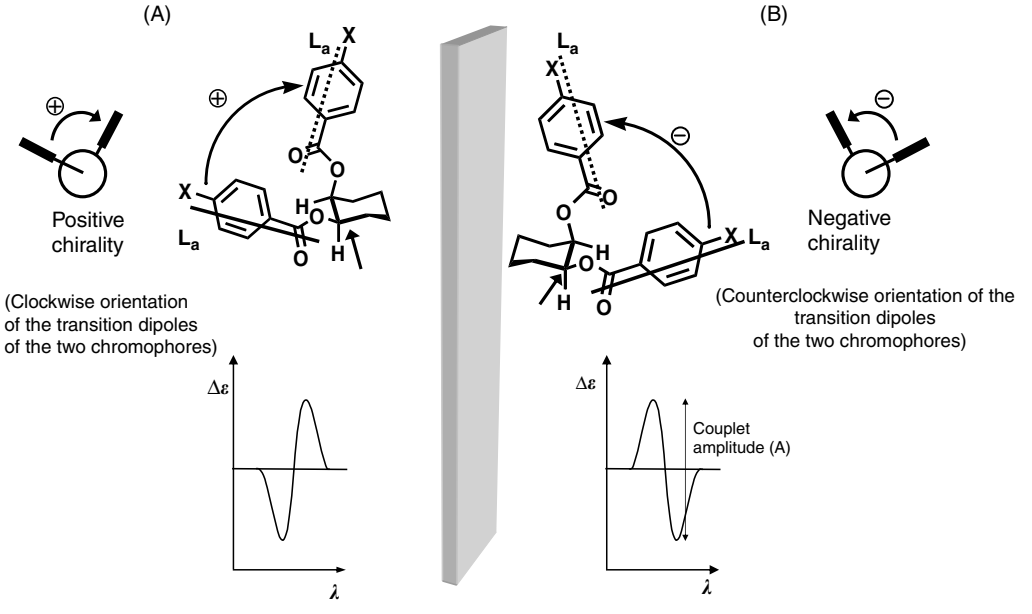

FIGURE 6.2 Clockwise (A) and counterclockwise (B) orientation of two identical exciton coupling chromophores leading to positive (A) and negative chirality (B) and to mirror image CD spectra.

(destabilized dipole–dipole interaction) and the signal appears as a single absorption maximum with double intensity (Figure 6.1D). In cases where the energy difference between the α- and the β-states is substantial, the UV/Vis spectra show two maxima. In the CD spectrum the α- and the β-states are reflected by a positive and a negative CE resulting in the typical bisignate or split CD curves with a positive first CE at higher wavelength and a negative second CE at lower wavelength (Figures 6.1E and 6.2A). In the case of the dibenzoate moiety shown in Figure 6.1A, the "total" electronic transition moments, which can be obtained by simple vector addition, are orientated along (μ_B, β-state) and perpendicular (μ_A, α-state) to the chromophoric C_2 axis (Figure 6.1F).[13,14] The charge rotation associated with the transition dipoles results in a magnetic transition (*m*) moment. Parallel arrangement of the electric (μ) and magnetic (*m*) transition moments leads to a positive CE, while an antiparallel arrangement leads to a negative CE (Figure 6.1F).[19]

The corresponding mirror image compound with a counterclockwise orientation of the two chromophores (negative chirality) (Figure 6.2B) results in a mirror image CD curve with a negative first CE at higher wavelength and a positive second CE at lower wavelength (Figure 6.2B). The amplitude (*A*) of a CD spectrum, which is defined as the distance between the peak and the trough of a split CD curve (see Figures 6.1E and 6.2), is: (i) inversely proportional to the square of the interchromophoric distance, (ii) proportional to the absorption coefficient of the coupling chromophores, and (iii) dependent on the interchromophoric projection angle with a maximum at approximately 70° and minima at 0° and 180°. For example, no exciton coupling will be observed when the transition dipoles of two interacting chromophores are orientated coplanar.[13,14]

6.2.2 Special Features of the Exciton Chirality Method

6.2.2.1 Chromophores Used for Exciton Coupling

Any chromophore with a large ε-value and known direction of the electric transition moment (μ) is useful for exciton chirality (Figure 6.3). The *p*-substituted benzoates (**1–3**) or cinnamate chromophores (**4, 5**) are widely used for exciton coupling and the direction of the electric transition moment is along the long axis of the molecule and almost parallel to the C−O bond (bold lines in Figure 6.3). Although there might be free rotation around the two C−O bonds, ester bonds are known to be *S-trans* (see Figure 6.1A) and furthermore X-ray data and calculations show that the ester carbonyl is *syn* with respect to the carbinyl hydrogen (Figure 6.1A).[13,14]

To enhance the sensitivity and to avoid overlapping with preexisting absorption bands, other powerful chromophores with intense and red-shifted absorbance have been developed. Characteristic examples shown in Figure 6.3 include 2-napthoate (**6**), 2-anthroate (**7**), 9-anthroate (**8**), and 9-methylanthryl groups (**9**) with absorption bands below 300 nm and *p*-dimethylamino cinnamate (**5**) or tetraarylporphyrin (**10**) with absorption bands above 300 nm.[13,14,20–25] These chromophores are primarily used for the derivatization of primary and secondary −OH and $-NH_2$ groups. For primary amino groups, neutral or protonated Schiff bases are also useful red-shifted chromophores.[26,27]

As a characteristic example Figure 6.4 shows the exciton coupling CD spectra of 1(*R*), 2(*R*)-*trans*-cyclohexanediol derivatized with either the benzoate (**1**) or 2-naphthoate (**6**) chromophores at the hydroxyl groups. In the case of the 1(*R*),2(*R*)-*trans*-cyclohexanediol *bis*(benzoate), (**13**) the coupling of the two benzoate chromophores leads to a negative split CD curve with a negative first CE at 236 nm ($\Delta\varepsilon = -25.7\,dm^3/(mol\,cm)$) and a positive CE at 221 nm ($\Delta\varepsilon = +9.7\,dm^3/(mol\,cm)$) with an amplitude *A* of −35.4. Compared to **13**, the 2-naphthoate derivative (**14**) exhibited a bisignate CD with the same sign and extrema at 241 nm ($\Delta\varepsilon = -339\,dm^3/(mol\,cm)$) and 228 nm ($\Delta\varepsilon = +223\,dm^3/(mol\,cm)$) but with a >15-fold stronger *A* value of −562.[28] This can be explained by the larger absorption coefficient of the 2-naphthoate chromophore (Figure 6.3) and is a basic principle of exciton coupling: the stronger the absorption of the chromophores, the larger the *A* values.

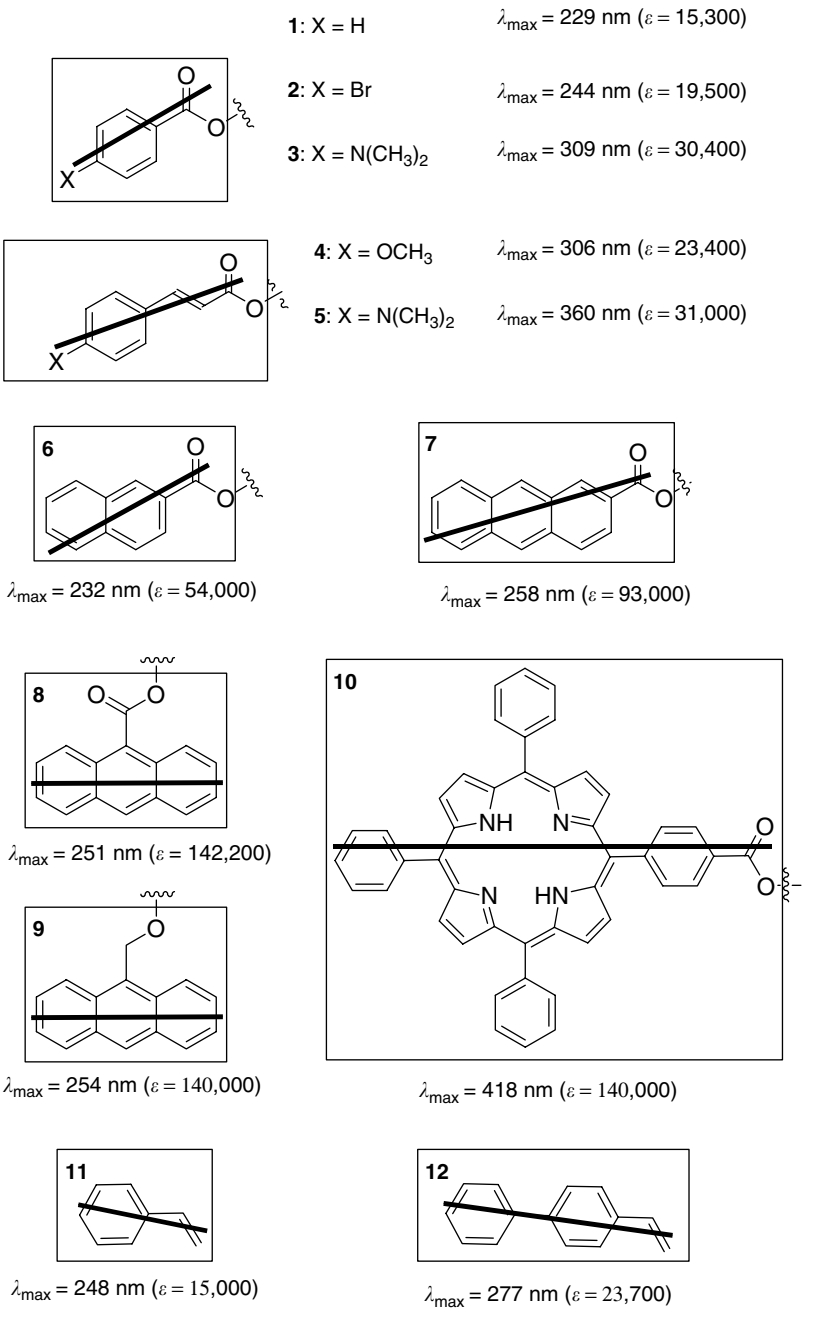

FIGURE 6.3 Selected chromophores useful for exciton chirality applications (bold lines represent the direction of the transition dipoles).

As can be seen from the following examples, the chromophores can be introduced by O- or N-acylation of hydroxyl or amino groups, derivatization of carboxylic acid or sulfanyl groups, cross-metathesis at C=C double bonds (chromophores **11** and **12**, Figure 6.3), or could already be preexisting in the molecule (e.g., enone, diene, etc.). Exciton chirality can also be extended to non-degenerate systems consisting of two different chromophores (Section 6.2.3.1).[13,14]

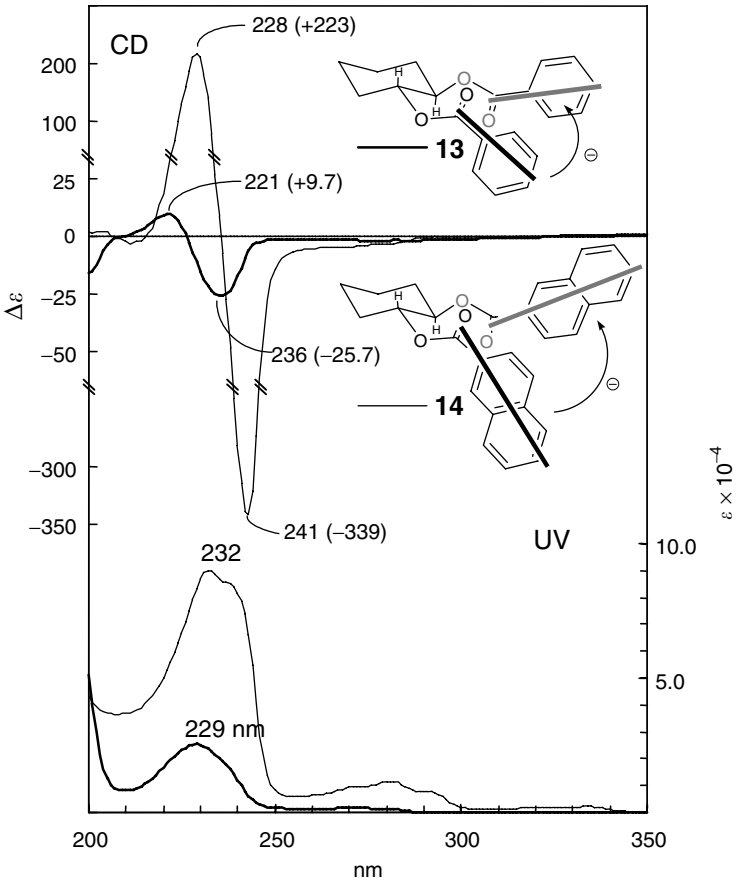

FIGURE 6.4 CD and UV spectra of 1(*R*),2(*R*)-*trans*-cyclohexanediol *bis*(benzoate) (**13**) and 1(*R*),2(*R*)-*trans*-cyclohexanediol *bis*(2-naphthoate) (**14**) in acetonitrile (1-cm cell).

6.2.2.2 Additivity of CD Amplitudes

The amplitude (*A*) of a split CD curve for a compound containing three or more identical chromophores can be approximated by the sum of each interacting chromophore pair. As illustrated in Figure 6.5, the CD amplitude of a pyranose derivatized with *p*-bromobenzoate can be calculated from the three bischromophoric pairs: $A_{total} = A_1 + A_2 + A_3$.[29,30] The additivity applies not only for the monochromatic amplitude but also for the entire CD curve when two different chromophores are present, as was shown for glucose, galactose, and mannose derivatized with *p*-bromobenzoate and *p*-methoxycinnamate.[31–33] The bischromophoric CD spectra obtained are characteristic for each substitution pattern and type of pyranoside.[31] The additivity relation allows the interpretation of complex CD spectra of multichromophoric molecules. The reverse principle, extracting stereochemical information from the difference CD curve, was applied for the stereochemical assignment of natural products such as nemadectin,[34] malonofungin,[35] and fumonisin FB_3.[36]

6.2.3 Applications of the Exciton Chirality Method

6.2.3.1 Exciton Coupling between Different Chromophores

At least two chromophores are required in a substrate for the application of the exciton chirality method and in most applications the chromophores are introduced by derivatization as described above. However, the coupling chromophore(s) could already be preexisting in the molecule,

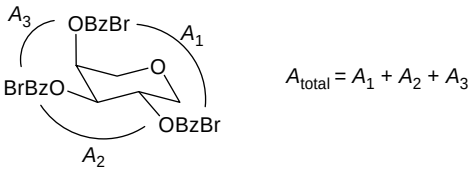

FIGURE 6.5 Additivity in CD amplitudes (A) of a pyranose tri-*p*-bromobenzoate.[29,30]

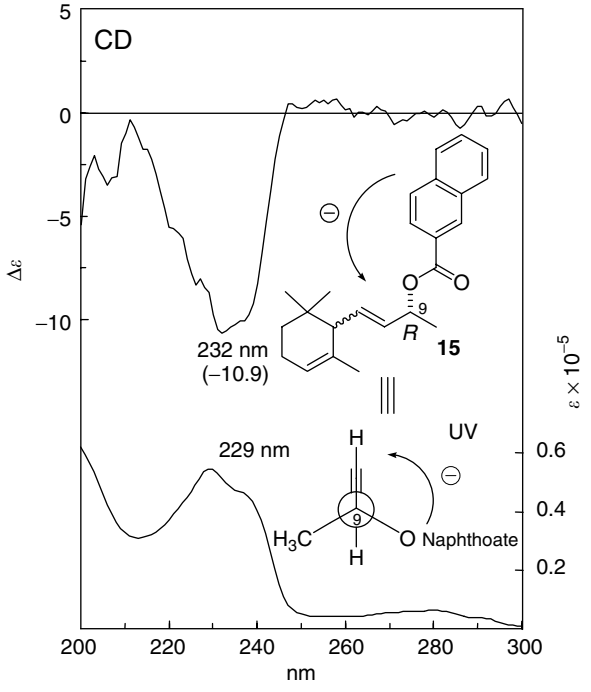

FIGURE 6.6 CD and UV spectra of α-ionyl-2′-naphthoate (**15**) in acetonitrile (1-cm cell) and preferred conformation of **15**. (Reprinted from Humpf, H.-U., *Carotenoid-Derived Aroma Compounds, ACS Symposium Series*, 802, 56, 2002. With permission. Copyright 2001 American Chemical Society.)

for example, enones, dienes, and aromatic heterocycles. Examples for the stereochemical assignment of natural products on the basis of exciton coupling between preexisting chromophores are vinblastine, quassin, calycanthine, periplanone B, and abscisic acid.[13,14] In the case of different chromophores, exciton coupling is still observed even when the absorption maxima of the chromophores are 100 nm apart.[13,14]

Another example is the so-called "allylic benzoate method".[37–39] In the case of allylic or homoallylic alcohols (e.g., α-ionol) the double bond can be used as a chromophore for exciton coupling and the second chromophore (e.g., benzoate[37–39] or 2-naphthoate[40,41]) has to be introduced at the hydroxyl group. Figure 6.6 shows the UV and CD spectra of α-ionol derivatized with the 2-naphthoate chromophore. The CD spectrum of chromophoric derivative **15** with a negative CE at 232 nm ($\Delta\varepsilon = -10.9\,\mathrm{dm^3/(mol\,cm)}$) (Figure 6.6) arises from exciton coupling between the transition moments of the two chromophores. In particular, the π–π* transition of the double bond at ca. 195 nm couples with the ^1B_b transition band of the 2-naphthoate at ca. 232 nm. If the two axes of the 2-naphthoate and the double-bond chromophore possess the sense of a left-handed screw, the first CE is negative, as observed for **15**, and it is not necessary to measure the second CE below ca. 200 nm. In addition

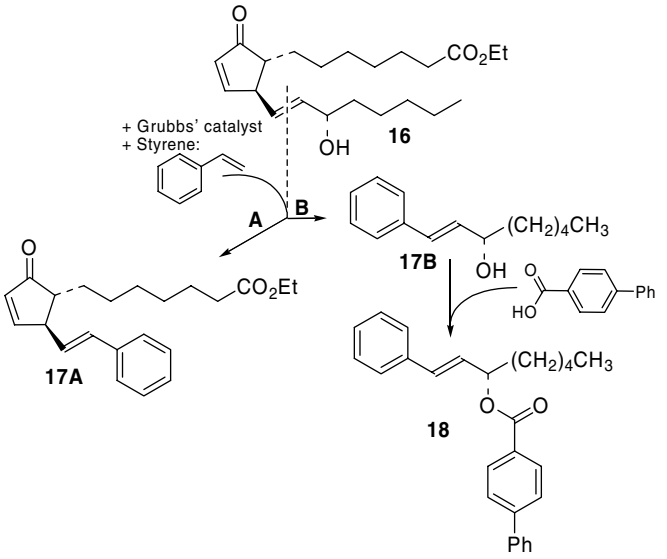

FIGURE 6.7 Derivatization of prostaglandin A_1 (**16**) to styrenoids by cross-olefin metathesis using Grubbs' Ru catalyst. (Adapted from Tanaka, K., Nakanishi, K., and Berova, N., *J. Am. Chem. Soc.*, 125, 10802, 2003.)

to the intense π–π* transition, the double bond has other weak transitions in the same region and these may give rise to additional CEs at around 205 nm. Since the 2-naphthoate CE at 232 nm is isolated and not perturbed by other transitions, the sign of the first CE at 232 nm reflects the chirality in a straightforward manner.[40,41] As shown earlier for a series of acyclic allylic alcohols, of the three limiting conformers, the one is the most favored in which H-9 in α-ionol is eclipsed with the allylic double bond (Figure 6.6).[38] From the obtained negative first CE at 232 nm, the 9(*R*)-configuration for **15** was determined. Allylic amines have also been studied by conversion of the primary amino group into phthalimides.[42] A similar method is also useful for the stereochemical assignment of acyclic hydroxylated dienes, as was shown for a series of lipoxygenase-catalyzed oxygenation products of 1(*Z*),4(*Z*)-unsaturated fatty acids.[43,44]

However, the "allylic benzoate method" is limited since many natural products (e.g., prostaglandins) contain other chromophores that might overlap with the CE at around 230 nm, e.g., the twisted enone chromophore of prostaglandin A_1. To overcome this problem, Nakanishi and coworkers[45–48] recently developed another straightforward method for allylic alcohols, amines, and other ene moeties. This method is based on cross-metathesis using Grubbs' catalyst.[49] As shown with prostaglandin A_1 (**16**, Figure 6.7), the double bond is converted by cross-metathesis into (*E*)-styrenoids **17A** and **17B** under mild conditions in high yield. Styrenoid **17B** is further acylated with *p*-phenylbenzoic acid to chromophore **18**. From the resulting exciton coupling CD spectra of **17A** (263 nm, $\Delta\varepsilon = +25.8\,dm^3/[mol\ cm]$; 226 nm, $\Delta\varepsilon = -23.8\,dm^3/[mol\ cm]$) and **18** (273 nm, $\Delta\varepsilon = +11.5\,dm^3/[mol\ cm]$; 248 nm, $\Delta\varepsilon = -9.9\,dm^3/[mol\ cm]$), it was possible to establish the absolute configurations of both moieties **17A** and **17B** and hence of prostaglandin A_1 (**16**) (Figure 6.7).[45] Other applications of this cross-metathesis/exciton chirality protocol can be found in the literature.[45–48]

Exciton coupling between different chromophores ("bichromophoric" method) has also been extensively used for the stereochemical assignment of acyclic compounds such as 1,2-diol; 1,2,3-triol; 1,2,3,4-tetraol; or 1,2,3,4,5-pentol moieties as well as amino alcohols.[14] The bichromophoric exciton chirality method requires two different chromophores that are selectively introduced at the primary and secondary hydroxyl groups. It turned out that the chromophoric combination of

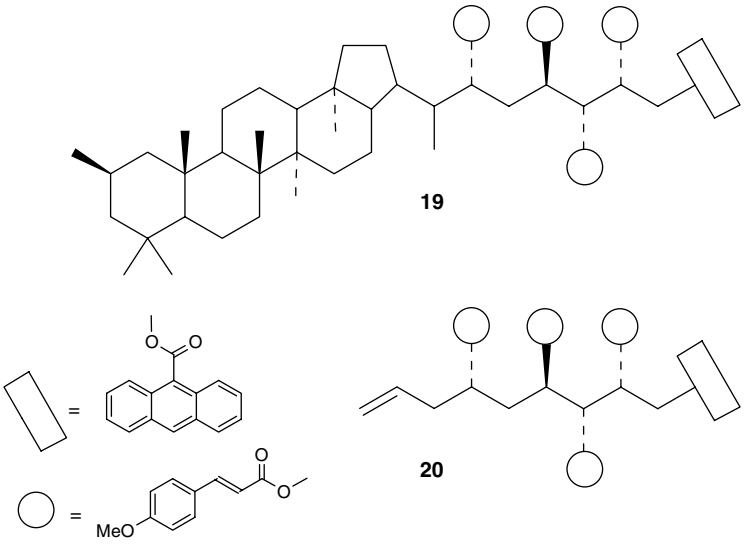

FIGURE 6.8 Structures of bacteriohopanoid (**19**) and a synthesized model compound (**20**).[51]

9-anthroate (**8**) and *p*-methoxycinnamate (**4**) is the method of choice.[14] In a two-step derivatization procedure the primary hydroxyl group is first acylated with the 9-anthroate chromophore, while the other chiral hydroxyl groups are derivatized in high yield with the *p*-methoxycinnamate in the second step. The 9-anthroate/methoxycinnamate coupling results in a strong positive or negative CE at 252 nm, which is diagnostic of the configuration at C2 or C3. For example, in the case of (*S*)-1,2-propandiol or (*R*)-1,3-butanediol a positive or negative CE at 252 nm is observed, respectively.[50] The CD curves of 1,2-polyols are much more complex and represent the absolute sense of twist between the interacting chromophores and the conformational population. The comparison of such CD curves with corresponding reference curves allows the stereochemical assignment of several stereogenic centers in a semiempirical manner on a microscale. The bichromophoric method has been described in detail in the literature and many "fingerprint" CD curves have also been published.[14] The method was applied to natural products such as bacteriohopanoid (**19**).[51] The comparison of the CD curves of **19** with the CD curve of synthesized model polyols (**20**) established the absolute configuration shown in Figure 6.8.

6.2.3.2 Other Applications of the Exciton Chirality Method

6.2.3.2.1 Stereochemical Assignment of Carboxylic Acid Groups

Carboxylic acid moieties and related structural units are widespread in bioactive natural products and play important roles in fundamental biochemical processes (e.g., lactic acid, sphingolipids), anticancer drugs (e.g., paclitaxel side chain), and antibiotics (e.g., amphotericin B).

The stereochemical assignment of α- and β-hydroxy acid moieties, as well as other carboxylic acid groups, still remains a difficult task that requires two chromophores suitable for exciton coupling. It was recently demonstrated that a two-step derivatization using the 9-methylanthryl (**9**) and the 2-naphthoate (**6**) or 2-anthroate (**7**) chromophores provides a general method for the absolute configurational assignment of α- and β-hydroxy carboxylic acids.[23,24,52] The chromophoric combination 9-methylanthryl/2-naphthoate or 9-methylanthryl/2-anthroate leads to strong bisignate mirror image CD curves with *A* values ranging from −149 to +168 for α- and β-hydroxy carboxylic acid enantiomers. The preferred sense of twist between the 9-methylanthryl and the 2-naphthoate or 2-anthroate groups follows the same CD pattern: negative chirality—(*S*) configuration and positive chirality—(*R*) configuration.[23,24,52] This general principle was also demonstrated with various

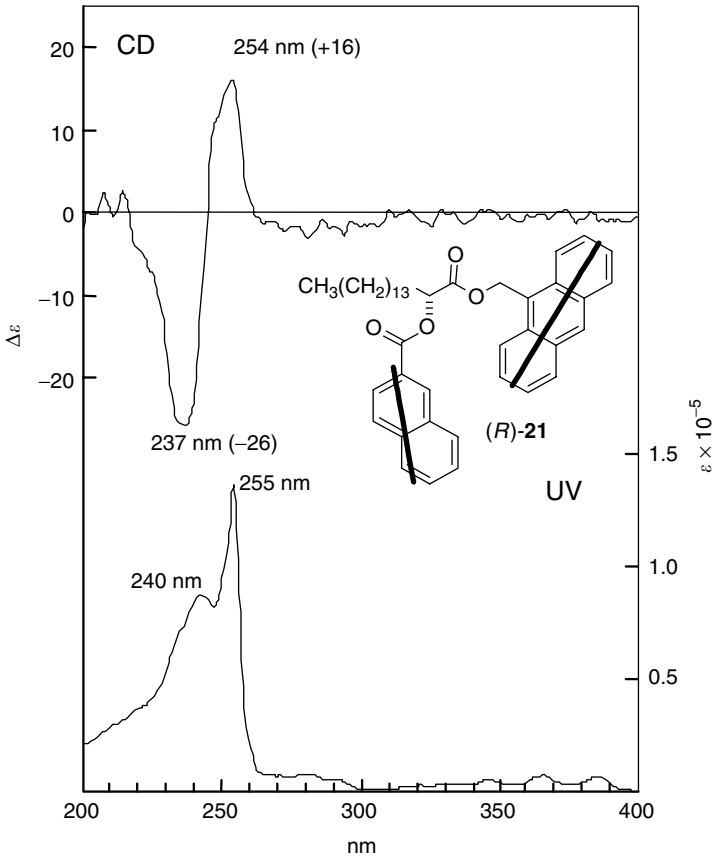

FIGURE 6.9 UV and CD spectra of the bichromophoric diester, (*R*)-**21** in acetonitrile (1-cm cell). The bold lines represent the direction of the transition dipoles. (Reprinted from Adam, W., Lazarus, M., Schmerder, A., Humpf, H.-U., Saha-Moeller, C.R., and Schreier, P., *Eur. J. Org. Chem.*, 9, 2013, 1998. With permission. Copyright 1998 Wiley-VCH.)

long-chain α- and β-hydroxy carboxylic acids.[53–56] As a representative example Figure 6.9 shows the CD of the bichromophoric diester of 2-hydroxypalmitic acid ((*R*)-**21**) that was obtained by kinetic resolution through lipase-catalyzed enantioselective acetylation.[55] In (*R*)-**21**, the long axis ^1B_b transition of the 9-methylanthryl chromophore with its quite intense absorption couples with the ^1B_b band of the 2-naphthoate chromophore to give a positive split CD curve with extrema at 254 nm ($\Delta\varepsilon = +16\,dm^3/[mol\,cm]$) and 237 nm ($\Delta\varepsilon = -26\,dm^3/[mol\,cm]$) and an amplitude *A* of +42.[55]

Another approach for the stereochemical assignment of α-hydroxy carboxylic acids was developed on the basis of the intramolecular stacking properties of a bisporphyrin derivative.[57,58] This approach consists of amidation of the carboxyl group with ethanolamine followed by derivatization with porphyrin (**10**, Figure 6.3) to form π,π-bisporphyrin derivatives that undergo intramolecular stacking. The sign of the observed bisignate CD couplet resulting from the favorable intramolecular π,π-stacking conformer reflects the absolute configuration of the stereogenic center.[58]

Another strategy for the stereochemical assignment of carboxylic acid groups is the direct esterificaton with 2-naphthol ($\lambda_{max} = 222$ nm, $\varepsilon = 54{,}000$, acetonitrile). For the derivatization of carboxylic acid groups, in a one-pot reaction and in quantitative yield, *N, N-bis*(2-oxo-3-oxazolidin yl)phosphorodiamidic chloride was used for the activation of the carboxyl group.[52,59] The diterpene abietic acid, a well-known major constituent of gum or wood resin, was used as model compound. The molecule consists of the typical hydrophenanthrene system and contains an (*R*)-configured

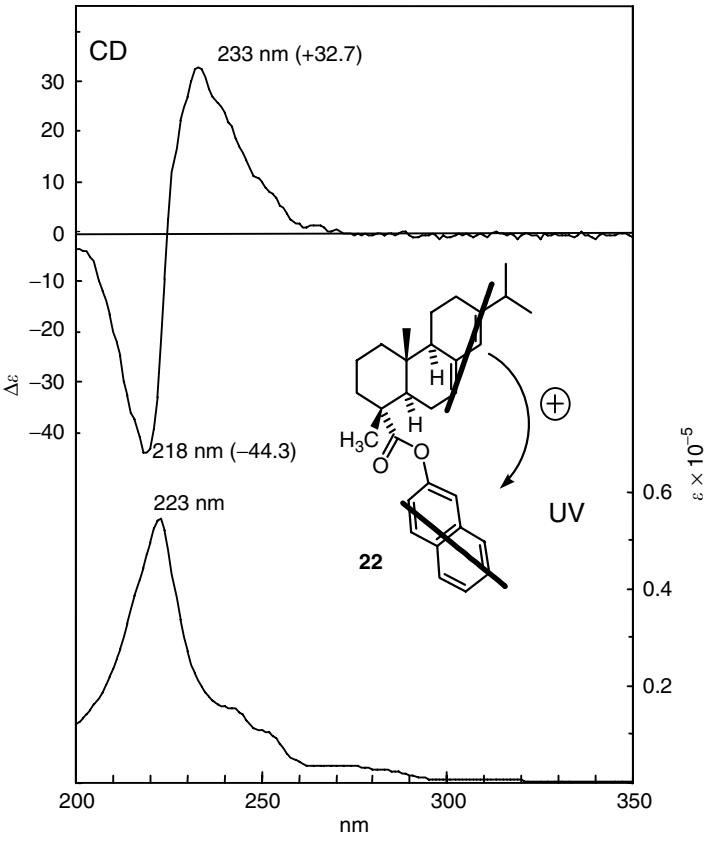

FIGURE 6.10 UV and CD spectra of abietic acid derivative **22** in acetonitrile (1-cm cell). The bold lines represent the direction of the transition dipoles. (Reprinted from Hartl, M. and Humpf, H.-U., *Tetrahedron Asymmetry*, 11, 1741, 2000. With permission. Copyright 2000 Elsevier.)

carboxylic group in position 1 (Figure 6.10). This group was derivatized with 2-naphthol to yield a mononaphthylester (**22**). In **22**, the ^1B_b band of the naphthoyl chromophore interacts with the diene moiety at ca. 235 nm (ε = 21,500) already preexisting in the molecule. The resulting CD of **22** (Figure 6.10) is characterized by a positive CD couplet with extrema at 233 nm ($\Delta\varepsilon$ = +32.7 dm^3/[mol cm]) and 218 nm ($\Delta\varepsilon$ = −44.3 dm^3/[mol cm]), constituting a positive chirality, just as expected from the clockwise orientation of the two transition moments.[52,59] This method was recently applied for the stereochemical assignment of the tricarballylic acid side chain of fumonisins,[60] a class of mycotoxins with carcinogenic activity.[61]

6.2.3.2.2 Stereochemical Assignment of Sulfanyl Groups

As already mentioned, chromophores used for exciton coupling are mainly introduced by O- or N-acylation of hydroxyl or amino groups. However, it is also possible to extend the exciton chirality method to the stereochemical assignment of sulfanyl groups that can also be derivatized with chromophores. This principle was applied for the first time to a cyclic sulfanyl compound[62] and recently a method for the stereochemical assignment of acyclic 2- and 3-sulfanyl-1-alkanols has been described.[63] Sulfur compounds play an important role as flavor compounds because many of them have very low odor thresholds and contribute considerably to the overall odor impression even at low concentrations.[64] Most sulfur compounds are also optically active and the odor properties of diastereomers can differ tremendously. In the case of 2- and 3-sulfanyl-1-alkanols both functional groups were derivatized with the 9-anthroate chromophore (**8**) in a one-step reaction.[63]

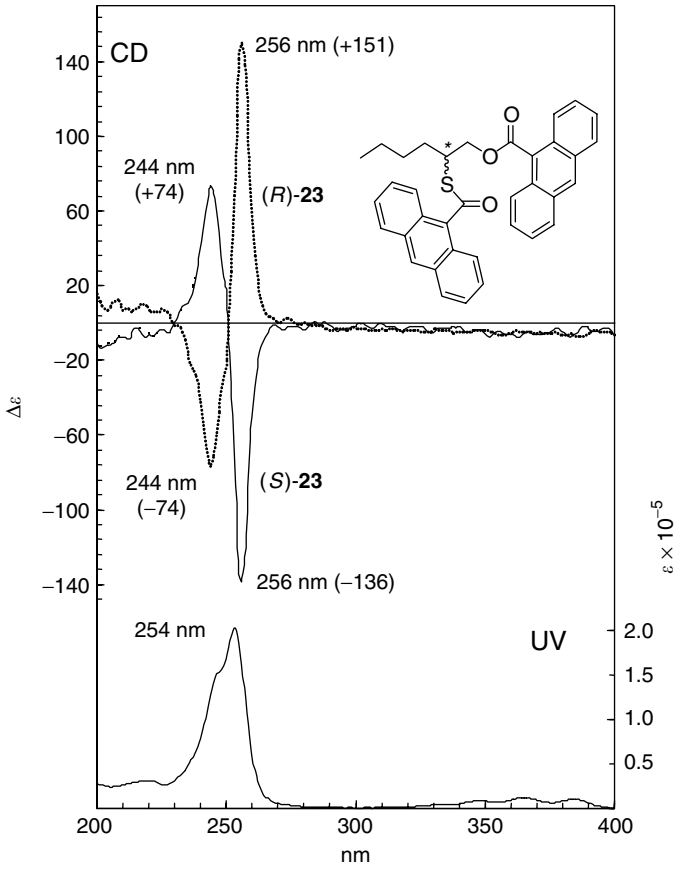

FIGURE 6.11 UV and CD spectra of 1-*O*-(9-anthroyl)-2-*S*-(9-anthroyl)-2-sulfanyl-1-hexanol (**23**) in acetonitrile (1-cm cell). (Reprinted from Weckerle, B., Schreier, P., and Humpf, H.-U., *J. Org. Chem.*, 66, 8160, 2001. With permission. Copyright 2001 American Chemical Society.)

The resulting exciton coupling CD curve unequivocally allows the determination of the absolute configuration as the example of derivatized 2-sulfanyl-1-hexanol (**23**) in Figure 6.11 demonstrates. Although 9-anthroate is sterically hindered and the derivatization of both functional groups proceeds only in approximately 10% yield, the method is very useful since only one derivatization step is necessary and the chromophoric derivatives can easily be isolated by thin layer chromatography (TLC) due to the highly fluorescent 9-anthroate. Furthermore, the intense CD spectra allow applications in the microgram range.[63]

6.2.3.3 Absolute Configurational Assignment of Compounds with a Single Stereogenic Center

The application of the exciton chirality method needs at least two interacting chromophores with well-defined conformation. The absolute configuration of compounds with a single chiral center cannot in general be determined by this method. The exception is for cases in which preexisting chromophores function as an interacting partner (e.g., allylic benzoate method) or the second interacting chromophore could be introduced at another nonchiral functional group (e.g., carboxylic acid group in the case of α-hydroxy carboxylic acids). However, in recent years exciton chirality-based methods for the stereochemical assignment of compounds having just one functional group

(e.g., OH, NH_2) have been developed by several groups. Recent advances in this field were summarized in an excellent review.[16] Only a few examples will be discussed in this chapter.

A very practical solution is based on the derivatization of the functional group with an axially helical bichromophore. As a result, diastereomeric conformers are formed and the resulting exciton coupling CE is determined by the dominant diastereomer.[16,65,66] A similar concept is based on the intramolecular porphyrin π,π-stacking as already described for the stereochemical assignment of α-hydroxyl carboxylic acids.[58] This concept is also useful for other acyclic compounds with OH or NH_2 groups.[57]

Most recently a different approach has been developed by Nakanishi, Berova, and coworkers by using a zinc porphyrin tweezer, which consists of two zinc porphyrin complexes connected by a pentanediol spacer. The zinc porphyrin tweezer was originally developed to determine the absolute configuration of chiral diamines and the application was extended to α-amino acids, α-amino alcohols, primary and secondary amines, as well as secondary monoalcohols.[67–71] This method is based on a host/guest complexation mechanism in which the chiral substrate is complexed to the dimeric Zn porphyrin tweezer. The bisporphyrin host is forced into a chiral arrangement with the intramolecular twist controlled by the stereochemistry of the guest molecule. The sign of the resulting exciton CD couplet correlates with the absolute configuration of the substrate. As a representative example, Figure 6.12 shows the adduct formed between the zinc porphyrin tweezer and (*R*)-1,2-diaminopropane, the favored conformation of the host/guest complex, and the resulting negative split CD curve.[67] Recently, a magnesium tetraarylporphyrin tweezer was used for the absolute configurational assignment of α-chiral carboxylic acids.[72]

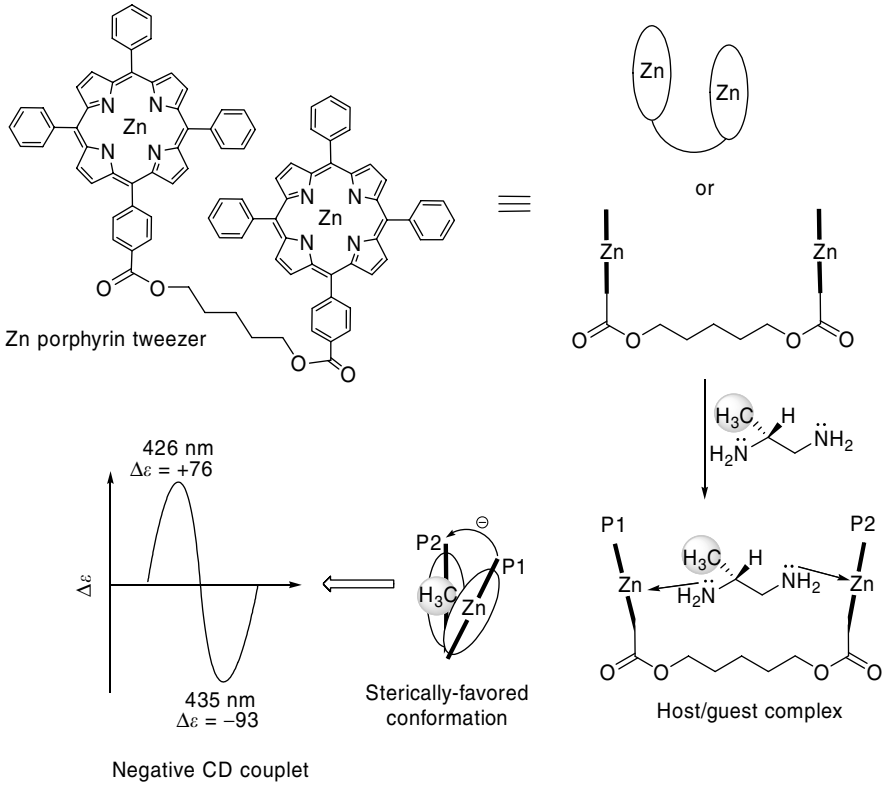

FIGURE 6.12 Host/guest complex formed between Zn porphyrin tweezer and (*R*)-1,2-diaminopropane, sterically favored conformation, and resulting negative CD couplet.[67]

6.2.3.4 Fluorescence-Detected Exciton-Coupled Circular Dichroism

An interesting alternative to conventional CD detection is fluorescence-detected circular dichroism (FDCD) by using fluorescent chromophores such as 2-naphthoate.[73,74] While CD measures the differential absorption in transmission, FDCD detects the difference in the fluorescence intensity for left and right circularly polarized excitations. Under standard conditions, when the emission of a sample is directly proportional to the absorbance, the same dichroic information can be obtained from both processes. Since fluorescence directly measures the amount of light emitted against zero background while absorbance is determined from an intensity difference of transmitted light, the fluorescence signal typically can be observed at much lower concentrations than absorbance. This enhanced sensitivity allows the measurement of fluorescence-detected exciton-split CD spectra at submicromolar concentrations. However, the application of FDCD has remained limited for many years due to instrumental problems and only the recent development of FDCD detectors for standard CD instruments has opened new perspectives for this technique. For a detailed description of FDCD and practical applications see the excellent review articles by Nehira[75] and Tanaka et al.[47]

6.3 CONCLUSIONS

The exciton chirality CD method is a powerful microscale technique that allows the determination of the absolute configuration and conformation of organic molecules and has been widely used for various types of natural and synthetic products. The method is based on the "through space" coupling of two or more chromophores in chiral substrates giving rise to bisignate CD curves that allow the determination of the absolute configuration in a straightforward, nonempirical manner. Although the exciton chirality method has been widely used for more than three decades, only during recent years has the development of new chromophores and derivatization techniques for several functional groups, the improvement of the sensitivity, and the application to compounds with a single stereogenic center allowed a broadened applicability of this method. Further developments are expected in the field of fluorescence-detected exciton-coupled CD since this technique has the potential to further increase the sensitivity and the selectivity.

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7 Separation of Enantiomeric Mixtures of Alkaloids and Their Biological Evaluation

Stephen T. Lee, Russell J. Molyneux, and Kip E. Panter

CONTENTS

7.1 INTRODUCTION

7.1.1 Enantiomers

Chiral chemical compounds that are otherwise identical but have nonsuperimposable mirror image structures are known as enantiomers. The terms enantiomer and chiral are derived from the Greek words for opposite and handedness, respectively. Enantiomers have the same physical and chemical properties as each other with two exceptions: they rotate plane-polarized light in opposite directions and they react and interact differently with other chiral compounds, including biological molecules.

In biological systems, chirality is the rule rather than the exception, controlling the utilization of nutrients, influencing the functioning of genes and enzymes, and regulating interactions at receptor sites. For example, the human body can only utilize (−)-amino acids but not the unnatural (+)-enantiomers. Many examples of different toxicological and pharmacological activity of enantiomers have been reported. Thus, humans can metabolize (+)-glucose and not (−)-glucose, and (+)-leucine tastes sweet while (−)-leucine is bitter. Aroma characteristics are highly dependent on chirality; for example, 4*S*-(+)-carvone, which occurs in caraway, dill, and certain citrus fruits, smells of caraway

whereas 4*R*-(−)-carvone, the main constituent of spearmint, has a minty odor. Similar considerations apply to synthetic compounds. The nonsteroidal antiinflammatory drug, ketoprofen, is sold "over the counter" as the (*S*)-enantiomer, dexketoprofen, because the therapeutic activity resides only in this enantiomer.[1] Perhaps the most notorious example of differential physiological activity between isomers is the drug thalidomide, with the (*R*)-enantiomer having sedative properties, whereas the (*S*)-enantiomer is teratogenic. Thalidomide was synthesized as a racemic mixture, but purification to obtain only the (*R*)-enantiomer was not a viable solution as a liver enzyme converts the *R*-enantiomer into *S*-enantiomer.[2] In recent years, there has been increased interest in the administration of drugs as the single enantiomer rather than the racemic mixture. In the year 2000, chiral drugs represented one third of all drug sales worldwide,[3] and in 2005 nine of the top ten drugs had chiral active ingredients.[4]

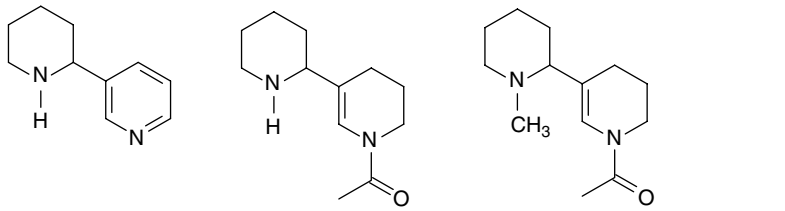
The fundamental rationale for natural products chemistry involves isolating and characterizing bioactive chemical constituents produced by plants and other organisms. Often, compounds with one or more chiral centers are extracted, isolated, and characterized by modern instrumental techniques such as MS, IR, chromatography, NMR, etc., but it has become quite commonplace to neglect to determine the optical rotation, even though the structural elucidation indicates the presence of chiral centers. Even when optical rotation is measured for a new natural product, there are no assurances that the values obtained are consistent with a single enantiomer. Furthermore, the existence of an enantiomeric mixture will generally not be revealed by spectroscopic or spectrometric techniques because each enantiomer will have an identical spectrum. The only means to establish the absolute optical rotation values for a given enantiomeric pair is by stereospecific synthesis, by chromatographic separation using chiral columns, or occasionally by preferential crystallization of one of the enantiomers. For these reasons, it is often the case that no effort is made to determine if a natural product is in reality a single enantiomer, a racemic mixture, or a mixture with unequal amounts of the two enantiomers. Because of the differential toxicity and pharmacological activity of enantiomers, it is essential to describe the stereochemical structure of any chiral centers and accurately determine the optical rotation and purity with respect to each enantiomer prior to their biological evaluation.

Plant secondary metabolites are often pure enantiomers. The tobacco plant *Nicotiana tabacum* produces solely (−)-nicotine[5] and the poppy plant *Papaver somniferum* only biosynthesizes the pain-relieving (−)-(5*R*,6*S*,9*R*,13*S*,14*R*)-morphine.[6] Similarly, the antimalarial drugs febrifugine and isofebrifugine found in *Dichroa febrifuga* have much higher activity than their synthetic enantiomers.[7] However, there are several examples of plants producing enantiomeric mixtures of compounds. Both (+)- and (−)-enantiomers of alpha-pinene, a major monoterpene of the pine tree, occur in ponderosa pine resin, but the (−)-enantiomer predominates in California.[8] Similarly, germacrene D is synthesized as one or both enantiomers in various plants, fungi, and animals; however, the (−) configuration is the most common enantiomer in higher plants.[9] The monocyclic monoterpene (+)- and (−)-limonene enantiomers have been shown to be present in orange peel and other plants and are used as fragrances in household products and components of artificial essential oils.[10] The (+)-limonene isomeric form is more abundantly present in these plants than the racemic mixture and (−)-isomeric form.[11] Recently, enantiomeric ratios of (+)- and (−)-gossypol have been measured in the leaves, stems, and roots of selected accessions of the cotton (*Gossypium hirsutum*) variety Marie Galante. The (−)-gossypol content was much lower (<8%) than in other cotton varieties and cultivars in which the (+):(−) ratio is ca. 3:2.[12] This is a significant finding because (−)-gossypol is much more toxic to chickens and other nonruminants than the (+)-enantiomer,[13] limiting the use of cottonseed as a feed.

The following examples illustrate the way in which alkaloids can vary in enantiomeric composition in plant collections from different locations and at different times, and outline a methodology to separate the individual enantiomers so that their respective biological activities can be evaluated. Other classes of compounds will require approaches tailored to their specific functional groups and chromatographic properties but the same general principles apply.

7.1.2 Anabasine and Ammodendrine Enantiomers

Ingestion of *Nicotiana* and *Lupinus* species by pregnant livestock at specific gestational periods can result in newborns with multiple congenital contractures (MCC) and cleft palates.[14–20] Anabasine (**1**), a piperidine alkaloid, has been shown to be the predominant alkaloid in *N. glauca*. Anabasine is present in other *Nicotiana* spp. and has been identified as the responsible teratogen (Figure 7.1).[19] Ammodendrine (**2**), another piperidine alkaloid, found in several *Lupinus* spp., is also a reported teratogen (Figure 7.1).[21,22] Anabasine (**1**) and ammodendrine (**2**) both have chiral centers. Anabasine (**1**) is present in *N. glauca* as a racemic mixture[19,23] and ammodendrine (**2**) has been reported as a racemic mixture in several genera of the Leguminosae.[24] The variability of reported optical rotation values for ammodendrine (**2**) isolated from *Lupinus* species indicated that lupine plants may be producing enantiomeric mixtures of ammodendrine (Table 7.1).[25–33]



Anabasine (**1**) Ammodendrine (**2**) *N*-Methylammodendrine (**3**)

FIGURE 7.1 The chemical structures of anabasine (**1**), ammodendrine (**2**), and *N*-methylammodendrine (**3**).

TABLE 7.1
Optical Rotation and Toxicity Data for Anabasine (1), Ammodendrine (2), and *N*-Methylammodendrine (3)

Compound	Observed or Reported Optical Rotations	LD_{50} ± CI[a]	Source
D-Anabasine[b] (72–78% pure)	$[\alpha]^{20}_D$ +35.5° (*c* 1.99, $CHCl_3$)	10.9 ± 0.80	*Nicotiana glauca*
L-Anabasine[b] (80–89% pure)	$[\alpha]^{20}_D$ −48.1° (*c* 1.25, $CHCl_3$)	16.2 ± 1.0	*N. glauca*
L-Anabasine[25]	$[\alpha]_D$ −84° (*c* 0.043, 95% EtOH)		NA
L-Anabasine[26]	$[\alpha]^{24}_D$ −79.2° (*c* 0.5, MeOH)		Synthetic
L-Anabasine[27]	$[\alpha]^{20}_D$ −80° (*c* 0.91, MeOH)		Synthetic
D-Ammodendrine[b]	$[\alpha]^{24}_D$ +5.4° (*c* 1.27, MeOH)	94.1 ± 6.8	*Lupinus formosus*
L-Ammodendrine[b]	$[\alpha]^{24}_D$ −5.7° (*c* 1.66, MeOH)	115 ± 7.0	*L. formosus*
Ammodendrine[28]	$[\alpha]^{24}_D$ +6.65° (*c* 3.9, EtOH)		*L. formosus*
Ammodendrine[29]	$[\alpha]^{24}_D$ +7.5° (*c* 0.22, EtOH)		*L. varius*
Ammodendrine[30]	$[\alpha]^{24}_D$ +7.1° (*c* 0.08, MeOH)		*L. varius*
Ammodendrine[31]	$[\alpha]^{23}_D$ −20° (*c* 0.004, $CDCl_3$)		*Castilleja miniata*
Ammodendrine[32]	$[\alpha]^{24}_D$ +15° (EtOH)		
Ammodendrine[33]	$[\alpha]^{24}_D$ +11.8° (*c* 0.288, EtOH)		*L. hirsutus*
D-*N*-Methylammodendrine[b]	$[\alpha]^{23}_D$ +62.4° (*c* 0.51, MeOH)	56.3	*L. formosus*
L-*N*-Methylammodendrine[b]	$[\alpha]^{23}_D$ −59.0° (*c* 1.17, MeOH)	63.4 ± 4.7	*L. formosus*
N-Methylammodendrine[28]	$[\alpha]^{24}_D$ +40.5° (*c* 2.0, EtOH)		*L. formosus*
N-Methylammodendrine[31]	$[\alpha]^{23}_D$ −44° (*c* 0.02, $CDCl_3$)		*C. miniata*

[a] CI (95% confidence intervals).
[b] This study.

7.1.3 Optical Rotation Measurements

The problem of occurrence of enantiomeric mixtures in plant samples is compounded by the difficulties of obtaining comparable optical rotation values. Polarimeters have become much less common as standard equipment in laboratories than in the past, so that many chemists have little practical experience of their use. For this reason the factors limiting accuracy of such instruments are reviewed here in relation to the overall topic of this chapter, namely, the separation of enantiomers.

The rotatory power of a solution of any particular compound is dependent on wavelength of the light used, the length of the sample tube, concentration of the solution, and measurement temperature and solvent. Some of these factors, particularly temperature, are likely to vary both intra and interlaboratory and there may be insufficient sample available to duplicate concentrations used in earlier reports. Thus, in the absence of an authentic sample from a previous determination, it is unlikely that a subsequent rotation determination will yield an identical value.

The specific rotation (α; positive or negative) of a compound is derived from the following equation:

$$\alpha = \frac{\text{observed rotation}}{lc}$$

where l is the length of the sample tube in decimeters (dm) and c the concentration of the sample in grams per milliliter (g/mL).

Typically, for most laboratory polarimeters, $l = 1.0$ dm (i.e., 10 cm) and the i.d. of the cell is 4 mm, so that the volume of solution required to fill the cell is ca. 1.25 mL. It is obvious that significant quantities of pure compound are necessary to give a measurable value for the observed rotation. When the specific rotation is small, as for D- and L-ammodendrine (ca. 5.5°), the potential for large errors in the observed value is great, introduced by weighing errors or presence of impurities that may or may not have rotatory power themselves. It is therefore imperative that adequate criteria of purity be applied to the compound being measured. For comparison with literature values, it is essential that the measurement temperature and solvent be the same as those reported.

Optical rotations are invariably reported for λ 546 nm, the sodium D line, and the instrument may also be capable of recording rotations for the mercury lines at λ 578, 546, 436, and 365 nm; if the solution is significantly colored, the shorter wavelength rotations may not be recorded with an acceptable degree of accuracy. However, specific rotations for all measurable rotations should be reported to give several points of comparison. If ORD/CD data can be obtained, this is much more desirable as it provides data over a range of wavelengths rather than at specific points and can be generated with very low sample concentrations. Polarimetry data should be reported in the form shown in Table 7.1.

7.2 ISOLATION OF ANABASINE AND AMMODENDRINE ENANTIOMERS

7.2.1 Synthesis and Separation of Anabasine and Ammodendrine-Based Diastereomers

Anabasine (0.55% yield) and ammodendrine (0.47% yield) were extracted and isolated from *N. glauca* and *Lupinus formosus*, respectively.[19,34] *N*-Methylammodendrine (**3**) has also been found in *L. formosus* material.[21,22,35,36]

Anabasine enantiomers were converted into diastereomers by a peptide-coupling reaction utilizing 9-fluorenylmethoxycarbonyl-L-alanine (Fmoc-L-Ala-OH) in the presence of *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC) and hydroxybenzotriazole (HOBT) dissolved in *N,N*-dimethylformamide (DMF) (Figure 7.2).[23] The formation of Fmoc-L-Ala-anabasine (**4**, MH^+ 456) was monitored by electrospray ionization mass spectrometry (ESIMS). The conversion of **1** to **4** allowed reverse-phase HPLC analysis of the diastereomers and confirmed that the anabasine enantiomers were present in *N. glauca* plant material (Figure 7.3).

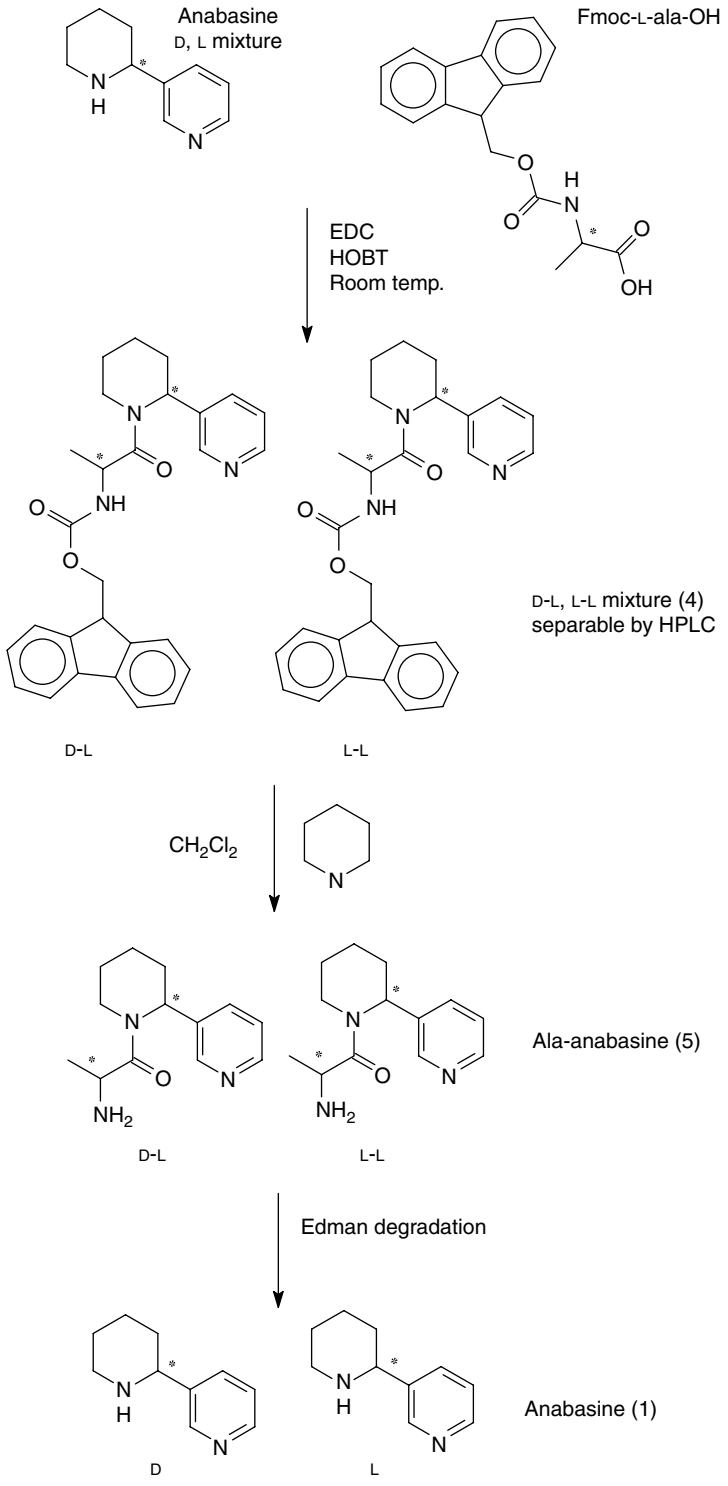


FIGURE 7.2 Conversion of anabasine (**1**) into diastereomers for separation and conversion of diastereomers back to enantiomerically rich isomers. The "*" denotes a chiral center.

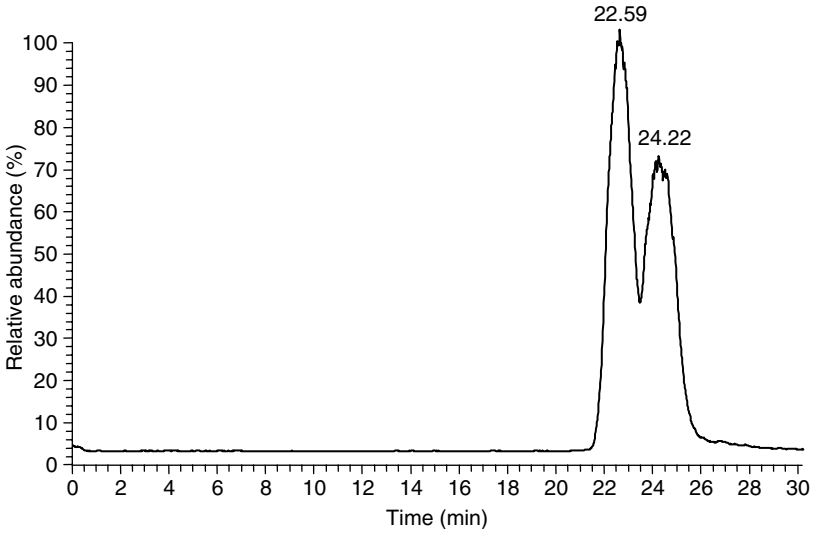


FIGURE 7.3 Reconstructed HPLC ion chromatogram (*m/z* 456.3) for Fmoc-L-Ala-anabasine-based diastereomers from *Nicotiana glauca* obtained by reverse-phase HPLC using a 100 mm × 2 mm i.d., 5 µm Betasil C-18 column with a mobile phase of 20 mM ammonium acetate/methanol (45:55, v/v) at a flow rate of 0.5 mL/min. The detector was a Finnigan LCQ mass spectrometer operating in ESI mode.

Treatment of the Fmoc-L-Ala-anabasine diastereomers (**4**) with piperidine in methylene chloride was monitored using ESIMS for the formation of L-Ala-anabasine (**5**, MH^+ 163). The L-Ala-anabasine diastereomers were separated and isolated in milligram quantities using a semi-preparative scale 250 mm × 10 mm i.d., 5 µm, Betasil C-18 HPLC column.[23]

Fmoc-L-Ala-ammodendrine diastereomers (MH^+ 502) were synthesized and converted to L-Ala-ammodendrine diastereomers (MH^+ 280) and subsequently isolated using the same methods described for anabasine.[34]

7.2.2 Conversion of Diastereomers to Enantiomers

Conversion of the isolated L-alanine-based diastereomers of anabasine and ammodendrine to their respective enantiomeric forms of anabasine (**1**) and ammodendrine (**2**) was accomplished by an Edman degradation procedure to remove the L-alanine.[23]

N-Methylammodendrine (**3**) was present in previous collections of *L. formosus* and has been found in other *Lupinus* species.[21,22,35–37] The correct optical rotation values for the enantiomers of *N*-methylammodendrine (**3**) also needed to be determined. Thus, the *N*-methyl derivatives were obtained by treating D- and L-ammodendrine (**2**) with iodomethane and then separated from their respective reaction mixtures using silica gel column chromatography.[34]

7.2.3 Enantiomeric Purity

Once synthesized and isolated from their respective reaction mixtures, the chiral purity of each enantiomers should be determined prior to any further testing.

7.2.3.1 HPLC Measurements

A sample of the isolated enantiomers was individually converted back to its Fmoc-L-alanine derivatives and reexamined using reverse-phase HPLC. Thus, the putative L-anabasine enantiomer (L-**1**)

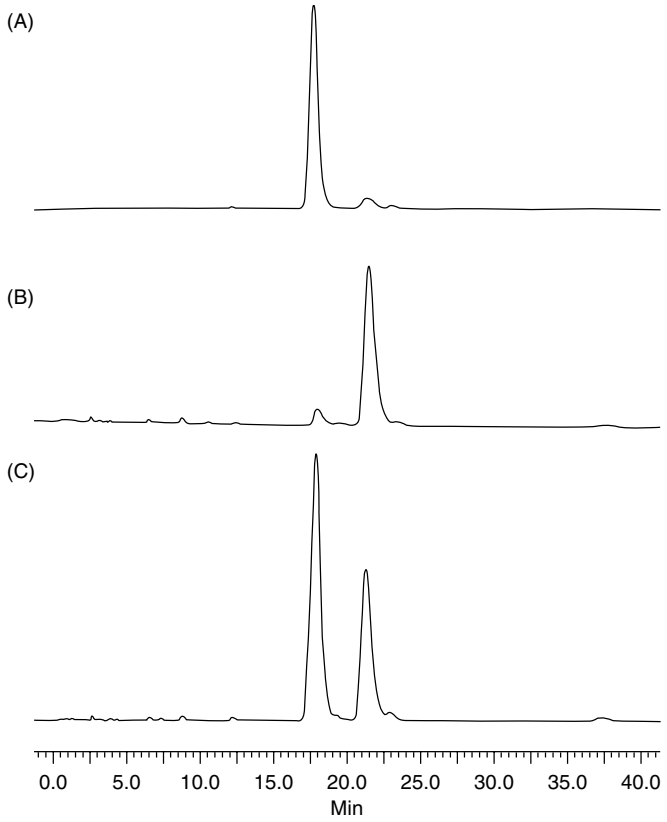


FIGURE 7.4 Chiral HPLC analysis of the two anabasine enantiomer samples obtained by cleavage of the Fmoc-Ala-coupled diastereomers separated using RP-HPLC. (A) The *S*-enriched anabasine fraction, (B) the *R*-enriched anabasine fraction, and (C) equal volumes of the two fractions were coinjected. Commercially available *S*-anabasine was also shown to elute with a retention time corresponding to the *S*-enriched fraction major peak. The chromatography was performed using a 250 mm × 4.6 mm i.d., Daicel chiral OJ-H column with an *n*-hexane/ethanol/diethylamine/trifluoroacetic acid (97:3:0.1:0.1, v/v/v/v) mobile phase at a flow rate of 1 mL/min. The compounds were detected with a UV/Vis diode array detector at 260 nm.

was measured to be 89% pure and the D-anabasine enantiomer (D-**1**) to be 78% pure with respect to contamination from the complementary enantiomer. In addition, the chiral purity of the D- and L-anabasine enantiomers *per se* was also estimated using a chiral HPLC column (Figure 7.4). Using this method, the enantiomeric purities of the L- and D-anabasine preparations were measured to be 93% and 92%, respectively.[23]

In the same way, reverse-phase HPLC of the isolated ammodendrine enantiomers after conversion back to the Fmoc-L-Ala-ammodendrine derivatives established the D-ammodendrine enantiomer to be 91% and L-ammodendrine to be 98% enantiomerically pure.[34]

7.2.3.2 Optical Rotation Measurements

The optical rotation of L-anabasine has been variously measured as $[\alpha]_D$ −84°, −79.2°, and −80°.[25–27] The optical rotations of the L-anabasine and D-anabasine samples prepared in this study were measured as $[\alpha]^{20}_D$ −48.1° and $[\alpha]^{20}_D$ +35.5°, respectively. Using the optical rotation measurements from the fractions prepared by separation of their Fmoc-L-Ala diastereomers and assuming the optical

rotation of L-anabasine to be $[\alpha]_D$ −80°, the enantiomeric purity of the prepared enantiomers can be calculated according to the following equation:

$$\text{percentage enantiomeric purity} = 100 - \left|\left[\frac{OR^{100} - OR^{x}}{OR^{r}}\right] \times 100\right|$$

where OR^{100} is the optical rotation at 100% enantiomeric purity, OR^{x} the optical rotation measured experimentally, and OR^{r} the range in optical rotation between the pure enantiomers.

Thus, the L-anabasine sample can be calculated to be 80% enantiomerically pure while the enantiomeric purity of the D-anabasine enantiomer can be calculated as 72% with respect to contamination from the complementary isomer.[23]

The observed optical rotations of D-ammodendrine ($[\alpha]^{24}_D + 5.4°$) and L-ammodendrine ($[\alpha]^{24}_D$ −5.7°), estimated to be 91% and 98% enantiomerically pure, respectively, are consistent with expected optical rotations of isolated enantiomers, that is, equal but opposite in sign.[34] However, these optical rotations differ from previously reported values (Table 7.1) where no measures were taken to ensure enantiomeric purity of the isolates. The greater magnitude of the previously reported optical rotations than those determined for the separated pure enantiomers indicates that prior optical rotations for ammodendrine (**1**) must be incorrect and that the larger rotations are possibly due to contamination of the samples by other alkaloids with higher rotation values.

The optical rotations of D-*N*-methylammodendrine and L-*N*-methylammodendrine were $[\alpha]^{23}_D$ +62.4° and $[\alpha]^{23}_D$ −59.0°, respectively. As in the case of the ammodendrine enantiomers, these measurements are consistent for pairs of enantiomers and they are also substantially different from the measurements previously reported (Table 7.1).[34]

7.3 BIOACTIVITY

7.3.1 Mouse Toxicity

The toxicity of the anabasine (**1**), ammodendrine (**2**), and *N*-methylammodendrine (**3**) enantiomers was evaluated using an established mouse lethality (LD_{50}) bioassay.[34] Briefly, weanling Swiss Webster mice (15–20 g body weight) were fasted overnight, weighed, and injected intravenously with a sterile solution of alkaloid in buffered saline using a tuberculin syringe with a 27-gauge needle. Clinical signs were immediate and included depression, muscular weakness, tremors, and death. Death occurred rapidly (within 5 min after injection) or there was a complete recovery within 1 h.

Thus, the murine LD_{50} values of the L- and D-anabasine samples were determined to be 16.2 ± 1.0 mg/kg, $n = 26$, and 10.9 ± 0.8 mg/kg, $n = 26$, respectively.[23] The LD_{50} of L-ammodendrine in mice was determined to be 115.0 ± 7.0 mg/kg, $n = 20$, and that of D-ammodendrine was 94.1 ± 7.8 mg/kg, $n = 23$. Similarly, the murine LD_{50} values of L- and D-*N*-methylammodendrine were determined to be 63.4 ± 4.7 mg/kg, $n = 15$, and 56.3 mg/kg (no CI), $n = 7$, respectively (Table 7.1).[34]

7.3.2 Human Fetal Nicotinic Acetylcholine Receptor

Enantiomeric mixtures of anabasine are teratogenic and cause skeletal defects and cleft palates in livestock.[19] It is not known whether the D- or the L-enantiomer of anabasine is more potent in its ability to activate the fetal nicotinic acetylcholine receptors (nAChR), thereby resulting in teratogenicity. The D-enantiomer of anabasine was more toxic in a mouse bioassay compared to the L form, but teratogenic comparisons have not been determined.

Therefore, to investigate the neuromuscular nAChR agonist properties of these enantiomers, a human tumor cell line (TE671), known for expressing the fetal human muscle type nAChR, was used. Cell membrane depolarization responses to sudden application of a nicotinic agonist toxin (D- or L-anabasine) were measured by changes in fluorescence of a membrane

TABLE 7.2
Nicotinic Agonist Actions of Anabasine Enantiomers on the Fetal Human nAChR

Compound	D-Anabasine	L-Anabasine
EC_{50} (μmol)	2.6 ± 0.56	7.1 ± 1.3
Max. (%)	97 ± 6.9	101 ± 9.1

potential-sensitive dye.[38] The rank order of potency (EC_{50}) for activation of the fetal skeletal muscle nicotinic receptor is D-anabasine > L-anabasine, implying the more potent teratogenic activity of the D-enantiomer (Table 7.2).[23]

7.4 CONCLUSIONS

There is a statistical difference ($p < .05$) in the measured murine lethality (LD_{50}) between the D- and L-anabasine fractions.[23] This difference in lethality as well as the rank order comparison of nicotinic agonist potency in the D and L forms would suggest that there might be a difference in teratogenic activity between the D and L forms. There is also statistical difference in the murine toxicity between the D- and L-ammodendrine.[34] The differences between these pairs of enantiomers are not as large as is often the case with enantiomers where one form is primarily responsible for the observed physiological activity.[7,39] It is possible that the receptor site responsible for toxicity is not chirally highly discriminatory. It is also possible that these compounds are not the proximate toxins and that they are metabolized into compounds in which the asymmetric center is lost. This could occur either by isomerization of the existing double bond to a position between the two piperidine rings or by an oxidative process in the liver, resulting in the introduction of a double bond in the piperidine ring. An analogy for the latter process exists with the pyrrolizidine alkaloids, which are not toxic *per se* but are converted to toxic "pyrrolic" metabolites by P450 liver enzymes.[40]

It is recognized that acute mouse toxicity is not a surrogate for teratogenicity. However, the structure–activity relationship for toxicity and teratogenicity of related piperidine alkaloids,[41] and the significant differences in toxicity and differences in activity of the human fetal neuromuscular nAChR by D-anabasine and L-anabasine would suggest that there may be significant differences in the teratogenic potentials of these compounds. If the teratogenic effects of these alkaloids are due to their ability to cause sustained contracture of the skeletal muscles of the neck and back of the embryo, one would predict, assuming similar pharmacokinetic properties, that the rank order of teratogenic potency would be D-anabasine > L-anabasine and D-ammodendrine > L-ammodendrine. Limitations in the amount of isolated enantiomeric material available only permitted initial possible differences in toxicity based on a mouse model to be investigated. However, a true measure of teratogenicity will need to be tested in a proper system such as a fetal goat model.[20]

7.5 IMPLICATIONS

The above examples were investigated because of the observed differences in optical rotation data for the alkaloids reported in the literature. The obvious conclusion had to be either that the isolated alkaloids were contaminated with other compounds with significant optical rotation values, or that the alkaloids occurred as enantiomeric mixtures, differing in proportion with geographic location, growth stage, or season of collection. The procedures illustrated here, that is, synthesis of diastereoisomers separable by conventional HPLC and conversion back to the individual enantiomers, or direct separation of the enantiomers by chiral HPLC, and subsequent biological comparison of each enantiomeric pair, should be applied whenever the enantiomeric purity is in question. It should be

noted that whenever a new natural product is isolated, it is just as important to take measures to establish the optical purity of the compound as to establish its compositional purity. In the absence of such proof, the biological activity data will remain compromised.

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8 Dereplication and Discovery of Natural Products by UV Spectroscopy

Thomas O. Larsen and Michael A.E. Hansen

CONTENTS

8.1 INTRODUCTION

8.1.1 The New Chemoinformatic Era of Natural Product Chemistry

A major challenge in the discovery of natural products is to implement a good strategy for rapid detection of previously isolated compounds. This dereplication process is to ensure that isolation, structure elucidation, and pharmacological investigations can be focused on novel compounds.[1] In this scenario, chromatography combined with high-resolution mass spectrometry,[2] diode array detection (DAD) of UV/Vis absorbance, or nuclear magnetic resonance (NMR) spectroscopy[3–5]

plays an important role. The data generated from these techniques are then often combined with searching of databases such as Chemical Abstracts Service using SciFinder,[6] Antibase,[7] or MarinLit.[8]

For most of the last century, dereplication was something that was limited to "inhouse" knowledge about the chemistry and spectroscopic properties of individual compounds usually from a limited group of organisms or plants that were of particular interest for an academic group or a pharmaceutical company. This scenario has, however, changed quite dramatically within approximately the last ten years during which natural product research has been highly inspired and influenced by the great advances in bioinformatics and the new discipline of chemoinformatics. Common to these two disciplines is an informatics-based approach where advanced computer-based search tools are used to extract all relevant information from globally accessible databases such as the large genomic databases. Compared to bioinformatics, chemoinformatics is in its infancy, but it has entered a new era bringing new life and hopes for the future of natural products chemistry. A new chemoinformatic-based approach, among others dealing with construction of easily accessible databases containing the enormous amounts of chromatographic and spectroscopic data that can be generated using modern instrumental equipment, will therefore hopefully be the basis for both enhanced academic and industrial research efforts aimed at the discovery of the many new natural products that are still to be discovered.[1] It has recently been estimated that only 5–7% of all existing fungi have so far been cultured by researchers with the implication that there is obviously still much new chemistry to discover.[9,10]

8.1.2 The Chemotaxonomic Screening Approach

Traditionally, spectroscopic analysis has been performed at the single-compound level; however, more recently the application of dereplication has started also at the crude-extract level, comparing all the extractable chemistry from an organism simultaneously to information in databases.[1] Two excellent techniques for the direct "fingerprinting" of complex mixtures of biofluids or raw extracts are electrospray ionization mass spectrometry (ESI MS),[11–13] and NMR spectroscopy.[14] Complementary to this, a modern dereplication strategy needs to involve dereplication at several levels such as in the chemotaxonomic-based screening approach (Figure 8.1).[1]

Thus, for studies of filamentous fungi, a natural initial step is to investigate the macro- and micromorphology of a given set of fungi. This allows an initial selection of a number of representative strains either by experts or by automated image analysis methods.[15,16] Thereafter, we start to look into the "global chemistry" of the fungi (step 2, Figure 8.1). Extracts are made by microextraction of only a few agar plugs from one fungal culture,[17] and the metabolites in the extract are analyzed simultaneously (step 3, Figure 8.1) by direct injection mass spectrometry (DIMS)[18] or by a simple thin layer chromatography (TLC) setup.[19] The mild electrospray ionization conditions allow the detection of numerous protonated (or sodiated), but otherwise usually nonfragmented, metabolites present in the extract in what we call a mass profile. In most cases, the production of secondary metabolites is very consistent from isolate to isolate in a species.[20] This means that clustering of such mass profiles by chemometric methods usually leads to very distinct groups of extracts representing species or chemotypes of the fungi investigated (step 4, Figure 8.1). The relatively few isolates/strains per species that are selected from looking at the global chemistry are subsequently grown on a larger number of media to generate conditions that will allow the expression of as broad a range of secondary metabolites as possible, according to the "one strain many compounds" (OSMAC) philosophy.[21]

The subsequent steps of the screening approach are aimed at generation of single natural product compound libraries to be tested for bioactivity in relevant bioassays. Extracts from the optimal media conditions are separated into microtiter plates (step 5, Figure 8.1) using an LC-DAD-MS system coupled with a microfractionation system. This setup (step 6, Figure 8.1) allows linking of

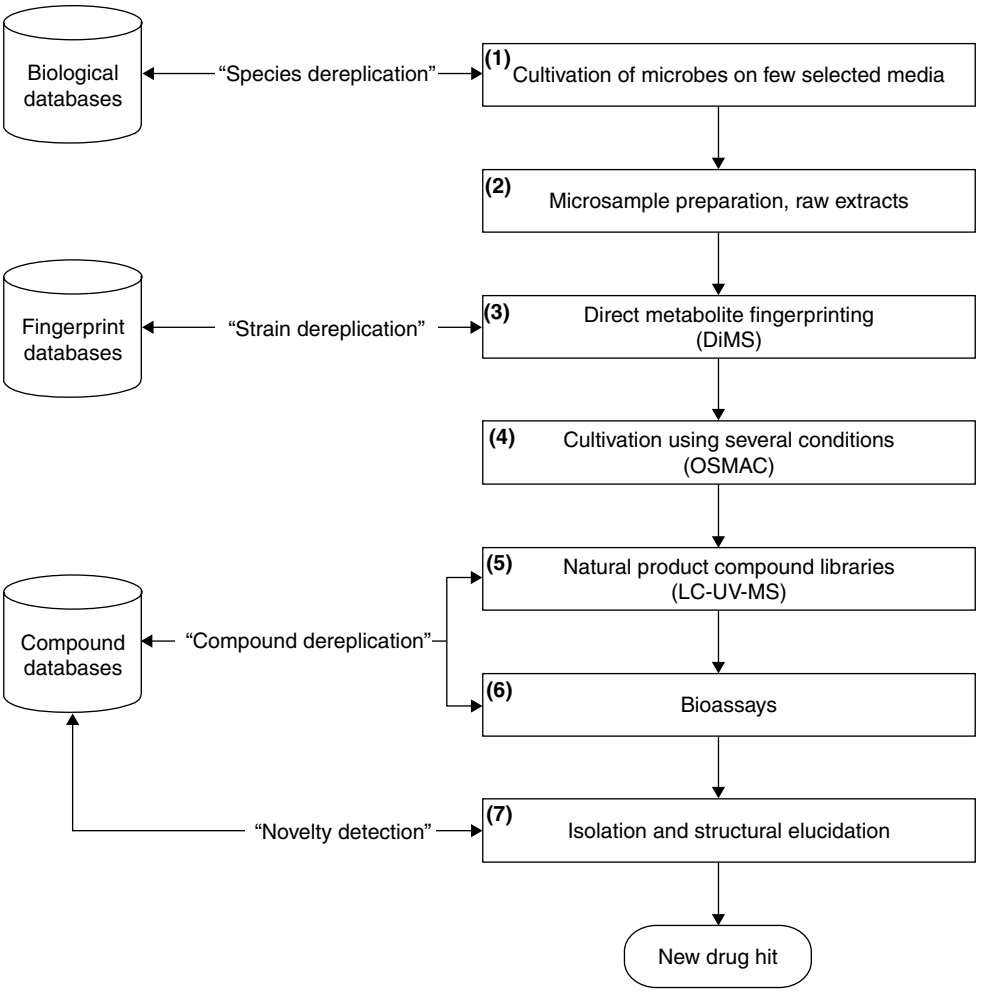

FIGURE 8.1 The chemotaxonomy-based screening approach. See text for detailed description of the individual steps. (Reproduced from Larsen, T.O., Smedsgaard, J., Nielsen, K.F., Hansen, M.E., and Frisvad, J.C., *Nat. Prod. Rep.*, 22, 672, 2005. With permission of The Royal Society of Chemistry.)

biological activity of a compound in a particular microtiter well directly to UV and MS data. This leads to direct and fast dereplication as recently demonstrated by the finding of penicillic acid and patulin as quorum-sensing inhibitors.[22] The UV/Vis and MS data are complementary whereby a characteristic UV/Vis spectrum of a given compound can often link the compound to a specific class of compounds (Section 8.4), and the high-resolution mass spectrometry data usually give only few possible combinations of the molecular formula. This therefore facilitates the task of database searching (Section 8.5) in the dereplication process.

Despite the importance of MS and NMR in the dereplication process, the purpose of the rest of this chapter is to illustrate the possibilities and scope of UV spectroscopy in combination with chemoinformatics as part of modern analytical natural product chemistry. One aim of this chapter, therefore, is to illustrate that even though the nature of a typical UV spectrum is less individually defining when compared to an accurate mass spectrum, more structural information can often be extracted from an UV spectrum than from a mass spectrum.

8.2 POTENTIAL AND LIMITATIONS OF UV SPECTROSCOPY

With the recent development in modern instrumental equipment, most natural products laboratories now have access to benchtop LC-DAD-MS equipment as well as to "state-of-the-art" NMR facilities. In particular, the advancement of high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) combined with fast UV diode array detectors and much better columns for high-resolution separation has made it possible to acquire the UV spectrum of practically every single component from a natural product extract. As a consequence, the UV spectrum is one of the most readily accessible pieces of information related to the structure of natural products and there is increased interest in exploiting its usefulness.[23]

In practice, no organic chemist would rely on UV absorption spectroscopy as a primary tool for structural elucidation, even though some rules exist for calculation of simple chromophoric systems.[24] A major disadvantage of UV is the fact that the absorption frequencies of C−C, C−H, and isolated C=C groups cannot be observed in the easily accessible region of the UV spectrum. This means that highly saturated natural products will all have noninformative and very mundane "end-absorption-type" UV spectra. Examples of such compounds are some terpenoids and cyclic peptides, the latter biosynthesized from only aliphatic amino acids. The limitation in scope of UV spectroscopy therefore lies more in the nature of the compounds to be studied than in the actual instrumental technique.

Fortunately, the structures of many other types of natural products contain several conjugated double bonds making these compounds absorb both ultraviolet light (200–400 nm) as well as visible light (400–800 nm). This is mainly due to the mobile π-electrons, which are part of such polyunsaturated functionalities, for example, aromatic ring systems, one or more carbonyl groups, carboxylic acid, and ester or amide functional groups. Each separated system of conjugated double bonds constitutes a chromophore in the molecule.

As illustrated in Section 8.4, many of the known types of fungal bioactive metabolite have distinct UV spectra. This was efficiently demonstrated for the first time about 20 years ago when HPLC-DAD was used to detect the production of important mycotoxins and other secondary metabolites as part of chemotaxonomic and food safety studies.[25] Nielsen and Smedsgaard[2] extended this fungal metabolite database to 474 metabolites and also included MS data. Similarly, other research groups have developed "inhouse" HPLC-DAD databases for dereplication purposes.[26] For example, Fiedler et al.[27] used their database containing ca. 750 UV spectra to investigate a set of 600 marine actinomycete strains for the production of known antibiotics and also for the discovery of possible new metabolites. Larsen et al.[28–30] used a knowledge-based, UV-guided approach for detection and isolation of novel alkaloids from fungal extracts. To systematize and keep track of data and structural knowledge related to UV spectra of both known and possible new fungal metabolites, we recently developed a new algorithm called "X-hitting" to be used in combination with a learning-based database.[31,32] In "X-hitting", known metabolites are tracked by the feature "cross-hitting", whereas potential new metabolites are indicated by the "new-hitting" feature of "X-hitting". Section 8.5.3 describes the nature and potential of the "X-hitting" algorithm.

8.3 A FEW IMPORTANT FUNGI AND THEIR METABOLITES

Filamentous fungi such as *Aspergillus, Fusarium*, and *Penicillium* produce some of the most well-known and economically important microbial natural products (Figure 8.2). This includes famous pharmaceutically related compounds such as the antibacterial penicillin (**1**) produced by *Penicillium chrysogenum*, the cholesterol-lowering mevinolin (**2**) produced by various *Aspergillus* species, mycophenolic acid (**3**) produced by, among others, *Penicillium brevicompactum*, and the antifungal and anticancer compound griseofulvin (**4**).[33]

Of similar importance to mankind are mycotoxigenic metabolites that can often be found in our food and feedstuffs.[34] Some fungi such as *Fusarium, Alternaria*, and *Stachybotrys* grow mainly on plants and crops in the fields, whereas other fungi such as penicillia are considered generally to be

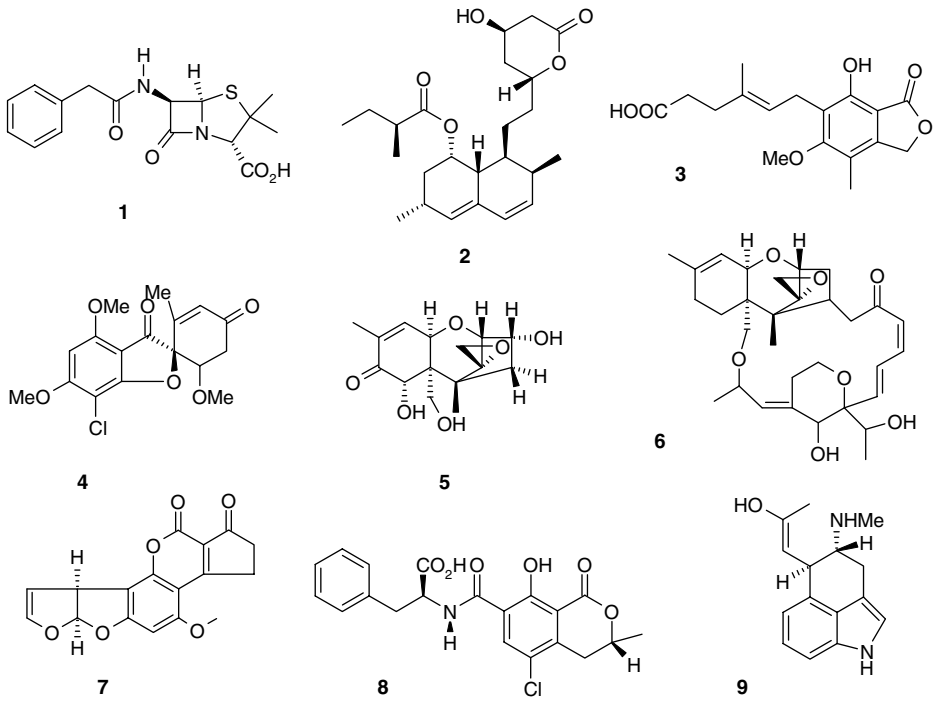

FIGURE 8.2 Some important fungal metabolites.

"storage molds" since they mainly grow on products such as stored cereals, bread, cheese, and meat-based products. Finally, species of other genera such as *Aspergillus* seem to be ubiquitous, including *A. fumigatus*, which is a very successful saprophyte with a worldwide distribution. *A. fumigatus* is able to attack and damage a wide range of stored fruits, crops, nuts, and hay. In tropical areas, it often causes deterioration of cotton and woollen fabrics and the species is extremely important as an infective organism causing systemic mycoses in humans.[33]

As a consequence, regulatory authorities around the world are analyzing, on a daily basis, a large number of foods and feed products for the presence of extremely toxic compounds. Important "field toxins" are trichothecene toxins (sesquiterpenes) such as deoxynivalenol (**5**, Figure 8.2) produced by a number of *Fusarium* species and the macrocyclic satratoxin (**6**) produced by *Stachybotrys chartarum*. Trichothecenes as a group show a wide range of biological activities ranging from antimicrobial and phytotoxic properties, as well as acute toxic and immunosuppressive effects in man. Other important mycotoxins are the extremely carcinogenic aflatoxins, such as aflatoxin B_1 (**7**, Figure 8.2), that are produced on maize and nuts in tropical regions by *Aspergillus flavus* in particular.[34] Aflatoxins are polyketides like the nephrotoxic ochratoxin A (**8**), produced by *Penicillium verrucosum* on stored grain and by *Penicillium nordicum* on cheese and meat products.[35] Other important toxins are ergot alkaloids, such as chanoclavine I (**9**), produced by the fungus *Claviceps purpurea* and which were a frequent source of misery and death during the Middle Ages through contamination of rye bread.[36]

8.4 CHARACTERISTIC UV SPECTRA OF FUNGAL POLYKETIDES, ALKALOIDS, AND TERPENOIDS

In general, secondary metabolites are biosynthesized by using many of the small metabolic products or intermediates of primary metabolism. The major secondary metabolite pathways lead to biosynthesis of polyketides, terpenoids, shikimic acid-derived metabolites, and alkaloids. In the

following section, the nature of some of these types of compounds is discussed along with illustrations of their UV spectra.

8.4.1 Alkaloids

Alkaloids and small nonribosomal peptides are primarily biosynthesized from amino acids. They can be divided into a large number of subclasses that are often named after their common molecular precursors, for example, indole alkaloids, piperidine alkaloids, or the type of ring system that is generated, for example, diketopiperazines, benzodiazepines, and quinazolines. Since many alkaloids incorporate into their structure one or more aromatic amino acids, such as tryptophan, phenylalanine, tyrosine, and histidine, or derived compounds such as anthranilic acid, they are "born with" a chromophore thereby generally making many alkaloids excellent targets for dereplication and "novelty hitting" by UV approaches. The large structural diversity seen between the different types of alkaloids is likewise reflected in the variation in the shape of the UV spectra that can be observed (Figure 8.3), as also illustrated by Singh et al.[37]

On the other hand, the spectra of compounds within the same subclass of compounds are often very similar. This means that several of the compounds in a fungal or plant extract will often have

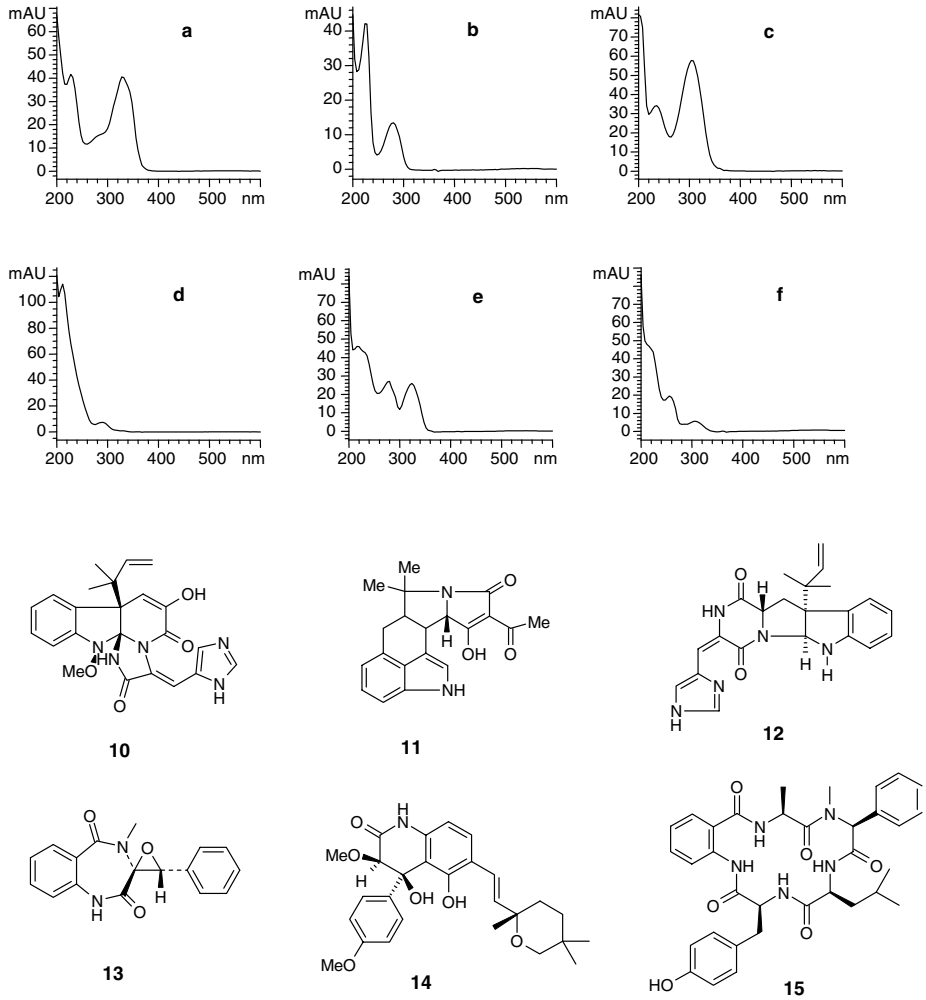

FIGURE 8.3 UV spectra and structures of selected fungal alkaloids: (a) meleagrin (**10**), (b) cyclopiazonic acid (**11**), (c) roquefortine C (**12**), (d) cyclopenin (**13**), (e) peniquinolone A (**14**), and (f) cycloaspeptide A (**15**).

almost identical UV spectra since they are either intermediates or end products of the same biosynthetic family/pathway. Likewise, compounds of the same class are often produced by different organisms and can therefore be observed in different natural product extracts.[20] This means that one can search for a certain class of interesting compounds within a library of files containing raw UV data. One example of compounds that are broadly distributed within *Penicillium* and *Aspergillus* are the quinazoline-like compounds such as anacine (**16**), verrucine A (**17**), alantrypinone (**18**), and fumiquinazoline C (**19**) (Figure 8.4). Owing to the incorporation of anthranilic acid into their structures, they all have very distinctive UV spectra with characteristic and similar fine structures, a feature that they share with benzodiazepines such as sclerotigenin (**20**) and auranthine (**21**) (Figure 8.4).[1]

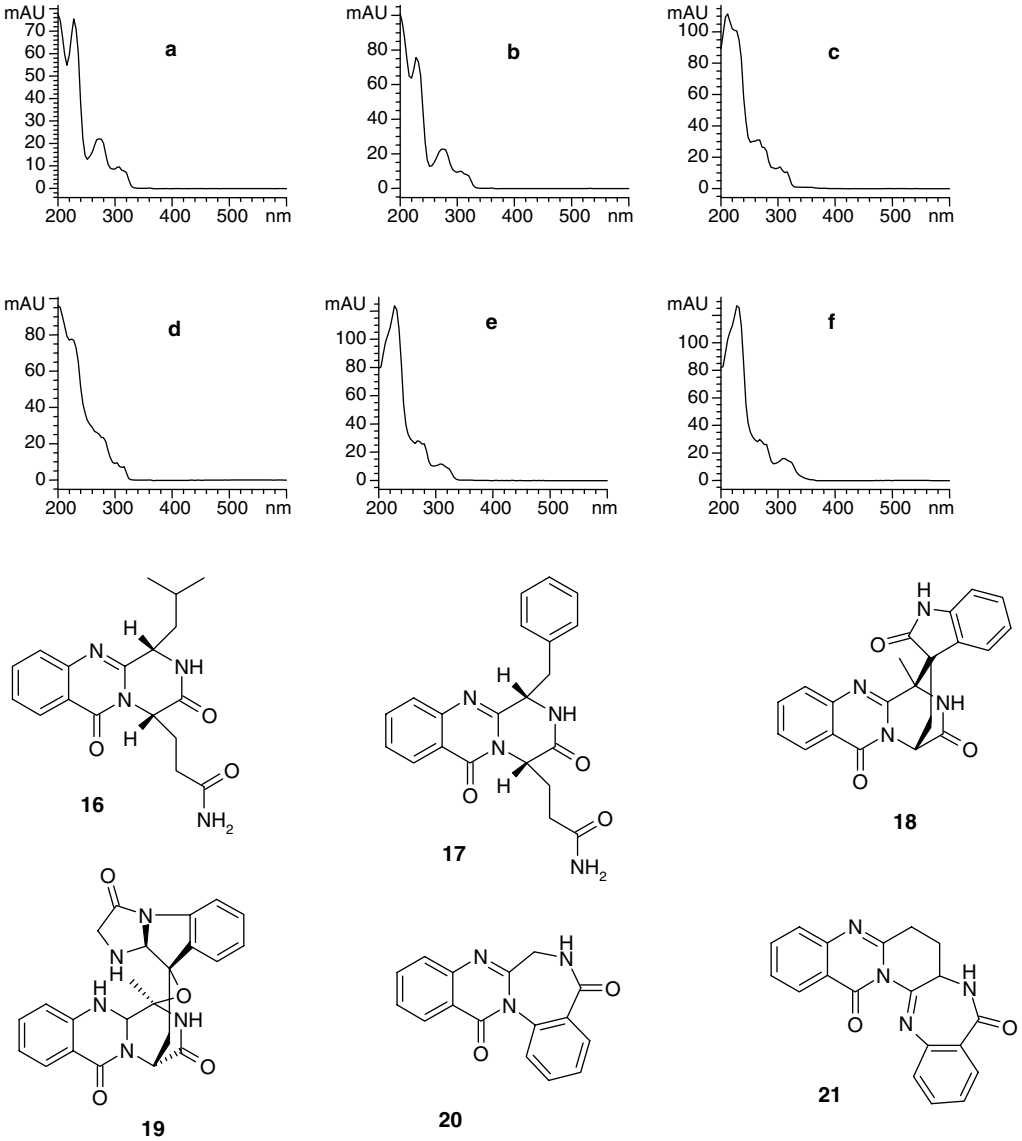

FIGURE 8.4 UV spectra and structures of four quinazoline alkaloids: (a) anacine (**16**), (b) verrucine A (**17**), (c) alantrypinone (**18**), and (d) fumiquinazoline C (**19**); and the two benzodiazepines: (e) sclerotigenin (**20**) and (f) auranthine (**21**).

8.4.2 Polyketides

Polyketides constitute a large class of natural products that are, simplistically, derived from poly-β-keto chains that are formed by coupling of acetic acid (C_2) units via condensation reactions very similar to the initial steps in the biosynthesis of fatty acids. In reality, this involves more complex conjugation reactions of small activated organic acids such as acetyl coenzyme A together with malonyl coenzyme A, which at the same time lose carbon dioxide generating a four-carbon unit (diketide). This unit can likewise be extended by repeated conjugation with several additional malonyl coenzyme A units. While the carbonyl groups are reduced before attachment of the next malonate group during fatty acid biosynthesis, highly reactive poly-β-keto chains are formed during polyketide biosynthesis, eventually leading to all kinds of both simple and highly complex cyclization reations.[36] Usually oxygen atoms are lost during cyclization and, similar to fatty acid biosynthesis, carbonyl groups can also be reduced to alcohols, double bonds, or to aliphatic moieties.

An example of a compound that has undergone several reduction steps during its biosynthesis is compactin (**22**, Figure 8.5), which is a nonaketide. The structure of compactin (**22**) thus contains

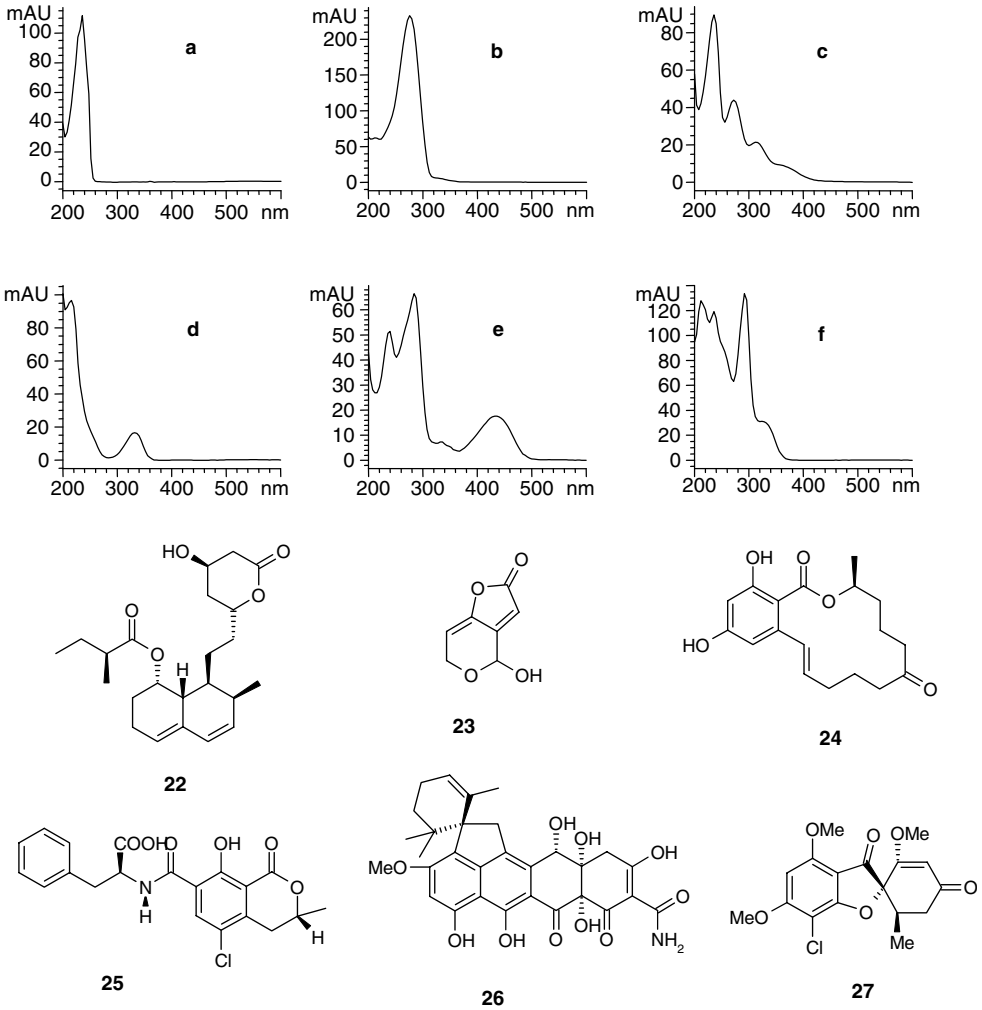

FIGURE 8.5 UV spectra and structures of some common fungal polyketides: (a) compactin (**22**), (b) patulin (**23**), (c) zearalenone (**24**), (d) ochratoxin A (**25**), (e) viridicatum toxin (**26**), and (f) griseofulvin (**27**).

only one carbonyl group and two conjugated double bonds. This is reflected in the UV spectra of compactin and other statins (such as the mevinolins), which all have a similar major absorption close to 237 nm with two tiny shoulders. This minor feature actually makes it possible to differentiate the UV spectra of the statins from other polyketides such as patulin (**23**), zearalenone (**24**), ochratoxin A (**25**), the tetracycline metabolite viridicatum toxin (**26**), and griseofulvin (**27**) (Figure 8.5). Polyketides (**24**–**27**) all have more conjugated chromophore systems than the statins or patulin due to one or more aromatic ring systems leading to other characteristic UV spectra of these compounds (Figure 8.5). Thus, aromatic and other types of polyketides comprise a very large group of natural products that are excellent targets for an UV-based dereplication approach.

8.4.3 Terpenoids

Terpenoids comprise a large structurally diverse group of natural products derived from C_5 isoprene units. In brief, the two key isoprene units dimethylallyl dipyrophosphate (DMAPP) and isopentenyl diphosphate (IPP) are biosynthesized from three activated acetyl coenzyme A moieties. Two or more DMAPP and IPP isoprenoid units can then be subsequently added in a head-to-tail way to generate hemiterpenes (C_5), monoterpenoids (C_{10}), sesquitepenoids (C_{15}), and higher orders of terpenoids including the steroids.[36] Many of the smaller terpenoids (mono and sesquiterpenes), which are generally volatile in nature, do not contain any oxygen atoms, but those that do, such as geosmin or 2-methyl-isoborneol, usually have very distinctive odors that can contribute to the moldy characteristic of growing fungi.[38] The larger, more oxygenated terpenoids, such as many of the *Fusarium* trichothecene toxins including deoxynivalenol (**5**), (Figures 8.2 and 8.6), are not volatile.

Examples of useful UV spectra of compounds with terpenoid character are shown in Figure 8.6. In general, terpenoids *per se* (in contrast to secondary metabolites of mixed, for example, terpenoid and alkaloidal, origin) contain few double bonds, and therefore few chromophoric systems, usually resulting in end-absorption type of UV spectra less suitable for UV-based dereplication approaches as seen for deoxynivalenol (**5**, Figure 8.6). An exacerbating factor is the fact that the terpenoid building blocks, that is, the isoprene units, do not have carbonyl groups, as is the case for most alkaloids and all polyketides. One example of a terpenoid containing a conjugated double bond system resulting in a characteristic UV spectrum is the steroid ergosterol (**28**) that is produced by all fungi.

Many natural products are of mixed biosynthetic origin. Thus, many alkaloids or polyketides can contain, for example, a hemiterpene unit. The meroterpenoids, such as hesseltin A (**29**), are formed from a polyketide and terpenoid whereas verrucologen (**30**), penitrem A (**31**), and pyripyropene A (**32**) are biosynthesized from an amino acid and a terpenoid moiety.

8.5 DATABASES AND ALGORITHMS

8.5.1 Structure of HPLC-DAD Data

HPLC-DAD instruments yield 2D data for each sample that is being analyzed. One dimension is represented by the retention time and the other by the absorbance spectrum. Each data point in the matrix is therefore the absorbance of what is eluted from the column at a given retention time at a certain wavelength. Usually, the raw data matrices are stored on the hard disk, allowing reprocessing of the data after the run is finished. Data should be organized and structured in such a way that for each data file all available information about the sample origin and analytical conditions are registered in an accompanying database. This should include, for example, the geographic and seasonal details for plants, and the strains and growth conditions for microbes. Seen as a whole, the database therefore is a "snapshot" of the ability of a plant or a defined microbial strain to produce chemical substances under certain conditions, as seen through the "eyes" of an HPLC system.

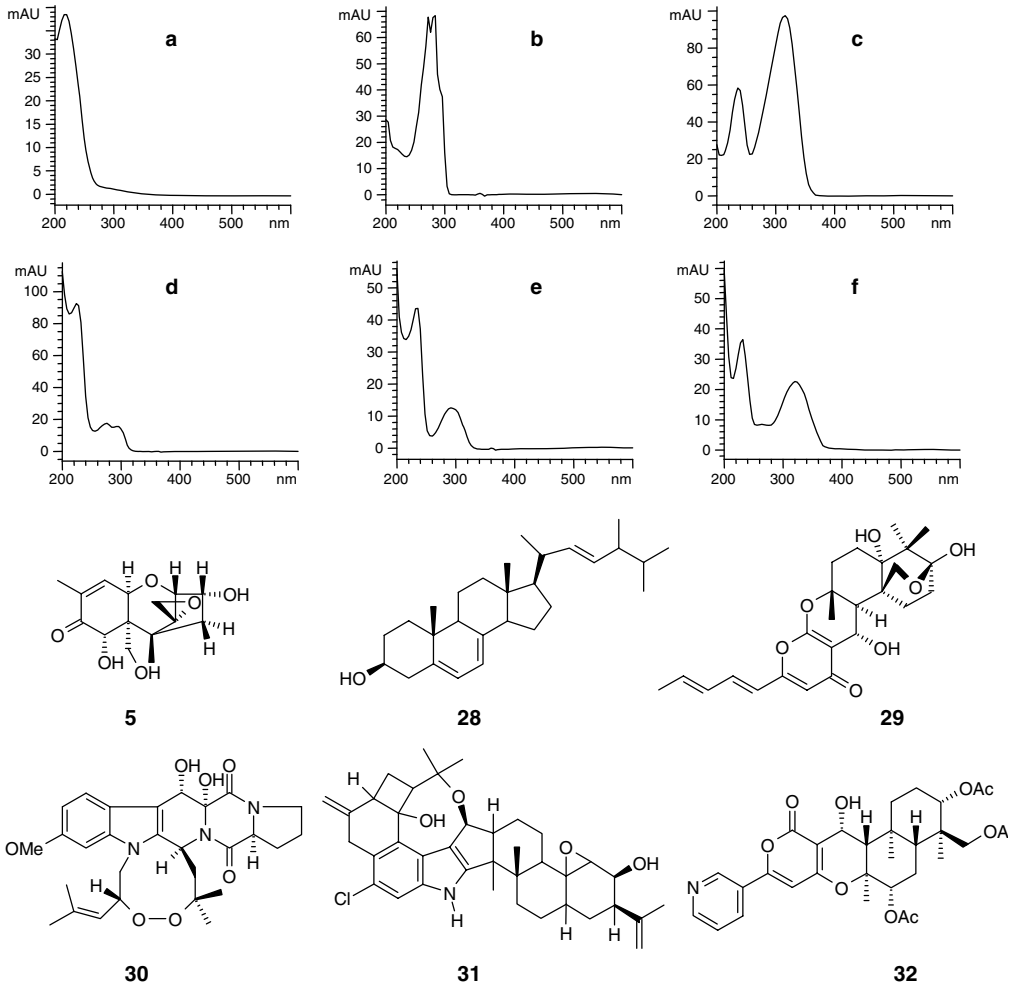

FIGURE 8.6 UV spectra and structures of some common terpene-derived fungal metabolites: (a) deoxynivalenol (**5**), (b) ergosterol (**28**), (c) hesseltin A (**29**), (d) verrucologen (**30**), (e) penitrem A (**31**), and (f) pyripyropen A (**32**).

8.5.2 Data Processing/Analysis

There are several important steps involved in the processing and analysis of UV/Vis data, that is, noise reduction (or signal enhancement), baseline (or background) correction, chromatographic alignment, peak detection, deconvolution of chromatographic peaks, peak purity, and standard library search methods.

8.5.2.1 Noise Reduction

Noise is usually defined as the random, sample-unassociated variations that can occur in an UV chromatographic trace. It can also be defined in a much broader sense as a spectrum artifact that is caused by drift in internal instrument parameters, for example, the drift in baseline where the true peak signal is superimposed upon a more slowly varying surface.

The most common way of removing noise, especially the stochastic noise, is based on a so-called "moving window" filter.[39,40] This can be represented as a window of a given size moving along the profile to be filtered (Figure 8.7). The middle element of the window is replaced with the

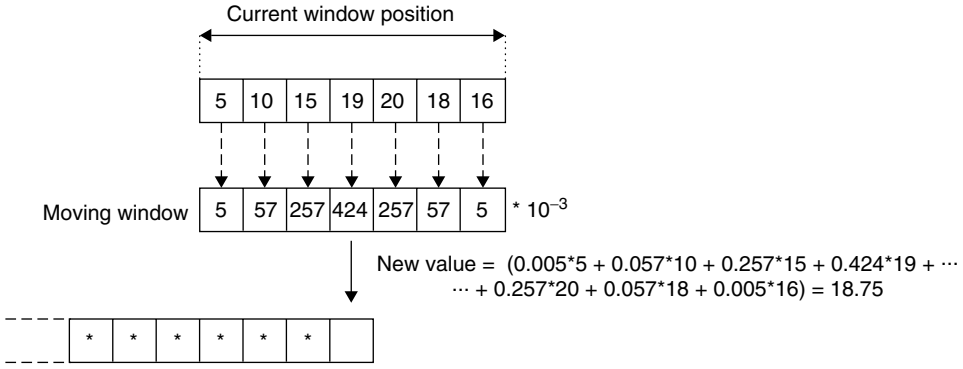

FIGURE 8.7 Illustration of a moving window filter with Gaussian weights, $N(\mu = 0, \sigma = 1)$.

weighted average of all elements in the window. The size of the window and the weights chosen in the window are important for the properties of the filter.[41] The window illustrated in Figure 8.7 shows a window with Gaussian weights, $N(\mu = 0, \sigma = 1)$.

If all weights were chosen to be one over the length of the filter, that is, 1/7 in the example shown in Figure 8.7, the window would have calculated the mean of the overlaid cells in the profile, and hence the filter would have been called a "mean filter". Other types of filters are based on this moving principle having different properties.[42,43] Some calculate the dot-product between the window weights and the profile values, as shown in Figure 8.7, whereas others estimate the median, a quantile, or other (weighted) measure derived from the profile values within the window.

It is important to remember the value of new elements and not to make the replacement until the window has passed. This must be done since all calculations are based on the original data in the array. When the ends of the profile are filtered and parts of the window are outside the spectrum, the calculation must be done on fewer elements than when the entire window is inside the array. This implementation leaves the ends of the array unfiltered. For a 7-point filter, this means that when n elements are filtered, elements 1, 2, 3, and $n - 2$, $n - 1$, n remain unchanged when filtering is complete. For many applications that involve long profiles and short filters, this is not a problem. Alternatively, the profiles can be padded with the values found at the end, or padded with zeros.

Unfortunately, smoothing with these fixed filters does not preserve the height and width, that is, the area, of a peak and the (centroid) position if the peak is skewed. Some of the existing filter algorithms can be made adaptive on the basis of measured peak properties, such as intensity or width. To accommodate for this problem, the spectrum can be approximated locally by a higher-order polynomial (of some order) within a moving window. This filtering method is closely related to the so-called Savitzky–Golay filter[42] available in most of the instrumental software packages, for example, MassLynx and HP Chemstation.

8.5.2.2 Baseline (Background) Correction

While the moving window removes stochastic noise, other artifacts that are introduced to the signal during acquisition are considered to be deterministic noise. As described above, most of the chromatographic data can be regarded as "real information" added on to a "noisy" background or baseline.[44] In the case of chromatographic data, the IUPAC compendium of technical terminology defines the baseline as the part recorded when only carrier gas or solvent elutes from the column.

The baseline can be either flat, linear with a positive or negative slope, curved, or a combination of all three. In most correction algorithms, it is the goal to estimate the baseline $g(t)$, which then is subtracted from the original chromatogram (Figure 8.8).

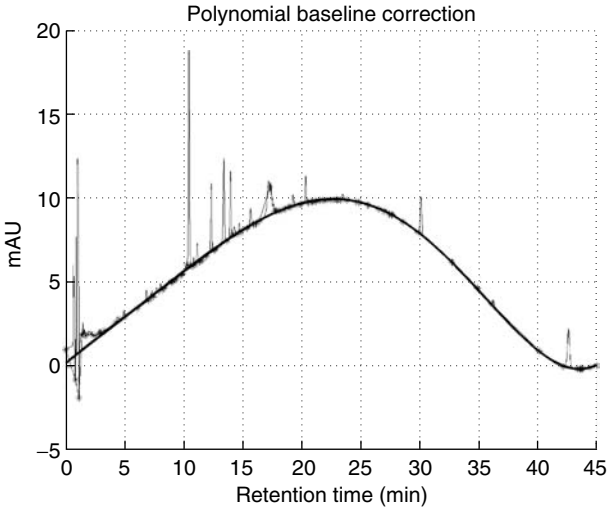

FIGURE 8.8 Illustration of baseline correction. The heavy black line is the estimated background $g(t)$ that can be subtracted from the signal.

The methods available for estimating the background vary in complexity.[45] Some of the methods work on the whole chromatogram at once, and some are local methods that break the chromatogram into relevant smaller sections where the background function is estimated better. In addition, some methods are parametric methods that try to estimate a function (piecewise or global) to the background. The simplest are the locally applied linear correction methods[46] followed by the more complex nonlinear methods. In most software packages, such as Origin (by OriginLab) or PeakFit (by Systat Software), as well as for many published papers, the background is estimated by a least-squares polynomial fitting performed on a user-defined subset of points, which should belong to the background. A new tool for removing the background from chromatographic data was introduced with the wavelet transform approach.[47–50]

8.5.2.3 Chromatographic Alignment

Retention time variations are a serious impediment to the successful application of automated comparison of chromatographic data.[51–54] These variations are due to subtle, random, and often unavoidable changes and variations over time in instrument parameters. Column aging and fluctuations in pressure, temperature, solvent composition, and flow may cause an analyte to elute at different retention times in replicate runs. Even with the implementation of advanced instrumentation control such as electronic pressure control, subtle run-to-run retention time shifts can be small but always present and must be taken into account to successfully apply chemometric methods. Matrix effects and stationary phase decomposition may also cause variation in retention time. The main reason is that most pattern recognition techniques and chemometric methods are based on point-to-point comparison for successful analysis. Many of the available alignment algorithms do not require knowledge or identification of peaks.

The main goal for all "warping" algorithms is the same, that is, to align chromatograms so that they correct the random differences between chromatographic runs. These algorithms typically search to find the optimal "warp" according to some criteria. Warping is the process of stretching or shrinking one profile of peaks to make it match with another profile of peaks. A simple warp example is the stretching or shrinking of the profiles in a linear manner just by moving the ends (like an elastic band). Unfortunately, this simple approach cannot be used since the difference between the profiles might vary locally along the retention time axis. Therefore, all of the warping

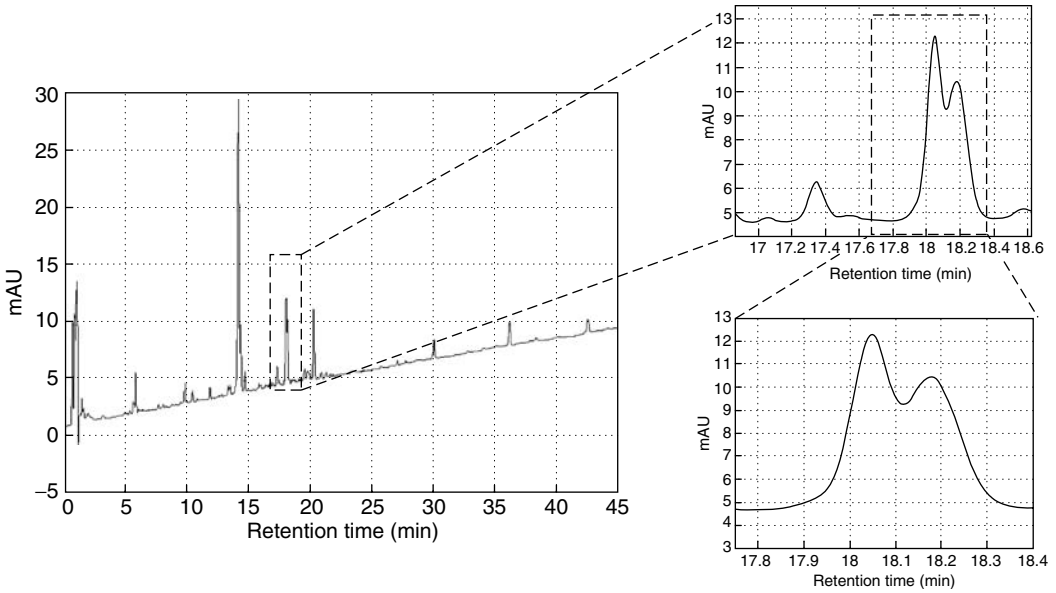

FIGURE 8.9 An HPLC-UV DAD chromatographic profile with several peaks. See Figure 8.10 for the first- and second-order derivatives of the selected peak.

algorithms try to warp the profiles to each other in such a way that they are fitted or warped locally. The criterion used for judging a good local fit is how well the "locally warped" pieces correlate to each other.

Many attempts have been made to increase the speed and performance of warping algorithms for finding the best match between two chemical profiles. Pravdova et al.[55] and Tomasi et al.[56] have reviewed and evaluated two competing state-of-the-art warping methods, that is, dynamic time warping (DTW)[57] and correlation optimized warping (COW).[46] Other warping methods include peak alignment with genetic algorithm (PAGA),[58] local warping (LW),[59] parametric time warping (PTW),[60] a hidden Morkov model-based approach,[61] and fuzzy warping (FW).[62]

One of the conclusions that can be made on the basis of all existing literature is that no matter what method is chosen, it will be based on a trade-off between performance and speed. For example, some methods, such as DTW, are relatively fast but have been found to perform not as well as the COW algorithm.[55,56]

8.5.2.4 Peak Detection

One of the crucial steps in analyzing chromatographic data is the detection of peaks. Traditionally, peaks are detected in chromatographic profiles as illustrated in Figure 8.9 in which the average trace of an HPLC-UV DAD is plotted. Figure 8.10 illustrates how the first- and second-order derivatives (Figure 8.10b) of the profile (Figure 8.10a) can be used to detect peaks in the profile. Thus, information about the profile topology can be obtained and utilized to identify peaks and valleys as well as saddle points (shoulders).

While the above example illustrates peak detection along the retention time axis, the information available in the second dimension given by the wavelengths of absorption in the UV spectrum can be included as illustrated in Figure 8.11. Under the assumption that a peak is a peak in both dimensions, a comparison of the derivatives for both the retention time and wavelength will reveal information about the peak locations.

It is important to emphasize that the presence of noise will make the task of detecting peaks using the derivative approach difficult. To accommodate the presence of noise, filtering techniques

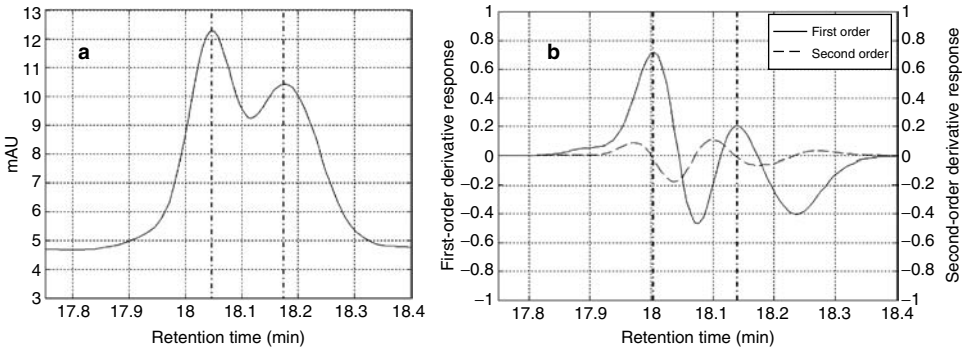

FIGURE 8.10 Illustration of the detection of peaks in the profile representing the peak selected in Figure 8.9 (a), using first-order (solid line) and second-order derivatives (dashed line) (b).

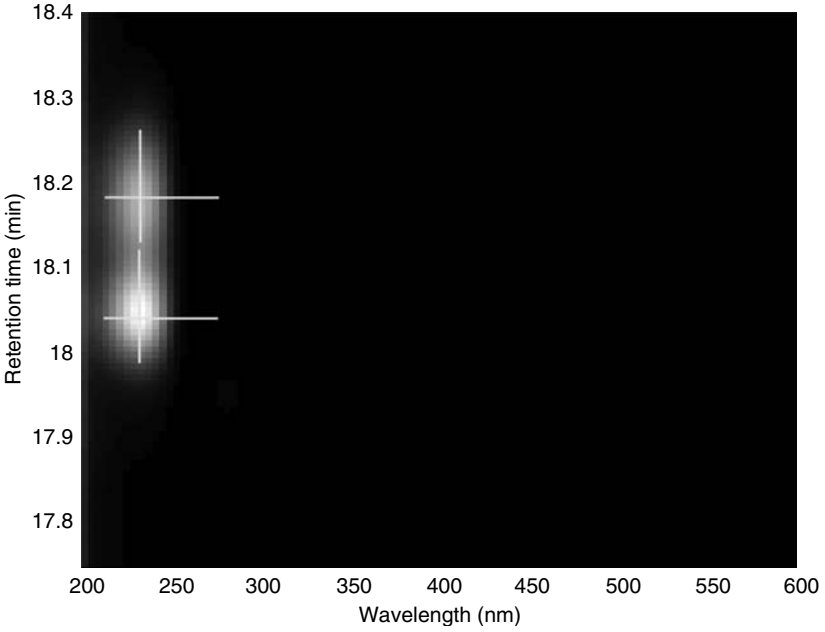

FIGURE 8.11 Illustration of the detection of peaks utilizing the 2D structure (retention time and wavelength of absorption) in HPLC-UV DAD.

and peak consistency analysis can be applied before or together with the estimation of the derivatives, which will reduce the number of erroneously assigned peaks to a minimum.

8.5.2.5 Deconvolution of Chromatographic Peaks

Chromatographic techniques often give rise to situations where reaching complete resolution is not possible. Deconvolution is the process of separating target peaks from overlapping peaks.[63,64] Together with the development of high-performance instruments, the number of algorithms and tools for deconvolution of chromatographic data has been increasing steadily. As a result, compounds hidden within a peak cluster can now be quantified with relatively small errors.

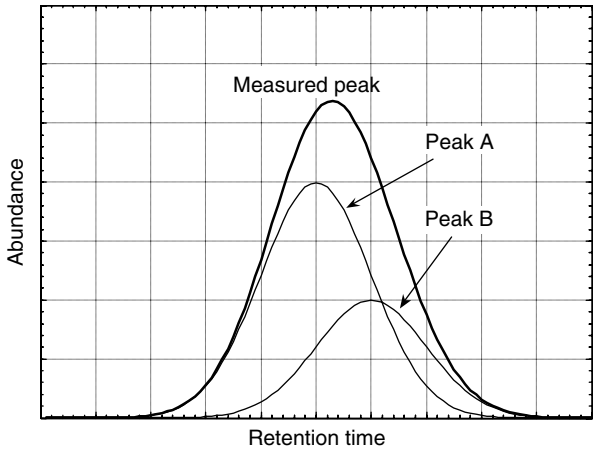

FIGURE 8.12 Coelution of two compounds, A and B, in the chromatographic peak. No shoulders, valleys, or excessive tailing are seen.

Deconvolution methods can be divided into two fundamental categories, that is, those that use single (1D) profiles[65,66] and those that are based on higher-dimensional chromatographic data.[67,68] Most 1D approaches, for example, retention time profiles, rely on fitting a "sum of peak" model to the profile.[69,70] However, two peaks eluting at almost the same time might differ in the MS spectrum or UV absorbance spectrum. Therefore, adding more dimensions (such as UV absorption wavelength or mass spectra) to the deconvolution algorithm makes the task easier.

Deconvolution can be achieved either in an automated fashion using the software packages provided with most GC-MS instruments (e.g., Pegasus, Leco, St. Jospehs, MI) or separate software can be applied, such as AMDIS.[71] The AMDIS software is originally based on the algorithm developed by Stein.[64]

8.5.2.6 Peak Purity

Peak purity analysis is designed to measure the degree of impurities that are coeluting with a given peak. Using a single wavelength UV/Vis detector, one must see a shoulder, valley, or excessive tailing to suspect the presence of an impurity. The absence of these features on the chromatographic peak is not a foolproof assurance of peak purity. The impurity may not be seen simply because the chromatographic resolution is low (Figure 8.12). A photodiode array detector can provide additional information by using the acquisition of UV/Vis spectra to determine peak purity.

One way of uncovering contributions due to impurities in an HPLC peak is to overlay peak profiles acquired at several wavelengths. As two different compounds are unlikely to exhibit identical absorption over multiple wavelengths, the presence of an impurity is revealed by the deviation of the profiles. To compensate for the differences in spectroscopic intensities at different wavelengths, the signals to be compared are first normalized to the maximum absorbance value or to equal areas. Peaks free of impurities exhibit good overlap but the presence of an impurity is indicated by a shift in the retention time maximum at different wavelengths (Figures 8.13a and 8.13b). This signal overlay method is not considered to be very sensitive and is highly dependent on the resolution of analyte and impurity peaks. However, if care is taken to correct for solvent background and if the signals are normalized to the highest absorbance value in the time range plotted, then the ratios of signals acquired at different wavelengths can be calculated and plotted. The resulting ratiograms are good indicators of peak purity. Any significant distortion of the ratiograms from the ideal rectangular form is an indication of differential absorption and the presence

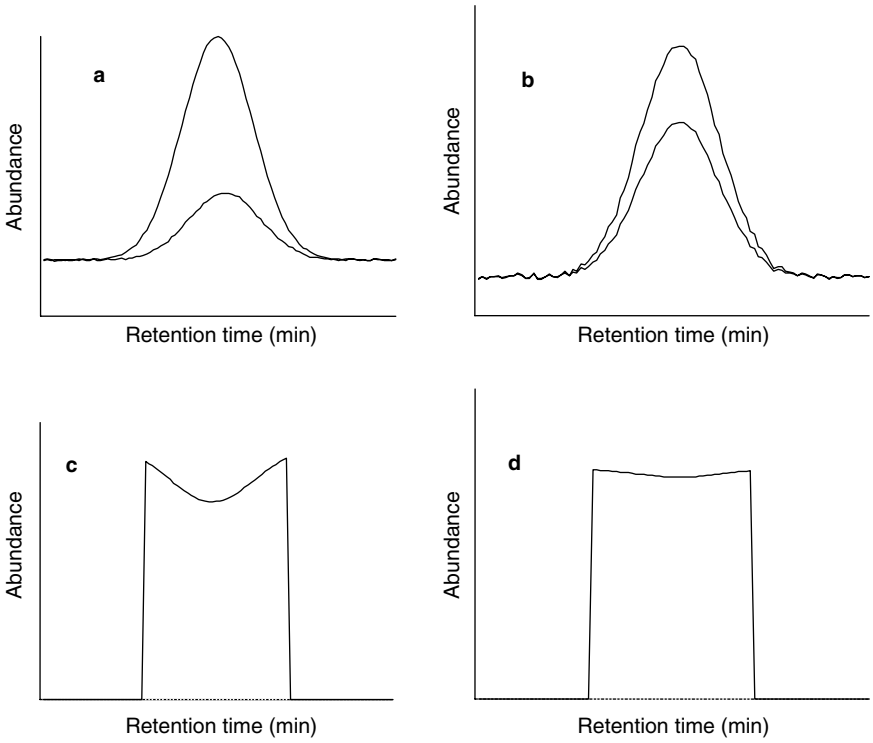

FIGURE 8.13 Ratiograms (c) and (d) taken from (a) an impure and (b) a pure peak.

of an impurity (Figures 8.13c and 8.13d). The ratiogram is usually recommended as an additional qualifier in conjunction with other peak purity analysis methods.

8.5.2.7 Standard Library Search Methods

The conventional way of comparing spectra is based on their UV_{max} or several other local extrema. Alternatively, comparing two spectra with absorbance values (again, at different wavelengths) is equivalent to comparing two vectors, and several simple metrics can be applied. Suitable metrics could be calculating the angle between the vectors, their correlation coefficient, or the Euclidian distance, but other examples of measuring the similarity between vectors exist.

Over the years there has been little effort to use UV/Vis spectra for compound identification. The reason for this is that UV/Vis spectra of most organic molecules consist of a few broad, similar absorption bands. Moreover, most of the absorptions appear at the very short wavelengths between 200 and 300 nm. Thus, it is not always easy to visually distinguish the spectrum of one compound from that of another. Still, the importance of UV/Vis spectroscopy for quantitative analysis is well known, as described above.

8.5.3 X-Hitting

The first step of the "X-hitting" algorithm[31,32] is to create two databases, both on the basis of UV spectra extracted from HPLC-UV DAD analyses of standards and culture extracts. The compound database contains information about the reference spectra obtained from the HPLC-UV DAD analysis of standards or samples where a well-known target metabolite can be identified. The sample database contains information about the spectra obtained from HPLC-UV DAD analysis of

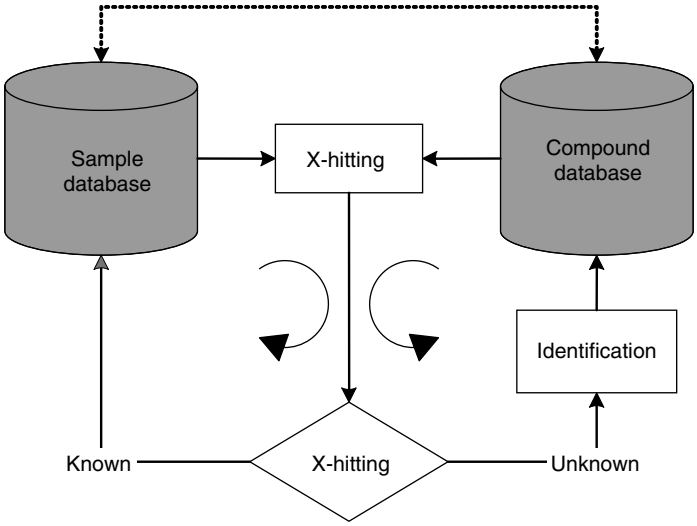

FIGURE 8.14 The principle of "X-hitting".

unknown samples. Each database is made individually, so that changes can be done independently (Figure 8.14).

The compound database contains the reference spectra. This information is extracted from a set of samples, that is, HPLC-UV DAD data from analyses of isolates or of standards, in which the presence of the known compound(s) has been verified by LC-MS, NMR analysis, or both. The information stored includes compound names, source (or physical location) of the samples or producer organisms, retention times, retention indices, and other relevant information about retention intervals. A retention interval indicates the acceptable elution window of a given compound.

The sample database is composed of the HPLC-UV DAD data from analyses of samples and contains links to the HPLC-UV DAD data file locations, and important isolate information (metadata) such as isolate name, collection reference number, and culture media. To avoid any confusion in the following analyses and evaluation, we emphasize that the samples from which the reference spectrum is extracted are excluded from the sample database in cases where this spectrum is under investigation.

In "X-hitting", known metabolites are tracked by the feature "cross-hitting", whereas potentially new metabolites are indicated by the "new-hitting" feature of "X-hitting". The main idea of the algorithm behind X-hitting is to compare the shape between two UV spectra and thereby return a similarity index describing the statistical similarity of the two spectra. To capture information about the actual shape of each of the profiles at different scales (coarser or finer details), a linear combination of the correlations between higher orders of derivatives is used. In addition to the algorithm requiring to be computationally fast, a main criterion is to retain relationships between "neighboring" absorbance values in the spectra (shape-preserving metric). By using derivatives, such relationships can be incorporated by measuring the differences and the positions of topologies and extreme points. Finally, filtering techniques are applied to the derivatives to reduce the sensitivity to potential noise fragments in the spectra.

With respect to the drug discovery process, the great potential of "cross-hitting" is its ability to support the choice of the optimal organism for production of a given natural product. This is the case when detecting strains producing mycotoxins but may be even more important for the tracking of new species producing a desired compound (in this scenario a new drug lead). In this way "cross-hitting" of known compounds can be applicable to "new-hitting" of organisms. Very often

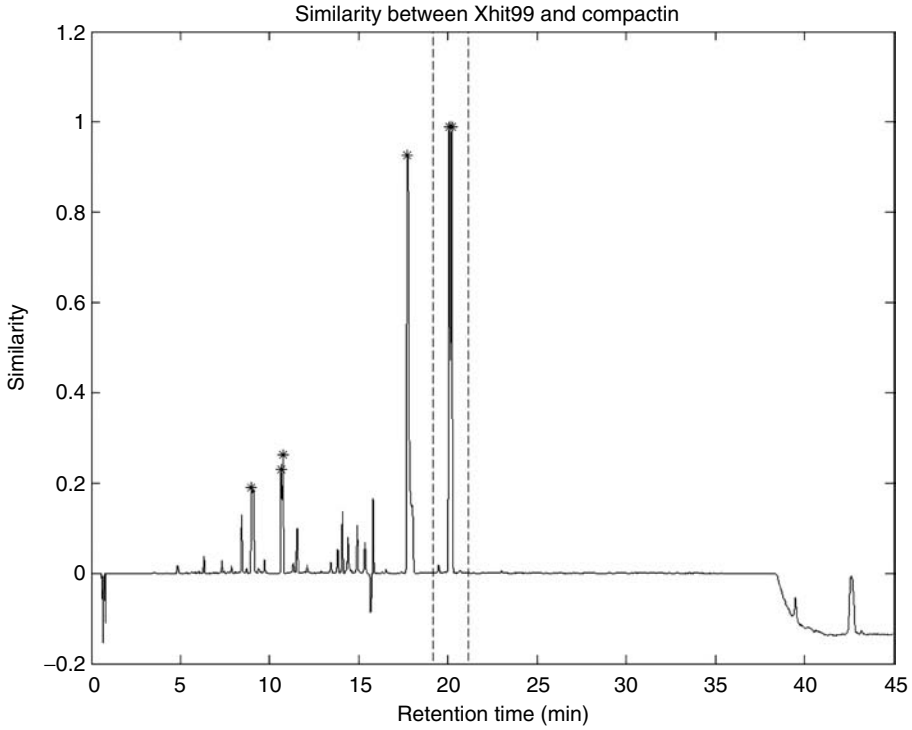

FIGURE 8.15 Example of a similarity profile between a sample (code: Xhit99) and a standard (compactin) spectrum. The vertical dotted lines indicate the retention time interval in which compactin is expected to elute. The significantly higher peak close to the expected retention time has an UV spectrum very similar to compactin (Figure 8.16).

a certain compound is produced as a minor metabolite by one species (under certain conditions), whereas it appears as one of the major metabolites produced by another, completely different species.

