2.01 Overview and Introduction

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Extending knowledge and principles of structural chemical diversity is one important outcome derived from the comprehensive study of natural products. The 19 chapters of this volume provide an eye-catching glimpse of some past landmark discoveries, a synopsis of the current discovery strategies, and prospects for future advances. The perspectives provided here are rich in overviews of unusual scaffolds produced from terrestrial and marine organisms that are especially robust in machinery to biosynthesize unusual compounds. We are confident that substantial rewards will be gained for readers engaged in the careful study of the synopses that follow. It is also noteworthy that this collection provides an orthogonal view of the natural products structural diversity presented in Volume 1, which was organized largely on biosynthetic grounds.

The potential of molecular structures to populate structural chemical space is diverse and the challenge is to make new discoveries that add to the accumulated knowledge base. An important milestone was achieved during the assembly of this volume and it involves the registration, in 2008 by Chemical Abstracts Service (CAS), of the 40 millionth chemical substance. However, a rather surprising outcome was deduced by a CAS team lead by Lipkus; his team analyzed these frameworks and concluded that "half of the compounds can be described by only 143 framework shapes". The rigorous study of natural products provides an optimal way to expand such understanding and such a prospect is amply illustrated by the 19 chapters in this volume.

The rate of new compound discovery is on an upward trajectory; worldwide more than 200 new compounds are discovered per hour. A topic of substantial general interest to chemistry and biology professionals involves assessment and utilization as tools for further inquiry of the structural diversity present in both synthetic and natural products. Each chapter in Volume 2 illustrates the pathways and methods useful in the discovery of natural products, many of which possess previously unexplored molecular structure domains. Each chapter presents accounts on biosynthetic products possessing high atom diversity, intriguing elements of chirality, or structures densely sprinkled with functionality. Overall, these chapters depict hundreds of molecular structures and are roughly divided into eight topical areas.

The traditional targets for natural products discovery – terrestrial plants, marine macro algae, and arthopods – are examined in Chapters 2.02–2.04. The important role played by plant natural products in the development of therapeutics to treat a wide range of diseases is outlined in Chapter 2.02. More than 29 significant natural products are discussed, including such well-known compounds as taxol, the vinca alkaloids, prostratin, resveratrol, and the ginkgolides. The last two of these three chapters are sharply focused on marine and terrestrial natural products. Descriptions of new connectivity patterns are highlighted, but even more important, are the fascinating contrasts in marine versus terrestrial chemical ecology mechanisms that can be gleaned from side-by-side reading of these chapters.

Significant natural products continue to be derived from microorganisms and this is the focus of five successive chapters, Chapters 2.05–2.09. The pioneering work of Professor Waksman on soil-derived actinomycetes, a source of the majority of natural antibiotics, provides the launching point for the rich discussions that follow in Section 2 of this volume. The so-called 'antibiotic of last resort', vancomycin ($C_{66}H_{75}Cl_2N_9O_{24}$), discovered in the 1950s, is representative of complex structures elaborated by filamentous bacteria. Similarly significant milestone natural products serve as anchor points for the remaining chapters. Some examples include (1) cyrptophycin ($C_{35}H_{43}ClN_2O_8$) from cyanobacteria, (2) epothilone A ($C_{26}H_{39}NO_6$ S) from myxobacteria, (3) the diketopiperize NPI-2538 being explored in a US anticancer clinical trial, whose structure is based on halimide isolated from a marine-derived fungus, and (4) the structurally complex amphidinolide M

($C_{43}H_{66}O_6$), a potent cytotoxin ($IC_{50} = 0.05\ ng\ ml^{-1}$ vs. L1210 murine lymphoma cells) and lead structure among signature compounds of marine dinoflagellates.

The prolific sources of marine natural products, sponges, and gorgonians are explored in the next section, Chapters 2.10–2.11. A large percentage of marine natural products are sponge-derived and attention is devoted to five major groups of secondary metabolites obtained from sponges. Another important topic involves structures that are leads for human disease therapeutics. At the top of this list are polyketides such as halichondrin B, spongiastatin-1, and discodermolide. A flavor of the diverse array of natural products that can be encountered from octocorals can be gained from the chapter dealing with just one genus, *Pseudopterogorgia*. A total of 243 substances divided into 19 chemotypes have been discovered from this source between 1968 and 2008.

The application of molecular genetics to natural products discovery offers rich rewards. This topic, alongside the results of employing synergistic stimuli to upregulate natural product biosynthetic pathways, is explored in the next set of three chapters, Chapters 2.12–2.14. Genome sequencing offers a glimpse into the molecular blueprint of an organism and by reading and decoding this information, chemists can employ cleaver methods to extract new metabolites from cultured organisms (Chapter 2.12) as well as from uncultured environmental communities (Chapter 2.13). For instance, the genome sequence of *Streptomyces coelicolor* A3(2) deduced in the year 2002 revealed that this model actinomycete bacterium has the genetic capacity to synthesize two dozen natural products, most of which at the time were of unknown molecular composition but have since succumbed to structure elucidation to yield new chemical entities. This powerful combination of genomics and natural product chemistry has furthermore been successfully applied to probe intimate symbiont interactions in insects and marine organisms (Chapter 2.14).

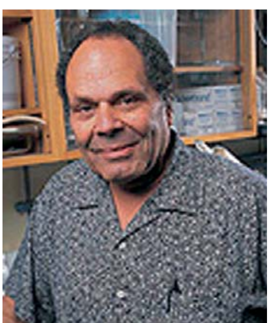
Great structural diversity is possible from the combinatorial combination of natural and unnatural amino acids. Nature has used this as a template to form powerfully active natural products. The richness of molecular structures coupled with physiological activity is the focus of Chapters 2.15 and 2.16. A dazzling array of peptide toxins from the venoms of cone snails and sea anemones is illustrated in Chapter 2.15 that will provide the reader with a comprehensive appreciation for their distribution, diversity, neuropharmacology, and therapeutic applications. Marine tunicate cyclic peptides were the inspiration for the discovery of a new family of ubiquitous peptides from symbiotic as well as free-living cyanobacteria called cyanobactins that complement the fascinating assortment of highly modified ribosomal peptides derived from microorganisms.

The final sections of this volume treat three complementary topics. Chapter 2.17 outlines the application of insights from bioinformatics and molecular genetics to explore new chemical structural space relative to macrolides and cyclic peptides. The task of total structure elucidation is a core activity of natural products chemistry and it is important that this be completed in an unequivocal fashion. The trials and tribulations of this task are explored in Chapter 2.18 through the discussion of illustrative case examples of structure elucidations gone bad. Somewhat surprising is the following passage from Chapter 2.18: "By the end of 2008, more than 200 structure revisions for marine natural products have been reported." The final two chapters, Chapters 2.19 and 2.20, return to natural products and therapeutics. These ideas were explored in the opening chapter and intriguing new dimensions are explored therein.

The topics of this volume amply illustrate the structural diversity of both terrestrial and marine natural products. We know that the reader will gain insights from the study of past milestone developments as well as pitfalls in experimental design. The critical role of dereplication in modern structure elucidation is also amply illustrated. Once the knowledge base is in hand from these chapters, we trust that many readers will be inspired to design and apply new experimental directions for future studies.

Biographical Sketches

Bradley S. Moore is currently professor of oceanography and pharmaceutical sciences at the Scripps Institution of Oceanography and the Skaggs School of Pharmacy and Pharmaceutical Sciences at University of California, San Diego. He was first introduced to natural product research as a chemistry undergraduate student at the University of Hawaii, where he explored the chemistry and biosynthesis of cyanobacterial natural products with the late Professor R. E. Moore. Fascinated by the beauty and complexity of natural product structures, he went on to conduct graduate (Ph.D. 1994 in bioorganic chemistry with Professor H. G. Floss at the University of Washington) and postdoctoral research (1994–95 with Professor J. A. Robinson at the University of Zürich) on the biosynthesis of bacterial natural products in order to explore how nature assembles complex organic molecules. Prior to moving to the University of California at San Diego in 2005, he held academic appointments at the University of Washington (1996–99) and the University of Arizona (1999–2005). His research interests involve exploring and exploiting marine microbial genomes to discover new biosynthetic enzymes, secondary metabolic pathways, and natural products for drug discovery and development.



Phillip Crews is currently distinguished professor of chemistry and biochemistry at the University of California at Santa Cruz. His training included a B.S. in chemistry from the University of California at Los Angeles, and a Ph.D. in organic chemistry from the University of California at Los Angeles. He engaged a year of postdoctoral research at Princeton University, supported by a National Science Foundation Fellowship. His entire independent academic career has been at the University of California at Santa Cruz. Early in his term as an assistant professor he began, without prior training, a program in marine natural products chemistry that required a 10-year incubation to become successful.

Research in the Crews lab emphasizes innovative approaches to the study of marine natural products chemistry. During the thirty years his program has matured and is regarded, on an international level, as among the most active and productive in this subject area.

The effectiveness of his efforts continues to be based on a foundation of multifaceted discoveries. The Crews group emphasizes a field-driven approach to explore and discover inspirational chemical structures often accompanied by significant biological activity derived from marine sponges. Several years ago the lab expanded investigations to encompass the study of marine-derived fungi.

The most important new compounds that the Crews lab have published are those that exhibit anticancer activity and/or action against relevant molecular targets, or function as important molecular probes. Significant examples (but not an exhaustive list) of these new biomolecules first discovered at the University of California at Santa Cruz include bengamides A and B, jasplakinolide, fijianolides A and B, mycothiazole, plakinidines A and B, the psammaplins, asparazine, the milnamides, psymberin, leucosolenamines A and B, the RHMs, and efrapeptin polypeptides. Several of these compounds have been the seed for further therapeutic development. The synthetic compound, LAF380, developed as a bengamide A analogue entered cancer clinical trials in 2000. Though the trials were subsequently suspended, other bengamide congeners are being evaluated for their potential as anticancer drugs. The fijianolides, psymberin, and efrapeptin G have shown positive in vivo responses at the Ford Cancer Center and a broad-based campaign has begun to further exploit the clinical potential of these agents. One additional noteworthy development is our lab continues to supply important actin inhibitors, jasplakinolide and latrunculin A, to investigators throughout the world to facilitate its use as a molecular probe. For the last decade, more than 200 publications have appeared annually based on the use of both compounds in cell biology research.

2.02 Terrestrial Plants as a Source of Novel Pharmaceutical Agents

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2.02.1 Introduction

The study of natural products, or 'Nature's Combinatorial Library', has had a long history as a source of drugs, and plants have historically been at the forefront of natural product drug discovery. In the anticancer area, for example, vinblastine and vincristine, etoposide, paclitaxel (Taxol), docetaxel, topotecan, and irinotecan, among others, are all plant-derived natural products or modified versions of plant compounds, while antimalarial therapy would be much poorer without quinine and artemisinin and the drugs derived from these plant products. This chapter provides an overview of the major medicinal agents that are themselves natural products isolated from plants or are chemical modifications of such lead compounds. It covers the therapeutic areas of cancer, HIV, malaria, cardiovascular, and central nervous system (CNS) diseases. Natural plant products have also made contributions in areas such as immunomodulatory[1–3] and antibiotic activities,[4–6] and the reader is referred to the cited reviews for information on these areas.

2.02.2 Plants as a Source of Bioactive Compounds

Plants have historically been the most important source of novel bioactive natural products, for obvious practical reasons. It is much easier to identify and collect biomass from a tree or shrub than to culture and identify a microbial species, or to dive into the ocean to collect marine organisms. It is thus thoroughly understandable that the vast majority of natural products discovered before about the mid-twentieth century were of plant origin. In addition to the relative ease of collection of plant biomass, plants often have a tradition of use as phytomedicines, and this tradition can guide the selection of plant materials to be investigated. The importance of plants as a source of anticancer agents has been summarized recently[7] and a discussion of the value of natural products to future pharmaceutical discovery points out that "natural products have been the major source of chemical diversity for starting materials for driving pharmaceutical discovery over the past century."[8] Finally, a comprehensive review with over 800 references to natural product-derived compounds in clinical trials has also appeared recently.[9] Although the reviews cited cover natural products from all sources, they do include many examples of plant-derived compounds in clinical use or as leads to clinical development, and thus serve to highlight the importance of plant natural products as a source of bioactive compounds.

2.02.3 Anticancer Compounds

2.02.3.1 Vinca Alkaloids

The vinca alkaloids vinblastine (**1**) and vincristine (**2**) were the first natural products to enter clinical use as anticancer agents. They were isolated independently from the plant *Catharanthus roseus* G. Don (then known as *Vinca rosea* L., whence the common name of the alkaloids came) by two research groups in the late 1950s and early 1960s. One group was that of Robert Noble and Charles Beer at the University of Western Ontario and the other was that of Gordon Svoboda at Eli Lilly and Company. Interestingly, the Canadian group was actually looking for antidiabetic agents and discovered the anticancer activity of vinblastine by a combination of good luck and astute reasoning.[10]

1 R = CH_3, X = OCH_3 Vinblastine
2 R = CHO, X = OCH_3 Vincristine
3 R = CH_3, X = NH_2 Vindesine

4 Vinorelbine *n* = 1
5 Anhydrovinblastine *n* = 2

6 Vinflunine

The mechanism of action of the vinca alkaloids is that of the inhibition of the polymerization of tubulin to microtubules. The cellular protein tubulin, which occurs in α- and β-forms, is essential for proper cellular function. During mitosis tubulin polymerizes to form microtubules, which are long tube-shaped protein polymers. The equilibrium between unpolymerized α- and β-tubulin and microtubules is an important one and any disruption of this equilibrium can send dividing cells into mitotic block and apoptosis.[11] The vinca alkaloids bind to β-tubulin at a different site from paclitaxel (Taxol) and act to prevent tubulin assembly.

Vinblastine and vincristine have been used clinically for many years. The major importance of vinblastine is as part of a combination treatment for Hodgkin's disease, while vincristine is used in combination chemotherapy of acute lymphoblastic leukemias and lymphomas.[12] Several analogues of the vinca alkaloids have entered clinical use or clinical trials, including vindesine (**3**),[13] vinorelbine (**4**),[14] the naturally occurring anhydrovinblastine (**5**), and vinflunine (**6**).[10,15] Several synthetic approaches to the vinca alkaloids have been developed, especially by Kuehne,[16] while studies of the chemistry of the alkaloids in super acidic media have yielded new alkaloids such as vinflunine.[15]

2.02.3.2 Podophyllotoxin, Etoposide, and Teniposide

The resin product obtained by extraction of the dried roots and rhizomes of the North American plant *Podophyllum peltatum* L. (the American mandrake or mayapple) and of the related Indian species *Podophyllum emodi* Wall. Ex Royle is known as podophyllin and has long been known to possess medicinal properties. The major active substance in podophyllin is the lignan lactone podophyllotoxin (**7**) although a variety of other lignans and lignan glycosides have also been isolated from podophyllin.[17]

7 Podophyllotoxin

8 Etoposide R = CH_3

9 Teniposide R =

10 Etopohos

Podophyllotoxin has potent cytotoxic activity and also acts as an inhibitor of tubulin polymerization but it is too toxic to be useful as an anticancer agent. Fortunately, scientists at Sandoz, Ltd. studied the chemistry of podophyllotoxin glycosides and these studies led to the discovery of the semisynthetic podophyllotoxin analogues etoposide (**8**) and teniposide (**9**). Both these compounds are characterized by having a 4′-hydroxyl group and a glycoside substituted at the 4-*epi*-position. Etoposide is approved for the treatment of testicular cancer and both drugs are used for a variety of cancers.[18] Both etoposide and teniposide are only sparingly soluble in water and so the prodrug etopophos (**10**) was developed to provide a more soluble form of etoposide; it has a similar pharmacological profile to etoposide.[19]

Interestingly, the modes of action of etoposide and teniposide differ markedly from that of the parent compound podophyllotoxin. These compounds arrest cells in the late S and G2 phase of the cell cycle and have no effect on tubulin assembly. Instead, they induce single-strand breaks in DNA (etoposide) or in the DNA in L1210 cells (teniposide).[20–22] In the case of teniposide, these breaks are predominantly double-stranded. These effects are due to the ability of these compounds to inhibit DNA topoisomerase II (topo II).[23] DNA topoisomerases are enzymes that allow DNA to coil and uncoil (i.e., change its topology), which is a necessary prelude to mitosis. The topo II mediates double-strand breaks by forming a complex with DNA, the so-called cleavable complex. Etoposide stabilizes this complex and inhibits the enzyme, thus leading to double-strand breaks and ultimately to cell death.[24]

Numerous podophyllotoxin analogues have been prepared in attempts to develop improved drugs and the three new derivatives GL-331 (**11**),[25] NK 611 (**12**),[26] and TOP-53 (**13**)[27] illustrate some of the leading candidates to emerge from this work. The development of these and other podophyllotoxin analogues has been reviewed.[28,29] In spite of extensive synthetic work, the natural product podophyllotoxin remains the preferred source of all the analogues developed to date.

11 GL-331 **12** NK611 **13** TOP-53

2.02.3.3 Camptothecin and Analogues

The alkaloid camptothecin (**14**) was discovered in the mid-1960s by the team of Monroe Wall and Mansukh Wani at the Research Triangle Institute in North Carolina; this was the same team that discovered taxol (now known as paclitaxel) a few years later. Camptothecin was obtained from extracts of the Chinese tree, *Camptotheca acuminata* Decne., 1873, and it showed good activity against L1210 leukemia. It was however very insoluble in water and this led to the clinical studies that were carried out on its water-soluble ring-opened sodium salt. Unfortunately, these trials revealed numerous problems, connected in large part with the lack of activity of the ring-opened form. The trials were thus suspended[30] and the development of this compound was delayed for several years.

Fortunately, studies on camptothecin continued in some laboratories and it was discovered that it had a previously unknown mechanism of action, namely the ability to inhibit topoisomerase I (topo I).[31] The topoisomerases I and II are enzymes that allow chromosomal DNA to undergo changes in topology (i.e., relaxation) prior to replication. Camptothecin was found to inhibit topo I but not topo II, and to do so

by binding to the topo I covalent binary complex.[31] Camptothecin was the first compound found to inhibit topo I as opposed to topo II, and it thus complements drugs such as the podophyllotoxin analogues etoposide and teniposide, which inhibit topo II (Section 2.02.2). This mechanism is consistent with the fact that camptothecin is capable of inhibiting DNA synthesis leading to cell death during the S phase of the cell cycle.[32] It is noteworthy that camptothecin shows remarkable specificity in binding only to the cleavable complex formed between topo I and DNA; it does not bind to DNA alone or to topo I alone. Although topo I is an enzyme found in all cell types, its levels are elevated in tumors of the colon, ovary, and the prostrate, and this is presumably a significant part of the reason for the effectiveness of the camptothecin analogues against the first two of these tumors.[33]

14 Camptothecin **15** Topotecan **16** Irinotecan

This discovery significantly increased interest in the compound and led to the synthesis of a number of water-soluble analogues, which ultimately led to the development of the camptothecin analogues topotecan (Hycamtin) (**15**) and irinotecan (Camptosar) (**16**), both of which have been approved for clinical use. Topotecan is used as second-line therapy for advanced ovarian cancer in patients who have failed to respond to treatment regimens that include platinum or paclitaxel, while irinotecan has been approved for the treatment of advanced colorectal cancer.

Many other analogues of camptothecin have been prepared. Studies of compounds modified on the quinoline ring system have shown that substitutions at C-11 and C-12 normally result in a reduction of activity, while substitutions at C-7, C-9, and C-10 can lead to enhanced activity.[34] The E-ring lactone is important for activity and almost all modifications to this ring have led to less active compounds; the homocamptothecins, with an expanded ring E, represent an important exception.[34]

A survey of the distribution of camptothecin and its metabolites has been published.[35] The compounds are still obtained from the bark and seeds of *C. acuminata* and *Nothapodytes foetida*. Recent studies with hairy root cultures of *C. acuminata* and *Ophiorrhiza pumila* indicate that plant tissue culture methods of production may prove feasible in the future.[35]

2.02.3.4 Combretastatins

The combretastatins, such as combretastatin A-4 (**17a**), are a family of stilbenes, which were isolated from the South African 'bush willow' *Combretum caffrum* (Eckl. & Zeyh.) Kuntze, collected in Southern Africa in the 1970s as part of a random collection program for the US National Cancer Institute (NCI) by the US Department of Agriculture (USDA), working in collaboration with the Botanical Research Institute of South Africa.[36] They act as antiangiogenic agents, causing vascular shutdown in tumors and resulting in tumor necrosis.[37] Poor solubility of combretastatin A-4 in aqueous media precluded its advanced development but the water-soluble analogue, combretastatin A-4 phosphate (CA-4P; Zybrestat; R = PO_3Na_2, **17b**), has received orphan drug status from the US Food and Drug Administration (FDA) for the treatment of a range of thyroid cancers and ovarian cancer (http://www.fda.gov) and is in advanced clinical trials against anaplastic thyroid cancer, in combination with paclitaxel and carboplatin (http://www.clinicaltrials.gov).

17a Combretastatin A-4, R = H
17b CA-4P, R = PO_3Na_2

18a A-305754 W =

18b A-259745 W =

18c A-289099 W =

The synthesis of many combretastatin analogues (e.g., **18a**, **18b**, and **18c**) clearly illustrates the power of a relatively simple natural product structure to spawn a prolific output of medicinal and combinatorial chemistry.[36,38,39] Most synthetic congeners contained the essential trimethoxy aryl moiety linked to substituted aromatic moieties through a variety of two or three atom bridges including heterocyclic rings and sulfonamides. A number of combretastatin mimics are being developed and three analogues are in clinical trials[40] while 11 are in preclinical development as potential anticancer agents.

2.02.3.5 Homoharringtonine

Homoharringtonine (HHT) (**19**) is an ester derivative of the parent alkaloid cephalotaxine (**20**), which has an unusual tetracyclic ring system. Cephalotaxine was isolated from two *Cephalotaxus* species[41] and its final structure was assigned by X-ray crystallography.[42] HHT was isolated in 1970 by workers at the USDA laboratories in Peoria[43] but its anticancer activity was first recognized by Chinese investigators.

19 Homoharringtonine

20 Cephalotaxine

HHT functions as an inhibitor of protein synthesis and this appears to be its major mechanism of action. It is active against several murine tumors, including L1210 and P388 leukemias and B26 melanoma, and it was selected for development because of its activity and its relatively higher availability in plants. It has advanced to several Phase II clinical studies but it has not yet been approved for clinical use in the United States or Europe. Its synthesis, medicinal chemistry, and mechanism of action have been reviewed.[44] Its clinical development and use have also been reviewed.[45] The latter review concludes, "However, the promising activity of HHT in patients with CML, MDS, APL, central nervous system leukemia, and polycythemia vera, as well as the development of pure semisynthetic HHT and HHT derivatives, should reinvigorate research to establish the value of HHT in hematologic malignancies as well as other tumors."[45]

2.02.3.6 Taxol and Its Analogues

The diterpenoid **21** was isolated from the bark of the western yew, *Taxus brevifolia* Nutt, in the late 1960s by Monroe Wall and Mansukh Wani as part of a systematic search for anticancer compounds from plant sources,

and its structure was published in 1971.[46] Compound **21** was named taxol by Wall and Wani, but the name Taxol was later trademarked by BMS for their formulation of compound **21**, and the name paclitaxel is now used for compound **21**. Because of the historical nature of this section, the name taxol is used throughout; no infringement of the BMS trademark is intended. It has an unusual structure, with a complex tetracyclic ring system coupled to a substituted phenyl isoserine side chain. The tetracyclic ring system had been isolated previously as the diterpene baccatin III (**22**), although the correct structure of this compound was not published until 1975.[47]

21 Taxol

22 R = Ac Baccatin III
23 R = H 10-Deacetylbaccatin III

24 β-Lactam

The original Wall and Wani publication clearly indicated the promising anticancer activity of taxol but in spite of this its development was slow and almost did not occur at all. This was due to three major reasons. In the first place, taxol is a complex compound that would obviously be very difficult to synthesize, and it only occurred in low amounts in the thin bark of the scarce western yew; later calculations indicated that it would take the bark of 3–6 trees to provide the taxol needed to treat one patient. Compound supply was thus going to be a major problem. In the second place, taxol is very insoluble in water and so its formulation presented significant challenges. Finally, it had an unknown mechanism of action and the only *in vivo* activities it showed were against leukemias for which good drugs such as the vinca alkaloids already existed.

Fortunately, its initial activity, both *in vitro* and *in vivo*, was sufficiently interesting for the National Cancer Institute to provide the funding to produce enough taxol to carry out additional biological testing on the then new human tumor xenograft assays in nude mice. These studies indicated that taxol had strong reproducible activity against the B16 melanoma, meeting the NCI criterion for development, and the NCI Decision Network Committee approved it as a development candidate in April 1977.[48] This decision paved the way for it to enter formulation studies, and a Cremophor–ethanol surfactant formulation was developed and approved in 1980. This decision had both positive and negative effects. On the negative side, the large doses of Cremophor necessary for successful formulation caused some hypersensitivity reactions in some patients and nearly resulted in taxol being dropped as a clinical candidate. On the positive side, this problem caused taxol to be administered as a slow infusion, which probably enhanced its activity, and the Cremophor itself appears to have some positive effects on taxol's activity.[48]

A further and very important discovery was made by Susan Horwitz in 1979 when she reported that taxol had a totally new mechanism of action. The importance of tubulin as an anticancer target was discussed briefly in Section 2.02.3.1 and it was well recognized that compounds such as the vinca alkaloids, which inhibit the polymerization of α- and β-tubulin to microtubules, had the potential to be effective anticancer agents. It was not anticipated that another whole class of compounds existed that could promote the assembly of α- and β-tubulin to microtubules. It was this activity that Susan Horwitz and her associates reported in a key publication in *Nature*.[49] This discovery gave a significant boost to taxol's prospects and proved to be crucial in maintaining interest in taxol when it encountered hypersensitivity reactions in early clinical trials.[48]

Clinical trials of taxol were initiated in 1984 and the first report of clinical activity, against ovarian cancer, was published in 1989.[50] This aroused enormous interest in taxol as a drug and the NCI provided funding for large-scale production and also initiated a Cooperative Research and Development Agreement (CRADA) with BMS. The report by Holmes *et al.*[51] in 1991 that taxol had excellent activity against primary metastatic breast cancer created a huge demand for the drug. BMS met the challenge of successfully providing enough drug, initially by an intensive program of bark collection and extraction, and later by semisynthesis from 10-deacetylbaccatin III (**23**), which is available from yew needles in relatively large amounts,[52] and a β-lactam

such as **24**.[53] Taxol is currently produced by several companies by isolation and by semisynthesis and BMS also produces it by plant tissue culture methods.[54]

Taxol's tubulin-polymerization activity has been shown to result from its binding to microtubules with a stoichiometry of approximately 1 mol of drug to 1 mol of tubulin dimer.[49] At high concentrations the drug stabilizes microtubules and increases the total polymer mass[55] but these concentrations are higher than those needed to inhibit microtubule functions.[56] At clinically relevant concentrations, however, taxol interferes with the formation of the mitotic spindle, thus preventing the chromosomes from separating[57] and leading to mitotic arrest.[58] Further details of the tubulin-binding action of paclitaxel can be found in various reviews.[59,60]

The importance of the tubulin-polymerization activity of taxol has led to numerous studies of the taxol–tubulin interaction. The nature of the binding site of taxol on tubulin could not be determined by X-ray crystallography because the complex cannot be crystallized, but stabilized zinc sheets of 'microtubules' can be prepared and the structure of these sheets has been determined by electron crystallography at a resolution of 3.7 Å.[61] These studies indicated that taxol occupies a hydrophobic cleft on β-tubulin and this binding converts it into a hydrophilic surface.[62,63]

The binding conformation of taxol on tubulin has been studied by several investigators and several binding conformations have been proposed. Recent results, however, indicate that the most probable binding conformation is the 'T-taxol' conformation.[64] This conclusion is supported both by rotational-echo double resonance (REDOR) NMR experiments, which allow direct measurement of internuclear distances in the bound polymer[65] and by the synthesis of taxol analogues constrained to the T-taxol conformation.[66] Bridged analogue **25**, for example, is 20-fold more potent than taxol toward the A2780 ovarian cancer cell line and is also more active toward taxol-resistant cell lines.[67] This work has been reviewed.[68]

25 **26** Docetaxel

In the chemistry area virtually every position on the ring and on the side chain of taxol has been subjected to structural modifications and ring contractions and ring expansions have also been employed in attempts to generate improved analogues. This work has been described in several reviews[69–71] and the interested reader is referred to these for more information.

In addition to taxol, the taxol analogue docetaxel (**26**) is in clinical use. Docetaxel was developed by the Potier group in Paris and was originally discovered as the result of an approach to the synthesis of taxol by carrying out a hydroxyamination reaction on the side chain of a 13-cinnamoylbaccatin III.[72] Docetaxel is the drug of choice for the treatment of advanced non-small-cell lung cancer (NSCLC) that is refractory to primary chemotherapy.[73] Taxol itself is approved for the treatment of breast and ovarian cancers and Kaposi's sarcoma but it is also used for the treatment of lung cancer and various other cancers. It is increasingly used in combination therapy with other agents such as cisplatin for the treatment of ovarian cancer.[74]

New developments in the use of taxol include improved delivery methods for taxol itself and the use of taxol analogues with improved activities. The only improved delivery method currently in clinical use is Abraxane, which is an albumin nanoparticle-based formulation that has been shown to induce higher response rates than the Cremophor-based formulation.[75] Other methods of drug delivery are being developed, however. One approach is the use of antibody targeting,[76] which will be especially valuable when used with potent second- or third-generation taxanes. Another approach is the use of gold nanoparticles loaded with tumor necrosis factor (TNF) as the tumor-targeting agent; preliminary studies of these derivatives have been promising.[77]

Many taxol analogues have been developed and are in various stages of clinical trials, but none has yet been approved for clinical use. Four compounds are however in advanced Phase III clinical trials. TXD258 (**27**) is a

dimethoxy derivative of docetaxel that is in Phase III trials for the treatment of hormone refractory metastatic prostate cancer and in Phase II trials for prostate cancer. It is not a substrate for P-glycoprotein[78] and it can cross the blood–brain barrier, so it has some potential advantages as compared to taxol. Larotaxel dihydrate (**28**)[79] is in Phase III trials for the treatment of breast and pancreatic cancers; it has a cyclopropane ring in place of the C-7 hydroxyl group. Paclitaxel poliglumex (Xyotax) (**29**) is a conjugate of paclitaxel with a biodegradable polyglutamic acid; this feature was designed to increase water solubility and improve its pharmacokinetic profile. It is in Phase III trials by Cell Therapeutics for the treatment of NSCLC and ovarian cancer.[80] Taxoprexin (Luitpold Pharmaceuticals) (**30**) is a 2′-acyl paclitaxel and is in Phase III trials for the treatment of NSCLC and in Phase II trials for several other cancers.[81]

27 TXD258 XRP-6258

29 Paclitaxel poliglumex (Xyotax)
a + *b* averages 10

28 Larotaxel dihydrate

30 Taxoprexin

In conclusion, the discovery of taxol 40 years ago marked the beginning of a significant advance in cancer chemotherapy, the results of which are still being worked out in laboratories around the world. Taxol has been a huge success in the clinic and although it is far from a perfect drug it has brought significant benefit to many patients. It is safe to predict that the combination of improved taxol analogues with improved methods of drug delivery will maintain the importance of this natural product well into the twenty-first century.

2.02.3.7 Plant Saponins

Plant saponins are not normally considered to be promising anticancer lead compounds, partly because of problems with general toxicity. One exception to this rule is the acylated cholestane diglycoside OSW-1 (**31**), which was isolated from the bulbs of *Ornithogalum saundersiae* by Sashida and coworkers in 1992.[82] In addition to OSW-1 three related compounds were also isolated and two were tested for inhibitory activity on cyclic AMP phosphodiesterase. OSW-1 and the related compound **32** were the most active, with IC_{50} values of 55 and 5 μmol l^{-1}, respectively.[82]

31 R = H OSW-1
32 R = OMe

Subsequent studies by the same investigators in collaboration with the National Cancer Institute indicated that OSW-1 had potent cytostatic activity against human promyelocytic leukemia cells (HL-60) and a comparison of OSW-1 with clinically used anticancer agents such adriamycin and taxol showed that OSW-1 was more potent than any of the comparison clinical agents and showed little toxicity.[83] These properties made OSW-1 an interesting lead compound and several syntheses of the compound and its analogues have been reported[84–87] and reviewed.[88] A detailed study of its activity concluded, "In summary, we found that OSW-1 possesses highly potent anticancer activity against several human malignant cell lines and primary leukemia cells from patients with CLL.[89]" This compound exhibited a unique mechanism of action in which structural and functional damage to mitochondria triggers activation of the Ca^{2+}-dependent apoptosis pathway. Moreover, OSW-1 appeared less toxic to normal or nonmalignant cells than to tumor cells *in vitro*. The exact mechanisms responsible for such selectivity remain unclear. It is possible that cancer cells have alterations in mitochondria and in calcium regulation that are not found in normal cells, making them more vulnerable to OSW-1. It should also be noted that although nonmalignant cells appeared less sensitive to OSW-1 than cancer cells, the IC_{50} values for the normal cells were in the nanomolar range, suggesting that this compound is still toxic to normal cells. Thus, it is essential to perform rigorous animal toxicology studies before considering clinical evaluation of this compound. Targeting strategies such as antibody-mediated drug delivery may improve therapeutic selectivity and should be considered in any future development of this potent compound as a potential novel anticancer agent.[89]

The future clinical use of OSW-1 is thus unclear at the present time but it is very probable that a combination of the compound with an appropriate targeting strategy will ultimately prove to be successful since antibody targeting in particular works best with highly potent warheads.

2.02.3.8 Triptolide

The unusual triepoxide triptolide (**33**) was first isolated from *Tripterygium wilfordii* Hook F. or the 'thunder god vine' by the late Morris Kupchan as part of his systematic investigation of natural products as potential anticancer agents.[90] *T. wilfordii* is used in traditional Chinese medicine for the treatment of arthritis and related diseases[91] and has yielded a large number of other terpenoids in addition to triptolide, some of which have antitumor-promoting activity.[92]

33 Triptolide (PG490)

34 PG490-88

Triptolide has been shown to induce apoptosis of several human cancer cell lines grown in culture[93] and to inhibit tumor development in a murine breast cancer model[94] but has shown toxicity at high doses. The 14-succinyl sodium salt of triptolide (**34**), known as PG490-88, suppresses tumor growth without toxicity and has entered Phase I clinical trials; the data is summarized as follows: "Our results suggest a potential role of PG490-88 alone and in combination with chemotherapy as a novel antineoplastic regimen for the treatment of patients with solid tumors."[95]

Other activities of triptolide and of *T. wilfordii* extracts are discussed in a recent review;[92] these include immunosuppressive and anti-inflammatory effects as well as antiproliferative effects.[96,97] The potential as well as the chemical complexity of triptolide have led to several synthetic approaches to the compound[98–103] but current supplies are still provided by isolation from *T. wilfordii*. Some investigations of plant tissue culture production methods have been carried out[104] but this approach is not yet practical on a large scale, so compound supply could be an issue in the event that PG490-88 or a related compound is approved for clinical use. Triptolide is thus a versatile lead compound and it will be interesting to find out if its diverse activities lead to clinical benefits to cancer patients.

2.02.3.9 Protopanaxadiol

Ginseng (*Panax ginseng* C. A. Meyer) has a long history of use for medical purposes, including the treatment of diabetes, cancer, and heart disease.[105,106] Isolation of its root constituents has yielded many compounds, with the saponin glycosides known collectively as ginsenosides showing the most activity; over 60 different ginsenosides have been isolated.[107] Many ginsenosides have also been isolated from the related plant *Panax notoginseng*, also known as *Panax pseudoginseng*.[108] Protopanaxadiol (PPD, **35**) is the aglycone of the saponins Rb_1 and Rd, two of the major saponins in ginseng, while its analogue protopanaxatriol (**36**) is the aglycone of ginsenosides Re and Rg_1.[109]

35 R = H 20(*S*)-protopanaxadiol
36 R = OH 20(*S*)-protopanaxatriol

37 25-Methoxy-20(*S*)-protopanaxadiol

A study of the *in vitro* activity of PPD and related compounds indicated that it was one of the most effective inhibitors of cell growth of the group examined, with IC_{50} values in the 20–70 $\mu mol\,l^{-1}$ range for growth-inhibitory activity against several different human cancer cell lines.[110] The related compound 25-methoxy-protopanaxadiol (25-MeO-PPD, **37**) was also investigated and found to be effective as an adjunct therapy for androgen-independent prostate cancer in mice; thus docetaxel alone decreased tumor growth by 69% but docetaxel in combination with 25-MeO-PPD decreased tumor growth by 94%.[111] In another study, PPD was shown to cause apoptosis through multiple pathways, which is a clear advantage in cancer therapy given the heterogeneous nature and genetic instability of cancer cell populations.[112] No clinical studies on PPD or 25-MeO-PPD have yet been reported but the results described above indicate that these compounds have good potential as anticancer agents.

2.02.3.10 Ingenol-3-Angelate (PEP005)

Plants of the Euphorbiaceae family and particularly members of the *Euphorbia* genus have long been known to yield latexes with irritant and carcinogenic properties and these properties have been associated with diterpenes of the phorbol class.[113] Members of the *Euphorbia* genus also contain diterpenes with other skeletons, among them the ingenane derivative PEP005 (**38**).[114,115]

38 Ingenol 3-angelate

The sap of the plant *Euphorbia peplus* is used in Australia for the treatment of skin cancers and there is preliminary evidence to support its efficacy.[116] The active agent of this sap was identified as PEP005[117] and topical applications of this compound, also known as PEP005, cured a series of subcutaneous mouse and human tumors on mice. Its mechanism of action was identified as a protein kinase C (PKC) activator and it also had potent antileukemic effects in addition to its topical effects.[118] Another study of the differential effects of PEP005 on PKCα and PKCδ suggested that the drug induced apoptosis through this pathway, suggesting that targeting PKC isoforms is a valid approach to cancer therapy.[119] These promising results have led to the initiation of clinical trials and topical PEP005 is in Phase II development for the treatment of actinic keratoses and basal cell carcinoma.[120]

2.02.3.11 Phenoxodiol

Phenoxodiol (**39**) is not strictly speaking a natural product but it is modeled after the plant hormones daidzein (**40**) and genistein (**41**). Flavonoids are known to have diverse biological effects, including the regulation of the plant cell cycle and apoptosis, and some flavonoids show similar effects in animals. A study of flavonoid derivatives indicated that phenoxodiol (3-(4-hydroxyphenyl)-2H-1-benzopyran-7-ol) inhibited a wide range of cancer cell lines and was more potent than its lead compound genistein (**41**).[121] It was particularly encouraging to find that it induced apoptosis in chemoresistant ovarian cancer cells.[122]

39 Phenoxodiol
(3-(4-Hydroxyphenyl)-
2H-1-benzopyran-7-ol)

40 R = H Daidzein
41 R = OH Genistein

These encouraging results allowed phenoxodiol to move into clinical trials even though its mechanism of action was unclear and it is currently in Phase III clinical trials for drug-resistant ovarian cancer. It inhibits topo II and induces both caspase-dependent and caspase-independent apoptosis as well has having effects on the proteosome and Akt signal transduction, but recent results indicate that its primary site of action is on plasma membrane electron transport.[123]

2.02.3.12 Flavopiridol

Although it is totally synthetic the model for the novel flavonoid structure of flavopiridol (**42**) is a natural product, rohitukine (**43**), the constituent responsible for anti-inflammatory and immunomodulatory activity isolated from *Dysoxylum binectariferum* Hook. f. (Meliaceae). *D. binectariferum* is phylogenetically related to the Ayurvedic plant *Dysoxylum malabaricum* Bedd. used for rheumatoid arthritis.

42 Flavopiridol **43** Rohitukine

From over 100 analogues synthesized during structure–activity relationship studies, flavopiridol was selected for preclinical and clinical development based on tyrosine kinase activity, potent growth-inhibitory activity against a series of breast and lung carcinoma cell lines, and broad spectrum *in vivo* activity against human tumor xenografts in mice.[124] Although initially thought to be a specific inhibitor of cyclin-dependent kinases, the mechanisms by which it mediates antitumor activity remain undefined and are still being studied.[125] It is currently in 20 Phase I and Phase II clinical trials, either alone or in combination with other anticancer agents, against a broad range of tumors, including leukemias, lymphomas, and solid tumors (http://www.clinicaltrials.gov). Of particular interest is the observation of significant activity against chronic lymphocytic leukemia (CLL), a cancer currently lacking efficacious treatment.[126,127] For further discussion on the potential of such inhibitors, the reviews by Fischer[128,129] and the report by Mayer *et al.*[130] should be consulted.

2.02.3.13 β-Lapachone (ARQ 501)

Species of the genus *Tabebuia* have been used historically in the Amazonian region for the treatment of several diseases, including syphilis, fevers, malaria, cutaneous infections, and stomach disorders. Starting in the 1960s, claims for clinical efficacy in the treatment of cancers, particularly in Brazil, resulted in widespread sales of the stem bark and trunk wood of *Tabebuia impetiginosa* (Mart. ex DC.) Standl. (synonym *Tabebuia avellanedae* Lorentz ex Griseb.), *Tabebuia rosea* (Bertol.), and *Tabebuia serratifolia* (Vahl) Nichols. in health food stores under various names such as ‘pau d’arco’ or ‘lapacho’. Of the many bioactive compounds isolated, the naphthoquinones, particularly lapachol (**44**) and β-lapachone (**45**), have received most attention.

44 Lapachol **45** β-Lapachone

Observation of significant *in vivo* antitumor activity for lapachol in some early mouse models resulted in its advancement to clinical trials by the NCI in the 1970s but the trials were terminated because of the unacceptable levels of toxicity.[131] Interest in β-lapachone was stimulated by its activity against a range of tumor cell lines, including breast, leukemia, and prostate lines and several multidrug resistant lines.[132–134] It was developed by ArQule under the code name ARQ 501 and has completed six clinical trials against a range of solid tumors, including pancreatic cancer in combination with gemcitabine.

2.02.3.14 Adenine Derivatives: Olomucine, Roscovitine, and Analogues

Olomucine (**46**), originally isolated from the cotyledons of radish (*Raphanus sativus* L.A.) provided the natural product model for the synthetic agent roscovitine (**47**).[135] Olomucine (**46**) inhibits cyclin-dependent kinases, proteins that play a major role in cell cycle progression.[136] R-Roscovitine is currently in Phase II clinical trials

in Europe as seliciclib.[137,138] The basic structural motif led to the purvalanols (**48**, **49**), which are even more potent[139] and which have now led to even more selective agents such as NU6140 (**50**), which target survivin, thus acting synergistically with paclitaxel.[140]

46 Olomucine

47 Roscovitine (CYC202)

48 Purvalanol A (R = H)
49 Purvalanol B (R = CO_2H)

50 NU6140

2.02.3.15 Other Active Compounds in Preclinical Development

A number of naturally derived agents have been entered into clinical trials which have been terminated due to lack of efficacy or unacceptable toxicity. One of these, maytansine (**51**), was isolated in the early 1970s from the Ethiopian plant, *Maytenus serrata* (Hochst. ex A.Rich.) R. Wilczek.[141] Despite very low yields (2×10^{-5}% based on plant dry weight), its extreme potency in testing against cancer cell lines prompted the production of sufficient quantities to pursue preclinical and clinical development.

51 Maytansine

Unfortunately, promising activity in preclinical animal testing failed to translate into significant efficacy in clinical trials and it was dropped from further study in the early 1980s. Maytansine has, however, been revived through the application of targeting technology. The isolation of closely related compounds, the ansamitocins, from a microbial source, *Actinosynnema pretiosum*, has permitted the production of larger quantities of this class of compounds and this factor, together with their extreme potency, has stimulated continued interest in pursuing

their development. This microbial production has posed the question as to whether the maytansines are actually plant products or are produced through an association between a microbial symbiont and the plant, a question which is a topic of continuing study.[142] A derivative of maytansine, DM1, has been coupled to huC242, a Mab directed against the *muc*1 epitope expressed in a range of cancers, to produce a conjugate known as SB408075 or huC242-DM1 (also known as Cantuzumab Mertansine), which is in Phase I clinical trials against various cancers, including pancreatic, biliary, colorectal, and gastric cancers.[143] Other conjugates include huC242-DM4, currently in clinical trials targeting various solid tumors (http://www.clinicaltrials.gov),[144] huN901-DM1 being developed for the treatment of small-cell lung cancer[145] and natural killer/T cell malignancy,[146] and the J591-DM1 immunoconjugate, which demonstrated activity against prostate-specific membrane antigen (PSMA) positive cells *in vitro* and *in vivo*.[147]

Another example of an 'old' drug in the process of revival is bruceantin (**52**), which was first isolated from *Brucea antidysenterica* J. F. Mill., a tree used in Ethiopia for the treatment of 'cancer'. As often happens, activity observed in animal models bearing a range of tumors was not matched by any objective responses in clinical trials, resulting in termination of further development.[148]

52 Bruceantin

Interest has been rekindled by the recent observations of significant activity of compound **52** against panels of leukemia, lymphoma, and myeloma cell lines, as well as in animal models bearing early and advanced stages of the same cancers. Association of this activity with the down-regulation of a key oncoprotein (c-*myc*) is considered strong evidence supporting the development of bruceantin as an agent for the treatment of hematological malignancies.[148] It has also been reported to have potent antimalarial activity.[149]

A plant-derived compound with a long history is the lupane-type triterpene, betulinic acid (**53**), which has been isolated from many taxonomically diverse plant genera.[150] A major source is the birch tree, *Betula* spp., which is also a primary source of its C-28 alcohol precursor, betulin, the isolation of which was first reported in 1788.

53 Betulinic Acid

The observation of cytotoxicity for **53** against a range of cancer cell lines and significant *in vivo* activity in animal models bearing human melanoma xenografts have prompted the development of systemic and topical formulations of the agent for potential clinical trials. Reduction of UV-C-induced DNA breakage in congenital melanocytic naeval cells by betulinic acid has suggested a potential role as a chemopreventive agent[151] and a 20% betulinic acid ointment is currently being evaluated in the treatment of dysplastic nevi (moderate to severe dysplasia; http://www.clinicaltrials.gov). Additional biological activities, including antibacterial, anti-inflammatory, and antimalarial, have been reported for betulinic acid and several derivatives, but the most important activities have been associated with inhibition of the replication of strains of HIV (Section 2.02.4.4).

Other common plant-derived triterpenoid acids, such as oleanolic and ursolic acid, exhibit weak anti-inflammatory and antitumor activities, and studies have been directed at the synthesis of new analogues having increased potencies. These studies have led to the synthesis of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) (**54**) and its methyl ester (**55**), which exhibit potent *in vitro* and *in vivo* antitumor activity against a wide range of tumors, including breast and pancreatic carcinomas and leukemias.[152]

54 CDDO; R = H
55 CDDO-Me; R = CH_3

Significant activity shown by CDDO against epithelial ovarian carcinoma cell lines, including lines that were resistant to clinically used agents such as cisplatin, has resulted in further evaluation of CDDO in the treatment of these cancers, which are leading causes of death from gynecologic cancers.[153] Evaluation of C-28 derivatives of CDDO such as CDDO methyl ester (CDDO-Me **55**), CDDO imidazolide (CDDO-Im), CDDO ethyl amide (CDDO-EA), CDDO trifluoroethyl amide (CDDO-TFEA), and CDDO diethylamide (CDDO-DE) against pediatric solid tumor cell lines indicate their potential for the treatment of high-risk pediatric solid tumors.[154] The mechanisms of action and molecular targets of these compounds are the focus of ongoing research. Reported effects include blocking of the synthesis of inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2), two enzymes involved in inflammation and carcinogenesis, and inhibition of the interleukin-1 (IL-1)-induced expression of the proinflammatory proteins matrix metalloproteinase-1 (MMP-1) and matrix metalloproteinase-13 (MMP-13). *In vitro* and *in vivo* studies of CDDO-Me have indicated that it has potent antiangiogenic activity.[155] CDDO has been in Phase I clinical trials against solid tumors and lymphomas (http://www.clinicaltrials.gov).

Mu Lan (*Indigofera tinctoria* L.), a product from the Chinese *Materia Medica*, is used for the treatment of chronic myelogenous leukemia (CML). The main constituents are the family of bisindoles, known generically as indirubins (**56**), and while indole-derived molecules are found in a large number of indigo-producing plants, they are also produced by bacteria and are found in gastropod mollusks, where they are the source of the purplish-red dye known from antiquity as 'Tyrian Purple'. Indirubins were the first human-used compounds to be identified as inhibitors of cyclin-dependent kinases. Substituted indirubins have been synthesized (as a result of data from work with a mollusk) and the 6-bromo derivative (**57**) and its 3′-monooxime (**58**) show comparable activity to other known cyclin-dependent kinases inhibitors, such as flavopiridol and roscovitine, and are candidates for preclinical development.[156,157] The 3′-substituted 7-halogenoindirubins (**59**) have also been synthesized and despite the observation that steric hindrance appears to prevent interaction with the kinases targeted by other indirubins, such as cyclin-dependent kinases and glycogen synthase kinase-3, they exhibit significant cytotoxicity against a diversity of human tumor cell lines and thus represent a new promising family of antitumor agents.[158]

56 Indirubin; X = O; R^1 = H; R^2 = H
57 6-Bromoindirubin; X = O; R^1 = Br; R^2 = H
58 Oxime of bromoindirubin; X = N-OH; R^1 = Br; R^2 = H
59 7-Bromoindirubin; X = O; R^1 = H; R^2 = Br

Consumption of *Veratrum californicum* by pregnant sheep has long been associated with birth defects in lambs, including cyclopia in severe cases. These teratogenic effects are due to the specific inhibition of vertebrate cellular responses to the hedgehog family of secreted growth factors resulting from the presence of alkaloids of the jervine class, in particular cyclopamine (**60**).[159,160] The hedgehog cell-signaling pathway is normally quiescent in adult cells but aberrant activation of the pathway in adults has been implicated in many cancers, including cancers of the pancreas, prostate, lung (small cell), and brain (glioma). Activation of this pathway is blocked by cyclopamine, and analogues and prodrugs are in various stages of preclinical development.[161–163] The potential for the use of combinations of cyclopamine with other agents is also being investigated.[162] In addition, early preclinical studies indicate that cyclopamine may be effective in the treatment of psoriasis.[164]

60 Cyclopamine

The schweinfurthins (e.g., schweinfurthin B, **61**), isolated from the African plant *Macaranga schweinfurthii* Pax., display significant selective activity against CNS and renal and breast cancer cell lines in the NCI 60 cell line anticancer assay.[165] The spectrum of anticancer activity does not match that of any currently used agent, indicating that these compounds may be acting at a previously unrecognized target or through a novel mechanism. The isolation of larger samples of the schweinfurthins from the natural source have met with limited success and synthetic strategies have been developed to provide a reliable source of natural schweinfurthins and synthetic analogues for further biological testing.[166,167] In the case of schweinfurthin F (**62**),[168] the total synthesis of the (*R,R,R*)- and (*S,S,S*)-enantiomers and comparisons of spectral data, optical rotations, and bioassay data with those reported for the natural product have resulted in assignment of the natural material as the (*R,R,R*)-isomer.[169]

61 Schweinfurthin B; R = $CH_2CH_2CH{=}CMe_2$
62 (*R,R,R*)-Schweinfurthin F; R = CH_3

Curcumin (**63**) is the principal active constituent of the traditional medicine and spice, turmeric (*Curcuma longa*). It is reported to possess an extensive range of biological activities, including anticarcinogenic, antidiabetic, anti-inflammatory, antimicrobial, antioxidant, antiviral, cardioprotective, gastroprotective, neuroprotective, immunomodulatory, and activities related to the treatment of cystic fibrosis and wound healing.[170–172] These multiple activities are derived from pleiotropic effects on genes and cell-signaling pathways at multiple levels and are discussed in some recent reviews.[171,172] Thus, curcumin has significant potential as a therapeutic agent and is currently in human clinical trials for the treatment of Alzheimer's disease

(AD)[173] as well as the treatment of several cancers, including colon and pancreatic cancers (in combination with agents such as gemcitabine and celebrex), as a chemopreventive agent against colorectal cancer, and in the prevention of oral mucositis in children receiving chemotherapy (http://www.clinicaltrials.gov).

63 Curcumin

64 EF24

A large number of curcumin analogues have been synthesized and one structural modification commonly used has been the modification of the central conjugated β-diketone to the monocarbonyl dienone. Many such compounds exhibit cytotoxicities against a wide range of cancer-related cell lines while retaining rodent toxicity profiles comparable to the parent natural product, while some analogues (e.g., EF24; **64**) exhibit improved pharmacological properties, including good oral bioavailability in mice.[174]

Parthenolide (**65**), a major sesquiterpene lactone from *Tanacetum parthenium*, possesses significant tumor-specific cytotoxicity against human leukemia, lung, lymphoma, breast, and prostate cancer cells, as well as antiangiogenic activity and *in vivo* activity.[175–177] Parthenolide targets nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and has recently been shown to inhibit the growth of multiple myeloma (MM) cell lines, including drug-resistant cell lines and primary cells.[178] Novel analogues of parthenolide, with a high oral bioavailability, have now been developed and are undergoing preclinical testing.[179] Such analogues are exemplified by dimethylaminoparthenolide (DMAPT; **66**)

65 Parthenolide

66 DMAPT

DMAPT (**66**) is highly active against primary human leukemia stem cells (LSCs) from both myeloid and lymphoid leukemias and mechanistic studies indicate that it promotes oxidative stress responses, inhibition of NF-κB, and activation of p53. DMAPT is about 70% orally bioavailable and *in vivo* activity has been demonstrated in both mouse xenograft models and spontaneous acute canine leukemias, indicating that it has the potential to target human LSCs *in vivo*,[180] providing support for the clinical development of parthenolide in MM therapy.

2.02.4 Anti-HIV Agents

Between 1987 and 1996, the NCI tested over 30 000 plant extracts in an *in vitro* cell-based anti-HIV screen (http://www.niaid.nih.gov), which determined the degree of HIV-1 replication in treated infected lymphoblastic cells versus that in treated uninfected control cells. Several natural products showed *in vitro* activity and michellamine B, the calanolides, and prostratin are discussed below.

2.02.4.1 Michellamine B

Michellamine B (**67**) is the main *in vitro* active anti-HIV constituent isolated from the leaves of the liana, *Ancistrocladus korupensis*, a new species[181] collected in the Korup region of southwest Cameroon.[182] It showed

in vitro activity against an impressive range of HIV-1 and HIV-2 strains[182] and based on the observed activity and the efficient formulation of the diacetate salt, michellamine B progressed to advanced preclinical development. *In vivo* studies, however, indicated that effective anti-HIV concentrations could only be achieved at close to neurotoxic dose levels[183] and further studies aimed at clinical development were discontinued. However, the discovery of novel antimalarial agents, the korupensamines, for example, korupensamine A (**68**), from the same species,[184] adds further promise for this species. Michellamine B and some related compounds have been synthesized through the dimerization of korupensamine A[185,186] and patents have been issued for general approaches to their synthesis[187] The michellamines have also been found to exhibit other biological activities.[188–190] Thus, despite the termination of development of michellamine B as a potential anti-HIV agent, the range of biological activities observed for this class of compounds and the strategies available for their synthesis[191] offer the potential for the synthesis of more biologically effective analogues.

67 Michellamine B

68 Korupensamine A

2.02.4.2 The Calanolides

(+)-Calanolide A (**69**) was isolated as the major *in vitro* active anti-HIV agent from the leaves and twigs of the tree, *Calophyllum lanigerum*, collected in Sarawak, Malaysia in 1987,[192] but later collections of other specimens of the same species gave only trace amounts of the compound. A detailed survey of *C. lanigerum* and related species showed that the latex of *Calophyllum teysmanii* yielded (−)-calanolide B (**70**), which, though being slightly less active than (+)-calanolide A, has the advantage of being readily available from the latex that is tapped in a sustainable manner without causing any harm to the trees.[193] The calanolides were licensed by NCI/NIH to Medichem Research, Inc., which, as required by the NCI Letter of Collection (http://ttc.nci.nih.gov), negotiated an agreement with the Sarawak State Government. The development of the drugs was initiated by Sarawak Medichem Pharmaceuticals, a joint venture company formed between the Sarawak State Government and Medichem Research, Inc. The lead role in the development is now being undertaken by Craun Research Sendirian Berhad, a company incorporated in Sarawak. (+)-Calanolide A, synthesized by Medichem chemists,[194] has shown an acceptable level of safety and a favorable pharmacokinetic profile in healthy, HIV-negative individuals,[195,196] and is currently in further clinical trials, while (−)-calanolide B is in preclinical development. Synthetic studies have led to the preparation of the 11-demethyl-12-oxo analogue (**71**) having comparable *in vitro* anti-HIV-1 activity to calanolide A.[197] This structure has been used as a template for the synthesis and structure–activity studies of a library of over 100 congeners, with the corresponding 10-bromomethyl derivative (**72**) showing greater potency against HIV-1 (2.85 nmol l^{-1} compared to 100 nmol l^{-1} for calanolide A) and a greatly improved therapeutic index.[197] Additional biological activities have also been observed.[198,199]

69 (+)-Calanolide A

70 (–)-Calanolide B

71 11-Demethyl-12-oxo-cal A; R = H
72 10-Bromomethyl analogue; R = Br

2.02.4.3 Prostratin

Prostratin (**73**) was isolated as the active constituent from an extract of the wood of the tree, *Homalanthus nutans*,[200] a plant identified by ethnobotanist Paul Cox as being used by traditional healers in Samoa for the treatment of yellow fever (subsequently identified as viral hepatitis). Interviews were conducted with traditional healers under terms of a covenant negotiated with the chiefs and orators in the village of Falealupo and with the concurrence of the Samoan Prime Minister and members of parliament.[201]

73 Prostratin

Mechanistic studies indicated that prostratin interacts with a cellular target necessary for viral entry, thereby inhibiting the entry step of the replication cycle of HIV.[202] Subsequently, prostratin was shown to be a potent activator of HIV expression in latently infected T-cell lines[203] and its potential value in HIV therapy lies more in its possible utility as a viral activator rather than as an anti-HIV agent.[204–206] The mechanism whereby prostratin exerts its viral activation effect is reported to involve activation of NF-κB.[207,208] Using a Jurkat T-cell line containing latent HIV proviruses, other phorbol-13-monoesters have also been shown to reactivate HIV latency, with activity rapidly decreasing with shortening of the acyl side chain; prostratin and phorbol-13-stearate activate HIV-1 gene expression in these latently infected cells, with the latter being at least 10-fold more potent than prostratin.[209] Prostratin and the proteasome inhibitor bortezomib have been reported to induce Kaposi's sarcoma-associated herpesvirus (KSHV) gene expression from two lymphoma cell lines *in vitro*, suggesting their further investigation as therapeutic agents for KSHV-associated malignancies.[210] The further development of prostratin is being undertaken by the AIDS Research Alliance of America (ARA; http://www.aidsresearch.org), which has negotiated an agreement with the Government of Samoa allowing for benchmark payments to the Government of Samoa, the village, and the families of the healers. In addition, ARA will endeavor to obtain prostratin from Samoan plant sources as long as it can be produced in a cost-effective manner and will strive to ensure that the drug will be distributed at minimal profit in developing nations where use of the drug is approved.

2.02.4.4 Betulinic Acid

The anticancer activity of betulinic acid (**74**) was briefly discussed in Section 2.02.3.15 where it was also noted that some of its most important activities have been associated with the inhibition of the replication of strains of HIV. Several 3-*O*-succinyl derivatives have been synthesized and exhibit potent anti-HIV-1 activity[211] and the 3,3′-dimethylsuccinyl derivative, named Bevirimat (**75**), has successfully completed Phase IIa clinical trials, and is currently in Phase IIb trials. Bevirimat represents a new class of HIV drugs called maturation inhibitors

and its novel mechanism of action offers potential for use either alone or in combination with current anti-AIDs.[212] While derivatives with a side chain at C-3 can inhibit HIV-1 maturation, derivatives with a side chain at C-28 can block HIV-1 entry and two entry inhibitors, IC9564 (76) and A43D, have been found to exhibit a broad spectrum of anti-HIV-1 activity.[213] The potential of bifunctional (C-3 and C-28) derivatives is being investigated and significant enhancement of potency relative to either of the monofunctional derivatives has been reported, with activity shown against both HIV entry and maturation.[214]

74 R = H Betulinic acid

75 R = Bevirimat

76 IC9564

2.02.5 Antimalarial Compounds

It can be argued quite successfully that the most important parasitic infection worldwide is malaria. Although there are many other debilitating diseases caused by parasites in various parts of the developing world, the number of deaths annually due to malaria is over one million, with the vast majority (80% plus) being children under the age of 5 (Centers for Disease Control statistics for 2005).

2.02.5.1 Quinine

The first compound used against malaria was quinine (77), isolated in 1820 by Cavetou and Seturner[215] from the bark of *Cinchona* spp., long used by the indigenous people of Amazonia for the treatment of fevers. Building on part of the structure of quinine (led by lack of access to the natural product in World War II), synthetic molecules containing the isoquinoline core present in quinine were investigated and the first of these analogues, chloroquine (78), was introduced in 1943 by the Allies.[216] Ironically, the same molecule had been synthesized in Germany in 1934 under the name Resochin but had been dropped due to toxicity. Subsequently, a number of similar molecules have been introduced into clinical use, but in all cases, parasitic resistance to these molecules has risen relatively rapidly after introduction.

77 Quinine

78 Chloroquine

2.02.5.2 Artemisinin

Roughly 400 years after the recognition of the activity of extracts of *Cinchona* spp. came the next antimalarial drug, which was identified directly from the Chinese *Materia Medica.* This was the previously unknown molecule artemisinin (79) isolated from extracts of the Wormwood tree, *Artemisia annua*, which like the source plant of quinine, had a long history of use in traditional medicine. Artemisinin was originally discovered by Chinese scientists in 1972 and was reported in a 1998 review to be present in other species of the same genus.[217] A slightly more soluble derivative, artesunate (80), was launched in 1987 by the Guilin No. 2 Pharmaceutical Factory in China; rights were subsequently obtained by both the US Army and Sanofi-Aventis.

79 Artemisinin **80** Sodium artesunate **81** Arterolane tosylate

In the last few years, variations on the basic structure have been launched in combination with other antimalarials (usually variations on the chloroquine structure) such as dihydroartemisinin and piperaquine phosphate (Artekin), artemether and lumefantrine (Coartem), artesunate/mefloquine (Artequin) and artesunate, sulfamethoxypyrazine, and pyrimethamine (Co-Arinate).[218] Currently, there is another fixed dose combination with an artemisinin derivative in clinical trials, pyronaridine/artesunate (Pyramax in Phase III). However, the tri-oxo scaffold system in artemisinins has led to the synthesis of not only artemisinin variations[219] but to totally synthetic molecules with the trioxane moiety included, such as arterolane tosylate (81). This compound is in Phase II trials as a single agent under Ranbaxy and is in Phase I trials in combination with piperaquine phosphate, also under Ranbaxy.

Over the last few years, artemisinin and more soluble derivatives have altered the treatment of resistant malaria, but the cost of collection and the quantities of plants required are significantly more expensive than the countries where the drug is required can afford for general treatment. In an attempt to reduce the price and to make the production not dependent upon wild or even cultivated plant collections, Keasling's group at Berkeley in conjunction with the Gates Foundation and Amyris Pharmaceuticals have transferred the genes from the producing plant into *Escherichia coli* and also *Saccharomyces cerevisiae.* They have successfully expressed the base terpene (amorpha-4,11-diene) and followed up with modification of the base structure both chemically and to some extent, biochemically via P450 enzymes.[220–222] Although not yet economically viable when compared to direct plant collection and extraction, the process is yet to be optimized for production and with the experience in the manipulation of *E. coli* or *S. cerevisiae*, this is definitely a feasible project.

The mechanism of action of both artemisinin and related compounds has been linked to inhibition of the parasite-encoded sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) PfATP6,[223] which was supported by decreased resistance in organisms with lower copy numbers of the gene[224] and of polymorphism in the genes in the field.[225] Further work into the mechanism of action of artemisinin is ongoing in many laboratories around the world[226] and there is evidence for involvement with chelation of nonheme iron by most if not all of the tri-oxo-containing compounds, be they modified natural products or synthetics.[227]

2.02.6 Cardiovascular and Metabolic Diseases

2.02.6.1 Resveratrol

Resveratrol (82) is a polyphenolic antioxidant found in many plants, including grapes, nuts, and berries. First isolated in 1940 from the roots of white hellebore (*Veratrum grandiflorum*), it has gained prominence through its association with the highly publicized 'French Paradox' linked to the drinking of red wine.[228] There is an extensive literature on the multiple health benefits attributed to resveratrol and interested readers are referred to several recent reviews that summarize the available data.[229]

82 Resveratrol

Resveratrol has been shown to possess cancer chemopreventive properties through affecting the three stages of carcinogenesis, namely, tumor initiation, promotion, and progression, as well as suppressing angiogenesis and metastasis.[228] These anticarcinogenic effects appear to be closely associated with its inhibition of several molecular targets including kinases, cyclooxygenases, ribonucleotide reductase, and DNA polymerases, which are involved in cancer development.[228] There is mounting evidence supporting its role in the protection of the cardiovascular system, again operating by multiple mechanisms.[230] In this case, inhibition of apoptotic cell death at very low concentrations is reported to provide protection from various diseases, including myocardial ischemic reperfusion injury, atherosclerosis, and ventricular arrhythmias, the so-called preconditioning effect.[230] Other beneficial effects are achieved through increased production of nitric oxide, down-regulation of vasoactive peptides, lowering of the levels of oxidized low-density lipoprotein, and inhibition of COX-2.[231] Promising data have also been obtained related to its potential role in the modulation of progressive neurodegenerative afflictions such as Alzheimer's, Huntington's, and Parkinson's diseases.[232,233] Resveratrol's potential efficacy in the treatment of metabolic diseases such as type 2 diabetes is attributed to its activation of Sirtuin 1 (SIRT1), a member of the sirtuins, a novel family of enzymes that reportedly help regulate nutrient sensing and utilization, and metabolic rate. Activation of SIRT1 leads to a decrease in glucose levels, increased insulin sensitivity, an increase in mitochondrial number and function, decreased adiposity, improved exercise tolerance, and potential lowering of body weight.[234] A proprietary formulation of resveratrol having enhanced bioavailability (SRT-501) and the first of a novel class of SIRT1 activators, has been reported to be safe and well tolerated in humans and is currently in clinical trials for the treatment of type 2 diabetic patients.[234]

2.02.6.2 Hoodia Compounds

Hoodia gordonii has a history of traditional use by the San people in Southern Africa as an appetite suppressant. The South African Council for Scientific and Industrial Research (CSIR) studied *H. gordonii* and *H. pilifera* as sources of appetite suppressants and isolated the oxypregnane steroidal glycoside, code named P57AS3 (P57; **83**), as the sole active constituent.[235] The compound was patented and in 2003 an agreement was reached between the San people and the CSIR for the sharing of any royalties that might result from the sale of products derived from *H. gordonii*;[236] the compound was licensed to the British company, Phytopharm, and sublicensed to Pfizer, and later to Unilever. Thus far no commercial product has been developed. Profit-sharing agreements were also signed with the South African Hoodia Growers (Pty) Ltd. and complaints have been lodged with the governments of Switzerland and Germany concerning sale of Hoodia products outside the terms of the agreement.[236] Several other pregnane glycosides have been isolated from *H. gordonii* but no appetite suppressant activity has been reported.[237–239]

83 P57AS3

2.02.6.3 *β*-Adrenergic Amines: Ephedrine, Propranolol, Atenolol, and Metoprolol

In the early 1920s, ephedrine (**84**) was isolated from *Ephedra sinica* and *Ephedra equisetina*, plants used for millennia in traditional Chinese medicine for the treatment of asthmatic and other bronchial conditions.[240] Pure ephedrine was shown to have physiological actions that were very similar to adrenaline, causing elevation of blood pressure plus inotropic and chronotropic actions on the heart and became the first of the bronchodilators. These eventually became known as the sympathomimetic amines. Other amino compounds based on the ephedrine structure, such as benzedrine and methamphetamine, became widely used as stimulants during WWII, but thereafter were generally tightly regulated because of their abuse potential. This was followed by the synthesis of isoprenaline (Isoprel, **85**), which showed excellent activity as a bronchodilator, having little action on blood pressure but having cardiac stimulant effects. Subsequent work of Black demonstrated the existence of two basic types of *β*-receptors, the *β*1 being predominately cardiac and the *β*2 predominately tracheal/lung. This led to the development of the first true *β*-blocker, propranolol (**86**).[241] At first (late 1960s–1980s), the compounds developed had the potential for both agonist and antagonist activities, but chemical modifications, coupled with the use of isolated receptor assay techniques, led to the synthesis of compounds such as atenolol and metoprolol (**87**) having no detectable sympathomimetic activities. Improved *β*-blockers also having vasodilating activity are labetalol (**88**), carvedilol, and nebivolol (**89**).[242,243]

84 Ephedrine

85 Isoprenaline

86 Propranolol

87 Metaprolol

88 Labetalol

89 Nebivolol

2.02.6.4 Digoxin and Related Cardiac Glycosides

Digoxin (**90**) and related cardiac glycosides such as digitoxin and ouabain are noted for their efficacy in the treatment of congestive heart failure and as antiarrhythmic agents. A possible new role in the area of cancer prevention and/or treatment is emerging and recent research showing their effects on mechanisms involving cell-signal transduction leading to selective control of the proliferation of human tumor compared to normal cells indicates potential as a means of targeted cancer chemotherapy.[244]

90 Digoxin

2.02.7 CNS Active Agents

Plant extracts have been used for their effects on the brain and the CNS since time immemorial as evidenced by the use of opium for at least 4000 years,[245] by the long-standing practice of South American Indians to chew coca leaves,[246] and by the widespread use of Indian hemp (*Cannabis sativa*).[247] This section of the review will not discuss these older and well-established agents and will instead focus on four newer classes of compounds with genuine potential for beneficial effects on such important diseases as AD.

2.02.7.1 Huperzine A

Huperzine A (**91**) was isolated from the club moss *Huperzia serrata* (Thunb.) Trev., also known as *Lycopodium serratum* Thunb.,[248] and was found to be a potent inhibitor of acetyl cholinesterase (AChE) with significant selectivity for AChE over butyrylcholinesterase.[249] Since AD is characterized by cholinergic dysfunction resulting from a deficiency of the neurotransmitter acetylcholine,[250] AChE inhibitors play a major role in the treatment of this devastating disease. Currently used AChE inhibitors include tacrine, donepezil (Aricept), rivastigmine, galanthamine, and memantine. The biological effects of huperzine A have been reviewed on several occasions[251,252] and the compound is currently in clinical trials for the treatment of AD. A review of these trials states, "Thus, it appears that HupA (huperzine A) is more effective than other drugs that affect the cholinergic system and that are currently on the market in the USA for the treatment of mild to moderate AD. Because HupA was discovered in China during the years just after the Cultural Revolution, however, no patents were filed and all of this information rests in the public domain, rendering HupA non-viable as a commercially developable drug."[253]

91 Huperzine

92 E12E

93 ZT-1

94 Hopeahainol A

This situation has spurred a large number of investigations to improve the activity and selectivity of huperzine A, while at the same time producing a viable commercial drug candidate. These studies include

total syntheses of huperzine A and various analogues[254,255] and a cocrystal X-ray structure of huperzine A in complex with *Torpedo californica* AChE.[255] A particularly interesting approach is the use of dimeric huperzine A analogues such as E12E (**92**). E12E consists of two simplified huperzine A analogues linked by a 12-carbon chain, designed to allow simultaneous binding with the catalytic triad and the peripheral site in AChE.[256] E12E is more potent than huperzine A as an AChE inhibitor but is not as selective, so it is not yet fully optimized. This approach does however hold the promise of the discovery of economical and effective huperzine A analogues. An alternate candidate is the prodrug ZT-1 (**93**), which is in Phase II clinical trials for the treatment of AD.[257]

Huperzine A is not the only AChE inhibitor available from nature's storehouse. As one example, the unusual polyphenol hopeahainol A (**94**) was recently reported from *Hopea hainanensis*.[258] It has a comparable IC_{50} to huperzine A against AChE and was shown to be a reversible inhibitor of the enzyme. It is thus another example of the versatility of the plant kingdom in providing novel structures with pharmacological relevance.

2.02.7.2 Ginkgolides

The ginkgolides are a unique class of diterpenoids isolated from the 'fossil' tree *Ginkgo biloba* L. This tree is the only remaining species of the Ginkgoales, once a very large order, and its extreme botanical position is matched by the unusual chemistry of its constituents. The early chemistry of the ginkgolides was worked out primarily by the groups of Nakanishi[259] and Sakabe,[260] leading to the assignment of structure **95** for ginkgolide B. The ginkgolides are diterpene trilactones and are very stable to most chemical conditions; they are also the only natural products that contain a *tert*-butyl group. They have been synthesized by several investigators, most notably by Corey, who described his retrosynthetic approach in his Robinson lecture.[261]

95 Ginkgolide B

The pharmacological effects of ginkgolide B and *G. biloba* extracts are varied, but a key effect is their activity as antagonists of platelet-activating factor (PAF). This led to extensive investigations of their biology and pharmacology, leading to the registration of the extract EGb-761 as a phytomedicine; this and similar *Ginkgo* extracts are now among the most used drugs in continental Europe with worldwide sales estimated at half a billion dollars in 2000.[262] EGb-761 is a standardized mixture of 24% flavonol glycosides and 6% terpene trilactones but their activity is believed to be due in large measure to the latter constituents. The CNS effects of EGb-761 have been reviewed and it is concluded, "The evidence that EGb-761 has cognition-enhancing properties in healthy humans and animals appears inconclusive and controversial at this stage."[263] The authors do however add, "These data suggest that short-term treatment with EGb-761 may facilitate some cognitive functions in older adults who are not cognitively impaired."[263]

Studies of ginkgolide B are also discussed in the above-cited review, with effects noted on ischemia, cerebrovascular injury, and inflammation and it is noted that the free radical scavenging effects of the flavonoid component of EGb-761 are in all probability important contributors to the overall activity of this product.[263] It can also be noted that *G. biloba* extracts have other activities than those mentioned above and that these extracts also have cytostatic activities that might render them valuable adjuncts to cancer chemotherapy.[264]

2.02.7.3 St. John's Wort

A second major herbal medicine is St. John's wort, which consists of the leaves and flowering parts of *Hypericum perforatum* L. (Clusiaceae). It is sold as an antidepressant in both the United States and Europe and its sales in

Europe are impressive at $6 billion in 1999, outselling Prozac by a factor of 4.[265] The constituents of St. John's wort include naphthodianthrones such as hypericin (**96**) and pseudohypericin (**97**), the phloroglucinol derivative hyperforin (**98**),[266] and various flavonoids such as quercetin (**99**), rutin (**100**), quercitrin (**101**), and biapigenin.[265]

96 R = H Hypericin
97 R = OH Pseudohypericin

98 Hyperforin

99 R = H Quercitin
100 R = rutinose Rutin
101 R = rhamnose Quercitrin

The pharmacological activities of the various constituents of St. John's wort have been studied extensively. Hyperforin appears to be the major bioactive constituent and it acts in several ways: serving as a neurotransmitter reuptake inhibitor with a broad selectivity, as a ligand for the pregnane X receptor, and as an antibacterial and antitumor agent.[267] It also has a rapid effect on amyloid precursor protein processing, indicating a possible role in the prevention of AD.[268] Hypericin and the flavonoid constituents may have some beneficial effects but a study in which St. John's worts with high and low hyperforin concentrations were tested, only the high-content sample was more effective than a placebo in treating depression.[269] St. John's wort has been reported to be "one of the safest known psychotherapeutic agents with proven clinical efficacy."[270]

2.02.7.4 Salvinorin A

The neoclerodane diterpene salvinorin A (**102**) was isolated from the leaves of the psychoactive mint *Salvia divinorum*,[271,272] together with salvinorin B (**103**) and other compounds. Several related compounds, including salvidivin A (**104**), salvidivin C (**105**), and salvinorin H (**106**) have recently been isolated from *S. divinorum*.[273] Salvinorin A has been synthesized.[274]

102 R = OAc Salvinorin A
103 R = OH Salvinorin B

104 Salvidivin A

105 Salvidivin C

106 Salvinorin H

Salvinorin A was found to be the major psychoactive compound of this plant, with significantly higher potency than even the closely related salvinorin B.[275] Although initial attempts to discover its molecular target were unsuccessful, it was later found to be a potent and selective κ-opioid receptor agonist;[276] it is this agonist activity on the κ-opioid receptor that is most probably the reason for salvinorin A's psychoactive effects. Salvinorin A is the first nonnitrogenous compound to be detected with this activity.

Because of its psychoactive effects salvinorin A does not have immediate application as a therapeutic agent, but it is valuable as a probe in opioid pharmacology.[275] Its selective agonist activity also raises the hopes that a selective antagonist of the κ-opioid receptor could be developed; such an antagonist would be of interest as a potential therapeutic agent for AD.[277]

2.02.8 Outlook and Future Prospects

In this chapter we have highlighted the tremendously important role that plants have played as a source of novel drugs for the treatment of a variety of serious diseases. As we (Gordon M. Cragg and David J. Newman) noted in Chapter 3.01, "it is clear that Nature will continue to be a major source of new drug leads." We also noted that "plant endophytes also offer an exciting new resource, and research continues to reveal that many of the important drugs originally thought to be produced by plants are actually products of endophytic microbes residing in the tissues between living plant cells." Several important anticancer agents have been isolated in small quantities from endophytic fungi isolated from their original source plants. These include Taxol, camptothecin, podophyllotoxin, an epimer of the precursor to the anticancer drug etoposide, and vinblastine and vincristine. Recently, hypericin has been isolated from an endophytic fungus from *H. perforatum*. Such discoveries bode well for the development of methods for greatly increased production of key bioactive natural products and readers are referred to the aforementioned chapter for more details. It is also apparent from our discussions in this chapter that synthesis has been all important in the elaboration of the structures of many plant-derived lead molecules to yield more effective drugs. Nature is the source of a multitude of unique 'privileged structures' and we foresee multidisciplinary collaboration playing an ever-increasing role in expediting the optimization of natural drug leads through the application of total and diversity-oriented synthesis and combinatorial chemistry and biochemistry, combined with good biology.

