Bioactive Compounds from Natural Sources

Isolation, characterisation and biological properties

Edited by
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Also available as a printed book
see title verso for ISBN details

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LONDON AND NEW YORK

First published 2001
by Taylor & Francis
11 New Fetter Lane, London EC4P 4EE

Simultaneously published in the USA and Canada
by Taylor & Francis Inc,
29 West 35th Street, New York, NY 10001

Taylor & Francis is an imprint of the Taylor & Francis Group

This edition published in the Taylor & Francis e-Library, 2004.

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British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

Library of Congress Cataloging in Publication Data
Bioactive compounds from natural sources : isolation, characterisation, and biological properties / edited by Corrado Tringali,
p. cm.
"Simultaneously published in the USA and Canada."
Includes bibliographical references and index.
ISBN 0–7484–0890–8 (alk. paper)
1. Pharmacognosy. 2. Natural products. I. Tringali, Corrado, 1950–
RS160 .B55 2000
615'.19—do21

00–033765

ISBN 0-203-21231-2 Master e-book ISBN

ISBN 0-203-26972-1 (Adobe eReader Format)
ISBN 0-7484-0890-8 (Print Edition)

To Graziella and Paolo

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Preface

In recent years, a renewed interest in obtaining biologically active compounds from natural sources has been observed, notwithstanding the impressive progress of new competing methodologies, as for example, combinatorial chemistry and high-throughput screening or genetical engineering. Contributing to this world-wide attention towards formulations based on natural products are their low or absent toxicity, their complete biodegradability, their availability from renewable sources, and, in most cases, their low-cost if compared with those of compounds obtained by total chemical synthesis. In developed countries this could be connected with the trend favourable to the so-called 'sustainable development', and to some extent with the observed decline of patent applications in Organic Chemistry, paralleled by the rise of Life Sciences applications. This 'may reflect the switch in interest of the chemical industry from synthetic to biologically derived molecules and processes' (A. Abbott, 1995, *Nature 375*, 619). In developing countries, this is sustained by the search for biologically active compounds obtainable from locally available plants, particularly with a view to reducing public health costs which have significantly been raised due to acquisition of synthetic drugs from industrialised countries. The study of active principles involved in Traditional Medicine treatments can also lead to an improvement of these remedies.

A further drive to the study of compounds obtainable from natural sources is the increasing consciousness that destruction or severe degradation of rain forests and other wild habitats, including seas and oceans, will unavoidably result in the loss of unexamined species and consequently of potentially useful compounds. In fact, individual plant species may contain over one thousand chemical substances and only a minor fraction of the estimated total of 250,000 to 300,000 plant species has been studied for biomedical application; on the other hand, the marine ecosystem is still, to a large extent, unexplored. Thus, the urgent need for protection of biodiversity is, at molecular level, a need for protection of the chemical diversity, that is the variety of natural 'libraries' of compounds not yet identified and characterised.

Owing to this renewed attention to pharmaceuticals, agrochemicals and nutraceuticals (functional foods) obtained from natural sources, the study of bioactive secondary metabolites, traditionally carried out mainly by chemists, has increasingly attracted the attention of pharmacologists, biologists, botanists, agronomists, etc., stimulating cooperative work. This book is aimed at reinforcing the interdisciplinary approach to the study of bioactive natural products, hopefully suggesting new research projects to scientists already involved with bioactive natural products and possibly attracting further researchers to this field.

The book includes chapters focused on general methods, such as that on screening methods in the search for pharmacologically active compounds (Chapter 1) and the

surveys on modern HPLC hyphenated techniques (Chapter 2) or about NMR methods in the structural elucidation (Chapter 3). These are followed by more specific chapters, focused on main topics in the research field of bioactive natural products, with emphasis on the biological properties of the compounds cited. The importance of ethnobotanical approach is exemplified by Chapter 4, discussing studies on Mexican medicinal plants. The growing interest in cancer chemoprevention is represented here by Chapter 5, focused on flavonoids. Chapter 6 presents a detailed review of important anti-tumor drugs obtained from plant metabolites and includes examples of chemical synthesis of bioactive natural products or their analogues. The recent claim on the anti-tumor properties of paclitaxel (taxol) and the interest in related compounds suggested the inclusion of Chapter 7. Other surveys on compounds displaying important pharmacological activities are presented in Chapters 8 (anti-HIV), 9 (Antioxidative), 10 (Antimalarial) and 11 (Antiinflammatory).

Chapter 12 is devoted to fungal phytotoxins, of main agrarian and forestall interest. A further attention to the agronomic field has been given in Chapter 13 – treating of limonoids – a class of compounds known for their properties against insects.

The last three chapters have been devoted to marine metabolites. Research on secondary metabolites from marine organisms, stimulated by the discovery of a prostaglandin derivative in gorgonians in 1968, has showed an impressive development to date, affording new lead compounds for pharmaceutical industry as well as intriguing knowledge on the chemical ecology of marine habitat. As representative examples of this research field, immunomodulating marine glycolipids are discussed in Chapter 14, and surveys of bioactive compounds from marine opistobranchs and Japanese soft corals are presented respectively in Chapters 15 and 16.

We hope that this work can offer an overview on the modern methodology in the search for bioactive compounds from natural sources, and will be useful to advanced students and research scientists from various disciplinary sectors related to natural products.

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CHAPTER

1

Biological Screening Methods in the Search for Pharmacologically Active Natural Products

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Contents

1.1 INTRODUCTION

Natural products have served as an important source of drugs since ancient times and about half of the useful drugs today are derived from natural sources. Chemodiversity in nature, e.g. in plants, microorganisms and marine organisms, still offers a valuable source for novel lead discovery, but rapid identification of the bioactive compounds of natural product mixtures remains a critical factor to ensure that this tool of drug discovery can compete with recent developed technologies such as chemical compound libraries and high-throughput screening of combinatorial synthetic efforts. Rapid screening of natural product mixtures requires the availability of a library of reference of natural compounds and methods for simple identification of putative lead structural classes avoiding, to a large extent, the potential for false-positive results. The coupling of chromatographic methods such as high pressure liquid chromatography (HPLC) with diode array detection, mass spectrometry (MS) or nuclear magnetic resonance spectroscopy (NMR) or, and with, on-line bioactivity assays, is an important tool for high throughput screening of natural product mixtures. The introduction of a dereplication step after extraction by using a reproducible preseparation method would enable the rapid elimination of false positives (Verpoorte, 1998). The effective use of automated procedures and databases in the isolation, identification and biological profiling of bioactive compounds from natural sources will be the best guarantee to the continued discovery of novel chemotypes from nature (Hook *et al.*, 1997).

Structure-activity studies of these leads, preferentially combined with computer-graphic model building, should result in molecules with optimal activity and bioavailability, fewer side effects and an acceptable therapeutic index and, consequently in good candidates, for the development to new drugs.

1.2 ROLES OF BIOASSAYS

It is self-evident that in order to exploit natural product mixtures fully, an endeavour should be made to design as many screening programmes as possible. These screening programmes will of course be substantially different, whether they are organized by a major pharmaceutical company, a consortium group consisting of several university research groups, all or not in combination with a research group of a large pharmaceutical company, or by a small pharmaceutical firm or an individual university research group. It is therefore necessary to put the bioassays, to be discussed, in the context of their usage before one can evaluate them or understand their benefits and weaknesses.

According to Suffness and Pezzuto (1991) four major roles of bioassays can be distinguished, i.e. prescreens, screens, monitors and secondary testings. In a prescreen a bioassay is applied to large numbers of initial samples to determine whether or not they have any bioactivity of the desired type. Such bioassays must have high capacity, low cost,

and must give rapid answers. They need not be quantitative. A bioassay in a screen is used to select materials for secondary testing, whereas, in a monitor, a bioassay is used to guide fractionation of a crude material towards isolation of the pure bioactive substances. It must, therefore, be fast and cheap, have high capacity, and be readily available to the phytochemist. In the secondary testing, lead compounds are evaluated in multiple models and test conditions to select candidates for development towards clinical trials. Secondary testing is consequently characterized by a low capacity and expensive and slow bioassays.

Not all screening programmes contain separate prescreens and screens, since if the screening assay has enough capacity and is selective enough to reduce actives to a manageable number for the secondary testing to follow, prescreens are not needed. The monitor may be the same as the prescreen or the screen or may be a bench-top bioassay, which can be carried out using simple facilities in a chemistry laboratory. Special considerations should be taken into account for screening and/or monitoring plant extracts. The methodology should be adaptable to highly colored, tarry, in water poorly soluble, and chemically complex materials. Besides general requirements, such as validity, lack of ambiguity, accuracy, reproducibility, simplicity and reasonableness of cost, the bioassays should be highly selective to limit the number of leads for secondary testing, highly specific to eliminate false positives and highly sensitive to also detect low concentrations of active compounds (Vanden Berghe and Vlietinck, 1991).

Most of the aforementioned requirements are better met by *in vitro* testing, so that in most screening programmes the typical pattern is that *in vitro* screens feed into *in vivo* tests, the latter mostly being introduced during the secondary evaluation. In all cases, however, a screening process must reduce the test substances for secondary evaluation to a manageable size, but hopefully not so few that no useful development candidates come out. The stage at which *in vivo* tests will be introduced, largely depends on the hit rate of the screen. If there are a substantial number of actives, *in vivo* testing will be early, whereas if actives are rare, they may go to final isolation and structure elucidation prior to *in vivo* testing (Suffness and Pezzuto, 1991).

The concept of a hit rate is, however, a difficult issue in many pharmaceutical companies. If a hit rate is set too high, a plethora of actives will result which will have to go through a second screening stage, i.e. a successive combination of a subcellular and a cellular assay or vice-versa. If the hit rate is set too low, insufficient lead structures for isolation and structure-activity studies may be generated (Cordell, 1995).

1.3 CLASSIFICATION OF BIOASSAYS

The methods for the detection of biological activity of natural product mixtures can best be divided into two groups for screening purposes: general screening bioassays and specialized screening bioassays. Depending on the aims of the screening programme, either

a general screening which can pick up many different effects, or a specific assay which is directed at finding some effect against a specific disease, has to be performed. A broad screening bioassay is probably most useful if one is randomly screening chosen organisms for any kind of pharmacological activity. The alternative to using broad screening would be the setting up of a battery of specific test methods, which is cumbersome and expensive. Another drawback of using a general test for screening and monitoring is that one does not know, until the active compound has been isolated, if the work was worth doing. Since, in most phytochemical laboratories engaged in the bioassay-guided isolation of actives from natural product mixtures no complex bioassays can be used, efforts have been made to introduce single, inexpensive 'front-line' or 'bench-top' bioassays for the rapid screening of extracts and fractions. Care must be taken in the interpretation and predictive ability of these screening bioassays, but in general, they provide interesting preliminary information on the pharmacological potential of the plant extracts under study (Ghisalberti, 1993). Whereas primary screening bioassays can be applied in-house by chemists, pharmacognosists, botanists and others, who lack the resources or expertise to carry out more elaborated bioassays, many specialized screening bioassays have been, or are being, developed; however, they are increasingly sophisticated and require the skills and expertise of biologic scientists.

Specialized screening bioassays can be subdivided according to the target organisms which are used in the model (Hostettmann *et al.*, 1995). These can be lower organisms, e.g. microorganisms, insects, molluscs, protozoa, helminths, isolated subcellular systems, e.g. enzymes, receptors, organelles, isolated intact cells of human or animal origin, isolated organs or vertebrates, or whole animals.

In any case, specialized bioassays have to be relevant which means they should predict the intended therapeutic indications. To be relevant or 'correlational' a bioassay has to fulfil some basic criteria. Firstly, the bioassay must be sensitive in a dose-dependent fashion to standard compounds that are known to possess the desired therapeutic property. Secondly, the relative potency of known active agents in the bioassay should be comparable to their relative potency in clinical use. Thirdly, the bioassay should be selective, i.e. the effects of known agents in this therapeutic indication should be distinguishable from effects of drugs for other indications (Vogel and Vogel, 1997).

It is clear that the degree of relevance increases from subcellular systems (molecular assays) to cellular systems (cellular assays), to organs up to conscious animals and human volunteers. Although considerable discussion is going on about the necessity of animal experiments, it is without any doubt that they are necessary for the discovery and evaluation of drugs. However, they should be performed only if they are necessary and well conceived (Williamson *et al.*, 1996).

Molecular bioassays, which use isolated subcellular systems, have some contrasting characteristics with cellular bioassays which use intact cells. The primary feature of molecular bioassays is their high specificity. It is possible to find a small number of new

compounds with a specific activity in a selective way in a relatively short period of time using a high capacity receptor binding or enzyme inhibition assay. The hit rate will be quite low, however, and a large number of diverse samples need to be screened. All compounds working by a mechanism unrelated to the assay will be missed, whereas agents that do not enter the cells, or are rapidly metabolised, will be recorded as false positives. The cost of screening, however, is low but the cost of the large number of required samples must be considered. This type of screening is ideal, in the first step, in testing a specific hypothesis about the potential of agonists or antagonists of a particular molecular target to demonstrate pharmacological activity (Suffness and Pezzuto, 1991).

1.4 SURVEY OF BIOASSAYS

1.4.1 General screening bioassays

1.4.1.1 Broad screening bioassays

Broad assaying procedures can be performed by Hippocratic screening (Malone, 1983) or by the use of the isolated guinea-pig ileum (Samuelson, 1991; Vlietinck *et al.*, 1991). The Hippocratic method is performed on intact rats and involves observation of about 30 parameters at 3- to 5-dose levels. It is a time-consuming and expensive method, which requires much experience in observation and comparatively large amounts of test substances, but the screening of a wide variety of biological activities is possible. The large amount of extract and the time factor makes the method unsuitable for monitoring the isolation of an active component from plant extracts. Substituting mice for rats and observation of only the most pronounced effect in a limited number of animals could potentially overcome the drawback of using the Hippocratic screening for monitoring purposes (Samuelson, 1991).

The isolated organ method, using, e.g. guinea pig ileum for the observation of contraction or inhibition of contraction, has been used as a non-specific screen for pharmacological effects of crude plant extracts by different research groups. Two tests are performed for each extract. First, the extract's ability to contract the ileum is tested. In a second test, the ileum is induced to contract by electrical stimulation and the extract is tested for ability to inhibit these contractions (Figure 1.1a). As the ileum is a piece of smooth muscle, innervated by many different nerve systems, all of which can be electrically stimulated at the same time, a positive response can indicate many different mechanisms for the pharmacological activity of the extract. The most pronounced advantage of this test is that it is very simple and quick to perform and that it requires only small amounts of substance to be tested. The test is very appropriate for monitoring a subsequent procedure for isolation of pharmacologically active compounds. Although some information can be obtained by skilful application of known agonists and antagonists, the major drawback of being rather non-specific is still valid, so that

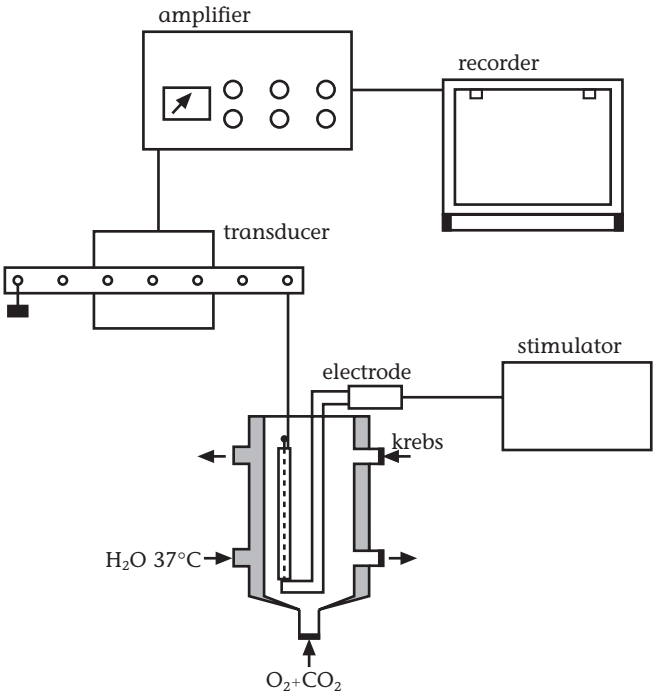


Figure 1.1a: Isolated organ bath for coaxial stimulation experiments.

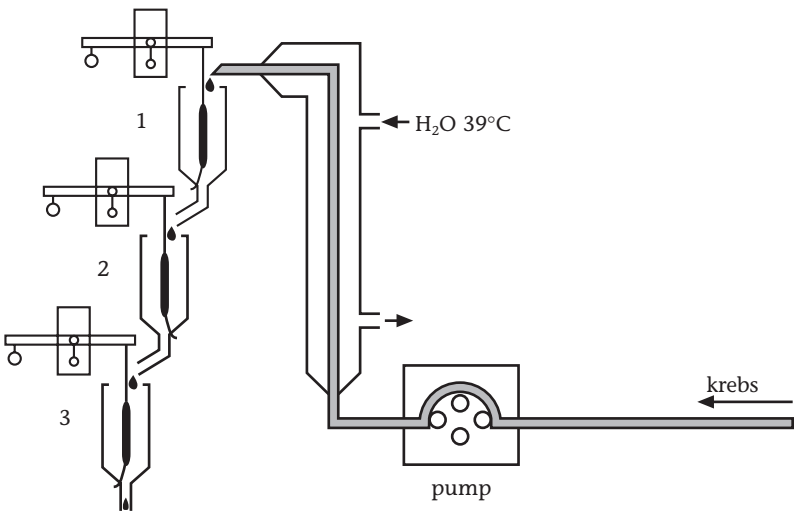
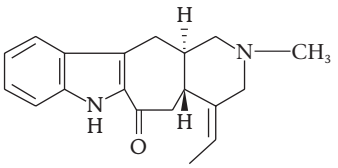
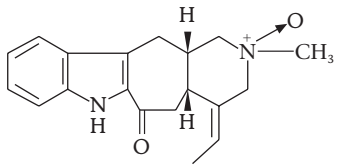


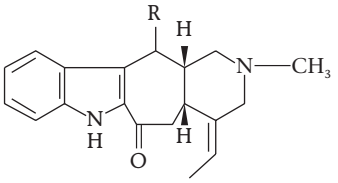
Figure 1.1b: Superfusion technique using a cascade of isolated organs.



16-epimethuenine



methuenine-*N*-oxide



methuenine R = H, H
6-oxomethuenine R = O

Figure 1.2: Structures of alkaloids isolated from the leaves of *Pterotaberna inconspicua.*

much time can be spent on isolation of compounds which turn out to be of limited pharmacological value (Samuelson, 1991).

We investigated the pharmacological properties of several 2-acylindole alkaloids, isolated from the leaves of *Pterotaberna inconspicua*, which are used in Central Africa to treat hypertension and gastro-intestinal upsets. Cumulative dose-response experiments and coaxial stimulation of the guinea pig ileum in an organ bath showed that methuenine is a non-competitive antagonist against acetylcholine and histamine, whereas 6-epimethuenine behaved as a weak antihistaminic. The other isolated alkaloids 6-oxo-methuenine and methuenine N-oxide were almost devoid of these pharmacological activities (Bakana *et al.*, 1985) (Figure 1.2).

1.4.1.2 Primary screening bioassays

The two most popular primary screening bioassays include the brine shrimp lethality test and the crown-gall tumour inhibition test (Ghisalberti, 1993; McLaughin, 1991). The first technique is an *in vivo* lethality test in a tiny crustacean, the brine shrimp (*Artemia salina*). Since its introduction in 1982 (Meyer *et al.*, 1982), this test has been used for the isolation of *in vivo* active antitumour agents and pesticides produced by plants. It can, however, also be used to evaluate plants for different pharmacological activities, taking into account the basic premise that pharmacology is simply toxicology at a lower dose. Toxic substances might indeed elicit, at lower non-toxic dose, interesting pharmacological effects (McLaughin, 1991).

The second technique is the inhibition of the development of crown gall tumours on

discs of potato tubers (Ferrigni *et al*., 1982). Crown-gall is a neoplastic disease induced by the gram-negative bacterium, *Agrobacterium tumefaciens*, a bacterium known to infect a number of crop plants, and is due to the transfer of a tumour-inducing plasmid from the bacterium in the plant genome. The bioassay is fairly accurate in predicting 3PS (P388) *in vivo* murine antileukemic activity. It is not meant to replace the P388 assay, but it is a convenient, rapid, inexpensive, safe and statistically reliable prescreen for 3PS antitumour activity. The assay also circumvents mouse toxicity, which is an inherent disadvantage of the 3PS assay (McLaughin, 1991). Another primary bioassay which is useful for determining which substance should be investigated for *in vivo* antineoplastic activity is the starfish or sea urchin assay. The eggs of the starfish, *Asterina pectinifera*, and the sea urchin, *Strongylocentrotus purpuratus*, have cell membranes permeable to a variety of substances. Exposure of the fertilized eggs to different antineoplastic agents will lead to different outcomes, which will allow to detect DNA and RNA synthesis inhibitors, microtubuli assembly inhibitors, and protein synthesis inhibitors. The sea urchin assay appears to be less selective than the starfish egg assay and relatively insensitive to a series of antineoplastic agents (Ghisalberti, 1993).

Other primary screening bioassays involve the detection of antibacterial and antifungal activities. There are a number of simple tests for antibiotic activity that can be carried out with simple equipment and a minimum of microbiologic expertise. 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. The introduction of the microtitre plate, containing, e.g. 96 holes, allows the simultaneous testing of three dilutions of one or two extracts against not less than 24 or 12 microorganisms, respectively (Figure 1.3) (Vanden Berghe and Vlietinck, 1991).

Representative microrganisms responsible for human infections of significance can be chosen for screening. A prototype of a microbial battery for screening plant extracts consists of one or more representatives of the different groups of bacteria, including

	Different microorganisms												
Extract dilution	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
control	C	C	C	C	C	C	C	C	C	C	C	C	A
1/2													B
1/8													C
1/32													D
control	C	C	C	C	C	C	C	C	C	C	C	C	E
1/2													F
1/8													G
1/32													H

Figure 1.3: Scheme of a microtitre plate used in the agar dilution antimicrobial test method.

gram-positive and gram-negative cocci and rods, and one or more representatives of yeasts, spore-producing fungi, and dermatophytes.

Some simple and quick bioassays monitor the inhibitory effects of extracts on seed germination. The etiolated wheat coleoptile bioassay has been used, not only to detect plant growth regulating substances, but also to find compounds that have mycotoxic, immunosuppressant and antifungal activity (Kubo, 1989). It is important, however, to be aware of the fact that some compounds that are inhibitory at high concentrations can be growth promoting at lower concentrations, and vice versa. Also herbicidal-, insect-antifeedant, larvicidal- and molluscicidal activities can been determined by simple bioassays, which can function as surrogate assays to isolate bioactive compounds from plant extracts. It should, however, be remembered that primary bioassays provide only preliminary information, which should always be checked in more appropriate specialized bioassays.

1.4.2 Specialized screening bioassays

As described above, after having found a certain type of activity, it will be necessary to study this activity in more detail by using one or several specialized bioassays for screening and/or monitoring purposes. These *in vitro* or *in vivo* tests are more sophisticated than the primary screening bioassays and require the expertise of biochemists or pharmacologists. They can, consequently, only be performed in a multidisciplinary team.

We have classified the many existing specialized screening bioassays according to the target organisms which are used. Some of these bioassays are described in this section.

1.4.2.1 Lower organisms

Numerous research programmes to detect and isolate antibacterial-, antifungal-, antiviral- and antiparasitic compounds from plants and other natural sources have been designed and are performed. Large batteries of bacteria, yeasts, fungi, viruses, insects, molluscs, protozoa, and helminths, are thereby used in a broad range of *in vitro* and/or *in vivo* bioassays.

Relatively few antiviral drugs are available, since they must meet a number of criteria. They must inhibit at least one of the propagation steps of the virus, have a broad range of activity, and not be immunosuppressive. The methods commonly used for evaluation of *in vitro* antiviral activities are based on the different abilities of viruses to replicate in cultured cells. Some viruses can cause cytopathic effects (CPE) or form plaques. Others are capable of producing specialized functions or cell transformation. Virus replication in cell cultures may also be monitored by the detection of viral products, i.e. viral DNA, RNA or polypeptides. Thus, the antiviral test selected may be based on inhibition of CPE, reduction or inhibition of plaque formation, and reducing virus yield or other viral functions (Vlietinck and Vanden Berghe, 1991b).

TABLE 1.1

In vitro antiviral screening assays

Determination of the viral infectivity in cultured cells during virus multiplication in the presence of a single compound (A-S) or a mixture of compounds, e.g. plant extracts (A-M) or after extracellular incubation with a single compound (V-S) or a mixture of compounds (V-M).
[1] *Plaque inhibition assays* Only for viruses which form plaques in suitable cell systems. Titration of a limited number of viruses in the presence of a non-toxic dose of the test substance. Applicability: A-S.
[2] *Plaque reduction assay* Only for viruses which form plaques in suitable cell systems. Titration of residual virus infectivity after extracellular action of test substance(s). Cytotoxicity should be eliminated, e.g. by dilution, filtration etc., before the titration. Applicability: V-S; V-M.
[3] *Inhibition of virus-induced cytopathic effect (CPE)* For viruses that induce CPE but do not readily form plaques in cell cultures. Determination of virus-induced CPE in monolayers, cultured in liquid medium, infected with a limited dose of virus and treated with a non-toxic dose of the test substance(s). Applicability: A-S; A-M.
Virus yield reduction assay Determination of the virus yield in tissue cultures, infected with a given amount of virus and treated with a non-toxic dose of the test substance(s). Virus titration is carried out after virus multiplication by the plaque test (PT) or the 50% tissue culture dose end point test (TCD_{50}). Applicability: A-S; A-M.
[4] *End point titration technique (EPTT)* Determination of virus titer reduction in the presence of two-fold dilutions of test compound(s). Applicability: AS; A-M. This method has been especially designed for the antiviral screening of crude extracts (Vanden Berghe and Vlietinck, 1991).
[5] *Assays based on measurement of specialized functions and viral products* For viruses that do not induce CPE or form plaques in cell cultures. Determination of virus specific parameters, e.g. hemagglutination and hemadsorption tests (myxoviruses), inhibition of cell transformation (EBV), immunological tests detecting antiviral antigens in cell cultures (EBV, HIV, HSV and CMV). Reduction or inhibition of the synthesis of virus specific polypeptides in infected cell cultures, e.g. viral nucleic acids, determination of the uptake of radioactive isotope labelled precursors or viral genome copy numbers. Application: A-S; A-M; V-S; V-M.

A survey of the various *in vitro* antiviral tests and their possible suitability for the antiviral and/or virucidal screening of natural products and their mixtures is presented in Table 1.1. It should be emphasized that the toxic effects of the antiviral agent on the host cells must be considered, since a substance may exhibit an apparent antiviral activity by virtue of its toxic effects on the cells. The cytotoxicity assay on cell cultures is usually done by the cell viability assay and the cell growth rate test, although other parameters such as destruction of cell morphology under microscopic examination or

measurement of cellular DNA synthesis have been used as indicators of compound toxicity (Hu and Hsiung, 1989).

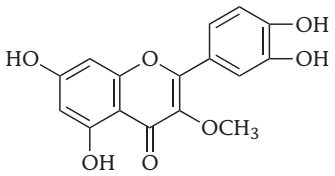
The US National Cancer Institute (NCI) has, in 1987, implemented AIDS-screening and antiviral development programmes in parallel with its long standing anticancer drug programme. The effects of HIV and potential antiviral agents on human CEM T4-lymphocytes are followed by cell survival, which is determined by means of the vital stain XTT (2,3-bis[1-methoxy-4-nitro-5-sulfonylphenyl]-5-[(phenylamino)carbonyl]-2H-tetrazoliumhydroxide), a soluble version of the tetrazolium salt MTT. Dose-response data are produced automatically from the optical densities obtained on the plates in an automatic plate reader using especially developed software. The drug zidovudine (AZT) is used as a positive control for quality control purposes and as a standard for comparisons (Lednicer and Narayanan, 1993). Increasing attention is being focused on inhibitors of several HIV-enzymes such as reverse transcriptase, integrase and protease (De Clercq, 1995).

Animal models for a number of virus infections are available. They are helpful in detecting, not only if the candidate compound is an effective viral inhibitor without inducing viral resistance but also if it has a good bioavailability, resists, and not adversely effects the immune system, and not interferes with the normal metabolic processes of uninfected cells. Some viral infections, such as herpes simplex in guinea-pig and mice and cytomegalo- or poliovirus infections in mice, mimic the natural diseases very closely. These animal models allow continuous observation of the infected animals, and virus spread and pathogenesis at local sites can be studied thoroughly (Sidwell, 1986).

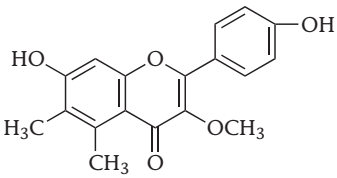
We investigated several plant preparations which are used in traditional medicine against viral diseases. Bioassay-guided isolation of an extract, prepared from the leaves of *Euphorbia grantii*, which is used in Rwanda to treat childhood diseases such as poliomyelitis, afforded several antiviral 3-methoxyflavones. They are active against a wide range of picornaviruses, except Mengo virus, and interfere with an early step in the viral RNA synthesis. Structure-activity studies resulted in the synthesis of 4′,7-dihydroxy-3-methoxy-5,6-dimethylflavone possessing a very high *in vitro* activity against all polio and rhinovirus serotypes tested and inducing no resistance (Figure 1.4) (De Meyer *et al.*, 1991).

Protozoal diseases are a major threat to world health and they include malaria, leishmaniasis, trypanosomiasis, amoebiasis and giardiasis. In addition, cryptosporidosis, pneumocystis and toxoplasmosis are becoming more prevalent in developed countries due to suppression of the immune system.

Two major antimalarial screening tests using the rodent parasite *Plasmodium berghei* in mice, may be used for the screening of plant extracts, the 4-day suppressive test of blood schizontocidal action (Peter's test), and the Rane test of blood schizontocidal activity. Neither of these tests is suitable for the identification of long acting



3-O-methylquercetin (*Euphorbia grantii*)



4',7-dihydroxy-3-methoxy-5,6-dimethylflavone (synthetic analogue)

Figure 1.4: 4'-Hydroxy-3-methoxyflavones with potent antipicornavirus activity: from bioactive substance isolated from *Euphorbia grantii* leaves to potent synthetic analogue.

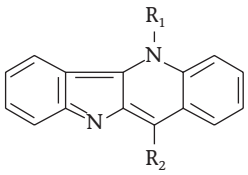
compounds. Useful *in vitro* tests have become available only recently. The inhibition of the incorporation of ^{3}H-hypoxanthine into plasmodia of the human malaria parasite, *Plasmodium falciparum*, can be quantitatively measured in microplates and has been adapted to a semi-automated procedure. The data obtained with this method has been shown to correlate with *in vivo* data obtained with *P. berghei* (Phillipson, 1991).

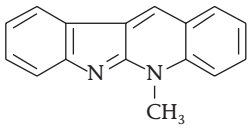
Since *in vivo* testing for amoebiasis is notoriously difficult, unpleasant to perform, expensive, and time consuming, several *in vitro* models have been developed. In their initial tests with *Entamoeba histolytica* Phillipson and coworkers (1995) used flat sided culture tubes and assessment of activity was obtained by counting the amoebae. Subsequently, a more sensitive microplate was developed and amoebal growth was measured by staining with eosin. Similarly, a new microplate assay was developed for determining antigiardial activity *in vitro* utilizing *Giardia intestinalis* and measuring soluble formazon production from a tetrazolium reagent.

Although it is not possible to develop antimalarial and amoebicidal drugs without resort to *in vivo* procedures, the *in vitro* tests which are now available, can be used to select new drug leads from plants. By combining *in vitro* cytotoxicity testing with *in vitro* amoebicidal and antiplasmodial testing, it is possible to obtain some measure of selectivity of action (Phillipson, 1991).

We assessed several extracts and alkaloids from the root bark of *Cryptolepis sanguinolenta in vitro* against *Plasmodium falciparum* (D-6) (chloroquine sensitive strain), K-1 and W-2 (chloroquine-resistant strains). Cryptolepine and its hydrochloride, 11-hydroxycryptolepine and neocryptolepine showed a strong antiplasmodial activity against D-falciparum chloroquine-resistant strains. Quindoline was less active. *In vivo* tests on infected mice showed that cryptolepine, when tested as its hydrochloride, exhibited chemosuppressive activity against *Plasmodium berghei yoelii* and *Plasmodium berghei berghei* (Figure 1.5) (Cimanga *et al.*, 1997).

Schistosomiasis, known as bilharzia, is a parasitic disease, propagated by three species of schistosomes, *viz. Schistosoma mansoni* and *S. japonicum* (intestinal bilharzia) and





	R_1	R_2
Crytolepine base or hydrochloride	CH_3	H
Hydroxycryptolepine	CH_3	OH
Quindoline	H	H

Neocryptolepine

Figure 1.5: Structures of alkaloids with antiplasmodial activity isolated from *Cryptolepis sanguinolenta*.

S. haematobium (urogenital bilharzia). The transmission of schistosomiasis requires the presence of an intermediate host such as a fresh water snail of the genera *Biomphalaria* and *Bulinus*. Plant extracts can be investigated for their toxicity to *Schistosoma mansoni* in mice. Schistosomes are isolated from mice by a simple perfusion technique and collected, after sedimentation, in saline. For counting, adult worms can be recovered on a mesh screen. The livers of the mice are examined for dead worms, and the eggs in the intestines and livers are counted. The overall procedure lasts at least 12 weeks and is obviously not suitable for bioassay-guided isolations (Baldé *et al.*, 1989). The experimental procedure is schematised in Figure 1.6.

1.4.2.2 Isolated subcellular systems

Molecular assays look for activity using isolated systems, including enzymes and receptors. Though already known for many years in pharmacology and frequently used in industrial drug development, in studies of natural products the systematical use of these methods for screening purposes has only started.

Only a limited number of the many existing enzyme assays have been utilized in the screening of extracts (Vogel and Vogel, 1997). Examples of such biochemical test systems include macrophage-associated carboxypeptidases such as trypsin and kallikrein, enzymes of the arachidonic pathway such as cyclooxygenase, 5-lipoxygenase and thromboxane-synthetase, and enzymes of the hypertensive proteolytic cascade such as renin and angiotensin-converting enzyme (ACE) and the genital proteolytic system such as acrosin.

Other enzyme assays, which have been used for the screening of natural substances include monoamine oxidase (antidepressant activity), H^+/K^+-ATP-ase (anti-gastric ulcer activity), polysaccharide degrading enzymes (amylase, glycosidase) and aldose reductase (anti-diabetic activity), HMG-CoA reductase (anti-atherosclerotic activity), 5α-reductase

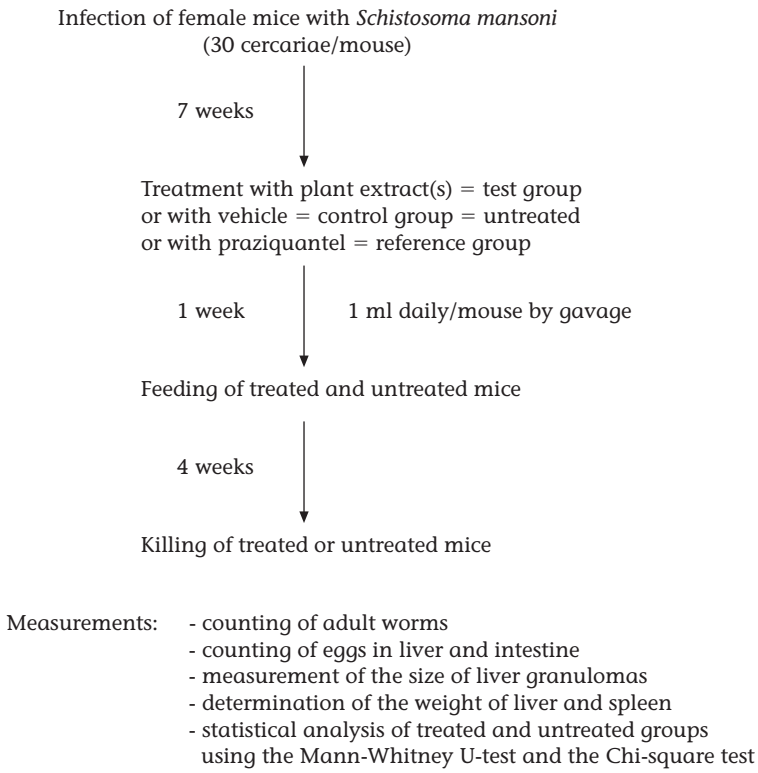


Figure 1.6: Determination of schistosomicidal effects of plant extracts on mice infected with *Schistosoma mansoni*: experimental procedure.

(anti-adrogenic activity), and xanthine oxidase (anti-oxidative activity). The inhibitory activity of the test compounds is mostly determined after chromatographic separation of the enzymatic reaction products by means of UV or fluorescence spectrophotometric detection. An example, representing the aims and the procedure of the assay of trypsin and trypsin-like enzymes, is shown in Figure 1.7.

Many drug discovery programmes have been based on the utilization of radioligand receptor binding technology to identify lead compounds which interact with receptors likely to be important in neuronal, immunological, gastrointestinal, and cardiovascular function/dysfunction. Ligand binding studies may be carried out on whole tissue, on single or dispersed cells, or on cell membranes. Disperse cell preparations can be made from the appropriate cell type, but the availability of such cells may be limited. Therefore, cultured cells may be used, which reduces the necessary number of experimental animals. However, the culture procedure itself is complicated, and may result in some

Trypsin-activation
- debriding agents: cleaning of necrotic wounds, ulcers, abscesses
- liquefaction of tenacious sputum in bronchial disorders such as bronchitis, bronchial asthma and thrombophlebitis

Trypsin-inhibition (prototype of proteases)
- Thrombin-blockers: antithrombotic agents
- Kallikrein-blockers: block the endogenous kinin system: anti-inflammatory agents, treatment of acute pancreatitis, carcinoid syndrome
- Acrosin-blockers: male antifertility agents

Figure 1.7a: Aims of the enzyme assay of trypsin and trypsin-like enzymes.

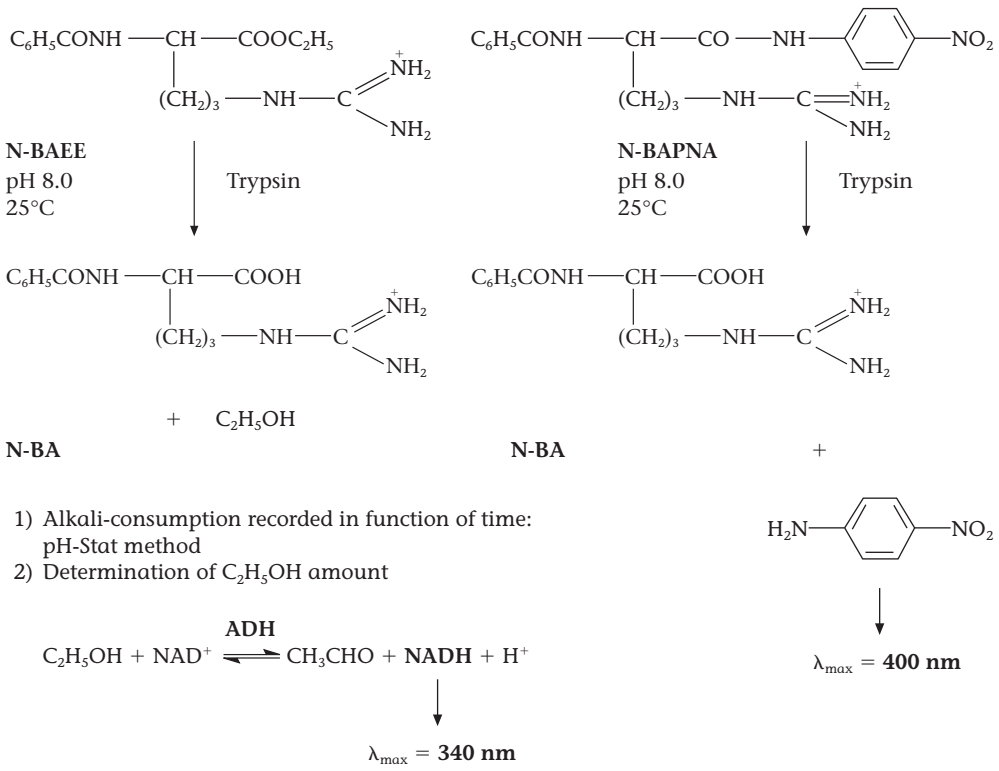


Figure 1.7b: Procedure of the enzyme assays of trypsin and trypsin-like enzymes.

transformation of the cells. Finally, one may use transfected cells, in which the appropriate receptor has been introduced (Coleman, 1997).

The object of ligand binding is to determine the competition between the test molecule and a radio-labelled molecule which has high affinity for the binding site in question. If the test compound has affinity for the binding sites, it will inhibit the degree of binding obtained with the radioligand in a concentration-related fashion, and the concentration of the compound required to inhibit radioligand binding will give some idea of its affinity for the ligand binding sites.

The filtration-based radioligand binding is a simple, rapid, sensitive, and specific technique, which makes it very suitable as bioassay for screening and monitoring of natural product mixtures. Typically, a specific binding of at least 75% is needed for high volume screening and should accurately reflect the interaction between the compound and the receptor, and not be a non-specific binding interaction, for it is important that the assay is run at equilibrium. A drawback with this method is that it is not easy to distinguish whether a compound is an agonist or an antagonist, so that subsequently functional assays in whole animals or with isolated organs should be carried out.

Sweetnam *et al.* (1993) have described the role of receptor binding in drug discovery and illustrated this by presenting the comparative data from 38 different primary assays including receptors for adenosine, catecholamines, amino acids, biogenic amines, channel proteins, acetylcholine, opiates, prostanoids, reuptake sites, and second messengers.

Two screening studies of plant extracts, used in traditional medicine in China and Suriname respectively, for receptor binding activity have recently been published (Zhu *et al.*, 1996; Hasrat *et al.*, 1997a). In both studies the results provide partial scientific support for a number of plant species for their use in traditional medicine for the relief of pain (Zhu *et al.*, 1996), against proteinuria (Hasrat *et al.*, 1997b,c) and for their tranquillizing effects (Hasrat *et al.*, 1997d).

Recently, the on-line coupling of reversed-phase liquid chromatography to a biochemical detection system based on receptor ligand interactions has been realized. The receptor-affinity detection (RAD) is performed using a post-column reaction detection system with open-tubular reaction coils. When combined with structure-activity elucidating methods like (MS) the RAD-system should be able to give two-dimensional spectra on structure and activity. Using a combined LC-RAD/MS method, the biochemical activity and structure elucidation can be performed in one run. It will, however, be a great challenge to implement other biological affinity interactions in LC-RAD systems, e.g. membrane bound receptors and cell-bound receptors, since, up till now, only soluble receptors have been tested in the post-column detection system (Irth *et al.*, 1995; Oosterkamp *et al.*, 1996).

1.4.2.3 Isolated cellular systems

Cellular assays utilizing intact cells of human or animal origin have been used for more than half a century to screen for cytotoxic agents from natural sources. Cytotoxicity, or toxicity to cells in culture, can be subdivided into cytostatic activity, i.e. stopping cell growth (often reversible) and cytocidal activity, i.e. killing cells. A large number of cytotoxicity-based bioassays have been used as prescreens for antitumour and antineoplastic activity. These short-term *in vitro* growth inhibition assays with cultured cells include Walker carcinosarcoma 256, mouse L-120 leukemia, Ehrlich murine ascites tumour, sarcoma 180 and mouse P-388 leukemia cell lines (Geron *et al.*, 1972). While these traditional cell-cytotoxicity assays are indicative for activity in leukemia, lymphoma, or a few rare tumours, their efficacy in finding products with activity in the predominantly occurring slow growing solid tumours of humans is strictly limited. Therefore, the US National Cancer Institute (NCI) has, in 1989, developed a disease-oriented strategy in which an *in vitro* primary screen employing a tumour cell line panel consisting of a total of 60 known tumour cell lines, derived from seven cancer types, i.e. lung, colon, melanoma, renal, ovaria, brain and leukemia, is used (Boyd, 1989). The objective is the discovery of agents that demonstrate selective cytotoxicity with a cell line derived from a single type of primary human tumour. This *in vitro* disease-oriented prescreen can be followed by an *in vivo* screen, since the cells can be carried as solid tumours in athymic mice. Some practical considerations and applications of this *in vitro* anticancer drug discovery screen have recently been reviewed (Boyd and Paull, 1995).

Several mechanism-based *in vitro* bioassays can be designed by analogy with the types of molecular responses mediated by known, clinically effective antitumour agents. Thus, as examples, monitoring effects similar to those known to be mediated by vinblastine (tubulin depolymerization), taxol (tubulin stabilization), camptothecin (topoisomerase inhibition), 2-methyl-9-hydroxy-ellipticinium (topoisomerase II inhibition) and bleomycin (DNA cleavage) are all reasonable avenues towards novel drug discovery. To establish the true efficacy of promising anticancer agents, subsequent evaluation in more advanced testing systems is required, followed by (pre)clinical trials (Suffness and Pezzuto, 1991).

Chemoprevention of cancer may be defined as the deliberate introduction of selected substances into the diet for the purpose of reducing cancer incidence. A large number of plant metabolites may, indeed, have cancer-preventive properties (Verhagen and Feron, 1994). It can thereby be anticipated that anticarcinogens act on any of the transition states between exposure to a carcinogenic agent and the final outcome (disease: cancer). According to Wattenberg (1992) cancer-preventing substances can be classified into three categories according to the phase time in carcinogenesis when they are effective, *viz.* compounds that prevent the formation of carcinogens from precursor substances, compounds that prevent carcinogenic agents from seeking or reacting with critical

target sites in tissues (blocking agents), and agents that act subsequent to exposure to carcinogens, once the initiating damage has been irreversibly done (suppressing agents). With respect to the onset of carcinogenesis initiation, Hartman and Shankel (1990) describe various levels at which antimutagenesis is effected like desmutagenesis (destruction/inactivation of mutagens), interference with error-prone and stimulation of DNA-repair, and several other possibilities.

The establishment of a number of short-term tests for genotoxicity, e.g. chromosome aberration test, micronucleus test has provided the possibility to predict whether or not a substance possesses an intrinsic mutagenicity and thus the potential to affect the initiation phase of carcinogenesis. These assays are then valuable to select potential mutagens. Conversely, these assays can also be used to get an impression of the possible antimutagenic potential of a compound by assessing its modulatory effects on the results of established mutagens in these assays: a decrease in the response of these positive controls may point towards potential antimutagenicity. The tests can be conducted either *in vitro* with prokaryotes or eukaryotes cell systems or *in vivo* with experimental animals. Based upon *in vitro* and *in vivo* data activity profiles of antimutagens can be developed (Waters *et al.*, 1996). The most valuable data on cancer-preventing effects of plant products may come from studies on humans. They can be performed in two ways, *viz.* epidemiology studies based on dietary questionnaires and experimental biomarker research, which links toxicological and epidemiological studies (Verhagen and Feron, 1994).

Several nutritive dietary anticarcinogens include vitamins A, C, E and β-carotene, all well-known antioxidants and/or radical scavengers, which could serve as an explanation for this mode of action. Many experiments have indeed shown that reactive oxygen species (ROS), including radicals and oxidants, play various roles in the development of cancer. ROS can directly damage DNA, activate transcription factors, or active kinases and each of these changes may activate various genes including oncogenes. Gene activation may also be mediated by cytokines released in response to ROS or from phagocytic cells (Kehrer and Smith, 1994).

There is compelling evidence that oxidative stress is implicated in the pathogenesis of many disorders besides cancer, although a consensus for such involvement has been reached only in a few cases. A role for free radicals has been postulated for some cardiovascular diseases such as atherosclerosis and reperfusion injury, several neurological disorders such as Alzheimers' and Parkinsons' diseases, some lung diseases such as fibrosis and emphysema, cataract, rheumatoid arthritis and other inflammatory diseases and also aging.

The finding of new antioxidative leads which are capable of preventing and/or eliminating oxidative stress and development of suitable screening bioassays merits, consequently, high priority and would be very welcomed. A survey on *in vitro* and *in vivo* assays for ROS has been published by Halliwell *et al.* (1992). These techniques

include trappic assays, in which the radicals are allowed to react with a trap molecule to give one or more stable products, which are then measured, or with a spin trap to form a more-stable radical, which is detectable by electron spin resonance (ESR). Other useful screening bioassays are fingerprint assays including the 'DNA fingerprinting' approach, in which the DNA damage that occurs when cells and tissues are subjected to oxidative stress is chemically determined, and the measurement of the end products of lipid peroxidation such as lipid hydroperoxides and certain aldehydes. Measurements of some of the end products of oxidative damage to proteins can be achieved by the protein carbonyl assay. GC, HPLC, HPLC/MS, HPLC/antibody techniques, light emission, fluorescence, and several enzymatic reactions, are the methods used to detect and measure biological lipid peroxidation (Halliwell *et al.*, 1992).

Recently, we investigated the structure-activity relationship of flavonoids as inhibitors of xanthine oxidase and as scavengers of the superoxide radical, produced by the action of the enzyme xanthine oxidase. They could be classified into six groups: superoxide scavengers without inhibitory activity on xanthine oxidase (category A), xanthine oxidase inhibitors without any additional superoxide scavenging activity (category B), xanthine oxidase inhibitors with an additional superoxide scavenging activity (category C) xanthine oxidase inhibitors with an additional pro-oxidant effect on the production of superoxide (category D), flavonoids with a marginal effect on xanthine oxidase but with a pro-oxidant effect on the production of superoxide (category E), and finally, flavonoids with no effect on xanthine oxidase or superoxide (category F). A prototype flavonoid of each category is shown in Table 1.2 (Cos *et al.*, 1998).

Another important field for which isolated cellular systems are used in many screening bioassays is that of immunostimulation. It has to be emphasized that immunostimulation primarily implies stimulation of the non-specific immune system, i.e. stimulation of the functions and thus the efficiency of granulocytes, macrophages, Kupfer cells, monocytes, natural killer cells, complement factors and certain T-lymphocyte popula-

TABLE 1.2
Summary of the classification of flavonoids into six categories according to their inhibition of xanthine oxidase and superoxide scavenging activity

category	inhibition of xanthine oxidase[a]	superoxide scavenging activity[a]	example
A	0	+	(–)-epigallocatechin
B	+	0	baicalein
C	+	+	myricetin
D	+	–	galangin
E	0	–	7-hydroxyflavanone
F	0	0	naringenin

[a]Key: 0, no effect; +, effect; –, pro-oxidant effect

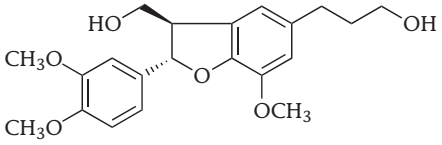
tions. This also includes the stimulation of a series of mediators such as mono- and lymphokines secreted by these cells in response to any induction (Wagner and Jurcic, 1991; Labadie, 1993). Since many recurrent infections and malignant diseases are caused by a decreased number of immune competent cells, it is clear why granulocytes, monocytes, macrophages and T-lymphocytes are the preferred target cells for screening. These cells can be obtained from human donor blood or animal organs. A survey of assays for immunomodulation is described by Wagner and Jurcic (1991).

In vitro tests such as the granulocyte phagocytosis assay (smear test), the chemoluminescence assay, the chemotaxis assay, the lymphocyte proliferation assay, the assay of natural killer activity and the assay for tumour necrosis factor (TNP) production as well as *in vivo* tests such as the carbon clearance assay for phagocytosis have extensively been utilized in the screening and monitoring of active plant compounds. The *in vivo* assay for anticomplement activity has not only a relevance for the processing of antigens but also for inflammatory processes and can be used as a screening or monitoring for anti-inflammatory active plant extracts (Lasure *et al.*, 1994; Cimanga *et al.*, 1995; Huang *et al.*, 1998). Recently, a series of surveys on immunomodulatory agents from plants has been edited by Wagner (1999).

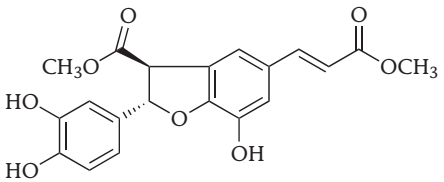
Other *in vitro* assays which have been utilized by our research group to screen and monitor active plant extracts include the inhibition assay of platelet aggregation as experimental model for evaluating the antithrombotic and anti-inflammatory potential of the isolated compounds (Rasheed *et al.*, 1984; Laekeman *et al.* 1985) and the proliferation assay of human umbilical vein endothelial cells (HUVEC) as experimental *in vitro* model for wound healing (Vanden Berghe *et al.*, 1993; Pieters *et al.*, 1993). Bioassay-guided isolation of dragon's blood (Sangre de Drago), a traditional South American drug used for wound healing, ulcers and against cancer, afforded the dihydrobenzofuran lignan 3′,4-O-dimethylcedrusine as active compound. The lignan not only improves wound healing *in vivo* by stimulating the formation of fibroblasts and collagen, but also acts as an inhibitor of cell proliferation (Pieters *et al.*, 1993, 1995). Structure-activity studies of a series of synthetic 2-phenyldihydrobenzofuran lignans showed that methyl (E)-3-[2-(3,4-dihydroxyphenyl)-7-hydroxy-3-methoxycarbonyl-2,3-dihydro-1-benzofuran-5-yl]prop-2-enoate, obtained by dimerization of caffeic acid had the highest activity in a screening panel consisting of 60 human tumour cell lines. These products constitute a new group of antimitotic agents that inhibit tubulin polymerization (Figure 1.8) (Pieters *et al.*, 1999).

1.4.2.4 Isolated organs of vertebrates

Organ-based assays are more and more replaced nowadays as the front-line primary screen, but often retain signification as secondary screens to confirm the results of, e.g. radioligand-binding bioassays and, hence, to assist in prioritization of active extracts or compounds. They represent, consequently, an essential connection between the high



3',4-O-dimethylcedrusin
(Sangre de Drago)



methyl(E)-3[2-(3,4 dihydroxyphenyl)-7-hydroxy-3-methoxycarbonyl-2,3-dihydro-1-benzofuran-5-yl]-prop-2-enoate
(synthetic analogue)

Figure 1.8: Dihydrobenzofuran lignans with wound healing properties and antitumoral activity: from bioactive substance isolated from Sangre de Drago to potent synthetic analogue.

technology of the primary screens and the realities of the pharmacological effectiveness (Cordell, 1995).

Segments of the gastro-intestinal tract or spirally cut strips of vascular tissue are mainly used in the organ bath method for isolated organs. Since the guinea-pig ileum gives contractions to many agonists including acetylcholine, angiotensin, arachidonic acid, bradykinin, histamine (H_1-receptors), prostaglandins (type E), serotonin and substance P, this isolated organ has been used as a broad screening bioassay for non-specific antispasmodic activity and for parasympathicomimetic or parasympathicolytic activity (see under 1.4.1.1). For the study of adrenergic mechanisms the isolated rabbit jejunum is appropriate, whereas the rat phrenic nerve-diaphragm preparation is utilized for the study of the action of muscle relaxants (Williamson *et al.*, 1996). With all these models, single-dose experiments, cumulative dose-response experiments and coaxial stimulation experiments can be carried out (Laekeman *et al.*, 1993; Bakana *et al.*, 1995).

The tracheal spiral from sensitized guinea-pigs is often used to represent the large airways; lung parenchyma strips, prepared from the same animals are then utilized to represent peripheral airways. Contractions can be induced with histamine, carbachol, LTD_4, PAF and arachidonic acid, and the effects of plant extracts, or tested against these directly (Williamson *et al.*, 1996).

Possible vasodilating effects can be studied using the rabbit central ear artery perfusion model (Laekeman *et al.*, 1986). In the rat mesenteric artery model, the perfusion system is connected to a suitable device for detecting changes in perfusion pressure to indicate vasoconstriction or vasodilation. Cardiotonic properties can be studied by using the spontaneously beating right atrium or the stimulated left atrium of the guinea-pig. The superfusion technique, which consists of bathing an isolated tissue with a stream of artificial salt solution at 37 °C and a constant flow rate allows the testing of several tissues in cascade, generally up to six arranged in two banks. This arrangement allows a parallel assay of individually injected samples. We have used such an experimental set-

up for the detection of prostaglandin-like activities of plant extracts. The cascade consisted of two vascular smooth muscle preparations, *viz.* the rabbit coeliac and mesenteric arteries and one non-vascular smooth muscle preparation, *viz.* the rat stomach strip. Prostaglandin-E like activity was detected as vascular smooth muscle relaxing and non-vascular smooth muscle stimulating activities in the cascade model (Figure 1.1b) (Üstünes *et al.*, 1985).

A cascade system consisting of rabbit coeliac and mesenteric arteries and rabbit aortic tissue was used for the detection of serotoninergic activity in plant extracts. The system can even be refined by fixing two separate aortic ring preparations, one with denuded endothelium, and the other with intact endothelium on the cascade. In this way, relaxation due to the release of endothelium-derived relaxing factor (EDRF) or nitric oxide can be determined (Üstünes *et al.*, 1991).

1.4.2.5 Whole animals

Biomedical research needs animals. This is most obvious in the case of *in vivo* animal experiments. However, for other scientific reasons, e.g. *in vitro* studies, biological material is also needed to study enzymes, membranes, receptors, cells, tissues or organs which are obtained from dead animals. Therefore, animals have to be sacrificed in biomedical laboratories, (i) at the end of *in vivo* experiments; (ii) during experiments where sacrifice of the animals is not part of the study but must be done when pain, distress and suffering exceed acceptable levels or if it is likely for the animal to remain in pain or distress after cessation of the experiment; and (iii) to provide biological material for *in vitro* studies (Vogel and Vogel, 1997).

Most large pharmaceutical companies dispose of a large battery of *in vivo* tests for the secondary evaluation of selected leads. Animals used in these tests include mice, rats, hamsters, guinea-pigs, rabbits, cats, dogs, ferrets, cattle, sheep, goats, horses, pigs, and primates.

In vivo tests for the detection of activity of the cardiovascular system, the gastrointestinal tract, the liver and the biliary system, the respiratory system, the renal system, and the endocrinological system, as well as for the determination of anti-inflammatory, analgesic, psychotropic, neurotropic, immunomodulating and antidiabetic activity, and effects on learning and memory, have recently been reviewed (Vogel and Vogel, 1997). *In vivo* tests also remain, in many cases, the stepping-stone between antimicrobial, antiviral, antiparasitic and anticancer *in vitro* tests, and the demonstration of a corresponding activity in human clinical trials. These models should predict efficacy in man, and must therefore mimic the natural disease as closely as possible. The challenge for the investigator will always be to correlate *in vitro* data with *in vivo* findings, bearing in mind the old saying '*in vitro* simplicity, *in vivo* verity'.

1.5 CONCLUSIONS

An attempt has been made to outline the most important aspects of the empirical approach to find new lead compounds from natural product mixtures such as plants. It is thereby apparent, and promising, to state how much progress has been made in the development of sensitive and simple primary and specialized bioassays for screening and/or monitoring purposes of plant extracts. The pharmaceutical industry now has at its disposal automated high-throughput *in vitro* screens for biological activities which are capable of examining thousands of compounds or extracts in very short periods of time.

As a result of these new technologies, it has become current fashion to guide fractionation of plant extracts towards rapid isolation and identification of the pure bioactive compounds. Since more and more approaches to automating the dereplication of these bioactive natural products are developed, it is not too presumptuous to expect that in the near future several new lead chemical entities from nature will be placed in the research and development pipelines for new drugs.

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CHAPTER

2

Applications of Liquid Chromatography/UV/MS and Liquid Chromatography/ NMR for the On-line Identification of Plant Metabolites

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Contents

2.1 INTRODUCTION

Innovations in analytical technology have often played an important role in the progress of natural product chemistry. The characterisation of metabolites in complex mixtures requires sophisticated hyphenated techniques, which should provide good sensitivity and selectivity as well as structural information on the constituents of interest. With the introduction of high throughput screening programmes, there is an urgent need for even more efficient and sensitive methodologies, which yield adequate on-line information for metabolite structure determination.

High performance liquid chromatography (HPLC) is used routinely in phytochemistry to 'pilot' the preparative isolation of natural products (optimisation of the experimental conditions, checking of the different fractions throughout the separation) and to control the final purity of the isolated compounds (Kingston, 1979; Hostettmann *et al.,* 1998). The development of hyphenated techniques related to this efficient separation technique in the past 20 years has provided powerful new tools such as LC/UV-photodiode array detection (LC/UV-DAD) (Huber and George, 1993), LC/mass spectrometry (LC/MS) (Niessen, 1999) and very recently LC/nuclear magnetic resonance (LC/NMR) (Spraul *et al.,* 1993). The combination of the high separation efficiency of HPLC with these different detectors has made possible the acquisition of on-line complementary spectroscopic data on an LC peak of interest within a complex mixture. As crude plant extracts represent very complex mixtures containing up to hundreds of constituents, these new LC-hyphenated techniques have been rapidly integrated for the study of crude plant extracts (Wolfender *et al.,* 1998b).

In this paper, several examples of applications of LC/UV, LC/MS and LC/NMR in the characterisation of natural products will be presented and the possibilities and limitations of these new techniques as on-line identification tools for plant metabolites, will be discussed. The techniques themselves will not be described in detail here; explanations for basic principles can be found in many books or reviews (LC/UV-DAD (Huber and George, 1993), LC/MS (Niessen, 1999; Niessen and Tinke, 1995), LC/NMR (Albert, 1995; Sudmeier *et al.,* 1996; Lindon *et al.,* 1997; Wolfender *et al.,* 1998a)).

2.2 ROLE OF LC-HYPHENATED TECHNIQUES IN THE EARLY RECOGNITION OF PLANT METABOLITES

The plant kingdom represents an extraordinary reservoir of novel molecules. Of the estimated 400,000–500,000 plant species around the globe, only a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even lower. There is thus currently a resurgence of interest in the vegetable kingdom as a possible source of new lead compounds for introduction into therapeutical screening programmes. The rapid disappearance of tropical forests and

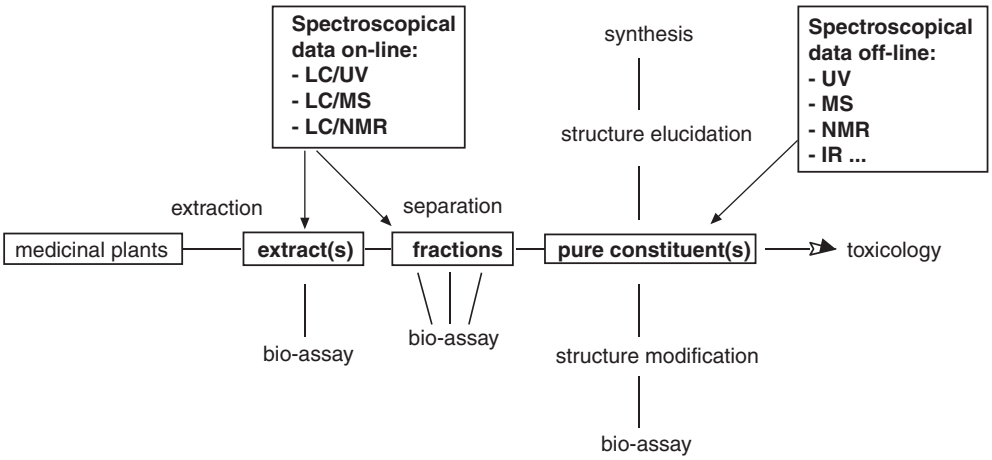

Figure 2.1: Procedure for obtaining the active principles from plants and use of LC-hyphenated techniques as strategic analytical screening tools during the isolation process.

other important areas of vegetation have meant that it is essential to have access to methods which lead to the rapid isolation and identification of bioactive natural products. The approach adopted to obtain an exploitable pure plant constituent involves interdisciplinary work in botany, pharmacognosy, pharmacology, chemistry, toxicology and can be formulated as in Figure 2.1 (Hamburger and Hostettmann, 1991).

By following only a bioactivity-guided fractionation procedure, there is a risk of unnecessarily isolating known plant constituents with recognised activity. Furthermore, interesting lead compounds, which do not exhibit the tested activity, will simply be missed. In order to avoid the time-consuming isolation of known constituents, LC-hyphenated techniques are used at the earliest stage of separation (Figure 2.1) (Hostettmann *et al.,* 1997). This chemical screening is valuable to detect compounds with interesting structural features and to target their isolation.

2.3 PRACTICAL POSSIBILITIES AND LIMITATIONS OF LC/UV, LC/MS AND LC/NMR FOR ON-LINE STRUCTURE DETERMINATION

In natural product chemistry, the combination of UV, MS and NMR spectroscopic data of pure constituents has often permitted their unambiguous structure determination. Other techniques such as IR or X-ray crystallography have been used less often and mainly when the other spectroscopic methods failed to give a complete structure assignment. Thus by analogy, the combination of the LC-hyphenated techniques LC/UV, LC/MS and LC/NMR should ideally enable the complete structure characterisation of any plant metabolites directly in an extract, provided that its corresponding LC peak is

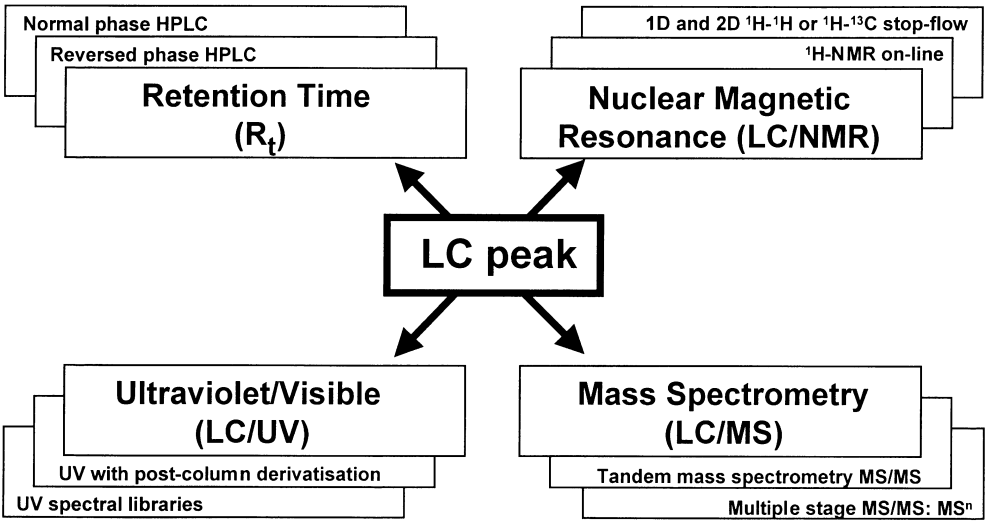

Figure 2.2: Summary of all types of on-line information obtained by LC-hyphenated techniques.

clearly resolved. In practice, however, such a statement is not fully true and many factors may hinder on-line detection and structure determination of a plant metabolite. Often only partial structure information will be obtained, but this on-line data will already provide very precious information for targeting the isolation of new compounds or for the dereplication of known constituents. A summary of the different LC-hyphenated techniques used in our laboratory for plant extract screening is presented in Figure 2.2.

One of the main problems is the inherent sensitivity of the spectroscopic techniques used for LC-detection. For example, LC/NMR gives, in general, far more structural information than LC/MS but it is many order of magnitudes less sensitive. Several problems and limitations of the use of the LC-hyphenated techniques will be discussed here. The potential of LC-hyphenated techniques for on-line identification will be demonstrated through different examples.

2.3.1 Liquid chromatography/UV-photodiode array detection (LC/UV-DAD)

LC/UV-DAD (Huber and George, 1993) has been used for almost two decades by phytochemists for screening extracts (Hostettmann *et al.,* 1984; Yoshimura *et al.,* 1994; Bramley, 1992) and is now widely spread in many laboratories. LC/UV-DAD provides mainly on-line information for natural products having strong chromophores. For compounds having extended double bond systems such as polyphenols, for example,

information on the type of constituents or their substitution is obtained. The choice of the LC solvents has to be made according to their inherent UV cutoff, to avoid interferences, but this is practically the only limitation of this technique for its application in plant extract analysis. New instruments allow the recording of UV spectral libraries and an automatic computer search can be performed provided that UV natural products databases have been built up. The UV spectra measured are, however, dependent on the composition of solvent systems used and matching with spectra measured in other solvents shows differences.

2.3.2 Liquid chromatography/mass spectrometry (LC/MS)

At present, MS is one of the most sensitive methods of molecular analysis. Moreover, it has the potential to yield information on the molecular weight as well as on the structure of the analytes. Due to its high power of mass separation, very good selectivities can be obtained. LC/MS has been used in analytical chemistry since the mid '80s and now numerous LC/MS systems are available on the market and different LC/MS interfaces have been built up. These LC/MS interfaces must accomplish nebulisation and vaporisation of the liquid, ionisation of the sample, removal of the excess solvent vapour, and extraction of the ions into the mass analyser. Now the most commonly used LC/MS interfaces are electrospray (ES) (Whitehouse *et al.,* 1985) and atmospheric pressure chemical ionisation (APCI) (Bruins *et al.,* 1987). Older type of interfaces such as thermospray (TSP) (Blakley and Vestal, 1983) or continuous flow fast atom bombardment (CF-FAB) (Caprioli, 1990) have, however, been revealed to be very efficient for natural products ionisation (Wolfender *et al.,* 1995a). In conjunction with these interfaces, various types of analysers such as quadrupole (Q), ion traps (IT) or time of flight (TOF) can be used and each of them will have their own characteristics in terms of mass accuracy, resolution and MS/MS possibilities (Mosi and Eigendorf, 1998).

The main problem of LC/MS is that the response is strongly dependant on the nature of the compounds to be analysed, the solvent and buffer used for the separation, the flow rate and the type of interface used. Thus, as a crude plant extract represents a complex mixture of metabolites, having various physico-chemical properties, it will be difficult to find LC/MS conditions that are optimum for the ionisation of all constituents (Wolfender *et al.,* 1994). Often, it will be necessary to analyse the extract under different ionisation conditions. On the other hand, the specific detection of given constituents can be performed at very low detection limits, provided that the correct ionisation method is used (Ndjoko *et al.,* 1998). Comparison of different interfaces and ionisation conditions, in the case of crude plant extract analysis, has been discussed in several papers (Wolfender *et al.,* 1994; Wolfender *et al.,* 1995b; Wolfender *et al.,* 1998b).

Another characteristic of LC/MS is soft ionisation. Indeed, the spectra acquired in LC/MS, unlike electron impact ionisation (EI), display mainly the molecular ion species

and only very few fragments are observed. The structure information given by a single LC/MS spectrum alone is thus often rather poor. This problem may be overcome by the use of tandem mass spectrometry (MS/MS) which will provide fragments through collision-induced dissociation (CID) of the molecular ions produced (Busch *et al.,* 1988; Herderich *et al.,* 1997). In order to obtain EI-like spectra, the particle beam (PB) (Wiloughby and Browner, 1984) interface can be used but its ionisation range will be restricted mainly to low molecular weight compounds. The general aspect of the LC/MS spectra, the intensity, as well as the type of molecular species observed, will thus differ according to the type of conditions used, hindering the efficient use of LC/MS databases of spectra of natural products. LC/MS/MS spectra are, however, more reproducible and the use of MS/MS databases of natural products can be considered for dereplication. New search algorithms have been commercialised very recently.

As will be demonstrated, LC/MS can be an extremely powerful technique for screening crude plant extracts, but the right ionisation conditions will have to be carefully optimised. At present, with the appropriate LC/MS configuration, the analysis of small non-polar metabolites to very large polar constituents such as proteins is possible (Niessen, 1999).

2.3.3 Liquid chromatography/nuclear magnetic resonance (LC/NMR)

As mentioned earlier, NMR is the spectroscopic technique which will provide by far the most useful information for the identification of natural products (Albert, 1995). The coupling between HPLC and NMR should thus ideally give important information online for the direct identification of plant metabolites. The recent progress in pulse field gradients and solvent suppression, the improvement in probe technology and the construction of high field magnets have given a new impulse to LC/NMR, a technique which has already been known for over 15 years (Watanabe *et al.,* 1979).

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 (Spraul *et al.,* 1993). This problem has even worsened in the case of typical LC-reversed phase operating conditions, where more than one protonated solvent was used and where the resonances changed frequencies during the analysis in gradient mode. Furthermore, the continuous flow of sample in the detector coil complicated solvent suppression. These problems have now been overcome, thanks to the development of fast, reliable, and powerful solvent suppression techniques such as WET (Water suppression Enhanced through T_1 effects) (Smallcombe *et al.,* 1995), which produced high quality spectra in both on-flow and stop-flow modes. These techniques consist of a combination of pulsed field gradients, shaped rf pulses, shifted laminar pulses and selective ^{13}C decoupling and are much faster than classical presaturation techniques previously used in this field (Albert, 1995). Thus, in typical reversed HPLC conditions, non-deuterated solvents such as MeOH or

MeCN can be used. Water is usually replaced by D_2O, which is a relatively 'cheap' deuterated solvent, and gives better quality spectra.

In the on-flow mode, the LC/NMR spectra are acquired continuously during the separation and are stored as a set of scans in discrete increments. The on-flow data are processed as for a 2D NMR experiment. One dimension of this 2-D plot represents the NMR ppm scale and the other the time scale (see, for example, Figure 2.5). According to the analysis, a compromise between the number of scans per increment and the LC resolution has to be made. As mentioned before, however, one of the drawbacks of LC/NMR is its inherent relatively low sensitivity. In our experience, a rough estimation of the detection limit in the on-flow mode with a 60 µl cell on a 500 MHz instrument was realised for the secoiridoid glycoside swertiamarin (MW 374). An injection of 20 µg of this compound on-column was necessary in order to obtain an S/N ratio of 3 for the olefinic proton H-3 (16 scans/increment 1 ml/min) (Wolfender *et al.,* 1998a). These limits can be reduced, however, if proper digital treatment of signals is achieved with state-of-the-art techniques available for modern NMR instruments. Thus, practically on-flow LC/NMR measurements will be mainly restricted to the direct measurement of the main constituents of a crude extract and this often under/overloaded LC-conditions. Typically 1–5 mg of crude plant extract will have to be injected on the column.

One way to improve the detection limit is to work in the stop-flow mode. Operation in stop-flow requires that the retention times of the analytes of interest are known, or that a sensitive method of detection such as LC/UV or LC/MS is used prior to LC/NMR to trigger the detection (Holt *et al.,* 1998). In practice, one of these detectors is connected on-line 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. The stop-flow mode permits longer acquisition times than in on-flow and satisfactory LC/^{1}H-NMR spectra of compounds present in the low µg range can be obtained. In this mode, the measurement of various 2D correlation experiments such as COSY, NOESY, HSQC and HMBC is also possible provided that the concentration of the metabolite is high enough (generally more than 100 µg of sample is required).

If LC/NMR is now practically applicable for the direct observation of metabolite resonances in LC-reversed phase systems, this does not mean that all the information obtained by conventional measurement in standard deuterated NMR solvents will be obtained. 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. This can be a major drawback when dealing with unknown constituents. In order 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 (Ndjoko *et al.,* 1999). Another problem is that the chemical shifts recorded in a reversed phase solvent will slightly differ from those reported in standard deuterated NMR sol-

vents. This can be a drawback if precise comparison with literature data have to be performed (Cavin *et al.,* 1998).

In spite of these practical problems, the progress realised in LC/NMR during these recent years is impressive and this technique provides invaluable on-line information in crude plant extract analysis.

2.4 LC-STRATEGIES FOR THE SCREENING OF POLYPHENOLS

Polyphenols are widely distributed among plant constituents, and often occur, both as aglycones and glycosides. In this category, constituents such as flavonoids have long been recognised as one of the largest and most widespread classes of compounds (Harborne, 1988).

All polyphenolic constituents possess a strong chromophore system. Thus LC/UV-DAD spectra of these constituents are very characteristic and provide already important structural information about the type of polyphenols (e.g. flavones, xanthones, anthraquinones, simple phenols), as well as on their oxidation pattern (Markham, 1982; Wolfender and Hostettmann, 1993; Hostettmann and Hostettmann, 1989). The systematic recording of reference LC/UV spectra of polyphenol standards and the constructions of in-house libraries of spectra permit a reliable searching for many known constituents (Rodriguez, 1997). Furthermore LC/UV-DAD analysis can be run several times with post-column addition of classical UV shift reagents. This method allows the recording of the all set of shifted UV spectra and provides much information on the positions of free OH groups (Hostettmann *et al.,* 1984).

LC/MS provides molecular weight information for both polyphenol aglycones and glycosides. These compounds are well ionised in the positive ion mode using thermospray or APCI interfaces (Wolfender *et al.,* 1994). With these techniques, **O**- and **C**-glycosides will also display characteristic fragments. If a high number of hydroxyl groups are present, analysis in the negative ion mode will be preferred. LC/MS/MS is helpful, especially if complementary information on the aglycone moiety are necessary.

LC/^{1}H-NMR provides important complementary information such as aromatic proton patterns for the confirmation of the type of substitution (Wolfender *et al.,* 1997a). More detailed information can be obtained with stop-flow 2-D experiments provided that the LC peaks are highly concentrated.

2.4.1 LC/UV and LC/MS for localisation of related xanthone glycosides in *Halenia corniculata*

The first example treated here demonstrates what information can be generated by a single HPLC run performed with on-line LC/UV-DAD and LC/TSP-MS detection for series of xanthone aglycones and glycosides. This analysis has been performed on the

dichloromethane extract of *Halenia corniculata*, a Gentianaceae from Mongolia, in order to screen its xanthone composition. These compounds are indeed interesting for their potential antidepressive activity as specific inhibitors of monoamine oxidase (Suzuki *et al.,* 1981).

The LC/UV-DAD chromatogram of this plant presented more than 19 peaks having UV spectra characteristic for xanthones (Rodriguez *et al.,* 1995). LC/TSP-MS was performed in the positive ion mode with the filament off and by using ammonium acetate as buffer. Under these conditions, the total ion current trace recorded with LC/TSP-MS showed ionisation of all the peaks recorded in the UV trace of the extract (254 nm). Two types of xanthone **O**-glycosides as well as xanthone aglycones were detected in this extract. A display of the selective ion traces of the molecular ions of the aglycones and the glycosides of these xanthones was used for their specific detection. As shown in Figure 2.3, for example, three xanthones of *H. corniculata,* **1**, **2** and **3** appeared in the single ion trace *m/z* 363, which indicated the presence of a common hexasubstituted aglycone (one hydroxyl and five methoxyl groups) in each case. This was confirmed by similar UV spectra for the three xanthones. The glycosides **1** and **2** were detected separately at *m/z* 709 and 679 Da, respectively. These ions corresponded to weak molecular sodium adducts $[M + Na]^+$ of the xanthone glycosides. Important fragments corresponding to the aglycone moiety $[A + H]^+$ were also observed in the LC/TSP-MS spectra (Figure 2.3). The difference of 316 Da between the ions $[M + Na]^+$ and $[A + H]^+$ in the TSP-MS spectrum of **2** was characteristic of the loss of a disaccharide moiety constituted of hexosyl and pentosyl units (-pentosyl (132) -hexosyl (162) -Na (23) +H (1)). Likewise, a difference of 346 Da (-hexosyl (162) -hexosyl (162) -Na (23) +H (1)) for **1** indicated the presence of a dihexosyl derivative. As primeverosyl and gentiobiosyl are the only disaccharide residues corresponding to these masses which have been found to date in the Gentianaceae family, **1** and **2** were, respectively, the gentiobiosyl and the primeverosyl derivative of the corresponding free aglycone **3**. The UV spectra of the three compounds were very similar but did not correspond to any of the available data. Thus, a rare oxidation pattern was indicated by these on-line data and subsequent isolation of these compounds was undertaken. Following this approach, other series of aglycones and corresponding glycosides were detected and 17 xanthones have been characterised. The isolation of the most interesting xanthones was undertaken (Rodriguez *et al.,* 1995).

This example of LC/UV/MS analysis shows that, for simple known compounds like xanthones, a precise identification can be performed on-line, provided that some information about the type of the constituents and their occurrence in given plant families is already available. Furthermore, series of related aglycones and glycosides can be easily localised.

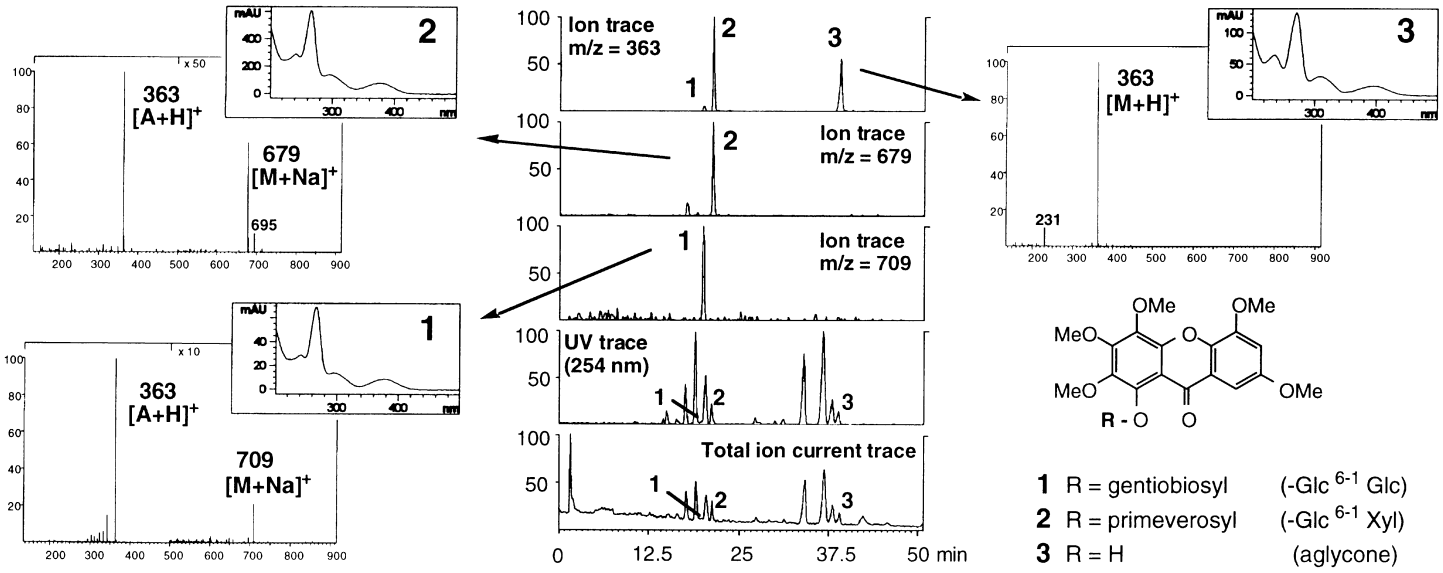

Figure 2.3: LC/UV and LC/TSP-MS analysis of the crude dichloromethane extract of *Halenia corniculata* (Gentianaceae) (Rodriguez *et al.,* 1995). HPLC: C18 Nova-Pak (4 µm, 150 × 3.9 mm i.d.); gradient, CH_3CN-H_2O 5:95 → 65:35 in 50 min (1 ml/min). Injection: 400 µg. The UV and MS spectra of three related xanthones (aglycone **3** and its two glycoside derivatives **1** and **2**) are displayed. The ion *m/z* 363 is the main fragment and corresponds to the aglycone moiety $[A+H]^+$. The ion traces displayed at *m/z* 679 and 709 correspond to the molecular adduct ions $[M+Na]^+$ of gentiobioside **2** and primeveroside **1**, respectively. (Reproduced with permission of Elsevier Science.)

2.4.2 LC/UV with combined use of UV shift reagents and LC/MS for the identification of flavonols in *Epilobium* species

As shown in the previous example, a simple LC/UV/MS analysis already provides interesting data on related xanthone aglycones and glycosides but did not give precise information on the substitution pattern of these polyphenols. In order to determine the position of the hydroxyl groups on the polyphenol skeleton, LC/UV with the aid of post-column addition of UV shift reagents (Wolfender and Hostettmann, 1993) can be performed. These UV reagents have indeed been extensively used for the characterisation of pure constituents (Markham, 1982). In this method, extracts are first analysed by LC/UV/MS and then the separation is repeated five times by LC/UV-DAD, each time by using another type of UV shift reagent. This procedure has been used for the characterisation of numerous xanthones in the extracts of different Gentianaceae species from the genus *Chironia* (Wolfender and Hostettmann, 1993) and it has also been efficient for the characterisation of flavonol glycosides in *Epilobium* species (Onagraceae) (Ducrey *et al.*, 1995). Plant of the genus *Epilobium* are indeed of increasing interest due to their use in folk medicine as herbal tea for the treatment of benign prostatic hyperplasia (BPH). Extracts of various *Epilobium* species have indeed demonstrated important activity against 5-α-reductase and aromatase, two enzymes which are involved in the aetiology of BPH, and the actives principles were identified as two macrocyclic ellagitannins (Ducrey *et al.*, 1997).

In order to screen the flavonol composition of various *Epilobium* species LC/MS and LC/UV-DAD with shift reagents has been used. To illustrate this approach, the analysis of the MeOH extract of *Epilobium angustifolium* is presented here. For one of the flavonols of the extract, isomyricitrin (**4**), the LC/TSP-MS spectrum as well as all the UV shifted spectra have been displayed (Figure 2.4). The LC/TSP-MS of **4** presented a clear $[M + H]^+$ molecular ion at *m/z* 481 and aglycone fragment ion $[A + H]^+$ at *m/z* 319. The mass difference (162 Da) was indicative for an hexosyl derivative, most probably a glucoside. From the mass of the aglycone, it could be deduced that the flavonol was bearing five hydroxyl groups. The interpretation of the shift induced by the post-column addition of the specific reagents gave interesting additional information. The shift of band II by 11 nm with the weak base 0.1 M Na_2HPO_4 was characteristic for a non-substituted 7-hydroxyl group. A 15 nm shift with boric acid was typical for *ortho*-dihydroxyl groups on the B ring. The shift of 42 nm of band I obtained with $AlCl_3$ without neutralisation was specific for a 5-hydroxyl group. Addition of $AlCl_3$ after neutralisation gave a 56 nm shift of band I. This was due to a combination of an *ortho*-dihydroxyl group (C-3′ and C-4′) and the C-4 keto function with the 5-hydroxyl complexing with Al^{3+}. With these data the identification of isomyricitrin (**4**) was confirmed since UV data showed an *ortho*-dihydroxyl group on the B ring and free hydroxyl groups at C-5 and C-7.

A similar procedure was followed, combining TSP/LC-MS and LC-UV, to identify the

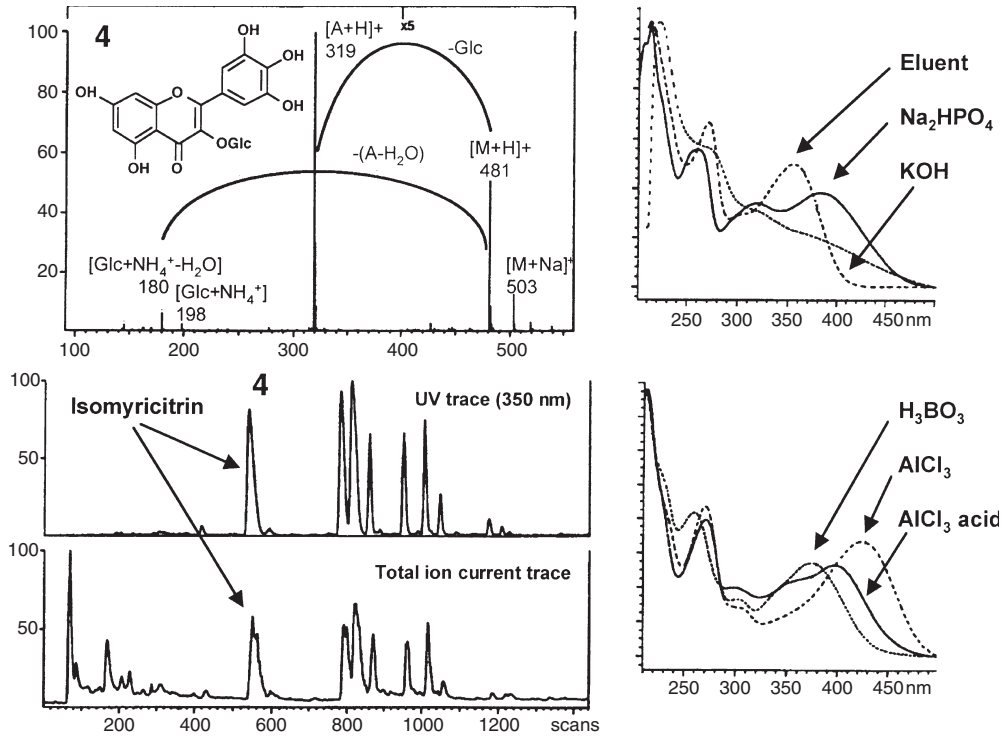

Figure 2.4: LC/UV and LC/TSP-MS analysis of the MeOH extract of *Epilobium angustifolium* (Onagraceae) (Wolfender *et al.,* 1997b). HPLC: C18 Nova-Pak (4 µm, 150 × 3.9 mm i.d.); step-gradient, CH_3CN-H_2O 0:100 → 25:25 in 30 min (1 ml/min). The LC/TSP-MS spectrum of **4** as well as its corresponding UV and UV shifted spectra are displayed. (Reproduced with permission of Research Signpost.)

other flavonol glycosides in different *Epilobium* species and 13 different flavonol derivatives were identified (Ducrey *et al.,* 1995).

2.4.3 On-flow LC/^{1}H-NMR for the analysis of *Swertia calycina*

UV and MS data alone, in many cases, do not provide enough structural information for the characterisation of phenolic constituents, often information on atom connectivity has to be provided. In this respect, complementary data can be obtained by on-flow LC/^{1}H-NMR, if the metabolites of interest are present in high concentrations in the extract.

This type of analysis was used, for example, to identify the antifungal principle of the dichloromethane extract of another plant from the Gentianaceae family, *Swertia calycina*. The LC/UV chromatogram of dichloromethane extract of *S. calycina* presented

three main peaks (**5–7**) (Figure 2.5). The LC/UV and LC/MS data, together with chemotaxonomical considerations, allowed the identification of most xanthones and secoiridoids of this plant. The UV spectrum of **6**, however, was not attributable to a common polyphenol of the Gentianaceae such as flavones or xanthones. It was very weakly ionised in LC/TSP-MS, but a protonated molecular ion was nevertheless found at *m/z* 189. This small molecular weight (188 Da) and the UV spectrum suggested that **6** could be a quinonic compound, but as no metabolite of this type was previously found in the Gentianaceae family, it was not possible to identify it on-line. In order to confirm these attributions and to obtain more structure information on-line, the extract was submitted to an on-line LC/^{1}H-NMR analysis on a 500 MHz instrument (Wolfender *et al.*, 1997). The same LC conditions as for the LC/UV/MS analysis were used except that the water of the LC gradient system was replaced by D_2O. However, the quantity of extract injected onto the column was increased to 1 mg to obtain at least 20 µg for each peak of interest. For the suppression of the solvent signal of MeCN and its two ^{13}C satellites, as well as the residual HOD peak, a fast sequence called WET was run before each acquisition (Wolfender *et al.*, 1997a). Under these conditions, the on-line LC/NMR analysis of *S. calycina* provided ^{1}H-NMR spectra for all the major constituents. A plot of the retention time (y axis) versus the NMR shifts (x axis) permitted the localisation of the resonances of compounds **5**, **6** and **7** (Figure 2.5). On this 2-D plot, strong signals of aromatic methoxyl groups were observed around 4 ppm for **6** and **7**. Xanthone **7** exhibited two pairs of aromatic protons, while the quinonic compound **6** presented five other low fields protons. The more polar secoiridoid **5** showed different signals between 3 and 6 ppm. The important trace starting from 4.8 ppm (at 0 min) and ending to 4 ppm (at 30 min) was due to the change of the chemical shift of the residual negative water (HOD) signal during the LC gradient. The traces between 1 and 2.6 ppm were due to residual MeCN signal and solvent impurities.

A slicing of this bidimensional plot in single on-line LC/^{1}H-NMR spectra for each constituent allowed a precise assignment of their specific resonances. The ^{1}H-NMR data of **5** and **7** confirmed their on-line identification by LC/UV/MS as sweroside and decussatin, respectively. On the LC/^{1}H NMR spectrum of **6**, two signals (2H, δ 8.11, *m*, H-5,8 and 2H, δ 7.89, *m*, H-6,7) were characteristic of four adjacent protons of an aromatic ring with two equivalent substituents. The low field shift of the H-5,8 signal indicated that these two protons were in *peri* position of carbonyl functions suggesting most probably the presence of a naphthoquinone nucleus. The strong bands recorded in UV at 243, 248, 277, 330 nm confirmed this deduction. The singlet at 4.35 ppm was attributed to H-3 and the remaining methoxyl group was thus at position C-2. With this on-line data and the molecular weight deduced from the LC/TSP-MS spectrum (MW 188), **6** was finally identified as a known 2-methoxy-1,4-naphthoquinone, which was responsible for the antifungal activity of the extract and was reported for the first time in the Gentianaceae family (Wolfender *et al.*, 1997a).

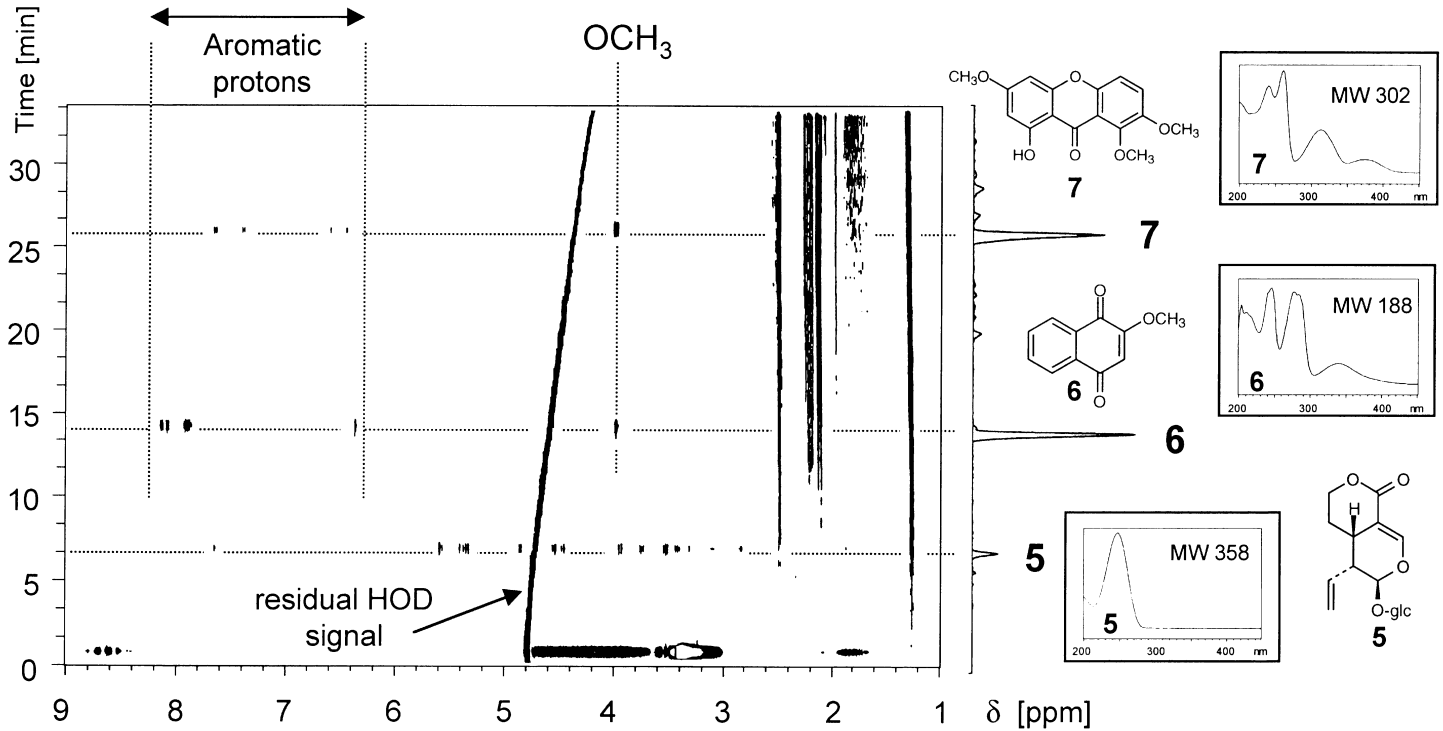

Figure 2.5: Bidimensional LC/^{1}H-NMR chromatogram of the crude CH_2Cl_2 extract of *Swertia calycina* (Gentianaceae) (Wolfender *et al.*, 1997a). Methoxyl groups and aromatic proton signals of **6** and **7** are clearly visible together with all the resonances of the monoterpene glycoside **5**. The signal of HOD is negative and was continually shifted during the LC gradient. HPLC: same conditions as in Figure 2.3 but water was replaced by D_2O, Injection: 1 mg. LC/NMR: 16 scans/increment, flow cell (60 µl, 3 mm i.d.), 500 MHz. (Reproduced with permission of John Wiley & Sons Limited.)

2.4.4 LC/MS/MS and stop-flow LC/^{1}H-NMR for the analysis of xanthones and flavones in *Gentiana ottonis*

Often, in crude plant extracts, the compounds of interest are not present in very high concentrations and their analysis cannot be directly performed by on-flow LC/NMR. In these cases, LC/NMR has to be performed in the stop-flow mode and LC/^{1}H-NMR spectra are obtained for all the peaks of interest by performing longer acquisition than in the on-flow mode. This type of analysis was necessary for the complete characterisation of the xanthone and flavone constituents from *Gentiana ottonis*, a Gentianaceae species from Chile (Wolfender *et al.,* 1997a).

The LC/UV analysis of this extract presented peaks with UV spectra characteristic of secoiridoids (**8**), flavones (**10** and **12**) and xanthones (**11** and **13–15**). The LC/TSP-MS analysis of this extract allowed the attribution of molecular weights of all these compounds. Compounds **9**, **10** and **12** presented fragments characteristic for **C**-glycosides (losses of 90 and 120 Da). According to their UV spectra, **10** and **12** (MW 448 and 446) were, respectively, tri- and tetraoxygenated flavones **C**-glycoside. In order to obtain satisfactory LC/^{1}H-NMR spectra, this extract was analysed in the stop-flow mode (Figure 2.6).

The secoiridoid glycoside (**8**) presented signals characteristic for glucose in the 3–4.5 ppm region and typical resonances of the monoterpene moiety in the 5–8 ppm range. These data, together with the molecular weight of **8** (MW 374), enabled its unambiguous identification as swertiamarin, a widespread bitter principle in the Gentianaceae family.

Xanthone **O**-glycoside **13** (MW 436) presented signals characteristic for a **O**-glucosyl moiety (δ 3.3–5.1) and one methoxyl group (δ 3.96). A pair of *meta* coupled aromatic protons (1H, δ 6.70, *d*, J = 2.0, H-4 and 1H, δ 6.47, *d*, J = 2.0, H-2) indicated a 1,3-disubstituted A-ring. The B-ring exhibited a pair of *ortho* coupled protons (1H, δ 7.34, *d*, J = 8.8, H-6 and 1H, 7.17, *d*, J = 8.8, H-7). This information, together with UV data, confirmed a 1,3,5,8-substitution pattern for **13**. The LC/^{1}H-NMR of **15**, the aglycone of **13**, also exhibited a pair of *meta* and *ortho*-coupled protons, as well as a methoxyl group. However, with respect to **13**, one of the *ortho* protons was shifted upfield, indicating glycosylation on the B-ring (at C-5 or C-8). A careful comparison of the LC/UV data with those of authentic samples allowed, finally, the identification of **13** and **15** respectively as 1,5,8-trihydroxy-3-methoxyxanthone (bellidifolin) and 1,5-dihydroxy-3-methoxy-8-**O**-glucosylxanthone (8-**O**-glucosylbellidifolin). Similar deductions allowed the identification of **11** and **14** as 1,3,5,8-tetrahydroxyxanthone (desmethylbellidifolin) and 1,3,5-trihydroxy-8-O-glucosylxanthone (8-**O**-glucosyldesmethybellidifolin).

The stop-flow LC/^{1}H-NMR of the flavone **C**-glycoside **12** (MW 446) (Figure 2.6) gave signals for six aromatic protons (δ 6.8–8.1) and one methoxyl group (δ 4.0), together with those of the **C**-glycoside moiety (δ 3.5–5.0). A pair of symmetric *ortho*-coupled

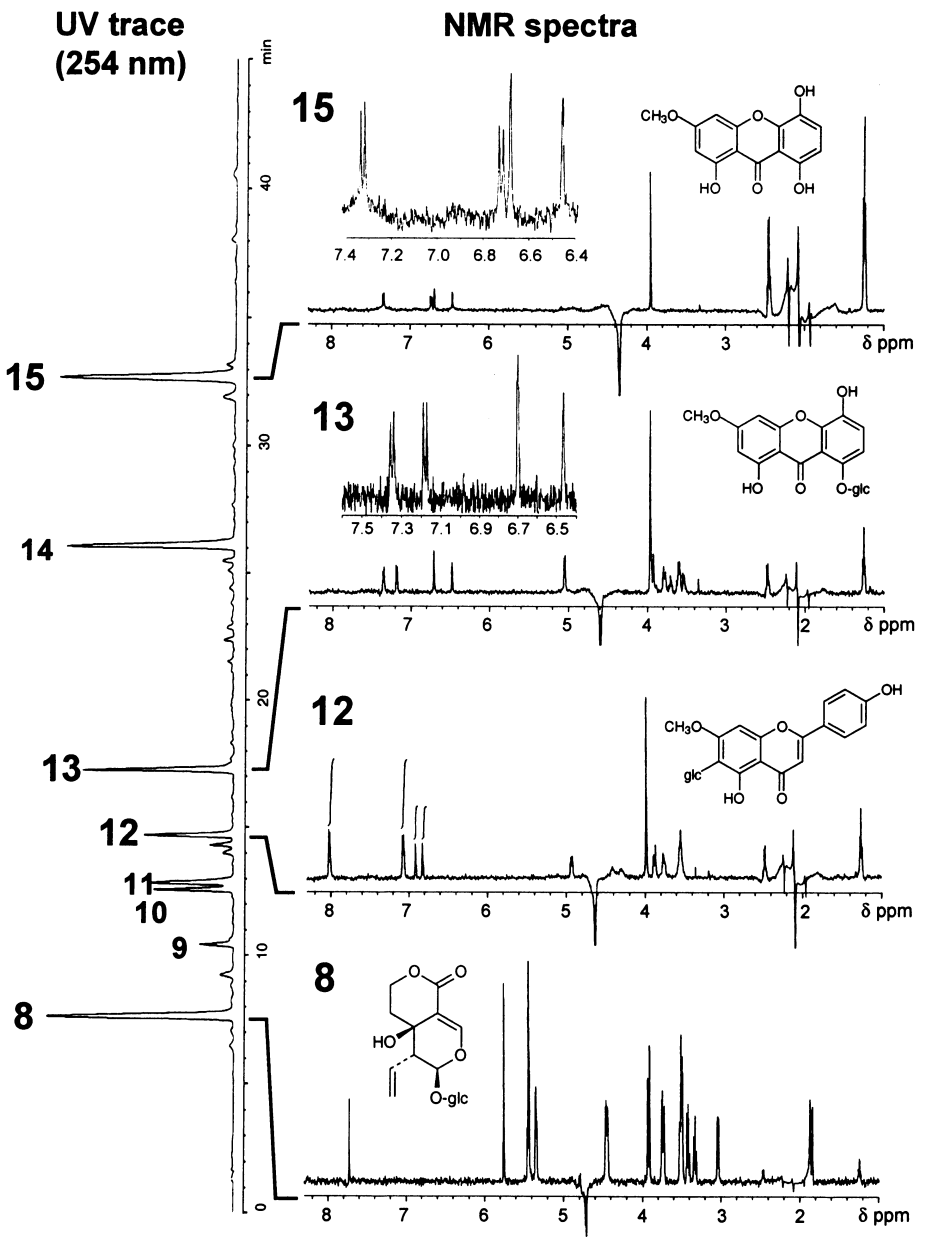

Figure 2.6: LC/UV chromatogram of the root MeOH extract of *Gentiana ottonis* (Gentianaceae) (vertical display) with the stop-flow LC/^{1}H-NMR spectra of **8**, **12**, **13** and **15** (Wolfender *et al.,* 1997a). On these LC/^{1}H-NMR spectra, the coupling constants and the integration of the aromatic signals were easily measured. HPLC: Same conditions as in Figure 2.5. Injection: 0.4 mg. LC/NMR: stop-flow 256 to 1024 scans/peak according to the concentration, flow cell (60 µl, 3 mm i.d.), 500 MHz. (Reproduced with permission of John Wiley & Sons Limited.)

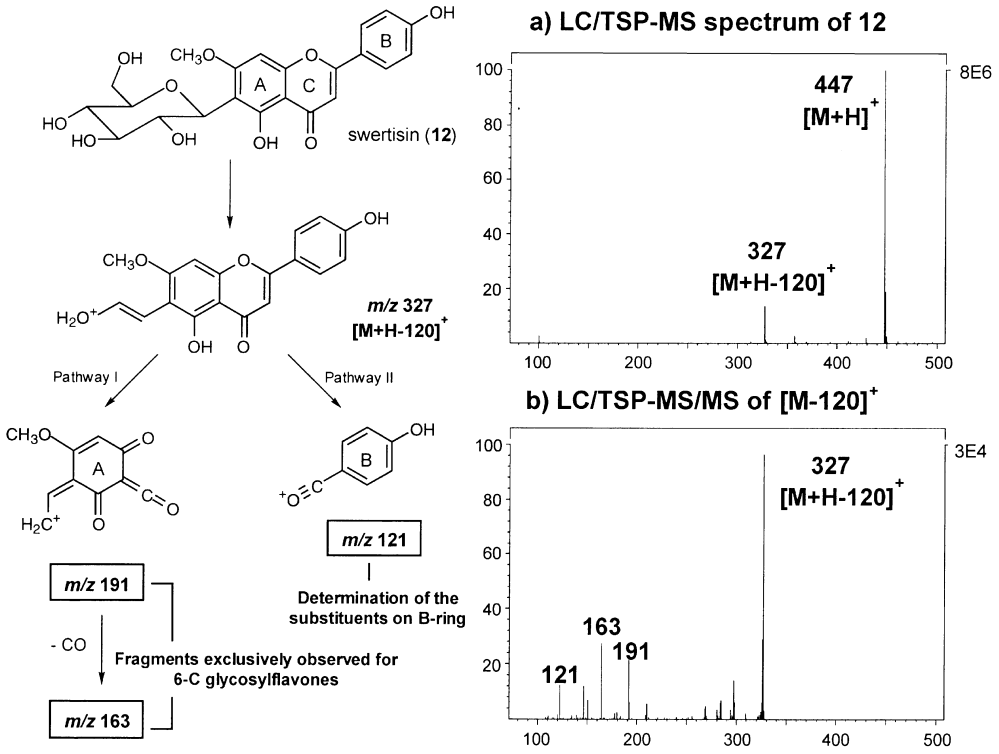

Figure 2.7: LC/TSP-MS (a) and LC/TSP-MS/MS (b) spectra of swertisin (**12**) in the root MeOH extract of *Gentiana ottonis* (Gentianaceae) (Wolfender *et al.,* 1997a). The LC/MS/MS analysis was performed using the fragment $[M+H-120]^+$ (spectrum a) as parent ion. Characteristic daughter ions at *m/z* 121, 163 and 191 were observed, indicating the substitution on the A- and B-ring of the C-glycosylflavone (**12**). (Reproduced with permission of John Wiley & Sons Limited.)

protons (2H, δ 7.06, *d*, *J* = 8.3, H-3′,5′ and 2H, δ 8.00, *d*, *J* = 8.3, H-2′,6′) was characteristic for a B-ring substituted in C-4′. The singlet at δ 6.8 was attributable to H-3. The other singlet at δ 6.9 was due to a proton, either at position C-6, or C-8 on the A-ring. LC/UV/MS and stop-flow LC/NMR data were not sufficient in this case to fully ascertain the structure of **12**. In order to ascertain the position of C-glycosylation, a LC/MS/MS experiment was performed. As shown in Figure 2.7, the LC/TSP-MS/MS spectrum of **12**, obtained by choosing $[M + H-120]^+$ as parent ion, exhibited fragments at *m/z* 191 and 163, characteristic for 6-C-glycosylated flavones. Indeed, it is known that TSP-MS/MS spectra of the $[M + H-120]^+$ fragments of isomeric C-glycosylflavones show different specific daughter ions (Rath *et al.,* 1995). The A-C-ring fragments from a *retro* Diels-Alder cleavage are only observable for the C-6 position isomers, as was the case for **12**. Furthermore the fragment observed at *m/z* 121 was indicative of a monohydroxylated B-ring, confirming the position of the methoxyl group on the A-ring. These UV, MS,

stop-flow LC/^{1}H-NMR and MS/MS data allowed the identification of **12** as the known 5,4′-dihydroxy-7-methoxy-6-**C**-glucosylflavone (swertisin). Similarly, the flavone **C**-glycoside **10** (MW 448) was identified as the common 5,7,3′,4′-tetrahydroxy-6-**C**-glucosylflavone (isoorientin) (Wolfender *et al.,* 1997a).

2.4.5 LC/^{1}H-NMR and stop-flow 2D LC/NMR for the characterisation of a new flavanone from *Monotes engleri*

As has been demonstrated, the combination of LC/UV, LC/MS, LC/MS/MS and LC/^{1}H-NMR data permits in many cases the structure elucidation of various known polyphenols. These data, however, are not always sufficient for a full on-line identification of new constituents. If a complete structural identification has to be carried out, information on the ^{13}C signals is necessary and different 2D correlation experiments have to be performed. These types of experiments can be run by LC/NMR in the stop-flow mode. Indeed LC/NMR in the stop-flow mode is not only more sensitive for single ^{1}H-NMR measurements but also enables a series of 2D correlation experiments to be run, provided that the sample concentration in the LC/NMR cell is high enough (typically hundreds of µg 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 experiments such as HSQC or HMBC.

The possibilities of LC/NMR in the stop-flow mode are exemplified by the structure determination of a flavanone from an enriched fraction of the CH_2Cl_2 extract of *Monotes engleri* (Dipterocarpaceae) (Garo *et al.,* 1998). This plant showed antifungal activity against the yeast *Candida albicans* in a bioautographic TLC assay. This activity was linked to a compound, which gave a strong UV absorbing spot on TLC at 254 nm. On LC/UV analysis at 254 nm, this corresponded to the main compound **16** of the extract (Figure 2.8). The UV spectrum of this compound was characteristic for a flavanone and its LC/TSP-MS spectrum showed a molecular ion $[M + H]^+$ at 357 which exhibited a loss of 68 Da ($[M + H\text{-}C_4H_8]^+$), characteristic for an **O**-prenyl substituent. Based on these data and according to the molecular weight of a flavanone skeleton, **16** was most probably a flavanone with one **O**-prenyl unit and three hydroxyl substituents. This hypothesis was confirmed by the stop-flow LC/^{1}H-NMR spectra measured on this peak.

The presence of a flavanone skeleton with a prenyl unit could indeed be established: two methylene protons at δ 2.9 and δ 3.2 ppm suggested the presence of a γ-dihydropyrone ring. The deshielded doublet for the methylene of the prenyl unit at δ 4.7 ppm confirmed the O-connection of the prenyl unit, as deduced from the LC/MS data. These LC/^{1}H-NMR data were, however, not sufficient for a complete identification and the position of the prenyl unit could not be deduced. Stop-flow LC/2D-NMR correlation experiments were thus performed on an enriched fraction containing 1 mg of **16**: GHSQC (gradient heteronuclear single quantum coherence), GHMBC (gradient heteronuclear multiple quantum coherence), 1D TOCSY and WET-NOESY (2D Nuclear

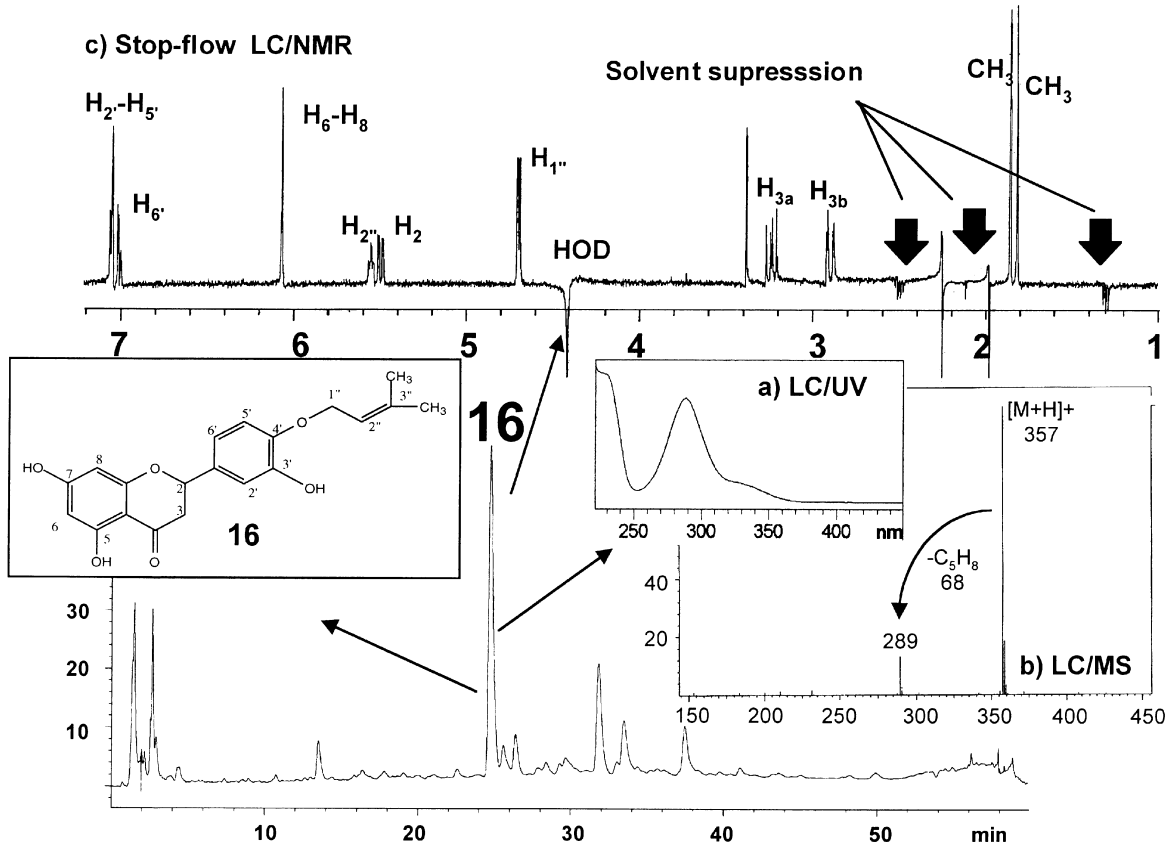

Figure 2.8: LC/UV chromatogram (254 nm) of the crude CH_2Cl_2 extract of *Monotes engleri* (Dipterocarpaceae) (Garo *et al.*, 1998). Insert: a) UV spectrum of the flavanone **16**; b) LC/TSP-MS spectrum of **16** and c) stop-flow LC/^{1}H-NMR of **16**. HPLC: C18 Nova-Pak (4 µm, 150 × 3.9 mm i.d.); MeCN-D_2O gradient (20:80 to 70:30 in 50 min); 1 ml/min. Injection: 1 mg. LC/NMR: 24 scans/increment, flow cell (60 µl, 3 mm i.d.), 500 MHz. (Reproduced by permission of Verlag Helvetica Chimica Acta AG.)

Overhauser Effect) NMR experiments were recorded in a total acquisition time of 9.6 hours. The LC/GHSQC showed all C-H connectivities. In the LC/GHMBC, the long-range couplings of the γ-dihydropyrone ring were visible and the position of the prenyl unit on the B-cycle could be determined. The combination of the data obtained with these two inverse ^{1}H-^{13}C correlation experiments allowed assignment of all the ^{13}C resonances of **16** on-line, directly in the LC eluent (Garo *et al.,* 1998). In order to determine the exact position of the prenyl unit on the flavanone skeleton of **16**, 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-cycle in the MeCN:D_2O solvent system did not permit the differentiation of H-C(2′) and H-C(5′), preventing the full structural assignment of **16** (Figure 2.9b). A second stop-flow LC/NMR analysis of the active fraction X was then performed in MeOH:D_2O. In this latter solvent system, the three aromatic protons of the B-cycle were observed at distinct chemical shifts. Two doublets (J = 7.8 Hz) at δ 6.90 and δ 6.95 were attributable to the *ortho*-coupled protons H-C(6′) and H-C(5′), and one broad singlet at δ 6.97 was due to H-C(2′). A second LC-WET-NOESY was performed in this latter solvent system (Figure 2.9a). First, an irradiation of H-C(2) gave enhancement of the two H-C(2′) and H-C(6′) aromatic protons at δ 6.97 and δ 6.90. A correlation with the H-C(2) and the two protons on C(3) was also shown (Figure 2.9). Secondly, irradiation of H-C(1′′) gave enhancement of the *ortho*-coupled proton at δ 6.95 (H-C(5′)) (Figure 2.9). Other NOE effects were also measured on the prenyl unit, particularly between H-C(1′′) and H-C(2′′), H-C(1′′) and H-C(5′′), as well as between H-C(2′′) and H-C(4′′) (Figure 2.9). According to these observations, the attachment of the **O**-prenyl substituent at position C(4′) was confirmed and **16** could thus be identified on-line 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. Pure compound **16** showed a negative optical rotation ($[\alpha_D] = -24.7$), and the absolute configuration at carbon C(2) has been determined by CD experiments (Garo *et al.,* 1998).

2.5 LC-STRATEGIES FOR THE SEARCH FOR NEW IRIDOIDS AND SECOIRIDOIDS

Iridoids represent a large, and still expanding, group of cyclopentan-(c)-pyran monoterpenoids. They are found as natural constituents in a large number of plant families, usually, but not invariably, as glucosides. Secoiridoids represent another type of monoterpene glycosides that are derived from secologanin, their biosynthetic precursor. They represent the bitter principles of many plant families especially in the Cornales, Dipsacales and Gentianales orders (Rodriguez *et al.,* 1999). They may occur as esters with different acids, and especially with biphenylcarbonic acids, which enhance strongly

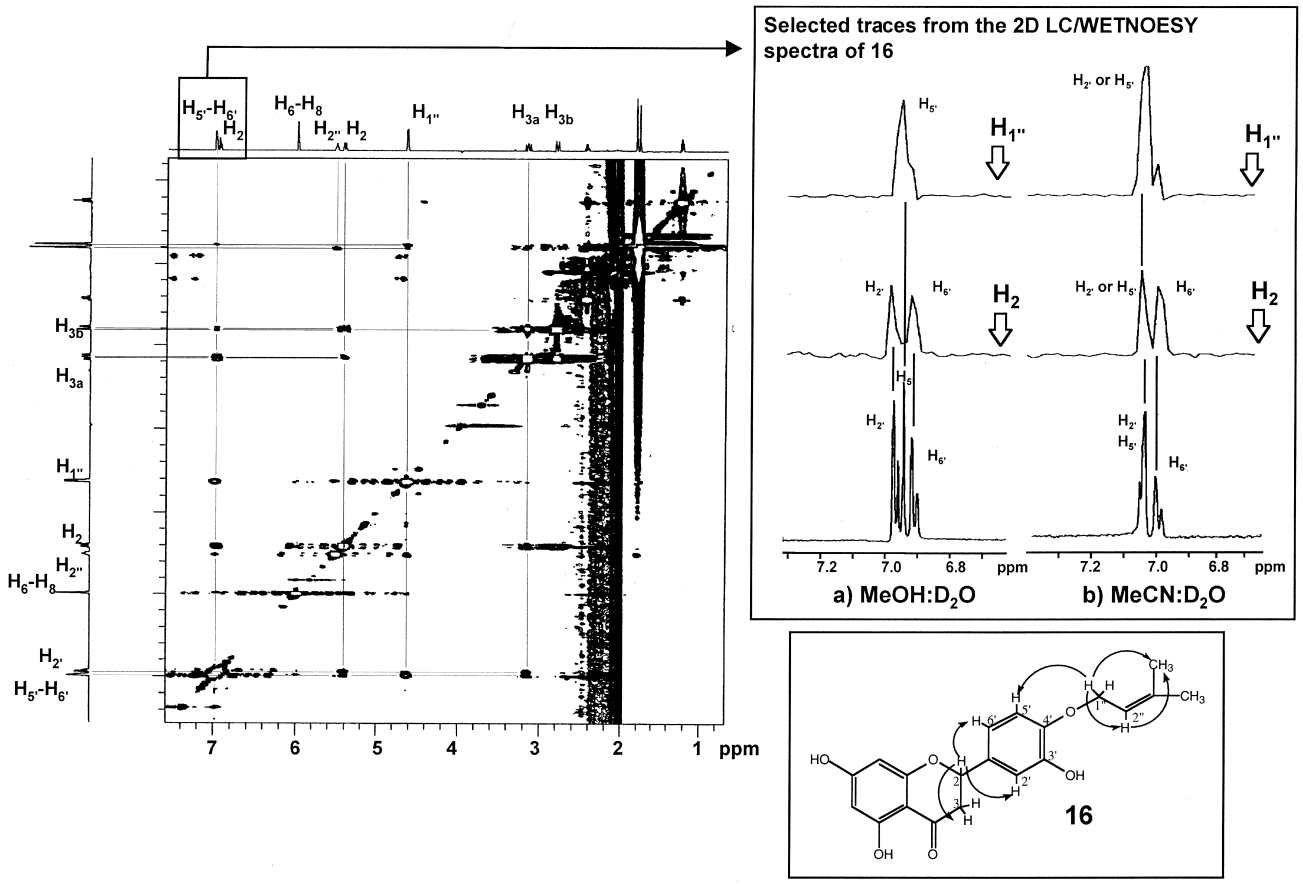

Figure 2.9: Stop-flow LC/WET-NOESY spectrum of **16** (solvent MeOH-D_2O) (Garo *et al.,* 1998). Insert: Selected traces showing the main NOE correlations in both MeCN-D_2O and MeOH-D_2O solvent systems. LC/WET-NOESY: transients 8, increments 256. Time: 2.5 hr. HPLC: same conditions as in Figure 2.8. Injection: 1 mg of the enriched fraction. (Reproduced by permission of Verlag Helvetica Chimica Acta AG.)

their bitter taste. Plants containing these compounds are used in many herbal preparations for their stomachic properties.

Unlike for polyphenols, the LC/UV spectra of most iridoids and secoiridoids are often of limited use because these constituents lack important chromophores. LC/UV spectra, however, are important for the characterisation of acylated iridoids, where the acyl groups are aromatic derivatives such as coumaroyl, feruloyl or benzoyl groups (Rodriguez *et al.,* 1999). Usually for iridoids and secoiridoids, the partial structural identification relies mainly on LC/MS data. These compounds can be generally analysed by TSP, APCI or ES/LC/MS in the positive ion mode. As these compounds are thermolabile and occur often as glycosides, the choice of the ionisation conditions are of great importance as this will be exemplified below. For the analysis of aglycones such as valepotriates, for example, parameters such as the temperature of the source in LC/TSP-MS play an important role in the control of their fragmentation (Wolfender *et al.,* 1994). LC/NMR has been only scarcely used for the characterisation of these constituents because the most useful information is obtained in ^{13}C-NMR and ^{13}C can not be directly detected in LC/NMR because of its lack of sensitivity. This technique, however, can be efficiently used to differentiate closely eluting isomers provided that other data are available on the mixture through classical NMR experiments.

2.5.1 Combination of LC/TSP-MS and LC/CF-FAB-MS for the screening of new secoiridoids in *Gentiana rhodantha*

As mentioned, LC/MS is a very important technique for the dereplication and screening of secoiridoids. This technique was used, for example, for the analysis of a Chinese Gentianaceae *Gentiana rhodantha*, which presented an original secoiridoid composition. The LC/UV analysis of *G. rhodantha* showed the presence of only one predominant xanthone **18** and different secoiridoids. Compound **18** was rapidly identified as the widespread xanthone **C**-glycoside mangiferin by its TSP-MS spectrum and the computer fitting of its UV spectrum with our in-house UV spectral library. With the help of the LC/TSP-MS spectra recorded on-line, chemotaxonomical considerations and comparison with pure standards, the secoiridoids with retention times less than 10 min were easily identified (Ma *et al.,* 1994). Among them a very minor secoiridoid, sweroside (**17**) (MW: 358), was found to be present. The slower running peaks (**19** and **20**) also exhibited the same UV spectra of secoiridoids (one band at around 240 nm) (Figure 2.10). These compounds, which were less polar than the common secoiridoids, were studied in more details. The LC/TSP-MS analysis of **19** and **20** gave in each case a spectrum identical to that obtained for sweroside **17**; all exhibited an intense ion at 359 Da and no ion at higher masses as it was shown by the display of the ion trace *m/z* 359 of the extract. However, the chromatographic behaviours of **19**, **20** and sweroside **17** were quite different. In order to obtain complementary information on these constituents, a second

LC/MS analysis with CF-FAB was achieved, using the same HPLC conditions. The total ion current recorded for the whole chromatogram showed a very important MS response for compounds **19** and **20** while the more polar metabolites were only weakly ionised. The CF-FAB spectrum of **19** recorded on-line exhibited a very intense pseudo-molecular ion $[M\text{-}H]^-$ at *m/z* 913 together with a weak ion at *m/z* 555 corresponding to the loss of a 'sweroside like' unit $[M\text{-}H\text{-}358]^-$ (CF-FAB spectrum of **19**, Figure 2.10b). This complementary information indicated clearly that the molecular weight of **19** was 914 Da. For the same compound, only a fragment corresponding to a 'sweroside like' unit *m/z* 359 was recorded during the LC/TSP-MS analysis (TSP spectrum of **19**, Figure 2.10a). According to the different results obtained on-line for **19** in the HPLC screening of the extract of *G. rhodantha,* it was concluded that **19** was probably a type of moderately polar large secoiridoid containing at least one unit very similar to sweroside (**17**). The CF-FAB spectrum of **20** exhibited an intense $[M\text{-}H]^-$ pseudomolecular ion at *m/z* 1629 indicating a molecular weight of 1628 for this compound. Fragments at *m/z* 1271, 913 and 555 showed, respectively, the consecutive losses of 'sweroside like' units (−358 Da). These on-line LC/MS results suggested that **20** was probably similar to **19** with two more 'sweroside like' units attached to it (Ma *et al.,* 1996).

Following the LC/MS screening results, a targeted isolation of **19** and **20** was undertaken. A full structure determination of **19** and **20** with the help of 1D and 2D NMR experiments as well as with different chemical reactions confirmed the hypothesis made on-line. These two compounds were found to be natural products of a new type (Ma *et al.,* 1994; Ma *et al.,* 1996).

2.5.2 LC/UV, LC/MS and LC/^{1}H-NMR for the detection and characterisation of acylated secoiridoids from *Lisianthius seemannii*

In another case, a Gentianaceous plant *Lisianthius seemannii* collected in Panama was investigated (Rodriguez *et al.,* 1998). The LC/TSP-MS analysis of the MeOH extract indicated the presence of high molecular mass secoiridoids. The main constituent was identified on-line as lisianthioside (**21**) a secoiridoid dimer already isolated from another species *L. jefensis* (Hamburger *et al.,* 1990). Two minor peaks eluting after lisianthioside in this LC/MS analysis presented, however, interesting UV and MS characteristic features. According to the LC/UV/MS results, these compounds were two isomers (MW: 862) related to lisianthioside (MW: 716) but possessing, most probably, an additional coumaroyl moiety. According to these preliminary results, **22** and **23** were found to be potential new acylated dimeric secoiridoid glycosides and their targeted isolation was decided. Pure **22** could be obtained, while **23** rapidly converted in **22**, to give a mixture of the two isomers. The structure of **22** was consistent with the hypothesis made from the LC/UV/MS data of the crude extract and was established as (*E*)-4′-*O*-(*p*-coumaroyl) lisianthioside, called seemannoside A. As compound **23** was found to be unstable in a

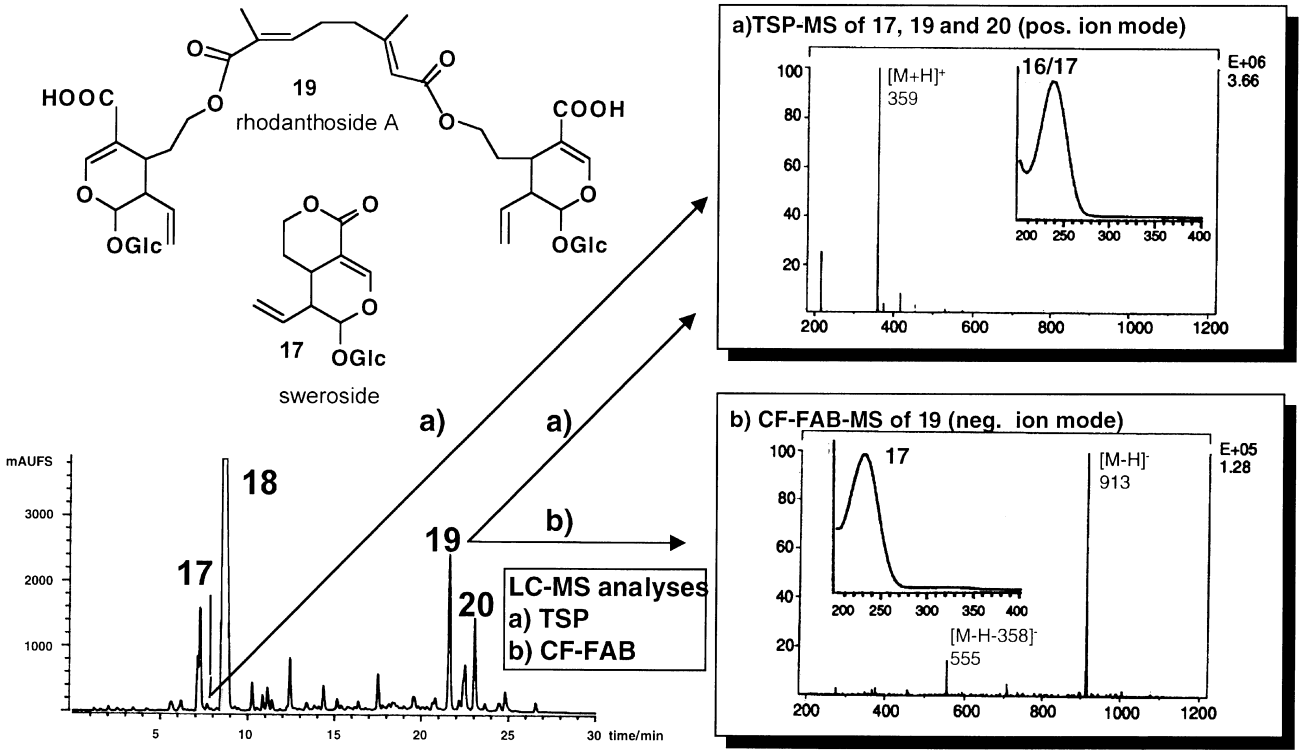

Figure 2.10: Combined TSP (a) and CF-FAB (b) LC-MS of the enriched BuOH fraction of the methanolic extract of *Gentiana rhodantha* (Gentianaceae) (Ma *et al.*, 1994). HPLC: C18 NovaPak (4 µm, 150 × 3.9 mm i.d.); gradient, CH_3CN-H_2O (0.05% TFA) 5:95 → 50:50 in 30 min; 0.9 ml/min. TSP (a): Positive ion mode; filament off; vaporiser, 90°C; source, 230°C; $AcONH_4$ 0.5 M (0.2 ml/min post-column). CF-FAB (b): Negative ion mode; FAB tip 50°C; source, 100°C; glycerol 50% (v/v) (0.15 ml/min post-column); LC flow post column split 1:100. (Reproduced by permission of Verlag Helvetica Chimica Acta AG.)

pure form, a stop-flow LC/NMR of a fraction containing both isomers **22** and **23** was performed in order to obtain unambiguous ^{1}H-NMR data for these isomers.

The separation of these constituents needed a good LC resolution and the column could not be overloaded. Thus, long acquisition times of around 30 minutes were necessary to obtain a signal-to-noise ratio around 10 for the aromatic protons of each **22** and **23** (Figure 2.11). The LC/^{1}H-NMR spectrum of **22** (Figure 2.11a) was in total accord with the one obtained by the conventional method, although slight differences could be noticed for the chemical shift values due to the solvent (MeCN-H_2O mixture for on-line measurements). The LC/^{1}H-NMR spectrum recorded for **23** (Figure 2.11b) was very similar to that measured for **22**, as far as the monoterpenic and glycosidic parts of the molecule are concerned. However, the resonances corresponding to the acyl moieties (between δ 5 and 8 ppm) reflected the structural differences of the two isomers **22** and **23**. Indeed, both olefinic protons at δ 7.78 (1H, *d*, J = 16.1 Hz) and 6.47 (1H, *d*, J = 16.1 Hz) ppm were shifted upfield to δ 7.11 (1H, *d*, J = 12.2 Hz) and 5.95 (1H, *d*, J = 12.2 Hz) ppm, respectively. These chemical shifts, together with the values of the coupling constants were characteristic of *cis*-olefinic protons. For both compounds **22** and **23**, four aromatic protons, forming an AA'XX' system, were clearly discernible and corresponded to the phenyl part of a *p*-coumaroyl unit. The presence of a (*Z*)*p*-coumaroyl moiety in compound **23** was thus established and **23** was finally identified as (*Z*)-4′-*O*-(*p*-coumaroyl) lisianthioside, or seemannoside B.

2.6 LC-STRATEGIES FOR THE SCREENING OF SAPONINS

The triterpenoids constitute a large and diverse group of natural products derived from squalene. More than 4000 triterpenes have been isolated so far and almost 40 skeletal types have been identified (Connolly and Hill, 1991). They are widespread in the plant kingdom. Glycosides of triterpenes, also named saponins, commonly occur in higher plant. They are highly polar, non-volatile and thermally labile compounds (Hostettmann and Marston, 1995).

These constituents lack UV chromophore and their partial identification has been almost exclusively performed with LC/MS techniques. These methods provide interesting information about the molecular weight of both the aglycone and glycoside parts. According to the type of LC/MS technique used, different on-line information may be obtained. In this respect, a comparison of LC/ES-MS versus LC/CF-FAB-MS and LC/TSP-MS has been carried out (Wolfender *et al.,* 1995b). The results have shown that LC/TSP-MS provided mainly molecular weight information for saponins bearing up to two sugar units and produced intense aglycone ions but failed to analyse larger glycosides. LC/ES-MS versus LC/CF-FAB-MS were both well adapted to the analysis of large saponins but almost no fragment information could be deduced from the LC/ES/MS spectra, while CF-FAB was providing sugar sequence information. In order to generate both molecular

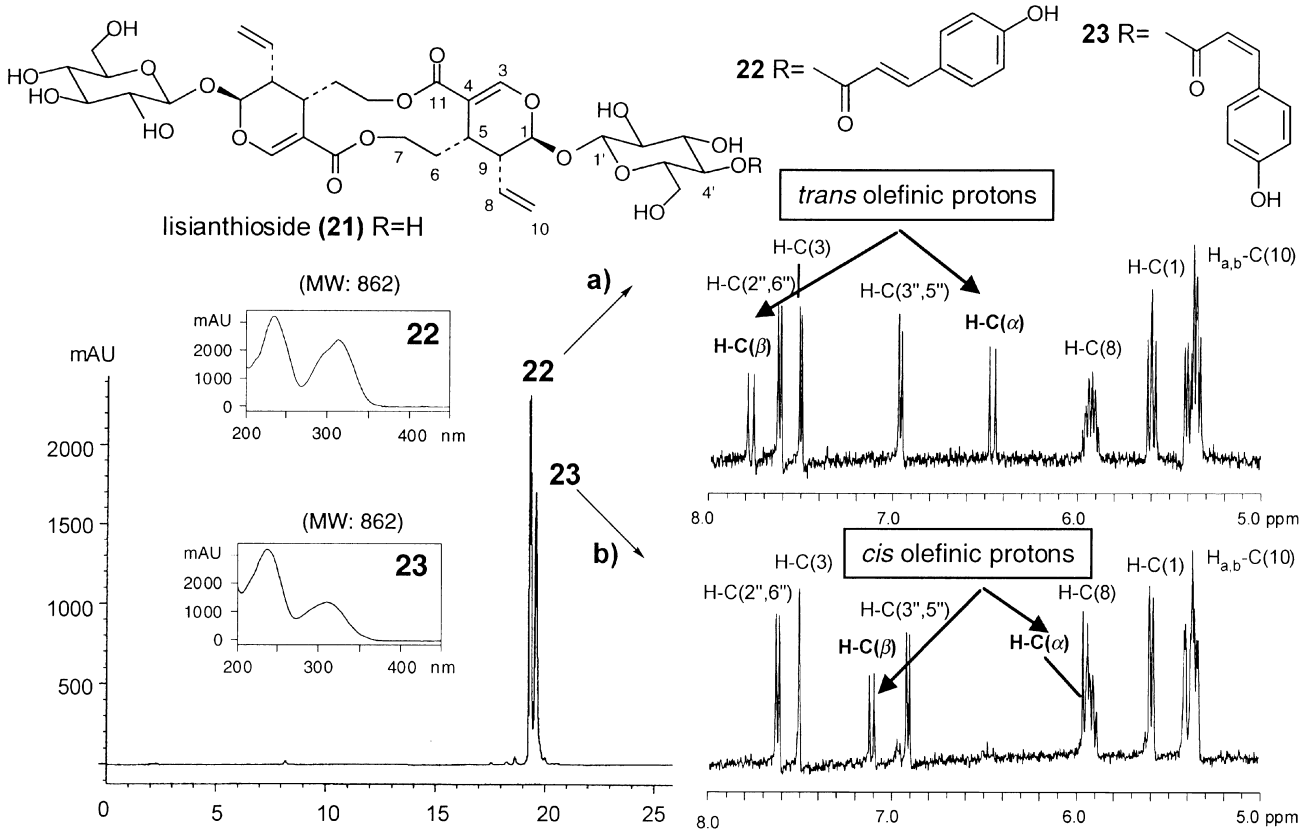

Figure 2.11: LC/UV and LC/^{1}H-NMR analysis of a fraction of the MeOH extract of *Lisianthius seemanii* (Gentianaceae) aerial parts containing both isomers **22** and **23** (Rodriguez *et al.*, 1998). HPLC, same conditions as in Figure 2.5. Insert: expansion of the aromatic region of the stop-flow LC/^{1}H-NMR of **22** and **23**. (Reproduced by permission of Verlag Helvetica Chimica Acta AG.)

information and sugar sequence fragment, the combined use of LC/ES/MS in the negative ion mode and multiple stage MS/MS (MS^n) has been particularly useful.

2.6.1 LC/MS^n analysis of saponins from *Phytolacca dodecandra*

The LC/ES-MS analysis of saponins in the negative ion mode provide mainly molecular weight information, with MS response in the ng range, but almost no fragment ions. Thus, in order to obtain more structure information, MS/MS experiments have to be performed. Multiple stage MS/MS experiments (MS^n) can be performed with an ion-trap mass analyser on compounds such as glycosides to determine their sugar sequence (Wolfender *et al.,* 1995b). With such an instrument, only one ion of interest can be isolated and excited. At the same time, the other consecutive reactions usually observed in a high-pressure collision cell are reduced. By adjusting the collision energy, it is possible to cleave only one sugar at a time, making the interpretation of the spectra very simple.

For example, a MS^n experiment was performed on a pentaglycosylated saponin **24** from *Phytolacca dodecandra* (Phytolaccaceae), a plant used for the local control of schistosomiasis (Figure 2.12) (Wolfender *et al.,* 1995b). The TFA anion adduct at *m/z* 1363 (Figure 2.12a) was easily fragmented in the ion trap giving the deprotonated ion *m/z* 1249 (Figure 2.12b). This latter $[M-H]^-$ ion yielded a first fragment at *m/z* 1087 (-Glc) (Figure 2.12c) which then cleaved into ions at *m/z* 925 or 941 showing the simultaneous loss of a glucosyl or a rhamnosyl unit respectively. These losses were characteristic of a branched sugar chain. The ion *m/z* 779 (Figure 2.12e) issued from the fragmentation of *m/z* 925 (-Rha) or 941 (-Glc). Finally, the *m/z* 617 ion and the aglycone ion at *m/z* 455 were observed from cleavage of *m/z* 779.

This type of MS^n experiment was found to be very helpful for clarifying the sugar sequence of saponins. It can also be performed on-line in scan-dependent experiments where the ion trap automatically isolates the molecular ion of the eluting saponins and carried out automatically several MS/MS experiments on it, before analysing another LC peak (Perret *et al.,* 1999).

2.7 LC-STRATEGIES FOR THE RESOLUTION OF CLOSELY ELUTING ISOMERS

As shown, LC/NMR has not often been used for the total screening of all constituents of a crude plant extract, especially because its inherent lack of sensitivity has hindered the satisfactory detection of minor constituents. This technique, however, is of great interest for investigating enriched fractions or for obtaining distinct proton spectra when closely related compounds can not be isolated.

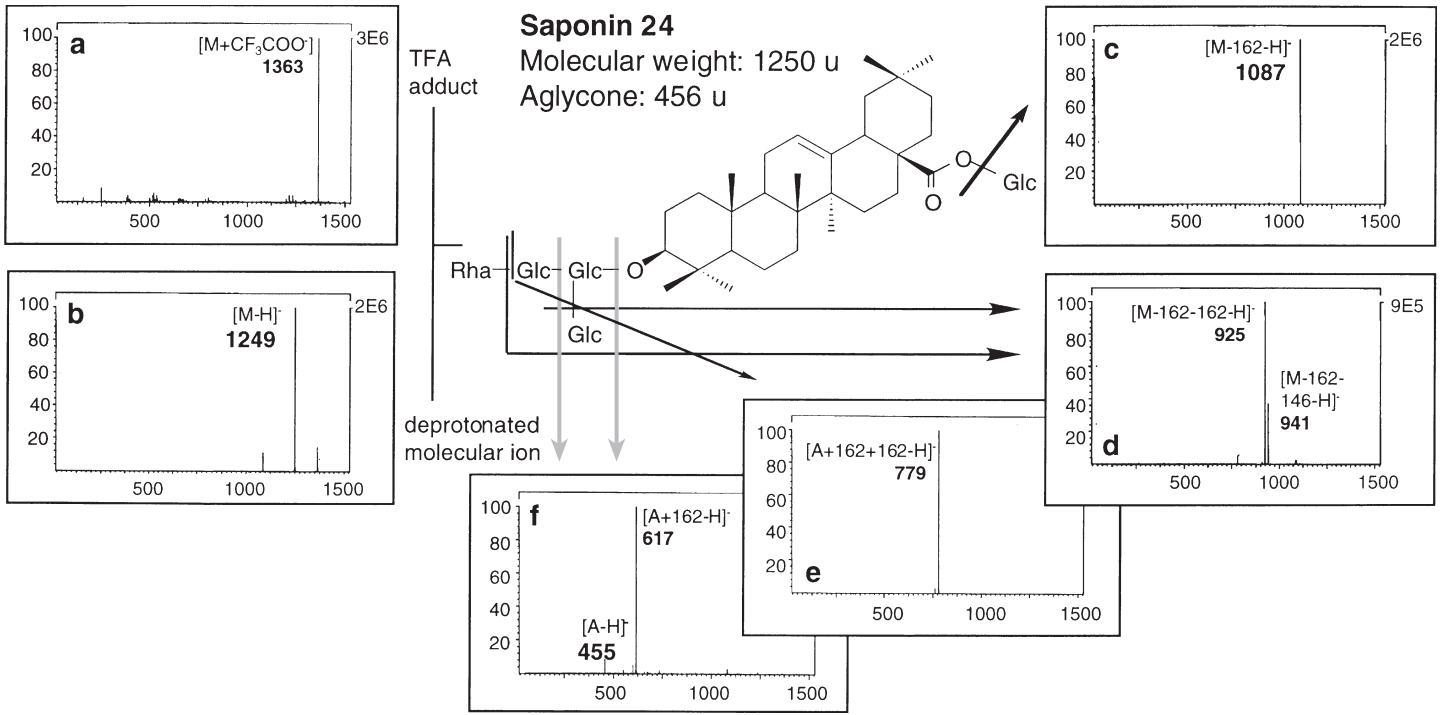

Figure 2.12: LC/ES-MS^n spectra of the pentaglycosylated saponin **24** from the methanolic fruit extract of *Phytolacca dodecandra* (Phytolaccaceae) (Wolfender *et al.*, 1995b). Sample (1 mg/ml) injected by a syringe pump (5 µl/min) (infusion experiment), buffer: TFA 0.05%, negative ion mode. This experiment allowed a sequential fragmentation of the saponin sugar chain (cleavage of only one sugar at each MS/MS step), clarifying the structure determination. (Reproduced with permission of John Wiley & Sons Limited.)

2.7.1 In-mixture 2D NMR and LC/^{1}H-NMR analysis of naphthoquinones from *Cordia. linnaei*

As an example, LC/UV/MS and LC/NMR was performed on a fraction of the root dichloromethane extract of a Panamanian shrub *Cordia linnaei* (Boraginaceae) (Ioset *et al.,* 1999). This plant was indeed interesting for its antifungal and larvicidal properties and its phytochemical investigation yielded several 6-alkyl-substituted naphthoquinones (Ioset *et al.,* 1998). While the main constituents of this plant were isolated successfully, 2 minor closely related naphthoquinones (**25** and **26**) could not be separated satisfactorily, even after several trials. LC/UV/MS and LC/NMR analyses of the fraction containing the mixture **25**/**26** were thus performed in order to differentiate efficiently these closely related naphthoquinones. LC/UV/MS data indicated that **25** and **26** were probably isomers of naphthoquinones with relatively large substituents. Indeed, for both compounds, intense ammonium adducts at *m/z* 424 $[M + NH_4]^+$ were recorded. The possible presence of a weak bond such as an ester bond was suggested by the fragments observed at *m/z* 307 for **25** and **26**. Confirmation of the molecular weights was obtained in the EI-MS analysis of the fraction (*m/z* 406 $[M]^{+\cdot}$). On-flow LC/^{1}H-NMR analysis were performed on 400 µg of this fraction using a MeCN-D_2O gradient on a C-18 column (Figure 2.13).

Clear LC/^{1}H-NMR spectra for **25** and **26** were obtained. Both compounds were found to show identical signals in the aromatic region: two protons at δ 7.09 (2H, H-2, 3) and three protons forming an AMX system (1H, δ 8.07, H-8; 1H, δ 7.96, H-5 and 1H, δ 7.76, H-7) characteristic of 6-substituted naphthoquinones. A careful study of the two spectra indicated the presence of supplementary resonances at δ 6.91 (1H, *q, J* = 6.8 Hz, H-22), δ 1.87 (3H, *s*, CH_3-23) and δ 1.82 (3H, *d*, *J* = 7.2 Hz, CH_3-24) in the case of **25** and of an extra singlet at δ 5.76 (1H, *bs*, H-21) for **26** (Figure 2.13). For this latter compound, two more methyl groups appeared at δ 1.90 (3H, *s*, CH_3-24) and δ 2.13 (3H, *s*, CH_3-23) after reinjection of the fraction using a MeOH:D_2O gradient (MeOH, δ 3.35). These signals were not visible when the spectra were measured under the MeCN:D_2O conditions because of their location under the suppressed peak of the solvent (MeCN, δ 2.10). The on-flow LC/^{1}H-NMR data of fraction E suggested **26** to be cordiaquinone C and indicated that the difference between the two compounds was probably due to the nature of the acidic unit esterified to the cyclohexane ring.

As the ^{1}H-NMR resonances of **25** and **26** were clearly assigned due to their individual LC/^{1}H-NMR spectra, 2D NMR experiments were performed directly on 40 mg of fraction E without further separation. This approach permitted the measurements of rapid NMR experiments on a concentrated sample avoiding time-consuming 2D stop-flow LC/NMR experiments of analytical LC quantities. For **26**, correlations between the methyl groups (δ 1.90 and δ 2.13) and the broad singlet at δ 5.76 were observed in a selective TOCSY

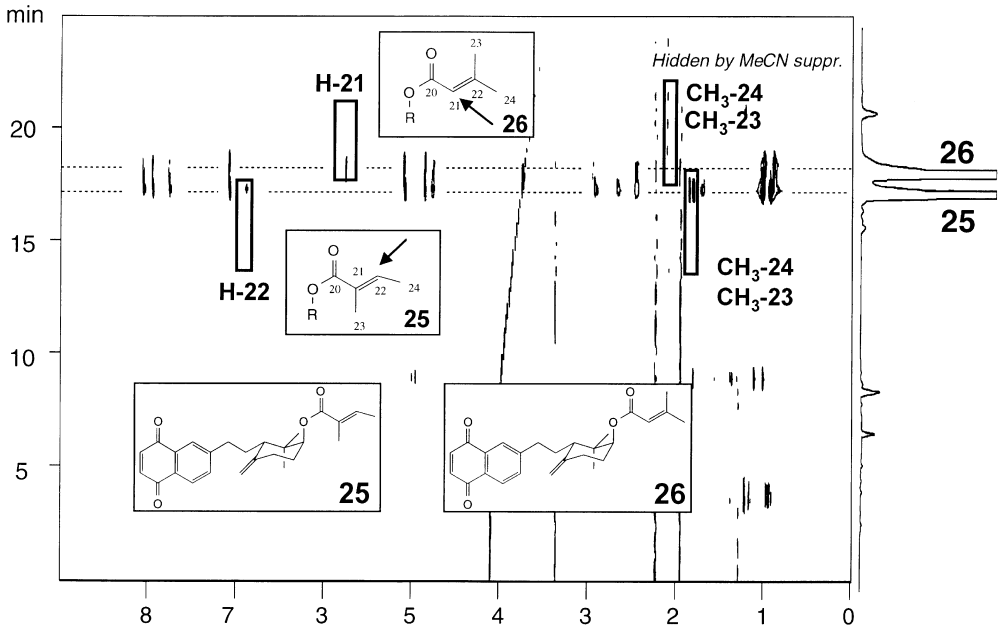

Figure 2.13: Bidimensional on-flow LC/^{1}H-NMR chromatogram of fraction E from the dichloromethane extract of *Cordia linnaei* (Boraginaceae) (Ioset *et al.,* 1999). HPLC: C18 Nova-Pak (5 µm, 250 × 3.9 mm i.d.); MeCN-D_2O gradient (35:65 to 95:5 in 30 min); 1 ml/min. Injection: 0.4 mg. LC/NMR: 24 scans/increment, flow cell (60 µl, 3 mm i.d.), 500 MHz. (Reproduced with permission of John Wiley & Sons Limited.)

experiment on H-21 (δ 5.76) (Figure 2.14b). The final attribution of the ^{1}H and ^{13}C signals of **26**, based on the combination of LC/^{1}H-NMR of **26** and COSY, HSQC, HMBC and ^{13}C spectra of fraction E, were in perfect agreement with the literature data of cordiaquinone C, indicating that the acidic unit esterified to the cyclohexane ring was, in this case, senecioic acid. For **25**, a selective TOCSY experiments on H-22 at δ 6.91 of fraction E gave correlations between this signal and the two methyl groups at δ 1.87 and δ 1.82, suggesting the substituent in **25** to be a tiglic acid moiety (Figure 2.14a). As for **26**, this result was confirmed by COSY, HSQC and HMBC experiments. Compounds **25** and **26** were thus found to differ only in the nature of the ester moiety. **25** was found to be a new naphthoquinone and has been named cordiaquinone H (Ioset *et al.,* 1999).

2.8 CONCLUSIONS

LC-hyphenated techniques are playing an increasingly important role as a strategic tool to support phytochemical investigations. Indeed, these techniques provide a great deal of preliminary information about the content and nature of constituents of crude plant

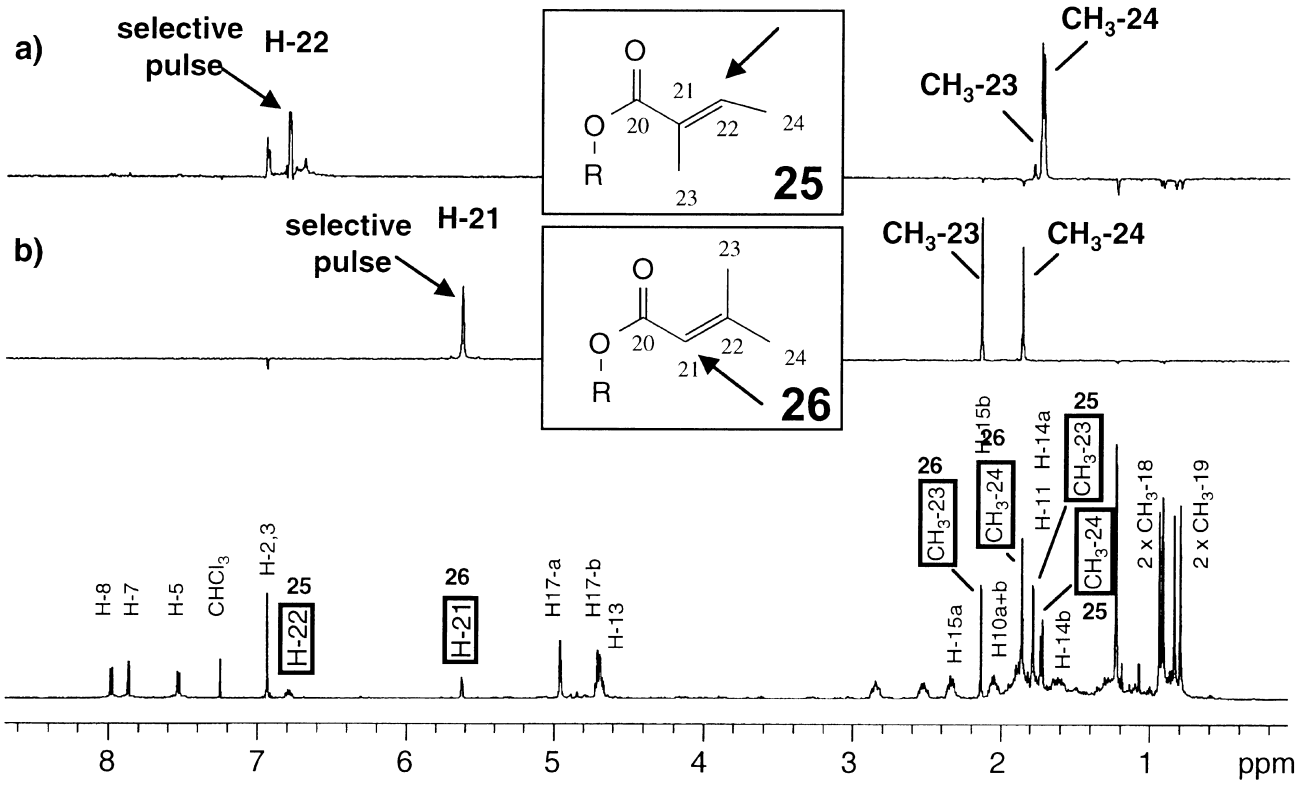

Figure 2.14: In mixture ^{1}H-NMR spectrum of fraction E (40 mg, $CDCl_3$) and TOCSY 1-D selective pulse experiments performed on the specific protons at δ 6.78 of **25** and δ 6.52 of **26** (Ioset *et al.*, 1999). (Reproduced with permission of John Wiley & Sons Limited.)

extracts. In certain cases, combination with a spectral library and pre- or post-column derivatisation allows structure determination on-line. This is very useful when large numbers of samples have to be processed because unnecessary isolation of known compounds is avoided. Once the novelty or utility of a given constituent is established, it is then important to process the plant extracts in the usual manner, to obtain samples for full structure elucidation and biological or pharmacological testing.

In this chapter, the potential of LC/UV, LC/MS, LC/MS-MS and LC/NMR has been discussed through various examples of secondary plant metabolite analysis. It has been also shown that, according to the type of natural product to be analysed, one or the other of these hyphenated techniques will be more valuable.

LC/MS analysis of crude plant extracts is not straightforward due to the great variety of their constituents. As has been shown, no interface allows an optimum ionisation of all the metabolites within a single crude plant extract. Often, different ionisation modes or different interfaces are necessary to obtain a complete picture of the extract composition.

The recent introduction of LC/NMR for the crude plant extract screening will probably make another breakthrough in the on-line structural determination of natural products. This hyphenated method allows the recording of precious complementary on-line structure information when LC/UV/MS data are insufficient for an unambiguous peak identification. Indeed, LC/NMR has proven to be very effective in obtaining 1D spectra on both flowing and non-flowing samples, as well as stop-flow 2D spectra. However, compared with UV or MS, NMR remains a rather insensitive detection method and the need for solvent suppression in conventional LC/NMR restricted the observable NMR range. In the on-flow mode, this technique is, at present, limited to the characterisation of the major constituents of crude plant extracts. In order to obtain ^{1}H-NMR spectra of minor metabolites or for bidimensional experiments, the use of the stop-flow mode is necessary.

With the full set of spectroscopic information obtained by LC/UV, LC/MS and LC/NMR, the phytochemist will be able to characterise rapidly the main constituents of a given plant and to choose carefully which metabolites are to be isolated for in-depth structural or pharmacological study. The chemical screening of extracts with such elaborate hyphenated techniques generates a huge amount of information. In order to rationalise this approach and to use it efficiently with a high sample throughput, the next challenge will be to find a way to centralise all these data for rapid pattern recognition by reference to standard compounds. With such an analytical system, phytochemists will then be able to concentrate their efforts on finding new biological targets. This aspect still remains the more difficult problem to solve when searching for new leads.

ACKNOWLEDGEMENTS

Financial support was provided by the Swiss National Science Foundation.

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CHAPTER

3

Applications of Modern NMR Techniques in the Structural Elucidation of Bioactive Natural Products

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Contents

3.1 INTRODUCTION

Nuclear Magnetic Resonance (NMR) techniques have proved to be powerful tools for the structural determination of organic compounds since the beginning of their applications in chemistry. They are particularly useful in the field of natural products, where frequently the problem occurs of elucidating the structure of a completely novel compound, sometimes possessing unusual or unprecedented structural features.

Early NMR spectrometers, equipped with permanent magnets working at low magnetic fields (1.41 Tesla, corresponding to a 60 MHz carrier frequency for ^{1}H nuclei), exclusively allowed the acquisition of ^{1}H NMR spectra, affording structural information complementary to that available from chemical analysis or well-established physical methods like Ultraviolet (UV) or Infrared (IR) spectroscopies, or from the technique of Mass Spectrometry (MS), at that time routinely used by chemists. Nowadays modern NMR spectroscopy performed with cryospectrometers operating at high magnetic field (up to 21.14 Tesla, i.e. 900 MHz for ^{1}H) and capable of executing a variety of sophisticated, multipulse and multidimensional experiments, has largely superseded the above methods. Thus, it has become the main choice for establishing chemical structures, particularly when X-ray crystallography is inapplicable. It requires only minute amounts of substance and frequently also allows the determination of stereochemical details, or even preferred conformation in solution. In addition, the variety of experiments available on modern NMR instruments has considerably reduced the time needed for the identification of known compounds, thus giving valuable help to the chemical knowledge of previously unexamined natural sources.

The present importance and widespread adoption of NMR analysis, coupled with the growth of interdisciplinary research focused on bioactive secondary metabolites, justify the present Chapter, devoted to applications of the modern NMR techniques mainly used in natural products chemistry. Part of this chapter will briefly describe the more common experiments on the basis of a practical approach, setting aside any theoretical treatment of the relevant physical phenomena (for the physical principles of NMR *see:* Abragam, 1961; Ernst *et al.*, 1987; Kessler *et al.*, 1988; Ernst, 1992). Examples of applications to structural determination and spectral assignment of bioactive natural products will be included, with emphasis on the strategy/methodology, rather than on experimental details. A large number of books, reviews and articles are today available for fully understanding NMR experiments as well as for carrying out most of them (Derome, 1987; Sanders and Hunter, 1987; Martin and Zektzer, 1988a; Braun *et al.*, 1996). Several of these are rich in data and applications on natural products (*see* for example: Sadler, 1988; Breitmaier and Voelter, 1987; Derome, 1989; Nakanishi, 1990; Martin and Crouch, 1991; Breitmaier, 1993; Friebolin, 1993; Atta-ur-Rahman, 1996).

3.2 BASIC PRINCIPLES

NMR spectroscopy originates from the fact that nuclei (like electrons) possess an angular momentum, described by a spin quantum number $\boldsymbol{I}$, which gives rise to a nuclear magnetic dipole when $\boldsymbol{I} \neq 0$. Consequently, when these nonzero-spin nuclei are immersed in a magnetic field of strength $\boldsymbol{B_0}$ their nuclear magnetic dipoles assume two (or more) relative orientations with respect to it, thus producing two (or more) corresponding energy levels. Between these levels a spectroscopic transition may occur by absorption of electromagnetic radiation of appropriate frequency (ν, known as the Larmor frequency, which usually falls in the radiofrequency region, *ca.* 1–1000 MHz), in accordance with the following fundamental equation

$$\nu = \gamma B_0/2\pi$$

where γ is a constant (called *gyromagnetic ratio*) for the type of nucleus considered.

The most important nuclei in the field of natural products have spin number $\boldsymbol{I} = \frac{1}{2}$ and are (in ranking order) ^{1}H, ^{13}C, ^{15}N, ^{31}P, and ^{17}O, whose properties are reported in Table 3.1. Among these, ^{1}H is considerably more sensitive because of its high natural abundance and high gyromagnetic ratio, and therefore it is the first-choice nucleus to be investigated. It is followed by ^{13}C, whose importance is obviously linked to the carbonious nature of the skeleton of natural products, while the remaining nuclei are less common and hence only used to solve specific problems.

Four fundamental properties are associated with the magnetic resonance for a given nucleus, namely **chemical shift**, **intensity**, **scalar coupling** (*spin–spin coupling*), and **dipolar coupling** (evidenced in the nuclear Overhauser effect), which are considered in the following sections.

TABLE 3.1
Properties of some nuclei occurring in natural products

Isotope	Spin I	Natural abundance (%)	Gyromagnetic Ratio γ (10^7 rad T^{-1} s^{-1})	NMR frequency (MHz) at 11.74 Tesla	Relative sensitivity[a]
^{1}H	1/2	99.985	26.752	500.00	1.0
^{2}H	1	0.015	4.107	76.75	9.65×10^{-3}
^{12}C	0	98.89	—	Not active	—
^{13}C	1/2	1.11	6.728	125.72	1.59×10^{-2}
^{14}N	1	99.63	1.934	36.12	1.01×10^{-3}
^{15}N	1/2	0.37	−2.713	50.66	1.04×10^{-3}
^{31}P	1/2	100.00	10.839	202.40	6.63×10^{-2}
^{16}O	0	99.96	—	Not active	—
^{17}O	5/2	0.04	−3.628	67.79	2.91×10^{-2}

[a]For constant field and equal number of nuclei. The actual relative intensity of an NMR signal can be obtained from the product of the relative sensitivity and the natural abundance.

3.2.1 Chemical shift

The exact resonance frequency of a nucleus is strongly dependent on the electron density on it, which induces a diamagnetic shielding of the nucleus from the external magnetic field. Therefore, nuclei in different chemical environments will resonate at different frequencies with respect to a reference nucleus. This difference is called *chemical shift* (δ) and can be expressed in part-per-million (ppm) by the following equation

$$\delta = (\nu - \nu_{\text{reference}})10^6/\nu_{\text{reference}} \text{ (ppm)}$$

The reference resonance for both ^{1}H and ^{13}C NMR is that of tetramethylsilane [TMS, $Si(CH_3)_4$] to which is assigned the δ value 0.00.

Often two or more nuclei may show the same chemical shift and they are called *isochronous*. This may occur if they experience the same chemical environment, because of symmetry relationship or fast rotation, and, therefore, they are called 'chemically equivalent'. Alternatively, diverse nuclei (i.e. chemically non-equivalent) may be isochronous by a casual coincidence of their chemical shifts, so we speak of 'accidental isochrony'.

From the above definition it is obvious that chemical shift values are independent of the external magnetic field at which the spectrum is acquired. Usually ranging from 0 to 12 ppm for ^{1}H and from 0 to 230 ppm for ^{13}C, they give a strong indication of the functional groups present in a molecule. As an example, Figure 3.1 gives some typical resonances for ^{1}H (Jackman and Sternhell, 1969). A similar trend is observed for ^{13}C (Breitmaier and Voelter, 1987; Kalinowski *et al.*, 1988) and other nuclei (Mason, 1987).

3.2.2 Intensity

The intensity of a ^{1}H NMR signal (the area underneath the peak, obtained by integration) is proportional to the number of protons that originate it, and therefore it gives useful information in assessing the structural formula. The intensity of ^{13}C NMR resonances is (usually) not directly proportional to the number of carbon nuclei because of various phenomena (different relaxation times and different NOE enhancement) but, if wanted, an acceptable proportionality can be imparted by acquiring the spectrum under particular conditions (Shoolery, 1977).

3.2.3 Scalar coupling

Two nuclei (e.g. ^{1}H/^{1}H or ^{1}H/^{13}C) that are chemically not equivalent can influence each other through the intervening bonding electrons because of their mutual spin-pairing tendency. This results in the splitting of both signals, which will appear as two lines (*doublet*, d) with identical separation. This separation (frequency difference) is expressed

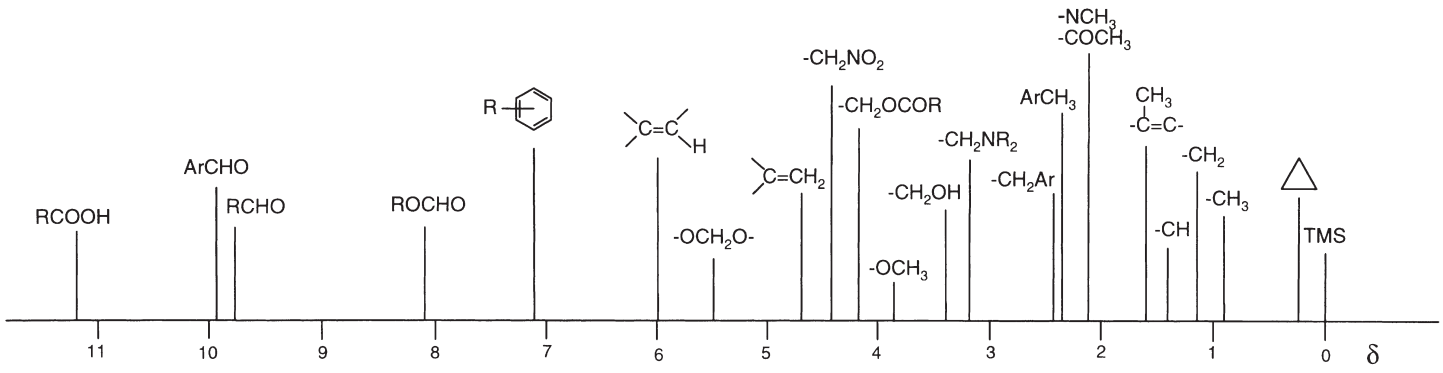


Figure 3.1: ^{1}H NMR chemical shift for typical functional groups. The vertical lines indicate an average value for each generic group.

in hertz (Hz) and is called the *coupling constant*, *J*. The coupling of nuclei (also called **spin–spin coupling**) is effective when the two nuclei are linked through 1, 2 or 3 bonds (^1J, ^2J or ^3J constant, respectively), whereas at longer distances it usually becomes negligible. The *J* value is independent of the applied magnetic field strength, but it is dependent on the nature of the intervening bonds and on the angular relationship of the nuclei. In particular, ^3J constants depend on the dihedral angle between the nuclei and follow the 'Karplus equation', thus giving useful stereochemical information (Karplus, 1959; Haasnoot *et al.*, 1980; Marshall, 1983). Long-range coupling (^4J or longer), when occurring, indicates multiple-bond conjugation or peculiar stereochemical arrangement.

A nucleus coupled to n other equivalent nuclei will appear as a *multiplet* of $n + 1$ lines, separated by the same *J* constant (e.g. for $n = 3$ it will appear as a *quartet*, q). Instead, if the n nuclei are not equivalent it will appear as a '*multiple-multiplet*' with n different *J* constants (e.g. for $n = 3$ it will appear as a *double-double-doublet*, ddd, with three *J* constants and $2^n = 8$ lines). Therefore, the analysis of a multiplet structure affords useful information on the environment of the given nucleus. This analysis is somewhat impeded when the chemical shift difference ($\Delta\nu$) of the coupled nuclei is comparable with the *J* constant ($\Delta\nu/J \leq 10$, *strongly coupled* nuclei), thus giving rise to 'second order' multiplets no longer obeying to above 'first order' rules. In these cases a detailed quantum-mechanics-based analysis can be used for the interpretation and to extract the correct ν e *J* values (Günther, 1980; Becker, 1980), which may be achieved by using a computer simulation program (Diehl *et al.*, 1972; Haegele *et al.*, 1987). The alternative (when available) is the acquisition of the ^{1}H NMR spectrum at a higher magnetic field. In this case the difference in hertz between two signals becomes larger (because $\Delta\nu$ is directly proportional to $\boldsymbol{B}_0$) while the coupling constant is unmodified, thus giving a higher $\Delta\nu/J$ and hence a first-order multiplet more amenable to interpretation. For this reason and for the concomitant gain in sensitivity there is a continuous demand for spectrometers with higher operating field.

Scalar coupling between two protons or between a proton and a carbon can be evidenced in several ways, thus probing the structural connections of a molecule. These methods, constituting the large majority of tools of modern NMR analysis, will be detailed in the following sections.

3.2.4 Dipolar coupling

In addition to scalar coupling, two nuclei, not necessarily directly bonded but close spatially, can mutually interact by a through-space dipole–dipole mechanism (Noggle and Schirmer, 1971; Neuhaus and Williamson, 1989). This interaction does not give any effect in a normal spectrum. It can only be observed when a perturbation of one nucleus, in this case by radiofrequency irradiation, stimulates an alternative relaxation path for the other, as a result of which a corresponding increase in signal intensity is

observed (nuclear Overhauser enhancement, NOE). The magnitude of this enhancement is strongly dependent on the distance r between the two nuclei, being proportional to r^{-6}. The maximum NOE enhancement factor is

$$\eta = \gamma_{\text{irradiated}}/2\gamma_{\text{observed}}.$$

Therefore, the resulting intensity I of the observed signals, with respect to the original unperturbed intensity I_0, will be

$$I = I_0 + \eta I_0 = (1 + \eta)I_0.$$

Consequently, in homonuclear NOE a maximum increase of 50% can be observed, whereas the enhancement for ^{13}C upon ^{1}H irradiation can reach up to 200%. In this latter case, a strong variation of signal intensity is observed in relation to the number of hydrogens directly bonded to the carbon atom.

In contrast to scalar coupling, the effectiveness of dipolar coupling is strongly dependent on the strength of magnetic field and on the so-called *correlation time* of the molecule, which, in turn, is related to the speed of molecular tumbling in solution and to molecular weight. In fact, low molecular weight (short correlation times) and weak magnetic fields (the so-called 'extreme narrowing' conditions) originates a positive NOE, whereas high molecular weight (long correlation times) and strong magnetic fields (the so-called 'spin diffusion' conditions) give rise to negative NOE. In the intermediate situation the NOE enhancement can be very weak or even null. NOE has found widespread application in the determination of geometrical and stereochemical relationships in natural products.

3.2.5 Multipulse and multidimensional NMR

In the early decades NMR spectra were acquired in the *Continuous Wave* (CW) mode, that is by immersion of the sample in a magnetic field and recording of its radiofrequency absorption, while the rf (or the magnetic field) was sweeping in the chemical shift range. The CW acquisition was rather slow and required a large amount of sample, thus precluding the observation of less sensitive nuclei (e.g. ^{13}C). Instead, modern NMR spectrometers work in the *pulsed Fourier Transform* mode (FT NMR) by which the sample, immersed in the magnetic field, is irradiated for a short time period (a rf pulse of μs length) with all the frequencies of the range. Then the resonating frequencies are detected in a receiver coil as the envelope of oscillating signals as function of time [$\mathbf{S}(t)$, the so-called *Free Induction Decay*, FID]. A mathematical Fourier transformation of this FID converts the signals as function of time to signals as function of frequency

$$\mathbf{S}(t) \xrightarrow{FT} \mathbf{S}(\nu),$$

thus affording the usual spectrum. In order to perform the FT mathematical treatment the FID has to be stored in digitized form in a computer memory, thus allowing the summing up of the signals coming from repeated scans. In this way, the NMR spectrum can be acquired with minute amount of sample or for rather insensitive nuclei (e.g. ^{13}C).

In conclusion, the acquisition of a FT NMR spectrum can be divided into the following blocks (Figure 3.2a):

preparation – pulse (excitation) – **detection** (t_2)

A better intuitive understanding of the FT NMR can be obtained by using a pictorial vector model. Let us consider a sample containing nuclei with spin $\boldsymbol{I} = \frac{1}{2}$ (^1H, for example). Each individual nucleus, when immersed in a magnetic field, can assume only two quantized orientations, called α and β, respectively aligned or *anti*-aligned to the central $\boldsymbol{B}_0$ field and rotating around it with a precessional motion (Figure 3.2b).