The "X-hitting" algorithm is a semiautomated, iterative algorithm where the knowledge about existing compounds is added to the database manually. Based on the knowledge of the UV absorbance characteristics (sample database) of the unknown compound, similar and identical compounds are found and identified in the dataset of full HPLC-UV DAD matrices (compound database).

8.5.3.1 Cross-Hitting

"Cross-hitting" is based on the fact that structurally identical compounds are expected to elute at the same retention time (or index) as well as having the same UV/Vis spectrum. The "cross-hitting" checks for high values in the similarity profile within a predefined elution window specified by the elution properties of the known compound.

By comparing the UV spectra and chromatographic characteristics of the sample in the sample database against the same data for the specified standard (known compound being searched for) available in the compound database, a similarity profile is derived for a sample and any specific standard. For example, Figure 8.15 shows a search for compactin (the standard) in the unknown sample labeled Xhit99. The profile shows low similarity values except within the expected retention time window and another peak just outside the window. The UV spectra of the peaks marked with an "*" in the similarity profile (Figure 8.15), compared to the UV spectrum of compactin (**22**), are shown in Figure 8.16 in the order (a to d) of decreasing similarity.

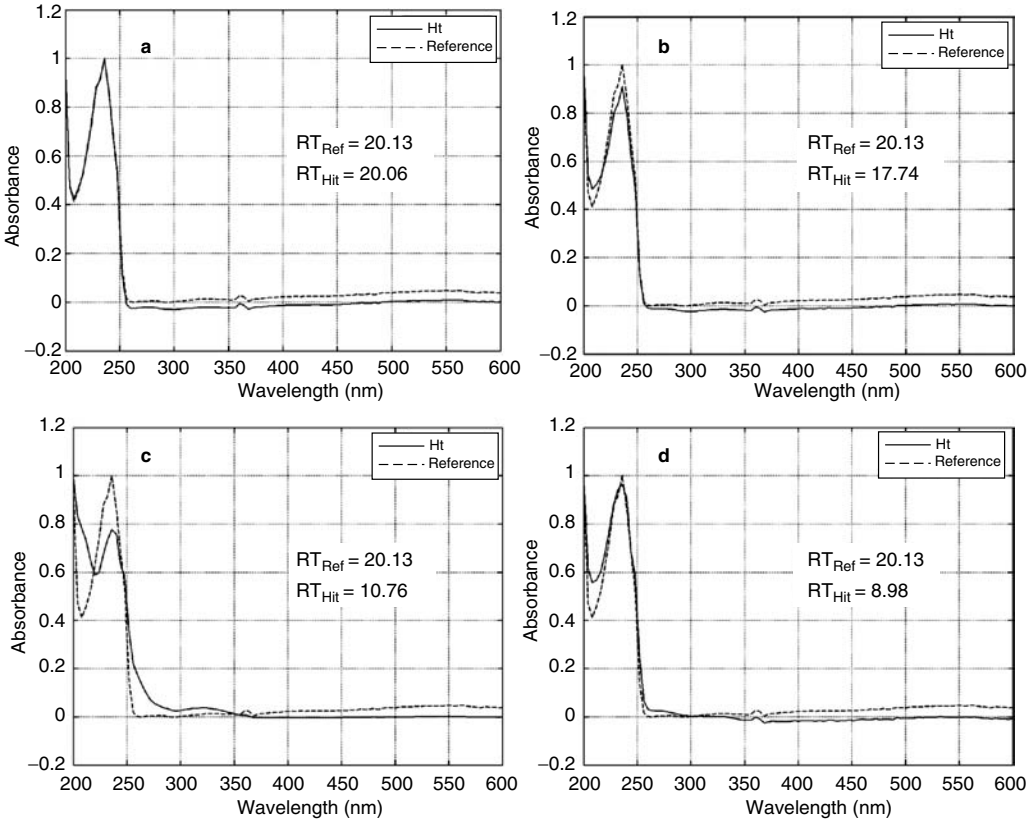

FIGURE 8.16 The result of the "X-hitting" algorithm. (a) The UV spectrum of the compound within the retention time window that shows a high similarity index to compactin (Figure 8.15). (b–d) Some of the peaks found outside the expected retention time interval (marked with an * in Figure 8.15). The dashed line in each subfigure is the UV absorption spectrum for compactin.

8.5.3.2 New-Hitting

In many cases (as exemplified in Figures 8.15 and 8.16), a similarity profile illustrates a positive cross-hit outside the expected elution window for the standard compound being sought. The UV spectra of these additional peaks are all almost identical to that of the target compound (Figure 8.16) and therefore likely represent analogs and biosynthetically related compounds. The detection of these related compounds in the samples, by virtue of their similar UV spectra to the specific standard, is referred to as "new-hitting".

Figures 8.17 and 8.18 show an example of the "new-hitting" approach searching for alantrypinone and analogs.[31,32] When using the UV spectrum of alantrypinone as a target spectrum for a cross-search of all the UV data contained in approximately 124 sample data files, the similarity profile generated from a *Penicillium lapatayae* extract showed several peaks (n1–n8, Figure 8.17) with similarities significantly above the noise level and strongly indicating the presence of quinazoline-like compounds in the extract. In addition, a compound was identified as a cross-hit (c1, Figure 8.17) since it eluted within the retention time interval (dashed vertical lines in Figure 8.17) expected for the target compound alantrypinone. However, since the similarity of the n1 spectrum toward the spectrum of alantrypinone was larger than that of c1 (Figure 8.18), the compound appearing at c1 could not be alantrypinone. On the basis of these findings, it was decided to isolate and elucidate the structure of these two compounds, likely to have structures very similar to that of alantrypinone.

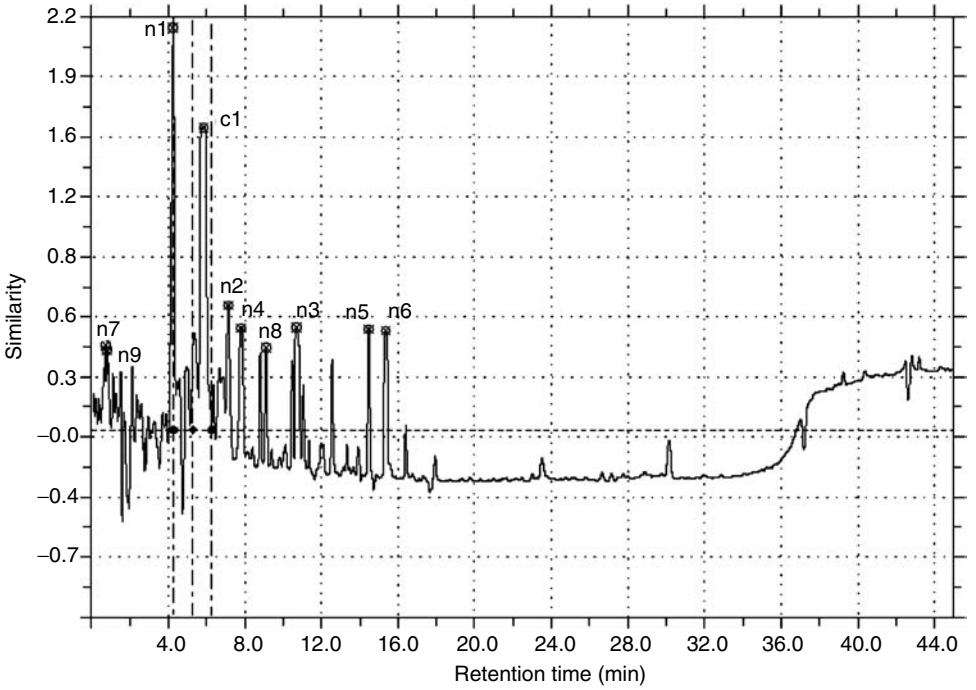

FIGURE 8.17 Similarity profile for *P. lapatayae* Xhit97 versus alantrypinone showing one "cross-hitting" peak (c1) and nine "new-hitting" peaks (n1–n9). (Reproduced from Larsen, T.O., Petersen, B.O., Sørensen, D., Duus, J.Ø., Frisvad, J.C., and Hansen, M.E., *J. Nat. Prod.*, 68, 871, 2005. With permission of The American Chemical Society.)

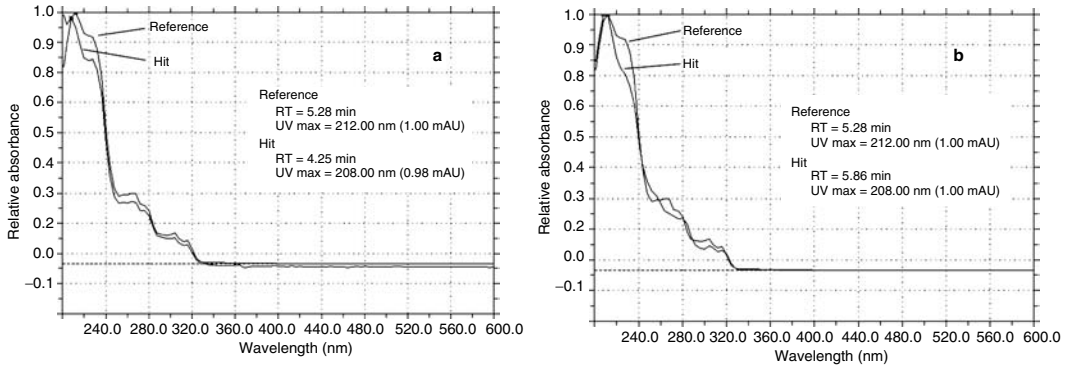

FIGURE 8.18 UV spectrum of the reference (and target) compound alantrypinone (labeled as "Reference") compared to the UV spectra (labeled as "Hit") of (a) lapatin A (c1 in Figure 8.17) and (b) lapatin B (n1 in Figure 8.17). (Reproduced from Larsen, T.O., Petersen, B.O., Sørensen, D., Duus, J.Ø., Frisvad, J.C., and Hansen, M.E., *J. Nat. Prod.*, 68, 871, 2005. With permission of The American Chemical Society.)

Careful investigation of the LC-MS data of the extract from Xhit97 (MS results not shown) revealed that the molecular formulae for the two compounds, n1 and c1, were likely to be $C_{23}H_{19}O_3N_5$ and $C_{20}H_{14}O_3N_4$, respectively. On searching these formulae in SciFinder, they matched 190 and 220 compounds, respectively. One of the SciFinder matches for the former compound (n1, $C_{23}H_{19}O_3N_5$) was spiroquinazoline, which contains a chromophore corresponding to an anthranilic acid derivative. The dereplication process therefore suggested that n1 could be spiroquinazoline (**33**) and the

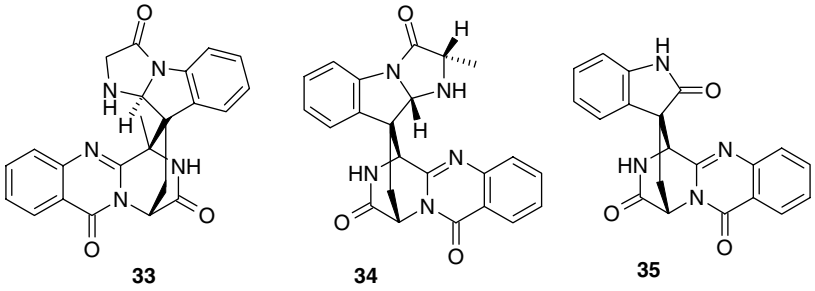

FIGURE 8.19 Structures of spiroquinazoline (**33**), lapatin A (**34**), and lapatin B (**35**).

other was likely to be a novel compound. The two compounds were isolated and the structures elucidated. The compounds were determined to be novel spiroquinazoline metabolites lapatin A (**34**) and B (**35**) (Figure 8.19), structurally very similar to spiroquinazoline (**33**) and alantrypinone (**18**), proving the concept of the "new-hitting" by the "X-hitting" approach. This case illustrates the scope of the X-hitting approach since both major (lapatin A) and minor components (lapatin B) are equally easily recognized by their UV spectra. This is important since minor products of a biosynthetic pathway are just as likely to have an interesting bioactivity as major ones, therefore being equally important compounds to discover.

8.6 CONCLUSIONS AND PERSPECTIVES

With the present chapter we have emphasized the scope of using UV spectroscopy as an important part of a dereplication process in modern analytical natural product chemistry highly influenced by chemoinformatics. Thus, we have illustrated that many of the alkaloid and polyketide natural products have characteristic spectra that can often be associated to a certain subclass within these two major types of natural products, thereby demonstrating that a lot of structural information can be extracted from an UV spectrum. We have also described the new algorithm "X-hitting", which allows for automated searching of full UV spectra in learning-based databases. Algorithms of this type not only can be an excellent part of a dereplication process but might even be used directly in a discovery process since compounds with similar but different spectra are also identified on the basis of similarity. UV approaches should, however, not stand-alone but naturally be part of a hyphenated approach ultimately using a combination of UV, MS, and NMR detection.

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9 Liquid Chromatography-Mass Spectrometry in Natural Product Research

Ka-Wing Cheng, Feng Chen, and Mingfu Wang

CONTENTS

9.1 INTRODUCTION

In the modern context, "natural products" collectively refer to the reservoir of metabolites supplied by nature, mainly from plants and microbes. For decades, natural products have been a wellspring of drugs and lead compounds for drug discovery. The primary driving forces for the long-lasting interest in natural product research have been the continuously emerging evidence supporting the health-promoting and pharmaceutical potentials of many of these natural gifts. Statistically,

plant-derived pharmaceuticals have consistently comprised a significant market share of prescribed medicines and "over the counter" products in developed countries.[1–3] The use of plant materials for combating diseases can be dated back to ancient times. The roles of traditional herbal medicines are particularly prominent in Asian countries, such as China, Japan, and India, and integration of these herbal medicines into the modern medical practice is quite common.

Recently, many households in Western countries, especially the United States, have also begun to use complementary medicines along with conventional medical care. In 2001, U.S. consumers alone spent 4.2 billion dollars on herbal medicines. This does not include purified natural products, which traditionally are considered as pharmaceuticals.[4] The multiple constituents in these herbs may act synergistically to exert the beneficial effect, as opposed to single agents often encountered in conventional pharmaceutical agents. One example is milk thistle extract, which was found to be an effective hepatoprotectant.[5] However, the speed with which a new phytogenic product is put on the market and the number of these herbal products have been increasing explosively. Associated with such rapid product development is the increasing incidence of adverse effects or even fatalities.[6,7] Although these cases are relatively rare, they undermine customers' confidence in herbal medicines and would eventually hurt the reputation of the industry. Therefore, there is a worldwide urge for different parties, especially legal authorities and manufacturing companies, to strictly follow or develop methodologies, in addition to reinforcing regulations, that help ensure the safety and efficacy of these products. These would include the accurate profiling or "fingerprinting" of multicomponent products, rigorous control of the levels of ingredients known to possess inherent toxicity, removal of adulterants, pharmacokinetic studies of potential drug candidates, and so forth. As natural products are metabolites obtained from a plethora of coexisting compounds and are usually surrounded by complex matrices, fulfillment of these tasks entails the availability of highly selective, sensitive, precise, accurate, and reproducible analytical techniques.

In association with natural product research, numerous analytical tools have been developed for the separation and characterization of prospective compounds. Application of mass spectrometry (MS) and its coupling to liquid-phase separation techniques, especially liquid chromatography (LC-MS), have been recognized as a revolutionary breakthrough in the analysis and characterization of natural products. High-performance LC (HPLC) is capable of accomplishing delicate separation of compounds from a variety of samples. MS, especially multistage MS, can be used to detect analytes with superior sensitivity and selectivity, on the basis of its capability of forming, separating, and detecting ions that match with the preset mass-to-charge ratio (*m/z*) extrapolating to their molecular weight. Although UV-photodiode array detectors (LC-UV-DAD) and fluorescence detectors have enabled HPLC separation with very sensitive detection, insufficient selectivity and interferences from molecules with similar chromatographic behavior remain its major drawbacks. This is visualized as clusters of background peaks and seriously fluctuating baselines in the chromatograms. On the contrary, MS alone cannot directly identify analytes present in very complex matrices in which the MS response may be perturbed, resulting in a reduced or enhanced response in the spectrum.[8] By combining LC and MS, a synergistic analytical capacity can thus be realized in the sense that they mutually complement each other's shortcomings. For instance, LC presents MS with cleaner samples, whereas MS helps reduce laborious sample preparation steps required before proceeding to analyte identification.[9]

In its infancy, the most important hurdle to overcome was the incompatibility between the LC eluate and the MS system. The former is in liquid phase, whereas the latter can only detect analytes in the gas phase. Due to the development and continuous improvement of various interfaces, most commonly electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), for bridging these two analytical tools, LC-MS has become a state-of-the-art technology. The contribution of LC-MS to natural product research is illustrated by its incomparable performance in various areas, including preliminary screening, isolation and characterization, quality control/fingerprinting and metabolism and pharmacokinetic studies for obtaining parameters relating to the absorption, distribution, metabolism, and excretion of bioactive natural products. The concern that ESI and APCI are soft ionization techniques that result in very little molecular fragmentation and

thus offer insufficient structural information is removed by the use of tandem MS, which provides supplementary information of fragments dissociated from the molecular ions.[10]

This chapter will briefly describe the incorporation of LC-MS into a natural product isolation–characterization system. The majority of the chapter will then concentrate on summarizing, discussing, and illustrating the applications of LC-MS (particularly ESI and APCI) in selected areas of natural product research. Major limitations of this hyphenated technique and how such shortcomings may be overcome will also be discussed.

9.2 MASS SPECTROMETRY AS AN INTEGRAL PART OF AN ISOLATION–CHARACTERIZATION SYSTEM

9.2.1 Interfaces between LC and MS

In the early days, ionization of analytes introduced into an MS system occurred in gas phase *in vacuo*, mostly via collision with energetic particles such as high-energy electrons (in electron impact, EIMS) and neutral atoms (in fast atom bombardment, FABMS). Although mass spectrometers do separate gas-phase ions according to their *m/z* ratios, in natural product research, which often involves samples containing chemical entities of diverse properties, the role of separation has largely been taken up by different techniques, especially chromatography. Analytical chemists struggled for years attempting to configure the mass spectrometer as an integral part of an isolation–characterization system. The fact that analytes are already in the gas phase after gas chromatographic separation gave them strong inspiration for direct coupling between these two previously "stand-alone" tools. This started the first golden age of MS in the analysis of organic compounds. In natural product research, the most representative role of GC-MS is probably in the analysis of essential oils.[11] In spite of its power in online separation and identification of analytes, the penetration of GC-MS into the field of natural product research has been limited, as the majority of prospective natural molecules possess some polar and ionic characteristics and are insufficiently volatile unless derivatized.[12] Furthermore, gas-phase ionization (also known as hard ionization) is prone to lead to extensive fragmentation of analyte ions, and excessive heat of ionizing particles could cause thermal degradation of labile compounds.[13]

The consideration that eluates from LC columns are in liquid phase, and the potential consequences associated with the strong heat or highly energetic particles of the hard ionization techniques, led to the development of soft ionization interfaces, primarily ESI and APCI.[14,15] Their applications have realized the unprecedented capacity of MS as an online detector in the fields of organic chemistry, natural product chemistry, and combinatorial chemistry.

Both ESI and APCI are atmospheric pressure ionization (API) processes. In contrast to EI ionization, API sources cause protonation or deprotonation of analyte molecules to generate even-electron charged species, which give them much greater stability against fragmentation.[16] Provided that the mass range of the target analytes is limited to about 2000 Da and that they have some degree of polarity and are soluble in low-boiling solvent(s), either ESI or APCI can be employed to accomplish quantitative or qualitative analysis.[17]

9.2.1.1 Electrospray Ionization

In ESI, ionization occurs in solution phase. Participation from the solvent molecules is required in the process. As the solvent carrying the analytes emerges from the metal capillary that is at a preset potential difference relative to a counter electrode, the high voltage (a few thousand volts) causes solvent vaporization with concurrent creation of charged droplets.[17] Progressive solvent evaporation increases charge density, causing Coulombic repulsion. The size of the droplets is eventually reduced to an extent that favors charge transfer from solvent molecules to the analytes, generating gas-phase ions.[18] These are focused into the high-vacuum region of the instrument for mass analysis, whereas the originally accompanying liquid effluent and its vapor are excluded. In many cases, this

nebulization process can be pneumatically assisted, typically by applying a coaxial flow of nitrogen gas.[19] Before the introduction of this strategy, the delivery rate of the LC effluent to the ESI chamber was limited to less than 10 μL/min to permit sufficient desolvation. Improved desolvation in this modified version of ESI thus allows for a much larger range of flow rates and, more importantly, much higher flow rates compatible with standard analytical LC procedures.[12]

Polarity of the ions formed in ESI depends on the mode of operation. For example, in the positive-ion mode for analysis of basic compounds, the electrospray needle is usually held at a positive potential of a few kilovolts relative to the potential at the counter electrode and vice versa in the negative-ion mode for analysis of acidic compounds.[12,20] In analyses of high-molecular-weight molecules, the capability of ESI to form multiply-charged ions $[M + nH]^{n+}$ provides distinct advantages over APCI.[21,22] As a result, the effective mass limit of conventional mass spectrometers is well extended and, since multiple mass measurements of a molecular ion are acquired in the mass spectrum, mass accuracy could be improved through statistical averaging, provided that some knowledge about the number of charges is already in hand or assumptions about the charge stage are made for unknown analytes.[12,17]

9.2.1.2 Atmospheric Pressure Chemical Ionization

APCI is a close relative of ESI, yet significant differences exist between their ionization mechanisms. Like ESI, a coaxial nebulizing gas may be used to aid the nebulization process.[17] However, for APCI, conversion of the solvent into an aerosol is brought about by thermal energy, in contrast to the strong electric field desolvation of ESI. Continuous vaporization of the aerosol gives rise to gas-phase molecules before ionization is initiated.[12] High static voltage (2–5 kV) applied to a corona discharge needle placed between the nebulizer and the vacuum orifice induces a discharge, which in turn leads to the generation of a charged plasma through a combination of collisions and charge transfer reactions.[17] Protonation or deprotonation reactions then follow, to produce analyte ions when sample molecules pass through this region.[23] In general, APCI is more effective than ESI for molecules of mass range limited to about 2000 Da, and especially for less polar compounds.[24] Another advantage is its tolerance to higher flow rates.[25]

9.2.1.3 Dual Ionization Mode Capability

Owing to the distinctive functionality inherent with ESI and APCI, many commercial mass spectrometers dedicated for LC-APIMS applications are now equipped with both interfaces that can be programmed to operate either alternately or simultaneously.[26] The complementary nature of these two interfaces thus implies that a single MS system is endowed with the capacity to analyze a tremendous range of molecules with diverse properties.

9.2.1.4 Limiting Considerations

Unquestionably, ESI and APCI have enabled the elegant combination between LC and MS to accomplish numerous challenging tasks that were frequently issues of concern for many analytical chemists. In spite of the many merits, several limitations have been recognized in the history of their applications in natural product research and some of these remain as research questions for further studies.

First is the barrier between the atmospheric region for ion generation and the vacuum region for mass analysis. This problem is largely solved with a differential pumping system consisting of a series of pumping stages with increasing vacuum orifice but decreasing pumping capacities and lower operating pressure.[12] Differential pumping permits ion enrichment because of the fact that the paths of the ions and neutrals are differentially influenced by the pumping dynamics and the electronic fields (mostly RF based) of the tuning lenses.[12] Differential pumping has also alleviated the situation (especially in ESI) where there is competition between analytes and surrounding sample molecules for ionization resulting in a compromised ionization efficiency (matrix ion

suppression).[26] This susceptibility to matrix ion suppression is particularly serious for samples containing high levels of salts, buffers, acids, or ion pair agents.[25] Chromatographic separation of target analytes from the solvent front is thus suggested as the most effective way for improving molecular ion signals, that is, by preventing the first 10–20% of the HPLC eluant from entering the MS system.[24] Effective sample cleanup is by no means less important, and Tiller and Romanyshyn[27] showed how a meticulous HPLC protocol can be employed to avoid the problem of matrix effect.

Some shortcomings associated individually with ESI or APCI are also notable. As mass spectrometers carry out mass analyses in a cycle-wise manner, application of constant-flow ESI implies significant wastage of samples.[23] Development of mass spectrometers with higher duty cycle and availability of pulsed ESI interfaces have been the most important remedies for this drawback.[28] Volatility and thermal stability are more a matter of concern for APCI than for ESI, since the former involves ionization in the gaseous phase.[24] Furthermore, presence of charged species, such as very basic molecules from salts or buffers, is detrimental to the APCI process, and in some cases, complete suppression of analyte ionization can be observed.[23]

9.2.2 Common Mass Analyzers for LC-MS

After separation, vaporization, and ionization, the gas-phase analyte ions are then ready to enter the high vacuum region of the mass analyzer. Inside, electric and magnetic fields are manipulated to differentially influence the tracks of ions and focus them onto the detector. Except for large molecules ionized with ESI, most ions generated in a mass spectrometer are singly charged. The *m/z* values shown in the mass spectrum thus represent the masses of the corresponding ions. There are mainly three types of mass analyzers commonly employed in natural product research: quadrupole, ion trap, and TOF mass analyzers.

9.2.2.1 Quadrupole Analyzer

The quadrupoles consist of two pairs of parallel, equally spaced electrodes around a central axis. Each pair carries an identical radio frequency and a direct current voltage, and, for a given RF/DC voltage, only ions of a selected *m/z* would pass through onto the detector, whereas ions of a higher or lower *m/z* would be filtered out.[12]

A single quadrupole can operate in two modes, that is, full scan and selected ion monitoring (SIM). The former scans every *m/z* within a preset mass range, whereas the latter is configured to only allow ions of specific *m/z* to pass through. Thus, the SIM mode is more sensitive and is commonly employed for quantitative analysis.

9.2.2.2 Ion Trap Analyzer

An ion trap mass analyzer consists of one ring electrode and two end cap electrodes. Depending on the RF voltage and frequency applied to the ring electrode, ions of a desired range of *m/z* are trapped. To scan the trapped ions and produce a mass spectrum, the RF is sequentially increased to eject ions of different *m/z* through a hole in the end cap onto the detector.[29] Alternatively, an ion trap can isolate an ion of a particular *m/z*, whereas ions of other *m/z* are ejected. Once isolated, the ion can be fragmented for MS/MS analysis. For MS^n analysis, a selected product ion can then be further fragmented and the cycle can be repeated for a number of times. The sequential MS/MS analysis by ion trap is known as "tandem-in-time", and it provides rich structural information for structural elucidation.

9.2.2.3 Time-of-Flight Analyzer

The TOF analyzer is simple in design. The ions generated from the ion source are accelerated, using a high voltage, along the vacuum flight tube. Since all ions acquire the same amount of kinetic energy in the accelerator, the small mass ions would have a higher velocity compared to the ones

with a larger mass. Thus, the ions arrive at the detector at different times depending on their *m/z*. Newer instruments are often equipped with a reflectron to compensate for energy differences of initial ions and improve the mass resolution. Since TOF has virtually no limits on the mass range, it is often the method of choice in the analysis of high-molecular-weight compounds such as proteins.

9.2.2.4 Tandem-in-Space Analyzers

Tandem-in-space analyzers consist of two or more mass spectrometer units connected physically together. The most popular tandem mass spectrometer is the triple-quadrupole (QqQ).[30] A number of modes are available in QqQ mass analyzers including selected reaction monitoring (SRM), which is the basic mode of quantification in QqQ. Depending on the purpose of the study, product ion scans, precursor ion scans, or neutral loss scans may be used to verify the LC eluate.

In addition, there are a growing number of hybrid mass spectrometers. One example is the quadrupole-TOF (Q-TOF) tandem MS in which a quadrupole analyzer is coupled to a TOF analyzer. Since these instruments embody two mass analyzers, they are much more expensive compared to single analyzer mass spectrometers.

9.3 APPLICATIONS OF LC-MS IN THE SCREENING AND CHARACTERIZATION OF NATURAL PRODUCTS

Nature's tremendous generation and regeneration capabilities give rise to an apparently unlimited unique source of biological molecules. Even though natural resources have been exploited for thousands of years and through various approaches, only a minor proportion has been investigated on the basis of their profiles and content of biologically active constituents. For a single structural backbone, many different derivatives can be biosynthesized depending on factors such as the host biological species and the growing conditions.[31] The different patterns and nature of the structural moieties in these molecules interact to affect their *in vitro* and *in vivo* activities. Moreover, recent advances in molecular biology have revealed new or more promising drug targets for combating or preventing different human health disorders.[32] Thus, there has been an incentive for natural product chemists to continuously devote their efforts to the search for novel compounds that have the potential to elicit a beneficial pharmacological response when administered to the human physiological system.

Another approach is the identification of alternative sources rich in known bioactive compounds.[33] For both qualitative and quantitative investigations, the availability of representative pure natural products as standards is of crucial importance. For some classes of biogenic compounds, only a limited number of synthetic standards are commercially available. Identification of rich, alternative sources therefore provides an economic and efficient approach to obtaining pure standards in sufficient quantity. These in turn can serve as standards for accurate quantification of a more diverse range of structurally related components and supply enough pure compounds for detailed pharmacological studies. Although various databases, including taxonomic databases, and previous literature may provide useful clues to prospective sources, it is the complexity of biological samples and the probability of the occurrence of many alternative sources that place a strong demand for powerful analytical tools to efficiently screen, isolate, and finally characterize the target chemical entities.

In recent years, simple yet highly indicative bioassays have been utilized in the screening or isolation process to narrow down the number of potential hits on the basis of some preselected bioactivities. However, fractionation procedures guided by a single bioactivity have been criticized for two major reasons: reisolation of known compounds with recognized activity (i.e., replication) and the potential to miss constituents showing no activity in the test but possessing interesting structural features.[1] It is therefore suggested that high-efficiency, LC hyphenated techniques should come into play preferably as early as in the screening process to avoid these pitfalls.[34]

The combination of LC separation with UV spectrophotometric detection and MS (Figure 9.1) enables the identification of known compounds, that is, dereplication, in extracts by comparing

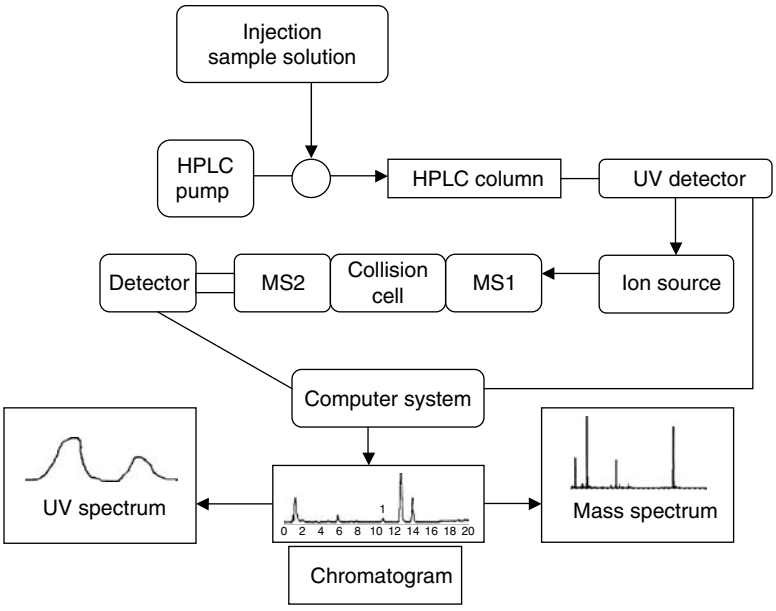

FIGURE 9.1 Diagrammatic representation of an HPLC-UV-MS/MS system.

their LC retention time and UV and MS spectra with those obtained from reference compounds. The combined capacities of separation efficiency and detection sensitivity and selectivity of these hyphenated techniques have been utilized in many laboratories for prescreening of natural products. This usually involves the simultaneous analysis of different classes of compound within a complex matrix. As an example, using LC-ESIMS/MS, Han and coworkers[35] simultaneously identified 10 major compounds including five diterpenoids and five flavonoids in *Isodon rubescens*, a traditional Chinese medicine (TCM) for treatment of cancers. The structures were tentatively assigned from the LC-MS/MS data and by comparison with literature data. In another approach to identify secondary metabolites (polyketides) from *Streptomyces violaceoruber* TU22,[36] UV/Vis diode array detection of the LC eluate revealed seven main peaks, corresponding to its principal polyketide constituents. Comparison with spectra produced from reference compounds quickly divided them into either granaticin or nongranaticin polyketides. Combination with MS data enabled tentative assignment of the known structures.

The characterization and identification of natural products in various plant materials have recently been achieved by us using LC-ESIMS/MS.[37–40] By using both positive and negative MS modes, and a water (0.1% formic acid) acetonitrile gradient solvent system, various compounds from plant materials have been identified, and the ESIMS/MS data were very suitable for characterization of the structures of phenolic compounds (flavonoids, isoflavonoids, lignans, tannins, etc.) and saponins. For most phenolic compounds and saponins, MS under the negative mode will give a clear $[M - 1]^-$ molecular ion peak and MS and MS/MS under positive mode will give more ion peaks for structural elucidation. A representative positive ESIMS is shown in Figure 9.2.

Despite the power of UV and MS detection for locating specific structural features and for providing information about the molecular weight and nature of the substituents, the structure assigned up to this stage can only be considered tentative. Full elucidation regarding the configuration of the substituents on the skeletal structure usually requires complementary information from NMR experiments that can be facilitated using the LC-NMR online technique.[41] In many cases, for known compounds, the proton NMR is adequate. For novel structures, additional information from ^{13}C-NMR and different 2D NMR experiments are often necessary for elucidation.[42]

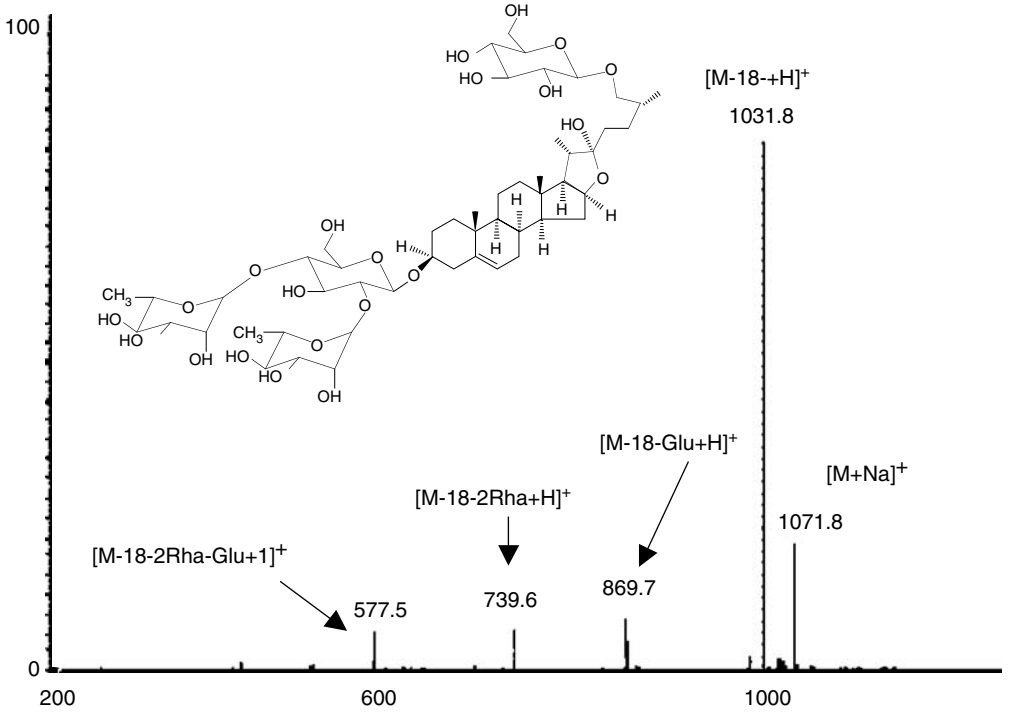

FIGURE 9.2 The positive ESI-MS of protodioscin, a saponin from asparagus (*Asparagus officinalis*) and tribulus (*Tribulus terrestris*).

In summary, each hyphenated technique has its own unique capacity. Mutual complementation by recruiting a set of these tools (LC-DAD [photodiode array], LC-DAD-MS, LC-MS^n, LC-NMR, etc.) will enable challenges confronted at different stages of the product/drug development process to be managed efficiently.

9.4 ROLES OF LC-MS IN QUALITY CONTROL OF HERBAL PRODUCTS

Although herbal medicines are well accepted by the general public, the majority of the clinicians remain skeptical of using herbal therapy. Concerns arise from the poor quality control of herbal products, in particular the fact that lack of consistency of the levels of the active ingredients in these products contributes to variations in efficacy, demonstrated in the clinical trials. As pointed out by Wolsko et al.,[43] the actual dosage used in three clinical trials varied from 80% to 113% of the expected dosage. These studies highlighted the fact that the quality control of these herbal products should be improved. Thus, it is imperative that herbal products be properly defined and standardized by various means to ensure consistent chemical composition at all stages of production so as to achieve reliable clinical efficacy. Otherwise, the credibility of herbal medicines will be continually questioned by clinicians and these medicines cannot be incorporated into mainstream medical care.

Most herbal medicines can be regarded as safe due to their long history of use in health care. However, adverse reactions arising from the consumption of herbal medicines have occurred occasionally and have been a subject of several reviews.[44,45] These adverse reactions can be relatively mild effects such as allergy; however, severe toxicity[6] and liver failure[7] have led to several fatalities. Therefore, the inherent toxicity of some herbal plants should be emphasized and the toxic ingredients removed from the herbal preparation. In the case that the toxic ingredient is also the active ingredient, stringent regulations are urgently needed to control the levels of the constituent(s) to prevent overdosing. Additionally, cases of intoxication have occurred as a result of misidentification of botanical

materials.[46] The authentication of plant materials, especially in the form of dried extracts or fine powders, is very difficult, leading to the possibility of misidentification by the manufacturer. Morphological identification is often unreliable and is costly,[47,48] and PCR-based molecular authentication techniques, although rapid and less expensive, provide no information regarding the phytochemical contents of the plant materials in question. In this regard, chemical fingerprinting is able to give accurate species identification and phytochemical profiling in a single run. One further stumbling block to the more widespread acceptance of herbal medicines is irresponsible manufacturers, a factor that is not helped by the lax regulations on herbal products. In the United States, herbal medicines are sold as dietary supplements under the Dietary Supplement and Health Education Act (DSHEA), and no premarket review of the ingredients is required by the FDA. The FDA can prohibit the sale of an herbal drug only if it is proved unsafe. As a result, irresponsible manufacturers may include synthetic drugs to improve the efficacy of their products.[49–53] Such adulteration with cheaper ingredients has occurred in some ginseng products.[54] These adulterated products could put the health of consumers at risk. With hundreds of new herbal products entering the market every year, fast and robust methods are critical for the regulatory authorities to sieve out adulterants from these products.

Addressing the issues of safety and quality of herbal products is by no means an easy task. Chromatographic fingerprinting presents a formidable analytical challenge since each single herbal plant contains myriads of compounds with different structures and a wide range of polarities, many of which would only be present in trace amounts. Fortunately, the powerful separation ability of current chromatography techniques and the availability of an array of detection methods allow the generation of chemical fingerprints for authentication and quality evaluation of herbal medicines. There are several chromatography techniques commonly used in pharmaceutical analyses, including thin-layer chromatography (TLC), gas chromatography (GC), and HPLC. TLC is still a standard technique for providing a "fingerprint" of herbal medicines in the pharmacopoeia of many countries, such as China and the United States. TLC is simple to use and is inexpensive; however, its separation power is limited and its sensitivity and selectivity are not as good as hyphenated techniques such as GC-MS, HPLC-DAD, and LC-MS. Use of diode array (alternatively referred to as photodiode array) detection may cause a bias in favor of compounds with strong chromophores, whereas weakly UV/Vis absorbing compounds may not be detected. The presence of coeluting impurities together with target compounds also prevents quantification. GC combined with MS can yield very good separation and mass spectra for compound identification. However, HPLC hyphenated techniques (especially LC-MS) are preferred methodologies in many cases due to their versatility and the limitations of GC-MS as previously discussed (Section 9.2.1). In some cases, where standard compounds are not available, either commercially or by laboratory purification, LC-MS/MS is essential to provide data for tentative structural assignments of unknown peaks.[55] The following section of the review will focus on the applications of LC-MS in fingerprinting and standardization, as well as in the detection of toxic components and synthetic adulterants.

9.4.1 Fingerprinting for Authentication of Plant Materials

The quality of the raw materials is fundamental to the quality of the final products. Therefore, proper authentication of plant materials is a critical first step to screen out misidentified plant materials and, possibly, contaminants. An emerging role for LC-MS is in the chemical fingerprinting of plant materials for species identification and authentication, and to differentiate it from related species with similar phytochemical profiles.[56] Indeed, chromatographic fingerprinting is recommended as a means of quality control by the U.S. FDA and the WHO. Such a fingerprint should be as comprehensive as possible, and the peaks should be identified to highlight possible active ingredients as well as toxic compounds. This fingerprint profile, with its characteristic markers, can be employed in quality control at a later stage.

LC-MS can achieve rapid separation and detection of multiple components in herbal plants. However, the fact that phytochemical contents could vary, even for plant materials of the same

species, should be accounted for when interpreting the chromatographic fingerprints. Fortunately, it is observed that the variability is mainly quantitative, whereas qualitatively it is relatively consistent. On the basis of this observation, peak distribution and morphology should be emphasized, whereas individual peak heights may serve as a secondary criterion for authentication.[57]

In the past, often one or relatively few markers have been used for chromatographic fingerprinting.[58] However, it is becoming apparent that multicomponent analysis is necessary.[59] *Psoralea corylifolia* is a popular TCM, and its extracts have been found to possess antitumor, antioxidant, and antibacterial activities. Its chemical constituents include coumarins and flavones such as psoralen, isopsoralen, and psoralidin.[60,61] A chromatographic fingerprint was developed using LC-ESIMS^n.[62] The chromatographic conditions involved an Alltima C18 reverse-phase column eluted with a linear gradient of water–acetic acid (100:0.1, v/v) and acetonitrile from 40% to 70% in 55 min at a flow rate of 1.0 mL/min. Ten batches of *P. corylifolia* samples from two different parts of China were analyzed. The fingerprint patterns revealed the presence of 12 common peaks for *P. corylifolia*, for which the identities of 10 were determined from the MS and MS/MS^n data. Further analysis of *P. corylifolia* from 20 different sources also revealed the presence of the 12 common peaks.

Another well-known TCM, *Salvia miltiorrhiza*, commonly known as Danshen, has been investigated in detail by Hu and coworkers.[57] This plant is used in the United States, Europe, and China for the treatment of cardiovascular and cerebrovascular diseases, and its main chemical constituents are nonpolar diterpenoids and water-soluble phenolics, for example, rosmarinic, lithospermic, and salvianolic acids.[63] An LC-ESIMS/MS^n method has been developed for the simultaneous determination of these two classes of compound. Separation was performed on a C18 column with a gradient of water–acetonitrile–formic acid (90:10:0.4, v/v) and pure acetonitrile. Negative-ion mode was used for detection of phenolics, whereas nonpolar diterpenoids were analyzed in the positive-ion mode. Eighteen characteristic peaks identified in the fingerprint profile were tentatively assigned by analysis of the MS/MS spectrometric data and several of them further confirmed by comparison with reference standards. Analysis of seven samples collected from different parts of China showed good repeatability of this fingerprint pattern. These spectra may be used as fingerprints for reliable authentication and quality evaluation of the corresponding herbal medicine.[57]

A more challenging analytical problem is presented by TCM formulations consisting of several medicinal herbs. Although a mixture of herbs is not common in the United States, it is widely employed in China and other Asian countries. A mixture of herbs presents a very complex chemical profile of thousands of constituents. Several TCM formulations have been profiled using LC-MS. Gan-Lu-Yin is a TCM formulation consisting of ten herbs. It relieves side effects of radiation therapy in the treatment of nasopharyngeal cancer and may be used as a health tonic. LC-DAD-ESIMS analysis of this formula was carried out on a Lichrospher C18 column with a mixture of 0.01% phosphoric acid, acetonitrile, and water as the mobile phase.[64] Several LC-MS fingerprints were obtained using total ion chromatogram (TIC) and SIM techniques, with the latter giving a much better signal-to-noise ratio. In total, 15 marker compounds were identified on the basis of their retention time and mass spectra in comparison with reference standards.

The analytical power of LC-MS is further manifested in the differentiation of closely related plant species. Although in many traditional medicine practices, closely related plant species may substitute for each other, it is recommended to identify plant materials using their scientific names in the view of achieving chemical uniformity.[65] An example is Radix Stemonae. The three species of *Stemona*, that is, *S. tuberosa, S. japonica*, and *S. sessilifolia*, are all recognized as the genuine source material for the herb Radix Stemonae. A method is thus required for their differentiation and authentication. These plants contain Stemona alkaloids with antitussive activities.[66] Stemona alkaloids are not sensitive to direct UV detection due to the absence of UV-absorbing chromophores; thus, a sensitive analytical method was developed using LC-ESIMS/MS.[67] Using a C18 column with a gradient solvent system of water and acetonitrile with 0.1% triethylamine, eight peaks were found in the fingerprint. Among them, six were identified as Stemona alkaloids. An analysis of 30 samples of *S. tuberosa* revealed wide variation in the abundance of each type of the alkaloids

present. Thus, individual peak heights were not very informative. Fortunately, LC-MS analysis showed that *S. japonica* had four additional peaks (tentatively assigned on the basis of MS^n spectra), that can serve as unique markers. Furthermore, *S. sessilifolia* had a unique peak that was not present in either *S. tuberosa* or *S. japonica*. Therefore, each of the species could be differentiated using LC-MS fingerprinting.

Similarly, several species of *Cimicifuga* were distinguished from each other using LC-MS with a turbo ion spray (TIS) interface.[68] Root and rhizomes of *C. racemosa* (black cohosh) have been traditionally used for treating fevers, menstrual cramps, arthritis, and insomnia.[69] Since triterpene glycosides of *C. racemosa* have weak UV absorption, the authors used LC-TIS-MS to examine the fingerprint profile of *C. racemosa* and related herbs. The soft ionization characteristic of TIS enabled assignment of many saponins by their unique molecular weights. For isomers, identification was achieved by comparing the retention time with reference standards. It was found that all the species had some distinctive peaks, which gave them distinctive profiles. Many peaks of *C. racemosa* and about half of the peaks in the three Chinese species could be assigned as known compounds. On the contrary, the Japanese species also contain unique saponins ($MW > 700$) that can be used as markers in LC-MS profiling. Thus, this LC-MS method can help resolve closely related plant species.

9.4.2 Standardization of Herbal Medicines

With the public demanding safe, effective, and high-quality products, there is a pressing need for the standardization of herbal extracts. A chemically consistent product in an appropriate dosage is a prerequisite for achieving clinical effectiveness as well as ensuring safety. This implies that the chemical components in the extract have to be properly defined qualitatively and quantitatively. Practical evaluation of herbs is often based on the measurement of marker compounds, which ideally are the chemical constituents whose levels correlate with the activity in a bioassay and in clinical testing.[58] These marker compounds should be employed to monitor the quality of raw materials, manufacturing intermediates, and the final products. In addition, they are useful in assisting the development and validation of the manufacturing process. For example, the extraction protocol can be optimized to concentrate bioactives while minimizing potential toxic ingredients. In this section, the applications of LC-MS to accomplish batch-to-batch consistency of a large number of samples will be summarized on the basis of the approach adopted for quantification of the marker constituents, that is, using an online UV detector for quantification with MS for identification, SIM mode in MS, and SRM mode in tandem MS/MS.

9.4.2.1 UV Detector for Quantification with MS for Identification

LC-UV is the most widely employed method for the quantitative analysis of herbal products. It often gives excellent reproducibility and is well suited for routine analytical work. Thus, it is not surprising that a review of recent literature indicated that UV detectors are still widely employed for the quantification of marker compounds, with the MS detectors mainly used for qualitative evaluation.

As an example, an LC-UV and LC-MS method was developed for the quality evaluation of Alismatis Rhizome, a traditional Chinese and Korean medicine.[70] Alisol B 23-acetate is a major component in this herb and it showed promising cytotoxic activity against several tumor cell lines.[71] Thus, alisol B 23-acetate was chosen as a marker for quality evaluation of Alismatis Rhizome.[71] LC was performed using a Zorbax 300SB C18 column with 75% acetonitrile as the mobile phase and UV detection at 215 nm. Reference standards were available for peak confirmation. Regression analysis revealed good linear relationship ($R = 0.9996$) between the peak area and the concentration of alisol B 23-acetate over the range of 0.05–1 mg/mL. The limit of detection (LOD) was 0.5 μg/mL and reproducibility was 1.53 and 2.98% for intra and interday analysis, respectively.

Generally, UV absorbance-based quantification, accompanied by good HPLC separation, is good enough for compounds with strong UV absorption such as phenolic compounds, and MS

detection really does not add much advantage. However, the sensitivity is insufficient for compounds with poor chromophores, such as saponins and terpenoids. In addition, UV detection is nonspecific in nature. Thus, it cannot easily distinguish an analyte from a coeluting interference, which may contribute to misleading results.[72] Finally, UV quantification has little tolerance to matrix effects, which necessitates the baseline separation of all the marker analytes. This can result in long run times (>60 min) and a consequent low throughput.

9.4.2.2 Selected Ion Monitoring Mode in MS

Although measurement precision is more variable depending on the type of MS detector chosen, MS detection is more selective compared to UV detection. However, LC-MS equipment can be more expensive and requires more operational skills. Thus, it is recommended that LC-MS be used in nonroutine work[72] and for applications where UV detection is not satisfactory. One major method employed for MS-based quantification is the SIM mode. In this mode, the mass spectrometer behaves as a selective detector that records one or more preselected ions. In contrast to the TIC, the time spent on scanning unwanted *m/z* values is effectively reduced. As a result, sensitivity is also enhanced by a factor of 10^2–10^3. LC-MS in SIM mode often gives superior selectivity compared to UV detectors for many natural products, although in some cases, interference with ions with identical *m/z* may occur.[12]

Recently, an LC-MS method with SIM was developed for the analysis and standardization of artemisinin in *Artemisia annua* L.[73] Using an Agilent 1100 system with an ODS3 column, the mobile phase was water (0.1% formic acid) and acetonitrile in gradient with an 11 min run time per sample for the direct detection and quantification of artemisinin, a sesquiterpene lactone with antimalarial activity. To accurately analyze artemisinin, SIM was used to record the abundance of the $[M - 18 + H]^+$ ion peak at *m/z* 265.3 under positive-ion MS mode, with a scan range between *m/z* 250 and 270. Quantification was based on the LC-MS peak area of artemisinin compared to a standard curve generated with pure artemisinin. The linearity range of detection was found to be 5.141–82.25 μg/mL for artemisinin, suggesting that this protocol is a very sensitive method for its quantitative analysis.

LC-MS using SIM mode is also applicable for sensitive and selective quantification of compounds in complex, multiherb mixtures. For the quality control of the injectable TCM formulation Naodesheng, an LC-APCIMS method was developed for the simultaneous identification and quantification of ten major bioactive components.[74] Injectable Naodesheng consists of five herbs and it is used in the treatment of stroke. Separation was performed using a Luna C18 column with a stepwise gradient of water and methanol (30–70% methanol in 0–8 min, and 70–90% methanol in 12 min). To enhance the stability of the molecular ion, 0.1% formic acid was added to the mobile phase. Ten flavones and ginsenosides were resolved and identified by comparison with reference standards. A SIM method was established by first obtaining the fragmentation pattern using direct APCIMS, and then the abundant fragment for each compound was selected for SIM quantification. Good linearity ($R > 0.99$) was obtained from the calibration curves, and the reproducibility of the results was acceptable with an intraday RSD of less than 5% for all the compounds. The sensitivity of detection was high and all compounds had a limit of quantitation (LOQ) of <0.2 μg/mL. This LC-MS method is particularly suitable for quantifying ginsenosides as these compounds often have poor UV chromophores.

9.4.2.3 Multiple-Reaction Monitoring Mode in Tandem MS/MS

Multiple-reaction monitoring (MRM), as a quantitative technique, provides even greater selectivity than SIM. It is usually performed using a tandem MS-capable instrument such as a QqQ MS. In MRM, one or more preselected precursor ions, often the most abundant ones, are allowed to pass through the first mass selector and are fragmented by CID. For each precursor ion, one unique product ion is selected and allowed to pass the next analyzing stage to be detected.[12] The combination

of two selection events confers greater selectivity as well as excellent sensitivity due to improvement in signal-to-noise ratio.

Ginkgo biloba is one of the most popular herbal medicines around the world. It has a wide range of pharmacological activities, and clinical studies indicate that it has a positive impact on cognitive functions in Alzheimer's disease.[75] Bioactive components in ginkgo extracts include flavonoid glycosides, ginkgolides, and bilobalide.[76] As ginkgolides lack UV chromophores, an LC-MS/MS method was subsequently developed for their analysis.[77] To optimize the MRM monitoring conditions, tandem MS/MS analysis of pure standards was carried out to determine the most abundant product ions. Using negative-ion electrospray MS, abundant $[M-H]^-$ ions were formed. Each deprotonated ion was then subjected to CID, and the subsequent tandem mass spectra revealed that the loss of two carbon monoxide molecules $[M-H-2CO]^-$ was the dominant fragmentation pathway. Thus, for quantification purpose, the $[M-H]^-$ and $[M-H-2CO]^-$ ions of ginkgolides were chosen as the precursor and product ions, respectively. LC separation was achieved using a Waters Xterra C18 column with a linear gradient of 10–70% aqueous methanol in 14 min. Good linear correlation coefficients ($R > 0.995$) were achieved and the reproducibility was acceptable (RSD <5% intraday and <8% interday). Furthermore, this tandem MS method demonstrated 10-fold higher sensitivity than previous LC-MS methods,[78] with a LOD ranging from 1.6 to 50 pg and a LOQ of 4–150 pg.

The selectivity of MS/MS in the MRM mode is also valuable for surmounting challenges presented by complex, multiherb preparations. Two typical examples are the Chinese herbal formulations Shiau-feng-saan and Dang-guei-nian-tong-tang. An LC-MS/MS method was configured for detection of five marker compounds, that is, matrine, oxymatrine, ferulic acid, mangiferin, and glycyrrhizin.[79] These markers are major constituents of Sophorae Radix, Angelica sinensis Radix, Anemarrhenae Rhizoma, and Glycyrrhizae Radix that are present in both formulations. LC separation was achieved using a C18 column with step gradients of 0.005% trifluoroacetic acid and acetonitrile as the mobile phase. Instead of monitoring five transitions simultaneously across the entire chromatographic run, the MS scan was divided into four segments in which the MRM scan was selectively optimized to monitor one or two markers for a fixed time period corresponding to their expected elution time, thereby maximizing the sensitivity. Quantification of all the markers was not perturbed by interference from the complex matrix. Regression equations revealed good linearity ($R > 0.997$) over a wide concentration range. The LOD was 20 ng/mL except for glycyrrhizin (60 ng/mL), and recoveries of the marker compounds were all >90%.

Although a higher start-up cost for a tandem-capable MS/MS system is required to perform MRM quantification compared with single-stage MS, with the increasing accessibility to tandem mass spectrometers, this technique will likely see its golden age for standardization of herbal products in the near future.

9.4.3 LC-MS for Detection of Toxic Components and Synthetic Adulterants

Despite the fact that many traditional herbal medicines have a long history of human consumption, inherent toxicity of some of their constituents should not be underestimated. Intoxication from consumption of herbal medicines has also been reported as a result of misidentification and adulteration. The roles of hyphenated LC techniques in assisting proper identification of herbal species have previously been discussed (Section 9.4.1). On the contrary, even nontoxic herbs can have side effects if inappropriately dosed, such as the excessive consumption of ginseng, which may cause nervousness and depression.[80] Thus, precise species identification and formulation, and detection of trace levels of potent toxicants are of critical importance.

9.4.3.1 Detection of Aristolochic Acids I and II in Herbal Products

A well-known result of misidentification is the "Chinese herb nephropathy", which was caused by replacement of *Stephania tetrandra* with *Aristolochia fangchi* in a slimming formulation.[81,82] *A. fangchi* contains aristolochic acids I and II (Figure 9.3) which are known as nephrotoxins and

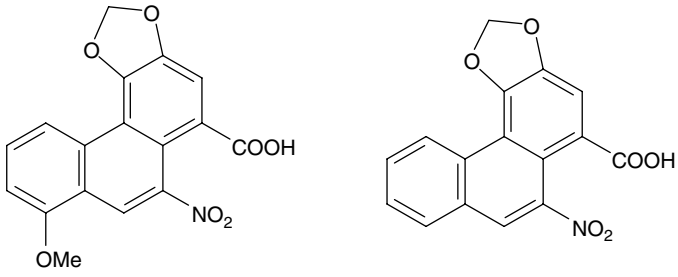

FIGURE 9.3 The structure of aristolochic acid I and II.

carcinogens.[83] A case study of 104 patients revealed that 43 of them developed end-stage renal disease and another 17 had urothelial carcinoma.[83] Since the outbreak of the "Chinese herb nephropathy", several LC-MS methods have been developed for the detection and quantification of aristolochic acids I and II in herbal medicines. The primary aim of these studies was to achieve the lowest possible detection limits, since traces of aristolochic acids pose a health risk.[84]

9.4.3.1.1 Solid-Phase Extraction

The combination of solid-phase extraction (SPE) for clean-up of extracts followed by LC-MS analysis in the SIM mode has enhanced the sensitivity of detection of aristolochic acids I and II. SPE was accomplished by first extracting aristolochic acids from the methanolic herbal extracts onto a Chromabond C18 Hydra column, followed by column washing (water) and elution (methanol: tetrahydrofuran, 80:20, v/v). Reverse-phase LC analysis was completed within 21 min employing a linear gradient of methanol (0.5% acetic acid)–water (0.5% acetic acid), from 60 to 100% methanol. Using APCI as the ionization source, a strong characteristic fragment was found at *m/z* 295, which corresponds to the $[M - NO_2]^+$ ion. Compared to full-scan mode (LOD = 15 ng), the SIM mode at *m/z* 295 achieved considerably higher sensitivity (LOD = 2 ng).[84] Lee and coworkers[85] also employed a SPE-LC-MS approach for the detection of both aristolochic acids I and II, but in this case, a two-step SPE was applied using a reverse-phase C8 bonded cartridge and an anion exchange QMA cartridge in sequence with different washing and cleaning conditions. Aristolochic acids I and II were baseline resolved in HPLC analysis using a step gradient of 20 mM ammonium acetate and acetonitrile. ESI-MS was employed in the SIM mode, monitoring *m/z* 359 $[M + NH_4]^+$, *m/z* 324 $[M + H - H_2O]^+$, *m/z* 298 $[M + H - CO_2]^+$, and *m/z* 296 $[M + H - NO_2]^+$ for aristolochic acid I and *m/z* 329 $[M + NH_4]^+$, *m/z* 294 $[M + H - H_2O]^+$, and *m/z* 268 $[M + H - CO_2]^+$ for aristolochic acid II. A comparison of samples with or without SPE treatment revealed that SPE significantly improved detection by eliminating the majority of interferences from the plant matrix, resulting in a much better signal-to-noise ratio. Thus, SPE significantly facilitated the analysis of the aristolochic acids in complex herbal mixtures.

9.4.3.1.2 Tandem Mass Spectrometric Analysis

Another approach to circumvent the problem of interferences is to take advantage of the unique selectivity offered by LC-tandem MS/MS analysis.

To enhance the sensitivity of detection, various ESI- and APCI-based approaches were investigated to maximize the ion current of either the $[M + H]^+$ or $[M + NH_4]^+$ ions for subsequent CID experiments.[86] It was found that APCI offered the better ion response. Using a C18 column with a gradient mixture of methanol and 0.1% ammonium acetate at basic pH (7.4), aristolochic acid I and II were baseline-separated and both formed abundant $[M + NH_4]^+$ ions (aristolochic acid I, *m/z* 359; aristolochic acid II, *m/z* 329). High-pressure liquid chromatography-MS/MS analysis revealed that monitoring a single product ion formed from $[M + NH_4]^+$ ions did not result in

unambiguous detection due to the presence of coeluting impurities with similar product ions. Thus, a full product ion scan was necessary for confirmation, and quantification was carried out by analyzing the extracted single ion chromatograms of $[(M + NH_4) - NH_3 - CO_2]^+$, that is, *m/z* 298 and *m/z* 268 for aristolochic acids I and II, respectively. In a sample of low interference, this method was acceptable. However, it was observed that in some samples, high concentrations of coeluting impurities of *m/z* 359 were present, which suppressed product ion formation from aristolochic acid I. Instead, product ions from the impurities dominated, which prevented meaningful interpretation. In this case, confirmation was achieved by MS^3 analysis. Thus, CID of the $[(M + NH_4) - NH_3 - CO_2]^+$ ion (*m/z* 298) resulted in diagnostic losses of 17, 30, and 46 amu and gave a single peak for the *m/z* 359 → 298 → 268 MS^3 transitions. Depending on the level of interferences, the LOD ranged from 250 pg to 2.5 ng.

In another study, Jong and coworkers[87] exploited an acidic mobile phase (a gradient of 0.005% trifluoroacetic acid and acetonitrile) for LC separation in combination with ACPI-MS/MS for detection. Under acidic conditions, the aristolochic acids were better separated from impurities, and incorporation of trifluoroacetic acid greatly enhanced the ion count of $[M + H - H_2O]^+$. For aristolochic acid I, $[M + H - H_2O]^+$ (*m/z* 324) was fragmented by CID to give two product ions at *m/z* 280 ($[M + H - H_2O - CO_2]^+$) and *m/z* 265 ($[M + H - H_2O - CO_2 - CH_3]^+$). However, $[M + H - H_2O]^+$ of aristolochic acid II could not be efficiently fragmented. Instead, the base peak $[M + H + CH_3OH - H_2O]^+$ was subjected to CID to give product ions at *m/z* 294 ($[M + H - H_2O]^+$) and *m/z* 266 ($[M + H - H_2O - CO]^+$). Subsequently, MRM analysis was employed to monitor the transitions *m/z* 324 → 280 for aristolochic acid I and *m/z* 326 → 294 and *m/z* 326 → 266 for aristolochic acid II. Detection limits were at the lower ng/mL levels, which were similar to those reported by Kite and coworkers.[86]

9.4.3.2 Sieving out Adulterants from Herbal Products

The final issue concerning the safety of herbal medicines that will be addressed in this review is the presence of synthetic adulterants. The inclusion of prescription drugs in herbal products could be the result of irresponsible manufacturers' attempts to boost the clinical efficacy of their products. Analysis of 2609 TCM samples collected from major Taiwan hospitals revealed that 23.7% of the TCMs were adulterated.[50] In another report from Singapore, 4.5% of 2080 samples of TCMs investigated were found to contain undeclared drugs.[88] Other small-scale surveys from the United States,[89] the United Kingdom,[90] and Japan[91] all indicated that such adulterations are a common occurrence around the world. Adverse reactions may arise and diagnosis is difficult as the patients involved frequently hide their use of herbal medicines from the physicians.[92] The followings are a few examples illustrating the potential of LC-MS for safeguarding health of consumers by rapidly sieving out harmful adulterants.

One Indonesian powdered herbal preparation was reported to be adulterated with the synthetic drugs phenylbutazone, caffeine, and oxyphenbutazone. Accordingly, an LC-MS/MS method was developed for their detection and quantification.[93] The $[M + H]^+$ ions of the LC isolates were selected as precursor ions, which were fragmented to produce product ions. Transitions monitored were *m/z* 309.3 → 120.0 for phenylbutazone, *m/z* 195.1 → 138.0 for caffeine, and *m/z* 325.1 → 204.0 for oxyphenbutazone. Excellent linear correlations were observed for phenylbutazone and caffeine, $R = 0.9999$ for 0.1–10 μg/mL and $R = 0.9975$ for 1–50 μg/mL concentration ranges. The LODs for the three compounds were found to be 3.69, 0.84, and 2 ng/mL, respectively. Using this method, it was found that each sachet of the powdered herb contained 43.17 mg phenylbutazone and 3.23 mg caffeine.

In another study, LC-MS/MS was applied to determine nine common illegal adulterants, such as sildenafil and famotidine, in herbal medicines.[53] Highly selective MS/MS analysis was necessary to minimize interferences. For each drug, the three most intensive transitions were selected and monitored simultaneously by MRM to ensure specific detection and quantification. Results were considered positive if well-defined peaks occurred in all the three transitions with similar

retention time, and the peak ratios were within a given threshold. The LODs of the adulterants were within the range of 0.05–1.5 ng/mL. This method is rapid (5 min) and is capable of detecting multiple adulterants in a run by adjusting the MRM settings. Over 200 samples have been screened, with 74 of them being determined positive for adulterants.

Perhaps the most comprehensive method for the detection of illegal drug adulterants was proposed by Bogusz et al.[94] Using LC-ESIMS/MS, 80 common synthetic adulterants including analgesics, antibiotics, antidiabetics, antiepileptics, and weight-reducing drugs, were analyzed and quantified. The compounds were resolved on a Superspher 100 RP-18 column with a stepwise gradient of 10 mM ammonium formate (pH 3.0) and acetonitrile in 75 min. Sixty of them were analyzed in the positive-ion ESI mode with the rest analyzed using the negative-ion mode. To ensure the specificity of detection, for most of the drugs, two transitions were registered in MRM mode, a feature considered to be sufficient for unequivocal identification. Although some drugs eluted together, their quantification was not hindered as the product ions were quite different. The LODs ranged from 5 pg to 1 ng per sample and the recoveries ranged from 63% to 100%. Analyses of herbal medicines have revealed the presence of undeclared drugs such as tadalafil, sildenafil, testosterone decanoate, and caffeine.

9.5 ROLES OF LC-MS IN METABOLISM AND PHARMACOKINETIC STUDIES OF NATURAL PRODUCTS

Establishing the pharmacological basis for efficacy of an herbal product is a constant challenge due to their complex composition and the ever-increasing list of their potential active constituents. *In vitro* bioassays normally are cheap and relatively easy to perform. However, the relevance of the findings from these assays to their real efficacy in humans and animals is a premise based on the requirement that a sufficient concentration of active constituents can reach the targeted site(s) of action. Thus, the bioavailability, metabolic pathways and elimination routes, and pharmacokinetics of bioactive natural products are important issues to link data from pharmacological assays with clinical effects. Although there is usually detailed information available about the pharmacokinetics of synthetic drugs, this is rarely the case for herbal medicines due to the complexity of their extracts and the lack of knowledge of the active principles in these products. As the concentrations of single compounds in the finished herbal products are usually in the lower milligram range per dose and the resulting plasma concentrations are in the picograms-per-liter range, the analytical methods used in bioavailability and pharmacokinetic studies need to be sufficiently sensitive and potentially more sensitive than those for synthetic drugs. Another important issue is selectivity, as often one or several groups of compounds need to be analyzed and there is an increased chance of encountering interferences from plasma-derived compounds. The third issue is the potentially extensive metabolism and biotransformation of natural products. The bioactives are often subject to metabolism by the intestinal microflora prior to absorption, making the analytical work more challenging.[95]

Today, increasingly advanced techniques are used in metabolism and pharmacokinetic studies of natural products; among them, LC-MS/MS is found to be very suitable for quantitative analysis in pharmacokinetic studies of natural products and also for determining their metabolic pathways and elimination routes. As an example, the metabolism of silibinin, the main isomer of a group of flavanoids extracted from the seeds of the milk thistle weed, a common herb that is widely used to maintain liver health and for the treatment of liver disorders, was investigated using HPLC-ESI-ion trap MS.[96] Silibinin was found to produce one major metabolite and at least two minor metabolites when incubated with human liver microsomes. Detailed LC-MS/MS studies confirmed that the major metabolite is demethylated silibinin and the two minor metabolites are monohydroxy- and dihydroxy-silibinin. Another example is the quantitation of picroside I and kutkoside (Figure 9.4), the active constituents of the herbal hepatoprotectant "Picroliv", in rabbit plasma by a LC-MS/MS method.[97] In this research, MRM was performed on an API-4000 LC-MS/MS mass spectrometer. The analytes and internal standard (amarogentin) were first extracted using Oasis HLB SPE

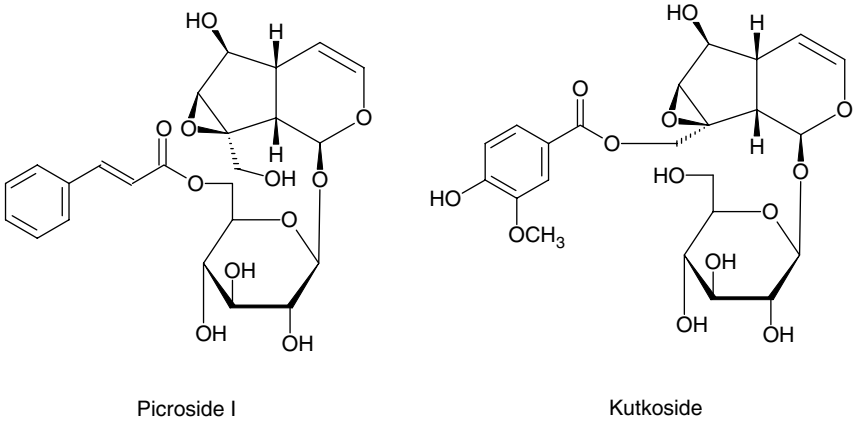

FIGURE 9.4 The structures of picroside I and kutkoside.

cartridges and then separated on an Spheri RP-18 column (100 mm × 4.6 mm i.d., 10 μm) coupled with a guard column using acetonitrile:MilliQ water (50:50, %v/v) as the mobile phase at a flow rate of 1 mL/min. Quantitation was carried out with negative-ion ESI in the MRM mode. The precursors to product ion transitions for picroside I, kutkoside, and amarogentin were *m/z* 491 → 147, 199; 511 → 167, 235; and 585 → 227, respectively. The linearity of response in plasma was observed over a concentration range of 1.56–400 ng/mL with a LOD of 0.5 ng/mL for both picroside I and kutkoside. The recoveries from spiked control samples were >60% and >70%, respectively. Finally accuracy and precision of the validated method were within the acceptable limits of <20% at low and <15% at other concentrations.

9.6 SUMMARY AND CONCLUSIONS

The availability of different ionization interfaces has enabled the marriage of two previously standalone techniques, LC and MS. Through the past several decades of frequent participation in overcoming numerous analytical challenges in a wide range of scientific disciplines, LC-MS and related hyphenated techniques have been considered invaluable, especially in the field of natural product research where their role as a dereplication tool is manifested to the greatest extent. Although a combinatorial approach to the synthesis of chemically diverse compound libraries has been highly valued in recent years, direct acquisition from natural sources has not yet waned, due to the irreplaceable advantages associated with it and may in fact be experiencing a resurgence. At the present time, the extraordinarily complex nature of most herbal extracts and the possibility of the coexistence of toxicants remain the major hindrance to their incorporation into mainstream medical care in many Western countries. With the development of LC-MS and related hyphenated techniques, the quality of natural products and herbal medicines will be improved dramatically and we should see a brighter future for them worldwide.

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10 Application of High-Speed Countercurrent Chromatography to the Isolation of Bioactive Natural Products

Huabin Li and Feng Chen

CONTENTS

10.1 INTRODUCTION

Natural products continue to play an important role in pharmaceutical, cosmetics, and food industries. About 50% of the drugs introduced into the market during the last 20 years are derived directly or indirectly from natural sources. In the future, natural products will continue to play a part as protagonists for discovering new drugs.[1] Discovery of the well-known antitumor drug taxol and

the antimalarial drug artemisinin further stimulated the interest in searching for biologically active compounds from natural sources. For lead compound discovery, or for scientific validation of a traditional medicinal plant or a phytopharmaceutical, the bioactive components in natural sources need to be identified. How to access these diverse compounds effectively from natural sources is an exciting challenge. The isolation of bioactive compounds from natural sources, such as plant extracts, microbial fermentation products, or animal tissues, presents a number of practical difficulties because target compounds are often present as minor components of extremely complex mixtures.

High-speed countercurrent chromatography (HSCCC) is a relatively new, all-liquid separation technique. Because there is no solid support matrix in HSCCC column, it eliminates irreversible adsorptive loss, denaturation, and contamination of samples from the solid support matrix used in the conventional chromatographic column.[2] HSCCC is fundamentally a preparative separation technique and has now been widely used for isolation and purification of various natural and synthetic products.[3] Several monographs and reviews have been published,[4–12] in which examples of separations in the various fields can be found. This chapter reviews recent applications of HSCCC to the isolation and purification of bioactive natural products. A comprehensive review of the literature on the use of HSCCC in isolation of natural products is not intended; only selected examples are presented and discussed to illustrate the capabilities of this technique and to serve as an entry point for further applications of the various HSCCC methods.

10.2 HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

10.2.1 Principle

HSCCC is a liquid–liquid partition chromatography with a support-free liquid stationary phase. To retain the liquid stationary phase when the liquid mobile phase is pushed through it, a centrifugal force field is used.[11] Separation in HSCCC is based purely on the partition of the solutes between the two liquid phases since no solid support matrix is employed to retain the stationary phase. The advantage of a liquid stationary phase is that the solutes have access to the whole volume of the stationary phase, rather than the interface between the mobile phase and the surface of the solid stationary phase as in many other chromatographic techniques, leading to a high efficiency of extraction. In addition, either phase of the two-phase solvent system can be used as the mobile phase, and the phase role can even be changed during a run to elute all the injected species from the column while, unlike backflush, the separation is still progressing after phase reversal. HSCCC is mainly a preparative purification technique, and crude extracts or semipure fractions can be chromatographed with sample load ranging from milligrams to multigrams. Aqueous and nonaqueous solvent systems can be used, and the possibilities for the selection of a two-phase solvent system are almost limitless. The separation of compounds with a wide range of polarities is possible.

10.2.2 Apparatus

A typical HSCCC system consists of a mobile phase reservoir, a pump, an injection valve, a column, a detector, a fraction collector, and a recorder or data processor, which is very similar to an HPLC unit except for the column (Figure 10.1). The liquid stationary phase is held in an inert, coiled tubular column by a centrifugal force field while the immiscible mobile phase passes through. The whole HSCCC machine could be considered as a column of an HPLC system.[11] The use of HSCCC instrumentation requires some simple but special technical knowledge because the selection of the experimental conditions and the practical separation procedure are quite different from those of HPLC.

10.2.3 Methodology

The selection of the two-phase solvent system is the most important, and is also the most difficult step because any change of the mobile phase composition is likely to change the stationary phase composition or volume; it is estimated that about 90% of the entire work in HSCCC is spent on this

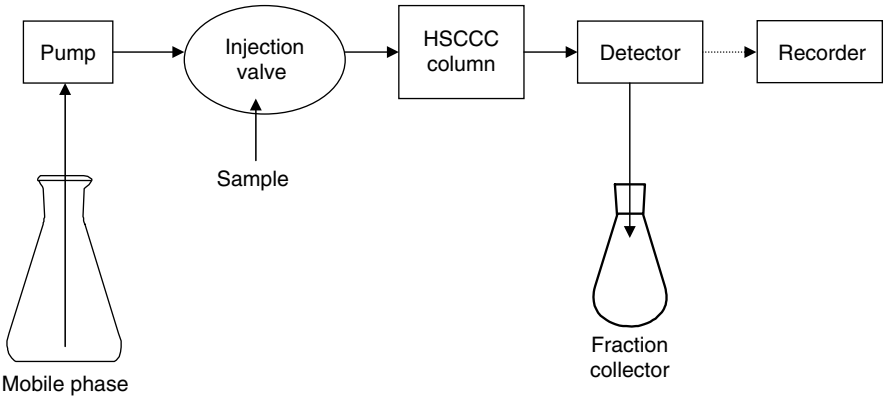

FIGURE 10.1 Schematic diagram of HSCCC system.

process. To select a suitable two-phase solvent system, previous literature on HSCCC should be carefully consulted, and some rules need be considered. For example, the target compound should be soluble and stable in the solvent system, the settling time of the solvent system should be short (<30 s), the partition coefficient (K) of the target compound should fall within a suitable range (usually between 0.5 and 2), and the retention of the stationary phase should be satisfactory.[3,4] The higher the retention of the stationary phase, usually the better the peak resolution. A small K value usually results in a poor resolution, while a large K value tends to give better resolution but broader peaks and more dilute peak fractions due to a longer elution time.[7,11]

Either phase of the two-phase solvent system can be used as the mobile phase. However, when the lower phase is used as the mobile phase, the retention of the stationary phase is usually more stable, but the lower phase should be pumped into the column in the head-to-tail elution mode. If the upper phase is used as the mobile phase, it should be pumped into the column in the tail-to-head elution mode. A lower flow-rate of the mobile phase usually gives a higher retention level of the stationary phase, which will improve the peak resolution although a longer separation time is required. A lower revolution speed of the HSCCC column will reduce the volume of the stationary phase retained in the column leading to lower peak resolution. The sample may be dissolved in either phase or in a mixture of the two phases, and injection volume is usually less than 5% of the total column capacity.[3,7,11]

10.3 APPLICATION OF HSCCC TO THE ISOLATION OF BIOACTIVE NATURAL PRODUCTS

The versatility of HSCCC makes it an ideal method for the isolation of bioactive natural products. One of the main aims of HSCCC application is to obtain bioactive natural products in quantities sufficient for the study of their biological, pharmacological, and clinical effects, both *in vitro* and *in vivo*. The production of bioactive compounds as pure reference standards for quality control purposes is another essential aspect of HSCCC application.[12–14] Many HSCCC methods have been developed for the isolation and purification of bioactive natural products from complex crude extracts or semipurified fractions. Different HSCCC methods will be discussed in detail with a number of examples of HSCCC application for the isolation of bioactive natural products.

10.3.1 Isocratic Elution

Since HSCCC is an all-liquid chromatographic technique, there is no solid support matrix in HSCCC column and any change of the mobile phase composition is likely to change the stationary phase composition or volume. Therefore, isocratic elution is used most widely to develop HSCCC methods.

10.3.1.1 One-Step HSCCC Separation Using Isocratic Elution

10.3.1.1.1 Carotenoids and Squalene from Microalgae

The beneficial effects of carotenoids have been well documented from the numerous clinical and epidemiological studies in various populations. Carotenoids have been proposed as cancer prevention agents, life extenders, and the inhibitors of ulcers, heart attack, and coronary artery disease. With the rising global concern to avoid the undesirable effects of synthetic food additives, such as allergy, hypersensitivity, intolerance, and childhood hyperactivity, a number of attempts have been made to find new natural sources of carotenoids. Certain microalgae may serve as a continuous and reliable source of carotenoids because they can be cultivated in bioreactors on a large scale. Furthermore, the qualities of the microalgal cells can be so controlled that they may contain no herbicides, pesticides, or any other toxic substances by using clean nutrient media for growing the microalgae.[15,16] Some microalgae may also produce squalene, a highly unsaturated aliphatic hydrocarbon, which is normally used in its natural form as a moisturizing or emollient agent in pharmaceuticals and cosmetic preparations. More importantly, it is a potential oxidation inhibitor, and can protect cells against free radicals, strengthen the body's immune system, and decrease the risk for various cancers. Several HSCCC methods have been developed in our laboratory for the isolation and purification of these bioactive compounds from the microalgae. In the following figures, panel (a) shows the analytical and panel (b) the preparative separation by HSCCC.

For isolation and purification of lutein (**1**) from the microalga *Chlorella vulgaris*, an analytical HSCCC instrument was first used for the preliminary selection of a suitable two-phase solvent system composed of *n*-hexane–ethanol–water (4:3:1, v/v) (Figure 10.2a). Then, preparative HSCCC was successfully performed using the above solvent system, which yields lutein in 98% purity from 200 mg of the crude extract in a one-step separation (Figure 10.2b).[17]

Crude astaxanthin (**2**) was obtained from the microalga *Chlorococcum* sp. by extraction with organic solvents after the astaxanthin esters were saponified. Preparative HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (5:5:6.5:3, v/v) was successfully performed yielding astaxanthin in 97% purity from 250 mg of the crude extract (Figure 10.3).[18]

Zeaxanthin (**3**), a structural isomer of lutein, could be isolated and purified from the cyanobacterium *Microcystis aeruginosa* by HSCCC with a two-phase solvent system composed of

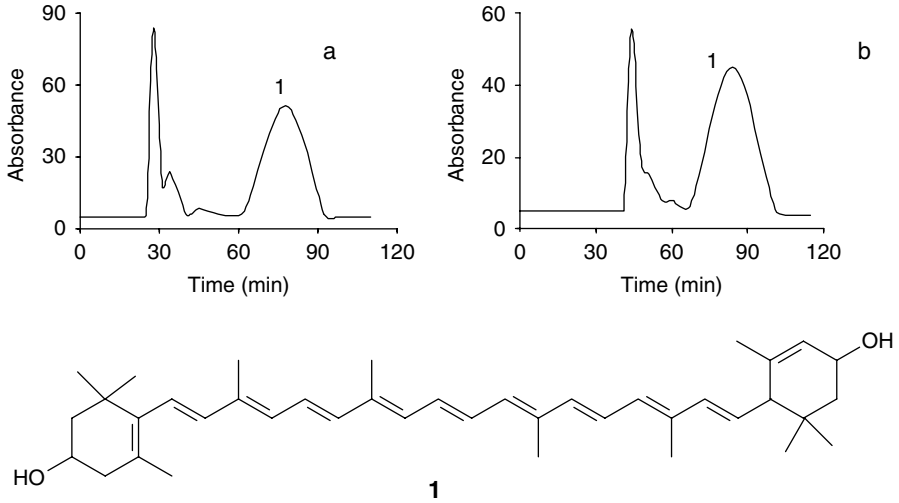

FIGURE 10.2 Chromatogram of crude lutein from the microalga *Chlorella vulgaris* by (a) analytical HSCCC separation with a 30 mL capacity column and 10 mg sample and (b) preparative HSCCC separation with a 230 mL capacity column and 200 mg sample. **1**: lutein. (Reprinted from Li, H.B., Chen, F., Zhang, T.Y., Yang, F.Q., and Xu, G.Q., *J. Chromatogr. A*, 905, 151, 2001. With permission.)

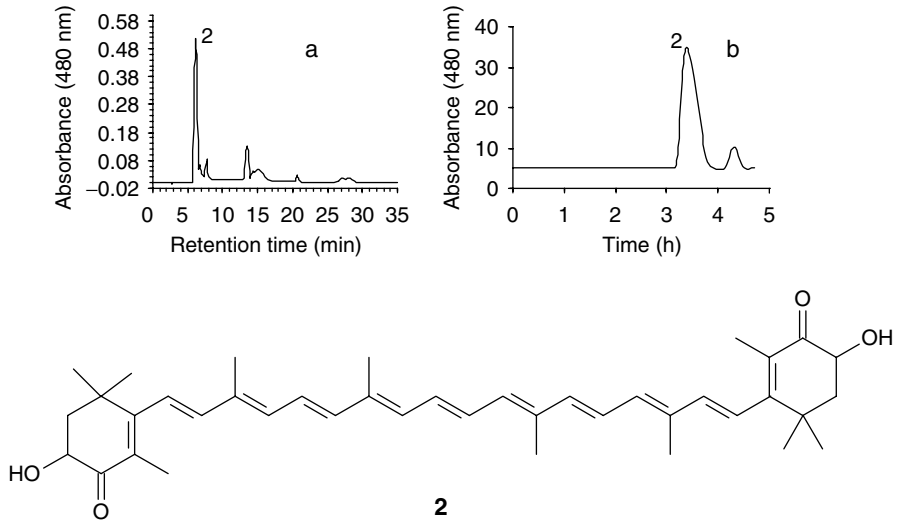

FIGURE 10.3 (a) HPLC chromatogram of crude astaxanthin from the microalga *Chlorococcum* sp. (b) HSCCC chromatogram of crude astaxanthin from the microalga *Chlorococcum* sp. **2**: astaxanthin. (Reprinted from Li, H.B. and Chen, F., *J. Chromatogr. A*, 925, 133, 2001. With permission.)

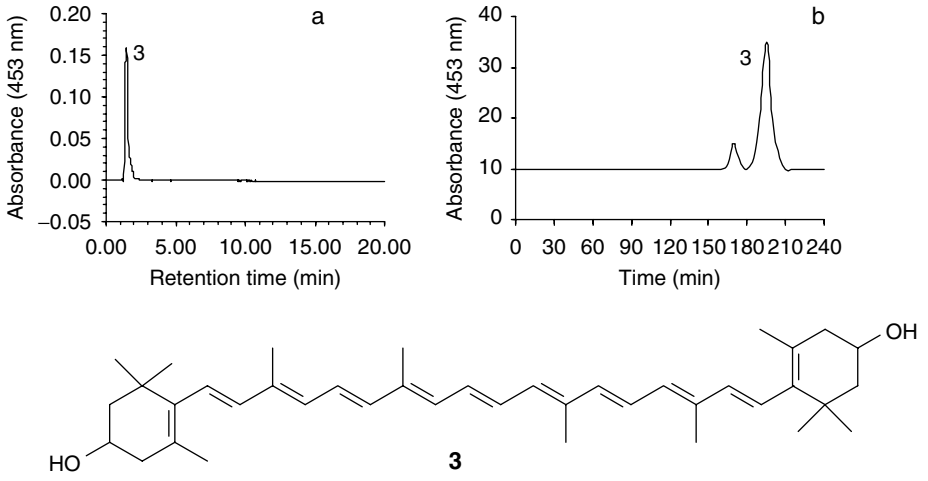

FIGURE 10.4 (a) The HPLC chromatogram of purified zeaxanthin from (b) the HSCCC of crude zeaxanthin from the microalga *Microcystis aeruginosa*. **3**: zeaxanthin. (Reprinted from Chen, F., Li, H.B., Wong, R.N.S., Ji, B., and Jiang, Y., *J. Chromatogr. A*, 1064, 183, 2005. With permission.)

n-hexane–ethyl acetate–ethanol–water (8:2:7:3, v/v) (Figure 10.4). The purity of zeaxanthin was approximately 96.2%, and the recovery was 91.4%. This was the first report of zeaxanthin being successfully separated and purified from microalgae.[19]

Saponification and organic solvent extraction of the microalga *Chlorella zofingiensis* provided a crude extract of canthaxanthin (**4**). Preparative HSCCC, with a two-phase solvent system composed of *n*-hexane–ethanol–water (10:9:1 v/v), was successfully performed yielding canthaxanthin in 98.7% purity from 150 mg of the crude extract containing 2.1% canthaxanthin in a one-step separation (Figure 10.5). The recovery of canthaxanthin was 92.3%.[20]

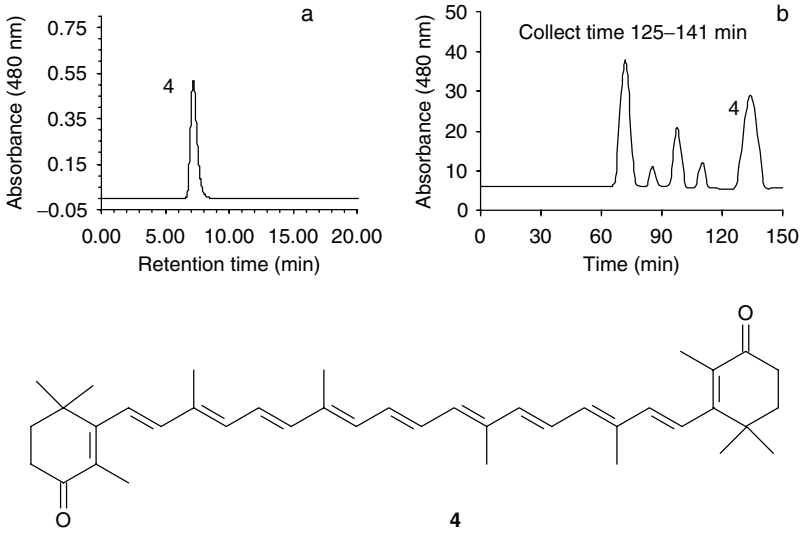

FIGURE 10.5 (a) The HPLC chromatogram of purified canthaxanthin from (b) the HSCCC of crude canthaxanthin from the microalga *Chlorella zofingiensis*. **4**: canthaxanthin. (Reprinted from Li, H.B., Fan, K.W., and Chen, F., *J. Sep. Sci.*, 29, 699, 2006. With permission.)

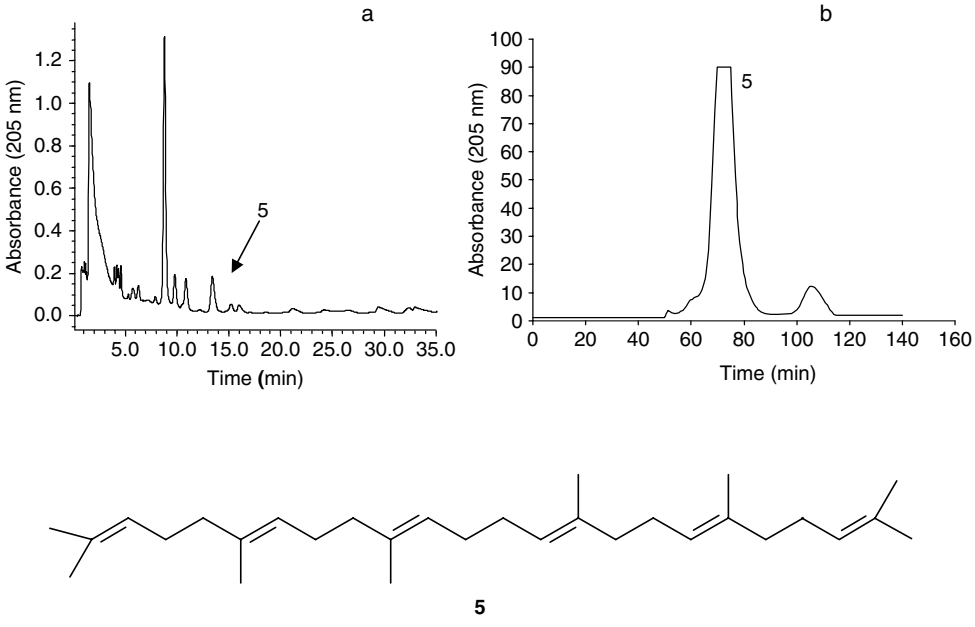

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FIGURE 10.6 (a) HPLC chromatogram of crude squalene extracted from the microalga *Thraustochytrium* ATCC 26185. (b) HSCCC chromatogram of crude squalene extracted from the microalga *Thraustochytrium* ATCC 26185. **5**: squalene. (Reprinted from Lu, H.T., Jiang, Y., and Chen, F., *J. Chromatogr. A*, 994, 37, 2003. With permission.)

Crude squalene (**5**) was obtained from the microalga *Thraustochytrium* ATCC 26185 by extraction with organic solvents. Further separation was achieved using HSCCC with a waterless two-phase solvent system composed of *n*-hexane–methanol (2:1, v/v) (Figure 10.6). The upper, mobile phase was pumped into the column in the tail-to-head elution mode. The method yielded 0.2 mg squalene in 96% purity from 150 mg of the crude extract containing 0.14% squalene, with a recovery of 95%.[21]

These examples indicate that HSCCC is a powerful tool for the separation and purification of biologically active substances in algae and has potential applications in other biotechnological downstream processes.

10.3.1.1.2 Extraction of Active Components from Herbal Medicines

Traditional herbal medicines have been used to treat human diseases in the East for millennia, and people are becoming increasingly interested in these herbs because of their low toxicity and good therapeutic performance. Traditional herbal medicines have demonstrated significant activities in antitumor, anti-inflammatory, analgesia, immunomodulation, allergy, and antiviral assays. To further study the biological activities and to control the quality of the herbal medicine, a large quantity of pure materials is urgently needed. Although the crude extract from a medicinal herb could contain a high number of different compounds with a broad range of polarity, most often only one component needs to be separated from the others. Therefore, HSCCC using isocratic elution can be an effective choice. The following examples from our laboratory illustrate this approach.

Rhodiola sachalinensis A. Bor. possesses several medical functions such as resistance to anoxia, microwave radiation, and fatigue. As a drug of "source of adaptation to environment" in Chinese traditional medicine, it has been used by divers, astronauts, pilots, and mountaineers to enhance the body's ability to survive in adverse environments. Furthermore, its effect on extending human life was also proposed. The major active constituent of this medicinal herb is salidroside (**6**). Crude salidroside was obtained from this herb by extraction with methanol. Preparative HSCCC with a two-phase solvent system composed of *n*-butanol–ethyl acetate–water (2:3:5, v/v) was successfully performed yielding salidroside (32 mg) in 98% purity from 250 mg of the crude extract in a one-step separation (Figure 10.7).[22]

Gastrodia elata Blume is a famous Chinese medicinal plant that has been widely used for the treatment of rheumatism, epilepsy, paralysis, hemiplegia, lumbago, headache, and vertigo. Gastrodin (**7**) is the bioactive component of *G. elata* and has sedative and anticonvulsant actions, neuroprotective effects, facilitating memory consolidation and retrieval, and antioxidant and free radical scavenging activities. The crude gastrodin was obtained by extraction with ethanol from the dried roots of *G. elata* under sonication. Preparative HSCCC with a two-phase solvent system

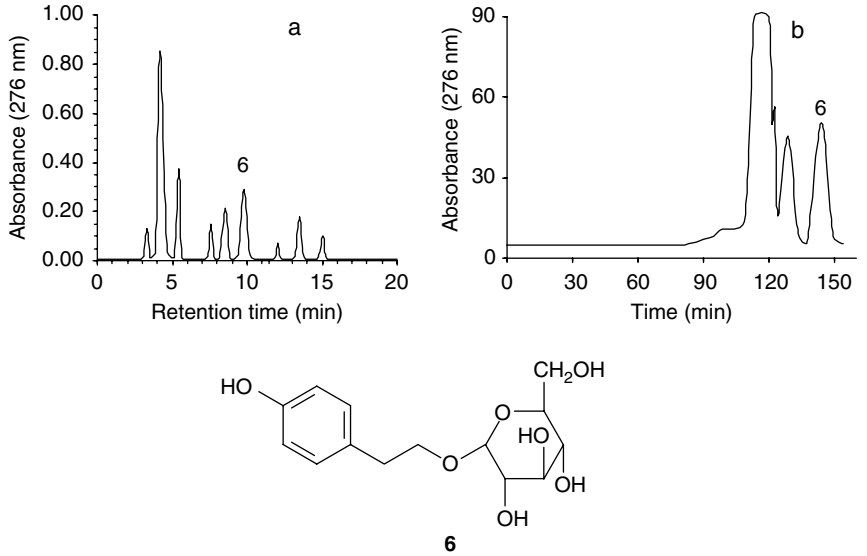


FIGURE 10.7 (a) HPLC chromatogram of crude salidroside from *Rhodiola sachalinensis* A. Bor. (b) HSCCC chromatogram of crude extract from *R. sachalinensis* A. Bor. **6**: salidroside. (Reprinted from Li, H.B. and Chen, F., *J. Chromatogr. A*, 932, 91, 2001. With permission.)

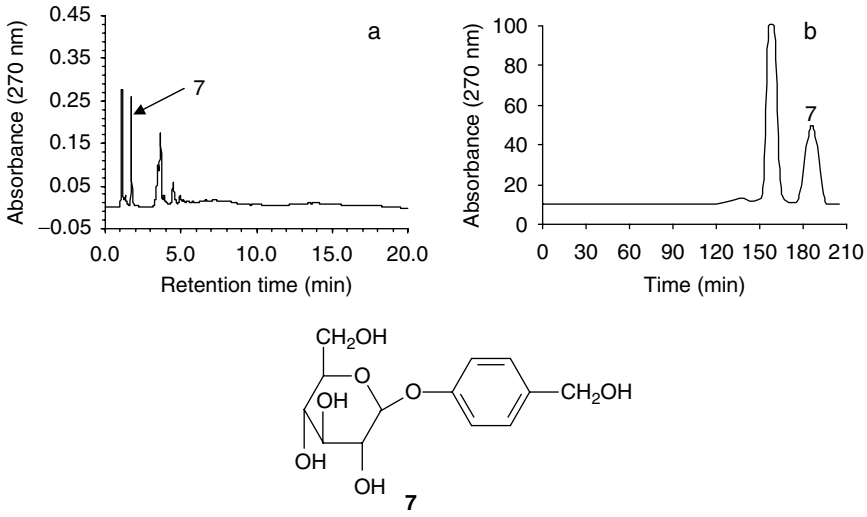


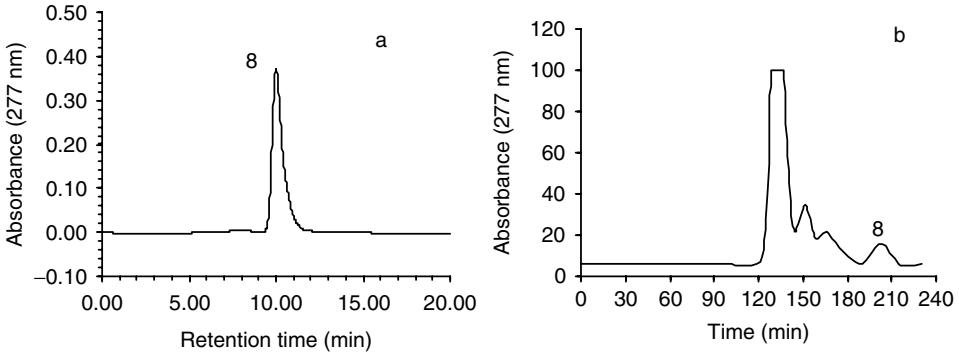
FIGURE 10.8 (a) HPLC chromatogram of crude gastrodin from *Gastrodia elata* Blume as well as the chemical structure of gastrodin. (b) HSCCC chromatogram of crude gastrodin from *G. elata* Blume separation. **7**: gastrodin. (Reprinted from Li, H.B. and Chen, F., *J. Chromatogr. A*, 1052, 229, 2004. With permission.)

composed of *n*-butanol–ethyl acetate–water (2:3:5, v/v) was performed, yielding 48 mg gastrodin in 96% purity from 500 mg of the crude extract containing 10.3% gastrodin, with a recovery of approximately 90% (Figure 10.8).[23]

Forsythia suspensa (Thunb.) Vahl. has been used widely in traditional medicines to treat gonorrhea, erysipelas, inflammation, pyrexia, and ulcers. It has also shown antioxidant activity, as well as antibacterial, antiviral, choleretic, and antiemetic effects. The crude phillyrin (**8**) was obtained by extraction with 50% ethanol from the dried fruits of *F. suspensa* under sonication. Preparative HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (1:9:1:9, v/v) was developed for the isolation and purification of phillyrin (Figure 10.9). The method yielded 5.6 mg phillyrin in 98.6% purity from 500 mg of the crude extract containing 1.2% phillyrin, with the recovery of 92%.[24]

The root of *Paeonia lactiflora* Pall. is a commonly used Chinese herbal medicine that has been shown to possess antispasmodic, anti-inflammatory, and analgesic effects. Paeoniflorin (**9**), a water-soluble compound from *P. lactiflora*, is the principal bioactive component and has been reported to exhibit anticoagulant, neuromuscular blocking, cognition-enhancing, immunoregulating, and antihyperglycemic effects. HSCCC was successfully used for the preparative separation and purification of paeoniflorin using a two-phase solvent system composed of *n*-butanol–ethyl acetate–water (1:4:5, v/v) (Figure 10.10). From 160 mg of the crude extract containing 22.0% paeoniflorin, 33.2 mg of paeoniflorin was obtained in 98.2% purity; the recovery of paeoniflorin was 94.3%.[25]

The dried roots of *Salvia miltiorrhiza* Bunge have been used widely for the treatment of various kinds of disorders including coronary artery disease and angina pectoris. The effective components of the roots can be classified as lipid soluble and water soluble. The lipid-soluble components are mainly tanshinones (diterpenoid quinones) while the water-soluble ones are mainly phenolic compounds. Recently, much attention has been paid to the pharmacological activities of the water-soluble phenolic compounds. Among these phenolic compounds, salvianolic acid B (lithospermic acid B) (**10**) exhibits endothelium-dependent vasodilation in the aorta and may be useful in the treatment of hypertension. Magnesium salvianolic acid B has a potent hepatoprotective activity and shows an improvement effect on uremic symptoms. The crude salvianolic acid B was obtained



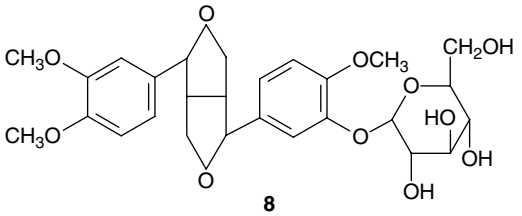


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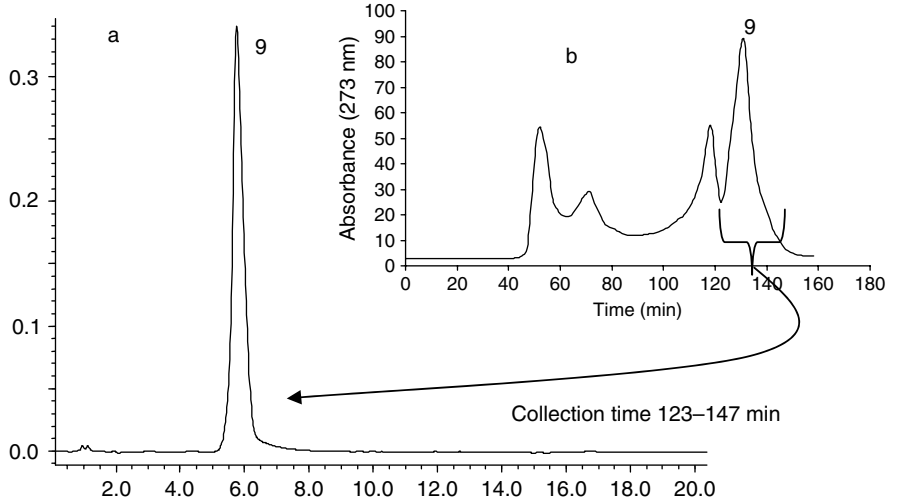
FIGURE 10.9 (a) The HPLC chromatogram of the purified phillyrin from (b) the HSCCC of the crude extract from *Forsythia suspensa* (Thunb.) Vahl. by HSCCC. **8**: phillyrin. (Reprinted from Li, H.B. and Chen, F., *J. Chromatogr. A*, 1083, 102, 2005. With permission.)

by extraction with ethanol–water from *S. miltiorrhiza* (Figure 10.11a). Preparative HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (3:7:1:9, v/v) was successfully performed yielding 342 mg salvianolic acid B in 98% purity from 500 mg of the crude extract (Figures 10.11b and 10.11c).[26]

Glycyrrhizin (**11**) is one of the main bioactive components in the liquorice plant (*Glycyrrhiza uralensis* Fisch). Liquorice has been used as a sweetening and flavoring agent and for the treatment of a variety of health problems for thousands of years. Recently, glycyrrhizin has been found to be highly active in inhibiting replication of the severe acute respiratory syndrome (SARS)-associated virus as well as a potential therapeutic agent for chronic hepatitis and acquired immunodeficiency syndrome (AIDS). Moreover, glycyrrhizin has many other pharmacological uses that include anti-inflammatory, antiulcerous, antihepatotoxic, antioxidative, and antiallergic activities. The separation and purification of glycyrrhizin from a methanol–water (70:30, v/v) extract of liquorice roots was achieved using HSCCC with a two-phase solvent system composed of ethyl acetate–methanol–water (5:2:5, v/v) (Figure 10.12). The method yielded 42.2 mg glycyrrhizin in 96.8% purity from 130 mg of the crude exact with 95.2% recovery.[27]

Lithospermum erythrorhizon Sieb. et Zucc., a boraginaceous medicinal plant, is the main source of shikonin that has many beneficial effects in wound healing. It also has anti-inflammatory, antibacterial, antitumor, antidiabetes, and antiviral activities. Shikonin (**12**) was successfully isolated and purified from the crude extract of this plant by preparative HSCCC using a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (16:14:14:5, v/v) (Figure 10.13). A total amount of 19.6 mg of shikonin in 98.9% purity was obtained from 52 mg of the crude extract containing 38.9% shikonin with 96.9% recovery. The preparative isolation and purification of shikonin by HSCCC was completed in 200 min in a one-step separation.[28]

Ligusticum chuanxiong Hort has been used widely in traditional medicines for the treatment of various kinds of disorders such as cardiovascular and cerebrovascular diseases. Chuanxiongzine



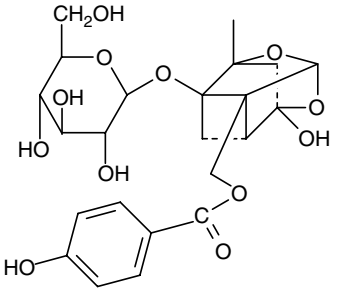


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FIGURE 10.10 (a) The HPLC chromatogram of the purified paeoniflorin from (b) the HSCCC of the crude sample from *Paeonia lactiflora* Pall. **9**: paeoniflorin. (Reprinted from Chen, F., Lu, H.T., and Jiang, Y., *J. Chromatogr. A*, 1040, 205, 2004. With permission.)

(i.e., tetramethylpyrazine) (**13**) is the bioactive component of this herb, which improves the cerebral blood flow through inhibiting thrombus formation and platelet aggregation during ischemic attack. Chuanxiongzine can significantly improve central cholinergic system function and enhance learning and memory ability. In addition, chuanxiongzine has antibacterial properties, and a prophylactic effect on mice with endotoxemia. It scavenges superoxide anion and decreases nitric oxide production in human polymorphonuclear leukocytes. The crude chuanxiongzine was obtained by extraction with ethanol from the dried roots of *L. chuanxiong* under sonication. Preparative HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (5:5:3:7, v/v) was successfully performed yielding 11.5 mg chuanxiongzine in 96.8% purity from 300 mg of the crude extract containing 4.2% chuanxiongzine, with a recovery of 91% in a one-step separation (Figure 10.14).[29]

The above studies indicate that HSCCC using an isocratic elution is a useful technique for the separation and purification of bioactive components from Chinese medicinal herbs, even if the content of target compound is very low. In fact, HSCCC using an isocratic elution has been used widely for the isolation and purification of bioactive compounds from natural products, examples of which are shown in Table 10.1.

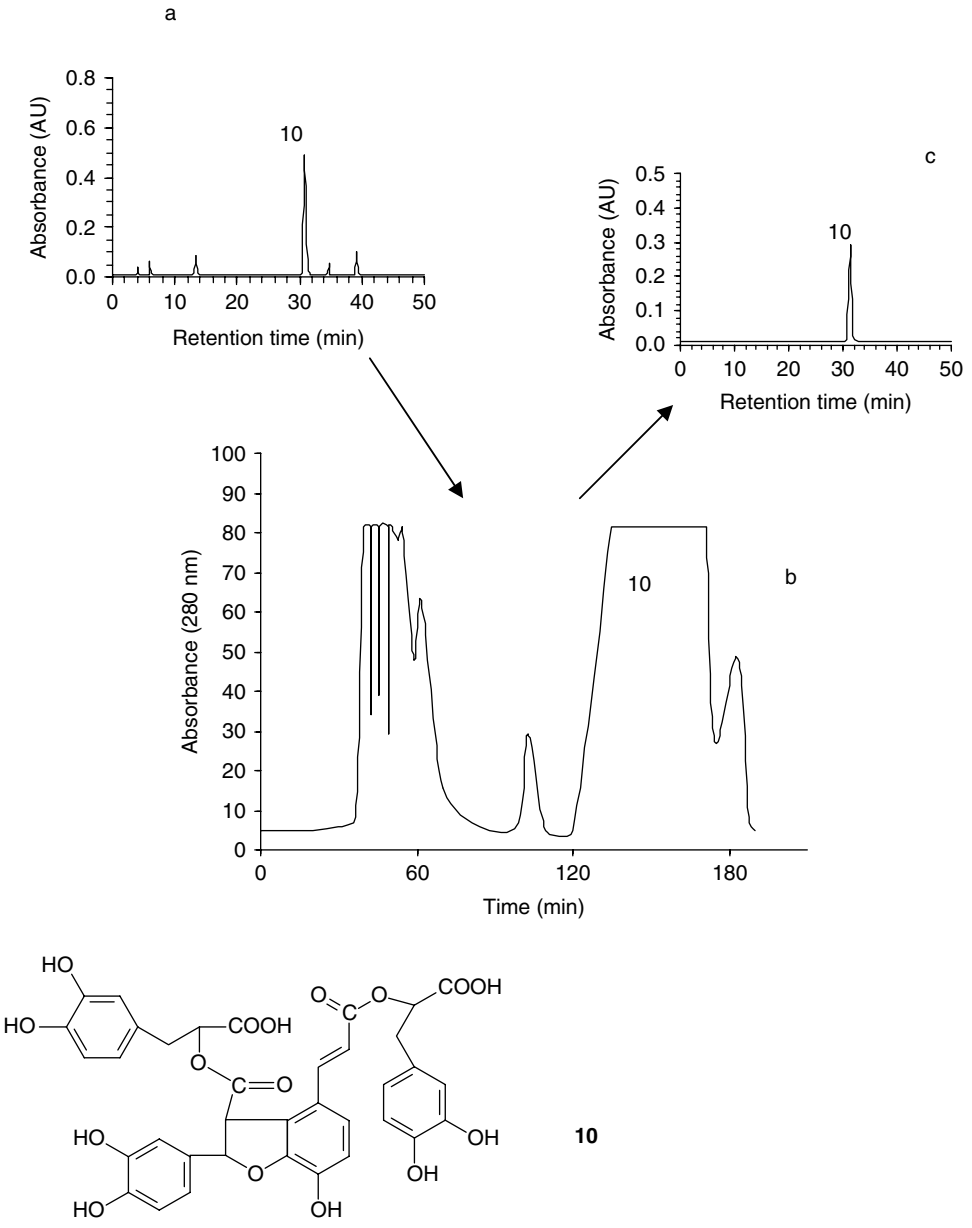


FIGURE 10.11 (a) Chromatogram of crude salvianolic acid B from *S. miltiorrhiza* Bunge by HPLC analysis. **10**: salvianolic acid B. (b) Chromatogram of crude salvianolic acid B from *S. miltiorrhiza* Bunge by HSCCC separation. (c) HPLC chromatogram of salvianolic acid B purified by HSCCC from *S. miltiorrhiza* Bunge. (Reprinted from Li, H.B., Lai, J.P., Jiang, Y., and Chen, F., *J. Chromatogr. A*, 943, 235, 2002. With permission.)

10.3.1.2 Two-Step HSCCC Separations Using Isocratic Elution

For some samples, HSCCC using an isocratic elution needs to be used repeatedly with the same or different two-phase solvent system to obtain compounds of suitable purity for structural analysis and bioactive testing. Several examples are given below.

Chlorogenic acid (**14**) is one of the major phenolic compounds found in the traditional Chinese medicinal herb Flos Lonicerae. It can suppress the N-nitrosating reaction and inhibit hepatic

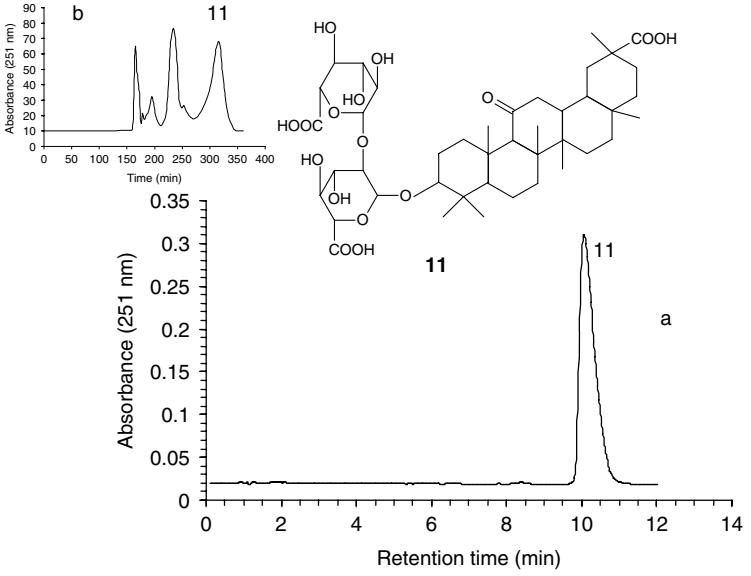


FIGURE 10.12 (a) The HPLC chromatogram of purified glycyrrhizin from (b) the HSCCC of the crude sample extracted from the root of liquorice. **11**: glycyrrhizin. (Reprinted from Jiang, Y., Lu, H.T., and Chen, F., *J. Chromatogr. A*, 1033, 183, 2004. With permission.)

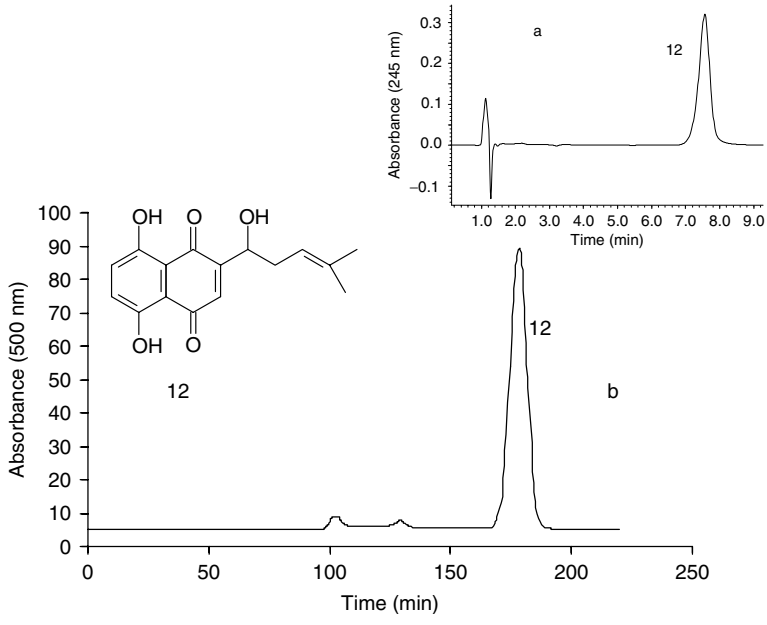


FIGURE 10.13 (a) The HPLC chromatogram of purified shikonin from (b) the HSCCC of the crude shikonin extracted from *Lithospermum erythrorhizon* Sieb. et Zucc. **12**: shikonin. (Reprinted from Lu, H.T., Jiang, Y., and Chen, F., *J. Chromatogr. A*, 1023, 159, 2004. With permission.)

glucose 6-phosphatase, which may be a significant factor in the abnormal diabetic state. Moreover, it may serve as an antioxidant, antitumor, antimutagenic, and anticarcinogenic agent. The separation and purification of chlorogenic acid from the crude extract of Flos Lonicerae was achieved by HSCCC (Figure 10.15). A high acid, highly polar two-phase solvent system containing

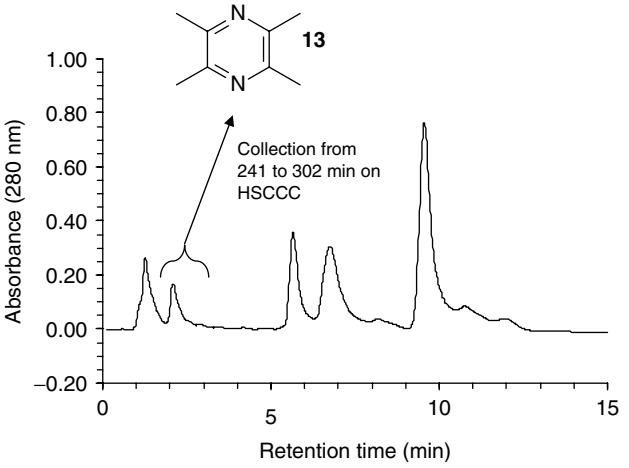


FIGURE 10.14 HPLC chromatogram of crude chuanxiongzine from *Ligusticum chuanxiong* Hort. **13**: chuanxiongzine. (Reprinted from Li, H.B. and Chen, F., *J. Chromatogr. A*, 1047, 249, 2004. With permission.)

n-butanol–acetic acid–water (4:1:5, v/v) was run on a preparative scale. The upper phase was used as the mobile phase in the head-to-tail elution mode. A 300 mg quantity of the crude extract containing 5.97% chlorogenic acid was loaded into the HSCCC column. The purity of chlorogenic acid after the first run separation was only 73.3% and there were still impurities in the product (Figure 10.15a). This product was purified again by HSCCC with the same solvent system (Figure 10.15b), which yielded 16.9 mg chlorogenic acid in 94.8% purity with approximately 90% recovery.[58]

Scutellaria baicalensis Georgi has been used for antiinflammation, anticancer, treating bacterial and viral infections of the respiratory and the gastrointestinal tracts, "cleaning away heat", moistening aridity, "purging fire", detoxifying toxicosis, reducing the total cholesterol level, and decreasing blood pressures. This herb also possesses cholagogic, diuretic, and cathartic actions. Some concentrated composite herbal preparations that contain *S. baicalensis* as a major ingredient in their prescriptions are widely used in oriental countries. Its dried roots contain over 30 different flavonoids, such as baicalin, baicalein, wogonin, wogonin 7-*O*-glucuronide, oroxylin A, and oroxylin A 7-*O*-glucuronide. Baicalin is the most abundant component, and has antiallergic, anti-inflammatory, anti-HIV, antitumor, antioxidant and free radical scavenging, and anti-SARS coronavirus effects.[59,60] The crude baicalin (**15**) was obtained by extraction with methanol–water (70:30, v/v) from *S. baicalensis*. The "first run" HSCCC was performed with a two-phase solvent system composed of *n*-butanol–water (1:1, v/v) and yielded 48.5 mg of baicalin in 82.0% purity from 200 mg of the crude extract containing 21.6% baicalin. This partially purified fraction was purified again by HSCCC with the same solvent system. After this "second run", 37.0 mg of baicalin at 96.5% purity was obtained, with 86.0% recovery (Figures 10.16a and 10.16b).[61]

The roots of *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao have been used as an antiperspirant, adiuretic, or atonic in traditional medicine. It has immunostimulant, cardiotonic, and antiaging activities. Its isoflavones showed antimicrobial and superoxide anion scavenging activities. Calycosin (**16**) is the main isoflavone component of this herb, which has proved to be active against *Giardia intestinalis*, a potent protozoan agent for enteric disease. It also showed *in vitro* activity against the chloroquine-sensitive strain poW and the chloroquine-resistant clone Dd2 of *Plasmodium falciparum*, the parasite responsible for malaria. The HSCCC was performed with a two-phase solvent system composed of *n*-hexane–chloroform–methanol–water (1:3:3:2, v/v), which yielded calycosin at 75% purity, after the first run separation, from 200 mg of the crude ethyl acetate extract. This partially purified fraction was further purified by HSCCC with the same solvent

TABLE 10.1
Isolation of Bioactive Compounds from Natural Sources by HSCCC Using Isocratic Elution

Sample	Component	Purity (%)	Solvent System	References
Aconitum sinomontanum	Lappaconitine, ranaconitine, *N*-deacetyllappaconitine, and *N*-deacetylranaconitine	>98.5	Chloroform–methanol–0.3 M HCl (4:1.5:2)	30
Ampelopsis grossedentata	(+)-Dihydromyricetin	99	*n*-Hexane–ethyl acetate–methanol–water (1:3:2:4)	31
A. grossedentata	Two flavonoid glycosides	Not reported	*n*-Hexane–ethyl acetate–methanol–water (1:6:1.5:7.5)	32
Andrographis paniculata	Andrographolide and neoandrographolide	99.9 and 98.5	*n*-Hexane–ethyl acetate–methanol–water (1:4:2.5:2.5)	33
Astragalus membranaceus	Calycosin	99	*n*-Hexane–chloroform–methanol–water (1:3:3:2)	34
Broccoli, arugula, and horseradish	Glucosinolates	>95	*n*-Propanol–acetonitrile–saturated aqueous ammonium sulfate–water (1:0.5:1.2:1)	35
Byrsonima crassa	Three flavonoids	>95	Ethyl acetate–*n*-propanol–water (140:8:80)	36
Chlorella vulgaris	Lutein	98	*n*-Hexane–ethanol–water (4:3:1)	17
Chlorella zofingiensis	Canthaxanthin	98.7	*n*-Hexane–ethanol–water (10:9:1)	20
Chlorococcum sp.	Astaxanthin	97	*n*-Hexane–ethyl acetate–ethanol–water (5:5:6.5:3)	18
Cistanches salsa	Two phenylethanoid glycosides	>98	Ethyl acetate–*n*-butanol–ethanol–water (4:0.6:0.6:5)	37
Edgeworthia chrysantha	Syringin and edgeworoside C	>96	Ethyl acetate–ethanol–water (15:1:15)	38
Epimedium segittatum	Icariin	85.7	*n*-Hexane–*n*-butanol–methanol–water (1:4:2:6)	39
Gastrodia elata	Gastrodin	96	*n*-Butanol–ethyl acetate–water (2:3:5)	23
Glycyrrhiza uralensis	Glycyrrhizin	96.8	Ethyl acetate–methanol–water (5:2:5)	27
Forsythia suspensa	Phillyrin	98.6	*n*-Hexane–ethyl acetate–ethanol–water (1:9:1:9)	24
Ligusticum chuanxiong	Chuanxiongzine	96.8	*n*-Hexane–ethyl acetate–ethanol–water (5:5:3:7)	29
Linum usitatissimum	Secoisolariciresinol diglucoside	93	*t*-Butyl methyl ether–*n*-butanol–acetonitrile–water (1:3:1:5)	40
Lithospermum erythrorhizon	Shikonin	98.9	*n*-Hexane–ethyl acetate–ethanol–water (16:14:14:5)	28

Magnoliae officinalis	Honokiol and magnolol	99.2 and 98.2	*n*-Hexane–ethyl acetate–methanol–water (1:0.4:1:0.4)	41
M. officinalis	Honokiol and magnolol	98.7 and 99.5	Light petroleum–ethyl acetate–tetrachloromethane–methanol–water (1:1:8:6:1)	42
Marigold flower petal	Lutein	98.5	*n*-Heptane–chloroform–acetonitrile (10:3:7)	43
Microcystis aeruginosa	Zeaxanthin	96.2	*n*-Hexane–ethyl acetate–ethanol–water (8:2:7:3)	19
Monascus purpureus	Mevinolinic acid	99	*n*-Hexane–ethyl acetate–methanol–water (1:1:1:1)	44
Onion	Five anthocyanins	Not reported	*t*-Butyl methyl ether–*n*-butanol–acetonitrile–water (2:2:1:5)	45
Oroxylum indicum	Five flavonoids	85–98	Chloroform–methanol–water (8:10:5)	46
Paeonia lactiflora	Paeoniflorin	98.2	*n*-Butanol–ethyl acetate–water (1:4:5)	25
Paepalanthus microphyllus	Four naphthopyranone glycosides	92–98	Water–ethanol–ethyl acetate–hexane (10:4:10:4)	47
Panax notoginseng	Four dammarane saponins	Not reported	*n*-Hexane–*n*-butanol–water (3:4:7)	48
Phyllanthus urinaria	Corilagin and ellagic acid	>95	*n*-Butanol–acetic acid–water (4:1:5)	49
Polygonum cuspidatum	Resveratrol, anthraglycoside A, and anthraglycoside B	>98	Chloroform–methanol–water (4:3:2)	50
Rabdosia rubescens	Oridonin	97.8	*n*-Hexane–ethyl acetate–methanol–water (1:2:1:2)	51
Rhodiola sachalinensis	Salidroside	98	*n*-Butanol–ethyl acetate–water (2:3:5)	22
Salvia miltiorrhiza	Salvianolic acid B	98	*n*-Hexane–ethyl acetate–ethanol–water (3:7:1:9)	26
S. miltiorrhiza	Przewaquinone A	93.7	Carbon tetrachloride–methanol–water–*n*-hexane (3:3:2:1)	52
Silybum marianum	Silycristin, silybin, and isosilybin	93.1, 95.7, and 89.7	*n*-Hexane–ethyl acetate–methanol–water (1:4:3:4)	53
Soybean	Four isoflavones	>90	Hexane–ethyl acetate–*n*-butanol–methanol–acetic acid–water (1:2:1:1:5:1)	54
Streptomyces ambofaciens	Spiramycins I, II, and III	98.2, 92.3, and 97.4	*n*-Hexane–ethyl acetate–methanol–water (3:6:5:5)	55
Thraustochytrium ATCC 26185	Squalene	96	*n*-Hexane–methanol (2:1)	21
Tomato	Lycopene	98.5	*n*-Hexane–dichloromethane–acetonitrile (10:3.5:6.5)	56
Vaccinium myrtillus	Delphinidin-3-*O*-sambubioside and cyanidin-3-*O*-sambubioside	Not reported	Methyl *tert*-butyl ether–*n*-butanol–acetonitrile–water–trifluoroacetic acid (1:4:1:5:0.01)	57

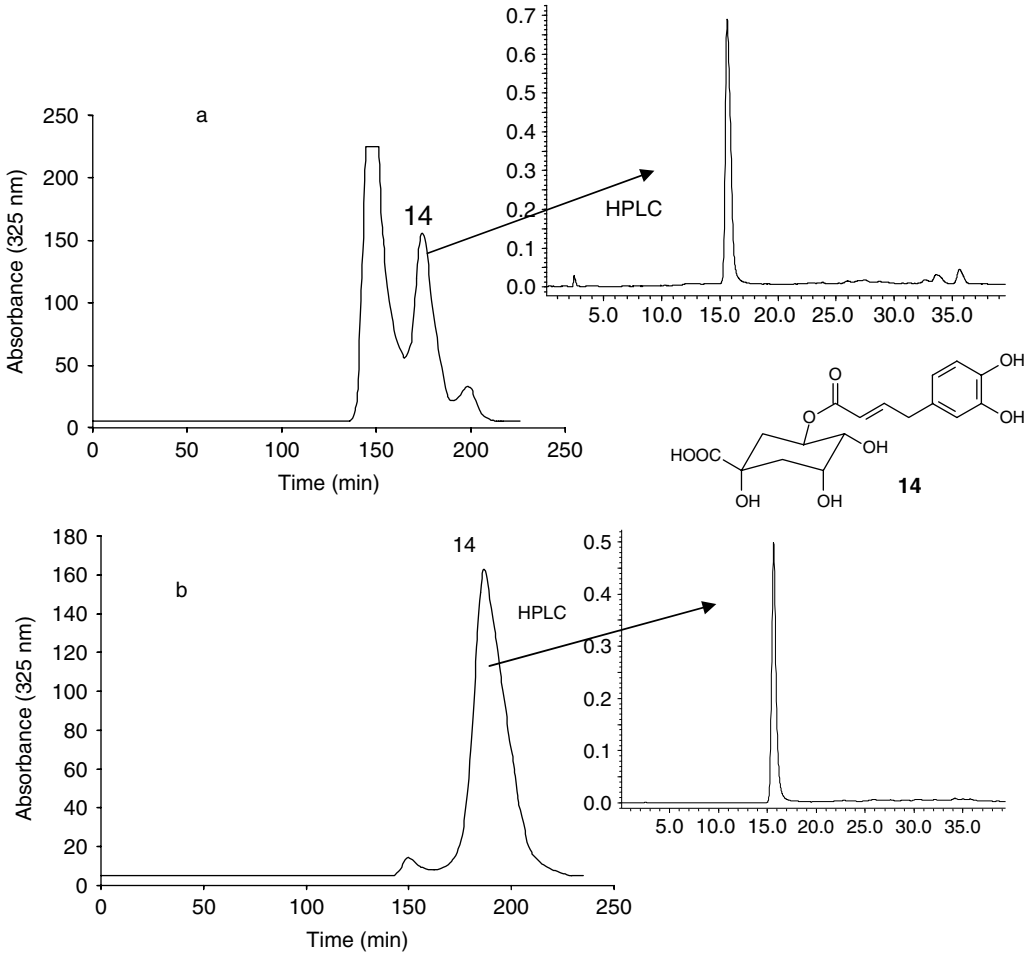


FIGURE 10.15 HSCCC chromatograms of the crude sample extracted from Flos Lonicerae: (a) the first separation and (b) the second separation. **14**: chlorogenic acid. The insets are the respective HPLC chromatograms of the HSCCC product. (Reprinted from Lu, H.T., Jiang, Y., and Chen, F., *J. Chromatogr. A*, 1026, 185, 2004. With permission.)

system. The second separation yielded 14.8 mg of calycosin in 99% purity. The chemical structure of the purified calycosin was confirmed by MS, ^{1}H, and ^{13}C NMR.[34]

Resveratrol (**17**) and piceid (**18**) are the major polyphenols in the root of *Polygonum cuspidatum*, which is used as a folk medicine for the treatment of atherosclerosis and for other therapeutic purposes. They inhibit the copper-catalyzed oxidation of low-density lipoprotein, platelet clotting and arachidonate metabolism, reduce liver injury from peroxidized oil, and have cancer-chemopreventive activities. The crude methanol extract of *P. cuspidatum* was partitioned between ethyl acetate and water. Crude resveratrol was obtained from the ethyl acetate phase while crude piceid was obtained from the water phase. A solvent system consisting of chloroform–methanol–water (4:3:2, v/v) was used for the HSCCC purification of the crude resveratrol, which yielded 72.5 mg of resveratrol. The crude piceid was first purified using HSCCC with ethyl acetate–ethanol–water (10:1:10, v/v), which yielded 58.5 mg of the partially purified piceid. Then, the same solvent system at the modified volume ratio of 70:1:70 was used for further purification of the partially purified fraction, and 35.45 mg of piceid was obtained. The chemical structures of the purified resveratrol and piceid were confirmed by electrospray ionization MS and ^{1}H NMR.[62]

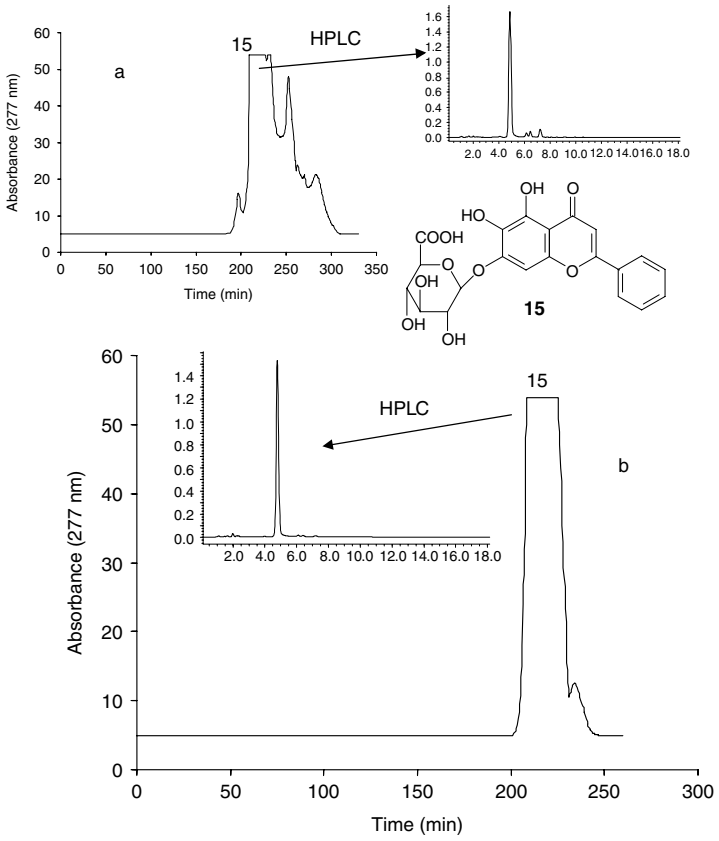
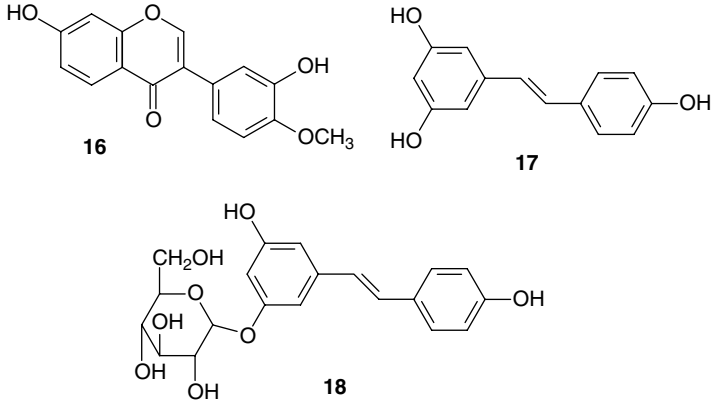
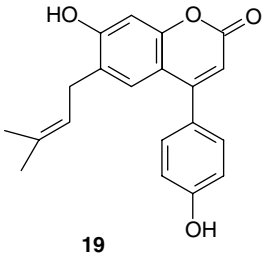


FIGURE 10.16 HSCCC chromatograms of the crude baicalin extracted from *Scutellaria baicalensis* Georgi: (a) the first separation and (b) the second separation. **15**: baicalin. The insets are the respective HPLC chromatograms of the HSCCC product. (Reprinted from Lu, H.T., Jiang, Y., and Chen, F., *J. Chromatogr. A*, 1017, 117, 2003. With permission.)



Glycyrrhiza inflata Bat. is an important species of licorice that has been used to treat diseases such as phthisis, contagious hepatitis, bronchitis, and ague. Inflacoumarin (**19**) and licochalcone (**20**) are the bioactive components in this herb. The ethanol extract was separated by HSCCC with a two-phase solvent system composed of *n*-hexane–chloroform–methanol–water (5:6:3:2, v/v).