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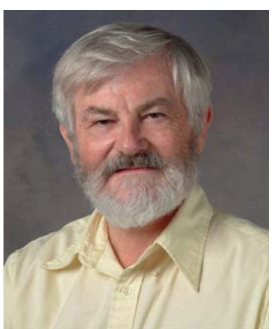
Biographical Sketches

Gordon Cragg completed his undergraduate training in chemistry at Rhodes University, South Africa and his D.Phil. (organic chemistry) from Oxford University in 1963. After 2 years of postdoctoral research at the University of California, Los Angeles, he returned to South Africa to join the Council for Scientific and Industrial Research (CSIR). In 1966, he joined the Chemistry Department at the University of South Africa and moved to the University of Cape Town in 1972. In 1979, he returned to the United States to join the Cancer Research Institute at Arizona State University. In 1985, he moved to the National Cancer Institute (NCI) in Bethesda, Maryland, and was appointed Chief of the Natural Products Branch in 1989. He retired in December, 2004, and is currently serving as an NIH Special Volunteer. His major interests lie in the discovery of novel natural product agents for the treatment of cancer and AIDS, with an emphasis on multidisciplinary and international collaboration. He has delivered over 100 invited talks at conferences in many countries worldwide and has been awarded NIH Merit Awards for his contributions to the development of taxol (1991), leadership in establishing international collaborative research in biodiversity and natural products drug discovery (2004), and contributions to developing and teaching NIH technology transfer courses (2004). In November 2006, he was awarded the William L. Brown Award for Plant Genetic Resources by the Missouri Botanical Garden. Missouri Botanical Garden also named a recently discovered Madagascar plant in his honor, *Ludia craggiana*. He has established collaborations between the NCI and organizations in many countries promoting drug discovery from their natural resources.

David G. I. Kingston was born in London and received his B.A and Ph.D. degrees in chemistry under Lord Todd and D. W. Cameron from Cambridge University in 1960 and 1964, respectively. After postdoctoral appointments at M.I.T. and Cambridge, he moved to the United States, where he is currently a University Distinguished Professor at Virginia Polytechnic Institute and State University. His research is centered around studies of bioactive natural products, and he is currently investigating the chemistry and tubulin-binding properties of paclitaxel, epothilone B, and discodermolide, as well as carrying out biodiversity conservation and drug discovery work in Madagascar, where he leads the Madagascar International Cooperative Biodiversity Group. He received the Research Achievement Award of the American Society of Pharmacognosy in 1999, and the Ernest Guenther Award in the chemistry of natural products from the American Chemical Society in 2008. He was Virginia Scientist of the Year in 2002. Two plants have been named in his honor; the South American plant *Cordia kingstoniana* J. S. Miller and the Asian yew *Taxus kingstonii* Spjut (the 'Kingston yew'). He also serves as one of several lay pastors in his local church, the Blacksburg Christian Fellowship.



David Newman is the current chief of the Natural Products Branch (NPB) in the Developmental Therapeutics Program at the National Cancer Institute in Frederick, Maryland. He was born in Grays, Essex, United Kingdom in 1939. In 1963, he received an M.Sc. in synthetic organic chemistry from the University of Liverpool working under Professor George Kenner, FRS on pyrrole and porphyrin syntheses. Following time as a synthetic chemist at Ilford, Ltd., he joined the ARC's Unit of Nitrogen Fixation at the University of London and then Sussex, as a research assistant in metallo-organic chemistry with Professor J. Chatt, FRS., moving to the microbial biochemistry group in early 1966 as a graduate student under Professor John Postgate, FRS, and was awarded a D. Phil. in 1968 for the work on microbial electron transport proteins from *Desulfovibrio*. Following a move to the United States in September 1968, he did 2 years of postdoctoral work at the Biochemistry

Department at the University of Georgia working on protein sequencing of *Desulfovibrio* ferredoxins, and then in 1970 joined SK&F in Philadelphia as a biological chemist. At SK&F, most of his work was related to biological chemistry and antibiotic discovery and he left SK&F in 1985 when the antibiotic group was dissolved. For the next 6 years, he worked in marine and microbial discovery programs (Air Products, SeaPharm, and Lederle) and then in 1991, joined the NPB as a chemist responsible for marine and microbial collection programs. He was given the NIH Merit Award in 2003 for this work and following Gordon Cragg's retirement from the position of Chief, NPB at the end of 2004, he was acting chief until appointed chief in late 2006. He has been the author and coauthor of over 110 papers, reviews, book chapters (and an editor, with Gordon Cragg and David Kingston, of *Anticancer Agents from Natural Products*), and holds 18 patents.

2.03 Marine Macroalgal Natural Products

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2.03.1 Introduction

The ocean covers nearly 70% of the Earth and represents 90% of the biosphere, providing a home for over 30 phyla and 500 000 species of marine organisms.[1] These organisms have evolved over millions of years to produce a vast diversity of unique chemical compounds that fulfill varied functions. Among these are molecules with potent biological activities that may have evolved as biochemical warfare between organisms in order to persist in an aggressive environment. Marine organisms produce secondary metabolites that are structurally distinct from those produced by terrestrial organisms, possibly due to factors unique to marine environments such as high salinity and pressure and a relatively constant temperature.[1,2] Unusual functional groups, such as isocyanate, isonitrile, dichloroimine, and halogenated functionalities, occur predominantly in marine metabolites.[2]

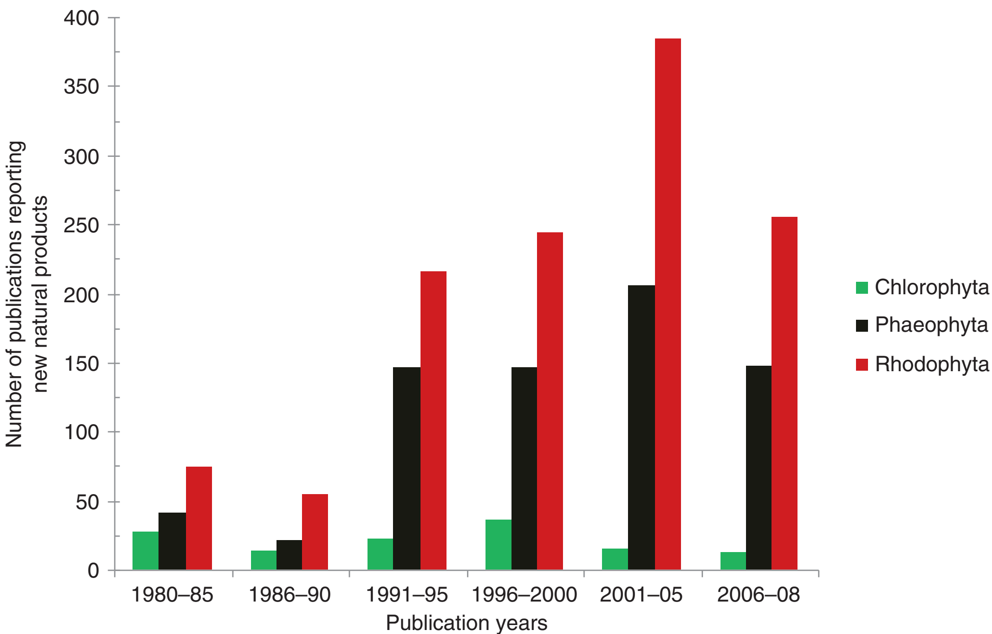

Figure 1 Reports of novel natural products from marine macroalgae since 1980. The search was conducted using Marinlit and ISI Web of Science, limiting output to chemistry journal research articles and notes, using a number of keywords. Hits were verified to ensure that natural products reported were new and discoveries were not double-counted. This figure may underrepresent the actual discovery rate, if natural products were reported in nonchemistry journals and/or if keywords were different from those used for the literature search.

Natural products from terrestrial plants and soil microbes have traditionally played an important role in drug discovery and were the basis of most early medicines.[3] The ocean, in contrast, was left virtually untapped of its vast resources until the early 1970s. Over the last several decades, scientists have discovered many distinctive types of biologically active secondary metabolites with unusual and exciting carbon skeletons from Chlorophyta (green algae), Phaeophyta (brown algae), and Rhodophyta (red algae). Scientific reports of new natural products from marine macroalgae have steadily increased since the 1980s (**Figure 1**). This chapter contextualizes studies on marine macroalgal natural products, with particular attention on structurally diverse natural products with ecological relevance and pharmaceutical potential, highlighting biosynthetic implications of structurally diverse metabolites. Preference is given to recently discovered natural products, as well as to compounds that are particularly promising for future development as drugs in pharmacological studies.

A series of excellent reviews on marine natural product chemistry, organized phylogenetically, are published annually in *Natural Product Reports*.[4] In addition, many recent review articles have explored particular marine natural products based on their specific biological activities, such as antitumor and cytotoxic effects,[5] therapeutics for tuberculosis and malaria,[6] and antifoulants.[7] Furthermore, review articles are also available based on compound class, such as the review written by Gross and König[2] on terpenoids from marine organisms with pharmacological activity. Other reviews of interest are cited throughout the chapter.

2.03.2 Isoprenoids

Marine macroalgae produce a wide variety of intriguing and diverse isoprenoid structures derived from C_5 isoprene units,[8] and many reports have been published on the ecological roles of these metabolites.[9,10] Marine terpenoids are frequently found with halogenated functionalities and one or more rings, which can have important implications for their biological activities. Isoprenoid metabolites are derived via the classical mevalonate pathway or the more recently discovered deoxyxylulose phosphate pathway.[11] Isoprenoids are

the dominant class of secondary metabolites known from macroalgae, representing 59% of the metabolites isolated to date from green algae, 46% from red algae, and 68% from brown algae.[12] Due to the substantial representation of isoprenoids among macroalgae, this review will focus mainly on novel isoprenoid carbon connectivities, ecological roles, and isoprenoids that have gained pharmaceutical interest.

2.03.2.1 Novel Connectivity and Cyclization Patterns

Due to the immense effort already made in the discovery of isoprenoid natural products from macroalgae, uncovering novel carbon skeletons is now rare. However, several reports have recently been published on macroalgal isoprenoids that possess novel connectivities and cyclizations.

2.03.2.1.1 Labdanes

Labdane-based diterpenes (**Figure 2**) have been isolated from tissues of fungi, insects, and marine organisms, from essential oils and resins, and from tissues of higher plants, and have been shown to possess a broad spectrum of biological activities.[13]

Two labdane-type brominated diterpenes (**1** and **2**) containing unprecedented seven- and eight-membered ether rings were isolated from the red alga *Laurencia obtusa*, collected in Mitikas Bay, Greece.[14]

OH Cl O Br H H O O Br H H

1 **2**

Metabolites **1** and **2** were proposed to share a common decalin precursor, formed from geranyllinalool (**3**) via cyclization and bromination, and the relative stereochemistry was assigned using ^{1}H–^{1}H scalar couplings and nuclear Overhauser effect spectroscopy (NOESY) experiments.[14] Enzyme-catalyzed dehydration, followed by double-bond transposition, allylic chlorination, and epoxidation, gives rise to the intermediate **4**. Nucleophilic attack of the C-8 hydroxyl group on either C-15 or C-14 leads to metabolites **1** and **2** (**Scheme 1**).

Figure 2 Labdane carbon skeleton.

OH Br⁺ ⁻OH **3** Br OH OH O a b Cl a b Br OH **4** a b **1** **2**

Scheme 1 Hypothesized biosynthetic pathway of **1** and **2**.[14]

Laurencia paniculata, collected from Qatari, Arabian Gulf, was found to produce (−)-paniculatol (5), an unusual labdane with a tetrahydropyran ring, and absolute stereochemistry was determined by X-ray crystallography.[15] More recently, two additional labdanes (6 and 7) with tetrahydropyran rings were identified from an unknown Okinawan species of *Laurencia*, and absolute stereochemistry also established by a combination of two-dimensional nuclear magnetic resonance (2D NMR) spectral data and X-ray crystallographic data.[16] While labdanes originating from macroalgae have intriguing structures, no biological tests were reported for these metabolites.

5 **6** **7**

2.03.2.1.2 Dactylomelane metabolites

Cyclization between C-6 and C-11 is an unusual skeletal characteristic of diterpenes (**Figure 3**), with only four marine species reported to utilize this pattern in secondary metabolism.

Two dactylomelane metabolites were isolated from sea hares *Aplysia dactylomela*[17] (dactylomelol, 8) and *Aplysia punctata* (puctatene acetate, 10)[18] and, more recently, similar metabolites were found from two red algal species, *Sphaerococcus coronopifolius* (sphaerolabdadiene-3,14-diol, 9)[19] and *Laurencia* sp.[20] *Laurencia* sp. (Tenerife, Canary Islands) produced a number of novel and relatively unstable hydroperoxide metabolites, such as dactylohydroperoxide C (12), in addition to 8 and puctatene (11).[20] Isolation of 8 and 11 from *Laurencia* suggests a macroalgal biogenesis for this class of diterpenes. The relative stereochemistries of 8–12 were established by analysis of nuclear Overhauser effect (NOE) data. No biological activities were reported for these metabolites.

8 **9**

10 R = Ac
11 R = H

12

Figure 3 Dactylomelane cyclization.

2.03.2.1.3 Meroditerpenes

Novel metabolites of mixed biogenesis are more likely to contain novel carbon skeletons than are the better-explored isoprenoid secondary metabolites. Therefore, hybrid metabolites from macroalgae seem to become front-runners for exciting natural product discovery. Uncovering novel skeletons for new scaffolds remains an integral part of drug discovery.

Since 2005, 28 novel bioactive metabolites (e.g., bromophycolides A and B (**13 and 14**), callophycoic acid C (**15**), and callophycol A (**16**)), representing 8 new carbon skeletons derived from mixed isoprenoid–shikimate biosynthesis, have been isolated from the red alga *Callophycus serratus* (Fiji Islands).[21–24] The absolute configurations of **13 and 14** were determined by X-ray crystallography, and absolute stereochemistries of subsequent bromophycolides were established by analysis of NOE data and from inferring a common biogenesis as **13 and 14**. An X-ray crystal structure of callophycoic acid A (structure not shown) provided its configuration from which the absolute stereochemistries of other callophycolic acids were inferred, and relative stereochemistry of **16** was determined through analysis of NOESY data.[23] Extracts yielding callophycoic acids and callophycols showed no signs of bromophycolides, which were found from collections at different sites, suggesting population-level variation in secondary metabolism leading to two chemotypes.[23] The most cytotoxic metabolite was **13**, with moderate *in vitro* cytotoxicity against a broad range of tumor types (mean $IC_{50} = 6.7\ \mu mol\ l^{-1}$; IC_{50} = half maximal inhibitory concentration). The G1 phase of the cell cycle was arrested when human ovarian cells were exposed to **13**, suggesting that apoptosis stemmed from cells arrested in G1.[21] Interestingly, the callophycoic acids and callophycols were significantly less active in pharmacological assays than **13 and 14**, suggesting the importance of the macrocyclic lactone functionality.[23] Nevertheless, compounds of both chemotypes suppressed growth of the algal pathogen *Lindra thalassiae* at and below natural concentrations, suggesting a potent antifungal chemical defense.[25]

13 **14**

15 **16**

An extract of the brown alga *Cystoseira* sp. (Montaña Clara Island, Mediterranean Sea) exposed to acetylation reaction conditions yielded two novel meroditerpenes, cystoseirone diacetate (**17**), with an unusual C-6–C-12

ether linkage, and amentol chromane diacetate (18), and whose relative stereochemistry was proposed from analysis of rotating-frame Overhauser effect spectroscopy (ROESY) data.[26]

17

18

The biosynthesis of cystoseirone (the unacetylated, likely natural product) was hypothesized to result from an oxidation of the enol–ether system found in 18, with subsequent rearrangement.[26] This was tested by treating 18 with *m*-chloroperbenzoic acid (MCPBA) in dichloromethane, which afforded two isomers, one of which was identical to 17 (**Scheme 2**).

2.03.2.2 Chemical Ecology

Secondary metabolites have long been assumed to enhance the survival of macroalgae by providing defenses against consumers, competitors, or parasites.[27] Many field and laboratory studies have tested antifeedant and antifouling effects of macroalgal metabolites.[28,29] Fewer ecological studies have addressed antimicrobial or other antiparasitic defenses in macroalgae, despite the fact that marine organisms are frequently exposed to high concentrations of potentially harmful marine microbes.[30]

18

17

Isomer of **17**

Scheme 2 Proposed mechanism for chemical transformation of 18 to 17 and its diastereomer.[26]

2.03.2.2.1 Antiherbivore metabolites

Numerous reports and reviews focus on macroalgal–herbivore interactions and the mechanisms by which macroalgae tolerate or resist herbivory.[31,32] Many algal species deter herbivores by morphological, structural, or chemical defenses[33] or by associating with unpalatable algae or other benthic organisms.[34,35] Due to the extensive literature available on these interactions, the aim of this section is to highlight specific antiherbivore terpenes from macroalgae.

Three new diterpenoids, acutilol A (**19**), acutilol A acetate (**20**), and acutilol B (**21**) from the brown alga *Dictyota acutiloba*, were found to be potent feeding deterrents against both temperate and tropical herbivorous fishes and sea urchins, and their relative stereochemistry was determined by NOESY experiments.[36] Common tropical and temperate herbivores were deterred by the acutilols at 20% of their natural concentrations, suggesting that these secondary metabolites provide an efficient chemical defense for *D. acutiloba*.[37] These compounds are structurally related to the common pachydictyane carbon skeleton, but the $\Delta^{1,10}$ double bond is an unusual feature of this compound class.[36] The pachydictyols and dictyols, first isolated in the 1970s, have also been shown to possess potent deterrent effects. Cruz-Rivera and Hay[38] tested the antifeedant effects of dictyols against six mesograzers, and found that amphipods were deterred by dictyols, but the isopod *Paracerceis caudata* was not deterred.

10
1
H
O
RO H
19 R = H
20 R = Ac
H
O
HO H
21

Halimeda spp. are well-studied green algae found in tropical areas characterized by high levels of herbivory.[39] Major metabolites from *Halimeda* include the diterpenes halimedatetraacetate (**22**) and halimedatrial (**23**),[40] which act as feeding deterrents and allow the alga to persist in areas of intense herbivory.[41] Additional studies established that upon damage, levels of **22** decreased while the concentration of **23** increased, suggesting that upon wounding, the alga quickly converts **22** into the more deterrent **23** via a putative enzyme-mediated pathway.[42] One difficulty of working with activated defenses is that the use of extraction solvents may lead to spontaneous activation of reactive precursors, preventing assessment of the true 'inactivated' state of defenses (J. Kubanek and M. E. Hay, unpublished). Nevertheless, activated defenses appear to be common among tropical macroalgae. Cetrulo and Hay[43] found that upon wounding, 17% of species tested exhibited changes in palatability, consistent with activated chemical defenses.

OAc
O
AcO
OAc
OAc
22
O
H
O
H
CHO
23

In a more recent example of activated macroalgal chemical defenses, caulerpenyne (**24**), from the invasive green alga *Caulerpa taxifolia*, was suggested to rapidly transform into oxytoxin 2 (**25**) upon wounding.[44] However, due to the labile nature of **25**, the authors were unable to test the hypothesis that **25** is more deterrent than **24**. Other studies testing the reactivity of 1,4-dialdehyde metabolites found in higher plants demonstrated that these compounds readily react with nucleophiles, making it difficult to use them in manipulative experiments.[45]

24 **25**

2.03.2.2.2 Antifouling metabolites

Solid surfaces exposed to seawater can undergo a series of changes leading to the accumulation of marine organisms, mainly consisting of microbial slimes, diatoms, barnacles, tunicates, bryozoans, and spores of marine algae.[46,47] Macroalgae, being a prolific source of bioactive natural products, may produce secondary metabolites to inhibit this process of fouling.[48] Since the 1970s, the antifouling effects of *Laurencia* spp. isoprenoids have been noted.[49] These metabolites, however, are generally toxic to many marine organisms and so their commercial development is not feasible.[50]

Caulerpa prolifera, found in the shallow waters of Saronicos Gulf, Greece, is abundant and exhibits minimal fouling.[51] Fifteen acetylene sesquiterpenoid esters (**26a–i, 27a–f**) from this alga inhibited fouling in a manner similar to the biocide bis-(tributylin) oxide (TBTO).[51] Similar to *Laurencia* antifouling metabolites, **26** and **27** and TBTO are generally toxic, thus limiting the further development of these compounds.

26 **27**

26a, 27d R = $CH_3–(CH_2)_4–(CH=CH–CH_2)_2–(CH_2)_6CO–$
26b, 27c R = $CH_3–(CH_2)_{14}CO–$
26c R = $CH_3–CH_2–(CH=CH–CH_2)_3–(CH_2)_6CO–$
26d R = $CH_3–CH_2–(CH=CH–CH_2)_5–(CH_2)_2CO–$
26e R = $CH_3–(CH_2)_{16}CO–$
26f R = $CH_3–(CH_2)_7–CH=CH–(CH_2)_9CO–$
26g R = $CH_3–(CH_2)_4–(CH=CH–CH_2)_4–(CH_2)_2CO–$
26h, 27e R = $CH_3–CH_2–(CH=CH–CH_2)_3–(CH_2)_4CO–$
26i R = $CH_3–CH_2–(CH=CH–CH_2)_4–(CH_2)_3CO–$
27a R = $CH_3–(CH_2)_{12}CO–$
27b R = $CH_3–(CH_2)_5–CH=CH–(CH_2)_7CO–$
27f R = $CH_3–(CH_2)_7–CH=CH–(CH_2)_7CO–$

To date, no macroalgal terpenes have been utilized commercially as antifoulants. Macroalgal furanones, however, are currently in development to prevent fouling (see Section 2.03.3.3). Macroalgae that are generally unfouled in the field are strong leads for the discovery of novel antifoulants, especially if found in habitats where other organisms are highly fouled.

2.03.2.2.3 Antimicrobial metabolites

Despite a large body of literature describing the antimicrobial activities of macroalgal secondary metabolites, little evidence exists to date to support the hypothesis that algal secondary metabolites target marine pathogens at realistic natural concentrations.[52] Only a handful of studies demonstrate the ability of macroalgal secondary metabolites to effectively deter or suppress ecologically relevant pathogens (see Sections 2.03.3.1.2 and 2.03.3.3). Two novel triterpene sulfate esters, capisterones A and B (**28** and **29**) from the green alga *Penicillus capitatus*, suppressed growth of the marine fungal pathogen *L. thallasiae* when tested at and below whole-tissue natural concentrations.[53] Assignment of the relative stereochemistries of **28** and **29** relied on interpretation of 2D NMR spectral data. While work in this area of chemical ecology is progressing, the few studies reported thus far propose ecologically relevant assays as guides for future studies.[30]

28 R = OAc
29 R = H

2.03.2.3 Metabolites with Pharmacological Potential

2.03.2.3.1 Halomon

Halogenated monoterpenes have been known from red algae since the mid-1970s, but it was not until the early 1990s that a metabolite with pharmaceutical potential moved to preclinical drug development. The pentahalogenated monoterpene halomon (**30**) was isolated from extracts of the red alga *Portieria hornemannii* (Batan Island, Philippines) and its X-ray crystal structure, absolute stereochemistry, and complete ^{1}H and ^{13}C NMR assignments were reported.[54] Halomon (**30**) was associated with a novel cytotoxicity profile against diverse tumor types in the National Cancer Institute's (NCI) primary screening panel.[54] Several reports have been published on the total synthesis of **30** to aid its progress and development through preclinical trials.[55,56] Unfortunately, research and development of **30** as an anticancer agent has been limited due to its failure to exhibit significant *in vivo* effects.[57]

30

2.03.2.3.2 Polyether triterpenes

Squalene-derived polyether triterpenes (e.g., **31–34**) are a structurally exciting group of marine algal natural products exhibiting a great diversity of ring sizes and intriguing biological activities. The first reported macroalgal polyether metabolite, thyrsiferol (**31**), from *Laurencia thyrsifera*, collected from New Zealand, was assigned the absolute structure **31** by X-ray crystallographic analyses based on thyrsiferol 18-acetate (**32**).[58,59] Following this discovery, several other structurally related polyether metabolites were reported from *Laurencia* spp., including dehydrothyrsiferol (**33**)[60] and thyrsiferyl 23-acetate (**34**).[61] The latter exhibited superior activity against P-388 lymphoid neoplasm cells, with $IC_{50} = 0.47\ \mathrm{nmol\,l^{-1}}$. Furthermore, **34** potently and selectively inhibited protein phosphatase 2A (PP2A), with no effect on protein phosphatases 1, 2B, 2C, or protein tyrosine phosphatase, making **34** a potential probe for identification of cellular processes dependent on PP2A.[62]

31 $R^1 = R^2 = H$
32 $R^1 = H$, $R^2 = Ac$
34 $R^1 = Ac$, $R^2 = H$

33

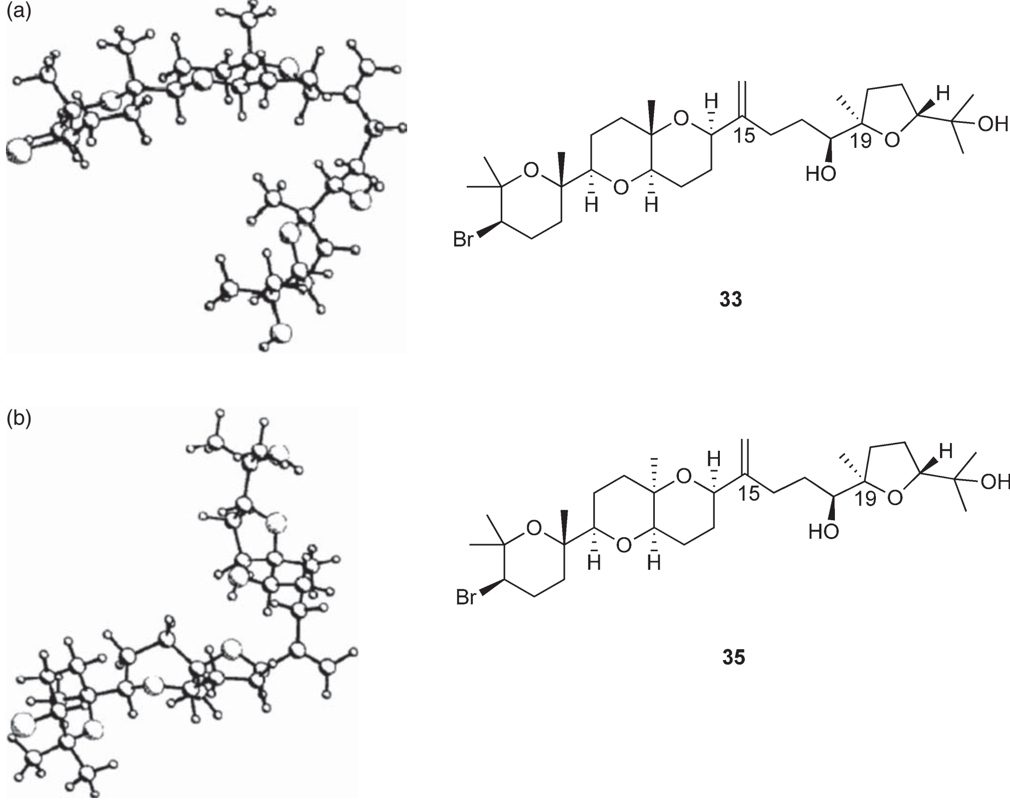

Figure 4 Stereoviews of proposed 3D structures of (**a**) **33** with downward side chain and (**b**) **35** with upward side chain. Reprinted from J. J. Fernandez; M. L. Souto; M. Norte, *Bioorg. Med. Chem.* **1998**, *6*, 2237–2243, with permission from Elsevier.

The potent and selective effects of **34** propelled further studies of this compound class. After discovering many new analogues from *Laurencia* spp., structure–activity relationships were established based on a combination of isolated natural products and synthetic analogues.[63,64] Fernandez *et al.*[63] proposed that the spatial arrangement of the flexible chain affected metabolite bioactivity. From calculations of stable conformations of isolated polyethers using distance constraints established from NOE data, it was proposed that polyethers with C-15–C-19 chain turned downward were more potent than those with the side chain turned upward, as illustrated by comparing **33** (downward side chain, $IC_{50} = 0.01\ \mu g\ ml^{-1}$) versus **35** (upward side chain, $IC_{50} > 1\ \mu g\ ml^{-1}$) (**Figure 4**). The original structures and pharmacological activities drove synthetic efforts toward producing these compounds, and a thorough review of the total synthesis strategies is available.[65]

2.03.2.3.3 Dolabellanes

Dolabellanes (**Figure 5**) are constituents of mollusks, coelenterates, and brown algae, and they are also found in terrestrial mold, liverwort, and higher plant species.[66] Several dolabellanes have shown potential for pharmaceutical application, exhibiting cytotoxic, antibacterial, antifungal, antiviral, and antimalarial activities.[66]

Figure 5 Dolabellane carbon skeleton.

Dolabellanes **36–39** from the brown alga *Dictyota dichotoma* in Cortadura (Cádiz, Spain) exhibited mild activity in *in vitro* cytotoxicity assays against P-388 mouse lymphoma, A549 human lung carcinoma, HT-29 human colon cancer carcinoma, and MEL-28 human melanoma tumor cell lines.[67] Dolabellane **37** exhibited $IC_{50} = 1.2\ \mu g\ ml^{-1}$ against P-388 and A549 tumor cell lines and $IC_{50} = 2.5\ \mu g\ ml^{-1}$ against HT-29 and MEL-28 tumor cell lines.[67] Relative stereochemical assignments were made upon analysis of a series of NOE difference spectroscopy experiments.

36 $R^1 = OH$; $R^2 = H$
37 $R^1 = R^2 = H$

38

39

From *D. dichotoma* collected near Sicily, Italy, four dolabellanes (**40–43**) were isolated, displaying activity against Gram-positive and Gram-negative bacteria.[68] Moreover, **40** exhibited significant *in vitro* activity against influenza and adenoviruses.[69] As with **36–39**, the relative stereochemistries of dolabellanes **40–43** were determined by NOE.

40 **41**

42 **43**

The diverse array of functionalities and stereocenters has made dolabellanes a challenge for synthetic chemists, who have helped to establish the absolute configurations of these natural products.[66] Several distinct strategies have been tackled, with macrocyclization as the key reaction.[66] Although macroalgal dolabellane diterpenes exhibit a range of pharmacologically relevant activities, no dolabellane metabolite has been pursued so far for further pharmaceutical development.

2.03.3 Fatty Acid and Polyketide Metabolites

The condensation of C_2 acetate units and their subsequent modification lead to a vast number of polyketide (acetogenin) and fatty acid-based metabolites. Complex structures are biosynthesized via reactions involving alkylation, phenolic oxidative coupling, oxidative cleavage of aromatic rings, cyclization, and the use of starter units other than acetate.[11] These secondary metabolites constitute the second most abundant class found in macroalgae, accounting for 19% of green algal metabolites and 38% of red algal metabolites.[12]

2.03.3.1 Polyketides

2.03.3.1.1 C_{15} acetogenins

Although C_{15} acetogenins have been isolated from various algal species since the 1970s, novel derivatives are continually being reported, with mono-, di-, tri-, and tetracyclic structures possessing enyne and bromoallene functionalities. However, the discovery of acetogenins with new carbon skeletons is now rare. The biological activities of C_{15} acetogenin are generally weak to moderate.

The red alga *L. obtusa* (Scanlon's Island, Ireland) was found to contain scanlonenyne (**44**), a novel acetogenin with a ketone at C-7.[70] No biological data were reported. A new brominated acetogenin, chinzallene (**45**), was isolated from an unknown species of *Laurencia* in Japanese waters (Chinzei, Saga Prefecture) and is structurally related to okamurallene (**46**), which is known from *Laurencia intricata*.[71,72] Relative stereochemistries of **44–46** were established by analysis of ^1H–^1H scalar couplings in combination with 2D NOE spectral data.

44 **45** **46**

While acetogenins have frequently been isolated from *Laurencia* spp., it is unusual to see bromoallene and enyne metabolites co-occurring. Four novel acetogenins, laurendecumallenes A and B (**47** and **48**) and laurendecumenynes A and B (**49** and **50**), were discovered in *Laurencia decumbens* (Weizhou Island of Guangzi Province, China), suggesting that these functional groups share a common biosynthetic pathway (**Figure 6**).[73] Stereochemical assignments are relative for **47–50**, as established through NOESY experiments.

While structurally diverse, these known macroalgal acetogenins do not possess exciting pharmacological activities. Moreover, the ecological relevance of this compound class has not been well documented. However,

47 **48**

49 **50**

Figure 6 Proposed biogenesis of bromoallene from terminal enyne. Adapted from N. Y. Ji; X. M. Li; B. G. Wang, *J. Nat. Prod.* **2007**, *70*, 1499–1502.

due to the large database of acetogenins isolated from various species of *Laurencia*, many studies have used these metabolites for chemotaxonomic identification of species.[71]

2.03.3.1.2 Lobophorolide

Strong antifungal activity against the marine pathogen *L. thalassiae* led to the isolation of lobophorolide (**51**), a polyketide from the brown alga *Lobophora variegata*.[74] Throughout the bioassay-guided fractionation, only **51** or fractions containing it inhibited fungal growth, suggesting that this compound accounts for all of the antifungal activity.[74] Significant antifungal effects were also observed against the human pathogen *Candida albicans* ($IC_{50} = 1.3\ \mu g\ ml^{-1}$) and against human colon cancer cells ($IC_{50} = 0.03\ \mu g\ ml^{-1}$). The structural similarity of **51** to tolytoxin[75] from cyanobacteria suggests that **51** is likely the product of a microbial symbiont; however, a putative symbiont has not been discovered to date. Compound **51** was proposed to share relative and absolute stereochemistry with that of tolytoxin, with the exception of C-6, for which Kubanek *et al.* argued an *R* configuration, as opposed to the *S* configuration proposed by Carmeli *et al.* in tolytoxin. A putative microbial origin was also supported by the patchy geographic distribution of **51** among collections of *L. variegata.*

51

2.03.3.1.3 Manauealides

Several successive food poisonings reported from ingestion of the Hawaiian red alga *Gracilaria coronopifolia* (Waiehu, Maui) led to the identification of manauealides A–C (**52–54**), aplysiatoxin (**55**), and debromoaplysiatoxin (**56**), with the last two implicated as causative agents.[76] To date, **51–56** are the only macrocyclic polyketide metabolites isolated from macroalgae. The absolute configurations of **52–54** were established by comparison of circular dichroism (CD) spectra of these metabolites with that of **56**, whose absolute configuration was previously established.[77] As with the case of **51**, Nagai *et al.* suggested that cyanobacteria could be the true producer of these toxins, since **55** and **56** were previously reported from cyanobacteria and a sea hare known to feed on cyanobacteria, and that cyanobacteria were sometimes found growing on *G. coronopifolia.*[78,79]

52 $R^1 = Cl$, $R^2 = H$
54 $R^1 = H$, $R^2 = COCH_3$

53 $R^1 = H$, $R^2 = Br$
55 $R^1 = Br$, $R^2 = H$
56 $R^1 = H$, $R^2 = H$

2.03.3.2 Fatty Acids

Acetylenic functionalities, as in peyssonenynes A and B (57 and 58) from the red alga *Peyssonnelia caulifera* (Yanuca Island, Fiji),[80] are rare for macroalgae, with only one previous report of lipid acetylenes, produced by the red alga *Liagora farinosa*.[81] Both 57 and 58 showed similar activity in a DNA methyltransferase (DNMT-1) enzyme inhibition assay, with an $IC_{50} = 16$ and $9\,\mu mol\,l^{-1}$, respectively, but peyssopyrone (59), also from *P. caulifera*, was inactive.[80]

57 *E* isomer
58 *Z* isomer

59

2.03.3.3 Furanones

Many in-depth ecological studies have been performed using the red alga *Delisea pulchra*, which is known to produce a series of structurally related brominated furanones (60–63).[82,83] These furanones inhibit fouling and bacterial attachment, acting as antagonists of bacterial communication normally mediated by acylated homoserine lactones, which regulate bacterial swarming and biofilm formation.[84,85] Dworjanyn *et al.*[86] demonstrated chemically mediated antifouling effects of furanones using surface extracts of *D. pulchra*, as well as pure furanones tested at natural surface concentrations. These metabolites also act as strong deterrents to many herbivores at natural concentrations.[87] It seems reasonable to suggest that *D. pulchra* is able to compensate for the cost of producing these secondary metabolites by utilizing these furanones as a defense against multiple threats, such as biofouling, antimicrobial colonization, and herbivory.

60 $R^1 = H$, $R^2 = Br$
61 $R^1 = H$, $R^2 = H$
62 $R^1 = OAc$, $R^2 = H$
63 $R^1 = OH$, $R^2 = H$

2.03.4 Shikimate Metabolites

Shikimic acid (64) is the biosynthetic precursor to an array of aromatic compounds, including benzoic and cinnamic acids.[11] This pathway is utilized by microorganisms and plants, but not by animals, which obtain essential shikimate building blocks like phenylalanine from their diets.[11] Red algae are known to be a prolific source of halogenated phenolic metabolites derived from shikimic acid, comprising approximately 5% of known algal metabolites.[12]

64

2.03.4.1 Halogenated Phenols

A number of bromophenols have been isolated from the genus *Rhodomela*, exhibiting various biological functions such as feeding deterrents.[88] Antibacterial assays led to the isolation of two new (65 and 66) and three known (67–69) bromophenols from the red alga *Rhodomela confervoides* (Qingdao, China).[89] Metabolite 69 exhibited moderate activity against *Staphylococcus epidermidis*, with a minimum inhibitory concentration of 35 $\mu g\,ml^{-1}$.[89]

65 66 67

68 69

Three new (70–72) and three known (73–75) bromophenols from the red alga *Polysiphonia urceolata* (Qingdao, China), whose absolute configurations were determined by CD spectra, displayed significant radical scavenging activity when compared to known antioxidants, with IC_{50} values of 6–36 $\mu mol\,l^{-1}$.[90] The radical scavenging activities of 70–72 were 10- to 13-fold more active than the known synthetic antioxidant butylated hydroxytoluene (BHT), making these metabolites potential leads as antioxidant drugs.

70 **71** **72**

73

74 R = OH
75 R = Br

2.03.5 Nonribosomal Peptide Metabolites

Many natural peptides are synthesized by a sequence of enzyme-controlled processes carried out by a multifunctional enzyme of modular arrangement, similar to some polyketide synthases.[11] These nonribosomal peptide synthetases (NRPSs) typically consist of an adenylation domain, a peptidyl carrier protein domain, and a condensation or elongation domain in order to carry out amide bond formation and some derivations of amino acid residues.[11]

2.03.5.1 Depsipeptides

Depsipeptides are a class of nonribosomal peptides cyclized via an ester bond and often contain nonprotein amino acids. Several bioactive depsipeptides, ranging from a C_{31} tripeptide to a C_{75} tridecapeptide, were isolated from a sacoglossan mollusk, *Elysia rufescens*, and from its algal diet, *Bryopsis* sp. Collectively known as kahalalides, these peptides display a range of biological activity. Kahalalide A (**76**) displays moderate antimalarial activity and has been noted for its *in vitro* effects against *Mycobacterium tuberculosis*, while kahalalide E (**77**) selectively inhibits herpes simplex virus II (HSV II).[91] Kahalalide F (**78**), whose absolute stereochemistry was determined by chemical degradation,[92] exhibits activity against select solid tumors and some AIDS-opportunistic infections, and is currently undergoing phase II clinical trials.[93,94] Hill *et al.*[95] reported strains of kahalalide-producing *Vibrio* sp. bacteria associated with *Bryopsis* sp. and *E. rufescens*, which suggests a microbial origin of these depsipeptides; however, natural product chemists await full description of this work in the peer-reviewed literature.

Kahalalide G (**79**), the linear peptide form of **78**, has so far been found to be inactive in all bioassays.[91] To date, 17 kahalalides have been isolated and characterized from *Bryopsis* sp., but **78** remains the only kahalalide to advance to clinical trials for the possible treatment of lung cancer, other tumors, and AIDS.[96–100]

76

77

78

79

2.03.6 Alkaloid Metabolites

Algal compounds are rarely nitrogenous, possibly because macroalgae are often nitrogen-limited.[101] However, a handful of nonpeptide, nitrogen-containing metabolites that possess interesting biological activity has been reported from macroalgae.

2.03.6.1 Indole Metabolites

Red algae of the genus *Chondria* are known for the production of cyclic polysulfides[102] and terpenoids.[103] An unidentified species of *Chondria* (Buenos Aires, Argentina) was found to produce novel indolic metabolites, chondriamides A and B (80 and 81), with antiviral, antifungal, and cytotoxic activities.[104] Chondriamide A (80) exhibited antiviral activity against HSV II with $IC_{50} = 1\ \mu g\ ml^{-1}$, while 81 displayed antifungal activity against *Aspergillus oryzae* and *Trichonphyton mentagrophytes*.[104] Furthermore, 80 and 81 displayed moderate cytotoxicity against KB cell lines, with IC_{50} values of 0.5 and $<1\ \mu g\ ml^{-1}$, respectively.[105] Chondriamide C (82), from *Chondria atropurpurea*, exhibited *in vitro* anthelmintic activity against *Nippostrongylus brasiliensis* with an EC_{80} of $90\ \mu mol\ l^{-1}$.[106] Kuramochi *et al.*[107] reported on the total synthesis of 80 and 82 using a newly developed approach based on the Curtius rearrangement and acylation of alkenylcarbamate.

80 R = H
81 R = OH

82

Almazoles A and B (83 and 84) were isolated from an unknown red algal species of *Haraldiophyllum* (Dakar, Senegal) containing a rare 2,5-disubstituted oxazole ring.[108] After recollection of *Haraldiophyllum* sp. some years later, 83 and 84 were not observed, perhaps because of geographic variation or compound instability, but another novel indole bearing an oxazolone ring, almazolone (85), was discovered.[109] Almazolone (85) was isolated as an 88:12 mixture of Z/E isomers, due to the photochemical and thermal instability of the compound. Guella *et al.*[109] also synthesized 85 by condensation of indole-3-carboxaldehyde with 3-phenylpropionyl glycine.

83 R = CHO
84 R = H

85

2.03.6.2 2,7-Naphthyridines

Gross *et al.*[110] discovered two novel 2,7-naphthyridine metabolites, lophocladines A and B (86 and 87), from an unknown and understudied red algal species *Lophocladia* (Savusavu, Fiji Islands). The only other naturally occurring representative of this compound class comes from the terrestrial plant *Valeriana officinalis*.[111] Lophocladine B (87) exhibited moderate activity against lung and breast cancer cells, with IC_{50} values of 64.6 and $3.1\ \mu g\ ml^{-1}$, respectively, whereas 86 was found to be inactive in all assays.[110] NCI-H460 lung cancer

cells showed morphologic changes when treated with 87, depolymerizing 85% of the microtubules at 45 μmol l^{-1}, but the potency of 87 is rather moderate when compared to other tubulin depolymerizing compounds.[110]

86 87

2.03.7 Glycolipids

Carbohydrates are one of the most common components of plants, animals, and microorganisms, with six-carbon sugars (hexoses) and five-carbon sugars (pentoses) being the most commonly encountered carbohydrate unit.[11] Medicinally important natural products are frequented with various sugar units to form a class of compounds known as glycosides.[11] Macroalgae are known to produce a handful of biologically active glycosides, representing another exciting class for pharmacological research.

The red alga *Gracilaria asiatica* (Indonesia) was found to produce gracilarioside (88) and gracilamides (89 and 90), possessing unusual cyclopropane-containing alkyl chains.[112] This was the first report of naturally occurring marine ceramides with a cyclopropane ring. Fatty acid chain lengths and cyclopropane ring positions were determined using microscale chemical degradation. Furthermore, absolute configurations of 88–90 were established through a combination of chemical degradation and CD spectral analyses. These metabolites exhibited weak activity against melanoma cells, with 18.2% cell death at 20 μg ml^{-1} for 88 and 11.7% cell death at 30 μg ml^{-1} for 89 and 90.[112]

88

89

90

Human intoxication resulting after ingestion of the red alga *Polycavernosa tsudai* (Tanguisson Beach, Guam, previously called *Gracilaria edulis*) led to the isolation of toxic glycosidic macrolides, polycavernosides (**91–97**), with polycavernoside A (**91**) reported as the illness-causing agent.[113–115] The toxicity of **91** in mouse bioassays was established as 0.2–0.4 mg kg^{-1}, with human symptoms including diarrhea, vomiting, paresthesia, loss of consciousness and, in extreme cases, death.[113,116] Total synthesis of **91** was reported by Fujiwara and Murai[117] and thus the absolute configuration of **91** was determined by CD analysis. No reports have been published on the ecological role of these toxins in the producing organism.

91 R^1 = H, R^2 = Me, R^3 = H
92 R^1 = H, R^2 = H, R^3 = H
93 R^1 = Me, R^2 = Me, R^3 = H
94 R^1 = Me, R^2 = Ac, R^3 = H
95 R^1 = Me, R^2 = H, R^3 = H
96 R^1 = Me, R^2 = Ac, R^3 = Me
97 R^1 = Me, R^2 = Me, R^3 = Me

A highly active antimitotic extract from the green alga *Avrainvillea nigricans* (Portsmouth, Dominica) yielded two novel glycolipids, nigricanosides A and B (**98 and 99**), representing a new class of ether-linked glycoglycerolipids.[118] Nigricanoside A dimethyl ester (**100**) was found to arrest human breast cancer cells in mitosis with IC_{50} = 3 nmol l^{-1}, stimulating polymerization of tubulin.[118] Hydrogenation of the alkenes significantly reduced activity against the breast cancer cell line, establishing their importance. The ability of the potent nigricanosides to promote tubulin polymerization is without precedent among previously known glycoglycerolipids, making these metabolites an exciting anticancer drug lead.[118]

98 R^1 = H, R^2 =
99 R^1 = H, R^2 =
100 R^1 = Me, R^2 =
101 R^1 = Me, R^2 =

2.03.8 Conclusion

Structurally diverse, bioactive metabolites are continually being reported from macroalgae. The rate of new natural product discovery increased substantially in the 1990s and has continued to increase through the 2000s, particularly from red macroalgae (e.g., Figure 1). However, it should be noted that most recent discoveries are featured in more specialized journals than in the early years and the discovery of new structural classes of secondary metabolites from macroalgae has become rare. Properties of macroalgal natural products range from pharmacological activities, such as antitumor, antimicrobial, and antiviral effects, to ecological roles such as herbivore deterrence and antimicrobial defenses. The large numbers of known isoprenoids and phenolic compounds lacking significant pharmacological activities make these structural classes less stimulating for future discoveries of pharmaceutical leads. However, rare and/or understudied macroalgal species could be promising for the discovery of new and exciting secondary metabolites, including compounds of mixed biogenesis. Some of the most structurally complex and biologically active molecules reported recently from macroalgae may in fact originate from marine microbes, which are a yet-underexplored source of chemical innovation. Continued research on bioactive macroalgal natural products could stimulate the identification of structurally novel compounds as well as intriguing syntheses of compounds for biological and pharmacological research.

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Abbreviations

Ac acetyl
BHT butylated hydroxytoluene
2D two-dimensional
CD circular dichroism
DNMT-1 DNA methyltransferase
MCPBA *meta*-chloroperbenzoic acid
Me methyl
NCI National Cancer Institute
NMR nuclear magnetic resonance
NOE nuclear Overhauser effect
NOESY nuclear Overhauser effect spectroscopy
NRPS nonribosomal peptide synthetase
PP2A protein phosphatase 2A
ROESY rotating-frame Overhauser effect spectroscopy
spp. species
TBTO bis-(tributylin) oxide

Nomenclature

^{1}H	proton
^{13}C	carbon-13
μg ml^{-1}	micrograms per milliliter
μmol l^{-1}	micromole per liter
nmol l^{-1}	nanomole per liter
Δ	delta

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2.04 Insect Natural Products

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2.04.1 Introduction

With greater than 1 million described species, arthropods comprise more than 80% of all known animal species, and by some estimates make up roughly two-thirds of all extant species.[1,2] The phylum Arthropoda includes insects, spiders, ticks, lice, centipedes, shrimp, and crabs, as well as several less well-known groups. Arthropods are virtually ubiquitous worldwide, and many species play dominant roles in the ecology of their habitats. One commonly cited factor in the arthropod's 'phyletic dominance,' as termed by Meinwald and Eisner,[3] is their extensive use of small-molecule chemical signals. Arthropods use chemical signals for mate attraction and selection, for defense against predators and pathogens, and for the acquisition of prey. In fact, the extent to which the ecological interactions of arthropods are facilitated by small-molecule metabolites is only now becoming clear, and many novel types of chemical interactions remain to be identified.

Arthropod natural products are structurally diverse, including compounds derived from fatty acid, polyketide, terpenoid, nucleoside, and amino acid pathways, although the biosynthesis of most of these compounds has not yet been studied in detail (**Figure 1**). The biosynthesis of defensive metabolites among beetles and ants has been reviewed recently,[4] and a monograph devoted to insect natural product biosynthesis, *Biosynthesis in Insects*, by Morgan,[5] has recently become available. In addition to covering biosynthetic aspects, Morgan's text provides

Figure 1 Examples of arthropod natural products from spiders (1, 4), mites (6), ants (5, 7, 10), fireflies (3), termites (7), grasshoppers (9), and beetles (2, 8, 11, 12).

an excellent overview of insect natural products, including many older examples that are not dealt with in this chapter. Compared to fungi and bacteria, arthropods generally produce less polar compounds, which mostly are of polyacetate or fatty acid origin, with structures derived from polyketide (or polypropanoid) pathways being less common. Furthermore, arthropod-derived compounds are more likely to possess carbocyclic ring

systems and chemically reactive functional groups such as ketones or enamines. An additional layer of structural complexity is introduced in the biosynthesis of complex arthropod alkaloids such as the chilocorines and myrmicarins or the macrocyclic polyamines. These compounds are derived from oligomerization of several polyacetate- or fatty acid-derived building blocks resulting in unique oligocyclic or macrocyclic structures, for example, chilocorine B (**2**), myrmicarin 663 (**5**), and PAML908 (**8**) (**Figure 1**). Arthropod metabolic capabilities often vary greatly, even among closely related species, as well as within individual species between different life stages. In coccinellid beetles, for example, larvae, pupae, and adults are often found to produce very different types of defensive alkaloids (see Section 2.04.6.1).

Today, our knowledge of the structures and functions of small-molecule secondary metabolites in arthropods remains uneven. A few groups of organisms, including some species of ants, beetles, butterflies, moths, and spiders, have been chemically scrutinized in considerable detail, and as a result a relatively great number of structures have been identified from these species. However, most species of insects and other groups of arthropods, for example, crustaceans, remain largely unexplored. It is worthy of note that even for those cases where chemical analyses have led to the identification of new and often chemically fascinating structures, the biological roles of the identified compounds, perhaps with the exception of sex pheromones, have rarely been explored comprehensively. In most cases, the identified compounds have been assigned generic attributes such as 'irritant' or 'defensive,' without a detailed analysis of either their full ecological context or their molecular mode of action. In what Blum has termed 'semiochemical parsimony,' individual arthropod secondary metabolites frequently serve multiple ecological functions,[6] and our current understanding of many seemingly well-studied chemical–ecological interactions involving arthropods may in fact be largely incomplete. Furthermore, our understanding of the biochemical pathways involved in arthropod secondary metabolite regulation lags far behind that for other groups, such as plants, bacteria, and fungi. Only recently have chemical ecologists and chemical biologists begun to fully address these questions, and perhaps the current state of arthropod natural product chemistry can be best described as one of the change, where purely structure-oriented chemical prospecting is being supplanted by a focus on elucidation of the detailed molecular mechanisms underlying chemical–ecological interactions.[7]

The most well-known functions of secondary metabolites among arthropods include the use of pheromones for intraspecific communication, the employment of antipredatorial defensive agents, and the offensive use of paralytic and/or toxic agents, such as in the form of venoms, for the acquisition of prey. In recent years, considerable insight has been gained in all three categories, as well as in the discovery of heretofore unknown interactions (see Section 2.04.4.1). Fossil evidence of chemical defense in the insects reaches as far back as the Early Cretaceous period.[8] The chemical defense of insects has been reviewed as recently as 2005,[9] and earlier works dealing with chemical defense of ants,[10] beetles,[11–13] as well as of arthropods in general,[14–16] also exist.

In this chapter, arthropod pheromones and hormones will not be considered, except for a few example structures of important compound classes. These topics are dealt with in detail in Chapters 4.03 and 4.04. Our primary focus will be the defensive and venom chemistry of terrestrial arthropods, as well as any additional structures discovered as a result of general chemical prospecting in these animals. Biosynthetic origin and biological roles of the described compounds, as far as they are known, will be summarized briefly; however, for more extensive information on ecological functions or biosynthesis the reader should consult some of the many excellent reviews and monographs referenced. Throughout we will make an effort to point out indirect effects and benefits that can or have been gained through the study of arthropod natural products.

This chapter is organized primarily based on a classification of arthropod-derived compounds according to their putative biogenetic origin ('terpenoids,' 'polyketides') or specific structural features ('alkaloids,' 'nucleosides'). This approach allowed us to emphasize chemical characteristics and peculiarities that distinguish arthropod-derived compounds from other groups of natural products. Phylogenetic relationships are discussed only in specific cases where they directly relate to similarities in natural product profiles. With respect to our classification of arthropod natural products as terpenoids, fatty acid derivatives, or polyketides, it should be noted that for most arthropod natural products, assignments of biogenetic origin remain tentative at best because few biosynthetic routes have been confirmed experimentally.

2.04.2 Challenges in Arthropod Natural Products Chemistry

One of the oldest known human uses of an insect natural product is that of carminic acid (**13**) as the active color ingredient of the natural dye cochineal.[17] Produced from the scale insect, *Dactylopius coccus*, cochineal dye was once a geopolitically important commodity. The semisynthetic derivative carmine later found applications as a biological stain and as a food coloring. As was generally the case with early use of natural products, little to no consideration was given to the compound's ecological significance. Only much later, scientific studies showed that carminic acid benefits *D. coccus* by acting as a deterrent to predation,[18] but could also be sequestered by predaceous insects that feed on *D. coccus* for their own defense.[19] The ability to sequester defensive metabolites is particularly widespread among arthropods, although sequestration of dietary toxins has also been observed for marine gastropods,[20] as well as for some birds,[21] reptiles,[22] and amphibians. In the case of many tropical poisonous frogs, the sequestered toxins are in fact derived from arthropod prey species, and extensive research in this area has had the indirect effect of revealing much about the defensive alkaloids of many species of arthropods (**Figure 2**).[23–27]

The widespread occurrence of sequestration adds to the challenges intrinsic to arthropod natural product research. The sheer number of species promises a virtually unlimited pool of organisms for future analyses, whose genetic diversity – or that of associated microorganisms – probably encodes a correspondingly diverse collection of small-molecule metabolites. Frequently, different life stages of species have vastly different metabolomes, and careful inspection of a species' lifecycle and ecology can often lead to the discovery of additional groups of metabolites. However, precise identification of species and life stages often requires the enlistment of collaborators with specific taxonomic expertise. This poses a considerable challenge for the systematic screening of arthropod species for new natural products, because for many, if not most groups of arthropods biological knowledge is extremely limited.

Another problem is posed by the fact that the number of known species that can be collected in large quantities (often those species considered pests) represents only a small fraction of overall arthropod species diversity. Most arthropod species are rare, and can only be collected sporadically, often with dramatic seasonal or yearly variations in availability. It is unfortunately all too common to identify what appears to be a promising species for further research, often with considerable investment of time in collecting preliminary data, only to realize subsequently that additional specimens cannot be obtained. This problem is compounded by the fact that many insect species are difficult or even impossible to raise in captivity. Such instances bring to the foreground what may be the biggest challenge in insect natural products research: lack of sufficient sample amounts for detailed analyses.

Because arthropods, like most other vertebrates and invertebrates, live in close, often symbiotic association with various types of microorganisms, it cannot generally be assumed that the isolated compounds have been biosynthesized by the arthropod. Some compounds isolated from arthropods may be of microbial origin, or result from mixed biogenesis including participation of both the host organism and its associated microfauna. In a few cases, for example, the coleopteran defensive polyketide pederin (**14**),[28] bacterial origin has been demonstrated, and it is probable that many more so-called arthropod natural products are in fact the products of microbial symbionts.[29,30] Furthermore, as previously mentioned, many arthropods sequester small-molecule metabolites from their diet – plants, other arthropods, or fungi. Such sequestered compounds frequently undergo additional modification. The ultimate biosynthetic origin of compounds isolated from arthropods is

Figure 2 Carminic acid (**13**) and pederin (**14**).

thus sometimes difficult to discern. For example, chrysomeline leaf beetles, over the course of their evolutionary history, appear to have employed *de novo* synthesis, dietary sequestration, and mixed insect–plant biogenetic routes toward the acquisition of defensive metabolites.[31]

In spite of these challenges, the extent to which arthropods have developed extremely specialized adaptations, many of which are dependent on secondary metabolites, suggests that arthropods, along with their specifically adapted microfauna, should continue to be a primary source of chemical diversity. Furthermore, the overall focus of research on insect chemical ecology continues to expand from a relatively narrow analysis of the involved secondary metabolites to a more comprehensive approach aimed at elucidation of associated biomolecular mechanisms and their effect on the ecological fitness of the interacting organisms. Recent progress in the area of plant–insect interactions is illustrative of this new direction.

Arthropod evolution, especially that of insects, is widely accepted to be inextricably linked to the evolutionary history of plants.[32] In the years since the landmark study by Ehrlich and Raven concerning coevolution among butterflies and their angiosperm food plants,[33] there has been much progress toward understanding these interactions at the molecular level. One of the most studied systems concerns lepidopteran species that have developed specific detoxification mechanisms aimed at the glucosinate–myrosinase defense system found within plants of the order Brassicales.[34] A detailed understanding of the biomolecular mechanisms of plant–insect ecological interactions holds promise in many areas. For example, there are potentially great environmental benefits associated with the ability to move from blanket pesticide use in agricultural pest control to finely tuned, chemically mediated, biorational approaches based on a firm understanding of the chemical signals used by all participants in the overall interaction, including the target pest, the plant, and often the pest's natural predators. Strategies for insect pest control based on insect chemical–ecology have been reviewed recently.[35,36]

2.04.3 Terpenoids

Terpenoids play many different ecological roles, for example, as pheromones or defensive agents.[37] Arthropod terpenoids also function in hormonal signaling, for example, as juvenile and moulting hormones. As with other classes of arthropod metabolites, the extent to which arthropod-derived terpenoids have been obtained directly from dietary sources, produced from the modification of ingested metabolites, or synthesized *de novo* is in many cases unknown. Even for those examples where *de novo* terpenoid biosynthesis has been demonstrated, for example, through isotopic labeling experiments, knowledge of the biosynthetic pathways remains incomplete. The biosynthetic origin of terpenoids in particular is often uncertain because plants, serving as host or food source for many arthropod species, are prolific producers of various kinds of terpenoid natural products. It is widely accepted that insects as a group lack the capability to synthesize steroids and other triterpene-derived, or higher, terpenoid structures *de novo*. Specifically, the ability to combine two farnesyl moieties to produce squalene appears absent in all insect species.[38] The distinction between C_{10}-C_{25} terpenoids and C_{30} and greater terpenoids is thus particularly relevant for the arthropods (Chapters 1.15–1.18, 1.21).

Although the pace of discovery for novel arthropod-derived terpenoids continues to lag behind that for plants, several new structures have been described within the last 10–15 years, and the extent to which arthropod natural products may represent a largely untapped source of chemical diversity is illustrated by several recent additions to this ecologically important family of natural products. In addition to continuing progress toward the characterization of new structures, there are noteworthy examples among terpenoids where considerable progress has been made with respect to the biosynthetic, physiological, or ecological aspects of some previously well-known classes, for example, the iridoids. Progress in arthropod terpenoid research during the past 10–15 years is thus best characterized as a shift in focus to an in-depth analysis of the molecular biology and chemical ecology of some well-known structural classes.

2.04.3.1 Monoterpenes

Monoterpenoid-derived natural products have been described from a large number of arthropod species, and many examples of their use as defensive agents and pheromones have been reported. Monoterpenoids have furthermore attracted considerable attention in recent years because of the central role they play in

insect–plant interactions, specifically those involving sequestration of plant-derived defensive terpenoids by phytophagous insect species. In fact, monoterpenoids now appear as the one group of arthropod natural products for which knowledge of their biochemical, physiological, and ecological roles is most complete. To date, the two most well-studied examples are the iridoid monoterpenoids associated with *Chrysomelid* beetles and monoterpenes associated with bark beetles (family *Scolytidae*). The ecological and biochemical aspects of bark beetle monoterpenes have been recently reviewed and will not be dealt with in this chapter.[39]

The iridoids, a family of cyclopentanoid monoterpenes, are widespread in nature (**Figure 3**). Nepetalactone (**15**), the active component of catnip was the first example to be fully characterized,[40,41] and hundreds of structures have subsequently been described from a variety of plant and animal sources.[42] In addition to their roles as defensive agents and pheromones, iridoids participate in the biosynthesis of certain alkaloids.[43] A simple iridoid alkaloid is represented by the pyridine derivative actinidine (**16**).[44] An early example of an iridioid characterized from an arthropod source is anisomorphal (**17**), isolated from the defensive secretion of the southern walking stick, *Anisopmorpha buprestoides*.[45] Such defensive use of iridoids is widespread among insects, and many of the same defensive iridoids have been identified in several different insect groups. For example, dolichodial (**18**), a diastereomer of anisomorphal, has been found in ants,[46] beetles,[47] thrips,[48] sawflies,[49] and walking sticks.[50]

Iridoids have long been recognized as important defensive metabolites among larvae and adults of some chrysomelid beetle species,[51] and general biosynthetic pathways have been worked out for some predacious, as well as phytophagous species within this family. Most striking, however, are the emerging biochemical details of iridoid production and regulation within the context of the interaction between phytophagous species and their plant hosts. For example, studies of iridoid production in larvae of some phytophagous chrysomelina beetle species have recently revealed a complex system involving the incorporation of the iridoid precursor 8-hydroxygeraniol-8-*O*-B-D-glucoside from both a dietary source as well as from *de novo* biosynthesis (**Figure 4**). The common precursor is transported from either the gut, in the case of sequestration, or the fat body, where *de novo* production occurs, to a defensive glandular reservoir where biosynthesis is completed.[52] The enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase has been identified as the apparent regulator for the input of precursor from the two converging streams,[53] a mechanistic feature precedent in bark beetle isoprenoid biosynthesis,[54] as well as steroid biosynthesis in vertebrates.[55] In the case of chrysomelid beetles, 8-hydroxygeraniol, generated through the sugar cleavage of dietary 8-hydroxygeraniol-8-*O*-B-D-glucoside, was found to inhibit HMG-CoA reductase, shutting down *de novo* biosynthesis when dietary precursor is readily available. Interestingly, several related beetle species possess a similar defense system based on the conversion of both host–plant- and insect-derived *O*-glucoside salicilin into salicylaldehyde, a finding which seems to hold additional implications for the coevolution of plant and insect chemical defense.[56] The iridoids thus appear as one of relatively few families of arthropod defensive secondary metabolites for which a more detailed insight of their biochemical, physiological, and ecological relationships is emerging.

Although the pace of discovery of novel monoterpenoid structures from arthropod sources has seemingly decreased in recent years, new examples continue to emerge. An example is parectadial (**19**), a monoterpenoid dialdehyde characterized from the defensive secretion of the walking stick, *Parectatosoma mocquerysi*.[57] Expanding on previous analyses by Meinwald *et al.*,[45] who first reported the presence of defensive monoterpenes such as the iridoid anisomorphal (**17**) in these insects, parectadial provides an example of the close relationship between progress in arthropod natural products research and advances in analytical technology, especially with respect to instrument sensitivity. Using recently developed 1 mm microsample NMR probe technology, spectroscopic data leading to the characterization of parectadial was collected for extracts obtained from only 10 μl of the native secretion (**Figure 5**).

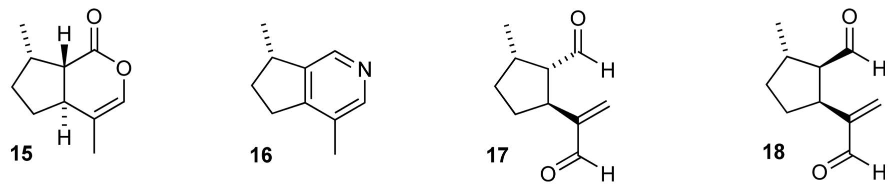

Figure 3 Nepetalactone (**15**), actinidine (**16**), anisomorphal (**17**), and dolichodial (**18**).

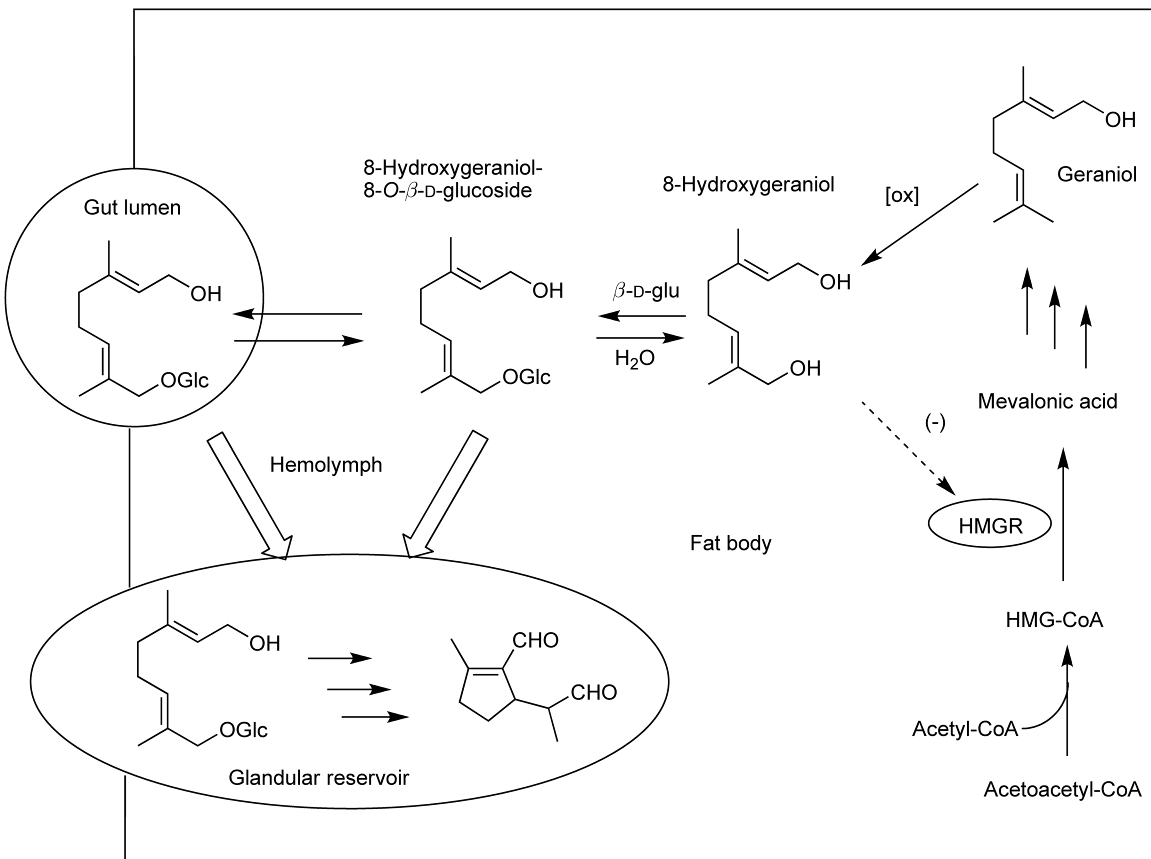

Figure 4 Iridoid biosynthesis in chrysomelids.

Figure 5 Parectadial (**19**) from the defensive secretion of the walking stick, *Parectatosoma mocquerysi.*

2.04.3.2 Sesquiterpenes and Diterpenes

One of the important examples for medicinally significant insect natural products is the defensive agent cantharidin (**20**), a simple terpene carboxylic diacid anhydride which is produced by several beetle species from the families *Meloidae* and *Oedemeridae.* Cantharidin has been the subject of considerable historical notoriety due to its purported aphrodisiac properties, with preparations of meloid beetles commonly known as 'Spanish Fly.' In reality, cantharidin is highly toxic to humans, and the aphrodisiac myth has resulted in several documented cases of cantharidin poisonings. Nonetheless, cantharidin has also found medicinal use as a vesicant.[58] Although the structure of cantharidin suggests it to be a simple monoterpene, labeling studies have demonstrated that it actually derives from the degradation of the sesquiterpene farnesol.[59]

First isolated in 1810, cantharidin has received considerable attention in recent years for the complex ecological interactions that it facilitates. For example, males of the beetle *Neopyrochroa flabellate* are attracted to

and will consume cantharidin. Females of this species, in turn, selectively mate with cantharidin-endowed males, during which time a controlled portion of cantharidin is transferred to the female and subsequently to the eggs. The compound is thus integral to courtship and mating in this species, serving first as a copulatory enticing agent, and subsequently as a nuptial gift, whose base value lies in its antipredatorial properties.[60,61] This use of defensive metabolites as a type of 'chemical currency' is widespread among arthropods and is one of the hallmarks in their chemical ecology.

Recent additions to the collection of arthropod sesqui- and diterpenoids include both variants of previously identified frameworks, as well as examples of more novel structures. Stenotarsol (**21**) is a putative defensive agent, which represents the first secondary metabolite to be isolated from the mycophagous family of beetles, *Endomychidae*.[62] The apparent sesquiterpene structure of stenotarsol comprises a carbon framework previously unknown among the terpenoids. Cantheronone (**22**), a diterpene enone, was isolated from the total body extracts of the soldier beetle *Cantharis livida* (**Figure 6**).[63]

A large number of secondary metabolites have been characterized from ants. Although ants seem to rely primarily on alkaloids for chemical defense (Section 2.04.6), recent investigations of ants from the genus *Crematogaster* have revealed novel defensive strategies involving both fatty acid-derived agents (Section 2.04.4) as well as terpenoids. Seven new furanocembranoids were characterized from two subspecies of the ant *Crematogaster brevispinosa*.[64,65] The compounds, which are stored in the Dufour gland, were shown to be toxic to the ant *Myrmica sabuleti* when applied topically. The authors speculate that the furan moiety may play a key role in the observed toxicity, as many plant species produce alkyl furans that may act as feeding deterrents for phytophagous insects.[66] Two representative structures (**23**) and (**24**) are shown in **Figure 7**.

Termites are also well-known as prolific producers of defensive natural products, including many of apparent terpenoid origin.[67,68] Termite soldiers of the subfamily *Nasutitermitinae* deploy complex mixtures of terpenoids from cephalic frontal glands. A variety of cembrene-derived diterpenes, including structures based on the kempane,[69,70] longipane,[71] rippertane,[72] secotrinervitane,[73] and trinervitane[69] skeletons have been previously characterized from this subfamily. The most recent additions include six new diterpenes based on the trinervitane skeleton characterized from soldiers of species *Nasutitermes guayanae* and *N. surinamensi*.[74] Representative structures (**7** and **25**) are shown in **Figure 8**.

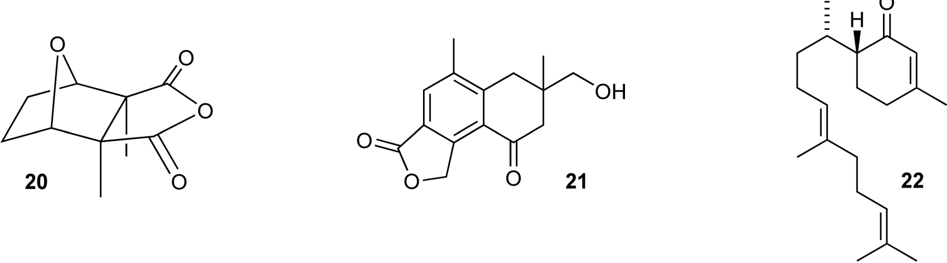

Figure 6 Cantharidin (**20**), stenotarsol (**21**), and cantheronone (**22**).

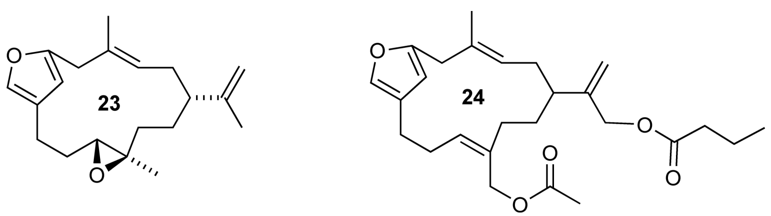

Figure 7 Furanocembranoids from *Crematogaster brevispinosa*.

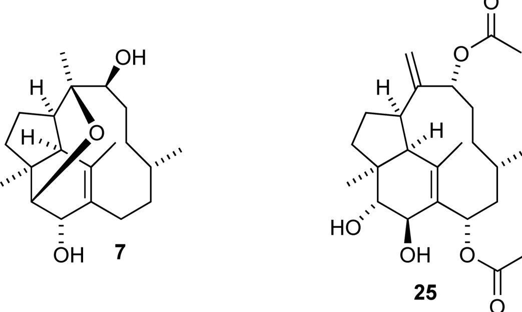

Figure 8 Trinervitane diterpenes from *Nasutitermes* termites.

Analyses of the cinnibar moth, *Tyria jacobaeae*, revealed an interesting example for sequestration of defensive terpenoids. As with many other moths found within the family *Actiidae*, larvae of this species are able to sequester pyrrolizidine alkaloids[75] (also see Section 2.04.6.4) from their plant host, in this case species from the genus *Senecio*. A population of *T. jacobaeae* was recently identified, however, which lives on plants from the genus *Petasites*, from which it instead sequesters terpenoids such as petasol (**26**) and related compounds (**Figure 9**).[76]

2.04.3.3 Steroids and Triterpenes

A variety of steroidal natural products have been isolated from insects, even though, as mentioned previously, insects are not able to carry out *de novo* steroid biosynthesis. The steroidal nucleus, as it occurs in insect primary and secondary metabolites thus must ultimately come from dietary or symbiotic microbial sources. For many phytophagus insects, C_{28} and C_{29} phytosterols are converted into cholesterol (C_{27}) through a series of dealkylation pathways, with cholesterol subsequently serving as the 'starting point' for further metabolic transformations, and resulting in a wide variety of steroid-based natural products.[77] In other cases, dietary phytosterols are sequestered and deployed unmodified, and as with other compound classes, the relative importance of dietary sequestration versus modification is not always clear.

The earliest documented example of the sequestration of any plant metabolite for defensive purposes among the insects involved cardiotonic steroidal furanones commonly known as cardenolides. Pioneering work by Rothschild and coworkers demonstrated that certain species of monarch butterflies (genus *Danaus*),[78] as well as the grasshopper *Poekilocerus bufonius*,[79] sequester injested cardenolides such as calotropin (**27**) (**Figure 10**) from their milkweed food source (family Asclepiadaceae). The sequestered toxins were shown to provide a potent deterrence to predation by various vertebrate and invertebrate predators. The interaction between monarch butterflies and milkweed comprises one of the most well-studied systems within the field of chemical ecology,[80] although many more examples of toxin sequestration among the Lepidoptera have subsequently been documented.[81] More recently several species of chrysomelid beetles have been shown to biosynthesize cardenolides from cholesterol.[82]

HO
O
26

Figure 9 Petasol.

Figure 10 Cardiotonic steroids isolated from insects. Calotropin (27) from *Danaus* butterflies and three representative firefly lucibufagins (3, 28, 29).

Fireflies of many species are a source of a family of defensive steroidal pyrones known collectively as lucibufagins (3, 28, 29). The lucibufagins, which are characterized by the presence of a pentadienolide moiety at C-17 of the steroid nucleus, are a subset of the cardiotonic steroids known as bufadienolides. Lucibufagins and bufadienolides are often grouped with the cardenolides as 'cardiotonic steroids' stemming from their apparently similar mode of action in inhibiting vertebrate Na^+, K^+ ATPase. Cardiotonic steroids were originally identified in plants, most prominently digitoxin from *Digitalis* spp.[83] Furthermore, a variety of bufadienolides has been identified from skin secretions of toads from the genus *Bufo*, as well as in the snake *Rhabdophis tigrinus*, which sequesters bufadienolides from toads for defensive purposes.[84] The great variety of bufadienolides identified from plant and animal sources has been reviewed.[85] In fireflies, lucibufagins act as potent deterrents toward a number of predators, including arthropod and vertebrate species. It is, however, the ecological flux of the lucibufagins through the ecosystem that is particularly noteworthy. Females of the genus *Photuris*, the so-called 'femmes fatales,' mimic the light patterns of *Photinus* females, luring in *Photinus* males who are immediately devoured. The *Photinus* males' lucibufagins are sequestered by the *Photuris* female for her own defense. Recently, diurnal fireflies of the species *Lucidota atra* were found to contain a complex array of lucibufagins, which are structurally distinct from those found in *Photinus* and *Photuris*.[86] When placed together in a laboratory setting, however, *Photuris* females were found to also devour *L. atra*, sequestering the *L. atra* lucibufagins, and leading to the speculation that species such as *L. atra* may have been driven to adopt diurnal behavior at least in part to avoid predation by the nocturnal predator *Photuris*.[86]

The details of lucibufagin biosynthesis, including the dietary source of the steroid skeleton for species such as *Photinus* and *L. atra* remain unknown. To date, over 20 unique lucibufagins have been characterized from three firefly genera, and it seems probable that lucibufagins are widespread among fireflies. Females of the genus *Photuris* have been demonstrated to carry out further metabolic transformation of lucibufagins sequestered from their *Photinus* prey.[87] The clear structural similarity of the lucibufagins to the bufadienolides found in toads suggests that fireflies could be a dietary source for bufadienolides in toads, but such a connection has not been demonstrated to date. The recent report of at least one amphibian-hunting reptilian predator, the snake *R. tigrinus*, that sequesters bufadienolides from its amphibian prey, raises the possibility that

bufadienolides may be transmitted through multiple trophic levels. Such movement of defensive natural products through several trophic levels is not without precedent. For example, the predaceous ladybird beetle *Coccinella 7-punctata* sequesters pyrrolizidine alkaloids from its prey, the aphid *Aphis jacobaeae*, which in turn has sequestered them from its plant food.[12]

The ecdysteroids provide an example of arthropod metabolites with multiple ecological functions at different hierarchic levels.[88] On the intraindividual level, ecdysteroids, such as 22-*O*-acetyl-20-hydroxyecdysone (**30**), are believed to function as moulting hormones for all arthropod species.[89] Some arthropod species, however, additionally employ some of the same ecdysteroids in a defensive capacity. In what is one of relatively few documented cases of chemical defense of marine arthropods, the sea spider, *Pycnogonum litorale*, discharges a mixture of ecdysteroids, the most abundant component being 22-*O*-acetyl-20-hydroxyecdysone (**30**), when threatened.[90] The same steroid was identified in the defensive glandular secretion of the chrysomelid leaf beetle, *Chrysolina carnifex*,[91] and is furthermore found in various plant species (**Figure 11**).