Because of the thermal Boltzmann distribution, the more stable α state will be slightly more populated than the β state and a macroscopic net magnetization $\boldsymbol{M}_z$ aligned with the $\boldsymbol{B}_0$ field (which by convention is assumed as the *z*-axis) will result. At

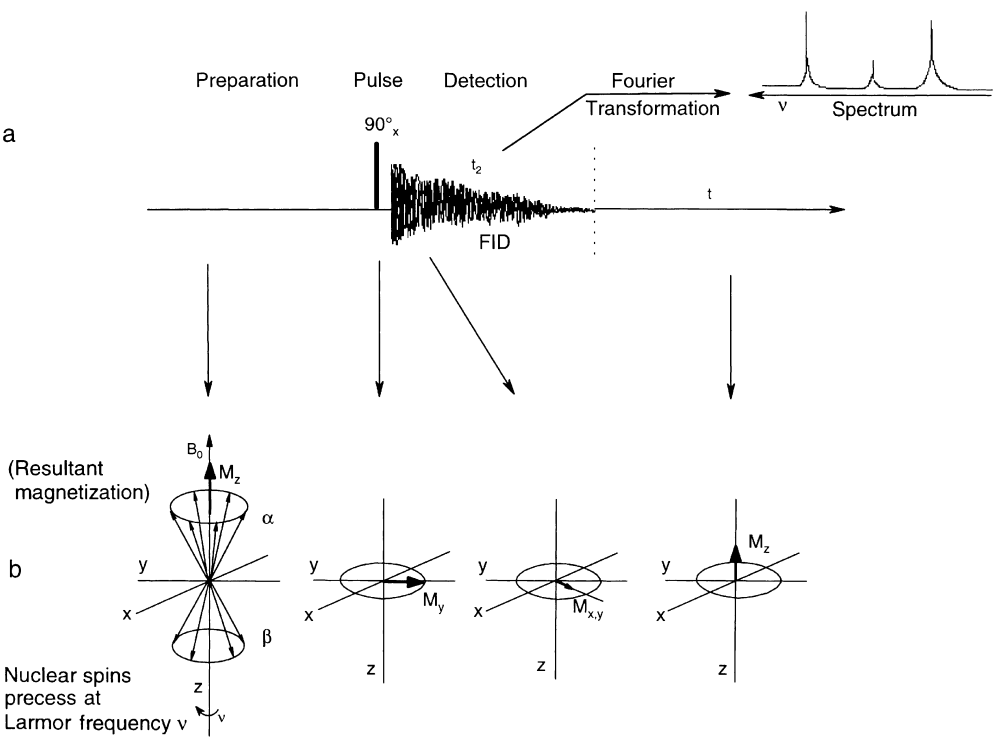


Figure 3.2: Principle of FT NMR spectroscopy: (a) sequence of events in the acquisition of a simple 1D NMR spectrum and (b) corresponding representation of the evolution of the initial steady-state magnetization vector.

this point, the application of a radiofrequency pulse along the *x*-axis will induce, by means of its magnetic component, the rotation of $\boldsymbol{M}_z$ around the *x*-axis in the *z*-*y* plane. The extent of this rotation depends on the duration of the rf pulse therefore, by using a pulse of proper length (a 90° pulse) the original $\boldsymbol{M}_z$ magnetization can be tilted by 90°, thus lying in the *y*-axis. Immediately after the pulse, this $\boldsymbol{M}_y$ magnetization will be detected by a receiver coil situated in the *y*-axis, which will give a signal oscillating as a function of time (the FID). This signal is exponentially decaying because of relaxation phenomena, namely energy exchanges with other nuclei (*spin–spin* or *transverse* relaxation) or with neighboring molecules (*spin–lattice* or *longitudinal* relaxation). The $\boldsymbol{M}_y$ magnetization is continually decreasing and, after some time, will be nullified, while the original $\boldsymbol{M}_z$ vector will be slowly restored. When the equilibrium distribution is recovered, a second excitation pulse can be applied to give a new FID which can be added to the previous one, and so forth.

Soon after the advent of pulsed FT-NMR, it was recognized that nuclear spins can be 'manipulated' by a proper sequence of suitable pulses and time intervals leading to a transfer of magnetization (or, more generally speaking, *coherence*) from a nucleus to another coupled to it, either by scalar or by dipolar coupling. In this way, it is possible to detect which nuclei are coupled to a given one by exciting it and observing where the magnetization has gone.

Indeed, nowadays several pulse sequences are available which allow, literally, the 'driving' of the magnetization through the nuclear spins. This 'driving' is normally obtained through the so-called *phase-cycling* by a suitable change of sign and direction of pulses and position of the receiver, in order to select the wanted transfers and concomitantly to suppress the co-occurring unwanted ones. To this end, the use of additional pulsed (magnetic) field gradients (PFG) has recently been introduced, which requires a reduced phase-cycling (Baker and Freeman, 1985; Hurd, 1990; Keeler *et al.*, 1994). In a few cases the magnetization transfer can be understood by using the above pictorial model, but a more general description has to resort to quantum-mechanical treatment (Ernst *et al.*, 1987) or simplified *product operator formalism* (Kessler *et al.*, 1988). A detailed description of multipulse experiments is beyond the scope of the present chapter, which is focused on their practical use in structural elucidation.

The tracking of magnetization transfers in multipulse experiments can be achieved by using a frequency-selective excitation of a given nucleus with selective pulses (*shaped soft pulses*) (Kessler *et al.*, 1991) and recording NMR spectra in the usual way, i.e. one-dimensional spectra. In 1D spectra the frequency (chemical shift) is the dimension to which we refer, the intensity of the signals being ignored. An alternative and very useful way to observe these magnetization transfers is a 2D spectrum, constituted by two frequency dimensions and a third one related to signal intensity, thus giving a 3D surface. For easy inspection, a 2D NMR spectrum is usually plotted as a map where the signal intensity appears as contour lines. In a typical 2D NMR spectrum the magnetization transfer produces a correlation signal (*cross-peak*) at the crossing of frequencies of the

two interacting nuclei. A pulse sequence for 2D NMR can be conceptually divided into the following blocks

preparation – evolution (t_1) – **mixing** (coherence/magnetization transfer) – **detection** (t_2).

Technically speaking, the 2D NMR spectrum is obtained by acquiring a series of 1D FIDs (usually 256 or 512) in which a time interval (t_1, *evolution time*) of the pulse sequence is progressively incremented (Figure 3.3).

In this way the signal will be a function of the two time-variables t_1 and t_2 [$\mathbf{S}(t_1,t_2)$]. Therefore, two mathematical Fourier transformations can be consecutively applied to it

$$\mathbf{S}(t_1,t_2) \xrightarrow{FT(t_2)} \mathbf{S}(t_1,\nu_2) \xrightarrow{FT(t_1)} \mathbf{S}(\nu_1,\nu_2)$$

to give the 2D spectrum [i.e., a signal as function of two frequencies, $\mathbf{S}(\nu_1,\nu_2)$]. In other words, the series of FIDs is converted, by the first FT, into a series of 1D spectra in which any given signal (having a ν_2 frequency) is 'modulated' by the frequency of the nucleus from which the magnetization transfer was originated. Thus, for each ν_2 frequency, a signal as function of t_1 (a virtual FID) is obtained, whose Fourier transformation furnishes the second frequency (ν_1) of the 'modulating' nucleus.

The advantage of a 2D spectrum over the corresponding 1D spectrum is the simultaneous observation of many magnetization transfers at once. In addition, the 2D spectrum allows the resolving of ambiguities due to accidental isochronies by observing the coupled counterpart in the other dimension. However, since its acquisition usually requires more time, the 1D spectrum will be preferred when only limited information is required.

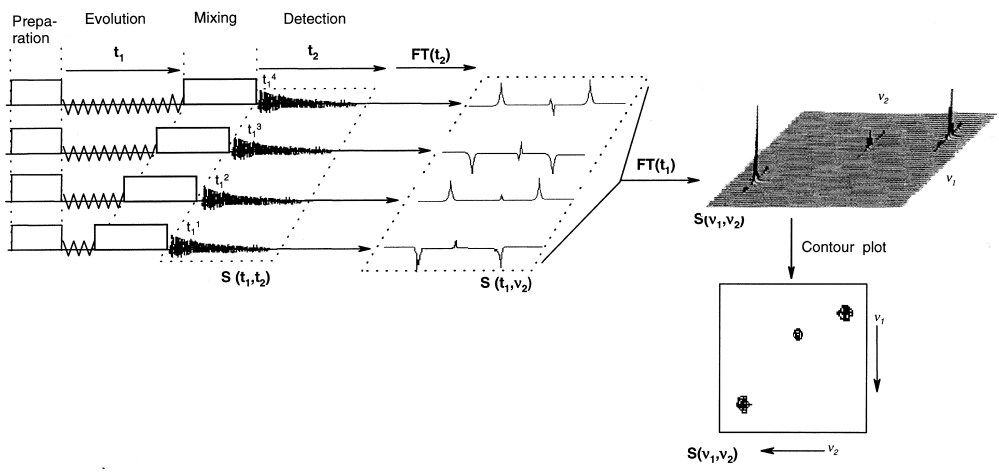


Figure 3.3: Principle of 2D NMR spectroscopy. A generic unspecified 2D correlation experiment is used for the illustration.

The concept can be extended beyond the 2D spectrum can be further extended by introducing one or two additional evolution times and magnetization transfers, hence a third and a fourth dimension, to give, respectively, 3D and 4D NMR spectra, which have found fundamental applications in the 3D-structure determination of proteins (Clore and Gronenborn, 1993).

As has been said before, magnetization transfer in a pulse sequence can be obtained by exploiting any kind of scalar or dipolar coupling, either homonuclear or heteronuclear. Therefore, all pulse sequences may be divided into two broad classes related to scalar or dipolar coupling. Each of them can then be further divided into homonuclear and heteronuclear experiments. Each experiment is usually known by an acronym (sometime quite picturesque), which is explained in the following, just after it is given. Because of their large number, these acronyms may sometime be a source of confusion for beginners.

Homonuclear scalar experiments are employed to trace the network of coupled spins of the same isotope. For example, ^{1}H-^{1}H couplings can be evidenced with these common pulse sequences, either in 1D or 2D form: COSY (*COrrelation SpectroscopY*), Long-Range COSY, TOCSY (*TOtal Correlation SpectroscopY*, also known as HOHAHA, *HOmonuclear Hartmann Hahn*). Analogously ^{13}C-^{13}C couplings can be traced using 1D or 2D INADEQUATE *(Incredible Natural Abundance DoublE QUAntum Transfer Experiment*) sequences.

Heteronuclear scalar experiments are used to trace the coupling between ^{1}H and a heteronucleus, usually ^{13}C. In these cases, the experiments can be performed, either in direct or 'inverse' mode, by directly acquiring, respectively, the ^{13}C or ^{1}H nucleus. Direct experiments: HETCOR (*HETeronuclear CORrelation*, also called H,X-COSY), Long-Range HETCOR, COLOC (*COrrelation spectroscopy via LOng-range Couplings*). Inverse experiments: REVINEPT (*REVerse Insensitive Nuclei Enhanced by Polarization Transfer*), HSQC (*Heteronuclear Single Quantum Correlation*), HMQC (*Heteronuclear Multiple Quantum Correlation*), HMBC (*Heteronuclear Multiple Bond Correlation*).

Homonuclear dipolar experiments evidence the space proximity of two homonuclei, and the most common sequences are: 1D NOEDS (*Nuclear Overhauser Effect Difference Spectroscopy*), NOESY (*Nuclear Overhauser Effect SpectroscopY*) and ROESY (*Rotating-frame Overhauser Effect SpectroscopY*). For the **heteronuclear dipolar** correlation the HOESY (*Heteronuclear Overhauser Effect SpectroscopY*) experiment has to be mentioned.

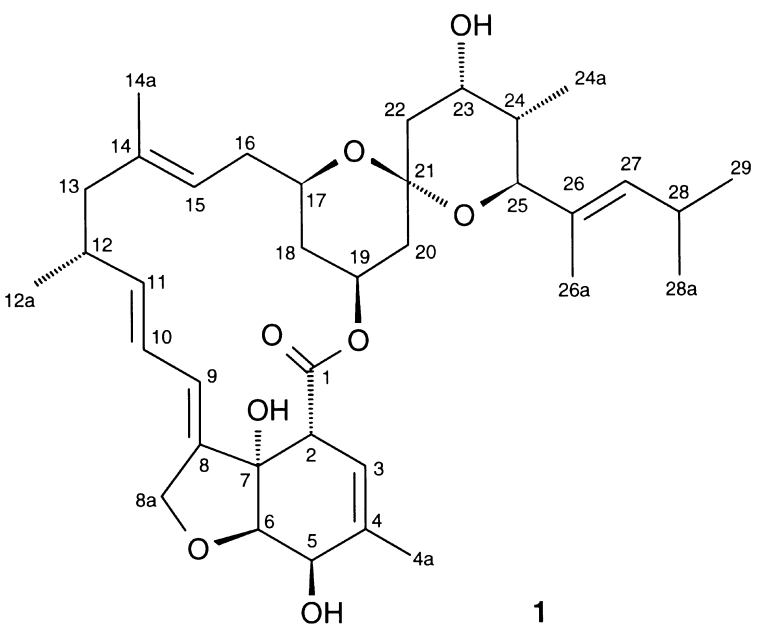
A detailed description of the use of these experiments in structure elucidation as well as proper referencing will be given in the sequel.

3.3 MOLECULAR FORMULA AND MAIN SPECTRAL DATA

A rational approach to the determination of the structure of an unknown natural product should start with the determination of the molecular formula (MF). This is frequently acquired on the basis of accurate mass measurement (high-resolution mass

spectrum, HRMS). The standard electron-impact (EIMS) method is normally used for low-molecular weight, easily vaporizable compounds, while other soft-ionization techniques like fast atom bombardment (FABMS), are frequently used for non-volatile or labile compounds (Bloor and Porter, 1993). Actually, the first step in the analysis is frequently the recording of a ^{1}H NMR spectrum. This may occur for various reasons, among them, the nowadays-widespread availability of NMR spectrometers, sensitivity of ^{1}H nuclei, as well as variety of information acquirable with a single proton NMR spectrum. The subsequent step is normally the execution of BB and DEPT ^{13}C NMR spectra (*see* section 3.3.2). As will be detailed in the following, a careful analysis of ^{1}H and ^{13}C NMR spectra frequently suggests a molecular formula and allows structural analysis to begin. Of course, accurate mass measurement or elemental analysis is undoubtedly required for a sound determination of the structure when the compound under study cannot be rapidly identified as previously known.

In the following, we will discuss applications of modern NMR experiments performed as a case study on a bioactive natural product, LL-F28249-α (**1**), originally isolated by Carter and co-workers (Carter *et al*., 1987, 1987a) from the culture broth of *Streptomyces cyaneogriseus* ssp. *noncyanogenus*. LL-F28249-α belongs to the important milbemycin family of macrolide antibiotics, whose members display antiparasitic and insecticidal properties (Davies and Green, 1986) and are currently marketed as antiparasitic drugs (Fisher, 1997). Further examples, and more specialized applications, will be discussed in sections 3.7 and 3.8, by referring to different compounds.



1

3.3.1 1D ^{1}H NMR

The first analysis of a ^{1}H NMR spectrum requires the determination, for each separate signal, of chemical shift value, multiplicity, coupling constants and intensity, i.e. the number of hydrogens (integral). This cannot be easily obtained for signals due to strongly coupled nuclei, or those overlapped with other peaks, or those involved in complex spin systems. Nevertheless, by means of the methods detailed in the following sections, the assignment of all the ^{1}H resonances is, to date, a reasonable goal for low or medium molecular weight molecules. If a low magnetic field spectrometer is used, some advantage in signal separation may be obtained by the use of 'shift reagents' (*see* section 3.6.3), or simply by changing the solvent. More conveniently, if a spectrometer working at high magnetic fields (500–900 MHz for ^{1}H) is available, the analysis is sensibly aided by the larger separation of the signals as well as the 'first order' appearance of many multiplets. As an example of this gain, in Figure 3.4 two ^{1}H NMR spectra of **1**, registered respectively at 200 MHz and 800 MHz (in $CDCl_3$) are reported. It can be clearly seen that, in the 800 MHz spectrum, many signals are clearly resolved while others have improved appearance.

All the spectra discussed in the sequel have been performed at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Figure 3.5a shows the ^{1}H NMR spectrum of **1**, registered in $CDCl_3$. All the resonances have been assigned to the relevant positions of the structure, on the basis of the NMR experiments reported in the sequel, and are in agreement with previous studies (Tsou *et al.*, 1989; Rajan and Stockton, 1989). The majority of peaks are well separated, thus allowing direct measurement of many chemical shifts and coupling constants. The expansion of the 4.5–6.0 ppm region (Figure 3.5b) shows examples of integral ratios, indicating the number of protons for each multiplet.

Unresolved signals may require further experiments to obtain unambiguous assignment. For instance (*see* Figure 3.5), the resonances of H-19 and H-11 (chemically very different) are overlapped in a complex 2H signal centered at δ 5.34. Similarly, H-9 and H-10 appear as a 2H signal at δ 5.74. The methylene protons H-8a (δ 4.65) and H-8a′ (δ 4.68) are a typical example of 'second order' spin system (AB system) where a distortion of the doublet structure for each proton is observed, due to the low ratio $\Delta\nu/J$. In fact, these protons are not equivalent but their chemical shifts are very close ($\Delta\nu$ = 15 Hz) with respect to their geminal coupling constant (J = 15 Hz).

A list of the observed signals and related integral ratios should, in principle, allow the determination of the number of hydrogens in the molecule. Nevertheless, exchangeable protons, like those of —OH or —NH, may give rise to elusive resonances, broad or even apparently absent from the spectrum. These can be more easily observed by lowering the proton exchange rate by removal of water or acidic impurities or using purified $(CD_3)_2CO$ or $(CD_3)_2SO$ as solvent, which cause a downfield shift because of the formation of hydrogen bonds. Their identification can be confirmed by their disappearance

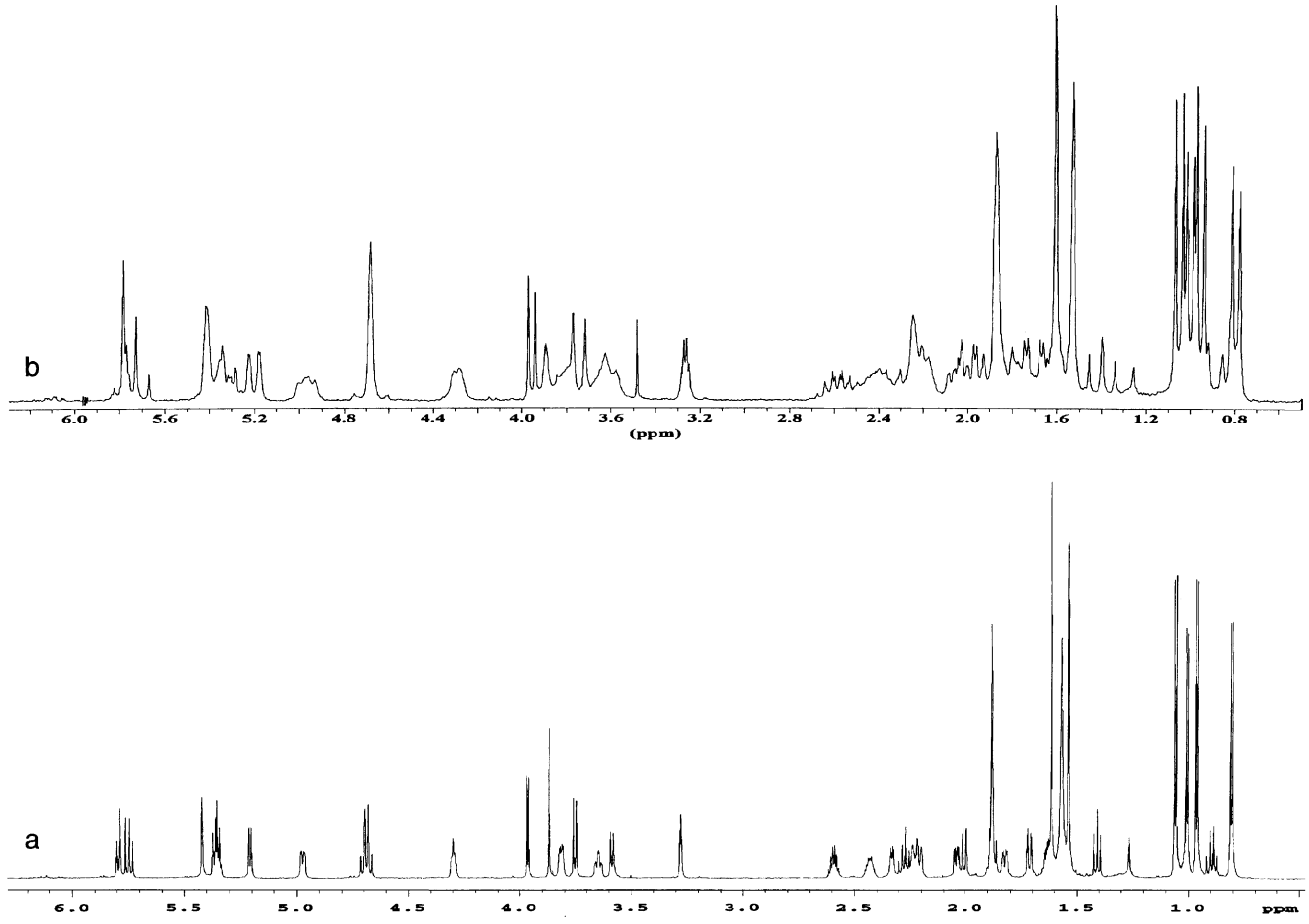


Figure 3.4: Comparison of the ^{1}H NMR spectra of **1** (in $CDCl_3$) acquired at (a) 800 MHz and (b) 200 MHz.

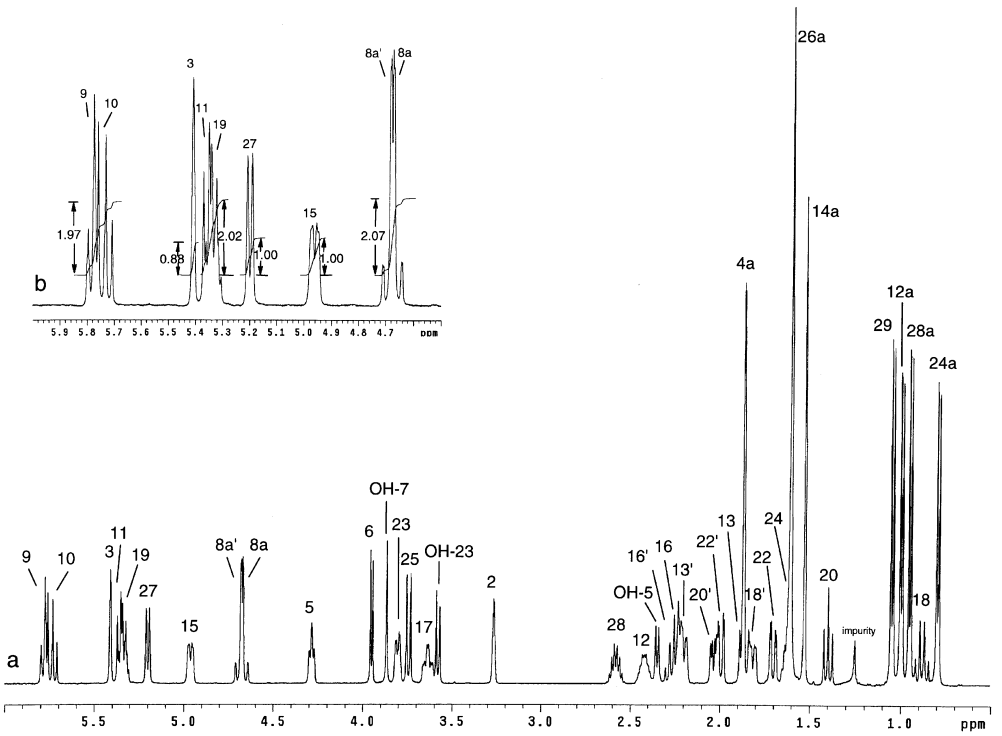


Figure 3.5: 500 MHz ^{1}H NMR spectrum of **1** (in $CDCl_3$) including: (a) the signal assignment and (b) the expansion of the 4.5–6.0 ppm region with integration values.

upon deuterium exchange, normally obtained by shaking the sample solution with D_2O (Silverstein and Webster, 1998). In Figure 3.5, signals due to OH groups (not reported in the literature) are observable as sharp doublets at δ 2.35 (OH-5, d) and 3.58 (OH-23, d), and a singlet at δ 3.88 (OH-7, s). Their appearance and the reproducibility of their chemical shifts indicate that they are involved in hydrogen-bonding (Abraham and Loftus, 1980). Figure 3.6 shows how D_2O exchange allowed identification of OH-23, OH-7 and OH-5.

More generally, the ^{1}H NMR spectrum easily directs the analysis towards a specific class of natural products. For instance, a crowded high-field region of the spectrum, rich in methyl signals, suggests an isoprenoid compound. Aromatic compounds (flavonoids, phenylpropanoids, lignans, etc.), in contrast, show a number of signals in the low-field region of the spectrum. The presence of a glycosidic moiety, as well as of saccharide-derived structure (sugar alcohols, cyclitols, etc.), is easily inferred by the crowded region of CHOH resonances (around δ 3–5).

A first look at the ^{1}H NMR spectrum of **1** suggests a complex structure with a number of carbinol or vinylic resonances and seven methyl groups. A total of 52 hydrogens may be accounted on the basis of integral analysis.

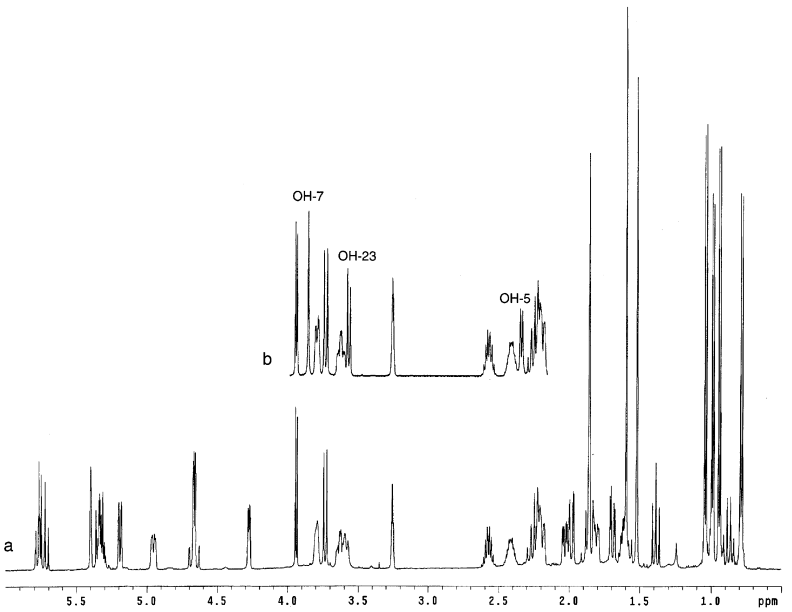


Figure 3.6: Comparison of (a) the ^{1}H NMR spectrum of **1** (in $CDCl_3$) acquired after shaking with a few drops of D_2O with (b) the region containing OH resonances before the H/D exchange.

3.3.2 1D ^{13}C NMR

^{13}C NMR spectroscopy is very important in the analysis of an unknown natural compound, since it affords key information about the carbon skeleton of the molecule. The simultaneous presence in the molecule of ^{1}H and ^{13}C nuclei, both possessing $\boldsymbol{I} = \frac{1}{2}$ spin, give rise to their mutual scalar coupling. Therefore, ^{13}C resonances in a conventional spectrum are split into multiplets with a large $^1J_{CH}$ (120–170 Hz) as well as with smaller $^2J_{CH}$ (0–8 Hz) and $^3J_{CH}$ (2–13 Hz). Undoubtedly, these multiplets may give useful information, but their analysis is somewhat difficult and usually complicated by extensive overlapping (Johnson and Jankowski, 1972). Therefore, a fully ^{1}H-decoupled ^{13}C NMR spectrum (sometimes referred to as ^{13}C-{^{1}H}) is normally registered, in which each resonance appears as a single line. The most common ^{1}H-decoupling is obtained by a *Broad-Band* (or 'noise'-modulated) high power irradiation over the entire ^{1}H frequency range (BB-decoupling). Alternatively, a lower power *Composite Pulse Decoupling* (CPD) can be used, which has the advantage of minimizing heating of the sample and possible decomposition (Shaka *et al.*, 1983).

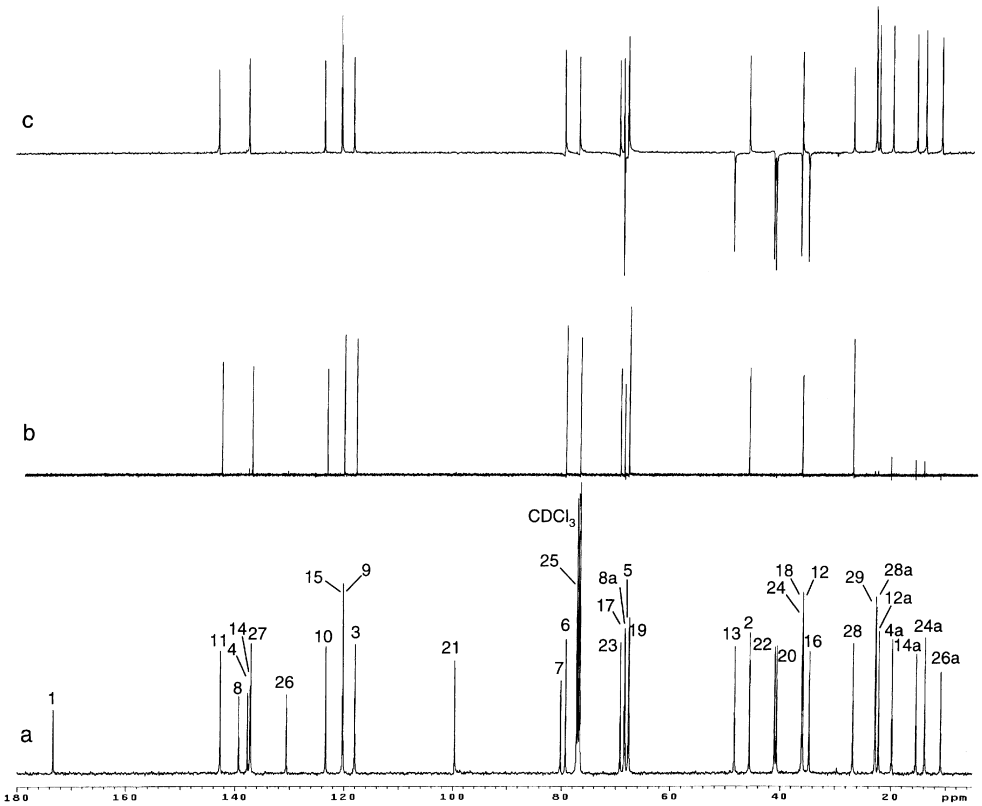


Figure 3.7: 125 MHz ^{13}C NMR spectra of **1** (in $CDCl_3$): (a) CPD-decoupled, (b) DEPT-90, and (c) DEPT-135.

The routine CPD-decoupled ^{13}C NMR spectrum of macrolide **1** (registered at 125.3 MHz in $CDCl_3$) is reported in Figure 3.7a. In this example, the number of peaks (36) exactly matches the number of carbons in the molecule. This may not occur in some ^{13}C NMR spectra, due to symmetry elements in the structure or to lack of resolution of peaks. Thus, a careful integrated analysis of MS, ^{1}H and ^{13}C NMR data is normally needed to determine the correct number of carbon atoms in the molecule, and this may be aided by running a second BB decoupled spectrum in a different solvent or by resorting to further experiments, discussed in the following sections. A chemical shifts analysis (Breitmaier and Voelter, 1987) of the reported spectrum indicates the presence of ten sp^2-hybridized carbons (δ 118–143) in addition to a carboxyl function (δ 173.5, C-1). The majority of signals are due to sp^3-hybridized carbons, eight of them (δ 66–81) clearly deshielded by oxygen atoms. The peak, at δ 99.7 (C-21), appears as a strongly deshielded carbon, and may be tentatively assigned to a ketal function.

The ^{1}H-coupling information (i.e. peak multiplicities related to the number of protons directly attached to each C atom) lost in the BB ^{13}C spectrum can be re-obtained by using multipulse experiments such as APT [*Attached Proton Test* (Patt and Shoolery, 1982)] based on a spin-echo experiment and INEPT [*Insensitive Nuclei Enhancement by Polarization Transfer* (Morris and Freeman, 1979)] or DEPT [*Distortionless Enhancement by Polarization Transfer* (Doddrell *et al.*, 1982)] sequences, both based on a $^{1}H \rightarrow {}^{13}C$ magnetization transfer. The DEPT sequence offers several advantages (higher sensitivity because of the polarization transfer step and higher tolerance for imperfect pulses or delays) and therefore is the most common method currently used for determining the carbon multiplicities. In the DEPT sequence the tilting angle of the final ^{1}H pulse (Θ) can be chosen among three common values: 45°, 90°, and 135°. For each of them, quaternary carbons will not be observed, while a different appearance of ^{13}C signals according to their multiplicity will occur. In a DEPT-45 spectrum all protonated carbons (CH, CH_2, and CH_3) appear as positive. A DEPT-90 spectrum only exhibits CH resonances, while all others are null. Finally, a DEPT-135 spectrum affords CH_3 and CH signals as positive while CH_2 is negative. In conclusion, by combining the information from the three experiments it is possible to unequivocally deduce the multiplicity of each carbon signal. In current spectrometers a program capable of performing the automated complete spectral editing of ^{13}C DEPT spectra is usually available.

By comparison of the BB decoupled ^{13}C NMR spectrum of **1** with the DEPT-135 experiment (Figure 3.7c), six methylenes as well as seven quaternary carbons were easily identified. The ambiguity between CH and CH_3 groups was resolved by the DEPT-90 spectrum (Figure 3.7b), which distinguished sixteen methines from seven methyls. DEPT experiments may resolve isochronous peaks due to carbons with different multiplicities, thus aiding the correct determination of the number of carbon atoms. If one signal is obscured by the solvent peak this will be clear from the DEPT editing, where the deuterated carbons do not appear. For instance, in Figure 3.7a, it is hard to distinguish, at a glance, two separate peaks at δ 68.4 and 68.5 (C-8a and C-17) but it becomes easy by looking at Figure 3.7c. Moreover, the peak at δ 76.6 (C-25), partly obscured by the $CDCl_3$ resonance, is clearly seen in Figure 3.7c. Once the multiplicity of each carbon signal has been determined, the number of protons bound to carbon atoms should match at least the number of unexchangeable protons deduced from the ^{1}H NMR spectrum. These data have to be compared with MS or elemental analysis results, in order to acquire or corroborate the MF and consequently to determine the 'degree of unsaturation'. Macrolide **1** has MF $C_{36}H_{52}O_8$, hence 11 formal unsaturations.

A careful inspection of the ^{13}C NMR spectra frequently allows one to establish the number of multiple bonds and, consequently, the number of rings. In our example, the number of sp^2 signals, accounting for one carboxyl and five double bonds, strongly suggest for the molecule a pentacyclic structure.

3.3.3 1D NMR of other nuclei

In addition to ^{1}H and ^{13}C, many other magnetically active nuclei may be studied with high-magnetic field spectrometers (Mason, 1987). Nevertheless, this is not a routine step in natural product analysis, essentially due to the low sensitivity of the most commonly occurring nuclei in secondary metabolites, i.e. ^{15}N (Levy and Lichter, 1982) and ^{17}O. Proton decoupled ^{15}N NMR spectra are increasingly used for typical nitrogenous metabolites, like peptides, provided that the available amount and the solubility of the substance do not preclude the analysis.

3.4 PARTIAL STRUCTURES: ANALYSIS OF ONE-BOND (H,C), GEMINAL AND VICINAL (H,H) CORRELATIONS

3.4.1 One-bond heteronuclear (H,C) correlations

Once the analysis of ^{1}H and ^{13}C NMR spectra has been completed, the next logical step is the connection of proton signals with the resonances of the carbon atoms to which they are directly attached through a single bond. This information is obtained with any of the **heteronuclear scalar correlation experiments** that exploit the magnetization transfer through $^{1}J_{CH}$ coupling. In any experiment, magnetization goes in the more advantageous $^{1}H \rightarrow {}^{13}C$ direction, but detection can be with either of the two nuclei. The choice is usually made on the basis of technical limitations of the spectrometer and/or sample amount, ^{1}H-detection (**inverse-detection**) being 8–16 times more sensitive than ^{13}C-detection (**direct-detection**). With modern instruments equipped with inverse detection, the best choice is the 2D HSQC (Bodenhausen and Ruben, 1980) spectrum, which gives a higher resolution. The analogous 2D HMQC (Bax and Subramanian, 1986) is performed when amount of sample or time limitation require better sensitivity. To the same end, many other inverse-detected sequences can also be used (REVINEPT, REV-DEPT), which have found a limited diffusion among natural product chemists.

When inverse detection is not available in the accessible spectrometer, experiments with ^{13}C direct detection have to be used. The most common sequence is HETCOR (Bax and Morris, 1981; Wilde and Bolton, 1984), based on an 'INEPT-like' polarization transfer. Alternatively, the DEPT-COSY experiment (Bendall and Pegg, 1983) can be used, which gives the ^{13}C-multiplicity phase information.

The analysis of heteronuclear correlation spectra is carried out simply by tracing the coordinates of each cross-peak with the proton chemical shift scale (normally reported on the ordinate) as well as the carbon chemical shift scale (normally on the abscissa). Thus, a list of correlations between protonated carbons and the attached protons is constructed. In addition, methylenes with diastereotopic protons will show separate ^{1}H resonances correlating with a single ^{13}C signal, thus allowing the identification of geminal

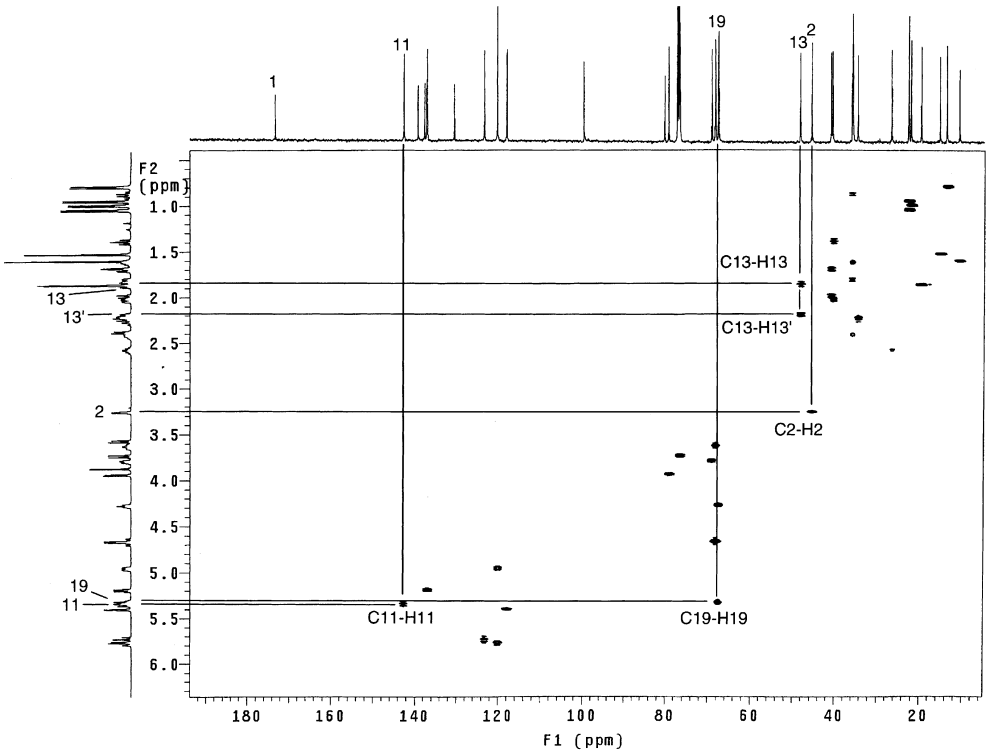


Figure 3.8: ^{1}H-^{13}C HSQC spectrum of **1**.

H,H relationships. Crowded regions of complex ^{1}H NMR spectra are more easily analyzed because of the spreading of proton resonances along the whole ^{13}C chemical shift scale. On the other hand, unresolved ^{13}C peaks may also take advantage of two-dimensional analysis.

Figure 3.8 illustrates the contour plot diagram of a HSQC (H,C) experiment performed on **1**. The 1D ^{13}C and ^{1}H spectra are normally imposed on the corresponding edges of the diagram (in this case, on the top and side, respectively) to aid the assignments. As an example, the correlation of some carbon signals with that of the directly bonded protons is indicated: δ 45.7 (C-2) correlates with δ 3.26 (H-2); δ 48.4 (C-13) with both δ 1.88 (H-13) and δ 2.18 (H-13b'); the overlapped δ 5.35 (H-11) and δ 5.32 (H-19) signals are clearly assigned to separate carbons resonating respectively at δ 142.9 (C-11) and δ 67.8 (C-19).

In summary, the global 'chemical information' extractable from a one-bond heteronuclear correlated experiment will undoubtedly be much larger than that obtainable from the simple chemical shift analysis of 1D proton and carbon spectra.

3.4.2 Geminal and vicinal (H,H) connectivity

As was said in section 3.2.3, the scalar coupling between ^{1}H nuclei indicates the through-bond closeness of the given protons; therefore, the exploitation of this information is particularly useful to start the drawing of partial structures of the molecule under study. Many ^{1}H-^{1}H scalar couplings can be simply deduced by the analysis of the splitting pattern of each resonance and can find confirmation with simple spin-decoupling experiments. However, more effective are ^{1}H-^{1}H correlation experiments.

3.4.2.1 ^{1}H signal multiplicity and spin decoupling

The signal multiplicity or 'splitting' may help preliminary analysis of the ^{1}H NMR spectrum, indicating the presence of easily recognizable spin systems, like terminal alkyl chains or typically substituted aromatic rings (Chamberlain, 1974). For instance, the series of methyl doublets at δ 0.79–1.04 in the proton spectrum of **1** (Figure 3.5) clearly suggests the presence of $CHCH_3$ groups.

The coupled protons can be identified, in very simple spectra, by the equal values of coupling constants, but a direct physical proof is usually required. The simplest approach is the spin decoupling experiment based on the irradiation of a ^{1}H signal during the acquisition (*double resonance*). In this way, all the couplings of that proton are removed and consequently the multiplets of coupled partners are simplified. Figure 3.9 shows that the irradiation of the signal at δ 2.58 (H-28) of **1** changes the methyl signals at δ 0.94 (H_3-28a) and δ 1.04 (H_3-29) into singlets, thus proving the presence of an isopropyl group. As additional information, the simplification of the signal at δ 5.19 (H-27) shows its vicinal relationship with the irradiated proton.

3.4.2.2 Geminal and vicinal (H,H) correlations

The spin-decoupling method suffers the disadvantage that several experiments have to be performed to elucidate spin systems with numerous couplings. In addition, ambiguity arises when close or overlapping multiplets are irradiated. Both these problems are alleviated using 2D homonuclear scalar experiments. The most typical of these, historically also the first 2D NMR experiment, is 2D COSY (Aue *et al.*, 1976). Actually, there are many variants of the COSY experiment (COSY-45 and COSY-90, having, respectively, a tilting angle of 45° or 90° for the last pulse; DQF-COSY [Piantini *et al.*, 1982], E.COSY [Griesinger *et al.*, 1987], β-COSY [Bax and Freeman, 1981b], etc.), each of them having some useful characteristic, but essentially they give the same basic structural information.

The usual appearance of a contour plot map obtained from a COSY spectrum is a square diagram, roughly symmetrical in respect of the diagonal from lower left to upper right. Coupled protons are recognized by an off-diagonal peak at the coordinates corre-

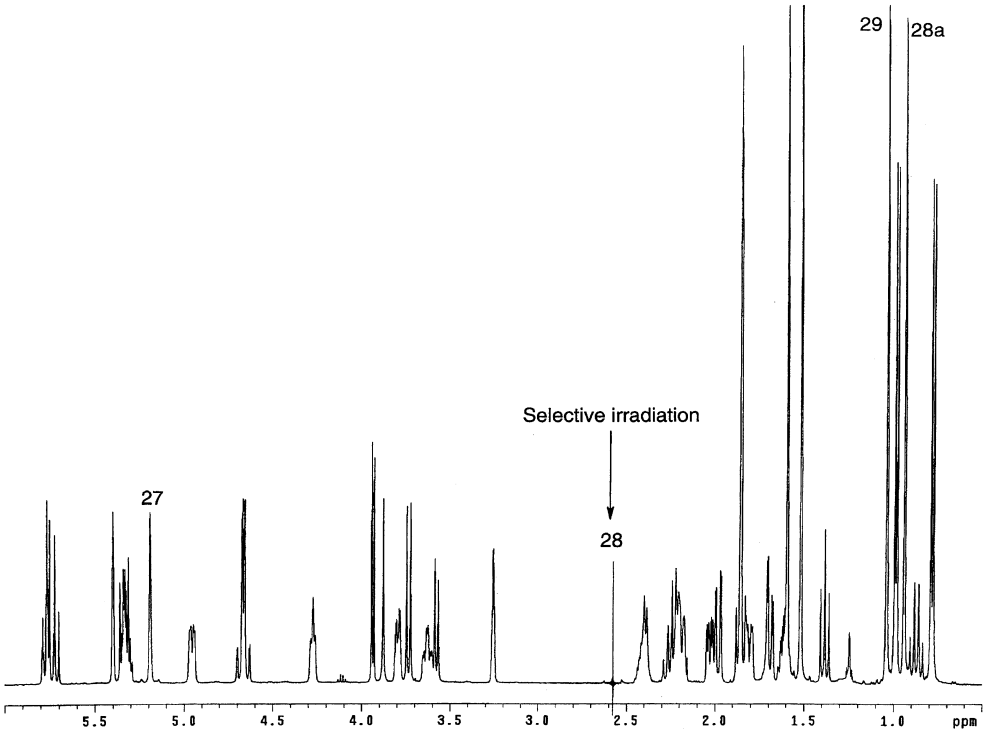


Figure 3.9: ^{1}H double resonance experiment on **1**. The selective irradiation of H-28 signal (at 2.58 ppm) is indicated.

sponding to the chemical shift of the two coupled protons. The diagonal is a contour plot version of the 1D spectrum.

A COSY spectrum is currently used for establishing homonuclear geminal ($^{2}J_{HH}$) and vicinal ($^{3}J_{HH}$) scalar coupling, this latter case being very important for elucidating partial structures. In Figure 3.10, the COSY-90 spectrum of macrolide **1** is shown. The 1D ^{1}H spectrum is imposed on the top and side to aid the assignments. The analysis of a COSY contour plot generally starts from an easily assignable peak of a spin system. For example, starting from the signal at δ 5.19 (H-27) and tracing a vertical line from the diagonal peak, a cross-peak at δ 2.58 is encountered, that is the chemical shift of a coupled proton (H-28). This, in turn, correlates with both δ 1.04 (H_3-29) and 0.94 (H_3-28a). From these data, partial structure C-27/C-29 is elucidated. Analogously, the connectivity from δ 3.74 (H-25) to δ 0.79 (H_3-24a) through δ 1.61 (H-24) can be established, determining the structural fragment C-24a/C-25.

In our example, a careful analysis of COSY vicinal correlations establishes a series of partial structures, namely C-2/C-3, C-5/C-6, C-10/C-13, C-15/C-20, C-22/C-25, C-27/C-29.

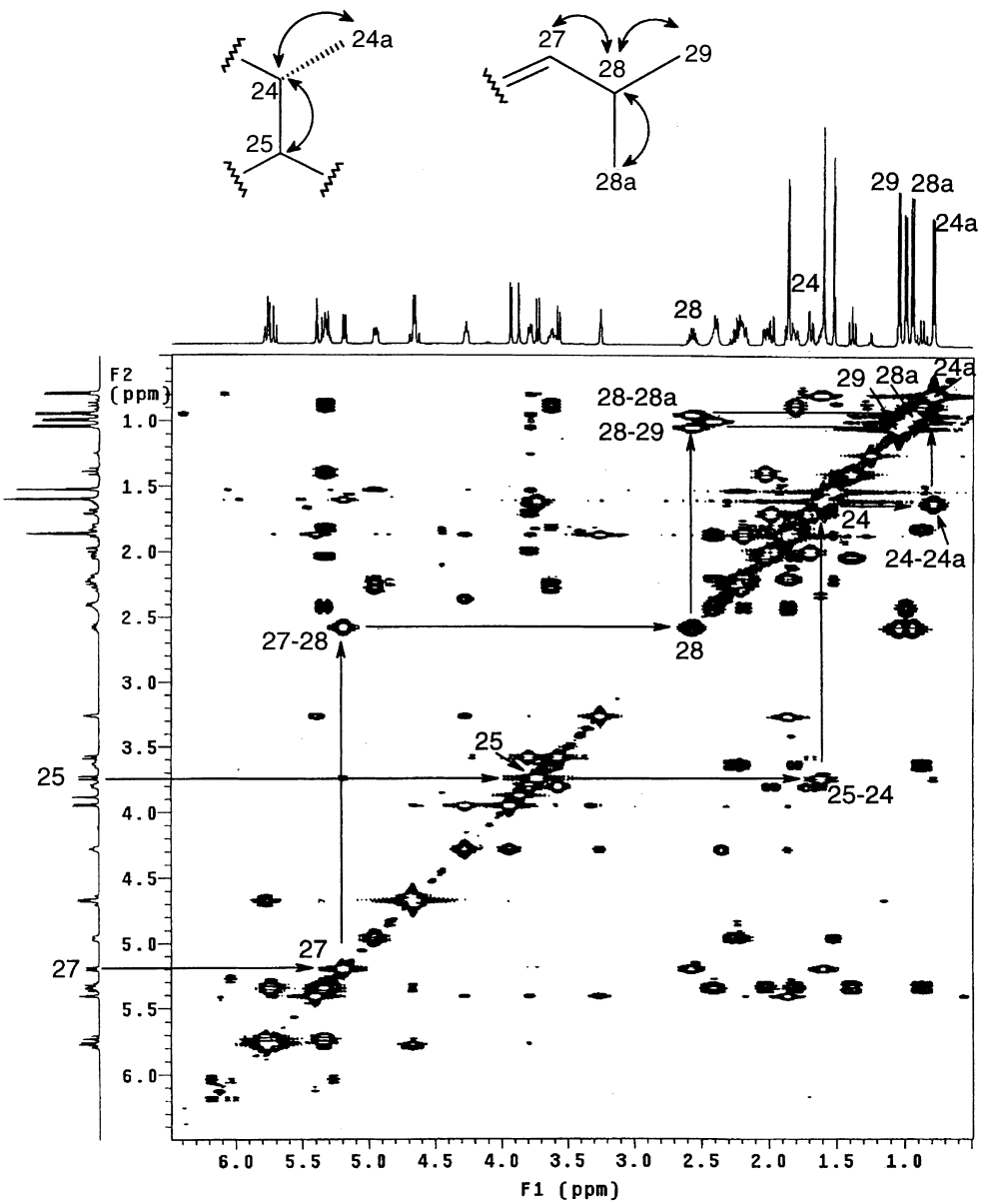


Figure 3.10: COSY-90 spectrum of **1**.

Interestingly, the 5-OH and 23-OH signals are unambiguously assigned on the basis of their relevant vicinal correlations.

In principle, analysis of a COSY spectrum should allow the construction of partial structures for all the spin systems with geminal or vicinal correlations, even if some

vicinal correlations may be absent because of instrumental conditions as well as from *J* values close to zero. Conversely, the COSY spectrum frequently shows cross-peaks due to spin coupling over more than three bonds (long-range coupling), generally less intense than those due to $^2J_{HH}$ or $^3J_{HH}$. These may be emphasized through the LR-COSY experiment and profitably used for joining partial structures (*see* section 3.5.2). Nevertheless, in the initial analysis of a new and unusual compound, long-range correlations may complicate the structural elucidation because of the difficulty in distinguishing vicinal from long-range connectivity. Some problems may also arise due to intense singlet peaks obscuring cross-peaks in close proximity to the diagonal. The above-cited difficulties can normally be circumvented by the use of further NMR experiments, for instance DQF-COSY (*see* below) or 1D-TOCSY (*see* section 3.4.3).

The double-quantum filtered version of the COSY experiment (DQF-COSY) is frequently preferred to the normal COSY in spite of the minor sensitivity and consequently the longer time required. Actually, DQF-COSY offers particular advantages when a careful analysis of a region of the spectrum including intense singlets is desired. In a DQF-COSY, these intense signals are substantially reduced and a reduction in the intensity of the whole diagonal is obtained. An expansion (high-field region, δ 0.5–3.0) of the phase-sensitive DQF-COSY spectrum of **1** is reported in Figure 3.11a, in comparison with the same region of the normal COSY spectrum (Figure 3.11b). For the sake of an immediate comparison, half of each contour plot is put beside the other.

3.4.3 Complete elucidation of spin systems

The information so far collected should allow the drawing of all partial structures involving hydrogen-bearing carbons. However, when doubts arise because of ambiguities in assigning correlation peaks, one may resort to experiments aimed at elucidating the complete spin system. One of these is the TOCSY experiment (Davis and Bax, 1985), in which increasing the value of the 'mixing-time' parameter allows the occurrence of two, three or more successive ^{1}H-^{1}H magnetization transfers through the entire spin system. Therefore, the closeness of two protons can be estimated by acquiring a few TOCSY spectra with increasing mixing time. In fact, correlations appearing with shorter mixing time will be due to vicinal or geminal protons (as in COSY spectra), whereas those appearing only with longer mixing times will be due to more distant protons in the spin system. A less convenient alternative is given by the H-Relayed-COSY experiment (Eich *et al.*, 1982; Bax and Drobny, 1985).

When overcrowding occurs in the ^{1}H NMR spectrum the dispersion of the ^{13}C signals can be conveniently used in a series of heteronuclear spectra in which a $^1J_{CH}$ transfer is associated with a COSY or TOCSY experiment. As mentioned in section 3.4.1, in these instances the acquisition can be in either direct (^{13}C) or inverse mode (^{1}H). The best-known experiments are H-Relayed-H,C-COSY (Sarkar and Bax, 1985),

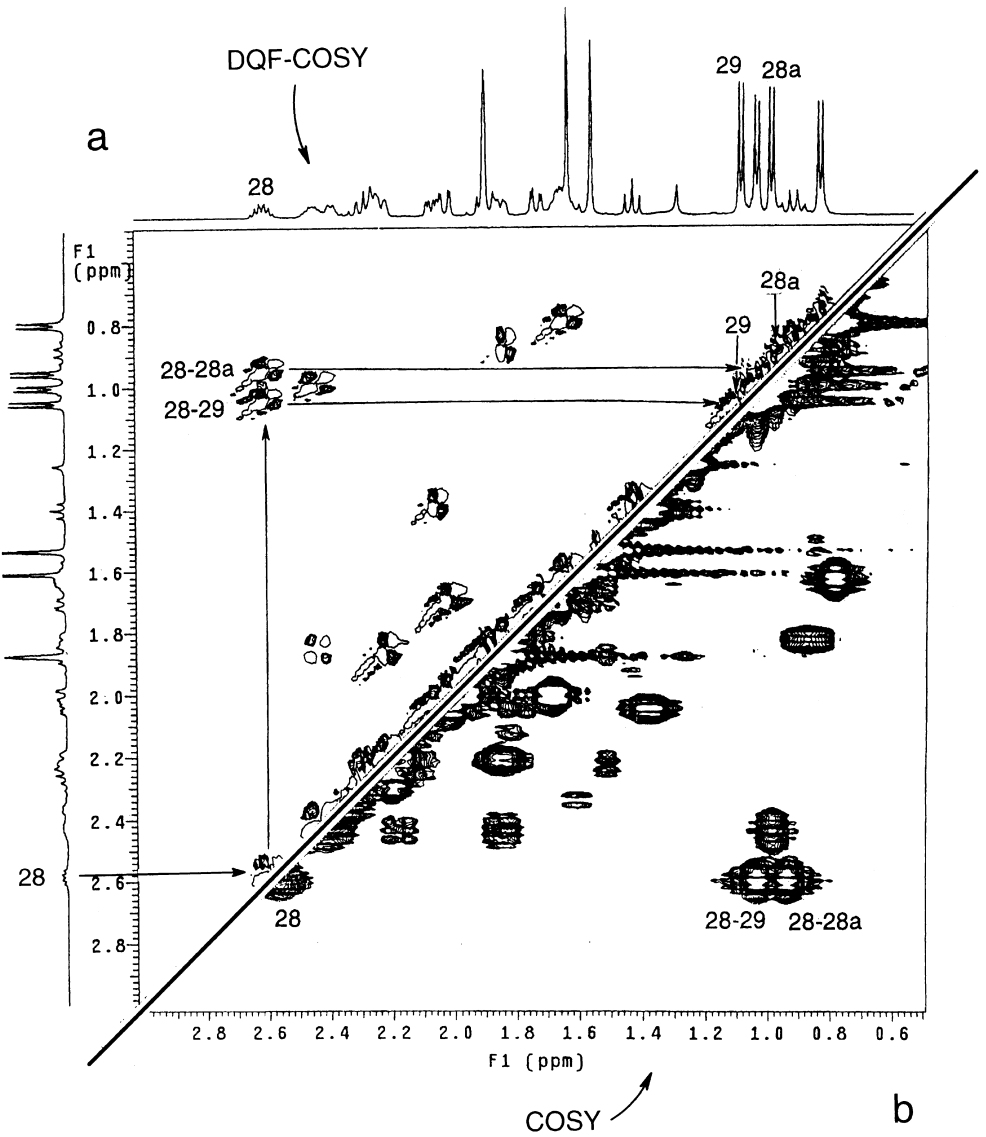


Figure 3.11: Comparison of corresponding portions of (a) DQF-COSY and (b) COSY-90 spectra of **1**.

Hetero-TOCSY (Bax *et al.*, 1985), HMQC-COSY (Gronenborn *et al.*, 1989), HMQC-TOCSY (Davis, 1989).

The general appearance of a 2D TOCSY contour plot, symmetrical with respect to a diagonal, is very similar to that of the COSY but shows a larger number of cross-peaks. Indeed, this richness of information may complicate the first step analysis. Thus, it is normally preferable to run a COSY spectrum before performing the TOCSY experiment.

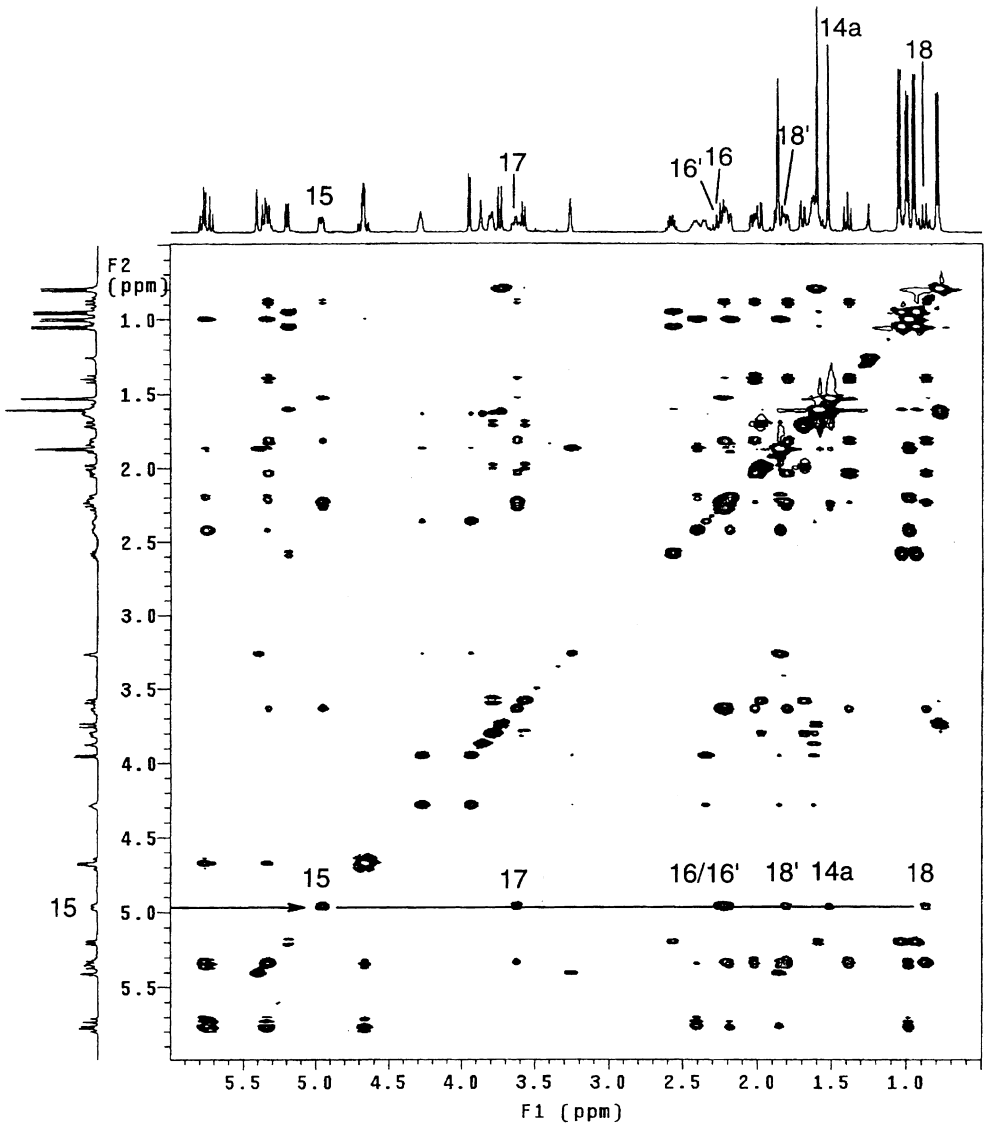


Figure 3.12: 2D TOCSY spectrum of **1** (mixing time = 80 ms).

The additional information may be profitably used to resolve ambiguities or corroborate the previously established partial structures. Also, some long-range correlations can be observed in a TOCSY spectrum, and this information will be useful in the further steps of structural elucidation, as detailed in the following sections.

The TOCSY spectrum of compound **1**, registered with a mixing time of 80 ms, is shown in Figure 3.12. The spectrum should be analyzed exactly as a COSY. Nevertheless,

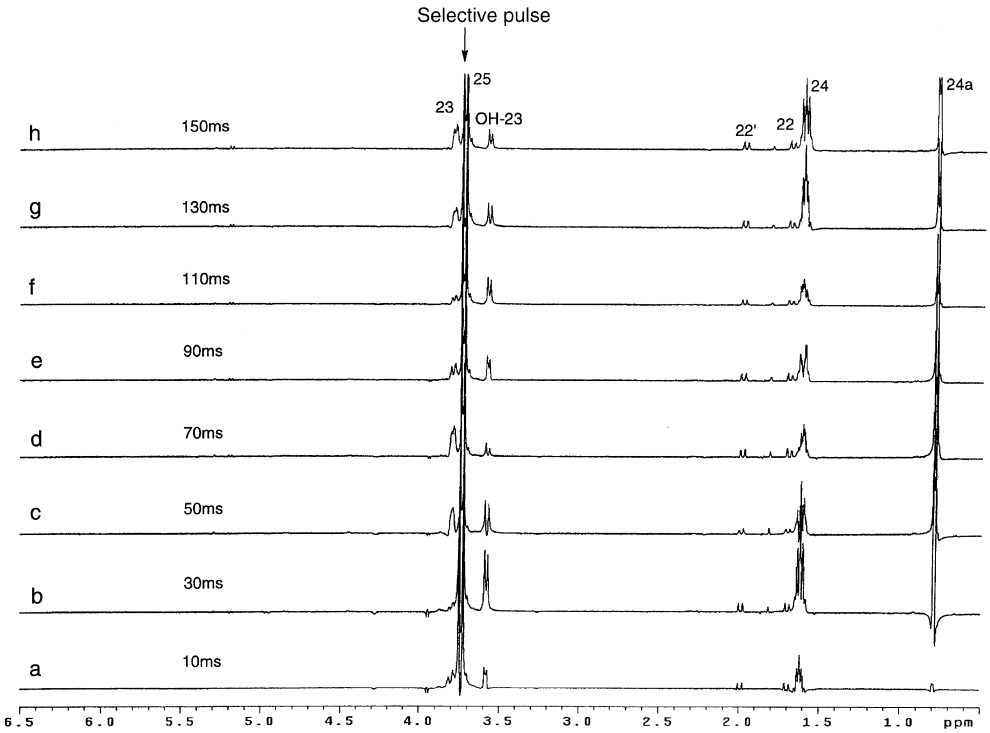


Figure 3.13: 1D TOCSY experiments of **1** performed with increasing mixing times from (a) to (h) and by selective excitation of H-25 resonance (at 3.74 ppm).

in our example, for the sake of simplicity only one row has been traced, starting from the H-15 (δ 4.95) peak on the diagonal and connecting the cross-peaks of the correlated protons, namely H-17 (δ 3.63), H-16′ (δ 2.25), H-16 (δ 2.21), H-18′ (δ 1.82), H_3-14a (δ 1.52) and H-18 (δ 0.88). In some cases, the analysis may be aided by plotting a 'slice' of the three-dimensional diagram corresponding to a row (or a column). In fact, in this way a clear picture of the entire spin system isolated from the rest of the ^1H spectrum can be obtained, thus allowing also the analysis of signals completely buried by other resonances.

A similar result can be obtained with the 1D version of the TOCSY experiment (Kessler *et al.*, 1989), in which a signal is excited by a selective pulse, allowing the successive ^1H-^1H magnetization transfers within its spin system. Indeed, the 1D TOCSY is the equivalent of a single slice of the 2D TOCSY. The 1D version is preferred over the 2D one when only a few spin systems need to be studied in detail.

An application of the 1D TOCSY experiment to compound **1** is shown in Figure 3.13. A selective 'Gaussian' soft pulse has been centered on H-25, and an array of 1 D TOCSY experiments with gradually increasing mixing times from 10 to 150 ms has been performed, affording, respectively, the spectra a-h. The propagation of correlation to

H-24 (δ 1.61), H-24a (δ 0.79), OH-23 (δ 3.55), H-22 (δ 1.69) and H-22′ (δ 1.99) is clearly discernible. Of course, only signals of correlated nuclei appear, and this is particularly useful for the analysis of crowded spectra, provided that an isolated proton of the spin system is discernible in the conventional ^{1}H spectrum. In our example, it is worth noting that the multiplet structure of H-24, hidden by the intense peak due to H-26a (even at 800 MHz!), can be observed and analyzed.

3.5 CONNECTING PARTIAL STRUCTURES: HOMO- AND HETERONUCLEAR LONG-RANGE CORRELATIONS

A critical step in the strategy for the complete elucidation of the structure of an unknown natural compound is the connection of the hydrogen-bearing partial structures or fragments, obtained through the previously described experiments, to at least a hypothetical full structure. This should be corroborated by further physical measurements or chemical conversions. Obviously, when the molecule does not contain quaternary carbons or heteronuclei (commonly, O or N), the connection of the partial structures is just a continuation of the above studies. In the alternative case the NMR repertoire furnishes additional experiments which can address different problems.

3.5.1 One-bond (C,C) correlations

When quaternary atoms are interposed between hydrogen-bearing partial structures their connection can be obtained by using a homonuclear scalar $^{1}J_{CC}$ correlation experiment called INADEQUATE (Freeman and Frenkiel, 1982). This experiment appears to be one of the most powerful for structure elucidation, since all the C-C linkages, i.e. the skeleton of the molecule, can be traced with a single experiment. When a single information is required the SELective 1D version of INADEQUATE, called SELINQUATE (Berger, 1988), can be used. However, the extremely low sensitivity of INADEQUATE, due to the low probability (less than 10^{-4}) of finding a molecule having two ^{13}C nuclei directly bonded, has severely limited its applications. Thus, it is hard to include the 2D INADEQUATE spectrum within the routine protocol for structure elucidation of natural products, particularly in a search for natural compounds possessing potent biological activity and isolated in minute amounts. Consequently, for the sake of brevity, we will not discuss here the INADEQUATE spectrum of macrolide **1**, which has already been reported (Rajan and Stockton, 1989).

3.5.2 Long-range homonuclear (H,H) correlations

The introduction of a delay into the COSY pulse sequence produces the Long-Range-COSY (LR-COSY or delayed-COSY) experiment (Bax and Freeman, 1981a), in which the cross-peaks due to $^{4}J_{HH}$, $^{5}J_{HH}$, or even longer-range spin–spin coupling, are emphasized.

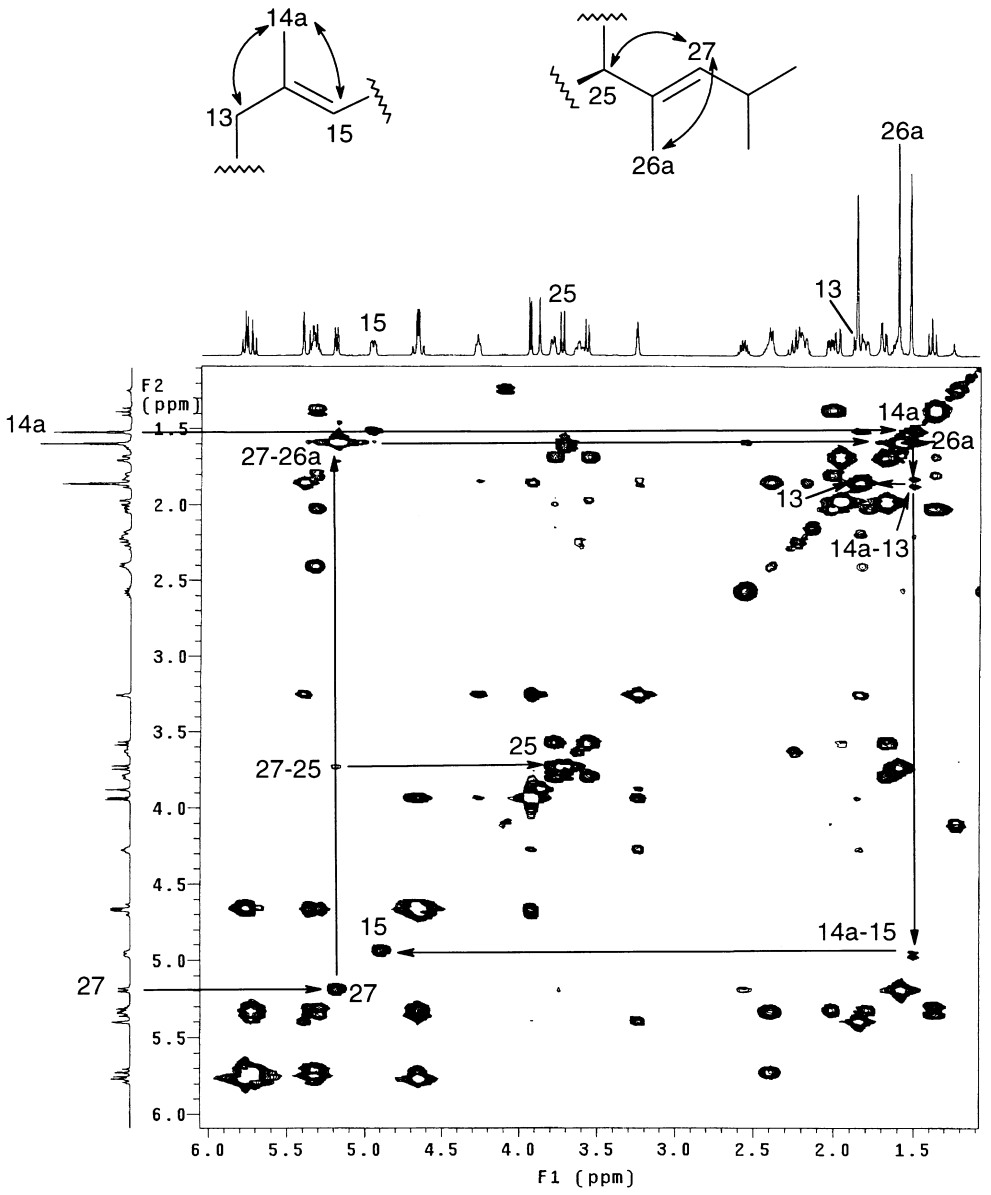


Figure 3.14: Portion (1.1–6.0 ppm) of LR-COSY spectrum of **1**.

An expansion (δ 1.1–6.0) of the LR-COSY spectrum of **1** is shown in Figure 3.14. Clear LR-COSY cross-peaks are observed, allowing the connection of the previously elucidated partial structures. In particular, the C-27/C-29 fragment is connected to C-22/C-25 through the long-range coupling of H-27 (δ 5.19) with both H-25 (δ 3.74) and H_3-26a (δ 1.60). The partial structures C-15/C-20 and C-9/C-13 are linked *via* the long-range

coupling of H_3-14a (δ 1.52) with both H-15 (δ 4.95) and H-13 (δ 1.88). Nevertheless, the presence of quaternary carbons or oxygen atoms does not allow the unambiguous connection of the extended substructures mentioned above, and further NMR experiments are required for a sound elucidation of the structure.

3.5.3 Long-range heteronuclear (H,C) correlations

The method of choice for connecting hydrogen-bearing partial structures is that of exploiting magnetization transfer through ^{1}H-^{13}C long-range couplings ($^{2}J_{CH}$ and $^{3}J_{CH}$) (Martin and Zektzer, 1988b). Also, in this case, the corresponding 2D heteronuclear experiments can be acquired either direct using [Long-Range-HETCOR (Wynants *et al.*, 1984), COLOC (Kessler *et al.*, 1984a,b)] or inverse detection [HMBC (Bax and Summers, 1986)].

There are two intrinsic problems in analyzing long-range heteronuclear correlated experiments. Firstly, the final result may be significantly affected by the selection of a parameter related to the mean J_{CH} value. This may be circumvented by performing more than one HMBC experiment, setting different mean J_{CH} values, for instance 4 Hz and 8 Hz. A further difficulty may arise in distinguishing between $^{2}J_{CH}$ and $^{3}J_{CH}$. For this reason, the deductive analysis based on the long-range heteronuclear correlated spectra has to be carried out very carefully and corroborated by all the available physical, chemical and literature data.

In Figure 3.15 an expansion of the HMBC spectrum (low-field ^{1}H region) of macrolide **1** is reported. This contour plot is similar to the HSQC spectrum (*see* Figure 3.8) and has to be analyzed in the same manner. Of course, the cross-peaks in this spectrum correlate carbons and protons not directly bound (even if some residual $^{1}J_{CH}$ could be observed). As examples, the correlations of the carboxyl signal (C-1, δ 173.5) with the signals of H-2 (δ 3.26, $^{2}J_{CH}$), H-19 (δ 5.32, $^{3}J_{CH}$) and H-3 (δ 5.40, $^{3}J_{CH}$) are shown, as well as those of carbon C-21 (δ 99.7) with both H-25 (δ 3.74) and H-23 (δ 3.80). In the whole contour plot, further correlations of C-21 with both H-20, H-20′, H-22 and H-22′ are observable. These key correlations afford new information. In fact, the carboxyl is evidently in a bridge position between the C-2/C-8a moiety and the C-15/C-20 substructure. On the other hand, the ketal carbon C-21 is a spiranic connection between this latter portion and the C-22/C-25 partial structure. The majority of the other cross-peaks in the HMBC spectrum are useful in confirming the above cited partial structures, at the same time allowing the assignment of signals due to quaternary carbons. In particular, the bicyclic moiety C2/C-8a can be defined in detail through a number of long-range heteronuclear correlations. Among them, the quaternary carbon C-8 (δ 139.4) is particularly important, showing correlations with H-2 (δ 3.26), OH-7 (δ 3.88), H-6 (δ 3.94), H-8a (δ 4.65) and H-8a′ (δ 4.68). In addition, C-8 shows correlations with H-10 (δ 5.73) and H-9 (δ 5.77), which corroborate its contiguity with the conjugated diene moiety.

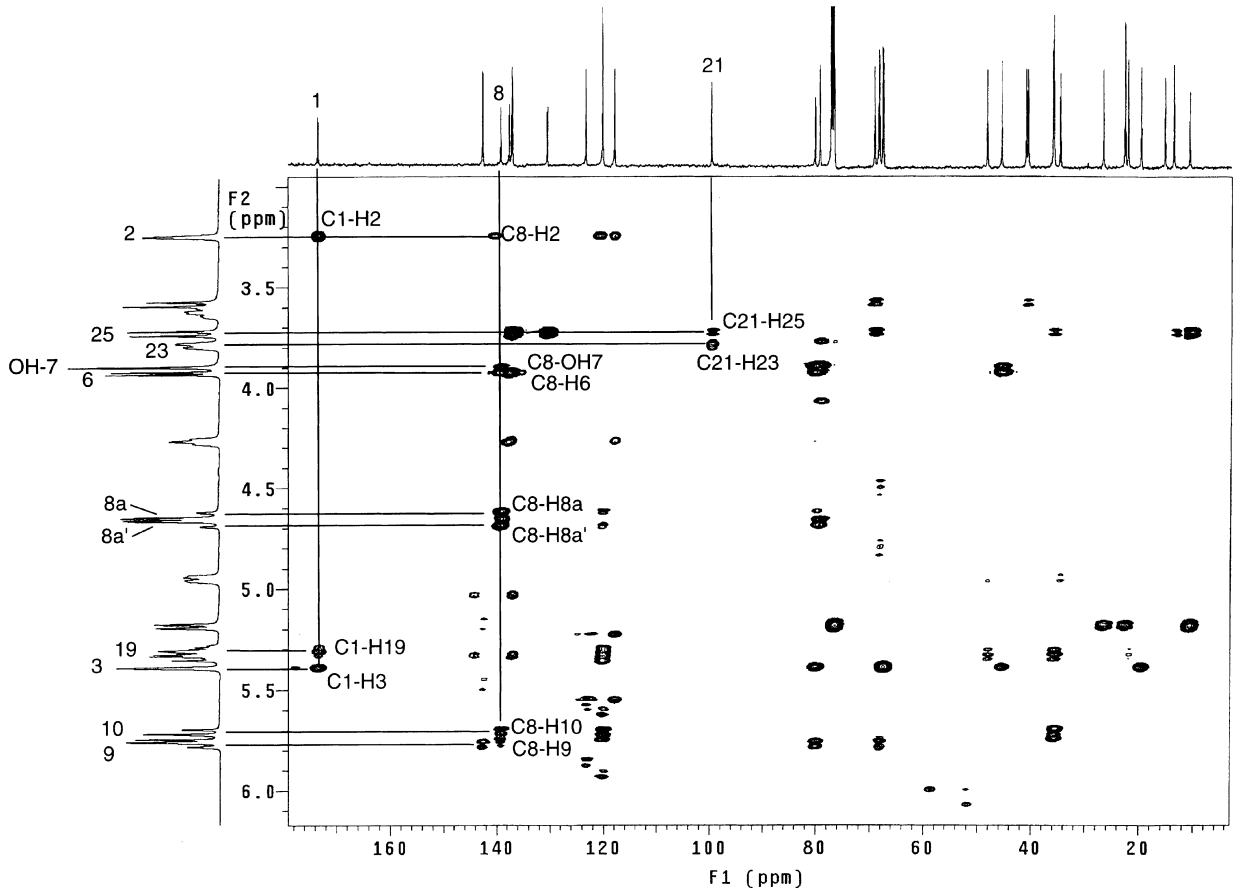


Figure 3.15: Portion of ^{1}H-^{13}C HMBC spectrum of **1**.

3.5.4 A summary of the strategy for structure elucidation

As outlined in the previous sections, a strategy for structure elucidation of unknown natural products may be summarized in a few steps, which we set out in this section, again with reference to the model compound **1**.

First, the molecular formula has to be determined, thus allowing the degree of unsaturation to be calculated. The ^{1}H and ^{13}C NMR (aided by DEPT) should corroborate it and indicate the presence of typical functional groups, as well as the number of multiple bonds. On this basis, the number of cycles in the structure can be determined. In the example, a pentacyclic structure including five double bonds, a carboxyl function, and several oxygen-bearing carbons, could be established.

The 2D one-bond HETCOR (or HSQC if available) is normally useful in assessing unambiguously the C-H connectivity of protonated carbons. The diasterotopic methylene protons frequently occurring in polycyclic structures are conveniently identified.

One or more of the available versions of the 2D COSY spectrum, or, in simple cases, 1D double resonance experiments, should allow the definition of partial structures, corresponding to spin-systems based on geminal or vicinal coupling. This may be aided by TOCSY experiments. Cross-peaks attributable to long-range coupling should be evaluated carefully and possibly emphasized through a LR-COSY experiment. Thus, the partial structures previously defined could be connected in larger substructures. In our example, this allowed the fragments C-9/C-20 (open at positions: 17, CHO-; 19, CHO-; 20, CH_2-) C-22/C-29 (open at: 22, CH_2-, and 25, CHO-) and C2/C-8a, to be established, even if with some ambiguity. The carboxyl function C-1 remained to be located.

Frequently the HMBC experiment is the final step in determining the gross structure (excluding stereochemical details) of the molecule under study. The careful analysis of this spectrum must validate the previous assessments and generally allows these substructures to be unambiguously connected. In our case, the analysis of the C-2/C-8 moiety confirmed the presence of two rings and extended this part up to C-20. The two rings C-17/C-20 and C-22/C-25 can be closed through the spiranic carbon C-21. At this point, the remaining degree of unsaturation is undoubtedly due to the bridge ester between C-2 and C-19. Thus, the framework of macrolide **1** is complete.

Of course, in some cases, the above protocol does not allow the partial structures coming from the NMR spectral analysis to be unambiguously joined. Nevertheless, these results will be integrated by literature search, UV, IR and MS data, and, if possible, by chemical conversions and the study of these derivatives. Also, the analysis of dipolar correlations, as well as Molecular Modeling studies, discussed in the following, may be useful to corroborate or complete the structural analysis, and are, of course, of critical importance in defining the stereochemistry of the compound under study.

3.6 STEREOCHEMICAL AND CONFORMATIONAL STUDIES

Quite often, when the entire molecular structure of a natural product has been established, a few stereochemical details remain to be defined, which can be related to *cis-trans* (*Z-E*) geometrical relationships as well as to the relative or absolute configuration at stereogenic centers. In some cases, stereochemical data may integrate or parallel the unambiguous determination of the structure. Thus, a variety of methods have been employed to afford stereochemical information, and a selection of these is briefly discussed below.

3.6.1 Chemical shift and coupling constant analysis

Some stereochemical questions can be addressed with chemical shift data based on closely related systems. For example, the *E* or *Z* geometrical relationship of a trisubstituted double-bond moiety, common in terpenoid derivatives, can be assigned by using the ^{13}C chemical shift value of a methyl group bound to the relevant sp^2-carbon. It is generally found below or above 20 ppm in the *E* or *Z* geometry, respectively (Johnson and Jankowski, 1972). Many of these empirical rules can be found, in the literature, to solve specific problems. In the case of macrolide **1**, the ^{13}C resonances of vinyl methyls 14a (15.4 ppm) and 26a (11.1 ppm) confirm the *E* geometry of the pertinent double bonds, while the 4a signal (20.0 ppm) indicates a *Z* configuration for 3,4-double-bond as it is imposed by the 6-membered ring.

Additional stereochemical information is included in the value of coupling constants. Thus, it has been demonstrated that the value of $^3J_{HH}$ (Karplus, 1963) or $^3J_{CH}$ (Marshall, 1983) is dependent on the dihedral angle θ between the coupled atoms, in accordance with the Karplus equation (Karplus, 1959)

$$^3J = A\cos^2\theta + B\cos\theta + C$$

where *A*, *B*, and *C* coefficients are structure-dependent parameters (Haasnoot *et al.*, 1980). From several studies, it can be generalized that $^3J_{HH}$ value will be very small (*ca.* 0 Hz) when $\theta \approx 90°$, while it will be large for $\theta \approx 0°$ or 180°, with $J_{180°}$ usually larger than $J_{0°}$. Consequently, on the basis of ^3J, the axial–axial spatial relationship ($^3J_{aa} \approx 7$–9 Hz) of two protons in six-membered rings can be easily distinguished from the axial–equatorial or equatorial–equatorial ones ($^3J_{ea} \approx {}^3J_{ee} \approx 2$–5 Hz). In addition, *cis* or *trans* relationship for olefinic protons can also be determined with acceptable confidence ($^3J_{cis} \approx 6$–14 Hz; $^3J_{trans} \approx 14$–20 Hz).

$^4J_{HH}$ couplings are usually very small, but they can have significant values when the two protons are stereochemically fixed in the so-called 'W' (or 'M') arrangement (particularly, if the four H—C—C—C—H bonds are coplanar). In addition, they can also be large if a double bond is present (i.e. allylic systems of type H—C=C—C—H) (Jackman and Sternhell, 1969).

Because of their relevance in stereochemical and conformational analysis, several 1D to 3D NMR experiments have also been devised to measure coupling constants even in complex biological macromolecules (Eberstadt *et al.*, 1995).

For compound **1** the *trans* geometry of the C-10/C-11 double bond is clearly deducible on the basis of the pertinent vicinal coupling constant ($J_{10,11}$ = 15.0 Hz). Analogously, the large *J* values (10.8–12.1 Hz) associated with H-17, H-19, H-24, and H-25 methine signals establish their 'axial' orientation. In contrast, H-23 has to be equatorial on the basis of its small *J* values (2.6–3.0 Hz). From this data the relative *cis* and *trans* configurations can be assigned, respectively, to H-23/H-24 and H-24/H-25 couples.

3.6.2 Establishing spatial proximity: NOE-based experiments

A wealth of stereochemical information can be obtained from the study of ^{1}H homonuclear dipolar coupling which occurs between nuclei in spatial proximity, but not necessarily scalarly coupled. The dipolar coupling can be evidenced using the 1D NOEDS experiment (Sanders and Mersh, 1982) as well as 2D NOESY (Jeener *et al.*, 1979; Kumar *et al.*, 1980) and ROESY [previously also called CAMELSPIN (Bothner-By *et al.*, 1984; Bax and Davis, 1985)].

In the 1D NOEDS experiment a spectrum acquired when a resonance is selectively irradiated is subtracted from a reference with irradiation in an empty zone of the spectrum. In the resulting difference spectrum all unperturbed signals disappear, while those of protons close in space appear with higher intensity. The irradiated signal will be seen as an intense negative peak. With respect to the area of this latter peak, the percentage of enhancement can be calculated, which is proportional to the spatial closeness of the two protons. In cases of close resonances, it is difficult to selectively irradiate only one of them, and hence undesired NOE originated from the close signals can be observed, giving rise to ambiguity or confusion.

In Figure 3.16 an example of NOEDS on macrolide **1** is reported. Irradiating at the frequency of H-27 (δ 5.19), a net enhancement at δ 3.74 (H-25) is observed, as well as lower responses at δ 0.79 (H_3-24a), 0.94 (H_3-28a), 1.04 (H_3-29), 1.60 (H_3-26a) and 2.50 (H-28). This evidence confirms the closeness of substructure C-27/C-29 to the C-22/C-25 ring.

Similar information can be obtained by the 2D NOESY spectrum, which offers the advantage that several NOE interactions are obtained in a single experiment and that ambiguities due to unselective irradiation in NOEDS are absent. In this experiment an important parameter is the *mixing time*, the duration of the dipolar magnetization exchange. The volume of three-dimensional cross-peaks (frequently they are simply classified as strong, medium or weak) in the NOESY spectrum can be used to extract the actual interproton distances.

As mentioned in section 3.2.4, the effectiveness of dipolar NOE interaction is strongly dependent on the correlation time of the molecule. The situation is especially

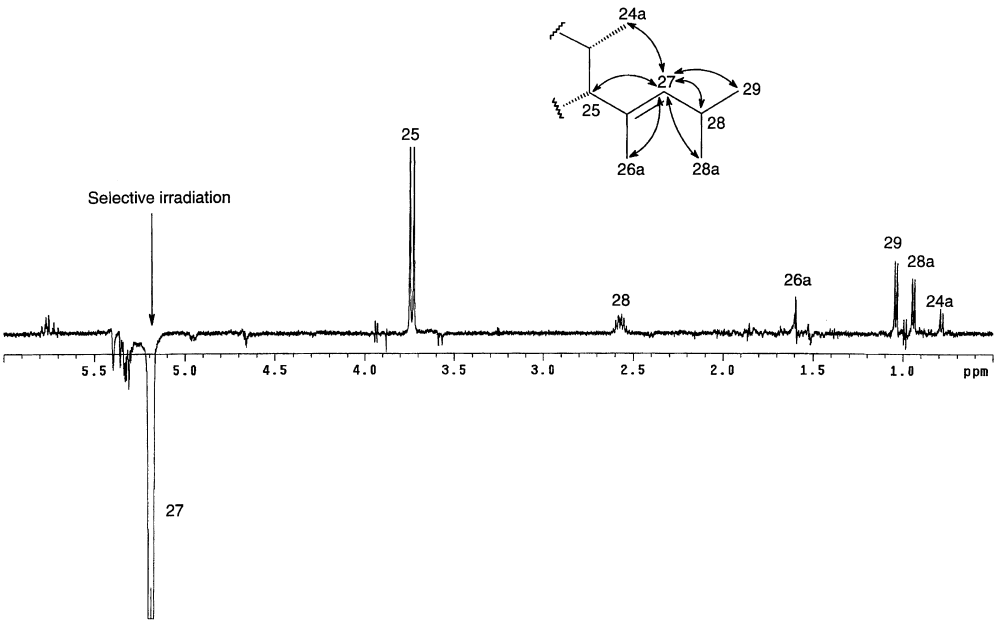


Figure 3.16: NOEDS experiment on **1**. The selective irradiation of the H-27 resonance (at 5.19 ppm) is indicated.

bad for medium-sized molecules, in which NOE can be very weak or even zero. In order to overcome these problems, the ROESY experiment has been devised, in which the *mixing* is obtained by a *spin-lock* rf irradiation. The duration of the mixing time in ROESY, as well as in NOESY spectra, allows the build-up of dipolar cross-peaks (ROE [*Rotating-frame Overhauser Effect*] or NOE) and hence it may influence the appearance of some correlations. Therefore, a few experiments with two to four different mixing times are usually performed in order to evidence all possible correlations.

The phase-sensitive ROESY spectrum performed on **1** with a mixing time of 250 ms is shown in Figure 3.17. It has to be analyzed in a similar way to COSY or TOCSY spectra. Several important correlations can be observed such as the ROE between H-17 and H-19, clearly indicating their closeness and hence their *cis* relative configuration, in accordance with the axial orientation previously deduced from *J* values. As expected from the NOEDS experiments discussed above, the dipolar interactions of H-2 with H-3 and H-8a are clearly demonstrated by the relevant ROE cross-peaks. Additional correlations between H-6/H-8a′, H-10/H-12, H-12/H_3-14a, H-15/H-17 and H-25/H-27 are easily observed and their relevance in pinning down stereochemical and conformational details will be discussed in section 3.6.4.

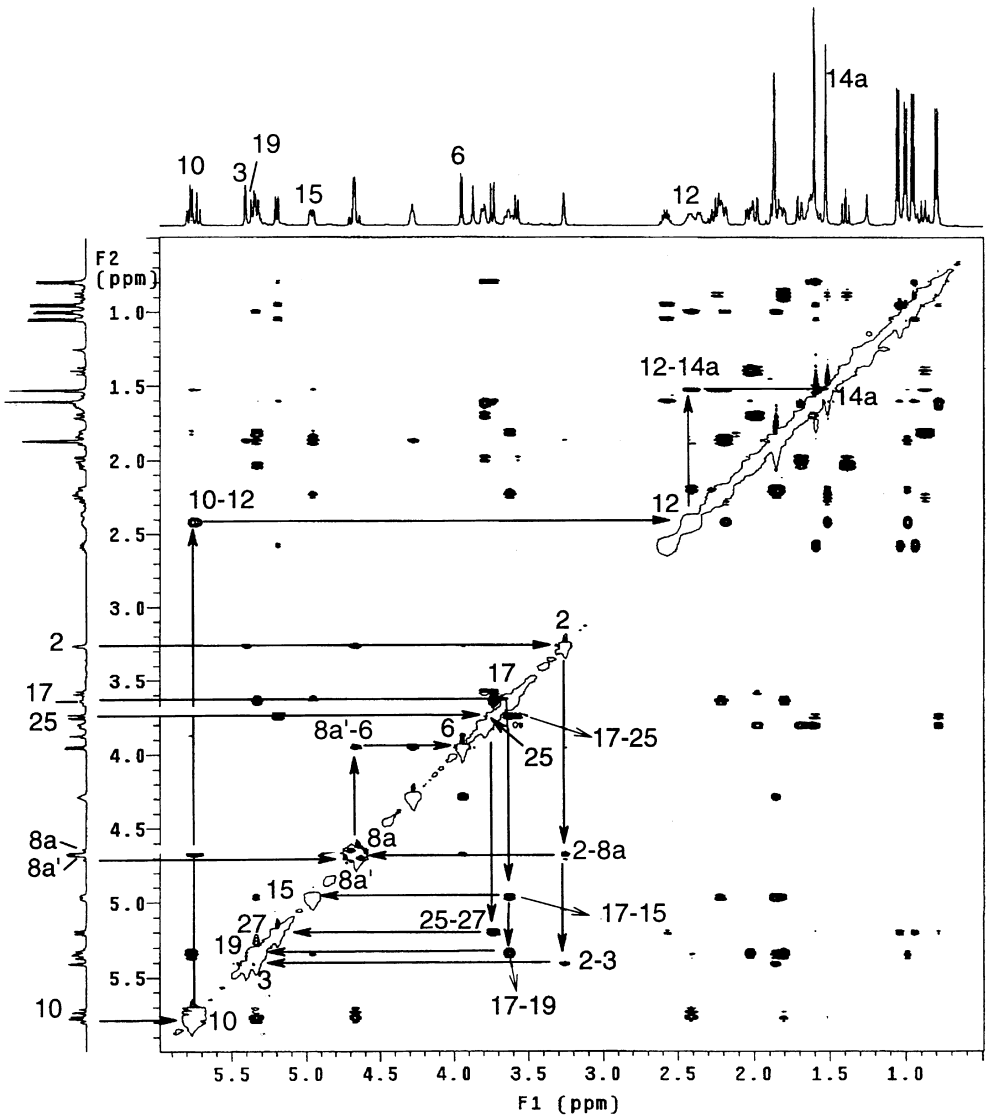


Figure 3.17: ROESY spectrum of **1** (mixing time = 300 ms).

3.6.3 Lanthanide shift reagents (LSR)

For molecules containing one (or few) O- or N-atom, additional stereochemical information can be derived from the use of a paramagnetic complex of a lanthanide metal (Eu, Pr) (Hofer, 1976; Morill, 1986). This, by interacting with the heteroatom, causes a distance-dependent shift of ^{1}H resonances through the so-called 'pseudocontact mechanism', in

accordance with the McConnell-Robertson equation: $\Delta\nu = K[(3\cos^2\theta\text{-}1)r^{-3}]$, in which $\Delta\nu$ is the molar lanthanide induced shift (LIS), r is the Eu–H distance and θ the O–Eu–H angle. The best fitting of LIS data with geometrical parameters can be obtained with iteration programs using a proper three-dimensional model of the molecule. The more common LSR complex are $Eu(fod)_3$ [tris-(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-3,5-octanedionato)europium] and $Eu(dpm)_3$ [tris-(dipivalomethanato)europium] which cause a downfield shift, and the analogue diamagnetic complexes of Pr, which produce upfield shifts (Sievers, 1973). Because of the large shifts caused, LSR have been used to simplify the spectra, as a 'surrogate' of a high-field spectrometer, in early stages of structural elucidation of natural products and more recently even in conjunction with 2D NMR (*see* for example, Amico *et al.*, 1991a; Caccamese *et al.*, 1991).

3.6.4 Three-dimensional models and conformational studies

Often, the qualitative analysis of *J*, NOE, ROE or LIS data allows the assignment of stereochemistry just by using the gross structure, however, their correct interpretation usually requires a three-dimensional model of the molecule. In this regard, conventional molecular models, are quite useful, such as the 'Dreiding', which allow the comparison of interproton distances in different stereoisomers and their fitting with experimental NOE or LIS data. However, the increasing power of computers has made routine the use of molecular modeling programs. By means of these, a realistic model of the molecule, usually corresponding to the most probable 3D-structure, is obtained by energy minimization using a convenient theoretical treatment (in decreasing order of accuracy and computer-power requirement): *ab-initio* quantum-mechanical [for example, HF/STO-3G or FCI/∞ (Pople, 1999)], semi-empirical quantum-mechanical [for example, AM1 (Dewar *et al.*, 1985)] or empirical molecular-mechanical [MM2 or MM3 (Allinger *et al.*, 1989); CHARMM (Brooks *et al.*, 1983); AMBER (Weiner *et al.*, 1986)]. A direct comparison of the fitting of energy-minimized 3D-models of different stereoisomers with experimental data is sufficient when few conformational degrees of freedom occur.

However, when several stable conformations are possible, a more accurate conformational search has to be performed for each stereochemistry using an appropriate algorithm: Monte Carlo method (Saunders *et al.*, 1990), Molecular Dynamics (van Gunsteren and Berendsen, 1990), or Distance Geometry (Crippen and Havel, 1988). The result of conformational search carefully compared with all experimental data can simultaneously give the best compatible stereochemistry and conformation.

In many instances, all available experimental data can be directly included in the conformational search as 'restraints', by using a so-called 'penalty function'. This, if the number of restraints are sufficient, will then directly give the best fitting 3D-structure having the minimum deviation from the experimental data. This procedure has become

the standard one for determining the 3D-structure (conformation) of proteins (Clore and Gronenborn, 1993) and nucleic acids (Wüthrich, 1986).

In the case of macrolide **1**, by assuming the relative stereochemistry of the 11 stereogenic centers as established, we will show how the use of molecular modeling in conjunction with experimental data allows the determination of its conformation in solution.

The presence of an elevated number of rotatable single bonds in **1** indicates that a large number of conformations are possible for it. Therefore, a Monte Carlo conformational search was performed using MM2 force-field and chloroform-model-solvent as implemented in *MacroModel* V4.5 program (Mohamadi *et al.*, 1990). This search indicates that within 1.5 kcal/mol from the global minimum five very similar conformations are found, having a superimposable geometry of the macrocyclic ring and only differing in the relative orientation of the freely-rotating groups of the side-chain attached at C-25.

The lowest-minimum-energy structure (Figure 3.18) was then used as the most representative one to compare the experimental data. Indeed, a very good fitting of key data was found. For example, the H-17/H-25 ROE correlation, observable in Figure 3.17, was justified by a distance of 2.53 Å measured in the computer model between the two protons. Similarly, other ROE interactions were explained: H-2/H-8a (2.85 Å), H-6/H-8a′ (2.67 Å), H-10/H-12 (2.42 Å), H-12/H_3-14a (2.85 Å), H-15/H-17 (2.59 Å), H-17/H-19 (2.64 Å), H-25/H-27 (2.26 Å). Of course, all the experimental axial or equatorial orientations were also found in the model. In addition, a *pseudo*-axial upward orientation of 14-Me, H-12 and H-10 with respect to the average plane of the macro-ring was also observed.

From these data it can be concluded that the lowest-energy conformation depicted in Figure 3.18 is preferred in solution, while the side-chain at C-25 remains relatively free-rotating. This conformation is very similar to those previously reported, either in solution (Rajan and Stockton, 1989), or in solid state (Carter *et al.*, 1987), indicating that it is driven by intramolecular forces which play a major role with respect to crystal lattice or solvent effects. As a confirmation of this conclusion, in our study three intramolecularly bonded OH groups have been assigned, which may constitute a relevant portion of these intramolecular forces. On the other hand, these H-bonds are easily observable in the lowest-energy structure (Figure 3.18) which allows their confident location at the following positions: 23-OH···O-17 (δ 3.58, O···O distance 2.79 Å), 7-OH···O=C-1 (δ 3.88, O···O distance 2.60 Å), 5-OH···O-6 (δ 2.35, O···O distance 2.60 Å).

3.6.5 Absolute configuration

Natural products frequently possess several stereogenic centers whose relative stereochemistry can be easily elucidated using the above methods. However, as the last step, it often remains a problem to assign their absolute configuration, which remains largely

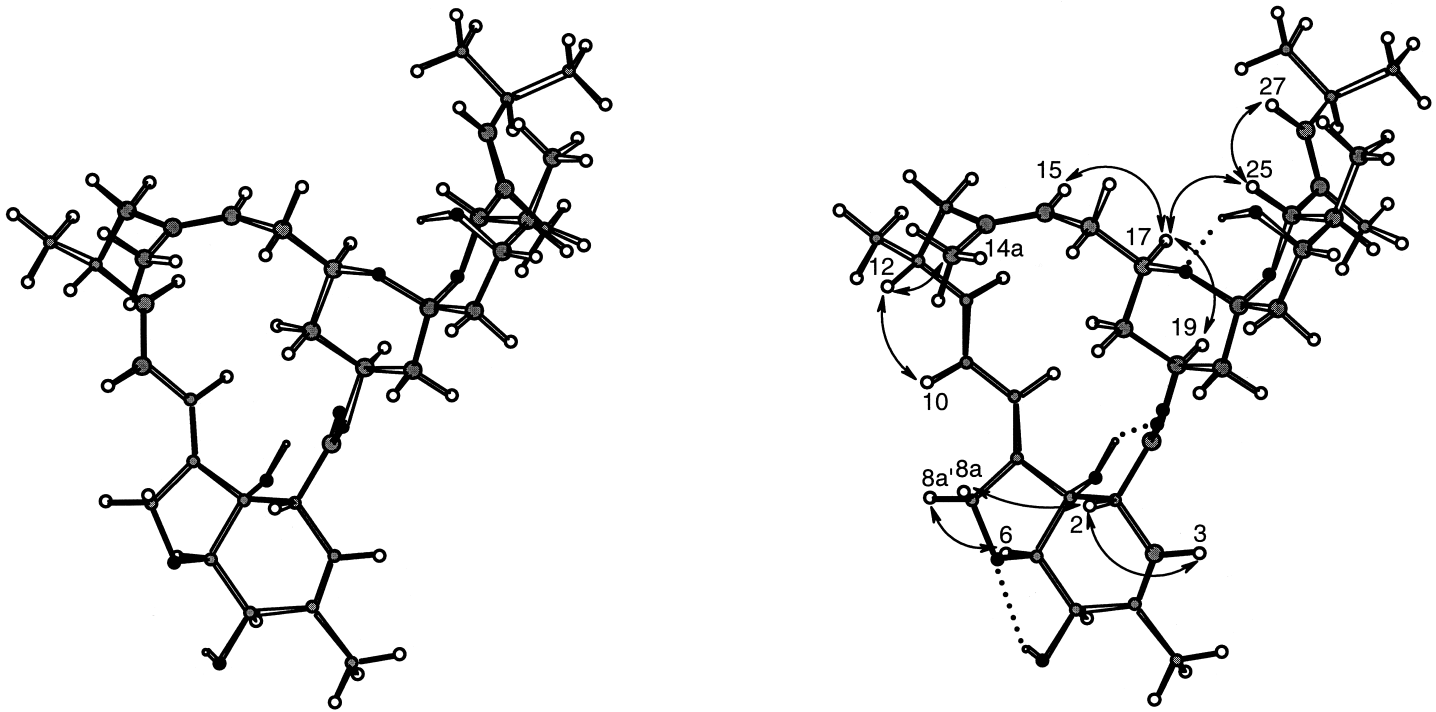


Figure 3.18: Stereoview of the lowest-energy conformation of **1** obtained by Monte Carlo conformational search. Selected ROE interactions are indicated by arrows, while hydrogen bonds are represented by dotted lines.

unsolved because of its intrinsic difficulty. A few methods are currently available to solve this question, namely, the Bijovet anomalous X-ray scattering (Bijovet *et al.*, 1951), methods based on asymmetric synthesis like the Horeau (Horeau, 1962) and Mislow (Green *et al.*, 1966) procedures, and others based on circular dichroism (Harada and Nakanishi, 1983; Zhou *et al.*, 1991). In some instances this problem can also be solved with the aid of modern NMR techniques.

In fact, when a secondary alcohol function is present, the Kakisawa-Kashman modification (Ohtani *et al.*, 1991) of the Mosher method (Dale and Mosher, 1968; Dale *et al.*, 1969) can be reliably applied. It requires the esterification of the alcohol with each enantiomer of Mosher acid [2-methoxy-2-phenyl-2-(trifluoromethyl)acetic acid, MTPA]. Then, assignments of as many ^{1}H resonances as possible are made, possibly with the aid of 2D NMR techniques, for each of the (*R*)- and (*S*)-MTPA esters. Next, the upfield shielding effect of MTPA-phenyl ring is evaluated as $\Delta\delta = \delta_{S\text{-ester}} - \delta_{R\text{-ester}}$ for corresponding sets of protons in the two esters. The distribution of all positive and negative $\Delta\delta$, respectively, on the right and left sides of the so-called MTPA-plane (containing the H—C—O—C=O moiety) of a 3D molecular model of the ester warrants the *R*-configuration, while the opposite distribution proves the *S*-configuration. The reliability of this empirical method is based on the comparison of many points ($\Delta\delta$ of all assigned resonances) and, therefore, several efforts have been made to extend it to different classes of compound (e.g. amines [Seco *et al.*, 1997]) and to different derivatizing agents (Latypov *et al.*, 1998).

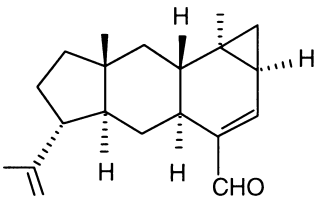
3.7 MISCELLANEOUS APPLICATIONS

In addition to the previous 'case study', in this section we report some 'real-life' applications of NMR experiments derived from our research work. This has been largely devoted to the study of seaweed secondary metabolites, which include diterpenoids and meroditerpenoids. Among them, several products have shown important biological activities (Tringali, 1991, 1997). Further examples are also considered which pertain to compounds from other natural sources.

The structure of the algal diterpenoid fasciola-7,18-dien-17-al (**2**), isolated from *Dilophus fasciola* and possessing a new tetracyclic carbon skeleton, has been determined essentially on the basis of spectral data, including homonuclear 2D NMR methods (Tringali *et al.*, 1986). The conclusive step of this work was to distinguish between two gross structures with different carbon skeletons as well as to determine the stereochemistry of the chiral centers. Both these goals were obtained by a careful NMR study including NOEDS, NOESY and LIS data, facilitated by the almost complete lack of conformational flexibility of both policyclic frameworks. In particular, the relative orientation of protons and methyls at the chiral centers was established through NOE studies. At this stage, Eu(fod)$_3$ induced shifts were measured for selected protons and the

so-called $1/r^2$ method (Cockerill *et al.*, 1973) was applied to correlate these data with r values measured on Dreiding stereomodels of the two competing stereostructures. A comparison between the correlation coefficients for the two plots of LIS vs $10^2 r^{-2}$ clearly defined the correct structure, including relative stereochemistry.

The major bioactive component of the extract obtained from the brown alga *Dictyota dichotoma*, dolabellane **3**, together with four co-occurring diterpenoids of the same family, were studied with the aim of determining their preferred conformation in solution (Piattelli *et al.*, 1995). The initial step was to assign, unambiguously, all of the peaks in the ^{1}H and ^{13}C NMR spectra, largely on the basis of COSY, one-bond and long-range HETCOR experiments. This study prompted us to a re-examination of previous stereochemical assignments, in particular at C-3 and C-4 (the carbons of the epoxide cycle). To discriminate between the 3*S*,4*S* or 3*R*,4*R* configurations (this latter depicted in structure **3**), we used NOESY data in comparison with the results of a conformational search performed on both diasteroisomers by means of molecular mechanics (MM2) calculations. We obtained clear evidence in favor of the 3*R*,4*R* stereochemistry.



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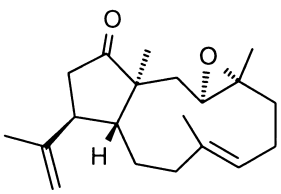

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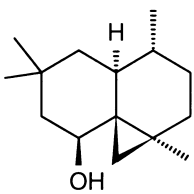

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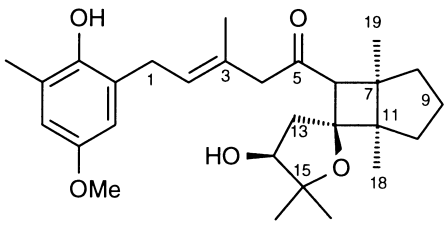

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A further example of the use of lanthanide shift reagent for both structure and relative stereochemistry determination is illustrated by the case of laurobtusol (**4**) isolated from the red alga *Laurencia obtusa* (Caccamese *et al.*, 1991). Because of extensive signal overlapping in the 1.00–1.90 ppm region, its structure could not be elucidated even with the use of several 2D NMR experiments. Therefore, we resorted to the incremental addition of Eu(fod)$_3$, monitored by the acquisition of ^{1}H and ^{13}C NMR spectra, until a complete resolution was observed for proton resonances. At this point a new set of doped 2D NMR spectra was acquired. In addition, long-range H/C-correlations were evidenced using the 1D INAPT experiment (Bax *et al.*, 1985) (they were unobservable in

the 2D experiment because of the short relaxation time caused by LSR). Using these data the final structure was obtained, apart from stereochemistry. To solve this last question, the preferred conformation for each of the eight possible diastereoisomers was obtained by MM2 calculations and used for the comparison of simulated versus experimental LIS data. The calculated agreement factor allowed the assignment of reported stereochemistry at an acceptable level of confidence.

Applications of homonuclear ^{13}C-^{13}C correlation experiments are quite rare within the natural products field, thus we want to cite here the use of symmetrized 2D INADEQUATE (Mareci and Freeman, 1982) to assign the quaternary carbon signals at 52.56 and 45.96 ppm of the metabolite balearone (**5**) isolated from the brown algae *Cystoseira stricta*, *C. mediterranea* and *C. tamariscifolia* (Amico *et al.*, 1991b). From this spectrum (acquired on a 620 mg sample) the resonance at δ 52.56 is seen to correlate with those at 81.20 (C-12), 35.43 (C-10), 18.79 (C-18), and 45.96 ppm. This last is, in turn, correlated with the resonances at 40.45 (C-8), 16.77 (C-19), and 59.71 ppm (C-6). Therefore, the signals at 52.56 and 45.96 ppm were unambiguously assigned to C-11 and C-7, respectively.

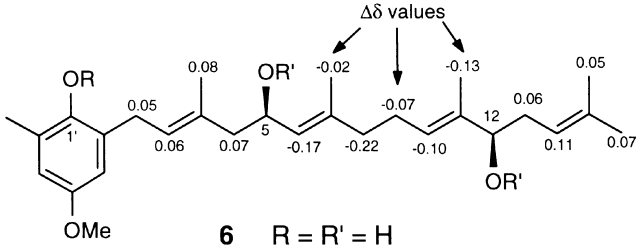
The absolute configuration of some meroditerpenoid metabolites isolated from Mediterranean algae of the genus *Cystoseira* was determined by using the Kakisawa-Kashman modification of Mosher's method (Amico *et al.*, 1997). From this work it is worth mentioning the case of compound **6**, previously isolated from *C. adriatica* (Amico *et al.*, 1988), which possesses two stereogenic centers (C-5 and C-12) of unknown — at that time — relative and absolute configuration. To solve this question, **6** was protected by methylation at 1′-OH, and converted to the corresponding Mosher esters (*R*)-MTPA-**6a** and (*S*)-MTPA-**6a**, whose complete ^1H NMR resonance assignment was obtained by means of 2D COSY spectra. The evaluation of their Δδ values ($\Delta\delta = \delta_{S\text{-ester}} - \delta_{R\text{-ester}}$) afforded the data reported on structure MTPA-**6a**, which shows the systematic arrangement of positive and negative Δδ values for protons surrounding the two chiral centers. This allowed the confident attribution of the absolute *R* stereochemistry at both positions 5 and 12.

An application of a peculiar NMR technique is given by the structure elucidation of tamariscolone **7** isolated from *Cystoseira tamariscifolia* (Amico *et al.*, 1989). At the last stage of structure determination the alternative closures of the ether bridge to 14 or 15 positions could not be excluded on the basis of all available 2D NMR data. Therefore, we resorted to a *Differential Isotope Shift* (DIS) experiment (Pfeffer *et al.*, 1979) aimed to observe the small shift of the hydroxyl-bearing carbon resonance upon deuterium substitution of the free OH group. The DIS spectrum was acquired using a coaxial dual cell containing the same amount of compound **7** in the OH and OD forms, respectively, in the inner and outer tube. Thus, a DIS value of 0.12 ppm was observed for C-15 quaternary carbon, whereas no shift was detected for C-14. This indicated that C-15 bears the hydroxyl group, while C-14 is involved in the ether bridge, thus proving the presence of an oxygenated eight-membered ring.

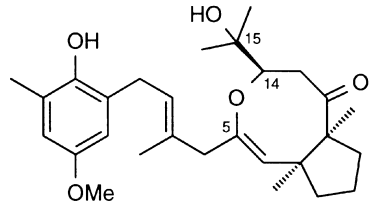
As an example of the critical importance of a single NMR datum, it is worth citing here the cross-correlation peak in a long-range heteronuclear correlation experiment as the key to discriminating between two alternative structures of the fungal metabolite **8**, isolated in the frame of a study on bioactive tetraprenylphenols from the fruiting bodies of the basidiomycete *Suillus granulatus* (Tringali *et al.*, 1989a, 1989b). In fact, a long-range HETCOR experiment performed on the more stable diacetate **8a** allowed the observation of a $^{3}J_{CH}$ coupling of C-5 with H-4, compatible with the reported structure and not plausible for the alternative, in which the dihydropyran ring is reversed with respect to the aromatic ring and the cited nuclei would be separated by four bonds.

We have recently established the structure of an unusual cytotoxic compound, sarcodonin (**9**), isolated during a reinvestigation of the fruiting bodies of the basidiomycete *Sarcodon leucopus* (Geraci *et al.*, 2000). The main part of this study has been carried out on the more stable peracetate **9a**. For this compound, in addition to the usual ^{1}H and ^{13}C NMR spectra we found it useful to run a ^{15}N NMR, which showed the presence of two nitrogen atoms (δ 387.0 and 187.2) with very different shielding effects. Compound **9a** was subjected to an array of NMR experiments including DQF-COSY, TOCSY, HSQC and HMBC. Hydrogenolysis of **9** afforded a *p*-terphenyl product as well as cyclo-L-isoleucyl-L-isoleucine, thus allowing the determination of the absolute *S* configuration at C-4α, C-4β. A key step in the determination of the structure was the assessment of the joining mode between the *p*-terphenyl moiety and the 1,4-diazinic ring, that is with the aliphatic β-chain close to the central ring of the terphenyl (up), or in the opposite geometry, with the β-chain in the outer position (down). Taking into account the stereogenic centers N-1β, C-2β, eight different stereostructures were considered a possibility for **9a**. To discriminate among them, as well as to establish the configurations at N-1β and C-2β, we resorted to an integrated use of ROESY data and molecular mechanics study of the above-cited eight structures. We used the lowest-energy geometry of each stereoisomer as preferred conformation in solution in order to compare selected interproton distances with experimental ROE interactions. These latter could be adequately satisfied only by the 1β*R*, 2β*R* configuration.

Selective excitation experiments may be particularly useful for the study of natural products with complex structures showing very crowded NMR spectra, even at higher magnetic fields, for example, polar compounds with an oligosaccharide moiety. To test the potentiality of this technique, we used the 1D TOCSY experiments during an NMR study of a semisynthetic derivative of a β-cyclodextrin, including a cyclopeptide functionality (Impellizzeri *et al.*, 1996). This unsymmetrical molecule (MW 1417) gives rise, in principle, to 49 distinct ^{1}H NMR signals, disregarding the exchangeable protons. A series of 1D TOCSY experiments corroborated the assignments of the modified glucopyranose unit, allowing the observation of the multiplet structure for overlapped protons. More recently, we had a further opportunity to employ the 1D TOCSY experiment in establishing some details of the structure of a long-chain ester of ferulic acid isolated from the bark of the medicinal African plant *Parkia biglobosa* (Tringali *et al.*, 2000).



6 R = R' = H
6a R = Me, R' = H
MTPA-**6a** R = Me, R' = MTPA



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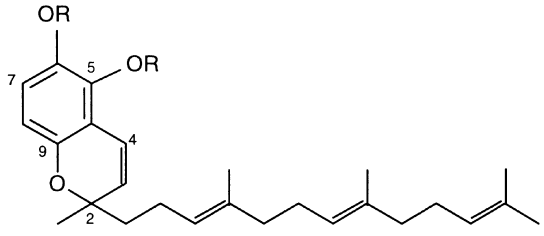
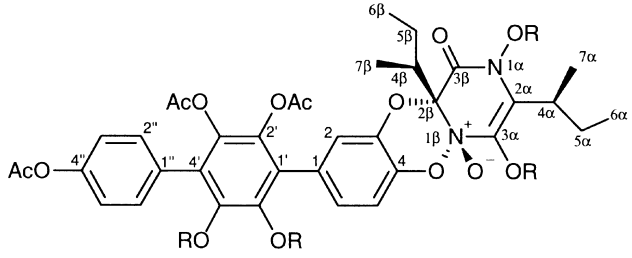


8 R = H
8a R = Ac



9 R = H
9a R = Ac

3.8 FUTURE OUTLOOK AND CONCLUDING REMARKS

From the above discussion, it is evident that the present modern NMR spectroscopy occupies a prominent role in the structure elucidation of bioactive natural products. However, it appears that a further expansion of its horizons can be achieved through additional technical improvements. These are related to the issues of sensitivity, molecular complexity, speed of characterization, analysis of mixtures and detection of biological activity.

As concerns the sensitivity, it is known that often the isolable amount of a given natural product is so small as to preclude its full characterization by NMR, but it could still be enough to elicit biological activity. A lower limit of 1 μmol (that is 0.2–1 mg of compound with MW 200–1000 Da) is reachable with 200–600 MHz spectrometers equipped with inverse detection, but, obviously, it is strongly dependent on the molecular complexity.

A simple way to perform structural elucidation using a lower amount of product is the use of ultra-high field spectrometers working from 750 to 900 MHz which are currently produced by few manufacturers, and are still only available at few sites worldwide. Alternatively, this can be accomplished by the use of special NMR probes which require a very limited amount of solvent (down to 2 μl, Olson *et al.*, 1995). For example, a complete NMR study (including the less-sensitive HMBC experiment) was performed on a *ca.* 0.04 μmol sample of cryptolepinone using a SubMicro-Inverse-Detection-Gradient (SMIDG) NMR probe and a Shigemi NMR microcell of 1.7 mm external diameter (Martin *et al.*, 1998). Of course, the sensitivity gain can be profitably extended to other less-accessible experiments and, in fact, with the same technique even ^{1}H-^{15}N long-range correlations can be detected at a low-level concentration (*ca.* 1 μmol [Hadden and Martin, 1998]).

The major problem associated with increasing molecular complexity is the extensive overlapping of NMR resonances which can be coupled to a pronounced line broadening. In fact, this latter is correlated to the increasing of the decay rate of magnetization through transverse relaxation (or spin–spin relaxation, *see* section 3.2.5) upon the increase of molecular size. Reduction of signal overlapping can be obtained using the spectral dispersion of ultra-high field spectrometers or exploiting the 'spreading' of multidimensional experiments (in particular heteronuclear correlations). Thus, by using 3D (and 4D) heteronuclear experiments the three-dimensional structure of ^{13}C and ^{15}N labeled proteins of MW up to 40,000 Da is currently determined (Fesik and Neri, 1993). It appears that this limit can be moved to 100,000–200,000 Da with the introduction of the TROSY (*Transverse Relaxation-Optimized SpectroscopY*) technique (Pervushin *et al.*, 1997).

However, in the case of natural products, these limits are quite low because of the higher degree of complexity associated with their very diverse and unpredictable structural moieties. This problem is further aggravated by the (usually insuperable) difficulty

of working at natural ^{13}C abundance. Because of these and other difficulties, the upper limit presently appears to be in the 10–15,000 Da range. In this regard, an impressive example is given by the structure elucidation of maitotoxin (Murata *et al.*, 1993), having a molecular weight of 3422 Da, which represents the largest natural non-biopolymer product known to date. Its structure is composed of 32 6-to-8-membered rings and contains 98 chiral centres (Cook *et al.*, 1997).

The characterization of a natural product may be a quite time-consuming step particularly relevant for molecules of a certain complexity. On the other hand, because of the great and detailed amount of information obtainable with modern NMR techniques, structure elucidation has became a problem more easily solvable using a systematic approach. Therefore, several efforts have been devoted to the development of computer programs which may assist or even automate the entire process (from spectral peak-picking to structure generation) (Lindsay *et al.*, 1980; Gray, 1986; Ley *et al.*, 1994). Several recent approaches appear to be successful (Steinbeck, 1996; Peng *et al.*, 1998; Lindel *et al.*, 1999), but their universal applicability and usefulness remain to be confirmed. In any case, it is conceivable that, with the aid of artificial intelligence, further progress in this direction will be made which, in conjunction with efficient database retrieval, could lead to faster structure elucidations.

In the quest for bioactive natural products the isolation and purification step is highly demanding. Modern NMR may give significant aid by means of a combination of the so-called 'hyphenated techniques' (LC-UV, LC-MS and LC-NMR), in which the raw extract of natural products is separated by HPLC and on-line analyzed through UV, MS or NMR spectral methods (*see* Chapter 2 of this book). In this respect, some help could also come from the application of the DOSY (*Diffusion-Ordered 2D NMR SpectroscopY*) experiment, in which pulsed field gradients (PFG) are used to select the spectra on the basis of the diffusion coefficients of the molecules (Morris and Johnson, 1993). It allows the analysis of mixtures by separation of NMR spectral data in accordance with the different molecular sizes of the components (Lin and Shapiro, 1996).

Exploiting the DOSY principle, PFG NMR can also be used in the screening or evaluation of biological activity of natural products upon observation of their binding to a biological receptor (when it is available in sufficient amount) (Bleicher *et al.*, 1998). In fact, upon binding to the receptor the diffusion behavior of an active molecule will be profoundly modified, thus allowing its identification by a proper use of PFG-based experiments (Lin *et al.*, 1997). Analogously, the change in relaxation rate can also be used (Hajduk *et al.*, 1997). Alternatively, the binding can be ascertained with *Bio-Affinity NMR Spectroscopy* (Henrichsen *et al.*, 1999) by exploiting the so-called *transferred NOE* effect (trNOE) (Ni, 1994). In this case, the signals of substrate are selectively observable because they undergo a large negative NOE enhancement during the time that it is bound to the receptor. Using a similar principle, the binding substrate can be characterized using *Saturation Transfer Difference NMR Spectroscopy* (Mayer and Meyer, 1999). To the same end,

the quite different 'SAR-by-NMR' (*Structure–Activity Relationship by Nuclear Magnetic Resonance*) approach, can be used (Shuker *et al.*, 1996). In this case, the binding of a molecule to a ^{15}N-labeled protein receptor is determined by the observation of ^{15}N or ^{1}H chemical shift changes in HSQC spectra. The procedure is particularly useful in the development of new target-directed drugs.

From the examples reported throughout this chapter, it is evident that not only is NMR spectroscopy of central importance for the structure elucidation of natural products (in which it is probably beating X-ray crystallography thanks to its flexibility), but it is also an important tool for several other aspects of the search for bioactive products from natural sources.

3.9 TECHNICAL NOTE

All the NMR experiments and molecular modeling calculations herewith reported for the macrolide **1** have been performed purposely for this work. The 800 MHz ^{1}H NMR spectrum was recorded on a Varian Inova instrument (GRA-UGA 800 MHz NMR Regional Facility, CCRC-UGA, Georgia, USA). The 200 MHz ^{1}H NMR spectrum was run on a Bruker AC-200 spectrometer (ICTMP-CNR, Catania, Italy).

The 500 MHz spectra were acquired at constant temperature (298 K) on a Varian Unity Inova spectrometer (Dipartimento di Scienze Chimiche, University of Catania, Italy) operating at 499.86 (^{1}H) and 125.70 MHz (^{13}C), equipped with gradient-enhanced, reverse-detection 5 mm probe, using software supplied by the manufacturer. The 90° ^{1}H and ^{13}C transmitter rf pulse width was 6.9 μs and 12.0 μs, respectively, while the corresponding 90° decoupler pulse width at the maximum power was 6.1 μs and 13.4 μs, respectively. A solution of 30 mg of **1** in 0.6 ml of $CDCl_3$ was used for all experiments, except for HMBC, run with a solution of 60 mg/0.6 ml.

Homonuclear 2D spectra were typically acquired with 2K data points for 512 t_1 increments of 16 scans (32 for DQF-COSY), resulting in a total acquisition time of *ca.* 2 h (*ca.* 8 h for DQF-COSY). DQF-COSY and ROESY spectra were acquired using the TPPI phase-sensitive mode. 2D TOCSY spectra were acquired using an 8 kHz spin-locking field (MLEV-17 sequence) and a mixing time of 40, 80 or 120 ms. 1D TOCSY spectra were performed using a 100 ms Gaussian soft pulse and an array of mixing times from 10 ms to 150 ms. The ROESY experiment was performed with a 2 kHz spin locking field (MLEV-17 sequence) using a mixing time of 250 ms. HSQC and HMBC were acquired with 2K data points for 512 t_1 increments of 32 and 160 scans, with a total time of *ca.* 5 and 14 h, respectively. Polarization transfer delays were optimized respectively for an average J_{CH} = 135 Hz (HSQC) and J_{CH} = 7.5 Hz (HMBC).

Molecular modeling was performed by using the *MacroModel* V4.5 program running on a Silicon Graphics Indigo XS 24 workstation. All structures were energy minimized with the Polak-Ribiere conjugate gradient algorithm, until an energy-gradient lower

than 0.05 kJ/(Å mol) was reached, using MM2 force-field and GB/SA chloroform-model-solvent. Conformational search was performed with the Monte Carlo Multiple Minimum (MCMM) procedure (5000 steps) allowing a 0–180° torsion angle to all rotatable single bonds.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Dr. O. Emaldi and Dr. G. Miraglia (Wyeth-Lederle SpA, Catania, Italy) for the biological material source of macrolide LL-F28249-α, in the frame of a Cooperative project with the University of Catania. Thanks are due to Dr. J. Glushka (CCRC-UGA, Georgia) for running the 800 MHz ^{1}H NMR spectrum as well as to Dr. G. Impallomeni (ICTMP-CNR, Catania, Italy) for the 200 MHz ^{1}H NMR. Dr. O.D. Longo and Dr. C. Spatafora [Dipartimento di Scienze Chimiche (DSC), Università di Catania, Italy] are also acknowledged for the purification and preparation of the samples. We thank also Prof. M. Piattelli (DSC) for reviewing the manuscript, as well as Dr. V. Calì (DSC) and Dr. Grazia M.L. Consoli (ISSN-CNR, Valverde, Italy) for valuable help in the preparation of this work. Financial support was obtained from the CNR (Roma, Italy) and the Ministero della Pubblica Istruzione, Università e Ricerca Scientifica e Tecnologica (PRIN, Roma, Italy) as well as by grants from the Università degli Studi di Catania (Progetti di Ricerca di Ateneo) and from the Università degli Studi di Salerno.

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CHAPTER

4

*Bioactive Secondary Metabolites from Selected Mexican Medicinal Plants: Recent Progress**

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Contents

*Dedicated to the memory of Professor Lidia Rodriguez-Hahn who made major contributions to the successful development of natural products chemistry in México.

4.1 INTRODUCTION

Current estimates indicate that Mexico is the third ranked megadiversity country after Brazil and Colombia, with flora from both temperate and tropical climates. According to a recent nationwide survey, there are about 21,600 species of vascular plants in Mexico. More than 3,350 from this stock form part of the medicinal flora, and a good testimony of the efficacy and cultural value of most of these species is their persistence in contemporary Mexican markets (Bye *et al.*, 1995).

Regardless of its richness and variety, only a small percentage of the Mexican medicinal flora has been investigated from the phytochemical or pharmacological point of view. Thus, the potential of Mexican medicinal plants as a source of bioactive compounds remains largely unexplored; mostly in view that many drugs and pest control agents have come to us from the use of plants by indigenous cultures. In this scenario, we have established a multidisciplinary research program to obtain phytochemicals of medicinal and agrochemical interest from Mexican plants used in folk medicine. In the medicinal area, our efforts have been focused on the discovery of active principles useful for the treatment of gastrointestinal disorders, which are a major health problem in Mexico (Mata, 1993; Mata *et al.*, 1999). Recently, we have also initiated a systematic investigation on Mexican Annonaceous as a source of cytotoxic acetogenins. With regard to pesticides, our investigations have been directed to the discovery of (i) herbicide agents, mostly affecting the process of photosynthesis (Lotina Hennsen *et al.*, 1998), and (ii) new insecticides from the Neotropical Meliaceae (Jiménez *et al.*, 1998 and references cited therein). The search for insecticides has been carried out in collaboration with Professor John T. Arnason from the University of Ottawa in Canada.

Our studies have been conducted following the experimental approach regarded as most successful for the discovery of active principles, i.e. bioassay-guided isolation. Once the plant materials are selected according to the ethnomedical and chemotaxonomic criteria, organic soluble extracts are prepared. The resulting extracts are submitted for biological testing using appropriate bioassay systems. After experimental confirmation of biological activity, the active extracts are fractionated, monitoring the biological activity at each step of the fractionation stage. This process continues until pure active compounds are isolated. The active isolates are subjected to structure elucidation and, when possible, to further biological studies.

The purpose of this chapter is to illustrate the potential of Mexican medicinal flora as a source of useful biologically active products through examples stemming from our research program. Brief considerations of the botanical sources, and of the isolation, the structure elucidation, and the biological properties of the active phytochemicals, are presented.

4.2 SMOOTH MUSCLE RELAXING CONSTITUENTS FROM *SCAPHYGLOTTIS LIVIDA* (ORCHIDACEAE)

Scaphyglottis livida (Lindley) Schltr. is an epiphytic orchid widely distributed along the tropical forest of the State of Veracruz, Mexico. Indigenous people refer to the orchid as 'parasita' and employ the whole plant medicinally. The ground herb is applied topically to the body of humans to eliminate ectoparasites. On the other hand, the decoction is employed for the treatment of stomach aches and to avoid abortion. These popular uses suggested the presence of smooth muscle relaxant agents in the plant. Consequently, we decided to validate this hypothesis with a pharmacological test employing the rat ileum model. The pharmacological investigation employing the rat ileum was performed as previously described, using two different types of experiments. In the first one, the crude extract, primary fractions and isolated compounds were evaluated for their ability to relax the spontaneous ileum contractions (Rojas *et al.*, 1995). In the second, the effect of pure compounds on cholinergic, histaminergic, nitrergic, and ion induced-smooth muscle contractions was investigated (Rojas *et al.*, 1996). The aim of the last set of experiments was to obtain information about the mode of action of the active compounds.

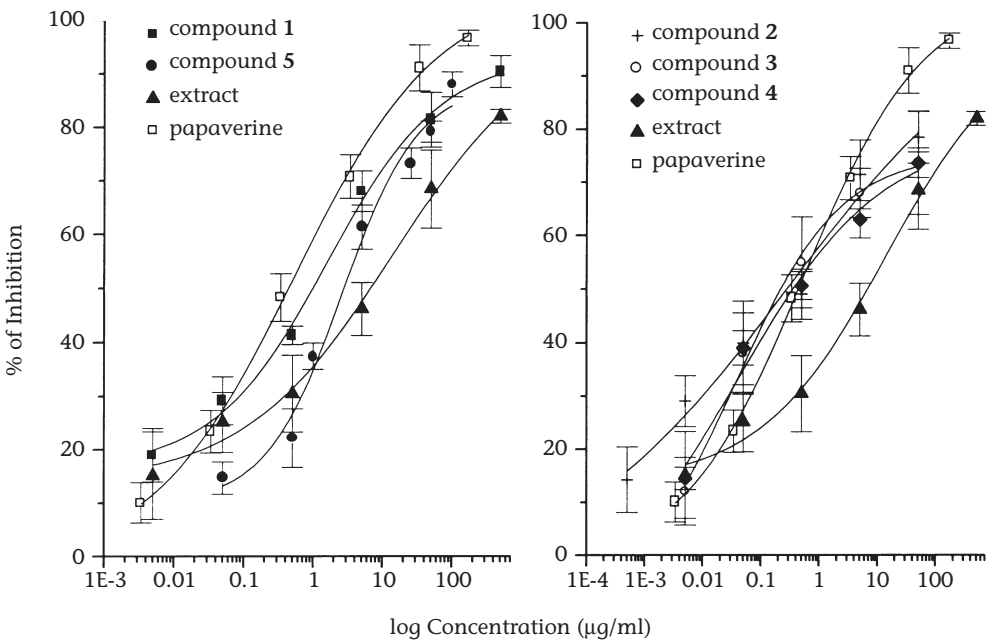


Figure 4.1: Concentration-response curves showing the relaxatory effect of the extract and compounds **1–5** from *Scaphyglottis livida* on isolated rat ileum. Values are expressed as the percentages of inhibition of contractile response calculated as the mean from six data ± S.E.M. $p < 0.05$. (Adapted from Estrada *et al.*, 1999, *Planta Med.* **65**, 109–114.)

TABLE 4.1
Inhibition of the spontaneous contraction of isolated rat ileum induced by the extract and compounds from *S. livida*

substance	Emax[a,c]	IC_{50} (M)[c]	potency[b]
Papaverine	96.70 ± 5.02	$1.55 \times 10^{-6} \pm 0.12$	1
extract	82.10 ± 6.02	6.06 ± 1.02*	—
compound **1**	93.50 ± 3.02	$5.83 \times 10^{-6} \pm 0.55$	0.2658
compound **2**	85.20 ± 2.08	$7.37 \times 10^{-7} \pm 0.07$	2.1031
compound **3**	80.00 ± 1.98	$9.50 \times 10^{-7} \pm 0.03$	1.6315
compound **4**	83.59 ± 1.30	$6.66 \times 10^{-7} \pm 0.01$	2.3273
compound **5**	88.05 ± 1.80	$7.13 \times 10^{-6} \pm 0.42$	0.2173

* The IC_{50} of the extract is expressed in μg/ml.
[a] Indicates the percentage of maximum inhibition.
[b] Potency was obtained by the formula: IC_{50} (M) papaverine/IC_{50} (M) compound, assuming a value of 1.00 for papaverine.
[c] Means ± S.E.M.; n = 6; $p < 0.05$.

In the preliminary pharmacological evaluation, it was found that a $CHCl_3$-MeOH (1:1) extract of *S. livida* displayed a significant inhibition of the tone and amplitude of the spontaneous contractions of the rat ileum. The effect was concentration dependent and the calculated IC_{50} (concentration value producing 50% inhibition) was 6.06 μg/ml (Figure 4.1 and Table 4.1). Bioactivity-guided fractionation of the active extract by column chromatography over silica gel yielded eleven primary fractions (F1 to F11). Two fractions (F5 and F6) decreased the spontaneous contractions of the rat ileum when tested at the IC_{50} of the original extract (Figure 4.2). HPLC separation (silica gel, hexane-*i*-PrOH-MeOH 94:3:3) of active fractions F5 and F6 led to the isolation of five active aromatic compounds, two bibenzyls [3,4′-dihydroxy-5,5′-dimethoxybibenzyl (**1**) and batatasin III (**2**)] and three phenanthrene derivatives [coelonin (**3**), 3,7-dihydroxy-2,4-dimethoxyphenanthrene (**4**) and 3,7-dihydroxy-2,4,8-trimethoxyphenanthrene (**5**)] (Figure 4.3). The five phytochemicals turned out to be known natural products and were characterized by spectroscopic and spectrometric methods (Estrada *et al.*, 1999).

Compounds **1–5** induced noted concentration-dependent inhibition of the spontaneous contractions of rat ileum (Figure 4.1). The highest inhibitory activities were observed for compounds **2–4**, which were more potent than papaverine used as a positive control (Estrada *et al.*, 1999).

In the second set of experiments, it was demonstrated that the relaxant activity elicited by the isolates is mediated, at least in part, by neuronal release of nitric oxide (NO). Addition of **1–5** directly to the organ bath abolished the smooth muscle contractile response (Figure 4.4) induced by NG-nitro-L-arginine methyl ester (L-NAME, 5×10^{-3} M), an inhibitor of nitric oxide synthase. Furthermore, addition of L-NAME

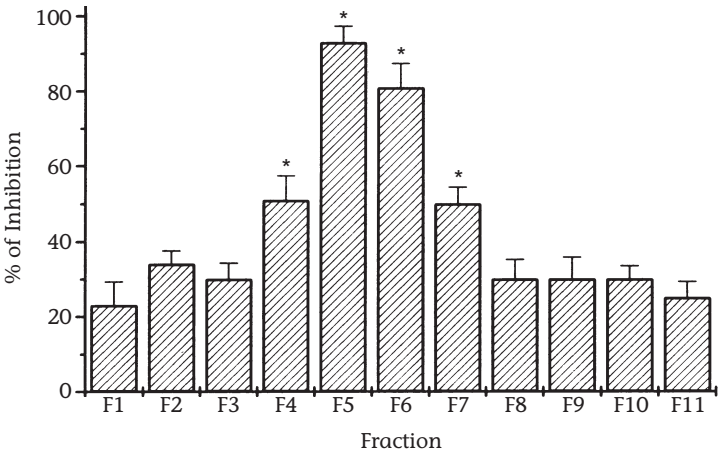


Figure 4.2: Effect of the chromatographic fractions from the chloroform-methanol (1:1) extract of *Scaphyglottis livida* on the spontaneous contractions of the rat ileum. Values are expressed as the percentages of inhibition of contractile response obtained at the IC_{50} of the original extract calculated as the mean from six data ± S.E.

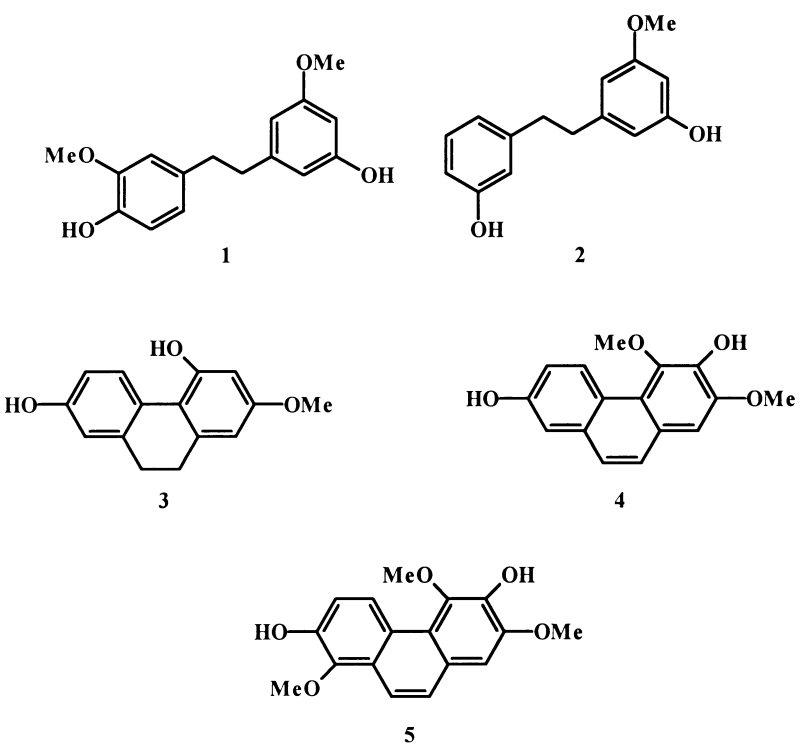


Figure 4.3: Smooth muscle relaxing constituents from *Scaphyglottis livida* (Orchidaceae).

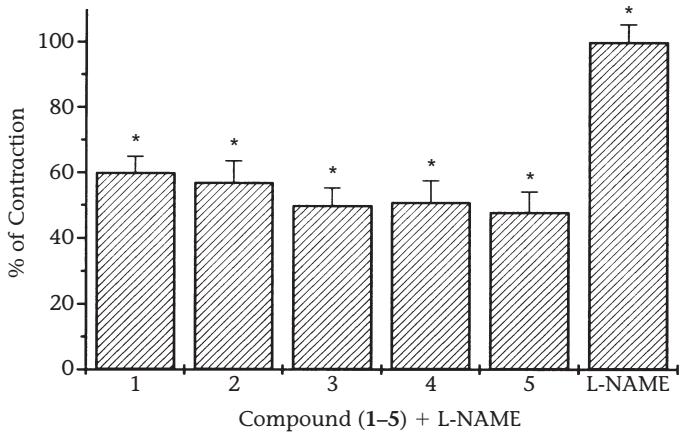


Figure 4.4: Effect of the isolated compounds **1–5** from *Scaphyglottis livida* on the contractions induced by L-NAME on the rat ileum. *p < 0.05 (ANOVA).

(5×10^{-3} M) to the preparation reverted the relaxatory effect evoked by **1–5**. As an example, the effects induced by bibenzyl **1** are shown in Figure 4.5 (Estrada *et al.*, 1999).

NO brings about smooth muscle relaxation by binding to iron in the hem that is part of soluble guanylyl cyclase, stimulating the formation of cGMP, a second messenger. cGMP, in turn, stimulates a protein kinase, which phosphorylates the light chain of myosin, eliciting relaxation (Bredt and Snyder, 1992). To establish the signaling pathway which mediates the relaxing effect of **1**, the major active principle isolated, compound **1**-provoked NO/cGMP formation in rat whole ileum rings was measured by using a radio-immunoassay procedure (Leurs *et al.*, 1991). The results revealed that **1**

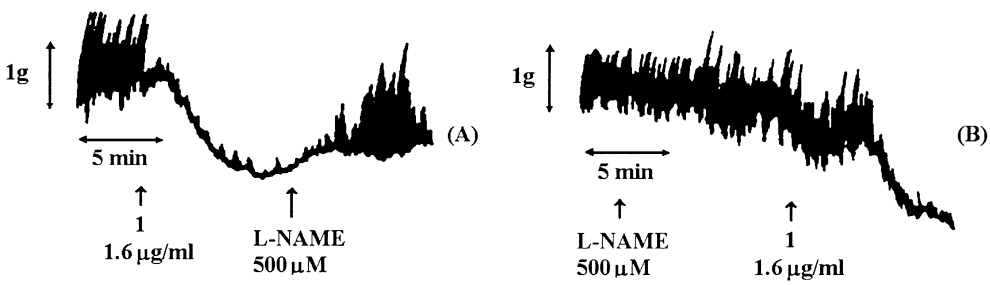


Figure 4.5: (A) Tracing of rat ileal muscle showing that the relaxatory response induced by compound **1** was reversed in the presence of L-NAME. (B) Tracing of the rat ileal muscle showing the relaxatory response evoked by compound **1** after treatment with L-NAME during 10 min. The arrows indicate the treatments added to the bathing fluid. (From Estrada *et al.*, 1999, *Planta Med.* **65**, 109–114, with permission.)

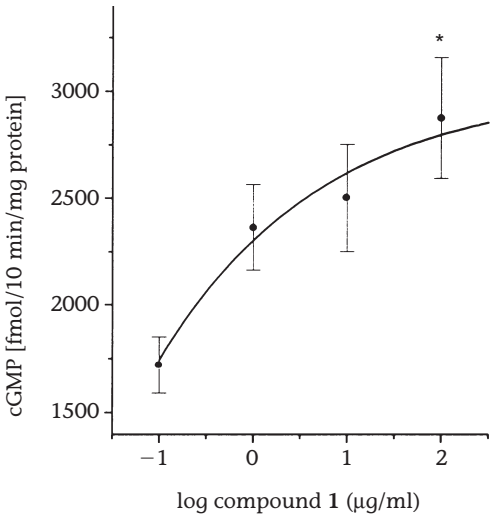


Figure 4.6: Concentration-response curve showing compound 1-induced cGMP generation in rat whole ileum rings. Results are means ± S.E.M. of n = 8. *$p < 0.01$ compared with basal value. (Adapted from Estrada *et al.*, 1999, *Planta Med.* **65**, 109–114.)

induces cGMP generation in a concentration-dependent manner (Figure 4.6). Furthermore, when the ileum rings were stimulated with a single concentration of bibenzyl **1** (100 μg/ml) in the presence of L-NAME or 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ), a potent selective inhibitor of the soluble guanylyl cyclase, compound **1**-induced cGMP production was abolished (Table 4.2). These experiments clearly indicated that compound **1** exerts its relaxing action through the stimulation of nitric oxide synthase and the generation of cGMP (Estrada *et al.*, 1999). The stimulation of cGMP formation induced by compound **1** was almost completely dependent on the presence

TABLE 4.2

Effect of compound **1**-induced cGMP (fmol/10 min/mg protein) generation in the presence of L-NAME and ODQ, and in the absence of calcium

Basal (n = 26)	1620.85 ± 86
Compound **1** (n = 24)	2260.18 ± 88*
L-NAME (n = 16)	1760.62 ± 146
Compound **1** + L-NAME (n = 18)	2090.53 ± 124
ODQ (n = 11)	1770.25 ± 209
Compound **1** + ODQ (n = 11)	1480.40 ± 194
Basal (-Ca^{2+}) (n = 18)	1900.63 ± 188
Compound **1** (-Ca^{2+}) (n = 11)	1430.49 ± 158

Concentration of compound **1**: 100 μg/ml. *$p < 0.01$ compared to basal.

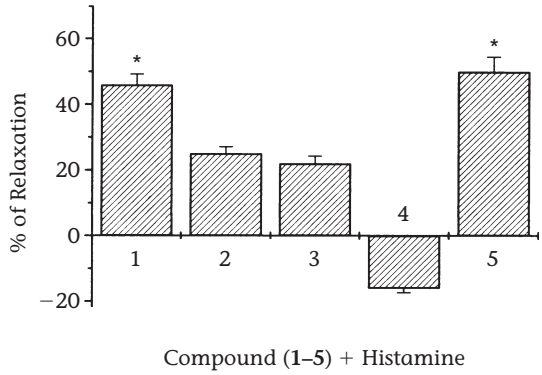


Figure 4.7: Effect of the isolated compounds **1–5** from *Scaphyglottis livida* on the contractions induced by histamine on the rat ileum. *p < 0.05 (ANOVA).

of the extracellular calcium. Removal of this cation from the medium and the simultaneous addition of an excess of EGTA resulted in a blockade of compound **1**-induced cGMP accumulation (Table 4.2).

It is important to point out that the relaxant activity of the isolates does not involve a direct anticholinergic mode of action or an interference with calcium influx in the smooth muscle cells (Estrada *et al.*, 1999). However, compounds **1** and **5** exhibited a moderate antihistaminergic effect since they significantly antagonized the contractions induced by histamine (1×10^{-3} M) (Figure 4.7).

In conclusion, the potent spasmolytic action shown by the extract and aromatic compounds **1–5** isolated from *S. livida* could be related to the ethnomedical use of this species in Mexican folk medicine for the treatment of stomach aches. The pharmacological investigation on compounds **1–5** indicated that the production of nitric oxide in ileal tissue is involved in the spasmolytic effect. In the case of compound **1**, nitric oxide/cGMP formation constitutes the signaling pathway in the spasmolytic action. The moderate antihistaminergic effect exhibited by **1** and **5** could also be related with the antispasmodic response exerted by these compounds. Finally, it is important to indicate that aromatic compounds **1–5** represent new leads for the development of smooth muscle relaxing agents of therapeutic interest.

4.3 ANTIPROTOZOAL PROANTHOCYANIDINS FROM *GERANIUM NIVEUM* (GERANIACEAE)

Geranium niveum S. Watson (Geraniaceae) is a silvery canescent-leaved herb, which grows along the dry stream banks and grassy meadows of the pine-oak forests in the high mountains of western Chihuahua, Mexico. The Tarahumara Indians call this

perennial herb 'makiki' and employ the decoction of the roots as an antifebrile, a purgative and as a remedy for kidney pain and gastrointestinal ailments.

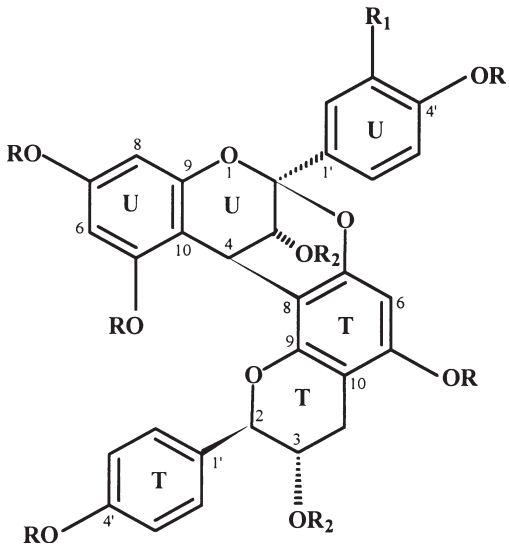
In a preliminary screening conducted to evaluate the *in vitro* antiprotozoal activity against axenically grown trophozoites of *Giardia lamblia* and *Entamoeba histolytica* of some Mexican medicinal plants *G. niveum* was selected for fractionation (Calzada *et al.*, 1998). After the initial observation of the significant antiprotozoal activity induced by a $CHCl_3$-MeOH (1:1) extract prepared from the roots of *G. niveum* (Table 4.3), large-scale extraction and fractionation was undertaken. The crude active extract was suspended in water and subjected to consecutive solvent partition with $CHCl_3$ and EtOAc. The resulting fractions were tested for their ability to inhibit the growth of trophozoites of *G. lamblia* and *E. histolytica*. The highest level of activity was found in the EtOAc fraction (IC_{50} = 6.6 and 18.7 μg/ml for *E. histolytica* and *G. lamblia*, respectively). The active fraction was separated by sequential column chromatography using silica gel with increasing solvent polarity, gel permeation (Sephadex LH-20) and HPLC (Spherisorb S5ODS2) to yield two novel antiprotozoal A-type proanthocyanidins, along with several known compounds which were inactive. The new proanthocyanidins were characterized as *epi*-afzelechin-(4β → 8, 2β → *O* → 7)-afzelechin (**6**), *epi*-catechin-(4β → 8, 2β → *O* → 7) afzelechin (**7**) and were given the trivial names of geranins A and B (Figure 4.8), respectively (Calzada *et al.*, 1999). The approach for elucidating the structures of both compounds was identical, and to illustrate the most relevant aspect of this process, the identification of geranin A (**6**) will be briefly discussed.

Geranin A (**6**) was found to have a molecular formula of $C_{30}H_{24}O_{10}$ based on HRFABMS. The A-type proanthocyanidin nature of compound **6** was evident from the ^1H-, ^{13}C- (Figure 4.9) and COSY NMR spectra. Thus, the ^1H-NMR spectrum contained the characteristic signals for the dihydropyran rings of the upper (U) and terminal (T) flavan-3-ol units. The resonances for the U dihydropyran ring appeared as an AB system at δ 4.08 (d, *J* = 3.5 Hz, H-3U) and 4.26 (H-4U, d, *J* = 3.5 Hz). Those of the T unit were observed as an A_2XY system at δ 4.80 (d, *J* = 8.0 Hz, H-2 T), 4.17 (ddd, *J* = 8.5, 8.0 and

TABLE 4.3
Antiprotozoal activity of the MeOH-$CHCl_3$ (1:1) extract and geranins A (**6**) and (**B**) from *G. niveum*

compound	IC_{50} μg/mL (CI)[a]	
	Giardia lamblia	*Entamoeba histolytica*
extract	20.6 (20.7–20.5)	8.7 (8.9–8.5)
6	2.4 (2.6–2.1)	184.7 (186.1–183.4)
7	6.0 (7.0–5.9)	13.6 (14.0–13.0)
Metronidazole[a]	0.21	0.04

[a] Positive control



	R	R_1	R_2
6	H	H	H
6a	Me	H	H
6b	Me	H	(*S*)-MTPA
6c	Me	H	(*R*)-MTPA
7	H	OH	H

Figure 4.8: Structures of the antiprotozoal A-type proanthocyanidins from *Geranium niveum* (Geraniaceae).

5.5 Hz, H-3T), 2.93 (dd, J = 16.5, 5.5 Hz, H-4Ta) and 2.58 (dd, J = 16.5, 8.5 Hz, H-4Tb). The J (8 Hz) value between H-2T and H-3T revealed the *trans* relationship of these protons. In addition, the aromatic region of this spectrum indicated the presence of four aromatic rings in the molecule. The correspondent resonances appeared as a singlet at δ 6.10 (H-6T), two A_2B_2 and one AB systems consistent with a penta-, two *p*-di- and one tetra-substituted benzene rings, respectively. The ^{13}C-NMR (Figure 4.9) data and the HMQC correlations supported the above ^{1}H-NMR assignments.

Once the nature of the compound was determined, the next step in the structure elucidation process was to define the linkage between the two flavonoids moieties. Biogenetically, the two flavonoid units can be linked to build up the basic skeleton of an A-type proanthocyanidin through three type of linkages, namely (4 → 8, 2 → *O* → 7), (4 → 6, 2 → *O* → 5), and (4 → 6, 2 → *O* → 7). A NOESY experiment allowed to discriminate between these possibilities, thus, the correlations H-4U/H-2T, H-3U and H-6U/H-2′T,

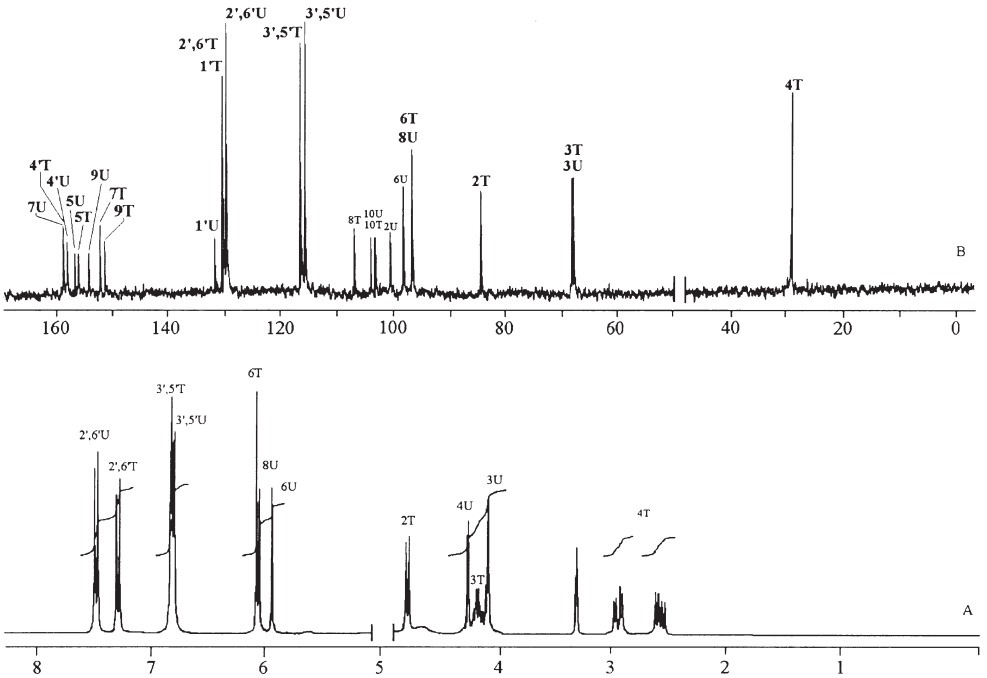


Figure 4.9: ^{1}H (A, 500 MHz) and ^{13}C (B, 125 MHz) NMR spectra of geranin A (**6**) in MeOH-d_4.

H-6′T defined the ($4 \rightarrow 8$, $2 \rightarrow O \rightarrow 7$) linkage. The correlations H-2T/H-3U and H-4U were also observed in the COSY spectrum of **6**.

With the planar structure resolved, the absolute configuration at the chiral centers was established by combining CD measurements and ester Mosher methodology. According to previous reports proanthocyanidins with 4*R*- configuration all show a positive large Cotton effect ~200–220 nm, whilst those with 4*S*-absolute stereochemistry exhibit a negative couplet in this zone (Barret *et al.*, 1979). The CD spectrum of geranin A (**6**) displayed a strong positive Cotton effect at 220 nm ($[\theta] = 3.26 \times 10^3$), consequently the stereochemistry at C-4U was *R*. Automatically the stereochemistry at C-2U was assigned as *R*. To determine the absolute stereochemistry at C-3U and C-3T, the *S*-(+) and *R*-(−)-MTPA esters **6b** and **6c**, respectively, were prepared from the methyl derivative **6a** which, in turn, was obtained by treatment of **6** with dimethyl sulfate. The analysis of the $\Delta\delta_{R\text{-}S}$ data of the *R*-(+) and *S*-(−)-MTPA esters **6c** and **6b** showed positive differences for H-2′, 6′U [$\Delta\delta_{R\text{-}S} = +0.13$] and H-3′, 5′U [$\Delta\delta_{R\text{-}S} = +0.20$] and a negative difference for H-6U [$\Delta\delta_{R\text{-}S} = -0.12$] indicating that the absolute stereochemistry of the chiral center at C-3U was *R*. The positive difference found for H-4T*ax* [$\Delta\delta_{R\text{-}S} = +0.13$] and the negative differences for H-2T [$\Delta\delta_{R\text{-}S} = -0.10$] and H-2′, 6′T [$\Delta\delta_{R\text{-}S} = -0.10$] revealed that the absolute stereochemistry of the chiral center at C-3T was *S*. Thereafter,

the absolute configuration at C-2T was determined as *S* because of the *trans* relationship between H-3T and H-2T.

Geranins A (**6**) and B (**7**) exhibited moderate antiprotozoal properties, compared with the standard, against *G. lamblia* and *E. histolytica* (Table 4.3). In both cases, *G. lamblia* was the most sensitive protozoan. None of the geranins displays cytotoxic activity against three different cell lines (MCF-7 breast carcinoma, HT-29 colon adenocarcinoma, and A-549 lung carcinoma) in a 7-day MTT test (Calzada *et al.*, 1999). To the best of our knowledge, this is the first report not only of antiprotozoal properties for proanthocyanidins but also for the presence of this type of compound in the genus *Geranium*. Finally, our findings could provide some scientific support for the ethnomedical use of the roots of this species.

4.4 CYTOTOXIC ACETOGENINS FROM *ROLLINIA MUCOSA* AND *ANNONA PURPUREA* (ANONNACEAE)

The Annonaceus acetogenins are powerful antitumor and pesticidal products that are found only in the plant family Annonaceae. These compounds exhibited their potent bioactivities through depletion of ATP levels via inhibiting complex I of the mitochondria and inhibiting the NADH oxidase of plasma membranes of tumor cells. Applications as pesticides and antitumor agents hold excellent potential, especially in the thwarting of resistance mechanisms, which require an ATP-dependent efflux (Alali *et al.*, 1999 and references cited therein). The Annonaceae family is richly distributed in Mexico, mainly in the tropical lowlands, however, the potential of the Mexican species as a source of new acetogenins remains unexplored. Therefore, using the brine shrimp lethality test (BST), a reliable biological test to detect Annonaceous acetogenins (McLaughlin, 1991), we began screening seeds, leaves and bark samples of all available Annonaceae from the region of Los Tuxtlas, State of Veracruz, and accordingly selected *Rollinia mucosa* (Jacquin) Baillon and *Annona purpurea* Moc. & Sessé ex Dunal (Annonaceae) for fractionation.

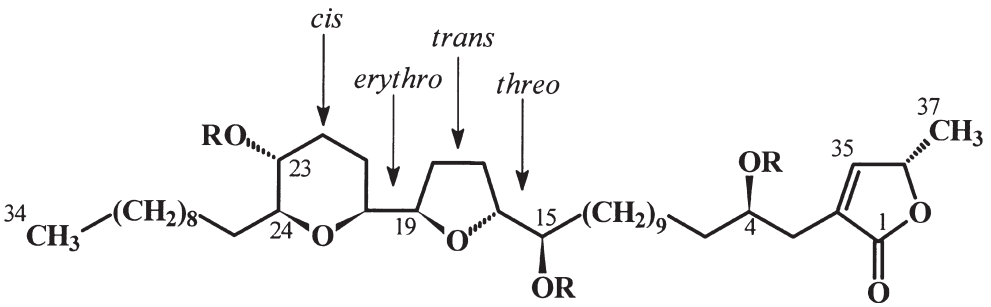
4.4.1 Chemical investigation of *Rollinia mucosa*

Rollinia mucosa is a tree widely distributed in Tropical America. The fruit of this plant, commonly known in Mexico as 'anonillo', 'anonita del monte' and 'cherimoya', is edible and employed in folk medicine for the treatment of cancer and gastrointestinal disorders. Previous chemical investigations of the fruit, leaves, bark and seeds of *R. mucosa* led to the isolation and identification of a number of cytotoxic and insecticide acetogenins (Alali *et al.*, 1999 and references cited therein; Cave *et al.*, 1997 and references cited therein).

From the seeds of *R. mucosa*, we have isolated a novel cytotoxic acetogenin,

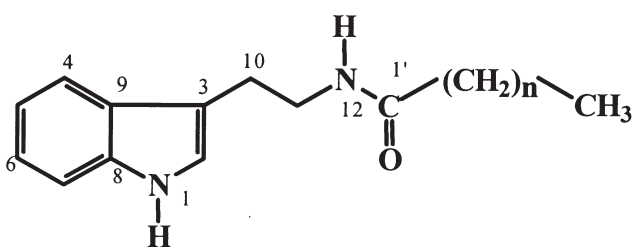
jimenezin (**8**) (Figure 4.10), and eight novel amides derived from tryptamine, N-palmitoyltryptamine (**9**), N-stearoyltryptamine (**9a**), N-arachidoyltryptamine (**9b**), N-behenoyltryptamine (**9c**), N-tricosanoyltryptamine (**9d**), N-lignoceroyltryptamine (**9e**), N-pentacosanoyltryptamine (**9f**) and N-cerotoyltryptamine (**9g**), (Figure 4.11) (Chávez *et al.*, 1998, 1999).

The dried seeds of *R. Mucosa* were extracted with CH-Cl_3-MeOH (1:1). The resulting crude extract exhibited potent activity in the brine shrimp lethality test and against human solid tumor cell lines (Table 4.4). The active extract (LC_{50} = 0.41 μg/ml) was subjected to solvent partition with hexane and 10% H_2O in MeOH. The most active fraction as evaluated in the brine shrimp lethality test was the methanolic (brine shrimp



compound	R
8	H
8b	(*S*)-MTPA
8c	(*R*)-MTPA

Figure 4.10: Structure of jimenezin (**8**).



compound	n	compound	n
9	14	**9d**	21
9a	16	**9e**	22
9b	18	**9f**	23
9c	20	**9g**	24

Figure 4.11: Novel Tryptamine amide derivatives from *Rollinia mucosa*.

TABLE 4.4
Brine shrimp lethality and cytotoxicity data for the extract and compounds from *A. purpurea* and *R. mucosa*

compound	[μ]	A-549[b]	MCF-7[c]	HT-29[d]	A-498[e]	PC-3[f]	PACA-2[g]
A. purpurea extract	1.1	$<10^{-2}$	1.53	1.47	3.53	1.16	$<10^{-2}$
R. mucosa extract	0.41	>10	>10	1.69	$<10^{-3}$	$<10^{-3}$	6.95
purpurediolin (**10**)	7.0×10^{-2}	4.43×10^{-1}	9.16×10^{-1}	$<10^{-7}$	1.36	3.53×10^{-1}	1.44
purpurenin (**11**)	2.9×10^{-2}	1.29	1.67	3.16×10^{-1}	1.25	1.07	1.98
purpuracenin (**12**)	3.0	4.8×10^{-2}	>10	>10	$< 10^{-3}$	$< 10^{-3}$	>10
annoglaucin (**13**)	2.2×10^{-2}	1.08	1.56	$<10^{-7}$	1.01	3.56×10^{-1}	1.45
jimenezin (**8**)	5.7×10^{-3}	1.64×10^{-2}	$> 10^{-1}$	4.25×10^{-3}	4.94×10^{-2}	2.77×10^{-4}	1.69×10^{-4}
adriamycin		4.47×10^{-3}	8.97×10^{-2}	1.62×10^{-2}	1.10×10^{-3}	2.13×10^{-2}	2.88×10^{-3}

[a] Brine shrimp lethality test; data are expressed as LC_{50}. [b] Human lung carcinoma. [c] Human breast carcinoma. [d] Human colon adenocarcinoma. [e] Human kidney carcinoma. [f] Human prostate adenocarcinoma. [g] Human pancreatic carcinoma

lethality test $LC_{50} = 6 \times 10^{-2}$ μg/ml). This fraction was further separated by open column chromatography using silica gel with increasing solvent polarities to yield 14 secondary fractions (F1–F14). Repeatedly HPLC [silica gel, hexane-*i*PrOH-MeOH, (92:4:5)] of the most active fraction F6 (brine shrimp lethality test $LC_{50} = 7 \times 10^{-3}$ μg/ml) afforded jimenezin (**8**) (Chávez *et al.*, 1998). HPLC separation of the less active fraction F4 (brine shrimp lethality test $LC_{50} = 41.9$ μg/ml) yielded amides **9–9g**. Finally, HPLC purification of the remaining active fractions F5 and F7 (BST $LC_{50} = 2.2$ μg/ml and 4.5×10^{-1} μg/ml, respectively) afforded several known acetogenins (membranacin, desacetyluvaricin, rolliniastatin 1, bullatacin, squamocin, and motrilin) (Chávez *et al.*, 1999).

Jimenezin (**8**), which possess a novel skeleton, belongs to the rare type acetogenins containing a tetrahydropyran (THP) ring. Its structure elucidation was pursued by spectroscopic, chiroptical and chemical methods (Chávez *et al.*, 1998). The molecular formula was established as $C_{37}H_{66}O_7$ by HRFABMS. The IR spectrum contained absorptions for hydroxyl (3418 cm^{-1}) and α,β-unsaturated lactone (1715 cm^{-1}). Sequential losses of three molecules of H_2O from the MH^+ in the FABMS as well as the formation of the tri-TMSi derivative **8a** confirmed the existence of three hydroxyl groups in compound **8**. The presence in jimenezin (**8**) of an α,β-unsaturated methyl γ-lactone with a hydroxyl group at C-4 was suggested by the ^1H NMR (Figure 4.12) resonances at δ 7.18 (H-35), 5.06 (H-36), 3.85 (H-4), 2.53 (H-3a), 2.40 (H-3b), and 1.43 (H-37) corresponding in the ^{13}C NMR spectrum (Figure 4.13) to the resonances at δ 174.6 (C-1), 151.7 (C-35), 131.2 (C-35), 78.0 (C-36), 70.0 (C-4), 33.3 (C-3) and 19.1 (C-37). In addition to the resonance attributable to H-4 the ^{1}HNMR spectrum exhibited six well-defined signals in the range of δ H 3.0–4.0. Those at 3.75 (H-15), 3.90 (H-16) and 3.94 (H-19) were assigned to the THF ring with a flanking hydroxyl group. The remaining signals at

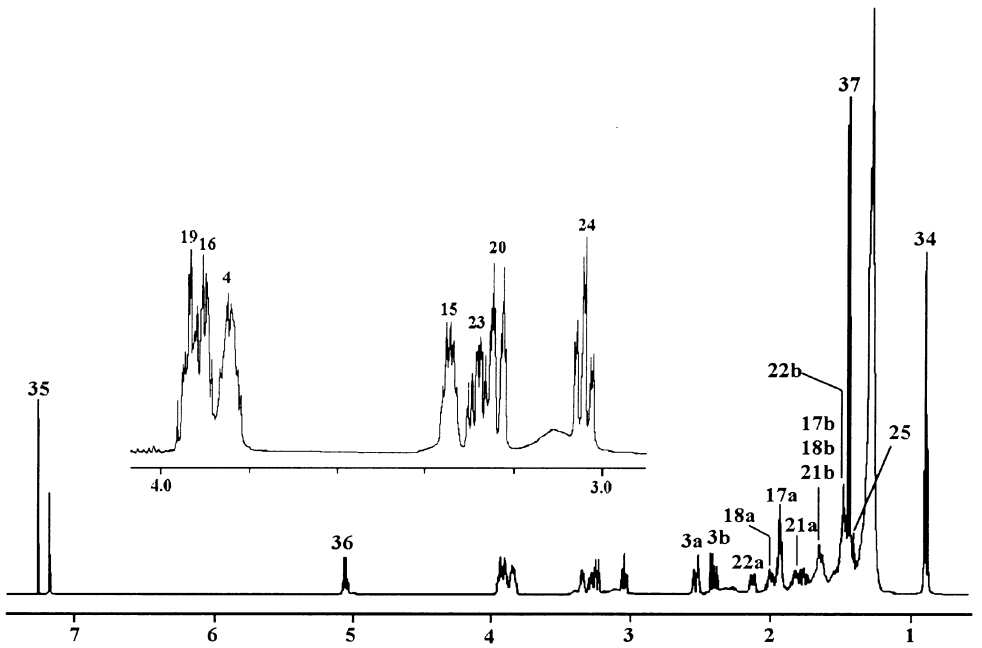


Figure 4.12: ^{1}H NMR (500 MHz) spectrum of jimenezin (**8**) in $CDCl_3$.

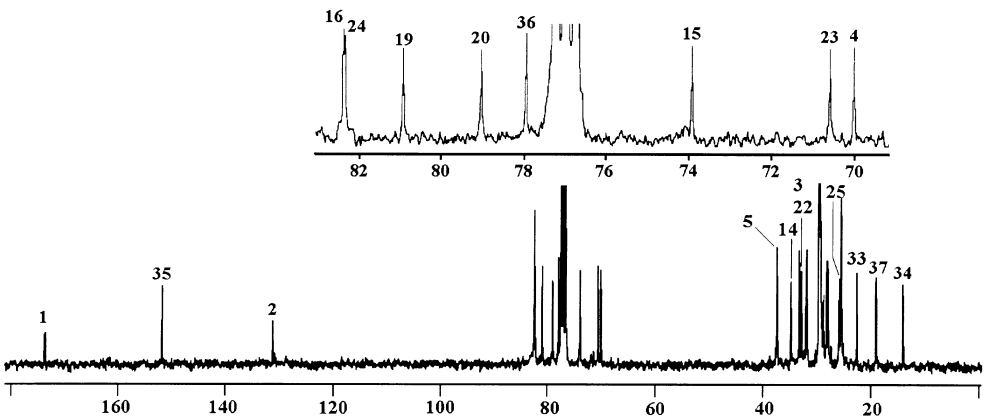


Figure 4.13: ^{13}C NMR (125 MHz) spectrum of jimenezin (**8**) in $CDCl_3$.

δ 3.00 (H-24), 3.24 (H-20) and 3.28 (H-23) were consistent with the presence of a hydroxylated THP ring in the molecule. The COSY NMR relationships found between H-16/H-15, H-20/H-19, H-24/H-23, H-23/H-22, H-22/H-21 and H-21/20, not only confirmed the presence of a hydroxylated THP moiety but also established that this was adjacent to the THF ring that was itself flanked by a hydroxyl group.

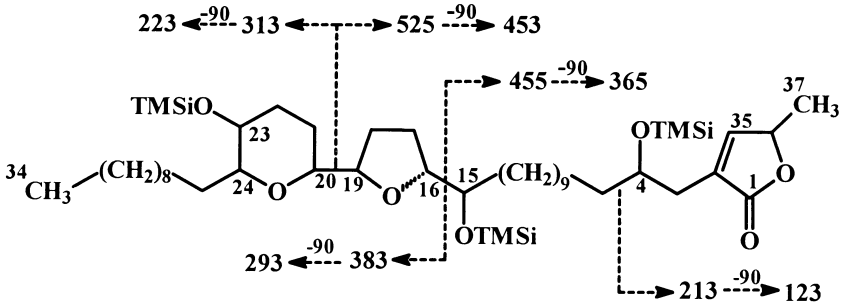


Figure 4.14: Diagnostic EIMS fragment ions of the TMSi derivative **8a**.

The disposition of the adjacent THF-THP unit along the aliphatic chain was determined by the analysis of the fragmentation pattern displayed by the tri-TMSi derivative **8a** (Figure 4.14). Thus the intense fragment ion peaks at m/z 455 (cleavage at C-15/C-16) and m/z 525 (cleavage at C-19/20) allowed placement of the THF ring with a flanking hydroxyl between C-15 and C-19 and of the hydroxylated THP ring between C-20 and C-24.

The relative stereochemistry at C-15/C-16 was assigned as *threo* based on the chemical shift values of both C-15 (δ 73.9) and H-15 (δ 3.35) (Born *et al.*, 1990). On the other hand, the relative configuration of the chiral centers of the THF moiety was determined as *trans* by comparing the NMR information with that of other acetogenins. The *cis* stereochemistry of the THP ring was established by interpreting the NOESY spectrum (Figure 4.15) that exhibited an intense cross-peak between H-20 (δ 3.24) and H-24 (δ 3.00). On the other hand, the *trans*-diaxial relationship of H-23 and H-24 was determined by a homodecoupling NMR experiment (Figure 4.16). Thus, irradiation of the resonance at δ 1.42 (H-25) simplified the signal assignable to H-24 to a doublet (J = 9.2 Hz). The magnitude of the coupling constant observed for this doublet was consistent with the *trans* relationship between H-23 and H-24 and, therefore, with the equatorial orientation of the hydroxyl group at C-23.

The absolute configuration of the stereogenic carbinol centers was established using Mosher ester methodology. Analysis of the $\Delta\delta_{S\text{-}R}$ data (Table 4.5) of the per-(*S*)- and per (*R*)-MTPA Mosher ester derivatives **8b** and **8c** showed that the absolute stereochemistry of the chiral centers at C-15, C-23 and C-4 were *R*, *R*, and *R*, respectively. Therefore, the absolute stereochemistry for C-16, C-19, C-20 and C-24 was deduced as *R*, *R*, *S* and *S*, respectively. Finally, the *S* configuration at C-36 was established according to the Hoye's Mosher ester method (Hoye *et al.*, 1994). Thus, the $\Delta\delta_{S\text{-}R}$ values for H-35 and H-36 were −0.25 and −0.06, respectively, suggesting an unlike relative configuration for C-4/C-36. Since C-4 has *R* configuration, C-36 must possess the *S* configuration. The negative Cotton effect at 238 nm in the CD spectrum of jimenezin (**8**) provided further evidence for the *S* configuration at C-36.

The structural identification of the novel amides was accomplished by a combination

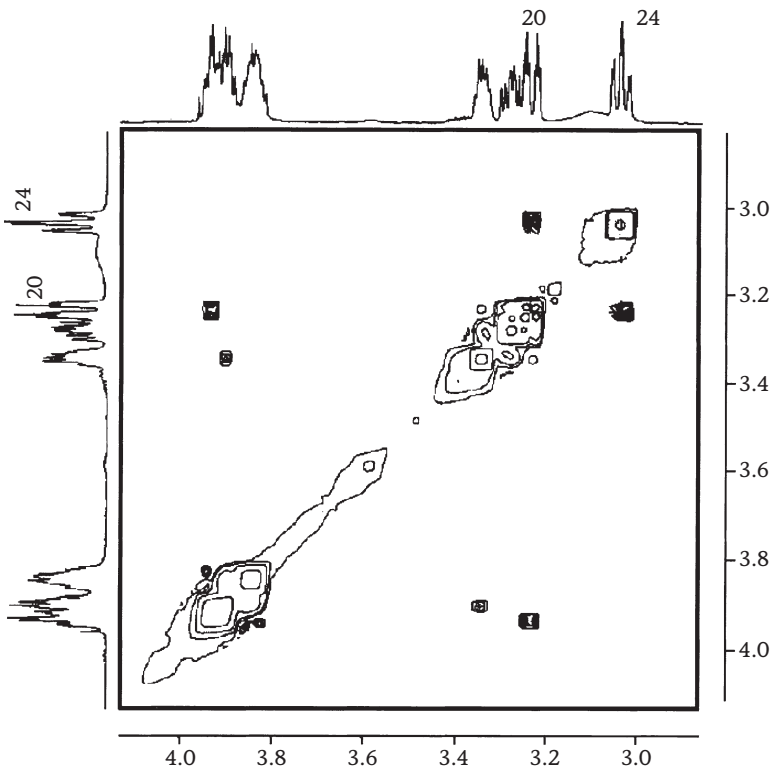


Figure 4.15: Partial view of the NOESY spectrum (500 MHz, $CDCl_3$) of jimenezin (**8**) showing the correlation H-20/H-24.

of chemical, spectrometric and spectroscopic methods (Chávez *et al*., 1999). It is relevant to indicate that this is the first report of tryptamine derived alkaloids from a member of the Annonaceae, a family well known as a source of a variety of isoquinoline alkaloids.