The partially purified fractions containing inflacoumarin and licochalcone were, respectively, purified with the same solvent system at the modified volume ratio of 1.5:6:3:2. The purities of inflacoumarin A and licochalcone A were, respectively, 99.6% and 99.1% after the second run.[63]



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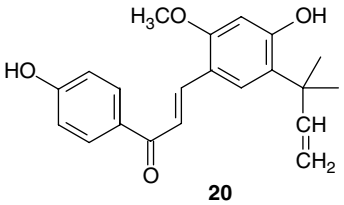

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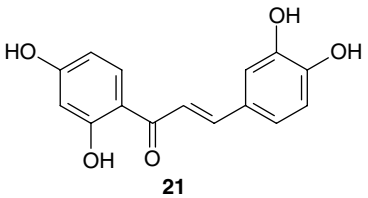
The seeds of *Vernonia anthelmintica* Willd are used as a vermicide and for the treatment of vitiligo and breast cancer. To separate and identify the effective components for vitiligo, the seeds of the herb were extracted with light petroleum and 60% ethanol successively, and then the ethanol extract was further extracted with ethyl acetate. Pharmacological tests revealed a high antivitiligo activity in the ethyl acetate extract, which was separated by HSCCC with a solvent system composed of chloroform–dichloromethane–methanol–water (2:2:3:2, v/v). The partially purified fraction with the highest antivitiligo activity was further purified using HSCCC with another solvent system composed of 1,2-dichloroethane–methanol–acetonitrile–water (4:1.1:0.25:2, v/v), and the purified compounds were characterized by MS and NMR. Three flavonoids, 2′,3,4,4′-tetrahydroxychalcone (**21**), 5,6,7,4′-tetrahydroxyflavone (**22**), and butin (**23**) were obtained in purities of 95–97%. They all showed antivitiligo activity, and butin had the highest activity.[64]



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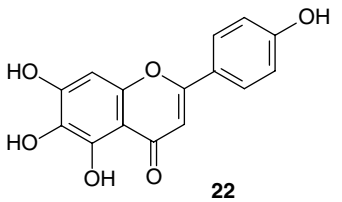

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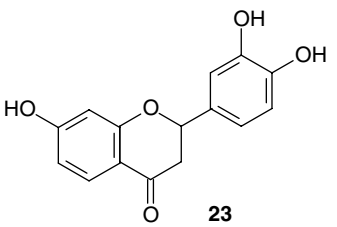


23

10.3.2 Stepwise or Gradient Elution

Extracts from plant materials usually contain a high number of different compounds with a broad range of polarities. If only one component needs to be separated from the others, then an isocratic HSCCC method using a constant composition of the mobile phase or a constant flow-rate of the mobile phase can be used. To separate more components with a large difference in their polarity, stepwise or gradient elution as well as stepwise increasing the flow-rate of the mobile phase might be adopted. With these methods, which also may reduce running time, two-phase solvent systems and operating parameters should be chosen carefully because of the risk of disturbing liquid–liquid equilibria and causing a severe loss of the stationary phase.

As previously mentioned (Section 10.3.1.2), *S. baicalensis* Georgi is one of the most widely used traditional Chinese herbal medicines, and baicalin, baicalein, wogonin, and oroxylin A are its main active components.[59,60] HSCCC with a two-phase solvent system composed of *n*-butanol–water (1:1, v/v) has been used for the isolation and purification of baicalin alone.[61] To isolate baicalein (**24**), wogonin (**25**), and oroxylin A (**26**) from the herb, an HSCCC method with a two-phase solvent system composed of *n*-hexane–ethyl acetate–*n*-butanol–water (1:1:8:10, v/v) was successfully used by increasing the flow-rate of the mobile phase stepwise from 1.0 to 2.0 mL/min after 4 h (Figure 10.17). The method yielded 144.8 mg of baicalein in 95.7% purity, 50.2 mg of wogonin in 98.5% purity, and 12.4 mg of oroxylin A in 93.2% purity from 500 mg of the crude extract in a one-step separation. The recoveries of baicalein, wogonin, and oroxylin A were 92.7, 91.6, and 92.5%, respectively.[65]

Cnidium monnieri (L.) Cusson is one of the most popular traditional Chinese medicinal herbs. Its fruits have been used for the treatment of impotence, frigidity, and skin-related diseases, and have exhibited strong antiallergic, antipruritic, antidermatophytic, antibacterial, antifungal, and antiosteoporotic activities.[66,67] A group of coumarins, osthol (**27**), imperatorin (**28**), bergapten (**29**), isopimpinellin (**30**), xanthotoxin (**31**), and xanthotoxol (**32**), are the main bioactive constituents of *C. monnieri*.[68] Bergapten possesses anti-inflammatory and analgesic activities, while imperatorin exhibits strong cytotoxic activity on human leukemia, chemopreventive effects on hepatitis and skin tumors, and anti-inflammatory activity. The crude extract was obtained by ethanol extraction of the dried fruits of this herb under sonication (Figure 10.18a). Preparative HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (5:5:5:5, v/v) was developed by increasing the flow-rate of the mobile phase stepwise from 1.0 to 2.0 mL/min after 180 min for the isolation and purification of bergapten (**29**) and imperatorin (**28**) from the crude extract (Figure 10.18b). The method yielded 45.8 mg (92.1% yield) of bergapten in 96.5% purity and 118.3 mg (93.7% yield) of imperatorin in 98.2% purity from 500 mg of the crude extract in a single run.[66] In addition, an HSCCC method was successfully used for isolation and purification of osthol (**27**) and xanthotoxol (**32**) from this herb using stepwise elution with a pair of two-phase solvent

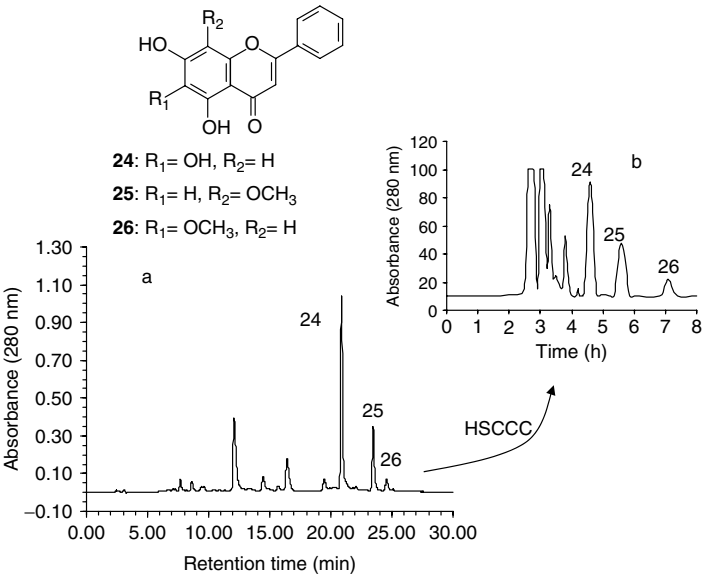


FIGURE 10.17 (a) HPLC chromatogram of the crude extract from *Scutellaria baicalensis* Georgi and (b) HSCCC chromatogram of the crude extract from *S. baicalensis* Georgi. **24**: baicalein, **25**: wogonin, and **26**: oroxylin A. (Reprinted from Li, H.B. and Chen, F., *J. Chromatogr. A*, 1074, 107, 2005. With permission.)

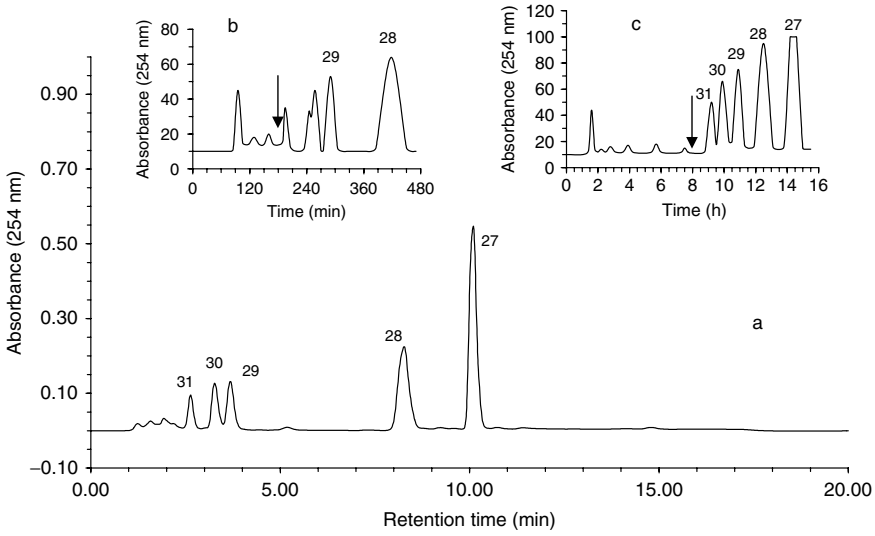
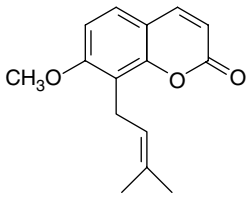


FIGURE 10.18 (a) Chromatogram of the crude coumarins from *Cnidium monnieri* (L.) Cusson by HPLC analysis. (b) Chromatogram of crude extract from *C. monnieri* (L.) Cusson by HSCCC separation using stepwise increases of the flow-rate of the mobile phase. Stationary phase, the upper phase; mobile phase, lower phase; flow-rate, 0–180 min, 1 mL/min, and 180–480 min, 2 mL/min. (c) Chromatogram of the crude coumarins from *C. monnieri* (L.) Cusson by HSCCC separation using stepwise elution with solvent systems A and B. Stationary phase: the upper phase of solvent system A; mobile phase: 0–480 min, the lower phase of solvent system A, and 480–780 min, the lower phase of solvent system B. **27**: osthol, **28**: bergapten, **29**: imperatorin, **30**: isopimpinellin, and **31**: xanthotoxin. The arrows indicate the time of stepwise change in flow-rate (b) or mobile phase composition (c). (Modified from Li, H.B. and Chen, F., *J. Sep. Sci.*, 28, 268, 2005. With permission.)

systems composed of *n*-hexane–ethyl acetate–methanol–water at 1:1:1:1 (v/v) and 5:5:6:4 (v/v), which were selected using an analytical HSCCC instrument. About 308 mg of the crude extract was separated, yielding 88.3 mg of osthol and 19.4 mg of xanthotoxol in high purity of over 98%.[67] A refined preparative HSCCC with the two-phase solvent systems *n*-hexane–ethyl acetate–ethanol–water (5:5:4:6, v/v) and *n*-hexane–ethyl acetate–ethanol–water (5:5:6:4, v/v) was developed using stepwise elution (Figure 10.18c). The five relatively pure coumarins were obtained from 500 mg of the crude extract in a single run. Their purities were 90.6–98.9% and the recoveries were 85.7–94.2%. Compared with the previous methods,[66,67] this method could be used to purify more coumarins in a single run and in larger amounts.[68]



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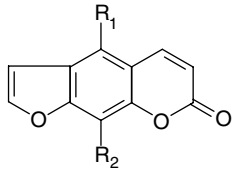
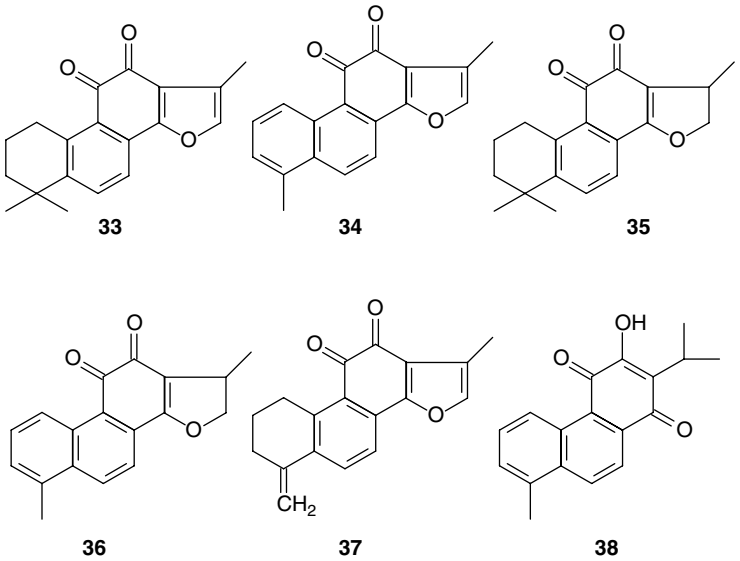


28: R_1= H, R_2= $OCH_2CH{=}C(CH_3)_2$
29: R_1= OCH_3, R_2= H
30: R_1= OCH_3, R_2= OCH_3
31: R_1= H, R_2= OCH_3
32: R_1= H, R_2= OH

S. miltiorrhiza Bunge is a well-known Chinese medicinal plant (Section 10.3.1.1.2).[26] The methanol extract from the roots of the herb was partitioned between light petroleum and water. The crude tanshinones were obtained from the organic phase. A set of three two-phase solvent systems composed of *n*-hexane–ethanol–water at the different volume ratios of 4:1.8:2 (A), 4:2.3:2 (B), and 4:3:2 (C) were selected using analytical HSCCC. Preparative HSCCC was performed using a stepwise elution in sequence as follows: the upper organic phase of solvent system A was used as stationary phase, and the lower aqueous phase was used as mobile phase. After 2 h, the mobile phase was switched to the lower phase of system B to elute the column for 50 min. Then, the lower phase of system C was used as the mobile phase to complete the separation. Tanshinone IIA (**33**) (7 mg), tanshinone I (**34**) (3 mg), and cryptotanshinone (**35**) (4 mg) in purities of over 95% were obtained from 50 mg of the crude extract in a single run.[69] Another preparative HSCCC method was also developed for the separation and purification of six diterpenoids from the herb. The crude diterpenoids were obtained by extraction with ethanol–*n*-hexane (1:1, v/v) from this herb. Preparative HSCCC with the two-phase solvent systems A, composed of *n*-hexane–ethanol–water (10:5.5:4.5, v/v), and B, composed of *n*-hexane–ethanol–water (10:7:3, v/v), was successfully performed in a stepwise elution yielding six relatively pure diterpenoids from 300 mg of the crude extract in a one-step separation (Figure 10.19). The purities of dihydrotanshinone I (**36**), cryptotanshinone, methylenetanshiquinone (**37**), tanshinone I, tanshinone IIA, and dannshenxinkun B (**38**) were 88.1, 98.8, 97.6, 93.5, 96.8, and 94.3%, respectively. Compared with the previous method,[69] this method could be used to obtain more tanshinones in a single run and in larger amounts.[70]



33 **34** **35**

36 **37** **38**

Centella asiatica L. has been used widely for treatment of leprosy, open wounds, and mental retardation. A preparative HSCCC method was developed for the isolation and purification of the principal bioactive compounds in this herb using a mobile phase gradient with a stepwise increase of elution strength. The lower phase of the two-phase solvent system composed of *n*-hexane–*n*-butanol–0.05 M NaOH (5:1:6, v/v) was used as the stationary phase, and the upper phase was used as the initial mobile phase. Then, the mobile phase was changed, stepwise, into the upper phase of *n*-hexane–*n*-butanol saturated by 0.05 M NaOH at different volume ratios of 1:1 (after 50 min), 1:2 (after 120 min), and 1:4 (after 220 min). Simultaneously, the flow-rate was also decreased stepwise from 4.0 to 3.0 and 2.0 to 1.5 mL/min to minimize loss of the stationary phase. The retention of

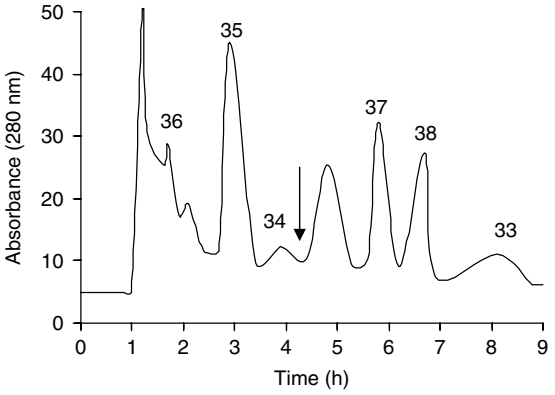
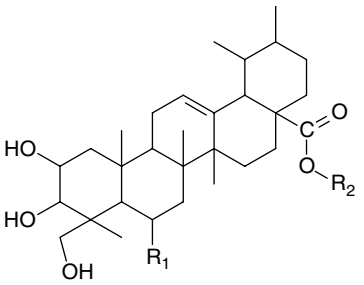


FIGURE 10.19 Chromatogram of crude tanshinones from *Salvia miltiorrhiza* Bunge by HSCCC separation using stepwise elution. The time of the stepwise change in solvents is indicated by the arrow. Peaks—**36**: dihydrotanshinone I, **35**: cryptotanshinone, **34**: tanshinone I, **37**: methylenetanshiquinone, **38**: danshenxinkun B, and **33**: tanshinone IIA. (Reprinted from Li, H.B. and Chen, F., *J. Chromatogr. A*, 925, 109, 2001. With permission.)

stationary phase during the gradient steps decreased from 75 to 67, 65, and 64% at the end of the separation. The method yielded asiatic acid (**39**) (18 mg), madecassic acid (**40**) (13 mg), asiaticoside (**41**) (140 mg), and madecassoside (**42**) (75 mg) in high purities (resulting in single HPLC peak) from 600 mg of the crude extract. This study indicated that HSCCC using step-gradient elution was a versatile and economic technique to separate complex crude extract containing a wide polarity range of compounds.[71]



39: R_1= H, R_2= H
40: R_1= OH, R_2= H
41: R_1= H, R_2= trisaccharide unit
42: R_1= OH, R_2= trisaccharide unit

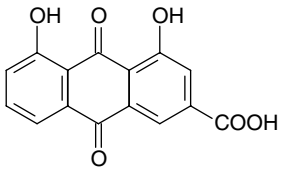
10.3.3 Multidimensional CCC

Overlapping of the components in a complex chromatogram can be a very serious problem to overcome. Increasing the peak capacity is one of the best strategies to improve the resolution, and this is readily achieved by a multidimensional separation. In fact, multidimensional chromatography is a frontier of contemporary chromatographic science. Comparative investigation on the separability between single and serially coupled dual high-speed countercurrent chromatographs showed that the separability of serially coupled dual instrument was four times as much as that of the single one.[72]

The loss of the stationary phase and its adverse influence on the separation in HSCCC 1 would spread to HSCCC 2 when HSCCC systems 1 and 2 were simply connected in series. However, the peak resolution of multidimensional CCC system may be improved by the use of two solvent systems, one for HSCCC 1 and the other for HSCCC 2.[73] Therefore, a peak fraction eluted from the column of the first HSCCC with insufficient resolution is directly introduced into the column of the second HSCCC to elute with a different two-phase solvent system. In this way, any serious loss of the stationary phase caused by the sample would be limited to HSCCC 1. The multidimensional CCC system could be considered as online, two-step HSCCC separations with the same or different two-phase solvent systems.

A multidimensional CCC technique was used for the separation of tanshinones from *S. miltiorrhiza* Bunge. Cryptotanshinone (**35**), tanshinone I (**34**), and tanshinone IIA (**33**) could be well resolved in the first HSCCC with light petroleum–ethyl acetate–methanol–water (2:3:2.5:1.7, v/v), but the resolution of dihydrotanshinone I (**36**) was unsatisfactory. The peak fraction of the first separation containing dihydrotanshinone I was introduced into the second HSCCC system with light petroleum–ethyl acetate–methanol–water (2:3:2.5:1.8, v/v). Consequently, dihydrotanshinone I was successfully isolated from other components from which it was not resolved in the stepwise elution.[69] Tanshinone IIA (16 mg), tanshinone I (10 mg), dihydrotanshinone I (7 mg), and cryptotanshinone (11 mg) in high purities of over 95% were obtained from 100 mg of the crude extract of this herb using multidimensional CCC.[74]

Rhein (**43**) has a strong antibacterial action on *Bacteroides fragilis*, and may be useful in cancer chemotherapy as a biochemical modulator. It also retards the progression of type 2 diabetic nephropathy. Rhein was separated and purified from the traditional Chinese herb *Rheum officinale* Baill using multidimensional CCC with the same two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (3:7:5:5, v/v), which yielded 6.7 mg of rhein in a purity of 97% from 500 mg of the crude extract.[75]



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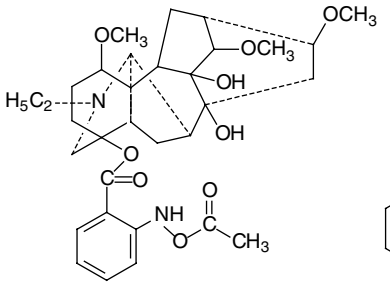
10.3.4 pH Zone-Refining CCC

In the pH zone-refining CCC technique, a retainer (acid for acidic analytes or base for basic analytes) is added into organic stationary phase to retain the analytes in the column, and an eluter (base for acidic analytes or acid for basic analytes) is added into aqueous mobile phase to elute the analytes according to their pK_a values and hydrophobicities. The technique produces a succession of highly concentrated rectangular peaks with minimum overlap, and has several advantages over the conventional CCC method. For example, the loading capacity of sample could be increased remarkably and the target compounds are eluted at extremely high concentrations. The minor ionic compounds could be concentrated and collected at the zone boundaries. In addition, the separation could be monitored by the pH value of the effluent when there are no chromophores. However, pH zone-refining CCC is only applicable to ionizable compounds, which will somewhat limit its use in the natural products field. In addition, the sample must be stable over a wide range of pH values and removal of acid or base from the collected fractions can be difficult.[3,12]

Lappaconitine (**44**) is the major bioactive component in the medicinal herb *Aconitum sinomontanum* Nakai and is used as a pain reliever, mainly for cancer patients. A pH zone-refining CCC

method was developed using a two-phase solvent system composed of methyl *tert*-butyl ether–tetrahydrofuran–distilled water (2:2:3, v/v) for the purification of lappaconitine from a prepurified sample containing lappaconitine at about 90% purity. Triethylamine (10 mM) was added to the upper organic stationary phase as a retainer, and hydrochloric acid (10 mM) was added to the aqueous mobile phase as an eluter. The separation of 10.5 g of the crude sample yielded 9.0 g of lappaconitine at a high purity of 99%.[76]

Sophora flavescens Ait is a typical traditional Chinese medicinal herb and is used as an antifebrile, diuretic, anthelmintic, and antidote agent. Its roots contain more than ten kinds of alkaloids, and sophocarpine and matrine are the main effective constituents used for cancer treatment. The pH zone-refining CCC was applied to the separation and purification of alkaloids from a crude extract of the root of this herb using a two-phase solvent system composed of methyl *tert*-butyl ether–water (1:1, v/v). Triethylamine (10 mM) was added to the upper organic phase as a retainer and hydrochloric acid (5–10 mM) was added to the aqueous phase as an eluter. Sophocarpine (**45**) (170 mg) and matrine (**46**) (600 mg) in purities of over 98% were obtained from 1.0 g of the crude extract.[77]



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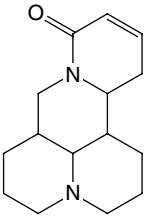

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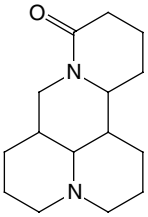

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10.3.5 Cross-Axis CCC

Cross-axis CCC is considered a variety of HSCCC but based on a different type of synchronous planetary motion. The coil holder revolves around the central axis of the centrifuge and synchronously rotates about its horizontally oriented axis. The axes of the rotation and revolution form a 90° angle. This technique provides reliable retention of the stationary phase of viscous, aqueous polymer two-phase systems used for protein separations. The polymer phase systems polyethylene glycol–inorganic salt and polyethylene glycol–dextran are often used. The polyethylene glycol–salt systems have relatively high interfacial tension and low viscosity, and provide suitable partition coefficient values for many proteins.[2,11]

The mushroom *Morchella esculenta* (L.) contains glycoproteins that showed anticarcinogenic and immune modulation activities. A cross-axis CCC method was developed for the isolation and purification of glycoproteins from fermentation media of the mushroom using a polymer phase system composed of 12.5% (w/w) polyethylene glycol 8000 in distilled water as the upper, stationary phase, and 25% (w/w) potassium dihydrogen phosphate (pH 9.0) in distilled water as the lower, mobile phase. Three glycoprotein components of *M. esculenta* were resolved.[78]

A cross-axis dye–ligand affinity CCC method was developed for the purification of alcohol dehydrogenase from a crude extract of bovine liver using a 16% polyethylene glycol 1000–12.5% potassium phosphate system at pH 7.3 containing 0.05% Procion Red dye as an affinity ligand. The upper polyethylene glycol 1000-rich phase containing Procion Red was used as the stationary phase, and bovine liver proteins in the crude sample were eluted from the column using the potassium phosphate-rich lower phase. Then, the alcohol dehydrogenase that was retained in the column due to its affinity to the dye was reverse-eluted using the polyethylene glycol 1000-rich upper phase.

In this way, alcohol dehydrogenase could be purified directly from the crude extract of bovine liver with minimum loss of its enzymatic activity.[79]

The cross-axis CCC was applied to the purification of glucosyltransferase from cell lysate of *Streptococcus mutans* using an aqueous polymer two-phase system composed of 4.4% (w/w) polyethylene glycol 8000–6% (w/w) dextran T500 containing 10 mM phosphate buffer at pH 9.2. After elution of the impurities in the cell lysate using the upper phase, the apparatus was stopped, and glucosyltransferase was still retained in the dextran-rich lower stationary phase. Then, the column contents were extruded (see Section 10.3.6), by air pressure, onto a hydroxyapatite column that removed the polymers from the glucosyltransferase fraction. The purity of glucosyltransferase in the final product was increased about 87 times over that in the cell lysate with a good recovery rate of about 79%.[80]

Some polysaccharides from traditional Chinese medicinal herbs are used for the treatment of hepatitis, malignancy, gastric ulcer, intoxication, and immunodeficiency. A cross-axis CCC method was developed for the purification of polysaccharides from the dried roots of *Achyranthes bidentata* B1, which are used for the treatment of soreness of the lumbar and knee joints with weakness in the legs, amenorrhea with mass formation in the abdomen, and dizziness due to hyperactivity of the liver. The separation was performed using an aqueous polymer two-phase system composed of 12.5% (w/w) polyethylene glycol 1000, 8% (w/w) potassium dihydrogen phosphate, and 8% (w/w) dipotassium hydrogen phosphate buffer at pH 6.8, which yielded 1.1 g of polysaccharides from 2 g of the crude sample.[81]

10.3.6 Other Methods

Other HSCCC methods or techniques, such as dual-mode CCC, elution–extrusion CCC, foam CCC, HSCCC with an evaporative light scattering detector or a mass spectrometer detector, and small analytical CCC instruments, as well as industrial CCC instruments, will be discussed briefly.

As mentioned in Section 10.1, because of the liquid nature of the stationary phase in HSCCC it is possible to perform unique operations, which are not possible in classical liquid chromatography with a solid stationary phase. For example, irreversible retention is impossible in HSCCC because one can empty the column to recover the solutes trapped in the stationary phase or reverse the elution mode, where the initial mobile phase will become the stationary phase, and *vice versa*.

10.3.6.1 Dual-Mode CCC

If the retention volumes of some solutes are too high, the dual-mode CCC method could be used, where the phase role is reversed during the separation. All the injected compounds could be eluted from the column by reversing the phases. This is unlike a column backflush because the separation is still progressing after phase reversal.[82,83] The dual-mode CCC method was used for the purification of alcohol dehydrogenase from the crude extract of bovine liver (Section 10.3.5).[79] In addition, the dual-mode CCC method also gave improved yields in the purification of antibiotics compared with previous results using normal-mode CCC, and various applications showed that it is very useful to reduce separation time in preparative CCC.[82]

10.3.6.2 Elution–Extrusion CCC

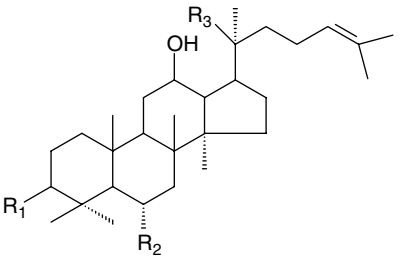
Elution–extrusion CCC is another way to elute all the injected compounds from the column. Some solutes are eluted from the column using the mobile phase, leaving a higher concentration of other solutes in the stationary phase. The stationary phase can then be extruded out of the column in a continuous way using the liquid stationary phase.[83] The purification of glucosyltransferase from cell lysate of *S. mutans* (Section 10.3.5) can be considered as an example of the elution–extrusion CCC application, where the column contents were fractionated by air pressure instead of the liquid stationary phase.[80]

10.3.6.3 Foam CCC

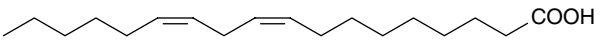
With a foam CCC system, the sample mixture is separated according to the foaming capability. Compounds with a foam-producing capacity or foam affinity quickly move with the foaming stream and are collected through the foam outlet, whereas the remaining compounds are carried with the liquid stream in the opposite direction and eluted through the liquid outlet.[2,84] Foam CCC was used for the fractionation of the antibiotic bacitracin.[85,86] A recycling foam CCC system was developed in which the effluent from the liquid outlet was directly returned into the column through the sample feed line. The sample solution was then continuously recycled for repetitive foam fractionation. The recycling foam CCC was applied to separations of microcystin extract and bacitracin complex. The foam-active components were eluted in the order of their hydrophobicity. The recycling foam CCC could be applied to separation and enrichment of various foam-active components from crude natural products.[84]

10.3.6.4 Eluate Detection Systems

The UV detector is usually used for HSCCC; however, evaporative light scattering detectors and mass spectrometric detectors are also used to monitor HSCCC separation process, especially for compounds without UV absorption. HSCCC with an evaporative light scattering detector was applied to the separation of saponins from the root of *Panax notoginseng* (Burk.) F.H. Chen. Five saponins, ginsenoside-Rg-1 (**47**), ginsenoside-Rd (**48**), notoginsenoside-R-1 (**49**), ginsenoside-Re (**50**), and ginsenoside-Rb-1 (**51**), were isolated with the solvent systems composed of chloroform–methanol–2-butanol–water (5:6:1:4, v/v) and ethyl acetate–*n*-butanol–water (1:1:2, v/v), successively.[87] HSCCC with evaporative light scattering detection was also used for the separation and purification of fatty acids in grape seed oil, which yielded 430 mg of linoleic acid (**52**) in 99% purity from 1.0 g of the oil.[88] The coupling of HSCCC with ESI-MS/MS was utilized to separate, purify, and detect tanshinone IIA from the crude extract of *S. miltiorrhiza* Bunge. The purity of recovered tanshinone IIA was 98%.[89]



47: R_1 = OH, R_2 = –O-Glc, R_3 = –O-Glc
48: R_1 = –O-Glc–Glc, R_2 = H, R_3 = –O-Glc
49: R_1 = OH, R_2 = –O-Glc–Xyl , R_3 = –O-Glc
50: R_1 = OH, R_2 = –O-Glc–Rha, R_3 = –O-Glc
51: R_1 = –O-Glc–Glc, R_2 = H, R_3 = –O-Glc–Glc
(Glc: β-D-glucopyranosyl, Xyl: β-D-xylopyranosyl, Rha: α-D-rhamnopyranosyl)



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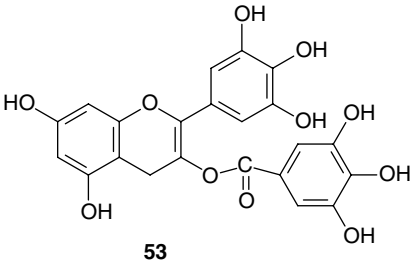
10.3.6.5 Small Coil-Volume CCC

One of the major factors restricting the use of HSCCC as an analytical tool is the slow separation speed. A new small coil-volume CCC instrument was developed, which was as rapid as HPLC, achieving high resolutions in minutes as opposed to hours, with the capability of linking with a

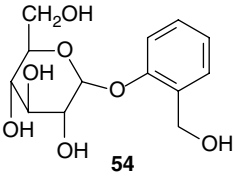
mass spectrometer. Its volume was 4.6 mL, and the maximum rotation speed was 2100 rpm. Stationary phase retention factor higher than 60% could be obtained with 1500 rpm and 1 mL/min, which could separate compounds with distribution coefficient of 1 in less than 5 min.[90]

10.3.6.6 Large-Scale CCC

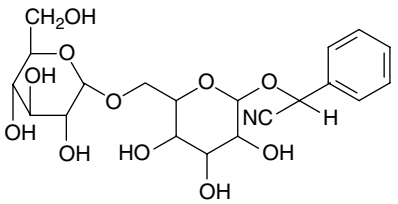
Although HSCCC is widely used for preparative separation in laboratories, the scaling-up of CCC for industrial use is challenging but very promising. A large-scale CCC system with a 10 L capacity column was prepared using a slowly rotating helical device. Using this system, 40 g of epigallocatechin gallate (**53**) in a purity of 92.7% could be obtained, with a recovery rate of 82.6%, from 150 g of the crude tea extract in a single run.[91] A larger scale CCC system with a 40 L capacity column was also constructed and used for semi-industrial separations of salicin (**54**) from the bark extract of *Salix alba* and amygdalin (**55**) from the fruit extract of *Semen armeniacae*. The process yielded 63.5 g of salicin in a purity of 95.3% from 500 g of the crude extract containing salicin at 13.5%, and 221.2 g of amygdalin in a purity of 94.1% from 400 g of the crude extract containing amygdalin at 55.3%.[92]



53



54



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10.4 CONCLUSIONS

HSCCC is a liquid–liquid partition technique that avoids irreversible adsorptive loss, denaturation, and contamination found in solid–liquid systems. HSCCC is mainly a preparative purification technique, and complex crude extracts or semipurified fractions can be separated with sample loads ranging from milligrams to multigrams. The separation of compounds with a wide range of polarities is possible. HSCCC is one of the most useful techniques for the isolation and purification of bioactive compounds from natural sources, such as plant extracts, microbial fermentation products, or animal tissues.

A number of HSCCC methods have been developed for the isolation and purification of bioactive natural products. HSCCC using isocratic elution is used most widely. Sometimes, HSCCC with an isocratic elution needs to be used repeatedly with the same or a different two-phase solvent system to obtain suitably pure compounds. To separate bioactive components with a large difference in their polarity, stepwise or gradient elution as well as stepwise increasing the flow-rate of the mobile phase might be adopted. Multidimensional CCC system could be considered for online two-step HSCCC separations with the same or a different two-phase solvent system. The pH

zone-refining CCC is applicable to ionizable compounds and allows remarkable increases in the loading capacity. Cross-axis CCC provides reliable retention of the stationary phase of viscous aqueous polymer two-phase systems used for the separation of proteins as well as polysaccharides. On the basis of the liquid nature of the stationary phase, dual-mode CCC and elution–extrusion CCC have been developed that could elute all the injected compounds from the CCC column and remarkably reduce separation times. Foam CCC system can be used for the separation and enrichment of compounds with a foam-producing capacity or foam affinity.

The UV detector is widely used for HSCCC. Evaporative light scattering detectors and mass spectrometric detectors are also used to monitor HSCCC separation process, especially for compounds without UV absorption. A small coil-volume CCC instrument with a 4.6 mL capacity column has been prepared to use HSCCC as an analytical tool, which could separate compounds with distribution coefficient of 1 in less than 5 min, and has the capability of linking with a mass spectrometer. On the contrary, a large-scale CCC system with a 40 L capacity column has been constructed for industrial use, and 500 g of the crude extract could be separated in a single run.

The versatility of HSCCC makes it an ideal method for the separation and purification of bioactive natural products. A number of bioactive compounds, such as carotenoids, flavonoids, alkaloids, saponins, terpenes, coumarins, polysaccharides, antibiotics, and proteins, have been separated and purified from plant extracts, microbial fermentation broths, or animal tissues by HSCCC. Usually, milligram to multigram quantities of compounds in high purities can be obtained in a single run from the crude extracts or semipurified fractions. Sometimes, prepurification techniques such as solvent partition or column chromatography can be adopted before HSCCC to improve the loading capacity and separation efficiency. On some occasions, further purification after HSCCC may be necessary to provide compounds of suitable purity for structural analysis and bioactive testing, which can be accomplished by appropriate techniques such as recrystallization or HPLC.

To further extend the capabilities of HSCCC in isolating bioactive natural products, existing strategies of HSCCC should be modified or improved. The combination of HSCCC with solvent partition or column chromatography may find application in the systematic separation and purification of multicomponents from medicinal plants. A microscale HSCCC instrument may be developed for handling small quantities of highly valuable natural products. Also the combined use of HSCCC-MS, HSCCC-NMR, and HSCCC-IR may be efficient for the separation and identification of bioactive natural compounds in complex matrices. With respect to the direct application of HSCCC technology for large-scale purification of bioactive molecules in the pharmaceutical or food industry, development of a robust industrial-scale CCC instrument will definitely be a challenge.

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11 Biosensing Approach in Natural Product Research

Maria Minunni and Anna R. Bilia

CONTENTS

11.1 INTRODUCTION

The analysis of natural constituents of herbal drugs, herbal drug preparations, and herbal medicinal products is generally carried out on a complex matrix where a target compound or a target class of constituents (active principles or markers) must be evaluated. This evaluation is a crucial step

both in quality control and stability testings of well-known herbal drugs, herbal drug preparations, and herbal medicinal products, but it is also important in terms of chemical screening of bioactive constituents from plants in the search for new drugs.

Biosensors represent new analytical devices that appear to be an analyst's dream: they are able to operate directly on complex matrices, in many cases; to be selective and sensitive enough for the required application; to be portable and sometimes also disposable; and to have fast analysis times.[1] Biosensors have mainly been used for analytical purposes in environmental chemistry, clinical practice, and analysis of food, but recently several examples dealing with natural compounds have also been reported in the literature.

In this chapter the main biosensor technologies, based on different transduction principles, are described and examples of their applications to natural product analysis are reported. The different examples are grouped by target analytes containing different functional groups or in terms of their bioactivity, such as the ability to intercalate DNA or to act as antioxidants.

11.2 BIOSENSORS

As defined by IUPAC in 1999, a biosensor is an integrated device able to give qualitative and quantitative or semiquantitative specific information through the use of a biological element of recognition in close spatial contact with a transducer. The biological element is responsible for the biological recognition of the target analyte and thus for the sensor specificity. The biomolecule is immobilized on a physical transducer that translates the biorecognition event into a useful electrical signal (Figure 11.1). Biosensors can be divided mainly into two categories: catalytic and affinity biosensors.[1]

11.2.1 Catalytic Biosensors

Catalytic biosensors involve a catalytic event in which a substrate is converted into a product. Catalysis occurs at the transducer interface, and substrate depletion or product formation is measured by a transducer. The well-known enzyme-based sensor for glucose, widely used in clinical practice for glycemia measurements and marketed by many different companies, belongs to this category. In this specific example, only one enzyme is immobilized, but it is also possible to use a set of enzymes, a whole organism (e.g., bacterial cell) or a tissue slice as the catalytic element. Table 11.1 shows some biological elements that could be used in conjunction with various forms of transducers.

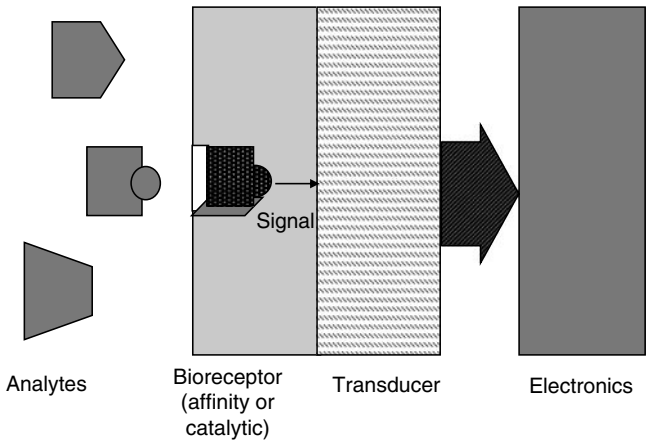


FIGURE 11.1 Schematic of a biosensor.

TABLE 11.1
Various Types of Biological Elements That Can Be Used in Conjunction with Different Transducer Technologies

Biological Elements	Transducers
Enzymes	Electrochemical
Organelles	Optical (including surface plasmon resonance and fluorometric)
Microorganisms	Thermometric
Tissues	Piezoelectric
Cellular receptors	
Antibodies	
Nucleic acids	
Biomimetic receptors (i.e., MIP, aptamers)	

11.2.2 Affinity Biosensors

In affinity biosensors, recognition of the analyte in solution by the immobilized biological element is based on an affinity reaction, e.g., an antigen–antibody binding, nucleic acid hybridization, or receptor–ligand interaction (Table 11.1). On the basis of these different interactions, the affinity biosensors can be divided into immunosensors, DNA sensors, aptasensors, etc.[2]

In recent years, nucleic acids have received increasing interest because this system can be applied for studying DNA–protein interactions or to show the ability of different chemical compounds to bind DNA (e.g., intercalators).[2,3]

11.3 TRANSDUCTION PRINCIPLES

11.3.1 Electrochemical Detection

Electrochemical transduction makes use of electrodes as the sensing element and the measured parameter is current or potential. The great advantage of electrochemical biosensors is represented by inexpensive components and the possibility of developing small inexpensive devices with disposable transducers. Much literature is available for catalytic sensors. In this chapter, we report examples of enzyme-based electrochemical sensors for the detection of different functional groups.

Since the 1960s, when ion-selective, membrane electrodes were introduced, there has been a continuous evolution in electrode design. In the case of voltammetric-based detection (amperometry), current is measured at a fixed potential, while in potentiometry the change in the potential occurring at a sensing electrode is observed. The transducer can be a pH-electrode if H^+ is formed, an amperometric one if H_2O_2 is formed, or the corresponding sensor if a gas, such as ammonia, is formed (Figure 11.2a). In our case with amperometric electrodes, we employed the functional relationship between the intensity of the current (I), measured at a certain potential (E), and the concentration of the analyte (c), that is, $I = f(c)$. In potentiometric electrodes, the functional relationship between the potential, measured at $I = 0$, and the activity (a_i) of the ion, is $E = f(-\log a_i)$. Usually the ionic strength is kept constant by the addition of a strong electrolyte. In this way, the activity can be substituted with the concentration and for an ion M^{z+}, $pM = -\log C_{M^{z+}}$ is used. The electrode function is $E = f(pH)$ for H^+-selective membranes (e.g., glass electrode), when the H_3O^+ concentration has to be determined in solution.

Modern electrochemical techniques can be coupled with new electrode designs in the development of biosensors. For example, disposable electrodes using screen-printing technology, based on

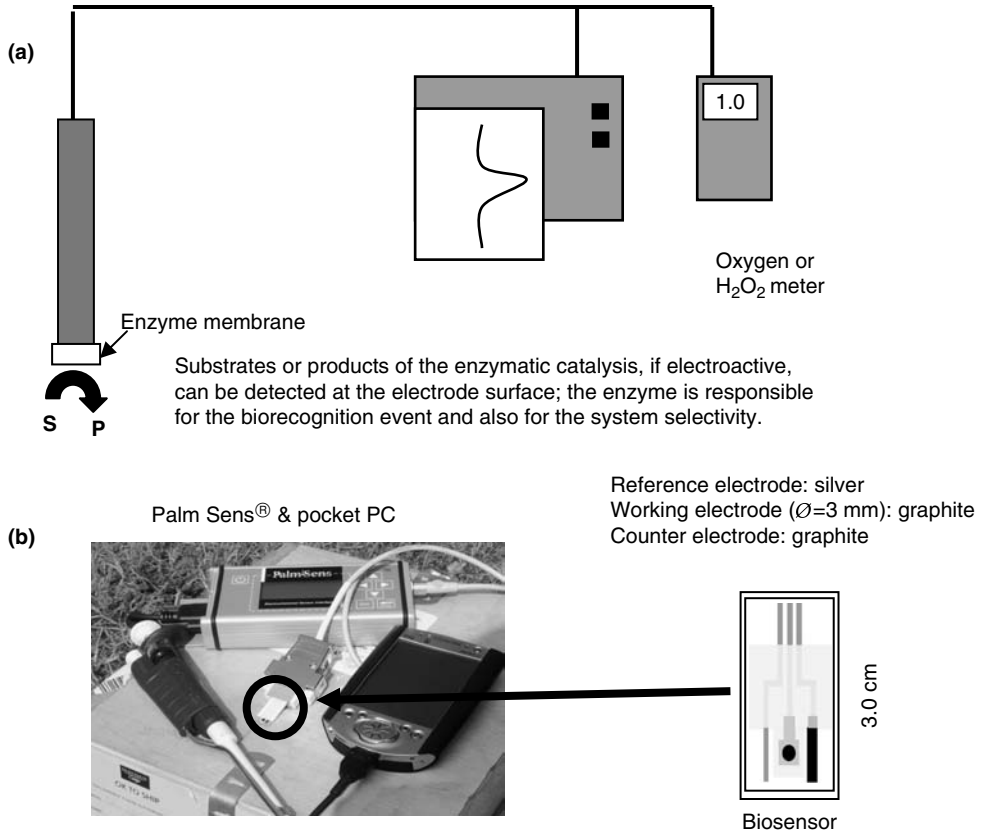


FIGURE 11.2 (a) Scheme of an electrochemical catalytic biosensor. The substrate conversion by the enzymes is accompanied by O_2 consumption or by H_2O_2 production that can be revealed amperometrically. (b) Portable electrochemical detector coupled to single-use, screen printed electrode (SPE). The system is a three-electrode device (pseudoreference, working, and counter electrodes). The working electrode surface could be modified for the development of both catalytic and affinity sensors. A few microliters of the solution to be tested is deposited on the SPE surface to form an electrochemical cell or, alternatively, the electrode can be dipped into a beaker for measurements in batch mode.

film deposition, are available on the market. Screen-printing technology, one of the oldest forms of graphic art reproduction, consists of depositing inks on a film substrate in a controlled pattern and thickness. An example of a single-use, screen printed electrode (SPE) is shown in Figure 11.2b.

11.3.2 Piezoelectric Detection (Mass-Sensitive Devices)

DNA-based biosensors rely on the use of quartz crystal microbalance (QCM) transducers. They are extremely sensitive mass-measuring devices, which allow dynamic monitoring of binding events, using an oscillating crystal, carrying a receptor (molecular probe) immobilized on its surface. The addition of a target compound (analyte), interacting with the receptor, causes a mass increase at the sensor surface associated with biospecific interaction, which results in a decrease in the oscillating frequency (Figure 11.3).

Analysis time is only a few minutes, and the cost per analysis is very competitive (a few US dollars per analysis), when compared to traditional methods. Furthermore, the cost of the instrumentation is very affordable.

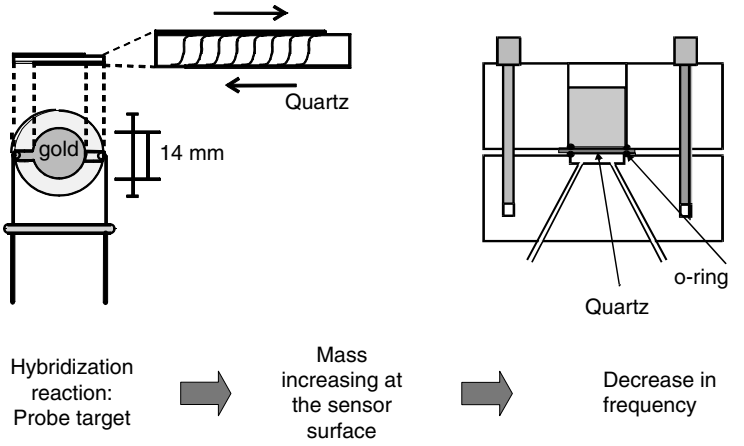


FIGURE 11.3 Piezoelectric sensing utilizing a 10 MHz AT-cut quartz crystal with gold electrodes and measurement cell for analysis in liquid phase.

11.3.3 Optical Detection: Surface Plasmon Resonance–Based Sensing

Surface plasmon resonance (SPR) is a widely used optical method for biospecific interaction analysis since it is possible to monitor the affinity interactions in real time. It is also very easy to obtain the relative affinity constants (K_A).

The SPR phenomenon is influenced by changes in refractive index (RI), occurring when affinity interaction takes place at the interface between the immobilized receptor and its target in solution. Variations in the RI influence the resonant angle at which SPR occurs. The recorded signal is the resonant angle change versus time. The affinity interaction can be followed in real time without the use of any facilitating label such as a chromophore or fluorophore.

Commercially available bench-top instruments are perfectly suitable for laboratory use. Some offer a high degree of automation, contributing significantly to very good analytical performance. More recently, portable devices based on SPR have also been marketed demonstrating that the technology is also suitable for in-field measurements.

11.4 CATALYTIC BIOSENSORS FOR THE DETECTION OF SPECIFIC FUNCTIONAL MOIETIES

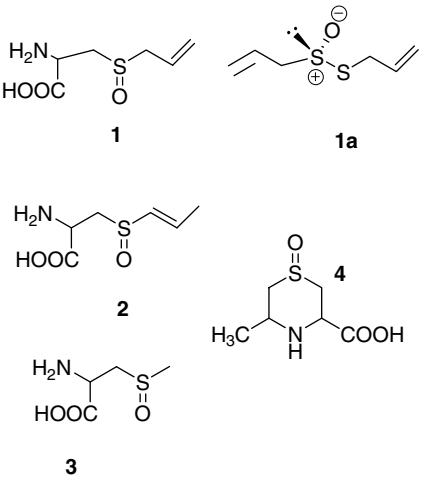
Catalytic sensors use enzymes that convert a substrate into a product that can be recognized and quantified. The selectivity and specificity of the system rely on the enzyme coupled to the transducer. Thus, by immobilizing different enzymes on the sensing elements, a wide spectrum of analytes can be detected. The enzymes can respond only to a specific functional group rather than the whole molecule, and therefore recognize a class of compounds.[1]

Regarding the analysis of natural products, a few examples are reported in the literature, grouped on the basis of the chemical class of the analytes detected, for example, cysteine sulfoxides, glucosinolates, cyanogenic glycosides, and polyphenols. It should be noted that in some reported works the term biosensor is also used for systems that do not fulfill the IUPAC definition (Section 11.1). These systems are often based on the use of a column with the immobilized enzymes (the bioreactor) distal to the detection system, that is, not in contact as in the IUPAC definition.

11.4.1 Cysteine Sulfoxides

Plants of the genus *Allium* such as garlic (*Allium sativum*), onion (*A. cepa*), and leek (*A. porrum*) gain their characteristic taste and odor from a variety of volatile compounds containing several sulfur atoms. These compounds, which are responsible for the health benefits of these plants, are

formed from the enzymatic reaction of cysteine derivatives. A cholesterol-lowering effect, an antiatherosclerotic activity, and a cancer-protective activity have been shown for cysteine sulfoxides and other sulfur-containing substances derived from these plants. The most common compounds are alliin (**1**), isoalliin (**2**), methiin (**3**), and cycloalliin (**4**). Alliin ((+)-*S*-allyl-L-cysteine sulfoxide) is the flavor component generally used for the standardization of garlic-containing remedies. However, it is the enzymatically formed metabolite allicin (**1a**, 2-propenyl-2-propenethiolsulfinate) that is generally quantified.



The first approach in the analysis of such constituents using biosensors was reported by Krest et al.,[4] who proposed the use of alliinase, catalyzing the conversion of alliin (**1**) into allicin (**1a**), pyruvic acid, and ammonium ions (NH_4^+). This system uses a column (bioreactor) made of a cartridge filled with immobilized alliinase (EC 4.4.1.4) from *A. sativum*. Ammonium ions are enzymatically formed in the reactor, and the consequent concentration is proportional to the alliin present in the sample. In a basic environment (by adding sodium hydroxide) the NH_4^+ is converted into NH_3 and, to realize photometric detection, NH_3 passes through a semipermeable membrane into a solution containing a pH indicator, whose color change is monitored at 605 nm. This system is equipped with a flow injection analysis valve. The analysis time is around 5 min per sample. An alternative approach, with comparable results to photometric detection, is the use of commercially available gas-sensing electrodes for NH_3.

An improvement of such systems for the determination of alliin is represented by a flow-through apparatus using alliinase dispersed in a cartridge which is distal to the ammonia electrode, or immobilized on Con A–agarose inside the flow-through cell and nearly in contact with the ammonia electrode. In the first case, the detection limit for alliin was found to be 5×10^{-6} M and long analysis times (15–20 min) were required for a single measurement.[5,6] In the second case of enzyme immobilization into a small, replaceable cartridge, the detection limit was estimated as 3.7×10^{-7} M, with a very good correlation coefficient for the calibration curve (0.999). The substrate specificity of alliinases has also been investigated by analyzing real samples. The results obtained with the sensor (corrected for intrinsic free ammonium ion) correlated very well with the HPLC-based analysis used as a reference technique.[7]

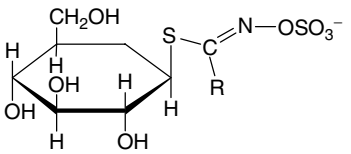
Recently, to simplify and miniaturize the analytical setup, a new sensing approach, coupled again to alliinase, has been reported.[6,8] The enzymatically-formed ammonia is again detected by a potentiometric biosensor based on a pH-sensitive electrolyte/insulator/semiconductor (EIS) layer structure made of Al/p-Si/SiO_2/Si_3N_4. The EIS biosensor also detected other cysteine sulfoxides that are common in wild *Allium* species. The sensor was sensitive to alliin concentrations as low as

5×10^{-5} M, which, although not as good as previously reported, still represents an interesting and promising approach for alliin detection.

A conventional chromatography-based analysis of alliinase activity and the composition of the sulfur-containing compounds, depending on harvesting time, is an important reference when biosensing is applied for screening purposes.[9]

11.4.2 Glucosinolates

Glucosinolates are thioethers, generally consisting of a sugar entity, β-D-thioglucose, with a thioester bond to an organic aglycone that yields isothiocyanate, nitrile, thiocyanate, or a similar compound upon hydrolysis. Glucosinolates have only been detected in a restricted number of dicotyledons with over 80% of all glucosinolates so far identified found in the family Brassicaceae. The best known compounds are sinigrin (**5**), gluconapin (**6**), and glucobrassicin (**7**).



5 R = 2-propenyl
6 R = 3-butenyl
7 R = 3-indolylmethyl

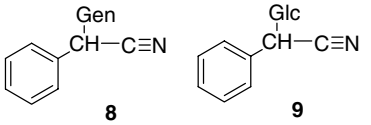
These compounds often contribute a bitter, "hot" taste to condiments, for example, mustard and horseradish, and may exhibit goitrogenic or antithyroid activity. However, they also show beneficial effects, and research indicates that glucosinolates and their derivatives may have potential for fighting human cancers.

A biosensor based on electrochemical transduction has been developed for determination of such molecules.[10] The system consisted of two enzymes, myrosinase and glucose oxidase, immobilized onto an eggshell membrane and with a dissolved oxygen electrode as a transducer. The detection scheme was based on the enzymatic reactions of glucosinolates leading to the depletion of the dissolved oxygen level in the base solution. The decrease in the oxygen level was monitored and related to the glucosinolate concentration. The effects of the ratio of the two enzymes, pH, ascorbic acid concentration, phosphate buffer concentration, and temperature on the response of the glucosinolate biosensor were investigated. Storage and repeatability of the biosensor were also evaluated. The analytical range of the biosensor varied from 2.5×10^{-5} to 7.5×10^{-4} M glucosinolates and the time taken to reach a steady signal was about 100 s. The biosensor is very specific since common matrix interferents such as tartaric acid, glycine, oxalic acid, succinic acid, D, L-alanine, adipic acid, D,L-cysteine, calcium chloride, and sodium chloride did not give any significant response.

The glucosinolate biosensor has successfully been applied to determine the concentration of total glucosinolates in seeds of commonly consumed vegetables and exhibits excellent stability with a long shelf life of at least three months. The system is a reliable and accurate method for the determination of total glucosinolates in real samples.

11.4.3 Cyanogenic Glycosides

Cyanogenic glycosides are widespread in nature and are known to occur in more than 2500 plant species, many of which are used medicinally or as food. Approximately 25 cyanogenic glycosides are known, the most common being amygdalin (**8**, almonds), prunasin (**9**, stone fruits of *Prunus* species), linamarin, and lotaustralin (**10** and **11**, cassava and lima beans).



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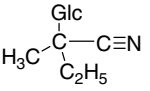

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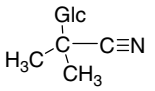

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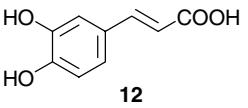
Gen = gentiobiose
Glc = glucose

If plant material containing these compounds is eaten, the glycosidase enzyme hydrolyzes the cyanogenic glycosides to produce hydrogen cyanide, carbohydrate, and ketones or benzaldehyde. Thus, plant products (notably cassava, an important source of carbohydrates for people in Africa and South America), if not adequately detoxified during processing or preparation of the food, are toxic because of the release of this hydrogen cyanide. Recently, electrochemical sensors have been developed based on an ammonia gas electrode and a potentiometric biosensor based on a pH-sensitive EIS layer structure made of Al/p-Si/SiO2/Si_3N_4. These devices were applied, for screening purposes, to check on the levels of cyanogenic glycosides in plant material.[11] The systems were similar to those described for cysteine sulfoxides (Section 11.4.1), developed by the same research group, but use immobilized cyanidase (EC 3.5.5.1). Cyanidase hydrolyzes cyanide to formic acid and ammonia, the latter detected by the gas sensor. However, determination of cyanogenic glycosides includes two enzymatic steps: the conversion from cyanogenic glycosides to HCN by plant enzymes, and then the conversion of HCN into NH_3 and formic acid by cyanidase. Ammonia can be detected either by the ammonia electrode or by a p-semiconductor device. In the case of the ammonia sensor, the cyanidase was immobilized on an activated NHS-activated Sepharose. In the p-semiconductor, cyanidase was directly loaded onto the surface of the pH-sensitive layer of Si_3N_4 and trapped with a dialysis membrane. In both cases the samples were delivered in flow mode. The detection limit of the ammonia sensor for cyanide was 6 μM, with a linear range of 20–300 μM. The sensitivity of the sensor can be increased by using recombinant cyanidase from a bacterial source. The stability of the cartridge was several weeks or more. The EIS device is based on the pH-sensitive layer of Si_3N_4. The detection of CN^- ion, based on changes in pH, is dependent on the ammonia (pK_a 9.2) and formic acid (pK_a 3.8) formed from the catalysis with cyanidase. An equimolar mixture of both, as obtained by the enzymatic reaction, shows a weak acidic pH. Therefore, an increase in cyanide concentration corresponds to a pH decrease, detected by the pH-sensitive layer of Si_3N_4. The sensor is in contact with an Ag/AgCl reference electrode. The operative range is from 1×10^{-6} to 1×10^{-4} M, with an estimated detection limit of 1 μM. The response time is 1 min. Both methods show sufficient sensitivity toward cyanide at subtoxic concentrations in samples from plant material and the environment (soil or water).

11.4.4 Polyphenols

Polyphenols are a very complex group of molecules commonly found in various medicinal plants and also in plant-derived food and beverages such as olive oil, apple and citrus juices, chocolate, tea, coffee, and wine. Several thousand molecules have been identified so far and the main classes include simple phenolic acids, which are ubiquitous, mainly represented by hydroxycinnamic acids that occur free (e.g., caffeic acid, **12**) or as esters of quinic acid (e.g., chlorogenic acid, **13**) or of sugar moieties (e.g., verbascoside, **14**). A second, very abundant class is represented by the flavonoids with a C6–C3–C6 backbone structure. Flavonols (e.g., quercetin, **15**), isoflavones (e.g., genistein, **16**),

catechins (e.g., epicatechin, **17**), and anthocyanins (e.g., malvin, **18**) are characteristic flavonoid derivatives. Less common but with therapeutic value are stilbenes (e.g., resveratrol, **19**), lignans (e.g., pinoresinol, **20**), and several tannins. Polyphenols play important roles in long-term health and reduction of the risk of chronic and degenerative diseases by acting as natural antioxidants, but they also influence the sensorial properties of many foods.



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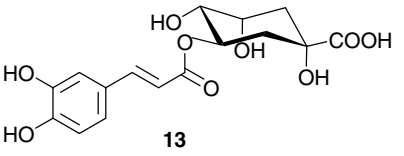

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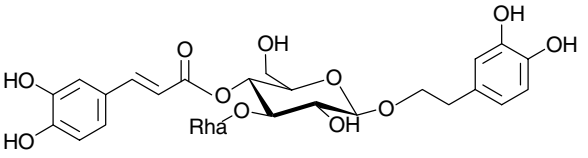
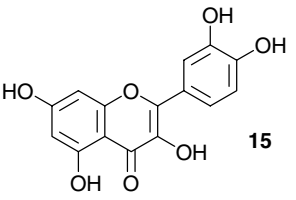


Rha = rhamnose **14**



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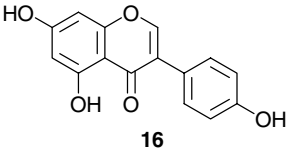

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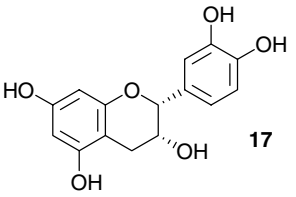

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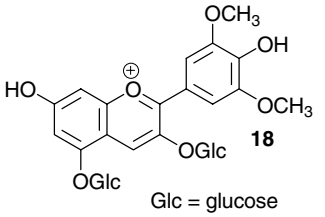

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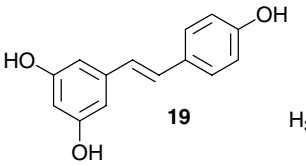
Glc = glucose



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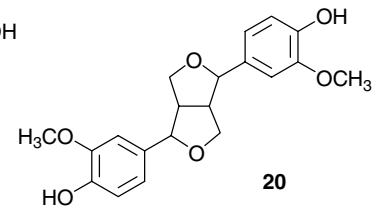

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Many different sensors have been developed in the last 15 years for polyphenol detection, and electrochemical transduction is the approach most applied. The catalytic sensors reported in the literature use mostly the enzyme tyrosinase (also called polyphenol oxidase). The enzyme catalyzes the oxidation of the phenolic substrate to a quinonic form that is reduced at the electrode polarized at a fixed potential. In the presence of oxygen, this enzyme is capable of catalyzing

ortho-hydroxylations of monophenols and oxidation of the consequent *ortho*-dihydroxyphenols to *ortho*-quinones. Measurements can be carried out by recording the signal variation related to the dissolved oxygen consumption or to the formation of the relative quinone.

Behavior of the tyrosinase enzyme electrode has been investigated under different experimental conditions.[12–15] Several authors[16–21] have tested the performance of the tyrosinase electrode in different organic solvents and the effect of different additives has also been investigated.[22] A very exhaustive review on the use of organic phase enzyme electrodes, including tyrosinase biosensors, is given by Campanella et al.[23] Organic phase enzyme electrodes constitute a new class of biosensor applicable to the analysis of substrates or matrices insoluble or scarcely soluble in aqueous media.

Recently, the use of SPEs has been proposed to evaluate polyphenol content. Screen-printing technology is used for the production of disposable sensors that are very useful because during the oxidation process a polymeric film is formed on the electrode surface leading to electrode surface "inactivation" ("electrode fouling"), one of the main drawbacks of common graphite-based electrodes. SPEs have also been used for screening natural products using bare graphite.[24] Different compounds, including flavones, flavonols, catechins, tannins, and phenylpropanoids, were tested with this system. Calibration was performed in a range between 20 and 80 μM of the polyphenol catechin. HPLC analysis was used as reference analytical method. This method can be useful for a rapid and sensitive screening of polyphenols in plant extracts.[24]

In some cases, plant tissue containing polyphenol oxidase (e.g., banana, potato, apple, burdock) has been coupled to electrodes and used for the detection of catechol-related components such as flavonols and catechins in beers.[25–27] The linear detection range of the plant-tissue electrodes was, on average, between 2 and 12 μM catechins.[25] A burdock tissue-based biosensor was applied for measuring total catechins in green tea infusions. As the catechins in a sample are oxidized, the oxygen electrode measures the amount of oxygen consumption, as a decrease in electric current. One limitation of this approach, however, is that the biosensor is inadequate for accurate quantification of total catechins because of the severe variability in the relative biosensor response to the different catechins.[27]

11.5 EVALUATION OF ANTIOXIDANT PROPERTIES

The experimental evaluation of defense properties against free radicals represents an extremely interesting heuristic and applicable objective. Research on different natural matrices has been carried out to experimentally evaluate the free-radical scavenging properties of several fruits, plants, wine, etc.

11.5.1 Superoxide Dismutase–Based Biosensor

Biosensors based on superoxide dismutase (SOD) target the superoxide radical and this system has proven to be useful in determining the antioxidant capacity of different types of synthetic or natural substances capable of scavenging the superoxide radical.[28] The antioxidant capacity is evaluated using the enzyme SOD, which determines essentially the action of scavengers on the superoxide radical generated *in situ* in the measuring cell using the xanthine:xanthine oxidase system. In particular, the antioxidant capacity was checked using an amperometric SOD biosensor. The superoxide radical is produced by oxidation of xanthine in aqueous solution. The subsequent disproportionation reaction of the superoxide radical, catalyzed by the SOD immobilized on the H_2O_2 electrode, releases oxygen and hydrogen peroxide. The hydrogen peroxide released is oxidized at the anode and consequently generates an amperometric signal variation (of the order of nanoamperes) that is proportional to the concentration of superoxide radical in solution.

$$\text{Xanthine} + 2O_2 + 2OH^- \xrightarrow{\text{Xanthine oxidase}} \text{Uric Acid} + 2H^+ + O_2^{\cdot -}$$

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

The addition of samples possessing antioxidant properties produces a decrease in the signal strength, as the antioxidant species react with the superoxide radical, thus reducing its concentration in solution. There is a consequent reduction in the H_2O_2 released and thus also in the intensity of the amperometric signal.

The same sensor was applied to different plant products sold by herbalists (e.g., ginger, dog rose, ginseng, and chamomile), several types of tea, and also to different fresh aromatic herbs, several varieties of olives and fresh fruit.[29,30] The precision of antioxidant capacity measurements for herbal products and for nondiluted samples was generally with an RSD of 5–10%.[29] The precision of this analysis method was found to be good for aromatic herb samples (RSD $\leq$ 8%), acceptable for homogenized fruit samples (RSD $\leq$ 13%), and less good for homogenized and centrifuged samples (RSD $\leq$ 20%).[30] Finally, the same authors applied this sensor successfully to evaluate the total antioxidant capacity of several algae.[31]

11.5.2 Laccase-Based Biosensor

A different amperometric biosensor based on the enzyme laccase has been presented for 30 different phenolic compounds with different structures.[32] Laccase (EC 1.10.3.2) is able to oxidize many different substrates with the concomitant reduction of oxygen to water, and its specific affinity for oxygen as an electron acceptor is very high. The specificity of laccase is rather low since it can oxidize both phenolic and nonphenolic compounds. Laccase catalyzes the removal of a hydrogen atom from the hydroxyl group of *ortho*- and *para*-substituted mono- and polyphenolic substrates. Transformation processes catalyzed by laccase can be accompanied by the appearance of electrochemically active products, which enable the use of standard electrochemical techniques for their determination.

Laccase-based sensors for phenolic compounds have the advantage that the applied potential is within the optimum potential range, where contributions to the response from compounds usually interfering in enzyme-based biosensors are small and the background current takes its smallest value. Another advantage with laccase-based sensors is that their reoxidizing agent, molecular oxygen, is already present in the carrier solution and need not be added.

11.5.3 Cytochrome *c*–Based Biosensor

The use of a different superoxide-scavenging electrochemical cytochrome *c* (Cyt *c*) sensor has been reported for testing methanolic extracts of Chinese tonifying herbs.[33] The system correlated very well with *in vivo*-estimated antioxidant properties, showing again the potential of such devices for screening purposes.

11.5.4 DNA-Based Biosensor

A very recent application for antioxidant properties of several plant extracts involves a biosensor consisting of a double strand of DNA immobilized on an SPE surface.[34] DNA damage is promoted by the generation of the OH radicals via a Fenton-type reaction. Hydroxyl radicals can interact with DNA bases as well as with deoxyribose residues. The antioxidant properties of the extracts are determined by changes that occur within the DNA layer by means of guanine oxidation peak evaluated by a sensitive electrochemical technique (square wave voltammetry) (Figure 11.4). The scavenging ability can also be evaluated using electron spin resonance (ESR) spectroscopy on the basis that the DPPH (1,1-diphenyl-2-picrylhydrazyl) signal intensity is inversely proportional to the antioxidant concentration and the reaction time.

A Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) solution was used as a standard substance to compare the radical scavenging efficiency of several plant aqueous extracts (i.e., *Peumus boldus*, *Baccharis genstelloides*, *Cymbopogon citrate*, *Foeniculum vulgare*, *Mentha piperita*, and *Camellia sinensis*). The antioxidant activities obtained with the DNA-based biosensor

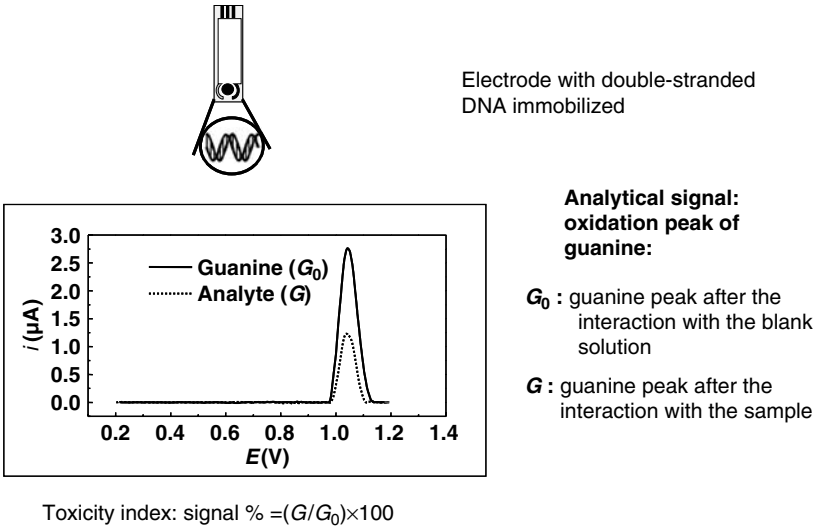


FIGURE 11.4 The interaction of DNA with oxidative agents modifies the oxidation of the DNA guanine base. Guanine oxidation signal recorded at $E_p = +1.0$ V versus Ag-SPE (screen-printed electrodes). An index of the interaction between the agents interacting with DNA is calculated as percentage decrease in the guanine oxidation peak. A toxicity index is given by: signal % $= (G/G_0) \times 100$.

method were lower than those obtained by the ESR-DPPH method but indicated that the biosensor can be applied in the antioxidant screening analysis of natural samples.

11.6 AFFINITY SENSORS

Affinity sensing is based on the biospecific interaction between a surface-immobilized receptor and its ligand in solution. Affinity interactions are, for example, immunochemical reactions between an antibody (Ab) and the relative antigen (Ag), an interaction between nucleic acids (hybridization interaction), or the interaction between a DNA-binding molecule and DNA nucleic acid. As with a catalytic sensor, the receptor is immobilized on the transduction surface, but in this case the interaction leads to the formation of a complex (Ag–Ab, DNA–DNA, or ligand–DNA) instead of a catalysis product, which will be revealed by the transduction event.

Different transduction principles are reported for affinity sensing. Many devices make use of optical (with or without facilitating labels) and mass sensors. Applications of affinity sensors include concentration measurements of a target analyte or estimation of the ability of a ligand to bind the immobilized receptor. Sometimes, depending on the devices, the affinity constant of the biospecific interaction occurring at the interface can also be easily evaluated. In the case of an immunochemical interaction the system is called an immunosensor; when the affinity interaction is based on DNA interaction the system is a nucleic acid-based sensor.

11.6.1 Immunosensors

11.6.1.1 Optical Sensing for Glycyrrhizin and Paclitaxel

11.6.1.1.1 Surface Plasmon Resonance

The optical instrument based on SPR (Section 11.3.3) has been applied to the development of an immunosensor for selective and sensitive detection of glycyrrhizin (**21**), a saponin that represents the active principle of licorice (*Glycyrrhiza* spp.) root, one of the most common herbal medicines used in European, Middle Eastern, Asian, and Chinese traditions and having antiulcer and antiviral activity.

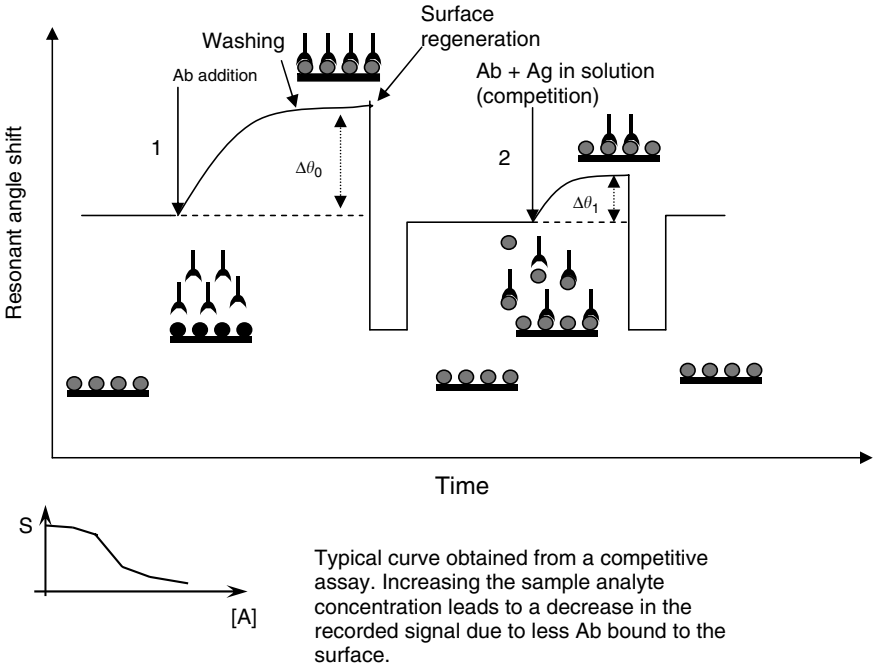
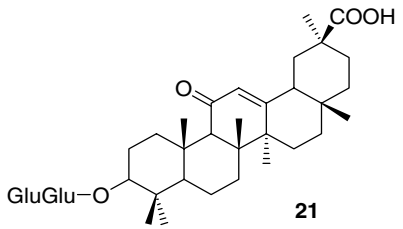


FIGURE 11.5 In the competitive assay, a higher response is observed when the antibodies (Abs) are alone in solution (or in the presence of a noncompetitor) and consequently all the antibodies (present at a limiting concentration) will bind to the surface. When antibodies are incubated in the presence of the relative antigen (Ag) then a competition exists between the antigen and the surface and a lower signal ensues. A typical competition curve is displayed on the left.[35]



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Glu = glucuronic acid

To develop the immunosensor, the antigen (glycyrrhizin) was conjugated with bovine serum albumin (BSA). This glycyrrhizin–BSA conjugate was immobilized on the sensing surface and a competitive assay was developed (Figure 11.5).

With this approach, the lowest detection limit found for glycyrrhizin was 5 μg/mL (corresponding to 3.3×10^{-8} M) with an RSD of 1.29 calculated over three measurements. Detection of glycyrrhizin was also carried out by indirect competitive ELISA. The results observed by both SPR and ELISA are comparable, including similar lowest detection limits.[35]

11.6.1.1.2 Fluorometric

A different approach, based on affinity chromatography coupled with fluorimetric detection, was adopted for paclitaxel (**22**), a complex diterpenoid molecule isolated from the bark of the Pacific Yew *Taxus brevifolia* L. Paclitaxel represents one of the most active anticancer agents introduced in clinical oncology practice.[36]

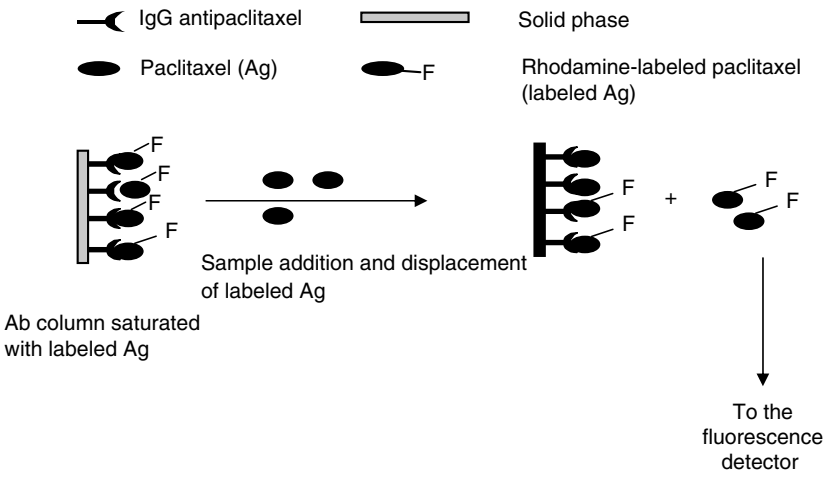
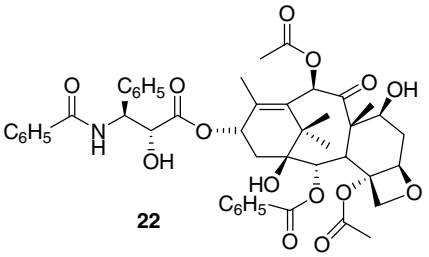


FIGURE 11.6 The antibodies (Ab) in the column are saturated with the corresponding fluorescent-labeled antigen (Ag) (in this case, rhodamine-labeled paclitaxel). The assay is based on the displacement and detection downstream of the rhodamine-labeled paclitaxel, by a flow-through spectrofluorometer, as a result of the competition with paclitaxel introduced as a pulse into the stream of carrier buffer flowing through the system. The newly introduced, unlabeled paclitaxel (Ag) competitively displaces the labeled paclitaxel from the immobilized antibodies on the column. The displaced, labeled paclitaxel is detected using the spectrofluorometer. The peak height of the fluorescence intensity profile of the displaced rhodamine-labeled paclitaxel was directly proportional to the concentration of paclitaxel applied.[36]



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Because of the low abundance of this molecule, alternative sources have been sought, such as the exploitation of roots, needles, foliage, stems, and clippings from other members of the Taxacae family, and culturing of *Taxus* plant cells, *Taxomyces* fungi, and *Erwinia taxi* bacteria in bioreactors. To aid production optimization, rapid screening methods for paclitaxel are needed. Continuous flow, affinity-based (immunochromatography) techniques, and instruments using fluorescence labels could speed up the screening process. The configuration employed by Sheikh and Mulchandani[36] is based on the use of a glass capillary column containing antipaclitaxel antibodies immobilized on protein A and placed upstream to a fluorescence detector (Figure 11.6).

The lower detection limit was found to be 1 ng/mL at a flow rate of 0.1 mL/min. The system response was very reproducible (RSD 4.8%; $n = 10$) and linear up to 100 ng/mL. The assay time is 8 min at 0.1 mL/min flow. Reuse of the system was possible by resaturating the affinity column with new rhodamine-labeled paclitaxel. Paclitaxel was also successfully detected in human plasma, showing the potential for monitoring paclitaxel in body fluids of patients undergoing paclitaxel therapy.[36]

11.6.1.2 Piezoelectric Sensing for Cocaine

Cocaine (**23**) is one of the most commonly used drugs of abuse. It is an alkaloid present in the leaves of the South American shrub *Erythroxylon coca* and has a powerfully reinforcing psychostimulant

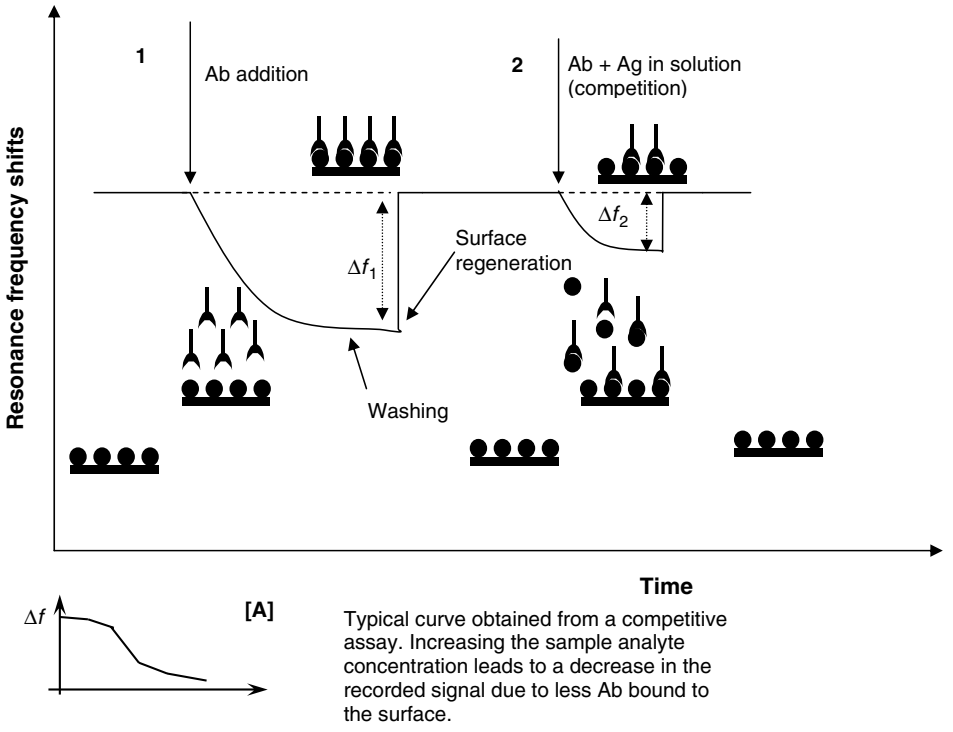


FIGURE 11.7 Responses of the piezoelectric immunosensor for cocaine. The antigen BZE-DADOO is immobilized on the surface. (1) Addition of Ab in the absence of Ag in solution (no competition); (2) addition of Ab in the presence of Ag in solution (competition). Higher responses are found in the absence of the competitor. A typical competition curve is displayed on the left.[37]

action. If overdosed, it acts as an antimuscarinic drug inhibiting gastric motility and increasing the likelihood of gastric ulceration and perforation.

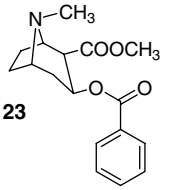
For detection of low molecular weight–compounds with a mass sensor such as a piezoelectric device, a competitive assay is the best choice (Figure 11.7). Halamek et al.[37] immobilized α-benzoylecgonine-1,8-diamino-3,4-diaxaoctane (BZE-DADOO) on the surface of a 10-MHz quartz crystal. An optimized concentration (14 μg/mL) of affinity-purified, sheep polyclonal antibodies against BZE-DADOO was incubated for a selected time (3 min) with the cocaine analyte and a competitive assay performed. The incubated mixture was introduced to the flow system with the crystal, and the binding curve was recorded. The calibration curve obtained indicated that 100 pmol/L of cocaine can be detected.

11.6.2 Synthetic Receptors: Molecular Imprinted Polymers for Caffeine Detection

The molecular recognition capabilities of molecular-imprinted polymers (MIPs) have been described in a wide range of applications. MIPs are synthetic materials "built" to specifically bind the target molecules for which they are designed. In sensor application, polypyrrole (PPy) is one of the most explored conduction polymers and has been used to develop a caffeine (**24**) biosensor.

Quantitation of caffeine was achieved through change of the resonance frequency of the piezoelectric quartz crystal. A surface containing nonmolecular imprinted PPy was used as a control. The measurements were carried out in a liquid matrix with a steady state achieved in 10 min. The sensor response was very reproducible (RSD 9%, $n = 6$) for a solution containing 0.5 mg/mL caffeine. Regeneration of the sensor was possible in a few minutes. The detection limit was found to be 5 μg/mL.

The sensor response was affected by the presence of molecules with structures related to caffeine (**24**), particularly theophylline (**25**) and xanthine (**26**). This cross-reactivity was not negligible. These molecules are able to fit into the pores of the MIP tailored for caffeine since they have a fewer number of methyl groups. However, the sensor was applied to coffee samples and the comparison with standard spectrophotometric analysis showed good correlation between the two methods.[38]



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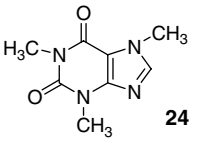

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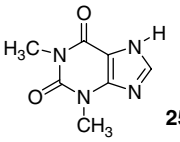

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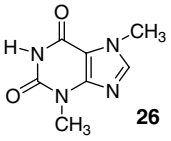

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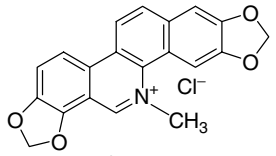
11.6.3 DNA-Based Sensors

A different approach in affinity sensing is based on the use of nucleic acids as receptors. The ability of different molecules to bind DNA can thus be studied by looking at the affinity interaction between the nucleic acid immobilized on the sensor surface and the molecule of interest in solution.

In this regard, optical sensing (SPR-based and reflectometer interference spectroscopy) has been applied to natural compounds to screen their ability to bind DNA. Reflectometer interference spectroscopy was applied first in a study of the binding of well-known intercalators, that is, actinomycin D, nogalamycin, and doxorubicin, to surface-immobilized DNA, which were then compared with the intercalating capacity of two natural alkaloids, sanguinarine (**27**) and chelerythrine (**28**).[39] Compounds **27** and **28** are benzo[*c*]phenanthridinium alkaloids with many activities, including antimicrobial, anti-inflammatory, antiproliferative, antiviral, and antifungal, and can be found in many plants such as *Sanguinaria canadensis* L. (bloodroot) and *Chelidonium majus* L. (great celandine).



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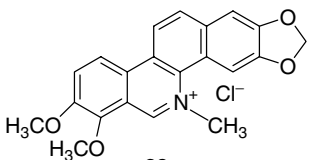

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The results were compared with those obtained from reference techniques including DNA melting studies. A good agreement was found for all of the investigated compounds. Monitoring of the binding event at various intercalator concentrations allowed both kinetic and thermodynamic characterizations of the interaction with immobilized DNA. This system, applied to pure

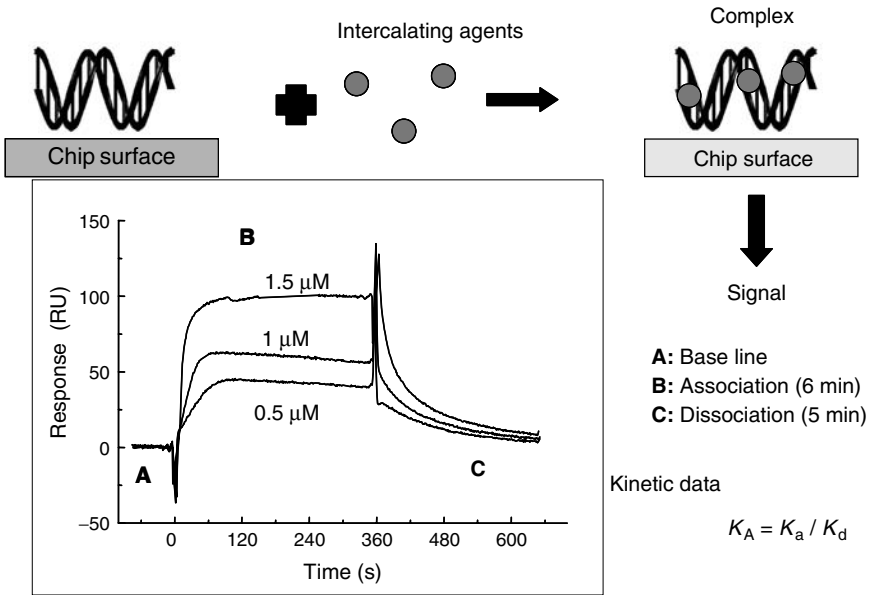


FIGURE 11.8 Surface plasmon resonance (SPR)–based sensing. The response occurring from the interaction between the binder (intercalating agent) and the relative receptor (nucleic acid) on the surface is displayed in real time and without the use of any label. In the association phase (B) the ligand interacts with the DNA forming a complex. In the dissociation phase (C), as the chip is washed with buffer, the complex resulting from the interaction starts to dissociate. The results are reported as resonance shift (RU), which is the difference between the final values of resonance units at the end of the association or dissociation phase and the initial value with buffer.[40]

compounds, has recently been proposed for the analysis of some alkaloidal fractions obtained by chromatographic separation of an extract of *C. majus*.[40]

The ability of these molecules to interact with the double-stranded nucleic acid (dsDNA) immobilized on the sensor surface has been investigated using an SPR-based optical biosensor (Figure 11.8). The biointeraction between the ligand and the immobilized DNA was followed in real time, allowing estimation of the association (k_a) and dissociation (k_d) phases of the interaction. From these values the affinity constant (K_A), that is, the "strength" of the binding between the active compounds and immobilized DNA, of the different compounds was evaluated (Figure 11.8). A good agreement was found between the general behavior of the eight fractions on the biosensor and the relative presence of alkaloids as estimated by HPLC-MS.[40] The results obtained with this biosensor demonstrate the potential of this device in drug screening and bioassay-guided fractionation of active constituents from plants.

11.6.4 Optical Sensing for Antiendotoxins

Another optical sensor, based on resonant mirror technology, was applied for screening traditional Chinese herbs for their ability to bind lipid A, an evolution-conserved region of lipopolysaccharide (LPS), the principal component of the outer membrane of gram-negative bacteria, and a common trigger in the pathogenesis of sepsis.[41] One of several adjuvant therapeutic approaches for severe sepsis is currently focusing on the neutralization of LPS. Agents that bind LPS and neutralize its activities could have potential clinical applications. In traditional Chinese medicine, there is a large range of herbs available reported to have antiendotoxin properties. However, herbs possess a large number of complex constituents and it is extremely difficult to identify which is the antiendotoxin component. Affinity-sensing could help in this search as demonstrated by the work reported by Genfa et al.[41] Results obtained from the analysis of 42 aqueous extracts of Chinese herbs, eight of

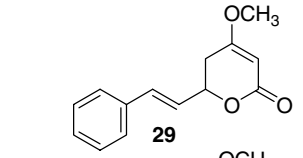
which, that is, *Belaminda chinensis*, Colla Corii Asini, Radix Paeoniae Rubras, Radix et Rhizoma Rhei, Fructus Aurantii Immaturus, Chinese White Olive, Fructus Gardeniae, and *Aconitum carmichaeli* Debx., possessed constituents with the ability to bind with lipid A immobilized on the sensor. The biosensor findings were supported by the use of standard methods applied to the plant giving the highest biosensor signal, that is, Radix Paeoniae Rubras, including silica gel chromatography and HPLC, to purify pentagalloylglucose, the lipid A-binding product. Subsequent *in vitro* and *in vivo* studies confirmed this biological activity.

11.7 BIOSENSOR BASED ON THE MODULATION OF THE BIOLOGICAL ACTIVITY OF THE RECEPTOR

11.7.1 Tissue Biosensors

Tissue biosensors incorporate plant or animal tissue into the transducing element that is then able to translate some biological responses from the tissue into a measurable analytical signal. The use of plant tissues has already been described for the detection of polyphenols (Section 11.4.4). Animal tissue, in particular nerve tissue, can also be employed for screening, for example, neuroactive agents. It is possible to use nerve tissue-based biosensors for studying natural products.[42] Nerve tissue displays high sensitivity to many drugs and toxins that affect the nerve tissue conduction by binding to the ion channels along the surface of the nerve membrane.

The nerve tissue used to test Hawaiian plants was the large abdominal ganglion of the crayfish, cut and fixed to the electrode. As the neuromodulatory constituents are introduced into the system, they bind to ion channels of the nerve membrane and the nerve is prevented from forming an action-current response to the applied stimulus. Using this nerve tissue biosensor, extracts were tested from various native and introduced plant species in Hawaii, which are traditionally used for medical and religious purposes. The investigation was carried out on species having known neuroactive substances such as the kava-kava plant (*Piper methysticum* G. Forster) that contains kavalactones, that is, kavain (**29**), dihydrokavain (**30**), methysticin (**31**), and yangonin (**32**), and also on species such as the hutu tree (*Barringtonia asiatica* (L.) Kurs.) whose active principles are not known.



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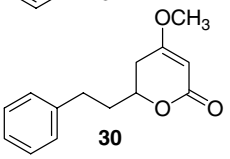

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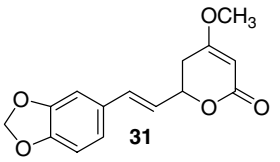

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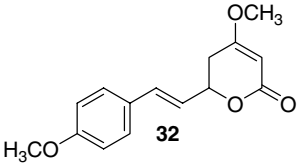

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Different parts of the plants, for example, stems, roots, and leaves, were tested since the concentration of the active compound can be different in different parts of the plant. Dose–response curves can be constructed to assay the relative potency of the chemicals. The effectiveness of this tissue-biosensor approach is interesting, considering the rapidity of the response.

11.7.2 Microphysiometer to Monitor the Cell Response to Compounds

The microphysiometer is a device that studies the influence of compounds on membranes of living, cultivated cells, and the resulting effect on cellular metabolism. The system is aspecific, in the sense that it monitors the reaction of the cell to different stimuli. By measuring the rate of acidification of cultivated cells, changes in metabolism can be investigated. The rate at which the cell excretes H^+ ions is very closely linked to the rate at which they convert nutrients into energy. By maintaining the cell in a minimally buffered environment, small quantities of excreted acid, a by-product from energy metabolism, can be measured. Since energy metabolism is coupled to cellular ATP usage, any event that perturbs cellular ATP levels, such as receptor activation or membrane disruption, will cause a change in energy metabolism and therefore an alteration of acid excretion. In addition to ATP, other alterations, such as membrane integrity, affect acidification by altering the Na^+/H^+ exchange.

The cells in their culture media are sealed inside disposable polycarbonate cell capsules (12 mm in length) and incubated at 37°C in a humidified environment containing 3% CO_2 for 2 days, to allow membrane attachment. Then, the cell culture is loaded into a specific chamber of the silicon-based instrument. The acidification rate measurement gives an indication of the effect of the added analyte on the cell culture.

This system has been applied to study the effect of saponins on endothelial cells from calf aorta.[43] Saponins are the active principles of many medicinal plants and show a variety of biological effects, but sometimes have toxic properties. Despite being quite unspecific, this system could give important indications on the effect of a compound on cell metabolism. This approach is general and can be applied to any kind of natural product to investigate the relative biological activity.

11.7.3 Enzyme Biosensors

Acetylcholinesterase (AChE) enzyme amperometric biosensors have been widely reported for environmental applications; however, they can be useful in the screening for AChE inhibitors, with potential application for Alzheimer's disease research.[44,45] A sensor developed by Lenigk et al.[46] is based on a two-enzyme (AChE and choline oxidase) sensor coimmobilized on the electrode surface (platinum working electrode). Acetylcholine chloride (AChCl) is used as the substrate for AChE. The following reactions then take place at the enzyme-coated electrode:

1. Hydrolysis of acetylcholine

$$\mathrm{AChC1} + \mathrm{H_2O} \xrightarrow{\mathrm{AChE}} \mathrm{Acetate} + \mathrm{Choline} + \mathrm{H^+}$$

2. Reaction of choline

$$\mathrm{Choline} + 2\mathrm{O_2} + \mathrm{H_2O} \xrightarrow[\longleftarrow]{\text{Choline oxidase}} \mathrm{Betaine} + 2\mathrm{H_2O_2}$$

Measurements are performed by adding the substrate (AChCl) to the buffer and the relative current is recorded. After the steady state is reached, the inhibitor is added. After the current drops and reaches a steady state again, the decrease in current is recorded. The response time is a few

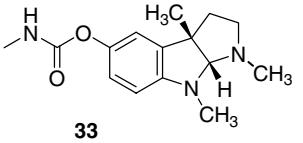
minutes, depending on the concentration of the inhibitor. After the measurements, the electrode is washed and placed in the buffer solution until the next measurement. The system can be applied to reversible inhibition where the magnitude of the current generated by the substrate was the same before and after the experiment. Quantitative determination of the IC_{50} of a well-known, reversible AChE inhibitor, that is, 9-amino-1,2,3,4,-tetrahydroacridine, and two structurally-related compounds was carried out. The results were compared with spectrophotometric-based reference methods and a good correlation was found.[46]

It should be noted that in the case of irreversible inhibition, different biosensor configurations should be used.[44,45] It is preferable, in fact, when dealing with these inhibitors to add the AChE in solution. In this way the irreversible inhibition does not occur on the electrode surface, allowing the reuse of the biosensor, and thus it is only necessary to replace the AChE after each measurement cycle. An index of the inhibition (*I*%) produced by the analyte on AChE is given by the following equation:

$$I\% = \frac{I_1 - I_2}{I_1} \times 100$$

where I_1 and I_2 stand for the currents recorded in absence and presence of the inhibitor, respectively.

These approaches have been very successfully applied to organophosphate and carbamate pesticide detection in different environmental and food matrices.[45] The same system was further applied to the analysis of natural compounds. Scopolamine, eserine, and atropine were tested for their ability to inhibit AChE activity versus well-known synthetic inhibitors, carbofuran, and acridine. As expected, scopolamine and atropine did not exert any inhibition on AChE because their activity is related to the competition with the AChE receptor, while eserine (**33**) was able to inhibit AChE at nanomolar concentrations extending to 100% inhibition at micromolar levels.[47] This example demonstrates the utility of this inhibition biosensor in the search for new natural AChE inhibitors, which represent a very important class of drugs active in many relevant diseases including Alzheimer's.



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11.8 FUTURE PERSPECTIVES

The application of biosensor technology to natural product research has been reported and discussed. Special attention has been focused on the applications of these devices to the quality control of herbal drugs, herbal drug preparations, and herbal medicinal products. Biosensors applied for analytical purposes (screening and quantification of target compounds in complex mixtures, e.g., plant extracts) have been classified on the basis of the class of constituents with a characteristic moiety (e.g., sulfoxides, cyanides). Biosensors used for activity studies (e.g., evaluation of antioxidant properties, modulation of cell response in terms of pH changes, enzyme inhibition) have also been reported. Both catalytic (e.g., enzyme- and tissue-based sensors) and affinity (immuno, nucleic acid, molecular-imprinted membranes) sensors have been discussed in their application to natural product research.

One challenge in biosensor application is the possibility to couple it with chemical analysis, as in hyphenated technologies, to speed up the discovery of new molecules with different biological activities. This application could be very useful, for example, in the search for bioactive constituents from natural sources. In fact, biological and pharmacological screening must deal with a large

numbers of samples, asking essentially "yes/no" questions in automated analysis protocols. When dealing with extracts and fractions obtained from plants, the approach is generally based on bioactive-guided fractionation to obtain the active constituents. Thus, the preliminary identification of these compounds, at the earliest stage of separation, is a strategic element for guiding selective isolation procedures. To rationalize this approach, it would be important to couple the purification–fractionation assay with simple online tests, which could give fast and reliable information about the bioactivity of the compound of interest. Biosensors could represent this connection and applications in this sense are encouraged and expected.

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12 Anticancer Drug Discovery and Development from Natural Products

Gordon M. Cragg and David J. Newman

CONTENTS

12.1 INTRODUCTION

Throughout the ages humans have relied on nature for their basic needs and not least, medicines. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence

for thousands of years. The first records, written on clay tablets in cuneiform, are from Mesopotamia and date from about 2600 BCE, whereas Egyptian medicine dates from about 2900 BCE, with the best-known Egyptian pharmaceutical record being the *Ebers Papyrus* dating from 1500 BCE.[1] The Chinese *Materia Medica* has been extensively documented over the centuries, with the first record (Wu Shi Er Bing Fang), containing 52 prescriptions, dating from about 1100 BCE,[2] and documentation of the Indian Ayurvedic system dates from about 1000 BCE (*Susruta* and *Charaka*).[3,4] In the ancient Western world, the Greeks contributed substantially to the rational development of the use of herbal drugs. The philosopher and natural scientist, Theophrastus (~300 BCE), in his *History of Plants*, dealt with the medicinal qualities of herbs, and Dioscorides, a Greek physician (100 CE), during his travels with Roman armies, recorded the collection, storage, and use of medicinal herbs. Galen (130–200 CE), who practiced and taught pharmacy and medicine in Rome, published no less than 30 books on these subjects and is well known for his complex prescriptions and formulas used in compounding drugs, sometimes containing dozens of ingredients ("galenicals").

During the Dark and Middle Ages (fifth to twelfth centuries), it was the Arabs who were responsible for the preservation of much of the Greco-Roman expertise and for expanding it to include the use of their own resources, together with Chinese and Indian herbs unknown to the Greco-Roman world. The Arabs were the first to establish privately owned drug stores in the eighth century and the Persian pharmacist, physician, philosopher, and poet Avicenna, contributed much to the sciences of pharmacy and medicine through works such as *Canon Medicinae*, regarded as "the final codification of all Greco-Roman medicine". A comprehensive review of the history of medicine may be found on the National Library of Medicine's History of Medicine homepage.[5]

12.1.1 The Role of Traditional Medicine in Drug Discovery

The use of plants in the traditional medicine systems of many other cultures has been extensively documented.[6] These plant-based systems continue to play an essential role in health care, and it has been estimated by the World Health Organization that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care.[7] Plant products also play an important role in the health care systems of the remaining 20% of the population, mainly residing in developed countries. An analysis of data on prescriptions dispensed from community pharmacies in the United States from 1959 to 1980 indicated that about 25% contained plant extracts or active principles derived from higher plants and, at that time, at least 119 chemical substances, derived from 90 plant species, could be considered as important drugs in use in one or more countries.[7] Of those 119 drugs, 74% were discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine. A more recent study using U.S.-based prescription data from 1993 demonstrated that natural products from all sources were still playing a major role in drug treatment; over 50% of the most-prescribed drugs in the United States had a natural product either as the drug or as a model in the synthesis or design of the agent.[8]

12.1.2 The Role of Marine Organisms in Drug Discovery

Although marine organisms do not have a significant history of use in traditional medicine, the ancient Phoenicians employed a chemical secretion from marine mollusks to produce purple dyes for woollen cloth and seaweeds have long been used to fertilize the soil. The world's oceans, covering more than 70% of the earth's surface, represent an enormous resource for the discovery of potential chemotherapeutic agents. Of the 33 animal phyla listed by Margulis and Schwartz, 32 are represented in aquatic environments, with 15 being exclusively marine, 17 being marine and nonmarine (with five of these having more than 95% of their species only in marine environments), and only one, *Onychophora*, being exclusively nonmarine.[9] Before the development of reliable scuba diving techniques some 40 years ago, the collection of marine organisms was limited to those obtainable by skin diving. Subsequently, depths from approximately 10 to 120 ft became routinely attainable. The marine environment has been increasingly explored as a source of novel bioactive agents. Deep water

collections can be made by dredging or trawling, but these methods may suffer from disadvantages such as environmental damage and nonselective sampling. These disadvantages can be partially overcome by using manned submersibles or remotely operated vehicles (ROVs); however, the high cost of these forms of collecting precludes their extensive use in routine collection operations.

12.1.3 The Role of Microorganisms in Drug Discovery

The serendipitous discovery of penicillin from the filamentous fungus *Penicillium notatum* by Fleming in 1929, and the observation of the broad therapeutic use of this agent in the 1940s, ushered in a new era in medicine, "the Golden Age of Antibiotics" and promoted the intensive investigation of nature as a source of novel bioactive agents.[10,11] Microorganisms are a prolific source of structurally diverse bioactive metabolites and have yielded some of the most important products of the pharmaceutical industry, including antibacterial agents, such as the penicillins, cephalosporins, aminoglycosides, tetracyclines, and other polyketides of many structural types; immunosuppressive agents, such as the cyclosporins and rapamycin; cholesterol lowering agents, such as mevastatin and lovastatin; and anthelmintics and antiparasitic drugs, such as the ivermectins.[12] The investigation of plant endophytic fungi and microbes from relatively unexplored habitats, such as extreme environments (the source of so-called extremophiles) and marine sediments, is yielding novel bioactive metabolites of potential chemotherapeutic value. For a more detailed discussion, interested readers are referred to a recent review.[13]

Until recently, microbiologists were greatly limited in their study of natural microbial ecosystems due to an inability to cultivate most naturally occurring microorganisms. It has been estimated that less than 1% of microorganisms seen microscopically have been cultivated and that "a handful of soil contains billions of microbial organisms".[14] Advances in the understanding of the gene clusters that encode the multimodular enzymes, such as polyketide synthases or nonribosomal peptide synthetases, both involved in the biosynthesis of a multitude of microbial secondary metabolites, have enabled the detailed analysis (genome mining) of the genomes of long-studied microbes such as *Streptomyces avermitilis* and, in particular, *Streptomyces coelicolor.* These studies have revealed the presence of additional polyketide synthase and nonribosomal peptide synthetase clusters for as yet unidentified secondary metabolites not detected in standard fermentation isolation processes.[15,16] In addition, procedures based on the extraction of nucleic acids (the metagenome) from environmental samples permit the identification of uncultured microorganisms through the isolation and sequencing of ribosomal RNA or rDNA (genes encoding for rRNA). Samples from soils and seawater are currently being investigated,[17,18] and these methods may be applied to other habitats, such as the microflora of insects and marine animals.[13] Valuable products and information are certain to result from the cloning and understanding of the novel genes that will be discovered through these processes.

12.1.4 Microbial Symbionts

There is mounting evidence that many bioactive compounds isolated from various macroorganisms are also produced by associated microbes and may actually be metabolites synthesized by symbiotic bacteria.[19–21] They include the anticancer maytansanoids, originally isolated from several plant genera of the Celastraceae family (Section 12.5), and a range of antitumor agents isolated from marine organisms, which closely resemble bacterial metabolites. Some of these will be discussed in more detail in subsequent sections.

12.1.5 The Ongoing Role of Natural Products

The continuing valuable contributions of nature as a source of potential chemotherapeutic agents have been reviewed by Newman et al.[12] This was subsequently extended in an analysis of approved drugs from the aspect of their structural sources using the following definitions:

"B" Biological; usually a large (>45 residues) peptide or protein either isolated from an organism/cell line or produced by biotechnological means in a surrogate host.

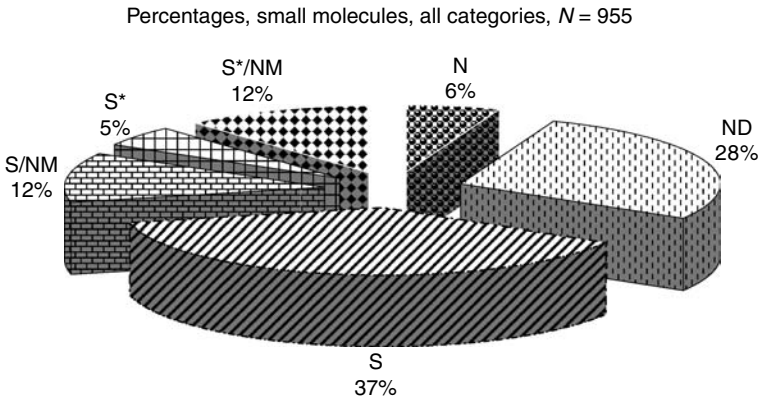


FIGURE 12.1 Sources of new chemical entities, 1981–2005.

"N" Natural product.
"ND" Derived from a natural product and is usually a semisynthetic modification.
"S" Totally synthetic drug, often found by random screening/modification of an existing agent.
"S*" Made by total synthesis, but the pharmacophore is/was from a natural product.
"V" Vaccine.

In addition, a subcategory of "NM" (natural product mimic) was added to the S and S* primary categories: for compounds that fell into the category of "designed from knowledge gained from a natural product" or, in some cases, "discovered by using an assay whereby the compound was designed to displace the natural substrate in a competitive fashion", and are thus "natural product mimics or NM". In practice, both methods, and other information such as X-ray binding studies (*ab initio* or *in silico*), may well have been involved in the derivation of the final drug.

Using these definitions, the sources of new drugs over the period 1981–2005 (data from a 2003 report[22] and unpublished data through December 2005) indicate that although 66% of the 955 small molecule, new chemical entities are formally synthetic, 17% correspond to synthetic molecules containing pharmacophores derived directly from natural products with 12% of the remainder either modeled on a natural product inhibitor of the molecular target of interest, or mimicking (i.e., competitively inhibiting) the endogenous substrate of the active site, such as ATP. Thus, only 37% of the 955 new chemical entities can be classified as truly synthetic in origin (Figure 12.1).

In the area of cancer treatment, over 60% are other than truly synthetic; all approved anticancer drugs from the beginning of chemotherapy are listed in Table 12.1, organized according to their source and in alphabetical order by their generic names within the source. Readers interested in agents currently in clinical trials may refer to the National Cancer Institute's (NCI's) listing of trials in progress.[23]

For the purposes of this chapter, the discussion is divided into sections on the basis of the source organisms. As noted above (Section 12.1.4), however, in several instances it has been determined that the active agent is produced by a microbe associated with the host macroorganism from which the agent was originally isolated.

12.2 ANTICANCER AGENTS FROM PLANT SOURCES

Plants have a long history of use in the treatment of cancer,[24] though many of the claims for the efficacy of such treatment should be viewed with some skepticism because cancer, as a specific disease entity, is likely to be poorly defined in terms of folklore and traditional medicine.[25]

TABLE 12.1
Sources of Approved Antitumor Drugs (from 1930s to June 2006) Ordered by "Source" and Alphabetized within "Source" by "Generic Name"

Generic Name	Source	Year Introduced
Aldesleukin	B	1992
Alemtuzumab	B	2001
Bevacizumab	B	2004
Celmoleukin	B	1992
Cetuximab	B	2003
Denileukin diftitox	B	1999
Interferon alfa-2a	B	1986
Interferon alfa-2b	B	1988
Interferon gamma-1a	B	1992
Interleukin-2	B	1989
Mobenakin	B	1999
Pegaspargase	B	1994
rhAd-p53	B	2005
Rituximab	B	1997
Tasonermin	B	1999
Teceleukin	B	1992
Tositumomab	B	2003
Trastuzumab	B	1998
Aclarubicin	N	1981
Actinomycin D	N	1964
Angiotensin II	N	1994
Arglabin	N	1999
Asparaginase	N	1969
Bleomycin	N	1966
Carzinophilin	N	1954
Chromomycin A3	N	1961
Daunomycin	N	1967
Doxorubicin	N	1966
Leucovorin	N	1950
Masoprocol	N	1992
Mithramycin	N	1961
Mitomycin C	N	1956
Neocarzinostatin	N	1976
Paclitaxel	N	1993
Paclitaxel nanoparticles	N	2005
Pentostatin	N	1992
Peplomycin	N	1981
Sarkomycin	N	1954
Solamargine (aka BEC)	N	1987
Streptozocin	N	Pre-1977
Testosterone	N	Pre-1970
Vinblastine	N	1965
Vincristine	N	1963
Alitretinoin	ND	1999
Amrubicin HCl	ND	2002
Belotecan hydrochloride	ND	2004
Calusterone	ND	1973
Cladribine	ND	1993
Cytarabine ocfosfate	ND	1993

(*continued*)

TABLE 12.1 (Continued)
Sources of Approved Antitumor Drugs (from 1930s to June 2006) Ordered by "Source" and Alphabetized within "Source" by "Generic Name"

Generic Name	Source	Year Introduced
Dexamethasone	ND	1958
Docetaxel	ND	1995
Dromostanolone	ND	1961
Elliptinium acetate	ND	1983
Epirubicin HCI	ND	1984
Estramustine	ND	1980
Ethinyl estradiol	ND	Pre-1970
Etoposide	ND	1980
Exemestane	ND	1999
Fluoxymesterone	ND	Pre-1970
Formestane	ND	1993
Fosfestrol	ND	Pre-1977
Fulvestrant	ND	2002
Gemtuzumab ozogamicin	ND	2000
Goserelin acetate	ND	1987
Hexyl aminolevulinate	ND	2004
Histrelin	ND	2004
Hydroxyprogesterone	ND	Pre-1970
Idarubicin hydrochloride	ND	1990
Irinotecan hydrochloride	ND	1994
Leuprolide	ND	1984
Medroxyprogesterone acetate	ND	1958
Megesterol acetate	ND	1971
Methylprednisolone	ND	1955
Methyltestosterone	ND	1974
Miltefosine	ND	1993
Mitobronitol	ND	1979
Nadrolone phenylpropionate	ND	1959
Norethindrone acetate	ND	Pre-1977
Pirarubicin	ND	1988
Prednisolone	ND	Pre-1977
Prednisone	ND	Pre-1970
Teniposide	ND	1967
Testolactone	ND	1969
Topotecan HCl	ND	1996
Triamcinolone	ND	1958
Triptorelin	ND	1986
Valrubicin	ND	1999
Vapreotide acetate	ND	2003
Vindesine	ND	1979
Vinorelbine	ND	1989
Zinostatin stimalamer	ND	1994
Amsacrine	S	1987
Arsenic trioxide	S	2000
Bisantrene hydrochloride	S	1990
Busulfan	S	1954
Carboplatin	S	1986
Carmustine (BCNU)	S	1977
Chlorambucil	S	1956
Chlortrianisene	S	Pre-1981

TABLE 12.1 (Continued)
Sources of Approved Antitumor Drugs (from 1930s to June 2006) Ordered by "Source" and Alphabetized within "Source" by "Generic Name"

Generic Name	Source	Year Introduced
cis-Diamminedichloroplatinum	S	1979
Cyclophosphamide	S	1957
Dacarbazine	S	1975
Diethylstilbestrol	S	Pre-1970
Flutamide	S	1983
Fotemustine	S	1989
Heptaplatin/SK-2053R	S	1999
Hexamethylmelamine	S	1979
Hydroxyurea	S	1968
Ifosfamide	S	1976
Lenalidomide	S	2006
Levamisole	S	Pre-1981
Lobaplatin	S	1998
Lomustine (CCNU)	S	1976
Lonidamine	S	1987
Mechlorethanamine	S	1958
Melphalan	S	1961
Mitotane	S	1970
Nedaplatin	S	1995
Nilutamide	S	1987
Nimustine hydrochloride	S	Pre-1981
Oxaliplatin	S	1996
Pamidronate	S	1987
Pipobroman	S	1966
Porfimer sodium	S	1993
Procarbazine	S	1969
Ranimustine	S	1987
Razoxane	S	Pre-1977
Semustine (MCCNU)	S	Pre-1977
Sobuzoxane	S	1994
Sorafenib mesylate	S	2005
Thiotepa	S	1959
Triethylenemelamine	S	Pre-1981
Zoledronic acid	S	2000
Anastrozole	S/NM	1995
Bicalutamide	S/NM	1995
Bortezomib	S/NM	2003
Camostat mesylate	S/NM	1985
Dasatinib	S/NM	2006
Erlotinib hydrochloride	S/NM	2004
Fadrozole HCl	S/NM	1995
Gefitinib	S/NM	2002
Imatinib mesilate	S/NM	2001
Letrozole	S/NM	1996
Nafoxidine	S/NM	Pre-1977
Sunitinib maleate	S/NM	2006
Tamoxifen	S/NM	1973
Toremifene	S/NM	1989
Aminoglutethimide	S*	1981

(*continued*)

TABLE 12.1 (Continued)
Sources of Approved Antitumor Drugs (from 1930s to June 2006) Ordered by "Source" and Alphabetized within "Source" by "Generic Name"

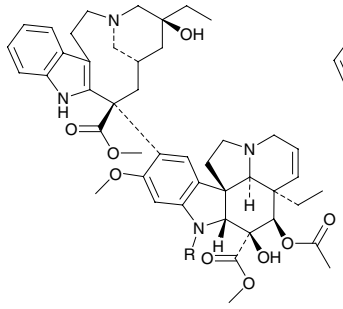
Generic Name	Source	Year Introduced
Azacytidine	S*	Pre-1977
Capecitabine	S*	1998
Carmofur	S*	1981
Clofarabine	S*	2005
Cytosine arabinoside	S*	1969
Decitabine	S*	2006
Doxifluridine	S*	1987
Enocitabine	S*	1983
Floxuridine	S*	1971
Fludarabine phosphate	S*	1991
Fluorouracil	S*	1962
Ftorafur	S*	1972
Gemcitabine HCl	S*	1995
Mercaptopurine	S*	1953
Methotrexate	S*	1954
Mitoxantrone HCI	S*	1984
Nelarabine	S*	2005
Thioguanine	S*	1966
Uracil mustard	S*	1966
Abarelix	S*/NM	2004
Bexarotene	S*/NM	2000
Pemetrexed	S*/NM	2004
Raltitrexed	S*/NM	1996
Tamibarotene	S*/NM	2005
Temozolomide	S*/NM	1999
BCG live	V	1990
Melanoma theraccine	V	2001

Note: Where no formal date is given (i.e., pre-date), this is the earliest date that the drug can be found in a clinical use situation in the literature. There are three "pre-dates" obtained by inspection of lists of approved drugs from various government sources and a drug is listed only once, under the earliest listing that mentions it.

12.2.1 Plant-Derived Drugs in Clinical Use

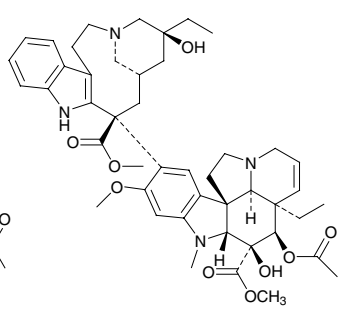
The structures of some plant-derived anticancer drugs currently in clinical use are shown in Figure 12.2. The best known are the so-called vinca alkaloids, vinblastine (**1**) and vincristine (**2**), isolated from the Madagascar periwinkle, *Catharanthus roseus* G. Don. This plant was used by various cultures for the treatment of diabetes, and vinblastine and vincristine were first discovered during an investigation of the plant as a source of potential oral hypoglycemic agents.[26] Therefore, their discovery may be indirectly attributed to the observation of an unrelated medicinal use of the source plant. It is interesting to note that though the plant was originally endemic to Madagascar, the samples used in the discovery of vinblastine and vincristine were collected in Jamaica and the Philippines. More recent semisynthetic analogs of these agents are vinorelbine (**3**) and vindesine (**4**). These agents act through the inhibition of tubulin polymerization and are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers, including leukemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma.

The two clinically active agents etoposide (**5**) and teniposide (**6**), which are semisynthetic derivatives of the natural product epipodophyllotoxin, may be considered as being more closely linked to a plant originally used for the treatment of cancer.[27] The *Podophyllum* species, *P. peltatum* Linnaeus

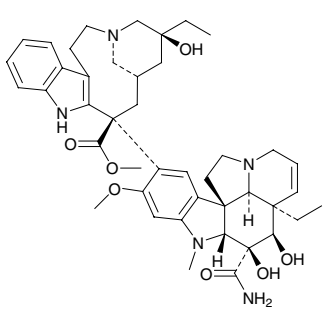


1 Vinblastine (R = CH_3)

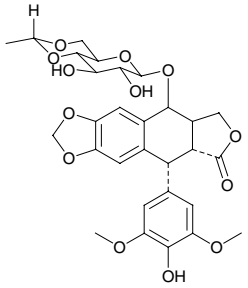
2 Vincristine (R = CHO)



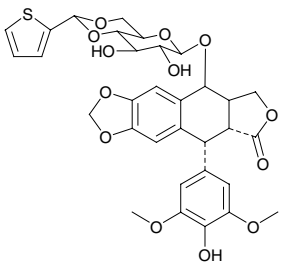
3 Vinorelbine



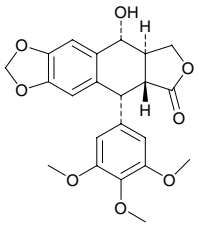
4 Vindesine



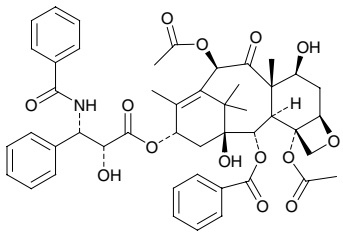
5 Etoposide



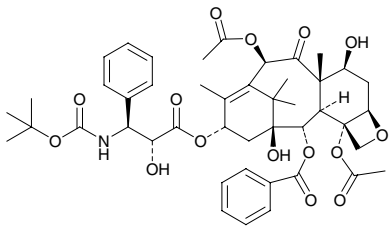
6 Teniposide



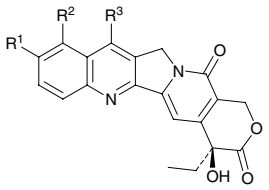
7 Podophyllotoxin



8 Paclitaxel (Taxol™)

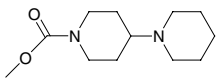


9 Docetaxel (Taxotere™)



10 Topotecan (R^1 = OH; R^2 = $CH_2N(CH_3)_2$; R^3 = H)

11 Irinotecan R^1 =



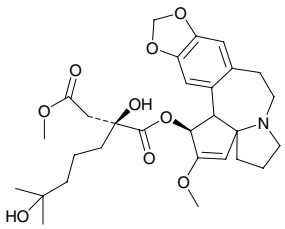
R^2 = H

R^3 = CH_2CH_3

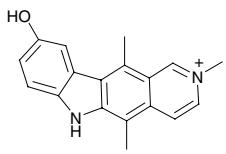
12 R^1=R^3= H; R^2 = NH_2

13 R^1=R^3= H; R^2 = NO_2

14 Camptothecin R^1 = R^2 = R^3 = H



15 Homoharringtonine



16 Elliptinium

FIGURE 12.2 Plant-derived drugs in clinical use.

(commonly known as the American mandrake or Mayapple) and *P. emodii* Wallich, from the Indian subcontinent, have a long history of medicinal use, including the treatment of skin cancers and warts.[24] Epipodophyllotoxin is an isomer of podophyllotoxin (**7**), which was isolated as the active antitumor agent from the roots of various species of the genus *Podophyllum*, but has now also been found to be produced by an endophytic fungus isolated from *P. peltatum*.[28] Many closely related podophyllotoxin-like lignans were also isolated and several of them were introduced into clinical trials, only to be dropped due to lack of efficacy and unacceptable toxicity. Extensive research led to the development of etoposide and teniposide as clinically effective agents, which are used in the treatment of lymphomas and bronchial and testicular cancers and which act through inhibition of topoisomerase II, an important enzyme involved in the replication pathway of DNA during cell cycle progression.

More recent additions to the armamentarium of naturally derived chemotherapeutic agents are the taxanes and camptothecins. Paclitaxel (Taxol®) (**8**) initially was isolated from the bark of the Pacific yew, *Taxus brevifolia* Nutt., collected in Washington State, USA as part of a random collection program by the U.S. Department of Agriculture for the NCI.[29] The use of various parts of *T. brevifolia* and other *Taxus* species (e.g., *T. canadensis*, *T. baccata*) by several Native American tribes for the treatment of some noncancerous conditions has been reported.[25] The leaves of *T. baccata* are used in the traditional Asiatic Indian (Ayurvedic) medicine system,[4] with one reported use in the treatment of cancer.[24] Paclitaxel, along with several key precursors (the baccatins), occurs in the leaves of various *Taxus* species, and the ready semisynthetic conversion of the relatively abundant baccatins to paclitaxel, as well as active paclitaxel analogs, such as docetaxel (Taxotere®) (**9**),[30] has provided a major, renewable natural source of this important class of drugs. Taxol has also been isolated from many endophytic fungi.[31] The elucidation of its unique mechanism of action, involving the promotion of tubulin polymerization and stabilization of the resultant microtubules was, at the time, a landmark discovery in cancer chemotherapy. This led to intensive research aimed at the discovery of other chemotypes operating by a similar mechanism.[32] Several of these discoveries are discussed in later sections of this chapter. Paclitaxel is used in the treatment of breast, ovarian, and nonsmall cell lung cancers and has also shown efficacy against Kaposi's sarcoma, whereas docetaxel is primarily used in the treatment of breast and nonsmall cell lung cancers. Paclitaxel has also attracted attention in the potential treatment of multiple sclerosis, psoriasis, and rheumatoid arthritis and as a constituent of stents. In addition, 23 taxanes are in preclinical development as potential anticancer agents.

The clinically active agents topotecan (Hycamptin®) (**10**), irinotecan (Camptosar®; CPT-11) (**11**), 9-amino- (**12**), and 9-nitro-camptothecin (**13**) are semisynthetically derived from camptothecin (**14**), isolated from the Chinese ornamental tree *Camptotheca acuminata* Decne.[33] Camptothecin has also been reported to be produced by an endophytic fungus of the family Phycomycetes, subsequently identified as *Entrophospora infrequens*,[34] isolated from the inner bark of *Nothapodytes foetida*.[35] Camptothecin (as its sodium salt) was advanced to clinical trials by NCI in the 1970s, but was dropped because of severe bladder toxicity. However, extensive research led to the development of more effective derivatives, topotecan (**10**, Hycamptin®) and irinotecan (**11**, CPT-11; Camptosar®). Topotecan is used for the treatment of ovarian and small cell lung cancers, whereas irinotecan is used for the treatment of colorectal cancers. This class of agents acts through inhibition of topoisomerase I, another important enzyme involved in the replication pathway of DNA during cell cycle progression and, to date, remains by far the most important class of topoisomerase I inhibitors.[32]

Other plant-derived agents in clinical use are homoharringtonine (**15**), isolated from the Chinese tree *Cephalotaxus harringtonia* var. *drupacea* (Sieb and Zucc.),[36] and elliptinium (**16**),[25] a derivative of ellipticine isolated from species of several genera of the Apocynaceae family including *Bleekeria vitensis* A. C. Sm., a Fijian medicinal plant with reputed anticancer properties. A racemic mixture of harringtonine and homoharringtonine has been used successfully in China for the treatment of acute myelogenous leukemia and chronic myelogenous leukemia. Purified homoharringtonine has shown efficacy against various leukemias, including some resistant to standard treatment, and has been reported to produce complete hematologic remission in patients with late chronic phase chronic myelogenous leukemia. Elliptinium is marketed in France for the treatment of breast cancer.

12.2.2 Plant-Derived Agents in Clinical Development

The structures of some plant-derived agents currently in clinical development are shown in Figure 12.3. Flavopiridol (**17**) is totally synthetic, but the basis for its novel flavonoid structure is a natural product, rohitukine (**18**), isolated as the constituent responsible for antiinflammatory and immunomodulatory activities from *Dysoxylum binectariferum* Hook. f. (Meliaceae), which is phylogenetically related to the Ayurvedic plant *D. malabaricum* Bedd. used for rheumatoid arthritis. Flavopiridol was one of over 100 analogs synthesized during structure–activity studies and was selected for preclinical and clinical development on the basis of tyrosine kinase activity, potent growth inhibitory activity against a series of breast and lung carcinoma cell lines, and broad-spectrum *in vivo* activity against human tumor xenografts in mice.[37] It is currently in 19 phase I and phase II clinical trials, either alone or in combination with other anticancer agents, against a broad range of tumors, including leukemias, lymphomas, and solid tumors. Added interest has been stimulated by observation of significant activity against chronic lymphocytic leukemia, a cancer currently lacking efficacious treatment.[38,39] For further discussion about potential of such inhibitors, the recent reviews by Fischer[40,41]and the report by Mayer et al.[42] should be consulted.

The combretastatins (e.g., **19**) were isolated from the South African "bush willow" *Combretum caffrum* (Eckl. & Zeyh.) Kuntze, collected in Southern Africa in the 1970s as part of a random collection program for the NCI by the USDA, working in collaboration with the Botanical Research Institute of South Africa.[43] The combretastatins are a family of stilbenes, which act as antiangiogenic agents, causing vascular shutdown in tumors and resulting in tumor necrosis. A water-soluble analog, combretastatin A-4 phosphate (**20**), has shown promise against thyroid cancer in early clinical trials. This chemical class has served as a model for the synthesis of a host of analogs containing the essential trimethoxy aryl moiety linked to substituted aromatic moieties through a variety of two or three atom bridges including heterocyclic rings and sulfonamides. It also provides an impressive display of the power of a relatively simple natural product structure to spawn a prolific output of medicinal and combinatorial chemistry.[44] A number of combretastatin mimics are being developed. Three analogs are in clinical trials, whereas 11 are in preclinical development.

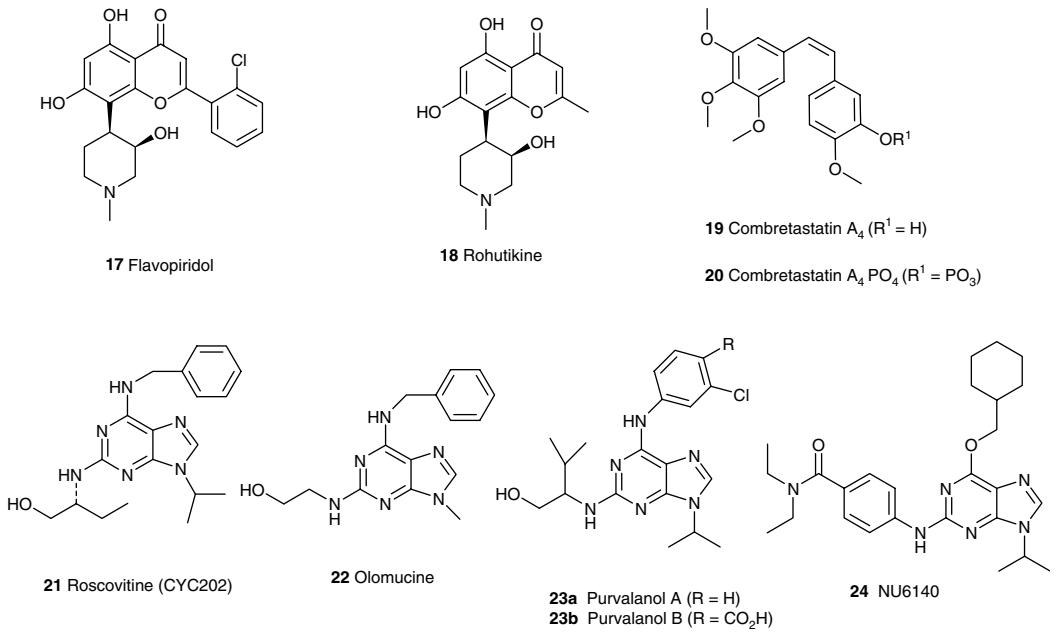


FIGURE 12.3 Plant-derived agents in clinical development.

Another synthetic agent based on a natural product model is roscovitine (**21**), which is derived from olomucine (**22**), originally isolated from the cotyledons of the radish, *Raphanus sativus* L.[45] Olomucine (**22**) was shown to inhibit cyclin-dependent kinases, proteins which play a major role in cell cycle progression. Roscovitine is currently in phase II clinical trials in Europe as CYC202. The basic structural motif led to the purvalanols (**23a** and **23b**), which are even more potent[46] and have now led to even more selective agents such as NU6140 (**24**) that targets survivin, thus acting synergistically with paclitaxel.[47]

12.2.3 Plant-Derived Agents in Preclinical Development

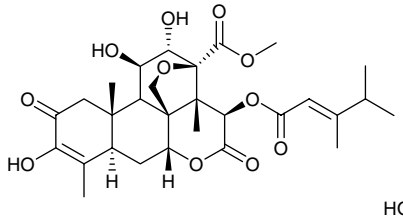
A number of naturally derived agents have been entered into clinical trials that have been terminated due to lack of efficacy or unacceptable toxicity. One of these, maytansine, has been revived through the application of targeting technology (Section 12.5). Another example of an "old" drug in process of revival is bruceantin (**25**, Figure 12.4), which was first isolated from *Brucea antidysenterica* J. F. Mill., a tree used in Ethiopia for the treatment of "cancer".[48] As often happens, activity was observed in animal models bearing a range of tumors but no objective responses were observed in clinical trials and so further development was terminated. Interest has been rekindled by the recent observations of significant activity against panels of leukemia, lymphoma, and myeloma cell lines, as well as in animal models bearing early and advanced stages of the same cancers. Association of this activity with the downregulation of a key oncoprotein (c-*myc*) is considered strong evidence supporting the development of bruceantin as an agent for the treatment of hematological malignancies.

Betulinic acid (**26**), another plant-derived compound with a long history, is a lupane-type triterpene that has been isolated from many taxonomically diverse plant genera.[49] A major source is the birch tree, *Betula* spp., which is also a primary source of its C28 alcohol precursor, betulin, the isolation of which was first reported in 1788. Cytotoxicity against a range of cancer cell lines and significant *in vivo* activity in animal models bearing human melanoma xenografts have prompted the development of systemic and topical formulations of the agent for potential clinical trials. Additional biological activities, including antibacterial, antiinflammatory, and antimalarial, have been reported for betulinic acid and several derivatives, but the most important activities have been associated with inhibition of the replication of strains of the human immunodeficiency virus (HIV).[50]

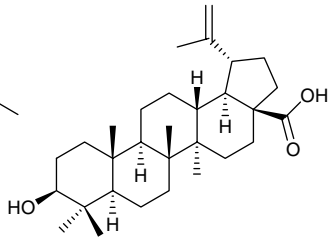
Other triterpenoid acids, such as oleanolic and ursolic acid, which are common plant constituents, are associated with weak antiinflammatory and antitumor activities. Programs directed at the synthesis of new analogs having increased potencies have led to the synthesis of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) (**27**) and its methyl ester, which exhibit potent *in vitro* and *in vivo* antitumor activity against a wide range of tumors, including breast and pancreatic carcinomas and leukemias.[51] Significant activity shown by CDDO against epithelial ovarian carcinoma cell lines, including lines which were resistant to clinically used agents such as cisplatin, has resulted in further evaluation of CDDO in the treatment of these cancers, which are leading causes of death from gynecologic cancers.[52]

The family of bis-indoles, known generically as indirubins (**28**), are the main constituents of Mu Lan (*Indigofera tinctoria* L.), a product from the Chinese *Materia Medica* used for the treatment of chronic myelogenous leukemia. Indole-derived molecules are found in a large number of indigo-producing plants and are also produced by bacteria and are found in gastropod mollusks, where they are the source of the purplish-red dye known from antiquity as "Tyrian purple". They were the first human-used compounds identified as inhibitors of cyclin-dependent kinases. Substituted indirubins have been synthesized (as a result of data from work with a mollusk) and the 6-bromo derivative (**29**) and its 3′-monooxime (**30**) show comparable activity to other known cyclin-dependent kinases inhibitors, such as flavopiridol and roscovitine, and are candidates for preclinical development.[53,54]

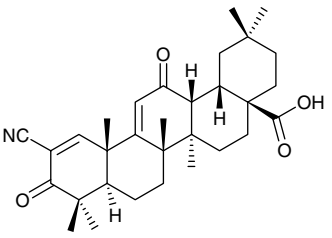
Species of the genus *Tabebuia* have a history of use in the Amazonian region for the treatment of several diseases, including syphilis, fevers, malaria, cutaneous infections, and stomach disorders.



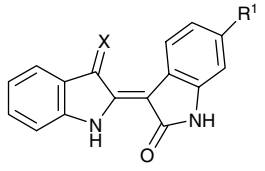
25 Bruceantin



26 Betulinic acid



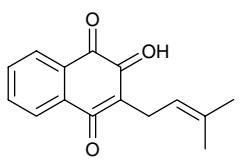
27 CDDO



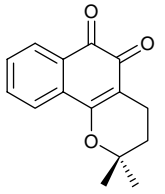
28 Indirubin (X = O; R^1 = H)

29 Bromoindirubin (X = O; R^1 = Br)

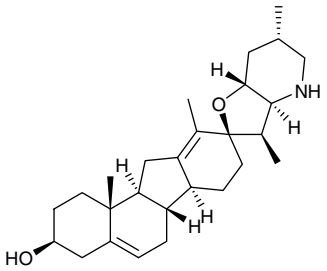
30 Oxime of bromoindirubin (X = N-OH; R^1 = Br)



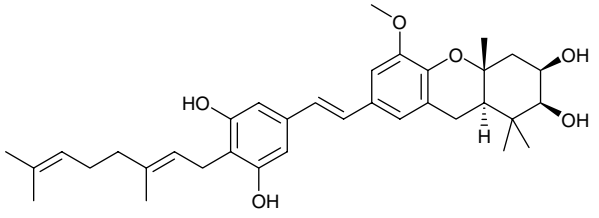
31 Lapachol



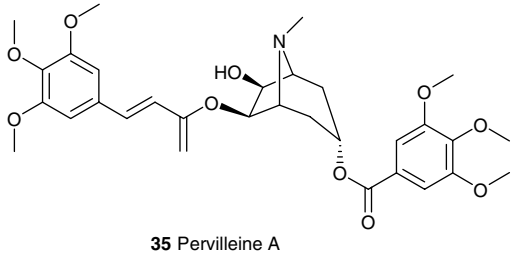
32 β-Lapachone



33 Cyclopamine



34 Schweinfurthin B



35 Pervilleine A

FIGURE 12.4 Plant-derived agents in preclinical development.