Water beetles employ a variety of corticosteroids for defense against vertebrate predators. Pioneering work by Schildknecht *et al.*[92] demonstrated the presence of large amounts of cortexone in the glandular defensive discharges of the water beetles, *Dytiscus marginalis*, as well as the presence of testosterone in the defensive secretion of two species of the genus *Ilybius*.[93] The steroids, some of which are known vertebrate hormones, function as endocrine disrupters in would-be vertebrate predators, often with profound effects. Subsequent work has resulted in the identification of a wide variety of steroidal defensive agents in water beetles.[5] A more recent addition to the family of water beetle defensive steroids is mirasorvone (**31**), isolated from the defensive secretion of the sunburst diving beetle, *Thermonectus marmoratus*. Mirasorvone comprises the first 18-oxygenated pregnane to be isolated from an insect source.[94] The defensive steroids of water beetles provide another example in which associated gut microorganisms probably play a role in biosynthetic transformations. For example, *Bacillus* strains isolated from the foregut of the water beetle *Agabus affinis* were demonstrated to effect the *in vitro* transformation of pregnenolone into a variety of oxidized derivatives.[95]

Triterpene saponins are a well-known class of plant terpenoids for which occurrence in the defensive secretions of insects has only recently been reported. Pentacyclic triterpenoids have been shown to occur widely as major components of the defensive secretions of several genera of New World chrysomelids, including *Platyphora*, *Desmogramma*, *Leptinotarsa*, and *Labidomera*. For example, the defensive secretion of the chrysomelid beetle *Platyphora ligata* was found to contain the two new oleanane triterpene saponins, ligatosides A (**11**) and B, in addition to chlorogenic acid and a mixture of phosphatidylcholines.[96] Subsequent analyses of the glandular defensive secretions from other chrysomelids have revealed four additional oleanane triterpene saponins, as well as quaternary ammonium derivatives of cadaverine and nicotinamide.[97] In the case of the leaf beetle *P. kollari*, the oleanane triterpene β-amyrin (**32**), present in the cuticular wax of the beetle's food plant, was shown to be the dietary source of the oleanane framework,[98] and it is probable that the oleanane triterpenoids found in other leaf beetles are elaborated from β-amyrin or other plant-derived precursors as well (**Figure 12**).

Figure 11 Defensive steroids isolated from arthropods.

Figure 12 Ligatoside A (**11**) and β-amyrin (**32**).

2.04.3.4 Tetraterpenes

There are few examples of tetraterpene natural products among the arthropods aside from carotenoids, which commonly play a role in arthropod pigmentation. An interesting example of a nonpigmentary tetraterpene is poduran (**33**), recently identified from whole body extracts of the springtail *Podura aquatica*.[99] The unusual structure of poduran, consisting of a tricyclic head region connected to a tail comprising the head-to-tail linking of five isopentyl moieties has not previously been observed from a natural source. The biosynthetic and ecological aspects of poduran remain to be elucidated (**Figure 13**).

2.04.4 Fatty Acid and Other Polyacetate Derivatives

Arthropods produce a wide range of metabolites derived from simple fatty acid or polyacetate precursors. The most well-studied fatty acid-derived natural products are no doubt the volatile sex pheromones prevalent among the Lepidoptera, although fatty acids and derivatives also function in additional roles, including defense, as well as in their recently discovered capacity as elicitors of plant defense mechanisms. Fatty acids furthermore serve as biosynthetic precursors to some important families of defensive alkaloids, such as the coccinellines and polyazamacrolides (PAMLs) (Section 2.04.6.1). As mentioned earlier, volatile pheromones are discussed in Chapter 4.04.

Examples of fatty acid-derived defensive compounds are widespread among arthropods. In the simplest cases, long-chain fatty acids themselves have been shown to serve as repellents that deter predators such as ants.[100] A classic example is the defensive secretion of the whip scorpion, *Mastigoproctus giganteus*, which contains an aqueous mixture of acetic and caprylic acids.[101] More recent examples include the mayolenes (**34**), isolated from the European cabbage butterfly, *Pieris rapae*.[102,103] The mayolenes, which comprise a series of *O*-acylated 11-hydroxylinolenic acid derivatives, were identified as the chief components of droplets present at the tips of glandular hairs running the length of *P. rapae* larvae. Further analyses demonstrated the plant-derived lignin pinoresinol (**35**) to comprise a minor, yet potent, component of the *P. rapae* defensive secretion. The biosynthetic details of the mayolenes remain unknown, although it is noteworthy that animals are believed incapable of making linolenic acid (**Figure 14**).[5]

Ants of the genus *Crematogaster* employ a defensive strategy in which a spatulate sting is used to apply a topical venom to the integument of attackers. As previously mentioned, in two subspecies of *C. brevispinosa* the venom contains a mixture of furanocembranoid diterpenes.[64] In other species of this genus however, the active

Figure 13 Poduran from the springtail *Podura aquatic*.

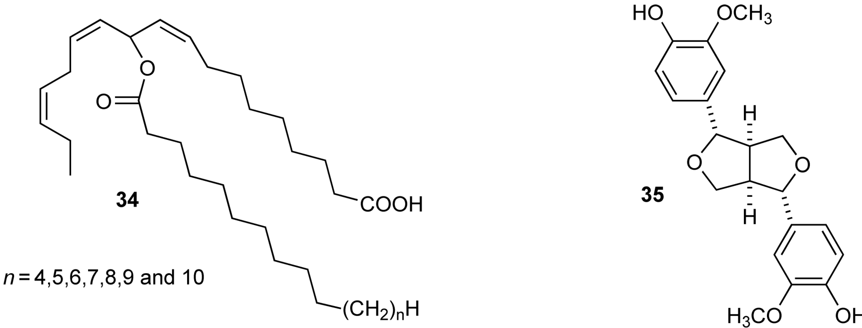

Figure 14 Mayolenes (**34**) and pinoresinol (**35**) characterized from the defensive secretion of the cabbage butterfly, *Pieris rapae.*

components of the venom seem to derive from fatty acid origin. In the European species *C. scutellaris*, a mixture of several latent long-chain aldehydes containing an (*E,E*)-cross-conjugated dienone moiety are stored in the Dufour gland as their primary acetate precursors. The individual mixture components vary by both chain length, as well as by the position and number of isolated double bonds. Upon discharge, the acetate precursors are mixed with an acetate esterase and an oxygen-dependent alcohol oxidase, leading to rapid formation of the active defensive aldehydes with the simultaneous liberation of acetic acid, which functions as an alarm pheromone. The chemically labile 4-oxo-2,5-dienals undergo further oxidation and rearrangement, resulting in a highly complex mixture containing additional structures, including the corresponding carboxylic acids, α-angelica lactones, and lactols of the apparent 5,6-dihydro analogues.[104–106] A representative acetate precursor (**36**), along with its liberated aldehyde (**37**) and subsequent forms (**38–40**) is shown in **Figure 15**.

Fourteen additional long-chained fatty acid derivatives were subsequently identified from the venom of three unidentified New Guinean *Crematogaster* species.[107] In addition to further homologues of the 4-oxo-2,5-dienals as

Figure 15 Chemical defense in the ant *Crematogaster scutellaris.*

41

42

43

44

45

Figure 16 Fatty acid derivatives from New Guinean and Brazilian *Crematogaster* species.

observed for *C. scutellaris*, the mixture contains six furan derivatives varying in chain length and double bond position, as well as two polyunsaturated β-hydroxy ketones (**41** and **42**, shown with a representative furan **43** in **Figure 16**). As with the European *Crematogaster* secretion, some of the identified mixture components, for example, the furan derivatives, are probably not native to the glandular contents, but rather result from further reaction following emission. Similar examples of post-secretory chemical reactions of defensive components, leading to increased chemical diversity, have been observed for the PAMLs, a group of macrocyclic polyamines from ladybird beetles[108–110] (Section 2.04.6.1.4(i)).

Additional analyses carried out on other *Crematogaster* species have revealed further chemical diversity among this group of ants. Analysis of the defensive secretion obtained from an unidentified *Cremotagaster* species collected in Brazil yielded a highly unsaturated long-chain acetate (**44**),[111] and most recently the Dufour gland from the African ant species, *C. nigriceps* was found to contain a mixture of mono- and di-unsaturated, long-chained 1-alkenyl-1,3,5-trihydroxycyclohexane derivatives (**45**).[112] Neither the ecological function nor the biosynthetic origin for these derivatives has been determined, although it seems likely that they also derive from fatty acids.

Sawfly larvae have been shown to possess a variety of chemical defenses, including many of apparent fatty acid origin. In many cases, the defensive agents were shown to have been sequestered from a plant host.[113] For two species from the genus *Hoplocampa*, the defensive ventral glandular secretion was determined to comprise of a complex mixture of aromatic compounds and terpenes, as well as several polyacetate-derived components.[114] The polyacetate-derived components were represented mainly by unsaturated aldehydes, such as (*E*,*Z*,*Z*)-2,4,7-decatrienal, but also included acids, esters, and alcohols.

The novel polyene peroxide, mycangimycin (**46**), was recently identified from a previously unknown actinomycetous bacterium associated with the Southern Pine Beetle, *Dendroctonus frontalis*.[115] Beetles of this species engage in a mutualistic relationship with the fungus *Entomocorticum* sp. A, which serves as a food source for the developing *D. frontalis* larvae. Mycangimycin was shown to selectively inhibit growth of the antagonistic fungus *Ophiostoma minus*, which threatens larval development by competing with *Entomocorticum* sp. A (**Figure 17**).[116]

46

Figure 17 Mycangimycin.

Macrocyclic lactones are well-known from a variety of insect groups, in which they have been shown to play a number of roles including use as defensive agents in termites,[117] aggregation pheromones in beetles[118] and ants,[119] and as a raw material for the construction of brood cells in bees.[120] The novel dimethylfuran lactone (**47**) was recently identified as an aggregation pheromone for the Chrysomelid beetles, *Galerucella calmariensis* and *Galerucella pusilla*. Males of these species emit **47** when feeding on host foliage.[121] The pupae of some *Coccinelid* beetles have recently been shown to employ a diverse collection of nitrogen-containing macrolides as potent feeding deterrents (Section 2.04.6.1). Further examples of defensive macrolides come from the African fungus-growing termite *Pseudacanthotermes spiniger*.[122] Soldiers of this species deploy a defensive salivary secretion containing macrocyclic lactones, for example (**48**), which were demonstrated to be toxic to ants. The secretion also contains polysaccharides, which seem to augment the defensive response through the formation of an immobilizing film on potential predators (**Figure 18**).

2.04.4.1 Fatty Acid–Amino Acid Conjugates and Related Elicitors

Fatty acid–amino acid conjugates and other surfactant-like fatty acid derivatives play important roles in insect–plant interactions. When feeding on their host plant, many herbivores, for example, moths' larvae or grasshoppers, release regurgitants that come into contact with injured plant tissue.[123] Fatty acid derivatives present in the feeding herbivore's regurgitant then trigger localized or systemic defense responses by the plant, culminating in the release of volatile organic compounds (VOCs) that may attract natural predators of the herbivore. Although fatty acid–amino acid conjugates had been known to occur in the gut of various species of Lepidoptera, the first fatty acid–amino acid conjugate shown to induce a defense response in plants, was volicitin (**49**) (**Figure 19**), a hydroxylated *N*-acyl glutamine isolated from the regurgitant of the beet army-worm, *Spodoptera exigua*.[124] *S. exigua* caterpillars feed on corn (*Zea mays*), which responds to the inflicted injury with the systemic emission of a characteristic blend of VOC's that attracts females of the parasitic wasp *Cotesia marginiventris*, a natural predator of *S. exigua*. Several of the emitted volatiles, including indole and several terpenoids, are produced only when *S. exigua* regurgitant comes into contact with damaged plant tissue, and are absent when the plants are simply mechanically wounded. Furthermore, the timing of emission of these volatile compounds corresponds to the period of day when the parasitic wasps forage for their hosts. The plants are thus able to distinguish between mechanical wounding and damage caused by insects. Similar tritrophic interactions have been described for species from various other plant families, including cotton (*Gossypium hirsutum*),[125] beans (*Phaseolus lunatus*),[126] and cabbage (*Brassica oleracea*).[127] In the case of the interaction of *S. exigua* and corn plants, volicitin has been shown to contribute significantly to the induction of VOC biosynthesis following insect damage. Since the initial identification of volicitin, several other *N*-acyl glutamines were isolated from the regurgitant of *S. exigua* and closely related species, including oxygenated conjugates such as the epoxide (**50**),[128] as well as phosphorylated analogues such as (**51**),[129] which comprise the first examples of naturally occurring phosphorylated long-chain fatty acids. Further analyses of oral secretions from other lepidopteran larvae led to the identification of four related compounds, including (**52**), representing 17-*O*-acylated derivatives of volicitin and analogues.[130] Most recently, a whole new class of ω-functionalized sulfated α-hydroxy fatty acid derivatives, the caeliferins, have been shown to act as elicitors.[131] Found in the regurgitant of the American bird grasshopper, *Schistocerca americana*, the caeliferins, including (9) and (53), have been shown to

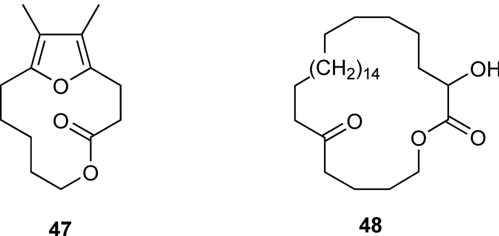

Figure 18 Macrocyclic lactones from *Galerucella* spp. and *Pseudacanthotermes spiniger*.

Figure 19 Fatty acid conjugates characterized from insect regurgitants.

induce the release of VOCs when administered to the leaves of damaged corn seedlings. To date, the predator of *S. americana* that is presumably recruited by the corn plants' VOC release has not been identified.

Generally, it is not clear what kind of adaptive benefit *S. americana* and other insects derive by producing fatty acid conjugates such as the caeliferins or volicitin. Recent work by Mori *et al.* suggests that *N*-acyl glutamines may play a role in nitrogen assimilation among Lepidopteran larvae.[132]

There is evidence suggesting that, in some species, fatty acid conjugates are produced by symbiotic microorganisms present in the insect gut. Multiple bacterial strains cultured from gut segments of lepidopteran larvae, including *S. exigua*, were demonstrated to synthesize various *N*-acylamino acids from the corresponding precursors, although hydroxylation at C-17 as necessary for the production of volicitin was not observed.[133] Although the precise role of gut bacteria in the biosynthesis of volicitin and related compounds remains to be determined, this study is noteworthy as it provides the first evidence for the involvement of gut bacteria in the biosynthesis of lepidopteran natural products. There are many examples of natural products deployed through regurgitation or excretion, and symbiotic gut bacteria may be involved in the production of these metabolites to a much greater extent than is currently appreciated. Interestingly, arthropod feces in several cases have been shown to contain defensive metabolites, some of which could be of bacterial origin as well.[134,135]

2.04.4.2 Bicyclic Acetals and Spiroacetals

Bicyclic acetals and spiroacetals based on cryptic ketodiols of polyacetate or, less frequently, polypropanoid origin are widespread in nature. They play important roles as pheromones in intraspecific and interspecific communication in many insect species, and thus their chemistry is discussed in greater detail in Chapter 4.04.

Exo-brevicomin, **54** Frontalin, **55** Multistriatin, **56**

57 **58** **59** **60**

Figure 20 Bicyclic acetals from insects.

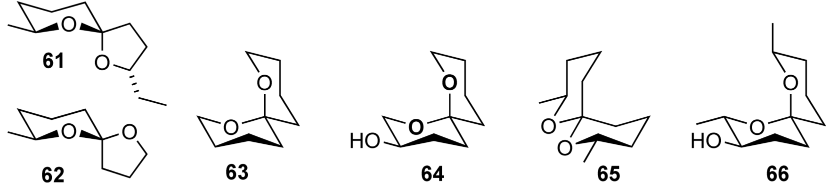

Figure 21 Spiroacetals from insects. Reproduced from W. Francke; W. Kitching, *Curr. Org. Chem.* **2001**, *5*, 233–251.

Examples include the alkylated 2,8-dioxabicyclo[3.2.1]octane (*exo*-brevicomin, **54**), 6,8-dioxabicyclo[3.2.1]-octanes (frontalin, **55**, and multistriatin, **56**), and the 2,9-dioxabicyclo[3.3.1]nonane (**57**).[136] Brevicomin, frontalin, and multistriatin are important components of the male or female pheromones of several bark beetle species. More recently, several hydroxylated 6,8-dioxabicyclo[3.2.1]octanes, including **58–60**, have been identified from the mountain pine beetle, *Dendroctonus ponderosae*.[137] Interestingly, frontalin (**55**) also represents an important component of the Asian elephant's male pheromone (**Figure 20**).[138]

Spiroacetals are equally widespread among insects. About 30 different spiroacetals (not including stereoisomers) have been identified from several families of beetles, ants, bees, wasps, bugs, and fruit flies. In some insect species, spiroacetals have been shown to act as chemical signals; however, in most cases the biological role of the identified spiroacetals remains unknown. Their chemistry has recently been reviewed by Francke and Kitching.[139] A few example structures, including hydroxylated derivatives are shown in **Figure 21**.

2.04.5 Polyketides

A variety of natural products of apparent polyketide origin, including both pheromonal and defensive agents, have been characterized from arthropods, although in most cases the exact biosynthetic origin of these metabolites is not clear. In a recent review, Pankewitz and Hilker[140] point out that even though there are many insect natural products that have structural aspects suggesting polyketide biosynthesis, no genes encoding polyketide synthases corresponding to polyketide production have ever been detected in the genome of an insect. There are several cases in which arthropod natural products originally suspected to be of polyketide origin were later shown to derive from other pathways. For example, the defensive alkaloids adaline (**103**) and coccinelline (**75**) (Section 2.04.6.1) were shown, through *in vitro* labeling experiments, to derive from a fatty acid, rather than polyketide, pathway.[141] In other cases, polyketide-derived structures isolated from insects have been shown to result from dietary sequestration or microbial symbionts. In such instances, the identification of identical or closely related polyketide natural products from unrelated organisms may provide a clue that microbial symbionts are the ultimate source. The coleopteran defensive metabolite pederin (**14**)

Figure 22 Pederin (**14**) and theopederin A (**67**).

(**Figure 22**) is exemplary. First characterized from beetles of the genus *Paederus*,[142] the subsequent characterization of closely related structures such as theopederin A (**67**), isolated from a marine sponge fostered the speculation of a common bacterial source for this family of polyketides.[143] Subsequent studies by Kellner[144] and Piel[28] identified a bacterium from the genus *Pseudomonas* as the likely source of pederin. Natural product production by symbiotic bacteria has been reviewed by Piel[29] (Chapter 2.14).

The defensive deployment of noxious benzoquinones and napthoquinones is widespread among arthropods, especially beetles[145] and millipedes.[146] A classic example is the explosive discharge of benzoquinones such as (**68**) and (**69**) observed among the bombardier beetles.[147] The defensive secretion of the tenebrenoid beetle *Argoporus alutacea* contains a mixture of 6-alkyl-1,4-napthoquinones, including (**70**) and (**71**).[148] Many quinone-derived compounds could reasonably be predicted to derive from polyketide biosynthetic pathways; however, in most cases their biosynthesis has not been thoroughly investigated.[140] A recent comparison of the biosynthesis of the polyketide-derived anthraquinone chrysophanol (**72**) in prokaryotic and eukaryotic organisms, including the leaf beetle *Galeruc tanaceti*, however, has revealed that chrysophanol biosynthesis in *G. tanaceti* follows the folding pattern typical of eukaryotes, and not that of prokaryotes, as would be expected if a bacterial endosymbiont were involved.[149] Earlier studies have shown that this beetle does not sequester chrysophanol from its plant host,[150] strongly suggesting that this polyketide is biosynthesized *de novo* by the insect (**Figure 23**).

Many arthropod pheromones are of putative polyketide origin, which are discussed in greater detail in Chapter 4.04. One noteworthy example is the novel 2,3-disubstituted β-lactone, vittatalactone (**73**), recently characterized as a suspected male-produced aggregation pheromone for the striped cucumber beetle, *Acalymma vittatum*.[151] Vittatalactone is the first β-lactone to be isolated from a beetle, and is only the third such structure to be characterized from an insect (**Figure 24**).

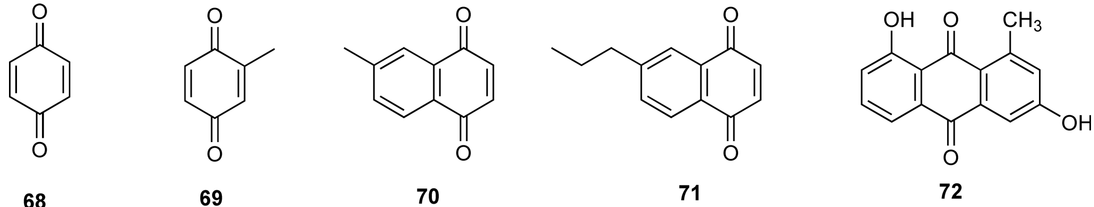

Figure 23 Defensive quinones from arthropods.

Figure 24 Vittatalactone (**73**) from the striped cucumber beetle, *Acalymma vittatum*.

2.04.6 Alkaloids and Amines

Alkaloids play an important role in the chemical defense of many arthropod species. They were among the first more complex organic compounds isolated from insects, and their discovery provided motivation for further extensive study of insect natural products chemistry. Over the past 40 years, a very large number of alkaloids have been identified from insects and other arthropods, from simple alkylamines, pyrrolidines, and piperidines, to highly complex steroidal and other oligocyclic structures. Many of these more complex structures are without precedent in nature, both with regard to their chemical architecture as well as with respect to their putative biosynthetic origin. Interestingly, some of the alkaloids identified from arthropods have also been found in the skin secretions of poison frogs and toads, which spawned the hypotheses that the remarkable diversity of bioactive lipophilic alkaloids identified from these vertebrates might actually be of arthropod origin. Recent work by Daly and coworkers strongly suggests that most (if not all) of the more than 800 known poison frog alkaloids are in fact of dietary origin, which has sparked a resurgence of interest in arthropod alkaloids[24,25,27] (Chapter 1.25).

In this chapter, we will focus primarily on new types of structures discovered in the past 10–15 years. Older literature on insect alkaloids has been reviewed comprehensively by Glisan King and Meinwald[11] and Daloze *et al.*[12] and is summarized briefly where relevant. For the purpose of organizing the stunning structural diversity of arthropod alkaloids, we will again rely primarily on structural features or putative biosynthetic origin, because we believe that such a representation provides a more systematic introduction to the chemistry of insect alkaloids than traditional approaches based on phylogenetic relationships. We will start with consideration of alkaloids of fatty acid or polyketide origin, followed by a section on the pumiliotoxins, polypropanoid-derived alkaloids originally identified in frogs, sections describing alkaloids of amino acid and terpenoid origin, and finally two brief sections on plant-derived pyrrolizidine and other miscellaneous alkaloids.

2.04.6.1 Fatty Acid or Polyacetate-Derived Alkaloids

Among insects, beetles and ants have been shown to be the most prolific producers of alkaloids, including many intriguing structures that are unique among known natural products. Most of these compounds are derived from simple straight chains of carbon atoms, and therefore appear to originate from fatty acid or, perhaps less likely, polyketide biosynthesis. Unfortunately, the biosynthesis of only very few insect-derived alkaloids has been investigated so far. The first examples for alkaloids of this general type were identified in ants and beetles almost simultaneously. In 1970, MacConnell *et al.*[152,153] described *trans*-2-methyl-6-alkylpiperidines such as **74** as the major components of fire ant venom (*Solenopsis saevissima*), while Tursch *et al.*[154] identified the tricyclic alkaloid *N*-oxide coccinelline, a 2-methylperhydro-9b-azaphenalene (**75**), from the blood of the European ladybird beetle, *C. 7-punctata*. These initial discoveries spawned great interest in the alkaloids of ants, ladybird beetles, and, by extension, many other types of arthropods (**Figure 25**).[10–12]

2.04.6.1.1 Acyclic amines

Among acyclic amines characterized from arthropods, relatively few appear to be fatty acid derived. The diaminooctadecene, harmonine (**76**), was originally identified from the ladybird beetle *Harmonia leis conformis*,[155] and has since been found in the blood of many other ladybird beetle species.[11,12,156] More recently, Wang *et al.*[157] identified signatipennine (**77**) from the New Guinean ladybird beetle *Epilachna signatipennis*. Biosynthetically, signatipennine appears to be derived from stearic acid and two molecules of the amino acid serine (**Figure 26**).

74

Coccinelline, 75

Figure 25 Early examples of insect-derived alkaloids.

Figure 26 Acyclic alkaloids from ladybird beetles.

A much greater number of acyclic polyamines have been described from spiders. These compounds feature short linear chains consisting of three to five carbons atoms, which are probably derived from amino acids. Spider-derived polyamines will therefore be discussed together with other amino acid-derived alkaloids and amines in Section 2.04.6.5.

2.04.6.1.2 Mono-, di-, and tricyclic alkaloids from ants

Following the initial discovery of alkyl-substituted piperidines in *Solenopsis* venom, a very large number of structurally related alkaloids were identified in the venom from ants of the subfamilies Myrmicinae and Pseudomyrmicinae,[10] all of which share a carbon skeleton based on a single unbranched chain. Analyses of ants from the genera *Solenopsis*, *Monomorium*, and *Diplorhoptrum* revealed 2,5-dialkylated pyrrolidines, 2,6-dialkylated piperidines, corresponding to 1-pyrrolines and 1-piperideines, as well as alkenyl-substituted variants. In some species, these monocyclic alkaloids are accompanied by corresponding 3,5-dialkylated pyrrolizidines and indolizidines.[10] Recent studies have shown that, at least for some ant species, the alkaloid content is caste specific.[158,159] For example, analyses of *Solenopsis maboya* workers revealed (*5E,9E*)- and (*5Z,9Z*)-3-butyl-5-methylindolizidine (**78 and 79**) as well as *trans*-2-methyl-6-nonylpiperidine (**74**), whereas analysis of queens of this species revealed one single alkaloid, *cis*-2-heptyl-6-methylpiperidine (**80**) (**Figure 27**).[160]

More recently, analyses of the venom of the African ant, *Myrmicaria eumenoides* revealed the side-chain oxygenated indolizidines myrmicarin 237A and 237B (**81 and 82**) as major components (**Figure 28**).[161] *Myrmicaria* venom consists of a mixture of alkaloids and monoterpene hydrocarbons, including limonene and terpinolene. Another oxygenated indolizidine was described by Jones *et al.*,[162] who identified the 8-hydroxyindolizidine **83** from the venom of the Southeast Asian species *Myrmicaria melanogaster*, where it occurs along with alkyl- and alkenyl-substituted pyrrolidines (**86**), indolizidines (**85**), and the 3-butyl-5*H*-octahydropyrrolo[1,2-*a*]azepine (3-butyl-lehmizidine, **84**), one of the few ant alkaloids featuring a seven-membered ring. Pyrrolo[1,2-*a*]azepanes had originally been found in skin secretions of the poison frog, *Dendrobates lehmanni*; however, the recent discovery of this type of alkaloid in *Myrmicaria* ants suggests that these dendrobatid pyrrolo[1,2-*a*]azepanes are probably of dietary origin, as has been suggested for several other groups of poison frog alkaloids, including many of the pyrrolidines, piperidines, pyrrolizidines, and indolizidines discussed in this section.[24,27]

Venom of ants of the African species *Myrmicaria opaciventris* revealed a group of tricyclic alkaloids, the pyrrolo[2,1,5-cd]indolizines myrmicarin 215A, 215B, and 217 (**87–89**) (**Figure 29**).[163] Similar to the indolizidines from *Myrmicaria*, these pyrrolo[2,1,5-cd]indolizines are based on an unbranched chain of 15-carbon atoms, and it has been shown that the *Myrmicaria* indolizidines and pyrrolo[2,1,5-cd]indolizines can be derived synthetically from side-chain oxygenated 2,6-disubstituted piperidines in a biomimetic fashion.[164] In addition to the bicyclic and

Figure 27 Piperidine and indolizidine alkaloids from *Solenopsis* ants.

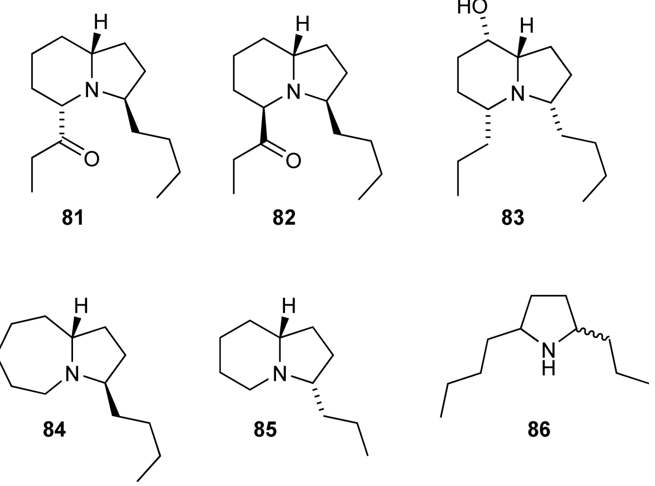

Figure 28 Alkaloids from *Myrmicaria* ants.

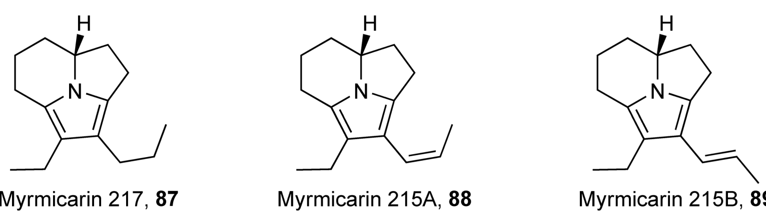

Figure 29 Tricyclic *Myrmicaria* alkaloids.

tricyclic derivatives 81–82 and 87–89, African *Myrmicaria* ants produce an array of structurally unusual oligocyclic alkaloids, which are derived from oligomerization of the 3,5-disubstituted indolizidines and pyrrolo[2,1,5-cd]-indolizines described here. These oligomeric compounds are described in Section 2.04.6.1.4.

The catalogue of ant alkaloids based on straight-chain carbon skeletons has been expanded further by Jones *et al.*,[165] who identified 4-methyl-6-propylquinolizidine (**90**) and the two decahydroquinolines **91** and **92** in the venom of the Brazilian ant species *Solenopsis picea*. In addition, the 19-carbon decahydroquinolines **93** and **94** were identified from virgin queens of *Solenopsis azteca* (**Figure 30**).[159] 2,5-Disubstituted decahydroquinolines, including 5-*epi*-stereoisomers of **93** and **94** have been identified in skin extracts of several frog species and one toad species, providing yet another example for a class of alkaloids that occur in both arthropods and amphibians.[166]

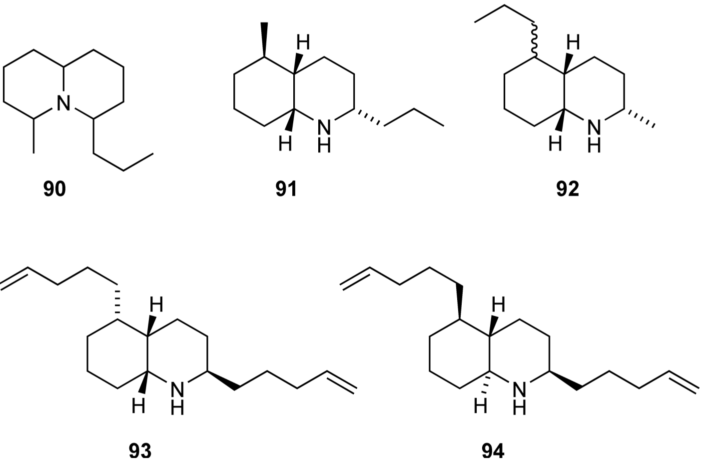

Figure 30 Alkaloids from *Solenopsis picea* and *S. azteca*.

In some cases, arthropod alkaloid biosynthesis relies on the combination of polyacetate-derived chains with building blocks from other pathways. Venom samples collected from several species of ants from the genus *Tetraponera* were shown to contain intriguing tricyclic alkaloids based on the decahydropyrrolo[1,2-*c*]-pyrrolo[1′,2′-*a*]pyrimidine and decahydropyrido[1,2-*c*]pyrrolo[1′,2′-*a*]pyrimidine systems, the tetraponerines (**10, 95–99**).[167,168] Their structural features suggested that the tetraponerines are derived from combination of a polyacetate chain and a separate five-carbon building block. This hypothesis was confirmed by Devijver *et al.*,[169] who showed through ^{14}C-labeling experiments that these compounds are produced from ornithine-derived putrescine and polyacetate chains (**Figure 31**).

Recently, oribatid mites (arthropods that, such as spiders, belong to the class Arachnida) were shown to also produce many of the bicyclic ant alkaloids described in this section including twenty-five 5,8-disubstituted or 5,6,8-trisubstituted indolizidines, one 1,4-disubstituted quinolizidine, as well as several pumiliotoxins, polypropanoid alkaloids described in detail in Section 2.04.6.2.[27]

2.04.6.1.3 Mono-, di-, and tricyclic alkaloids from ladybird beetles

Ladybird beetles produce a wide variety of polyacetate or fatty acid-derived alkaloids, many of which closely resemble the ant venom alkaloids described in the previous section. The original discovery of coccinelline (**75**) and precoccinelline (**100**) (**Figure 32**) in blood of the European ladybird beetle, *C. 7-punctata* had been motivated in part by these beetles' aposematic coloration and their tendency to reflex-bleed when molested. Many ladybird beetle species share these characteristics and correspondingly are chemically protected by alkaloids as well. The carbon skeletons of almost all of these alkaloids are based on simple unbranched chains. The structures, biology, and laboratory syntheses of ladybird beetle alkaloids have been reviewed previously by Daloze *et al.*[12] and by Glisan King and Meinwald.[11]

Recent additions to this growing family of compounds include 2-dehydrococcinelline (**101**) from the European ladybird beetle *Anatis ocellata*,[170] and (*S*)-3-hydroxypiperidin-2-one (**102**), which was isolated along with harmonine (**76**) from *Harmonia axyridis* and *Aiolocaria hexaspilota*.[156] Adalinine (**104**), a congener of the earlier-identified adaline (**103**), a 9-azabicyclo[3.3.1]nonane, was identified from the blood of *Adalia 2-punctata* and *Adalia 10-punctata*.[171] Because adaline appears to be derived from a single straight chain of 13 carbon atoms, it seems probable that adalinine is formed through retro-aldol condensation of a hypothetical 4-dehydroadaline. Another type of bicyclic alkaloid derived from a 13-carbon chain was recently identified from *Hyperaspis campestris*: the 2-pyrrolecarboxy-substituted 3-oxaquinolizidine, hyperaspine (**105**).[172] Most bi- and tricyclic ladybird alkaloids are derived from straight chains consisting of an odd number of carbons; however, there are also a few examples for bicyclic alkaloids based on even-numbered carbon chains. Supporting the notion that these alkaloids are polyacetate derived, alkaloids based on even-numbered chains usually terminate in a carboxylic acid or, as in the case of the open-chained harmonine (**76**), feature other terminal functionalization. Two examples for alkaloids based on 12-carbon chains are the piperidinic lactones calvine (**106**) and 2-epicalvine (**107**), isolated from *Calvia 14-guttata and C. 10-guttata*.[173] Using *in vitro* labeling experiments, the fatty acid origin of adaline (**103**) as well as for coccinelline (**75**) has recently been confirmed.[141]

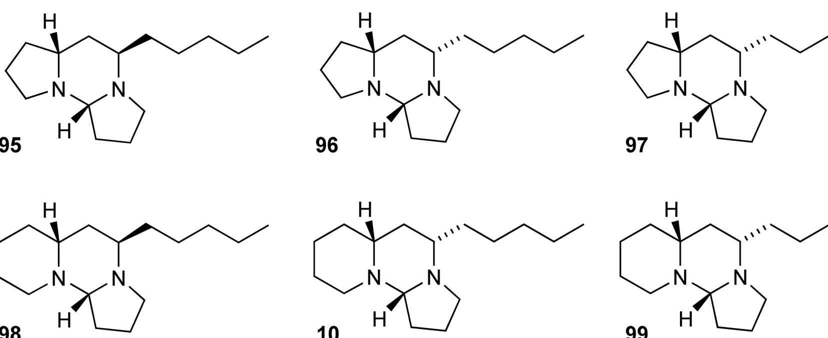

Figure 31 Alkaloids from *Tetraponera* ants.

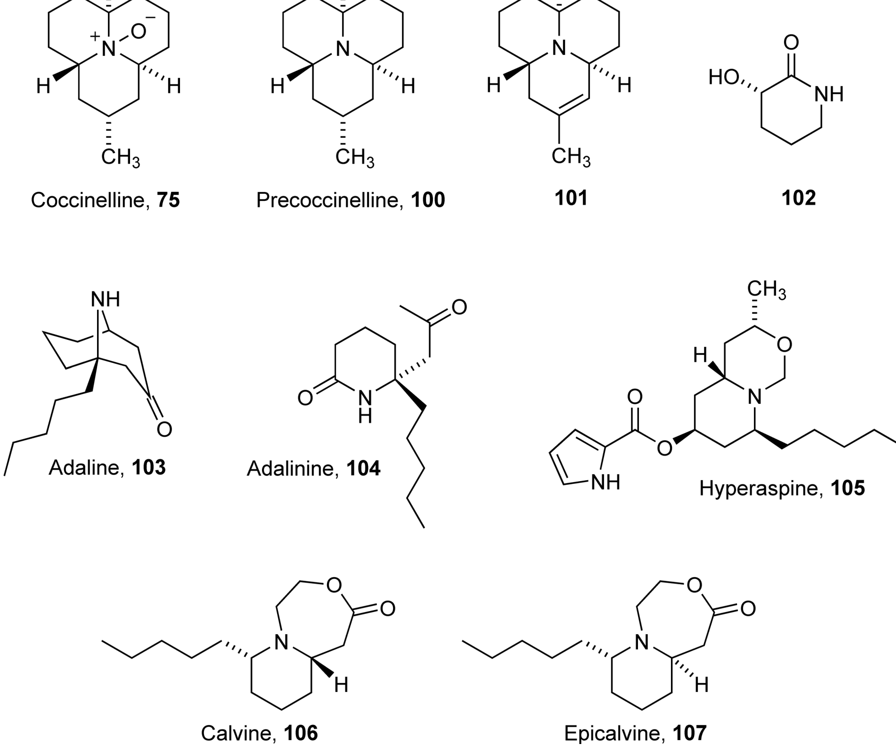

Figure 32 Ladybird beetles alkaloids.

2,6-Dialkylated piperidines and pyrrolidines (109–112, 114), some of which featuring oxygenated or aminated side chains, as well as another 9-azabicyclo[3.3.1]nonane, euphococcinine (108), have been identified from the blood of the Mexican bean beetle *Epilachna varivestis*.[174–176] In both *Epilachna varivestis* and *E. borealis*, the alkaloid patterns vary significantly between larval, pupal, and adult life stages. Adults of *E. varivestis* and the related squash beetle, *E. borealis*, further contain *N*-hydroxyethyl-pyrrolidines (115) and the corresponding pyrrolidino-oxazolidinines (116) (Figure 33).[177]

The pupae of these two species of *Epilachna* produce one of the most fascinating groups of insect natural products, the azamacrolides and PAMLs. *Epilachna* pupae are covered with a dense coating of glandular hairs, which secrete small droplets of a lipophilic fluid with deterrent properties. In the case of *E. varivestis*, the pupal defensive secretion consists of a mixture of five macrolides, the epilachnenes 117–121, which are derived from

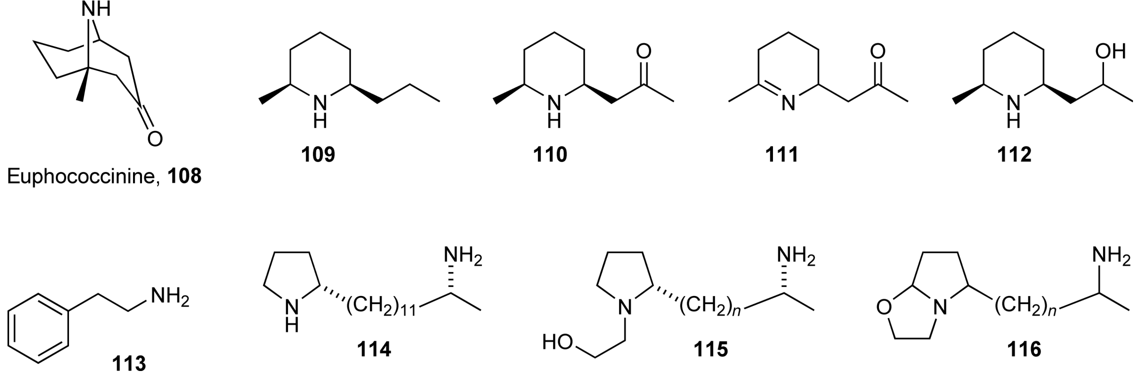

Figure 33 Alkaloids from *Epilachna* spp.

Epilachnene, **117** **118** **119**

120 **121**

Figure 34 Macrocyclic lactones from *Epilachna varivestis*.

lactonization of hydroxyethylamino-substituted fatty acids, forming 14- or 15-membered macrocycles. Biosynthetic studies showed that epilachnene (**117**) is derived from oleic acid and L-serine (**Figure 34**).[178]

In the case of *E. borealis*, the pupal secretion was shown to consist of a combinatorial library of several hundred macrocyclic polyamines, the PAMLs. These compounds are described in more detail in the following section.

2.04.6.1.4 Oligomeric derivatives

The amazing structural diversity of natural products results from the assembly of simple building blocks derived from primary metabolism, such as acetate, malonate, amino acid, or isoprene units, with concurrent or subsequent further chemical modification. As already pointed out, the ant and ladybird beetle alkaloids described in the preceding sections all appear to be more or less directly derived from polyacetate chains, with occasional inclusion of additional building blocks such as amino acid-derived ethanolamine or putrescine. In this section, we will discuss two groups of insect alkaloids whose biosynthesis involves an additional stage of assembly: the oligomerization of several polyacetate-derived building blocks producing entirely novel types of natural products. In the three known examples for insects taking advantage of this strategy, the macrocyclic PAMLs from ladybird beetles,[179] the oligocyclic *Myrmicaria* alkaloids,[163,180] and the dimeric coccinelline derivatives,[181–184] the oligomerization step affords new levels of structural diversity in different ways. In the case of the PAMLs, oligomerization results in formation of a very large number of structurally similar compounds of a very wide range of molecular weights, whereas in the case of the dimeric coccinelline derivatives and *Myrmicaria* alkaloids oligomerization produces a few distinct oligocyclic carbon skeletons.

The oligomerization of polyacetate chains generating the structurally more complex PAMLs, *Myrmicaria* alkaloids, and coccinelline dimers is somewhat reminiscent of the oligomerization of tryptamine-derived building blocks in plant alkaloid biosynthesis. For example, the great variety of cyclotryptamine alkaloids such as hodgkinsine or calycanthine are derived from oligomerization of pyrrolidino[2,3-b]indoline (or equivalent) building blocks.[185]

2.04.6.1.4(i) Polyazamacrolides Initial spectroscopic analyses of *E. borealis* pupal secretion produced confusing results.[110,179] Whereas NMR-spectroscopic analyses suggested that the secretion should represent a relatively straightforward mixture of vitamin E acetate and derivatives of simple (ω-1) (2-hydroxyethylamino)alkanoic acids, mass spectroscopic analyses revealed compounds of a very wide range of molecular weights from around MW 400 to above MW 2000. Moreover, the mass spectrometric analyses revealed the presence of several isomeric compounds for each molecular weight observed. Eventually, it was determined that *E. borealis* produces a combinatorial library of macrocyclic oligomers based on the three (ω-1)*R*-(2-hydroxyethylamino)alkanoic acids **124–126**.[110,179,186,187] The major components of this 'polyazamacrolide' (PAML) library are series of homologous trimers (**122**), tetramers (**8**), pentamers (**123**), and hexamers, along with smaller amounts of dimers, heptamers, and larger oligomers. The most abundant compounds in the series of trimers, tetramers, and pentamers are derived from incorporation of three, four, or five units of the most

abundant building block, 10-(2′-hydroxyethylamino)undecanoic acid (126). For the series of oligomers consisting of more than six units, statistical incorporation of the less abundant building blocks leads to increasingly broad distributions of oligomers. Additional structural diversity is introduced by the presence of amide isomers of the predominant ester linkages. For example, the dimer PAML454 (127) is accompanied by the two amide isomers 128 and 129. Interestingly, fresh *E. borealis* pupal secretion does not contain detectable amounts of monomeric azamacrolides and only very low concentrations of the open-chain building blocks 124–126 or open-chained oligomers. The mechanisms by which this very wide range of exclusively cyclic oligomers is biosynthesized have not yet been investigated; however, results from the analysis of the pupal secretion of a closely related ladybird beetle species, *Subcoccinella 24-punctata*, suggests that the oligomerization process can be precisely controlled (**Figure 35**).

Tetramers, **8**

Trimers, **122**

Pentamers, **123**

Building blocks, **124–126**, *n* = 5, 6, or 7

PAML454, **127**

128

129

PAML530, **130**

PAML532, **131**

PAML534, **132**

Building blocks, **133** and **134**

Figure 35 Polyazamacrolides ('PAMLs') from *E. borealis* and *Subcoccinella 24-punctata*.

The *Su. 24-punctata* pupal secretion consists almost entirely of the three PAMLs **130–132**, which represent the three possible dimers of the two (ω-3)*S*-(2-hydroxyethylamino)-substituted fatty acids **133 and 134**.[108,186] As in the case of *E. borealis,* these three PAMLs were accompanied by the corresponding amide isomers. Interestingly, only very small quantities of the four possible trimers of **133 and 134** were observed and higher oligomers were entirely absent. Similarly, epilachnene (**117**), the monomeric lactone of **134** which constitutes the major component of *E. varivestis* pupal secretion was absent from that of *Su. 24-punctata.* Therefore, it appears that oligomerization of the fatty acid-derived building blocks in *Epilachna* spp. and *Su. 24-punctata* is carefully regulated: whereas in *E. varivestis*, the building blocks **133 and 134** are used to produce exclusively monomeric azamacrolides, the very same building blocks are used by *Su. 24-punctata* to produce almost exclusively dimers (**130–132**). Finally, in the case of *E. borealis* the oligomerization of the similar building blocks **124–126** is geared toward production of a very wide range of ring sizes.

2.04.6.1.4(ii) Dimeric coccinelline derivatives The first examples for insect alkaloids derived from more than one polyacetate-derived chain were identified from ladybird beetles of the genera *Exochomus* and *Chilocorus.* Timmermans *et al.*[183] initially isolated the hexacyclic alkaloid exochomine (**135**) from *Exochomus quadripustulatus*, and the structurally related chilocorines A–D (**2, 136–138**) were subsequently identified from *Chilocorus cacti* and *Chilocorus renipustulatus.*[184,188–190] Exochomine and the chilocorines appear to be derived from condensation of two types of tricyclic subunits, both based on linear 13-carbon chains. For exochomine and chilocorines A, B, and D, one of the two 13-carbon chains forms a coccinelline-like 2-methylperhydro-9b-azaphenalene tricyclus, whereas chilocorine C features a 1-(hydroxymethyl)-7-methylperhydro-8b-azaacenaphthylene subunit instead. The occurrence of a branched carbon skeleton in chilocorine C seems surprising; however, given that – similar to all other subunits in these alkaloids – the branched subunit consists of 13 carbon atoms, it seems probable that the 1-(hydroxymethyl)-7-methylperhydro-8b-azaacenaphthylene system and the 2-methylperhydro-9b-azaphenalene subunits share a common polyacetate-derived (linear 13-carbon) precursor.[189] The second 13-carbon chain in exochomine and the chilocorines forms an octahydro-azaacenaphthylene tricyclus, which, in the case of chilocorine D, is modified to include a seven-membered ring.[190]

The two tricyclic subunits are linked through one or two carbon–carbon bonds, resulting in hexacyclic or heptacyclic structures, respectively. Although exochomine (**135**) and the chilocorines (**2, 136–138**) do not constitute exact dimers of any one specific building block, they are nonetheless frequently referred to as 'dimeric' ladybird beetle alkaloids because they are derived from two similarly functionalized 13-carbon subunits. However, almost exact dimers of coccinelline have been identified as well. *Psyllobora 22-punctata* was shown to contain the heptacyclic coccinelline-'dimer' psylloborine (**139**),[181] whereas *Halyzia 26-guttata* and *Vibidia 12-guttata* were found to contain the double-bond isomer isopsylloborine (**140**) (**Figure 36**).[182]

2.04.6.1.4(iii) **Oligocyclic *Myrmicaria alkaloids*** The great majority of alkaloids from ladybird beetles and ants described in the previous sections was originally detected using combinations of gas chromatography and mass spectrometry (GC–MS) and subsequently characterized further using NMR-spectroscopy and other techniques. This approach was very successful, because most ant and ladybird beetle alkaloids are fairly nonpolar and of relatively low molecular weight. An exception is represented by the above-discussed PAMLs from *Epilachna* and *Subcoccinella* ladybird beetles, which due to their high molecular weights are unsuitable for GC–MS and thus were detected and characterized primarily through HPLC–MS.[110,179] Another case is presented by ants from the genus *Myrmicaria.* Conventional GC–MS analyses of venom samples obtained from the African species *M. eumenoides* had revealed the side-chain oxygenated indolizidines myrmicarin 237A and 237B (**81 and 82**).[161] Additional GC–MS analyses of venom from another African *Myrmicaria* species revealed the pyrrolo[2,1,5-cd]indolizines myrmicarin 215A, 215B, and 217 (**87–89**).[163] However, the composition of *M. opaciventris* venom samples collected from different African locations was found to vary significantly: for some locations, GC–MS analysis reproducibly revealed large quantities of myrmicarin 215A, 251B, and 217, whereas venom from other locations contained only small amounts of these compounds, and venom samples from yet another group of locations was lacking volatile alkaloids entirely. Venom samples from *M. opaciventris* lacking the known myrmicarins were then analyzed directly by NMR spectroscopy, in order to determine whether these ants produce alkaloids whose higher molecular weight or

Precoccinelline, **100** Exochomine, **135** Chilocorine A, **136**

Chilocorine B, **2** Chilocorine C, **137** Chilocorine D, **138**

Psylloborine A, **139** Isopsylloborine A, **140**

Figure 36 'Dimeric' coccinelline derivatives from ladybird beetles.

polarity might have precluded their detection by GC–MS. These analyses led to the identification of the dimeric and trimeric alkaloids myrmicarin 430A (**142**), myrmicarin 663 (**5**), and myrmicarin 645 (**144**) featuring structurally unique tetracyclic and heptacyclic ring systems (**Figure 37**).[180,191]

Despite their structural complexity, it appears that these compounds derive from fairly straightforward dimerization or trimerization of a simple 15-carbon precursor. Although their biosynthesis has not been investigated yet, it seems probable that unsaturated derivatives of myrmicarin 237 (**81**) such as the hypothetical **141** could represent an immediate precursor, which through a series of intra- and/or intermolecular condensation steps could give rise to myrmicarins 215A/B, 430A, 645, and 663.[180] In fact, synthetic studies have shown that the 3,5-disubstituted indolizidine **145** is a suitable precursor for myrmicarin 217 (**87**).[192]

All of the oligomeric alkaloids identified from *Myrmicaria* to date are fairly labile. They constitute enamines and as such may undergo additional condensation and polymerization and are additionally prone to oxidative degradation. Therefore, chromatographic isolation of the oligomeric myrmicarins has been challenging, and only for myrmicarin 663, the most abundant and stable oligomers, has such isolation been successful. Myrmicarin 430A and 645 have never been isolated – they are among the first complex natural products identified that have never been obtained in pure form.[180,191] Their structural characterization and identification thus had to rely largely on NMR-spectroscopic analyses of unfractionated *Myrmicaria* venom samples, which typically represented fairly complex mixtures consisting of several monoterpene hydrocarbons, the oligomeric myrmicarins, as well as (in some cases) 'monomeric' indolizidines or pyrrolo[2,1,5-cd]indolizines. Experience gained during these analyses subsequently encouraged the use of NMR spectroscopy for other cases where the isolation of individual compounds could not be accomplished or was deemed impractical. A recent example is the identification of the bacillaenes, a family of highly unstable products of the NRPS/PKS gene cluster in *Bacillus subtilis*.[193]

Myrmicarin 237A, **81**
Myrmicarin 215A, **89**
141
?
Myrmicarin 430A, **142**
Myrmicarin 663, **5**
143
Myrmicarin 645, **144**
145
Myrmicarin 217, **87**

Figure 37 Dimeric and trimeric *Myrmicaria* alkaloids and putative precursors **141 and 155**. Structure **143** highlights the three 15-carbon chains in myrmicarin 663 (**5**).

2.04.6.2 Pumiliotoxins

The pumiliotoxins, a group of alkyl and hydroxyl-substituted indolizidine alkaloids, are distinguished from most other arthropod-derived alkaloids in that they feature highly branched carbon skeletons that suggest a polypropionate or polyketide origin. They were originally identified from the skin of poison frogs of the genus *Oophaga* (formerly *Dendrobates*) *pumilio*.[194–196] As pointed out in the preceding section, analyses of skin extracts of poison frogs have revealed a very large number of lipophilic alkaloids, all of which are now believed to originate from dietary arthropods.[24,194] The putative arthropod origin of the pumiliotoxins remained unclear until recently, when pumiliotoxins 307A and 323A (**6 and 146**) were detected in formicine ants of the genera *Brachymyrmex* and *Paratrechina*.[24] The further observation of ants of these species in the stomachs of sympatric *O. pumilio* strongly suggested that indeed *Brachymyrmex* and *Paratrechina* are one source of pumiliotoxins in frogs. Interestingly, ants are probably not the only source of pumiliotoxins. Subsequent studies of oribatid mites (arthropods that, like spiders, belong to the class Arachnida) by Saporito *et al.*[27] revealed a stunningly large variety of alkaloids, including 41 alkaloids previously identified in sympatric poison frogs. These include twenty-five 5,8-disubstituted or 5,6,8-trisubstituted indolizidines, one 1,4-disubstituted quinolizidine, three pumiliotoxins, including PTX 307A (**146**), which had also been found in formicine ants, and the homopumiliotoxin hPTX 251R (**147**) (**Figure 38**). Therefore, it appears that ants and mites essentially produce the very

Figure 38 Pumiliotoxins identified from frogs (6, 146, and 147), ants (6 and 146), and mites (146 and 147).

same families of alkaloids, even though, phylogenetically, these two groups of arthropods are only distantly related. This raises the question whether these so-called arthropod alkaloids are perhaps of microbial origin, as has been shown to be the case for many complex natural products from other phyla, especially marine animals.[29] One example for an arthropod natural product for which a bacterial origin has recently been demonstrated is the polyketide pederin, originally isolated from beetles of the genus *Paederus* (see Section 2.04.5).[28]

2.04.6.3 Terpenoid and Steroidal Alkaloids

Only a few examples for alkaloids of terpenoid origin have been identified from arthropods. The ant *Monomorium fieldi* was shown to contain (2*E*) and (2*Z*)-farnesylamine (**148**), which, surprisingly, constitutes the first identification of farnesylamine or any of its derivatives in nature.[197] The defensive agent actinidine (**16**) has been found in a number of insect species and probably derives from an iridoid precursor, although the details of actinidine biosynthesis in insects have not yet been fully clarified.[44]

Among the most significant recent discoveries in arthropod alkaloid chemistry ranks the identification of batrachotoxin derivatives (**151–152**) from arthropods.[25] Batrachotoxins were originally discovered in the skin extracts of South American poison-dart frogs (the name derived from Greek 'batrachos' for 'frog').[198] Surprisingly, batrachotoxins were subsequently also found in the skin and feathers of New Guinean bird species of the genera *Pitohui* and *Ifrita*.[21,199,200] Batrachotoxins are among the most toxic natural products known; for example, they are more than 250 times more toxic than strychnine.[25] The occurrence of batrachotoxins in different vertebrates as distantly related as birds and frogs strongly suggested that they are of dietary origin, which is now believed to be the case for most, if not all alkaloids identified from frogs.[24,25,27,195,196,201] However, the batrachotoxins' origin remained a mystery, until analyses of New Guinean beetles of the little-studied genus *Choresine* (family Melyridae) revealed large quantities of batrachotoxin, batrachotoxin A, and crotonic acid esters of batrachotoxin A (**Figure 39**). Since beetles from the family Melyridae are widely distributed, they constitute a possible source for the batrachotoxins found in both birds and frogs. This hypothesis is supported further by analyses of the stomach content of *Pitohui* birds, which revealed *Choresine* beetles as part of these birds' diet.[25]

Occasionally, plant alkaloids of terpenoid origin are sequestered by insects. For example, larvae of many species of sawfly are chemically protected by toxic metabolites they sequester from their host plants. This includes iridoid glycosides (see Section 2.04.3) and a group of steroidal alkaloids produced by plants of the genus *Veratrum*, such as zygadenine (**149**). In some cases, sawfly larvae have been shown to further metabolize sequestered *Veratrum* alkaloids; for example, zygadenine is derived from hydrolysis of the ester functionalities in sequestered protoveratrine A (**150**).[202,203]

Zygadenine, **149**

Protoveratrine A, **150**

R = H (batrachotoxin A)
R = *cis*-crotonoyl
R = *trans*-crotonoyl

R′ = H (batrachotoxin)
R′ = CH_3 (homobatrachotoxin)

Figure 39 Alkaloids of terpenoid or steroidal origin.

2.04.6.4 Plant-Derived Pyrrolizidine Alkaloids

Aposematic moths and butterflies (Lepidoptera) are often associated with poisonous plants, and these insects' preponderance for sequestering toxins from their plant hosts is well established.[204–206] Among plant-derived secondary metabolites, pyrrolizidine alkaloids play particularly important roles in the chemical ecology of many butterfly and moth species. Plant-derived pyrrolizidines such as monocrotaline (**153**) are used as chemical defense agents and, in some cases, are metabolized into volatile derivatives that serve as pheromones, such as danaidone (**154**) and hydroxydanaidal (**155**).[75,207,208] In the case of the moth *Cosmosoma myrodora*, males acquire intermedine (**156**) and lycopsamine (**157**) from plants such as *Eupatorium capillifolium*. The alkaloids are incorporated systemically, which has been demonstrated to confer protection against predators. The sequestered pyrrolizidine alkaloids are further deposited onto a dense mass of fine cuticular filaments, which are discharged onto the female during courtship, embellishing her with alkaloid as a result. In addition, the males transfer some of the acquired pyrrolizidine alkaloids to the female by seminal infusion. The female moths probably derive some chemical protection from this nuptial gift, and further bestow some of the received alkaloids on the eggs.[209] More recently, the role of pyrrolizidine alkaloids in the ecology and behavior of the butterfly *Euploea mulciber* was studied.[210] Pyrrolizidine alkaloids have also been shown to get sequentially sequestered through several trophic levels. The predaceous ladybird beetle *C. 7-punctata* sequesters pyrrolizidine alkaloids from its prey, the aphid *A. jacobaeae*, which in turn has sequestered them from its plant food (**Figure 40**).[12]

2.04.6.5 Amino-Acid Derived Alkaloids and Amines

Various groups of arthropods have been shown to produce alkaloids that incorporate amino acid-derived building blocks. Examples include the incorporation of serine-derived ethanolamine or ornithine-derived putrescine into polyacetate-derived structures in ladybird beetles, as described in the preceding sections.

Most prominently, many spider species utilize amino acid-derived acylpolyamines as part of their venom. Similar to other venomous animals, spiders produce complex mixtures of biologically active compounds in their venoms, which in addition to acylpolyamines often contain free amino acids, peptides, enzymatically active proteins, or nucleosides (see Section 2.04.7).[211–213] More than 100 different acylpolyamines have been

Figure 40 Pyrrolizidine alkaloids and derivatives.

identified from spider venoms to-date, primarily from the families Agelenidae and Araneidae. Their structures are based on α,ω-polyazaalkane chains that usually consist of one to nine aminopropyl, aminobutyl, or aminopentyl units, all of which are probably derived from amino acid metabolism.[211] One of the termini in these polyamine chains is usually capped with an aromatic acyl group derived from tyrosine or tryptophan, for example, indolylacetyl, 4-hydroxybenzoyl, 2,5-dihydroxybenzoyl, or 2,5-dihydroxyphenylacetyl groups. A number of representative examples are shown in **Figure 41**. Occasionally, amino acids such as β-alanine or lysine are incorporated in the polyamine chains, as is the case in structures **1 and 160**, respectively. Additional amino acids sometimes cap the tail end of the polyazaalkane chain (most frequently L-arginine) or form a small peptide insert between the aromatic acyl group and the polyazaalkane chain. Some spider species produce complex mixtures of polyamines, including *N*-methylated or *N*-hydroxylated derivatives as well as compounds featuring dimethylammonium bridges. Two recent additions to this family of natural products, the acylpolyamines **161 and 162** identified in *Coelotes pastoralis* venom feature a terminal 2,5-dihydroxybenzoyl group.[214] A nonacylated polyamine – bis(agmatin)oxalamide – has been identified from the primitive hunting spider, *Plectreurys tristis*.[215] Interestingly, bioassays with this compound did not reveal any insecticidal activity, in sharp contrast to the strong toxicity of almost all acylated polyamines.

Acylpolyamines are potent neurotoxins that have been shown to inhibit calcium-ion channels and selectively block glutamate, NMDA (*N*-methyl-D-aspartate), or AMPA (α-amino-3-hydroxy-5-methylisoxazol-4-propionate) receptors, which is probably the basis of their insecticidal properties.[216] As a result, acylpolyamines have received considerable attention as tools for studying neurotransmitter function and may be of therapeutic use.[212,217,218]

Acylpolyamines have also been identified from the poison gland secretion of a solitary wasp, *Philanthus triangulum*.[219] As in the case of spiders, this wasp's venom represents a complex mixture which contains several acylpolyamines as major components. The 'philanthotoxins,' for example, PhTX433 (**163**) inhibit glutamate as well as nicotinic acid receptors (**Figure 42**).

Similar to many other types of arthropod natural products, acylpolyamines frequently occur as complex mixtures, of which only small quantities are available, and thus their chemical characterization and identification is often challenging. In the case of the acylpolyamines, additional difficulties arise from strong signal overlap in the NMR spectra, which results from the repetitive nature of the polyazaalkane chains. Therefore, NMR-spectroscopic analyses often did not permit complete structural assignments, and acylpolyamine identification had to rely to a considerable extent on mass spectrometric analyses. Using MS–MS techniques, the positions of the nitrogen atoms in the polyamine chains could often be determined based on observing the characteristic fragments that arise from breakage of the carbon–nitrogen bonds.[220,221]

In addition to the acylpolyamines, many simple alkaloids and biogenic amines of amino acid origin have been identified from arthropods, including L-hypaphorine (**164**), a simple tryptophan derivative identified along with choline (**166**) and signatipennine (**77**) from the New Guinean ladybird beetle *E. signatipennis*,[157] as well as N_{α}-quinaldyl-L-arginine (**165**), identified from adult *Su. 24-punctata*.[222] Choline and acetylcholine were also identified from caterpillars of the family Saturniidae, along with the corresponding tertiary amines. When disturbed, the saturniid caterpillar *Attacus atlas* defends itself by spraying a highly irritating secretion, which in addition to choline derivatives (**166–169**) contains a variety of other amino acid-derived compounds (**170–176**) (**Figure 43**).[223]

Figure 41 Typical acylpolyamines identified in spider venom, including Agel464 (**158**) from the funnel web spider *Agelenopsis aperta*, NPTX10 (**1**) and joramine (**160**) from *Nephila clavata* and pseudoargiopinine 1 (**159**) from *Argiope lobata*. **161** and **162** were identified recently from *Coelotes pastoralis* venom.

Figure 42 PhTX433, an acylpolyamine from the wasp, *Philanthus triangulum*.

2.04.6.6 Miscellaneous *N*-Heterocyclic Compounds

Simple pyridine, pyrazine, and pyrrolo derivatives have been described from many different types of arthropods. Nicotine (**177**), nicotinamide, nicotinic acid, 2-pyrrolidone, *N*-methylpyrrolididone (**178**), 8-hydroxyquinoline (**179**), and 2-isobutyl-3-methoxypyrazine (**180**) have been detected in the defensive secretion and blood of larvae of the moth *Lymantria dispar*.[224] The nicotine isomer anabasine (**181**) and its congener anabaseine (**182**) have been identified as venom components in several ant species,[225–227] whereas

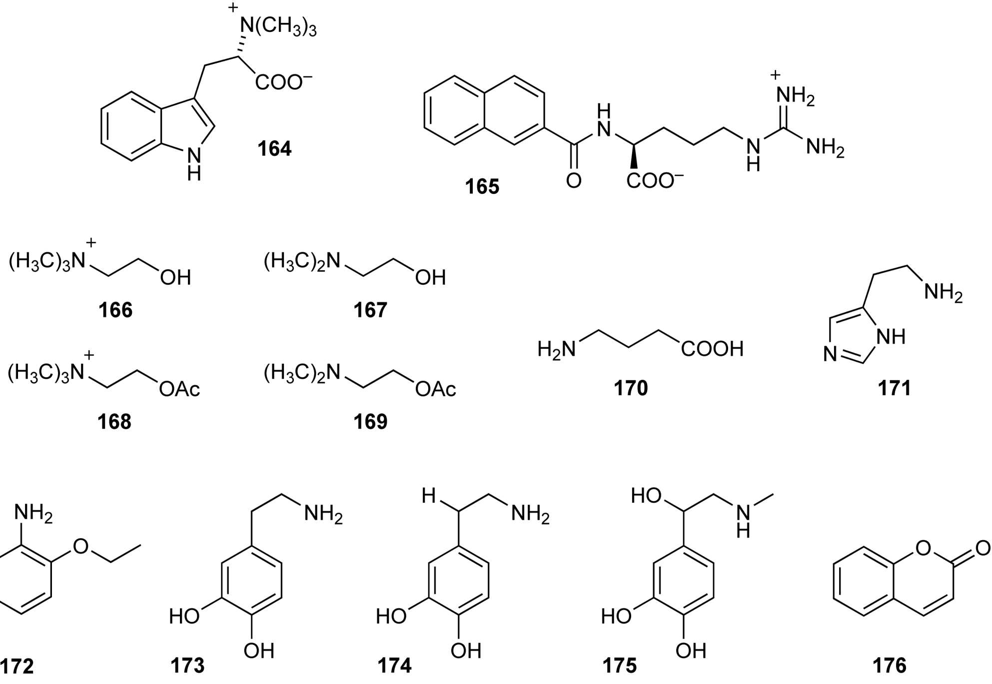

Figure 43 Simple amino acid derivatives from arthropods.

cephalic extracts of ants from the genus *Anochetus* were shown to contain 3-methyl-4-phenylpyrrole (**183**) and 2,5-dimethyl-3-isoamylpyrazine (**184**).[228] Methoxy- and alkyl-substituted pyrazines play an important role as volatile deterrents for many insect species. These compounds are the principal components of the 'warning odor' of many aposematic insects.[229–231]

The pyrido[2,3-*b*]pyrazines, **185–187**, have been identified in the defensive secretion of the giant springtail, *Tetrodontophora bielanensis*.[232] The secretion appears to confuse and strongly disorient potential predators. An unusual quinoline-derived betaine (**188**) with defensive properties was recently identified from the blood of *Photuris versicolor* fireflies. In addition, these fireflies' chemical defense relies on sequestered cardiotonic steroids, the lucibufagins (see Section 2.04.3.3) (**Figure 44**).[233]

2.04.7 Nucleoside Derivatives

Spider venoms are generally a complex mixture of hydrophilic small-molecule neurotransmitters, acylpolyamines (discussed in Section 2.04.6.5), peptides, and proteins.[211,234] More recent studies resulted in the identification of an entirely new family of natural products, the sulfated nucleosides. Although nucleosides are ubiquitous in nature, the occurrence of sulfated derivatives had not previously been reported. The first example from this family, HF-7 (**189**) was identified in venom from *Hololena curta* as part of an assay-guided fractionation, and its structure was subsequently confirmed through total synthesis.[235] Later work demonstrated that sulfated nucleosides were more widespread, but had probably escaped detection by classical analytical protocols. Using a novel approach based on direct NMR spectroscopic analysis of native, unpurified venom samples, six additional sulfated nucleosides, including **4 and 190**, were identified in venom collected from the hobo spider *Tegenaria agrestis*.[236] Most recently, sulfated nucleosides were shown to be present in four additional species, including the brown recluse, *Loxosceles recluse*.[214] The brown recluse is among the few spider species whose bite can inflict severe tissue damage in humans. The two sulfated guanosine derivatives **191 and 192** comprise the major small-molecule components of *Loxosceles* venom; however, their biological role is not known (**Figure 45**).[214]

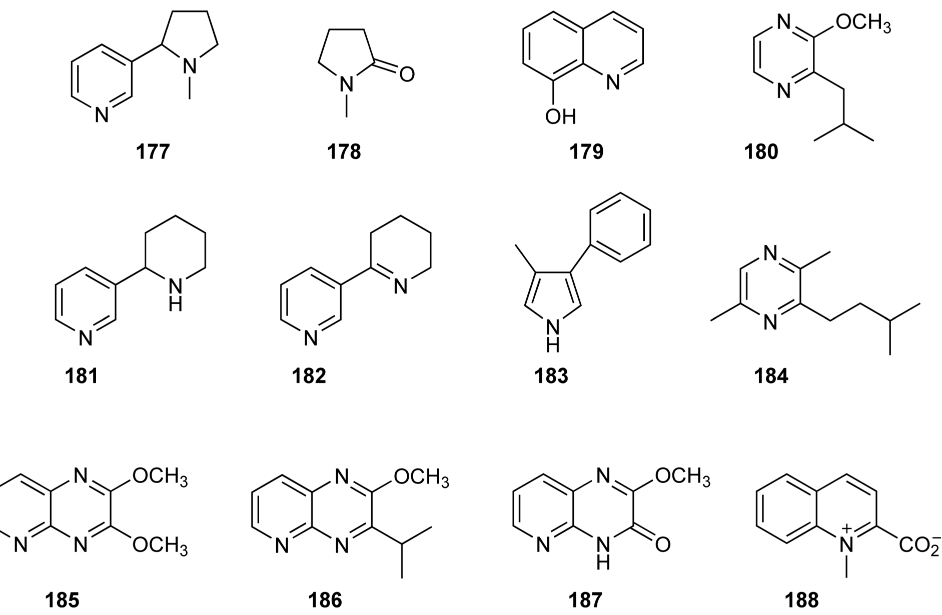

Figure 44 Miscellaneous *N*-heterocyclic compounds from arthropods.

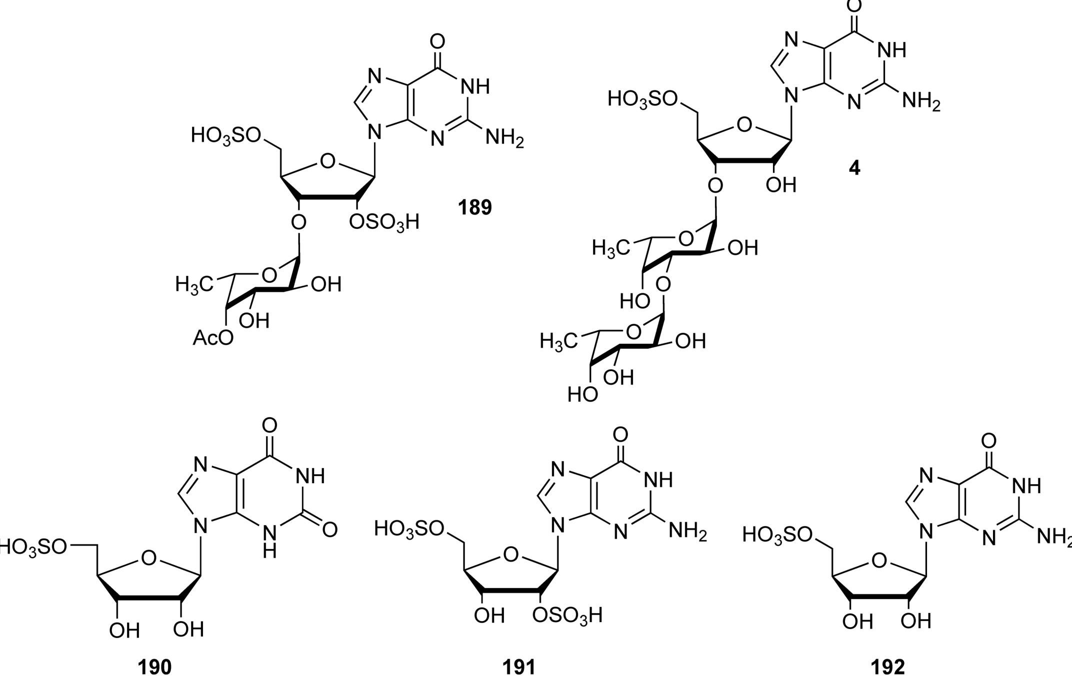

Figure 45 Sulfated nucleosides from spiders.

2.04.8 Miscellaneous Compounds

Beetles from several genera of the family Chrysomelidae (leaf beetles) have been shown to contain large quantities of the 3-nitropropanoyl-isoxazolinone glucosides **193–195** in their defensive secretions.[237,238] Upon emission of the defensive secretion, these glycoside esters come into contact with an esterase resulting in release of free 3-nitropropanoic acid, a well-known vertebrate toxin (**Figure 46**).[9]

Figure 46 3-Nitropropanoic acid esters (**193–195**) from chrysomelid beetles and pinoresinol (**35**) from caterpillars of the cabbage butterfly, *Pieris rapae*.

Pinoresinol (**35**) was found as a minor constituent of the defensive secretion produced by caterpillars of the cabbage butterfly, *P. rapae*. Pinoresinol enhances the deterrent properties of the secretion's major components, the fatty acid-derived mayolenes (see Section 2.04.4). Interestingly, pinoresinol could not be detected in the caterpillar's cabbage diet, suggesting that perhaps other plant lignans serve as precursors.[102]

2.04.9 Outlook

The scope and pace of discovery in arthropod natural product chemistry has markedly increased over the past two decades, no doubt largely due to advances in analytical technology. Because of the specific challenges inherent to arthropod natural products research, such as frequently limited samples sizes and complex ecological interactions (discussed in detail in Section 2.04.2), arthropod natural product chemistry was bound to benefit considerably from the recent advances in NMR spectroscopy and mass spectrometry. Particularly relevant in this regard are improvements of sensitivity (especially for NMR spectroscopy) and data analysis techniques (for both NMR and MS). For example, bombykol (**196**) (**Figure 47**), the silkworm pheromone, was identified in 1959 after extensive chemical and spectroscopic studies based on milligram amounts of pheromone painstakingly obtained from more than 500 000 female moths.[239] Today, similar pheromones are routinely characterized based on samples of 1 μg or less.[240] Of course, factors such as the structural complexity and novelty of the discovered compounds must also be considered when making such comparisons.

In another example, identification of the first bufadienolides from an invertebrate source required extraction of 28 000 *Photinus pyralis* fireflies (Section 2.04.3.3). The crude extract was fractionated into five pure fractions, representing amounts from over 1 g down to 70 mg, which were then characterized by a combination of chemical and spectroscopic methods. Key structural information was afforded by NMR spectroscopic analyses using a modest 250 MHz NMR spectrometer, which nonetheless permitted detailed structural characterization of the firefly-derived bufadienolide **197**.[241] Just over 25 years later, a similar analysis was carried out using a partially purified extract obtained from only 50 fireflies of the rare species, *L. atra*. A 600 MHz spectrometer equipped with a microcoil probe was used,[242] allowing the characterization of 13 new bufadienolides present in amounts ranging from only 20–75 μg, which corresponds to a decrease in sample requirement of roughly four orders of magnitude.[243] Furthermore, isolation of individual components through lengthy chromatographic separation schemes was no longer required for full identification of the new structures, greatly simplifying the analytical process. In these analyses, the use of two-dimensional NMR spectroscopy for the characterization of mixtures played a central role. Several subsequent studies, including the identification sulfated nucleosides from spider venom,[214,236] and the

Bombykol, **196**

197

Myrmicarin 430A, **142**

Bacillaene, **198**

17

14

Figure 47 Representive natural products of arthropod (**17,142,196,197**) and microbial (**14,198**) origin.

identification of the highly unstable polyketide bacillaene (**198**) from *B. subtilis*,[193] have shown that using state-of-the-art NMR spectroscopy even minor components of complex small-molecule mixtures can be characterized. Such NMR-spectroscopic analyses of complex mixtures may not always permit complete structural assignments; however, additional results from mass spectroscopic analyses frequently allow proposing complete structures. Reducing the need for chromatographic separations can not only accelerate compound discovery, but also offers distinct advantages for the discovery of chemically unstable metabolites. It seems probable that the pervasive use of chromatography in natural products chemistry has skewed our knowledge of metabolite production by arthropods (and other organisms), because sensitive compounds often do not survive extended exposure to solvents or chromatography media. In fact, the original motivation to explore the utility of high-resolution two-dimensional NMR spectroscopy for the characterization of small-molecule mixtures arose because alkaloids present in the poison gland secretion of *Myrmicaria* ants were found to be highly unstable for chromatographic isolation.[180,191] Myrmicarin 430A (**142**), the most unstable of the *Myrmicaria* alkaloids identified so far, thus represents one of the first members of a growing class of natural products that have never been isolated in pure form.

Improvements in NMR spectroscopic sensitivity will also benefit studies aimed at elucidation of the chemical–ecological interactions of arthropods. For example, NMR spectroscopic analysis of insect metabolites traditionally necessitated the pooling of material collected from multiple individuals, effectively eliminating NMR spectroscopy as a technique that could be used for the detailed analysis of metabolite dynamics in ecological studies. Recent studies by Dossey *et al.*,[50] however, indicated that an increase in NMR spectroscopic sensitivity enabled analyses of metabolite mixtures within individual walking sticks, *Anisomorpha buprestoides*, permitting complete characterization of the iridioid anisomorphal (**17**) from a single insect specimen. Using the *A. buprestoides* secretion as a model system, subsequent work by Zhang *et al.*[244] demonstrated the application of total correlation spectroscopy (TOCSY) for this type of analysis. The ability to analyze individual specimens by both NMR spectroscopy and MS holds considerable promise for future ecological studies. Advanced processing of spectroscopic data, taking advantage of statistical tools originally developed for metabolomics studies,[245–248] will further enhance the utility of NMR spectroscopy and MS for ecological and other biological studies. However, to-date there have been few reports on the application of metabolomics techniques to natural products research.[249]

In contrast to plant, microbial, and marine sources, there are relatively few examples of arthropod metabolites serving as pharmaceutical agents or lead structures. Whether this reflects an intrinsic unsuitability of arthropod metabolites for medicinal applications, or is more a manifestation of other factors remains to be determined. It seems probable that arthropod microbial defenses represent an untapped source for antibiotics, largely passed over in favor of high-yielding sources, such as plants and fungi. However, these traditional sources for lead structures may now be nearing exhaustion, while there is a dire need for new approaches to control drug-resistant pathogens.[250] Recent and future advances in analytical techniques, screening methodology, and not least chemical synthesis may motivate increased inclusion of arthropod natural products in broad pharmaceutical screening programs.