4.4.2 Chemical investigation of *Annona purpurea*

Annona purpurea Moc. & Sessé ex Dunal (Anonnaceae) is a small tree up to 7 m high. The fruit of this plant, commonly known as 'ilama' in Mexico, is edible. It is also used in folk medicine as a remedy for fevers and colds. From the leaves of this species, Hostettmann and co-workers obtained six acetogenins, namely, bulatacin, rolliniastatin 1, purpureacin 2, cherimoline, sylvaticin, and purpureacin (Cepleanu *et al*., 1993).

Following the same isolation procedure as for *R. mucosa*, three novel adjacent bis-THF acetogenins, purpurediolin (**10**), purpurenin (**11**) and purpuracenin (**12**), were obtained from the seeds of *A. purpurea* (Figures 4.17 and 4.18). In addition, the known compounds

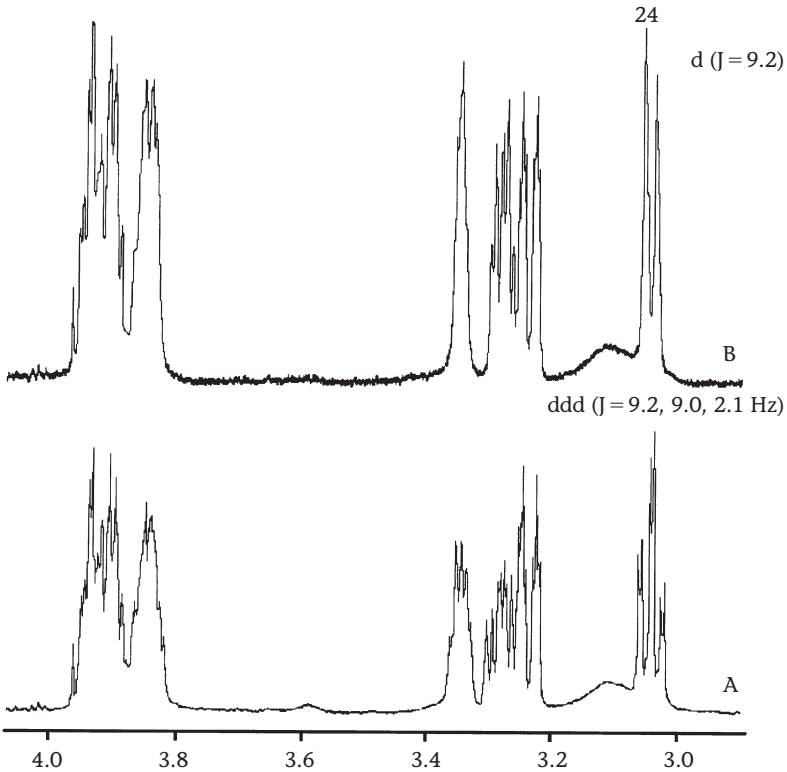


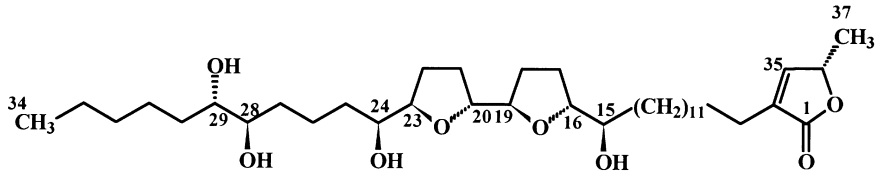
Figure 4.16: Homodecoupling NMR experiment on jimenezin (**8**). (A) Normal spectrum (only the zone δ_H 3.0–4.0 is shown). (B) Irradiation at δ_H 1.42 (H-25).

TABLE 4.5

Partial ^{1}H NMR[a] data of the (*S*)- and (*R*)- Mosher esters **8b** and **8c**

proton	δ_H **8b**	**8c**	$\Delta\delta_{S-R}$	proton	δ_H **8b**	**8c**	$\Delta\delta_{S-R}$
14	1.62	1.51	+0.11	22a	2.32	2.24	+0.08
16	4.01	4.02	–0.01	22b	1.62	1.41	+0.21
17a	1.81	1.91	–0.10	24	3.23	3.26	–0.03
17b	1.46	1.70	–0.24	25	1.30	1.49	–0.19
18a	1.90	1.91	–0.01	35	6.78	7.03	–0.25
18b	1.52	1.79	–0.27	36	4.86	4.92	–0.06
20	3.28	3.21	+0.07	3a	2.54	2.62	–0.08
21a	1.81	1.61	+0.20	3b	2.62	2.69	–0.07
21b	1.54	1.53	+0.01	5	1.69	1.65	+0.04

[a] Obtained in $CDCl_3$ at 500MHz.



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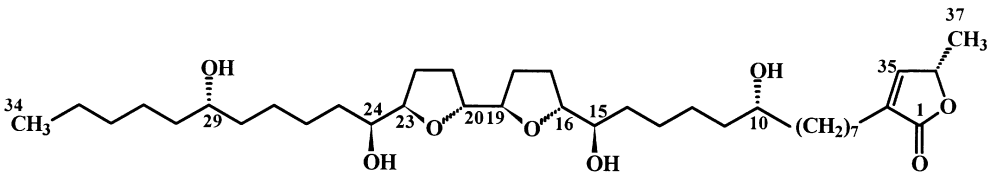

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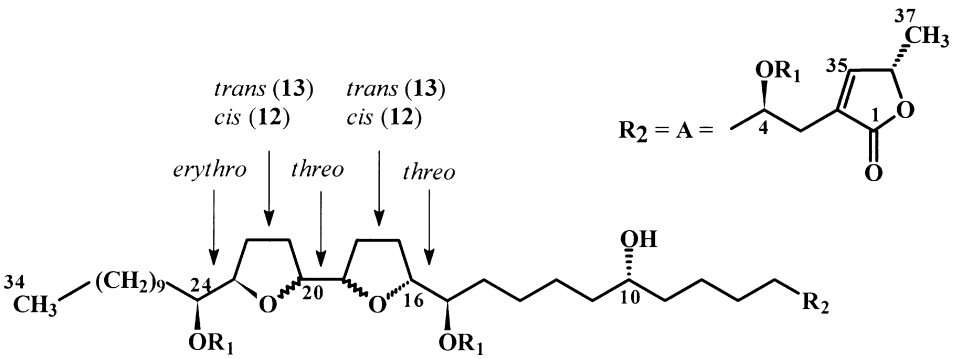
Figure 4.17: Structures of purpurediolin (**10**) and purpurenin (**11**) from *Annona purpurea*.



	R_1	R_2		R_1	R_2
12	H	A	**13**	H	A
12a	H	B	**13a**	H	B
12b	(*S*)-MTPA	A	**13b**	(*S*)-MTPA	A
12c	(*R*)-MTPA	A	**13c**	(*R*)-MTPA	A
12d	(*S*)-MTPA	B	**13d**	(*S*)-MTPA	B
12e	(*R*)-MTPA	B	**13e**	(*R*)-MTPA	B

Figure 4.18: Structures of purpuracenin (**12**), annoglaucin (**13**) and derivatives.

bullatacin, squamocin, motrilin, annoglaucin (**13**), xylomatenin, and annonacin A were isolated (Chávez and Mata, 1998, 1999). The new acetogenins vary in the location of the hydroxyl groups along the aliphatic chain and in the stereochemistry around the bis-THF rings.

The structure elucidation of the novel compounds from *A. purpurea* was accomplished following the standard strategies for identifying this type of acetogenins (Gu *et al.*, 1995; Cave *et al.*, 1997; Alali *et al.*, 1999 and references cited therein). Once more, the application of the Mosher ester method and CD measurements established the absolute stereochemistry of the stereogenic centers in compounds **10–13**. The absolute configuration at C-10 in acetogenins **12** and **13** was not solvable by spectral analysis of the (*S*)- and (*R*)-per-Mosher ester derivatives of the natural products (**12b**, **12c**, **13b** and **13c**) because the chemical shifts of the protons at C-9 and C11 were indistinguishable. Therefore, both compounds were converted into their respective ketolactones **12a** and **13a** (Figure 4.18). The translactonization was accomplished by treating acetogenins **12** and **13** with diethylamine. Analysis of the $\Delta\delta_{S\text{-}R}$ of the (*S*)- and (*R*)-per-Mosher ester derivatives of the resulting C-2/C-4 *cis* and *trans* translactonized isomers (**12d**, **12e**, **13d** and **13e**) showed positive differences for H-4. Accordingly, the stereochemistry at C-10 was *R* (Chávez and Mata, 1999).

4.4.3 Cytotoxic effects of the acetogenins from *Rollinia mucosa* and *Annona purpurea*

The novel acetogenins **8** and **10–12** as well as annoglaucin (**13**) were significantly active in the brine shrimp lethality test and were also cytototoxic for six human solid tumor cell lines in a 7-day MTT test using adryamicin as the positive control (Table 4.5). The level of activity displayed by acetogenins **9–13** is comparable to that previously described for similar compounds (Gu *et al.*, 1995; Cave *et al.*, 1997; Alali *et al.*, 1999 and references cited therein). Purpureodiolin (**10**) and annoglaucin (**13**) showed high selectivity against HT-29 (Human colon adenocarcinoma). The stereochemistry around the adjacent bis-THF makes a notable difference in the cytotoxic activities of compounds **12** and **13.** Purpuracenin (**12**) with a stereochemistry *threo*/*cis*/*threo*/*trans*/*erythro* configuration showed selectivity against A-549, A-498 and PC-3 cell lines, while annoglaucin (**13**) with *threo*/*trans*/*threo*/*trans*/*erythro* configuration displayed selectivity against HT-29. The tryptamine amide derivatives were inactive against the six human tumor cell lines tested (ED_{50} > than 10 μg/ml) and were not toxic to *Artemia salina* (LC_{50} > 500 μg/ml).

4.5 PESTICIDAL LIMONOIDS FROM *SWIETENIA HUMILIS* (MELIACEAE)

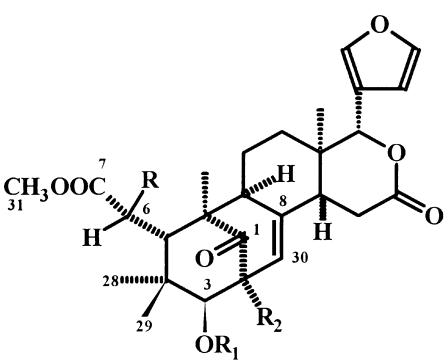
The Meliaceae family is characterized by the presence of limonoid triterpenes, many of which are biologically active against insects. From the Asian species *Azadirachta indica*

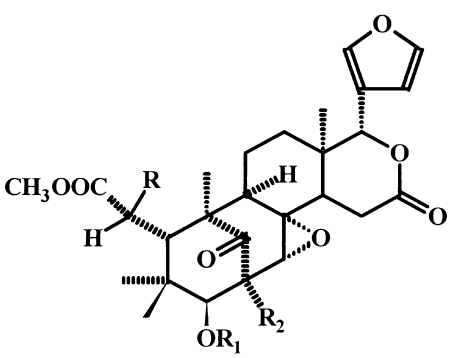
and *Melia azederach*, two limonoids have been commercialized: azadirachtin in the US and toosendanin in China (Isman *et al.*, 1997 and references cited therein, *inter alia*).

In an effort to find practical insecticides, Arnason and coworkers have screened some extracts of Mexican Meliaceae for effects against the European Corn Borer (ECB) *Ostrinia nubilalis* Hubner, a highly polyphagus species, which attack important economic crops such as, corn, sweet pepper, beet, bean, potato, oat, soybean and wheat. The significantly active extracts included *Swietenia humilis* Zucc.

S. humilis, locally known as 'zopilote', 'cóbano', 'flor de venadillo' and 'caoba', is a tree up to 20 m high which grows in the tropical areas of Mexico. Decoctions or infusions of the ground seeds of this plant are highly valued for the treatment of worms, amebiasis, cancer, chest pains and coughs.

Chromatographic fractionation of the $CHCl_3$ prepared from dry seeds of *S. humilis* allowed the isolation of six new limonoids, humilinolides A-F (**14–19**), possessing the mexicanolide skeleton (Segura-Correa *et al.*, 1993; Jiménez *et al.*, 1998a). In addition, the known limonoids humilin B (**20**), methyl-2-hydroxy-3-β-isobutyroxy-1-oxomeliac-8 (30)-enate (**21**), methyl-2-hydroxy-3-β-tigloyloxy-1-oxomeliac-8 (30)-enate (**22**), swietenin C (**23**), and swietemahonin C (**24**) were obtained (Figure 4.19) (Jiménez *et al.*, 1998). Compounds **23** and **24** were new to this species, while compounds **20–22** were





	R	R_1	R_2		R	R_1	R_2
16	H	Tigloyl	Ac	**14**	OH	Isobutyryl	OH
17	OAc	Ac	OH	**15**	OAc	Isobutyryl	OH
18	OAc	Tigloyl	OH	**19**	OAc	Tigloyl	OAc
21	H	Isobutyryl	OH	**20**	H	Isobutyryl	OH
22	H	Tigloyl	OH	**24**	OAc	Isobutyryl	H
23	OH	Isobutyryl	H				

Figure 4.19: Limonoids from *Swietenia humilis* (Meliaceae).

previously isolated from the seeds of *S. humilis* by Okorie and Taylor in 1971. At that time, mexicanolides **21** and **22** were obtained as a mixture that was very difficult to separate, and their structures were proposed based on the ^{1}H NMR and MS analyses of the mixture. In our investigation, limonoids **21** and **22** were separated by preparative HPLC on silica gel-RP.

The mexicanolides from *S. humilis* can be categorized into two structural subclasses by considering the degree of oxidation at C-8/C-30 of the basic methyl-1-oxomeliacate nucleus. The compounds in the first group have an 8,30 double bond, while those in the second possess an 8,30-epoxide function. The compounds in each subclass differ in the number and position of the oxygenated substituents but all are characterized by the presence of an ester moiety at C-3. The acid residues esterifying the hydroxyl group at C-3 is variable. In compounds **14**, **15**, **20**, **21**, **23** and **24** this residue corresponds to isobutyric acid, in compounds **16**, **18**, **19** and **22**, to tiglic acid, and in compound **17**, to acetic acid.

The basic strategy for the elucidation of the molecular structures of the new limonoids from *S. humilis* involved, first, the analysis of the IR, MS and unidimensional NMR spectra. These classical methods allowed establishing the basic mexicanolide skeleton and the nature of the acid residues esterifying the hydroxyl groups present in the basic nucleus. The location of the ester residues along the skeletal structure was confirmed by the study of the correlations observed in the HMBC experiment. For example, the placement of the acetoxy and tigloyloxy groups at C-6 and C-3, respectively, in humilinolide E was carried out unambiguously, based on the HMBC correlations H-6/C-5, C-7, 6-COMe and H-3/C-28, C-29, carbonyl carbon of the tigloyl unit. The relative stereochemical relationship among the stereogenic carbons was determined by the observation of the NOE correlations in the phase-sensitive NOESY spectra. Finally, the comparison of the CD curves of the isolated compounds with those of swietenin of known chirality enabled the assignment of the absolute stereochemistry at the stereogenic centers. In all cases, a negative Cotton effect was observed at 330 nm. The structures of humilinolides A (**14**) and D (**17**) and swietemahonin C (**24**) were confirmed by single crystal X-ray diffraction studies (Segura-Correa *et al.*, 1993, 1996; Jiménez *et al.*, 1998a).

Limonoids **14–17** were evaluated for their effect on the growth and developmental parameters of European corn borer in comparison with toosendanin, a commercial insecticide (Jiménez *et al.*, 1997, 1998). Humilinolides A-D (**14–17**) caused significant growth reduction in ECB larvae when incorporated at 50 ppm into diets fed to larvae beginning at the neonate stage (Figure 4.20). The highest growth reduction was elicited by humilinolide C (**16**), which produces an effect similar to that of toosendanin. However, at 5 ppm only humilinolide C and toosendanin showed significant growth inhibition. Toosendanin at 50 ppm induced only moderate larval mortality (<37%), but humilinolides A-D (**14–17**) produced higher mortality (>50%). The percentage of larvae

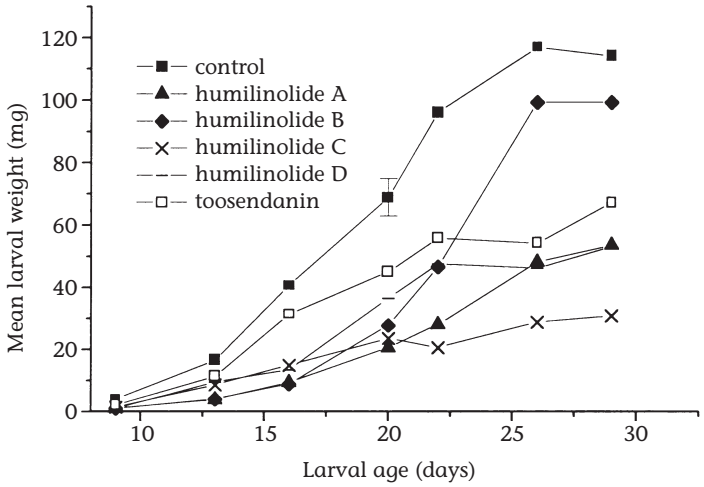


Figure 4.20: Growth of ECB larvae fed to control diets or diets treated with 50 ppm humilinolides A-D (14–17) or toosendanin.

which reached pupation, decreased compared to the control group for all the test compounds. The most important effect was observed with humilinolides D (**17**) and C (**16**), which at 50 ppm resulted in only 10 and 13% of pupation, respectively. Survival to adult stage, as compared to the pupal stage, showed no further reductions in toosendanin; however, humilinolides A-D (**14–18**) produced additional mortality and lower survivorship to adult stage. Finally, the tested compounds produced significant delays in mean time to adult stage.

The use of the seeds of *S. humilis* for the treatment of cancer and amebic dysentery in folk medicine prompted us to evaluate the cytotoxic and the antiprotozoal potential, respectively, of the extract and isolated compounds. These evaluations were performed using the same bioassay systems as for *G. niveum*. Neither the extracts nor the phytochemicals had significant cytotoxic activity against the cell lines tested or antiprotozoal activity against *G. lamblia* and *E. histolytica*.

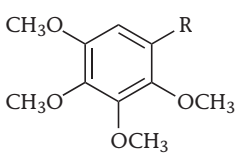
In summary, the limonoids of *S. humilis* caused larval mortality, as well as growth reduction, and increased the development time of survivors when incorporated into artificial diets of neonates of *O. nubilalis* at 50 ppm. The level of activity exhibited by these compounds at 50 ppm suggests that they are potent naturally occurring insect defenses in the seeds of *S. humilis* where they can be found in concentrations from 299 to 470 ppm. Given that humilinolides A-D (**14–18**) showed comparable activity to toosendanin and that the compounds are biosynthesized in high amounts in the seeds of *S. humilis*, we believe this species is a promising lead for the production of a practical insecticidal standardized extract.

4.6 PHYTOTOXIC COMPOUNDS FROM *MALMEA DEPRESSA* (ANNONACEAE)

Malmea depressa (Baill.) R. E. Fries (Annonaceae), commonly known by the Maya people as 'elemuy', 'sufricaya', 'elemuy-box' and 'nazareno prieto', is a tree up to 10 m high. This species is known to occur in Central America and Mexico (from Veracruz to the Peninsula of Yucatan). The tree is used by local people as an analgesic agent and for the treatment of several diseases including pellagra, liver and kidney gallstones (Jiménez *et al*., 1996 and references cited therein).

Preliminary testing of *M. depressa* against *A. salina* revealed that this Annonaceus did not contain acetogenins. However, in our screening subprogram for potential herbicidal agents we found that a $CHCl_3$ extract prepared from the stem bark of *M. depresa* inhibited radical growth of seedlings of *A. hypochondriacus* and *E. crusgalli* [IC_{50} (concentration producing 50% inhibition) values of 134.0 and 457 μg/ml, respectively)]. Fractionation of the active extract by column chromatography over silica gel, using a bioautographic phytogrowth-inhibitory bioassay at each step for activity directed fractionation, led to the isolation of the known phenylpropanoids 1,2,3,4-tetramethoxy-5-(2-propenyl) benzene (**25**), 2,3,4,5-tetramethoxycinnamaldehyde (**26**), *trans*-isomyristicin (**27**), 2,3,4,5-tetramethoxycinnamyl alcohol (**28**) and the novel C_6C_1 derivative 2,3,4,5-tetramethoxybenzaldehyde (**29**) (Figure 4.21) (Jiménez *et al*., 1996). All the compounds have in common the tetraoxygenation of the benzene ring. Tetraoxygenation of simple aromatic compounds is rare in nature, and their presence in *M. depressa* could be of chemotaxonomic significance.

Compound **25** produced significant inhibition of radicle growth in *A. hypochondriacus* (IC_{50} = 43.0 μg/ml) but it was less active against *E. crusgalli* (IC_{50} = 810 μg/ml). The new natural product **29** and phenylpropanoids **26–28** were markedly less active in the phytogrowth-inhibitory bioassays than **25** (Jiménez *et al*., 1996).



R = CH_2—CH═CH_2 (**25**)
R = CH═CH—CHO (**26**)
R = CH_2—CH═CH_3 (**27**)
R = CH═CH—CH_2—OH (**28**)
R = CHO (**29**)

Figure 4.21: Phytogrowth-inhibitory compounds from *Malmea depressa* (Annonaceae).

The effect of natural product **25** on several photosynthetic activities associated with the light reaction phase was also studied. The photosynthetic activities evaluated included proton uptake, ATP synthesis, electron transport rate (basal, phosphorylating and uncoupled) and partial reactions of the photosystems I (PS I) and II (PS II). The assays were carried out on freshly lysed spinach chloroplasts following an experimental approach recently reviewed (Lotina-Hennsen *et al.*, 1998). The results indicated that this compound significantly inhibits proton uptake, light induced ATP synthesis, and electron flow, in three different conditions (basal, phosphorylating and uncoupled) in a concentration-dependent manner (Figures 4.22 and 4.23), therefore acting as a Hill's reaction inhibitor. The concentration values producing 50% inhibition (I_{50}) for each electron transport flow (basal, phosphorylating and uncoupled) were 3.6, 5.0 and 2.7 μM, respectively. The I_{50} for ATP-synthesis and H^+-uptake inhibition were 1.40 and 2.30 μM, respectively. In order to localize the site of inhibition on the electron transport pathway, the effect of compound **25** on partial reactions of the PS I and PS II was measured using artificial electron donors, electron acceptors and appropriate inhibitors. The last set of experiments demonstrated that the phenylpropanoid inhibited electron flow at the PS II in the span from P680 to QA (Jiménez *et al.*, 1998b).

The significant level of activity of phenylpropanoid **25** as a Hill reaction inhibitor prompted us to explore its accessibility in the plant. The knowledge of the location of active principles within the plant might be crucial to determine real possibilities of practical applications. In this regard, we found that compound **25** is present not only in the stem-bark of the plant but also in the wood and the leaves. GC-MS analyses of the essential oils obtained from the leaves, stem-bark and wood of this species, by water and

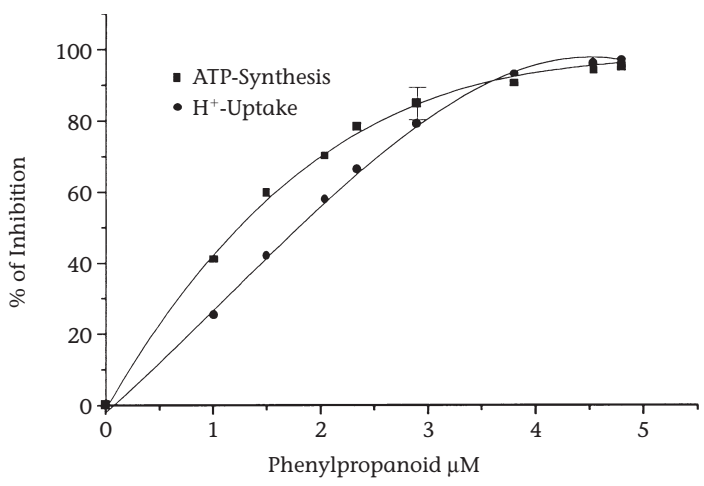


Figure 4.22: Effect of different concentrations of 1,2,3,4-tetramethoxy-5-(2-propenyl) benzene (**25**) on ATP synthesis and proton uptake.

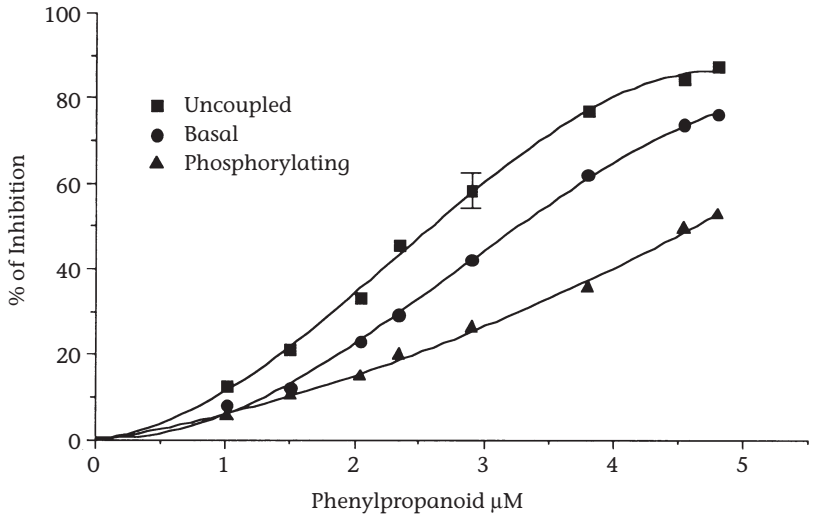


Figure 4.23: Effect of different concentrations of 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene (**25**) on electron transport (basal, phosphorylating and uncoupled) from water to methylviologen.

steam distillation, revealed that the active compound **25** is present in the three oils in high proportions (96, 97.8 and 95%, respectively). Thus, the presence of phenylpropanoid **25** in such a high yield in the leaves of the plant could grant a reliable and economic source of this compound (Jiménez *et al.*, 1998b).

In conclusion, the major phytotoxic phenylpropanoid from M. *depressa* blocks the photosynthesis process in isolated spinach chloroplasts by inhibiting one or more of the enzymes involved in the electron transport chain in the range from P680 to QA.

4.7 CONCLUDING REMARKS

Selected Mexican plants from the Annonaceae *(Rollinia mucosa, Annona purpurea and Malmea depressa*), Meliaceae (*Swietenia humilis*), Orchidaceae (*Scaphyglotys livida*) and Geraniaceae (*Geranium niveum*) families were identified as a source of potential pesticidal or medicinal agents. Bioassay-guided isolation of these species, based on different *in vitro* bioassay systems, yielded five aromatic compounds with noted spasmolytic activity from *S. livida*; four novel cytotoxic actogenins from *R. mucosa* and *A. purpurea*; four novel insecticidal limonoids from *S. humilis*; two new antiprotozoal proanthocyanidins from *G. niveum*; and one phytotoxic phenylpropanoid from *M. depressa*. In addition, our investigations on *S. livida* and *G. niveum* provide scientific evidence supporting the major reputed medicinal properties of these plants. This short survey clearly indicates that the potential of Mexican medicinal flora as a source of bioactive compounds is

enormous. The results also demonstrated that the preliminary efforts carried out in our program have proven to be worthy although much work remains to be done on the Mexican medicinal flora.

ACKNOWLEDGEMENTS

This work has been conducted under the auspices of several project grants funded by Consejo Nacional de Ciencia y Tecnología (CONACyT, Convenio 27978-N) and Dirección General de Asuntos del Personal Académico UNAM (DGAPA, Proyecto IN205197). R. Mata is very grateful to all colleagues and Ph.D. students whose collaboration has contributed to the results described in this chapter, in particular to Fernando Calzada, Adelina Jiménez, Laura Acevedo and Samuel Estrada. Their faith and enthusiasm have been the basis of this work.

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CHAPTER

5

Flavonoids as Cancer Chemopreventive Agents

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Contents

5.1 INTRODUCTION TO CANCER CHEMOPREVENTION

Cancer is undoubtedly one of our most serious human health problems, with there being high mortality rates worldwide of more than seven million deaths per year (Weinstein, 1991). In 1999, in the United States alone, it was estimated that 1,221,800 persons will have been diagnosed with invasive cancer, and an additional one million people would also contract basal or squamous cancers of the skin. Over 1,500 persons per day (or over 563,100 Americans) will have died in 1999 from various manifestations of cancer (Landis *et al.*, 1999). The relative five-year survival rate for cancer patients is about 56% in the United States (Anonymous, 1997). Among those organ sites with exceptionally low five-year survival rates are esophageal, lung, pancreatic, stomach (all at 2%), ovarian (39%), and breast cancers (20%) (Anonymous, 1997). Thus, the prevention rather than the treatment of cancer has become of increasing importance in recent years.

Cancer chemoprevention is a relatively new concept. The pioneering work to reduce cancer incidence by chemical intervention was initiated by the groups of Wattenberg and Sporn in the early 1960s and 1970s (Wattenberg, 1985; Sporn, 1993). Later, scientists have embraced the concept of cancer chemoprevention as a distinct new discipline of oncology (Kelloff *et al.,* 1994a; Greenwald *et al.,* 1995; Hong and Sporn, 1997; Stoner *et al.,* 1997). As part of his work concerning retinoids and cancer prevention, Professor Michael B. Sporn has defined the term 'cancer chemoprevention' as 'the prevention or delay of the process of carcinogenesis in humans by ingestion of dietary or pharmaceutical agents' (Sporn *et al.,* 1976).

Based on the results of animal studies and epidemiological data (Bjelke, 1975; Hirayama, 1982; Knekt, 1992), various groups of compounds have been classified as cancer chemopreventive agents (Kelloff *et al.,* 1994b), and clinical intervention studies are underway in human subjects at high risk of developing specific types of cancers (Boone *et al.,* 1990; Kelloff *et al.*, 1994a,b). Thus far, large intervention trials on two synthetic compounds [tamoxifen (Novaldex®) and finasteride (Proscar®)] are being performed (Powles and Hickish, 1995; Thompson and Coltman, 1996). In recent years, there have been intensive research efforts to investigate the cancer chemopreventive effects of dietary constituents such as beverages, culinary herbs, fruits, spices, and vegetables. Carcinogenesis inhibition studies in animal models have been performed on dietary compounds known to act as either anti-initiating agents (e.g. diallyl sulfide, ellagic acid, and certain isothiocyanates) or antipromotion/antiprogression agents (e.g. epigallocatechin gallate, limonene, and quercetin) (Ho *et al.*, 1994; Huang *et al.*, 1994). Moreover, a number of plant-derived natural products are under development at various levels of preclinical and clinical trial as chemopreventive agents (such as curcumin, β-carotene, ellagic acid, and 18β-glycyrrhetinic acid) (Kelloff *et al.*, 1994b, 1995; Steele *et al.*, 1998). Thus, it seems likely that there will be a continued need for the discovery and development of novel plant-derived cancer chemopreventive agents. In this chapter, a

brief overview of the previous literature on flavonoids as cancer chemopreventive agents in experimental models will be provided, and some experimental strategies that might be employed for the discovery of further agents of this type are described. Finally, some aspects of our phytochemical work on the isolation and structure elucidation of flavonoids as potential cancer chemopreventive agents will be highlighted.

5.2 ACTIVITY OF FLAVONOIDS IN FULL-TERM CARCINOGENESIS INHIBITION EXPERIMENTS

Plant-derived flavonoids are a large group of naturally occurring phenylchromones found in fruits, vegetables, tea, and wine. The daily human intake in western countries of flavonoids was recently estimated to be about 23 mg/day (Hertog *et al.*, 1993). However, this may be elevated to as much as 2–3 g/day with a high dietary intake of herbs and spices (Hertog *et al.*, 1992). Flavonoids have been shown to have a wide range of biological activities, including antiallergic, antibacterial, antiinflammatory, antimutagenic, antioxidant, antiproliferative, antithrombotic, antiviral, and hepatoprotective effects (Bors and Saran, 1987; Robak and Gryglewski, 1988; Wall, 1992; Middleton and Kandaswami, 1994).

Recently, there has been a tremendous increase in the number of studies on flavonoids as potential cancer chemopreventive agents. Certain flavonoids have been shown to interact in the genesis of cancer in both of the defined stages of initiation and promotion/progression (Ito and Imaida, 1992). For example, they have been shown to prevent carcinogens from reacting with cells at the initiation stage (Wattenberg and Leong, 1970) and to block the promotion stage by inhibiting ornithine decarboxylase (ODC) synthesis (Fujika *et al.*, 1986). The major subclasses of flavonoids so far demonstrated with this type of biological activity include chalcones, flavanones, flavanols, flavones, flavonols, and isoflavones. Examples will be discussed in turn for each of these flavonoid subclasses of compounds shown to exhibit cancer chemopreventive activity in experimental animal models in the following paragraphs, with a summary provided in Table 5.1.

Chalcones have been reported to possess antifungal, antiinflammatory, antitumorigenic, and bacteriostatic properties (Pappano *et al.*, 1985; Tsuchiya *et al.*, 1994; Iwata *et al.*, 1997). Recently, several *in vivo* anticarcinogenesis effects of chalcones have been documented. For example, inhibitory effects in rats on oral carcinogenesis initiated with 4-nitroquinoline-1-oxide (4-NQO) resulted from the administration of chalcone (**1**) and 2-hydroxychalcone (**2**) (Makita *et al.*, 1996). Isoliquiritigenin (**3**) (Yamamoto *et al.*, 1991) and 3′-methyl-3-hydroxychalcone (**4**) (Satomi, 1993) have been reported to inhibit the incidence of papilloma formation produced in a 7,12-dimethylbenz(*a*)anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA) two-stage carcinogenesis mouse skin model, using CD-1 and ICR mice, respectively.

TABLE 5.1

Examples of flavonoids with cancer chemopreventive activity in experimental animals

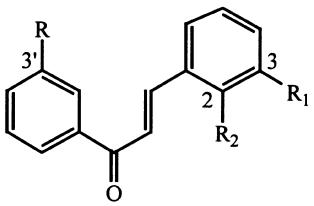
Compound/code	Model			Species (and strain)	Ref.
	Initiation	Promotion	[Carcinogen]		
Chalcones					
Chalcone (**1**)	Initiation, oral carcinogenesis [(4-NQO)][a]			F344 rats	Makita *et al.,* 1996
2-Hydroxychalcone (**2**)	Initiation, oral carcinogenesis [(4-NQO)][a]			F344 rats	Makita *et al.,* 1996
Isoliquiritigenin (**3**)		Promotion, epidermal ODC induction [(TPA)][c]		CD-1 mice	Yamamoto *et al.,* 1991
		Promotion, skin papillomas [(DMBA)/(TPA)] two stage carcinogenesis		CD-1 mice	Yamamoto *et al.,* 1991
3′-Methyl-3-hydroxy-chalcone (**4**)		Promotion, skin papillomas [(DMBA)/TPA)] two stage carcinogenesis		ICR mice	Satomi, 1993
Flavanone					
Hesperidin (**5**)	Initiation, colon carcinogenesis [AOM][a]			F344 rats	Tanaka *et al.,* 1997
	Initiation, urinary-bladder carcinogenesis [NNN][a]			ICR mice	Yang *et al.,* 1997
Flavanols					
(−)-Epicatechin (**6**)		Promotion, duodenal carcinogenesis ([ENNG])[b]		Mice	Fujita *et al.,* 1989
		Promotion, lung tumorigenesis (NNK)[a]		Mice	Chung *et al.,* 1992
(−)-Epigallocatechin (**7**)		Promotion, duodenal carcinogenesis ([ENNG])[b]		Mice	Fujita *et al.,* 1989
		Promotion, lung tumorigenesis (NNK)[a]		Mice	Chung *et al.,* 1992
(−)-Epicatechin gallate (**8**)		Promotion, duodenal carcinogenesis ([ENNG])[b]		Mice	Fujita *et al.,* 1989
		Promotion, lung tumorigenesis (NNK)[a]		Mice	Chung *et al.,* 1992
(−)-Epigallocatechin gallate (**9**)		Promotion, duodenal carcinogenesis ([ENNG])[b]		Mice	Fujita et al., 1989
		Promotion, skin carcinogenesis [(DMBA)/(okadaic acid)][c]		Mice	Yoshizawa, 1996
Flavones					
Apigenin (**10**)		Promotion, skin papillomas [(DMBA)/(TPA)][c] two stage carcinogenesis		SENCAR mice	Wei *et al.,* 1990
Diosmin (**11**)	Initiation, colon carcinogenesis [AOM][a]			F344 rats	Tanaka *et al.,* 1997
	Initiation, urinary-bladder carcinogenesis [NNN][a]			ICR mice	Yang *et al.,* 1997
Luteolin (**12**)		Promotion, skin carcinogenesis [(DMBA)/TPA] [c]		Swiss albino mice	Elangovan *et al.,* 1994a
Nobiletin (**13**)	Initiation, skin carcinogenesis [(BP)][a]			Mice	Attaway, 1994
Tangeretin (**14**)	Initiation, skin carcinogenesis [(BP)][a]			Mice	Attaway, 1994
Flavonols					
Quercetin (**15**)		Promotion, colonic neoplasia [AOM][a]		CF-1 mice	Deschner *et al.,* 1991
		Promotion, fibrosarcoma [20-MC][a]		Swiss albino mice	Elangovan *et al.,* 1994b
	Initiation, skin papillomas (DMBA)[a]			Hamster	Balasubramaniam and Govindasamy, 1996
		Promotion, skin carcinogenesis [(DMBA)/(TPA)][c]		Mice	Korkina and Afanas'ev, 1996
	Initiation, oral carcinogenesis [(4-NQO)][c]			F344 rats	Makita *et al.,* 1996
Rutin (**16**)	Initiation, colonic neoplasia [(AOM)][a]			CF-1 mice	Deschner *et al.,* 1991
Isoflavones					
Genistein (**17**)	Initiation, colon carcinogenesis [AOM][a]			Mice	Steele *et al.,* 1995
		Promotion, skin tumorigenesis [(DMBA)/(TPA)]		CD-1 mice	Bowen *et al.,* 1993
Biochanin A (**18**)	Initiation, mammary carcinogenesis [(MNU)][b]			CD/Crj rats	Gotoh *et al.,* 1998

[a] Indirect chemical carcinogens: AOM, azoxymethane; BP, benzo(*a*)pyrene; DMBA, 7,12-dimethylbenz(*a*)anthracene; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; 4-NQO, 4-nitroquinoline-1-oxide; 20-MC, 20-methylcholanthrene; NNN, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine.

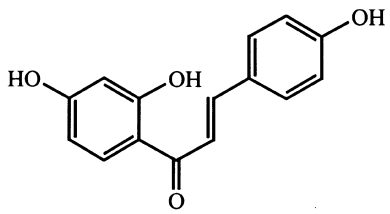
[b] Direct chemical carcinogens: ENNG, *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine; MNU, *N*-nitroso-*N*-methylurea.

[c] Tumor promoters: 12-*O*-tetradecanoylphorbol 13-acetate (TPA); okadaic acid.

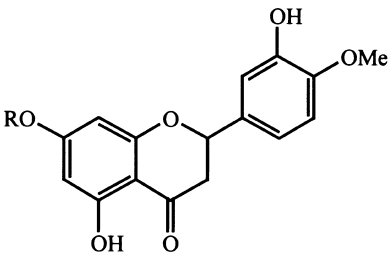
Citrus fruits contain variable quantities of flavonoids, as exemplified by the flavanone glycoside hesperidin (**5**) (hesperetin 7-rutinoside). This is an abundant and inexpensive by-product of *Citrus* fruit cultivation and can be isolated in large amounts from the discarded rinds of the common orange, *Citrus aurantium* L. Hesperidin (**5**) has shown several biological activities including antiinflammatory and antioxidant effects, and



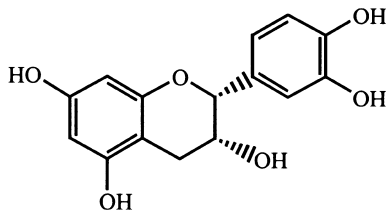
1 R = H, R_1 = H, R_2 = H
2 R = H, R_1 = H, R_2 = OH
4 R = CH_3, R_1 = OH, R_2 = H



3



5 R = *O*-rutinosyl



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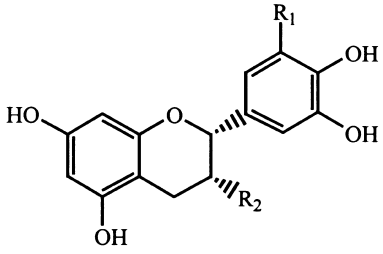
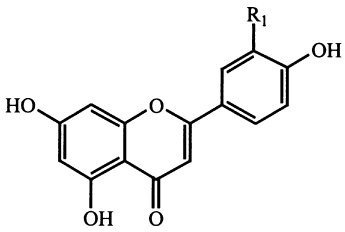


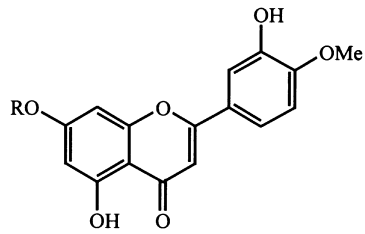
7 R_1 = OH, R_2 = OH
8 R_1 = H, R_2 = galloyl
9 R_1 = OH, R_2 = galloyl

Figure 5.1: Examples of flavonoids with cancer chemopreventive activity in experimental animals.

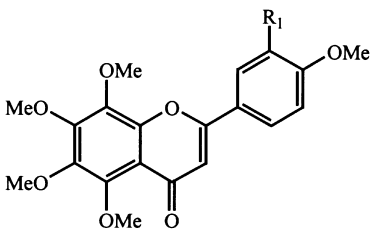
inhibition of prostaglandin synthesis (Damon *et al.*, 1987; Lonchampt *et al.*, 1989; Galati *et al.*, 1994). Hesperidin (**5**) was found to inhibit azoxymethane (AOM)-induced colon carcinogenesis in rats (Tanaka *et al.*, 1997), and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary-bladder carcinogenesis in mice (Yang *et al.*, 1997).



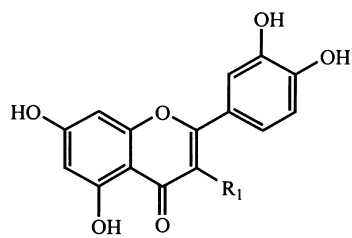
10 $R_1 = H$
12 $R_1 = OH$



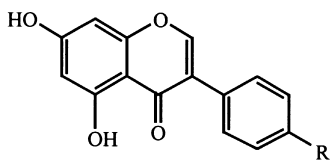
11 R = *O*-rutinosyl



13 $R_1 = OCH_3$
14 $R_1 = H$



15 $R_1 = OH$
16 R_1 = *O*-rutinosyl



17 R = OH
18 $R = OCH_3$

Figure 5.1: *(continued)*

Tea (the leaves of *Camellia sinensis* L.) is grown in about 30 countries, and is the most widely consumed beverage in the world. Tea is manufactured as either green, black, or oolong; black tea represents approximately 80% of all tea products consumed. In recent years, there has been an intensive research effort on the study of green tea and its major flavanol constituents, (−)-epicatechin (EC) (**6**), (−)-epigallocatechin (EGC) (**7**), (−)-epicatechin gallate (ECG) (**8**), and (−)-epigallocatechin gallate (EGCG) (**9**) for their cancer chemopreventive properties, as has been documented in several reviews (Agarwal and Mukhtar, 1996; Lin *et al.*, 1997; Ahmad *et al.*, 1998; Katiyar and Mukhtar, 1998). Green tea has shown nonspecific and broad spectrum anticarcinogenic effects in many different animal models. For example, oral administration of green tea inhibited *N*-nitrosodiethylamine (NDEA)-induced forestomach tumors in mice (Wang *et al.*, 1992b), *N*-nitrosomethylbenzylamine (NMBzA)-induced esophageal tumors in rats (Xu and Chi, 1990), 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced tumorigenesis in mouse skin (Wang *et al.*, 1991; 1992a), and azoxymethane (AOM)-induced colon carcinogenesis in rats (Yamane *et al.*, 1997). As listed in Table 5.1, there have been several reports of *in vivo* carcinogenesis inhibitory effects of (−)-epigallocatechin gallate (EGCG) (**9**), including serving as an inhibitor of the promotion stage of duodenal carcinogenesis induced by *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine (Fujita *et al.*, 1989), and inhibition of the tumor-promoting activity of okadaic acid in a two-stage carcinogenesis experiment in mouse skin (Yoshizawa, 1996). The mechanism of cancer chemoprevention by EGCG may involve the inhibition of tumor necrosis factor-α (TNF-α) release by blocking the interaction of the tumor promoter to its receptor as well as inhibition of proteolysis of the TNF-α precursor protein (Suganuma *et al.*, 1997). EGCG treatment resulted in arrest in the G_0-G_1 phase of the cell cycle and dose-dependent apoptosis in human carcinoma cells (Ahmad *et al.*, 1997).

Food-derived flavonoids from edible plants such as the flavones found in *Citrus* juices including nobiletin (**13**) and tangeretin (**14**) have shown inhibitory activities against carcinogen-induced tumors in rats and in mice (Deschner *et al.*, 1991; Korkina and Afanas'ev, 1996). Polymethoxylated flavonoids, such as nobiletin (**13**) and tangeretin (**14**) are more potent inhibitors of tumor cell growth than hydroxylated flavonoids (Attaway, 1994). The polymethoxyflavones, nobiletin (**13**), and tangeretin (**14**), were found to enhance carcinogen removal by inducing benzo(*a*)pyrene hydroxylase activity in the liver and lungs of rats (Wattenberg, 1975; Attaway, 1994). In addition, both nobiletin (**13**) and tangeretin (**14**) have shown antiproliferative effects on human squamous carcinoma cells (Kandaswami *et al.*, 1991).

Quercetin (**15**), a flavonol, exists in many fruits and vegetables in the diet, with the average human daily intake estimated to be 16–25 mg/person (Hertog *et al.*, 1992). This substance has been studied widely and found to inhibit TPA-induced tumors (Korkina and Afanas'ev, 1996), and has antioxidant (Cotelle *et al.*, 1996) activity that enables it to scavenge active oxygen and electrophiles (Saija *et al.*, 1995). Moreover, quercetin (**15**)

has been reported to inhibit the incidence of papillomas and tumors induced by 7,12-dimethylbenz(*a*)anthracene (DMBA) in a hamster buccal pouch model (Balasubramaniam and Govindasamy, 1996). Several other *in vivo* anticarcinogenesis effects of quercetin (**15**) have been documented, including inhibition of azoxymethane (AOM)-induced colonic neoplasia in CF-1 female mice (Deschner *et al.*, 1991), 20-methylcholanthrene (20-MC)-induced fibrosarcoma in male Swiss albino mice (Elangovan *et al.*, 1994b), and 4-(NQO)-initiated oral carcinogenesis (Makita *et al.*, 1996). Several possible mechanisms for the activity of quercetin in *in vivo* cancer chemopreventive interactions have been proposed, such as the potent inhibition of certain biochemical events associated with tumor promotion (e.g. alterations in protein kinase C) (Ferriola *et al.*, 1989), inhibition of lipid peroxidase and cytochrome P-450 (while increasing levels of glutathione *S*-transferase) (Elangovan *et al.*, 1994a), and induction of apoptosis (Wei *et al.*, 1994). Moreover, quercetin (**15**) was found to down-regulate signal transduction in human breast carcinoma cells (Singhal *et al.*, 1995).

Recently, due to their potential for reducing the risk of coronary heart disease and chemopreventive properties in various forms of cancer, isoflavone phytoestrogens, which are found in a wide variety of plant products, have attracted widespread interest (Anderson *et al.*, 1995; Steele *et al.*, 1995). Several epidemiological studies related to the consumption of soybeans have shown a relatively low incidence of breast, colon, and prostate cancers (Lee *et al.*, 1991; Messina *et al.*, 1994). The major presumed cancer chemopreventive agents contained in soybeans (containing 1 to 3 mg per gram of these phytoestrogens) are isoflavones such as genistein (**17**). Laboratory results have indicated that genistein (**17**) can inhibit phorbol ester-type tumor promoter-induced H_2O_2 formation both *in vitro* and *in vivo* and 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced proto-oncogene expression (*c-fos*) in mouse skin, and can suppress TPA-promoted skin tumorigenesis (Jha *et al.*, 1985; Wei *et al.*, 1993, 1995). In addition, genistein (**17**) reduced the number of skin carcinomas induced by 7,12-dimethylbenz(*a*)anthracene (DMBA) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in CD-1 mice when it was applied topically (Bowen *et al.*, 1993). Another isoflavone derivative, biochanin A (**18**), proved able to reduce the incidence of mammary carcinogenesis in rats induced by *N*-nitroso-*N*-methylurea (Gotoh *et al.*, 1998).

5.3 APPROACHES TO THE DISCOVERY OF CANCER CHEMOPREVENTIVE AGENTS FROM PLANTS

Laboratory work on the discovery of naturally occurring plant-derived cancer chemopreventive agents at the College of Pharmacy, University of Illinois at Chicago has been supported for a number of years by the National Cancer Institute (NCI), National Institutes of Health, Bethesda, Maryland. The overall design of the botanical, biological, and chemical aspects of this cancer chemoprevention project has been described previously (Pezzuto,

1993, 1995; Pezzuto *et al.*, 1998, 1999). The plant materials investigated in this program have been obtained by utilizing the resources of the University of Illinois Pharmacognosy Field Station and the herbarium of the Field Museum of Natural History, Chicago, as well as from commercial sources and by field collection through interactions with international botanist collaborators. Priority in plant collections has been given to species which are known to be edible. Initially, a small amount (100–500 g) of each plant species is collected for preliminary studies. Non-polar and polar extracts are prepared from each plant obtained, which are then evaluated using a panel of about 10 short-term *in vitro* bioassays (Pezzuto, 1995; Pezzuto *et al.*, 1998, 1999). Selected extracts are then evaluated in a mouse mammary organ culture model as a secondary discriminator (Mehta and Moon, 1991; Mehta *et al.*, 1994). The panel of *in vitro* assays has been established to monitor inhibition of carcinogenesis at the stages of initiation (antimutagenic activity, antioxidant activity, and induction of quinone reductase activity in cell culture), promotion (inhibition of phorbol ester-induced ornithine decarboxylase activity in cell culture, inhibition of cyclooxygenase activity, inhibition of phorbol dibutyrate receptor binding [PDBu]), and progression (induction of HL-60 cell differentiation, aromatase inhibition, Ishikawa cell lines for antiestrogenic and estrogenic activities, estrone sulfatase inhibitors) (Pezzuto *et al.*, 1999). Selected plant extracts, showing potency and/or selectivity in the *in vitro* assay panel, are selected for bioassay-guided fractionation. Plant extracts and active fractions are subjected to standard chromatographic separation techniques using silica gel, Sephadex gels, ion-exchange resins, and other column support materials, based on polarity. Separation procedures include flash column, medium-pressure liquid chromatography (MPLC), and partition procedures, such as droplet counter-current chromatography (DCCC). In several cases, semi-preparative HPLC has been applied to separate more complex constituents and isomers (Kinghorn *et al.*, 1998). Recently, a LC-MS dereplication procedure was incorporated into our program, in order to rapidly to detect already known active compounds in plant extracts, especially in the case of antioxidants, since a relatively large number of plant constituents have been described with this activity (Lee *et al.*, 1998). Pure active compounds are then evaluated in all of the *in vitro* assays, with selected compounds then progressing to the mouse mammary organ culture model (Mehta *et al.*, 1994). The *in vivo* cancer chemopreventive activity of highly promising pure plant constituents is then evaluated in a two-stage mouse skin and/or a rat mammary carcinogenesis model (Pezzuto, 1995; Udeani *et al.*, 1997).

5.4 ISOLATION OF FLAVONOIDS WITH POTENTIAL CANCER CHEMOPREVENTIVE PROPERTIES

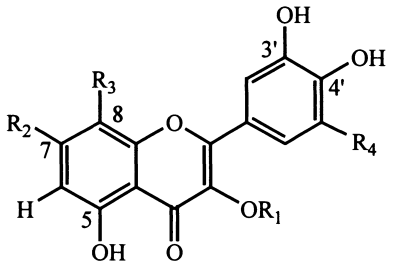
In our laboratory, the isolation and identification of many plant-derived compounds with potential cancer chemopreventive properties has been carried out to date. These active constituents represent a wide range of major plant secondary metabolites, inclusive of compounds of the alkaloid, aryl polysulfide, benzenoid, diterpenoid, flavonoid,

furocoumarin, lignan, monoterpenoid, quinone, rotenoid, simaroubolide, stilbenoid, triterpenoid, withanolide, and xanthone classes (Kinghorn *et al.*, 1998). Of these active compounds, flavonoids have constituted the largest single group. Examples of flavonoids obtained in our program on cancer chemoprevention to date, and details of their structure elucidation are reviewed in the following paragraphs.

5.4.1 *Chorizanthe diffusa*

Chorizanthe diffusa Benth. (Polygonaceae), collected in California, represented one of the initial plant extracts found to exhibit biological activity when evaluated with a panel of *in vitro* assays (Pezzuto *et al.*, 1999). The ethyl acetate-soluble extract of the entire plant exhibited significant antioxidant activity based on the scavenging activity of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals (IC_{50}: 12.8 μg/mL) and inhibition of 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced free radical formation with cultured HL-60 cells (IC_{50}: 22.0 μg/mL) (Chung *et al.*, 1999). Moreover, the extract demonstrated 57% inhibition in 7,12-dimethylbenz(*a*)anthracene-(DMBA)-induced preneoplastic lesion formation with a mouse mammary organ culture model (Mehta *et al.*, 1994). No previous phytochemical work had been performed on this species. Bioassay-guided fractionation of the active extract of *C. diffusa* using the DPPH antioxidant assay led to the isolation of one novel (**19**) and four known (**15**, **20–22**) flavonoids.

Compound **19**, 5,8,3′,4′,5′-pentahydroxy-3,7-dimethoxyflavone, was assigned a molecular formula of $C_{17}H_{14}O_9$ from its HREIMS data. The UV and ^{1}H- and ^{13}C-NMR data suggested that **19** is a flavone (Agrawal *et al.*, 1989). It was apparent that **19** contains five hydroxyl groups (δ_C 126.2, 137.1, 145.9 [double intensity], 152.8) and two OMe groups (δ_C 56.1, 59.1; δ_H 3.81, 3.93). The relative locations of these functionalities were established from the following observations. The ^{1}H-NMR spectrum suggested that the A ring was tri-substituted (a singlet was observed at δ_H 6.57), and an aromatic proton singlet integrating for two protons at δ_H 7.27 was assigned for H-2′ and H-6′ in ring B, respectively. By using shift reagents, the absence of any free hydroxyl group at the C-7 position (Mabry *et al.*, 1970) was indicated, since no bathochromic shift in the UV spectrum occurred on the addition of sodium acetate, whereas a large bathochromic shift was observed upon addition of sodium methoxide, indicating the presence of a hydroxyl group at C-4′ in compound **19** (Markham and Mabry, 1975). In addition, the presence of a chelated hydroxyl group at C-5 was inferred from the UV spectral bathochromic shift produced with $AlCl_3$ (Mabry *et al.*, 1970). Unambiguous assignments of the ^{1}H- and ^{13}C-NMR spectra of **19** were achieved by analysis of a combination of the ^{1}H-^{13}C heteronuclear shift correlation (HETCOR) and ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC) NMR techniques. The OCH_3 group (δ_H 3.93) could be assigned at C-7 based on a 1D-NOE experiment, in which irradiation at δ_H 6.57 (H-6) resulted in a NOE for OCH_3-7 (δ_H 3.93). Thus, the δ_H 6.57 resonance showed cross peaks with signals at



19 $R_1 = CH_3$, $R_2 = OCH_3$, $R_3 = OH$, $R_4 = OH$
20 $R_1 = CH_3$, $R_2 = OH$, $R_3 = H$, $R_4 = H$
21 $R_1 = CH_3$, $R_2 = OCH_3$, $R_3 = OH$, $R_4 = H$
22 R_1 = rha-3-Ac, $R_2 = OH$, $R_3 = H$, $R_4 = H$

Figure 5.2: Flavonoids isolated from *Chorizanthe diffusa.* (Rha = L-rhamnopyranosyl.)

δ_C 104.5 (C-10), 152.8 (C-5), and 126.2 (C-8), which strongly supported this being the H-6 signal, rather than those at H-7 or H-8. Furthermore, the δ_H 7.27 (H-2′ and H-6′) signal exhibited connectivities with signals at δ_C 145.9 (C-3′), 137.1 (C-4′), 107.9 (C-2′ and C-6′), 145.9 (C-5′), and 156.1 (C-2). The resonance at (δ_H 12.27) indicated the location of an OH group at C-5, because correlations were observed with the δ_C 152.8 (C-5) and 104.5 (C-10) signals. Confirmation of the structure of **19** was obtained by LC-MS and LC-MS-MS studies using electrospray detection. The known flavonoids, 5,7,3′,4′-tetrahydroxy-3-methoxyflavone (**20**), 5,8,3′,4′-tetrahydroxy-3,7-dimethoxyflavone (**21**), quercetin (**15**), and 3′′-*O*-acetylquercitrin (**22**) were also isolated from *C. diffusa* in our investigation (Chung *et al.*, 1999).

Flavonoids **15**, **19**, and **22** were comparable in activity as free-radical scavengers in the DPPH assay, when compared with that exhibited by several standard antioxidants [e.g. ascorbic acid (IC_{50} 22 μg/mL); 2(3)-*tert*-butyl-hydroxyanisole (IC_{50} 21 μg/mL); caffeic acid (IC_{50} 12 μg/mL); gallic acid (IC_{50} 5 μg/mL); nordihydroguaiaretic acid (IC_{50} 12 μg/mL)], respectively. Compounds **20** and **21** were only moderately active in this assay, as well as in a free-radical quenching assay utilizing HL-60 cells. Three of the isolates were also evaluated for antimutagenic potential in a forward mutation assay with *Salmonella typhimurium* strain TM677 (Shamon *et al.*, 1994), and compounds **19** and **20** were found to be highly active in this regard. Further, compounds **15** and **19–22** were evaluated for their potential to inhibit DMBA-induced preneoplastic lesions with mouse mammary glands in organ culture (MMOC assay). 5,7,3′,4′-Tetrahydroxy-3-methoxyflavone (**20**) and quercetin (**15**) mediated significant inhibitory activity (67% inhibition) and thus were rated as good candidates for full-term evaluation in an experi-

mental carcinogenesis model (Chung *et al.*, 1999). The cancer chemopreventive activity of quercetin (**15**) in several animal models has been mentioned earlier in this chapter.

5.4.2 *Daphniphyllum calycinum*

Daphniphyllum calycinum Benth. (Daphniphyllaceae) is a species native to the People's Republic of China. This plant was selected for activity-guided fractionation in our project since its ethyl acetate-soluble extract was found to significantly inhibit DMBA-induced preneoplastic lesions formation in the mouse mammary gland organ culture, to the extent of 100% at a concentration of 10 μg/mL (Mehta and Moon, 1991; Mehta *et al.*, 1994). However, in the various *in vitro* assays available to the project, the only activity shown was *in vitro* antioxidant activity (IC_{50} of the initial extract 84.0 μg/mL) in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical assay (Pezzuto *et al.*, 1999). Bioassay-guided fractionation led to isolation of a novel compound, namely, 5,6,7,4′-tetrahydroflavonol 3-*O*-rutinoside (**23**), and a known constituent, kaempferol 3-*O*-neohesperidoside (**24**), which exhibited IC_{50} values of 43.2 and 79.6 μg/mL, respectively, in the DPPH assay. The weak activity of these constituents seems to account for the original antioxidant activity of the extract of *D. calycinum* (Gamez *et al.*, 1998). Compound **23** exhibited an elemental formula of $C_{27}H_{30}O_{16}$ from its HRFABMS data. Preliminary analyses of its UV, IR, NMR, and LC-MS data indicated that it was a flavonoid glycoside. The flavonoid portion of the molecule of **23** was readily established as kaempferol, and two anomeric protons were observed in the ^{1}H-NMR spectrum at δ_H 5.12 (doublet, J = 7.3 Hz; H-1 of β-glucose) and δ_H 4.51 (broad singlet; H-1 of α-rhamnose) (Agrawal, 1992). The glucose proton was shown to be directly attached to the C-3 carbon (δ 135.5) of the aglycone by the observation of a correlation peak in the HMBC spectrum. A three-bond HMBC correlation between the anomeric proton of the rhamnosyl moiety

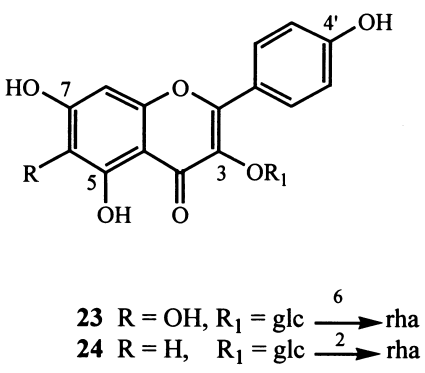


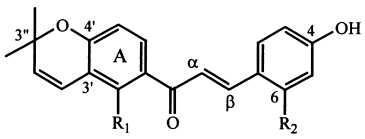
Figure 5.3: Flavonoids isolated from *Daphniphyllum calycinum*. (Glc = D-glucopyranosyl; rha = L-rhamnopyranosyl.)

and C-6 of the glucosyl unit, along with the downfield shift of C-6′′ in the ^{13}C NMR spectrum (δ_C 68.6), suggested a $1 \rightarrow 6$ linkage between the rhamnosyl group and the glucosyl unit, consistent with a rutinoside structure (Agrawal *et al.*, 1989). Due to their low potency as antioxidants, no further studies are planned on compounds **23** and **24**.

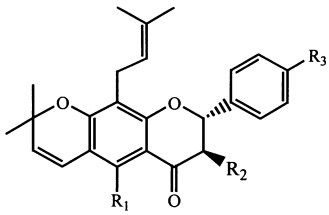
5.4.3 *Mundulea sericea*

Mundulea sericea (Willd.) A. Chev. (syn. *M. suberosa* Benth.) (Fabaceae) is a legume distributed throughout parts of Africa and India, and the plant has had extensive ethnobotanical use as an aphrodisiac, fish poison, and insecticide (Luyengi *et al.*, 1994). There have been several phytochemical studies on this species prior to our own investigation, leading to the isolation of chalcones, flavonones, isoflavonones, rotenoids, and an imidazole derivative (Luyengi *et al.*, 1994). The ethyl acetate extract of the bark of *M. sericea* exhibited very potent inhibitory effects against phorbol ester-induced ornithine decarboxylase (ODC) activity in cell culture (Luyengi *et al.*, 1994). Work up of this extract guided by the ODC bioassay led to the isolation and spectroscopic characterization of twelve active flavonoids (**25–36**), comprising two chalcones [4-hydroxylonchocarpin (**25**) and munsericin (**26**)], two flavanones [mundulin (**27**) and lupinifolin (**29**)], two flavanols [mundulinol (**28**), lupinifolinol (**30**)], two isoflavones [mundulone (**31**) and munetone (**32**)], and four rotenoids [deguelin (**33**), tephrosin (**34**), (−)-13α-hydroxydeguelin (**35**), and (−)-13α-hydroxytephrosin (**36**)], of which three of these substances are new constituents (**26**, **35**, **36**) (Mbwambo *et al.*, 1996; Luyengi *et al.*, 1994; Lee *et al.*, 1999). Munsericin (**26**), a novel chalcone with an elemental formula of $C_{20}H_{18}O_4$, was assigned as a positional isomer of 4-hydroxylonchocarpin (**25**), which was also isolated from *M. sericea* bark in our investigation. The ^1H NMR spectrum of **26** exhibited two uncoupled methyl groups at δ_H 1.45, and evidence for a *cis*-ethylenic moiety (signals at δ_H 5.67 and 6.34; d, J = 10.0 Hz), and a *gem*-dimethyl pyran ring (at δ_H 1.35 and 1.43). Also apparent from its ^1H NMR spectrum were protons of an enone functionality occurring at δ_H 7.40 and 7.82 (d, J = 13.0 Hz) for H-α and H-β, respectively, as well as a 1,4,6-trisubstituted aromatic ring, in which proton signals were observed at δ_H 6.42 (1H, d, J = 8.1 Hz) and δ_H 7.82 (1H, d, J = 8.1 Hz) (ring B). Selective INEPT and COLOC NMR studies were performed to confirm the structure proposed for munsericin (3′′,3′′-dimethylpyrano[3′,4′]4,6-dihydroxychalcone) (Luyengi *et al.*, 1994). Selective INEPT and COLOC NMR experiments supported the position of the two phenolic groups in ring B, and confirmed that the *gem*-dimethyl pyran ring was attached to ring A (Luyengi *et al.*, 1994).

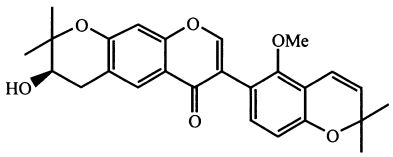
Compound **35** was assigned an elemental formula of $C_{23}H_{22}O_7$ (by high-resolution EIMS), and preliminary analysis of its spectral data indicated that **35** was a C-13 hydroxylated deguelin derivative. The coupling constant between H-7a and H-13a (J = 3.8 Hz) and the close similarity of the CD spectra of **35** with that of deguelin



25 R_1 = OH, R_2 = H
26 R_1 = H, R_2 = OH



27 R_1 = OH, R_2 = R_3 = H
28 R_1 = R_2 = OH, R_3 = H
29 R_1 = R_3 = OH, R_2 = H
30 R_1 = R_2 = R_3 = OH



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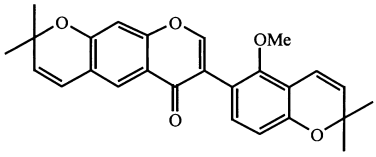

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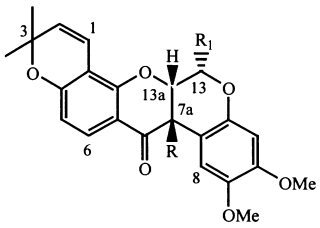


33 R = R_1 = H
34 R = OH, R_1 = H
35 R = H, R_1 = OH
36 R = R_1 = OH

Figure 5.4: Flavonoids isolated from *Mundulea sericea*.

confirmed the *cis* B/C ring stereochemistry of this novel compound. Soft irradiation of the H-13 methine proton (δ 5.81) in a selective INEPT NMR experiment resulted in enhancements of the ^{13}C NMR signals corresponding to C-7a and C-11a of **35**. The small coupling constant of 1.8 Hz between H-13 and H-13a in the ^{1}H NMR spectrum suggested a β-configuration for H-13, and hence an α-configuration for the hydroxyl group attached to C-13. Further supportive data for **35** as 13α-hydroxydeguelin were obtained from molecular modeling experiments (Luyengi *et al.*, 1994). Compound **36** was assigned a molecular formula of $C_{23}H_{22}O_8$ from its HREIMS, and was established structurally as 13α-hydroxytephrosin in a similar manner (Luyengi *et al.*, 1994).

Among the isolates obtained from *M. sericea*, the rotenoids deguelin and tephrosin exhibited extremely potent activity in the ODC assay (EC_{50} values of <1 ng/mL in each case), whereas the hydroxylated compounds **35** and **36** were approximately an order of magnitude less active than their respective parent compounds in this assay. Several of the isolates from *M. sericea* were significantly active in the mouse mammary organ culture assay (Gerhäuser *et al.*, 1995), and deguelin was selected for *in vivo* testing, and was found to both reduce the formation of papillomas in a two-stage DMBA/tetradecanoylphorbol 13-acetate (TPA) skin carcinogenesis model with CD-1 mice and mammary tumors in a *N*-methylnitrosourea carcinogenesis model with Sprague Dawley rats (Udeani *et al.*, 1997). The mechanism of action of deguelin (**33**) involves regulation of ODC activity at transcriptional levels, as well as inhibition of TPA-independent c-Myc-induced ODC activity, and inhibition of NADH dehydrogenase activity in cell culture (Gerhäuser *et al.*, 1995, 1997a). In addition, munetone (**32**) inhibited 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced ornithine decarboxylase activity (IC_{50} = 46 ng/mL) in cultured mouse epidermal ME 308 cells, and inhibited TPA-independent c-Myc-induced ODC activity with cultured BALB/c c-MycER cells, as well as 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced preneoplastic lesion formation in our mouse mammary gland organ culture (MMOC) system (Lee *et al.*, 1999). These data suggest the potential of munetone (**32**) to serve as a cancer chemopreventive agent by blocking the process of tumor promotion (Lee *et al.*, 1999). Therefore, from this work on *M. sericea*, considerable structural diversity within the flavonoid class was demonstrated, and some of these compounds exhibit very promising biological activity.

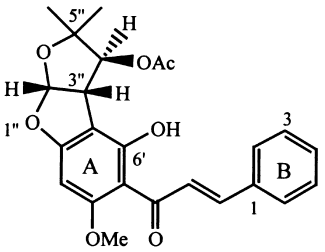
5.4.4 *Tephrosia purpurea*

Tephrosia purpurea Pers. (Leguminosae) is a widely distributed small erect perennial plant, occurring in southern Africa, and in Asia, from Pakistan to Vietnam, and eastward through Malaysia to tropical Australia (Chang *et al.*, 1997). This plant has had many medicinal uses, including as an anthelmintic and a blood purifier, and in the treatment of asthma, diabetes, diarrhea, dyspepsia, edema, gonorrhea, rheumatism, and urinary disease. Moreover, the plant has documented use as an insecticide and fish poison. Previous phytochemical investigations on *T. purpurea* have resulted in the isolation of coumarins, flavanones, and rotenoids (Chang *et al.*, 1997). In preliminary screening, an ethyl acetate extract of the entire plant of *T. purpurea* gave positive results in one of the *in vitro* assays, namely, the induction of quinone reductase (QR) activity with cultured Hepa 1c1c7 mouse hepatoma cells (Gerhäuser *et al.*, 1997b). Induction of Phase II enzymes, such as NAD(P)H:quinone reductase and glutathione *S*-transferase, is an important mechanism of certain cancer chemopreventive agents (Zhang *et al.*, 1994; Gerhäuser *et al.*, 1997b). The role of these enzymes is to inhibit multiple activation processes of procarcinogens by Phase I enzymes, by catalyzing conjugating reactions,

thus leading to detoxification pathways (Zhang *et al.*, 1994; Talalay *et al.*, 1995; Gerhäuser *et al.*, 1997b). Bioassay-monitored fractionation of the extracts led to the isolation of eight biologically active compounds using this QR assay system, inclusive of two novel flavonoids, namely, (+)-tephropurpurin (**37**) and 7,4′-dihydroxy-3′,5′-dimethoxyisoflavone (**38**), and six known flavonoids, pongamol (**39**), (+)-purpurin (**40**), lanceolatin B (**41**), (−)-maackiain (**42**), (−)-4-methoxymaackiain (**43**), and (−)-medicarpin (**44**) (Figure 5.5, **37–44**). In the following paragraphs, the steps involved in the structure elucidation of the two novel compounds will be described briefly.

(+)-Tephropurpurin (**37**) exhibited a molecular formula of $C_{24}H_{24}O_7$ from its high-resolution mass spectral data. In its ^{1}H NMR spectrum, doublets (J = 15.5 Hz) at δ_H 7.85 (H-α) and 7.78 (H-β) indicated that these protons were related to one another in a *trans* arrangement, and, along with the presence of carbonyl signal at δ_C 193.0 (C-β′), suggested that compound **37** was a chalcone unit in which ring B was unsubstituted (Gupta *et al.*, 1980; Venkata Rao and Ranga Raju, 1984). The remaining functionalities in the molecule could be assigned as a methoxy substituent (δ_C 56.2; δ_H 3.94), a hydroxyl group (δ_H 14.28), an acetate group (δ_C 169.8 and δ_C 20.9; δ_H 2.16), and a *gem*-dimethyl unit (ν max 1334–1234 cm^{-1}; δ_C 27.4, 23.2; δ_H 1.12, 1.27), and were inferred by close comparison with the spectral data of (+)-purpurin (**40**) (Venkata Rao and Ranga Raju, 1984), a compound which co-occurred with **37** in this investigation. The stereochemistry of (+)-purpurin (**40**) was assigned initially by chemical derivatization, in which (+)-purpurin has been found to undergo decetylation under mild alkaline conditions to produce a chalcone that retains its positive optical rotation (Venkata Rao and Ranga Raju, 1984). Accordingly, the stereochemistry of **40** at H-2′′ and H-3′′ was confirmed as *cis*, based on the comparison of the coupling constants of the resonances at δ_H 6.47 (1H, d, J = 6 Hz, H-2′′) and δ_H 4.03 (1H, d, J = 6 Hz, H-3′′) with literature values (Chang *et al.*, 1997). However, a more recent study has provided the absolute stereochemistry of (+)-purpurin as 2*S*,7a*R*,10a*S*,10*S* (equivalent to 2*S*,2′′*R*,3′′*S*,4′′*S*) based on the synthesis of (−)-purpurin ($[\alpha]_D$ −30.0°, $CHCl_3$) from (−)-semiglabrin in conjunction with X-ray crystallographic analysis (Pirrung *et al.*, 1998). The H-2′′ and H-3′′ protons of (+)-tephropurpurin (**37**) were mutually coupled (J = 6.2 Hz), and NMR experiments supported their occurrence in a *cis*- and β-orientation. Thus, a 15.7% NOE effect was observed at δ_H 4.03 (H-3′′) on irradiation of H-2′′ at δ_H 6.47, while irradiation at δ_H 4.03 (H-3′′) gave a 20.2% NOE at δ_H 6.47 (H-2′′). However, irradiation at δ_H 5.43 (H-4′′) did not produce any NOE effects, consistent with H-4′′ being of α stereochemistry, as in the case of (+)-purpurin (**40**).

Unambiguous assignments of the ^{1}H and ^{13}C-NMR spectra of **37** were achieved by analysis of a combination of the (HETCOR) and (HMBC) NMR spectra. Moreover, in a 2D-NOESY experiment, H-β at δ_H 7.78 showed a NOE enhancement with a signal at δ_H 7.61 (H-2). Finally, the initial relative stereochemistry of (+)-tephropurpurin (**37**) proposed has been revised so as to be consistent with a recent X-ray structural study on an



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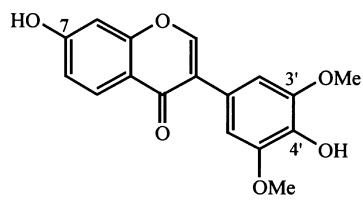

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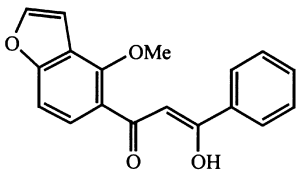

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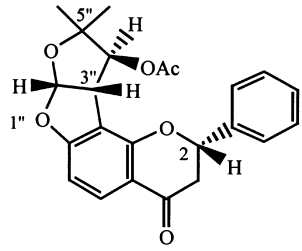

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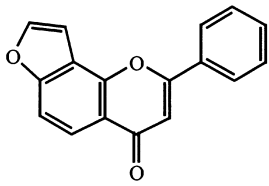

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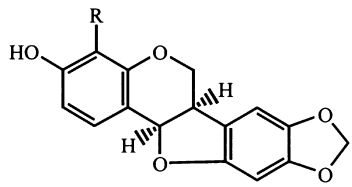

42 R = H
43 R = OCH_3

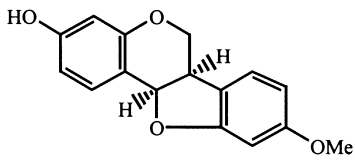

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Figure 5.5: Flavonoids isolated from *Tephrosia purpurea.*

enantiomer of (+)-purpurin, and is (+)-5′′,5′′-dimethyl-4′′-β-acetoxytetrahydrofurano(2′′,3′′-*b*)-dihydrofurano(4′,5′-*h*)-2′-methoxy-6′-hydroxychalcone (Chang *et al.*, 1997; Pirrung *et al.*, 1998).

Compound **38**, 7,4′-dihydroxy-3′,5′-dimethoxyisoflavone, exhibited a molecular formula of $C_{17}H_{14}O_6$ from its high-resolution mass spectral data. Comparison of its UV, IR, and ^{1}H-and ^{13}C-NMR data with literature values indicated it was an isoflavone (Yahara *et al.*, 1989). The functionalities apparent in the molecule were two hydroxyl groups (IR, ν max 3600–3400 cm^{-1}; δ_C 162.5, 135.6) and two magnetically equivalent OMe groups (δ_C 56.1, δ_H 3.78). In its ^{1}H-NMR spectrum, a doublet of doublets (J = 8.7 and 1.4 Hz) at δ_H 6.94 (H-6), a doublet (J = 1.4 Hz) at δ_H 6.87 (H-8), and a doublet (J = 8.7 Hz) at δ_H 7.97 (H-5) were observed as an ABX system, suggesting that ring A was functionalized only at C-7, and that the other three oxygenated substituents were all present in ring B. Unambiguous assignments of the ^{1}H- and ^{13}C-NMR spectra of **38** were achieved by analysis of a combination of the ^{1}H-^{13}C (HETCOR) NMR and a selective-INEPT NMR experiments. In a 1D NOE NMR experiment on **38**, irradiation at δ_H 6.87 (H-2′ and H-6′) gave 6.9% and 5.3% NOE effects for the resonances at δ_H 8.32 (H-2) and δ_H 3.78 (OMe), respectively. Irradiation at δ_H 3.78 gave a 6.8% NOE effect at the δ_H 6.87 signal only. Thus, the two OMe groups in the molecule of **38** could be assigned tentatively, in turn, to the C-3′ and C-5′ positions (Chang *et al.*, 1997). The ring-B ^{13}C-NMR chemical shifts of **38** were comparable with published values for those of a model compound with a symmetrical 4′-hydroxy-3′,5′-dimethoxy substituted B ring, pumilaisoflavone D (Yenesew *et al.*, 1989).

(+)-Tephropurpurin (**37**), 7,4′-dihydroxy-3′,5′-dimethoxyisoflavone (**38**), pongamol (**39**), (+)-purpurin (**40**), lanceolatin B (**41**), (−)-maackiain (**42**), and (−)-4-methoxymaackiain (**43**), all significantly induced QR activity in a murine hepatoma cell line (Hepa 1c1c7) with CD (concentration to double QR activity) values observed ranging from 0.15–17.2 μM. Compound **37** gave the most interesting biological data, since it exhibited a CD (concentration to double QR activity) value of 0.15 μM, and an IC_{50} (50% cell survival after 2 days of incubation) value of 13.4 μM, with a resultant Chemoprevention Index (IC_{50}/CD) of 89.0. The analogous data for sulforaphane (a known inducer of QR activity from broccoli) (Zhang *et al.*, 1992) obtained in the same experiment were: CD 0.43 μM, IC_{50} 11.0 μM, and CI 25.0 (Chang *et al.*, 1997). Accordingly, compound **37** may be considered a promising lead for further evaluation as a cancer chemopreventive agent.

5.5 CONCLUSIONS

There is a large and growing interest in the investigation of secondary metabolites from foodstuffs and other plants as potential cancer chemopreventive agents. A multidisciplinary collaborative approach has been made in an attempt to discover novel

plant-derived cancer chemopreventive agents. Thus far, a large number of structurally diverse active compounds has been found with flavonoids representing the largest single group. To date, deguelin (**33**), a rotenoid, has been shown to mediate cancer chemopreventive activity in full-term carcinogenesis studies (Gerhäuser *et al.*, 1995; Udeani *et al.*, 1997), and is currently being evaluated in additional test systems. Several other promising flavonoid leads have been identified in this investigation so far, including 5,7,3′,4′-tetrahydroxy-3-methoxyflavone (**20**), munetone (**32**), and (+)-tephropurpurin (**37**), which will be evaluated in additional biological test systems in the future.

ACKNOWLEDGEMENTS

The authors wish to acknowledge support of the phytochemical work described herein by Program Project grant P01 CA48112, funded by the National Cancer Institute (NCI), NIH, Bethesda, MD (Dr. J.M. Pezzuto, overall project Principal Investigator). We also wish to thank several faculty, postdoctoral, and graduate student colleagues for their excellent contributions in this project and whose names are included in the references below.

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CHAPTER

6

Antitumor Drugs from the Secondary Metabolites of Higher Plants

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Contents

6.1 INTRODUCTION

There are about 500,000 species of plants growing on the earth and it is estimated that at least 5000 different chemical compounds of secondary metabolites are present in a single species of plant. It is apparent that the secondary metabolites of plant origin constitute a tremendous resource for exploring useful drugs. In plants, the primary metabolites, including proteins, lipids, nucleic acids, enzymes, and coenzymes, etc., come from the metabolism of carbohydrates with the incorporation of nitrogen and mineral elements. By utilizing primary metabolites and numerous infinite molecules, plants synthesize the secondary metabolites for the purpose of survival and well-being. Taxonomically related plants generally produce chemically similar secondary metabolites and, therefore, may have similar pharmacological effects.

In the early 1950s, a research program screening for antitumor drugs of plant origin was initiated mainly by the National Cancer Institute (NCI) in the USA. Large-scale screening procedures were made available, plant materials were procured, and crude extracts were put through preliminary screening. Fractionation, isolation and purification of the active antitumor principles followed, in parallel with laboratory identification and characterization of the antitumor properties. Basic pharmacological and toxicological studies in animals ensued, and finally, a number of promising compounds were selected for clinical studies, with the ultimate goal of finding the active antitumor drugs from plants. This program represented a combined effort mobilizing many biomedical research organizations in the government and in medical, pharmaceutical, and chemical institutes and industries. The achievements during the past few decades have been very rewarding.

This chapter will briefly review the bioassay methods used in cancer chemotherapy research to detect anticancer activity. Secondly, anticancer secondary metabolites of particular interests, including podophyllotoxin, camptothecin, harringtonine, homoharringtonine, vincristine and vinblastine, ellipticine, colchicine, bruceantin, acronycine, maytansine, tetrandrine, thalicarpine, indicine, and some of their derivatives, are to be discussed. The discussion will focus on their isolation, structural elucidation, pharmacology and mechanism of action. Besides spectroscopic methods, chemical synthesis for some of these compounds will also be reviewed. Thirdly, well-known anticancer agents of plant origin mainly developed in China, namely curcumol, curdione, curzerenone, gossypol, indirubin, lycobetaine, monocrotaline, oridonin, triptolide, and tripdiolide, will also be briefly described. In addition, two antitumor agents from higher plants, tylocrebrine and lapachol, selected by NCI under clinical trial, will also be briefly noted.

6.2 ANTITUMOR SCREENING METHODS

6.2.1 Collection and extraction of plant sample for primary antitumor screening

Freshly collected juicy samples, such as fresh fruits or soft tubules, leaves etc., may be extracted directly with ethanol, otherwise those samples collected from the open field or from plantation garden have to be dried properly before extraction. Voucher specimens for each sample should be properly prepared and authenticated by a botanical taxonomist. The voucher specimen should bear detailed information concerning location, time of collection, weather condition during collection, and description of whole plant, preferably by photograph attached to the specimen to facilitate the latter collection.

6.2.2 Preliminary screening test

6.2.2.1 Cytotoxicity test

This assay procedure is based on the cytotoxicity of the test drug, KB-cell culture method (Geran *et al.*, 1972). The result is determined on the concentration of the test material in the culture medium required to inhibit protein synthesis to 50% as compared to that of control cultures. For preliminary screening an $ED_{50} \leqq 10\ \mu g/ml$ is judged to be active and warrants further study (Geran *et al.*, 1972; Boyd, 1993).

6.2.2.2 Tumor weight test

In this test the homogenate of tumor cells taken from a propagating animal is inoculated subcutaneously into the thigh of one of the hind legs of another animal. The animal is then treated with the test drug extract. The tumor growth (in terms of weight) may be determined by dissecting both hind legs along the hip joints, one bearing tumor and the other without. The difference in weights of both legs represents the weight of the tumor. The activity of the test drug is expressed in terms of % of control group ($T/C \times 100$). A value of $T/C \leqq 44\%$ is judged to represent that the test material is active and warrants further study. The solid tumor used for preliminary screening is WM256 in the albino rat.

6.2.2.3 Increase of life span test

The assay is performed by transplanting a certain number of tumor cells intraperitoneally into test and control animals. Test group animals are then treated with the plant extract, and if the plant extract is active it will inhibit the tumor cells from growing; therefore, the animal will live longer than the untreated control group animals. The activity of plant extract is judged by the life prolongation effect in terms of days, so that if $T/C \times 100 \geqq 125\%$ on non-toxic dose, the test material is judged to be active and pursued further. For preliminary screening, P388 and/or L1210 leukemias in mice are most generally used.

Disease-oriented *in vitro* anticancer drug screening procedure

The National Cancer Institute has implemented a new *in vitro* disease-oriented drug discovery screening system which started in 1990. This system employs 60 human tumor cell lines arranged in seven sub-pads that represent diverse histologies: leukemia, melanoma, lung, colon, kidney, ovary, and brain. For routine evaluation, each sample is tested in a 2-day continuous drug exposure protocol. 100 μg/ml of crude extracts or fractions of the panel lines are selected for further testing (Boyd, 1993, 1997; Michael *et al.*, 1992).

Clinical test

A new agent, developed through careful research, has been demonstrated to have encouraging antitumor activity and, with a documented toxicity profile, it may be a candidate for clinical testing. Phase I clinical trials test the tolerance of patients to the drug and try to establish an appropriate dose for the drug in humans. Phase II clinical trials seek to demonstrate efficacy of the new drug as a single agent. Phase III clinical trials incorporate the new agent into current therapeutic regimens and seek to demonstrate that the addition of the new agent to the combinations leads to better treatment outcomes than the original regimen (Devita, 1977; Marson *et al.*, 1984).

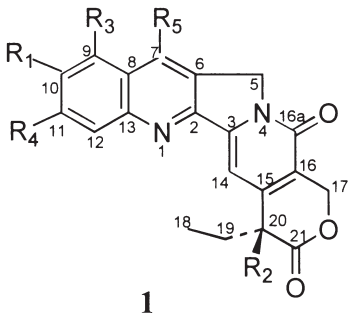
6.3 ALKALOIDS

6.3.1 Camptothecin

Camptothecin (CPT, **1**) is a pyrano-indolizinoquinoline alkaloid, originally isolated from a sample of wood and bark of *Camptotheca acuminata*, Decne (Nyssaceae) (Wall *et al.*, 1966) and other parts of the same plant. Camptothecin was also isolated in higher yield (0.3%) from *Nothapodytes foefida* (Icacinaceae) (Ku and Tang, 1980; Govindachai *et al.*, 1972), *Ophiorrhiza mungos* (Rubiaceae) (Ku and Tang, 1980; Tafur and Tang, 1976), and *Ervatamia heyneana* (Apocyanaceae) (Ku and Tang, 1980; Gunasekera *et al.*, 1979).

Beside camptothecin, related alkaloids, 10-hydroxycamptothecin (**2**), 10-methoxycamptothecin (**3**) (Wani *et al.*, 1969; Hsu *et al.*, 1977) and 11-hydroxycamptothecin (**4**) (Wall *et al.*, 1986), desoxycamptothecin (**5**), and 20-hexanoyl-10-methoxycamptothecin (**6**) (Advaovics *et al.*, 1979) have been reported to be isolated from *Camptotheca acuminata*.

The anticancer activity of the leaf extract of *C. acuminata* was found in 1966, by Wall, in collaboration with NCI's Hartwell. During the 1950s USDA had a mass screening program to search for potential precursors for cortisone synthesis from plant sources and kept a large number of frozen crude extracts. When NCI started to screen natural products for anticancer activity in 1957, it was agreed that a selection of 1000 of the ethanolic plant extracts held at the USDA should be sent to the NCI for testing in anticancer screening and *C. acuminata* was one of those extracts (Wall *et al.*, 1996). This



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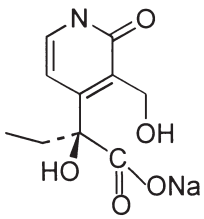


1- Sodium salt

Natural Products	R_1	R_2	R_3	R_4	R_5
(**1**) camptothecin (CPT)	H	OH	H	H	H
(**2**) 10-hydroxy-CPT	OH	OH	H	H	H
(**3**) 10-methoxy-CPT	OCH_3	OH	H	H	H
(**4**) 11-hydroxy-CPT	H	OH	H	OH	H
(**5**) desoxy-CPT	H	H	H	H	H
(**6**) 20-hexanoyl-10-methoxy-CPT	OCH_3	$—OC(CH_2)_4CH_3$	H	H	H
Semi-Synthetic Drugs	R_1	R_2	R_3	R_4	R_5
(**14**) Topotecan	H	OH	a	H	H
(**13**) Irinotecan	b	OH	H	H	CH_2CH_3
(**12**) SN-38	OH	OH	H	H	CH_2CH_3
(**15**) 9-aminocamptothecin	H	OH	NH_2	H	H

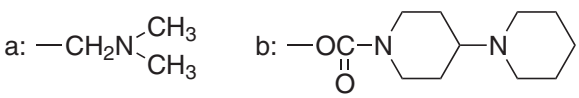


Figure 6.1: Camptothecin and analogues.

movement gave momentum to NCI's natural product program from the beginning and also brought in from USDA, an established chemist-botanist cooperative research model (Perdue, 1976).

Isolation

The powdered sample of wood and bark of *C. acuminata* (18.5 kg) was extracted by heptane under reflux. After heptane extraction, the residue was extracted with 90% of ethanol under reflux, the alcoholic extract was concentrated, diluted with 5% alcohol and then extracted twice with 4 vol. of chloroform. Combined chloroform extracts were evaporated to dryness to yield a total of 254 g, and this extract was fractionated by counter current distribution procedure using a CCl_4–MeOH–H_2O (12:10:14) system into a total of 11 tube fractions. Each fraction was evaporated to dryness and was assayed

against KB (*in vitro*) and L1210 (*in vivo*). The results showed that the activity was concentrated in fractions 2, 3, and 4 and yielded 22.8 g of crude camptothecin. The crude camptothecin was purified by silica gel column chromatography and by recrystallization (Wall *et al.*, 1996).

Camptothecin does not form stable salts with mineral acids. NaOH disrupts the lactone ring and converts camptothecin to its sodium salt, which, on acidification, yields camptothecin again. The sodium salt of camptothecin has approximately one tenth of the antitumor activity, against P388 leukemia in mice. The synthetic (±)-camptothecin has about half the activity of natural camptothecin (Wani *et al.*, 1980).

Structural elucidation

The determination of physical properties and exhaustive spectral analysis of camptothecin and X-ray crystographic study on 20-(*S*)-iodoacetyl-camptothecin led to the assignment of structure (**1**) for 20-(*S*)-camptothecin. The CD-determination of **1**, showing a negative Cotton effect curve in the region 300–400 nm, confirms the *S*-configuration at C-20. Although the ^{1}H NMR spectrum had been established previously (Wall *et al.*, 1966; Govindachar *et al.*, 1972; Hutchinson *et al.*, 1974), the complete chemical shift assignments of NMR (^{1}H and ^{13}C) were recently reported and are summarized in Figure 6.2 (Pirillo *et al.*, 1995).

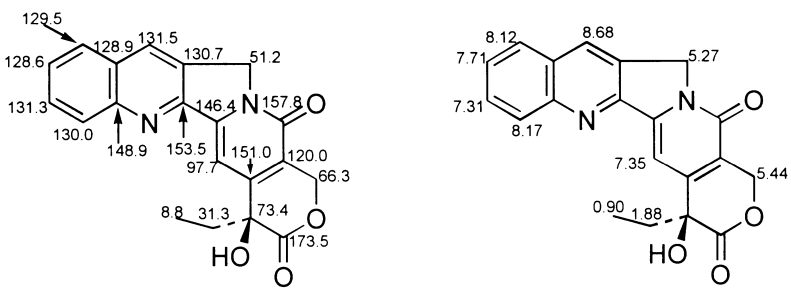
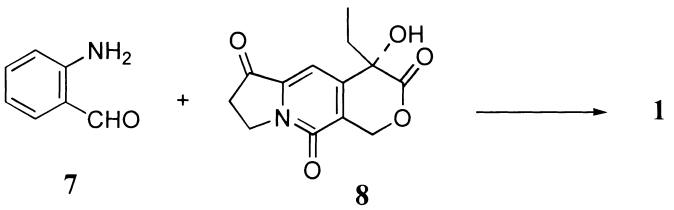


Figure 6.2: Chemical shifts of camptothecin.

Synthesis and structure–activity relationships

A number of reviews (Perdue, 1976; Wall *et al.*, 1996; Sawada *et al.*, 1996; Wang *et al.*, 1997) and papers (Wani *et al.*, 1980, 1987a,b,c; Wall *et al.*, 1986, 1993; Akihiro *et al.*, 1998) describing total synthesis of camptothecin and analogues have appeared. One on efficient synthesis of 20-(*S*)-camptothecin and some of its analogues utilizing Friedlander reaction from appropriately substituted *o*-aminobenzaldehyde (**7**) with tricyclic synthon (**8**) by Wall and Wani, is summarized in Scheme 6.1.



Scheme 6.1: *Synthesis of camptothecin.*

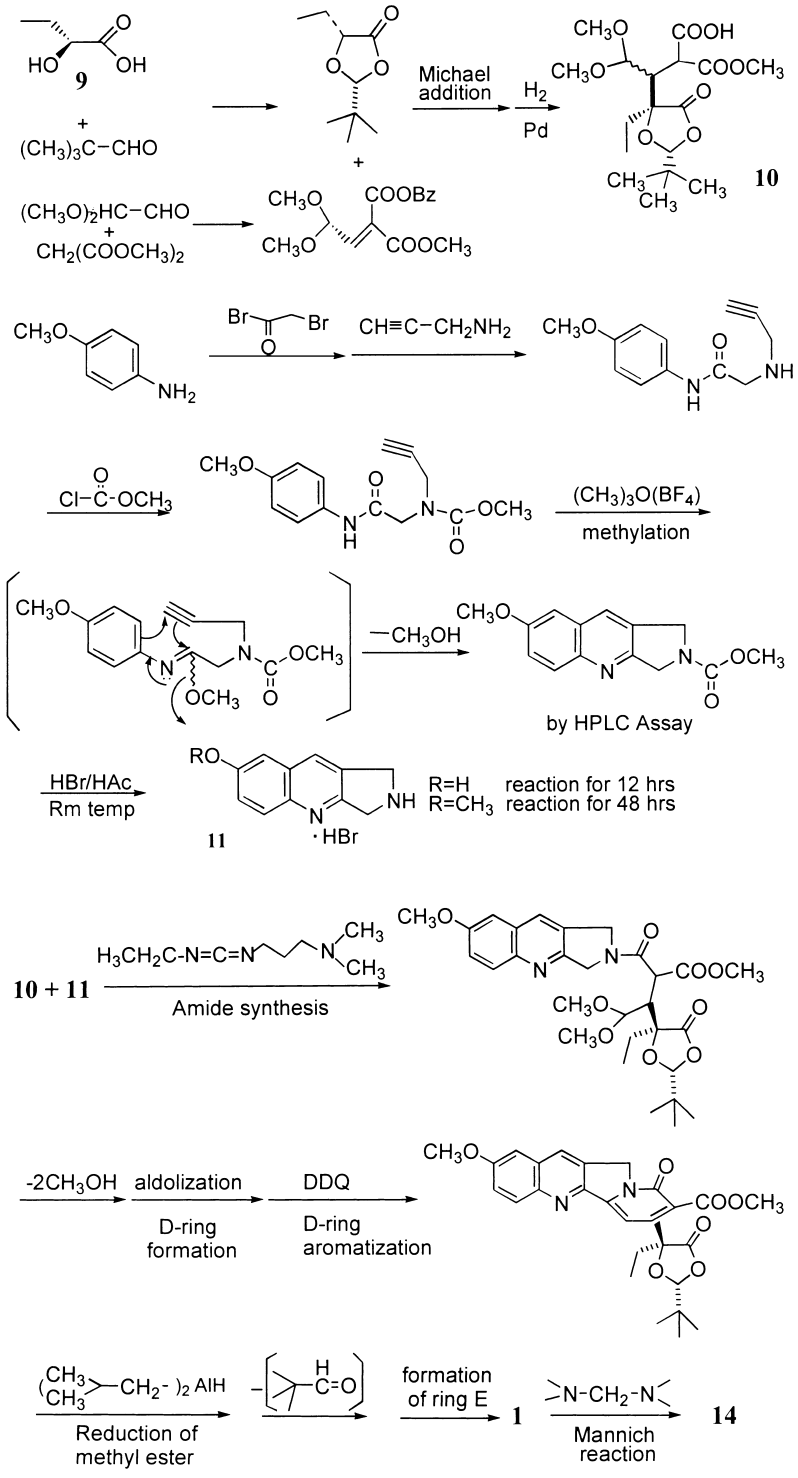
Recently, an efficient total synthesis of camptothecin and topotecan has been accomplished using an enolate equivalent of non-racemic 2-hydroxybutyric acid (**9**) to introduce C-(18–21) of camptothecin in natural absolute configuration (Scheme 6.2) (Fortunak *et al.*, 1996).

Camptothecin and its analogues, possessing the δ-lactone E-ring and the *S* configuration of C-20 hydroxy group, are essential for activity. According to SAR studies (Perdue, 1976), in the series of camptothecin and its derivatives (*see* Figure 6.1), the 7-ethyl derivatives SN-38 (**12**) is 10 times more active than that of camptothecin and 6 times more active than that of 10-hydroxyl CPT (**2**). In addition, SN-38 is comparatively less toxic and therefore it is being used clinically in cancer chemotherapy.

Due to the water insolubility for compounds **1** and **12**, developments of the new water-soluble camptothecin analogues are in progress. Significant activity was observed for 9-amino and 10-carbamate derivatives. Irinotecan (**13**, CPT-11), prepared by the reaction of chlorocarbonyl amide (Figure 6.1b) with SN-38 in pyridine (Sawada *et al.*, 1991), which is water soluble and metabolized in the body to give SN-38, is therefore a prodrug of SN-38 and is in clinical use. Topotecan (**14**), possessing a dimethylaminomethyl group at C-9, has been synthesized by the reaction of bis-(dimethylamino)-methane with (*S*)-10-hydroxycamptothecin (**2**). However, studies to develop more potent and low toxicicity water-soluble camptothecin derivatives, as inhibitors of topoisomerase I, are still in progress (Wang *et al.*, 1994, 1997; Iyer and Ratain, 1998; Luzzio *et al.*, 1995; Yaegashi *et al.*, 1994; McCabe *et al.*, 1991). Attempts to incorporate dual activities of camptothecin with 4′-*O*-demethyl epipodophyllotoxin derivatives in one single molecule through an imine linkage (Bastow *et al.*, 1997) are an interesting new development. These conjugates are designed to display dual target specificity and a broad spectrum of cytotoxic activity against drug-resistant tumors.

Pharmacology

Camptothecin (**1**) and several of its semisynthetic analogues are applied as clinical anticancer drugs in the USA, Europe, and Japan. 9-Nitro-CPT, a prodrug of 9-amino-CPT (**15**), and CPT (**1**), which are water-insoluble drugs and are administered orally, are both in Phase I/Phase II clinical trials by the Stehlin Institute. Two 10-hydroxycamptothecin



Scheme 6.2: Total synthesis of camptothecin (**1**) and topotecan (**14**).

derivatives, 10-hydroxy-9-dimethylaminomethyl-CPT (**14**, Topotecan) and 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-CPT (**13**, Irinotecan, CPT-11) have received FDA approval for use in ovarian and breast cancer. During S-phase of cell replication, DNA topoisomerase I catalyzes parent DNA relaxation *via* a transient single-stranded DNA break, by the formation of a reversible [DNA-topo-I] complex, and allows the replication fork to proceed down the DNA strand which serves as a template for synthesis of a new strand of daughter DNA. Camptothecin perturbs the reversible equilibrium by the formation of [topo-I-CPT-DNA] ternary complex which blocks the reversibility and leads to G2 phase arrest and apoptosis (Hsiang *et al.*, 1985). In addition, camptothecin overcomes MDR1-mediated drug resistance which is one of the many mechanisms of drug resistance in tumor cells (Chen and Liu, 1994).

7-Ethyl-10-hydroxy-camptothecin (SN-38) (**12**) is an active metabolite of CPT-11 and is effective against lung cancer. The results of clinical trials on the combination therapy of SN-38 with other anticancer agents showed excellent additive effects if SN-38 was, respectively, combined with cisplatin, VP-16, taxol, bleomycin, Adramycin, and 5-fluorouracil, for non-small cell lung cancer (NSCLC). For small cell lung cancer (SCLC), cisplatin, VP-16 and taxol are the most suitable for combination with SN-38 (Pei *et al.*, 1997).

6.3.2 Ellipticine

Ellipticine (**16**) (Figure 6.3) is an antineoplastic alkaloid with a green fluorescence property, which has been reported to be present in many Apocyanaceae plants, including *Ochrosia elliptica* Labill. and *Strychnos dinklagei* Gilg. 9-Methoxyellipticine (**17**) was found by Goodwin *et al*. (1959) from *O. elliptica*. Olivacine (**18**) (Figure 6.5) was isolated from the bark and stem of *Aspidosperma olivaceum*, *A. longepetiolatum* and *A. ulei* (Apocyanaceae) (Marini-Bettolo and Schmutz, 1959; Schmutz and Hunziker, 1958; Suffness and Cordell, 1985).

Several ellipticine derivatives possessing a pyridocarbazole nucleus have been found to have a marked selectivity against eight brain tumor cell lines of the disease-oriented NCI *in vitro* screen systems (Kenney *et al.*, 1995).

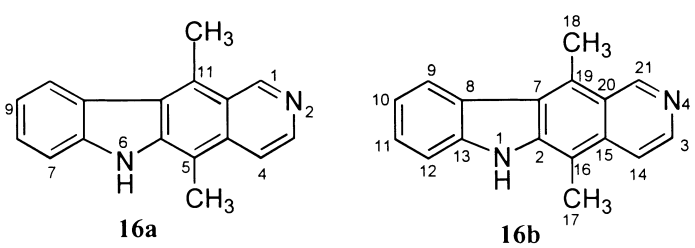


Figure 6.3: Numbering systems of ellipticine (**16**).

Isolation and structural elucidation

One kg of powdered *Ochrosia elliptica* was extracted thoroughly with 1% alcoholic tartaric acid solution at 55°C. The alcoholic extract was concentrated to 2500 ml; to this, 1500 ml of 2N HCl was added and the solution was filtered through supercel to remove part of the unwanted substances. The aqueous alcoholic filtrate 3 was further purified by extraction with chloroform and then alkalinized with Na_2CO_3 and extracted with ethyl acetate. The alkaloidal EtOAc extract was diluted by adding approximately one half volume of EtOH and extracted with 15% dilute HAc. The alkaloids in HAc solution were extracted with EtOAc after alkalinization with Na_2CO_3 and the EtOAc layer, after proper treatment, yielded 6 g of dried crude alkaloids which, following treatment by warm chloroform, yielded 186 mg of methoxyellipticine. 3 vol. of benzene were added to the chloroform mother liquid, and the resulting solution was chromatographed on acid-washed Al_3O_3 and eluted with Bz-$CHCl_3$. Fractions 22–29, eluted with 550 ml of

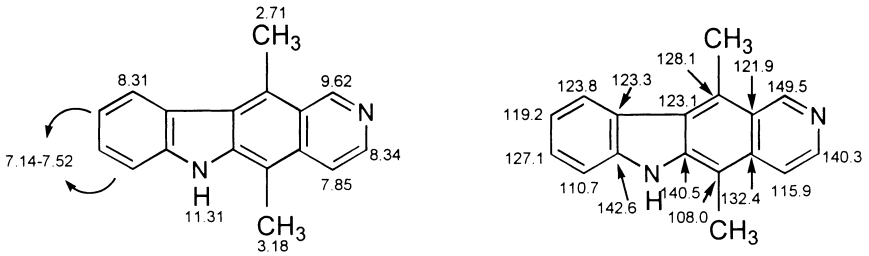
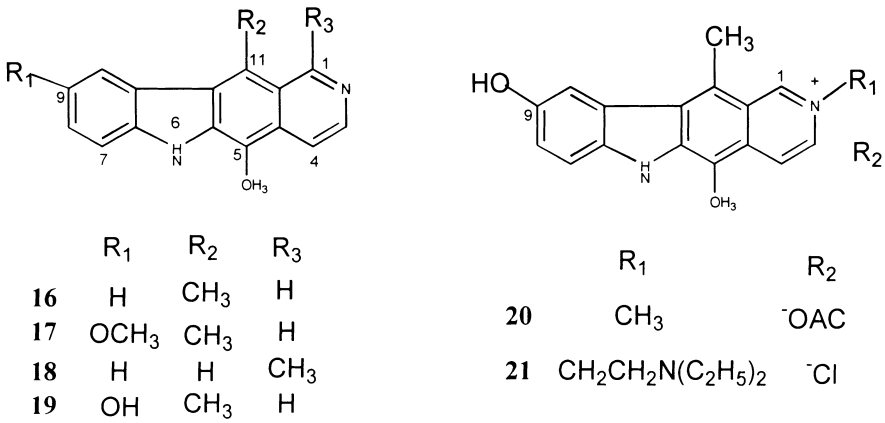


Figure 6.4: ^1H NMR and ^{13}C NMR of elipticine (**16**).



	R_1	R_2	R_3
16	H	CH_3	H
17	OCH_3	CH_3	H
18	H	H	CH_3
19	OH	CH_3	H

	R_1	R_2
20	CH_3	^-OAC
21	$CH_2CH_2N(C_2H_5)_2$	^-Cl

Figure 6.5: Some ellipticine analogues.

Bz:$CHCl_3$ (1:2), contained most of the ellipticine, which was recrystallized from chloroform to yield 0.203 g of crystalline ellipticine (**16**).

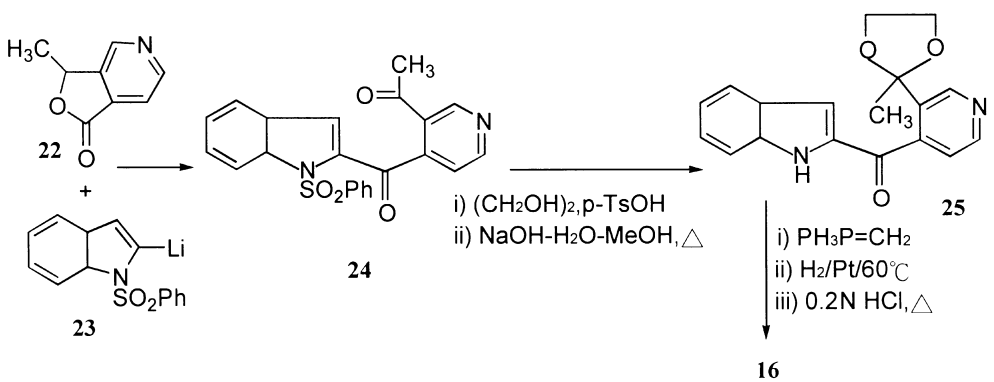
There are two numbering systems for ellipticine derivatives, which are shown as **16a** based on the pyrido[4,3-*b*]carbazole and **16b** on the biogenetic pathway (Figure 6.3). The ^{1}H-NMR (Michel *et al.*, 1980) and ^{13}C-NMR (Sainsbury *et al.*, 1982; Mansour *et al.*, 1983) spectra of ellipticine have been elaborated recently (Figure 6.4).

Synthesis and structure–activity relationships

The total syntheses of ellipticine has been briefly reviewed (Suffness and Cordell, 1985) and one of the precise synthetic processes developed by Taylor and Joule (1979) is presented below. Briefly, the process starts with condensation of pyridine lactone (**22**) with 2-lithio-1-phenylsulfonylindole (**23**) to yield **24**, followed by oxidation to give **25**, and then the Witting reaction, catalytic reduction, and acid hydrolysis to yield ellipticine (Scheme 6.3) (Taylor and Joule, 1979).

Recently, *N*-2-(diethylaminoethyl)-9-hydroxyellipticinium chloride (**21**, DHE) was developed and exhibited about 100-fold more cytotoxic activity than ellipticine (Djuric *et al.*, 1992). This increased cytotoxicity was not accompanied by greater amounts of DNA strand breakage or protein-DNA cross-linking effects, but it showed that the type of DNA damage that resulted in topoisomerase II inhibition by **21** was much more persistent than the DNA damage elicited by ellipticine (Djuric *et al.*, 1992).

Suffness and Cordell (1985) reviewed the SAR profiles and summarized that the absence of methyl groups on C-16 and C-19 of ellipticine (**16b**), or absence only on C-16, lead to the loss of activity. Derivatives with methoxy or thiomethyl group at C-16 also resulted in loss of activity and they also concluded that the substitution of a hydroxy group, methoxy or ester group or amino at C-10 (formula **16b**) would enhance the binding affinity for DNA. When the N-4 (formula **16b**) of ellipticines was substituted



Scheme 6.3: Taylor-Joule synthesis of ellipticine (**16**).

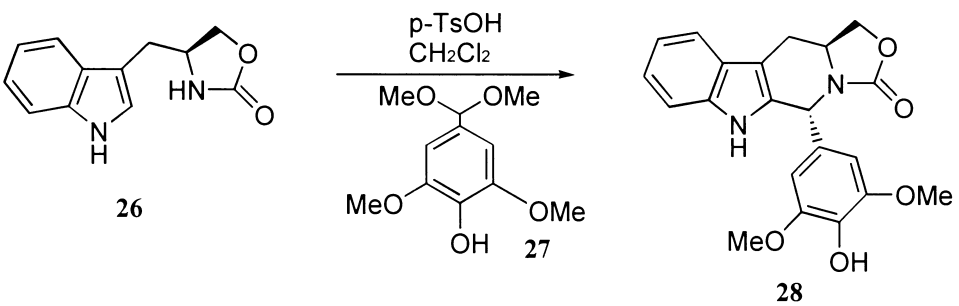
with various alkyl, hydroxyalkyl, and aminoalkyl groups, these derivatives showed a higher degree of DNA-binding. Recently, Fosse *et al.* (1994) pointed out that the hydroxy group at position 9, or the ethyl group at position 5 (formula **16a**), will enhance the DNA–drug complex stabilization. From the above evidence, together with recent reports by Moron *et al.* (1993), it may be concluded that the position of the pyridinic nitrogen atom, as well as of the C-5, C-9, and C-11 (formula **16a**), play a crucial role in antitumor activity.

It is of interest to combine different parts of two anticancer molecules with different mechanisms of action. For example, fusing partial structure **26** of ellipticine with partial structure **27** of etoposide enabled azatoxin (**28**) to bind DNA and inhibit the growth of tumor cells (Cline *et al.*, 1997). Moreover, azatoxin (**28**) was found to be as active as etoposide (Leteurtre *et al.*, 1992) and its derivatives, with increased steric crowding around the 4′-position, exhibited increased activity (Macdonald *et al.*, 1991). An efficient method for the synthesis of **28** has been developed recently by the Macdonald group accomplished by cyclization of oxazolidinone **26**, with syringaldehyde dimethyl acetal **27** in the presence of *p*-TsOH (15 mol%) in CH_2Cl_2 (Scheme 6.4) (Madalengoitia *et al.*, 1997).

Pharmacology

The ability of ellipticine derivatives to induce breakage in DNA containing apurinic sites is related to the nucleophile substituent at C-9 position (Malvy *et al.*, 1986; Bertrand *et al.*, 1989). The cleavage site on DNA generated by ellipticine treatment in human oncogene c-*myc* appears to be mostly localized in the 5′ end of the gene locus (Vilarem *et al.*, 1987).

Celiptium (**20**) (N-2-methyl-9-hydroxyellipticinium acetate, **NMHE**) and detalliptinium (**21**) (N-2-diethylaminoethyl-9-hydroxyellipticinium chloride, **DHE**) are two ellipticine derivatives (Figure 6.5) clinically used against breast cancer. They exhibit the highest activity on the topo II DNA cleavage reaction and show site specificity in pBR322



Scheme 6.4: Synthesis of azatoxin (28).

plasmid DNA and in a human c-*myc* gene inserted in a lambda phage DNA. These results are identical for ellipticines (Multon *et al.*, 1989).

Both ellipticine and 9-hydroxyellipticine (**19**) caused selective inhibition of p53 protein phosphorylation in Lewis lung carcinoma and SW480 (human colon cancer cell line). A correlation was observed between p53 phosphorylation inhibition and cytotoxic activity of these agents, suggesting that inhibition of p53 protein phosphorylation *via* kinase inhibition may be involved in the anticancer mechanism of ellipticines. Moreover, it has also been demonstrated that brief exposure to **19** caused apoptosis of cancer cells and suggested the dephosphorylated mutant p53 may induce apoptosis (Ohashi *et al.*, 1995). Further antitumor mechanism of 9-hydroxyellipticine (**19**) was reported by Sato *et al.* (1998), who concluded that compound **19** inhibits telomerase in human pancreatic cancer cells through inhibition of protein kinase rather than through restoration of functional wild-type p53.

Unlike ellipticines which promote DNA-breaking activity, the mechanism of some aza-ellipticines are due to DNA intercalation, and consequently induce protein-linked DNA breaks in NIH 3T3 cells. These aza-ellipticines did not impair DNA-synthetic activity in isolated nuclei, while ellipticine largely decreased this activity, indicating that lesions induced in DNA by aza-ellipticines are mediated by topo II (Vilarem *et al.*, 1986).

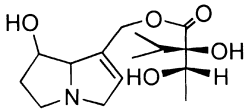
A recent clinical phase II trial of celiptium (**20**) in 33 patients with metastatic breast cancer revealing that one patient achieved complete remission, four achieved partial responses, and six achieved minor responses (Buzdar *et al.*, 1990).

6.3.3 Indicine and indicine *N*-oxide

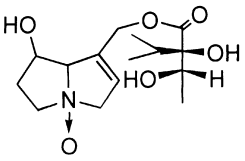
Indicine (**29**) and indicine oxide (**30**, INDI) (Figure 6.6) are pyrrolizidine alkaloids, first isolated from *Heliotropium indicum* L. (Boraginaceae) (Mattocks *et al.*, 1961) and implicated in the hepatoxicity of this plant. The interest in antitumor activities of pyrrolizidines was due to the isolation of monocrotaline (**31**) from *Crotalaria spectabilis* Roth. (Leguminosae) (Kupchan *et al.*, 1964), and senecionine (**32**) and senecionine *N*-oxide (**33**) from *Senecio triangularis* Hook (Compositae) (Kupchan *et al.*, 1964). Compounds **31**, **32**, and **33** were isolated through antitumor bioassay-directed procedures.

Heliotropium indicum was reinvestigated 15 years after the first isolation of indicine, this time using an antitumor bioassay-directed procedure.* Indicine *N*-oxide was found to be active against P388 and L1210 mouse leukemia, and Walker 256 carcinosarcoma in rats (Kugelman *et al.*, 1976).

*Assay based on tumor weight reduction test, result is considered active if $T/C \times 100 \leqq 44\%$

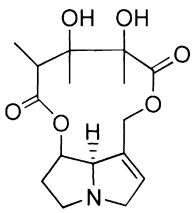


(**29**) indicine

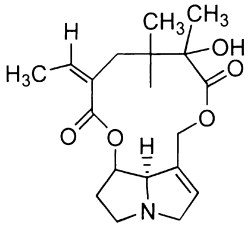


(**30**) indicine N-oxide

(2,3-dihhydroxy-2-isopropyl-butyric acid 7-hydroxy-5, 6, 7, 7 α-tetrahydro-3H-pyrrolizin-1-ylmethyl ester)



(**31**) monocrotaline
(Adenocarcinoma 755: 140 mg/kg, T/C=28%)



(**32**) senecionine (WM 256: 25 mg/kg, T/C+16%)
(**33**) senecionine N-oxide (WM 256: 40mg/kg, T/C 21%)

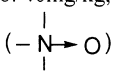


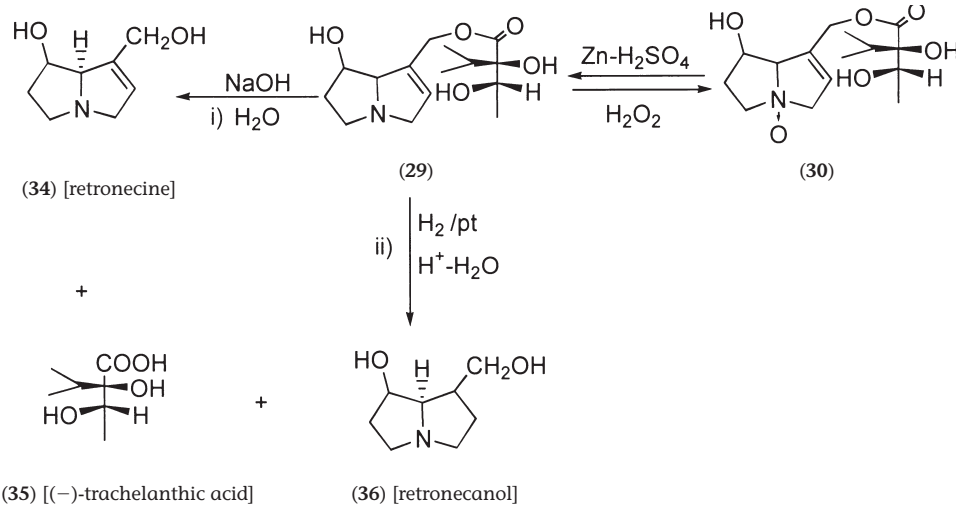
Figure 6.6: Pyrrolizidine alkaloids and their antitumor activities.

Isolation

Indicine alkaloids were extracted from *Heliotropium indicum* (2.04 kg) with ethanol at room temperature by percolation, the alcoholic extract was chromatographed through Dowex ion exchange resin C (acid form 100 g, damp) and eluted by 0.8 N NH_4OH solution (300 ml). The *N*-oxide(s) in the alkaloidal elute was reduced with Zn-dust after the solution was acidified with dilute sulfuric acid. The mixture was then alkalinized and extracted with chloroform to give a pale brown gum (8.8 m) of mixed bases. Indicine (**29**) was crystallized from alcohol as its hydrochloride (m.p. 131–132°C, $[\alpha]_D$ + 11.25°). Indicine *N*-oxide was obtained by oxidation of indicine with 30% H_2O_2 in ethanol at room temperature for 3 days, and was crystallized from MeOH-acetone. (m.p. 130–131°C; $[\alpha]_D$ + 34.0°).

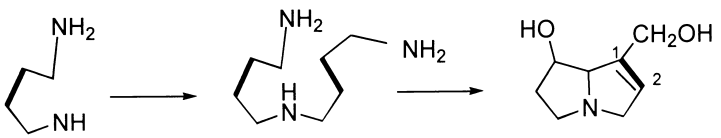
Structural elucidation

The structure of indicine (**29**) was deduced by alkaline hydrolysis and hydrogenolysis. Both reactions yielded a common product (−)-trachelanthic acid (**35**). In addition, retronecine (**34**) and retronecanol (**36**) were obtained, respectively. Compounds **29** and **30** were convertible as shown in Scheme 6.5 (Mattocks *et al.*, 1961).

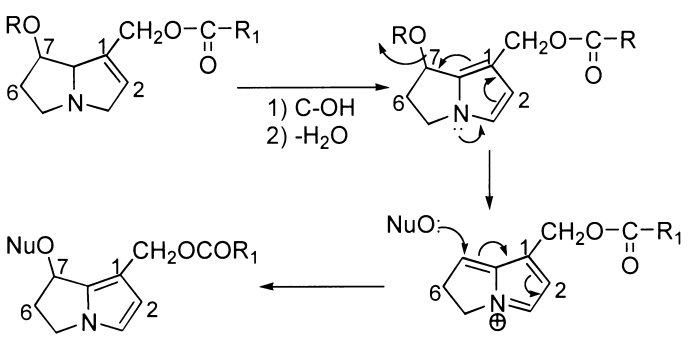


Scheme 6.5: Reaction of indicine alkaloids.

Retronecine (**34**) was demonstrated to be derived biogenetically from putrescine through a symmetric intermediate, *N*-(4-aminobutyl)-1,4-diaminobutane (homospermidine) (Khan and Robins, 1981a,b) as shown in the following scheme:



Pharmacology



Scheme 6.6: Metabolism transformation of pyrrolizidines.

TABLE 6.1
Antitumor activity of indicine N-oxide

Tumor system	Regimen	T/C %	Evaluation
B16 melanoma (ip)	64 mg/kg ip q.3h. on days 1, 5, 9	217	Evaluation
	256 mg/kg ip q.3h on day 1	158	Highly active
MX-1 mammary xenograft at subrenal capsule	800 mg/kg, sc. Daily for 10 days.	(−65)	Highly active
M5076 sarcoma (ip)	600 mg/kg ip, q.4d for 5 days	230	Highly active
P388 (ip)	400 mg/kg ip, daily for 9 days	222	Highly active

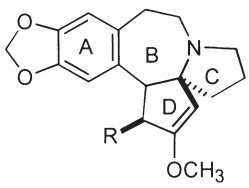
The hepatotoxicity of pyrrolizidine alkaloids was demonstrated to be due to transformation into reactive nucleophiles in the body, after metabolism, as represented by Scheme 6.6.

Indicine *N*-oxide (**29**) shows high activity in different animal tumor systems and xenograft tests (*see* Table 6.1 for Summary).

Based on promising activity against different tumor systems and favorable pharmacological results, indicine *N*-oxide has been selected as a candidate for clinical trials (Mattocks, 1968; Jago *et al*. 1970).

6.3.4 Homoharringtonine and harringtonine

In recent years, much attention has been paid to the alkaloids isolated from *Cephalotaxus* spp.: *C. haringtonia* var. *drupaceae* SIEB. et ZUCC. and *C. fortunei*



cephalotaxine (**37**) R : OH

harringtonine (**38**) R : $H_3C-\overset{OH}{\underset{H_3C}{C}}-(CH_2)_2-\overset{HO}{\underset{CH_3OOCCH_2}{C}}-\overset{O}{\overset{\|}{C}}O-$ (2' 1' 3')

homoharringtonine (**39**) R : $H_3C-\overset{OH}{\underset{H_3C}{C}}-(CH_2)_3-\overset{HO}{\underset{CH_3OOCCH_2}{C}}-\overset{O}{\overset{\|}{C}}O-$

isoharringtonine (**40**) R : $H_3C-\overset{H}{\underset{H_3C}{C}}-(CH_2)_2-\overset{HO}{\underset{HO}{C}}-\overset{O}{\overset{\|}{C}}O-\overset{C}{\underset{HO}{H}}-CO_2CH_3$

deoxyharringtonine (**41**) R : $H_3C-\overset{H}{\underset{H_3C}{C}}-(CH_2)_2-\overset{HO}{\underset{CH_2CO_2CH_3}{C}}-\overset{O}{\overset{\|}{C}}O-$

Figure 6.7: Harringtonine type compounds.

(Cephalotaxaceae). All those compounds have the unusual tetracyclic spiro-benzazepin structure and most of them have antitumor properties. The first known cephalotaxine (**37**) has a basic structure with a free alcohol group and is devoid of antitumor activity. The rest of the alkaloids **38**, **39**, **40**, and **41** (Figure 6.7) are esters of cephalotaxine, which contain alkylated and hydroxylated succinic acid monomethyl esters and are therefore designated harringtonine type alkaloids. The intensity and type of antitumor activity of these ester alkaloids is apparently strongly dependent on the nature of the organic acid component. Among these alkaloids, homoharringtonine (**39**) is most active and, therefore, was selected as a candidate for clinical trials in the United States.

Isolation

More than 20 alkaloids which possess the cephalotaxine nucleus have been isolated from seven species of *Cephalotaxus* genus (Suffness and Cordell, 1985). The minor, but active, alkaloids, harringtonine (**38**) and homoharringtonine (**39**), are esters of cephalotaxine with differently substituted succinic acid residues, and were isolated first by Powell *et al*. in 1969 and 1970, respectively. Harringtonine, homoharringtonine, isoharringtonine (**40**), and deoxyharringtonine (**41**) have recently been isolated from *Cephalotaxus* species by Takano *et al*. (1996, 1997). In summary, dried leaves and stems were extracted with MeOH and were partitioned between EtOAc and 3% aqueous tartaric acid. Then, the aqueous phase was made more alkaline with saturated Na_2CO_3 solution and extracted with $CHCl_3$. The $CHCl_3$-solution phase was concentrated to give crude alkaloid extract. This crude extract was subjected to ODS (C_{18}) Column using 0.03 M aq. $(NH_4)_2CO_3$–MeOH mixtures of increasing MeOH concentration (0–100%) to obtain 13 fractions. Fractions (eluted with 30% MeOH) were collected and processed on ODS (C_{18}) column or RP-HPLC, using 0.03 M aq. $(NH_4)_2CO_3$–MeCN solvent systems to yield compounds **37**, **38**, **39**, **40**, and **41** (Zhou *et al*., 1995; Fang *et al*., 1998).

Structural elucidation

The complete structure of cephalotaxine (**37**) was primarily established through spectroscopic elucidation (Powell *et al*., 1969, 1970) and the absolute configuration was determined as 3*S*,4*S*,5*R* by X-ray crystallography on cephalotaxine, *p*-bromobenzoate (Arora *et al*., 1974) and cephalotaxine itself (Arora *et al*., 1976). The structures of harringtonine (**38**) and homoharringtonine (**39**) have been confirmed unambiguously by the ^{1}H and ^{13}C NMR data (Powell *et al*., 1972; Weislerder *et al*., 1980; Huang and Xue, 1984). The chemical shifts of ^{13}C NMR for homoharringtonine (Figure 6.8) are presented herein. Recent reports on several new harringtonine-type alkaloids also provided the detailed spectral analysis, including 2D NMR, as well as the cytotoxic data of these compounds (Takano *et al*., 1996; Wang *et al*., 1992; Takano *et al*., 1997). Delfel and Rothfus (1977) used the tissue culture technique to yield major homoharringtonine.

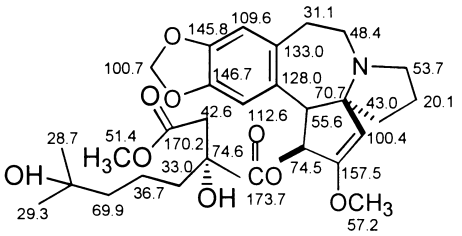
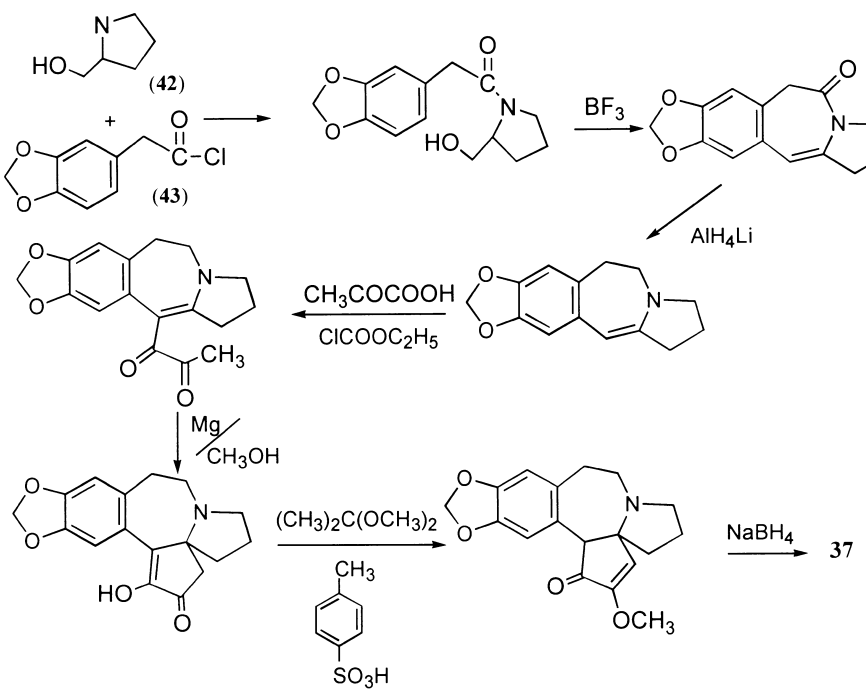


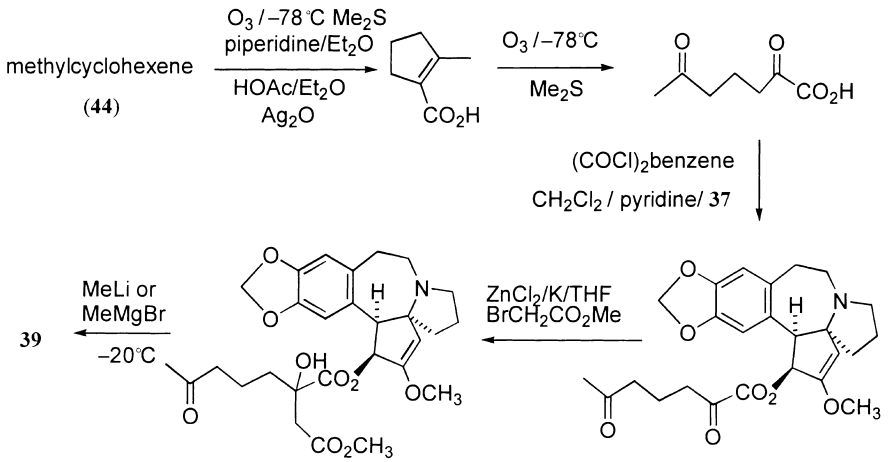
Figure 6.8: The chemical shifts of ^{13}C NMR for **39**.

Synthesis

The total syntheses of cephalotaxine and its esters, including homoharringtonine, have been extensively reviewed (Smith *et al.*, 1980; Tang and Eisenbrand, 1992; Huang and Xue, 1984). By employing acylating prolinol (**42**) and aryl acid chloride (**43**) as starting materials, Weinreb and Auerbach (1975) developed a sophisticated total synthesis of (**37**), as shown in Scheme 6.7.



Scheme 6.7: Total synthesis of cephalotaxine (**37**) by Weinreb and Auerbach.



Scheme 6.8: The synthesis of homoharringtonine (**39**) from cephalotaxine (**37**).

Esterification of **37** to homoharringtonine (**39**) starting from methylcyclohexene (**44**) was accomplished by Hiranum and Hudlicky (1982) (Scheme 6.8).

On the SAR studies, the length of R_1 moiety at C-2' position in harringtonine-type alkaloids (Figure 6.9) may change the cytotoxicity, no matter what its *R* or *S* form configuration (Wang *et al.*, 1992).

The synthesis of deoxyharringtonine (Mikolajczak *et al.*, 1974) and isoharringtonine (Li *et al.*, 1984) from cephalotaxine leads to optical isomeric mixtures which may be separated by HPLC.

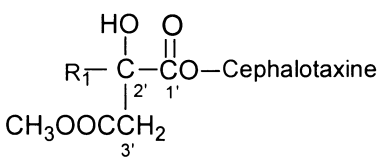


Figure 6.9

Pharmacology

The principal mechanism of action of homoharringtonine is the inhibition of DNA and protein synthesis (Wu *et al.*, 1981, 1982) and cell growth (Liu *et al.*, 1994). By acting on the ribosomes of cancer cells, homoharringtonine was shown to inhibit the polypeptide chain elongation in eukaryotic cells (Tujebajeva *et al.*, 1992) and block the progression of cells from G1 phase into S phase and from G2 phase into M phase (Zhou *et al.*, 1995). Recently, both harringtonine and homoharringtonine have been reported to rapidly

induce apoptosis in leukemia (HL-60) cells (Fung *et al.*, 1998; Fung *et al.*, 1994; Li *et al.*, 1994; Liu *et al.*, 1994). In addition, homoharringtonine induces heat protection and facilitates dissociation of heat shock transcription factor and heat shock element complex (Lee *et al.*, 1993). Studies combining homoharringtonine and the other anticancer agents, cytosine arabinoside (Ara-C) and interferon (IFN-α), suggest that there may be inhibition of chronic myeloid leukemia colony growth (Visani *et al.*, 1997). In a recent report, He *et al.* (1996) presented overexpression of the *mdr*-1 gene and P-glycoprotein in the harringtonine-resistant HL-60 cell.

Homoharringtonine has relatively mild toxicity and no anthracycline-like cardiac toxicity, which makes it a suitable agent for the treatment of aged patients (Coonley *et al.*, 1983). The results of phase I clinical trials recommended a suitable dosage of 2–4 mg/patient/day infusion for leukemia and other solid tumors (Suffness and Cordell, 1985). A clinical report in China showed that 16 of 18 patients with acute promyelocytic leukemia who were treated in combination with harringtonine, adriamycin, thioguanine and prednisone and with Chinese traditional medications resulted in complete remission (88.8%) (Guo *et al.*, 1993). Favorable results on combination therapy of homoharringtonine with other anticancer drugs were also reported (Feldman *et al.*, 1996; Coonley *et al.*, 1983). With treatment of chronic myelogenous leukemia with homoharringtonine infusion at a daily dose of 2.5 mg/m^2, of a total of 58 patients evaluated for hematologic response, 42 had complete hematologic remission (He *et al.*, 1995). In a phase II trial, of 43 patients with relapsed acute myelogenous leukemia (AML), seven achieved complete remission, and two of three patients resistant to cytarbine achieved complete remission (Feldman *et al.*, 1992a). Recently, in a review by Cheson (1998) on homoharringtonine for acute myeloid leukemia in phase II evaluation, the complete remission was less than 30%. Homoharringtonine was ineffective in the treatment of advanced renal cell carcinoma in phase II trial (Witte *et al.*, 1996), while it was recommended for the chemotherapy of head and neck cancer (Xu and Liang, 1996). In addition, clinical use of homoharringtonine was reported to have favorable effects in glaucoma particles (Peng *et al.*, 1995; Shi *et al.*, 1995).

6.3.5 (+)-Tetrandrine

(+)-Tetrandrine (**45**), present in the Chinese drug han-fang-chi, the root of *Stephania tetrandra* S. Moore (Menispermaceae), is a bisbenzylisoquinoline alkaloid, in which the two benzylisoquinoline moieties are connected via two ether linkages (Chen *et al.*, 1935; Chuang *et al.*, 1939; Kondo and Yano, 1928; Tomita *et al.*, 1967). Phaeanthine, the *l*-isomer of (+)-tetrandrine has been isolated from *Gyrocarpus americanus* Jacq. (Gyrocarpaceae) (McKenzie and Price, 1953).

Isolation and structural elucidation

Tetrandrine (**45**) has also been isolated from *Cyclea barbata*, *Cyclea peltata*, *Triclisia*

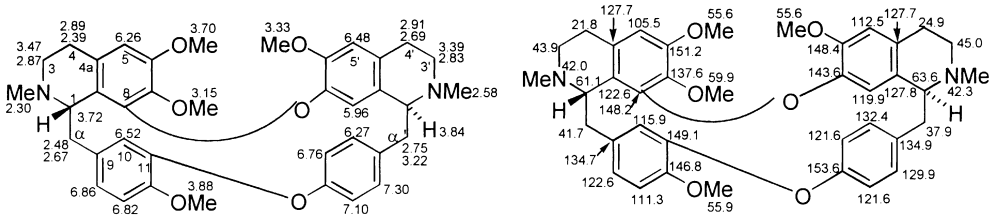


Figure 6.10: The ^{1}H and ^{13}C NMR chemical shifts of tetrandrine.

subcordata, *Cocculus pendulus*, *Pachygone dasycarpa*, and *Isopyrum thalictroides* (Suffness *et al.*, 1985; Kupchan *et al.*, 1973; Lin *et al.*, 1993; Dwuma-Badu *et al.*, 1975; Hussain *et al.*, 1984; Guinaudeau *et al.*, 1977; Philipov and Istatkova, 1977). For the isolation of **45** from *C. barbata* reported in 1993, the powdered roots were extracted with MeOH three times, and the MeOH solution was evaporated in vacuo. The residue was then treated with 1% HCl, and the acidic solution was treated with NH_4OH to regulate pH value to 9. After extracting with $CHCl_3$ and evaporation of the $CHCl_3$ solution, the crude alkaloid extract (1.2% yield) was dissolved in Me_2CO and gave crystalline tetrandrine (**45**) (Lin *et al.*, 1993).

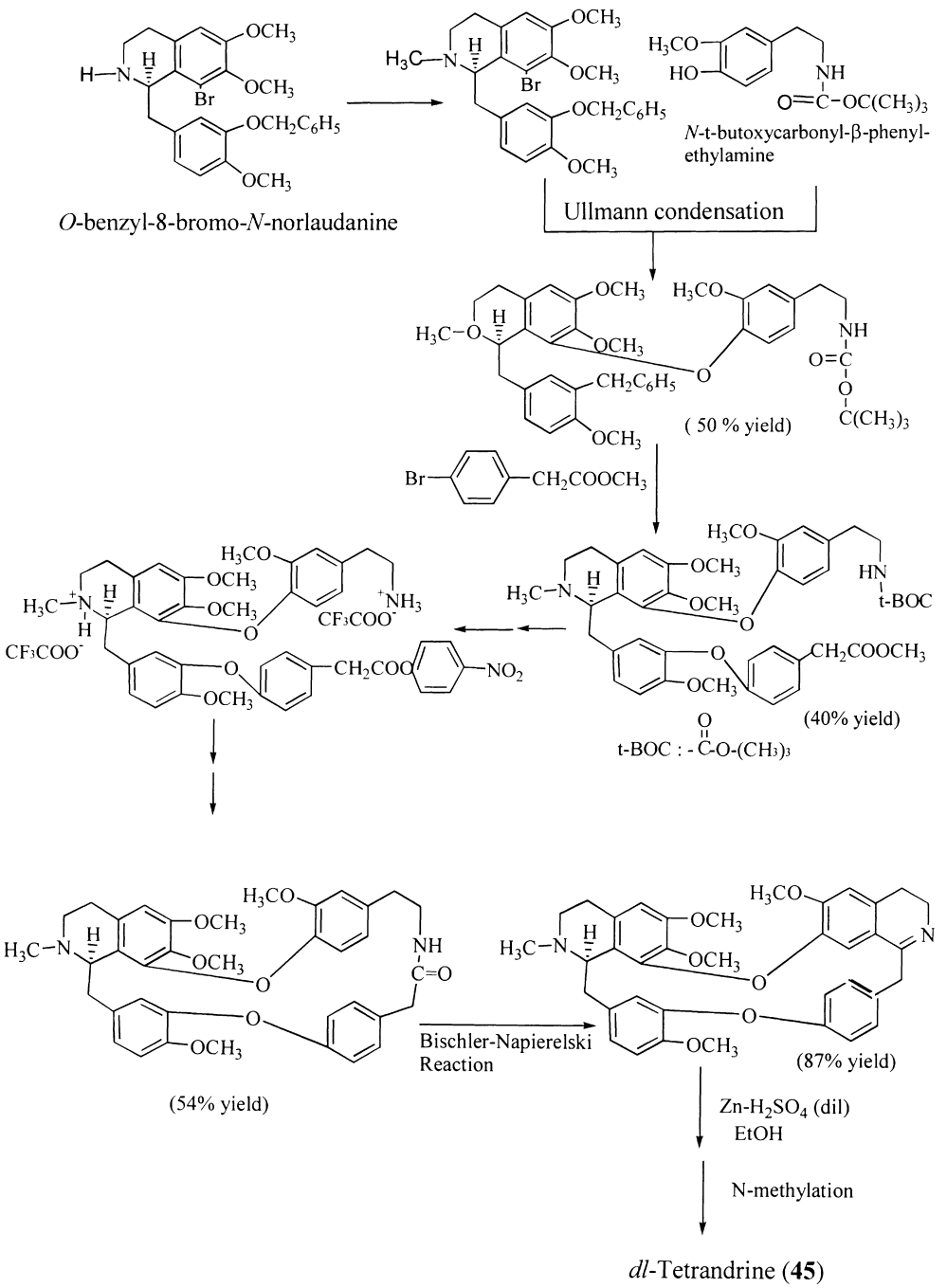
Although the stereochemistry of tetrandrine was determined by X-ray analysis in 1976 (Gilmore *et al.*, 1976). The complete and unambiguous assignments of ^{1}H- and ^{13}C-NMR chemical shifts, especially for the four methoxy groups and the chemical shifts of C-3, 4, 3′, 4′ and α, and α′ protons and carbon, were made by high resolution NMR (Figure 6.10) and 2D-NMR techniques (Kupchan and Altland, 1973b; Lin *et al.*, 1993).

Synthesis

Both *d*- and *l*-forms of tetrandrine have been synthesized via Ullman condensation to form two ether linkages using *O*-benzyl-8-bromo-*N*-norlaudanine as starting material which was synthesized from *O*-benzylhomoisovanillic acid and β-3-bromo-4,5-dimethoxyphenylethylamine (Scheme 6.9) (Inubushi *et al.*, 1968, 1969).

Pharmacology

Tetrandrine was selected for development at the National Cancer Institute due to its significant activity against the Walker 256 carcinoma (Hartwell and Abbot, 1969). The investigation of structure requirements for antitumor activity, Kupchan and Altland (1973b), concluded that monomeric benzylisoquinolines and aporphines were inactive, while bisbenzylisoquinolines were active. Additional evidence offered by them indicated that a conformationally restricted macrocyclic ring and *N*-methyl groups are not required.



Scheme 6.9: Total synthesis of *dl*-tetrandrine (**45**).

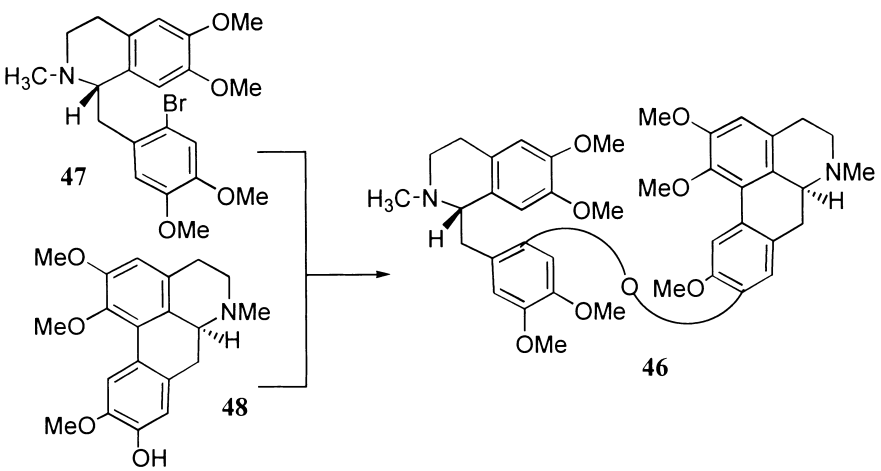
Investigational New Drug application was approved for tetrandrine in 1973. After phase I trial was completed, results suggested that there were no antitumor effects in a limited patient sample. Tetrandrine was recently found to exhibit *in vitro* anticancer activity against the drug-resistant cancer cells by the modulation of P-glycoprotein (Choi *et al.*, 1998; Ye *et al.*, 1996). Interestingly, the search for the mechanism of inhibition in human leukemia U937 and HL-60 cells revealed that tetrandrine inhibited these cells *via* induction of apoptosis (Lai *et al.*, 1998; Dong *et al.*, 1997). In addition to its antitumor activity, tetrandrine was also shown to exert anti-inflammatory, immunosuppressive (Qian *et al.*, 1983), hypotensive (Li *et al.*, 1989), and to have cardiovascular effects (Sutter and Wang, 1993).

6.3.6 Thalicarpine

Isolation and structural elucidation

Thalicarpine (**46**), which is a dimeric aporphine-benzylisoquinoline alkaloid, was first isolated from *Thalictrum dasycarpum* Fisch. & Lall. (Ranunculaceae) (Kupchan *et al.*, 1963). It was also present in other species of plants such as *T. minus*, *T. revolutum*, *Hernandia ovigera* L., and *H. peltata* L. (Hernandiaceae) (Mollov and Duschewka, 1964; Tomita *et al.*, 1966; Furukawa *et al.*, 1972; Wu *et al.*, 1977; Chalandre *et al.*, 1986; Suffness and Cordell, 1985).

An efficient extraction and isolation procedure for thalicarpine is briefly presented here. The ethanol extract of *T. revolutum* was evaporated to dryness to produce a brown residue, which was extracted with 2% aqueous citric acid and then filtered. After adding $CHCl_3$ to the filtrate, the acidic and neutral substances were removed. The resulting



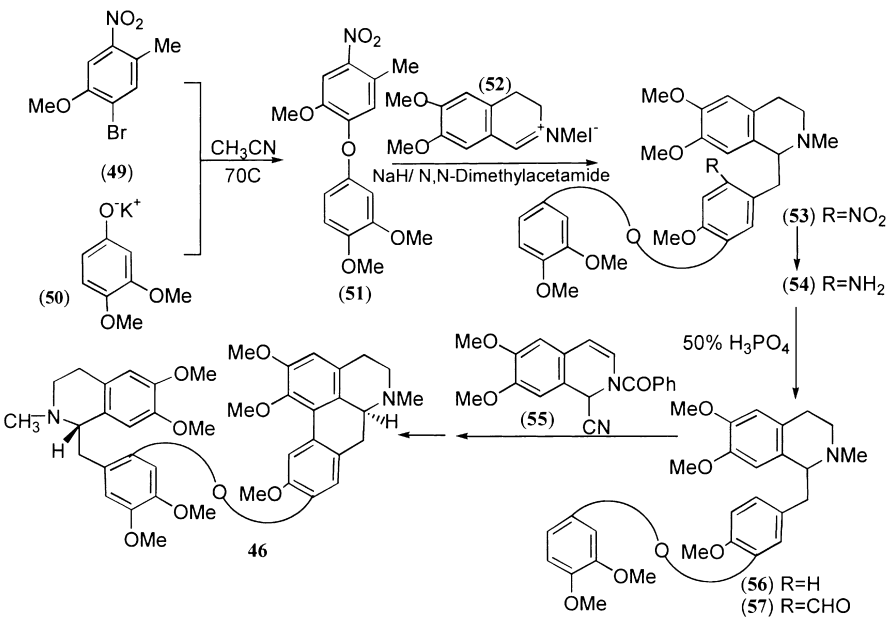
Scheme 6.10: *Synthesis of thalicarpine* (**46**).

citric acid solution was processed as described in a previous paper (Wu *et al.*, 1976) to give an ether-soluble nonphenolic base fraction, which was chromatographed on a silicic acid column. Fractions per 100 ml were collected by gradually increasing methanol to chloroform as eluting solvents. Most of thalicarpine was found under the mixtures of methanol in chloroform by 2.5 (3 liters), 5.0 (3 liters), and 10% (1 liter). The residue from column fractions 29 to 32 was dissolved in methanol and concentrated to a volume which produced crystalline needles. Finally, recrystallization from methanol yielded thalicarpine as colorless needles.

The structure of thalicarpine was deduced through the analysis of NMR spectra, while an Ullman-type ether synthesis of L-6′-bromolaudanosine (**47**) and *N*-methyllauroteta-nine (**48**) can identify natural thalicarpine (Scheme 6.10) (Tomita *et al.*, 1965; Tomita *et al.*, 1967).

Synthesis

The total synthesis of thalicarpine has been developed by the Kupchan group (Kupchan and Liepa, 1971; Kupchan *et al.*, 1973). An efficient total synthesis of thalicarpine by proceeding to the diaryl ether (**51**) *via* the alkaloid hernandaline (**50**) was briefly presented here (Kupchan and Liepa, 1971). In summary, diaryl ether was prepared from the activated aryl halide (**49**) and the phenoxide (**50**). By condensation of **51** and 3,4-dihydroisoquinoline (**52**), the aporphine precursor (**53**) was produced. The diamine (**54**)



Scheme 6.11: Partial pathway of the total synthesis of thalicarpine (**46**).

was obtained from **53** by hydrogenation with 5% Pd-C in ethanol. Diazotization of **54**, followed by cyclization in 50% aqueous phosphoric acid, gave the aporphine (**56**) and the phenol (**55**). The remaining sequent steps for producing thalicarpine from the aporphine (**56**) were described in the paper (Kupchan and Liepa, 1971) and shown as Scheme 6.11.

Pharmacology

In the 1960s, thalicarpine had been reported to have antitumor activity against the Walker 256 carcinosarcoma in the rat and to be cytotoxic to KB cells. Due to the recommendation of the tolerable dose from phase I clinical trial, thalicarpine was evaluated in a phase II clinical trial treating fourteen advanced malignant patients, however, there were no complete or partial objective responses, leading to the cancellation of further clinical investigation (Leimert *et al.*, 1980). Recent research on thalicarpine (**46**) (=thaliblastine, NSC-68075, CAS-5373-42-21) revealed higher cytotoxicity in a cisplatin-resistant rat ovarian tumor cell line than in a parental sensitive line (Chen *et al.*, 1993a,b). Chen *et al.* (1993c) also demonstrated that thalicarpine circumvents multidrug-resistant tumor cells by direct binding to P-glycoprotein. Furthermore, Seifert *et al.* (1996) reported that the same drug affected the cell cycle progression of an ovarian tumor line and its cisplatin-resistant subline. More evidence was provided by Seifert *et al.* (1996), where a G2/M block in the cell cycle was observed; thereafter, cell-cycle arrest in G1 became prominent, while S-phase cells finished DNA replication.

6.3.7 Emetine

Emetine (**58**) is a benzoquinolizine-isoquinoline dimeric alkaloid. It was isolated from *Cephaelis ipecacuanha* (Brotero) Richard or from *Cephaelis acuminata* Karsten (Rubiaceae).

Isolation and structural elucidation

Emetine (**58**) may be extracted from the ipecac powder with benzene–heptane mixture. The organic extract was then shaken with dilute HCl, and the aqueous layer containing hydrochloride salts of mixed alkaloids was made more alkaline with ammonia and extracted with ether. The ether layer was shaken with diluted NaOH to remove phenolic alkaloid cephaline (**59**). The ether solution was then concentrated and emetine (**58**) was purified by recrystallization as its hydrochloride (Battersby *et al.*, 1959a).

The structure of emetine was mainly determined by Hoffmann degradation of *N*-acetylemetine (**60**) (Scheme 6.12) (Battersby *et al.*, 1959b).

Emetine has four asymmetric centers at positions 2, 3, 12_b and 1′. The stereochemistry of emetine was deduced by van Tamelen (1959) and by Battersby (1960), respectively. Characteristic chemical shifts of the indole benzylic carbons of the tetrahydrocarboline unit were used to determine the conformation of benzo-substituted

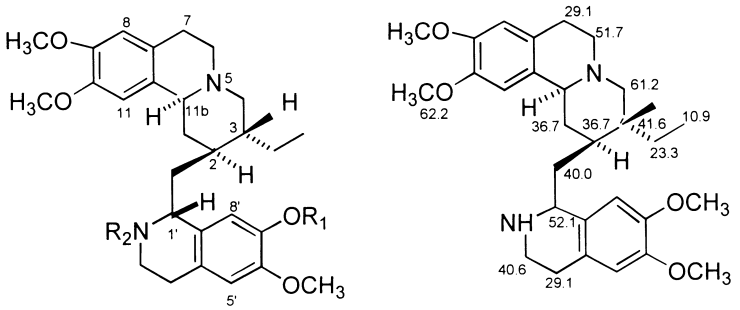
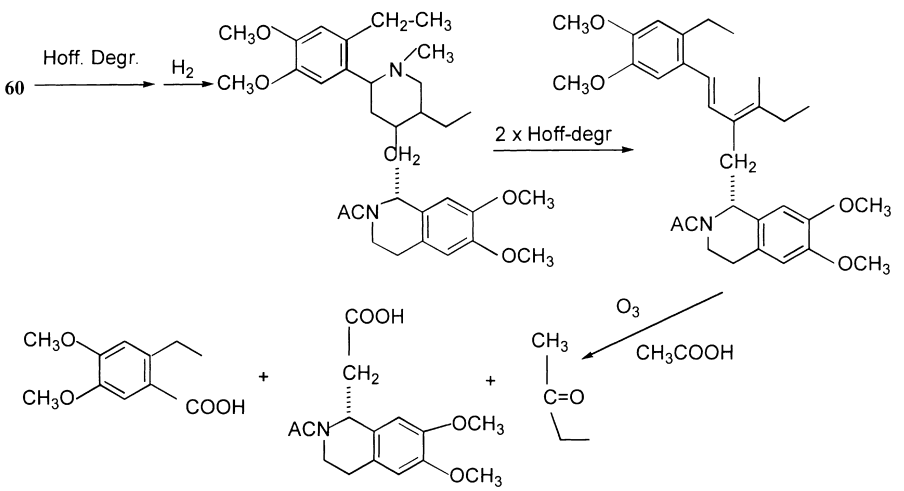


Figure 6.11: Compound **58**, **59** and **60** and ^{13}C NMR of Emetine (**58**).

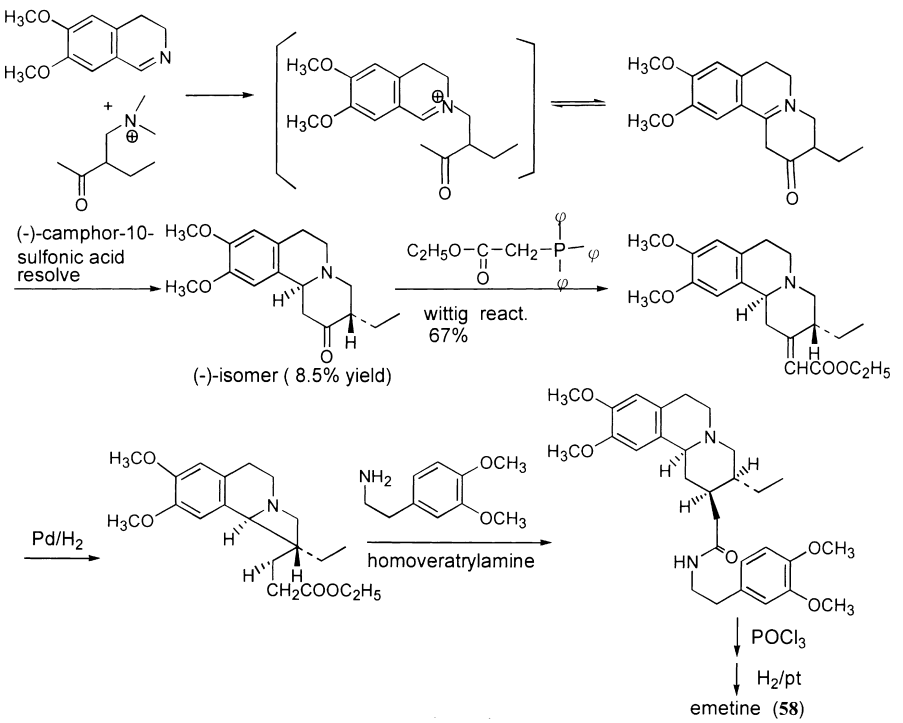
quinolizidine, although the assignment of the benzylic carbon is somewhat more difficult owing to its occurrence at lower field. The ^{13}C-NMR spectrum of emetine is shown in Figure 6.11. (Tourwe and Binst, 1978).

Synthesis and structure-activity relationships (SAR)

Among various total synthetic methods, the method developed by Openshaw and Whittaker (1963a,b, 1969) (Scheme 6.13) is a high-yield commercial process producing four asymmetric centers, as in natural emetine (Whittaker, 1969). Recent synthetic methods have also been described by Popp *et al.* (Popp and Watts, 1977) and by Saraf *et al.*



Scheme 6.12: The chemical degradation of acetylemetine (**60**).



Scheme 6.13: Total synthesis of emetine (**58**).

(Saraf, 1981); in addition to the SAR studies, together with previous reports, they conclude that positions 2 and 3 in the C ring are not absolutely critical for activity, but that position 1′ in the tetrahydroisoquinoline unit is critical (Grollman and Jarkovsky, 1975).

Pharmacology

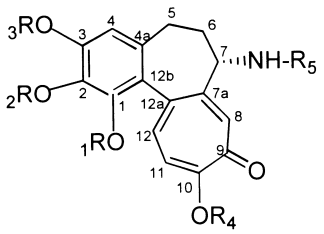
Emetine prevented protein synthesis by inhibiting the translocation of peptidyl-tRNA from the receptor site to the donor site on the ribosome (Huang and Grollman, 1970). After SAR studies of structurally-related compounds, the antiamebic, as well as the protein synthesis inhibitory effect of emetine and its analogues, was found to result from their possessing the partial structure previously described as a topochemical requirement (Grollman, 1967).

Emetine is a useful systemic amebicide for the treatment of severe invasive intestinal and extraintestinal amebiasis (Tracq and Webster, 1996). It has been demonstrated to have nonspecific antigranulomatous effect (Grollman, 1965) in several experimental animal systems, including L1210, P388, sarcoma 180, and Yoshida sarcoma in mice (Aulutta *et al.*, 1974; Jondorf *et al.*, 1971). In recent reports, emetine is widely used in cytology and cancer research as a protein biosynthesis inhibitor (Anderson *et al.*, 1996;

Lee and Wurster, 1995; Scott and Dawson, 1995; Bicknell *et al.*, 1994; Schweighoffer *et al.*, 1993; Kochi *et al.*, 1993).

6.3.8 Colchicine

Colchicine (**63**) was isolated from corms or seeds of *Colchicum autumnale* Linne (Liliaceae) and was also reported to be present in many other species of Liliaceae plants (Boit, 1961; Wildman, 1960).



	R_1	R_2	R_3	R_4	R_5
Colchiceine (**62**)	CH_3	CH_3	CH_3	H	CH_3CO
Colchicine (**63**)	CH_3	CH_3	CH_3	CH_3	CH_3CO
N-Methyl-*N*-deacetylcolchicine (**64**) (DMC)	CH_3	CH_3	CH_3	CH_3	CH_3
N-Deacetyl-colchicine (**65**) (TMCA)	CH_3	CH_3	CH_3	CH_3	H

Isolation

Colchicine and related alkaloids may be isolated from the corms of *Colchicum autumnale*. The viscous oily alcoholic extract obtained from 20 kg of dried powder was diluted with 4 liters of water, and this was extracted exhaustively with ether. The combined ether extract was extracted in sequence with H_2O (A) and then with 3% HCl (B).

The fraction (A) was acidified with dilute HCl to pH 2–3 and then extracted with chloroform (80 ml × 6). The combined chloroform extract was evaporated to dryness to yield 48 g (Aa); this was redissolved in a minimum quantity of ethanol and to this solution an adequate quantity of NaCl was added to salt out apigenin (**61**) (24.7 g) from solution.

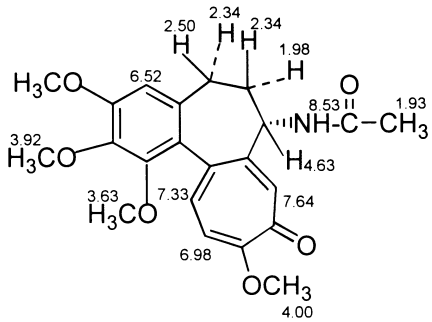
The mother liquid from isolation of apigenin (**61**) was extracted with chloroform (200 ml × 5), and after concentration, crude colchicine (**62**) was crystallized out. The mother liquid, after crystallization of **62**, was evaporated to dryness and was chromatographed on aluminum oxide and eluted with ether–chloroform of increasing polarity. This yielded colchicine (**63**) along with *N*-deacetylcolchicine (substance B, TMCA) (**65**) and other minor alkaloids. The total yield of colchicine was 32 g after recrystallization from EtOAc-ether. The water layer (Ab), which, after separation of Aa, was made alkaline with dilute NH_4OH and then extracted by chloroform, yielded 1.5 g of *N*-methyl-*N*-deacetylcolchicine (DMC) (**64**)

after concentration. The mother liquid, after crystallization of DMC, was evaporated to dryness, chromatographed (Al_2O_3) and yielded, in sequence, a further quantity (5 g) of DMC and other minor alkaloids (Wildman, 1960).

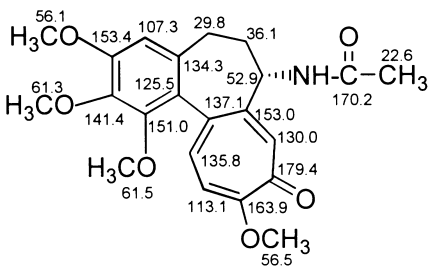
The dilute HCl-extract B was neutralized with NH_4OH, and extracted with chloroform; a further quantity (5.42 g) of **64** was obtained by Al_2O_3 chromatography. Colchicine, recrystallized from EtOAc, has a molecular formula of $C_{22}H_{25}O_6N$. (MP 155°C, [α] −119.9 (in chloroform)).

Structural elucidation

The structure of colchicine has been elucidated from various spectral data. Thus, the ^{13}C NMR and ^{1}H NMR data (**63a** and **63b**) provided the unambiguous evidence to support the structural assignments (Sedmera *et al.*, 1979; Freyer *et al.*, 1987).



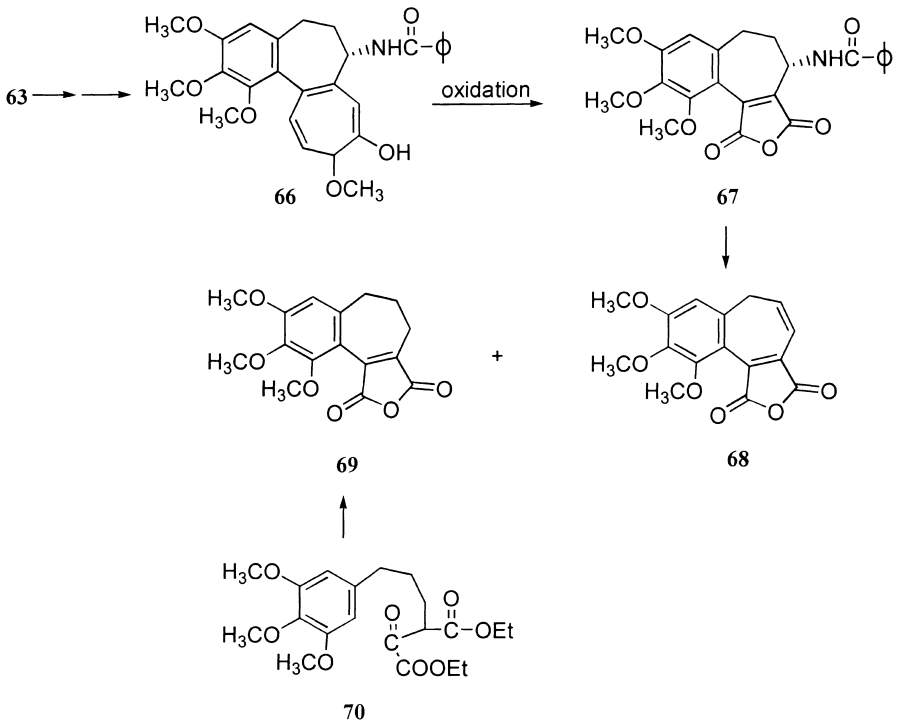
63a



63b

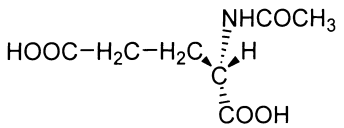
Colchicine and its analogues have an unusual tricyclic structure containing a tropolone ring. The aromatic ring A and seven-membered ring B structure was confirmed by synthesis of a degradation product **69** of colchicine (**63**) (Scheme 6.14) (Horning *et al.*, 1950; Koo, 1953). The final proof of the seven-membered tropolone structure of ring C with the assignment of the carbonyl and methoxyl groups at C-9 and C-10, respectively, in colchicine was arrived at by catalytic reduction to *N,N*-dimethylaminocolchicide (**71**) to tetra-and hexahydrodemethoxylcolchicine (**72** and **73**, respectively) (Scheme 6.15) (Rapoport *et al.*, 1954).

Hydrolysis of **71** produced colcheicine **62** and the sequence of reactions proved that the $N(CH_3)_2$ and C=O groups occupied the same two positions of ring C as that occupied by the OCH_3 and C=O groups in colchicine. Dimethylaminocolchicide **71** absorbed 3 moles of hydrogen rapidly and then, more slowly, added 2 moles of H_2 to yield, in sequence, **72** and **73**. Hydrogenation of colchicine **63** also gave **73**. The sequence of reactions that proved the C=O groups of these compounds are in the same position. The positions of ring C double bonds of **72** and **73** and the basic ring skeleton



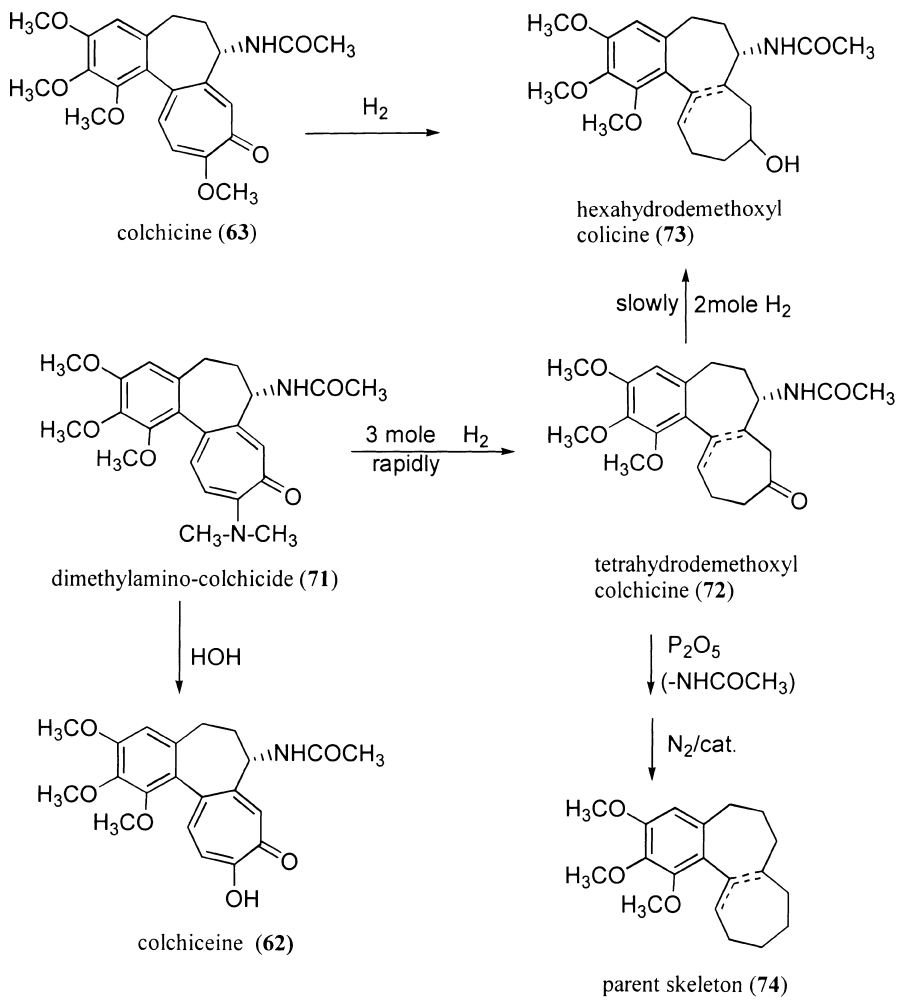
Scheme 6.14: Chemical elucidation of ring A and B.

compound **74** may be assigned to 7a,12a or 12,12a in conjugation with aromatic ring A. Both assignments are compatible with the UV and IR spectra. The absolute configuration of C_7 was elucidated by oxidation of colchicine to yield *N*-acetyl-L-glutamic acid (**75**) and the stereochemistry of C_7 of colchicine (**63**) is thus related to D-glyceraldehyde (Corrodio and Hardegger, 1955).



N-acetyl-L-glutamic acid (**75**)

One of the unambiguous proofs of colchicine structure represented as formula **63** is achieved by Van Tamelen and his group (van Tamelen *et al.*, 1959, 1961) by complete synthesis. Excellent reviews are available (Capraro and Brossi, 1984; Kato *et al.*, 1974; Evans *et al.*, 1978, 1981).



Scheme 6.15: Elucidation of tropolone ring-C.

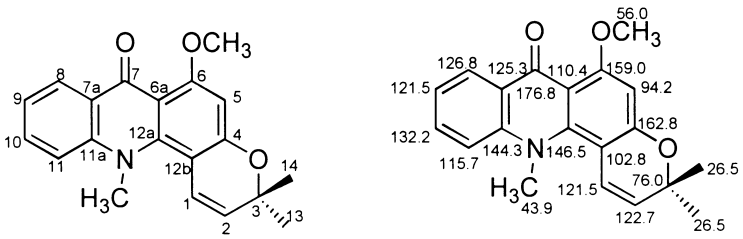
Pharmacology

Colchicine has long been known for its antitumor activity. However, it is not used clinically for this purpose, due to its high toxicity. In cancer research, colchicine is widely employed as a reference standard for evaluating if other drugs have a similar mechanism of anti-microtubule activity (Lin *et al.*, 1988).

Demecolchicine (*N*-deacetyl-*N*-methylcolchicine) (DMC, **64**) is now used clinically in cancer therapy for myelocytic leukemia (Scott, 1957; Spiers *et al.*, 1975). The result of clinical trials of *N*-deacetylcolchicine (TMCA, **65**) indicated it might be of value in melanoma and chronic granulocytic leukemia (CGL) (Stolinsky *et al.*, 1967). Further, the

binding of colchicine to tubulin results in positive enthalpy and entropy changes, and a relatively large favorable free-energy change suggesting that the binding site is located in a hydrophobic or nonpolar pocket (Bryan, 1972).

6.3.9 Acronycine



(**76**) Acronycine

Acronycine is a hemiterpene acridone alkaloid that was first isolated from *Acronychia baueri* Schott (Rutaceae), and was selected as a candidate for a phase I clinical trial (Hughes *et al*., 1948; Gerzon and Svoboda, 1983; Svoboda *et al*., 1966). Most interesting is its activity against numerous solid tumors including sarcoma, myeloma, carcinoma, and melanoma (Suffness and Cordell, 1985; Dorr *et al*., 1989). Acronycine is a broad-spectrum antitumor agent whose development as a clinically useful agent has been hindered, partly due to its poor solubility characteristics. Early clinical studies of acronycine by oral administration gave rise to significant nausea and vomiting as the major toxicity (Scarffe *et al*., 1983).

Isolation and structural elucidation

The major and active alkaloid, acronycine, was isolated first from *A. baueri* in 1948. A hemiterpene acridone alkaloid from ground bark of the above plant was extracted by stirring with two portions of n-hexane. Concentration *in vacuo* produced a nondrying oil which was dissolved in ether and chilled and ethylene dichloride and HCl were added to produce a red-orange amorphous hydrochloride. Suspending the salt in H_2O and extracting with ethylene dichloride obtained amorphous free bases. After crystallization from acetone, three crops of crystals were yielded. Recrystallization of the second crop and the third crop from hot methanol yielded normelicopidine and acronycine, respectively. The separation of normelicopidine from acronycine was then made, since the former is quite insoluble in methanol (Svoboda *et al*., 1966).

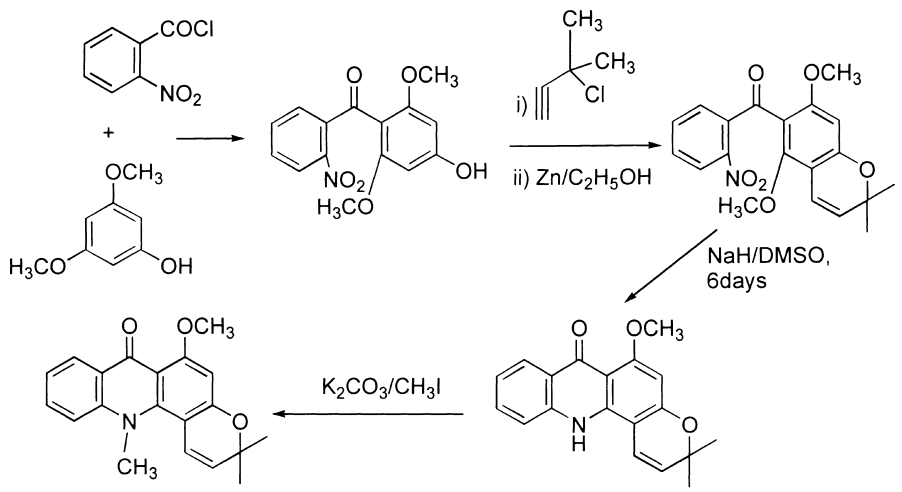
Acronycine is an acridone alkaloid with an additional hemiterpene unit attached at C-4 of the parent nucleus and cyclized to form a pyran ring. Its structure was deduced from chemical and spectroscopic evidence.

The complete correct structure of acronycine was not confirmed by spectroscopic elucidation (UV, IR, MS, NMR) until 1966. The ^{1}H-NMR data of acronycine have been

reported as follows: ($CDCl_3$) δ 1.52 (6H, s, $-C(CH_3)_2$), 3.77 (3H, s, $-NCH_3$), 3.96 (3H, s, $-OCH_3$), 5.47 (1H, d, J = 9.7 Hz, H-2), 6.30 (1H, s, H-5), 6.50 (1H, d, J = 9.7 Hz, H-1), 7.06–7.69 (3H, m, H-9, H-10 and H-11), and 8.37 (1H, dd, J = 1.5, 7.5 Hz, H-8). Proof of the angular structure for acronycine was also established by an X-ray crystallographic analysis of 5-bromo-1,2-dihydroacronycine which showed that the parent compound had the 3*H*-pyrano[2,3-c]-7-one nucleus (Gougoutas and Kaski, 1970).

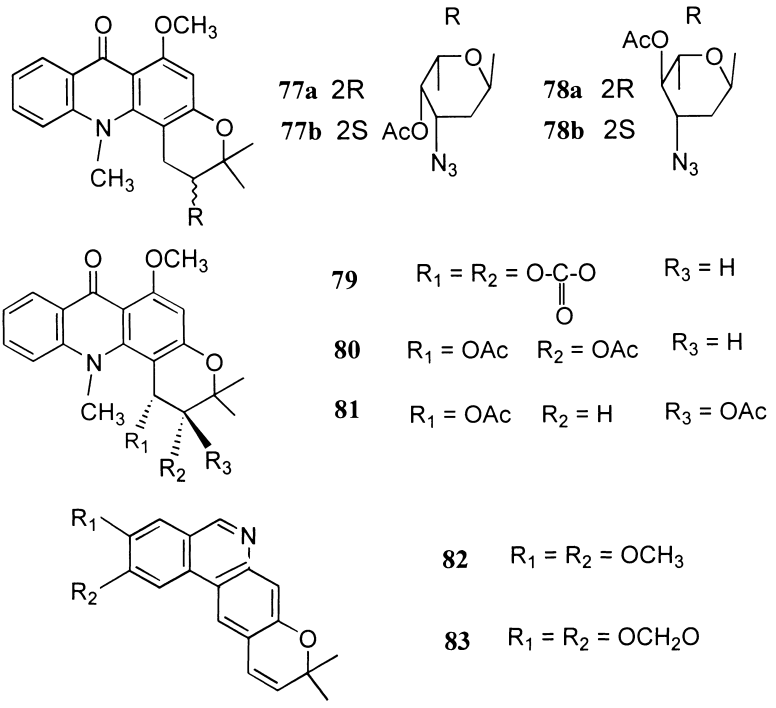
Synthesis and structure–activity relationships (SAR)

Acronycine (**76**) can be synthesized (Suffness and Cordell, 1985; Adams *et al.*, 1976; Adams *et al.*, 1981) by Friedel-Crafts condensation of 2-nitrobenzoyl chloride and 3,5-dimethoxyphenol.