Claims for clinical efficacy in the treatment of cancers, starting in the 1960s, particularly in Brazil, led to widespread sales of the stem bark and trunk wood of *T. impetiginosa* (Mart. Ex DC.) Standl. (synonym, *T. avellanedae* Lorentz ex Griseb.), *T. rosea* (Bertol.) and *T. serratifolia* (Vahl) Nicholson in health food stores under various names such as "pau d'arco" or "lapacho". Numerous bioactive

compounds have been isolated, but the naphthaquinones, particularly lapachol (**31**) and β-lapachone (**32**), have received most attention. Significant *in vivo* antitumor activity observed for lapachol in some early mouse models resulted in its advancement to clinical trials by the NCI in the 1970s, but the trials were terminated due to unacceptable levels of toxicity.[55] Interest in β-lapachone has been stimulated by its activity against a range of tumor cell lines, including breast, leukemia and prostate lines, and several multidrug resistant lines,[56] and it is currently in phase I clinical trials.

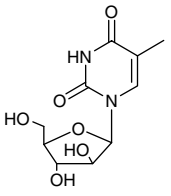
Consumption of *Veratrum californicum* by pregnant sheep has long been associated with the development of birth defects in lambs, including cyclopia in severe cases. These teratogenic effects have been found to be due to presence of alkaloids of the jervine class, in particular cyclopamine (**33**), and the specific inhibition of vertebrate cellular responses to the hedgehog family of secreted growth factors.[57] Although the hedgehog cell signaling pathway normally is quiescent in adult cells, aberrant activation of the pathway in adults has been implicated in many cancers, including cancers of the pancreas, prostate, lung (small cell), and brain (glioma). Cyclopamine blocks the activation of this pathway, and analogs are in various stages of preclinical development.

The schweinfurthins (e.g., schweinfurthin B [**34**]), isolated from the African plant *Macaranga schweinfurthii* Pax, display significant activity in the NCI 60 cell line anticancer assay, with selectivity against central nervous system, renal, and breast cancer cell lines.[58] The spectrum of anticancer activity shows no correlation with any currently used agent and suggests that these compounds may be acting at a previously unrecognized target or through a novel mechanism. Repeated attempts to isolate larger samples of the schweinfurthins from the natural source have met with limited success, and synthetic strategies are being developed to provide a reliable source of natural schweinfurthins and synthetic analogs for further biological testing.[59,60]

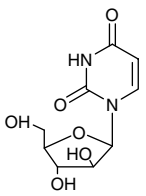
The drug resistance developed by many cancers in patients treated with standard anticancer agents is a serious problem encountered in cancer chemotherapy and may develop in a cell population through repeated exposure to treatment with that particular drug. This cell population may subsequently show broad cross-resistance to other anticancer agents even though it has never been exposed to those agents and this phenomenon is called multidrug resistance (MDR). MDR may be related to the presence of an *MDR1* gene encoding a phosphoglycoprotein (known as either Pgp or P-glycoprotein in the literature), which effectively pumps the drugs out of the cell, thereby precluding their antitumor actions. Several compounds that reverse this effect *in vitro* in cell line studies (so-called MDR inhibitors) have been discovered, but their effectiveness in the clinic has been disappointing in many cases, so there is a continuing search for more effective MDR inhibitors. The pervilleines (e.g., pervilleine A [**35**]) isolated from the Madagascar plant *Erythroxylum pervillei* Baillon (Erythroxylaceae) have shown promising MDR activity both *in vitro* and *in vivo.* Pervilleine A is currently in preclinical development.[61]

12.3 ANTICANCER AGENTS FROM MARINE SOURCES

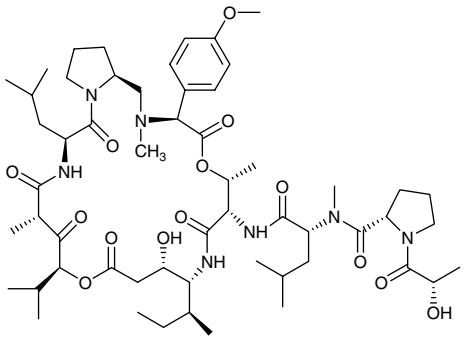
The marine environment has proven to be a very rich source of extremely potent compounds that have demonstrated significant antitumor activity. The initial discoveries from the marine environment can be traced to the reports of Bergmann on the discovery and subsequent identification of spongothymidine (**36**, Figure 12.5) and spongouridine (**37**, Figure 12.5) in the early 1950s from the Caribbean sponge *Tethya crypta*.[62–64] These reports actually led to a complete reversal of the then current dogma that for a nucleoside to have biological activity, it had to have ribose or deoxyribose as the sugar, but that the base could comprise a multiplicity of heterocycles and even carbocycles. These discoveries led to the identification of a close analog, cytosine arabinoside, as a potent antileukemic agent.[12] Despite the significant number of marine-derived agents that has been, or is currently, in clinical trials for the treatment of cancer, as yet, none has reached the stage of commercialization.



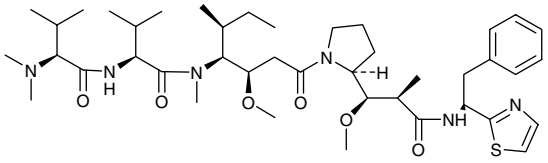
36 Spongothymidine



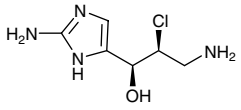
37 Spongouridine



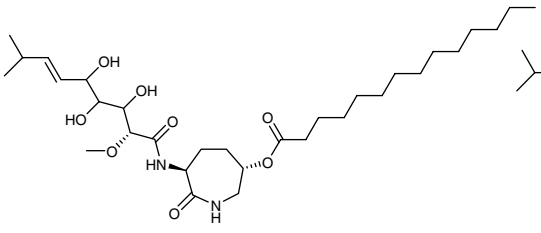
38 Didemnin B



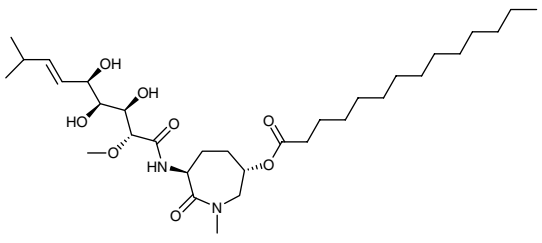
39 Dolastatin 10



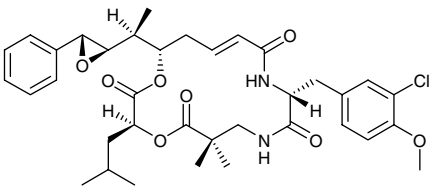
40 Girolline (Girodazole)



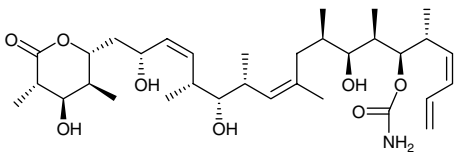
41 Bengamide A



42 Bengamide B



43 Cryptophycin 52



44 Discodermolide

FIGURE 12.5 Marine-derived agents now withdrawn from clinical trials.

12.3.1 Clinically Studied Marine-Derived Anticancer Agents

12.3.1.1 Marine-Derived Agents Now Withdrawn from Clinical Trials

Didemnin B (**38**, Figure 12.5), isolated from the tunicate *Tridemnum solidum*, demonstrated excellent antiviral activity and subsequent cytotoxic activity against P388 and L1210 murine leukemia cell lines.[65] It advanced into preclinical and clinical trials in the early 1980s as the first-defined chemical compound *directly* from a marine source to go into clinical trials for any major human disease. Despite many different treatment protocols and testing against many types of cancer, the

compound turned out to be too toxic for use and trials were officially terminated in the middle 1990s. Although didemnin B was not successful, a very close chemical relative is currently in clinical trials (cf. aplidine below). The background to these compounds has been reviewed as part of a discussion of antitumor compounds from tunicates.[66]

Dolastatin 10 (**39**) is a linear member of a series of cytotoxic peptides that were originally isolated in very low yield from the Indian Ocean mollusk *Dolabella auricularia*.[67,68] On the basis of its potency and mechanism of action as a tubulin interactive agent binding close to the vinca domain,[69] together with the development of viable syntheses, dolastatin 10 was selected for phase I clinical trials in the 1990s. It progressed to phase II trials as a single agent against metastatic melanoma[70] and prostate cancer,[71] but although tolerated at the doses high enough to give the expected levels *in vivo* to inhibit cell growth, it did not demonstrate significant anticancer activity. There are other dolastatins and molecules related to them that are still in clinical and preclinical trials; these are discussed in later sections.

Girolline (Girodazole [**40**]), a substituted imidazole isolated from the sponge *Pseudaxinyssa cantharella*, was shown to be an inhibitor of protein synthesis, acting preferentially on the termination step in eukaryotic protein synthesis, in contrast to other known protein synthesis inhibitors such as emetine, homoharringtonine, anguidine, and bruceantin, which generally act at either the initiation or elongation steps.[72] It proceeded to phase I clinical trials, but the trials were terminated due to significant hypertensive effects. A thiazole derivative of girolline, 5-deazathiogirolline, was synthesized in the hope of altering these effects, but was effectively inert.[73]

Bengamides A (**41**) and B (**42**) were first reported in 1986 as anthelmintic compounds (together with some antibiotic and cytotoxic activities),[74] and further analogs, together with antitumor activities, were reported in several subsequent papers.[75–77] A derivative of bengamide A, shown to be an inhibitor of methionine aminopeptidases, was entered into phase I clinical trials in 2000, but was withdrawn in 2002.

Cryptophycins were isolated from a nonmarine *Nostoc* cyanophyte species[78] and a semisynthetic analog, cryptophycin 52 (**43**), was selected for clinical trials in the mid-1990s. It was advanced to phase II trials, which were terminated in 2002 due to toxicities and lack of efficacy.[79] Similar compounds possessing the same activities have been isolated from the Okinawan sponge *Dysidea arenaria*.[80,81]

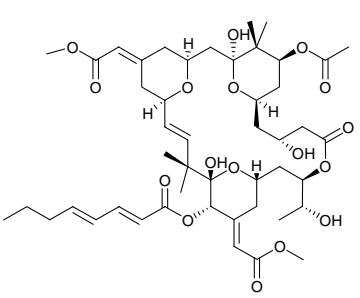
Discodermolide (**44**) was isolated from the Caribbean sponge *Discodermia dissoluta* and was originally considered to be a new immunosuppressive agent with incidental cytotoxicity. The latter activity was emphasized by a later observation that discodermolide bound to microtubules more potently than Taxol®.[82] There have been numerous syntheses reported,[83,84] and 60 g of discodermolide prepared according to current Good Manufacturing Practice (cGMP) was produced for clinical studies.[85–90] It entered phase I clinical trials as a potential treatment against solid tumors. However, no objective responses were seen, though stable disease was reported with 20% of the patients all of whom had advanced solid malignancies. Phase I trials have now ceased with discodermolide due to toxicity, though other analogs are being evaluated preclinically.

12.3.1.2 Marine-Derived Agents Currently in Clinical Trials

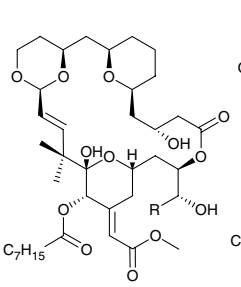
Structures of some marine-derived agents currently in clinical trials are shown in Figure 12.6. The bryostatins are a series of macrocyclic lactones originally isolated in minute yields from the bryozoan *Bugula neritina*.[91–93] Bryostatin 1 (**45**) of cGMP quality has been isolated in sufficient quantities to permit more than 80 clinical trials to date, with 20 being completed at both phase I and phase II levels. There have been some responses to the compound as a single agent with effects ranging from complete remission, to partial remission, or to stable disease. However, its use as a single agent is probably not the optimal application for this compound. When administered in combination with other cytotoxins, such as the vinca alkaloids or nucleosides, in the treatment of carcinomas that are leukemic in nature, the response rates, even in phase I trials, begin to demonstrate greater efficacy worthy of further investigation.[94]

Since the publication of the first structure by Pettit in 1982, the bryostatins have been the target of many synthetic chemistry groups.[91,95,96] In addition, extensive studies have been performed on the synthesis of simpler analogs possessing comparable or better activity, particularly related to binding to some of the molecular targets, protein kinase C (PKC) isozymes, resulting in the preparation of compounds, bryologs (e.g., **46a**–**e**), with greater potency than bryostatin 1 in *in vitro* cell line assays.[97–107]

An interesting question arising from the search for bryostatin sources was why so few colonies of the apparently ubiquitous source organism, *B. neritina*, actually produce detectable levels of bryostatins 1–3 and why are they so geographically widespread? A possible explanation is that the bryozoan is actually the host to a symbiotic microorganism that may well be the actual producer of

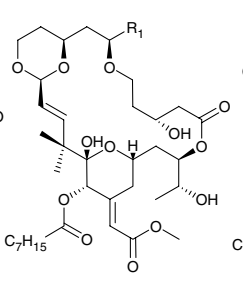


45 Bryostatin 1



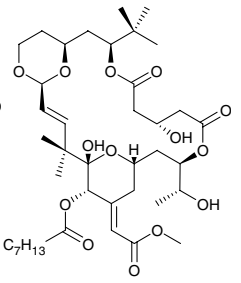
46a (R = CH_3)

46b (R = H)

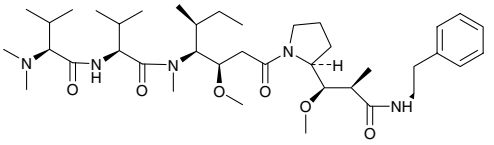


46c (R = *t*-Bu)

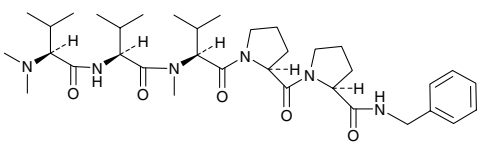
46d (R = H)



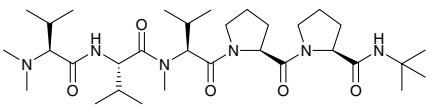
46e



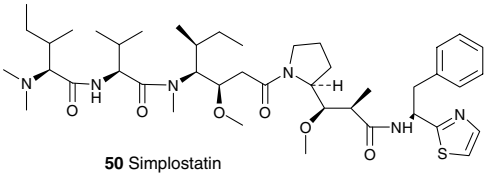
47 TZT-1027(Auristatin PE or Soblidotin)



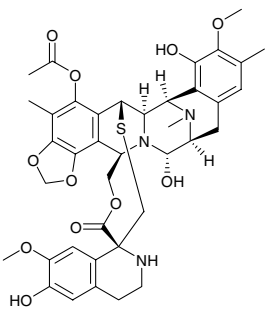
48 Cematodin (LU-103793)



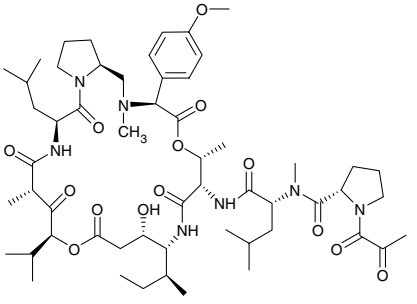
49 ILX-651 (Synthatodin or Tasidotin)



50 Simplostatin



51 Ecteinascidin (ET743)



52 Aplidine

FIGURE 12.6 Marine-derived agents currently in clinical trials. (*Continued*)

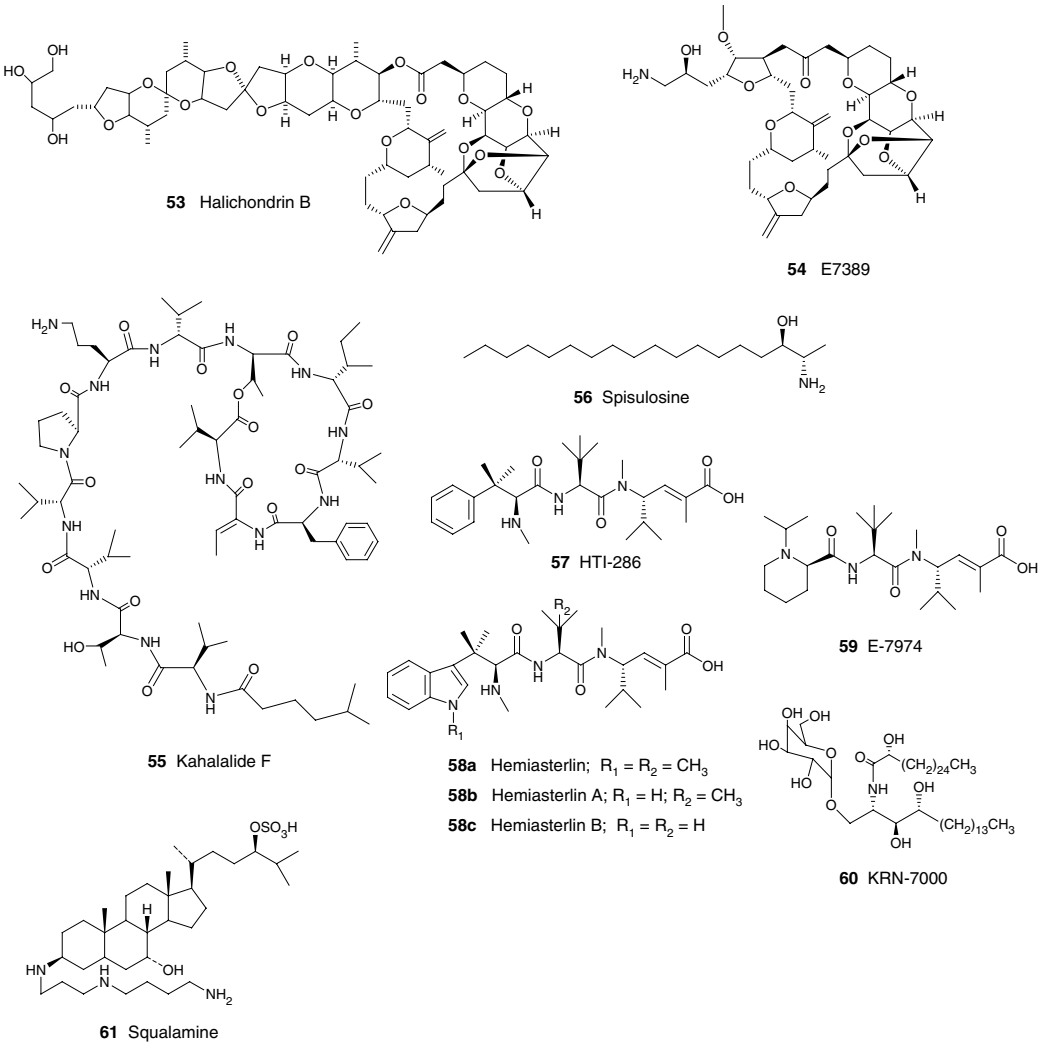


FIGURE 12.6 (*Continued*).

the compound. Use of molecular probes demonstrated the presence of a putative type I polyketide synthase gene fragment in the microbial flora (*Candidatus Endobugula sertula*) of colonies that produced bryostatin but which was absent in the corresponding flora of nonproducers.[108,109] If successful, the cultivation of the organism, or a surrogate with the bryostatin polyketide synthase system expressed, would potentially solve any supply problems if bryostatin 1 becomes a commercial drug.[110]

Although clinical trials of dolastatin 10 have been terminated (see Section 12.3.1.1), several other dolastatin analogs are currently in clinical development.[67] The synthetic derivative TZT-1027 (auristatin PE or soblidotin) (**47**) is in phase II clinical trials and has been shown to exhibit potent antivascular effects in addition to antitubulin activity, suggesting that a dual mechanism might well be possible with this agent.[111,112] The analog cematodin (LU-103793) (**48**) has progressed into phase II clinical trials against malignant melanoma, metastatic breast cancer, and nonsmall cell lung cancer. Although no objective responses have been reported, there are reports of stable disease being seen in both the melanoma and breast cancer trials and a subjective increase in a quality of life measure in the lung trial.[113–115] Phase II studies in melanoma, breast, and nonsmall cell

lung cancers have been initiated with ILX651 (synthadotin or tasidotin) (**49**), which is an orally active third-generation dolastatin 15 analog. There have been two published reports on phase I studies with this agent, and a profile of the compound showing that it is in phase II trials.[116–118]

It is interesting that the dolastatins have also been shown to be of microbial origin, with the isolation of a dolastatin analog, symplostatin 1 (**50**), from the marine cyanobacterium *Simploca hynoides*.[119] Subsequently, dolastatin 10 was isolated from another marine cyanobacterium that was known to be grazed on by *D. auricularia*, the original source nudibranch.[120] It has also been isolated from this nudibranch following feeding of the cyanophyte (personal communication, Dr. V.J. Paul). There have been two further reports of the isolation of dolastatin-like peptides from different collections of the ubiquitous cyanophyte *Lyngbya majuscula*,[121,122] providing further evidence for the microbial source of these peptidic cytotoxins.

Ecteinascidin 743 (ET743; Yondelis™) (**51**) was originally isolated in very low yields from the ascidian *Ecteinascidia turbinata*[123,124] and the conduct of basic *in vitro* and *in vivo* testing and mechanism of action studies required the collection of large amounts of the ascidian from Caribbean locations. Although the synthesis of ET743, and a phthalimido analog having a similar activity profile, has been achieved,[125,126] the acquisition of sufficient quantities of ET743 for advanced preclinical and clinical studies was initially achieved by very large-scale aquaculture of *E. turbinata* in open ponds followed by isolation and then, for later supplies, by means of a 21-step semisynthetic conversion of cyanosafracin B, a metabolite isolated through the large-scale fermentation of the marine microbe *Pseudomonas fluorescens*.[127,128] Ecteinascidin 743 is currently in a number of phase II/III clinical trials for ovarian, soft tissue sarcoma, breast, endometrial, prostate, nonsmall cell lung, and pediatric cancers, and has been granted orphan drug status by the European Commission for soft tissue sarcoma and ovarian cancer,[127] with submission to the EU regulatory authorities for registration made on August 1, 2006 as a treatment for sarcoma. Ecteinascidin 743 is the first of a novel class of DNA-binding agents and details of its complex, transcription-targeted mechanism of action are discussed by Henríquez et al.,[127] and if approved for marketing, it will be the first, direct-from-sea, antitumor agent to reach this stage.

Aplidine (dehydrodidemnin B) (**52**) was isolated from the Mediterranean tunicate *Aplidium albicans*[129,130] and is currently in phase II clinical trials for a range of cancers, including melanoma, pancreatic, head and neck, small cell and nonsmall cell lung, bladder, and prostate cancers, as well as non-Hodgkin lymphoma and acute lymphoblastic leukemia.[127] Orphan drug status has been granted by the European Commission for the treatment of acute lymphoblastic leukemia, which is a leading cause of death for persons under 35 years of age.[127] The precise mechanism of action of this agent is not yet known, but details of studies to date are discussed by Henríquez et al.[127] What is very interesting, both chemically and pharmacologically, is the significant difference in the toxicity profile resulting from the change of the lactyl side chain in didemnin B (Section 12.3.1.1) to a pyruvate side chain in the dehydro derivative (aplidine).[131]

Halichondrin B (**53**) has been isolated from several sponges, including *Halichondria okadai* from Japan,[132] an *Axinella* sp. from the Western Pacific,[133] *Phakellia carteri* from the Eastern Indian Ocean,[134] and a deep water *Lissodendoryx* sp. off the East Coast of South Island, New Zealand.[135] Sufficient quantities for early preclinical studies were isolated from large-scale collections of the *Lissodendoryx* sp. and similar yields could also be obtained from this sponge grown by aquaculture in shallow waters off the coast of New Zealand. Halichondrin B and norhalichondrin B were successfully synthesized,[136] and the synthetic strategy was adapted for the synthesis of a large number of structurally simpler analogs, some of which maintained the biological activity but were intrinsically more chemically stable, due to the substitution of a ketone for the ester linkage in the macrolide ring.[137] One of these, E7389 (**54**), was selected for further development and is now in phase III clinical trials, particularly against refractory breast carcinoma.

The cyclic depsipeptide kahalalide F (**55**) was isolated from the Sacoglossan mollusk *Elysia rufescens* following grazing by the mollusk on a green macroalga, *Bryopsis pennata*.[138,139] Following isolation and identification, it was discovered that the depsipeptide also occurs in the

alga, though in much lower concentration, and thus it appears that the mollusk concentrates the depsipeptide significantly. An efficient synthesis was developed for the compound[140] and it entered phase I clinical trials in Europe in December 2000 for the treatment of androgen-independent prostate cancer. Activity has also been reported in the treatment of androgen-resistant prostate cancer patients[141,142] and against other advanced solid tumors.[143] Studies have also commenced in the treatment of liver carcinoma.[110] The primary mechanism of action for kahalalide F has not been fully delineated but progress in studies in this area is discussed by Henríquez et al.[127]

Spisulosine (**56**), isolated from the marine clam *Spisula polynyma*, was observed to exhibit substantial selective activity against tumor cells compared to normal cells[144] and appears to interact with the endothelial cell differentiation gene receptors.[145–147] This agent was in phase I trials against solid tumors,[110] but was withdrawn in late 2006.

HTI-286 (**57**) is a synthetic analog of hemiasterlin (**58a**–**c**) that was originally isolated from the South African sponge *Hemiasterella minor* and shortly thereafter from a Papua New Guinea sponge *Cymbastela* sp.[148] These compounds interact with tubulin to produce microtubule depolymerization in a manner similar to the vinca alkaloids, the cryptophycins and the dolastatins.[149] HTI-286 was found to be the most effective of a large number of synthetic analogs and went into phase I clinical trials, and was scheduled to go into phase II to investigate its potential in the treatment of nonsmall cell lung cancer[148] but was then suspended. However, another closely related compound, synthesized by chemists at Eisai, is E7974 (**59**), which is currently in phase I clinical trials and is quoted as not being a good substrate for the MDR complex in tumor cells.[150] Hopefully, it will proceed further along the development pathway than the earlier variations on these structures.

The first *R*-galactosylceramides from natural sources, the agelasphins, were obtained from the marine sponge *Agelas mauritianus* and exhibited antitumor and potential immunostimulatory activities.[151,152] These molecules were found to show potent *in vivo* activity against the murine B16 melanoma and the synthesis of various derivatives culminated in the production of KRN-7000 (**60**),[153,154] which entered a phase I clinical trial in 2001 for cancer immunotherapy. No objective responses were reported but the observation of biological effects on a few patients with high levels of natural killer T cells[155] suggested that a preselection of patients with high natural killer T-cell populations might give objective responses in other trials. Further work was recently reported where although no objective results were recorded, two patients were stabilized for a significant time period,[156] and recently, the compound was entered into trials as an antiviral agent against hepatitis B and C following the reports of activity of the base molecule and simpler variations by Mehta et al.[157]

Squalamine (**61**) isolated from the common dogfish shark, *Squalus acanthias*, collected off the New England coast,[158] was shown to be a fairly simple aminosterol with broad-spectrum antibiotic activity but was also found to exhibit significant antiangiogenic activity.[159] Although it has not shown promise as a single agent, it has now progressed into phase II clinical trials in combination with agents such as carboplatin or paclitaxel for the treatment of patients with advanced nonsmall cell lung cancer, where partial responses were observed in 12 (28%) of patients, with 8 (19%) more having stable disease.[160] Later reports of activity in randomized phase II trials have been given in abstract form.[161] However, it is in the treatment of wet macular degeneration where significant activity is now being seen, with phase III trials under way using squalamine as a single agent[162] utilizing its antiangiogenic activity to stop the unrestrained capillary growth that is the underlying cause of this disease.

Neovastat (AE-941) is a standardized liquid extract comprising the $<$500 kDa fraction from shark cartilage prepared under cGMP conditions from taxonomically identified shark species harvested under sustainable conditions. Details of its angiostatic and antitumor activities and possible mechanism of action have been reported.[163] The preparation has been in many phase II/III clinical trials in Canada, Europe, and the United States, and though initial details of the pivotal studies in phase III renal carcinomas were reported,[164,165] the renal carcinoma trials ceased recruitment at $\sim$300 patients. To date no further reports have been published in the literature on these renal carcinoma trials, and currently it is reported to be in a long-term phase III trial against nonsmall cell lung carcinoma.[166,167]

12.3.2 Marine-Derived Agents in Preclinical Development

In this section, a selection of marine-derived compounds (Figure 12.7) exhibiting promising levels of preclinical antitumor activity is presented to illustrate the rich diversity of chemotypes that has common mechanisms of actions and that constitute promising lead compounds or scaffolds for further development through application of techniques such as combinatorial chemistry. The promise of natural products in this regard has been addressed in several reports and reviews.[168–174]

12.3.2.1 Tubulin Interactive Agents

The discovery of the mechanism of action of paclitaxel resulted in extensive studies of marine natural products as a source of novel agents having a similar mechanism of action using tubulin-related assays.[110] Using these assay systems, a wide variety of marine-derived compounds have now been identified as tubulin-interactive agents.[32]

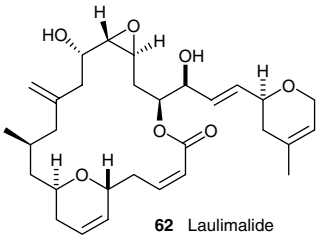
Laulimalide (**62**) and isolaulimalide (also known as fijianolides B and A, respectively) were isolated from the Pacific Ocean sponge *Cacospongia mycofijiensis*[175] and have been reported in other sponge genera, including *Hyatella*, *Fasciospongia*, and *Dactylospongia*, and also from a chromodorid nudibranch grazing on the sponge. Although this agent is a microtubule-stabilizing agent, it might bind at a site different from the taxanes.[176] It is reported to have an additional mechanism independent of mitotic arrest whereby G1 aneuploid cells are formed due to aberrant mitotic events at 5–7 nM, concentrations approximately 30% of the levels required for mitotic accumulation. The many synthetic routes to laulimalide, as well as methods of synthesis of sections of the overall molecule, have been reviewed.[177] Subsequent papers have reported both the synthesis of the base molecule[178] and of modified molecules (mainly 11-desmethyl variants) on the basis of modeling studies.[179,180] Similar molecules were reported from investigators at the Eisai Research institute in the United States in a comparable time frame.[181,182]

The syntheses of five laulimalide analogs ("laulilogs"), with modifications in the epoxide (**63a**), the C_{20} alcohol (**63b**), and the C_1–C_3 enoate of the base natural product structure, have been reported.[183] These were poor substrates for the phosphoglycoprotein that is a major mechanism of drug resistance in mammalian cells, and thus these compounds were active against paclitaxel-resistant cell lines. Further biological data on the five laulilogs demonstrated that better activities were shown by **63a** and **63b**.[184]

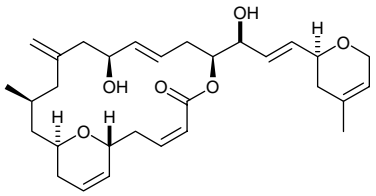
Curacin A (**64**), isolated from the cyanobacterium *L. majuscula*,[185] is a potent cytotoxin but no *in vivo* activity has been reported, probably due to its effective insolubility in any formulation. The application of combinatorial chemistry around the basic structure has enabled the synthesis of more soluble variants that are undergoing evaluation.[186] Relatively recently, the complete biosynthetic gene cluster was identified and cloned, which may permit modification via combinatorial biosynthetic techniques in due course.[187]

Eleutherobin (**65**) was originally isolated from the Australian octacoral *Eleutherobia* sp.,[188] later from the Caribbean octacoral *Erythropodium caribaeorum*,[189] and has been reported to be produced by whole organism aquaculture.[190] Interest in this molecule was stimulated by its demonstrating microtubule-stabilizing activity similar to that of paclitaxel and laulimalide, but supply problems have hindered its development. The closely related sarcodictyins (**66**), isolated from the Mediterranean corals *Sarcodictyon roseum* and *Eleutherobia aurea*,[191,192] were originally reported as lacking biological activity but later found to interact with tubulin.[193] Combinatorial libraries have been synthesized around the sarcodictyins yielding a compound having weak interaction with tubulin but antiproliferative activity matching that of eleutherobin.[194] Synthetic chemists have still continued their work on novel methods to produce these agents and analogs, even though aquaculture might be a feasible route. Thus, in 2004, Chandrasekhar et al.[195] reported the synthesis of a simplified oxy-analog of eleutherobin and were able to model it onto porcine β-tubulin and

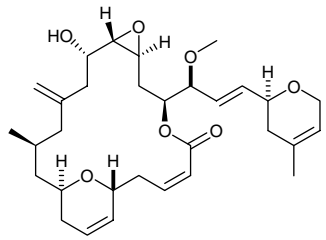
reported that the compound had some cytotoxicity. This work was followed in 2005 by two papers from Castoldi's group in Italy, one[196] reporting a newer method of synthesis of eleutherobin involving a novel synthesis of a key intermediate in the Danishefsky synthetic scheme and the other, a complete discussion of their work.[197] In the latter paper, they reported their syntheses of a number of C-7 modified eleutheside analogs that still retained paclitaxel-like activity and in the case of one particular molecule, the tubulin interactions were comparable to those of paclitaxel, but the



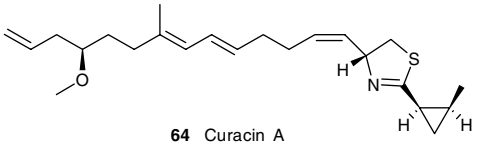
62 Laulimalide



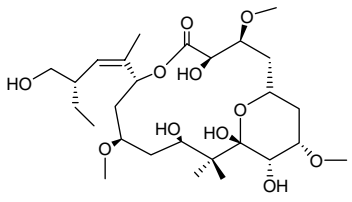
63a $C_{16}-C_{17}$-desepoxy-laulimalide



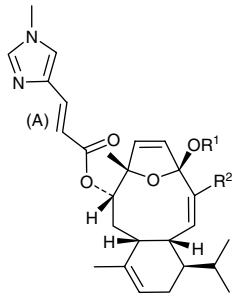
63b C_{20}-methoxy-laulimalide



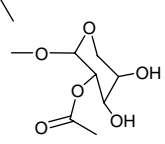
64 Curacin A



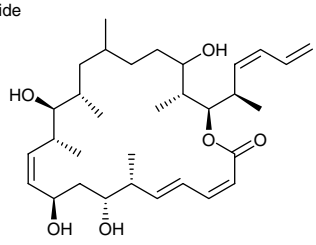
67 Peloruside



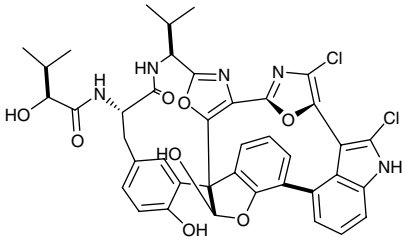
65 Eleutherobin (A) = *E*, $R_1 = CH_3$, R^2 =



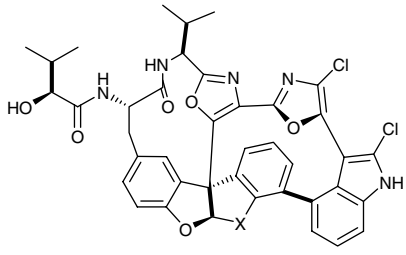
66 Sarcodictyin (R^1 = H, $R^2 = CO_2CH_3$)



68 Dictyostatin 1



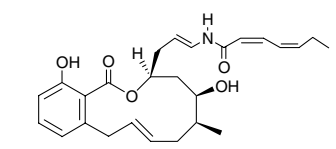
69 Original diazonamide structure



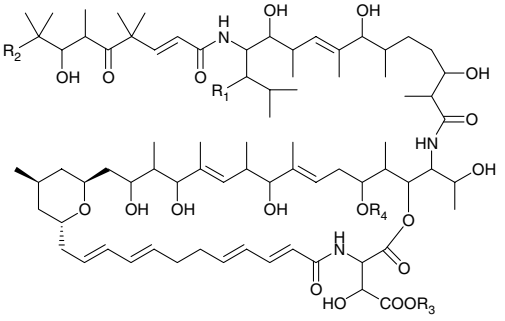
70 ''OXO''-analog, X = O

71 Revised diazonamide structure, X = NH

FIGURE 12.7 Marine-derived agents in preclinical development.



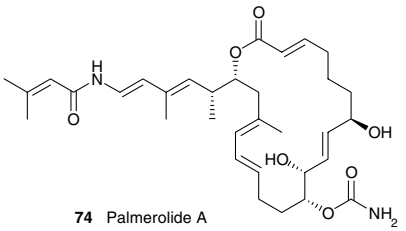
72 Salicylihalimide A



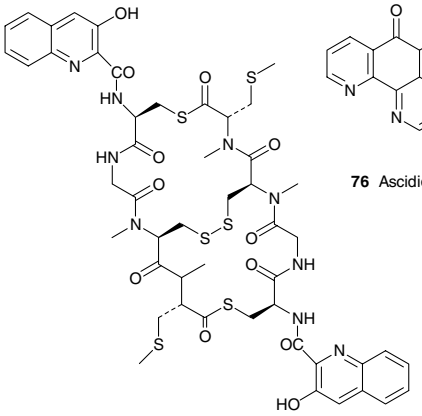
73a Chondropsin A (R^1 = OH, R^2 = $COOCH_3$, R^3 = H)

73b 73-Deoxychondropsin A (R^1 = H, R^2 = $COOCH_3$, R^3 = H)

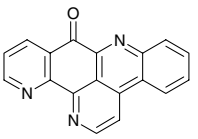
73c Chondropsin C (R^1 = H, R_2 = H, R^3= H)



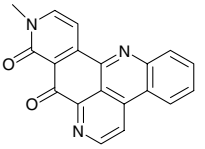
74 Palmerolide A



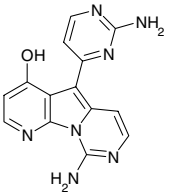
75 Thiocoraline



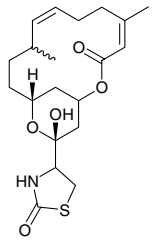
76 Ascididemnin



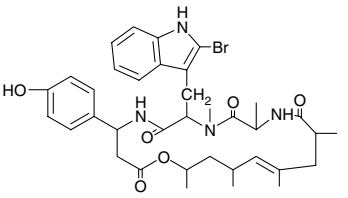
77 Neoamphemidine



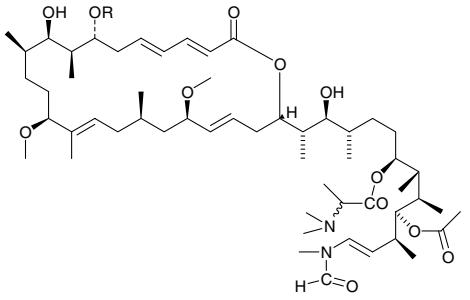
78 Variolin B



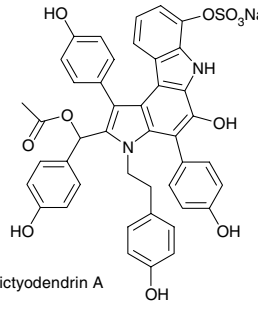
79 Latrunculin



80 Jaspamide



81 Aplyronine (R = 2-$N(CH_3)_2$-3-methoxypropanoyl)



82 Dictyodendrin A

FIGURE 12.7 (*Continued*).

compound was at least two orders of magnitude less cytotoxic. As stated in their discussion, such a molecule may well have utility in other pharmacologic areas and they point to the papers discussing the utility of paclitaxel-like molecules as protective agents against β-amyloid toxicity in primary neurons.[198–200] This work illustrates the considerable value of applying combinatorial and modern

synthetic chemistry techniques to optimization of natural product–led discoveries and interested readers should consult the recent review by Newman and Cragg[201] for further details.

In 2000, West and Northcote[202] reported the isolation of the cytotoxic macrolide peloruside A (**67**) from the New Zealand marine sponge *Mycale hentscheli*. This initial report was then followed two years later by a paper by Hood et al.[203] that demonstrated that this compound was another in the series of marine-derived cytotoxins with a mechanism of action similar to that of Taxol. In a similar fashion to Taxol, peloruside A demonstrated induction of apoptosis following G_2/M arrest and its relatively simple structure may well lend itself to synthetic modifications.[203]

Further biological work related to the potential mechanism has now demonstrated that peloruside A, like the epothilones, discodermolide, and laulimalide, is a weaker substrate for the phosphoglycoprotein efflux pump than Taxol and it also appears to bind at the same or a similar site to that reported for laulimalide, one significantly different from the taxoid binding site.[204] Evidence of another potential mechanism related to the *ras* status of the cell lines/tumors, and hence added evidence for further development, was reported by Miller et al.[205] when it was shown that peloruside A enhanced apoptosis in H-*ras*-transformed cells but appeared not to have any immunosuppressive effect in contrast to the two other unrelated cytotoxins also found in the same sponge, mycalamide A and pateamine.

Peloruside A has been difficult to reisolate in any significant quantity from nature, though there are reports of successful isolation from aquacultured sponge fragments.[204] Consequently, it has been the target of a number of synthetic groups over the last few years. Recently, Jin and Taylor[206] reported the total synthesis of the (+) or natural epimer of the compound. This and other syntheses, coupled to the potential for aquacultural production of the natural metabolite, will significantly aid in the production of enough material to further evaluate the full potential of this compound and analogs.

Two other promising marine-derived discoveries exhibiting tubulin-interactive properties are dictyostatin 1 (**68**) from a deep water lithistid sponge and diazonamide A (**69**) from a *Diazona* species. In both cases, the absolute structures were determined by synthesis and in so doing some very interesting discoveries were made in the case of diazonamide A. The dictyostatin story also had an unusual aspect to it in that the compound had originally been discovered as a cytotoxin from a Maldivian *Spongia* sp. by Pettit et al.,[207] and then rediscovered using a tubulin-interaction assay by Isbrucker et al.[208] roughly ten years later. The absolute structure was proven by syntheses by Paterson et al.[209] and Shin et al.[210] with both synthetic schemes confirming that the ten stereo centers that discodermolide (44) shares with dictyostatin were as postulated from extensive NMR and molecular modeling.[211] These syntheses now open up the possibilities for production of significant quantities of this molecule and closely related ones for further investigations, and the two papers should be consulted for the various proposed structures and for the diagrams that demonstrate the very close similarities between discodermolide and dictyostatin in their conformations when the X-ray structure of the former is compared to the solution conformation of the latter.

Diazonamide A (**69**) was first reported in 1991 from the ascidian *Diazona angulata*[212] and, subsequently, a number of formal syntheses were published. During this time frame, the original structure was questioned by Harran causing a reassessment of the original data, culminating in the publication of syntheses of the original structure (**69**), an oxo analog (**70**), and then their revised structure (**71**).[213] This was followed by a series of syntheses by Nicolaou's group[214–216] culminating with the comment that without the full diazonamide architecture, no compound broke the low micromolar level barrier as a cytotoxin.

Using the molecules provided by the Harran group, Hamel and colleagues[217] investigated the interactions with tubulin and reported that both diazonamide A and the oxygen analog (where the dihydropyrrole ring of the revised structure had been replaced by a dihydrofuran moiety) were potent inhibitors of microtubule assembly, comparable to dolastatin 10 in their responses. It is possible that they have a specific, but as yet unrecognized binding site, or they may bind at the so-called "peptide binding site", but only when this site is at the end of growing molecules.

12.3.2.2 Vacuolar-ATPase Inhibitors

ATPase enzymes occur throughout eukaryotes and their prime function is to pump hydrogen ions from one side of a membrane to the other. These vacuolar (Vo)-ATPases perform this function within vacuoles in the cell and are dependent upon ATP for the necessary energy to perform the function.

Salicylihalimides A (**72**) and B, isolated from the Western Australian marine sponge *Haliclona* sp., exhibited potent activity in the NCI cancer cell line screen and showed patterns of activity similar to the bafilomycin and concanamycin derivatives, compounds known to exhibit Vo-ATPase inhibitory activity.[218] Subsequent work from the same laboratory expanded the range of the structures to include another marine-derived product series, the lobatamides, and both were shown to be specific inhibitors for the higher eukaryotic Vo-ATPases but not the fungal equivalents. Subsequently, similar molecules have been reported from bacteria and fungi.[219,220] Although syntheses have been published,[221–223] *in vivo* studies were delayed due to lack of sufficient material but are now ongoing in collaboration with Australian scientists. This class of compounds is of interest not only as antitumor agents but also in bone resorption and may have utility in osteoporosis.[219,220] In addition to the agents referred to, two other classes with similar activities have recently been described, the macrocyclic chondropsins (**73a**–**c**)[224] and a smaller ring macrolide from Antarctic waters, palmerolide A (**74**).[225]

12.3.2.3 DNA Polymerase α Inhibitors

Thiocoraline (**75**), originally reported from two strains (ACM2-092 and ML1) of a marine actinomycete *Micromonospora* sp. (possibly *marina*) cultured from both a soft coral and a mollusk collected off the Mozambique coast, demonstrated selective activity against breast, colon, renal, and melanoma cell lines in the NCI screen and has been reported to have *in vivo* activity.[226,227] The mechanism of action was shown to be inhibition of DNA polymerase α.[228] In addition to a total synthesis,[229] the complete biosynthetic pathway has been cloned and sequenced.[230]

12.3.2.4 Reductive DNA-Cleaving Agents

Synthetic studies around the ascididemnin (**76**) structure have led to semisynthetic compounds that exhibit submicromolar activity against 12 human tumor cell lines.[231] Interestingly, another member of this structure class, neoamphimedine (**77**), but not its regioisomer, amphimedine, shows *in vitro* and *in vivo* activity comparable to etoposide and, like etoposide, appears to interact with topoisomerase II, but does not stabilize cleavable complexes, unlike all other currently used topoisomerase II inhibitors.[232–234]

12.3.2.5 Potential Cyclin-Dependent Kinase (Cdk) Inhibitors

The variolins, isolated from the Antarctic sponge *Kirkpatrickia variolosa*, are unique chemotypes with potent *in vitro* activity reported against a variety of cancer cell lines.[235,236] The most active, variolin B (**78**), has been synthesized[237] and, together with other, more recent derivatives,[238] appears to exert its activity through inhibition of the kinase activity of at least three different cyclin-dependent kinase/cyclin complexes.[239]

12.3.2.6 Actin-Active Agents

Actin is an essential component of the cell cytoskeleton and its interactive role(s) with tubulin/microtubules in maintaining cell shape and influencing cell division is beginning to emerge.[240] While conducting these studies, latrunculin B (**79**), isolated from the marine sponge *Latruncula magnifica*, was used as a probe. Although prior work had been performed with fungal (cytochalasins) and plant (cucurbitacins) metabolites, the latrunculins were the first actin-interactive agents to be reported from marine sources.[241] Since then a number of very different structural motifs,

almost all from marine sources, have been shown to interact with actin and, in a number of cases, significant chemistry has been performed around the basic structures to determine the structure–activity relationships. Examples include recent syntheses of simplified variations on jaspamide (**80**)[242] and earlier modifications around the basic aplyronine A (**81**) structure.[243,244] Details on the syntheses of swinholide A, scytophycin C, aplyronine A, mycalolide A, and a diastereomer of ulapualide A, all highly cytotoxic compounds and all acting on various forms of actin, have been reviewed.[245] However, despite extensive research on the isolation, identification, synthesis, and pharmacology of such agents, no successful demonstration of realistic *in vivo* activity in animals has yet been reported.

12.3.2.7 Telomerase Inhibitors

Although there had been earlier reports of the *in vitro* and *in vivo* inhibition of telomerases by synthetic compounds[246] and some natural products,[247] the first marine metabolites reported exhibiting this activity were the dictyodendrins A–E (A, **82**), isolated from the Japanese sponge *Dictyodendrilla verongiformis*, which demonstrated 100% inhibition of human telomerase at 50 μg/mL.[248]

12.4 ANTICANCER AGENTS FROM MICROBIAL SOURCES

12.4.1 Microbial-Derived Drugs in Clinical Use

Antitumor antibiotics (Figure 12.8) are among the most important of the cancer chemotherapeutic agents, which include members of the anthracycline, bleomycin, actinomycin, mitomycin, and aureolic acid families. Except for the semisynthetic compounds, all were isolated from various *Streptomyces* species.

The first microbial-derived agent to be clinically used was actinomycin D (**83**) (chemically it is named as D-actinomycin C_1 and generically known as dactinomycin), which was introduced in the early 1960s. Despite extensive research into the preparation of analogs, no derivatives have been approved for clinical use outside of trial protocols.[249] In recent years however, there have been a series of reports that actinomycin D has activities related to perturbation of the signal transduction cascade(s) distinct from its well-known intercalating ability, and it will be interesting to see if these activities rejuvenate interest in this class of molecules.[250]

One of the most important classes of microbial-derived agents is the anthracyclines, with daunorubicin (**84**) and its derivative doxorubicin (adriamycin) (**85**) being the best known of these agents currently in clinical use, and it is still a major component of the treatment regimen for breast cancer.[251] The mechanism of action of these molecules, aside from their formal identification as intercalators into the DNA helix, is now known to be inhibition of topoisomerase II, one of the important enzymes in the replication pathway of DNA during cell cycling. Derivatives of doxorubicin, such as epirubicin, idarubicin, pirirubicin, and valrubicin, have also been approved for clinical use and the expansion of the efficacy of doxorubicin is being explored through use-targeted delivery techniques (Section 12.5).

Another important class is the family of glycopeptolide antibiotics known as bleomycins (e.g., bleomycin A_2 [**86**], Blenoxane®) and initially, the closely related structural class, the phleomycins.[252] Bleomycin was originally thought to act through DNA cleavage, but recent studies suggest that the major mechanism of action may be inhibition of t-RNA.

The mitomycins (mitosanes) were discovered in the late 1950s and mitomycin C (**87**) was approved for clinical use in Japan in the 1960s and in the United States in 1974. Its serious bone marrow toxicity has led to extensive synthetic studies aimed at developing a less toxic analog, but, despite this, it remains the only clinically used member of this class.[253]

Other microbial products still in use include mithramycin and streptozotocin.[254] Calicheamicin (**88**), an extremely potent antitumor member of the enediyne structural class, is the

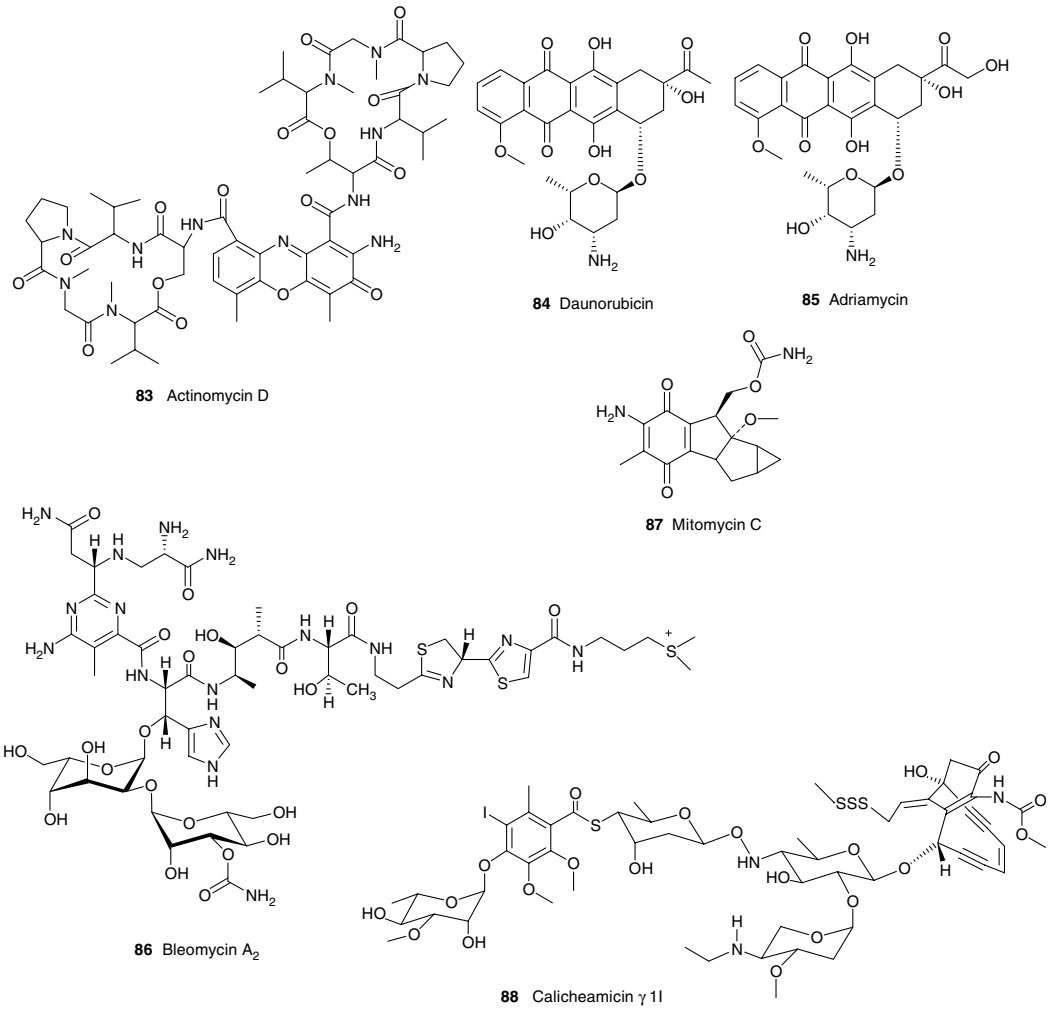


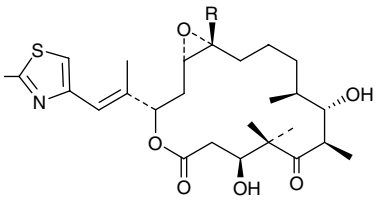
FIGURE 12.8 Microbial-derived drugs in clinical use.

most important recently approved anticancer drug and is used in the form of a targeted therapy, Mylotarg® (Section 12.5), for the treatment of acute myelogenous leukemia.[255]

12.4.2 Microbial-Derived Agents in Clinical Development

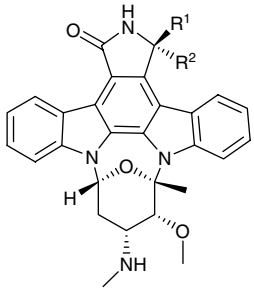
In addition to the agents listed above, there are some extremely interesting structural classes of agents isolated from microbes, or modified from such agents, in various phases of clinical trials (Figure 12.9). There are perhaps two major structural types that evince significant interest on the part of both chemists and cell biologists. These are the structures based on the epothilones (**89a** and **89b**) from the extremely prolific Myxomycetales,[256] and the indolocarboxazoles such as staurosporine (**90**) from the Actinomycetales.

The epothilones are of great interest as potential antitumor agents due to their mechanism of action being the same as that of paclitaxel (*vide infra*) though having at first glimpse, quite a different topology. Molecular modeling, however, has shown that there are significant common structural features in the two basic molecules.[257–259] Originally, the epothilones were difficult to obtain in any



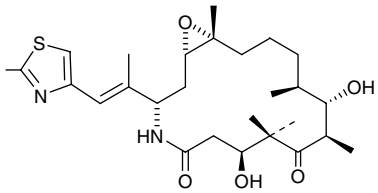
89a Epothilone A (R = H)

89b Epothilone B (R = CH_3)

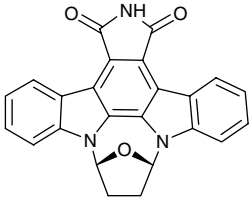


90 Staurosporine ($R^1 = R^2 = H$)

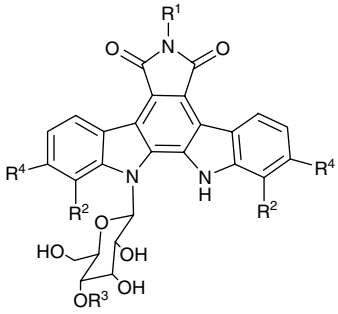
92 UCN 01 ($R^1 = H$; $R^2 = OH$)



91 16-Azaepothilone B (Ixabepilone)



93 SB 218078

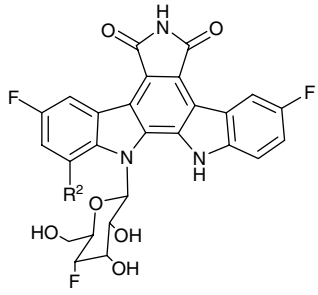


94 Rebeccamycin ($R^1 = H$; $R^2 = Cl$; $R^3 = CH_3$; $R^4 = H$)

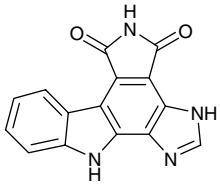
95 NB 506 ($R^1 = NHCHO$; $R^2 = OH$; $R^3 = H$; $R^4 = H$)

96 Edotecarin ($R^1 = NHCH(CH_2OH)_2$; $R^2 = H$; $R^3 = H$; $R^4 = OH$)

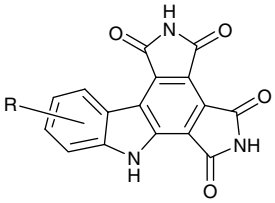
97 BMY 27557 ($R^1 = CH_2CH_2N(CH_2CH_3)_2$; $R_2 = Cl$; $R_3 = CH_3$; $R^4 = H$)



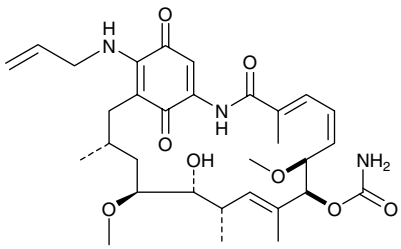
98 BMS-250749



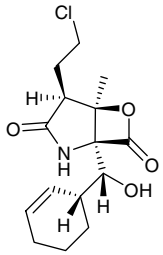
99 Granulatimide



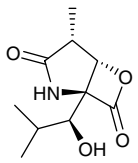
100 Base structure



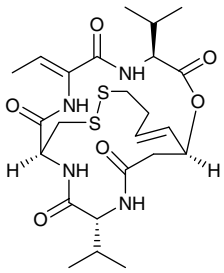
101 17-AAG



102 Salinosporamide A



103 *clasto*-Lactacystin-β-lactone (Omuralide)



104 Depsipeptide FK-228

FIGURE 12.9 Microbial-derived agents in clinical development.

quantity and a significant amount of time and effort was spent by both academics and industry in efforts to synthesize both epothilones A and B and their precursors, C and D. During this time period, Frykman et al.[260] cloned and expressed the polyketide gene cluster that produced epothilones A and B. Removal of the terminal gene for the P_{450} enzyme, which epoxidized the macrolidic double bond in the precursor epothilones C and D, and transfer to a different host produced crystalline epothilone D from a 100 L fermentation.[260]

The major impetus behind all of these studies was the realization that the epothilones were active against paclitaxel-resistant cell lines.[261] The aza-analog of epothilone B (**89b**) (ixabepilone [**91**] synthesized by Bristol-Myers), hepothilone B (patupilone), a water-soluble analog of epothilone B, epothilone D (KOS-862), and ZKEPO are in phase I or phase II clinical trials, though the recent comments by de Jonge and Verweiji[262] on the epothilones as a class should be borne in mind when assessing their future potential. However, in spite of the varied clinical results there have been significant recent efforts expended (early 2006) on the total synthesis of this class of compounds both by chemical means involving modifications around the basic epothilone skeleton,[263–266] and by use of biosynthetic modifications using the synthetic gene cluster.[267,268] The overarching aim of this research is to overcome some of the pharmacological and toxicological problems reported during clinical trials.

The indolocarboxazoles first came into prominence with the identification of staurosporine (**90**) and its simple derivative UCN-01 (**92**) as potential inhibitors of components of the eukaryotic cell cycle and of phosphokinase C.[269] The differences in the potential mechanisms of action of staurosporine and simple derivatives versus synthetic analogs were shown by a comparison with the synthetic compound SB-218078 (**93**). Although not a natural product, this compound is definitely derived from the indolocarbazole base structure; a reduced furan ring replaces the substituted pyranose sugar found in staurosporine, and the maleimide ring of the didemnimides replaces the pyrrolidone ring of staurosporine.[270] This compound demonstrated at least a 15-fold selectivity for checkpoint kinase 1 (Chk1) inhibition versus cyclin-dependent kinase (Cdk1, also known as cdc2) and a 65-fold selectivity over PKC (IC_{50} values of 15, 250, and 1000 nM, respectively). In contrast, staurosporine is active against all three kinases at the 5–8 nM level and UCN-01 (**92**) has a 15-fold selectivity for Chk1 versus Cdk1, but has comparable activity against PKC (IC_{50} values of 7, 100, and 4 nM, respectively). These workers also confirmed the findings of Shao et al.[271] about the synergistic effect of UCN-01 (**92**) on camptothecin (**14**) cytotoxicity and demonstrated that SB-218078 (**93**) had comparable activity, implying that the mechanism of this abrogation with UCN-01 is due to Chk1 inhibition. Concomitantly with this work, Graves et al.[272] reported that UCN-01 inhibits Chk1, thus causing the phosphatase Cdc25c to be dephosphorylated and hence allowing activation of Cdk1 (Cdc2) and abrogating the G2 checkpoint.

The compounds related to the cytotoxin rebeccamycin (**94**) are extremely interesting from a mechanistic aspect since relatively simple modifications of the base indolo-carbazole generate molecules that have both topoisomerase I- and topoisomerase II-inhibitory activities. The findings have led to the synthesis of water-soluble analogs, such as NB-506 (**95**), and second- and third-generation products, all of which show excellent preclinical *in vivo* activity. The topoisomerase I activity of this class of compounds has been extensively studied[273] and one of the most interesting discoveries about this structural class has been the derivative whose generic name is edotecarin (J-107088, **96**), which is reportedly in phase III trials for therapy of brain carcinomas,[274] although currently (April 2006) it is still listed as being in phase II in the Prous Integrity® database. If it is ultimately commercialized, it may be the first noncamptothecin-based topoisomerase I inhibitor. Alteration of the substitution pattern around the indolocarbazole moiety of rebeccamycin (**94**) gives a molecule known as BMY-27557 (BMS-181176, NSC 655649, or becatecarin, **97**) that now exhibits selectivity for topoisomerase II rather than topoisomerase I. Currently, this compound is in phase II/III trials for a variety of carcinomas, with a number of reports about clinical findings now being published.[275–277]

The continuing potential for development of this base structure is shown by some recent papers. First, active agents can still be made by modification of the base rebeccamycin structure using fluorine substitution, giving rise to BMS-250749 (**98**) that is headed for phase I trials as a topoisomerase I inhibitor.[278] Second, modification of the base skeleton to include other heterocyclic and carbocyclic rings extends the fermentation products into previously unexplored chemical space, as demonstrated by asymmetric phenyl substitution producing compounds with significant cytotoxic activity in cell lines, blocking at G_2/M or S phase in the cell cycle.[279]

Although not yet reported from a microbe, the marine compounds known as the granulatimides (**99**), originally reported in 1998 from the ascidian *Didemnum granulatum*, are most probably of microbial origin and much improved four-step synthetic methods for the production of molecules of the related general structure class (**100**), using both parallel syntheses and microwave techniques, have been reported.[280] Although biological activities have not yet been reported, by analogy with the indolocarbazole equivalents, they will probably demonstrate some interesting biological properties.

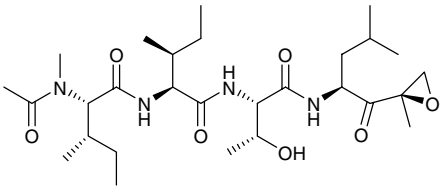
It is noteworthy that the first signal transduction modulator, other than a formal cyclin-dependent kinase or PKC inhibitor, to enter clinical trials was a microbial product. Thus, 17-allylamino-geldanamycin (17-AAG, **101**) entered phase I trials in 2001 and is currently in phase I/II trials using a variety of treatment modalities. This compound and other geldanamycin derivatives bind at the major ATP-binding site of the protein chaperone HSP-90. Although now known not to be suitable for use as a single agent in humans due to its predominately cytostatic response,[281] it appears to be effective when used in combination with cytotoxins such as paclitaxel. In late 2003,[282] it was shown that the HSP-90 in BT474 breast tumor cells binds 17-AAG (**101**) much more strongly (approximately 100-fold higher affinity) than that in "normal" cells (human renal epithelial cells and dermal fibroblasts), thus giving a partial answer to the question, why should HSP-90 inhibitors not significantly affect normal cells as well? Similarly, a recent report demonstrates that activated mutant forms of the protein kinase B-RAF are more sensitive than wild type to 17-AAG, thus demonstrating further that subtle changes in mutated proteins found in cancer cells can help the targeting of this agent.[283] There are many further avenues to explore in the inhibition of the protein chaperones that can be seen from the recent publications of a variety of investigators on the potential for HSP90 inhibitors.[284–286] Thus, the initial work with geldanamycin derivatives has led to simpler synthetic molecules with similar actions, including relatively simple substituted purines that mimic ATP (as do the geldanamycins) but that were derived without the use of computer modeling. Water-soluble derivatives based on this skeleton have recently been reported.[287,288] One or more of these compounds are now being directed toward early clinical trials.

The marine bacterial metabolite salinosporamide A (**102**) was isolated from a totally new genus, *Salinispora* that mapped to the Micromonosporaceae, found in marine sediments across the tropics. It demonstrated activity as a cytotoxic proteasome inhibitor[289] similar to that observed for a structurally related compound, omuralide (**103**) (which was synthesized by Corey and Li[290,291]), which resulted from a spontaneous rearrangement of the microbial metabolite lactacystin in neutral aqueous media.[292,293] Salinosporamide has been synthesized,[294] and also prepared by large-scale (1000 L), saline fermentation of *Salinospora* sp. under cGMP conditions, and entered phase I clinical trials in May 2006.

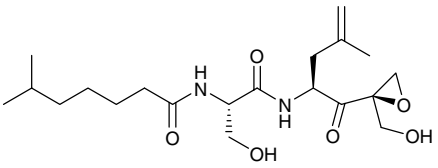
Depsipeptide (FR-901228, FK-228, **104**), originally isolated from *Chromobacterium violaceum* strain 968 on the basis of its antitumor activity and currently in phase II and phase III clinical trials,[295] is a four-amino acid-containing cyclic macrolide with a second ring system containing a dithio linkage. It was originally identified as a result of its potent antitumor activity and is now known to be active in signal transduction as a result of inhibition of histone deacetylases.[296]

12.4.3 Microbial-Derived Agents in Preclinical Development

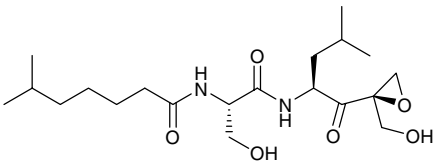
In 1999, Crews' group at Yale reported that the epoxyketone microbial metabolites epoxomicin (**105**, Figure 12.10)[297] and eponemycin (**106**)[298] exhibited their cytotoxic activities as a result of proteasome inhibition. Similar to the omuralide (**103**)-based inhibitors, epoxomicin reacted predominately with



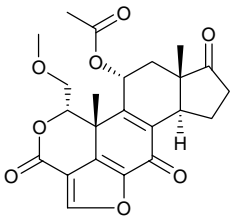
105 Epoxomicin



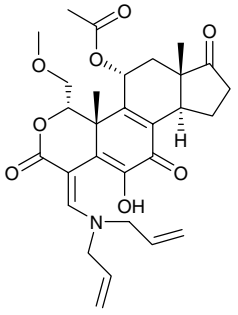
106 Eponemycin



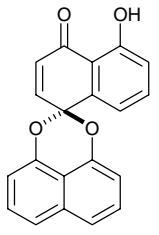
107 Dihydroeponemycin



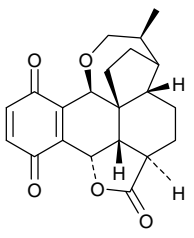
108 Wortmannin



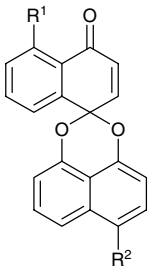
109 PX-866



110 Palmarumycin CP_1



111 Pleurotin



112 S11 (R^1 = H; R^2 = OH)

113 S12 (R^1 = OH; R^2 = OH)

114 C12 (R^1 = OH; R^2 = $OCOCH_2NH_2$)

FIGURE 12.10 Microbial-derived agents in preclinical development.

the chymotrypsin-like site, whereas the less potent eponomycin and its synthetic analog, dihydroeponemycin (**107**), had roughly equal activity against both the chymotrypsin-like and the caspase-like sites. In contrast to the "regular" peptidic inhibitors such as the aldehydes, boronates, or vinyl sulfones, these peptidic epoxyketones reacted with both the hydroxyl and amino groups of the N-terminal threonine, forming a morpholino ring system as a result. These expoxyketones are the most selective proteasome inhibitors so far reported. The synthesis of these compounds and derivatives as a means to develop proteasome inhibitors with novel activities has been reviewed.[299]

There are other microbial products that are acting as scaffolds around which to synthesize novel agents that are designed to overcome some of the problems inherent in the original natural product. In some cases, this is simply straightforward chemistry, but in other cases it uses combinatorial processes to optimize the lead structure. Wortmannin (**108**), an antifungal product originally isolated from *Penicillium wortmanni* Klocker in 1957 was found, many years later, to be an excellent, but unstable, nanomolar inhibitor of phosphoinositol-3-kinase and, very recently, to also inhibit mammalian polo-like kinase, again with nanomolar potency.[300] Owing to the instability of the parent compound (lactone ring opening), a significant amount of basic chemistry has been performed[301]

leading to the identification of C_{20} derivatives such as PX-866 (**109**) that has demonstrated *in vivo* activity in human xenografts.[302] Also very interesting from a chemical perspective is the report by Yuan et al.[303] that wortmannin is quite stable, with a half-life of almost 58 h in phosphate buffered saline. However, addition of simple amino acids or albumin to the phosphate buffered saline reduced the half-life to between 8 min and almost 4 h depending upon the mixture. Using this data, Yuan et al. synthesized C_{20}-modified wortmannin derivatives, some of which had the capability to undergo intramolecular attack to generate wortmannin *in situ*, whereas others would bind at the kinase site and then be activated by peptidic amino groups. If confirmed, then compounds such as this may well be a method of delivery of wortmannin that may overcome the chemical stability issues and perhaps even some of the toxicity issues. In any case, these results imply that a significant amount of earlier literature data might well be worth reinvestigating.

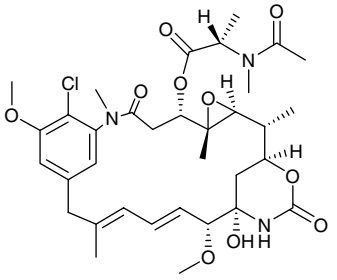
An example of how a base structure from microbes may be modified systematically via combinatorial techniques is shown in a series of papers covering the evolution of the use of the naphthoquinone spiroketal moiety of the palmarumycins.[304,305] The palmarumycins and derivatives initially reported by Lazo et al.[304] were exposed to many pharmacological screens but, although they showed potent cytotoxicity, their potential targets were unidentified at that time. Approximately 1 year after the submission of that paper, Wipf et al.[305] reported that palmarumycin CP_1 (**110**) had inhibitory activity comparable to that of pleurotin (**111**), with IC_{50} values in the 170–350 nM range when assayed against thioredoxin–thioredoxin reductase (Trx-1-TrxR), and demonstrated that certain aspects of the base structure, in particular the enone system, were required for activity in this assay.

The link between inhibition of the Trx-1-TrxR system by the fungal products pleurotin and the palmarumycins and, by inference, effects of these upon hypoxia induction factor 1α was made in 2003 when Welsh et al.[306] reported evidence for direct inhibition of hypoxia induction factor 1α by pleurotin (shown to be a Trx-1-TrxR inhibitor by use of the NCI's COMPARE algorithm in 1997 by Kunkel et al.[307]). Work by Wipf's group,[308] using data derived from studies with other palmarumycins isolated from a Costa Rican-sourced ascomycete, led to further modifications of the base structure yielding the simple analogs S-11 (**112**) and S-12 (**113**) that exhibited biological activities comparable to pleurotin in both the thioredoxin enzyme system and (most importantly) *in vitro* cytotoxicity assays. Extension of the structures by formation of prodrugs led to the identification of compound C-12 (**114**) as a lead structure to follow for subsequent *in vivo* studies due to its solubility and easy release of the active material.[309]

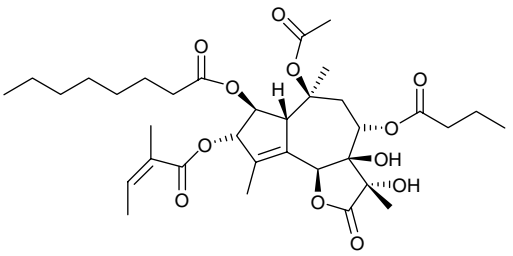
12.5 TARGETED DELIVERY OF NATURAL PRODUCTS

A recurring liability of natural products, at least in the area of cancer chemotherapy, is that, though often very potent, they have limited solubility in aqueous solvents and exhibit narrow therapeutic indices. These factors have resulted in the initial demise of a number of pure natural products, such as the plant-derived agents bruceantin (**25**) and maytansine (**115**) (Figure 12.11), but an alternative approach to utilizing such agents is to investigate their potential as "warheads" attached to monoclonal antibodies or polymeric carriers specifically targeted to epitopes on tumors of interest.[310,311]

A promising case is that of maytansine (**115**) that was isolated in the early 1970s from the Ethiopian plant, *Maytenus serrata* (Hochst. Ex A. Rich.) Wilczek.[312] Despite very low yields (2×10^{-5}% on the basis of plant dry weight), its extreme potency in testing against cancer cell lines permitted the production of sufficient quantities to pursue preclinical and clinical development. Unfortunately, very promising activity in preclinical animal testing did not translate into significant efficacy in clinical trials and it was dropped from further study in the early 1980s. Related compounds, the ansamitocins, were subsequently isolated from a microbial source *Actinosynnema pretiosum* posing the question whether the maytansines are actually plant products or are produced through an association between a microbial symbiont and the plant. This latter question is a topic of continuing study.[313] The microbial source of closely related compounds has permitted the production of larger quantities of this class of compounds and this factor, together with their



115 Maytansine



116 Thapsigargin

FIGURE 12.11 Targeted drugs.

extreme potency, has stimulated continued interest in pursuing their development. A derivative of maytansine, DM1, conjugated with a monoclonal antibody (Mab) targeting small cell lung cancer cells, is being developed as huN901-DM1 for the treatment of small cell lung cancer. Another conjugate of DM1 to J591, a Mab targeting the prostate-specific membrane antigen, is in clinical trials against prostate cancer. A conjugate known as SB408075 or huC242-DM1 (also known as Cantuzumab Mertansine), produced by the coupling of DM1 to huC242, a Mab directed against the *muc*1 epitope expressed in a range of cancers, including pancreatic, biliary, colorectal, and gastric cancers, is currently in phase I clinical trials in the United States.[313]

Another case of considerable interest is that of thapsigargin (**116**) isolated from the umbelliferous plant *Thapsia garganica* L., collected on the Mediterranean island of Ibiza.[314] Thapsigargin induces apoptosis in quiescent and proliferating prostate cancer cells and, though it does not show selectivity for prostate cancer cells, it has been conjugated to a small peptide carrier to produce a water-soluble prodrug that is specifically activated by prostate-specific antigen protease at metastatic prostate cancer sites. Treatment of animals bearing prostate cancer xenograft tumors demonstrated complete tumor growth inhibition without significant toxicity. Given that the prodrug is stable in human plasma, it holds promise as a treatment for human prostate cancer.

The potential for a directed delivery of the dolastatin derivative TZT-1027 (**47**) to prostate cancer cells, by using the upregulation of the adhesion molecule, E-selectin, found in the epithelium of prostate carcinomas, has been demonstrated. A Mab directed to E-selectin, with auristatin linked via a cathepsin B-labile linker gave more than 85% inhibition of growth of prostate carcinoma cell lines in mouse models.[315,316]

The targeting of daunorubicin (**84**) and doxorubicin (**85**) through liposomal encapsulation as a means of ameliorating cardiotoxicity and improving efficacy has been investigated. Encouraging results have been observed in the treatment of a number of cancers, including metastatic breast cancer.[251,317] Clinical trials have also been reported using polymeric delivery systems.[310,318,319]

The first antigen-targeted chemotherapeutic agent approved by the U.S. Food and Drug Administration for human use is Mylotarg (gemtuzumab ozogamicin), in which calicheamicin (**88**) is conjugated to an antibody targeting the CD33 antigen expressed on differentiated myeloid cells. It is used in the treatment of acute myelogenous leukemia.[255]

12.6 CONCLUSIONS

Nature continues to be the most prolific source of biologically active and diverse chemotypes. Although relatively few of the actual isolated compounds advance to become clinically effective drugs in their own right, these unique molecules often serve as models for the preparation of analogs using chemical methodology such as total or combinatorial synthesis, or manipulation of biosynthetic pathways. Such concepts are becoming part of the "mainstream thoughts of medicinal

chemists" is shown by the recent review by Costantino and Barlocco,[320] which demonstrates these principles.

Many of these analogs have superior drug-like properties in terms of better pharmacological properties (more potent activity, reduced toxicity, or both) or pharmaceutic properties, such as improved ease of formulation or enhanced bioavailability, and may then advance into clinical trials. In addition, conjugation of toxic natural molecules to monoclonal antibodies or polymeric carriers specifically targeted to epitopes on tumors of interest can lead to the development of efficacious targeted therapies. The tremendous value of natural products as mechanistic probes is also abundantly illustrated.

12.7 PERSONAL INSIGHT AND FUTURE DIRECTIONS

It can be seen from the discussions above that natural products from all sources still have the potential to lead chemists of all types into areas of drug discovery and development that would never have been considered if the "privileged structures from nature" had not been isolated, purified, and used as probes of cellular and molecular mechanisms. In spite of the discussions in the late 1980s to late 1990s about the vast potential of combinatorial chemistry as a discovery tool, it is now quite evident that this technique, except in the very special cases of peptides and nucleosides (which are actually "privileged structures" in their own right), is not the panacea that it was thought to be. To us, a multidisciplinary approach to drug discovery, involving the generation of truly novel molecular diversity from natural product sources, combined with total and combinatorial synthetic methodologies, and including the manipulation of biosynthetic pathways (so-called combinatorial biosynthesis), provides the best solution to the current productivity crisis facing the scientific community engaged in drug discovery and development.

Once more, we strongly advocate *expanding*, not decreasing, the exploration of nature as a source of novel active agents that may serve as the leads and scaffolds for elaboration into desperately needed efficacious drugs for a multitude of disease indications.

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13 Sourcing Natural Products from Endophytic Microbes

Gary Strobel and Uvidelio Castillo

CONTENTS

13.1 INTRODUCTION

The need for new and useful compounds to provide assistance and relief in all aspects of the human condition is ever growing. Drug resistance in bacteria, the appearance of new, life-threatening viruses, the recurrent problems of diseases in persons with organ transplants, and the tremendous increase in the incidence of fungal infections in the world's population all underscore our inadequacy to cope with these medical problems. Environmental degradation, loss of biodiversity, and spoilage of land and water also add to problems facing mankind, and each of these in turn can have health-related consequences.

Endophytes, microorganisms that reside in the tissues of living plants, are relatively unstudied as potential sources of novel natural products for use in many aspects of human society. However, some of the most extensive and comprehensive work on natural products produced by endophytes has been done on the *Neotyphodium* sp. found on grasses.[1] Alkaloids synthesized by this fungus in its grass hosts have been implicated in fescue toxicosis in rangeland animals.[1] The chemistry and

biology of this and other grass endophytes are reviewed elsewhere.[2] Unfortunately, because this work is so comprehensive, one may be led to the conclusion that endophytes only produce toxic compounds in their respective hosts and that they hold no promise for any medicinal applications. It turns out that this is simply not the case. Endophytes examined from a plethora of sources show that an overwhelming number of them produce natural products with promising potential for many applications. This is especially true when sourcing endophytes from higher plants existing in the world's rainforests since so much microbial diversity exists in these regions.

Within the approximately 300,000 higher plant species that exist on the earth, each individual plant, of the billions that exist, is most likely a host to one or more endophytes. Only a handful of these plants, primarily grass species, have ever been completely studied relative to their endophytic biology.[2] Consequently, the opportunity to find new and interesting endophytic microorganisms among the myriad of plants in different settings and ecosystems is very great. The intent of this chapter is to provide insights into the occurrence of endophytes in nature, the products that they make, and how some of these organisms show some potential for human use. The majority of this chapter discusses rationale for study, methods used, and examples of a number of endophytes isolated and studied in the author's (GS) laboratory over the course of many years. This review, however, also includes some specific examples that illustrate the work of others in this emerging field of bioprospecting and sourcing the microbes of the world's rainforests.

13.2 THE NEED FOR AND PURSUIT OF MICROBIAL PRODUCTS

13.2.1 New Medicines

There is a general call for new antibiotics and chemotherapeutic agents that are highly effective and possess low toxicity. This search is driven by the development of resistance in infectious microorganisms (e.g., *Staphylococcus, Mycobacterium, Streptococcus*) to existing drugs and by the menacing presence of naturally resistant organisms. The ingress to the human population of disease-causing agents such as AIDS, EBOLA, and SARS requires the discovery and development of new drugs to combat them. Not only do diseases such as AIDS require drugs that target them specifically, but also new therapies are needed for treating ancillary infections that are a consequence of a weakened immune system. Furthermore, others who are immunocompromised (e.g., cancer and organ transplant patients) are at risk of infection by opportunistic pathogens, such as *Aspergillus, Cryptococcus*, and *Candida*, that normally are not major problems in the human population. In addition, more drugs are needed to efficiently treat parasitic protozoan and nematodal infections such as malaria, leishmaniasis, trypanomiasis, and filariasis. Malaria, by itself, is more effective in claiming lives each year than any other single infectious agent with the exception of AIDS and TB.[3] However, the enteric diseases, as a group, claim the most lives each year than any other diseases and, unfortunately, the victims are mostly children.[3]

Novel natural products, and the organisms that make them, offer opportunities for innovation in drug discovery. Exciting possibilities exist for those who are willing to venture into the wild and unexplored territories of the world to experience the excitement and thrill of engaging in the discovery of endophytes, their biology, and potential usefulness.

It is evident that natural product-based compounds have an immense impact on modern medicine. For instance, about 40% of prescription drugs are based on them, and well over 50% of the new chemical products registered by the U.S. Food and Drug Administration (FDA) as anticancer agents, antimigraine agents, and antihypertensive agents are natural products or derivatives thereof in the time frame of 1981–2002.[4] Excluding biologics, between 1989 and 1995, 60% of approved drugs and pre–new drug application (NDA) candidates were of natural origin.[5] From 1983 to 1994, over 60% of all approved and pre-NDA stage cancer drugs were of natural origin as were 78% of all newly approved antibacterial agents.[6] Many examples abound that illustrate the value and importance of natural products from plants and microorganisms to modern civilizations. The discovery

and development of taxol is a modern example of a natural product that has made an enormous impact on medicine.[7–9]

Recently, however, natural product research efforts have lost popularity in many major drug companies and, in some cases, have been replaced entirely by combinatorial chemistry, which is the automated synthesis of structurally related small molecules. In addition, many drug companies have developed interests in making products that have a larger potential profit base than anti-infectious drugs. These synthetic compounds are ones that may provide social benefits, relieve allergenic responses, reduce the pain of arthritis, or ones that can sooth the stomach. It appears that this loss of interest in natural products is not only an economically driven decision but can also be attributed to the enormous effort that is required to rationally select a biological source, isolate active natural products, decipher their structures, and begin the long road to product development. It is also apparent that combinatorial chemistry and other synthetic chemistry methodologies revolving around certain basic chemical structures are now serving as a never-ending source of products to feed the screening robots of the drug industry. Within many large pharmaceutical companies, progress of professionals is based primarily on the number of compounds that can be produced and sent to the screening machines. This tends to work against the numerous steps needed even to find one compound in natural product discovery. It seems important to emphasize that the primary purpose of combinatorial chemistry should be to complement and assist the efforts of natural product drug discovery and development, not to supersede it.[5] Consequently, only a few larger companies still retain an interest in natural products chemistry.

Natural products often provide lead molecules or platform structures whose activity can be enhanced by manipulation through combinatorial and synthetic chemistry. Natural products have been the traditional pathfinder compounds with an untold diversity of chemical structures unparalleled by even the largest combinatorial libraries.

13.2.2 Potential Agricultural and Industrial Needs

Although the various needs for new medicines seem apparent, it is less obvious that there are also needs for new microbial products and processes in agriculture and industry. As an example, one of the pressing needs in agriculture is a suitable replacement for methyl bromide as a soil sterilant. It not only is harmful to the farm operators but also has been implicated in the degradation of the ozone layer that protects the earth from harmful UV radiation. Can an endophyte or the bioactive natural products they produce possibly be used to replace methyl bromide? Also, can endophytes be found that produce active products that can be used to control plant and animal diseases?

A major industrial problem is the biofouling of structural surfaces once they become wet. A number of fungal species readily grow on structural surfaces especially gypsum-based dry wall once they become wet. In fact, a major problem in New Orleans after hurricane Katrina is the development of moldy structures that are still sound but rendered useless due to the growth of toxic fungi that have taken over structural surfaces. Is it possible that one or more endophytes may be found to assist in the amelioration of these problems?

13.3 ENDOPHYTIC MICROBES

It may also be true that a reduction in interest in natural products for use in drug development has happened as a result of people growing weary of dealing with the traditional sources of bioactive compounds, including plants of the temperate zones and microbes from a plethora of soil samples gathered in different parts of the world by armies of collectors. In other words, why continue to do the same thing when robots, combinatorial chemistry, and molecular biology have arrived on the scene? Furthermore, the logic and rationale for time and effort spent on drug discovery using a target-site-directed approach has been overwhelming.

Although combinatorial synthesis produces compounds at random, secondary metabolites, defined as low molecular weight compounds not required for growth in pure culture, are produced as an adaptation for specific functions in nature.[10] Shutz points out that certain microbial metabolites seem to be characteristic of certain biotopes, both at an environmental as well as an organismal level.[11] Accordingly, it appears that the search for novel secondary metabolites should center on organisms that inhabit unique biotopes. Thus, it behooves the investigator to carefully study and select the biological source before proceeding, rather than to take a totally random approach in selecting the source material. Careful study also indicates that organisms and their biotopes that are subjected to constant metabolic and environmental interactions should produce even more secondary metabolites.[11] Endophytes are microbes that inhabit such biotopes, namely higher plants, which is why they are currently considered as a wellspring of novel secondary metabolites offering the potential for exploitation of their medical benefits.[12]

The investigator interested in exploiting endophytes should have access to, or have some expertise in, microbial taxonomy, that includes modern molecular techniques involving sequence analyses of 16S and 18S rDNA. Currently, endophytes are viewed as an outstanding source of bioactive natural products because there are so many of them occupying literally millions of unique biological niches (higher plants) growing in so many unusual environments. Thus, it would appear that a myriad of biotypical factors associated with plants can be important in the selection of a plant for study. It may be the case that these factors govern which microbes are present in the plant as well as the biological activity of the products associated with these organisms.

What are endophytes? Bacon and White[2] give an inclusive and widely accepted definition of endophytes: "Microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects". Although the usual lack of adverse effect nature of endophyte occupation in plant tissue has prompted focus on symbiotic or mutualistic relationships between endophytes and their hosts, the observed biodiversity of endophytes suggests they can also be aggressive saprophytes or opportunistic pathogens.

Both fungi and bacteria are the most common microbes existing as endophytes. It would seem that other microbial forms most certainly exist in plants as endophytes such as mycoplasmas, rickettsia, and archebacteria; however, no evidence for them has yet been presented. In fact, it may be the case that the majority of microbes existing in plants are not culturable with common laboratory techniques, making their presence and role in plants even more intriguing.

The most frequently isolated endophytes are the fungi.[13] However, at the outset, it is important to note that the vast majority of plants have not been studied for any endophytic association. Thus, enormous opportunities exist for the recovery of novel fungal forms, including genera, biotypes, as well as species in the myriad of plants yet to be studied. Hawksworth and Rossman[14] estimated there may be as many as 1 million different fungal species, yet only about 100,000 have been described. As more evidence accumulates, estimates keep rising as to the actual number of fungal species. For instance, Dreyfuss and Chapela[15] estimated there may be at least 1 million species of endophytic fungi alone. It seems obvious that endophytes are a rich and reliable source of genetic diversity and may represent many previously undescribed species.

Finally, in our experience, novel microbes (as defined at the morphological and molecular levels) often have associated with novel natural products. This fact alone helps eliminate the problems of dereplication in compound discovery.

13.4 RATIONALE FOR PLANT SELECTION

It is important to understand that the methods and rationale used to guide plant selection seem to provide the best opportunities to isolate novel endophytic microorganisms at the genus, species, or biotype level. Since the number of plant species in the world is so great, creative and imaginative strategies must be used to quickly narrow the search for endophytes displaying bioactivity.[16]

A specific rationale is used for the collection of each plant for endophyte isolation and natural product discovery. Several hypotheses govern this plant selection strategy:

1. Plants from unique environmental settings, especially those with an unusual biology, and possessing novel strategies for survival are seriously considered for study.
2. Plants that have an ethnobotanical history (used by indigenous peoples) and that are related to the specific uses or applications of interest are selected for study. These plants are chosen either by direct contact with local peoples or via local literature. Ultimately, it may be learned that the healing powers of the botanical source, in fact, may have nothing to do with the natural products of the plant, but of an endophyte inhabiting the plant.
3. Plants that are endemic, having an unusual longevity, or that have occupied a certain ancient landmass, such as Gondwanaland, are also more likely to lodge endophytes with active natural products than other plants.
4. Plants growing in areas of great biodiversity also have the prospect of housing endophytes with great biodiversity.

Just as plants from a distinct environmental setting are considered to be a promising source of novel endophytes and their compounds, so too are plants with an unconventional biology. For example, an aquatic plant, *Rhyncholacis penicillata*, was collected from a river system in southwest Venezuela where the harsh aquatic environment subjected the plant to constant beating by virtue of rushing waters, debris, and tumbling rocks and pebbles.[17] These environmental insults created many portals through which common phytopathogenic oomycetes could enter the plant. Still, the plant population appeared to be healthy, possibly due to protection provided by an endophytic product. This was the environmental biological clue used to pick this plant for a comprehensive study of its endophytes. Eventually, an unusual and potent antifungal strain of *Serratia marcescens*, living both as an epiphyte and an endophyte, was recovered from *R. penicillata*. This bacterium produces oocydin A, a novel antioomycetous chlorinated macrocyclic lactone (Figure 13.1).[17] It is conceivable that the production of oocydin A by *S. marcescens* is directly related to the endophyte's relationship with its higher plant host. Currently, oocydin A is being considered for agricultural use to control the ever-threatening presence of oomycetous fungi such as *Pythium* spp. and *Phytophthora* spp. Oocydin A also has activity against a number of rapidly dividing cancer cell lines.[17]

Plants with an ethnobotanical history also are likely candidates for study since the medical uses for which the plant was selected may relate more to its population of endophytes than to the plant biochemistry itself. For example, a sample of the snake vine, *Kennedia nigriscans*, from the Northern Territory of Australia was selected for study because its sap has traditionally been used as a bush medicine. In fact, this area was selected for plant sampling since it has been home to the world's longest standing human civilization, the Australian Aborigines. The snake vine is harvested, crushed, and heated in an aqueous brew by local Aborigines in southwest Arnhemland to treat cuts, wounds, and infections. As it turned out, the plant contains an entire suite of streptomycetes.[18]

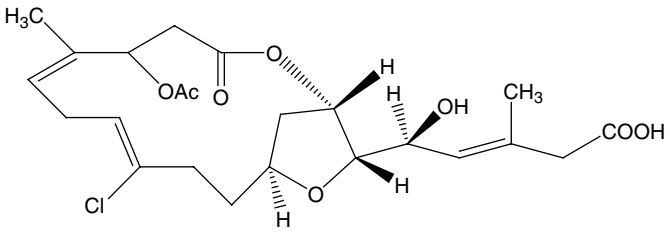

FIGURE 13.1 Oocydin A, a chlorinated macrocyclic lactone isolated and characterized from a strain of *Serratia marcescens*, obtained from *Rhyncholacis penicillata* (absolute stereochemistry unknown).

One in particular, designated *Streptomyces* NRRL 30562, has unique partial 16S rDNA sequences when compared to those in GenBank.[19] It produces a series of actinomycins including actinomycins D, Xo_{β}, and X_2, which will be described in Section 13.6.2.2.2. It also produces novel, broad-spectrum peptide antibiotics called munumbicins that are discussed in Section 13.6.2.2.2. This appears to be an excellent example illustrating the potential that some of the healing properties in plants, as discovered by indigenous peoples, might be facilitated by compounds produced by one or more specific plant-associated endophytes as well as the plant products themselves.

Furthermore, some plants generate bioactive natural products and have associated endophytes that produce the same natural products. One such example is the case with taxol, a highly functionalized diterpenoid and famed anticancer agent that is found in *Taxus brevifolia* and other yew species (*Taxus* spp.).[8] In 1993, a novel taxol-producing fungus *Taxomyces andreanae* was isolated and characterized from *T. brevifolia*.[20]

13.5 ENDOPHYTES AND BIODIVERSITY

Of the myriad of ecosystems on the earth, those having the greatest general biodiversity of life seem to be the ones also having the greatest number of and most diverse endophytes. Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems. The most threatened of these spots cover only 1.44% of the land's surface; yet, they harbor over 60% of the world's terrestrial biodiversity.[16] In addition, each of the 20–25 areas identified as supporting the world's greatest biodiversity also support unusually high levels of plant endemism.[16] As such, one would expect, with high plant endemism, there also should exist specific endophytes that may have evolved with the endemic plant species. Biological diversity implies chemical diversity because of the constant chemical innovation that is required to survive in ecosystems where the evolutionary race to survive is most active. Tropical rainforests are a remarkable example of this type of environment. Competition is great, resources are limited, and selection pressure is at its peak. This gives rise to a high probability that rainforests are a source of novel molecular structures and biologically active compounds.[21]

Bills et al.[22] describe a metabolic distinction between tropical and temperate endophytes through statistical data that compare the number of bioactive natural products isolated from endophytes of tropical regions to the number of those isolated from endophytes of temperate origin. Not only did they find that tropical endophytes provide more active natural products than temperate endophytes, but they also noted that a significantly higher number of tropical endophytes produced a larger number of active secondary metabolites than did fungi from other substrata. This observation suggests the importance of the host plant as well as the ecosystem in influencing the general metabolism of endophytic microbes.

13.6 ENDOPHYTES AND NATURAL PRODUCTS

It has been suggested that the reason some endophytes produce certain phytochemicals, originally characteristic of the host, might be related to a genetic combination of the endophyte with the host that occurred in evolutionary time.[12] This is a concept that was originally proposed as a mechanism to explain why *T. andreanae* may be producing taxol.[23] Thus, if endophytes, and especially cultured endophytes, can produce the same rare and important bioactive compounds as their host plants, this would not only reduce the need to harvest slow-growing and possibly rare plants, but also help to preserve the world's ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a high-value product may be easier and more economical to produce effectively, thereby reducing its market price.

All aspects of the biology and interrelationship of endophytes with their respective hosts is a vastly underinvestigated and exciting field.[24–27] Thus, more background information on a given plant species and its microorganismal biology would be exceedingly helpful in directing the search for bioactive products. Presently, no one is quite certain of the role of endophytes in nature and what appears to be their relationship to various host plant species. Although some endophytic fungi appear to be ubiquitous

(e.g., *Fusarium* spp., *Pestalotiopsis* spp., and *Xylaria* spp.), one cannot definitively state that endophytes are truly host specific or even systemic within plants any more than assume that their associations are chance encounters. Frequently, many endophytes of the same species are isolated from the same plant, and only one or a few biotypes of a given fungus will produce a highly biologically active compound in culture.[28] A great deal of uncertainty also exists about what an endophyte produces in culture and what it may produce in nature. It seems possible that the production of certain bioactive compounds by the endophyte *in situ* may facilitate the domination of its biological niche within the plant or even provide protection to the plant from harmful invading pathogens. Furthermore, little information exists relative to the biochemistry and physiology of the interactions of the endophyte with its host plant. It would seem that many factors changing in the host as related to the season, and other factors including age, environment, and location, may influence the biology of the endophyte. Indeed, further research at the molecular level must be conducted in the field to study endophyte interactions and ecology. All of these interactions are probably chemically mediated for some purpose in nature. An ecological awareness of the role these organisms play in nature will provide the best clues for targeting particular types of endophytic bioactivity with the greatest potential for bioprospecting.

13.6.1 Isolation, Preservation, and Storage of Endophytic Cultures for Product Isolation

Detailed techniques for the isolation of microbial endophytes are outlined in a number of reviews and technical articles.[17–20,24–27]

If endophytes are being obtained from plants growing in polar regions, the dry tropics, or some temperate areas of the world, one can expect to acquire from none to only one or two endophytic cultures per plant sample (0.5–10.0-cm limb piece). However, from the wet tropics this number may rise to 20–30 or even more microbes per plant piece. Given limited fermentation capabilities, it is critical to label and store cultures of freshly isolated microbes for work in the future and for patent and publication purposes.

Generally, preservation in an aqueous 15% glycerol solution at −70°C is an exceedingly good procedure for saving cultures until work on them can proceed at a later date.[24–27] Growing fungal cultures on sterilized barley or other grains with subsequent storage at −70°C is an effective alternative to the glycerol storage solution.

13.6.2 Some Examples of Bioactive Natural Products from Endophytes

The following section shows some examples of natural products obtained from endophytic microbes and their potential in the pharmaceutical and agrochemical arenas. Many of the examples are taken from our own work, and thus, this review is by no means inclusive of all natural product work in endophytes.

13.6.2.1 Endophytic Fungal Products as Antibiotics

Fungi are the most commonly isolated endophytic microbes. They usually appear as fine filaments growing from the plant material on the agar surface. Generally, the most commonly isolated fungi are in the group Fungi Imperfecti or Deuteromycetes. Basically, they produce asexual spores in or on various fruiting structures. Also, it is quite common to isolate endophytes that produce no fruiting structures whatsoever such as Mycelia Sterilia.

Quite commonly endophytes produce secondary metabolites when placed in culture. However, the temperature, the composition of the medium, and the degree of aeration will affect the amount and kind of compounds that are produced by an endophytic fungus. Sometimes endophytic fungi produce antibiotics. Natural products from endophytic fungi have been observed to inhibit or kill a wide variety of harmful microorganisms including, but not limited to, phytopathogens, as well as bacteria, fungi, viruses, and protozoans that affect humans and animals.

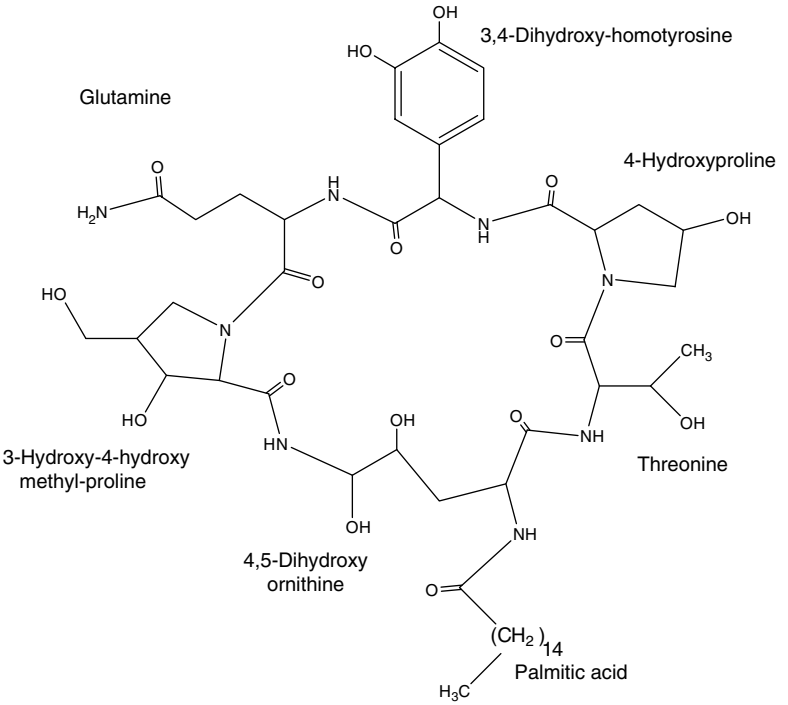

FIGURE 13.2 Cryptocandin A, an antifungal lipopeptide obtained from the endophytic fungus, *Cryptosporiopsis* cf. *quercina* (no stereochemistry is intended).

Cryptosporiopsis cf. *quercina* is the imperfect stage of *Pezicula cinnamomea*, a fungus commonly associated with hardwood species in Europe. It was isolated as an endophyte from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia.[29] On Petri plates, *C. quercina* demonstrates excellent antifungal activity against some important human fungal pathogens including *Candida albicans* and *Trichophyton* spp. Cryptocandin, a unique peptide antimycotic related to the echinocandins and the pneumocandins,[30] was isolated and characterized from *C. quercina*.[29] This compound contains a number of peculiar hydroxylated amino acids and a novel amino acid, 3-hydroxy-4-hydroxymethyl-proline (Figure 13.2). It is generally true that not one but several bioactive and related compounds are produced by an endophytic microbe. So other antifungal agents related to cryptocandin are also produced by *C. quercina*. Cryptocandin is also active against a number of plant pathogenic fungi including *Sclerotinia sclerotiorum* and *Botrytis cinerea*. Cryptocandin and its related compounds are currently being considered for use against a number of fungi causing diseases of the skin and nails.

Cryptocin, a unique tetramic acid, is also produced by *C. quercina* (Figure 13.3).[31] This unusual compound possesses potent activity against *Pyricularia oryzae*, the causal organism of "rice blast", one of the worst plant diseases in the world, as well as a number of other plant pathogenic fungi.[31] The compound was generally ineffective against a general array of human pathogenic fungi. Nevertheless, with minimum inhibitory concentrations against *P. oryzae* at 0.39 μg/mL, this compound is being examined as a natural chemical control agent for rice blast and is being used as a model to synthesize other antifungal compounds.

Pestalotiopsis microspora is a common rainforest endophyte.[24–26] It turns out that enormous biochemical diversity exists in this endophytic fungus, and many secondary metabolites are produced by various strains of this widely dispersed organism. One such secondary metabolite is ambuic acid, an antifungal agent, which has been recently described from several isolates of

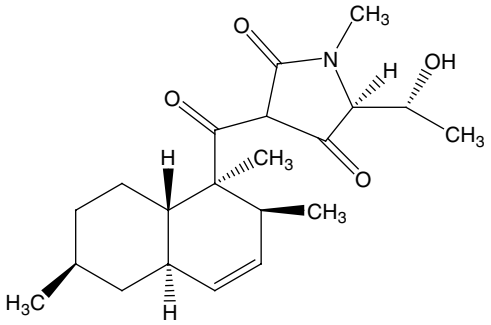

FIGURE 13.3 Cryptocin, a tetramic acid antifungal compound found in *Cryptosporiopsis* cf. *quercina*.

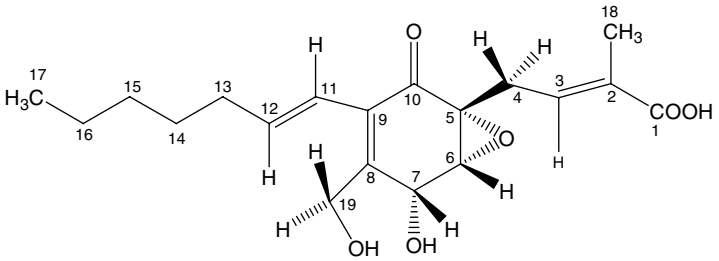

FIGURE 13.4 Ambuic acid, a highly functionalized cyclohexenone epoxide produced by a number of isolates of *Pestalotiopsis microspora* found in rainforests around the world. This compound possesses antifungal activity and has been used as a model compound for the development of solid-state NMR methods for the structural determination of natural products.

P. microspora found as representative isolates in many of the world's rainforests (Figure 13.4).[32] As an interesting spin-off to endophyte studies, ambuic acid and another endophyte product, terrein, have been used as models to develop new solid-state NMR tensor methods to assist in the characterization of molecular stereochemistry of organic molecules.[33,34]

A strain of *P. microspora* was isolated from the endangered tree *Torreya taxifolia* and produces several compounds having antifungal activity including pestaloside, an aromatic β-glucoside, and two pyrones, pestalopyrone and hydroxypestalopyrone.[35] These compounds also possess phytotoxic properties. Other newly isolated secondary products obtained from *P. microspora* (endophytic on *T. brevifolia*) include two new caryophyllene sesquiterpenes, pestalotiopsins A and B,[36] 2α-hydroxydrimeninol, and a highly functionalized humulane.[37,38] Variation in the amount and kind of products found in this fungus depends on both the culture conditions and the original plant source from which it was isolated.

Pestalotiopsis jesteri is a newly described endophytic fungal species from the Sepik river area of Papua New Guinea, and it produces the highly functionalized cyclohexenone epoxides, jesterone and hydroxy-jesterone, which exhibit antifungal activity against a variety of plant pathogenic fungi.[39] Jesterone has subsequently been synthesized with complete retention of biological activity (Figure 13.5).[40] Jesterone is one of only a few products from endophytic microbes for which total synthesis has been successfully accomplished.

Phomopsichalasin, a metabolite from an endophytic *Phomopsis* sp., represents the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring. This metabolite exhibits antibacterial activity in disk diffusion assays (at a concentration of 4 μg/disk) against *Bacillus subtilis*, *Salmonella gallinarum*, and *Staphylococcus aureus*. It also displays a moderate activity against the yeast *Candida tropicalis*.[41]

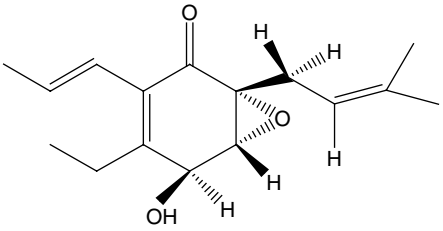

FIGURE 13.5 Jesterone, a cyclohexenone epoxide from *Pestalotiopsis jesteri*, has antioomycete activity.

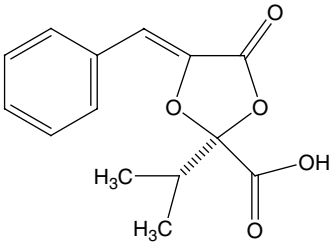

FIGURE 13.6 Guignardic acid from *Guignardia* sp. obtained from *Spondias mombin*, an anacardiaceaeous plant in Brazil.

An endophytic *Fusarium* sp. isolated from *Selaginella pallescens* collected in the Guanacaste Conservation Area of Costa Rica was screened for antifungal activity. A new pentaketide antifungal agent, CR377, was isolated from the culture broth of the fungus and showed potent activity against *C. albicans* in agar diffusion assays.[42]

Colletotric acid, a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus isolated from *Artemisia mongolica*, displays antibacterial activity against bacteria as well as against the fungus, *Helminthsporium sativum*.[43] Antimicrobial products have been identified from another *Colletotrichum* sp. isolated from *Artemisia annua*, a traditional Chinese herb that is well recognized for its synthesis of artemisinin (an antimalarial drug) and its ability to inhabit many geographically different areas. Not only did the *Colletotrichum* sp. found in *A. annua* produce metabolites with activity against human pathogenic fungi and bacteria, but also metabolites that were fungistatic to plant pathogenic fungi.[44]

A novel antibacterial agent, guignardic acid, was isolated from the endophytic fungus *Guignardia* sp. Interestingly, the organism was obtained from the medicinal plant *Spondias mombin* of the tropical plant family Anacardiaceae found in Brazil. The compound was isolated by UV-guided fractionation of the fermentation products of this fungus and is the first member of a novel class of natural compounds containing a dioxolanone moiety formed by the fusion of 2-oxo-3-phenylpropanoic acid and 3-methyl-2-oxobutanoic acid that are products of the oxidative deamination of phenylalanine and valine, respectively (Figure 13.6).[45]

13.6.2.2 Endophytic Bacterial Products as Antibiotics

There are only a limited number of bacterial species known to be associated with plants including species of *Pseudomonas* and *Streptomyces*.

13.6.2.2.1 Endophytic Pseudomonas *Species*

One of the most common endophytic bacterial genera encountered is *Pseudomonas*, which has representative biotypes and species that are epiphytic, endophytic, and pathogenic. They have been reported from every continent including Antarctica. Some of these species produce phytotoxic compounds as well as antibiotics.

The ecomycins, active against human pathogenic fungi such as *Cryptococcus neoformans* and *C. albicans*, are produced by *Pseudomonas viridiflava* that is generally associated with the leaves of many grass species and is located on and within the tissues.[46] These novel lipopeptides have masses of 1153 and 1181 and contain, in addition to the common amino acids such as alanine, serine, threonine, and glycine, some unusual amino acids including homoserine and β-hydroxyaspartic acid.[46]

The pseudomycins, produced by a plant-associated pseudomonad, are another group of antifungal peptides.[47,48] They are active against a variety of plant and human pathogenic fungi, including *C. albicans* and *C. neoformans*, and a variety of plant pathogenic fungi including *Ceratocystis ulmi* (the Dutch Elm Disease pathogen) and *Mycosphaerella fijiensis* (causal agent of Black Sigatoka disease in bananas). The pseudomycins are cyclic depsipeptides that include several nontraditional amino acids including L-chlorothreonine, L-hydroxyaspartic acid, and both D- and L-diaminobutryic acids. Cyclization is formed by acylation of the OH group of the N-terminal serine with the terminal carboxyl group of L-chlorothreonine. Variety in this family of compounds is imparted via *N*-acetylation by one of a series of fatty acids including 3,4-dihydroxydecanoic and 3-hydroxytetradecanoic acids.[48] The molecules are candidates for use in human medicine especially since structural modification by chemical synthesis has successfully removed mammalian toxicity.[49] The author (GS) has shown that pseudomycins are also effective against a number of ascomycetous fungi and are being considered for the control of the Black Sigatoka disease in bananas.

13.6.2.2.2 Endophytic Streptomyces *Species*

Streptomyces spp. are filamentous bacteria, belonging to the group Actinomycetales, that live in widely diverse ecological settings. Generally, the actinomycetes are Gram-positive organisms with a high G + C content and do not have an organized nucleus. To date, actinomycetes have been the world's greatest source of natural antibiotics with just one genus, *Streptomyces*, the source of 80% of these compounds.[50] The majority of the antibiotic producers are known from soil sources and, until recently, it was not realized that these organisms can exist as endophytes. One of the first endophytic *Streptomyces* spp. isolated was that from perennial ryegrass (*Lolium perenne*).[51] This isolate produces methylalbonoursin, a weak antibiotic diketopiperazine.[51] Other streptomycetes with activities against plant pathogens have been isolated from a number of plant species in Japan and are being considered as biocontrol agents.[52]

Using the ethnobotanical approach to plant selection, the snake vine plant *Kennedia nigriscans* was chosen as a possible source of endophytic microbes, because of its long traditional use by Australian Aborigines to treat cuts and open wounds resulting in reduced infection and rapid healing. This plant, collected near the Aboriginal community of Manyallaluk in the Northern Territory of Australia, consistently produced an endophytic actinomycete designated *Streptomyces* NRRL 30562.[19] The organism was not found in several tree species supporting the vine suggesting a host-selective or specific association of the endophyte with a specific plant genus. This streptomycete produces a family of actinomycins including D, Xo_{β}, and X_2. These compounds may not only protect the plant from fungal and bacterial infections, but also have unknowingly served the Aborigines as a source of bush medicine.

The snake vine-derived *Streptomyces* NRRL 30562 also produces the novel antibiotics munumbicins E-4 and E-5, which possess widely differing biological activities, depending on the target organism.[53] These compounds appear to be related to the actinomycins but have a mild yellowish coloration and unique NMR spectra, and both possess masses of 1445. The munumbicins are effective against a wide range of fungal pathogens of plants and certain Gram-negative and Gram-positive bacteria. The compounds are currently being more completely chemically characterized.

More recently, endophytic streptomycetes have been discovered in the upper Amazon of Peru, an area of the world claimed to be one of the most biologically diverse. The inner tissues of the follow-me vine, *Monstera* sp., commonly yield a verticillated streptomycete with outstanding

inhibitory activities against pythiaceous fungi as well as the malarial parasite *Plasmodium falciparum*. The bioactive component is a mixture of lipopeptides and is named coronamycin.[54]

13.6.2.3 Volatile Antibiotics from Endophytes

Muscodor albus is a newly described endophytic fungus obtained from small limbs of *Cinnamomum zeylanicum* (cinnamon tree).[55] This xylariceaous (nonspore-producing) fungus effectively inhibits and kills certain other fungi and bacteria by producing a mixture of volatile compounds.[56] The majority of these compounds have been identified by GC/MS, synthesized or acquired, and then ultimately formulated into an artificial mixture that mimicked the antibiotic effects of the volatile compounds produced by the fungus.[56] Individually, each of the five classes of volatile compounds produced by the fungus had some antimicrobial effects against the test fungi and bacteria but none was lethal. However, collectively they acted synergistically to cause death in a broad range of plant and human pathogenic fungi and bacteria. The most effective class of inhibitory compounds was the esters, of which isoamyl acetate was the most biologically active; however, to be lethal, it needs to be combined with other volatiles.[56] The composition of the medium on which *M. albus* is grown dramatically influences the kind of volatile compounds that are produced.[57]

The ecological implications and potential practical benefits of the "mycofumigation" effects of *M. albus* are very promising given the fact that soil fumigation utilizing methyl bromide will soon be illegal in the United States. Methyl bromide not only is a hazard to human health but also has been implicated in causing destruction of the ozone layer. The potential use of mycofumigation to treat soil, seeds, and plants will soon be a reality as AgraQuest of Davis, California, has EPA approval to release this organism for agricultural uses. The artificial mixture of volatile compounds may also have usefulness in treating seeds, fruits, and plant parts in storage and while being transported. In addition, *M. albus* is already in a limited market for the treatment of human wastes. Its volatile components have both inhibitory and lethal effects on such fecal-inhabiting organisms as *Escherichia coli* and *Vibrio cholera*. Studies are under way that show its promise to fumigate buildings, thus removing the potential for fungi to contaminate building surfaces and causing health risks.

Using *M. albus* as a screening tool, it has now been possible to isolate other endophytic fungi producing volatile antibiotics. The newly described *M. roseus* was twice obtained from tree species found in the Northern Territory of Australia. This fungus is just as effective in causing inhibition and death of test microbes in the laboratory as *M. albus*.[58] Other interesting *M. albus* isolates have been obtained from several plant species growing in the Northern Territory of Australia and the jungles of the Tesso Nilo area of Sumatra, Indonesia.[59,60]

A non *Muscodor* species i.e., *Gilocladium* sp., that produces volatile antibiotics has also been discovered. The volatile components of this organism are totally different from those of either *M. albus* or *M. roseus*. In fact, the most abundant volatile inhibitor is [8]-annulene, formerly used as a rocket fuel and discovered here for the first time as a natural product. However, the bioactivity of the volatiles of this *Gliocladium* sp. is not as good or as comprehensive as that of the *Muscodor* spp.[61]

13.6.2.4 Antiviral Compounds from Endophytes

Another fascinating use of products from endophytic fungi is the inhibition of viruses. Two novel human cytomegalovirus (hCMV) protease inhibitors, cytonic acids A and B, have been isolated from solid-state fermentation of the endophytic fungus *Cytonaema* sp. Their structures were elucidated as *p*-tridepside isomers using MS and NMR methods.[62] It is apparent that the potential for the discovery of compounds having antiviral activity from endophytes is in its infancy. The fact, however, that some compounds have been found already is promising. The main limitation to compound discovery to date is probably related to the absence of common antiviral screening systems in most compound discovery programs.

13.6.2.5 Endophytic Fungal Products as Anticancer Agents

Taxol and some of its derivatives represent the first major group of anticancer agents that are produced by endophytes (Figure 13.7). Taxol, a highly functionalized diterpenoid, is found in each of the world's yew (*Taxus*) species but was originally isolated from *T. brevifolia*.[8] The original target diseases for this compound were ovarian and breast cancers, but now it is used to treat a number of other human tissue-proliferating diseases. The presence of taxol in yew species prompted the study of their endophytes. By the early 1990s, however, no endophytic fungi had been isolated from any of the world's representative yew species. After several years of effort, a novel taxol-producing endophytic fungus *T. andreanae* was discovered in *T. brevifolia*.[20] The most critical line of evidence for the presence of taxol in the culture fluids of this fungus was the electrospray mass spectrum of the putative taxol isolated from *T. andreanae*. In electrospray mass spectrometry, taxol usually gives two peaks, one at *m/z* 854 $[M + H]^+$ and the other at *m/z* 876 $[M + Na]^+$. Fungal taxol had an identical mass spectrum to authentic taxol, and ^{14}C labeling studies showed the presence of fungal-derived taxol in the culture medium.[23] This early work set the stage for a more comprehensive examination of the ability of other *Taxus* species and many other plants to yield taxol-producing endophytes.

Some of the most commonly found endophytes of the world's yews, and many other plants, are *Pestalotiopsis* spp.[24–27] One of the most frequently isolated endophytic species is *P. microspora*.[24] An examination of the endophytes of *Taxus wallichiana* yielded *P. microspora*, and a preliminary monoclonal antibody test indicated that it might produce taxol.[30] After preparative TLC, a compound was isolated and shown by spectroscopic techniques to be taxol. Labeled (^{14}C) taxol was produced by this organism from several ^{14}C precursors that had been administered to it.[63]

Several other *P. microspora* isolates were obtained from a bald cypress tree (*Taxodium distichum*) in South Carolina (USA) and also shown to produce taxol.[28] This was the first indication that endophytes residing in plants other than *Taxus* spp. were producing taxol. Therefore, a specific search was conducted for taxol-producing endophytes on continents not known for any indigenous *Taxus* spp. This included investigating the prospects that taxol-producing endophytes exist in South America and Australia. In Australia, taxol-producing *Pestalotiopsis guepini* was isolated from the extremely rare, and previously thought to be extinct, Wollemi Pine (*Wollemia nobilis*).[64] Also, quite surprisingly, a rubiaceous plant *Maguireothamnus speciosus* yielded a novel fungus *Seimatoantlerium tepuiense* that was shown to produce taxol. This endemic plant grows on the top of the tepuis on the Venezuelan–Guyana border in southwest Venezuela.[65] Taxol production has also been noted in *Periconia* sp. and the novel endophytic fungus *Seimatoantlerium nepalense*.[66,67]

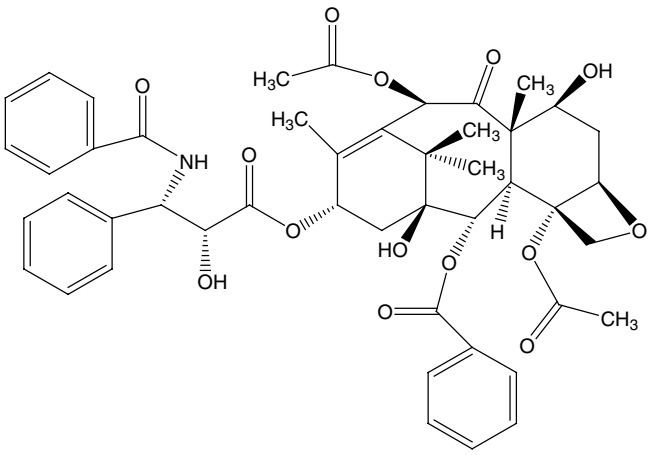

FIGURE 13.7 Taxol, the world's first billion dollar anticancer drug, is produced by many endophytic fungi. It too possesses outstanding antioomycete activity.

Simply, it appears that the distribution of taxol-producing fungi is worldwide and is not confined to endophytes of yews. The ecological and physiological explanation for this seems to be related to the fact that taxol is a fungicide, and the most sensitive organisms to it are plant pathogens such as *Pythium* spp. and *Phytophthora* spp.[68] These pythiaceous organisms are some of the world's most important plant pathogens and are strong competitors with endophytic fungi for niches within plants. In fact, their sensitivity to taxol is based on their interaction with tubulin in an identical manner as in rapidly dividing human cancer cells.[9,68] Thus, *bona fide* endophytes may be producing taxol and related taxanes to protect their respective host plant from degradation and disease caused by these pathogens.

Other investigators have also made observations on taxol production by endophytes, including the discovery of taxol production by a *Tubercularia* sp. isolated from the Chinese yew (*Taxus mairei*) in Fujian province of southeastern mainland China.[69] At least three endophytes of *T. wallichiana* produce taxol, including *Sporormia minima* and *Trichothecium* sp.[70] Using HPLC and ESI-MS, taxol has been discovered in the hazelnut (filbert) tree (*Corylus avellana* cv. Gasaway).[71] Several fungal endophytes of this plant produce taxol in culture.[71]

It is important to note, however, that taxol production by all endophytes in culture is in the range of submicrogram to microgram per liter. Also, commonly, the fungi will attenuate taxol production in culture, increasing the possibility for recovery of the taxol, if certain activator compounds are added to the medium.[66] Efforts are being made to determine the feasibility of making microbial taxol a commercial possibility. The greatest prospect may be the discovery of endophytes that make large quantities of one or more taxanes that could then be used as platforms for the organic synthesis of taxol or one of its anticancer relatives.

Torreyanic acid, a selectively cytotoxic quinone dimer and potential anticancer agent, was isolated from a *P. microspora* strain (Figure 13.8). This strain was originally obtained as an endophyte associated with the endangered tree *T. taxifolia* (Florida torreya).[72] Torreyanic acid was tested in several cancer cell lines, and it demonstrated 5–10 times more potent cytotoxicity in those lines that are sensitive to protein kinase C agonists and causes cell death by apoptosis. Recently, torreyanic acid has been successfully synthesized by application of a biomimetic oxidation/dimerization cascade.[73]

Alkaloids are also commonly found in endophytic fungi. The fungal genera *Xylaria*, *Phoma*, *Hypoxylon*, and *Chalara* are representative producers of a relatively large group of substances

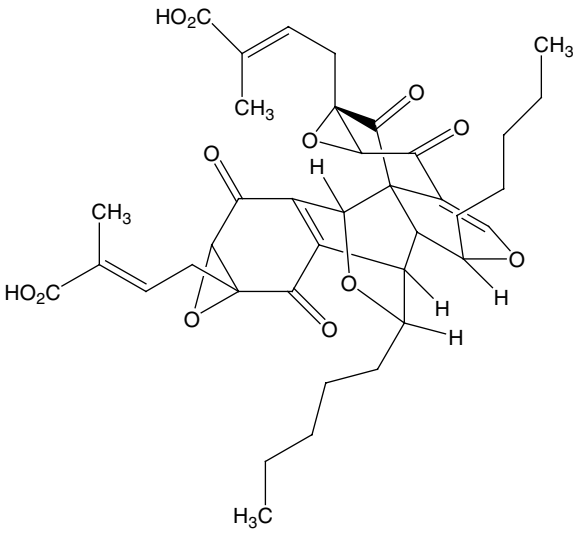

FIGURE 13.8 Torreyanic acid, an anticancer compound, from *Pestalotiopsis microspora*.

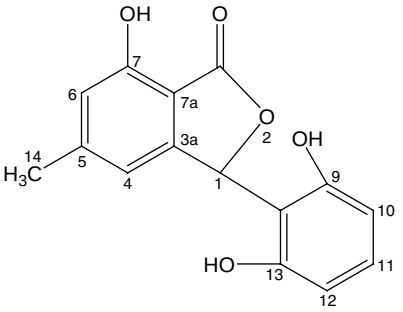

FIGURE 13.9 Isopestacin, an antioxidant produced by an endophytic *Pestalotiopsis microspora* strain, isolated from *Terminalia morobensis* growing on the north coast of Papua New Guinea.

known as the cytochalasins of which over 20 are now known. Many of these compounds possess antitumor and antibiotic activities, but, because of their cellular toxicity, they have not been developed into pharmaceuticals. Three novel cytochalasins have recently been reported from a *Rhinocladiella* sp. as an endophyte on *T. wilfordii*. These compounds have antitumor activity and have been identified as 22-oxa-[12]-cytochalasins.[74] Thus, it is not uncommon to find one or more cytochalasins in endophytic fungi. This provides an example of the fact that redundancy in discovery occurs, making dereplication an issue even for these underinvestigated sources.

13.6.2.6 Endophytic Fungal Products as Antioxidants

Two compounds, pestacin and isopestacin, have been obtained from culture fluids of *P. microspora*, an endophyte isolated from a combretaceaous plant, *Terminalia morobensis*, growing in the Sepik River drainage system of Papua New Guinea.[75,76] Both pestacin and isopestacin display antimicrobial as well as antioxidant activity. Isopestacin was attributed with antioxidant activity on the basis of its structural similarity to the flavonoids (Figure 13.9). Electron spin resonance spectroscopy measurements confirmed this antioxidant activity showing that the compound is able to scavenge superoxide and hydroxyl free radicals in solution.[75] Pestacin occurs naturally as a racemic mixture and also possesses potent antioxidant activity, at least one order of magnitude more potent than that of Trolox, a vitamin E derivative.[76] The antioxidant activity of pestacin arises primarily via cleavage of an unusually reactive C–H bond and to a lesser extent, through O–H abstraction.[76]

13.6.2.7 Endophytic Fungal Products as Immunosuppressive Compounds

Immunosuppressive drugs are used today to prevent allograft rejection in transplant patients, and in the future they could be used to treat autoimmune diseases such as rheumatoid arthritis and insulin-dependent diabetes. The endophytic fungus, *Fusarium subglutinans*, isolated from *T. wilfordii*, produces the immunosuppressive, but noncytotoxic, diterpene pyrones, subglutinols A and B.[77] Subglutinols A and B are equipotent in the mixed lymphocyte reaction (MLR) assay and thymocyte proliferation (TP) assay with an IC_{50} of 0.1 μM. The famed immunosuppressant drug cyclosporin A, also a fungal metabolite, was roughly as potent in the MLR assay and 10^4 more potent in the TP assay. However, the lack of toxicity associated with subglutinols A and B suggests that they should be explored in greater detail as potential immunosuppressants.[77]

13.6.3 Surprising Results from Molecular Biological Studies of *P. microspora*

Of some compelling interest is an explanation about how the genes for taxol production may have been acquired by *P. microspora*.[78] Although the complete answer to this question is not at hand, some other relevant genetic studies have been performed on this organism. *P. microspora* Ne 32 is

one of the most easily genetically transformable fungi that has been studied to date. *In vivo* addition of telomeric repeats to foreign DNA generates extrachromosomal DNAs in this fungus.[78] Repeats of the telomeric sequence 5′-TTAGGG-3′ were appended to nontelomeric-transforming DNA termini. The new DNAs, carrying foreign genes and the telomeric repeats, replicated independently of the chromosome and expressed the information carried by the foreign genes. The addition of telomeric repeats to foreign DNA is unusual among fungi. This finding may have important implications in the biology of *P. microspora* Ne 32 since it explains at least one mechanism as to how new DNA can be captured by this organism and eventually expressed and replicated. Such a mechanism may begin to explain how the enormous biochemical variation may have arisen in this fungus.[28] Also, this initial work represents a framework to aid in the understanding of how this fungus may adapt itself to the environment of its plant hosts and suggests that the uptake of plant DNA into its own genome may occur. In addition, the telomeric repeats have the same sequence as human telomeres which points to the possibility that *P. microspora* may serve as a means to make artificial human chromosomes, a totally unexpected result.

13.7 CONCLUDING STATEMENTS

Endophytes are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of applications. The mechanisms through which endophytes exist and respond to their surroundings must be better understood in order to be more predictive about which higher plants to seek, study, and employ in isolating their microfloral components. This may facilitate the natural product discovery process.

Although work on the utilization of this vast resource of poorly understood microorganisms has just begun, it has already become obvious that an enormous potential for organism, product, and utilitarian discovery in this field holds exciting promise. This is evidenced by the discovery of a wide range of products and microorganisms that present potential as described in this chapter.

It is important for all involved in this work to realize the importance of acquiring the necessary permits from governmental, local, and other sources to collect and transport plant materials (especially from abroad) from which endophytes are to be eventually isolated. In addition to this aspect of the work is the added activity of producing the necessary agreements and financial-sharing arrangements with indigenous peoples or governments in case a product develops an income stream.

Certainly, one of the major problems facing the future of endophyte biology and natural product discovery is the rapidly diminishing rainforests that hold the greatest possible resource for acquiring novel microorganisms and their products. The total landmass of the world that currently supports rainforests is about equal to the area of the United States.[16] Each year, an area the size of Vermont or greater is lost to clearing, harvesting, fire, agricultural development, mining, or other human-oriented activities.[16] Presently, it is estimated that only a small fraction (10–20%) of what were the original rainforests existing 1000–2000 years ago is currently present on the earth.[16] The advent of major negative pressures on them from these human-related activities appears to be eliminating entire megalife forms at an alarming rate. Few have ever expressed information or opinions about what is happening to the potential loss of microbial diversity as entire plant species disappear. It can only be guessed that this microbial diversity loss is also happening, perhaps with the same frequency as the loss of megalife forms, especially since certain microorganisms may have developed unique symbiotic relationships with their plant hosts. Thus, when a plant species disappears, so too does its entire suite of associated endophytes. Consequently, all of the capabilities that the endophytes might possess to provide natural products with medicinal potential are also lost. Multistep processes are needed now to secure information and life forms before they continue to be lost. Areas of the planet that represent unique places housing biodiversity need immediate preservation. Countries need to establish information bases of their biodiversity and at the same time begin to make national collections of microorganisms that live in these areas. Endophytes are only

one example of a life-form source that holds enormous promise to impact many aspects of human existence. The problem of the loss of biodiversity should be one of concern to the entire world.

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14 Isolation of Bioactive Natural Products from Myxobacteria

Makoto Ojika

CONTENTS

14.1 INTRODUCTION

Myxobacteria are gram-negative unicellular bacteria that inhabit soil and aquatic environments globally.[1] They are characterized by their life cycle in which, upon starvation, they gather by gliding to form the structurally diverse and colorful multicellular fruiting bodies. During the last few decades, the myxobacteria have become established as proficient producers of novel secondary metabolites.[2–4] The most significant secondary metabolites are epothilones, which are promising candidates for anticancer drugs with a paclitaxel-like action mechanism.[5] However, one of the critical disadvantages is that they are hard-to-culture microorganisms since they are difficult to isolate from nature and are slow growing. Recently, researchers have tried to clone and heterologously express the biosynthetic genes of myxobacterial secondary metabolites to produce them more effectively.[6]

Our group has been studying secondary metabolites of a few myxobacterial species. One of our strategies to discover novel antimicrobials is based on a paper-disk assay using a plant pathogen of the genus *Phytophthora*. During our screening research, this filamentous fungus-like microorganism, though not very popular in antibiotic screening,[7] turned out to be a good indicator

for inhibitors of the respiratory chain electron transport system. One of the specific observations of myxobacterial secondary metabolite research is an unusually high rate of discovery of electron transport inhibitors,[3] for example, β-methoxyacrylates such as myxothiazole A,[8] melithiazoles,[9] cystothiazoles,[10] haliangicins,[11] and cyrmenins.[12] Therefore, the combination of myxobacteria (as sources) and *Phytophthora* (as indicator) is advantageous for the discovery of novel antibiotics of this type. This review describes the isolation, structures, and biological activities of several myxobacterial metabolites that we detected using the *Phytophthora*-based assay methods.

14.2 SCREENING METHOD

In the screening for myxobacterial antimicrobials, we conducted a simple paper-disk assay using the plant pathogen *Phytophthora capsici*. Since the genus *Phytophthora* is notorious for aggressively infecting important crops such as potato and tomato, anti-*Phytophthora* substances would be promising leads for agrochemicals. *Phytophthora* is a fungus-like filamentous microorganism (Figure 14.1). Sporulation is a rare event during the life cycle, making it difficult to determine the minimum inhibition concentration (MIC) through the common dilution method. Therefore, the diffusion method using paper disks was adopted in this research.

A piece (5 mm × 5 mm) of mycelia of *P. capsici* precultured on a synthetic agar medium is placed in the center of an agar plate and incubated for 2 days with 60% humidity at 25°C until the colony grows to 3–4 cm in diameter (Figure 14.2). A paper disk containing a sample at an appropriate dose is placed 1 cm away from the colony front. After incubation for an additional 1 day when

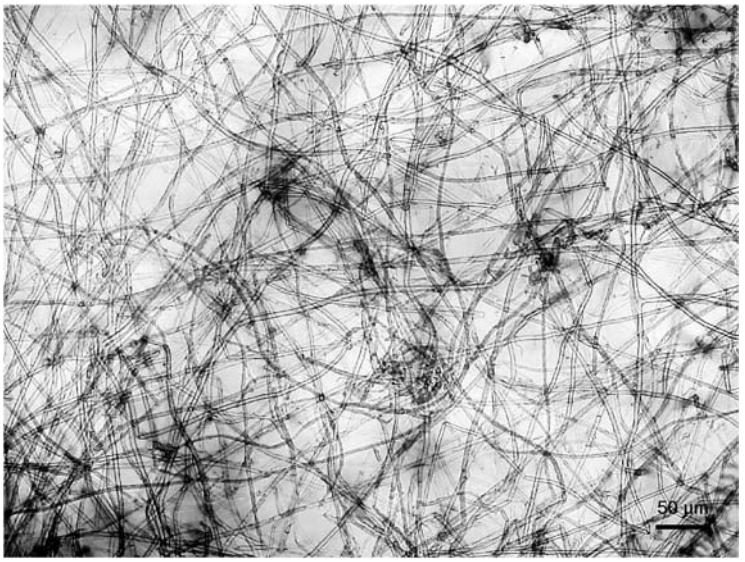


FIGURE 14.1 Mycelia of *Phytophthora capsici* (bar = 50 µm).

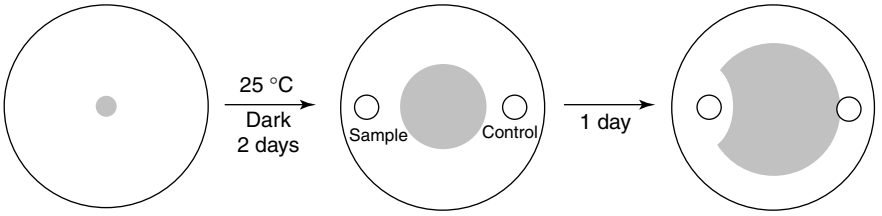


FIGURE 14.2 Assay method for anti-*Phytophthora* compounds.

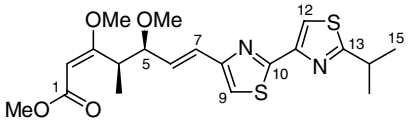
the colony front reaches the control disk, the inhibition zone formed around the sample disk is observed. The activity is represented by the minimum dose per disk to express a significant inhibition zone.

14.3 CYSTOTHIAZOLES

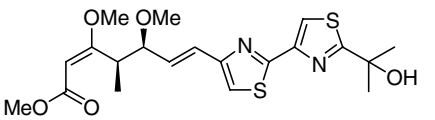
To search for novel myxobacterial antimicrobials, hundreds of organic and aqueous extracts of myxobacteria isolated from soils, barks, and rotting plants, as well as stocked strains, were screened using the paper disk method described. One of the most active anti-*Phytophthora* samples was an organic extract of *Cystobacter fuscus* strain AJ-13278, which was isolated from a soil sample collected at Kamakura, Kanagawa, Japan. After large-scale fermentations, cystothiazoles and myxalamide (Section 14.4) derivatives were discovered.