Arthropods represent a vast, yet largely untapped source of chemical diversity, and the potential for further discovery is great. For example, there are ~45 000 described species of mites, which, by some estimates, may only account for ~5% of the actual number. Of the described species, only a handful has been chemically scrutinized.[27] With the advent of evermore sensitive methods of analysis and biological assays capable of screening smaller amounts of material, even well-studied species of arthropods may warrant a second look. For example, a reexamination by Daloze *et al.*[12] of the alkaloid content of ladybird beetle species originally studied 25 years earlier revealed additional alkaloids that had escaped detection in the initial analyses. The discovery that arthropods including ants, beetles, and mites are the source of the enormous diversity of alkaloids identified from poison frogs,[24,25,27,166] along with the finding that pederin (**14**) from *Paederus* beetles is actually of microbial origin,[28] suggest that the sequestration of small molecules plays a much greater role in nature than previously acknowledged.[29] As pointed out earlier, it seems probable that many more of the arthropod natural products described in this chapter will turn out to be of microbial origin.

As more detailed knowledge of the biological functions of arthropod natural products emerges, an increasingly complex web of chemical interactions is revealed. For many systems, the veneer of simplicity has been peeled away to reveal complex ecological relationships and metabolic pathways, or the unexpected participation of additional trophic levels. In a recent commentary, Jerrold Meinwald characterized our current understanding of arthropod chemical signaling by suggesting that "Future opportunities far outweigh present accomplishments, which are best viewed as a promising start."[251] We could not agree more.

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Biographical Sketches

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Dr. Schroeder's research aims to develop NMR spectroscopy-based approaches that complement or enhance traditional methodology by enabling detailed characterization of small-molecule metabolites in complex biological samples, with regard to both chemical structure and biological function. His current work focuses on a comprehensive structural and functional annotation of the metabolome of the model organism *Caenorhabditis elegans*.

2.05 Terrestrial Microorganisms – Filamentous Bacteria

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2.05.1 Introduction

The recognition of the potential of soil-dwelling organisms in general and actinomycetes in particular as prolific producers of antibiotics is undoubtedly attributed to the work of Selman Waksman at Rutgers University. Although the first examples of antibiotics were produced by other organisms such as fungi (e.g., penicillin G, 1) or bacteria from the genus *Bacillus* (thyrothricin, a mixture of compounds), it soon became evident to Waksman and coworkers that the filamentous bacteria called actinomycetes presented an amazing source for the discovery of antibiotic substances, superior to that shown by any other group of living organisms. Waksman's group discovered more than 20 antibiotics between 1940 and 1970, four of which found practical application.[1,2] Their example was soon followed by other groups around the world, giving rise to what has been called the

'Golden Age' of antibiotics. Most of the antibiotics currently used in the clinic are semisynthetic derivatives of the natural molecules discovered in the 'Golden Age' period. In spite of this success, the use of microbial sources for screening for drugs has declined in the last two decades. It is generally perceived that natural product discovery efforts now provide a diminishing return on investment and, as such, have been mostly eliminated in favor of synthetic compounds and combinatorial libraries. Although the latter approaches have provided a large number of compounds for screening, success rate continues to suffer due to inherently poor diversity. Exclusive use of these libraries has not increased drug discovery. In fact, many people argue that many of the early combinatorial libraries have been a distraction. Clearly, chemical diversity offered by natural products and their drug-like properties are actively sought to complement existing compound libraries.[3]

The first section of this chapter describes some of the older antibiotics, together with other bioactive molecules that have positively impacted therapeutics and that were discovered in the first four decades since the start of the early efforts. Newer compounds, discovered mostly in the last two decades, are described in the second section, focusing on compounds that have progressed to the late stages of clinical development and/or those whose mode of action has been elucidated. This section also includes some of the key older compounds whose mode of action has been recently studied.

Penicillin G (**1**)

2.05.2 Historical Bacterial Metabolites

Actinomycin was the first antibiotic isolated by Waksman and Woodruff[4] in 1940. Although not useful in antibiotic therapy, it remains a commonly used chemotherapeutic agent for the treatment of a variety of cancers. Actinomycin was first isolated from *Streptomyces antibioticus* and is produced by many *Streptomyces* strains.[5] The actinomycins are a family of bicyclic chromopeptide lactones sharing the chromophoric phenoxazinone dicarboxylic acid to which are attached two pentapeptide lactones of nonribosomal origin.[6] Actinomycin D (2) acts as a transcription inhibitor, binding to DNA duplexes at the transcription initiation complex and preventing RNA polymerase elongation.[7] Conformation of the molecule is extremely well adapted for intercalation into a right-handed DNA helix, favoring the establishment of hydrophobic interactions that stabilize the DNA/antibiotic complex.[8–11] More than 41 actinomycins have been reported, mainly with variations in the peptide portion of the structures. The class is represented by actinomycin D (2), which is perhaps the most deeply studied member of the series.

Actinomycin D (**2**)

Streptomycin (**3**)

Surely the most relevant antibiotic discovered at Rutgers University in those early days was streptomycin (3), the first member of the aminoglycoside class of antibiotics. The discovery of this broad-spectrum antibiotic in 1944 was highly relevant due to its application as the first antibiotic useful to treat tuberculosis.[12] This discovery was followed by successive discoveries of many aminoglycosides such as neomycin, kanamycin, and gentamycin for the treatment of Gram-negative infections. These compounds were subject to large chemistry efforts leading to the development of many semisynthetic derivatives. All aminoglycosides inhibit protein synthesis by binding to a specific site of 30S subunit of the bacterial ribosome and perturbing the elongation of the nascent peptide chain.[13] The use of aminoglycosides in the clinics has declined due to the emergence of resistance and suboptimal toxicological profiles, in particular nephrotoxicity.

Erythromycin (**4**)

Azithromycin (**5**)

Telithromycin (**6**)

The 1950s brought other families of new antibiotics that were highly useful for clinical practice. Erythromycin (4) was the first macrolide introduced in the clinic[14] and was originally used as an alternative therapy to β-lactams to treat infections with Gram-positive pathogens such as *Staphylococcus* spp. and *Streptococcus* spp. This compound, produced by a strain of *Saccharopolyspora erythraea*, consists of a 14-membered macrocyclic lactone. Macrolide antibiotics inhibit bacterial protein synthesis by binding specifically to the 23S rRNA in the 50S ribosomal subunit, blocking translation by stimulating the dissociation of peptidyl-tRNA during translocation of the nascent chain.[2] The rapid development of resistance (by methylation of the target 23S rRNA and efflux mechanisms), poor pharmacokinetic properties, and undesirable adverse effects profile of erythromycin prompted the development of the semisynthetic analogues azithromycin (5) in 1992 and clarithromycin in 1994. These compounds have improved pharmacokinetic properties and provided expanded spectrum, including Gram-negative bacilli.[15] More recently, ketolides represent a new generation of macrolide antibiotics that were developed to overcome macrolide-resistant respiratory pathogens. These molecules bind to another region of the 23S rRNA and show an improved activity, even against macrolide-resistant strains.[16] Telithromycin (6) is one of the most advanced ketolides and was first approved in Europe in 2001 and, subsequently, in the United States in 2004 for oral use to treat Gram-positive infections including multidrug-resistant *Staphylococcus* strains as well as *Haemophilus influenzae* and *Moraxella catharrhalis*.[17]

Vancomycin (**7**)

Teicoplanin (**8**)

Oritavancin (**9**)

Telavancin (**10**)

Dalbavancin (**11**)

Vancomycin (7) is a glycopeptide antibiotic that was isolated by E.C. Kornfeld (working at Eli Lilly) in 1955 from *Amycolatopsis orientalis* (*Nocardia orientalis*) and remains a drug of last resort to treat serious Gram-positive infections resistant to other antibiotics, including methicillin-resistant *Staphylococcus aureus* (MRSA). This antibiotic blocks the synthesis of the peptidoglycan (PG) component of bacterial cell wall by recognizing the dipeptide motif D-Ala-D-Ala of PG and inhibiting both the transglycosylation and the transpeptidation steps.[2] Widespread use of vancomycin has led to significant to resistance in *Enterococcus faecium* (vancomycin-resistant *Enterococcus faecium* (VRE)) and some resistance to *S. aureus* (vancomycin-resistant *S. aureus* (VRSA)). Teicoplanin (8) is a related glycopeptide discovered in 1984 from *Actinoplanes teichomyceticus*, displaying the same mode of action. Structurally, it differs from vancomycin by virtue of the substitution of two aromatic amino

acids and one additional sugar residue, leading to an alternation in physical properties and differential activities. This antibiotic is widely used in Europe but not approved in the United States.[18,19] The glycopeptide class of compounds continue to serve as starting points for the synthesis of derivatives with improved antibiotic properties, as exemplified by oritavancin (**9**), telavancin (**10**), and dalbavancin (**11**). Oritavancin and telavancin are vancomycin analogues presenting a second mode of action, that of inhibiting the transglycosylation step, which ensures the efficacy of oritavancin and telavancin against VRE.[19–21] Dalbavancin, a semisynthetic analogue of a teicoplanin congener A40926, produced by a strain of *Nonomurea* sp., exhibited high *in vivo* efficacy against Gram-positive bacteria, especially MRSA and vancomycin-sensitive *Enterococcus faecium* (VSE), but not against vanA *Enterococcus* strains. On the contrary, oritavancin showed activity against vanA vanB enterococci.[19–22]

Besides producing antibiotics useful to fight against bacterial infections, actinomycetes were soon recognized as producing antagonistic substances against other types of organisms, such as fungi. Many *Streptomyces* spp. are known with produce polyene macrolides, which are potent antifungal agents. Amphotericin B (**12**) is one of the best known polyene macrolides produced by *Streptomyces nodosus*. It has been the drug of choice for the treatment of serious systemic fungal infections for more than 45 years until the recent introduction of caspofungin, a cyclic lipopeptide produced by a fungus. Amphotericin B produces pores in fungal cell membranes by high–affinity binding to sterols in general and ergosterol in particular. This binding disrupts the integrity of the membrane, causing the loss of ions, small molecules, and oxidative enzymes, resulting in cell death. The interaction of amphotericin B with phospholipids to form nonbilayer lipid phases has also been associated to this pore formation.[23] Amphotericin B shows a broad-spectrum antifungal activity and has been useful for the treatment of candidiasis, cryptococcosis, histoplasmosis, coccidiodomycosis, and aspergillosis. Resistance to amphotericin B is uncommon.[24] Unfortunately, it also exhibits a high degree of nephrotoxicity, which limits its utility. This toxicity has been somewhat ameliorated by liposomal formulation.

Amphotericin B (**12**)

Nikkomycin Z (**13**)

Nikkomycin X (**14**)

Nikkomycins are nucleoside amide antibiotics produced by *Streptomyces tendae* Tü 901 and are known to show antifungal, anti-insecticidal, and acaricidal activities. They are competitive inhibitors of chitin synthase. Nikkomycins are produced as a complex mixture, with nikkomycin Z (**13**) and nikkomycin X (**14**) representing the major components.[25]

Avermectins (**15, 16**) are 16-membered lactones produced by *Streptomyces avermitilis*, and they exhibit potent anthelmintic activity without significant antibacterial or antifungal activity.[26] These compounds are produced as a mixture of eight components, with avermectin B_{1a} (**15**) being the most abundant. Avermectins interact with glutamate-gated chloride channels with high affinity and specificity, leading to hyperpolarization of neuronal membranes, which cause paralysis and death in nematodes and arthropods.[27] Catalytic reduction of avermectin led to the synthesis of the 22,23-dihydro derivative ivermectin (**17**), which was the first avermectin approved as an antiparasitic agent.[28] Ivermectin has been the treatment of choice for nematode and arthropod infections in cattle and pets. Although ivermectin was discovered and first developed for the treatment of parasitic infections in animals, it was subsequently approved for the treatment of river blindness in humans and has been tremendously successful in eliminating this debilitating disease in parts of Africa.[29–31]

Avermectin B_{1a}, R = $CH(CH_3)CH_2CH_3$, $\Delta^{23,24}$ (**15**)
Avermectin B_{1b}, R = $CH(CH_3)_2$, $\Delta^{23,24}$ (**16**)
Ivermectin R = $CH(CH_3)CH_2CH_3$ + $CH(CH_3)_2$, (**17**)

Doxorubicin (**18**) produced by *Streptomyces peucetius* is a member of the anthracycline family of antibiotics that have been successful treating various forms of cancer.[32–34] These compounds have been used in the clinic, but the mode of action is still uncertain. The mechanisms proposed include DNA intercalation and free radical formation leading to DNA damage, DNA binding, alkylation and cross-linking, as well as initiation of DNA damage via inhibition of topoisomerase II.[35] Bleomycins, represented by bleomycins A2 and B2 and **19 and 20**, are glycopeptides produced by *Streptomyces verticillus* and are approved as anticancer drugs.[36] They present a unique mode of action mediated by dioxygen activation and sequence selective degradation of DNA. Mitomycin C (**21**) is a member of the mitomycin family of natural products produced by *Streptomyces caespitous*, which also have potent antitumor activity.[32,37]

Doxorubicin (**18**)

Mitomycin C (**21**)

Bleomycin A_2, R = $CH_2CH_2CH_2S^+(CH_3)_2$ (**19**)
Bleomycin B_2, R = $CH_2CH_2CH_2CH_2NHC(NH)NH_2$, (**20**)

2.05.3 Newer Compounds or Compounds with Recently Described Mechanism of Action

This section covers compounds that were reported relatively recently. The mechanism of action of these compounds is reasonably understood. The compounds in this section have been sorted by their biological activity and their potential utility in the clinic. The class of compounds include immunosuppressants (rapamycin and FK506), antitumor agents (geldanamycin to echinomycin), anti-inflammatory agents (efomycin), antiobesity agents (lipstatin), and antibiotics (streptogramins to platensimycin).

2.05.3.1 Rapamycin and FK506

Rapamycin (sirolimus) (**22**), a 29-membered lactone, is a potent immunosuppressant produced by *Streptomyces hygroscopicus*, and it also possesses antifungal and antineoplastic activity. This macrocyclic lactone inhibits the lymphocyte T activation and proliferation that occurs in response to antigenic and cytokine stimulation.[38] Rapamycin binds to immunophilin, the cytosolic FK506-binding protein (FKBP)-12, a peptidyl-prolyl *cis–trans* isomerase, to form the FKBP-12 complex. This complex binds a key regulatory kinase, mammalian target of rapamycin (mTOR), suppressing cytokine-driven T-cell proliferation and suppressing the progression from G1 to the S phase of the cell cycle.

FK506 (tacrolimus) (**23**) is a 23-membered macrocyclic lactone isolated from *Streptomyces tsukubaensis* and is structurally related to rapamycin. It displays antifungal and immunosuppressive activities. It is marketed as an immunosuppressant that can be used in transplant therapy and several autoimmune disorders. Rapamycin and FK506 share the same common cellular receptor FKBP, but they present a different mechanism of action. Similar to cyclosporine A, FK506 suppresses T-cell activation at the level of lymphokine production and prevents the expression of the interleukin 2 receptor (IL-2R).[38,39] In contrast, rapamycin has little effect on lymphokine production but markedly suppresses IL-2 T-cell proliferation.

Rapamycin (**22**)

FK506 (**23**)

2.05.3.2 Geldanamycin

Geldanamycin (**24**) is an antitumor agent produced by a strain of *S. hygroscopicus* var. *geldanus*. It belongs to the group of benzoquinone class of compounds known as ansamycins (e.g., herbimycin A, macbecin, and ansatrienins). Geldanamycin inhibits the ATPase activity of chaperone heat shock protein 90 (Hsp90), which maintains conformation, stability, and function of oncogenic protein kinases involved in signal transduction cascades leading to proliferation and progression of cell cycle and apoptosis. The Hsp90 is overexpressed in several tumor types, making it a potential target for antitumor therapy, and therefore makes geldanamycin an attractive candidate for drug development. A derivative of geldanamycin (17-allylamine,17-demethoxigeldanamycin) showed good activity and selectivity in preclinical mice models, having progressed into human Phase I trials with promising results.[40]

Geldanamycin (**24**)

2.05.3.3 Indolocarbazols

Rebeccamycin (**25**) is an indolocarbazol alkaloid produced by the actinomycete *Lechevalieria aerocolonigenes* and it inhibits DNA topoisomerase I with an IC_{50} in the micromolar range and impairs the growth of lung adenocarcinoma tumor cells. A water-soluble tartrate salt of the semisynthetic analogue of rebeccamycin, NSC 655649 (**29**), entered clinical trials for colon carcinoma but did not progress beyond Phase II.[41,42]

Staurosporine (**26**), another indolocarbazol, is produced by several actinomycetes species but shows a very different mechanism of action, as it is a nonselective inhibitor of various protein kinases.[43] Lack of selectivity for a particular protein kinase has significantly hampered the development of this compound as a useful drug. Recently, however, a number of compounds derived from this lead have entered the clinic for the potential treatment of cancer. These include 7-deoxystaurosporine (**27**) and CGP41251 (**28**).[44,45] CGP41251 shows multiple modes of action, including inhibition of angiogenesis *in vivo*.

Rebeccamycin (**25**)

Staurosporine, R = H (**26**)
7-Hydroxystaurosporine, R = OH (**27**)

CGP41251 (**28**)

NSC 655649 (**29**)

2.05.3.4 Enediynes

Natural enediynes were first discovered in 1987. They are represented by two classes of antitumor compounds, esperamycins and calicheamicins (**30**), produced by strains of *Actinomadura verrucosospora* and *Micromonospora echinospora*, respectively.[46] These compounds present a unique mode of action. They contain a (*Z*)-1,5-diyn-3-eno moiety that has been proposed to intercalate in the minor groove of DNA, triggering a number of aromatization reactions and the transitory formation of benzenic biradicals, producing breaks in both strands of DNA. Recent years, a number of other enediynes have been reported (dinemycins, kedarcidin, C-1027, maduropeptin), produced by several actinomycetes strains. These compounds are potent cytotoxic agents (IC_{50} values in the picomolar range) and act by breaking the DNA double helix, inducing the inhibition of replication and the activation of DNA-dependent protein kinase.[47] Strong, but undifferentiated, cytotoxicity of these compounds between tumor and normal cells prevented their development as clinical drugs until the development of tumor-specific selective targeting agents. Monoclonal antibodies specific for epitopes overexpressed in tumor cells can be used as selective targeting agents. Calicheamicin (**30**) was conjugated with recombinant humanized IgG_4 kappa antibody to produce Mylotarg (**31**), which binds to CD33 antigens expressed on the surface of leukemia blasts. Mylotarg was approved for the treatment of myeloid leukemia.[48]

Calicheamicin (**30**)

Mylotarg^R (**31**)

2.05.3.5 Leinamycin

Leinamycin (**32**), a macrocyclic lactam produced by a strain of *Streptomyces atroolivaceus*, was discovered in 1989.[49,50] It shows broad-spectrum antibacterial and antitumor activities, particularly against drug-resistant tumor cell lines. This compound is a hybrid of a polyketide and a nonribosomal peptide. Leinamycin alkylates DNA in the presence of thiol groups and induces the break of single-stranded DNA.[51] It shows a potent antitumor activity in several *in vivo* tumor models resistant to other agents. More stable thioester derivatives of leinamycin that show antiproliferative activity in the nanomolar range *in vitro* with *in vivo* activity have been reported. KF22678 (**33**) showed an improved activity compared to cisplatin and a broad spectrum of activity against lung, colon, ovary, and prostate carcinoma models.[52,53]

Leinamycin (**31a**)

KF22678 (**32**)

2.05.3.6 Cyclopropilindols

Cyclopropilindols are cytotoxic compounds that act as alkylating agents, exemplified by the duocarmycins (e.g., duocarmycin A, **33**) and the compound CC-1065 (**34**), both of which are produced by *Streptomyces* strains. These molecules are very potent antitumor agents.[54] They bind to the minor groove of DNA and specifically alkylate DNA following a nucleophilic attack. All natural compounds of this class show cytotoxicity in the picomolar range against leukemia L1210 cell lines. The compound CC-1065 showed even better antitumor activity in an *in vivo* model. Although it cannot be used in humans due to its toxicity, it has served as a template to design new compounds with a better profile in the *in vivo* models.[55,56]

Duocarmycin A (**33**)

CC–1065 (**34**)

2.05.3.7 Trichostatin

Trichostatin A (**35**) is a hydroxamic acid produced by *S. hygroscopicus*.[57] This compound is a reversible inhibitor of histone deacetylase (HDAC), one of the most promising and already validated targets for the development of antitumor agents.[58] HDAC inhibitors are capable of inducing morphological reversion of cells transformed with an oncogene to their normal phenotype. Many tumor cell lines are susceptible to HDAC inhibitors, and mouse models have shown that these compounds reduce tumor growth and metastasis *in vivo*.[59] Acetylation and deacetylation of histones are critical in the regulation of transcription in eukaryotic cells.

Trichostatin A (**35**) is structurally related to suberoylanilide hydroxamic acid (SAHA), a molecule that has reached the market as a therapy for the treatment of cutaneous T-cell lymphoma (CTCL), marketed as vorinostat.[60] Hybrid derivatives have been synthesized combining features of trichostatin A and fungal-derived cyclic tetrapeptides such as trapoxin and apicidin, which show subnanomolar activities.[58,61–64]

Trichostatin A (**35**)

2.05.3.8 Elsamicins

Elsamicins A (**36**) and B (**37**) were isolated in 1986 from an unidentified actinomycete.[65] These compounds are analogues of chartreusin (**38**), a cytotoxic compound discovered in 1964. Mechanistically, the elsamicin class of compounds exert their cytotoxic effect by strongly binding to DNA, specifically recognizing C + G rich sequences, inducing strand scission and single-strand breaks in the presence of reducing agents. In addition, elsamicin A is one of the most potent inhibitors of topoisomerase II discovered to date, but it does not inhibit topoisomerase I. Elsamicin A also inhibits transcription, apparently as a result of its binding to DNA.[66]

Elsamicin A (BMY-28090, Elsamutricin) showed cytotoxicity against a broad panel of murine and human tumor cell lines, representing diverse histological types. It showed equally good activity in mice models against several cancer types, such as P388 and L1210 leukemia, B16 melanoma, and M5076 sarcoma.[67] Elsamicin A has an improved water solubility that allowed it to be entered into clinical development. In Phase I clinical trials elsamicin A showed a good safety profile and proceeded to Phase II clinical studies, where it failed to show activity in patients with metastatic breast cancer, colorectal cancer, non-small cell lung cancer, or ovarian cancer, but it did show some modest activity in patients with relapsed or refractory non-Hodgkin's lymphoma.[66]

Other natural analogues of elsamicins and chartreusin have been discovered in the last decades (e.g., chrymutasins), but none showed better properties than elsamicin.[66] The semisynthetic derivative IST-622 (**39**) has entered clinical trials in Japan, but no therapeutic effects have been reported.[68] This compound appears to inhibit both topoisomerase II and topoisomerase I.[66]

Elsamicin A (**36**)

Elsamicin B (**37**)

Chartreusin (**38**)

IST-622 (**39**)

2.05.3.9 ECO-4601

ECO-4601 (**40**) is a novel farnesylated dibenzodiazepinone discovered by genomic scanning.[69] The compound is produced by a *Micromonospora* sp. isolated from soil.[70] This compound was also simultaneously and independently discovered (as diazepinomicin) from a marine *Micromonospora* sp.[71] ECO-4601 selectively binds with submicromolar affinity to the peripheral benzodiazepine receptors but not to central benzodiazepine (gamma amino butyric acid A ($GABA_A$)) receptors. The peripheral receptors are critical components of the mitochondrial permeability transition pore, which is involved in the initiation and regulation of apoptosis. Lack of binding to the central receptors avoids the anxiolytic and anticonvulsant properties of benzodiazepines. ECO-4601 has been shown to be cytotoxic against many tumor cell lines and to have moderate antitumor activity against several human cancer xenograft rat models.[72] The specific mode of action of ECO-4601 is not yet well understood, but it was reported recently that it also inhibits the Ras–mitogen-activated protein kinase (Ras–MAPK) pathway.[73] This compound has entered a Phase I clinical trial for treatment of cancer and has been shown to be well tolerated.[73]

ECO-4601 (**40**)

2.05.3.10 Echinomycin

Echinomycin (**41**) is a cyclic peptide from the quinoxalin family. It is produced by a strain of *Streptomyces echinatus* and was initially discovered in the 1950s as an antibacterial agent.[74] Although its antitumor properties were described in the 1960s, its mode of action emerged when it was described as the first DNA *bis*-intercalator in the 1970s.[75,76] Echinomycin recognizes and binds to specific DNA sequences, mostly containing CpG steps. It is a potent inhibitor of transcription, more potent than actinomycin D. Echinomycin shows cytotoxicity over diverse cell lines, and has been subjected to a number of Phase II clinical trials in a broad range of cancer types (colorectal cancer, soft tissue sarcoma, non-small cell lung cancer, and others). Unfortunately, it did not show any efficacy.[77–79] Echinomycin was recently shown to inhibit hypoxia-inducible factor-1 (HIF-1).[80]

Echinomycin (**41**)

2.05.3.11 Efomycins (Elaiophylins, Niphimycins)

Efomycins, elaiophylin, and niphimycins (e.g., efomycin G, **42**) are a series of related dimeric *bis*-lactone macrolides produced by strains of *Streptomyces* sp.[81] and have attracted some attention due to their ability to

inhibit the selectin-mediated leukocyte adhesion to endothelial cells, *in vitro* as well as in mice models. The selectin-mediated adhesion as initiator of leukocyte recruiting seems to be relevant in several pathologies, such as psoriasis, rheumatoid arthritis, and myocardial infarction. In addition, efomycin M (**43**), a semisynthetic derivative in which glycosidic units have been removed,[82] showed efficacy in reducing skin inflammation in two rodent models of psoriasis, matched with good pharmacokinetic properties and low toxicity.[82–85]

Efomycin G (**42**)

Efomycin M (**43**)

2.05.3.12 Lipstatin

Lipstatin (**44**) is a fatty acid β-lactone produced by a strain of *Streptomyces toxytricini*[86,87] and it inhibits the activity of pancreatic lipases. Lipstatin was hydrogenated to produce the tetrahydro analogue orlistat (**45**), which has been developed as the first of the new classes of antiobesity agents.[88,89] The compound is not absorbed and acts in the lumen of the intestine, reducing fat absorption by almost 30%, by inhibiting gastric and pancreatic lipases. Orlistat is used for weight control in obese and obesity-dependent type II diabetic patients under the trade name Xenical.[90–94]

Lipstatin (**44**)

Orlistat (**45**)

2.05.3.13 Streptogramins

Streptogramins represent a unique class of antibacterial agents that occur as pairs of structurally unrelated molecules and include pristinamycins, virginiamycins, oestreomycins, and mikamycins. Group A (or M) (virginiamycins-M and pristinamycins-II) consists of polyunsaturated macrolactones, whereas group B (or S) (virginiamycins-S and pristinamycins-I) consists of cyclic hexadepsipeptides. Pristinamycin II_A/virginiamycin M_1 is coproduced with pristinamycin I_A by *Streptomyces pristinaespiralis* and with virginiamycin S_1 by *Streptomyces virginiae*. They are produced as natural mixtures by different *Streptomyces* species, but none of these compounds have been found to be produced alone. Both groups of molecules inhibit bacterial protein synthesis at the peptidyl transfer step and are bacteriostatic when tested alone but bactericidal when tested in combination. They show synergistic activity *in vivo* against many bacteria, leading to reduction of emergence of resistance. Synergy between type A and B components originates from the initial conformational changes in peptidyl transferase caused by type A streptogramins, which not only inhibits protein synthesis but more importantly increases ribosomal affinity for type B streptogramins by 40-fold.[95,96] The semisynthesis of water-soluble derivatives of pristinamycin I_A (**46**) and II_B (**47**) allowed the development of injectable formulations containing a combination of quinupristin (**48**)/dalfopristin (**49**) used to treat multidrug-resistant infections including VRSA and VRE.[97]

Pristinamycin I_A (**46**)

Pristinamycin II_B (**47**)

Dalfopristin (**48**)

Quinupristin (**49**)

2.05.3.14 Muraymycins

Muraymycins were reported in 2002 as a family of novel antibiotics produced by *Streptomyces* sp.[98] This class of compounds consists of more than 19 members showing structural variations of the ester group at the hydroxy leucine and the second aminofuranosyl residue, as highlighted in the structure of muraymycin A_1 (**50**). These compounds are structurally related to a number of uridyl peptide antibiotics (mureidomycins,[99–106] pacidamycins,[107–109] napsamycins,[110] liposidomycins,[111,112] and others) inhibiting PG synthesis at the level of the translocase, the product of the MraY gene. Muraymycins have been shown to inhibit the synthesis of lipid II and PG. The compounds are active against Gram-positive and Gram-negative bacteria and show *in vivo* efficacy in a mouse model of *S. aureus* infection.[98] A number of analogues with improved properties have been reported.[113]

Muraymycin A_1 (**50**)

2.05.3.15 GE81112

GE81112 is a mixture of tetrapeptide factors A (**51**), B (**52**), and B_1 (**53**). It was discovered by using an *in vitro* translation screen driven by a model mRNA containing natural initiation signals. They are produced by a strain of *Streptomyces* sp. and are specific for the inhibition of bacterial protein synthesis. They are shown to target the 30S ribosomal subunit specifically interfering with the binding of fMet-tRNA to the P-site and thereby selectively inhibiting the formation of the 30S initiation complex. GE81112 has been reported as the most potent inhibitor of initiation of protein synthesis in bacteria known to date, above other antibiotics such as pactamycin or edeine.[114]

The *in vitro* antibiotic activity of this compound was highly media-dependent and the best activity was observed only in minimal media. This phenomenon renders this compound less useful on its own as an antibiotic for clinical use, but its attractive *in vitro* properties, highly selective mechanism of action, and structural features make it a good starting point as lead candidate for derivatization or rational design to improve its activity against whole cells.[115]

Factor A: R = H, X = O (**51**)
Factor B: R = NH_2, X = O (**52**)
Factor B_1: R = NH_2, X = NH (**53**)

2.05.3.16 Actinonin

Actinonin (**54**) is a modified tripeptide that was originally isolated in 1962 from a *Streptomyces* sp.[116] No attention was paid to this compound until the elucidation of its mechanism of action as a potent and reversible inhibitor of bacterial peptide deformylase (PDF),[117] which was validated by both genetic and biochemical approaches. The hydroxamate group of actinonin is essential for its activity, acting as the chelating agent to bind the Fe^{2+} ion of the enzyme.[117] The simplicity of actinonin structure led to the synthesis of therapeutically useful compounds. One of them (LBM415, BB-83698) has entered the early stages of human clinical trials as an antibiotic.[118]

Actinonin has also shown activity against a broad panel of tumor cell lines and various cancer mice models, but the mechanism of action is not well understood.[119] Actinonin has also been investigated in other therapeutic areas where metalloproteases play a role. For example, the presence of PDF in *Plasmodium falciparum* led to its investigation as an antimalarial agent.[119] Likewise, its capacity to inhibit other metalloproteases such as meprin has been related to its ability to prevent ischemic acute kidney injury in rats.[120]

Actinonin (**54**)

2.05.3.17 Ramoplanins

Ramoplanins are new members of the glycolipodepsipeptide class of antibiotics and were discovered in 1984. They are produced as a mixture of ramoplanins A_1, A_2, and A_3 by a strain of *Actinoplanes* sp.[121] The structural difference is in the length of the N-terminal acyl chain. Ramoplanins act by inhibiting the late stage assembly of the PG synthesis, involving complexation of lipid II and inhibiting the action of the MurG translocase and transglycosylases. Ramoplanins recognize and bind to a PG-binding locus different from the D-Ala-D-Ala targeted by vancomycin and show no cross-resistance with other glycopeptides. Ramoplanin A_2 (**55**) is structurally related to enduracidins and janiemycin produced by *Streptomyces fungicidicus* and *Streptomyces macrosporeus*, respectively.[122,123] It has been shown that enduracidins, like ramoplanin, bind to PG lipid intermediates.[124]

Ramoplanin A_2 is a promising clinical candidate for the treatment of MRSA and VRE infections. It is more potent than vancomycin (minimum inhibitory concentration (MIC) values ≤ 2 and 1 μg ml^{-1}, respectively). It showed a broad-spectrum activity against Gram-positive pathogens both *in vitro* and *in vivo*, including *Enterococci*, *Staphylococci*, *Bacilli*, *Streptococci*, *Listeria monocytogenes*, and Gram-positive anaerobes such as *Clostridrium difficile*. Like vancomycin, it shows no activity against Gram-negative pathogens. Currently, ramoplanin is in Phase III clinical trials. This antibiotic received a Fast Track status from the Food and Drug Administration (FDA) for the treatment of *C. difficile*-associated diarrhea (CDAD) in 2004 and is currently in advanced stages of development.[21]

Ramoplanin A_2 (**55**)

2.05.3.18 Daptomycin

Daptomycin (**56**) is a lipopeptide produced by a strain of *Streptomyces roseosporus* and was originally discovered in the 1980s.[125] It was targeted for development but abandoned during Phase II studies due to its potential toxicity. With the increased need for newer antibiotics, this compound was recently reevaluated as a broad-spectrum agent against multiresistant Gram-positive pathogens.[126] Mechanistically, it has been shown to disrupt membranes of Gram-positive bacteria. The activity of daptomycin is dependent on Ca^{2+} ions.[127] The compound showed *in vivo* efficacy against most Gram-positive pathogens, including MRSA and VRE. Daptomycin was approved by FDA in 2003 for the treatment of patients with Gram-positive infections.[21,22]

2.05.3.19 Friulimicin

Friulimicins (e.g., friulimicin A, **57**) were isolated from *Actinoplanes friuliensis* DSM 7358. Friulimicin A is a cyclic peptide with lipophilic chain. Friulimicin A and daptomycin are cyclic peptides but differ mechanistically. Unfortunately, details of the mechanism of friulimicin are not fully understood.[127,128] Friulimicin has also recently entered human clinical trials but was suspended recently.

Daptomycin (**55**)

Friulimicin A (**56**)

2.05.3.20 Everninomycins

Everninomycin (57) is a member of a family of oligosaccharides produced by a strain of *Micromonospora carbonacea* var. *africana* that were used as the starting point for the development of SCH-27899 as an antibiotic.[129–133] Unfortunately, this compound was abandoned after Phase III studies due to its poor efficacy/safety profile.[134,135] Everninomycins showed an excellent activity against Gram-positive bacteria, including *S. pneumoniae*, MRSA, VRE, and *E. faecalis*.[136–138] They inhibit protein synthesis by interacting with the 30S subunit of ribosome and ribosomal protein L16.[139,140]

Everninomicin (**57**)

2.05.3.21 Glycylcyclines

Glycylcyclines (e.g., tigecycline, **58**) are semisynthetic derivatives of tetracycline (**59**) and they inhibit protein synthesis. They were designed to avoid the efflux-mediated resistance mechanisms that have plagued the tetracycline class.[141] Glycylcyclines are active against MRSA and methicillin-resistant *Staphylococcus epidermidis* (MRSE), penicillin-resistant *Streptococcus pneumoniae* (PRSP), and VRE.[141] Tigecycline is a 9-*tert*-butyl-glycylamido derivative of minocycline (**60**) and was synthesized from tetracycline.[142] Tygecycline acts by blocking the entry of the aminoacyl-tRNA to the binding site A, blocking the elongation of nascent peptide chain. This compound, approved by the FDA in 2005, is more efficacious than vancomycin against MRSA and methicillin-sensitive *Staphylococcus aureus* (MSSA) models and shows no cross-resistance with tetracyclines.[143] In spite of its broad-spectrum activity against Gram-positive bacteria and many Gram-negative bacteria, it maintains an adverse effect profile similar to other tetracyclines.[143]

Tigecycline (**58**)

Tetracycline (**59**)

Minocycline (**60**)

2.05.3.22 Mannopeptimycins

Mannopeptimycins α–ε (e.g., mannopeptimycin α, **61**) are novel cyclic glycopeptides produced as a complex mixture by a strain of *Streptococcus hygroscopicus* LL-AC98.[144] Mannopeptimycins were discovered in the 1950s but were shelved until recently, when they were reexamined due to the emergence of multidrug-resistant Gram-positive pathogens. Mannopeptimycins showed good antibacterial activity against Gram-positive bacteria, including methicillin-resistant *Streptococci* and VRE. These compounds inhibited the cell wall by targeting lipid II.[145] Interaction with lipid II leads to accumulation of UDP-MurNAc-pentapeptide and blocking of lipid II-dependent PG maturation steps. In competitive penicillin-binding protein (PBP) experiments, it was shown that mannopeptimycins do not bind to the staphylococci or *Escherichia coli* PBPs, suggesting that the inhibition of PG biosynthesis is most likely caused by the interference of transglycosylation by binding to this transglycosylase substrate.[146,147] Competition studies suggested that mannopeptimycins do not bind the D-Ala-D-Ala residue region on lipid II, the binding site of vancomycin, although the exact mode of binding to lipid II has not been determined.[148]

Mannopeptimycin α (**61**)

2.05.3.23 Thiazolyl Peptides

GE2270A (**62**) was isolated from a strain of *Planobispora rosea* and belongs to the thiazolyl peptide family of antibiotics inhibit protein synthesis. It binds to the 23S rRNA and blocks the action of the elongation factor EF-Tu.[149–151] The compound exhibits excellent activity against Gram-positives, including resistant strains, in experimental mice models of *Streptococci* endocarditis.[152]

Nocathiacins (e.g., nocathiacin I, **63**) and thiazomycin (**64**) are newly discovered thiazolyl peptides isolated from *Amycolatopsis fastidiosa* and are potent antibacterial agents both *in vitro* and *in vivo* against Gram-positive pathogens. Although they are also protein synthesis inhibitors, their mode of action is different from GE2270A, acting through binding to the 23S RNA and L11 ribosomal protein.[153–156]

GE2270A (**62**)

Nocathiacin I (**63**)

Thiazomycin (**64**)

2.05.3.24 Acyldepsipeptidolactones

Acyldepsipeptidolactones (ADEPs) were already reported in 1985 as a complex produced by *Streptomyces hawaiiensis* NRRL 15010 and have been recently reexamined.[157] ADEP1 (**65**) is an *N*-acylhexapeptidolactone from which the derivative ADEP2 (**66**) was synthesized, with 10-fold increased activity. ADEP2 is active against Gram-positive pathogens, and shows bactericidal activity against *S. aureus*, *E. faecalis*, and *S. pneumoniae* in murine infection models. The compound has a novel mode of action in that it targets the caseinolytic protease ClpP, the catalytic subunit of a proteasomelike bacterial protease. ADEPs are allosteric activators in the absence of the ATPase subunit, which ensures conformational changes allowing for opening of the channel for proteins to enter into the proteolytic chamber. ADEPs alter the highly regulated intracellular protease activity of ClpPs, promoting cell degradation processes and causing cell death. The high frequency of resistance (10^{-6}) of ADEP precludes its use in monotherapy, but it could be used in combination with other antibiotics.

ADEP 1 (**65**)

ADEP 2 (**66**)

2.05.3.25 Arylomycins

Arylomycins (e.g., arylomycin A_2, **67**) are a series of 12 biaryl-bridged lipopeptide antibiotics produced by *Streptomyces* sp. Tu 6075.[158,159] Arylomycin A_2, together with its glycosylated analogues produced by another *Streptomyces* species (ATCC PTA-3546), represents a new group of lipoglycopeptides that specifically target the periplasmic type I signal peptidase (SPase).[159,160] This molecule forms a β-sheet structure that mimics the binding of SPase substrate.[161] These peptides are potent and competitive inhibitors of SPase I with K_i values of 50–158 nmol l^{-1}. They block protein secretion in *S. aureus* whole cells[160] but show modest activity against Gram-positive and Gram-negative pathogens.

Arylomycin A_2 (**67**)

2.05.3.26 Tiacumicins

Tiacumicins are an antibiotic complex produced by *Dactylosporangium aurantiacum* spp. *hamdenensis* NRRL 18085.[162,163] Tiacumicin B (OPT-80, **68**) is the major component of the antibiotic complex, and its antibacterial activity is due to the inhibition of RNA synthesis. OPT-80 (**68**) is a narrow-spectrum agent active against *C. difficile* and is in Phase II clinical trials for the treatment of CDAD and VRE infections. CDAD indication received 'Fast Track' status from the FDA.[164,165]

Tiacumicin B (**68**)

2.05.3.27 GE23077

GE23077 (e.g., GE23077A_1, **69**) is a new cyclic heptapeptide complex produced by a strain of *Actinomadura* and was discovered in the course of a screening program for inhibitors of RNA polymerase. The complex is a mixture of four factors—A_1, A_2, B_1, and B_2—that differ only in the structure of the acyl group. These antibiotics are potent inhibitors of *E. coli* RNA polymerase with an IC_{50} of 0.02 $\mu g\,ml^{-1}$, including rifampicin-sensitive and rifampicin-resistant polymerases.[166] However, they show very weak antimicrobial activity due to poor penetration attributed to their strong hydrophilicity. To improve penetration of GE23077 across membranes, different parts of the molecule were modified to alter its physicochemical properties, identifying moieties for RNA polymerase activity but without much success.[166,167]

GE23077A_1 (**69**)

2.05.3.28 ECO-0501

The new glycosidic polyketide antibiotic ECO-0501 (**70**) was discovered from the vancomycin producer *A. orientalis* ATCC 43491, using a genome-scanning approach for the discovery of novel biosynthetic pathways capable of producing novel metabolites.[168] ECO-0501 exhibited activity against Gram-positive bacteria including MRSA and VRE with MICs comparable to those of vancomycin (2 $\mu g\,ml^{-1}$). The compound is effective in a mouse model of *S. aureus* infection and showed a good safety profile. It has been suggested that the compound acts through a novel membrane or cell wall target.[169]

ECO-0501 (**70**)

2.05.3.29 Platensimycin and Platencin

Platensimycin (71) and platencin (72) are produced by various strains of *Streptomyces platensis*.[170–173] They were discovered by using an innovative mechanism of action-based screening based on an antisense whole-cell differential sensitivity assay.[174] Platensimycin and platencin consist of two structural units, a common 3-amino-2,4-dihydroxy-benzoic acid and a different and quite unusual diterpenoid (tetracyclic enone acid (platensimycin) and tricyclic enone acid (platencin)).[175] Platensimycin is a potent and selective inhibitor of the bacterial elongation condensing enzyme FabF, whereas platencin is a balanced inhibitor of both initiation and elongation-condensing enzymes, FabH and FabF. More specifically, they bind to the malonyl-binding site and inhibit the acyl enzyme intermediate. Both compounds are potent and broad-spectrum Gram-positive antibacterial agents and also show *in vivo* efficacy.[170–173]

Platensimycin (**71**) Platencin (**72**)

In summary, the examples mentioned in this chapter are a good representation of the outstanding chemical diversity exhibited by bioactive molecules produced by terrestrial actinomycetes discovered during the last few decades. This overview, necessarily limited in size, has omitted many other novel compounds with different modes of action that never reached preclinical development stages. These compounds could be the subject of another review.

Actinomycetes have been a tremendous source for natural products of biological significance and have been prolific producers of large numbers of medicines and drug candidates. About 60% of all known microbial products have shown some type of biological activity, and the majority of those products are of actinomycetes origin.[176,177] Unfortunately, the rate of discovery of new metabolites from actinomycetes has been declining during the last decades, after reaching a peak in the 1970s.[178] Moreover, most of the compounds discovered in recent times, except those reported here notwithstanding, do not represent truly new paradigms in the sense of new chemistry and biological activity, but rather they represent variations on known scaffolds and/or biological activities. The limitations of conventional natural product screening processes, including poor strain diversity, inability to exploit the full metabolic potential of these strains with appropriate culture conditions, inadequate primary and secondary screens, hyperabundance of known metabolites leading to the absolute requirement of efficient and highly sensitive dereplication tools, and technical limitations of the isolation chemistry may partially explain this disappointing outcome. Despite the unlimited potential, the lack of routine discovery of novel drugable compounds has been partly responsible for the negative impression of this field by the pharmaceutical industry and, along with noncontrollable,[179] has prompted most large industrial drug discovery groups to move away from natural products research. Although some of these groups have been able to reinvent themselves as small biotechnology companies, and many continue to be very active in the field, the overall effort

dedicated currently to natural products drug discovery is considerably less than it was 30 years ago. Obviously, this reduced effort has also had a negative impact on the rate of the discovery of new molecules.

However, the lack of the effort by the pharmaceutical industry notwithstanding, it is also true that this field has experienced considerable progress in recent years, not only from a technological perspective, but also in understanding of the factors underlying the success of the efforts in natural products discovery.[180,181] Furthermore, it has been estimated that only a minor proportion of the antibiotics produced from actinomycetes has been discovered.[182] Numerous reports have highlighted that bacterial diversity is largely underexplored, and there is a need to efficiently mine and evaluate additional terrestrial bacterial groups using both traditional and novel metagenomic approaches.[183–185] Clearly, the potential for structural diversity is not limited to terrestrial bacteria, and efforts should be extended to filamentous bacteria from other underexplored habitats, such as marine environments, which have already delivered novel compounds with interesting properties.[186,187]

There is little doubt that bacteria in general, and actinomycetes in particular, in combination with the novel technologies and approaches for natural products discovery reported in the last years[188] will continue to provide high levels of structural diversity and leads required for the development of future innovative drugs.

Abbreviations

ADEP acyldepsipeptidolactone
CDAD *Clostridium difficile*-associated diarrhea
CTCL cutaneous T-cell lymphoma
FDA Food and Drug Administration
FKBP FK506-binding protein
GABA gamma amino butyric acid
HDAC histone deacetylase
HIF-1 hypoxia-inducible factor 1
IL-2R interleukin 2 receptor
MAPK mitogen-activated protein kinase
MRSA methicillin-resistant *Staphylococcus aureus*
MRSE methicillin-resistant *Staphylococcus epidermidis*
MSSA methicillin-sensitive *Staphylococcus aureus*
mTOR mammalian target of rapamycin
PBP penicillin-binding protein
PDF peptide deformylase
PG peptidoglycan
PRSP penicillin-resistant *Streptococcus pneumoniae*
SAHA suberoylanilide hydroxamic acid
SPase signal peptidase
VRE vancomycin-resistant *Enterococcus faecium*
VRSA vancomycin-resistant *Staphylococcus aureus*
VSE vancomycin-sensitive *Enterococcus faecium*

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Biographical Sketches

Dr. Singh received his Ph.D. in organic chemistry from Avadh University in India. He held postdoctoral appointments at the University of Glasgow (1982–83) studying synthesis and biosynthesis and at Arizona State University. He continued his work at Cancer Research

Institute of ASU for next 4 years as assistant and associate research professor studying natural products chemistry including total syntheses. Dr. Singh codiscovered combretastatins and synthesized dolastatins, 10 and 15. Many of these compounds have been used in human clinical studies for the treatment of cancer. In 1989, he joined Merck Research Laboratories and has rapidly risen through the ranks to the position as Director and head of natural products. His major accomplishments at Merck are the discoveries of actinoplanic, chaetomellic, clavaric, oreganic, and zaragozic acids, fusidienols, and cylindrols as FPTase inhibitors; apicidins as histone deacylase inhibitors, many ion channel modulators, over 20 classes of HIV-1 integrase inhibitors, HIV-1 RT inhibitors, nodulisporic acids as insecticides, and, most importantly, platensimycin and platencin as novel broad-spectrum antibiotics with a novel mode of action. Dr. Singh has published over 175 peer-reviewed original papers covering all aspects of natural products, including structure, biology, medicinal chemistry and structure–activity relationships, biosynthesis, and total synthesis. He has published over 18 review articles and book chapters. He has given many invited talks at national and international meetings, presented contributory talks, and is an inventor and/or coinventor of over 35 issued patents. He is a member of the advisory boards of the *Journal of Natural Products*, the *Journal of Antibiotics*, and the National Center for Natural Products Research of University of Mississippi. He is a recipient of Percy E. Julien Memorial Award (2007) and 2005 American Society of Pharmacognosy's (ASP) Arthur E. Schwarting Award for best *Journal of Natural Products* paper of 2004. He received the honorary doctor of philosophy degree in chemical biology from Stevens Institute of Technology (2006). Currently, he is Director of Medicinal Chemistry in External Basic Research at Merck Research Laboratories.

Olga Genilloud received her BS degree in biochemistry (1983) and her Ph.D. degree in biochemistry and molecular genetics (1988) from the Universidad Complutense de Madrid, Spain. After obtaining her Ph.D. with a project on the regulation of naturally produced antibiotics by *Enterobacteria* (Department of Molecular Genetics, Hospital Ramón y Cajal, Madrid), which she completed at the Harvard Medical School in Boston, she joined the Centro de Investigación Básica de España (CIBE), the basic research site of Merck Sharp & Dohme in Madrid, Spain, in 1989. She has been leading a research group focused on the discovery of natural products produced by actinomycetes as new leads for drug development. She has actively explored alternative strategies to address the diversity and isolation issues associated with the discovery of novel producing strains, and developed new molecular and metabolic profiling tools for their rapid evaluation. In February 2009, she was appointed Scientific Director of the recently created Fundación Centro de Excelencia MEDINA, a public–private partnership that ensures continuation of the discovery efforts from microbial natural products that were previously developed at CIBE. She authored many publications, book chapters, and patents derived from her contributions to the discovery of novel compounds produced by actinomycetes and the application of new molecular tools in her field.

Fernando Peláez was born in Madrid, Spain, in 1962. He received his B.S. degree in biology from the Universidad Complutense de Madrid in 1984. He received his Ph.D. degree in 1989 at the Centro de Biología Molecular (Universidad Autónoma de Madrid), working on the control of protein synthesis in eukaryotic cells. He joined the Centro de Investigación Básica de España (CIBE), the basic research site of Merck, Sharp & Dohme in Spain, to take responsibility over the fungal research area within a screening group dedicated to the discovery of natural products with biological activity as a source of new leads for drug development. In 1999, he was appointed CIBE Director and led this group for the next 9 years. During this time, the group expanded his research interests to other aspects of the drug discovery process, including the support to lead optimization projects by implementing *in vitro* automated screens to assess safety and potential drug–drug interactions of candidate molecules. In December 2008, he joined the Spanish National Cancer Research Center (CNIO) as the Director of the Biotechnology Programme. He is an author or coauthor of 130 original papers, reviews, and book chapters and a coinventor of more than 40 patents on bioactive natural molecules.

2.06 The Natural Products Chemistry of Cyanobacteria

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2.06.1 Introduction

Cyanobacteria, also known botanically as Cyanophyta or 'blue-green algae', are fascinating organisms because of their abundance and variety, their impact on the ecology of aquatic systems, and their exceptional capacity to produce structurally diverse and highly bioactive secondary metabolites. This exceptional capacity has raised both popular and scientific interests in these organisms as they contribute to toxicity events, both in marine and freshwater environments, and their capacity to yield lead molecules for drug discovery efforts. Much of the recognition of this biosynthetic talent of cyanobacteria owes to the 30-year effort of Richard E. Moore who pioneered the investigation of marine blue-green algae beginning in 1977 with the discovery of majusculamides A and B.[1] However, freshwater species were reported in the scientific literature as early as the 1930s to produce toxins that negatively impacted human as well as animal health.[2] As a result, both marine and freshwater cyanobacteria have been actively pursued for their unique and bioactive components; however, the motivations have differed with most marine investigations looking for 'remedies' whereas freshwater ones have focused on the 'risks'.[3]

Because of this considerable interest, the natural products chemistry of cyanobacteria has been relatively frequently and thoroughly reviewed. In addition, the natural products of marine cyanobacteria are reviewed yearly in the long-standing comprehensive review of marine natural products.[4] For example, more than 200 reviews of the search terms 'cyanobacteria toxin review' are retrieved in SciFinder with most of these focusing on freshwater species. Of marine reviews on cyanobacteria, something less than 50 exist; however, several have occurred recently and provided comprehensive, insightful, and broad coverage of their chemistry,[5–9] biological properties,[10–15] and biosynthetic pathways.[16,17]

Hence, in constructing this perspective review of the natural products chemistry of cyanobacteria, we have attempted to dissect, analyze, and make understandable the metabolites of these gifted organisms by applying a biosynthetic reasoning to their presentation. We have accessed the natural products of marine, freshwater, and terrestrial cyanobacteria by considering diverse sources of information, including proprietary databases such as MarinLit and SciFinder, the various literature reviews described above in this introduction, and our personal knowledge of the field. We begin the chapter with a detailed analysis of the sources of reported cyanobacterial secondary metabolites at the genus level, and for the more prolific genera, the species level as well. Since the marine natural products literature is readily accessed through the MarinLit database program, we have analyzed the molecular weight ranges of the described marine cyanobacterial metabolites. Next, we have analyzed the types of metabolites that have been isolated from these life-forms in terms of their structural classes and major modifications. We also looked in some detail as to the types of amino acids that are used by cyanobacteria in producing their natural products and have produced a series of figures that details these findings. These charts are accompanied by a discussion of the trends in secondary metabolism by cyanobacteria and give some insights that are followed up by examples in the ensuing discussion of specific compounds.

The section of the chapter describing examples of specific cyanobacterial metabolites begins with a description of the fatty acid-derived compounds, which generally lack recognizable amino acid components and follow this with those possessing a terpene-deriving section. This theme of primarily carbon-based frameworks is continued in the next section of metabolites, which are derived exclusively from polyketide biosynthetic pathways. Metabolites of a pure peptide origin are presented next; however, there are substances that are both alkaloidal and of pure peptide origin (e.g., a number of cyanobacterial peptides possess an *N,N*-dimethylvalyl terminus).

Next, peptides and polyketides are joined to several classes of lipopeptide metabolites, and this constitutes a major biosynthetic theme in cyanobacteria. Indeed, cyanobacterial lipopeptides come in two distinctive categories: polyketide sections which transition into a nonribosomal peptide synthetase (NRPS) section, thus forming amide or in some cases ester bonds (e.g., with α-hydroxy acids), and the reverse wherein the carboxyl function of an amino acid serves as the starter unit for one or more polyketide extensions. These are fundamental and significant biosynthetic variants, and hence, are a good basis for further dissection of cyanobacterial lipopeptides. As such, new terminology is herein presented so as to facilitate the discussion of these two classes; polyketides transitioning to peptides are described as 'ketopeptides' (e.g., **79–84, 93**), whereas the reverse, peptides modified by polyketide extension, are to be known as 'peptoketides' (e.g., **1**); in each case, the root word order describes the biosynthetic sequence. However, cyanobacterial natural products often possess elements of both of these two motifs, occurring either as polyketide synthetase PKS–NRPS–PKS or NRPS–PKS–NRPS constructs; because further new terminology seems cumbersome, these will be described simply as 'complex ketopeptides' or 'complex peptoketides'.

2.06.2 Trends in the Structures of Cyanobacterial Natural Products

2.06.2.1 Taxonomy

The order Oscillatoriales accounts for 58% of all isolated secondary metabolites from marine cyanobacterial sources while the Nostocales accounts for 24%, Chroococcales 10%, Stigonematales 8%, and Pleurocapsales <1%. Hence, a majority of the unique natural products of cyanobacteria are derived from filamentous forms with generally larger genome sizes (6.0–10 Mbp) than the unicellular forms (1.7–4.0 Mbp), and this matches the deduced capacity to produce natural products from genome sequence information (**Figure 1**).[18] The genus that has yielded the most reported structures is *Lyngbya*, accounting for 35% of all cyanobacterial secondary metabolites. Other genera with significant contributions are *Nostoc* (11%), *Oscillatoria* (9%), *Microcystis* (7%), and *Schizothrix* (6%). Genera with smaller contributions are *Anabaena* (4%), *Hapalosiphon* (4%), *Tolypothrix* (4%), *Symploca* (3%), *Phormidium* (2%), *Kyrtuthrix* (2%), *Scytonema* (2%), and *Synechocystis* (2%). The remaining 9% of compounds come from 23 different genera (**Figure 2**).

Microcystis aeruginosa (order Chroococcales) accounts for 92% of the compounds isolated from the genus *Microcystis* (49 compounds total) with 6% coming from *Microcystis viridis* and 2% from undetermined species. However, it is likely that some variants of the microcystin structure were not tallied in our analysis.

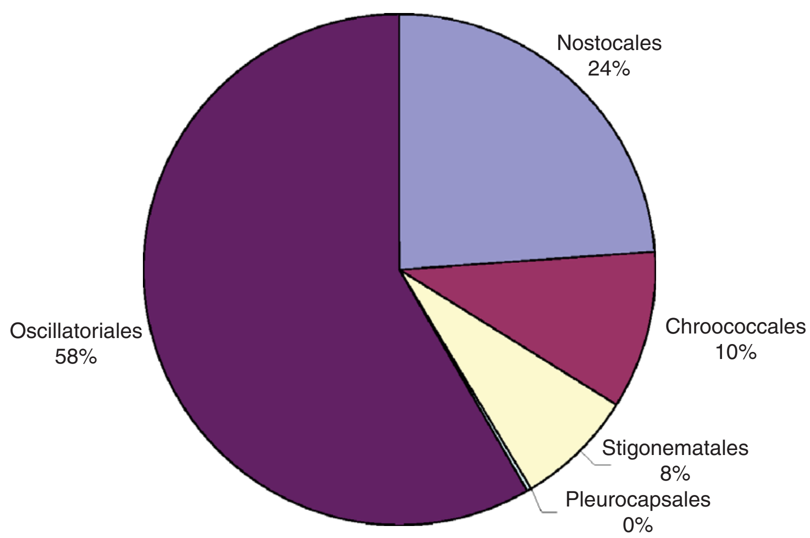

Figure 1 Percentage of isolated marine cyanobacterial natural products by taxonomic order. ($n = 678$)

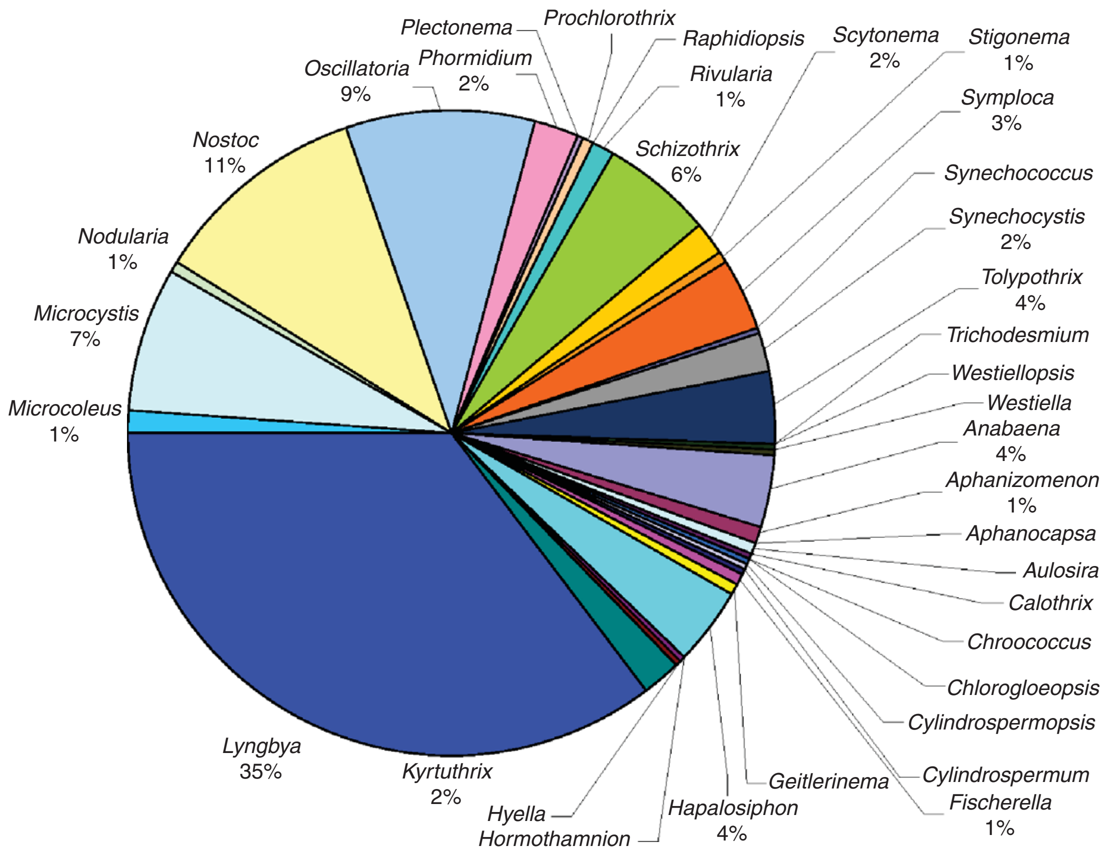

Figure 2 Percentage of isolated marine cyanobacterial natural products by genus. ($n = 678$)

Compounds isolated from the genus *Nostoc* (order Nostocales, 73 compounds) have predominantly been isolated from unknown species (91%). *Nostoc commune* accounts for only 6% of the isolated compounds with *Nostoc linckia*, *Nostoc muscorum*, and *Nostoc spongiaeforme*, each responsible for about 1%.

The genus *Oscillatoria* (order Oscillatoriales, 64 compounds total) has a much broader distribution of isolated compounds among different species as compared to the other genera of cyanobacteria; 30% comes from undetermined species, 28% from *Oscillatoria agardhii*, 19% from *Oscillatoria nigroviridis*, and 13% from *Oscillatoria spongelia*. The species *Oscillatoria raoi* and *Oscillatoria acutissima* are responsible for 3% each and *Oscillatoria rosea* and *Oscillatoria amphibia* each accounts for 2%.

Within the genus *Lyngbya* (order Oscillatoriales, 240 compounds total), the species *Lyngbya majuscula* accounts for 76% of the compounds isolated to date. *Lyngbya semiplena* accounts for 5%, *Lyngbya bouillonii* for 3%, 14% comes from undetermined species, and the remaining 2% comes from *Lyngbya aestuarii*, *Lyngbya aerugineo-coerulea*, *Lyngbya confervoides*, and *Lyngbya gracilis*. However, it is possible that some of these species identifications are erroneous as phylogenetic determinations have become widely employed quite recently. Additionally, phylogenetic analysis has revealed that *L. aestuarii* is likely not a member of the *Lyngbya* genus (N. Engene, personal communication) (**Figure 3**).

2.06.2.2 Molecular Weight

The molecular weight distribution of compounds isolated from marine cyanobacteria shows a bell-shaped distribution, somewhat skewed to higher molecular weight compounds, with the peak occurring at the 400–500 range. This is interesting because drug-like molecules tend to group in this molecular weight range.[19] However, the average molecular weight of all marine cyanobacterial secondary metabolites is 645 whereas the median molecular

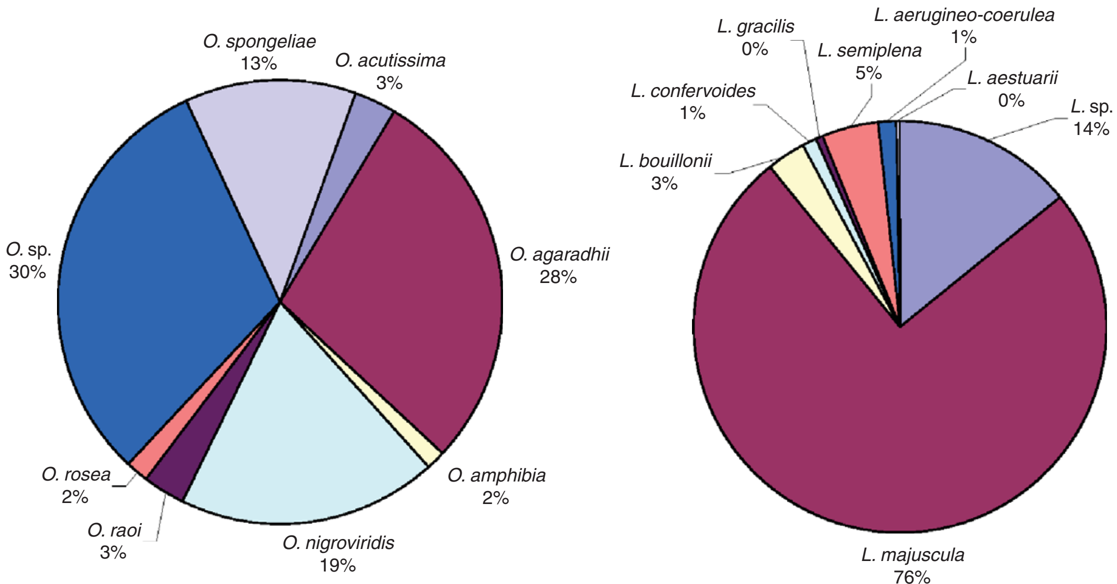

Figure 3 Percentage of isolated marine cyanobacterial natural products from *Oscillatoria* (left, $n = 64$) and *Lyngbya* (right, $n = 240$) by species.

weight is 604, as determined from the unique entries in the MarinLit database. This graph of sizes of cyanobacterial metabolites is skewed to the higher molecular weight range by a number of quite large cyanobacterial natural products. The largest structure considered in this review (e.g., excluding proteins and other biopolymers, including cyanovirin-N, which is 11 kDa)[20] is microviridin D of molecular weight 1801 kDa (**Figure 4**).[21]

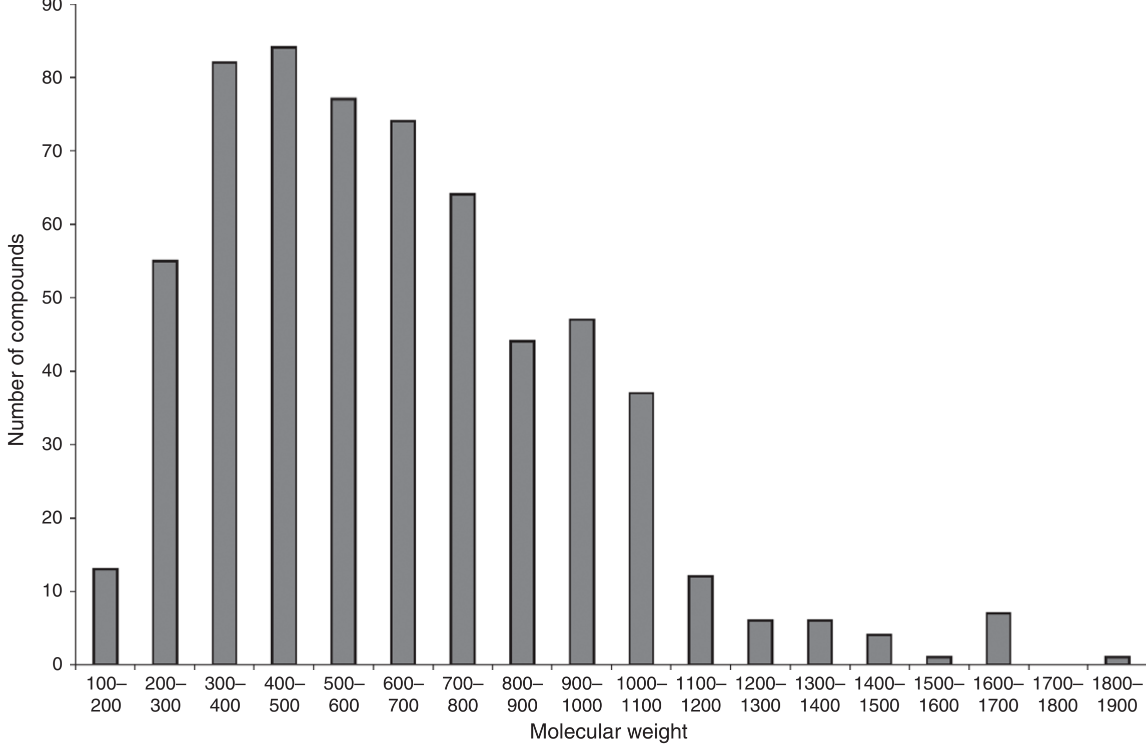

Figure 4 Molecular weight distribution of marine cyanobacterial natural products.

2.06.2.3 Structural Classes

The most prominent structural class of cyanobacterial secondary metabolites is the mixed ketopeptides (PKS–NRPS compounds) starting from a polyketide chain and then transitioning to amino acids (251 compounds total). A substantial proportion of ketopeptides are cyclic (60%), which is about the same proportion for peptoketides (NRPS–PKS compounds). Halogenation occurs in roughly 36% of the ketopeptides; interestingly, this is the same percentage of regular polyketides that are halogenated, but the numbers are much lower for peptoketides (8%). Slightly more than one-third of ketopeptides show a complex biosynthetic origin involving multiple transitions between PKS and NRPS domains and vice versa; peptoketides compounds show about the same percentage in this regard.

Approximately 70% of the pure peptide secondary metabolites from cyanobacteria are cyclic and only about 9% are halogenated. Polyketides show an equal distribution among linear, cyclic, and polycyclic structures. Interestingly, 10% of polyketides are glycosylated while more than one-third are halogenated. Terpenes, whether they are pure terpenes or composed of isoprenoid units in combination with other biosynthetic components, occur primarily as fused polycyclic structures (>50% for both terpene classes). However, terpenes are not a very abundant class of secondary metabolites in cyanobacteria (52 compounds total in both classes) and often tend to be glycosylated; an example is the bacteriohopanepolyols (e.g., **23**). Fatty acids (32 compounds) also show a high degree of glycosylation (50%) with the majority being of an overall linear constitution. Alkaloids isolated from cyanobacteria (66 compounds) show a high degree of halogenation (47%) and roughly a quarter are glycosylated (23%). A distinctive sugar derivative, a dimethoxypentose, is present in many of these glycosides. Finally, as is typical with alkaloids, fused polycyclic structures predominate (~70%) (**Figure 5**).

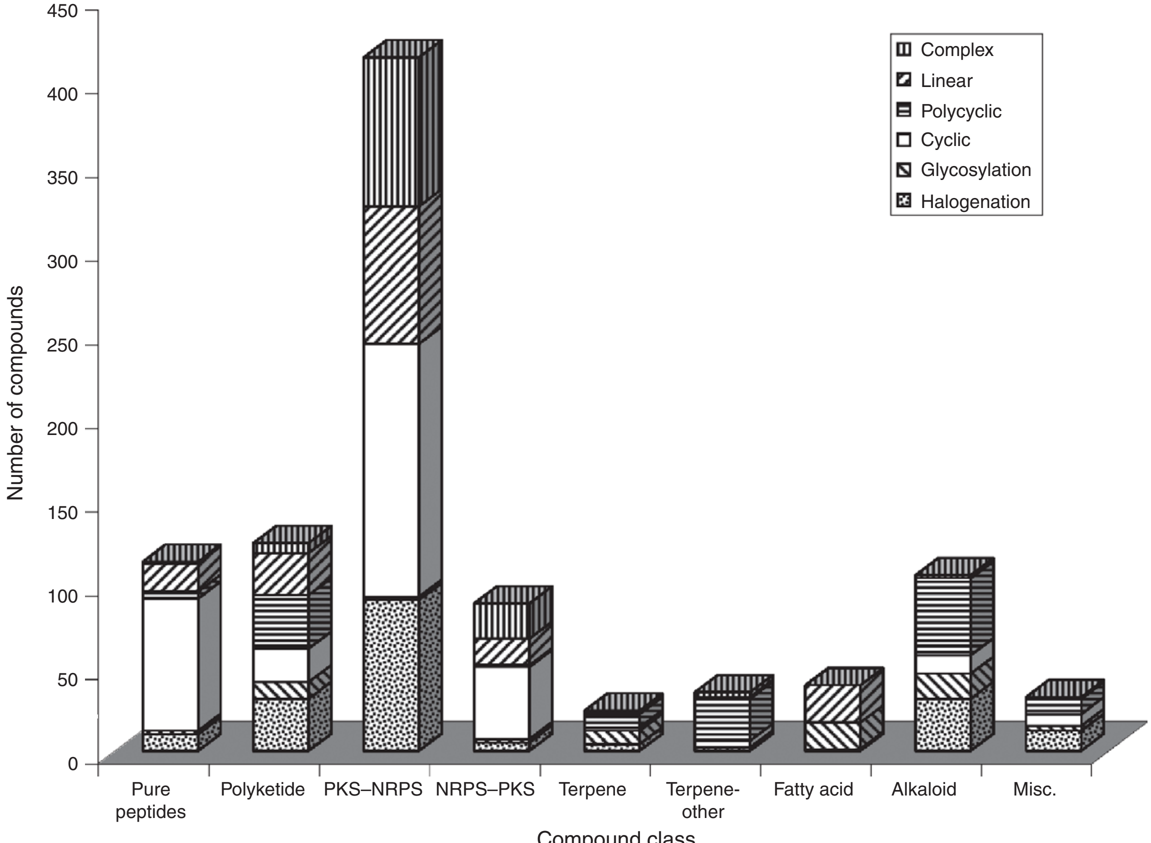

Figure 5 Structural characterization of cyanobacterial natural products. A single compound could be tallied multiple times for different structural features.

2.06.2.4 Amino Acids

The most commonly occurring amino acid is valine with 273 occurrences followed by tyrosine (215), alanine (190), proline (187), threonine (186), isoleucine (173), leucine (158), phenylalanine (141), and glycine (126). It is interesting to note that the majority of amino acids incorporated into cyanobacterial secondary metabolites are nonpolar with the second highest class being polar noncharged amino acids. About one half of the time (49%), amino acids are incorporated into cyanobacterial secondary metabolites without modification. However, when modified, they are most commonly altered by N-methylation (17%) or N,N-dimethylation (3%), incorporation into a heterocyclic ring (13%), polyketide extension (6%), hydroxylation, typically in the β position (4%), and O-methylation (2%). Valine is the most common amino acid to be N,N-dimethylated and as such forms the N-terminus of a number of highly bioactive peptoketides (e.g., dolastatin 10 (**1**)) (**Figure 6**).

Valine is incorporated most commonly with either no modification or N-methylation and is primarily present as the L-form. Tyrosine undergoes a wide variety of modification, including N-methylation, O-methylation, N,O-dimethylation, and halogenation with the L-form predominating over the D-form. Glycine and isoleucine show the highest prevalence of polyketide extension, followed by alanine, valine, and proline. Cysteine and threonine are involved in the most heterocyclic rings due to their ability to form five-membered rings with the carbonyl of adjacent amino acids (usually alanine, valine, phenylalanine, or isoleucine). It is interesting to note that while cysteine tends to form thiazole rings, thereby losing its chiral center, threonine tends to form oxazoline rings, which retain the original stereocenter of the amino acid. *Allo*-isoleucine shows an equal distribution between L- and D-forms whereas *allo*-threonine tends to be mainly L-form. Lysine (nine occurrences) is found exclusively in the metabolites of freshwater cyanobacteria, and to date it is always found in the D-form. 3-Amino-6-hydroxy-2-piperidone (Ahp) is of relatively common occurrence in cyanobacterial metabolites, and homologated amino acids such as homotyrosine (29 occurrences) and homophenylalanine (four occurrences) have a rare but distinctive incidence. *N,O*-dimethyltyrosine is a common form of this amino acid and represents a distinctive metabolic signature for compounds of cyanobacterial origin, whether isolated from a cyanobacterium or an invertebrate host (e.g., compound **48**) (**Figures 7–11**).

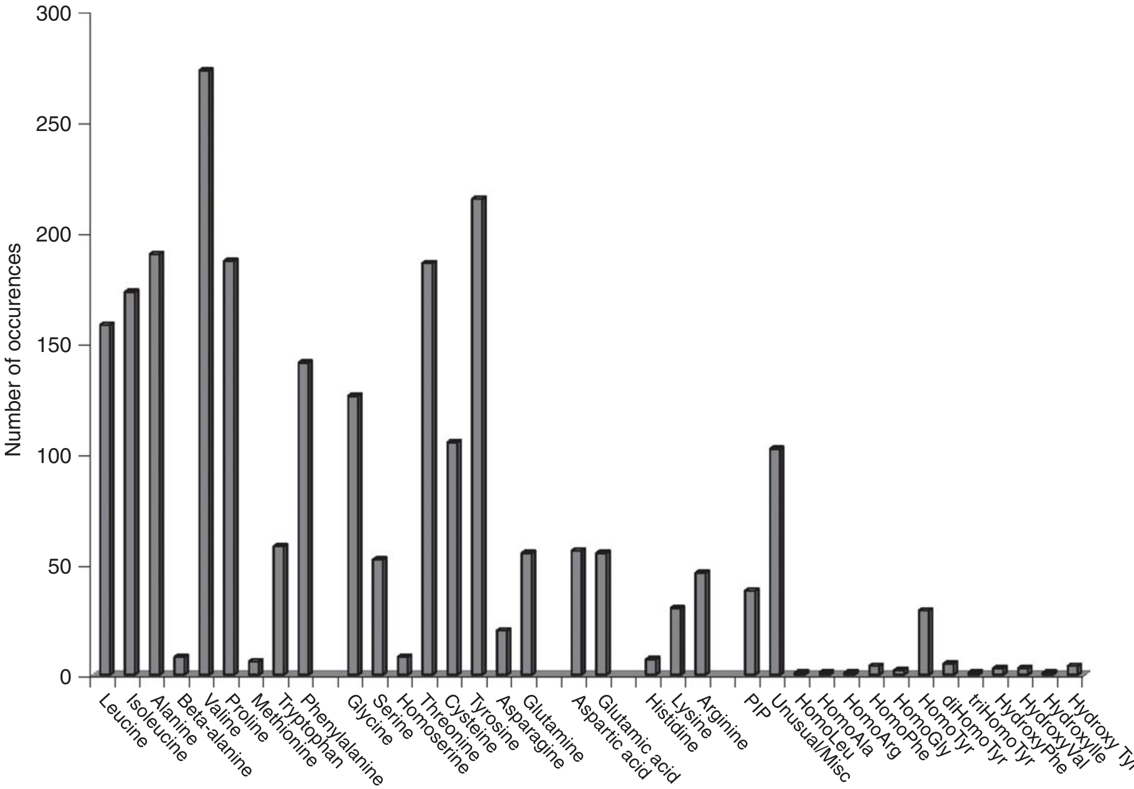

Figure 6 Occurence of amino acids in cyanobacterial natural products.