Modification of the structure of acronycine aimed to increase its water solubility, thus Mitaku *et al.* (1996) combined the acronycine pharmacophore with various sugar units. In addition to elevating water solubility, this produced derivatives with differing bioavailability and selective toxicity towards tumor cells. Thus, a series of 2-hydroxy-1,2-dihydroacronycine glycosides were synthesized by condensation of the racemic aglycone with appropriate glycoside donors. Compounds **77a**, **77b**, and **78a**, **78b**, bearing a 2,3,6-trideoxy-3-azido-L-*lyxo*- and L-*arabino*-hexopyranose unit, respectively, were significantly more potent than acronycine in inhibiting cell proliferation.

In addition, several reports, including the studies of SAR in the acronycine series, were published (Elomri *et al.*, 1996; Magiatis *et al.*, 1998; Razafimbelo *et al.*, 1998). Among these papers, Elomri *et al.* (1996) reported that *cis*-1,2-di-*O*-carbonyl-1,2-dihydroxy-1,2-dihydroacronycine (**79**) was 75-fold more potent than acronycine in inhibiting L1210 cell proliferation, and *cis*-1,2-diacetoxy-1,2-dihydroacronycine (**80**) was 16-fold more active than acronycine.



Magiatis *et al.* (1998) obtained the esters of *trans*-1,2-dihydroxy-1,2-dihydroacronycine. Permanganate oxidation of acronycine (**76**) led to the keto alcohol which could be reduced to *trans*-1,2-dihydroxy-1,2-dihydroacronycine using $NaBH_4$. These ester compounds were more potent than acronycine when tested against L-1210 cells *in vitro*. The diacetate compound *trans*-1,2-diacetoxy-1,2-dihydroacronycine (**81**) was evaluated *in vivo* against murine P-388 leukemia and was markedly active at a dose 16-fold lower than acronycine itself (Magiatis *et al.*, 1998). Following the above discovery, the studies of SAR concluded that the biological activities of the acronycines were in the order 1,2-hydroxy < 1,2-esters < diacetates. An additional paper by Razafimbelo *et al.* (1998) described the syntheses of the potent active analogues of acronycine, 2,3-dimethoxy- (**82**) and 2,3-methylenedioxy-9,9-dimethyl-9H-pyrano[3,2-*b*]phenanthridine (**83**).

Pharmacology

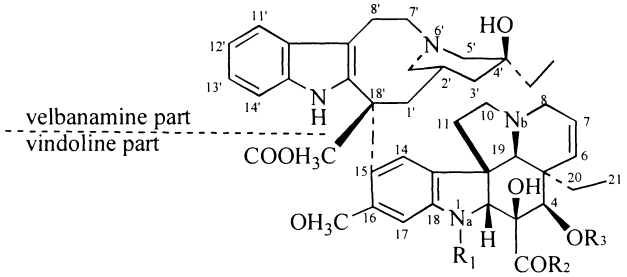
The mechanism of action and antiumor activity of acronycine have been partially reviewed (Funayama *et al.*, 1983; Shieh *et al.*, 1992). It has the ability to inhibit the incorporation of uridine, cytidine, and other nucleosides that lead to the inhibition of nucleoside transport across plasma membranes. Acronycine and its derivatives exhibited cytotoxic activity with several human tumor cell lines (breast, colon, lung, melanoma, KB-3, and drug-resistant KB-V1), and murine tumor cells (L1210, HT29, and P-388)

(Mitaku *et al.*, 1996; Elomri *et al.*, 1996; Magiatis *et al.*, 1998; Razafimbelo *et al.*, 1998).

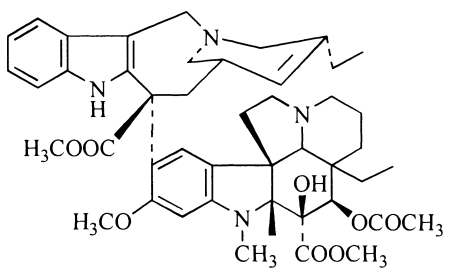
Further investigation of acronycine and **83** demonstrated that these two drugs could induce a partial accumulation of cells in the G2 + M phase of the cell cycle (Magiatis *et al.*, 1998; Razafimbelo *et al.*, 1998). In early clinical studies of acronycine by oral administration, one of 16 patients had a clear response for 72 weeks and two of them showed little improvement. The drug therefore showed minimal activity in multiple myeloma (Scarffe, 1983).

6.3.10 Vincristine and vinblastine

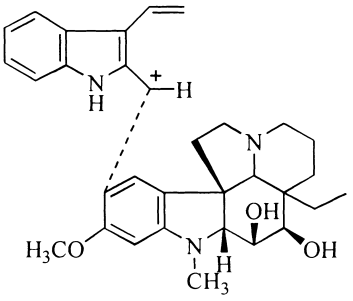
Vincristine (**84**) and vinblastine (**85**) are indole-indoline dimeric alkaloids isolated from *Catharanthus roseus* (L.) Gon. (Apocyanaceae) (Figure 6.12). The plant, commonly known as Madagascan periwinkle, has long been used as a hypoglycemic agent for the treatment of diabetes. In the course of evaluation of carbohydrate metabolism, periwinkle



84. Vincristine
R_1=CHO, R_2=OCH_3, R_3=CH_3CO
85. Vinblastine
R_1=CH_3, R_2=OCH_3, R_3=CH_3CO
86. Vindesine
R_1=CH_3, R_2=NH_2, R_3=H



87. Vinorelbine
(nor-7'-anhydrovinblastine, Navelbine)



88. m/z = 509

Figure 6.12: Vinca alkaloids and related compounds.

extract was found to be devoid of such effect. However, it was found to have granulocytopenic toxicity in laboratory animals. Guided by this toxic effect, Noble was able to isolate a new antileukemic drug, vincaleukoblastine (vinblastine, **85**) (Noble *et al.*, 1958, 1959). Independently, during the same period, Johnson and Svoboda (Johnson *et al.*, 1959; Svoboda *et al.*, 1962, 1975) also isolated vincaleukoblastine in addition to vincristine (**84**), from the same plant.

Through intensive structure–activity relationship studies on **84** and **85**, semisynthetic congeners vindesine (**86**) and vinorelbine (**87**) were developed. Compounds **85**, **86**, and **87** are now in clinical use (Mehta, 1998; Chabner *et al.*, 1996).

Isolation and structural elucidation

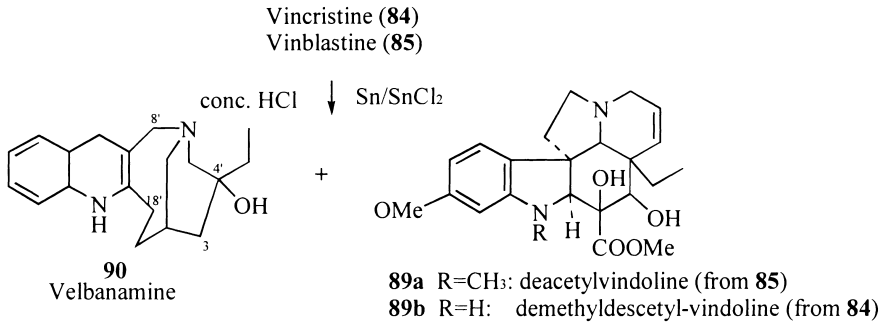
Isolation of vinblastine (85) (Noble *et al.*, 1959)

The dried *Vinca rosea* leaf powder (600 g) was extracted with EtOH–H_2O–AcOH (9:1:1) mixture; after evaporation, the dried extract was dissolved in 2% dilute hydrochloric acid and insoluble material was filtered off. The filtrate was then adjusted to pH 4 and extracted with benzene to yield benzene extractable Fr-A (1440 mg). The acidic water layer (pH 4) was neutralized with NH_4OH and again extracted with benzene to yield 1200 mg of Fr-B. The aqueous layer was adjusted to pH 8.5 and was further extracted with chloroform to yield Fr-C. The fractionation procedure was guided by using the depression in leukocyte count of normal rat peripheral blood taken from the tail of the test animal. The results showed that most of the active substance was concentrated in Fr-B. Thus Fr-B was purified by absorption chromatography [Adsorbent: Woehlem Al_2O_3 grade IV–V 120 g; eluent: start with Bz:CH_2Cl_2 = 65:35% (v/v), 2700 ml; add gradually 2500 ml of pure CH_2Cl_2 in linear gradient manner, finally the column is washed with alcohol]. The bioassay results indicated most leukopenic activity resided in the eluted fraction 9 (27.5 mg), which was purified by preparative TLC chromatography and vincaleukoblastine was crystallized as a sulfate. The analytical results of vincaleukoblastine indicated the empirical formula: $C_{24}H_{35}N_2O_7 \cdot 1/2H_2SO_4$ (melts with decomposition at 285–290°C; [α] −36.9; UV: λ_{max} 262 (log ϵ 3.69), 298 (3.58), 297 (sh., 3.55). IR:3450 (>NH); 1725, 1235 (C=O carbonyl and ester), 750 (disubstituted benzene)).

Structural elucidation

The structures of vincristine (**84**) and vinblastine (**85**) were elucidated by classical analysis coupled with spectral determinations and chemical reactions. The dimeric structures of vinblastine and vincristine were deduced from a reductive cleavage by Sn/$SnCl_2$ to give indole and indoline halves (Scheme 6.16).

In addition to NMR analysis, the positions of attachments of indole-indoline halves of the VLB molecule were discovered by the presence of the prominent *m*/*z* 509 fragment **88** (Figure 6.12) of the mass spectrum of **85**. The final establishment of the



Scheme 6.16: Reductive cleavage of **84** and **85**.

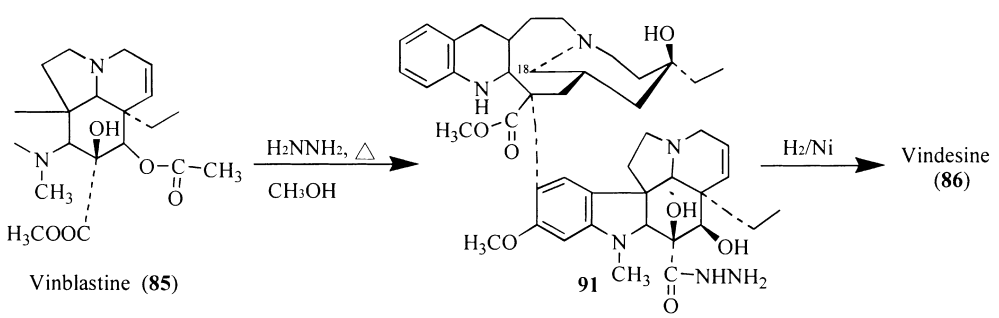
positions of attachment of the two parts at C-15 of vindoline and C-18′ of velbanamine was established by using DCl-Sn/$SnCl_2$ reductive cleavage in the same reaction as indicated in Scheme 6.16. Combined mass and NMR evidence of the degradation products thus confirmed the attachment of C-15, C-18′.

Synthesis

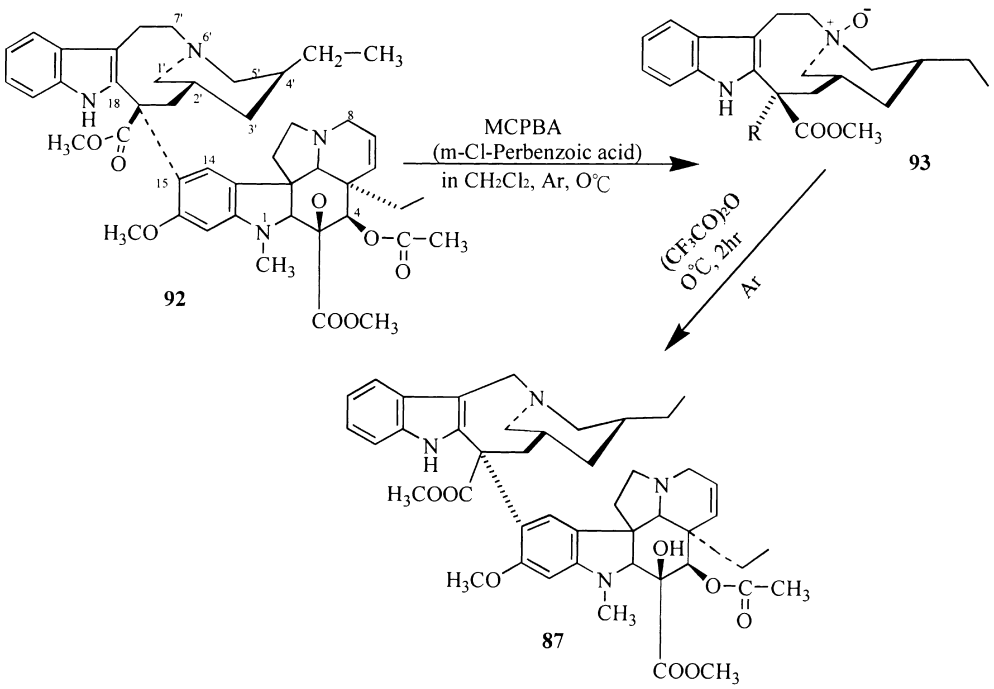
Structure modification of vinblastine (**85**) yielded two new anticancer drugs: vindesine (**86**, deacetylvinblastine amide, VDS) (Scheme 6.17) (Barnett *et al.*, 1978) and vinorelbine (**87**, 3′,4′-dehydro-7′-nor-vinblastine) (Scheme 6.18) (Mangeney *et al.*, 1979b). Both **86** and **87** are now in clinical use.

Vindesine (**86**) was synthesized by preferential hydrazinolysis of the C-3 ester of the vindoline moiety of VLB (**85**), followed by hydrogenolysis of the resulting deacetyl vinblastine hydrazide (**91**) to yield vindesine (Scheme 6.17).

Vinorelbine (**87**) is nor-7′-3′,4′-anhydrovinblastine obtained by applying modified Polonovski reaction on anhydrovinblastine $N_{6'}$-oxide (**93**) resulting from the action of



Scheme 6.17: Synthesis of vindesine (**86**).



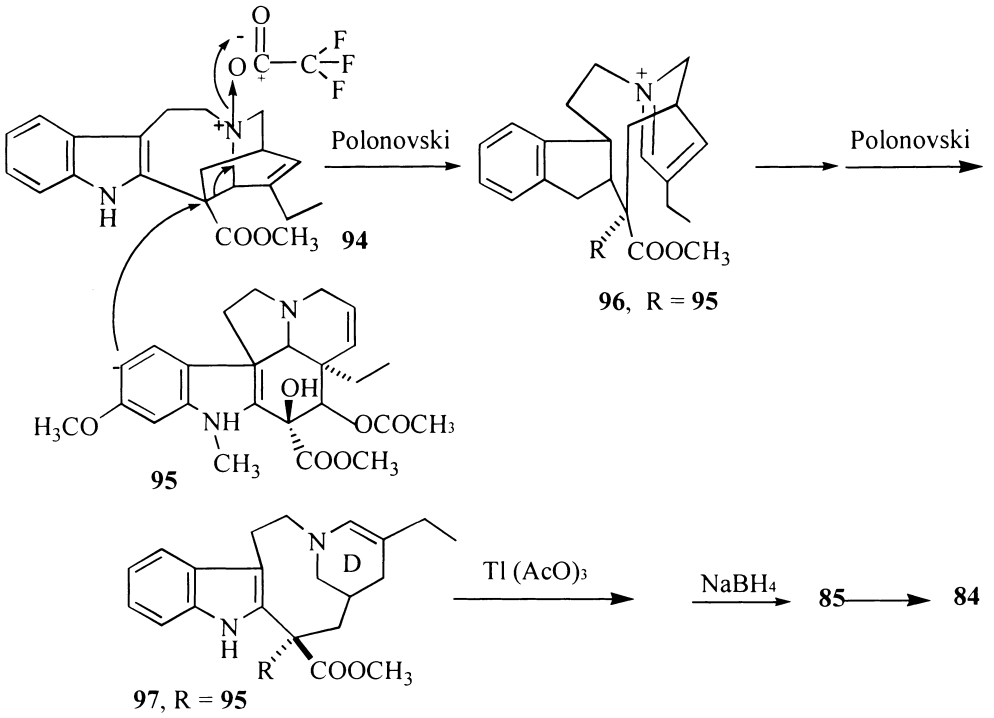
Scheme 6.18: Synthesis of vinorelbine (**87**).

m-chloroperbenzoic acid (MCPBA) on anhydrovinblastine (**92**) (Mangeney *et al.*, 1979a) (Scheme 6.18).

Synthesis of vinblastine and vincristine was achieved by Mangeney (1979b) and Richter (1975). Vinblastine (**85**) has been synthesized by the coupling of $N_{6'}$-oxide of catharanthine (**94**) and vindoline (**95**) through a modified Polonovski reaction. The resultant immonium salt **96** was reduced and passed through a second Polonvski reaction leading to enamine **97**. Stereoselective oxidation by thallium oxide of this unstable compound **97** gave axial −OH with natural stereochemisty as in vinblastine (**85**) (Scheme 6.19).

It has been reported that VLB can be converted to vincristine by *N*-demethylation followed by formylation of vindoline-N^a (Neuss *et al.*, 1974; Brannon *et al.*, 1975). Therefore, the synthesis of vinblastine is also a formal synthesis of vincristine (**84**).

Anhydrovinblastine (**98**) has been reported to be obtained biologically from vindoline (**95**) and cantharanthine (**94**) by incubation with cell free extract of *C. roseus* (Stuart *et al.*, 1978). Compound (**98**) can be converted to vinblastine by incubation with cell-free homogenates of *Catharanthus roseus* suspension culture (McLauchlan *et al.*, 1983).



Scheme 6.19: *Synthesis of vinblastine (**85**) and vincristine (**84**).*

Pharmacology

Vinca alkaloids, like colchicine and podophyllotoxin, are tubulin inhibitors. These agents bind specifically to tubulin during the cell cycle, blocking the polymerization of tubulin into microtubules, thus blocking spindle formation and arresting cells in metaphase. When cells are incubated with vinblastine, dissolution of microtubules occurs and high regular mole to mole tubulin and vinblastine crystals are formed. Cells undergo changes characteristic of apoptosis (Smets, 1994).

Vinblastine, vincristine and vindesine are used to treat the acute leukemias, lymphomas, and breast and lung cancer. Vinorelbine has recently been introduced for advanced breast cancer and for advanced non-small cell lung cancer (Mehta, 1998; Chabner *et al.*, 1996).

6.3.11 Maytansine

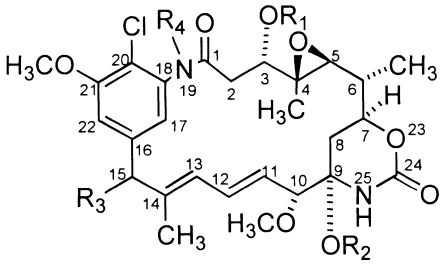
Maytansine (**99**) (Figure 6.13) is the first ansa macrolide with significant antitumor activities originally isolated from *Maytenus serrata* (Hochst. ex A. Rich) R. Wilczek (*M. ovatus* Loes) (Celastraceae) guided by bioassay against KB (*in vitro*) and P-388 leukemia in

mice tumor systems (Kupchan *et al.*, 1972a). Its broad spectrum of antitumor activity, very high potency, and favorable therapeutic index in animal tumors have stimulated the search for the other rich plant sources of maytansine (Table 6.2).

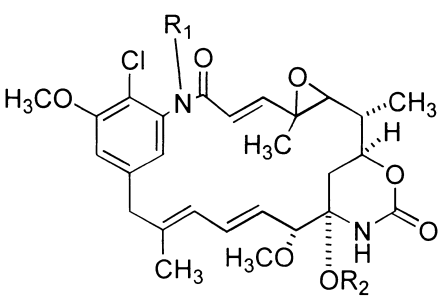
Isolation

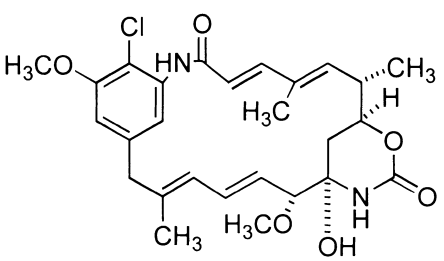
Maytansine (**99**) was originally isolated from *M. ovatus* guided by bioassay methods in a tedious process (Kupchan *et al.*, 1972a). An efficient isolation procedure using *M. buchananii* was therefore established to supply sufficient quantities for clinical trials (Kupchan, 1977; Suffness, 1979).

The alcohol extract (107 kg) from 2728 kg of powdered wood (with bark) of *Maytanus buchananii* was partitioned by vigorous stirring between EtOAc and H_2O (240 liters:180 liters)



99. maytansine R_1=CO.CHCH$_3$.NCH$_3$.COCH$_3$; R_2=H, R_3=H, R_4=CH$_3$
100. maytanprine R_1=CO.CHCH$_3$.NCH$_3$.COCH$_2$CH$_3$; R_2=H, R_3=H, R_4=CH$_3$
101. maytanbutine R_1=CO.CHCH$_3$.NCH$_3$.COCH(CH$_3$)$_2$; R_2=H, R_3=H, R_4=CH$_3$
102. maytanvaline R_1=CO.CHCH$_3$.NCH$_3$.COCH$_2$CH(CH$_3$)$_2$; R_2=H, R_3=H, R_4=CH$_3$
103. maytansinol R_1=R_2=R_3=H; R_4=CH$_3$
104. maytanacine R_1=CO.CH$_3$; R_2=R_3=H; R_4=CH$_3$
105. 3-bromopropyl -maytansine R_1=CO.CHCH$_3$.NCH$_3$.COCH$_3$; R_2=-CH$_2$CH$_2$CH$_2$Br; R_3=H, R_4=CH$_3$





106. maysine R_1=CH$_3$, R_2=H
107. normaysine R_1= R_2=H

108. maysenine

Figure 6.13: Natural and semisynthetic maytansinoids.

TABLE 6.2
Natural occurrence of maytansine

Plant Name	Plant Part[a]	Isolated Content (mg/kg)	References
Maytenus ovatus (Celastraceae)	fr-rt-st	0.2–0.3	Kupchan, 1972a, 1975a,b, 1977a,b
M. buchananii (Loes)	st-bk	1.5	Kupchan, 1975a, Kupchan *et al.*, 1977
R. Wilczek		2.5	Suffness and Douros, 1979
M. diversifolia (Gray) Hou	st	0.374	Lee *et al.*, 1982
M. acuminata	st	0.13	Suffness and Douros, 1979
M. confertiflora Luo et Chen	st		Wang *et al.*, 1981
M. emarginata	rt-st-lf	0.5	Suffness and Douros, 1979 Kuo *et al.*, 1990
M. heterophylla	st	0.3	Suffness and Douros, 1979
M. mossambicensis	st	0.05	Suffness and Douros, 1979
M. nemorsa	st-lf	0.01	Suffness and Douros, 1979
M. polycantha	st-bk	0.08	Suffness and Douros, 1979
M. senegalensis	st	0.05	Suffness and Douros, 1979
M. rothiana	rt-st-lf	5.6	Suffness and Douros, 1979
Putterlickia pyracantha	st-lf	1.8	Suffness and Douros, 1979
Szyszyl.	st-bk	4.5	
(Celastraceae)	fr	5.5	
P. verrucosa	st	12.3	Kupchan, 1975b, 1977
	st	6.6	Suffness and Douros, 1979
	st	7.5	Suffness and Douros, 1979

[a] fr = fruit, rt = root, st = stem, bk = bark, lf = leaf.

for 8 hours. After sedimentation was complete, the supernatant liquids were decanted and filtered. The solid sediment was treated again with EtOAc:H_2O (120:60 liters each $\times$3). The liquid filtrates were combined and the layers were separated. The aqueous layer was extracted with EtOAc (60 liters each $\times$3). EtOAc layers, containing maytansine, were combined (840 liters) and washed thoroughly with 5% dilute NaOH solution (240 liters, then 60 liters each $\times$4) to eliminate inactive acidic substances. The EtOAc layer was then washed again with 2N HCl (40 liters each $\times$2) to remove inactive basic components. The EtOAc layer was finally washed with 800 liters 4.2% NaOAc solution to eliminate any residue of hydrochloric acid and was then evaporated under vacuum to yield maytansine concentrated Fr-A (13.23 kg). Fr-A was treated with $(AcO)_2$-Pyr. (12.25:12.25 liters), stirring for 19 h. Then, the mixture was evaporated under reduced pressure to yield Fr-B. The acetylation procedure was aimed to render those constituents containing reactive OH groups less polar to facilitate later purification. Fr-B was partitioned between CCl_4 and 20% aqueous MeOH (60 liters:60 liters). The aqueous methanol layer was again treated in the same manner with CCl_4 (24 liters $\times$4). The MeOH layers containing maytansine were combined (total ~208 liters) and diluted with an adequate amount of water (~36 liters) to adjust the final strength of H_2O–MeOH to 38:62 (v/v). This aqueous methanol solution

was then extracted with $CHCl_3$ (60 liters, followed with 24 liters ×4). The $CHCl_3$ extracts were combined and evaporated to dryness to yield Fr-C (1758 gm) — Fr-C contains almost all of the maytansine present in the 2728 kg of plant powder. The above process was guided by KB-cell culture assay. Fr-C (1758 g) was separated by column chromatography on silica gel (10 kg silica gel, col. diam = 4 in gradient eluent: isopropanol in CH_2Cl_2, at 80 psig at a rate of *ca.* 4.24 liters/h.). The fractions were monitored by TLC and/or HPLC. Maytansine rich fractions (eluted with 4.5% of isopropanol in CH_2Cl_2) were combined and evaporated to yield 15 g of crude maytansine (Fr-D). Fraction D (15 g) was rechromatographed under similar conditions using 1 inch diameter column and 10 ml of each fraction were collected. The maytansine containing fractions were combined to yield 2.11 g of pure maytansine after recrystallization using CH_2Cl_2–EtOEt (0.0001% yield based on the weight of the dried plant powder).

Structure elucidation

Maytansine (**99**) was assigned the molecular formula $C_{34}H_{46}ClN_3O_{10}$, m.p. 169–170°C, $[\alpha]_D^{20}$ −125°, (*c.* 0.056, $CHCl_3$) on the basis of elemental analysis and by electron impact mass spectrometry. The spectrum showed prominent peak *m/z* 630.26 $C_{33}H_{43}ClN_2O_8$ due to M^+-61 (H_2O + HNCO) and other major peaks at *m/z* 485 ($M^+-(H_2O$ + HCNO)$-R_1OH$), 470 (485−CH_3), 450 (185−Cl), 128 (R′OH—OH), 100 (128-COOH). Maytansine has the following functionalities as determined by IR: 1740 cm^{-1} (ester C═O), 1724 cm^{-1} (carbamate C═O), 1661 (lactone C═O), and 1570 (C═C). Maytansine was converted to 3-bromopropyl ester at C_9-OH (**105**). The structure of (**105**) was solved by heavy-atom X-ray crystallography and led to the structure assignment of formula (**99**) for maytansine. The absolute configurations of **99** were found to be 3*S*, 4*S*, 5*S*, 6*R*, 7*R*, 9*S*, 10*R* and 2′*S*. The structure was determined to have epoxide, carbinolamine and aryl halide functions. The H^1- and C^{13}-NMR assignments of **99** are summarized in Figure 6.14 (Kupchan *et al.*, 1972, 1977a,b; Bryan *et al.*, 1973).

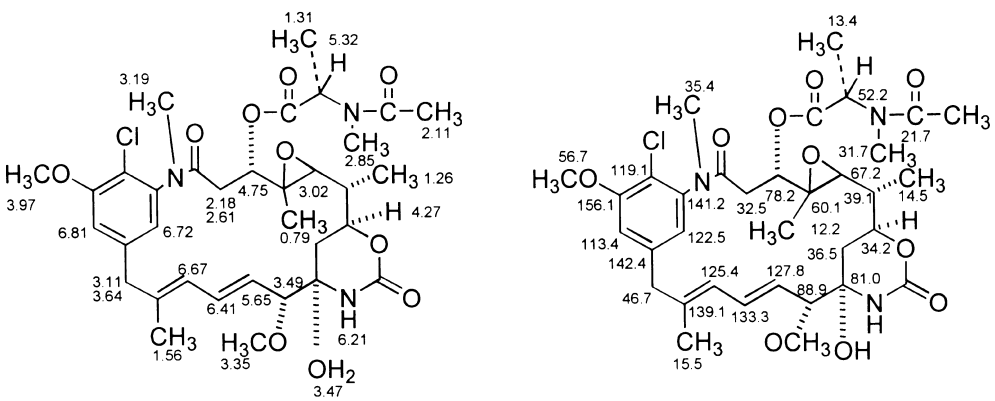
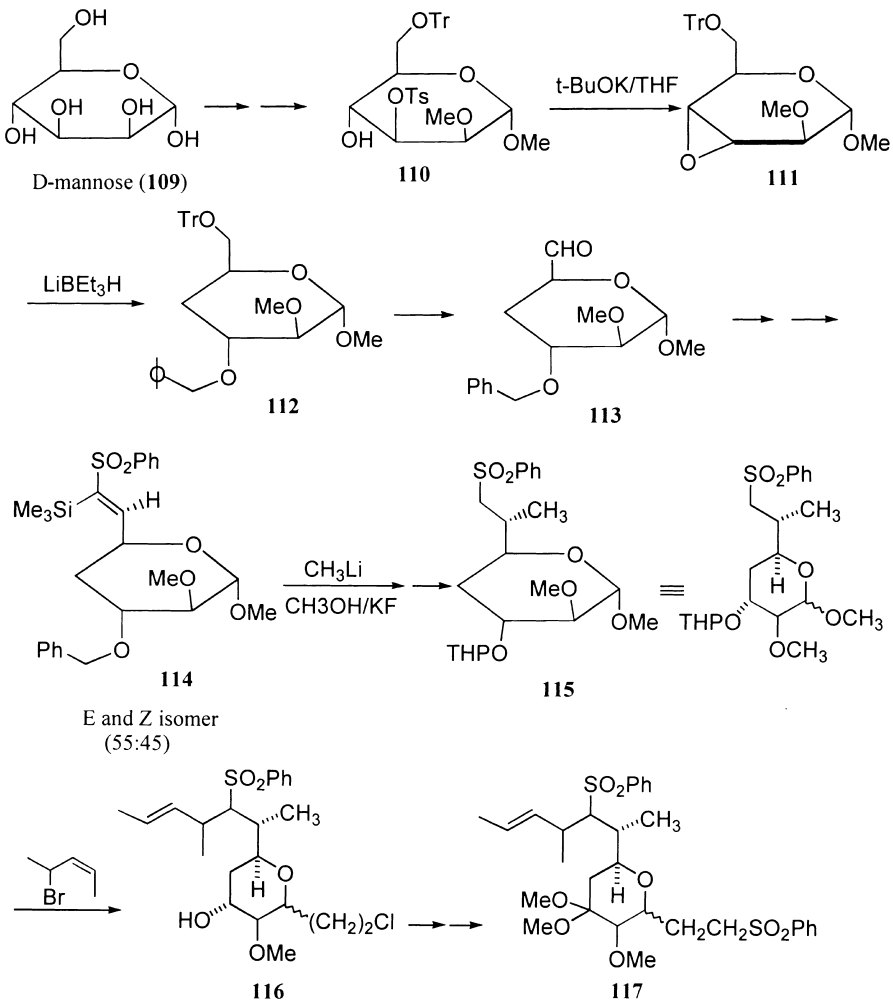


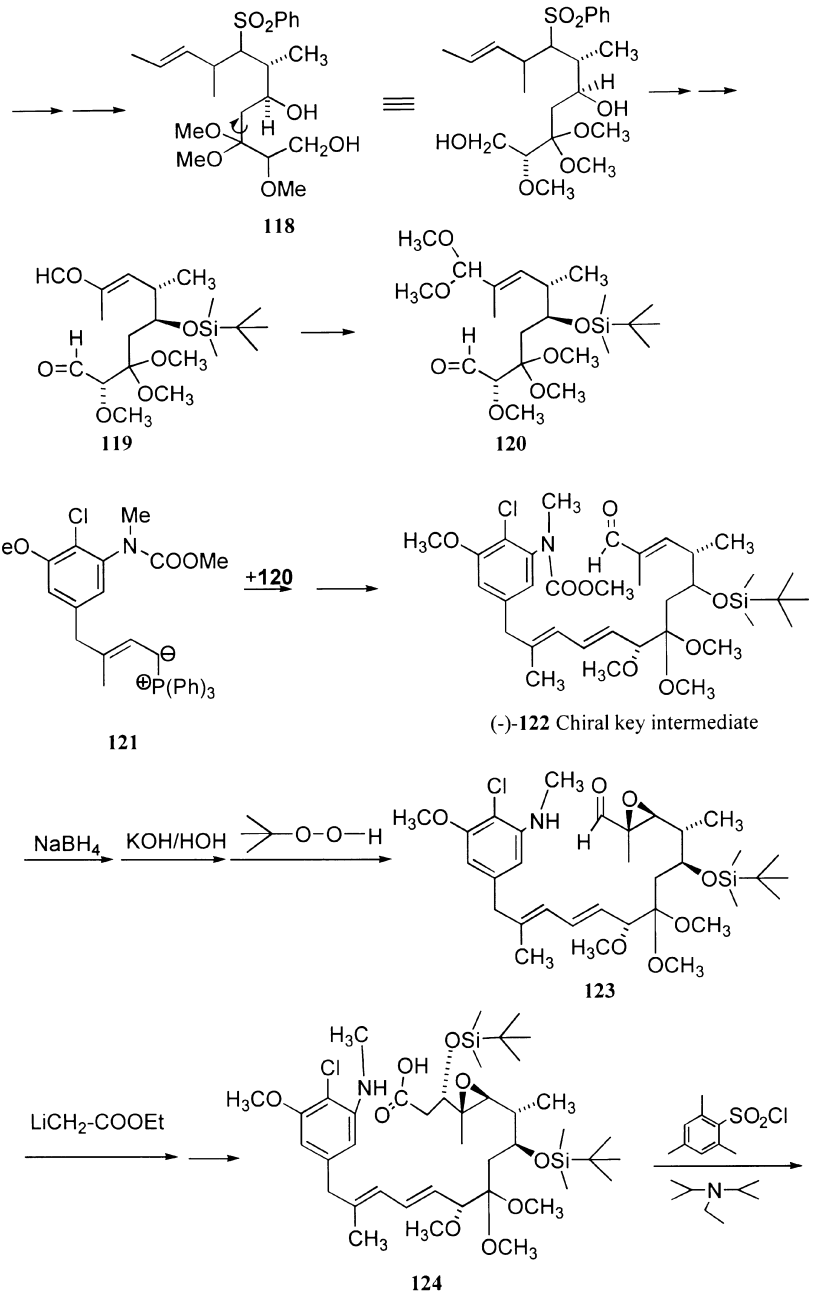
Figure 6.14: H^1 and C^{13}-NMR assignment of maytansine.

Total synthesis of maytansine

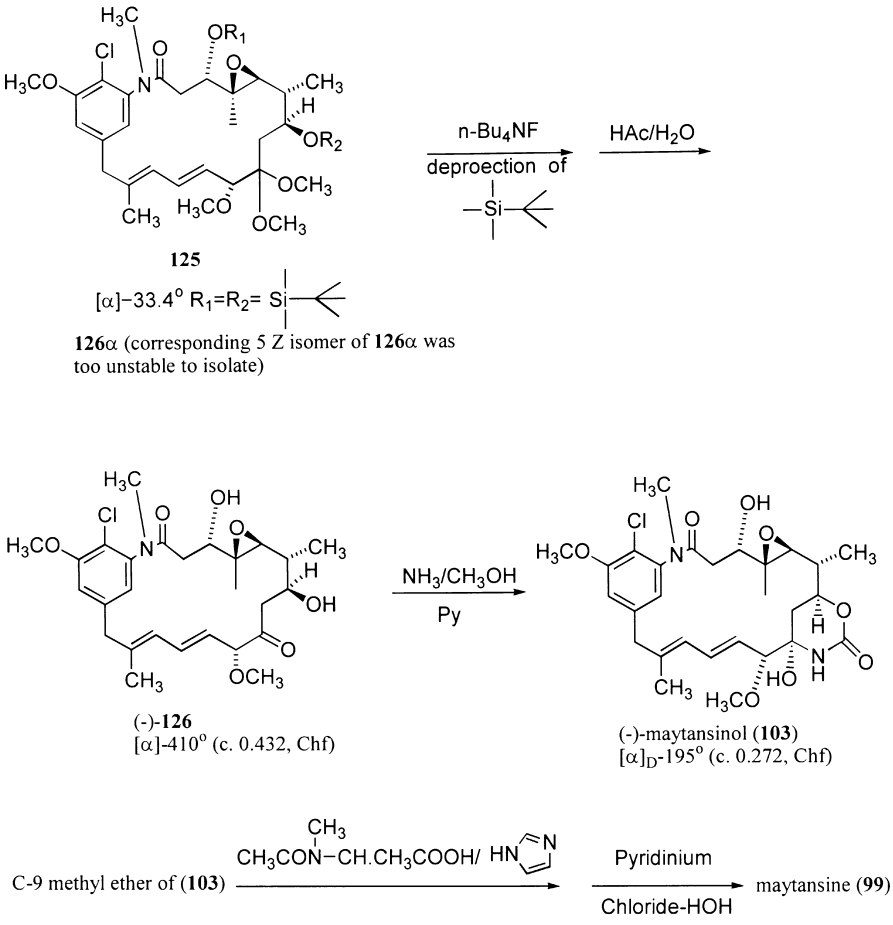
Maytansine (**99**) is an *N*-acetyl-*N*-methyl-L-alanine ester of maytansinol (**103**) (Figure 6.13). Maytansinol is a natural product, isolated in addition to **99**, from *Putterlickia verrucosa* (Kupchan, 1975a). Chiral (−)-maytansinol has been synthesized stereoselectively through chiral key intermediate (−)-**122** obtained by Wittig reaction of aromatic fragment (**121**) (Kitamura *et al.*, 1984b) and the chiral fragment **120** derived from D-mannose (**109**). In the series of reactions D-mannose (**109**) served as the chiral starting material and as chiral template for the induction of the asymmetric centers. The conversion of maytansinol (**103**) to maytansine (**99**) has been reported in the literature (Corey *et al.*, 1980; Kitamura *et al.*, 1984a) (Scheme 6.20).



Scheme 6.20: Total synthesis of maytansine.



Scheme 6.20: (*contd*).



Scheme 6.20: (*contd*).

Pharmacology

Maytansine is an exceptionally interesting antineoplastic agent with a very broad spectrum and a favorable therapeutic index at μg/kg effective dose ranges (Table 6.3).

The structure–activity relationship of maytansine (**99**) and natural and semi-synthetic maytansinoids have been evaluated (Kupchan *et al.*, 1972c, 1974, 1975b, 1977b, 1978); the results indicated the following:

1 C-3 ester function is essential for antileukemic activity against P388 lymphocytic leukemia in mice and cytotoxicity in KB cell culture test. Compounds **103** (maytasinol), **106** (maysine), **107** (normaysine) and **108** (maysenine) are all devoid of the

TABLE 6.3
Maytansine *in vivo* antitumor activity (Suffness, 1979)

Tumor	Dose (μg/kg)	ILS(%)[a]
B16 melanoma	16	59
Colon 26	64	31
L1210 leukemia	180	49
Lewis Lung	32	32
P388 leukemia	256	14

[a] Increase in life span in test group vs. control group.

said functional group and are thus inactive in the *in vivo* P388 system as well as in KB cell culture.

2 The alcoholic function of C_9 carbinolamide has to be free. Blockage of C-9 OH, as in the case of 3-bromopropylmaytansine (**105**), resulted in reduction of antileukemic activity and cytotoxicity.

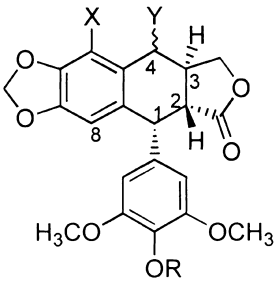
3 It has been suggested that C-9 free carbinolamide acting as an alkylation group is involved in the antitumor activity of maytansine (**99**).

The results of clinical trials of maytansine (**99**) are not very promising (Gastrointestinal Tumor Study Group 1985; Thigpen *et al.*, 1983a,b). However, recent development of targeted drug delivery research on **99** is encouraging. Folate-maytansinoid complex has been reported to display high cytotoxicity and remarkable selectivity on certain folate-receptor overexpressed carcinoma cells lines (Ladino *et al.*, 1997).

In addition, antibody–maytansine conjugate has recently been reported to show high antigen-specific cytotoxicity for cultured human cancer cells (IC_{50} = 10–40 pM) (Chari, 1992). Maytansine at 6×10^{-8} M inhibited cell division in the eggs of sea urchins and clams and caused the disappearance of mitotic apparatus, or prevented it from forming when added at an early stage (Remillard *et al.*, 1975). Maytansine is widely used as a reference compound, taxol and colchicine in cancer research (Jordan *et al.*, 1998).

6.4 PODOPHYLLOTOXIN AND ITS DERIVATIVES

Podophyllotoxin (**127**) (Figure 6.15) is a dimeric phenylpropanoid lignan isolated from *Podophyllum peltatum* L. (Berberidaceae) in 1880. The resin precipitated from the alcohol extract of the crude rhizoma by adding water is termed podophyllin. Podophyllin has been used as a cathartic and for local treatment of venereal warts. Both podophyllum rhizoma and podophyllum resin are offical in the US pharmacopoeia (USP 2000) and the fruit of *podophyllum emodi* Wall is official in PROC pharmacopoeia (PROCP, 1999) (for comprehensive review of early literature *see* Kelly and Hartwell, 1954). Podophyllotoxin



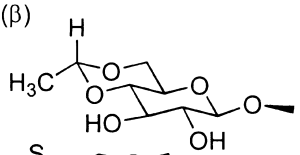
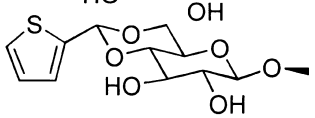
podophyllotoxin (**127**)	R=Me,	X=H,	Y=OH (α)	
α-peltatin (**128**)	R=H,	X=OH,	Y=H	
β-peltatin (**129**)	R=Me,	X=OH,	Y=H	
4'-demethylpodophyllotoxin (**130**)	R=H,	X=H,	Y=OH (α)	
epipodophyllotoxin (**131**)	R=Me,	X=H,	Y=OH (β)	
etoposide (**132**)	R=H,	X=H,	Y= (β)-	
teniposide (**133**)	R=H,	X=H,	Y= (β)-	

Figure 6.15: Podophyllotoxin and congeners.

was isolated as an antitumor substance guided by bioassay procedures against experimental tumor systems from *Podophyllum peltatum* (Hartwell *et al.*, 1948a,b, 1950, 1951; Nadkarni *et al.*, 1953) and from *Juniperus virginiana* L. (Cupressaceae) (Kupchan *et al.*, 1965). Podophyllotoxin (**127**) and some naturally occurring and semisynthetic congeners are listed in Table 6.4 among which the semisynthetic compounds etoposide (**132**) and teniposide (**133**) are now in clinical use (Chabner *et al.*, 1996; Mehta, 1998).

Isolation and structural elucidation

Podophyllin (podophyllum resin) from Podophyllum rhizoma

1000 g of podophyllum powder was extracted, exhaustively, by percolation with alcohol. The alcoholic extract was evaporated to syrupy consistency and was poured into 100 ml of ice-cold dilute HCl (water:conc. HCl = 1000:10) with constant stirring. The supernatant liquid was decanted and the precipitate was washed thoroughly with portions of water free from acid (Hartwell and Betty, 1950).

Podophyllotoxin from podophyllin

1000 g of dried podophyllin was shaken with 7000 ml of chloroform in separated fractions. The chloroform extracts were combined and evaporated to gummy consistency. It

TABLE 6.4
Some natural and semisynthetic podophyllotoxins

Name of compound	Sources	Reference
Podophyllotoxin (**127**)	*Podophyllum emodi* Wall	Von Warburg *et al.*, 1957 Hartwell *et al.*, 1952, 1953
	Podophyllum peltatum L. (Berberdiaceae)	Hartwell, 1948a; Hartwell and Detty, 1948b
	Juniperus virginiana L. (Cuperssaceae)	Kupchan and Hemingway, 1965
	J. lucayana	Hartwell *et al.*, 1953
	J. scopulorum	Hartwell *et al.*, 1953
	J. sabina var. *tamariscifolia*	Hartwell *et al.*, 1953
α-Peltatin (**128**)	*P. peltatum*	Hartwell, 1948a; Hartwell and Detty, 1948b, 1950
α-Peltatin glucoside	*P. peltatum*	Von Wartburg *et al.*, 1957
β-Peltatin glucoside	*P. peltatum*	Von Wartburg *et al.*, 1957
β-Peltatin (**129**)	*P. peltatum*	Hartwell, 1948a; Hartwell and Detty, 1948, 1950
4′-Demethylpodophyllotoxin (**130**)	*P. hexandrum* Royle (*P. emodi* Wall ex Royle)	Nadakarni *et al.*, 1952, 1953
4′-Demethylpodophyllotoxin glucoside	*P. peltatum*	Stoll *et al.*, 1954a Von Wartburg *et al.*, 1957
8-*O*-(β-D-glucopyranosyl)-β-peltatin	*P. emodi*	Stoll *et al.*, 1954c
	P. peltatum	Stoll *et al.*, 1954a, 1955
Podophyllotoxin-β-D-glucoside	*P. peltatum*	Stoll *et al.*, 1954 Von Wartburg *et al.*, 1957
	P. emodi Wall	Stoll *et al.*, 1954a,b
4′-Demethylepipodophyllotoxin glucoside	*P. emodi*	Stahelin *et al.*, 1991
Deoxypodophyllotoxin	*Thujopsis dolabrata* (L.) Sieb. & Zucc. (Cupressaceae)	Akahori *et al.*, 1972
	Bursera morelensis (Bruseaceae)	Jolad *et al.*, 1977
5′-Desmethoxy-deoxypodophyllotoxin	*Bursera morelensis*	Jolad *et al.*, 1977
3′-Demethylpodophyllotoxin	*Linum album* (Linaceae)	Weiss *et al.*, 1975
Silicicolin (1-desoxy-podophyllotoxin)	*Juniperns silicicola* (small) Baiky (Pinaceae)	Hartwell *et al.*, 1952, 1953
Epipodophyllotoxin (**131**)		Hartwell and Schrecker, 1951
Etoposide (**132**)		Pelsor *et al.*, 1978 Keeler-Juslen *et al.*, 1971
Teniposide (**133**)		Keeler-Juslen *et al.*, 1968, 1970

was then purified by column chromatography (alumina, benzene–absolute alcohol (1:1) (Hartwell and Delly, 1950).

The structure of podophyllotoxin was proposed in 1880 and 1932, and after fine revision it was shown to have structure **127** (Lee and Wang, 1995; Hartwell and Schrecker, 1958). A review of the *Podophyllum* compounds including the isolation and their chemical properties, structural determination (mainly basing on chemical reaction),

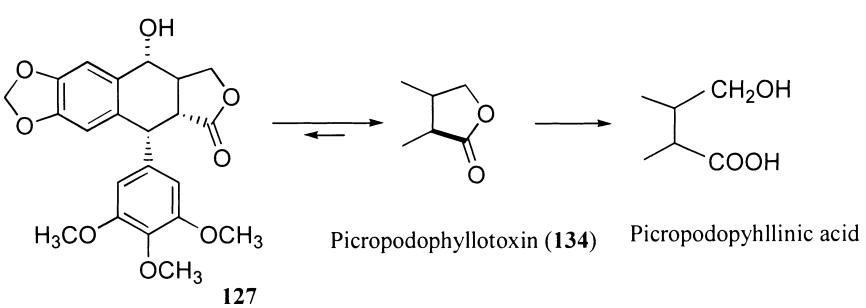
stereochemical configuration, and antitumor activity, is available (Hartwell and Schrecker, 1958). The antimitotic activity of podophyllotoxins was associated with their unique configuration at C-2, C-3, and C-4, with *trans*-fused γ-lactone ring. From X-ray analysis for the structural studies of one of these lignans, 5′-demethoxy-β-peltatin was first noted in 1972 (Bate and Wood, 1972).

Rithner *et al.* (1983) investigated the structural stability by dynamic NMR (DNMR) time scale at different temperatures, suggesting that the stable conformation of **127** was related to the E-ring and its substituents. This NMR evidence not only provided the exact chemical shifts of podophyllin lignans, but was also helpful for the explanation of the stereochemistry of the molecule (see Figure 6.15 and Scheme 6.21) (Brewer *et al.*, 1979). In comparison with the ^{1}H-NMR spectra of podophyllotoxin (**127**) and picropodophyllotoxin (**134**), the overlap signal of H-2 and H-3 at the γ-lactone ring in **127** in contrast to lower chemical shift of H-2 in **134** supported the *trans*-fused γ-lactone ring in the former, and the *cis* form in the latter. Also, the 4β hydroxy group in epipodophyllotoxin (**131**) has lower chemical shift than the 4α hydroxy group in **134**.

Synthesis and structure-activity relationships

In 1954, an isomer of podophyllotoxin glycoside with a *cis*-fused lactone, the picropodophyllotoxin glycoside, was found (Liu, 1989a). Gensler and Gatsonis (1966a) found that the epimerization of podophyllotoxin to picropodophyllotoxin (**134**) by mild catalysis (Scheme 6.21) decreased cytotoxicity, and they also achieved the total synthesis of podophyllotoxin in the same year (Gensler and Gatsonis, 1966b). In 1968, Kuhn and von Wartburg (1968) established the synthesis of podophyllotoxin-β-D-glucopyranoside. The glycosides of podophyllotoxin, α- and β-peltatin and 4′-demethylpodophyllotoxin were found to be 10^2–10^4 times less active when tested in tumor cell culture than corresponding aglycones (Jardine, 1980).

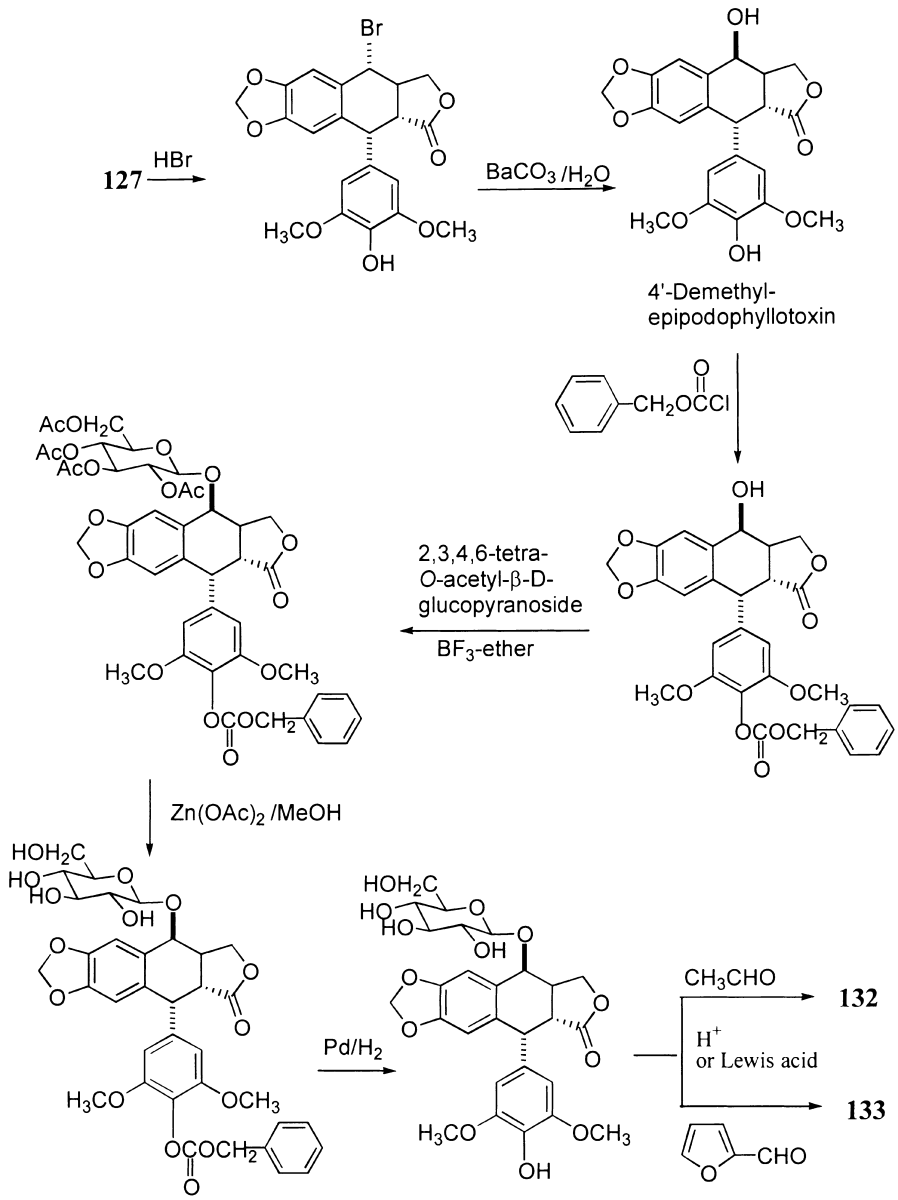
However, some epipodophyllotoxin glucoside derivatives are more active than aglycone. The most promising epipodophyllotoxin glucoside acetals are the benzylidene derivatives (**135**), acetaldehyde derivative (etoposide VP-16, **132**) and thiophen-2-



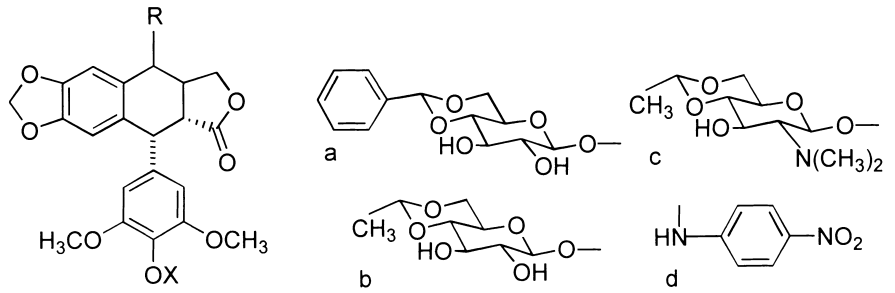
Scheme 6.21: The epimerization of podophyllotoxin (**127**) to **134**.

aldehyde derivative (teniposide VM-26, **133**) — of these three derivatives, VM-26 and VP-16 are in clinical use (Mehta, 1998; Chabner *et al.*, 1996).

Epipodophyllotoxin may be obtained from podophyllotoxin using hydrogen bromide and barium carbonate as the first step for the synthesis of **132** and **133** (Scheme 6.22) (Kuhn *et al.*, 1968, 1969a,b; Keller-Juslen *et al.*, 1971).



Scheme 6.22: Synthesis of etoposide (**132**) and tenoposide (**133**) from **127**.



	X	R
4′-demethylepipodophyllotoxin-benzylidene glucoside (**135**)	H	a
etoposide phosphate (**136**)	H_2PO_3	b
NK-611 (**137**)	H	c
GL-331 (**138**)	H	d

Although compounds **132** and **133** have been shown to be useful drugs through clinical trials, structural modification is still an important way to solve problems, including poor water solubility, myelosuppression, poor bioavailability, and drug resistance by tumor cells (van Maanen *et al.*, 1988). Hundreds of new derivatives have been synthesized, focusing not only on the inhibition of topo II but also on the enhancement of therapeutic index, extensive therapeutic spectrum, and higher water solubility. Several reviews on such studies are available (Wang *et al.*, 1997; Lee and Wang, 1995; Zhang and Lee, 1994; Terada *et al.*, 1993). To improve the water solubility, the etoposide phosphate (**136**) and NK-611 (4′-demethylepipodophyllotoxin-9(2-deoxy-2-dimethylamino-4, 6-*O*-ethylidene)-β-D-glucopyranoside (**137**) were produced by Bristol-Myers Squibb Co. and Nippon Kayaku, respectively (Wang *et al.*, 1997; Machida *et al.*, 1993). Etoposide phosphate (**136**) is a prodrug in plasma and will be hydrolyzed to etoposide by phosphatase within a few minutes (Sessa *et al.*, 1995). Also, compound **137** was shown to be less potent than **127** but more convenient in clinical medication. Furthermore, a new epipodophyllotoxin derivative, which contains a *p*-nitroanilino moiety at the 4β-position of etoposide (GL-331, **138**), was prepared and has encouraging pharmacological properties (Wang *et al.*, 1990).

Pharmacology

The mechanism of action of podophyllotoxin is similar to that of colchicine, having the ability to disrupt the assembly of microtubules and therefore producing metaphase arrest in dividing cells. It was first suggested that podophyllotoxin prevented the binding of colchicine, as tested with grasshopper embryo tubulin (Wilson and Friedkin,

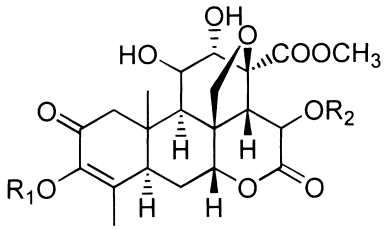
1967). Interestingly, in contrast to the arrest of cells in metaphase produced by podophyllotoxin, epipodophyllotoxin derivatives VP-16 (**132**) and VM-26 (**133**) can prevent cells from entering mitosis and arrest cells in the late S or G_2 phase of the cell cycle (Loike *et al.*, 1976a). Previous reports suggested that these epipodophyllotoxin compounds could not bind to microtubules but induced single-stranded breaks in DNA (Horwitz and Loike, 1977; Huang *et al.*, 1973; Loike and Horwitz, 1976b). Further investigations reported that VP-16 and VM-26 inhibit the catalytic activity of DNA topoisomerase II (topo II), the essential enzyme catalyzing the breakage of double-strand DNA in cell mitosis (Wang, 1985; Osheroff *et al.*, 1991; Hande, 1998). Topo II is also a constituent of the mitotic chromosome scaffold and has an essential role in the segregation of replicated DNA at mitosis (Earnshaw *et al.*, 1985; Holm *et al.*, 1985). It has been reported that the cytotoxicity of epipodophyllotoxin, VP-16 and VM-26 is due to the formation of a stable and irreversible covalent bond with DNA-topo II complex, the latter is a normal transient intermediate in the topo II catalytic cycle (Osheroff *et al.*, 1991; Hande, 1998; Earnshaw *et al.*, 1985; Holm *et al.*, 1985).

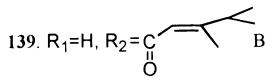
Owing to a lower toxicity than podophyllotoxin, VP-16 and VM-26 were approved by the US Food and Drug Administration (FDA) for the treatment of small-cell lung cancer, testicular cancer, melanoma, acute leukemia, Hodgkin's disease, non-Hodgkin's disease, gastric cancer, breast cancer, and ovarian cancer in 1984 and 1992 (Hande, 1996, 1998; Chabner *et al.*, 1996; Mehta, 1998). Recently, Sessa *et al.* (1995) and Zucchetti *et al.* (1994) have reported the clinical and pharmacokinetic studies of these drugs.

GL-331 (**138**) showed topo II inhibition and caused DNA double-strand breakage and G2 phase arrest. It could induce cell death by stimulating protein tyrosine phosphatase activity and apoptotic DNA formation (Huang *et al.*, 1996). GL-331 was also shown to be active in many multidrug-resistant cancer cell lines (Wang *et al.*, 1990). Due to good stability and biocompatability, and its favorable pharmacokinetic profiles, similar to those of VP-16, compound **138** has completed phase I clinical trials at M.D. Anderson Cancer Center, and is currently in phase II clinical trials in Taiwan (Lee, 1999).

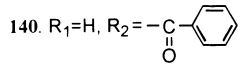
6.5 BRUCEANTIN

Bruceantin (**139**) (Figure 6.16) was originally isolated with closely related molecules **140** and **141** from the stem bark of *Brucea antidysenterica* Mill (Simaroubaceae), guided by bioassay against KB (*in vitro*) and P388 mouse leukemia (*in vivo*) experimental tumor systems. Bruceantin also showed inhibitory activity against L-1210 lymphoid leukemia, WM 256 carcinosarcoma and other murine tumor systems, the Lewis lung carcinoma, and B-16 melanocarcinoma (Kupchan, 1973c, 1975c). Bruceantinoside-B (**143**) and -A (**144**) (Okano *et al.*, 1981) were reported to be isolated from the wood of the same plant. Upon acid hydrolysis, bruceatinoside-A & -B both yielded bruceantin (**139**).

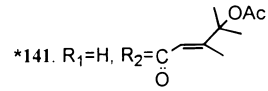




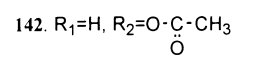
139. R_1=H, R_2= Bruceantin



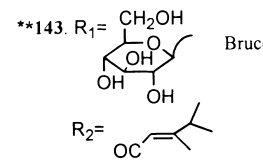
140. R_1=H, R_2= Bruceantarin



*141. R_1=H, R_2= Bruceantinol



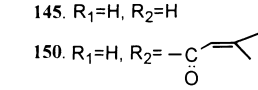
142. R_1=H, R_2= Brucein B



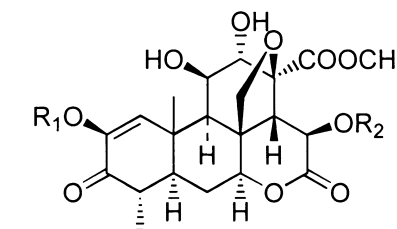
**143. R_1= Bruceantinoside B

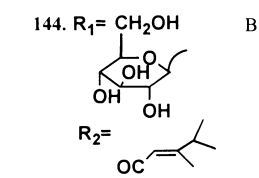
R_2=

145. R_1=H, R_2=H Bruceolide



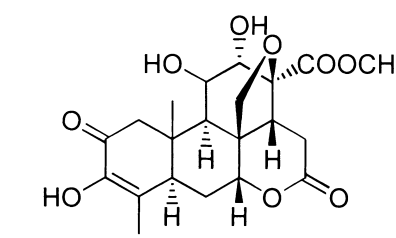
150. R_1=H, R_2= Brusatol





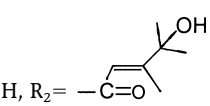
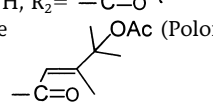
144. R_1= Bruceantinoside A

R_2=



149. (-)-15-deoxybruceolide

Figure 6.16: Bruceantin and analogues.

* The originally assigned formula **141**, R_1=H, R_2=  for bruceantinol was confirmed for brucein C, for bruceantinol, R_2 should be  (Polonsky, 1980).

** This proposed formula of bruceantinoside B was confirmed to be the formula for yadanzioside P (Sakaki, 1986). Therefore, the site of attachment of glucose moiety in bruceantinoside B is at C-3.

Isolation

Bruceantin (139)

10 kg of stem bark of *Brucea antidysenterica* was extracted with 95% alcohol and under reflex yielded alcohol extract A (1180 g, ED_{50} = 0.45 μg/ml). A was partitioned between water (6 liters) and chloroform (6 liters × 2). The chloroform layer was evaporated to yield B (385 g, ED_{50} = 0.34 μg/ml) and the aqueous layer was evaporated to dryness to yield Fr-C (630 g, ED_{50} = 18.5 μg/ml). Fr-B was partitioned between 90% methanol (6 liters) and petroleum ether (4 liters × 4). Concentration of petroleum ether extract gave Fr-D (189 g, ED_{50} = 17.0 μg/ml). The 10% aqueous methanol layer was diluted with further amounts of water to adjust the final concentration of H_2O:MeOH (20:80) and was extracted with CCl_4 (3.8 liters × 4). The CCl_4 extracts were combined and evaporated to dryness to give Fr-E (70 g, ED_{50} = 1.6 μg/ml). The aqueous-methanol layer was further diluted with an adequate amount of water to adjust the methanol:water to 60:40. This methanol layer was then extracted with chloroform (2.4 liters × 5). The combined chloroform extract was evaporated to give Fr-F (90 g, ED_{50} = 0.021 μg/ml), and the aqueous methanol layer was evaporated to dryness to yield Fr-G (10 g, ED_{50} = 38.0 μg/ml). The most active fraction Fr-F was chromatographed on a column of silica SR (5.4 kg) and eluted with chloroform to methanol/chloroform in the manner of gradient dilution. The fractions were combined according to TLC similarities (Chrom AR, 2:3 ether in benzene, $FeCl_3$-vanillin spray), and the 1% methanol/chloroform eluted Fr-H (8.1 g, ED_{50} = 0.031 μg/ml) and Fr-I (3.6 g, ED_{50} = 0.05 μg/ml) showed highest activity. Rechromatography of Fr-H was done on silica AR (600 g) with benzene followed by continuing addition of ether. The fractions eluted with ether:benzene = 20:80 yielded bruceantin (**139**) (2.0 g, 0.02%) m.p. 225–226°C; $[\alpha]_D^{25}$–43°(*c*. 0.31, pyr.).

Bruceantinoside A (144) and conversion of 144 *to* 139

The *Brucea antidysenterica* wood powder (4228 lbs) was thoroughly extracted by percolation with methanol. The methanol extract was concentrated to 440 lbs and was treated by adding aqueous-methanol (22:78, 116 gal) and extracted successively with CH_2Cl_2 (85, 55, 55 gal). The combined CH_2Cl_2 extract was evaporated to approximately 32 gal and was partitioned between 10% aqueous-methanol (232 gal) and 186 gal of *n*-hexane in divided fractions. The aqueous methanol layer was diluted with water (63 gal) and was then extracted with CCl_4 (136, 110, and 110 gal successively). The aqueous-methanol layer was further diluted with water (83 gal) and extracted with $CHCl_3$ (55 gal ×4) to yield 181 g of chloroform extract.

The crude chloroform soluble fraction (181 g) was chromatographed on silica gel (1 kg, 7 × 60 cm column; eluent: chloroform–methanol–water = 50:14:3, v/v) to yield Fr 1 (30.5 g), Fr 2 (68.6 g), Fr 3 (51.1 g) and Fr 4 (12.0 g). Fraction 4 was indicated to contain glucoside (IR 3400 cm^{-1}, 1060 cm^{-1}, and 1040 cm^{-1}). It was purified by

preparative TLC on silica gel, and the band with the same Rf as reference bruceantinoside A was scraped, extracted, and purified by Sephadex column chromatography to yield 2.1 g amorphous bruceantinoside A (**144**), m.p. *ca.* 200° (dec), $[\alpha]_D - 3.6°$ (*ca.* 0.5, pyr.).

Bruceantinoside A was hydrolyzed in dilute methanolic H_2SO_4 (10 ml 3N H_2SO_4: 10 ml methanol) by refluxing. Bruceantin (**139**) in the reaction mixture was extracted with chloroform and was purified by TLC.

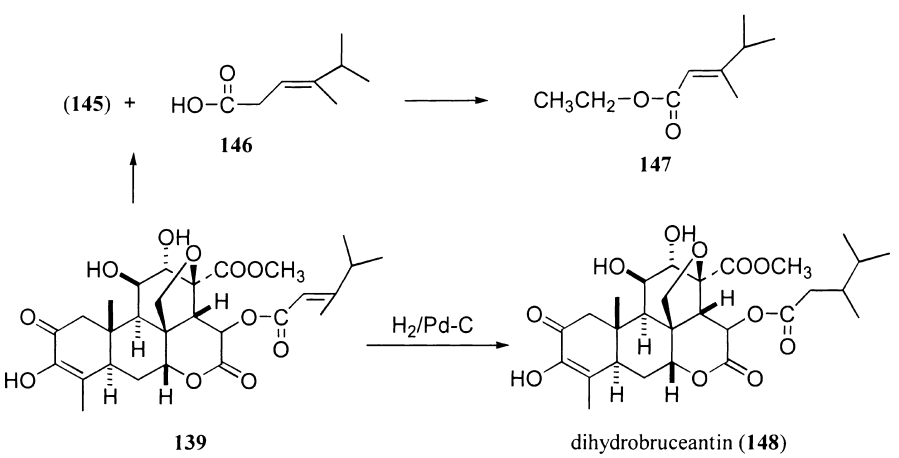
Structural elucidation (Kupchan *et al.*, 1975c)

Spectroscopic elucidation of bruceantin (139)

The structure of bruceantin was elucidated by spectroscopic examination, and was displayed in UV absorption with 50 nm bathochromic shift from 280 to 330 nm, and with KOH showed the presence of diosphenol function. Mass spectra of bruceantin displayed primary fragmentation peaks corresponding to a loss of $C_7H_{22}O$ (*m/z* 438) and base peak corresponding to $C_7H_{11}O$ (*m/z* 111). The whole mass spectrum is almost identical to that of a known reference brucein B (**142**) (Polonsky *et al.*, 1967). The major differences between NMR spectra of bruceantin (**139**) and brucein B (**142**) were the additional signals for (**139**) of a 6- proton doublet (J = 6.5 Hz) at δ 8.88, a vinyl methyl signal at δ 7.82 and a vinyl proton singlet at δ 4.39. These data, and the base peak at *m/z* 111 in the mass spectrum, supported formulation of bruceantin as the 3,4-dimethyl-2-pentenoic acid ester of bruceolide (**145**).

Chemical degradation of 139

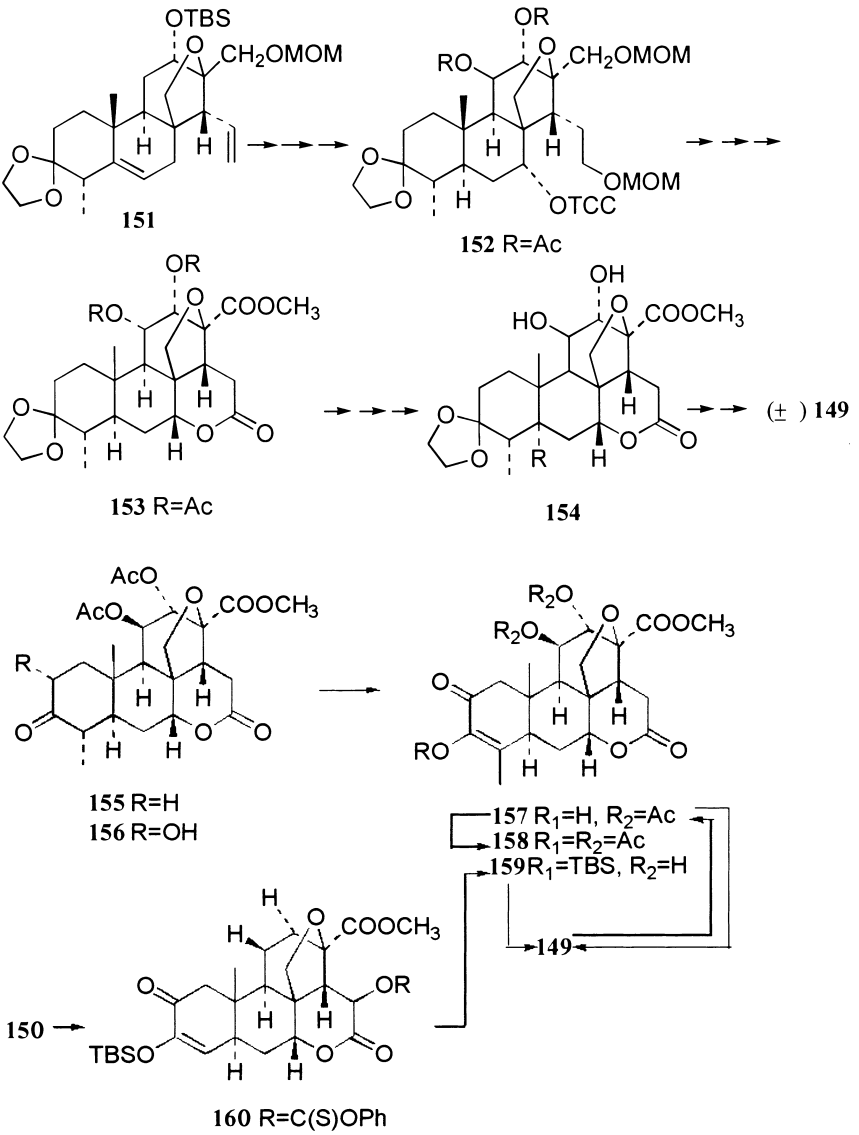
Bruceantin on alkaline hydrolysis yielded bruceolide (**145**) and *trans*-3,4-dimethyl-2-pentenoic acid (**146**), which was identified as its ethyl ester by reacting with diazoethane to yield ethyl *trans*-3,4-dimethyl-2-pentenoate (**147**). Hydrogenation of **139** (H_2/Pd) gave dihydrobruceantin (**148**) (Scheme 6.23).



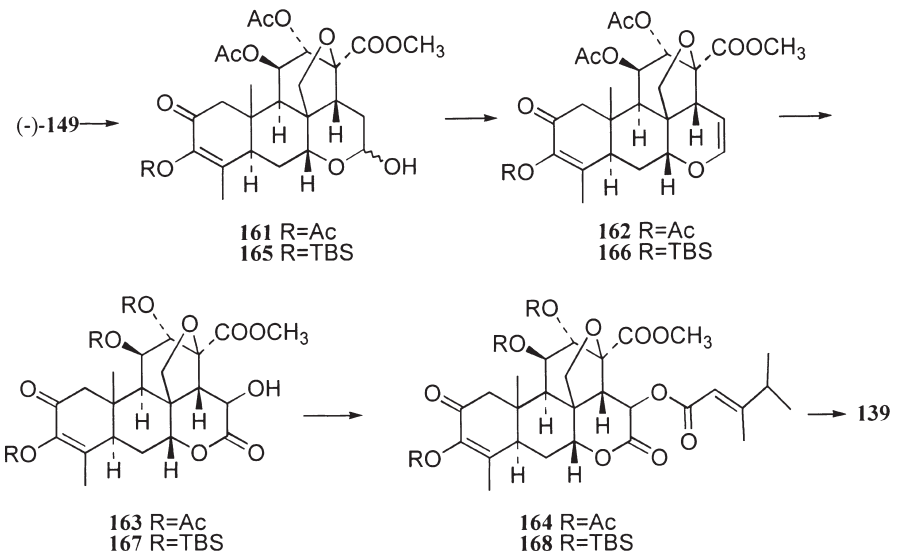
Scheme 6.23: Chemical reaction of bruceantin (**139**).

Synthesis

The total synthesis of (−)-bruceantin was achieved by the synthesis of (±)-15-deoxybruceolide and relayed to the conversion of (−)-15-deoxybruceolide (**149**) derived from naturally occurring brusatol (**150**) (Sasaki *et al.,* 1990 and references cited therein) (Schemes 6.24 & 6.25).



Scheme 6.24: Synthesis of (±)-15-deoxybruceolide (**149**).



Scheme 6.25: Synthesis of (−)-bruceantin (**139**) from (−)-15-deoxybruceolide (**149**).

Specimens (−)-**149**, (−)-**157**, (−)-**158** were prepared from brusatol (**150**), isolated from *Brucea javanica* (L.) Merr. The removal of the C-15-oxygen function of **150** was achieved by converting its TBS-ether to phenyl thiocarbonate **160**. Compound **160** was then converted to **159** by radical-mediated deoxygenation using tributyltin hydride ($(C_4H_9)_3SnH$). Acetylation of **159** gave (−)-**149** which was convertible to (−)-**157** and (−)-**158** by acetylation.

(−)-Bruceantin was synthesized from (−)-15-deoxybruceolide **149** in the form of its triacetate **161**, or 3-TBS-11,12-diacetate **165** through the following steps: (i) selective reduction of C-16 carboxy group to hemiketal **161** (or **165**); (ii) conversion of hemiacetal **161** (or **165**) into corresponding vinyl ether **162** (or **166**) by reaction with $POCl_3$-Py; (iii) oxidation of vinyl ether to corresponding bruceolide was performed first by OsO_4 to C-15,16-diol (**162** → **163**) or by MCPBA (*meta*-chloroperbenzoic acid) (**166** → **167**). The diol was further oxidized by Ag_2O and the bruceolide **163** or **167** was finally converted to (−)-bruceantin (**139**) by esterification with (*E*)-$(CH_3)_2CHCH_3C{=}CH$-COOH in the presence of DCC and DMAP.

Pharmacology

Antitumor activity

At very low doses, bruceantin exerts antitumor activity against L1210 lymphoid leukemia, P388 lymphocytic leukemia, Lewis lung carcinoma and B16 melanoma in mice (Kupchan *et al.*, 1973c, 1975c). Because of its broad spectrum of antitumor activity

in experimental systems, bruceantin (**139**) has been selected for clinical trial in human cancer (NCI, 1976; Garnick *et al.*, 1979; Bedikian *et al.*, 1979; Wiseman *et al.*, 1982).

Bruceantin irreversibly inhibits protein synthesis at its initiation step at low concentrations and at its elongation step at higher concentrations, the ID_{50} determined is 0.03 μM on Hela cells and 0.04 μM on reticulocytes (Lias, 1967). The IC_{50}, as assayed on P388 cells was <5 μM and 15 μM on P388-UNC strain (Hall *et al.*, 1983). Bruceantin exerts an inhibitory effect on phosphorylation *in vitro* in P388 cells at a concentration of 0.015 μM, and inhibits DNA, RNA and protein synthesis (Hall *et al.*, 1982; Eigebaly *et al.*, 1979).

Antimalaria activity of bruceantin

Using the inhibition of incorporation of [^{3}H]hypoxanthine as an index of viability of malaria parasites, it has been shown that the IC_{50} value of bruceantin was 0.013 μM in chloroquine-resistant strain (K_1) of *Plasmodium falciparum* and 0.008 μM in chloroquine-sensitive *P. falciparum* strain (T9-96) (Ekong *et al.*, 1990). The inhibition activity is likely to be due to the effect upon the ribosome, rather than upon nucleic acid metabolism (Kirby *et al.*, 1989).

Anti-amebic activity of bruceantin

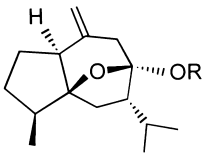
Bruceantin killed *Entamoeba histolytica in vitro* at IC_{50} 0.018 g/ml. The ID_{50} is the concentration of drug which decreased the number of colonies to half that of the control test (Gillin *et al.*, 1982).

6.6 OTHER ANTITUMOR DRUGS FROM HIGHER PLANTS

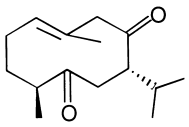
In addition to the compounds discussed in the preceding sections of this chapter, twelve anticancer drugs which were derived from the higher plants, including curzerenone, curdione, curcumol, gossypol, indirubin, lycobetaine, monocrotaline, oridonin, triptolide and tripdiolide, mainly developed in China with limited clinical usefulness, are to be briefly introduced. Moreover, two natural secondary metabolites, tylocrebrine and lapachol which were entered in phase I clinical trials in US will also be simply noted here.

Curzerenone (169) curdione (170) and curcumol (171)

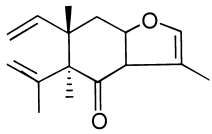
The essential oils of the rhizomes of the Zingiberaceae plants, *Curcuma zedoaria* (Berg.) Rose, *C. wenyujin*, *C. wenchowensis* Sp. Nor., and *C. xanthorrhiza*, have been used clinically for uterus cancer in China (Hsu, 1980). The purification of such oils showed that curzerenone (**169**) curdione (**170**) and curcumol (**171**) are the main constituents. Compound **169**, possessing a benzofuran sesquiterpene skeleton and isolated from *C. zedoaria* (Fukushima *et al.*, 1970), exhibited inhibitory activity on Ehrlich carcinoma,



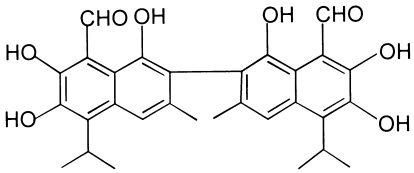
Curcumol (**171**)



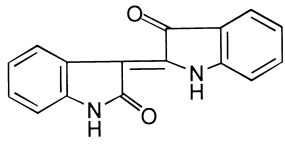
(+)-Curdione (**170**)



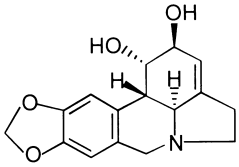
Curzerenone (**169**)



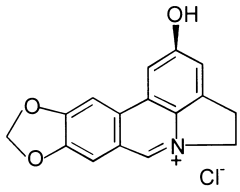
Gossypol (**172**)



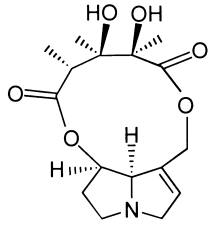
Indirubin (**173**)



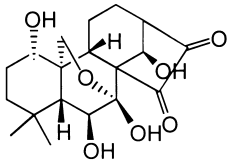
Lycorine (**176**)



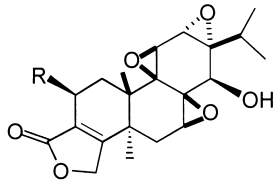
Lycobetaine (**175**)



Monocrotaline (**177**)

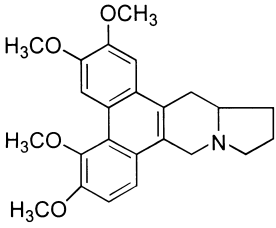


Oridonin (**178**)

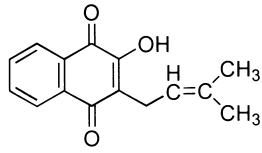


Triptolide (**179**): R = H

Tripdiolide (**180**): R = OH



Tylocrebrine (**181**)



Lapachol (**182**)

Figure 6.17: Anti-cancer natural products of limited interests.

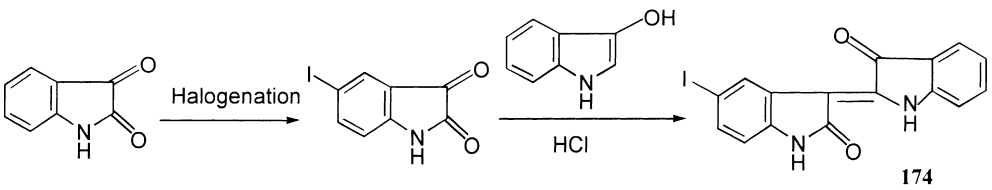
L615 and other tumors, and was used as a clinical drug for carcinoma of the cervix and skin carcinomas (Hsu, 1980; Hsu and Han, 1979). Two germacrane-type sesquiterpenoids, curdione (**170**) and curcumol (**171**), were first isolated from *C. zedoaria* (Hikino *et al.*, 1967). They were found to show potent protective effect on D-galactactosamine (D-GalN)/lipopolysaccharide (LPS)-induced acute liver injury in mice and to exhibit inhibitory activity on D-GalN-induced cytotoxicity in primary cultured rat hepatocytes (Matsuda *et al.*, 1998).

Gossypol (172)

Gossypol was isolated from cotton plants, such as *Gossypium hirsutum* (Stipanovic *et al.*, 1975), *G. barbadense* (Seshadri and Sharma, 1975), and *Hibiscus tiliaceus* (Ali *et al.*, 1980) and *Montezuma speciosissima* (Jolad *et al.*, 1975). Gossypol is known to be an effective oral contraceptive drug for males. Pharmacologically, it has antispermatogenic effects by prohibiting the spermatozoa from entering the oocyte (Kennedy *et al.*, 1983). It is used clinically in benign gynecological conditions and in cancer patients, including stomach, esophageal, liver, breast, and bladder tumors (Lee, 1987). Gossypol shows minimal side-effects and no myelosuppression toxicity (Shelley *et al.*, 1999). The Shelley group also pointed out that the *l*-enantiomer of gossypol induced a dose-dependent cell-killing effect in melanoma, lung, breast, cervix, and leukemia tumor cells *in vitro* with mean IC_{50} of 20 μM and was significantly more potent than the *d*-enantiomer of gossypol (Shelley *et al.*, 1999). The SAR studies suggested that (−)-gossypol is significantly more active in melanoma cell lines compared with the (+)-isomer, and more cytotoxic than common cancer drugs such as cisplatin, dacarbazine, mephalan, etc. (Blackstaffe *et al.*, 1997). In contrast to the *d*- & *l*-isomers of gossypol, (+)-gossypolone showed up to threefold greater inhibitory effect against MCF-7 malignant cells than (−)-gossypolone (Liang *et al.*, 1995).

Indirubin (173)

Indigo naturalis Adans, *Indigofera tinctoria* L. (Leguminosae), and *Baphicacanthes cusia* Bremek (Acanthaceae), have been used in traditional medicine in China for a long time (Hsu, 1980; Lee, 1987; Han, 1994). After systematic analysis by the bioassay-guide purification, biindolylidene linking 2-3′, such as in indirubin (**173**), was isolated and found to display inhibitory effects on chronic granulocytic leukemia when used clinically (Hsu, 1980; Lee, 1987; Han, 1994). Interestingly, the main molecule biindolylidene linking 2-2′ as in indigo has no antitumor activity; nor does the analogue linking 3-3′, as in isoindigotin. SAR studies showed that substitution of the N_1-position in indirubin caused inactivation, except for $N_1,N_{1'}$-dimethylindirubin which exhibits some anticancer activity (Wu *et al.*, 1985). Of those halogen substituted analogues (Scheme 6.26), compound **174** was shown to increase the life span of mice by 41–73% in a leukemia system, and to have a marked inhibitory action against W256 in rats with inhibition rates of 48–83% (Gu *et al.*, 1989).



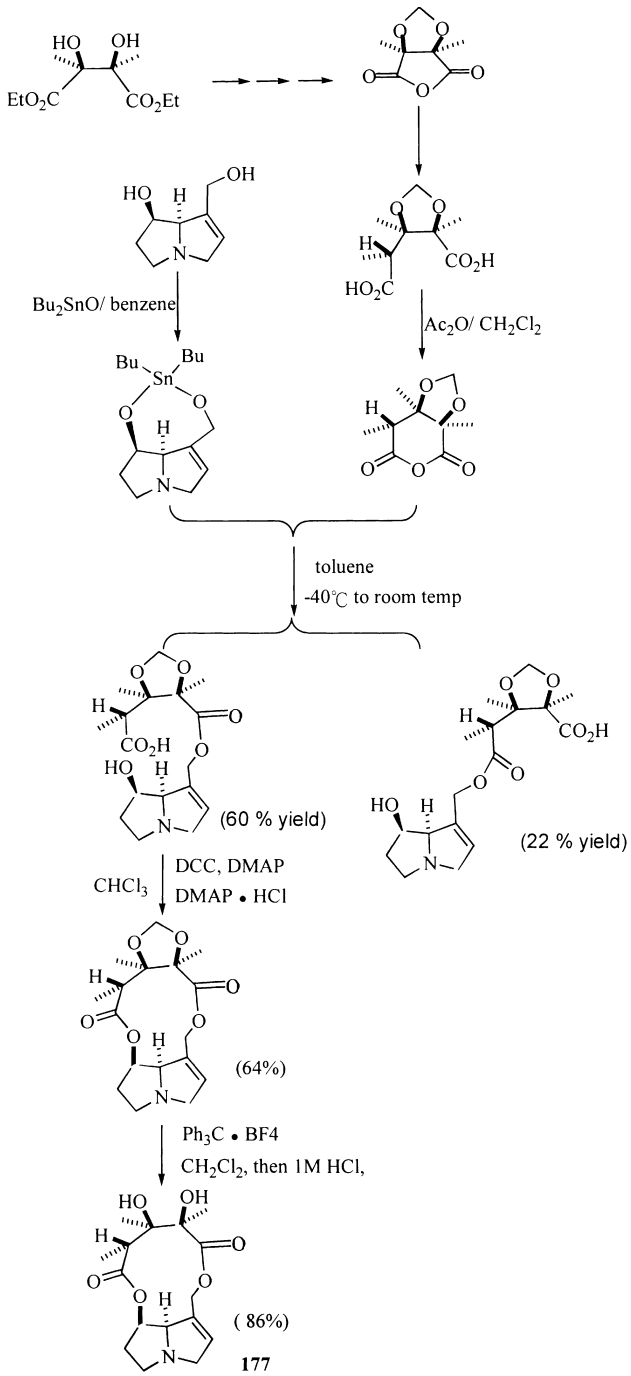
Scheme 6.26: Synthesis of indirubin derivative **174**.

Lycobetaine (175)

Lycobetaine (AT-1840) was derived semisynthetically from natural lycorine (**176**), which was isolated from Amaryllidaceae, such as *Lycoris radiata*, *Crinum amabile*, *Narcissus pseudonarcissus*, and *Haemanthus multiflorus* etc. The main Amaryllidaceae lycorine alkaloids of *L. radiata* are inactive. However, lycobetaine exhibited antitumor effects against several animal tumors such as Ehrlich carcinoma, ascites hepatoma, sarcoma 180, and Yoshida sarcoma (Hsu, 1980). In addition, the *in vitro* cytotoxicity test showed that **175** inhibited several human tumor cell lines, including breast cancer, fibrosarcoma, lung cancer, colon cancer, oral epidermoid carcinoma, as well as vinblastine-resistant KB cells (Lin *et al.*, 1995). Clinical reports show this drug is effective in gastric carcinoma and ovarian carcinoma (Hsu, 1980). Lycobetaine did not bind to DNA covalently and did not cause DNA alkylation, so that it is neither a DNA strand break nor an interstrand cross-link agent (Liu *et al.*, 1989). The SAR studies revealed that the changes in the distance between phenolic oxygen and quaternary nitrogen of betaine in lycobetaine affect its antitumor activity (He and Weng, 1989).

Monocrotaline (177)

In the 1930s, Neal *et al.* first isolated the pyrolizidine alkaloid, monocrotaline, from *Crotalaria spectabilis*. Monocrotaline was also isolated from other *Crotalaria* species. The structure of **177** was elucidated by using ^{1}H and ^{13}C NMR spectral analyses (Bredenkamp *et al.*, 1987; Molyneux *et al.*, 1982). Although it has been reported that *Crotalaria sessifolora* has been used clinically for skin and cervical carcinoma (Hsu, 1980) the best biological data related to its hepatotoxicity and carcinogenicity (Hooson *et al.*, 1976) and the inhibitory effect on the drug-metabolizing enzymes of rat liver, due to its active component (Dalvi, 1987). The recent reports on monocrotaline mostly concern its toxicity, causing veno-occlusion in the liver, pulmonary arterial hypertension, and right ventricular hypertrophy, as well as disturbing glutathione metabolism (Yan and Huxtable, 1996). Thus, due to its serious toxicities, monocrotaline was only employed for skin cancer. Structure–activity–relationship studies revealed that the semisynthetic products, dehydromonocrotaline (Kim *et al.*, 1993) and lycopsamine (Zalkow *et al.*, 1985) were more effective in inhibiting colony formation than their parent compound. Total



Scheme 6.27: Total synthesis of optically active monocrotaline (**177**).

synthesis of optically active monocrotaline has been reported recently (Scheme 6.27) (Niwa *et al.*, 1992).

Oridonin (178)

Rabdosia rubenseus Hamst was used in folk medicine for esophageal and stomach cancer in China. Oridonin was isolated from this plant and shown to be active on sarcoma **180** and on ascites hepatoma (Hsu, 1980). Compound **178** was also reported to be isolated from other *Rabdosia* spp. (Meng *et al.*, 1989; Osawa *et al.*, 1994; Takeda *et al.*, 1997) and *Isodon* spp. (Fujita *et al.*, 1976). Clinical trials revealed that this antitumor drug exhibited activity on late-stage esophageal cancer (Hsu, 1980). Spectral analysis of NMR evidence enabled Fujita *et al.*, to assign formula **178**, possessing kaurene-type diterpene, for oridonin (Fujita and Taoka, 1972; Meng *et al.*, 1989; Osawa *et al.*, 1994; Takeda *et al.*, 1997). The SAR studies of oridonin and its derivatives revealed that the α-methylene cyclopentanone and spirolactone aldehyde moieties are the active sites of the oridonin molecule (Fujita *et al.*, 1976; Node *et al.*, 1983).

Triptolide (179) and tripdiolide (180)

These two active diterpenes showed inhibitory effect on L-1210 leukemia; both were first isolated from Taiwan plants, *Tripterygium wilfordii* Hook f. (Celastraceae) by Kupchan *et al.* (1972a). The original *T. wilfordii* has been used in Chinese medicine, mainly as an insecticide, for hundreds of years. Notably, this plant has been reported in recent literature to exhibit significant anti-fertility, anti-rheumatic, and also immunosuppressive effects, (Zhang *et al.*, 1993).

Tylocrebrine (181)

Tylocrebrine is a phenanthroindolizidine alkaloid isolated from *Tylophora crebrifolia* (Asclepiadaceae) (Gellert *et al.*, 1962, 1964). It exhibited antileukemic effect in P388 and H210 in mice. Phase I trial was discontinued due to CNS toxicity.

Lapachol (182)

Lapachol was isolated from *Stereospermum suaveolens* cham. (Bignoniaceae) (Rao *et al.*, 1968). It exhibited antitumor activity, as tested by WM 256, in rats. Phase I trial was halted in 1970 due to unfavorable results.

ACKNOWLEDGEMENTS

We wish to thank Professor Chieh-Fu Chen, Director of the National Research Institute of Chinese Medicine, Taiwan, for encouraging this work, and Professor Kuo-Hsiung Lee, Director of Medicinal Chemistry and Natural Products, University of North Carolina, USA for kindly providing the available references. We are also grateful to many

colleagues and students, including Hui-Chi Huang, Hsiu-Ling Liu, Li-Ming Yang Kuo, Chien-Chang Shen, Hsiou-Yu Ding, Yi-Ling Lee, Shu-Ling Wu, Ming-Der Wu, Yi-Pin Chang, Yuh-Maan Yu, and Ang-Su Yu, for contributing their time in many ways to help the completion of the manuscript of this chapter.

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CHAPTER

7

Bioactive Taxoid Production from Natural Sources

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Contents

7.1 INTRODUCTION

The emergence of paclitaxel (**3**) (Taxol®) in 1982 as a highly effective drug for the treatment of refractory ovarian cancer has been rapidly followed by a serious problem related to the adequate supply of the drug (Cragg and Snader, 1991). Paclitaxel (**3**) (Figure 7.1)

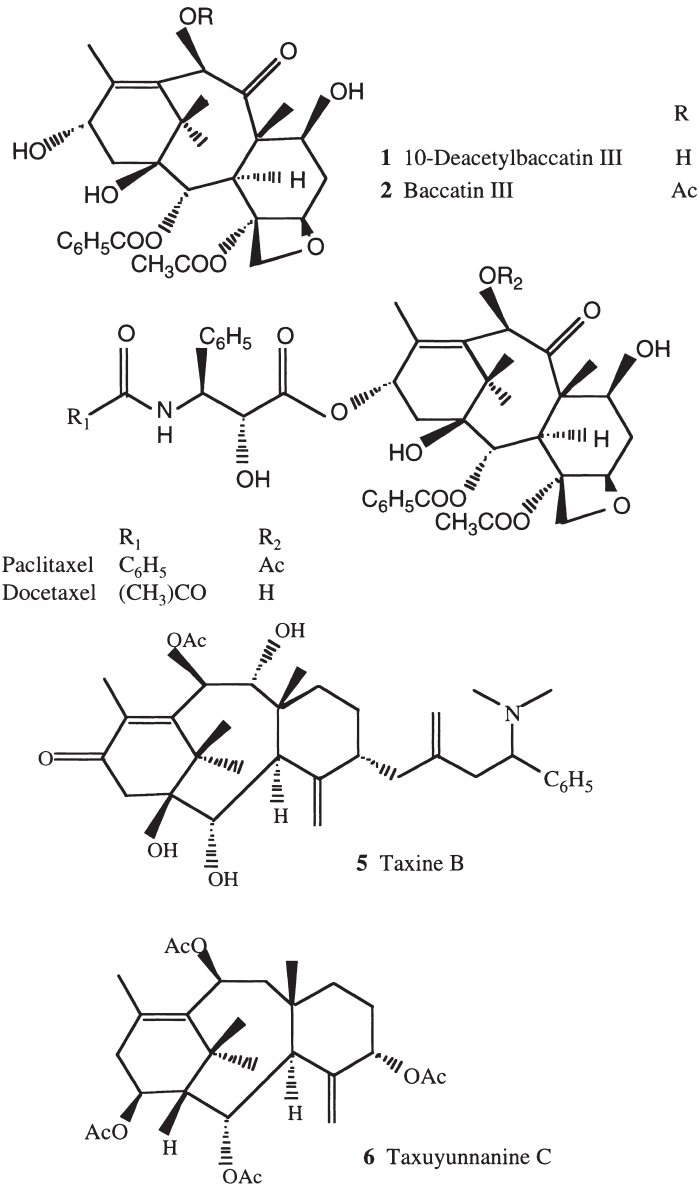

Figure 7.1: Structures of taxoids cited in the text.

is a complex diterpenoid which was first isolated from the bark of the Pacific yew, *Taxus brevifolia* (Taxaceae), a slow-growing tree mainly found in the Pacific Northwest (Wani *et al.*, 1971). Paclitaxel (**3**) is found in the yew's bark in minute quantities, about 0.017% dry weight basis (Witherup *et al.*, 1990; Wheeler *et al.*, 1992). The sacrifice of a 100-year-old tree yielding about 3 kg of bark containing some 300 mg of **3** is required to obtain approximately a single cancer chemotherapy drug dose (Cragg and Snader, 1991). In 1992, Hauser Chemical Research reported that their current production capacity had reached 130 kg of **3** which was extracted from 730,000 kg of Pacific yew bark collected in 1991 (Suffness and Wall, 1995). These data indicate that the extraction of **3** from naturally growing *Taxus* trees is quite limited compared to the large number of trees which are needed to obtain a sufficient amount of **3**.

Taxus baccata (L.) (Taxaceae).

Several alternative sources of **3** have been identified and are currently the subject of numerous world-wide investigations. They include: agricultural supplies of taxoids from various organs of *Taxus* species; hemisynthesis, i.e. the binding of a side chain to biogenetic precursors of **3** such as baccatin III (**2**) or 10-deacetylbaccatin III (**1**), or the conversion of taxine B (**5**) into bioactive taxoids; total synthesis; biotechnological approaches, i.e. the production of taxoids by *Taxus* cell and tissue culture, and the prospecting of new natural sources of **3** such as microorganisms.

The aim of this chapter is to summarize and discuss the progress achieved in the following fields, especially within these last years:

- Phytochemical investigations related to the isolation and structure elucidation of new taxoids from several Taxaceae species. A summary of the numerous studies related to the isolation of new taxoids from 1997 up to now will be given to demonstrate the wide structural diversity of the taxoid within the *Taxus* genus and of the numerous current researches in this field.
- Cultivation of yew for production of **3**. The possibility of growing millions of selected *Taxus* cultivars to produce biomass for the extraction of **3** is now considered in response to the environmentalists concerned about long-term impacts of intensive harvesting and its effects on the biodiversity of the ancient forests.
- The use of *in vitro* cultures as alternative sources for taxoid production. These include *Taxus* cell tissue and organ cultures and the prospecting of microorganisms producing taxoids. The point covering specifically the identification of biochemical or genetic markers for biosynthesis of **3** will be discussed. Indeed, the identification of specific key enzymes in the taxane biosynthetic pathway is a prerequisite for the rational elaboration of a genetic engineering program.

For each of these research fields, an appropriate strategy for the fractionation of plant constituents and for the active constituent discrimination from crude extracts as well as for the quantification of the target metabolite, is essential. Therefore, a short overview of the most advanced analytical methods for taxoid analysis is first presented.

7.2 TAXANES FRACTIONATION AND ANALYSIS

In order to evaluate taxoid production in different *Taxus* plants, tissue cultures and microorganism materials, several analytical methods have been reported including chromatography, immunological methods, cell bioassay and tubulin assembly assays.

Among the chromatographic methods, high performance liquid chromatography (HPLC) remains the most frequently used method for the quantitative determination of taxoids. Reverse phase chromatography on octadecyl, phenyl, cyano or pentafluorophenyl-bonded columns are usually used. A specific new reverse phase HPLC column for rapid isocratic separation has been developed for analysis of **3** (Ketchum and Gibson, 1993). UV detection at 227 nm is generally used for the identification and the quantitative determination of taxoids. More sophisticated chromatographic procedures such as micellar electrokinetic chromatography (Chan *et al.*, 1994), liquid chromatography-tandem mass spectrometry (LC-MS-MS) (Volk *et al.*, 1997), high-speed counter-current chromatography initiated by Vanhaelen-Fastré *et al.* in 1992 have also been developed for taxoid analysis and preparative fractionations. More recently, Yang *et al.* (1998) have

developed an efficient method for the preparative purification of **3** from crude chloroform extracts of *T. yunnanensis*, using an industrial preparative liquid chromatograph (IPLC) and a polymeric stationary phase (D956 resin). Using a single-system apparatus, about 5 kg of the crude extract could be loaded onto the column, and about 50 g of **3** (>99% pure) could be recovered after three chromatographic runs within 155 h without organic solvent waste. The screening of a large population of *Taxus* cultivars and the optimization of taxoid production by *Taxus* tissue cultures or microorganisms require rapid, sensitive and specific methods. Immunological procedures such as enzyme-linked immunosorbent assay (ELISA) (reviewed by Jaziri *et al.*, 1996) and fluorescence polarization immunoassay (FPIA) (Bicamumpaka and Pagé, 1998) greatly simplify the laborious and time-consuming pre-purification step required for the HPLC analysis of taxoids. Both polyclonal and monoclonal antibodies against **3**, **1**, **2** or against the so-called 'taxane diterpene skeleton' have been produced. Immunoassay using polyclonal antibodies may be useful for the rapid screening of taxoid-producing strains, but the possibility of cross-reactivity makes further chromatographic analysis essential. Nevertheless, the monitoring of chromatographic fractionation of plant or cell culture extracts through this method revealed to be a powerful way to identify new taxoids (Jaziri *et al.*, 1997). Indeed, taxoids lacking side chain substituent at C-13 are of particular interest, since they are potent precursors for the hemisynthesis of **3** and analogues as docetaxel (**4**) (Taxotere®). In addition, their structure could contribute to a better understanding of the complex biosynthetic pathway of **3**.

In view of the cytotoxic activity of **3**, a cell bioassay using Chinese hamster ovary cells has also been developed for the screening of bioactive taxoids in *Taxus* cultures (Shuler *et al.*, 1992). However, this cell bioassay lacks specificity, as cytotoxic compounds are selected instead of compounds showing specifically microtubule stabilization. Jaziri *et al.* (1997) have described a new concept for the isolation of bioactive taxoids from natural sources. The approach, developed for the detection of new bioactive taxoids from *Taxus baccata* stem bark extracts, was based on the use of two complementary biological tools: antibodies (anti-**3** and anti-**1**) and naturally occurring receptor(s) for taxoids (complex of tubulin-microtubule). The combination of the immunoassay with microtubule bioassay show very promising results: several known taxoids, and even new taxoids, were detected and isolated. This screening strategy can also be applied to other microtubule-stabilizing agents and, therefore, proves to be effective for the discovery of new microtubule-stabilizing agents derived from molecular features of compound **3**. Both assays associated with chromatographic methods are powerful tools for the screening of natural product collections, for the study of additional sources of **3** and related taxoids, and of new compounds showing the same biological properties as **3**.

7.3 NEW TAXOIDS FROM TAXACEAE

Paclitaxel (**3**) was discovered, thanks to a very ambitious screening program initiated by the National Cancer Institute (US) between 1958 and 1980, which resulted in the investigation of more than 110,000 compounds extracted from 35,000 plant species for anticancer activity monitored by several bioassay systems (Appendino, 1993; Nicolaou *et al.*, 1994; Tubbing and McDowell, 1995).

Paclitaxel (**3**) was originally isolated from the bark of the Pacific yew (*T. brevifolia*) using a bioassay based on the cytotoxic activity against the L1210 leukemia cells to monitor its activity during the fractionation of extracts (Suffness and Wall, 1995). The structure of **3** (originally named taxol) was published in 1971 (Wani *et al.*, 1971); it was not patented at that time, probably because its concentration in the bark of *T. brevifolia* was found to be very low (0.017% dry weight) (Witherup *et al.*, 1990, Wheeler *et al.*, 1992).

Paclitaxel (**3**) has also been isolated from other species of the genus *Taxus*, including *T. baccata*, *T. cuspidata*, *T. canadensis*, *T. wallichiana* and *T. x media*, and from different parts of the plant (seeds, needles, young stems, woody stems, wood, bark and roots) (Wani *et al.*, 1971; Miller *et al.*, 1981; Witherup *et al.*, 1990; Vidensek *et al.*, 1990; Fett-Neto and DiCosmo, 1992; Wickremesinhe and Arteca, 1994; El Sohly *et al.*, 1995). In addition to **3**, many other closely related taxoids have been isolated from the genus *Taxus*. Although taxoids seems to be restricted to the genus *Taxus*, Ettouati *et al.* (1989) have isolated numerous taxoids from the needles and trunk bark of *Austrotaxus spicata*, another genus of the Taxaceae originating from New Caledonia, and Luo *et al.* (1994) have detected by HPLC, **3** and **2** in needles of *Torreya grandis*. However, Guo *et al.* (1995) failed to find taxoids in extracts of *T. nucifera* needle. Taxoids were also detected by immunoassay in cell aggregates derived from explants of various *Taxus* and *Torreya* genera (Smith, 1994).

Few reviews on the taxoids isolated from *Taxus* sp. have been reported. However, a very complete work has recently been published by Parmar *et al.* (1999); up to 270 taxoids, including taxane and taxine derivatives isolated from 1907 to December 1997 are described.

In the present review, we have selected 13 studies related to the phytochemical investigations on *Taxus* sp, carried out from 1997 until March 1999. This literature review is summarized in Table 7.1. Up to 30 new taxoids have been described but only three of them were studied for their biological activity. It is regrettable that such a situation is so frequently encountered in the natural product studies.

7.4 YEW CULTIVATION

Paclitaxel (**3**) and its key precursors have been detected in the needles of various *Taxus* species. The investigation of these sources is actively promoted by the NCI and also by

TABLE 7.1
Overview of phytochemical investigations on *Taxus* species from 1997 to March 1999

Plant species	Geographical origin	Tissue	Compounds	Number of new taxoids	Yield	Biological activity assay	Reference
T. wallichiana	Himalaya	needle	Abeotaxane	1	75 mg from 60 kg	unspecified	Chattopadhyay *et al.*, 1999
T. cuspidata var. nana	Japan	stem	Taxuspinananes H, I, J and K	4	respectively 11,15,1 and 24 mg from 20 kg	P388 cells	Morita *et al.*, 1998
T. yunnanensis	China	bark	Taxayuntin H and J	2	unspecified	unspecified	Zhou *et al.*, 1998
T. baccata	Italy	unspecified	N-debenzoyl-N-butanoyl and N-debenzoyl-N-propanoyl-10-deacetylbaccatine	2	minor constituents	unspecified	Gabetta *et al.*, 1998
T. brevifolia	Portland (USA)	bark	4 new taxoids belong to the 11(15-1)-abeobaccatin VI group and one to the brevifoliol group	5	unspecified	unspecified	Rao and Juchum, 1998
T. baccata	Spain	needle	10-(β-hydroxybutyryl)-10-deacetylbaccatine and 5-cinnamoylphototaxicin	2	unspecified	unspecified	Sotao and Castedo, 1998
T. mairei	China	root	Taxumairol K	1	35 mg from 60 kg	Hela tumor cells	Shen *et al.*, 1998
T. chenensis	China	needle	Bicyclic 3,8-secotaxane	3	respectively 7, 3 and 9 mg from 7.1 kg	unspecified	Shi *et al.*, 1998
T. cuspidata var. nana	Japan	stem	Taxuspinanane C	1	0.000009%	unspecified	Morita *et al.*, 1997
T. wallichiana	Himalaya	needle	5-deacetyl-1-hydroxybaccatin	1	34 mg from 5 g of the mother liquors (?)	unspecified	Barboni *et al.*, 1997
T. mairei	Taiwan	root	Taxumairol C, D and E	3	respectively 3, 40 and 29 mg from 60 kg	unspecified	Shen and Chen, 1997
T. yunnanensis	China	root	1-β-hydroxy-10-deacetylbaccatine I, 9 and 10-deacetylbaccatine VI	3	unspecified	unspecified	Hongjie *et al.*, 1997
T. cuspidata var nana	Japan	stem	Taxuspinanane A and B	2	respectively 72 and 75 mg from 20 kg	P-388 cells	Morita *et al.*, 1997

private pharmaceutical industries using 10-desacetylbaccatin III as a starting precursor for **3** or **4** hemisynthesis. Nowadays, the availability of millions of ornamental *Taxus* species and cultivars in some major US and Canada nurseries, as well as abundant supply of several other wild *Taxus* species in other countries, make the isolation of **3** from the needles a most attractive proposition for long-term bulk production. Nevertheless, the cultivation of yew for **3** production still presents a number of technical and production challenges. Indeed, despite the fact that the yew is also grown for ornamental use on a large scale, very little information is available about the optimization of plant growth, and even less about the genetic and the environmental factors that affect taxoid production. *Taxus* x. *media* 'Hicksii' was selected as the most appropriate suitable cultivar for large-scale cutting culture in nursery conditions. The needle yields a **3** content equivalent to that of the bark; in addition, this cultivar is immediately available in large numbers and its growth habit made it ideal for mechanical harvesting (Donovan, 1995).

Plantlets of this *Taxus* sp. are growing for six to more than twelve years in nurseries before sale for landscape plantings and annual shaping of the plants generates stem and especially needles clippings.

Research reports from several laboratories have shown that dried *Taxus* leaves from ornamental *Taxus* exhibit extremely variable **3** contents. Several authors have demonstrated that these variations are not only related to genetic, epigenetic and environmental conditions, but also to different methods of sampling, drying, and of extraction procedures. For instance, the effects of drying intact clippings of *Taxus* on the recovery of **3** and related taxoids have been studied under different drying conditions. The processing time has been found to be the most critical factor in preserving the taxoids content of the needles of intact *Taxus* clippings during drying. Conditions which would allow drying of the clippings in less than 6 days at a temperature up to 50°C would preserve the taxoid content from the clippings (ElSohly *et al.*, 1997).

7.5 *TAXUS* SP. CELL, TISSUE AND ORGAN CULTURES AS ALTERNATIVE SOURCES FOR TAXOIDS PRODUCTION

The manipulation of plant cell and tissue culture systems has resulted in an enhanced production of various secondary metabolites. *Taxus* cell and tissue cultures could potentially provide, either sufficient quantities of **3** itself, or of taxoids useful for the hemisynthesis of derivatives, such as **4** (Taxotere®), also suitable for chemotherapeutical purposes. Several approaches have been employed for the establishment of various *Taxus* culture materials, including, undifferentiated (callus or cell suspension) and differentiated (organ) cultures. Genetic transformation of *Taxus* cells was also reported. Wild strains of *Agrobacterium tumefaciens* and *A. rhizogenes* have been successfully used to deliver foreign DNA into the *Taxus* genome (literature reviewed by Gibson *et al.*, 1995; Jaziri *et al.*, 1996).

Several parameters have been reported to affect both the growth rate and taxoid production. A rapid, reproducible and economic production of significant quantities of taxoids in cell culture has still not been demonstrated until now, but several advances in the field of *Taxus* sp. cell growth have been reported. An optimized medium has been used to grow three cell lines of *T. brevifolia* (Ketchum *et al.*, 1995). The authors assessed the effects of various components of the culture medium, and established a new medium formulation (TM5 medium). According to the data previously published on the *Taxus* sp. growth cycle, the TM5 medium was found superior, since a significant improvement of callus growth rate was reported. The time for doubling the cell fresh weight was reduced from the 13 to 20 days previously observed, to 3.5 to 5.6 days.

Environmental and nutritional factors have been shown to influence taxoid accumulation in cultures. Fett-Neto *et al.* (1992) have studied the effect of nutrients and other effectors on **3** production by *T. cuspidata* cell cultures; a 0.02% yield of **3** (dry weight basis) was observed in callus cultures. Srinivasan *et al.* (1995) have studied the kinetics of biomass accumulation and production of **3** by *T. baccata* cell suspension cultures. Paclitaxel (**3**) was found to accumulate at high yields (1.5 mg/l) exclusively in the second phase of growth. A similar yield of **3** from cell suspension cultures of *T. brevifolia* was obtained after 10 days of culture in an optimized medium containing 6% fructose (Kim *et al.*, 1995). The optimization of taxoid production has been the subject of many other reports. Significant results were published by Mirjalili and Linden (1995) who demonstrated that the gas phase composition affects the timing and rate of **3** production in cell suspension culture of *T. cuspidata*. The most effective gas mixture composition in terms of **3** production was 10% (v/v) oxygen, 0.5% (v/v) carbon dioxide and 5 ppm ethylene. Under these conditions, the cultures produced 6.5 mg/l of **3** within 21 days. Interestingly, the addition of fructose (1%), on day 11, increased the final concentration of **3** up to 12.2 mg/l which corresponds to a maximum volumetric productivity of 0.64 mg/l/day. However, more investigations are needed since it has been estimated that the economic viability of production of **3** using plant cell culture technique requires a production of 1–2 mg/l/day (Gibson *et al.*, 1995; Mirjalili and Linden, 1995). In addition to **3**, several other taxoids have been identified in both cells and culture medium of *Taxus* cultures (Jaziri *et al.*, 1996; Menhard *et al.*, 1998). More recently, Vanek *et al.* (1999) succeeded in isolation of **3** and eight of its analogues from a *T. baccata* cell suspension cultivated in a 6.2 l bioreactor. The authors stated that the total taxoids concentration both in the cells and in the medium was still rather low for placitaxel industrial production. However, the feasability of the scaling up of taxoids production by *T. baccata* cell was clearly demonstrated and represents an important step in the development of biotechnological production of **3**.

Secondary metabolite accumulation is a result of a dynamic equilibrium between product formation, transport, storage, turnover and degradation. The low yield of taxoids in cell cultures could be explained by the following reasons: lower degree of cell

differentiation, inappropriate developmental state, diversion of common precursors from secondary metabolism into primary metabolism or in other pathways of the secondary metabolism, suppressed or reduced expression of key enzymes in undifferentiated cells, or lack of appropriate storage sites.

Paclitaxel (**3**) is found in virtually all parts of the *Taxus* plant (Videnseк *et al.*, 1990; Fett Neto and DiCosmo, 1992). The precise localization of **3** in the plant tissue or cell culture presents numerous problems, as **3** is not soluble in aqueous solutions and is displaced during classical fixation procedures used for cytological examination. Recently, by using polyclonal anti-**3** antiserum, Russin *et al.* (1995) have developed an immunohistochemical technique for localization of **3** in *Taxus cuspidata* tissues. Using cryotechniques and a water-soluble melanine resin (Nanoplast), **3** was localized almost exclusively in the cell walls of phloem, vascular cambium, and xylem. According to these results, and in view of the lipophilic nature of **3**, the authors concluded that taxoids are most likely excreted into the extracytoplasmic compartment (the cell wall) and not sequestered within the cellular components (e.g. vacuoles, plastids). As **3** affects microtubules in a wide variety of organisms, including plants, exclusion from the protoplast by excretion into the cell wall should be considered as a good strategy to avoid the toxic effect of bioactive taxoids within *Taxus* cells. According to these observations, more appropriate taxoid extraction procedures are required for the improvement of taxoid recovery. However, this immunolocalization study does not clearly reveal whether **3** itself is excreted into the cell wall, or whether its precursors are excreted and then modified.

In this perspective, Durzan and Ventimiglia (1994) have reported that *Taxus* tissue or cell cultures extracted exhaustively with methanol and then treated with xylanase, could release compounds having taxoid antibody reactivity. An additional amount of unidentified taxoids (300% of the extracted amount of free **3**) was recovered from the xylanase-treated tissue culture material versus the control. This study clearly shows that bound compounds displaying an immunocytochemical reaction typical of the taxoid ring are associated with the cell wall and its extracellular membranes.

Furthermore, little research has been conducted on cellular, physiological and developmental regulation of taxoid biosynthesis. However, recent results obtained in the field of the taxoid biochemistry could ultimately lead to an improvement of taxoid production by genetic modification of *Taxus* plants and cell cultures. It is, therefore, essential to carefully study the biosynthesis of taxoids, especially the identification and characterization of the rate-limiting steps of the pathway. The biosynthetic pathway involved in the formation of **3** and its closely related taxoids bearing a side chain at C-13, can be divided into three major biosynthetic steps, including: the formation of the taxane ring skeleton; the synthesis of the side chain; and, finally, the esterification of the taxane ring system with the side chain (Rohr, 1997).

It was previously claimed that the taxane ring system was built in *Taxus canadensis*

from acetate and mevalonate. However, the reported incorporation rates of the radioactive precursors were very low (from 0.02 to 0.12%), and none of the labeled taxoids obtained (**2** and **3**) was subjected to chemical degradation reactions in order to localize the position of the label sites within the molecules. In recent studies devoted to the biosynthesis of the taxane carbon skeleton in *Taxus chinensis*, Eisenreich *et al.* (1996) used a selected cell line producing taxuyunnanine C (**6**) as a major taxoid. This compound has the advantage, for biosynthetic studies, of containing four acetyl residues that can be used as internal reference standards. By using these experimental conditions, the authors clearly demonstrated that the labeling patterns, after feeding the cell culture with radioactive glucose and acetate, were inconsistent with the mevalonate pathway. The authors concluded that the taxane carbon skeleton is not of a mevalonate origin. The taxoid data obtained by Eisenreich *et al.* (1996) share important features with the alternative pathway of isoprenoid biosynthesis operating in some eubacteria.

On the other hand, three early steps of **3** biosynthesis have been discovered: the cyclization of geranylgeranyl diphosphate (GGPP) to taxa-4(5),11(12)-diene, the hydroxylation to taxa-4(20),11(12)-dien-5a-ol and the conversion of this alcohol to the corresponding acetate ester. The GGPP synthase from *T. baccata* cell suspension culture has been purified and characterized (Laskaris *et al.*, 1999, personal communication). Taxadiene synthase, the enzyme catalysing the cyclization of GGPP, has been purified, its mechanism of action elucidated, and the corresponding cDNA cloned (Koepp *et al.*, 1995; Hezari *et al.*, 1995; Lin *et al.*, 1996; Wildung and Croteau, 1996). Laskaris *et al.* (1999) demonstrated that the GGPP synthase activity, catalyzing the condensation of IPP and FPP forming the universal precursor of diterpenes GGPP, parallels taxane production in *T. baccata* cell suspension culture elicited by methyl jasmotate. The enzyme activity induction was followed by a sharp increase in taxane accumulation in cell cultures.

The next step, following the formation of GGPP and leading to the taxane skeleton, includes the cyclization of the GGPP to taxa-4(5),11(12)-diene catalyzed by taxadiene synthase. Hezari *et al.* (1997) have studied the production of **3** and taxadiene synthase activity in *T. canadensis* cell suspension culture. The results obtained from the analysis of the enzyme activity levels during the time course of **3** accumulation indicated that rate-limiting transformation step lay farther down the pathway than the cyclization step. Little is known about the diterpene pathway regulation in *Taxus*, including the dynamic process of interconversion of the intermediates. Laskaris *et al.* (1999) pointed out the fact that, unlike taxadiene synthase, GGPP synthase is inducible by methyl jasmonate, and that the induction pattern is followed by a similar accumulation pattern of taxanes. This observation points towards a regulatory role of GGPP synthase in taxane formation. The manipulation of its activity might be of importance for controlling the flux of intermediates towards **3**.

The *Agrobacterium tumefasciens*-mediated transformation of *Taxus* species has already

been described (Han *et al.*, 1994; Plaut-Carcasson, 1994) and taxadiene synthase cDNA is available. In addition, specific isoforms of GGPP synthase from *Taxus* cell culture have been purified and will be cloned in the near future. Therefore, it will soon be possible to evaluate, by sense or antisense experiments, the impact of genetic modification of such a regulative enzyme on taxoid production.

Finally, the side chain of **3** is formed from phenylalanine via β-phenylalanine and phenylisoserine (Fleming *et al.*, 1993). The benzoylation of the phenylisoserine nitrogen is the last step after attachment of the side chain to the C-13 position of **2** (Floss, 1995). Studies on the enzymatic esterification of the taxane structure to the side chain has not been reported yet.

7.6 THE SEARCH FOR TAXOID-PRODUCING MICROORGANISMS

Several research groups have addressed the **3** supply dilemma in many different ways. In 1993, Stierle *et al.* (1993) have discovered a new biological source of **3**: an endophytic microorganism colonizing the yew tree. Within two years, they have isolated more than 300 fungi from the bark and needles of *Taxus brevifolia*, a yew genus growing in Montana, Washington, Idaho, and Oregon. The promising producers of **3** (about 10% of the total fungi they isolated) have been studied, using a variety of different methods, including chromatography, mass spectrometry, antibody-based immunoassay, and feeding experiment with radioactive precursors followed by the isolation of labelled **3**. *Taxomyces andreanae* was the first described endophytic fungus of pacific yew that produce **3** in a semi-synthetic liquid medium. The yield initially measured with *T. andreanae* grown in mycological broth for 3 weeks reached 24 to 50 ng of **3**/liter of culture medium. Strobel *et al.* (1996) have also been interested in the search for other yew-associated microorganisms able to produce **3**. In this respect, they isolated and identified a second endophytic fungus, *Pestalotiopsis microspora*, from *Taxus wallachiane*, a Himalayan yew growing on steep, moist mountain slopes at altitudes of about 1500–3000 m. These geographic areas were found to have been preserved from agricultural practices with the rationale that yews growing in these areas would have a maximum advantage to form host-microbe relationships that might be involved in taxoid biosynthesis. Paclitaxel (**3**) isolated from *P. microspora* was demonstrated to be biologically active against 26 cancer cell lines, spectroscopically identical to authentic **3** and it accumulated in cultures at microgram levels per liter. Considering the enormous reproductive potential of the endophytic fungi via spore formation, Strobel and coworkers have postulated that paclitaxel-producing endophytes might be found in plants showing an ecology similar to that of yew but which doesn't produce taxoids. Confirming this hypothesis, these authors have isolated from *Taxodium distichum* (bald cypress) many strains of endophytic *Pestalotiopsis microspora*; the production of **3** was indeed confirmed in some of these fungi (Li *et al.*, 1996).

The results obtained by Strobel *et al.* (1996) indicate that endophytic fungi are excellent candidates for consideration in fermentation technology and open up a promising alternative route to solve the supply of **3**. Indeed, from a practical point of view, microbial fermentation as a means of bioactive taxoids production, compared to plant material, shows several advantages: large biomass production (microorganisms typically respond favorably to routine culture conditions), productivity amplification and stability, availability of less sophisticated and time consuming methods for genetic manipulation of microorganisms.

On the trail of fungi, a bacteria named *Brevibacterium* sp. has been patented for its potential to produce taxoids. Unfortunately, the origin of this microorganism and the yields were not clearly stated in the patent (Tahara, 1995).

A number of benefits could be expected from the production of **3** or precursors from microorganisms. They are related to environmental problems as well as to a better availability of a drug whose interest in cancer chemotherapy is ever-growing and still required more pharmaceutical and pharmacological investigations.

In addition, the fact that taxonomically different microorganisms associated with plants are able to synthetize some identical secondary metabolites, support the hypothesis of a possible horizontal gene transfer between *Taxus* species and their endophytic microorganisms and raise important biological questions.

7.7 GENERAL REMARKS AND PROSPECTS

The literature review related to bioactive taxoids from natural sources allowed the identification of three natural sources for production of **3** as summarized in Figure 7.2: extraction from natural growing or nursery-cultivated *Taxus* sp., hemisynthesis of bioactive taxoids using appropriate precursors extracted from *Taxus* needles, and biotechnological processes based on either *Taxus* cell culture or fermentation of taxoid-producing microorganisms. The long-term impact of yew harvesting on the environment, combined to the very low yield of the target compounds and the taxoids production instability by cell and microorganisms, represent the major limiting factors for bioactive taxoid supply.

According to estimations of yields of **3** expressed theoretically by day, it could be assumed that the best value obtained until now in cell cultures by Mirjalili and Linden (1995) (0.2% of dried weight) is much higher than those obtained with yew bark or needle (Table 7.2). However, the general problem associated with the production stability over subcultures is not solved yet. Consequently, no conclusion could be drawn from the production of **3** by microorganisms as the average content of **3** expressed in % dry weight, as well as the microorganisms growth rate, are not available.

Therefore, the biotechnological approach remains a potent way for bioactive taxoids production. In addition, these alternative methods offer opportunities to discover new

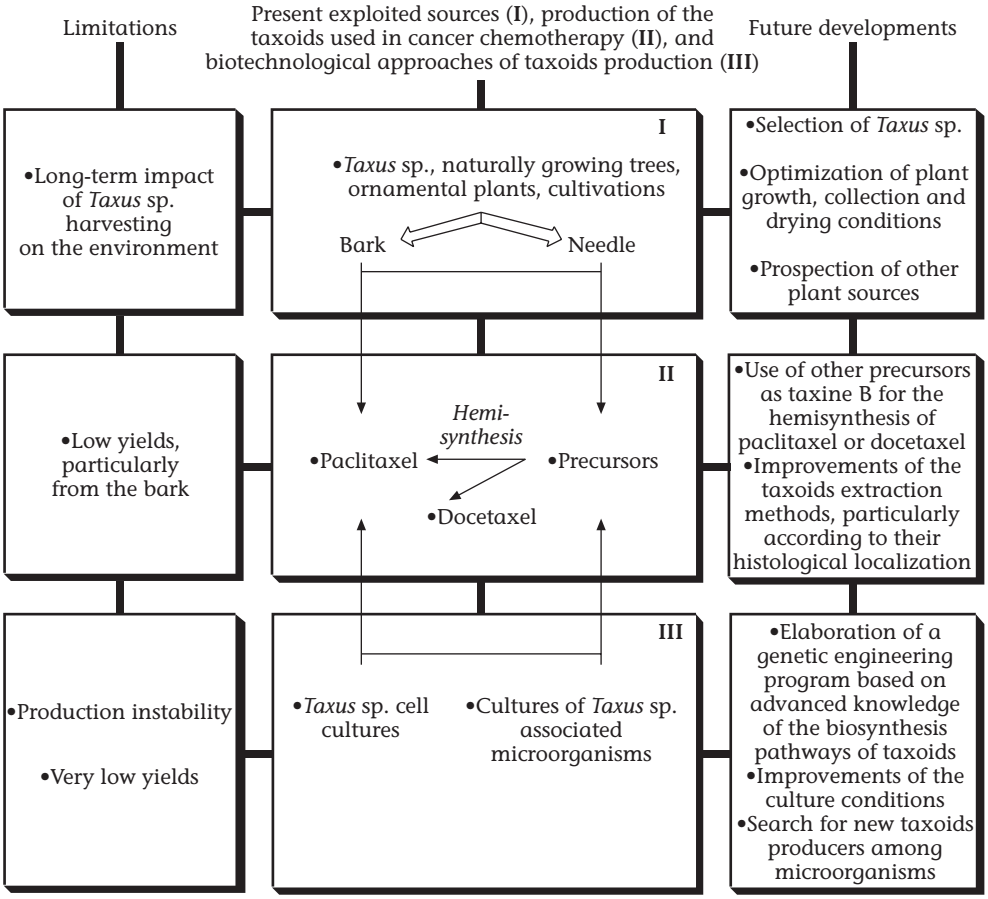

Figure 7.2: Exploited or considered natural sources for the production of paclitaxel and docetaxel, two taxoids extensively used in chemotherapy.

TABLE 7.2

Paclitaxel productivity from various natural sources

Source of paclitaxel	Specific biosynthetic rate	Average paclitaxel content (% dry weight)
	(mg/g/day)	
Bark of mature tree (100 years old) (Gibson *et al.*, 1995)	$4.70\ 10^{-6}$	0.017
Plantation (4 years old) (Gibson *et al.*, 1995)	$0.34\ 10^{-6}$	0.005
	(mg/l/day)	
Taxus cell culture (Mirjalili and Linden, 1995)	0.64	0.2
Taxomyces andreanae (Stierle *et al.*, 1993)	$2{,}4\ 10^{-6}$	unspecified

active metabolites and provide a powerful tool for the elucidation of the taxoid biosynthetic pathway.

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CHAPTER

8

Anti-HIV Aromatic Compounds from Higher Plants

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Contents

8.1 INTRODUCTION

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS) and is related to lentiviruses, a subfamily of retroviruses. HIV infects T-lymphocytes (Dagleish *et al.*, 1984), macrophages, dendritic cells and microglia cells of the central nervous system (Price, 1988; He *et al.*, 1997), rendering patients severely immunodeficient and sensitive to opportunistic infections.

HIV is an enveloped RNA virus encoding many proteins required for its growth in a host cell (Figure 8.1): surface glycoprotein gp120; transmembrane glycoprotein gp41

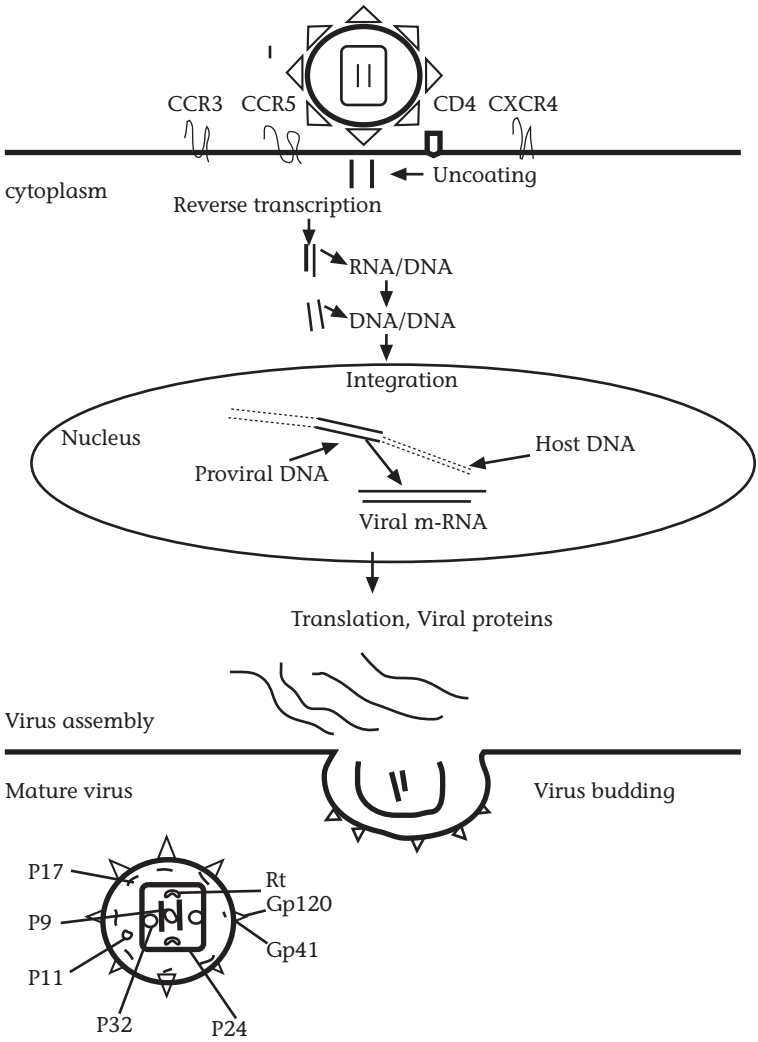


Figure 8.1: Replication cycle of HIV.

(linked together with a non covalent bond); matrix protein p17; capsid protein p24; nucleocapsid protein p9; enzymes: protease p11, integrase p32 and reverse transcriptase, RT; regulatory proteins Tat, Rev and Nef; accessory proteins Vpr, Vif and Vpu (Vpx for HIV-2). HIV-2 was first identified in 1985; although it is genetically similar to HIV-1, the genome is most homologous to the SIV which causes AIDS in macaque monkeys. HIV-2 is less pathogenic and fewer carriers develop full blown AIDS.

HIV has a very complex replication process in T-cells; the infection can be either lytic, which results in death of the host cell or persistent, where the host cell becomes chronically infected and continues to grow while producing virus.

8.2 HIV LIFE CYCLE (LYTIC INFECTION)

8.2.1 Cell/virus interaction

The envelope glycoprotein of HIV-1 is synthetised as a precursor product, which is cleaved by proteolysis to produce gp120 and gp41 (Allan *et al.*, 1985). Mature gp120 mediates virus infection of target cells and also the spread of virus by cell to cell fusion. It binds to main cellular receptor CD4 (the cell surface differentiation antigen) and undergoes a conformational change exposing hydrophobic fusion peptide at the N-terminus of gp41 to induce fusion of viral and host membranes. Co-receptors, CXCR4 in T-cell lines (Feng *et al.*, 1996), CCR3 and CCR5 in primary T-lymphocytes, macrophages and microglia cells are also critical for the infection (Deng *et al.*, 1996; Alkhatib *et al.*, 1996; He *et al.*, 1997).

8.2.2 Virus uncoating and reverse transcription

After the fusion, the viral genome is released into the cellular cytoplasm, and the viral RNA is converted into double-stranded DNA in order to allow integration into the host genome. The process of reverse transcription using enzyme reverse transcriptase (RT) is specific to retroviruses. The RT has three enzymatic functions: RNA-dependent polymerase converts viral RNA into RNA/DNA hybrid; RNAase activity degrades original viral RNA; and DNA-dependent polymerase activity adds a complementary strand to produce double-stranded DNA (Barber *et al.*, 1990; Varmus, 1988).

8.2.3 Integration

The enzyme integrase catalyses the cleavage and strand exchange reactions to integrate the proviral double-stranded DNA into the cellular DNA (Bushman and Craigie, 1991).

8.2.4 Transcription

At this stage, cellular DNA-binding proteins are utilised to make mRNA. However, HIV

replication is controlled by two RNA binding proteins: Tat, the transactivator and Rev, the regulator (Emerman and Malim, 1998, and references therein).

8.2.5 Packaging of viral genome and release of progeny virus

The process of virus assembly is not fully understood. The virus is released by budding from the cellular membrane and the HIV-1 protein Vpu appears to facilitate the final release of nascent viral capsid, and also the processing and transport of the viral envelope glycoproteins (Emerman and Malim, 1998).

8.2.6 Virus maturation

Viral protease belongs to the family aspartyl protease and has unique cleavage specificity. It is responsible for the cleavage of large precursor proteins into mature functional proteins, which is necessary for the production of infectious virus (Kohl *et al.*, 1988). Cellular enzymes (glucosidases and mannosidases) carry out the viral glycoprotein trimming and impaired glycosylation of envelope proteins also leads to the production of non-infectious virus. Myristoylation of the N-terminus of the viral gag, gag-pol and nef proteins is also catalysed by cellular enzyme N-protein myristoyl transferase (Gottlinger *et al.*, 1989).

8.2.7 Chronic infection

Once the viral DNA is integrated into the host genome and the infection has been established in a cell, a number of cell lines become chronically infected and resistant to new infection.

The complex growth cycle of HIV in its host cell as outlined above, has many potential targets for therapeutic interventions. Many drugs are being used for the treatment of AIDS, however, due to the emergence of drug-resistant mutants and the establishment of persistent infection, they have not been successful. Combination therapy, using drugs targeting different stages of virus infection is currently in use and it is proving to be better for the long-term survival of AIDS patients.

Medicinal plants, traditionally used for the treatment of various diseases, provide a rich source of novel compounds possessing diverse biological activities. Therefore, compounds derived from plants have been included in the drug-discovery programmes all over the world and, to-date, many have been reported to have anti-HIV properties.

The anti-HIV activity of compounds is generally measured in T-cell lines infected with different strains of HIV. Briefly, several concentrations of compounds are made in 96 well plates and incubated with cells and virus (HIV-1, HIV-2, or SIV), or with chronically infected cells, for 5–6 days at 37°C. The inhibition of infection is monitored by

examining syncytia formation, by measuring the cell viability using the XTT-Formazan method and by estimating viral antigen gp120 using ELISA or, finally, by titrating progeny virus released. Only the last two assays are used in chronically infected cells and for calculating $EC50_{50}$ values in all cell lines.

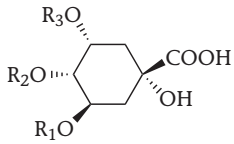
8.3 NATURAL PRODUCTS AS ANTI-HIV AGENTS

Natural products represent a rich and largely untapped source of structurally novel chemicals which are worth investigating as possible anti-HIV agents. A number of proteins and polysaccharides isolated from plants have been shown to have potent inhibitory activity against HIV-infection *in vitro*. Unfortunately, these macromolecules are not ideal therapeutic agents because they are not properly absorbed if taken orally, and are likely to cause allergic reactions if administered intravenously. Hence, in the following text, emphasis will be placed on anti-viral properties of aromatic compounds, which are the most commonly found constituents of many medicinal plants and have diverse biological properties.

8.3.1 Phenolics

Large phenolic compounds and tannins are capable of binding to proteins of diverse origins, sometimes in a non-specific manner (Moore and Pizza, 1992). They have been reported to inhibit viral enzymes (Brinkworth *et al.*, 1992; Fesen *et al.*, 1994; Nonaka *et al.*, 1990; Tan *et al.*, 1991; Robinson *et al.*, 1996) and infection (Nakashima *et al.*, 1992; Kashiwada *et al.*, 1995) as well as cellular DNA polymerases (Parker *et al.*, 1989).

However, quinic acid derivatives, 4,5-di (**2**) and 3,4,5-tri-O-caffeoylquinic acid (**1**) purified from *Securidaka longipedunculata* and 3,4,5-tri-O-galloylquinic acid (**3**) from *Guiera senegalensis,* inhibited HIV infection in a different manner (Mahmood *et al.*, 1993a). The antiviral activity of the purified compounds **1–6** (Figure 8.2) was tested in HIV- and SIV-infected C8166 cells and herpes simplex virus type 1-infected VERO cells and the results are presented in Table 8.1. The EC_{50} values for HIV-1 infection were comparable for compounds **1–3**, but more significant differences were seen in terms of toxicity. The lower toxicity of compound **1** gave a higher selectivity index (SI) of over 300, more than threefold that of compounds **2** (SI = 67) or **3** (SI = 94). At the non-toxic concentration of 40 μg/ml compound **1** suppressed completely the formation of sincytia and production of p24 antigen as well as the cytopathic effect of HIV-1 infection. Non-toxic concentration of compounds **4** and **5** and quinic acid had no effect on virus infection, whereas compound **6** exhibited only a slight selective anti-HIV-1 activity (Table 8.1). Compounds **1** and **2** were much less inhibitory to HIV-2 infection, the EC_{50}'s being more than 10-fold greater than for HIV-1 infection, and they exhibited intermediate activities against SIV infection. The inhibition of HIV infection was more pronounced



	R_1	R_2	R_3
1	Caffeoyl	Caffeoyl	Caffeoyl
2	-H	Caffeoyl	Caffeoyl
3	Galloyl	Galloyl	Galloyl

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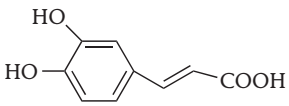

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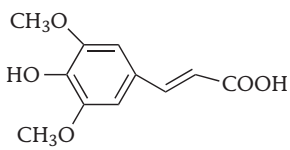

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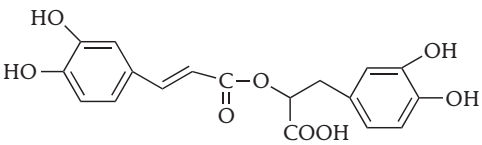


Figure 8.2: Chemical structures of anti-HIV plant metabolites **1–6**.

TABLE 8.1

Antiviral activity of compounds **1–6**

		$HIV\text{-}1_{IIIB}$	$HIV\text{-}2_{ROD}$	SIV_{MAC}	Herpes	
Compounds	TC_{50}	EC_{50}[a]	EC_{50}	EC_{50}	TC_{50}	EC_{50}
1	100	0.32	20	2	200	0.08
2	40	0.6	8	2	100	0.16
3	15	0.16	ND	ND	ND	ND
4	200	200	>200	>200	200	>50
5	200	200	ND	ND	ND	ND
6	100	40	100	80	150	20
AZT	>1000	0.01	0.016	0.02	ND	ND
Ganciclovir	ND	ND	ND	ND	100	0.08

[a] EC_{50} values are the concentration in μg/ml which inhibited by 50% the production of gp120 of HIV or SIV, or herpes simplex virus type 1 surface antigens; TC_{50} values represent the concentrations which reduce by 50% the growth of uninfected cell controls.

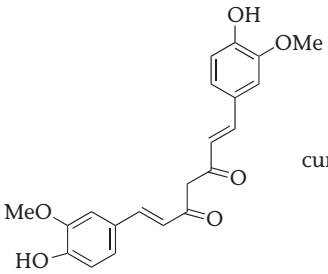
when compounds were present during virus adsorption than when added one or more hours after infection, suggesting that they influence binding of virus to cells. This conclusion was reinforced by the ability of the compounds to inactivate virus infectivity, to inhibit the binding of gp120 to CD4, and to reduce fusion between chronically infected and uninfected H9 cells. Brief treatment of infected cells with 10 μg/ml compound **1** reduced syncytium formation by more than 50%. Under the same conditions, dextran sulphate enhanced virus infection, and syncytium formation was only inhibited when the compound was present during the mixing of chronically infected and uninfected H9 cells. A similar difference between dextran sulphate and compounds **1** and **2** was observed in their effects upon the binding of soluble CD4 (sCD4) to gp120. The inhibition of gp120-sCD4 interaction by compounds **1** and **2** was only reduced slightly by removal of compound prior to addition of CD4, whereas to be effective, dextran sulphate needed to be present during binding. The interaction of compounds **1** and **2** with gp120 thus appeared largely irreversible (Mahmood *et al.*, 1993a).

The 'specific' interaction of compounds **1** and **2** with gp120 was further illustrated by their ability (like dextran sulphate) to prevent binding of monoclonal antibodies 358 and 388, known to interfere with gp120-CD4 interaction and neutralize virus infectivity. Antibody 358 recognises the V3 loop and antibody 388 the CD4 region (Cordell *et al.*, 1991) of HIV-1 gp120. The compounds had no effect on the interactions of monoclonal antibodies 323 and 360 with the C-terminal and N-terminal regions, respectively, of gp 120. The galloyl derivatives were previously shown to inhibit the *in vitro* activities of both HIV RT and cellular DNA polymerases, in particular DNA polymerase α (Nishizawa *et al.,* 1989; Parker *et al.,* 1989). However, the ability of the non-ionic detergent Triton(−)X-100 as well as BSA to neutralize the inhibitory effects of **1** and **2** suggests that inhibition of the viral enzyme *in vitro* is non-specific.

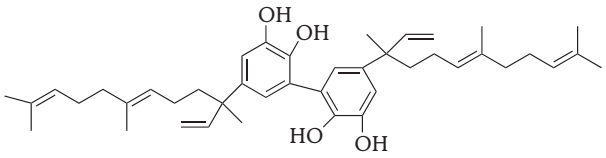
Therefore, it is unlikely that RT represents the main site of antiviral action, although we cannot exclude some effect even of impaired host cell DNA polymerase activity. Although the presence of serum also reduced the inhibition of gp120-CD4 interaction (data not shown), the compounds retained their ability to inhibit binding of virus to cells under these conditions. Recently, Robinson *et al.* (1996) have reported inhibition of enzyme integrase *in vitro* by three compounds, 3,5-dicaffeoylquinic acid, 1-methoxyoxalyl-3,5-dicaffeoylquinic acid and L-chicoric acid at concentrations ranging from 0.06–0.66 μg/ml.

Two aryl units, one of which contains the 1,2-dihydroxy pattern, separated by an appropriate linker segment seem to be necessary for the HIV-IN inhibiting activity. Curcumin (**7**) (Figure 8.3), which bears strong similarity to caffeic acid phenyl ethyl ester, afford an example of a moderately potent HIV-1-1N inhibitor which does not contain the catecol pattern (Burke *et al.*, 1995; Zhao *et al.*, 1997).

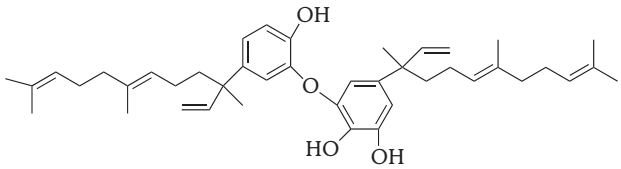
Chemical studies on *Pothomorpha peltata,* growing in the Dominican Republic, afforded three new cathecol, namely peltatols A (**8**), B (**9**), and C (**10**) (Figure 8.3). These



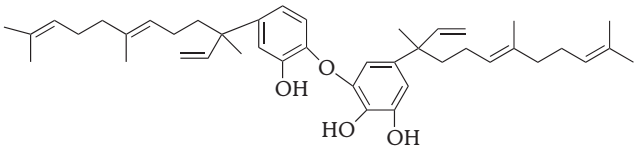
curcumin **7**



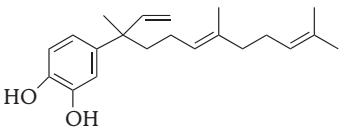
Peltatol A (**8**)



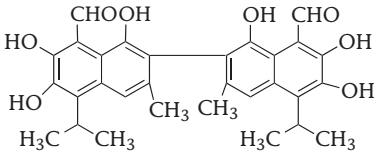
Peltatol B (**9**)



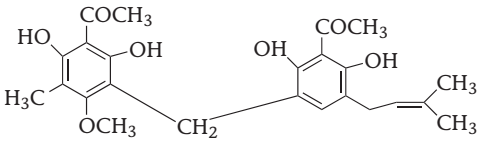
Peltatol C (**10**)



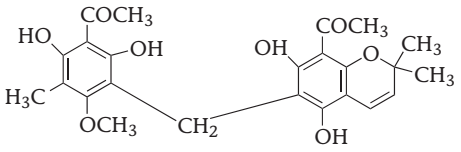
4-nerolidylcatechol (**11**)



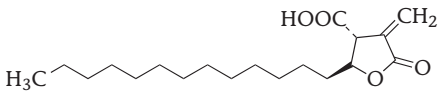
gossypol (**12**)



Mallotojaponin (**13**)



Mallotochromene (**14**)



protolichesterinic acid (**15**)

Figure 8.3: Chemical structures of anti-HIV plant metabolites **8–15**.

compounds inhibit HIV-1 induced cell killing at subtoxic concentrations of 1–10 μg/ml. The derivative 4-nerolidylcatechol (**11**) (Figure 8.3) was inactive in the anti-HIV screen, however, it weakly blocked forbol receptor binding (Gustafson *et al.*, 1992).

Gossypol (**12**) (Figure 8.3), a polyphenolic aldehyde extracted from cottonseed, is active against enveloped viruses, including HIV. Evaluation of the pure enantiomers showed the (−)-enantiomer of gossypol to be very inhibitory (EC_{50} = 1.0–5.0 μM), whereas the (+)-enantiomer was much less active (EC_{50} = 50–100 μM) (Lin *et al.*, 1989). Since gossypol was quite cytotoxic in cell cultures, several analogues were prepared, in order to increase the SI (Lin *et al.*, 1993). Although differences in inhibition of cytotoxicity were found, none was of the magnitude to justify further study, so that its potential application is only to be considered as an external virucidal agent.

Other plant-derived phenolics with anti-HIV RT activity include several phoroglucinol derivatives such as mallotojaponin (**13**) and mallotochromene (**14**) (Figure 8.3), both isolated from the pericarps of *Mallotus japonicum* (Nakane *et al.*, 1991), which inhibited, at a concentration of 10 μg/ml, the enzyme activity by 67 and 75%, respectively, with (rA)n $(dT)_{12–18}$ as the template primer. The degree of inhibition was dose-dependent; in the case of mallotojaponin, a concentration of 25 μg/ml caused more than 90% inhibition. The enzyme activity was also inhibited, though to lesser extent, by these compounds in the presence of initiated MS-2 phage RNA as the template primer.

Furthermore an aliphatic α-methylene-γ-lactone, protolichesterinic acid (**15**) (Figure 8.3) isolated from the lichen *Cetraria islandica* was shown to inhibit HIV-RT. Compound **15** was evaluated for its potential to inhibit nucleic acid polymerases HIV-1 RT, HIV-2 RT, DNA polymerase and RNA polymerase. It inhibited DNA polymerase β most effectively (IC_{50} = 7.4 μM), with the remaining polymerases being inhibited with IC_{50} in the 20–30 μM range (Pengsuparp *et al.*, 1995). Since the inhibitory activity was greatly reduced by increasing the concentration of bovine serum albumine (BSA), it is likely that this compound interacts with the enzyme non-specifically to exert its inhibitory activity (Pengsuparp *et al.*, 1995).

8.3.2 Flavonoids

Flavonoids structurally resemble nucleosides, isoalloxazine and folic acid. They have strong affinity for binding to proteins and heavy metals and are able to scavenge free radicals. A review by Havsteen (1983) describes properties of flavonoids in detail. Flavonoids have been purified from a number of medicinal plants (Aquino *et al.*, 1990, 1991) and have been studied in some detail in our laboratory (Table 8.2) for their inhibitory properties against HIV-infection of C8166 cells and also in chronically

TABLE 8.2
Anti-HIV activity of flavonoids **16–43**

No.	Compound	Source	EC_{50}[a]	TC_{50}[b]	SI[c]
16	Chrysin (5,7-dihydroxyflavone)	Sigma	20	50	2.5
17	Myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone)	Sigma	2	40	20
18	Myricetin-3-O-rhamnoside	*Befaria cinnamomea*	100	>200	>2
19	Kaempherol (3,4′,5,7-tetrahydroxyflavone)	*Rosa damascena*	4	70	17.5
20	Kaempherol-3-O-glucoside	*R. damascena*	8	>200	>25
21	Kaempherol-7-O-β-D-glucopyranoside	*R. damascena*	>250	>250	1
22	Kaempherol-3-O-(6-O-trans-p-coumaroyl)-β-glucopyranoside	*R. damascena*	50	>100	>2
23	Quercetin (3,3′,4′,5,7-pentahydroxyflavone)	*R. damascena*	20	100	5
24	Quercetin-7-O-β-D-glucopyranoside	*R. damascena*	>250	>250	1
25	Quercetin-3-O-rhamnoside	*B. cinnamomea*	50	>100	>2
26	Quercetin-3-O-arabinoside	*B. cinnamomea*	>100	>100	1
27	Quercetin-3-O-galactoside	*Myntostachis setosa*	>100	>100	1
28	Quercetin-3-O-glucoside	*M. setosa*	50	>100	>2
29	Isorhamnetin (3,4′,5,7-tetrahydroxy-3′-methoxyflavone)	*M. setosa*	>100	>100	1
30	(+)Gallocatechin (3,3′,4′,5,5′,7-hexahydroxyflavan)	*Croton draconoides*	5	>80	>16
31	(−)epigallocatechin	*C. draconoides*	>100	>100	1
32	(−)epicatechin (3,3′,4′,5,7-pentahydroxyflavan)	*Detarium microcarpum*	2	>100	>50
33	(+)Catechin	*D. microcarpum*	4	>100	>25
34	(−)epicatechin-3-O-gallate	*D. microcarpum*	1	>100	>100
35	(+)catechin-7-O-gallate	*D. microcarpum*	10	>100	>10
36	4′-methoxy-5,7-dihydroxyflavanone-7-O-rutinoside	*M. setosa*	>40	40	>1
37	Aromadendrin, 3,5,7,4′-tetrahydroxyflavanone	*Cuscuta reflexa*	10	>200	>20
38	Aromadendrin-7-O-β-D-glucopyranoside	*C. reflexa*	20	200	10
39	3,5,7,8,4′-pentahydroxyflavanone	*C. reflexa*	40	>100	>2.5
40	Taxifolin (3,5,7,3′,4′-pentahydroxyflavanone)	*C. reflexa*	50	100	2
41	Taxifolin-7-O-β-D-glucopyranoside	*C. reflexa*	100	350	3.5
42	Prunin-7-O-β-D-glucopyranoside	*C. reflexa*	40	>100	>2.5
43	Coccinoside B (5,7,2′,5′-tetrahydroxy-7-O-β-D-glucopyranosl flavanone)	*C. reflexa*	20	>100	>5

[a] EC_{50} values are the concentration in μg/ml which inhibited by 50% the production of gp120.
[b] TC_{50} values represent the concentrations which reduce by 50% the growth of uninfected cell controls.
[c] SI is a ratio of the TC_{50} value/EC_{50} value.

infected H9 cells using methods previously described (Mahmood, 1995). It was found that small differences in their structures had great influence on their inhibitory properties and specificity (Mahmood *et al.*, 1993b, 1996, 1997).

Flavans exhibited the most selective anti-HIV activity. The galloyl derivative (−)-epicatechin 3-*O*-gallate (**34**) consistently exhibited the greatest activity, EC_{50} of 1 μg/ml and selectivity index >100, and was somewhat more active than (−)-epicatechin (**32**). Differences between isomers were noted in the lower activity of (+)-catechin (**33**) and particularly dramatically in the absence of any selective activity of (−)-epigallocatechin (**31**) in contrast to (+)-gallocatechin (**30**). Substitution of the hydroxyl at position 7 of (+)-catechin (**33**) by a gallate moiety in **35** caused a reduction of the SI (Table 8.2).

Like quinic acid derivatives, compound **34** inhibits virus absorption to cellular receptors by binding to gp120, as pre-incubation of free virus with the compound prior to the addition of cells results in total inactivation of viral-infectivity, suggesting irreversible interaction with gp120 (Mahmood *et al.*, 1993b).

Of all flavones tested, myricetin (**17**) (SI = 20), kaempferol (tetrahydroxyflavone) (**19**) and its 3-O-glycosides (**20** and **22**), caused significant inhibition of HIV-1 infection at non-toxic concentration (Table 8.2).

The mechanism of action of flavones **17**, **20**, **22**, **23** and flavanones **37**, **38** and **40** is similar to that of flavans inhibiting gp120/CD4 interaction in a dose-dependent manner. However, quercetin (**23**), myricetin (**17**), taxifolin (**40**) and flavans are less specific and inhibit viral enzymes protease (Mahmood *et al.*, 1996; Brinkworth *et al.*, 1992) and RT as measured by specific methods using viral enzymes. Conversely, kaempherol (**19**) is highly specific for inhibiting protease, only at concentrations which are not toxic to cells (EC_{50}, 2 μg/ml). It also inhibits viral infectivity from chronically infected H9 cells and processing of precursor proteins, as judged by sodium dodecyl sulphate (SDS) gel electrophoresis and immunoblotting analysis, confirming that it is a protease inhibitor (Mahmood *et al.*, 1996). Quercetin (**23**) and related pentahydroxyflavones have also been reported to inhibit enzyme integrase (Fesen *et al.*, 1993) but chrysin (**16**), a dihydroxyflavone was inactive. It was a potent inhibitor of TNF-α (tissue necrosis factor) or PMA (phorbol ester) stimulated expression of HIV-1 from latently infected cells. Chrysin (**16**), acacetin (**44**) and apigenin (**45**) (Figure 8.4) inhibited the expression of HIV-1 with selectivity indices of 9.6, 8.5 and 5.1 respectively (Critchfield *et al.*, 1996).

Recently, four new flavonol 7-O-glycosides purified from *Bidens leucantha* were inactive against HIV-infection of C8166 cells, whereas four chalcone ester glycosides inhibited infection with selectivity indices ranging from 5 to over 10 (De Tommasi *et al.*, 1997).

One flavone, i.e. baicalein (**46**) (Figure 8.4) and three flavonols namely quercetin (**23**), myricetin (**17**) and quercetagetin (**47**) (Figure 8.4), have been shown to inhibit *in vitro* RTs of certain retroviruses, including Ruascher murine leukemia virus (RLV) and HIV, as well as cellular DNA polymerase (Ono *et al.*, 1989). Comparative studies with

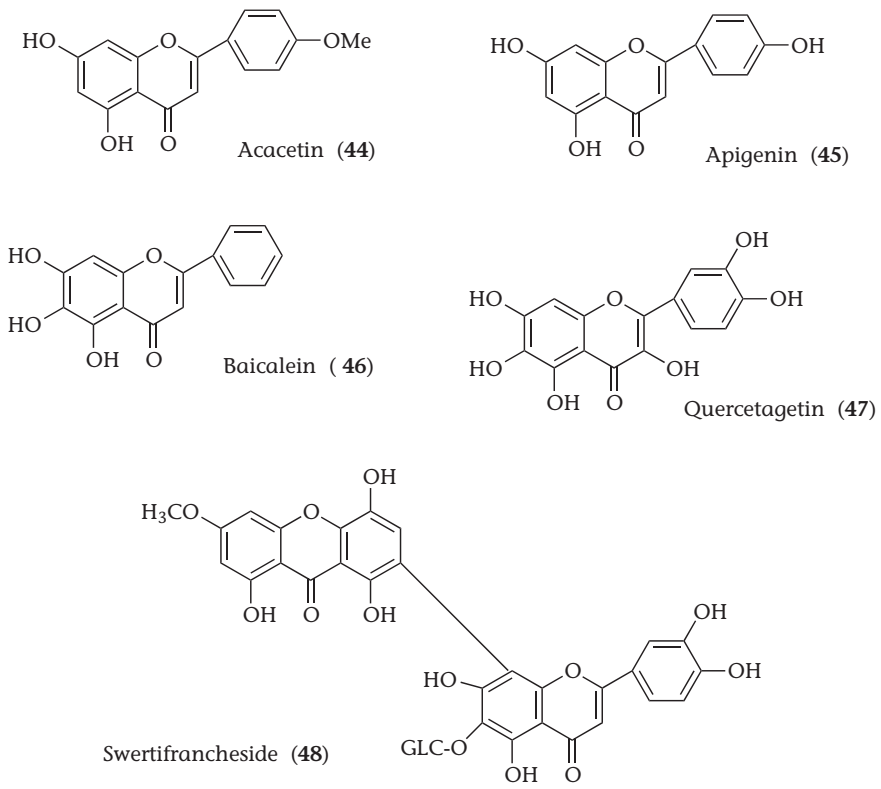


Figure 8.4: Chemical structures of anti-HIV plant metabolites **44–48**.

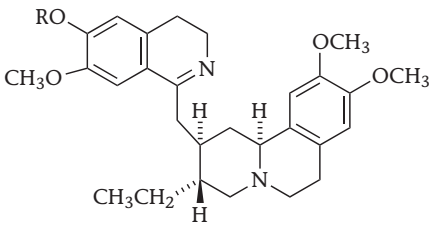
other flavonoids revealed that the presence of both double bond between positions 2 and 3 of the flavonoid pyrone ring, and the three hydroxy groups introduced on positions 5, 6, and 7 (i.e. baicalein), were a prerequisite for the inhibition of RT-activity. Removal of the 6-hydroxy group of baicalein (**46**) required the introduction of three additional hydroxyl groups at positions 3,3′ and 4′ (quercetin) to afford a compound still capable of inhibiting the RT-activity. Quercetagetin (**47**) which contains the structure of both baicalein (**46**) and quercetin (**23**), and myricetin (**17**) which has the structure of quercetin (**23**) with an additional hydroxy group on the 5′ position also provide strong inhibitors of RT activity. The inhibition by baicalein (**46**) of RT is highly specific, whereas quercetin (**23**) and quercetagetin (**47**) were strong inhibitors of DNA polymerase β and DNA polymerase I, respectively. Myricetin (**17**) was also a potent inhibitor of both DNA polymerase α and DNA polymerase I. These facts suggested that baicalein (**46**) might be less toxic to the retroviruses-infected host cell DNA and RNA polymerases than the flavonols (Ono *et al.*, 1989). Spedding *et al.* (1989) found the biflavonoid amentoflavone besides scutellarein and quercetin to be the most active inhibitors of

AMV-RT, Rous-associated virus-2 (RAV-2)-RT and Moloney murine leukemia virus (MMLV)-RT out of eighteen flavonoids tested.

Also, swertifrancheside (**48**), a new flavanone-xanthone glucoside isolated from *Swertia frachetiana*, was found to be a potent inhibitor of DNA polymerase activity of HIV-1 RT with IC_{50} of 43 μM (Pengsuparp *et al.*, 1995).

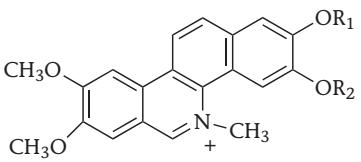
8.3.3 Alkaloids

Various alkaloids exhibited anti-HIV activity in different test systems. Psychotrine dihydrogen oxalate (**49**) and *O*-methylpsychotrine sulfate heptahydrate (**50**), the salts of isoquinoline alkaloids from ipecac (Figure 8.5), were found to be potent inhibitors of the



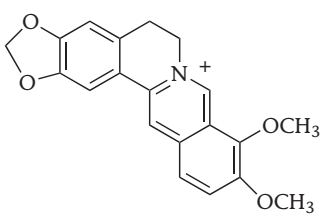
R = H Psychotrine (**49**)

R=CH_3 *O*-Methylpsychotrine (**50**)

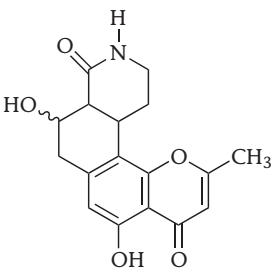


R_1=H R_2=CH_3 Fagaronine (**51**)

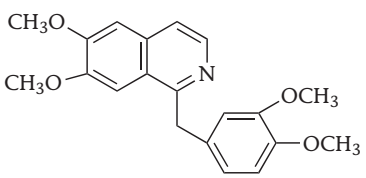
R_1= R_2= -CH_2- Nitidine (**52**)



Berberine (**53**)



Schumannificine (**54**)



Papaverine (**55**)

Figure 8.5: Chemical structures of anti-HIV plant metabolites **49–55**.

DNA polymerase activity of human immunodeficiency virus-1 reverse transcriptase (HIV-1 RT) (Tan *et al.*, 1991).

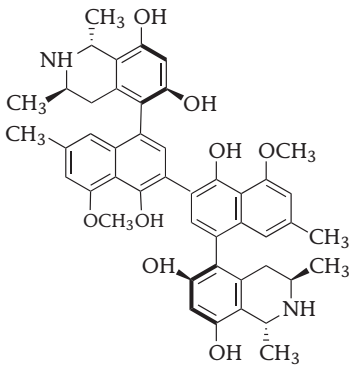
The plant families Rutaceae and Papaveraceae are well known for possessing an interesting class of isoquinoline alkaloids with noticeable biological properties. Fagaronine chloride (**51**), nitidine chloride (**52**) and berberine chloride (**53**) (Figure 8.5) (a typical representative of protoberberine alkaloids) inhibit HIV RT (Tan *et al.*, 1991) as well as other enzymes, including several mammalian polymerases (Sethi, 1976). A unique group of alkaloids, containing a chromone ring system purified from *Schumanniophyton magnificum*, exhibit activity against HIV-1 infection of C8166 cells. The compound piperidone schumannificine (**54**) (Figure 8.5) shows the highest selectivity and binds to the envelope protein interfering with its interaction with the cellular receptor. However, it also inhibits herpes simplex type 1, with high selectivity (Houghton *et al.*, 1994).

Papaverine (**55**) (Figure 8.5), found in the opium poppy *Papaver somniferum* and commonly used as a smooth muscle relaxant, has also been reported to inhibit HIV-envelope protein expression, hence virus infection *in vitro* (Turano *et al.*, 1989). In a report involving a limited number of patients (Basetti *et al.*, 1989), recovery of immunological cutaneous responsiveness was noted after administering papaverine orally for 4 weeks. This was also associated with 50% improvement in their absolute T_4 cells counts. Preliminary experiments indicated that the combination of suboptimal concentration of both papaverine and AZT enhanced their inhibitory effects on HIV replication and HIV-induced cytophatic effects. The fact that papaverine has potent antiviral effects against CMV *in vitro* might make it attractive for use in combination therapy of AIDS patients with CMV disease.

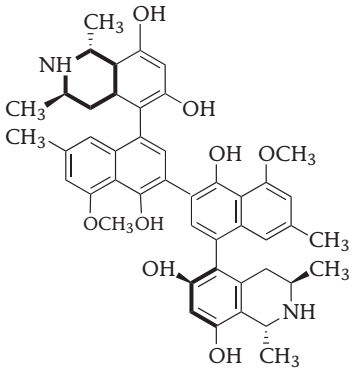
Michellamines A (**56**), B (**57**) and C (**58**), dimeric naphthylisoquinoline alkaloids (Figure 8.6) from *Ancistrocladus korupensis* have been studied in various cell lines and against a number of biologically diverse laboratory and clinical strains of HIV-1, including the AZT-resistant strains and HIV-2. Biochemical studies of the mechanism of action of the most potent and abundant member of the series, Michellamine B (**57**) (Boyd *et al.*, 1994), showed that this alkaloid acts, both at an early stage of the HIV-life cycle by inhibiting reverse transcriptase (RT), as well as at later stages by inhibiting cellular fusion and syncytium formation. Michellamine B (**57**) has been committed to preclinical drugs development by the National Cancer Institute.

In the course of a scale-up isolation of Michellamine B (**57**), several related 'monomeric' alkaloids, korupensamines and three new dimeric alkaloids Michellamine D (**59**), E (**60**), F (**61**) were isolated and identified. Michellamine D-F, in preliminary comparative testing against several strains of HIV-1 in various infected host cells, exhibited cytoprotection comparable to Michellamine B (EC_{50} 2–6 μM), but Michellamine D and E appeared to be one order of magnitude more cytotoxic to the host cells (Hallock *et al.*, 1997).

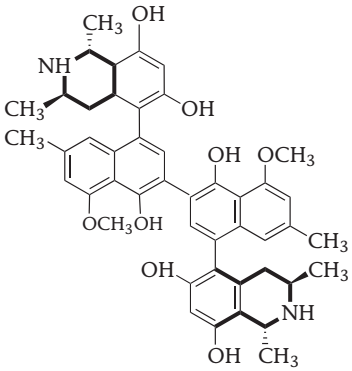
The inhibition of HIV-infectivity has been reported for some alkaloids through the



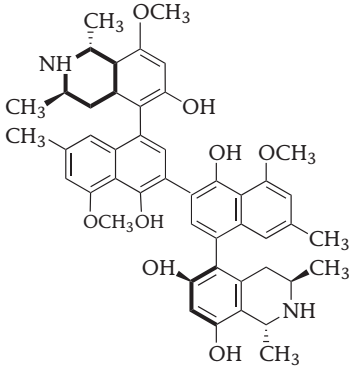
Michellamine A (**56**)



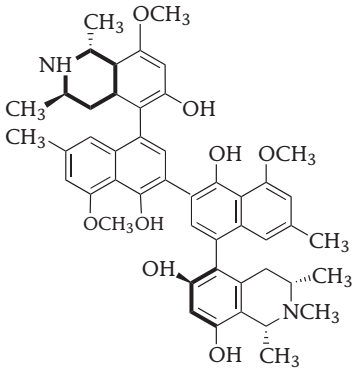
Michellamine B (**57**)



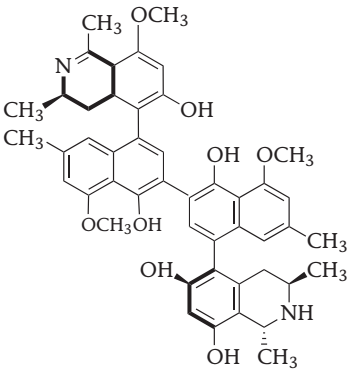
Michellamine C (**58**)



Michellamine D (**59**)



Michellamine E (**60**)



Michellamine F (**61**)

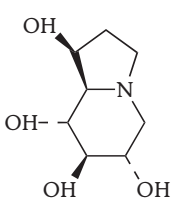
Figure 8.6: Chemical structures of anti-HIV plant metabolites **56–61**.

inhibition of the nitrogen-linked glycosidation process of HIV. The most representative alkaloid which showed this mechanism of action is castanospermine (**62**), a not aromatic compound which we mention here for the peculiarity of the mechanism of action. Castanospermine (1,6,7,8, tetrahydroxyoctahydroindolizidine) (**62**) (Figure 8.7) is a plant alkaloid isolated from the seeds of an Australian chestnut tree, *Castanospermum australe* (Nash *et al.*, 1988). This alkaloid has been shown to be a potent inhibitor of the endoplasmic reticulum enzyme α-glucosidase I, thereby preventing removal of glucose residues during the normal processing of glycoproteins. The resultant proteins contain incompletely processed carbohydrate chains composed of Glc3-Man7-9(GlcNAc)2, and this alteration has had profound effects on cell surface expression and function of some glycoproteins. It has been shown to display *in vitro* antiviral activity against retroviruses, including Rauscher murine leukemia, Moloney murine leukemia virus, and HIV (Sunkara *et al.*, 1987). Castanospermine (**62**) can inhibit both envelope glycoprotein-mediated syncytium formation and HIV infection of $CD4^+$ cells. In acute HIV infection of susceptible $CD4^+$ lymphocytes, the presence of castanospermina dramatically inhibits the spread of virus. Walker *et al*. (1987) suggest that profound inhibitory effects of castanospermina on HIV infection can be attributed to reduction of the virion infection and to a reduction in cell-to-cell spread of virus action by inhibition of cell fusion events. These observations suggested that castanospermine, as well as other drugs that modify the glycosylation of the HIV envelope glycoprotein, may be of use in the treatment of HIV infections.

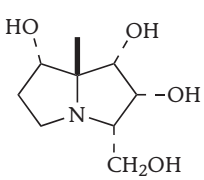
In addition to castanospermine, (**62**) also 6,7-diepicastanospermine and several polyhydroxylated pyrrolizidine alkaloids such as australine (**63**) (Figure 8.7), and analogues, showing varying degrees of inhibitory activity against glycosidases, have been isolated from *Castanospermum australe* and *Alexia leopetala* (Nash *et al.*, 1988).

8.3.4 Quinones

Hypericin (**64**) and pseudohypericin (**65**) (Figure 8.8) are polycyclic diones, occurring in plants of the genus *Hypericum*, the most well known being *H. perforatum*. Both of the

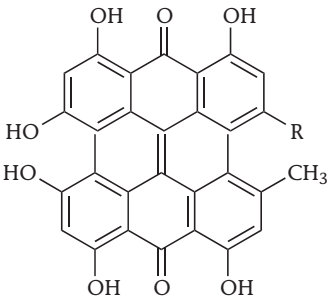


Castanospermine (**62**)



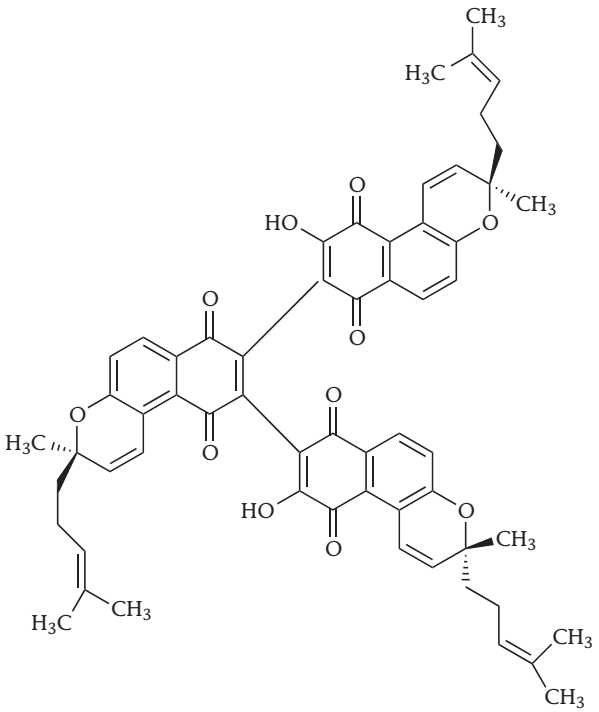
Australine (**63**)

Figure 8.7: Chemical structures of anti-HIV plant metabolites **62–63**.



R=CH_3 Hypericin (**64**)

R=CH_2OH Pseudohypericin (**65**)



Conocurvone (**66**)

Figure 8.8: Chemical structures of anti-HIV plant metabolites **64–66**.

above compounds have been studied in some detail. Hypericin (**64**) and pseudohypericin (**65**) seem to interfere with proper assembly and maturation of virus cores of particle budding from cells (Meruelo *et al.*, 1992). Visible light is essential for all of the antiviral effects of the two compounds. Upon illumination, these compounds inactivate enveloped but not unenveloped viruses, acting as virucidal agents (Lenard *et al.*, 1993).

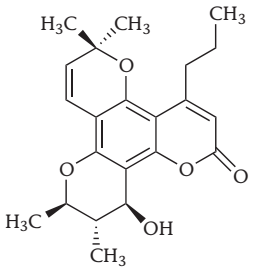
Only when the concentration of hypericin (**64**) approached the cytotoxic level could an apparent light-independent antiviral effect be observed. These compounds also inactivate both the infectivity and reverse transcriptase activity of intact viruses that have undergone proper assembly and budding (Lavie *et al.*, 1989). Several explanations might account for the interference with proper assembly and/or maturation of retroviruses by hypericin. Alternatively, hypericin (**64**) could interfere with important steps occurring in virus assembly at the cell membrane. For example, an hypothesis has been proposed suggesting that gag and gag-pol polyproteins become associated at the cell surface membrane to interact at their amino termini with a transmembrane segment of the envelope-glycoprotein complex and interact at their carboxyl termini with genomic RNA. As stated above, hypericin (**64**) and pseudohypericin (**65**) treatment also results in complete inactivation of reverse transcriptase of HIV in murine and human viruses. It is important to clarify that this effect is not due to interference by hypericin (**64**) or pseudohypericin (**65**) with the reverse transcriptase enzymatic reaction because hypericin (**64**) or pseudohypericin (**65**) do not affect reverse transcriptase activity when commercially purified enzyme is used. An explanation for this direct inactivation of virus consistent with the interference with polyprotein processing by hypericin that leads to improper assembly and/or maturation of retrovirus particles would be as follows (for which no data are yet available). Reverse transcriptase within the core of intact virus would be assumed to be an inactive enzyme or proenzyme kept nonfunctional, either by tight association with another viral core component, or by its tertiary structure within the virion core. Such an inactive form might be rendered active by viral-encoded proteases or by other mechanism (e.g. kinases) during infection of cells or whenever virions are disrupted. Hypericin (**64**) could then interfere with this activation or release of reverse transcriptase. Purified reverse transcriptase is not affected by hypericin (**64**), because enzyme activation or release had already occurred during purification of the enzyme from virus particles.

The difference in activities between hypericin (**64**) and pseudohypericin (**65**) is an important additional observation. The availability of two structurally related compounds that differ in a single hydroxyl group side-chain substitution, yet have significantly different biological activities, enables a preliminary insight into structure-function relationships. With a main common structural feature of a flat core of eight fused aromatic rings encircled by six phenolic hydroxyls, the substitution for the seventh hydroxyl group that occurs in pseudohypericin clearly diminishes antiretroviral activity. They are also potent inhibitors of the enzyme protein kinase C (Takahashi *et al.*, 1989).

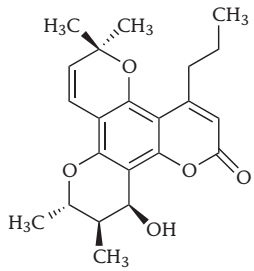
Recently, a trimeric naphtoquinone, conocurvone (**66**) (Figure 8.8), isolated from an extract from a *Canospermun* species has been shown to be an anti-HIV principle by preventing the replication of HIV in human T-lymphoblastic cells in a concentration range of 0.02–50 μM. The related monomer teretifolione isolated from the same extract was found to be inactive against HIV (North *et al.*, 1993).