14.3.1 Production and Isolation

A medium-scale culture of *C. fuscus* was first carried out.[10] A seed culture was inoculated into a 500 mL Erlenmeyer flask containing 100 mL of a production medium and 2% (w/v) adsorber resin (Sepabeads® SP207) for promoting the production of secondary metabolites. The growth of this myxobacterium is relatively fast. The fermentation was done on a rotary shaker (180 rpm) at 28°C for 4 days. The bacterial cells and the absorber resin were collected from 2.5 L of culture broth and extracted with a mixture of acetone and methanol. The extract was partitioned between 60% aqueous methanol and dichloromethane, and the dichloromethane fraction was chromatographed on silica gel. The ethyl acetate-eluted fraction, which was active against *P. capsici* at 1 μg per disk, was subjected to silica gel flash chromatography. The most active fraction was purified by recrystallization to yield 31.6 mg of cystothiazole A (**1**) as colorless needles. A more polar active fraction, which was eluted after **1**, was further purified using preparative TLC to give an active fraction, which was subsequently subjected to normal-phase HPLC (hexane–dichloromethane–methanol, 70:29:1) to give 1.7 mg of cystothiazole B (**2**) as a colorless powder.



Cystothiazole A (**1**)

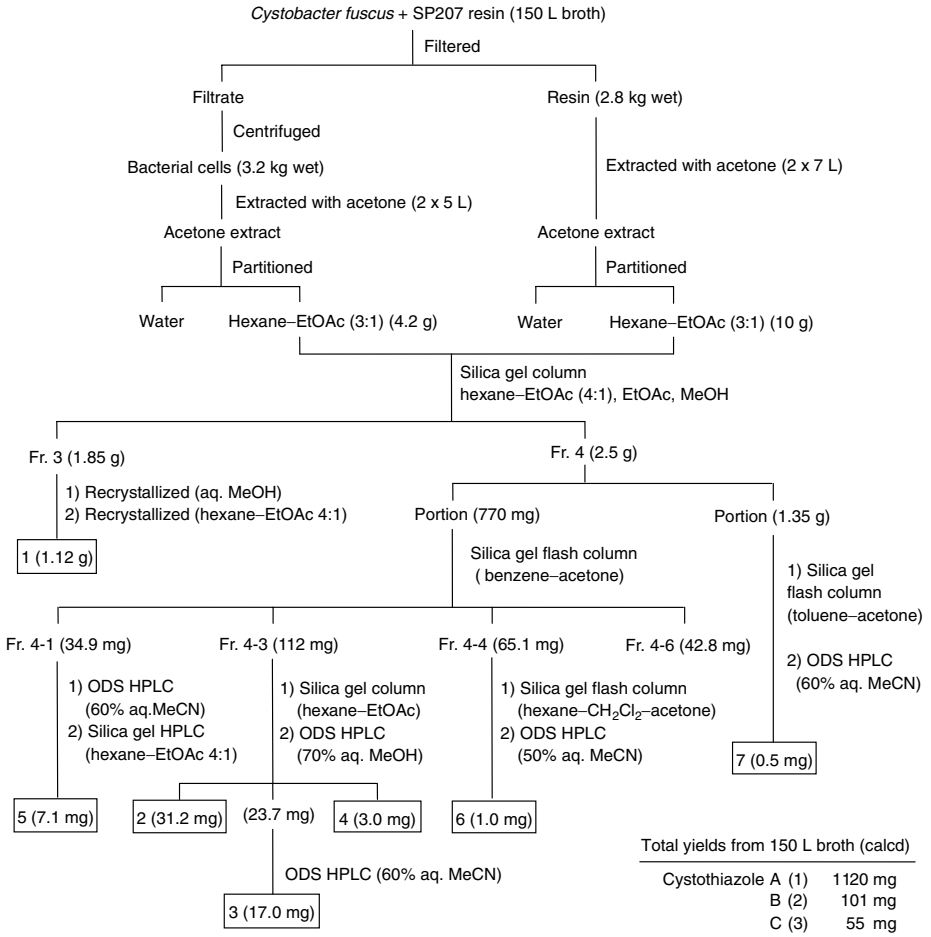


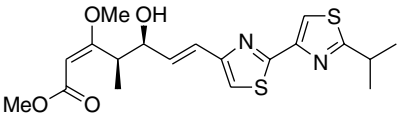
Cystothiazole B (**2**)

14.3.2 Production of Derivatives

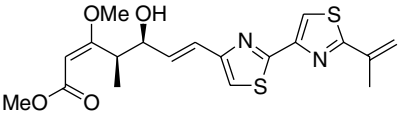
14.3.2.1 Large-Scale Fermentation

Structural diversity of antimicrobials is generally important for examining structure–activity relationships. To obtain other congeners of cystothiazole A (**1**), a much larger-scale fermentation of *C. fuscus* was performed by employing a 300 L jar fermenter.[13,14] After being cultured at 28°C for 4 days, the bacterial cells and the adsorber resin were separately collected from the 150 L culture, and both were extracted with acetone (Figure 14.3). Each extract was partitioned between hexane–ethyl acetate (3:1) and water. The organic fractions from the bacterial cells and resin were combined and sequentially separated by normal-phase and reverse-phase silica gel columns, and reverse-phase HPLC to give five new congeners, cystothiazoles C–G (**3**–**7**), as well as cystothiazoles A (**1**) and B (**2**). From this large-scale experiment, 1.12 g of cystothiazole A (**1**) was obtained as crystals and used for biological tests. The calculated yields of the congeners were 0.9–101 mg (Figure 14.3). Another class of antimicrobials, 2′-*O*-methylmyxalamides, was obtained from fraction Fr 4-6 (Figure 14.3) as described in Section 14.4.1.

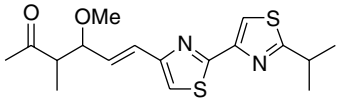




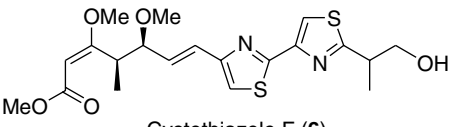
Cystothiazole C (**3**)



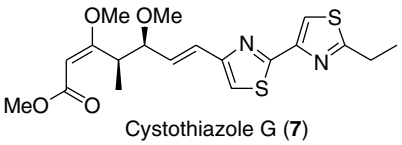
Cystothiazole D (**4**)



Cystothiazole E (**5**)



Cystothiazole F (**6**)



Cystothiazole G (**7**)

FIGURE 14.3 Isolation scheme for cystothiazoles.

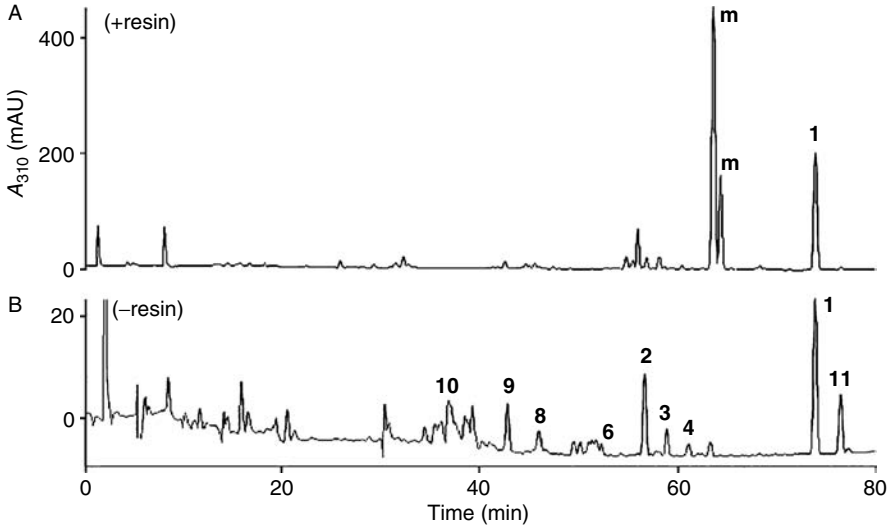


FIGURE 14.4 HPLC analysis of ethyl acetate extracts of the myxobacterium *Crystobacter fuscus*. (A) Cultured for 4 days with the resin; (B) cultured for 4 days without the resin. **m**: 2′-*O*-methylmyxalamide D compounds (see Section 14.4).

14.3.2.2 Biotransformation

Another reliable technique to obtain new congeners of mother (major) compounds might be biotransformation. It was observed that the production of unidentified polar congeners became relatively higher in nonresin cultures than in the resin cultures, though the yield of cystothiazole A (**1**) was decreased from 8 to 0.8 mg/L (Figure 14.4).[15] These findings suggest that **1** is accumulated and protected in the presence of the resin by adsorption and is biochemically transformed to polar congeners by the producer myxobacterium in the absence of the resin. To promote the production of such novel congeners, cystothiazole A (**1**) (10 mg/L) was externally added into a culture of *C. fuscus* in the absence of the resin. The time-dependent production profiles of the congeners were analyzed by HPLC using a photodiode array detector. The novel peaks were chromatographically isolated, identifying three additional cystothiazoles **8**–**10** (Figures 14.4 and 14.5). The externally added **1** underwent rapid oxidative metabolism such as hydroxylation, dehydrogenation, and *O*-demethylation to afford cystothiazole B (**2**), melithiazole B (**11**, Figure 14.5),[9] and cystothiazole C (**3**), within 3 h.[15] With longer time (6 h or more), the biotransformation of **1** proceeded further to produce more congeners at higher oxidative stages that were more polar (**4** and **8**–**10**, Figure 14.5).

14.3.2.3 Chemical Synthesis

The third method to increase the structural diversity of antimicrobials is chemical synthesis/transformation. Cystothiazole A (**1**) possesses two asymmetric carbons with 4*R*,5*S* stereochemistry. Needless to say, the unnatural stereoisomers (enantiomer and diastereomers) of **1** can be created only by chemical synthesis. If the stereochemistry is not important for activity, then the synthesis of active derivatives would become much easier. To investigate the importance of the stereochemistry, three stereoisomers, (4*R*,5*R*)-, (4*S*,5*S*)-, and *ent*-**1** as well as natural **1**, were stereoselectively synthesized.[16] The key reactions were the C–C bond formation between C-4 and C-5 by the asymmetric aldol and anti-aldol additions. In spite of all possessing the β-methoxyacrylate moiety, all three stereoisomers were inactive even at a dosage 2500-fold greater than that of **1** (Section 14.3.3).

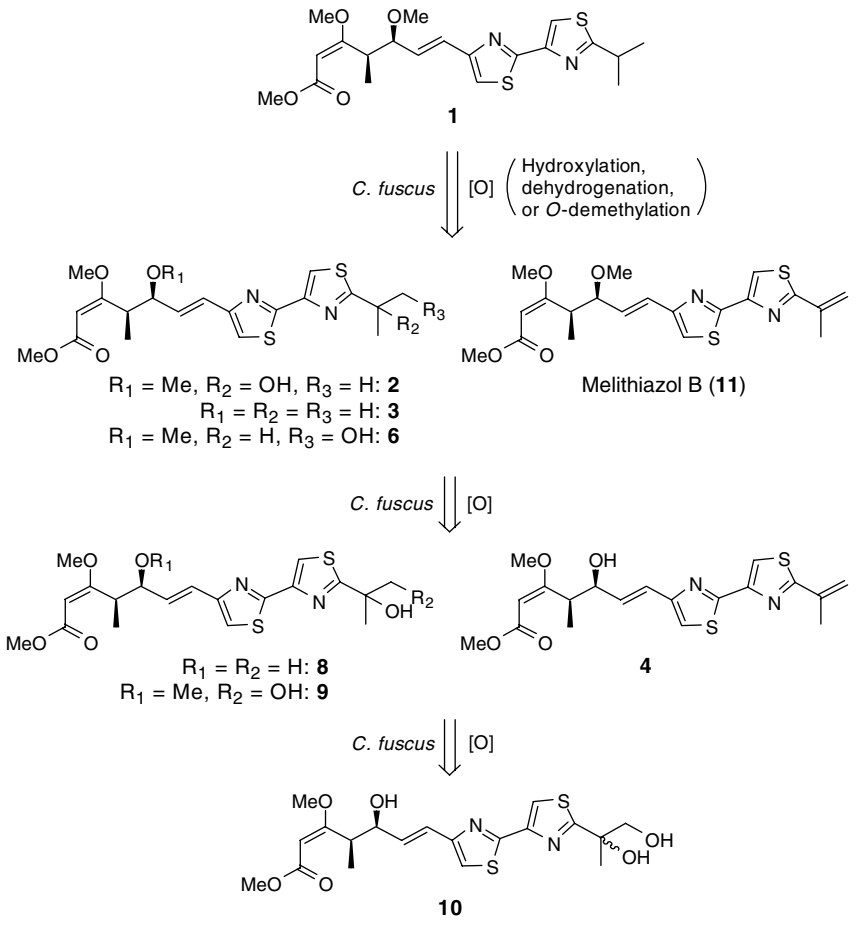
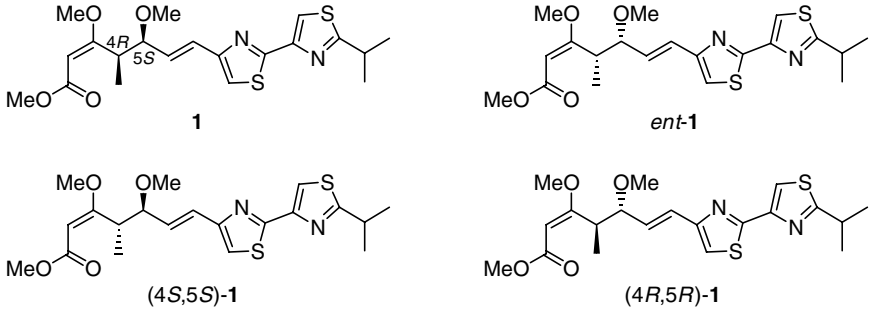
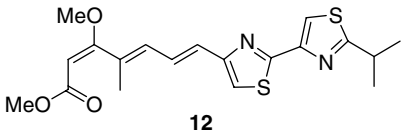


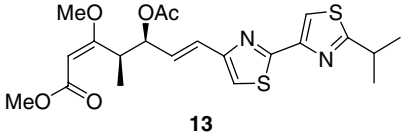
FIGURE 14.5 A plausible pathway of oxidative biotransformation of cystothiazole A (**1**) by the producer.



The hydroxylated derivative cystothiazole C (**3**) is a propitious starting material for further derivatives. Mesylation of **3** was followed by spontaneous β-elimination to give the nonchiral conjugated derivative (**12**) and acetylation of **3** provided acetate **13** under usual conditions.[17] *O*-Methylation of **3** under basic conditions (MeI, NaH) to **1** was unsuccessful but provided the δ-lactone **14** as the major product by intramolecular cyclization.[13]



12



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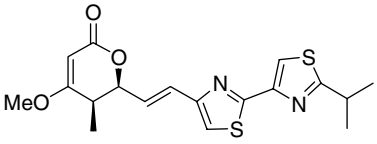


14

14.3.3 Biological Activity

The bioactivity of all the natural and unnatural cystothiazoles described in the previous sections was investigated as follows.

14.3.3.1 Anti-*Phytophthora* Activity

The minimum doses to form an inhibition zone on the agar plate disk bioassay (Section 14.2) are summarized in Table 14.1. The major substance cystothiazole A (**1**) showed the highest activity, inhibiting *P. capsici* at the minimum dose of 40 ng per disk (Figure 14.6). The dehydro derivative,

TABLE 14.1
Minimum Doses of Cystothiazoles and Derivatives for Anti-*Phytophthora* Activity

Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Dose (μg per disk)	0.04	1	5	5	5	0.2	0.2	>25	>25
Compound	**10**	**11**	***ent*-1**	**(4*S*,5*S*)-1**	**(4*R*,5*R*)-1**	**12**	**13**	**14**	
Dose (μg per disk)	>25	0.04	>100	>100	>100	>100	5	>100	

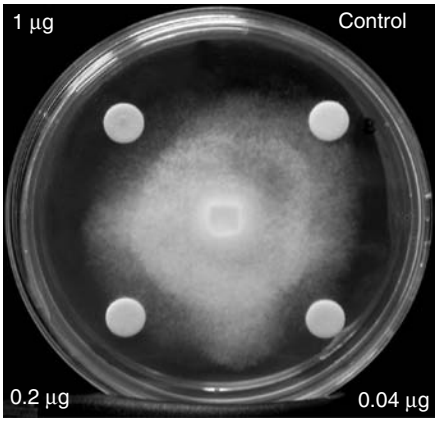


FIGURE 14.6 Anti-*Phytophthora* activity of cystothiazole A (**1**).

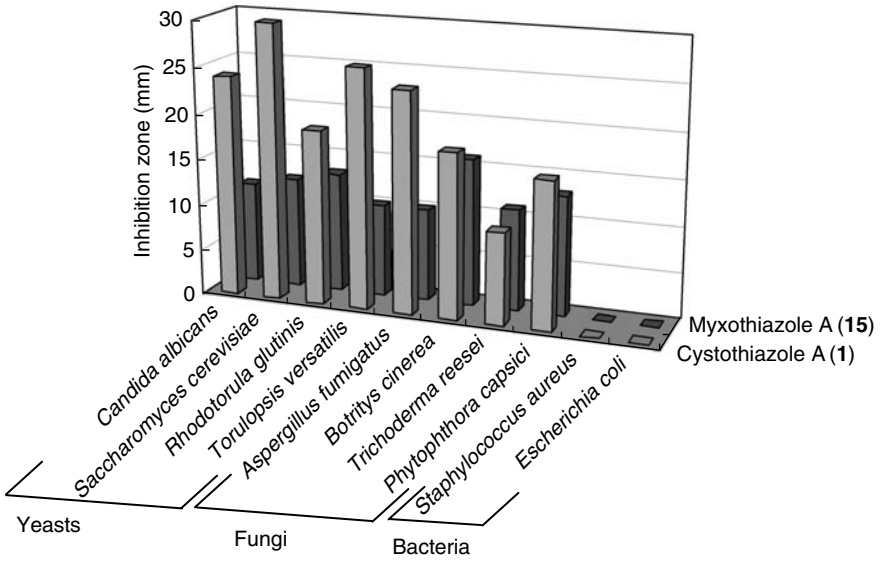
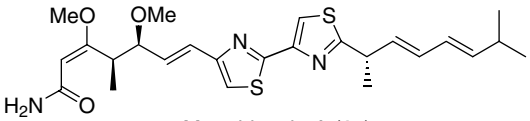


FIGURE 14.7 Antimicrobial spectrum of cystothiazole A (**1**) in comparison with myxothiazole A (**15**).

melithiazole B (**11**, Figure 14.5), showed a similar activity to **1**. Results (Table 14.1) suggested the following structure–activity relationships: (1) the β-methoxyacrylate moiety is important; (2) 4*R*,5*S* stereochemistry is essential; (3) the 5-methoxy group is important; and (4) a lipophilic alkyl group on bithiazole is important.

14.3.3.2 Antimicrobial Spectrum

The antimicrobial spectrum of cystothiazole A (**1**) was determined against several yeasts, fungi, and bacteria in comparison with a previously isolated, related bioactive substance, myxothiazole A (**15**) (Figure 14.7).[10] Yeasts and fungi were sensitive to both **1** and **15**, but bacteria were not affected. This tendency is attributable to the presence of the mitochondrial respiratory chain system in the eukaryotes, which is the target of the β-methoxyacrylates. Cystothiazole A (**1**) showed higher inhibitory activity than myxothiazole A (**15**) against most of the microorganisms.



Myxothiazole A (**15**)

14.3.3.3 Cytotoxicity

Cystothiazole A (**1**) showed lower cytotoxicity than myxothiazole A (**15**) against two human tumor cell lines (Figure 14.8).[10] This suggests that **1** is a better pesticide candidate than **15**. A further detailed evaluation of the cytotoxicity of **1** was then carried out using the 60-cell line panel at the National Cancer Institute. The cytotoxicity spectrum, with 50% growth inhibition concentrations that ranged from 0.023 to 100 μM, and an average concentration of approximately 2 μM, did not

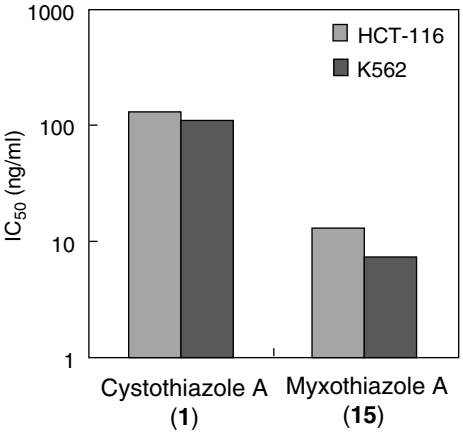


FIGURE 14.8 Cytotoxicity of cystothiazole A (**1**) and myxothiazole A (**15**) against two human tumor cell lines.

show any significant similarity to those of known anticancer drugs. However, further pharmaceutical studies were halted because of *in vivo* toxicity when evaluated with an NCI mouse model.

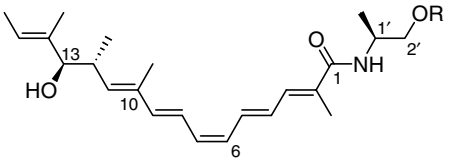
14.4 MYXALAMIDE DERIVATIVES

14.4.1 Isolation and Structures

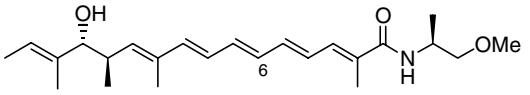
Myxalamides are polyene amide metabolites originally isolated from the myxobacterium *Myxococcus xanthus* Mx x12.[18,19] During the isolation of the cystothiazoles by a large-scale culture (150 L) of the myxobacterium *C. fuscus* (Section 14.3.2.1), weak anti-*Phytophthora* metabolites were isolated from a chromatographic fraction and identified as 2′-*O*-methylated derivatives of myxalamide D (**16**).[20] The fraction Fr. 4-6, which was obtained via acetone extraction of the bacterium, solvent partition, and chromatography twice on silica gel (Figure 14.3), was separated by reverse-phase HPLC to afford 2′-*O*-methylmyxalamide D (**17**, 1.9 mg) and two geometric isomers, (6*E*)-**17** (7.8 mg) and (6*E*,10*Z*)-**17** (1.9 mg), from 150 L of culture.

These metabolites are so susceptible to light, air, and trace acids that they gradually decomposed during spectroscopic analyses and completely disappeared after being stored for several months even at –30°C. The actual contents of these metabolites in the extract must be much higher (>10-fold) than the isolated amounts judging from their proportion in a mixture before the HPLC purification. In addition, the ratio of three isomers altered after preparative HPLC since **17** was the major isomer in the pre-HPLC isomeric mixture. This instability was also demonstrated in the HPLC analyses of crude extracts of *C. fuscus* cultured with or without the adsorber resin (Figure 14.4). The 2′-*O*-methylmyxalamide D compounds are major metabolites in the presence of resin, whereas they are trace products in the nonresin culture.

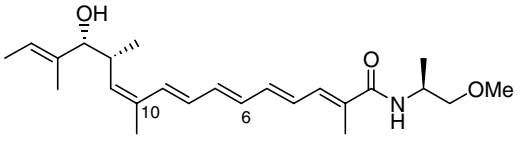
The structures, including stereochemistry, were determined by spectroscopic analyses and chemical derivatizations. The stereochemistry was the same as those of the previously isolated myxalamides.[18,19] Although the myxalamides have also been discovered from other sources[2] including *Stigmatella aurantiaca*,[21] the methyl ether-type derivatives such as **17** were the first examples of myxalamides. It was recently demonstrated that the genome of *C. fuscus* contains a unique gene encoding a methyltransferase as well as the gene cluster responsible for biosynthesis of myxalamide D (**16**), which is converted by the methyltransferase into **17**.[22]



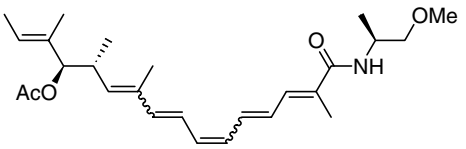
R = H: myxalamide D (**16**)
R = Me: 2′-*O*-methylmyxalamide D (**17**)



(6*E*)-**17**



(6*E*,10*Z*)-**17**



18 (mixture of acetates of **17**)

TABLE 14.2
Minimum Doses of 2′-*O*-Methylmyxalamide D (17) and Derivatives for Anti-*Phytophthora* Activity

Compound	17	(6*E*)-17	(6*E*,10*Z*)-17	18
Dose (μg per disk)	2	2	2	5

14.4.2 Biological Activity

The anti-*Phytophthora* activities of 2′-*O*-methylmyxalamide D (**17**), (6*E*)-**17**, (6*E*,10*Z*)-**17**, and their acetates (**18**, mixture) were evaluated using the paper disk assay method (Table 14.2). The minimum doses to form a significant inhibition zone were determined to be 2 μg per disk for all three isomers. Their acetate mixture (**18**) still showed activity at 5 μg per disk. Since all the three isomers showed the same anti-*Phytophthora* activity, the olefin geometry plays no crucial role for their antifungal activity. The activity of the acetate mixture (**18**) suggests that the free 13-OH group is not essential. In addition, the free 2′-OH group of the known myxalamides might not be very important, although a direct comparison of the activities was not performed between the myxalamides and the new 2′-*O*-methyl ether derivatives. Based on a structural similarity to the myxalamides, the mode of action of these methyl ether derivatives could be blockage of the mitochondrial respiratory chain by the inhibition of NADH oxidation at complex I.[18,23]

14.5 HALIANGICINS

The myxobacteria had been regarded as terrestrial microorganisms until recently, when some myxobacterial strains were successfully isolated from marine environments.[24] Since then, several

more strains have been occasionally isolated, though they are still rare.[11,25–27] From such a marine myxobacterial species, *Haliangium ochraceum* AJ-13395, the novel β-methoxyacrylate-type polyene antifungal compound named haliangicin (**19**) was isolated, and its bioactivities and planar structure were reported.[11,28] This marine myxobacterium was tentatively named *H. luteum* in previous papers,[11,28] and the new species name *H. ochraceum* was then given after a detailed taxonomic description.[25] An NMR-based reexamination of the structure revealed that the substance was a mixture of stereoisomers. The author's research group tried to purify them and determine the exact structures by a large-scale culture of the myxobacterium *H. ochraceum*.[29]

14.5.1 Production and Isolation

This slowly growing myxobacterium, *H. ochraceum* AJ-13395, was cultured for 17 days at 27°C. The optimum salt content for growth in the culture medium is 2–3% NaCl. The addition of the adsorber resin SP207 produced a threefold increase of haliangicin (**19**).[11]

The culture broths (total 9 L) were centrifuged to collect the bacterial cells and the resin, which were then extracted with acetone (Figure 14.9).[29] The extract was chromatographed twice on silica gel. The haliangicin-containing fraction was purified by preparative HPLC to obtain haliangicin (**19**),

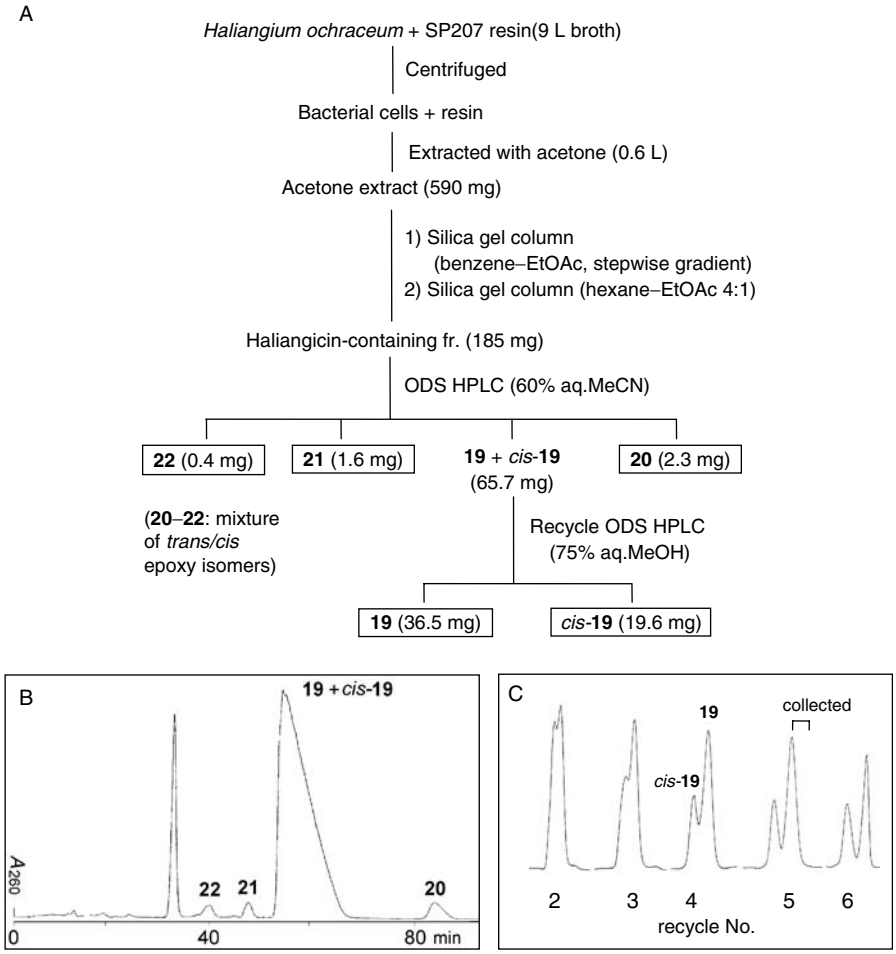
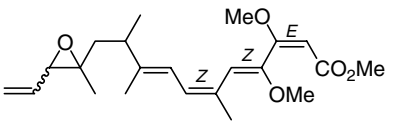
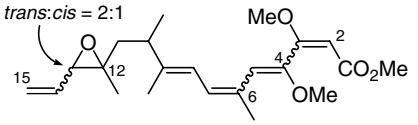


FIGURE 14.9 Isolation of haliangicins (**19**–**22**). (A) Isolation scheme, (B) first ODS HPLC, and (C) recycle HPLC.

which turned out to be a 2:1 mixture of isomers based on NMR analysis (Figure 14.9B). Three geometrical isomers, haliangicin B (**20**), haliangicin C (**21**), and haliangicin D (**22**), were also obtained. After NMR analysis, like haliangicin, all the mixtures were found to consist of *trans*- and *cis*-epoxy isomers in the ratio of 2:1, respectively. The major haliangicin fraction was further subjected to recycle HPLC to obtain pure haliangicin (**19**) and *cis*-haliangicin (*cis*-**19**) (Figure 14.9C). Owing to the high lability, the haliangicins gradually decomposed even when stored in benzene at –20°C, and an effort to purify **20** by recycle HPLC was not successful. The stereochemical analyses were performed mainly by NOESY experiments. For example, haliangicin (**19**) showed the NOESY correlations of H11/H13 and H14/12-Me, suggesting the *trans* stereochemistry of the epoxy moiety.



trans-epoxide : haliangicin (**19**)
cis-epoxide : *cis*-haliangicin (*cis*-**19**)
(**19**:*cis*-**19** = 2:1)



2*Z*,4*Z*,6*Z* : haliangicin B (**20**)
2*E*,4*E*,6*Z* : haliangicin C (**21**)
2*E*,4*E*,6*E* : haliangicin D (**22**)
(*trans*/*cis* mixtures)

Thus, a combination of the large-scale fermentation and the recycle HPLC separation made it possible to reveal the accurate structural information and diversity of the haliangicins.

14.5.2 Biological Activity

The anti-*Phytophthora* activity of the haliangicins was evaluated by using the paper disk assay method described in Section 14.2.[29] The major metabolites, haliangicin (**19**) and *cis*-haliangicin (*cis*-**19**), were most active, indicating minimum doses of 8 ng per disk for growth inhibition. This activity is the highest of the myxobacterial antifungals that we have discovered. The minimum doses of haliangicins B and C (**20** and **21**) were 40 ng per disk while that for haliangicin D (**22**) was 200 ng per disk. The results suggest that the geometry of the conjugated tetraene moieties is important for the activity but the epoxide geometry is not.

An antimicrobial spectrum was evaluated for a mixture of haliangicin (**19**) and *cis*-**19** (Figure 14.10).[11] The haliangicins showed inhibitory activity against fungi comparable to amphotericin B and were inactive against bacteria. Interestingly, oomycete microorganisms, which are insensitive to amphotericin B, are potently inhibited by the haliangicins.

The use of the oomycete *P. capsici* in our screening program seems to be reasonable for the selective detection of β-methoxyacrylate-type antimicrobials.

Since the haliangicins are a class of the β-methoxyacrylates, the mechanism of action was likely to involve the inhibition of mitochondrial respiratory chain like the cystothiazoles (Section 14.3). This was demonstrated by the fact that haliangicins (a mixture of **19** and *cis*-**19**) inhibited beef heart mitochondrial NADH oxidase activity at an IC_{50} of 2.5 nM.[11] In addition, the inhibition site in the electron transport system was determined as cytochrome *b-c1* complex (complex III) by different spectra of reduced and oxidized mitochondrial membranes. Haliangicins were also reported to show cytotoxicity at an IC_{50} of 0.21 μM against the P388 cell line.[11]

14.6 SUMMARY

In the search for novel myxobacterial antimicrobials that inhibit a phytopathogenic oomycete of the genus *Phytophthora*, three classes of metabolites were discovered and found to possess a similar mechanism of action related to the mitochondrial respiratory chain blockage (Table 14.3). Two classes of them, the cystothiazoles and the haliangicins, are new members of the β-methoxyacrylates,

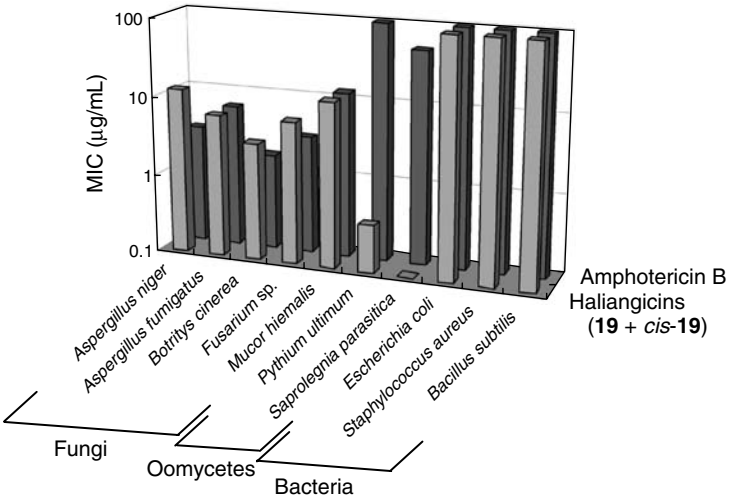


FIGURE 14.10 Antimicrobial spectrum of haliangicins. MICs > 100 μg/mL are indicated as 100 μg/mL.

TABLE 14.3
Three Classes of Antimicrobials from Two Myxobacteria

Sources	Compound Class	No. of Members	Anti-*Phytophthora*[a] Activity	Target	Ref.
C. fuscus	Cystothiazoles	10	0.04	Electron transport (complex III)	10, 13–15
	2′-*O*-methyl-myxalamides D	3	2	Electron transport (complex I)	20
H. ochraceum	Haliangicins	8	0.008	Electron transport (complex III)	11, 28, 29

[a] Minimum doses (μg per disk) of the major member to inhibit the oomycete *P. capsici*.

some of which are used as pesticides in the marketplace. Another class, 2′-*O*-methylmyxalamides, targets different machinery in the same electron transport system, though the anti-*Phytophthora* activity is lower than that of other two classes. Recently, new additional β-methoxyacrylates have been isolated from a slightly halophilic myxobacterium by this assay system.[30] Therefore, the hit rate of electron transport inhibitors from the myxobacteria through this simple method has ended up to be 100% in our studies.

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15 Naturally Occurring Glycosidase Inhibitors

Robert J. Nash

CONTENTS

15.1 INTRODUCTION

15.1.1 The Definition of Naturally Occurring Glycosidase Inhibitors

This chapter is concerned specifically with structurally simple alkaloids that have two or more hydroxyl groups and which function as sugar analogs, enabling them to cause glycosidase inhibition or allowing them to interact with sugar receptors. These alkaloids can be extremely specific and potent inhibitors of glycosidases but can also induce biological responses through interactions with specific sugar receptors.[1,2] Given a number of names such as polyhydroxylated alkaloids, azasugars, imino sugars, or sugar analogs, in their most simple form the ring oxygen of the sugar is replaced by a nitrogen (Figure 15.1).

There are five major classes of glycosidase inhibitors (Figure 15.2) produced by plants and microorganisms, that is, pyrrolidines (resembling furanose sugars), piperidines (resembling pyranose sugars), and the bicyclic pyrrolizidine, indolizidine, and *nor*-tropane (and tropane) alkaloids. The bicyclic alkaloids seem to function as analogs of monosaccharides with the second ring often appearing to bestow chemical and biological stability. Most of the natural glycosidase inhibitors have been reported from plants but this probably reflects the interests of the few main research groups working on such compounds and they may be more abundant in microorganisms than currently known. A large number of the natural alkaloids are shown in the review by Watson et al.[1]

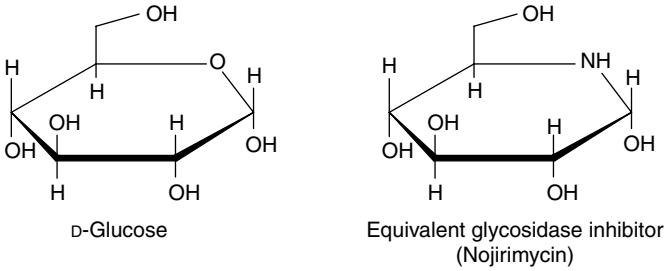

FIGURE 15.1 Structural comparison of D-glucose with its nitrogen analog, nojirimycin.

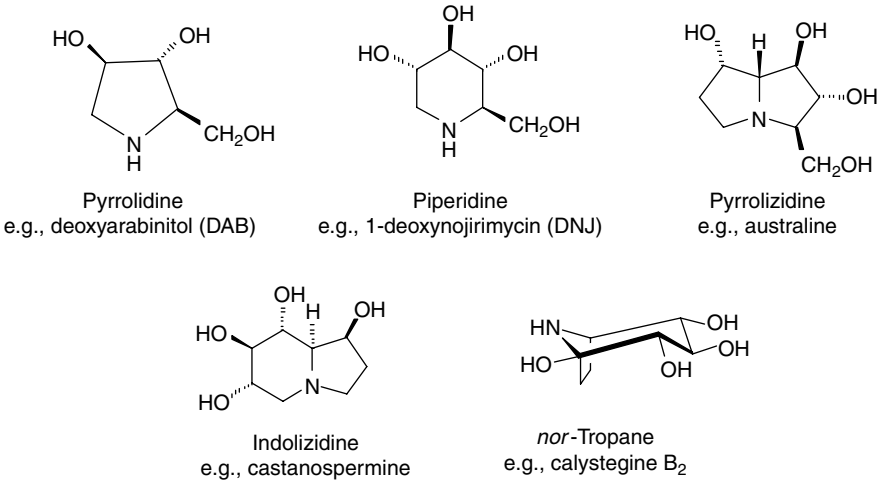

FIGURE 15.2 The five major classes of glycosidase inhibitors.

The alkaloids often occur in plants as glycosides and the presence of the sugars frequently changes the glycosidase inhibition activity and specificity of the alkaloid.

There are other natural products that can inhibit glycosidases, such as many aromatic compounds, especially phenolics, but these are usually not specific in binding to glycosidases and will often bind or precipitate a range of proteins. Acarbose is a well-known pseudo-tetrasaccharide (comprising the amino-sugar acarviosine and one maltose unit), which inhibits digestive α-glucosidase. Where there may be some specificity amongst other classes of compounds, often they have not been put through a wide range of assays and many are not competitive inhibitors, for example, the flavone baicalein and several compounds from marine organisms including macrolides and polyacetylenes.[3]

15.1.2 The Roles of Naturally Occurring Glycosidase Inhibitors

One of the prerequisites for utilization of sugars by mammals, insects and microorganisms is that complex carbohydrates and glycoproteins are broken down by a range of enzymes including glycosidases. Plants usually contain high concentrations of sugars and, therefore, are a good source of nutrition. The ability of potent glycosidase inhibitors in plants to prevent herbivores from utilizing the sugars efficiently would seem to be their main role. The protective role of glycosidase inhibitors is supported by the fact that a number of aposematic insects sequester these compounds, for example, *Urania* and *Acherontia* moths and Colorado beetles.[4,5] Microorganisms that produce these compounds, such as strains of *Bacillus, Streptomyces*, and *Metarhizium*, probably derive benefit from inhibiting glycosidases released by competing microorganisms.[5] Organisms that produce the inhibitors presumably have

enzymes that are more able to distinguish between the sugar analog and the correct substrate and, therefore, are inhibited less. The presence of such specific enzymes has been shown with insect species that specialize in feeding on plants with high concentrations of glycosidase inhibitors.[6]

Plants contain many glycosides and it may well be that the glycosidase inhibitors also serve to prevent the removal of sugars from glycosides. For example, in our laboratory we have found that *Lotus corniculatus* (Fabaceae) contains not only cyanogenic glucosides but also glucosidase inhibitors.[7] The inhibitors may prevent the release of cyanide in the plant prior to attack by a herbivore.

The alkaloidal glycosidase inhibitors appear, in general, to be readily taken up into the blood of mammals and distributed around the body where they can then adversely affect the health of the herbivore and their ability to feed for prolonged periods on those plants, for example, *Swainsona* and *Astragalus* species, *Ipomoea carnea*, and *Hyacinthoides non-scripta*.[1,8,9] The main toxic effects once absorbed into blood appear to be via inhibition of glycosidases involved in lysosomal degradation of glycoproteins and glycolipids causing intense vacuolation in cells of the central nervous system (CNS) in particular.[10] However, some have also been shown to affect utilization of glycogen, for example, deoxyarabinitol (DAB) and castanospermine.[11,12] Plants that contain alkaloidal glycosidase inhibitors usually have a complex mixture of related structures, which gives the plants a very broad range of glycosidase inhibition that may reduce the development of resistance in herbivores.

15.1.3 Distribution of Glycosidase Inhibitors

The alkaloidal glycosidase inhibitors have been primarily reported from plants with a few from microorganisms and insects.[1] The glycosidase inhibitors in insects appear to have been sequestered from their food plants.[6,7] Microorganisms are probably major producers of this group of alkaloids and indeed the first one reported, nojirimycin, was found in a broth of *Streptomyces* in 1966.[13] Since then, a small number of bacteria (principally Actinomycetes) and a fungus (*Metarhizium anisopliae*) have been shown to produce alkaloidal glycosidase inhibitors. However, the water-soluble components, including any alkaloidal glycosides, of microbial broths are not often analyzed in great detail due to problems with components in the media obscuring low molecular weight–water-soluble metabolites.[1,5]

Within the Plant Kingdom the alkaloidal glycosidase inhibitors more commonly accumulate in higher plants but also occur in lower plants, though usually at low concentrations, for example, in the fern *Arachniodes standishii*.[14] Certain glycosidase inhibitors are very common, such as DAB, deoxynojirimycin (DNJ), and dihydroxymethyldihydroxypyrrolidine (DMDP). Some or all of these latter three compounds will usually be present as part of a complex mixture of related compounds wherever glycosidase inhibitors are found. They often occur in related taxa, for example, the *nor*-tropane calystegine alkaloids seem almost ubiquitous in the Solanaceae and the Brassicaceae,[15] but in the genus *Nicotiana* they are of very sporadic occurrence. Perhaps most remarkable is that the calystegines are quite major metabolites of foods such as potatoes and aubergines. However they were not reported in these foods until 1993, despite routine analysis of these foods in so many laboratories for decades before then, and claims that there were unknown, possibly toxic metabolites, especially in potatoes.[6,16,17] The calystegines are also of interest because they seem to occur sporadically in other plant families such as the Moraceae that were not previously known to contain tropane alkaloids. This latter observation may indicate an endophytic source rather than the plants *per se*.

It may well be that in some cases, such as is suspected (personal observation) with the calystegines, the alkaloidal glycosidase inhibitors detected in plants are the products of endophytes or the uptake of microbial products from the soil. The cycads are a primitive group of plants not known for alkaloid production and yet *Lepidozamia perofskyana* was found in our laboratory to contain the indolizidine alkaloid swainsonine. Whether the swainsonine is a *Lepidozamia* product or produced by the nitrogen-fixing, blue-green bacteria in its roots is not known. Many plant taxa, not generally thought to be alkaloid producing, do contain alkaloidal glycosidase inhibitors, for example, the distribution of polyhydroxylated pyrrolizidine alkaloids is much greater in Myrtaceae[18] and Hyacinthaceae[19] than are the classic nonpolar pyrrolizidine alkaloids. It is probable that the biosynthetic routes for most of

the polyhydroxylated alkaloids are different from the more classic, fairly nonpolar alkaloids and they are probably derived from hydroxylated prolines and pipecolic acids.[20]

15.1.4 The Therapeutic Value of Alkaloidal Glycosidase Inhibitors

It is known that water-soluble components such as carbohydrates and glycosides are important biomolecules, which are sparingly soluble in dry methanol and less polar solvents. The glycosidase inhibitors (alkaloidal and nonalkaloidal) that affect utilization of carbohydrates and alter carbohydrate biology within organisms are widely distributed but frequently overlooked.

Many have an ability to inhibit glycosidase enzyme activity both *in vivo* and *in vitro*.[1] Glycosidase enzymes are essential to a wide range of cell functions including digestion, glycan synthesis, and degradation. Glycosidase enzymes are essential for the correct formation of glycoproteins and glycolipids.[1] Transformation of normal cells to cancer cells is associated with a change in glycosidase activity and changes in glycans on the cell surface.[21] Many cancer cells have increased *N*-linked glycosylation and this seems to be correlated with a poor prognosis for patients.[21] Cancer cells have been shown to upregulate certain glycosidases, such as hexosaminidases and β-mannosidase, which may help to degrade the extracellular matrix during metastasis.[22] Compounds that are able to specifically inhibit the enzymes that are upregulated during cancer cell transformation may be able to prevent aberrant glycan formation and reduce tumor proliferation.

Despite an assumption by many in the pharmaceutical industry that very water-soluble compounds have poor pharmacokinetic characteristics, for example, the Lipinski Rules,[23] the carbohydrate analogs are often orally available, probably due to sugar transport systems, and can show great specificity of biological activity.[1] Such selectivity is due to the chemical and biological diversity of the structures of small sugars. For instance, there are 2^9 stereoisomers of sucrose alone and even glucose has 2^6 possible isomers in the pyranose and furanose forms. This amazing diversity in such small molecules completely surpasses, in molecular weight terms, anything achieved by the amino acids. Carbohydrates help determine the three dimensional structure of proteins, which is inherently linked to their function and efficacy. Chemicals that are able to affect carbohydrate biochemistry can affect protein glycosylation patterns and so influence cell-to-cell signaling and cell-type specificity. Such modifiers of glycosylation can increase viral and cancer cell recognition by the immune system and some also appear to be able to directly modulate the immune system. The modulation of the immune response appears to be independent of glycosidase inhibition and seems to involve the interaction of the alkaloidal carbohydrate analogs with cell surface C-type lectin receptors at concentrations below that likely to cause significant glycosidase inhibition.[2,24]

The therapeutic selectivity of the glycosidase inhibitors seems to be in part due to the fact that healthy cells have efficient processes to quality control glycosylation whereas infected or cancerous cells have altered quality control, allowing more aberrant glycans to become expressed at the cell surface.[25] In this respect, the more specific the glycosidase inhibition the best chance there is of reducing side effects. Almost all of the commercial investigation of glycosidase inhibitors has been on the first compounds of this type discovered, namely, DNJ, castanospermine, swainsonine, and derivatives of them. These early compounds have aroused considerable interest in the potential of these compounds as therapeutic agents for a wide range of disorders but much more specific natural compounds and their derivatives are now known. Table 15.1 summarizes the current knowledge of the therapeutic applications of the glycosidase inhibitors.

15.1.5 Importance of Alkaloidal Glycosidase Inhibitors in Herbal Medicines and Foods

It is amazing that there is still very limited knowledge of the active principles of most herbal medicines. Even those widely used such as Echinacea have many contradictory claims regarding the active constituents. One of the major remaining mysteries in the knowledge of herbal medicines (and "healthy" foods) is in the characterization of their water-soluble secondary components, as

TABLE 15.1
Mechanism of Action and Therapeutic Applications of Glycosidase Inhibitors

Target/Mechanism	Therapeutic Application
Modification of carbohydrate processing enzymes	Treatment of metabolic disorders, e.g., Bayer's Miglitol for diabetes
Chaperones to produce functional glycoproteins	Treatment of enzyme deficiencies, e.g., Tay Sachs disease, Fabry disease
Glycosylation trimming inhibition	Antiviral and anticancer activity, e.g., Migenix' Celgosivir for Hepatitis C
Binding to immune cell surface receptor	Immunotherapy, e.g., Summit's SMT 14400 as an anticancer and anti-infective agent
	Immune modulation, e.g., Summit's SMT 15000 as a vaccine adjuvant

these are the most difficult to analyze. Traditionally, most medicinal herbs were prepared in hot water. However, these days very few commercial herbal products are prepared or analyzed in water and consequently determination of the active principles might be elusive because the right components of the plant have not been extracted for use or analysis. It is also the case that plant-based drug discovery programs have typically relied on intermediate or nonpolar solvents such as methanol, chloroform, or hexane for extraction, due to ease of processing. There are herbal products that are standardized for classic alkaloids such as Belladonna but the glycosidase inhibitors, calystegines in the case of Belladonna, can be dominant in concentration and are not considered. A more thorough knowledge of the water-soluble components (including glycosidase inhibitors) of herbal products such as Echinacea, which gives conflicting clinical results, will allow clinical trials to be conducted on well-characterized extracts and lead to good therapeutic products, which can attract premium prices.

15.2 EXTRACTION AND PROCESSING OF ALKALOIDAL GLYCOSIDASE INHIBITORS

The classical method used to isolate alkaloids from plants involves macerating the tissue with dilute aqueous acid, filtering off the solids, making the filtrate alkaline and reextracting the unionized alkaloid from the filtrate into an immiscible organic solvent such as ether or chloroform. The efficiency of this procedure depends upon the distribution coefficients of the ionized and unionized forms of alkaloid with respect to the aqueous and nonaqueous phases. The presence of hydrophilic groups in an alkaloid molecule will increase the relative solubility of the unionized form in the aqueous layer and molecules containing several such groups are likely to be highly insoluble in ether or chloroform, even at high pH values. This chapter is concerned with polyhydroxylated alkaloids that often function as glycosidase inhibitors but which have been overlooked in the past on account of their hydrophilic nature and the inability of conventional high performance liquid chromatography (HPLC) systems to separate or detect them. Most also fail to be detected by the reagents commonly used for alkaloids such as Dragendorff's and iodoplatinate reagents and they give weak reactions with ninhydrin, which are easily masked by co-occurring amino acids.

The most commonly used solvent for extraction of glycosidase inhibitors is 25–50% aqueous ethanol, which gives complete extraction of the glycosidase inhibitors without also extracting most proteins and very nonpolar components. Water will also extract all of the known glycosidase inhibitors and proteins can then be precipitated. Dry alcohols are selective as to which glycosidase inhibitors they will extract but do not offer great advantages as many sugars and amino acids are still extracted along with the alkaloids.

While it is often possible to detect the glycosidase inhibitors using HPLC-MS/MS or GC-MS in the crude aqueous alcohol extracts, their presence is largely obscured by sugars and amino acids,

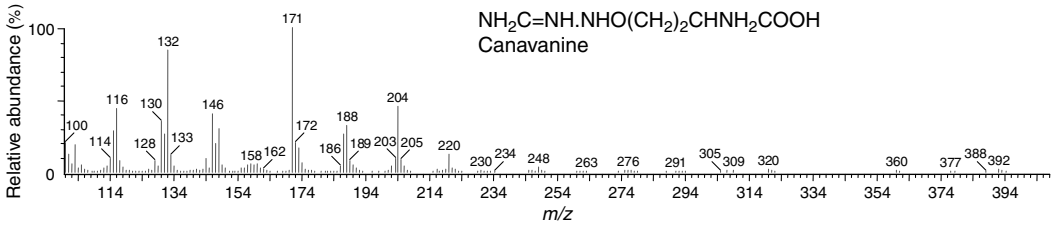

FIGURE 15.3 Electron impact ionization mass spectrum of trimethylsilylated canavanine.

which will almost always be present at very high concentrations. Ion exchange resins are extremely useful in concentrating alkaloidal glycosidase inhibitors from extracts for analysis. Strongly acidic cation exchange resins such as the widely available IR-120 and Dowex 50 are very efficient at retaining the full range of known alkaloidal glycosidase inhibitors, including their glycosides, when used in columns. A large bead size, for example, 25–50 mesh, is ideal for a crude extract to avoid the column blocking. Unbound material such as sugars and phenolics can be removed using copious amounts of water. The bound alkaloidal glycosidase inhibitors and amino acids can be removed using 2 M ammonium hydroxide and the ammonia removed by rotary evaporation or by centrifugal evaporations systems.

The known alkaloidal glycosidase inhibitors are remarkably stable in extreme acidic or alkaline conditions but the prolonged exposure to the resins or strong ammonia is likely to result in loss of some glycosides. Alternatively, though less effectively, the resin may be simply added to the extract and then washed with water or placed in dialysis tubing. The main problem with the latter methods is that the resin or the tubing often becomes coated with precipitated proteins and phenolics and so efficiency is greatly reduced. The strongly acidic cation exchange resins require a wash in 2 M HCl prior to use even if they are supplied in the H^+ form; they are then washed to neutrality with water before the samples are loaded.

Alkaloidal glycosidase inhibitors from plants can be purified for full structural analysis by combinations of cation and anion exchange chromatographic columns. It has been shown that very closely related glycosidase inhibitors can be fully separated by ion exchange chromatography alone. In some cases, glycosidase inhibition has been used to follow fractionation but typically this is difficult unless the inhibitors are extremely potent or the extracts have been concentrated by the cation exchange methods described above. The two most frequently used ion exchange resins for isolation of alkaloidal glycosidase inhibitors are the weakly acidic cation exchange resin Amberlite CG50 used in the NH_4^+ form and the strongly basic anion exchange resin Amberlite CG400 used in the OH^- or acetate form. The alkaloidal glycosidase inhibitors can be fractionated on columns of both resins by elution with water alone, followed by ammonia, on CG50 and acetic acid on CG400. The anion exchange resin is useful in removing most neutral and acidic amino acids, which can be major components of plant and microbial aqueous preparations. Analysis of the amino acid components, however, can also yield interesting and unusual nonprotein amino acids such as canavanine (Figure 15.3 shows the trimethylsilyl [TMS] derivative of canavanine), which is present in many medicinal plants including *Astragalus membranaceus* and *Sutherlandia frutescens* and may explain the therapeutic claims for these plants.

15.3 ANALYSIS

15.3.1 Gas Chromatography-Mass Spectroscopy

GC-MS has proved extremely robust in determining the polyhydroxylated alkaloidal glycosidase inhibitors as their TMS derivatives in complex extracts even before extract clean-up using cation exchange chromatography. It can result in excellent resolution of closely related structures.

Preferably, weak trimethylsilylation reagents are used to avoid partial derivatization of the nitrogen of the monocyclic alkaloids. All samples need to be dried before derivatization. TMS derivatives can be prepared using a mixture of hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in pyridine, for example, the Pierce "Tri-Sil" silylation reagent comprised of HMDS/TMCS/pyridine in a ratio of 2:1:10. Samples react quickly at room temperature but can be heated at 60°C for 15 min and then left at room temperature. Insoluble reaction products can be sedimented by centrifugation and the supernatant transferred to fresh vials. Molyneux et al.,[26] have also used *N*-methyl-*N*-(TMS)-fluoroacetamide (MSTFA), which can derivatize the secondary amino groups of the monocyclic glycosidase inhibitors although this was not found to be very consistent.

A suitable GC method uses, for example, a high polarity fused-silica column (e.g., Varian "Factor Four" VF-5 ms column, 25 m × 0.25 mm i.d., 0.25 μm phase thickness) with a carrier gas (helium) flow rate of 1 mL/min. TMS derivatives can be separated using a temperature program starting at 160°C for 5 min, followed by a linear increase to 300°C at a rate of 10°C/min. The temperature is held at 300°C for an additional 10 min with a total analysis time of 29 min.

Electron impact mass spectrometry of the column eluant has been carried out using a Perkin Elmer "TurboMass Gold" mass spectrometer, with a quadrupole ion filter system, which was run at 250°C constantly during analysis. The detector mass range was set to 100–650 amu. The temperature of the transfer line (GC to MS) was held at 250°C. Samples (1 μL) were injected onto the column via a split vent (split ratio 50:1) through a fused silica narrow bore injection liner packed with deactivated quartz wool and the injection port temperature was maintained at 200°C.

Typically, monocyclic alkaloids are eluted in the first 10 min, bicyclic compounds with four or more hydroxyls elute in the range of 9–12 min and glycosides are seen between 14 and 18 min. Glycosides usually fragment with the major ions representing the sugar and the molecular ion of the free glycosidase inhibitor. The protein amino acids have very short retention times while more interesting nonprotein amino acids such as canavanine can often be distinguished by longer retention times or "unusual" spectra.

As previously mentioned, the most common glycosidase inhibitors of plants are DAB, DNJ, and DMDP. The mass spectra of DAB, DNJ, and swainsonine (an important toxin and still with potential as an anticancer drug) as TMS derivatives are shown in Figure 15.4.

If any alkaloidal glycosidase inhibitors are present in plant or microbial samples then most often they will be present as complex mixtures of related structures, for example, the complex mixture of alkaloidal glycosidase inhibitors present in the English bluebell (*H. non-scripta*, Hyacinthaceae) as shown by GC-MS analysis of TMS-derivatives (Figures 15.5 and 15.6). The bluebell is interesting in that it is very consistent, with respect to the glycosidase inhibitors that are present, their concentrations and their ratios, from diverse locations and from all parts of the plant. This plant is well known to be toxic to livestock in the UK and causes lethargy, coldness, and can cause death in cattle and horses.[27] Sheep appear to avoid eating it. Of native mammals, only the badger is said to eat them and will dig up bulbs. The traditional protection for livestock is to fence areas of woodland containing bluebells.

15.3.2 High Performance Liquid Chromatography

There are major hurdles to overcome in detecting alkaloidal glycosidase inhibitors in extracts by conventional HPLC systems. These include their high water solubility, co-occurrence in aqueous extracts with dominant sugars and amino acids and lack of a suitable chromophore. We have carried out extensive comparisons of HPLC columns in attempts to find HPLC systems suitable for preparative chromatography of underivatized alkaloidal glycosidase inhibitors and have only found systems designed for carbohydrates to work well. Chen et al.,[28] successfully used a combination of cation exchange fractionation and thermospray LC-MS to determine castanospermine and related compounds in extracts of *Castanospermum australe*. They conducted preparative LC using a Phenomenex 1B-SIL NH_2 column and a step gradient of MeCN–H_2O (90:10) as mobile phase A and

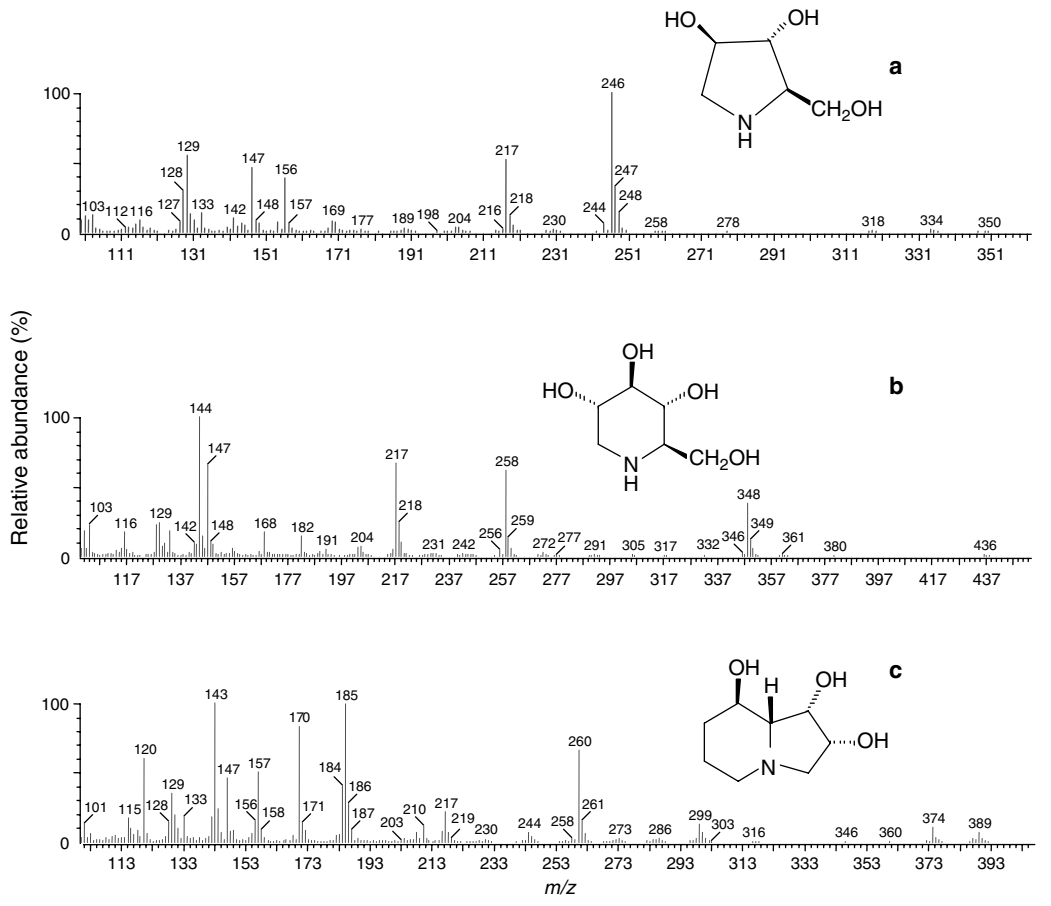

FIGURE 15.4 Trimethylsilylated mass spectra of some common glycosidase inhibitors: **(a)** DAB, **(b)** DNJ, and **(c)** swainsonine.

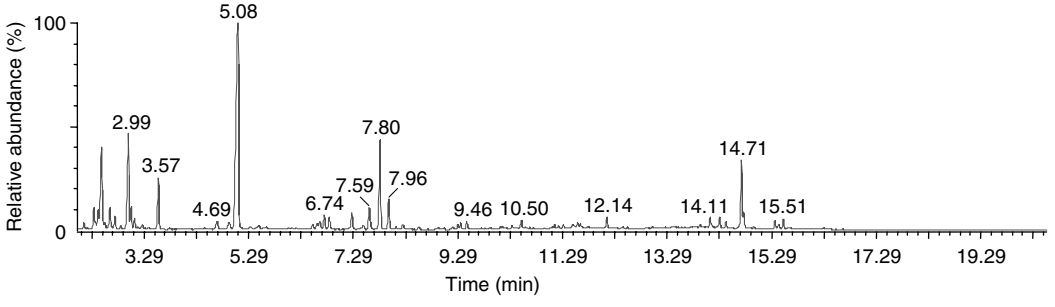

FIGURE 15.5 GC-MS total ion chromatogram of Bluebell (*H. non-scripta*) extract showing the complex glycosidase inhibitor profile. Here run as the trimethylsilylated derivatives.

MeCN−H_2O (50:50) as mobile phase B. Thermospray LC-MS was carried out using further purified fractions on a Whatman Partisil 10 SCX column (and an amino column of IBM Carbo) using MeCN−H_2O (80:20) as the mobile phase. While this method works well for purified samples it seems more labor intensive and difficult than GC-MS methods. Our experience has also been that some HPLC columns reported in the literature to work with alkaloidal glycosidase inhibitors such

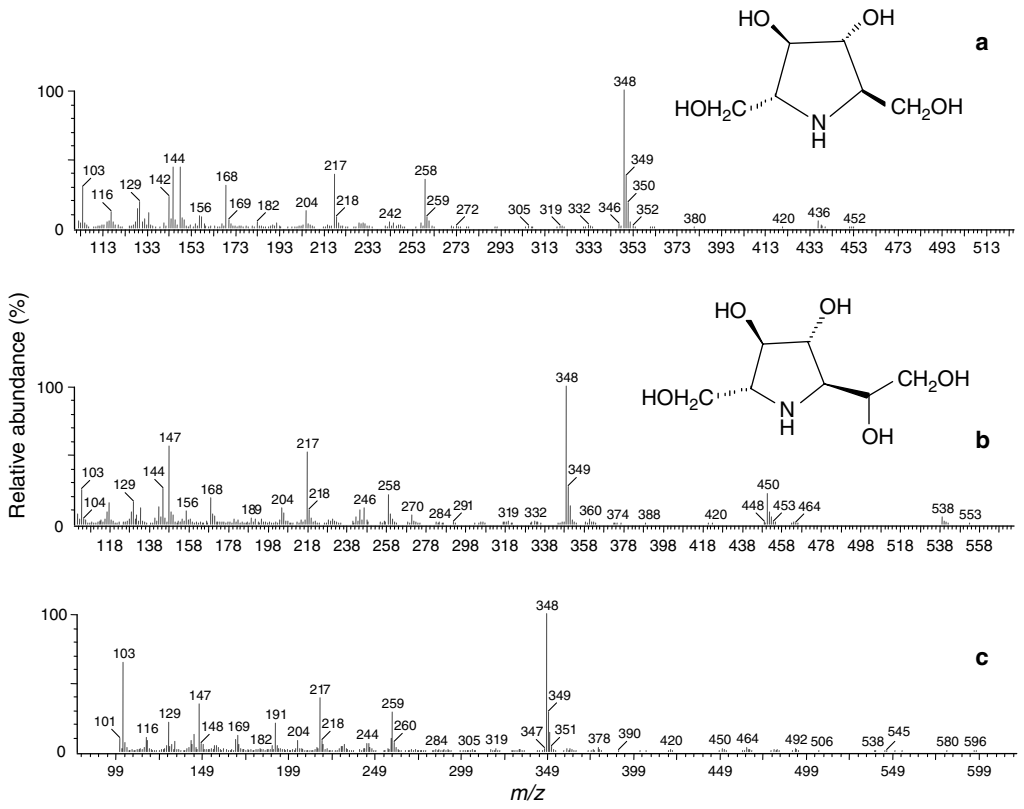

FIGURE 15.6 Mass spectra derived from the GC-MS ion chromatogram in Figure 15.5. **(a)** TMS-DMDP, **(b)** TMS-α-homo-DMDP, and **(c)** the TMS-homo-DMDP-xyloside showing ions characteristic of TMS-homo-DMDP **(b)**.

as the high affinity anion exchange (HAAX) columns gave very poor reproducibility and high losses. We have determined, however, that evaporative light scattering detectors do give good sensitivity on conventional HPLC systems.

By far the best results in terms of resolution, sensitivity, and reproducibility have been obtained by our laboratory using the Dionex-type carbohydrate HPLC systems that use electrochemical detection. Such a system was used by Donaldson et al.,[29] in 1990 for detection of castanospermine, swainsonine, DNJ, and DMJ. We experimented with their method and found that it gave excellent results for a wide range of glycosidase inhibitors (Figure 15.7) using a Dionex cation exchange column (Ion-Pac CS-3, a moderate capacity, high efficiency sulfonated cation exchange column) with elution by dilute HCl. It was useful in combination with GC-MS analysis in that sugars and glycosides were resolved but eluted before the free alkaloids. Larger glycosides not detected by GC-MS are also detected.

For preparative purposes, we use an Ion-Pac CS-10 column (22 mm × 250 mm) and a mobile phase of 40 mM methanesulfonic acid with the column maintained at a temperature of 35°C. The flow rate is 20 mL/min and the typical loading capacity of a partially purified alkaloid mixture is 10–20 mg. The electrochemical detector requires a basic solution and so, after splitting 1:100 with most of the eluant going to a fraction collector, 300 mM NaOH is added to the eluant directed to the detector using a pneumatic controller (approx 1.5 mL/min at 60 psi). The specificity of the Dionex as an analytical system is good in that common protein amino acids that are detected by GC-MS as TMS derivatives are not detected.

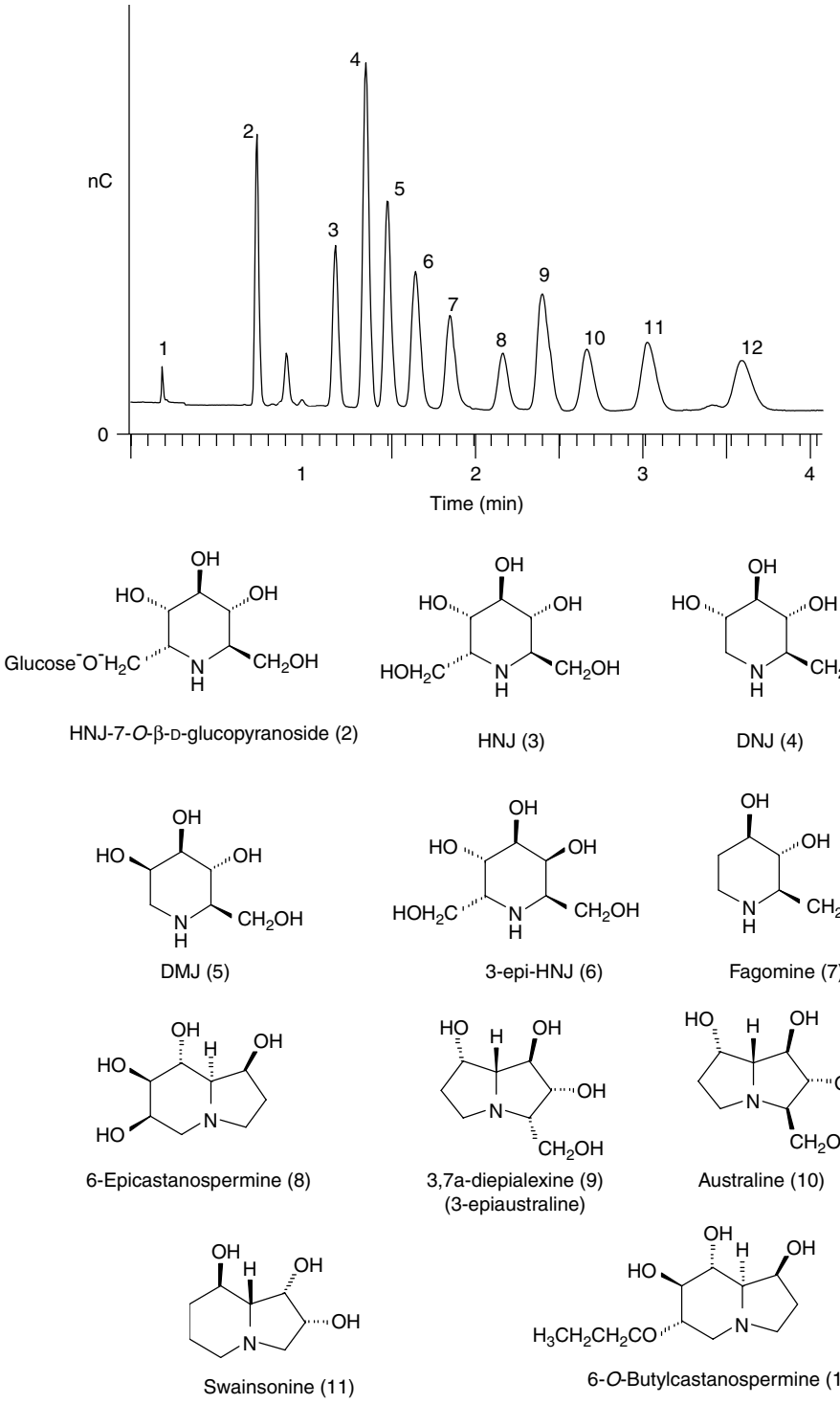

FIGURE 15.7 Example of Dionex HPLC chromatogram of alkaloid glycosidase inhibitors using CS3 CS3 Ionpac and ED40 PED (time in minutes). 1, sugars; 2, α-homonojirimycin-7-*O*-β-D-glucopyranoside; 3, α-homonojirimycin (HNJ); 4, 1-deoxynojirimycin (DNJ); 5, 1-deoxymannojirimycin (DMJ); 6, 3-epi-α-homonojirimycin; 7, fagomine; 8, 6-epicastanospermine; 9, 3,7a-diepialexine; 10, australine; 11, swainsonine; and 12, 6-*O*-butylcastanospermine (Celgosivir).

15.3.3 Thin Layer Chromatography

The alkaloids will run on cellulose and silica gel $60F_{254}$ plates in solvents mixtures such as butan-1-ol/acetic acid/water (12:3:5) and propan-2-ol/acetic acid/water (4:1:1). In addition to the use of iodine vapor, there are a number of other suitable reagents for visualizing the alkaloids.

o-Tolidine reagent will permit visualization of many of the primary and secondary amine alkaloids as blue-black spots. The developed plates are sprayed with a 2% solution of sodium hypochloride and dried using a hairdryer. They are then sprayed with *o*-tolidine reagent (320 mg *o*-tolidine, 30-mL glacial acetic acid made up to 500 mL with water and mixed 2:1 with 0.4% potassium iodide in water) and heated using a hairdryer.

Naphthoresorcinol reagent will also work with many of the alkaloids when the plate is sprayed with a solution of 2 mg of 1,3-dihydroxynaphthalene in 500 mL H_2O and heated using a hairdryer. The reagent takes approximately 2 min to develop color (red/brown/yellow). However, none of these reagents works with all the known alkaloidal glycosidase inhibitors. Automated multiple development thin layer chromatography (AMD-TLC) has been used for the detection of the calystegines and their precursors on high performance thin layer chromatography (HPTLC) plates.[30]

15.3.4 High-Voltage Paper Electrophoresis

Screening using high-voltage paper electrophoresis is an extremely quick way of determining whether glycosidase inhibitors or other unusual nitrogen-containing metabolites such as nonprotein amino acids may be present in extracts.[31] The calystegines were first located by this method using silver nitrate as the visualizing reagent while DMDP and castanospermine were detected using ninhydrin (0.2% in 95% aqueous acetone). Buffers such as formic acid/acetic acid/water (30:60:910, pH 1.9) work very well since the glycosidase inhibitors move away from the neutral and acidic amino acids and can be seen, following ninhydrin treatment, as gray or yellow spots running near to arginine and lysine which are mauve. Whatman paper No. 1 or 3 are usually used and good separations can be achieved after 15–30 min at 3–6 kV.

15.3.5 Glycosidase Inhibition Assay

Detecting the presence of alkaloidal glycosidase inhibitors in plants by using glycosidase assays is possible if the compounds are potent inhibitors or are present at high concentrations.[32,33] However, plants in particular have components such as phenolics that can bind fairly nonspecifically to glycosidases making the detection of specific inhibitors impossible without a preliminary ion exchange step. This step also removes sugars which themselves can give confusing results. It is becoming apparent that some plant components other than alkaloids can have reasonably specific glycosidase inhibition but typically they are not very potent. Therapeutically, the less specific inhibitors of glycosidases are not very interesting since they will tend to bind to many proteins on ingestion, including the gut wall and other components in the diet.

It is important, therefore, early in the process of screening extracts for alkaloidal glycosidase inhibitors to prove that the inhibition caused by binding has some specificity for glycosidases. To prove the specificity, a range of cheap commercially available glycosidases may be chosen, for example, two α-glucosidases, a β-glucosidase, and an α-mannosidase. If all glycosidases are strongly inhibited it may indicate nonspecific binding to proteins. Two α-glucosidases are suggested because often yeast glucosidase is used but this gives different results from most other glucosidases and, for example, is not greatly inhibited by castanospermine despite this alkaloid being extremely potent against almost all other α-glucosidases. Other glycosidase activities of interest can be added to these. Fairly crude homogenates of insects or tissues from mammals can also be used as the enzyme source.

There are a number of simple glycosidase assays using synthetic substrates, for example, methylumbelliferyl and *p*-nitrophenyl, that release a fluorescent or colored product and are good for a first screen.[34] It is essential in such assays to keep the color intensity of the plant or microbial sample

low to avoid obscuring the spectrophotometric determination of the released colored product, for example, *p*-nitrophenol measured at 405 nm. Enzyme activity is measured spectrophotometrically and compared against suitable controls. One of the complications in determining precise glycosidase specificity of alkaloidal glycosidase inhibitors in mixtures is that very minor alkaloids may be extremely potent inhibitors with inhibition constants (K_i) of 10^{-8} or 10^{-9}.

15.3.6 NMR

Determination of the structures of the glycosidase inhibitors by the use of chemical shift parameters has proved in a number of cases to be unreliable.[35] However, the use of $^1H-^1H$ three bond coupling constants ($^3J_{HH}$) allows easy, direct comparison between samples and extraction of the relative configurations for the ring protons even for the pyrrolizidine alkaloids, which have proven the most difficult. Controlling the pH of the NMR solvent has been found to be important for obtaining good NMR data. NMR analysis of the pyrrolizidine alexines (australines) and casuarines has shown that the change in configuration of single positions in the rings can have profound effects on the conformations of apparently very similar structures and probably helps to explain the dramatic changes in biological activities of these alkaloids.

Mass spectrometric and NMR data can be misleading since often enantiomers can be present. In some cases, the only way to prove which compound is inhibiting the enzyme in question is by synthesis.

15.4 PERSONAL INSIGHT

Occasionally, a novel chemical class is discovered, which leads to a new area of intense pharmaceutical research and exploitation. In the early 1980s, this seemed to be the case with the alkaloidal glycosidase inhibitors. Although two glycosidase inhibitors, Bayer's Miglitol® for diabetes and UCB/Actelion's Zavesca® for Gaucher disease, have reached the market, realization of the full potential of this class of compounds has been frustrated by side-effect problems and challenges in compound isolation. Almost all of the commercial developments have been made on the very first glycosidase inhibitors identified, that is, DNJ, castanospermine, and swainsonine, all of which suffered in one way or another from a lack of specificity in glycosidase inhibition leading to side effects. Recently, however, the earlier limitations of scarcity and undesirable side effects have been resolved, in particular, by new developments in isolation and synthesis techniques that have led to the discovery of novel imino sugar templates with enhanced glycosidase specificity and reduced toxicity. It is also now clear that glycosidase inhibitors are extremely widespread in plants and microorganisms and many are ingested daily in foods with little or no knowledge of their significance to health or nutrition. Perhaps most exciting is the discovery that carbohydrate analogs such as the casuarines can retain biological activity as immune modulators without necessarily being glycosidase inhibitors.[2] The alkaloidal glycosidase inhibitors now appear well positioned to rekindle pharmaceutical interest in a number of therapeutic areas.

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16 Bioassay-Directed Isolation and Identification of Antiaflatoxigenic Constituents of Walnuts

Russell J. Molyneux, Noreen Mahoney, Jong H. Kim, and Bruce C. Campbell

CONTENTS

16.1 INTRODUCTION

Aflatoxins are polyketide metabolites most commonly produced by certain strains of the fungi *Aspergillus flavus* and *A. parasiticus*, which can infect a number of major agricultural crops, including corn, peanuts, cotton, and tree nuts. The most common of these metabolites are aflatoxins B_1 (**1**) and B_2 (**2**), and G_1 (**3**) and G_2 (**4**), with aflatoxins B_2 and G_2 being the 8,9-dihydro derivatives of (**1**) and (**3**), respectively (Figure 16.1). The B and G groups differ in the presence of cyclopentanone and δ-lactone moieties, respectively. In general, *A. flavus* produces the B group aflatoxins

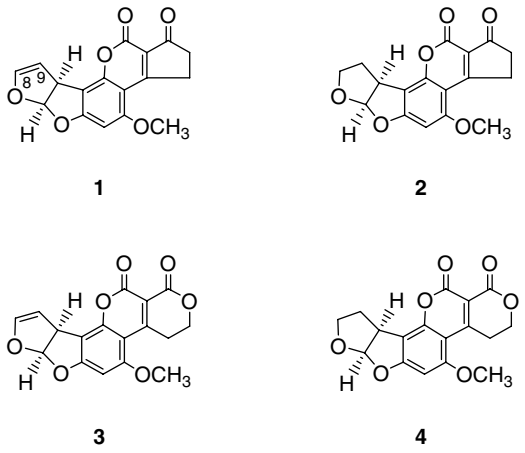

FIGURE 16.1 Structures of aflatoxins B_1 (**1**) and B_2 (**2**), and G_1 (**3**) and G_2 (**4**).

whereas *A. parasiticus* produces both B and G groups. Although not all strains of *A. flavus* and *A. parasiticus* are capable of producing aflatoxins (i.e., are "aflatoxigenic") the contamination of human and animal food crops by these compounds is of great concern because they are classified as carcinogens, particularly in humans infected with hepatitis.[1,2] On occasion these compounds can be acutely toxic, as illustrated by a recent episode in rural Kenya where contaminated maize led to 317 cases of poisoning and 125 deaths.[3]

Tree nuts, especially almonds, pistachios, and walnuts, are unique because, unlike other crops, they are consumed as foods entirely by humans and cannot be diverted to animal feed or industrial uses if they are found to be contaminated. Consequently, they are extremely valuable agricultural crops worldwide and particularly in California, where their aggregate value exceeds $3.1 billion.[4] A large proportion of this value is from export markets, in which aflatoxin levels are highly regulated due to food safety and quality concerns. Thus, whereas the Food and Drug Administration maximum guidance level limit for tree nuts intended for human consumption within the United States is 20 ng/g (i.e., 20 ppb),[5] in the European Community an extremely low tolerance level of 2 ng/g for aflatoxin B_1 and 4 ng/g total aflatoxins is applied.[6] This presents a serious constraint for producers and exporters because of the need to ensure that shipments do not exceed regulatory limits and the threat of rejection or even destruction if they do so. It is therefore essential to find acceptable methods for limiting or eliminating production of aflatoxins by the fungus in order to protect both consumers and producers.

Treatment with fungicides both pre and postharvest can reduce fungal infection. Postharvest processing to destroy aflatoxins can be applied although the expense would probably make it impractical. However, neither of these methods is likely to be acceptable to consumers or regulators because they either involve the application of toxic chemicals or would change the delicate, organoleptic properties of the product. It is therefore essential to develop natural control methods that will enhance resistance to *Aspergillus* colonization and growth, or suppress aflatoxin biosynthesis. Of these two approaches, the latter is likely to be the most acceptable since reduction in aflatoxigenic fungi may merely result in their ecological niche being occupied by other toxic or deleterious microorganisms.

One indicator that aflatoxin biosynthesis is susceptible to control is that a number of strains of *Aspergillus* exist that do not produce the mycotoxins, suggesting that aflatoxigenesis is not absolutely essential for functioning or survival. In fact, displacement of toxigenic strains by an atoxigenic strain has been applied in Arizona to prevent contamination of cotton seed with aflatoxins,[7] although it is unlikely that a similar approach would be acceptable in food crops.

Another indicator is that plants differ between both species and cultivars in their ability to become contaminated, suggesting that phytochemical factors are present that can influence the extent of aflatoxin biosynthesis. Discovery of such factors would enable them to be enhanced through selection of cultivars in which their presence is highest or through directed crop breeding programs. Identification of antiaflatoxigenic phytochemicals therefore presents an ideal situation for bioassay-directed isolation and identification.

16.2 BIOASSAY FOR ANTIAFLATOXIGENIC ACTIVITY

Bioassay-directed fractionation of phytochemical inhibitors of aflatoxin biogenesis depends upon a simple, reproducible method for measurement of aflatoxins produced when exposed to known amounts of plant tissue or extracts. This approach requires that tested materials be incorporated into growth media on which a suitable fungal strain is cultured. Biosynthesis of aflatoxin B_1 is a complex process, starting with cyclization of a polyketide chain to norsolorinic acid (**5**) and proceeding through a series of anthraquinonoid intermediates, culminating in versicolorin A (**6**), which possesses the difuranoid moiety characteristic of the aflatoxins. Versicolorin A then undergoes rearrangement of the ring system to sterigmatocystin (**7**) and ultimately to aflatoxin B_1 (**1**) (Figure 16.2).

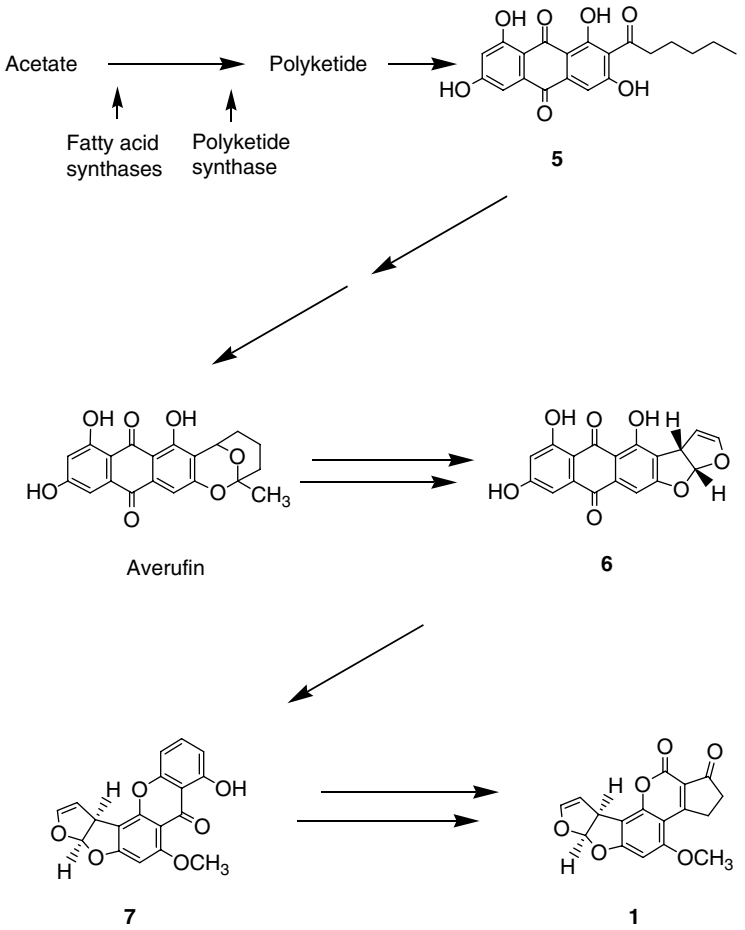

FIGURE 16.2 General outline of the biosynthetic pathway to aflatoxin B_1 (**1**). The initial aromatic product, norsolorinic acid (**5**), formed by cyclization of the polyketide chain, is progressively transformed through the anthraquinones averufin and versicolorin (**6**) to the xanthone sterigmatocystin (**7**) by a series of fatty acid synthases, oxidoreductases, and methyl transferases to the aflatoxins.

The genetics, molecular biology and enzymology of this process have been thoroughly investigated and the details are substantially defined.[8,9] Inevitably, the intricacy of aflatoxin biosynthesis requires that a considerable time has to elapse between inoculation of the fungus onto the media and maximum aflatoxin production. This period, amounting to several days, cannot be avoided but, on the other hand, numerous methods have been developed for analysis of aflatoxins and these can be rapidly performed with great accuracy.

16.2.1 Fungal Cultures and Inoculation of Media

Many strains of aflatoxin-producing *Aspergillus* exist, isolated from numerous sources, and these are available from culture collections such as the American Type Culture Collection (ATCC)[10] or the Dutch Centraalbureau voor Schimmelcultures (CBS).[11] In studies involving investigation of aflatoxin formation on tree nuts, the strain NRRL 25347, which was originally collected from a pistachio orchard, is one of the most suitable. In experiments conducted on tissues from other aflatoxin-susceptible crops, such as corn, cotton or peanuts, it would be more appropriate to use isolates from those crops. The reasoning behind such choices is that exposure of the fungus to nutrients from plant tissues to which they are not adapted could cause gross distortions in the aflatoxin profile, drastically enhancing or decreasing the amounts normally produced. For example, *A. flavus* grown on nut-based media is exposed to high levels of lipids whereas artificial media such as potato dextrose agar (PDA) are sugar-based. This results in aflatoxin levels being much lower when the fungus is grown on PDA than when grown on pistachio medium.

16.2.1.1 Preparation of Fungal Cultures

A. flavus NRRL 25347, grown on PDA for 7 days, has been used for preparation of spore suspensions. Analysis is simplified by using this strain because it produces almost entirely aflatoxin B_1 (**1**) with only trace levels of aflatoxin B_2 (**2**) and no aflatoxin G_1 (**3**) or G_2 (**4**) (Figure 16.1). Spores were collected on a swab, transferred to 0.05% Tween 80 and the spore concentration determined using a Neubauer counting chamber. A single point inoculation (5 µL, equivalent to ca. 200 spores) was inoculated onto the appropriate medium (10 mL) on a 60 mm Petri plate. For time-course experiments, triplicate sets of plates were incubated under the same conditions and analyzed daily from day 0 (control) to day 11. The effect on fungal growth can be evaluated by visual assessment of the extent of mycelium radial expansion at each time point. For antiaflatoxigenic activity, plates were prepared in triplicate and incubated at 30°C in the dark and analyzed after 7 days, the approximate growth period for maximum aflatoxin production determined from the time-course experiments.[12,13]

16.2.1.2 Preparation of Media

Media for testing of comparative antiaflatoxigenic activity of almond, pistachio, or walnut cultivars, respectively, consisted of 5% of the appropriate ground whole nut kernels, including seed coat, dispersed in 1.5% plain agar. When seed coat alone is used, plain agar does not contain sufficient nutrients to support fungal growth, so these samples were mixed with the artificial media, PDA, or Vogel's medium N (VMN).[14,15]

Although PDA is readily available from suppliers, VMN is a more satisfactory material since it is a defined medium whereas PDA may vary somewhat between batches. For testing antiaflatoxigenic activity over a range of concentrations, ground seed coat or ground kernel without seed coat were added at 10, 25, 50, 100, 200, and 400 mg per 10 mL of media. Control plates consisted of medium with no nut material incorporated. To test the combined effect of individual parts of nuts, ground seed coat was added to a media of 5% ground kernel without seed coat in 1.5% agar at 10, 20, 30, 40, 50, and 60 mg per 10 mL of media. Media were autoclaved and Petri plates (10 mL of media per 60 mm diameter) were prepared in replicates of four for the comparison of tree nut cultivars, and in triplicate for all others.

To test various solvent extracts of plant material, the residues remaining after evaporation to dryness were incorporated into VMN medium at 0.25% w/v. Individual isolated compounds were added at various levels (w/v) and autoclaved with the media. Control samples contained none of the added extracts or compounds.

16.2.2 Analysis for Aflatoxins

Numerous procedures have been developed for analysis of aflatoxins, in particular high-performance liquid chromatography (HPLC), combined with various detection techniques, and immunoassay methods.[16] The primary focus of aflatoxin analysis method development has been to analyze for them as contaminants in crop plants or food samples where levels are generally quite low. Commercial immunoassay kits are available and convenient for such use. Plant samples represent extremely complex matrices and extensive cleanup may be required prior to analysis but for experimental purposes, where the composition of the fungal growth media is relatively simple, HPLC analysis with fluorescence detection is ideally suited. After treatment to produce the fluorescent derivative, the actual analysis can be automated and provides accurate, sensitive values for replicated samples.

16.2.2.1 Extraction of Fungal Cultures and Growth Media

The total contents of the Petri plate, including the fungal mat, spores, and media, were extracted with methanol (50 mL) and each replicate analyzed individually. Methanol was removed from an aliquot (1 mL) of the extract by evaporation with a stream of nitrogen at 40°C. The residue was derivatized by treatment for 10 min with hexane (200 µL) and trifluoroacetic acid (200 µL) at room temperature. Each treated sample was evaporated to dryness with nitrogen at 40°C and redissolved in water–acetonitrile (9:1, 1 mL) for HPLC analysis.

16.2.2.2 HPLC Analysis for Aflatoxin B_1

Aliquots (20 µL) of the analytical samples prepared from the extracts were analyzed for aflatoxin by reverse-phase HPLC and fluorescence detection, with excitation at 365 nm and detection of emission at 455 nm.[17] The HPLC system consisted of a 250 mm × 4.6 mm i.d. Vydac C18 reverse-phase column, eluted isocratically with water/methanol/acetonitrile/acetic acid (60:35:5:0.5, v/v/v/v) at a flow rate of 1.0 mL/min. As a result of the hexane/trifluoroacetic acid treatment, aflatoxin B_1 is detected as the hemiacetal derivative, aflatoxin B_{2a} (2-hydroxy-aflatoxin B_2),[18] eluting at a retention time of 4.9 min. The lower detection limit is 0.02 µg per Petri dish or 10 mL media. Using *A. flavus* NRRL 25347, aflatoxin B_2 was detected at levels that were insignificant relative to aflatoxin B_1 (ca. 0.1%) and therefore did not need to be quantitated. Each data point is calculated as the average of three replicates. Other strains could have significantly higher levels of aflatoxin B_2 in which case the amounts should be determined. Similar considerations apply to aflatoxins G_1 and G_2 produced by strains of *A. parasiticus*.

16.3 IDENTIFICATION OF AFLATOXIN RESISTANCE FACTORS IN TREE NUTS

To identify specific constituents in tree nuts that confer resistance to aflatoxin biosynthesis, that is, antiaflatoxigenic factors, it is necessary to establish the species, varieties, and specific tissues that have the greatest effect on aflatoxigenesis, since such materials are likely to have the highest concentrations of bioactive compounds. Isolation and identification of individual components should thereby be simplified and sufficient amounts obtained for biological testing. It is important to note that plants have two broad classes of antifungal phytochemicals, namely phytoalexins and phytoanticipins.[19,20] Phytoalexins are inducible metabolites that are only produced in significant amounts in response to fungal attack, whereas phytoanticipins are constitutive plant metabolites, already present in the plant in their bioactive form or as precursors from which they can be easily generated.

Phytoalexins suffer from the disadvantage that the locations where they are generally produced are in the immediate vicinity of fungal attack and amounts vary with the extent of infection, by which time sufficient aflatoxin may have been produced to cause contamination. On the other hand, phytoanticipins are a genetic character of any particular plant species or variety, and are therefore capable of being manipulated for optimum amounts and possibly even structure-activity features by conventional breeding or genetic engineering. The general approach to identifying antiaflatoxigenic compounds in tree nuts is therefore to determine the most resistant nut species, then the varieties within that species and finally the location of the factors within the individual nuts.

16.3.1 Comparison of Tree Nut Species and Varieties

In studies focused on major commercial tree nut crops in California, three species are of concern: almonds (*Prunus dulcis*), pistachios (*Pistacia vera*), and English walnuts (*Juglans regia*). Almonds and walnuts consist of at least 23 and 26 cultivars, respectively. In addition, there are numerous breeding lines available from agricultural experiment stations. However, pistachio production in California, in contrast to other producer countries, relies upon a single cultivar, 'Kerman'.

Measurement of aflatoxin production on commercial cultivars of these three tree nut species, together with black walnut (*Juglans nigra*), showed a definitive trend in ability to support aflatoxigenesis with almonds, on average, being 22 times more susceptible than English walnuts. Pistachios and black walnuts were intermediate in value, being 10 times and 11 times more

TABLE 16.1
Aflatoxin B_1 Production *in vitro* on Ground Kernels of Tree Nut Species and Cultivars Inoculated with *Aspergillus flavus*

Species/Cultivar	Aflatoxin B_1 (μg/g nut)
Almond (*Prunus dulcis*)	
(Range; 23 cultivars/11 breeding lines)	40–384
Mission	82
Monterey	118
Butte	170
Padre	170
Nonpareil	172
Carmel	176
Fritz	200
Sonora	246
Pistachio (*Pistacia vera*)	
Kerman	80
Walnut, Black (*Juglans hindsii*)	
Rawlins	90
Walnut, English (*Juglans regia*)	
(Range; 26 cultivars)	n.d.– 56
Tulare	n.d.
Vina	1.0
Eureka	3.2
Payne	7.0
Chandler	11.2
Serr	16.4
Hartley	38.0
Chico	56.0

Note: n.d.: <0.04 μg/g nut; all values are averages of quadruplicates for each cultivar.

susceptible, respectively. Table 16.1 shows the average values for all cultivars and values for some of the more common commercial varieties. It is obvious from these results that 'Tulare' walnut, which completely suppressed aflatoxin formation, would be the best variety to investigate, since it is likely to have the highest level of antiaflatoxigenic constituents.

16.3.2 Tissue Localization of Resistance Factors

The edible portion of tree nuts consists of two distinct types of tissues: the kernel itself or endosperm, which comprises the majority of the nut by weight, and the seed coat or pellicle, a papery tissue enclosing the kernel. To determine whether resistance factors are located in either or both of these tissues, samples from 'Tulare' nuts were manually separated and aflatoxin production measured *in vitro* when endosperm or pellicle was incorporated into PDA medium. Figure 16.3 shows that the endosperm itself caused no inhibition of aflatoxin formation and in fact produced a dramatic increase in levels when incorporated above 100 mg, probably due to increased availability of nutrients. The effect of the pellicle was completely different, exhibiting almost complete suppression (3% of control) of aflatoxin at incorporation levels in PDA of 1% and above, while as little as 0.1% incorporation reduced the aflatoxin to ca. 36% of control.

When pellicle was added back to a medium consisting of 5% endosperm in agar, the ability of kernel to support or enhance aflatoxin production was overridden. Aflatoxin production was inversely proportional to the amount (10–60 mg) of added pellicle. At 30 mg of incorporated pellicle, approximating the proportion of seed coat to endosperm in whole nuts, the aflatoxin was barely above the detection limit (0.02 μg) at 0.8% of control. These results establish that the antiaflatoxigenic activity is therefore restricted entirely to the pellicle and that inclusion of kernel material would only complicate isolation of the bioactive constituent(s) by adding extraneous material to the overall extract.

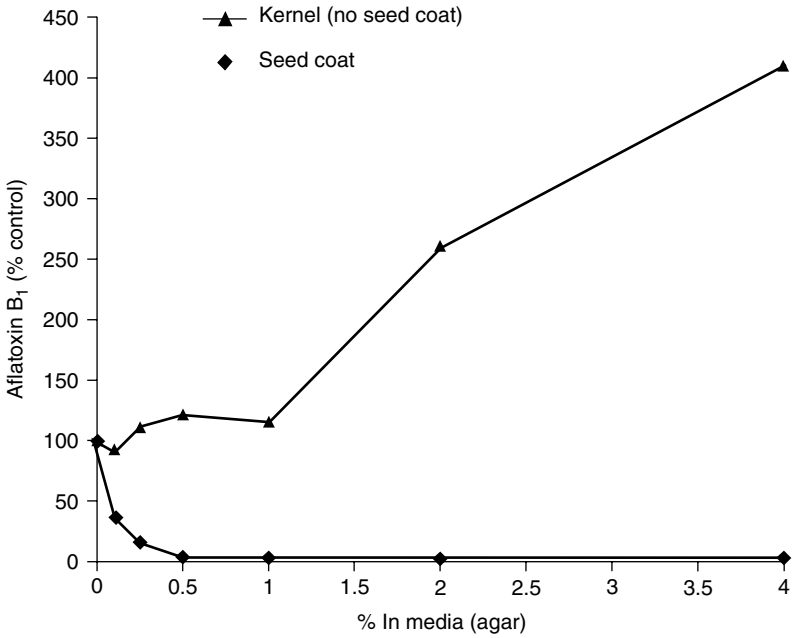

FIGURE 16.3 Inhibitory activity of the walnut variety 'Tulare' seed coat on aflatoxigenesis. Kernel with seed coat removed exhibited no inhibitory activity.

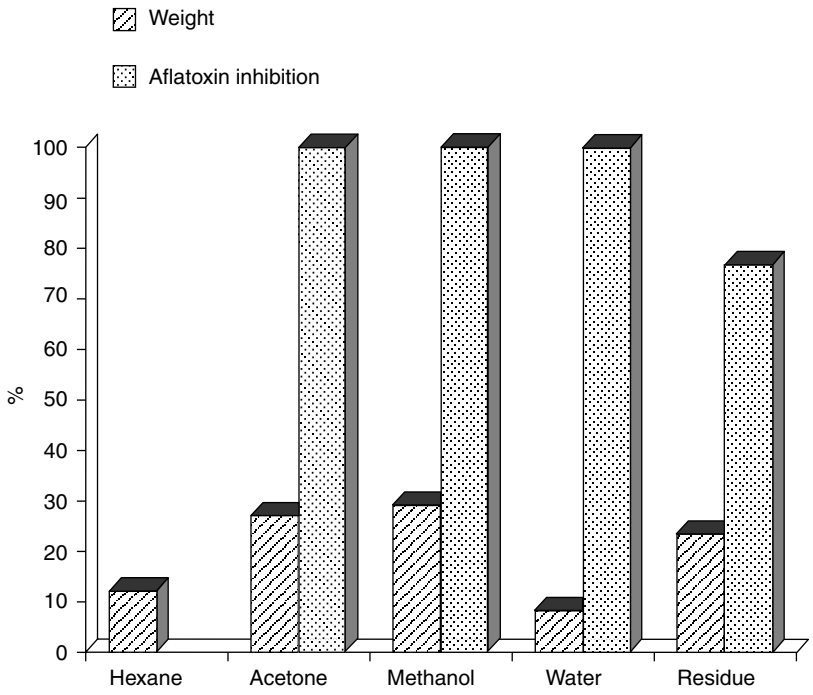

FIGURE 16.4 Aflatoxin inhibitory activity and weights of extractable constituents of 'Tulare' seed coat, expressed as percentages of the original, for a series of extracts produced by sequential extraction with solvents of increasing polarity.

16.4 ISOLATION AND STRUCTURE DETERMINATION

16.4.1 Sequential Extraction and Bioassay of Fractions

The extraction and fractionation of 'Tulare' pellicle followed conventional techniques for isolation of bioactive natural products. Ground pellicle was sequentially extracted in a Soxhlet extractor with hexane, acetone, methanol, and water. After removal of solvent, the extracted material was tested *in vitro* for antiaflatoxigenic activity. The weight percent of extracted material and aflatoxin inhibition of extracts, and residue after extraction by the complete sequence of solvents, are shown in Figure 16.4. In contrast to the situation usually observed with such an approach, where most of the bioactivity is confined to one or two fractions, all of the fractions, with the exception of the hexane extract, showed significant antiaflatoxigenic activity.

When the residue after hexane extraction was tested, the activity was found to be retained. Analysis of the individual solvent extracts by GC/MS showed that hexane extracted a complex of lipid materials but the other solvents contained no low-molecular-weight compounds, with the exception of trace amounts of methyl gallate in the water extract. These results indicate that the constituents with activity against aflatoxin formation must be comprised of a series of polar, relatively high-molecular-weight substances.

This finding was consistent with early work by Jurd [21,22] on the composition of walnut pellicle, showing that large amounts of hydrolyzable tannins are present, one of which, juglanin, is isomeric with corilagin, the tannin occurring in pods of the legume *Caesalpinia coriaria*.[23,24] Corilagin (1-*O*-galloyl-3,6-(*R*)-hexahydroxydiphenoyl-β-D-glucopyranose) is one of the simplest hydrolyzable tannins, consisting of one glucose unit esterified with single hexahydroxydiphenic acid and gallic acid moieties. Despite the same gross structure, the differences in melting point and optical rotation values between juglanin and corilagin shows that juglanin must have a different positional

or stereochemical configuration. Recently, 16 new and known hydrolyzable tannins have been isolated from walnut pellicle.[25] In this case, and in the case of the earlier work,[22] the specific cultivar was not identified. It is emphasized that it is always important to identify not only the plant species being examined but also the variety or cultivar in the case of commercial crop plants. For example, the considerable differences in bioactivity exhibited by the walnut cultivars indicate that these correlate with the presence of specific constituents.

Taken together, the biological activity of the various solvent extracts, in combination with previous knowledge about walnut pellicle constituents, suggests that suppression of aflatoxin production has to be related to the hydrolyzable tannin constituents of the pellicle. The presence of activity in all fractions, and even in residue after complete sequential extraction, is consistent with a series of tannins of increasing complexity and polarity. Even the most simple hydrolyzable tannin would not be expected to be extractable with hexane, given the polyphenolic nature of this class of compound.

Hydrolyzable tannins are biosynthesized from pentagalloyl glucose (**8**), which then undergoes a series of hydrolytic and oxidative coupling reactions to give simple tannins such as strictinin (**9**) (Figure 16.5) and more complex structures in which phenolic groups have undergone esterification or ether formation with additional gallic acid moieties. This is exemplified by the variety of compounds isolated from walnuts by Fukuda et al.[25] At first sight, the extraordinary structural variety of hydrolyzable tannins would appear to make the question of which ones are most likely to be antiaflatoxigenic an intractable one. However, *Aspergillus* species are known to be capable of growing in tanning vats containing extremely high levels of hydrolyzable tannins[26] and at least one strain of *A. flavus* has been shown to excrete an extracellular tannase.[27] This enzyme has been shown to hydrolyze tannic acid, a commercial hydrolyzable tannin, to give gallic acid. The strain used in our experiments, *A. flavus* NRRL 25347, was screened for tannase activity by growing it on tannic acid/agar medium. This simple plate assay showed a visible zone of clearing, which has been shown to be consistent with tannase production.[28]

16.4.2 Identification of Antiaflatoxigenic Constituents

Tannase hydrolysis of walnut tannins would be expected to give three products, that is, glucose, gallic acid (**10**), and hexahydroxydiphenic acid (**11**). However, the latter cannot be isolated and spontaneously lactonizes to the dilactone, ellagic acid (**12**) (Figure 16.5). Since gallic and ellagic acids are the ultimate products of the action of the fungus on hydrolyzable tannins, either one or both of these compounds must be responsible for inhibition of aflatoxin formation. The availability of the pure acids either from commercial sources or by acid hydrolysis of crude tannin enables them to be tested as aflatoxigenesis inhibitors. When this was done over an 11-day time course, gallic acid reduced aflatoxin levels by 96%, relative to control on day 6, the time of maximum production, whereas the reduction by ellagic acid was only 16% (Figure 16.6).

The activity of hydrolyzable tannins in inhibiting aflatoxin formation therefore does not appear to be dependent on the structure of individual tannin components but rather upon the relative amounts of gallic acid present and, to a much lesser extent, ellagic acid. As biosynthesis of the tannins from pentagalloyl glucose proceeds, the proportion of ellagic acid would be expected to increase and antiaflatoxigenic efficacy should decline with maturity. However, those walnut samples with gallic acid content above a certain level, as yet undefined, would maintain inhibitory activity.

16.4.3 Analysis of Gallic and Ellagic Acid Content in Walnuts

16.4.3.1 HPLC Analysis of Gallic and Ellagic Acids

To correlate gallic and ellagic acid inhibition of aflatoxin with their respective contents in walnut pellicle at various growth stages or with specific varieties, it is necessary to use a rapid analytical method adaptable to large sample numbers. The ability of tannase to hydrolyze the tannins into these separate components suggests that an analogous chemical method should be applicable.

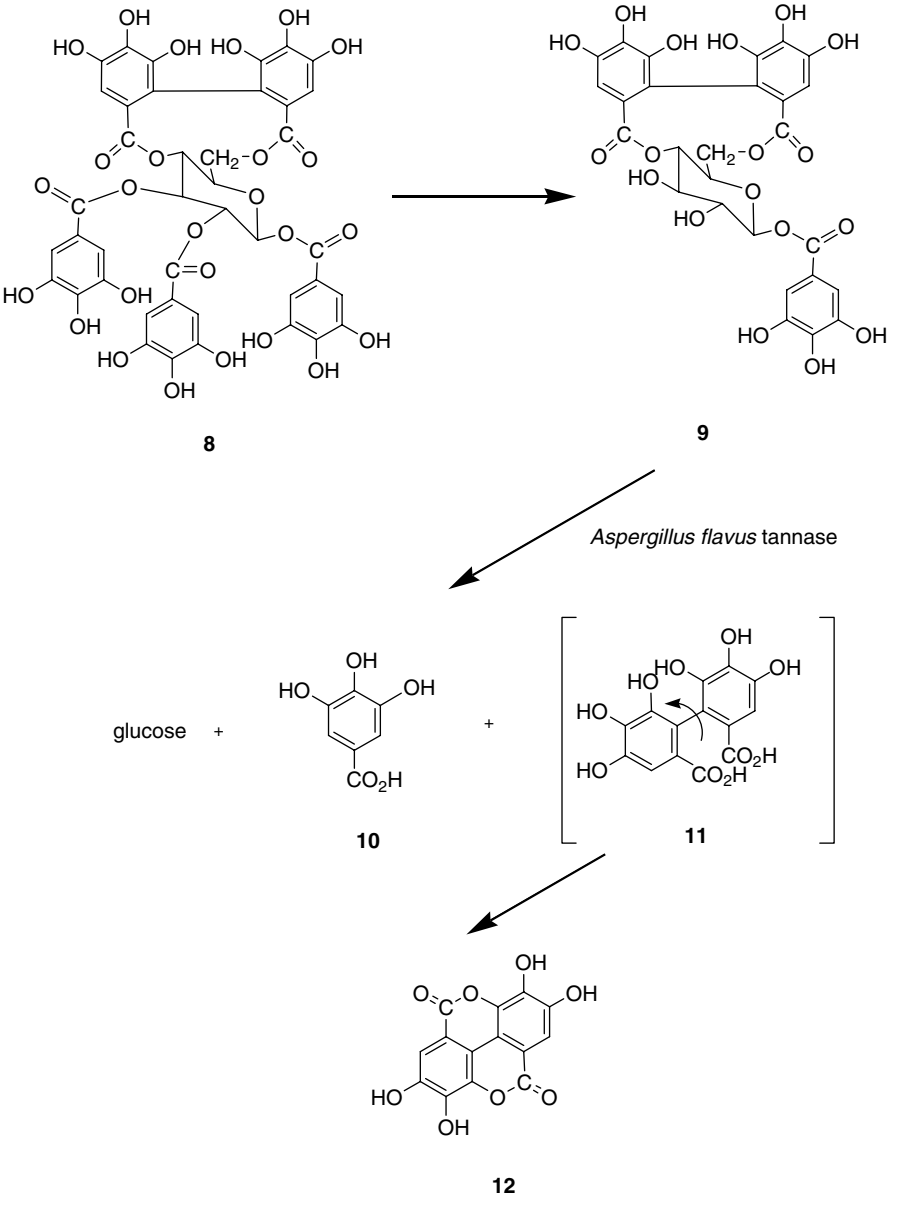

FIGURE 16.5 Formation of the simple representative hydrolyzable tannin, strictinin (**9**), from pentagalloyl glucose (**8**), the biosynthetic precursor of all hydrolyzable tannins. Tannins can be hydrolyzed either chemically or by *Aspergillus flavus* tannase to glucose, gallic acid (**10**), and hexahydroxydiphenic acid (**11**), which spontaneously lactonizes to ellagic acid (**11**).

It is generally easier to analyze acids as ester derivatives and consequently a methanolysis technique has been developed by Lei et al.[29] that can be used to analyze both acids simultaneously using HPLC in either food products or wood samples. Gallic acid moieties are esterified to methyl gallate while hexahydroxydiphenic acid units are lactonized directly to ellagic acid, with detection of each compound at 280 and 252 nm, respectively. Using this procedure, ground walnut pellicle samples (20 mg) were treated with methanolic HCl and stirred at 100°C for 1 h. Aliquots (200 μL) of the reaction mixture supernatants were evaporated to dryness under N_2 at 40°C, redissolved in

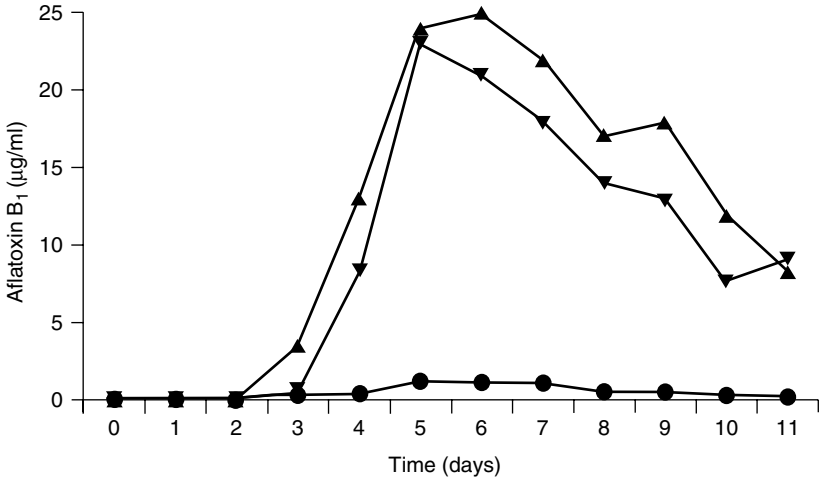

FIGURE 16.6 Time-course for aflatoxin production *in vitro* in the presence of gallic (●) or ellagic (▼) acids, and without added constituents (▲) (control).

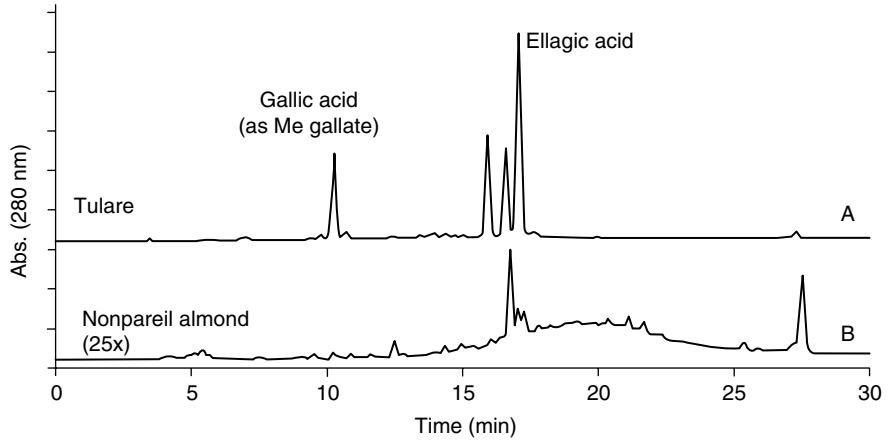

FIGURE 16.7 HPLC analysis of gallic and ellagic acids in methanolic HCl hydrolysate of (**A**) 'Tulare' walnut seed coat, or (**B**) 'Nonpareil' almond seed coat. The almond analysis is amplified 25 times relative to that of the walnut analysis.

methanol (1.0 mL), and filtered through 0.2 µm nylon syringe filters. Samples of the solution (10 µL) were analyzed for methyl gallate and ellagic acid using HPLC on a 250 mm × 4.6 mm i.d. C18 reverse-phase column, using a gradient from 100% water containing 0.2% TFA to 100% methanol over 25 min at a flow rate of 1.0 mL/min, with detection at 252 and 280 nm using a multiple wavelength or diode array detector. Linear calibration curves can be generated for methyl gallate and ellagic acid over concentration ranges of 0.1–4.0 and 0.1–1.0 mg/mL, respectively. Use of a diode array detector ensures that each compound is correctly identified by both retention time matching and comparison of their UV spectra. An example of the HPLC analysis of pellicle from 'Tulare' walnut, compared with that of 'Nonpareil' almond, is shown in Figure 16.7. The methyl gallate and ellagic acid peaks in the walnut sample are well-resolved, whereas the almond sample, which contains no hydrolyzable tannins, shows no peaks corresponding to either of these compounds, even at signal amplification 25 times that of the walnut sample.

16.4.3.2 Contents of Gallic and Ellagic Acid in Walnut Cultivars

The HPLC analysis provides a convenient method for measuring changes in gallic and ellagic acid levels with growth stage and for comparing levels in different cultivars. As illustrated for the 2003 growing season (Figure 16.8), starting at the earliest "jelly" stage before the kernel has started to become firm, gallic acid levels are already high, increasing approximately threefold over the first month of kernel development and then declining quite rapidly before settling at a fairly constant level over the remainder of the growing season. In contrast, ellagic acid levels show an increasing trend over the whole period of kernel development. This is consistent with what is known about hydrolyzable tannin biosynthesis, with the initially formed pentagalloyl glucose gradually losing gallic acid due to partial hydrolysis and dimerization to hexahydroxydiphenic acid moieties (Figure 16.5). It is particularly noteworthy that whereas the ellagic acid levels are quite similar in both 'Tulare' and 'Chico' varieties, the levels of gallic acid in 'Tulare' are consistently higher than in 'Chico' throughout the growing season. Furthermore, the 'Tulare' gallic acid levels are maintained at a much higher level, attaining 3.2% dry weight (dw) at maturity, compared with 1.8% dw for 'Chico'.

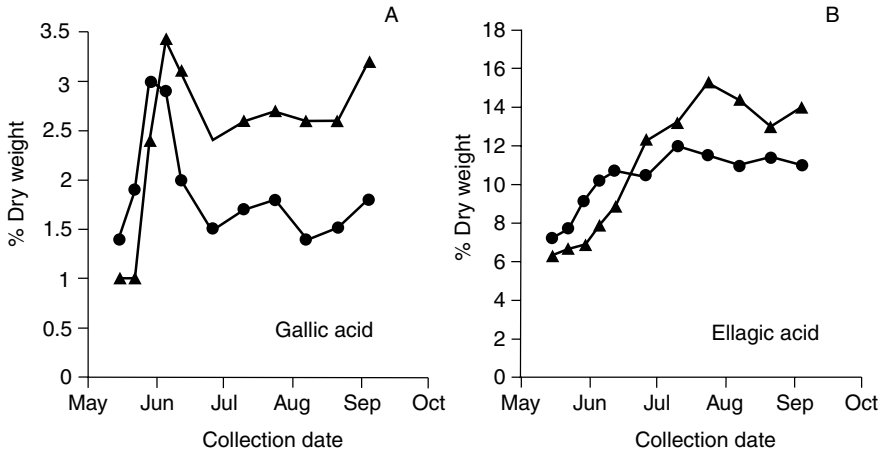

FIGURE 16.8 Analysis of (A) gallic acid, and (B) ellagic acid in 'Tulare' (▲) and 'Chico' (●) walnut seed coat on a biweekly basis throughout the growing season.

TABLE 16.2
Gallic Acid Content (Free and Bound) of Seed Coat from Nut Species and Aflatoxin B_1 Production *In Vitro* on Ground Kernels Inoculated with *A. flavus*

Species/Cultivar	Gallic Acid (% dw)	Aflatoxin B_1 (μg/plate)
Walnut, English (*Juglans regia*)		
Tulare	3.2	0
Chico	1.8	28
Walnut, Black (*Juglans hindsii*)		
Rawlins	1.0	44
Pistachio (*Pistacia vera*)		
Kerman	0.5	40
Almond (*Prunus dulcis*)		
Nonpareil	<0.1	172
Mission	<0.1	202

These values correlate with the relative ability of the two varieties to suppress aflatoxin production *in vitro* and are consistent with those measured for other nut species (Table 16.2). Thus, the black walnut (*Juglans hindsii*) cultivar 'Rawlins' and the pistachio cultivar 'Kerman' have gallic acid contents of 1.0% and 0.5% dw, with aflatoxin production levels of 44 and 40 μg/plate, respectively. As noted previously, almonds are much more capable of supporting aflatoxin biosynthesis and, correspondingly, the cultivars 'Nonpareil' and 'Mission' contain essentially no detectable gallic acid. It should be noted that a linear correlation between gallic acid content and aflatoxin suppression cannot be expected because other natural products, including ellagic acid, phenolic acids, and flavonoids that may be present, could also contribute to the overall antiaflatoxigenic activity. However, especially in the case of walnuts, the hydrolyzable tannins comprise such a large proportion of the kernel natural products that the gallic acid therefrom should be expected to be the primary bioactive constituent. Pistachios also contain hydrolyzable tannins, although primarily in the hull rather than the seed coat, but almonds contain none. In the latter case, therefore, any aflatoxin inhibitory activity must derive from other compounds, most probably flavonoids.[30,31]

16.5 HYDROLYZABLE TANNIN STRUCTURE–ACTIVITY CONSIDERATIONS

The bioactivity-directed assays, described above, demonstrate quite conclusively that the ability of walnuts to suppress aflatoxin formation derives from the hydrolyzable tannins in the seed coat, and that it is the gallic acid content of these tannins that is the major contributing factor. Nevertheless, gallic acid can be bound in several different forms, particularly as gallate esters of the carbohydrate core, as depsides of the primary gallate, or hexahydroxydiphenate moieties. Hydrolysis of such ester linkages would be expected to proceed at different rates and even the substitution position on the carbohydrate and extent of steric hindrance should affect hydrolysis. It has been shown that the fungal tannase consists of several isozymes[32] but fractionation into separate esterase and depsidase activities has not been achieved. Measurement of gallic acid content in seed coat can therefore only be regarded as an approximate prediction of ability to inhibit aflatoxin production. It is essential that relative concentrations of different structural types of hydrolyzable tannins be determined and the facility with which they undergo hydrolysis be assessed.

Although the bioassay used in these studies provides a convenient tool for evaluating different fractions and compounds for antiaflatoxigenic activity, it cannot be regarded as a model for the situation *in vivo*. For example, in determining the activity of gallic acid, the compound is distributed evenly throughout the media where a considerable portion will never be accessible to the fungus. In contrast, *A. flavus* growing on the seed coat will be directly exposed to the hydrolyzable tannin and the fungal tannase will generate gallic acid at sites where the metabolic activity, including aflatoxin biosynthesis, is likely to be greatest. Since the effective *in vitro* inhibitory concentration in the media need only be reproduced at the point of contact *in vivo*, the level of gallic acid required to inhibit aflatoxin formation should be considerably lower than that measured in the bioassay experiments.

16.6 MECHANISM OF ANTIAFLATOXIGENIC ACTIVITY

The role of hydrolyzable tannins and their gallic acid moiety in producing antiaflatoxigenic activity is an important practical finding for tree nut production because it suggests ways of controlling aflatoxin contamination, either through conventional breeding or genetic manipulation. However, of even more importance from a scientific perspective is the provision of a tool to investigate the reasons for aflatoxin production by the fungus, a question that has never been resolved.

The fungicidal properties of certain phytochemicals are well established but the suppression of metabolite biosynthesis without affecting fungal growth is unparalleled. The mechanism by which gallic acid affects aflatoxin formation is not yet established but particularly noteworthy is the lack of fluorescent compounds extractable from the culture plates during aflatoxin analysis as well as an absence of colored compounds in the media. This is indicative of a suppression of the formation of

all aromatic biosynthetic precursors of aflatoxins, including norsolorinic acid (**5**), the orange-colored metabolite that is initially formed by cyclization of the polyketide chain, and not merely a block of the pathway more immediately prior to aflatoxin formation (Figure 16.2). It is therefore most likely that gallic acid affects genes controlling fatty acid or polyketide synthases involved in the earliest stages of aflatoxin biosynthesis. An important corollary of this hypothesis is that it overcomes concerns that elimination of aflatoxin could result in accumulation of other toxic metabolites such as sterigmatocystin (**7**), as would be the case if there were only a partial block of aflatoxin biosynthesis.

Both hydrolyzable tannins and gallic acid itself are known to be potent antioxidants and scavengers of free radicals.[25] It is therefore reasonable to surmise that such properties may play a role in alleviating oxidative stress on the fungus, induced by generation of reactive oxygen species and hydrogen peroxide produced by plant defense responses.[33,34] Environmental conditions capable of promoting aflatoxin production, such as drought and high temperatures, are also known to induce oxidative stress signaling pathways in both plant and microbial cells.[35,36] This suggests a close association between aflatoxin biosynthesis and oxidative stress, possibly through formation of peroxides and epoxides formed by lipid peroxidation.[37] If this is so, antioxidants could be used as tools to investigate functional genomics with respect to oxidative stress responses and aflatoxigenesis.

While the lack of a practical gene transformation system prevents functional elucidation directly in *A. flavus*, numerous stress response pathways have been characterized for the yeast *Saccharomyces cerevisiae*.[38] This system can therefore be used as a model to evaluate phenotypic response to oxidative stress induced by hydrogen peroxide on selected *S. cerevisiae* strains, including mutants with single gene deletions.[39] On exposure to 3.3 mM hydrogen peroxide, growth was completely inhibited for six 10-fold serial dilutions of wild-type *S. cerevisiae* cells, with the exception of undiluted ($\sim 10^6$) cells. Under control conditions in the absence of H_2O_2, all colonies from serially diluted cells were visible when grown on tannic acid, a commercially available representative hydrolyzable tannin, or on gallic acid, up to 0.4% w/v (data not shown), indicating that these compounds *per se* are not toxic to the yeast. However, the growth inhibition induced by H_2O_2 was circumvented by addition of the antioxidants tannic acid or gallic acid in a concentration dependent manner over the range of 0.05–0.4%. Glutathione, included as a positive antioxidant control, showed a similar effect.[39]

To identify the functional role of particular *S. cerevisiae* genes in antioxidative stress responses, cell growth of 22 specific deletion mutants, included strains defective in gene regulation (*yap1*Δ), DNA damage control (*rad54*Δ), signal transduction (*sho1*Δ), and antioxidative stress response (*cta1*Δ) have been examined under oxidative stress and potential alleviation. Figure 16.9 illustrates the effect on these four selected gene deletion strains in comparison to wild-type *S. cerevisiae* when exposed to 2.5 mM H_2O_2 in the presence and absence of 0.4% antioxidant. The mutant *yap1*Δ, a strain defective in a transcription factor for gene regulation of the antioxidative stress response,

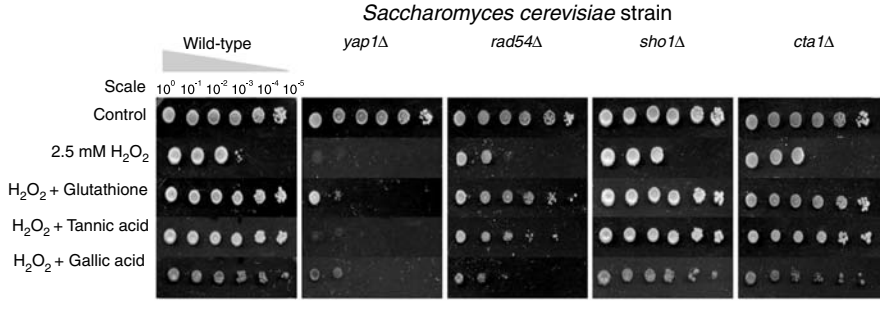

FIGURE 16.9 Growth suppression by oxidative stress induced with 2.5 mM hydrogen peroxide of wild-type yeast (*Saccharomyces cerevisiae*) and selected deletion mutants. Differential growth recovery by addition of the antioxidants, glutathione, tannic acid, and gallic acid is shown, indicating the relative sensitivity of specific deletions to oxidative stress.

showed little appreciable recovery on treatment with the antioxidants, suggesting that it is extremely sensitive to oxidative stress. The strain *rad54*Δ, deficient in a DNA-dependent ATPase, showed partial recovery, but the strains *sho1*Δ and *cta1*Δ, deficient in transmembrane osmosensor signal transduction and catalase-dependent antioxidant stress response, respectively, exhibited complete recovery.

Whereas hydrogen peroxide was moderately to severely lethal to all yeast strains, the antioxidants fully alleviated oxidative stress in the wild type. However, the degree of alleviation varied among the deletion mutants, depending upon the deleted gene and compound tested and thus differential responses to various antioxidants were observed. For example, strain *gsh2*Δ, deficient in glutathione synthetase, showed full recovery on treatment with glutathione but only partial recovery with tannic or gallic acids.[39] Similarly, not all mutants deficient in antioxidation stress genes responded to treatment with antioxidants. Thus, strain *sod1*Δ, lacking a Cu/Zn superoxide dismutase, showed no recovery with tannic acid and only slight recovery with gallic acid or glutathione. These variable effects are advantageous in that they provide specificity in treatments, allowing individual genes to be targeted and specific antioxidant natural products to be used as "molecular scalpels".

The relationship between oxidative stress and aflatoxin biosynthesis can be further explored by identification of orthologs of *S. cerevisiae* genes using the Expressed Sequence Tag (EST) database for *A. flavus*.[40] By this procedure, 43 genes involved in gene regulation, signal transduction, and antioxidation have been cataloged.[39] The validity of this procedure was established by successful functional complementation of the mitochondrial superoxide dismutase gene, *sodA*, an antioxidative stress gene from *A. flavus*, in a *sod2*Δ yeast mutant lacking the ortholog. This demonstrates the potential for *S. cerevisiae* to serve as a high-throughput analysis model system to identify involvement of candidate genes from an *A. flavus* EST library in signaling pathways. The use of functional complementation analysis and knockout mutants should enable the relationship between aflatoxin biosynthesis and oxidative stress to be elucidated. The availability of *A. flavus* genomic microarrays will further help identify the genes involved in the oxidative stress/aflatoxin biosynthesis relationship. Through differential expression, microarray analysis (unpublished results) has already shown that treatment of *A. flavus* with antioxidants regulates genes far upstream from the aflatoxin biosynthetic gene cluster.

16.7 CONCLUSIONS

The approach described in this chapter for defining the localization of aflatoxin resistance factors by progressively identifying the specific localization within plant species, varieties, organ, and finally specific tissue by bioactivity-guided assay can be applied to many other such problems, particularly in the plant world. It should be pointed out that in this case the hydrolyzable tannins are not phytoanticipins, since they do not markedly affect the growth of the fungus, except at relatively high concentrations. Rather, we would coin the word "phytoantigenins", because they function as plant constituents acting against the genesis of specific fungal natural products.

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17 Bioactive Peptides in Hen Eggs

Yoshinori Mine and Icy D'Silva

CONTENTS

17.1 INTRODUCTION

Many of the properties of food proteins with physiological significance beyond the nutritional requirements of nitrogen for growth and maintenance are attributed to physiologically active peptides encrypted in the proteins.[1] Within the sequence of the parent protein the peptides are inactive but during *in vivo* gastrointestinal digestion or *in vitro* enzyme treatment and food processing, bioactive peptides or "food hormones" are released. Depending on the amino acid sequence, the peptides exhibit various functions such as opioidergic, immunostimulatory, antihypertensive, and antimicrobial activity.[2,3] A number of peptides with different biological activities have been described[4] since the discovery in 1970 of bradykinin-potentiating peptides possessing angiotensin 1-converting enzyme (ACE)-inhibitory activity.[5,6] The observation that functional peptides can be generated through proteolytic digestion of a parent protein, which may have a different physiological function in the organism, suggests that multifunctionality may be an intrinsic property of most proteins, and that bioactive peptides can be "tailored and modeled" to achieve a desired function.[7]

Common structural properties of bioactive peptides include short sequences; hydrophobic amino acids in addition to proline, lysine, or arginine groups; and resistance to digestive peptidases.[8] However, structure–function relationships of most bioactive peptides have not yet been completely elucidated. The endogenous and exogenous (e.g., exorphins, casomorphins, and rubiscolins from partially digested food) opioid peptides mimic opiates in the brain. Exorphins such as α-, β-, or κ-casein, α-lactalbumin, β-lactoglobulin, wheat gluten, and rice albumin, have structures similar to endogenous opioid peptides with a tyrosine residue located at the amino terminal or bioactive site.[8] Although they represent a considerable subset of bioactive peptides with *in vitro*- or *in vivo*-demonstrated opioid agonist activity, only a few of their bioactive effects have been observed following oral or intragastric administration in animal models,[9] necessitating the study of optimum utilization of such peptides during passage through the gastrointestinal tract.

There is increasing commercial interest in the production of bioactive peptides with minimal destruction of activity, as well as with enhanced activity for various purposes such as therapeutics, functional foods, nutrigenomics, and specific nutraceutical applications.[1] Furthermore, bioactive peptides can have several clinical advantages over traditional small-molecule chemotherapeutics, including specificity, selectivity, and potency.[10] Industrial-scale production of such peptides involves their separation by automated and continuous systems but is hampered by the lack of suitable technologies. However, selective column chromatography methods are replacing batch methods of salting out. At the laboratory scale for biochemical, biological, and biophysical studies, ion exchange chromatography is widely used because of the low resulting denaturation, release of a nonaltered by-product, and easy scale-up, as reported for hen albumen (egg white).[11] Phage display is a well-established approach for the rapid generation of peptide libraries, which can be "panned" for specific activities leading to "targeted therapeutics", a current clinical paradigm of choice in the field of oncology.[10]

Most bioactive peptides, to date, have been isolated from milk and milk-based products.[4] The hen egg, consisting of the shell, shell membrane, albumen, and egg yolk, is an important source of nutrients, containing all of the essential components such as proteins, lipids, vitamins, minerals, carbohydrates, and growth factors required by the developing embryo, as well as defense factors against bacterial and viral infection.[12] Proteins, the source of bioactive peptides, are distributed throughout the egg, with the majority in the albumen, few in the egg yolk, and a small proportion in the eggshell and shell membrane.[13,14] The proteins can occur both as free and conjugated forms. The latter are formed when the protein is modified by prosthetic groups such as lipids and carbohydrates to yield lipoproteins and glycoproteins, respectively.[15]

Proteomic analysis of hen albumen using two dimensional electrophoresis and mass spectrometry has revealed the presence of three yet-to-be identified proteins, and sixteen known proteins, two of which were not previously detected, *viz*., Tenp, a protein with strong homology with a bacterial permeability-increasing protein family; and VMO-1, a vitelline membrane protein.[16] Egg proteins

are an important source of bioactive peptides.[15,17] With the sequencing of the 1200 Mb genome of the domesticated chicken (*Gallus gallus*)[18] and the availability of powerful tools for proteomics, the study of known and yet-to-be identified bioactive egg peptides will be further facilitated. Diverse biological activities, including antimicrobial, protease inhibitory, vitamin-binding, anticancer, and immunomodulatory activity have been attributed to egg peptides,[15] and is the subject of discussion of this chapter, with pertinent references to general advances in the field of bioactive peptides.

17.2 ALBUMEN PEPTIDES

The various biological activities of albumen-derived peptides are summarized in Table 17.1.

17.2.1 Antimicrobial Activity

Antimicrobial peptides represent an important component of innate immunity and nutritional immunity.[7] Albumen peptides from proteins with demonstrated antimicrobial activities, act as part of the natural defense system of the egg. These antimicrobial effects may be attributed to several mechanisms, including bacterial cell lysis, metal-binding, and vitamin-binding.[19] Antimicrobial peptides derived from eggs offer the advantage of safety for use in medicine and in the food industry.

17.2.1.1 Lysozyme

Lysozyme, acts as a mucopeptide *N*-acetylmuramyl hydrolase, which exerts bacteriolytic activity by hydrolyzing the β(1–4) linkage between *N*-acetylmuraminic acid and *N*-acetylglucosamine of peptidoglycan, the structural component of bacterial cell walls.[20] It is most effective against Gram-positive bacteria such as *Clostridium sporogenes* and *Bacillus* spp.[21] When used in conjunction with other compounds such as nisin and EDTA, lysozyme is also effective against other spoilage and pathogenic organisms such as *Listeria monocytogenes*.[22]

Modification of the lysozyme molecule with a hydrophobic moiety, through fatty-acid acylation[23,24] or by the genetic fusion of hydrophobic peptides to the C-terminus of lysozyme[25–27] enhanced the bactericidal activity of lysozyme against Gram-negative bacteria, possibly by mediating its interaction and insertion into the bacterial membrane.[28] Enhanced antimicrobial activity against Gram-negative bacteria has also been demonstrated by lysozyme–polysaccharide conjugates.[29–31] Ibrahim et al.[32] also found that perillaldehyde (Figure 17.1), a naturally occurring phenolic aldehyde, coupled to lysozyme, markedly enhanced the antibacterial activity against both *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative).