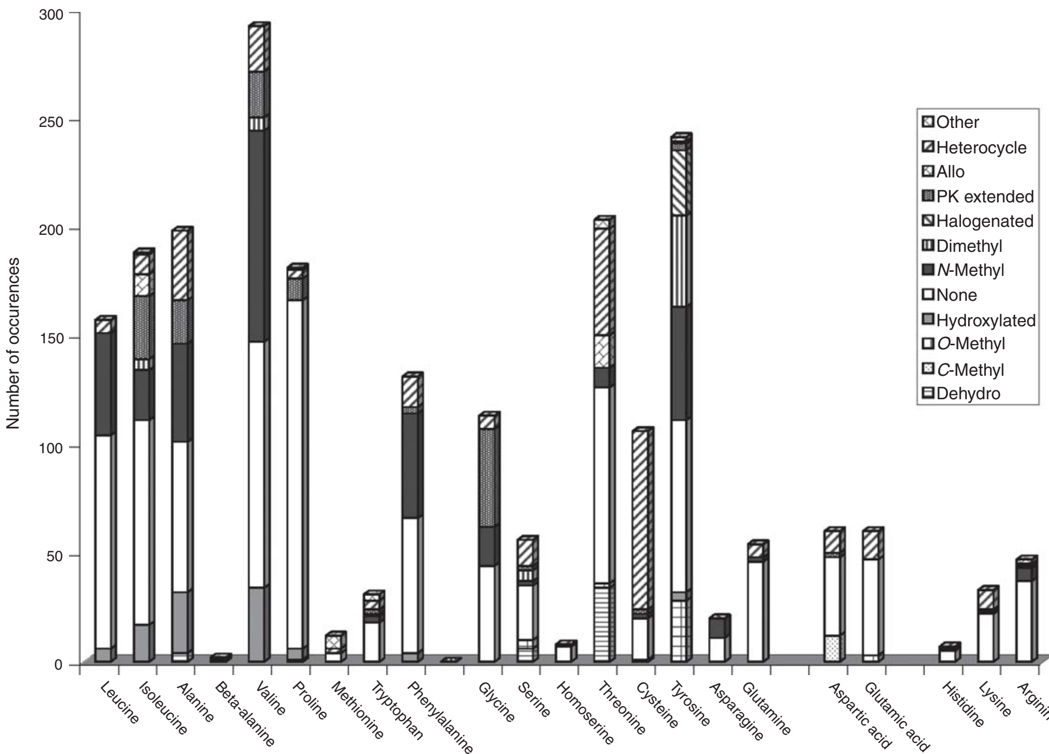

Figure 7 Modification of amino acid units found in cyanobacterial natural products.

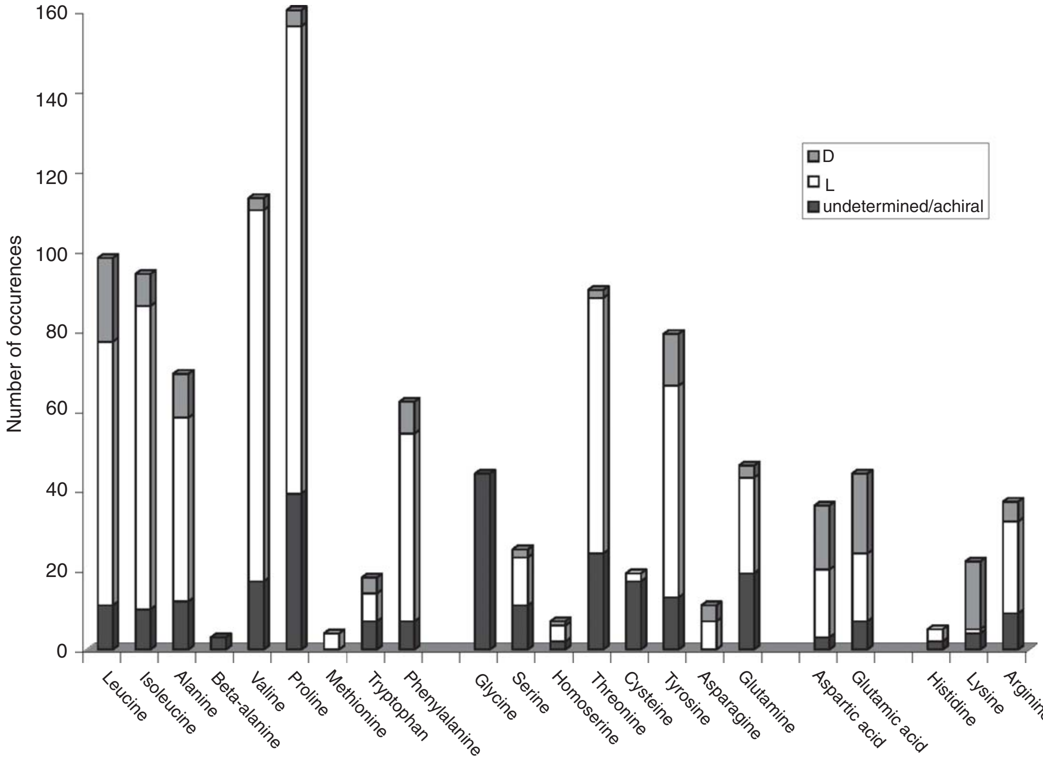

Figure 8 Chirality of unmodified amino acids found in cyanobacterial natural products.

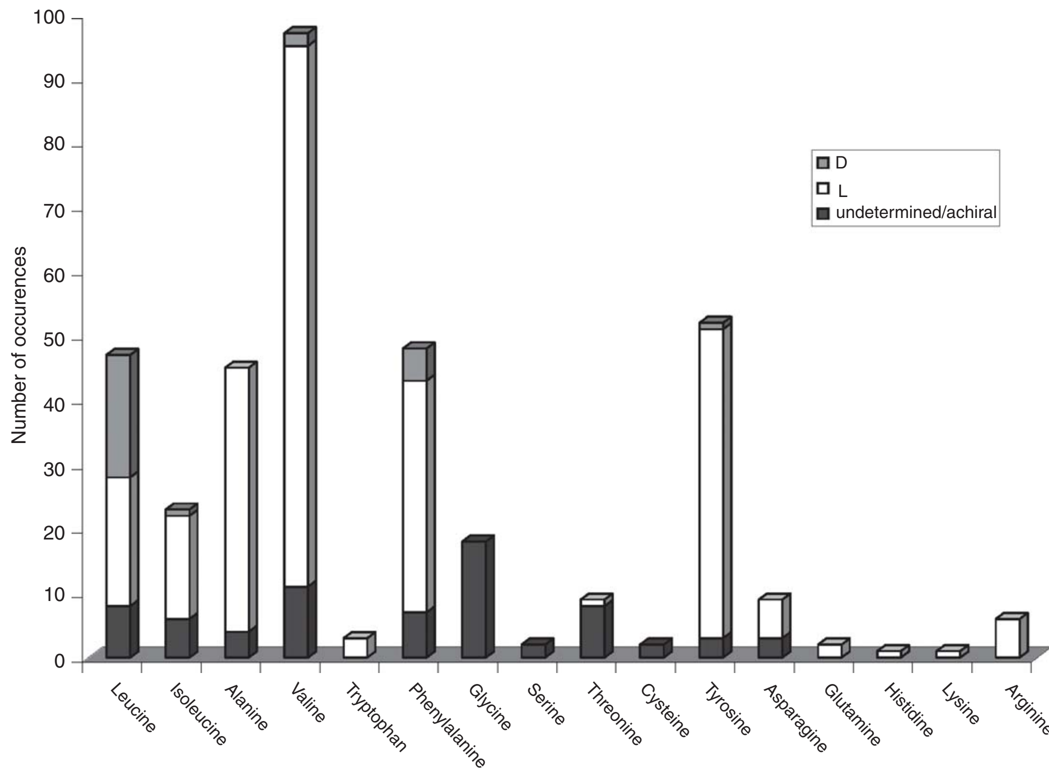

Figure 9 Chirality of N-methylated amino acids found in cyanobacterial natural products.

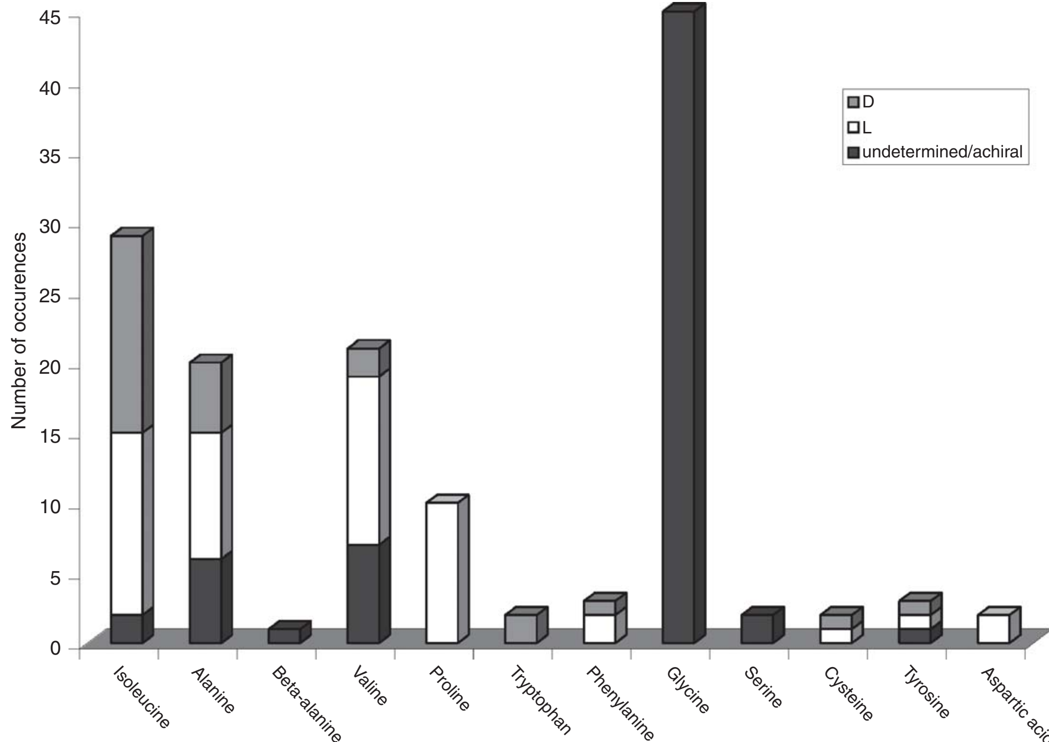

Figure 10 Chirality of polyketide extended amino acids found in cyanobacterial natural products.

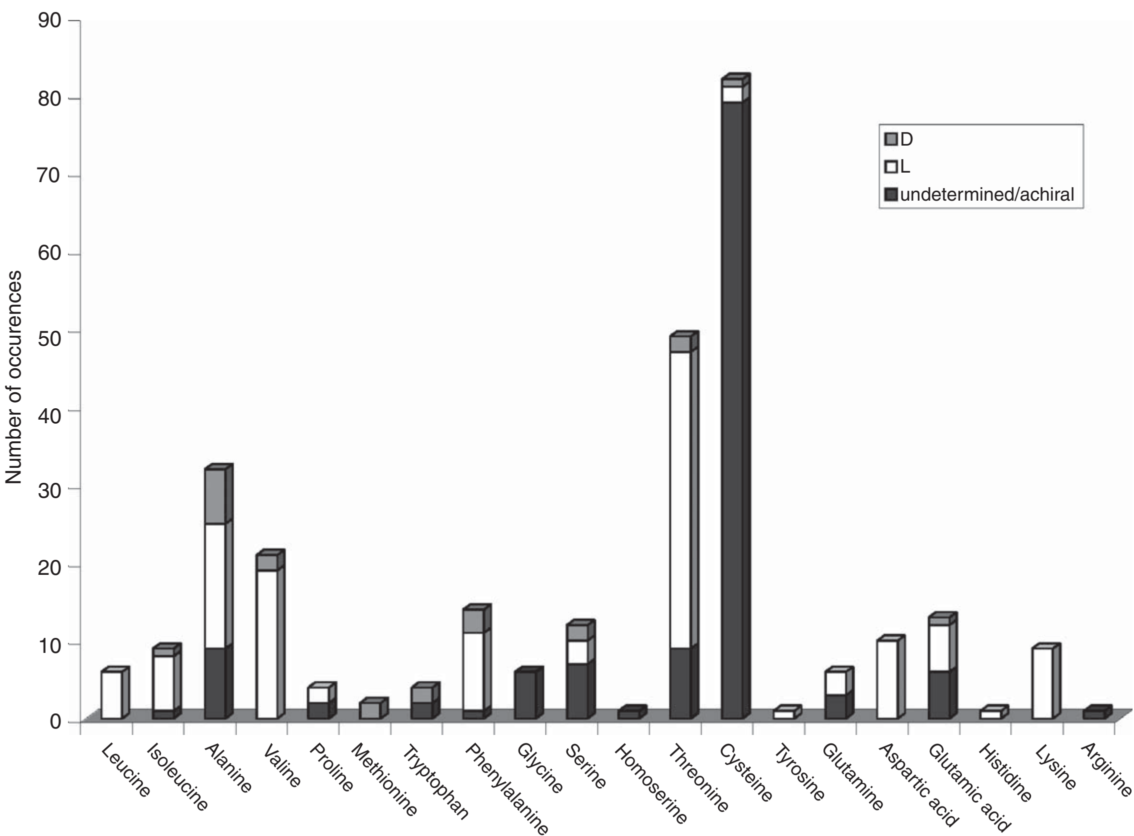

Figure 11 Chirality of amino acids involved in forming heterocycles found in cyanobacterial natural products.

2.06.2.5 Other Trends

While we did not tally marine and freshwater/terrestrial cyanobacterial metabolites separately, we have some perceptions that are worthy of note. Marine cyanobacterial metabolites tend to have more lipophilic character, possessing a greater degree of polyketide nature and possessing more hydrophobic amino acids and fewer with charged side chains. Freshwater cyanobacterial metabolites distinctively possess a greater incidence of acidic (aspartate and glutamate) and basic (arginine, lysine, histidine) amino acids.

Similarly, we did not tally the biological activity of cyanobacterial natural products as these data appear in diverse publications that are often separate from the discovery and structure elucidation papers. Nevertheless, it is quite clear that cyanobacterial natural products are a very biologically active group, with a preponderance showing toxicity to mammalian cells.[6] Moreover, a substantial number exert their toxicity to cells through inhibition of either tubulin or actin polymerization.[22] A growing number show activity in neurotoxicity assays and exert their effects through interaction at the voltage-gated sodium channel (VGSC).[11] The natural biological function of marine cyanobacterial metabolites has been studied to some extent and appears largely to constitute a chemical defense against predation. As their growth form provides essentially no protection against being consumed by a variety of classes of would-be predators, and because of their macroscopic stature, the necessity for a chemical means of defense is readily apparent.[23]

2.06.3 Fatty Acid Derivatives from Cyanobacteria

The fatty acids of cyanobacteria are quite an interesting group of compounds in that they show interesting structural modifications, several possess potent biological properties, and some are used as building blocks in more complex cyanobacterial natural products. Surveys of the fatty acids of some species of cyanobacteria show them to

be diverse in terms of size, degree of oxidation and alkylation, and their patterns of unsaturation.[24] A few dominant themes emerge from considering these fatty acid-derived metabolites: (1) many show a terminal decarboxylation with resulting formation of a terminal olefin, a modification seen later in this chapter in a number of lipopeptide natural products (e.g., kalkitoxin **2** and curacin A (**3**); (2) several show repetitive patterns of oxidation to produce isotactic polymethoxy alkanes; (3) a number of unusual functional groups are created by the addition of other elements (vinyl halides,[25] isonitriles); (4) interesting carbon additions to form methyl- or cyclopropyl-containing fatty acids; and (5) oxylipins, which appear to result from lipoxygenase-mediated oxidation of polyunsaturated fatty acid precursors. In some cases, a single metabolite shows more than one of these unusual modifications. Below are specific examples of some of these more interestingly modified fatty acid metabolites from cyanobacteria.

One of the more bioactive fatty acid derivatives from cyanobacteria, (–)-*trans*-7(*S*)-methoxytetradec-4-enoic acid, known by the trivial name 'lyngbic acid' (**4**), is a simple derivative of myristic acid. This was first reported as a product of the marine cyanobacterium *L. majuscula* collected in Hawaii,[26] but has been subsequently isolated from strains collected in many locations in the Pacific, Atlantic, and Indian oceans. Although this fatty acid derivative, in free form, possesses low micromolar antifungal and cancer cell cytotoxic properties, it is a frequent component of a number of more complex cyanobacterial natural products, including the malyngamides (e.g., malyngamides A **5** and C **6**)[27] and the hermitamides.[28] It has been synthesized in chiral form several times, both as an individual target[29] and as a portion of a larger metabolite (e.g., malyngamide X).[30] However, in this latter regard it is surprising that not more synthetic effort has been expended in the synthesis of malyngamide derivatives as there are many features of structural interest,[31] and some malyngamides possess biomedically useful properties such as cancer cell cytotoxicity[32] and anti-HIV properties.[33]

1

2

3

4

5

6

7

H
N
O
8

O
O
OAc
Br
9

NC
OMe OMe OMe OMe
10

OH OH O
OH
OH
11

O
O
H
O
H
12

A simple cyclopropyl fatty acid is found in amide or ester linkage with other subunits in the two related cyanobacterial metabolites, grenadadiene (7) and grenadamide (8).[34] The structures of these two metabolites were developed from normal nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis, and subsequently, asymmetric synthesis of grenadamide showed it to possess the *R* configuration at both chiral centers.[35] Grenadamide was moderately active in a brine shrimp toxicity model. These natural products are intriguing for their connection of an unusual fatty acid unit with a biogenic amine or other small and densely functionalized structural unit. Other alkylated fatty acids of a related overall molecular topology have been reported from other cyanobacteria, such as a 2,5-dimethyldodecanoic acid derivative from Australian collections (9).[36] Long-chain fatty acids acylated to the amine group of amino acids are emerging as a new class of bioactive microbial metabolite with important cell–cell communication properties.[37] Interestingly, another class of microbial cell communication chemical has been isolated from a marine cyanobacterium, a classic 'homoserine lactone'; however, its biological role and functioning have not yet been demonstrated.[38]

A series of modestly cytotoxic isonitrile natural products (e.g., mirabilene-A isonitrile) was obtained from cultures of the freshwater cyanobacterium *Scytonema mirabile*.[39] These are structurally and biosynthetically interesting as they possess several of the features identified in the structural summary given above, including a series of 1,3-methoxy groups that is on the same stereochemical face of the molecule (isotactic), a terminal olefin likely resulting from decarboxylation, and pendant methyl groups that appear to derive from *S*-adenosyl methionine (SAM) methylation (e.g., are located at predicted C-2 positions of the polyketide backbone). Isotactic polymethoxy alkenes have been isolated from several species of cyanobacteria[40] and have also been isolated from sponges,[41] although they almost certainly derive from the metabolic processes of associated cyanobacteria.[42] Finally, the mirabilenes (e.g., mirabilene B isonitrile 10) also possess the intriguing isonitrile functionality at a vinylic site. From previous work with terpene isonitriles produced by the cyanobacterium *Hapalosiphon fontinalis*, it appears that this unusual appendage derives from the nitrogen and C-2 of the amino acid glycine.[43]

Oxylipins are also produced by cyanobacteria and include simple hydroxyl acids such as 9-hydroxy-10,12,15-octadecatrienoic acid and 9-hydroxy-10,12,15-octadecadienoic acid from *Anabaena flos-aquae* f. *flos-aquae*.[44] Additionally, hepoxillin-type metabolites have been obtained from cyanobacteria and logically result from rearrangements of the hydroperoxide formed by an ω6-lipoxygenase acting on an 9,12,15-octadecatrienoic acid (α-linolenic acid) precursor to produce malyngic acid (11).[45] Similarly, an epoxy ester has also been isolated from various cyanobacteria (mueggelone = gloeolactone 12)[46,47] and likely owes its origin to the intermediacy of an epoxy allylic carbocation.[48]

2.06.4 Terpenes

Terpenes are a large class of hydrocarbon compounds constructed from five-carbon isoprene units that are combined to produce a great variety of skeletons, which are then acted upon by various enzymes to add functionality and altered oxidation. Terpene-derived compounds possess a broad array of activities and are produced by animal, plant, and bacterial sources. This pathway has been reviewed extensively elsewhere[49] but, briefly, involves chain extension of isoprene units into phosphorylated chains in multiples of five carbons. The initially formed linear prenyl chains are then acted upon by other enzymes resulting in cyclization and additional modifications. For example, one class of modification involves oxygenation on the ring before cyclization to yield lactone and furan moieties, or postcyclization to yield carbonyl and alcohol functionalities.

Sterols comprise a very important class of compounds in plant and animal systems. Bacteria and other organisms utilize cholesterol or related sterols in the synthesis and maintenance of their cell walls. True sterols are present in cyanobacteria to only a very small degree; they are largely replaced with a steroid-like class of triterpenoid known as the hopanoids.[50] The carbon frameworks of sterols and bacterial hopanoids are synthesized in essentially the same manner as other terpenes, however, the cyclization reactions to yield their polycyclic skeletons are unique.[51,52] A variety of sterols and hopanoids are found in cyanobacteria and they are believed to have important cellular functions in cell wall structure and function.[52–54] As discussed in Section 2.06.5, many of the hopanoids also possess a polyalcohol chain derived from a sugar.[55,56]

OH HO O O O **13**

HO OH O HO H H **14**

HO OH O O **15**

O O O O I H O H OH Br O O O **16** and its proposed diterpene skeleton

Another special class of terpene formed by the isoprenoid biosynthetic pathway are the carotenoids.[57] Carotenoids are important compounds in photosynthesis for harvesting of light energy as well as in the protection of cyanobacteria from excessive amounts of UV radiation. The carotenoids present in cyanobacteria are similar to those found in higher life-forms along with some glycosylated derivatives[58] (see Chapters 1.15–1.18).

2.06.4.1 Tolypodiol

Tolypodiol (**13**) is a meroditerpene that was isolated from a cultured sample of *Tolypothrix nodosa* originally collected in a soil sample from Nan Madol, Pohnpei.[59] Its structure was determined by a combination of mass spectral analysis and NMR of the parent compound as well as its acetylated analogue. Tolypodiol was found to possess anti-inflammatory activity in the mouse ear edema assay with an $ED_{50} = 30\ \mu g$ per ear, which rivals that of the standard, hydrocortisone (20 μg per ear).[59]

2.06.4.2 Noscomin/Comnostins

Bioassay-guided fractionation of a cultured sample of *N. commune* yielded the meroditerpenes, noscomin (**14**)[60] and the comnostins (e.g., **15**).[61] Noscomin was found to consist of a dodecahydrophenanthrene core while the comnostins possess the less common dodecahydrocyclopentanaphthalene. Structural determination of noscomin was achieved using 1D and 2D NMR techniques. For comnostin A, an X-ray diffraction study confirmed the NMR-based structural assignments. The structures of comnostins B–E were then determined through NMR data comparisons with comnostin A. All of the compounds isolated were found to be antibacterial with differential selectivity among compounds for different bacterial strains. Comnostin B was also found to be molluscicidal and cytotoxic against human epidermoid carcinoma cell line (KB) and human colonic carcinoma cell lines (CACO-2). While these two metabolites share an overall resemblance to tolypodiol, the configuration of the migrating methyl and hydride groups (apparent Wagner–Meerwein 1,2-shifts) are opposite to those of **13**, and suggests that an alternately folded precursor is the substrate for initial cyclization that leads to **14** and **15**.

2.06.4.3 Tasihalide

From the lipophilic extract of a field collection of *Symploca* sp., a highly unusual iodinated diterpene (**16**) was isolated.[62] Subsequently, the voucher sample was found to contain both cyanobacteria and a rhodobacterium, and thus the ultimate source of this natural product is uncertain. While halogenation is quite common in cyanobacterial metabolites (see Section 2.06.2), the incorporation of iodine is, in general, very rare and in this example unique for a naturally occurring iodinated diterpene. Tasihalide A (**16**) contains a novel tetracyclic core that is both highly oxygenated and incorporates iodine and bromine atoms. The structure of tasihalide A was elucidated using a standard combination of 1D and 2D NMR and mass spectral data. While there was no biological activity reported for this compound, it is nevertheless a highly interesting and significant finding because of its unique tetracyclic core and pattern of halogenation.

2.06.5 Saccharides and Glycosides

To date, cyanobacteria have rarely been reported to produce exclusively sugar-based secondary metabolites, such as the aminoglycosides or aminocyclitols typical of the actinomycetes. However, it should be noted that part of the reason for their underrepresentation may be the fact that many natural products chemists do not focus on water-soluble metabolites, and thus standard extraction and isolation protocols strongly select against the isolation of polar, sugar-based compounds.

Nevertheless, cyanobacteria have been reported to produce a number of sugar-containing secondary metabolites with diverse structural features. Glycosidated peptides include several aeruginosins (**17–19**),[63,64] which are described later in this review, the related glycolipopeptide suomilide (**20**) from *Nostoc spumigena*,[65] and the antifungal hassallidins (**21, 22**).[66,67] The bacteriohopanepolyols are a group of prokaryotic terpenoids that incorporate a sugar-derived C_5 unit, examples isolated from cyanobacteria include **23** from a *Nostoc* strain,[56] and **24**, which incorporates an additional anhydrogalacturonic acid moiety, from *Prochlorothrix hollandica*.[55]

17

18

19

20

21 R = H
22 R = rhamnose
(predicted configuaration)

A wide range of cyanobacterial glycolipids have also been isolated. One of the most structurally interesting of these is the only-known glycosidated malyngamide, malyngamide J (**25**), which is substituted by a dimethoxyxylose residue.[68] Glycosidated polyketides include lyngbyaloside, a 16-membered macrolide (**26**) with an appended trimethyldeoxymannose group.[69] Several closely related metabolites have been isolated from sponges and are likely of cyanobacterial origin.[70,71] Two cytotoxic swinholide derivatives, ankaraholides A (**27**) and B (**28**) were reported from *Geitlerinema* sp. from Madagascar and possess di-*O*-methyl-lyxopyranoside residues.[72] Biological studies showed that **27** acts in a very similar fashion to swinholide A, inhibiting cancer cell growth and disrupting the actin cytoskeleton.[73]

Other glycosidated metabolites include the unusual chlorinated alkaloid **29**, with an attached dimethoxyxylose residue.[74] It has been speculated that such alkaloids are biosynthetically related to the malyngamides, although this has not yet been supported experimentally. Another unusual class is the tolyporphins, glycosidated porphinoids typified by tolyporphin A (**30**), which was reported from *T. nodosa* in 1992,[75] although the structure was later revised after its total synthesis was completed.[76] These metabolites can reverse multidrug resistance in cancer cell lines and act as potent photosensitizers.[77,78] The polycarvernosides are another group of polyketide–glycosides, which were isolated as the toxic metabolites from a fatal poisoning in Guam.[79] While the consumed seaweed in this poisoning event was the red alga *Polycavernosa tsudai*, these toxic glycosides almost certainly are derived from cyanobacteria that are epiphytic upon these macroalgae.

In spite of this diversity of sugar-containing metabolites, there are only a few solely carbohydrate-based secondary metabolites reported from cyanobacteria. The following are interesting examples.

2.06.5.1 Iminotetrasaccharide

The iminotetrasaccharide **31** was isolated from an *Anabaena* strain collected from New South Wales, Australia.[80] Purification was carried out by reversed-phase (RP) column chromatography and high performance liquid chromatography (HPLC). High resolution fast atom bombardment mass spectroscopy (HRFABMS) suggested a molecular formula of $C_{28}H_{45}NO_{20}$, while peracetylation yielded a product with molecular formula $C_{44}H_{61}NO_{28}$, suggesting the presence of eight free OH/NH groups. The structure was solved primarily by extensive NMR analysis, using correlation spectroscopy (COSY) to establish the fourspin-systems, which were subsequently linked by heteronuclear multiple-bond correlation (HMBC) and rotating-frame nuclear overhauser effect spectroscopy (ROESY) data. Comparison of coupling constants and ^{13}C NMR shifts of the various sugar moieties with literature values identified the three sugar units as glucuronic acid, 3,4-diacetylarabinopyranose, and 3-*O*-methylglucopyranose, which was confirmed by acid hydrolysis of **31**. The configuration of the amino sugar was finally resolved by X-ray analysis.

Iminosugars such as the nojirimycins have been shown to possess potent activities as glycosidase inhibitors, an activity that has proven useful in anticancer applications.[81,82] Metabolite **31** was tested against a range of glycosidases but possessed only moderate activity against *Escherichia coli* glucuronidase. Interestingly, another of the rare cyanobacterial sugar-based metabolites, di(hydroxymethyl)-dihydroxypyrrolidine (**32**) from *Cylindrospermum* sp., also acted as a glycosidase inhibitor.[83]

2.06.5.2 Cyclodextrin

Another intriguing sugar-based metabolite from a cyanobacterium is cyclodextrin **33**, isolated from a Hawaiian *Tolypothrix byssoidea* strain.[84] A range of related metabolites were produced; these proved inseparable and were analyzed as a mixture. However, acetylation of the mixture yielded peracetate derivatives that could be separated by HPLC and analysis of both the purified peracetate and the unmodified mixture was used in the elucidation of the major component **33**. MS and extensive NMR analysis led to the structure shown. 2D NMR analysis was crucial in the determination of the individual sugar units, while their sequence was solved by difference nuclear overhauser effect (NOE) experiments. The configurations of all sugar units were presumed to be D on the basis of comparison with related cyclodextrins. The *Tolypothrix* cyclodextrins were found to block the activity of some of the other toxic metabolites produced by this cyanobacterium.[84]

25

26

27 R = H
28 R = Me

29

31

30

32

33

2.06.6 Peptides

Cyanobacteria produce a wide range of peptides and other amino acid-derived metabolites. The diversity of cyanobacterial peptides has been reviewed recently, and the introductory section of this chapter gives some further insights into the metabolic trends of cyanobacterial peptides.[8,85] They range in size from compounds derived from a single pair of amino acids to multicyclic polypeptides of 1800 Da, and a number of them possess unusual structural features. A particular characteristic is the incorporation of nonprotein amino acids, including D-amino acids, as well as many more exotic examples, some of which are described below. These amino acids, both standard and nonstandard, are often further modified by N- and O-methylation, sulfation, halogenation, glycosidation, oxidation, dehydration, heterocyclization, prenylation, ketide extension (covered in Section 2.06.8), and various other modifications.

Such structural features are typical of nonribosomal peptides and indeed the majority of cyanobacterial peptides are believed to be synthesized by NRPS systems. Nonribosomal peptides, in contrast to ribosomal peptides, are synthesized by protein complexes without the involvement of ribosomal RNA. The genes coding for these huge complexes are some of the largest known.[86] These catalytic systems operate in a modular fashion, similar to modular PKSs (described in Section 2.06.7) with each module catalyzing the activation and covalent linkage of a single amino acid to the growing peptide chain. In general, the linear organization of the protein modules corresponds to the order of the amino acids in the final peptide (concept of 'colinearity'), and as such, reasonable predictions of structure can be made based on knowledge of the gene cluster sequence alone. Cyanobacterial nonribosomal peptide biosynthesis has been reviewed recently[85,87] and more general reviews on NRPSs are also available.[86,88]

Minimally, each module requires three domains – an adenylation domain (A), which activates a specific amino acid for incorporation; a thiolation domain (T), also known as a peptidyl carrier protein (PCP), which transfers the growing peptide from one module to another; and the condensation (C) domain, which carries out the condensation of two residues. Analysis and comparison of the primary sequences of numerous adenylation domains has led to the establishment of a code that can be used to predict the amino acid activated by a particular adenylation domain.[89,90] Genetic analysis of these sequences has shown that they clade together based on the amino acid encoded, rather than by species of origin.[91]

Thiolation domains contain a conserved serine residue, to which a 4′-phosphopantetheinyl group is attached – it is this 'arm' to which the growing peptide is attached. The phosphopantetheinyl group is itself attached by a specific enzyme known as a phosphopantetheinyl-transferase (PPTase), the gene for which is often found within the NRPS cluster.[92] Thiolation and condensation domains are also known to influence the selection of amino acids, although to a lesser extent than the adenylation domains.[90,93] Finally, each NRPS cluster usually possesses a single thioesterase domain, which cleaves the peptide from the protein complex, often cyclizing the released peptide in the process.

In addition to these crucial domains, other modifying domains are often found in NRPS modules. These include heterocyclization domains, which catalyze the formation of heterocyclic rings from cysteine, serine, and threonine residues (see Section 2.06.2). Epimerization domains are structurally very similar to condensation domains[94] but catalyze the epimerization of the activated amino acid. N-methylation is very common in cyanobacterial peptides and thus N-methylation domains are commonly found in cyanobacterial NRPS modules, integrated within the adenylation domains.[95] Other modifying domains are known, including oxidation, reduction, and formyltransferase domains. In addition to domains integrated within the NRPS proteins themselves, many tailoring enzymes are found within the gene clusters. These may be involved in the formation of unusual amino acids, in addition to halogenation, sulfation, glycosylation, O-methylation, as well as other modifications. Finally, it should be noted that cyanobacteria produce many hybrid peptide/polyketide structures, and as such many NRPS systems contain PKS elements. These hybrid molecules, classified as lipopeptides, are discussed in more detail in Section 2.06.8 of this chapter.

Following are several examples of cyanobacterial peptides that depict their structural diversity, illustrate some of the trends in metabolism identified in Section 2.06.2, and outline their biological activities and biosynthesis (see Chapters 2.16 and 5.19).

2.06.6.1 Lyngbyatoxins

Lyngbyatoxin A (**34**) was isolated from a Hawaiian *L. majuscula* strain by a combination of size exclusion chromatography and HPLC, and its structure elucidated by the analysis of ^{1}H and ^{13}C NMR data, spin–spin decoupling experiments, and chemical derivatization to the acetate and the tetrahydro derivative.[96] Two oxidized derivatives, lyngbyatoxins B (**35**) and C (**36**), were reported in 1990 from another *L. majuscula* specimen collected from the same beach as the initial lyngbyatoxin A producer.[97] Interestingly, **34** was also isolated from *Streptomyces* strains and is known as teleocidin A-1.[98]

The biology of **34** has been studied in some detail. It causes dermatitis known as 'swimmer's itch'[99] and has been implicated in several poisonings and even deaths due to the consumption of contaminated turtle meat.[100] Subsequent studies on the toxicity of **34** in mice have shown that it causes damage to the gastrointestinal tract, and at high enough levels, death from internal bleeding (i.p. lethal dose of 250 $\mu g\ kg^{-1}$).[101] Mechanistic studies have indicated that **34** also acts as a tumor promoter by binding to protein kinase C, an activity that may suggest its possible use in anticancer therapy.[102] Within their natural environment, the lyngbyatoxins are believed to confer a selective advantage to producing strains by deterring grazing by a range of consuming species.[103]

The lyngbyatoxin biosynthetic cluster was isolated and characterized to span 11.3 kb and consists of four genes *ltxA–D*.[104] *ltxA* is a two-module NRPS that condenses L-Val and L-Trp in a typical fashion. However, rather than being released by a thioesterase, the peptide fragment is reductively cleaved by a reductase (Red) domain to give the primary alcohol. This was confirmed experimentally by heterologous expression of the C-terminal PCP-Red domains of *ltxA*. The purified protein did indeed reduce the synthetic substrate analogue **37** to the corresponding primary alcohol **38** in the presence of nicotinamide adenosine dinucleotide phosphate (NADPH).[105]

ltxB codes for an unusual cytochrome P-450 monooxygenase that was implicated in the oxidation and cyclization of the tryptophan moiety, while *ltxD* encodes an oxidoreductase that is putatively involved in the oxidation of **34** to **35** and **36**. The sequence of *ltxC* was not similar to any characterized gene product, and thus it was believed to be involved in the prenylation of **34**. The LtxC protein was heterologously expressed in *E. coli*, purified by affinity chromatography, and confirmed to produce lyngbyatoxin A (**34**) from geranyl diphosphate and indolactam V (**39**).[104]

2.06.6.2 Aeruginosins

The aeruginosins are a class of cyanobacterial peptide incorporating two characteristic structural features: an N-terminal hydroxyphenyllactic acid residue and the highly unusual amino acid 2-carboxy-6-hydroxyoctahydroindole (Choi). The structural diversity, synthesis, and biological activity of this structure class have recently been reviewed.[13] The first example was aeruginosin 298-A (**40**), isolated from *M. aeruginosa* NIES-298.[106] The structure was elucidated primarily by 2D NMR; however, the absence of any NMR

correlations between the leucine and Choi residues led to some uncertainty. Acetylation caused a characteristic downfield shift in the resonances for the C-6 protons, leading to the structure shown. The configuration of the leucine moiety was initially determined to be L by gas chromatography (GC) analysis, however, it was later reassigned as D by synthetic studies.[107,108] Since the initial discovery of **40**, over 20 additional aeruginosins have been isolated from cyanobacteria, such as microcin SF608 (**41**)[109] and the chlorinated, glycosidated aeruginosin 205A (**17**).[63] Related structure classes include the dysinosins (e.g., dysinosin A, **42**),[110] isolated from sponges but believed to be of cyanobacterial origin.

The aeruginosins and their derivatives are of biological interest due to their activity as inhibitors of serine proteases, in particular those involved in blood coagulation. Metabolite **40** has been cocrystallized with thrombin while aeruginosin 98-B (**43**) has been crystallized with trypsin, shedding some light on their mechanisms of action; in both cases, the guanidinium group occupies the crucial S1 site.[111,112] Chlorodysinosin has been patented for its potent activity as an inhibitor of thrombin (factor IIA), factor VIIA, and factor XA, which are important serine proteases in the blood coagulation pathway.[113]

The biosynthesis of the aeruginosins has only recently been investigated. Screening of the genome of a *Planktothrix* strain for NRPS sequences led to the isolation of a gene fragment, which was subsequently used to screen a genomic library.[64] This led to the sequencing of the entire gene cluster and flanking regions (35 kb total) containing 19 open reading frames (ORFs), 9 of which were believed to be involved in aeruginoside biosynthesis. *AerA* encodes a PKS-like module including a reductase domain, which was proposed to activate phenylpyruvate and then reduce it to the phenyllactate starter unit. *AerB* coded for a single NRPS module with an epimerase domain, expected to attach the D-leucyl group. *AerC–F* were similar to several genes coding for proteins involved in bacilysin biosynthesis and are believed to construct the Choi moiety, while *AerG* codes for another NRPS protein that incorporates the Choi unit into the peptide. *AerH* is implicated in synthesis of the 1-amidino-2-ethoxy-3-amino piperidine (Aeap) moiety, and the glycosyltransferase produced by *AerI* was proposed to attach the sugar to the Choi unit. In the same study, the authors isolated and elucidated the structures of two new aeruginosides (**18**, **19**) associated with the cluster.

2.06.6.3 Cyanopeptolins

Cyanobacteria produce a wide range of cyclic depsipeptides containing an 3-amino-6-hydroxy-2-piperidone (Ahp) group. One of the most common structure classes incorporating such a residue are the cyanopeptolins, which also contain a characteristic lactone linkage involving the β-OH group of threonine. Cyanopeptolins A (**44**) –D were isolated from *Microcystis* sp. PCC7806 by anion exchange and HPLC. Structures were solved by a combination of amino acid analysis, GC, and 2D NMR, with total correlation spectroscopy (TOCSY) experiments proving crucial in the structural determination of the unusual Ahp residue.[114] Numerous further examples of this structure class have since been isolated, including nostocyclin (**45**),[115] the phosphorylated micropeptin T20 (**46**),[116] and aeruginopeptin-95B (**47**), which incorporates the highly unusual amino acid tetrahydrotyrosine.[117] Many cyanopeptolins act as protease inhibitors, with micropeptin T20 possessing an IC_{50} of 2.5 nmol l^{-1}, and numerous others possessing IC_{50}s in the low micromolar range.[116,118]

The biosynthesis of this structure class has been relatively well studied: the first gene cluster to be sequenced was from *Anabaena* strain 90, coding for the production of the formylated anabaenopeptilides 90A (**48**) and 90B (**49**).[119] The depsipeptide is constructed using the standard NRPS machinery by genes *apdA*, *B*, and *D*, which contained two, four, and one NRPS modules, respectively. The gene product of *apdA* contains a formyltransferase domain, which is used to attach the formyl group at the N-terminus of the peptide. ApdC shows some sequence similarity to bacterial halogenases and is believed to carry out the halogenation of the tyrosine residue. The gene *apdE* codes for a methyltransferase that putatively methylates the tyrosine residue, while *apdF* shows significant similarity to a ketoreductase that was initially believed to be involved in the formation of the Ahp residue; however, more recent studies have contradicted this proposal.[120] Based on established knowledge of NRPS machinery, structures **48** and **49** were predicted for the new metabolites, although they were not isolated. A knockout mutant in which the *apdA* gene was interrupted by insertion of a *cat* cassette did not produce **48** or **49**, as determined by MS analysis. In a subsequent study on the same strain, **48** and **49** were isolated and the structures elucidated by standard techniques, confirming the predictions made on the basis of genetic information alone.[121]

The structure of a new cyanopeptolin-984 (**50**) was determined by MS–MS from *Microcystis* cf. *wesenbergii* and the gene cluster responsible for its biosynthesis cloned and sequenced.[120] Its cluster showed significant similarities to that previously isolated and described above. Phylogenetic analysis of numerous cyanobacterial NRPS clusters revealed clustering of adenylation domains based on amino acid selectivity rather than origin, while condensation and thiolation domains grouped according to operon position. A third cluster was sequenced from a *Planktothrix* strain, coding for the sulfated cyanopeptolin 1138 (**51**).[122] This cluster lacked a halogenase gene but included a putative glyceric acid-activating domain and a sulfotransferase. Phylogenetic analysis did not reveal any clear evidence for horizontal gene transfer, suggesting that the clusters had evolved independently within the individual genera. Alternately, the evidence of gene transfer may no longer be obvious due to an ancient gene transfer event.

A number of related cyclic peptides containing the Ahp group (e.g., somamide A (**52**)) have been isolated from marine cyanobacteria of the genera *Symploca*, *Lyngbya*, and *Schizothrix*, and several of these also show interesting profiles of protease inhibition.[123–125]

42

43

44

45

46

(proposed configuration)

47

48 R^1 = Me, R^2 = H
49 R^1 = H, R^2 = Cl

50

2.06.6.4 Cyclamides

The cyclamides are small cyclic peptides that characteristically contain multiple thiazole, thiazoline, oxazole, and oxazoline rings, which are derived from cysteine, serine, and threonine residues. Some of the first examples of this class to be described were the patellamides (**53–55**) from the tunicate *Lissoclinum patella*, although it was later determined they were produced by the symbiotic cyanobacterium *Prochloron* sp. The structures were solved by a combination of acid hydrolysis and GC analysis, coupled with 2D NMR.[126] Smaller cyclic peptides from this class include the hexapeptides westiellamide (**56**)[127] and microcyclamide (**57**) from *M. aeruginosa*.[128] Many members of the class possess cytotoxic properties, although their biological function or mechanism of action is not fully understood. In contrast to many cyanobacterial peptides, aside from the unusual heterocyclic residues, these peptides generally contain only ribosomal amino acids.

The reason for this exclusive use of ribosomal amino acids became apparent when the biosynthetic gene cluster coding for the patellamides was reported in 2005. The patellamides are in fact biosynthesized by a ribosomal process. Initial attempts to locate a NRPS cluster in the genome of the producing cyanobacterium, *Prochloron* sp., were unsuccessful; a single cluster was isolated but it did not correlate with patellamide production.[129] Hence, a basic local alignment search tool (BLAST) search of the *Prochloron* genome was carried out targeting ribosomal peptides, using all eight of the possible sequences leading to patellamide A. A single hit was obtained and eight genes, *patA–G*, were identified that could be responsible for patellamide biosythesis. The entire cluster was heterologously expressed in *E. coli* and the production of patellamides A (**53**) and C (**55**) was confirmed by liquid chromatography-coupled mass spectroscopy (LCMS) analysis. The patellamide gene cluster was also elucidated independently using shotgun cloning and heterologous expression techniques in *E. coli*.[130] *patE* coded for a peptide containing the amino acid sequences for both

compounds 53 and 55; these sequences are subsequently cleaved by the *patA* protease to give the eight-amino acid long precursor peptides. *patD* was implicated in either the cyclization of threonine and cysteine to give oxazoline and thiazoline rings or the cyclization of the overall peptides, while *patG* coded for a protein with homology to several oxidoreductases, and was proposed to oxidize the newly formed heterocycles to the fully oxidized thiazoles.[131] No function was proposed for the other *pat* genes; however, further work has shown that small alterations within the cassettes encoding for patellamide synthesis results in a wide variety of related analogues both naturally[132] and from single point mutations.[133] A subsequent theoretical study proposed that the macrocyclization and epimerization steps occur spontaneously because of the inherent properties of the precursor peptides, rather than being enzymatically controlled.[134]

BLAST searches using *pat* genes revealed the existence of homologues in *Trichodesmium erythraeum*, suggesting the presence of a patellamide-like cluster. A detailed analysis of the sequences enabled the prediction of a potential structure (**58**), and MS/MS analysis of cultures of this strain was consistent with the presence of such a peptide.[135] Analysis of the genome of *M. aeruginosa* NIES298, a microcyclamide producer, revealed the presence of another gene cluster similar to that producing the patellamides. Sequences from this new cluster were used to analyze the genome of another *Microcystis* strain by BLAST, leading to the discovery of yet another cyclamide-producing gene cluster and the isolation of two new microcyclamides (**59**, **60**).[136]

51
proposed configuration

52

53

54 R = isobutyl
55 R = isopropyl

56

57

58
(predicted configuration)

59

2.06.6.5 Anatoxin-a(s)

Anatoxin-a(s) (**61**) is a potent neurotoxin, first isolated from the cyanobacterium *A. flos-aquae*.[137] Despite the name of this metabolite, it is structurally unrelated to anatoxin-a (**62**). The structure was not solved until 1989 through a combination of ^{1}H, ^{13}C, ^{15}N, and ^{31}P NMR experiments on a sample of anatoxin-a(s) that had been biosynthetically enriched in ^{13}C and ^{15}N. In order to solve the absolute configuration at C-5, *R* and *S* isomers of **63**, a degradation product of **61**, were synthesized from D- and L-asparagine. The circular dichroism (CD) curves of the two enantiomers were recorded and compared with those from **63** derived from natural **61**, conclusively proving the configuration of natural **61** to be *S* at C-5.[138]

Anatoxin-a(s) acts as an irreversible inhibitor of acetylcholinesterase,[139] reducing blood pressure and heart rate in rats dramatically.[140] It has been identified as the active agent in a number of animal poisonings, including the deaths of numerous birds in 1993, which coincided with huge cyanobacterial blooms in freshwater lakes.[141] As a result, a biosensor has recently been developed for the detection of **61** in environmental samples.[142]

The biosynthesis of **61** has been investigated by several feeding studies. Studies with ^{14}C-labeled amino acids proved that C-2, C-4, C-5, and C-6 were derived from L-arginine, while the *N*-Me and *P*-OMe groups were determined to originate from the tetrahydrofolate C-1 pool.[143] Subsequent studies with [3,3,4,4,5,5-$^{2}H_6$]-arginine showed that only three deuterium atoms were retained in the product, two at C-4 and one at C-5. This suggested the involvement of 4-hydroxyarginine (**64**) as an intermediate in the biosynthesis of **61**, a hypothesis confirmed by feeding labeled 4-hydroxyarginine to the cyanobacterium.[144]

2.06.7 Polyketides

Polyketides are a diverse class of compounds that are often created by a series of modular enzymes which condense and then modify chains of acetate or propionate units primarily through reduction, dehydration, cyclization, and aromatization reactions. Polyketides, with their enormous structural variety, show a broad range of biological activities (see Chapters 1.10 and 1.11).

2.06.7.1 Biosynthesis of Polyketides

Polyketide biosynthesis has been well studied and there are several reviews regarding the general construction of these metabolites.[145,146] Generally, polyketides are initiated with acetate (or propionate) derived from either acetyl-CoA (or propionyl-CoA) or malonyl-CoA (or methylmalonyl-CoA). The chain is extended by further addition of acetate units by a ketosynthase and acyltransferase domain. The acetate units can then be altered by the inclusion of a variety of domains within the PKS pathway. Common examples of alterations are ketoreductase,

dehydratase, enoyl reductase, and methyl transferase domains. Ketoreductase domains stereoselectively reduce the β-carbonyl to an alcohol functionality. Following ketoreduction, a dehydratase catalyzes dehydration to form an α, β-unsaturated double bond. An enoyl reductase is then optionally deployed to reduce the double bond resulting in the overall conversion from a carbonyl unit to a methylene unit. Methylation of the α-carbon is another common modification of polyketides and can occur through a couple of different pathways. Transfer of the methyl group from SAM to the α-carbon involves a methyl transferase acting before reduction of the β-carbonyl functionality. Alternatively, a polyketide can form with incorporation from a methylmalonate unit (deriving from propionate) rather than malonate (deriving from acetate), thus producing a pendant methyl group at the α-carbon. After assembly of the complete polyketide chain, cleavage occurs via operation of a thioesterase resulting in either a linear polyketide chain or sometimes directly forming a cyclized macrolide polyketide. In cyanobacteria, there is little if any utilization of propionate in polyketide pathways, but variable levels of reduction and methylation, including O-methylation of a β-hydroxy group, are quite common.

2.06.7.2 Oscillatoxin/Aplysiatoxin

Aplysiatoxin (**65**) was first isolated from the gut of *Stylocheilus longicauda*, a sea hare, by Kato and Scheuer[147] but was later shown by Moore and coworkers[148] to be a secondary metabolite of *L. majuscula*, a major component of the sea hare's diet. Aplysiatoxin and related compounds, debromoaplysiatoxin and oscillatoxin A (**66**), were initially isolated due to their proinflammatory actions, known from algal blooms that result in contact dermatitis known as 'swimmer's itch'.[149] A number of analogues with altered bromination and oxygenation have been isolated from cyanobacterial sources; for example, oscillatoxin A was first isolated from *O. nigroviridis* in 1978 by Mynderse and Moore[149] with its structure being elucidated by MS and NMR comparison to aplysiatoxin. The absolute stereochemistry of these compounds was not fully elucidated until 1984 using CD analysis of the natural products and degradation products.[149] The tetrahydropyran–tetrahydropyran (THP–THP) spiro system attached to a macrolactone in these compounds is unique and has presented a challenge in both structure elucidation and synthetic efforts. It is interesting to note that oscillatoxin D contains a hexane–THP spiro system and is lacking the macrolactone, suggesting that its biosynthetic pathway utilizes an alternate cyclization domain or a single cyclization domain that may be capable of facilitating both the THP–THP spiro and hexane–THP spiro cyclizations.

This family of metabolites has been shown to be potent tumor promoters, making them useful pharmacological tools for the study of cancer.[150] The total synthesis of aplysiatoxin and debromoaplysiatoxin was first accomplished by the Kishi group at Harvard in 1987.[151] No other synthetic routes have been reported to date but newer chemistry has allowed for the synthesis of some of the fragments more efficiently.[152–154] Investigation of the biosynthetic pathway leading to the aplysiatoxins has not occurred to date but certainly holds some interesting surprises as it relates to formation of the unique pyran ring system, high level of methylation, and combination of two polyketide units to form a macrolactone.

2.06.7.3 Acutiphycin

Acutiphycin (**67**) was isolated from *O. acutissima* found in a freshwater pond in Oahu, Hawaii.[155] This compound was found to possess cytotoxic and antineoplastic activity along with *in vivo* activity against murine Lewis lung carcinoma.[155] The structure of this macrolide was elucidated by NMR in combination with degradation studies using ozonolysis and acid hydrolysis. Since its initial isolation, however, the cyanobacterium no longer produces acutiphycin (**67**), thus requiring total synthesis for any further investigation of its chemical and biological properties. The first total synthesis was reported in 1995[156] with another reported in 2006 that capitalized on a convergent strategy to decrease the number of linear steps while also utilizing different connection strategies.[157] No studies have investigated the biosynthesis of acutiphycin.

2.06.7.4 Scytophycin/Tolytoxin/Swinholide

An example of a compound displaying both cytotoxic and antifungal activity is given by scytophycin A (**68**). The scytophycins were initially isolated from the terrestrial cyanobacterium *Scytonema pseudohofmanni* in 1986 by Ishibashi *et al.*[158] whereas tolytoxin (**69**) was initially isolated in 1977 from *Tolypothrix conglutinate*, but due to

small amounts of material the structure was not fully elucidated until 1990.[159] Tolytoxin was found to be a closely related analogue, differing from scytophycin B by a single methoxy group, but retaining cytotoxic and fungicidal activity. The biosynthetic pathway of tolytoxin has been fully explored with stable isotope feeding experiments.[160] The starting unit is a glycine residue, which is then chain elongated by 15 actetate units to form a 32-member polyketide chain. This chain then undergoes two cyclizations resulting in the formation of dihydropyran and macrolactone rings. This pathway displays the versatility of PKS pathways for incorporating a variety of starting materials in order to synthesize diverse molecular frameworks, and in this regard, formally represents an example of a peptoketide (see Section 2.06.8.1). The pathway to the scytophycins is likely to be nearly identical to that of the tolytoxins but with a few altered domains that result in a different oxygenation and methylation pattern.

Swinholide A (**70**), a compound initially isolated from a red sea sponge known to associate with symbiotic cyanobacteria, is structurally similar to the scytophycins, appearing to be a dimer of two polyketide chains. While it was initially isolated from the sponge *Theonella swinhoei*, collections of the cyanobacteria *Symploca* cf. sp. from the Fiji Islands also yielded swinholide A, suggesting that it is likely a cyanobacterial metabolite.[72] Moreover, a collection of the cyanobacterium *Geitlerinema* sp. from Madagascar also yielded glycosylated swinholides, named ankaraholide A (**27**) and ankaraholide B (**28**), which were discussed earlier in Section 2.06.5.

60 **61** **62** **63**

L-arginine → **64** → **61**

65 **66**

67 **68**

69

2.06.7.5 Nakienone

A family of small polyketide compounds, named the nakienones A–C (**71**) as well as nakitriol, were isolated from a mat of *Synechocystis* that had overgrown a section of coral near the island of Okinawa. The cyanobacterial overgrowth was believed to be the cause of death of this section of coral.[161] The nakienones were shown to possess cytotoxic properties varying among different tumor cell types and specificity between the various analogues. Structures were determined using 2D NMR techniques and by comparison of fragments made synthetically. They are structurally similar to the didemnenones, which were the first non-nitrogenous compounds isolated from the didemnid tunicates *Didemnum voeltzkowi* and *Trididemnum* cf. *cyanophorum*. The didemnenones display cytotoxic activity in addition to antifungal and antibacterial properties.[162]

The total synthesis of nakienone A was reported by Negishi in 1997.[163] Negishi's synthesis used a Pd-mediated cross-coupling reaction to install the pentadiene functionality on the cyclopentene ring. The total synthesis not only proved the original stereochemical determinations to be correct but also represented a facile method for the synthesis of the entire series of natural compounds as well as analogues for structure activity relationship (SAR) studies.

2.06.7.6 Nostocyclophanes

Paracyclophanes were initially known only from synthetic sources until 1990 when bioassay-guided fractionation of the extracts of two cyanobacteria, *Cylindrospermum licheniforme* and *N. linckia*, resulted in the isolation of cylindrocyclophane A and nostocyclophane D (**72**), respectively.[164] Three other additional glycosylated derivatives were also isolated from the *N. linckia* extract and all four compounds showed cytotoxic activity with the most potent being D with an $IC_{50} = 0.5\,\mu g\,ml^{-1}$.[165] Cylindrocyclophane lacks the chlorination present in the nostocyclophanes but is composed of the same basic polyketide skeleton and also shows cytotoxic activity. Structures of the nostocyclophanes were determined by NMR and mass spectral methods along with CD; an X-ray crystal structure of nostocyclophane D confirmed these assignments. Culturing of *N. linckia* allowed for feeding studies to help elucidate the biosynthetic pathway to these cyclophanes.[166] Two potential pathways were initially envisioned, one involving shikimic acid and the other involving a polyketide logic. Stable isotope feeding studies indicated that the nostocyclophanes arise from a nonaketide. Feeding experiments with ^{13}C- and ^{2}H-labeled acetate precursors followed by 2D NMR analysis revealed that all of the carbons present in the paracyclophane core, including the aromatic ring, arose from acetate. By using double-labeled ^{18}O and ^{13}C acetate, they were able to show that the phenolic hydroxyls were acetate derived, thus confirming the ^{13}C-labeled acetate studies. While the source of the carbon atoms of the nostocyclophanes is clear, there are still a number of intriguing questions regarding the mechanisms and timing of dimerization and halogenation.

2.06.7.7 Caylobolide

Isolated from a Bahamian collection of *L. majuscula*, caylobolide (**73**) is a unique 36-membered macrolactone that shows cytotoxicity to human colon cancer cell line HCT-166.[167] Caylobolide contains a 1,3,5-triol and a repeating 1,5-diol within the macrocycle. The 1,5-diol had not been reported before the isolation of caylobolide and points to a unique biosynthetic pathway. Additionally, this compound is interesting because of its being atypical of the metabolites usually isolated from cyanobacteria. The 1,5-diol repeat is likely produced from PKS modules that catalyze full reduction of the β-ketone alternating with those that simply reduce the β-ketone to an

alcohol functionality. The secondary methyl group β to the lactone carbonyl is at a site predicted to derive from C-1 of acetate, and hence, likely involves an 3-hydroxy-3-methyl glutarate-CoA (HMG-CoA) synthase-type reaction.[167] The relative stereochemistry of the 1,3,5-triol was determined by means of Kishi's universal NMR database, allowing comparison of the observed NMR shifts with those of previously known compounds. Absolute stereochemistry of these three positions as well as the alcohol at C-33 was determined using Mosher's analysis of the methoxy trifluoromethyl phenyl acetic acid (MTPA) esters. The *in vitro* cytotoxicity was found to be 9.9 $\mu mol\,l^{-1}$ with no antifungal activity against two different strains of *Candida*.

2.06.7.8 Oscillariolide/Phormidolide

A sample of *Oscillatoria* sp., collected in Gokashowan Bay, Japan, was cultured and found to produce the polyketide compound **74** and given the trivial name oscillariolide.[168] Oscillariolide consists of a 14-member macrolactone with an attached tetrahydrofuran ring and a polyketide chain containing a terminal bromoalkene. The structure was elucidated using MS and 2D NMR techniques especially COSY, heteronuclear multiple quantum coherence (HMQC), HMBC, and homonuclear Hartmann-Hahn (HOHOHAHA) for connectivity and spatial orientation. Oscillariolide inhibited cell division in fertilized starfish eggs at 0.5 $\mu g\,ml^{-1}$.[168]

A related compound, phormidolide (**75**), was isolated from *Phormidium* sp. collected in Indonesia using the brine shrimp assay to guide its isolation.[169] Phormidolide contains a 16-member macrolactone ring with fused tetrahydrofuran and polyketide chain with a terminal bromoalkene and additionally has a long-chain fatty acid attached to one of the hydroxyls of the polyketide chain. The initial extract showed activity at inhibiting the Ras–Raf protein interaction, which is upregulated in many cancer cell types; however, this activity was traced to a tetrapyrrole metabolite related to chlorophyll (Singh and Gerwick, unpublished). Its structure was determined with a variety of 2D NMR techniques including the development of a new HSQMBC technique, which allowed for the determination of long-range heteronuclear coupling constants even for overlapping complex multiplets, and in combination with NOE data from GROESY experiments, these were valuable for stereochemical determination.[170] Absolute stereochemical assignments were made using Mosher's ester analysis. While the biosynthetic pathways of neither of these compounds have been reported, they likely have a number of interesting features as they possess several pendant methyl groups at C-1 positions of the acetate building blocks and an intriguing terminal bromoalkene functionality.

2.06.7.9 Borophycin

A culture of the same collection of *N. linckia* that contained the nostocyclophanes also yielded an interesting boron-containing compound (**76**). A lipophilic extract displayed cytotoxicity toward human epidermoid carcinoma and colorectal adenocarcinoma and subsequently yielded borophycin (**76**) as the active compound.[171] Its structure was determined by mass spectral and NMR techniques and was found to be similar to the known boron-containing antibiotics boromycin and aplasmomycin. Absolute stereochemical assignments were made by X-ray crystallography as well as comparisons with boromycin and aplasmomycin. Biosynthetic feeding studies with the cultured cyanobacterium were able to show that the entire borophycin skeleton arises from the incorporation of acetate units with the pendant methyl groups deriving from methionine. This is dissimilar to boromycin and aplasmomycin in which the pendant methyl groups have been shown to derive from the incorporation of methylmalonyl units that derive from propionate.[171]

2.06.8 Lipopeptides

2.06.8.1 Ketide-Extended Amino Acids (Peptoketides)

2.06.8.1.1 Dolastatin 10

The extracts from a large collection of *Dolabella auricularia* sea hares from the Indian Ocean were evaluated at the National Cancer Institute and showed an anticancer effect in the P388 lymphocytic leukemia mouse model. After 15 years of intense effort, an exceptionally potent toxin known as dolastatin 10 was isolated and its structure determined using extensive degradation by hydrolysis in concert with 2D NMR and various MS methods.[172] The peptide was

present in the sea hare in very low yield, which made its characterization particularly challenging. Ultimately, it was shown that dolastatin 10 and others in the compound class derive from the sea hare's diet of marine cyanobacteria, in particular, *Symploca* sp.[173] Dolastatin 10 (1) and its analogues have generated much excitement due to their potent *in vivo* anticancer properties. Dolastatin 10 has been shown to act via disruption of cancer cell microtubule networks, thus disturbing the normal cell division process (i.e., it is antimitotic). Although this natural product progressed into Phase II clinical trials, it was ultimately dropped as a single agent due to undesired peripheral toxicity. Chemical modification efforts to reduce toxicity resulted in the synthesis of TZT-1027 (77, Auristatin; Soblidotin), which recently completed a Phase II clinical trial in patients with advanced or metastatic soft tissue sarcomas.[174]

Dolastatin 10 is composed of several interesting amino acid residues, including a terminating *N,N*-dimethyl valine, which is present in quite a number of bioactive cyanobacterial peptides (e.g., coibamide),[175] and an adjacent pair of ketide-extended residues (one isoleucine and one proline). In both cases, these ketide-extended residues are reduced in their β-ketone functionality to the alcohol level, and then O-methylated; in the latter residue, C-methylation also occurs at the α-carbon. It is intriguing to speculate that these tandem sequences may have arisen through a gene duplication event. The C-terminus of the linear peptide is composed of a condensed phenylalanine and cysteine residue, termed 'dolaphenine', in which the thiazoline ring formed by condensation of the cysteine sulfur and phenylalanine carbonyl followed by dehydration is oxidatively decarboxylated to the monosubstituted thiazole ring. The same or similar C-terminus modification is seen in other cyanobacterial metabolites, such as barbamide (**78**).[176]

2.06.8.1.2 Barbamide

The same Curaçao collection of *L. majuscula* that yielded curacin A (**3**) also gave the curious lipopeptide barbamide (**78**).[176] Barbamide was isolated as a snail toxic compound from the active extract with an LD_{50} of $10\,\mu g\,ml^{-1}$ to *Biomphaleria glabrata*. As such, it possibly contributes to the chemical defense of this cyanobacterium by inhibiting feeding by marine mollusks. Its structure was deduced from a series of subunits composed of a thiazole, *N*-methyl phenylalanine-like unit, a β-methoxy amide, a CH_2–CH–CH_3 spin system, and a deshielded carbon atom at 105 ppm. These partial structures were assembled by HMBC and through comparison with a related series of compounds known from the tropical sponge *Dysidea herbacea*.[177] Stereochemistry was completed later by a combination of degradation routes and biosynthetic incorporations,[178] and ultimately confirmed by synthesis.[179]

A series of papers gave an outline of barbamide biosynthesis through the feeding of various stable isotope-labeled precursors to cultures of the barbamide-producing *L. majuscula*.[178] This was further explored through cloning of the barbamide biosynthetic gene cluster, which was composed of 13 ORFs of 26 kb overall length.[180] The molecular logic for barbamide biosynthesis became clear with the initiation being selection of leucine as substrate, its chlorination by a tandem pair of novel radical halogenases,[181,182] the first catalyzing dichlorination and the second completing the conversion to a trichloromethyl group. This is followed by transamination and subsequent transfer to the PCP domain of BarE, a PKS module, wherein the carboxyl carbon of the leucine unit is lost via an unknown process, and then extended by two carbons through a PKS extension. An enolic β-methoxy group is produced using an *O*-methyl transferase, and then passage to a bimodular NRPS occurs for the incorporation of phenylalanine and cysteine. Barbamide's structure is completed by heterocyclization of the terminal cysteine, dehydration, and oxidative decarboxylation to the corresponding thiazole. Truly, barbamide (**78**) has a fascinating biosynthesis with many mechanistic features awaiting future investigation. Of note here, it represents a 'minimal' lipopeptide with a single acetate extension contributing just two carbon atoms from this source.

2.06.8.2 Simple Ketopeptides

2.06.8.2.1 Hectochlorin

A culture of *L. majuscula* deriving from collections in Jamaica was examined for biologically active and structurally unusual constituents.[183] Two such classes of compounds were isolated, each of a mixed polyketide and peptide origin. The first of these reported, hectochlorin (**79**), was isolated as a potent brine shrimp toxin, and subsequently shown to induce hyperpolymerization of eukaryotic actin.[183] This mechanism also explains the drug's potent *in vitro* cytotoxicity to cancer cells ($LD_{50} = 20\,nmol\,l^{-1}$ in CA46 human Burkitt lymphoma cells). The planar structure was deduced by NMR methods and because diffraction quality crystals were formed, an X-ray experiment was used to confirm structure and establish both relative and absolute stereochemistries. The structure of hectochlorin (**79**) is intriguing on several accounts and falls into a subclass of cyanobacterial compound populated by nearly a dozen compounds isolated from other collections of *L. majuscula* or gastropod mollusks, which feed on this cyanobacterium (i.e., *D. auricularia*).[184–186] Interesting structural features include the gem-dichloro functionality at the penultimate carbon of the polyketide section and the occurrence of two 2,3-dihydroxyisovaleric acid units alternating with thiazole–carboxylate moieties. Moreover, the two hydroxy acid units are incorporated into hectochlorin in alternate orientations (one forms a

lactone ester with its secondary hydroxyl group whereas the other employs the tertiary hydroxyl in this regard). Hectochlorin (**79**) shows potent antifungal activity to several crop disease fungi, and this provided motivation for its total chemical synthesis by an agrochemical company.[187]

Further insight into the biosynthetic origin of hectochlorin was obtained from characterization of the putative gene cluster encoding for its biosynthetic enzymes.[188] The gene cluster was located on two fosmids through library screening with probes developed to the PKS and cysteine heterocyclization domains and revealed a 38 kb cluster with all of the predicted domains for hectochlorin biosynthesis. Thus, the deduced molecular logic for hectochlorin assembly involves activation and acyl carrier protein tethering of a hexanoic acid unit, which is subsequently dichlorinated by a radical-type halogenase.[189] This is subsequently chain extended with malonyl-CoA, C-methylated at the α-carbon withSAM, reduced in its β-carbon to an *S*-alcohol, and then passed on to a bimodular NRPS. The first of these NRPS modules incorporates 2,3-dihydroxyisovaleric acid (likely as the keto acid, which is reduced to the secondary alcohol in trans) followed by cysteine; the latter unit is subsequently heterocyclized and oxidized to a thiazole ring. A nearly identical second bimodular NRPS catalyzes the same sets of reactions; however, as noted above, the orientation of the 2,3-dihydroxyisovaleric acid in the growing molecule is opposite; the molecular basis for this change in orientation is not understood. A thioesterase appears to catalyze both macrocyclization and product release from the tethering ACP.

2.06.8.2.2 Antanapeptin A

Another common class of lipopeptide from cyanobacteria includes a starter PKS lipid section, almost always found as an eight-carbon chain (exceptions being jamaicamide,[190] carmabin,[191] and palau'amide[192]), in which oxidation of the ω-terminus occurs to typically produce a terminal alkyne functionality. Such terminally oxidized PKS motifs can be further dissected into those with an overall linear structure, such as the jamaicamides,[190] apramides,[191] dragonamides,[193] carmabins,[194] and dragamabin,[195] and those possessing a 3-hydroxy or 3-amino group that is used to produce an overall cyclic structure. Cyclic examples of this type of metabolite are more plentiful and include the yanucamides,[196] georgamide,[197] the antanapeptins,[198] pitipeptolide,[199] palau'amide,[192] ulongapeptin,[200] malevamide C,[201] the guineamides,[202] and wewakpeptin.[203] Another trend in this class is that they all possess either one or two *C*-methyl groups at the 2-position of the lipid chain, giving rise to either a 3-hydroxy-2-methyloctynoic acid (Hmoya) or a 2,2-dimethyl-3-hydroxyoctynoic acid (Dhoya) unit. A number of variations on this theme occur with regard to the type and degree of terminal oxidation, with examples being a terminal alkene (jamaicamide C) or bromoalkyne (jamaicamide A (**93**)),[190] a terminal methyl ketone (carmabin B),[194] a penultimate gem-dichloro function (hectochlorin (**79**)),[183] or a completely saturated terminus (e.g., a methyl group as in antanapeptin C).[198]

An example of this terminal acetylene-containing lipopeptide is given by antanapeptin A (**80**), isolated from a Madagascar collection of *L. majuscula*.[198] The distinctive lipid chain in antanapeptin A was initially suggested by acetylenic carbons at δ83.5 and 68.9 and normal 2D NMR techniques used to connect this spin system to form a Hmoya unit. HMBC connected this substructure to a series of amino acids, some of which were N-methylated. Marfey's analysis was used to determine the amino acid stereochemistry and GC–MS versus standards used for the lone hydroxyacid; the Hmoya unit configuration was not established. The related antanapeptins B–D were variants in the lipid chain oxidation level or possessed simple aliphatic amino acid exchanges (e.g., a valine in antanapeptin D replaces the isoleucine in antanapeptin A). To date, a significant biological property has not yet been associated with these substances, leaving in question their natural biological role.

Additionally, the biosynthetic logic for the acyclic compounds of this class are related to a well-represented set of metabolites from the freshwater cyanobacteria *M. aeruginosa*, *O. agardhii*, and *Nostoc* sp. and are known as the microginins (e.g., microginin T1, **81**),[204] oscillaginins,[205] and nostoginins.[206] These latter compounds are initiated by a PKS-derived section composed of 10-carbons in length (except for the nostoginins, which are still 8-carbons) that then transitions into a linear peptide of 3–5 residues in length. All of these lipid portions possess an α-hydroxy group and a β-amino function, so it is perhaps surprising that no cyclic forms of these compounds have yet been reported. The lipid section of these molecules is either completely reduced or uniquely oxidized on their ω-terminus with either a mono- or dichloro methyl group. This type of halogenation is intriguing because of its resemblance to that found in barbamide with its trichloromethyl group that has been

shown to be oxidized by two tandem radical halogenases, the first catalyzing dihalogenation and the second only operating on the dichloro product to form the trichloromethyl group.[181,182] Hence, in the microgenins, it appears that radical halogenases are operating which have different reactivity to form both mono- and dihalogenated products.

Finally, there are a few other miscellaneous lipids of this general origin but which possess differently functionalized lipid chains. For example, jamiacamide B (82) possesses the terminal acetylene functionality, however, the chain is further elongated and elaborated with pendant vinyl chloride and secondary methyl groups.[190] Viridamide (83) is another extended PKS example of this biosynthetic logic but contains a methoxy group at the $\omega 6$ position.[207] A shorter version of this 'starter' logic is found in micromide (84), which is composed of a hexanoic acid with a β-methoxy group.[208] In fact, many other cyanobacterial lipopeptides are initiated by a parallel logic (e.g., the somamides (e.g., 52)[124]) in which a short lipid chain is found at the terminus; whether this is constructed by PKS extension of an acetate initiator unit or selected for directly as butyrate or hexanoate is unknown at present.

2.06.8.2.3 Makalika ester

Some sea hares are well-known specialist feeders on cyanobacteria (e.g., *D. auricularia*) and in Hawaii this includes the mollusk *S. longicauda*. A collection of this mollusk from Black Point near Diamond Head in Oahu yielded a number of well-recognized *Lyngbya* metabolites, including several malyngamides, lyngbyatoxin A acetate, and two new and related polyketides of interesting structure, makalika ester (85) and makalikone ester.[209] They were both isolated in small yield and structures determined by various NMR methods with chemical shift reasoning and HMBC data playing prominent roles. Makalika ester showed modest cytotoxicity to several cancer cell lines (2.5–5 $\mu g\ ml^{-1}$).

These two makalika natural products are biosynthetically interesting for several reasons. First, they illustrate the *tert*-butyl starter group in common with several other interesting cyanobacterial products (e.g., apratoxin A (86)[210] and antillatoxin (87)[211]), which if incorporated as *tert*-pentanoic acid or its equivalent, would place the pendant *exo*-olefin at a C-1 position from a first acetate extension. This would imply the production of the carbon branch by an HMG-CoA synthase-like mechanism, as discussed elsewhere in this review.[212,213] Next, it appears that three further acetate extensions occur; however, there is little biosynthetic precedence or knowledge of how the terminal vinyl chloride is formed. Finally, the mode of assembly of the lipopeptide is interesting for it is an alcohol that derives from the incomplete reduction of the starter unit carbonyl that becomes esterified with a modified proline residue (N-methylated in 85). With this nonstandard juncture between ketide and amino acid subunits, the biosynthetic logic for makalika ester formation is not obvious.

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2.06.8.2.4 Microcystin LR

The microcystins are a family of nearly 100 related cyclic peptides from cyanobacteria of the genera *Microcystis*, *Aphanisomenon*, *Anabaena*, *Nostoc*, and *Planktothrix*, which show inhibitory activity to mammalian protein phosphatases PP1 and PP2a.[214] It is these biochemical targets and the resulting hepatoxicity that led to their original discovery from *M. aeruginosa*.[215] Toxic response to microcystins have been documented in both livestock and human populations.[216,217] Structural diversity in the series is created by amino acid substitutions at several positions; the second and fourth residue downstream of the carboxyl side of the ADDA group (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid) in microcystin LR are particularly variable. Microcystin LR (**88**) is the dominant compound in the family and its biosynthesis has been studied through precursor feeding studies, a molecular genetic approach, as well as a PCR-based approach.[218–220] The ADDA group arises from activation of a phenylproponoid, followed by loss of the carbonyl carbon, and then progressive lengthening by four PKS extensions, three of which also add a methyl group to the α-carbon from SAM.[218,221] A variable level of reduction is observed during processing of the intermediate β-carbonyl function to form the remnant oxidations and unsaturations observed in the ADDA unit. A glutamic acid unit is next added, followed by serine, however, with amide bond formation occurring through reaction with the side chain carboxyl functionality of the glu residue (see discussion of largazole (**89**) Section 2.06.8.3.10). The serine is modified by both N-methylation and dehydration. Alanine and leucine are next added, followed by an aspartic acid residue. The aspartate residue is modified by β-methylation and then condenses through its side chain carboxyl to form an amide with arginine. Macrocyclization and release from enzyme tethering occurs through reaction with the β-amino functionality of the ADDA group. The ADDA group and related residues are found in several other metabolites, including the nodularins (**90**) from *Nodularia* sp.,[222] nostophycin (**91**) from *Nostoc* sp.,[223] and motuporin (**92**) from the sponge

T. swinhoei.[224] Because human and animal health are adversely affected by this family of cyclic peptides, there are monitoring and surveillance programs for the microcystins in several countries.[225]

2.06.8.3 Complex Ketopeptides

2.06.8.3.1 Jamaicamide A

Two distinctive series of secondary metabolites were isolated from a cultured Jamaican *L. majuscula*, hectochlorin (**79**) and the jamaicamides.[190] The structures of the jamaicamides were assembled by normal spectroscopic methods in addition to being an early test case for a new NMR pulse sequence, the Accordion 1,1-Adequate, which facilitates detection of all two-bond J_{CCH} couplings in a molecule.[226] The major metabolite, jamaicamide A (**93**), was unique in its possession of an acetylenic bromide functionality, only previously observed once before in nature.[227] The jamaicamides show moderate neurotoxicity in a cellular model with low micromolar inhibition of the VGSC.

A series of isotope-labeled acetate and amino acid feeding experiments identified the structural units comprising jamaicamide A (**93**), with the most notable features being the highly integrated combination of amino acids with polyketide fragments and a pendant vinyl chloride functionality deriving from C-2 of acetate.[190] The latter feature was recognized to be consistent with a recently described pathway involving a cassette of genes with an HMG-CoA synthase-like core motif, which adds acetate to a β-carbonyl functionality in a growing polyketide.[212,213] This observation was used in designing an efficient cloning strategy for the jamaicamide biosynthetic gene cluster.[190] Bioinformatic analysis of this cluster was highly revealing of an overall progressive biosynthetic logic for assembly of jamaicamide A, an analysis facilitated by a high degree of colinearity between the gene cluster and utilization of the biosynthetic proteins. The pathway begins by activating hexanoic acid, desaturating this to a terminal alkyne, bromination, and then one round of PKS extension. At this point, the β-branch is introduced and by chemistry not yet understood, converted to a vinyl chloride functionality. This is progressively followed by additional PKS extensions, β-alanine incorporation, PKS extension, α-alanine incorporation, and one final PKS extension. Again, by transformations not yet understood, the product jamaicamide A is completed with formation of a pyrrolinone ring. While the structure and biosynthesis of the jamaicamides are unique, several features are seen in a number of other cyanobacterial natural products. For example, the pendant vinyl chloride functionality is typical in the malyngamides (e.g., malyngamide C (**6**)[27]) while the pyrrolinone ring is common to a diverse array of cyanobacterial compounds including the microcolins (e.g., **96**)[228] and majusculamides.[1]

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2.06.8.3.2 Mirabimide E

The extract of a cultured terrestrial cyanobacterium, *S. mirabile* from Hawaii, was found to produce a solid tumor-selective cytotoxin that was isolated by HPLC and named mirabimide E (**94**).[229] Structure elucidation of this new metabolite was complicated by nonstandard behavior both by MS and by ^{13}C NMR, the former giving only fragment ion masses in normal matrices used in FABMS, and the latter only giving the full complement of carbon atom signals when the delay time between pulses was extended to 5 s. These and other challenges in the structure elucidation were overcome by enriching mirabimide E with 80% ^{13}C and 90% ^{15}N using $NaH^{13}CO_3$ and $Na^{15}NO_3$, respectively, in the culture media, and then access to additional NMR experiments, including the INADEQUATE experiment. Stereochemistry in mirabimide E (**94**) was determined by a series of degradation reactions yielding fragments that were analyzed either by chiral GC–MS (an alanine unit cleaved from the pyrrolinone ring) or by Mosher's NMR analysis (the tetrachloro-3-hydroxy-2-methyl decanoate). This lead paper on mirabimide E also completed a total enantiospecific chemical synthesis of mirabimide E so as to confirm structure and provide material for biological assay.[229] Mirabimide E showed strong solid tumor-selective cytotoxicity at 5 $\mu g\,ml^{-1}$ to colon adenocarcinoma 38 using solid agar conditions.