8.3.5 Coumarins

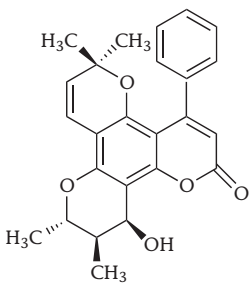
Coumarin derivatives are another class of polycyclic compounds reported to have anti-HIV properties *in vitro*. Inophyllums purified from *Calophyllum inophyllum*, are inhibitors of RT and HIV replication in cell cultures (Patil *et al.*, 1993). Calanolides A (**67**) and B (**68**) (Figure 8.9), isolated from the leaves of *Calophyllum lanigerum* inhibit the *in vitro* replication of HIV-1 in lymphoblastic cells, but are inactive against HIV-2 (Kashman *et al.*, 1992). They inhibit the DNA-dependent DNA polymerase and RNA-dependent DNA-polymerase activities of HIV-1 RT. Moreover, calanolide A (**67**) was not only active



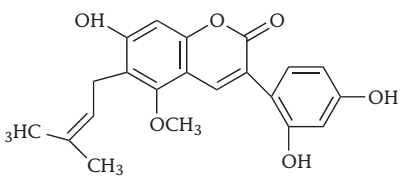
(+)- Calanolide A (**67**)



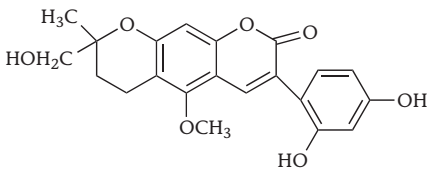
(-)- Calanolide B (**68**)



Soulattrolide (**69**)



Glycocoumarin (**70**)



Lycopyranocoumarin (**71**)

Figure 8.9: Chemical structures of anti-HIV plant metabolites **67–71**.

against AZT-resistant G-9106 strain of HIV-1 but also against the pyridone-resistant A17 strain, the latter being highly resistant to HIV-1 specific non-nucleoside RT inhibitors such as TIBO and nevirapine (Kashman *et al.*, 1992). On the basis of these findings (+)-calanolide A (**67**) has been committed to preclinical drug development by the US NCI. It has been shown that only (+)-calanolides A (**67**) and (−)-calanolide B (**68**) are potent HIV-inhibitors (EC_{50} 0.2 and 0.3 μM, respectively) while (−)-calanolide A and (+)-calanolide B are inactive against the virus (Cardellina *et al.*, 1995). While (+)-calanolide A (**67**) is only a minor compound of the leaves of *Calophyllum lanigerum,* (−)-calanolide B (**68**) is abundantly present in the latex of *Calophyllum teysmanmii*, which also contains a related coumarin, soulattrolide (**69**) (Figure 8.9) (Fuller *et al.*, 1994). (−)-Calanolides B (**68**) and soulattrolide (**69**) could represent alternative molecules to calanolides A (**67**) (Pengsuparp *et al.*, 1996).

Suksdorfin from the fruit of *Lomatium suksdorfii* inhibits HIV infection in H9 cells with an EC_{50} of 2.6 μM but the mode of action of this coumarin appears to be different from inophyllums as it has no inhibitory effect on the enzyme RT (Lee *et al.*, 1994). Two coumarins isolated from *Glycyrrhiza glabra*, namely glycocoumarin (**70**) and licopyranocoumarin (**71**) (Figure 8.9), were found to inhibit giant cell formation in HIV-infected cell cultures without any cytotoxicity (Hatano *et al.*, 1988).

8.3.6 Lignans

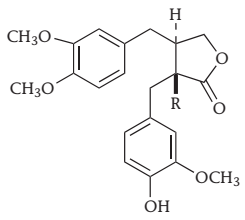
Two lignanolides of the dibenzylbutyrolactone type (−)-arctigenin (**72**) and (−)-trachelogenin (**73**), isolated from *Forsythia intermedia* and *Ipomea cairica*, respectively, have been shown to inhibit replication of HIV-1 in infected human cell systems (Eich *et al.*, 1990). These compounds were found to inhibit the integration of proviral DNA into cellular genoma. Recently, bioactivity-directed fractionation of the ethanolic extract of *Kadsura interior* led to the isolation of 12 lignans (**74–85**) (Chen *et al.*, 1997) (Figure 8.10). All the compounds were evaluated for their inhibitory activity against HIV-1 replication in acutely infected H9 cells. Seven of these compounds (**74**, **79**, **81–85**) were active with selectivity index values > 5 (Table 8.3). The active natural lignans all contain a cyclooctane ring linking a biphenyl ring system, which is substituted with methoxy and/or methylenedioxy groups. Since the biphenyl substitution pattern seems to be important for the anti-HIV activity, structure-activity correlations for related synthetic biphenyl compounds that also contain bismethylenedioxy and methoxy groups (**86–90**) (Figure 8.10), were performed. To determine the optimal pattern of biphenyl substitution, five isomeric biphenyl compounds were evaluated, including compounds (**91–93**) (Figure 8.11) and two synthetized derivatives (**94–95**) (Figure 8.11). The anti-HIV activity of the brominated derivatives **96–100** was also evaluated (Figure 8.11). The anti-HIV activities of the natural lignans (**74–85**) and their brominated derivatives (**86–90**) are reported in Table 8.3. Among these compounds, gomisin-G (**84**) exhibited

the most potent anti-HIV activity with EC_{50} and SI values of 0.006 μg/ml and 300, respectively. Schisanterin-D (**79**), kadsuranin (**81**), and schisandrin-C (**83**), showed good activity with EC_{50} values of 0.5, 0.8 and 1.2 μg/ml, and SI values of 110, 56 and 33.3, respectively. Compounds **74**, **82**, and **85** were also active, but not so potent as the former compounds. Comparison of the anti-HIV activity of compounds **74–85** suggests that both 6-benzoyl and 7-hydroxy groups might enhance the anti-HIV activity. Among the synthetic byphenyl compounds **91–100**, the relative order of potency was **96** > **97** > **99** > **91** > **93**. The other five compounds (**92**, **94**, **95**, **98**, **100**) had no, or significantly less, anti-HIV activity (Table 8.3). Comparison of the anti-HIV activities of **91–95** and **96–100** suggested that, like the natural lignans, the relative position and types of substituents on the phenolic hydroxy groups are very important for the activity. In particular the 2- (or 2′)-methoxycarbonyl and 4- (or 4′)-methoxy groups on the

TABLE 8.3
Anti-HIV activities of compounds 74–100

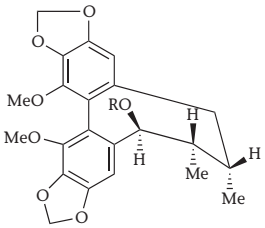
Compound	[a]IC_{50} (μg/ml)	EC_{50} (μg/ml)	[b]SI
74	50	3	13
75	50	20	2.5
76	>100	35	2.9
77	50	12	2.3
78	40	11	3.6
79	55	0.5	110
80	9	–	–
81	45	0.8	56
82	9	1.5	6
83	40	1.2	33.3
84	1.8	0.006	300
85	9	1	9
86	9	–	–
87	25	–	–
88	20	4	5
89	>100	–	–
90	25	20	1.3
91	>100	5	>20
92	>100	65	>1.2
93	>100	7.5	>13
94	>100	70	>1.4
95	1.8	No inhibition	–
96	>100	0.23	>480
97	100	0.52	190
98	>100	90	>1.1
99	>100	2.1	>48
100	45	No inhibition	–

[a]IC_{50} refers to the concentration of drug that causes 50% reduction in total cell number. Drugs that have IC_{50} values >100 μg/ml cannot be tested at higher concentrations for a more exact [a]IC_{50} value due to the effect of the solvent, DMSO.
[b]SI is a ratio of the IC_{50} value/EC_{50} value.

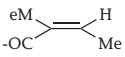


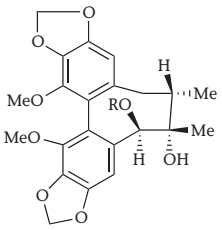
R=H (-)-Arctigenin **72**

R=OH (-)-Trachelogenin **73**

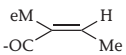


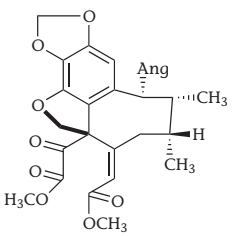
74 R=



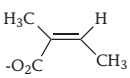


75 R=



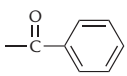


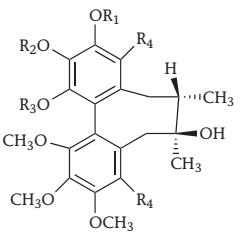
76 Ang=



78 R=

79 R=



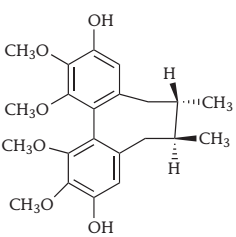


77 $R_1+R_2=CH_2$, $R_3=CH_3$, $R_4=H$

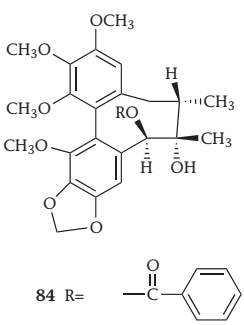
80 $R_1=R_2=R_3=CH_3$, $R_4=H$

86 $R_1+R_2=CH_2$, $R_3=CH_3$, $R_4=Br$

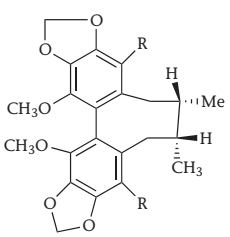
87 $R_1=R_2=R_3=CH_3$, $R_4=Br$



82

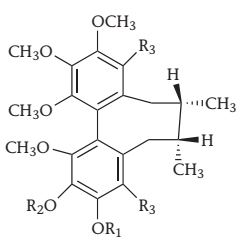


84 R=



83 R=5

89 R=Br



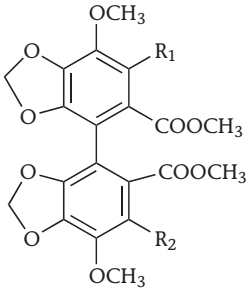
81 $R_1+R_2=CH_2$, $R_3=H$

85 $R_1=R_2=CH_3$, $R_3=H$

88 $R_1+R_2=CH_2$, $R_3=Br$

90 $R_1=R_2=CH_3$, $R_3=Br$

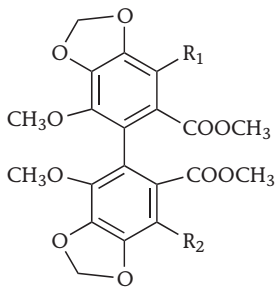
Figure 8.10: Chemical structures of anti-HIV plant metabolites **72–90**.



91 R_1=R_2=H

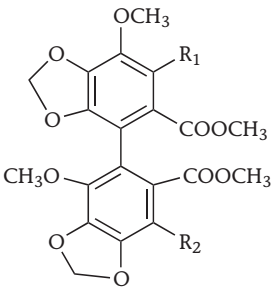
96 R_1=R_2=Br

97 R_1=Br R_2=H



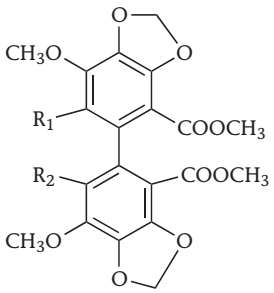
92 R_1=R_2=H

98 R_1=R_2=Br



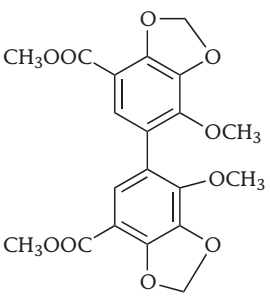
93 R_1=R_2=H

99 R_1=R_2=Br



94 R_1=R_2=H

100 R_1=R_2=Br



99

Figure 8.11: Chemical structures of anti-HIV plant metabolites **91–100**.

biphenyl ring, as found in **91** and **93**, were an essential anti-HIV structural feature. Furthermore, activity could be enhanced greatly by a bromine introduced at the 3- (or 3′)-position as found in **96**, **97** and **99**. Preliminary studies on the mechanism of action of **96** and **97** showed these compounds to be template-primer HIV-1 reverse transcriptase (RT) inhibitors. Compounds **96** and **97** also showed potent inhibitory effects against HIV-1 RT-associated DNA polymerase and RNAase activities. Further studies on the mechanism of action and further structural modification of the lead compounds are in progress (Chen *et al.*, 1997).

In conclusion, in the last ten years considerable attention has been given to the plant kingdom as a source of new anti-HIV agents. Investigations have resulted in the isolation of a large variety of compounds which interfere with different viral targets ranging from adsorption of the virus to the host cell to release from it. Sometimes it is possible to observe differences in the mechanism of action of compounds which are closely related. In some cases, the anti-HIV activity of a crude extract seems to be due to the combinatory effects of a number of compounds of different specifities. Then, an extract can act as a cocktail of molecules interfering with different viral targets and inhibiting the virus cycle in different stages.

As reported above, many are the aromatic compounds from vegetal sources which have shown a good *in vitro* anti-HIV activity, but there is very little data to prove their usefulness in patients. Among the molecules being further investigated for their potential in the systematic therapy and prophylaxis of HIV infections are (+)-calanolide A, inhibitor of reverse transcriptase and mischellamina D, inhibitor of cellular fusion and syncitium formation which have been committed to preclinical drug development. Other interesting aromatic compounds for the development of anti-HIV agents are virus absorption inhibitors such as phenolic plant components, virus transcriptase inhibitors such as castanospermine and budding inhibitors such as hypericin. On the basis of the above reports, it appears evident that the plant kingdom represents a rich and promising source of new possible anti-HIV leads which should be further explored.

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CHAPTER

9

Antioxidative Plant Constituents

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Contents

9.1 INTRODUCTION

There has been an increasing interest in the contribution of free radical reactions participating in reactive oxygen species to the overall metabolic perturbations that result in tissue injury and disease. Reactive oxygen species are generated in specific organelles of cells under normal physiological conditions.

Phagocytic cells ingest and kill invading pathogens with free radicals including superoxide anion, hydrogen peroxides, nitric oxide and hypochlorite (Moslen, 1994). Peroxisomes generate hydrogen peroxide as a byproduct in the process of β-oxidation of fatty acids, however, this molecule is locally decomposed by high concentration of catalase (Beckman and Ames, 1997). The reduction of molecular oxygen (O_2) to water (H_2O) proceeds by a series of single electron transfers, therefore, highly reactive intermediates such as superoxide anion (O_2^-), hydrogen peroxides (H_2O_2) and hydroxyradical (HO·) are generated in mitochondria (Cadenas, 1989). Some microsomal cytochrome P-450 enzymes also reduce O_2 to O_2^- directly (Goeptar *et al.*, 1995). The defence mechanisms against these reactive oxygen species include radical scavenging enzymes and cellular antioxidants. Superoxide dismutase (SOD) catalyzes the dismutation of O_2^- to O_2 and H_2O_2. Catalase and peroxidase scavenge H_2O_2 to metabolize O_2 and H_2O. Vitamin E serves to minimize HO· concentration in cell membranes. A critical balance exists between the generation and detoxification of reactive oxygen species in cells. However, diseases, aging and chemical environments such as drugs, pesticides, herbicides and various pollutants can disrupt this balance by inhibition of the cellular antioxidant defences and/or by stimulation of the formation of reactive oxygen species. These reactive oxygen species can damage DNA, so as to cause mutation and chromosomal damage, oxidize cellular thiols (resulting in inhibition of key enzymes), and abstract hydrogen atoms from unsaturated fatty acids to initiate the peroxidation of membrane lipids.

It is suggested that lipid peroxidation may be a common pathogenic mechanism because it is considered a basic mechanism involved in reversible and irreversible cell and tissue damage (Dargel, 1992). Lipid peroxidation of biological membranes damages the membrane structures and functions, not only by degrading the highly unsaturated fatty acids, but also by forming breakdown products that can result in other types of membrane damage and disturbances elsewhere. Lipid hydroperoxides, hydroxy fatty acids and epoxy fatty acids are the major products of lipid peroxidation, and they have powerful biological effects. For example, 4-hydroxy alkenals (hydroxy fatty acids) inhibit DNA synthesis, glucose-6-phosphatase and adenyl cyclase, and react with polyamines and thiols such as glutathione (Slater and Cheesemann, 1988). Cellular damage, due to lipid peroxidation, causes serious derangements such as ischemia-reperfusion injury (Omar *et al.*, 1991), coronary arteriosclerosis (Jackson *et al.*, 1993), diabetes mellitus (Sugawara *et al.*, 1992) and neurodegenerative diseases (Simonian and Coyle, 1996). It is also associated with aging (de Quiroga *et al.*, 1992) and carcinogenesis (Smith, 1985).

One of the major reactions of lipid peroxidation is autooxidation, which is slow oxidation of lipids by $^3O_2$. Rancidity and spoilage of foodstuffs are the result of the autooxidation of fats. Especially in muscle foods, the oxidation of lipids is implicated in the development of off-flavors (Gray and Pearson, 1987), loss of fresh meat color (Faustman *et al.*, 1989), and the formation of harmful lipid peroxidation products (Monahan *et al.*, 1992). The autooxidation process is represented as consisting of initiation, propagation and termination steps. Oxygen acts, not only as an oxidant to initiate chain reactions, but also as a substrate for the propagation reaction (Porter and Wujek, 1988). The formation of lipid peroxides by enzyme reaction in living cells is catalyzed by lipoxygenase and cyclooxygenase. The former is considered to catalyze the initial reaction of arachidonate cascade, which synthesizes various bioactive lipids (Samuelsson, 1983). The latter is known as prostaglandin endoperoxide synthase, which introduces two molecules of oxygen to arachidonic acid to form prostaglandin G_2 (Samuelsson, 1983). The most serious lipid peroxidation processes are concerned with cellular oxidation-reduction reactions, which are the normal metabolism in the cells.

Monoamine oxidase, which catalyzes the oxidative deamination of a variety of monoamines, such as catecholamines and serotonin, yields hydrogen peroxide (H_2O_2) as a normal byproduct (Kalgutkar and Castagnoli Jr., 1995). Hydrogen peroxide forms highly reactive hydroxy radical (OH·) in the presence of transition metals such as iron (Fe) or copper (Cu) (McCord, 1985). Hydroxy radicals are able to abstract methylene hydrogen atoms from polyunsaturated fatty acids in biological membranes to initiate lipid peroxidation (Chen *et al.*, 1995). Increased lipid peroxidation in the substantia nigra of Parkinsonian patients supports the notion that free radicals have damaged the dopaminergic neurons in the nigrostriatal pathway (Kopin, 1993).

It has been proposed that xanthine oxidase is an important source of oxygen-derived free radicals in reperfused tissues. The elevated cytosolic calcium in ischemic cells activates a calcium-dependent protease, which catalyzes the conversion of xanthine dehydrogenase to xanthine oxidase. Ischemia causes the degradation of ATP to hypoxanthine and xanthine, which form the substrates of xanthine oxidase (Omar *et al.*, 1991). Reperfusion introduces molecular oxygen into tissues and initiates the xanthine oxidase reaction, resulting in the production of superoxide anion and other toxic oxygen species, which attack cellular constituents including cell membrane lipids (Mayumi *et al.*, 1993).

In living systems, dietary antioxidants such as α-tocopherol, ascorbic acid, carotenoids, flavonoids, and other phenolics, may be effective in protection from oxidative damage (Namiki *et al.*, 1993; Stavric and Matula, 1992). A number of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been developed, but their use has begun to be restricted due to their toxicity (Ito *et al.*, 1983; Namiki, 1990). Vitamin E (α-tocopherol) is an effective natural antioxidant but has limited usage (Fang and Wada, 1993). As a result, there is considerable

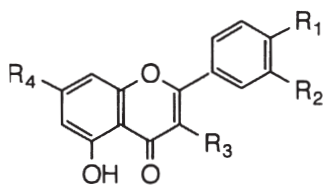
interest in the food industry and in preventive medicine in the development of natural antioxidants from botanical sources (Schuler, 1990; Okuda *et al.*, 1993; Andersson *et al.*, 1996).

Our continuous search for antioxidative plant constituents also revealed various types of antiperoxidative phytochemicals, which are effective in preventing lipid peroxidation in mitochondria and microsomes. Mitochondria (Paraidathathu *et al.*, 1992) and microsomes (Morehouse and Aust, 1988) are the most common sources of reactive oxygen species. These antioxidative plant constituents would protect mitochondrial enzyme activities against oxidative stresses and scavenge reactive oxygen species in microsomes.

9.2 FLAVONOIDS

Flavonoids are naturally occurring phenolics which are widely distributed in a variety of plants at high levels and are commonly ingested from vegetables, fruits and beverages (tea and wine). Antioxidative (Das and Ramanathan, 1992) and radical scavenging activities (Bors *et al.*, 1990) of flavonoids are well studied. In our earlier work, sulfated, methylated and glycosidal flavonoids were isolated from *Polygonum hydropiper* (Haraguchi *et al.*, 1992, 1996a; Yagi *et al.*, 1994), a medicinal herb used as a spice in Japanese cuisine. They were effective in inhibiting linoleic acid peroxidation and in preventing the generation of superoxide anion. Among them, quercetin (**1**), isorhamnetin (**3**) and rhamnazin (**6**) showed potent inhibition against microsomal lipid peroxidation induced by Fe(III)-ADP/NADPH.

The peroxidation of polyunsaturated fatty acids can proceed through nonenzymic autoxidative pathways or through processes that are enzymically catalyzed (Slater, 1984). The initial step of lipid peroxidation is the abstraction of a hydrogen from a polyunsaturated fatty acid (LH) giving rise to a carbon-centered free radical (L·). The



	R_1	R_2	R_3	R_4
1	OH	OH	OH	OH
2	OH	OH	OSO_3K	OH
3	OH	OCH_3	OH	OH
4	OH	OCH_3	OSO_3K	OH
5	OH	OCH_3	OSO_3K	OSO_3K
6	OH	OCH_3	OH	OCH_3
7	OH	OCH_3	OSO_3K	OCH_3
8	OH	OH	O-Glc	OH
9	OCH_3	OH	O-Glc	OSO_3K
10	OH	OH	O-Glucur	OH
11	OH	OH	O-Gal	OH

Figure 9.1: Antioxidative flavonoids isolated from *Polygonum hydropiper*.

rearrangement of diene conjugation is followed by an interaction with an oxygen molecule to form a peroxy radical (LOO·). This can abstract a hydrogen atom from an adjacent fatty acid, resulting in the formation of lipid hydroperoxide (LOOH) (Ernster, 1993). The most susceptible cellular location for lipid peroxidation is the microsome (Kappus, 1985). Microsomes, especially smooth-surfaced endoplasmic reticulum, easily produce lipid peroxides, and are thought to supply the peroxidation products to other tissues (Roders, 1978). NADPH-cytochrome P-450 reductase is involved in NADPH-dependent enzymical lipid peroxidation in microsomes (Aust and Svingen, 1982), which is illustrated in Figure 9.2.

In our recent work searching for antioxidative plant phenolics, different types of flavonoids (flavonol, flavanonol, flavone, flavanone, and so on) having the same 5,7,3′,4′-hydroxy substitutions (quercetin type), were isolated from edible plants: luteolin (**12**) from *Rosmarinus officinalis* (Okamura *et al.*, 1994), taxifolin (**13**) from

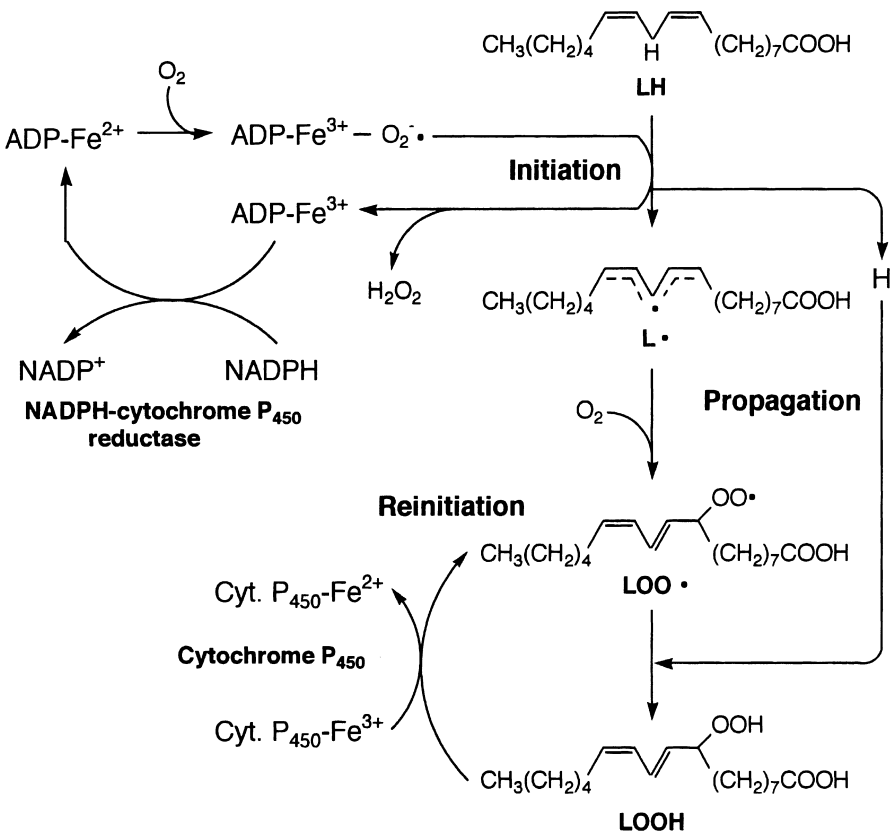
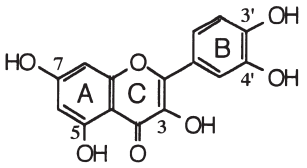
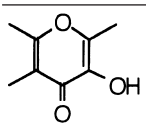
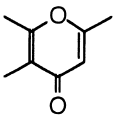
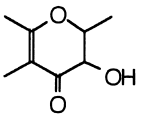
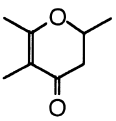
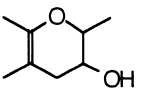


Figure 9.2: NADPH-dependent lipid peroxidation in microsomes. The illustration shows the formation of 9-trans, cis-LOOH of linoleic acid. Other hydroperoxides (13-trans, trans-LOOH, 13-trans, cis-LOOH and 9-trans,trans-LOOH) are also formed.

Engelhardtia chrysolepis (Haraguchi *et al.*, 1996b), and eriodictyol (**14**) from *Thymus vulgaris* (Haraguchi *et al.*, 1996c). Their inhibitory effect on lipid peroxidation, which occurs when rat liver microsomes are incubated with Fe(III)-ADP/NADPH (Pederson *et al.*, 1973), are shown in Table 9.1. All types of flavonoids showed potent inhibition of

TABLE 9.1
Inhibition of microsomal lipid peroxidation and superoxide anion generation by various flavonoids having same hydroxy substitutions



Structure of C-ring	Compound	IC_{50} (μM)[a]		
		microsomal[b] lipid peroxidation	O_2^- generation[c] in microsome	O_2^- generation[d] by xanthine oxidase
	**1** quercetin (flavonol)	20.7	2.5	53.8
	**12** luteolin (flavone)	7.3	>100	3.3
	**13** taxifolin (flavanol)	13.1	>100	5.9
	**14** eriodictyol (flavanone)	7.3	>100	8.8
	**15** catechin (flavan-3-ol)	14.5	>100	0.8

[a] Expressed as the mean of 50% inhibitory concentration of triplicate determination, obtained by interpolation of concentration-inhibition curves.
[b] NADPH-dependent lipid peroxidation was determined by thiobarbituric acid method.
[c] Determined by measuring the reduction rate of succinoylated cytochrome *c*.
[d] Detected by the reduction of nitroblue tetrazolium.

NADPH-dependent lipid peroxidation in liver microsomes. The hydroxyls at C-3′ and C-4′ in flavonoid B-rings have been reported as structural determinants of antioxidative activities in non-enzymatic lipid peroxidation (Ratty and Das, 1988).

It is well established that lipid peroxidation is one of the reactions set in motion as a consequence of the formation of free radicals in cells and tissues. The one-electron reduction products of O_2, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxy radical (OH·) actively participate in the initiation of lipid peroxidation (Mayumi *et al.*, 1993). Several oxidative enzymes, such as xanthine oxidase, produce the O_2^- radical as a normal product of the one-electron reduction of oxygen, resulting in tissue injury (Mayumi *et al.*, 1993). It is reported that, for a high superoxide scavenging activity in a xanthine-xanthine oxidase system, a hydroxyl group at C-3′ in the B-ring and at C-3 in the C-ring, were essential (Cos *et al.*, 1998). The effect of various types of flavonoid on the generation of superoxide anion by xanthine oxidase system are also shown in Table 9.1. Luteolin (**12**) and eriodictyol (**14**) do not have an hydroxyl group at C-3 in the C-ring, however, they were as effective as other flavonoids in scavenging the superoxide anion.

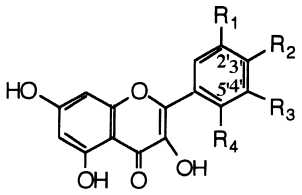
The cellular sources of superoxide anion in mammalian cells include the microsomal electron transfer chain, entailing a slow electron transfer to O_2 via NADPH-cytochrome P-450 and NADPH-cytochrome b_5 reductase (Cadenas, 1995a). Superoxide anions produced on incubation of liver microsomes with NADPH, can be detected by reduction of succinoylated ferricytochrome *c* (Kuthan and Ullrich, 1982). As shown in Table 9.1, quercetin (**1**) was an effective scavenger of superoxide anion in microsomes, however, other flavonoids had no effect on microsomal O_2^- generation.

It is reported that free hydroxyl groups in the A-ring (C-5 and C-7) participate in the inhibition of lipid peroxidation, while the presence of hydroxyls in the B-ring is not necessary, though it increases the activity, with some differences according to the structure type (Cholbi *et al.*, 1991). Table 9.2 shows antioxidative activities of flavonols with 5,7-dihydroxy substituted A-ring. Kaempferol (**16**), quercetin (**1**), morin (**17**) and myricetin (**18**) are effective in preventing NADPH-dependent lipid peroxidation in microsomes.

Antioxidative compounds effective in preventing NADPH-dependent lipid peroxidation may inhibit enzymic reduction of Fe^{3+}-ADP. Furthermore, addition of NADPH to microsomes activates cytochrome P-450, so added antioxidants could be metabolized to products more or less active in inhibiting peroxidation (Halliwell, 1995). However, lipid peroxidation started by Fe^{3+}/ascorbate avoids these problems (Halliwell, 1995). Table 9.2 also shows the effect of flavonols on ascorbate-induced microsomal lipid peroxidation, which is stimulated non-enzymatically (Slater, 1984). These flavonols also showed potent inhibition on non-enzymic lipid peroxidation in microsomes, as shown in Table 9.2. There was a little difference in efficiency of antioxidative activity of these flavonols among peroxidation systems employed. Whatever the mode of lipid peroxidation

TABLE 9.2

Antioxidative activity of flavonols having different hydroxy substitutions in B-ring



	Substitution				IC_{50} (μM)[a]			
	R_1	R_2	R_3	R_4	NADPH[b] dependent	ascorbate[c] induced	CCl_4[d] induced	*t*-BuOOH[e] induced
16	H	OH	H	H	9.0	3.8	6.6	8.4
1	H	OH	OH	H	21.8	1.9	5.7	7.8
17	H	OH	H	OH	19.0	3.2	11.2	12.3
18	OH	OH	OH	H	47.6	1.5	7.0	7.2

[a] Determined by thiobarbituric acid method, and expressed as the mean of 50% inhibitory concentration of triplicate determination, obtained by interpolation of concentration-inhibition curves.
[b] Lipid peroxidation was induced by Fe(III)-ADP/NADPH.
[c] Lipid peroxidation was induced by Fe(III)-ascorbate.
[d] Lipid peroxidation was induced by CCl_4/NADPH generating system.
[e] Lipid peroxidation was induced by tertiary butyl hydroperoxide.

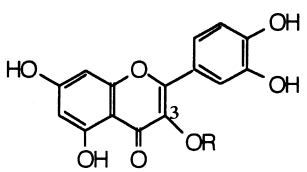
induction, the localization of radical formation centres in the membrane can be different. Hence, the efficiency of peroxidation inhibitors can also be different (Kagan *et al.*, 1990).

Various xenobiotics, including chemotherapeutic agents, pesticides and environmental pollutants, are concerned with the production of reactive oxygen species and initiation of lipid peroxidation. In particular, many lipid soluble xenobiotics can interact directly and in an unmetabolized state with plasma membranes to sufficiently alter membrane integrity, and thus enhance membrane permeability and alterations in calcium homeostasis (Stohs, 1995). Halogenated compounds, which are widely used in industrial and agricultural purposes, are the most common xenobiotics with respect to human exposure. Many halogenated compounds have been shown to be potent inducers of lipid peroxidation (Fraga *et al.*, 1987). In particular, metabolites of carbon tetrachloride (CCl_4) initiate membrane lipid peroxidation which results in cell necrosis (Orrenius *et al.*, 1992). Reductive dehalogenation of CCl_4, catalyzed by cytochrome P-450 in microsomes, generates trichloromethyl and trichloromethylperoxyl radicals, which initiate membrane lipid peroxidation (Recknagel *et al.*, 1989). Organic hydroperoxides, such as tertiary butyl hydroperoxide (*t*-BuOOH), also liberate reactive oxygen species by cytochrome P-450 catalyzed reaction, and initiate microsomal lipid

peroxidation (Minotti, 1989). This cytochrome P-450-dependent lipid peroxidation in microsomes was also inhibited by flavonols, as shown in Table 9.2.

Lipid peroxidation is initiated by active oxygen species attacking unsaturated fatty acids, and is propagated by a chain reaction cycle involving lipids, peroxy radicals and lipid hydroperoxides (Witting, 1980). Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxy radical (OH·), actively participate in the initiation of lipid peroxidation (Mayumi *et al.*, 1993). Superoxide anions were generated enzymatically in a xanthine-xanthine oxidase system and non-enzymatically in a phenazine methosulphate-NADH system, and assayed by reduction of nitro blue tetrazolium. Flavonoids lowered the concentration of superoxide anion in both enzymatic and non-enzymatic systems (Robak and Gryglewski, 1988). Naturally occurring flavonoids mainly exist as glycosides in plants (Macheix *et al.*, 1990), and are suggested to be absorbed as glycosides from human intestine (Hollman *et al.*, 1996). Table 9.3 shows the superoxide anion scavenging activity of quercetin (**1**) glycosides, isoquercitrin (**8**), quercitrin (**19**) and rutin (**20**). These flavonoid glycosides are effective in scavenging both enzymatically and non-enzymatically generated superoxide anions. Lipid peroxidation is a typical free radical oxidation and proceeds via a cyclic chain reaction (Witting, 1980). The radical scaveng-

TABLE 9.3
Radical scavenging activity of quercetin-type flavonol glycosides



	R	IC_{50} (μM)[a]		
		superoxide anion[b]		DPPH[e] radical
		enzymatically[c] generated	non-enzymatically[d] generated	
1	H	53.8	18.2	3.0
8	Glc	11.4	8.8	4.1
19	Rha	16.8	17.5	5.2
20	Glc-Rha	11.7	8.6	4.0

[a] Expressed as the mean of 50% inhibitory concentration of triplicate determination, obtained by interpolation of concentration-inhibition curves.
[b] Detected by the reduction of nitroblue tetrazolium.
[c] Superoxide anions were generated by xanthine–xanthine oxidase system.
[d] Superoxide anions were generated by phenazine methosulphate and NADH.
[e] Radical scavenging activity on diphenyl-*p*-picrylhydrazyl was measured colorimetrically.

ing activity, which can be measured as decolorizing activity following the trapping of the unpaired electron of nitrogen-centered radical, diphenyl-*p*-picrylhydrazyl (DPPH), was also examined. These flavonoid glycosides effectively scavenged DPPH radical, as shown in Table 9.3.

It is widely accepted that exogenous quinone compounds produce their cytotoxicity through the formation of reactive oxygen species (Afanas'ev *et al.*, 1990). The one-electron reduction of quinone derivatives to the semiquinone free radicals is catalyzed by several flavoenzymes, including microsomal NADPH-cytochrome P-450 reductase and mitochondrial NADH-ubiquinone oxidoreductase (Powis and Apple, 1980; Thor *et al.*, 1982). Most semiquinones reduce molecular oxygen to form superoxide anion and, subsequently, other toxic reactive oxygen species, resulting in various tissue damages (Figure 9.3). For example, adriamycin, a quinone-containing anthracycline antibiotic,

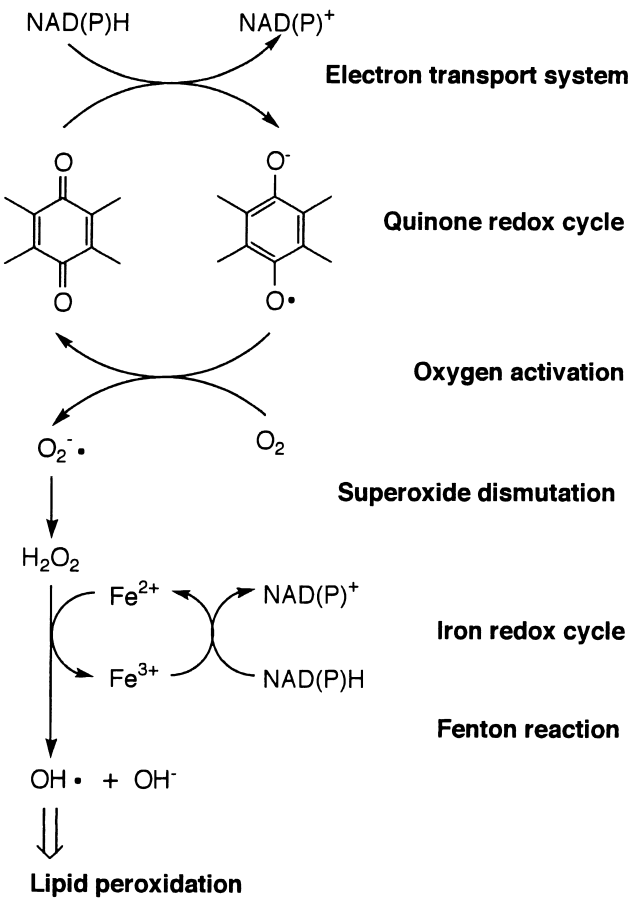


Figure 9.3: One-electron redox cycling of quinone compounds and subsequent generation of toxic active oxygen species.

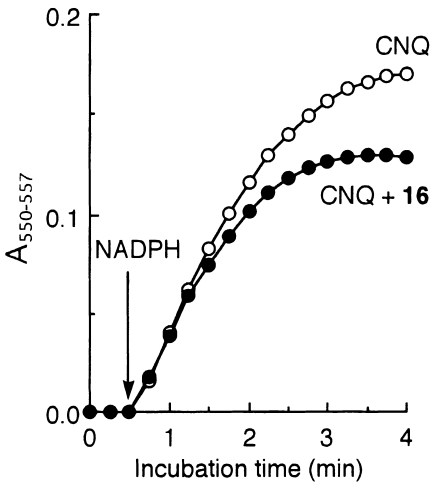


Figure 9.4: Effect of flavonol (**16**) on quinone-induced generation of superoxide anion in rat liver microsomes.

has been demonstrated to induce lipid peroxidation in rat liver microsomal suspension containing NADPH-generating system (Fukuda *et al.*, 1992). 2,3-Dichloro-1,4-naphthoquinone (CNQ), a seed fungicide and foliage protectant applied to agricultural products, mediates the generation of superoxide anion and hydrogen peroxide (Pritsos *et al.*, 1982). When rat liver microsomes were incubated with CNQ, the addition of NADPH induced the generation of superoxide anion (Figure 9.4). Kaempferol (**16**), a potent antioxidative flavonol (Table 9.2), suppressed the quinone-induced superoxide generation, as shown in Figure 9.4. This effect was also observed when superoxide dismutase (SOD) was added to the reaction mixture.

The production and regeneration of superoxide anion induced by quinone compounds leads to the stimulation of oxygen consumption (Haraguchi *et al.*, 1996d, 1997a). Naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) is the basic unit of tetracyclic antitumour antibiotics, such as daunorubicin, adriamycin, and carminomycin (Cadenas, 1995b). When this quinone was added to the microsomal suspension, oxygen consumption was induced. The addition of SOD and catalase both decreased the rate of oxygen consumption induced by some organic xenobiotics (Hodnick *et al.*, 1994). As shown in Figure 9.5, the addition of kaempferol (**16**) and catalase effectively inhibited quinone-induced microsomal oxygen consumption.

Chalcones are a group of phenolic compounds belonging to the flavonoid family, widely occurring in nature as pigments. Retrochalcones, licochalcone A (**21**), B (**22**), C (**23**), D (**24**) and echinatin (**25**), were isolated from the roots of *Glycyrrhiza inflata*. They

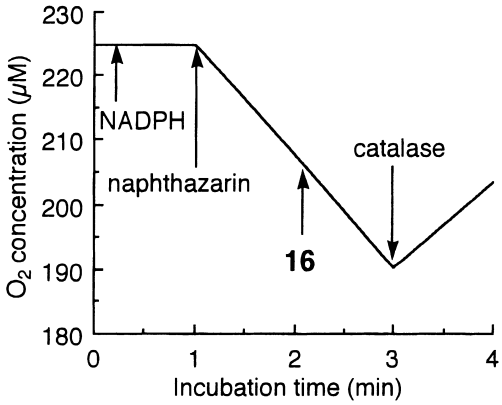


Figure 9.5: Effect of flavonol (**16**) in combination with catalase on quinone-induced oxygen consumption in rat liver microsomes.

were effective in preventing microsomal lipid peroxidation induced by Fe(III)-ADP/NADPH (Haraguchi *et al.*, 1998a). In particular, licochalcones B and D showed potent antioxidative activity. These two compounds strongly inhibited superoxide anion production in the xanthine-xanthine oxidase system and showed potent scavenging activity on DPPH radical (Table 9.4). Furthermore, licochalcones B and D scavenged superoxide anion in microsomes. The catechol is generally very sensitive to oxidation, especially the one mediated by free radicals, and the formation of relatively stable *ortho*-semiquinone radical through donation of catechol hydrogen can be ascribed. The majority of potent antiperoxidative activity exhibited by licochalcone B and D would be

TABLE 9.4
Antioxidative activity of retrochalcones in *G. inflata*

Compound	IC_{50} (μM)[a]		
	Lipid peroxidation[b]	DPPH radical[c]	O_2^- generation[d]
21	18.1	44.1	64.8
22	2.5	6.5	7.0
23	44.1	>100	71.2
24	2.0	5.7	9.9
25	61.0	>100	>100

[a] Expressed as the mean of 50% inhibitory concentration of triplicate determination, obtained by interpolation of concentration-inhibition curves.
[b] Microsomal lipid peroxidation was induced by Fe(III)-ADP/NADPH.
[c] Radical scavenging activity on diphenyl-*p*-picrylhydrazyl was measured colorimetrically.
[d] Superoxide anion was generated by xanthinine–xanthine oxidase.

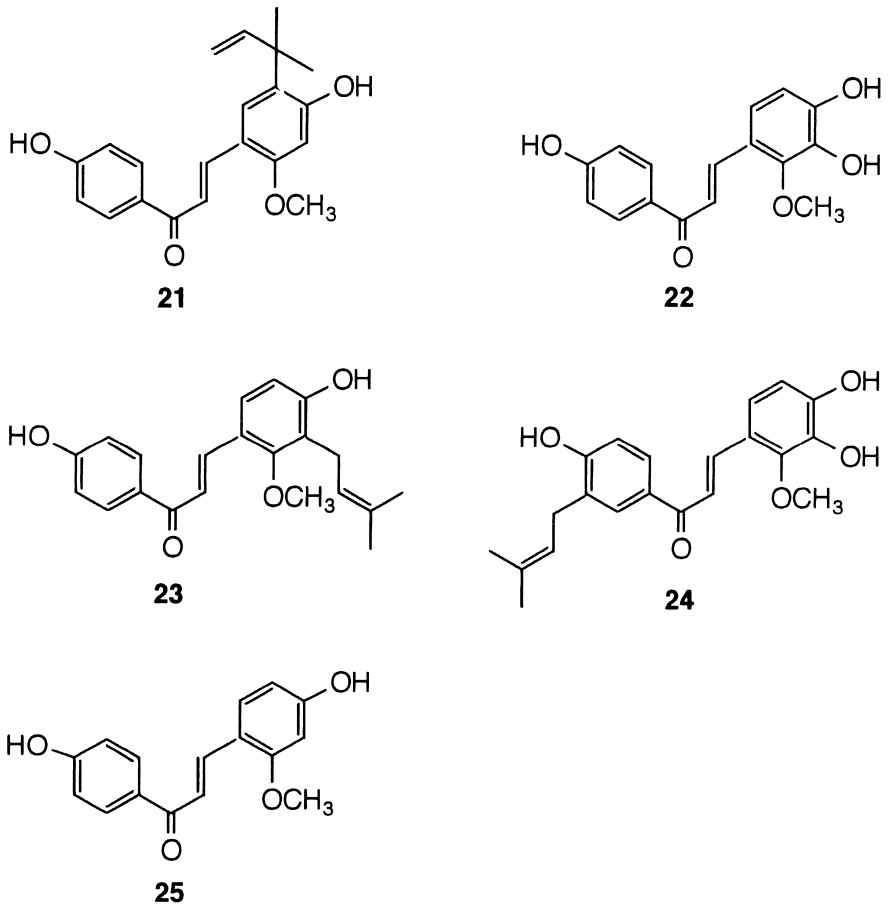


Figure 9.6: Antioxidative retrochalcones isolated from *Glycyrrhiza inflata*.

based on this mechanism, though the *ortho*-dihydroxy group (catechol moiety) can disturb lipid peroxidation by the trapping of an iron ion, which is an essential cofactor of the peroxidation process, through its divalent chelation ability. One of the earlier works pointed out that the presence of this functionality is necessary for inhibitory activity on non-enzymic peroxidation by flavones (Ratty and Das, 1988). The most potent activity was observed in compounds having a free catechol group at 3′- and 4′-position of flavone skeleton (Mora *et al.*, 1990). In the case of licochalcones B and D, where an *ortho*-dihydroxy group occurs in B-ring, their antiperoxidative activity strikingly increased by one order of magnitude as compared to other chalcones lacking such a functional group. The presence of a methoxyl *ortho* to 3′,4′-dihydroxy in these two retrochalcones will mostly contribute to increases in antiperoxidative activity, since such a strong electron-donating group would further stabilize rather stable *ortho*-

semiquinone radicals. These structural features will be the basis for potent inhibitory activity of licochalcones B and D on lipid peroxidation and radical scavenging activity. Some naturally occurring polyhydroxyflavones have been proved to be effective inhibitors of lipid peroxidation in cell-free systems. However, their potential efficiency as therapeutic agents will be in doubt, since they are not hydrophobic enough to penetrate into the cell and reach tissues, where they are expected to inactivate free radicals involved in some diseases. Licochalcones in *G. inflata* are more hydrophobic than polyhydroxyflavones such as quercetin, and so on. In particular, licochalcones A, C and D have prenyl groups (C_5 units) in either A- or B-ring. The introduction of such lipophilic substitution into these molecules will increase their hydrophobicity, which is expected to help improve their biochemical and pharmacological properties through enhanced affinity for the plasma membrane. This lipophilic part of antioxidative compounds such as α-tocopherol is considered to be important for proper orientation of the molecule in the membrane (Kagan and Quinn, 1988).

Isoflavones are also major members of naturally occurring flavonoids. Isoflavan derivatives, glabridin (**26**), hispaglabridin A (**27**), hispaglabridin B (**28**), 4′-*O*-methylglabridin (**29**), 3′-hydroxy-4′-*O*-methylglabridin (**30**), were isolated from *Glycyrrhiza glabra*. They inhibited lipid peroxidation in rat liver microsomes (Haraguchi *et al.*, 1997b). Hispaglabridin A (**27**), especially, showed potent antioxidative activity against peroxidation induced by Fe-ascorbate (Table 9.5). This compound also has prenyl moiety between two hydroxyls, which would provide an affinity for hydrophobic environment to this isoflavan. 3′-Hydroxy-4′-*O*-methylglabridin (**30**) strongly inhibited Fe-ADP/NADPH dependent lipid peroxidation. This isoflavan has an electron-donating methoxy group in the *ortho*-position of a phenolic hydroxy function, which would contribute to the stability of phenoxy radicals generated in the antiperoxidation process.

TABLE 9.5
Antioxidative activity of isoflavans from *G. glabra* in rat liver microsomes

Compound	IC_{50} (μM)[a]	
	NADPH-dependent[b]	ascorbate-induced[c]
26	8.4	24.5
27	3.5	2.0
28	21.6	>100
29	10.8	21.2
30	0.4	11.2

[a] Expressed as the mean of 50% inhibitory concentration of triplicate determination, obtained by interpolation of concentration-inhibition curves.
[b] Lipid peroxidation was induced by Fe(III)-ADP/NADPH.
[c] Lipid peroxidation was induced by Fe(III)-ascorbic acid.

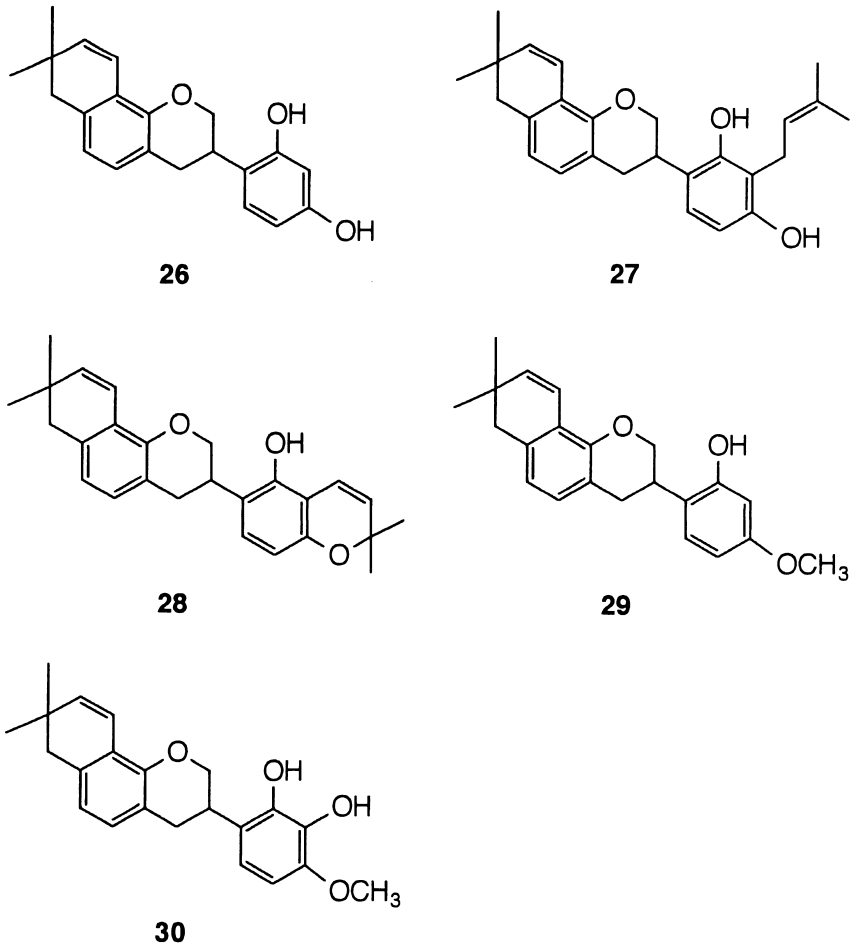
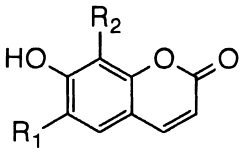


Figure 9.7: Antioxidative isoflavans isolated from *Glycyrrhiza glabra.*

9.3 COUMARINS AND XANTHONES

Coumarins, which are also benzopyrones (α-pyrone), form a large class of phenolic compounds occurring in plants. Payá *et al.* (1992) investigated effects of coumarins with various hydroxyl and other substitutions on lipid peroxidation and some oxygen radicals. Among the plant-derived coumarins, *o*-dihydroxy substitutions, fraxetin (**31**), esculetin (**32**) and daphnetin (**33**), were effective inhibitors of Fe^{3+}-ascorbate induced microsomal lipid peroxidation.

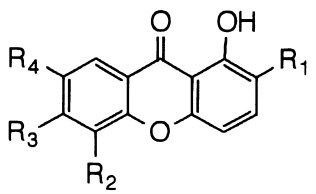
Xanthones consist of γ-pyrone like flavonoids and exhibit a wide range of biological activities. Minami *et al.* (1994) isolated hydroxyxanthones from the woods of *Garcinia*



	R_1	R_2
31	OCH3	OH
32	OH	H
33	H	OH

Figure 9.8: Antioxidative naturally occurring coumarins.

subelliptica and their antioxidative properties were evaluated. Among them, 1,2-dihydroxy-5,6-dimethoxyxanthone (**35**) and 1,8-dihydroxy-6-dimethoxyxanthone (**36**), were effective in preventing lipid peroxidation in rat brain homogenates. 1,2,5-trihydroxyxanthone (**34**) was a potent scavenger against DPPH radical and O_2^- derived from xanthine-xanthine oxidase system. Globuxanthone (**37**) was also effective in scavenging O_2^- and preventing lipid peroxidation.



	R_1	R_2	R_3	R_4
34	OH	OH	H	H
35	OH	OMe	OMe	H
36	H	H	OMe	OH

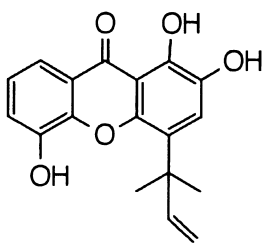


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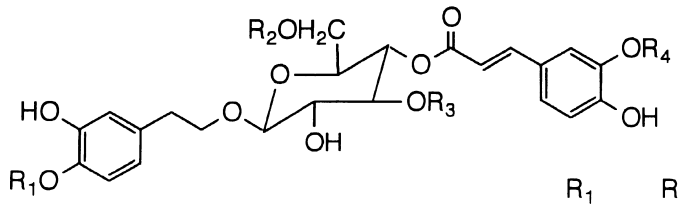
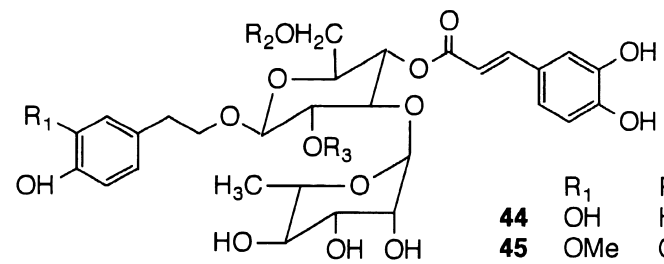
Figure 9.9: Antioxidative xanthones isolated from *Garcinia subelliptica*.

9.4 PHENYLPROPANOIDS

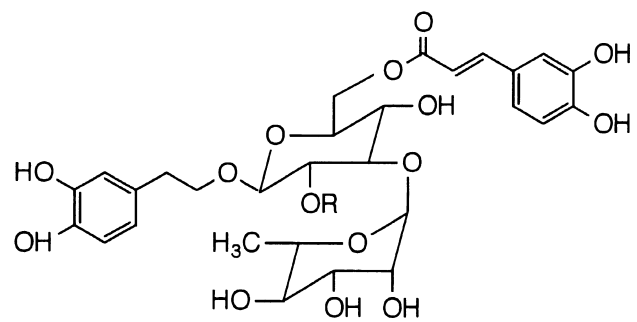
Phenylpropanoids are also widely distributed in edible plants and foodstuffs derived from plants. Recently, Wang *et al.* (1996) studied the scavenging activities of phenylpropanoid glycosides, verbascoside (**38**), pedicularioside A (**39**), M (**40**), N (**43**), leucosceptoside A (**41**) and martynoside (**42**), isolated from *Pedicularis* plants, on superoxide anion and hydroxyl radicals. The results demonstrated that the number of phenolic hydroxyl groups in the structures is related to their radical scavenging activities. Xiong *et al.* (1996) also isolated phenylpropanoid glycosides, 2′-acetylacetoside



	R_1	R_2	R_3	R_4
38	H	H	H	H
39	H	Rha	Api	H
40	H	Rha	Api	Me
41	H	H	Rha	Me
42	Me	H	Rha	Me
43	Me	Rha	Api	Me



	R_1	R_2	R_3
44	OH	H	Ac
45	OMe	Glc	H
46	OH	Glc	Ac
47	OH	Glc	H
48	OH	H	H
49	H	H	H



	R
50	Ac
51	H

Figure 9.10: Antioxidative phenylpropanoid glycosides.

(**44**), cistanoside A (**45**), tubuloside A (**46**), echinacoside (**47**), acetoside (**48**), syringalide A 3′-α-rhamnopyranoside (**49**), tubuloside B (**50**) and isoacetoside (**51**), from *Cistanche deserticola*, and investigated their antioxidative activities. These compounds exhibited significant inhibition on both ascorbate-Fe^{2+} and Fe^{3+}-ADP/NADPH induced lipid peroxidation in rat liver microsomes. Their antioxidative activity was also found to be potentiated by an increase in the number of phenolic hydroxyl groups in the molecule.

Phenylpropanoids are derived from *trans*-cinnamic acid (**52**) and *p*-coumaric acid (**53**). Further oxidation of *p*-coumaric acid generates caffeic acid (**54**), and its methylation leads to ferulic acid (**55**). Among the most widely distributed phenylpropanoids in plant tissues are hydroxycinnamic acids (Rice-Evans, 1996). Toda *et al.* (1991) investigated the effects of these phenolcarboxylic acids on the generation of superoxide anion and production of lipid peroxide induced by superoxide anion. Ferulic acid (**55**) scavenged superoxide anion, and caffeic acid (**54**) and ferulic acid (**55**) inhibited lipid peroxidation. Carboxylic acids of phenylpropanoids are reduced to produce corresponding aldehydes, alcohols and olefins. Eugenol (**56**) is one of the reduced phenylpropanoids, a major component of clove oil. Eugenol (**56**) is reported to decrease lipid peroxidation induced by CCl_4 in microsomes (Kumaravelu *et al.*, 1995). This compound also inhibits Fe^{2+}-ascorbate and Fe^{2+}-H_2O_2-induced lipid peroxidation in rat liver mitochondria (Nagababu and Lakshmaiah, 1992).

Redox reactions frequently occur in mitochondria, which are constantly susceptible to oxidative stress (Wiswedel *et al.*, 1989). Inner membranes of mitochondria are especially at risk for lipid peroxidation, because mitochondria utilize oxygen at a high rate and inner membranes have a large content of polyunsaturated fatty acids, together with peroxidation catalysts such as iron and copper (Hingh *et al.*, 1995). In mitochondrial membranes, electron transport chain is involved in the enzymatic lipid peroxidation (Kappus, 1985). It has been reported that NAD(P)H support enzymatically induced lipid peroxidation in submitochondrial particles in the presence of an iron chelate (Glinn *et al.*, 1991). Electrons are transferred from NADH to Fe^{3+}, converted Fe^{2+} makes an addition to O_2, and then Fe-O_2 complex initiates lipid peroxidation (Takayanagi *et al.*, 1980). In addition, electron donors such as NADH and succinate generate CoQ·, which reacts with O_2 to produce O_2^- (Sugioka *et al.*, 1988). Superoxide anions would reduce iron ions and initiate lipid peroxidation (Figure 9.11).

Table 9.6 shows the antioxidative activities of phenylpropanoids on microsomal and mitochondrial lipid peroxidation. Eugenol (**56**) and isoeugenol (**57**), especially, were

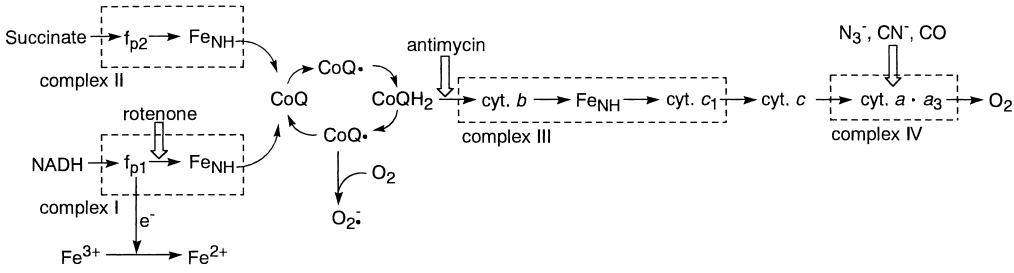
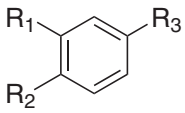
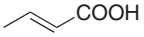
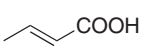
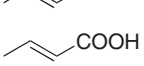
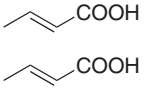


Figure 9.11: Mitochondrial electron transport chain and generation of initiators of lipid peroxidation. fp, flavoprotein; Fe_{NH}, nonheme iron; CoQ, coenzyme Q_{10}; cyt, cytochrome. White arrows indicate the action site of respiratory inhibitors.

TABLE 9.6
Antioxidative activity of phenylpropanoids in microsomes and mitochondria



	Substitution			IC_{50} (μM)[a]	
	R_1	R_2	R_3	microsomal[b] lipid peroxidation	mitochondrial[c] lipid peroxidation
52	H	H		>100	>100
53	H	OH		>100	>100
54	OH	OH		58.8	>100
55	OCH_3	OH		>100	>100
56	OCH_3	OH		11.6	7.6
57	OCH_3	OH		14.8	3.2
58	H	OCH_3		72.5	93.0
59	—O—CH_2—O—			54.5	82.5
60	—O—CH_2—O—			29.2	52.0

[a] Determined by thiobarbituric acid method, and expressed as the mean of 50% inhibitory concentration of triplicate determination, obtained by interpolation of concentration-inhibition curves.
[b] Lipid peroxidation was induced by Fe(III)-ADP/NADPH.
[c] Lipid peroxidation was induced by Fe(III)-ADP/NADH.

potent antioxidants against Fe(III)-ADP/NADH induced lipid peroxidation in mitochondria.

Oxidative coupling of two phenylpropanoid units (C_6-C_3 unit) results in lignan and neolignan. Lu and Liu (1992) investigated antioxidative activity of dibenzocyclooctene lignans isolated from *Schisandra* plants. Among them, schisanhenol (**61**) isolated from *S. rubriflora* was the most active lignan, which inhibited iron/cysteine-induced lipid peroxidation of rat liver microsomes and prevented the decrease of membrane fluidity of microsomes induced by iron/cysteine.

Honokiol (**62**) and magnolol (**63**) are the constituents of the bark of *Magnolia obovata*. These neolignans exhibited free radical scavenging activity on DPPH (Lo *et al.*, 1994) and inhibition of microsomal and mitochondrial lipid peroxidation (Haraguchi *et*

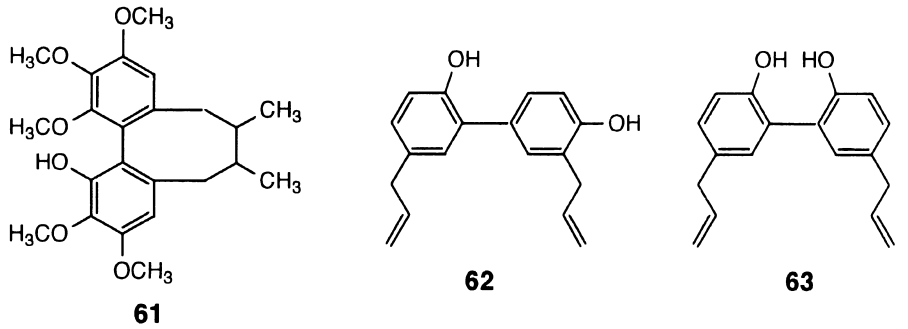


Figure 9.12: Antioxidative lignan and neolignan.

al., 1997c). Figure 9.13 shows the effect of honokiol (**62**) on oxygen consumption induced by lipid peroxidation in mitochondria. In submitochondrial particles in which the electron transport chain is blocked by rotenone, oxygen uptake is not observed. The addition of Fe^{3+}-ADP/NADH to mitochondrial suspension resulted in a rapid oxygen uptake. The electron flow from NADH to Fe^{3+}-ADP forms Fe^{3+}-ADP-O_2^- complex, which causes lipid peroxidation. Once lipid peroxy radicals have been generated, oxygen consumption can proceed continuously, since lipid peroxidation is a typical chain reaction. Honokiol (**62**) showed time-dependent inhibition against mitochondrial oxygen consumption. This indicates honokiol (**62**) inhibits radical chain reaction of lipid peroxidation, but not the formation of active Fe^{3+}-ADP-O_2^- complex.

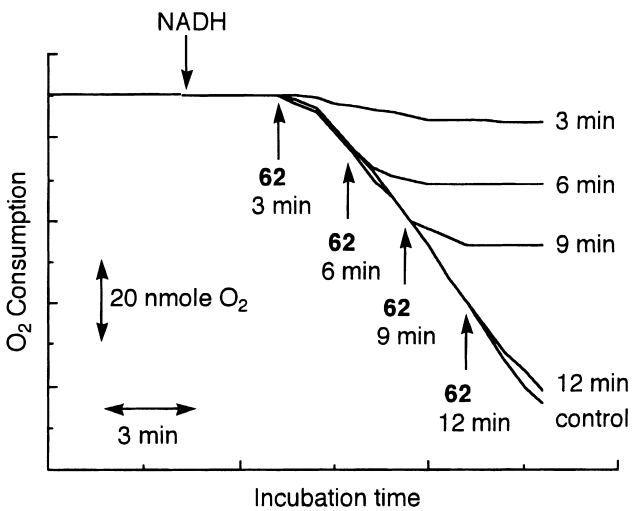


Figure 9.13: Effect of neolignan (**62**) on oxygen consumption in mitochondria. The arrows indicate the time of addition of compound **62**.

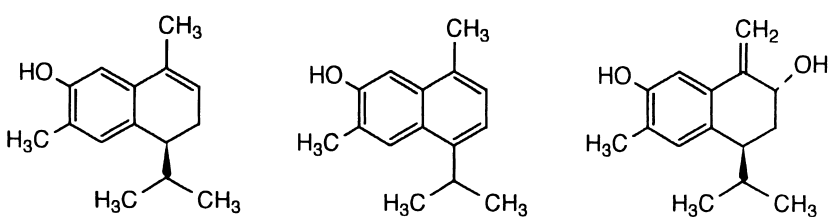
9.5 TERPENOIDS

Terpenoids are also widely distributed in a variety of plants. In comparison to hydrophilic flavonoids, lipophilic terpenoids have been revealed to possess potent antioxidative activities and protective effects against oxidative stresses in mitochondria.

Sesquiterpenoids, 7-hydroxy-3,4-dihydrocadalin (**64**), 7-hydroxycadalin (**65**), and 2,7-dihydroxy-β-calacoren (**66**), were isolated from the dried flowers of *Heterotheca inuloides*, a medicinal plant used for the treatment of postoperative thrombophlebitis and pathicus in Mexico. These sesquiterpenoids showed potent antioxidative activities against linoleic acid autoxidation, mitochondrial lipid peroxidation induced by Fe(III)-ADP/NADH and microsomal lipid peroxidation induced by Fe(III)-ADP/NADPH, and radical scavenging activity on DPPH (Haraguchi *et al.*, 1997d; Kubo *et al.*, 1996). Especially, 7-hydroxy-3,4-dihydrocadalin (**64**) showed complete inhibition of mitochondrial lipid peroxidation at a concentration of 15 μM (Haraguchi *et al.*, 1996e).

Rosemary (*Rosmarinus officinalis*) is a herb used as a food additive. Abietane-type diterpenes, carnosol (**68**), rosmanol (**69**), isorosmanol (**71**), and rosmariquinone (**72**) were isolated, and their antioxidative activities on linoleic acid peroxidation were reported (Inatani *et al.*, 1983). Recently, our group also isolated carnosic acid (**67**), carnosol (**68**), rosmanol (**69**), and epirosmanol (**70**) as effective antioxidants in biological systems. These diterpenoids inhibited the generation of superoxide anion. The 50% inhibitory concentrations were in the range of 5–15 μM. Carnosic acid (**67**) was most effective in inhibiting the generation of superoxide anion by xanthine oxidase system; 70% inhibition was observed at 9 μM. Diterpenoids isolated from *R. officinalis* inhibited the production of lipid peroxides induced by microsomal NADPH oxidation. Rosmanol (**69**) and epirosmanol (**70**) were most effective in preventing the microsomal lipid peroxidation; complete inhibition was observed at 3 μM. Furthermore, carnosic acid (**67**) and carnosol (**68**) completely inhibited mitochondrial lipid peroxidation linked to the respiratory chain at 9 μM (Haraguchi *et al.*, 1995).

Totarane-type diterpenes, totarol (**73**), totaradiol (**74**), 19-hydroxytotarol (**75**), totaral (**76**), 4-β-carboxy-19-nortotarol (**77**), and sugiol (**78**), were isolated from the root bark of



64 **65** **66**

Figure 9.14: Antioxidative sesquiterpenes isolated from *Heterotheca inuloides*.

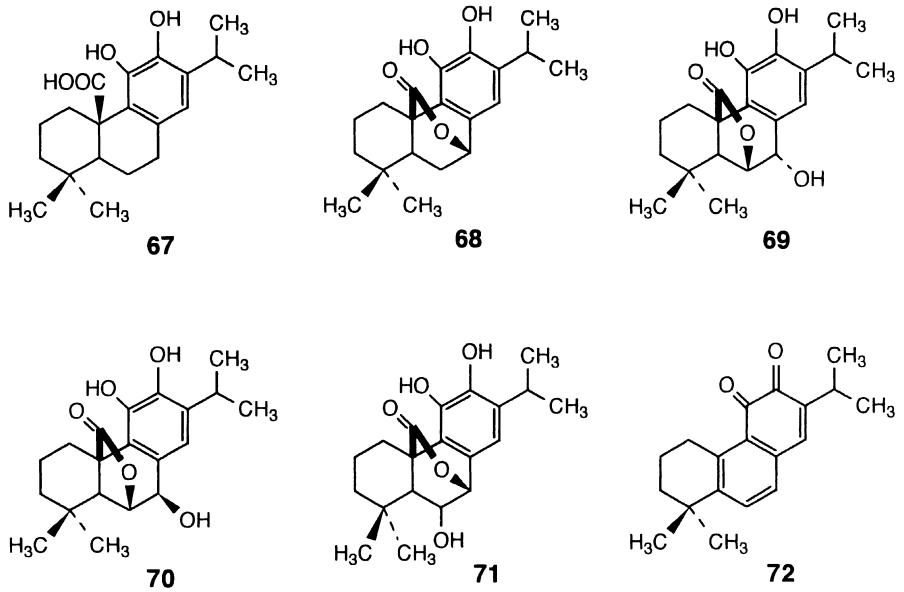


Figure 9.15: Antioxidative diterpenes isolated from *Rosmarinus officinalis*.

Podocarpus nagi, and they showed potent antiperoxidative activities (Haraguchi *et al.*, 1997e). Totarol (**73**), totaradiol (**74**) and 19-hydroxytotarol (**75**) exhibited almost complete inhibition of microsomal lipid peroxidation at 10 μM. They also strongly inhibited mitochondrial lipid peroxidation; almost complete inhibition was obtained at 1 μM. The difference in antioxidative activity among these diterpenes may be caused by their affinity for the lipophilic environment due to C-4 conjugated functions. Totarol (**73**) protected human red blood cells against oxidative hemolysis and protected mitochondrial enzyme activities against peroxidative stress (Haraguchi *et al.*, 1996f).

Biphenyl compounds (**79–83**), which would be oxidative coupling products of monoterpenes, were isolated from thyme (*Thymus vulgaris*), and their inhibitory activities on linoleic acid peroxidation were evaluated (Miura and Nakatani, 1989). Recently, we isolated a flavonoid and one of the biphenyl compounds, 3,4,3′4′-tetrahydroxy-5,5′-

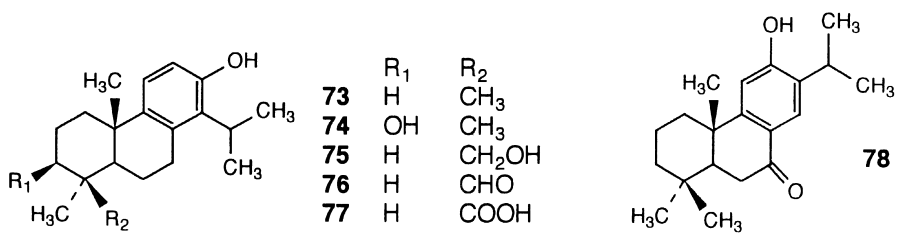


Figure 9.16: Antioxidative diterpenes isolated from *Podocarpus nagi*.

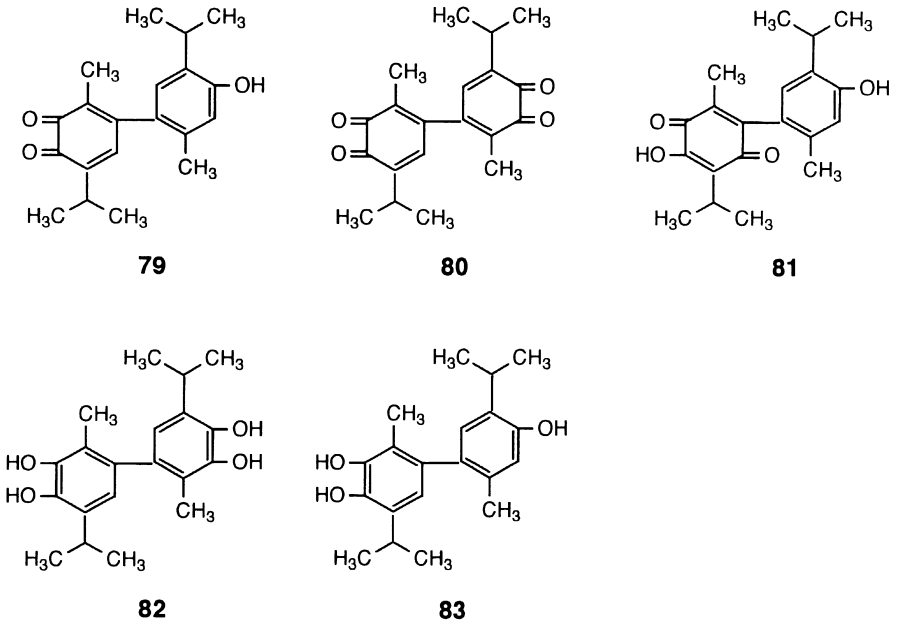


Figure 9.17: Antioxidative biphenyl compounds isolated from *Thymus vulgaris*.

diisopropyl-2,2′-dimethylbiphenyl (**82**), from the leaves of *T. vulgaris* as active components for inhibiting mitochondrial and microsomal lipid peroxidation (Haraguchi *et al.*, 1996c).

Various oxidative stresses affect the mitochondrial enzyme activities (Veitch *et al.*, 1992). Some proteins are loosely attached to the surface of membranes, but most are tightly attached. Therefore, lipid peroxidation can damage membrane proteins as well as the lipid. NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase are the most sensitive sites to mitochondrial peroxidative injury. NADPH-dependent lipid peroxidation in submitochondrial particles results in a remarkable loss of these enzyme activities (Narabayashi *et al.*, 1982). When rat liver mitochondria were incubated with Fe(III)-ADP/NADPH, membrane lipids were peroxidized and NADH- and succinate-cytochrome *c* reductase activities decreased. As shown in Figure 9.18, monoterpene dimer **82** protected both enzyme activities against NADPH-dependent peroxidation.

The mitochondrial respiratory chain generates a superoxide anion (O_2^-), and subsequently, hydrogen peroxide (H_2O_2) at the level of complex I and at the ubiquinone-cytochrome *b* segment (Boveris, 1984). Lipid peroxides produced by hydroxy radical (OH·) derived from H_2O_2 and O_2^- affect mitochondrial function (Forman and Boveris, 1982). The autoxidation of dihydroxyfumarate (DHF) generates superoxide O_2^- and H_2O_2. Once formed, O_2^- leads to the generation of OH· through non-enzymatic

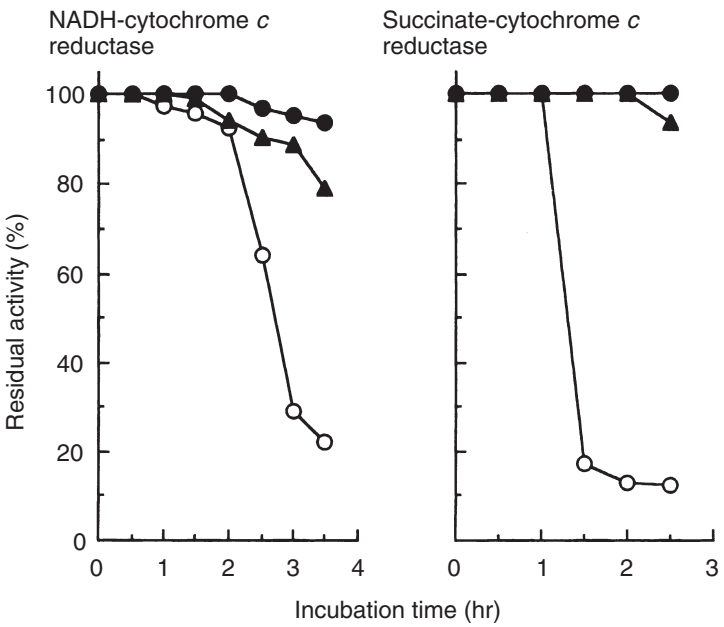


Figure 9.18: Effects of compound **82** on NADPH-dependent oxidative injury in mitochondrial respiratory chain. Each plot is the mean of triplicate determinations. ●: 9.8 μM, ▲: 3.3 μM, ○: control.

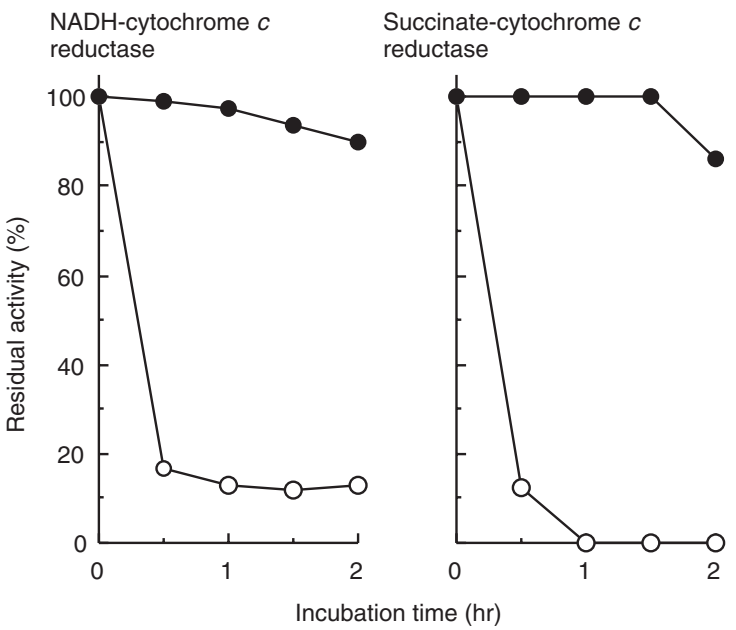


Figure 9.19: Effects of compound **82** on DHF-induced oxidative injury in mitochondrial respiratory chain. Each plot is the mean of triplicate determinations. ●: 3.3 μM, ○: control.

dismutation, which is catalyzed by Fe^{3+}-ADP (Kukreja *et al.*, 1988). When mitochondrial suspensions were incubated with DHF, respiratory enzyme activities decreased. The addition of Fe^{3+}-ADP to the incubation mixture accelerated the loss of enzyme activities. As shown in Figure 9.19, compound **82** protected the enzyme activities of NADH- and succinate-cytochrome *c* reductase against DHF-induced peroxidation.

A dienone-phenolic triterpene, celastrol (**84**) (Figure 9.20), has been isolated from *Tripterygium wilfordii*, and its antioxidative activity in mitochondria was examined by Sassa *et al.* (1990). This terpenoid inhibited oxygen consumption due to peroxidation of mitochondrial membranes induced by ADP and Fe^{2+}, prevented the formation of lipid peroxides, and scavenged DPPH radicals.

Yun *et al.* (1999) isolated pentacyclic triterpene esters (**85**, **86**) (Figure 9.20) from the root bark of *Hibiscus syriacus*, and showed their antioxidative activity. These two triterpene esters inhibited Fe-ascorbate-induced lipid peroxidation in rat liver microsomes. Their antioxidative activity would be due to the antiperoxidative action of caffeate moieties conjugated to the triterpene aglycones.

Table 9.7 shows the characteristics of the antioxidative activities of typical terpenoids isolated in our group. Many plant secondary metabolites exhibit antioxidative activity, and mechanisms of antioxidant action have been proposed (Cuvelier *et al.*, 1992; Ohnishi *et al.*, 1994). Sesquiterpenoids from *H. inuloides* (e.g. **64**) and diterpenoids from *P. nagi* (e.g. **73**) have an electron-donating methoxy or isopropyl group in the *ortho* position of a phenolic hydroxy function. First, phenolic hydroxyl may act as H-atom donor to peroxy radicals (LOO·). Phenoxy radicals which would be generated in the process of antiperoxidation may be stabilized, not only by their aromatic character, but also by the

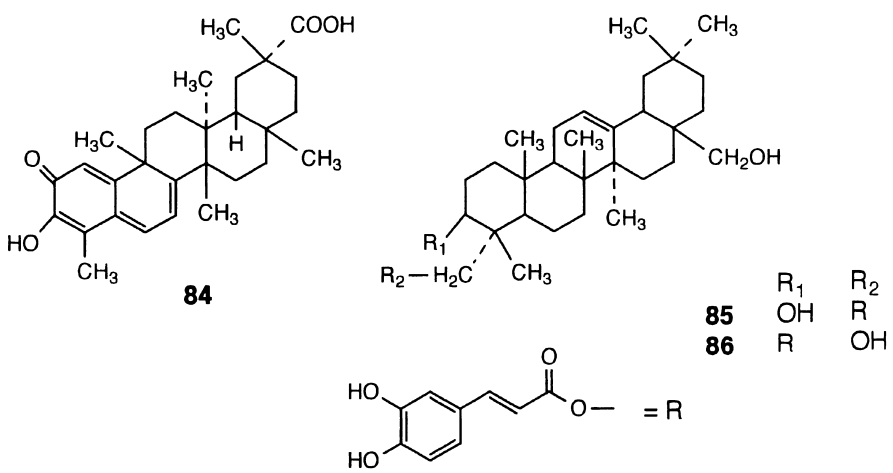
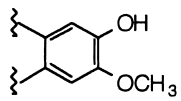
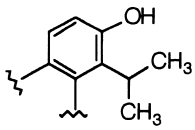
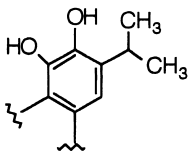
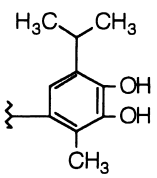


Figure 9.20: Antioxidative triterpenes.

TABLE 9.7
Antioxidative and superoxide anion scavenging activities of various types of terpenoids

Possible part for antioxidative action in the structure	Compound	IC_{50} (μM)[a]		
		lipid peroxidation[b]		O_2^- generation[e] by xanthine oxidase
		microsomal[c]	mitochondrial[d]	
	**64**	9.3	3.0	>100
	**73**	4.8	0.5	>100
	**67**	0.8	4.6	6.5
	**82**	0.6	4.6	1.7

[a] Expressed as the mean of 50% inhibitory concentration of triplicate determination, obtained by interpolation of concentration-inhibition curves.
[b] Determined by thiobarbituric acid method.
[c] Lipid peroxidation was induced by Fe(III)-ADP/NADPH.
[d] Lipid peroxidation was induced by Fe(III)-ADP/NADH.
[e] Detected by the reduction of nitroblue tetrazolium.

presence of the electron-donating group (Weng and Gordon, 1992). The phenoxy radical may act with another peroxy radical, converting it to much less reactive product (Figure 9.21). They showed more potent antioxidative activity against mitochondrial peroxidation than microsomal peroxidation, and no effect on superoxide anion generated by xanthine oxidase system. On the other hand, terpenoids from *R. officinalis* (e.g. **67**) and *T. vulgaris* (e.g. **82**) showed both inhibitory activity on the formation of lipid peroxide and scavenging activity of superoxide anion. These terpenoids have *ortho*-dihydroxy group (catechol part), which would be able to trap and stabilize various radical molecules (Figure 9.21).

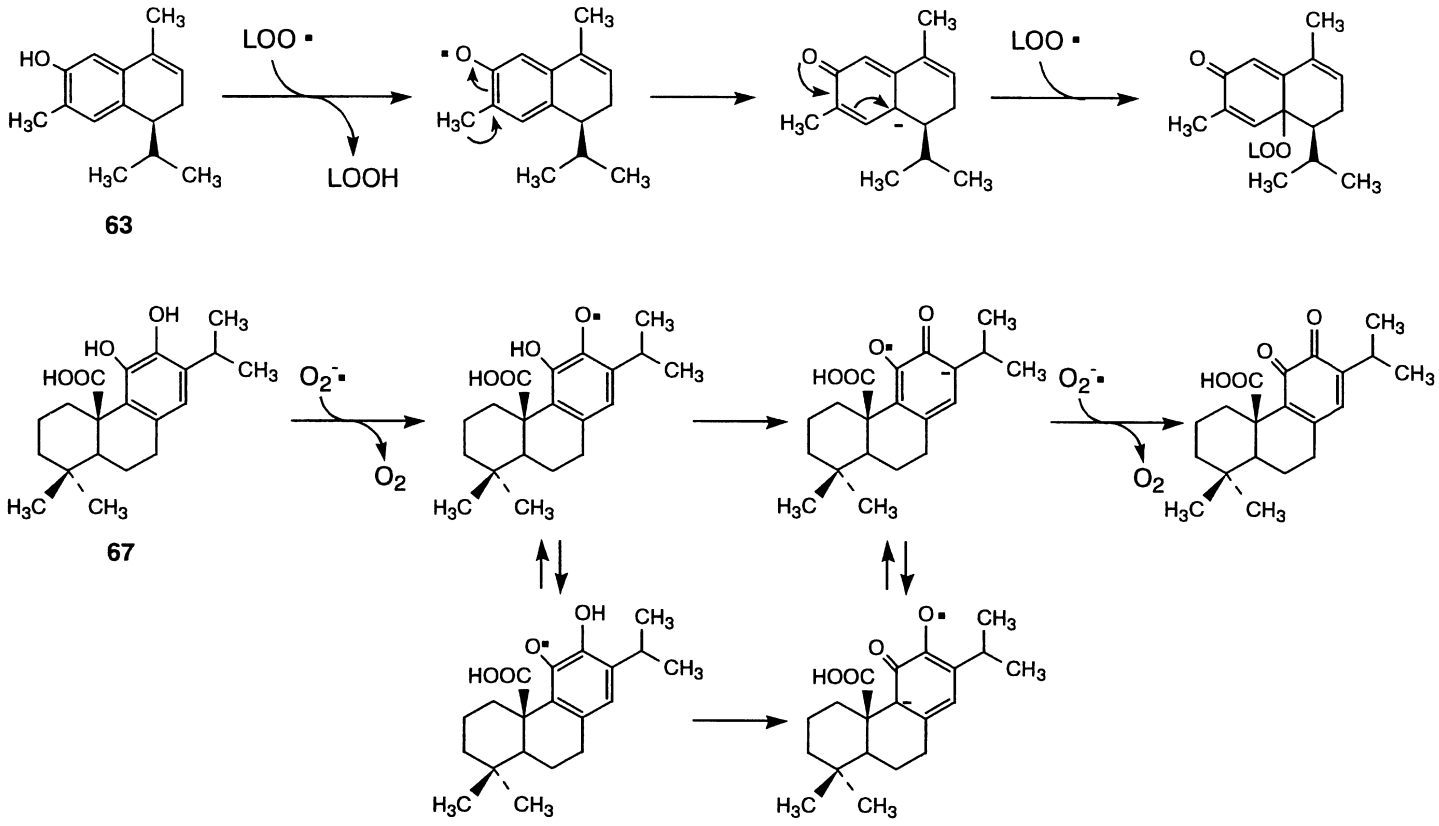


Figure 9.21: Proposed mechanisms of antioxidative action of terpenoids.

9.6 CONCLUDING REMARKS

In living systems, various reactive oxygen species are generated and can cause cell damage. A major form of cellular oxidative damage is lipid peroxidation, which is initiated by reactive oxygen species through the extraction of a hydrogen atom from unsaturated fatty acids of membrane phospholipids (Farber *et al.*, 1990). Membrane lipids are particularly susceptible to oxidation, not only because of their high polyunsaturated fatty acid content, but also because of their association in the cell membrane with enzymic and non-enzymic systems capable of generating free radical species (Halliwell and Gutteridge, 1990a). Peroxidation of membrane lipid is a cardinal feature of oxyradical toxicity (Cross *et al.*, 1987). The chain reaction of lipid peroxidation yields several types of secondary free radicals and a large number of reactive compounds, resulting in the destruction of cellular membranes and other cytotoxic responses (Bus and Gibson, 1979). Oxidative degradation of polyunsaturated fatty acids occurs in two sequential steps (Svingen *et al.*, 1979). The initiation reaction involves reactive oxygen species such as hydroxy radical as initiators, forming a conjugatively stabilized carbon centered radical (L·). This reacts rapidly with oxygen to form peroxy radical (LOO·), which abstracts a hydrogen atom from another fatty acid to form lipid hydroperoxides (LOOH) and a new carbon centered radical (L·) until the chain reaction is terminated (propagation). Therefore, antioxidative materials acting in living systems are classified as preventive antioxidants and chain-breaking ones (Halliwell and Gutteridge, 1990b). In this article, some characteristics of antioxidative plant constituents were mentioned, placing the focus on mitochondria and microsomes. Some of these compounds prevented the formation of lipid peroxide and scavenged superoxide anions. They would be classified as preventive antioxidants. Others strongly inhibited lipid peroxidation but showed little effect on superoxide. They would be classified as chain-breaking ones (Figure 9.22).

Mitochondrial damage, due to lipid peroxidation, causes various diseases and is associated with aging (Guarnieri *et al.*, 1992; Sohal and Weindruchi, 1996). Mitochondria are considered the most important sites where peroxidative processes can arise, causing damage to electron transfer activities (Guarnieri *et al.*, 1985). Some antioxidative terpenoids not only prevented lipid peroxidation in mitochondria but also protected mitochondrial functions against oxidative stresses. On the other hand, some antioxidative plant components, which are effective in preventing electron transport chain linked lipid peroxidation, are reported to inhibit mitochondrial electron transport system itself (Hodnick *et al.*, 1986a; Bohmont *et al.*, 1987) or generate reactive oxygen species in electron transport chain (Hodnick *et al.*, 1986b, 1994). In the case of microsomes, some flavonoids also inhibit cytochrome P-450 activity (Sousa and Marletta, 1985). Antiperoxidative action and inhibitory activity on these normal reactions would be due to independent mechanisms. To clarify the

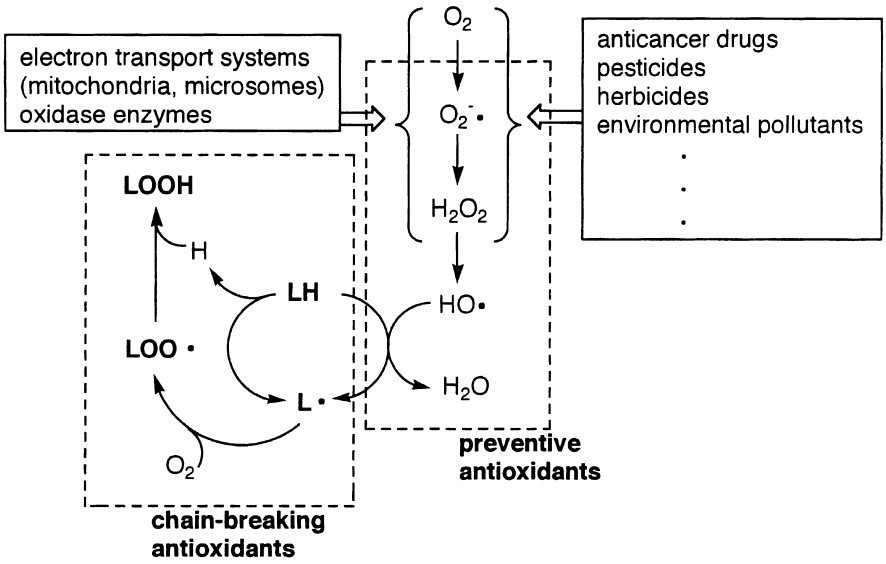


Figure 9.22: Summarization of membrane lipid peroxidation and antioxidants.

differences in these mechanisms of action may lead to the effective utilization of plant-derived antioxidants.

Many of the plants mentioned here, especially in our original works, contain different structure types of antioxidative compounds; flavonol glycosides and sesquiterpenoids in *H. inuloides* (Haraguchi *et al.*, 1997d), flavanones and monoterpene dimers in *T. vulgaris* (Haraguchi *et al.*, 1996c), flavones and abietane-type diterpenes in *R. officinalis* (Okamura *et al.*, 1994; Haraguchi *et al.*, 1995), flavan-3-ols and totarane-type diterpenes in *P. nagi* (Tan and Kubo, 1990; Haraguchi *et al.*, 1997e). Flavonoids actively scavenge oxygen radicals, and terpenoids strongly prevent lipid peroxidation. The combined effects of the different nature in hydrophobic terpenoids and hydrophilic flavonoids would contribute to the total antioxidative action of these plants.

Antioxidative constituents in plants have other various biological activities, especially in the case of flavonoids (Middleton and Kandaswami, 1993). Flavonols in *P. hydropiper* (Haraguchi *et al.*, 1996a) and flavanols in *E. chrysolepes* (Haraguchi *et al.*, 1996g, 1997f) mentioned in this article, show potent inhibition against lens aldose reductase, which has been implicated in the pathogenesis of diabetic complications, such as cataracts (Ammon *et al.*, 1996). Oxidative damages and lipid peroxidation are also concerned with diabetes (Asayama *et al.*, 1989) and cataracts (Spector, 1991). These additional activities of antioxidative phytochemicals would contribute to their pharmaceutical uses. Antioxidative diterpenoids from *P. nagi* (Haraguchi *et al.*, 1996h) and

retrochalcones from *G. inflata* (Haraguchi *et al.*, 1998b) have antimicrobial activities. Both antioxidative and antimicrobial actions of plant materials are required in the food industry. Many kinds of plant have been used for prevention and treatment of various diseases and food preservation. Explication of their active constituents and mechanisms of their action would lead to further utilization of plant materials.

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CHAPTER

10

Antimalarial Natural Products

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10.1 THE BURDEN OF MALARIA

Malaria is estimated to kill between 1.5 and 2.7 million persons every year. Most are African children younger than five years of age. More than 300 million suffer from malaria and it has been estimated that a single bout of malaria costs a sum equivalent to over 10 working days. In 1987 the total economic cost of malaria: health care, treatment, lost production etc., was estimated to be US$ 800 million for tropical Africa; this figure is expected to be more than US$ 1,800 million by 1995. Malaria thus has disastrous social consequences and is a heavy burden on economic development (Casteel, 1997). Figure 10.1 depicts the geographical distribution of malaria in 1997 (WHO).

Malaria in humans is caused by four species of parasites belonging to the genus *Plasmodium*, *P. falciparum*, *P. ovale*, *P. malaria*, and *P. vivax*. *P. falciparum* is the parasite causing most deaths. The life cycle of this parasite is depicted in Figure 10.2. The British major, Ronald Ross, discovered the vector function of mosquitoes belonging to the genus Anopheles more than 100 years ago. The discovery made Ross enthusiastically write a poem in which he claimed:

I know this little thing
A myriad men will save.
O Death, where is thy sting.
Thy victory. O grave!

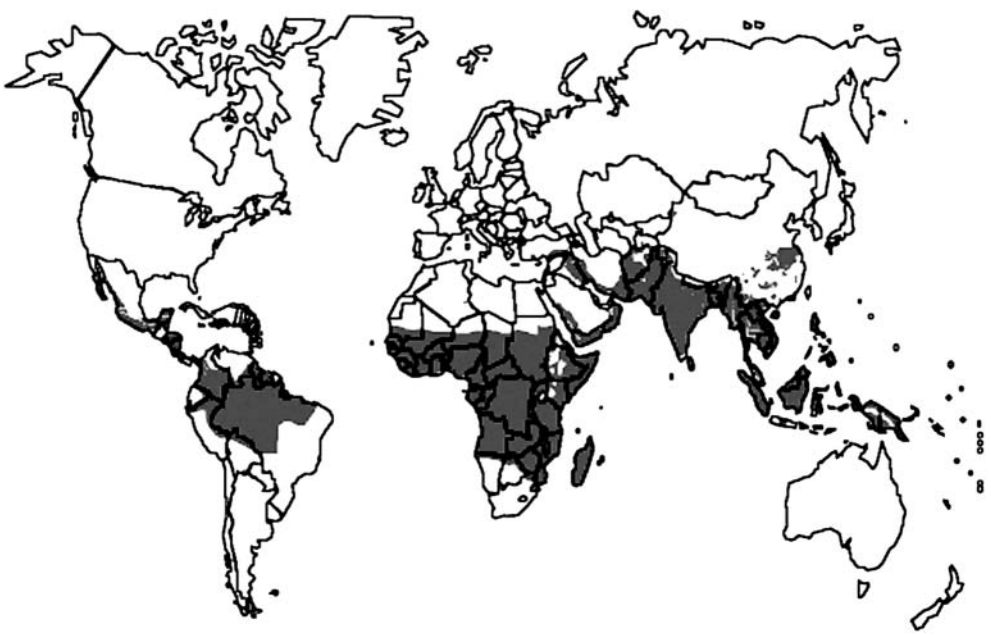


Figure 10.1: The geographical distribution of malaria in 1997 (WHO).

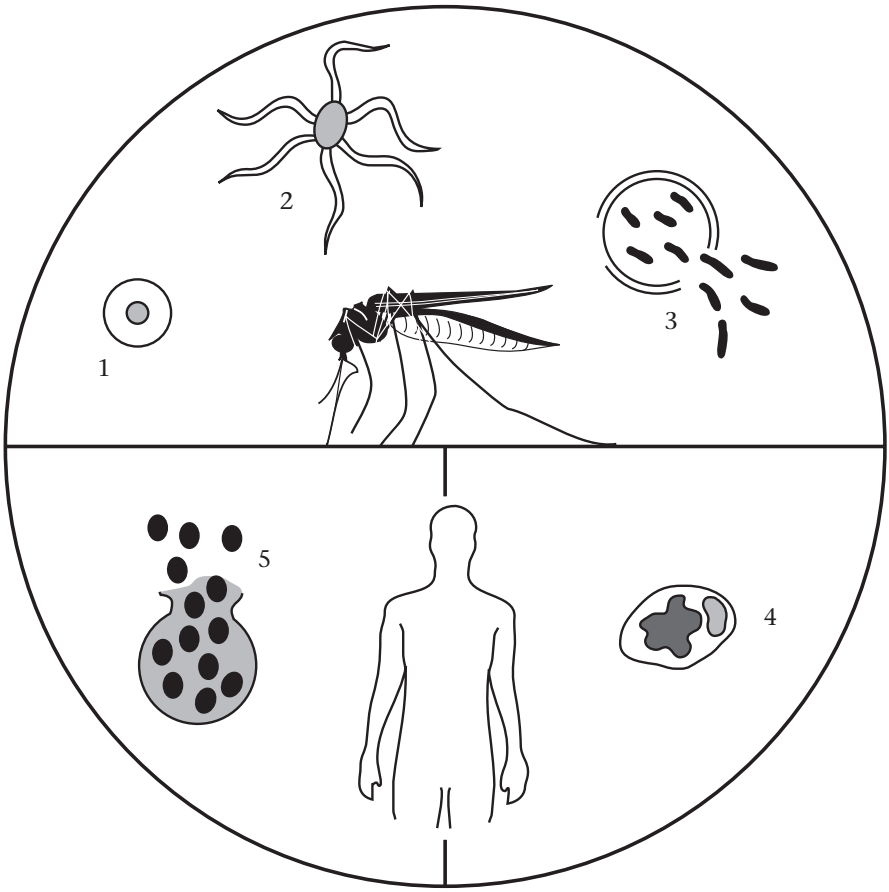


Figure 10.2: Simplified presentation of the life cycle of the malaria parasite.

When a parasite takes a blood meal from an infected person it swallows some female or male parasites, gametocytes (1). The gametocytes undergo sexual reproduction in the digestive tract of the mosquito. Ultimately, sporozoites (3) form, which are injected with the saliva of the mosquito the next time it bites a human victim. In the human the sporozoites invade the parenchymal cells of the liver. During development in the liver the patient remains asymptomatic but after a variable period of time, 6–8 days for vivax, 9 days for ovale, 12–16 days for malaria, and 5–7 days for falciparum, merozoites (4) are released from the liver. Some parasites might differentiate into a dormant stage (hypnozoites), providing a reservoir that can be activated for up to five years after the initial infection. The merozoites invade the erythrocytes, where they feed on the haemoglobin. After proliferation the erythrocyte rupture and the liberated merozoites (5) invade other erythrocytes. Some merozoites are converted into gametocytes (Casteel, 1997).

The 'little thing' was the just-discovered parasites in the stomach of the mosquitoes. In the late 1950s and 1960s it appeared that the optimistic statement of Ross would become true. An intensive use of insecticides — predominantly DDT — and the use of chloroquine, eradicated malaria from a large part of the tropical world and almost completely from the non-tropical world, and afforded a considerable decrease in many parts of the tropical world (Day, 1998; Jayaraman, 1997). Development of resistance to DDT amongst the mosquitoes, together with resistance of the parasites against chloroquine has dramatically aggravated this situation. Another problem is the effect on the environment of the extended use of DDT, even though it has been debated whether human or environmental welfare should be given the higher priority (Laird, 1985). Today, malaria has again become one of the three most fatal diseases in the world.

As is illustrated in Figure 10.1, malaria occurs mainly in the poor part of the world. This implies a poor motivation of the medical companies for developing antimalarial drugs, since the purchasing power of the patients is limited (Briefing, 1997). A review of the history of malaria reveals that the major efforts in the fight against it are always done by military researchers, mostly connected to wars in the infected areas (Foley and Tilley, 1998). As a consequence, WHO have given malaria a high priority and a number of programmes to control malaria have been initiated (WHO). Three important attempts are: a) development of a vaccine against malaria (Facer and Tanner, 1997; Kumar *et al.*, 1999; Riley, 1997); b) vector control (Greenwood, 1997); and c) development of new antimalarial drugs (Bruno *et al.*, 1997). Mapping of the genome of the parasite might reveal new possibilities for the control of malaria.

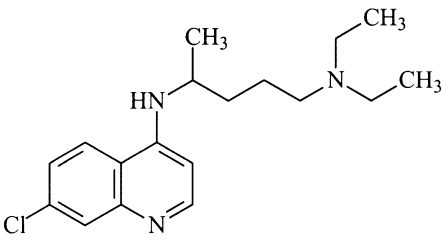
10.2 DRUGS AVAILABLE FOR TREATMENT OF MALARIA

At the present, a number of drugs are available for the treatment of malaria (Casteel, 1997; Murray and Perkins, 1996). Some of these are listed below, according to their mechanism of action.

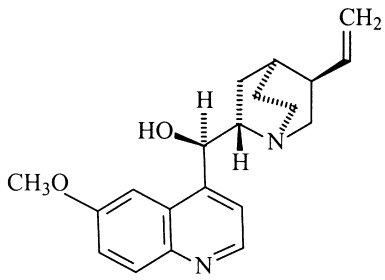
10.2.1 Haem detoxification

The haem remaining after digestion of the protein part of haemoglobin is toxic to the parasites, maybe because of the strong reducing power of iron(II) haem (Foley and Tilley, 1998; Ridley, 1997a). Even after oxidation to iron(III) haem the complex remains toxic as it is membrane-interactive and potentially lytic (Ridley, 1997a). To avoid the toxic effects, the parasites convert haem to the polymeric haemozoin also known as β-haematin or malaria pigment. Inhibition of haem polymerisation is believed to be the mechanism of action of the quinoline antimalarials such as chloroquine (**1**), quinine (**2**), mefloquine (**3**), and halofantrine (**4**) (Bray *et al.*, 1998; Casteel, 1997; Fitch and Chou, 1997; Foley and Tilley, 1998; Ridley, 1997a). The mechanism of action may

indicate that the quinoline antimalarials might be without side effects. This is almost true for chloroquine, which has very few side effects. Rapid spreading of resistance, however, limits the continued use of this drug (Casteel, 1997). In contrast, overdosing of quinine can induce tinnitus, vomiting, nausea, visual disturbance, central nervous system disturbance and cardiotoxicity (Casteel, 1997). The symptoms vanish when administration of the drug ceases. Resistance towards quinine has been reported in South East Asia (Casteel, 1997). Serious neurological or psychiatric reactions have been associated with mefloquine (Casteel, 1997).



(**1**)



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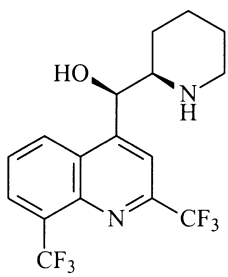

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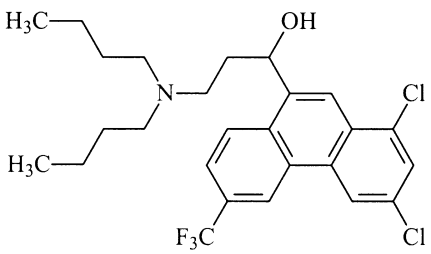

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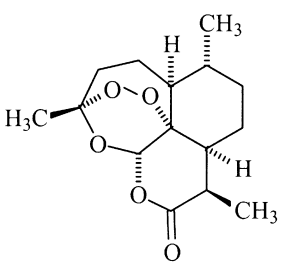
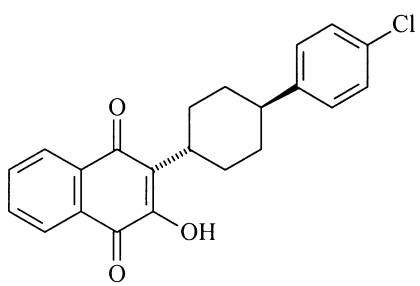
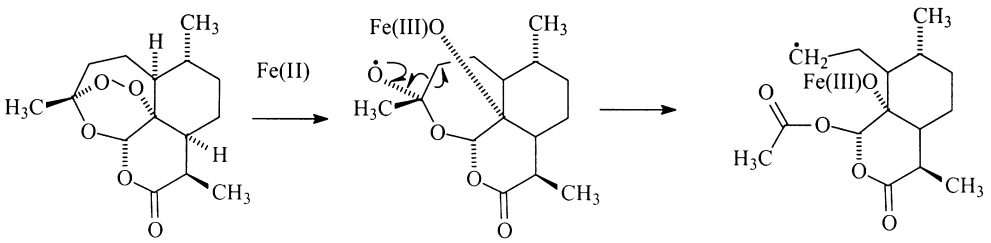


(**5**)



(**6**)

Also, the peroxide antimalarials such as artemisinin (**5**) appear to be able to inhibit this polymerisation by alkylating the haem (Scheme 10.1) (Posner, 1997; Robert and Meunier, 1998b; Robert and Meunier, 1998a). According to the mechanism suggested in Scheme 10.1, the iron(II) in the haem remaining after proteolysis of the protein part of haemoglobin, cleaves the peroxide bridge forming a reactive radical which, after some rearrangements in the molecule, alkylates one of the pyrazole rings in the porphyrin nucleus of haem. This alkylation is suggested to inhibit the haemazoin formation. In the body this reaction sequence can only be induced by degraded haemoglobin. Thus, it is tempting to conclude that the peroxide antimalarials do not have side effects. Even though the peroxide antimalarials are found to have very few side effects (Park *et al.*, 1998), artemisinin (**5**) is known to have neurotoxic activities (Edwards, 1997). A problem with the peroxide antimalarials, however, is recrudescence, i.e. after an initial clearance of the parasites the parasitemia increases again. Artemisinin is, therefore, often used in combination with other antimalarials such as mefloquine or tetracyclines (Casteel, 1997).



(**5**) (**5a**)

Scheme 10.1: The reductive activation of the peroxide bridge by iron (II).

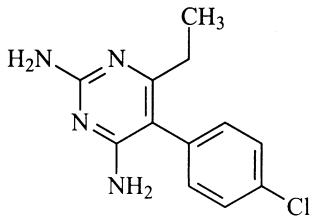
The reductive activation of the peroxide bridge by iron (II) induces the homolytic cleavage of the O—O bond. The resulting alkoxyl radical isomerises quickly by homolytic cleavage of the C—C bond to produce the reactive C-centred radical **5a**. The radical **5a** has, in model experiments, alkylated a porphyrin nucleus (Robert and Meunier, 1998b).

10.2.2 Pyrimidine metabolism and electron transport

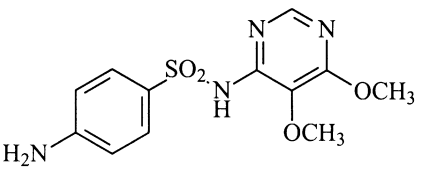
Whereas the host is able to salvage and to synthesise pyrimidines *de-novo*, the parasites have to synthesise the nucleosides *de-novo* (Olliaro and Wirth, 1997; Rathod, 1997). Thus, inhibitors of pyrimidine syntheses would be expected to be selective inhibitors of the growth of the parasites. Atovaquone (**6**) appears to block pyrimidine synthesis by inhibition of the respiratory chain of malaria mitochondria at complex III (Hudson, 1993), even though it has also been suggested that the compound inhibits haemazoin formation (Casteel, 1997).

10.2.3 Folate metabolism

Some of the most widely used antimalarials are mixtures of sulfonamides and pyrimethamine. Sulfonamides prevent formation of dihydropterate and pyrimethamine is an inhibitor of dihydrofolate reductase. Both types of compounds thus inhibit the



(**7**)



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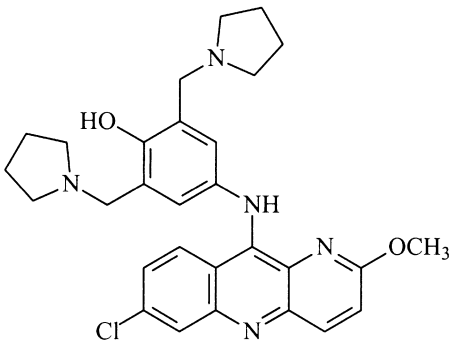
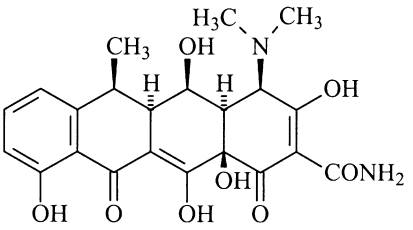
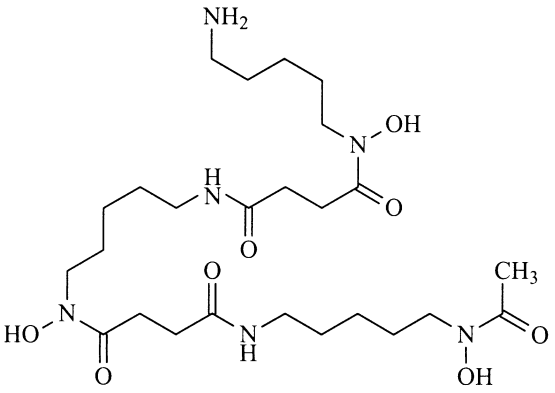


(**9**)



(**10**)



(**11**)

formation of dihydrofolate, which is necessary in the biosynthesis of pyrimidines. A frequently used drug is fansidar, which is a mixture of pyrimethamine (**7**) and sulfadoxine (**8**) (Casteel, 1997). Pyrimethamine is an antifolate. A number of other antimalarial drugs are, likewise, mixtures of sulfonamides and antifolates (Casteel, 1997). Resistance against folate inhibitors has been reported (Casteel, 1997).

10.2.4 Topoisomerase II inhibitors

Clinical experiments have been performed in China with the topoisomerase inhibitor pyronaridine (**9**) (Casteel, 1997).

10.2.5 Protein synthesis

Tetracyclines, such as doxycycline (**10**), are used in the treatment of malaria, however, due to their slow-acting nature, they are almost always used in combination with fast-acting agents such as chloroquine (**1**) or quinine (**2**). The mechanism of action is only poorly understood, but they might inhibit mitochondria protein synthesis (Casteel, 1997).

10.2.6 Iron chelation

A number of iron(III) chelators have antimalarial activity *in vitro*, apparently through the mechanism of withholding iron from vital metabolic pathways of the intra-erythrocytic parasite (Hider and Liu, 1997; Mabeza *et al.*, 1999; Pradines *et al.*, 1996). Other iron chelators appear to inhibit malaria parasites by forming toxic complexes with iron (Mabeza *et al.*, 1999). Desferrioxamine (**11**) has clinical activity in both uncomplicated and severe malaria in humans (Mabeza *et al.*, 1999).

10.3 DEVELOPMENT OF ANTIPARASITIC DRUGS

The rapid spread of resistance against the presently used antimalarial drugs has created a desperate need for new drugs. In the light of the burden of the disease, WHO has given development of new antimalarial drugs a high priority (WHO). Successful drug development entails a highly co-ordinated interaction of numerous groups, working in disciplines ranging from basic sciences to the medical professions (Yevich, 1996). There is roughly a 1 to 10,000 chance that a compound will make its way from the laboratory to the market place. The average cost of bringing a drug to the market is in excess of US$ 300 million, indicating the need for the involvement of pharmaceutical companies in the process (Yevich, 1996). Realising that private companies have to be involved, also stresses the importance of protecting any lead compound by patent, since this is a

minimum prerequisite for protecting a possible drug against plagiarism and, consequently, decreased interest by the investors. Drug development, from initial discovery to commercialised drug, involves a number of steps which are illustrated in Table 10.1 (Ridley, 1997b). As is illustrated in this table, the process might be divided into three stages: discovery, development, and clinical use. The goal of the discovery stage is to develop a drug candidate, which will be taken first into Clinical Phase 1. In Clinical Phase 1 a few healthy volunteers will be treated with the drug to verify that the compound is not toxic. The clinical proof of principle will be given in Clinical Phase 2 by treatment of a few selected patients. Finally, in Clinical Phase 3, a large group of patients will be tested to verify the qualities of the drug. Unacceptable side effects in the clinical phases will cause the drug candidate to be discharged.

10.3.1 Rational design of lead compound

Drug discovery can be performed in two ways. The process can be started in a rational way by identifying a biological target molecule (Gutteridge, 1997). An interesting target would be an enzyme, which is essential for the parasites but not present in the host organism. Alternatively an enzyme can be chosen, which is distinctly different in the host and the parasite, enabling design of molecules, which selectively interacts with the parasite enzyme (Hunter, 1997). A number of such targets are mentioned above, under the discussion of the different drugs presently used. It is fair to mention, however, that none of these drugs have been developed on a rational basis. All of them have been developed in an empirical way and the targets have been identified afterwards — if at all. A number of additional potential targets have been mentioned in some recent reviews (Olliaro and Wirth, 1997; Subbayya *et al.*, 1997; Wang, 1997).

10.3.1.1 Protease inhibitors

The malaria parasites use haemoglobin as the principal source for amino acids (Olliaro and Wirth, 1997). Haemoglobin is degraded in the digestive vacuole and a number of proteases are involved in its digestion. Significant differences from the proteases of the host identify the enzymes of the digestive vacuole as potential targets for drugs (Chen and Li, 1992; Coombs and Mottram, 1997; Westling *et al.*, 1997; McKerrow *et al.*, 1999). Based on the expected three-dimensional structure of the parasite proteases, a series of inhibitors have been designed by, for example, computational chemistry. A number of the inhibitors were synthesised and tested *in vitro* as potential antimalarial drugs. A series of these were derivatives of benzaldehyde benzoylhydrozone (Li *et al.*, 1994) and chalcones (Cohen *et al.*, 1997; Li *et al.*, 1995), and these were able to kill the parasites at low concentrations. Similarly, a series of phenothiazonones have been shown to efficiently inhibit the growth of *P. falciparum* and to inhibit falcipain, a protease isolated from the vacuole of the parasite (Dominguez *et al.*, 1997). A series of low-molecular-weight

TABLE 10.1

The drug discovery and development process, slightly modified after Ridley (1997b). (Reproduced with permission of Academic Press Inc.)

Discovery			Development				Registration	Clinical use
Exploratory biology	Discovery of lead	Optimisation of activity	Pre-clinical	Clinical phase 1	Clinical phase 2	Clinical phase 3		Post marketing surveillance
Rational	Empirical		Non-clinical Process chemistry, formulation, pharmacokinetics, toxicology					
Target identification	Molecular screening	Medicinal chemistry	Availability	Safety/ tolerance in humans	Clinical proof of principle	Clinical efficacy	Satisfying regulatory agencies of efficacy and safety	Safety
Target validation	Parasitology screening	Mechanism of action studies	Pharmacokinetics	Pharmacokinetics	Dose-range finding			Efficacy
	Traditional medicine	Structure activity relationships	Safety/ toxicity *in vitro* *in vivo*		Safety/ tolerance in humans	Safety/ tolerance in humans		Effectiveness

non-peptide plasmepsin II aspartyl protease inhibitors has been synthesized by combinatorial chemistry. Plamepsin II is one of the enzymes parasites use for degradation of the haemoglobin. Some of the inhibitors show high activity and a significant selectivity towards plasmepsin II (Haque *et al.*, 1999) and some falcipain inhibitors have been shown to have some activity *in vivo* (Olson *et al.*, 1999)

10.3.1.2 Drug transport inhibitors

Resistance towards some quinoline antimalarials is caused by expression in the parasite of a pump, which removes the drug. Inhibitors of these pumps, such as verapamil and desipramine, reverse the resistance (Batra and Bhaduri, 1997; Casteel, 1997). This strategy, however, is complicated by unacceptable side effects (Foley and Tilley, 1998; Olliaro and Wirth, 1997).

10.3.1.3 Phospholipid metabolism inhibitors

Blockage of the choline transporter which provides the parasite with choline — a precursor required for synthesis of phosphatidylcholine, the major phospholipid of the parasites — efficiently controls the growth of *P. falciparum in vitro* (Ancelin and Vial, 1986; Calas *et al.*, 1997) and in mice and monkeys (Panthong *et al.*, 1994).

10.3.1.4 Shikimate inhibitors

Shikimate pathway, conserved in plants, algae, bacteria, and fungi, but not in animals, has recently been detected in intracellular protozoan parasites such as *Plasmodium*. Inhibitors of the shikimate pathway have been found to modestly inhibit the growth of malaria parasites (McConkey, 1999).

10.3.2 Empirical discovery of lead structure

Even though the advancements of computational chemistry and the increased knowledge of three-dimensional structures of the enzyme make it still more accessible to perform a docking of a ligand into the binding site, discovery of lead compounds is still mainly based on screening of libraries of chemicals or plant extracts. The use of the target molecules for screening enables high throughput screening, if the target molecules can be made available outside the parasites.

An alternative approach is to screen the ability of the compound to inhibit the growth of parasites. This approach was, in the 1970s, facilitated by development of methods for *in vitro* cultivation of *Plasmodium* parasites, enabling screening of a large number of compounds for growth inhibitory activity (Khalid *et al.*, 1986; Trager and Jensen, 1976).

10.3.3 Bioassays used for antimalarial drug discovery

10.3.3.1 *In vitro* screening assays

10.3.3.1.1 Growth inhibition

Usually, two strains of *P. falciparum* are used, a chloroquine sensitive such as 3D7 and a chloroquine resistant such as Dd2. Human peripheral blood erythrocytes are used for the *in vitro* screening studies. Parasites are added to the erythrocytes and cultured at 37°C in an atmosphere of 2% oxygen, 5% carbon dioxide, and 93% nitrogen (Trager and Jensen, 1976). The effect of plant extracts or fractions on the growth of *P. falciparum* is tested by adding 50 μl of a solution of the test sample to 50 μl of parasitised erythrocytes (5×10^8 cells/ml with a parasitemia of approximately 1%). It is important to keep the concentration of the solvent used for dissolving the plant extract low (typically below 0.5%) if an organic solvent such as dimethyl sulfoxide (DMSO) has been used. The parasites are grown in wells of flat-bottomed microtitre plates. The cultures are then incubated for 24 hours; 0.8 μCi of ^{3}H-hypoxanthine or a radiolabelled amino acid such as phenylalanine is added to the wells and the cultures are cultivated for an additional 24 hours. The cultures are harvested and the amount of radiolabelled metabolite, taken up in the parasites, counted. A smaller uptake in the parasites incubated with the test samples than that observed in the controls, gives a measure of inhibition of the parasite growth (Chen *et al.*, 1994a; Jensen *et al.*, 1982). Another method, which can be used for determination of inhibition of parasite growth, is spectrophotometric measurement of parasite lactate dehydrogenate activity. The assay utilises conversion of nitroblue tetrazolium to formazan.

Alternatively, the growth can be measured by microscopic counting of the parasites. Thin smears of the parasite cultures, obtained after incubation with different concentrations of plant extracts or test samples, are stained with Giemsa, and parasitemia is determined as the percentage of infected erythrocytes.

Stage specific assay

In order to test the activity of the test sample on different erythrocytic stages the cultures are synchronised to young ring stage by sorbitol lysis. The parasitised erythrocytes are centrifuged and the pellet resuspended in aqueous 5% D-sorbitol for 5 minutes. They are isolated by an additional centrifugation and addition of erythrocytes to the parasites yields a culture in which at least 90% of the parasites are at the same stage of development. It is then possible to test the samples against the different erythrocytic stages of the parasites by adding them to the cultures at the appropriate time (Lambros and Vanderberg, 1979).

10.3.3.1.2 Selectivity of malaria assays

It is especially important to realise that the *in vitro* method only reveals if a given compound inhibits the growth of the parasites. It does not tell if the compound is generally

cytotoxic, i.e. if the compound kills all types of cells including the cells of the hosts (Angerhofer *et al.*, 1992a). To elucidate this problem, additional test systems must be used. Very often a poor ability to inhibit the growth of cancer cells (e.g. KB cells) or brine shrimps, is taken as an indication of the selectivity of the tested compound. A compound which highly efficiently prevents the growth of the parasites *in vitro* but not the growth of the cancer cells or brine shrimps, is considered of interest for additional studies. The development of a procedure for stimulating lymphocytes with phytohaemagglutinin A *in vitro* (Bygbjerg and Flachs, 1986) has enabled screening of the compounds against a cell type belonging to the immune system. An advantage of this protocol is that it reveals if the compounds might inhibit the immune system, which would be a drawback for drugs to be used for treatment of infections such as parasitic diseases.

10.3.3.2 *In vivo* screening

10.3.3.2.1 Mouse models

Parasites

Different species of *Plasmodium* are used in these assays. The most common ones are *P. berghei* K173, *P. yoelii* YM, *P. chabaudi*, and *P. vinckei*. These strains are lethal to mice and kill the infected untreated animal within 7 to 12 days. Different species of mice are used.

A modified 4-day suppressive test

In the most common assay the animals are inoculated intraperiotoneally with 10^6 parasitised erythrocytes suspended in 0.2 ml. The test sample is administered, either intraperitoneally, or orally. The first administration usually starts three hours after infection and treatment can be given for three to five days depending on the potency of the test sample and the half life in the animals of the active compound. Control mice receive normal saline. Chlorquine, or another drug, ought to be used as a positive control. From day four of infection, thin blood smears are made from tail blood of the mice for determination of parasitemia. The mortality of the mice is determined up to 28 days following the last treatment (Peters, 1980).

For determination of the curative efficacy of the treatment, blood from the treated mice is drawn 28 days after treatment and injected intraperitoneally into new mice. Parasitemia and survival in these animals are determined; lack of parasite detection in the blood 14 days after infection is indicative of definite cure.

10.3.3.2.2 Rat model

Rats infected with 10^7 parasitised erythrocytes infected with *P. berghei* ANKA strain are used. Otherwise, the experiments are performed as described above for experiments in mice.

10.3.3.2.3 Disadvantages of murine models

Drug metabolism in these animals is different from humans and there is a high detoxifying serum activity in them. Biology of the parasite species, tested in these models, is

different from that of human parasites, e.g. there is no hypnozoite formation in any of the murine parasites. Drug susceptibility of murine parasite is different from that of humans. Finally, the interplay between the drug and the host immune system in the murine system is different from that of humans.

10.3.3.2.4 Monkey models

New World primates, such as *Aotus lemurinus* and *Saimiri* spp. monkeys, are commonly used to test the *in vivo* antimalarial efficacy of potential antimalarial drug candidates against infections with *P. falciparum*. The cost and the availability of the monkeys limits the use of these models to drug candidates which have shown promising properties in the murine models.

Non-human primates, such as Macaques, are used to test drugs against human parasites *P. vivax*. In this model, *P. knowlesi* and/or *P. cynomolgi*, are used to infect the animals. These parasites are closely related to *P. vivax*.

The advantage of these models is that they are used for testing drugs against human parasites such as *P. falciparum* and *P. vivax*. When the parasitemia reaches 1% the infected monkeys are treated with the test compound, either intraperitoneally or orally. Parasitemia is determined daily by thick smears and, whenever necessary, by a polymerase chain reaction, identifying parasite DNA. The compounds will be tested at higher parasitemias if they show good effect at a parasitemia of 1%

10.3.3.3 *In vitro* versus *in vivo* models

A promising lead compound should be highly potent (preferentially an IC_{50} value below 1 μM, i.e. the concentration which causes 50% inhibition of the growth of the parasites should be below 1 μM) and should be soluble in pharmaceutically suitable solvents and the compound should be active *in vivo*. As will be illustrated below, a number of compounds are known, which very efficiently kill parasites *in vitro*, but show no activity *in vivo*. Some reasons for this discrepancy are that: a) the compound is unable to penetrate the cell membrane, keeping the compound distant from the intraerythrocytic *P.* parasites; b) the compound is quickly metabolised by the host organism; or c) the compound is distributed in the host organism in a way which prevents it from reaching the blood and thereby the erythrocytes. Compounds meant for oral administration have to be absorbed from the alimentary canal and compounds meant for injection have to be sufficiently soluble in pharmaceutically suitable solvents. Identification of a lead compound does not mean that a drug candidate has been developed. The previously mentioned *in vivo* screening assays, only reveal if the test sample is efficient in the chosen animal, with no pronounced toxic effects. The assay does not disclose a long-term toxic effect such as cancer promotion and it does not disclose how the compound will act in humans. Long-term toxicity tests are performed in other animal studies. The problem concerning the effect of a compound on humans can only be investigated in clinical trials. Before the

compound can go into clinical trials it has also to be verified that it is available in sufficient amounts, i.e. it has to be easily synthesised or easily isolated from a natural source which can be collected in a sustainable way. Optimisation of lead structures again can be done in a rational or in an empirical way. If the target molecule for the lead structure is known, a model of the molecule bound to the target molecule, can be constructed, and from this model a structure with improved properties can be designed (Liljefors and Petterson, 1996). If the target molecule is not known, the optimisation has to be performed in an empirical way. To make this optimisation more efficient Quantitative Structure Activity Relationship analyses can be performed (Högberg and Norinder, 1996).

An example of a drug which was developed by a screening procedure, is chloroquine (**1**). The starting point for this drug was methylene blue. The optimisation procedure, in this case, was very empirical, which is illustrated by the fact that the drug developed initially, was thought too toxic for clinical use. Later findings, however, led to a re-evaluation (Foley and Tilley, 1998).

10.3.4 Natural products

The importance of natural products should not be underestimated. They are still an important source of lead compounds, especially for development of chemotherapeutics (Buss and Waigh, 1995; Cragg *et al*., 1997; Cragg, 1998; Pandey, 1998; Senior, 1996; Shu, 1998; Wall, 1998). It should be mentioned that the fact that a product is isolated from a plant which has been used for centuries, or even millennia, does not diminish the requirements for extensive toxicological studies, since many natural products have severe toxic effects or may be cancerogenic (Hecker, 1984).

Even today, the majority of drugs used against malaria have been developed from, or are, natural products. Among these is the oldest and still-used drug, quinine, which is obtained from the bark of trees belonging to the genus *Cinchonae*. The Europeans brought the bark from South America. For some time it was known as Jesuit bark. The active alkaloids were already isolated in a pure state in 1820, but their structures were not finally elucidated until 1967 (Eiden, 1998). The exact mechanisms of action of these alkaloids are still debated in the literature (Foley and Tilley, 1998). Quinine (**2**) also illustrates that excellent lead structures might be obtained from nature, but, in probably all cases, optimisation of the structure can lead to better drugs. The clinically used quinolinemethanols developed from quinine, such as mefloquine (**3**) possess a better therapeutic ratio than the natural products.

10.3.4.1 Selection of plant material

The selection of plant material for screening of natural products can be based on: a) random collection; b) ethnopharmacology; c) chemotaxonomy; and d) collection of unusual and not-investigated taxa.

10.3.4.1.1 Random collection

Random collection was used by the National Institute of Health for development of chemotherapeutics for treatment of cancer. Only a very few drug candidates were discovered by this approach, which led to changes in the concept of the programme (Frei, 1982).

10.3.4.1.2 Collection based on ethnopharmacology

Knowledge of healing plants has been handed down from father to son and mother to daughter all over the world. In the western world, self-medication, with plants or plant extracts have only a limited importance at the present, although the discipline has undergone a renaissance recently. A major part of the world population, however, still mainly relies on traditional medicine. Deforestation and a changed way of life quickly reduce the survival of the plants and thus, the transmitted knowledge. Drug development, based on this ancient knowledge, has therefore, to be initiated quickly. Investigations into the antiparasitic principles of *Artemisia annua*, used in traditional Chinese medicine for millennia, led to the discovery of artemisinin (**5**), one of the most promising recent antimalarial drugs and lead compounds (Angerhofer *et al.*, 1992a; Casteel, 1997; Klayman, 1985). An ethical problem, however, arises if ethnopharmacological information is used as a starting point. If the information leads to a new drug, how should the income from this drug be shared between the inventor of the drug, the medical company developing the drug, and the informant originally offering the information leading to the drug? All of the three parties have to be involved if this approach is taken and, consequently, an agreement for a fair sharing of the income has to be made. The fear of losing natural resources without getting any part of the profit has already made some countries restrict, or even ban, export of botanicals for chemical studies (Elisabetsky, 1991). Attempts to solve these problems have been the topic for international conferences (Baker *et al.*, 1995). A concrete example of a solution has been found by Merck and Costa Rica (Reid *et al.*, 1996).

10.3.4.1.3 Collection based on chemotaxonomy

The chemotaxonomic approach is based on the fact that botanically related plants very often produce chemically related secondary constituents. If a promising agent of a rare structure is found in a species belonging to a certain genus, other species belonging to the same genus offer a good chance of generating related compounds (Mabry and Mears, 1970). As an example, quinine and closely related alkaloids, have until now only been found in the bark of *Cinchona* or *Remijia*, species both belonging to Rubiaceae (Glasby, 1991). For an overview of chemotaxonomy, the series of Hegnauer can be used (Hegnauer, 1992)

10.4 NATURAL OCCURRING ANTIPLASMODIAL COMPOUNDS

A number of reviews discussing naturally occurring antiplasmodial agents, have been published (Mukherjee, 1991; Murray and Perkins, 1996; Phillipson and O'Neill, 1986; Phillipson and Wright, 1991a,b; Wright *et al.*, 1996). The discussion below will emphasise recently reported examples.

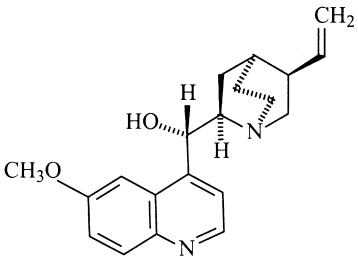
10.4.1 Alkaloids

The alkaloids are a heterogeneous group of naturally occurring compounds containing nitrogen. No exhaustive definition exists, but many alkaloids have an amino acid as precursor and many have pharmacological activity.

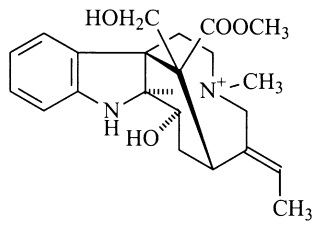
10.4.1.1 Tryptophane derived alkaloids

10.4.1.1.1 Quinolines

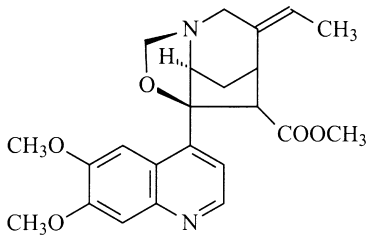
The alkaloid which has been used as an antimalarial drug for the longest period of time, is the quinolinecarbinolamine quinine (**2**). This alkaloid has been of tremendous importance as a drug, either as a constituent of the cinchona bark, which for centuries was the only available drug against malaria, either in the form of an extract of the cinchona bark or as the pure compound. It has been stated that quinine is the drug that has saved most human lives (Casteel, 1997). A problem with quinine is that the difference between the therapeutic dose and a toxic dose is very small, which makes it difficult to give an adequate dose. Examples of drugs in which this drawback has been diminished are mefloquine (**3**) and halofantrine (**4**). Another drawback of quinine is development of resistance among the parasites. The drug is believed to act by an inhibition of haem polymerisation. The *in vitro* IC_{50} values of quinine against a number of different *P. falciparum* strains are 100–440 nM (Nontprasert *et al.*, 1996; Wright *et al.*, 1993). The potency of dihydroquinine, which generally is found as a contaminant in quinine, is of the same order of magnitude, whereas the metabolite 3-hydroxyquinine is one order of magnitude less potent (Nontprasert *et al.*, 1996). Other quinolinecarbinolamines found in the cinchona bark, especially quinidine (**12**), are also used in the clinic (Casteel, 1997). Quinidine is a diastereomer of quinine in which the carbinol carbon and the neighbouring sterogenic carbon have opposite configuration. Several species of the genus *Alstonia* are used in traditional medicine for treatment of, among others, malaria. The major alkaloid, echitamine (**13**), only showed a little antimalarial activity, whereas two minor quinoline alkaloids, corialstonine (**14**) and corialstonidine (**15**), showed some activity against malaria *in vitro* (Wright *et al.*, 1993). Except for the alkaloids present in traditional medicine, the quinolinecarbinolamines, are the only alkaloids which are still used clinically.



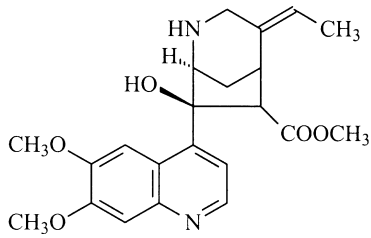
(**12**)



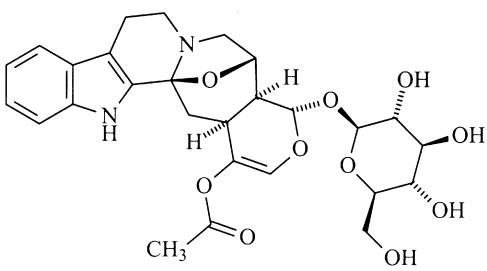
(**13**)



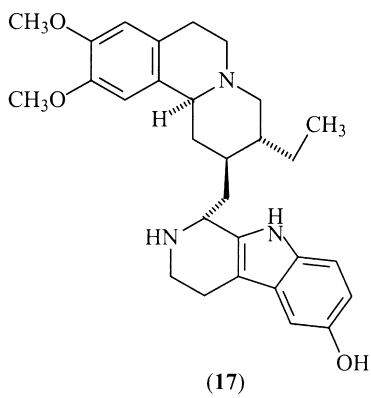
(**14**)



(**15**)



(**16**)

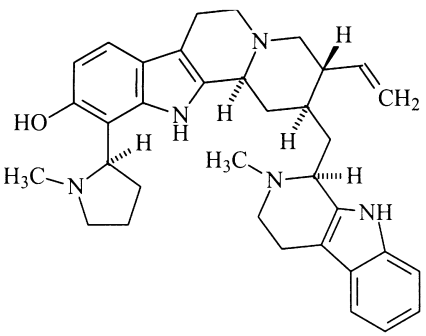


(**17**)

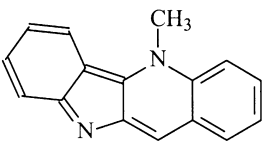
10.4.1.1.2 Indole alkaloids

The indole alkaloid, glucoside cadambine (**16**), shows activity in contrast to a number of related compounds (Kitagawa *et al.*, 1996). The indole alkaloid, tubulosine (**17**), has been shown to have high activity against *P. falciparum in vitro* and to cure malaria infections in mice (Sauvain *et al.*, 1996). In contrast, usambarensine, 3′,4′-dihydrousambarensine, N_b-methylusambarensine, usambarine, 18,19-dihydrosambarine,

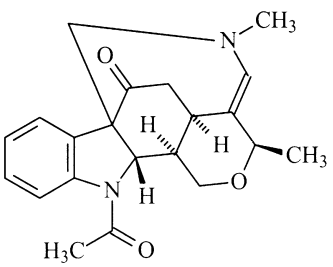
strychnopentamine, isostrychnopentamine, akagerine, tubulosine, and 10′-hydroxy-usambarensine were all very active *in vitro*, but neither strychnopentamine (**18**) nor 18,19-dihydrousambarine cleared infections of *P. falciparum* in mice (Wright *et al.*, 1991; Frédérich *et al.*, 1999). 3-Farnesylindole also inhibits the growth of *P. falciparum* (Nkunya *et al.*, 1991). Cryptolepine (**19**) is highly active *in vitro*, but the *in vivo* activity is debated (Kirby *et al.*, 1995; Cimanga *et al.*, 1997). Neither of the strychnos alkaloids, strychnobrasiline (**20**), nor malagashanine (**21**), inhibit the growth of chloroquine resistant *P. falciparum*, but both of them potentiate the effect of chloroquine *in vitro* as well as *in vivo* (Rasoanaivo *et al.*, 1994; Trigalo *et al.*, 1999). Decoctions of *Strychnos myrtoides* are used as adjuvants in traditional Madagascan medicine for treatment of malaria (Rasoanaivo *et al.*, 1994). The strong effect of strychnos alkaloids on the central nervous system, however, limits their use as antimalarial drugs.



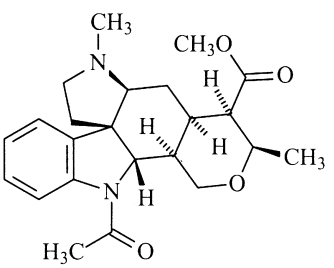
(**18**)



(**19**)

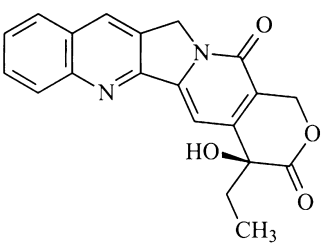


(**20**)

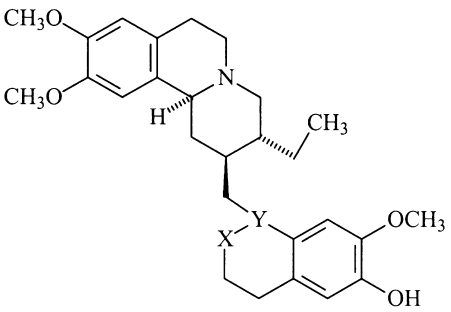


(**21**)

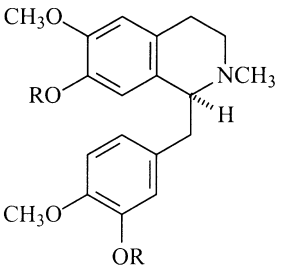
The topoisomerase inhibitor, camptothecine (**22**), moderately inhibits the growth of *P. falciparum in vitro* (Bodley *et al.*, 1998).



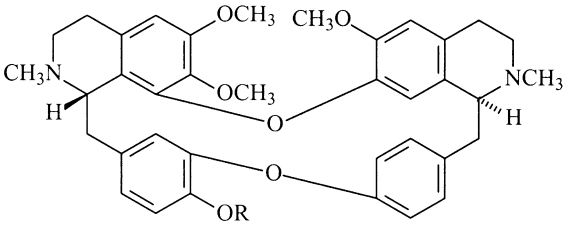
(**22**)



(**25**) X-Y = N=CH
(**26**) X-Y = HN-CH_2



(**23**) R = H
(**24**) R = CH_3



(**27**) R = H
(**28**) R = CH_3

10.4.1.2 Phenylalanine derived alkaloids

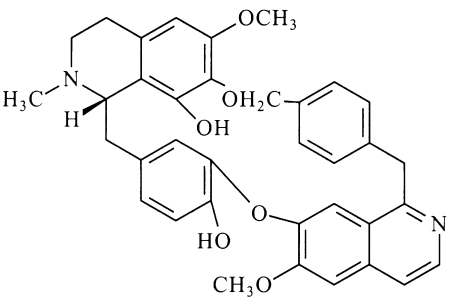
10.4.1.2.1 Benzylisoquinoline alkaloids

The benzylisoquinoline alkaloids reticuline (**23**) and laudanosine (**24**) have a moderate antimalarial activity *in vitro* and laudanosine also has some effect *in vivo* (Rasoanaivo *et al.*, 1998). Psychotrine (**25**) and cephaeline (**26**) have been shown to have high activities against *P. falciparum in vitro* and to cure malaria infections in mice (Sauvain *et al.*, 1996).

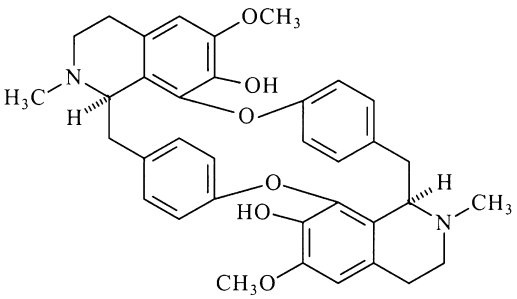
10.4.1.2.2 Bisbenzylisoquinolines

A group of phenylalanine-derived alkaloids, which has received enormous interest as antiplasmodial agents, are the bisbenzylisoquinoline alkaloids such as penduline (**27**) and tetrandrine (**28**) (Valentin *et al.*, 1997). Both of these alkaloids are representatives of bisbenzylisoquinoline alkaloids, a number of which have low nanomolar IC_{50} values *in vitro* (Böhlke *et al.*, 1996; Guinaudeau *et al.*, 1997; Likhitwitayawuid *et al.*, 1993a; Lin *et al.*, 1993, 1994). Screening of large numbers of bisbenzylisoquinoline alkaloids have

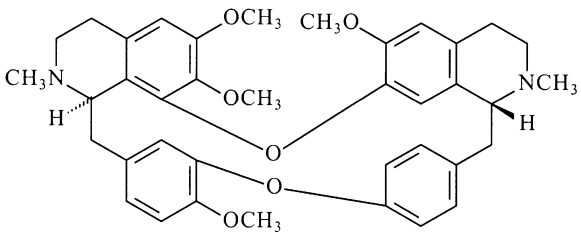
revealed a high potency and selectivity towards malaria parasites (Angerhofer *et al.*, 1999; Marshall *et al.*, 1994). Some of the more promising alkaloids are cycleatjehine (**29**) and cycleanine (**30**) (Angerhofer *et al.*, 1999). The performed structure activity studies were not performed as a three-dimensional analysis of the structures of the molecules, which makes it difficult to draw solid conclusions from the data (Angerhofer *et al.*, 1999). In spite of the reported pronounced activities no results concerning the *in vivo* activities of this type of alkaloids have been reported. Even a very high *in vitro* activity is of no interest unless the compounds are also able to cure an infection *in vivo*. An unexpected observation is that phaeanthine (**31**), antagonizes the *in vitro* effect of chloroquine towards chloroquine sensitive *P. falciparum* (Ekong *et al.*, 1991).



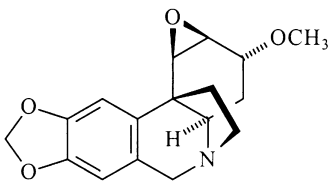
(**29**)



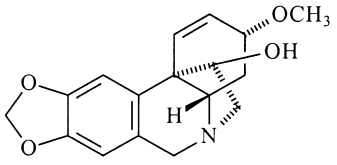
(**30**)



(**31**)



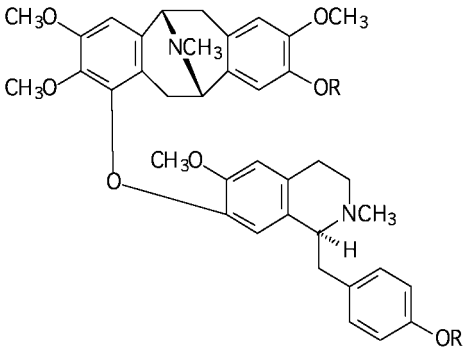
(**32**)



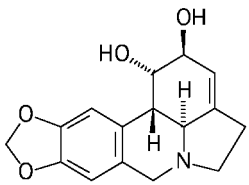
(**33**)

10.4.1.2.3 Alkaloids from Amaryllidaceae

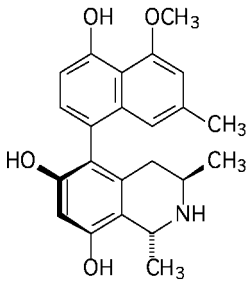
Augustine (**32**), crinamine (**33**), and lycorine (**34**) are examples of isoquinoline alkaloids isolated from *Crinum amabile* belonging to Amaryllidaceae (Likhitwitayawuid *et al.*, 1993b). The Amaryllidaceae alkaloids show a wide range of biological activities, and augustine and crinamine were also potent inhibitors of *P. falciparum in vitro*. The selectivity of the compounds, however, appeared to be poor (Likhitwitayawuid *et al.*, 1993b).



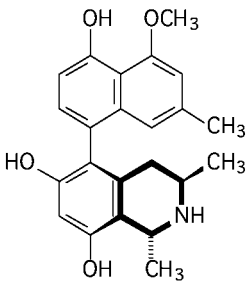
(**35**) R = CH_3
(**36**) R = H



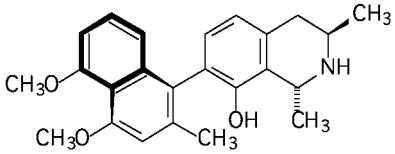
(**34**)



(**37**)



(**38**)



(**39**)

10.4.1.2.4 Hervelines

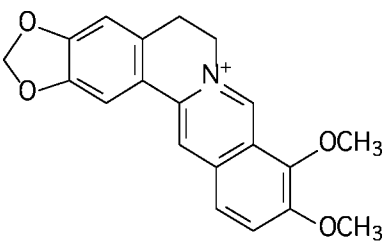
The hervelines are a type of pavine-benzylisoquinoline alkaloids obtained from *Hernandia voyoronii*. Herveline C (**35**) has a strong *in vitro* antimalarial activity and synergistically increases the activity of chloroquine. Herveline D (**36**) also has a strong *in vitro* antimalarial activity but antagonises the activity of chloroquine *in vivo* (Rasoanaivo *et al.*, 1998).

10.4.1.2.5 Naphtylisoquinoline alkaloids

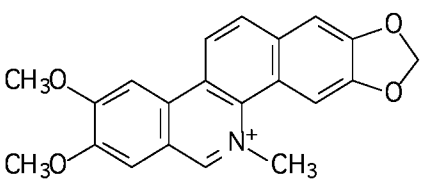
Naphtylisoquinoline alkaloids are a group of alkaloids found in Ancistrocladaceae, some of which, including korupensamine A (**37**), korupensamine B (**38**) and 5-*O*′-demethyldionchophylline, have been shown to possess pronounced antiplasmodial activity *in vitro* (Bringmann *et al.*, 1998; Hallock *et al.*, 1994). The activity of the 7-naphtylisoquinoline alkaloid dioncophylline A (**39**) was doubled by converting it into the corresponding quaternary ammonium ion (Bringmann *et al.*, 1997). Some dimeric naphtylisoquinoline alkaloids showed only a very limited activity (Hallock *et al.*, 1994).

10.4.1.2.6 Protoberberine alkaloids

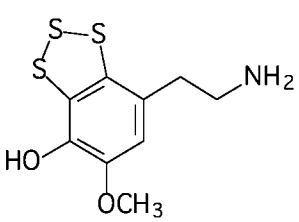
The protoberberines are characterised by the presence of an isoquinoline skeleton, annelated with a naphtalene nucleus. The nitrogen is typically quaternised. Many protoberines such as berberine (**40**) very potently inhibit the proliferation of malaria parasites *in vitro* but are without effect *in vivo* (Iwasa *et al.*, 1998; Vennerstrom and Klayman, 1988).



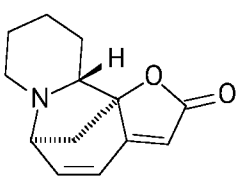
(**40**)



(**41**)



(**42**)



(**43**)

10.4.1.2.7 Other isoquinoline alkaloids

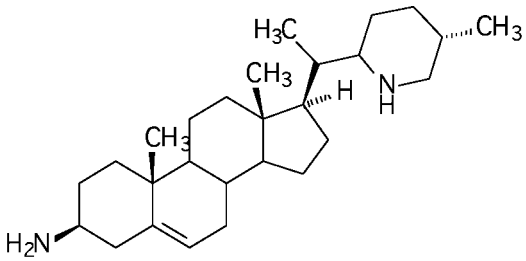
The nucleus of nitidine (**41**) is also formed by an annelation of naphtalene and isoquinoline, but the two moieties are annelated differently from the protoberberine alkaloids. As with the protoberberines, nitidine is very potent *in vitro*, which might be related to its ability to inhibit topoisomerase (Gakunju *et al.*, 1995). Clinical trials with nitidine as an antileukemic agent have been terminated, indicating that the compound might be toxic (Gakunju *et al.*, 1995).

10.4.1.2.8 Lissoclinotoxins

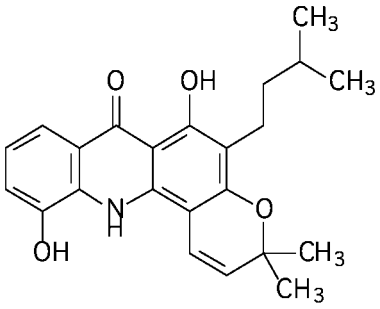
A series of sulfur-containing alkaloids has been isolated from the tunicate *Lissoclinum perforatum*. Among these, the isoquinoline Lissoclinotoxin A (**42**), showed high activity against a number of organisms, including *P. falciparum*. No studies involving mammalian cells revealed if lissoclinotoxin A was generally cytotoxic (Litaudon *et al.*, 1994).

10.4.1.3 Pseudoalkaloids

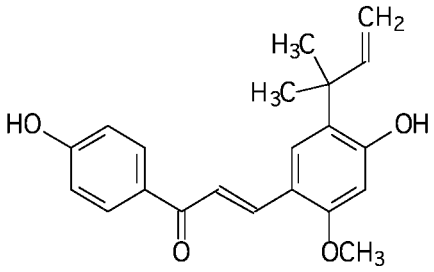
Alkaloids, in which the carbon skeleton has not been formed from an amino acid, are known as pseudoalkaloids. Securinine (**43**) is an example of a pseudoalkaloid with moderate antimalarial activity *in vitro* (Weenen *et al.*, 1990). The steroid alkaloid sarachine (**44**), isolated from *Saracha punctata*, strongly inhibits the growth of malaria parasites *in vitro* and controls infections of *P. berghei* in mice (Moretti *et al.*, 1998).



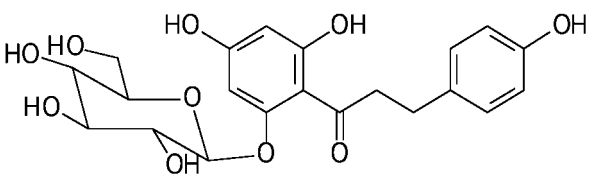
(**44**)



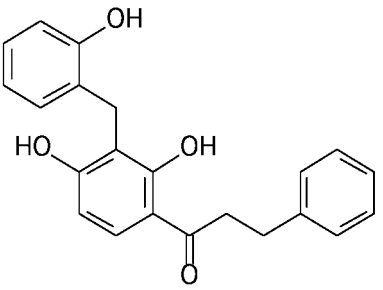
(**45**)



(**46**)



(**47**)



(**48**)

Acridone alkaloids are found in the plant family Rutaceae. A series of 30, most of which were isolated from species belonging to the genus *Citrus*, *Glycosmis*, and *Severinia*, were tested for their growth inhibition of *P. yoelii in vitro*. Of the seven potent alkaloids, atalaphillinine (**45**) was tested *in vivo* and shown to be able to clear infections of *P. berghei* and *P. vinckei* in mice (Fujioka *et al.*, 1989). No toxic effects were observed in the mice.

10.4.2 Flavanoids

Flavanoids are a complex group of natural products composed of a C_6C_3-moiety having shikimic acid as a precursor and a C_6-moiety of polyketide origin. Some examples are known in which the two moieties have been alkylated or the polyketide moiety might contain seven carbons.

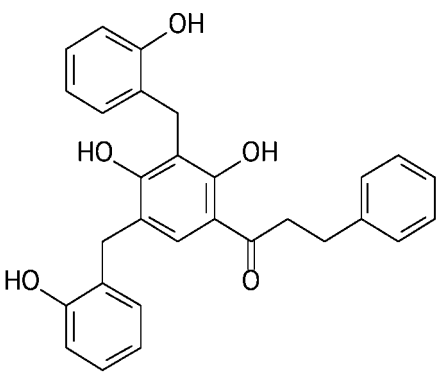
10.4.2.1 Chalcones

The chalcone skeleton, 1,3-diphenylprop-2-enone-1, only consists of two carbocyclic rings. As mentioned above, a series of chalcones was tested as antimalarial agents because they were expected to be protease inhibitors (Li *et al.*, 1995). Prior to this attempt, however, an extract of Chinese liquorice roots was found to potently inhibit the growth of *P. falciparum* as well as the parasites causing leishmaniasis, *Leishmania donovani*. A bioguided fractionation led to the isolation of licochalcone A (**46**) (Chen *et al.*, 1994a,b). Only trace amounts of licochalcone A were found in later batches of Chinese liquorice roots, which might reflect a different biological origin of the roots. Chinese liquorice can be obtained from three species belonging to the genus *Glycyrrhiza: G. glabra* (European liquorice), *G. uralensis*, or *G. inflata*. The antimalarial activity of a sample, in which licochalcone A only was present in trace amounts, was related to the presence of other chalcones (Christensen *et al.*, 1994). The problem of a reliable source of licochalcone A was solved by total synthesis. Simple modification of this synthesis also enabled synthesis of related licochalcones, which, however, turned out to be less

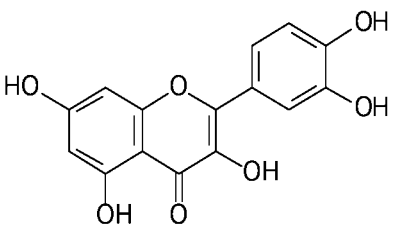
potent antiparasitic agents (Nielsen *et al.*, 1995). Even though licochalcone A shows promising antiparasitic properties *in vitro* as well as *in vivo*, attempts have been made to increase the selectivity. A three-dimensional quantitative structure activity relationship analysis was performed, which revealed how to optimise the antileishmanial activity and diminish the activity against human lymphocytes (Nielsen *et al.*, 1998). Chalcones are α,β-unsaturated ketones, which are known to easily make a conjugated addition to nucleophiles such as thiolo groups in proteins (Nadelmann *et al.*, 1997). The remaining activity of dihydrochalcones, in which this double bond has been reduced, and the activities of β-substituted chalcones, make it unlikely that the reactivity of the double bond is the major reason for the activities. Studies on the mechanism of action revealed that licochalcone A severely damaged the mitochondria of leishmania parasites without affecting the mitochondria of the host cells (Chen *et al.*, 1993). More detailed studies have revealed that the chalcones are inhibitors of fumarate reductase. Since fumarate reductase has a crucial importance for the energy metabolism in the parasites, whereas it has no importance for the host cells, this enzyme might be a promising target for new antimalarial drugs. No drug-targeting fumarate reductase has been developed, indicating that fumarate reductase might be a new pharmacological target.

10.4.2.1.1 Dihydrochalcones

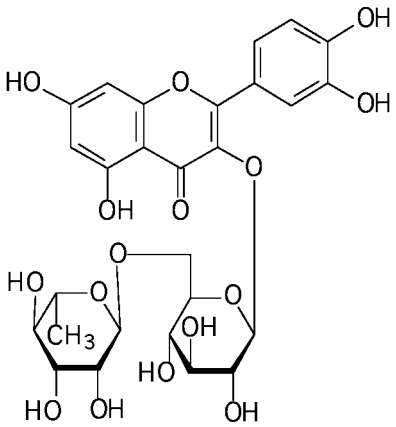
Besides the liquorice chalcones, some dihydrochalcones are known as antiparasitic agents. The oldest known examples are phlorizin (**47**) and its aglycone phloretin (Kutner *et al.*, 1987a; Silfen *et al.*, 1988). Phlorizin cannot penetrate the membranes of healthy erythrocytes, whereas the drug can penetrate the pores which are induced by the parasite in infected cells (Kutner *et al.*, 1987a). The phlorizin-induced inhibition of the pores arrests the parasite growth (Kutner *et al.*, 1987b; Kutner *et al.*, 1987a). A side effect of the phlorizin analogues is their inhibition of the sodium-glucose cosystem



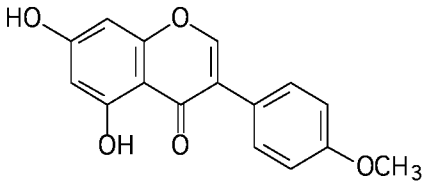
(**49**)



(**50**)



(**51**)



(**52**)

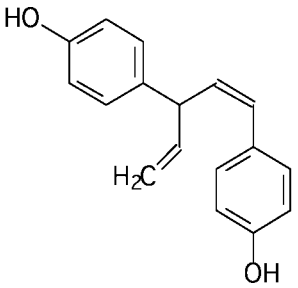
whereby they induce glycosuria and inhibit other transport systems (Kutner *et al.*, 1987a). Attention has to be paid to these side effects, when developing antiparasitic drugs using phlorizin as a template. Other dihydrochalcones, including diuvaretin (**48**) and uvaretin (**49**), have also been shown to be potent antimalarial agents (Nkunya *et al.*, 1991).

10.4.2.2 Flavones and isoflavones

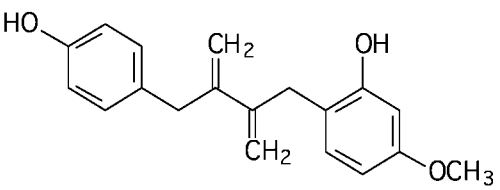
The flavones, which are characterized by the 2-phenylbenzopyran nucleus, in general, are only modest antimalarial agents. Quercetin (**50**) has an IC_{50} value of 21 μM, whereas the glycoside rutin (**51**) has no activity (Khalid *et al.*, 1986). In contrast, the isoflavanoids — 3-phenylbenzopyrans — in general, have a markedly higher antimalarial activity. Biochanin C (**52**) has the same activity as the chalcones (Nielsen, 1997). Similar IC_{50} values have been found for other isoflavanoids and, in addition to antimalarial activities, isoflavanoids also are known to be estrogenic (Sheeman, 1998), which is a problem for drug development.

10.4.3 Lignans

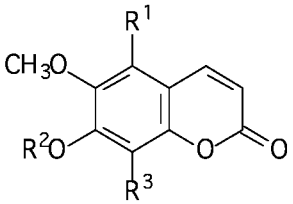
The lignans are biogenetically built by dimerisation of two C_6C_3-moieties originating from shikimic acid. The neolignan nyasole (**53**) significantly inhibits the growth of chloroquine-resistant as well as chloroquine-susceptible *P. falciparum* strains in concentrations in which it only moderately affects phytohaemmaglutinin A stimulated proliferation of lymphocytes (Oketchrabah *et al.*, 1997). Termilignan (**54**) shows the same antimalarial activity as nyasol (Valsaraj *et al.*, 1997).



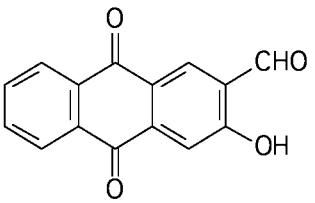
(**53**)



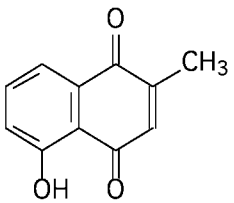
(**54**)



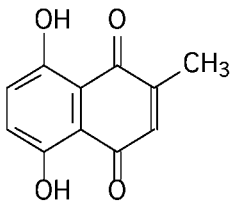
(**55**) $R^1 = OCH_3$, $R^2 = CH_3$, $R^3 = H$
(**56**) $R^1 = H$, $R^2 = H$, $R^3 = OCH_3$



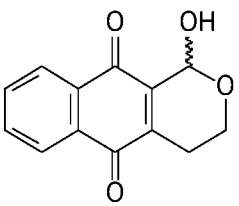
(**57**)



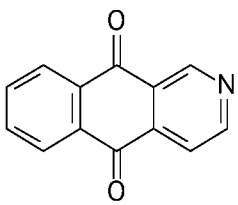
(**58**)



(**59**)



(**60**)



(**61**)

10.4.4 Coumarins

The coumarins contain the 2-oxobenzopyrane skeleton. The *in vitro* antimalarial IC_{50} values for 5,6,7-trimethoxycoumarin (**55**) and isofraxidin (**56**) were 10–35 μM, whereas scopoletin and pectachol showed significantly less activities (Cubukcu *et al.*, 1990; Khalid *et al.*, 1986).

10.4.5 Anthraquinones

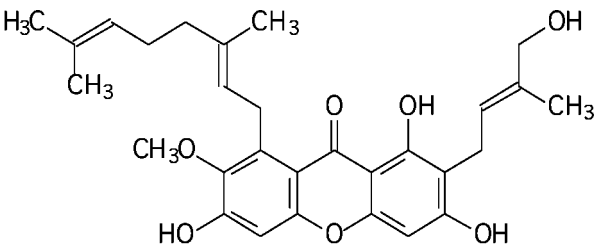
Anthraquinone may either be formed via the acetate pathway or by a sequence involving shikimate and mevalonate (Dewick, 1998). A number of anthraquinones have been isolated from the tree *Morinda lucida*, which is widely used for treatment of malaria in West Africa (Makinde *et al.*, 1994; Wijnsma and Verpoorte, 1986). The poor activities of anthraquinones, e.g. (**57**) isolated from the bark, make it unlikely that any antimalarial activity is caused by these compounds (Sittie *et al.*, 1999).

10.4.6 Naphtoquinones

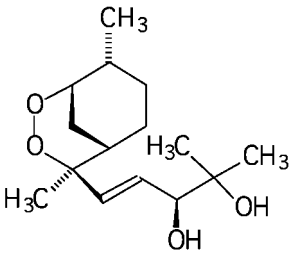
The potent antimalarial effects of plumbagin (**58**), and to a smaller extent 2-methylnaphtazarin (**59**) (Likhitwitayawuid *et al.*, 1998a), violate the statement that a 2-hydroxy group is essential for activity (Hudson, 1993). The tetrahydroderivative isoshinanolone was approximately 100 times less active. Derivatives of plumbagin were found to be less potent (Likhitwitayawuid *et al.*, 1998a). The two naphtoquinones (**60**) and (**61**) have also been found to be potent antimalarial compounds *in vitro*, but activities towards KB cells, however, was only three times smaller (Solis *et al.*, 1995). Extensive medicinal chemical studies, in which attention was made to high activity and metabolic stability in man, eventually led to atovaquone (Hudson, 1993). Atovaquone (**6**), and possibly other naphtoquinones, inhibit the mitochondria cytochrome C reductase, and thereby the mitochondria electron transport, by competing with ubiquinone (Hudson, 1993). Mammalian mitochondria are considerably less sensitive.

10.4.7 Xanthones

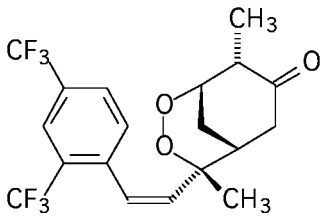
Xanthones, e.g. cowanol (**62**) isolated from *Garcinia cowa*, which has been used as an antipyretic in traditional medicine in Thailand, are potent antimalarial compounds *in vitro* (Likhitwitayawuid *et al.*, 1998b). The mode of action might be based on the ability of the xanthones to complex with haem and thereby preventing haem-polymerisation (Ignatushchenko *et al.*, 1997). A model for docking xanthones on haem was constructed (Ignatushchenko *et al.*, 1997). This model can also be used for docking acridones (Fujioka *et al.*, 1989) to haem, indicating that they may have the same mode of action.



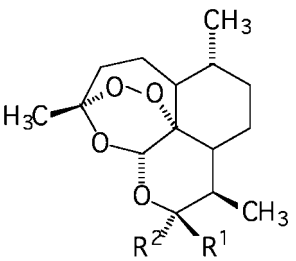
(**62**)



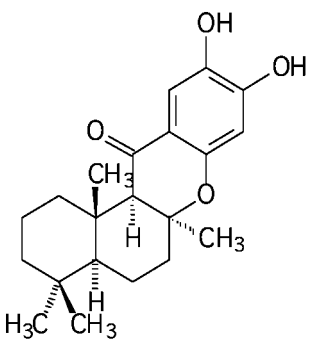
(**63**)



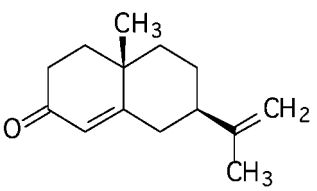
(**64**)



(**65**) $R^1 = OCH_3$, $R^2 = H$
(**66**) $R^1 = OC_2H_5$, $R^2 = H$
(**67**) $R^1 = H$, $R^2 = OOCCH_2CH_2COO^-$



(**68**)



(**69**)

10.4.8 Terpenoids

The terpenoids are a heterogeneous group of natural products, which have all been formed from mevalonic acid. They are characterised by the presence of the isoprene building unit in the skeleton, even though this unit might be severely changed by rearrangement in the molecule or carbon atoms might be lost (Dewick, 1998). Depending on the number of isoprene units, the terpenoids are classified as monoterpenoids (2 units ~ 10 carbons), sesquiterpenoids (3 units ~ 15 carbons), diterpenoids (4 units ~ 20 carbons), sesterpenoids (5 units ~ 25 carbons), triterpenoids (6 units ~ 30 carbons), tetraterpenoids (8 units ~ 40 carbons), or higher terpenoids.

10.4.8.1 Sesquiterpenoids

10.4.8.1.1 Peroxides

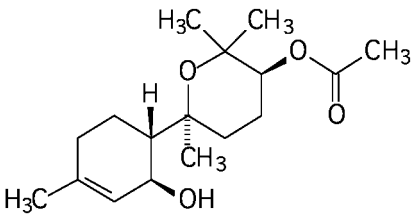
The antimalarial activity of the natural endoperoxide yingzhaosu A (**63**), isolated from *Artabotrys unicinatus* used in Southern China for malaria (Desmet, 1997), has prompted a drug development programme at Hoffmann-La Roche, which eventually lead to arteflene (**64**) (Ro 42-1611) (Hofheinz *et al.*, 1994). The pharmacokinetic of yingzhaosu A prevented its use as a drug, but clinical studies with arteflene have revealed that this drug might be used for treatment of mild malaria (Salako *et al.*, 1994; Somo-Moyou *et al.*, 1994).

Artemisinin (**5**) is another example of a natural peroxide. In the case of artemisinin, the compound itself has been used as a drug, but poor solubility in pharmaceutical suitable solvents or poor chemical stability has also initiated medicinal chemical studies for the development of better drugs. Examples of such drugs are artemether (**65**), arteether (**66**), and sodium artesunate (**67**) (Casteel, 1997; Murray and Perkins, 1996).

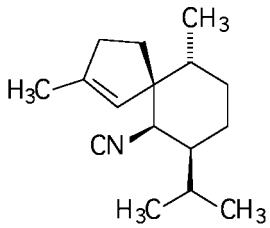
A series of additional natural peroxides has been screened for antimalarial activity (Rucker *et al.*, 1991, 1992). Most of the peroxides were terpenoids, mainly sesquiterpenoids, but some examples of other origin were also investigated. Most of the compounds were potent, but no studies on the selectivity of the compounds were reported. The field is also investigated by synthesis of different peroxides (Peters *et al.*, 1993; Pitt *et al.*, 1998; Posner *et al.*, 1992).

10.4.8.1.2 Sesquiterpenoids without peroxide groups

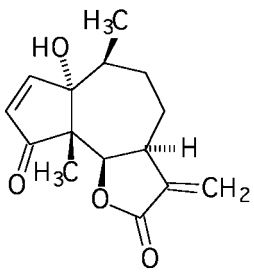
15-Oxopuupehenone (**68**) is a representative of a distinctive family of marine sponge metabolites consisting of a sesquiterpene joined to a C_6-shikimate moiety, which possesses antiplasmodial and antitumour activity (Nasu *et al.*, 1995). α-Cyperone (**69**), which, like α-methylene sesquiterpene lactones, is an α,β-unsaturated ketone, also show antimalarial activity *in vitro* (Weenen *et al.*, 1990). 1-Hydroxy-α-bisboloxide A acetate (**70**) also inhibits the growth of *P. falciparum in vitro* (Cubukcu *et al.*, 1990).



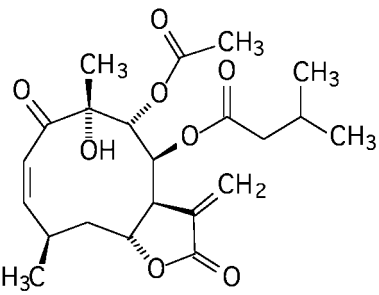
(**70**)



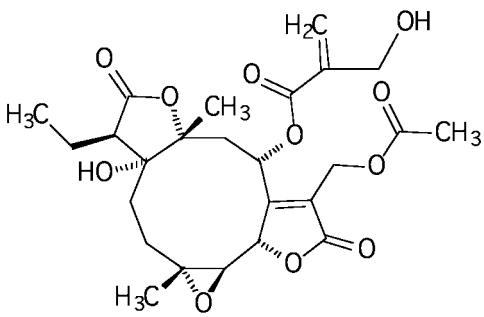
(**71**)



(**72**)



(**73**)



(**74**)

Axisonitrile 3 (**71**) is an example of a sesquiterpenoid isonitrile, from a marine sponge, which possesses high and selective antimalarial selectivity (Angerhofer *et al.*, 1992b). Also, a number of diterpenoid isonitriles with interesting properties have been described.

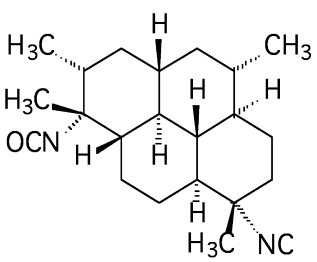
10.4.8.1.3 Sesquiterpene lactones without peroxide groups

Parthenin (**72**), neurolenin B (**73**), and a series of other sesquiterpene lactones, have been shown to have some antimalarial activities (Francois *et al.*, 1996; Hooper *et al.*, 1990; Ohigashi *et al.*, 1994). A characteristic for the active sesquiterpene lactones in these studies is the presence of a double bond conjugated with carbonyl groups, either as a α-methylene lactone or as a α,β-unsaturated ketone or ester. Sesquiterpene lactones with these features are known to easily undergo conjugated additions with nucleophiles such as thiolo groups in cysteine residues of proteins and this reaction is assumed to explain the general cytotoxicity of the lactones (Kupchan *et al.*, 1970). Sesquiterpene lactones were, for several years, studied intensively as potential chemotherapeutic agents for treatment of cancer (Cassady and Suffness, 1980; Misra and Pandey, 1981; Pettit *et al.*, 1985). Unfortunately the sesquiterpene lactones appear not to be significantly more toxic against the malaria parasites than the cancer cell lines, indicating that intensive medicinal chemical studies have to be performed before antimalarial drugs can be designed on this basis. 16,17-Dihydrobrachycalyxolide (**74**) was found to be more toxic towards lymphocytes than malaria parasites (Oketchrabah *et al.*, 1998).

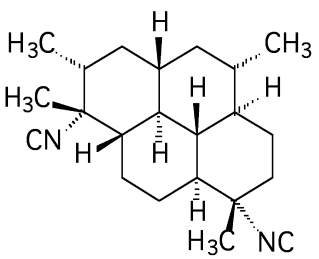
10.4.8.2 Diterpenoids

A series of 15 diterpenoids, containing isonitrile, isothiocyanante, or isocyanate groups, have been isolated from the tropical marine sponge *Cymbastela hooperi* (Konig *et al.*, 1996; Wright *et al.*, 1996). Two of these diterpenoids (**75**, **76**), especially, were very potent antimalarial compounds and showed higher activity towards malaria parasites than against KB cells. *In vivo* studies may reveal if these compounds are promising new lead compounds for antimalarial drugs.

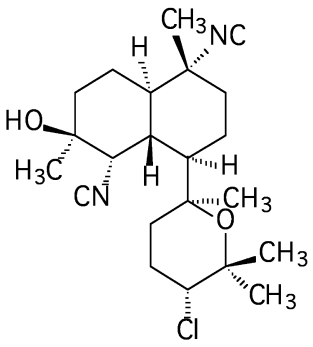
Kalihinol A (**77**), isolated from a marine sponge belonging to the genus *Acanthella*, is an additional example of an isonitrile which very potently inhibits the growth of *P. falciparum* (Miyaoka *et al.*, 1998). The compound shows a high selectivity towards parasites.



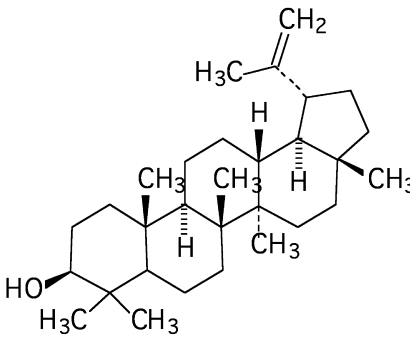
(**75**)



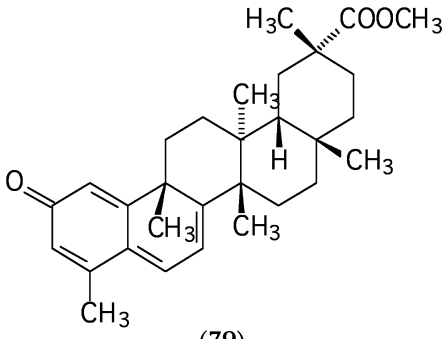
(**76**)



(77)



(78)



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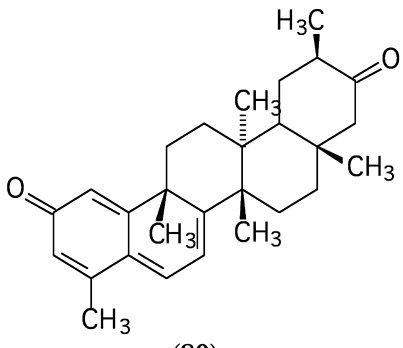


(80)

10.4.8.3 Triterpenoids

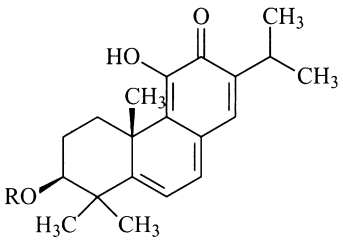
10.4.8.3.1 Lupeol

Lupeol (**78**) is a weak *in vitro* antimalarial (Alves *et al.*, 1997).