Through the strategy of "tailoring and modeling", a number of short peptides with high bactericidal activity have been developed from the bactericidal domain of lysozyme.[7] Enzymatic hydrolysis of lysozyme has been found to enhance its activity, by exposing and generating antibacterial portions of the protein.[33,34] Peptides corresponding to amino acid residues 98–112, 98–108, and 15–21 possessed antimicrobial activity against *E. coli* and *S. aureus*,[35,36] and synthetic bactericidal lysozyme polypeptides were found to not only damage bacterial outer membranes, but to also inhibit DNA and RNA synthesis.[35,37] Peptide IVSDGNGMNAWVAWR-NH2 (residues 98–112), derived from chicken lysozyme exhibited antimicrobial activity against *E. coli* and *S. aureus* but interacted very weakly with membrane lipids when compared with peptide RAWVAWR-NH2 derived from human lysozyme.[38] The rapid passage of peptides into bacteria suggested that the main bacterial site of action may be located inside bacteria.[39] However, not all segments of the peptide were active. Ile98-Met105 was inactive while Asn106-Arg112 was weakly active. The C-terminal segment NAWVAWR showed improved antimicrobial activity by exchanging the asparagine (Asn: N) with arginine (Arg: R). A requirement of two Arg and two tryptophans (Trp: W) was essential to maintain activity. Lysozyme was also found to prevent antibiotic-induced bacteriolysis and subsequent endotoxin release, while retaining antibiotic efficacy, suggesting its use for the prevention of endotoxemia in Gram-negative sepsis induced by antibiotic treatment.[40]

TABLE 17.1
Bioactive Albumen Peptides

Activity	Sequence	Source	Reference
Antimicrobial	N/A	Lysozyme	20–44
	IVSDGNGMNAWVAWR	Lysozyme	35–39
	Oligopeptides	Ovalbumin	45
	N/A	Ovotransferrin	46–48, 50, 51
	92-amino acids	Ovotransferrin	49
	N/A	Avidin	52–54
	Oligopeptides	Ovomucin	55–60
	N/A	Cystatin	61–63
	Oligopeptides	Cystatin	64
Antiadhesive	Glycopeptides	Ovomucin	66
Immunomodulating	N/A	Ovalbumin	69
	Oligopeptides	Ovalbumin	70–73
	Oligopeptides	Ovomucin	74, 75
	N/A	Lysozyme	76, 77
	Oligopeptides	Lysozyme	78–79
	N/A	Ovotransferrin	80–83
	N/A	Cystatin	84–87
Hypocholesterolemic	Oligopeptides	Ovomucin	88
Anticancer	N/A	Lysozyme	89–96
	Glycopeptides	Ovomucin	97, 98
	N/A	Avidin	99–105
	N/A	Cystatin	106–112
Antihypertensive	FRADHPFL Ovokinin	Ovalbumin	113, 114
	RADHPF (Ovokinin[2–7])	Ovalbumin	115–118
	RPLKPW	Ovalbumin	118, 119
	Oligopeptides	Ovalbumin	120
	FGRCVSP	Ovalbumin	121, 122
	ERKIKVYL	Ovalbumin	121, 122
	FFGRCVSP	Ovalbumin	121, 122
	LW	Ovalbumin	121, 122
	FCF	Ovalbumin	121, 122
	NIFYCP	Ovalbumin	121, 122
	YAEERYPIL	Albumen	123–126
	RADHPFL	Albumen	123–126
	IVF	Albumen	123–126
	Oligopeptides	Albumen	118, 127–129
Antioxidant	Oligopeptides	Albumen	113
	YAEERYPIL	Albumen	113
	Oligopeptide	Lysozyme	131
	N/A	Ovalbumin	31
Protease inhibition	N/A	Ovomacroglobulin	134–145
	N/A	Cystatin	146–154
	N/A	Ovomucoid	155–159
	N/A	Ovoinhibitor	146, 160–163
Biospecific ligand	N/A	Avidin	105
	N/A	Ovomucoid	164

Note: N/A, not applicable.

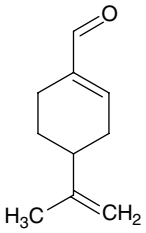

FIGURE 17.1 Structure of perillaldehyde.

Lysozyme has also demonstrated antiviral activity, reportedly associated with its charge rather than with its lytic ability.[21] Oral and topical applications of lysozyme were found to prevent and control herpes simplex and chicken pox by virtue of its anti-inflammatory action.[41] Albumen lysozyme has been reported to possess activity against HIV type 1.[42] The antibacterial properties of lysozyme have led to its use in oral health care products such as toothpaste, mouthwash, and chewing gum, and to protect against periodontis-causing bacteria and infections in the oral mucosa.[41,43] Gram-negative bacteria such as *E. coli* were sensitized by high pressure to a peptide corresponding to amino acid residues 96–116 of lysozyme. This may contribute to the development of more efficient technology for cold high-pressure pasteurization.[44]

17.2.1.2 Ovalbumin

Peptides produced by the tryptic and chymotryptic digestion of ovalbumin (OA), and their synthetic counterparts, were found to be strongly active against *Bacillus subtilus* and to a lesser extent against *E. coli*, *Bordetella bronchiseptica*, *Pseudomonas aeruginosa*, and *Serratia marcescens*, as well as *Candida albicans*.[45]

17.2.1.3 Ovotransferrin

Ovotransferrin, a member of the transferrin family of iron-binding proteins widely distributed in various biological fluids, has the capacity to reversibly bind iron.[46] It is suggested to function as an iron scavenger, thereby preventing iron use by microorganisms, and as an iron delivery agent.[47] It has been found to exert antibacterial activity against a wide spectrum of bacteria by permeating bacterial outer membranes, reaching the inner membrane and causing the selective permeation of ions and dissipation of electrical potential.[48] A 92-amino acid ovotransferrin peptide, OTAP-92, was found to be capable of killing Gram-negative bacteria by crossing the bacterial outer membrane through self-promoted uptake and damaging the cytoplasmic membrane.[49] It has also been shown that ovotransferrin possesses both antiviral activity against Marek's disease virus in chicken embryo fibroblasts[50] and antifungal activity against species of *Candida*.[51]

17.2.1.4 Avidin

Avidin possesses the unique ability to specifically bind the water-soluble vitamin biotin and has been found to inhibit the growth of biotin-requiring bacteria and yeasts.[52,53] Its antimicrobial activity has been attributed to its ability to bind to various Gram-negative and Gram-positive bacteria, including *E. coli K-12*, *Klebsiella pneumoniae*, *S. marcescens*, *P. aeruginosa*, *S. aureus*, *and S. epidermis*.[54]

17.2.1.5 Ovomucin

Ovomucin and ovomucin-derived peptides, besides their physical functions such as maintaining the structure and viscosity of the albumen and thus preventing the spread of microorganisms,[55] have demonstrated antiviral activity against Newcastle disease virus, bovine rotavirus, and human influenza virus *in vitro*.[56–60] Ovomucin-derived peptides, produced by treatment of ovomucin with

the enzyme pronase, showed increased solubility, compared with native ovomucin, while still retaining virus-binding activity.[58,60]

17.2.1.6 Cystatin

Albumen cystatin has been shown to possess antibacterial activity, preventing the growth of group A *Streptococcus*,[61] *Salmonella typhimurium*,[62] and the periodontis-causing *Porphyromonas gingivalis*.[63] Blankenvoorde et al.[64] found that peptides derived from cystatin were also capable of inhibiting the growth of *P. gingivalis*.

17.2.2 Antiadhesive Properties

The adhesion of microorganisms to host tissues is the first step in the infection process. In many cases, the adhesion is mediated by an interaction between components on the surface of the microorganism and carbohydrates on the mucosal surface of the host.[65] It has been suggested that oligosaccharides and glycoconjugates (glycoproteins and glycolipids) on the mucosal surface of the intestine, competitively inhibit microorganism–carbohydrate adhesion, thereby preventing microbial infection.[66]

Kobayashi et al.[66] found that enzymatic digestion of the highly glycosylated ovomucin produced ovomucin glycopeptides (OGP) with *E. coli* O157:H7-specific binding sites consisting of sialic acid. Based on these findings, it was suggested that OGP may be protective against *E. coli* O157:H7 infection *in vivo*, and is a potentially novel probe for the detection of the bacteria.

17.2.3 Immunomodulating Activity

The immune system responds to antigenic stimulation with a complex array of molecular events involving antigen-presenting cells, B cells, T cells, and phagocytes. Cytokines play a significant role in regulating such immune responses.[67] Cytokines and growth factors mediate a wide range of physiological processes including hematopoiesis, immune responses, wound healing, and general tissue maintenance. They are concomitantly involved in the pathology of a wide range of diseases and have potential use in replacement and immunomodulatory therapy.[68] Several albumen proteins and peptides have demonstrated immunomodulating activity.

17.2.3.1 Ovalbumin and Ovomucin

OA, when modified with dicarbonyl methylglyoxyl, has been found to induce the release of tumor necrosis factor-alpha (TNF-α) in a dose-dependant manner *in vitro*.[69] Immunogenic OA peptides have been used to enhance immune responses for cancer immunotherapy.[70–72] Tezuka and Yoshikawa[73] found that the phagocytic activity of macrophages was increased by the addition of the OA peptides OA 77–84 and OA 126–134 derived from peptic and chymotryptic digestions, respectively.

Ovomucin-derived peptides may also act as immunomodulators, showing macrophage-stimulating activity *in vitro*.[74] Synthetic ovomucoid peptides have also demonstrated immunomodulating activity, inducing T cell secretion of cytokines interleukin (IL)-4, IL-6, IL-10, IL-13, and interferon-gamma (IFN-γ).[75]

17.2.3.2 Lysozyme

When combined with immunotherapy, lysozyme was effective in improving the treatment of chronic sinusitis[76] and normalizing humoral and cellular responses in patients with chronic bronchitis.[41] Lysozyme, as an immunoglobulin production stimulating factor, was found to enhance antibody production in hybridomas and lymphocytes.[77] It has also been shown to regulate and restore the immune responses in immune-depressed patients undergoing anticancer treatments.[41]

It has been suggested that the antibacterial activity of lysozyme might also occur via stimulation of the macrophage phagocytic function. The hydrolysis products of peptidoglycans obtained by the

lytic activity of lysozyme may act as an adjuvant or immunomodulator[19] by enhancing immunoglobulin productivity and regulating and restoring the immune responses in immune-depressed patients undergoing anticancer treatments.[41,77] Posttranslational modifications, such as nitration of tyrosines (Tyr: Y) and modifications of tryptophans induced by antigen-presenting cells, in the T cell contact residues of the peptides DGSTDYGILQINSRW (residues 48–62) and YTGYSLGNWYCAAKFE (residues 20–35) of hen albumen lysozyme, and in synthetic peptides with Tyr replaced by nitrated Tyr, resulted in the recognition of the peptides by $CD4^+$ T cells on binding to class II histocompatibility molecule $I\text{-}A^k$.[78,79]

17.2.3.3 Ovotransferrin

Ovotransferrin is an acute-phase protein in chickens, the serum levels of which increase in inflammation and infections.[80] It acts as an immunomodulator, modulating macrophage and heterophil functions *in vitro*. Further immunomodulating effects of ovotransferrin have also been shown, including the inhibition of proliferation of mouse spleen lymphocytes[81] and the enhanced phagocytic response of peripheral blood mononuclear cells and polymorphonuclear cells in dogs.[82] Ovotransferrin was also found to facilitate the recovery of chick eyes from induced myopia.[83]

17.2.3.4 Cystatin

Cystatins may also be involved in inflammation and immune responses through the cytokine network, through mechanisms unrelated to the known protease inhibitory regions of the molecule.[84] Verdot et al.[85,86] found that chicken cystatin induced the synthesis of TNF-α and IL-10, resulting in an up-regulation of nitric oxide in mice *in vivo* as well as in mouse peritoneal macrophages *in vitro* thereby greatly reducing parasite numbers in a mouse model of visceral leishmaniasis.[87] Cystatin was also found to up regulate the production of IL-6 by human gingival fibroblast cells and murine splenocytes, and the IL-8 production of gingival fibroblasts.[84]

17.2.4 Hypocholesterolemic Activity

Ovomucin peptides were found to inhibit cholesterol uptake *in vitro* by Caco-2 cells, and reduce serum cholesterol in rats, that is, displaying an hypocholesterolemic action.[88]

17.2.5 Anticancer Activity

17.2.5.1 Lysozyme and Ovomucin

Lysozyme has been studied extensively as an anticancer agent. It has been shown to inhibit tumor formation and growth, both *in vitro* and *in vivo*, when administered orally.[89–94] It was also found to enhance the efficacy of chemotherapy treatments[95] and have a preventative effect when administered to normal mice.[96]

Evidence suggests that the anticancer effects of orally administered lysozyme may rely heavily upon the host-mediated immune response, including activation of the spleen and macrophages.[91] However, more recent data indicates that lysozyme may also exert action on the tumor cells themselves.[92,94]

Pronase-prepared glycopeptides of ovomucin have also demonstrated antitumor effects in a double-grafted tumor system in mice.[97] These effects have been suggested to be related to the antiangiogenic activity of ovomucin, thereby inhibiting tumor growth.[98]

17.2.5.2 Avidin

Avidin has been used in cancer treatment to localize and image cancer cells and to pretarget drugs to tumors. Owing to its tetrameric structure, tight biotin binding, and signal amplification, it leads to the accumulation of higher effective doses and increased persistence of biotinylated anticancer

drugs, as compared with other immunotherapeutic procedures.[99] Tumor pretargeting with avidin has also been found to be effective in increasing the uptake of TNF-α conjugated to biotin *in vitro*, improving the antitumor activity of TNF-α.[100–102] Yao et al.[103] found that radiolabeled avidin bound to lectins expressed on the surface of tumor cells and rapidly localized at high levels in various types of tumors in mice, thereby reducing radioactivity accumulation in other organs.

Avidin has also been found to be essential for the activity of adoptively transferred T cells at tumor sites.[104] The utilization of avidin in drug delivery through the blood–brain barrier has been demonstrated, facilitating delivery of therapeutics to the brain.[105]

17.2.5.3 Cystatin

Several proteases, including cysteine proteases, are required for tumor progression and metastasis.[106] Increased levels of cysteine proteases, and a decrease in cystatin, have been observed in various cancers.[107,108] Cystatin, a protease inhibitor, has been shown to inhibit tumor invasion in ras-transformed breast epithelial cells.[109] Multifunctional inhibitors, composed of chicken cystatin in conjunction with other protease inhibitors, have been suggested for the therapy of solid tumors.[106,110,111] Cystatins also produce less intensive side effects than other synthetic protease inhibitors currently used in medical treatments.[112]

17.2.6 Antihypertensive Activity

It has been reported that certain albumen-derived peptides can play a role in controlling the development of hypertension by exerting vasorelaxing effects.[113] An antihypertensive and vasorelaxing octapeptide, Phe-Arg-Ala-Asp-His-Pro-Phe-Leu (FRADHPFL) corresponding to fragment 358–365 of OA and named ovokinin, was isolated by peptic digestion; its effect being mediated by B_1 receptors, which stimulated the release of prostacyclin.[114] Oral administration of high doses of ovokinin, in the form of an emulsion of egg yolk, to spontaneously hypertensive rats (SHR) was highly effective, possibly due to the yolk phospholipids that improved intestinal absorption and protected the peptide from intestinal peptidases. Ovokinin (2–7), a hexapeptide, Arg-Ala-Asp-His-Phe-Leu (RADHPF) produced by chymotrypsin digestion of OA and corresponding to OA fragment 359–364, was also found to possess vasorelaxing activity but mediated by nitric oxide[115] and bradykinin B_2 vascular receptors[116] in SHR rats but not in normotensive Wistar–Kyoto (WKY) rats. Both peptides were found to significantly lower the systolic blood pressure in SHR rats, in a dose-dependent manner, when administered orally.[117]

The replacement of amino acids in the ovokinin (2–7) peptide has resulted in enhanced antihypertensive activity, with the most potent derivative Arg-Pro-Leu-Lys-Pro-Trp (RPLKPW) resulting in 100-fold more potent antihypertensive activity.[118] The RPLKPW peptide was genetically introduced into homologous sequences in soybean β-conglycinin α′ subunit, and the recombinant product expressed in *E. coli*. Oral administration of RPLKPW-incorporated α′ subunit in SHR resulted in antihypertensive activity,[119] making it the first example of a genetically modified food protein possessing physiological activity based on a bioactive peptide.

Two ACE-inhibitory peptides, that is, OA 183–184 and OA 200–218, were identified in OA by peptic and tryptic digestions, respectively.[120] Six ACE-inhibitory peptides were isolated from a pepsin digest of OA with IC_{50} values in the range of 0.4–15 μmol/L, but only one dipeptide, Leu-Trp (LW), showed antihypertensive activity in SHR rats.[121,122]

Miguel et al.[123] examined peptides with ACE-inhibitory properties produced by enzymatic hydrolysis of crude albumen but which were mainly derived from OA. Peptide sequences Arg-Ala-Asp-His-Pro-Phe-Leu (RADHPFL), Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu (YAEERYPIL), and Ile-Val-Phe (IVF) with IC_{50} values of 4.7, 6.2, and 33.1 μmol/L, respectively, exhibited potent ACE-inhibitory activity.[124] Studies simulating gastrointestinal digestion indicated that the sequences YAEERYPIL and RADHPFL hydrolyze when administered orally[125] while the IVF sequence acts directly.[126] Termination of oral administration resulted in arterial blood pressure values similar to that of untreated rats.

Improvements in antihypertensive activity have been reported resulting from: incorporation of peptides into liposomes to protect them through the gastrointestinal system; modification of the structure of the peptides by cyclization;[127] genetic modification to incorporate peptide sequences; incorporation of antihypertensive egg peptide sequences into soybean protein using controlled mutagenesis; and modification of residues close to the active peptide to facilitate its release *in vivo*.[118,128,129]

17.2.7 Antioxidant Activity

Reactive oxygen species and other free radicals cause oxidative damage to DNA, proteins, and other macromolecules. They have also been implicated in a number of multifactorial degenerative diseases including diabetes, cancer, and cardiovascular disease.[130]

Davalos et al.[113] reported that the enzymatic hydrolysis of crude albumen proteins with pepsin resulted in the production of peptides with strong antioxidant activities. The peptide Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu, which was shown previously to possess ACE-inhibitory activity, also exhibited a high radical-scavenging activity. These results would suggest that the combined antioxidant and ACE-inhibitory properties of albumen hydrolysates, or the corresponding peptides, would make a useful multifunctional preparation for the control of cardiovascular diseases, in particular, hypertension. An 18-amino acid domain of lysozyme was reported to suppress reactive oxygen species and oxidative stress genes.[131] The antioxidant effects of OA were also found to be enhanced by glycosylation, via the covalent attachment of galactomannan, suggested to be a result of an increase in lipid affinity.[31]

17.2.8 Protease Inhibition

Proteases play key roles in several physiological processes, including intracellular protein degradation, bone remodeling, and antigen presentation. Protease activities are increased in pathophysiological conditions such as cancer metastasis and inflammation. They are also required for invasion by microorganisms.[132] Therefore, protease inhibitors represent an important class of compounds of therapeutic significance. Four protease inhibitors have been identified in albumen, that is, cystatin, ovomucoid, ovomacroglobulin (also known as ovostatin), and ovoinhibitor.[19] Microbial proteases are involved in the mechanism of penetration of tissues by bacteria, in the proteolytic cleavage of precursor proteins for virus replication, and in the facilitation of host invasion by parasites.[133]

17.2.8.1 Ovomacroglobulin

Ovomacroglobulin possesses broad-spectrum inhibitory activity against various types of proteases, including serine proteases, cysteine proteases, thiol proteases, and metalloproteases,[134,135] and has demonstrated *in vitro* and *in vivo* antimicrobial activity against *S. marcescens* and *P. aeruginosa*.[135–138] It was found to reduce corneal destruction in an experimental keratitis model in rabbits and to accelerate wound healing.[138–140] Ovomacroglobulin enhanced periodontal wound healing in rats by accelerating fibroblast growth, collagen deposition, and capillary formation in tissue;[141] suppressed *P. aeruginosa* and *Vibrio vulnificus* septicemia due to the inhibition of kinin-generating proteases;[142,143] inhibited the inflammatory proteinase medullasin *in vitro*;[144] and suppressed metalloproteinases and vascular permeability in skin tissues, which play a role in tumor metastasis.[145]

17.2.8.2 Cystatin

Both cystatin and ovoinhibitor (Section 17.2.8.3), a serine protease inhibitor,[146] have been found to prevent rotavirus infection in mice,[147] and cystatin inhibits the action of poliovirus protease, thereby effectively inhibiting virus replication *in vitro*.[148,149]

Albumen cystatin, part of a "superfamily" of cystatins, belongs to the Type 2 cystatins[150] and contains two disulfide bonds but no carbohydrate.[150] It inhibits most cysteine proteinases, including

ficin; papain; and cathepsins B, C, H, and L.[151] Low contents of cystatins in natural resources may limit their applications.[62] However, genetic modification and elevated expression of cystatin has been examined, not only providing a source for increased quantities of cystatin, but also resulting in the production of recombinant cystatin with enhanced proteinase inhibitory activity.[152]

Cystatins inhibited the tumor-associated activity of intracellular cysteine proteases and have been suggested as potential anticancer drugs.[153] Cystatin was found to reduce the activity of the key proteolytic enzymes responsible for the growth of gastric cancer *in vitro*.[154]

17.2.8.3 Ovomucoid and Ovoinhibitor

Ovomucoid, a serine protease inhibitor, has been shown to be particularly useful to enhance the oral delivery of protein/peptide therapeutics that are otherwise subjected to extensive proteolytic degradation in the gastrointestinal tract.[155] For example, because ovomucoid inhibits digestive enzymes, such as trypsin, β-chymotrypsin, and elastase, it improves the oral delivery of insulin,[156–158] and has been examined for coadministration with calcitonin, a polypeptide associated with calcium homeostasis and bone remodeling, which is often used in the management of osteoporosis.[155] In addition, ovomucoid has been used as a model for the design of therapeutic, inhibitory peptides.[159]

Ovoinhibitor inhibits enzymes such as trypsin, chymotrypsin, and elastase, as well as various bacterial and fungal proteinases.[146,160] It prevents the development of rotavirus-related gastroenteritis in a mouse model of rotavirus infection,[161] and inhibits the formation of active oxygen species by human polymorphonuclear leukocytes, which are associated with inflammatory diseases, mutagenicity, and carcinogenicity.[162] It has also been used to study models of autoimmune arthritis in mice.[163]

17.2.9 Biospecific Ligand Activity

Avidin is utilized as a biospecific ligand to aid drug delivery through the blood–brain barrier thereby facilitating delivery of therapeutics to the brain.[105] Ovomucoid promotes the targeting of drugs to the blood by acting as a biospecific ligand to lectins on the walls of the gastrointestinal tract.[164]

17.3 EGG YOLK PEPTIDES

The biological activities of egg yolk–derived peptides are summarized in Table 17.2.

17.3.1 Antimicrobial Activity

The antimicrobial activity of egg yolk lipoproteins against *Streptococcus* spp. increased upon treatment with digestive enzymes,[165] indicating that the molecule may require degradation in order to release the maximal levels of the active component. Fatty acids, with activity against *Streptococcus mutans* were enzymatically released from lipoproteins extracted from egg yolk.[166]

Egg yolk phosvitin, an iron-binding phosphoprotein, has demonstrated antibacterial activity against *E. coli* under thermal stress, causing disruption of cells and DNA leakage, suggested to be a result of the synergistic effect of its high metal-chelating ability and high surface activity.[167,168] Sialyloligosaccharides, present as glycoproteins or glycopeptides[169] have been found to inhibit *Salmonella* infection by inhibiting the entry of bacteria through the gut.[170]

17.3.2 Antiadhesive Properties

Kassaify et al.[171] identified high-density lipoproteins in nonimmunized egg yolk that have antiadhesive activity against *Salmonella enteritidis*, *S. typhimurium* and *E.coli* O157:H7. Egg yolk–derived sialyloligosaccharide, and its asialo derivative, as well as a sialylglycopeptide of egg yolk–derived sialyloligosaccharide, inhibited the binding of *S. enteritidis* and *E. coli* to human intestinal cells *in vitro*, and prevented *Salmonella* infection when orally administered to mice.[170] The effects of the

TABLE 17.2
Bioactive Egg Yolk Peptides

Activity	Sequence	Source	Reference
Antimicrobial	N/A	Lipoproteins	165, 166
	N/A	Phosvitin	167, 168
	Glycopeptides	Sialyloligosaccharides	169, 170
Antiadhesive	N/A	High-density lipoproteins	170–173
Immunomodulating	N/A	Low density lipoproteins	174–176
Antihypertensive	Oligopeptides	Yolk hydrolyzate	177
Antioxidant	Enzymatic digests	Phosvitin	178
	N/A	Phosvitin phosphopeptides	179
Nutrient bioavailability	N/A	Phosvitin phosphopeptides	168, 180, 181

Note: N/A, not applicable.

sialyloligosaccharides and their derivatives on macrophage activation suggested that these yolk-derived compounds exerted their antibacterial action by preventing bacterial adhesion and entry through the intestine, rather than via immunomodulatory effects.[170] Similar results were observed using sialylglycopeptides from egg yolk, conjugated to nondigestive polysaccharides in order to increase retention time in the gut.[172] Egg yolk proteins also significantly inhibited the ability of fluorescent *S. mutans* to adhere to hydroxyapatite, an *in vitro* model surface used to mimic adhesion of oral bacteria to saliva-coated surfaces.[173]

17.3.3 Immunomodulating Activity

The immunomodulating activity of low-density lipoproteins (LDL) has been demonstrated using human histocytic lymphoma cells (U-937), human monocytic leukemia cells (THP-1), and U-937-derived macrophage-like cells (U-M),[174] promoting the growth of all three cell types. LDL was also shown to enhance the production of IgM in human–human hybridomas.[175] Jolivet et al.[176] have identified apovitellenin 1 and apolipoprotein B in the LDL component of egg yolk.

17.3.4 Antihypertensive Activity

ACE-inhibitory oligopeptides with antihypertensive activity were produced by enzymatic hydrolysis of egg yolk.[177]

17.3.5 Antioxidant Activity

Phosvitin and its enzymatic digests were found to protect against iron-catalyzed hydroxyl radical formation, and protect DNA against oxidative damage induced by Fe(II) and peroxide. These observations suggested that phosvitin may be useful for the prevention of iron-mediated oxidative stress related diseases, such as colorectal cancer.[178] Phosvitin phosphopeptides (Section 17.3.6) possess antioxidative activity against hydrogen peroxide–induced oxidative stress in intestinal epithelial cells.[179]

17.3.6 Nutrient Bioavailability

Phosphopeptides derived from the tryptic hydrolysis of partially dephosphorylated phosvitin have been found to enhance calcium-binding capacity and inhibit the formation of insoluble calcium phosphates.[180,181] Furthermore, these phosvitin phosphopeptides demonstrated better calcium-solubilizing activity than commercial casein phosphopeptides.[168,180]

17.4 EGGSHELL AND MEMBRANE PEPTIDES

Chicken eggshell is a mineralized structure consisting of approximately 95% calcium carbonate by weight, the rest made up of an organic matrix consisting mainly of glycoproteins and proteoglycans.[182,183] A recent study demonstrated that a 21,000 Da protein present in the soluble eggshell matrix proteins may play an important role in increasing calcium transport across intestinal epithelial cells *in vitro*.[184]

Eggshell membrane is composed of collagen-like proteins, which are largely located in the inner membrane.[185] Eggshell membrane–derived peptides, prepared by alkaline treatment, were shown to stimulate human skin fibroblasts *in vitro*.[186] Eggshell membranes also contain antimicrobial substances, with lysozyme,[187,188] ovotransferrin,[188] and β-*N*-acetylglucosaminidase[188,189] activity, but the components responsible for the antimicrobial activity have not been conclusively identified.[188]

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18 Biological Fingerprinting Analysis: Strategy for Screening Bioactive Compounds in Traditional Chinese Medicines

Xingye Su, Hanfa Zou, Xiaoyuan Lei, Liang Kong, and Lianghai Hu

CONTENTS

18.1 INTRODUCTION

Chemical substances derived from natural products such as plants, animals, and microbes have been invaluable as a source of therapeutic agents for all kinds of diseases since the dawn of medicine.[1–5] Even now, natural products still play a dominant role in the discovery of leads to drugs for the treatment of human disease. During the period 1981–2002, of the 877 small molecule New Chemical Entities introduced, nearly half (49%) were natural products, semisynthetic natural product analogues, or synthetic compounds based on natural-product pharmacophores.[6] Traditional Chinese

medicines (TCMs), as an important group of natural remedies, are gaining increased attention in drug lead discovery. TCMs have a long history dating back several thousands of years.[7] The origin of TCMs was associated with the legendary testing of many herbs for their medicinal properties by the folk hero Shen Nong.[8] His experience and work in these areas was eventually recorded in *Shen Nong Ben Cao Jing* (*The Herbal Classic of the Divine Plowman*) in about 2700 BCE. Up until the second and first centuries BCE, the establishment of the "Yin and Yang" doctrine under the influence of Confucius helped to reinforce the use of herbal materials for relieving illnesses and the first major medical text, *Huang Di Nei Jing* (*Yellow Thearchy's Inner Canon*), appeared in China. With the development of theory and clinical practice, China accumulated a rich body of empirical knowledge about the use of medicinal plants for the treatment of various diseases. In the Ming Dynasty (1368–1644), a major medical literature *Ben Cao Gang Mu* (*The Comprehensive Herbal Foundation*) written by Li Shi-Zhen in 1590 discussed 1892 medicinal substances and contained over 1000 illustrations and over 10,000 medicinal formulas, which indicates that the contemporary Chinese had grasped comprehensive pharmaceutical knowledge and clinical experience. So far, there have been 12,806 medical resources discovered in China, including 11,145 medicinal plants, 1581 medicinal animals, and 80 medicinal minerals.[9] The clinic medicinal experience of more than 4000 years and a modern integrated theory system for diagnosis and treatment open a potential shortcut for discovering new bioactive compounds from these natural products.

Screening, analysis, and identification of bioactive compounds are always a challenge in natural products research, including TCMs, since each may contain hundreds or thousands of components, of which only a few show pharmacological activity.[10] In the past decades, many effective methods have been used for analyzing the components of TCMs to screen and identify the bioactive compounds. The bioactivities of crude and semipurified extracts or distilled fractions of TCMs have been screened using classic pharmacological means.[11–13] It is still an important approach in drug discovery despite its high cost and low efficiency. Pharmacological screening with organ and tissue models overcomes some disadvantages of using whole animal models, such as large amounts of compound needed for screening, but it has rarely been applied in screening of TCMs.[14,15] Cell-based screening[16,17] is rapidly expanding, as innovations in target selection and instrumentation increase the number of targets that can be efficiently screened in cellular formats and hence it has become an important means in the screening and evaluation of TCMs.

Molecular screening methods have gained attention because they are rapid, economical, highly sensitive, and specific. Many drug targets have been discovered and identified with the development of biotechnology.[18,19] Despite the complex components and unclear reaction mechanisms, the pharmacological effects of the active constituents in TCMs should also be based on the molecular mechanisms. Since Wang et al.[20,21] used receptor and enzyme models for the screening of more than 400 extracts from 150 TCMs, many molecular-based models have been developed for screening TCMs for activities.[22–24]

In essence, molecular screening is based on the affinity interaction of the bioactive compounds in TCMs with the biomolecular targets. Unlike synthetic drugs, TCMs are often complex natural molecular libraries. Purification of each component is impractical and hence effective screening of the library is necessary. With the development of genomic and proteomic technologies, and the advances in combinatorial chemistry, recognition studies based on library screening have been applied to the identification of interacting counterparts against known or unknown libraries. A range of small molecule–biomacromolecule interactions have been studied ranging from the chemically simple to complex interaction systems.[25] Based on chromatographic (including affinity-based chromatography with immobilized macrobiomolecules) and mass spectrometric methods, biological fingerprinting analysis (BFA) was proposed as a potential approach to screen for bioactivities in extracts of natural products. Thus, BFA provides chromatographic and mass spectrometric data that are obtained simultaneously with information on the bioactivity of the natural products in potentially complex extracts obtained from TCMs.

Knowledge of absorption, distribution, metabolism, elimination, and toxicity (ADME/Tox) properties of drugs is critical to drug discovery and development.[26] In the drug discovery industry,

approximately 50% of candidates failed because of poor ADME/Tox properties.[27] To increase the efficiency and reduce the cost of pharmaceutical research, moving ADME/Tox evaluations into early discovery stages has been widely accepted in the pharmaceutical industry.[28] A variety of experimental assays have been developed to characterize each aspect of the ADME/Tox properties. The tools involved include physicochemical methods and biological assays using biomolecules, subcellular fractions, primary cell culture, immortalized cell lines, tissues, and whole organs.[28] When applied to the screening of complex natural product extracts, a challenge faced by these assays in the discovery phase is the simultaneous assessment of multiple compounds, hence the need for efficient biological fingerprinting strategies.

18.2 BIOLOGICAL FINGERPRINTING ANALYSIS OF TRADITIONAL CHINESE MEDICINES

As an effective separation tool, chromatography has played a major role in the analysis of complex mixtures of natural products. Analytes are separated on the basis of different interaction abilities with the stationary and mobile phases.[29] Chromatographic separation, followed by pharmacological assessment of analytes, is the basic approach to discover bioactive components in TCMs. BFA is aimed at speeding up this bioactivity discovery process and follows two major strategies. First, the crude natural product extracts are allowed to interact with the target macrobiomolecule prior to the chromatography. Reverse-phase high-performance liquid chromatography (reverse-phase HPLC) has been used widely due to its high separation efficiency.[15,30–32] Comparison of the target-treated sample chromatograms and samples that were not treated with the target indicates those components of the extract with the targeted bioactivity. In the second approach, the biomolecular target is immobilized as the stationary phase and chromatographic process then directly reflects the interaction of the analytes and the target on-column.

18.2.1 Biological Target-Interaction Chromatography

BFA, as a tool for screening and analysis of the multiple bioactive compounds in TCMs, was originally defined as the comparison of "fingerprint" chromatograms of the extracts of TCMs before and after the interaction with biological systems (DNA, protein, cell, etc.).[29] A microdialysis-based technique was employed whereby a solution of a TCM extract mixed with a target macrobiomolecule was perfused through a microdialysis probe. The TCM components that interacted with the macrobiomolecules (the "interactive components") were retained within the microdialysis membrane. These "interactive components" can be characterized with association constants between them and the target macrobiomolecules. The chromatographic peak areas of the "interactive components" after interaction with the macromolecule are decreased, whereas for "noninteracting components" there is almost no change in their peak areas before and after the interaction. Thus, macromolecule-binding compounds in the extract of the TCM can be easily distinguished from those not binding.

18.2.1.1 Targeting HSA and Human Serum

Most synthetic drugs show various degrees of binding to plasma proteins.[33,34] Human serum albumin (HSA) is the most abundant blood plasma protein (ca. 30–50 g/L) and plays an important role in the transport of both endogenous and exogenous compounds such as metabolites, fatty acids, steroids, metal ions, and drugs.[35] Reversible noncovalent binding to HSA controls the active plasma concentration of a drug and provides a reservoir for a long-term action, which affects the pharmacokinetic, pharmacodynamic, and toxicological properties. Binding of drugs to plasma proteins, mostly to albumin and α_1-acid glycoprotein (AGP), is one of many factors that influences drug distribution.[36]

HPLC of the extracts of Rhizoma Chuanxiong (*Ligusticum chuanxiong* Hort.), which is used to treat angiocardiopathy, nephropathy, and menstrual disorders, revealed that there were more than

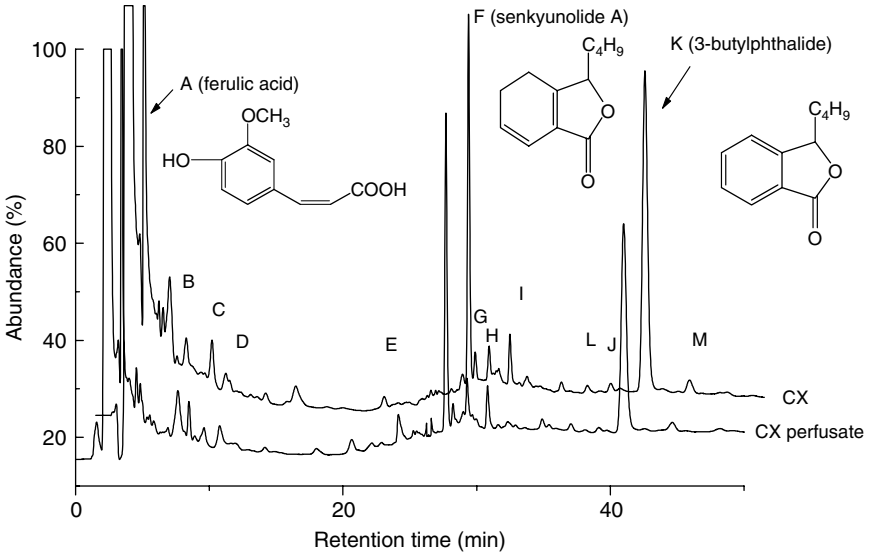


FIGURE 18.1 Chromatograms of components in Rhizoma Chuanxiong extract and in dialysate separated on C18 column. "CX" represents the Rhizoma Chuanxiong, and "CX perfusate" represents the dialysate after microdialysis probe. Peaks with letters, shown for the "CX" trace, represent the components in Rhizoma Chuanxiong. (From Guo, M., Su, X.Y., Kong, L., Li, X., and Zou, H.F., *Anal. Chim. Acta*, 556, 183, 2006. With permission.)

20 components (Figure 18.1), with major peaks marked with letters A–M.[32] Peaks A, F, and K have been identified as ferulic acid, senkyunolide A, and 3-butylphthalide (Figure 18.1), respectively, by comparison of their UV and mass spectra with those of standard samples and experimental data in the literature.[37,38]

At perfusion rates typically used for microdialysis, equilibrium is not established across the dialysis membrane; rather a steady-state situation is rapidly set up. The concentration of analyte collected in the dialysate is, therefore, some fraction of the actual concentration in the perfusate solution. Therefore, the first step in applying microdialysis to the BFA of TCMs is to determine the recovery efficiency for various components. The microdialysis probe recovery efficiency (R) is defined as the ratio between the concentration of a compound in the dialysate (C_d) and the original concentration in the perfusate solution (C_s) (Equation 18.1).

$$R = \frac{C_d}{C_s} \tag{18.1}$$

Recovery efficiencies of compounds in Rhizoma Chuanxiong are listed in Table 18.1. During microdialysis sampling, some factors influence the recovery, such as the type of microdialysis membrane, length of dialysis membrane, the geometry of the probe, system temperature, and the perfusion flow rate.[39,40] Strict control of perfusion flow rate and system temperature is critical to the precision of recovery. The values of relative standard deviation of recoveries for all peaks from Rhizoma Chuanxiong were below 3.1% (Table 18.1) validating the use of microdialysis coupled with HPLC.

When the Rhizoma Chuanxiong extract, mixed with HSA, is perfused through the microdialysis apparatus, the individual components may bind to the protein. The unbound components will then diffuse through the semipermeable membrane at rates corresponding to their recovery efficiencies. Rearrangement of Equation 18.1, and insertion of the derived recovery efficiency and the measured concentration in the dialysate, provides the concentration in the original extract

TABLE 18.1
Microdialysis Recovery of Compounds in the Extract of Rhizoma Chuanxiong

Peaks	Retention Time (min)	Relative Recovery (%)	RSD (%) ($n = 5$)
A (ferulic acid)	4.3	58.7	1.5
B	7.1	89.1	0.3
C	8.0	85.2	2.1
D	9.8	95.7	3.0
E	20.6	87.3	2.2
F (senkyunolide A)	27.9	88.8	0.5
G	28.5	84.0	1.1
H	29.2	98.4	1.2
I	31.6	91.3	0.4
L	37.1	36.7	0.8
J	38.3	87.4	1.7
K (3-butylphthalide)	41.2	68.9	0.7
M	45.6	69.5	3.1

Note: RSD represents relative standard deviation.
Source: Guo, M., Su, X.Y., Kong, L., Li, X., and Zou, H.F., *Anal. Chim. Acta*, 556, 183, 2006. With permission.

(C_s) (Equation 18.2). This concentration is an "apparent" concentration since some of the original components may be bound to the protein target. The difference between the concentration (C_0) of the components determined before any interaction with the protein, that is, CX in Figure 18.1, and the new "apparent" concentration (C_s) calculated from the recovery efficiencies and the concentration in the dialysate according to Equation 18.2, represents the amount of component bound to the protein. The degree of binding is then calculated using Equation 18.3.

$$C_s = \frac{C_d}{R} \tag{18.2}$$

$$\text{Binding degree} = \frac{C_0 - C_s}{C_0} \tag{18.3}$$

The microdialysates of Rhizoma Chuanxiong before and after interaction with HSA at concentrations of 0.3 and 0.6 mM and pH 7.4 were analyzed by HPLC (Figure 18.2). Quantitation of the peaks and insertion into Equations 18.2 and 18.3 provided the degree of binding of components to the HSA (Figure 18.3). For example, it can be seen (Figure 18.3) that the binding degrees of ferulic acid (peak A) were 23.0% (using 0.3 mM HSA) and 36.7% (using 0.6 mM HSA).

It has been reported that most drug ligands are bound to HSA reversibly.[41,42] Although it is generally recognized that there are a small number of distinct binding locations, controversy remains about the exact number of discrete binding locations on albumin. The general consensus is that there are two principal binding sites, I and II, for small heterocyclic or aromatic carboxylic acids such as aspirin, warfarin, and ibuprofen; two long-chain fatty acid sites, III and IV; and two metal-binding sites, V and VI.[43] As an aromatic carboxylic acid, ferulic acid may be expected to bind relatively strong to HSA. The rather low binding degrees of senkyunolide A (peak F), 6.5% at 0.3 mM HSA and 5.4% at 0.6 mM HSA, may be due to the presence of a reduced aryl ring that does not favor binding to sites on HSA. The greater degrees of binding of 3-butylphthalide (peak K), namely 27.7% and 30.2% with 0.3 and 0.6 mM HSA, respectively, may be explained by its aromatic and more hydrophobic nature and the presence of binding sites on HSA for such compounds.

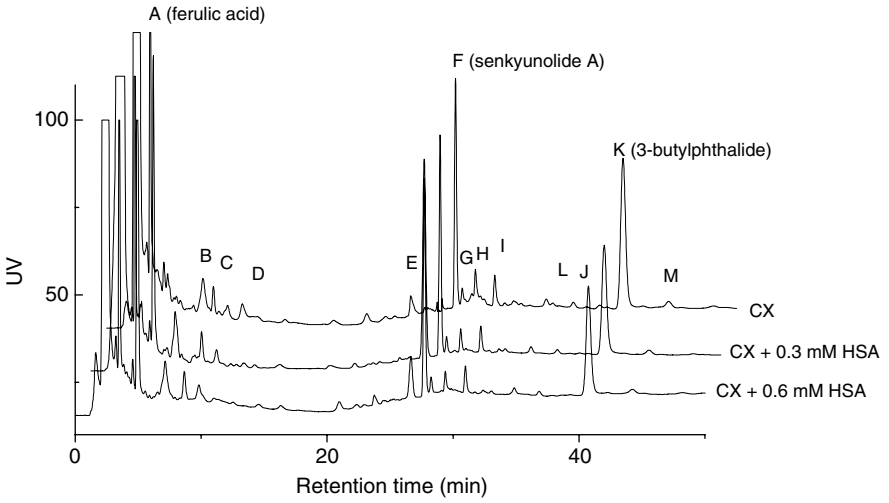


FIGURE 18.2 Chromatograms of Rhizoma Chuanxiong extract reaction with HSA. CX represents the Rhizoma Chuanxiong, and CX + 0.3 mM HSA or CX + 0.6 nM HSA represents the interaction of Rhizoma Chuanxiong extract with HSA at the concentration of 0.3 or 0.6 mM, respectively. (From Guo, M., Su, X.Y., Kong, L., Li, X., and Zou, H.F., *Anal. Chim. Acta*, 556, 183, 2006. With permission.)

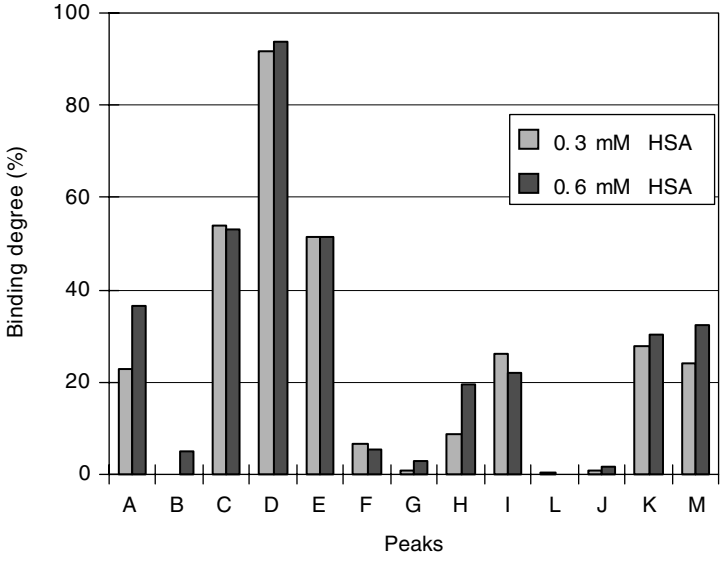


FIGURE 18.3 Binding degrees of Rhizoma Chuanxiong extract with human serum albumin (HSA). The left bar in each pair represents the binding degree of Rhizoma Chuanxiong with 0.3 mM HSA whereas the right bar represents binding with 0.6 mM HSA. (From Guo, M., Su, X.Y., Kong, L., Li, X., and Zou, H.F., *Anal. Chim. Acta*, 556, 183, 2006. With permission.)

The pH value is a critical factor, affecting the molecular state of both biopolymers and drugs, especially for ionizable compounds. When the pH is higher than the isoelectric point of HSA (pH 4.0), HSA bears negative charge. Therefore, the effect of pH value on the binding degrees of compounds in Rhizoma Chuanxiong was investigated. The binding degree of ferulic acid with 0.3 mM HSA decreased with an increase in pH (23.0% at pH 7.4, 12.1% at pH 6.0, and 11.5% at pH 8.9).

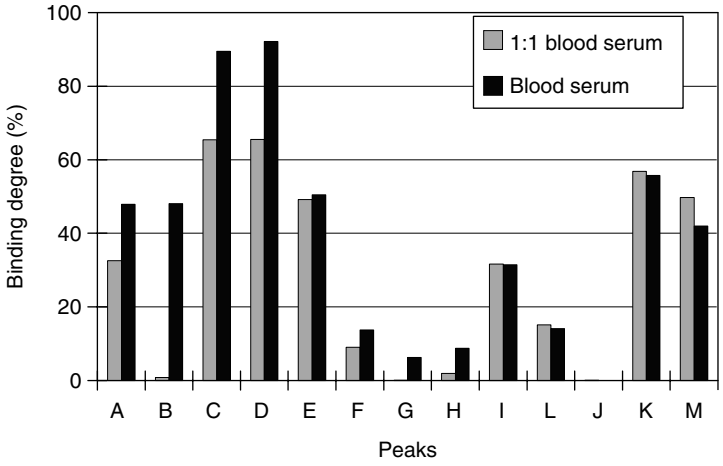


FIGURE 18.4 Binding degrees of components in Rhizoma Chuanxiong extract with human blood serum. The left bar represents the binding degree of Rhizoma Chuanxiong with human blood serum diluted 1:1 with phosphate buffer (pH 7.4, 0.5 M NaCl). The right bar represents binding with whole human blood serum. (From Guo, M., Su, X.Y., Kong, L., Li, X., and Zou, H.F., *Anal. Chim. Acta*, 556, 183, 2006. With permission.)

This can be attributed to the changes in the structure of the binding site of HSA with pH and also the ionic state of ferulic acid. For similar reasons, binding of 3-butylphthalide to HSA was also observed to decrease with increasing pH. The binding degrees of other peaks were changed at different pH values. Thus, it can be concluded that pH influences the interaction between compounds in Rhizoma Chuanxiong and HSA as a result of changes in the binding site structures of HSA and the molecular state of active compounds.

It is widely accepted in the pharmaceutical industry that the overall distribution, metabolism, and efficiency of many drugs can be altered, based on their affinity to serum albumin. Many promising new drug candidates are rendered ineffective because of their unusually high affinity for this abundant protein.[44] Therefore, compounds with higher or lower binding degrees in TCMs would not contribute to their bioactivity. In Figure 18.3 it can be seen that peaks C, D, and E have strong binding affinities for HSA while the binding degrees (with 0.6 mM HSA) of peaks I (22.2%) and M (32.5%) were similar to peaks A (ferulic acid) and K (3-butylphthalide). Since ferulic acid and 3-butylphthalide are well-known active compounds in Rhizoma Chuanxiong,[45] peaks I and M should be worthy of further study as additional possible bioactive compounds in Rhizoma Chuanxiong.

The binding degrees of compounds in Rhizoma Chuanxiong to blood serum at pH 7.4 were also determined using BFA (Figure 18.4). From Figures 18.3 and 18.4 it can be seen that all compounds, except for peak E, have a higher degree of binding with the blood serum than with HSA alone. For example, the binding degree of ferulic acid was 33.3% in 1:1 blood serum (i.e., blood serum that has been diluted 1:1 with phosphate buffer, pH 7.4, containing 0.5 M NaCl) and 47.9% with whole blood serum. This is presumably due to the complement of other proteins such as AGP and IgG, etc., present in blood serum. The binding degrees of peak E to HSA were 51.5% (0.3 mM HSA) and 51.6% (0.6 mM HSA) while binding degrees to 1:1 blood serum and whole blood serum were 49.2% and 50.5%, respectively. This observation of the same degree of binding indicates that the compounds of peak E bind only to HSA and not to other proteins in blood serum.

The BFA method was also applied in a study of the binding degree of compounds in Rhizoma Chuanxiong to blood serum at different pH values. It was observed that the binding degree of ferulic acid decreased with increasing pH from 6.0 to 8.9, as seen with HSA. Possibly this implies that HSA

is the major binding protein of ferulic acid in blood serum. A similar trend of decreased binding was observed for 3-butylphthalide. However, for 3-butylphthalide, the binding degree to 1:1 blood serum was 58.6% at pH 7.4, 25.5% at pH 6.0, and 23.4% at pH 8.9. For binding to HSA, the results were 27.7%, 2.9%, and 6.5% to 0.3 mM HSA, respectively. Thus, binding degrees on blood serum were not decreased to the same extent as with HSA. Presumably, there are other binding proteins in blood serum for 3-butylphthalide as greatly influenced by the solution pH values as was HSA.

18.2.1.2 Targeting DNA

BFA was applied to the screening of the DNA-binding agents from TCMs. DNA is the molecular target of many antimicrobial, antiviral, and antitumor active drugs.[46] The formation of adducts between genotoxic carcinogens and DNA is believed to be the first step in chemically induced carcinogenesis.[47,48] Understanding the interaction of drugs with DNA is the first necessary step in elucidating the molecular basis for the potent therapeutic or toxic activities of the compounds. Interaction properties of TCM extracts of *Coptis chinensis* Franch., with the effects of clearing heat, drying up dampness, purging toxicosis, and detoxification, and *Phellodendron amurense* Rupr., used as an antistomachic, anti-inflammatory, and antipyretic agent, with calf thymus DNA (ct-DNA) have been investigated,[30] and their biofingerprinting chromatograms are shown in Figure 18.5. Peak areas showed that seven peaks in *C. chinensis* and three in *P. amurense* decreased after the interaction with DNA. Comparison of retention times and UV spectra with authentic compounds showed that three of them were jatrorrhizine (P1), palmatine (P2), and berberine (P3) [Figure 18.5].

It was observed that the ct-DNA-binding degrees of berberine (P3 in Figure 18.5) in the extracts of *C. chinensis* and *P. amurense* were 48.2% and 29.1%, respectively, despite similar concentrations in the two samples (0.014 mM in the former, 0.015 mM in the latter). As there are many other components present in these extracted samples with variable DNA-binding abilities, synergistic or antagonistic effects should influence the binding behaviors of berberine to DNA, which may account for the large discrepancies in binding degrees of different extracted samples. This phenomenon is common in the interaction of TCMs with macrobiomolecular targets.[49]

Changes in DNA structure by the selective binding of small molecules can play a role in the control of gene expression and may present attractive targets for small-molecule therapeutics.[50] A knowledge of structural preferences will also help in understanding the binding mechanisms of small molecules with DNA. To evaluate the DNA structural binding preferences for multiple unidentified compounds in the TCM extracts, centrifugal ultrafiltration sampling has been applied as an alternative to microdialysis. The BFA of TCMs was improved for samples of low concentration and low volume, and developed for the evaluation of DNA structural preferences for multiple small molecules simultaneously without purification.[31] In each HPLC run, the binding degrees of every resolved compound in the sample can be obtained simultaneously. The six DNA-binding compounds in the extract of the TCM *Rheum palmatum* L. were analyzed and each of them had unique structural preference to DNA.

18.2.1.3 Targeting Tublin and Microtubules

Microtubules are long, filamentous, tube-shaped protein polymers that are essential components of the cytoskeleton in all eukaryotic cells.[51] Microtubules and their dynamics are a target for anticancer drugs[52–54] since they are extremely important in the process of mitosis, during which the duplicated chromosomes of a cell are separated into two identical sets before cleavage of the cell into two daughter cells.

Figure 18.6 shows the biological fingerprinting chromatograms of *Taxus chinensis* extract targeting microtubules. Comparison of the chromatograms before and after the interaction shows that there are five peaks (P1, P2, P3, P4, and P5) with an obvious decrease in area. Comparison with authentic standards indicated that P3 was cephalomannine and P2 and P5 were the known[55,56] microtubule-binding agents baccatin III and taxol, respectively. With the help of the mass and UV

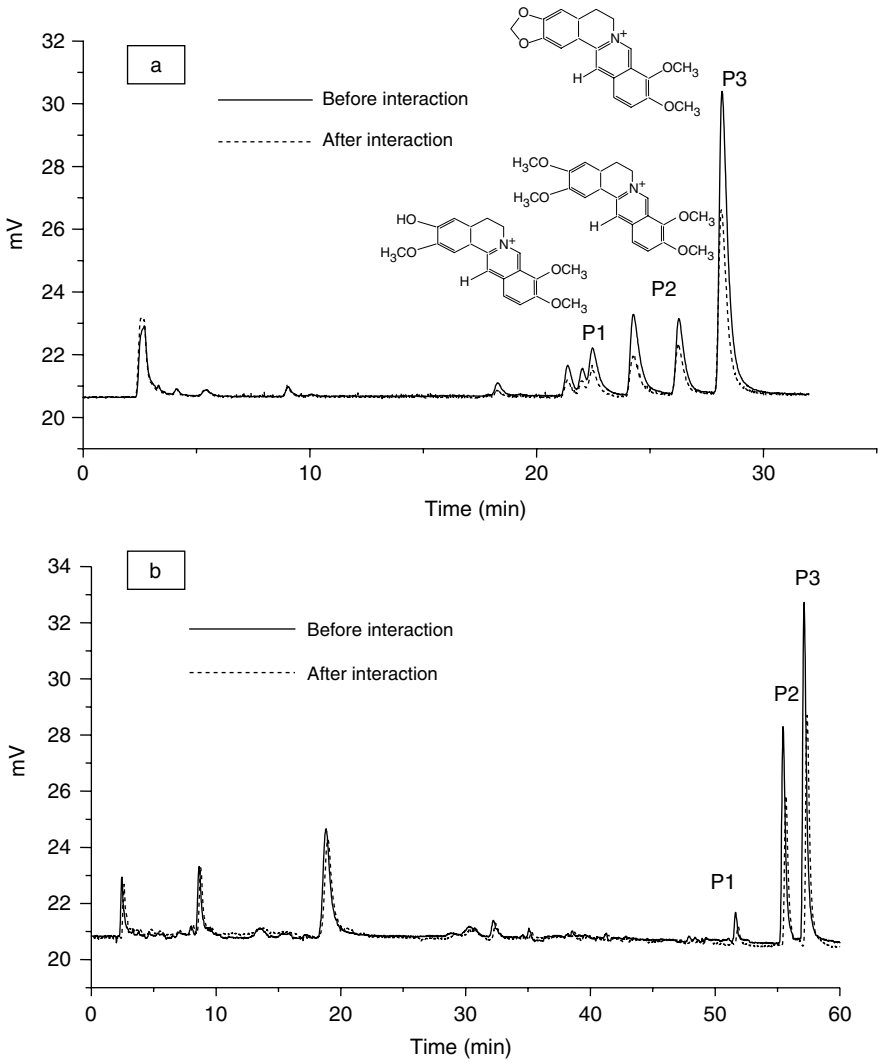


FIGURE 18.5 Biofingerprinting chromatograms for the extract of (a) *Coptis chinensis* Franch. and (b) *P. amurense* Rupr., before and after the interaction with ct-DNA. Peak identifications: (P1) jatrorrhizine, (P2) palmatine, (P3) berberine. (From Su, X.Y., Kong, L., Li, X., Chen, X.G., Guo, M., and Zou, H.F., *J. Chromatogr. A*, 1076, 118, 2005. With permission.)

spectra, and the comparison of the retention time with identified compounds, P1 and P4 were tentatively identified as 10-deacetylbaccatin III and 7-epi-10-deacetyltaxol, respectively. These compounds were promising microtubule-binding agents and their binding properties are under further investigation.

18.2.2 Biological Fingerprinting Analysis by Affinity Chromatography with Immobilized Target Molecules

Separation of TCMs by conventional chromatography such as gas chromatography (GC), reverse-phase HPLC, and normal-phase HPLC is based on the physicochemical interactions between the analytes and the mobile and stationary phases. Therefore, there is no correlation between their retention and bioactivities. Affinity chromatography is based on the biological interactions between

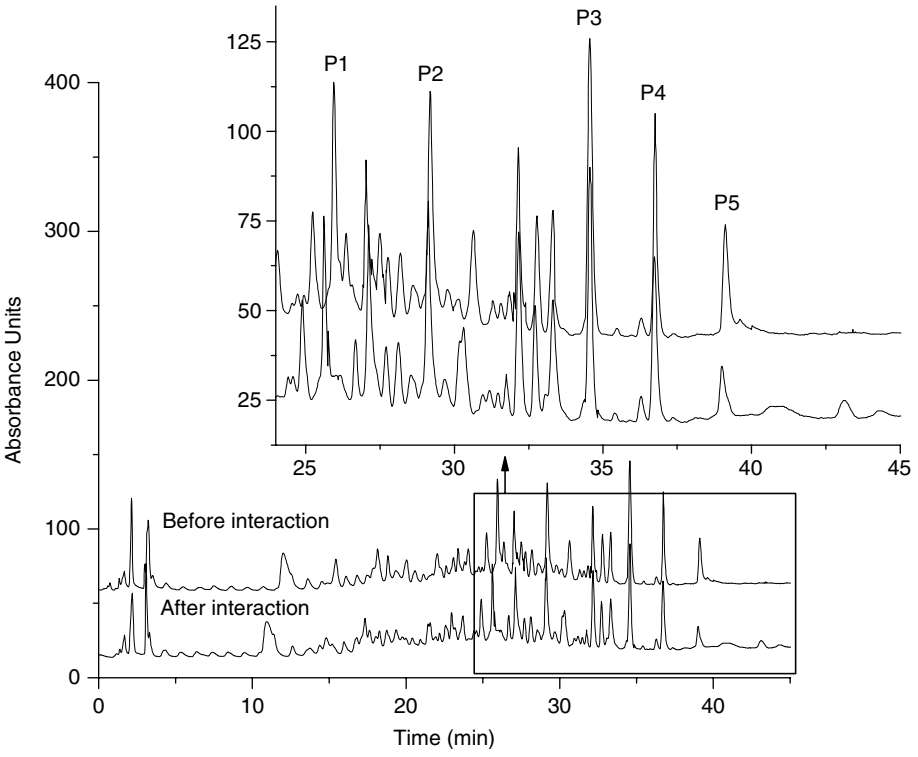


FIGURE 18.6 Biofingerprinting chromatograms for the extract of *Taxus chinensis* before and after the interaction with microtubules. Peak identification: (P1) 10-deacetylbaccatin III, (P2) baccatin III, (P3) cephalomannine, (P4) 7-epi-10-deacetyltaxol, (P5) taxol.

biologically active compounds and immobilized target macromolecules such as proteins, liposomes, and DNA, and has been successfully applied to rapidly probe drug–target binding and to study anticooperative, noncooperative, and cooperative protein–ligand interactions.[57–59]

Application of affinity chromatography to studies of TCMs and natural products has significant advantages. First, the interactions of biologically active compounds with proteins, enzymes, and DNA can be probed, even if these compounds have not yet been identified. Second, the biologically active components and their biochemical change during the processing of TCMs can be rapidly evaluated and monitored. Third, the competitive interactions occurring between biologically active compounds can be studied by adding some effective components screened from Chinese remedies or endogenous compounds to the mobile phase. Therefore, it might be expected that affinity chromatography should play an important role in unraveling the mysteries of TCMs.

18.2.2.1 Immobilized Liposome and Biomembrane Chromatography

The activity, toxicity, distribution, and other processes of orally administered drugs in the human body depend initially on their intestinal absorption across the epithelial cell membrane. Therefore, the permeability of drugs across biological membranes has been considered as one of the most important coefficients to evaluate their bioactivity.[60–62] It may become a quick and reliable way to identify the bioactivity of drugs by investigating drug–biological membrane interactions. Reverse-phase HPLC can be used to model solute–membrane partitioning, but it primarily models the hydrophobic interactions between solutes and membrane. It does not address the hydrophilic interactions between solutes and the polar head groups of membrane lipids, which is also believed to be important for the partition of drugs to biological membranes. A novel chromatography method,

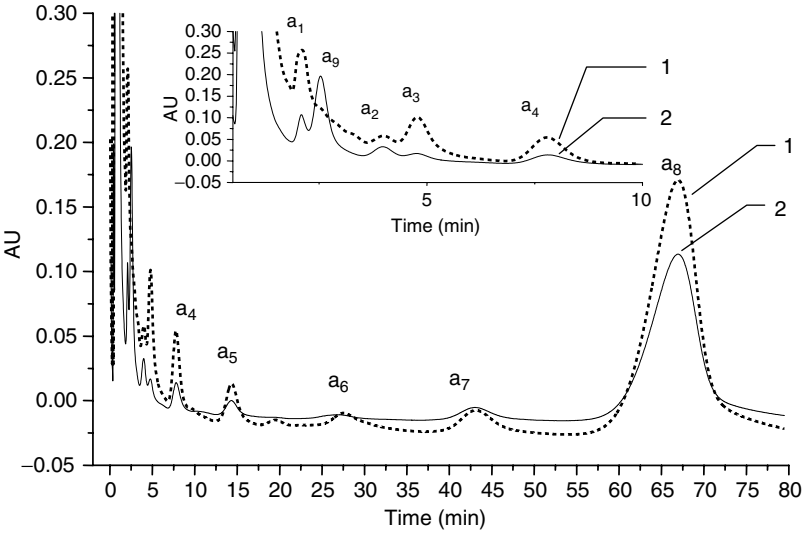


FIGURE 18.7 Immobilized liposome chromatography of Radix *Angelica sinensis*. Detection wavelength was 210 nm for chromatogram 1 and 280 nm for chromatogram 2. The inset shows an expansion of the first 10 min of the separation. (From Mao, X.Q., Kong, L., Luo, Q.Z., Li, X., and Zou, H.F., *J. Chromatogr. B*, 779, 332, 2002. With permission.)

immobilized biomembrane chromatography (IBMC), has been developed as a powerful model to study drug–membrane interactions *in vitro*.[63–65] IBMC can be subdivided into cell membrane chromatography (CMC) and immobilized liposome chromatography (ILC).

Liposomes formed from phosphatidylcholine can mimic the lipid bilayer structure and the fluidity of biological membranes. Therefore, ILC can be used for probing the ability of drugs to penetrate biological membranes. Mao et al.[66] first introduced the technique for screening and analyzing permeable compounds in TCMs. Radix *Angelica sinensis* has been used to modulate the immune system and to treat cardiovascular and cerebrovascular diseases for a long time. More than ten peaks were resolved from the methanol extract of roots of Radix *A. sinensis* based on their interactions with liposome-coated stationary phase as shown in Figure 18.7. Two of them, ligustilide and ferulic acid, which are well documented as the primary bioactive components in Radix *A. sinensis*, were identified from their mass spectra and comparison with standards.

This method can be a powerful tool for screening and analyzing the bioactive components in TCMs. However, soft gel beads such as Sepharoses and Sephadexes that were still the main supports of liposome immobilization in liquid chromatography research impeded further development of the technique because they all suffer from some drawbacks, such as large particle size, wide size distribution, and poor mechanical strength, which resulted in the low column efficiency and low separation speed. For effectively avoiding these problems, Mao et al.[67] proposed a covalent coupling method for preparing an immobilized unilamellar liposome stationary phase on porous silica. Experimental results indicated that the stability of covalently coupled liposome columns was obviously superior to that of liposome-coated columns, but the selectivity of both columns was basically identical. Both types of columns were applied in the separation and analysis of a crude extract of the TCM *L. wallichii*, which is widely used in the treatments of many ischemia disorders. Six main peaks can be distinguished in both chromatograms with comparable retentions. Better repeatability was achieved on the covalently coupled column than on the coated liposome column after three continuous injections, due to the facile loss of coated phosphatidylcholine from the coated liposome column.

Because biomembrane chromatography, with the immobilized liposome as stationary phase, could closely mimic the interaction of analytes with biological membranes, including intestine epithelial cells, it can be used as a tool for prediction of drug absorption across the intestine. Mao et al.[68] found that the weighed retention value of drugs at three pH values on the ILC correlated well with their cell absorption rate constants (P_{app}). This further supported the use of biomembrane chromatography as a model to study absorption and distribution of TCMs in the human body. Sheng et al.[69,70] expanded the application of the ILC to the analysis of the biomembrane-permeable compounds in a combined prescription of TCMs.

CMC is another important type of biomembrane chromatography. Immobilized, biological cell membranes on silica supports can apparently reflect the interaction between analytes and cell membrane or membrane receptors. He et al.[71,72] introduced the technique for the study of TCMs. By changing the different cell membranes, the bioactive components of TCMs for specific targets can be screened. This technique has been applied to study the bioactive components against vasodilatation in *A. sinensis*,[73] *Cladonia alpestris*,[74] *Herba epimedii*,[75] *Semen cuscutae*,[76] and *Leontice robustum*[77] by immobilized rabbit vascular cell membrane as stationary phase. For example, with *L. robustum*, used to treat arthralgia and myalgia, it was found that its *n*-butanol extract (HMQ-4) showed affinity to rabbit vascular cell membrane, whereas the ethyl ether, ethyl acetate, and petroleum ether extracts did not show affinity. Therefore, it was tentatively confirmed that the *n*-butanol extract contained the bioactive components against vasodilation. Subsequently, the *n*-butanol extract HMQ-4 was separated into five fractions on a silica column using ethyl acetate/methanol as the eluent. These fractions were further tested by the immobilized rabbit vascular CMC. The fractions, HMQ-42 and HMQ-44, exhibited the affinity with HMQ-44 eventually being shown to be the predominant effective active fraction in an *in vitro* pharmacological test.

18.2.2.2 Immobilized Plasma Proteins Chromatography

Wang et al.[78,79] analyzed the biologically active components in *Angelica sinensis* (Oliv.) Diels using immobilized HSA on silica as the stationary phase. Ten peaks (A1 to A10, Figure 18.8a) were obtained from the methanol extract of *A. sinensis* with an optimized mobile phase of 50 mM phosphate buffer (pH 7.4)/acetonitrile (95/5, v/v). Among them, two peaks identified as ferulic acid (A1) and ligustilide (A9) were the principal biological active components, which agrees very well with the reports in the literature. This result supported the application of immobilized plasma protein affinity chromatography as an effective way of analyzing and screening biologically active components in TCMs. Furthermore, a quality-control method for TCMs, which is very important in the identification and characterization of medicine materials and crude drugs, was developed based on the quantitative determination of ferulic acid and ligustilide in methanolic and water extracts of *A. sinensis*. It was observed that the concentrations of ferulic acid and ligustilide in methanolic extracts were 2 and 53 times higher than those in water extracts, respectively.

In the study of the methanol extract from *Artemisia capillaris* Thunb., which has been used to cure hepatitis A for a long period of time, Wang et al.[80] obtained five major peaks and several minor peaks (Figure 18.8b) using affinity chromatography with HSA immobilized on the stationary phase. Two of them were identified as scoparone (6,7-dimethoxycoumarin; SCO in Figure 18.8b) and capillarisin (CAP in Figure 18.8b).

Further demonstrating the usefulness of this affinity chromatography technique, Kong et al.[81] resolved the aqueous extracts from four kinds of TCMs on an HSA column, resulting in different chromatographic profiles.

In addition to serum albumin, AGP is another important carrier, mainly binding basic drugs,[82] and perhaps could be used as a complement to serum albumin for the immobilized protein of the stationary phase in affinity chromatography for the BFA of TCMs. Wang et al.[83] studied the methanol extract from Radix Salviae Miltiorrhizae (*Salvia miltiorrhiza*), a TCM used for the treatment of cardiovascular diseases, by affinity chromatography with AGP as the stationary phase, and more

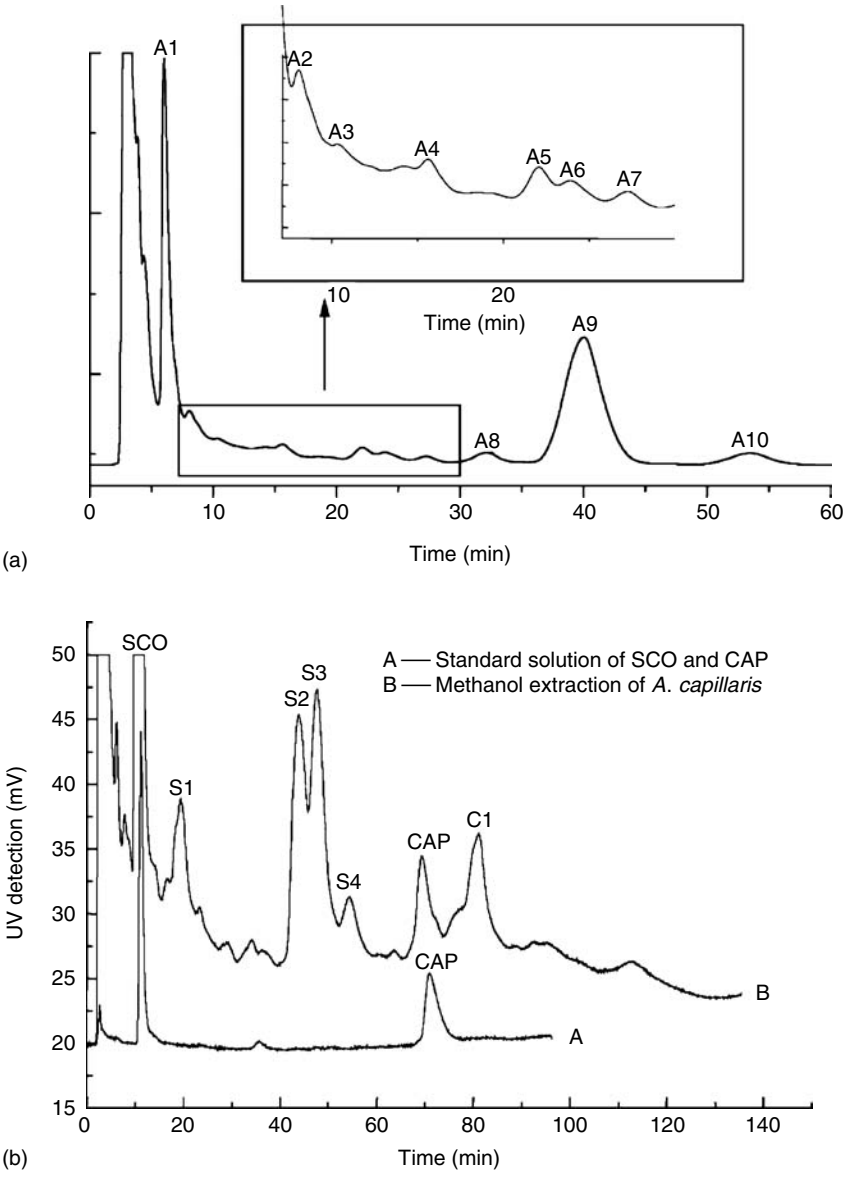


FIGURE 18.8 Chromatograms for methanol extract of (a) *Angelica sinensis* detected at 205 nm and (b) *Artemisia capillaris* Thunb. detected at 238 nm using the immobilized human serum albumin stationary phase. (From Wang, H.L., Kong, L., Zou, H.F., Ni, J.Y., and Zhang, Y.K., *Chromatographia*, 50, 439, 1999 and Wang, H.L., Zou, H.F., Ni, J.Y., Kong, L., Gao, S., and Guo, B.C., *J. Chromatogr. A*, 870, 501, 2001. With permission.)

than ten peaks were obtained. Tanshinone IIA was ultimately identified as one of the principal bioactive components. Moreover, Wang et al.[84] applied the same method to analyze the biologically active components of Rhizoma Chuanxiong. Five major peaks and a number of small peaks were resolved based on their affinity for AGP and HSA, and three of them were identified as ferulic acid, chuanxiongzine, and ligustilide, all regarded as effective components.

The possibility for fast differentiation of the TCM sources by the comparison of the fingerprint of chromatograms for six typical TCMs on the AGP stationary phase was investigated.[83] It was

observed that different TCMs, even for Rhizoma cimicifugae from three different geographical sources, showed different fingerprint characteristics under the same operating conditions thereby providing a distinguishing characteristic.

18.2.2.3 Immobilized DNA Chromatography

An immobilized DNA stationary phase on silica has been developed for the BFA of bioactive components in TCM extracts.[85] In this approach, ct-DNA was bound onto the surface of amino silica by the formation of a phosphoramidate bonding between the 5′ terminal phosphate group and the amino silica, and the prepared column was applied to the BFA of *C. chinensis* Franch. extract. It was observed that six main peaks were separated based on their affinity to DNA. By comparison of the retention time and UV/Vis spectrum with standards, one of the peaks was identified as palmatine and another was a combination of berberine and jatrorrhizine.

18.2.3 2D-HPLC: Coupling Affinity Chromatography to Reverse-Phase HPLC

Although affinity chromatography can provide high separation selectivity for BFA of TCMs, its separation efficiency is lower in comparison to reverse-phase HPLC. Peak overlapping often occurs if the overall number of compounds in a sample exceeds the peak capacity of the column. TCMs are very complex samples in themselves, and generally contain up to hundreds or even thousands of different compounds. Therefore, any single peak may contain several compounds during separation of TCMs by affinity chromatography. Multidimensional separation techniques provide dramatic improvements in peak capacity. In a two dimensional (2D) separation, the total capacity is equal to the product of the peak capacities in both dimensions, resulting in much higher resolution. Two dimensional liquid chromatography has been widely used, since it appeared in 1990, to characterize and separate biomolecules, polymers, and other complex mixtures due to its high peak capacity, powerful separation, and resolution ability.[86–89] A comprehensive 2D-HPLC separation system has been developed for the analysis of TCMs.[38,90]

Recently, the performance of comprehensive 2D-HPLC has been improved for analysis of TCMs.[91] First, the speed of the analysis in the second dimension was increased by using a silica monolithic ODS column. Second, a software-based method to increase the intensity of the weak signals by normalization of peak heights was developed to observe the low-abundance compounds in TCMs. This improved system was tested using a methanol extract of Rhizoma Chuanxiong by using a CN column for the first dimensional separation and a silica monolithic ODS column for the second dimensional separation. There was a marked improvement in peak resolution, with about 120 components separated with UV detection within 130 min. This result meant that the number of peaks detected doubled and the analysis time halved in comparison with previously reported results obtained without the use of the silica monolithic ODS column.[37]

A combination of an immobilized HSA affinity column and a silica monolithic ODS column was applied to the analysis of the extract of *R. palmatum* L.,[92] which is commonly used for the treatment of hemorrhage of the digestive system, acute hepatitis, gallstone, inflammation of sweat glands, and for inhibition of *Helicobacter pylori*.[92] The affinity chromatography with HSA-immobilized stationary phase was applied to separate the bioactive components according to their affinity to protein in the first dimension. Then, the unresolved bioactive components retained on the HSA column were further separated on the silica monolithic ODS column in the second dimension. By coupling the 2D separation system with diode array and MS detectors, the UV and molecular weight information of the separated compounds were also obtained. Most components had weak interactions with the HSA stationary phase and were eluted in the first ten cycles (Figure 18.9). The later eluted components, which exhibited a single peak on the HSA column, also contained several low-abundance components. Because the relative abundance of components in extracts of TCMs is usually quite different, some of the peaks were not baseline separated and could not be clearly seen

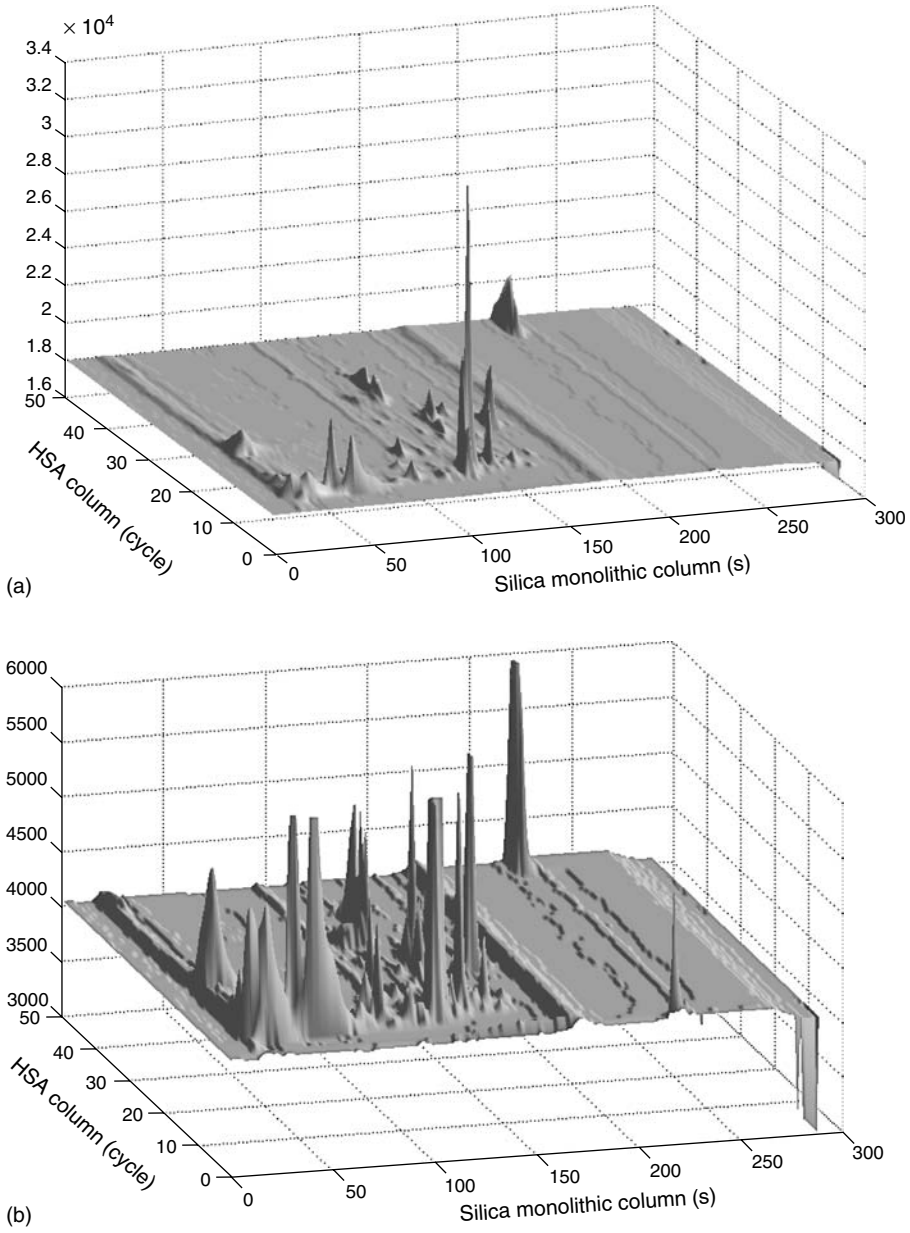
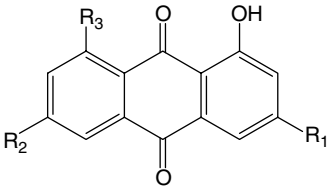


FIGURE 18.9 Three dimensional landscape images of *Rheum palmatum* L. (a) Original image without normalization and (b) after normalization by a value of one-eighth of the highest peak. Detection wavelength, 250 nm. (From Hu, L.H., Li, X., Feng, S., Kong, L., Su, X.Y., Chen, X.G., Qin, F., Ye, M.L., and Zou, H.F., *J. Sep. Sci*., 29, 881, 2006. With permission.)

with the blurred spots in the 2D chromatogram. Therefore, the chromatograms were manipulated to increase the intensity of the weak signals by normalization of peak heights.[91] The three dimensional landscape images of the *R. palmatum* L. extract before and after normalization of peak heights to one-eighth of the highest peak height is shown in Figure 18.9. It can be clearly seen in Figure 18.9b that more possibly bioactive components can be separated from a single peak from the HSA column after the second dimensional separation by the silica monolithic ODS column and normalization



R_1	R_2	R_3	
CH_3	H	OH	Chrysophanol (MW 254.23)
CH_2OH	H	OH	Aloe-emodin (MW 270.23)
CH_3	H	O-$C_6H_{11}O_5$	Chrysophanol-8-*O*-glucopyranoside (MW 416.38)
CH_2OH	H	O-$C_6H_{11}O_5$	Aloe-emodin-8-*O*-glucopyranoside (MW 432.38)
CH_3	OH	O-$C_6H_{11}O_5$	Emodin-8-*O*-glucopyranoside (MW 432.38)
CH_3	OCH_3	O-$C_6H_{11}O_5$	Physion-8-*O*-glucopyranoside (MW 446.41)

FIGURE 18.10 Molecular structures and weights of the identified compounds in the extract of *Rheum palmatum* L. (From Hu, L.H., Li, X., Feng, S., Kong, L., Su, X.Y., Chen, X.G., Qin, F., Ye, M.L., and Zou, H.F., *J. Sep. Sci*., 29, 881, 2006. With permission.)

of peak heights. The comprehensive 2D liquid chromatography system herein shows its high peak capacity, sensitivity, and powerful resolving potential for BFA of the bioactive components in TCMs and natural products.

The main bioactive compounds reported for *R. palmatum* are anthraquinones and their glycosides (Figure 18.10).[93–96] By comparing the UV and mass spectra of detected peaks in the extract with those of standards and literature reports, six of them can be primarily identified as aloe-emodin-8-*O*-glucopyranoside, chrysophanol-8-*O*-glucopyranoside, physion-8-*O*-glucopyranoside, aloe-emodin, emodin-8-*O*-glucopyranoside, and chrysophanol.

18.2.4 Biological Fingerprinting Analysis Based on *In Vitro* Metabolism

A drug absorbed orally is transported via the portal circulation to the liver, where it is usually subjected to hepatic metabolism followed by elimination as bile or via the kidneys. Because the liver is the major organ for drug metabolism, increased throughput screening assays have been developed to determine the metabolic stability of drugs. A method based on HPLC by *in vitro* metabolism with liver homogenate for screening of TCMs was designed.[15] This can be readily scaled up by an easily controlled process with good analytical reproducibility. Figure 18.11 shows the HPLC-UV fingerprinting chromatogram of Radix *A. Sinensis* extract, which was incubated with homogenized liver from Sprague–Dawley rats.

Comparison of the chromatograms shows that some peaks disappeared and some new ones appeared during incubation. The structural information on these compounds can then be determined primarily by MS analysis. A metabolite of coniferyl ferulate, that is, ferulic acid ethyl ester, was identified using HPLC-MS, UV/Vis, and IR. Antineoplastic activity of coniferyl ferulate and ferulic acid ethyl ester was detected using the MTT assay. It was observed that coniferyl ferulate has considerable acute inhibitory activity to HeLa cell culture whereas its metabolite did not show any antineoplastic activity.

Pan et al.[97] developed oxidized carbon nanotubes as a matrix of MALDI-TOF MS for analysis of small molecules. Reliable quantitative analysis of jatrorrhizine and palmatine with a wide linear range and good reproducibility of relative peak areas was achieved using this matrix. MALDI-TOF MS with this unique matrix was adopted for the BFA of several TCMs before and after metabolism with mouse liver homogenate *in vitro*. As in the fingerprinting obtained by HPLC, some ion peaks in the mass spectrum disappeared and some new ones appeared after metabolism. These ion peaks can be primary identified by their molecular weight. The change of the concentration of each

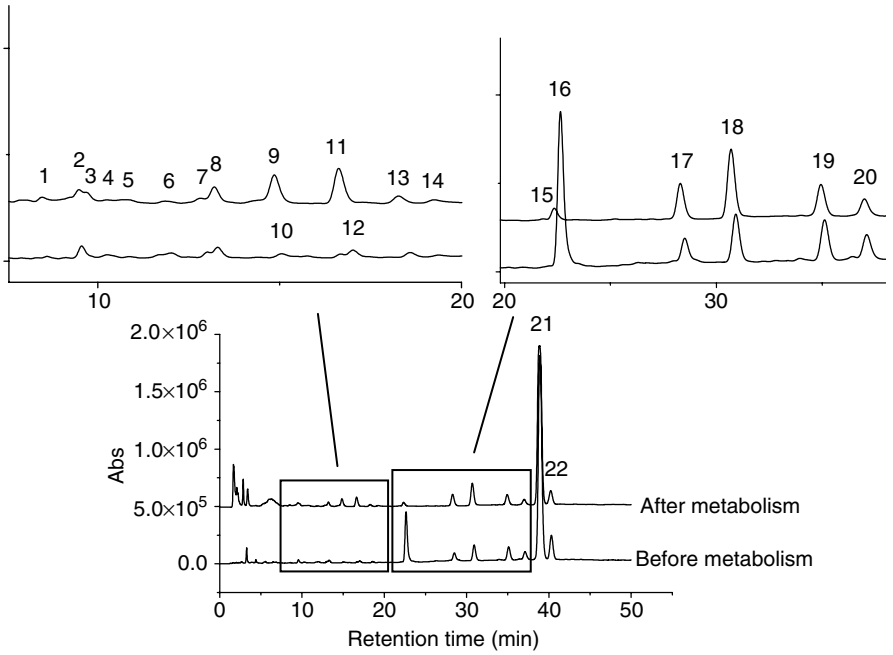


FIGURE 18.11 HPLC-UV-based biological fingerprinting chromatograms for extract of Radix *Angelica sinensis* before and after *in vitro* metabolism with Sprague–Dawley rat liver homogenate. Detection wavelength, 205 nm. Peak 9, ferulic acid ethyl ester; peak 16, coniferyl ferulate; peak 21, ligustilide.

compound can also be obtained by quantitative analysis. Owing to the different selectivity and sensitivity, the result obtained by the MS analysis is a complement to chromatographic methods.

18.3 PHARMACOLOGICAL SCREENING WITH ANIMAL MODELS

The routine process for screening is to extract a single ingredient or single distilled fraction from TCMs, and then to determine its bioactivity using classic pharmacological means. The whole animal model is the most classical pharmacological screening model, and is a very important aspect of medicinal evaluation because it can apparently determine the efficacy, side effects, and toxicity of medicines as a whole. Although this method is expensive and inefficient, at present it is still a primary means of drug discovery and evaluation. In recent years, some improved methods have been developed for pharmacological screening of TCMs with animal models.

18.3.1 Pharmacological Screening in Biofluids

In conventional pharmacological screening of TCMs, some components are directly extracted from a single medicine or formulas by a specific separation method, and then pharmacological evaluation is carried out to determine the bioactivity of these components. Although the method is very straightforward and objective, some deficiencies have been found in screening of TCMs: generally, TCMs are taken orally and some components are changed by gastrointestinal or liver metabolism; therefore, the actual active components are not always the components in original TCMs; and, because the components of TCMs are very complicated, many inactive compounds interfere with the *in vivo* experiment results so that authentic evaluation for efficacy is very difficult. In 1988 and 1992, Homma et al.[11] proposed a serum pharmacological screening strategy, subdivided into serum pharmacology and serum pharmacochemistry, based on the hypothesis that the active compounds

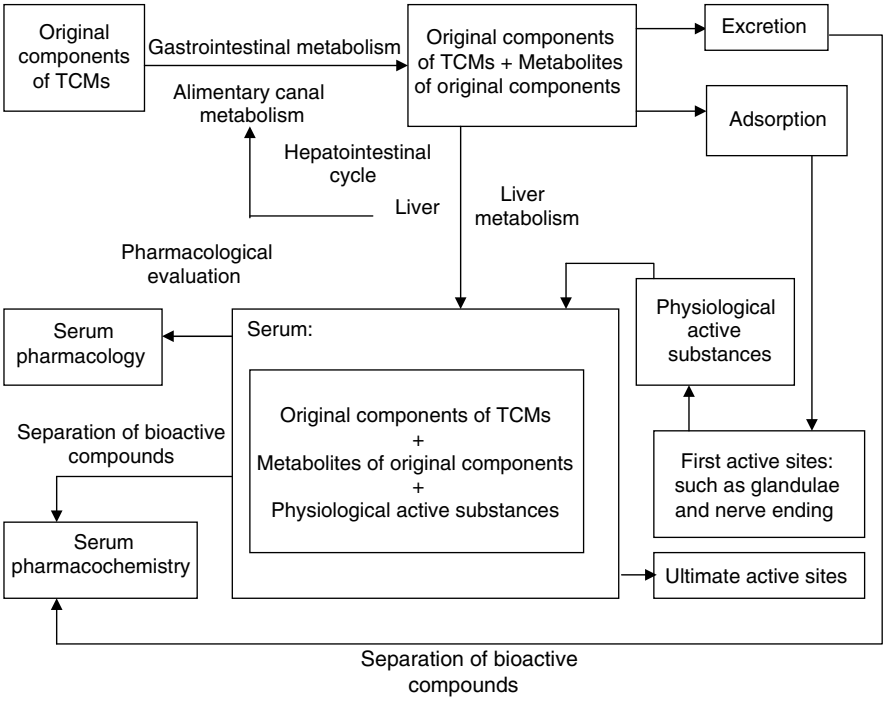


FIGURE 18.12 Schematic of serum pharmacology and serum pharmacochemistry. (From Huang, X.D., Kong, L., Li, X., Chen, X.G., Guo, M., and Zou, H.F., *J. Chromatogr. B*, 812, 71, 2004. With permission.)

should appear in blood and urine with appropriate concentrations and urinary excretion rates after the administration of medicines (Figure 18.12).

Serum pharmacochemistry utilizes the physiology of animals to separate the bioactive components of TCMs into animal fluids, and then these bioactive components in the fluids can be subsequently extracted and identified by common techniques. The method is very straightforward and is helpful in determining the real active components in TCMs. Furthermore, it separates the possible bioactive components from numerous components of TCMs.

Saiboku-To, a very popular Chinese medicine in Japan consisting of ten different plant extracts, is used for the treatment of bronchial asthma. β-D-Glucuronidase-treated urine samples, collected before and after the administration of Saiboku-To to healthy and asthmatic subjects, were applied to a rapid-flow fractionation (RFF) apparatus to afford three pH-dependent fractions. HPLC with a multichannel ultraviolet absorption detector identified three new peaks in the postadministration urine that were eventually identified as magnolol, a major component in *Magnolia officinalis*, and 8,9-dihydroxydihydromagnolol and liquiritigenin, components of *M. officinalis* and *Glycyrrhiza glabra*, respectively. These chemicals had been suggested as possible candidates for antiasthmatic agents in Saiboku-To.

In 1997, Homma et al.[12] further improved the method for analyzing the Saiboku-To. First, normal-phase chromatography was used to detect magnolol, the most hydrophobic component among the β-D-Glucuronidase-treated urinary products of Saiboku-To. Then, reverse-phase HPLC was performed to find other more polar components than magnolol. In addition to magnolol, 8,9-dihydroxydihydromagnolol, and liquiritigenin, five other components were found in the postadministration Saiboku-To urine, namely medicarpin from *G. glabra*, baicalein, wogonin, and oroxylin A from *Scutellaria baicalensis*, and davidigenin of unknown origin. Furthermore, they identified seven flavonoids and two anthraquinone derivatives in urine specimens following administration

of the TCMs Daisaiko-To and Shosaiko-To used for the treatment of hyperlipidemia and chronic hepatitis.[98] Dihydrooroxylin A with *S*-configuration (80% ee), the first example of stereoselective hydrogenation of a herbal flavonoid by some of the metabolizing enzymes of intestinal bacterial flora or the human liver, was also isolated from human urine collected after oral administration of Daisaiko-To and Shosaiku-To.[99] The observation that the amount of dihydrooroxylin A in the herbal medicines was much less than that excreted into the urine indicated that the *S*-isomer was principally formed in the human body through stereoselective transformation of oroxylin A by intestinal bacterial flora or hepatic enzymes.

In China, Wang[100] studied the active components of TCMs Radix Polygalae (*Polygala tenuifolia* L., used as a tonic, sedative, expectorant, and anti-inflammation agent), *A. capillaris* Thunb., *Vaccinium vitis-idaea* L. (used as anti-inflammatory folk medicine to treat respiratory system infections), and Rhizoma Atractylodis Macrocephalae (*Atractylodes macrocephala* Koidz., used for treating abdominal pain and gastroenterology disease) by serum pharmacochemical screening. He found that the primary components in Radix Polygalae, namely onijisaponins A-G and tenuifoliose A-P, had no efficacy with respect to the sedative or apophlegmatisant effects of Radix Polygalae. However, from the serum collected after oral administration of Radix Polygalae he obtained 3,4,5-trimethoxycinnamic acid and its metabolite methyl 3,4,5-trimethoxycinnamic acid that were identified as the actual bioactive components.

Furthermore, Wang[100] found that the trimethoxycinnamic acid in serum was from the gastrointestinal metabolism of tenuifoliside A and C, components of Radix Polygalae, and that methyl 3,4,5-trimethoxycinnamic acid was the metabolite of trimethoxycinnamic acid in liver. Therefore, tenuifoliside A and C are the prodrugs. Moreover, when he studied the Rhizoma Atractylodis Macrocephalae, it was found that atractylone and atractylenolide I and II, the generally considered active components, could not be found in blood, whereas (6*E*,12*E*)-tetradecatriene-8,10-diyne-1,3-diol from the metabolism of another original component of Rhizoma Atractylodis Macrocephalae, (4*E*,6*E*,12*E*)-tetradecatriene-8,10-diyne-1,3-diol diacetate, was obtained in serum. Both of these tetraene-diyne-diols were efficacious in eliciting the antianabrosis and improvement of the digestive actions of Rhizoma Atractylodis Macrocephalae. Thus, (6*E*,12*E*)-tetradecatriene-8,10-diyne-1,3-diol is the real bioactive component of Rhizoma Atractylodis Macrocephalae *in vivo* while the diacetate is a prodrug (Figure 18.13). Continuing the work further, Wang also obtained the bioactive components arbutin and fraxin in *V. vitis-idaea* L.[101] and 6,7-dimethylesculetin in *A. capillaris* Thunb.[100] by using the method.

Ding et al.[102] made a preliminary serum pharmacochemistry study of the TCM Radix *Rehmannia glutinosa*, which has been used in the therapy of dementia. First, they established an HPLC fingerprint of the plant material and then recorded HPLC fingerprints of rat serum samples obtained after orally taking different extracts of the TCM (including aqueous extract, liposoluble extract, and alcoholic extract) and those of control rat serum samples. They found that the HPLC fingerprint of rat serum sample obtained after orally taking an aqueous extract was significantly different from those of rat serum samples obtained after orally taking the other extracts and control rat serum samples. Although the aqueous macromolecular fraction was the principal active fraction of Radix *R. glutinosa*, they did not continue the research in order to identify the bioactive components.

A similar investigation of *L. chuanxiong* Hort. to determine HPLC fingerprints of rat serum samples indicated that ferulic acid, which is generally considered an active component in *L. chuanxiong*, was not the real bioactive compound for antirelease of serotonin from platelets and inhibition of Ca^{2+} transport toward rabbit blood endotheliocytes.[103] However, for some TCMs, especially for formulas composed of many original medicines, it is still difficult to separate all components from biofluids by current separation techniques. Therefore, a number of current studies of TCMs in China are focusing on the use of the animal serum collected after oral administration as a preliminary pharmacological screening for specific pharmacological evaluation, that is, serum pharmacology. As mentioned in the text above, compared with conventional pharmacological experiments,

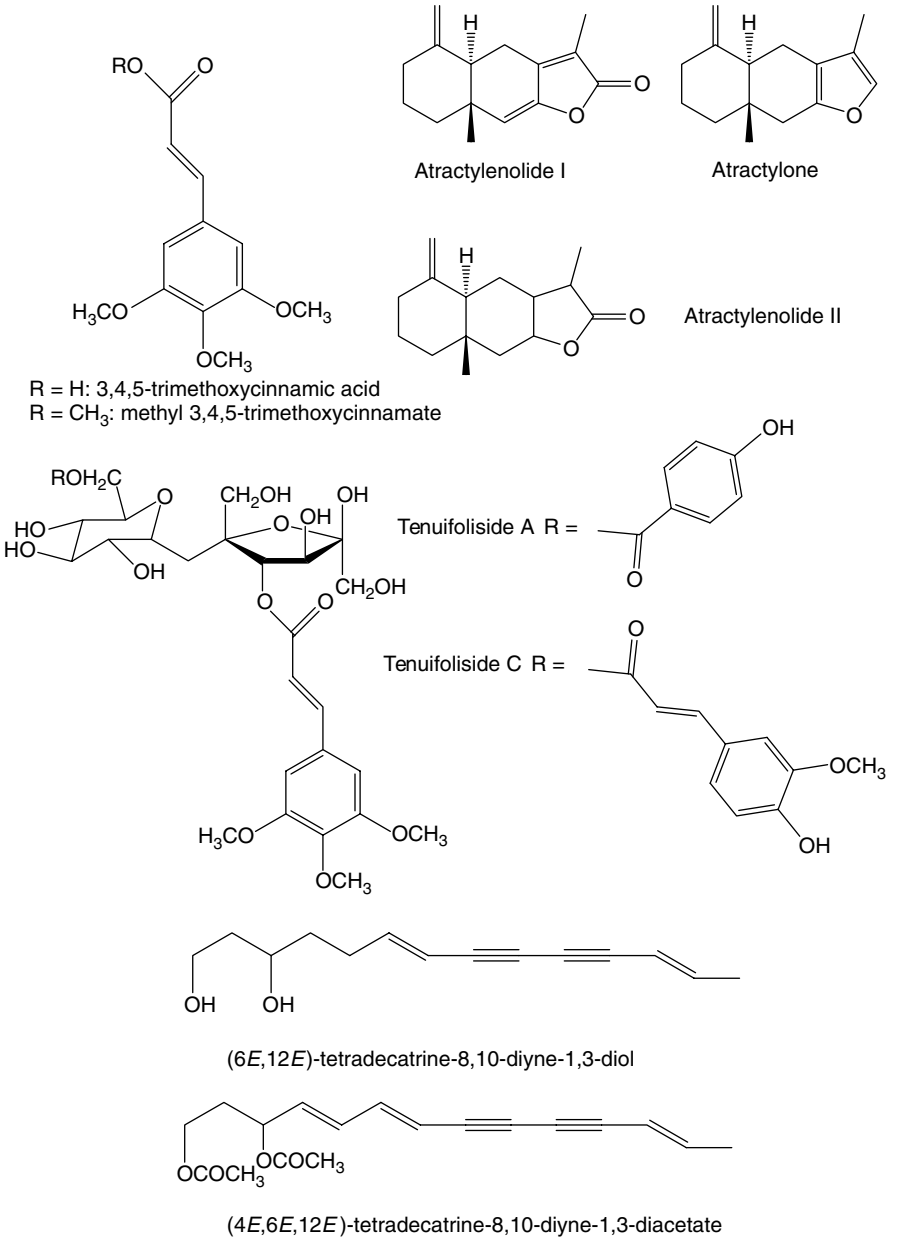


FIGURE 18.13 Molecular structures of compounds in Rhizoma Atractylodis Macrocephalae and Radix Polygalae.

the method excludes the interference of many inactive compounds in TCMs and embodies the actual efficacy of TCMs because the serum contains the real bioactive components. The screening of TCMs with antitumor activity and antihepatofibrosis activity by serum pharmacology has been reviewed.[13,104]

One of three possible results can usually be found in serum pharmacology experiments: (1) the original medicines have the specific efficacy, but the serum collected after oral administration of the original medicines does not have the efficacy;[105] (2) the original medicines are not efficacious

in vivo, but the serum after oral administration does have efficacy,[106] which indicates that the real bioactive compounds arise from the transformation of some components in original medicines by absorption and metabolism *in vivo*; and (3) both the original medicines and the serum after oral administration are bioactive,[107] which indicates that the bioactive compounds might exist in the original medicines. The method can be associated with modern high-throughput screening methods by specific drug targets such as cell, bacterial, tissues, or organs, and gives a preliminary pharmacology evaluation of TCMs. Furthermore, if necessary, the bioactive components in the serum with specific pharmacological function can also be obtained by the method of serum pharmacochemistry described earlier.

18.3.2 Pharmacological Screening with Organ and Tissue Models

As is generally known, many original components of TCMs are usually effective only after biological transformation *in vivo*, namely, their metabolites result in the efficacy.[14] Recently, a method for screening *in vitro* metabolism of TCMs using liver tissues was designed by Kong et al.[15] In contrast to studying *in vivo* metabolism using the whole animal model, *in vitro* metabolism can be scaled up easily by providing enough liver tissues and thus larger amounts of the metabolites can be obtained for study. Furthermore, the *in vitro* process can be readily controlled and good reproducibility can be obtained.

With the development of modern medicine and pharmacology, more and more animal tissues and organs have been selected as drug screening models, such as *in vitro* blood vessels, heart perfusion, and tissue culture. These models can monitor the effects of drugs under normal physiological pathological conditions. They overcome some deficiencies of the whole animal models. First, the amount of sample required for screening is reduced; usually the amount of sample in the whole animal model needs more than 1–5 g (according to the dosage and the size of the animal used), whereas the amount of sample used in the tissue and organ model is as low as one-tenth of the whole animal model or less. Second, the labor intensity is reduced and the scale of screening is enlarged. Multisample screening can be performed at the same time so that the efficiency is improved and the cost is reduced. Third, it decreases the interference of other *in vivo* factors and is more likely to provide an authentic evaluation of pharmacology.

Although the organ and tissue models have been widely used in drug screening, a few examples can be seen that have been used in screening of TCMs. Su et al.[108] carried out the preliminary screening of 12 TCMs for inhibition of blood vessel growth in chicken embryos, and it was found that the Rhizoma Curcumae and Radix Curcumae were active. A screening method was proposed for the pharmacological screening of TCMs that studied the distribution of components to organs and tissues which, similarly to the serum pharmacological screening approaches, is still based on the hypothesis that the active compounds should appear *in vivo* with appropriate concentrations after the administration of medicines.[109] After feeding the TCMs Rhizoma Chuanxiong, Herba Ephedrae (utilized for perspiratory, antitussive, antipyretic, and anti-inflammatory purposes) and Rhizoma Gastrodiae (used to treat vertigo, blackout, headache, and hemiplegia) to rats, various tissues such as the brain, stomach, liver, and kidney, and serum and urine were analyzed by HPLC, LC-MS, and GC-MS.[109] It was found that seven and four components of Rhizoma Chuanxiong entered the blood and brain, respectively. Thus, vanillin, senkyunolide H, senkyunolide I and its isomer, ferulic acid, and two undetermined compounds were detected in serum, and senkyunolide H, senkyunolide I and its isomer, and ferulic acid were detected in brain, which was in agreement with previous reports on the dominant active components in Rhizoma Chuanxiong. Moreover, in the study of Rhizoma Gastrodia, it was observed that the distribution of gastrodin and gastrodigenin in the organs was liver > stomach > kidney, and stomach > kidney > liver, respectively. Gastrodin and gastrodigenin were also detected in brain and urine by GC-MS, which further verified the previous results that the active components in Rhizoma Gastrodia are gastrodin and gastrodigenin, and gastrodin is prone to metabolize into gastrodigenin *in vivo*.

18.4 SCREENING METHODS WITH CELLULAR MODELS

In recent years, the field of cell-based screening has expanded rapidly as innovations in target selection and instrumentation increase the number of targets that can be efficiently screened in cellular formats. Cell-based screens can be configured to provide a broad range of data on drug activity, mechanism of action, and drug ability. Compared with the whole animal models, the cellular models based on different diseases and mechanisms are more adaptable to large-scale drug screening because the resource and culture of cells are relatively economical and easy. It has become a very important means in screening and evaluation of TCMs. On the basis of the selection of cells, it can be subdivided into normal and pathological cell models. For example, lymphocytes, splenocytes, and neutrophil granulocytes have been used for evaluating the anti-inflammatory and immune modulation efficacy of TCMs.[110] Certainly, the most widely used models are tumor cells and other simulated pathological cell models. Zhang et al.[16] screened the ethanolic extracts of 100 common traditional Chinese drugs, which are widely used in many prescriptions in the treatment of cancer in China, for multidrug resistance of the KBV200 cell line *in vitro* with the MTT method. The results showed that extracts of Fructus Lagenariae Sicerariae, Radix Glycyrrhizae, Poria, Herba Andrographitis, Radix Sophorae Tonkinensis, Caulis Mahoniae, Folium Artemisiae Argyi, Rhizoma Curcumae, and Fructus Cnidii had multidrug resistance reversal activity. Another five extracts were cytotoxic to the KBV200 cell line.

Twenty-nine of fifty herbs usually used in Guangxi Province were positive in a screen for their antiliver tumor activity using the MTT method and cultured liver cells.[111] Lu et al.[112] studied the selective inhibition of a polysaccharide from *Acanthopanax giraldii* Harms var. *hispidus Hoo*, which has been used in the treatment of rheumatism as well as a tonic, on human gastric cancer cells, human embryonic tenocytes, and human lymphocytes. The morphology of the cells was observed by optical microscopy and the MTT method was used to examine their activity and functions. The polysaccharide exerted selective inhibition on the activity and function of human gastric cancer cells, slight inhibition on human embryonic tenocytes, and no inhibition on healthy human lymphocytes, which indicated that the polysaccharide may be used safely to prevent or treat gastric cancer due to its selective inhibition. The *in vitro* antitumor activity of the total steroidal glycoside fraction from the root of *Cynanchum auriculatum* was investigated by determining cell growth curve, MTT test, protein content assay, and morphological observation.[17] The results showed that the fraction exhibited potent cytotoxic effect on all four solid tumor cell lines, Hce-8693, PC_3, HeLa, and PAa, in a concentration-dependent manner.

It has been estimated that more than 4000 components in Chinese medicinal plants have been screened by *in vitro* tumor cell models or *in vivo* animal–transplanted tumor models (xenografts).[113,114] In addition to the well-known taxol, camptothecin, and vincristine anticancer compounds, β-elemene from Rhizoma Curcumae, matrine from Radix Sophorae Flavescentis, colchicines, etc., exhibited excellent antitumor or anticancer activity in clinical practice.[115,116]

Xiong et al.[117] reported the screening of 27 TCMs for inhibition of HIV infection of the H_9 cell line and found that 11 TCMs including Radix Sophorae Flavescentis, Flos Lonicerae, and Spica Prunellae showed activity. Progress on the screening of TCMs for antihepatitis B virus (HBV) by *in vitro* 2.2.15 cell line has been reviewed,[118] and Mi et al.[119] carried out the screening of 21 Chinese medicinal herbs for antiviral agents by means of 2.2.15 cell model. The extracts of *Phyllanthus urinarin* and *Polygonum cuspidatum* exhibited obvious effects on duck HBV and human HBV, whereas the extract of *Eclipta alba* showed limited inhibition on HBV DNA polymerase.

18.5 SCREENING BASED ON THE ACTIVITY OF RECEPTORS AND ENZYMES

In essence, the efficacy of drugs is closely associated with the *in vivo* binding to critical biomacromolecules such as enzymes, receptors, DNA, RNA, and proteins. This provides an important path to screen drugs at the molecular level. Because the requirement for amount of sample is very low

in these models, one drug may be evaluated with multiple targets. So far, about 600 drug targets have been found and identified, and with the development of molecular biology, recombinant DNA techniques, molecular pharmacology, biochemistry, genomics, and proteomics, it can be expected that more drug targets will be gradually revealed in the future. Therefore, the target-based screening models will become more and more popular due to their diversity and specificity. Since most of the drug targets are receptors and enzymes, drug discovery based on the binding of drugs and receptors as well as their strength of interaction or the change of enzyme activity is the mainstream of target-based screening. The receptor and enzyme models have become fundamental to modern high-throughput screening of drugs. The receptor and enzyme models are rapid, economical, highly sensitive, and specific. The models remedy some deficiencies in whole animal models and provide the possibility of functional screening. In discovery of Western drugs, they have become a routine screening means. As for Chinese medicines, although their treatment theory is different from the Western medicines, they are identical in molecular mechanism. As early as the 1980s, Wang et al.[20,21] had used the receptor and enzyme models for screening TCMs. More than 400 extracts from 150 common TCMs were systematically screened against 11 receptors and enzymes. Bioactive compounds such as alantolactone, medicarpin, and ursolic acid were found. Gao et al.[120] established and optimized the receptor model based on human muscarinic 1 receptor (M1) for high-throughput screening of 400 aqueous extracts of TCMs. By using this technology, three extracts of TCMs were identified as potential agonists to the M1 receptor.

Sun et al.[121] studied *N*-methyl-D-aspartate receptor (NMDAR) antagonist activity in traditional Chinese stroke medicines. They used the patch-clamp technique to screen 22 TCM stroke drugs for NMDAR antagonist activity in cultured cortical neurons. The drugs were also screened for their ability to abate NMDA-induced neurotoxicity. Aqueous extracts of *S. baicalensis*, *Stephania tetrandra*, and *S. miltiorrhiza* blocked currents induced by NMDA, but none of these extracts blocked NMDA-induced neuronal death. These TCM drugs may exert therapeutic effects due to their Mg^{2+} content. An aqueous *Uncaria rhynchophylla* extract blocked NMDA-evoked currents by $54.98 \pm 8.61\%$ even at $+60$ mV and reduced NMDA-induced neuronal death by $59.13 \pm 3.52\%$. NMDAR antagonist activity may underlie the neuroprotective effects of this TCM.

The platelet activating factor (PAF) receptor has been used to study inhibition by ginkgolide B from Folium Ginkgo.[122] A methanol extract of "Gouteng" (*U. rhynchophylla*) (commonly used as antihypertensive, sedative, and anticonvulsant agent) hooks and stems was assessed for its ability to inhibit the binding of radioligands to 13 different receptors.[22] The extract inhibited ligand–receptor binding by more than 60% to α_2-adrenoceptors, dopamine 1, 5-HT1A (serotonin), opiate, and $GABA_A$ and $GABA_B$ (γ-aminobutyric acid) receptors. Bioassay-guided fractionation resulted in the isolation of ursolic acid (muscarinic and sulfonylureas activities), husutine (α_2- and β-adrenoceptor, 5-HT1A and 5-HT2, opiate and sulfonylureas activities), and epiallocorynanthine (β-adrenoceptor, 5-HT1A and 5-HT2, and opiate receptor activities).

In addition to receptors, some potential drug candidates can be found directly from the inhibitory activity on a specific enzyme. Thus, huperzine A, a selagine-type alkaloid, was isolated from *Huperzia serrata*, a plant used in Chinese folk medicine for treatment of some mental disorders. Pharmacological studies have revealed that it has powerful and reversible anticholinesterase activity and its inhibitory effect is three times as potent as that of physostigmine, a well-known cholinesterase inhibitor (ChEI). Huperzine A has been used for the treatment of myasthenia gravis, with an improvement in 98% of the 128 cases treated, and for improving memory in the case of senile dementia and senile or presenile simple memory disorders. Clinical trials indicated that huperzine A was a promising candidate as a ChEI in the treatment of Alzheimer's disease.[123] Some huperzine derivatives have been synthesized including isovanihuperzine A (IVHA) that deserves further study as a novel ChEI due to its potent acetylcholinesterase inhibition and its lower toxicity than huperzine A.[124]

The inhibitory activity of 35 Chinese medicines, including extracts of traditional medicines or clinically useful recipes, herbs, and isolated compounds, against human immunodeficiency virus reverse transcriptase was investigated *in vitro*.[125] Fourteen of the thirty-five medicines were found

to be active. Baicalin, in particular, was studied in great depth. It was found to be a noncompetitive inhibitor of HIV RTase, with an effective concentration (IC_{50}) of 22 mM.[125]

El-Mekkawy et al.[126] isolated a new highly oxygenated triterpene named ganoderic acid α from a methanol extract of the fruiting bodies of *Ganoderma lucidum* together with 12 known compounds. The regular consumption of *G. lucidum* in the form of tea or mushroom powder was believed to preserve the human vitality and to promote longevity. *G. lucidum* has also been used for the prevention or treatment of a variety of diseases including cancer.

The anti-HIV-1 and anti-HIV-1-protease activities of these compounds were studied. Ganoderiol F and ganodermanontriol were found to be active as anti-HIV-1 agents with an inhibitory concentration of 7.8 mg/mL for both, and ganoderic acid B, ganoderiol B, ganoderic acid C1, 3-β,5-α-dihydroxy-6-β-methoxyergosta-7,22-diene, α-ganoderic acid, ganoderic acid H, and ganoderiol A were moderately active inhibitors against HIV-1 protease with a 50% inhibitory concentration of 0.17–0.23 mM.

Prolyl endopeptidase (PEP) is an enzyme that plays a role in the metabolism of proline-containing neuropeptides, for example, vasopressin, substance P, and thyrotropin-releasing hormone (TRH), which have been suggested to be involved in learning and memory processes. In a systematic screening for PEP inhibitors from TCMs, Fan et al.[23] found that methanol extract from the underground part of *Rhodiola sacra* shows significant inhibitory activity against PEP derived from *Flavobacterium meningosepticum*. *R. sacra* is used as a natural health product with medicinal properties with regard to antiaging and antidementia effects. Examination of the constituents of the extract resulted in the isolation of 19 known compounds. Among these, seven compounds, namely protocatechuic acid, gallic acid, (–)-epigallocatechin 3-*O*-gallate, 3-*O*-galloylepigallocatechin-(4β → 8)-epigallocatechin 3-*O*-gallate, sacranoside A, arbutin, and 4-*O*-(β-D-glucopyranosyl)-gallic acid, showed PEP inhibition. A kinetic study indicated that they are noncompetitive inhibitors, except for protocatechuic acid that is a competitive inhibitor.

Hepatitis C virus (HCV) causes chronic infection, frequently leading to liver cirrhosis and hepatocellular carcinoma. In recent research, HCV NS3-NS4A protease is often used as a useful enzyme target for screening of anti-HCV compounds. Luo et al.[24] immobilized polyclonal antibodies of a synthetic HCV NS3-NS4A protease inhibitor (IC_{50} 8.5 μg/mL) for mimicking HCV NS3-NS4A protease to screen affinity compounds from an extract of the traditional Chinese Herb *P. urinaria* L. by frontal affinity chromatography (FAC) coupled with mass spectrometry. The screening of extract resulted in brevifolin, brevifolin carboxylic acid, corilagin, ellagic acid, and phyllanthusiin U with high inhibitory activity.

Owing to poor durability of receptors and enzymes to organic solvents, a technique of molecular imprinting has been created for artificial receptors.[127–129] A majority of cases have proved its practicability as a complementary substitute for natural receptors. It has been successfully used in extraction and binding assays of some drugs.[130] Xie et al.[131] have introduced the technique for extracting active compounds from TCMs. Quercetin, a typical active compound of the flavonoid family in gingko leaves, was used as the template for preparing the molecularly imprinted polymer (MIP). The resultant MIP could directly trap a specific class of compounds including quercetin and kaempferol from the hydrolyzate of gingko leaves. The result demonstrated the possibility of direct extraction of certain pharmacophoric constituents from herbs by the molecular imprinting technique. Moreover, they also obtained two antitumor active components, harmaline and harmine, from the crude extract of *Peganum nigellastrum* by this technique.[132]

18.6 APPLICATION OF MASS SPECTROMETRY

Electrospray ionization mass spectrometry (ESIMS) has been widely applied in most investigations of macromolecular interaction with small molecules since they are introduced into the mass spectrometer directly from solution. When used in natural products analysis, ESIMS rapidly evolved to powerfully complement other HPLC detection systems. As a result, the interface of HPLC with ESIMS has provided a method that has effectively facilitated the integration of complex mixtures

with the rapid drug discovery processes. Some strategies were developed using ESIMS method for the screening of natural products.[133–138] De Boer et al.[135] screened enzyme inhibitors by an ODS column coupled to a continuous-flow enzymatic assay with ESIMS as the detector. Using cathepsin B as the model enzyme, three compounds from red clover (*Trifolium pratense* L.) extract showed their binding activity. The affinity of more than 67,000 putative ligand–substrate pairs were evaluated in a 24 h screening run by multiplexing both targets and compound collections based on the electrospray ionization-Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR) method.[136] After that, this method was also used to evaluate the noncovalent interactions between multiple RNA-based drug targets and components derived from a bacterial natural product library.[133]

18.7 CONCLUSIONS AND PERSPECTIVES

The screening and analysis of the multiple bioactive compounds in complex systems of natural products, such as in TCMs, is a formidable challenge. However, the rapid development of modern analysis and separation methods offers a variety of means for unraveling the mystery of TCMs. Even so, some strategies have to be designed specifically for TCMs. The whole animal model is very important for therapeutic evaluation because it allows assessment of the efficacy, side effects, and toxicity of medicines *in vivo*, but its high cost and low efficiency are incompatible with the requirements of modern high-throughput screening. In contrast, the cellular models and receptor and enzyme models allow screening with the specific targets based on the cellular and molecular levels, respectively. They are rapid, economical, highly sensitive, and specific. Therefore, compared with the animal models, they are more adaptive to high-throughput and large-scale drug screenings due to their diversity and low cost. However, because most diseases involve multitarget action, eventually, whole animal, *in vivo* models have to be used to confirm results indicated by the high-throughput screenings.

BFA based on the chromatographic and MS methods provides a valuable alternative for this task. The comparison of the chromatograms before and after the interaction with the free targets can distinguish the active ones quickly from the large number of compounds in the natural products library within an extract. Affinity chromatography simulates the interaction of multiple small molecules with the immobilized biomolecules. Coupled to reverse-phase HPLC in a 2D-HPLC mode, the resolution of affinity chromatography can be increased. ESIMS is a powerful complement to other HPLC detection methods. The strategies based on both HPLC-ESIMS and MALDI-TOF MS can be expected to play more important roles in the BFA in future. The BFA displays several advantages in library screening, including the direct analysis of crude extracts of the natural products without further purification of each compound. Also, biological interaction information can be deduced from the chromatograms or spectra, from which a certain compound can be estimated as active or inactive and the relative binding ability of the active ones can also be obtained. Finally, the approach has a wide applicability to the cases of nearly all the interaction systems of small molecules and macromolecules. Besides the ADME properties, the application of the BFA can be extended to other screening targets. In addition, it is fast, simple, highly efficient, and exhibits high throughput.

Because of the great need in biological research and drug discovery, the drug screening system is becoming increasingly complex, from single drug–single target to drug library–target, even to the drug library–target library. Therefore, more rapid and more efficient methods are required to directly obtain binding information from multiple systems. Thus, BFA shows great promise for future development as a drug discovery tool in TCMs research.

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19 Antimalarial Compounds from Traditionally Used Medicinal Plants

Lengo Mambu and Philippe Grellier

CONTENTS

19.1 INTRODUCTION

Parasitic infections caused by the protozoa *Plasmodium* are responsible for malaria, a severe disease with 300–515 million cases and that kills 2.7–3 million humans per year; a majority are children (<5 years old) in the tropical and subtropical regions of the world.[1] More than 90% of the deaths are in sub-Saharan Africa. Four species of *Plasmodium*, namely, *P. ovale, P. malariae, P. vivax*, and *P. falciparum*, cause human malaria. The most virulent, *P. falciparum*, is responsible for severe clinical malaria and death. A dramatic increase in malaria is observed today resulting mainly from the widespread use of insecticides, which has led to the selection of *Anopheles* mosquitoes (vectors) resistant to insecticides, and the increasing prevalence of parasite resistance to the standard antimalarial drugs like chloroquine. This situation and the complexity in developing efficient vaccines require an urgent need for new antimalarial drugs to replace ineffective drugs.[2] The struggle against malaria in developing countries, which is rivaled only by AIDS and tuberculosis as the world's most pressing health problem, constitutes an important challenge for the twenty-first century.

In many malaria-endemic countries, available drugs are often unaffordable or inaccessible and treatments are mainly based on the use of traditional herbal remedies. Indeed, indigenous plants play an important role in the treatment of many diseases, and more than 80% of people worldwide are estimated to use medicinal plants.[3] However, few data are available to assess their efficiency and their safety. Validation of these traditional practices could lead to new strategies in malaria control. In other respects, despite the progress in chemistry and the development of combinatorial chemistry, natural products from plants or other organisms remain an inexhaustible reservoir of molecules, with many still unexplored, which can constitute original lead molecules for new antimalarial drugs. Historically, plants have provided two major drugs for the treatment of malaria, namely, quinine, isolated from *Cinchona* species and which served as template for the synthesis of the quinoline antimalarial drugs[4,5] and, more recently, artemisinin isolated from *Artemisia annua*,[6] a plant traditionally used in Chinese medicine against malaria. Artemisinin derivatives constitute the foundation of the new chemotherapeutic strategy developed by the World Health Organisation (WHO) against malaria (artemisinin-based combination therapy). Thus, traditional medicines as potential sources of new drugs must continue to be investigated.

19.2 DEVELOPMENT OF BIOASSAYS FOR ANTIMALARIAL ACTIVITY

19.2.1 The Life Cycle of Malaria Infection

P. falciparum has a complex life cycle involving two hosts, the *Anopheles* female mosquito, the vector, where the sexual phase of the parasite development occurs, and humans where the parasite undergoes two phases of extensive asexual proliferation. During the bite by an infected *Anopheles*, the parasites in the sporozoite stage enter human blood and are carried to the liver where they invade hepatocytes in what is called the hepatic or exoerythrocytic phase. During this asymptomatic period, which lasts 5–7 days for *P. falciparum*, the sporozoites develop within the hepatocytes

and, after several rounds of mitosis, produce several thousands of new infective forms, the merozoites, that are released into the bloodstream invade the red blood cells. This is the intraerythrocytic cycle, which lasts 48 h for *P. falciparum* and causes the malaria disease. During this asexual blood cycle, the parasite undergoes a successive development into the ring stage (0–20 h) and the trophozoite stage (20–36 h), and then undergoes several mitoses (schizont stage, 36–48 h) that lead to the differentiation of 16–24 new infective merozoites. Erythrocyte lysis releases the merozoites into the bloodstream, and a new intraerythrocytic cycle can be initiated. For some still not well-understood reasons, some merozoites differentiate into male and female gametocytes that are taken up by the *Anopheles* during the blood meal. Sexual reproduction occurs in the digestive tract of the mosquito and the zygote formed produces, after several divisions, thousands of sporozoites that migrate to the salivary glands and can be transmitted to humans during a bite.

Different antimalarial bioassays have been developed on the basis of either *in vitro* or *in vivo* inhibition of parasite growth or, more recently, the inhibition of potential parasite targets, allowing the screening of plant extracts or isolated compounds.

19.2.2 Bioassays for the Erythrocytic Stage of *Plasmodium*

Because the intraerythrocytic cycle is responsible for the symptoms of the malaria disease, the efforts for antimalarial drug development were (and are still) essentially directed against this particular phase of parasite development.

Initially, drug screenings were time-consuming, costly, and limited to the use of animal malaria models (rodent, chicken, or monkey models) and the ethically questionable trials in humans. The development of the continuous culture of *P. falciparum* on human erythrocytes *in vitro* by Trager and Jensen[7] in 1976 was a critical advance, allowing drug evaluation on well-established laboratory strains (e.g., K1, W2, dd2, 3D7, and FcB1) and also on fresh isolates of blood from patients.[8] Typically, parasites are maintained on leukocyte-free erythrocytes at 2–5% hematocrit, in RPMI culture medium supplemented with 5–10% human serum at 37°C under a reduced percentage of oxygen.

Standard protocols of drug and resistance evaluation are recommended by the WHO to facilitate comparison of data from around the world. They generally involve evaluation by using Giemsa-stained smears and counting the parasitemia or the distribution of the different parasite stages in treated and nontreated cultures. These assays have the advantage of requiring minimal equipment and can be easily applied in the field. However, they lack precision and are particularly time-consuming thereby preventing rapid, large-scale screening of molecules. Several methods have been developed for the screening of large numbers of compounds in 96-well plates, even in 384-well plates, for high-throughput screening.[9] The concentrations causing 50% inhibition (IC_{50}) and also 90% inhibition (IC_{90}) are easily obtained from the drug concentration–response curve by nonlinear regression.[10]

19.2.2.1 Incorporation of Radiolabeled Precursors

These assays are based on the incorporation of metabolic precursors by the parasite that reflects its growth. They take advantage of the fact that the host cell, the red blood cell, possesses a reduced basic metabolism. Uptake of [^{3}H]-hypoxanthine, a precursor for nucleic acids, was the first described and is still the most widely used, but other precursors, such as [^{3}H]-phenylalanine, an indicator of protein biosynthesis, or [^{3}H]-ethanolamine, an indicator of lipid metabolism, have also been described.[11–13]

Although very sensitive and reproducible, these assays have several potential disadvantages. They are costly, involve radioactivity, with all the problems linked to the manipulation of radioactive compounds and the treatment of radioactive wastes, can be quite complex, and require special equipment (e.g., cell harvester, liquid-scintillation counter). Therefore, they can be problematic to set up in remote locations or those locations with poor resources.

19.2.2.2 Colorimetric, Fluorometric, and Flow Cytometry–Based Assays

Nonradioactive and low-cost alternative assays have been developed but are usually less standardized and often less sensitive than the [^{3}H]-hypoxanthine uptake method.

Colorimetric assays include the detection of parasite lactate dehydrogenase (LDH),[14] by its preferential metabolism of the substrate 3-acetylpyridine adenine dinucleotide, which is only weakly metabolized by the erythrocyte LDH, or the microculture tetrazolium assay.[15] Good correlations were observed with the radioactive assays, but compared to the radioactive assays, more readily accessible equipment (a spectrophotometer) is required.

Fluorescence-based assays have also been described using DNA-binding fluorophores or flow cytometry techniques, but they require more sophisticated equipment (a spectrofluorimeter or fluorescence cell analyzer).[16]

19.2.2.3 Interlaboratory Variation

Discrepancies have often been observed between results from a same plant extract or a pure compound between laboratories. They can be due to the type of antimalarial assay used (e.g., radioactive versus colorimetric assays) or due to the *P. falciparum* strains used as well as their drug resistance status. However, data may also be influenced by the following laboratory-specific modifications of the basic *in vitro* conditions of screening that have been introduced:

- The use of asynchronized or synchronized cultures. *P. falciparum* has an asynchronous intraerythrocytic development *in vitro*. The synchronous development that was observed *in vivo* in humans can be restored by treatments taking into account the biological properties of the different parasite stages.[17] Synchronous cultures allowed further study of the drug effects on specific stages of the parasite development (ring stage, trophozoite, and schizont stage).[18]
- The duration of the drug incubation. This can vary from the 48 h assays that are widely used to incubation assays of 24 or 72 h that have been reported, especially for evaluating fast- or slow-acting drugs, respectively.[19]
- The use of human serum or serum substitute. Variability of quality of human serum batches as well as access to nonimmune human sera in endemic malaria countries can be serious problems for data reproducibility. Albumax was proposed as a substitute for human serum and is now used by many laboratories.[20] It is mainly composed of fatty acid–rich bovine serum albumin. However, in addition to its high cost, a major disadvantage is that compounds can bind to Albumax constituents and therefore show weaker antimalarial activity than when tested in the presence of human serum.
- The culture conditions. Initial parasitemia, hematocrit, and atmosphere under which cultures are maintained (e.g., a 5% CO_2 atmosphere versus a well-defined atmosphere such as 6% O_2, 3% CO_2, 91% N_2) are all factors that may be the cause of discrepancies.

To minimize variations due to experimental procedures, several standardized protocols have been proposed.[21]

19.2.2.4 *In Vivo* Methods

For *in vivo* antimalarial evaluation, *Plasmodium* species infecting humans are unfortunately unable to grow in nonprimate models and the use of monkeys is limited by the high cost. Therefore, they are generally used for final drug evaluation in preclinical development.

Fortunately, several rodent malaria models are available, such as *P. berghei, P. yoelii*, *P. chabaudi*, and *P. vinckei*, that have been used in the discovery of several antimalarial drugs. The biology of these parasite species has been well characterized, and a range of standardized assays

are available that provide indications of the efficacy and the bioavailability of the studied drug, for example, the "4-day suppressive test", the "dose-ranging test", and the "onset/recrudescence test".[22]

As with the *in vitro* screens, interlaboratory discrepancies between results for the same drug can be observed. These are mainly due to the choice of the rodent malaria species or the mouse strain, and the route of drug administration and the adjuvant used. Rodent malaria species differ in terms of infectious potency, host cell preference (e.g., reticulocytes versus mature erythrocytes), lethality, and synchronization of parasite development, all of which can affect results. It must be noted that a sophisticated mouse model, using immunocompromised BXN mice, has been developed for *P. falciparum*–infected human erythrocytes. This opens new perspectives for *in vivo* drug evaluation against *P. falciparum*.[23]

19.2.3 Bioassays for the Hepatic Stage of *Plasmodium*

In humans, after bite and infection, parasite multiplication begins by an asymptomatic phase of proliferation in liver cells. Development of drugs against this particular parasite stage has two main advantages in fighting malaria. Firstly, such drugs could be used as prophylactic agents by preventing the access of the parasite to the blood and, in consequence, the development of the disease. Secondly, the human-infecting species *P. vivax* and *P. ovale*, unlike *P. falciparum*, have latent forms (hypnozoites) in liver responsible for relapsing infections, sometimes years after the primary infection. *P. vivax* is the most widely distributed human malaria parasite causing 70–80 million clinical cases per year, and in some parts of the world, it is the most prevalent *Plasmodium* species infecting humans. Hypnozoite forms are unaffected by the drugs used to eliminate the erythrocytic infection. Drugs active against hypnozoites could then be used as antirelapse agents limiting the recrudescence of *P. vivax* and *P. ovale*.

The number of available drugs that act against the hepatic phase is limited. 8-Aminoquinolines, such as primaquine and tafenoquine, are the main and the most efficient, but their use is restricted due to serious hematological toxicity. They cause hemolysis in patients with G6PD deficiency, and in cases of severe forms of G6PD deficiency, the risks of primaquine treatment might exceed the benefits.

Evaluation of drugs against the hepatic stage has been mainly performed using *in vivo* models of rodent parasites, for example, *P. berghei* and *P. yoelii*. However, such drug screening is time-consuming and costly due to the need to reproduce the complete life cycle of the parasite to obtain viable sporozoites in insects. Difficulties increase greatly if screening is performed using monkey models for human malaria parasites. The ability to culture *Plasmodium* in hepatocytes has opened new perspectives in the research of drugs and natural products active against the hepatic stage.[24–26]

In vitro screenings are usually performed using sporozoites of rodent *Plasmodium* infecting primary rodent hepatocytes or hepatoma cell lines. The sporozoite development within the hepatocyte into a schizont stage is followed by either Giemsa staining or immunofluorescence assay using specific antibodies or a genetically manipulated fluorescent parasite.

Assays based on primary culture of human hepatocytes infected with *P. falciparum* sporozoites have also been described. A high-throughput *in vitro* screening of drug activity on *Plasmodium* liver stages was recently developed on the basis of a sophisticated infrared fluorescence scanning system.[27] The technique allows rapid, automatic counting of *Plasmodium*-infected hepatocytes. Although very promising, such assays are, however, greatly dependent on the production of a large number of sporozoites in insects, a limiting step for rapid, high-throughput screening of drugs against *Plasmodium*-infected liver cells.

19.2.4 Bioassays for Parasite Targets

The understanding of the biochemistry of malaria parasites has increased considerably over the past two decades and has allowed the identification of many potential targets for new drugs. In addition, the recently decrypted genomes of several *Plasmodium* species have enabled the rapid identification of

putative targets that are homologous to validated targets in other organisms.[28] Usually, target validation in *Plasmodium* requires demonstrating that a specific inhibitor of the target shows antimalarial activity *in vitro* or *in vivo*. However, such an approach has limits. It is often difficult to demonstrate that the inhibitor kills the parasite by acting specifically on the selected targets and not by inhibition of unrelated biochemical mechanisms.

Recent advances in genetic understanding of *Plasmodium* have greatly increased the ability to validate potential drug targets. Methodologies are now available to transfect rodent *Plasmodium* and *P. falciparum,* and to replace or knock out genes that code for potential target proteins.[29,30] Phenotype analysis of the resultant mutant parasite allowed a study of the importance of the target in each phase of development of the complex life cycle of *Plasmodium* (infection and growth in liver and red blood cells, production of sexual forms, ookinete and sporozoites). Furthermore, access to new transcriptomic and proteomic technologies offers new opportunities to study the impact of a drug treatment on the entire parasite metabolism at either the RNA or the protein level.[31,32] This should lead to a better understanding of the mechanism(s) of action of drugs and the biochemical pathways involved in killing the parasites.[33]

These recent advances have allowed development of bioassays based upon validated targets, or those targets still in the process of validation, for drug or natural product screening.[34,35]

19.2.4.1 Inhibition of Heme Polymerization

Heme polymerization (Figure 19.1) is a natural process that occurs in the acidic food vacuole of the parasite where hemoglobin, a major source of amino acids for the parasite, is degraded by specific proteases. Heme, which is toxic for the parasite, is detoxified by polymerization into a pigment, the hemozoin. Inhibition of hemozoin formation is considered as an attractive target for new antimalarial drugs, and high-throughput assays are available.[36] 4-Aminoquinolines such as chloroquine and amodiaquine act by interfering with this polymerization process. Cysteine and aspartic parasite proteases (falcipains and plasmepsins, respectively), involved in the degradation of hemoglobin (Figure 19.1), are also interesting targets, and the design of specific inhibitors may benefit other research directed against human proteases involved in different pathologies.[37,38]

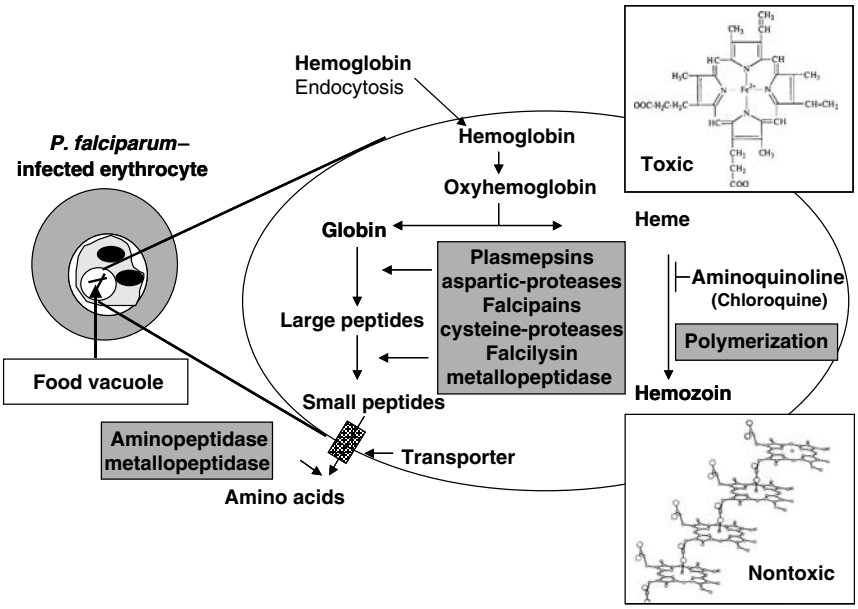


FIGURE 19.1 Mechanisms of hemoglobin degradation and heme detoxification in *P. falciparum*–infected blood cell.