Some features of the biosynthesis of mirabimide E (**94**) were examined through feeding experiments with [1,2-$^{13}C_2$]acetate and [methyl-^{13}C]methionine, which showed the decanoate and C-4 and C-5 of the pyrrolinone unit to derive from six units of acetate, and the C- and O-methyl groups to derive from methionine. Considering the models provided by jamaicamide A (**93**)[190] and barbamide (**78**),[176] it seems likely that the decanoate may derive from hexanoate, which is multiply halogenated by a radical halogenase related to that described from barbamide. This would be extended by two rounds of PKS extension, C-methylation with SAM and simple ketoreduction of the β-carbonyl formed after the second extension. An NRPS with specificity for L-alanine is likely responsible for amide bond formation and this product is then extended by one more acetate

unit. While the mechanism of conversion of this tethered product to a methoxypyrrolinone ring is unknown, this functional group, as well as analogues formed by inclusion of other amino acids, is characteristic of a number of cyanobacterial compounds, including the jamaicamides[190] and microcolins.[228]

2.06.8.3.3 Madangolide

A collection of *L. bouillonii* from Papua New Guinea yielded an extract that was intensely investigated for new natural products, yielding madangolide (**95**) and related substances.[230] The structure of madangolide was established from straightforward NMR and MS analysis; however, some complexities were encountered due to signal degeneracy. Nevertheless, through careful spectroscopic analysis with selective irradiations, a complete planar structure was deduced with the two olefinic bonds both being established as *Z*. Madangolide (**95**), as a representative of these complex ketopeptide macrolides from *L. bouillonii*, is predicted to arise from a starter *tert*-pentanoic acid (or alternatives as discussed for apratoxin (**86**)) followed by three rounds of acetate extension. The methyl group at C-5 is predicted to arise from an HMG-CoA synthase-like mechanism,[212,213] whereas that at C-2 is consistent with a SAM origin. Next, an NRPS incorporation of glycine is suggested with N-methylation occurring. The conjugated dienamide represents an unusual feature of madangolide (**95**); both olefinic bonds in this lipid chain are 'misplaced' in the chain relative to normal PKS unsaturations and both are of the less common Z-geometry. Biosynthetic studies of these functional groups will be informative. The molecule is completed by three additional rounds of PKS extension, the last of which is also C-methylated from SAM.

2.06.8.3.4 Kalkitoxin

Extracts of *L. majuscula* from the Curaçao beach known as Playa Kalki showed pronounced fish and brine shrimp toxicity and bioassay-guided isolation efforts resulted in the isolation of kalkitoxin (**2**) as the active material.[231] Its essentially linear structure was relatively easily assembled from NMR and MS data and defined a complex lipid with a high level of methylation, a terminal olefin, and a lone thiazolene ring. Absolute stereoconfiguration at C-3 was determined as *R* by Marfey's analysis following ozonolysis and hydrolysis, and relative stereochemistry at the adjacent methyl groups (C-7, C-8, C-10) established using *J*-based configurational analysis. Absolute configuration of these three centers plus the remote C-2′ center was determined by careful comparison of ^{13}C NMR and CD data with all possible synthetic stereoisomers, thereby identifying natural kalkitoxin as the 3*R*,7*R*,8*S*,10*S*,2′*R* isomer. Pure kalkitoxin was found to be exceptionally toxic in several model systems, including the brine shrimp (LC_{50} 170 nmol l^{-1}), fish toxicity (LC_{50} 700 nmol l^{-1}) and two neurochemical assays that collectively indicated it was nearly 10-fold more potent than saxitoxin as a blocker of the mammalian VGSC (EC_{50} 1 nmol l^{-1}).

Kalkitoxin (**2**) is a fascinating molecule for its proposed alternating PKS and NRPS sections, as well as a degree of uncertainty over the origin of the initiating unit. On the one hand, the isopentyl chain could derive from an isoleucine fragment; α-decarboxylation of aliphatic amino acids is reported in the biosynthesis of other cyanobacterial metabolites (e.g., leucine incorporation into barbamide).[178] Alternatively, it could arise from a diketide in which there is both C-methylation and full reduction of the second acetate unit. The next unit incorporated appears to be glycine, which is then extended by three acetate extensions. Methylation from SAM occurs on the first and second acetate units (C-10 and C-8) and a C-1 methylation, presumably from an HMG-CoA synthase-like mechanism, also occurs on the second acetate unit (C-7). An NRPS with specificity for cysteine logically follows and undergoes a heterocyclization–dehydration sequence to form a thiazolene. The carboxyl group of cysteine is then likely homologated by a single round of acetate extension but then undergoes decarboxylation, as shown in the biosynthesis of curacin A (**3**, see Section 2.06.8.3.8),[232] to form the terminal olefin group. Given the biological potency and proposed biosynthetic uniqueness of kalkitoxin, it would be fascinating to understand its biosynthesis at the genetic and enzymological level.

2.06.8.3.5 Microcolin

Southern Caribbean collections of *L. majuscula* are a common source of a highly antiproliferative and immunosuppressive lipopeptide known as microcolin A (**96**).[228] Activity in a mixed lymphocyte assay was originally used to direct the isolation of this metabolite and structure elucidation followed from normal procedures. Toxicity apparently prevented its development as a therapeutic.[233] Stereochemistry at the two aliphatic methyl groups was problematic and finally succumbed to comparisons with synthetic materials of defined configurations.[234] Microcolin illustrates a broad class of marine cyanobacterial metabolite, which includes several closely related microcolins,[235] majusculamide D, and deoxymajuscualmide D.[236]

Its predicted biosynthesis begins with a polyketide chain with appended methyl groups, most likely deriving from SAM (e.g., they appear at C-2 derived carbons in the polyketide). This motif transitions to an NRPS, which is progressively responsible for incorporation of five amino acid residues, the last of which is alanine. Next, a single PKS module extends this final amino acid by two carbons, reduction and dehydration forms a *cis*-olefin and cyclization to the preceding amide nitrogen occurs to produce the five-membered pyrrolinone ring. As noted elsewhere in this review, the mechanistic chemistry and enzymology catalyzing this terminating ring-forming process remains unknown at present.[190] Variations in the structures of microcolin A (**96**) analogues involve the degree of oxidation of the proline ring and acetylation of the threonine and hydroxyproline residues. It may be that these latter elements are introduced post PKS–NRPS assembly.

2.06.8.3.6 Apratoxin

The red-pigmented cyanobacterium *L. bouillonii* occurs throughout the Indo-Pacific as thin veil-like coverings over holes in the reef, usually occurring between 7 and 35 m deep. Upon collecting these thin veils, a small shrimp, *Alpheus frontalis*, emerges from behind to vigorously defend this protective covering of its home. For years the Moore laboratory in Hawaii had detected an exceptionally potent cytotoxicity associated with extracts from these collections and eventually was able to isolate this substance from a Guam collection and define its complete molecular structure, giving it the trivial name of apratoxin A (**86**).[210] A normal interplay of NMR and MS data were used to piece together the planar structure and stereochemistry examined for the peptide section by hydrolysis and chiral chromatographic analysis in combination with standards. For the larger lipid portion, *J*-based configuration analysis followed by Mosher ester/NMR studies established the absolute configuration whereas for the smaller section the double-bond geometry was established from ROESY data. The pure compound showed exceptional cytotoxicity to the KB and LoVo cell lines (0.52 and 0.36 nmol l^{-1}, respectively), and hence, was evaluated *in vivo*. These latter evaluations with the mouse colon adenocarcinoma model used IV administration at 1.5 and 3.0 mg kg^{-1} dosing, and little to no *in vivo* activity was observed despite significant animal toxicity. A functional genomic study of apratoxin A's mechanism of cytotoxicity has shown that it causes G1 cell cycle arrest and apoptosis, at least in part through fibroblast growth factor receptor signaling and STAT3.[237]

Subsequent isolations of the related compounds apratoxin B and C showed that the biosynthetic pathway had some capacity for variance in N-methylation and C-methylation associated with the *t*-butyl group.[210] Recently, a new analogue, apratoxin D (**97**), was obtained from Papua New Guinea collections of *L. bouillonii*, which shows more substantial modification of the PKS-derived lipid chain.[238] Its planar structure was largely constructed by data comparisons with apratoxin A with the lone area of modification being the insertion of an additional PKS unit with C-methylation that intervenes between the starter unit and the incompletely reduced second unit (in apratoxin A) that forms the site for eventual macrocyclization. The configuration of the new methine center in apratoxin D was deduced by NOE and coupling constant analysis, which related this center to the adjacent lactone methine. This latter center and the remaining stereocenters in apratoxin D (**97**) were assigned based on very similar chemical shifts to those of apratoxin A (**86**).

Biosynthetically, the apratoxins have a number of intriguing features, including the starter *t*-butyl unit, a methyl group attached to a C-1 position of acetate and thus predicted to derive through an HMG-CoA synthase-like reaction, and a ketide-extended cysteine, which has undergone cyclization and dehydration to form a thiazolene ring. The *t*-butyl group may come from an unusual *t*-pentanoic acid starter, or alternatively from acetate, propionate, or *iso*-butyrate undergoing one to three SAM-derived methylations. With different apratoxin analogues identified from various strains of *L. bouillonii*, it becomes of interest to understand the molecular genetic basis for this molecular diversity; to this end, efforts to locate and characterize the apratoxin biosynthetic gene cluster in *L. bouillonii* are underway in the author's laboratory.

2.06.8.3.7 Ypaoamide

A popular tourist beach on the island of Guam was closed in May 1994 when a massive bloom of marine cyanobacteria coincided with an extensive killing of larval rabbitfishes. The cyanobacterial mix was composed predominately of *L. majuscula* and *Schizothrix calcicola*, and an extract of this mixed material was found to be deterrent to fish feeding. A major unique lipid constituent was isolated (0.27% of dry biomass weight), although not shown to necessarily explain the rabbitfish die-off, and given the trivial name ypaoamide (**98**).[239]

Ypaoamide was analyzed by standard techniques with a critical realization being the presence of a *t*-butyl group along with a 1,4-disubstituted aromatic ring and two α, β-unsaturated amides. Further use of COSY and HMBC led to five partial structures and these could be assembled by further HMBC experiments using conditions optimized for $\mathcal{J} = 5$ Hz. While geometry could be deduced based on NOE data, the lone stereocenter was not investigated.

The biosynthetic origin of ypaoamide was studied through culture studies followed by GC–MS analysis. However, of the two dominant species in the original mixture, only *L. majuscula* filaments survived and grew. Fortunately, extraction of three separate cultures of this *L. majuscula* and analysis showed a peak indistinguishable from that of ypaoamide in all three, thereby identifying this as the producing species. Ypaomaide (**98**) shows several interesting biosynthetic features. First, it possesses the distinctive *t*-butyl group seen also in antillatoxin (**87**) and the apratoxins (**86**). This starter unit is extended by two rounds of PKS extension, which then transitions to an NRPS with specificity for β-alanine, similar to what is observed for jamaicamide (**93**). A PKS extension of this amino acid unit with corresponding conversion to a β-methoxyenoate is predicted to next occur and is a parallel motif to what is observed in both the jamaicamides (**81, 93**), barbamide (**78**), and malyngamides A (**5**)[240] and R (**100**).[241] Finally, a terminal pyrrolinone ring is produced, in similarity to many cyanobacterial metabolites including the jamaicamides, malyngamides A and R, and the microcolins (e.g., **96**),[228] by NRPS-based incorporation of phenylalanine, PKS extension, ketone reduction, and dehydration, possibly to the *cis*-olefin, and cyclization that may be coincident with product release. When biosynthetically dissected in this manner, ypaoamide (**98**) appears to possess a number of the distinctive motifs observed in other cyanobacterial metabolites, albeit in new combinations, thus giving a keen insight into the evolutionary strategy for structure diversification in this marine cyanobacterium.

2.06.8.3.8 Curacin A

Curacin A (**3**) is the name given to a complex ketopeptide obtained from a Curaçao collection of *L. majuscula* with powerful cancer cell toxicity properties.[242] Samples found growing as trellises from mangrove roots were exceptionally rich in this new natural product and while its planar structure was assembled very efficiently from 2D NMR data, configuration at the four stereocenters took several years of additional effort. Ultimately, these chiral centers were assigned on the basis of comparisons of degradative fragments with the authentic materials produced by enantioselective synthesis.[243] Curacin A showed low nanomolar cytotoxic properties to cancer cells *in vitro* and this was shown to result from the drugs interference with tubulin polymerization through strong noncovalent binding at the colchicine site.[244] *In vivo*, the molecule was essentially inactive as a result of its water insolubility and instability. Analogue structures with improved pharmacokinetic parameters have been produced by synthesis of a focused combinatorial library of analogues.[245]

Curacin A (**3**) is a deceptively simple structure with a fascinatingly complex origin. From a number of conceptually very different biosynthetic origins, a general scheme of a complex ketopeptide emerged from stable isotope precursor feeding experiments.[232] The molecule is initiated as a short polyketide, elaborated by a pendant carbon from C-2 of acetate to form a cyclopropyl ring. Next, cysteine is joined in amide linkage and heterocyclized/dehydrated to a thiazoline ring. The carboxyl carbon of this cysteinyl residue is then modified by seven rounds of ketide extension, each with various levels of reduction or methylation. A final step results in decarboxylation to yield the distinctive terminal olefin group, which derives from C-2 of acetate. Insights gained from these precursor incorporation experiments then helped to develop an efficient cloning strategy of the 86 kb biosynthetic gene cluster encoding for curacin A biosynthesis.[232] Several interesting perceptions into this unique biosynthetic assembly have resulted. First, a new mechanism of ketide initiation was deduced and explored by biochemical experiments and X-ray crystallography. A novel GCN5-related *N*-acetyl transferase (GNAT) enzyme directly accepts acetate loading onto a phosphopantetheinyl arm from an acetyl-CoA precursor.[246] This is converted to a diketide, which is then modified by a β-branching reaction using a core HMG-CoA synthase-like domain, which adds C-2 of acetate to the β-carbonyl functionality.[212] At some stage, the methylene group of this new branch is chlorinated by a radical halogenase. A tandem set of enoyl-CoA hydratases progressively catalyze dehydration to the α, β-unsaturated thioester and then decarboxylation with proton addition to yield an α, β-unsaturated γ-chloro species. This sequence of reactions is consistent with the results of isotope feeding experiments wherein a single ^{2}H atom is attached to the methylene carbon of the cyclopropyl ring from a [2-$^{2}H_3$, 2-^{13}C]acetate precursor.[232] The next enzymatic domain in the cluster, an enoyl

reductase, is proposed to catalyze protonation of the quaternary β-carbon with the double bond electrons then flowing from the α-carbon to displace chloride from the γ-carbon, forming the cyclopropyl ring of curacin A (**3**). Additional unusual elements of curacin A biosynthesis not yet studied at the biochemical level include the formation of the C3–C4 *cis*-double bond and terminal olefin, which concludes the pathway.

2.06.8.3.9 Malyngamide R

The malyngamides are another example of complex ketopeptides, which are both broadly represented and highly varied within various strains of the marine cyanobacterium *L. majuscula*. More than 25 variants of this structure type are known from collections made around the world.[5] Most of these derive from a 14-carbon monounsaturated methoxy fatty acid moiety, which also occurs in free form, and is known as 'lyngbic acid' (**4**).[5] The lyngbic acid portion is always found as an amide, sometimes N-methylated, which has a further appendage usually containing a vinyl chloride function and an oxidized cyclohexyl ring (Type A malyngamides). Variations include chain length of the fatty acid, secondary versus tertiary amide, carbon chain lengths, presence or absence and geometry of the vinyl chloride function, oxidation pattern on the cyclohexyl ring, and nature of ring substituents, which include acetate esters, methyl groups, and sugar glycosides. An example of a Type A malyngamide is the antifungal metabolite malyngamide C acetate (**99**).[27] However, a second subgroup of malyngamides (Type B) has a rather different structure for the amine portion of the structure, represented by malyngamides A (**5**),[240] B, Q, and R (**100**).[241] These malyngamides are acyclic in their initial amine-linking portion and terminate with a pyrrolinone ring with various substituents.

A likely biosynthesis of these two classes of malyngamide can be deduced using malyngamides C acetate (**99**, Type A) and A (**5**, Type B) as examples. For the Type A malynamides, lyngbic acid is passed from a PKS element to an NRPS, which incorporates glycine (or occasionally β-glycine), which is then extended by one acetate extension. The β-carbonyl is then predicted to be modified by the HMG-CoA synthase-like enzymology to produce a β-branch, which in analogy to jamaicamide A (**93**), is converted to a vinyl chloride function. The pathway continues with two additional ketide extensions, cyclization, and variable levels of reduction/oxidation. Alternatively, the Type B malyngamides vary in that the initial NRPS-mediated glycine extension of lyngbic acid is followed by PKS extension and β-branch formation, and this is followed by a single PKS extension before transition back to an NRPS, which incorporates glycine (**5** or serine **100**), and finally once again ketide extended and cyclized to a pyrrolinone ring by unknown mechanisms.

2.06.8.3.10 Largazole

A Florida collection of the marine cyanobacterium *Symploca* sp. yielded a cytotoxic extract that was progressively fractionated to yield a novel cancer cell-selective cytotoxin named largazole (**89**).[247] The planar structure of largazole was determined by standard NMR methods, prominently COSY, HMBC, and nuclear overhauser effect spectroscopy (NOESY) and the sequence of residues confirmed by detailed MS–MS analysis. The chirality was established in the three asymmetric centers by a reaction sequence involving ozonolysis, oxidative workup, acid hydrolysis, and then chiral HPLC in comparison with chemical standards. Largazole (**89**) shows exceptional antiproliferative activity with GI_{50} values to transformed cell lines in the low nanomolar range. Interestingly, nontransformed cells were much less sensitive to the effects of the drug, suggesting a drug target unique to cancerous cells. Largazole possesses several highly unusual structural elements, including two polyketide sections connected through a thioester, a condensed cysteinyl–alanyl unit, which forms a thiazole ring, and a 4-methyl thiazoline ring formed from a second cysteine residue. While this latter modification has been seen previously in cyanobacterial metabolites (e.g., in didehydromirabazole),[248] this is the first such thioester linkage observed in the natural products of these organisms. Presumably, its origins derive from nucleophilic attack by the side chain sulfur atom of cysteine, rather than the more normal use of the α-amino group in such a condensation, with subsequent loss of the amino group and two rounds of ketide extension to form this unprecedented 3-hydroxy-7-mercaptohept-4-enoic acid unit. It will be interesting to discover if this alternate condensation using a side chain group rather than the α-amino group occurs while the respective substrates are tethered to the proteins of a modular PKS–NRPS–PKS construct. Indeed, some precedence for condensation chemistry to occur using amino acid side chain functional groups is observed in other cyanobacterial metabolites, such as microcystin LR (**88**); however, in this latter case this is accomplished through

tethering of the side chain carboxyl functionality of glutamic acid to the NRPS thiolation domain for subsequent condensation with the α-amino group of serine[219] (for more details on Lipopeptides, see Chapters 1.10 and 1.11).

2.06.9 Conclusion

In this chapter, we have attempted to point out the structural trends in the natural products of cyanobacteria and then give significant examples, which illustrate and make these metabolic trends tangible. The dominant theme is one of integrated NRPS and PKS pathways to produce lipopeptide compounds and these come in two orientations. The first and more prevalent are those metabolites initiated by a PKS and then transitioning to a NRPS portion, a type we have named here as ketopeptide. Less prevalent but still quite common among cyanobacterial metabolites is the reverse orientation wherein an NRPS-initiated pathway transitions into a PKS-produced portion; this we have termed a peptoketide. We feel it useful and insightful to dissect the broad class of lipopeptides along these fundamental features of their construction. Indeed, further dissection of ketopeptides and peptoketides, in terms of their degrees of halogenation, cyclization, and glycosylation, shows that they are subject to different types of metabolism. Often, however, there are multiple switches back and forth between NPRS and PKS elements and this leads to more complex substances in which the ancillary metabolic patterns are less clear.

Halogenation is another theme of importance and of special note in the cyanobacteria. Chlorine is the dominant halogen employed by cyanobacteria in covalent modification of organic natural products and this fact contrasts with some other groups of marine life-forms, which tend to utilize bromine to a greater degree (e.g., the Rhodophyta or red algae).[249] The types of halogen-containing functional groups found in cyanobacterial natural products are unique and suggest the presence of several classes of enzymes for their incorporation. For example, chloro- and bromo-tyrosine derivatives are well represented among cyanobacterial metabolites (e.g., cryptophycin A[250] and symplocamide A[123]) and these are likely produced by the action of a haloperoxidase. Alternately, cyanobacteria produce a multitude of unique halogenations at unactivated centers, including the trichloromethyl group of barbamide (**78**), the monomethyl group of microginin T1 (**81**), and the vinyl chloro group of jamaicamide A (**93**) and malyngamide C (**6**). In these latter cases, a new type of enzymatic halogenation occurs, which involves high energy iron (IV) oxides that catalyze radical reactions.[181,182]

The biological properties of cyanobacterial natural products are conveniently separated into those that constitute risks to human health and those that may offer remedies to treat disease.[3] Cyanobacteria contribute substantially in both dimensions and hence, a robust primary literature as well as review literature are available on these topics. In general, a substantial number of the toxins of cyanobacteria appear to influence ion channel function and thus exert a neurotoxic action.[11] And while not exclusively of freshwater occurrence, marine cyanobacteria are less well known for their overtly toxic metabolites, and only a few of these have been shown to work by ion channel mechanisms. Rather, marine cyanobacteria produce natural products that are little encountered because these organisms grow attached as macrobacterial colonies and are not eaten by humans or by most herbivorous marine organisms. This latter point addresses the natural function of marine cyanobacterial metabolites, which appears to mainly involve the repulsion of would-be generalist predators by being distasteful and toxic. The pharmacological trend in marine cyanobacterial metabolites is toward the production of very potent inhibitors of tubulin or actin polymerization. This is itself interesting as these prokaryotes lack the biochemical targets for these defensive substances and thus suffer little ill effect from their accumulation, sometimes in huge quantities (e.g., curacin A (**3**) constitutes up to 11% of the lipid extract of Curaçao strains of *L. majuscula*).[251]

Recent progress in understanding the biosynthesis of cyanobacterial natural products has been enormously enhanced through gene cloning and genomic approaches. The gene clusters for a number of cyanobacterial natural products are now known, primarily those involving PKS or NRPS elements, and in some cases detailed mechanistic and enzymological studies have started to shed light on the more novel and intriguing transformations.[212,246] At the same time, a growing number of cyanobacterial genomes have been sequenced and these are helping to reveal the wealth, diversity, and architecture of gene clusters encoding for secondary metabolites.[18] With the advent of techniques allowing single cell genome sequencing, such as the combination of micromanipulation of single cells followed by multiple displacement amplification (MDA) of the DNA from such an isolated cell and then 454 sequencing, access to genomic information and these natural product pathways is being dramatically enhanced.[252]

The future holds much excitement for natural products studies with cyanobacteria. Understanding the molecular basis for the regulation of natural product gene expression is pivotal to both a number of environmental issues involving harmful blooms and controlled expression in biotechnological and biomedical applications. A more detailed knowledge of the formation of the unique molecular frameworks and functional groups in cyanobacterial metabolites, at the genetic as well as protein level, will enable the effective capture of these biosynthetic processes and utilization in diverse aspects of biotechnology. Another promising area for significant scientific advance is in the area of molecular pharmacology. Cyanobacterial natural products represent some of the most potent and biologically active metabolites available from nature; however, only through a precise knowledge of how they function in terms of target cells, target proteins, and target atom motifs, will we really gain control of how to intelligently design analogues and rationally apply these for therapeutic benefit. A similar detailed molecular level understanding of how cyanobacterial compounds exert their ecological functions could be insightful and potentially build new bridges between the natural functions of secondary metabolites and their application in pharmacology and medicine.

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Biographical Sketches

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2.07 Myxobacteria – Unique Microbial Secondary Metabolite Factories

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2.07.1 Myxobacteria as Producers of Bioactive Secondary Metabolites

The Gram-negative myxobacteria, which are members of the δ-group of proteobacteria, are fascinating microorganisms that exhibit many unusual characteristics.[1,2] Myxobacteria move by gliding or creeping on surfaces, and they show a unique cooperative social behavior based on a complex communication system. One notable aspect of their life cycle is morphological differentiation, which occurs upon starvation and culminates in the formation of fruiting bodies and the production of myxospores (**Figure 1**).[1,2] The most relevant biotechnological feature of myxobacteria is their ability to produce novel classes of bioactive secondary metabolites.[3–5] More than 500 derivatives of at least 100 different core structures have been characterized to date, and most of those secondary metabolites represent novel molecular skeletons. Approximately 5% of known microbial compounds are myxobacterial in origin, and therefore only the intensively studied actinomycetes (70%), fungi (18%), and bacilli strains (6.5%) are better sources of natural products.[3] Most of the myxobacterial metabolites are polyketides (PKs) or nonribosomally made peptides, or hybrids of the two structural classes, and they often contain functionalities that are found infrequently in other natural products. Exploiting this chemical resource is of significant interest, as the compounds isolated to date exhibit a wide array of biological activities (**Figure 2**). It is particularly remarkable that the observed modes of action are rarely exhibited by other microbial secondary metabolites, which makes myxobacteria a promising source of novel drug leads.

An example of particular interest is epothilone from *Sorangium cellulosum* So ce90, which has recently been approved for the treatment of breast cancer.[6–8] Epothilone inhibits microtubule depolymerization and is active against multidrug-resistant cancer cell lines and paclitaxel-resistant tumors. The most comprehensively characterized and so far only epothilone derivative, which received FDA approval for clinical use, is ixabepilone.[9] The compound is marketed in the US by Bristol-Myers Squibb (BMS) under the trade name Ixembra®.

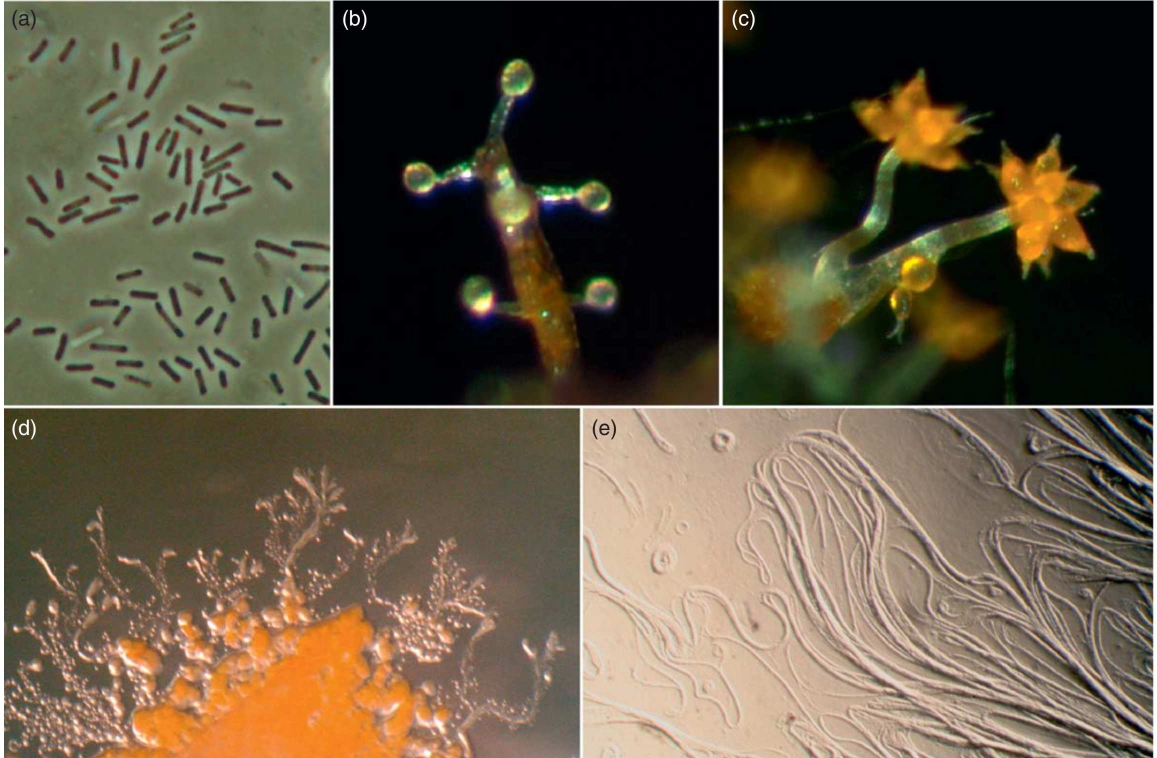

Figure 1 Different stages of the myxobacterial life cycle. (a) Phase-dark vegetative cells typical for Sorangiineae; (b) fruiting bodies of *Melittangium lichenicola* on a piece of rotting wood; (c) fruiting bodies of *Chondromyces apiculatus* in crude culture on rabbit dung; (d) swarming colony of a *Sorangium* sp. on mineral salts agar; (e) swarm colony edge of a *Cystobacter* sp. on an agar plate (pictures: Ronald O. Garcia).

Other promising myxobacterial anticancer compounds are tubulysin A from *Angiococcus disciformis* An d48 and disorazol A from *S. cellulosum* So ce12. In contrast to epothilone, both of these metabolites trigger the apoptotic process by inducing depletion of cell microtubules.[10–12] Argyrin B, isolated from *Archangium gephyra* Ar 8082, was found to be a potent inhibitor of T-cell-independent antibody formation and was recently shown to inhibit the proteasome.[13,14] Unusual mechanisms of action have also been identified for ambruticin, which affects osmoregulation in fungi,[15] sorangicin, which is one of the rare inhibitors of bacterial RNA synthesis,[16] and ratjadon, which blocks the export of proteins from the nuclear space into the cytoplasm.[17] Soraphen from *S. cellulosum* So ce12 was shown to interfere with a novel target, the fungal acetyl-CoA carboxylase;[18] today, this target is used in industry as a screening model for antifungal compounds.

Some myxobacterial compounds exhibit a biological function within and/or for the producing strains during the process of morphological differentiation.[1,2] Two examples have been reported to date, although exactly how the metabolites are involved in development remains poorly understood. For example, an early step in the differentiation process of *Stigmatella aurantiaca* depends on a signal secreted by the cells when nutrients become limiting.[19] A volatile compound named stigmolone was purified from the secreted material, and identified as a novel prokaryotic pheromone that is involved in starvation-induced aggregation and fruiting body formation.[19] More recently, the DKxanthene family of secondary metabolites was discovered; these compounds represent the major yellow pigment of the model strain *M. xanthus* DK1622 and several other *Myxococcus* species.[20] Using mutagenesis of the biosynthetic genes, the compounds were shown to play a role in morphological differentiation in these strains: Fruiting body formation was delayed and the amount of viable spores was significantly reduced in mutant strains lacking DKxanthene production. However, further experiments will be required to clarify the exact function of these pigments during the developmental life cycle of *M. xanthus*. In addition, compounds acting as siderophores – metabolites that are essential under iron-limiting conditions – were also

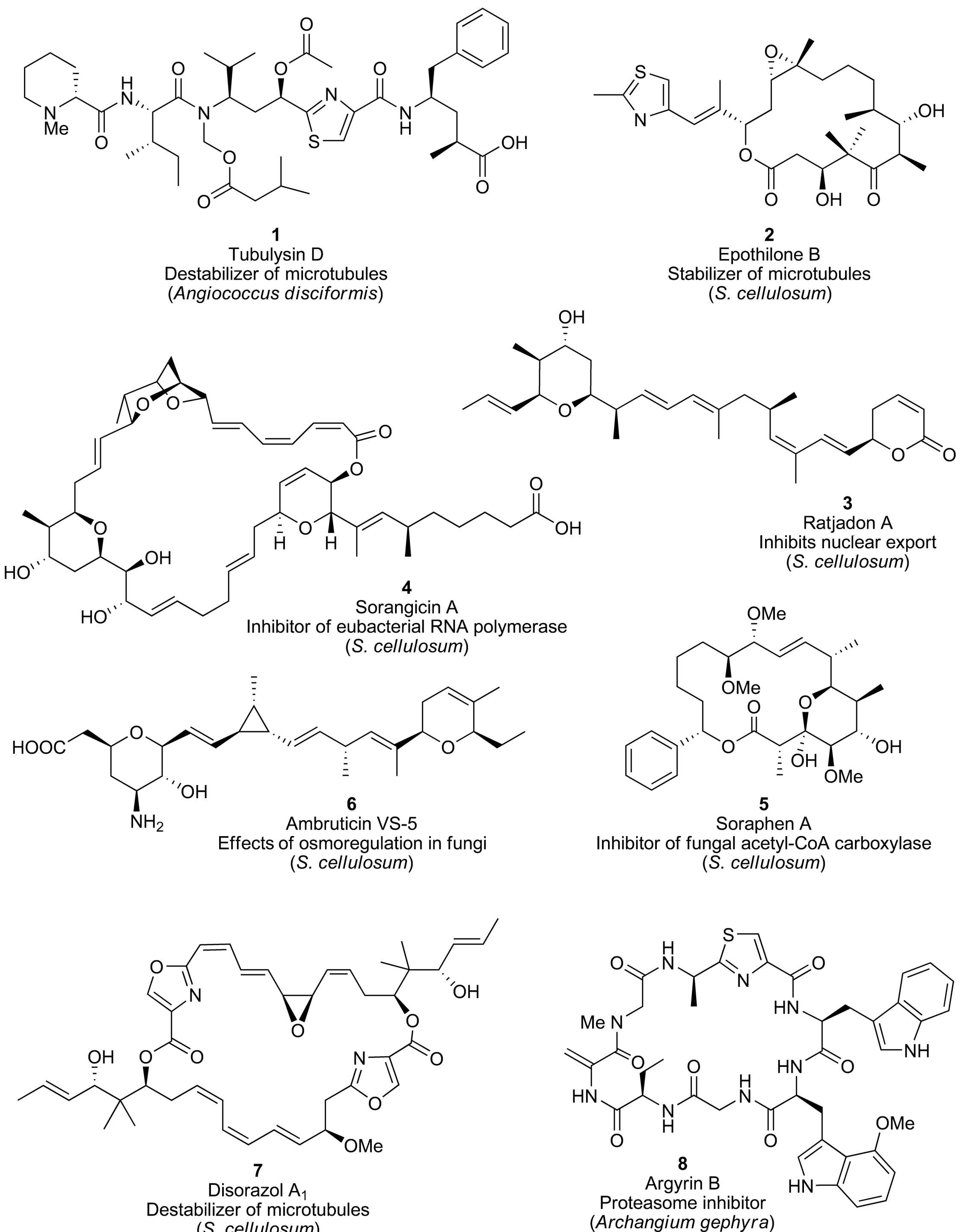

Figure 2 Myxobacterial secondary metabolites with biological activity. The structure, name, mechanism of action, and producer organism are given.

identified in a number of myxobacteria. The most prominent examples of this class of compounds are the catecholate-type siderophore myxochelins, which are produced by a range of myxobacterial species (*S. cellulosum*, *S. aurantiaca*, *A. disciformis*, and *M. xanthus*).[21–23]

2.07.2 Approaches to Exploring the Biosynthetic Potential of Myxobacteria

A number of different strategies have been described to exploit the biosynthetic potential of myxobacteria and other microorganisms.[24] As the production of secondary metabolites is often a specific response of the producing organism to the environmental conditions,[25] a simple way to trigger the production of new compounds is to vary the cultivation conditions. Indeed, growth conditions for strains of different myxobacterial species in liquid culture have been established, but even today these procedures are far from routine, and have to be developed specifically for each strain.[3]

2.07.2.1 Biodiversity-Based Approaches

As a complement to classical screening programs, several novel approaches are being explored to further extend the biosynthetic potential of the myxobacteria. One strategy is to identify strains with novel physiological properties, as previous research has focused largely on aerobic and mesophilic strains.[3,26] Psychrophilic myxobacteria,[2] which grow only at 4 °C, were recently isolated from antarctic soil samples, while improved cultivation methods have yielded thermophilic strains, which grow rapidly at high temperatures (42–44 °C).[27] Myxobacterial species capable of growing under anaerobic conditions have been obtained from soils and river sediments. These biodiversity-based efforts have also revealed genuine alkaliphilic strains, which swarm and form fruiting bodies at pH 9.5 but do not grow at pH 7.2,[2] as well as true halophilic myxobacteria from the marine environment.[2,28,29] New families of myxobacteria have even been isolated from standard soil samples, once growth conditions were suitably modified; some of these novel strains produce as yet unidentified secondary metabolites[30] (R. O. Garcia and R. Müller, unpublished data). The success of these approaches makes it likely that further groups of myxobacteria will be identified in future.[3]

2.07.2.2 Improved Chemical Screening

Known species remain promising candidates for novel secondary metabolites, as most strains have only been screened by UV-based analysis, and bioactivity screens have only been applied in a limited fashion. Therefore, high-performance liquid chromatography–mass spectrometry (HPLC–MS)-based chemical screening is expected to reveal numerous novel compounds, if used in conjunction with rigorous statistical evaluation. In fact, an initial study based on chemical screening of 98 *M. xanthus* species by high-resolution MS coupled to HPLC suggested that novel secondary metabolites were present in almost all of these highly related isolates.[31] Although eight compounds were known previously from *M. xanthus*, this study revealed another 37 candidates for new secondary metabolites.

2.07.2.3 Genetic Approaches

2.07.2.3.1 Genome-independent approaches

Genetic information has also been established as a good starting point to explore the biosynthetic potential of myxobacteria, through the identification of natural product biosynthetic genes (**Figure 3**).[24,32] This strategy takes advantage of the fact that the genes for natural product biosynthesis are usually organized into clusters (20–200 kilobases (kb) in size) within the microbial genome.[24] Methods have been described to amplify polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) fragments from nonsequenced (myxo)bacteria, as these enzymes are employed most often for secondary metabolite biosynthesis in myxobacteria (see Section 2.07.3).[33] The obtained polymerase chain reaction (PCR) products can be used as probes to detect PKS and/or NRPS pathways in genomic libraries and to perform gene inactivation studies if the host strain can be manipulated genetically (**Figure 3**).[24,33] By comparative analysis of the metabolite profiles from gene inactivation mutants and the wild-type strain, natural products corresponding to known secondary metabolite biosynthetic genes can be identified. However, a prerequisite for this strategy is that the metabolites are produced (at least at the low levels required for detection by MS) by the wild-type strain under standard laboratory cultivation conditions. Three novel natural products (myxochelin[22] (unpublished data),

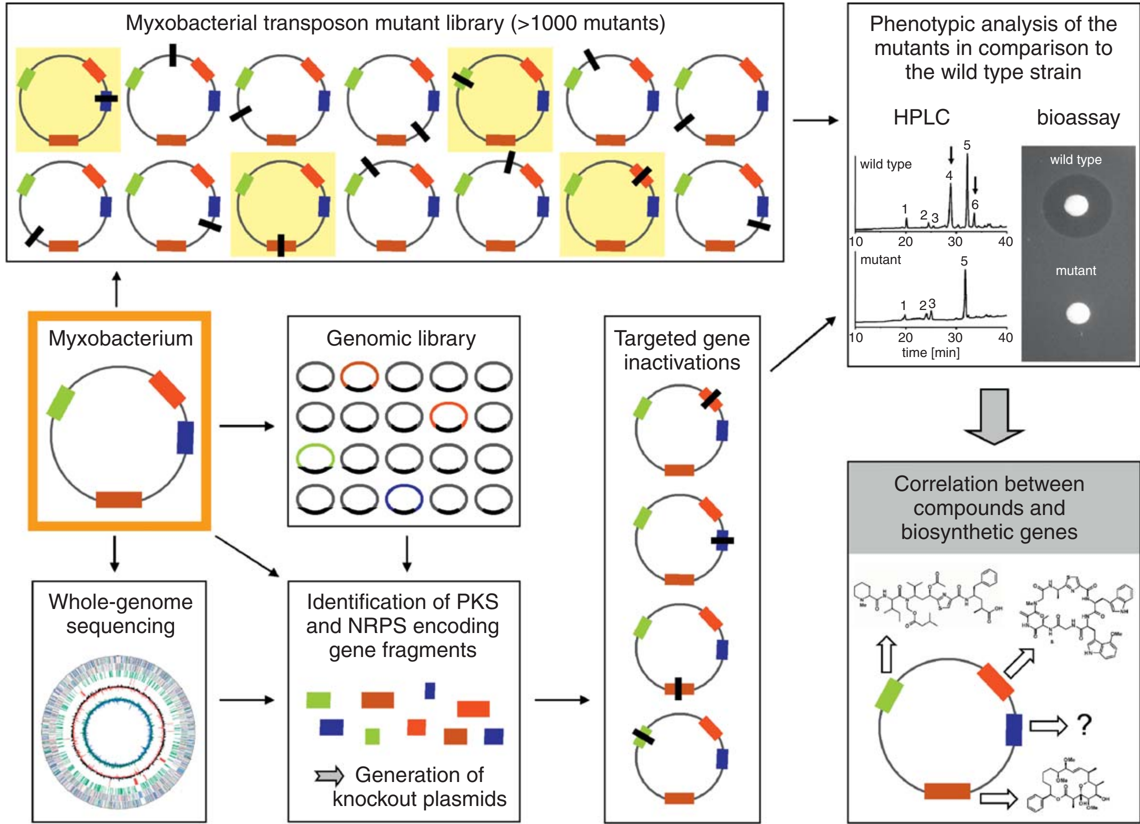

Figure 3 Identification of myxobacterial biosynthetic gene clusters and correlation to the secondary metabolites produced. Two different approaches are shown: random mutagenesis (transposon mutagenesis) and targeted gene inactivations after identification of secondary metabolite genes by genome scanning or genome sequencing. Loss of production in the mutant strains can easily be detected by analytical methods (e.g., HPLC) or compound-specific bioassays (e.g., specific inhibition of a test strain in an overlay experiment). PKS/NRPS biosynthetic gene clusters are shown as red, blue, brown, and green bars and sites of mutagenesis are indicated by black lines. In this example, three of the four biosynthetic gene clusters could be correlated to a compound. The biosynthetic product of the fourth cluster (shown in blue) could not be identified, suggesting that the pathway is silent under the cultivation conditions tested.

myxochromide,[34] and aurafuron[35]) were found in *S. aurantiaca* DW4/3-1 using this approach, and analogous experiments with other myxobacterial strains have yielded similar results.[33]

Transposon-based approaches have also been applied to myxobacteria (**Figure 3**). Mutants from transposon libraries can be analyzed chemically by comparison of their secondary metabolite profiles with the wild-type strain, or screened for altered bioactivity, for example, by overlay assays. This strategy was successfully used to identify the biosynthetic gene clusters responsible for the known metabolites tubulysin,[36] disorazol,[37,38] aurachin,[39] and DKxanthene[20] in the genomes of their respective producer strains, and has the potential to detect novel, as yet unidentified compounds and their corresponding biosynthetic pathways and regulatory elements (see Section 2.07.5).

2.07.2.3.2 Genome-dependent approaches ('genome mining')

Screening for new secondary metabolites and their biosynthetic gene clusters is considerably facilitated if the complete genome sequence of the producer organism is available. To date, two myxobacterial organisms, *M. xanthus* DK1622[40] and *S. cellulosum* So ce56,[41] have been completely sequenced, and the genomes of two other myxobacteria (*S. aurantiaca* DW4/3-1 and *Anaeromyxobacter dehalogenans* 2CP-C) are currently being analyzed (see microbial genome database from the J. Craig Venter Institute: http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi). Myxobacteria possess giant chromosomes (*M. xanthus*: 9.1 Mbp; *S. aurantiaca*: ~9.4 Mbp;

S. cellulosum: 13.1 Mbp), which are among the largest genomes yet known from bacteria.[40,41] Statistical analysis of all sequenced bacterial genomes revealed a positive correlation between genome size and the number of genes involved in the biosynthesis of secondary metabolites.[42] However, sequence information from more than 250 microbial genomes (including well-known producers of secondary metabolites) also shows that there is a significant discrepancy between the number of secondary metabolite gene clusters and the number of compounds that have been isolated from the strains.[24] The presence of such 'silent' natural product pathways, which likely generate additional compounds under native growth conditions, indicates that the genomic capacity of these microorganisms to synthesize secondary metabolites is much higher than originally appreciated.

The best studied examples of this phenomenon are *Streptomyces coelicolor* and *Streptomyces avermitilis*, which are known to produce three and four secondary metabolites, respectively, but actually contain 20 and 25 biosynthetic gene clusters in their genomes.[43,44] A detailed postgenomic examination of the production profile of *S. coelicolor* has already led to the isolation of two additional compounds, whose existence was postulated after genome annotation.[45,46] Recent results from the genome sequencing projects of *M. xanthus* and *S. cellulosum* show that the situation in myxobacteria is similar. Scanning of the *M. xanthus* DK1622 genome sequence for the presence of PKS- and NRPS-encoding genes revealed at least 18 biosynthetic gene clusters,[40] although none of the corresponding products were identified by screening until 2005. Postgenomic examination of *M. xanthus* extracts by HPLC–MS led to the identification of four compounds that were known from other myxobacteria, as well as several unknown secondary metabolites (**Figure 4**). By detailed sequence analysis and mutagenesis

Figure 4 Secondary metabolites produced by the myxobacterial model strain *Myxococcus xanthus* DK1622. Among the 18 putative PKS/NRPS biosynthetic pathways identified in the 9.1 Mb *M. xanthus* genome sequence, five biosynthetic gene clusters have been correlated to specific metabolites. Only one member of the identified compound families is shown: DKxanthene-534 (**9**), myxovirescin A_1 (**10**), myxochromide A_2 (**11**), myxalamid B (**12**), and myxochelin A (**13**).

experiments, the biosynthetic gene clusters for myxovirescin,[44] myxalamide,[48] myxochelin[31] (D. Krug and R. Müller, unpublished data), and myxochromide[34] biosynthesis were assigned, the latter three by correlating the gene sets with pathways already identified in *S. aurantiaca* strains.[22,34,49] Additionally, at least one new class of secondary metabolites was detected (DKxanthenes) and the structures elucidated.[20] Interestingly, a proteomic approach based on cells grown to the late logarithmic phase revealed the expression of biosynthetic proteins corresponding to 11 out of the 18 PKS/NRPS gene clusters, showing that most of the pathways are indeed expressed under laboratory conditions.[50] It is therefore highly likely that the additional pathways are active, but that the metabolites have to date escaped detection (detailed genetic studies do indeed show that some of these compounds belong to those detected in the HPLC–MS approach described above for *M. xanthus*; D. Krug, N. S. Cortina, and R. Müller, unpublished data). As these few examples illustrate, genome mining in myxobacteria appears to have a promising future.

2.07.3 Biosynthesis of Myxobacterial Secondary Metabolites at the Molecular Level

Myxobacterial secondary metabolites are frequently hybrid structures derived from the linkage of carboxylic acids and amino acids. In the majority of cases, the compounds are formed by complex, multistep biosynthetic processes catalyzed by giant multifunctional enzymes called PKSs and NRPSs.[51,52] Hybrid systems in which PKS and NRPS multienzymes cooperate are also known, and occasionally these machineries even appear within the same polypeptide.[53,54] A detailed understanding of the mechanisms involved in PKS, NRPS, or PKS–NRPS biosynthesis is a prerequisite for optimizing product yields and for manipulating the biosynthetic pathways in order to generate altered natural products.

The success of this approach depends on the cloning and subsequent genetic and biochemical characterization of the biosynthetic pathways. During the last decade, a large number of biosynthetic gene clusters from myxobacteria have been identified.[22,34,36–39,47,49,54–72] Almost without exception, the analyzed biosynthetic systems exhibit unusual or even unprecedented features, and therefore myxobacteria appear to be an enormous and fascinating resource for enzymes performing novel types of biochemistry.[32,73,74] It is also notable that the biosynthesis of myxobacterial compounds is frequently directed by mixed PKS–NRPS systems, while secondary metabolites derived from actinomycetes are typically either purely PK or nonribosomal peptide in nature. Among the 23 myxobacterial biosynthetic gene clusters identified to date, 8 encode purely PKS assembly lines, 2 are purely NRPS in character, while the remaining 13 are PKS–NRPS hybrid systems.

2.07.3.1 Biosynthesis of Polyketides

PKs are assembled from short-chain carboxylic acids such as malonate or methylmalonate using sequential decarboxylative condensations, in a process that is mechanistically similar to fatty acid biosynthesis. On the basis of their genetic architecture and the structure of their products, bacterial PKSs have been classified into three different types.[51,75,76] The majority of PKSs characterized to date from myxobacteria are type I, also referred to as modular PKSs. Modular PKSs consist of multienzymes and these are organized into several modules, each of which contains a set of catalytic domains.[51] The first module typically initiates the biosynthesis by loading the starter unit, while the last module usually contains a termination activity, for example, a thioesterase (TE) domain, to catalyze the release of the PK chain from the enzyme complex. For each chain extension step, a minimal set of three domains is required: an acyl transferase (AT) domain for extender unit selection and transfer, an acyl-carrier protein (ACP) for the covalent binding of the extender unit or growing intermediate to the enzyme complex, and a ketoacyl synthase (KS) domain for the decarboxylative condensation with the growing PK chain.[51] The resulting β-keto acid may subsequently be processed by additional domains within the module, including ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), and methyl transferase (MT) activities. The structural diversity observed in the resultant PKs arises in large part by variation in the domain composition of the modules. In general, type I PKSs produce nonaromatic, reduced PKs, either as linear chain or as cyclized macrolactones or macrolactams.

In addition to the multifunctional type I PKSs, two other bacterial PKS types (type II and type III) have been identified. Characteristic products of these types of PKS are (phenolic) aromatic compounds.[75] Type II PKS systems consist of a set of proteins each with a single catalytic function, which are employed iteratively during polyketide formation.[51,75] The biosynthetic complex minimally comprises an ACP as well as a KSα/KSβ heterodimer, which together produce a polyketo chain, which is subsequently released and cyclized after reaching a certain chain length, for example, by cyclases and aromatases. More recently, genes similar to plant chalcone synthases have been detected in bacteria, introducing 'bacterial type III PKS' systems into the nomenclature.[76,77] Usually, these iteratively operating condensing enzymes act directly on acyl-CoA substrates and are thus independent of ACPs. Type III PKSs function as homodimers and typically form small aromatic metabolites.

A number of myxobacterial polyketide biosynthetic pathways have been characterized (the corresponding products are shown in **Figure 5**). This analysis reveals that myxobacteria employ all three types of PKSs, although type I PKSs are used most frequently. The first myxobacterial PKS system identified was the soraphen biosynthetic machinery from *S. cellulosum* So ce12.[57,58] Two type I PKSs (SorA and SorB) are involved in assembling the antifungal macrolide from benzoyl-CoA and eight polyketide elongation units (**Figure 6**). As the biosynthesis of soraphen A (**5**) begins with a benzoyl-CoA starter unit, a CoA ligase (CL) domain for recognition and activation of the carboxybenzoate was anticipated to be present in the loading module. Instead, however, two AT domains are located within an intermixed loading module/module 1 architecture (ACP_L–KS_1–AT_L–AT_1–KR_1–ACP_1). This unusual domain organization was subsequently found in several other myxobacterial systems,[74] while biochemical studies have demonstrated that one of the AT domains loads the starter unit whereas the second one is responsible for selection of the first extender unit.[78] Presumably, a CL function is still required to activate the benzoate to the respective CoA ester, but as such an activity was not identified in the soraphen gene cluster region, it is presumably encoded elsewhere in the chromosome. Alternatively, the benzoyl-CoA may be derived directly from the degradation of cinnamic acid.[3]

Another striking feature of the soraphen PKS is the incorporation by modules 3 and 7 of methoxymalonate, a 'glycolate' extender unit (**Figure 6**). Recent studies have shown that this unusual extender unit is generated on an external ACP domain, and is most likely derived from a 1,3-bisphosphoglycerate precursor.[79,80] Correspondingly, the cluster contains a set of genes that can be assigned functions in the pathway to methoxymalonyl-ACP (*sorC*, *sorD*, and *sorE*).[57,79] Once product assembly on the PKS is complete, the chain is released by the terminal TE domain to form an 18-membered macrolide (soraphen X (**21**)); this enzyme-free intermediate then undergoes further modifications (pyran ring formation, dehydration, and O-methylation) to produce soraphen A (**5**) (**Figure 6**).

Stigmatella aurantiaca Sg a15 harbors the stigmatellin megasynthase.[62,81] Its product stigmatellin A (**14**) inhibits electron flow in the respiratory chain, and so the compound has been widely exploited in investigations of electron transport processes.[82,83] The involvement of a type I PKS in the biosynthesis of the chromone ring was unexpected, as aromatic polyketides in bacteria usually result from type II (or type III) PKS systems.[51,75–77] In contrast to all other myxobacterial type I PKS systems reported to date, each module of the stigmatellin megasynthase StiA–J is encoded on a separate gene (**Figure 7**). StiA contains an intermingled loading/extension module (ACP_L–KS_1–AT–AT–DH_1–KR_1–ACP_1) that exhibits a similar domain architecture to modules from the soraphen assembly line.[57,74] Molecular analysis of the stigmatellin gene cluster combined with feeding studies using labeled precursors revealed several novel biochemical features.[62] The most striking finding was the presence of an iteratively acting module, as the paradigm for type I PKS function was that of the single-use module. In this model, once a module has catalyzed its round of chain extension, the nascent acyl chain is passed to the downstream module. In this way, the structure of the product is determined directly by the number (and domain composition) of modules in the assembly line.[51] In stigmatellin biosynthesis, however, the number of modules in the synthase is inadequate to account for the observed number of chain extension cycles, and so either StiH or StiJ is likely to be used iteratively. This mechanism would seem to require an unusual transacylation of the biosynthetic intermediate from an ACP domain back to the preceding KS domain.[62] In support of this proposal, however, the recent characterization of several megasynthetases from myxobacteria and actinomycetes has revealed further examples of modular iteration.[74,84,85] Additionally, on the basis of feeding experiments and gene cluster analysis, a new PKS chain-release mechanism was proposed to account for the stigmatellin chromone ring, but this hypothesis awaits experimental support. The termination

(a)

14 Stigmatellin A (*Stigmatella aurantiaca*)

15 Aurafuron A (*S. aurantiaca*)

5 Soraphen A (*S. cellulosum*)

16 Spirangien A (*Sorangium cellulosum*)

17 Etnangien (*S. cellulosum*)

18 Jerangolid A (*S. cellulosum*)

6 Ambruticin VS-5 (*S. cellulosum*)

(b)

19 Aurachin D (*S. aurantiaca*)

(c)

20 Flaviolin (*S. cellulosum*)

Figure 5 Myxobacterial compounds generated by PKSs. Structure, name, and producer organism are given. (a) Type I PKS-derived products; (b) type II PKS product; (c) type III PKS product.

step is believed to be catalyzed by a novel C-terminal cyclization (Cyc) domain in StiJ instead of the common (terminal) TE domain (**Figure 7**). The resulting product is then further decorated by post-PKS steps, which include O-methylation of the two chromone ring hydroxyl groups by StiK and ring hydroxylation by a cytochrome P-450 monooxygenase, StiL.[62]

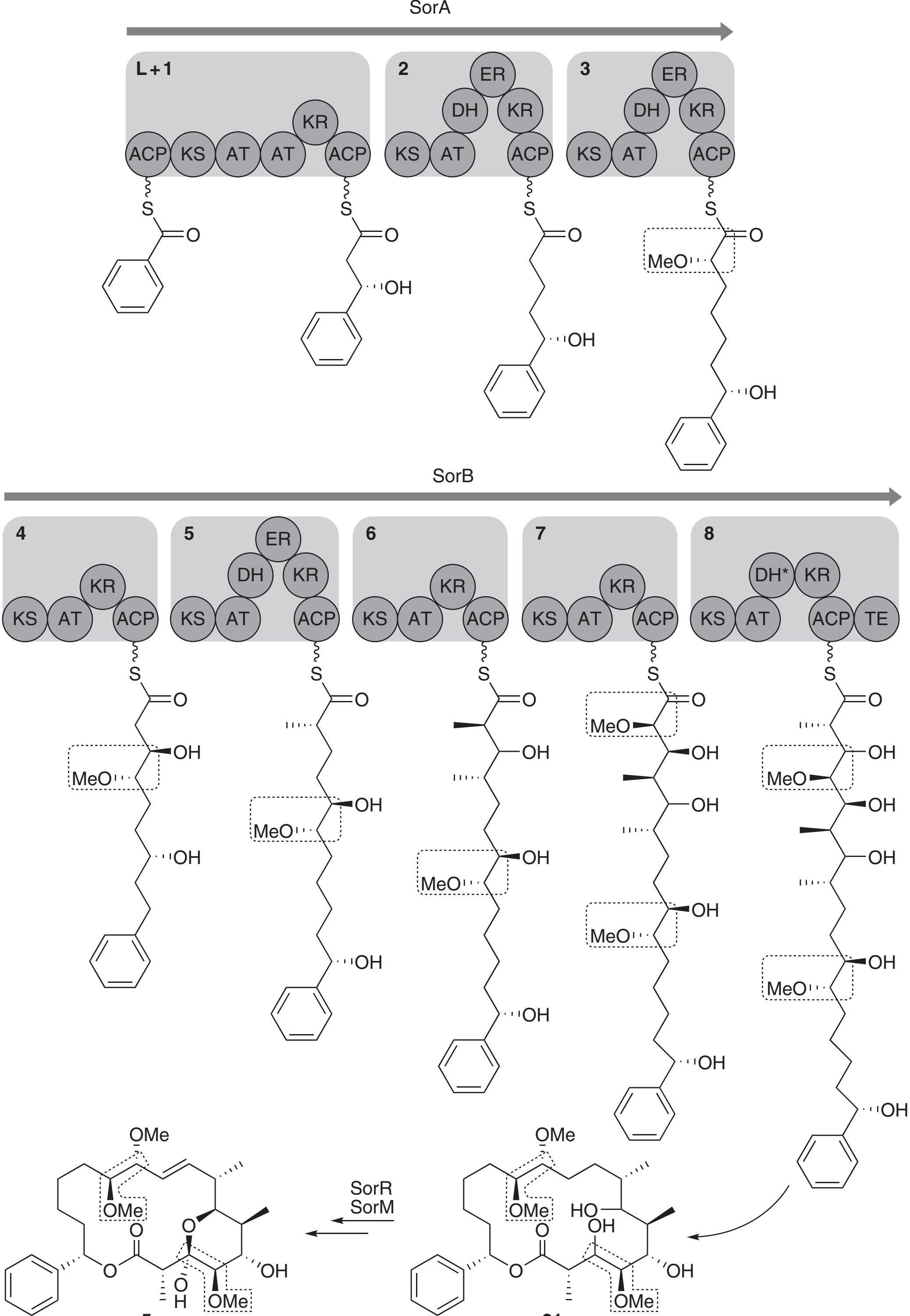

Figure 6 Type I PKS biosynthesis in myxobacteria (a). Biosynthesis of soraphen A (**5**) in *Sorangium cellulosum* So ce12. The DH domain from module 8 (marked with an asterisk) is most likely inactive. The unusual 'methoxymalonate' extender units incorporated by modules 3 and 7 are boxed. Most likely, macrocycle **21** released from SorB undergoes further post-PKS modifications (O-methylation, dehydration, and pyran ring formation) to form **5**. The stereochemistry of the linear intermediates bound to the enzyme complex was assigned based on the absolute configuration of **5** (and **21**).

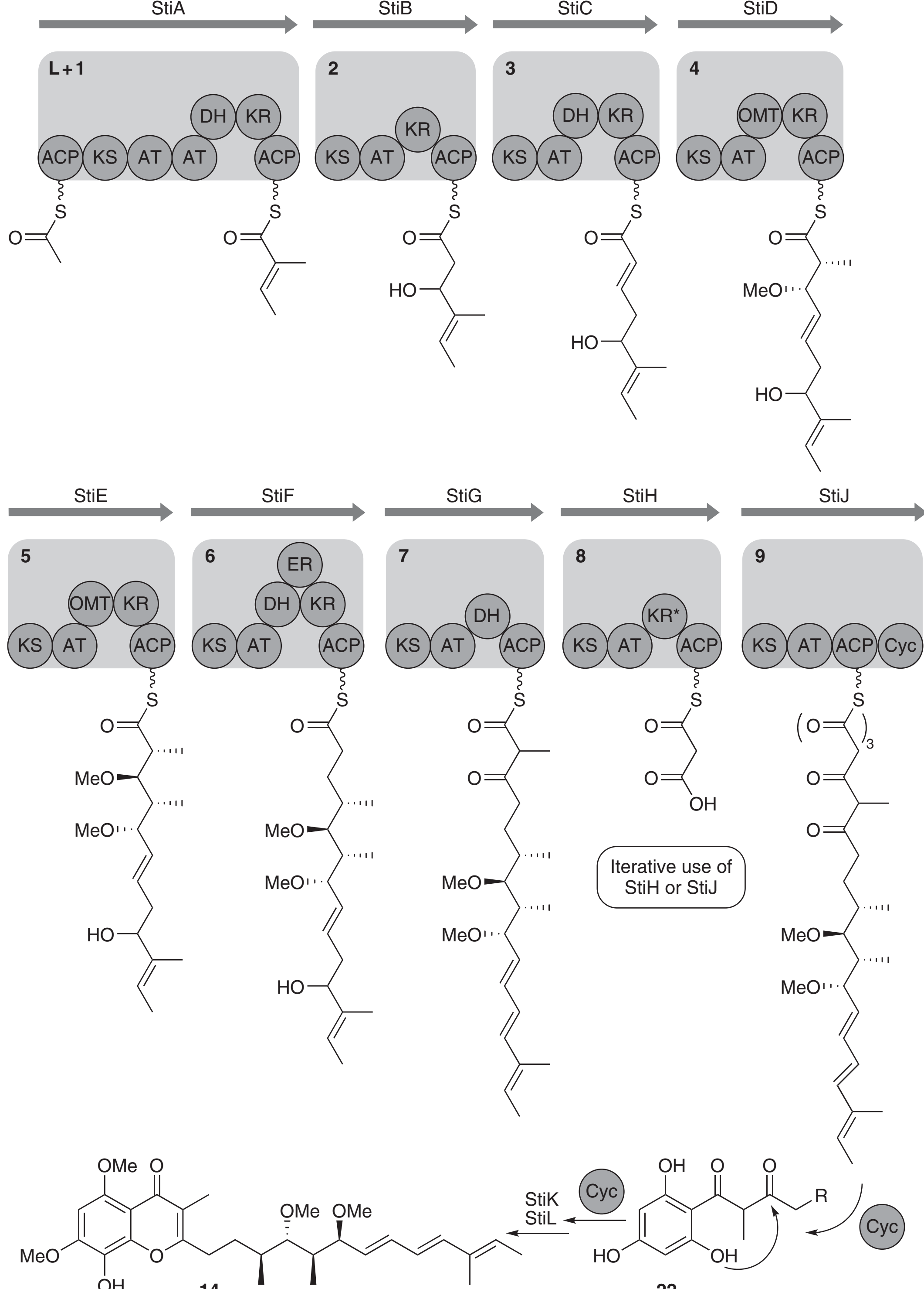

Figure 7 Type I PKS biosynthesis in myxobacteria (b). Biosynthesis of stigmatellin A (**14**) in *Stigmatella aurantiaca* Sg a15. The KR domain from module 8 (marked with an asterisk) is most likely inactive. The hydroxy group generated by module 2 by reduction of the first extender unit is assumed to be dehydrated by the module 7 DH domain. StiH and StiJ incorporate three malonyl-CoA extender units in total. Thus, one of these modules appears to function iteratively. The polyketide chain is released and cyclized by the terminal Cyc domain, most likely via intermediate **22**, and further decorated in post-PKS biosynthetic steps catalyzed by StiK and StiL. The stereochemistry of the linear intermediates bound to the enzyme complex was assigned based on the absolute configuration of **14**.

The collection of pure PKSs characterized from myxobacteria also includes the assembly lines for spirangien, aurafuron, jerangolid, ambruticin, and etnangien biosynthesis.[68–70,72] All of these systems belong to the type I class of megasynthases. Therefore, the recent discovery of a type II-like PKS directing aurachin biosynthesis in *S. aurantiaca* Sg a15 was unexpected.[39] Aurachins are isoprenylated quinoline alkaloids that act as electron transport inhibitors.[86] Previous feeding studies revealed that these structurally unique metabolites are biosynthesized from anthranilic acid (**23**) and acetate as building blocks.[87] The corresponding biosynthetic gene cluster contains a set of five genes (*auaA–E*), three of which (*auaB–D*) encode proteins typical for type II PKS systems[51,75,88] (an ACP (AuaB) and a presumed KSα (AuaC)/KSβ (AuaD) heterodimer).[39] Interestingly, AuaD lacks the active site Gln residue, which is usually essential for decarboxylation of the starter unit.[51] Therefore, it has been speculated that AuaD may be inactive, and thus not required for biosynthesis; alternatively, it may maintain its proposed role in chain length control.[89]

In addition to the PKS enzymes, the gene cluster encodes a prenyl transferase (AuaA) as well as a benzoate CoA ligase (AuaE). In the proposed biosynthetic scheme, anthranilic acid (**23**) is first activated as its CoA thioester (**24**) by AuaE and then transferred to the ACP (AuaB). The starter unit is then elongated with two malonyl-CoA units by the 'minimal PKS' (AuaBCD). Release of the polyketide intermediate and decarboxylation of the second extender unit would yield the quinoline structure (**25**) (**Figure 8**). Prenylation of the polyketide core with farnesyl pyrophosphate catalyzed by AuaA would then yield aurachin D (**19**). This compound is assumed to be transformed to further aurachin derivatives (aurachins C, B, and A) by enzyme activities that have not yet been identified and which are likely to be encoded in a different genomic region from the core gene cluster.[39]

The aurachin biosynthetic model is supported by mutagenesis experiments and feeding studies, which revealed that the quinoline structure (**25**) accumulates in *auaA* inactivation mutants. In addition, aurachin production was restored by feeding of the quinoline (**25**) to *auaD* and *auaE* inactivation mutants.[39] To our knowledge, the aurachin biosynthetic machinery was the first example of a type II PKS from a Gram-negative

Figure 8 Type II PKS biosynthesis in myxobacteria. Biosynthesis of aurachin D (**19**) in *Stigmatella aurantiaca* Sg a15. The biosynthetic steps that are catalyzed by the gene products (AuaA–AuaE) from the aurachin core cluster are shown (AuaA, prenyl transferase; AuaB, ACP; AuaC, KSα; AuaD, KSβ; and AuaE, benzoate CoA ligase). Binding of anthraniloyl-CoA to the ACP domain has not been proven experimentally, so direct binding of the starter unit to the KSα might be possible.

bacterium. Phylogenetic analysis further suggests that the aurachin PKS is undergoing an evolutionary transition from a type II PKS into a type III PKS system and/or fatty acid synthase or vice versa, and is thus the founding member of a new group of PKSs.[39] However, further experiments will be required to verify this hypothesis.

In addition to the type I PKS systems and the aurachin 'type II' PKS, type III PKSs have also been discovered during the ongoing genome sequencing projects (one type III PKS in *M. xanthus* DK1622 and two in *S. cellulosum* So ce56s).[90] Although the secondary metabolite profiles of both organisms and of a type III PKS inactivation mutant from *S. cellulosum* were extensively analyzed, no product could be correlated to any of these PKSs.[3,90] This finding suggests that these genes are 'silent' under the employed cultivation conditions. However, expression of one of the *Sorangium* type III PKSs in different *Pseudomonas* sp. resulted in red cultures (see **Figure 9**). The pigment responsible was identified as 2,5,7-trihydroxy-1,4-naphthoquinone (flaviolin (**20**)), the autooxidation product of 1,3,6,8-tetrahydroxynaphthalene (THN (**26**); **Figure 9**).[90] THN (**26**) is the primary product of type III PKS. The compound results from condensation of five malonyl-CoA units to form a pentaketide chain, which is then released from the enzyme by decarboxylative cyclization (**Figure 9**). Interestingly, neither **26** nor any known type III PKS product has ever been described from any myxobacterium. The production of flaviolin (**20**) in pseudomonads therefore represents the first example of the artificial activation of a myxobacterial secondary metabolite gene, and demonstrates the usefulness of heterologous expression techniques to discover (novel) compounds from silent natural product biosynthetic pathways (for more details on heterologous expression, see Section 2.07.5).

2.07.3.2 Biosynthesis of Nonribosomal Peptides

As with type I PKSs, NRPSs are built of repetitive catalytic units (modules), which are each responsible for the incorporation of one amino acid into the growing peptide chain.[52,91] Although different chemistries are employed for activation and condensation of the substrates, the basic steps of NRPS chain elongation show striking similarities to the type I PKS mechanisms: (1) recognition of the amino acid substrate and its activation as an aminoacyl adenylate; (2) covalent binding of the residue as a thioester to a carrier protein; and (3) condensation with the peptidyl residue attached to the upstream module.[52,91] Consequently, a typical NRPS elongation module minimally comprises an adenylation (A) domain responsible for amino acid activation, a thiolation (T) domain (also known as a peptidyl-carrier protein (PCP)) to which the activated amino acid is covalently attached, and a condensation (C) domain, which catalyzes peptide bond formation.[52,91] As in PKSs, a variety of optional domains, for example, MTs or epimerization (E) domains, further increase the structural

Figure 9 Type III PKS biosynthesis in myxobacteria. Biosynthesis of flaviolin (**20**) catalyzed by a type III PKS (CHS) from *Sorangium cellulosum* So ce56. The primary product released from the CHS, 1,3,6,8-tetrahydroxynaphthalene (**26**), is spontaneously oxidized to form **20**. Neither **26** nor **20** has been identified in the myxobacterial strain, indicating that the CHS-encoding gene is silent under the tested conditions. Activation of the type III PKS-encoding gene by heterologous expression in *Pseudomonas putida* led to the formation of the red pigment **20** in the *P. putida::CHS* strain (see picture).

diversity.[92] In the so-called 'linear NRPS' systems, the number and order of modules match the number and sequence of the amino acids incorporated into the peptide product. The first amino acid of the peptide product is selected by an initiation module, which lacks a C domain, while the terminal module usually contains a TE domain to release the full-length chain from the enzyme.[52,91]

To date, only two purely NRPS biosynthetic machineries have been reported from myxobacteria.[22,55,56] The first NRPS pathway to be characterized (and the first myxobacterial gene cluster to be identified) directs the biosynthesis of the DNA-binding antibiotic and antitumor agent saframycin Mx1 **30** in *M. xanthus*.[93] Its heterocyclic quinone structure originates from a linear peptide intermediate **27** (Ala-Gly-Tyr-Tyr), which is synthesized by a tetramodular assembly line composed of two multifunctional NRPSs, SafA and SafB (**Figure 10**).[55,56] It is likely that the tyrosine precursor is modified to 3-hydroxy-5-methyl-*O*-methyltyrosine through hydroxylation as well as O- and C-methylation reactions, before the monomer is loaded onto the NRPS complex. Once the tetrapeptide structure (**27**) is constructed, chain release by the last module of the assembly line should occur. However, in SafA, the typical C-terminal TE domain is substituted with a putative

Figure 10 Nonribosomal peptide biosynthesis in myxobacteria (a). Biosynthesis of saframycin Mx1 (**30**) in *Myxococcus xanthus* DM504/15. The tetramodular assembly line follows textbook biochemistry, except for the unusual reductive chain release by the terminal Red domain. The linear peptide chain (**27**) then undergoes several cyclization steps (the underlying mechanisms are not fully understood) and is further decorated with functional groups (highlighted in gray) by enzyme activities, which have not been identified to date. Based on the absence of E domains in the assembly line and the absolute configuration of the end product **30**, an L-configuration was assigned to the incorporated amino acids.

reductase functionality (Red domain). At the time of its discovery, such a domain was new to NRPS systems. However, a Red domain that participates in the biosynthesis of myxochelin has been characterized recently and shown to either release the chain from its thioester as a free aldehyde[94] or reduce it a second time to the corresponding alcohol.[95] An analogous reaction in the biosynthesis of saframycin (**Figure 10**) is proposed to release the PCP-bound peptide chain as a linear aldehyde (**27**). A series of intramolecular cyclizations (the underlying mechanisms are not yet understood) then lead to the formation of the ring structures characteristic of this compound family. The obtained peptide product has to undergo further modifications, including regiospecific hydroxylation, oxidation, and methylation reactions (the resulting functionalities are highlighted in gray in **Figure 10**) to produce saframycin Mx1 (**30**).

To date, the saframycin biosynthetic pathway from *M. xanthus* remains only partially characterized. A single modifying enzyme, the *O*-methyltransferase SafC, assumed to be involved in methyltyrosine formation, has been located adjacent to the two NRPSs (SafA and SafB).[96] Presumably, additional enzymes involved in postassembly line decoration are located adjacent to *safA–C*, but have not yet been identified. *Pseudomonas fluorescens* A2-2 and *Streptomyces lavendulae* NRRL 11002 produce the structurally related compounds safracin B (SAC-B) and saframycin A (SFM-A), and the complete biosynthetic pathways of these metabolites have been cloned and characterized.[97,98] Compared to the SafA/SafB NRPS complex from *M. xanthus*, both assembly lines consist of three NRPS proteins (SacA–C and SfmA–C) and, remarkably, different and individual biosynthetic strategies for the formation of the Ala–Gly–Tyr–Tyr tetrapeptide intermediate were proposed for each of these three systems.[98]

The NRPS-based biosynthesis of the myxochelins – catecholate-type siderophores produced by a number of myxobacterial strains – has also been investigated.[21] The biosynthetic pathway, the only known myxobacterial secondary metabolite gene cluster involved in iron acquisition, has been identified in *S. aurantiaca* Sg a15,[22] *S. cellulosum* So ce56,[23] and *M. xanthus* DK1622 (unpublished data), and six genes are found to be involved in myxochelin biosynthesis.

In contrast to the linear saframycin assembly line, the myxochelin biosynthetic complex operates in a nonlinear fashion. Such NRPSs deviate in domain organization from the standard (C-A-PCP)$_n$ architecture.[91] Initially, nonlinear NRPSs were regarded as rare exceptions to the colinearity rule, but with increasing sequence information on NRPS pathways, it has become clear that this type of system comprises a considerable fraction of nature's NRPS inventory. The mechanisms involved in myxochelin assembly have been elucidated, following reconstitution *in vitro* of the complete biosynthetic pathway by heterologous expression of the core NRPS biosynthetic proteins (MxcE, F, and G) as well as MxcL *in Escherichia coli*.[94,95] The loading module is split over two proteins – MxcE containing the adenylation domain, and MxcF containing the aryl-carrier protein (CP) domain connected to an isochorismate synthase (IC). 2,3-DHBA is activated by MxcE and transferred to the CP of MxcF (**Figure 11**). Overall, MxcF transfers two 2,3-DHBA units to MxcG for condensation with both the α- and ε-side-chain amino groups of the activated lysine; the double condensation activity of the MxcG C domain is very unusual. Another atypical feature of MxcG is the presence of a terminal NAD(P)H-dependent reductase (Red) domain (instead of the common TE domain), which catalyzes the release of the product from the enzyme complex. During this process, the PCP-bound thioester intermediate is reduced to yield an aldehyde (**31**),[94] which can undergo either a further round of Red-catalyzed reduction to generate the corresponding alcohol myxochelin A (**13**) or reductive transamination by MxcL to produce myxochelin B (**32**) (**Figure 11**). Similar reductive chain-release mechanisms have also been postulated for the biosynthesis of myxalamide[49] and saframycin.[55,56,98] The myxochelin assembly line represents the first example of a functional *in vitro* reconstitution of a complete myxobacterial NRPS assembly line, which set the stage for detailed analysis of the underlying biosynthetic mechanisms, especially the unusual reductive termination step.[94,95]

Interestingly, comparison of the myxochelin biosynthetic pathways from *S. aurantiaca* Sg a15 and *S. cellulosum* So ce56 reveals that the gene cluster organization is quite different,[23] which might reflect the evolutionary distance between the two strains (*S. aurantiaca* belongs to the myxobacterial suborder Cystobacterineae, and *S. cellulosum* to Sorangiineae). However, as the corresponding genes are quite similar in sequence (48–70% identity), it is tempting to speculate that the myxochelin pathway has diverged from a common ancestor.[23]

Figure 11 Nonribosomal peptide biosynthesis in myxobacteria (b). Biosynthesis of myxochelin A (**13**) and myxochelin B (**32**) in *Stigmatella aurantiaca* Sg a15. The biosynthetic complex represents a nonlinear NRPS system composed of a split loading module (MxcE and MxcF) and an elongation module (MxcG), which catalyzes two condensation steps. The terminal Red domain was recently shown to release the aldehyde **31**, which can undergo a further round of Red-catalyzed reduction to yield **13** or be reductively transaminated by MxcL to produce **32**.

2.07.3.3 Biosynthesis of PKS–NRPS-Derived Hybrid Structures

The structural and catalytic similarities between PKSs and NRPSs suggest that these modular enzymatic systems should be able to cooperate to form natural products – indeed many examples of hybrid PKS–NRPS systems are now known. The analysis of these biosynthetic machineries is of special interest for combinatorial biosynthesis, because creating new combinations of PKS and NRPS multienzymes could in principle lead to an enormous variety of structures.[53,99] Strikingly, the majority of myxobacterial biosynthetic gene clusters characterized to date (13 out of 23) direct the formation of polyketide/peptide hybrid metabolites (**Figure 12**). Among these compounds are the antifungal ajudazols, potent inhibitors of mitochondrial electron transport from *Chondromyces crocatus*.[100] The ajudazols are novel isochromanone derivatives, which incorporate an extended side chain containing an oxazole ring, a *Z,Z* diene, and a 3-methoxybutenoic acid amide.[101] Although the adjudazol backbone could be predicted straightforwardly to arise from a mixed PKS–NRPS system, the mechanism for generating some of the structural elements including the isochromanone ring and the *exo*-methylene group was not obvious from considerations of standard assembly line biochemistry.

In order to study the underlying biosynthetic processes, the corresponding biosynthetic gene cluster was cloned from *C. crocatus* Cm c5.[71] The assembly line includes eight type I PKSs (AjuA–AjuC, AjuE–AjuH, and AjuK), one NRPS (AjuD), and one NRPS–PKS hybrid enzyme (AjuL) (**Figure 13**). Adjudazol biosynthesis starts on AjuK, which displays the increasingly common intermixed chain initiation/extension module of the soraphen assembly line and other systems.[74] This module also contains an SAM-dependent *O*-methyl transferase; by analogy with the mechanism postulated for generation of the β-methoxyacrylate functionalities during myxothiazol and melithiazol biosynthesis,[54,63] this activity is assumed to methylate the enolized diketide intermediate. The remaining steps in adjudazol assembly are catalyzed by 12 extension modules (10× PKS and 2× NRPS modules), distributed among the AjuA–AjuH and AjuL subunits. Based on the location of double bonds in the adjudazol structure, DH activities were expected in modules 3, 5, 6, and 12. Interestingly, modules 3 and 5 both lack DH functions, and the DH domain from module 12 is mutated and so assumed to be inactive.[71] The missing DH activities in these three modules are most likely complemented by the iterative action of DH domains in the downstream modules 4, 6, and 13, as postulated for other myxobacterial systems.[74]

Figure 12 Myxobacterial compounds generated by PKS–NRPS hybrid systems. Thirteen PKS–NRPS biosynthetic gene clusters have been characterized to date. The structure of one representative member of the corresponding compound families is shown (except for the myxothiazol and cystothiazol structures, which are closely related to melithiazol A and the myxochromide A structure, which is similar to myxochromide S): tubulysin D (**1**), DKxanthene-534 (**9**), myxalamid B (**12**), disorazol A_1 (**7**), adjudazol A (**33**), chondramide D (**34**), melithiazol A (**35**), myxochromide S_1 (**36**), myxovirescin A_1 (**10**), and epothilone B (**2**).