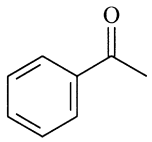
10.4.8.3.2 Quinone methides

Several species, belonging to the family Celastraceae, including the genera *Salacia* and *Maytenus*, are used for treatment of malaria in traditional medicine (Desmet, 1997; Gessler *et al.*, 1994; Sullivan *et al.*, 1998). Quinone methides such as pristimerin (**79**) and tingenone (**80**), have been isolated and found to potently inhibit the growth of *P. falciparum in vitro* (Figueiredo *et al.*, 1998). Quinone methides have previously been investigated as potential antitumour compounds (Gunatilaka, 1996; Misra and Pandey, 1981) but they appear to have some selectivity towards parasites (Figueiredo *et al.*, 1998).

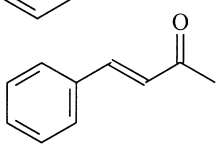
3-*O*-Benzoylhosloppone (**81**) and 3-*O*-cinnamoylhosloppone (**82**), both of which also contain a quinoid moiety, have also been found to be potently antimalarial *in vitro* (Geethva and Varalakshmi, 1998).

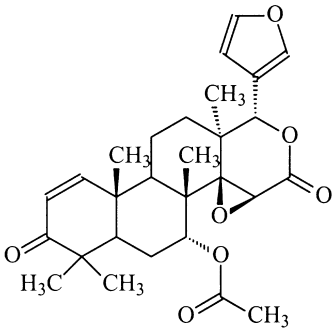


(**81**) R =

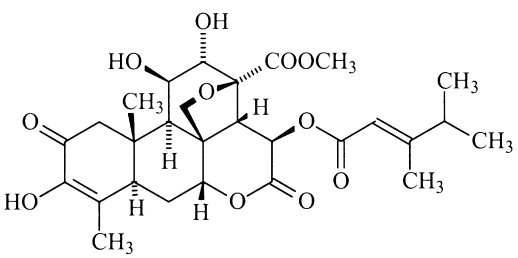


(**82**) R =





(**83**)



(**84**)

10.4.8.3.3 Limonoids

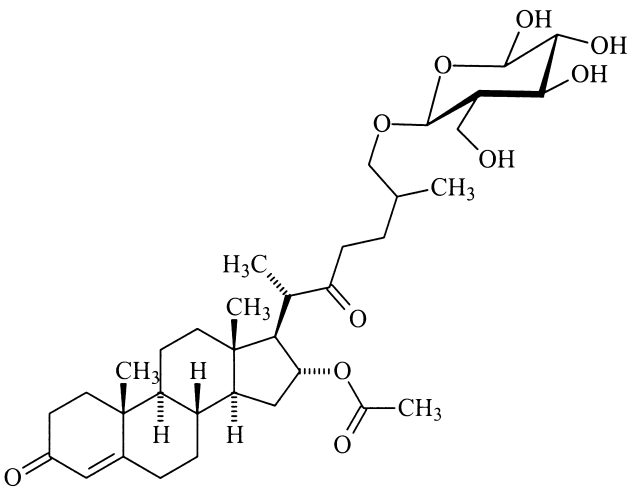
Limonoids are a group of triterpenoids characterised by a C-17-side chain which has been transformed into a furane ring (Dewick, 1998; Dreyer, 1968; Taylor, 1984). Gedunin (**83**) was recognised early as a potent antimalarial compound (Khalid *et al.*, 1986) which shows some selectivity (MacKinnon *et al.*, 1997). Attempts to modify the structure of gedunin or to find other limonoids with better activities have not led to more promising lead structures (Khalid *et al.*, 1998; MacKinnon *et al.*, 1997; Phillipson and Wright, 1991b). The neem tree, *Azadirachta indica*, has a strong reputation throughout India, Asia and Africa, as an antimalarial (Desmet, 1997; Phillipson and Wright, 1991a). A number of limonoids have been isolated from this tree (Phillipson and Wright, 1991a).

10.4.8.3.4 Quassinoids

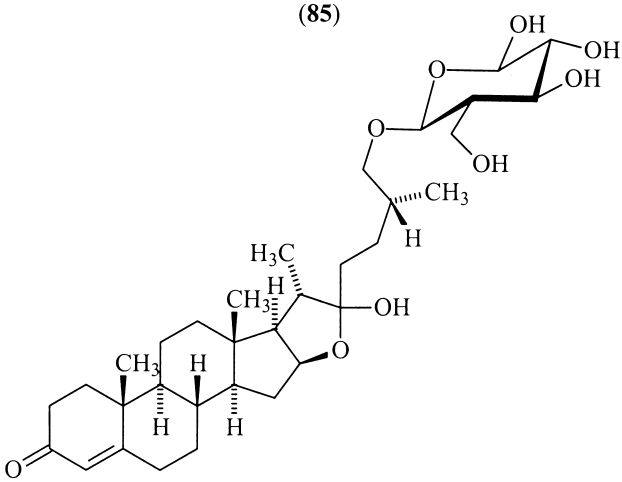
Quassinoids are another group of triterpenoids in which the C-17-side chain has been transformed (Dewick, 1998). Some quassinoids, including bruceantin (**84**), are more potent as antimalarials than chloroquine (Phillipson and Wright, 1991a) and most show high activity (Cabral *et al.*, 1993). The quassinoids have previously been investigated as potential antitumour compounds (Cassady and Suffness, 1980; Misra and Pandey, 1981; Ozeki *et al.*, 1998) indicating that selectivity might be a problem.

10.4.8.4 Steroids

Two steroidal saponins, tumacoside A (**85**) and tumaquenone (**86**), displayed some antimalarial activities *in vitro*, whereas other saponins were inactive (Saez *et al.*, 1998).



(**85**)



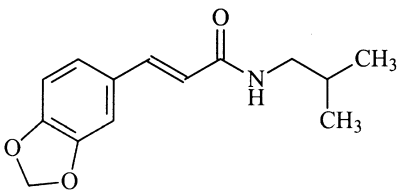
(**86**)

10.4.9 Fatty acids

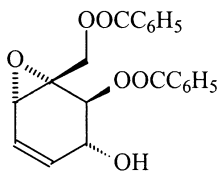
Oleic acid is reported to be a very weak *in vitro* antimalarial (Krugliak *et al.*, 1995).

10.4.10 Alkamides

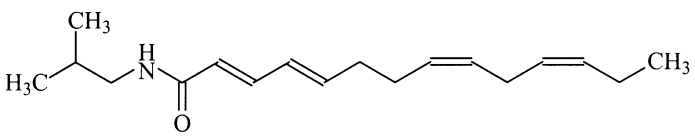
Alkamides are naturally occurring amides of especially unsaturated acids. Fagaramide (**87**), *N*-isobutyl-2,4-decadienamide, 2,4-octadienamide, 2,4-decadienamide, and hazaleamide (**88**), are modest antimalarials *in vitro* (Shibuya *et al.*, 1992; Sittie *et al.*, 1998; Weenen *et al.*, 1990).



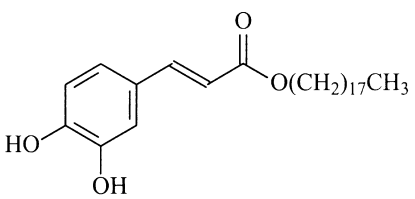
(**87**)



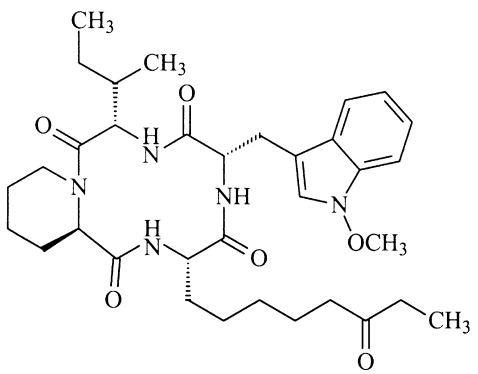
(**89**)



(**88**)



(**90**)



(**91**)

10.4.11 Esters

Pipoxide (**89**) (Nkunya *et al.*, 1991) and octadecyl caffeate (**90**) (Likhitwitayawuid *et al.*, 1998a) are modest antimalarials *in vitro*.

10.4.12 Peptides

The previously discussed antimalarials are all typical examples of secondary metabolites. Recent reports have called to attention cyclic peptides, which also show antimalarial properties. Apicidin, a cyclic peptide isolated from *Fusarium pallidoroseum*, controls infections of *P. berghei* in mice in concentrations at less than 10 mg/kg (Singh *et al.*, 1996). Cyclosporins are potent antimalarials *in vitro* (Kocken *et al.*, 1996), whereas the cyclooctapeptide, chevalierin A, was only found to be moderately potent (Baraguey *et al.*, 1998). A series of other chevalierins were not able to inhibit the growth of malaria parasites.

10.5 CONCLUSION

In the last two decades malaria has become an extremely serious threat to the health and economic prosperity of the world. It is currently primarily restricted to the developing countries in the tropical and subtropical regions of the world, which cannot afford to pay for the high cost of research and drug development. It is hoped that, in the not-too-distant future, funding of malaria research will come to reflect the major thread that this disease represents to the world's population. Strategies to control the disease involve vector control, development of vaccines and of new drugs, to overcome the serious spread of resistance against the presently available drugs. Nature has proven to be a good source for lead structures, but in no cases a source for good drug candidates. Several projects, starting with botanicals used in traditional medicine, have led to active principles, which are used as templates for development of drug candidates. Research, performed in the last decade, has revealed that marine organisms also offer new lead structures. Cyclic peptides could also reveal new possibilities for drug development. The present review has called attention to some new lead structures and we sincerely hope that these new paths for drug development might lead to the new effective and safe drugs which are so urgently needed.

ACKNOWLEDGEMENT

We wish to acknowledge the financial support from Statens Serum Institute, Copenhagen, Danida, The Danish Ministry of Foreign Affairs, and the European Commission's INCO-DC programme.

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CHAPTER

11

Search for Natural Products which Affect Cyclooxygenase-2

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Contents

SUMMARY

This review summarises recent developments in the search for natural products with effect on the isoenzyme cyclooxygenase-2 (COX-2). Two strategies are described, whose objective is to find substances of natural origin, which either affect the *in vitro* prostaglandin biosynthesis which is catalysed by COX-2, or which affect the expression of COX-2. The effect on COX-2 of a number of plant extracts and of 65 isolated compounds with origins in terrestrial plants, microorganisms and marine organisms, are summarised and discussed. Examples are also given of how inhibitors of cyclooxygenase catalysed biosynthesis *in vitro* can be isolated using bioassay guidance. The COX-2 regulators which are identified, are of many different origins and represent a molecular diversity which includes stilbenes, antrhaquinones, flavonoids, alkaloids, fatty acids and terpenoids. All of these have different selective activities on COX-2. The search for regulators of COX-2 from natural sources can reveal novel structure-activity relationships which can be used in the drug discovery process, such as the development of improved anti-inflammatory drugs and use as pharmacological probes in inflammatory research.

11.1 INTRODUCTION

Many of today's important drugs are derived from natural products. The objectives of the drug discovery process are to find new targets and new leads. Two major approaches are currently used in lead finding, one uses the chemical diversity of nature and the other applies combinatorial chemistry. In order to discover new leads from nature, suitable bioassays need to be used and developed for the detection of bioactivity, guidance of the isolation procedure, and a preliminary biological characterisation of isolated compounds.

The enzyme cyclooxygenase-1 (COX-1), also named prostaglandin endoperoxide H synthase-1 (PGHS-1), has been used extensively in assays for studying the anti-inflammatory activity of plant extracts and plant constituents. Interest in this area has increased dramatically in recent years due to the discovery of an isoform of COX-1, cyclooxygenase-2 (COX-2), also known as prostaglandin endoperoxide H synthase-2 (PGHS-2). COX-1 is considered to be constitutively expressed and to be important for homeostatic functions such as maintaining the integrity of the gastric mucosa, regulating renal blood flow and mediating normal platelet function. The COX-2 isoform, on the other hand, is induced by inflammatory mediators, such as cytokines, in different inflammatory states (Vane and Botting, 1998). In a recent review by Marnett *et al.* (1999), developments in this area, concerning structure, function and inhibition of the prostaglandin endoperoxide H synthases are excellently described.

The motivation for research in this area is, of course, to develop more selective anti-inflammatory drugs, but recent research also points to possible applications for selective COX-2 inhibitors as drugs for cancer and Alzheimer's disease (Vane and Botting, 1998).

11.2 ASSAYS FOR NATURAL COX-2 REGULATORS

There are two principal strategies in the search for natural compounds which regulate the activity of COX-2. Both are based on the fact that COX-2 is the inducible isoform of the enzyme and on the hypothesis that the process of induction causes the unwanted symptoms of inflammation and related conditions. The first strategy identifies the effect on the COX-2 enzyme, while the second strategy investigates the gene expression of COX-2, i.e. suppression of protein or mRNA.

11.2.1 Inhibition of the COX-2 enzyme

In general, the assays for inhibition of prostaglandins catalysed by COX-2 measure the conversion of arachidonic acid (AA) to prostaglandins, especially PGE_2 which is the major metabolite detected in inflammation (Vane and Botting, 1996).

There are several sources of the enzyme. Purified sheep placenta enzyme and human recombinant enzyme have been used by, for example, Kase *et al.* (1998) and Noreen *et al.* (1998b). Aspirin and lipopolysaccharide (LPS) treatment leads to exclusive synthesis of COX-2 in recovered cells (Lee *et al.*, 1992; Payá *et al.*, 1996) which means that the enzyme can also be extracted from microsome fractions of human or rat leukocytes or macrophages which have been treated with aspirin and LPS. Hwang *et al.* (1996) have also used LPS-stimulated macrophages for studying the PGE_2 formation in whole cells, but without isolating the microsome fraction. Hiermann *et al.* (1998), used another model with Ca ionophore to induce ^{14}C-prostaglandin synthesis in a perfused rabbit ear.

11.2.1.1 Methods of quantifying PGE_2

The most common way to quantify PGE_2, formed by COX-2, is to use a radio immunoassay (RIA), which is based on the competitive binding between unlabelled (sample) and labelled molecules to specific antibodies against PGE_2 (Moroney *et al.*, 1988). RIA kits for the quantification are commercially available. An alternative way is to use an enzyme immunoassay (EIA), which uses an enzyme instead of radioactivity as the label (Kase *et al.*, 1998). A method using a tracer with scintillation counting has also been used for determining the COX-2 inhibitory effect (Ringbom *et al.*, 1998). This approach uses a labelled substrate for the enzyme, ^{14}C-arachidonic acid being the most common one. Consequently, enzymatic products of the reaction, including PGE_2, are also labelled and can be quantified in a scintillation counter, after the excess unreacted AA has been removed by column chromatography (Noreen *et al.*, 1998b). This method has the disadvantage that prostaglandins are not separated but are analysed as a group. A more specific and selective method can be developed with HPLC detection of the products formed. Subbaramaiah *et al.* (1998a) have extracted the reaction products with ethyl acetate and applied a sample of the extract on a reversed phase HPLC column, combined with an on-line radiochemical detector.

The conversion of AA to PGE_2 consumes oxygen and this fact can be used to quantify the product formation using oxygen electrodes (Kulmacz and Lands, 1987). Both instantaneous and continuous measurement of the enzyme reaction can be carried out with this method (Wang *et al.*, 1999).

11.2.2 Suppression of COX-2 gene expression

The expression of COX-2 is highly restricted in most tissues. Exceptions include the macula densa of the kidney and the brain (Zhang *et al.*, 1997). However, in certain conditions, including rheumatoid arthritis (Spangler, 1996) and some forms of colon cancer (Subbaramaiah *et al.*, 1997), the gene expression is upregulated. This can occur in response to a number of stimuli, including lipopolysaccharide (LPS), phorbol esters (TPA), tumour necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β) (Appleton *et al.*, 1996). Battistini *et al.* (1994) showed that these stimuli affect prostanoid production and COX-2 expression in a variety of cell types. The induction of expression is rapid, which indicates that the COX-2 gene belongs to the immediate early gene family (Herschman, 1991).

Numerous growth factors and cytokines are involved in inflammation, and they act via signal transduction pathways which alter transcription (Herschman, 1996; Smith *et al.*, 1996). This means that the COX-2 message is regulated to a large extent by transcriptional control. The promoter/enhancer region of the human COX-2 gene contains several binding-sites for recognised transcription factors, including nuclear factor-kappa B (NF-κB) and the cyclic AMP responsive element binding protein (CREBP) (Crofford, 1996). Unlike the COX-1 gene, the COX-2 gene also contains a glucocorticoid regulatory region, which explains why the gene expression is inhibited by glucocorticoids such as dexamethasone (Vane and Botting, 1996).

Another major means of controlling COX-2 expression is believed to be post-transcriptional regulation. The human COX-2 mRNA contains 22 copies of the RNA instability sequence, AUUUA, and this may cause the short half life (≈30 min) of the transcripts (Morris and Richards, 1996). Interestingly, these transcripts can be made less stable, for example by IL-1β, or they can be made more stable, for example by dexamethasone (Ristimäki *et al.*, 1994). This raises the possibility of using this mechanism as a drug target.

11.2.2.1 Methods to induce the COX-2 gene expression

Various stimuli have been used to induce the COX-2 gene expression, including chenodeoxycholate (CD) and phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), UV irradiation and TNFα via the transcription factor NF-κB (*see*, for example, Zhang *et al.*, 1999; Tao *et al.*, 1998; Soriani *et al.*, 1998; Holloway *et al.*, 1998, respectively). COX-2 gene expression has also been induced *in vivo*, with the potential inhibitor and the inducer being administered to the animal, and then the gene expression quantified (Jang and Pezzuto, 1998; Wu *et al.*, 1998).

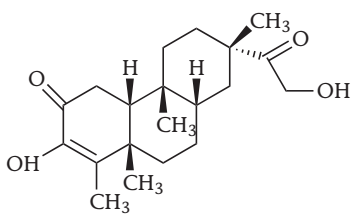
11.2.2.2 Detection of the products of COX-2 gene expression

Blotting techniques are most commonly used to quantify COX-2 gene expression, for example, Northern blot is used to analyse the amount of mRNA. The analysis begins by isolating mRNA, which is electrophoresed on an agarose gel. The gel is then blotted on an electrostatically charged paper and hybridised with a labelled probe which is specific to the COX-2 mRNA (Roshak *et al.*, 1997). Western blotting (also called immunoblotting) is an analogous method to analyse the amount of proteins. Proteins are electrophoresed on a gel which separates molecules according to size (usually by one-dimensional SDS-PAGE), blotted onto a membrane, and then hybridised with an antibody against COX-2 (Glaser and Lock, 1995). Another method for detecting COX-2 gene expression uses the reverse transcriptase polymerase chain reaction (RT-PCR). The mRNA is isolated and then converted to cDNA by reverse transcriptase. After PCR amplification, the gene segment for COX-2 is identified using agarose gel electrophoresis (Holloway *et al.*, 1998). Finally, RIA methods can be used to analyse the amount of COX-2 protein, e.g. Pang and Hoult (1997).

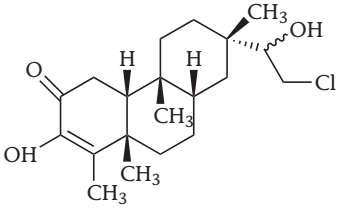
11.3 NATURAL PRODUCTS WHICH AFFECT COX-2 CATALYSED PROSTAGLANDIN BIOSYNTHESIS *IN VITRO*

11.3.1 Akendo 1, 2 and 3

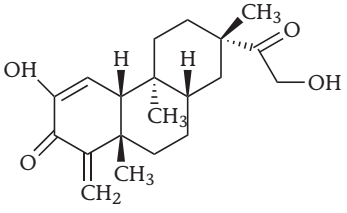
The dolabrane derivatives, akendo 1, 2 and 3 (**1–3**) were tested on COX-2 catalysed PGE_2 synthesis in LPS-stimulated microsomes from human leukocytes. RIA was used for quantification of the produced PGE_2. Akendo 3 (**3**) inhibited the activity of COX-2 by 43%



Akendo 1 (**1**)



Akendo 2 (**2**)

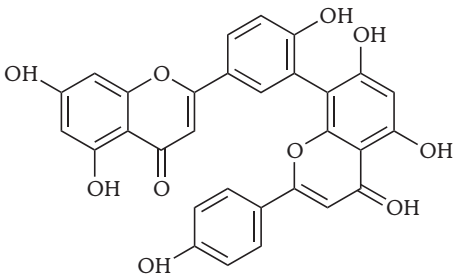


Akendo 3 (**3**)

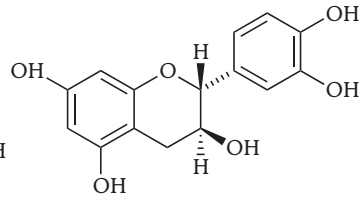
(tested at 100 μM), while **1** and **2** had no effect. The thromboxane B_2 synthesis, which is catalysed by COX-1 in the same cells, was inhibited by **2** and **3** with an IC_{50} value of 49.7 μM and 33.7 μM, respectively. Compound **1** had no effect. The IC_{50} values of the reference compounds, NS-398 on COX-2 and indomethacin on COX-1, were 2.0 μM and 0.01 μM, respectively (Payá *et al.*, 1996).

11.3.2 Amentoflavone, (+)-Catechin, (+)-Gallocatechin, 4′-MeO-(−)-Gallocatechin, Mearnsitrin, Myricitrin, Ouratea-catechin, Ouratea-proanthocyanidin, Quercitrin, Vavain and Vavain-3′-O-β-D-glucoside

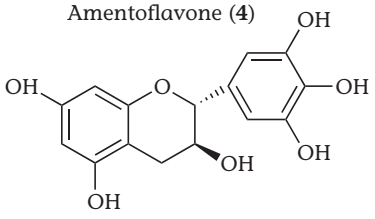
The inhibitory effects of the flavonoids, amentoflavone (**4**), (+)-catechin (**5**), (+)-gallocatechin (**6**), 4′-MeO-(−)-gallocatechin (**7**), mearnsitrin (**8**), myricitrin (**9**), ouratea-catechin (**10**), ouratea proanthocyanidin (**11**), quercitrin (**12**), vavain (**13**) and vavain-3′-O-β-D-glucoside (**14**) on COX-2 and COX-1 catalysed prostaglandin biosynthesis are described in Section 11.6.



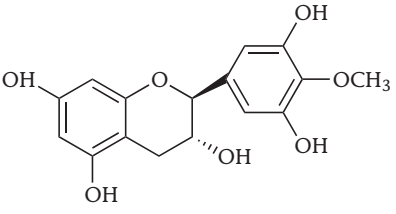
Amentoflavone (**4**)



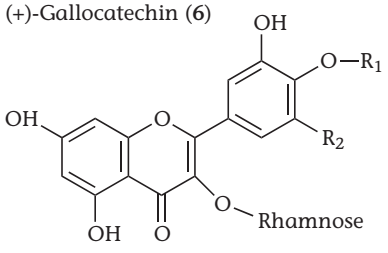
(+)-Catechin (**5**)



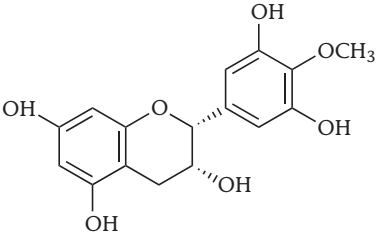
(+)-Gallocatechin (**6**)



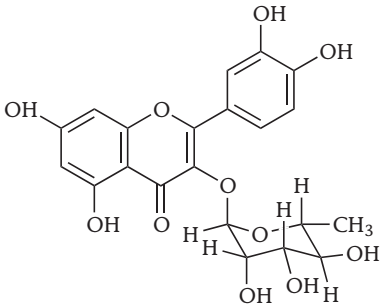
4'-MeO-(−)-gallocatechin (**7**)



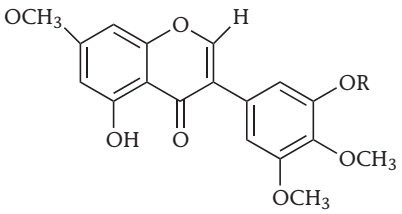
Mearnsitrin R_1=CH_3, R_2=OH (**8**)
Myricitrin R_1=H, R_2=OH (**9**)
Quercitrin R_1=H, R_2=H (**12**)



Ouaratea-catechin (**10**)



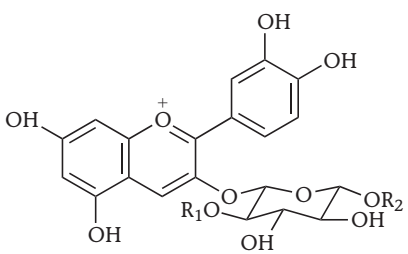
Ouaratea-pro-anthocyanidin A (**11**)



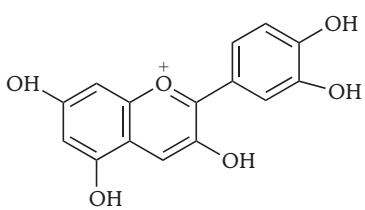
Vavain R=H (**13**)
Vavain 3'-O-β-D-glucoside R=glucose(**14**)

11.3.3 Anthocyanin 1–3 and Cyanidin

The effects of anthocyanins 1–3 (**15–17***)* and the cyanidin aglycon (**18***)*, were tested on COX-1, obtained from ram seminal vesicles, and human recombinant COX-2. The enzyme activities were measured by the rate of O_2 uptake. The three anthocyanins (**15–17**) showed little or no effect on the enzyme activity at 300 μM although higher concentrations of **15** and **16** increased the activity. The ratio of the activity of COX-2 to that of COX-1 (COX-2/COX-1 ratio) was 0.56 for **18**. Compound **18** had IC_{50} values of 60 μM and 90 μM, for COX-2 and COX-1, respectively. The rate of inhibition was time-independent. The reference compound, aspirin gave an IC_{50} value of 1050 μM for both enzymes (Wang *et al*., 1999).



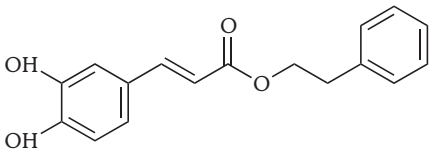
Anthocyanin 1: R_1=glucose, R_2=rhamnose
Anthocyanin 2: R_1=H, R_2=rhamnose (**16**)
Anthocyanin 3: R_1=H, R_2=H (**17**)



Cyanidin (**18**)

11.3.4 Caffeic acid phenethyl ester

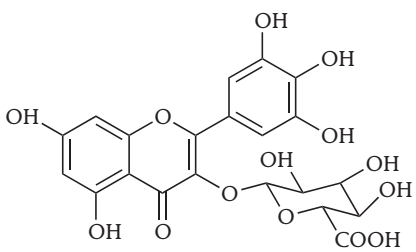
The enzyme activities of recombinant COX-2 and COX-1, expressed in baculovirus, were inhibited by caffeic acid phenethyl ester (**19**), yielding IC_{50} values of 82 μM and 58 μM, respectively (Michaluart *et al.*, 1999).



Caffeic acid phenethyl ester (**19**)

11.3.5 Myricetin-3-O-β-D-glucuronide

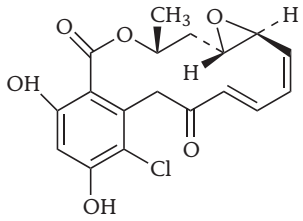
Myricetin-3-O-β-D-glucuronide (**20**), a flavonol glycoside, showed an equipotent inhibition of COX-2 and COX-1 with IC_{50} values of 8 and 10 μM, respectively. The COX-2 enzyme was obtained from sheep placenta and the COX-1 from ram seminal vesicles. The IC_{50} values of indomethacin were 2.4 and 9.2 μM, respectively. The cyclooxygenase activity was measured at 530 nm. A more potent inhibition was observed by **20** for the production of 12-HHT catalysed by COX-1 in human platelets (IC_{50} = 0.5 μM), measured by RP-HPLC with UV-detection at 232 nm. Moreover, the inhibition of the production of PGI_2, PGD_2, and PGE_2 in perfused rabbit ear *in vivo* by **20** (1 μg) was measured, using RIA and/or TLC (Hiermann *et al.*, 1998).



Myricetin-3-O-β-D-glucuronide (**20**)

11.3.6 Radicicol

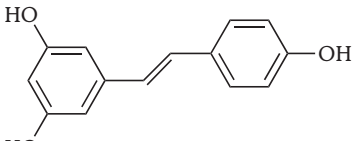
The enzyme activities of COX-2 and COX-1 were unaffected by the fungal antibiotic radicicol (**21**). The enzymes were purified from sheep placenta and ram seminal vesicles and the amount of radioactive PGE_2 produced was determined by scintillation (Chanmugam *et al,* 1995).



Radicicol (**21**)

11.3.7 Resveratrol

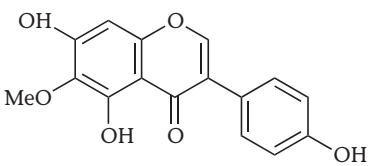
Johnson and Loo (1998) found, by monitoring oxygen consumption, that resveratrol (**22**) (100 μM) increased COX-2 activity by a factor of two, and inhibited COX-1 in a non-competitive manner with a K_i of 26 μM. They also reported that the peroxidase activities of COX-2 and COX-1, measured by reduction of 5-phenyl-4-pentenyl hydroperoxide, were inhibited by **22** (IC_{50} values of >200 μM and 15 μM, respectively). They obtained lower IC_{50} values of **22** when they measured peroxidase activity following the oxidation of N,N,N′,N′-tetramethyl-p-phenylenediamine of ovine COX-2 and COX-1 or human recombinant COX-2 enzyme (45 μM, 4 μM and 85 μM, respectively). Furthermore, **22** inhibited the hydroperoxidase activity of COX-2 with an ED_{50} of 85 μM (Jang *et al.*, 1997). Subbaramaiah *et al.* (1998a) showed that resveratrol (**22**) (30 μM) inhibited basal PGE_2 synthesis by 70% through inhibition of COX-2 activity in HME cells. Jang *et al.* (1997) measured oxygen consumption and reported that **22** had no effect on COX-2 activity. However, both the cyclooxygenase and hydroperoxidase activity of COX-1 was inhibited by **22** (ED_{50} of 15 μM and 3.7 μM, respectively) (Jang *et al.*, 1997).



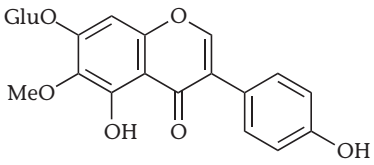
Resveratrol (**22**)

11.3.8 Tectoridin and Tectorigenin

No inhibitory effect was seen of either of the isoflavonoids, tectorigenin (**23**) and tectoridin (**24**), (3–30 μM) on the PGE_2 production catalysed by COX-2 or COX-1, obtained from sheep placenta and sheep seminal vesicle. Dose-dependent inhibition was observed for the reference compounds: NS-398 (0.01–1 μM) on COX-2 and indomethacin (0.01–1 μM) on COX-1. The amount of PGE_2 produced was measured by RIA (Kim *et al.*, 1999).



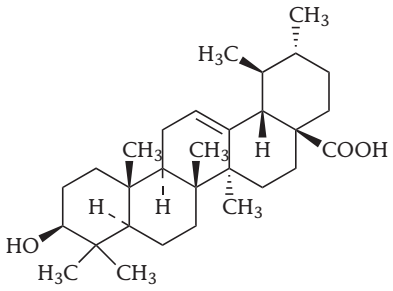
Tectorigenin (**23**)



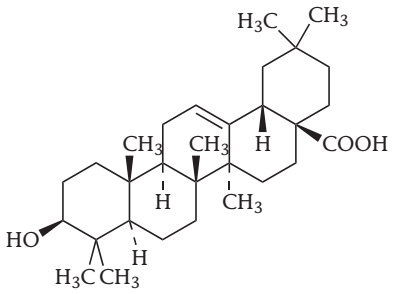
Tectoridin (**24**)

11.3.9 Ursolic acid, Oleanolic acid and Glycyrrhetinic acid

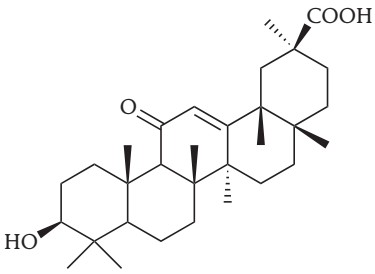
The effects of the triterpenes, ursolic acid (**25**), oleanolic acid (**26**) and glycyrrhetinic acid (**27**), on COX-2 and COX-1 catalysed prostaglandin biosynthesis, have been investigated (*see* Section 11.6).



Ursolic acid (**25**)



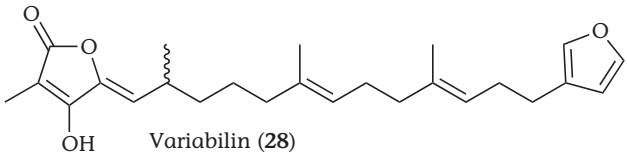
Oleanolic acid (**26**)



Glycyrrhetinic acid (**27**)

11.3.10 Variabilin

The inhibitory effects of the sesterterpene variabilin (**28**), on the COX-2 and COX-1 enzymes, from a microsomal fraction of human monocytes or J774 cells, were studied using RIA. Compound **28** did not affect the generation of PGE_2 of any of the enzymes up to 10 μg/ml. Furthermore, a dose of 10 μM **28** did not effect PGE_2 synthesis in rat cecum (Escrig *et al.*, 1997).

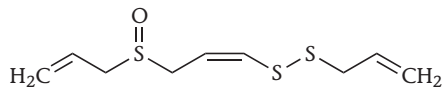


Variabilin (**28**)

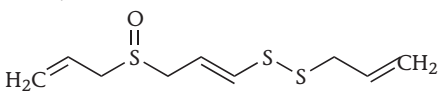
11.4 NATURAL PRODUCTS WHICH AFFECT COX-2 GENE EXPRESSION AND PRODUCTION OF PROSTAGLANDINS

11.4.1 Ajoene and Allicin

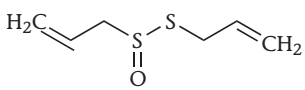
Ajoene (**29**) and allicin (**30**) had no effect on COX-2 mRNA but increased COX-2 protein levels in LPS-induced RAW 264.7 macrophage cells. The LPS-stimulated increase of PGE_2 was inhibited by **29** and **30** with IC_{50} values of 2.4 μM and 37.7 μM, respectively. Moreover, the PGE_2 production which is catalysed by COX-2 was inhibited by **29** with an IC_{50} value of 3.4 μM, while no effect was observed for **30** (Dirsch and Wollmar, 1998).



(Z)-Ajoene (**29**)



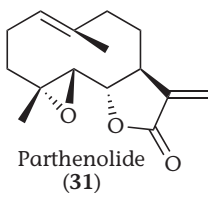
(E)-Ajoene (**29**)



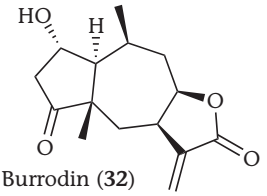
Allicin (**30**)

11.4.2 Burrodin, Confertiflorin, Costunolide, Encelin, Enhydrin, Leucanthin B, Melampodin A, Parthenolide, Psilostachyin A and Tenulin

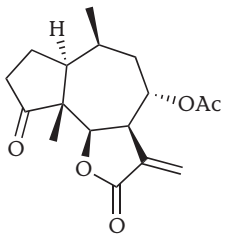
Parthenolide (**31**) inhibited the expression of COX-2 mRNA and COX-2 protein in LPS-stimulated alveolar macrophages in a dose-dependent manner. The *de novo* synthesis of COX-2, in the same cells, for **31** and for the related sesquiterpene lactones burrodin (**32**), confertiflorin (**33**), costunolide (**34**), encelin (**35**), enhydrin (**36**), leucanthin B (**37**), melampodin A (**38**), psilostachyin A (**39**) and tenulin (**40**), was investigated by measuring PGE_2 synthesis. The IC_{50} values are shown in Table 11.1 (Hwang et al., 1996).



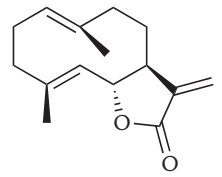
Parthenolide (**31**)



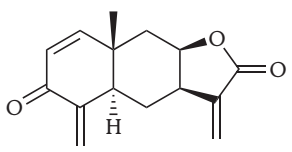
Burrodin (**32**)



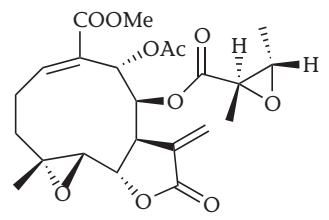
Confertiflorin (**33**)



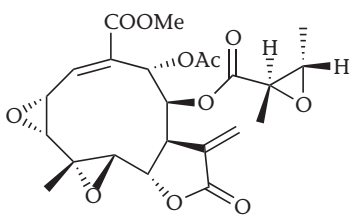
Costunolide (**34**)



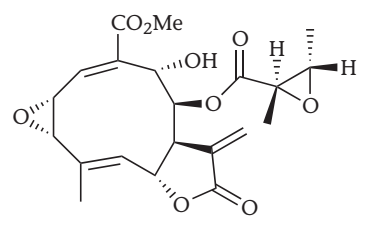
Encelin (**35**)



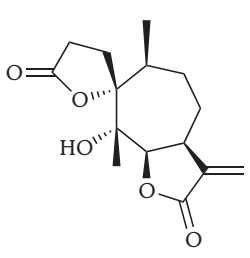
Enhydrin (**36**)



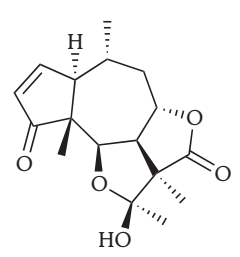
Leucanthin B (**37**)



Melampodin A (**38**)



Psilostachyin A (**39**)



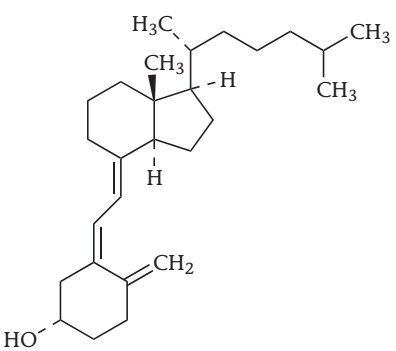
Tenulin (**40**)

11.4.3 Caffeic acid phenethyl ester

Caffeic acid phenethyl ester (**19**) (1–2.5 μM) suppressed 12-O-tetradecanoylphorbol-13-acetate (TPA) and Ca ionophore induced production of PGE_2, with or without excess of arachidonic acid, in oral epithelial 1483 cells. The COX-2 and COX-1 protein levels were not affected. The enzymatic activities of recombinant COX-2 and COX-1 expressed in baculovirus were inhibited by **19**, with IC_{50} values of 82 μM and 58 μM, respectively. Moreover, **19** (5–20 μg/ml) decreased TPA-mediated induction of COX-2 protein and mRNA levels in 1483 cells and in oral epithelial cells (MSK Leuk 1). The COX-2 promoter activity was also suppressed by **19** (15 μg/ml). A dose-dependent (tested between 5 and 7.5 μg/ml) inhibition of TPA and A23187 stimulated release of arachidonic acid was observed by **19**, although higher concentrations were needed than those required for suppression of the production of PGE_2. Administration of caffeic acid phenethyl ester (**19**) caused dose-dependent inhibition of the production of PGE_2 (ED_{50} 23 mg/kg) and 6-keto PGF1α in rat carrageenan air pouch fluid. A marked decrease in the level of COX-2 protein and of the amount of PG synthesised, was seen by **19** at 100 mg/kg, while indomethacin completely inhibited the production of PG but not of protein at 2 mg/kg (Michaluart *et al.*, 1999).

11.4.4 Cholecalciferol

Cholecalciferol (**41**) (vitamin D_3) significantly enhanced the expression of COX-2 mRNA and protein in human epidermal keratinocyte cell lines (tested at 30 μM). Compound **41** induced a dose-dependent increase in PGE_2 production, giving a 4-fold increase at 60 μM. Both effects may be mediated via protein kinase C (Kanekura *et al.*, 1998).

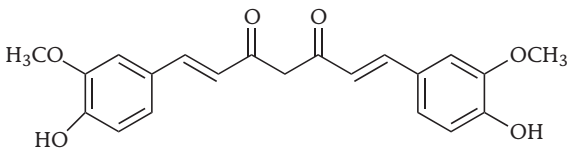


Cholecalciferol (**41**)

11.4.5 Curcumin

The phenolic antioxidant, curcumin (**42**), caused a dose-dependent inhibition of CD- and PMA-mediated induction of COX-2 gene expression in several gastro-epithelial cells.

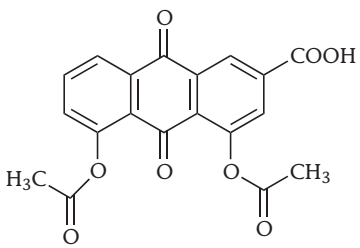
A dose range of 1–20 μM of **42** decreased CD- and PMA-stimulated COX-2 protein levels in human esophageal adenocarcinoma (SK-GT4), squamous carcinoma (SCC450), small intestine and colon carcinoma cells. The amount of COX-1 was not altered in SK-GT4 cells by **42**. Furthermore, the COX-2 mRNA levels induced by CD in SK-GT4 and in SCC450 cells were decreased by **42** (tested at 1–10 μM). Compound **42** (10 μM) also suppressed CD- and PMA-induced COX-2 transcription and PGE_2 synthesis in SK-GT4 cells (Zhang *et al.*, 1999). Pre-treatment with **42** (10–40 μM) of a human colon epithelial cell line before induction with TNF-α or fecapentaene-12 caused a dose-dependent inhibition of COX-2 mRNA expression (Holloway *et al.*, 1998). Zhang *et al.* (1999) suggest that curcumin (**42**) inhibits COX-2 mRNA expression via inhibition of protein kinase C activity, while Holloway *et al.* (1998) propose that this effect could be mediated through inhibition of NFκB activation.



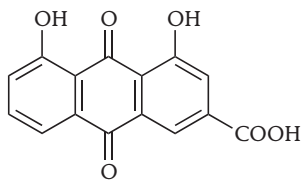
Curcumin (**42**)

11.4.6 Diacerhein and Rhein

The anthraquinones, diacerhein (**43**) and rhein (**44**) (5–20 μg/ml), produced a dose-dependent increase in COX-2 protein level in IL-1β-stimulated human osteoarthritis chondrocytes. An upregulation of PGE_2 synthesis was observed at 5 and at 10 μg/ml of **43** and of **44**, whereas no effect was observed for either of the compounds at a dose of 20 μg/ml (Pelletier *et al.*, 1998).



Diacerhein (**43**)

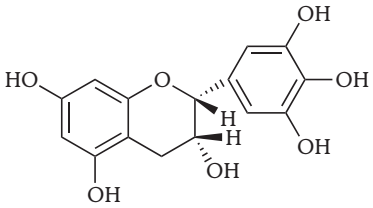


Rhein (**44**)

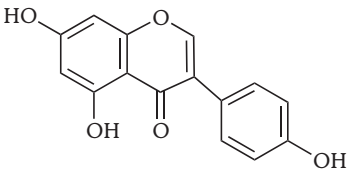
11.4.7 Epi-gallocatechin, Genistein, Daidzein, Hesperetin and Naringenin

Epi-gallocatechin (**45**) (10 μg/ml) decreased the ultraviolet-activated (UVA)-COX-2 gene expression by 40–50% in a human oral carcinoma cell line, while it increased it by

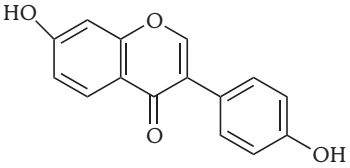
60–70% in a human skin fibroblast cell line at a dose of 15 μg/ml (Soriani *et al.*, 1998). The flavonoids, genistein (**46**), daidzein (**47**), hesperetin (**48**) and naringenin (**49**) (0–100 μM), induced a stress response in murine J774A.1 macrophages which resulted in the upregulation of COX-2 proteins (Johnson and Loo, 1998). Chanmugam *et al.* (1995) reported inhibition of LPS-induced COX-2 expression in macrophages by **46** with an IC_{50} value of 52 nM.



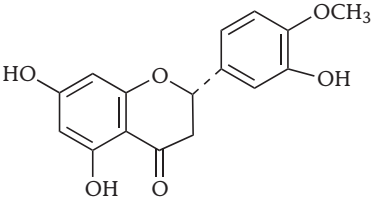
(−)-Epigallocatechin (**45**)



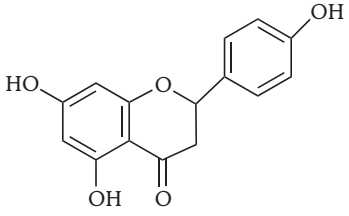
Genistein (**46**)



Daidzein (**47**)



Hesperetin (**48**)



Naringenin (**49**)

11.4.8 Fish oil and other fats

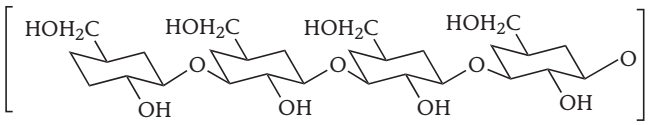
COX-2 and COX-1 protein levels were investigated in rats whose diet had been supplemented with corn and/or menhaden oil, and in which mammary tumours had been introduced by N-nitrosomethylurea. Menhaden oil (rich in n-3 fatty acids), but not corn

oil, significantly suppressed both COX-2 (−36%) and COX-1 (−28%) protein levels 31 weeks after tumour induction (Hamid *et al.*, 1999). In another set of experiments, both colonic mucosa and visible colonic tumours, induced by azoxymethane in rats, were investigated for COX-2 expression levels after 36 weeks of dietary supplementation with fish or corn oil. The data suggests that corn oil may promote colon tumourigenesis by upregulating the COX expression, whereas fish oil may exert its antitumour effect by inhibiting the COX-2 expression (Singh *et al.*, 1997). The relationship between the amounts of fats and fibre in the diet and the level of the COX-2 protein, was investigated in rats during azoxymethan-induced colonic tumourigenesis. Fish oil delayed and reduced the upregulation of COX-2 during tumourigenesis more than corn oil did (Chang *et al.*, 1998).

Liver fibrosis was induced in rats by ethanol in combination with fish oil, and it was found that it led to increased COX-2 mRNA levels. The mRNA levels returned to pre-experimental levels only when ethanol was discontinued and rats were fed a diet enriched in saturated fat (either palm oil or medium chain triglycerides) (Nanji *et al.*, 1997). Saito *et al.* (1997) reported that incubation of rat vascular smooth muscle cells with eicosapentaenoic acid triglyceride did not alter the amount of either COX-2 or -1 protein.

11.4.9 (1 → 3)-β-D-Glucan

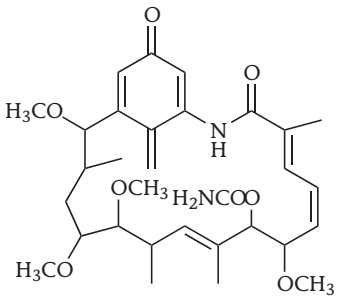
(1 → 3)-β-D-Glucan (**50**), a cell wall component of gram negative bacteria, increased the expression of COX-2 mRNA in rat alveolar macrophages in a dose-dependent manner. Compound **50** had the same effect in the murine monocyte/macrophages cell line, RAW 264.7. Both of these experiments used concentrations of **50** between 20 and 500 μg/ml, and the mRNA was detected with RT-PCR (Ljungman *et al.*, 1998).



(1→3)-β-D-Glucans (**50**)

11.4.10 Herbimycin A

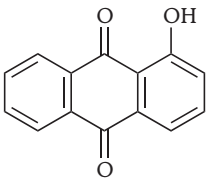
Herbimycin A (**51**) inhibited COX-2 expression in a macrophage-like (RAW 264.7) cell line, depending on which inducer had been used. When cells were stimulated with LPS, **51** strongly inhibited COX-2 expression, while stimulation of cells with PMA gave a weak inhibition (Chanmugam *et al.*, 1995).



Herbimycin A (**51**)

11.4.11 1-Hydroxy-anthraquinone

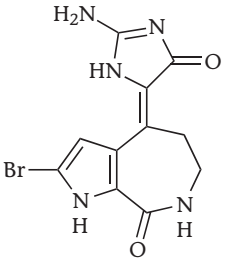
The mRNA expression of COX-2 increased significantly in non-neoplastic mucosa of both the colon and the cecum in rats fed with a diet consisting of 1% 1-hydroxy-anthraquinone (**52**) for 9 and 18 months (Yoshimi *et al.*, 1995).



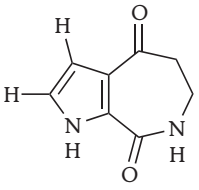
1-Hydroxy-anthraquinone (**52**)

11.4.12 Hymenialdisine and Aldisine

Hymenialdisine (**53**) (0.03–10 μM), which is a marine natural product, inhibited PGE_2 production in IL-1β-stimulated RSF (human rheumatoid synovial fibroblasts) in a dose-dependent manner through inactivation of the pro-inflammatory transcription factor



Hymenialdisine (**53**)

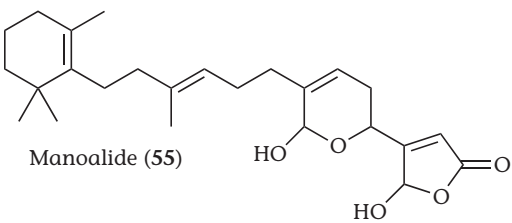


Aldisine (**54**)

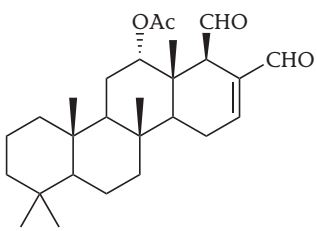
NFκB. Furthermore, **53** (3 μM) reduced the IL-1β-stimulated NFκB activation of COX-2 mRNA in RSF, to the same level as in the non-stimulated cells. The addition of **53** (3 μM) to cells induced by IL-1β reduced the COX-2 protein level to basal levels. In contrast, the analogue aldisine (**54**) (10 μM) showed only marginal effects in the systems studied (Roshak *et al.*, 1997).

11.4.13 Manoalide and Scalaradial

The marine natural products manoalide (**55**) and scalaradial (**56**) (both tested at 1–10 μM), dose-dependently inhibited the LPS-induced endogenous PGE_2 production, the COX-2 activity and the expression of COX-2 protein in human monocytes. The magnitude of all of these effects depended on the dose. Compound **56** (3–10 μM) inhibited both IL-1β-induced COX-2 protein expression and PGE_2 production. Compound **55** had less effect on COX-2 protein expression stimulated by the COX-2 inducers used in this study. Both **55** and **56** showed a dose-dependent inhibition of ionomycin- and PMA-stimulated PGE_2 production. Glaser and Lock (1995) suggest that the effect of scalaradial (**56**) is mediated through transcriptional regulation of COX-2 expression, and that the effects of both compounds are independent of lipid mediator production.



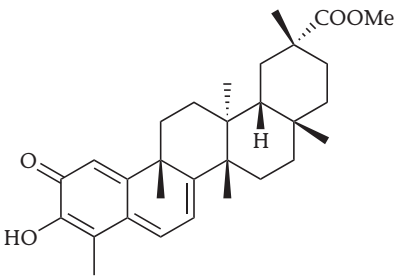
Manoalide (**55**)



Scalaradial (**56**)

11.4.14 Pristimerin

Pristimerin (**57**), which is a triterpene, did not reduce the steady-state mRNA levels of COX-2 in LPS-induced RAW 264.7 macrophages at a concentration of 1 μM (Dirsch *et al.*, 1997).



Pristimerin (**57**)

11.4.15 Radicicol

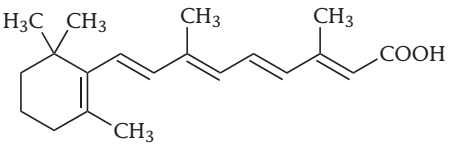
Radicicol (**21**) inhibited the COX-2 gene expression in LPS-stimulated macrophages (IC_{50} value of 27 nM), in IL-1β-induced rat smooth muscle cells and in human peripheral blood monocytes. Compound **21** did not inhibit the PMA-induced COX-2 expression in macrophages. Furthermore, **21** inhibited COX-2 expression *in vivo* in glomeruli of rats with experimental glomerulonephritis induced by anti-glomerular basement membrane antibodies. Radicicol (**21**) inhibited COX activity in rat alveolar macrophages, rat smooth muscle cells (SMS) and in peripheral human monocytes pre-treated with aspirin and stimulated with LPS, resulting in IC_{50} values of 10, 100 and 2 ng/ml, respectively. The IC_{50} value was 400 ng/ml of **21** when the SMS cells were stimulated with IL-1β. The amount of PGE_2 was determined with RIA (Chanmugam *et al.*, 1995).

11.4.16 Resveratrol

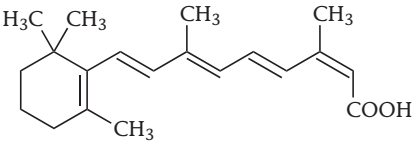
The stilbene derivative, resveratrol (**22**), had no effect on TPA-induced COX-2 or COX-1 mRNA expression in mouse skin (Jang and Pezzuto, 1998). The level of mRNA expression was determined by RT-PCR analysis in these experiments. On the other hand, Subbaramaiah *et al.* (1998a,b) reported that **22** suppressed the PMA-mediated induction of COX-2 mRNA and the induction of COX-2 transcription in human mammary and oral epithelial cell lines. Compound **22** inhibited recombinant human COX-2 enzyme activity and suppressed the PMA-mediated increase in the production of PGE_2, in a dose-dependent manner (Subbaramaiah *et al.*, 1998a). A decrease in basal production of PGE_2 (approximately 70%) by **22** has also been reported in human mammary and human oral epithelial cells and in leukoplakia cell lines by Subbaramaiah *et al.* (1998b). This reduction occurred by direct inhibition of COX-2 activity. Moreover, a PMA-mediated induction of COX-2 promoter activity was inhibited by **22** in a dose-dependent manner (Subbaramaiah *et al.*, 1998a). Jang and Pezzuto (1998) have suggested that the inhibitory effect of resveratrol (**22**) is an effect which is specific to particular species or to specific test systems.

11.4.17 Retinoids

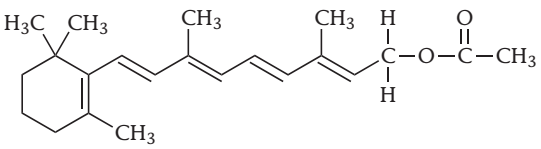
All-trans-retinoic acid (**58**) suppressed PMA-induced COX-2 mRNA expression in human oral epithelial cells, while 13-*cis*-retinoic acid (**59**) and retinyl acetate (**60**) partially inhibited the expression (all tested at 1 μM). The production of PGE_2 was also suppressed by the retinoids (**58–60**). Furthermore, **58** inhibited both PMA-mediated COX-2 transcription and promoter activity at 1 μM (Mestre *et al.*, 1997).



all-trans Retinoic acid (**58**)



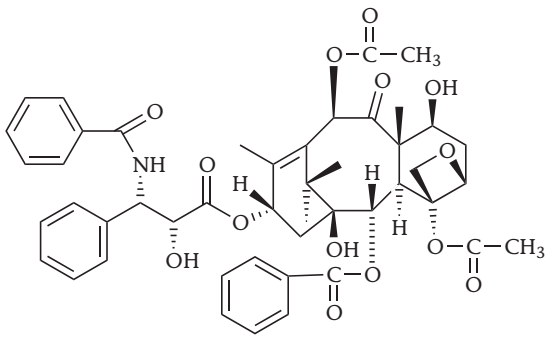
13-cis Retinoic acid (**59**)



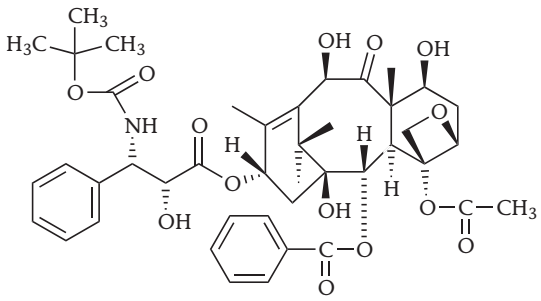
Retinyl acetate (**60**)

11.4.18 Taxol and Taxotere

Taxol (**61**) (10 μM) alone, or **61** (10 μM) and IFNγ increased the production of PGE_2, COX-2 mRNA and COX-2 protein expression in RAW 264.7 murine macrophages. The PGE_2 level was increased in a time-dependent manner. Neither **61** alone, nor **61** with IFNγ induced expression of COX-1. Taxotere (**62**), a semisynthetic analogue of **61**, did not alter the COX-2 expression or the COX-2 activity (Moos *et al.*, 1999). On the other hand, both **61** and **62** (0–30 μM) induced COX-2 expression in human monocytes suspended in 10% human serum (Moos and Fitzpatrick, 1998). Moos *et al.* (1999) have also described the same differential effects of **61** and **62** (10 μM) on COX-2 mRNA expression in RAW 264.7 murine macrophages. Moos and Fitzpatrick (1998) suggest that taxol (**61**) induces genes in murine macrophages by a mechanism other than stabilisation of microtubuli. They also propose that the taxol-induced expression of COX-2 is species-specific.



Taxol (Paclitaxel) (**61**)



Taxotere (Docetaxel) (**62**)

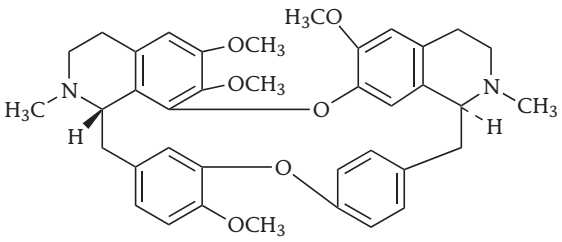
11.4.19 Tectoridin and Tectorigenin

Tectorigenin (**23**) (3–30 μM) significantly inhibited the PGE_2 production induced by TPA or thapsigargin in rat macrophages. The production of PGE_2 was measured by RIA. Tectoridin (**24**) was less active, but showed a significant inhibition at 10 and 30 μM. The induction of COX-2 protein by TPA or thapsigargin was inhibited in a dose-dependent manner by both the compounds, and **23** was more potent than **24**. The COX-1 protein level was not affected. Kim *et al.* (1999) suggest that the inhibition of PGE_2 by tectorigenin (**23**) and tectoridin (**24**) results from inhibition of the expression of COX-2 protein and not from inhibition of enzyme activity or phospholipase A_2.

11.4.20 Tetrandrine

Mouse macrophage-like J774 cells, when pre-treated with tetrandrine (**63**) (1–10 μM) and stimulated with LPS and IFNγ, showed a slight dose-dependent inhibition of the COX-2 protein level. Compound **63**, on its own, had no effect on COX-2 protein expression in non-stimulated cells. Moreover, **63** (10 μM) slightly inhibited PGE_2

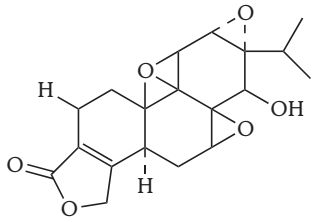
production in LPS-stimulated J774 cells, while the inhibition was total, when indomethacin was used as a reference (tested at 1 μM). On the contrary, PGE_2 was highly overproduced when no inducer was used. The authors concluded that the overproduction of PGE_2, caused by tetrandrine (**63**), was due to a cytotoxic effect on the macrophages (Pang and Hoult, 1997).



Tetrandrine (**63**)

11.4.21 Triptolide

Triptolide (**64**) inhibited the LPS-induced COX-2 mRNA expression of rheumatoid arthritis synovial tissue (RASF), human peripheral blood (HM), and human neonatal foreskin fibroblasts (HFF) with IC_{50} values of 0.04, 0.06 and 0.10 μM, respectively. Indomethacin gave IC_{50} values of greater than 2.80 μM in RASF and HFF cells. The subsequent PGE_2 synthesis was inhibited by **64** in RASF, HM, HFF and U937 cells, with IC_{50} values of 0.01, 0.02, 0.03 and >0.09 μM, respectively. An IC_{50} value of 2.79 μM was observed for indomethacin in all cells (Tao *et al.*, 1998).



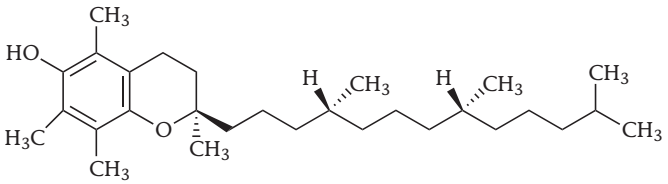
Triptolide (**64**)

11.4.22 Ursolic acid and Oleanolic acid

The triterpenes, ursolic acid (**25**) and oleanolic acid (**26**) (both tested at 10 μM), had no effect on COX-2 mRNA expression or on the subsequent PGE_2 production in LPS-induced RAW 264.7 mouse macrophages (Suh *et al.*, 1998).

11.4.23 Vitamin E

Vitamin E (**65**) supplementation completely inhibited the age-associated increase in PGE_2 synthesis and in the COX activity in LPS-induced murine macrophages. There was no inhibition of COX-2 or COX-1 mRNA expression or of protein levels in LPS-stimulated macrophages obtained from mice fed with **65** (30 or 500 ppm). Wu *et al.* (1998), conclude that vitamin E (**65**) inhibits the COX proteins at post-translational level.



Vitamin E (**65**)

11.5 PLANT EXTRACTS WHICH AFFECT COX-2 GENE EXPRESSION AND THE COX-2 CATALYSED PROSTAGLANDIN BIOSYNTHESIS

Hange-shashin-to, is a mixture of Pinelliae tuber, Scutellariae radix, Glycyrrhizae radix, Zizyphi fructus, Ginseng radix, Zingiberis siccatum rhizoma and Coptidis rhizoma. This mixture, tested at concentrations between 10 and 100 μg/ml, inhibited the PGE_2 biosynthesis which is catalysed by COX-2, but had no effect on the activity of COX-1. In these experiments the source of COX-1 was sheep seminal vesicles and that of COX-2 was sheep placenta. The amount of PGE_2 produced was measured by EIA. Of the seven extracts which were tested at 100 μg/ml, Scutellaria radix, Glycyrrhizae radix, and Coptidis rhizoma, inhibited COX-2 to a significant degree (Kase *et al.*, 1998).

The Chinese herbal remedy *Tripterygium wilfordii* Hook F was extracted in a mixture of methanol/chloroform and in ethyl acetate. Both extracts inhibited the synthesis of PGE_2 and COX-2 mRNA expression, in LPS-stimulated monocytes from HM, RASF and HFF, but did not affect U937 cells (Tao *et al.*, 1998). Decreased levels of PGE_2 were found in exudate cells in the air pouches of rats treated with an ethyl acetate extract of *Tripterygium wilfordii* Hook F (270 mg/kg body weight per day for 5 days). Exudate cells were obtained from rats which had been treated with *T. wilfordii* extracts and these cells were stimulated with IL-1 and LPS. The stimulated cells had the same PGE_2 content as non-stimulated cells. Both the PGE_2 and COX-2 mRNA levels were decreased in the air pouch lining in rats treated with the ethyl acetate extract, but the levels were not altered in the kidney or stomach. No significant difference in the production of PGE_2 in spleen cells from IL-1- and LPS-stimulated or non-stimulated rats treated with the extract was observed (Tao *et al.*, 1999).

Reininger and Bauer (1998) tested the n-hexane extracts of many herbal drugs for COX-2 and COX-1 inhibitory activity using an *in vitro* assay. Extracts of 50 μg/ml from Angelicae sinensis radix, Atractylodis lanceae rhizoma, Notopteryli rhizoma seu radix, Pieri samentosi fructus and Zingiberis rhizoma showed similar inhibitory effects on COX-2 and COX-1. Moon *et al.* (1998) reported the inhibition of PGD_2 generation in mast cells derived from bone marrow of 200 crude drugs. Methanol extracts of Bletillae rhizoma, Aconiti koreani rhizoma, Belamcandae rhizoma, Nelumbinis semen, Gleniae radix, and Auranti immatri pericarpum, inhibited COX-2 by more than 85% in this assay (tested at 2.5 μg/ml). Furthermore, Soo *et al.* (1998) screened 300 extracts of natural products for prostaglandin accumulation in LPS-induced mouse peritoneal macrophages. Methanol extracts of Zingiberis rhizoma, Alpinae officinarum rhizoma, Carophylli flos, Scutellariae radix, and *Dalbergia ordifera*, inhibited COX-2 by over 70% at a concentration of 1 μg/ml.

11.6 EXPERIENCE OF BIOASSAY-GUIDED ISOLATION OF INHIBITORS OF PROSTAGLANDIN BIOSYNTHESIS CATALYSED BY COX-2 AND COX-1

Bioassay-guided fractionation has been used in the search for selective COX-2 inhibitors of natural origin (Noreen *et al,* 1998c; Ringbom *et al.*, 1998). These experiments used a radiochemical enzyme assay to measure the prostaglandin biosynthesis catalysed by COX-2 and COX-1. Bioassay-guided fractionation of a hexane extract from *Plantago major* L. resulted in isolation of ursolic acid (**25**), a triterpene which inhibits COX-2 with an IC_{50} value of 130 μM (Ringbom *et al.*, 1998). Compound **25** has an IC_{50} value of 210 μM for the activity of COX-1, which means that the COX-2 selectivity ratio is 0.6. The same strategy has been used to isolate COX-1 inhibitors, resulting in the isolation of two new isoflavones, vavain (**13**) and vavain 3′-O-β-D-glucoside (**14**), and in the isolation of (+)-catechin (**5**) from the bark of *Ceiba pentandra* (IC_{50} values for COX-1: 97 μM, 381 μm, and 80 μM, respectively) (Noreen *et al.*, 1998a). The isoflavones (**13–14**) are specific inhibitors of the activity of COX-1, with less than 10% effect on COX-2, at concentrations of 900 μM and 1000 μM, for **13** and **14**, respectively. Compound **5** had a weak selectivity towards COX-1. The IC_{50} value was 130 μM on COX-2, which gives a COX-2 selectivity ratio of 1.3. Furthermore, bioactivity directed fractionation of *Atuna racemosa* and *Vantanea peruviana,* guided by the inhibition on COX-1, resulted in isolation of (+)-gallocatechin (**6**), 4′-MeO-(−)-gallocatechin (**7**), ouratea-catechin (**10**) and ouratea proanthocyanidin (**11**) (IC_{50} values: 95 μM, 12 μM, 3.3 μM, 138 μM, respectively) (Noreen *et al.*, 1998c). The IC_{50} values of **6**,**7**,**10** and **11** on COX-2 were not determined. The two gallocatechins (**6–7**) seem to be almost equipotent, since they showed 66% and 90% inhibition on COX-1 and 46% and 61% inhibition on COX-2, respectively, at 100 μg/ml. Both **10** and **11** were more selective for COX-1 at 100 μg/ml, with

77% and 81% inhibition on COX-1, and 23% and 37% for COX-2 catalysed prostaglandin biosynthesis. Since the commonly occurring flavonoid, (+)-catechin (**5**), has been isolated from *Ceiba pentandra*, *Syzygium corynocarpum*, and *S. malaccense*, following bioassay guided fractionation, a medium pressure liquid chromatography method has been developed to avoid repeated isolation (unpublished results).

A variety of extracts obtained from several plant families from Europe, Asia and South America, have been extracted with different solvents, and the effects of the extracts on both COX-2 and COX-1 catalysed prostaglandin biosynthesis have been investigated. About half of the 55 non-polar extracts (hexane and dichloromethane) showed more than 30% inhibition of COX-2 activity, while about 5% of 130 polar extracts did so — all were tested at 100 μg/ml (unpublished results). Our experience is that COX-2 selectivity is seldom observed in a crude extract, although further fractionation can reveal COX-2 selective compounds, e.g. isolation of ursolic acid (**25**) from *Plantago major* (Ringbom *et al.*, 1998).

We investigated further triterpenes and flavonoids in order to study the relationship between structure and activity. The triterpene oleanolic acid (**26**), inhibited both COX-2 and COX-1 catalysed prostaglandin biosynthesis with IC_{50} values of 295 μM and 380 μM, respectively, while glycyrrhetinic acid (**27**) did not inhibit either of the enzymes at concentrations of up to 425 μM (Ringbom *et al.*, 1998). Mearnsitrin (**8**) and myricitrin (**9**) showed less than 30% inhibition of both COX-2 and COX-1 at 100 μg/ml (Noreen *et al.*, 1998a). Furthermore, amentoflavone (**4**) did not inhibit COX-2 at maximum dissolved concentration (37 μM), although **4** showed an IC_{50} of 12 μM on COX-1 (Bucar *et al.*, 1998). Quercitrin (**12**) neither affected COX-2 nor COX-1 at 2000 μM (maximum dissolved concentration) (Noreen *et al.*, 1998b).

The COX radiochemical enzyme assay was originally described by White and Glassman, who used bovine seminal vesicles as a source of COX (White and Glassman, 1974). The discovery of COX-2 led to a further development of this assay with respect to both COX-2 and COX-1. As before, bovine seminal vesicles were used as a source of COX-1, while sheep placental cotyledons were used as a source of COX-2 (Noreen *et al.*, 1998b). The parameters that were considered were: time of pre-incubation, time of incubation, time of reaction, and concentrations of cofactors. The optimisation of these parameters resulted in a more reliable method and increased production of prostaglandins, but the inhibitors were less potent under the new conditions. For example, the IC_{50} value of (+)-catechin (**5**) for COX-1 increased from 130 μM to 546 μM at 0 min of pre-incubation (Noreen *et al.*, 1998b,c). Furthermore, the inhibition was measured, both after incubation of the enzyme and test compound, for 10 minutes, and without incubation, in order to detect time-dependent inhibition of the compounds. The inhibition of COX-2 by ursolic acid (**25**) and oleanolic acid (**26**) increased after 10 minutes, while that of (+)-catechin (**5**) decreased (Noreen *et al.*, 1998b; Ringbom *et al.*, 1998).

The COX catalysed biosynthesis assay can be further improved, for example, by using

non-radioactive labelled substrate and measuring PGE_2 specifically. However, the RIA assay of COX-catalysed synthesis is not fully satisfactory and alternative methods are being developed. In particular, these methods do not require the use of radioactive labelling, and they measure PGE_2 specifically. The two methods, scintillation proximity assay (SPA) and surface plasmon resonance (SPR), are particularly interesting. These allow the investigation of the specific PGE_2 production, and the affinity of natural products to COX-2 and COX-1, respectively (Huss *et al.*, 1999; Ringbom *et al.*, 1999). They also increase the screening capacity. The SPA method makes uses of the availability of specific antibodies against PGE_2 bound on anti-rabbit SPA beans and enables the assay to be transformed to a homogeneous format (Cook, 1996). The SPR technique detects binding events on a sensor surface (Fägerstam and Karlsson, 1994), i.e. binding of inhibitors to COX-2 or COX-1 immobilised on a surface.

11.7 CONCLUSION

A limited number of natural products which inhibit COX-2 have been isolated and characterised so far. The effects of these natural products on the suppression of COX-2 gene expression have most often been tested, but their effect on enzyme activity have only been investigated in a few cases. Most of the studies of natural compounds also lack a comparison between the effects on COX-1 and COX-2, which means that the selectivity-ratio of IC_{50} values cannot be calculated. Very few of the reported compounds in this review have been isolated using bioassay-guided isolation. This technique improves the search of COX-2 regulators with natural origin and reveals new structure-activity relationships, and its use should be extended and developed.

It has been reported, in several studies, that resveratrol (**22**) has a selective COX-2 activity. This phytoalexin is found in grapes, and has cancer chemopreventive activity *in vitro* (Jang *et al.*, 1997). Increased interest in foods which affect health (functional foods, nutraceuticals) can be seen in Japan, Europe and the USA. Continued research into natural product constituents of our diet with anti-inflammatory activities is therefore important.

In the January 1999 issue of TIPS, Wallace (Wallace, 1999) presents and discusses significant limitations of COX-2 inhibitors with respect to their efficacy and toxicity. Even if selective COX-2 inhibitors do not redeem their earlier promise for the development of new anti-inflammatory drugs, it is of great interest in identifying novel compounds of natural origin with a selective COX-2 activity for use as biochemical tools. Novel structure-activity relationships can help to answer some of the questions concerning the physiological and pathophysiological functions of the two isoenzymes, COX-1 and COX-2.

TABLE 11.1

Substances of natural origin investigated for an effect on COX-2 activity

Compound *Origin*	Method	Effect (IC_{50})	References
Ajoene (29) *Allium sativum*[1]	COX-2 enzyme activity	Inhibited (3.4 μM)	Dirsch and Wollmar (1998)
	COX-2 gene expression	Enhanced/ no effect	Dirsch and Wollmar (1998)
	Prostanoid production	Inhibited (2.4 μM)	Dirsch and Wollmar (1998)
Akendo 1 (1) *Endospermum diadenum* (*Euphorbiaceae*)[1]	COX-2 enzyme activity	No effect	Payá *et al.* (1996)
Akendo 2 (2) *Endospermum diadenum* (*Euphorbiaceae*)[1]	COX-2 enzyme activity	No effect	Payá *et al.* (1996)
Akendo 3 (3) *Endospermum diadenum* (*Euphorbiaceae*)[1]	COX-2 enzyme activity	Inhibited	Payá *et al.* (1996)
Aldisine (54) *Axinella verrucosa*,	COX-2 gene expression	No effect	Roshak *et al.* (1997)
Achanthella auratiaca[1]	Prostanoid production	No effect	Roshak *et al.* (1997)
Allicin (30) *Allium sativum*[1]	COX-2 enzyme activity	No effect	Dirsch and Wollmar (1998)
	COX-2 gene expression	Enhanced/ no effect	Dirsch and Wollmar (1998)
	Prostanoid production	Inhibited (37.7 μM)	Dirsch and Wollmar (1998)
Amentoflavone (4) *Biophytum sensitivum* DC. (*Oxalidaceae*)[1]	COX-2 enzyme activity	No effect	Bucar *et al.* (1998)
Anthocyanin 1 (15) Tart cherries[1]	COX-2 enzyme activity	No effect	Wang *et al.* (1999)
Anthocyanin 2 (16) Tart cherries[1]	COX-2 enzyme activity	No effect	Wang *et al.* (1999)
Anthocyanin 3 (17) Tart cherries[1]	COX-2 enzyme activity	No effect	Wang *et al.* (1999)
Burrodin[2] **(32)**	Prostanoid production	Inhibited (3.8 μM)	Hwang *et al.* (1996)
Caffeic acid phenethyl ester (19) Honeybee propolis[1]	COX-2 enzyme activity	Inhibited (82 μM)	Michaluart *et al.* (1999)
	COX-2 gene expression	Inhibited	Michaluart *et al.* (1999)
	Prostanoid production	Inhibited	Michaluart *et al.* (1999)

TABLE 11.1 *(continued)*

Compound *Origin*	Method	Effect (IC_{50})	References
(+)-Catechin (5) *Ceiba pentandra* Gaertner (*Bombacacea*), *Syzygium corynocarpum* C. Muell (*Myrtaceae*),	COX-2 enzyme activity	Inhibited (5910 μM/ 546 μM)	Noreen *et al.* (1998b)
Syzygium malaccense Merr. & Perry (*Myrtaceae*)[1]	COX-2 enzyme activity	Inhibited (130 μM)	Noreen *et al.* (1998c)
Cholecalciferol (41) Fish liver oil[3]	COX-gene expression	Enhanced	Kanekura *et al.* (1998)
	Prostanoid production	Enhanced	Kanekura *et al.* (1998)
Confertiflorin (33) *Ambrosia confertiflora*[4]	Prostanoid production	Inhibited (2.9 μM)	Hwang *et al.* (1996)
Costunolide (34) Common in plants[4]	Prostanoid production	Inhibited (6.9 μM)	Hwang *et al.* (1996)
Curcumin (42) *Curcuma longa* L.	COX-2 gene expression	Inhibited	Zhang *et al.* (1999)
(*Zingiberaceae*)[5]	COX-2 gene expression	Inhibited	Holloway *et al.* (1998)
	Prostanoid production	Inhibited	Zhang *et al.* (1999)
Cyanidin (18) Tart cherries[1]	COX-2 enzyme activity	Inhibited (60 mM)	Wang *et al.* (1999)
Daidzein (47) Common in plants[6]	COX-2 gene expression	Enhanced	Johnson and Loo (1998)
Diacerhein[2] (43)	COX-2 gene expression	Enhanced	Pelletier *et al.* (1998)
	Prostanoid production	Enhanced/ no effect	Pelletier *et al.* (1998)
Encelin (35) *Encelia farinosa*[4]	Prostanoid production	Inhibited (0.4 μM)	Hwang *et al.* (1996)
Enhydrin (36) *Enhydra fluctuans*[4]	Prostanoid production	Inhibited (0.6 μM)	Hwang *et al.* (1996)
Epi-gallocatechin (45) Green tea[1]	COX-2 gene expression	Inhibited/ enhanced	Soriani *et al.* (1998)
(+)-Gallocatechin (6) *Atuna racemosa* Raf. Ssp. Racemosa (*Chrysobalanaceae*)[1]	COX-2 enzyme activity	Inhibited	Noreen *et al.* (1998c)
4′-MeO-(−)-gallocatechin (7) *Atuna racemosa* Raf. Ssp. Racemosa (*Chrysobalancaceae*)[1]	COX-2 enzyme activity	Inhibited	Noreen *et al.* (1998c)

TABLE 11.1 *(continued)*

Compound *Origin*	Method	Effect (IC_{50})	References
Genistein (46) Common in plants[6]	COX-2 gene expression	Enhanced	Johnson and Loo (1988)
	COX-2 gene expression	Inhibited (22 μM)	Chanmugam *et al.* (1995)
(1 → 3)-β-D-Glucan (50) Common in fungi and yeasts[1]	COX-2 gene expression	Enhanced	Ljungman *et al.* (1998)
Glycyrrhetinic acid (27) *Glycyrrhiza glabra* L. (*Fabaceae*)[5]	COX-2 enzyme activity	No effect	Ringbom *et al.* (1998)
Herbimycin A[2] **(51)**	COX-2 gene expression	Inhibited (52 nM)	Chanmugam *et al.* (1995)
Hesperetin (48) *Persic vulgaris* (*Rosaceae*), *P. vulgaris* (*Rosaceae*) *Cordia obliqua* (*Boragineae*)[6]	COX-2 gene expression	Enhanced	Johnson and Loo (1998)
1-Hydroxy-anthraquinone[2] **(52)**	COX-2 gene expression	Enhanced	Yoshimi *et al.* (1995)
Hymenialdisine (53) *Axinella verrucosa*,	COX-2 gene expression	Inhibited	Roshak *et al.* (1997)
Achanthella auratiaca[1]	Prostanoid production	Inhibited	Roshak *et al.* (1997)
Leucanthin B (37) *Melampodium leucanthum*[4]	Prostanoid production	Inhibited (0.4 μM)	Hwang *et al.* (1996)
Manoalide (55) *Luffariella variabilis*[4]	COX-2 gene expression	Inhibited	Glaser and Lock (1995)
	Prostanoid production	Inhibited	Glaser and Lock (1995)
Mearnsitrin (8) *Syzygium malaccense* Merr. & Perry (*Myrtaceae*)[1]	COX-2 enzyme	No effect	Noreen *et al.* (1998c)
Melampodin A (38) *Melampodium leucanthum*[4]	Prostanoid production	Inhibited (1.1 μM)	Hwang *et al.* (1996)
Myricetin-3-O-β-D-glucuronide (20) *Epilobium angustifolium* L.[1]	COX-2 enzyme activity	Inhibited (8 μM)	Hiermann *et al.* (1998)
Myricitrin (9) *Syzygium corynocarpum* C. Muell (*Myrtaceae*), *S. malaccense* Merr. & Perry (*Myrtaceae*)[1]	COX-2 enzyme activity	No effect	Noreen *et al.* (1998c)
Naringenin (49) Common in plants[6]	COX-2 gene expression	Inhibited	Johnson and Loo (1998)

TABLE 11.1 *(continued)*

Compound *Origin*	Method	Effect (IC_{50})	References
Oleanolic acid (26) Common in plants[4]	COX-2 enzyme activity	Inhibited (295 μM)	Ringbom *et al.* (1998)
	Prostanoid production	No effect	Suh *et al.* (1998)
Ouratea-catechin (10) *Vantanea peruviana* Macbr. (*Humiriaceae*)[1]	COX-2 enzyme activity	No effect	Noreen *et al.* (1998c)
Ouratea-pro-anthocyanidin (11) *Vantanea peruviana* Macbr. (*Humiriaceae*)[1]	COX-2 enzyme activity	Inhibited	Noreen *et al.* (1998c)
Parthenolide (31) *Magnolia grandiflora,*	COX-2 gene expression	Inhibited	Hwang *et al.* (1996)
Tanacetum parthenium[1]	Prostanoid production	Inhibited (0.8 μM)	Hwang *et al.* (1996)
Pristimerin (57) Spp. in *Celastraceae* and *Hippocrateaceae* families[1]	COX-2 gene expression	No effect	Dirsch *et al.* (1997)
Psilostachyin A[2] **(39)**	Prostanoid production	Inhibited (4.6 μM)	Hwang *et al.* (1996)
Quercitrin (12) *Syzygium corynocarpum* C. Muell (*Myrtaceae*), *S. malaccense* Merr. & Perry (*Myrtaceae*)[1]	COX-2 enzyme activity	No effect	Noreen *et al.* (1998c)
Radicicol (21) Fungus strain KF91	COX-2 enzyme activity	No effect	Chanmugam *et al.* (1995)
	COX-2 gene expression	Inhibited (27 nM)/ no effect	Chanmugam *et al.* (1995)
	Prostanoid production	No effect	Chanmugam *et al.* (1995)
Resveratrol (22)	COX-2 enzyme	No effect	Jang *et al.* (1997)
Cassia quinquangulata Rich. (*Leguminosae*)[1]	activity	Enhanced	Johnson and Maddipati (1998)
		Inhibited	Subbaramaiah *et al.* (1998a)
	COX-2 gene expression	No effect	Jang and Pezzuto (1998)
		Inhibited	Subbaramaiah *et al.* (1998a)
		Inhibited	Subbaramaiah *et al.* (1998b)
	COX-2 hydroperoxidase activity	Inhibited (ED_{50} = 85 μM)	Jang *et al.* (1997)
		Inhibited (>200 μM, 45 μM, 85 μM)	Johnson and Maddipati (1998)

TABLE 11.1 *(continued)*

Compound *Origin*	Method	Effect (IC_{50})	References
	Prostanoid production	Inhibited	Subbaramaiah *et al.* (1998b)
all-trans-Retinoic acid[2] **(58)**	COX-2 gene expression	Inhibited	Mestre *et al.* (1997)
	Prostanoid production	Inhibited	Mestre *et al.* (1997)
13-cis-Retinoic acid[2] **(59)**	COX-2 gene expression	Inhibited	Mestre *et al.* (1997)
	Prostanoid production	Inhibited	Mestre *et al.* (1997)
Retinyl acetate[2] **(60)**	COX-2 gene expression	Inhibited	Mestre *et al.* (1997)
	Prostanoid production	Inhibited	Mestre *et al.* (1997)
Rhein (44) *Rheum* spp. *Cassia* spp.[3]	COX-2 gene expression	Enhanced	Pelletier *et al.* (1998)
	Prostanoid production	Enhanced/ no effect	Pelletier *et al.* (1998)
Scalaradial (56) *Cacospongia mollior*[4]	COX-2 gene expression	Inhibited	Glaser and Lock (1995)
	Prostanoid production	Inhibited	Glaser and Lock (1995)
Taxol (61) (Paclitaxel) *Taxus brevifolia* Nutt. (*Taxaceae*)[5]	COX-2 gene expression	Enhanced	Moos *et al.* (1999)
		Enhanced	Moos and Fitzpatrick (1998)
	Prostanoid production	Enhanced	Moos *et al.* (1999)
Taxotere (62) (Docetaxel) *Taxus baccata* L. (*Taxaceae*)[5]	COX-2 gene expression	No effect	Moos *et al.* (1999)
		No effect	Moos and Fitzpatrick (1998)
	Prostanoid production	No effect	Moos *et al.* (1999)
Tectoridin (24) *Belamacanda chinensis* (*Iridaceae*)[1]	COX-2 enzyme activity	No effect	Kim *et al.* (1999)
	COX-2 gene expression	Inhibited	Kim *et al.* (1999)
	Prostanoid production	Inhibited	Kim *et al.* (1999)
Tectorigenin (23) *Belamacanda chinensis* (*Iridaceae*)[1]	COX-2 enzyme activity	No effect	Kim *et al.* (1999)
	COX-2 gene expression	Inhibited	Kim *et al.* (1999)
	Prostanoid production	Inhibited	Kim *et al.* (1999)

TABLE 11.1 *(continued)*

Compound *Origin*	Method	Effect (IC_{50})	References
Tenulin (40) *Helenium tenuifolium* *H. elegans*[4]	Prostanoid production	Inhibited (18.0 μM)	Hwang *et al.* (1996)
Tetrandrine (63) *Stephania tetrandria*[1]	COX-2 gene expression	Inhibited	Pang and Hoult (1997)
	Prostanoid production	Inhibited/ enhanced	Pang and Hoult (1997)
Triptolide (64) *Tripterigum wilfordii* Hook F[1]	COX-2 gene expression	Inhibited (0.04 μM, 0.06 μM, 0.10 μM)	Tao *et al.* (1998)
	Prostanoid production	Inhibited (0.01 μM, 0.02 μM, 0.03 μM, >0.09 μM)	Tao *et al.* (1998)
Ursolic acid (25) *Plantago major* L. (*Plantaginaceae*)[1]	COX-2 enzyme activity	Inhibited (130 μM)	Ringbom *et al.* (1998)
	COX-2 gene expression	No effect	Suh *et al.* (1998)
	Prostanoid production	No effect	Suh *et al.* (1998)
Variabilin (28) *Ircinia variabilis* *Hemimycale columela*[1]	Prostanoid production	No effect	Escrig *et al.* (1997)
Vavain (13) *Ceiba pentandra* Gaertner (*Bombacacea*)[1]	COX-2 enzyme activity	Inhibited (>900 μM)	Noreen *et al.* (1998a)
Vavain 3′O-β-glucoside (14) *Ceiba pentandra* Gaertner (*Bombacacea*)[1]	COX-2 enzyme activity	Decreased (>1000 μM)	Noreen *et al.* (1998a)
Vitamin E (65) Common in plants[3]	COX-2 gene expression	No effect	Wu *et al.* (1998)
	Prostanoid production	No effect/ Inhibited	Wu *et al.* (1998)

[1] The origin of the compounds are reported as in the original article.
[2] The origin of the compound is not found.
[3–6] The origin of the compound is found in the following reference literature: [3]Budavari *et al.* (1996) [4]Connolly and Hill (1991) [5]Samuelsson (1999) [6]Harborne and Mabry (1982).

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CHAPTER

12

*Phytotoxins from Fungi, Pathogenic for Agrarian, Forestal and Weedy Plants**

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Contents

*Dedicated to the memory of Prof. G. Randazzo

12.1 INTRODUCTION

Toxins produced by phytopathogenic fungi have assumed great importance because of their involvement in several plant diseases. These pathogeneses have seriously damaged plants of agrarian, forestal and environmental interest. Frequently, the appearance of symptoms and the evolution of the disease, observed at distal part of the infection sites, suggests the involvement of translocable phytotoxins. Considering their social and economic impact, many efforts have been made to avoid losses in the agrarian production and to save the ornamental and forestal plants' patrimony. Several studies have tried to understand the role of bioactive microbial metabolites in the pathogenic process and, therefore, to use them against specific diseases. The chemical nature of these toxins ranges from low molecular weight compounds, includes all classes of natural products such as terpenes, chromanones, butenolides, pyrones, macrolides, aromatic derivatives, amino acids etc., to high molecular compounds such as proteins, glycoproteins and polysaccharides. As a result, many new phytotoxins, pesticides, fungicides, antibiotics, plant growth regulators, elicitins and mycotoxins, have been reported (Strobel, 1982; Graniti *et al.*, 1989; Ballio and Graniti, 1991; Tabacchi, 1994). In some cases, toxins have been used to obtain products for plant protection or, by genetic selection, plants resistant to specific disease (Durbin, 1981; Graniti *et al.*, 1989; Ballio and Graniti, 1991).

Plants infesting economically important crops are another important problem, causing marked losses in the agrarian production. Weed pests have always been recognized as one of the most serious agricultural and environmental problems. In agriculture, the control of weed diffusion is usually achieved by using agrochemicals belonging to different classes of organic compounds, often in large amounts. This causes serious problems to human and animal health and produces heavy environmental pollution. On the other hand, biological agents offer the advantage of being fully compatible with the environment, often with high specificity, and represent a long-term solution for controlling weeds which are particularly resistant to chemical herbicides. Therefore, many efforts have been made for weed biocontrol using their natural antagonists, mainly fungal pathogens, insects and, more recently, phytotoxins (Strobel *et al.*, 1987; Graniti *et al.*, 1989; Delfosse, 1990; Koltin *et al.*, 1993).

We report on the isolation, chemical and biological characterisation of several phytotoxins, herbicides, mycotoxins, fungicides, and antibiotics, which are produced by fungi pathogenic for plants with agrarian and forestall interest, and weeds. Studies on the structure-activity relationships and mode of action of some phytotoxins were also investigated by carrying out their stereoselective synthesis and the chemical derivatization. Their roles in the pathogenesis have also been hypothesized.

12.2 PHYTOTOXINS

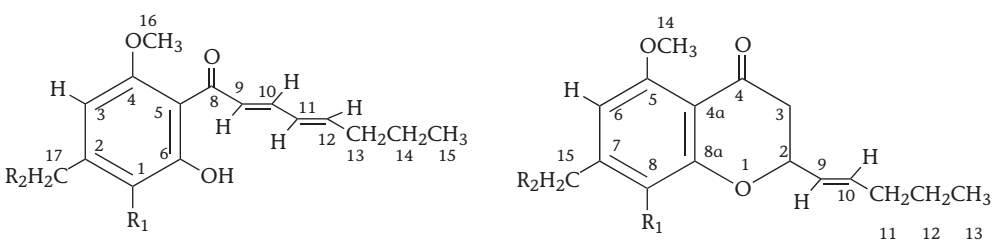
Studies on the involvement of toxins in plant disease caused by pathogenic microorganisms date from the second half of the 19th century. Phytotoxins are defined as microbial metabolites which are harmful to plants at very low concentrations. Most of the plant pathogenic fungi produce toxins in culture and in their hosts. Frequently, these compounds play a role in the pathogenesis and reproduce some, or even all, of the symptoms of the disease. In many cases, the toxins are low molecular weight compounds belonging to a variety of classes of natural products. They are able to diffuse from the site of the infection to surrounding tissues or are transferable within the plant. The virulence of the plant pathogen may depend on its capability to synthesize one or more toxins. Only a few phytotoxins are known as host-specific toxins, more frequently they are phytotoxic for a broad range of plant species. In some cases, studies on their mode of action and their role as 'vivo-toxin' have also been carried out (Strobel, 1982; Graniti *et al.*, 1989; Ballio and Graniti, 1991; Evidente, 1997; Upadhyay and Mukerji, 1997).

12.2.1 *Phoma* toxins

Preliminary studies of the toxin production of thirteen different species belonging to *Phoma* genera have indicated the presence of phytotoxic metabolites different from the fomenone (Bottalico *et al.*, 1983), the eremophilane sesquiterpenoid produced by *Phoma destructiva Plowr* (which is the causal agent of a wilt disease of tomato [*Lycopersicon esculentum* L.]) (Bottalico *et al.*, 1982), and *Phoma exigua* (Kinfield *et al.*, 1989). In particular, all of the strains have produced hydrophilic toxins, while the strain isolated from Chestnut synthesized lipophilic phytotoxins. Preliminary experiments showed two main toxins in the culture filtrate of this strain, the relative amounts of which depending upon the pH of the culture. Moreover, when assayed on tomato cuttings, only the more polar toxin showed wilting and necrosis similar to the symptoms induced on the same plant by the fungal culture filtrates (Evidente *et al.*, 1985a).

The isolate from Chestnut, identified as *Phoma cava* Schulzer, was cultured on semi-synthetic medium at room temperature. The two main metabolites were extracted by chloroform from the culture filtrates and purified as homogeneous oil by chromatography on Sephadex LH-20. They were both crystallized from EtOAc-petroleum and proved to be a toxic chalcone and its structurally related inactive chroman-4-one, respectively named cavoxin and cavoxone, (**1** and **2**, Figure 12.1 and Table 12.1). Assayed on tomato cuttings at 10^{-4} M, cavoxin caused a vascular browning and rapid wilting of leaves. Its structure, determined with extensive application of ^{1}H- and ^{13}C-NMR techniques and HR-EIMS, appeared to be that of a new tetrasubstituted benzoic acid derivative bearing an octadienoyl, an hydroxy, an hydroxymethyl and a methoxy group. The location of the substituents was determined by a gated decoupling ^{13}C-NMR experiment (Takeuchi

et al., 1977), which allowed the determination of the multiplicities and long-range ^{13}C-^{1}H coupling constants of the benzene ring carbons and those of the attached groups. Structure **1** assigned was further supported by Long-range Selective Pulse Decoupling (LSPD) (Takeuchi *et al.*, 1977; Seto *et al.*, 1978) and NOE-difference NMR spectra (Abraham and Loftus, 1978). Cavoxin structure was also confirmed by the corresponding methyl ester (obtained by reaction of the toxin with ethereal diazomethane) and the 8-deoxyhexahydroderivative (prepared by catalytic hydrogenation). Treatment of the toxin with diazomethan for a longer period (2 days) gave the partial and the total methylated derivatives of the cavoxin methyl ester (Evidente *et al.*, 1985b). Following the same spectroscopic approach, cavoxone (**2**) proved to be a new chroman-4-one and the structure was confirmed by preparing the corresponding methyl ester and the 15-*O*-acetyl derivatives. A longer treatment of **2** with diazomethane gave the 15-*O*-methyl derivative of the methyl ester, while catalytic hydrogenation yielded the 4-deoxy-tetrahydroderivative, which had a 2-alkyl-chroman-type structure. Furthermore, by refluxing with 6N HCl, cavoxin was quantitatively converted into **2**. These results confirmed the structure assigned to **2**. When left for a long period in moist air, or in solution, cavoxin spontaneously converted into **2**, suggesting that it is an artifact. In fact, added to culture medium in the absence of fungus, cavoxin was progressively transformed into cavoxone, which lacks optical activity (Evidente *et al.*, 1985b). Later, from the mother liquor of cavoxone crystallization, two minor metabolites were isolated. They were named cavoxinine and cavoxinone (**3** and **4**, Figure 12.1 and Table 12.1), and are structurally related to **1** and **2**, respectively, but show some differences in the pattern of substituents on the benzene ring. The structures of **3** and **4** were essentially established by comparing their spectroscopic properties to those of **1** and **2**, and by converting cavoxinine into **4**. These results, and some biosynthetic considerations, supported the structure **3** and **4** (Evidente, 1987). In fact, the location of the substituents on the



1 R_1=^{7}COOH, R_2=OH

2 R_1=^{16}COOH, R_2=OH

3 R_1=H, R_2=^{7}COOH

4 R_1=H, R_2=^{16}COOH

Figure 12.1: *Phoma cava* toxins.

TABLE 12.1
Toxins from fungi phytopathogenic for agrarian and forestal plants

Toxin	Source	Molecular formula	Molecular weight	IUPAC name	Reference
Ascosalitoxin	*A. pisi*	$C_{15}H_{20}O_4$	264	2,4-Dihydroxy-3-methyl-6-(1,3-dimethyl-2-oxopentyl)benzaldehyde	Evidente *et al.*, 1993b
Cavoxin	*Phoma cava*	$C_{17}H_{20}O_6$	320	2-Hydroxy-3-[(*2E,4E*)]-2,4-octadienoyl-4-methoxy-6-hydroxymethyl benzoic acid	Evidente *et al.*, 1985b
Cavoxinine	*Phoma cava*	$C_{17}H_{20}O_5$	304	2-(3-hydroxy-4-[(*2E,4E*)-2,4-octadienoyl])-4-methoxy)phenyl etanoic acid	Evidente *et al.*, 1987
Cavoxinone	*Phoma cava*	$C_{17}H_{20}O_5$	304	2-[(*1E*)-1-Pentenyl]-5-methoxy-7-carboxymethyl chroman-4-one	Evidente *et al.*, 1987
Cavoxone	*Phoma cava*	$C_{17}H_{20}O_6$	320	2-[(*1E*)-1-Pentenyl]-5-methoxy-7-hydroxymethyl-8-carboxy chroman-4-one	Evidente *et al.*, 1985b
Cyclopaldic acid	*S. cupressi*	$C_{11}H_{10}O_6$	238	3,5-Dihydroxy-4-formyl-7-methoxy-6-methyl-1(3H)-isobenzofuranone	Graniti *et al.*, 1992
Cytochalasin A	*A. heteromorpha* *A. lathyri* *P. e. heteromorpha*	$C_{29}H_{35}NO_5$	477	7-Hydroxy-16-methyl-10-phenyl-24-oxa-[14]cytochalasa-6(12),13,21-triene-1,20,23-trione (*7S, 13E, 16R, 21E*)	Evidente 1997, 1987; Vurro *et al.*, 1992, 1997
Cytochalasin B	*A. heteromorpha* *A. lathyri* *P. e. heteromorpha*	$C_{29}H_{37}NO_5$	479	7,20-Dihydroxy-16-methyl-10-phenyl-24-oxa-[14]cytochalasa-6(12),13,21-triene-1,23-dione (*7S, 13E, 16R, 20R, 21E*)	Evidente 1997; Vurro *et al.*, 1992, 1997
Dihydropinolidoxin	*A. pinodes*	$C_{18}H_{28}O_6$	340	2-(2,4-Hexadienoyloxy)-7,8-dihydroxy-9-propylnonan-9-olide	Evidente *et al.*, 1993a
Epipinolidoxin	*A. pinodes*	$C_{18}H_{26}O_6$	338	2-(2,4-Hexadienoyloxy)-7,8-dihydroxy-9-propyl-5-nonen-9-olide	Evidente *et al.*, 1993a
Episphaeropsidone	*S. sapinea* f. sp. *cupressi*	$C_7H_8O_4$	156	5-Hydroxy-4-methoxy-7-oxabicyclo-[4.1.0]hept-3-en-2-one (*5R*)	Evidente *et al.*, 1998d
Epoxypinolidoxin	*A. pinodes*	$C_{18}H_{26}O_7$	354	2-(2,4-Hexadienoyloxy)-7,8-dihydroxy-5,6-epoxy-9-propyl-nonan-9-olide	Evidente *et al.*, 1993a
Foeniculoxin	*P. foeniculi*	$C_{16}H_{20}O_4$	276	2-(6,7-Dihydroxy-7-methyl-3-methylen-1-ynyl)-hydroquinone	Evidente *et al.*, 1994
7′-Hydroxy*iso*-seiridin	*Seridium* spp.	$C_{12}H_{20}O_4$	228	3-Methyl-4-(1,5-dihydroxyheptyl)-2-(5H)-furanone	Evidente and Sparapano, 1994
7′-Hydroxyseiridin	*Seiridium* spp.	$C_{12}H_{20}O_4$	228	3-Methyl-4-(1,6-dihydroxyheptyl)-2-(5H)-furanone	Evidente and Sparapano, 1994
Iso-seiridin	*Seiridium* spp.	$C_{12}H_{20}O_3$	212	3-Methyl-4-(5-hydroxyheptyl)-2-(5H)-furanone	Evidente *et al.*, 1986; Sparapano *et al.*, 1986

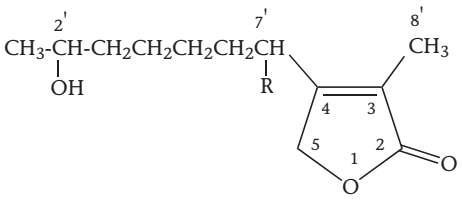
Pinolidoxin	*A. pinodes*	$C_{18}H_{26}O_6$	338	2-(2,4-Hexadienoyloxy)-7,8-dihydroxy-9-propyl-5-nonen-9-olide	Evidente *et al.*, 1993c
Sapinofuranone A	*S. sapinea*	$C_{10}H_{14}O_3$	182	4-(1-Hydroxy-2,4-hexadienyl)butan-4-olide (*1S, 2Z, 4E*)	Evidente *et al.*, 1999
Sapinofuranone B	*S. sapinea*	$C_{10}H_{14}O_3$	182	4-(1-Hydroxy-2,4-hexadienyl)butan-4-olide (*1R, 2Z, 4E*)	Evidente *et al.*, 1999
Seiricardine A	*Seiridium* spp.	$C_{15}H_{26}O_2$	238	Octahydro-1-isopropenyl-2,5-dihydroxy-3a,4,5-trimethyl-1H-indene	Ballio *et al.*, 1991
Seiricardine B	*Seiridium* spp.	$C_{15}H_{26}O_2$	238	Octahydro-1-(1-hydroxyisopropyl)-3a,4,5-trimethyl-5,8-epoxy-1H-indene	Evidente *et al.*, 1993d
Seiricardine C	*Seiridium* spp.	$C_{15}H_{26}O_2$	238	Octahydro-1-(1-hydroxyisopropyl)-3a,4,5-trimethyl-5,8-epoxy-1H-indene	Evidente *et al.*, 1993d
Seiricuprolide	*S. cupressi*	$C_{14}H_{20}O_5$	268	5,6-Epoxy-4,7-dihydroxy-13-methyl-2,8-tridecadien-13-olide (*2E, 8Z*)	Ballio *et al.*, 1988
Seiridin	*Seiridium* spp.	$C_{12}H_{20}O_3$	212	3-Methyl-4-(6-hydroxyheptyl)-2-(5H)-furanone	Evidente *et al.*, 1986; Sparapano *et al.*, 1986
Solanapyrone A	*A. rabiei; A. solani*	$C_{18}H_{22}O_4$	302	2H-Pyran-3-carboxaldehyde-4-methox-6-(1,2,4a,5,6,7,8,8a-octahydro-2-methyl-1-naphthalenyl)-2-oxo-[1*R*-(1α,2β,4aα,8aα)]	Ichihara *et al.*, 1983; Alam *et al.*, 1989
Solanapyrone B	*A. rabiei; A. solani*	$C_{18}H_{24}O_4$	304	2H-Pyran-2-one-3-(hydroxymethyl)-4-methoxy-6-(1,2,4a,5,6,7,8,8a-octahydro-2-methyl-1-naphthalenyl)-2-oxo-[1*R*-(1α,2β,4aα,8aα)]	Ichihara *et al.*, 1983; Alam *et al.*, 1989
Solanapyrone C	*A. rabiei; A. solani*	$C_{19}H_{25}NO_4$	331	2H-Pyran-3-carboxaldehyde-4[(2-hydroxymethyl)amino]-6-(1,2,4a,5,6,7,8,8a-octahydro-2-methyl-1-naphthalenyl)-2-oxo-[1*R*-(1α,2β,4aα,8aα)]	Ichihara *et al.*, 1983; Alam *et al.*, 1989
Sphaeropsidin A	*D. mutila* *S. sapinea* f. sp. *cupressi*	$C_{20}H_{26}O_5$	346	9H-10,4a-(Epoxymethano) phenanthrene-9,12-dione-7-ethenyl-1,2,3,4,4b,5,6,7,10,10a-decahydro-4b,10-dihydroxy-1,1,7-trimethyl	Evidente *et al.*, 1996b
Sphaeropsidin B	*D. mutila* *S. sapinea* f. sp. *cupressi*	$C_{20}H_{28}O_5$	348	9H-10,4a-(Epoxymethano) phenanthrene-12-one-7-ethenyl-1,2,3,4,4b,5,6,7,10,10a-decahydro-4b,9,10-trihydroxy-1,1,7-trimethyl	Evidente *et al.*, 1997b
Sphaeropsidin C	*S. sapinea* f. sp. *cupressi*	$C_{20}H_{28}O_4$	332	4a(9H)-Phenanthrenecarboxylic acid,9-one-7-ethenyl-1,2,3,4,4b,5,6,7,10,10a-decahydro-4b-hydroxy-1,1,7-trimethyl	Evidente *et al.*, 1997b
Sphaeropsidone	*S. sapinea* f. sp. *cupressi*	$C_7H_8O_4$	156	5-Hydroxy-4-methoxy-7-oxabicyclo-[4.1.0]hept-3-en-2-one (*5S*)	Evidente *et al.*, 1998d

benzene ring was in agreement with the cyclization of an appropriate acetate-derivative precursor via acylphloroglucinol, as proposed in the acetogenins biosynthesis (Richards and Hendrikson, 1964). Cavoxinone, obtained from **3**, was in all respect identical to **4** isolated from the culture filtrate of the fungus. The optical inactivity of natural cavoxinone suggests that it is a racemic mixture, probably formed from the open precursor **3**. Therefore, **4** may be considered an artifact as previously demonstrated for **2** produced from **1** (Evidente *et al.*, 1985b). However, **4** represents a further example of a chroman 4-one which are rare as natural products. The structure of cavoxinone was also confirmed by preparing the corresponding methyl ester by treatment with diazomethane (Evidente, 1987). The very low level of cavoxinine present in the culture filtrate of *P. cava* and the low amount of the pure compound available have not allowed phytotoxic activity tests.

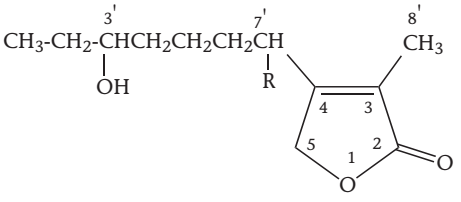
12.2.2 *Seiridium* toxins

Three species of *Seiridium*, namely *S. cardinale*, *S. cupressi* and *S. unicorne*, are associated with the canker diseases of cypress (*Cupressus sempervirens* L.). Since its first introduction in Europe (Barthelet and Vinot, 1944), the canker caused by the imperfect fungus *S. cardinale* (Wag.) Sutt *et* Gibbs has become the major disease of the Mediterranean cypress and other species of *Cupressaceae*. The cypress canker, first reported in the USA (Wagener, 1939), is a destructive disease which kills the trees, causing heavy losses to the nursery industry, to cypress plantations used for afforestation and wind-breaks, and to ornamental cypresses (Grasso and Raddi, 1979; Graniti, 1985). A similar disease, caused by strains of *S. cupressi* (Guba) Boesew. and *S. unicorne* Cooke *et* Ellis, although less serious, has spread in Greece (Kos) (Graniti, 1985; Xenopoulos, 1987) and Portugal (Graniti, 1985; Graniti and Frisullo, 1987), respectively. However, these fungi and the disease they induce also occur in other parts of the world.

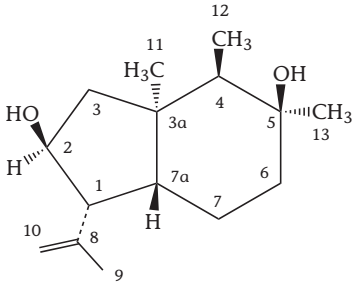
Evaluation of the damage caused by the strain of *Seiridium* to their host plant suggests that necrotic toxins are produced in the infected tissues and are possibly involved in the pathogenesis. From the *t*-BuOMe extract of the *in vitro* culture filtrates of all three fungi, seven phytotoxic metabolites have been isolated. Their purification was essentially achieved by column and preparative TLC chromatografic methods. Their characterisation was achieved by spectroscopic (UV, IR, 1D and 2D ^{1}H- and ^{13}C-NMR and HR-EIMS) techniques and by preparation of some key derivatives. The toxins belong to different classes of organic compounds and this is unusual in the field of fungi toxins. They proved to be four butenolides, namely seiridins (**5**, **6**, **7** and **8**, Figure 12.2), and three sesquiterpenes, named seiricardines A, B and C (**9**, **10** and **11**, Figure 12.2). In addition, specific to *S. cupressi*, a 14-macrolide, named seiricuprolide (**12**, Figure 12.2) and one substituted isobenzofuranone, identified as cyclopaldic acid (**13**, Figure 12.2), have been found. All the above phytotoxins were non selective and induced symptoms on host plants which were very similar to those observed in naturally infected cypress trees.



5 R=H; **7** R=OH



6 R=H; **8** R=OH



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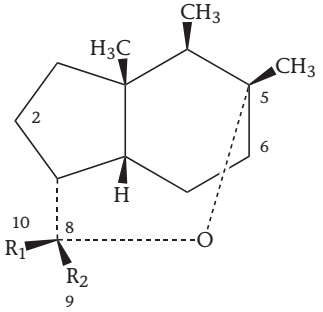
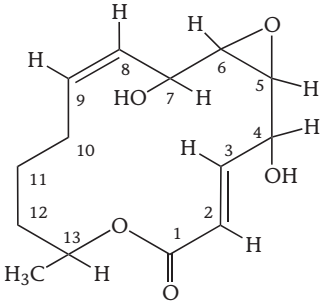


10 R_1=CH_3, R_2=CH_2OH

11 R_1=CH_2OH, R_2=CH_3



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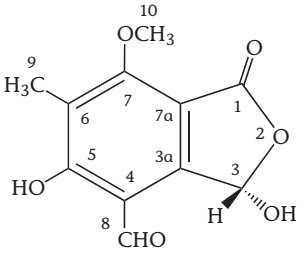

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Figure 12.2: *Seiridium* toxins.

The main phytotoxins produced by three species of *Seiridium*, seiridin and *iso*-seiridin, (**5** and **6**, Figure 12.2 and Table 12.1), are $\Delta^{\alpha,\beta}$-butenolides, characterised as 3-methyl-4-(6-hydroxyheptyl)-2-(5H)-furanone and its 4-(5-hydroxyheptyl) isomer, respectively (Evidente *et al.*, 1986; Sparapano *et al.*, 1986). But-3-enolides are relatively common natural compounds as fungi products (Turner and Aldridge, 1983), but the 3,4-dialkyl derivatives are rare. Both toxins caused chlorotic and necrotic symptoms on leaves of, either host (severed twigs of cypresses: *Cupressus sempervirens* L., *C. macrocarpa* Hartw. and *C. arizonica* Gr.) or non-host (cuttings of tomato and basil: *Lycopersicum esculentum* L. and *Ocynum basilicum* L.), test plants by absorptions of 0.3 mg/ml solutions. Preliminary experiments have also shown an antibacterial activity on *Pseudomonas* Mig and *Bacillus* Chon (Sparapano *et al.*, 1986). Being possibly involved in the pathogenesis, we have further investigated the biological activity of these toxins. The α,β-unsaturated-γ-lactone and the hydroxy heptyl side chain of seiridin and *iso*-seiridin were modified in order to gain information on the structure-activity relationship. In particular, the hydroxy group of the heptyl side chain was acetylated, while the double bond of the γ-lactone ring was hydrogenated. Furthermore, the α,β-unsaturated-γ-lactone ring was cleaved by reductive opening with hydrides; the last reaction also yielded a furane derivative of seiridin. The same derivatives were prepared by starting from *iso*-seiridin, that is a natural structural isomer of seiridin, with the hydroxy group of the side chain shifted at the adjacent C-3′ carbon. The effects of each compound on host and non-host plants (toxicity, electrolyte loss and seed germination), and their antimicrobial activity on bacteria, were assayed. We have found that the integrity of the α,β-unsaturated-γ-lactone, and the position and the presence of the hydroxy group in the heptyl side chain are important features for the biological activity of the two butenolides (Sparapano and Evidente, 1995b). They proved also to be essential in determining the specificity of the recognising reaction with the seiridin-antibodies used in a citofluorimetric method developed to detect the toxin by an immunochemical method. The antibodies were obtained by immunisation of rabbits with a seiridin-BSA (Bovine Serum Albumin) conjugated. The latter was obtained by coupling the ketone derivative, prepared by oxidation of the toxin, with the ε-NH_2 lysine groups of protein. When the ketone was converted into the corresponding dansylhydrazone, and both tested together with the above derivatives, it was shown that the presence of unmodified heptyl side chain and the integrity of the lactone are important features for a highly binding reaction. The citofluorimetric method could allow detection of seiridins present at a very low level in complex samples such as cypress tissues infected by the pathogen, and, therefore, it could be very useful to investigate their role as *vivo*-toxins (Evidente *et al.*, 1997a).

The virulence of 100 isolates of *S. cardinale* on three species of *Cupressus* was assessed and correlated to their capacity to produce seiridins in *in vitro* cultures. The analysis of the two toxins was performed by optimizing an HPLC method. The strains were classified as non-producers, scant producers, low producers, intermediate producers and high producers (Sparapano *et al.*, 1994).

Seiridins are available, at very low levels, after a long process of purification of fungi culture filtrates. Their biological importance and the possibility of using them in genetic selection to obtain a line of cypress which is resistant to the disease induced by *Seiridium* spp., prompted us to develop a total synthesis of these toxins. This was realised in a first enatioselective synthesis of seiridin by a short sequence based on five steps. The coupling of the 1-tosyl-*tert*-butylsilyl derivative of the enantio pure (*3R*)-1,3-butandiol (commercially available) with the appropriate Grignard reagent of the 4-pentenyl bromide afforded a reasonable yield of the expected olefin which was converted into the corresponding ketol by oxidation with acidic $KMnO_4$. The ketol, was first esterified in appropriate conditions, and then, by a Wittig intramolecular key olefination, followed by acid deprotection, converted into seiridin. The synthetic sequence confirmed the reported structure of the naturally occurring seiridin, but also provided large amounts of the optically pure **5** (Bonini *et al.*, 1995). Grigg *et al.* (1994) also reported a stereoselective synthesis of *iso*-seiridin (**6**), although this involved several steps, with an enantiomeric excess not exceeding 88%.

Finally, two new phytotoxic α,β-unsaturated butenolides, 7′-hydroxyseiridin and 7′-hydroxy*iso*-seiridin (**7** and **8**, Figure 12.2 and Table 12.1), have recently been isolated from culture filtrates of the three *Seiridium* spp. They are more polar and structurally related to seiridin and *iso*-seiridin from which differ by the presence of a further hydroxy group on the carbon of the side chain attached at the γ-lactone ring. The two new butenolides, tested at 50–100 μM and compared to **5** and **6**, showed a minor phytotoxicity when assayed on the above-cited host and non-host plants, as expected for the presence of a modified alkyl side chain at C-4 (Evidente and Sparapano, 1994).

From the culture filtrates of the above *Seiridium* species, three structurally related metabolites (seiricardines A, B and C) were isolated. They were characterised as new cyclic sesquiterpenes containing the octahydro-1H-indene system, a carbon skeleton found in plant and microorganisms, including well-known fungal metabolites showing phytotoxic and antifungal activity (Hikino *et al.*, 1966; Cole and Cox, 1981; Manitto, 1981; Turner and Aldridge, 1983). In particular, seiricardine A (**9**, Figure 12.2 and Table 12.1) is a new bicyclic sequiterpene, which, on the basis of extensive 1D and 2D NMR and HR-EIMS studies, was identified as octahydro-1-isopropenyl-2,5-dihydroxy-3a,4,5-trimethyl-1H-indene. Its structure was confirmed by preparing the corresponding 2-*O*-acetyl- and the 2-oxo-derivatives through a standard acetylation and mild acidic oxidation, respectively, while the catalytic hydrogenation of **9** yielded the 8,10-dihydroderivative. The spectral data of the derivatives were consistent with the structure attributed to **9**. As the toxin was withstanding crystallization, the relative stereochemistry of the six chiral carbons (C-1, C-2, C-3a, C-4, C-5 and C-7a), including that of two ring junction, was ascertained by a series of NOE differences spectra. They allowed the assignment of a (1,2)-*trans*-junction between the two rings and the relative stereochemistry with respect to the chiral centres as sketched in Figure 12.2 (Ballio *et al.*, 1991).

Seiricardines B and C (**10** and **11**, Figure 12.2 and Table 12.1), characterised by the use of the same methods, and proved to be two new tricylic sequiterpenes belonging to the same chemical class of **9**. They were characterised by the absence of the disubstituted double bond of the isopropenyl group at C-1 and the secondary hydroxy group at C-2. Furthermore, the presence of a methylene group (H_2C-2), located in the cyclopentan ring, and a quaternary oxygenated carbon and a hydroxymethyl group in the hydroxy-isopropyl side chain at C-1, was noted. Therefore, the third ring present in seiricardines B and C proved to be an ether bridge between C-5 and C-8, and the two toxins are epimeric diastereomers at C-8. This was also confirmed by an extensive 1D and 2D NMR investigation and supported by HR-EIMS data. Seiricardines B and C are two diastereomeric octahydro-1-(1-hydroxyisopropyl)-3a,4,5-trimethyl-5,8-epoxy-1H-indenes. The structure of both toxins was confirmed by resorting to the corresponding monoacetyl derivatives, converting the primary hydroxy of the isopropyl side chain at C-1. By reaction with an appropriate reagent for the acetylation of steric hindered alcohols, the two toxins yielded epimeric derivatives which, besides the acetylation of the primary hydroxy group, unexpectedly showed the cleavage of the ether bridge with the subsequent formation of a trisubstituted double bond between C-5 and C-6 and the presence of a hydroxy group at C-8. The latter proved to be acetylated in the other two derivatives of **10** and **11**, obtained from the same reaction (Evidente *et al.*, 1993d).

When adsorbed by severed twigs of cypress, a 0.3 mg/ml solution of seiricardine A produced leaf yellowing and browning (Ballio *et al.*, 1991), while seiricardines B and C caused leaf chlorosis followed by browning and chlorosis, respectively; whereas on tomato and bean cuttings they caused chlorosis and necrosis, and chlorosis, respectively (Evidente *et al.*, 1993d). Subperidermal injection of a 0.1 mg/ml solution of **9** into young cypress trees produced necrotic lesions on the stem and a diffuse yellowing of adjacent twigs (Ballio *et al.*, 1991); seiricardines B and C in the same test induced hypertrophic reaction of the tissue adjacent to the site of injection and a reddish discoloration of distal leaves, and reddening and longitudinal lesions, respectively (Evidente *et al.*, 1993d). In the agar diffusion assay, seiricardine A at concentration of 100 μg/ml showed a fungistatic effect on three test fungi (Ballio *et al.*, 1991), while seiricardine B, compared to seiricardine C, showed a higher inhibitory effect on the mycelia growth of the three test fungi (Evidente *et al.*, 1993d).

Together with seiridins and seiricardins, cyclopaldic acid, and a 14-macrolide, named seiricuprolide, were produced by *S. cupressi* only. Seiricuprolide, a new pentasubstituted 14-macrolide, was isolated in a small amount from the culture filtrate of this strain. It is a (*2E,8Z*)-5,6-epoxy-4,7-dihydroxy-13-methyl-2,8-tridecadien-13-olide (**12**, Figure 12.2 and Table 12.1). As a component of the mixture of fungal metabolites, **12** may contribute to the overall toxic activity of the pathogen. In fact, macrolides are quite common as naturally occurring substances and some are biologically active (Dean, 1963; Richards and Hendrikson, 1964; Manitto, 1981). Fungi are also known to produce

macrolides (Turner and Aldridge, 1983). Its structure determination was achieved by spectroscopic analysis of the toxins and of some key derivatives. In fact, **12** was converted into the corresponding 4,7-*O*,*O*′-diacetyl- and the 2,3,8,9-tetrahydro-derivatives by standard acetylation and catalytic hydrogenation, respectively. The epoxy ring located between the C-5 and C-6 of the macrocyclic ring was converted by a regioselective opening, operated with the dilithium tetrabromonickelate (II) complex (Dawe *et al.*, 1984), in the corresponding *trans*-bromidryn carrying the hydroxy group at C-5 and the bromine at C-6. Mechanistic considerations on the latter reaction, together with a series of proton decoupling NMR experiments and inspection of Dreiding models, suggest a *trans*-configuration of the hydroxy group on C-4 and C-7a, and a *cis*-junction between the epoxide and the 14-membered ring in **12** (Ballio *et al.*, 1988). The elucidation of the absolute configuration of seiricuprolide was carried out by X-ray crystallographic analysis. The absolute stereochemistry assigned to C-4, C-5, C-6, C-7 and C-13 was *R*, *S*, *R*, *S* and *S*, respectively, while the double bonds C(2)-C(3) and C(8)-C(9) showed *E* and *Z* configuration, respectively. The two hydroxy groups at C-4 and C-7 and the methyl group at C-13 protrude from the same face of the molecule, whereas the carbonyl oxygen lies on the opposite face of the macrolide ring. The epoxide and the 14-membered ring form a *cis*-junction. The assignment of the absolute configuration of the seiricuprolide also allows the determination of the absolute configuration of its *trans*-bromohydrin derivative, for which the absolute stereochemistry at C-5 is *R* (Bartolucci *et al.*, 1992).

When assayed on test plants (severed twigs of cypress or tomato and mung bean cuttings, at concentration of 0.5 or 0.4 mg/ml, respectively) seiricuprolide produced diffuse yellowing followed by browning on host plants, or chlorosis and necrosis on non-host plants (Ballio *et al.*, 1988).

Structural investigation on the main phytotoxin produced *in vitro* in relatively high concentration (ca. 40 mg/ml) by *S. cupressi* demonstrated that the substance was identical with cyclopaldic acid, an antibiotic known to be a metabolite of some species of *Penicillium*, *Aspergillus* and *Pestalotiopsis* (Achenbach *et al.*, 1982). In fact, the spectral (UV, IR, ^{1}H-NMR and EIMS) and the physical (m.p. and R_f) properties of the toxin (**13**, Figure 12.2 and Table 12.1) were in excellent agreement with those reported in the literature for the fungal antibiotic (Achenbach *et al.*, 1982). The same results were obtained by a direct comparison of compound **13** with an authentic sample of cyclopaldic acid. Furthermore, the spectroscopic data of the monoacetate of the hemiacetalic hydroxy group at C-3 and those of the isocyclopaldic acid, a structural isomer of **13**, which was obtained by its alkali treatment, were fully consistent with the structure of the toxin (Graniti *et al.*, 1992).

Absorbed by severed twigs of three species of *Cupressus* and by cuttings of two herbaceous non-host plants (tomato and mung bean), cyclopaldic acid at a concentration of 10–100 μg/ml induced leaf chlorosis and necrosis. Oat-seed germination and root

growth of oat seedlings were reduced to about 50% using 75 and 100 μg/ml cyclopaldic acid solutions, respectively. The compound showed antifungal activity towards species of *Botrytis*, *Fusarium* and *Geotrichum* when assayed at a concentration range from 10 to 100 μg/ml. Therefore, cyclopaldic acid was described as a non-selective fungal phytotoxin for the first time (Graniti *et al.*, 1992).

In order to investigate whether changes in the molecular structure of cyclopaldic acid might affect its biological activity, one structural isomer and six derivatives were prepared and assayed for toxicity to cuttings of three species of cypress, as well as to mung bean, oat, and tomato explants. Besides the monoacetate of the hemiacetalic hydroxy group at C-3 and the isocylopaldic acid, which correspond to oxidation and reduction of the two *ortho*-dialdehyde groups, some other derivatives were prepared. We transformed the aldehyde at C-4 into the corresponding phenylhydrazone of **13**, both the *ortho*-dialdeyde groups into the tetracetyl derivative of **13**, the diacetyl derivative of phenylhydrazone and the 1,3,8-trihydro-3-deoxy of **13**, the latter obtained by catalytic hydrogenation of the toxin, and the corresponding dimethyl derivative prepared by reaction with ethereal diazomethane. Toxicity for host and non-host plants of **13** derivatives having one or both aldehydic groups transformed, was less than that of **13**. Isocyclopaldic acid, **13** and, to a lesser extent, the monoacetylated, phenylhydrazone and hydrogenated derivatives of **13**, caused loss of electrolytes from cypress tissues. Isocyclopaldic acid, **13** and its monoacetyl derivative caused limited callus development of cypress tissue. Diacetylphenhyldrazone of **13** enhanced the yield of cypress callus tissue. Derivatives of **13**, having both aldehyde groups modified, induced root formation on cypress cuttings. The antifungal activity shown by **13** toward species of *Botrytis*, *Fusarium*, and *Geotrichum*, markedly decreased in its derivatives (Sparapano and Evidente, 1995b).

An immunochemical method was optimised to study the involvement of **13** in the canker disease of cypress. An antiserum against **13** was prepared by immunisation of rabbits with a cyclopaldic acid-bovine serum albumin conjugate (**13**-BSA). Antibodies recognising **13** were purified by immunoaffinity chromatography through an immunoadsorbent prepared by conjugating **13** to lysine-Sepharose 4B. The specificity of the antibodies was assayed against **13** and the above-mentioned derivatives. The results obtained were in agreement with the importance of the *ortho*-dialdehyde system for the biological activity of the toxin. The derivatives of **13**, modified on one of the two aldehyde groups, reacted with antibodies to a lesser extent compared with **13**-BSA and **13**. Isocyclopaldic acid and the tetracetylderivative of **13**, which have more than one modified functional group, showed a further decrease in their reactivity. Other derivatives, which have more substantial structural modifications of the carbon skeleton, did not react (Del Sorbo *et al.*, 1994).

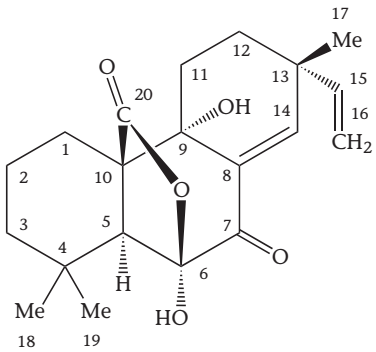
12.2.3 *Sphaeropsis* and *Diplodia* toxins

The fungi associated with canker disease of Italian cypress (*Cupressus sempervirens* L.) and other species of *Cupressus* in the Mediterranean area belong to the genera *Diplodia*, *Pestalotiopsis*, *Seiridium* and *Sphaeropsis* (Grasso and Raddi, 1979; Graniti, 1985; Graniti and Frisullo, 1987; Solel *et al.*, 1987; Madar *et al.*, 1989, 1991; Swart and Wingfield, 1991; Swart *et al.*, 1993).

Several phytopathogenic fungi belonging to the genus *Sphaeropsis* and *Diplodia* are responsible for severe diseases of agrarian and forest trees. *S. sapinea* f. sp. *cupressi* and *D. mutila* in cypress induce symptoms very similar to those produced by three phytopathogenic *Seiridium* species. It is well known that phytotoxins may be involved in pathogenesis and that microbial toxins may be used in the biological control of other pathogens which infected the same host plant of toxin-producer microorganism. This may be the case in the phytotoxins produced by *S. sapinea* f. sp. *cupressi* and *D. mutila* which could be used as antimicrobial substances against *Seiridium* spp.

The main toxin, called sphaeropsidin A (**14**, Figure 12.3 and Table 12.1) was isolated from the *in vitro* culture of *Sphaeropsis sapinea* f.s. *cupressi*. Spectroscopic data (essentially 1D and 2D ^{1}H- and ^{13}C-NMR and HR-EIMS) indicated that sphaeropsidin A was identical to a pimarane diterpenoid antibiotic previously isolated from some *Aspergillus* spp. (Ellestad *et al.*, 1972; Turner and Aldridge, 1983). The toxin proved to be a 9H-10,4a-(epoxymethano) phenanthrene-9-12-dione-7-ethenyl-1,2,3,4,4b,5,6,7,10,10a-decahydro-4b,10-dihydroxy-1,1,7-trimethyl, as also confirmed by an X-ray analysis still in progress to determine the absolute configuration of the toxin. Its complete spectral characterisation, as well as the antimicrobial and phytotoxic activities, have been reported (Evidente *et al.*, 1996b). In particular, when adsorbed by severed twigs of three species of *Cupressus* and cuttings of two herbaceous plants, sphaeropsidin A at 0.1 mg/ml produced leaf yellowing, browning and dieback on the host plants and yellowing of leaf, necrosis and epinasty of non-host plants. Subperidermal injection of a 0.1 mg/ml solution of sphaeropsidin A into young cypress trees caused longitudinal fissures and dark brown discoloration of cortical tissue. The compound showed antimicrobial activity towards twelve fungal species when assayed at a concentration range of 10 to 100 μg/ml. In particular, the fungistatic activity of sphaeropsidin A against *S. cardinale* and *S. cupressi*, both causal agents of a canker disease of cypress as *S. sapinea* f. sp. *cupressi*, is sufficient to account for the antagonistic action of *S. sapinea* f. sp. *cupressi* against *Seiridium* species. If sphaeropsidin A is produced *in vivo* by the fungus, it can be postulated that *S. sapinea* f. sp. *cupressi* spreading along the stem or branches may prevent the invasion of cortical tissue by *S. cardinale* or *S. cupressi* (Evidente *et al.*, 1996b).

A new examination of the organic extract (EtOAc) of the culture filtrates of the same fungus showed the presence of other minor metabolites. Two of them, called sphaeropsidins B and C (**15** and **16**, Figure 12.3 and Table 12.1), both structurally related to



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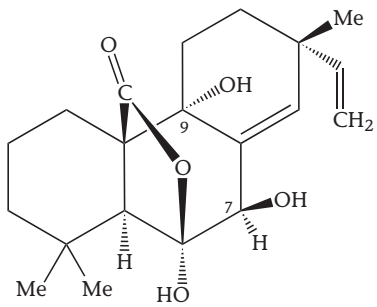

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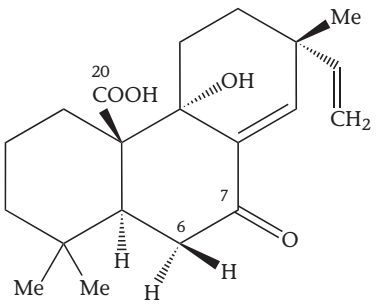

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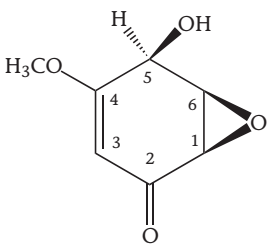

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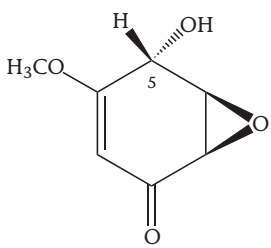

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Figure 12.3: *Sphaeropsis* and *Diplodia* toxins.

sphaeropsidin A, were isolated and characterised chemically and biologically. Extensive use of 1D and 2D NMR, and HR-EIMS associated to chemical work allowed us to identify sphaeropsidin B as the reduced derivative at C-7 of **14**, already known as an antibiotic metabolite of *Aspergillus* species (Ellestad *et al.*, 1972; Turner and Aldridge, 1983).

Sphaeropsidin C proved to be a new tricyclic acid pimarane diterpene, characterised as a 4a(9H)-phenanthrenecarboxylic acid, 9-one-7-ethenyl-1,2,3,4,4b,5,6,7,10,10a-decahydro-4b-hydroxy-1,1,7-trimethyl. Its structure was confirmed by converting **16** in the corresponding secondary alcohol at C-7 and methyl ester derivatives by a stereospecifical reduction and diazotation reaction, respectively; the ester derivative also showed, in agreement with Ellestad *et al.* (1972), the conversion of the double bond of the α,β-unsaturated ketone of **16** into a cyclopropane ring (Evidente *et al.*, 1997b). The stereoselectivity in the reduction of the toxin ketone group was not surprising because it had already been observed in the reduction of sphaeropsidin A to sphaeropsidin B, performed in the same conditions (Ellestad *et al.*, 1972). Sphaeropsidin C was also present in the cultural extract of *D. mutila* together with sphaeropsidin A, which was identified for the first time as the main phytotoxin produced by this fungus (Evidente *et al.*, 1997b).

Assayed on severed twigs of cypress and oak at 0.1 mg/ml, sphaeropsidins B and C caused dieback on *Cupressus macrocarpa*, browning and necrosis on *C. sempervirens* and yellowing on *C. arizonica*, necrosis on *Quercus cerri* and *Q. ilex*, and browning and necrosis on *Q. robur*. Injected into cortical tissues of cypress and oak seedlings, mentioned above, at 0.1 mg/ml, they caused dark brown discoloration, browning of internal tissues and browning on the former, and brown-blackish spots or necrotic lesions on the latter. On non-host plants such as tomato and oat, at the same concentration, sphaeropsidins B and C caused necrosis on cuttings and brown discoloration or stewing on stem. In the antimicrobial assay (tested at 10–100 μg/ml), both compounds showed an inhibitory effect on mycelial growth of seven test fungi. The inhibitory effects of both substances on two cypress pathogens *S. cardinale* and *S. cupressi* (as reported above for sphaeropsidin A) are promising for their potential use in the control of these two fungal pathogens of cypress to prevent early infections (Evidente *et al.*, 1997b).

The organic extracts of culture filtrates of *S. sapinea* f. sp. *cupressi* contained at least three other phytotoxic substances at lower concentration. Two of them appeared to be strictly related compounds, more polar than sphaeropsidins, from which they were structurally different. They were characterised as two new phytotoxic dimedone methyl ethers and therefore named sphaeropsidone and episphaeropsidone (**17** and **18**, Figure 12.3 and Table 12.1). The structure determination of the two toxins was performed using essentially spectroscopic (1D and 2D ^{1}H- and ^{13}C-NMR and HR-EIMS) methods. Sphaeropsidone and episphaeropsidone proved to be two disubstituted 7-oxabicyclo[4.1.0]hept-3-en-2-ones, which are epimers at C-5. Their structure and relative stereochemistry were confirmed by X-ray analysis, while absolute stereochemistry was deduced by circular dichroism measurements. The CD spectra of **17** showed a clear negative Cotton effect at 311 nm as already observed in other structurally close compounds containing the 5-hydroxy-7-oxabicyclo[4.1.0]hept-3-en-2-one moiety such as terremutin and panepoxydon (Miller, 1968; Fex and Wickberg, 1981). By contrast,

chaloxone, epoxydon, (+)-epiepoxidon and (+)-desoxyepiepoxidon (Nagasawa *et al.*, 1978; Fex and Wickberg, 1981) showed a positive Cotton effect in the same spectroscopic range. For all the compounds which are fungal metabolites, the absolute configuration has been reported (Miller, 1968; Nagasawa *et al.*, 1978; Fex and Wickberg, 1981). The clear positive Cotton effect observed at 260 nm in the CD curve of **17**, indicates the spatial *syn*-relation of the hydroxy group and the oxiran ring as already observed in some above-mentioned metabolites. In view of these findings, sphaeropsidone should have the absolute configuration depicted in **17**. The CD spectra of **18** compared to **17**, showed the expected negative Cotton effect at 312 nm and a different behaviour at 257 and 235 nm confirming, with respect to **17**, that the same orientation of the oxiran ring and that of the hydroxy group at C-5 had an opposite configuration as depicted in **18**. Such a conclusion was consistent with the results of a series of a NOE-difference spectra (Abraham and Loftus, 1978) performed on both **17** and **18**. As expected, in both metabolites a clear effect was observed between H-1 and H-6, and H-5 and the geminal OH group but the significant effect between H-5 and H-6 was only detected in **17** (Evidente *et al.*, 1998d).

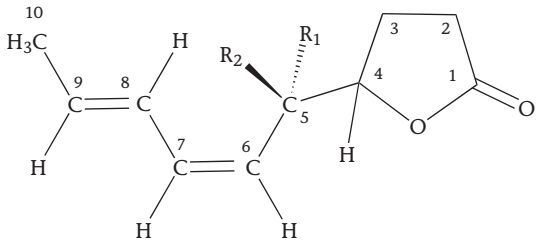
Assayed on severed twigs of cypress (at 0.2 mg/ml), sphaeropsidone caused browning and necrosis on *Cupressus macrocarpa*, no symptoms on *C. sempervirens* and chlorosis on *C. arizonica*. Epispaeropsidone at the same concentration caused necrosis on *C. macrocarpa*, browning and necrosis on *C. sempervirens* and necrosis on *C. arizonica*. On the non-host plant tomato at 0.1 mg/ml, both phytotoxins caused wilting. In a microbial assay, both compounds (tested at 25–100 μg/ml) showed an inhibitory effect on the growth of five fungal species tested. The growth of *Verticillium dahliae* was enhanced by both dimedone methyl ethers. In all cases, sphaeropsidone appeared to be less active than episphaeropsidone. In addition, the inhibitory effect elicited on *S. cardinale* and *S. cupressi*, the two cypress pathogens, was only shown by episphaeropsidone on the latter fungus (Evidente *et al.*, 1998d).

The taxonomy of *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton (*Sphaeropsidales*), an opportunistic pathogen of more than 30 species of *Pinus* in 25 countries (Swart and Wingfield, 1991), has been the subject of considerable confusion and conflicting reports. The pathogen occurs in coniferous forests throughout the world and has been associated with significant economic damage in exotic plantations in New Zealand, Australia and South Africa (Chou, 1976; Zwolinski *et al.*, 1990). *S. sapinea* also occurs in the Central and Eastern United States, causing severe damage on both native and introduced species. In Michigan, Minnesota and Wisconsin, two morphotypes of the pathogen were recognized (Palmer *et al.*, 1987). Isolates of the A morphotype were aggressive on both red and jack pine, but B morphotype isolates caused severe symptoms only on jack pine (Stanosz *et al.*, 1997). More recently, confirmation of two distinct populations of *S. sapinea* in the North Central United States was obtained by random amplified polymorphic DNA markers (RAPDs) (Smith and Stanosz, 1995). These observations opened a reconsideration of the nature of the host-pathogen interaction.

In order to contribute to understanding their physiology, we have undertaken a study on the production and identification of secondary metabolites produced by *Sphaeropsis* and *Diplodia* species. Usually, species belonging to the order of *Sphaeropsidales* produce *in vivo* and *in vitro* toxic substances (Uspenskaya and Reshetnikova, 1975). Furthermore, on the basis of the fungi metabolic behaviour, the metabolites can be used to better characterise biologically *S. sapinea* f. sp. *cupressi* from some strains of *S. sapinea* isolated from infected cypress trees. Recently, morphological, physiological, pathogenic and epidemiological studies on *S. sapinea*, *S. sapinea* f. sp. *cupressi* and *Diplodia mutila* suggested that a great morphological and physiological variability exists among different isolates of the fungal *taxa* examined (Frisullo *et al.*, 1997a,b). Moreover, the main toxic metabolites produced by three strains of *S. sapinea*, isolated from infected cypress trees proved to be chemically different from those produced *in vitro* by *S. sapinea* f. s. *cupressi* and *D. mutila* isolated from the same host plants.

Two 5-substituted dihydrofuranones, named sapinofuranones A and B (**19** and **20**, Figure 12.4 and Table 12.1), were isolated from liquid cultures of *Sphaeropsis sapinea*. A fungal strain isolated from *Cupressus macrocarpa* produced both **19** and **20** at concentrations higher than those produced by the two strains isolated from *C. sempervirens*. When assayed on host and non-host plants, **19** and **20** showed more toxic activity on inner bark tissues than on external ones. Phytotoxicity was observed, both on herbaceous non-host plants such as tomato, and on host plants such as cypress and pine trees. Symptoms appeared on severed twigs of cypress and pine species (at 0.1–0.2 mg/l), or on cuttings of herbaceous plants (at 0.05–0.1 mg/ml), after toxin absorption and after injection of a toxic solution (0.1 mg/ml) into the cortical tissues of two-year-old pine and three-year-old cypress trees. Browning or yellowing was observed on severed twigs of cypress and pine, respectively. Sapwood stain-like symptoms were observed on young active zone wood contiguous to cambium of cypress seedlings, while a severe withering of needles appeared on two-year-old pine seedlings. Tomato plants were also affected by both compounds; epinasty and brown discoloration appeared on petiols and leaves (Evidente *et al.*, 1999).

Compounds **19** and **20** were characterised, using spectroscopic methods (1D and 2D ^{1}H- and ^{13}C-NMR and HR-EIMS), as two new 4-[(*2Z,4E*)-1-hydroxy-2,4-hexadienyl]butan-4-olides, which are epimers at C-1 of the side chain. The chemical structure of **19** was also confirmed whilst preparing the corresponding 5-*O*-acetyl derivative (Evidente *et al.*, 1999). The absolute stereochemistry of this chiral centre, was determined by application of the Mosher's method (Dale *et al.*, 1969; Dale and Mosher, 1973) converting sapinofuranone A (**19**) into the diastereomeric *R*-(+)-α-methoxy-α-trifluorometylphenylacetate (MTPA) and the S-(−)-MTPA esters. Comparison of their ^{1}H NMR data showed a downfield shift (Δδ 0.16) of H-6 along with an upfield shift (Δδ 0.06) of H-4 allowing assignment of the *S*-configuration at C-5 in **19**. Whereas, comparing the ^{1}H NMR data of the *R*-(+)-MTPA and *S*-(−)MTPA esters obtained from **20**, an upfield shift (Δδ 0.23) of H-6



19 R_1=OH, R_2=H

20 R_1=H, R_2=OH

Figure 12.4: *Sphaeropsis sapinea* toxins.

along with a downfield shift (Δδ 0.04) of H-4 was observed. On the basis of reference data (Dale *et al*., 1969; Dale and Mosher, 1973), it was possible to assign the *R*-configuration at C-5 in **20**, which is opposite to that of **19**, and therefore, in agreement with their epimeric nature suggested by ^{1}H NMR data (Evidente *et al*., 1999).

The butanolide nature of **19** and **20** is rare in natural products (Turner and Aldridge, 1983). These compounds are closely related to butenolides and tetronic acids, which are well known as plant, fungal and lichen metabolites also exhibiting interesting biological activities (Dean, 1963). Among these are the 3,4-dialkylbutenolides isolated from culture filtrates of three species of *Seiridium* which produce canker disease on cypress (Evidente *et al*., 1986; Sparapano *et al*., 1986; Evidente and Sparapano 1994).

12.2.4 *Ascochyta* toxins

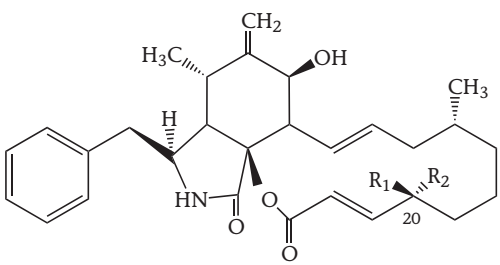
The genus *Ascochyta* includes a wide number of species having a high phytopathological importance. These species are responsible for severe diseases of important crops, both as foliar and pod diseases of legumes (i.e., *A. fabae* Speg. on faba bean, *A. pisi* Lib. and *A. pinodes* L.K. Jones on pea, *A. rabiei* [Pass.] Labrousse on chickpea) and foliar diseases of cereals (i.e., *A. avaneae* Sprague on oat and *A. hordei* Hara on barley). Considering the symptoms of the disease, usually expressed as necrosis and chlorosis, and also considering the possible problems for human and animal health due to the presence of fungal metabolites in food and feed, it seemed of interest to investigate the production of toxic metabolites by several *Ascochyta* species, and their occurrence in infected crops (Vurro *et al*., 1992).

Ascochyta heteromorpha (Schulzer *et* Sacc.) Curzi and then reclassified as *Phoma exigua* var. *heteromorpha* (Schulzer *et* Sacc.) Noordeloos *et* Boerema, the causal agent of a severe foliar disease of oleander (*Nerium oleander* L.) characterised by extensive brown-leaf

zonations, produced *in vitro* toxic metabolites. From the organic extracts of the culture of this fungus several new and known cytochalasins were isolated (for reviews *see* Evidente, 1997; Vurro *et al.*, 1997).

Cytochalasins are a large group of fungal metabolites produced by several species of fungi, having primarily cytological toxic effects on mammalian cells (Aldridge *et al.*, 1967), as well as other biological activities. The first two compounds of this group to be isolated and characterised were cytochalasins A and B (**21** and **22**, Figure 12.5 and Table 12.1) (Aldridge *et al.*, 1967; Rothweiler and Tamm, 1970). Up to now, more than 50 different cytochalasins from several species of fungi have been purified and identified. Investigation on the biological activity of cytochalasins, mainly referred to as cytochalasin B, indicated that these metabolites showed toxicity, both *in vitro* on animal cells, and *in vivo* towards animals, as well as toxicity on microorganisms such as bacteria, algae, fungi and protozoa. Furthermore, some cytochalasins were also observed to have phytotoxic activities. The chemical and biological interest in these compounds is evidenced by the numerous studies reported (for reviews *see* Tanenbaum, 1978; Cole and Cox, 1981; Natori and Yahara, 1991; Vurro *et al.*, 1997).

However, the main phytotoxic activity remained in the residue after CH_2Cl_2 extraction of liquid culture filtrates of *Phoma exigua* var. *heteromorpha*. Chemical work carried out to characterise the phytotoxic metabolites, indicated a hydrophilic and macromolecular nature for these substances. Further work to isolate and characterise these phytotoxins is still in progress. The exhaust aqueous phase obtained by culture filtrate after cytochalasin extraction was treated with cold ethanol and the precipitate dyalized to have a crude precipitate that proved to have essentially a polysaccharide nature. In fact, its chemical hydrolysis gave mannose, galactose and glucose in the 3:1:1 ratio as components. Moreover, the ^{13}C-NMR spectrum of this crude polysaccharide fraction indicated the presence of anomeric signals typical of both furanose and pyranose sugars,



21 $R_1+R_2=O$ **22** $R_1=H$, $R_2=OH$

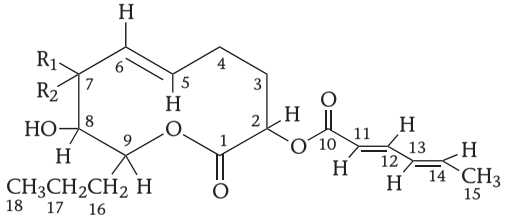
Figure 12.5: *Ascochyta* and *Phoma* toxins: cytochalasins A and B.

while the ^{31}P-NMR spectrum showed the presence of signals typical of phosphorylated saccharides. These results suggested a weak aqueous HF hydrolysis of the crude exopolysaccharide to obtain a dephosphorylated fraction, whose structure determination is still in progress. When assayed by infiltration on tobacco leaves, the crude exopolysaccharides, at concentration of 0.5 mg/ml, caused, after one day, a marked necrosis, which expanded in the successive days. The same fraction tested at 220 μg/ml on cellular suspension of poplar showed a significant elicitor activity, which induced a rapid increase of the phenylalanine ammonia-lyase activity (Vurro and Ellis, 1997), comparable to that of well known elicitor (Evidente *et al.*, 1997c).

The production of toxic metabolites by other 23 *Ascochyta* strains grown on autoclaved wheat grains, was investigated. Amongst the cultural organic extracts showing high toxicity, cytochalasins A and B were detected only in that of *A. lathyri*. This evidence clearly indicated the occurrence of other toxic metabolites (Vurro *et al.*, 1992).

From the culture organic extracts of *A. pinodes* Jones, the causal agent of the anthracnose of pea (*Pisum sativum* L.), characterised by severe lesions and necrosis of leaves and pods, the main phytotoxin, called pinolidoxin, was isolated. Pinolidoxin (**23**, Figure 12.6 and Table 12.1) was characterised using spectral (essentially 1D and 2D ^{1}H- and ^{13}C-NMR and HR-EIMS) and chemical methods, such as 2-(2,4-hexadienoyloxy)-7,8-dihydroxy-9-propyl-5-nonen-9-olide, a new phytototoxic tetrasubstituted nonenolide belonging to the well known family of macrolides. As mentioned above, they are quite common as naturally occurring compounds and some are biologically active (Dean, 1963; Richards and Hendrickson, 1964; Manitto, 1981; Turner and Aldridge, 1983). The toxin structure was confirmed by conversion of **23** in its 7,8-*O*,*O*′-diacetyl-, 7,8-*O*,*O*′-isopropylidene- and 5,6,11,12,13,14-hexahydro-derivatives (Evidente et al., 1993c). When assayed at 3×10^{-2} M on host (pea) and non-host (bean) plants, pinolidoxin was highly toxic, but tested at 6×10^{-4} M, only weakly toxic on brine shrimps (*Artemia salina* L.) (Evidente *et al.*, 1993c). Further chemical investigation on the same organic extract allowed the isolation of three new pinolidoxins, called epi-, dihydro- and epoxy-pinolidoxin (**24**, **25** and **26**, Figure 12.6 and Table 12.1). They were characterised as 7-epi-, 5,6-dihydro- and 5,6-epoxy-pinolidoxin, using the same approach employed for the structure determination of **23** (Evidente *et al.*, 1993a). Assayed on pea and bean leaves at 1 μg/μl, epipinolidoxin and dihydropinolidoxin caused necrotic lesion, whereas epoxypinolidoxin was inactive. Only the epi- and epoxyepipinolidoxin, tested at 50 μg/ml, were found to be active using the brine shrimp assay (Evidente *et al.*, 1993a).

The genus *Ascochyta* includes other species responsible for foliar and pod disease of pea such as *A. fabae* Speg. and *A. pisi* Lib. The latter microorganisms on the host-plant induced the same symptoms caused by *A. pinodes*. *A. pisi* produced toxic metabolites when grown on autoclaved wheat kernels. The main phytotoxin, called ascosalitoxin (**27**, Figure 12.6 and Table 12.1) was isolated and characterised, essentially by spectroscopic (^{1}H- and ^{13}C-NMR and EIMS) methods such as 2,4-dihydroxy-3-methyl-6-(1,3-



23 R_1=OH R_2=H

24 R_1=H R_2=OH

25

26

27

Figure 12.6: *Ascochyta* toxins: pinolidoxins and ascosalitoxin.

dimethyl-2-oxopentyl) benzaldehyde. The structure was consistent with the spectroscopic data of the 4-*O*-acetyl derivative prepared by standard acetylation of the toxin (Evidente *et al.*, 1993b). Ascosalitoxin, a new trisubstituted derivative of salycilic aldehyde, is structurally related to ascochitine the main toxic metabolite with antibiotic and phytotoxic activity isolated from liquid culture of both *A. pisi* and *A. fabae* (Iwai and Mishima, 1965; Colombo *et al.*, 1980). Ascosalitoxin, tested at 2 and 0.1 μg/μl, respectively, displayed phytototoxic activity on pea and bean leaves and pods, and on tomato seedlings. Assayed on brine shrimps at 0.2 mg/ml and on *Geotrichum candidum* at 100 μg/disk, the toxin showed no toxicity (Evidente *et al.*, 1993b).

Finally, *A. rabiei* (Pass.) Labrousse, is the fungal agent responsible for a severe disease of chickpea (*Cicerum arietinum* L.), which caused heavy crop losses in areas around the Mediterranean basin (Alam *et al.*, 1989; Mmbaga *et al.*, 1994). Infections of chickpea pods are very frequent, and *A. rabiei* is able to colonize extensively the seeds in the field. When the fungus was grown on a medium consisting of Czapek Dox nutrient and chickpea seed extract, the culture filtrates were toxic to cells isolated from leaves of the host plant. The toxins were isolated by partitioning with ethyl acetate and chromatographing the organic fraction on silica gel. Two compounds were separated and identified as solanapyrones A and C (**28** and **30**, Figure 12.7 and Table 12.1) by MS and NMR (Alam *et al.*, 1989). Using the same culture medium, supplemented with a range of amino acids, vitamins and cations, a new toxin named solanapyrone B (**29**, Figure 12.7 and Table 12.1) was isolated, together with solanapyrones A and C (Chen *et al.*, 1991). Solanapyrones A, B and C had previously been isolated as phytotoxic metabolites from the culture filtrates of *Alternaria solani*, the causal agent of early blight disease of tomato and potato (Ichihara *et al.*, 1983). The mechanism of action of these phytotoxins is not established, although the symptoms caused by the toxins (epinasty, chlorosis and necrosis), are consistent with the chickpea disease. Owing to the great importance of chickpea blight caused by *A. rabiei*, it seemed, therefore, of interest to investigate the presence of fungal toxic metabolites in naturally infected chickpea seeds. In fact, a toxic metabolite was isolated from the organic extract of a variety of these, collected near Aleppo (Syria). It is absent, both in healthy chickpea seeds, and healthy seeds from infected chickpea plants. The metabolite, toxic on brine shrimps (*A. salina* L., LD_{50} of 76 μg/ml), was identified by spectroscopic techniques (essentially 1D and 2D ^{1}H- and ^{13}C-NMR and EIMS) as the 3-hydroxy-9-methoxypterocarpan, which had been named medicarpin (**31**, Figure 12.7). Medicarpin together with pisatin and maackiain, other structurally related compounds, belong to the pterocarpanes subfamily of isoflavanoid known for their phytoalexin activity and, therefore, for their possible role in the disease-resistance mechanism of a variety of plants. This represents the first report on the presence of medicarpin in naturally infected chickpea seeds (Evidente *et al.*, 1996a).

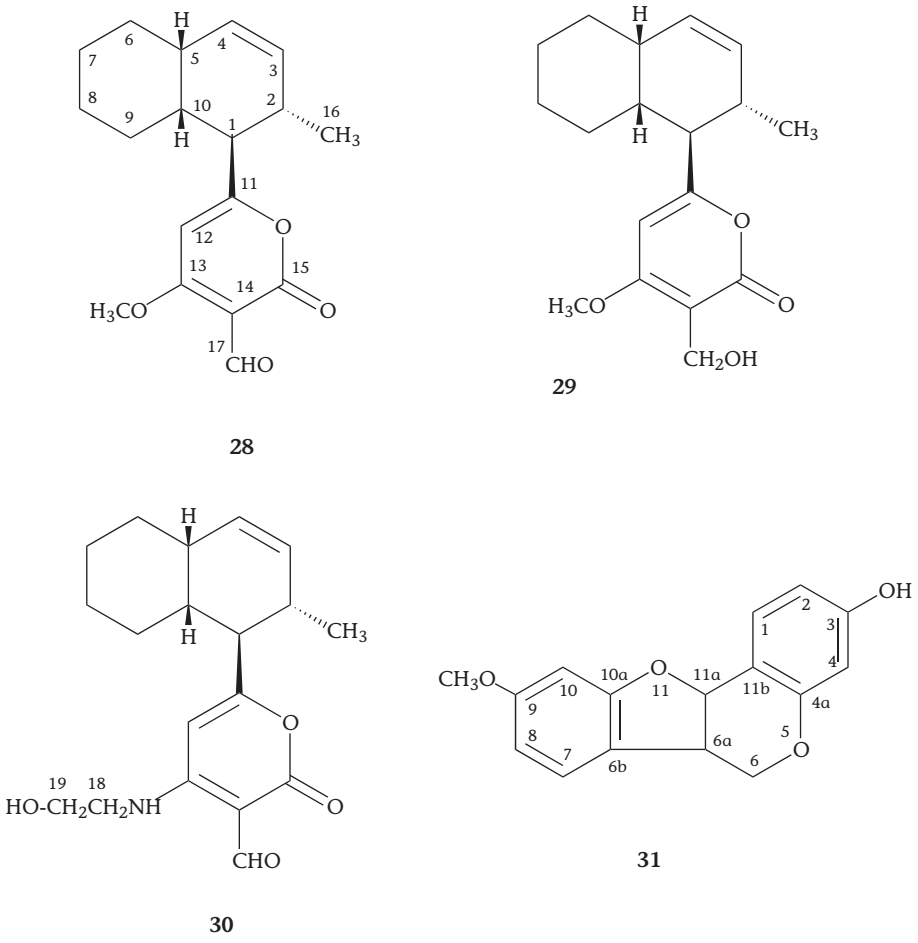


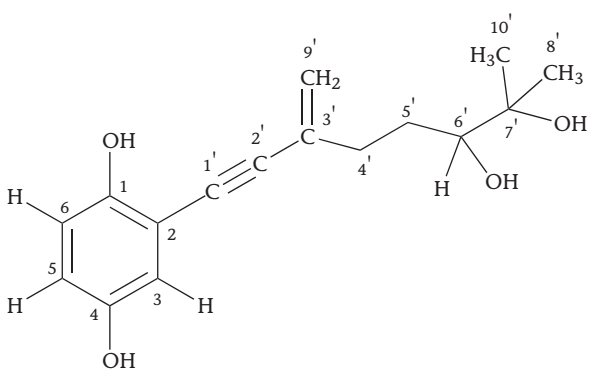
Figure 12.7 *Ascochyta rabiei* toxins.

12.2.5 *Phomopsis* toxins

Phomopsis foeniculi, was found to cause the necrosis of stems, leaves and inflorescences of fennel (*Foeniculum vulgare* subsp. *vulgare* var. *vulgare* [Mill.] Thell and var. *dulce* [Mill] Thell), a disease leading to marked decrease in fruit production in some areas of Italy and France. As fennel fruits are economically important in the phytotherapy and food industry, a research was planned to select lines of fennel which are resistant to the disease, using the toxin produced *in vitro* by *P. foeniculi*. The fungus biosynthesizes both hydrophilic macromolecular and lipophilic low molecular toxins; the latter were exhaustively extracted from the culture filtrates with ethyl acetate. One of the more

abundant phytotoxic metabolites, namely foeniculoxin, was isolated and characterised using spectroscopic methods (essentially 1D and 2D ^{1}H- and ^{13}C-NMR and EIMS) as 2-(6,7-dihydroxy-7-methyl-3-methylen-octan-1-ynyl)-hydroquinone (**32**, Figure 12.8 and Table 12.1). When assayed at a concentration of 3.6×10^{-3} M, foeniculoxin reduced root growth of the germinating seeds of both fennel and tomato, while it induced necrosis on tobacco leaves and wilting and/or isolated necrotic spots on the leaves of tomato cuttings. The toxin represents a new monosubstituted geranylhydroquinone with an unusual side chain (Evidente *et al.*, 1994). The terpenoid hydroquinones are a well-known family of natural substances, closely related to the corresponding *p*-benzoquinone into which are easily oxidized. Terpenoid hydroquinones, especially *p*-benzoquinones, have very important biological activities and are common as natural plant animal and microorganism (bacteria and fungi) metabolites (Whalley, 1963; Morton, 1965; Wiss and Gloor, 1970; Thomson, 1971). The structure of foeniculoxin was confirmed by preparing some key derivatives for converting **32** into the corresponding 1,4,6′-*O*,*O*′,*O*′′-triacetyl-, 1,4-*O*,*O*′-dimethyl- and 6′,7′-*O*,*O*′-isopropylidene-derivatives, by standard acetylation, methylation with diazomethane and chetalization with acid catalysed dry acetone. Catalytic hydrogenation converted foeniculoxin into the corresponding hexahydroderivative, while a mild oxidation yielded the corresponding *p*-benzoquinone and the products of the oxidative cleavage of the glycol system present in the side chain. As expected, the spectroscopic properties of all the derivatives were fully consistent with the structure assigned to the toxin. Furthermore, a series of NOE-difference spectra (Abraham and Loftus, 1978) carried out on the 1,4-*O*,*O*′-dimethyl-foeniculoxin confirmed the substituent pattern of the aromatic ring of the toxin (Evidente *et al.*, 1994).

We have also characterised the hydrophilic macromolecular phytotoxins, which have shown a polysaccharide nature. From the residual aqueous phase of the lipophilic



32

Figure 12.8: *Phomopsis* toxin.

metabolite extraction from culture filtrates, two polysaccharide fractions were isolated by more steps of organic solvent precipitation. In fact, from the crude mixture of polysaccharides, obtained by EtOH treatment, a precipitate of a pure mannan (fraction A) was produced by successive precipitation with propan-2-ol. The structure of this polysaccharide was determined by the application of the classic degrading chemical and analytical methods and spectroscopic techniques (essentially NMR and MS). The mannan proved to be constituted by a backbone of α-(1 → 6) linked D-mannopyranose units almost branched at 2-position, whereby the arms are made up of 2- or 3-linked α-D-mannopyranose residues. The other polysaccharide present in the 2-propanol supernatant (fraction B) contained galactose, as its main component, beside minor amounts of mannose and rhamnose. Its ^{1}H- and ^{13}C-NMR spectra were identical to those recently described for a galactan isolated from cell-wall of *Neosartorya* fungus (Leal *et al.*, 1995). These data, in agreement with the results of the methylation analysis indicate, for the more abundant polysaccharide of fraction B, a galactan structure with the following repeating unit (Corsaro *et al.*, 1998):

$$[\rightarrow 6)\text{-}\beta\text{-D-Gal}f\text{-}(1 \rightarrow 5)\text{-}\beta\text{-D-Gal}f\text{-}(1 \rightarrow 5)\text{-}\beta\text{-D-Gal}f\text{-}(1 \rightarrow]_n$$

No structural information could be obtained for the minor polysaccharide component in fraction B because any attempts to purify it were unsuccessful.

The phytotoxicity of crude exopolysaccharides, and of the A and B fractions from culture filtrates of *P. foeniculi*, was tested (at concentration of 0.5, 1.0, 2.5 and 5.0 mg/ml) on three different plants: the homologous host (uptake by cuttings from two-months' old plantlets of sweet fennel, cv. Scafati) and two non-hosts: tomato (uptake by four-leafed tomato cuttings from about one-month-old plantlets, cv. Marmande) and tobacco infiltration of the leaf mesophyll, cv. Samsun). All the three test plants showed phytotoxicity symptoms after treatment with crude exopolysaccharides, fraction A (mannan) and fraction B (impure galactan). In particular, necrotic symptoms were observed on leaves of tomato cuttings, wilt and basal stem flaccidity symptoms on fennel, and complete necrosis of the infiltrated area of tobacco leaves. Using the separated fraction, symptoms started to develop after 48 h and their intensity increased over the following 2–3 days (maximum intensity after 4–5 days) and at increasing concentrations. Infiltration of both polysaccharide fraction A and B together (in the same ratio as in the crude extract) gave a slightly stronger effect with all concentrations tested. Commercial mannan (Sigma-Aldrich) assayed as a control on all test plants gave no symptoms on tobacco and later and lighter symptoms on fennel and tomato cuttings (Corsaro *et al.*, 1998).

Data obtained on wilting and necrotic effects on the different test plants by uptake or infiltration of mannan and galactan purified fractions from *P. foeniculi* culture filtrates, yet to be more thoroughly investigated, can lead to hypothesize a role of the two polysaccharides in disease development. Following stem infection, damages to inflorescences

and foliage then occurs, and these long-distance effects could, in fact, be associated to production of substances with phytotoxic activity.

12.3 HERBICIDES

The weed pest is one of the most serious problems for agriculture and environment. Infesting plants generate a great obstacle to the normal flow of superficial waters, destroy the natural habitat, seriously damage the archaeological and monumental area, and cause heavy losses to crop production and to the pasture industry. Many plants of agrarian interest may die back when the weed grows in the same field, absorbing water, food substances, and sunlight. Furthermore, they represent a serious impediment to the normal agrarian activity. The diffusion of weed reduces the pasture areas, with consequent deterioration of animal food.

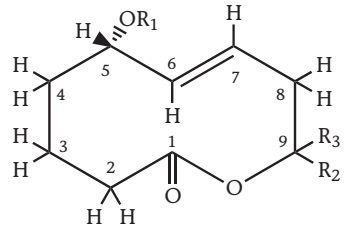
The control of weed diffusion has been achieved with agrochemicals belonging to different classes of organic compounds. They are usually used in very large amounts in agriculture, thus causing serious problems to human and animal health and producing heavy environmental pollution. In fact, these substances frequently have low specificity and are weakly, or not, biodegradable, accumulate in food plants and in layer, and in drinkable water. Furthermore, the chemical controls have a short-life and must usually be repeated on an annual or semi-annual basis. The biological agents offer the advantage of being compatible with the environment, often with high specificity, and also represent a long-term solution in the control of weeds which are particularly resistant to chemical herbicides. Therefore, many efforts have been made to biologically control the weeds using their natural antagonists as insect and/or microorganisms. Among the microorganisms, fungi are the most common pathogens of plants and, therefore, for weeds also. Some insects and fungi, which satisfy the criteria of efficacy, specificity and long-time persistence, have already been commercialized. Recently, researches have been started to isolate phytotoxins produced by some fungi pathogenic for weed and use them as natural herbicides. The goal of such a project is to use natural substances, their derivatives, or synthetic analogues, with increased efficacy and specificity, to avoid the release of microorganisms, and the possibility that they become host to other organisms. Since many phytotoxins isolated from fungi pathogenic for agrarian plants are not specific, they may be considered as potential natural herbicides in native forms or as derivatives and analogues (Graniti *et al.*, 1989; Delfosse, 1990; Strobel, 1991; Strobel *et al.*, 1991).

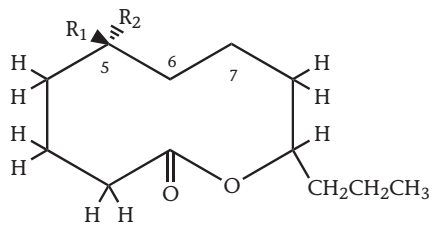
The first approach is the isolation of microorganisms from tissues of infected infesting plants, followed by selection of the strain with higher specificity and virulence. The second step is to find appropriate conditions for the *in vitro* growth of the fungus to obtain culture filtrates with high phytoxicity against the host plant. Next, the phytotoxins are isolated, characterized and in some cases derivatized before being tested as

potential herbicides. Finally, the knowledge of the chemical structure of these substances may allow the partial or total synthesis of the most appropriate natural herbicide, therefore avoiding the collection, preservation and growth of pathogen agents.

12.3.1 *Phoma* toxins

From infected leaves of *Erigeron annuus* L., a fungus, identified as *Phoma putaminum*, was isolated. *E. annuus*, commonly named annual fleabane, is an indigenous weed from North America widely found in field and pastures all over Europe, including Italy. When grown in liquid culture, *P. putaminum* produced phytotoxic metabolites, which were extracted and purified. The main phytotoxin present in the organic extract of culture filtrate, named putaminoxin, was characterised by spectroscopic methods (essentially 1D and 2D ^{1}H- and ^{13}C-NMR and HR-EIMS) as (5*S*)-5-hydroxy-9-propyl-6-nonen-9-olide (**33**, Figure 12.9 and Table 12.2). The structure of this new 10-macrolide was confirmed by conversion of the toxin into the corresponding 5-*O*-acetyl- and 6,7-dihydro-derivatives by standard acetylation and catalytic hydrogenation, respectively. The absolute stereochemistry of the secondary alcohol at C-5 was determined by applying GC Horeau's method (Evidente *et al.*, 1995). When assayed on host leaves using a puncture assay (20 μg/droplet), putaminoxin caused chlorosis, followed two days later by necrosis. Assayed on leaves of weed species and on non-host cultivated plants, it showed a range of toxicity (mandarine and sweet basil being among the less sensitive) and annual dog's mercury the most sensitive. However, the toxicity observed on annual fleabane was the most severe. The toxin showed weak toxicity (at 100 μg/disk) towards *G. candidum*, whereas when assayed up to 100 μg/disk against both *Escherichia coli* and *Bacillus subtilis* it was not toxic. Moreover, it showed no activity against *A. salina* L. (brine shrimps) larvae when assayed up to 2×10^{-4} M (Evidente *et al.*, 1995). Further investigation was carried out to ascertain whether associated toxins may be responsible for the high phytotoxicity of the organic culture extract. We identified at least four other structurally related metabolites. Two of them, namely putaminoxin B and C, were isolated and characterised as a (5*S*)-5-hydroxy-9-pentyl-6-nonen-9-olide and a 4-hydroxy-2-propyl-2-cyclononen-1,5-dione (**34** and **38**, Figure 12.9 and Table 12.2), respectively (Evidente *et al.*, 1997d). Putaminoxin B (**34**) differed from putaminoxin only for the length of the alkyl side chain attached to C-9 of the nonenolide macrocycle. When compared with **33**, putaminoxin C (**38**) had the same macrocyclic ring with a *trans*-disubstituted double bond and a propyl side chain, but it appeared differently functionalized. When assayed on punctured and detached leaves of several weeds and cultivated plants at 8 μg/droplet, putaminoxins B and C showed a wide range of toxicity. Putaminoxin C (**38**) showed semiselective toxic effects similar to those produced by **33**, while putaminoxin B showed no phytotoxicity. No zootoxic or antifungal activity was observed for either compound (**34** and **38**) when assayed up 5×10^{-5} M on *A. salina* and up





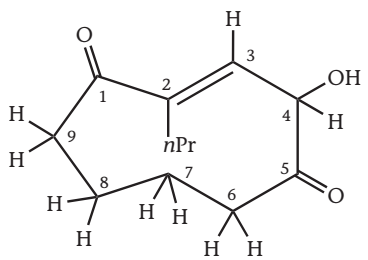
33 R_1=H, R_2=$\overset{10}{C}H_2\overset{11}{C}H_2\overset{12}{C}H_3$, R_3=H

34 R_1=H, R_2=$\overset{10}{C}H_2\overset{11}{C}H_2\overset{12}{C}H_2\overset{13}{C}H_2\overset{14}{C}H_3$, R_3=H

35 R_1=H, R_2=H, R_3=$CH_2CH_2CH_2CH_2CH_3$

36 R_1=HCO, R_2=$CH_2CH_2CH_3$, R_3=H

37 R_1=OH, R_2=H



38

Figure 12.9: *Phoma putaminum* toxins.

50 μg/disk on *G. candidum*. Assayed on *Bacillus megaterium* and *Pseudomonas* sp. at up 40 μg/disk, only putaminoxin C caused a clear inhibition halo of the growth of *B. megaterium* (Evidente *et al.*, 1997d).

More recently, two other metabolites were obtained from the same organic extract, and their chromatographic behaviour was very similar to that of **33**. The two metabolites, which were obtained as homogeneous compounds withstanding crystallization, were named putaminoxin D and E (**35** and **37**, Figure 12.9 and Table 12.2), being structurally related to putaminoxin and putaminoxin B. Putaminoxin D and E were characterised by chemical and spectroscopic methods as new disubstituted nonen- and non-anolides, respectively. The only substantial ^{1}H-NMR spectral difference of putaminoxin D, when compared with **33**, was the presence of more complex aliphatic proton systems. Furthermore, the two olefinic protons (H-6 and H-7) showed a complex signal. These results, together with ^{13}C NMR and EIMS data, suggested for putaminoxin D a structure very close to that of **34**, except for the different stereochemistry of the

TABLE 12.2
Toxins from fungi phytopathogenic for infestant plants

Toxin	Source	Molecular formula	Molecular weight	IUPAC name	Reference
Ascaulitoxin	*A. caulina*	$C_{14}H_{27}N_3O_{10}$	397	2,4,7-Triamino-5-hydroxyoctandioic acid 2-*N*-β-D-glucopyranoside	Evidente *et al.*, 1998c
Brefeldin A	*A. zinniae*	$C_{16}H_{24}O_4$	280	(4H-cyclopent[f]oxacyclotridecin-4-one-1,6,7,8,9,11a,12,13,14,14a-decahydro-1,13-dihydroxy-6-methyl	Vurro *et al.*, 1998
α,β-Dehydro curvularin	*A. zinniae*	$C_{16}H_{18}O_5$	290	4,5,6,7-Tetrahydro-11,13-dihydroxy-4-methyl-2H-3-benzoxacyclododecin-2,10(1H)-dione	Vurro *et al.*, 1998
9,10-Dehydro-fusaric acid	*F. nygamai*	$C_{10}H_{11}NO_2$	177	2-Pyridinecarboxylic acid, 5-(3-butenyl)	Capasso *et al.*, 1996
Fusaric acid	*F. nygamai*	$C_{10}H_{13}NO_2$	179	2-Pyridinecarboxylic acid, 5-butyl	Capasso *et al.*, 1996
Methyl ester of 9,10-dehydro-fusaric acid	*F. nygamai*	$C_{11}H_{13}NO_2$	191	2-Pyridinecarboxylic acid, 5-(3-butenyl) methyl ester	Capasso *et al.*, 1996
Methyl ester of fusaric acid	*F. nygamai*	$C_{11}H_{15}NO_2$	193	2-Pyridinecarboxylic acid, 5-butyl methyl ester	Capasso *et al.*, 1996
3-Nitropropanoic acid	*M. thelebola*	$C_3H_5NO_4$	119	3-Nitropropanoic acid	Evidente *et al.*, 1992
Putaminoxin	*P. putaminum*	$C_{12}H_{20}O_3$	212	5-Hydroxy-9-propyl-6-nonen-9-olide (*5S*)	Evidente *et al.*, 1995
Putaminoxin B	*P. putaminum*	$C_{14}H_{24}O_3$	240	5-Hydroxy-9-pentyl-6-nonen-9-olide (*5S*)	Evidente *et al.*, 1997d
Putaminoxin C	*P. putaminum*	$C_{12}H_{18}O_3$	210	4-Hydroxy-2-propyl-2-cyclononen-1,5-dione	Evidente *et al.*, 1997d
Putaminoxin D	*P. putaminum*	$C_{14}H_{24}O_3$	240	5-Hydroxy-9-pentyl-6-nonen-9-olide (*5S*)	Evidente *et al.*, 1998a
Putaminoxin E	*P. putaminum*	$C_{12}H_{22}O_3$	214	5-Hydroxy-9-propyl-nonan-9-olide (*5R*)	Evidente *et al.*, 1998a

double bond (C(6)=C(7)) of the macrocyclic ring. Such an hypothesis was ruled out by examining the ^{1}H-NMR spectrum of the 5-*O*-acetyl derivative of putaminoxin D. In fact, it differed from that of **35**, for the expected downfield shift of the H-5 doublet of double doublets, and for the olefinic protons unexpectedly appearing as two well-resolved systems. The coupling constants values measured for these systems allowed assignment of a *trans*-stereochemistry for the double bond of the macrocyclic ring located between C(6)-C(7) (Sternhell, 1969), and the same relative configuration for C-5 as indicated for this secondary hydroxylated carbon both in **33** and **34** (Evidente, *et al.*, 1995, 1997d). On the basis of these results the epimer structure at C-9 of **34** was suggested for putaminoxin D: it was supported by the EIMS data of **35** and its 5-*O*-acetyl derivative. Therefore putaminoxin D may be formulated as (5S)-5-hydroxy-9-pentyl-6-none-9-olide (Evidente *et al.*, 1998a).

Putaminoxin E (**37**) showed a molecular weight of 214, as deduced from its EIMS spectrum, differing from putaminoxin by the presence of two more hydrogen atoms. This is also evident from its ^{1}H-NMR spectrum. This substantially differed from the spectrum of **33** for the absence of the olefinic protons and a major complexity of the H-5 multiplet. These differences were similar to those already observed when the ^{1}H-NMR of 6,7-dihydroputaminoxin, obtained by catalytic hydrogenation of putaminoxin (**33**), was compared with that of **33**. The same evidence resulted from comparison of the ^{13}C-NMR spectrum of putaminoxin E with those of **33** and its dihydroderivative. Therefore, for putaminoxin E the structure of a 6,7-dihydroputaminoxin was supposed.