19.2.4.2 Apicoplast-Based Targets

Apicoplast, a particular organelle found in apicomplexan parasites including *Plasmodium*, possesses interesting properties for the development of antimalarial drugs. The apicoplast probably evolved as a result of endosymbiosis of a prokaryote, which seems to have a cyanobacterial origin, by an ancestor of the apicomplexans, leading to an organelle that maintains a separate genome and certain specific functions.

Apicoplasts possess biochemical pathways that are present in bacteria and plants but are absent in humans thereby providing specific targets for drugs. This is the case for the components of the type II fatty acid biosynthesis and the mevalonate-independent isoprenoid synthesis.[39] Both pathways are already targets for antibacterial drugs that constitute new lead compounds for antimalarial drugs.[40] Inhibitors of the prokaryotic protein synthesis, for example, tetracyclines and clindamycin, or of the prokaryotic DNA and RNA machinery, for example, quinolone antibiotics and rifampin, have antimalarial activity. These compounds probably act by targeting processes from bacterial origin in apicoplast or in mitochondria.[41]

19.2.4.3 Other Biochemical-Based Targets and Amelioration of Resistance

As potential parasite targets, one can also include the folate metabolism and the electron transport in mitochondria, for which highly effective inhibitors are available but that developed rapid parasite resistance,[42,43] the LDH enzyme of glycolysis,[44] the enzymes of the phospholipid synthesis,[45] the new permeability pathways of the infected erythrocyte membrane,[46] the protein kinases involved in signal transduction,[47] the proteases involved in erythrocyte invasion,[48] and the protein farnesyl transferases.[49]

A promising chemotherapeutic strategy that must also be emphasized is the use of compounds that reverse resistance to antimalarial drugs (Section 19.5.3.5). Combining such agents with antimalarial drugs for which resistance is observed could open new approaches to antimalarial chemotherapy, particularly for inexpensive, well-tolerated, and fast-acting antimalarial drugs such as chloroquine.

19.3 SELECTION OF PLANTS

Natural products isolated from plants present a great structural diversity and constitute an important source of original lead compounds for the development of new therapeutic agents.[50] Their chemical diversity arises from evolutionary selection pressures, enabling them to interact with a wide variety of proteins and other biological targets for specific purposes. This bioactivity forms the basis by which some of them become effective drugs in a wide range of therapeutic indications.[51]

Major drugs available for infectious disease, and in particular for malaria, have been obtained from plants. In malaria-endemic regions, people use traditional medicinal plants to treat malaria and the fever or symptoms associated with this pathology. This traditional medical knowledge has been accumulated over millennia. The ownership recognition of this knowledge and the biodiversity to which it is attached, and the sharing of the benefits that may arise as a result of its utilization, was enforced in the form of the United Nations Convention on Biodiversity.[52] The exploration of biodiversity using information from traditional knowledge must be done with respect to the laws of concerned countries and of the conservation of biological diversity.[53]

The collection of plant material for screening natural antimalarial activity can be done randomly or by using ethnobotanical, ethnopharmacological, chemotaxonomic, and endemic approaches.

Using the random approach, plants are collected without any consideration to traditional knowledge. This approach is laborious and needs a high level of investment. It was used by the National Cancer Institute (NCI) for the period of 1960–1982 in a search for anticancer agents, and few drugs were developed. In the ethnobotanical and ethnopharmacological approaches, surveys are performed among ethnic communities with the help of traditional healers. As the handing down

of this ancestral knowledge is mostly made by oral and not written communication, except for the Chinese *Materia Medica*, for example, loss of this information has been observed. Such enquiries are mainly conducted in subtropical and tropical countries where biodiversity is very large. It is generally accepted that a higher percentage of active extracts have been obtained from plants selected on the basis of traditional knowledge than from those randomly selected.

The chemotaxonomic approach is based on the observation that plants of the same genus produce related compounds. They could lead to a large natural diversity of derivatives of a compound of interest. The success of such an approach is illustrated by the anticancer agents that were derived from studies of *Taxus* species and *Podophyllum* species.[54] The study of tropical lianas, which belong to the very small palaeotropical families Dioncophyllaceae and Ancistrocladaceae, used traditionally for malaria treatment, may also be cited.[55] Similarly, since the powerful antimalarial drug artemisinin is isolated from *Artemisia annua*, which belongs to the Asteraceae family, other *Artemisia* species, and indeed other Asteraceae genera, might then produce related bioactive constituents and should be investigated for their antimalarial activity.

Interest in the endemic plant approaches arises from their particular evolutionary history with the expectation that they develop unique bioactive secondary metabolites with novel structures. For example, the disappearance of tropical forests in Madagascar or New Caledonia, islands with a high percentage of endemic plants, has led to a large investigation of unexplored plants before this natural resource is lost.

Quinine, a successful antimalarial isolated from the bark of Peruvian *Cinchona* spp. commonly known as "quinas", is characterized by its bitter taste, and consequently many plants used for the treatment of malaria have also been selected for their bitterness even if some of them were proved to be inactive *in vivo*.[56,57] The use of medicinal plants in combination with modern drugs is frequent when both of them are accessible and is consistent with the idea that "modern drugs cure, but plants heal".[58]

There are, however, limitations that need to be considered. Almost all natural compounds are produced as biological defense mechanisms, and their consequent potential cytotoxicity remains a limiting factor for the use of many compounds. Additionally, production of specific secondary metabolites can occur both at specific times in the growing cycle and in different parts of the organism, frequently leading to difficulties in sourcing sufficient material for study.

The antimalarial activity of plants used traditionally can be explained (Figure 19.2) by[59]:

- A direct effect of constituents on the parasite at erythrocytic stage, that is, intrinsic antimalarials, cytotoxic agents, and chemosensitizers

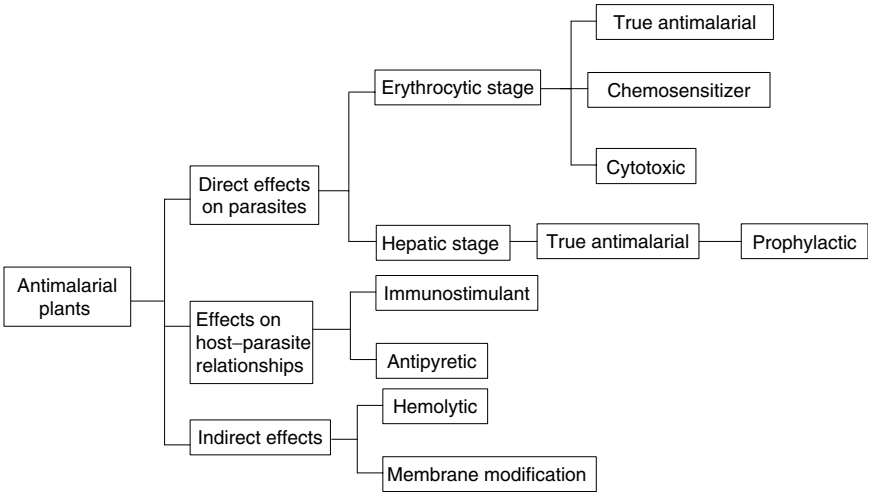


FIGURE 19.2 Different effects of molecules contained in antimalarial plants.

- A direct effect on the hepatic stage, that is, as intrinsic antimalarials and prophylactic or preventative agents
- Effects on host–parasite relationships such as antipyretics and immunostimulants
- Indirect effects as hemolytics or by membrane modification

19.4 EXTRACTION AND ISOLATION

19.4.1 Extraction

Drugs or remedies used in traditional medicine are commonly prepared in water at room temperature. In others cases, plants are boiled and the decoctions used after filtration.[59] Alcoholic macerations are also reported; for example, plants prepared in rum are currently used in French Guiana.[60] In the laboratory, however, extraction is preferably performed with organic solvents because it yields higher amounts of constituents than the traditional preparations. Furthermore, aqueous extracts, which contain more polar constituents, are frequently more difficult to work with. For screening of antimalarials in plants, three main processes are used to prepare extracts from air-dried and milled plant material, which take into account the polarity and ionizability of constituents:

1. Successive macerations first in non or low-polarity solvents, for example, cyclohexane and ethyl acetate and then in more polar solvents, for example, alcohol
2. Direct maceration in alcohol followed by partition of the crude alcoholic extract between water and then cyclohexane, ethyl acetate, and *n*-butanol successively, to yield the corresponding soluble extracts
3. For plants containing alkaloids, a selective acid–base extraction, which provides the crude alkaloid extract

19.4.2 Screening for Activity

Biological results obtained with nonpolar solvent extracts must be considered with care because in many cases, they are negated by the presence of lipid material that can induce false-positive responses. It is also known that tannins and polyphenols can induce false-positive activity on enzymes and should be removed from extracts by passage through a polyamide chromatography column.

Our general procedure to identify natural products with intrinsic antimalarial products is illustrated in Figure 19.3. It requires strong collaborations among traditional healers, botanists, chemists,

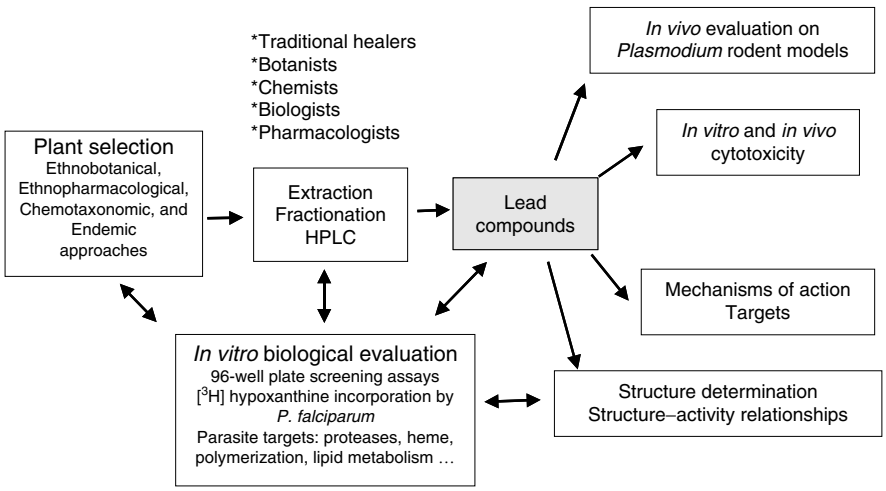


FIGURE 19.3 General procedure from plant to active compounds.

biologists, and pharmacologists. Biological assays were generally realized on the intraerythrocytic development of *Plasmodium* but could also be realized on purified parasite targets.

Extractions are initially performed on a small amount of each plant species. All the extracts obtained by the extraction process are tested *in vitro* against antimalarial drug-resistant strains of *P. falciparum* (mainly resistant to chloroquine) and eventually on sensitive strains. Extracts exhibiting an IC_{50} below 1 μg/mL, between 1 and 10 μg/mL, from 10 to 50 μg/mL, and over 50 μg/mL are considered as very good, good, moderate, and inactive, respectively. Extracts with IC_{50} values <10 μg/mL are preferentially selected and evaluated *in vitro* on mammalian cell lines to determine their cytotoxicity and also their selectivity toward *P. falciparum*.[61]

Cytotoxic compounds can inhibit parasite growth without intrinsic antimalarial property, and therefore cytotoxicity is evaluated to determine if the antiplasmodial activity observed is specific or due to a general toxicity. Selectivity is described by the selectivity index (SI), which is the ratio of the IC_{50} value on the mammalian cells to the IC_{50} value on *P. falciparum*. Extracts that demonstrate a high SI should offer the potential of safer therapy.

19.4.3 Isolation of Active Components

The extracts that have potent antimalarial activity and high selectivity are selected for bioassay-guided fractionation from large amounts of plant material. In this procedure, chromatographic separations are monitored with *in vitro* bioassays on *P. falciparum* until pure constituents responsible for the antimalarial activity are obtained.

Separation procedures are carried out using different chromatographic techniques including flash column, medium-pressure liquid chromatography (MPLC), high-pressure liquid chromatography (HPLC), and centrifugal countercurrent chromatography (CCC). Purification procedures combine different chromatographic supports to separate the compounds according to their physicochemical properties such as silica gel (normal and reverse phase) or alumina adsorbents for polarity-based separation, Sephadex size exclusion, filtration gels, such as LH-20, size-based separation, resins such as Amberlite XAD, hydrophilic affinity–based separation, and ion-exchange resins for charge-based separations.

In vivo assays on different species of rodent malaria parasites are conducted to confirm the antimalarial activity of pure compounds isolated from the plant extracts as well as *in vivo* toxicity studies for evaluating the potential therapeutic range of the molecule. In cases where there is abundant material or the possibility of semisynthesis, structure–activity relationship studies can be undertaken to increase the antimalarial activity and to decrease the toxicity of the derivatives.

19.4.4 Structure Determination

After isolation of pure compounds, their structures are determined on the basis of their spectroscopic properties using mass spectrometry, ultraviolet (UV) and infrared (IR) spectroscopy, and complete proton and carbon mapping using one and two dimensional NMR techniques (1D [^{1}H, ^{13}C] and 2D [COSY, HSQC, HMBC, NOESY]).

In some cases, chemical modifications or degradations are required to elucidate the complete structure. The absolute stereochemistry of optically active compounds can be established by various chemical procedures (using chiral anisotropic NMR reagents) and by circular dichroism (CD) spectroscopy analysis by comparison to similar compounds. If it is possible to obtain a suitable crystal of the molecule, the absolute stereochemistry is determined by X-ray crystallographic analysis.

Tandem techniques, or hyphenated techniques such as HPLC-MS and HPLC-NMR, are increasingly used to facilitate the analysis of plant extracts and the identification of individual components. Extending this further, a combination of HPLC, CD, tandem mass spectrometry, and NMR (i.e., HPLC-CD, HPLC-MS/MS, and HPLC-NMR) has been used for the online full stereochemical structural elucidation of extract components without any isolation from crude plant extracts.[62]

Hyphenated analysis is performed on microgram range to several milligrams of crude extract, which represents a complex mixture of constituents. This powerful technique also allows distinction between known compounds (dereplication) and new molecules. LC-NMR has also proved to be useful for the structural investigation of labile products.[63] However, the cost of such technologies, including high cost of materials and of reagents such as deuterated solvents required for LC-NMR, limits their use to specific purposes and the access to these technologies by laboratories of emerging countries. Furthermore, only a small amount of pure compound can be obtained, requiring a return to standard procedures of purification if a larger amount is needed for structural elucidations or biological investigations.

19.5 EXAMPLES OF ACTIVE COMPOUNDS

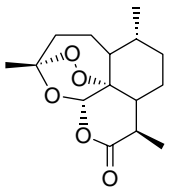
Several recent reviews have described natural antiplasmodial agents from plants that, in most cases, are used in traditional medicine.[64–68] In this chapter, we describe antimalarial compounds detected using the ethnobotanical and ethnopharmacological approaches.

In comparison with the most used antimalarial drugs, that is, chloroquine and artemisinin, which exhibit IC_{50} values in the nanomolar range, new compounds with IC_{50} values lower than 1 μM, between 1 and 10 μM, and higher than 10 μM are considered as potent, moderate, and weak antimalarial agents, respectively.

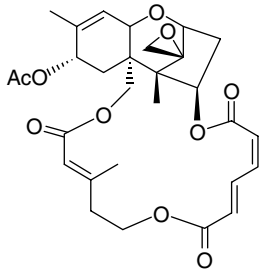
19.5.1 Terpenoids

19.5.1.1 Sesquiterpenoids

Since the discovery and the use of artemisinin **(1)**, an endoperoxide sesquiterpene lactone, particular attention has been directed to this class of compounds, the entity required for antimalarial activity being 1,2,4-trioxane.



1



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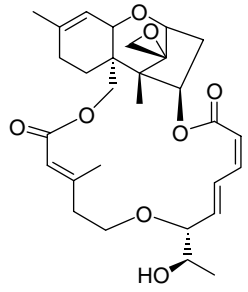
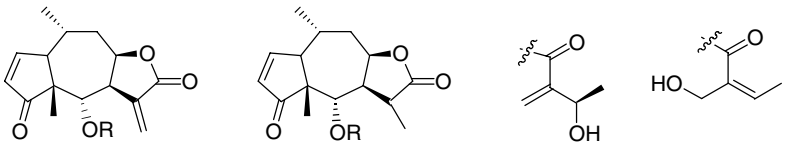


3

Verrucarin L acetate **(2)**, a macrocyclic trichothecene sesquiterpenoid, was isolated from the leaves and stem bark of *Ficus fistulosa* and a related compound, roridin E **(3)** from *Rhaphidophora decursiva*.[69] These two compounds were isolated as minor components and have been found to be very potent *in vitro* against *P. falciparum* with IC_{50} values below 1.5 nM on the chloroquine-sensitive D6 strain and the chloroquine-resistant W2 strain. The W2 strain was also reported to be resistant to other antimalarial drugs such as quinine and pyrimethamine.[70] Compound **3** showed a poor selectivity toward *P. falciparum*, whereas compound **2** appeared worthy of further evaluation *in vivo* on rodent malaria parasites. One major limitation for *in vivo* assays is the small quantity of compounds isolated from the starting material; for instance, only 0.36 mg of **2** was obtained from 11.8 kg of dried leaves and bark of *F. fistulosa*. In this case, total synthesis or semisynthesis from precursors will allow further investigation of the biological properties of these compounds. Curiously, trichothecenes are mycotoxins isolated mainly from fungal organisms. Their occurrence in these two plants, collected in the same area in Vietnam, suggests probable contamination of plant

material by fungus, including the possibility of an endophyte. In a previous work,[71] related macrocyclic trichothecenes isolated from the fungus *Myrothecium verrucaria* showed high antimalarial potency but were cytotoxic.

Three pseudoguaianolide sesquiterpene lactones including two helenalin derivatives **(4** and **5)** and 11,13-dihydrohelenalin-2-(1-hydroxyethyl)acrylate **(6)** were identified from the leaves of *Vernoniopsis caudata*, an endemic plant of Madagascar used traditionally for the treatment of pulmonary afflictions.[72] This plant was selected from a screening program of 190 medicinal plants of Madagascar as part of our collaboration with colleagues of the Malagasy Institute of Applied Research (IMRA).[73] Silica gel chromatography of the ethyl acetate extract (IC_{50} value of 1.6 µg/mL) yielded three compounds **(4**–**6)** that displayed potent *in vitro* antimalarial activity against the chloroquine-resistant FcB1 strain of *P. falciparum* in the nanomolar range for **5** and **6** and in the micromolar range for **4**.



4: R = A **6**: R = A A B

5: R = B

Compound **5** (IC_{50}: 190 nM) showed activity similar to that of chloroquine (IC_{50}: 110 nM), whereas compound **6** (IC_{50}: 410 nM) was two times less active toward this strain. Despite their *in vitro* antimalarial potency, these sesquiterpene lactones exhibited high cytotoxicity on KB cells. Compound **6** was more cytotoxic (IC_{50}: 50 nM) than andriamycin (IC_{50}: 270 nM) used as control, whereas compound **4** showed a similar activity (IC_{50}: 330 nM). The presence of α,β-unsaturated carbonyl structures, such as an α-methylene-γ-lactone, an αβ-unsaturated cyclopentenone, or a conjugated ester, contributed to the observed activities through alkylation of biological nucleophiles with thiol groups of cysteine residues by Michael-type addition.[74] The antiplasmodial activity observed for **4**–**6** could be attributed to their cytotoxicity. Interestingly, the most potent antiplasmodial, compound **5**, exhibited the lowest toxicity (IC_{50}: 930 nM), suggesting that other structural elements are involved in the selectivity toward *Plasmodium* and could be taken into account in the design of derivatives with higher therapeutic indexes. Owing to the low yield of the isolated compounds, no *in vivo* investigation was attempted.

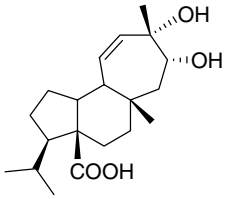
19.5.1.2 Diterpenoids

The investigation of the antimalarial activity of the roots of *Harpagophytum procumbens*, commonly known as Devil's claw and used in traditional medicine in South Africa to treat fevers, blood diseases, and digestive disorders, led to the isolation of the totarane and abietane diterpenes, 8,11,13-totaratriene-12,13-diol **(7)** and 8,11,13-abietatrien-12-ol **(8)**, respectively.[75]

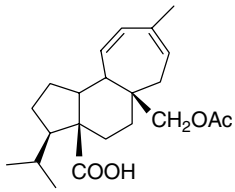
7 **8**

Compound **7** showed similar activity against the chloroquine-sensitive strain D10 and the chloroquine-resistant strain K1 of *P. falciparum* (IC_{50}: 2.5 and 2.7 μM, respectively). Compound **8** was slightly more active against the resistant strain than the sensitive one with IC_{50} values of 2.2 and 3.3 μM, respectively. They exhibited very little toxicity against mammalian cells as shown by their SI ($46 < SI < 82$). These compounds possess amphiphilic properties. It has been demonstrated that amphiphilic compounds can be incorporated into the lipid bilayer of erythrocyte membranes irreversibly and induce shape transformation of the membrane.[76] In this case, the inhibition of parasite growth observed *in vitro* could be attributed to indirect effects due to stomatocytic or echinocytic modifications of the host cell membrane. A good correlation between *in vitro* antiplasmodial activity of certain amphiphiles and their ability to modify the erythrocyte membranes was observed. However, it was shown that compounds **7** and **8** did not alter the erythrocyte membrane in contrast to the closely related abietane-type diterpene, dehydroabietinol, which exhibited antiplasmodial activity through modification of the erythrocyte membrane.[77] Both compounds were used to design a series of potential antiplasmodial lead compounds. Various derivatives were synthesized from totarol, a commercial analogue, to improve their selectivity, and two of them exhibited an SI of 180 and 207.[78]

Five mulinane-type diterpenes isolated from *Azorella compacta* were evaluated as potential *in vivo* growth inhibitors of *P. berghei* NK 65 in mice.[79] Under conditions where chloroquine exhibited an IC_{50} value of 2.5 mg/kg/day, two of the five, that is, 13,14-dihydroxymulin-11-en-20-oic acid **(9)** and 17-acetoxymulin-11,13-dien-20-oic acid **(10)**, exhibited 42% and 60% of parasite growth inhibition, respectively, for an intraperitoneal dose of 10 mg/kg/day. Thus, compounds **9** and **10** could be considered as potent antimalarial agents.

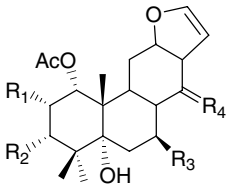


9

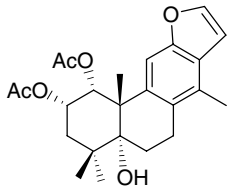


10

The dichloromethane extract of *Caesalpinia crista* possessed an interesting antimalarial activity in mice infected with *P. berghei*, with 98.6% inhibition of parasitemia at dose of 10 mg/kg/day. The seed kernel of this plant commonly known as "Bagore" in Indonesia is used as in anthelmintic and antimalarial remedies. The purification of the dichloromethane extract by successive silica gel column



	R_1	R_2	R_3	R_4
11:	H	H	OH	O
12:	H	OAc	H	O
13:	OAc	OAc	H	CH_2
15:	OAc	H	H	O



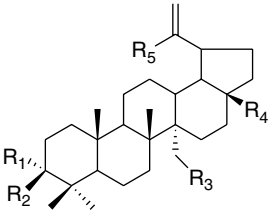
14

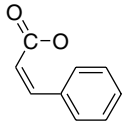
chromatography followed by normal and reverse-phase preparative TLC furnished 18 furanocassane- and norfuranocassane-type diterpenes.[80] The evaluation of the antimalarial activity of isolated compounds (except for two of them obtained in insufficient quantities) on *P. falciparum* FCR-3/A2 strain showed that 10 diterpenes were potent agents with IC_{50} values between 90 and 800 nM, five were moderate with IC_{50} values between 2 and 6.5 μM, and one was inactive. Compounds **11**–**14** were more potent than chloroquine; however, no cytotoxic investigation was reported for these compounds. In previous work, the *in vivo* activity of **15**, which exhibited an IC_{50} value of 800 nM, was determined by a 6-day suppressive test in mice infected with chloroquine-resistant strain NK 65 of *P. berghei*.[81] This compound reduced parasitemia by 48.0%, 40.9%, and 33% at doses of 10, 1, and 0.1 mg/kg/day, respectively. At the same concentrations, artemisinin, used as positive control, reduced parasitemia by 65.5%, 37.4%, and 26.9%, respectively.

19.5.1.3 Triterpenoids

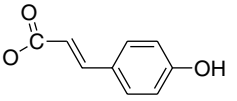
19.5.1.3.1 Pentacyclic Triterpenes

Pentacyclic triterpenes are ubiquitous constituents of plants, and several lupane triterpenoids, including betulinic acid and lupeol, had moderate antimalarial activity *in vitro* against *P. falciparum*. They were however ineffective *in vivo* against *P. berghei*.[76,82–84]





A
p-(*Z*)-coumaroyloxy



B
p-(*E*)-coumaroyloxy

16: R_1 = H, R_2 = OH, R_3 = A, R_4 = COOH, R_5 = CH_3

17: R_1 = H, R_2 = OH, R_3 = B, R_4 = COOH, R_5 = CH_3

18: R_1, R_2 = O, R_3 = H, R_4 = CH_3, R_5 = CHO

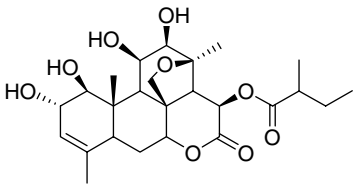
19: R_1 = H, R_2 = OH, R_3 = H, R_4 = CH_3, R_5 = CHO

Several triterpenes and the lupane triterpenes, messagenic acid A **(16)** and messagenic acid B **(17)**, were isolated from *Gardenia saxatilis* (Rubiaceae), a plant used in folk medicine in Thailand as a remedy for multiple diseases including malaria. Compounds **16** and **17** exhibited IC_{50} values of 2.4 and 4.6 μM, respectively, against the multidrug-resistant strain K1 of *P. falciparum*.[85] Under the same experimental conditions, artemisinin exhibited an IC_{50} value of 3.6 nM. Similar results were obtained from leaves of *Nuxia sphaerocephala* (Loganiaceae) used in traditional medicine in Madagascar to treat splenomegaly associated with malaria. Among the diterpenoids and triterpenoids isolated, 3-oxolupenal **(18)** and 3β-hydroxylupenal **(19)** inhibited the growth of chloroquine-resistant FcB1 and FcM29 strains of *P. falciparum in vitro* with IC_{50} values between 3.5 and 10.7 μM.[86] To test if these amphiphilic compounds might inhibit parasite growth through a modification of the erythrocyte membrane as described for betulinic and its analogues,[87] erythrocytes were incubated with increasing concentrations of compounds **18** and **19**. No cell lysis and no change of erythrocyte membrane shape in echinocytic forms were observed at concentrations up to 115 μM by phase

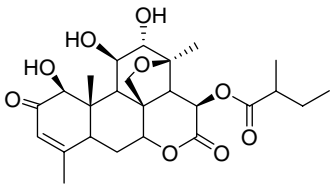
contrast microscopy. There was also no evidence of stomatocytic forms. However, this last modification might not be clearly detectable by photonic microscopy and will require further investigation using transmission electron microscopy.

19.5.1.3.2 Quassinoids

Quassinoids are related to triterpenoids and are found in the family of Simaroubaceae. They exhibited potent antimalarial activity but were cytotoxic in general, with a low SI. Orinocinolide **(20)**, isolated together with simalikalactone D **(21)** from the root bark of *Simaba orinocensis*, demonstrated a higher activity than chloroquine and artemisinin toward the chloroquine-sensitive clone D6 of *P. falciparum* (IC_{50}: 6.8 nM) and showed an activity similar to that of artemisinin toward the chloroquine-resistant clone W2 (IC_{50}: 17.8 nM).[88] The *in vitro* cytotoxic activity was evaluated against mammalian Vero cells and human cancer cells SK-MEL, KB, BT-549, and SK-OV-3. They exhibited activities comparable to the anticancer drug reference doxorubin, but compound **20** was found to be less cytotoxic than compound **21**. Thus, reduction of the carbonyl group to hydroxyl at position C-2 decreases the cytotoxicity (IC_{50} value of 20.8 µM for **20** versus 4.8 µM for **21** on the Vero cells). Despite cytotoxicity to mammalian cells, each of these compounds has a good SI toward *P. falciparum.* Compound **20** in particular is a good lead compound.

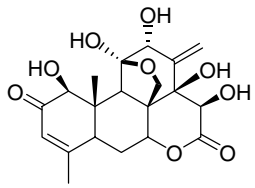


Orinocinolide (**20**)



Simalikalactone D (**21**)

Four quassinoids were isolated from the roots of *Eurycoma longifolia*, a traditional medicinal plant popularly used in Southeast Asia. Their antiplasmodial activity was evaluated *in vitro* by the LDH assay against the chloroquine-resistant Gombak A strain of *P. falciparum.* Eurycomanone **(22)** was the most potent of them; however, cytotoxicity evaluated against KB cells revealed its lack of selectivity toward the parasite.[89] To improve this selectivity, several acylated analogues were synthesized. Only 15-*O*-isovaleryleurycomanone **(23)**, obtained by mono-acylation of **22** with isovaleric acid at the C15−OH, exhibited comparable antimalarial activity to compound **22** and with a low toxicity against brine shrimp.[90] Compound **23** showed an SI of 109 when tested on the Gombak A strain of *P. falciparum.*



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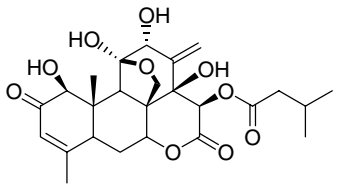
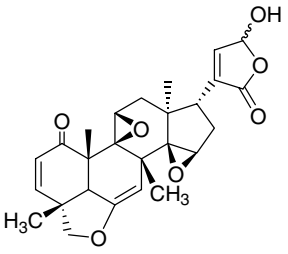


23

19.5.1.3.3 Limonoids

The leaves of *Trichilia rubescens* were studied after a survey of self-medicative behavior of wild chimpanzees in Uganda. Bioassay-guided fractionation yielded two active limonoids.[91] Trichirubine A **(24)** exhibited a significant antimalarial activity with an IC_{50} value of 0.66 µM against the

chloroquine-resistant strain FcB1 of *P. falciparum*. Trichirubine B **(25)** was obtained in a small quantity that enabled its structure determination but not its antimalarial activity with certainty. However, compound **25** was the major constituent of a fraction displaying an IC_{50} at 0.2 µg/mL. Neither the cytotoxicity on mammalian cells nor the *in vivo* activity against rodent *Plasmodium* was evaluated for compounds **24** and **25**. Several limonoids, including gedunin and nimbin, have been described with a large range of biological activities including good antimalarial activity.[92]



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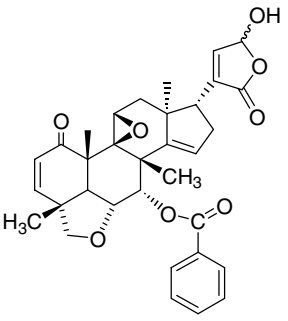
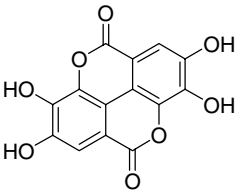


25

19.5.2 Phenolics

19.5.2.1 Simple

Ellagic acid **(26)** and 3,4,5-trimethoxyphenyl-(6′-*O*-galloyl)-β-D-glucopyranoside **(27)** were shown to be the active constituents of the methanolic extract of the bark of *Tristaniopsis calobuxus* (Myrtaceae).[93] Their antiplasmodial activities were similar on the chloroquine-sensitive clone D6 and the chloroquine-resistant clone W2 of *P. falciparum* (IC_{50} value of 0.5 µM for **26** and 3.2 µM for **27**). Under the same conditions, chloroquine and mefloquine showed IC_{50} values of 4.85 and 9.6 nM on D6, and of 112 and 3.4 nM on W2, respectively. The antimalarial potency of **26** and **27** could not be attributed to a general toxicity since an evaluation of the antiproliferative activities against a human skin fibroblast cell line and the hepatoma G2 cell line showed that at 100 µM, compound **27** was not cytotoxic for both cell lines. Compound **26** seems slightly more cytotoxic displaying 20% and 9% inhibition of the two cell lines, respectively, at the same concentration. Ellagic acid **(26)** is able to form π–π interactions with β-hematin, a synthetic compound spectroscopically identical to natural hematin. It was shown to inhibit β-hematin formation with an IC_{50} value three times higher than that of chloroquine, suggesting that ellagic acid could kill the parasite by inhibition of the heme polymerization process as proposed for chloroquine. Compounds **26** and **27** were also shown to inhibit the parasite protease, plasmepsin II, involved in the degradation of hemoglobin, with IC_{50} values of 4.02 and 35 µM, respectively.[94]



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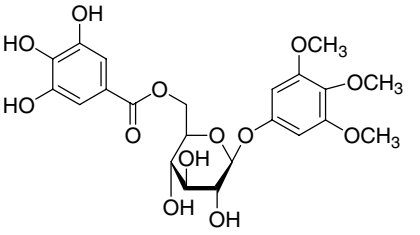
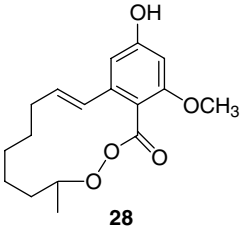


27

19.5.2.2 Lignans

The investigation of the chloroform extract of leaves and stems of *R. decursiva* illustrates a current case of the necessity to have access to a large amount of plant material.[95] The bioassay-guided fractionation of the extract yielded 0.49 mg of an active mixture from 5.03 kg of dried plant material. Large-scale recollection of the plant (20.1 kg) was required to yield six active compounds. The most potent were rhaphidecurperoxin **(28)**, a benzoperoxide, and polysyphorin **(29)**, a neolignan, with IC_{50} values of 1.37 and 0.88 µM, respectively, against the chloroquine-resistant strain W2 of *P. falciparum*. Despite the presence of a peroxide ester function in compound **28**, related to the endoperoxide bridge of artemisinin required for the antimalarial activity, its low selectivity when tested on mammalian cells was a limiting factor for further development.



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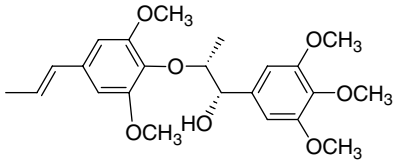
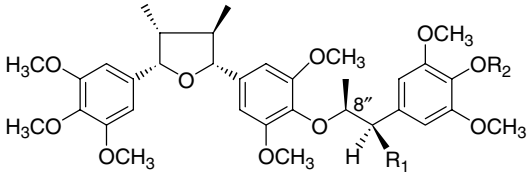


29

Among the lignans isolated from aerial parts of *Bonamia spectabilis*, three tetrahydrofuran-type sesquilignans, namely, bonaspectin C-4″-*O*-glucoside **(30)** and its aglycone **(31)**, and bonaspectin D-4″-*O*-glucoside **(32)**, exhibited antimalarial activity with IC_{50} values ranging from 1.3 to 6.5 µM on the chloroquine-sensitive strain PoW and from 1.7 to 4.6 µM on the chloroquine-resistant strain Dd2 of *P. falciparum*, **30** being the more active.[96] Treatments of synchronized cultures of *P. falciparum* with different concentrations of **30** demonstrated that it acts mostly on the trophozoite stage, inhibiting the formation of schizonts. Additionally, compound **30** exhibited low cytotoxicity against endothelial ECV-304 cells with an IC_{50} value of 52.8 µM.

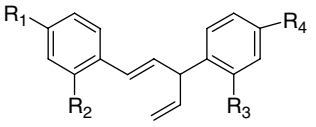


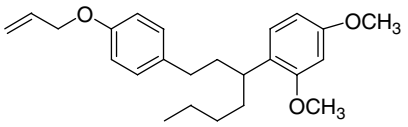
30: R_1 = H, R_2 = β-D-glucose (stereochemistry at 8″ not determined)

31: R_1 = H, R_2 = H

32: R_1 = OH, R_2 = β-D-glucose

To date, the antimalarial activity of natural lignans can be classified as good to moderate. The norneolignan hinokiresinol **(33)** was used as a template for the synthesis of analogues resulting in a 10-fold improvement of the IC_{50} value.[97] Hinokiresinol **(33)** and its *Z*-isomer, nyasol, were isolated from *Asparagus africanus* and exhibited IC_{50} values of 50 and 12 µM against chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*, respectively.[98] The *in vivo* antimalarial evaluation on mice infected with *P. berghei* of the two most active derivatives (**34** and **35**) showed significant reduction of parasitemia at day 5 without apparent toxicity.



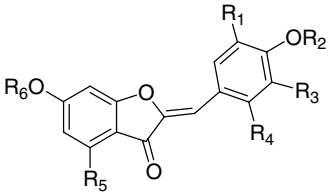


33: $R_1 = R_4 = OH$; $R_2 = R_3 = H$ Hinokiresinol **35**

34: $R_1 = R_2 = Cl$; $R_3 = R_4 = OCH_3$

19.5.2.3 Aurones

Aurones are tricyclic flavonoid-type phenolic compounds, isolated from the Rubiaceae and Cyperaceae and characterized by the presence of a benzylidine function. Since they were potent drugs against leishmania infections, their antimalarial activity was also evaluated.[99]



36: $R_1 = H$, $R_2 = H$, $R_3 = H$, $R_4 = OH$, $R_5 = H$, $R_6 = H$

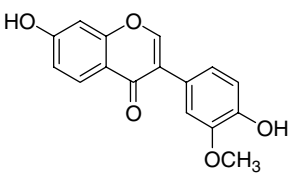
37: $R_1 = H$, $R_2 = H$, $R_3 = OCH_3$, $R_4 = H$, $R_5 = OH$, $R_6 = H$

38: bractein triacetate
$R_1 = OCH_3$, $R_2 = OAc$, $R_3 = OCH_3$, $R_4 = H$, $R_5 = OAc$, $R_6 = Ac$

Natural aurones were synthesized and tested *in vitro* against the NF54 and K1 strains of *P. falciparum*. Three aurones **(36–38)** exhibited high antimalarial activity on the multidrug-resistant K1 strain with IC_{50} values ranging from 7 to 30 nM. Surprisingly, they were less active against the chloroquine-sensitive strain NF54 (IC_{50} values ranging between 180 and 200 nM). However, they displayed a significant cytotoxic activity against KB and SKMel cells ($IC_{50} \leq 3.0\,\mu M$). Bractein acetate **(38)** was the most potent antimalarial with an SI of 543 on the multidrug-resistant strain K1 and is a promising antimalarial compound. Compounds **36** and **37** each displayed an SI between 100 and 120.

19.5.2.4 Flavonoids

Several studies showed that flavonoids could be generally considered as having weak-to-moderate antimalarial activity. For example, the investigation of ethyl acetate extract of the heartwood of *Dalbergia louvelii*, a species traditionally used to treat malaria and bilharzias, revealed four active flavonoids, including 7,4′-dihydroxy-3′-methoxyisoflavone **(39)** and isoliquiritigenin **(40)**, with IC_{50} values ranging from 5.8 to 8.7 μM against the chloroquine-resistant strain FcB1 of *P. falciparum*.[100]



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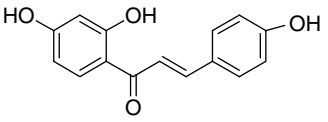

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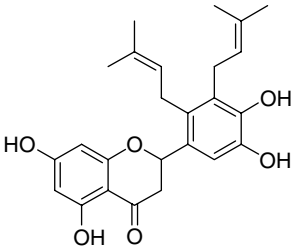
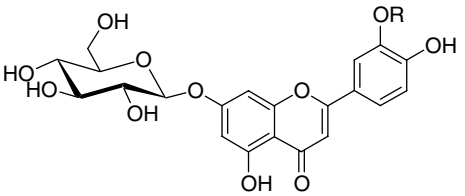


41

Similarly, five flavanones isolated from the stem bark of *Erythrina abyssinica* exhibited antimalarial activity against the D6 and W2 strains of *P. falciparum* with IC_{50} values ranging from 4.9 to 9.3 μM.[101] The prenylated flavanone abyssinin III **(41)** was the most active against the W2 strain. It should be noted that *E. abyssinica* is the most used plant for the treatment of malaria and microbial infections in Kenya.

Luteolin-7-*O*-β-D-glucopyranoside **(42)** and chrysoeriol-7-*O*-β-D-glucopyranoside **(43)**, isolated from *Phlomis brunneogaleata*, were shown to be the most active constituents of this plant.[102] They exhibited IC_{50} values of 5.4 and 12.8 μM, respectively, against the chloroquine-resistant strain K1. Compound **42** displayed a good enzyme-inhibitory potential toward enoyl-ACP reductase (FAB I) of *P. falciparum* with an IC_{50} value of 22.3 μM, whereas compound **43** was inactive. The test was performed spectrophotometrically *in vitro* using purified enzyme. The presence of an *ortho*-diphenol structure seems to be important for enzyme interaction. Compound **42** appears to be the first natural product targeting the plasmodial FAB I enzyme which is the key regulator of type II fatty synthases (FAS-II) in *P. falciparum*. Triclosan was identified as potent inhibitor of FAB I enzyme with an IC_{50} value of 50 nM. The two flavonoid glycosides **42** and **43** were both active against *Leishmania donovani* (IC_{50}: 2.5 and 8.9 μM).



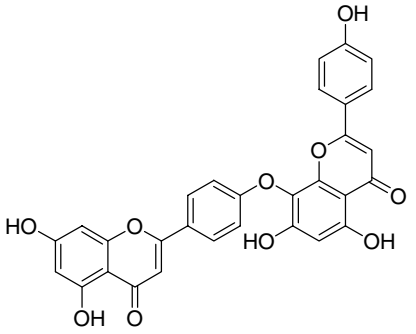
42: R = OH

43: R = OCH_3

In general, biflavonoids seem to be more potent than monoflavonoids. Lanaroflavone **(44)** isolated from the methanol extract of the aerial part of *Campnosperma panamense* inhibited the growth of the chloroquine-resistant strain K1 of *P. falciparum* with an IC_{50} value of 0.37 μM *in vitro*.[103] This potent antimalarial agent exhibited a moderate leishmanicidal activity against *L. donovani* amastigotes.

Bioassay-guided purification of the *n*-butanol fraction (IC_{50}: 7.7 μg/mL) of the roots of *Wikstroemia indica*, used in China for the treatment of scrofula, carbuncle, and traumatic injury, led to the isolation of the biflavonoids sikokianin B **(45)** and sikokianin C **(46)** previously identified from *Wikstroemia sikokiana*.[104] Compound **45** inhibited the growth of the chloroquine-sensitive strain FcR3 and the chloroquine-resistant strain K1 of *P. falciparum* in a similar way (IC_{50} value of about 0.9 μM), whereas **46** exhibited IC_{50} values of 1 and 0.61 μM, respectively. Since the activity of these two biflavonoids was similar against both strains, an absence of cross-resistance with

chloroquine can be concluded. They also showed a weak cytotoxicity on the MRC-5 cells (IC_{50}, SI: 40.5 μM, 41.7 and 20.2 μM, 20.0, respectively). It must be noted that the original *n*-butanol fraction initially showed a lack of selectivity (SI = 0.15, IC_{50} value of 1.2 μg/mL against the MRC-5 cells), indicating that the cytotoxic compounds were eliminated during the bioassay-guided purification. This is an example that illustrates that the SI of a mixture should not be a systematic criterion for rejection.



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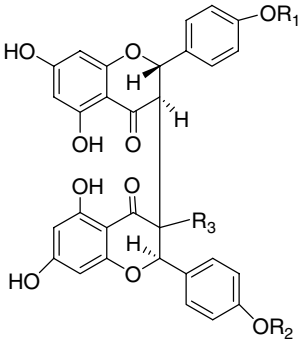


45: $R_1 = CH_3$, $R_2 = H$, $R_3 = \alpha H$

46: $R_1 = H$, $R_2 = CH_3$, $R_3 = \beta H$

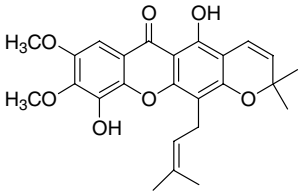
19.5.2.5 Xanthones

It has been proposed that xanthones kill *Plasmodium* by inhibiting heme polymerization in the acidic parasite food vacuole.[105] They effectively form soluble complexes with heme and prevent its polymerization *in vitro* at pH 5.2 in aqueous solution. Furthermore, a study on the stage-specific effects of xanthone derivatives, using synchronous parasite cultures, revealed that they preferentially inhibited the trophozoite stage of development. Hemoglobin degradation and, in consequence the heme polymerization process are maximum at this stage of development and essential for trophozoite growth into schizont.[106] Structure–activity relationships indicated that the presence of hydroxyl groups at positions 4 and 5, or higher degree of hydroxylation, is favorable for the antimalarial activity. However, hydroxylation at positions 1 or 8, leading to the formation of intramolecular hydrogen bond to the carbonyl moiety, probably decreased affinity for heme so that the activity diminished.

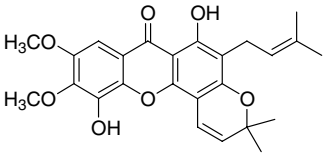
Symphonia globulifera is used in Cameroon, where resistance to chloroquine has reached 67% in some sentinel sites, to cure malaria and several diseases such as stomach and skin aches.[107] The bark is used for the treatment of malaria. From the seed shells, three active prenylated xanthones, that is, gaboxanthone **(47)**, symphonin **(48)**, and globuliferin **(49)**, were isolated along with a benzophenone, guttiferone A **(50)**.

These four compounds showed good-to-moderate activity against the chloroquine-resistant strain W2 of *P. falciparum*. Symphonin **(48)** was the most active with an IC_{50} value of 1.3 μM. IC_{50} values of the other compounds ranged from 3.2 to 3.9 μM. Cyclization of the isopentenyl group at position 4 of **49** led to the formation of a dimethylpyran ring attached to positions 3 and 4 of the xanthone nucleus (as for compound **48)** and an increase in the antimalarial activity.

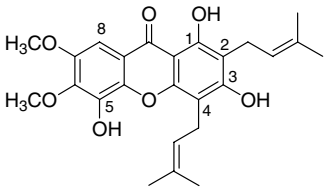
The influence of the pyranic ring and isopentenyl groups on the potency of xanthones was also reported from compounds isolated from *Calophyllum caledonicum*.[108] Dombakinaxanthone **(51)**, with an additional isopentenyl group, exhibited an IC_{50} value of 2 μM against chloroquine-resistant



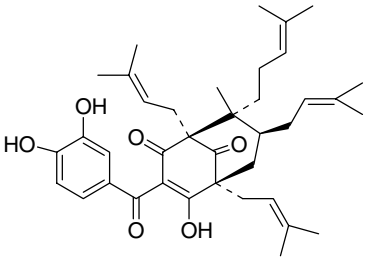
Gaboxanthone **47**



Symphonin **48**

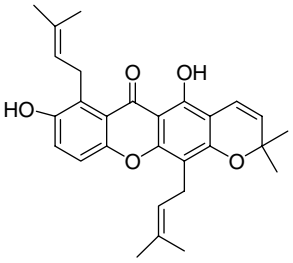


Globuliferin **49**



50

strain FcB1; however, the activity decreases when the pyranic ring is located in positions 7 and 8 for calothwaitesixanthone **(52)** (IC_{50} value of 7 μM).



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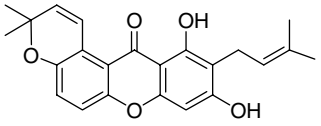

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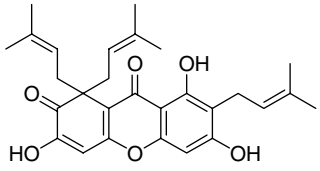

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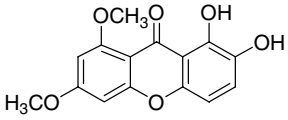


54

Six xanthones were purified from *Allanblackia monticola*, another medicinal plant used in Cameroon for the treatment of respiratory infections, diarrhea, and toothache. The most active was a triprenylated xanthenedione, namely, allanxanthone C **(53)**.[109] Compound **53** is structurally characterized by the presence of *gem bis*-prenyl groups at C1 and a third prenyl group at C7. It was more active on the chloroquine-resistant strain FcM29 of *P. falciparum* (IC_{50}: 1.3 μM) than on the

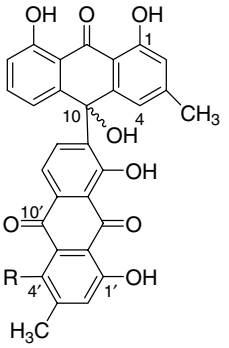
chloroquine-sensitive strain F32 (IC_{50}: 6.9 μM). This interesting compound exhibited no apparent cytotoxicity against the human melanoma A375 cells (IC_{50}: 180.6 μM).

Within the frame of a program for research and training in tropical diseases, investigation of *Andrographis paniculata* led to the isolation of four xanthones; among them was 1,2-dihydroxy-6, 8-dimethoxy-xanthone **(54)**, which exhibited moderate *in vitro* antimalarial activity and a lack of cytotoxicity on the MRC-5 cells.[110] Its *in vivo* antimalarial activity was evaluated on mice infected with *P. berghei* resulting in a reduction of 62.1% of parasitemia at a dose of 30 mg/kg/day.

19.5.2.6 Quinones

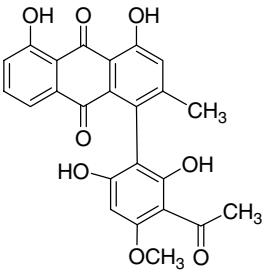
The dichloromethane extract of roots of *Kniphofia foliosa* was shown to be active against the 3D7 strain of *P. falciparum* with a weak cytotoxicity against KB cells.[111] The two most active compounds were identified as 10-(chrysophanol-7′-yl)-10-hydroxychrysophanol-9-anthrone **(55)** and chryslandicin **(56)**. Their absolute configurations were not specified. Compound **55** displayed the highest antimalarial activity with an IC_{50} value of 0.51 μM and an SI of 400, whereas compound **56** exhibited an IC_{50} value of 1.02 μM and an SI of 167.6. The hydroxyl group at position 4′ seems to be unfavorable for antimalarial activity and favorable for cytotoxicity. They were more active than the anthraquinone derivative knipholone **(57)**, which was shown to have a good antiplasmodial activity (IC_{50} values of 1.54 and 2.1 μM on the K1 and NF54 strains of *P. falciparum*, respectively) and a weak cytotoxicity (IC_{50}: 76 μM).[112]

A series of naphthoquinones and anthraquinones were isolated by bioassay-guided fractionation of a lipophilic extract of the root bark of *Stereospermum kunthiana*, a plant used to treat fever in Uganda. Among them, sterekunthal A **(58)** exhibited the best inhibitory activity against chloroquine-sensitive and resistant strains of *P. falciparum* (IC_{50}: 3.85 μM).[113] The potential use of this compound has been limited by its high toxicity against the endothelial ECV-304 cells (IC_{50}: 2.66 μM).



55: R = H

56: R = OH



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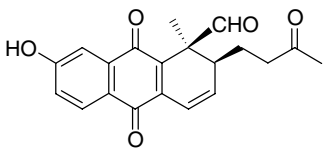


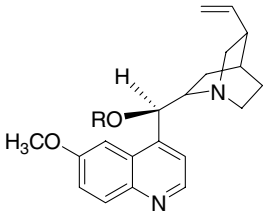
58

19.5.3 Alkaloids

Alkaloids represent a chemical group possessing a large number of potent antimalarial compounds. Quinine (**59**), isolated in the early 19th century from *Cinchona* (*succirubra*) species, has been used as an antimalarial, pure or as a decoction of the tree bark, for over 300 years. It has also served as a template for the synthesis of many quinoline-based antimalarial drugs.[4,5]

19.5.3.1 Quinolines

The esterification of the hydroxy group of quinine **(59)** with polyunsaturated and saturated fatty acids yielded lipid derivatives that showed lower antimalarial activity.[114] Only the acetyl derivative **(60)** exhibited antimalarial activity similar to that of quinine, but the cytotoxicity evaluated against FM3A cells was higher. In this case, the introduction of hydrophobic long-chain fatty acyl groups into quinine did not increase the activity against the parasite in spite of the expected more efficient interaction with the erythrocyte membrane due to the enhanced lipophilicity.

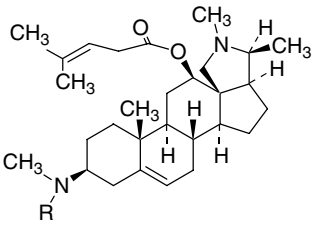


59: R = H, quinine
60: R = Ac

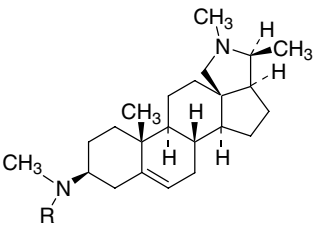
19.5.3.2 Steroidal Alkaloids

Funtumia elastica (popular name "*Ireh*") is used in traditional medicine in West Africa for the treatment of infectious diseases including malaria and bacterial and parasitic infections. The stem latex of *F. elastica* is also used for washing wounds, and the leaves are used for treating hemorrhoids and venereal diseases such as syphilis and gonorrhea. An ethanolic extract of the stem bark of *F. elastica* was found to exhibit *in vitro* inhibitory activity on the chloroquine-resistant strain FcB1 of *P. falciparum* (IC_{50}: 3.3 μg/mL) with no significant toxic effect on human MRC-5 cell lines (IC_{50} > 50 μg/mL).[61] TLC analysis of the crude extract using dichloromethane/methanol/NH_4OH (90/10/1) as the developing solution and visualizing spots with Dragendorff reagent showed the presence of alkaloids.[115] The classical acid–base extraction of alkaloids was not performed in this case, but the residue from the crude ethanolic extract was suspended in H_2O and partitioned successively with cyclohexane, ethyl acetate, and *n*-butanol to yield the corresponding soluble extracts. The antimalarial activity was concentrated in the aqueous and *n*-butanol extracts, which, at 10 μg/mL, exhibited, 98% and 89% inhibition of parasite growth, respectively. The cyclohexane and ethyl acetate extracts were devoid of antimalarial activity. The aqueous extract was consequently subjected to silica gel column chromatography, yielding six fractions (F1–F6) when eluted with dichloromethane/methanol/NH_4OH (90/10/1). Two fractions, F2 and F4, showed strong antimalarial activity with 98% inhibition at 10 μg/mL. Successive purifications of F2 yielded pure holarrhetine **(61)**, conessine **(62)**, and holarrhesine **(63)**. The fourth compound, isoconessimine **(64)** was obtained from F4. They showed significant inhibition of *P. falciparum* growth with IC_{50} values ranging from 0.97 to 3.39 μM and moderate cytotoxicity against the rat cell line L6. Compounds **61**–**64** possessed the same steroidal skeleton and differed by substituents on the amino group at position 3 and in the esterification at position 12. Elimination of an amino methyl group (compound **63**) does not significantly modify the antimalarial activity, but decreases the cytotoxicity slightly thereby increasing the SI. The same

observation is made when the 4-methyl-3-pentenoic acid substituent was removed (compound **62**). Removal of the ester at C12 and one of the amino methyl substituents decreases both the antimalarial and the cytotoxic activities (compound **64**). Compounds **61** and **63** were more toxic than **62** and **64**, from which they differ by the presence of the ester substituent in position C12. Conessine **(62)** and isoconessimine **(64)** showed the highest selectivity against *P. falciparum* (SI = 10–14). They could be used as lead compounds for the synthesis of novel derivatives with improved antimalarial activity and selectivity. Conessine **(62)**, isolated from *Holarrhena antidysenterica*, is also widely used in India as remedy in the treatment of amoebic dysentery caused by infection with *Entamoeba histolytica*.



61: R = CH_3



62: R = CH_3

63: R = H

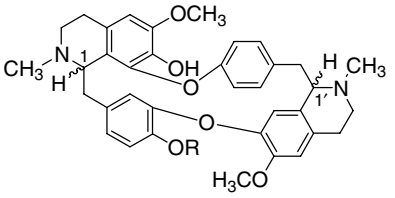
64: R = H

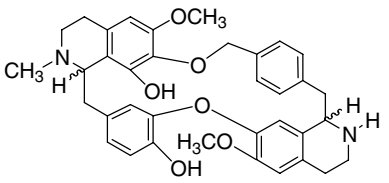
19.5.3.3 Bisbenzylisoquinolines

Bisbenzylisoquinolines are phenylalanine-derived metabolites isolated from many plant families including the Menispermaceae and Annonaceae. They exhibit a wide range of activity and some of them are known to have antimalarial activity that may or may not be associated with cytotoxicity.[116]

A decoction of the root of *Cissampelos mucronata* is widely used against malaria and fever in traditional medicine in the Congo.[117] The ethyl acetate and methanol extracts exhibited significant antimalarial activity against D6 and W2 strains of *P. falciparum*. Acid–base extraction of alkaloids yielded two potent crude alkaloid fractions, which inhibited parasite growth with IC_{50} values in the ng/mL range. The associated aqueous and neutral fractions exhibited no antimalarial activity.[118] Silica gel column chromatography yielded 17 fractions of which 5 showed interesting activity and provided sufficient material for further purification. After successive separations of the alkaloids by HPLC using both RP-18 and CN phases, 16 compounds were isolated including 13 bisbenzylisoquinoline alkaloids, 1 protoberberine alkaloid, 1 isoquinoline, and a new morphinane compound. The most promising ones were curine **(65)** and compound **66** (IC_{50} values around 101 nM) and *R,S*-tubocurine **(67)** and another conformer of curine (IC_{50} values around 168 nm). Although the antiplasmodial activity of curine **(65)**, isolated from *Isolona ghesquierei* (Annonaceae), has been previously reported,[119] the major contribution of the recent work was to develop a routine preparative HPLC separation. The HPLC method allows a more efficient separation of complex alkaloid mixtures compared to open column chromatography, which is performed with eluent systems requiring a high quantity of base. Considerable loss of compounds was observed using preparative normal-phase TLC, probably due to oxidative decomposition of products. Only one compound, a monomeric isococlaurine, was obtained after separation of a selected subfraction using HPLC on RP-C18 phase with mixtures of water, methanol, and trifluoroacetic acid as the mobile phase. The other alkaloids were isolated after passage through RP-C18 followed by HPLC separation on a CN stationary phase with a weak basic mobile phase comprising acetone, methanol, water, or ammonium hydroxide for reversed phase–type selectivity. The use of petroleum ether, hexane, acetone, methanol, and

ammonium hydroxide was adopted when normal-phase selectivity was needed. Curine **(65)** eluted as two distinctive peaks, and this phenomenon was also noticed for 12-*O*-methylcurine **(68)** as well as (−)-cissampetin **(69)**. Bisbenzylisoquinolines can form conformers in solution stable enough at room temperature to enable their isolation as distinct compounds. In the above case, the two conformers for each of **65, 68,** and **69** possessed identical spectroscopic data.





	H1	H1′	R
65: (-) curine	α	α	H
67: *R,S*-tubocurine	α	β	H
68: *S,S*-12-*O*-methylcurine	β	β	CH_3

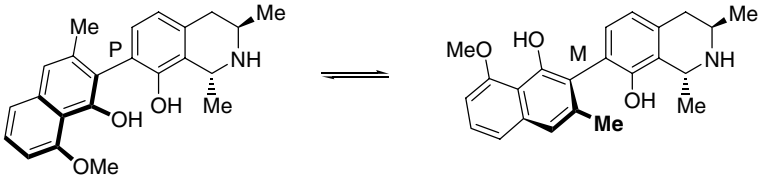
66

69: (-) cissampentin

The use of *Albertisia villosa* described in the Congolese Pharmacopoeia for the treatment of malaria and other infectious diseases is partly validated by the presence of three bisbenzylisoquinolines, that is, cycleanine (a major compound), cocsoline, and *N*-desmethylcycleanine.[120]

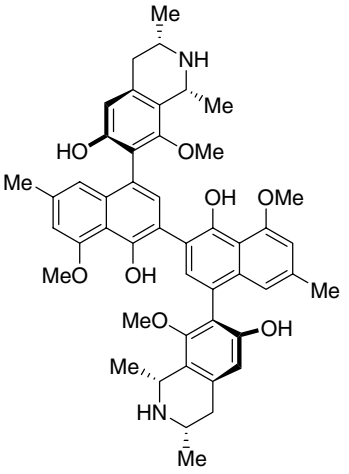
19.5.3.4 Naphthylisoquinolines

A rotationally hindered biaryl axis exists between the naphthalene and isoquinoline moieties of the naphthylisoquinolines that have been isolated from tropical lianas belonging to the closely related families Dioncophyllaceae and Ancistrocladaceae.[121] These plants are widely used in traditional medicine in Asia and in Africa. Dioncophylline E was isolated from *Dioncopyllum thollonii* as a pair of slowly interconverting atropo-diastereomers, (*P*)-**70** and (*M*)-**70**.[122] The absolute axial configuration of these two isomers was determined by a direct CD analysis of the HPLC separated compounds. The two HPLC peaks obtained by resolution of this compound yielded near-opposite CD-spectra, thus confirming the existence of two rotational isomers.

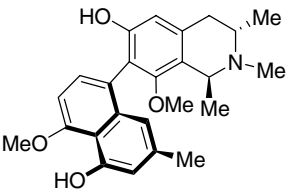


(*P*)-dioncophylline E **70** (*M*)-dioncophylline E **70**

Dioncophylline E **(70)** displayed high antimalarial activity *in vitro* against the chloroquine-sensitive NF54 and the chloroquine-resistant K1 strains of *P. falciparum* with IC_{50} values of 60.6 and 57.9 nM, respectively. Its antitrypanosomal activities were also evaluated against *Trypanosoma cruzi*, the agent of Chagas disease, and *T. brucei rhodesiense*, the agent of sleeping sickness, but it was less effective (IC_{50} values of 50.7 and 2 μM).



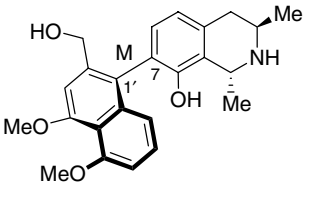
Ancistrogriffithine A **71**



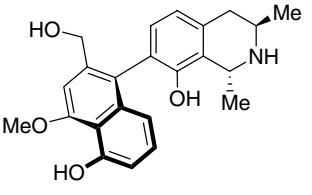
Ancistrogriffine A **72**

A dimeric naphthylisoquinoline alkaloid, ancistrogriffithine A **(71)**, along with ancistrogriffine A **(72)**, was isolated from *Ancistrocladus griffithii*.[123] The full structural elucidation of product **71** from both leaf and twig crude extracts, including relative and absolute configuration, had been previously investigated using an analytical "triad" consisting of a combination of HPLC-MS/MS, HPLC-NMR, and HPLC-CD.[124] The ruthenium-catalyzed oxidative degradation procedure was applied to leaf extract to complete the attribution of the absolute configuration. Compounds **71** and **72** each displayed an activity comparable to that of chloroquine against the chloroquine-resistant strain K1 but with some cytotoxicity toward L6 cells (IC_{50}, SI: 86 nM, 166 and 9.4 nM, 192, respectively)

Another promising alkaloid, habropetaline A **(73)**, has been identified from the crude stem extract of *Habropetalum dawei* (Dioncophyllaceae) using the analytical "triad", HPLC coupled to MS/MS, NMR, and CD. The full absolute stereostructure was established, but insufficient plant material prevented its preparative isolation. However, a directed search for this alkaloid in roots of the more available plant *Triphyophyllum peltatum*,[125] the most investigated species of Dioncophyllaceae, allowed its isolation in large amounts. This alkaloid is a methylated derivative of dioncopeltine A **(74)** that has also been isolated from this plant.



Habropetaline A **73**



Dioncopeltine A **74**

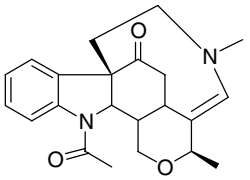
Habropetaline A **(73)** and dioncopeltine A **(74)** exhibited antimalarial activity comparable to artemisinin with IC_{50} values of 12.7 and 5.9 nM for **73** versus 12.6 and 8.7 nM for **74** on the chloroquine-resistant strain K1 and the chloroquine-sensitive strain NF54, respectively, without any apparent cytotoxicity on mammalian cells. Compound **73** was inactive against other protozoan parasites that cause tropical diseases, for example, *T. cruzi, T. brucei rhodesiense*, and *L. donovani*.

19.5.3.5 Resistance-Modulating Alkaloids

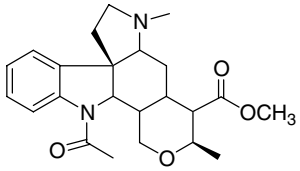
The ability of verapamil to reverse the resistance of *P. falciparum* to chloroquine has opened a new field of investigation for synthetic and natural products modulating antimalarial drug resistance.[126] Such compounds, referred to as chemosensitizers, reversing agents, or modulator agents, could be used in coadministration with existing antimalarial drugs.[127] They would be able to restore the susceptibility of resistant strains to some antimalarial drugs. Feasibility of such a strategy was demonstrated by using chlorpromazine in combination with chloroquine. The mixture has been found to reverse chloroquine resistance *in vivo* in the Aotus monkey *P. falciparum* model. Under the same experimental conditions, verapamil in combination with chloroquine was ineffective, probably due to drug toxicity.[128]

It was reported, from self-medication surveys in Madagascar, that populations living in chloroquine-resistant areas have been cured from malaria by using chloroquine in combination with a preparation of *Strychnos myrtoides*.[129] Two monoindole alkaloids, strychnobrasiline **(75)** (the major product) and malagashanine **(76)** (a minor product), were isolated from this plant. They were found to be devoid of both intrinsic antimalarial activity and cytotoxicity activity on mammalian cells. However, they exhibited significant chloroquine-potentiating actions on chloroquine-resistant strains of *P. falciparum*, malagashanine being the most potent compound. Surprisingly, these reversing agents did not enhance the activity of chloroquine on chloroquine-sensitive strains of *P. falciparum*, strongly indicating that they act by interfering with the mechanism(s) involved in chloroquine resistance. Malagashanine **(76)** was also found to exhibit significant enhancing action on other antimalarials including quinolines (quinine and mefloquine), aminoacridines (quinacrine and pyronaridine), and a structurally unrelated drug (halofantrine), all of which are reported to exert their antimalarial activity by inhibiting heme polymerization, as for chloroquine.[130]

The mechanism of action of malagashanine was recently investigated.[131] By following [^{3}H]-chloroquine accumulation in the presence of malagashanine **(76)**, it was found that this compound increased chloroquine accumulation at mild and old trophozoite stages of chloroquine-resistant strains. This effect was concentration dependent and not observed in chloroquine-sensitive strains. The different experiments undertaken by the authors provided strong evidence that malagashanine **(76)** prevents chloroquine efflux from the drug-resistant parasites and also stimulates chloroquine influx into these parasites. Thus, malagashanine seems to act through two different mechanisms, resulting in an increase of the intracellular chloroquine concentration in resistant parasites. Malagashanine **(76)** appears to be a useful lead compound for the design and the synthesis of more powerful resistance modulators.



75: Strychnobrasiline



76: Malagashanine

Drug interactions between chloroquine and natural products can be evaluated by the isobologram method (Figure 19.4).[10] Fractional inhibitory concentrations corresponding to the IC_{50} value of the drugs in mixture on the IC_{50} value of one drug alone are plotted. A curve above the diagonal, a curve falling below the diagonal, and a curve superimposed on the diagonal indicate, respectively, antagonism, synergism, and a simple additive effect of the natural product on chloroquine inhibition. A sum of the fractional inhibitory concentrations >1 indicates antagonism, equal to 1 addition, and <1 synergism.

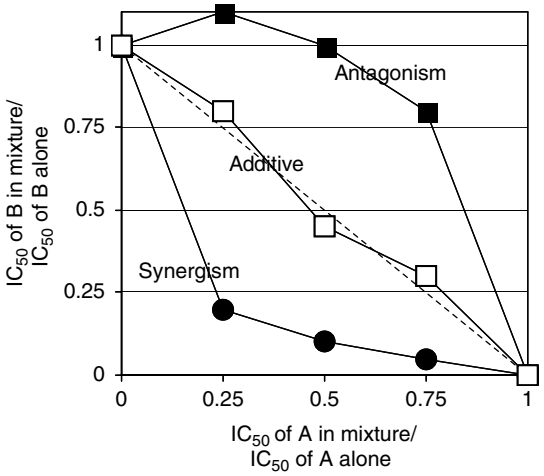
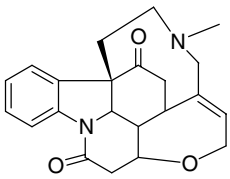
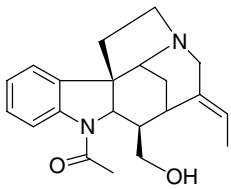


FIGURE 19.4 Isobologram of interaction between two compounds having antagonistic (full square), additive (open square), or synergistic (full circle) effects.

Two other indole alkaloids, icajine **(77)** and isoretuline **(78)**, devoid of *in vitro* antimalarial activity and toxicity against HCT-116 human cancer cell line, were isolated from African *Strychnos* spp. As for malagashanine **(76)**, they showed a marked synergistic effect with chloroquine on the chloroquine-resistant strain W2 of *P. falciparum*.[132] Icajine **(77)** was also demonstrated to share *in vitro* mefloquine-potentiating activity on the mefloquine-resistant F32 strain of *P. falciparum*, whereas isoretuline **(78)** showed little effect.



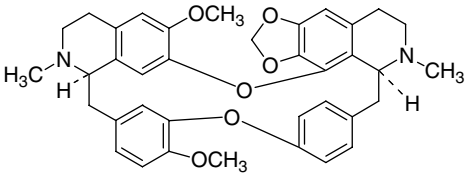
77: Icajine



78: Isoretuline

The potentiation of chloroquine activity was also described for indole alkaloids derived from *Aspidosperma* spp.[133]

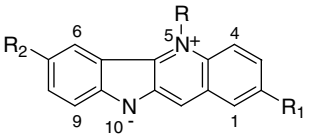
Stephania erecta is used in Thailand as an analgesic and a skeletal muscle relaxant. The bisbenzylisoquinoline alkaloid cepharanthine **(79)** was isolated from this plant and exhibited good *in vitro* antimalarial activity against the chloroquine-sensitive D6 and chloroquine-resistant W2 strains of *P. falciparum* with IC_{50} values of 450 and 590 nM, respectively. It preferentially inhibited the trophozoite stage development.[134] However, compound **79** showed significant cytotoxicity against KB cells (SI around 10), and despite a promising *in vitro* activity, it presented a weak *in vivo* activity against a murine model of malaria, with 46% of inhibition of parasitemia for the high dose of 100 mg/kg/day. Interaction studies with chloroquine further limited investigations on this compound. Cepharanthine was antagonistic to the action of chloroquine on the chloroquine-sensitive strain D6 and synergistic to the action of chloroquine on the chloroquine-resistant strain W2.

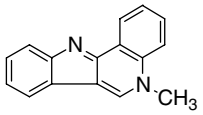


Cepharanthine **79**

19.5.3.6 Indoloquinolines

A decoction of the roots of *Cryptolepis sanguinolenta* is used in traditional medicine in Ghana for the treatment of malaria and other infectious and noninfectious diseases. Cryptolepine **(80)**, the major alkaloid present in the root, had potent antimalarial activity *in vitro* on *P. falciparum* and *in vivo* on rodent malaria parasite but was also cytotoxic, able to intercalate into DNA and to inhibit topoisomerase II as well as DNA synthesis.[135,136] Isocryptolepine **(81)**, also isolated from *S. sanguinolenta*, exhibited antimalarial activity comparable to compound **80**.





81: Isocryptolepine

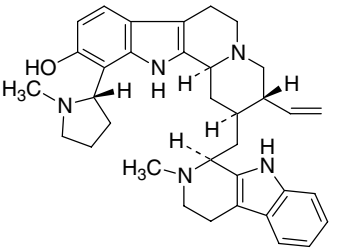
	R	R_1	R_2
80:	CH_3	H	H
82:	H	H	H
83:	CH_3	Br	Br
84:	CH_3	Cl	Br
85:	CH_3	Br	NO_2

The 5-methyl group appears necessary for the antimalarial activity since quindoline **(82)**, another compound isolated from this plant, is inactive against the chloroquine-resistant strain K1. To overcome the toxic side effects, several research groups have focused their efforts on the synthesis of cryptolepine analogues.[136,137] These analogues are expected to have reduced abilities to interact with DNA and thus to be less cytotoxic, but to retain potent antimalarial activity. Among several analogues synthesized, 2,7-dibromocryptolepine **(83)**, 7-bromo-2-chlorocryptolepine **(84)**, and 2-bromo-7-nitrocryptolepine **(85)** were the most potent *in vitro* and *in vivo* with a reduction of parasitemia >90% without any apparent toxicity to the mice.[136] They each exhibited a high selective index compared to cryptolepine **(80)**. The substitution at position 7 by halogen or nitro group in addition to halogen substituent in the quinoline ring enhanced the activity, whereas the presence of alkyl or alkoxy in the ring was detrimental.

19.5.3.7 Indolomonoterpenoid Alkaloids

Strychnos spinosa and *S. henningsii* are used traditionally in the treatment of fevers and malaria in Africa and Asia.[138] The *in vitro* antimalarial activity of 69 alkaloids isolated from various *Strychnos* species was investigated and revealed that bisindole alkaloids were the most potent and selective

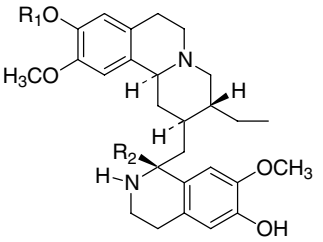
against chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*.[139] One of the most potent compounds, isostrychnopentamine **(86)** isolated from *Strychnos usambarensis*, exhibited an *in vitro* IC_{50} value of around 100 nM against all *P. falciparum* strains tested and was selective against human cancer cell lines (KB, WI-38, HCT-15, HCT-116) with an SI ranging from 15 to 421.[140] The evaluation of the stage specificity of **86** showed that it was active against all stages of the *Plasmodium* life cycle but preferentially active at the ring stage. This result suggested a different mode of action for isostrychnopentamine **(86)** compared to quinoline antimalarial drugs such as chloroquine and quinine, which are essentially active against the trophozoite stage. Furthermore, and unlike chloroquine and quinine, isostrychnopentamine **(86)** does not accumulate in the digestive vacuole. Compound **86** was active *in vivo* against the *P. berghei* NK 173 and *P. vinckei petteri* murine strains both sensitive to chloroquine. At a dose of 30 mg/kg/day, parasitemia is reduced by 61.8% on mice infected with *P. vinckei petteri*.



86: Isostrychnopentamine

19.5.3.8 Other Alkaloids

An ethanol extract of *Psychotria klugii* was found to possess an interesting antimalarial activity. Bioassay-guided fractionation led to the isolation of five benzoquinolizidine alkaloids; among them were two potent compounds, klugine **(87)** and cephaeline **(88)**.[141] They were equally potent against the chloroquine-resistant strain W2 (IC_{50}: 99 and 59.4 nM) and chloroquine-sensitive strain D6 (IC_{50}: 80.6 and 81.6 nM). They were each inactive against mammalian Vero cells with an SI of 217 and 193. Klugine **(87)** was devoid of cytotoxicity against the SK-MEL, KB, BT-549, and SK-OV-3 human cancer lines, whereas cephaeline **(88)** was more potent than doxorubicin against these cell lines.

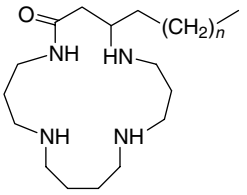


87: Kugline R_1= H, R_2 = OH

88: Cephaeline $R_1 = CH_3$, R_2 = H

The *Albizia* species (Leguminosae) *A. gummifera* and *A. schimperiana*, among others, are used for the treatment of parasitic infections including malaria and more generally against fever in traditional medicine in Kenya. Crude methanol extracts from the stem bark and leaves of *Albizia adinocephala* inhibited plasmepsin II, an indispensable enzyme for parasite survival.[142] This enzyme has

been well characterized and was available for assay development. Bioassay-guided fractionation of these extracts led to the isolation of two macrocyclic spermine alkaloids, budmunchiamines L4 **(89)** and L5 **(90)**. They inhibited plasmepsin II with IC_{50} values of 14 and 15 μM, respectively.



89: Budmunchiamine L4, $n = 11$

90: Budmunchiamine L5, $n = 13$

19.5.4 Plants and Natural Products Active at the Hepatic Cycle of Infection

Some plants widely used in traditional medicine to treat malaria were found to be devoid of activity against the erythrocytic stage of *P. falciparum in vitro* or in mice infected with *P. berghei*; nevertheless, they sometimes displayed exoerythrocytic antimalarial activity. For example, the hepatic schizontocidal activity of the ethanol extract of *Ampelozizyphus amazonicus*, known as "Indian beer", as well as the ethanolic extract of *Geissospermum argenteum* bark, might explain their prophylactic effect on human malaria.[56,60] Screening of plants used traditionally on the islands of Sao Tomé and Principe revealed, four, that is, *Struchium sparganophorum, Tithonia diversifolia*, *Pycnanthus angolensis*, and *Morinda lucida*, with antiplasmodial activity (IC_{50} values from 5 to 34 μg/mL) against *P. berghei* cultured *in vitro* on Hep G2 cells.[143]

Strychnopsis thouarsii is an endemic plant of Madagascar traditionally used against malaria. Previous work showed that the ethanolic extract was active *in vitro* against the erthryocytic stages of *P. falciparum* and led to the isolation of the active compounds, fangchinoline (7-*O*-demethyltetrandrine), a bisbenzylisoquinoline alkaloid, four aporphine alkaloids (isocorydine, predicentrine, liriotulipiferine, *N*-methyllindcarpine), and the morphinan alkaloid sinoacutine.[144] Fangchinoline was also demonstrated to be a chemosensitizer agent against chloroquine resistance of *P. falciparum* and vinblastine multidrug resistance of cancer cells.[10] The decoction of this plant was shown to inhibit growth of *P. yoelii* in hepatocyte cultures. Bioassay-directed fractionation of the stem bark decoction prepared as a traditional recipes led to the isolation of a new morphinan alkaloid named tazopsine.[145]

Only primaquine, atovaquone, and tafenoquine are currently used as prophylactic drugs. However, their use is limited by their cost or by their side effects (e.g., hematological toxicity for primaquine).

19.5.5 Other Sources of Natural Antimalarial Compounds

The isolation of antimalarial drug templates from the biodiverse marine environment has been reviewed elsewhere.[146] For example, an investigation of the antiparasitic potential of the marine natural product ascididemin, and related synthetic derivatives, revealed attractive antimalarial activities for five compounds with IC_{50} values for K1 and NF54 strains of *P. falciparum* ranging from 57 to 219 nM.[147] Xestoquinone, isolated from the marine sponge, *Xestospongia* sp., was found to be an inhibitor of Pfnek-1, a protein kinase of *P. falciparum*.[148]

Other organisms such as fungi also produce interesting bioactive compounds as shown by the example of macrocyclic trichothecenes.[71] Two inhibitors of the plasmodial FAS-II enzyme, cerulenin and thiolactomycin, were isolated from a fungus.[149]

Psalmopeotoxin I (PcFK1) and psalmopeotoxin II (PcFK2), two peptides identified in the venom of the Trinidad chevron tarantula, *Psalmopoeus cambridgei*, inhibited the growth of *P. falciparum* with IC_{50} values of 1.59 and 1.15 μM, respectively. This work demonstrated that peptides or toxins obtained from venom could be promising tools for antimalarial research and be the basis for the rational development of antimalarial drugs.[150]

19.6 CONCLUSION

Increasing resistance of the malaria parasites to currently available drugs is a major concern nowadays, limiting more and more the control of this serious disease affecting developing countries. This situation could worsen if the predicted global climate change occurs with a consequent displacement of the malaria vectors to areas where humans have not previously faced the parasite. Considering the limited arsenal of drugs, the WHO health policy recommends combinations of antimalarial drugs instead of single-drug treatments in order to reduce the emergence of parasites resistant to one of the drugs used in combination. Such combinations are based on the only drug for which no clear resistance has yet been reported, that is, artemisinin and its derivatives, a natural product derived from a traditionally used medicinal plant. This situation requires an urgent need for new antimalarial drugs, especially acting on the parasite through novel mechanisms of action.

As shown in this review, antimalarial properties have been described for numerous natural products. However, few compounds have been further investigated in preclinical studies or in clinical trials. Indeed, drug development is a long process with a high financial risk, and since antimalarial drugs compete poorly with other drug categories, few have been developed in the last decade. Perception of malaria by the public, the politicians, and the pharmaceutical companies is changing, and increasing financial contributions sustain discovery and development projects that offer hope for the development of new, affordable drugs. However, such efforts will not be fruitful if they do not also support research for a better understanding of the parasite biology at the molecular level. This is considered a prerequisite for identifying key molecules for the development of new chemotherapeutic strategies. With the sequencing of the genomes of malaria parasites, there is a revolution in parasitology. Postgenomic methodologies and technologies offer an increasing number of validated targets against malaria. High-throughput bioassays of molecules against these targets are becoming more accessible to the academic laboratories. By their large diversity, natural products from plants, marine organisms, or microorganisms constitute an inexhaustible reservoir of molecules of which only a few have been investigated. Access to these new bioassays will be an opportunity to explore this reservoir of molecules for their antimalarial properties, which may provide novel lead molecules for new antimalarial drugs.

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20 Germination Stimulant in Smoke: Isolation and Identification

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CONTENTS

20.1 INTRODUCTION

Toward the end of the 1980s, Hannes de Lange, a graduate student then at the University of Stellenbosch in South Africa, carried out a most unusual experiment on the plant that he had chosen to study for his dissertation. The plant, false heath (*Audouinia capitata*, Bruniaceae), grows in the fynbos, the magnificent botanical region in the south and southwest Cape of Africa. However, he faced a major problem. The seeds of false heath do not sprout under normal greenhouse conditions and the only references to seedlings of the plant came after a wildfire in the fynbos. In what appears to be a "suspension of logic", he devised an experiment in which he fumigated selected soil sites with plant-derived smoke. On revisiting the site some 7 months later, he found a considerable number of false heath seedlings growing on the smoke-treated plots, but not on the control sites. This finding sparked a great deal of interest and several groups from North America, South Africa, Europe, and Western Australia began the search for the germination stimulant(s) in plant-derived smoke.

Shortly after de Lange's discovery, smoke was found to elicit a remarkable increase in germination of hundreds of native Australian plant species[1–7] and a wide range of species from North America,[8,9]

South Africa,[10,11] and Spain.[12] In addition, smoke has been shown to promote the germination of selected crop species such as lettuce,[13] celery,[14] and sweet basil.[15] It has also been implicated in breaking seed dormancy of weed species such as wild oats (*Avenua fatua*)[16] and red rice (*Oryza sativa*),[17] shown to promote flowering[18] and enhance somatic embryogenesis[19] as well as stimulating root development[20] and increasing seedling vigor.[3,21–24]

The use of crude or semipurified smoke products has achieved some importance in a range of fields, including biodiversity conservation, horticulture, agriculture, and land restoration.[5,11,25] However, the identity of the bioactive chemical(s) in smoke that promotes germination remained unknown.[26–30]

In this chapter, the various strategies applied in the search for the proverbial "needle in the haystack" are presented. In the process, modern analytical and separation techniques were employed in the detection, isolation, and identification of the potent germination stimulant in smoke.

20.2 ISOLATION

20.2.1 Germination Bioassay

Because of the large number of chemicals contained in smoke, a key to identifying the active component(s) was the use of a rapid bioassay. A bioassay can be defined as a test that uses a biological indicator for the tracking of active components through a series of separation steps. To investigate the bioactive compound(s) in smoke, the bioassay had to meet a number of requirements. The most important was that the seeds of the test species should show highly significant germination response with smoke as compared to the control (water only). The species should also show consistency and reliability in its germination response and be readily available so as to allow continuity in the investigation.[28] In addition, the bioassay needed to be simple, easy to perform, and provide a quick turnaround of results.

In previous studies, seeds of *Syncarpha vestita* (Asteraceae)[31,32] and *Themeda triandra* (Panicoideae)[33,34] had been used in bioassays. However, these species are not readily available, are very small, and germinate over extended periods of time.[13] Baldwin et al.[26] used seeds of *Nicotiana attenuata* (Solanaceae) in a bioassay, but this species is known to produce seeds of varying dormancy with some genotypes germinating readily with only water.[35] Dixon et al.[28] had found that seeds of *Stylidium affine* (Stylidiaceae), although effective in many ways as a bioassay species, required 7 weeks incubation before final results became available. In addition, sterilization requirements made the assay time-consuming and difficult to perform.

A breakthrough came when Drewes et al.[13] reported that light-sensitive lettuce seeds (*Lactuca sativa* cv. Grand Rapids) responded to extracts obtained from smoke and could be used in a simple and rapid bioassay for the detection of germination-promoting compounds. Seeds of this species germinate within 24 h in the dark in response to a wide range of concentrations of a smoke-derived extract from *T. triandra*. Control germination for the experiment at 20°C was about 45%, whereas the smoke extract promoted germination to more than 90%. Since this discovery, the *L. sativa* bioassay has been used in a number of studies[27,32] and was adopted as a frontline bioassay in the current study. More statistically significant results were obtained at key points of the investigation with the highly smoke-responsive Australian species, *Conostylis aculeata* (Haemodoraceae)[3] and *S. affine*.[4]

20.2.2 Types of Smoke That Stimulate Germination

Previous studies had shown that the smoke derived by burning a variety of plant materials, cellulose and agar, produced the germination-active compound(s).[26,34,36] Plant-derived smoke contains a complex mixture of compounds many of which are derived as combustion products from lignin and cellulose. Smoke generated from cellulose does not contain the many aromatic compounds produced from lignin. Thus, the smoke obtained by pyrolysis of filter papers was used in the search for the active chemical(s).

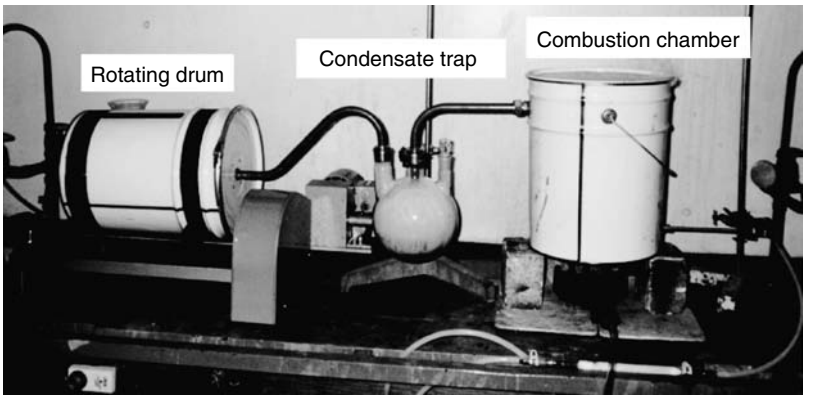


FIGURE 20.1 Apparatus for producing dry CSS.

Although water has been shown to be highly effective at trapping the active chemical(s) in smoke, aerosol smoke has consistently been reported as a more effective seed treatment for germination when compared to "smoke water".[1,5,25] In addition, soil or clean sand can be treated with aerosol smoke to produce "smoked sand" into which seeds can be sown in the conventional way.[1] Moreover, the stimulant(s) had been shown to remain active in the smoke-treated soil for more than a month under normal greenhouse conditions.[26] Ecologically, the stability of the active compound(s) in soil is extremely important, because following a fire event, the active compound(s) is deposited onto the soil surface and must survive the extremes of summer (typically when most fire events occur) and remain stable for periods of up to 6 months. The winter rains are able to solubilize the active compound(s) and deliver the germination stimulant(s) to the seed bank below. This important trait would suggest that the active compound(s) is not one of the more volatile components in smoke, otherwise its activity in soil would decrease appreciably with time. In terms of identifying the active agent(s), it was thought that sand could offer a selective method for trapping the chemicals in smoke and could provide a higher initial concentration of the active compound(s).

An apparatus developed to produce "cellulose-derived smoked sand (CSS)" is shown in Figure 20.1. The smoke generated in a 20-L combustion chamber was forced by positive pressure, through a round bottom flask that served as a "condensate trap" where the smoke was able to cool and the water present condensed. The now cool, drier smoke passed into a drum containing clean sand that was designed to rotate at slow speed to ensure uniform treatment of the sand. The purpose of the "condensate trap" was to prevent the sand from becoming damp from the water produced in the combustion process, which had been a major problem with an earlier apparatus. The damp "smoked sand" was found to be less selective in its trapping of smoke chemicals when compared with dry "smoked sand".

The material extracted with diethyl ether from the "smoked sand" and the "condensate trap" showed significant germination activity in the *L. sativa* bioassay. GC analysis of the two extracts showed that they had similar, albeit complex, profiles. This was interesting, as the "condensate trap" had been designed to enhance the selectivity of the "sand trap" by removing the water from the combustion process, yet the compounds that condensed with the water were similar to those adsorbed by the sand. Therefore, the apparatus was simplified to use only the "condensate trap" to contain the compounds. In this way, smoke could be generated continuously for hours without being limited by solubility or adsorptivity factors imposed by using water or sand as a trapping medium. In fact, the longer the apparatus was used, the greater the amount of "crude smoke" extract recovered. Once the smoke had passed through the "condensate trap", it was bubbled through deionized water to ensure maximum trapping of the smoke components.

20.2.3 Extraction of the Bioactive Agent

Attention now turned to the isolation of the active compound(s) from the aqueous condensate. A number of solvents, such as hexane, diethyl ether, ethyl acetate, chloroform, and dichloromethane, had previously been used to recover the organic material in smoke water.[11,28] In the present study, diethyl ether was chosen because of its low boiling point (37°C), an important consideration given that the chemical(s) of interest may be volatile. The smoke extracts obtained with ether showed activity in the bioassay, confirming previous observations.[28]

In classical natural products chemistry, and before the advent of modern preparative chromatography, it was common to fractionate the total organic extract from a plant sample into strong acids ($NaHCO_3$-solubles), weak acids (NaOH-soluble), basic (HCl-solubles), and neutral fractions. This separation on the basis of functional group greatly facilitated purification of compounds within a particular grouping. In this case, it was hoped that the bioactivity would be restricted to one of the four fractions. Since the basic fraction contained negligible mass, the strong acids, weak acids, and neutral fractions along with the extracted aqueous phase were all tested with the *L. sativa* bioassay (Figure 20.2). The fractions were tested at a series of dilutions to ensure the right concentration range for activity.

The majority of promotive activity was associated with the neutral fraction. The "strong acid" fraction also promoted germination as did the smoke water after extraction, but only at low dilution levels. At higher dilution (1/500), the neutral fraction promoted germination more effectively than the other fractions. Use of dichloromethane as the extracting solvent was later shown to decrease the germination response of the "strong acid" fraction and the extracted smoke water, with activity confined predominantly to the neutral fraction.

When the same fractions were tested with the *C. aculeata* bioassay, a similar germination pattern was found (Figure 20.3). The neutral fraction induced the highest levels of germination (ca. 50%) and the "strong acid" fraction also promoted a small level of germination, similar to that seen with the *L. sativa* bioassay (Figure 20.2).

These results provided clear evidence that the active compound(s) from cellulose-derived smoke (CS) was a neutral compound. This was of some significance, given that a large number of the compounds previously identified in smoke extracts and tested for germination activity had been

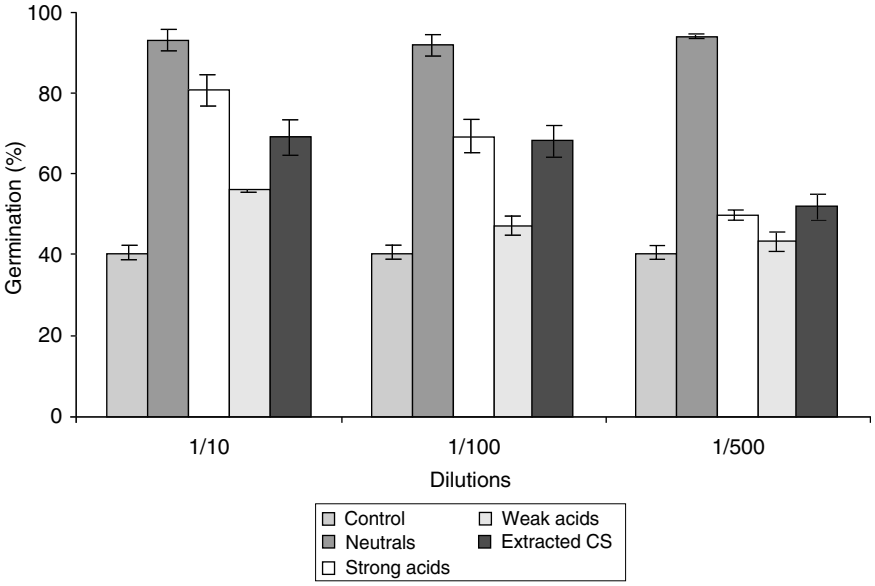


FIGURE 20.2 Germination of *Lactuca sativa* cv. "Grand Rapids" in response to fractions derived from the CS extract. Error bars represent standard errors of the mean (SEM). Water served as the control.

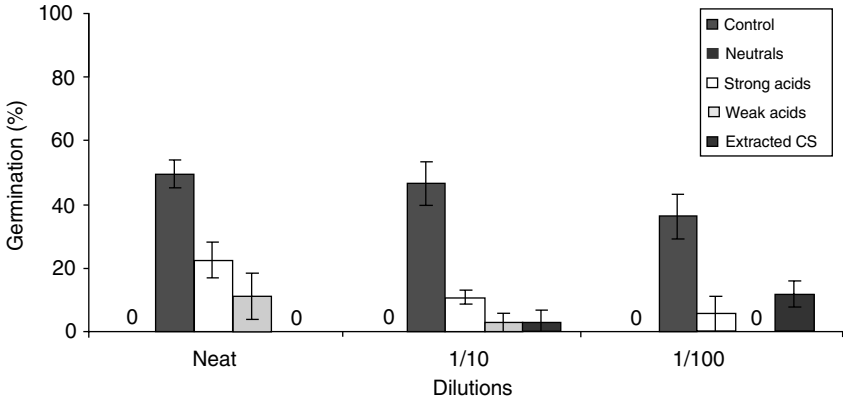


FIGURE 20.3 Germination of *Conostylis aculeata* in response to fractions derived from the CS extract. Control germination was 0%. Error bars represent SEM. Water served as the control.

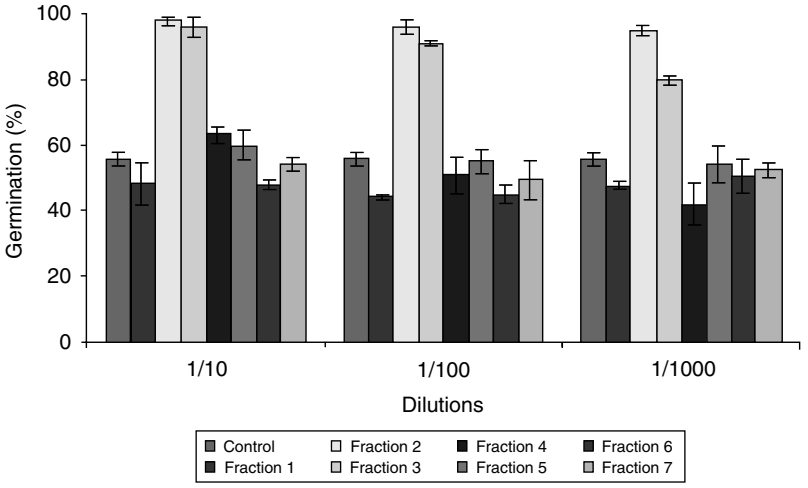


FIGURE 20.4 Germination of *Lactuca sativa* in response to the fractions derived from alumina chromatography of the CS neutral fraction. Error bars represent SEM. Water served as the control.

organic acids and phenolic compounds.[26–28] The acidic compounds could now be eliminated from consideration, thus reducing the number of potential candidates. In terms of mass return, the neutral fraction represented 40% of the original extract, further indicating the importance of this initial step.

20.3 SEPARATION

20.3.1 Column Chromatography

The next step was to separate the CS neutral fraction by chromatography using alumina with solvents of increasing polarity (light petrol → ether → ethyl acetate → methanol and mixtures thereof). Thus, the mixture of the neutrals was separated into seven fractions, a number dictated to a large extent by the bioassay. Separation into seven fractions and testing in triplicate at three dilutions (plus control) meant 66 samples would be tested, a manageable number.

Fractions CS 2 and CS 3, eluted with a mobile phase of 50% ether/light petrol and 100% ether, contained the majority of germination activity (Figure 20.4). These fractions were combined and

separated again on alumina initially eluted with light petrol and followed by incremental additions of ether up to 100% ether. Bioassay of the fractions (15 in total) showed that activity was associated with fractions CS 2-6, 2-7, and 2-8, which had eluted with approximately 30% ether/light petroleum as solvent (Figure 20.5).

GC analysis of fraction CS 2-7 revealed that the mixture was still very complex. However, analytical reverse-phase (RP) C18 HPLC of the active fraction CS 2-7 provided a good separation of the main components. Therefore, fraction CS 2-7 was applied to a C18-RP column and eluted, under reduced pressure, with water, followed by stepwise increases of acetonitrile into water up to 100% acetonitrile. A total of eight fractions were collected and an aliquot of each fraction was taken and tested with the *L. sativa* bioassay (Figure 20.6).

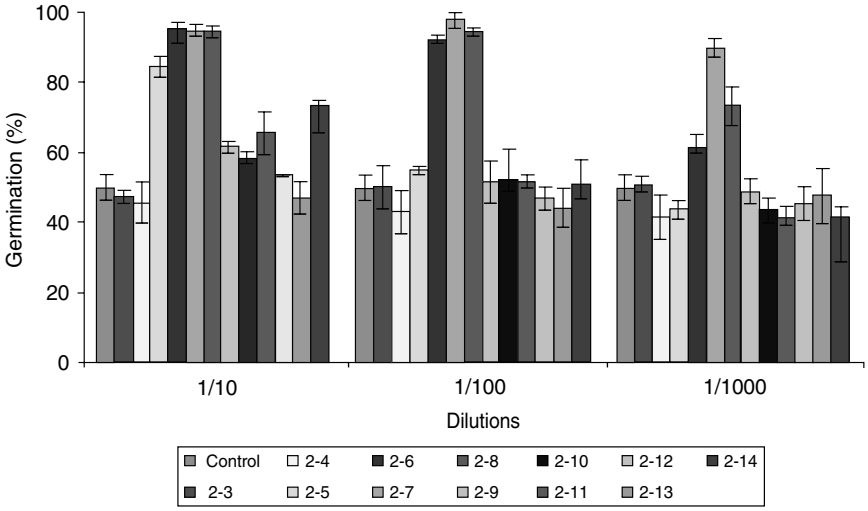


FIGURE 20.5 Germination of *Lactuca sativa* in response to the second alumina fractions. Error bars represent SEM. Water served as the control.

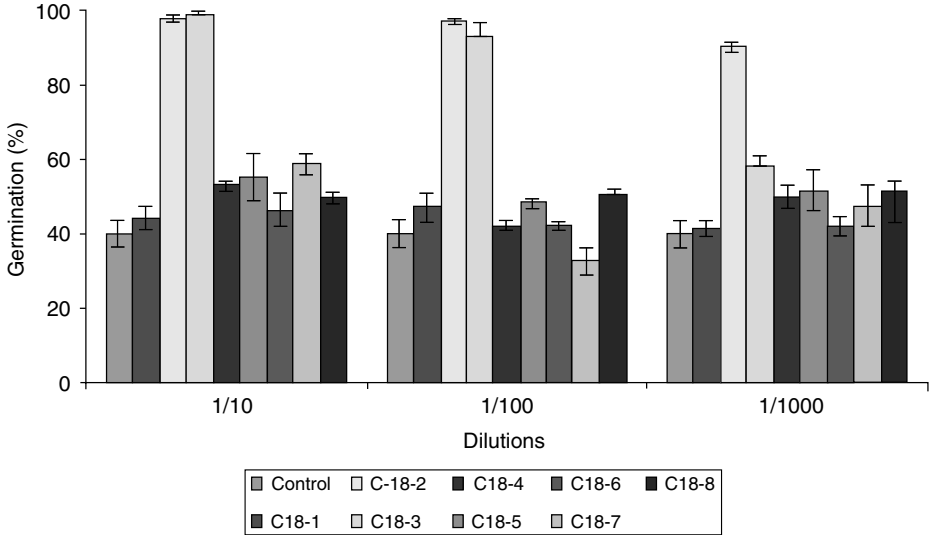


FIGURE 20.6 Germination of *Lactuca sativa* in response to the C18-RP separation of the alumina fraction CS 2-7. Error bars represent SEM. Water served as the control.

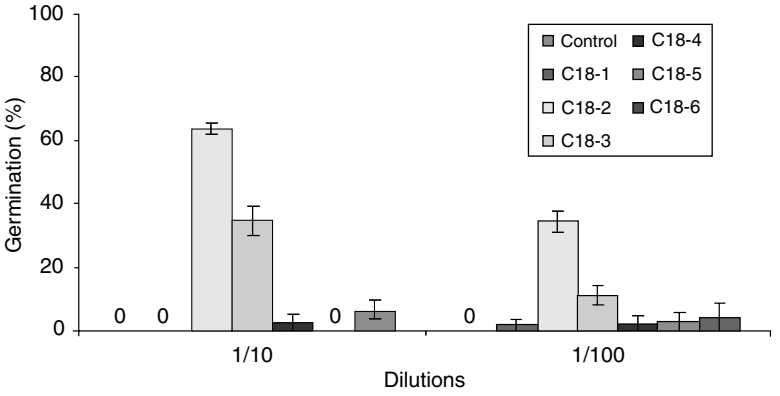


FIGURE 20.7 Germination of *Conostylis aculeata* tested with the C18 fractions. Error bars represent SEM. Water served as the control.

Fractions C18-2 and C18-3 had the highest activity (Figure 20.6). Furthermore, the active compound(s) had a low retention on the C18-RP column and eluted with 10–20% acetonitrile/water. As a precaution against losses of volatiles, care was taken to recover the compounds from the predominately aqueous fraction. Techniques such as evaporation *in vacuo*, freeze-drying, extraction (ether, dichloromethane), and evaporation with a stream of nitrogen were tested. The best method was found to be extraction of the aqueous residue, after evaporation of the bulk of acetonitrile under reduced pressure, with dichloromethane. The dichloromethane was then removed by distillation at atmospheric pressure.

Testing of the same C18 fractions with *C. aculeata* gave germination responses similar to those obtained with *L. sativa* (Figure 20.7), in which activity was confined to two fractions. Importantly, the results suggested that the same compound(s) was responsible for promoting germination in both of these different species. In addition, the activity of the C18-2 fraction was demonstrated at very low levels with bioassay concentrations of 10 ppb (i.e., 10 μg/L) showing activity with *L. sativa* and 100 ppb showing activity with *C. aculeata*.

Comparison of fraction CS 2-7 (from alumina fractionation) and the C18-2 fraction by HPLC (HP Hypersil ODS, 250 mm × 4 mm, 5 μm) showed that the majority of the peaks present in CS 2-7 were absent in the C18-2 fraction (Figure 20.8). Interestingly, the major peak and the bulk of CS 2-7 that eluted after R_t 20 min were not responsible for the activity, since they were absent in fraction C18-2. Comparison of the two active fractions C18-2 and C18-3 showed an area of overlap between R_t 18 and 20 min in the chromatograms, which should contain the active compound(s).

The HPLC chromatograms (Figure 20.8) showed that the C18 fractionation provided an efficient method for eliminating a significant number of compounds from the investigation. However, comparisons of the germination-active C18 fractions by GC-MS (Figure 20.9) showed that a considerable number of compounds were still present in the mixture.

20.3.2 Preparative HPLC Methods

After obtaining more of the C18-2 fraction, semipreparative HPLC was investigated. Half of the C18-2 fraction (ca. 15 mg) was applied to a C18-RP HPLC column (Alltech Econosil, 250 mm × 22 mm, 5 μm) and isocratically eluted with 30% methanol in water. Fractions were collected every minute for 40 min. A subsample from selected fractions was tested using the *L. sativa* bioassay. The results showed that fractions 21–24 (Prep21–Prep24; <1 mg each) had germination activity (Figure 20.10) with a >90% response.

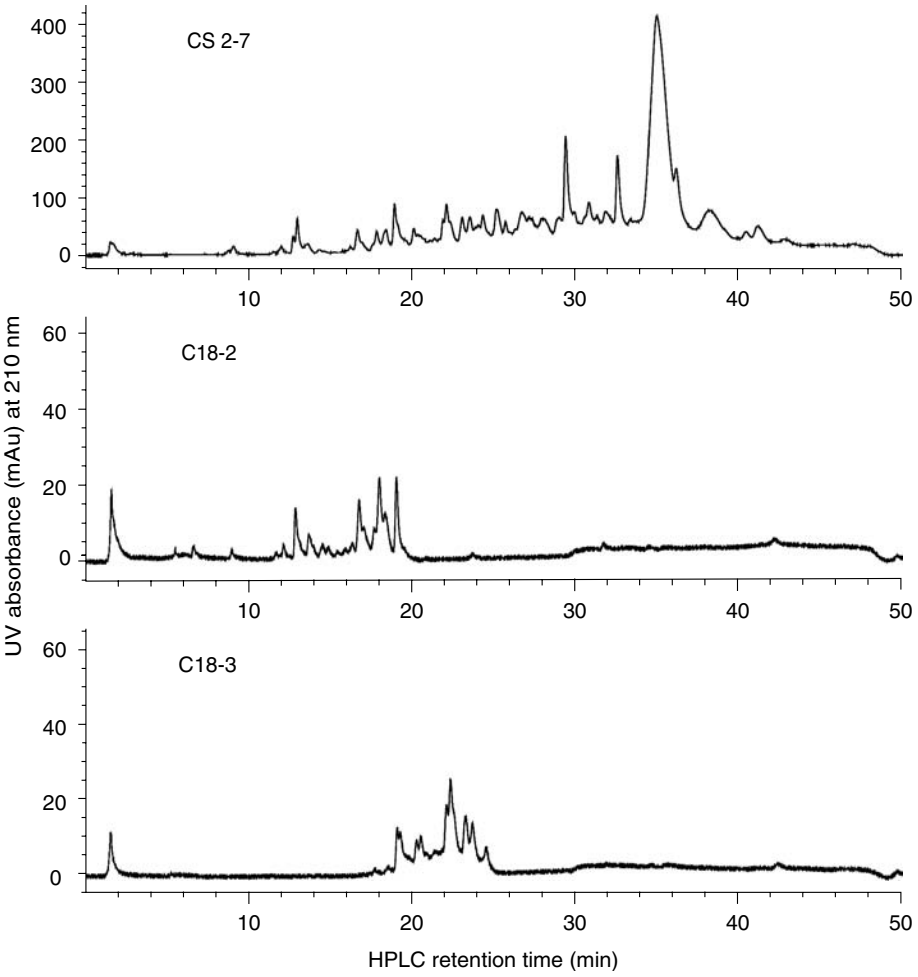


FIGURE 20.8 HPLC comparisons of the alumina fraction CS 2-7 and the C18 fractions, C18-2 and C18-3. *Note*: Different scales on *y*-axis.

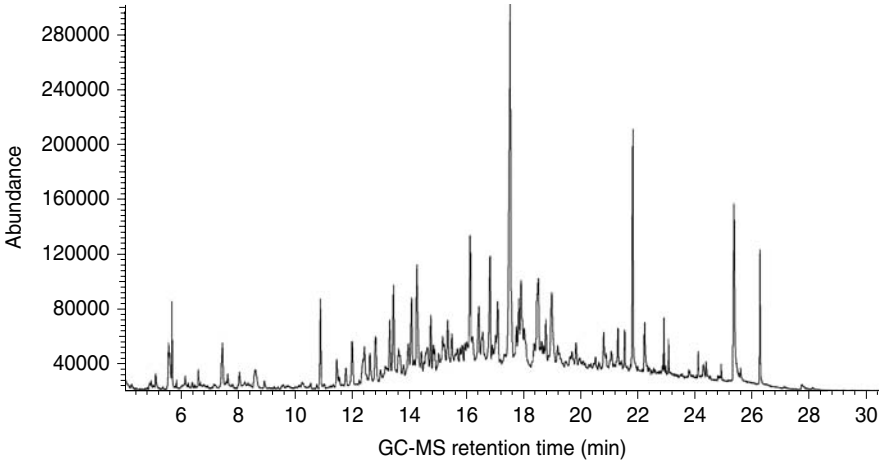


FIGURE 20.9 GC-MS chromatogram of fraction C18-2.

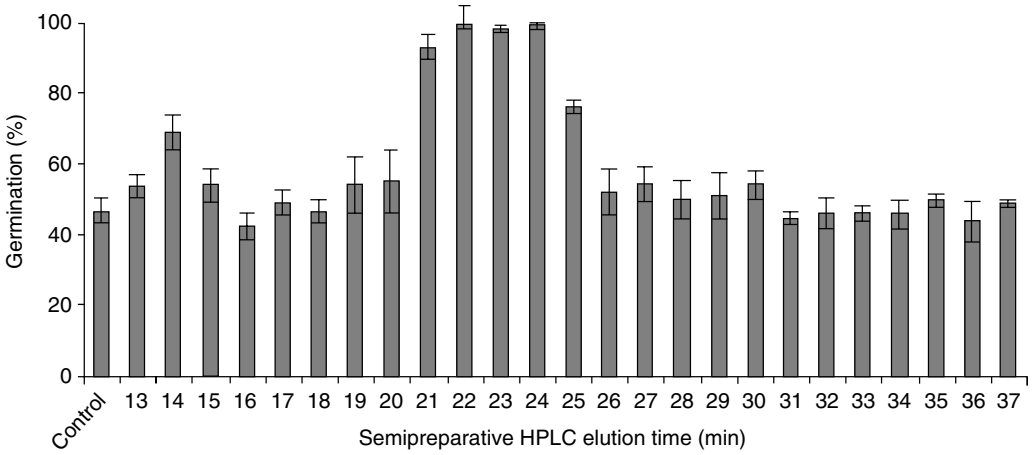


FIGURE 20.10 Germination of *Lactuca sativa* tested with the semipreparative HPLC fractions (Prep13–Prep37) derived from fraction C18-2. Numbers refer to the elution time of each fraction in minutes. Error bars represent SEM. Water served as the control.

Analysis of the active fraction Prep22 by HPLC using an isocratic 25% methanol/water mobile phase showed that the complexity of the mixture had been significantly reduced with approximately 12–15 main compounds now present (Figure 20.11a). Fraction Prep22 was reanalyzed by HPLC using an isocratic mobile phase of 15% acetonitrile/water (Figure 20.11b). Interestingly, the mobile phase of 15% acetonitrile/water altered the elution order of some of the compounds in fraction Prep22 relative to the elution seen with the methanol-based solvent system. In particular, the first main peak of the methanol separation (peak 1, Figure 20.11a), which absorbed strongly at 220 nm, was identified as the last peak in the separation using the acetonitrile solvent system (peak 1, Figure 20.11b). Peak 1 is clearly distinguished from the other two main peaks (peaks 2 and 5) since these last two also absorb strongly at 280 nm, whereas peak 1 does not.

Further HPLC method development led to a gradient elution method that gave sharper peaks and improved resolution. The fraction Prep22 was separated by HPLC (Hypersil) using both methanol (20–30% methanol/water over 30 min) and acetonitrile-based (10%–20% acetonitrile/water over 30 min) methods to further compare the elution pattern. One-minute fractions (1 mL) were collected after the eluent had passed through a photodiode array detector (DAD). The fractions collected were subjected to bioassay evaluation with *L. sativa* after removing the organic solvent with a stream of nitrogen and making up to 10 mL with ultrapure (Millipore) water (i.e., 1/10 dilution). Fractions collected during the semipreparative HPLC separation at points where no compounds were detected served as secondary controls in conjunction with the water control to ensure that any residual solvent had no effect on germination.

The results further indicated the sensitivity of the *L. sativa* seeds to the active compound(s). Fractions collected from the HPLC separation of Prep22, diluted 10-fold, still enhanced germination considerably. Fractions eluting after the most active fractions (15 and 16) for the methanol separation also showed activity, suggesting carryover of the active compound(s). Fractions derived from the acetonitrile separation showed that activity was restricted to fewer fractions, indicating the superior resolving ability of an acetonitrile-based method to separate the mixture.

The photodiode array spectra for both HPLC separations (Figure 20.12) can be directly compared to the germination results obtained with *L. sativa*. At the elution time that shows the highest germination activity, a characteristic UV absorbance maximum at ca. 330 nm (labeled with an "*" in Figure 20.12) was observed with both the methanol- and acetonitrile-based mobile phase systems (Figure 20.12).

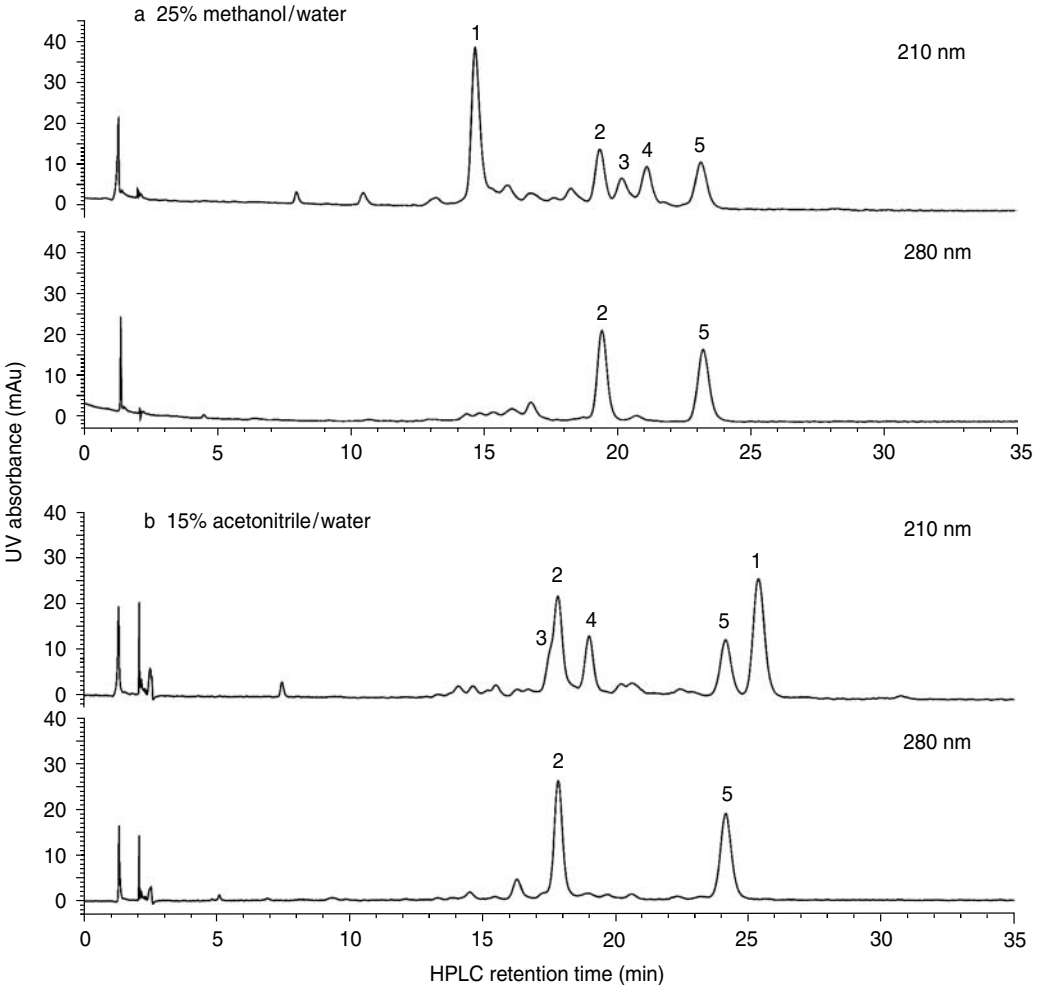


FIGURE 20.11 HPLC separation of fraction Prep22 using (a) methanol-based solvent system and (b) an acetonitrile-based solvent system with UV absorbance recorded at the wavelengths of 210 and 280 nm.

20.3.3 HPLC-MS Analysis

Analysis of the fraction Prep22 by HPLC-DAD-APCI MS using the gradient methanol-based mobile phase showed two quasi-molecular ions $[M + H]^+$ of *m/z* 151 and 137 corresponding to the UV absorbance maximum of 330 nm. HPLC-DAD-MS analysis using the gradient acetonitrile method showed the same two quasi-molecular ions with the UV absorbance maximum of 330 nm.

Of the two HPLC-DAD-MS separations, the acetonitrile solvent system gave better resolution of the active compound, as determined by the bioassay. Therefore, the remaining C18-2 fraction (15 mg) was separated by semipreparative HPLC (Econosil) using an isocratic mobile phase of 15% acetonitrile/water. Fractions were collected at 1-min intervals and subjected to bioassay evaluation, which showed that fractions 2Prep33 to 2Prep37 contained most of the activity. The mass recovered for the active fraction 2Prep35 was approximately 0.2 mg, which was much less than that obtained for the fractions derived from the methanol separation (ca. 1 mg). Analysis of 2Prep35 by HPLC (Hypersil) showed fewer compounds compared to fraction Prep22, which explained the lower yields. Use of the acetonitrile-based method for the semipreparative HPLC separation had significantly reduced the complexity of the fraction, providing the most purified fraction (2Prep35) obtained to date.

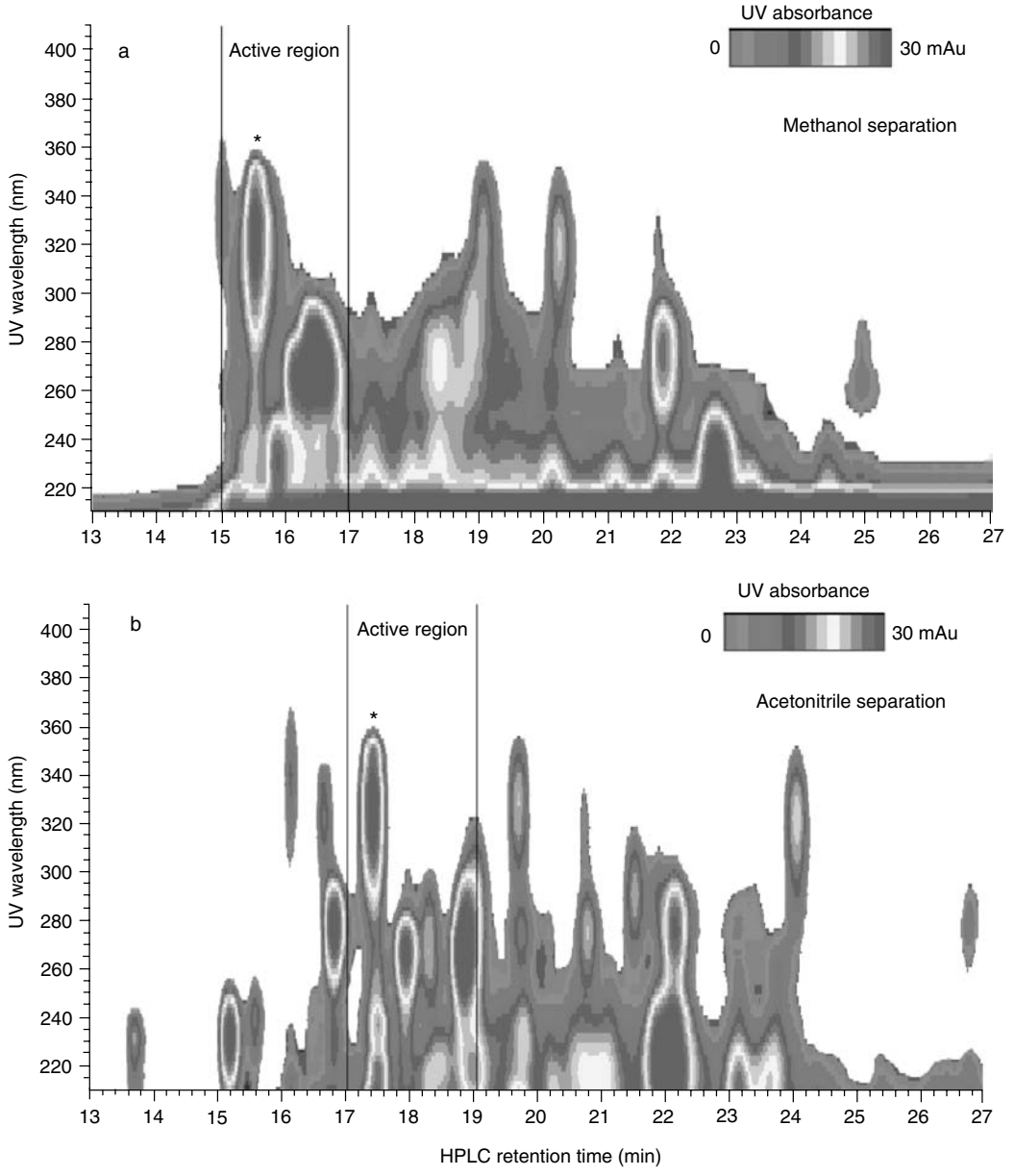


FIGURE 20.12 Photodiode array spectrum of the separation of fraction Prep22 achieved using the method: (a) 20% methanol/water to 30% methanol/water over 30 min and (b) 10% acetonitrile/water to 20% acetonitrile/water over 30 min. *y*-Axis shows the UV wavelengths recorded (210–400 nm) using a photodiode array detector (DAD).

HPLC analysis of fraction 2Prep35 using either methanol- or acetonitrile-based solvent mixtures failed to give adequate separation of the compounds present. Further HPLC method development using fraction 2Prep35 led to another solvent system based on tetrahydrofuran. Using the same C18-RP column (HP Hypersil ODS), an isocratic mobile phase of 5% tetrahydrofuran/water was found to give a reasonable separation of the compounds in contrast to the methanol and acetonitrile solvent systems. Analysis of the fraction 2Prep35 by HPLC-DAD-MS using the tetrahydrofuran solvent system showed the presence of four major peaks (Figure 20.13) with less than 10 compounds

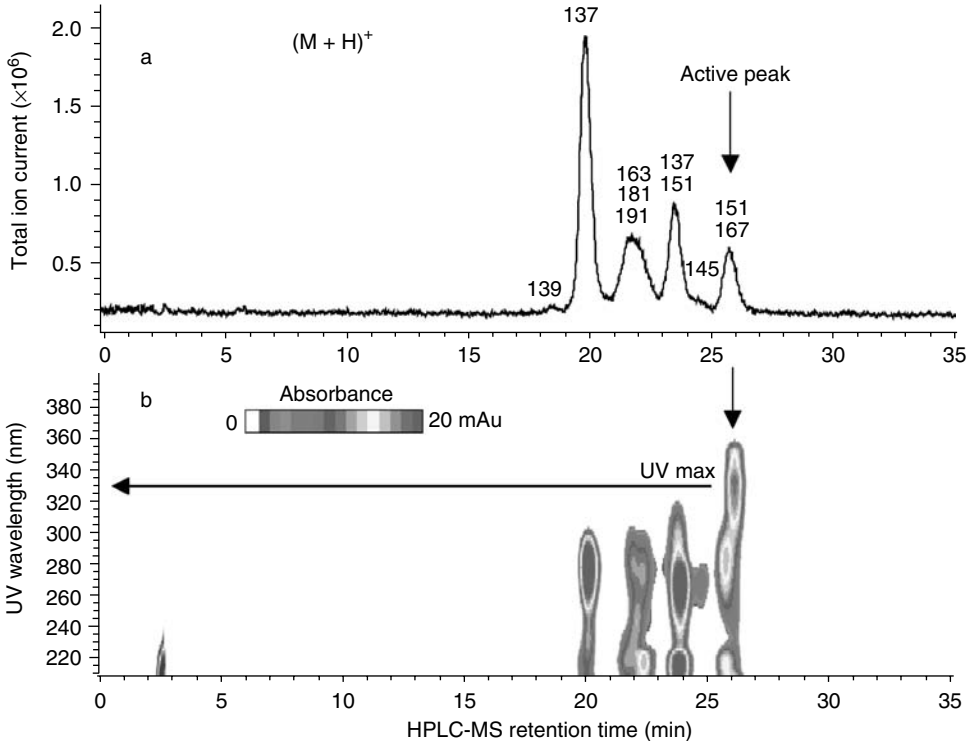


FIGURE 20.13 HPLC-DAD-APCI MS of fraction 2Prep35 separated using a C18-RP column eluted with 5% tetrahydrofuran/water. (a) Total ion chromatogram obtained with an ion trap MS operating in atmospheric pressure chemical ionization (APCI) mode; (b) corresponding photodiode array spectrum of fraction 2Prep35. (Flematti, G.R., Ghisalberti, E.L., Dixon, K.W., and Trengove, R.D., *Plant and Soil*, 263, 1, 2004. With permission.)

overall. The two compounds with the quasi-molecular ions $[M + H]^+$ of *m/z* 151 and 137, which had co-eluted in previous HPLC separations (Figure 20.12), were separated with the tetrahydrofuran mobile phase. Furthermore, the UV absorbance maximum of 330 nm now corresponded to compounds with the quasi-molecular ions of *m/z* 151 and 167. Interestingly, two compounds with an $[M + H]^+$ *m/z* 151 and two others with an *m/z* of 137 were observed (Figure 20.13).

One-minute fractions from the HPLC-DAD-MS separation were collected and tested for activity with *L. sativa*. Thus, a direct comparison of the germination activity of each fraction with the UV absorbance and mass spectra was obtained (Figure 20.13).

The activity was found to correspond to the last peak in the chromatogram (25–27 min, Figure 20.13). This correlated with the UV absorbance of 330 nm and the two quasi-molecular ions $[M + H]^+$ of *m/z* 151 and 167. Hence, the compound with molecular ion *m/z* 151 was confirmed as the active compound and the UV absorbance maximum of 330 nm was established as a characteristic feature of this compound. This represents the first time that a compound in smoke, which is responsible for germination activity, was isolated and some of its characteristic parameters were obtained.

This fraction (2Prep35) was analyzed by GC-MS (HP-5 ms, 30 m × 0.25 mm i.d., 0.25 μm) under electron impact (EI) conditions, and the mass spectra of the compounds were compared unsuccessfully to known compounds available in a mass spectrometric library (NIST 98). The GC-MS retention time for the active compound was determined to be 11.86 min based upon the similarity between the LC-MS and GC-MS data. In the EI mass spectrum of this compound, a strong molecular ion at *m/z* 150 (100%) and a major fragment ion of *m/z* 121 (71%) were observed. By optimizing the ionization voltage (45 eV) and using selective ion monitoring (SIM) mode, a good indication of

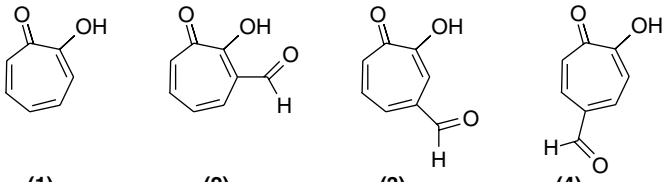
the isotope ratio for the molecular ion was obtained. Thus, the molecular ion cluster included an M + 1 ion at an abundance of 9.19 ± 0.03% and an M + 2 ion of 1.09 ± 0.01%. Because the compound (derived from cellulose) contains only C, H, and O,[29] comparison of this data with standard isotope tables[37] indicated a molecular formula of $C_8H_6O_3$.

20.3.4 Possible Identification: Tropolones

One of the more significant early pieces of evidence for the structure of the active compound was the UV absorbance at ca. 330 nm, indicative of a system with extended conjugation. A class of compounds with a characteristic UV chromophore in this region are the hydroxytropones. In particular, 2-hydroxy tropone (tropolone [**1**]) has a UV absorbance maximum between 300 and 380 nm.[38] The EI mass spectrum of tropolone is also interesting since an initial loss of 28 mass units is observed ($M^+ - CO$).[39] This is similar to that observed for the EI mass spectrum of the active compound (*m/z* 122 [25%]). The fragment ions at 65 and 66 in the tropolone mass spectrum are also present in the EI mass spectrum of the active compound (*m/z* 65 [14%], *m/z* 66 [16%]). In addition, if an aldehyde group is added to the tropolone ring, a molecular formula of $C_8H_6O_3$ as predicted for the active compound is obtained. Generally, aldehydes show strong ions corresponding to the loss of 1 and 29 in their mass spectra. For the active compound, only a low-intensity $[M - 1]^+$ ion (*m/z* 149 [2%]) was observed; however, a strong $[M - 29]^+$ ion (*m/z* 121 [71%]) was present in the EI mass spectrum. Given these points of correspondence, it seemed useful to investigate the formyl-substituted tropolones further.

The active compound had been shown in the early stages of this investigation to be essentially a neutral compound (Figure 20.2). The hydroxytropones, on the other hand, are known to function as both acids and bases, liberating carbon dioxide from sodium hydrogen carbonate and forming stable metal salts.[40] However, the properties of tropolone (**1**) are somewhat different due to the intramolecular bonding of the hydroxyl to the carbonyl. Hence, tropolone (**1**) is more volatile, has a lower melting point, has greater solubility in nonpolar solvents, and is less acidic than the 3- and 4-hydroxytropones.[40]

Since the hydroxy and the carbonyl groups of tropolone (**1**) exist as a tautomeric system and undergo rapid exchange, there are only three possible isomers of formyl tropolone available (**2**–**4**). Of these, 3-formyl tropolone (**2**) was the most interesting because the molecule has a unique hydrogen bonding arrangement that could be a significant feature of the active compound. Although 3-formyl tropolone (**2**) was known, its spectroscopic parameters such as UV absorbance and mass spectra had not been reported. Therefore, a method for preparing **2** was investigated. Thus, both 3-formyl tropolone (**2**) and 5-formyl tropolone (**4**) were synthesized from tropolone,[41–44] whereas 4-formyl tropolone (**3**) was commercially available.



(1) (2) (3) (4)

Initial comparison by GC-MS indicated that the compounds were similar to the active compound (Figure 20.14). In particular, and encouragingly, the EI mass spectrum of the active compound (R_t 11.85 min) was very similar to that of 4-formyl tropolone (**3**) (R_t 11.86 min). However, the excitement was short-lived, since none of the three regioisomers showed any activity in the *L. sativa* bioassay. Further to this, varying the GC operating conditions and spiking of the active fraction with 4-formyl tropolone clearly established that they were different. Retention times from HPLC and their UV spectrometric parameters were also different.

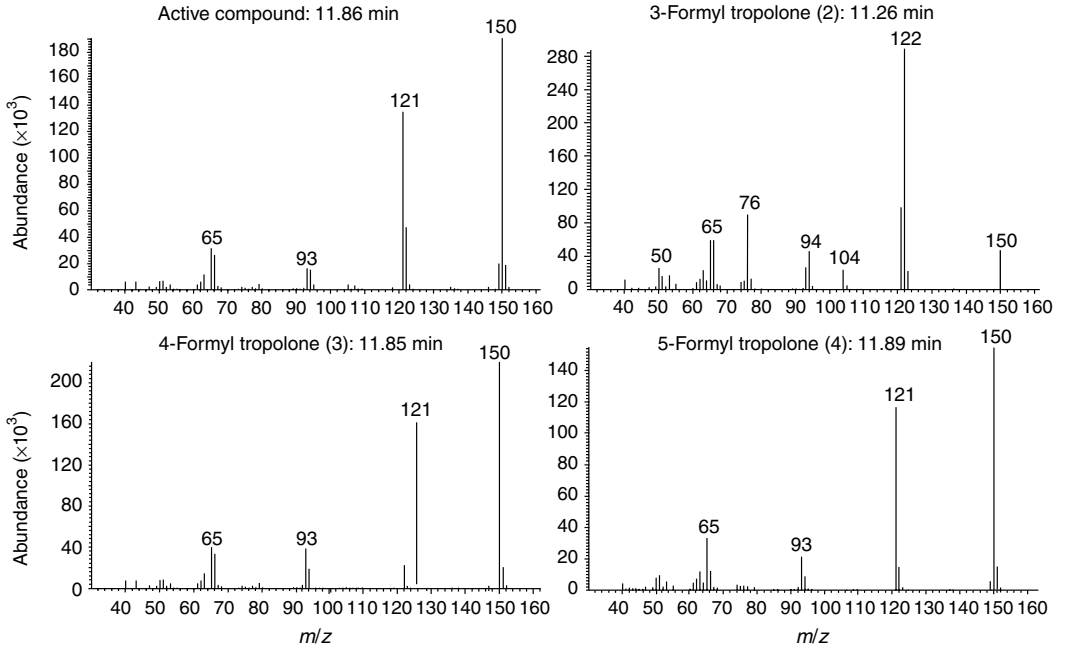


FIGURE 20.14 Comparison of retention times and mass spectra of the active compound and the three formyl tropolones by GC-MS.

20.3.5 Restart: Increase Initial Concentrations

It was evident that larger amounts of the active component were necessary to unambiguously identify the compound. Therefore, methods to increase the initial concentration of the active compound and improve the efficiency of the separation process were further investigated. When using the CS apparatus (Figure 20.1), it was noted that the combustion chamber developed a thick black tar residue on the inside of the drum. Since extracts of charred wood have previously been reported to stimulate germination similar to smoke extracts,[45] a small portion of the tar was extracted with water and tested for activity. The results showed that the tar extract had considerable activity and higher concentrations of the active compound than found in the "condensate trap".

At this stage, the initial separation methods developed (Figure 20.15) were successful at locating the active compound within a fraction (2Prep35) containing approximately ten compounds (Figure 20.13). Modification of the separation strategies on the basis of the experience accumulated could now be implemented to assist the isolation of the active compound. The first chromatography step using alumina was replaced by C18-RP silica, a support that had been more effective at reducing the complexity of the smoke extracts. Using an acetonitrile-based mobile phase, the drum neutral fraction was separated into six fractions. As in previous C18 separations, the second fraction eluted contained the majority of germination activity. This fraction (CSD C18-2) was separated by semipreparative HPLC (Econosil) using a mobile phase of 20% acetonitrile (instead of 15% acetonitrile), which gave shorter retention times and better resolution of the active compound. Although the yield of each active fraction (3Prep18–3Prep22) had increased (ca. 2 mg), the different separation process gave more complex fractions. Importantly though, the use of two RP separation methods had significantly reduced the complexity of the drum neutral extract and had provided enough material for further separation (Figure 20.15).