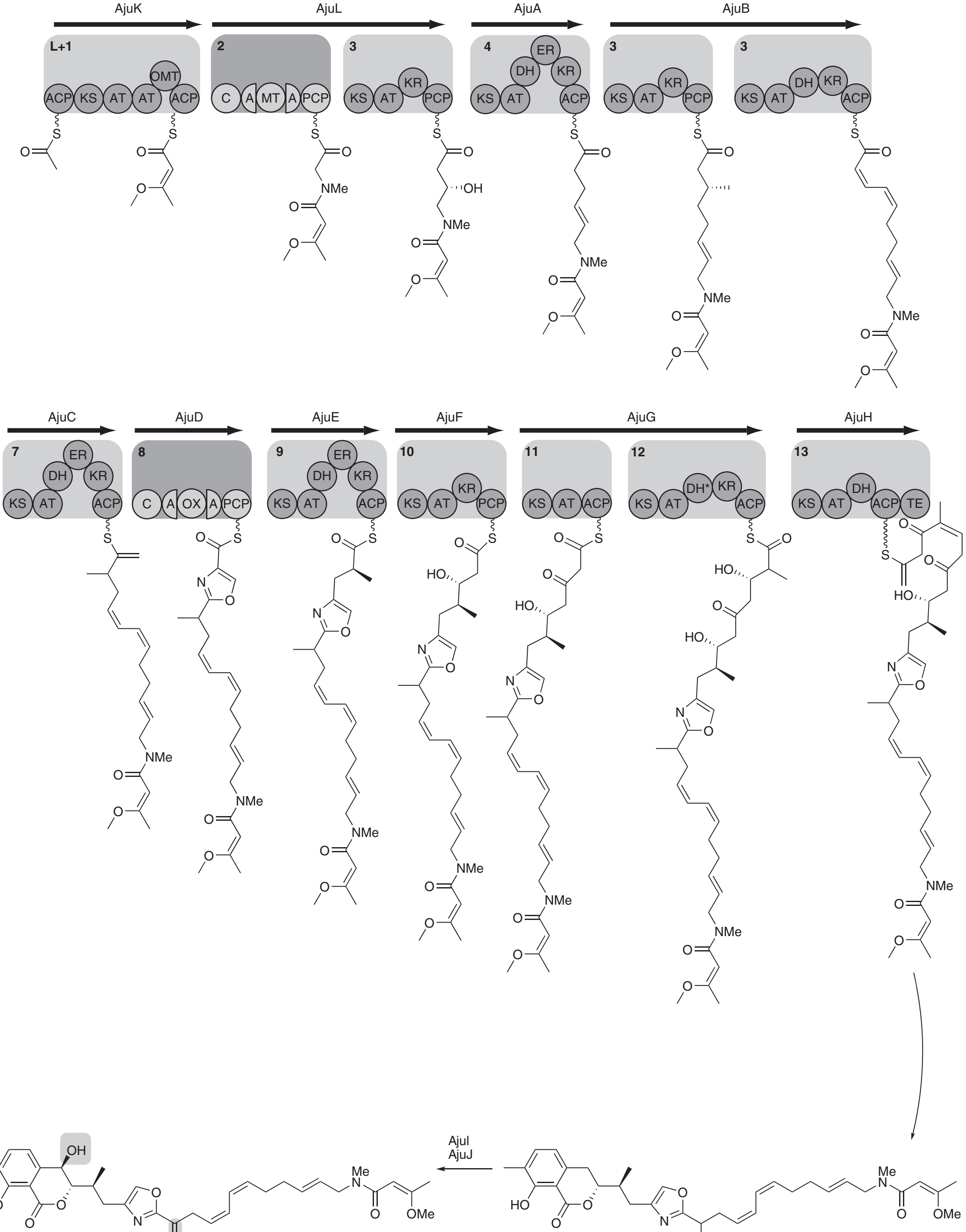

Figure 13 Biosynthesis of PKS–NRPS hybrid compounds in myxobacteria (a). Biosynthesis of adjudazol A (**33**) in *Chondromyces crocatus* Cm c5. The DH domain from module 12 (marked with an asterisk) is most likely inactive. The hydroxy groups generated by the KR domains from modules 3, 5, and 12 are assumed to be dehydrated by the DH functions in the downstream modules 4, 6, and 13, respectively. After the assembly of the polyketide chain, the unique isochromanone ring is possibly generated via TE-catalyzed lactonization as well as spontaneous aldol addition and aromatization processes. The obtained deshydroxyajudazol B (**37**) is further modified by two P-450 enzymes, AjuI and AjuJ, to yield adjudazol A (**33**) (modifications are highlighted in gray). The stereochemistry of the linear intermediates bound to the enzyme complex was assigned based on the configuration of **33**. The stereochemistries of the hydroxyl groups, which are subsequently dehydrated by downstream DH functions, were predicted based on the identified stereospecificity motifs in the respective KR domains.

A unique structural feature of the adjudazols is the aromatic isochromanone ring. A terminal cyclase functionality analogous to the cyclase of the stigmatellin PKS[62] could not be identified in the adjudazol assembly line. Instead, the PKS terminates in a TE domain (**Figure 13**). This observation might suggest that the isochromanone ring is generated by TE-catalyzed lactonization as well as spontaneous aldol addition and aromatization processes.[71] However, as the TE shows strongest homology to type II enzymes, the question was raised whether it is involved in the cyclization process at all. The obtained deshydroxyajudazol B (**37**) becomes the substrate of two modifying P-450 enzymes, AjuI and AjuJ, encoded within the biosynthetic gene cluster. Gene inactivation studies revealed that AjuJ is responsible for the hydroxylation of the isochromanone ring, whereas AjuI catalyzes the formation of the adjudazol A (**33**) *exo*-methylene group. This reaction represents the first P-450-catalyzed dehydrogenation process identified in bacterial metabolism.[71]

In addition to the adjudazols, *C. crocatus* Cm c5 produces a number of other secondary metabolites with potent and diverse biological activities.[102–106] These include the antitumor and antifungal chondramides A–D, which show striking similarities to a family of depsipeptides (e.g., jaspamide,[107,108] geodiamolides,[109] and neosiphoniamolide A[110]) isolated from diverse marine sponges. The chondramide PKS–NRPS biosynthetic machinery was recently identified, and shown to consist of two multifunctional PKSs (CmdA and CmdB) as well as two NRPS proteins (CmdC and CmdD) (**Figure 14**).[67] In common with several other myxobacterial PKSs, CmdA shows the arrangement of starter module/first extension module, which was initially thought to be atypical.[74] The biosynthesis begins with the selection and loading of an acetyl-CoA starter unit probably by the first AT domain. After elongation of the starter unit by module 1, the β-keto group is reduced to a hydroxyl function, which is needed for the final TE-mediated cyclization step. The dehydratase domain from this module shows low homology to functional dehydratases, and thus seems to be inactive.[67] The polyketide chain is elongated with two additional extender units and then further processed by the NRPS subunits CmdC and CmdD, which incorporate an alanine, a tryptophan, and finally a tyrosine residue into the structure. Although the stereochemistry of the tryptophan residue in the chondramide backbone is unknown, the presence of an epimerization (E) domain in module 5 argues for the epimerized D-configuration.[67] In some derivatives (chondramides B and D (**34**)), the tryptophan residue is chlorinated by the halogenase CmdE. The natural occurrence of unchlorinated chondramides indicates that CmdE modifies an NRPS-bound species or the end product of the NRPS, rather than the free tryptophan.

Another striking structural feature of the chondramides is the β-tyrosine moiety, as β-amino acids are relatively rare in nature. This functionality is thought to arise from intramolecular migration of the α-amino group, catalyzed by a so-called aminomutase.[111–114] A gene (*cmdF*) encoding a putative tyrosine amino mutase (TAM) was identified within the chondramide biosynthetic gene cluster. In recent biochemical studies, CmdF was shown to convert L-tyrosine (**38**) into R-β-tyrosine (**39**), which is then activated by the A domain of module 6.[115] However, *in vitro* studies have also shown that the A domain is able to activate α-tyrosine, although to a lesser extent. As all known chondramides incorporate R-β-tyrosine (**39**) in this position, the finding of promiscuous activation specificity by the A domain indicates that one or more of the C, PCP, or TE domains from module 6 perform a gatekeeper function.[67,115] Biosynthesis is terminated by the module 6 thioesterase, which catalyzes the concomitant macrocyclization and release of the PKS–NRPS chain from the assembly line.

Further PKS–NPRS hybrid compounds of interest from myxobacteria are the yellow pigment myxochromides A and myxochromides S, which consist of a cyclic peptide core decorated with a lipophilic side chain (**Figure 15**).[34,66] The myxochromide S biosynthetic complex was identified during a screening for PKS–NRPS hybrid pathways in *S. aurantiaca* DW4/3-1.[33] The assembly line comprises one PKS and two NRPS proteins (**Figure 15**), which both reveal interesting and novel biosynthetic features.[34] The PKS (MchA) involved in myxochromide side-chain biosynthesis was shown to be an iteratively acting enzyme, with an intrinsic capacity to produce polyunsaturated polyketide chains of varying length.[34,116] This type of PKS is capable of conducting multiple rounds of chain extension and was thought to be employed exclusively by fungi until Bechthold and coworkers verified the first example of an iteratively acting bacterial PKS module in 1997.[117] MchA accepts different starter units (acetyl- or propionyl-CoA) and occasionally performs additional chain extension cycles. Therefore, MchA represents a highly unusual iterative modular PKS that exhibits imperfect chain length control.[34,84,116] Sequence analysis reveals mutations in the active site of the MchA ER domain, which explains production of the highly polyunsaturated side chain.

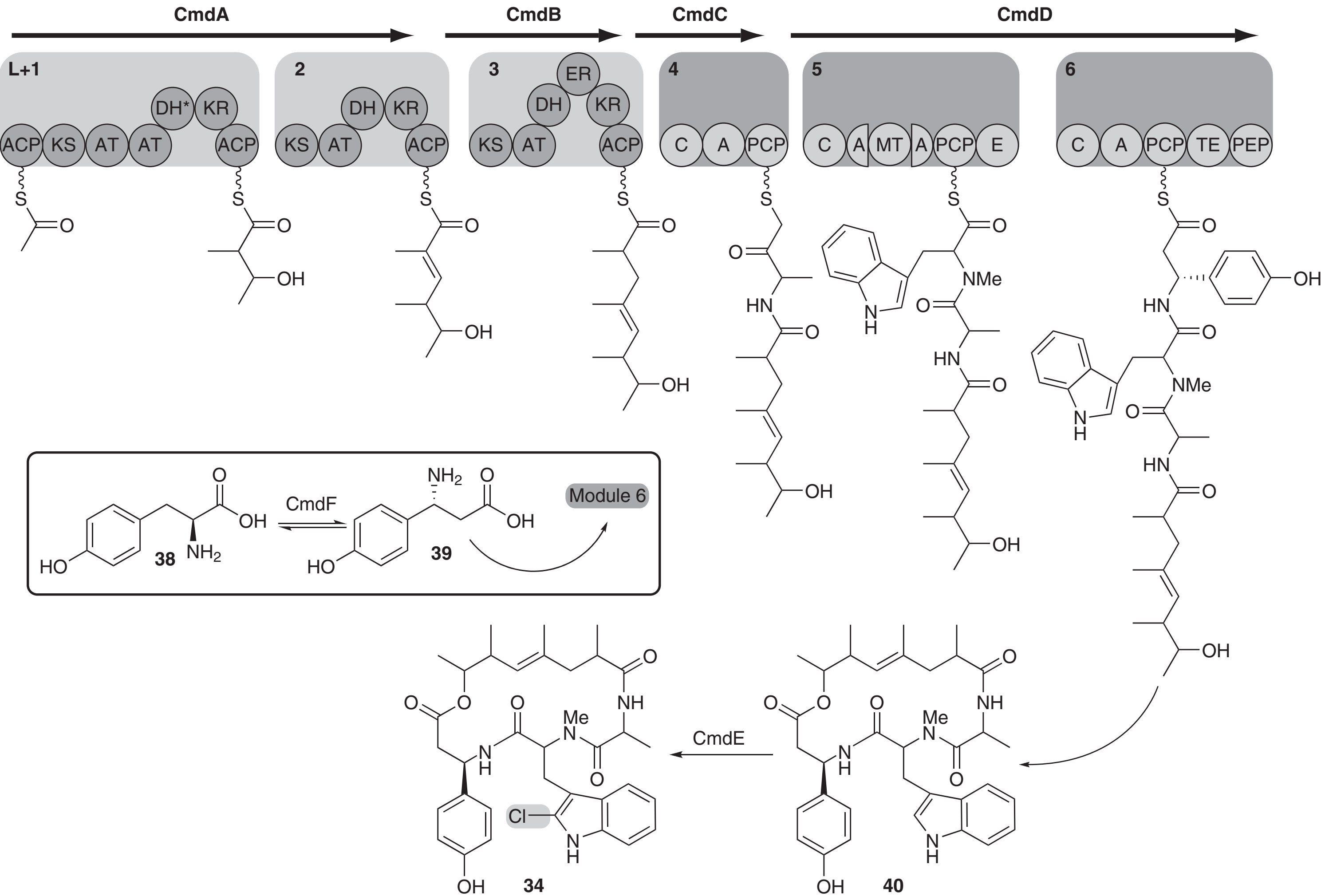

Figure 14 Biosynthesis of PKS–NRPS hybrid compounds in myxobacteria (b). Biosynthesis of chondramide D (**34**) in *Chondromyces crocatus* Cm c5. The DH domain from module 1 (marked with an asterisk) is most likely inactive. During the assembly of the chondramide backbone, an unusual extender unit – a β-amino acid – is incorporated by NRPS module 6. The precursor is generated by the tyrosine amino mutase (TAM) CmdF, which converts L-tyrosine into R-β-tyrosine (see box). The function of the terminal phosphoenolpyruvate synthase (PEP) domain is still unknown. Macrocyclization catalyzed by the TE domain yields chondramide C (**40**), which can be further transformed to the chlorinated derivative chondramide D (**34**). The halogenation process catalyzed by CmdE may also take place on the assembly line intermediate.

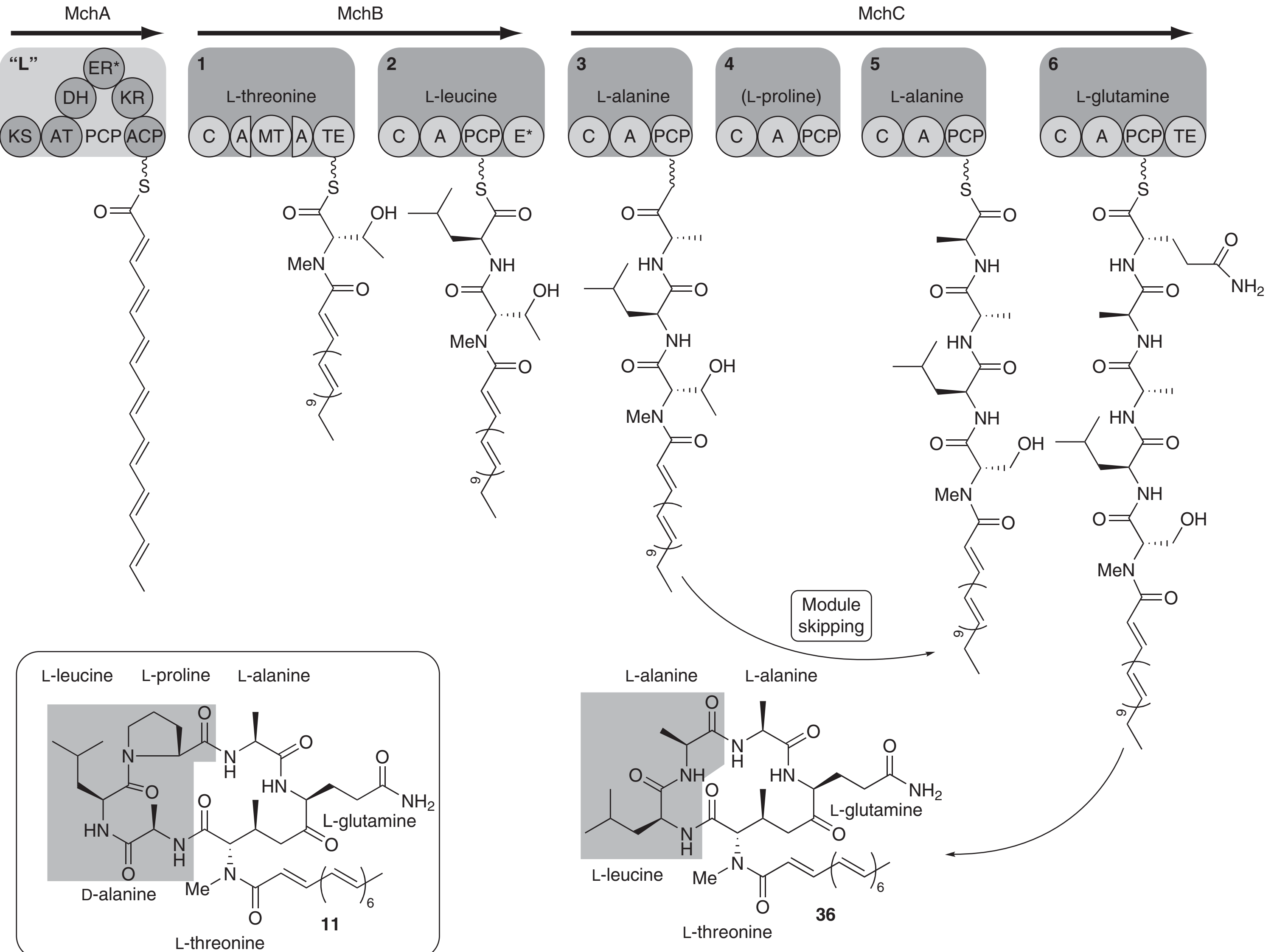

Figure 15 Biosynthesis of PKS–NRPS hybrid compounds in myxobacteria (c). Biosynthesis of myxochromide S_1 (**3**) in *Stigmatella aurantiaca* DW4/3-1. The iterative PKS MchA produces side chains of varying length, but only one product is shown. The ER domain from MchA and the E domain from MchB (all marked with an asterisk) are most likely inactive and/or not functional during biosynthesis. In addition, module 4 is skipped during peptide chain assembly, resulting in the formation of a pentapeptide core. Structurally related compounds (e.g., myxochromides A_2; see box) were isolated from *Myxococcus xanthus* DK1622 and arise from an evolutionarily similar assembly line. Experiments have revealed that point mutations in the NRPS proteins led to the observed differences in the peptide cores (highlighted in gray).

After polyketide chain formation, biosynthesis of the myxochromides S proceeds with the assembly of the peptide core, directed by the two NRPSs MchB and MchC. The N-terminal C domain from MchB most likely catalyzes condensation of the polyketide chain (bound to the MchA-ACP) with the amino group of threonine. This postulated role for the C domain resembles the proposed function of N-terminal C domains from several lipopeptide assembly lines.[118–121] The myxochromide chain is then elongated with an L-configured leucine by module 2; this observation indicates that the terminal E domain, which usually catalyzes the epimerization of the amino acid, does not act. The biosynthesis of the peptide chain continues with the incorporation of alanine by module 3, which is located on the tetramodular MchC (**Figure 15**). Intriguingly, MchB and MchC together contain six NRPS modules, although only five amino acids are incorporated into the myxochromide S peptide core (Thr–Leu–Ala–Ala–Gln). By detailed *in silico* analysis of the substrate specificity of the A domains combined with biochemical studies, it was shown that NRPS module 4, 'encoding' for proline, is skipped during peptide chain assembly.[34,66] The module skipping process in myxochromide S biosynthesis diverges from the paradigm of linear processive assembly, and is to date unique for nonribosomal peptide biosynthesis.[84] Finally, alanine and glutamine residues are incorporated by modules 5 and 6, and then the pentapeptide chain is cyclized and released from the biosynthetic complex by the terminal TE domain (**Figure 15**).

Intriguingly, the organization of NRPS modules and domains in the myxochromide S megasynthetase coincides (at least in theory) with the biosynthesis of the structurally similar myxochromide A compound family. Myxochromides A comprise a hexapeptide core containing the same amino acids (plus proline) as the myxochromides S, albeit in a different order.[66] To establish an evolutionary link between the myxochromide S and myxochromide A pathways, the genes directing myxochromide A biosynthesis were identified in the genome sequence of *M. xanthus* DK1622. Comparison of both PKS–NRPS megasynthetases shows an identical arrangement of modules and domains. By detailed sequence analysis of both assembly lines, together with biochemical investigations using purified NRPS domains, it was demonstrated that point mutations and module skipping have led to the chemical diversity found in this class of secondary metabolites.[66] Mutations in the A domains have resulted in different substrate specificities for modules 2 and 3 (Leu and Ala for myxochromide S compared to Ala and Leu for myxochromide A), which might also explain the nonoperation of the E domain during myxochromide S biosynthesis because it no longer recognizes the new substrate. Additionally, a number of mutations in module 4 of the myxochromide S NRPS, especially in the PCP core motif, have resulted in overall inactivation of this catalytic unit, and induced the unique module skipping process. The striking features of the myxochromide S assembly line (iterative use of the PKS module and module skipping) again illustrate that bacterial multimodular PKS and NRPS assembly lines have a much greater diversity in their biosynthetic operation than originally anticipated.

2.07.3.4 Prediction of Stereochemistry Based on Genetic Information

Detailed mechanistic studies of natural product pathways have greatly advanced the field. This knowledge can now be applied to predict, often with confidence, the stereochemical outcome of biosynthetic steps, yielding important information on the stereochemistry of natural products prior to their total synthesis. One of the most obvious successes of this approach has been through the analysis of modular PKS ketoreductases, which has allowed prediction of the direction of ketoreduction.[122,123] Application of this technique to the gene clusters for chivosazol, thuggacin, and etnangien, in combination with chemical degradation, allowed the full stereochemical assignment of the corresponding metabolites.[72,124,125] In addition, the presence or absence of epimerization domains in nonribosomal peptide synthetases can be used to predict the configuration of each amino acid in the biosynthetic product. This knowledge was applied to daptomycin after the biosynthetic genes were identified, resulting in correction of the original configurational assignment of L-Asn to D-Asn.[126] It was also used to establish the positions of the two alanines with opposite stereochemistry in myxochromide A.[34]

These applications will undoubtedly become more attractive in natural product chemistry as the stereochemical assignment is an essential prerequisite for total chemical synthesis and its establishment is often obstructed by the limited availability of many natural compounds for chemical degradation and partial synthetic methods required to establish the various stereocenters.

2.07.4 Deciphering Regulatory Mechanisms of Secondary Metabolism to Increase Productivity

Product yield is very often a factor that limits the development of a natural product as a lead compound. Traditionally, both chemical synthesis and classical strain improvement technologies have been applied to overcome this limitation. As natural products are often molecules of high structural and stereochemical complexity, their total synthesis is usually difficult, and yields are low. Classical strain improvement represents an equally time-consuming and rather undirected process, during which numerous rounds of mutagenesis and subsequent screening are applied to obtain strains with improved production titers.

Based on the knowledge gained from molecular biosynthetic studies and the recent progress in sequencing technology, valid alternatives for production titer improvement have recently been established. These studies rely on identifying regulators of secondary metabolite biosynthesis in the producing organisms. If the regulation is positive (i.e., production is induced), targeted overexpression as a means to increase productivity becomes possible. Alternatively, negative regulators may be identified, which after inactivation should result in mutant strains overproducing the respective compound. In discussing these approaches, we distinguish between genome sequence-independent methods and those strategies that rely on the availability of the DNA sequence of the producing organism.

2.07.4.1 Genome Sequence-Independent Approaches

Prior to the availability of genome sequences, only undirected transposon-based technology was established in myxobacteria to advance the classical strain improvement approach. The benefit of this technology is that it allows straightforward identification of the transposition target site, and therefore directly generates molecular knowledge correlated to altered metabolite profiles. In addition, transposon mutagenesis is ideally suited to identify target genes that do not show similarities to known regulators, and so offers the opportunity to discover completely novel regulatory mechanisms (**Figure 16**). Proof of principle for this approach was established by the identification of a novel positive regulator (StiR) of stigmatellin biosynthesis in *Cystobacter fuscus*, after screening 1200 transposon mutants.[127] Inactivation of *stiR* by transposon insertion resulted in a 20-fold reduction of stigmatellin production, as judged by HPLC analysis of mutant extracts, compared to the wild-type strain. As StiR shows sequence similarity only to proteins of unknown function from other bacteria (e.g., to two conserved hypothetical proteins in *Y. pseudotuberculosis* and *B. cereus*), this study might also trigger research to reveal the function of the homologue in the pathogenic strain.

In a subsequent study, genes encoding negative regulators of secondary metabolite biosynthesis in *A. disciformis* were analyzed. *Angiococcus disciformis* produces the highly efficient electron transport inhibitor myxothiazol and the tubulin destabilizer tubulysin.[36,128] Evaluation of extracts from 1200 transposon mutants by HPLC revealed six mutants in which myxothiazol production was increased by as much as 30-fold. Identifying the transposon integration sites coupled with sequencing of flanking regions showed that some of the inactivated genes encode proteins with similarity to known bacterial regulators, such as two-component systems and serine–threonine protein kinases. However, other identified gene products did not resemble any characterized proteins. Taken together, the data show that the transposon-based strategy is a valuable tool for identifying regulatory genes of secondary metabolism, including gene loci that cannot be detected using current *in silico* approaches. The results also demonstrate that targeted genetic manipulation of regulatory mechanisms is a valuable adjunct to standard strain improvement methods.

2.07.4.2 Impact of Genome Sequencing

Recently, two complete myxobacterial genome sequences have become available,[40,41] revealing that both species are multiproducers of secondary metabolites.[32,73] With the recent rapid progress in genome sequencing technologies, genomic data will undoubtedly become the starting point for studies of myxobacterial secondary metabolism. The availability of the genome sequence not only allows the identification of novel biosynthetic gene clusters and compounds (see Section 2.07.3), but also provides the opportunity to search the genome

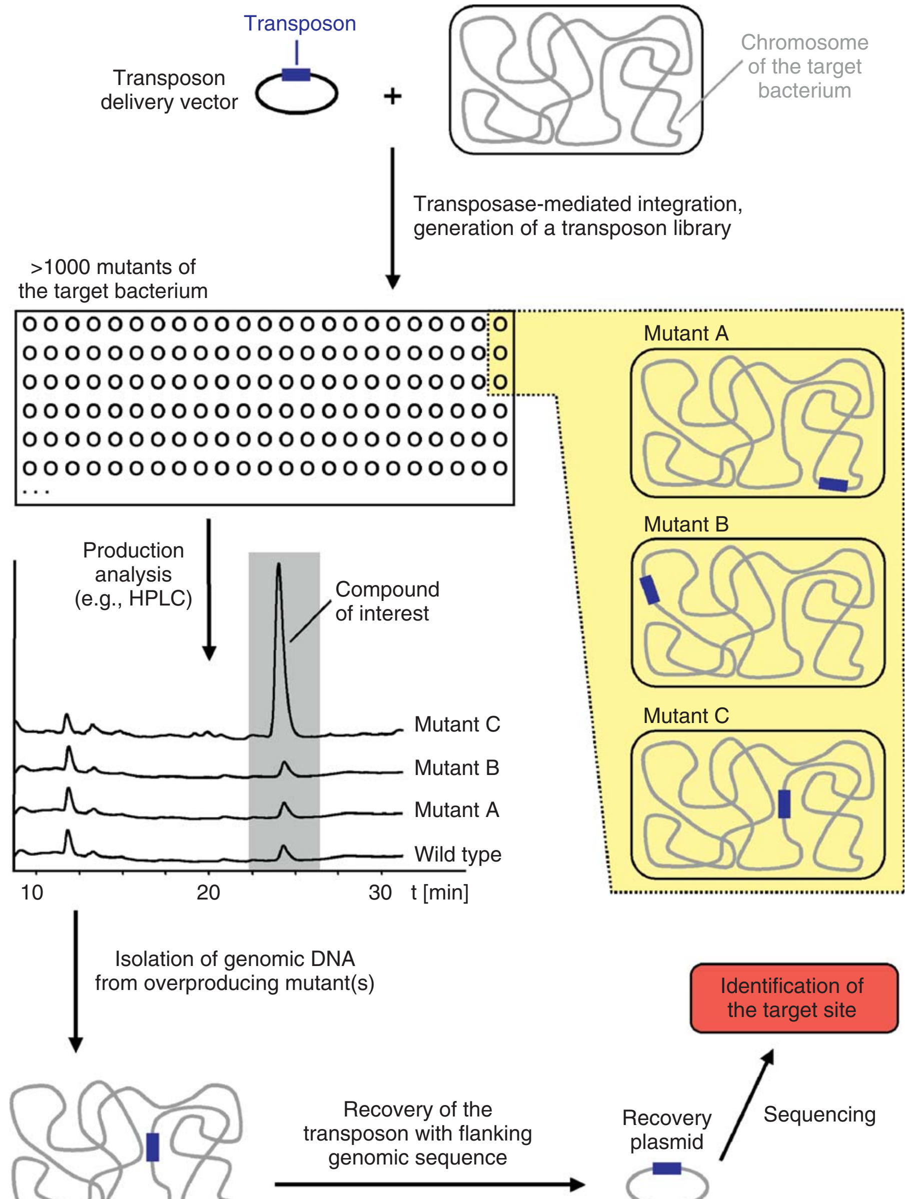

Figure 16 Overview of the transposon strategy. Transformation of the target bacterium with the transposon delivery vector generates mutants that harbor the transposable element (shown in blue) at various random locations in the chromosome. These mutants are analyzed for production of target compounds, and strains overproducing the metabolite (in the case of the search for negative regulators) are further characterized by identifying the target gene.

in silico for regulatory genes. This is especially important, because unlike in the actinomycetes, regulators of myxobacterial secondary metabolism are not typically colocalized with the biosynthetic genes. However, due to the presence of an enormous number of regulators in myxobacteria,[40,129] it is currently impossible to assign a regulator to a specific pathway on the basis of bioinformatics analysis alone.

To address this issue, biomagnetic separation technology has recently been applied to directly identify regulatory elements that interact with the control region(s) of biosynthetic gene clusters[130] (**Figure 17**). In this work, PCR was used to amplify the presumed promoter region of the chivosazol biosynthetic gene cluster in *S. cellulosum* So ce56. This DNA fragment was affinity-labeled during the PCR using biotinylated

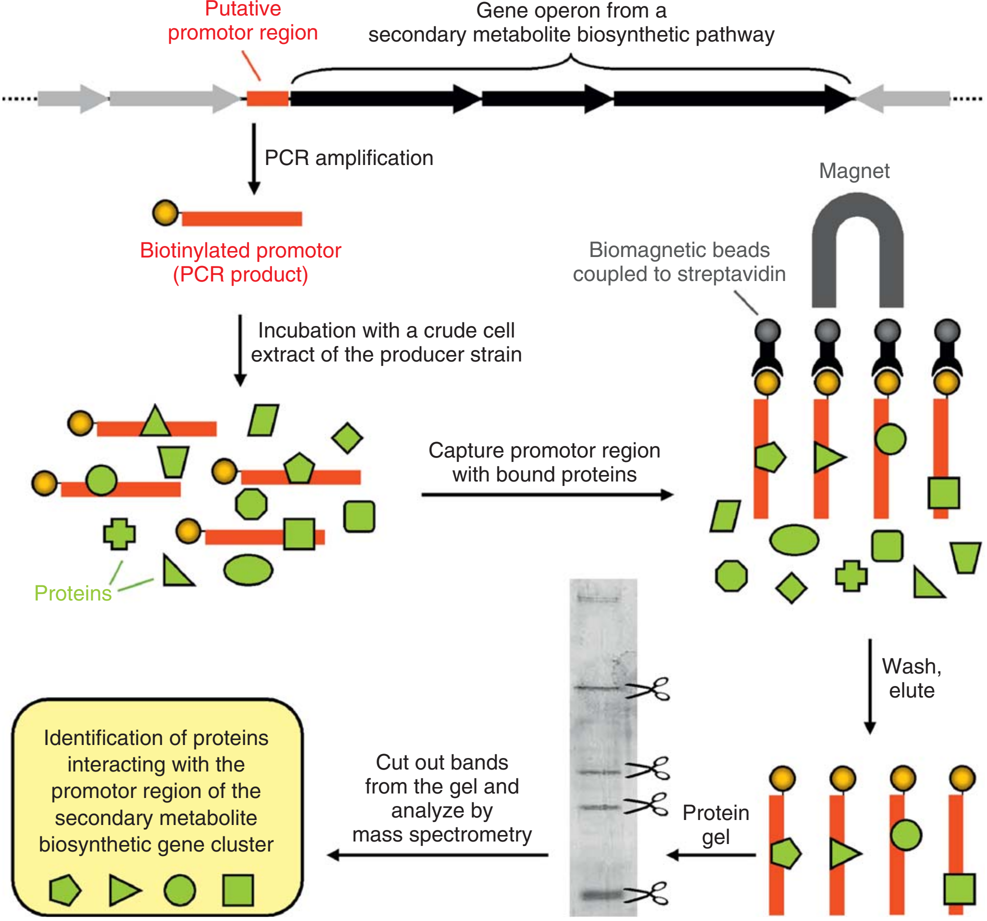

Figure 17 Biomagnetic separation strategy to identify direct regulators of secondary metabolism. After PCR amplification of the promoter region (shown in red), the biotinylated product is incubated with cell extract. Proteins (shown in green) specifically binding to the promoter are purified using biomagnetic separation and washing steps, and then analyzed by MS. Identification of the proteins is significantly enabled by the availability of the genome sequence.

oligonucleotides. Assuming that regulators of chivosazol biosynthesis would bind to the promoter region with high affinity, crude cell extracts of chivosazol-producing cells were incubated with the PCR product. Subsequently, streptavidin-coupled biomagnetic beads were used to capture the PCR product, together with the bound proteins from the crude cell extract. Washing steps were employed to purify specific regulators, which could next be eluted from the biotinylated DNA. Analysis by MALDI-ToF–ToF led to the identification of protein fragments, which were readily assigned to complete proteins encoded in the So ce56 genome. As expected, all subunits of the RNA polymerase were identified, as well as additional proteins including those that turned out to be regulators of secondary metabolite biosynthesis.

One of these proteins was ChiR. Inactivation of the encoding gene resulted in a dramatic decrease in chivosazol production, identifying ChiR as a direct positive regulator of chivosazol biosynthesis.[130] This finding was unexpected, because primary sequence analysis indicated that ChiR belongs to the DeoR family of proteins, known to function as negative regulators of diverse processes in other bacteria. Gel shift experiments using heterologously expressed ChiR identified the binding region within the promoter, and quantitative PCR analysis provided direct evidence that transcription of the chivosazol gene cluster is dependent on ChiR. The identification of ChiR as a positive regulator led to the expectation that increased expression of ChiR would result in improved productivity. Indeed, overexpression of the *chiR* gene in a merodiploid So ce56 mutant resulted in a fivefold increase in the production of chivosazol in a kinetic shake flask experiment and 2.5-fold

overproduction by fermentation. Yields of the other known secondary metabolites from So ce56 (etnangien and myxochelin) were not altered in the mutant strain. Notably, it would not have been possible to identify *chiR* by classical methods, as it is not colocated in the *S. cellulosum* genome with the chivosazol biosynthetic gene cluster.

In contrast, promoter fishing identified NtcA as a negative regulator of chivosazol biosynthesis.[130,131] NtcA is known to be involved in ammonium-dependent regulation of numerous processes in other bacteria. In general, an excess of ammonium salts in the growth medium of secondary metabolite producers is thought to accelerate growth, but in parallel to depress productivity.[132] In *S. cellulosum* So ce56, addition of ammonium to the growth medium indeed reduced the yield of chivosazol,[131,132] a regulatory process that seems to be directed, at least in part, by NtcA. Inactivation of *ntcA* gave rise to a mutant strain that overproduced chivosazol fivefold in a shake flask experiment (an increase from 150 to $\sim$700 mg L^{-1}). In addition, the inhibitory effect of ammonium on production was severely reduced in the mutant, as the mutant continued to produce a twofold excess of chivosazol relative to the wild-type strain, even in the presence of excess ammonium.

These data clearly demonstrate that targeted modification of regulatory mechanisms involved in secondary metabolite biosynthesis is an important tool for boosting production yields. The speed at which such mechanisms can be analyzed, sometimes in great detail, has been enormously accelerated by the availability of genome sequence information, as well as technologies for genetic manipulation. Novel regulators that would be transparent to standard bioinformatics analysis can be discovered by transposon mutagenesis. In addition, these studies provide significant insights into the complex regulatory networks of myxobacteria.

2.07.5 Biotechnological Strategies to Generate Modified Compounds

One of the simplest approaches toward trying to generate novel secondary metabolites is to feed unnatural precursor molecules to the culture broth of the producing organism. In this 'precursor-directed biosynthesis' strategy, feeding studies with isotopically labeled precursors are first used to identify specific building blocks in the natural product. This knowledge is then applied to select analogues of the building blocks to administer to growing cultures of the producer organism, in the hope that they will be incorporated into the final metabolite. For example, Hill and coworkers fed various halogenated forms of benzoic acid (the starter unit of soraphen biosynthesis (**Figure 6**)) to the native host *S. cellulosum*, and obtained chlorinated, brominated, and fluorinated derivatives of the soraphens.[133] However, production relative to soraphen itself was low, likely due to the preference of the PKS for the natural starter moiety. A similar strategy was recently used to generate novel chondramide derivatives, by feeding fluorinated tryptophan extender units to *C. crocatus*. The modified precursors were incorporated into the peptide chain by the NRPS, in parallel with the natural substrate.[67]

To increase the probability of producing unnatural compounds, a mixed genetic engineering/feeding approach termed 'mutasynthesis' has also been applied with myxobacteria. Here, genes directing the biosynthesis of the natural precursor or its incorporation into the assembly line are blocked by mutagenesis, and the mutant is grown in the presence of building block analogues. This approach was recently used to generate modified myxalamids in *M. xanthus*.[48] Myxalamids are widely occurring electron transport inhibitors that incorporate various starter units, including isobutyryl-CoA (found in the major metabolite myxalamid B). Isobutyryl-CoA is derived from the degradation of valine by the branched-chain keto acid dehydrogenase (BKD) complex (**Figure 18**). Consequently, by inactivation of the *bkd* gene *in M. xanthus*, it was possible to generate nine new myxalamid derivatives incorporating various cyclic and linear starter molecules, by feeding the appropriate precursors (**Figure 18**). Interestingly, this study also revealed that *M. xanthus* can transform a broad spectrum of short-chain carboxylic acid derivatives to their respective CoA esters, as such activation is necessary for the compounds to be recognized by the PKS biosynthetic machinery.

A more ambitious approach is to alter metabolite structures by genetic engineering of their respective biosynthesis gene clusters. This strategy, termed 'combinatorial biosynthesis', has been widely used with increasing success over the last decade.[134,135] This approach relies on the identification of specific gene clusters, detailed elucidation of the biosynthetic pathways, and the availability of genetic tools for manipulating the host organism. Although such work is difficult with the slow growing myxobacteria,[136] a number of pathways have been engineered successfully. For example, targeted inactivation of biosynthetic genes led to the formation of multiple derivatives of ajudazol and chondramide in *C. crocatus*,[67,71] stigmatellin and myxothiazol in

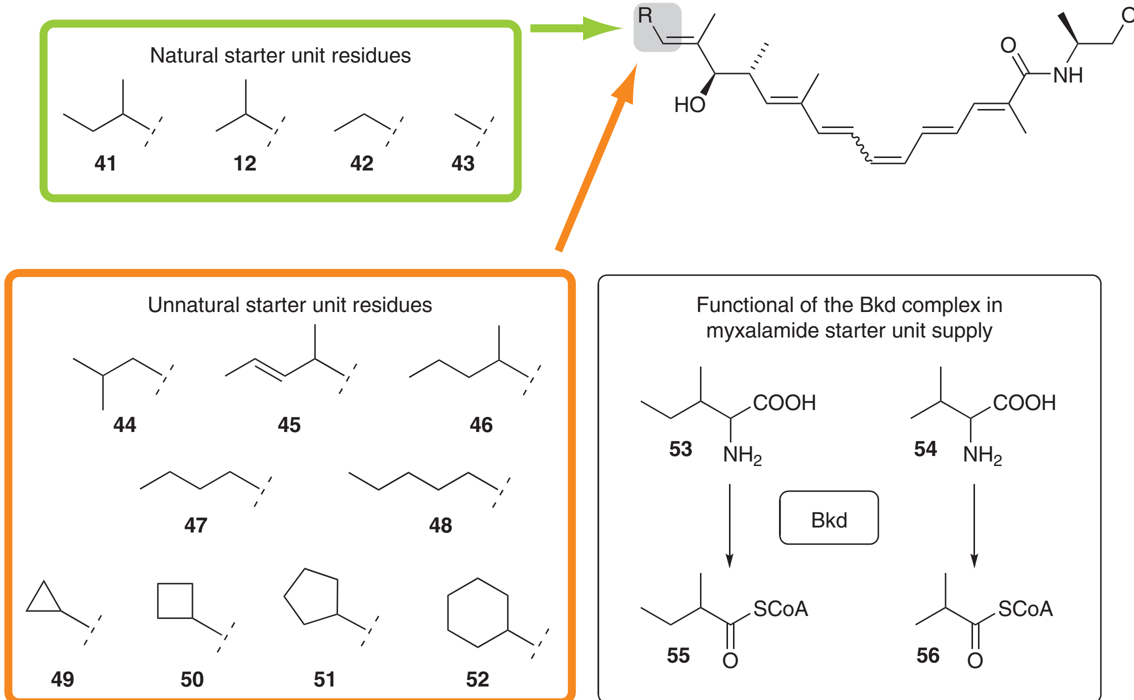

Figure 18 New myxalamids generated by 'mutasynthesis'. After inactivation of the branched-chain dehydrogenase (Bkd) complex, new derivatives (**44–52**, boxed in orange) were formed after feeding of the respective acids. Naturally occurring myxalamids are shown in the green box (**12**, **41–43**). The function of the Bkd complex is also illustrated. After transamination of valine (**54**) and isoleucine (**53**) to their α-keto acids (not shown), Bkd transforms the α-keto acids to the activated myxalamid starter moieties isovaleryl-CoA (**56**) and 2-methyl butyryl-CoA (**55**), respectively.

S. urantiaca,[54,62] and ambruticin[68] and spirangien[70] in *S. cellulosum*. However, as mutagenesis was restricted to the post-PKS processing enzymes (such as P-450s, methyl transferases, chlorinases, and hydrolases), the engineering did not introduce changes into the polyketide backbones of the compounds. To achieve such alterations, it is necessary to modify the PKS and NRPS genes themselves, typically by double homologous recombination – a technique that is only poorly established for any myxobacterial species.[137,138]

Alternatively, the complete set of genes may be transplanted into better suited host organisms, an approach called 'heterologous expression'. Advancements in this field have recently been reviewed.[139] Despite the appeal of this approach, cloning, modifying, and transferring complex gene sets encompassing up to 150 kbp remain far from routine, and yields in heterologous hosts are often disappointingly low. Nonetheless, there have been some notable successes, with yields far above the average for this technique (**Table 1**).

Table 1 Heterologously produced myxobacterial secondary metabolites

Compound	*Producing organism*	*Heterologous host strain*	*Yield*	*Reference*
Soraphen	*Sorangium cellulosum*	*Streptomyces lividans*	0.3 mg l^{-1}	140
Epothilone	*Sorangium cellulosum*	*Streptomyces coelicolor*	50–100 μg l^{-1}	141
		Escherichia coli[a]	10 μg l^{-1}	142
		Myxococcus xanthus	1–23 mg l^{-1}	143
Flaviolin	*Sorangium cellulosum*	*Pseudomonas putida*	10 mg l^{-1}	90
Myxochromide S	*Stigmatella aurantiaca*	*Pseudomonas putida*	40 mg l^{-1}	116
		Myxococcus xanthus	>1 g l^{-1}	144
Myxothiazol	*Stigmatella aurantiaca*	*Pseudomonas putida*[a]	0.8 mg l^{-1}	145
		Myxococcus xanthus	20 mg l^{-1}	146

[a] after genetic engineering of the host strain.

In terms of the successful modification of a megasynthetase, the epothilone pathway is currently the best example of a myxobacterial metabolite. The reason for the focus on epothilone is its commercial value as an anticancer compound.[147] The corresponding biosynthetic gene cluster has been studied intensively and different heterologous expression systems have been established in order to facilitate the generation of novel derivatives by genetic engineering. The epothilone PKS machinery produces different forms of epothilones, including the major components epothilone A and B, which arise from alternative incorporation of methylmalonyl-CoA or malonyl-CoA by module 4 (at positions C11 and C12; **Figure 19**). An astonishing variety of other epothilone derivatives was identified by Hardt *et al.*[148] by a detailed analysis of the culture broth from a 700 l fermentation broth. Overall, the domain organization of the epothilone biosynthetic gene cluster is highly consistent with the set of reactions required to build the PKS–NRPS hybrid molecule, except for the absence of a dehydratase function in module 4 and the presence of several domains in modules 8 and 9, which appear to be inactive.[60,61] The Kosan group reconstituted the complete gene set in the heterologous host *S. coelicolor*, obtaining the major epothilone derivatives in a very low yield.[141] The Kosan group also transplanted the gene cluster into *M. xanthus* [143] using a series of homologous recombination events. The resulting strain produced epothilones A (**58**) and B (**2**) in yields of 0.16 mg l^{-1}. Construction of a strain containing a mutation in *epoK*, encoding the P-450 epoxidase, resulted in the exclusive formation of epothilones C (**57**) and D. Further studies addressed yield improvement by media optimization.[149,150]

Heterologous expression in *M. xanthus* also allowed more straightforward genetic manipulation of the megasynthetase. Analysis of a strain engineered to produce epothilone D by deletion of *epoK* also revealed a new derivative, 10,11-didehydro-epothilone D.[149] Subsequently, the epothilone PKS was further modified by inactivating the ER domain of module 5 to produce 10,11-didehydro-epothilone D (**60**) as the major metabolite (**Figure 19**). Next, the same group engineered the epothilone PKS genes to generate new scaffolds for chemical modification.[151] Inactivation of the KR domain in module 6 resulted in accumulation of 9-oxo-epothilone D (**61**) and its isomer 8-*epi*-9-oxoepothilone D (**62**) (**Figure 19**). Modification of the KR domain in module 4 yielded the expected compound 12,13-dihydro-13-oxoepothilone C (**63**), albeit in trace amounts. Interestingly, the major product of the fermentation was 11,12-dehydro-12,13-dihydro-13-oxoepothilone D (**64**), an unexpected metabolite. The other predicted compound, 12,13-dihydro-13-oxoepothilone D, was not detected.

To elucidate the role of the dehydratase of module 5, three different deletions/replacements of this domain were generated.[152] None of the three recombinant strains produced detectable amounts of 11-hydroxy epothilones (the compounds expected if the dehydratase of module 5 did not participate in forming the double bond between carbons 12 and 13). Instead, the mutants produced compounds lacking the double bond at the positions introduced by module 4, which confirmed the role of the module 5 DH domain in dehydration. The novel compound (*E*)-10,11-didehydro-12,13-dihydro-13-hydroxy epothilone D (**59**) (**Figure 19**) found in these mutants was also unanticipated, because dehydration occurred to form the C10–C11 double bond in spite of the inactivation of the module 5 DH. Taken together, these data support an iterative function of the module 5 DH, but the generation of the novel metabolite cannot be explained by iteration alone.

Escherichia coli has also been engineered for epothilone production.[142] The genes encoding the entire gene cluster were redesigned *in silico* and then synthesized to enable optimal tuning of the codon usage for expression in *E. coli*. Expression of the cluster in a strain of *E. coli* modified to enable polyketide biosynthesis resulted in the production of epothilones C and D.

These experiments clearly demonstrate the feasibility of modification of PKS genes from myxobacteria to generate unnatural derivatives. However, yields have been low in most cases, and it will thus be of critical importance to establish better heterologous expression hosts to improve productivity and/or develop genetic tools for the producing organisms.

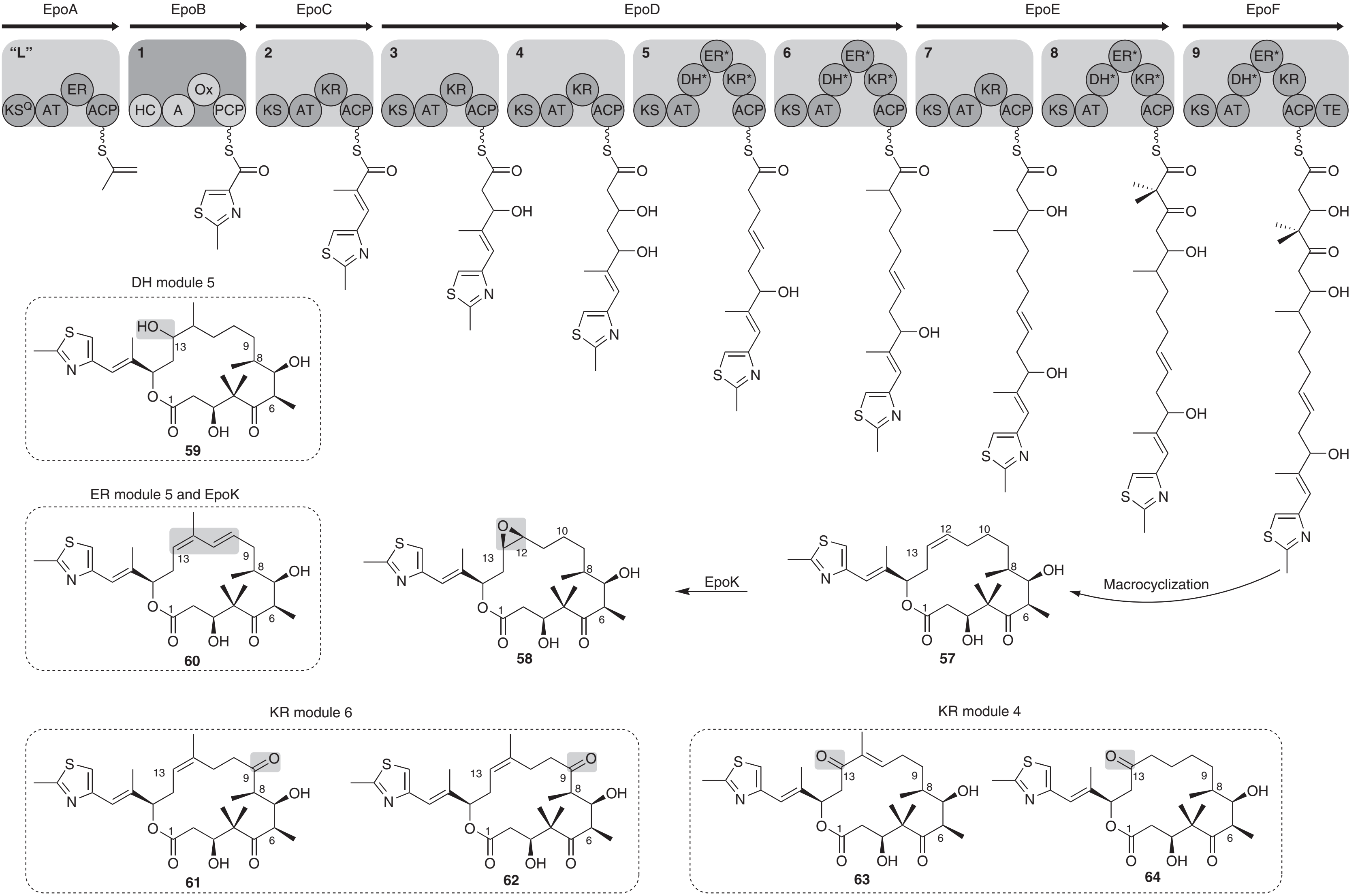

Figure 19 Epothilone biosynthesis and novel derivatives generated by genetic engineering. Domains with an asterisk are assumed to be inactive. The hydroxyl group formed by the KR domain in module 4 is dehydrated by the DH domain found in module 5. Module 4 incorporates either malonyl-CoA or methylmalonyl-CoA extender units (the figure only shows the incorporation of malonyl-CoA). Epothilone derivatives generated by genetic engineering are shown in boxes (see text) and the sites of mutagenesis are indicated.

2.07.6 Conclusions

Over the last three decades, myxobacteria clearly made their way to accepted valuable sources of natural products with significant potential in medical applications. Today, these bacteria are known to produce some 5% of known bacterial natural products although in depth analysis of the diversity of their secondary metabolism has just begun. The advent of the myxobacterial 'genomic era', as well as enormous progress in the isolation, fermentation and genetic manipulation of many myxobacterial strains, will most likely advance this research field way beyond the status it has reached today. In addition, newly discovered strains and their metabolites will continue to deliver novel chemistries and enzymatic mechanisms, especially because advances in analytical technology and method development will make it possible to identify novel metabolites, even if produced at low levels. In parallel, it is likely that additional myxobacterial lead structures will reach the clinic. Because of these expectations, the future of myxobacterial natural products research looks very promising.

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2.08 Natural Product Diversity from Marine Fungi

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2.08.1 Introduction

In recent years, secondary metabolites obtained from marine-derived fungi have drawn considerable attention as many of them are structurally unique and possess interesting biological and pharmacological properties.[1,2] Historically, the first secondary metabolite isolated from a marine-derived fungal strain was cephalosporin C, produced by a culture of a *Cephalosporium* sp., isolated in 1949 close to a sewage outlet off the Sardinian coast.[3] However, this was a more or less incidental discovery, and it took another 30 years until marine-derived fungi were analyzed more systematically. It was only in the late 1980s that sizable quantities of new secondary metabolites were discovered from this long neglected source.

The aim of this chapter is to give an overview of secondary metabolites from marine-derived fungi, focusing on the years 2007 and 2008. For earlier years, the reader is referred to a series of general reviews that focus on natural product chemistry of marine-derived fungi.[4–7] In addition, more specialized overviews give insights into individual topics, including biotechnological aspects,[8,9] screening strategies,[10] individual therapeutical areas,[11] and fungi from certain geographical areas.[12]

2.08.2 Biology of Marine-Derived Fungi

According to a classical definition, marine fungi cannot be defined on a strictly physiological basis, but rather an ecological one: obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat, while facultative marine fungi are those from freshwater or terrestrial milieus able to grow (and possibly also to sporulate) in the marine environment.[13] However, on a practical basis, it is challenging to separate indigenous marine species (obligate and facultative) from 'contaminants' (sometimes also designated 'transients'), which are terrestrial or freshwater species that are dormant in marine habitats, for example, in the form of spores or hyphal fractions. In principle, the best way of separating indigenous

from nonindigenous species would be to test their germination ability.[13] On the other hand, in many cases, it is difficult to obtain sexually reproducing forms, and there is a growing tendency to use molecular biology-based methods, for example sequencing of rDNA, instead of traditional approaches based on morphological characteristics.

As the next sections reveal, isolation attempts by natural product chemists and also by trained marine mycologists tend to yield many strains belonging to genera or species well known from terrestrial habitats, while truly obligate marine fungi are obtained on a much rarer scale. Thus, after some vigorous discussions in the earlier days about the true origin of fungal strains isolated from marine habitats, it has now become a common practice to designate these strains 'marine-derived fungi'. This neutral term does not imply any ecological role such as associates or true symbionts, and comprises any fungal strain obtained from marine environments using cultivation techniques with 'marine' media, but does not differentiate between facultative marine strains and contaminants from terrestrial habitats.

Chemically characterized marine-derived fungal strains have been obtained from virtually every possible marine habitat, including inorganic matter (soil, sediments, sandy habitats, artificial substrates, and the water column), marine microbial communities, marine plants (algae, sea grasses, driftwood, and other higher plants, especially mangrove plants), marine invertebrates (most notably sponges, but also corals, ascidians, holothurians, bivalves, and crustaceans), and vertebrates (mainly fishes). However, it is worth mentioning that it is expected that the fraction of culturable isolates is very low, that is, in the range of 1% or less, with regard to the overall estimated biodiversity, similar to the situation with bacteria.

While there is increasing evidence that marine-derived fungi are frequently encountered as associates of other organisms including animals and plants (algae and mangroves), comparatively little is known about free-living representatives found in the open seas, or their role in abiotic substrata such as marine sediments. A recent investigation indicates that the role of marine fungi in sediments probably has so far been underestimated considerably, simply because they tend to escape detection by microscopical techniques due to the formation of aggregates.[14] Virtually any isolation attempt yields new and sometimes novel strains, and the total biodiversity can only be estimated. Similar to the situation encountered with marine bacteria, molecular biological approaches very often give evidence for new taxonomic groups having no known closely related cultivated isolates, for example, when analyzing methane hydrate-bearing deep-sea marine sediments.[15] On the other hand, classical isolation techniques typically yield multiple fungal strains for one given biological source, exemplified by a recent report on fungal endophytes of the mangrove plant *Kandelia candel*, which resulted in more than 50 taxonomically distinct isolates for this one host species, sampled at a single nature reserve in Hong Kong.[16]

Fungal communities living in marine invertebrates are so far less characterized, although from a chemical point of view, they are among the most prolific producers of secondary metabolites. In the course of a study of marine ascomycetes, only one strain, *Abyssomyces hydrozoicus*, has been found to be associated with hydrozoans, while the remaining obligate marine fungi were mostly detected in marine algae.[17] Although a variety of fungal isolates can usually be obtained from most marine sponges using classical cultivation techniques, the true origin of these sponge-derived fungal strains remains a matter of debate. Since most genera encountered upon cultivation studies are well known from terrestrial habitats, and sponges are known to filter considerable amounts of seawater per day, it is difficult to decide whether a given isolate is truly a sponge associate or even symbiotic, or rather derives from spores washed into the sea and merely trapped inside the sponge during filter feeding. One major caveat is the fact that so far no unequivocal evidence of a fungal associate actively living inside a marine sponge, that is, as fungal hyphae, has been presented. It is interesting to note that this assumption with regard to the origin of sponge-derived fungi is in sharp contrast to the situation of sponge-associated bacteria. Even though sponges feed on bacteria accumulated through filtering of seawater, they have been shown to harbor large quantities of bacteria, some of which seem to be sponge-specific. The finding that some of these bacterial symbionts occur in different sponges collected independently from various geographical locations and are more closely related to each other than to any other known bacterial taxa[18,19] has led to the introduction of a new candidate bacterial phylum – Poribacteria.[20]

However, there is growing evidence that a true symbiotic association between sponges and fungi might indeed exist. On a molecular level, horizontal gene transfer of a mitochondrial intron from a putative fungal

donor to the sponge *Tetilla* sp. (Spirophorida) has recently been demonstrated.[21] In addition, it had been shown before, again by molecular biological methods, that sponges possess a cell surface receptor that recognizes $(1 \rightarrow 3)$-β-D-glucans and thus are able to detect fungi in their environment via the D-glucan carbohydrates on their surface.[22] By transmission electron microscopy, an endosymbiotic yeast was discovered in demosponges of the genus *Chondrilla* from various locations including the Mediterranean, the Caribbean, and the Australian Pacific.[23] This symbiont is a chitinous-walled fission yeast, and was interpreted as a yolk body in previous ultrastructural studies. Using immunocytochemical techniques, it could be demonstrated that symbiotic yeast cells were transmitted from the soma through the oocytes to the fertilized eggs, and thus are directly propagated by vertical transmission. However, this example represents a symbiotic relationship that appears to be rather specific for the sponge genus *Chondrilla*, since similar phenomena were not detected in other demosponge genera.[23]

The association of marine algae and their endophytic fungi has been studied for a number of years, also employing molecular techniques such as denaturing gradient gel electrophoresis (DGGE). One particularly well-characterized example is the brown alga *Fucus serratus*.[24] Previously, an endophytic fungus had been isolated and described as a new species, *Acremonium fuci*, which based on molecular data belonged to a clade that contained only isolates originating from marine sources or saline lakes.[25] This fungus was isolated independently from *Fucus distichus* in North America and *F. serratus* in Europe. Remarkably, the germination of its conidia occurred only in the presence of *F. serratus* tissue, or aqueous tissue homogenates, but not in seawater alone, suggesting a high level of adaptation of the fungus to its host. In a recently disclosed follow-up study, molecular techniques such as 28S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR)-DGGE were used, revealing that the predominant DGGE bands obtained from healthy algal thalli belonged to the *Lindra*, *Lulworthia*, *Engyodontium*, *Sigmoidea*/*Corollospora* complex, and *Emericellopsis*/*Acremonium*-like ribotypes.[26] In a parallel culture-based approach, 336 isolates representing 35 genera of the Ascomycota and Zygomycota were obtained, including *Sigmoidea marina* and *Dendryphiella salina*, together with members of the genera *Acremonium* (most of them representing *A. fuci*), *Cladosporium*, and *Fusarium*. By real-time quantitative PCR it was demonstrated that *A. fuci* colonized both healthy and decaying algal thalli, but the signal was stronger for the latter. Moreover, changes in the signature of the sequence types indicated that a change in fungal community structure occurred between healthy and decaying thalli.

In a recent systematic investigation of culturable fungi associated with the Hawaiian sponges *Suberites zeteki* and *Gelliodes fibrosa*, Guangyi Wang's group obtained a total of 20 independent fungal isolates from the former and 24 from the latter species.[27] All culturable strains belonged to Ascomycota, representing 7 orders and 22 genera, and even though they were differing in fungal species composition and structure, culturable communities of both sponges displayed similar phylogenetic diversity, but were significantly different from those in the water column. Representatives of the orders Pleosporales, Hypocreales, and Eurotiales were isolated from both sponge species, while two orders, Phyllachorales and Diaporthales, were found only in *S. zeteki*, and two other orders, Trichosphaeriales and Dothideales, only in *G. fibrosa*. At the genus level, only *Penicillium* and *Trichoderma* were present in both the species. Most of the genera cultured in this study were previously described from terrestrial habitats, including *Penicillium*, *Aspergillus*, *Trichoderma*, *Fusarium*, *Coniothyrium*, *Stilbella*, *Curvularia*, and *Myrothecium*, all of which have been reported before as producers of new secondary metabolites from the marine habitat. Remarkably, phylogenetic analysis revealed close affiliations of sponge-inhabiting fungi investigated in this study with cultured and uncultured fungi from both terrestrial and marine habitats, including pathogens to both animals and plants, leading the authors to speculate about a putative pathogenic role of some of the fungal associates inside the two sponges.[27] Based on their results, the authors believe that in general the percentage of culturable fungi in sponges is much higher than that of bacteria in sponges (about 1–3%), and that the culturable fungal community might be significant in the study of the fungal diversity of marine sponges. One possible role of this fungal community, which so far had not been so much in the focus of scientific investigations, may consist in decomposing marine organic materials such as particulate organic matter (POM) from the water column. This process, aided by the enormous filter capacity of their sponge hosts, could thus enhance the conversion of organic matter into nutrients for their hosts.[27]

In a similar study reported by the same group of authors, but comprising three Hawaiian sponges, *G. fibrosa* (the same species as studied before), *Haliclona caerulea*, and *Mycale armata*, an overall comparable picture was

obtained.[28] The internal transcribed spacer (ITS)-rDNA sequence analyses identified 86 independent isolates, belonging to 7 orders of Ascomycota, which further include, besides the ones mentioned above, Mycosphaerellales, Saccharomycetales, and Xylariales, and comprising at least 25 genera of Ascomycota and 1 genus of Basidiomycota. Three fungal genera, *Aspergillus*, *Penicillium*, and *Eupenicillium*, were found in all sponges and thus classified as 'sponge generalists'. Fungal genera such as *Ampelomyces*, *Tubercularia*, and *Clasoprorium*, which were identified in more than one sponge, were considered 'sponge associates', while genera such as *Didymella*, *Fusicoccum*, and *Lacazia*, which were found only in one sponge species, were designated 'sponge specialists'. Phylogenetic analysis revealed that 17% of strains were closely affiliated with fungal isolates from marine habitats, and the rest were related to terrestrial fungi. Moreover, 14% were closely related to fungal strains previously described in the literature as producers of natural products or enzymes, while 12% were closely affiliated with previously described pathogens from marine animals, humans, and plants.[29] Comparable results, at least in terms of the large number of fungal isolates per sponge, and also the dominance of genera from terrestrial habitats were also obtained in independent studies of sponges originating from Ireland[30] as well as Sakkhalin Island, Russia.[31]

Recently, fungal communities of the two Hawaiian sponge species mentioned above, *S. zeteki* and *M. armata*, were studied using molecular techniques, including DGGE upon amplification of total sponge genomic DNA by nested PCR.[32] Significant differences were found between the two sponges, and also between sponges and the surrounding seawater. Sequence analysis of the DGGE bands revealed that *S. zeteki* harbored 23 fungal species, while 21 species were obtained from *M. armata*. In total, fungal strains belonged to 11 taxonomic orders, comprising both Ascomycota and Basidiomycota, with 5 of these orders identified for the first time in marine sponges. On the species level, 13 sequences apparently indicated new species based on lack of similarity to published sequences in GenBank. Interestingly, phylogenetic analysis indicated that sponge-derived sequences were clustered into 'marine fungus clades' with those from other marine habitats, supporting the existence of 'marine fungal phylotypes'.

2.08.3 Natural Product Chemistry of Marine-Derived Fungi

2.08.3.1 General Aspects of Secondary Metabolites in Marine-Derived Fungi

The vast majority of chemical studies conducted so far deal with fungal strains that would be considered facultative, but not obligate marine fungi. While at least in many cases the latter seem to be difficult to cultivate, especially in large-scale fermentations required for the isolation and characterization of new chemical entities, seemingly ubiquitous strains including members of the genera *Aspergillus* and *Penicillium* are frequently encountered, and usually easily produced enough biomass for chemical studies. Nonetheless, even though their terrestrial counterparts have been the subject of chemical screening for at least five decades, marine-derived *Aspergillus* and *Penicillium* spp. continue to yield a broad array of new secondary metabolites, although in many cases, these are biogenetically closely related to natural products described previously from their terrestrial counterparts. Thus, the question arises whether there are significant differences between marine- and terrestrial-derived representatives of both genera with regard to their secondary metabolites chemistry.

Only a few reports exist in the literature that specifically address this question. Modern high-performance liquid chromatography (HPLC)-based methods for rapid dereplication of large numbers of strains in culture collections are now sufficiently sophisticated to detect minor quantities of congeners of a given class of secondary metabolites, provided suitable chromophores (or a tendency to ionize upon liquid chromatography–mass spectrometry (LC–MS)) are present.[33,34] For example, pyranonigrin A (**118**) was initially described from marine-derived strains of *Aspergillus niger*.[35,36] Later studies, based on extensive strain collections of *Aspergillus* species from various habitats, clearly demonstrated that **118** is widely distributed in terrestrial strains of *Aspergillus* belonging to section *Nigri*. Similarly, the discovery of the known citromycetin and a new (−)-2,3-dihydrocitromycetin (**65**) from the culture broth of a marine isolate of *Penicillium bilaii* prompted Robert Capon's group to screen their own in-house database, comprising HPLC–diode array/evaporative light scattering detected profiles for 6000 annotated microorganisms, for the characteristic UV–Vis spectra of these polyketides. A single 'hit' was obtained for a soil isolate of *Penicillium striatisporum*, which was subsequently shown to also produce citromycetin congeners including **65** and further new derivatives.[37]

1

Gliotoxin (**1**) is a well-known cytotoxic and immunosuppressive mycotoxin that was originally described from terrestrial fungi including *Gliocladium fimbriatum*, *Aspergillus fumigatus*, and *Penicillium* spp.[38,39] In the marine habitat, it was recently identified as a putative toxin that might be involved in shellfish toxicity phenomena. For example, a strain of *A. fumigatus* isolated from the sediments of a mussel bed in a shellfish-farming area situated at the Loire estuary (France) was shown to produce significant levels of **1** when cultured on a marine solid medium.[40] To test the relevance of this finding, the same group of authors conducted a systematic investigation of two marine-derived strains of *A. fumigatus*, and monitored the effects of growth conditions, especially the influence of salinity of the culture medium, in comparison with 13 terrestrial strains of the same species, obtained as clinical isolates from patients suffering from aspergillosis.[41] Remarkably, seawater salinity significantly reduced the growth rate of all the strains, and marine and terrestrial strains did not show significant differences with regard to their appearance, growth, and gliotoxin excretion. On the other hand, seawater salinity enhanced exudation and gliotoxin excretion by all strains of *A. fumigatus*, while the exudation of **1** by marine strains seemed to be less influenced by seawater salinity than that by terrestrial strains. Thus, this study apparently did not yield any evidence that there is a significant metabolic difference between strains of *A. fumigatus* of marine versus terrestrial origin, at least with regard to production of **1**.

On the other hand, the groups of Gerhard Bringmann and Johannes Imhoff have undertaken intensive studies of marine-derived strains of *Penicillium chrysogenum*, the source of sorbicillactone A (**108**).[42] So far, at least 19 different marine isolates of this fungus, capable of producing **108**, have been characterized (for a more detailed discussion, see below), whereas no reports exist that this ability also extends to terrestrial isolates of the same species. If this observation was correct, **108** could be considered a true 'marine' fungal secondary metabolite.

2.08.3.2 New Natural Products from Marine-Derived Fungi

In the following section, new natural products characterized from marine-derived fungal strains and reported in the years 2007 and 2008 are listed, together with details on their biological activity, if applicable. As in the previous years, the spectrum is clearly dominated by polyketides, which comprise roughly half of all new chemical entities. Further categories include prenylated polyketides (meroterpenoids), nitrogen-containing polyketides (often formed by the action of hybrid polyketide synthetase (PKS)/nonribosomal peptide synthetase (NRPS)), alkaloids, diketopiperazines, peptides, terpenes, lipids, shikimate-derived metabolites (phenylpropanoids), and miscellaneous natural products that are difficult to classify. It should be stated that assigning structures to any of these groups is sometimes arguable and based on structural analogies to natural products with established biosynthetic origin. The chapter finishes with a few selected examples of metabolically prolific fungi that produce a variety of secondary metabolites belonging to different biogenetic classes.

2.08.3.2.1 Polyketides

The fungus *Penicillium rugulosum* was isolated from the sponge *Chondrosia reniformis* collected off the island of Elba, Italy. Chemical investigation yielded a series of structurally unusual tricyclic and bicyclic pentaenes, prugosenes A1 (**2**), A2 (**3**), A3 (**4**), B1 (**5**), B2 (**6**), C1 (**7**), and C2 (**8**).[43]

	R^1	R^2
2	A	X
3	A	Y
4	A	Z
5	B	X
6	C	X
7	D	X
8	E	X

B (R^3 = H)
C (R^3 = CH_3)

D (R^4 = CH_3)
E (R^4 = CH_2OH)

Feeding studies with ^{13}C-labeled acetate and L-methionine revealed that 2 is an undecaketide, with all branching methyl groups being derived from *S*-adenosyl-methionine. The unusual oxabicyclo[2.2.1]heptane core present in 2–4 has so far been known only from shimalactone from the marine-derived fungus *Emericella variecolor*[44] and coccidiostatin A, characterized from a terrestrial isolate of *P. rugulosum*.[45] When 2 was treated with diluted NaOH, 5 and 7 were obtained as a major and a minor product, respectively, suggesting that 5 is formed by hydrolytic cleavage of the C-2/C-3 bond, while the formation of the cyclopentenone system of 7 and 8 should include decarboxylation of the bicyclic system of the A-type prugosenes (2–4). None of the compounds showed antimicrobial activity against one fungal and several bacterial test strains.

9
10 (10,11-Dihydro)

Bisorbicillinoids are thought to be biosynthesized via a Diels–Alder reaction involving two sorbicillinol or oxosorbicillinol moieties. A deep-sea isolate *Phialocephala* sp. collected from sediments at a depth of 5059 m produced two new congeners, oxosorbiquinol (9) and its 10,11-dihdro derivative (10).[46] Besides the hitherto reported sorbiquinol, 9 and 10 represent the only examples for bisorbicillinoids connected through the unsaturated side chain of one monomer and the six-membered ring of the other one. Compounds 9 and 10 displayed weak cytotoxic activity against five different cancer cell lines.

11 ($R^1 = CH_3$, $R^2 = \alpha$-OH)
12 ($R^1 = CH_3$, $R^2 = \beta$-OH)
13 (R^1 = H, $R^2 = \beta$-OH)

14

Chemical investigation of the fungus *Trichoderma* sp., isolated from the Caribbean sponge *Agelas dispar*, led to four novel sorbicillinoid polyketide derivatives trichodermanones A–D (**11–14**) with an unprecedented tricyclic ring system.[47] From a biogenetical point of view, **11–14** are unusual, since based on structural considerations, they are assumed to be produced via Diels–Alder cycloaddition of two different polyketide precursors, which seems to be a very rare case in nature. Compounds **11–13** proved inactive when tested for antimicrobial, antiparasitic, and cytotoxic properties, or inhibitory properties toward phosphatase, acetylcholine esterase, and trypsin, but were found to display moderate activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity test.

15 **16**

Dehydroxychlorofusarielin B (**15**) was obtained from an *Aspergillus* sp. isolated from the surface of the Korean marine brown alga *Sargassum horneri*.[48] The structure of **15** was secured by X-ray crystallography, and was found to be closely related to fusarielins A and B, known decalins previously described from a soil-borne *Fusarium* sp.[49] and were likewise detected in the culture broth. Fusarielin E (**16**) and the two known congeners exhibited a mild antibacterial activity against *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), and multidrug-resistant *S. aureus*. Shortly after this report, **16** was described from the culture broth of the marine-derived *Fusarium* sp. 05JANF165.[50] Even though **16** is claimed to differ from **15** with regard to the configuration at C-11, C-15, and C-16, their NMR data are virtually identical, so it is very likely that both compounds are actually identical. Compound **16** inhibited the conidia growth of *Pyricularia oryzae* by a swelling effect and induced curling deformation of the mycelia with a minimum inhibitory concentration (MIC) of 50 mg ml^{-1}.

17 **18**

Penisporolides A (**17**) and B (**18**) were characterized from the culture broth of the fungus *Penicillium* sp., purified from the mangrove plant *K. candel* that was collected in Hainan Island, China.[51] Their carbon framework displays close similarity to ascospiroketal B (**213**) from *Ascochyta salicorniae*,[52] including the biogenetically intriguing geminal dimethyl groups discussed below. When tested for inhibitory activity against xanthine oxidase, both compounds were found to be inactive.

19 **20**

Structurally closely related, but apparently differing with regard to the relative stereochemistry of the tetrahydro-3*H*,3′*H*-spiro[furan-2,2′-furo[3,2-*b*]furan]-5′(3*a*′*H*)-one core are cephalosporolides H (**19**) and I

(**20**). These compounds were described by the same group of authors, and were obtained from a fungal strain *Penicillium* sp. isolated from the mangrove plant *K. candel*,[53] although it is not clear whether or not both strains, that is, the producers of penisporolides (**17 and 18**) and cephalosporolides (**19 and 20**), were identical. Compounds **19 and 20** showed mild inhibitory activity toward xanthine oxidase and 3α-hydroxysteroid dehydrogenase.

21 **22**

8′-Hydroxyzearalanone (**21**) and 2′-hydroxyzearalanol (**22**) are two new resorcyclic acid-containing 14-membered lactones from the marine-derived fungus *Penicillium* sp., which was isolated from the surface of the drifting cotton clothing collected off Namhae Island, Korea.[54] They are structurally closely related to the known zearalanone, which was likewise detected in the culture broth of the fungus, together with three further known congeners of this structural type. Even though a variety of biological activities have been described previously for zearalanones, **21 and 22** proved inactive when tested for radical scavenging activity, antibacterial activity, or inhibitory properties toward tyrosinase.

23

Macrosphelides are a class of cyclic triesters acting as cell-adhesion inhibitors and were initially discovered in the fermentation broth of *Microsphaeropsis* sp. FO-5050, which was isolated from a soil sample.[55] Later, further congeners were obtained from a strain of *Periconia byssoides* isolated from the sea hare *Aplysia kurodai*.[56] The latest member of this class of compounds is macrosphelide M (**23**), which was also produced by *P. byssoides*, together with peribysin J (**188**).[57] Compound **23** was found to inhibit the adhesion of HL-60 cells to human umbilical vein endothelial cells (HUVECs) more potently than the positive control, herbimycin A.

24

25 (R^1 = OH, R^2 = H)
26 (R^1 = H, R^2 = OH)

27

The fungus *Curvularia* sp. produced four new 10-membered lactones **24–27**.[58] The strain was obtained from the red alga *Acanthophora spicifera* collected in Guam. Chemically, **24–27** are related to other lactones including modiolides[59] and decarestrictines,[60] previously reported from a marine-derived *Paraphaeosphaeria* sp. and from terrestrial *Penicillium* spp., respectively, but differ with regard to their oxygenation pattern. When tested in antibacterial, antifungal, or antialgal bioassays, none of the new congeners displayed any activity.

28 29 30

The strain *Phomopsis* sp. hzla01-1 produced two very closely related new 10-membered macrolides, phomolides A (28) and B (29), and one new benzofuran derivative (30).[61] All of them displayed antimicrobial activities against *Escherichia coli*, *Candida albicans*, and *Saccharomyces cerevisiae*, while 30 also showed strong activity against *Bacillus subtilis*. When tested for cytotoxicity, none of the compounds was active toward the HeLa cell line.