The structure of putaminoxin E was consistent with the fragmentation peaks observed in its EIMS spectrum. These findings, and the different chromatographic behaviour of **37** when co-chromatographed with 6,7-dihydroputaminoxin in three different systems, suggested for the toxin the structure of a 5- or 9-epimer of 6,7-dihydro derivative of **33**. However, a further comparison of the ^{1}H-NMR spectrum of **37** with that of 6,7-dihydroputaminoxin showed a very similar chemical shift value of the multiplet of H-9, while that of H-5 was shown to be very different. On the basis of these results putaminoxin E may be formulated as the 5-epi-6,7-dihydroputaminoxin (Evidente *et al.*, 1998a).

Furthermore, from the lesser polar fraction of the initial column, a compound was isolated (**36**, Figure 12.9), which showed a structure very similar to that of putaminoxin. In fact, its ^{1}H-NMR differed from that of **33** for the downfield shift of the H-5 doublet of the double doublets as a consequence of the esterification of the geminal hydroxy with a formyl group appearing as typical chemical shifts values in the ^{1}H- and ^{13}C-NMR spectra (Breitmaier and Voelter, 1987; Pretsch *et al.*, 1989). In agreement with the molecular weight of 240, compound **36** proved to be the 5-*O*-formylputaminoxin, as indicated by the fragmentation ions observed in its EIMS spectrum. It proved to be an artifact due to the extraction process. It was, in fact, the main component of the crude extract when extraction was made after acidification with HCOOH, but it was

absent when the culture filtrates were acidified with HCl and extracted in the same conditions.

Assayed on tomato and fleabane leaves at a concentration of 4×10^{-3} M, putaminoxins D and E, as well as 5-*O*-formylputaminoxin, showed no phytotoxicity, indicating that the presence of both the unalterated alkyl side chain at C-9, and both the double bond and the hydroxy group at C-5 are structural features important for the activity. These results agree with the lack of phytotoxicity previously shown by putaminoxin B (Evidente *et al.*, 1997d) which, together with **35**, showed a pentyl side chain at C-9 although with an opposite configuration. As already observed by testing both putaminoxin and putaminoxin B (Evidente *et al.*, 1995, 1997d), these metabolites were inactive on the brine shrimps (*A. salina*) assay (tested at 4×10^{-4} M) and on *G. candidum* (at 20 μg/disk). Putaminoxin C, being a disubstituted cyclononenedione and therefore quite different from putaminoxins, had shown toxicity on plants and bacteria (Evidente *et al.*, 1997d).

The nonenolides are macrolides which constitute a well-known group of naturally occurring compounds, including fungal metabolites with interesting biological activity (Dean, 1963; Richards and Hendrickson, 1964; Manitto, 1981; Turner and Aldridge, 1983). Among these, pinolodoxin (**23**, Figure 12.6) is the main toxin isolated from wheat cultures of *Ascochyta pinodes*, together with three minor structurally close metabolites (namely epi-, dihydro- and epoxy-pinolidoxin, **24**, **25** and **26**, Figure 12.6, respectively, *see* 12.2.4). Considering that pinolidoxin has shown aspecific phytotoxicity (Evidente *et al.*, 1993c) as did putaminoxin, a structure-activity relationship study was planned. This investigation was carried out using both toxins together with the above-cited natural analogs and some derivatives, prepared from **23** and **33** by modification of their functional groups. Their phytotoxicity, zootoxicity and antifungal activity was assayed to discover a derivative for possible use as natural pesticide having efficacy and a reduced environmental impact.

Putaminoxin (**33**) was transformed into the corresponding 5-*O*-acetyl and the 6,7-dihydro derivatives, while pinolidoxin (**23**), as reported in 12.2.4, was converted into the corresponding 7,8-*O*,*O*′-diacetyl and the 7,8-*O*,*O*′-isopropylidene derivatives; moreover **23**, by catalytic hydrogenation, gave the corresponding 5,6,11,12,13,14-hexahydro-derivative.

Assayed by leaf-puncture bioassay at 10^{-2} M on annual flaebane (*Erigeron annuus* L.), the host plant, as well as on some weedy and cultivated non-host plants at $3–4 \times 10^{-3}$ M, putaminoxin (**33**) proved to be the most toxic compound among putaminoxin analogs and derivatives. Putaminoxin natural analogs (**34–38**) and the 6,7-dihydro, 5-*O*-acetyl and 5-*O*-formyl (**36**) derivatives, were all inactive due to a modification of the nonenolide ring and the alkyl side chain. On the basis of these results the structural features which appeared of primary importance for the phytotoxic activity of putaminoxin were the presence of both unchanged hydroxy group at C-5 and alkyl side chain at C-9, as well as their stereochemistry. In addition, the functionalization

and conformational freedom of the nonenolide ring are also important (Evidente *et al.*, 1998b).

Many of these structural features appear to be important for the activity of pinolidoxins. Pinolidoxin (**23**), compared at 3×10^{-3} M to its analogs and derivatives, showed the highest phytoxicity on plants, both cultivated (pea and sugar beet) and weeds (showy crotalaria, fat hen and buttercup oxalis). Its natural analogues (**24–26**) and the 7,8-*O*,*O*′-diacetyl or the 7,8-*O*,*O*′-isopropylidene derivatives showed low or total loss of phytoxicity. However, 5,6,11,12,13,14-hexahydropinolidoxin showed the same strong activity as **23** on all tested plant species, probably due to the increased lipophilicity of this derivative, which could affect cell permeability. Therefore, also for the activity of pinolidoxin, primarily important features are the presence of an unmodified diol system between C-7 and C-8 with the correct stereochemistry and the functionalization and the conformational freedom of the nonenolide ring. The hexadienoyloxy residue at C-9 did not affect the activity (Evidente *et al.*, 1998b).

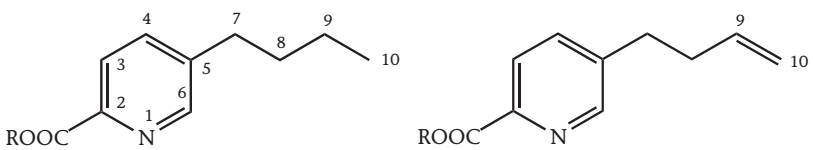
In view of its possible use as natural herbicide and fungicide, we assayed the zootoxic and the antifungal activity of both toxins (**23** and **33**) and some of their analogues and derivatives. On *G. candidum*, none of the compounds tested at 100 μg/disk (**33–37** and 6,7-dihydroputaminoxin and **24–26**) proved to be toxic, except **33**, which showed a weak toxicity (Evidente *et al.*, 1998b). With regard to the correlation between the structure and the toxicity on larvae of *A. salina*, the two toxins (**33**) and (**23**), their respective analogues (**34**, **35**, **37** and **24–26**), and three derivatives of **23** cited above, were tested in the $1–4 \times 10^{-4}$ M range. Only analogues **24–26** and 7,8-*O*,*O*′-diacetyl or the 7,8-*O*,*O*′-isopropylidene and the 5,6,11,12,13,14-hexahydro derivatives of pinolidoxin showed toxicity. In particular, the most toxic proved to be the 7,8-*O*,*O*′-diacetyl or the 7,8-*O*,*O*′-isopropylidene derivatives for their highest lipophilicity. This allows the two derivatives to enter the membrane of the larvae and then they are most likely activated by the hydrolysis of the protective group of the diol system between C-7 and C-8 (lethal metabolism). An indirect support comes from the 5,6,11,12,13,14-hexahydropinolidoxin which proved to be highly toxic because the hydrogenation of **23** renders the molecule highly lipophilic. This result, as well as the reduced toxicity shown by analogues **24–26**, should demonstrate that the toxicity on *A. salina* can be attributed, as invoked for the phytotoxicity, to the integrity of both the 7,8-diol system and the nonenolide ring of pinolidoxin. Modification of the side chain at C-2 does not affect any activity.

In conclusion, studies on structure-activity relationships provide useful information on the variability of biological properties, with respect to the chemical structure or presence/absence of active group/chain, also, in view of a possible use of natural compounds as pesticides, and on the possible chemical transformation of these compounds into more active or safer metabolites. From this viewpoint, putaminoxin (**33**), putaminoxin C (**38**) (Evidente *et al.*, 1997d), pinolidoxin (**23**) and its 5,6,11,12,13,14-hexahydro

derivative appear to be the most attractive. The availability of these metabolites (mainly of putaminoxin and pinolidoxin which appeared the most interesting nonenolides) in larger amounts, thank to optimize cultural conditions or use of large-scale production systems, as well as to their total synthesis now in progress (De Napoli *et al.*, 1998), could allow testing of these toxins in greenhouse or field experiments to evaluate their potential practical applicability, and to produce new and original modified natural compounds (Evidente *et al.*, 1998b).

12.3.2 *Fusarium* toxins

Striga hermontica (Del.) Benth, commonly called witchweed, is a parasitic weed which causes severe losses in many important cereal crops, mainly in sorghum, corn, millet, rice and sugar cane. The loss of grain-sorghum yield due to striga infestation may reach up to 70% and in cases of severe infestation there may be no yield at all. *S. hermonthica* is still very difficult to control, even using herbicides and fertilizers, cultural methods and resistant-crop varieties. Recently, Abbasher and Sauerborn (1992) reported a survey of microorganisms which are pathogenic to *S. hermonthica* and suggested that these could be useful for biologically controlling this weed. Among these, *Fusarium nygamai* Burgess and Trimboli, which causes large leaf and stem necrosis on the host, proved to be particularly promising. From the acid organic extract of culture filtrates, the main phytotoxins were identified, using an essentially spectroscopic method (^{1}H- and ^{13}C-NMR and FAB and EIMS), as fusaric and 9,10-dehydrofusaric acids (**39** and **41**, Figure 12.10 and Table 12.2). With respect to the free acids, the corresponding methyl esters (**40** and **42**, Figure 12.10 and Table 12.2) were also isolated for the first time as naturally occurring compounds at very low level (Capasso *et al.*, 1996). Fusaric acids (**39** and **41**) have already been described as toxic metabolites produced from other species of *Fusarium* (Turner, 1971; Turner and Aldridge, 1983; Luz *et al.*, 1990; Abraham and Hensenn, 1992). When assayed on tomato leaves and seedlings at 10^{-3} and 10^{-4} M, fusaric acids (**39** and **41**) and their methyl esters showed wide chlorosis evolving into necrosis as well as a strong root elongation, respectively. When assayed on brine shrimps at the same concentrations, both fusaric acids showed no zootoxic activity, while their methyl ester



39 R=H **40** R=CH_3 **41** R=H **42** R=CH_3

Figure 12.10: *Fusarium nygamai* toxins.

had a toxicity level of 50% expressed as mortality (Capasso *et al.*, 1996). The phytotoxic properties of fusaric acids were further investigated using biological assay on striga plants, seedlings, leaves and seeds, in order to test their possible use as natural herbicides. The application of very low amounts of toxins (10^{-6} M) caused a dramatic reduction in seed germination, while on punctured leaves, caused the appearance of large necrotic spots. The use of these metabolites against striga, possibly in combination with other cultural and biological methods, could be of assistance in controlling this noxious weed (Zonno *et al.*, 1996).

12.3.3 *Melanconis* toxins

Melanconis thelebola (Fr.) Sacc., later designated *Melanconis marginalis* (Peck) Wehmeyer (Doworth *et al.*, 1996), is the causal agent of cankers on the trunk and branches of many alder species (*Aldus cordata*, *A. glutinosa*, *A. incana* and *A. rubra*). The fungus induces extensive dieback of the crown, both in forest and spontaneous broadleafs such as *A. rubra* Bong. This species can be an important obstacle to successful reforestation programs, because of its rapid and unwanted growth. Therefore, the phytotoxins produced *in vitro* by *M. thelebola*, have been characterised with the aim of using them as natural herbicides. From the acid organic extracts of culture filtrates, the main toxin was purified and identified, using essentially spectroscopic methods (^{1}H- and ^{13}C-NMR and CI and EIMS), as the 3-nitropropanoic acid. The structure of the toxin was confirmed by preparing the corresponding methyl ester (Evidente *et al.*, 1992). The 3-nitropropanoic acid (Table 12.2), well known as toxin produced from some fungi including *Aspergillus* and *Penicillium* (Bush *et al.*, 1951; Raistrick and Stossl, 1958; Cole and Cox, 1981), is structurally related to the 3-methylthiopropionic acid, the toxic metabolite produced by the phytopathogenic bacteria *Xathomonas campestris* pv. *manihotis* (Perreaux *et al.*, 1982). When assayed on tomato cuttings and alder leaves at 2.5×10^{-2} M, 3-nitropropanoic acid caused a collapse of the petioles accompanied by complete wilting and colored necrosis, respectively (Evidente *et al.*, 1992). Experiments in the field have been made by Dr. C.E. Doworth, Pacific Forest Centre, Victoria B.C. Canada, to test the toxin as a potential herbicide against the host-plant (Doworth *et al.*, 1996).

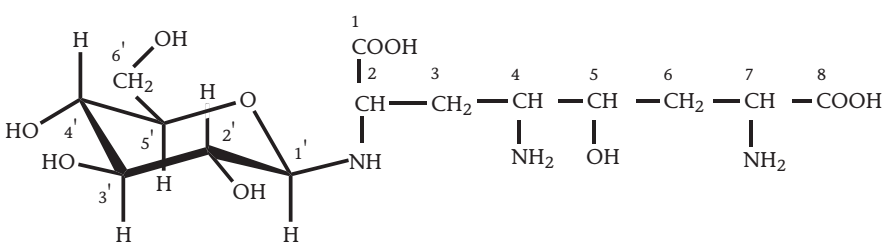
12.3.4 *Ascochyta caulina* toxins

The perthotrophic fungal species *Ascochyta caulina* (P. Karst.) v.d. Aa and v. Kest., has been proposed as a mycoherbicide against *Chenopodium album* (Kempenaar, 1995), also known as common lambsquarter or fat hen, a common world-wide weed of many arable crops such as sugar beet and maize (Holm *et al.*, 1977). The application of pycnidiospores of the fungus to *C. album* plants causes the appearance of large necrosis of leaves and stems and, depending on the amount of necrosis developed, plants show retarded growth or death.

Considering that *A. caulina* belongs to a well-known toxin-producing genus (Strange, 1997), and the possible use of fungal toxins as an alternative, or in addition to the use of pathogens in weed biocontrol (Strobel *et al.*, 1991), it seemed of interest to ascertain the production of toxic metabolites by *A. caulina*, and carry out their isolation, and chemical and biological characterization.

The culture filtrate of *A. caulina*, showing high phytototoxicity on leaves and cuttings, both of host and non-host plants, was examined to ascertain the chemical nature of the phytotoxic metabolites. They proved to be hydrophilic substances because they remained in the aqueous phase after exhaustive extraction was carried out on the culture filtrates with organic solvents of increasing polarity. The phytotoxic metabolites had a molecular weight lower than 1000 Da as deduced from dialysis experiments. These results prompted the purification of the crude culture filtrate by gel filtration. From a Biol-Gel P-2 column, eluted with ultrapure water, three phytotoxic fractions were obtained, one of which was a homogeneous compound, named ascaulitoxin (**43**, Figure 12.11 and Table 12.2).

Assayed on fat hen at 30 µg/droplet in the leaf-puncture assay, ascaulitoxin caused the appearance of necrotic spots surrounded by chlorosis. Particularly relevant in size was necrosis on sugar beet (*Beta vulgaris* L.). Clear necrosis also appeared, both on some weeds and on cultivated plants. Still clear, but of reduced size, were necrosis on tomato (*Lycopersicon esculentum* Mill.) and redroot pigweed (*Amaranthus retroflexus* L.). The speed of symptom development varied between two and five days, depending on the kind of leaves. Assayed on young fat hen cuttings at 8×10^{-4} M, the toxin caused the necrosis of cotyledons, starting from their edges. On young tomato cuttings, ascaulitoxin caused a clear marginal necrosis of cotyledons, and inhibited the production of adventitious roots, which appeared very well developed into the control. At 10^{-4} M the toxin caused 57 and 39% reduction of root elongation of fat hen and tomato seedlings, respectively. On the contrary, assayed up to 100 µg/disc on fungi (*G. candidum*) as well as on bacteria (*Pseudomonas syringae* and *E. coli*), ascaulitoxin showed no antimicrobial activity (Evidente *et al.*, 1998c).



43

Figure 12.11: *Ascochyta caulina* toxin.

The structure determination of ascaulitoxin was performed using spectroscopic and chemical methods. The chromatographic behaviour of the toxin and the product of its acid hydrolysis suggested the presence of a non-proteigenic amino acid and that of a glycid residue in **43**. This was consistent with the absence of absorption maxima in the UV spectra and with the NMR data. 1D and 2D ^{1}H- and ^{13}C-NMR studies showed the presence of a β-glucopyranosyl residue as well as that of the 2,4,7-triamino-5-hydroxy-octandioyl residue. However, the anomeric proton and carbon chemical shifts, compared to that of β-glucopyranoside (Breitmaier and Voelter, 1987; Agrawal, 1992) appeared significantly upfield shifted at a chemical shift value typical of β-*N*-glucopyranosides (Cuberta *et al.*, 1987; Laschat and Kunz, 1991). On the basis of these results, molecular weight of 397 and the molecular formula of $C_{14}H_{27}N_3O_{10}$ (measured by FAB mass spectrometry and from the HR-EI mass data of its penta-and hexa-acetyl derivatives), it is possible to hypothesize that ascaulitoxin is a glucopyranoside of the unusual 2,4,7-triamino-5-hydroxyoctandioic acid. The glycosylation site in **43** appeared to be the NH on C-2, as deduced from the COSY and HMBC data recorded in D_2O. The structure **43** assigned to ascaulitoxin was confirmed by chemical and spectroscopic methods as FAB MS spectrum. The methylalditol analysis confirmed that the sugar residue is glucose, which proved to be the D-stereomer from the results obtained in the GLC-MS analysis of the octanolyl hemiacetal derivative (Leontin *et al.*, 1978).

Considering that the presence of a stable hydrogen bond between the C-4 NH_2 and the C-5 hydroxy group does not allow their derivatization, the acylation is expected on the α amino group at C-2 and/or at C-7. In fact, **43** was converted in the two pentacetyl derivatives, having acylated the amino group at C-2 (the main product) or C-7, and a hexacetyl derivative, showing both amino group acetylated. Their ^{1}H-NMR spectra were very similar and all showed the expected downfield shifts of the geminal protons of the acetylable hydroxylated glucopyranosyl carbons, but differed for the allocation of the remaining acetyl group(s). This is confirmed by HR-EI MS and FAB MS data.

Therefore, ascaulitoxin may be formulated as N^2-β-D-glucopyranoside of the 2,4,7-triamino-5-hydroxyoctandioic acid (Evidente *et al.*, 1998c). We are planning the stereochemistry determination at C-2, C-4, C-5 and C-7 by a stereoselective synthesis of the toxin.

The finding of an *N*-glucoside of an atypical *bis*-amino acid is not surprising because nonproteigenic amino acids frequently occur as free or peptide components of animals, high plants, algae, and microorganisms, including fungi (Jones, 1969; Bycroft, 1970, 1971; Bycroft and Higton, 1985). Many of them have very unusual structures and interesting biological properties such as antibiotic and fungicide activities (Jones, 1969; Bycroft, 1970, 1971; Bycroft and Higton, 1985). Considering its interesting phytotoxicity on *C. album*, and the lack of activity against fungi and bacteria, further studies are in progress on the role of ascaulitoxin in the plant disease and on the mechanism of action. These aspects are important because the toxin could be used indirectly as a bio-

marker, for the improvement of *A. caulina* as mycoherbicide. In fact, if the toxin is a virulence factor, then more virulent strains of the pathogen could be more easily found, selecting the most toxigenic fungi. Moreover, these studies could permit the evaluation of the possible direct use of the metabolite as a natural herbicide, either in combination with toxic metabolites present in the culture filtrate of *A. caulina*, or with the pathogen itself, as well as with other control methods in the integrated weed management approach. In fact, considering that the toxin alone does not seem to be able to penetrate into the leaf, leaf applications of the toxin in combination with the pathogen (which otherwise is very specific) could result in a selective treatment. The pathogen could be helped by the toxin, during the penetration and colonization stage, to increase the disease level on the weed, without any effect on the non-host plant (Evidente *et al.*, 1998c). In this regard, the isolation and chemical characterization of the other toxic metabolites present in the culture filtrates of *A. caulina* are still in progress.

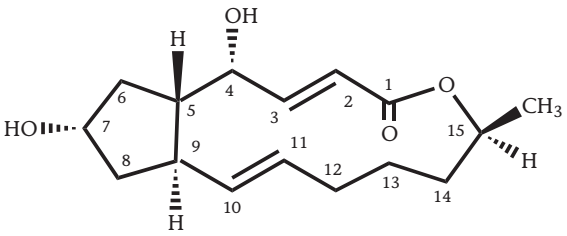
12.3.5 *Alternaria zinniae* toxins

Xanthium occidentale Bertol. is an exotic annual weed largely spread in Australia. Among the four species belonging to the genus *Xanthium* (namely, *X. occidentale*, *X. orientale* L., *X. italicum* Mor. and *X. cavanillesii* Schouw.), which constitute the Noogoora burr complex, *X. occidentale* was the first to be reported in Australia, and is becoming the most destructive and widespread species (Morin *et al.*, 1994).

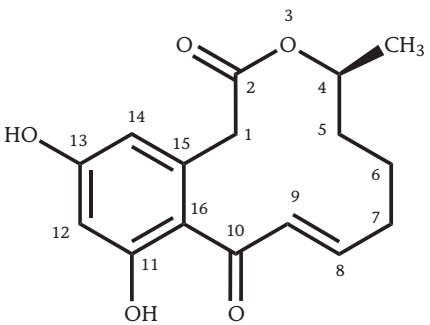
The biological control of this noxious weed, mainly with the use of plant pathogens has been proposed, using the classical and the inundative approach. In the classical approach, the particularly promising use of the rust *Puccinia xanthii* Schw. has been proposed since 1973 (Hasan). In the mycoherbicide or inundative approach, *Alternaria zinniae* has been considered a very good candidate, also to control *X. italicum*. In fact, the quantitative application of conidia suspensions caused the fast appearance of necrosis on host leaves (Morin *et al.*, 1996). Moreover, recently, in exploring new opportunities in the integrated weed management, possible additive or synergistic effects due to the interactions between rust and some facultative parasitic fungi have also been considered, mainly with regard to the use of *P. xanthii* and *Colletotrichum orbiculare* (Nehl and Brown, 1992; Auld *et al.*, 1997).

The genus *Alternaria* includes many species which are able to produce a wide array of secondary toxic metabolites belonging to several classes of chemicals, and possessing different biological activities, such as phytotoxicity, zootoxicity, cytotoxicity (Montemurro and Visconti, 1992). Moreover, most of the known host-specific fungal phytotoxins are produced by *Alternaria* species, and their structure and biological activities have been widely reviewed (Nishima and Nakatsuka, 1988).

Considering the possible use of fungal metabolites in weed biocontrol as more natural and safe herbicides (Strobel *et al.*, 1991), it seemed of interest to investigate the



44



45

Figure 12.12: *Alternaria zinniae* toxins.

possible production of bioactive metabolites by a strain of *A. zinniae* and their toxicity mainly toward *X. occidentale*, the host plant. The culture filtrates of *A. zinniae* were exhaustively extracted with $CHCl_3$ to give a brown oil having a high phytotoxic activity on host and non-host plants using the leaf puncture assay. The crude extracts were fractioned by column and preparative TLC chromatography to give the two main toxic metabolites (**44** and **45**, Figure 12.12 and Table 12.2). The structure of the two metabolites was determined by extensive application of spectroscopic methods (as 1D and 2D ^{1}H- and ^{13}C-NMR and HR-EIMS). The structure and the configuration of the two toxins have been confirmed by X-ray analysis.

From all the above data, **44** appears to be identical to brefeldin A, the macrocyclic antifungal cytotoxic and antiviral antibiotic independently isolated from microscopic fungi (including *Penicillium* and *Ascochyta* spp.) and also named decumbin, cyanein, ascotoxin and sinergisidin (Betina, 1992). Brefeldin A was also isolated from *Alternaria carthami* (Tietjen *et al.*, 1983), and *Curvularia lunata* (Coombe *et al.*, 1968). Similarly,

from all of the above data, **45** appears identical to the α,β-dehydrocurvularin, the octaketide lactone produced by a number of fungal species (*Penicillium*, *Curvularia*, *Cercospora* and *Stemphylium* spp.) (Caputo and Viola, 1977; Robeson and Strobel, 1981; Arai *et al.*, 1989; Lai *et al.*, 1989). In fact, **45** showed physical (mp, $[\alpha]^{25}D$) and spectroscopic (UV, IR, and the very few reported EIMS) properties and ^{13}C- and the partial ^{1}H-NMR data similar to those of α,β-dehydrocurvularin as reported in Munro *et al.* (1967) and Arai *et al.* (1989), respectively. This is the first report on the production of these two phytotoxins by a strain of *A. zinniae* — a good candidate for the biological control of *X. occidentale* with the inundative approach (Vurro *et al.*, 1998).

Even if some toxic properties of both compounds have already been reported (Suzuki *et al.*, 1970; Tietjen *et al.*, 1983; Robeson and Strobel, 1985; Betina, 1992), some aspects are new and seem to be of interest for a practical approach. Tietjen *et al.* (1983) demonstrated that brefeldin A was particularly active against species belonging to the *Asteraceae* family and only on two out of the twenty-two non-*Asteraceae* species tested. In this respect, in Vurro *et al.* (1998) the higher sensitivity of *X. occidentale* (belonging to the *Asteraceae*) to brefeldin A is also particularly noteworthy from the application point of view. In fact, the application of droplets containing around 0.3 μg of toxin caused the faster appearance of wider necrotic spots, both on host leaves and cotyledons. In contrast, the effect of this metabolite at the tested concentration on other non-host plants is lower or nil. Moreover, the toxin also caused severe necrosis when it was applied on host leaves and cotyledons without puncture, which was not observable for non-host plants. This unusual observed effect suggests the use of spray application. The very good activity of the culture filtrate against *Xanthium* plants would suggest its possible direct application for control, and also on account of its easy production by fungal fermentation and the fast symptom appearance (Vurro *et al.*, 1998). Further studies are under way to evaluate the possible direct use of *A. zinniae* culture filtrate and metabolites.

In the puncture assay, the effects caused by the culture filtrate of the fungus were always stronger, with respect to those caused by brefeldin A, α,β-dehydrocurvularin or both-toxins solutions. Considering that the used concentrations of brefeldin A and of α,β-dehydrocurvularin (10^{-4} and 10^{-3} M, respectively) were higher compared to their concentrations in the culture filtrate ($<10^{-5}$ and around 10^{-4} M, respectively), there must be other active metabolites in the culture filtrate which could have a sinergistic effect with brefeldin and α,β-dehydrocurvularin. Investigations are in progress for chemical and biological characterizations of still-toxic metabolites.

Recently, Vurro and Ellis (1997) showed that some fungal toxins, applied at a concentration which caused no macroscopic toxic effects, are able to suppress phenylalanine ammonia lyase induction, which can be one of the first steps in the mechanism of defence of plants from pathogen attack. Tietjen *et al.* (1983) also suggested that brefeldin A could be involved in the suppression of the defence mechanism of

Carthamus tinctorium from the pathogenic fungus *Alternaria chartami*. Thus, a suitable application could be the use of brefeldin A, at very low concentrations in a mycoherbicide suspension, together with *A. zinniae* conidia to block the defence reaction of *X. occidentale* and help the pathogen to cause a more severe disease, and hence to obtain a better control of the weed. Moreover, brefeldin A and α,β-dehydrocurvularin could also indirectly improve the mycoherbicide production, if used as biomarkers. As described above, if these toxins are virulence factors, which means that there may be a positive correlation between toxin production and aggressiveness of the candidate mycoherbicide, more virulent strains of the pathogen could be selected, simply by testing the *in-vitro* production of toxic metabolites (Vurro *et al.*, 1998).

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CHAPTER

13

Limonoids from Meliaceae and their Biological Activities

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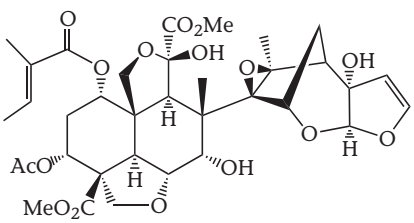
Contents

13.1 INTRODUCTION

The order Rutales, including the families Rutaceae, Meliaceae, Simaroubaceae and Cneoraceae, is among the richest and most diverse sources of secondary metabolites in the Angiospermae. Almost every part of the trees, leaves, bark and seeds, have long been used for medicinal and plant protection purposes. The most characteristic metabolites of the Rutales are limonoids, which are tetranortriterpenoids derived from tirucallane (H-20α) or euphane (H-20β) triterpenoids with a 4,4,8-trimethyl-17-furanylsteroidal skeleton. They have also attracted considerable interest because of their fascinating structural diversity and their wide range of biological activity. In particular, they characterize members of the family Meliaceae, where they are abundant and varied.

The family Meliaceae is known to contain about 1400 species. The neem tree, *Melia azadirachta indica* A. Juss, is part of Indian folklore and one of the meliacean species, which has interested many laboratories for a long time because of the variety of structures and biological activities of limonoid constituents. Most of the results have been presented in the international neem conferences (Schmutterer and Ascher, 1987). Azadirachtin, and related highly oxidized limonoids isolated from the neem seeds are the most potent insect antifeedants and their chemistry has been reviewed (Ley *et al.*, 1993).

On the other hand, the Chinaberry tree *Melia azedarach* Linn. is a native of Persia, India and China, but is naturalized in a number of continents including Africa, Australia and the Americas. Thus, the constituents of this tree from many regions have also been well studied. *M. azedarach* is known to produce azadirachtin-type limonoids such as the meliacarpinins, and C-19/C-29 bridged lactols, and its acyl acetals, such as azedarachins and trichilins, which are also potent antifeedants (Huang *et al.*, 1995). In this paper, I intend to review mainly the limonoid constituents of *M. azedarach* and the closely related tree *Melia toosendan*. Their antifeedant properties will also be discussed.



Azadirachtin

Figure 13.1: Azadirachtin.

13.2 BIOSYNTHESIS OF LIMONOIDS

Limonoids are thought to be derived from the tetracyclic triterpenoids tirucallane (20-*S*, H-20α) or euphane (20-*R*, H-20β). Many triterpenoids with the same ring structure have been observed in meliacean plants. Modification of the parent triterpene skeleton takes the form of oxidation, often with ring fission and recyclization involving any of the four rings, to give heterocyclic systems. According to the generally accepted scheme, the Δ^7-bond is epoxidized to a 7-epoxide, which is then opened, including a Wagner-Meerwein shift of the 14-Me to C-8, formation of the 7-OH, and introduction of a double bond at C-14/C-15.

Cyclization in the side chain most commonly proceeds through oxidation of the C-21 methyl and leads to a 21/23 or, more rarely, 21/24 oxides, from which the 17α-furan ring is formed with the loss of four carbons — a possible pathway is demonstrated in Figure 13.2. The last step is accomplished after formation of the 4,4,8-trimethylsteroid skeleton, as indicated by the occurrence of several protolimonoids with an intact C-8 side chain.

Azadirone (**2**), first isolated from *M. azadirachta* (Lavie and Jain, 1967), is a representative member of the limonoids (meliacins) in which four carbocyclic rings remain intact. Many meliacins also show the introduction of a 14,15β-epoxide, such as in acetyltrichilenone (**4**) and 6β-acetoxytrichilenone (**5**).

The wide variety of structural types found among the limonoids is generally the product of further oxidative ring opening and skeletal rearrangement (Figure 13.3). The rings A and D are sometimes oxidized to lactones (A and D-seco limonoids) by Baeyer-Villiger oxidation of the C-3 and C-16 keto groups. A,D-seco limonoids are found in all families of Rutales, especially in the Rutaceae. Rearrangement of the ring A leads to the formation of typical limonoids such as limonin, which is shown in Figure 13.4.

In the Meliaceae the degree of complexity in limonoid structures becomes greater, with fission of rings B and C occurring widely, as well as the rings A and D modifications mainly found in the Rutaceae. In particular, synthesis of the C-seco limonoids occurs only in the Meliaceae belonging to the tribe Meliaceae, in which they appear to be restricted to the genera *Melia*. The biosynthetic origin of the C-seco limonoids is less clear, because there is controversy about the mechanism by which the C-12/C-13 bond of the C-ring is opened. The series of sendanal (**10**), ohchinal (**47**), nimbolins (**52–56**) and nimbolidins (**60–65**) illustrates one possible process (Taylor, 1984) (Scheme 13.1) involved, which includes introduction of an oxygen function at C-12, followed by oxidation to a ketone. Cleavage of the C-12/C-13 bond is accompanied by the simultaneous opening of a 14,15-epoxide to generate the CHO-12 and allylic 15-OH functions as found in ohchinal (**47**). Rotation about the C-8/C-14 bond would allow the 15-OH to recyclize with CHO-12 to form a lactol C-ring as in **52–56**. Meliacarpinins (**69–78**), one of the most potent insect antifeedants from *M. azedarach*, are highly oxidized natural products of the C-seco class, similar to the azadirachtins. 1-Cynnamoylmelianolone (**68**) (Lee *et al.*, 1987), found in the fruits of the American variety, may be a precursor to the meliacarpinins.

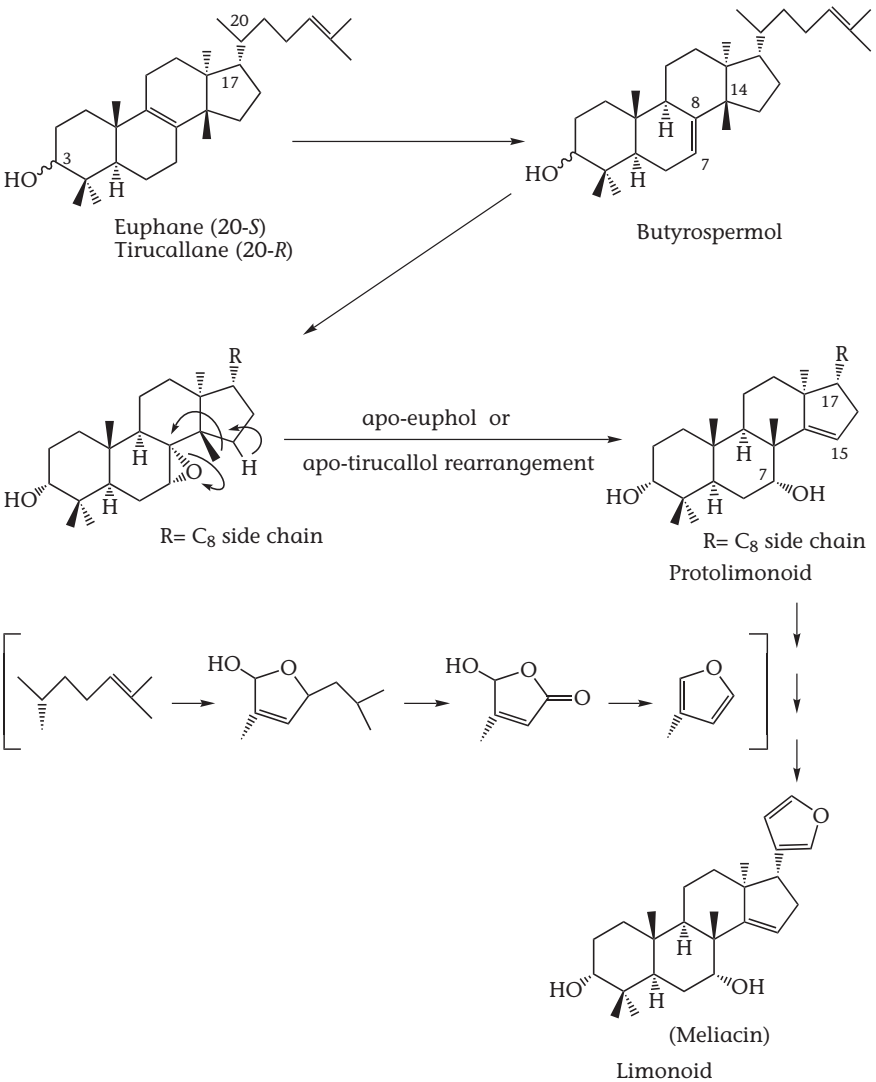
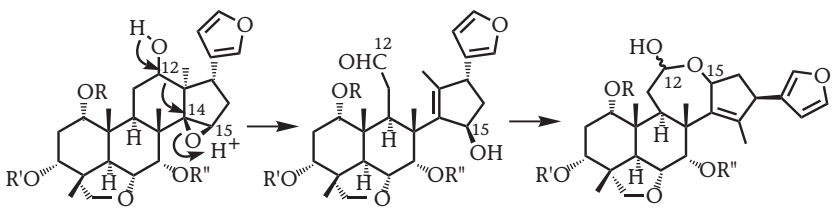


Figure 13.2: Biosynthetic pathway leading to the formation of a simple limonoid. (Modified from the scheme by Siddiqui *et al.*, 1988.)



Scheme 13.1: Formation of ring C-seco limonoids.

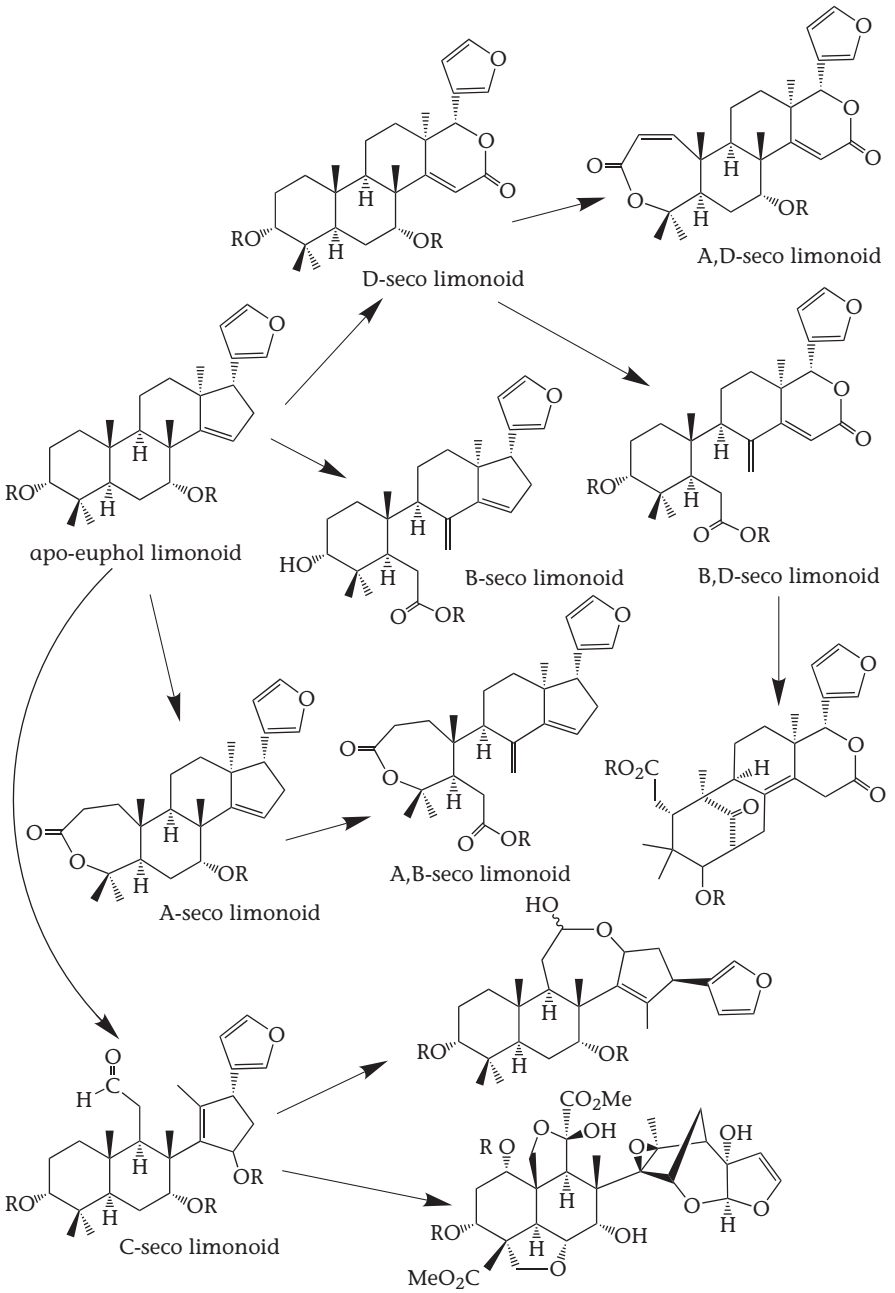


Figure 13.3: Major biosynthetic routes of limonoids. (Modified from the scheme by Champagne *et al.*, 1992.)

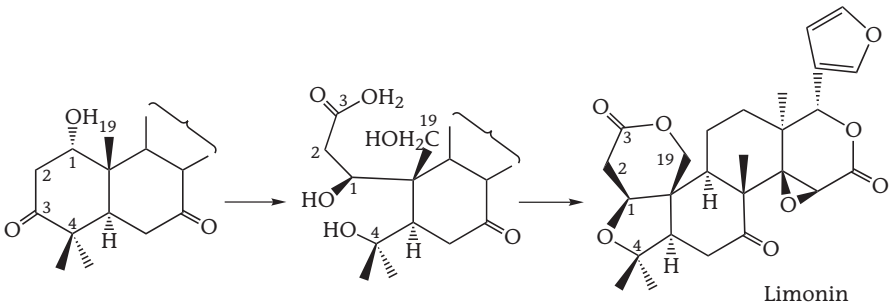


Figure 13.4: Fission of ring-A and formation of limonin.

Commonly, the B-seco compounds are observed in nature accompanying a fission of any of the rings A and D of the original limonoid skeleton. Rohitukin and methyl angolensate are typical compounds and the latter may be extensively rearranged to yield a complex phragmalin-type limonoid such as busseins from *Entandrophragma bussei* and *E. caudatum* (Haenni and Tamm, 1972; Haenni *et al.*, 1975).

13.3 BIOLOGICAL ACTIVITY OF LIMONOIDS

Many limonoids have a bitter taste. A number of Citrus juices develop a bitter taste gradually after the immediate bitterness due to the flavanone glycosides such as naringin. Limonin is found to be the causative factor, but structural features required for bitterness remain obscure. The wide occurrence of the limonoids in nature has evoked considerable interest in their biological activity. Recent work has established a wide range of biological activities for these compounds, including insecticidal, insect antifeedant and growth-regulating properties, a variety of medicinal effects in animals and humans, and antifungal, bactericidal, and antiviral activities. The range of biological

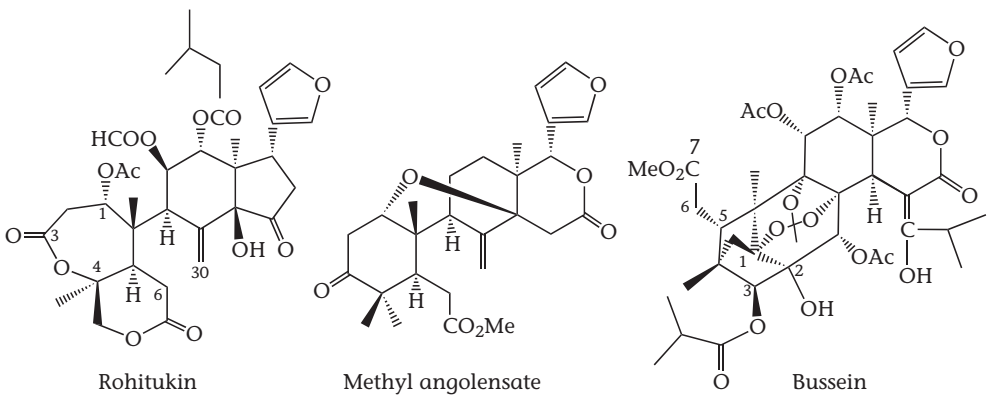


Figure 13.5: Some B-seco limonoids.

activity encountered in the limonoids has been reviewed (Champagne *et al.*, 1992).

Several species of Meliaceae have a history of use in traditional medicine for the treatment of cancer. The growth of the very representative P388 lymphocytic leukemia cells was exhibited by a number of limonoids, which were 19/29-bridged lactols and their 29-acyl derivatives with a 14,15-epoxide. 12-Hydroxyamoorastatin (**17**) and aphanastatin (**35**) from *Aphanamixis grandifolia* and toosendanin (**18**) from *M. azedarach* and its 29-acetate sendanin exhibited the activity of ED_{50} = 0.02, 0.065 (Polonsky *et al.*, 1978a,b), 0.026 (Itokawa *et al.*, 1995) and 0.01 μg/ml (Polonsky *et al.*, 1979), respectively. However, amoorastatone (**37**), having a 15-keto group, was marginally inactive (ED_{50} = 30 μg/ml) (Polonsky *et al.*, 1979). Apparently the 14,15β-epoxide is a very definite requirement for inhibition of neoplastic (P388) cell growth. The azadirachtin-type meliacarpinins (**69–78**) from *Melia azedarach*, also showed a significant cytotoxic activity of ED_{50} = 1.5 ~ 100 μg/ml against P388 cells (Takeya *et al.*, 1996), but to a lesser degree than the above sendanin-type limonoids. Although these compounds were active *in vitro*, they have been proved impossible to demonstrate a use *in vivo* activity.

Recently, a series of A,B-seco limonoids rubrins A-G (Figure 13.6), with hemiortho ester A-ring, from *Trichilia rubra* (Musza *et al.*, 1994) were found to exhibit a potent inhibitory activity in the LFA-1:ICAM-1 mediated cell adhesion assay with IC_{50} values of 10–25 nM, which may have therapeutic potential as antiinflammatory and antimetastatic agents, because none of the compounds show cytotoxicity.

Azadirachtin is one of the major insect antifeedants isolated from neem seeds and exhibits the antifeeding activity at 1.7 and <1 ppm concentrations (ED_{50} = 1.7 and <1 ppm) against the larvae of voracious insects *Epilachna varivestis* (Rembold, 1989) and *Spodoptera littoralis* (Ley *et al.*, 1993), respectively. However, 1 ppm corresponds to the concentration of about 0.02 μg/ml. The structure/activity relationships of azadirachtin and its derivatives, against the insect, have been reviewed (Ley *et al.*, 1993) and an azadirachtin extract of the tree has been commercially exploited, to some extent. In addition to this effect, it shows insecticidal and growth-inhibiting effect against insects and is responsible for many disturbances of insect metamorphosis, mortality and

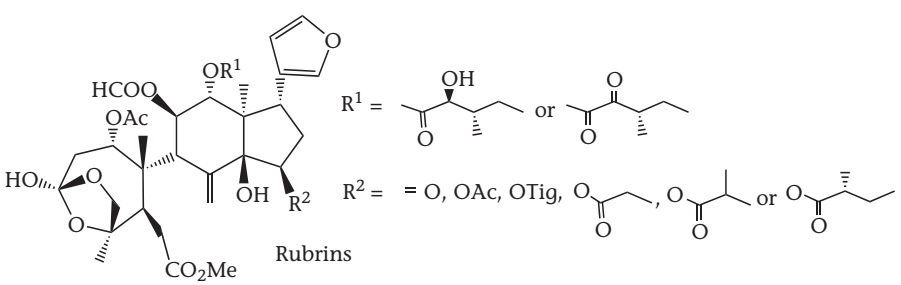


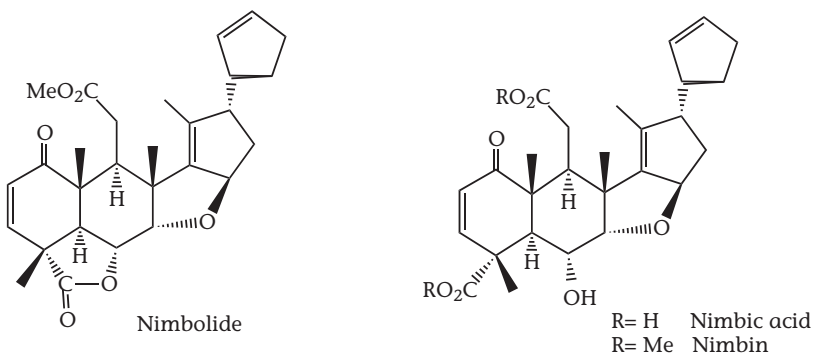
Figure 13.6: Cell adhesion inhibitors from *Trichilia rubra*.

decrease in fecundity. These aspects have been also reviewed in detail (Rembold, 1989; Rembold and Puhlmann, 1993). Recently, three meliacarpins (**79–81**) having the same ring structure with azadirachtin were isolated as potent insecticide against *S. littoralis* from the leaf of *M. azedarach* (Bohnenstengel *et al.*, 1999). The most active compound exhibited an ED_{50} of 0.48 ppm, which is comparable to that (ED_{50} = 0.32 ppm) of azadirachtin. Meliacarpinins (**69–78**), first isolated from *M. azedarach* by Nakatani *et al.* (1993), are also potent antifeedants (ED_{50} = 50 ppm). Aside from the azadirachtins and meliacarpinins, the most active antifeedants appear to be the 19/29-lactols described above. Structure-activity relationships in this class are described in detail in 13.6.

Some meliacean limonoids such as nimbolide, nimbic acid and nimbin (Figure 13.7) have been reported to have more ecological relevance, such as antifungal, bactericidal, and antiviral activities. The terpene lactone nimbolide inhibited *Penicilium falciparum* in culture with a moderate potency (ED_{50} = 2.0 μM) (Rochanakij *et al.*, 1985) and also exhibited antibacterial activity at 0.875 mg/disk against *Staphylococcus aureus*, *Bacillus subtilis* and *Staphylococcus* coagulase (plus and minus strains) (Rojanapo *et al.*, 1985). Nimbin inhibited potato virus X growth *in vitro* by <50% (Verma, 1974). Many other complex limonoids, including azadirachtin, trichilin and ruitukin are found to be inactive against *Echerichia coli* and some yeast, but the activity of nimbolide and nimbin suggests the possibility that some limonoids may function in defense against plant enemies other than insects.

13.4 LIMONOIDS FROM *MELIA AZEDARACH* AND *MELIA TOOSENDAN*

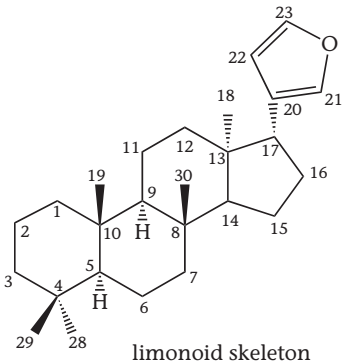
Limonoids may be found in all tissues of the plants, but different organs within an individual may produce different types of limonoids. *M. azedarach* and *M. toosendan* are large trees very similar to each other except that the fruit of *M. toosendan* is slightly bigger than that of *M. azedarach*, and they produce similar types of compounds. Since the initial isolation of azadirone (**2**) from the trunk wood of *M. azedarach* in 1967, about 60 limonoids have been isolated from the fruits, stem bark and root bark to-date.



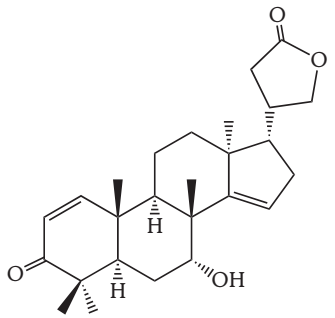
Nimbolide

R= H Nimbic acid
R= Me Nimbin

Figure 13.7: Antifungal, bactericidal and antiviral limonoids from *Melia azedarach*.

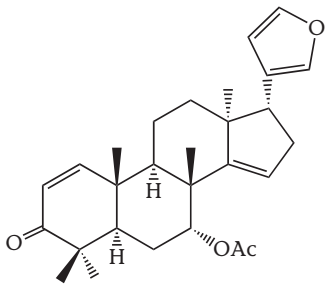


limonoid skeleton

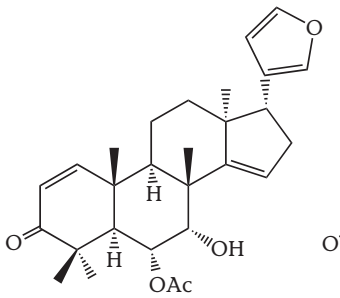


Tetranortriterpenoid (**1**)

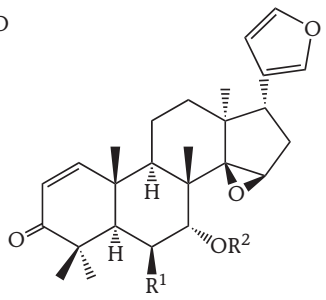
Group 1: Meliacins and derivatives



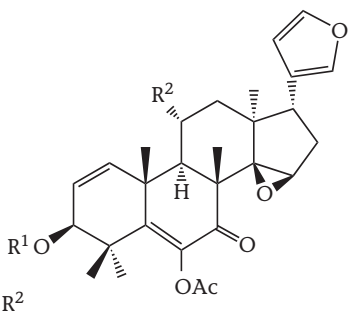
Azadirone (**2**)



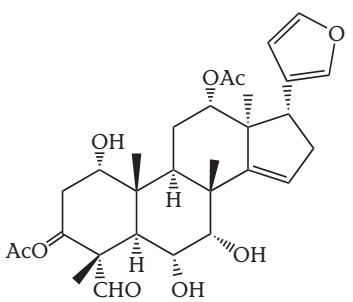
Meldenin (**3**)



R^1	R^2	
H	OAc	Acetyltrichilenone (**4**)
OAc	H	6β-Acetoxytrichilenone (**5**)



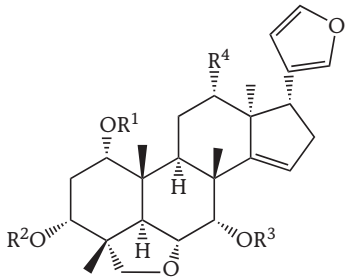
R^1	R^2	
β-D-Glu	H	6-Acetoxy-3β-hydroxy-7-oxo-14β,15β-epoxymeliac-1,5-diene 3-*O*-β-D-Glucopyranoside (**6**)
β-D-Xy	H	6-Acetoxy-3β-hydroxy-7-oxo-14β,15β-epoxymeliac-1,5-diene 3-*O*-β-D-Xylopyranoside (**7**)
α-L-Rham	OH	6-Acetoxy-3β,11α-dihydroxy-7-oxo-14β,15β-epoxymeliac-1,5-diene 3-*O*-α-L-Rhamnopyranoside (**8**)
β-D-Glu	OAc	6,11α-Diacetoxy-3β-hydroxy-7-oxo-14β,15β-epoxymeliac-1,5-diene 3-*O*-β-D-Glucopyranoside (**9**)



Sendanal (**10**)

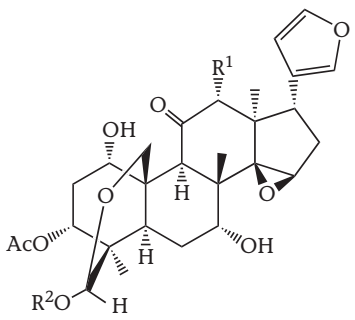
Figure 13.8: Limonoids from *M. azedarach* and *M. toosendan*.

Group 1: Meliacins and derivatives (continued)



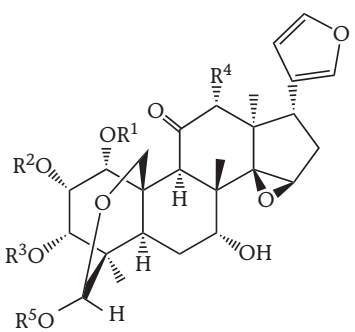
R^1	R^2	R^3	R^4	
Ac	Ac	Cin	H	Nimbolin A (**11**)
Tig	Ac	H	OAc	Trichilinin B (**12**)
Ac	Tig	H	H	Trichilinin C (**13**)
Cin	H	H	OAc	Trichilinin D (**14**)
Cin	Ac	H	OAc	3-*O*-Acetyltrichilinin D (**15**)
Bz	H	H	OAc	Trichilinin E (**16**)

19/29 bridged acetals



R^1	R^2	
OH	H	12-Hydroxyamoorastatin (**17**) (and 29-epimer)
OAc	H	Toosendanin (**18**) (and 29-epimer)
OH	X	Azedarachin A (**19**)
OAc	X	12-*O*-Acetylazedarachin A (**20**)
OH	Y	Azedarachin B (**21**)
Ac	Y	12-*O*-Acetylazedarachin B (**22**)
H	Y	Azedarachin C (**23**)

X=$COCH(CH_3)CH_2CH_3$
Y=$COCH(CH_3)_2$



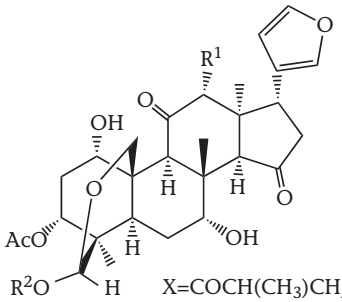
R^1	R^2	R^3	R^4	R^4	R^5	
H	Ac	Ac	β-OH	X		Trichilin A (**24**)
H	Ac	Ac	OH	X		Trichilin B (**25**)
H	Ac	Ac	OAc	X		12-*O*-Acetyltrichilin B (**26**)
Ac	Ac	Ac	OAc	X		1,12-di-*O*-Acetyltrichilin B (**27**)
H	Ac	Ac	H	X		Trichilin D (**28**)
H	Ac	Ac	OAc	Y		Trichilin H (**29**)
Ac	Ac	Ac	OAc	Y		1-*O*-Acetyltrichilin H (**30**)
H	H	Ac	OAc	X		Trichilin I (**31**)
H	H	Ac	H	X		Trichilin J (**32**)
H	H	Ac	H	Y		Trichilin K (**33**)
H	Ac	H	H	X		Trichilin L (**34**)
Ac	H	Ac	OH	X		Aphanastatin (**35**)
Ac	Ac	Ac	OH	Y		Meliatoxin A (**36**)

X=$COCH(CH_3)CH_2CH_3$
Y=$COCH(CH_3)_2$

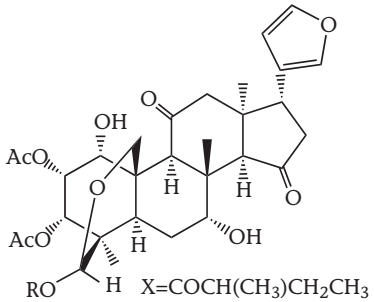
Figure 13.8: *(continued)*.

A variety of oxidation and skeletal rearrangements of the basic skeleton are found in the limonoids from *M. azedarach* and *M. toosendan* (Figure 13.8). A characteristic oxidation of the skeleton produces C-19/C-29 bridged acyl acetals with a 14,15-epoxide as in the azedarachins (**19–23**) and trichilins (**24–34**), or their D-ring keto compounds **37–44**. Furthermore, many C-seco limonoids **47–67**, which are major ring-seco limonoids of *M. azadirachta*, *M. azedarach* and *M. toosendan* in the family Meliaceae and mainly found in

Group 1: Meliacins and derivatives (continued)

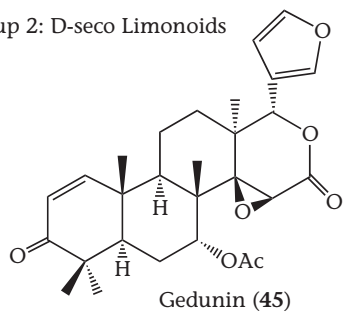


R^1	R^2	
H	H	Amoorastatone (**37**) (and 29-epimer)
OH	H	12α-Hydroxyamoorastatone (**38**) (and 29-epimer)
OAc	H	*Iso*-chuanliansu (**39**) (and 29-epimer)
H	X	Neoazedarachin A (**40**)
H	Y	Neoazedarachin B (**41**)
H	Me (endo)	Neoazedarachin D (**42**)

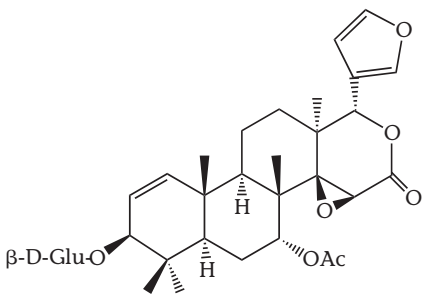


R	
X	Meliatoxin B_1 (**43**)
Y	Meliatoxin B_2 (**44**)

Group 2: D-seco Limonoids

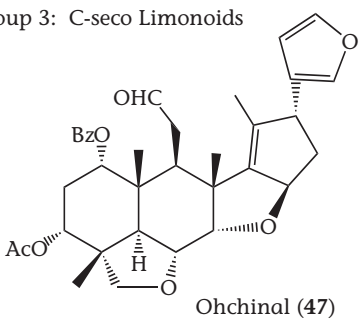


Gedunin (**45**)

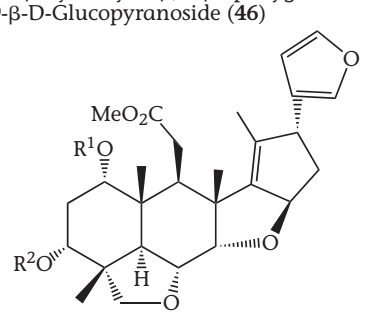


7α-Acetoxy-3β-hydroxy-14β,15β-epoxygedunan -1-ene 3-*O*-β-D-Glucopyranoside (**46**)

Group 3: C-seco Limonoids



Ohchinal (**47**)



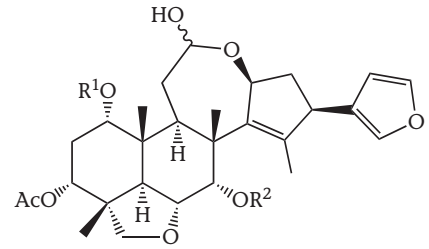
R^1	R^2	
Tig	Ac	Salannin (**48**)
Tig	H	Deacetylsalannin (**49**)
Cin	H	Ohchinin (**50**)
Cin	Ac	Ohchinin acetate (**51**)

Figure 13.8: *(continued)*.

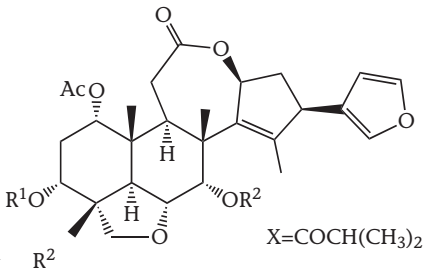
these three related species, are usually observed as CHO-12 and CO_2Me-12, and their lactols and lactones.

1-Cynnamoylmelianolone (**68**), meliacarpinins (**69–78**) and meliacarpins (**79–81**) are the more oxidized natural products of this class, similar to the azadirachtins. It is of

Group 3: C-seco Liminoids (continued)

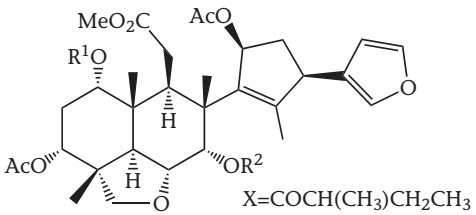


R^1	R^2	
Ac	Cin	Nimbolin B (**52**)
Ac	Bz	Nimbolinin A (**53**)
H	Bz	1-Deacetylnimbolinin A (**54**)
Ac	Tig	Nimbolinin B (**55**)
H	Tig	1-Deacetylbimbolinin B (**56**)



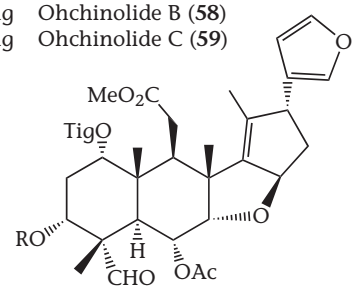
X=$COCH(CH_3)_2$

R^1	R^2	
Ac	Bz	Ohchinolide A (**57**)
Ac	Tig	Ohchinolide B (**58**)
X	Tig	Ohchinolide C (**59**)

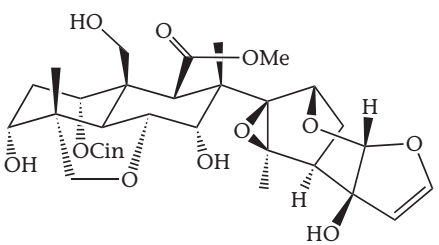


X=$COCH(CH_3)CH_2CH_3$
Y=$COCH(CH_3)_2$

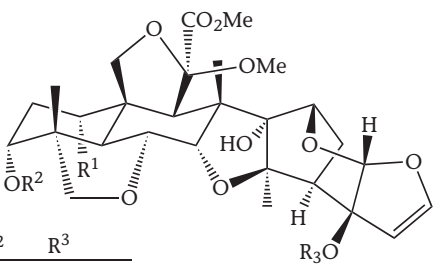
R^1	R^2	
Ac	Bz	Nimbolidin A (**62**)
Ac	Tig	Nimbolidin B (**63**)
Ac	Y	Nimbolidin C (**64**)
Tig	Tig	Nimbolidin D (**65**)
Tig	Y	Nimbolidin E (**66**)
X	Tig	Nimbolidin F (**67**)



R= H Ohchinolal (**60**)
R= Ac Acetylohchinolal (**61**)



1-Cinnamoylmelianolone (**68**)

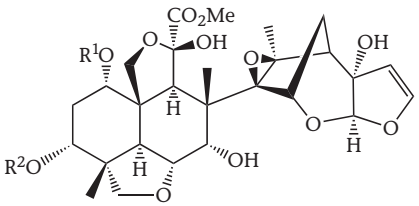


R^1	R^2	R^3	
OCin	Ac	H	Meliacarpinin A (**69**)
OCin	H	H	Deacetylmeliacarpinin A (**70**)
H	Tig	H	Meliacarpinin B (**71**)
OAc	Tig	H	Meliacarpinin C (**72**)
OH	Tig	H	Deacetylmeliacarpinin C (**73**)
OAc	Tig	Ac	20-*O*-Acetylmeliacarpinin C (**74**)
OTig	Ac	H	Meliacarpinin D (**75**)
OTig	Ac	Ac	20-*O*-Acetylmeliacarpinin D (**76**)
OH	Tig	H	Meliacarpinin E (**77**)
H	X	H	1-Deoxy-3-methacrylyl-11-methoxymeliacarpinin (**78**)

X=$COC(CH_3)=CH_2$

Figure 13.8: (*continued*).

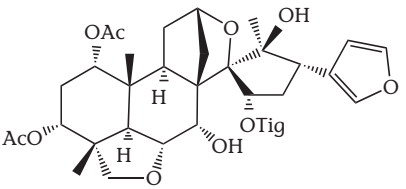
interest that none of the azadirachtin-type limonoids isolated from *M. azedarach* and *M. toosendan* possess a 4β-carboxylate like azadirachtin, but rather 4β-methyl group. The meliacarpins **69–78** differ from azadirachtin in that C-29 is not oxidized to a methoxy carbonyl group but is still present as an angular methyl. In addition, spirosendan (**82**), recently isolated from *M. toosendan* (Nakatani *et al.*, 1998a), is a novel spiro limonoid



R^1	R^1	
Cin	Cin	1,3-Dicinnamoyl-11-hydroxy-meliacarpin (**79**)
Cin	X	1-Cinnamoyl-3-methacrylyl-11-hydroxymeliacarpin (**80**)
Cin	OAc	1-Cynnamoyl-3-acetyl-11-hydroxymeliacarpin (**81**)

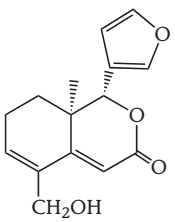
X = $COCMe=CH_2$

Group 4: Spiro Limonoid

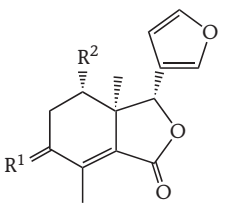


Spirosendan (**82**)

Group 5: Degraded Limonoids



Azedaralide (**83**)



R^1	R^2	
H_2	H	Fraxinellone (**84**)
H_2	OAc	12α-Acetoxyfraxinellone (**85**)
O	H	Fraxinellonone (**86**)

Figure 13.8: (*continued*).

possessing a C-12/C-30 bridged system. In limonoids from other origins, the rings A or D are commonly oxidized to lactones (A- or D-seco limonoids), but these derivatives have rarely been found in *M. azedarach* and *M. toosendan*. The exceptions are gedunin (**45**) (Ekong *et al.*, 1969) and its 3-glucosyl derivative (**46**) (Saxena and Srivastava, 1986).

In the frame of our studies on Meliaceae, four degraded limonoids **83–86**, exhibiting the ichthyotoxic activity (ED_{50} = 10–50 ppm) against a killifish, were isolated from *M. azedarach* collected at Guangzhou, China and at Okinawa, Japan (Nakatani *et al.*, 1998b). Degraded limonoids resulting from the biological degradation of limonoids are sometimes encountered in rutacean plants, but are rarely found in Meliaceae (Ekong *et al.*, 1969).

13.5 STUDY ON LIMONOID ANTIFEEDANTS OF *M. AZEDARACH* AND *M. TOOSENDAN*

We have investigated the limonoid constituents of *Melia azedarach* and the related meliacean plant, *M. toosendan*, and evaluated the antifeedant properties of more than fifty limonoids, which includes all of the types of limonoids isolated from *M. azedarach* and

M. toosendan, excluding glycosides. The structures and antifeedant activities of these compounds are detailed here.

13.5.1 Isolation of limonoids

The ether and methanol extracts of the root, stem bark, and fruits of Chinese *M. azedarach* and *M. toosendan*, contained a variety of limonoids which were detectable by the characteristic color upon treatment with Ehrlich's reagent on TLC. Limonoids from Meliaceae are commonly very sensitive to traces of acid and gradually decompose on a silica gel column. It was, therefore, necessary to use droplet countercurrent chromatography (DCCC), flash chromatography, preparative thin layer chromatography (prep TLC) and high performance liquid chromatography (HPLC) separation techniques. The isolation of the various congeners was accomplished as outlined in Figure 13.9.

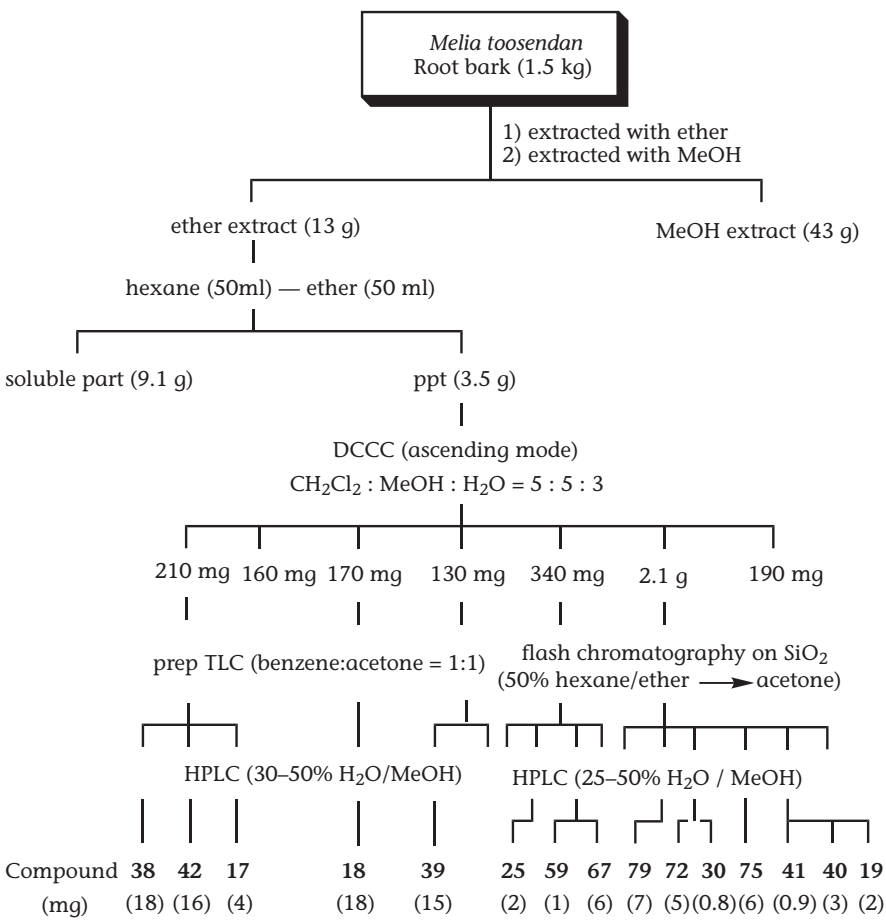


Figure 13.9: Isolation scheme for the limonoids from the root bark of *Melia azedarach*.

13.5.2 Structure elucidation

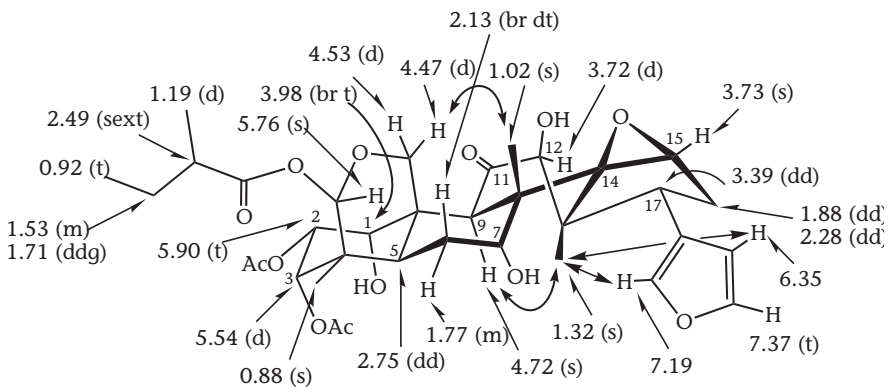
13.5.2.1 Structure of trichilins

A series of trichilins was first isolated from the root bark of the East African medicinal plant *Trichilia roka* (Meliaceae) (Nakatani *et al.*, 1981). Trichilins A (**23**) and its 12-epimer (trichilin B) (**24**) were first isolated by HPLC through a normal column using 1% $MeOH/CH_2Cl_2$ as solvent, but recently they were elucidated to be present as an equilibrium mixture in H_2O/MeOH solvent system. Their structures were elucidated by extensive ^1H (Figure 13.10) and ^{13}C (Table 13.1) nuclear magnetic resonance (NMR) and circular dichroism (CD) studies (Stonard *et al.*, 1983), and finally confirmed by chemical correlation as shown in Scheme 13.2.

13.5.2.2 Structure of spirosendan

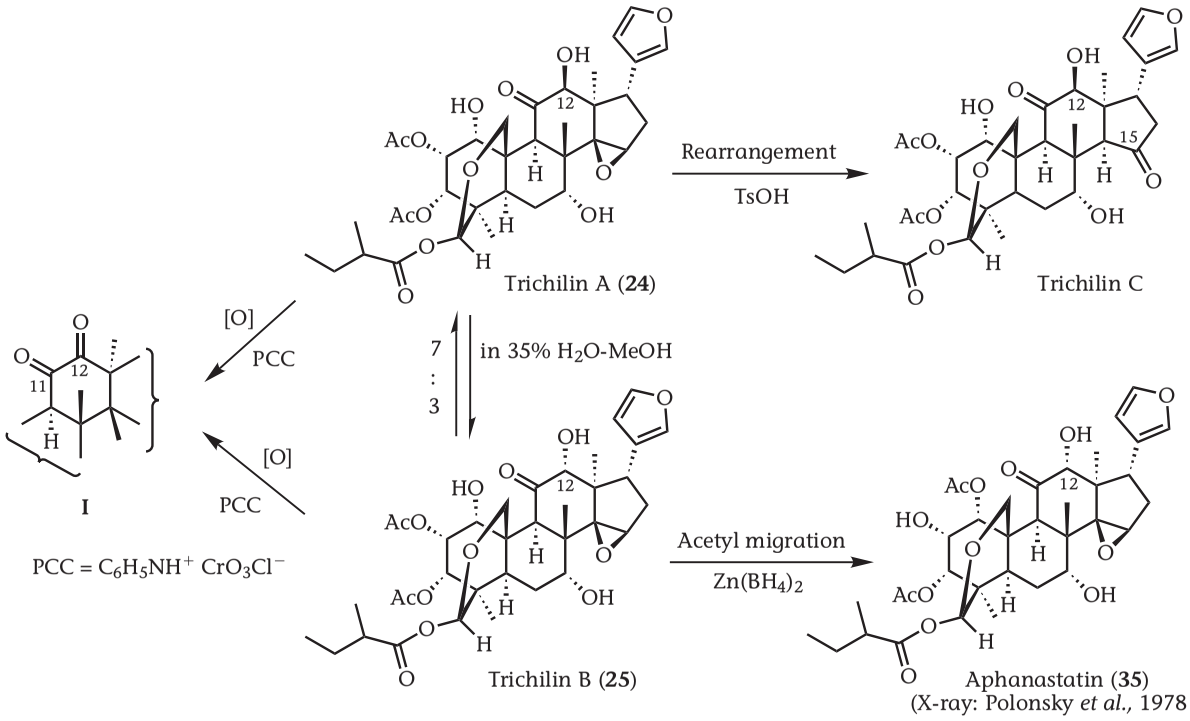
Spirosendan (**82**) is a new skeletal limonoid possessing a spiro-structure isolated from the root bark of *M. toosendan*. Some spiro limonoids have been isolated from Meliaceae plants, but in **82** the C-30 forms a carbon bridge between C-8 and C-12 in addition to the spiro structure, which is the first occurrence in limonoid. The structure and stereochemistry of spirosendan were elucidated by extensive NMR studies, as shown in Figure 13.11.

All protons directly bonded with carbon atoms were assigned by a ^1H-^{13}C NMR shift correlated (HSQC) measurement. After that, two major molecular fragments of C-1 to C-10 in the A/B ring system and C-15 to C-17 of the D-ring in limonoid skeleton, were deduced from the ^1H-^1H correlation (COSY) spectrum, decouplings, nuclear Overhauser

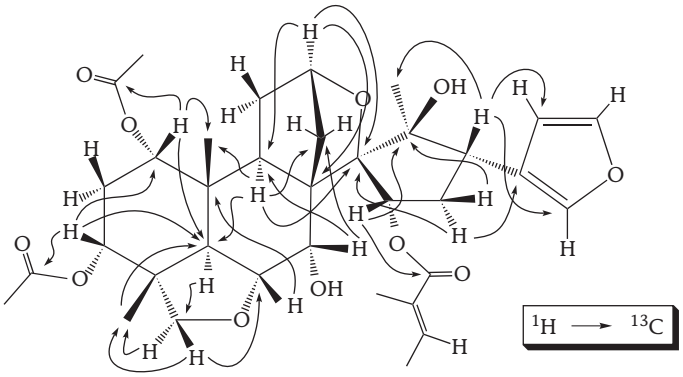


Trichilin A (**24**)

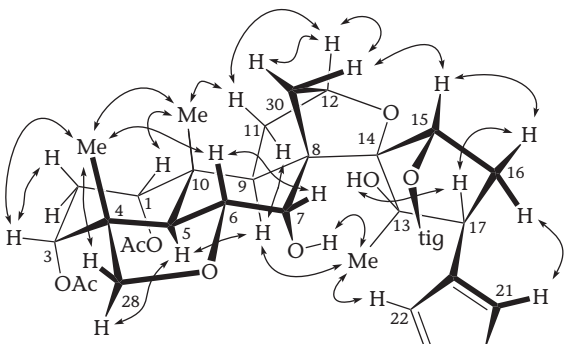
Figure 13.10: Selected ^1H NMR data ($CDCl_3$), 400 MHz, in δ (multiplicity), and nuclear Overhauser effect (NOE) correlations (double-headed arrows) of trichilin A (**24**).



Scheme 13.2: Chemical correlation of trichilins A (**24**), B (**25**) and C, and aphanastatin (**35**).



Selected HMBC correlations



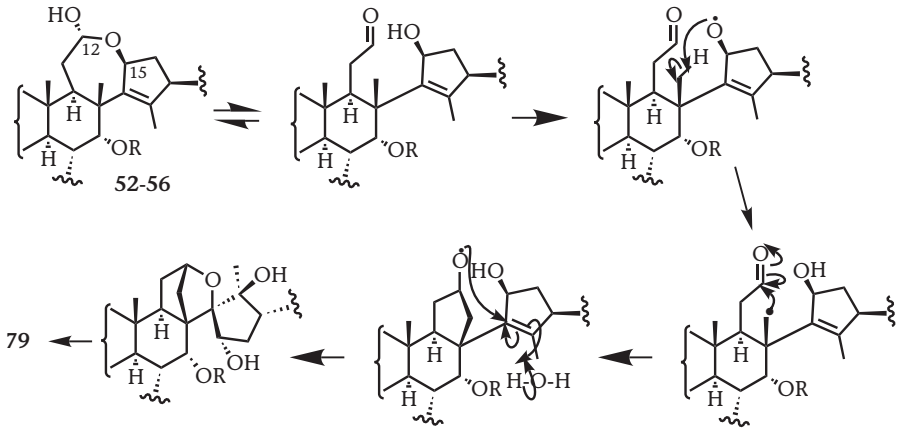
NOE correlations

Figure 13.11: Extensive NMR (selected HMBC and NOE) correlations in spirosendan (**82**).

effect (NOE) measurements and the ^1H-^{13}C heteronuclear long-range correlation (HMBC) spectrum.

The presence of three tertiary methyls at 4β (C-29), 10β (C-19) and 13α (C-18) and one methylene group at 4α (C-28) in the basic limonoid skeleton were easily confirmed. The absence of a methyl group to be C-8, which was changed to a methylene group directly attached to the 12-methine carbon, was confirmed by the HMBC spectrum. Thus, long-range ^1H-^{13}C couplings from H-7 and 9 to C-30 and between H_2-30 and C-8, 9, 11, 12 and 14, as well as between H_3-18 and C-14 and between H-12 and C-14, clearly established the C-12 to C-13 fragment through C-30, C-8 and C-14. Similarly, the C-13 to C-17 fragment was also established. Finally, all relative configuration was established from the NOESY data.

The evidence of the nucleus as spiro-ether rings can also be supported in biogenetic terms. A possible pathway leading to the formation of **79** can be proposed, as shown in



Scheme 13.3: Possible biogenesis of spirosendan (**79**) from the nimbolinins **52–56**.

Scheme 13.3. An inspection of the structure **79** suggests a derivation from nimbolin B (**52**), nimbolinins (**53–56**), whose cyclic hemiacetal/alcohol and aldehyde equilibrium yields an oxygen radical, which can then oxidize Me-30 to a radical and this can attack the aldehyde C-12, giving a second oxygen radical which finally attacks the 14,15-double bond, giving the bicyclo[$2^{11,9}.2^{14,O}.1^{12,8}$]-heptane-spiro^{14}[4.5]-decane system.

13.5.3 Selected ^{13}C NMR data of limonoids

Eighty-two limonoids have been isolated from *M. azedarach* and *M. toosendan* and their structures were mainly elucidated by NMR spectroscopic means. In particular, ^{13}C NMR was effective for the skeletal determination and carbon signals of a new compound are easily assignable by comparision with the data of known compounds. Then, the skeleton-wise compilation of the data of the isolated limonoids is presented in Table 13.1.

13.6 ANTIFEEDANT ACTIVITY OF THE LIMONOIDS FROM *M. AZEDARACH* AND *M. TOOSENDAN*

13.6.1 Antifeedant bioassay

The antifeeding assay results reported here were obtained with the Southern army worm *Spodoptera eridania* (Boisduval) and/or a Japanese insect pest *S. littoralis* (Boisduval) as the test species. *Spodoptera* species are distributed throughout the world and constitute a major agricultural threat. The feeding bioassay was carried out by the conventional leaf disk method (Wada and Munakata, 1968) using 2 cm-diameter leaf disks cut from the Chinese cabbage *Brassica campestris* L. var. *chinensis* (Cruciferae) with a cork-borer. Each

disk was dipped for 2 sec in an acetone solution of the sample; five treated disks were arranged alternately with another five control disks (immersed for 2 sec in acetone alone), all concentrically placed near the periphery in a Petri dish as illustrated (Figure 13.12). Subsequently, 10 third-instar larvae were placed in the center of the dish, and the treated and untreated leaves eaten by the larvae in 2–24 hr periods were evaluated at appropriate intervals. The bioassay was terminated after the larvae had eaten approximately 50% of the control disks, which usually took 6–12 hr. When the average eaten area of the treated disks was visually judged to be less than 50% of that of the control disks, the test compound was judged to be active. This bioassay was used to guide the isolations to the active limonoids, as well as to assess their relative antifeedant activities. To determine the minimum inhibitory concentration, this choice test was done at 50,

TABLE 13.1

^{13}C NMR Data of several types of limonoids

C	**12**	**14**	**18**(29-*R*)	**18**(29-*S*)	**24**	**25**	**40**	**42**	**53**
1	71.9	71.1	70.2	70.1	72.4	72.8	70.8	70.7	71.6
2	27.4	30.5	35.9	35.0	68.7	68.6	36.1	36.3	27.9
3	73.9	73.8	76.0	73.1	73.3	73.0	73.9	76.2	72.4
4	42.4	43.8	39.8	39.7	41.0	40.7	43.5	43.4	42.6
5	39.6	38.6	27.6	25.3	41.2	41.2	41.3	25.8	40.2
6	72.4	73.8	25.2	27.1	26.4	26.4	23.2	25.0	72.4
7	71.7	72.7	70.3	70.0	71.3	71.7	69.7	69.6	75.3
8	45.4	45.2	41.5	41.5	45.6	42.4	41.3	41.4	45.4
9	34.3	34.8	48.3	48.1	46.7	46.2	48.8	48.6	35.5
10	39.5	39.7	42.1	42.2	41.9	42.3	39.5	40.3	40.4
11	24.2	24.5	206.4	206.4	213.7	213.2	212.4	212.5	31.6
12	77.2	77.2	78.3	78.1	78.7	78.9	79.3	78.9	91.4
13	51.4	51.4	45.5	45.4	48.9	47.3	45.7	45.7	141.5
14	157.4	157.1	71.8	71.9	71.1	70.0	60.9	60.3	142.7
15	122.7	122.8	58.4	58.3	54.8	59.2	219.0	218.5	78.0
16	36.6	36.6	33.4	33.4	31.3	33.1	44.9	44.7	38.1
17	50.3	50.5	37.9	37.9	32.7	38.6	41.7	41.2	46.4
18	26.9	26.8	22.5	22.2	22.4	25.3	21.7	21.2	16.2
19	15.2	15.3	63.6	63.6	64.9	64.1	64.2	57.8	16.1
20	124.5	124.4	122.2	122.3	123.5	123.5	124.2	124.1	128.4
21	142.1	142.0	142.1	142.1	143.0	142.3	143.4	143.0	142.8
22	111.7	111.6	111.6	111.6	111.4	112.8	111.3	111.0	110.2
23	140.3	140.2	141.4	140.4	140.2	140.8	140.4	140.1	138.9
28	78.0	78.2	19.3	18.2	18.2	18.6	19.0	18.3	77.6
29	19.7	20.0	95.9	96.2	93.4	93.5	94.1	102.9	19.2
30	15.3	15.8	15.3	15.3	21.9	22.7	22.8	22.8	20.7
COR	Tig	Cin			2-MeBu	2-MeBu	2-MeBu		Bz
1′	166.5	165.3			175.2	175.3	175.6		164.7
2′	128.7	117.1			28.4	27.7	28.6		130.4
3′	138.0	146.4			16.2	16.3	16.8		129.3
4′	14.4	133.9			26.4	25.9	26.8		128.4
5′	11.9	128.3			11.2	11.3	11.7		133.0
6′		129.0							128.4
7′		130.8							129.3
8′		129.0							
9′		128.3							

TABLE 13.1

^{13}C NMR Data of several types of limonoids (*continued*)

C	**54**	**58**	**59**	**61**	**62**	**67**	**69**	**71**	**79**
1	71.3	71.7	71.5	71.7	71.5	70.8	70.8	24.9	72.2
2	29.3	27.6	27.8	27.3	27.2	30.5	28.0	33.5	28.4
3	72.5	71.8	71.8	72.0	72.8	75.1	70.9	70.7	71.8
4	42.5	42.4	42.4	42.7	42.6	47.1	42.4	42.7	43.0
5	39.3	40.6	40.6	40.3	40.0	39.5	35.0	39.7	39.4
6	72.6	71.8	70.8	72.9	71.8	69.1	71.1	71.0	74.9
7	75.4	75.6	73.9	75.6	75.2	85.3	83.5	84.3	67.3
8	45.3	45.1	45.0	47.5	47.4	47.5	51.5	51.0	60.3
9	36.0	37.0	37.3	38.8	38.7	35.9	47.8	54.8	35.9
10	41.1	40.1	40.8	40.9	40.7	41.9	49.9	49.5	39.3
11	31.1	32.5	32.7	32.0	32.0	25.0	106.7	106.7	34.0
12	92.0	171.2	171.2	173.6	173.6	172.5	170.1	170.1	76.6
13	142.4	138.4	138.6	133.4	133.5	135.7	94.9	94.8	81.6
14	143.0	147.9	146.8	148.6	148.5	146.1	93.1	93.0	96.7
15	77.9	83.9	85.4	82.2	82.0	87.6	81.2	81.1	80.7
16	38.0	37.7	37.6	37.1	36.7	41.2	29.8	29.8	32.6
17	46.7	47.0	47.0	47.7	47.5	49.6	50.8	51.3	45.2
18	16.4	16.0	16.0	17.5	16.9	17.0	26.4	26.3	20.7
19	16.2	15.4	15.5	16.0	16.0	16.7	70.4	71.0	16.9
20	128.1	126.5	126.5	128.0	127.8	126.9	86.3	86.2	123.9
21	138.9	139.2	143.5	142.9	143.2	143.0	109.3	109.4	142.7
22	110.3	110.0	110.0	110.2	109.8	110.6	108.0	108.0	111.4
23	142.7	143.4	139.2	139.5	139.5	138.9	145.7	146.0	139.9
28	78.0	78.0	78.0	78.1	77.8	204.3	76.4	76.8	79.1
29	18.8	19.2	18.9	19.8	20.0	14.4	18.3	18.4	20.8
30	20.7	20.1	20.5	20.2	20.1	13.0	17.7	17.2	42.6
COR′	Bz	Tig	*iso*-Bu	Tig	*iso*-Bu	Tig	Cin	Tig	Tig
1′	164.9	166.0	174.8	166.6	174.7	166.6	165.6	166.7	167.6
2′	130.5	128.6	34.7	129.4	33.5	128.9	117.3	128.4	129.3
3′	129.4	137.2	19.5	136.3	17.6	137.7	145.8	138.2	138.5
4′	128.5	14.5	19.5	14.4	19.9	14.1	133.9	14.4	15.0
5′	132.9	12.1		12.4		12.0	128.0	11.9	12.5
6′	128.5						129.1		
7′	129.4						130.8		
8′							129.1		
9′							128.0		

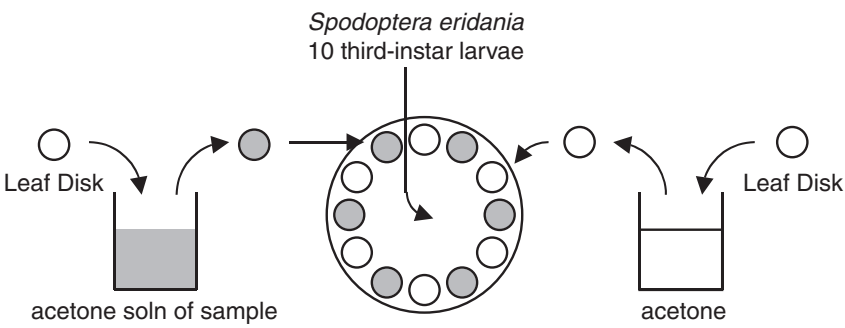


Figure 13.12: Strategy for the antifeedant bioassay.

100, 150, 200, 300, 500 and 1000 ppm, with 50 ppm corresponding to a concentration of *ca.* 1 μg/leaf-cm^2.

13.6.2 Limonoid antifeedants and their activities

The limonoids isolated from *M. azedarach* and *M. toosendan* and their effects on insect feeding are summarized in Table 13.1. To aid a quantitative comparison of structure-activity relationships among limonoids, the data are summarized primarily for the activity against the third-instar larvae of voracious pest insects *Spodoptera eridania* and/or *S. littoralis* (Boisduval). It is important to note that differences in the response of test insects when comparing different test species, or even different life cycle stages of the same species, can mask any meaningful observations of structure-activity relations. This is apparent from a comparison of the data reported against different insects for compounds **18**, **36**, **38–40**, **48**, **49** and **71** in the table.

Some quantitative trends are apparent from the table. The highly oxidized C-seco limonoids, meliacarpinins (**69**), (**71**), (**72**), (**75**) and (**78**), are the most active among the limonoids from *M. azedarach* and *M. toosendan*. Their activity is weaker than that of the azadirachtins from *M. azadirachta*, but they are much more potent antifeedants than the less oxidized class of C-seco limonoids exemplified by salannins (**48**) and (**49**), nimbolinins (**52–56**), ohchinolides (**57–59**) and nimbolidins (**61–65**). Aside from meliacarpinins, the most active compounds appear to be intact apo-euphol limonoids, amoorastatins (**17**) and (**18**), azedarachins (**19–23**) and trichilins (**24–36**), with a 14,15-epoxide and a C-19/C-29 acetal bridged system (effective dose 150–400 ppm), in which 12-hydroxyamoorastatin (**17**) is most active. In this limonoid class, some interesting structure-activity correlations were observed and they will be discussed in the following. The two members of the intact apo-euphol limonoid class without this C-19/C-29 acetal bridge, azadirones (**2**), (**4**) and (**5**) and trichilinins (**12–16**) showed little or no activity (effective dose >1000 ppm). Lastly, degraded limonoids (**83–86**) showed a little activity against *S. littoralis* (effective dose >700 ppm).

13.6.3 Structure-activity relationships in C-19/C-29 bridged acetals

During the course of our structural and chemical correlation studies, about 50 compounds belonging to C-19/C-29 bridged acetal class of limonoids were made available. Antifeeding assays with *S. eridania* by leaf disk method showed several interesting structure-activity correlations.

All natural C-19/C-29 bridged acetals possess the 11-keto group, and the configuration of the C-12 hydroxy group has a pronounced effect on the activity. The activity of compounds lacking the 12-OH is reduced. Acetylation or oxidation to ketones of 12- and/or 7-OH group also reduces the activities. Interestingly, activity is insensitive to sub-

TABLE 13.2

Antifeeding activity of limonoids from *M. azedarach* and *M. toosendan*

Limonoid	Origin[a]	Test insect	Effective conc. (ppm)
Protolimonoid			
Butanolide (**1**)	A, T	*S. eridania*	inactive
Group 1. Meliacins and derivatives			
Azadirone (**2**)	A, T	*S. eridania*	inactive
Meldenin (**3**)	A		–
Acetyltrichilenone (**4**)	T	*S. eridania*	inactive
6β-Acetoxytrichilenone (**5**)	T		–
Glucopyranoside (**6**)	A		–
Xylopyranoside (**7**)	A		–
Rhamnopyranoside (**8**)	A		–
Glucopyranoside (**9**)	A		–
Sendanal (**10**)	A		–
Nimbolin A (**11**)	A		–
Trichilinin B (**12**)	T	*S. eridania*	1000
Trichilinin C (**13**)	T	*S. eridania*	1000
Trichilinin D (**14**)	T	*S. eridania*	1000
3-*O*-acetyltrichilinin D (**15**)	T	*S. littoralis*	1000
Trichilinin E (**16**)	T	*S. eridania*	1000
C-19/C-29 bridged acetals			
(14,15-Epoxide)			
12-Hydroxyamoorastatin (**17**)	A, T	*S. eridania*	150
Toosendanin (**18**)	A, T	*S. eridania*	300
		S. littoralis	300
		O. furnacalis	200
Azedarachin A (**19**)	A, T	*S. eridania*	200
12-*O*-Acetylazedarachin A (**20**)	A, T	*S. eridania*	400
		S. littoralis	400
Azedarachin B (**21**)	A, T	*S. littoralis*	200
12-*O*-Acetylazedarachin B (**22**)	A, T	*S. eridania*	400
		S. littoralis	400
Azedarachin C (**23**)	A	*S. eridania*	400
Trichilin A (**24**)	A, T	*S. eridania*	300
Trichilin B (**25**)	A, T	*S. eridania*	200
		S. littoralis	200
12-*O*-acetyltrichilin B (**26**)	A	*S. eridania*	400
1,12-Di-*O*-acetyltrichilin B (**27**)	A	*S. eridania*	400
Trichilin D (**28**)	A	*S. eridania*	400
Trichilin H (**29**)	A, T	*S. eridania*	400
1-*O*-Acetyltrichilin H (**30**)	T	*S. littoralis*	400
Trichilin I (**31**)	T	*S. eridania*	400
Trichilin J (**32**)	T	*S. eridania*	400
Trichilin K (**33**)	T	*S. eridania*	400
Trichilin L (**34**)	T	*S. eridania*	400
Aphanastatin (**35**)	A	*S. eridania*	200
Meliatoxin A_2 (**36**)	A	*S. litura*	300
		S. eridania	400
(15-Keto)			
Amoorastatone (**37**)	A	*S. eridania*	400
12-hydroxyamoorastatone (**38**)	A, T	*S. eridania*	300
		S. littoralis	250
Iso-chuanliansu (**39**)	A, T	*S. eridania*	400
		S. littoralis	300
Neoazedarachin A (**40**)	T	*S. eridania*	500
		S. littoralis	400
Neoazedarachin B (**41**)	T	*S. littoralis*	400
Neoazedarachin D (**42**)	T	*S. littoralis*	400

TABLE 13.2 *(continued)*

Limonoid	Origin[a]	Test insect	Effective conc. (ppm)
Meliatoxin B_1 (**43**)	A	*S. eridania*	500
Meliatoxin B_2 (**44**)	A	*S. eridania*	500
Group 2. Ring D-seco limonoids			
Gedunin (**45**)	A	*O. nubilalis*	500
Glucopyranoside (**46**)	A		–
Group 3. Ring C-seco limonoids			
Ohchinal (**47**)	A	*S. frugiperda*	inactive
Salannin (**48**)	A, T	*S. eridania*	1000
		S. littoralis	1000
		E. insulana	100
Deacetylsalannin (**49**)	A	*S. eridania*	1000
		S. vorivestis	30
Ohchinin (**50**)	A		–
Ohchinin acetate (**51**)	A	*S. eridania*	1000
Nimbolin B (**52**)	A	*S. eridania*	1000
Nimbolinin A (**53**)	T	*S. littoralis*	1000
1-Deacetylnimbolinin A (**54**)	T	*S. littoralis*	1000
Nimbolinin B (**55**)	A	*S. eridania*	1000
1-Deacetylnimbolinin B (**56**)	A, T	*S. eridania*	1000
Ohchinolide A (**57**)	A		–
Ohchinolide B (**58**)	T	*S. eridania*	1000
Ohchinolide C (**59**)	A	*S. eridania*	1000
Nimbolidin A (**60**)	A		–
Nimbolidin B (**61**)	A, T	*S. eridania*	500
Nimbolidin C (**62**)	T	*S. eridania*	500
Nimbolidin D (**63**)	T	*S. eridania*	500
Nimbolidin E (**64**)	T	*S. eridania*	500
Nimbolidin F (**65**)	T	*S. eridania*	500
Ohchinolal (**66**)	A	*S. eridania*	1000
3-*O*-Acetylohchinolal (**67**)	T	*S. eridania*	1000
Highly oxidized C-seco-limonoids			
1-Cinnamoylmelianolone (**68**)	A		–
Meliacarpinin A (**69**)	A, T	*S. eridania*	50
		S. littoralis	50
Deacetylmeliacarpinin A (**70**)	A		–
Meliacarpinin B (**71**)	A, T	*S. eridania*	50
		S. littoralis	50
		S. exigua	150
Meliacarpinin C (**72**)	A, T	*S. eridania*	50
Deacetylmeliacarpinin C (**73**)	A		–
20-*O*-Acetylmeliacarpinin C (**74**)	A		–
Meliacarpinin D (**75**)	A, T	*S. eridania*	50
20-*O*-Acetylmeliacarpinin D (**76**)	A		–
Meliacarpinin Methacrylate (**77**)	A		–
Meliacarpinin E (**78**)	A	*S. eridania*	50
Meliacarpin (**79**)	A		–
Meliacarpin (**80**)	A		–
Meliacarpin (**81**)	A		–
Group 4. Spiro limonoid			
Spirosendan (**82**)	A, T	*S. littoralis*	1000
Group 5. Degraded limonoids			
Azedararide (**83**)	A	*S. littoralis*	1000
Fraxinellone (**84**)	A	*S. littoralis*	1000
12α-Acetoxyfraxinellone (**85**)	A	*S. littoralis*	1000
Fraxinellonone (**86**)	A	*S. littoralis*	700

[a] A: *M. azedarach*, T: *M. toosendan*.

stituent variation in the A-ring except for the nature of the hemiacetal structure. A hemiacetal bridge with a hydroxy group at C_{29} increases the activity in comparison to an acylated compound (compare the activities of **17** and **18** with **19–36**, Table 13.2), in which the activity is independent from the varieties or orientations of acyl groups. Replacement of the 14,15-epoxide with a C-15 ketone also results in reduced activity. These structure-activity relationships in the azedarachins and trichilins are summarized in Figure 13.13.

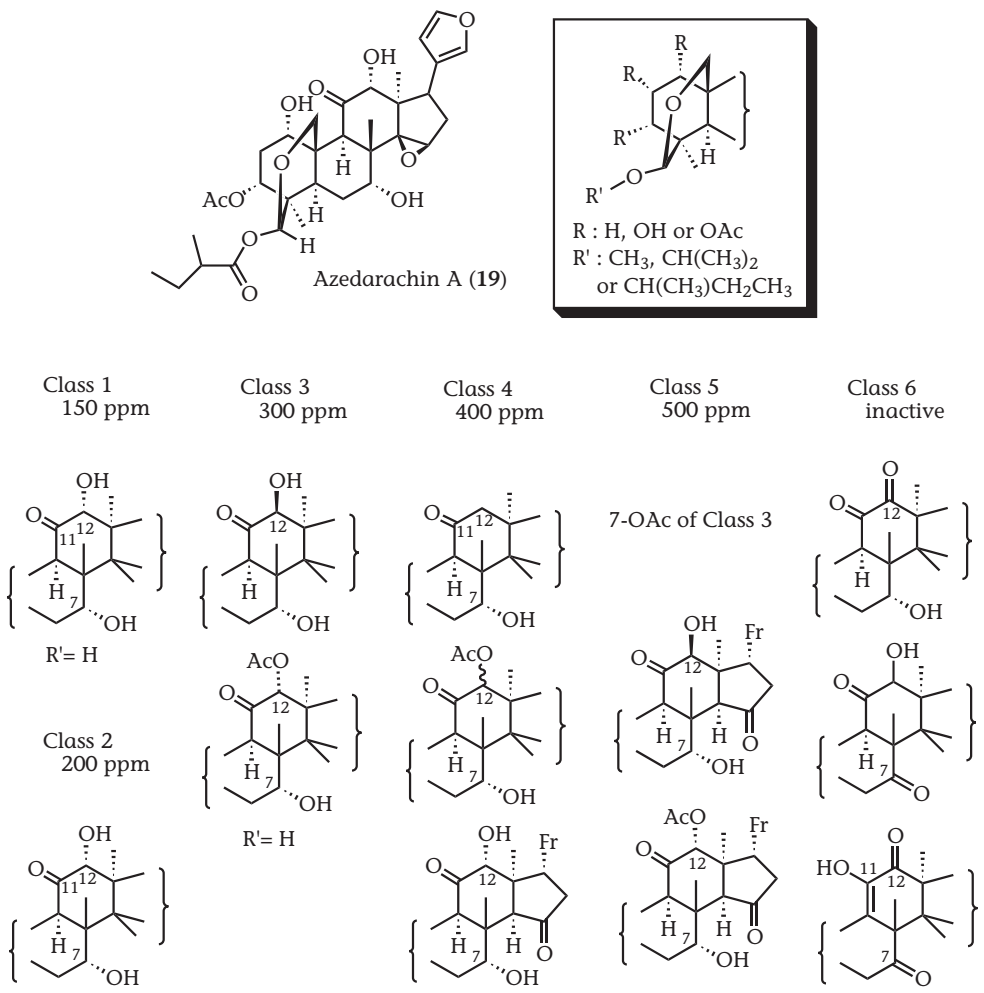


Figure 13.13: Structure-activity relationships of the C-19/C-29 bridged acetals (F = 3-furyl) against *Spodoptera eridania*, leaf disk method.

13.7 CONCLUSIONS

Fruits and bark of several species of Meliaceae have been used traditionally for treatment of a variety of diseases and their biologically active limonoid constituents have been researched to find useful compounds for specific agricultural or medicinal applications. As a result, a variety of bioassay designs and choice of bioassay species have been reported, but the common conclusion undoubtedly is that limonoids function primarily as 'antifeedant' in the host species. This suggests that the primary selective advantage of the production of limonoids by the host plant is protection against insect herbivory.

Limonoids may be found in all tissues of the plant and about 400 limonoids have been isolated to-date. The diversity and biosynthesis of limonoids is characterized by an evolutionary pattern of increasing oxidation and rearrangement of the original limonoid skeleton. Some relation of biogenesis of the limonoids in *M. azedarach* and/or *M. toosendan* with their antifeedant activity against the *Spodoptera* insects can be derived from the data in Table 13.2. The evolutionary trend of increasing oxidation correlates with increasing activity and the introduction of a bridged acetal structure may enhance the activity. However, conclusions about the significance of insect herbivory as a driving force in the evolutionary diversification of limonoids will have to await structure/activity studies with ecologically relevant insects.

ACKNOWLEDGEMENTS

I am greatly indebted to the large number of enthusiastic and dedicated colleagues whose names appear in the publications from my laboratory in the reference section. We would like to thank Dr. H. Naoki, Suntory for Bioorganic Research, and Mr. N. Nakayama, Nippon Roche Research Center, for extensive NMR and MS measurements. I am also grateful to Mr. Hasegawa and Mr. Takezaki, Kagoshima Prefectural Agricultural Experiment Station, for the supply of the insects.

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CHAPTER

14

Glycolipids with Immunomodulating Activity from Marine Sponges

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Contents

14.1 INTRODUCTION

Glycolipids are ubiquitous membrane constituents of animals and plants which have attracted broad interest in the last few decades because they play a major role as cell-surface-associated antigens and recognition factors. In particular, they are thought to be involved in asexual reproduction of sponges in which the cell recognition is of fundamental importance, and in which it is assumed that this reproduction process is mediated by the sugar heads of glycolipid molecules.

The structural investigation of natural glycolipids, as well as the study of their biological properties, is presently a complex matter, providing exciting challenges for chemists and biochemists. The aim of the present review is to illustrate some recent results in this field, throwing more interdisciplinary light on this subject. In particular, this chapter deals with the isolation and structure determination of glycolipids from marine sponges possessing immunomodulating activity.

Glycolipids are currently divided into three main classes. Glycoglycerolipids (GGLs) and glycosphingolipids (GSLs) are defined by whether they contain a glycerol or a sphingosyl backbone. Polyisoprenoid glycolipids are a third class of glycolipids which, among marine organisms, are mostly present in invertebrates belonging to phylum Echinodermata, although some have been found in sponges. The present review deals only with GSLs and some atypical glycolipids from sponges because glycoglycerolipids and polyisoprenoid glycolipids have not been reported to possess immunomodulating properties.

Recent results have shown that GSLs with immunomodulating activity are synthesized by sponges belonging to the genera *Agelas* and *Plakortis* while atypical glycolipids, formed with a sugar head glycosidically linked to a long alkyl chain, have been isolated from species belonging to the genera *Erylus* and *Plakortis*. From *Erylus placenta*, Fusetani's group isolated the erylusamines (Fusetani *et al.*, 1993; Sata *et al.*, 1994) while simplexides (Costantino *et al.*, 1997) have been isolated from *Plakortis simplex* by the authors of this chapter. It is noteworthy that both eylusamines and simplexides possess potent immunosuppressive activity with a non-cytotoxic mechanism.

14.2 GLYCOLIPIDS WITH IMMUNOMODULATING ACTIVITY

14.2.1 Glycosphingolipids

Glycosphingolipids (GSLs) are glycolipids built around a long-chain aminoalcohol known as a sphingoid base or long-chain base (LCB). The most commonly found sphingoid bases are sphingosine in higher animals and phytosphingosine in plants. Attachment of a fatty acid residue to the amino group of the sphingoid base gives rise to a ceramide. The saccharide head is glycosidically linked to the ceramide through the

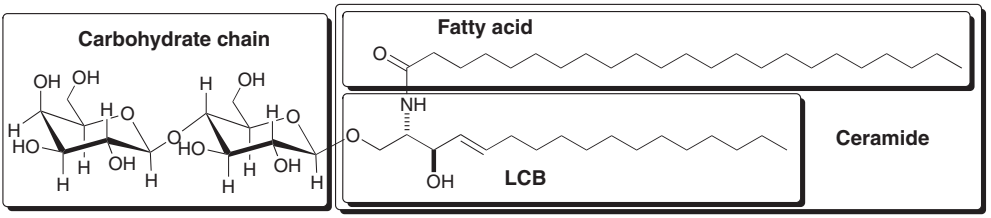


Figure 14.1: Structure of a representative glycosfingolipid.

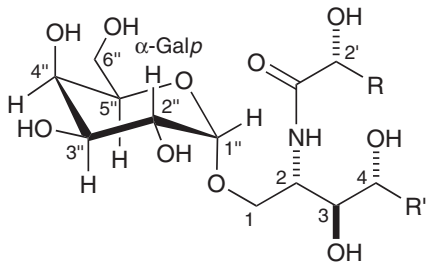
primary alcohol group of the sphingoid base. GSLs can be divided into two main classes: cerebrosides with a neutral sugar head, and gangliosides with a sugar head containing one or more residues of sialic acid.

A large number of different GSLs have been isolated, until now, mainly from organ tissues of higher animals. A significant portion of known GSLs come from marine organisms (Fattorusso and Mangoni, 1997), most of them being cerebrosides and gangliosides isolated from echinoderms (starfishes, sea urchins, holothurians or mollusks). In recent years, an increasing number of cerebrosides from marine sponges have been reported.

GSLs are membrane constituents of animals and plants. Because they are mainly located in the cell membrane, with the sugar head generally directed toward the outside of the cell, they are frequently involved in immunological processes. As an example, some complex fucose-containing GSLs possess the antigenic activity of the human ABH and Lewis blood group systems. As for marine glycolipids, gangliosides from the starfish *Anthocidaris crassispina* (Kubo *et al*., 1990) and phosphoglyspingolipids from the gastropod *Aplysia kurodai* (Abe *et al*., 1985) can induce an immune response when conjugated with macromolecules (haptene activity), and antibodies raised against these glycolipids have been used for their localization in the tissues.

Only recently, glycolipids capable of modulation of the immune system, have been reported to be present in marine sponges. Many of them are neutral GSLs (i.e. cerebrosides), while others are glycolipids with an atypical structure. Immunomodulators are substances which are not capable of raising antibodies by themselves, i.e. they are not antigens, but can either increase (immunostimulators) or decrease (immunosuppressors) the response of immune cells to an antigen, and are therefore also called biological response modifiers (BRMs). The first report of immunomodulating GSLs came from a Japanese group which, in 1994, isolated agelasphins from the sponge *Agelas mauritiana* (Natori *et al*., 1993). These metabolites are the first members of a new class of metabolites having an α-galactosylceramide structure that has no precedent in natural products.

Agelasphins are present in *Agelas mauritiana* as a mixture of homologues differing in the composition of the fatty acid (FA) and LCB chains. HPLC chromatography on a RP-18 column resulted in the separation of five pure cerebrosides. Structure elucidation



	Fatty acids:	LCBs:
agelasphin-7a	*n*-C_{24}	*n*-C_{16}
agelasphin-9a	*n*-C_{24}	*n*-C_{17}
agelasphin-9b	*n*-C_{24}	*iso*-C_{18}
agelasphin-11	*n*-C_{24}	*anteiso*-C_{19}
agelasphin-13	*n*-C_{25}	*anteiso*-C_{19}

Figure 14.2: Agelasphins from *Agelas mauritiana*, immunostimulating GSLs.

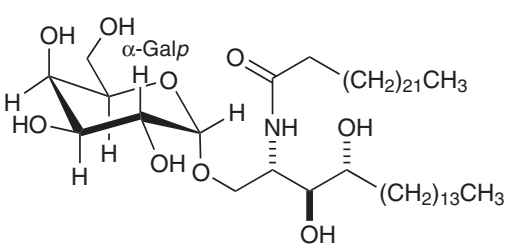
was accomplished using NMR experiments and chemical degradation, while the absolute stereochemistry was established by total synthesis (Akimoto *et al.*, 1993; Natori *et al.*, 1994). Agelasphins showed very interesting biological activities: they exhibited *in vivo* antitumor effects when tested on intraperitoneally implanted B16 murine melanoma. A cumulative effect was observed when these compounds were given in combination with adriamicin, a noted chemotherapeutic agent. In contrast, when the survival-period-prolonging effect of AGLs against intraperitonally implanted murine leukemia P388 cells was examined, no activity was observed. It is worth noting that chemotherapeutic agents generally show more potent antitumor activities against P388 than B16-bearing mice. Agelasphins were also tested for evaluating their stimulatory effects in the MLR (mixed lymphocyte reaction) assay showing a marked immunostimulating activity at concentrations below 1 μg/ml. In addition, agelasphins were shown to be not cytotoxic, so it is believed that these compounds act as biological response modifiers (BRMs) (Motoki *et al.*, 1995) and their marked antitumor activity is due to the activation of the immune system.

Because of these antitumor properties, the most active agelasphin (AGL-9b), was synthesized (Akimoto *et al.*, 1993). The synthesized compound showed the same activity of the natural AGL-9b on the growth of B-16 tumor and very similar stimulatory effects on the proliferation of lymphocytes (MLR assay). Various analogues of AGL-9b were synthesized by the same group (Morita *et al.*, 1995a) for studying the relationship between the structures and the immunomodulating activity, particularly the importance of the length of the lipophilic chains and the role of the three hydroxyl groups in the ceramide moiety. Analogues having the LCB and FA chains differing in length were synthesized and tested with an allogenic MLR assay. The results suggested that the length of

the lipophilic chain strongly affects the biological activity, and that C-18 and C-24 are the best lengths for LCB and FA chains, respectively. In addition, four new analogues, 2′-deoxy, 4-deoxy, 2′,4-dideoxy and 2′,3,4-trideoxy, were synthesized and their immunostimulating activity evaluated. The data demonstrated that only the 3-hydroxyl group plays an important role in the reactivity. These important results were also confirmed by testing the effects of the synthesized compounds on the proliferation of the lymphocytes of human umbilical cord blood (hUCB). The data obtained with the human cell assay resulted in re-evaluation of the role of the hydroxyl group at C-4 in biological activities.

While the above paper focused on the importance of the ceramide part of GSL on bioactivity, a successive report (Motoki *et al.*, 1995) from the same research group elucidated the importance of the stereochemistry of the sugar head of the molecule. To this end, isolated α- and β-monogalactosylceramides were tested with the MLR assay, and showed that the ceramides with α-Gal sugar head are better immunostimulating agents than those which possess β-Glu sugar. Similar results were obtained when the inhibitory effects of these compounds on the tumor growth of mice inoculated with murine melanoma B16 cells were examined. To confirm these hypotheses, four kinds of monoglycosilated ceramides (α-GalCer, β-GalCer, α-GluCer, and β-GluCer) having the same ceramide moiety, were synthesized (Morita *et al.*, 1995b) and tested. The data suggested that the activity of GSL is affected by the stereochemistry of the glycosyl linkage, α being more active than β, as well as by the type of sugar linked to the ceramide. Taking into account the above results, the agelasphin analogue KRN-7000 was proposed to be the best model for clinical application.

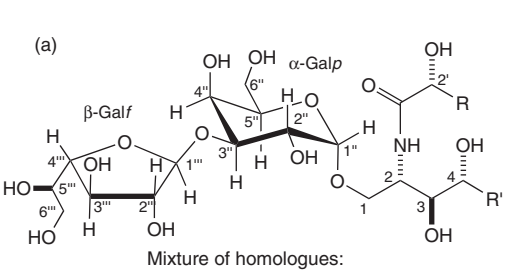
Further evidence, confirming the usefulness of KRN-7000 as a cancer therapy, was subsequently obtained. In particular, the relationship between the antitumor effects and the activation of natural killer (NK) cells was investigated and it was suggested that KRN-7000 enhances NK activity not directly, but *via* the activation of T cells. In addition, a tumor-specific immunity was acquired through treatment with KRN-7000 (Kobayashi, 1995). KRN-7000 also acts through activation of antigen-presenting cells, such as dendritic cells (Yamaguchi, 1996).

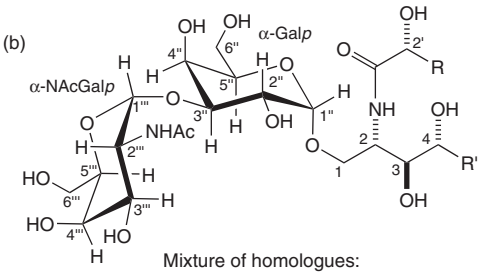


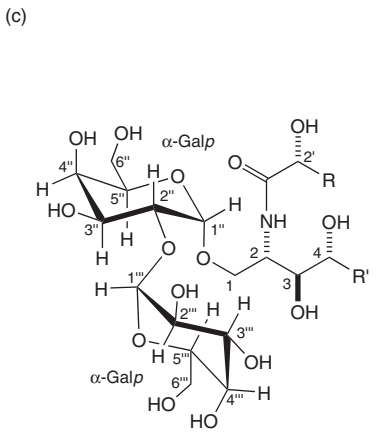
KRN-7000

Figure 14.3: The synthetic agelasphin analogue KRN-7000.

In 1996, Fattorusso's group reported a survey of the activity on the immune system of GSLs from porifera belonging to the genus *Agelas* collected along the coast of the Bahamas Islands (Costantino *et al.*, 1996). Previous studies by the same group (Costantino *et al.*, 1995a,b; Cafieri *et al.*, 1994, 1995, 1996) on *A. clathrodes*, *A. longissima*, *A. conifera*, and *A. dispar* showed that these sponges contain a large variety of GSLs having a more complex sugar chain relative to those of the agelasphins. All metabolites (Figures 14.4 and 14.5) isolated shared an interesting structural feature, namely, the α-galactosyl







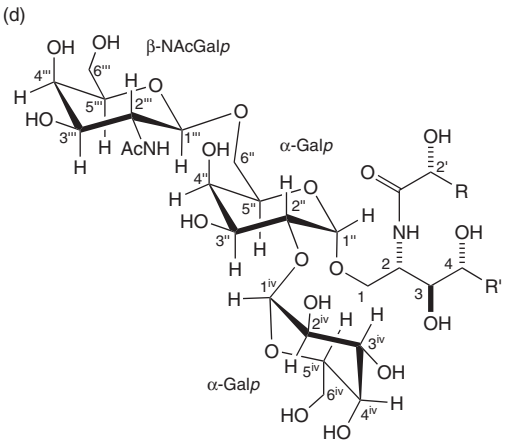
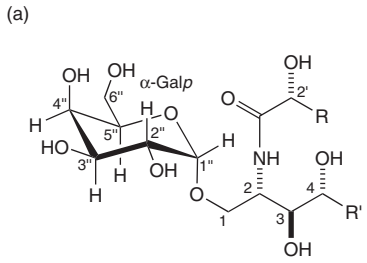
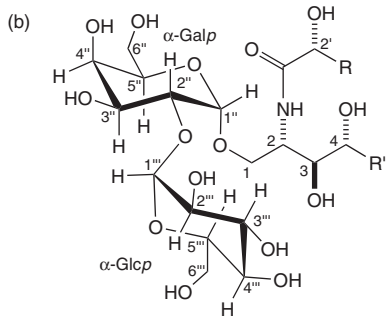


Figure 14.4: Glycosphingolipids with an inner α-galactose from *Agelas dispar*. T-cell proliferation assays showed that (a) and (b) are immunostimulating, whereas (c) and (d) are not active.



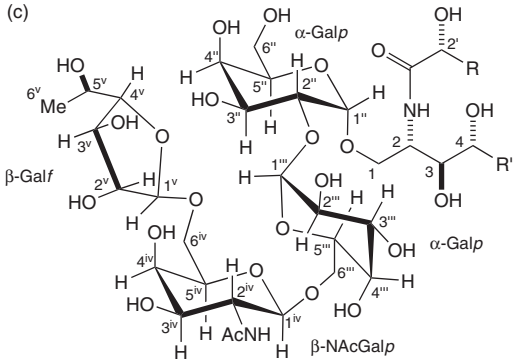
Mixture of homologues:

Fatty acids:	LCBs:
n-C_{22} (5.5%)	*n*-C_{17} (5.2%)
n-C_{23} (10.1%)	*iso*-C_{18} (27.6%)
n-C_{24} (78.3%)	*n*-C_{18} (16.8%)
n-C_{25} (6.1%)	*iso*-C_{19} (21.4%)
	n-C_{19} (23.1%)
	n-C_{21} (5.9%)



Mixture of homologues:

Fatty acids:	LCBs:
n-C_{22} (5.1%)	*n*-C_{17} (7.8%)
n-C_{23} (10.6%)	*iso*-C_{18} (29.7%)
n-C_{24} (77.2%)	*n*-C_{18} (12.3%)
n-C_{25} (7.1%)	*iso*-C_{19} (20.8%)
	n-C_{19} (22.0%)
	n-C_{21} (7.4%)



Mixture of homologues:

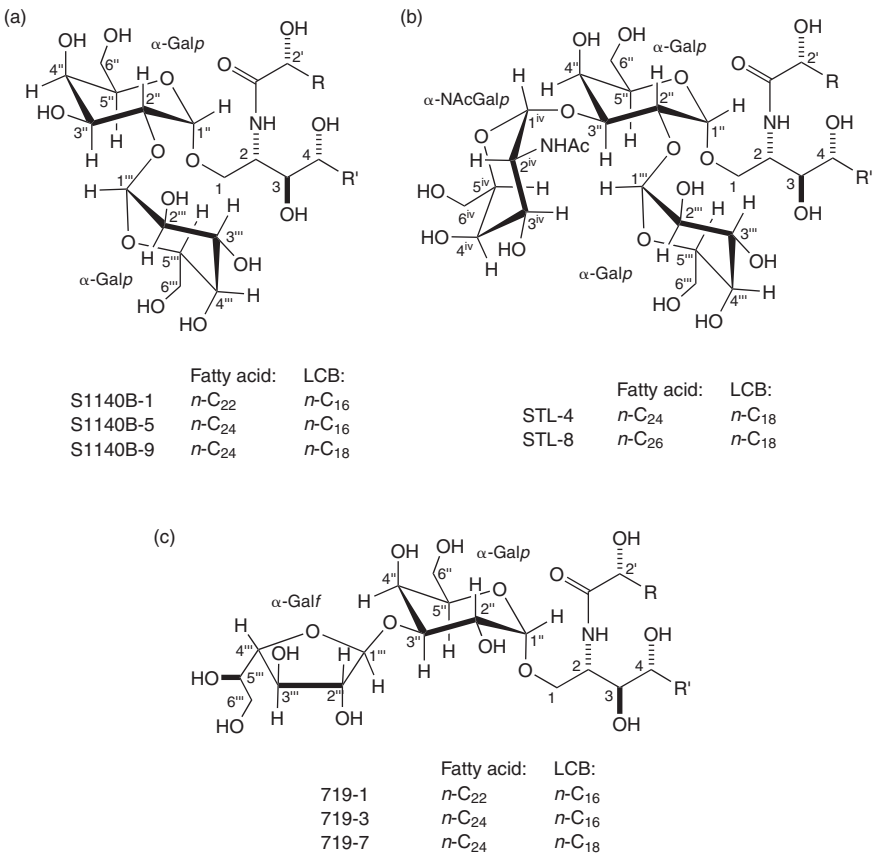
Fatty acids:	LCBs:
n-C_{21} (44.2%)	*iso*-C_{18} (28.1%)
n-C_{22} (2.5%)	*n*-C_{18} (28.8%)
n-C_{22} (30.3%)	*iso*-C_{19} (15.7%)
n-C_{23} (2.1%)	*n*-C_{19} (16.7%)
n-C_{24} (20.9%)	*n*-C_{21} (10.7%)

Figure 14.5: Glycosphingolipids with an inner α-galactose from *Agelas conifera* (a, b) and *Agelas longissima* (c). T-cell proliferation assays showed that (a) is immunostimulating and (b) is not active.

linkage between the first sugar and the ceramide. All the compounds were isolated as mixtures of homologues, differing in the length and in the branching of LCB and FA chains. Structural studies, as well as biological assays, were performed on the mixtures of homologues, while the nature of the fatty acid and the sphingoid bases was established by chemical degradation followed by GC/MS.

All compounds, except for the tetraglycosilated GSL, were tested for immunostimulatory activity using the T-cell proliferation assay (Ianaro *et al.*, 1995). This assay uses a pure population of lymphocyte, that is T-cells, from the lymph nodes removed from Swiss mice, and the stimulatory effect on the proliferation of lymphocytes is evaluated through [^{3}H]thymidine incorporation. Only three compounds exhibited a stimulatory effect, with the compound in Figure 14.5a being the most active at concentrations of 0.1 and 1 μg/ml. In contrast, compounds depicted in Figure 14.4c, 14.4d, and 14.5b did not show any stimulatory activity. These results seem to indicate that the position 2 of the inner sugar is a crucial point for the immunostimulating activity: glycosylation at this position results in the loss of the stimulatory effects on the proliferation of the lymphocytes.

Subsequently, Koezuka's group has reported the isolation of 2′′- or 3′′-monoglycosilated α-galactosyl ceramides and 2′′,3′′-diglycosilated α-galactosyl ceramides from three



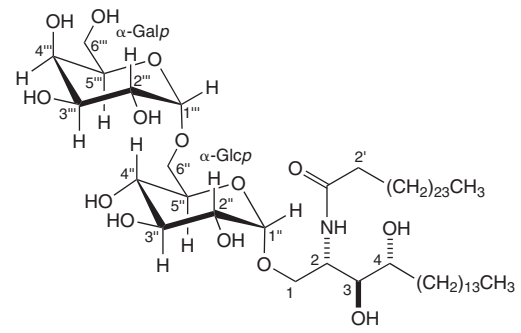
	Fatty acid:	LCB:
S1140B-1	*n*-C_{22}	*n*-C_{16}
S1140B-5	*n*-C_{24}	*n*-C_{16}
S1140B-9	*n*-C_{24}	*n*-C_{18}

	Fatty acid:	LCB:
STL-4	*n*-C_{24}	*n*-C_{18}
STL-8	*n*-C_{26}	*n*-C_{18}

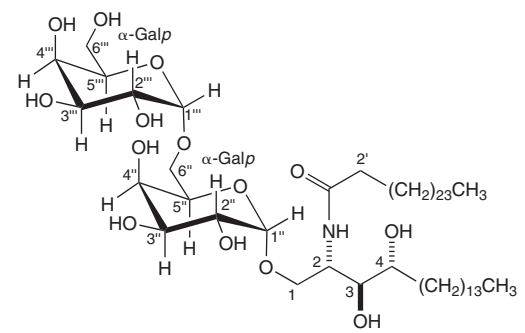
	Fatty acid:	LCB:
719-1	*n*-C_{22}	*n*-C_{16}
719-3	*n*-C_{24}	*n*-C_{16}
719-7	*n*-C_{24}	*n*-C_{18}

Figure 14.6: Glycosylceramides from (a) an unknown sponge, (b) *Stylissa flabelliformis*, and (c) *Agelas axisera*. These GSLs were used for evaluation of the influence of substitution at positions 2 and 3 of the inner sugar on immunostimulating properties of glycosylceramides.

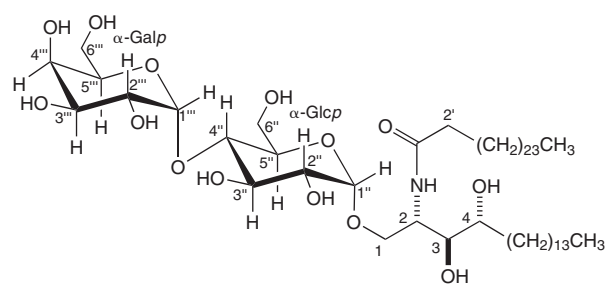
Japanese sponges, namely *Agelas axisera*, *Stylissa flabelliformis*, and an unidentified species (Uchimura *et al.*, 1997a). These metabolites, previously isolated by Fattorusso's group from *Agelas longissima* (Cafieri *et al.*, 1994, 1995) and *Axinella* sp. (Costantino *et al.*, 1994) as mixtures of homologues differing in length of LCB and FA chains, were obtained as pure compounds and their immunological activity was assayed with spleen cells and lymph-node cells. In contrast to previous results, it was found that the free 3′′-hydroxyl group plays a more important role in the immunostimulatory effects than the 2′′-hydroxyl group. It is, nevertheless, worth noting that these different results are



AGL-584



AGL-586



AGL-588

Figure 14.7: Three diglycosylceramides synthesized to evaluate the influence of substitution at positions 4 and 6 of the inner sugar on immunostimulating properties of glycosylceramides.

obtained using spleen cells instead of lymph-node cells. In addition, this report confirmed the importance of the length of the LCB and FA chains on the immunostimulatory effect, namely α-galactosyl ceramides having the longest carbon chains in the ceramide portion showed the strongest immunostimulating activity.

Most recently Uchimura *et al*. (1997b) compared the role of the free 4′′- and 6′′-hydroxyl of the inner pyranose ring in the immunostimulatory activity. Toward this end, they synthesized 6′′-glycosilated α-Gal Cer (AGL-586), 6′′-glycosilated α-Glu Cer (AGL-584) and 4′′-glycosilated α-Glu Cer (AGL-588).

These metabolites were tested in both previously mentioned assays, confirming that the configuration at C-4′′ affects the immunostimulatory activity: hence, the α-Gal Cer is more active than α-Glu Cer. Furthermore, this study reports that the free 4′′-hydroxyl groups play a more significant role than the free 6′′-hydroxyl groups: hence, AGL-586 is the most active compound.

In 1997, Costantino *et al*., reported the isolation and structural determination of two prenylated GSLs, the plakosides, from the Caribbean sponge *Plakortis simplex* (Costantino *et al*., 1997).

Plakosides are very interesting compounds, both for their structural features and biological activity. They are the first example of GSLs having a prenylated sugar and a cyclopropane-containing ceramide. Morever, the presence of a prenylated galactose is of great importance, with respect to their biological activity. In the T-cell proliferation assay, they demonstrated inhibition of the proliferation of lymphocytes stimulated with Concanavalin A, which does not appear to be related to cytotoxicity.

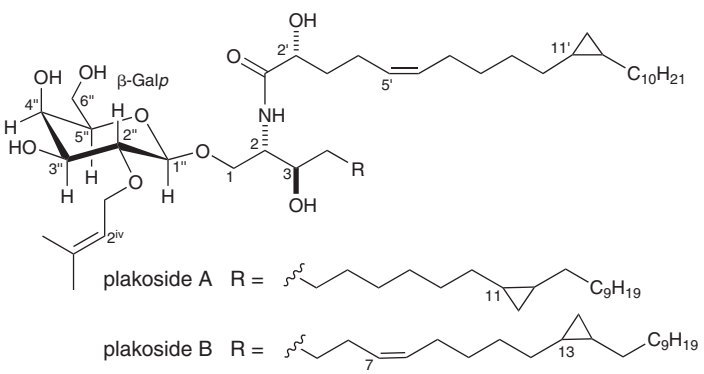


Figure 14.8: Plakosides from *Plakortis simplex*.

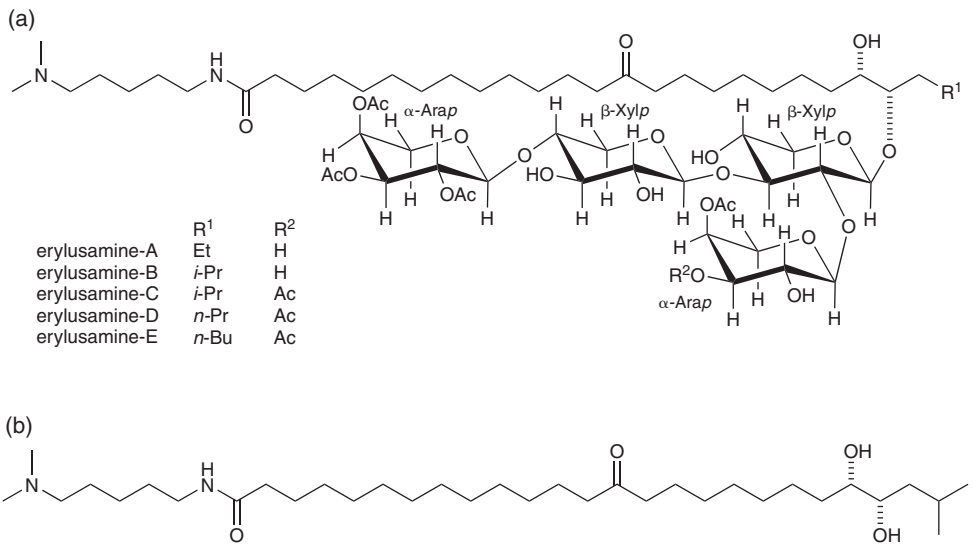
14.2.2 Atypical glycolipids

Erylusamines

A new class of marine natural products, erylusamines A–E (Figure 14.9a) were isolated from the Japanese marine sponge *Erylus placenta* (Fusetani *et al.*, 1993; Sata *et al.*, 1994). These metabolites are characterized by a polar head, constituting a tetrasaccharide moiety, glycosidically linked to an amide of a keto-hydroxy fatty acid.

The structure of the saccharide head was deduced from extensive 2D NMR experiments: COSY and HOHAHA data revealed the presence of four pentose rings while the linkage between the sugar units and the position of the glycoside bonds was established with HMBC cross-peak data. Concerning the aglycone moiety, in addition to NMR data, a mild acid hydrolysis was useful in obtaining the aglycone (Figure 14.9b), the molecular formula of which was secured by both FABMS and NMR data. The presence of a vicinal diol, inferred from cleavage of the C—C bond, was confirmed by synthesis of an acetonide upon treatment with 2,2-dimethoxypropane/*p*-TsOH. This derivative also allowed determination of the relative configuration of the two hydroxymethine carbon atoms.

Erylusamines exhibited an interesting biological activity as an interleukin-6 (IL-6) receptor antagonist. In particular, erylusamines B inhibited the binding of IL-6 to its receptor with an IC_{50} 66 μg/ml. Interleukin-6 is a multifunctional cytokine which exerts



	R^1	R^2
erylusamine-A	Et	H
erylusamine-B	*i*-Pr	H
erylusamine-C	*i*-Pr	Ac
erylusamine-D	*n*-Pr	Ac
erylusamine-E	*n*-Bu	Ac

Figure 14.9: (a) The five erylusamines, IL-6 receptor antagonists from *Erylus placenta*; (b) the aglycone of erylusamine B, used for determination of the relative configuration of the two hydroxymethine carbon atoms.

a variety of biological functions through the ligand-receptor interactions. Abnormal production of IL-6 causes development of an autoimmune state, such as rheumatoid arthritis or inflammation, whereas its constitutive production results in the disease states of HTLV-1 or HIV infections. Therefore, inhibitors of IL-6 are considered of therapeutical relevance.

Simplexides

Simplexides are the first example of a new class of atypical glycolipids with immunosuppressive activity. They have been isolated from the Caribbean sponge *Plakortis simplex* (Costantino *et al.*, 1999) as a complex mixture of homologues, and their structure has been elucidated using spectroscopic data and microgram-scale chemical degradation. The unusual lipid moiety of simplexides is a very-long-chain secondary alcohol composed of 34–37 carbon atoms. The hydroxyl group, located almost in the middle of the chain, is glycosylated by a disaccharide constituted by a β-galactose linked to an α-glucose through the hydroxyl group at position 4.

Simplexides were evaluated for their immunomodulating properties, and inhibited the lymphocyte proliferation at concentrations as low as 10 ng/mL. This immunosuppressive activity was not related to a cytotoxic activity, as shown by the negative response in the MTT assay (Mosmann, 1983) at all concentrations tested.

14.3 ANALYTICAL TECHNIQUES

14.3.1 Separation

The presence of long aliphatic chains linked to a polar head results in glycolipids being amphiphilic compounds and, therefore, are not very easy to isolate and separate.

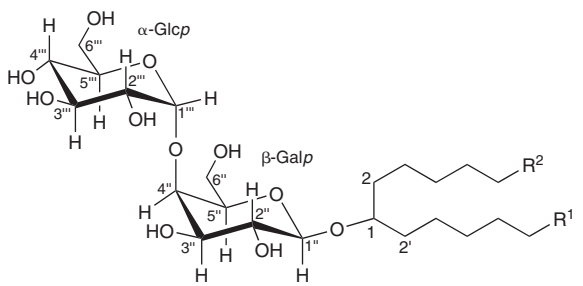


Figure 14.10: Simplexides from *Plakortis simplex*.

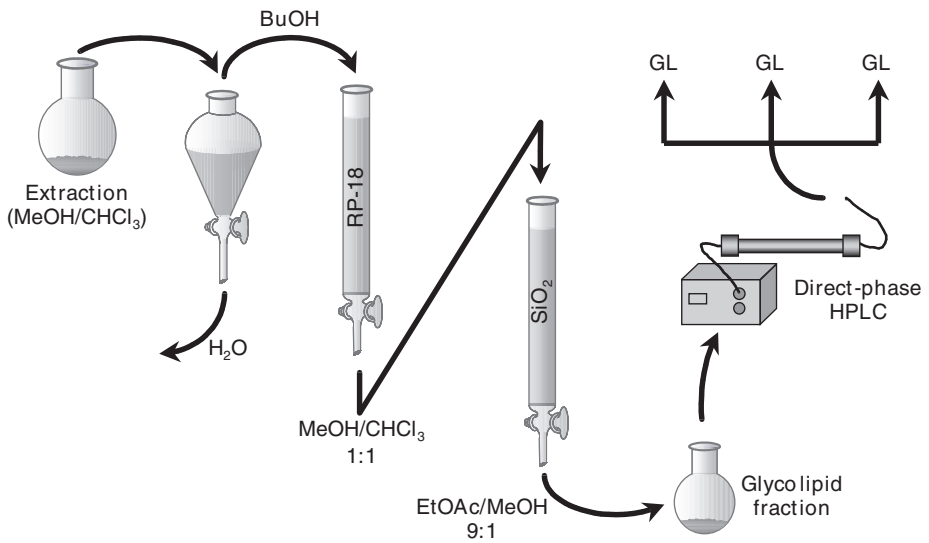


Figure 14.11: A typical procedure for isolation of glycolipids.

Glycolipids produce foams and emulsions and give rise to broad peaks when subjected to many kinds of HPLC separation. To make things worse, glycolipids very often are present in the organism as very complex mixtures of homologues, with the same polar moiety, but with alkyl chains differing in length and branching, so that separating pure compounds can be very difficult, or even impossible.

Glycolipid extraction from homogenized or lyophilized sponge tissue is generally performed using a mixture of chloroform and methanol. In some cases ethanol, followed by diethyl ether has been successfully used (Fusetani *et al.*, 1993). The crude extract is partitioned between water and *n*-butanol (Costantino *et al.*, 1994), or between the two phases of a ternary solvent system composed of water, methanol, and chloroform (the so-called Folch extraction, Folch *et al.*, 1957). Glycolipids, except for those with an exceedingly long saccharide chain, are found in the organic phase. The glycolipids are first purified by column chromatography on LH-20 (Kubo *et al.*, 1990) or RP-18 (Costantino *et al.*, 1994). The latter, stationary phase, takes advantage of the hydrophobic nature of aliphatic chains, so that glycolipids are strongly retained and eluted only with very non-polar eluents together with non-polar metabolites such as triglycerides, steroids, and terpenes. Unlike these metabolites, however, glycolipids are also strongly retained on silica gel because of their sugar moiety, so that a normal phase chromatographic separation can lead to a fraction mainly composed of glycolipids.

Separation of individual glycolipids from the crude glycolipid fraction can be a very difficult task. It is generally achieved by direct-phase HPLC using silica gel or DIOL columns with strong eluents such as *n*-hexane/*i*-PrOH/H_2O or $CHCl_3$/*i*-PrOH. Another

approach involves acetylation of the whole glycolipid mixture and subsequent HPLC separation on silica gel with less polar eluents, typically *n*-hexane/EtOAc mixtures. In complex cases, both techniques can be applied in sequence. Once a fraction of glycolipids with the same polar moiety has been obtained, reversed-phase HPLC with methanol or a mixture of methanol-water as mobile phase, depending on the polarity of the glycolipids isolated, can be used for the separation of individual homologues. When the isolated mixture of glycolipids is composed of only a few homologues, their separation into individual fractions according to both the side-chain length and unsaturation can be accomplished using a single purification step on RP-18 (Costantino *et al.*, 1997). However, when the mixture of glycolipids is more complex, as in the case of GSLs isolated from *Agelas* species, HPLC fractions can still be composed of several homologues. Although desirable, separation into individual homologues is not really necessary for structure determination of glycolipids, provided that the polar moiety is the same. Most of the experimental work on glycolipids has been performed on mixtures of homologues, since both chemical and NMR methods for structure elucidation of glycolipids can be used with a mixture of homologues as well, while the nature of the alkyl chains can be determined by chemical degradation.

14.3.2 Structural determination

A prerequisite for the study of natural immunomodulating glycolipids is the availability of an efficient and sensitive method for determining the structure of an unknown glycolipid. To this end, a variety of different techniques have been used, ranging from complex degradations procedures requiring grams of material, to exclusively spectroscopic analyses of 1-mg samples. Of course, immunomodulating glycolipids are by no means special from this point of view, so that the following brief discussion applies to glycolipids in general.

A distinctive feature of the structural determination of glycolipids is that the NMR methods that have been of widespread use for thirty years for organic molecules have been accepted in the glycolipid field only recently. In addition, structural determination of glycolipids often still requires chemical manipulation and degradation, particularly if the separation procedure could only provide a mixture of homologues (*see* earlier). As a general rule, determination of the structure of the saccharide chain and the non-polar aglycone are performed separately.

For characterization of the sugar chain, a well-established chemical procedure exists. The starting point is the identification of the monosaccharide units present in the molecule. This can be achieved by acid methanolysis of the glycolipid (Sweeley and Tao, 1972) followed by quantitative analysis of the obtained methyl monosaccharides using the chromatographic methods currently employed in carbohydrate chemistry. The subsequent step of the investigation is the identification of the position of the inter-unit

linkages and the sugar-aglycone bond, using Hakomori's method (Hakomori, 1964) or its modification by Sanford and Conrad (1966). The glycolipid is permethylated and subjected to acid methanolysis; the partially methylated monosaccharides are reduced to the corresponding alditols and acetylated, and then identified and quantitized through GC or GC-MS methods. Basically, this method allows discrimination between free hydroxyl groups (which are methylated in the alditol) and those involved in glycosidic bonds (acetylated in the alditol), providing useful data to determine glycosylation sites and furanose or pyranose structures of each monosaccharide in the carbohydrate chain. In simple cases, the sequence of the sugar units in the oligosaccharide chain can also be inferred. Otherwise, enzymatic or mild chemical partial hydrolysis of the glycolipid can be carried out, producing simpler glycolipids and oligosaccharides which are separated chromatographically and analyzed with the same methods. Alternatively, useful information on the sugar sequence has been obtained from the interpretation of fragmentation in the mass spectrum, usually performed on the native glycolipid using the FAB technique (Kawano *et al.*, 1990).

Finally, the configuration of the glycosidic bonds can be ascertained chemically by enzymatic hydrolysis with stereoselective glycosidases, or by oxidation with chromium trioxide, which for a sugar in the pyranose form is much more effective if the glycosidic bond is oriented equatorially (Laine and Renkonen, 1975).

This chemical procedure works well and has been widely used, but it is quite time-consuming and requires at least several milligrams of glycolipids, which are consumed in the analysis. In addition, reference samples of partially methylated alditols are required, and this can be a problem if uncommon sugars are present. Therefore, non-destructive NMR techniques, which do not require standards, have been developed.

The first reports of the use of NMR spectroscopy on glycolipids, long before a complete structural elucidation based on NMR became feasible, were directed toward the determination of anomeric configurations. In fact, anomeric protons can be readily identified even on a low-field spectrometer, and their coupling constants are a clear indication of anomeric configuration of a pyranose sugar. Since 1992, structural determinations of the whole hydrophilic portion of marine glycolipids (carbohydrate chain and functionalized part of the aglycon) performed through extensive NMR analysis appeared in the literature (Inagaki *et al.*, 1992). Determination of the planar (i.e. without stereochemistry) structure of the polar part of a glycolipid is made in the same way as for any other organic compound (except for the difficulties arising from overlapping signals, *see* below) and will not be discussed here. However, for a full elucidation of the structure of an oligosaccharide chain, determination of the stereochemistry is of crucial importance, because many sugars differ from each other only in their stereochemistry. In many cases, this can be unambiguously done from ^{1}H NMR experiments, provided that all proton resonances can be assigned to the relevant protons. The most favorable case is that of pyranose sugars, because they exist in the chair conformation so that axial

and equatorial protons can be readily distinguished on the basis of their coupling constants. Even sugars in the furanose form, however, can be characterized on the basis of NOE effects arising between protons in the *cis*-1,3 relationship, as shown by NOESY or ROESY experiments (Cafieri *et al.*, 1994).

A frequent problem with this approach is that sugar protons resonate in a quite narrow region of the spectrum, so that signal overlap is very likely. This is an obstacle to the assignment of resonances, and in addition, prevents the observation of signal multiplicities and the measurement of coupling constants. Signal overlap can be dramatically reduced using peracetylated glycolipids because of the better proton dispersion in their ^{1}H-NMR spectrum. An additional advantage of this technique is the easy discrimination between the resonances of ether and ester oxymethines proton because of their different chemical shift ranges (δ 3.5–4.5 and 4.7–5.7, respectively), thereby providing the same information as Hakomori's method (Costantino *et al.*, 1995a). Finally, if no further ester functionality is present in the glycolipid (as it happens for sphingolipids, but not for glycerolipids) the peracetylated glycolipid can be turned back to its natural structure using base-catalyzed methanolysis (Costantino *et al.*, 1997). An alternative (or additional) way to deal with signal overlap is the use of the HOHAHA experiment, which is particularly suitable for oligosaccharides, because every sugar gives rise to an isolated spin system. If the resonance of at least one proton of a monosaccharide is not overlapped, the slice of the HOHAHA spectrum at the chemical shift of that proton displays only resonances of protons belonging to the pertinent sugar, so that their multiplicities can be discerned and coupling constants measured (Costantino *et al.*, 1993). Because only small amounts of glycolipids are usually available, the determination of absolute stereochemistry of sugars would be difficult, at best. Moreover, spectroscopic methods to determine the absolute configuration of the sugars of a native glycolipid have not yet been developed, so it is often simply assumed that the sugars are in their naturally occurring configuration.

As for the structural determination of the hydrophobic portion of a glycolipid, it is strongly dependent on the class of glycolipids studied, but a few generalizations can be made. First of all, chemical degradation is unavoidable in many cases, particularly if the material to be investigated is a mixture of homologues differing in the lipid part of the molecule. NMR spectroscopy fails when used to study long alkyl chains, because, both in ^{1}H and in ^{13}C NMR spectra, signals of methylene groups are nearly coincident. However, NMR spectra provide valuable information about the presence (but not the position) of methyl branching on the chain. Mass spectroscopy can, of course, be used for determining the length of alkyl chains, but because most glycolipids have more than one alkyl chain, this technique allows determination only of the total number of carbon atoms in the chains, not their distribution along the chains. As an exception, the fragmentation peaks observed in the negative-ion FAB mass spectra of some glycosphingolipids have been used to address this point (Matsubara and Hayashi, 1993).

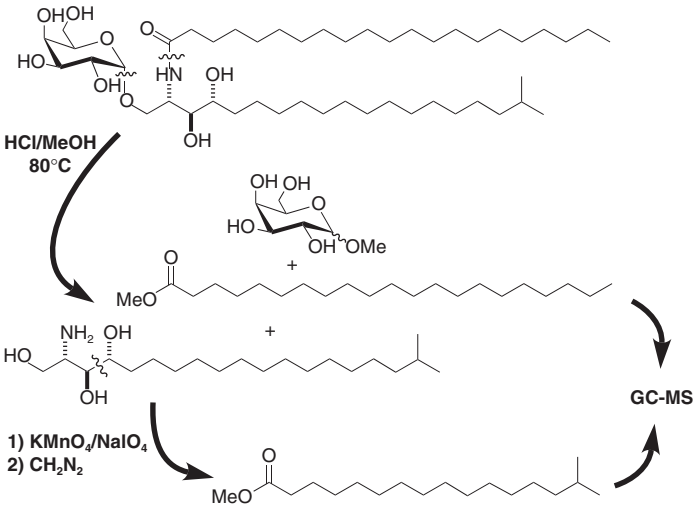


Figure 14.12: Degradation analysis for characterization of alkyl chains of glycosphingolipids.

Chemical degradation of glycosphingolipids, the most common immunomodulating glycolipids, follows a widely-accepted procedure. Treatment with acidic methanol causes the glycosphingolipid to be decomposed into the component sphingoid base, fatty acid methyl ester (often bearing a 2-OH group), and methyl glycosides, which are separated by partitioning and/or chromatography (Gaver and Sweeley, 1965). If a mixture of homologous GSLs is treated in the same manner, mixtures of sphingoid bases and fatty acid methyl esters will result. LCBs are identified and quantitized as appropriate derivatives by GC analysis. If reference compounds are not available, the identification can be accomplished by periodate/permanganate oxidation followed by methylation (Costantino *et al.*, 1994), or by periodate oxidation (Kubo *et al.*, 1992) of the sphingosine homologues, and gas-chromatographic identification of the resulting fatty acid methyl esters or aldehydes, respectively. GC or CG-MS analysis is also used for identification of fatty acid methyl esters.

For immunomodulating glycolipids with atypical structures, some rather complex microscale methods have been developed (e.g. Costantino *et al.*, 1997). The rationale behind all these methods is the attempt to convert the aglycone part of the glycolipid to a fatty acid methyl ester, which can be confidently identified by GC-MS because the fragmentation pattern of this class of compounds is very well known.

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CHAPTER

15

Metabolites of Marine Opisthobranchs: Chemistry and Biological Activity

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Contents

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15.1 INTRODUCTION

Opisthobranchs are marine gastropod molluscs, the shell of which is either reduced or completely absent. Many chemical studies have been performed in order to investigate their surprising immunity from predators. An annual summary of the chemical literature on marine organisms is provided by Faulkner (e.g. 2000) and includes studies of opisthobranch molluscs. The same author also discussed their chemical defense (Faulkner, 1992). Ecological aspects were discussed, first by Karuso (1987), and then by Cimino and Sodano (1989) for nudibranchs, by Carefoot (1987) and by Yamada and Kigoshi (1997) for anaspideans. Faulkner and Ghiselin (1983) were the first to link chemistry of opisthobranchs to evolution; more recently, evolutionary aspects were also discussed by Cimino and Ghiselin for sacoglossans (1998) and dorid nudibranchs (1999). Biosynthetic studies were summarized by Cimino and Sodano (1993), by Garson (1993) and by Davies-Coleman and Garson (1998). Dietary relationships with sponges were reviewed by Cimino and Sodano (1994), and finally, very recently, a complete overview of the chemistry and ecology of sacoglossans and dorids was published (Cimino *et al.*, 1999).

Monographs and faunas (Thompson, 1976; Riedl, 1983; Sabelli *et al.*, 1990) give somewhat different classifications of the Opisthobranchia. In this paper, as well as in our last review (Cimino *et al.*, 1999), we have followed the classification recently proposed by Rudman and Willan (1998), which is outlined in Figure 15.1. According to this arrangement, opisthobranchs are split into nine orders: Cephalaspidea, Anaspidea, Notaspidea, Sacoglossa, Nudibranchia, Acochlidea, Rhodopemorpha, Thecosomata and Gymnosomata. The main orders are the first five. In this paper we will present a general overview of the chemistry for Cephalaspidea, Notaspidea and Anaspidea. On the other hand, our review of Nudibranchia continues Karuso's coverage (1987) but excludes dorids, recently discussed (Cimino *et al.*, 1999) along with species belonging to the Sacoglossa. The pioneer chemical studies of Gymnosomata will be reported here. As in the previous review, general morphological and biological descriptions of the different groups of opisthobranchs have been reported here following Beesley *et al.* (1998).

15.2 ORDER CEPHALASPIDEA

Cephalaspidean opisthobranchs are characterized by a remarkable trapezoidal cephalic shield useful for burrowing in muddy buttoms. All specimens are shelled, even though the shell may be conspicuous, thin and fragile, or internal. Finally, some cephalaspideans lose the shell when mature. All these characteristics may support an evolutionary continuum from completely shelled snails to shell-less sea slugs. A majority of cephalaspideans are carnivorous, feeding upon molluscs, polychaetes and foraminiferans from shallow marine environments. However, within Cephalaspidea, species belonging to

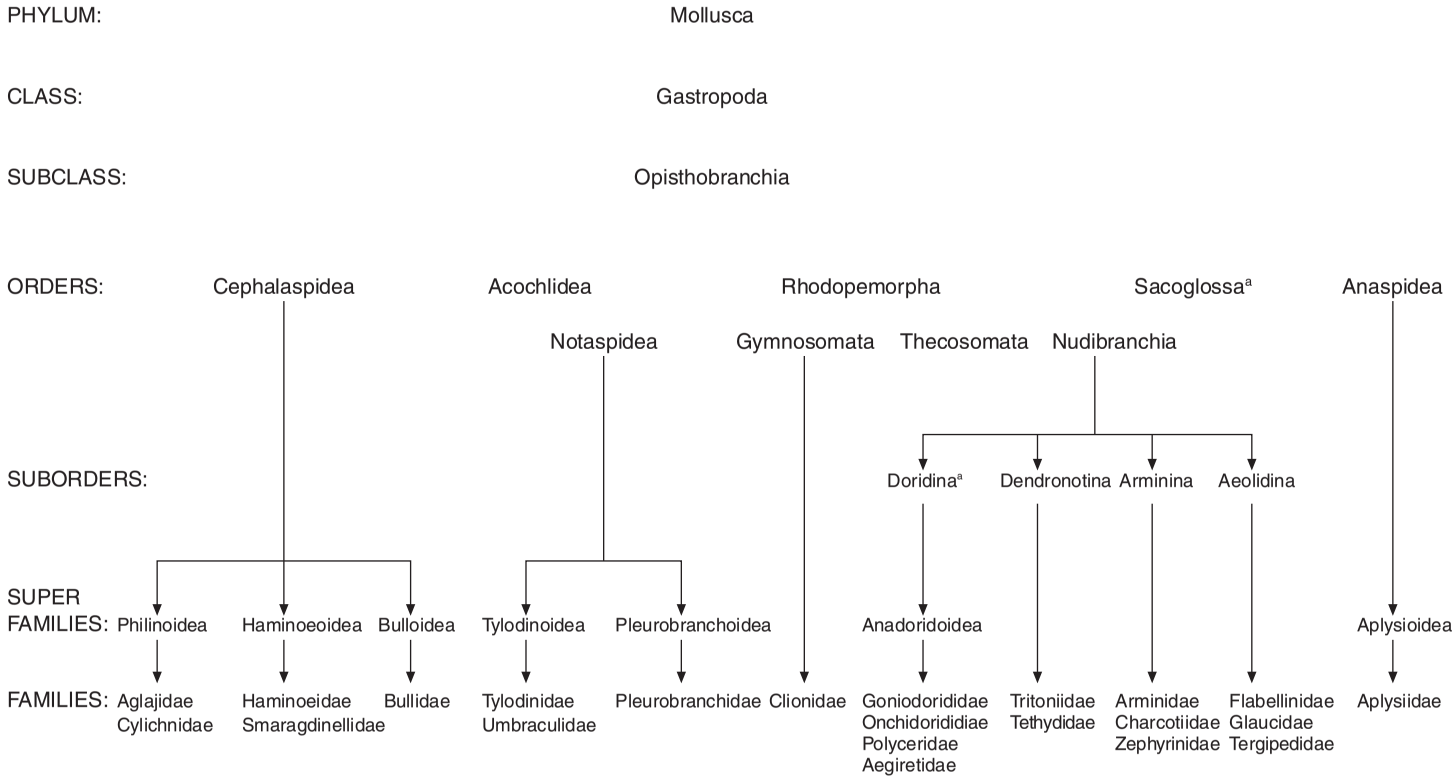


Figure 15.1: Summary of opisthobranch families discussed in this paper. The arrangement is according to Rudman and Willan (1998).

[a] The families belonging to the order Sacoglossa and to the superfamily Eudoridoidea have been treated in our previous work (Cimino *et al.*, 1999).

three of the eight superfamilies (Haminoeoidea, Bulloidea and Runcinoidea) are herbivorous and, because of their dietary habit, are often victims of the carnivorous species.

According to Burn and Thompson (1998), Cephalaspidea comprise eight superfamilies: Actenoidea, Ringiculoidea, Cylindrobulloidea, Diaphanoidea, Philinoidea, Haminoeoidea, Bulloidea and Runcinoidea. Even though chemical studies have been reported for species belonging to only three superfamilies (Philinoidea, Haminoeoidea and Bulloidea), they offer an intriguing picture of the fundamental ecological role played by chemical compounds in the marine environment.

15.2.1 Superfamily Philinoidea

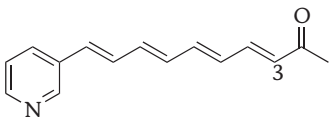
All philinoideans are carnivorous, and sometimes cannibalistic, molluscs. Some species are shelled, whereas others possess an extremely reduced shell. In this case, the absence of the encumbering weight gives an extraordinary agility to the molluscs, conducive to hunting. This peculiarity prompted the interest of chemists to investigate the secondary metabolites of molluscs in the family Aglajidae, some of which, e.g. *Navanax inermis*, are known for their great voracity (Paine, 1963).

15.2.1.1 Family Aglajidae

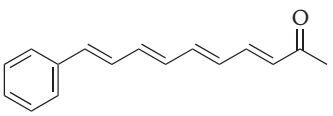
In 1977, Sleeper and Fenical published a milestone paper describing three chemical components, navenones A–C (**1–3**), from the Pacific aglajid *Navanax inermis* (Sleeper and Fenical, 1977). The three compounds were isolated from a specialized gland, the 'yellow gland', and displayed alarm pheromone properties. Collectively these compounds induce an avoidance-alarm response in a trail-following *Navanax* at the concentration of 1×10^{-5} M. In fact, navenones were secreted by the animals into their slime trail in order to communicate the presence of predators to their conspecifics. Subsequently, Fenical's group reported, along with the structures of the co-occurring 3-methyl derivatives of **1** and **2**, a biosynthetic experiment with ^{14}C-labelled sodium acetate which was injected into the food of *N. inermis*, and resulted, after two days, in moderate radioactivity of the navenones (Fenical *et al.*, 1979). Finally, the same group rigorously proved (Sleeper *et al.*, 1980) the alarm response induced by navenones and, since *Navanax* are cannibalistic, excluded, on the basis of relevant ecological experiments, the possibility that these molecules play a deterrent role against another trail-following *Navanax*. The poor incorporation observed in the biosynthetic experiments did not exclude the possibility that navenones were originated, and were biosynthetized, by other cephalaspideans, e.g. *Bulla gouldiana* and *Haminoea virescens*, which were used as food to maintain *Navanax* in aquarium.

In 1985, Scheuer's group published two papers on the chemical constituents of the Hawaiian aglajid *Philinopsis speciosa*. A pyridine derivative substituted at C-2 by a bicyclic polyketide-derived C_{16}-alkadienone, (−)pulo'upone (**4**), was characterized

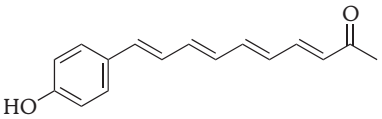
(Coval and Scheuer, 1985) together with a pair of polypropionate metabolites, niuhinone-A (**5**) and niuhinone-B (**6**) (Coval *et al.*, 1985). This latter compound exhibited toxicity at 24.6 ppm in a brine shrimp assay. The absolute stereochemistry of **4** was confirmed by a series of syntheses (Oppolzer *et al.*, 1988; Sugahara *et al.*, 1989; Matikainen *et al.*, 1993). Surprisingly, a metabolite, aglajne-1 (**7**), closely related to **6**, was also found (Cimino *et al.*, 1985) in the Mediterranean *Philinopsis* (=*Aglaja*) *depicta*. The dietary origin of **7** was proved by a comparative study of the secondary metabolites present in *P. depicta* and in its prey, the herbivorous shelled bulloid *Bulla striata* (Cimino *et al.*, 1987a). Both molluscs contained **7** and two other related polypropionates, aglajne-2 (**8**) and aglajne-3 (**9**). This finding prompted a reinvestigation of the predator–prey relationship between the Pacific pair *N. inermis* and *Bulla gouldiana* (Spinella *et al.*, 1993a). The parallelism with the Mediterranean pair was evident. In fact, both animals contained the known polypropionate **6**, the related γ-hydroxy-α-pyrone (**10**) and a 2-pyridine derivative, isopulo'upone (**11**), isomer of **4**. Compounds **10** and **11** were ichthyotoxic to mosquito fish *Gambusia affinis* at 10 ppm, whereas the toxicity (LD_{50}) to brine shrimp is 0.88 and 2.2 ppm, respectively. On the basis of these proven prey-predator relationships, *Philinopsis* and *Navanex* feeding on herbivorous *Bulla*, an analogous dietary habit could be suggested for other *Aglajidae* molluscs including *P. speciosa*.



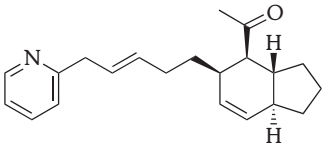
navenone-A (**1**)



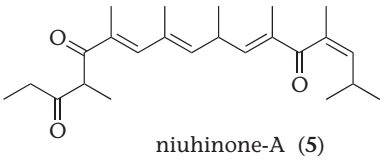
navenone-B (**2**)



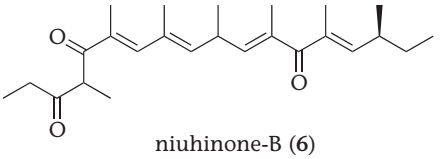
navenone-C (**3**)



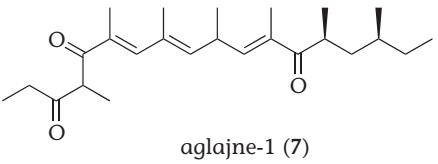
pulo'upone (**4**)



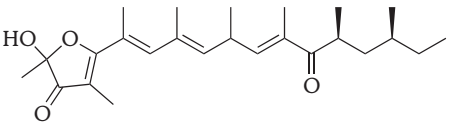
niuhinone-A (**5**)



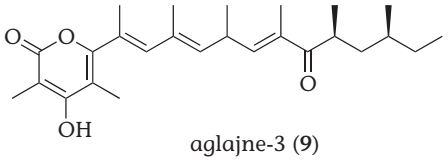
niuhinone-B (**6**)



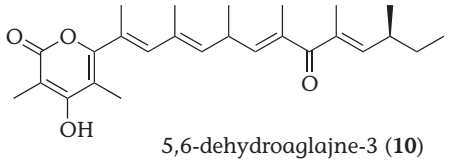
aglajne-1 (**7**)



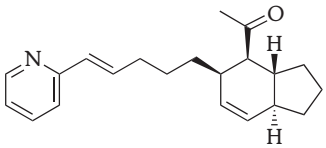
aglajne-2 (**8**)



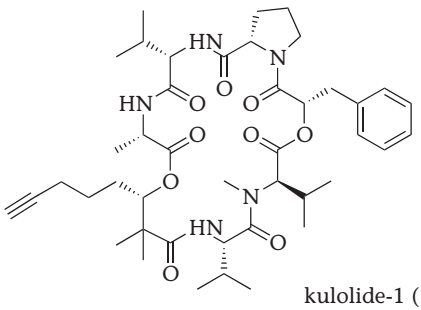
aglajne-3 (**9**)



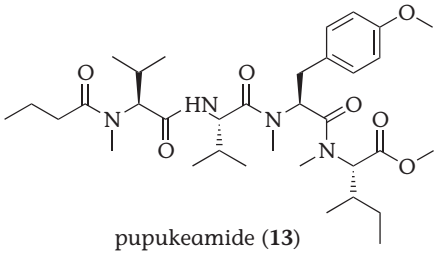
5,6-dehydroaglajne-3 (**10**)



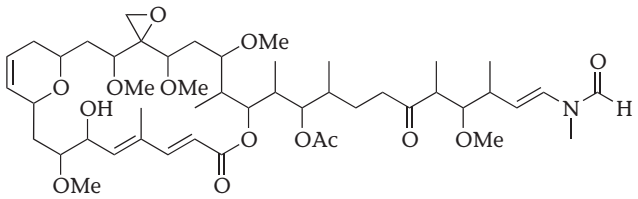
isopulo'upone (**11**)



kulolide-1 (**12**)



pupukeamide (**13**)



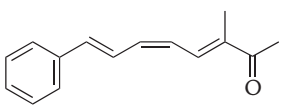
tolytoxin 23-acetate (**14**)

However, some recent studies by Scheuer's group have introduced intriguing news. First, a cytotoxic depsipeptide, kulolide-1 (**12**) (Reese *et al.*, 1996), then a linear tetrapeptide, pupukeamide (**13**) (Nakao *et al.*, 1996), and finally a series of metabolites related to **12** together with an unprecedented macrolide, tolytoxin-23-acetate (**14**) (Nakao *et al.*, 1998) were characterized from the polar extracts of *P. speciosa*. Kulolide-1 (**12**) possesses antitumor properties against L-1210 (0.7 μg/mL) and P388 (2.1 μg/mL) leukemia cells. The structural analogies with known metabolites suggested an origin for all these metabolites from a blue-green alga (cyanobacterium). It was proved that the transfer from the alga to the carnivorous mollusc was mediated by other herbivorous molluscs, i.e. the sea hares *Stylocheilus longicauda* and *Dolabella auricularia*. In fact, both animals were readily accepted by *P. speciosa* when feeding experiments were carried out in the laboratory (Nakao *et al.*, 1996).

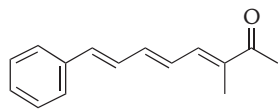
15.2.1.2 Family Cylichnidae

The family Cylichnidae displays some very primitive features. The shell, strongly calcified and external, generally offers a safe refuge to the animals accommodating their complete retraction. A unique exception is the shell of *Scaphander lignarius* which can only partially house the mollusc.

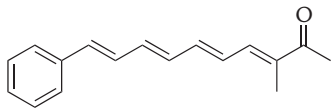
Until now, chemical studies of molluscs belonging to this family were limited to the Mediterranean *Scaphander lignarius* (Cimino *et al.*, 1989a). Two ω-phenyl conjugated trienones, lignarenone-A (**15**) and lignarenone-B (**16**), were isolated from the mantle extract of the mollusc. Both compounds are structurally related to 3-methylnavenone-B (**17**), a minor component of the alarm pheromone mixture of *N. inermis*. This analogy prompted interest in investigating the alarm pheromone properties of lignarenones. Unfortunately, aquarium experiments were unsuccessful, probably because of the difficult adaptation of the molluscs which were collected by dredging at a depth of 80–100 m. However, following a paper by Perrier and Fischer (1908), which described two defensive glands of *S. lignarius*, an anatomical study (Marin *et al.*, 1999) led to localization of lignarenones in a specific structure of the mantle, the intrapallial gland. This location should support the hypothesis that the carnivorous *S. lignarius* does not sequester navenones from its prey.



lignarenone-A (**15**)



lignarenone-B (**16**)



3-methylnavenone-B (**17**)

15.2.2 Superfamily Haminoeoidea

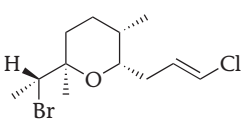
The shell of many Haminoeoidea molluscs is very reduced, thin and fragile, and shows clear evolutionary progression from heavy-shelled primitive forms. All haminoeoideans are herbivorous (Rudman, 1971). Until now, the species, which have undergone chemical studies, belong to only two families, Haminoeidae and Smaragdinellidae.

15.2.2.1 Family Haminoeidae

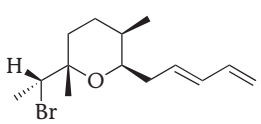
The chemical study of *Haminoea cymbalum* (Poiner *et al.*, 1989), from Guam, led to the characterization of the rearranged trisnorsesquiterpene, kumepaloxane (**18**), which is

exuded in the mucus of the animals, if molested, and possesses feeding deterrent properties against generalist carnivorous fishes. It is interesting to observe that halogenated tetrahydropyranyl sesquiterpenes are quite rare in the marine environment. Metabolites related to **18** were found in the red algal genus *Laurencia* and, surprisingly, also in the Australian sponge *Haliclona* sp. (Capon *et al.*, 1982). Both organisms seem to be an unlikely prey of *H. cymbalum*. Unpublished results of our group, in collaboration with an Indian team in Goa, led us to characterize, from a population of *H. cymbalum* which was collected off Mandapam, a metabolite (**19**) identical in all aspects with one of the described sponge metabolites (Capon *et al.*, 1982). This finding could suggest a misleading dietary relationship. In fact, it seems unlikely that **19** is sequestered by *H. cymbalum* from a dietary sponge.

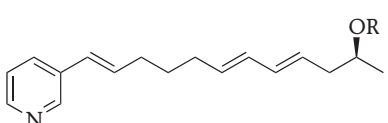
An extensive study (Cimino *et al.*, 1991a; Spinella *et al.*, 1993b; Marin *et al.*, 1999) of many Mediterranean species belonging to the genus *Haminoea* (*H. navicula*, *H. fusari*, *H. orteai*, *H. orbignyana*) led us to characterize a series of 3-alkylpyridines, named haminols, structurally related to the already described navenone-A (**1**). All haminols, e.g. haminol-A and -B (**20** and **21**), are characterized by a linear alkyl chain containing twelve carbons and an hydroxy, or acetoxy, function at C-2 and, in a single case, an acetoxy at C-1. The haminols are characterized by different distribution of the two (or three) unsaturations, along the chain. The absolute stereochemistry at C-2, determined by applying Mosher's method (Spinella *et al.*, 1993b), was *S* for all haminols. The metabolic patterns were characteristic for each species and this method could make a useful contribution to clarifying the complex taxonomy of this genus. Aquarium experiments rigorously proved that all haminols possess alarm pheromone properties. Sand (50 mg), containing haminols (0.1–1 mg), was placed on the trail of a *Haminoea* specimen. A clear escape reaction was recorded when a trail-following specimen reached the treated sand. Following up on this ecological role, an anatomical study localized haminols only in some external parts of the animals and, in particular, in the lateral parapodia (Marin *et al.*, 1999). The minor active concentrations of haminols ranged from 0.1 to 0.7 mg in 50 mg



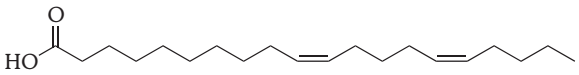
kumepaloxane (**18**)



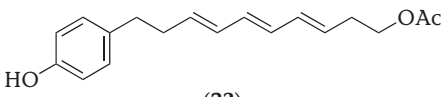
(**19**)



haminol-A R = H (**20**)
haminol-B R = Ac (**21**)



10,15-eicosadienoic acid (**22**)



(**23**)

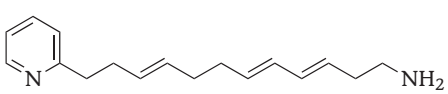
of sand. However, the acetyl derivatives were always two- to three-fold more active than the corresponding alcohols.

In conclusion, haminols seem to be chemical markers of all Mediterranean *Haminoea*. However, there are some conflicting data. They were not found in *Haminoea templadoi* (Carballeira *et al*., 1992), which contains the unusual 10,15-eicosadienoic acid (**22**), and in *Haminoea callidegenita* (Spinella *et al*., 1998), which contains a series of alkylphenols, e.g. **23**, structurally related to navenone-C (**3**). In the first case, the prolonged (1 year at −5°C) storage of the molluscs in acetone could have destroyed the unstable haminols. In the second case, it has to be pointed that, prior to this study, *H. callidegenita* was collected only off the Pacific coasts. Probably, it was recently transported from American coasts to Europe as a member of the fouling community on ships' bottoms.

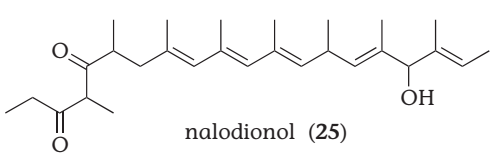
The evident parallelism between the chemical components of the genera *Scaphander* (**15**) and *Haminoea* (**20**, **23**) and those found in *N. inermis* (**1–3**) might justify an alternative origin of navenones without excluding dietary accumulation or bio-transformation of dietary metabolites.

15.2.2.2 Family Smaragdinellidae

Recently, Scheuer's group published the, until now, unique chemical study of a Smaragdinellidae mollusc (Szabo *et al*., 1996). The structural features of the two main metabolites of *Smaragdinella calyculata*, naloamine (**24**) and nalodionol (**25**), are of



naloamine (**24**)



nalodionol (**25**)

extreme interest. In fact, the co-occurrence of 2-alkylpyridines and polypropionates is similar to that recorded in *B. gouldiana* (Spinella *et al.*, 1993a). It might be suggested that, in Cephalaspidea, chemically different molecules play different ecological roles, intra-specific communication for the alkylpyridines, and inter-specific defensive action for the polypropionates. This suggestion seems to be supported by the significant cytotoxicity against P388 mouse leukemia cells (LD_{50} 3.5 μg/mL) possessed by the polypropionate **25**.

15.2.3 Superfamily Bulloidea

Bulloideans are herbivorous and are characterized by a large, thick, ovoid shell. This is the main distinctive characteristic between bulloideans and haminoeoideans, which prevents a common grouping. The superfamily includes only one family, Bullidae, and relatively few species.

15.2.3.1 Family Bullidae

Two unique chemical studies (Cimino *et al.*, 1987a; Spinella *et al.*, 1993a) of *Bulla* have already been discussed, reporting the predator–prey relationships between the two pairs *N. inermis–B. gouldiana* and *P. depicta–B. striata*. Polypropionates (**6–11**) seem to be the chemical marker of these molluscs. Dissection of both *Bulla* species localized the polypropionates in a white gland along the margin of the mantle (Marin *et al.*, 1999). The same paper reports a comparative study of the bioactivities of the main secondary metabolites from cephalaspideans: polypropionates and 3-alkylpyridines. Polypropionates, and in particular aglajne-3 (**9**) and dehydroaglajne-3 (**10**), were very toxic against the mosquito fish *Gambusia affinis* and the brine shrimp *Artemia salina*; in addition, they induced feeding deterrence to *Carassius auratus* but were completely inactive as alarm pheromones. By contrast, haminols were active only as alarm pheromones.