A number of different HPLC stationary phases were investigated using different mobile phases to find an efficient method for isolating the active compound from the 3Prep fractions. The column phases tested included an amino propyl column (both RP and normal phase mode), a phenyl

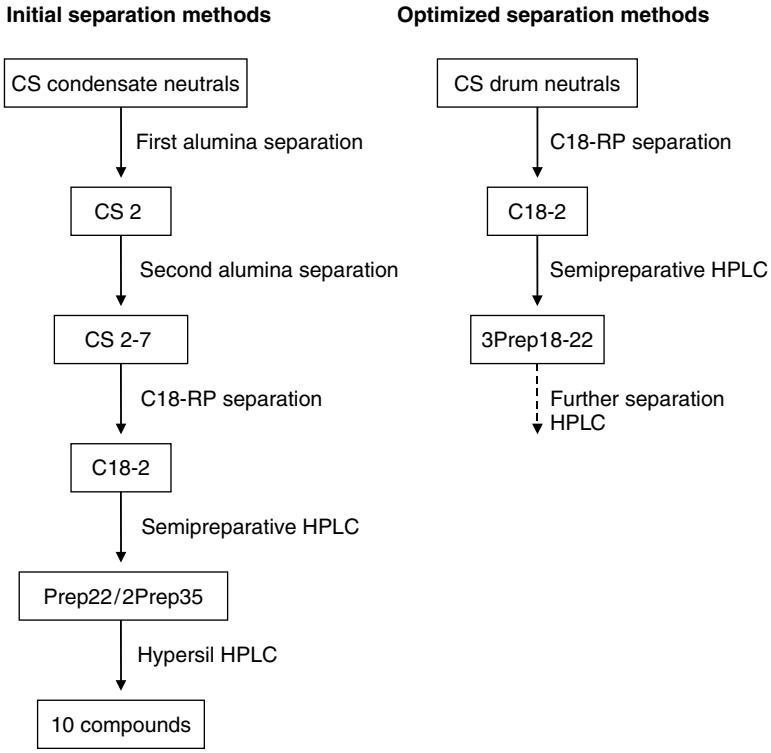


FIGURE 20.15 Flow diagrams illustrating the chromatography steps used in the initial separation attempt at isolating the active compound and the optimized separation methods used to generate more material for further separation.

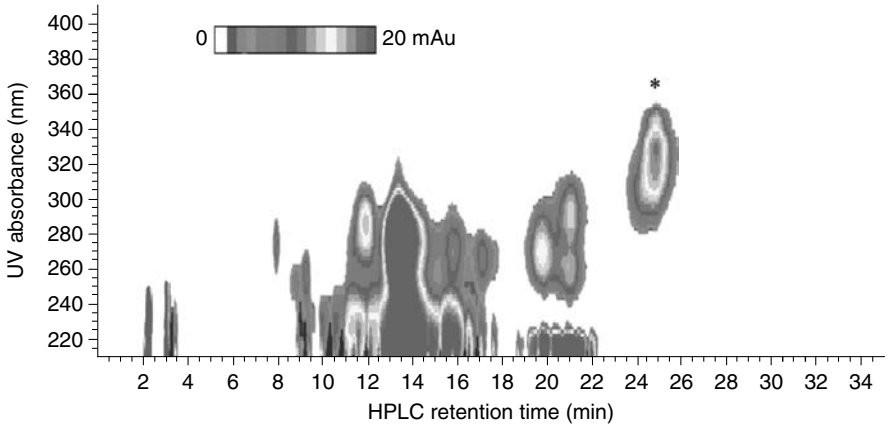


FIGURE 20.16 HPLC separation and photodiode array spectrum of fraction 3Prep20. The fraction was separated using an Asahipak ODP-50 HPLC column eluted with a mobile phase of 15% tetrahydrofuran/water. The active compound is indicated by (*).

column, a cyanopropyl column, and a C8 silica column. Unfortunately, none of these phases gave better separation than that achieved with the conventional C18 silica (Hypersil) column. However, use of an Asahipak ODP-50 column (250 mm × 4 mm i.d., 5 μm) gave increased retention of the active compound (labeled with an "*" in Figure 20.16). The Asahipak column differs from other stationary phases tested in being polyvinyl alcohol-based instead of silica-based.

To verify the retention time of the active compound, 1-min fractions were collected and tested for germination activity. The activity was confirmed for the peak eluting at 24–26 min and corresponding with the UV absorbance of ca. 330 nm (Figure 20.16). Significantly, when the active fraction was analyzed by HPLC-MS using the Asahipak column, only one ion (*m/z* 151) was observed for the active peak ("*" in Figure 20.16). The Asahipak column provided a method that cleanly separated the active compound from the other compounds present in the mixture. The semipreparative HPLC fractions containing the active compound (3Prep18–3Prep22) were separated using this method and the peak eluting at 24–26 min was collected.

The active fraction from the Asahipak column fraction was extracted with CH_2Cl_2. Evaporation of the solvent yielded approximately 0.3 mg of a light yellow residue (A-act). Analysis of A-act by HPLC (Hypersil) showed that more than one compound was present. Using 25% methanol/water mixture as the mobile phase resulted in good separation and revealed three main compounds (**5**, **6**, and **7**) absorbing at 325 nm (Figure 20.17). HPLC-DAD-MS analysis using the same separation conditions showed that the three compounds all had a molecular mass of 150 and a peak UV absorbance between 300 and 330 nm (Figure 20.18). In addition, the EI mass spectra obtained by GC-MS analysis showed that each compound had a strong molecular ion of *m/z* 150 and a major fragment ion of *m/z* 121 (Figure 20.18).

To determine which of the compounds (**5**, **6**, or **7**) was active, the three peaks were collected from the HPLC separation and tested for activity with *L. sativa*, *C. aculeata*, and *S. affine*. The results (Figure 20.19) showed that **6** was active with each of the bioassay species.

Thus, although the analytical data indicated that the three compounds were very similar, the bioassay showed that only one (compound **6**) was active.

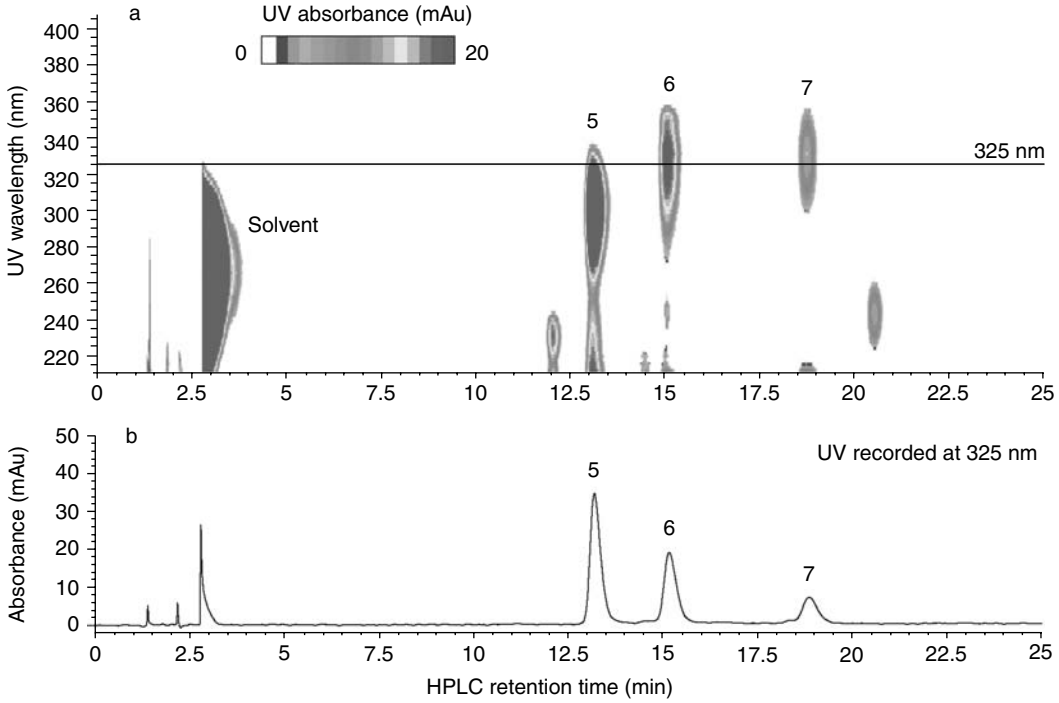


FIGURE 20.17 HPLC separation of A-act using a mobile phase of 25% methanol/water. The top spectrum (a) shows the photodiode array spectrum, and the bottom spectrum (b) shows the chromatogram recorded at a UV absorbance of 325 nm.

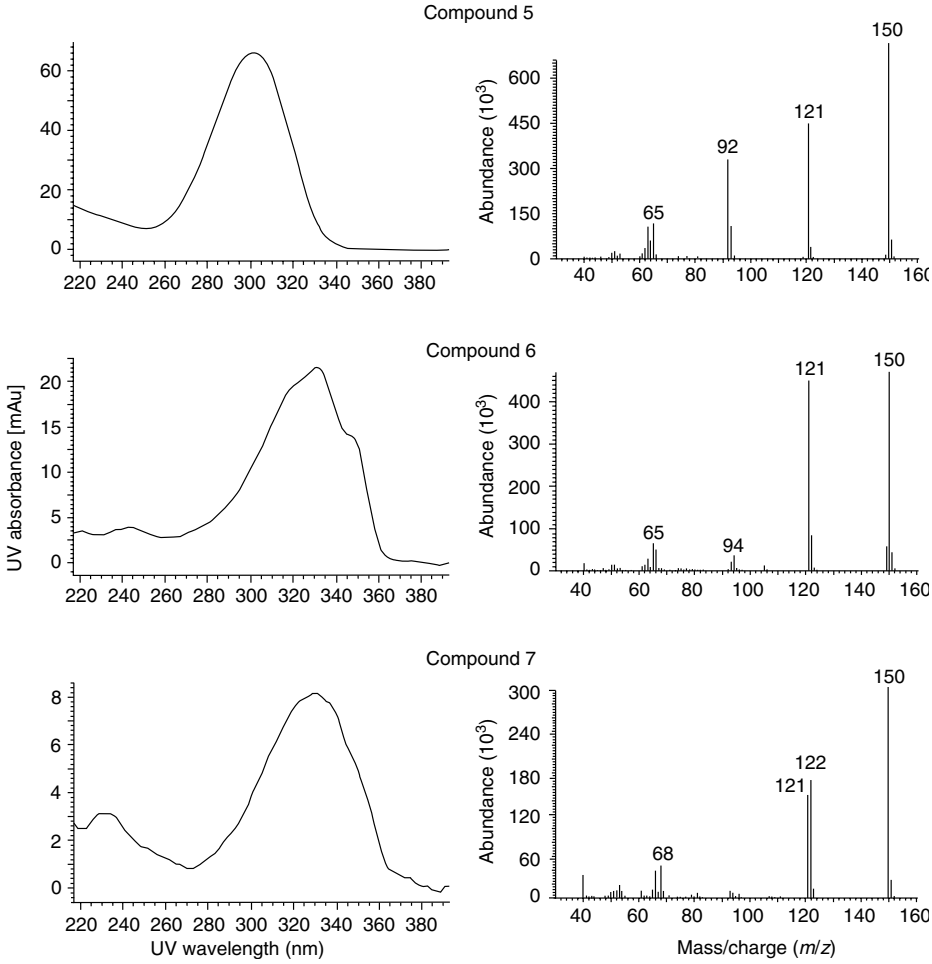


FIGURE 20.18 Comparison of the UV absorbance (from HPLC-DAD) and mass spectra (GC-MS) of the three main compounds present in the Asahipak fraction (A-act).

20.4 IDENTIFICATION

20.4.1 NMR Spectroscopy

The ^{1}H NMR spectrum of A-act (Figure 20.20) was considerably simpler than any obtained for different fractions so far. Careful consideration of integration values for the various signals and comparison of coupling constants to identify mutually coupled protons, as well as 2D NMR measurements (^{1}H–^{1}H COSY), allowed grouping of signals corresponding to each compound (Figure 20.20). Significantly, six protons were found for each compound, indicating that each had a molecular formula of $C_8H_6O_3$.

The small amount of material obtained in sample A-act (0.3 mg) prevented direct ^{13}C NMR measurement. Use of inverse 2D NMR methods (HSQC and HMBC) indirectly revealed the approximate chemical shifts of the carbon atoms for the major compound **5**. The presence of a 2-substituted furan was evident from the chemical shift and coupling constants (J) associated with the contiguous protons at C3 (δ 7.09, d, J = 3.6 Hz), C4 (δ 6.68, dd, J = 3.6, 1.8 Hz), and C5 (δ 7.85, d, J = 1.8 Hz). A two-proton signal at δ 5.22 included a long-range coupling (J = 1.8 Hz) from a deshielded proton at δ 6.22, best assigned to the α-proton of an α,β-unsaturated carbonyl system.

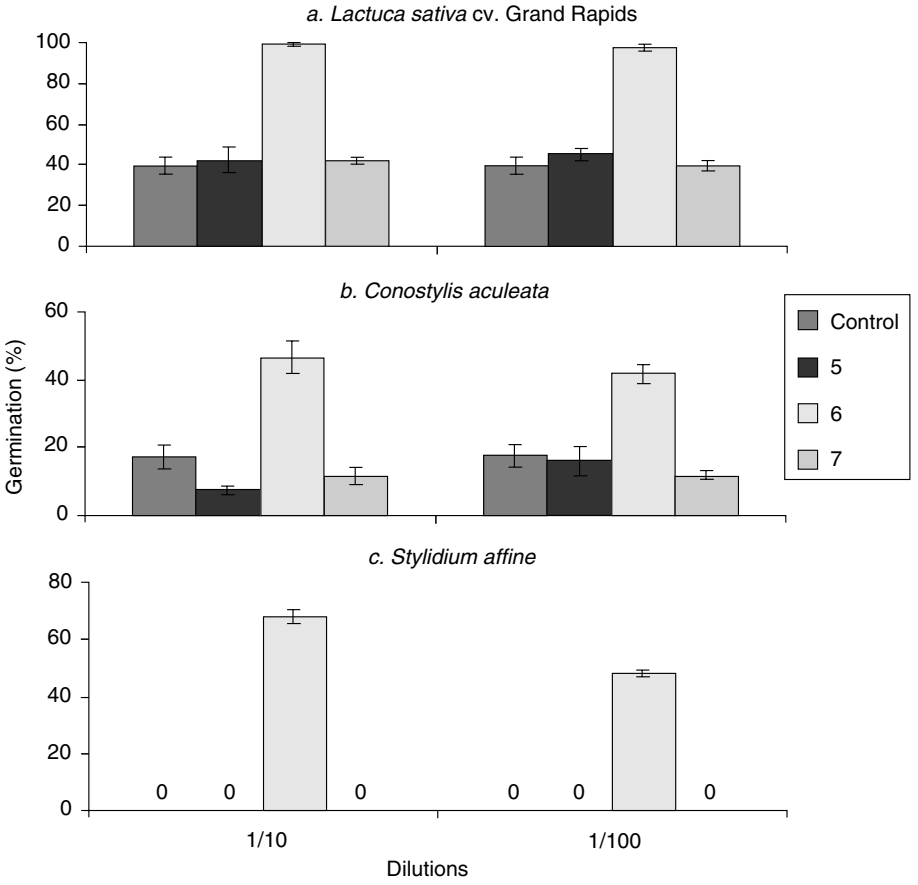
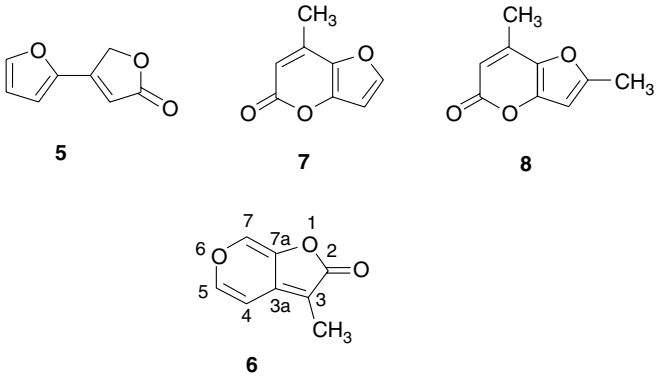


FIGURE 20.19 Germination of *Lactuca sativa, C. aculeata*, and *Stylidium affine* in response to compounds **5**, **6**, and **7**. Error bars represent SEM, and water served as the control.

From NMR measurement, eight carbons were detected. Given a molecular ion at *m/z* 150 in the MS of **5**, a formula of $C_8H_6O_3$ was inferred, requiring the presence of a C4-linked butenolide. A search of the literature revealed that this compound had been described previously.[46] Comparison of NMR (^{1}H and ^{13}C) parameters of the two samples confirmed its identity as 2,3′-bifuran-5′(2′*H*)-one (**5**).

The structural determination of compounds **6** and **7** required more material and further separation. Following the same separation procedure, fraction 2A-act was obtained containing the same three compounds. Repeated semipreparative HPLC (Hypersil) led to the isolation of compounds **5** (0.5 mg), **6** (1.3 mg), and **7** (1.6 mg).

The identity of compound **5** was confirmed as a known compound.[46] For compound **7** (MW 150), the chemical shift of each carbon atom and associated proton correlations were determined by ^{1}H NMR, ^{13}C NMR, and 2D NMR (HSQC and HMBC) techniques. Inspection of the NMR data showed that a 2,3-di-substituted furan system was present, indicated by the chemical shifts of C2 at 147.6 ppm and C3 at 109 ppm and a spin-coupling constant (*J*) of 2.1 Hz for H2 (δ 7.90) and H3 (δ 6.73). A lactone or ester functionality was also indicated by the carbonyl chemical shift at 162.0 ppm. IR absorption at 1724 cm^{-1} strongly suggested that an α,β-unsaturated δ-lactone[47] was present. On the basis of this information, **7** was assigned the structure of 7-methyl-5*H*-furo[3,2-*b*]pyran-5-one. The C2 homolog, 2,7-dimethyl-5*H*-furo[3,2-*b*]pyran-5-one (**8**), had previously been described.[48] The NMR spectra of **8** were in good agreement with those of **7** after allowance was made for the extra methyl group at C2 in **8**. The mass spectrum (M-28 and M-29 ions) and UV absorbance (λ_{max} 340 nm) of **8** lent further support to the similarity between **7** and **8**.



The small amount of material isolated made it difficult to identify compound **6**. The ^{1}H NMR, ^{13}C NMR, and 2D NMR (HSQC, HMBC, NOESY) measurements obtained required long acquisition times (60 h for ^{13}C and HMBC), and many of the correlations observed for the HMBC experiment (coupling measured at both 6 and 10 Hz) could only be tentatively assigned since they had low intensity and were difficult to distinguish from background noise. To overcome this, a 1D equivalent of the HMBC experiment known as the selective inverse multiple bond analysis (SIMBA)[49] experiment was conducted. In the SIMBA experiment, each ^{13}C signal was pulsed individually and the proton spectrum measured (Figure 20.21). Any proton signal with a two- or three-bond coupling to the carbon atom being pulsed was detected (similar to HMBC experiment). The SIMBA experiment is more sensitive than the HMBC experiment and enabled the tentative correlations to be confirmed.

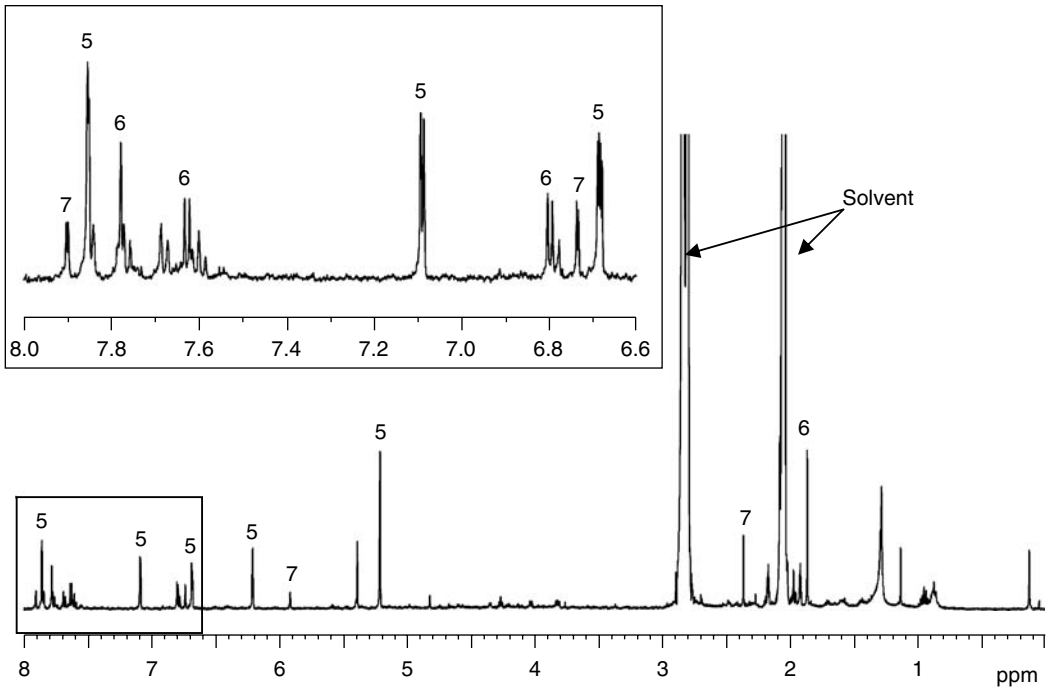


FIGURE 20.20 ^{1}H NMR (d_6-acetone) spectrum of A-act. Numbers indicate proton signals belonging to each compound **5**, **6**, and **7** as observed in Figure 20.19.

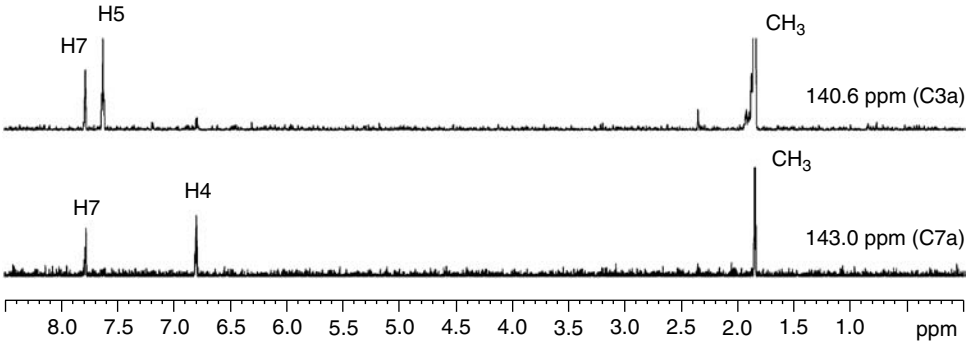


FIGURE 20.21 Example of the SIMBA experiment for compound **6**. The top spectrum shows the protons with two- or three-bond coupling to C3a (140.6 ppm), and the bottom spectrum shows protons with similar coupling to C7a (143.0 ppm). The spectrum is displayed in power spectrum mode as per convention.[49]

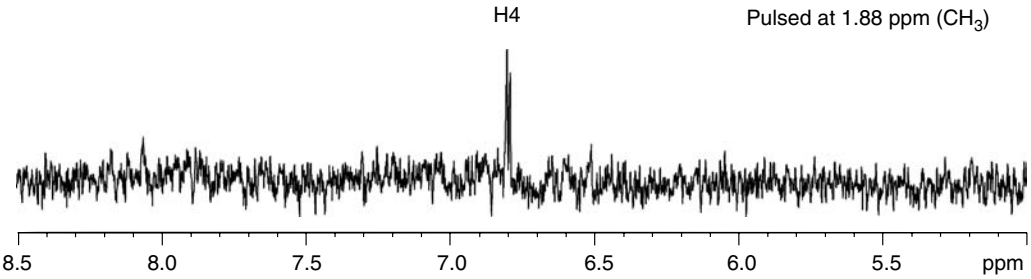


FIGURE 20.22 NOE difference experiment of compound **6** showing a through-space correlation of the methyl substituent to the proton at δ 6.8 ppm.

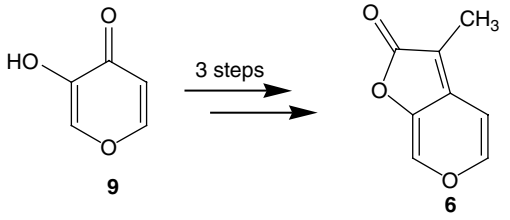
TABLE 20.1
^{1}H and ^{13}C NMR Assignments for Compound 6 with Associated HSQC and HMBC Correlations

No.	δ_C	δ_H (mult, *J*)	HSQC (H ⇒ C)	HMBC[a] (H ⇒ C)
2	171.1	—	—	—
3	100.0	—	—	—
CH_3	7.5	1.88 (br s)	CH_3	C3, C3a, C2
3a	140.6	—	—	—
4	104.1	6.80 (d, 5.5)	C4	C7a, C5
5	149.8	7.63 (d, 5.5)	C5	C4, C3a
7	128.0	7.78 (br s)	C7	C3a, C7a, C5
7a	143.0	—	—	—

[a] HMBC assignments were aided with the SIMBA experiment.[49]

Similarly, the 1D NOE difference experiment was superior to the 2D-NOESY and showed an NOE correlation of the methyl signal (δ 1.88 ppm) with the vinylic proton at δ 6.8 ppm (Figure 20.22).

As found for **5** and **7**, compound **6** also had a molecular formula of $C_8H_6O_3$. However, the furan moiety present in **5** and **7** was replaced by a pyran ring in **6** as indicated by characteristic proton chemical shifts (δ_H 6.80, 7.63) and coupling constant (J = 5.5 Hz) for the vicinal enol ether protons (Table 20.1). The chemical shifts of corresponding carbons (δ_C 149.8, 104.1) also supported this



SCHEME 20.1 Synthesis of 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (**6**) from pyromeconic acid (**9**).

assignment. A tetra-substituted double bond extended the conjugation (δ_C 140.6, 100.0), the distal carbon (C3) being substituted by a methyl group (δ_C 7.5, δ_H 1.88), which showed an NOE correlation with the β-enol ether proton (H4). The shielded nature of the methyl group carbon is consistent with it being part of an α,β-butenolide, the presence of which accounts for the UV absorption maximum λ_{max} 332 nm. With this data in hand, the structure 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (**6**) was proposed.

At this point, the only piece of evidence that was inconsistent with the proposed structure was the ^{13}C chemical shift for C7 at 128 ppm. Generally, vinylic carbons alpha to an oxygen atom have a chemical shift in the range 140–150 ppm (as observed for C5). However, it was noted that vinylic carbons beta to an oxygen atom are shielded approximately 30 ppm.[47] The C7 carbon of **6** has an oxygen atom at both the alpha (pyran oxygen) and beta (lactone oxygen) positions, for which an estimated chemical shift of 125.4 ppm can be calculated ($123.3_{vinyl} + 28.8_{alpha\ (ether)} - 26.7_{beta\ (ester)}$),[47] which is in reasonable agreement with the observed value of 128 ppm.

20.4.2 Synthesis

Since **6** was a new compound, confirmation of its structure was sought by synthesis. This was achieved by synthesis from pyromeconic acid[50] (**9**, Scheme 20.1), which gave a sample indistinguishable from **6** by spectroscopic methods (^1H NMR, ^{13}C NMR).

20.4.3 Confirmation of Activity

With the synthetic form of the butenolide (**6**) in hand, its activity was tested at a range of concentrations with the three bioassay species, *Lactuca sativa*, *Stylidium affine*, and *Conostylis aculeata* (Figure 20.23). Plant-derived smoke water (as opposed to cellulose-derived) was also tested at a number of dilutions to compare the efficacy of the synthetic compound to the response of these species with the crude smoke extract.

The results (Figure 20.23) confirm the germination-promoting activity of **6** for each of the bioassay species. Testing of a broader range of smoke-responsive species has further confirmed the activity of **6**.[51] In particular, the germination of the South African species *Syncarpha vestita* (Asteraceae) and the two North American species *Emmenathe penduliflora* (Hydrophyllaceae) and *Nicotiana attenuata* (Solanaceae), which have previously been used to test the activity of smoke extracts,[26,29,32] was also promoted by **6**. These results suggest that **6** is likely to be the main bioactive compound in smoke responsible for promoting seed germination.

20.5 CONCLUDING REMARKS

The discovery of the bioactive compound **6** is a useful example of a multidisciplinary approach toward identifying an unknown biologically active agent. The use of a rapid and reliable bioassay, *L. sativa* cv. Grand Rapids lettuce seed, which enabled hundreds of separations to be performed with a rapid turnaround in results (<2 days), was a key component of its isolation. The bioassay coupled with advanced analytical techniques (GC-MS and HPLC-DAD-MS) guided numerous separation attempts both in normal and in reversed phase, at low and high pressure, and with a variety of

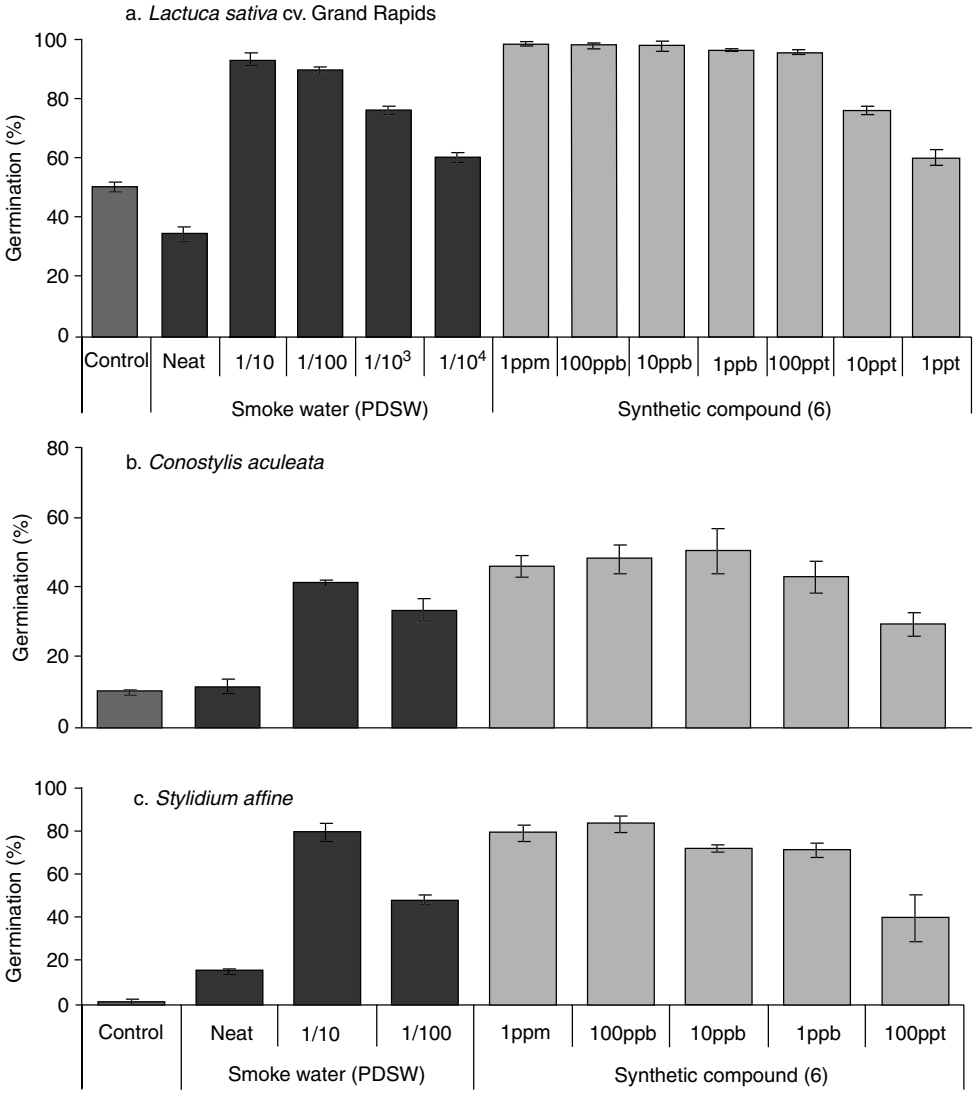


FIGURE 20.23 Germination of (a) *Lactuca sativa* cv. "Grand Rapids" lettuce seed, (b) *Conostylis aculeata*, and (c) *Stylidium affine* tested with plant-derived smoke water (PDSW) and the synthetic butenolide (**6**). Error bars represent SEM, and water served as the control. Flematti, G.R., Ghisalberti, E.L., Dixon, K.W., and Trengove, R.D., *Science*, 305, 977, 2004. With permission.

mobile phases. At times the active compound seemed impossible to isolate in pure form, but through persistence and perseverance the isolation of the "needle from the haystack" was achieved. Finally, extensive spectroscopic analysis (UV, IR, MS, ^{1}H NMR, ^{13}C NMR, and 2D NMR techniques) and synthesis of the proposed compound provided a sample whose spectroscopic parameters and bioactivity were identical to those obtained for the active agent in smoke.

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21 Plant-Associated Toxins: Bioactivity-Guided Isolation, ELISA, and LC-MS Detection

Steven M. Colegate

CONTENTS

21.1 INTRODUCTION

Plant-associated toxins are those bioactive metabolites that are derived from plants *per se*, or from microorganisms associated with the plants, for example, fungi (endophytes or saprophytes) and bacteria. Presumably evolved to deter herbivory or reduce competition for limited nutritional resources, the adverse effects of these bioactive natural products are varied, resulting in acute or chronic impacts on different tissues, organs, and biological systems.[1] The toxic metabolites can also enter the human food supply resulting in "primary" or "secondary" poisoning, depending on whether the plant itself or food derived from an animal exposed to the toxins, respectively, is ingested.[2]

As described in the first edition of this book, where the primary focus of plant-associated toxins is the effect on grazing livestock, there are some clearly defined, enabling requirements for the successful isolation of the toxic metabolites that actually cause the intoxication disease observed in the field.[3] These include a complete clinical and pathological description of the resultant intoxication such that any potential toxins, especially those isolated using a model bioassay rather than the disease development itself, can be verified in the target species. To develop this theme further, this chapter will present four case studies that collectively describe various approaches to the detection of some bioactive natural products that are toxic to humans and livestock, and, in some cases, their isolation and structural identification.

21.2 THE *IN VIVO* BIOACTIVITY-GUIDED ISOLATION OF TOXIC CUCURBITACIN STEROIDAL GLUCOSIDES FROM *Stemodia kingii*

This section describes the investigation of a disease in sheep that was associated with ingestion of *S. kingii* F. Muell. (Scrophulariaceae). This dicotyledonous angiosperm is unique to the Pilbara region of northwest Australia and has been tentatively associated with unknown numbers of sheep deaths since the mid-1990s. It is a short-lived (2–5 years) shrub (up to ca. 0.25 m) that can become dominant in heavily grazed areas.

Previous phytochemical investigations of *Stemodia* spp. have yielded terpenoid compounds, such as the diterpene stemodanes,[4] not associated with any related toxicity. Other *Stemodia* spp. have been used as medicinal plants, such as *S. durantifolia* as an antifertility treatment,[5] but there has been no literature mention of *S. kingii* prior to those associated with this intoxication.[6–8]

21.2.1 Clinical and Pathological Descriptions of the Intoxication of Sheep

The toxicity of the plant was confirmed via intraruminal administration of an aqueous slurry of the powered, dried aerial parts of the plant to penned sheep.[6] At a dose rate of 12 g milled plant/kg bodyweight (BW), death occurred within 24 h. At lower dose rates (down to 2 g/kg BW), animals ceased eating, developed a ruminal acidosis, and were in a depressed state when euthanized up to 4 days postdosing.

A severe watery, and sometimes mucoid, diarrhea was the principal clinical sign that developed within 24 h at all dose levels. In the terminal stages, the sheep became recumbent but continued to pass watery diarrhea accompanied by frequent urination. The consequent dehydration and low blood pressure made blood collection for clinical chemistry analysis difficult. Gross pathological and microscopic histological observations were recorded. Thus, pathologically, the intoxication was characterized by

inflammation of the entire gastrointestinal tract, and cardiomyopathy. Although the clinical signs of intoxication, associated clinical chemistry, and some of the pathological changes observed were consistent with the effects of cardiac bufadienolide or cardenolide steroidal glycosides,[9] some pathological changes observed had not previously been associated with cardiac glycoside intoxication.

21.2.2 Development and Validation of a Mouse Bioassay

Initial attempts to develop a toxicity bioassay using brine shrimp (*Artemia salina*)[10] were unsuccessful and so toxicity in mice was assessed. Aqueous slurries of the powdered plant were given to mice via an intragastric gavage needle to ensure an accurate estimate of the amount of plant administered. Clinical signs observed included lethargy, piloerection, a hunched posture, poor capillary refill, and cold extremities. Significantly, a watery diarrhea was also observed.[7]

At postmortem examination, there was significant evidence of antemortem diarrhea along with signs of other adverse effects on the lungs, liver, and heart. Microscopically, there were extensive changes in the jejunum and colon and some mice exhibited changes in the liver. Myocardial necrosis and vacuolation, similar but not as extensive as observed in the sheep, were also noted in *Stemodia*-treated mice.[7]

21.2.3 Isolation of the Toxins

Once the toxicity of the whole plant was confirmed in mice, and shown to be consistent with the clinical and pathological effects in sheep, various extracts of the plant could be assessed for toxicity using this bioassay. To facilitate and expedite the eventual discarding of the *in vivo* bioassay, every extract and resultant partition or fraction was examined, and compared, using thin layer chromatography (TLC) on normal phase silica gel plates. Extract component spots were visualized by spraying the developed plates with a solution of concentrated sulfuric acid in ethanol, and subsequent heating of the plate.[8]

21.2.3.1 Extraction

The milled, dried plant (aerial parts) was repeatedly extracted by shaking with methanol at room temperature until the methanol extract was colorless. Evaporation of the solvent under reduced pressure yielded a residue that caused clinical toxicity in the mouse bioassay. Subsequently, the dried methanolic extract was partitioned between petroleum ether and 10% water in methanol. The toxicity partitioned into the aqueous methanol fraction that was evaporated to dryness and reextracted with ethyl acetate. The TLC of the toxic ethyl acetate solubles revealed clusters of spots that turned pink/purple to purple in color when the acid-sprayed plate was warmed gently.

21.2.3.2 Chromatography

21.2.3.2.1 Column Chromatography

The crude ethyl acetate extract (an orange/yellow glass) was preadsorbed onto silica gel that was then added to the top of a larger column of silica gel prepared in chloroform. To elute the extract components, the column was washed with chloroform containing from 10% to 40% methanol. Each fraction was tested for toxicity in the mouse bioassay and examined using TLC. In this way, three groups of toxins were separated from the bulk of the extract. One group of toxins (the A group; pink/purple, R_f 0.7–0.8) was eluted within the first 60 mL of 20% methanol in chloroform while a second group (B group; purple, R_f 0.4–0.5) was eluted after about the first 100 mL of 20% methanol in chloroform. The third, most polar group of toxins (C group; purple, R_f 0.1–0.2) was eluted with 40% methanol in chloroform (Figure 21.1).

21.2.3.2.2 Radial Chromatography

The B group of toxins, containing five or more closely eluting spots, was selected for further purification using radial chromatography. Thus, TLC-selected fractions from the silica gel column chromatography were loaded onto a 2–4 mm thick, normal phase silica gel radial chromatography

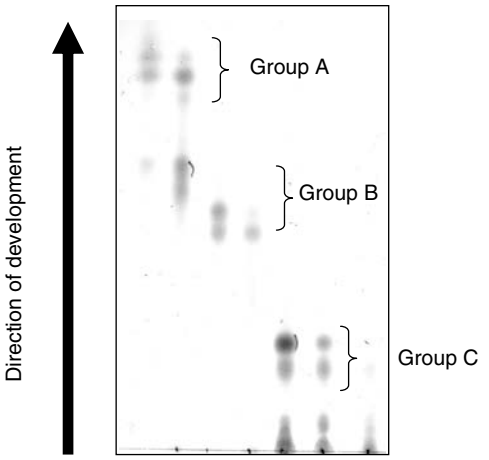


FIGURE 21.1 Thin layer chromatographic separation of the "A", "B", and "C" groups of toxins isolated from *Stemodia kingii*. The plate was developed in chloroform:methanol:0.88 ammonium hydroxide (68:30:2) and visualized by spraying with 2% sulfuric acid in ethanol and gentle warming (40–60°C).

plate that had been equilibrated with, and was eluted with, chloroform:methanol:0.88 ammonium hydroxide (79:20:1).

After each radial chromatographic run, TLC-selected fractions were pooled and rechromatographed. In this way, with repeated radial chromatography (up to three or four times), increasingly pure samples of the B3 and B4 toxins were obtained as amorphous, slightly hygroscopic, white/off-white solids.

21.2.4 Structural Elucidation

The structures of toxins B3 and B4 were elucidated using a combination of physicochemical methods, mass spectrometry, and NMR spectroscopy.[8] Only the B4 toxin was purified to the extent that allowed an accurate assessment of the optical rotation and the unusual melting point behavior. Fluffy white needles of toxin B4 were obtained by fractional recrystallization from warmed, B4-rich acetone solutions. The crystalline B4 ($[\alpha]_D = +86.5$, *c* 2.15 CH_3OH) apparently went through several degradation stages when heated to melting.[8]

21.2.4.1 Physicochemical Analysis

The lack of any changes in retention time for B3 and B4 on TLC analysis using developing solutions of differing pH suggested the absence of an alkaloidal character. This was confirmed by a combustion elemental analysis of the more pure B4 that indicated the presence of carbon, hydrogen, and oxygen only. Treatment of B3 or B4 with sulfuric acid in methanol resulted in the production of methyl glucoside (indicated by TLC and confirmed using NMR spectroscopy), thereby indicating a glucosidic component of the structures and giving rise to their trivial names, stemodioside B3 and stemodioside B4.

21.2.4.2 High Pressure Liquid Chromatography–Mass Spectrometry

Samples of B3 and B4 were analyzed using reverse-phase HPLC ion-trap MS in the electrospray ionization (ESI) mode. The ESI mass spectra of stemodiosides B3 and B4 (Figure 21.2) appeared to be characteristic of the B group of toxins since similar mass spectra were recorded for minor peaks observed during their purification. Ion clusters were observed that could be associated with $[M^+$–glucose] (ions labeled "a" in Figure 21.2), $[M^+]$ (ions labeled "b" in Figure 21.2), and $[2\,M^+]$ (ions labeled "c" in Figure 21.2). The ions within the clusters ("a", "b", and "c") were related by multiple losses of water (18 mass units).

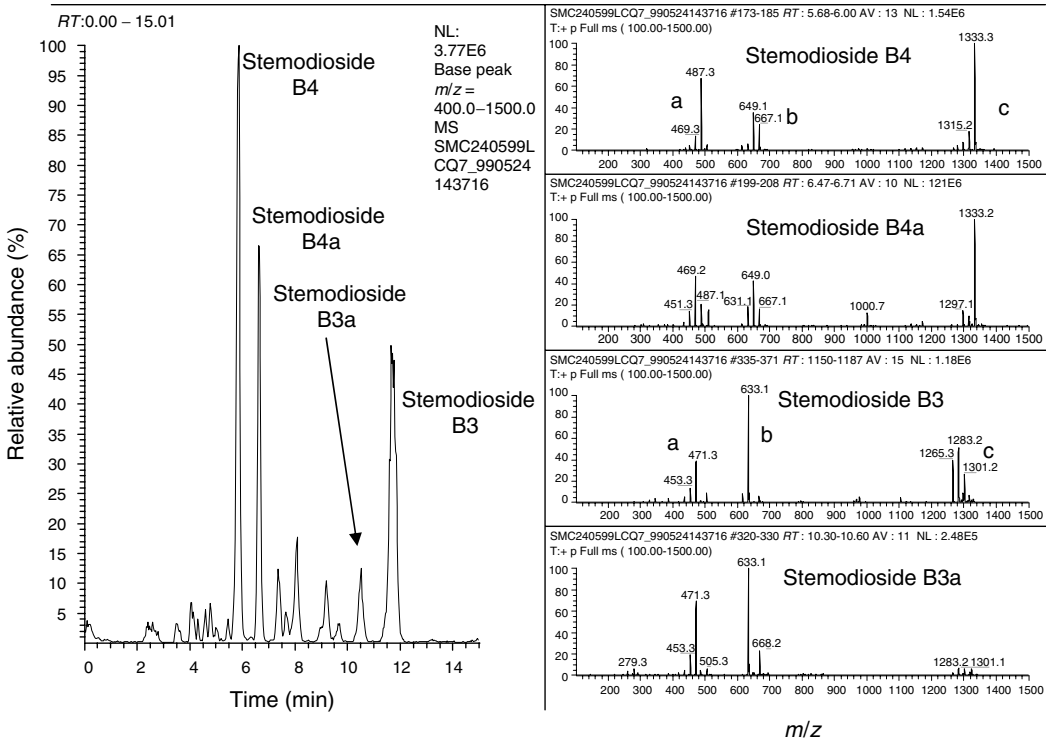


FIGURE 21.2 The HPLC-ESI ion-trap MS base ion chromatogram for a crude mixture of *Stemodia kingii* "B" group toxins. Mass spectra are shown for stemodioside B3, stemodioside B3a, stemodioside B4, and stemodioside B4a. The ion clusters labeled with "a" are associated with the [M^+ – glucose] fragments, "b" with the molecular ion, while "c" are associated with the molecular ion dimer. (Reproduced from Allen, J.G., Colegate, S.M., Mitchell, A.A., Mulder, R., and Raisbeck, M.F., *Phytochem. Anal.*, 17, 226, 2006. Copyright Wiley. With permission.)

The ESI mass spectra offered tantalizing insights into the relative structures of the B group components. For example, the ESI mass spectra for the peaks labeled stemodioside B4a and stemodioside B3a in Figure 21.2 are subtly different from those of stemodioside B4 and stemodioside B3, respectively, and seem to indicate isomeric relationships. By contrast, the differences between the mass spectra of B3 and B4 were more significant. A molecular weight of 666 Da for stemodioside B4 was supported by observation of ions at a mass to charge ratio (*m/z*) of 667 (20% relative abundance, $[MH]^+$), *m/z* 1333 (100%, $[2M + H]^+$), and *m/z* 487 (70%, $[MH]^+$–glucose). However, the mass spectrum of stemodioside B3 required more circumspect evaluation. The base ion at *m/z* 633, in association with an apparent dimer ion at *m/z* 1265 (40% abundance, $[2M + H]^+$), seemed to indicate a molecular weight of 632 Da. However, the observation of the $[MH]^+$–glucose ion at *m/z* 471 (40%) and an ion at *m/z* 1301 (20%, $[2M + H]^+$), in conjunction with analysis of the NMR data, indicated a molecular weight of 650 Da. For stemodioside B3, this seems to imply a very unstable molecular ion that readily loses a water molecule.

The high-resolution ESI-MS analysis of stemodiosides B3 and B4 returned a molecular ion adduct at *m/z* 673.389 and 689.3879, corresponding to $C_{36}H_{58}O_{10}Na^+$ (requires 673.393) and $C_{36}H_{58}O_{11}Na^+$ (requires 689.3877), respectively.

21.2.4.3 NMR Spectroscopy

Complete spin mapping of stemodiosides B3 and B4 was completed using one dimensional (1D) NMR experiments (i.e., ^1H, ^1H NOE-difference, ^{13}C, and ^{13}C-DEPT) and two dimensional (2D)

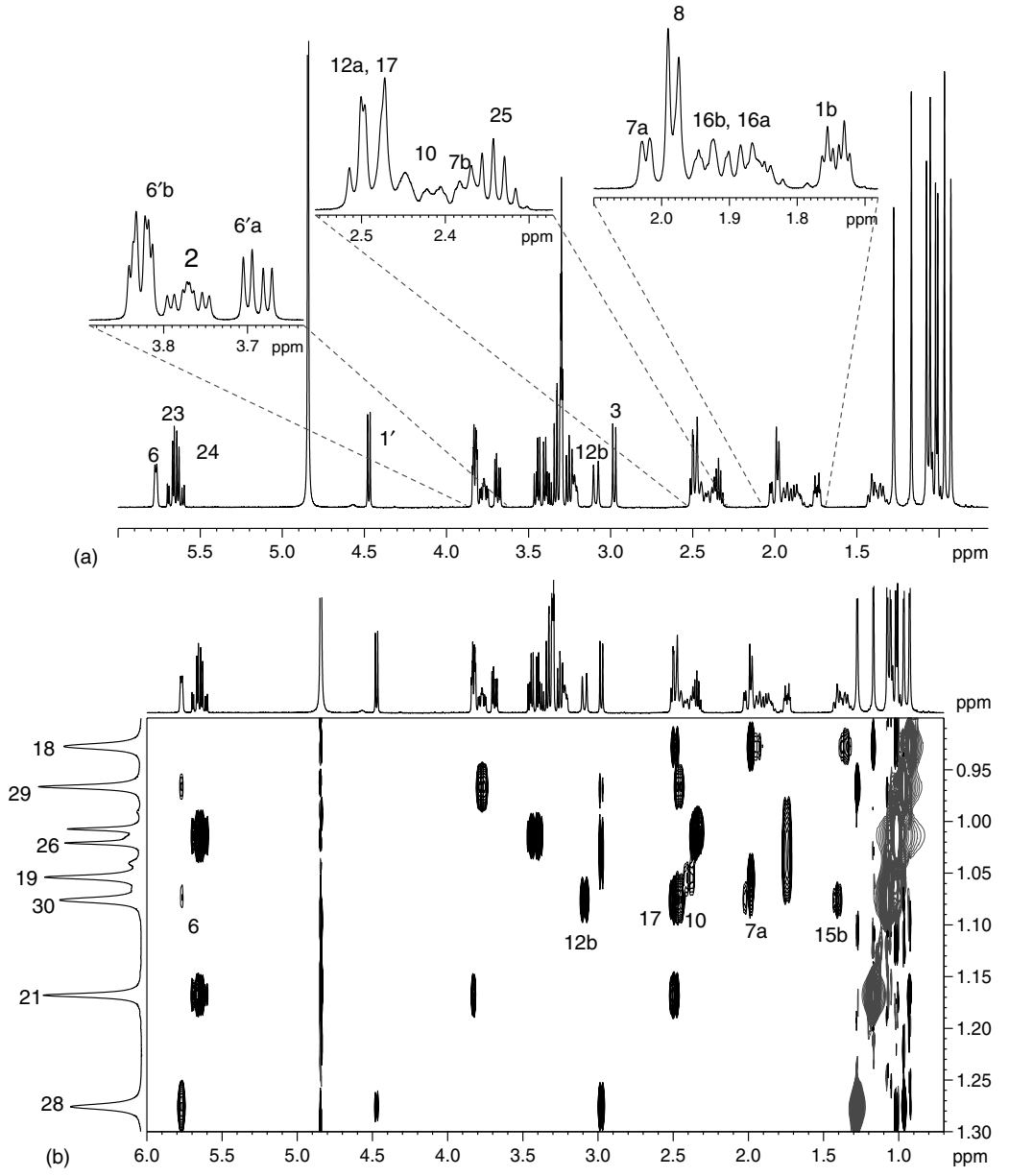


FIGURE 21.3 (a) The ^{1}H NMR spectrum for stemodioside B4 showing expansions for some areas (insets). Peak numbers refer to the carbon position of the proton and (b) a section of the gNOESY experiment expanded in the methyl resonance region (δ 0–1.3 ppm). As an example, the nOes observed for the CH_3-30 are labeled, i.e., H6, H12b, H17, H10, H7a, and H15b, indicating a spatial proximity of all these protons. (Reproduced from Allen, J.G., Colegate, S.M., Mitchell, A.A., Mulder, R., and Raisbeck, M.F., *Phytochem. Anal.*, 17, 226, 2006. Copyright Wiley. With permission.)

gradient-enhanced NMR experiments (i.e., ^{1}H–^{1}H COSY45, ^{1}H–^{1}H NOESY, ^{1}H–^{13}C HSQC, and ^{1}H–^{13}C HMBC). Nuclear Overhauser effect (nOe) measurements, to provide insight into the intramolecular spatial proximities, were obtained from a combination of 1D (steady-state NOE-difference) and 2D (phase-sensitive, gradient-selected ^{1}H–^{1}H NOESY) NMR experiments.

The NMR spectra, especially the ^{1}H NMR spectra (including the ^{1}H–^{1}H and ^{1}H–^{13}C 2D spectra), were complicated by signal overlap and multiple couplings requiring careful analysis of all

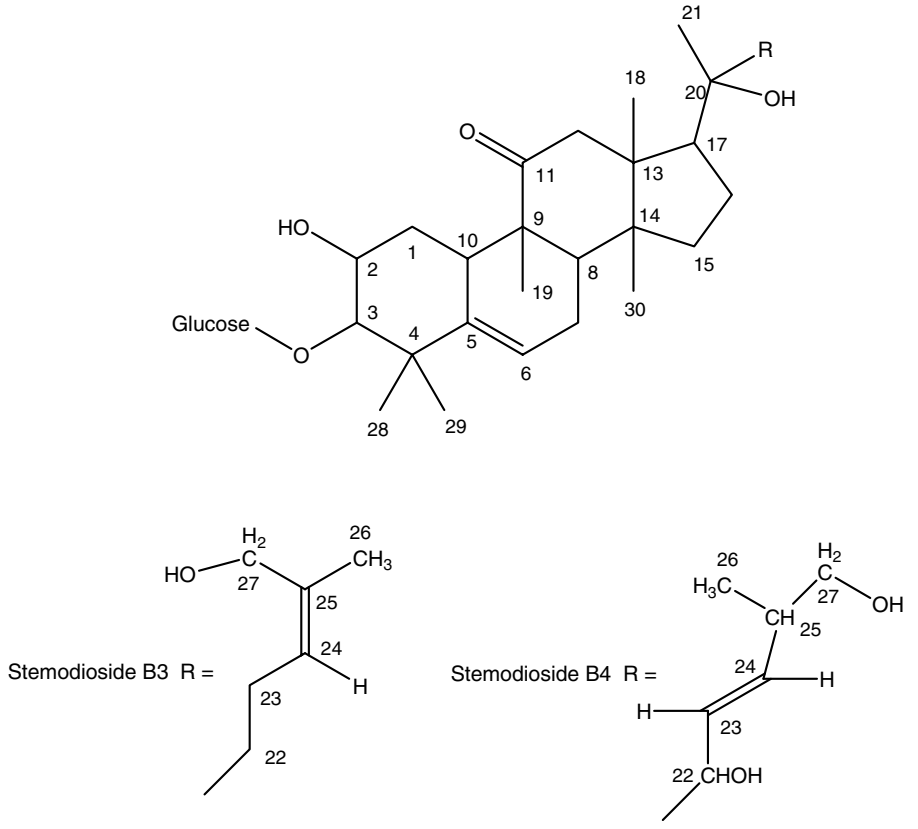


FIGURE 21.4 The core steroidal glucoside structure for stemodiosides B3 and B4 and the structures of the C17 side chain that differentiate the two toxins. (Reproduced from Allen, J.G., Colegate, S.M., Mitchell, A.A., Mulder, R., and Raisbeck, M.F., *Phytochem. Anal.*, 17, 226, 2006. Copyright Wiley. With permission.)

NMR experiments to deconvolute the data (Figure 21.3). The 2D spectra were particularly useful in differentiating overlapping signals and identifying coupled nuclei. Analysis of the ^{13}C NMR spectra, that is, determination of the number of and types (methyl, methylene, or quaternary) of carbons, in combination with the ESI-MS analysis, provided the molecular formulae of $C_{36}H_{58}O_{10}$ and $C_{36}H_{58}O_{11}$ for stemodiosides B3 and B4, respectively, thereby supporting the high-resolution mass measurements. Assimilation of the 1D and 2D NMR data enabled mapping of the steroidal glucoside core structure for B3 and B4 containing the deshielded carbonyl and one of the isolated alkene moieties. The other isolated alkene moiety, and the site of difference between stemodiosides B3 and B4, was included in the side chain on C17 (Figure 21.4).

The $^{1}H–^{1}H$ coupling constants and nOe interactions were used to determine the relative stereochemistry of the protons around the molecule. In some cases where the coupling constants were somewhat equivocal, spin–spin simulations were used to confirm the angular relationship between the protons concerned. The same approach, that is, proton coupling constants and nOe interactions, in addition to rational assignment of chemical shifts, was used to define the relative configuration around the alkene center in the C17 side chains (Figures 21.5 and 21.6).[8]

21.2.5 Conclusions and Future Research

The suspected toxicity of *S. kingii* was confirmed and fully described in experimental feeding trials with sheep. A toxicity model was validated in mice that was then used as a bioassay to guide the

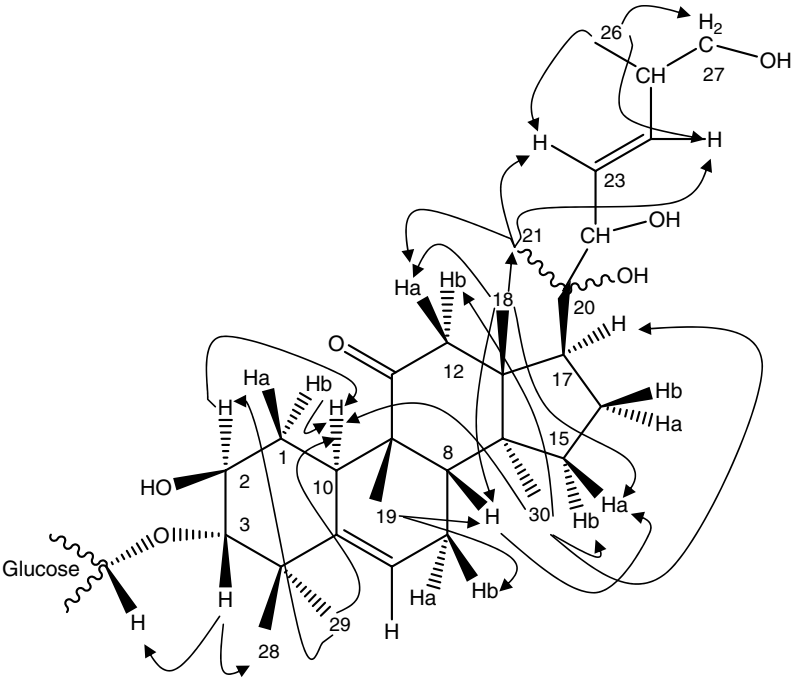


FIGURE 21.5 The deduced relative stereochemistry of stemodioside B4. Arrows depict observed nOe interactions. As an example, the interactions of the CH_3-30 substituent are shown in Figure 21.3b. (Reproduced from Allen, J.G., Colegate, S.M., Mitchell, A.A., Mulder, R., and Raisbeck, M.F., *Phytochem. Anal.*, 17, 226, 2006. Copyright Wiley. With permission.)

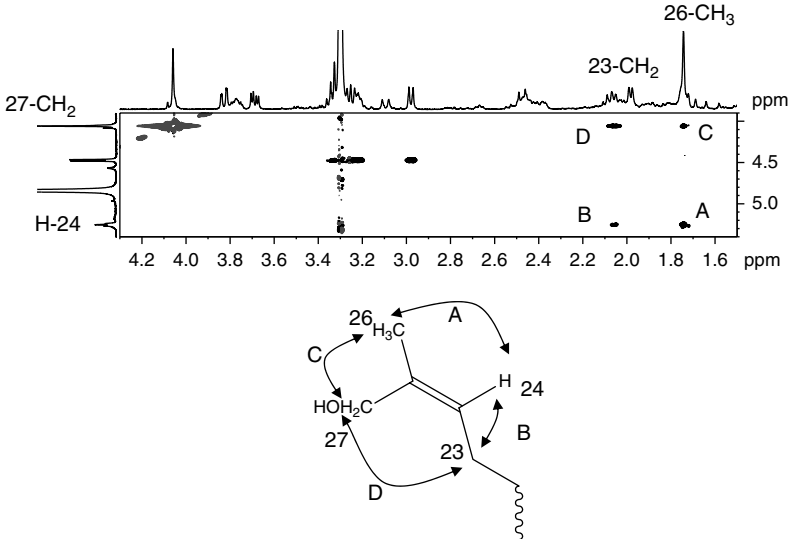


FIGURE 21.6 NOE interactions (gNOESY) confirming the *Z* configuration of the C24–C25 alkene in stemodioside B3. The crucial interactions are labeled A–D in both the contour plot and the partial structure. (Reproduced from Allen, J.G., Colegate, S.M., Mitchell, A.A., Mulder, R., and Raisbeck, M.F., *Phytochem. Anal.*, 17, 226, 2006. Copyright Wiley. With permission.)

isolation of stemodiosides B3 and B4 from a mixture of closely related compounds. Although the murine oral LD_{50} values of pure B3 and B4 were not determined rigorously, they were estimated, from the mortality data during the last stages of the bioactivity-guided purification, to be 99 and 42 mg/kg BW, respectively.

The core steroidal structure of stemodiosides B3 and B4 indicated that they belong to the cucurbitane type of triterpene steroids. This was confirmed by the deduced relative stereochemistry that defined the 5-ene, 9β-methyl, and 10α-H substitution characteristic of the cucurbitanes.[11] Stemodiosides B3 and B4 do not fit into any of the 12 major classes of cucurbitacins (A–T) described by Chen et al.;[11] however, stemodioside B3 formally seems to be the 2-hydroxy derivative of scandenoside R2 isolated from *Hemsleya panacis-scandens* (Cucurbitaceae),[12] while stemodioside B4 appears to be completely new.

Cucurbitanes have been isolated from medicinal plants and have a wide range of structures and reported bioactivities[11] including a bitter or sweet taste,[13] cytotoxicity and antitumor activity,[14] analgesic and antiinflammatory effects,[15] and ecdysteroid antagonism.[16] Cucurbitacins have also been implicated in cattle poisonings associated with *Cucumis* spp. and *Citrullus* spp., both of the Cucurbitaceae family.[17–19] In these cases, intoxication caused gastroenteritis (particularly affecting the forestomachs), hepatopathy, and cardiomyopathy, changes similar to those observed in poisoning by *S. kingii* and the stemodiosides.

Although stemodiosides B3 and B4 elicit clinical and pathological responses in mice similar to those observed in the experimental intoxication of sheep, the compounds have not yet been administered to sheep to definitively prove a causal relationship to the intoxication. Future research is aimed at isolating the other B group compounds and the groups A and C toxins. Such isolation and subsequent structural elucidation will allow an investigation of other potentially useful bioactivities.

21.3 THE *IN VITRO* DETECTION OF HEPATOCYTOTOXIC FUNGAL METABOLITES POTENTIALLY RELATED TO ACUTE BOVINE LIVER DISEASE

In the search for specific toxic natural products, it is not always convenient (or desirable) to utilize the affected animal species or an *in vivo* animal model. In this section the use of *in vitro*–cultured rat hepatocytes as a bioassay for plant-associated liver toxins will be described.

21.3.1 Description of the Intoxication

Acute bovine liver disease (ABLD) is a newly emerging, acute hepatopathy of beef and dairy cattle in southern parts of Australia.[20] It is uncertain whether sheep are truly resistant or whether differences in grazing behavior predispose cattle to the intoxication. Clinical signs include reduced milk production, acute photosensitization, profound morbidity, and even sudden death with some mortality rates in excess of 50% of affected animals. Deaths can occur within 12 h of access to a toxic paddock at the autumn break of the season. The causative factor(s) of ABLD are undefined but outbreaks have been consistently associated with the presence of the annual grass *Cynosurus echinatus* L. (Poaceae). Stands of *C. echinatus* within a pasture seemed to be indicative of a less well-managed pasture that also contained other weeds among the predominant pasture grasses.

Initial suspected involvement of the sporidesmin-producing *Pithomyces chartarum* or the microcystins produced by blue-green algae was dismissed on histological grounds. The pathology of the intoxication is quite unique involving hepatic periportal necrosis.[20] To address the possibility of mycotoxin involvement in the etiology of the disease, a mycological assessment of toxic pastures was conducted.[21] Samples of *C. echinatus* collected from the site and at the time of ABLD outbreaks have yielded a known, phytopathogenic fungus, *Drechslera biseptata*, which has a worldwide occurrence and, although none have been isolated, has been suspected of producing mycotoxins.[22]

Toxicity to rats and sheep has been previously associated with *D. campanulata* (syn. *Pyrenophora semeniperda*) with effects including anorexia, rumen stasis, diarrhea, and death.[23,24]

A preliminary feeding trial in which single 9-month-old steers were fed with oats or *C. echinatus*, both of which had been inoculated and incubated with a *D. biseptata* culture, failed to elicit any liver damage or clinical signs.[25] Therefore, a study was conducted to determine whether *D. biseptata*, or the *C. echinatus* with which it is associated, could adversely affect liver cells *in vitro*.[26]

21.3.2 Detection of Hepatocytotoxins

To investigate the possible involvement of *D. biseptata* in the liver damage characteristic of ABLD, a continuous line of rat hepatocytes was exposed to various extracts and fractions of extracts derived from cultured *D. biseptata* and from pasture plants collected from a toxic paddock.

21.3.2.1 *In Vitro* Culture of *Drechslera biseptata* and Rat Liver Hepatocytes

A sample of the stored source culture of *D. biseptata*, isolated from outbreaks of ABLD, was inoculated at a single central point onto V8 agar in 9 cm Petri plates.[26] The inoculated plates were placed in a plastic bag and the fungus left to grow at room temperature, exposed to daylight. The diurnal changes in light stimulated the sporulation of the fungus. After 7 days the mycelium formed a complete mat over the surface of the entire plate providing an ongoing source of the fungal isolate. For bioassay of fungal metabolites in the hepatocyte culture, the fungal growth was allowed to develop for about two weeks before processing.

The Clone 9 rat hepatocyte epithelial cell line (American Type Culture Collection accession number CRL-1439), originally isolated from a normal male Sprague–Dawley rat liver in 1968,[27] was used to evaluate *in vitro* toxicity. The cell line was routinely maintained in liquid culture according to the protocol supplied with the cells by the supplier and was then seeded into the OptiCell™ cell culture system for the bioassay. Because the cells continue to grow and are easily observed in the OptiCell system, a "real-time" assessment of cell health and morphology occurs when the cells are exposed to the test samples.

21.3.2.2 *In Vitro* Assessment of Extracts

Samples of the cultured *D. biseptata* (spores and mycelium), and toxic pasture samples of *C. echinatus* and other pasture grasses, were variously extracted with hexane, ethyl acetate, methanol, and water. The extracts were evaporated to dryness *in vacuo* and reconstituted in the cell culture media. The filtered (0.45 µm to ensure sterility) test samples were then added, at various doses, to the healthy hepatocytes growing within the OptiCell. After 4, 24, and 48 h, digital photomicrographs of the live, unstained cells within the OptiCell were captured using a microscope attached to a digital camera. After 48 h, excised OptiCell membranes, to which the cells were adhered, were stained with hematoxylin and eosin and mounted on microscope slides for further morphological assessment.

The untreated hepatocytes continued to grow and form a uniform layer of cells on the OptiCell membranes (Figure 21.7a). The ethyl acetate extract of the *D. biseptata* mycelium and spores had no effect on the hepatocytes while both the hexane and the water extracts elicited multinucleation (Figure 21.7b). The methanol extracts of the spores and mycelium, and the methanolic extracts of the residues after initial hexane, ethyl acetate, or water extraction, all resulted in severe, dose-dependent degeneration (Figure 21.7c) of the hepatocytes within 2 h of exposure. The same degenerative hepatocytotoxic effect was observed with culture media solubles from the methanolic extract of the toxic pasture-derived *C. echinatus* (Figure 21.7d).

Further purification of the hepatocytotoxic extracts was accomplished using reverse-phase C18 solid-phase extraction (SPE). Thus, the water-soluble components of the methanol extract of the

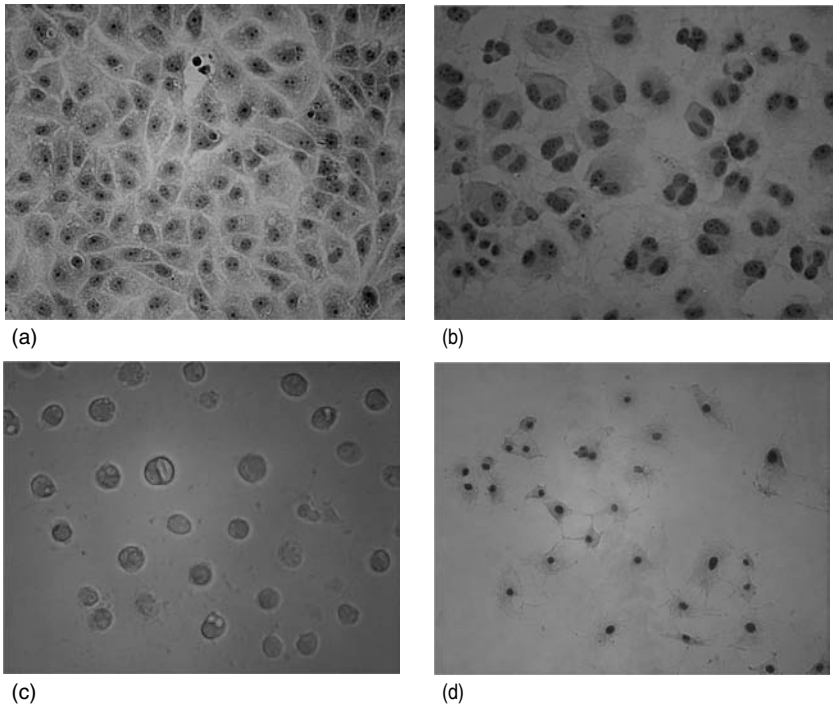


FIGURE 21.7 Cultured rat hepatocytes exposed to different extracts of *Drechslera biseptata* and to an extract of *Cynosurus echinatus*. All hematoxylin and eosin (HE)-stained cells were prepared at the completion of the 48-h observation period. (a) Untreated control showing normal hepatocyte morphology (HE, 400×); (b) binucleation associated with hexane or water extractables from *D. biseptata* cultures (HE, 400×); (c) "real-time" photomicrograph (400×) of degenerate hepatocytes exposed to the methanolic extract of *D. biseptata* for 2 h; and (d) cellular degeneration associated with exposure to the methanolic extractables from *C. echinatus* collected from a toxic paddock (HE, 400×). (Reproduced from Aslani, M.R., Pascoe, I., Kowalski, M., Michalewicz, A., Retallick, M.A.S., and Colegate, S.M., *Aust. J. Exp. Agric.*, 46, 599, 2006. Copyright CSIRO Publishing. With permission.)

D. biseptata cultures were applied to a C18 reverse-phase SPE column. Bioassay monitoring revealed that the hepatocytotoxicity was retained on the column and eluted with 70% methanol in water.

21.3.3 HPLC-MS of Hepatocytotoxic Extracts

Hepatocytotoxic fractions and the C18 reverse-phase SPE-purified fractions were analyzed using reverse-phase HPLC-ESI ion-trap MS. The methanolic extract of *D. biseptata* revealed two major peaks. These same two peaks were observed in the water extract of harvested spores (Figure 21.8a). The mass spectrum of peak 1 showed a molecular ion adduct ($[MH]^+$) at *m/z* 480 while peak 2 showed ions at *m/z* 478/496. It is presumed, but not conclusive from the current data set, that *m/z* 478 represents $[MH]^+$ while *m/z* 496 represents $[MNa]^+$. The mass spectrum of the smaller peak 3 showed an $[MH]^+$ ion at *m/z* 480.

Previous work has identified cytochalasins (Figure 21.9) from *D. campanulata* and *D. wirreganensis*.[28,29] Binucleation of barley root tip cells and mammalian cells has been reported following exposure to the cytochalasins.[28,30] HPLC-MS comparison of the hepatocytotoxic *D. biseptata* extracts with authentic samples of cytochalasins A and B (Figure 21.8b) indicated coidentity of peak 1 with cytochalasin B. However, the apparent discrepancy in the reported water insolubility of the cytochalasins A and B with the obvious water solubility of the hepatocytotoxic components from

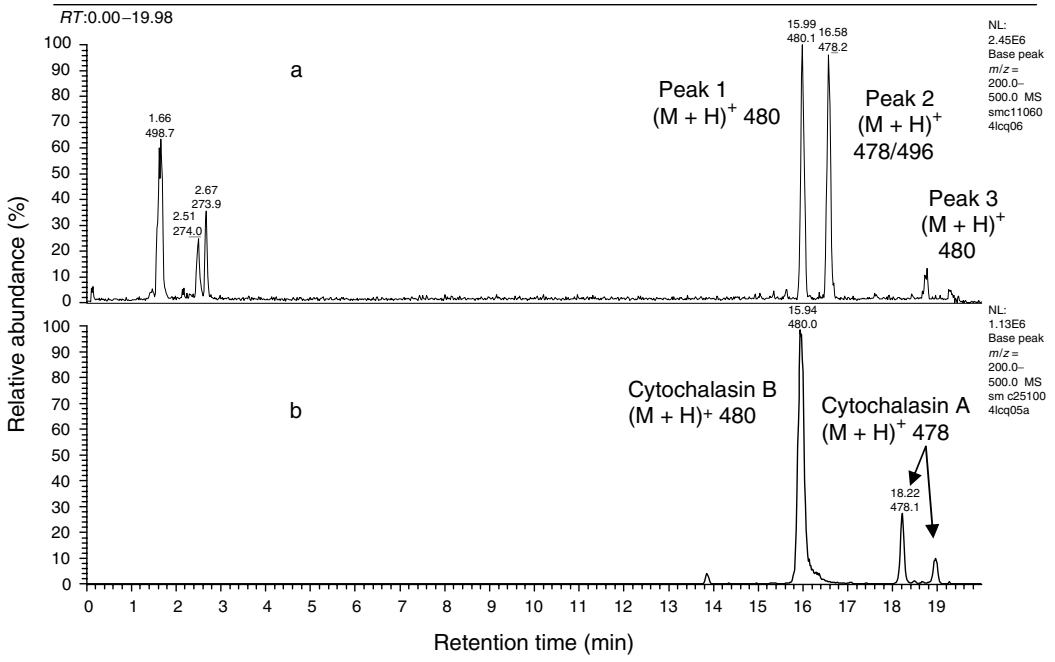


FIGURE 21.8 The HPLC-ESI ion-trap MS base ion (*m/z* 200–500) chromatograms for (a) a water extract of *Drechslera biseptata* culture spores and (b) authenticated standards of cytochalasins B and A. The *m/z* of the molecular ion adducts ($[MH]^+$) are shown beside the peaks. (Reproduced from Aslani, M.R., Pascoe, I., Kowalski, M., Michalewicz, A., Retallick, M.A.S., and Colegate, S.M., *Aust. J. Exp. Agric.*, 46, 599, 2006. Copyright CSIRO Publishing. With permission. http://www.publish.csiro.au/nid/73/issue/1426.htm.)

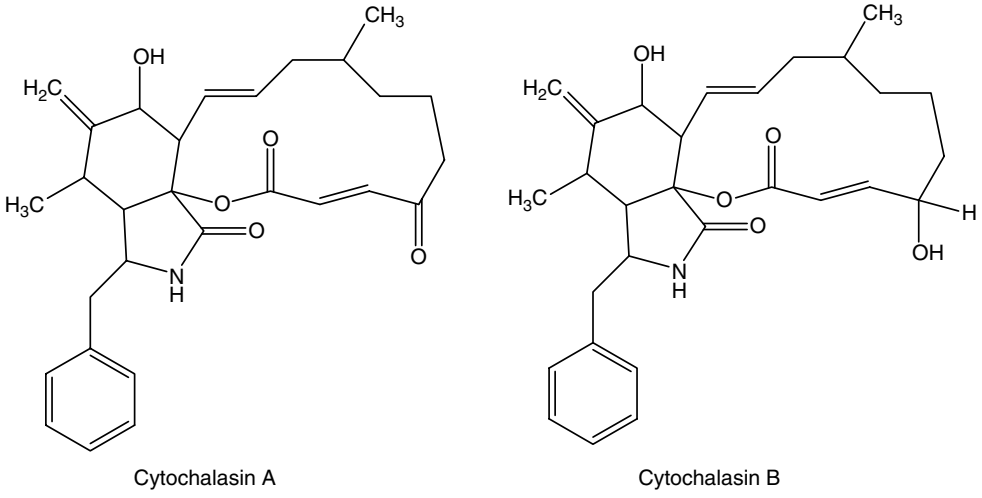


FIGURE 21.9 Structures of cytochalasins A and B.

D. biseptata requires rationalization. The later eluting peak ($[MH]^+$, *m/z* 478/496, Figure 21.8a) did not correspond to cytochalasin A ($[MH]^+$, *m/z* 478, Figure 21.8b).

HPLC-ESI-MS analysis of the eluates from the C18 reverse-phase SPE columns showed the presence of peak 1 in the hepatocytotoxic fraction eluted with 70% MeOH in water (Figure 21.10a)

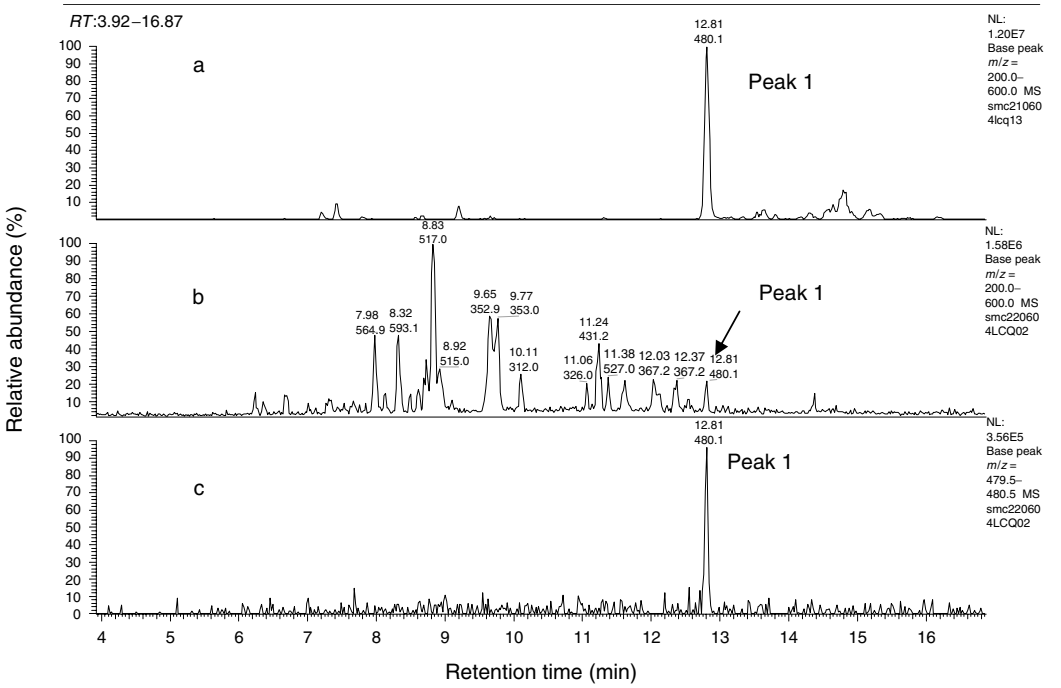


FIGURE 21.10 HPLC-ESI ion-trap MS ion chromatograms for (a) the hepatocytotoxic fraction of *Drechslera biseptata* extracted onto C18 reverse-phase SPE columns and eluted with 70% aqueous methanol; (b) the hepatocytotoxic extract from *Cynosurus echinatus* collected from a toxic paddock; and (c) a reconstructed ion chromatogram derived from (b), displaying *m/z* 480 consistent with peak 1. Peak 1 was shown to cochromatograph with, and have the same mass spectrum as, cytochalasin B (Figure 21.8b). (Reproduced from Aslani, M.R., Pascoe, I., Kowalski, M., Michalewicz, A., Retallick, M.A.S., and Colegate, S.M., *Aust. J. Exp. Agric.*, 46, 599, 2006. Copyright CSIRO Publishing. With permission.)

and also in the SPE-purified sample from the *C. echinatus* collected from the toxic paddock (Figures 21.10b and 21.10c). Unexpectedly, peak 2, although appearing to be less polar than peak 1 (Figure 21.8a), was eluted from the SPE column with 20% MeOH in water, that is, eluting from the SPE column in reverse order to the elution from the HPLC column.

21.3.4 Conclusions and Future Research

Secondary metabolites, isolated from a fungus derived from pasture samples collected at the site and time of outbreaks of ABLD, have been shown to be toxic to cultured hepatocytes. Preliminary HPLC-MS analysis indicated cytochalasin-like structures. The chemical identities of the postulated cytochalasins will require confirmation, perhaps by applying several different sets of chromatographic conditions that all result in coelution of the sample analytes with the cytochalasin standards available. Preferably, NMR mapping of the proton and carbon spin systems of the sample analytes should be compared to the cytochalasin standards. The latter approach will confirm identity or provide good data for *de novo* structural elucidation.

The isolated hepatocytotoxins will need to be assessed *in bovis* for their capacity to produce the clinical signs and pathology associated with ABLD. Full, and conclusive, identification and confirmation that the hepatocytotoxins do indeed cause ABLD will then allow further research to manage the disease, for example, rapid assays for pasture and stock management, and development of clinical management approaches.

21.4 ELISA DETECTION OF LOW-MOLECULAR-WEIGHT BIOACTIVE NATURAL PRODUCTS

The enzyme-linked immunosorbent assay (ELISA) relies on the interaction of the analyte with specific antibodies. The small size of the nonproteinaceous plant-associated toxins (and other low-molecular-weight bioactive natural products) means that the immune system does not recognize them, that is, they are nonimmunogenic.[31] Therefore, the toxin, or a modified version of the toxin (a hapten), is covalently bound to a carrier protein to form an immunogenic toxin (hapten)–protein conjugate. Vaccination of an animal with the conjugate, formulated with a suitable adjuvant, results in the *in vivo* production of a polyclonal suite of antibodies, some of which will be cross-reactive to the toxin (hapten) portion (epitope) of the immunogenic conjugate.

The toxin-specific antibodies thereby generated are incorporated into various ELISA formats depending on the specific use but, basically, they rely on the capture of the analyte by the antibodies and a resultant induction of a change, for example, color, that correlates to the level of analyte present in the sample. After optimization of the ELISA conditions, an intralaboratory validation against, for example, an HPLC-MS method of analysis should be rigorously conducted. Then, and especially if the ELISA is destined for use outside of the developing laboratory, an interlaboratory validation of the ELISA will be required. The optimized and validated ELISAs can be used to monitor natural sources for the presence of the specific analytes or for the presence of similar, cross-reacting natural products that may also have similar bioactivity.

This section will describe the development of ELISAs for two groups of bioactive natural products, the phomopsins and the corynetoxins. While these cases were primarily aimed at animal feed and human food safety considerations, the same approaches are valid for facilitating the search for other bioactive natural products.

21.4.1 Phomopsins

21.4.1.1 Source and Biological Activity of the Phomopsins

Produced by the fungus *Diaporthe toxica* (formerly *Phomopsis leptostromiformis*) growing on lupin (*Lupinus* sp.) seed and stubble, phomopsins cause the hepatic disease lupinosis.[32] The phomopsins are linear hexapeptide compounds (Figure 21.11) incorporating an ether-linked macrocycle between amino acids 1 and 3 in the linear chain.[32,33] Detection methods for the phomopsins have included an *in vivo* nursling rat bioassay[34] and HPLC and HPLC-MS methods.[35] The latter in particular is useful for detecting phomopsin-like compounds based on the mass spectra (Figure 21.12).

These compounds target the liver where they have a strong affinity for tubulin, a cytoskeletal protein involved in microtubule formation, thereby preventing microtubule formation with consequent effects on processes such as mitosis.[36] The tubulin-interactive activity of the phomopsins is reminiscent of that for colchicine and the *Vinca* alkaloids, vincristine and vinblastine, used in anticancer treatments.[37]

Because of the toxicity of the phomopsins, Australian food safety authorities have set an upper limit of 5 ppb of phomopsins in food destined for human consumption. This presented a challenge for physicochemical methods of analysis, despite the potential for the sensitivity to be improved (HPLC-MS/MS, e.g.), since the sample turnaround is slow and technological requirements are high.

21.4.1.2 Development of a Phomopsin ELISA

21.4.1.2.1 Antibody Production

Using protocols described by Carter and Meyerhoff,[38] an activated phomopsin-N-hydroxysuccinimidyl (NHS) ester was prepared and added slowly to keyhole limpet hemocyanin (KLH) in a bicarbonate

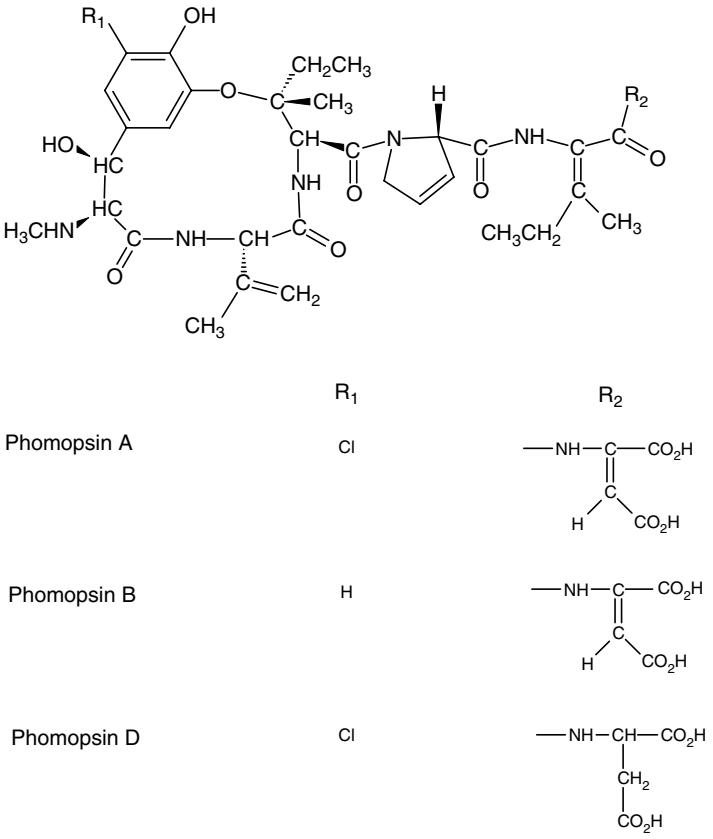


FIGURE 21.11 Structures of some phomopsins. (Reprinted from Than, K.A., Stevens, V., Knill, A., Gallagher, P.F., Gaul, K.L., Edgar, J.A., and Colegate, S.M., *Anim. Feed Sci. Technol.*, 121, 5, 2005. With permission from Elsevier.)

buffer. After the reaction period, unconjugated phomopsins were removed from the phomopsin–KLH conjugate by ultrafiltration (10,000 MW cutoff).[35,39]

Merino sheep wethers of approximately 12 months of age were subcutaneously immunized at the back of the neck with the phomopsin–KLH conjugate emulsified in an equal volume of Freund's complete adjuvant. Three subsequent booster injections, at 21-day intervals, of the same amount of conjugate emulsified in Freund's incomplete adjuvant, were followed by boosters at 6-month intervals when necessary to maintain antibody titers. Blood from the jugular vein was taken prior to, and 2–3 weeks after, each injection. The production of antibodies against phomopsins was assessed using an indirect ELISA in which the wells of a microtiter plate were coated with phomopsins and the captured antibodies detected using donkey, antisheep IgG horseradish peroxidase (HRP).[40]

21.4.1.2.2 Incorporation of Phomopsin Antibodies into an ELISA

A direct, competitive ELISA was developed by first coating the wells of a 96-well ELISA plate with the purified (Protein G affinity) ovine polyclonal IgG antibodies, which were equally cross-reactive with both phomopsin A and its deschloro analog phomopsin B (Figure 21.11). The coated wells were then incubated with a solution of the sample extract (or known calibration standards of phomopsin A) and a phomopsin–HRP conjugate (prepared in a similar way to the antigenic conjugate but using HRP instead of KLH as the conjugating protein). Thus, the free phomopsins and the phomopsin–HRP conjugate competed for binding to the immobilized phomopsin antibodies.

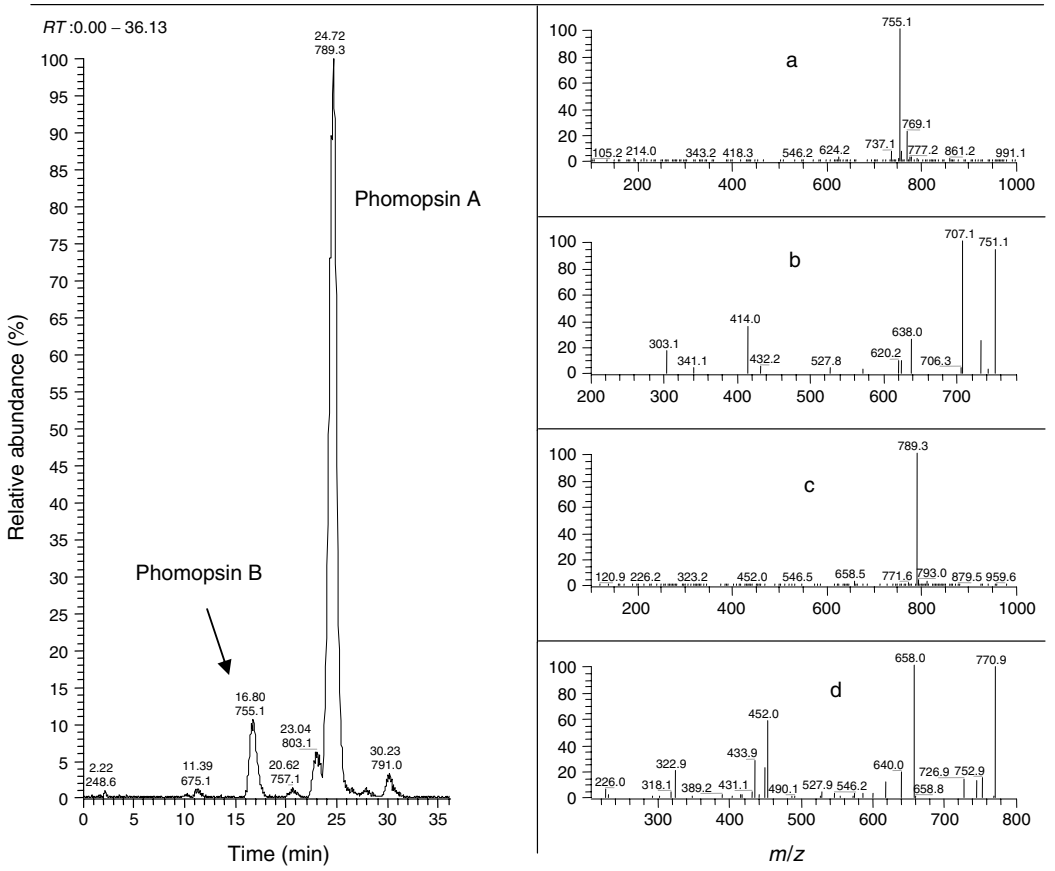


FIGURE 21.12 HPLC-ESI-MS base ion (*m/z* 100–1000) chromatogram of phomopsins. The full scan mass spectra (a) and (c) show the protonated molecular ions expected of phomopsin B and A, respectively. The MS/MS fragment ion spectra of phomopsin B (b) and phomopsin A (d) show a characteristic similarity that is useful in assigning phomopsin-like structural identity to the smaller peaks in the chromatogram. (Reprinted from Than, K.A., Stevens, V., Knill, A., Gallagher, P.F., Gaul, K.L., Edgar, J.A., and Colegate, S.M., *Anim. Feed Sci. Technol.*, 121, 5, 2005. With permission from Elsevier.)

Following postincubation washing of the wells, tetramethylbenzidine (TMB), the substrate for the HRP, was added to develop color in inverse proportion to the amount of phomopsins in the sample being analyzed. The resultant sensitivity (1 ppb lower level of detection), the simplicity of the assay, the rapid sample turnaround time, and high sample throughput make this assay suitable for monitoring animal feed and animal-derived food products for phomopsin contamination.

21.4.1.3 Application of the Phomopsin ELISA

The ELISA has been used to monitor lupin-based animal feeds and lupins for the human food supply for the presence of phomopsins. With each new sample matrix, i.e., other than lupins, the ELISA needs to be reoptimized to account for any potential matrix-associated effects on the detection. The ELISA has also been used to purposefully seek cross-reactive coextractives that, with similar antibody-recognizing epitopes, may have similar bioactivity to the phomopsins. Combined with HPLC, and even HPLC-MS, a specific "cross-reactivity fingerprint" of an extract can be determined (Figure 21.13).

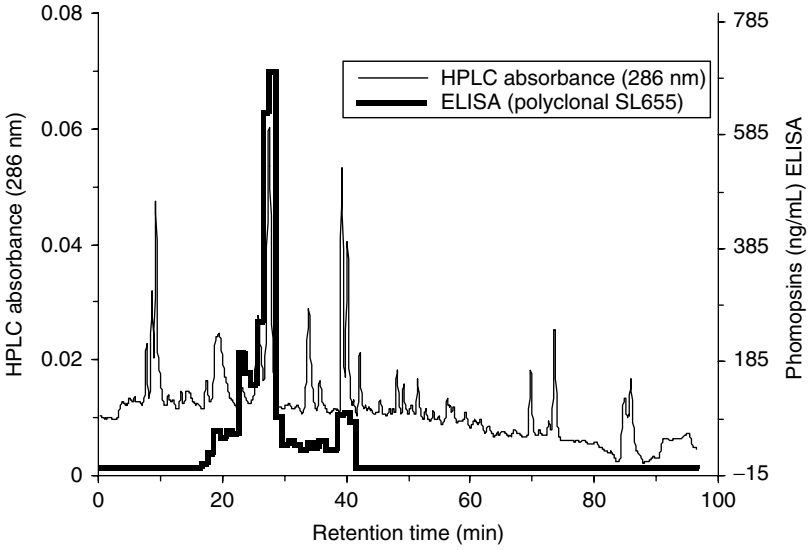


FIGURE 21.13 Correlation of an ELISA immunogram with the HPLC-UV chromatogram of an extract of *Diaporthe toxica*. Thus, fractions collected from the HPLC run were analyzed for cross-reactivity in the phomopsin ELISA. A positive response in the ELISA indicates phomopsin-like structural identity of unknowns. (Reprinted from Than, K.A., Stevens, V., Knill, A., Gallagher, P.F., Gaul, K.L., Edgar, J.A., and Colegate, S.M., *Anim. Feed Sci. Technol.*, 121, 5, 2005. With permission from Elsevier.)

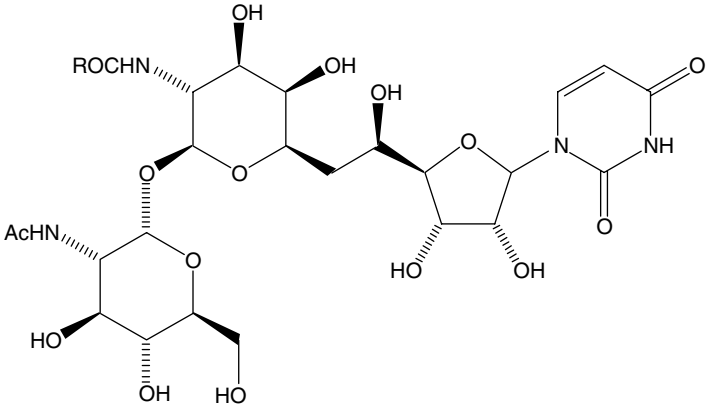


FIGURE 21.14 Structures of corynetoxins where R = C_{15}–C_{19} is an aliphatic chain that can be saturated, α, β-unsaturated, or β-hydroxylated, in addition to having different terminal hydrocarbon branches.[42] (Reprinted from Than, K.A., Stevens, V., Knill, A., Gallagher, P.F., Gaul, K.L., Edgar, J.A., and Colegate, S.M., *Anim. Feed Sci. Technol.*, 121, 5, 2005. With permission from Elsevier.)

21.4.2 Corynetoxins

21.4.2.1 Source of Corynetoxins

Corynetoxins are a family of *N*-acetylglucosamine tunicaminyluracil lipids that vary only in the length, terminal structure, and degree of β-hydroxylation of the fatty acid side chain (Figure 21.14).[41] An example of the HPLC-MS analysis of the corynetoxins is shown in Figure 21.15. The corynetoxins are related to other such families of compounds including the tunicamycins, antibiotic MM19290, and streptovirudins.[42,43] As potent inhibitors of *N*-acetylglucosamine-1-P transferase,

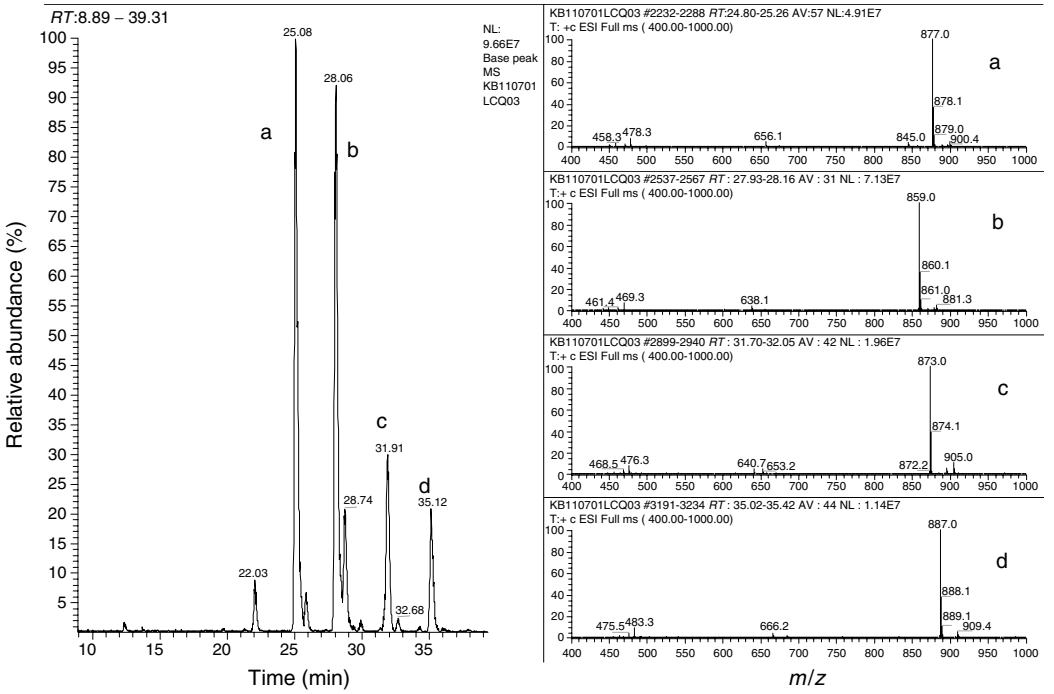


FIGURE 21.15 An HPLC-ESI ion-trap MS ion chromatogram of the corynetoxins in an extract of 15 individual bacterial "galls". For example, the mass spectra of corynetoxins H17a (a), U17a, (b), U18i (c), and U19a (d) are shown. For an explanation of the nomenclature of the corynetoxins, see Cockrum, P.A. and Edgar, J.A., *J. Chromatogr.*, 268, 245, 1983.

the corynetoxins prevent the formation of *N*-linked proteins by blocking the first step in the biosynthesis of the oligosaccharide moiety destined to be transferred to the nascent protein.[44]

The corynetoxins have been shown to be the causative chemicals of annual ryegrass toxicity that afflicts tens of thousands of livestock annually in Australia, mainly in Western Australia and South Australia. They are also responsible for flood plain staggers and Stewart's range syndrome when blown grass (*Agrostis avenacea*) and annual beard grass (*Polypogon monspeliensis*) are involved, respectively.[45] They are produced by the bacterium *Rathayibacter toxicus* once the infected plant starts to senesce,[46] presumably as an antibiotic, "anticompetition" reaction by *R. toxicus* as plant-derived nutrients become scarce. The bacterium infects the annual ryegrass by virtue of the nematode vector *Anguina funesta* and thereby forming bacterial colonies that take over the nematode galls. There is some suggestion that a bacteriophage is a fourth participant in this complex ecology.[47,48] Analysis of galls derived from fescue in New Zealand and in the United States has shown the presence of corynetoxin-like compounds in terms of physicochemical properties and bioactivity.[49]

21.4.2.2 Development of the Corynetoxin ELISA

In a similar way to the production of ovine antibodies against phomopsins (Section 21.4.1.2.1), antisera against corynetoxins were generated in sheep injected with an immunogenic conjugate prepared by chemically bonding a toxin-related hapten to fetal calf serum proteins using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as the linker.[50–52]

The ELISA can then be conducted in one of the two formats[35] but the indirect competitive ELISA format is currently preferred. Thus, the wells of a microtiter plate are first coated with the toxin-related hapten. This is in contrast to the direct competitive phomopsin ELISA (see Section 21.4.1.2.2) in which the wells were coated with the phomopsin antibodies. The coated wells are then incubated with sample extracts or calibration standard solutions (isolated corynetoxins or the

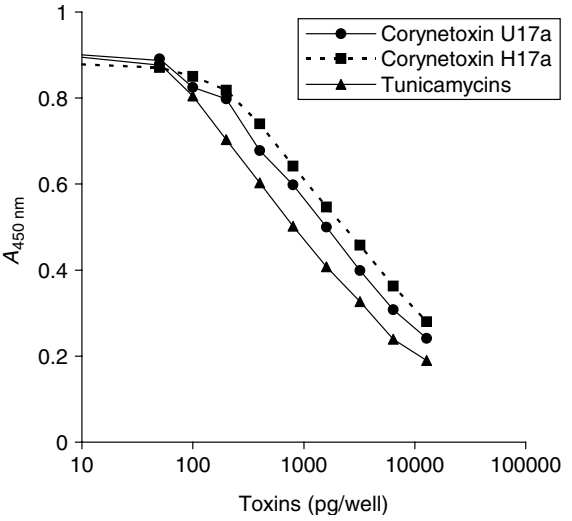


FIGURE 21.16 The indirect competitive ELISA format for *N*-acetylglucosamine tunicaminyluracil compounds showing the similarity of response for the tunicamycins and the individual corynetoxins H17a and U17a. (Reprinted from Than, K.A., Stevens, V., Knill, A., Gallagher, P.F., Gaul, K.L., Edgar, J.A., and Colegate, S.M., *Anim. Feed Sci. Technol.*, 121, 5, 2005. With permission from Elsevier.)

chemically similar and biologically equivalent tunicamycins) in the presence of the corynetoxin antisera. Following the incubation period and subsequent washing, the plate is incubated with donkey antisheep antisera conjugated to HRP. Addition of the enzyme substrate TMB then produces a color in inverse proportion to the amount of corynetoxins in the sample (Figure 21.16).

21.4.2.3 Application of ELISA

Thus far, the corynetoxin ELISA has been used for the detection of corynetoxins in animal feed stocks and in grains for human consumption. As with all ELISAs, whenever a new sample matrix is analyzed, the ELISA needs to be validated and reoptimized for that matrix to account for any modulating matrix effects.

It has been used in the immunogram mode (as with the phomopsins in Section 21.4.1.3) to show that ELISA cross-reactive material is present in the early eluting, polar fractions of an HPLC analysis. It is postulated that this may be a biosynthetic precursor to the corynetoxins, perhaps the *N*-acetylglucosaminyl-tunicaminyluracil core of the chemicals prior to fatty acid addition.[53]

21.5 HPLC-MS DETECTION AND TENTATIVE IDENTIFICATION OF TOXIC PYRROLIZIDINE ALKALOIDS

Once a class of bioactive metabolites has been identified, it then becomes possible to utilize technologies to facilitate the search for similar compounds. To exemplify this sort of approach, in this section, the application of HPLC-MS to the detection and tentative identification of toxic pyrrolizidine alkaloids, some of which have been candidates for antitumor agents (e.g., indicine-*N*-oxide), will be described.

21.5.1 Toxic Pyrrolizidine Alkaloids

The toxic pyrrolizidine alkaloids are a diverse class of naturally occurring mono- or diesters (including macrocyclic diesters) of 1-hydroxymethyl-7-hydroxy-1,2-dehydropyrrolizine.[54] These alkaloids, which lead to metabolites that are hepatotoxic and can be pneumotoxic, genotoxic, and carcinogenic,

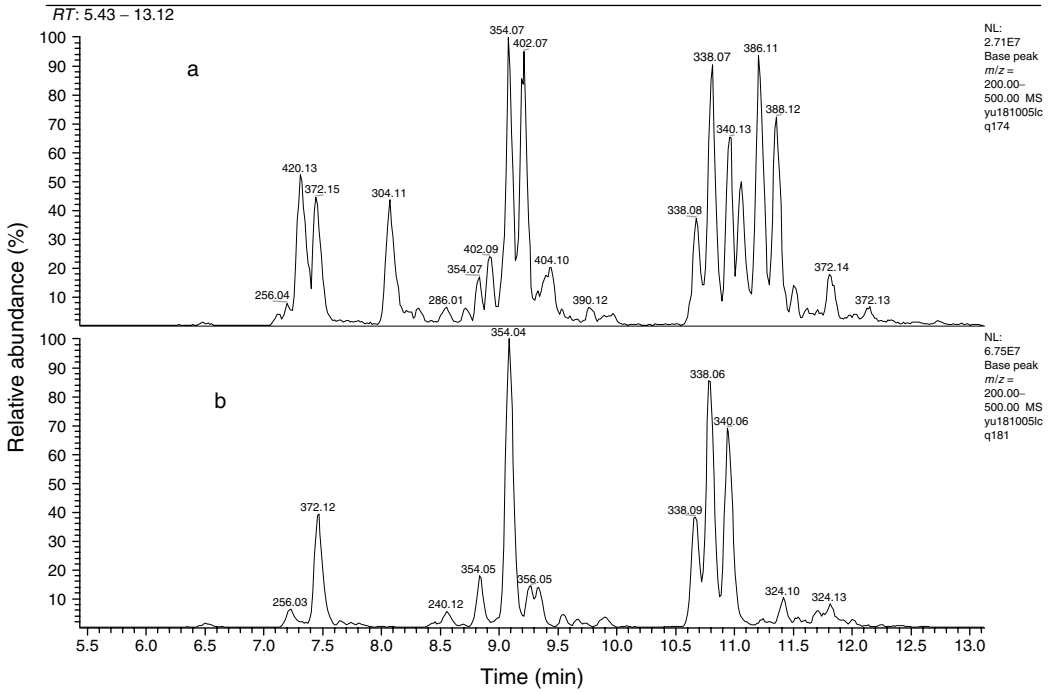


FIGURE 21.17 An alkaloid-focused clean-up of a reduced (zinc/sulfuric acid) extract of *Senecio ovatus* using strong cation exchange (SCX) and solid phase extraction (SPE). The reverse-phase HPLC-ESI-MS base ion (*m/z* 200–500) chromatograms of (a) methanolic solubles of the reduced extract and (b) the SCX SPE of the methanolic solubles of the reduced extract.

occur as natural components of many herbal preparations, cooking spices, and honey, and can contaminate other food crops and animal-derived food destined for the human food supply.[2] Consequently, regulations governing human exposure to these toxic pyrrolizidine alkaloids have been instigated by several countries.[55]

While the *N*-oxidation of pyrrolizidine alkaloids is a recognized detoxifying mechanism in mammals,[54] it has also been shown that ingested *N*-oxides are indeed toxic, presumably via *in vivo* reduction to the parent pyrrolizidine alkaloids and subsequent hepatic oxidation to the toxic "pyrrolic" metabolites.[56] Since the pyrrolizidine alkaloids are biosynthesized in plants as their *N*-oxides,[57] analysis of the *N*-oxide content is an important goal. Combined SPE and HPLC-MS approaches have provided methods for qualitative profiling and quantitative analysis of pyrrolizidine alkaloids and their *N*-oxides in plants and plant-derived products.[58–60]

21.5.2 Detection in Plant Extracts

Extraction of the pyrrolizidine alkaloids and their *N*-oxides from plant sources is a critical first step in the HPLC-MS analysis. Applied to bioactive natural products in general, the potential complexity of the "natural product cocktail" and the need for focused, "clean-up" stages have been discussed in several of the chapters in this book. In this present case, simple methanolic treatment of the plant will extract more than just the alkaloids. This can result in a more complicated chromatogram that may become a hindrance unless specific analytes are being searched for in the LC-MS total ion chromatogram. Insertion of an SPE-based cation exchange "clean-up" stage simplified the range of metabolites and thus interpretation of the HPLC-MS ion chromatogram (Figure 21.17).

The following case studies describe the alkaloidal profiling of some *Echium* and *Senecio* species. Methanolic extraction of a plant sample (or specific plant parts such as pollen, flowers, anthers,

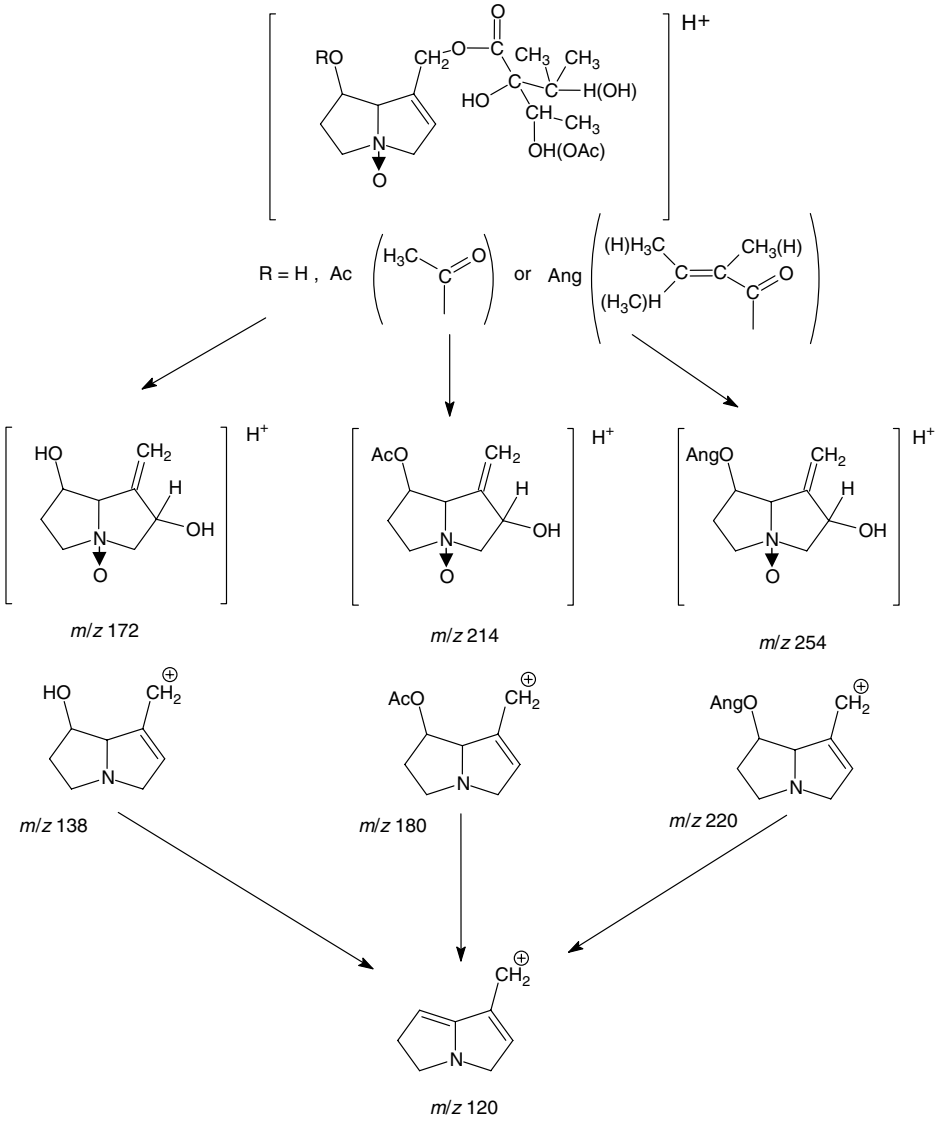


FIGURE 21.18 Some characteristic fragmentations observed for the 1,2-dehydropyrrolizidine alkaloids and their *N*-oxides. (Reproduced from Colegate, S.M., Edgar, J.A., Knill, A.M., and Lee, S.T., *Phytochem. Anal.*, 16, 108, 2005. Copyright Wiley. With permission.)

leaves, etc.) was repeated at room temperature until the extract was colorless. Gravimetric analysis of the extracted material showed that the lack of color corresponded to negligible extractives. Careful evaporation of the methanol (under vacuum and <40°C) yielded a green, gummy residue that was then extracted with dilute aqueous sulfuric acid that was subsequently applied to a strong cation exchange (SCX) SPE column. After water and methanol washes of the SPE column, the alkaloids were eluted with a saturated ammonia solution in methanol (ammoniated methanol). Immediate evaporation of the ammoniated methanol, to avoid unwanted hydrolysis of the pyrrolizidine alkaloid esters, yielded a residue that was reconstituted in methanol in preparation for HPLC-MS analysis.

Pyrrolizidine alkaloids can be tentatively recognized from the characteristic ions present in their mass spectra (Figure 21.18). With LC-MS involving soft ionization methods, that is, ESI and

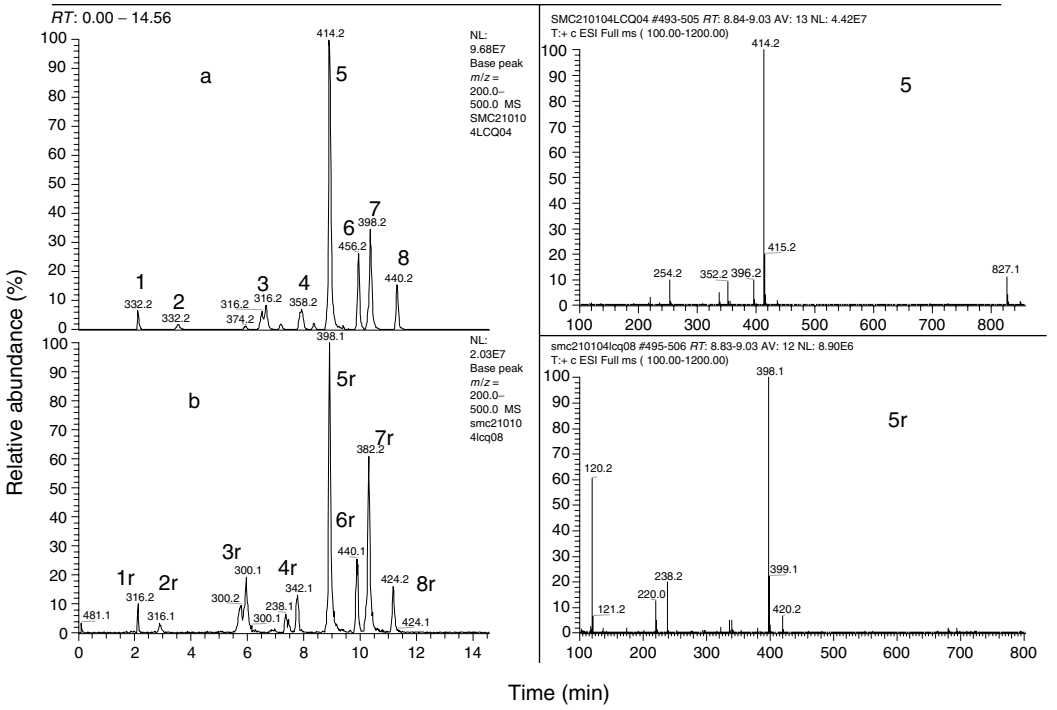


FIGURE 21.19 Confirmation of *N*-oxide character. The reverse-phase HPLC-ESI-MS base ion (*m/z* 200–500) chromatogram of an extract of *Echium plantagineum* petals shows the predominant presence of *N*-oxides (peaks 1–8). Treatment of the same sample with an indigocarmine-based redox resin resulted in "mirrored" peaks (1r–8r) with $[MH]^+$ 16 mass units less and eluting slightly earlier than their respective *N*-oxide peaks. As an example, the mass spectra for peaks 5 (echimidine-*N*-oxide) and 5r (echimidine) show the occurrence of the dimer ions for the *N*-oxides. Also evident is the increased fragmentation for the parent pyrrolizidine alkaloid (peak 5r).

atmospheric pressure chemical ionization (APCI), some form of collision-induced dissociation will need to be included into the MS setup, otherwise there is a lack of fragmentation of the molecular ion adduct.

During the HPLC-MS profiling of the pyrrolizidine alkaloid and *N*-oxide content of the *Echium*- and *Senecio*-derived samples, several apparently undescribed alkaloids were identified and assigned tentative structures based on their MS and MS^n data. Since the new alkaloids were isolated from the plant samples in concert with several known alkaloids, a basic assumption of biogenetic comparability was made in assigning tentative structures. This was an important concession to the usual rigor of structural elucidation, especially the need for NMR spectroscopic analysis, since much of the diversity in pyrrolizidine alkaloids involves isomeric changes not easily discernible using mass spectrometry.

Under the ESI-MS conditions used, the *N*-oxide character of eluted peaks was indicated by the appearance of a significant (5–10% relative abundance) dimeric molecular ion adduct ($[2M + H]^+$) that was absent, or of very low abundance, in the ESI mass spectrum of the parent tertiary pyrrolizidine alkaloids (Figure 21.19).[58–60] To confirm the suspected *N*-oxide character, a methanolic solution of the extracts was mixed with indigocarmine-based redox resin for about 2–4 h at 37°C and then reanalyzed using HPLC-ESI-MS. The formation of new peaks, usually eluting slightly earlier than the putative *N*-oxides, with molecular ion adducts 16 Da less than the *N*-oxides and no evidence of dimeric molecular ion adducts, strongly supported the pyrrolizidine alkaloid/*N*-oxide

relationship (Figure 21.19). As an example, the mass spectra of echimidine-*N*-oxide (peak 5, Figure 21.19) and its reduced partner, echimidine (peak 5r, Figure 21.19), are shown. The $[MH]^+$ ions (*m/z* 414 and 398) are clearly observed as the base ion peaks. A dimer ion peak at *m/z* 827 is observed for echimidine-*N*-oxide (peak 5) while the parent base (echimidine, peak 5r) exhibits far more fragmentation which is consistent with the characteristic patterns shown in Figure 21.18.

21.5.2.1 *Echium plantagineum* and *Echium vulgare*

E. plantagineum and *E. vulgare* are both of European origin but have become opportunistic weeds in other parts of the world with implications for livestock health, welfare, and productivity, as well as human health implications via the presence of their alkaloids in honey and other food products.

HPLC-MS analysis of *E. plantagineum* revealed *N*-oxides of the expected major pyrrolizidine alkaloids but has also revealed some minor pyrrolizidine-*N*-oxides previously unreported for *E. plantagineum* or that are apparently undescribed in the literature. A higher proportion of acetylated pyrrolizidine-*N*-oxides was observed in the flower heads relative to the leaves. The acetylated nature of pyrrolizidine alkaloids/*N*-oxides was indicated by the observation of peaks at *m/z* 180 for those acetylated at C7 and the facile loss of acetic acid ($MH^+ - 60$) for those acetylated at C3′ of the esterifying acid on C9. Six pyrrolizidine alkaloids or pyrrolizidine-*N*-oxides that have not previously been reported from *E. plantagineum* were tentatively identified on the basis of mass spectra (especially the MS/MS^n spectra) and biogenetic considerations. Three of these, 3′-*O*-acetylintermedine/lycopsamine (peak 3r, Figure 21.19), leptanthine-*N*-oxide (peak 1 or 2), and 9-*O*-angelylretronecine-*N*-oxide (peak 4), have been reported elsewhere while three others, 3′-*O*-acetylechiumine-*N*-oxide (peak 8), echimiplatine-*N*-oxide (peak 2 or 1), and echiuplatine-*N*-oxide (co-elutes with peak 5), appear unreported from any other source.

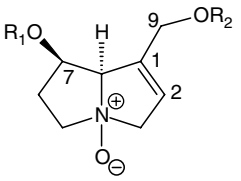
The alkaloidal profile of *E. vulgare* was similar to that of *E. plantagineum* but with the addition of new peaks. Close examination and rationalization of the MS and MS^n data revealed a suite of new alkaloids in *E. vulgare* in which the major *E. plantagineum* alkaloids were further esterified with angelic acid (or one of its configurational isomers) on the C9 esterifying acid (Figure 21.20).

21.5.2.2 *Senecio ovatus* and *Senecio jacobaea*

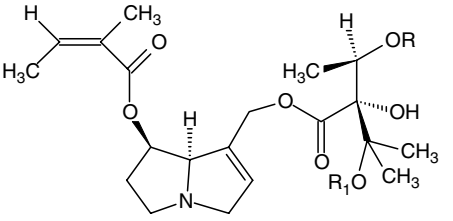
S. jacobaea, unlike *S. ovatus*, has been extensively studied around the world due to its weed-like propensity and the toxic effect on livestock. The HPLC-ESI ion-trap MS profiles for extracts of both plants (Figure 21.21) showed the presence (peaks a–g for *S. jacobaea* and 1–7 for *S. ovatus*) of pyrrolizidine-*N*-oxides.[61]

The mass spectrometric data of the major peak (peak a) in the profile of *S. jacobaea* (Figure 21.21b) were consistent with erucifoline-*N*-oxide (MH^+, *m/z* 366; $[2M + H]^+$, *m/z* 731, Figure 21.22). This and the absence of jacobine indicated that the *S. jacobaea* profiled was an erucifoline chemotype, in contrast to a jacobine chemotype.[62,63] The *N*-oxides of seneciphylline (peak d, MH^+, *m/z* 350, Figure 21.21 and 21.22) and senecionine (peak e, MH^+, *m/z* 352) were readily identified by their mass spectra and comparison with authenticated standards. The other peaks have not been unequivocally identified but all have mass spectra indicative of pyrrolizidine-*N*-oxides. For example, peaks b and c, both with an MH^+ at *m/z* 368, could correspond to the known alkaloids eruciflorine and any one of the hydroxylated senecionine-type alkaloids. The ESI-MS and MS/MS data for the latest eluting peak (peak g, MH^+, *m/z* 408) strongly supported an acetylated derivative of erucifoline-*N*-oxide (peak a). Pyrrolizidine alkaloid character was also indicated for the minor, unlabeled peaks in Figures 21.21a and 21.21b.

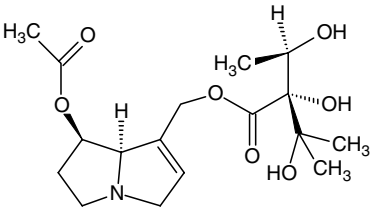
In contrast to *S. jacobaea*, very little research has been reported on *S. ovatus* (otherwise referred to *S. fuchsii* or *S. nemorensis*[64]). Consistent with the literature reports of pyrrolizidine alkaloids from *S. fuchsii*,[64] analysis of the HPLC-ESI-MS and MS/MS^n profiles (Figure 21.21a) and mass spectra data[61] clearly identified the presence of 1,2-dehydropyrrolizidine alkaloids (fragment ions



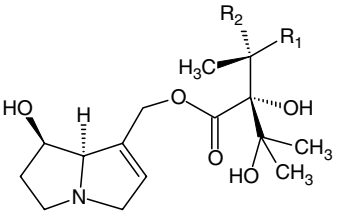
Pyrrolizidine-*N*-oxides



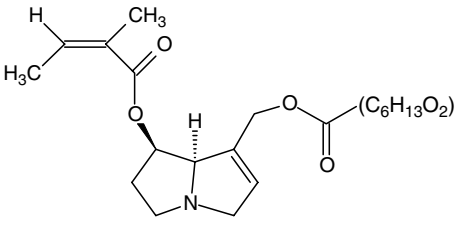
R = R_1 = H: echimidine
R = $COCH_3$, R_1 = H: 3′-*O*-acetylechimidine
R = H, R_1 = $COCH_3$: 5′-*O*-acetylechimidine*



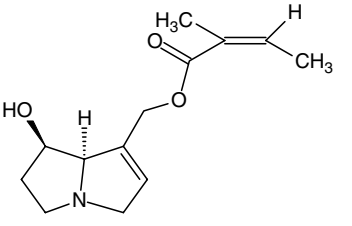
Uplandicine



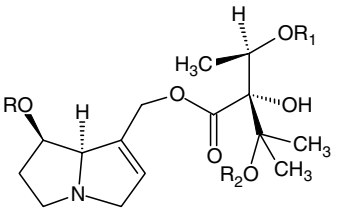
R_1 = OH, R_2 = H: leptanthine
R_1 = H, R_2 = OH: echimiplatine*



Echiuplatine*



9-*O*-Angelylretronecine



R_1 = angelyl, R_2 = H: 3′ esterification or
R_1 = H, R_2 = angelyl: 5′ esterification
R = H: vulgarine*
R = $COCH_3$: 7-*O*-acetylvulgarine*
R = angelyl: echivulgarine*

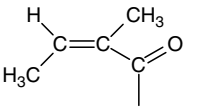
where angelyl = 

FIGURE 21.20 Structures of pyrrolizidine alkaloids identified using HPLC-ESI-MS in extracts of *Echium plantagineum* and *Echium vulgare*. The structures marked with an "*" are based on MS data and are tentative until confirmed using more rigorous structural elucidation approaches. The structures enclosed within the box are the new suite of alkaloids identified in *E. vulgare*.

at *m/z* 120, 138, 220) and their 1,2 saturated, pyrrolizidine analogs (fragment ions at *m/z* 122, 140, 222). Peak 7 was shown to be sarracine-*N*-oxide (Figure 21.22) and peak 6 was consistent with its 1,2-dehydro analog, triangularine-*N*-oxide (Figure 21.22). The MS and MS/MS data for peaks 4 and 2 were consistent with novel hydroxylated sarracine-*N*-oxides. Peak 4 is most likely the

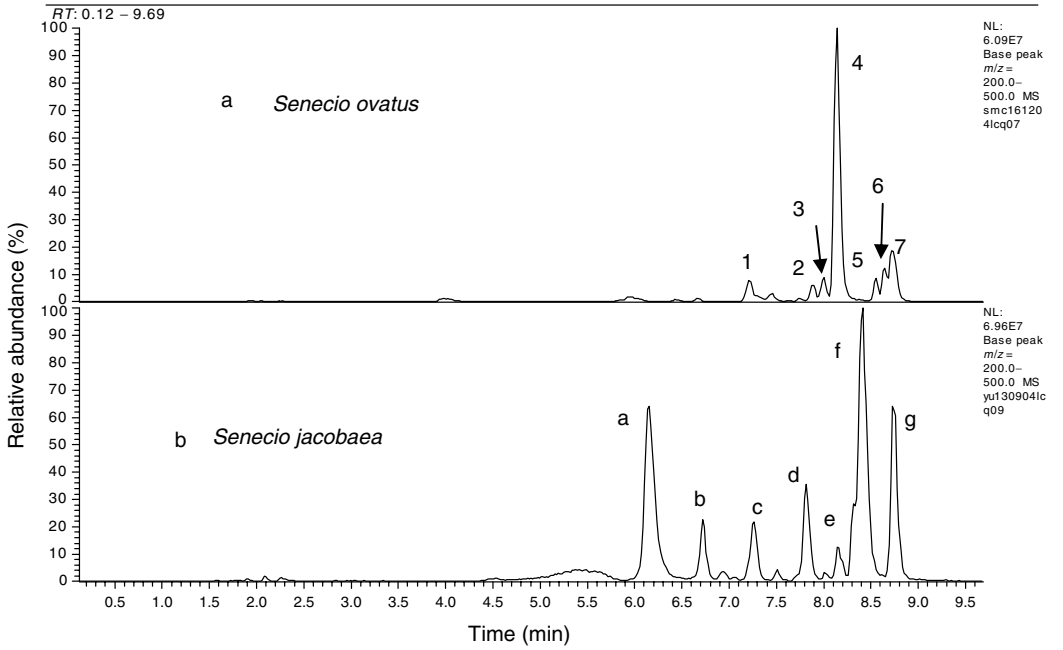
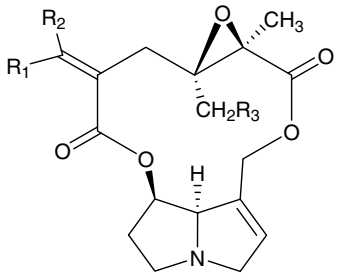
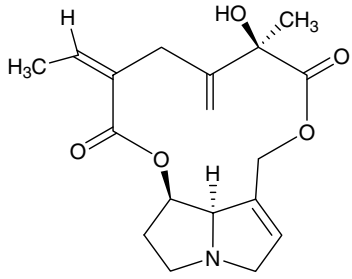


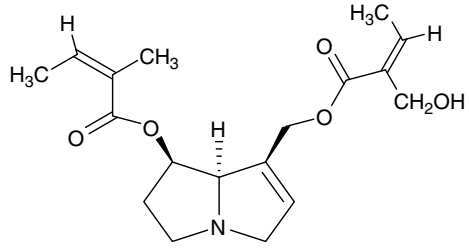
FIGURE 21.21 HPLC-ESI-MS base ion (*m/z* 200–500) chromatograms of SPE-treated extracts of: (a) *Senecio ovatus* and (b) *Senecio jacobaea*. Peaks (the number or letter labels are referred to in the text) were identified by comparison with authenticated standards and analysis of MS fragmentation data including MS^n.



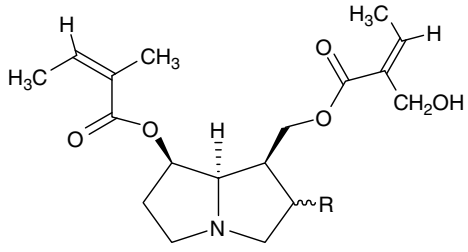
$R_1 = CH_3$, $R_2 = H$, $R_3 = H$: *Z*-erucifoline
$R_1 = CH_3$, $R_2 = H$, $R_3 = COCH_3$: acetyl-*Z*-erucifoline



Seneciphylline



Triangularine



R = H: sarracine
R = OH: hydroxysarracine

FIGURE 21.22 Some structures of pyrrolizidine alkaloids identified in extracts of *Senecio ovatus* and *Senecio jacobaea*.

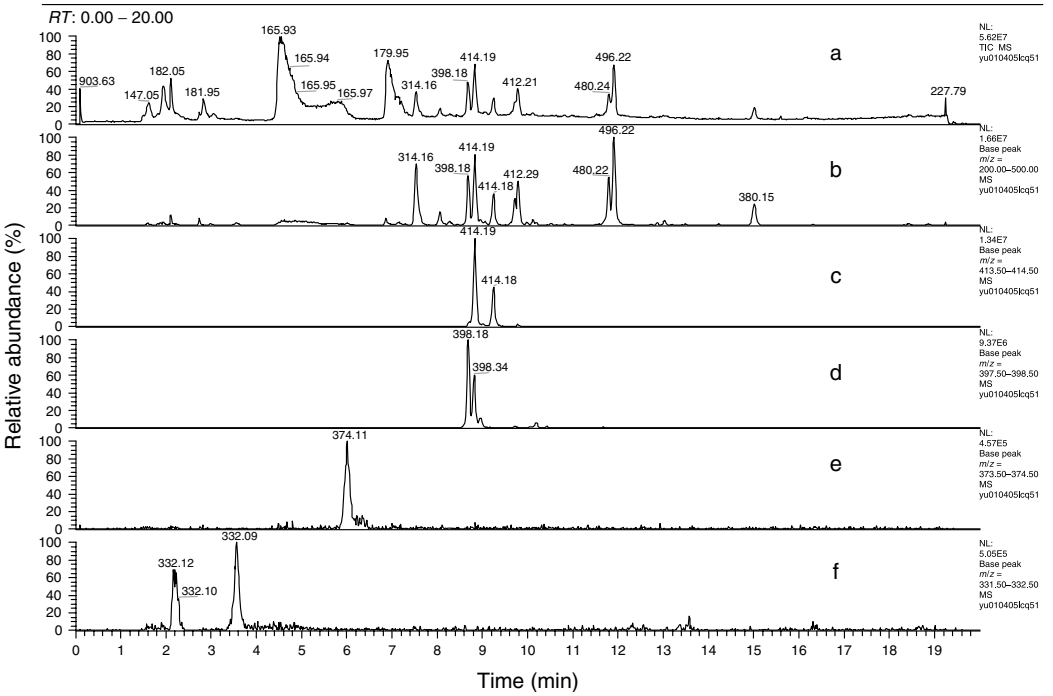


FIGURE 21.23 Reverse-phase HPLC-ESI-MS ion chromatograms of the strong cation exchange solid phase extract of an *Echium vulgare*-derived honey. (a) Total ion chromatogram, (b) base ion (*m/z* 200–500) chromatogram, and (c–f) reconstructed ion chromatograms displaying *m/z* 414, 398, 374, and 332, respectively.

2-hydroxysarracine-*N*-oxide (Figure 21.22) while the hydroxylation in peak 2 appears to be on the C9 esterifying acid. These compounds will require isolation and more rigorous structural identification involving NMR spectroscopy.

21.5.3 Detection and Quantitation in Honey

Honey, produced by bees that forage on the pyrrolizidine alkaloid–producing plants, also contains the pyrrolizidine alkaloids, sometimes in far exceeding those permitted in some countries in food and herbal products.[55,59] This detection of the pyrrolizidine alkaloids is just an example of how HPLC-MS profiling can be used to seek and monitor the presence of bioactive natural products in various matrices.

Briefly, the honey sample is diluted with aqueous sulfuric acid, filtered, and applied to SCX SPE column. The captured alkaloids are eluted and analyzed using the same SPE and HPLC-MS (and MS^n) techniques in much the same way as the plant extracts (Section 21.5.2) resulting in ion chromatogram profiles that can be quantitated for the pyrrolizidine alkaloids. Figure 21.23 shows the total ion chromatogram (a), and various reconstructed ion chromatograms (b–f), of an SCX SPE-treated *E. vulgare*-derived honey sample. The pyrrolizidine alkaloids and the corresponding *N*-oxides were consistent with those extracted from the plant itself (Section 21.5.2.1).[59] Figure 21.23b demonstrates selective display of the pyrrolizidine alkaloids (base ion range of *m/z* 200–500) from the bulk of the coextractives. Figures 21.23c and 21.23d show the utility of the reconstructed ion chromatograms to resolve overlapping peaks, while Figures 21.23e and 21.23f demonstrate the detection of minor pyrrolizidine alkaloids in the extract.

21.6 PERSONAL INSIGHT AND FUTURE DIRECTIONS

This chapter has described the detection, isolation, and structural identification of bioactive natural products that are primarily recognized for their mammalian toxicity and adverse effects on livestock management. However, the "oft-quoted" Theophrastus von Hohenheim (Paracelsus) reminds us that "All things are poison and nothing (is) without poison; only the dose makes that a thing is no poison". Therefore, as with many of the medicinally used bioactive natural products, there may be therapeutically valuable dose ranges for these "toxins". Indeed, other species-dependent activities may be "masked" by the dominant mammalian toxicity that stimulated the search. For example, the toxic cucurbitacins isolated from *S. kingii* (Section 21.2) may have useful insecticidal activity, or indeed many other useful activities.

There will always be a need to fully identify the natural products that lead to intoxication of livestock and humans. The isolation and structural elucidation requirements will continue to be met by techniques described in various chapters of this book (such as HPLC, NMR, MS, and combinations thereof) but it will be the method of detection and the resultant bioassay systems that will need to change. It will not be acceptable to use large numbers of animals for *in vivo* studies, and the restrictions on the unavoidable use of animals will become more stringent.

The use of cultured hepatocytes as an *in vitro* bioassay for the attempted detection of the liver toxins that cause ABLD (Section 21.3) is a step in this direction but the validity of this approach, not only for the specific case of ABLD but also for hepatotoxicities in general, is yet to be confirmed. Other cell lines could also be used in the "real-time" OptiCell system. Proteomic and genomic approaches, involving the detection of specific biomarkers of effect (toxic or otherwise) within an animal species, will also become part of the arsenal of techniques to be applied to the detection and guided isolation of bioactive natural products.

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