15.2.4 Conclusions

The overview of the chemical components in cephalaspideans opens intriguing perspectives with relevant relationships to many fields beyond chemistry and marine biology. In fact, the order Cephalaspidea, containing species at very different evolutionary stages and with different dietary habits, offers extraordinary models for the investigation of the ecology of marine invertebrates. The unusual metabolites present in cephalaspideans could be, according to Faulkner and Ghiselin (1983), the driving force to move the evolution in a certain direction. The structural features of many metabolites prompt further investigations of their bio-activities and their origin. Even though conclusive biosynthetic experiments are lacking, cephalaspideans could produce many of their chemical compounds. Until now, the chemical scenario was limited to only five of the twenty

cephalaspidean families. However, there is enough information about cephalaspideans to serve as a basis for understanding chemical defence in the other opisthobranch orders and recognizing some characteristics of their ancestral precursors.

15.3 ORDER NOTASPIDEA

The order Notaspidea contains a limited number of species which may directly derive from carnivorous cephalaspideans. The main distinctive feature is a dorso-lateral gill on the right side between mantle and foot of the molluscs. According to Willan (1998a), the order includes two superfamilies: Tylodinoidea and Pleurobranchoidea. The shell is conspicuous, external and limpet-like in the first superfamily, whereas it is either reduced, internal, or absent in the latter. All species are carnivorous, either specific for sponges, soft corals, or ascidians or opportunistic upon a wide range of soft-bodied invertebrates. Notaspideans are generally protected by strong acidic secretions which should exclude other chemical protection. Because of this, only a few chemical studies are reported in the literature. However, recent studies have shown that unusual molecules are present in the skin of many notaspideans.

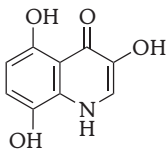
15.3.1 Superfamily Tylodinoidea

Tylodinoideans display very primitive features. All are shelled and feed on sponges. There are two families: Tylodinidae and Umbraculidae. Both have been subjected to chemical studies.

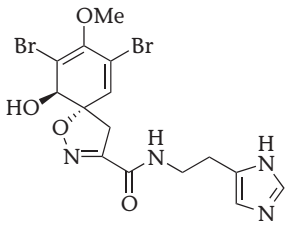
15.3.1.1 Family Tylodinidae

The main genus of this family is *Tylodina*, with four species distributed in restricted areas: *T. perversa* in the eastern Atlantic and Mediterranean, *T. americana* in the western Atlantic, *T. fungina* in the eastern Pacific, *T. alfredensis* in southern Africa.

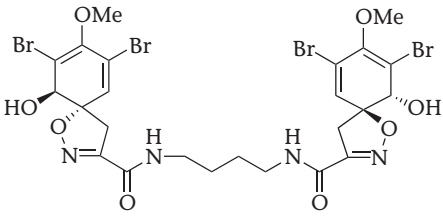
The Mediterranean *T. perversa* lives closely associated with the Aplysinellidae sponge *Verongia* (=*Aplysina*) *aerophoba*. The transfer of the main secondary metabolites from the sponge to the mollusc, includes (Cimino and Sodano, 1994) that of the pigment, uranidine (**26**) (Cimino *et al.*, 1984), which is responsible for the rapid blackening observed when the yellow sponge, or the nudibranch, is exposed to air. More recently (Teeyapant *et al.*, 1993), the secondary metabolites from the pair *T. perversa–V. aerophoba* from the Canary Islands, were analyzed. The pattern of brominated compounds, e.g. aerophobin-1 (**27**), was almost superimposable, with one exception due to the presence, only in the mollusc, of aerothionin (**28**). Although the authors suggested that the notaspidean could bio-modify brominated sponge constituents, an origin of **28** from another Aplysinellidae sponge cannot be excluded.



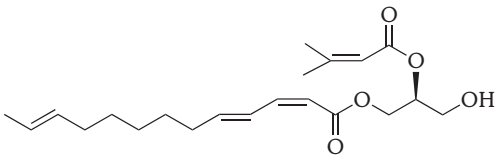
uranidine (**26**)



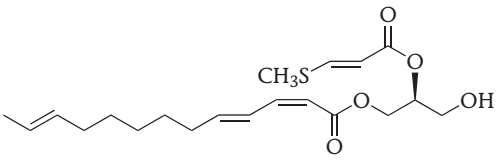
aerophobin-1 (**27**)



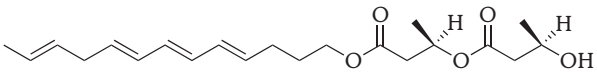
aerothionin (**28**)



umbraculumin-A (**29**)



umbraculumin-C (**30**)



umbraculumin-B (**31**)

15.3.1.2 Family Umbraculidae

This monotypic family comprises *Umbraculum umbraculum*. The notaspidean is very large, up to 28 cm in diameter, and is covered by a flattened shell. Its diet includes many demospongiid sponges with the surprising exception of those belonging to the orders Verongida and Dictyoceratida.

A large (20 cm in diameter) specimen of *Umbraculum umbraculum* (=*mediterraneum*) was found hidden in a specimen of the Mediterranean sponge *Geodia cydonium*. The skin of the mollusc contained three metabolites essentially absent in the sponge: two ichthyotoxic diacylglycerols, umbraculumin-A (**29**) and umbraculumin-C (**30**) (Cimino *et al.*, 1988), along with an inactive 3-hydroxybutyric ester, umbraculumin-B (**31**) (Cimino *et al.*, 1989b). Umbraculumin-A (**29**) and umbraculumin-C (**30**) proved to be very toxic to the mosquito fish *G. affinis* at 10 and 0.1 μg/mL, respectively. The absolute stereochemistry of **29** and **30** was proposed based on chemical methods (Gavagnin *et al.*, 1990) and confirmed by two elegant synthetic works (De Medeiros *et al.*, 1990, 1991).

15.3.2 Superfamily Pleurobranchoidea

The reduction of the shell is evident in notaspideans belonging to this superfamily. In fact, the shell is either internal or completely absent. The absence of physical protection

is balanced by a defensive acidic secretion characteristic of all pleurobranchoideans and well described for *Berthellina citrina* (Marbach and Tsurnamal, 1973). Epidermal cells all over the Notaspidean's surface secrete an acid mixture (pH 1) which protects living molluscs from potential predators (sea anemones, fishes, crabs). Conversely, dead specimens are immediately devoured.

15.3.2.1 Family Pleurobranchidae

This family includes two subfamilies: Pleurobranchinae and Pleurobranchaeinae. The shell is never present in species belonging to the latter subfamily. In spite of the strong acid secretion, chemical studies of three Mediterranean species led to the discovery of intriguing secondary skin metabolites, whereas a bioactive cyclic peptide was found in an Indonesian species.

15.3.2.1.1 Subfamily Pleurobranchinae

This subfamily includes three genera with different feeding habits: sponges for *Berthellina* species; sponges, soft corals and sea anemones for *Berthella* spp.; and ascidians for *Pleurobranchus* spp.

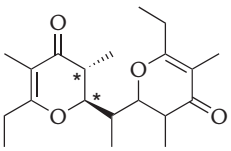
Chemical studies (Ciavatta *et al.*, 1993) of *Pleurobranchus membranaceus* led to the characterization of three unusual polypropionates, e.g. membrenone-C (**32**), present only in the skin of the mollusc. This location strongly suggested a protective role for these compounds even though the scarcity of specimens, which could be found only during nocturnal collections, limited a rigorous and complete evaluation of the bioactivities possessed by membrenones. Surprisingly, the structural features of membrenones are closely related to those of, for example, vallartanone-B (**33**), metabolites found (Manker and Faulkner, 1989; Arimoto *et al.*, 1996) in the Pacific pulmonate *Siphonaria maura*.

Analogously, the skin of *Pleurobranchus testudinarius* contains (Spinella *et al.*, 1997a) two unusual triterpenoids, testudinariol-A (**34**) and its C-10 epimer, structurally related to the defensive allomone, limatulone (**35**), of the limpet *Collisella limatula* (Albizati *et al.*, 1985). The defensive role of **34** was suggested by both its external anatomical location and its presence, as practically the sole component, in the lipid extract of the mucous secretion. It was shown to be active in the ichthyotoxicity test at 10 ppm.

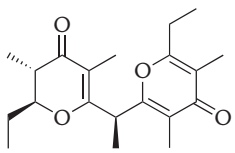
Another Indonesian species, *Pleurobranchus forskalii*, contains a series of cyclic peptides (Wesson and Hamann, 1996). Among these, keenamide-A (**36**), so far the only fully characterized compound, exhibited significant activities against the P-388, A-549 and MEL-20 at 2.5 μg/mL, and HT-29 at 5.0 μg/mL tumor cell lines. This finding supports the validity of our suggestion (Cimino *et al.*, 1999) that knowledge of the chemical components which control simple biological systems could be a productive strategy to detect bioactive natural products.

15.3.2.1.2 Subfamily Pleurobranchaeinae

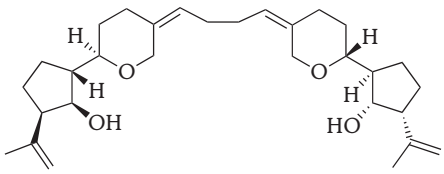
The Mediterranean *Pleurobranchaea meckelii* is a large shell-less notaspidean which accepts in its diet a wide range of soft-bodied invertebrates, hydroids, polychaetes, anemones and also molluscs. Careful extraction of the secondary metabolites from the skin of fresh animals living in different geographical areas, revealed the presence, in all extracts, of a pair of isomeric labdane aldehydes, **37** and its *Z* isomer (Ciavatta *et al.*, 1995), which exhibit clear structural analogies with diterpenoid **38**, a metabolite of the pulmonate *Trimusculus reticulatus* (Manker and Faulkner, 1987) which displays anti-predator properties (Rice, 1985).



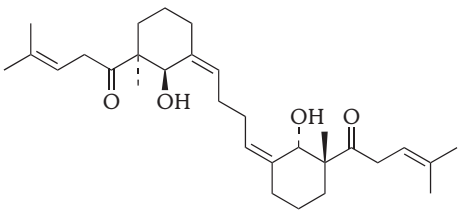
membrenone-C (**32**)
*relative stereochemistry



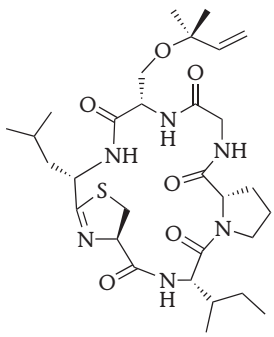
vallartanone-B (**33**)



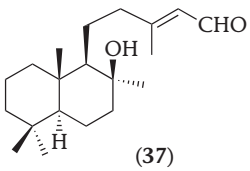
testudinariol-A (**34**)



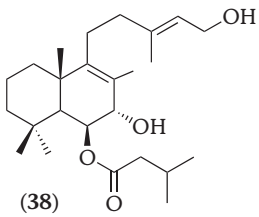
limatulone (**35**)



keenamide-A (**36**)



(**37**)



(**38**)

15.3.3 Conclusions

The order Notaspidea, possessing some very primitive characteristics, should be closely connected with the carnivorous cephalaspideans. The evolutionary reduction of the shell is evident, moving from hard-shelled molluscs to shell-less slugs. In parallel, the

chemical arsenal is constructed, either through the diet by bio-accumulation, or by *de novo* bio-synthesis. This hypothesis has to be proven, but the structural characteristics and the anatomical location of membrenones, testudinariols, and labdane aldehydes, seem to exclude a dietary origin. There could be a different origin for keenamide-A, which, analogously with the peptides found in the cephalaspidean *Philinopsis speciosa*, could be acquired through the food chain. Notaspideans need further investigation in order to clarify the origin of their unusual metabolites but, above all, their ecological role in the marine environment. Thompson (1976) said 'teleost fishes have a common but inexplicable abhorrence of anything tasting acidic'. However, even though all notaspideans are well protected by their acid secretion, recent chemical studies suggest that some of them have also elaborated alternative defensive strategies, which include the use of chemical weapons.

15.4 ORDER NUDIBRANCHIA

Nudibranchs are completely shell-less molluscs. They are classified in four suborders (Willan, 1998b), the holohepatic Doridina, with an unbranched digestive gland, the cladohepatic Aeolidina, with an always branched digestive gland, and finally Dendronotina and Arminina which include species possessing either unbranched or branched digestive glands. Doridina species are characterized by two antero-dorsal chemosensory rhinophores, and by some postero-dorsal gills, which are vital for the mollusc as its respiratory organ. Based on their ability to retract the gills, the Doridina are divided into two superfamilies, the cryptobranch Eudoridoidea and the more primitive phanerobranch Anadoridoidea. The chemical ecology of all nudibranchs was reviewed twelve years ago (Karuso, 1987) while we have more recently surveyed (Cimino *et al.*, 1999) the chemistry of cryptobranch Eudoridoidea, which, among the opisthobranchs, include the largest number of carnivorous, specifically spongivorous, representatives.

In this chapter we will review the chemistry of all nudibranchs published since Karuso's review.

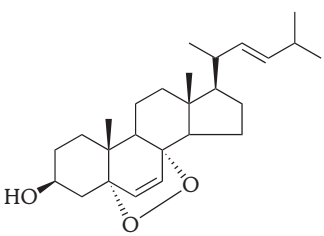
15.4.1 Suborder Doridina (superfamily Anadoridoidea)

As with the previously reported Eudoridoidea (Cimino *et al.*, 1999) all anadoridoideans are highly specialized carnivores, but their prey includes many marine invertebrates such as bryozoans, ascidians, sponges, polychaetes and other opisthobranchs. The molluscs are classified in seven families (Rudman, 1998a), but chemical studies have been reported only for species belonging to four families: Onchidorididae, Polyceridae, Aegiretidae and Goniodorididae.

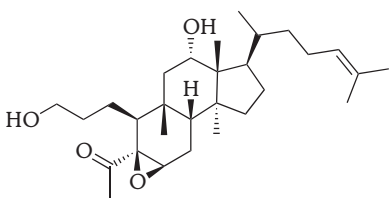
15.4.1.1 Family Onchidorididae

Many species belonging to this family are specialist bryozoan feeders. This is the case with the *Adalaria* genus. A series of sterol peroxides, e.g. **39**, was isolated (Stonard *et al.*, 1980; Gustafson and Andersen, 1985) from a Pacific *Adalaria* sp. More recently, the chemical study (Graziani *et al.*, 1995) of *Adalaria loveni* from Norway resulted in the isolation of lovenone (**40**), a degraded triterpenoid. The unusual structure of lovenone is well documented. However, it is of great interest to discover the origin of this metabolite which, being located only in the skin of the mollusc, should play a relevant ecological role. The authors suggest a dietary bryozoan origin, but a bio-transformation of dietary metabolites with subsequent transfer to the skin cannot be excluded. Lovenone (**40**) exhibited a modest *in vitro* cytotoxicity against human ovarian carcinoma HEY (ED_{50} 11 μg/mL) and human glioblastoma/astrocytoma U373 (ED_{50} 11 μg/mL) cell lines.

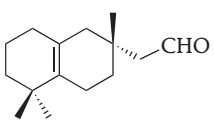
Andersen's group also isolated (Ayer *et al.*, 1984a,b) three odoriferous aldehydes, nanaimoal (**41**), acanthodoral (**42**) and isoacanthodoral (**43**) from *Acanthodoris nanaimoensis*. Acanthodoral (**42**) was also found in *Acanthodoris brunnea* (Faulkner *et al.*, 1990). Structure and absolute stereochemistry of **41** were recently proven by enantioselective total synthesis (Omodani and Shishido, 1994). Finding the three metabolites in all nudibranch populations from different British Columbia sites suggested a probable *de novo* biosynthesis if one accepts the premise that the composition of dietary metabolites should vary in different geographical sites (Faulkner *et al.*, 1990). This hypothesis has recently been confirmed (Graziani and Andersen, 1996a) by biosynthetic experiments with [1,2-$^{13}C_2$]acetate. Incorporation was proven by observing, in the ^{13}C-NMR spectra of aldehydes **41** and **43**, relatively intense doublets accompanying many resonances. This innovative methodology is a powerful tool, not only for the investigation of the



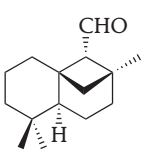
(**39**)



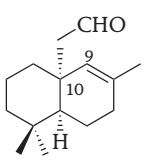
lovenone (**40**)



nanaimoal (**41**)



acanthodoral (**42**)



isoacanthodoral (**43**)

biosynthetic origin of secondary metabolites, but also to clarify some intermediate biosynthetic steps. On the basis of the relative incorporation in the single carbons of isoacanthodoral (**43**), Andersen's group suggested an unprecedented biosynthetic pathway of the sesquiterpenoid skeleton via cyclization of a monocyclofarnesane precursor and subsequent formation and opening of a cyclobutane ring which included the carbons at positions 9 and 10.

15.4.1.2 Family Polyceridae

The molluscs belonging to this family possess elongated, limaciform bodies with a greatly reduced mantle skirt. All species belonging to the two subfamilies, Polycerinae and Triophinae, feed on encrusting or erect bryozoans, whereas the Nembrothinae (subfamily) species exhibit different feeding habits, i.e. on arborescent bryozoans (*Tambja*), on ascidians (*Nembrotha*), and on the nudibranch *Tambja* (*Roboastra*).

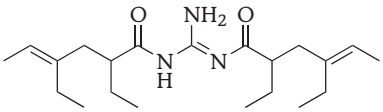
15.4.1.2.1 Subfamilies Polycerinae and Triophinae

Andersen's group found, in *Triopha catalinae* (Gustafson and Andersen, 1982) and *Polycera tricolor* (Gustafson and Andersen, 1985), the same symmetric diacylguanidins (triophamine, **44**) whose structure was confirmed by synthesis (Piers *et al.*, 1984). More recently, biosynthetic experiments with stable isotopes led to excellent incorporation in the acyl residues of **44** extracted from *T. catalinae* after treatment with sodium [1,2-$^{13}C_2$]acetate (Graziani and Andersen, 1996b), and sodium [2,3-$^{13}C_2$]butyrate (Kubanek and Andersen, 1997). Symmetrical diacylguanidines seem to be a chemical marker for many of these molluscs. In fact, a metabolite, limaciamine (**45**), closely related to **44**, is found in the skin of *Limacia clavigera* collected in Norway (Graziani and Andersen, 1998).

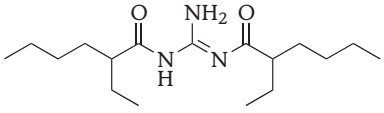
15.4.1.2.2 Subfamily Nembrothinae

Some alkaloids, possessing clear ichthyodeterrent properties, were found (Carté and Faulkner, 1983, 1986) in *Tambjia abdere* and *Tambjia eliora*. The skeleton of these alkaloids, named tambjamines A–D (**46–49**), is characterized by a differently substituted bipyrrole. The same metabolites were found in the preferred food of the mollusc, the bryozoan *Sessibugula translucens*, and also in the carnivorous polycerid *Roboastra tigris* which usually feeds on *Tambjia* spp. A series of rigorous ecological experiments revealed that low concentrations of tambjamines attract *R. tigris*, whereas high concentrations deter the same mollusc.

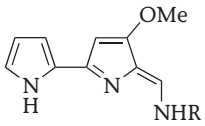
More recently, alkaloids **46** and **48** were found together with the related new tambjamines E–F (**50–51**) in the marine ascidian *Atapozoa* sp. (Lindquist and Fenical, 1991). The same mixture, with the absence of tambjamine-A (**46**) and the presence of the tetrapyrrole **52**, was detected in some nudibranchs of the genus *Nembrotha*, as well as *N. cristata* and *N. kubaryana*, which are well-known predators of the ascidian. An ecological



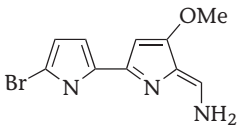
triophamine (**44**)



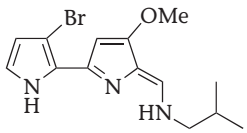
limaciamine (**45**)



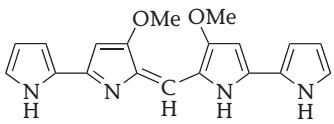
tambjamine-A R = H (**46**)
tambjamine-C R = (**48**)
tambjamine-E R = (**50**)
tambjamine-F R = (**51**)



tambjamine-B (**47**)



tambjamine-D (**49**)



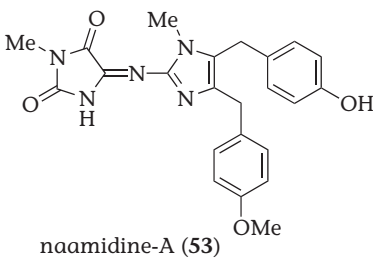
tetrapyrrole (**52**)

study (Paul *et al.*, 1990) of the ascidian *Atapozoa* sp. and its nudibranch predators, *Nembrotha* spp., from many different Indo-Pacific sites, rigorously proved that both animals were effectively protected against a variety of marine carnivorous fishes by tambjamines, whereas the tetrapyrrole **52**, present only in some *Nembrotha* spp., was the most ichthyodeterrent metabolite.

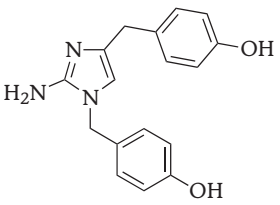
15.4.1.3 Family Aegiretidae

The two genera belonging to this family, *Notodoris* and *Aegires*, are both characterized by a heavily spiculed mantle, absence of a mantle skirt, and feeding habits specialized on calcareous sponges. The sponge–nudibranch relationships have been supported by a series of chemical studies of *Notodoris* species from tropical Indo-Pacific waters.

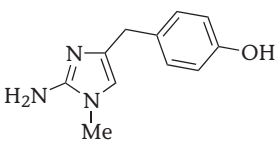
The predator–prey relationship between the nudibranch *Notodoris citrina* and the sponge *Leucetta chagosensis* was proven by the presence of the same mixture of unusual alkaloids in both animals (Carmely *et al.*, 1989). In fact, the chemical analysis of these two Red Sea invertebrates led to nine *p*-hydroxybenzyl derivatives of 2-aminoimidazole



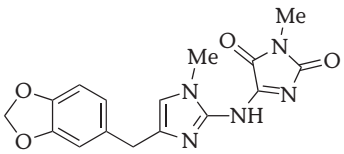
naamidine-A (**53**)



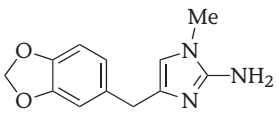
isonaamine-A (**54**)



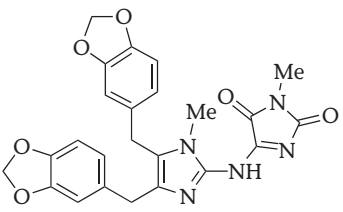
dorimidazole-A (**55**)



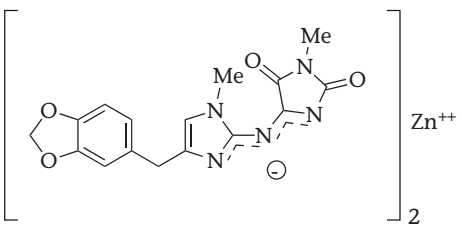
clathridine (**56**)



preclathridine-A (**57**)



clathridine-B (**58**)



Zn-clathridine (**59**)

(e.g. naamidine-A, **53**; isonaamine-A, **54**). Compound **54** was also found, along with the structurally related dorimidazole-A (**55**), in the anthelmintic extract of *Notodoris gardineri* from the Philippines (Alvi *et al.*, 1991). The structure of **55** was confirmed by synthesis but, surprisingly, compound **55** was active in the antiparasitic assay at 50 μg/mL, whereas the synthetic sample was completely inactive. As the NMR spectra of **55** revealed the presence of trace amounts of another compound, the observed bioactivity could be due to this contaminant (Alvi *et al.*, 1991). The selective chemistry of *N. gardineri* was confirmed by the study of a specimen from the Australian Great Barrier Reef (Carroll *et al.*, 1993), which contained clathridine (**56**), a 2-aminoimidazole alkaloid already described from the Mediterranean calcareous sponge *Clathrina clathrus* (Ciminiello *et al.*, 1989). Compound **56** was shown to be weakly cytotoxic to P388, CV1, HT29 and A549 cell lines (IC_{50} 10–20 μg/mL). A *Leucetta* sp. sponge, from the same marine habitat as *N. gardineri*, contained clathridine (**56**) together with a series of 2-aminoimidazole alkaloids (Carroll *et al.*, 1993). Finally, the expected metabolite pattern was also found in three specimens of *N. gardineri* from Papua New Guinea (Alvi *et al.*, 1993). Compound **56** was isolated, together with its simpler derivative **57**, its higher piperonyl homolog **58** and, for the first time in a mollusc, its organometallic congener Zn-clathridine (**59**), previously described for *Clathrina clathrus* (Ciminiello *et al.*, 1989, 1990).

15.4.1.4 Family Goniodorididae

There is only one paper in the chemical literature (McBeth, 1972) concerning members of this family. It reports the isolation and partial characterization of an acetylenic apocarotenoid, with the molecular formula $C_{31}H_{38}O_3$, from *Hopkinsia rosacea*. The pigment, named hopkinsiaxanthin, was also found in the food of the mollusc, the encrusting pink bryozoan *Eurystomella bilabiata*.

15.4.1.5 Remarks on Anadoridoidea

The superfamily Anadoridoidea is of remarkable interest from a chemical point of view. Different families display unique dietary habits but, in order to obtain bioactive molecules, they follow two main strategies, bio-accumulation and *de novo* bio-synthesis. It is difficult to prove, or to exclude, whether some species are also able to bio-modify dietary metabolites. Only some suspected examples, tetrapyrrole **52**, found in the extracts of some *Nembrotha* spp. but absent in their usual diet, could derive from tambjamine-A (**46**), present only in the diet, whereas the presence of compound **57** in *N. gardineri* could be due to bio-transformation of **56**, since **57**, absent in the sponge *Clathrina clathrus*, was obtained by hydrolysis of clathridine (**56**) (Ciminiello *et al.*, 1989).

Further studies are needed to investigate the effective biological role played by the chemical compounds present in the anadoridoidean molluscs; anatomical studies clarifying their distribution in the molluscs could offer extremely useful information.

15.4.2 Suborder Dendronotina

The size of dendronotinan molluscs ranges from a few millimeters to almost thirty centimeters. They can be either large and bulky or small and slender. Distinctive anatomical features are the sheath around the base of the rhinophores and the dorso-lateral appendages (cerata) which generally contain ramifications of the digestive glands and bear the gills. However, not all the dendronotinans are 'cladohepatic'; species belonging to the family Tritoniidae are 'holohepatic' and their cerata form dendritic gills. A majority of dendronotinans feed on cnidarians, in particular, hydroids and alcyonarian soft corals. Different dietary habits are exhibited by molluscs belonging to the family Tethydidae. These animals, generally large and bulky, are without radula and feed on epifaunal or planktonic small crustaceans by using the large mouth as an effective sieve. Ten families of Dendronotina have been recognized by Rudman (1998b), but chemical studies have been reported for only two, Tritoniidae and Tethydidae.

15.4.2.1 Family Tritoniidae

Tritoniids live closely associated with their selected food — alcyonarian soft corals or gorgonians, whose color and shape they often adopt, thus becoming cryptically

undistinguishable. To-date, all chemical studies have rigorously confirmed the dietary relationships between tritoniids and cnidarians.

The nudibranch *Tochuina tetraquetra* is a large dendronotid commonly found (Behrens, 1980) in the cold temperate waters from Alaska to California. Its name is due to the fact that this mollusc, known as 'Tochny', is a culinary delight, eaten raw or cooked, in the Kuril Islands. Two populations of *T. tetraquetra* were collected (Williams and Andersen, 1987) from two different British Columbia sites, Port Hardy and Barkley Sound. The first collections yielded two known soft coral metabolites, rubifolide (**60**) (Williams *et al.*, 1987) and pukalide (**61**) (also isolated from *Sinularia abrupta*; Missakian *et al.*, 1975), together with two new cuparane sesquiterpenoids, tochuinyl acetate (**62**) and its dihydro derivative **63**. The new metabolites (**62** and **63**), along with rubifolide, were also found in the extracts of the soft coral *Gersemia rubiformis* on which *T. tetraquetra* was often found feeding. The skin extract of a second collection contained two metabolites, the known ptilosarcenone (**64**) (from the sea pen *Ptilosarcus gurneyi*; Wratten *et al.*, 1977) and its butanoate analog **65**. Easy formation of **64** by elimination of butanoic acid from the sea pen metabolite **66**, raised suspicion that compounds **64** and **65** could be isolation artifacts. However, rapid work-up of the extracts of freshly collected molluscs failed to detect compound **66** in the skin of *T. tetraquetra*.

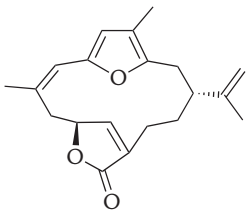
The octocoral *Telesto riisei* contains an impressive series of nineteen functionalized prostanoids (e.g. punaglandin-1, **67**) (Baker and Scheuer, 1994), which display promising anti-inflammatory, antitumor activities. Even though *T. riisei* generally appears to be free of predation, one specimen of a *Tritonia* sp. was collected on colonies of *Telesto* in Papua New Guinea. The ^{1}H-NMR spectrum of the crude methanol extract of the mollusc showed clear punaglandin signals.

The prey–predator relationship between the Antarctic shallow-water nudibranch *Tritoniella belli* and the octocoral *Clavularia frankliniana* was proven by the isolation (McClintock *et al.*, 1994) of chimyl alcohol (**68**) in the extracts of both animals. The authors demonstrated that **68** is an effective feeding deterrent (40% rejection at 0.04 mg/disk) against an omnivorous Antarctic predator, the sea star *Odontaster validus*.

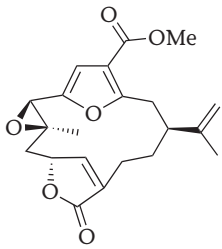
Ecologically relevant experiments proved (Cronin *et al.*, 1995) that the nudibranch *Tritonia hamnerorum* sequesters and concentrates, the furano-germacrene julieannafuran (**69**) from the sea fan *Gorgonia ventalina*, upon which it feeds. Compound **69** effectively protects the nudibranch from the attacks of the common predatory reef fish *Thalassoma bifasciatum*. It is relevant that compound **69** reduced bluehead wrasse feeding only 29% at its natural concentration found in *G. ventalina*, but it resulted in 73% of feeding inhibition at concentrations occurring in *T. hamnerorum*.

15.4.2.2 Family Tethydidae

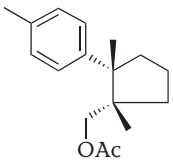
Only two genera, *Melibe and Tethys*, belong to this family. The molluscs are generally large and elongated, attaining twenty centimeters in length. The radula is absent, but



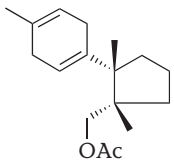
rubifolide (**60**)



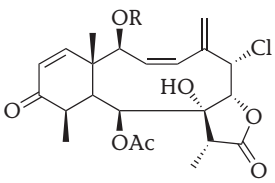
pukalide (**61**)



tochuinyl acetate (**62**)

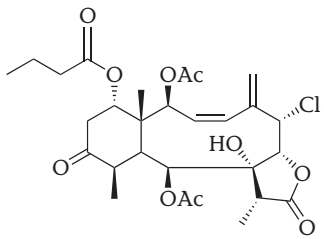


dihydrotochuinyl acetate (**63**)

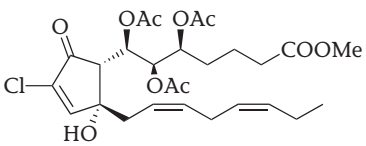


ptilosarcenone R = Ac (**64**)

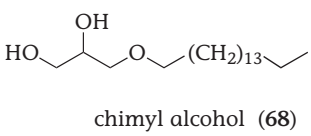
ptilosarcenone butanoate derivative (**65**) R = 



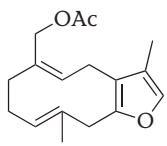
(**66**)



punaglandin-1 (**67**)



chimyl alcohol (**68**)



julieannafuran (**69**)

some chitinous thickening is present in the mouth of some species. The adult specimens are able to swim by lateral flexion of the body, which seems to be an escape strategy when the animal is molested, but the most spectacular response is linked to the detachment of the cerata. The body of these molluscs is surrounded by a series of dorso-lateral cerata, usually without branches of the digestive glands, which are easily lost. After the shedding, the cerata continue to wriggle for many hours thereby distracting predators.

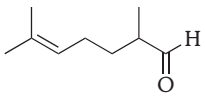
Ayer and Andersen (1983) reported two truncated monoterpenes, 2,6-dimethyl-5-

heptenal (**70**) and the corresponding acid **71** from British Columbian specimens of *Melibe leonina*. Compound **70** is responsible for the characteristic odor of the mollusc.

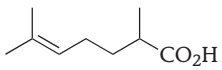
More recently, chemical studies of the Mediterranean *Tethys fimbria* led (Cimino *et al.*, 1989c, 1991b; Di Marzo *et al.*, 1991) to the characterization of many prostaglandin-1,15-lactones (PGL) belonging to the A_2, A_3, E_2, E_3, F_2 and F_3 series (**72–81**). Anatomical dissection revealed different distribution of the PGLs. The PGE lactones (**74–77**) were mainly compartmentalized in the cerata of the mollusc, whereas the mantle contained minor amounts of the PGE lactones and major quantities of the PGF lactones (**78–79**). Lactones **75** and **77** were highly unstable, giving the corresponding PGA lactones (**72–73**) which could be artifacts generated during work-up. Finally, mixtures of fatty-acid esters of the PGF lactones (**80–81**) were concentrated only in the ovotestis and in the eggmass.

A series of biosynthetic experiments (Di Marzo *et al.*, 1990, 1991) was performed by injecting [^{3}H]-labelled arachidonic acid (AA), prostaglandin-E2 (PGE_2) and prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) into the mantle near the upper right gill of a single specimen of *T. fimbria* for each experiment. After one or two days of incubation the metabolites from the mantle and from the cerata were separately analyzed. The experiments proved that the mollusc is able to lactonize AA yielding derivatives of the F and E series. Surprisingly, the PGF derivatives seem to be the precursors of the corresponding compounds of the E series by an unusual oxidation at C-9. Transfer of the PGE lactones from the mantle to the cerata was suggested by the observation (Di Marzo *et al.*, 1991) of opposite trends in the incorporations of the PGE_2 lactones in the mantle, decreasing with time, and in the cerata, increasing with time, when labelled PGE_2 was injected. Further experiments (Cimino *et al.*, 1991c) confirmed this transfer and attempted to investigate the biological role of the PG lactones present in the cerata. Because of their ichthyotoxicity against *Gambusia affinis* (ranging between 1 and 10 μg/mL) a deterrent activity against predators might be suggested but, more likely, these molecules serve more than one purpose. In fact, after detachment of the cerata, the PGE lactones were slowly transformed, by opening of the lactone ring to the corresponding PGE free acids which, by inducing contraction of invertebrate smooth muscle, should favour the prolonged contraction of the cerata. An histological study (Marin *et al.*, 1991) of *T. fimbria* cerata revealed the presence of both longitudinal and transverse smooth muscle fibers. The same paper reports the chemical composition of the mucous secretion which mainly contains PGE and PGA lactones.

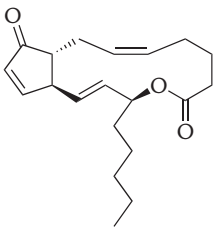
In conclusion, the study of *T. fimbria* is problematic. Starting from an intriguing biological observation, the autotomy of a marine mollusc, protected forms of the bioactive prostaglandins were discovered. In addition, their anatomical distribution suggested different roles for the main components of the cerata, PGE and PGA lactones, and for the related PGF lactones present mainly in the mantle and, as fatty acid esters, in the hermaphrodite gland (ovotestis) and in the eggmasses. A double defensive role, as ichthyotoxins and as protected forms of PGE free acids, was suggested for the PGE lactones. A



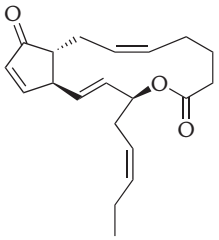
(**70**)



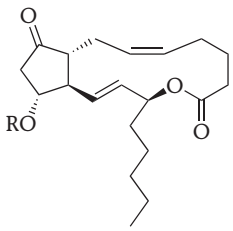
(**71**)



PGA_2-1,15-lactone (**72**)

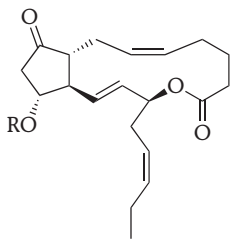


PGA_3-1,15-lactone (**73**)



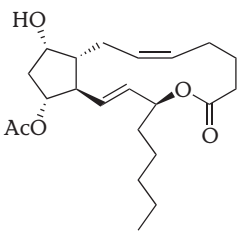
PGE_2-1,15-lactone (**74**) R = H

PGE_2-1,15-lactone-11-acetate (**75**) R = Ac

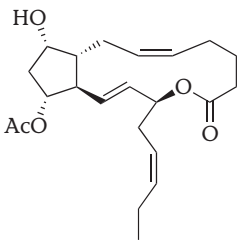


PGE_3-1,15-lactone (**76**) R = H

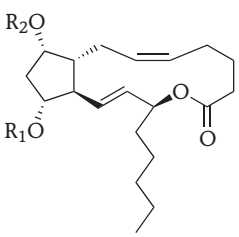
PGE_3-1,15-lactone-11-acetate (**77**) R = Ac



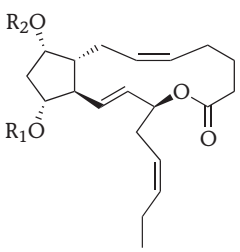
$PGF_{2\alpha}$-1,15-lactone-11-acetate (**78**)



$PGF_{3\alpha}$-1,15-lactone-11-acetate (**79**)



$PGF_{2\alpha}$-1,15-lactone
9- or 11-fatty acid esters (**80**)



$PGF_{3\alpha}$-1,15-lactone
9- or 11-fatty acid esters (**81**)

R_1 = H, R_2 = fatty acid

or

R_1 = fatty acid, R_2 = H

R_1 or R_2 = palmitic + arachidonic + eicosapenta-*cis*-5,8,11,17-enoic + *cis*-docosahexa-4,7,10,13,16,19-enoic acid

key role in the biosynthesis of all *Tethys* PG derivatives was assigned to the mantle PGF lactones. Furthermore, the mantle PGF lactones (**78–79**) might yield the mixtures of fatty acid esters of PGF lactones (**80–81**) present in the reproductive organ, the hermaphrodite gland, and in the eggmasses by acetyl-acyl trans-esterification. A study (Di Marzo *et al.*, 1992) of the early stages of oocyte development prior to formation of larvae (veligera) found that mixtures **80** and **81** were absent in the ovotestis of sexually immature nudibranchs. Furthermore, the PG composition of the eggmasses changed after the first week with decrease of the PGF fatty acid esters and corresponding increase of the PGE lactones **75** and **77**, whose levels were the highest immediately before hatching, sixteen days after the eggs were laid. The extracts of the veligers clearly contained only the mixtures of **80** and **81**.

15.4.2.3 Remarks on Dendronotina

Even though only two Dendronotina families have been studied, the results prompt further studies. Basically, in order to acquire their own chemical arsenal, tritoniids bioaccumulate from dietary soft corals bioactive metabolites, whereas *T. fimbria* is able to bio-synthetize via a unique prostaglandin pathway.

15.4.3 Suborder Arminina

The species belonging to this suborder are neither numerous nor homogeneous. There are six families (Willan, 1998c), but only three of them have been investigated chemically.

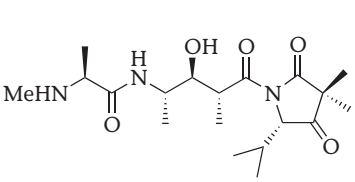
15.4.3.1 Family Zephyrinidae

The species belonging to this family display numerous dorso-lateral cerata which the animal, when disturbed, readily sheds. Generally, these nudibranchs feed upon bryozoans. The only species studied until now (Sodano and Spinella, 1986) is the Mediterranean *Janolus cristatus*, which contains the tripeptide janolusimide (**82**) toxic to mice. The structure of **82** was recently confirmed and its absolute stereochemistry determined by synthesis (Giordano *et al.*, 2000).

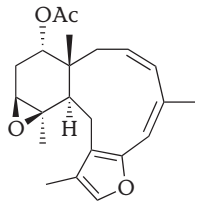
15.4.3.2 Family Arminidae

Arminids are nudibranchs without cerata. Homologously with Zephyrinidae spp., they are 'cladohepatic', their digestive glands extending to the mantle. Generally, they feed upon sea pens.

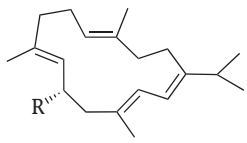
A study (Guerriero *et al.*, 1987) of the Mediterranean *Armina maculata* led to the isolation of the first briarane diterpenoid, verecynarmin-A (**83**), from a mollusc and also the first from a Mediterranean organism. Compound **83** is also present in the extracts of the pennatulacean octocoral *Veretyllum cynomorium*. However, the mollusc is able to concentrate the pennatulacean metabolite. In fact, verecynarmin-A is present in the mollusc at 0.02% dry weight *versus* 0.004% dry weight in the octocoral. Further studies (Guerriero *et*



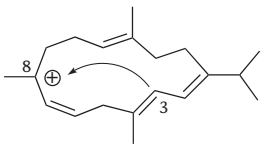
janolusimide (**82**)



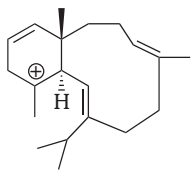
(−)-verecynarmin-A (**83**)



(+)-preverecynarmin (**84**) R = OAc
cembrene-C (**85**) R = H



(**86**)



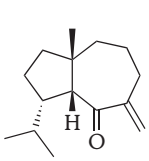
(**87**)

al., 1988, 1990) confirmed this dietary association, since six other additional related briarane diterpenoids were found in both animals. Surprisingly, a typical alcyonacean cembrenoid, preverecynarmin (**84**), was also detected in the pair *V. cynomorium–A. maculata*. In addition, the known compound cembrene-C (**85**) was detected in the extract of the nudibranch. The presence of **85** in the octocoral was strongly suspected even though it was not detected. However, hydrocarbon **85** may be the precursor of all diterpenoids in the pennatulacean, via the carbocation intermediate **86**, followed by nucleophilic attack at C-8 by C-3 leading, via **87**, to all briaranes. This biogenetic hypothesis was suggested by the chemical nature of the dietary metabolites concentrated by the nudibranch *A. maculata*.

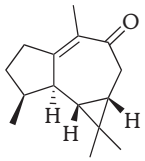
15.4.3.3 Family Charcotiidae

Charcothiid nudibranchs are a small 'cladohepatic' group with an elongated body, similar to that of arminids and tritoniids. The dietary habits of these molluscs are not clear, for instance, it is reported (Gosliner, 1987) that the food of the South African *Leminda millecra* was unknown.

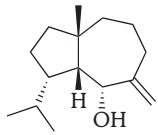
Pika and Faulkner (1994) found, in the extracts of *L. millecra*, four bioactive new sesquiterpenoids: millecrone A (**88**), millecrone B (**89**), millecrol A (**90**) and millecrol B (**91**), with structural features related to those of typical soft coral metabolites. Feeding on soft corals was confirmed by the analysis of the spicules contained in the digestive glands of the nudibranch. Spicules of the soft corals *Alcyonium foliatum*, *A. valdiviae*, and *Capnella thyrsoidea* were found. Millecrone A (**88**) inhibited the growth of *Candida albicans* at 50 μg/disk, millecrone B (**89**) inhibited the growth of both *Staphylococcus aureus* and *Bacillus subtilis* at 50 μg/disk, while millecrol B (**91**) was active against *B. subtilis* at 50 μg/disk.



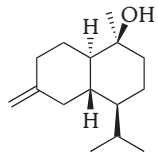
millecrone-A (**88**)



millecrone-B (**89**)



millecrol-A (**90**)



millecrol-B (**91**)

15.4.3.4 Remarks on Arminina

Chemical results from studying species belonging to this suborder establish some dietary habits. It might be interesting to further investigate *Janolus cristatus* in order to clarify if janolusimide (**82**) is obtained, either by bio-accumulation from an unidentified hydroid, or perhaps, from its symbionts, or by *de novo* bio-synthesis.

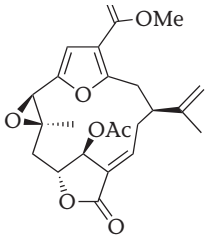
15.4.4 Suborder Aeolidina

Aeolid nudibranchs are elongated molluscs ranging in size from five millimeters to fifteen centimeters. All are 'cladohepatic', with branches of the digestive glands reaching to the top of the numerous cerata. Generally, they are protected by some urticant cells, the nematocysts, which are removed by the nudibranchs from cnidaria during feeding and transferred to the tip of the ceras into a terminal sac, the cnidosac. This is probably the reason that interest was aroused in investigating the chemical compounds from aeolid molluscs. Surprisingly, since Karuso's review (1987) only two chemical papers have been published. Both dealt with species belonging to the family Glaucidae, one of seven aeolid families (Rudman, 1998c).

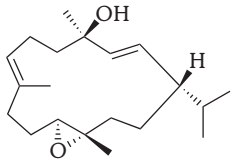
15.4.4.1 Family Glaucidae

Slattery *et al*. (1998) demonstrated, by a series of ecologically relevant experiments, that the aeolid nudibranch *Phyllodesmium guamensis* sequesters from its diet, the soft coral *Sinularia maxima*, diterpenoid **92**, structurally related to pukalide **61**, which is a feeding deterrent for the pufferfish *Canthigaster solandri*. The diterpenoid was mainly concentrated in the cerata. Previously, Coll *et al*. (1985) reported that *Phyllodesmium longicirra* sequesters, and concentrates in the cerata, three cembranoids (e.g. trocheliophorol **93**), from the alcyonarian coral *Sarcophyton trocheliophorum*.

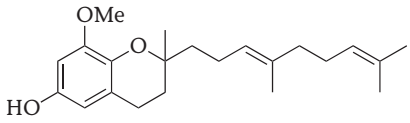
A second chemical study (Ciavatta *et al*., 1996) discovered, in the skin of the Mediterranean *Cratena peregrina*, a new prenylchromanol (**94**), which displayed ichthyotoxicity to *Gambusia affinis* at 10 ppm, along with two related, but previously known marine compounds. All three metabolites were completely absent in the extracts of the hydroid *Eudendrium racemosum*, upon which *C. peregrina* usually feeds.



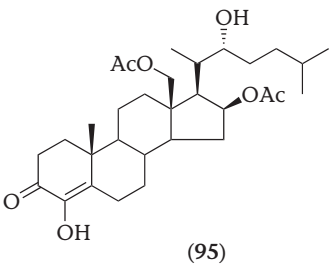
(**92**)



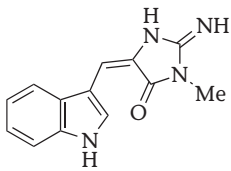
trocheliophorol (**93**)



(**94**)



(**95**)



(**96**)

15.4.4.2 Remarks on Aeolidina

In order to complete this general review of aeolid nudibranchs, something should be said about the previously reviewed chemical studies (Karuso, 1987) which were limited to the families Flabellinidae and Tergipedidae. Three Flabellinidae (Cimino *et al.*, 1980), *Hervia peregrina* (=*Cratena peregrina*, Glaucidae), *Flabellina affinis* and *Coryphella lineata* sequester several hydroxy and acetoxysterols (e.g. **95**) from three related hydroids, *Eudendrium racemosum*, *E. rameum* and *E. ramosum*. The Tergipedidae *Phestilla melanobrachia* contains (Okuda *et al.*, 1982) a series of indole alkaloids (e.g. **96**) derived from its prey, hard corals of the genus *Tubastrea*.

15.4.5 Conclusions

Nudibranchs are classified in four suborders and 34 families. Even though, the spongivorous superfamily Eudoridoidea (suborder Doridina) includes only five families, they account (Cimino *et al.*, 1999) for the majority of chemical studies. On the other hand, only 11 families of the remaining 29 have been studied. Because of this, it is too early to draw general conclusions. Analogously with notaspideans, the carnivorous nudibranchs should derive from ancestral cephalaspideans. Some species feed on infaunal organisms, sometimes also on conspecific molluscs, others on sessile encrusting or arborescent invertebrates, mainly cnidarians, but also sponges, bryozoans and tunicates. The bio-accumulation of dietary metabolites has been rigorously documented for many species in all suborders. Their protective role against common marine predators has been supported by

some ecologically relevant rigorous experiments (McClintock *et al.*, 1994; Cronin *et al.*, 1995; Marin *et al.*, 1991; Slattery *et al.*, 1998). The ability to construct, *de novo*, their own bioactive molecules, by bio-synthesis, has been proven for species belonging to the superfamily Anadoridoidea (families Onchidorididae and Polyceridae) and suborder Dendronotina (family Tethydidae) but probably this capability is also present in the other two superfamilies. Suspected bio-synthesized metabolites could be the tripeptide janolusimide (**82**) found in *J. cristatus* (Sodano and Spinella, 1986) and the prenylchromanol (**94**) from *C. peregrina* (Ciavatta *et al.*, 1996), both absent in the habitual diets of the molluscs.

No clear example of bio-transformation has been reported until now, even though the presence of the ichthyodeterrent tetrapyrrole **52** (Paul *et al.*, 1990) in some *Nembrotha* species, but absent in the dietary ascidians, yet closely related to their other metabolites, is very intriguing.

15.5 ORDER ANASPIDEA

Anaspideans are large molluscs in which the shell can either be external, but thin and fragile, or internal, or even completely absent. All these molluscs contain, in their mantle cavities, two glands: the opaline gland on the lower mantle, which secretes a repugnatory mucous fluid and the so-called purple gland, on the upper mantle, which produces a characteristic fluid derived from pigments of dietary Rhodophyta algae. Anaspideans are easily collectable in every Ocean, because of this, many papers have reported their chemical constituents. Since all anaspideans are strictly herbivorous, many chemical studies revealed typical algal metabolites. The order is split into two superfamilies, Akeroidea and Aplysioidea, both containing one family, Akeridae and Aplysiidae, respectively (Willan, 1998d). No chemical paper reports studies of species belonging to the family Akeridae. It is interesting to note that, moving from Akeridae to Aplysiidae molluscs, a progressive reduction of the shell is observed. In fact, Akeridae anaspideans possess an external, ovate, fragile and more or less transparent shell unable to enclose the whole animal. The shell of the Aplysiidae, always internal, ranges from thick and whorled in the genus *Dolabella*, to thin and plate-like in the genera *Aplysia* and *Syphonota*, to a calcareous wedge in the genera *Dolabrifera*, *Petalifera* and *Phyllaplysia*, and finally to the completely absent after metamorphosis in the genera *Stylocheilus*, *Bursatella* and *Notarchus*.

15.5.1 Family *Aplysiidae*

Among the nine genera belonging to this family, *Aplysia* and *Dolabella*, commonly named sea hares, have been those most extensively studied. An excellent overview by Carefoot (1987) comprehensively described the biology and ecology of *Aplysia* and summarized their chemical components with the dietary algal relationships. More recently, Yamada and

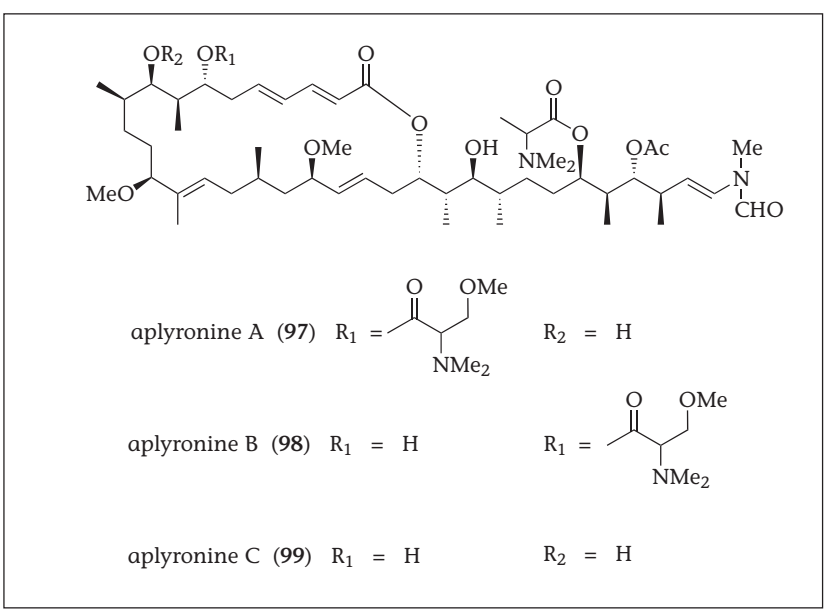
Kigoshi (1997) overviewed the bioactive compounds from both genera. These excellent reviews are our starting point. We will pay particular attention to the metabolites in the skin of the sea hares. This anatomical localization should lead to more specific molecules which are probably also involved in the defense mechanisms of the molluscs.

15.5.1.1 Genus *Aplysia*

Yamada and Kigoshi (1997) classify the bioactive compounds from *Aplysia* as: 1) polyketides; 2) terpenes; and 3) others.

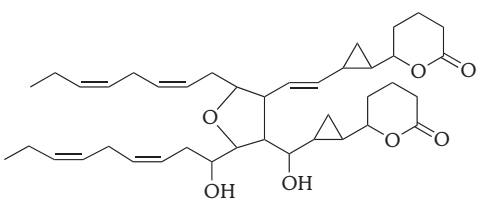
Polyketides

Three potent antitumor substances, aplyronines A–C (**97–99**) were isolated (Yamada *et al.*, 1993) from *Aplysia kurodai* by fractionation of the lipophilic extract guided by cytotoxicity against tumor cells ($HeLaS_3$, IC_{50} 0.039, 4.39, 159 mg/mL respectively). The three macrolides exhibited strong antitumor activity *in vivo* due to their ability to interact with actin, the protein in the cytoskeleton (Saito *et al.*, 1996). The absolute stereochemistry of **97** was secured (Ojika *et al.*, 1994) by a combination of NMR spectral and synthetic methods, and of **98** and **99** by enantioselective synthesis (Suenaga *et al.*, 1995). This series of excellent chemical papers strongly supports the basic idea that a study of opisthobranch molluscs can reveal bioactive molecules, which, though present in the marine organism in very small amounts, if of sufficient interest, can be synthetized. The origin of aplyronines is undetermined but, probably, they are produced by microalgae symbiotic either with the mollusc or with some algae upon which the mollusc feeds.

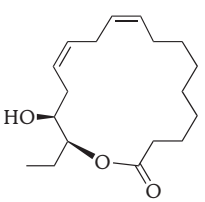


An unusual polyketide, aplydilactone (**100**), was isolated (Ojika *et al.*, 1990a) from the lipophilic extract of *A. kurodai*. The unusual metabolite seems to be biosynthesized from two eicosapentenoic acids by dimerization and oxidative cyclization leading to lactones and cyclopropanes. Aplydilactone is an activator of phospholipase A_2 *in vivo*, which is an important enzyme for prostaglandin biosynthesis. Other oxylipins were found (Spinella *et al.*, 1997b) in *Aplysia depilans*, collected from different Mediterranean and Atlantic sites. The skin extracts of all populations contained the same mixture, exhibiting almost identical HPLC profiles, of five unprecedented C-16 and C-18 fatty acid lactones (e.g. aplyolides B–C, **101–102**). Anatomical location and comparative distribution suggest that perhaps the sea hare is able to biosynthesize these lactones. It is interesting to observe that all lactones were ichthyotoxic at 10 ppm to *Gambusia affinis*, while the corresponding methyl esters were completely inactive.

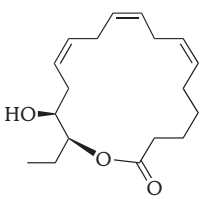
Many C_{15} halogenated cyclic ethers were found in different *Aplysia* species. Invariably, their origin is from the algae upon which the molluscs feed. The first to be isolated was dactylyne (**103**) (McDonald *et al.*, 1975) from *Aplysia dactylomela*, an anaspidean which can grow as large as 60 cm in length. A nine-membered ether ring was a structural feature of the fish-feeding deterrent brasilenyne (**104**) from *Aplysia brasiliana* (Kinnel *et al.*, 1979). Recently, polycyclic ethers were detected in the extracts of the digestive glands of *Aplysia parvula*, aplyparvunin (**105**) (Miyamoto *et al.*, 1995), and *A. dactylomela*, dactylallene (**106**) (Ciavatta *et al.*, 1997). Both compounds were toxic to *Gambusia affinis* even though their location, in the digestive gland, does not support a defensive role. The structure of dactylallene (**106**) is closely related to that of a known metabolite of the red alga *Laurencia obtusa*, obtusallene II (Öztunç *et al.*, 1991), differing from this only in the stereochemistries at C-4 and at the allene residue.



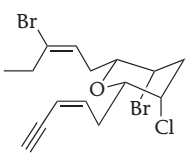
aplydilactone (**100**)



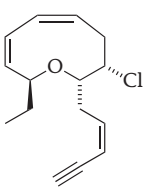
aplyolide B (**101**)



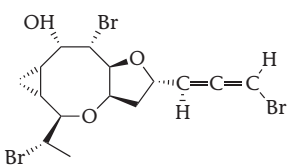
aplyolide C (**102**)



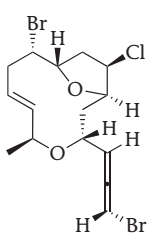
dactylyne (**103**)



brasilenyne (**104**)



aplyparvunin (**105**)



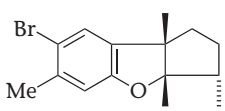
dactylallene (**106**)

Terpenes

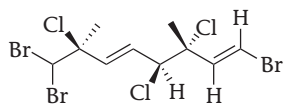
Yamamura and Hirata (1963) reported the structure of aplysin (**107**): it was the first terpenoid from a marine opisthobranch, the anaspidean *A. kurodai*. Since then, many chemical papers have described the structural features of halogenated mono-, sesqui-, and di-terpenoids, halogenated aromatic alkaloids coupled with sesquiterpenes and, lastly, degraded sterols.

The halogenated monoterpenes may be linear or cyclic. Generally, they are derived from red algae of the genus *Plocamium* on which the sea hares feed. The tribromotrichloromonoterpene **108** from *Aplysia californica* was the first of the series (Faulkner *et al.*, 1973). More recently (Kusumi *et al.*, 1987), a series of halogenated tetrahydropyrone sesquiterpenoids was isolated from *A. kurodai*. The general assumption that sea hares utilize these monoterpenoids for their own chemical protection, seems to be supported by the isolation (Miyamoto *et al.*, 1988) of the cyclic aplysiaterpenoid-A (**109**) and the linear aplysiaterpenoid-B (**110**), in a part of the body of *A. kurodai* where it would function effectively in defence. It appears that the mollusc is able to transfer defensive dietary metabolites from the digestive glands to the mantle.

Halogenated sesquiterpenoids, from sea hares, represent a wide variety of skeletons. Structures related to aplysin (**107**) and possessing antibacterial and ichthyotoxic properties, were found (Ichiba and Higa, 1986) in *A dactylomela* (e.g. cyclolaurenol, **111**). The same mollusc, collected in a different geographical area, surprisingly contained (Baker *et al.*, 1988) two antipodal *cis*-fused eudesmane sesquiterpenoids, lankalopuol-A and -B (**112** and **113**). Finding antipodal compounds in the same organism is very unusual. Perhaps the two sesquiterpenoids are the result of feeding on two closely related algae. The cytotoxic aplysistatin (**114**) was isolated (Pettit *et al.*, 1977) from *Aplysia angasi*. The nonisoprenoid skeleton of brasilenol (**115**) was found (Stallard *et al.*, 1978) in *Aplysia brasiliana*. Another sesquiterpenoid skeleton, chamigrene, is very common in both red

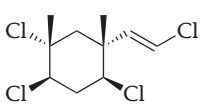


aplysin (**107**)

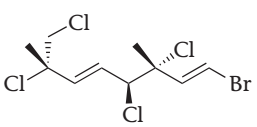


(**108**)

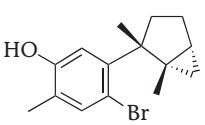
(3*R*,4*S*,7*S*)-*trans*,*trans*-3,7-dimethyl-1,8,8-tribromo-3,4,7-trichloro-1,5-octadiene



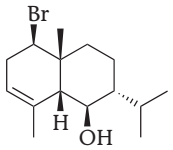
aplysiaterpenoid-A (**109**)



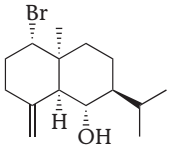
aplysiaterpenoid-B (**110**)



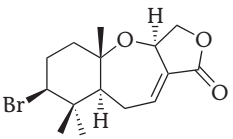
cyclolaurenol (**111**)



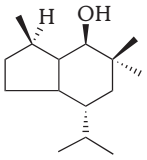
lankalapuol A (**112**)



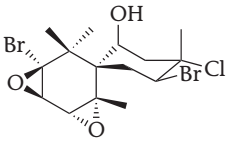
lankalapuol B (**113**)



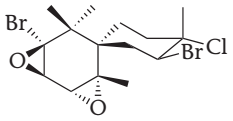
aplysistatin (**114**)



(+) brasilenol (**115**)



prepacifenol epoxide (**116**)

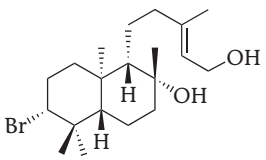


dehydroxyprepacifenol epoxide (**117**)

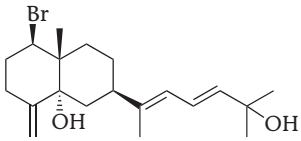
algae of the genus *Laurencia* and in sea hares which feed on it. Quite recently (Kaiser *et al.*, 1998), it was reported that *A. dactylomela*, from Brazilian waters, contains the known *Laurencia* metabolite prepacifenol epoxide (**116**) and its dehydroxy derivative (**117**).

The first halogenated diterpenoid, aplysin-20 (**118**), was found (Yamamura and Hirata, 1971) in *A. kurodai*. Recently, further studies of the same species led (Ojika *et al.*, 1990b) to the brominated diterpene aplysiadiol (**119**), which exhibits a rare extended sesquiterpene skeleton of a prenylated eudesmane. Two populations of *A. dactylomela*, from the Bahamas (Schmitz *et al.*, 1979), yielded bromoobtusenediol (**120**). From Puerto Rico, Schmitz *et al.* (1982) obtained five related cytotoxic diterpenoids, e.g. parguerol (**121**). Surprisingly, *A. dactylomela,* from the Canary Islands, yielded (Gonzalez *et al.*, 1987) a diterpenoid (**122**) with a dolabellane skeleton typical of Dictyotaceae algae and of *Dolabella* sea hares which feed upon them. This finding is of great interest as it supports a theory of possible competition between *Dolabella* and *Aplysia* postulating control of different marine habitats and adoption of highly selective dietary habits. However, the same species from the same waters also contains (Estrada *et al.*, 1989) a structurally unusual, halogenated diterpenoid, dactylomelol (**123**). Non-halogenated diterpenoids were found (Minale and Riccio, 1976) in the Mediterranean *Aplysia depilans* which contains a series of perhydroazulene diterpenes, e.g. dictyol-A (**124**), which are also found in algae of the family Dictyotaceae (Danise *et al.*, 1977). Non-halogenated diterpenoids were isolated from the Pacific *Aplysia vaccaria* (Midland *et al.*, 1983), which contains a series of metabolites, crenulides, typical of the brown alga *Dictyota crenulata* (Sun *et al.*, 1983). One of these, acetoxycrenulide (**125**) was highly toxic to a reef fish. Finally, an halogenated diterpenoid lactone, angasiol acetate (**126**), was a metabolite (Atta-ur Rahman *et al.*, 1991) of *Aplysia juliana* from the Indian coast of the Arabian Sea.

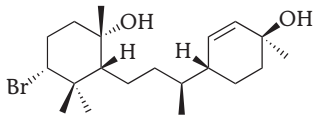
Other terpenoids with uncommon structure features include three cytotoxic alkaloids



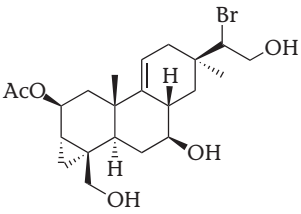
aplysin-20 (**118**)



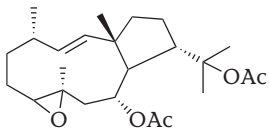
aplysiadiol (**119**)



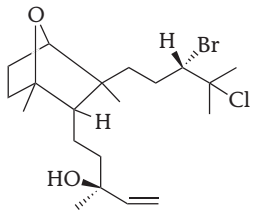
14-bromoobtus-1-ene-3,11-diol (**120**)



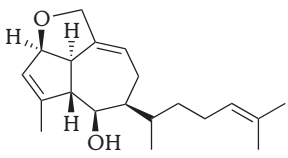
parguerol (**121**)



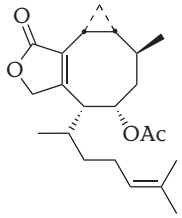
(**122**)



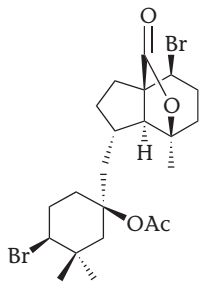
dactylomelol (**123**)



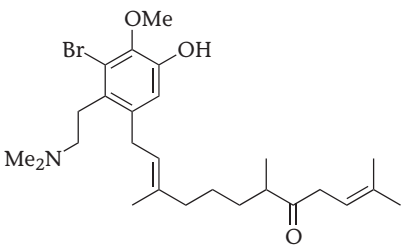
dictyol A (**124**)



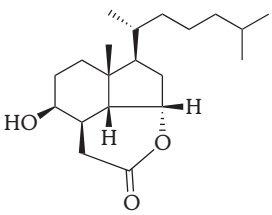
acetoxycrenulide (**125**)



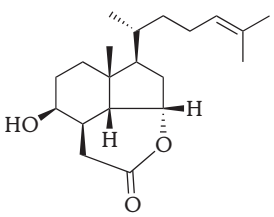
angasiol acetate (**126**)



aplaminone (**127**)

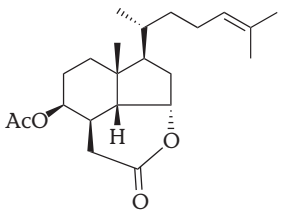


aplykurodin-A (**128**)

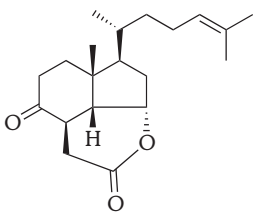


aplykurodin-B (**129**)

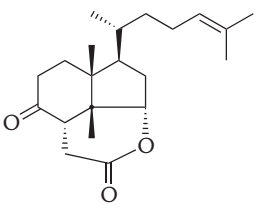
(e.g. aplaminone, **127**) from the extracts of *A. kurodai*. The biogenesis of the aplaminones may be envisaged by coupling a sesquiterpenoid with a brominated dopamine moiety (Kigoshi *et al.*, 1990). Two apparently degraded sterols, aplykurodin-A and -B (**128** and **129**) were recovered (Miyamoto *et al.*, 1986) from *A. kurodai*. Analogously, the mantle of the Mediterranean *Aplysia fasciata* contains two related ichthyotoxic metabolites, 4-acetylaplykurodin-B (**130**) and aplykurodinone-B (**131**) (Spinella



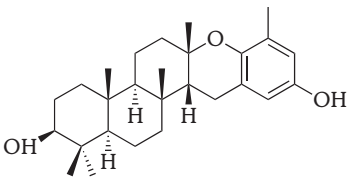
4-acetylaplykurodin-B (**130**)



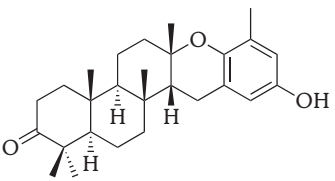
aplykurodinone-B (**131**)



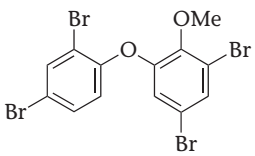
3-*epi*-aplykurodinone-B (**132**)



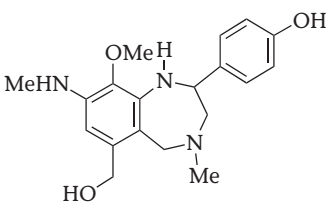
(+)-epitaondiol (**133**)



3-keto epitaondiol (**134**)



2-(2',4'-dibromophenoxy)-4,6-dibromo anisole (**135**)



aplysepine (**136**)

et al., 1992). More recently, a reinvestigation of the same mollusc led to the characterization of the C-3 epimer (**132**) of aplykurodinone-B, which was present in amounts comparable to those of **130** and **131**, in the mantle extracts (Ortega *et al.*, 1997). Finding aplykurodins in *Aplysia* species from different geographical sites suggests a fascinating hypothesis of the origin and ecological role of these compounds. These metabolites may derive from sterols or triterpenoids for the genesis of molecules useful for the protection of the molluscs against attacks by predators. However, no biosynthetic experiments have so far succeeded. Aplykurodins, not surprisingly, were completely absent in the digestive glands of those animals from which typical halogenated algal metabolites (Imperato *et al.*, 1977) were isolated.

Other ichthyotoxic metabolites of mixed biogenesis, containing a diterpenoid part linked to an aromatic ring, were found (Gerwick and Whatley, 1989) in the pair *A. dactylomela-Stypopodium zonale*. Numerous juveniles of the mollusc were observed feeding upon the brown seaweed *S. zonale* in Puerto Rico. The algal metabolites, stypoldione (Gerwick *et al.*, 1979) and (+)-epitaondiol (**133**), were concentrated by the

small juveniles (fresh weight per animal 130 mg) but, surprisingly, another compound, 3-ketoepitaondiol (**134**), was isolated in respectable quantity (approx. 3% of crude extract) only from the sea hare. Until now, this was the only paper which rigorously proved the transfer of metabolites from an alga to an anaspidean. In addition, it provided good evidence in favour of the capability by the sea hare to bio-accumulate dietary metabolites. Unfortunately, data regarding the anatomical distribution of the compounds in the sea hare is lacking.

Other metabolites

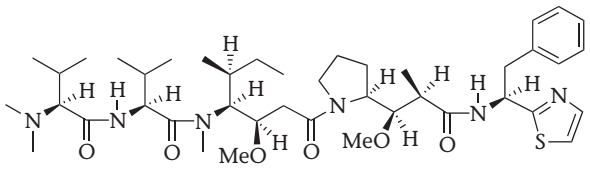
A polybrominated diphenyl ether (**135**) was detected (Kuniyoshi *et al.*, 1985) in the green alga *Cladophora fascicularis* and the anaspidean *Aplysia dactylomela*. The first marine-derived 1,4-benzodiazepine, aplysepine (**136**), was found (Ojika *et al.*, 1993a) in *A. kurodai*. The anomalous pattern of substitution suggested a biosynthetic origin from anthranilic acid and a β-phenethylamine, while other natural benzodiazepine are derived from anthranilic acid and α-amino acids.

15.5.1.2 Genus *Dolabella*

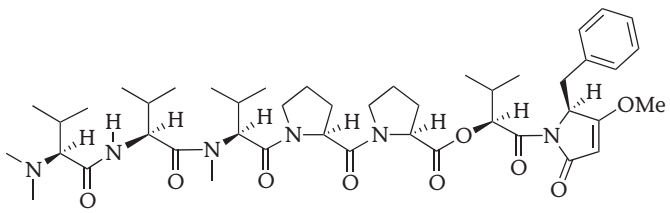
Yamada and Kigoshi (1997) classify the bioactive metabolites isolated from *Dolabella* spp. as: 1) peptides and depsipeptides; 2) polyketides; 3) terpenes; and 4) others.

Peptides and depsipeptides

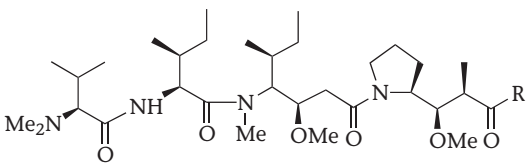
Since 1965 *Dolabella auricularia* from the Indo-Pacific has been an extraordinary source of bioactive peptides and depsipeptides, designated dolastatins, which possess strong cell growth inhibitory and antineoplastic properties. The chemical work has been conducted mainly by two groups, that of Pettit in Arizona and, more recently, that of Yamada in Japan. Among the most active antitumor compounds, dolastatin-10 (**137**) (Pettit *et al.*, 1987, 1989a) and dolastatin-15 (**138**) (Pettit *et al.*, 1989b) exhibited an ED_{50} value against P388 cells of 0.45 ng/mL and of 0.0024 μg/mL respectively. Dolastatin-10 (**137**) and dolastatin-15 (**138**) are in advanced preclinical development (Pettit *et al.*, 1997a; Aherne *et al.*, 1996). It is interesting to observe that two depsipeptides, dolastatin-H (**139**) and isodolastatin-H (**140**), which are closely related to **137**, were also isolated by Yamada's group (Sone *et al.*, 1996a) in trace amounts (0.3 mg each from 33 kg of *D. auricularia*). Because of this, evaluation of cytotoxicity was only possible by using synthetic samples. Surprisingly, *in vivo* antitumor activity against HeLa-S_3 cell line (0.0016 μg/mL), comparable to that of dolastatin-10 (**137**) was exhibited only by isodolastatin-H (**140**) while no significant activity was shown for its isomer, dolastatin-H (**139**) (Sone *et al.*, 1996a). This work is highly challenging. Generally, the structure determination is performed on amounts of less than 1 mg and synthetic work is absolutely necessary in order to confirm structure and stereochemistry and to have enough material for a complete evaluation of bioactivity. Some examples are significant:

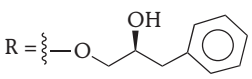


dolastatin 10 (**137**)

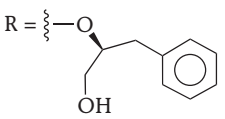


dolastatin 15 (**138**)

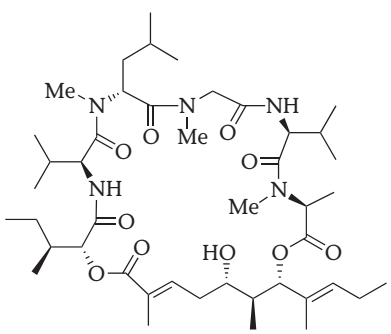




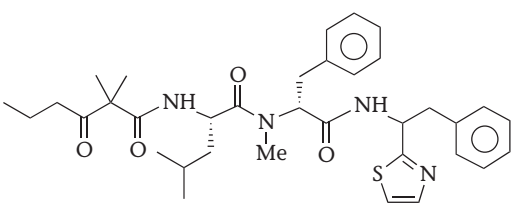
dolastatin-H (**139**)



isodolastatin-H (**140**)



aurilide (**141**)



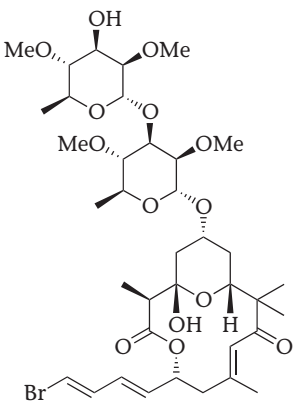
dolastatin 18 (**142**)

262 kg of *D. auricularia* yielded (Suenaga *et al.*, 1996a) 0.5 mg of aurilide (**141**) whereas 1.5 mg of dolastatin-18 (**142**) were obtained (Pettit *et al.*, 1997b) from 1000 kg of the same anaspidean. The origin of these molecules is a matter of conjecture; in fact, recently, analogues of dolastatin 10 and 13 have been isolated from the cyanobacterium

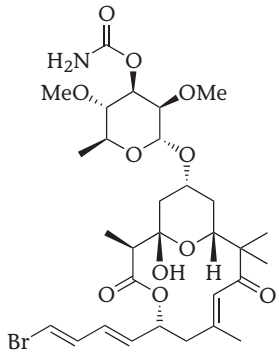
Symploca hydnoides (Harrigan *et al.*, 1998; Harrigan *et al.*, 1999) which could be associated with the molluscan tissues or with dietary algae. This supports the proposal that many compounds reported to come from the sea hare *D. auricularia* are most probably of cyanobacterial origin.

Polyketides

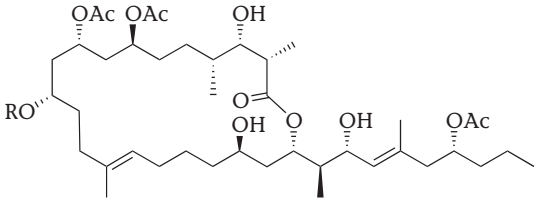
A series of cytotoxic macrolides were also detected in trace amounts in the extracts of *D. auricularia*. Auriside-A (**143**) and auriside-B (**144**) were separated (Sone *et al.*, 1996b) in sub-milligram amounts (0.8 and 0.7 mg, respectively) from 278 kg of the sea hare. These macrolides exhibited cytotoxicity against $HeLaS_3$ cells at 0.17 μg/mL and 1.2 μg/mL respectively. Moderately cytotoxic 22- and 24-membered macrolides (against $HeLaS_3$ cells ranging between 1.3 μg/mL and 6.3 μg/mL), dolabelides A–D (**145–148**), were characterized (Ojika *et al.*, 1995; Suenaga *et al.*, 1997) by chemical and spectral methods. Two C_{15} acetogenins containing cyclic ethers, doliculols A–B (**149–150**), were also found (Ojika *et al.*, 1993b) in the same mollusc. This is the first isolation of a non-halogenated C_{15} acetogenin from a marine organism. It is interesting to note that related cyclic ethers (but all halogenated) with a terminal enyne or allene are typical metabolites of the red



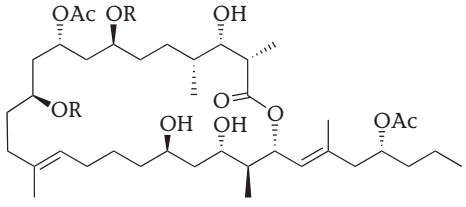
auriside-A (**143**)



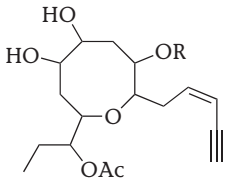
auriside-B (**144**)



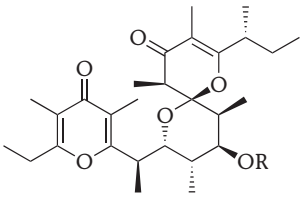
dolabelide-A (**145**), R = Ac
dolabelide-B (**146**), R = H



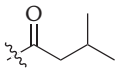
dolabelide-C (**147**) R = Ac
dolabelide-D (**148**) R = H



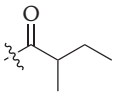
doliculol A (**149**) R = H
doliculol B (**150**) R = Ac

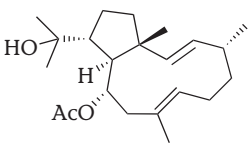


auripyrone A (**151**) R =

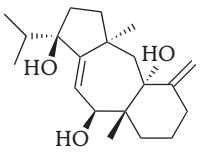


auripyrone B (**152**) R =

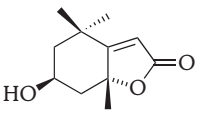




10-acetoxy-18-hydroxy-
2,7-dolabelladiene (**153**)



dolatriol (**154**)

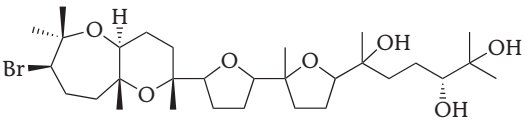


(−)-loliolide (**155**)

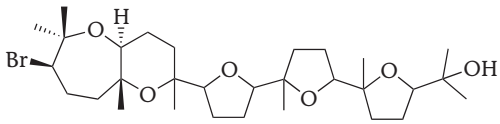
algal genus *Laurencia*, as well as of the sea hares which feed upon them. Because of this, the structural peculiarities of the doliculols prompt further studies in order to clarify the biosynthesis of halogenated and non-halogenated cycloethers. Two cytotoxic γ-pyrone polypropionates, auripyrone-A (**151**) and auripyrone-B (**152**) (Suenaga *et al.*, 1996b), were isolated from the methanol extract of the internal organs derived from 452 kg of Japanese specimens of D. auriculariae in extremely small amounts, 1.0 and 1.7 mg, respectively. These compounds exhibited cytotoxicity against $HeLaS_3$ cells at 0.26 and 0.48 μg/mL, respectively. Discovery of polypropionates in anaspideans is of great interest. In fact, polypropionates are quite rare in nature, but they characterize the metabolism of many opisthobranchs and marine pulmonates.

Terpenes

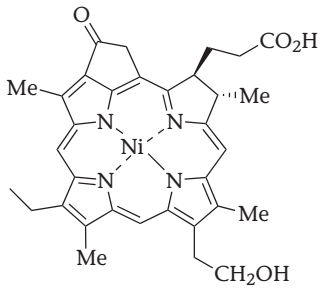
A series of diterpenoids with a *trans*-fused [9.3.0]-bicyclic carbon skeleton was isolated from the extracts of *Dolabella californica* (Ireland *et al.*, 1976; Ireland and Faulkner, 1977). The novel carbon skeleton of these compounds was named dolabellane and the first study led to the structure of 10-acetoxy-18-hydroxy-2,7-dolabelladiene (**153**) by single-crystal X-ray diffraction analysis (Ireland *et al.*, 1976). The algal dietary origin was successfully confirmed by finding related metabolites in many brown algae of the family Dictyotaceae (Piattelli *et al.*, 1995 and references therein). Another diterpenoid, dolatriol (**154**), exhibiting the related dolastane skeleton was characterized from *D. auricularia* (Pettit *et al.*, 1976). Dolatriol (**154**) was also found (Pettit *et al.*, 1980) in *Dolabella*



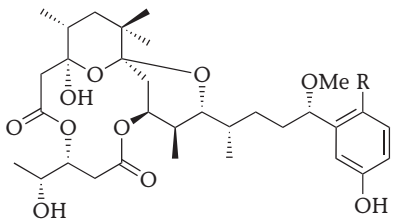
aurilol (**156**)



enshuol (**157**)



tunichlorinl (**158**)



aplysiatoxin (**159**) R = Br
debromo aplysiatoxin (**160**) R = H

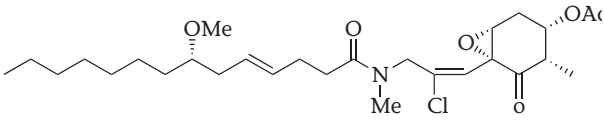
ecaudata together with (−)-loliolide (**155**), a well-characterized flowering plant constituent, which could either be a carotenoid artifact produced during isolation or a dietary by-product. Lastly, isolation (Suenaga *et al.*, 1998) of the cytotoxic bromotriterpene, aurilol (**156**), from *D. auricularia* is surprising. In fact, aurilol is structurally related to enshuol (**157**), an halogenated triterpenoid isolated from a red alga of the genus *Laurencia* which is not expected to suffer grazing by *D. auricularia*. Aurilol (**156**) is cytotoxic against $HeLaS_3$ cells at 4.7 μg/mL.

Other metabolites

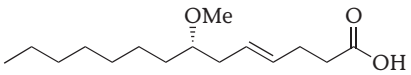
The nickel chelate tunichlorin (**158**) was found (Pettit *et al.*, 1993) in reasonable amounts (49 mg) from 500 kg of *D. auricularia*. Tunichlorin, which was known (Bible *et al.*, 1988) as the blue-green pigment of the tunicate *Tridemnum solidum*, may play an important role in electron transfer or other metabolic processes of the sea hare.

15.5.1.3 Genus *Stylocheilus*

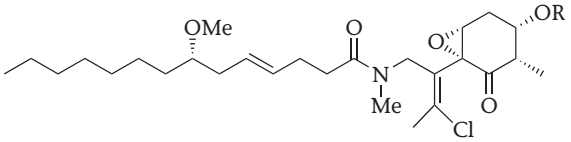
The small anaspidean *Stylocheilus longicauda* averages only about 27 mm in length, but offered chemists the opportunity to perform a series of interesting works. The chemical study of the digestive gland of *S. longicauda* led Kato and Scheuer (1974, 1975) to the isolation of two potent ether-soluble toxins (LD_{100} 0.3 mg/kg in mice), aplysiatoxin (**159**) and debromoaplysiatoxin (**160**). Their absolute stereochemistry was determined, some years later, by X-ray diffraction (Moore *et al.*, 1984). In the same paper it was reported that the two toxins possess highly inflammatory properties, since they induce severe contact dermatitis in swimmers and bathers during the summer months in



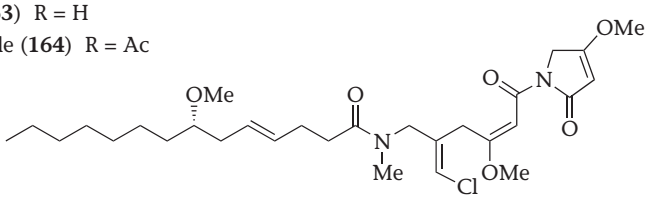
stylocheilamide (**161**) or (**164**)



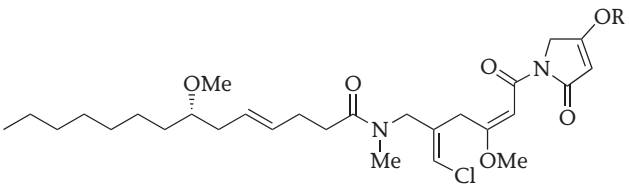
(**162**)



malyngamide-I (**163**) R = H
acetyl malyngamide (**164**) R = Ac



malyngamide-A (**165**)



malyngamide-B (**166**) R = H
acetyl malyngamide-B (**167**) R = Ac

Hawaii. The discovery (Mynderse *et al.*, 1977) of **160** in the blue-green alga *Lyngbya majuscula* (=*Microcoleus lyngbyaceus*) supported the dietary origin of the anaspidean toxins. Both toxins displayed interesting antileukemia activity. Further studies of *S. longicauda* (Rose *et al.*, 1978) led to the structural characterization of stylocheilamide (**161**, *see also* below), a non-toxic N-substituted amide of 7(*S*)-methoxytetradec-4(*E*)-enoic acid (**162**). The structural characterization of a related metabolite, malyngamide I (**163**), in the extracts of the cyanobacterium *L. majuscula* further supported the dietary relationship between the blue-green alga and the anaspidean and also led the authors to propose a probable structure revision of stylocheilamide from **161** to acetyl malyngamide I (**164**) (Todd and Gerwick, 1995).

Paul and Pennings (1991) demonstrated that *S. longicauda*: 1) prefers the filamentous cyanobacterium *L. majuscula* (=*M. lyngbyaceus*) over seven other diets; 2) concentrates the algal metabolites malyngamides A (**165**) and B (**166**); 3) converts **166** into its acetate **167**; and 4) is protected by the ichthyodeterrence of the malyngamides.

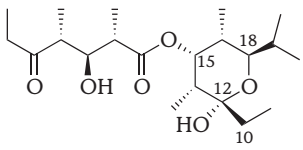
15.5.1.4 Genus *Dolabrifera*

Only one paper (Ciavatta *et al.*, 1996) reports chemical studies of species belonging to this genus. The skin of the small *Dolabrifera dolabrifera* (almost 30 mm in length) from Cuba contained a large amount (0.7 mg/animal) of a polypropionate named dolabriferol (**168**). The absence of dolabriferol (**168**) in the digestive gland of the mollusc indicates *D. dolabrifera* as a promising candidate for biosynthetic experiments. An attractive hypothesis is that the anaspidean bio-synthetizes its own chemical protection. Apparently, **168** is obtained by an uncommon coupling of a polypropionate acid with a polypropionate alcohol. An alternative origin from a polypropionate precursor, such as **169**, by cleaving of the tetrahydropyrone ring between C-9 and C-10 and subsequent formation of a cyclic hemiketal, by linking C-12 to the hydroxy group at C-18, was suggested.

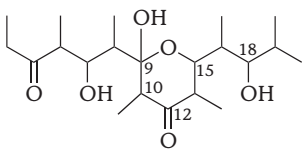
15.5.1.5 Genus *Bursatella*

Bursatella leachii has three subspecies, *B. leachii plei* from Puerto Rico (Gopichand and Schmitz, 1980), *B. leachii leachii* from the Thyrrenian Sea and *B. leachii savignyana* from the Adriatic Sea (Cimino *et al.*, 1987b) which contain an unusual metabolite, bursatellin (**170**), structurally related to chloramphenicol (**171**). Surprisingly, (+)-bursatellin is contained in *B. leachii plei*, whereas its enantiomer was present in *B. leachii savignyana*. The 1*R*, 2*R* absolute stereochemistry of **170** was ascertained by its synthesis (Racioppi *et al.*, 1990) starting from chloramphenicol D-base (**172**).

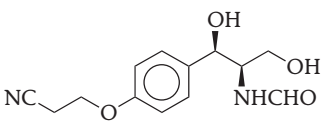
The absolute stereochemistry of the metabolite from *B. leachii leachii* is as yet



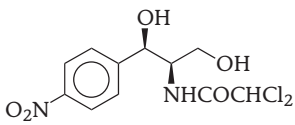
dolabriferol (**168**)



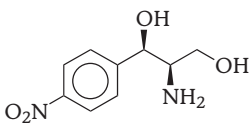
(**169**)



bursatellin (**170**)



chloramphenicol (**171**)



chloramphenicol D-base (**172**)

undetermined. The ecological role of **170** has been poorly investigated. Even the anatomical location of bursatellin is doubtful, since it was found in the skin of *B. leachii leachii* and in the digestive gland of *B. leachii plei*.

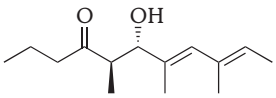
15.5.2 Conclusions

All chemical studies within the order Anaspidea are concentrated on species belonging to the family Aplysiidae. General rules for the dietary habits seem well supported by the experimental data. Almost all *Aplysia* molluscs contain typical halogenated red algae metabolites. The only exceptions seem to be *A. depilans* and *A. vaccaria*, which graze on brown algae containing non-halogenated cyclic diterpenoids. Analogous feeding habits of brown algae containing non-halogenated diterpenoids are observed in *Dolabella* molluscs. Another well-documented association exists between *S. longicauda* and blue-green algae. It is too early to draw general conclusions for two genera, *Bursatella* and *Dolabrifera*, even though the structural findings are not trivial and demand further investigation. However, it seems that anaspideans generally concentrate the dietary metabolites without defensive properties in their digestive glands. Only one study (Pennings and Paul, 1993) has rigorously treated this topic by offering customary and artificial diets to three anaspideans, *S. longicauda*, *D. auricularia* and *A. californica*, and by following the fate of the dietary metabolites. All three molluscs feed on different diets without optimally locating any metabolite for defense. A relevant aspect of these studies could be linked to the metabolites present only in the skin. Of course, a defensive molecule has to be located in the exposed and vulnerable parts of the mollusc. The unusual skin lactones in *A. kurodai*, *A. fasciata* and *A. depilans* are other excellent candidates for biosynthetic studies.

Lastly, the discovery of bioactive macrolides, peptides and depsipeptides is very impressive. The complexity of these molecules and their low concentration have forced chemists to develop advanced strategies in isolation, in structural analysis, in synthetic approaches, and in design of sophisticated bioassays.

15.6 ORDER GYMNOSOMATA

The opisthobranchs belonging to this order are represented by a few species in which shell, mantle, and mantle cavity, are lost in the adult stage (Newman, 1998). The Gymnosomata includes the four families Clionidae, Notabranchaeidae, Pneumodermatidae and Cliopsidae. Only one paper (Yoshida *et al.*, 1995) describes chemical studies on species belonging to this order. The pteropod *Clione antarctica* (family Clionidae) is a pelagic mollusc, that contains a polypropionate-derived compound, pteroenone (**173**), displaying feeding deterrence against the predatory fish *Pagothenia borchgrevinki* and *Pseudotrematomas bernacchii*.



pteroenone (**173**)

15.7 CONCLUDING REMARKS

This overview confirms the strategic role played by the opisthobranchs in the ecology of marine invertebrates. Probably starting from ancestral cephalaspideans, opisthobranchs evolved by conquering safe ecological niches. Some orders, Sacoglossa and Anaspidea, adopted herbivorous habits, selecting green, red, brown, and blue-green algae. Others, carnivorous Notaspidea and Nudibranchia, generally feed on sessile invertebrates, such as sponges, cnidarians, tunicates, and other molluscs. Opisthobranchs are often protected by chemical compounds which are acquired mainly by adopting three different strategies: bio-accumulation, bio-transformation, and bio-synthesis. All three strategies are well documented by numerous examples.

The basic idea that the study of these apparently unprotected molluscs might lead to the discovery of bioactive molecules is also well proven. If dolastatin 10 (**137**) and dolastatin 15 (**138**) are promising anticancer drugs, part of the credit has to be ascribed to the property of *D. auricularia* to contain among its wealth of secondary metabolites very bioactive molecules.

ACKNOWLEDEGMENTS

We are grateful to our colleagues at ICMIB, and in particular to Dr. V. Di Marzo, Dr. E. Mollo and Dr. G. Villani for the valuable contribution to the work from our Institute presented in this review. Thanks are also due to two colleagues, Prof. G. Sodano and Prof. A. Spinella, now at the University of Salerno, and to all the collaborators quoted in the references. We are also grateful to Prof. J. Ros, Prof. J. Ortea, Prof. M. Ballesteros, Dr. J. Espinosa, Prof. I. Fakhr, Dr. S. Wahidulla, Prof. A. Marin and Dr. J. Templado. We also thank Mr. R. Turco for the drawing of the formulae. Finally, we gratefully acknowledge Prof. M. Ghiselin and Prof. P. Scheuer for helpful comments on drafts of this chapter.

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CHAPTER

16

Bioactive Diterpenoids from Japanese Soft Corals

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Contents

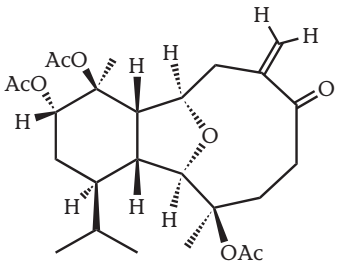
16.1 INTRODUCTION

Many kinds of living creatures, of which there are more than 30 phyla and 500,000 species, exist in the oceans, which cover almost two-thirds of the Earth's surface (Nienhuis, 1981). Sea water is a unique environment, therefore, it may be expected that many compounds with novel structures and differing bioactivities to those from terrestrial environments may be found among marine metabolites. Nevertheless, marine organisms did not attract chemists' and ecologists' attentions for a long time, probably because little was known about their ecology.

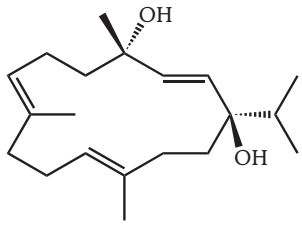
After the 1960s, the investigation and collection of marine organisms, previously very difficult, were facilitated by the availability of SCUBA diving equipment, thus allowing a remarkable development of these studies. Among the marine organisms living in the coral reefs, the marine animals, namely fishes, shrimps, sea stars, ascidians, sponges and so on, are the most rich and varied. The major life support systems for these diverse biota have been provided by coral reefs because it is important to have a good balance between these animals (Coll *et al.*, 1992). The ecological pressures on marine organisms include competition for space, maintenance of an unfolded surface, deterrence of predation, and the ability to successfully reproduce. Such factors may have led to the evolution of unique secondary metabolites responsible for the chemical components of these actions and interactions. These metabolites may also show physiological interactions with some terrestrial animals.

Since Weinheimer and Spraggins reported the isolation of the prostaglandin (15*R*)-PGA_2 from *Plexaura homomalla* in 1969 (Weinheimer and Spraggins, 1969), the soft corals have been shown to contain a fairly large variety of secondary metabolites, mainly diterpenoids, sesquiterpenoids, steroids, and prostanoids (Faulkner, 1984, 1986–1988, 1990–1999). Many of these constituents are structurally unique and exhibit interesting biological activities, e.g. ichthyotoxicity, toxicity to brine shrimp, cytotoxicity, antimicrobial action, Ca-antagonistic action, inhibition of cholinesterase, antiinflammatory activities, and so on. Recently, a number of diterpenoids have been isolated from the soft corals. Some selected examples are reported in Figure 16.1, namely labiatin B as a eunicellin-type diterpenoid (Roussis *et al.*, 1996), sarcophytol T as a cembrane-type diterpenoid (König and Wright, 1998), pseudopterosin as an amphilectane-type diterpenoid (Harvis *et al.*, 1988), xeniolide C as a xenicane-type diterpenoid (Hooper *et al.*, 1997), 2,9-diacetyl-2-debutyrylstecholide H as a briarein-type diterpenoid (Rodríguez *et al.*, 1998), asbestinin-11 as an asbestiane-type diterpenoid (Rodríguez *et al.*, 1994), and alanolide as a pseudopterane-type diterpenoid (Rodríguez *et al.*, 1996), 14,17-epoxyloba-8,10,13(15)-trien-18-acetate as an elemene-type diterpenoid (Edrada *et al.*, 1998), and finally sinulariadiolide with a new carbon skeleton (Iguchi *et al.*, 1996).

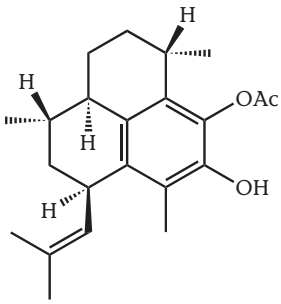
On the other hand, drug discovery programs are largely based on the screening of natural products possessing biological activities. The investigation of bioactive natural



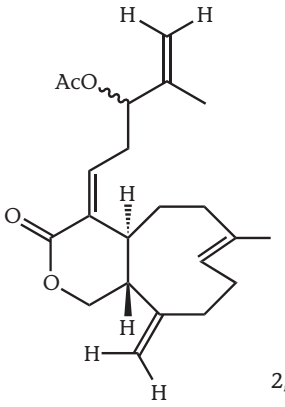
labiatin B



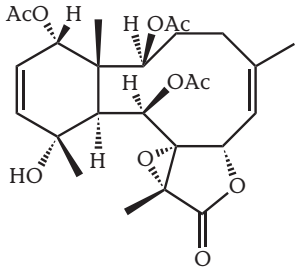
sarcophytol T



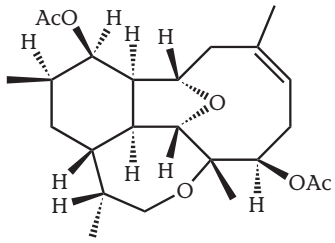
pseudopterosin



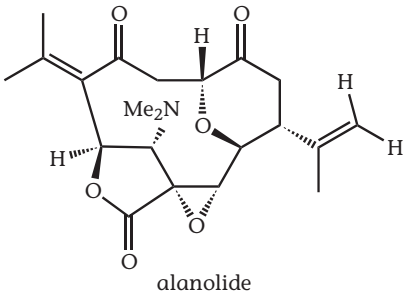
xeniolide C



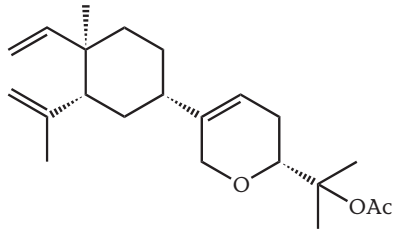
2, 9-diacetyl-2-debutyrylstecholide H



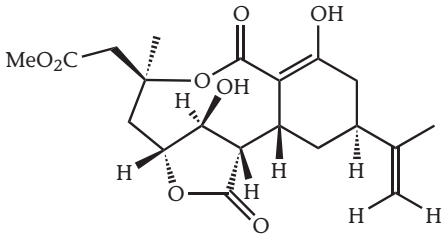
asbestinin-11



alanolide



14,17-epoxyloba-8, 10,13 (15)-trien-18-acetate



sinulariadiolide

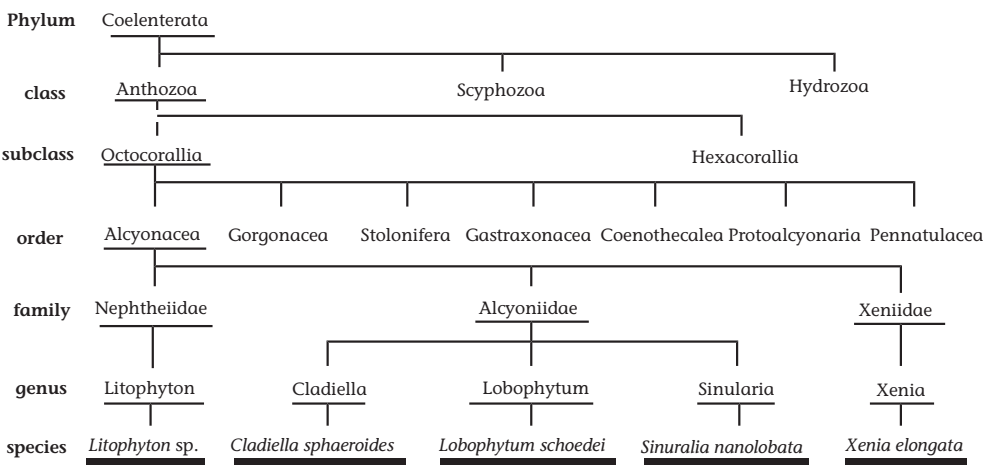
Figure 16.1: The diterpenoids containing some carbon skeletons obtained from soft corals.

products as potentially important leads in those programs is supported by improvements in bioassay techniques, especially *in vitro* procedures, which require only small amounts of material and are easy methods for the chemists, in addition to the development of highly specific assays. Our search for biologically active constituents aims to discover the natural resources to provide new drugs or chemical information that can be useful in facilitating the development of new therapeutic agents (König *et al.*, 1994). We have also searched for bioactive diterpenoids in Japanese soft corals, whose variety in the sea off the Pacific coast of Japan is well known. In this chapter, we explain techniques for isolating and determining structure including stereochemistry, and bioassay relating to our present studies of bioactive diterpenoids from the Japanese soft corals.

16.2 BACKGROUND

16.2.1 Octocoral taxonomy

The majority of corals are colonial marine invertebrates belonging to the phylum Coelenterata, class Anthozoa. Taxonomically, the Octocorallia comprise seven orders, namely, the Alcyonacea, Gorgonacea, Stolonifera, Gastraxonacea, Coenothecalea, Protoalcyonaria and Pennatulacea (Scheme 16.1). The Alcyonacea and Gorgonacea are particularly prominent in the sea off the Pacific coast of Japan. The five species which we will feature in this chapter belong to the order Alcyonacea.



Scheme 16.1: Major taxonomic relationships within the coelenterata.

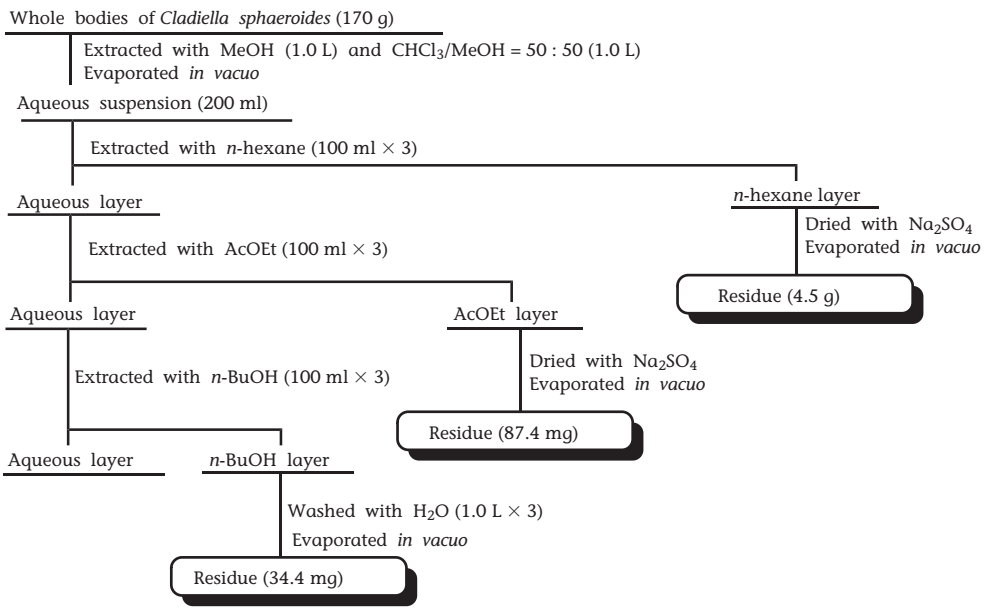
16.2.2 Collection, extraction, and isolation

16.2.2.1 Collection of the soft corals

Specimens were collected by hand using SCUBA at a depth of 2–5 m on the coral reef near Nichinan City, Miyazaki Prefecture, Japan, during spring or autumn, between 1989–1997. Usually, fleshy animals are frozen with dry ice immediately after collection. Specimens are carried to our laboratory as soon as possible and then kept in the freezer until just prior to extraction to prevent any oxidation and enzymatic reactions taking place.

16.2.2.2 Extraction and isolation

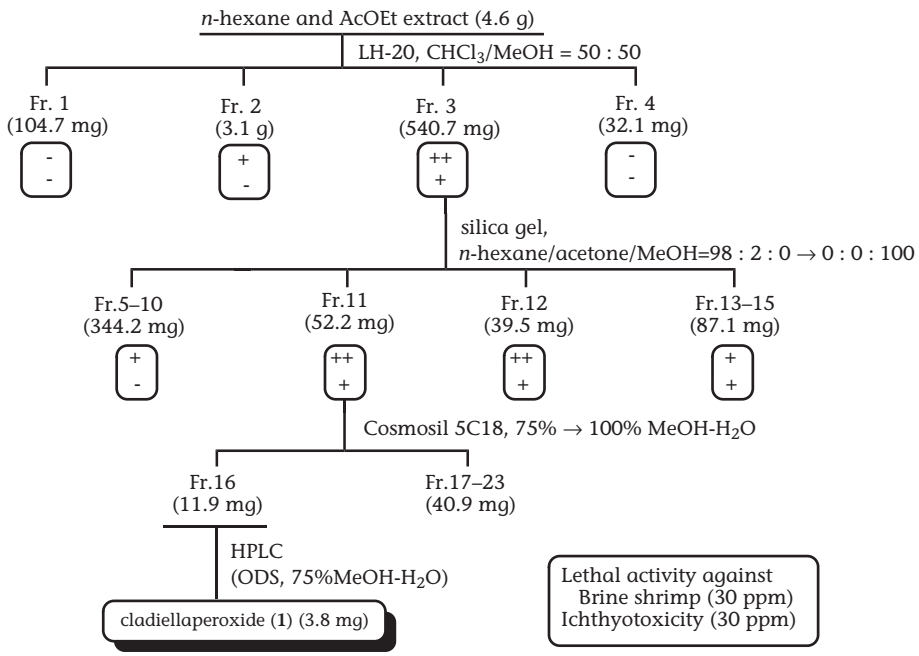
It is very important to screen specimens for biological activity when identifying bioactive compounds from natural sources. In our laboratory, the method shown in Scheme 16.2 for *Cladiella sphaeroides* is routinely applied to extracts of the aqueous suspension of specimens. The crude extracts thus obtained are subjected to a bioassay system (Yamada *et al.*, 1997) where fractions showing some bioactivity are further purified, thus providing rough fractions containing the bioactive constituents. In the following, we show an isolated example for illustration.



Scheme 16.2: Extraction and partition methods.

16.2.2.3 Extraction and isolation of the diterpenoids from the soft coral *Cladiella sphaeroides*

Initially, the typical extraction and isolation methods of bioactive diterpenoids from soft coral will be explained on the basis of an example from our studies on constituents of *Cladiella sphaeroides* (Yamada *et al.*, 1997). The outline for extraction and isolation are shown in Schemes 16.2 and 16.3, respectively. Chloroform ($CHCl_3$) is the preferred solvent for extraction, because of its ability to dissolve many organic compounds. However, MeOH is often employed to the first homogenate, followed by extraction with a $CHCl_3$-MeOH mixture, since the coral specimens usually contain large amounts of moisture. The resulting solutions are combined and concentrated *in vacuo* to an aqueous suspension. This suspension is extracted with successive equal volumes of *n*-hexane, EtOAc, and *n*-BuOH. The organic layers, other than *n*-BuOH, are subsequently dried separately over anhydrous Na_2SO_4, filtered, and evaporated to dryness. These extracts were then subjected to bioassays for ichthyotoxicity and toxicity to brine shrimp. The *n*-hexane and EtOAc extracts showed both bioactivities (Table 16.1), and were combined and chromatographed. Generally, the gel-filtration method is carried out as the first column chromatography in our procedure. We often employ the Sephadex LH-20 for gel-filtration with MeOH only or MeOH:$CHCl_3$ (1:1) mixed solvents. In most cases, the latter is used, because the crude extracts are not fully soluble in MeOH. It should be



Scheme 16.3: Bioassay-guided isolation methods for diterpenoids from *Cladiella sphaeroides*.

TABLE 16.1
Bioassay results of the extracts obtained from *Cladiella sphaeroides*

Extract	Lethal activity against brine shrimp LC_{100} (ppm)	Ichthyotoxicity LC_{100} (ppm)
n-hexane ext.	30.0	100.0
EtOAc ext.	30.0	100.0
n-BuOH ext.	inact.	inact.

noted that the gels for the gel-filtration are packed to a height 20 times over the diameter of the column tube. It is normally necessary to filter the extract through a filter paper before the solution is applied onto the column. The resulting crude fractions, i.e. four fractions in our example, are further examined for the toxic activity as previously described. The third fraction showed the most pronounced activities in both bioassays and was subsequently chromatographed on a silica gel column. Elution was performed with mixed solvents e.g. *n*-hexane/Et_2O, *n*-hexane/EtOAc, *n*-hexane/acetone, benzene/Et_2O, benzene/EtOAc, benzene/acetone, $CHCl_3$/MeOH, or $CHCl_3$/MeOH/H_2O. The selection and proportion of the eluate must be determined by preliminary TLC (thin-layer chromatography). The spots corresponding to the constituents of the sample can be observed on the TLC plate by treatment with suitable chromogenic reagents, e.g., 5%H_2SO_4–MeOH, anisaldehyde–H_2SO_4. The solvent which shows more definite spots on TLC is the most suitable and its proportion is adjusted to show 0.2–0.4 R_f (ratio of flow) values. Fractions obtained are further separated by normal phase open column chromatography with silica gel, reversed-phase open column chromatography with C8 and C18, and MPLC (middle pressure liquid chromatography) using the bioassay for guidance. MPLC is performed with flash or packed columns, and C8 or C18 gels are used in reversed-phase chromatography with MeOH/H_2O or MeCN/H_2O as the eluate (we often employ the more inexpensive MeOH). Finally, the resulting fractions containing bioactive constituents are purified by HPLC (high performance liquid chromatography). HPLC is conducted with C18 columns using MeOH/H_2O, and the detection is performed by the combined use of UV and RI (refractive index) detectors. As a result of such purification, the new diterpenoid cladiellaperoxide (**1**) (Figure 16.2) has been isolated from *Cladiella sphaeroides*. We will explain the structure elucidation methods applied to this compound and to some other diterpenoids isolated from soft corals in the next section.

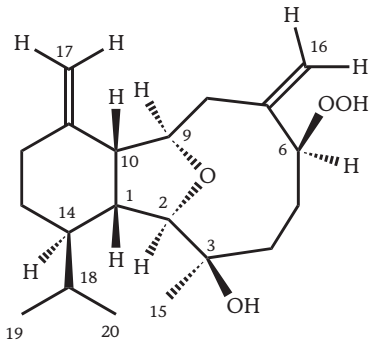


Figure 16.2: Structure of cladiellaperoxide (**1**).

16.3 STRUCTURE DETERMINATION OF THE DITERPENOIDS ISOLATED FROM SOFT CORALS

16.3.1 Structure of the eunicellin-type diterpenoid isolated from *C. sphaeroides*

The structures of constituents purified by HPLC are subsequently determined based on spectroscopic evidence. First, it is necessary to investigate their physical constants and chemical properties. The molecular formulas, molecular weights, optical rotations, melting points, and R_f values are shown in Table 16.2. The molecular formulas are determined by HR-MS (high resolution-mass spetrometry) or elemental analysis, but in many cases they are measured after the structures are almost deduced. Next, the functional groups are investigated by IR (infrared) and UV (ultraviolet) spectra. In particular, the presence of hydroxyl, olefin, carbonyl, amide, phenyl groups and so on, are clarified by IR spectra, alternatively conjugated systems are confirmed by UV spectra. In our laboratory, the measurement of IR spectra is carried out in solution (with $CHCl_3$ or CCl_4) or KBr methods in the wave number region of 400 to 4000 cm^{-1}, and UV spectra are measured with EtOH or MeCN in the wave length region of about 200 to 400 nm. The IR spectrum of cladiellaperoxide (**1**) indicated the presence of hydroperoxyl (3530 cm^{-1}), hydroxyl (3600 and

TABLE 16.2
The physical constants and chemical properties of cladiellaperoxide (**1**)

FAB MS $[M + H]^+$ MS	m/z	$[\alpha]_D$	mp.	R_f value *n*-hexane/EtOAc = 7:3
$C_{20}H_{33}O_4$	321	−27.8° (*c* 0.42, $CHCl_3$)	135.0–138.0°C	0.4

3300 cm^{-1}), and exocyclic methylene groups (3070, 1640, and 900 cm^{-1}). Once the functional groups have been revealed, a detailed structural elucidation can be carried out, particularly by NMR (nuclear magnetic resonance) spectroscopy. Initially, each component unit was examined by ^{1}H-NMR, ^{13}C-NMR, and ^{1}H,^{13}C-COSY (^{1}H-^{13}C correlation spectroscopy) spectra. The ^{1}H-NMR spectrum of **1** contained signals for two secondary methyls (δ_H 0.74 and 0.96), one quaternary methyl (δ_H 1.23), three oxygen-bearing methines (δ_H 3.67, 4.12, and 4.74), four olefinic protons (δ_H 4.68, 4.81, 5.26, and 5.52), and a hydroperoxide proton (δ_H 7.86). The ^{13}C-NMR spectrum of **1** suggested the presence of three methyl carbons (δ_C 15.1, 22.0, and 27.1), five methylene carbons (δ_C 25.3, 29.7, 31.8, 34.8, and 40.0), four methine carbons (δ_C 27.9, 44.0, 44.4, and 48.0), three oxygen-bearing methine carbons (δ_C 79.6, 86.3, and 91.8), one oxygen-bearing quaternary carbon (δ_C 91.8), and four olefinic carbons (δ_C 111.3, 118.0, 146.2, and 147.7). These data were confirmed by one-bond ^{1}H,^{13}C-COSY correlations between each proton and carbon. Next, the partial structures composed of three units were revealed by ^{1}H,^{1}H-COSY (^{1}H-^{1}H correlation spectroscopy) spectra for each proton. The ^{1}H,^{1}H-COSY spectrum of **1** clearly indicated the presence of seven partial structures, (**a–g**), in **1** as shown in Figure 16.3. In addition, the relationships between the partial structures were revealed and the planar structure was determined as a result. The connectivities of **1** were determined by the detailed analysis of the ^{1}H,^{13}C long-range COSY spectrum. Namely, ^{1}H,^{13}C long-range correlations of δ_C 34.8 (C-4) and 91.8 (C-2) to δ_H 1.23 (H-15) suggested the connectivities between C-2 and C-3, and between C-3 and C-4. Correlations of δ_C 40.4 (C-8) and 86.3 (C-6) to δ_H 5.26 and 5.52 (H_2-16) suggested the connectivities between C-6 and C-7, and between C-7 and C-8 (Figure 16.4). The

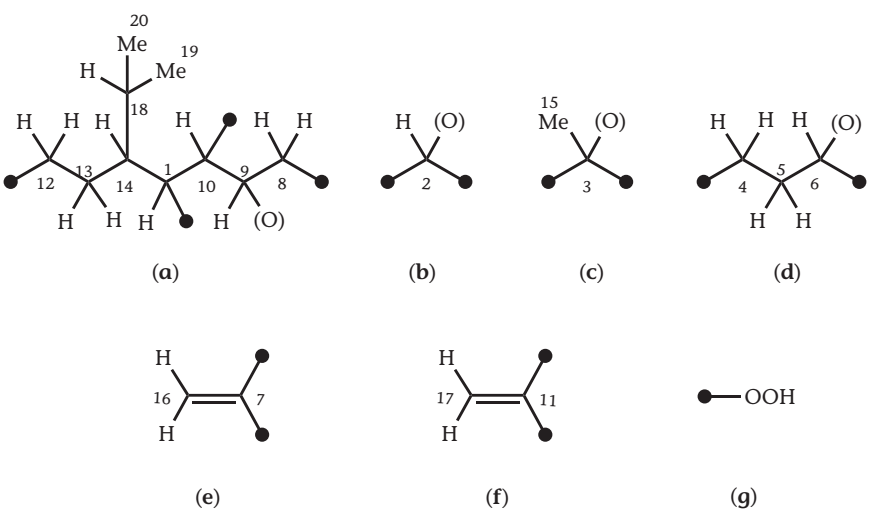


Figure 16.3: Partial structures of cladiellaperoxide (**1**).

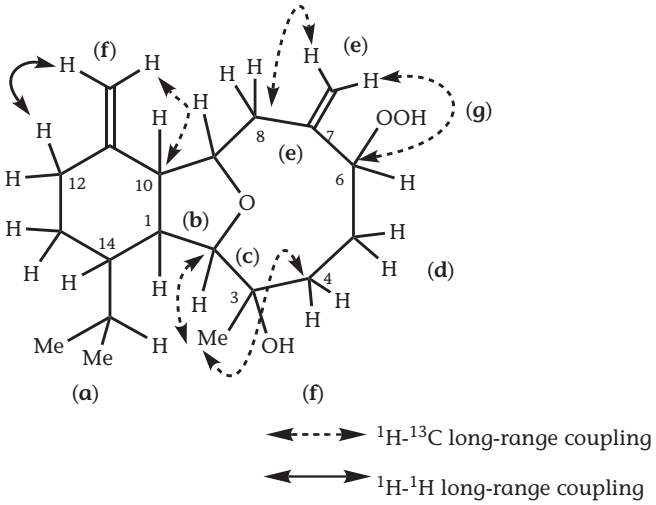
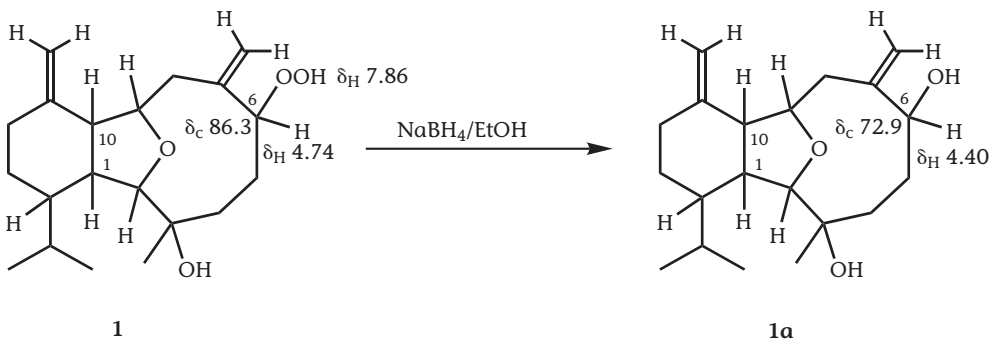


Figure 16.4: Planar structure of cladiellaperoxide (**1**).

connectivity between C-11 and C-12 was revealed by the ^{1}H,^{1}H long-range coupling between δ_H 4.68 (H_2-17) and δ_H 2.22 (H_2-12) (Figure 16.4). As a result, the residual connectivity between C-10 and C-11 was determined. By considering the previously mentioned data, the planar structure of **1** was determined to be a eunicellin-type diterpenoid. The location of the hydroperoxide group at the C-6 position was indicated by chemical conversion and NMR studies. Namely, **1** was converted to **1a** by means of $NaBH_4$ reduction (Scheme 16.4). The comparison of ^{1}H and ^{13}C-NMR spectra of **1** and **1a** showed that the signals due to H-6 (δ_H 4.74) and C-6 (δ_C 86.3) in **1** shifted to a lower field (δ_H 4.40 and δ_C 72.9) in **1a**, indicated that the C-6 position was peroxylated (Scheme 16.4).



Scheme 16.4: Conversion of **1** into **1a** and NMR data at the C-6 position.

Once the planar structure is determined, its stereochemistry can be elucidated. Initially, the relative configuration should be established, followed by determination of the absolute configuration. To determine the relative configuration, we often utilize NOESY (nuclear Overhauser effect correlation spectroscopy) or NOEDS (NOE difference spectroscopy) spectra, and *J* values between vicinal protons in the ^{1}H-NMR spectrum. In general, the NOE effects are observed for those protons which are spatially close (normally less than 3.5 Å). The NOESY spectrum of cladiellaperoxide (**1**) gave the correlations shown in Figure 16.5. Alternatively, the dihedral angles between vicinal protons are given by applying the Karplus rule (Karplus, 1959, 1963) to *J* values of each proton, that is $J_{5–6}$ = 3.2 Hz and 11.5 Hz, $J_{8–9}$ = 5.6, $J_{9–10}$ = 10.6 Hz, and $J_{13–14}$ = 3.4 and 11.8 Hz. As a result of the above-mentioned data, the relative stereochemistry of cladiellaperoxide (**1**) was determined as shown in Figure 16.5.

Once the relative stereochemistry is determined, the absolute configuration can be elucidated. The determinations of the absolute configuration are frequently achieved by applying the modified Mosher's method developed by Kusumi *et al.* (Ohtani *et al.*, 1991) using ^{1}H-NMR, the exciton chirality method by CD (circular dichroism) (Harada *et al.*, 1983), ORD (optical rotatory dispersion) spectra, the Bijvoet method by single crystal X-ray analysis (Bijvoet *et al.*, 1951) and so on. In particular, we frequently employed the modified Mosher's method, the exciton chirality method, and the octant rule by CD or ORD spectra. In this section we explain the method for the determination of the absolute configuration of cladiellaperoxide (**1**) on the basis of the modified Mosher's method and the exciton chirality method. The modified Mosher's method is an excellent method for elucidating the absolute configuration of organic compounds possessing a secondary alcohol moiety by measuring the ^{1}H-NMR spectra of their α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) esters. The detailed principle of this method is provided in the literature by Ohtani (Ohtani *et al.*, 1991). The MTPA esters **1S** and **1R**

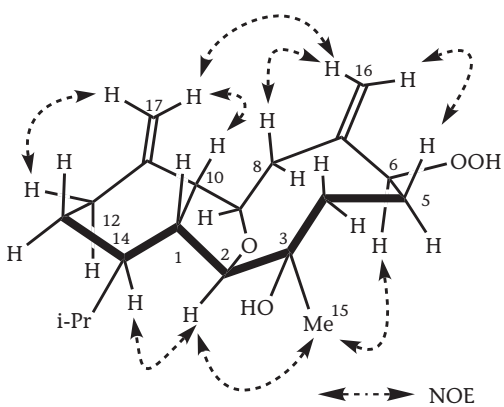
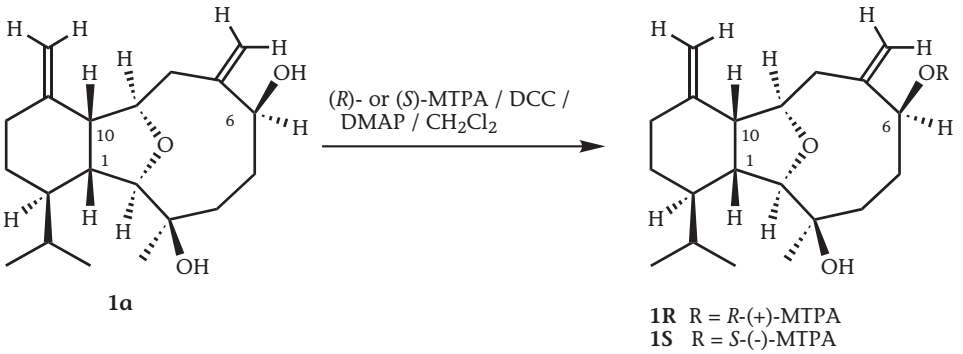


Figure 16.5: NOE correlations of cladiellaperoxide (**1**).



Scheme 16.5: Conversions of **1a** into **1R** and **1S**.

were prepared from **1a** by treatment with (*S*)-(−)-MTPA and (*R*)-(+)-MTPA, respectively (Scheme 16.5). The Δδ values are positive for protons on the right-hand side of the MTPA plane, and negative for those on the left-hand side as shown in Figure 16.6. Thus, it was considered that the absolute stereochemistry of C-6 had to be *S*. Nevertheless, because only the H-8α proton exceptionally showed an anomalous value, the above result had to be confirmed. Thus, we prepared the benzoate **1b** from **1a** by treatment with benzyl chloride/pyridine, as shown in Scheme 16.6, and measured the CD spectrum of **1b**. The absolute configuration was determined by an application of the CD exciton chirality method to **1b**. The detailed principle of this method is provided in the literature by Harada and Nakanishi (1983). In this method, two rules, the allylic benzoate rule and the dibenzoate chirality rule, are well known. Of these, the former rule was applied to **1b**. The UV and CD spectra of **1b** are presented in Figure 16.7, the CD

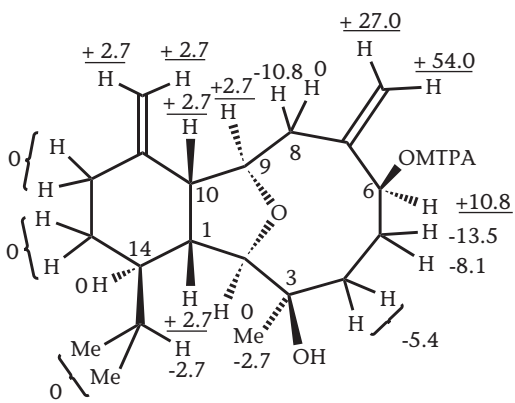
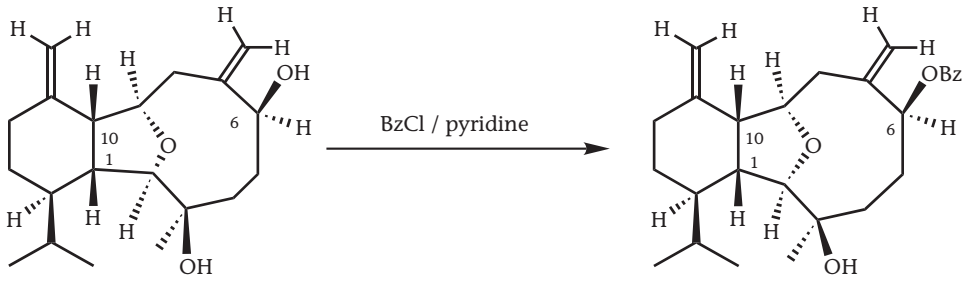


Figure 16.6: Δδ values (Δδ = $\delta_S - \delta_R$ at 270 MHz) obtained for MTPA esters **1S/1R**. The values are given in hertz.



Scheme 16.6: Conversions of **1a** into **1b**.

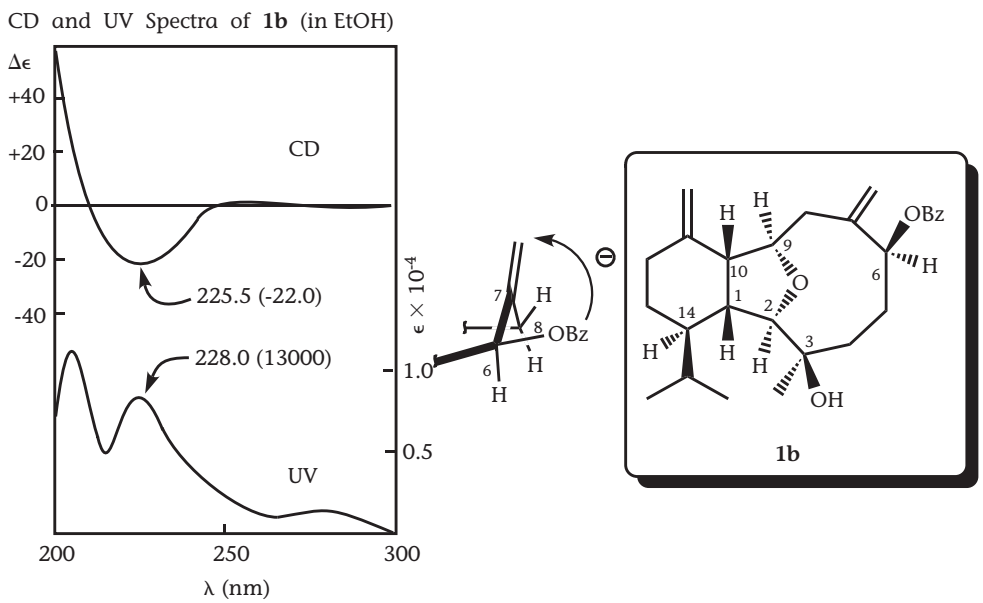


Figure 16.7: CD and UV spectra of **1b**.

spectrum shows a negative Cotton effect at 225.5 nm ($\Delta\epsilon$ −22.0). Taking account of this result, the negative chirality between exomethylene and the benzoyl group was designated as shown in Figure 16.7. Thus, the 6*S* configuration of **1b** was unambiguously demonstrated, proving that the result obtained through the modified Mosher's method was reliable. Consequently, the absolute configuration of 1*R*,2*R*,3*R*,6*S*,9*R*,10*R*,14*R* was established as shown in Figure 16.7. The only exception for H-8α can be explained by a deshielding effect of the benzene ring of the MTPA group on this proton, which is in

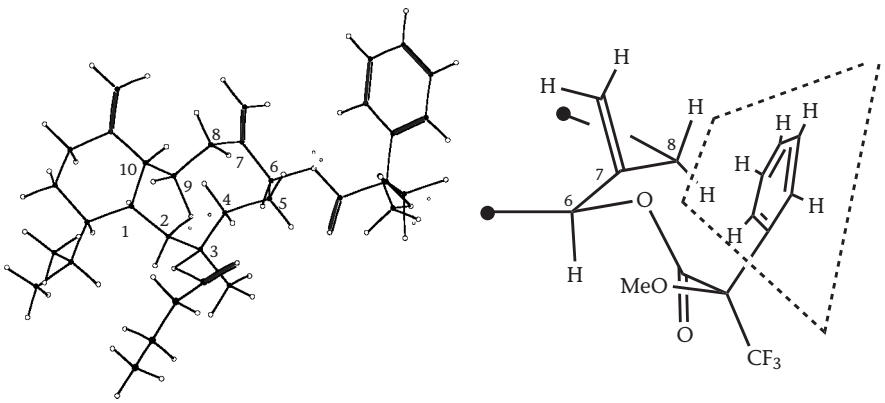


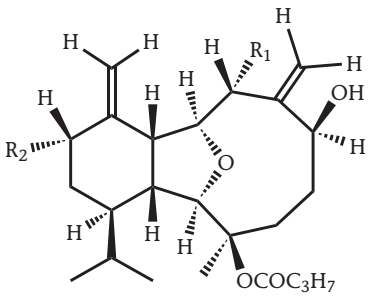
Figure 16.8: The calculated structure of **1R** by MM2.

approximately the same plane as the benzene ring, in **1R**. This fact was confirmed by an energy-minimized structure of **1R** calculated by MM2 (Allinger, 1977) based on the results of the NOESY experiment of **1R** as shown in Figure 16.8.

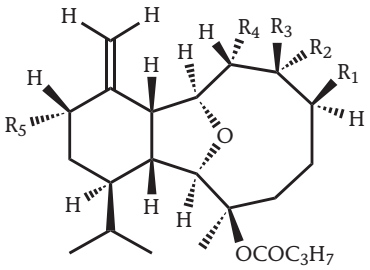
16.3.2 Structures of the eunicellin-type diterpenoids isolated from the mucus of *Litophyton* sp.

The litophynins, which are biologically active diterpenoids of the eunicellin class, have been isolated from the whole bodies of the soft coral *Litophyton* sp. by Ochi *et al*. (1987, 1988, 1990–1992). In addition, we have isolated two new eunicellin-type diterpenoids, litophynols A (**2**) and B (**3**), and three known diterpenoids (Figure 16.9) from the secreted surface mucus (Miyamoto *et al*., 1994) of the soft coral *Litophyton* sp. collected from the Nichinan Coast in Miyazaki Prefecture, Japan. In this section, the structure of these diterpenoids and their absolute configurations are explained. The extraction and isolation methods were carried out in the same manner as in the case of *Cladiella sphaeroides*, but EtOH and Et_2O were used at extraction.

Their structures have also been elucidated on the basis of spectral and single-crystal X-ray analyses, as shown in Figure 16.9. Among them, compounds **2** and **3** were new, and designated litophynols A (**2**) and B (**3**). Alternatively, compounds **4–6** were identified as litophynins E (Ochi *et al*., 1990), H (Ochi *et al*., 1991), and I monoacetate (Ochi *et al*., 1992), respectively. The relative configuration of litophynol A (**2**) was determined on the basis of single-crystal X-ray analysis of the diacetate **2a** prepared by acetylation of **2** with Ac_2O/pyridine. In addition, the determination of the absolute configuration of **2** was also conducted by application of the CD exciton chirality method to the dibenzoate **2b** derived from **2**. The CD spectrum of **2b** showed the two split Cotton effects at



litophynol A (**2**) R_1=OH, R_2=H
litophynin H (**5**) R_1=H, R_2=OH



litophynol B (**3**) R_1=OH, R_2=OH, R_3=Me, R_4=OH, R_5=H
litophynin E (**4**) R_1=OH, R_2=Me, R_3=OH, R_4=H, R_5=OH
litophynin I monoacetate (**6**) R_1=OH, R_2=H, R_3=OH, R_4=H, R_5=OAc

Figure 16.9: Diterpenoids isolated from the mucus of *Litophyton* sp.

234 nm ($\Delta\epsilon$ +23.2) and 224 nm ($\Delta\epsilon$ −16.1). Taking account of this result, the positive chirality between the C-6 and C-8 benzoyl groups was designated, and so the absolute configurations of C-6 and C-8 positions as 6*S* and 8*R* were established as shown in Figure 16.9.

As the absolute configuration of litophynin E (**4**), which was isolated by Ochi *et al.*, has not yet been determined, we applied the modified Mosher's method to (*S*)-(−)-MTPA ester (**4S**) and (*R*)-(+)-MTPA ester (**4R**) of **4** as in the case of **1**. Thus the absolute configuration of **4** was determined as shown in Figure 16.10.

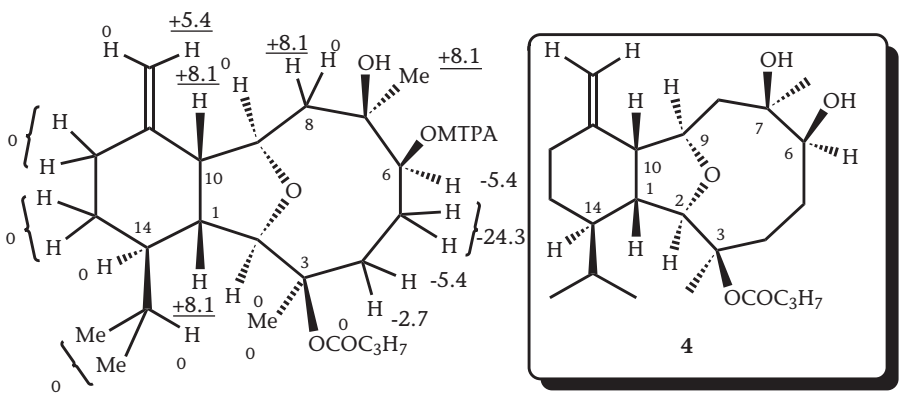
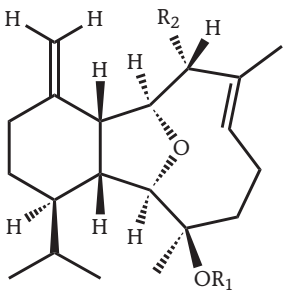
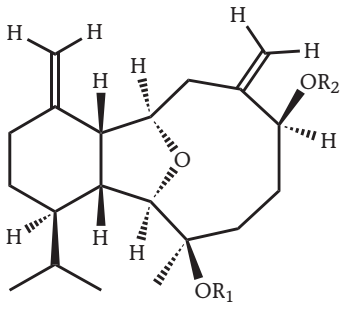


Figure 16.10: Δδ values (Δδ = δ_S–δ_R at 270 MHz) obtained for MTPA esters **4S**/**4R**. The values are given in hertz.



cladiellin (**7**) R_1=Ac, R_2=H
litophynin A (**8**) R_1=COC_3H_7, R_2=H
litophynin B (**9**) R_1=COC_3H_7, R_2=$OCOC_3H_7$
litophynin A acetate (**10**) R_1=COC_3H_7, R_2=OAc



11 R_1=Ac, R_2=H
12 R_1=R_2=Ac
litophynin F (**13**) R_1=COC_3H_7, R_2=H

Figure 16.11: Diterpenoids isolated from the whole bodies of *Litophyton* sp.

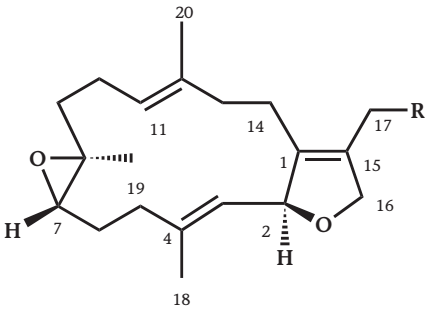
In addition to these compounds, we have isolated seven eunicellin-type diterpenoids **7–13** as shown in Figure 16.11. These compounds had already been isolated from the whole bodies of *Litophyton* sp. by Ochi *et al.*, but it is worth noting here that these compounds have been also obtained by us from the mucus of the same organism.

16.3.3 Structures of the cembrane-type diterpenoids isolated from *Lobophytum schoedei*

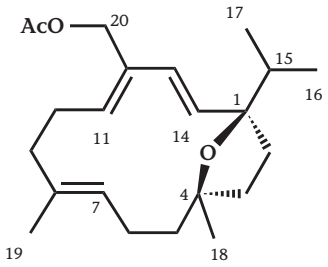
We have isolated two known diterpenoids, sarcophytoxide (**14**) (Kashman *et al.*, 1974) and isosarcophytoxide (**15**) (Bowden *et al.*, 1987), and three new diterpenoids, lobophynins A (**16**), B (**17**), and C (**18**) from *L. schoedei* collected in the same sea area as the previously discussed compounds (Figure 16.12) (Yamada *et al.*, 1997). We will briefly discuss the structure elucidation of these diterpenoids in this section. The extraction and isolation methods were also carried out in the same manner as in the case of *C. sphaeroides*, and diterpenoids **14–18** were obtained from the whole bodies (9.2 kg) of *L. schoedei*.

Initially, the compounds **14** and **15** were identified as sarcophytoxide (Kashman *et al.*, 1974), and isosarcophytoxide (Bowden *et al.*, 1987), respectively, on the basis of spectral analysis. It was proved that **14** and **15** were positional isomers for the epoxyl group. This fact was verified from the result that only reduction product **19** was obtained by treating both compounds **14** and **15** with a Zn-Cu couple in EtOH (Scheme 16.7) (Bowden *et al.*, 1987).

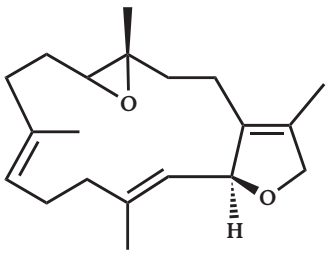
Lobophynin A (**16**) was isolated as a colorless oil. The molecular formula $C_{22}H_{34}O_3$ of **16** was determined by positive HR-FABMS (high resolution fast atom bombardment



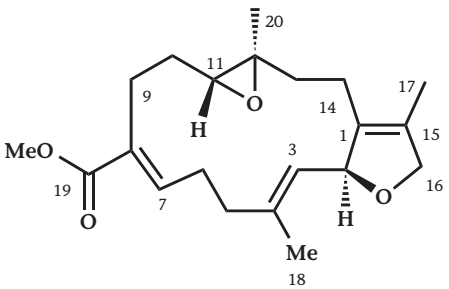
sarcophytoxide (**14**) R = H
lobophynin B (**17**) R = OAc



lobophynin A (**16**)



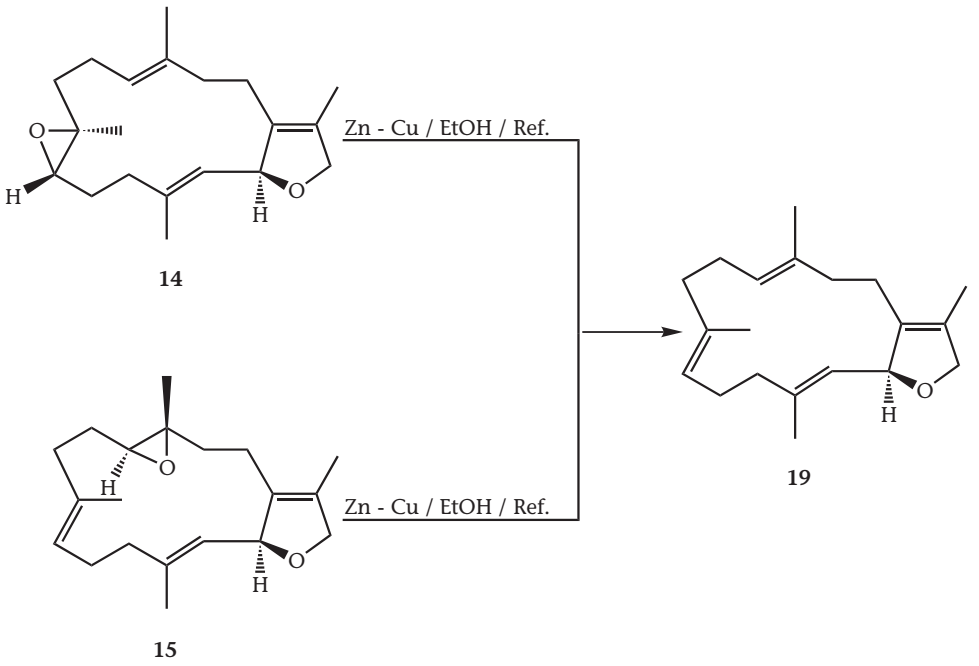
isosarcophytoxide (**15**)



lobophynin C (**18**)

Figure 16.12: Diterpenoids isolated from *Lobophytum schoedei*.

mass spectrometry). Its IR spectrum showed signals due to carbon–carbon double bonds (3010 and 910 cm^{-1}) and ester (1730 and 1240 cm^{-1}) groups. As the result of detailed examination of the ^{1}H-NMR, ^{13}C-NMR, and ^{1}H,^{13}C-COSY spectra of **16**, it was revealed that four secondary olefins [δ_H 5.91, 5.72, 5.59, 5.24; δ_C 137.4, 134.3, 129.9, 126.3], one oxygen-bearing methylene group [δ_H 4.66, 4.57; δ_C 61.2], one acetoxyl group [δ_H 2.07; δ_C 170.1, 21.0], one allyl methyl group [δ_H 1.67; δ_C 14.9], one quarternary methyl group [δ_H 1.33; δ_C 29.6], and one isopropyl group [δ_H 1.60, 0.85 (6H); δ_C 38.9, 17.7, 16.6] were present. Furthermore, the ^{13}C-NMR spectrum suggested the presence of two quarternary olefin carbons [δ_C 136.8, 131.6], two oxygen-bearing quarternary carbons [δ_C 78.6, 73.9], and six methylene carbons [δ_C 45.0, 36.2, 35.4, 33.2, 24.1, 23.3]. These data and the ^{1}H,^{1}H-COSY correlations of **16** clearly indicated the presence of seven partial structures as shown in Figure 16.13. Connectivities of these partial structures were determined by detailed analysis of the ^{1}H,^{13}C long-range COSY correlations shown in Figure 16.14, and the planar structure of **16** was determined to be a cembrane-type diterpenoid. The location of the acetoxyl group at the C-20 position was indicated by the values of chemical



Scheme 16.7: Conversions of **14** and **15** into **19**.

shifts in the ^{1}H-NMR and ^{13}C-NMR spectra and the NOESY correlations between the δ_H 2.07 (acetoxymethyl) and δ_H 4.57, 4.66 (H-20). The geometries of the olefin moieties were determined in the following manner. Initially, we determined the geometry of di-substituted olefin by the value of the coupling constant (*J*), i.e. *trans* olefin protons show *J* = 13–18 Hz, while *cis* olefin protons show *J* = 7–12 Hz. The value of coupling constant J_{13-14} being 15.5 Hz, the geometry between C-13 and C-14 was established as *trans*. In the case of methyl group-bearing tri-substituted olefin moiety, the geometry can be revealed by means of the chemical shift of the methyl carbon in the ^{13}C-NMR spectrum, i.e. chemical shifts of δ_C 15–20 usually suggest *E*-geometry and those of δ_C 23–26 suggest *Z*-geometry (Choi *et al.*, 1986). Consequently, the geometry between C-7 and C-8 was determined by the value of the chemical shift of the C-19 methyl carbon (δ_C 14.9) as shown in Figure 16.14. In addition, the cisoid conformation (from C-11 to C-14) was determined on the basis of NOESY correlations between δ_H 5.59 (H-11) and δ_H 5.91 (H-14), as well as examination of Dreiding stereomodels based on the NOESY correlations as shown in Figure 16.15. The relative stereochemistry and preferred conformation of lobophynin A (**16**) was thus determined as shown in Figure 16.15.

The structures of lobophynins B (**17**) and C (**18**) were also elucidated using the same method applied to lobophynin A (**16**), and the comparison of spectral data with those

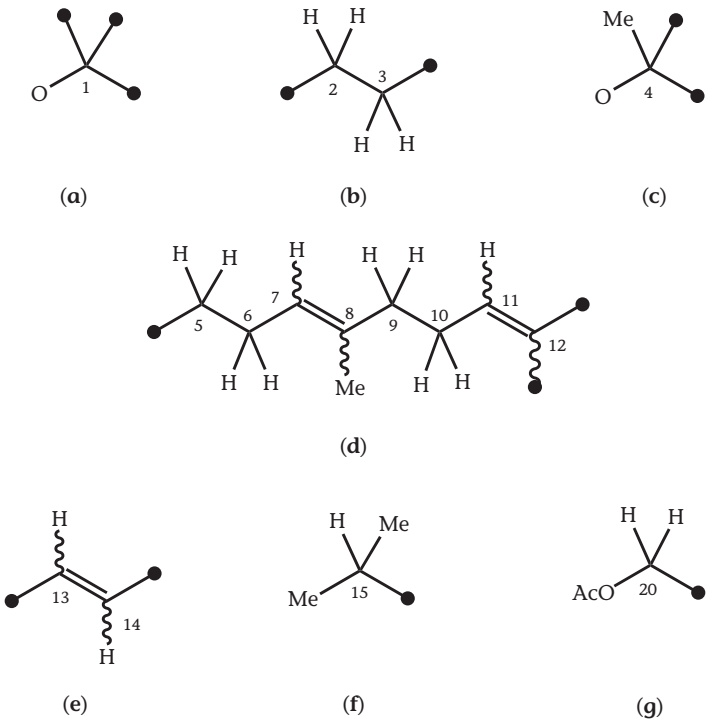


Figure 16.13: Partial structures of lobophynin A (**16**).

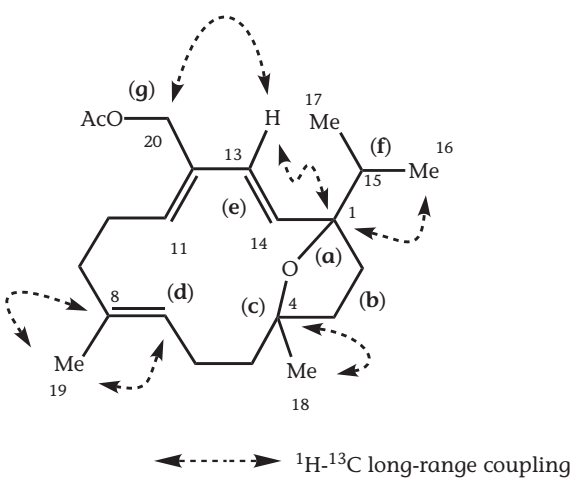


Figure 16.14: Planar structure of lobophynin A (**16**).

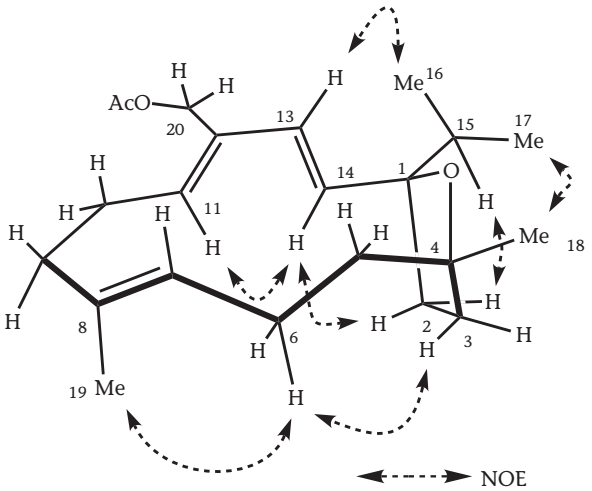


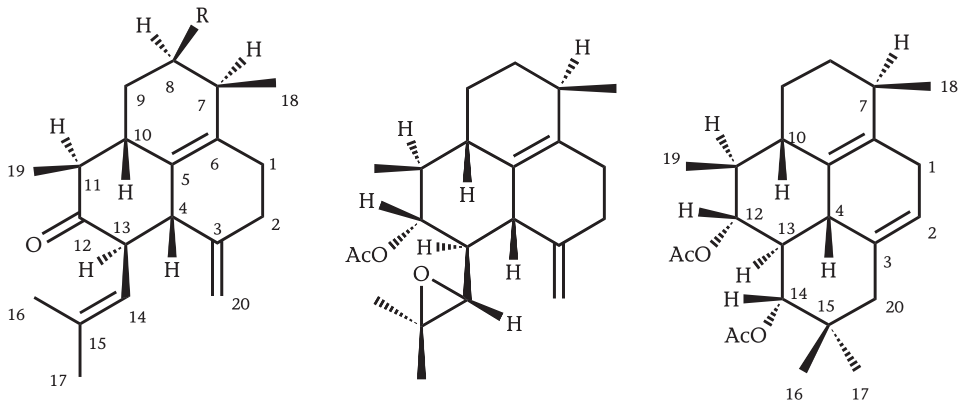
Figure 16.15: NOE correlations of lobophynin A (**16**).

of sarcophytoxide (**14**) and isosarcophytoxide (**15**). The position of the acetoxyl group of **17** was revealed by $^{1}H,^{13}C$ long-range COSY correlation between the ^{1}H-signal at δ_H 4.68 (H-17) and ^{13}C-signal at δ_C 139.0 (C-15). On the other hand, the location of the methyl ester group at the C-8 position of lobophynin C (**18**) was also indicated by $^{1}H,^{13}C$ long-range COSY correlations between the H-9 and C-8,C19. This location was also corroborated by the fact that ^{1}H-signal at δ_H 6.73 of H-7 in **18** shifted to a lower field than that of **15**. The 7*E*-geometry of **18** was assigned by means of the comparison of the chemical shift of H-7 with those of (7*Z*)-lobohedleolide (δ_H 5.76) and (7*E*)-lobohedleolide (δ_H 6.89) isolated by Uchio *et al.* (Uchio *et al.*, 1981).

16.3.4 Structures of the amphilectane-type diterpenoids isolated from *Sinularia nanolobata*

In the course of our continuing search for biologically active constituents from Japanese soft corals, we found that the *n*-hexane-soluble part (195.5 g) of the MeOH:$CHCl_3$ (50:50) extract of *Sinularia nanolobata* (26.0 kg) showed cytotoxicity against L1210 and KB cells *in vitro*. By means of the bioassay-directed fractionation of the extracts, we isolated four new amphilectane-type diterpenoids, designated sinulobatins A (**20**), B (**21**), C (**22**), and D (**23**) (Figure 16.16) (Yamada *et al.*, 1997). In this section, we wish to discuss briefly the structure determination of these compounds.

Sinulobatin A (**20**) was isolated as colorless prisms. The molecular formula $C_{22}H_{30}O_3$ was determined by positive HR-FABMS. The IR spectrum of **20** showed prominent peaks due to double bond (3040, 1660, and 900 cm^{-1}), carbonyl (1715 cm^{-1}), and ester (1735



sinulobatin A (**20**) R = OAc
sinulobatin B (**21**) R = H

sinulobatin C (**22**)

sinulobatin D (**23**)

Figure 16.16: Structures of sinulobatins isolated from *Sinularia nanolobata*.

and 1220 cm^{-1}) groups. The ^1H-NMR spectrum of **20** contained signals for two secondary methyls (δ_H 1.10 and 1.00), two vinyl methyls (δ_H 1.75 and 1.44), one exocyclic methylene (δ_H 4.78 and 4.56), and one olefinic proton (δ_H 5.13). The ^{13}C-NMR spectrum of **20** suggested the presence of three methylene carbons (δ_C 27.8, 28.8, and 32.1), five methine carbons (δ_C 38.1, 43.7, 50.9, 51.4, and 55.3), four quarternary olefinic carbons (δ_C 130.3, 132.2, 134.8, and 145.0), one ester carbon (δ_C 170.9), and one carbonyl carbon (δ_C 210.7). These data were confirmed by an HSQC (heteronuclear single-quantum correlation) 2D NMR spectrum. On the basis of these data and the molecular formula, **20** was considered to be a tricyclic C_{22} diterpenoid possessing acetoxyl, exocyclic methylene, tri-substituted olefin, and ketone groups. These facts and the ^1H,^1H-COSY 2D NMR spectrum of **20** clearly implied the presence of five partial structures (**a**–**e**) as shown in Figure 16.17. Their connectivities were determined by a detailed analysis of the HMBC (^1H-detected multiple-bond heteronuclear multiple quantum coherence) 2D NMR spectrum of **20**. In particular, HMBC correlations between the protons and carbons reported in Figure 16.18 suggested the connectivity of the partial structures (**a**–**e**) as shown in Figure 16.18. Thus, **20** was elucidated to be an amphilectane-type diterpenoid.

The relative stereochemistry of **20** was revealed by NOE correlations as shown in Figure 16.19 and *J* values from the ^1H-NMR spectrum, i.e. $J_{4–13}$ = 12.4 Hz, $J_{10–11}$ = 14.3 Hz, and $J_{13–14}$ = 10.0 Hz. Furthermore, we confirmed the structure by means of a single-crystal X-ray analysis. Next, the absolute configuration of **20** was established by studying its CD spectrum, which showed a positive Cotton effect at 294 nm ($\Delta\epsilon$ +6.3) due to an n → π* transition of the carbonyl group. The absolute configuration can

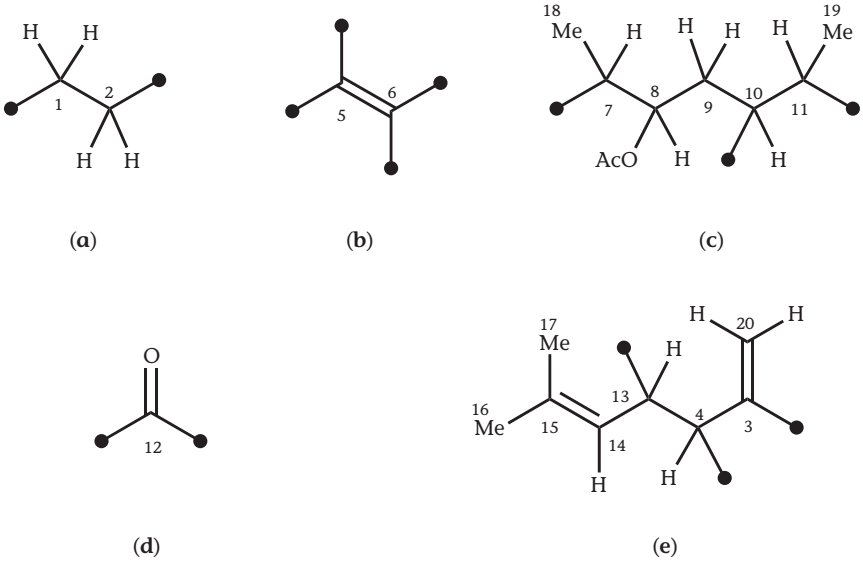


Figure 16.17: Partial structures of sinulobatin A (**20**).

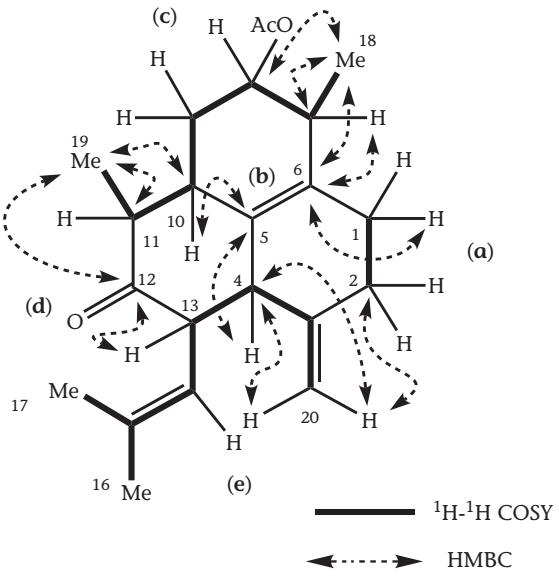


Figure 16.18: Planar structure of sinulobatin A (**20**).

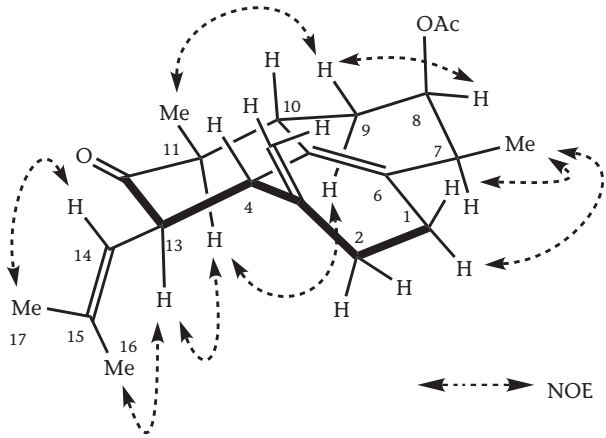
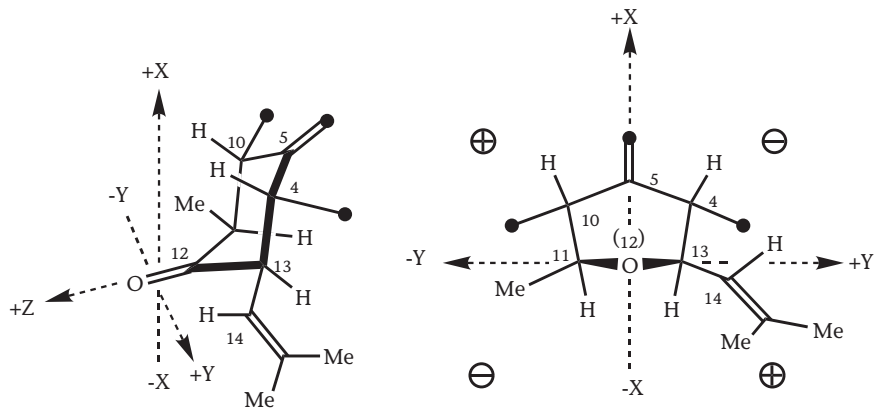


Figure 16.19: NOE correlations of sinulobatin A (**20**).



(a) octant projective view (b) back octant

Figure 16.20: Octant projective view and back octant of sinulobatin A (**20**).

be determined by applying the octant rule (Wratten *et al.*, 1978) to the cyclohexanone system. The octant rule is effective for the study of the stereochemistry of optically active substances. By applying the octant rule to the cyclohexanone system of **20**, it could be seen that the side chain at C-13 lay in the back-lower-right octant, which affords positive Cotton effect as shown in Figure 16.20. This fact suggested that the absolute stereochemistry of C-13 was *R*, and therefore, the absolute configuration of **20** as 4*S*,7*S*,8*R*,10*S*,11*S*,13*R* was established. Thus, the structure and absolute configuration of sinulobatin A (**20**) was elucidated.

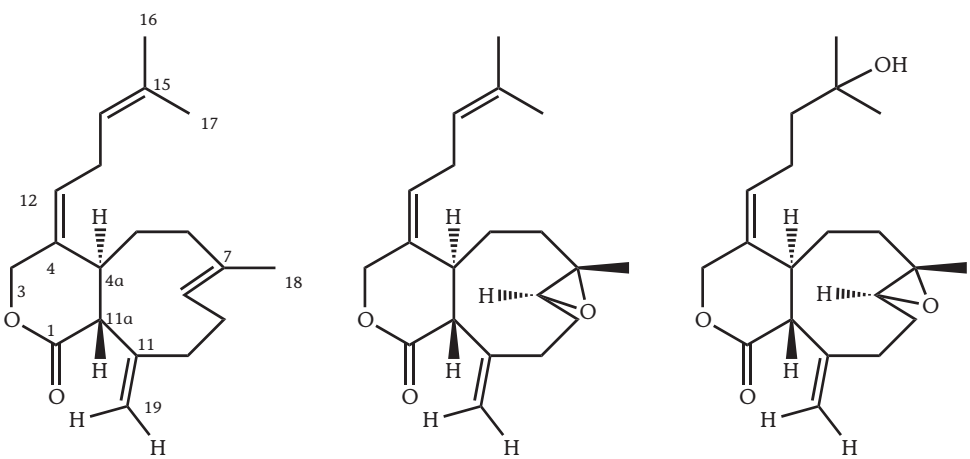
The structures of sinulobatins B (**21**), C (**22**), and D (**23**) were also elucidated by means of the same methods applied to sinulobatin A (**20**).

Amphilectane-type diterpenoids have been isolated from a blue coral (Look *et al.*, 1986), gorgonian corals (Molinski *et al.*, 1987), and marine sponges (Tamaka *et al.*, 1993). Thus, it is worth noting here that compounds **20**, **21**, **22**, and **23**, isolated by us, are the first amphilectane-type diterpenoids isolated from the alcyonacean corals.

16.3.5 Structures of the xenicane-type diterpenoids isolated from *Xenia elongata*

Miyamoto *et al.* have isolated a novel xenicane-type diterpenoid, designated deoxyxenolide B (**24**) and its oxidation products **25** and **26** from *Xenia elongata* collected in the same sea area cited in the preceding section (Miyamoto *et al.*, 1995). In this section, we discuss the structure of these diterpenoids and their absolute configurations. The extraction and isolation methods were carried out in the same manner as in the case of *Cladiella sphaeroides*. Their structures (shown in Figure 16.21) have also been elucidated on the basis of spectral analysis.

Two partial structures (**a**) and (**b**) of **24** were examined by ^{1}H-NMR, ^{13}C-NMR, and HSQC spectra as shown in Figure 16.22. Their connectivities were determined by means of HMBC correlations shown in Figure 16.22. As a result of these observations, the structure **24** was assigned to the new xenicane-type diterpenoid. The *E*-geometry of $\Delta^{4(12)}$ double bond was assigned based on the observed NOE correlation between H-3 and H-12. The 7*E*-geometry was determined by means of the value of the chemical shift of



deoxyxenolide B (**24**) **25** **26**

Figure 16.21: Structures of deoxyxenolide B (**24**) and its oxidation products **25** and **26**.

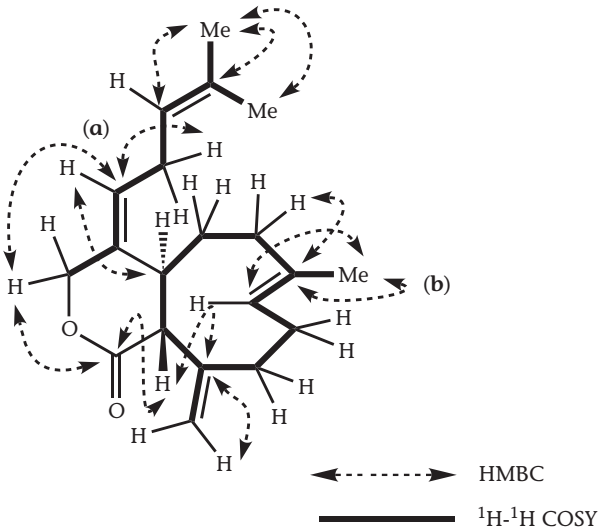


Figure 16.22: Planar structure of deoxyxenolide B (**24**).

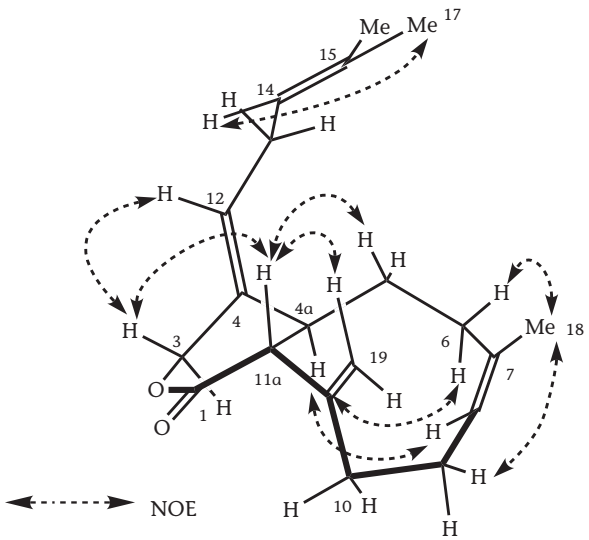


Figure 16.23: NOE correlations of deoxyxenolide B (**24**).

the C-18 methyl carbon (δ_C 17.9). The relative stereostructure of **24** was determined by NOESY correlations as shown in Figure 16.23. The absolute configuration of **24** was investigated by application of the lactone sector rule. The detailed principle of this rule is provided in the literature (Jennings *et al.*, 1965). It is similar to the octant rule except

for resolving the space into the sectoral spaces. The ORD spectrum of **24** showed a negative Cotton effect with a first extremum at 225 nm ([Φ] −16000). The octant and sector projections predicted a negative contribution, thus, the absolute stereochemistries of C-4a and C-11a of **24** were ascertained to be *S* and *R*, respectively.

In addition, it was revealed that deoxyxenolide B (**24**) is stable in the solid state but is oxidized in solution ($CHCl_3$, Me_2CO, H_2O), to give the oxidation products oxide-1 (**25**) and oxide-2 (**26**) as shown in Figure 16.21. The structures of these products were also determined on the basis of the spectral data. **26** was identified as compound previously isolated from the Japanese soft coral *Xenia florida* by Iwagawa *et al*. (1990).

16.4 BIOLOGICAL ACTIVITIES

We normally carry out the biological activity tests on the isolated compounds, focusing on lethal activity against brine shrimp, ichthyotoxicity against mosquito fish, and *in vitro* cytotoxic activity against murine lymphoma L1210 cells and human epidermoid carcinoma KB cells. These bioassay tests are relatively easy, therefore chemists can carry out them as bench bioassays. The detailed methods of those tests are provided in the related references and are summarized below.

16.4.1 Lethal activity against brine shrimp (Meyer *et al.*, 1982)

Lethal activity against *Artemia salina* L. (Brine Shrimp Test, BST) was performed using brine shrimp larvae, at 25°C for 24 h. All compounds were assayed by dissolving the appropriate amount in 1% of DMSO relative to the volume of test solution (sea water). Control tests were carried out with each test run. The toxicity was evaluated as LC_{100} (ppm).

16.4.2 Ichthyotoxicity (Yamada *et al.*, 1997)

Assays of ichthyotoxicity were conducted using the mosquito fish, *Oryzias latipes*, at room temperature for 24 h. Compounds were assayed at 30, 15, or 10 ppm by dissolving the appropriate amount in 1% of EtOH relative to the volume of test solution. Control tests were carried out with each test and the toxicity was evaluated as LC_{100} (ppm).

16.4.3 Cytotoxic activity (Carmichael *et al.*, 1987)

Cytotoxic activity *in vitro* was determined using murine lymphoma L1210 cells and human epidermoid carcinoma KB cells. Roswell Park Memorial Institute Medium 1640 supplemented with 10% FBS, and penicillin–streptomycin was used as the cell culture

medium. L1210 or KB cells (1×10^4 cells/ml) were cultured in a CO_2 gas incubator at 37°C for 72 h in 200 μl of medium using a 96-well microplate containing various concentrations of the test compound. Their viability, estimated using the MTT assay, was compared to that of control cells incubated in the identical medium without the compound. The cytotoxicity was evaluated as IC_{50} (μg/ml).

We now discuss the biological activities of diterpenoids **1–26** described in the preceding sections. The bioassay results are described in Table 16.3, and summarized as follows.

TABLE 16.3
Bioassay results of diterpenoids **1–26**

Compound	Lethal activity against brine shrimp LC_{50} (ppm)	Ichthyotoxicity LC_{100} (ppm)	Cytotoxicity [IC_{50} (μg/ml)] L1210	KB
1	>30.0	N.T.	1.5	2.9
1a	–	N.T.	26.4	53.1
2	N.T.	>20.0	N.T.	N.T.
3	N.T.	>20.0	N.T.	N.T.
4	–	>20.0	N.T.	N.T.
5	>10.0	>20.0	N.T.	N.T.
6	>10.0	>20.0	N.T.	N.T.
7	0.03	30.0	8.3	11.1
8	0.04	30.0	8.5	8.3
9	10.0	30.0	3.9	4.1
10	4.2	30.0	8.2	8.6
11	>20.0	30.0	12.0	11.3
12	7.5	N.T.	N.T.	N.T.
13	3.7	30.0	6.3	10.6
14	10.8	0.6	0.2	10.7
15	15.3	7.5	0.2	7.9
16	>30.0	N.T.	N.T.	N.T.
17	>30.0	N.T.	N.T.	N.T.
18	22.5	30.0	N.T.	N.T.
19	15.0	30.0	0.05	0.4
20	>30.0	–	3.0	5.1
21	>30.0	–	4.8	7.7
22	>30.0	–	3.2	4.5
23	>30.0	–	N.T.	N.T.
24	–	15.0	N.T.	N.T.
25	–	–	N.T.	N.T.
26	–	–	N.T.	N.T.

N.T.: not tested
–: inactive

16.4.4 Biological activities of eunicellin-type diterpenoids **1–13** and **1a**

Of the eunicellin-type diterpenoids, the less polar compounds **7–13** showed more pronounced activities for the lethal activity against brine shrimp than more polar compounds. In these diterpenoids, **2–11** and **13** exhibited ichthyotoxicity, and the concentrations (LC_{100}) were from 15 to 30 ppm. Furthermore, **1**, **1a**, **7–11**, and **13** showed cytotoxicity against L1210 cells and KB cells with IC_{50} values as shown in Table 16.3. In particular, it is interesting that compound **1**, which has a hydroperoxyl group, showed the most pronounced cytotoxicity.

16.4.5 Biological activities of cembrane-type diterpenoids **14–19**

Of these diterpenoids, **14** and **15** especially showed more pronounced ichthyotoxicity (LC_{100} 0.6 and 7.5 ppm, respectively), and these acute toxicities were observed after a few minutes. It is interesting that ichthyotoxicity of **19** is lower than those of **14** and **15**, but its cytotoxicity is higher. A large number of cembrane-type diterpenoids have been isolated from the soft corals. Many of them exhibit biological activities, e.g. cytotoxicity, inhibition of cholinesterase (Ne'eman *et al.*, 1974), Ca-antagonistic action (Kobayashi *et al.*, 1983), etc. In particular, the ichthyotoxic diterpenoids, sarcophine (Bernstein *et al.*, 1974) and lobolide (Kashman *et al.*, 1977), which have been isolated from the soft corals *Sarcophytum glaucum* and *Lobophytum* sp., respectively, are believed to play a role in the coral's protective mechanism against predators. Therefore, it is reasonable that cembrane-type diterpenoids **14–19** may also play a similar role.

16.4.6 Biological activities of amphilectane-type diterpenoids **20–23**

Diterpenoids **20–23** did not exhibit pronounced lethal activities against brine shrimp. However, they could not be tested for their ichthyotoxicity because of low yields, but **20–22** showed cytotoxicities with IC_{50} values as shown in Table 16.3.

16.4.7 Biological activities of xenicane-type diterpenoids **24–26**

Diterpenoid **24** exhibited ichthyotoxicity and its lethal concentration (LC_{100}) within 1 h was 15 ppm, but its oxidation products **25** and **26** did not show toxicity.

16.5 CONCLUSIONS

In this chapter, we have described the typical extraction, isolation, and structure elucidation methods of biologically active diterpenoids from soft corals. For efficient isolation of bioactive diterpenoids, it was shown to be important that extracts are partitioned

between solvents before their fractionation by column chromatography. Furthermore, it was also revealed that gel-filtration is effective for initial fractionation and that reversed phase HPLC is important for purification. In addition, it was proved that the 2D NMR spectra are the main methods for structure elucidation, and that a modification of Mosher's method and the CD exciton chirality method are excellent methods for the determination of absolute configuration.

A large number of eunicellin-type diterpenoids have been isolated from the soft corals and their bioactivities reported. In addition, Ochi *et al.*, have reported that some eunicellin-type diterpenoids isolated from the whole bodies of *Litophyton* sp. exhibited lethal and repellent activities against the muricid gastropod *Drupella fragum*, which was known to harm some hard corals (Ochi *et al.*, 1992). It is worth noting that we have obtained the same type of diterpenoids from the mucus of *Litophyton* sp. These facts make it possible that these eunicellin-type diterpenoids (in combination with a wide variety of other compounds) stored in the mucus on skin glands of *Litophyton* sp., which is devoid of a physical means of defence, could be used as a chemical defense system to survive in predator-rich environments.

Of the cembrane-type diterpenoids, lobophynin A (**16**) is unique for it has ether bonds between C-1 and C-4 and an acetoxyl group at C-20, while lobophynin B (**17**) is unusual as a dihydrofuran type cembranoid with an acetoxyl group at C-17.

The amphilectane-type diterpenoids have been isolated from a blue coral, the gorgonian corals, and the marine sponges. However, compounds **20**, **21**, **22**, and **23**, isolated by us, are the first amphilectane-type diterpenoids isolated from the alcyonacean corals.

The xenicane-type diterpenoids, which interestingly are oxidized in solution, have been isolated from *Xenia elongata*.

ACKNOWLEDGEMENTS

We are grateful to Mr. Y. Imahara, Wakayama of the Prefectural Museum of Natural History, for the identification of specimens, Dr. T. Komori, and Dr. T. Miyamoto, for their helpful discussions and suggestions, as well as to graduate students of our laboratory for their technical assistance. Thanks are also due to Mr. Y. Tanaka, and Miss Y. Soeda, of the Graduate School of the Pharmaceutical Sciences, Kyushu University, for the ^{1}H-NMR and ^{13}C-NMR measurements, and Dr. R. Isobe, of the Department of Industrial Chemistry, Faculty of Engineering, Towa University, for the MS measurement. The present work was supported in part by a Grant-in-Aid for Scientific Research (No. 3214 and 06453217) from the Ministry of Education, Science, Sports, and Culture, Japan, which is gratefully acknowledged.

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