31 (R = H)
32 (R = CH_3)
33 (R^1 = acetyl, R^2 = CH_3)
34 (R^1 = H, R^2 = CH_3)
35 (R^1 = R^2 = H)
36
37
38 (H-10,H-10' *trans*)
39 (H-10,H-10' *cis*)

Aspergiolide A (31) is a novel anthraquinone derivative with an unprecedented naphtho[1,2,3-*de*]chromene-2,7-dione skeleton isolated from the marine-derived fungus *Aspergillus glaucus*, which was cultured from the sediments around mangrove roots in China.[62] Its unique structure was secured by X-ray diffraction analysis, and its biosynthesis was postulated to involve condensation of an octaketide and a pentaketide precursor. In cytotoxicity assays, 31 was active at submicromolar concentrations toward A-549 and HL-60 cells, while activity toward BEL-7402 and P388 cell lines was decreased by approximately two orders of magnitude. A recent reinvestigation of the same fungal strain yielded a further congener, aspergiolide B (32),[63] together with seven new polyketides. Compounds 33–35 are unusual naphthyl furanosides, isoasperflavin (36) is a constitutional isomer of the known asperflavin from a terrestrial-derived *Aspergillus flavus*,[64] 37 was identified as the (+)-enantiomer of the recently described variecolorquinone A from the halotolerant Chinese fungus *Aspergillus variecolor*,[65] while 38 and 39 are new physicon–emodin bisanthrones. When tested against the most susceptible cell lines A-549 and HL-60 of the previous study, 32 displayed comparable activity to 31, whereas 38 and 39 exhibited moderate cytotoxic activity.

40

Monodictyquinone A (**40**) is a new antimicrobial anthraquinone that was isolated from the marine-derived *Monodictys* sp. obtained from the Japanese sea urchin *Anthocidaris crassispina*, together with three known congeners.[66] Compound **40** showed antimicrobial activity against *B. subtilis*, *E. coli*, and *C. albicans*, but was not cytotoxic toward HeLa cells even at high concentrations.

41 (R = OH)
42 (R = H)

Two new hexahydroanthrones, tetrahydrobostrycin (**41**) and its 1-deoxy congener (**42**), were isolated from a marine-derived fungus *Aspergillus* sp. strain 05F16 collected in Manado, Indonesia, together with the known pigment bostrycin and the plant hormone abscisic acid.[67] Compound **41** showed weak antibacterial activity against *S. aureus* and *E. coli*, while **42** was active against *S. aureus*. Both compounds displayed no antifungal activity against yeasts.

43 ($R^1 = CH_3$, $R^2 = OH$)
44 ($R^1 = H$, $R^2 = CH_3$)
45 ($R^1 = OCH_3$, $R^2 = CH_3$)

46

47

48

49

The fungus *Monodictys putredinis*, which was isolated from a marine green alga, collected in Tenerife, Spain, was subjected to an in-depth evaluation for potential cancer chemopreventive effects of its secondary metabolites.[68,69] Initially, chemical investigation led to the isolation of four new monomeric xanthones, monodictysins A (**43**), B (**44**), C (**45**), monodictyxanthone (**46**), and the benzophenone monodictyphenone (**47**), which are thought to be biogenetically derived from cleavage of a putative common anthraquinone precursor between C-4a/C-10a and carbonyl C-10.[68] A recent investigation of the same fungus yielded two novel dimeric chromanones monodictyochromes A (**48**) and B (**49**), consisting of two uniquely modified xanthone-derived units probably likewise derived from an anthraquinone precursor and coupled by phenol oxidative coupling in a regioselective manner. This coupling should also occur under strict stereoselectivity, since **48** was identified as the (*P*)-atropisomer, while **49** represents the (*M*)-stereoisomer as was deduced by comparison of the pertaining circular dichroism (CD) spectra with the model compounds (*P*)- and (*M*)-orsellinic acid camphanate.[69] All compounds were evaluated with regard to their cancer chemopreventive potential, evident as their ability to either inhibit or induce certain enzymes involved in biotransformation of

potential carcinogenic agents. Compounds **44**, **47**, and **49** were shown to inhibit cytochrome P-450 1A activity, which is involved in the metabolic conversion of procarcinogens into carcinogens, in the lower micromolar range. On the other hand, **44**, **45**, **48**, and **49** displayed moderate activity as inducers of NAD(P)H:quinone reductase, a carcinogen-detoxifying enzyme. Furthermore, **45**, **48**, and **49** showed weak inhibition of aromatase activity, essential for the biosynthesis of estrogens.

50 (R = OCH_3)
51 (R = H)

52

Chaetocyclinones A (**50**), B (**51**), and C (**52**) are new polyketides derived from the culture of *Chaetomium* sp. strain Gö 100/2, which was isolated from an undisclosed marine alga.[70] Compounds **50** and **52** display the same carbon skeletons as the known anhydrofulvic acid and vinaxanthone, metabolites previously described for *Carpenteles brefeldianum* (now named *Eupenicillium brefeldianum*)[71] and *Penicillium vinaceum*,[72] respectively, but differ with regard to their oxygenation patterns. Through labeling studies with ^{13}C-labeled acetate, **50** was shown to be biosynthesized via a linear heptaketide intermediate, undergoing oxidative cleavage and recyclization. The biosynthesis of **52** should accordingly involve an unusual twofold aldol condensation of two highly reactive heptaketide intermediates. However, since it proved difficult to control the production of **52** during fermentation and also to obtain complete labeling of this compound, the authors concluded that **52** might actually represent an artifact formed through dimerization of two highly reactive biosynthetic intermediates of **50** and **51**. Compound **52** displayed inhibitory activity against selected phytopathogenic fungi, but was not found to be cytotoxic.

53 (R = OH)
54 (R = H)

55

Chaetoxanthones A (**53**), B (**54**), and C (**55**) are structurally unusual xanthones that were obtained from the fungal strain *Chaetomium* sp. 620/GrK 1a isolated from an alga of unknown taxonomy off the island of Santorini in Greece.[73] Compounds **53** and **54** are substituted with a dioxane/tetrahydropyran moiety reminiscent of intermediates of aflatoxin biosynthesis, but rarely encountered in naturally occurring xanthones, while **55** is a chlorinated xanthone substituted with a tetrahydropyran ring. Compound **54** exhibited selective activity against *Plasmodium falciparum* with an IC_{50} (half-maximal inhibitory concentration) value of 0.5 μg ml^{-1} without being cytotoxic toward cultured eukaryotic cells, whereas **55** displayed moderate activity against *Trypanosoma cruzi* with an IC_{50} value of 1.5 μg ml^{-1}.

56 (R^1 = COOH, R^2 = H)
57 (R^1 = $COOCH_3$, R^2 = CH_3)
58 (R^1 = CH_3, R^2 = H)

The fungus *Penicillium* sp. (ZZF 32#) was isolated from the bark of the Chinese mangrove plant *Acanthus ilicifolius*.[74] Two new xanthones, 8-(methoxycarbonyl)-1-hydroxy-9-oxo-9*H*-xanthene-3-carboxylic acid (**56**) and dimethyl 8-methoxy-9-oxo-9*H*-xanthene-1,6-dicarboxylate (**57**), were obtained, together with one known congener, methyl 8-hydroxy-6-methyl-9-oxo-9*H*-xanthene-1-carboxylate (**58**). The structure of **58** was secured by X-ray crystallographic analysis, but comparison of its spectral data indicated that it is in fact identical to janthinone. The latter was reported initially as a lactone and is a metabolite from *Penicillium janthinellum*, isolated as an endophytic fungus from fruits of the Brazilean plant *Melia azedarach*.[75] Even though the crude extract of the fungus displayed cytotoxic properties, none of the isolated compounds **56–58** was active. However, **57** exhibited modest antifungal activity against *Fusarium oxysporum* f. sp. *cubense*.

O
O
OH
HO
O
59

The known depsidone unguinol (**59**) was identified as an inhibitor of the C_4 plant enzyme pyruvate phosphate dikinase (PPDK), a potential herbicide target, in the course of a systematic screening of more than 2000 extracts generated from ~450 marine-derived fungal strains.[76] Phylogenetic affiliation of the active fungal isolate F3000054 revealed a close relationship to *Emericella nidulans* (*Aspergillus nidulans*) based on 18S rRNA sequencing, while comparison of ITS regions demonstrated the highest identity to *Aspergillus unguis*. Compound **59** had previously been isolated from mycelia of *A. unguis*[77] and *A. nidulans*.[78] In the present study, **59** was found to selectively inhibit PPDK and to show mixed noncompetitive inhibition of PPDK with respect to the substrates pyruvate and ATP, but was uncompetitive with respect to phosphate. PPDK is potentially specific to C_4 plants, as was demonstrated by **59** having no effect on barley, a model C_3 plant, even at much higher concentrations.

OCH_3 OH O
H_3CO O CH_2OH
60
OH
OCH_3 O
O
H_3CO OH
H_3CO OCH_3
O OH
OH O
61
OCH_3 OH O
H_3CO O OH
HO OCH_3
OH
O
O
62

Three new naphtho-γ-pyrones, nigerasperone A (**60**), B (**61**), and C (**62**), were characterized from *A. niger* EN-13, an endophytic fungus isolated from the marine brown alga *Colpomenia sinuosa*.[79] Compound **60** is a linear naphtho-γ-pyrone, while **61** and **62** are dimers of two angular and two linear naphtho-γ-pyrones, respectively. Compounds **60–62** were devoid of cytotoxic activity, but **62** showed weak antifungal activity against *C. albicans* and moderate antioxidative activity in the DPPH assay.

63

64

Two new structurally related dimeric naphtho-γ-pyrones, 8′-*O*-demethylnigerone (**63**) and 8′-*O*-demethylisonigerone (**64**), were produced by the fungal strain *Aspergillus carbonarius* WZ-4-11, which was isolated from the marine sediments off Weizhou Island, China.[80] Compounds **63** and **64** exhibited weak inhibitory activities against *Mycobacterium tuberculosis* H37Rv, with MIC values of 43.0 and 21.5 $\mu mol\,l^{-1}$, respectively. The known 2,3-dihydronaphtho-γ-pyrones fonsecin and 10,10′-bifonsecin, which were likewise obtained, proved inactive, indicating that α,β-unsaturation in the pyrane ring of **63** and **64** is required for antimycobacterial activity.

65

(−)-2,3-Dihydrocitromycetin (**65**) is a new 2,3-dihydropyrano[3,2-*c*]chromen-4(5*H*)-one produced by the Australian fungus *P. bilaii* MST-MF667, described below as a source of bilains A–C (**163–165**).[37] Compound **65** is a congener of citromycetin, described first in 1931, but with its structure solved only 20 years later.[81] **65** was found to be inactive when tested for antibacterial, antifungal, antiparasitic, and cytotoxic properties.

66

67

68

69

Trichodermatides A–D (**66–69**) have been characterized from the fungus *Trichoderma reesei*, obtained from marine sediments in China.[82] Octaketide derivatives such as **67–69** with an α,β-unsaturated cyclohexenone fused to a pyran ring have repeatedly been reported from the genus *Trichoderma*,[83,84] but do not seem to occur elsewhere in nature. **66** is the first example of a pentacyclic polyketide with a ketal moiety. **66–69** exhibited weak cytotoxicity toward the A375-S2 human melanoma cell line.

70 (R = H)
71 (R = OCH_3)
72 (R = OH)

The endophytic fungus *Guignardia* sp. No. 4382 was isolated from the Chinese mangrove plant *K. candel*. Besides the known vermistatin (**70**), two new derivatives **71** and **72**, oxygenated at C-5′, were reported.[85] **70** was previously described as a metabolite from *Penicillium vermiculatum*.[86] When tested for cytotoxic properties, **70** was found to exhibit weak activity and **71** moderate activity, while both compounds were inactive when tested for antimicrobial activity toward one bacterial and two fungal strains.

73 **74**

Investigation of the marine-derived fungi *Acremonium* sp. and *Nodulisporium* sp. led to the isolation of two new biogenetically related pentaketides, acremonisol A (**73**) and (3*R*)-7-hydroxy-5-methylmellein (**74**).[87] Both fungal strains were endophytic to marine alga, in this case the red alga *Plocamium* sp. collected near Helgoland, Germany, and another algal species of unknown taxonomy originating from Corfu, Greece, respectively. **73** and **74** displayed no biological activity when tested against various bacteria, fungi, algae, or cancer cell lines.

75

(3*R*,4*aR*,5*S*,6*R*)-6-Hydroxy-5-methylramulosin (**75**) was isolated from a culture of a sterile mycelium that was derived from the Japanese green alga *Codium fragile*.[88] In addition, three further known mellein congeners were detected. **75** exhibited mild cytotoxic activity toward HeLa cells, whereas the remaining compounds were inactive.

76

Redoxcitrinin (**76**) was identified as a new polyketide produced by the marine-derived fungus *Penicillium* sp. MFA446.[89] **76** had so far only been reported as a synthetic product obtained during biosynthetic studies of the known citrinin,[90] a metabolite of *P. citrinum*. **76** and the structurally related known congeners phenol A and citrinin H2, which were likewise obtained from the culture broth of the fungus, exhibited moderate radical scavenging activity against DPPH with IC_{50} values ranging between 20 and 30 $\mu mol\, l^{-1}$.

77 (R = OH)
78 (R = H)

(3*S*,3′*R*)-3-(3′-hydroxybutyl)-7-methoxyphthalide (77) and (*S*)-3-butyl-7-methoxyphthalide (78) were isolated from the culture broth of the sponge-derived fungal strain CRIF2, belonging to the order Pleosporales.[91] 77 is a new fungal metabolite, while 78 was obtained synthetically before, but was isolated for the first time as a natural product. 77 and 78 are closely related to known phthalides such as (–)-3-butyl-7-hydroxyphthalide, a cytotoxic metabolite of *Penicillium vulpinum*.[92,93] 78 exhibited weak cytotoxic activity toward various cell lines.

79 80 81

The fungal strain *Exophiala* sp. MFC353 was isolated from the surface of the marine sponge *Halichondria panicea* collected off Bogil Island, Korea. Chemical investigation led to the discovery of two new polyketides, chlorohydroaspyrones A (1) and B (2).[94] The absolute configuration of 79 and 80 was deduced by careful analysis of hydrolysis products obtained from the known aspyrone (81) upon treatment with HCl. Workup of the resulting mixture yielded 79 and its 8-epimer in the ratio of approximately 2:1 besides trace amounts of 80, suggesting that acid-catalyzed ring opening of the epoxide proceeds via an S_N1 reaction. Compounds 79 and 80 exhibited mild antibacterial activity against *S. aureus*, MRSA, and multidrug-resistant *S. aureus*.

82 83

The strain *A. flavus* c-f-3, obtained from the Chinese marine alga *Enteromorpha tubulosa*, yielded two new 5-hydroxy-2-pyrones 82 and 83.[95] Compound 82 induced the production of cAMP on GPR12-transfected CHO and HEK293 cells in a dose-dependent manner, indicating that the compound might be a possible ligand for GPR12.

84

Ascochytatin (**84**) is a new spirodioxynaphthalene metabolite produced by the marine-derived fungus *Ascochyta* sp. NGB4 that was isolated from a floating scrap of festering rope in a Japanese fishing port.[96] The compound was discovered using a screening program focused on the bacterial two-component regulatory system (TCS), which consists of two proteins, a histidine kinase and a response regulator, and has received increasing attention as a novel antibacterial drug target, since it represents a fundamental system of bacterial response to environmental stress.[97] Even though **84** displays structural similarity to other fungal-derived spirodioxynaphthalenes, for example, the palmarumycins,[98,99] it represents the first member of this class of natural products featuring an oxidation at C-4′. Compound **84** exhibited stronger activity against the temperature-sensitive mutant *B. subtilis* CNM2000 than against the wild-type strain 168, suggesting that the compound inhibited the function of the YycG/YycF TCS in the bacterium.[96] Moreover, **84** exhibited strong antimicrobial activity against Gram-positive bacteria and *C. albicans*, and displayed cytotoxicity toward two mammalian cancer cell lines with IC_{50} values in the lower micromolar range.

The fungus *Nigrospora* sp. PSU-F5 was isolated from a sea fan *Annella* sp. collected near Similan Island, Thailand. Chemical investigation of this strain belonging to a genus that hitherto consisted exclusively of plant endophytes yielded three new structurally unusual epoxydon esters, nigrospoxydons A (**85**), B (**86**), and C (**87**), and one new pyrone, nigrosporapyrone (**88**).[100] The acid moieties in nigrospoxydons are abscisic acid, 2-hydroxy-4-methyl pentatonic acid, and 3-hydroxybenzoic acid for **85**, **86**, and **87**, respectively. It is noteworthy that besides further known compounds, also abscisic acid and epoxydon were detected in the culture broth of the fungus. Epoxydon and **85** exhibited mild antibiotic activity against *S. aureus* ATCC 25923 and MRSA.

A Chinese isolate of *Penicillium terrestre* derived from sediments yielded a series of new gentisyl alcohol polymers, including the trimeric terrestriol A (**89**) and the dimeric terrestrols B–H (**90–96**).[101] All compounds showed moderate cytotoxicity toward four different cancer cell lines as well as moderate radical scavenging activity in the DPPH assay. Furthermore, **95** displayed moderate inhibitory activity against protein tyrosine kinases Src and KDR.

HO OH OSO_3H

97

A new sulfoalkylresorcinol (**97**) was obtained from the culture extract of the marine-derived fungus *Zygosporium* sp. KNC52 originally isolated from a hard coral in Palau.[102] The compound was discovered in the course of a screening for antimicrobial substances targeting FtsZ, which is a structural homologue of eukaryotic tubulin and, similar to tubulin, is a GTPase that polymerizes in a GTP-regulated manner.[103] Compound **97** inhibited the GTPase activity of FtsZ by 50% at a concentration of 25 mg ml^{-1}, and almost completely inhibited FtsZ polymerization at this concentration. In addition, **97** also exhibited mild antimicrobial activity against various bacterial strains. Although alkylresorcinols are known from many different sources such as plants, algae, fungi, and bacteria, **97** is the first derivative with a sulfated side chain.

98 (R = H)
99 (R = OH)

100

101 (R = CH_2OH)
102 (R = OH)

Pichiafurans A–C (**98–100**) and pichiacins A (**101**) and B (**102**) have been characterized from the yeast *Pichia membranifaciens*, which was obtained from the Korean marine sponge *Petrosia* sp.[104] So far, no marine isolates of the genus *Pichia* had been described, and this yeast had not been studied with regard to its secondary metabolite chemistry. Compounds **98–100** are furyl ethers with 2-phenylethanol, while **101 and 102** are esters consisting of 2-phenylethanol and short-chain ω-hydroxy acids.

103 **104**

The fungus *Massarina* sp. CNT-016, isolated from a marine mud sample collected at low depths in the Palau Islands, was found to produce two new secondary metabolites, spiromassaritone (**103**) and massariphenone (**104**).[105] Compound **103** contains an unusual spiro-5,6-lactone, which has rarely been described for fungal secondary metabolites, and was identified as a stereoisomer of V214w, previously reported from an unidentified fungus.[106] For the related metabolite arthropsolide A, produced by *Arthropsis truncata*, it has been proposed that its biogenesis includes condensation of a pentaketide precursor and malic acid.[107] Compounds **103 and 104** were found not to display significant antimicrobial or cytotoxic activity.

2.08.3.2.2 Prenylated polyketides/meroterpenoids

Chemical characterization of the fungus *Eutypella scoparia* ICB-OBX, isolated from the marine pulmonate mollusk *Onchidium* sp., led to the discovery of cytosporin D (**105**) and cytosporin E (**106**).[108] Compounds **105 and 106** are structurally related to cytosporins A–C, inhibitors of angiotensin II binding previously described as being isolated from an endophytic *Cytospora* sp.[109] The cyclic carbonate functionality in **106** is rarely encountered in natural products and has so far been reported for phomoxides produced by fungi of the genera *Phoma*[110] and *Eupenicillium*.[111] Compounds **105 and 106** were found to be inactive when tested for antimicrobial activity toward *S. aureus*, *E. coli*, and *C. albicans*.

105

106

107

Chemical investigation of the endophytic fungus *Chaetomium globosum*, which was isolated from the inner tissue of the Chinese marine red alga *Polysiphonia urceolata*, resulted in the isolation of chaetopyranin (**107**), a new benzaldehyde secondary metabolite.[112] Compound **107** displayed moderate radical scavenging activity in the DPPH assay, and also exhibited moderate to weak cytotoxicity toward several tumor cell lines.

2.08.3.2.3 Nitrogen-containing polyketides

Sorbicillactone A (**108**), produced by marine-derived strains of *P. chrysogenum*, represents the first example of a sorbicillin-derived alkaloid,[113] and is of great pharmaceutical interest due to its promising activities in several mammalian and viral test systems, in particular a highly selective cytostatic activity against murine leukemic lymphoblasts (L5178y) and the ability to protect human T cells against the cytopathic effects of HIV-1.[114] For ongoing preclinical evaluation, large amounts of the compound are required, and thus an efficient process for its biotechnological production and isolation on a large scale has recently been developed.[42] By systematically optimizing parameters including media composition, growth conditions, preparation of spore suspension for inoculation, and especially screening for new sorbicillactone A producers, a 200-fold increase in the yield of **108** was achieved compared to the initial production rate of 25 $mg\,l^{-1}$ observed for the isolate E01-10/3* cultured from a sample of the Mediterranean sponge *Ircinia* sp., collected off Elba. In the meantime, at least 19 different marine isolates of sorbicillactone A-producing *P. chrysogenum* strains have been obtained from various sources including marine sponges, bryozoans, algae, and the marine sediments from Baltic Sea and Mediterranean Sea habitats. One particular problem is the co-occurrence of sorbicillactone B (the 2′,3′-dihydro congener of **108**), which is significantly less active, but shows very similar chromatographic properties. Extraction and purification of **108** thus requires a fine-tuned protocol involving adsorption to XAD, fast centrifugal partition chromatography, and gel chromatography on Sephadex LH20.[42] Although significant production of **108** by *P. chrysogenum* only succeeded in static surface cultures of the fungus, its biotechnological production in kilogram quantities sufficient for clinical trials now seems possible.

108

109 (R = CH_3)
110 (R = H)

11-*O*-Methylpseurotin A (**109**) was isolated by bioassay-guided fractionation of the culture both of an *A. fumigatus* isolate that was obtained from a deep water sediment collected off Vanuatu. The isolation procedure was monitored using a yeast halo assay with wild-type and cell cycle mutant strains of the budding yeast *S. cerevisiae*.[115] This assay was developed as a screening tool for the identification of bioactive small molecules, is easily automated, and allows quantitative assessment of inhibitory potencies based on a toxicity halo caused by active compounds.[116] **109** displayed the greatest activity differential versus the wild-type strain against the Hof1 (*hof1*Δ) haploid deletion strain, carrying a mutation in a gene involved in cytokinesis. In the same study, the previously known pseurotin A (**110**) was obtained, but was found to be inactive in the yeast halo assay.[115] Pseurotin A (**110**) is the parent compound of a series of highly functionalized 1-oxa-7-azaspiro[4.4]-nonanes including pseurotins, synerazol, and azaspirene produced by various fungal strains from terrestrial habitats. It was initially characterized from *Pseudoeurotium ovalis*[117] and shown to act as a competitive inhibitor of chitin synthetase,[118] and to represent a neuritogenic agent.[119] Through feeding experiments with ^{13}C-labeled precursors, it was shown that its biosynthesis involves L-phenylalanine, propionate, malonate, and L-methionine.[120] Recently, it has been demonstrated that the biosynthesis of **110** in *A. fumigatus* proceeds via a hybrid PKS/NRPS synthetase.[121]

111

The same 1-oxa-7-azaspiro[4.4]nonane framework as in pseurotins (**109**, **110**) is also present in cephalimysin A (**111**), isolated from a strain of *A. fumigatus* that was originally separated from the marine fish *Mugil cephalus*.[122] Its absolute stereostructure was elucidated after a series of chemical transformation, including reduction, formation of an acetonide, and generation of the corresponding MTPA esters according to the modified Mosher's method. Compound **111** exhibited significant cytotoxic activity against P388 and HL-60 cells.

112 ($R^1 = R^3 = OH$, $R^2 = R^4 = H$)
113 (R^1, $R^2 = O$, $R^3 = H$, $R^4 = OH$)

114 ($R^1 = R^3 = OH$, $R^2 = R^4 = H$)
115 (R^1, $R^2 = O$, $R^3 = H$, $R^4 = OH$)
116 ($R^1 = H$, $R^2 = OH$, R^3, $R^4 = O$)
117 ($R^1 = OH$, $R^2 = H$, R^3, $R^4 = O$)

The fungus *Spicaria elegans* was isolated from the marine sediments collected in Jiaozhou Bay, China. Chemical investigation led to the discovery of cytochalasins Z10–Z15 (**112–117**), representing the first cytochalasin congeners from nature with an open 8-carbon side chain instead of the usual 11–14-membered macrocyclic ring.[123] In general, cytochalasans have been described from a variety of fungal genera including *Phoma*, *Helminthosporium*, *Metarrhizium*, and *Zygosporium*[124] and have drawn considerable attention because of a wide range of biological activities, for example their ability to bind to actin filaments.[125] The tetrahydroisoindolinone subunit of cytochalasans was assumed to be formed via an intramolecular Diels–Alder reaction.[126] Recently, it was demonstrated by RNA silencing that a PKS/NRPS hybrid synthetase is involved in the biosynthesis of the cytochalasan chaetoglobosin A in *Penicillium expansum*,[127] connecting an amino acid to a straight chain polyketide, which would support the suggested intramolecular Diels–Alder reaction. From a biosynthetic point of view, the structures of **112–117** are thus intriguing, since they should require a twofold oxidative cleavage of the macrocyclic system. When evaluated for their cytotoxic activity against four cell lines, **112 and 113** displayed moderate cytotoxicity toward A-549 cells, whereas the remaining compounds were inactive.

118

The fungus *A. niger* LL-LV3020, from mangrove wood in the coastal environment of Hong Kong, yielded pyranonigrin A (**118**) as a major UV-active compound in its extract, after removal of large quantities of citric acid.[36] **118** had previously been reported from a sponge-derived *A. niger* strain,[35] but extensive analysis of its spectroscopic properties including ^{1}H–^{15}N HMBC led to the revision of its structure as depicted. Interestingly, since its initial detection in marine-derived fungi, **118** has been demonstrated to be a metabolite of rather widespread occurrence in terrestrial strains of *Aspergillus* belonging to section *Nigri*, including *A. carbonarius*, *A. costaricaensis*, *A. lacticoffeatus*, *A. niger*, *A. sclerotioniger*, and *A. tubingensis*.[128] In addition, **118** was also detected in rice mold starters used in the manufacturing process of fermented foods.[129,130]

119

A new amino-substituted dihydrostyrylpyrone (**119**) was obtained from the endophytic fungus *A. niger* EN-13 that was isolated from the Chinese marine brown alga *C.sinuosa*.[131] This type of fungal natural product seems to be restricted to *Aspergillus* section *Nigri*,[132] since the only derivatives reported so far include pyrophen[133,134] and aspernigrin B.[35,135] **119** displayed moderate antifungal activity against *C. albicans*.

120
121 (5-Epimer)
124 (D^{13})
125 (D^{13}, 5-Epimer)

122
123 (5,6-*E*)

The endophytic fungus *Penicillium* sp. GQ-7 was isolated from the inner bark of the Chinese mangrove plant *Aegiceras corniculatum*. Chemical investigation led to the discovery of a series of tetramic acids, penicillenol A_1 (**120**), A_2 (**121**), B_1 (**122**), B_2 (**123**), C_1 (**124**), and C_2 (**125**).[136] Interestingly, **120–123** were cytotoxic toward HL-60 cells with IC_{50} values ranging from 0.76 to 16.26 mmol l^{-1}, while congeners **124 and 125** with an additional double bond near the chain terminus proved inactive. As shown recently for equisetin[137] and fusarin C,[138] fungal-derived tetramic acids are biosynthesized via hybrid PKS/NRPS synthetases.

E-isomer **126** *Z*-isomer

E-isomer **127** *Z*-isomer

The tetronic acid nodulisporacid A (**126**) was isolated from *Nodulisporium* sp. CRIF1, a fungus obtained from a soft coral from Thailand, while the known tetramic acid vermelhotin (**127**) was produced by an unidentified sponge-derived fungal strain CRI247-01 belonging to the order Pleosporales.[139] Both compounds occurred as equilibrium *E*/*Z* mixtures. **126** shows structural similarity to lowdenic acid, previously described from a terrestrial *Verticillium* sp.,[140] while **127** is a metabolite of an unidentified fungal strain, likewise from a terrestrial habitat.[141] **126** was found to be inactive when tested for cytotoxic properties, but converting it into its methyl or benzyl ester resulted in a considerable increase in activity, similar in potency to underivatized **127**. In addition, **126 and 127** exhibited moderate antiplasmodial activity.

128 **129**

However, it is worth noting that not every nitrogen-containing polyketide is synthesized by hybrid PKS/NRPS synthetases. Recently, it was demonstrated that the biosynthesis of the 2-azaanthraquinone scorpinone (**128**) in the marine sediment-derived fungus *Amorosia littoralis* follows the 'classical' polyketide pathway with a linear heptaketide as biosynthetic intermediate, but does not incorporate amino acids such as alanine by means of a hybrid PKS/NRPS pathway.[142] Besides **128**, small amounts of herbarin (**129**) were detected. **129** possesses a cyclic hemiketal structure in which the nitrogen at position 2 in **128** is replaced by oxygen. Nevertheless, **129** exhibited the same pattern of enrichment from [2-^{13}C]acetate as observed in **128**, suggesting that both result from a common biosynthetic pathway. However, currently, it cannot be decided whether the nitrogen atom in **128** is derived from inorganic nitrogen or a nitrogen-containing organic precursor.

130 **131**

Carbonarone A (**130**) is a γ-pyrone, and the biogenetically obviously closely related carbonarone B (**131**) represents a α-pyridone derivative. Compounds **130 and 131** are new metabolites from *A. carbonarius*, isolated from the sediments collected off Weizhou island in China.[143] Both compounds exhibited moderate antiproliferative activity against K562 cells.

2.08.3.2.4 Alkaloids

Chemical examination of the endophytic fungus *Penicillium* sp. isolated from the Chinese mangrove plant *A. corniculatum* led to the discovery of eight new janthitrem-type indole triterpenes, shearinines D–K (**132–139**).[144] Shearinine D (**132**) is the 22-hydroxy derivative of the known shearinine A, which had previously been described from ascostromata of the terrestrial fungus *Eupenicillium shearii*.[145] Biogenetically, shearinines are suggested to arise from paspaline, which upon prenylation would undergo oxidative ring formation to yield paspalitrem A. This assumption was made on structural consideration, but was supported by the fact that paspaline, paspalitrem, together with shearinine A were likewise detected in the culture broth of the fungus under study.[144] The occurrence of **132–139** would then require a further prenylation step, oxidative cyclization to yield the pyrane ring, and various combinations of olefinic rearrangements or oxidative cleavage of the C-2–C-18 bond, the latter of which might occur spontaneously, since analogous reactions were observed during the NMR measurements. Shearinines D (**132**), E (**133**), and, to a lower extent, G (**135**) exhibited significant *in vitro* blocking activity on large-conductance calcium-activated potassium channels.

132 (R = H)
133 (R = CH$_3$)
134 (R^1 = R^2 = H)
135 (R^1, R^2 = O)
136
137
138
139

140 (H-22,H-23 *trans*)
141 (H-22,H-23 *cis*)

142

A marine isolate of the fungus *P. janthinellum* was obtained from the sediments collected at low depths from the Sea of Japan in Amursky Bay, near Vladivostok.[146] Its chemical characterization yielded three further janthitrem-type alkaloids besides shearinine A. Unfortunately, the names shearinines D (**140**), E (**141**), and F (**142**) were assigned, since the authors were obviously not aware of the publication discussed in the previous paragraph.[144] 'Shearinine F' is in fact identical to shearinine K (**139**), while **140** and **141** are obviously new compounds and thus should be renamed. Interestingly, the NMR data of the H-22,H-23-*cis* congener **141** differ significantly from shearinine D (**132**), suggesting that both H-22 and H-23 follow the β-orientation. Shearinine A, **140**, and **141** were found to induce apoptosis in human leukemia HL-60 cells, while **141** also inhibited the epidermal growth factor(EGF)-induced malignant transformation of murine JB6 P^+ Cl 41 cells, indicative of a potential cancer preventive effect.

143

The fungus *Exophiala* sp., isolated from the surface of the Korean marine sponge *H. panicea*, yielded a new alkaloid of the circumdatin class (see also **230–232**), circumdatin I (**143**).[147] In addition, the known circumdatins C[148] and G[149] were also obtained, previously reported as metabolites from a terrestrial- and a marine-derived strain of *Aspergillus ochraceus*, respectively. **143** and the two known circumdatins were found to exhibit UV-A protecting activity, with ED_{50} (effective dose, 50%) values in the range of 100 $\mu mol\,l^{-1}$, and thus more potent than the commercially used sunscreen agent oxybenzone (ED_{50} 350 $\mu mol\,l^{-1}$), which acted as a positive control.

2.08.3.2.5 Diketopiperazines

The fungus *Aspergillus* sp., which was isolated from the common mussel *Mytilus edulis*, yielded a series of complex prenylated diketopiperazines, notoamides A–D (**144–147**) and F–K (**148–153**) with diverse modifications in their backbones.[150,151] Compounds **144, 145, and 150** contain the same intriguing spiro-indolinone skeleton as encountered in sclerotiamide, previously reported as a metabolite from terrestrial isolates of *Aspergillus sclerotiorum*,[152] or in paraherquamide, a toxin produced by *Penicillium paraherquei*.[153] Compounds **148, 149, and 151** are related to stephacidin A, earlier described from an Indian isolate of *A. ochraceus*.[154] The remaining congeners could be considered biosynthetic intermediates, since all of them are characterized by the presence of a rearranged isoprenyl moiety at either C-2 or C-3 of the indole, but lack additional cyclization steps that link this substituent to the pyrrolo[1,2-*a*]pyrazine system in **144, 145, and 148–151**, possibly by

means of an intramolecular Diels–Alder reaction.[155] Compounds 144–146 and 151 showed moderate cytotoxicity against HeLa cells, while the remaining congeners proved inactive. Moreover, 146 was found to induce G2/M cell cycle arrest.

144 (R^1 = OH, R^2 = H)
145 (R^1 = R^2 = H)
150 (R^1 = R^2 = OH)

146

147 (R = α-H)
153 (R = β-OH)

148 (R^1 = R^2 = H, R^3 = OCH_3)
149 (R^1 = OH, R^2 = H, R^3 = OCH_3)
151 (R^1 = H, R^2, R^3 = O)

152

154

155 (R = H)
156 (R = OH)

157

158, **159**

160

A similar diverse suite of seven new prenylated indole diketopiperazine alkaloids was obtained when investigating the fungus *A. fumigatus* isolated from the holothurian *Stichopus japonicus*, collected off Qingdao, China.[156] Compound 154 was identified as a structurally unique spiro-3-indolinone derivative, while spirotryprostatins C (155), D (156), and E (157) were spiro-2-indolinones related to spirotryprostatins A and B, previously described as mammalian cell cycle inhibitors from a terrestrial isolate of *A. fumigatus*.[157,158] Compounds 158 and 159, obtained as an inseparable mixture of diastereomers, display the same skeleton as present in fumitremorgin, another well-known tremorgenic toxin from *A. fumigatus*.[159] In 160, identified as

13-oxoverruculogen, the two isoprene moieties present in 158 and 159 have been cross-linked by a peroxide bridge to yield a 1,2,4-dioxazocane ring system, similar to the known verruculogen reported from *Penicillium verruculosum*.[160] Compounds 154–160 displayed moderate cytotoxic activity against four different cell lines, with 157–160 being the most active congeners.

Chemical investigation of *Eurotium rubrum*, an endophytic fungus from the Chinese mangrove plant *Hibiscus tiliaceus*, resulted in the characterization of two new threefold prenylated diketopiperazine derivatives, dehydrovariecolorin L (161) and dehydroechinulin (162).[161] Compound 161 is the 8,9-dehydro derivative of variecolorin L, recently described from a halotolerant strain of *A. variecolor*, while 162 is the 8,9-dehydro congener of echinulin, described 50 years ago from *A. glaucus*.[162] When tested for biological activity, 161 and 162 were found to be devoid of cytotoxic or radical scavenging activity.

Bilains A (163), B (164), and C (165) are new members of the rare class of bis(methylthio)diketopiperazines, which have been identified as new metabolites from *P. bilaii* MST-MF667, obtained from a boat ramp on the Huon estuary in Tasmania.[37] Compounds 163–165 are new analogues of the known *cis*-bis(methylthio)silvatin, described from a terrestrial strain of *Aspergillus silvaticus*,[163] while the corresponding *trans*-bis(methylthio)silvatin had previously been characterized from a salt water culture of the terrestrial fungus *Coriolus consors*.[164] Compounds 163–165 were tested for antibacterial, antifungal, antiparasitic, and cytotoxic properties, but were found to be inactive.

The new diketopiperazine (*Z*)-6-benzylidene-3-hydroxymethyl-1,4-dimethyl-3-methylsulfanylpiperazine-2,5-dione (**166**) was isolated from the culture broth of the sponge-derived fungal strain CRIF2, belonging to the order Pleosporales.[91] Chemically and biogenetically, **166** is closely related to (3*R*,6*R*)-bisdethiodi(methylthio)-hyalodendrin, previously reported from a soil-borne *Penicillium turbatum*,[165] which was also obtained in this study. Both compounds exhibited weak cytotoxic activity toward various cell lines.

167 (R = OCH_3)
168 (R = OH)
169

The fungus *Penicillium aurantiogriseum* SP0-19, isolated from the Chinese sponge *Mycale plumose*, yielded three new quinazoline alkaloids, aurantiomides A (**167**), B (**168**), and C (**169**).[166] Structurally, **167–169** are closely related to anacine, a metabolite previously described from terrestrial strains of *P. aurantiogriseum* and *P. verrucosum*[167,168] and which based on its structure is most likely formed by incorporating anthranilic acid into a diketopiperazine composed of leucine and glutamine. Compounds **168 and 169** showed moderate cytotoxic activities toward various cell lines, while **167** was inactive.

2.08.3.2.6 Peptides

In an intriguing attempt to test the effects of competing coculture on secondary metabolites production, Bill Fenical's group investigated the fungus *Emericella* sp. CNL-878, which was previously isolated from the surface of a green alga of the genus *Halimeda*, collected in Papua New Guinea.[169] Upon fermentation in the presence of the marine actinomycete *Salinispora arenicola*, the fungus was induced to produce two new antimicrobial cyclic depsipeptides, emericellamides A (**170**) and B (**171**). Compound **170** contains an unusual hydroxylated fatty acid, 3-hydroxy-2,4-dimethyldecanoic acid (HDMD), the enantiomer of which was previously encountered in the lipodepsipeptide 15G256γ, reported from the marine-derived fungus *Hypoxylon oceanicum*.[170] On the other hand, the 3-hydroxy-2,4,6-trimethyldodecanoic acid (HTMD) moiety present in **171** has not yet been reported as a component of a lipopeptide. Compounds **170 and 171** were produced in low yields by *Emericella* sp. alone, that is, in yields that would not facilitate their isolation and structure elucidation, while in coculture the levels were enhanced by 100-fold. Both compounds showed modest antibacterial activities against MRSA. Recently, through genomic data mining, the biogenetic gene cluster, a PKS/NRPS hybrid synthetase, for the production of **170** has been identified in the fully sequenced genome of *A. nidulans*.[171] Interestingly, the levels of emericellamide production in *A. nidulans* cultured alone were comparable to the bacterium-stimulated marine fungus. According to the authors, replacement of the native promoter with either inducible or strong constitutive promoters appears feasible, and could provide a rational route to increase production levels even further, while the engineering of the PKS and NRPS for the production of novel analogues likewise seems an option.[171]

170　　**171**

172 **173**

Pseudodestruxin C (**172**) and β-Me-Pro destruxin E chlorohydrin (**173**) are two new cyclic depsipeptides that were obtained in the course of a multiscreening approach conducted by a consortium of Brazilian researchers, screening marine-derived fungal strains for antibacterial, antimycobacterial, and cytotoxic properties.[172,173] Among a total of 57 extracts from various strains, mainly derived from sediments and marine algae, the strain *Beauveria felina*, associated with the marine alga *Caulerpa* sp., was selected based on both biological and chemical characteristics, and chemical investigation of its culture broth ultimately led to the discovery of **172 and 173**. In general, most of the known members of the destruxin class of cyclic depsipeptides have been isolated from *Metarrhizium anisopliae*, and have been described to exhibit interesting biological activities including insecticidal, cytotoxic, antiviral, immunodepressant, and phytotoxic properties.[174]

174 (R = H)
175 (R = OH)

The fungal strain *Spicellum roseum* 193H15, isolated from the Caribbean sponge *Ectyplasia perox*, yielded two new cyclohexadepsipeptides, spicellamide A (**174**) and B (**175**).[175] Compounds **174 and 175** are structurally related to beauvericins, cytotoxic and insecticidal depsipeptides described from a range of fungi, including *Beauveria*, *Paecilomyces*, *Fusarium*, as well as higher fungi such as *Laetiporus*.[176] Since hydrolysis using Marfey's method revealed the presence of both D- and L-alanine in **174 and 175**, the position of this amino acid was determined from NOESY data in conjunction with molecular modeling calculations. Both compounds did not exhibit antimicrobial activity toward various test bacteria, fungi, and algae, but were found to exhibit moderate cytotoxicity against neuroblastoma cells.

176 (R = OH)
177 (R = H)

Two new cyclic depsipeptides, 1962A (**176**) and 1962B (**177**), were isolated from the fermentation broth of an unidentified fungal endophyte obtained from *K. candel* collected in Hong Kong.[177] Both **176 and 177** were found to contain one D-amino acid each. In the MTT bioassay, **176** displayed weak activity against human breast cancer MCF-7 cells.

178 (R^1, R^2 = O)
179 (R^1 = OH, R^2 = H)

Microsporins A (**178**) and B (**179**) are two new cyclic tetrapeptides isolated from the marine-derived fungus *Microsporum* cf. *gypseum*, obtained from a sample of the bryozoan *Bugula* sp. collected in the U.S. Virgin Islands.[178] Compounds **178 and 179** were identified as potent inhibitors of histone deacetylase (HDAC), and they also displayed cytotoxic activity against human colon adenocarcinoma (HCT-116), as well as against the National Cancer Institute 60 cancer cell panel. Compound **179** contains the unusual amino acid (2*S*)-2-amino-8-hydroxydecanoic acid, while **178** contains its oxidized derivative (*S*)-2-amino-8-oxodecanoic acid (Aoda). Aoda has been found in other fungal cyclic tetrapeptides including the potent protozoan HDAC inhibitors apicidin[179] and 9,10-deepoxy-chlamydocin,[180] described from *Fusarium pallidoroseum* and *Peniophora* sp., respectively. Compound **178** was synthesized by solid-phase synthesis using a sulfonamide linker resin.[178]

180 **181** **182**

183

The fungal strain *Acremonium* sp. MST-MF558a was isolated from a Tasmanian estuarine sediment sample, and based on its rDNA sequence was considered to represent a new species. From the culture broth, a novel family of lipodepsipeptides, acremolides A–D (**180–183**), was obtained, together with known chaetoglobosins.[181] The absolute stereochemistry of amino acid residues in **180–183** was determined using a new C_3 Marfey's method for amino acid analysis. Compounds **180–183** were neither cytotoxic in their own right nor

did they synergize the chaetoglobosin cytotoxicity, and furthermore they displayed no antibacterial or antifungal properties.

2.08.3.2.7 Terpenoids

The marine fungus *Penicillium* sp. BL27-2, isolated from sea mud in the Bering sea, yielded two new eremophilane sesquiterpenes, 3-acetyl-9,7(11)-dien-7*a*-hydroxy-8-oxoeremophilane (**184**) and 3-acetyl-13-deoxyphomenone (**185**).[182] The latter had been prepared in the course of the structure elucidation of sporogen A0 I from a mycophilic *Hansfordia* sp.,[183] but so far had not been reported as a natural product. When tested for cytotoxic activity, **185** was found to be active in the nanomolar range against three different cell lines, while **184** was less active by several orders of magnitude.

184 **185**

186 **187** **188**

Peribysins are a group of eremophilane-type sesquiterpenoids produced by *P. byssoides*, which was isolated from the sea hare *A. kurodai*, and are of interest because of their inhibitory effects on cell adhesion.[184] Recently, three new members of this class, peribysin H (**186**), I (**187**), and J (**188**), have been described.[57,185] Compounds **186–188** were found to inhibit the adhesion of HL-60 cells to HUVECs more potently than the positive control herbimycin A.

189 **190**

191 (R^1 = OH, R^2 = H)
192 (R^1 = H, R^2 = OH)
193 (R^1, R^2 = O)

194

Six new ergosterols, 3β-hydroxy-(22*E*,24*R*)-ergosta-5,8,22-trien-7,15-dione (**189**), 3β-hydroxy-(22*E*,24*R*)-ergosta-5,8,14,22-tetraen-7-one (**190**), 3β,15β-dihydroxy-(22*E*,24*R*)-ergosta-5,8(14),22-trien-7-one (**191**), 3β,15α-dihydroxy-(22*E*,24*R*)-ergosta-5,8(14),22-trien-7-one(**192**),3β-hydroxyl-(22*E*,24*R*)-ergosta-5,8(14),22-trien-7, 15-dione (**193**), and 5α,8α-epidioxy-23,24(*R*)-dimethylcholesta-6,9(11),22-trien-3β-ol (**194**), have been isolated from

the marine-derived fungus *Rhizopus* sp.[186] The fungus was isolated from the bryozoan *Bugula* sp. collected in Jiaozhou Bay, China. All compounds showed cytotoxic activity to varying degrees against four different cancer cell lines.

195

An unusual steroid derivative, ergosterimide (**195**), was characterized from the culture extract of *A. niger* EN-13, an endophytic fungus isolated from the Chinese marine brown alga *C. sinuosa*.[187] Chemically, **195** is a Diels–Alder adduct of a suitably substituted ergosteroid and maleimide. The latter has a widespread use in technical applications, and is commonly used as an adduct for Diels–Alder reactions due to its high reactivity. If maleimide indeed was a metabolite of the fungus under study, **195** would represent the first natural Diels–Alder adduct of this type.

2.08.3.2.8 Lipids

Asperamides A (**196**) and B (**197**) represent a sphingolipid and its corresponding cerebroside, respectively, and were discovered when analyzing the endophytic fungus *A. niger* EN-13 that was isolated from the Chinese marine brown alga *C. sinuosa*.[188] While sphingolipids containing a 9-methyl-C_{18}-sphingosine moiety have been reported frequently from natural sources,[189] **196 and 197** are characterized by a hitherto unreported 9-methyl-C_{20}-sphingosine moiety. **196** displayed moderate antifungal activity against *C. albicans*.

196 (R = H)
197 (R = β-D-glucopyranoside)

198 (R = β-D-glucopyranoside)

199 (R = β-D-glucopyranoside)

Structurally related cerebrosides asperiamides B (198) and C (199) were obtained from *A. niger* MF-16#, which was isolated from seawater collected in Quanzhou Gulf, China, together with two known intermediates of aflatoxin biosynthesis, averufin and nidurufin.[190] The latter compounds displayed moderate antiviral activity against tobacco mosaic virus, while 198 and 199 were inactive.

The fungus *Stilbella aciculosa*, isolated from the holothurian *Apostichopus japonica*, has been identified as a potential producer of prostaglandins.[191] In its culture liquid, prostaglandins of groups E and F were revealed, together with their biogenetic precursors, polyunsaturated eicosapentaenoic and docosahexaenoic acids, while the biomass of the fungus contained prostaglandins of group B. Oxylipins including prostaglandins have been implicated as a novel class of host–microbe signaling molecules,[192] also playing a role in pathogenesis.[193] Interestingly, the genome of most fungi, as exemplified by *C. albicans*, does not contain a cyclooxygenase homologue, but it has been demonstrated that a fatty acid desaturase homologue and a multicopper oxidase homologue play a role in their prostaglandin biosynthesis.[194]

2.08.3.2.9 Shikimate-derived metabolites

Prenylterphenyllin (200), 4′′-deoxyprenylterphenyllin (201), and 4′′-deoxyisoterprenin (202), together with the known 4′′-deoxyterprenin, were characterized from the culture of *Aspergillus candidus* IF1, isolated from the sediments collected off Gokasyo Gulf, Japan.[195] **200–202** are structurally related to terphenyllin[196] and terprenin,[197] previously described from terrestrial strains of *A. candidus*. Through labeling studies with phenylalanine, it has been demonstrated for terphenyllin and the related velucrisporin[198] that the terphenyl ring system is derived from self-condensation of two phenylpropanoid units.[196] 200–202 as well as 4′′-deoxyterprenin exhibited moderate cytotoxic activity against KB3-1 cells.

H_3CO OH OH R OCH_3 — H_3CO OH O OH OCH_3

200 (R = OH)
201 (R = H)

202

2.08.3.2.10 Miscellaneous

Bromomyrothenone B (203) and botrytinone (204) are two new cyclopentenones isolated from *Botrytis* sp., obtained from the surface of the Korean green alga *Enteromorpha compressa*.[199] Myrothenone B, the debrominated congener of 203, had previously been reported from two independent sources, an algicolous *Myrothecium* sp. from the same algal species (possibly the same collection),[200] and shortly thereafter from an endophytic *Streptomyces* sp. isolated from the Chinese mangrove plant *Aegiceras comiculatum*.[201] At first impression, 203 and 204 would appear to represent polyketides, but it is hard to spot a biogenetic relationship for both of them and, additionally, on at least two occasions they have been reported to co-occur with the known regular sesquiterpene cyclonerodiol. 203 and 204 were virtually inactive when assayed for radical scavenging, tyrosinase inhibitory, and antimicrobial activity.

NH_2 Br HO O — OH HO HO O

203 **204**

2.08.3.2.11 Individual fungal strains producing different classes of natural products

Some marine-derived fungal strains proved to represent producers of a diverse array of natural products belonging to different classes of natural products. This section highlights selected examples.

Gabriele König's group undertook extensive chemical characterization of the obligate marine and endophytic fungus *A. salicorniae*, which was obtained from the inner tissue of the marine green alga *Ulva* sp., collected from the North Sea, Germany. Ascosalipyrrolidinones A (**205**) and B (**206**) were characterized as deoxytetramic acid derivatives, which are only rarely encountered in nature.[202] Compound **205** displayed antimicrobial activity against *Bacillus megaterium*, *Mycotypha microsporum*, and *Microbotryum violaceum*, and also inhibited the enzyme tyrosine kinase. Furthermore, the compound showed moderate antiplasmodial activity against two strains of *P. falciparum* and exhibited significant activity against *T. cruzi* and *Trypanosoma brucei* subsp. *rhodesiense*, as well as cytotoxic activity against rat skeletal muscle myoblast cells and mouse peritoneal macrophages.[202] In the same investigation, a new α-pyrone, ascosalipyrone (**207**), was obtained, but was found to be devoid of significant biological activity. After being cultivated under different culture conditions, the same fungus produced two new epimeric lactones, ascolactones A (**208**) and B (**209**).[203] The absolute configurations of **208** and **209** were determined by comparing the experimental CD spectra with those calculated by employing time-dependent density functional theory,[204] and by chiral GC–MS subsequent to oxidative cleavage by Baeyer–Villiger oxidation of the side chain.[203] In addition, two known biogenetically related polyketides, ascochitin (**210**) and ascochital (**211**), were detected, the latter having previously been reported from the marine ascomycete *Kirschsteiniothelia maritima*.[205] Feeding experiments with [1,2-$^{13}C_2$]acetate and [Me-^{13}C]methionine revealed that **207** and **211** were derived from a single pentaketide and hexaketide chain, respectively, while all methyl groups in **207** (except for the terminal one in the side chain) were derived from *S*-adenosyl-methionine.[203] *In silico* screening using the PASS software identified some of the compounds **207–211** as potential inhibitors of protein phosphatases, which was experimentally confirmed for compound **211**, displaying inhibitory activity toward mycobacterial protein tyrosine phosphatase B (MPtpB) and protein tyrosine phosphatase 1B (PTP1B) with IC_{50} values of 11.5 and 38.5 μmol l^{-1}, respectively.[203] Recently, ascospiroketals A (**212**) and B (**213**) were described from *A. salicorniae*, representing biogenetically unique cycloethers, composed of methylated diketide attached to a highly modified octaketide via an ester link.[52] The most striking chemical feature of **213** from a biosynthetic point of view is the presence of two additional carbon atoms attached to C-2 of the octaketide part (C-16 and C-17). Extender units other than malonyl-CoA are not known from fungal polyketides, and these two carbons thus most probably arise from geminal biomethylation via *S*-adenosyl-methionine, a very rare process that seems to be unprecedented in the context of fungal polyketides.

The fungus *Gymnascella dankaliensis* OUPS-N134, isolated from the Japanese sponge *Halichondria japonica*, has been extensively studied by Atsushi Numata's group.[206–208] Following cell-based assay results, gymnostatins A–H, potent cytostatic polyketide alkaloids, were discovered. A reinvestigation of previously unexamined cytotoxic semipure fractions yielded a series of structurally unusual steroid-type compounds, the pattern of which varied depending on media composition.[209] Dankasterones A (**214**) and B (**215**) were obtained when glucose in the original medium was replaced by soluble starch, while gymnasterones A (**216**), B (**217**), C (**218**), and D (**219**) were isolated from malt–glucose–yeast media. Compounds **214** and **215** are the most unusual steroids possessing a 13(14 → 8)*abeo*-8-ergostane skeleton, which so far has been described only once from nature, resulting from a photochemical reaction of the insect molting hormone 20*R*-hydroxyecdysone.[210] On the other hand, **216** is structurally intriguing since it represents an unprecedented steroid alkaloid with an additional ring and an amide-linked side chain derived from gymnastatins (**220**, **221**). Compound **217** is a rare example of steroids with an epoxide-substituted D ring, while **218** and **219** contain an unusual 4,6,8(14)-conjugated triene system. **214**, **215**, and **217**–**219** exhibited significant growth inhibition against the murine P388 cancer cell line, whereas **214** also exhibited potent growth inhibition against human cancer cell lines. Recently, further analysis of the culture broth containing soluble starch instead of glucose revealed the presence of additional gymnastatin congeners, gymnastatins Q (**220**) and R (**221**), as well as the related dankastatins A (**222**) and B (**223**).[211] Compounds **220**–**223** displayed growth inhibition against the P388 cancer cell line and, furthermore, **220** inhibited growth of BSY-1 (breast) and MKN7 (stomach) human cancer cell lines.

224 (R^1 = OH, R^2 = H)
225 (R^1 = H, R^2 = OH)
226
227 (R^1 = CH_3, R^2 = H)
228 (R^1 = H, R^2 = CH_3)
229 (R^1 = CH_3, R^2 = H, $\Delta_{5,6}$)
230 (R = OCH_3)
231 (R = H)
232

Three pentaketides, aspinotriols A (224) and B (225) as well as aspinonediol (226), together with the known aspinonene and dihydroaspyrone, were obtained from the culture of an *Aspergillus ostianus* strain isolated from an unidentified marine sponge at Pohnpei, Micronesia.[212] Previously, the same strain had produced new chlorinated pentaketides when fermented in natural seawater.[213] Expecting that bromine-containing compounds might be obtained when a medium composed of a bromide solution in place of seawater was used, large amounts of bromine were added to the culture medium. However, no brominated compounds were detected, but the metabolite profile of the fungus changed considerably compared to the previous study. A further chemical investigation of the same fungus under identical culture conditions led to the discovery of three new 14-membered macrolides, named aspergillides A (227), B (228), and C (229).[214] While 14-membered macrolides in general have been described from fungi on several occasions, 227–229 are remarkable since they represent the first congeners with an additional tetrahydropyran or dihydropyran ring. Compounds 227–229 proved inactive when tested for antimicrobial activity toward MRSA; however, they exhibited moderate cytotoxicity toward mouse lymphocytic leukemia cells (L1210). Recently, the same fungus also yielded benzodiazepine-type alkaloids, including circumdatins A (230) and B (231) as well as the new congener circumdatin J (232).[215] Compounds 230 and 231 were initially described as metabolites from a terrestrial isolate of the fungus *A. ochraceus*, and their structures were reported as zwitterions.[148] In the present study, the structures of both the compounds were revised on the basis of X-ray crystallographic analysis, and 230 and 231 were found to contain a very unusual oxepin framework in the 'Southern' part. Compound 232 is the 15-deoxy congener of circumdatin D, likewise reported from *A. ochraceus*.[216] When tested for biological activity, 230–232 displayed neither antimicrobial nor cytotoxic properties.

233
234
235
236
237

A series of prenylated diketopiperazine alkaloids, 6-methoxyspirotryprostatin B (**233**), 18-oxotryprostatin A (**234**), and 14-hydroxyterezine (**235**), were isolated from a culture of the fungus *Aspergillus sydowi* PFW1, obtained from a driftwood sample collected from the beach of the island of Hainan in China.[217] Spirotryprostatin B and tryprostatin A have originally been described from *A. fumigatus* BM939, isolated from a sea sediment sample in Japan, and were identified as mammalian cell cycle inhibitors,[157,218] while terezine D is a metabolite of the coprophilous fungus *Sporormiella teretispora*.[219] In addition, the nitrogen-containing polyketide 14-norpseurotin A (**236**) was discovered, which is structurally closely related to pseurotin A (**110**) from *P. ovalis*.[117] Finally, a rearranged triterpenoid 6β,16β-diacetoxy-25-hydroxy-3,7-dioxy-29-nor-dammara-1,17(20)-dien-21-oic acid (**237**) was likewise obtained. In the MTT bioassay, **233–235** exhibited weak cytotoxicity against A-549 cells, while **236** and **237** displayed significant growth inhibition against *E. coli*, *B. subtilis*, and *Micrococcus lysoleikticus*.

2.08.4 Conclusions

Marine-derived fungi have already provided a variety of pharmacologically active novel secondary metabolites and thus represent a valuable resource for the discovery of new drug candidates. The most prominent example is the diketopiperazine halimide, previously characterized by Bill Fenical's group,[220] which is active as a tubulin-depolymerizing agent, and which served as lead structure for the simple analogue, NPI-2358, currently undergoing phase II clinical trials in patients with advanced non-small-cell lung cancer.[221,222] Even though, especially in the case of facultative marine species, the true origin of many strains is still difficult to trace, there is no doubt that ubiquitous genera such as *Aspergillus* or *Penicillium* contribute to the chemical diversity of the marine environment. In many cases, through alterations of the culture conditions, the metabolic spectrum of individual talented strains can be significantly enhanced. Currently, molecular biology-based studies are being undertaken, and there are first indications of truly specialized fungal communities associated with algae, mangrove plants, and marine invertebrates, displaying individual adaptations to their respective hosts. The recent years have seen a steady rise in interest from the part of the natural product community in marine-derived fungal strains, and especially in China a systematic evaluation of their metabolic diversity has begun on a very broad scale. Modern methods of rapid screening of large culture collections seem to render it more and more unlikely that exclusively 'marine' families of natural products exist, but on the other hand, they also contribute to the discovery of additional congeners, which are extremely valuable to investigate structure–activity relationships. A better understanding of the molecular basis of natural product biosynthesis and its mechanisms of regulation will contribute to make better use of the enormous chemical potential of marine-derived fungi, for example, in optimizing culture conditions, or, ultimately, in expressing entire biogenetic pathways in host systems better suited for industrial scale fermentation.

Given the discrepancy between the actual number of cultivated strains and the estimated biodiversity of fungi in marine habitats, marine-derived fungi are still a heavily underexplored source of new secondary metabolites, and thus it can be expected that this interesting group of organisms will continue to yield relevant discoveries in the future.

Glossary

circular dichroism Spectroscopic method measuring differences in the absorption of left-handed polarized light versus right-handed polarized light that arise due to structural asymmetry.

denaturing gradient gel electrophoresis Molecular fingerprinting method that separates polymerase chain reaction (PCR)-generated DNA products.

1,1-diphenyl-2-picrylhydrazyl Stable radical commonly used in a convenient colorimetric assay to assess antioxidative properties of compounds or extracts.

ED_{50} or effective dose, 50% Dosage that produces a desired effect in half of the test population.

facultative marine fungi Fungi originating from freshwater or terrestrial milieus that are able to grow (and possibly also to sporulate) in the marine environment.

IC_{50} or half-maximal inhibitory concentration Concentration required to inhibit a given biological process by half.
internal transcribed spacer Nonfunctional RNA situated between structural ribosomal RNAs on a common precursor transcript.
meroterpenoid A natural product that contains a terpenoid-derived moiety besides at least one other substructure derived by an alternative biosynthetic pathway, commonly a polyketide.
MIC or minimum inhibitory concentration The lowest concentration of an antimicrobial compound that will inhibit the visible growth of a microorganism.
nonribosomal peptide synthetase A family of enzymes or enzyme complexes that produce (cyclic) peptides in the cytosol (as opposed to regular protein biosynthesis, which occurs at the ribosomes).
obligate marine fungi Fungi that grow and sporulate exclusively in a marine or estuarine habitat.
polyketide synthetase A family of enzymes or enzyme complexes that produce polyketides.
ribosomal RNA Noncoding RNA, which is the essential component of the ribosome.

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Biographical Sketch

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2.09 Bioactive Metabolites from Marine Dinoflagellates

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2.09.1 Introduction

Dinoflagellates are a diverse group of unicellular eukaryotes with a large and unusual genome. The amounts of DNA per haploid nucleus are up to 60 times larger in comparison with humans. The chromosomes are permanently condensed and the chromatin structure is different from other eukaryotes because of the absence of nucleosomes.[1,2] Dinoflagellates have proved to be important sources of marine toxins such as fish and algal poisons as well as bioactive compounds and have been investigated worldwide by natural product chemists. In this chapter, topics include the isolation, structure elucidation, synthesis, biosynthesis, and bioactivity of macrolides, polyketides, and other bioactive metabolites from several genera of marine dinoflagellates. This chapter covers the literature published on a series of cytotoxic macrolides, designated amphidinolides, and long-chain polyketides isolated from marine dinoflagellates *Amphidinium* sp. and is a comprehensive review including our early reviews.[3–10] Synthetic work on amphidinolides were reviewed by Chakraborty[11] and Colby and Jamison.[12] Other reviews covering secondary metabolites from other genus of dinoflagellates have been published previously.[12–20]

2.09.2 *Amphidinium* sp.

2.09.2.1 Amphidinolides and their Related Compounds

2.09.2.1.1 Culture of Amphidinium *sp. and isolation of amphidinolides*

In our research for bioactive substances from marine organisms, we started the search for secondary metabolites from symbiotic marine microorganisms in 1986.[21,22] When the extracts of a number of microorganisms were subjected to several biological screens, the extracts of symbiotic dinoflagellates *Amphidinium* sp. collected at Okinawa, Japan, were found to exhibit potent cytotoxic activity (70–90% inhibition at 3 μg ml^{-1}) against murine lymphoma L1210 cells and human epidermoid carcinoma KB cells. The dinoflagellates *Amphidinium* sp. were isolated from the inner tissue of acoel flatworms *Amphiscolops* sp. living on algae or seaweeds in Okinawa coral reefs.[23]

Large-scale cultures of the dinoflagellates *Amphidinium* sp. in our laboratory have been performed using 3-l flat-bottomed glass flasks containing 2 l of seawater medium enriched with 1–3% Provasoli's Erd-Schreiber (ES) supplements[24,25] ($NaNO_3$: 300 mg, sodium glycerophosphate: 50 mg, FeEDTA: 2.5 mg, metal solution (including BO_3^{3-}, Mn^{2+}, Zn^{2+}, and Co^{2+}): 25 ml, vitamin B_{12}: 10 mg, vitamin B_1: 0.5 mg, biotin: 5 mg, TRIS: 500 mg in 100 ml distilled water, pH 7.8). Static incubation with 8000 luces of illumination in a cycle of 16 h of light and 8 h of darkness was carried out for 2 weeks at 25 °C. The cultures were harvested by removal of the supernatant by suction and then centrifugation to yield algal cells ranging from 0.1 to 0.3 g l^{-1} medium. Recently, 0.8-l glass tall dishes containing 500 ml of seawater medium have been used in place of 3-l glass flasks. The latter method was easier to handle, and the yields of the cells were improved to 0.7–1.0 g l^{-1} medium.

Harvested cells were extracted with methanol–toluene followed by partitioning between toluene and water. The toluene-soluble fractions were subjected to a systematic separation using silica gel column chromatography and C_{18} high performance liquid chromatography (HPLC). In total, 39 cytotoxic macrolides, designated amphidinolides A (**1**), B (**2a**), B4–B7 (**2d–2g**), C (**3a**), C2 (**3b**), D–F (**4–6**), G (**7a**), G2–G3 (**7b–7c**), H (**8a**), H2–H5 (**8b–8e**), J–S (**9–18**), T1–T5 (**19a–19e**), and U–Y (**20–24**), have been isolated so far (**Figure 1**).[10] From *Amphidinium* sp. (Y-5 strain, 25 μm in length and 20 μm in width) separated from the inside of a cell of a flatworm *Amphiscolops* sp. (0.5 mm in length and 0.2 mm in width, green in color) collected off Chatan beach, Okinawa, 15 macrolides, amphidinolides A–E (**1–5**), J–S (**9–18**), and V (**21**), and a linear polyketide, amphidinin A (**25**), have been isolated. Isolation yields of the amphidinolides are shown in **Table 1**. Furthermore, a variety of amphidinolides have been isolated from six strains (Y-42, Y-56, Y-71, Y-72, Y-100, and HYA002) of *Amphidinium* sp. obtained from different collections of flatworms *Amphiscolops* sp. as follows: (1) amphidinolides G–L (**7–11**) and amphidinolactones A (**27**) and B (**28**) from Y-25 strain separated from *Amphiscolops vreviviridis* collected off Sunabe beach, Okinawa, (2) amphidinolide F (**6**) was isolated together with amphidinolides B (**2a**) and C (**3a**) from a flatworm *Amphiscolops magniviridis* collected at Zampa, Okinawa, (3) amphidinolides G2 (**7b**), G3 (**7c**), H2–H5 (**8b–8e**), W (**22**), X (**23**), and Y (**24**) together with amphidinolides G (**7a**) and H (**8a**) from Y-42 strain separated from the Sunabe collection, (4) amphidinolides T1 (**19a**), T3–T5 (**19c–19e**), U (**20**), and a linear polyketide amphidinin B (**26**) together with amphidinolides B (**2a**) and C (**3a**) from Y-56 strain separated from the Zampa collection, (5) amphidinolides T1 (**19a**) and T2 (**19b**) together with amphidinolides B (**2a**), C (**3a**), and F (**6**) from Y-71 strain separated from the Sunabe collection, (6) amphidinolides G (**7a**) and H (**8a**) from Y-72 strain separated from the Zampa collection, (7) amphidinolides B4 (**2d**) and B5 (**2e**) from Y-100 strain separated from the Ma'eda Cape collection, and (8) amphidinolides B6 (**2f**) and B7 (**2g**) from HYA002 strain separated from the Sunabe collection. Recently, iriomoteolides 1a (**29a**), 1b (**29b**), 1c (**29c**), and 3a (**30**) were isolated from a benthic dinoflagellate *Amphidinium* sp. (HYA024), separated from benthic sea sand collected off Iriomote Island, Okinawa. Amphidinolides B1–B3 (**2a–2c**) and caribenolide I (**31**) were isolated from a free-swimming dinoflagellate *Amphidinium operculatum* ver nov *Gibbosum* (S1-36-5) isolated from the water at Brewers Bay, St. Thomas, US Virgin Islands by Shimizu and coworkers.

2.09.2.1.2 Structural features, biological activity, and total syntheses

Amphidinolides have a variety of backbone skeletons and different sizes of macrolactone rings (12- to 29-membered rings), and more than half of the amphidinolides have odd-numbered macrolactone rings (**Table 2**). Most of the amphidinolides contain a vicinally located one-carbon branch and *exo*-methylene unit, and some of them exhibit potent cytotoxicity and antitumor activity. Due to their diverse functionality, stereochemical complexity, low natural abundance, and encouraging bioactivity, amphidinolides have attracted great interest as challenging targets for total synthesis. Synthetic study of amphidinolides is also important to establish the structure of these macrolides in case the amount of natural compound was very limited.

In addition to the numerous efforts for total synthesis of these macrolides, 15 amphidinolides have been synthesized to date. The total synthesis of amphidinolides J (**9**), K (**10**), and P (**15**) have been reported by Professor Williams and colleagues.[26–28] Professor Fürstner's group accomplished the total synthesis of amphidinolides B1 (**2a**), B4 (**2d**), H (**8a**), H2 (**8b**), G (**9a**), T1 (**19a**), T3–T5 (**19c–19e**), V (**21**), X (**23**), and Y (**24**).[29–37] Professor Ghosh and colleagues achieved the total synthesis of amphidinolides T1 (**19a**) and W (**22**).[38–40] The total synthesis of amphidinolides A (**1**) and P (**15**) has been accomplished by Professor Trost's group.[41–45] Professor Dai and colleagues completed the total synthesis of amphidinolides

1

2a: $R_1 = CH_3$, R_2, R_4, R_6, $R_8 = OH$, R_3, R_5, R_7, $R_9 = H$, $\Delta^{2,3}$, $\Delta^{6,7}$
2b: $R_1 = CH_3$, R_2, R_3, R_6, $R_8 = OH$, R_4, R_5, R_7, $R_9 = H$, $\Delta^{2,3}$, $\Delta^{6,7}$
2c: $R_1 = CH_3$, R_2, R_4, R_6, $R_7 = OH$, R_3, R_5, R_8, $R_9 = H$, $\Delta^{2,3}$, $\Delta^{6,7}$
2d: $R_1 = CH_3$, R_2, R_3, R_5, R_7, $R_9 = H$, R_4, R_6, $R_8 = OH$, $\Delta^{2,3}$, $\Delta^{6,7}$
2e: R_1, R_4, R_5, R_7, $R_9 = H$, $R_2 = CH_3$, R_3, R_6, $R_8 = OH$, $\Delta^{2,3}$, $\Delta^{6,7}$
2f: $R_1 = CH_3$, R_2, R_3, R_5, $R_7 = H$, R_4, R_6, R_8, $R_9 = OH$, $\Delta^{2,3}$, $\Delta^{6,7}$
2g: $R_1 = CH_3$, R_2, R_3, R_5, $R_7 = H$, R_4, R_6, $R_8 = OH$, $\Delta^{2,3}$, 6,7-dihydro
4: $R_1 = CH_3$, R_2, R_4, R_5, $R_8 = OH$, R_3, R_6, R_7, $R_9 = H$, $\Delta^{2,3}$, $\Delta^{6,7}$

3a: R = OH
3b: R = OAc

5

6

7a: R_1, $R_4 = H$, $R_2 = CH_3$, $R_3 = OH$, $\Delta^{6,7}$
7b: $R_1 = CH_3$, R_2, $R_3 = H$, $R_4 = OH$, $\Delta^{6,7}$
7c: R_1, $R_4 = H$, $R_2 = CH_3$, $R_3 = OH$, 6,7-dihydro

8a: R_1, R_4, R_5, $R_7 = H$, $R_2 = CH_3$, R_3, R_6, $R_8 = OH$, $\Delta^{6,7}$
8b: $R_1 = CH_3$, R_2, R_4, R_6, $R_8 = H$, R_3, R_5, $R_7 = OH$, $\Delta^{6,7}$
8c: R_1, R_4, $R_8 = H$, $R_2 = CH_3$, R_3, $R_7 = OH$, $\Delta^{6,7}$
8d: R_1, R_4, $R_7 = H$, $R_2 = CH_3$, R_3, $R_8 = OH$, 6,7-dihydro
8e: $R_1 = CH_3$, R_2, R_3, $R_7 = H$, R_4, $R_8 = OH$, 6,7-dihydro

9

Figure 1 (Continued)

10

11

12

13

14: R = O, **15**: R = CH_2

16

17

18

19a

19b

Figure 1 (Continued)

19c: R_1, R_4 = H, R_2 = OH, R_3 = CH_3
19d: R_1 = OH, R_2, R_4 = H, R_3 = CH_3
19e: R_1 = OH, R_2, R_3 = H, R_4 = CH_3

20

21

22

23

24

25

26

27

28

Figure 1 (Continued)

Figure 1 Structures of amphidinolides A (**1**), B (**2a**) and B2–B7 (**2b–2g**), C (**3a**) and C2 (**3b**), D–F (**4–6**), G (**7a**) and G2–G3 (**7b–7c**), H (**8a**) and H2–H5 (**8b–8e**), J–S (**9–18**), T1–T5 (**19a–19e**), and U–Y (**20–23**); amphidinins A (**25**) and B (**26**); amphidinolactones A (**27**) and B (**28**); iriomoteolides 1a (**29a**), 1b (**29b**), 1c (**29c**), and 3a (**30**); and caribenolide I (**31**).

X (**23**) and Y (**24**).[46,47] Professor Zhao's group reported the total synthesis of amphidinolide T3 (**19c**).[48] Professor Lee and colleagues completed the total synthesis of amphidinolides E (**5**) and K (**10**).[49,50] Professor Jamison's group completed the total synthesis of amphidinolides T1 (**19a**) and T4 (**19d**).[51,52] Professor Roush and colleagues accomplished the total synthesis of amphidinolide E (**5**).[53,54] Professors Vilarrasa and Urpí and colleague reported the total synthesis of amphidinolide X (**23**).[55] Professors Cossy and Meyer and colleague completed the total synthesis of amphidinolide J (**9**).[56] Professor Carter's group completed the total synthesis of amphidinolides B1 (**19a**) and B2 (**19d**).[57] Professor Yadav and coworker accomplished the total synthesis of amphidinolide T1 (**19a**).[58] Professor Nicolaou's group reported significant progress toward the synthesis of amphidinolide N (**13**) and caribenolide I (**31**).[59,60]

2.09.2.1.3 Amphidinolide A

Amphidinolide A is a 20-membered macrolide possessing a dienoate chromophore, three *exo*-methylenes, two 1,2-diols, three branched methyl groups, and an epoxide, isolated from *Amphidinium* sp. (strain Y-5), which was separated from the marine acoel flatworm *Amphiscolops* sp. collected off Chatan beach, Okinawa. Originally, the stereochemistries of nine chiral centers in amphidinolide A (**1**) were proposed to be (**32**) on the basis of nuclear Overhauser enhancement and exchange spectroscopy (NOESY) correlations and *J* values.[61,62] However, the total synthesis of (**32**) and comparison of the nuclear magnetic resonance (NMR) data with those reported for natural amphidinolide

Table 1 Isolation yields for amphidinolides A–H (1–8) and J–Y (9–24); amphidinins A (25) and B (26); amphidinolactones A (27) and B (28); iriomoteolides 1a (29a), 1b (29b), 1c (29c), and 3a (30); and caribenolide I (31)

Isolation yields (10^{-4}%)											
Strain number[a]											
Compound	*Y–5*	*Y–25*	*Y–26*	*Y–42*	*Y–56*	*Y–71*	*Y–72*	*Y–100*	*S1–36–5*	*HYA002*	*HYA024*
1	20										
2a	10		0.8			17			1400[b]		
2b									240[b]		
2c									76[b]		
2d								8			
2e								2			
2f										30	
2g										30	
3a	15		0.3		9	12					
3b						1.5					
4	4										
5	4										
6			0.1		6						
7a		20		8			46				
8a		17		7			82				
9	60										
10	0.3										
11		2									
12	4										
13	9										
14	1										
15	2										
16	0.5										
17	5										
18	1										
19a					50	9.2					
20					2						
21	0.5										
22				90							
23[c]				4							
24				7							
25[d]	0.6										
26[d]					2						
27		0.14									
28		0.11									
29a											280[b]
29b											70[b]
29c											20[b]
30											150[b]
31									260[b]		

[a] *Amphidinium sp*.
[b] Dry weight.
[c] Macrodiolide.
[d] Linear polyketide.

A revealed that the proposed stereostructure was incorrect.[41,43,63] Reexamination of the ^{1}H and ^{13}C NMR data indicated that the correct stereostructure of amphidinolide A could be either (1) or an 11,12-epimer of (1). From a comparison of NMR data, optical rotations, and retention times from C_{18} HPLC of natural amphidinolide A, the synthetic (1) and 11,12-epimer of (1) revealed that (1) was the correct stereostructure of amphidinolide A (8*R*, 9*R*, 11*S*, 12*S*, 18*R*, 19*S*, 20*R*, 21*S*, and 22*S*).[44,64]

Table 2 Lactone ring size, stereochemistry, synthesis, and cytotoxicity data for amphidinolides A–H (**1**–**8**) and J–Y (**9**–**24**); amphidinins A (**25**) and B (**26**); amphidinolactones A (**27**) and B (**28**); iriomoteolides 1a (**29a**), 1b (**29b**), 1c (**29c**), and 3a (**30**); and caribenolide I (**31**)

				cytotoxicity (IC$_{50}$[a] *μg ml^{-1}*)				
Compound	*Lactone size*	*Stereochemistry*	*Synthesis*	*L1210*[b]	*KB*[c]	*HCT*	*DG–75*	*Raji*
1	20	Absolute	Total synthesis	2.0	5.7			
2a	26	Absolute	Total synthesis	0.000 14	0.0042	0.122		
2b	26	Unknown	Analog			7.5		
2c	26	Relative	No			0.206		
2d	26	Absolute	Total synthesis	0.000 12	0.001			
2e	26	Absolute	No	0.001 4	0.004			
2f	26	Absolute	No	0.005 8	0.004 6			
2g	26	Absolute	No	0.000 14	0.004 2			
3a	25	Absolute	Fragment	0.005 8	0.004 6			
3b	25	Absolute	No	0.8	3			
4	26	Relative	No	0.019	0.08			
5	19	Absolute	Total synthesis	2.0	10			
6	25	Relative	Fragment	1.5	3.2			
7a	27	Absolute	Total synthesis	0.005 4	0.004 6			
7b	27	Relative	No	0.3	0.8			
7c	27	Relative	No	0.72	1.3			
8a	26	Absolute	Total synthesis	0.000 48	0.000 52			
8b	26	Absolute	Total synthesis	0.06	0.06			
8c	26	Relative	No	0.002	0.022			
8d	26	Relative	No	0.18	0.23			
8e	26	Relative	No	0.2	0.62			
9	15	Absolute	Total synthesis	2.7	3.9			
10	19	Absolute	Total synthesis	1.65	2.9			
11	27	Unknown	Fragment	0.092	0.1			
12	29	Unknown	No	1.1	0.44			
13	26	Unknown	Analog	0.000 05	0.000 06			
14	15	Absolute	No	1.7	3.6			
15	15	Absolute	Total synthesis	1.6	5.8			
16	12	Absolute	No	6.4	>10			
17	15	Absolute	No	1.4	0.67			
18	16	Absolute	No	4.0	6.5			
19a	19	Absolute	Total synthesis	18	>20			
19b	19	Absolute	No	10	11.5			
19c	19	Absolute	Total synthesis	7.0	10			
19d	19	Absolute	Total synthesis	11	18			
19e	19	Absolute	Total synthesis	15	20			
20	20	Unknown	No	12	20			
21	14	Absolute	Total synthesis	3.2	7			
22	12	Absolute	Total synthesis	3.9	>10			
23	16[d]	Absolute	Total synthesis	0.6	7.5			
24	17	Absolute	Total synthesis	0.8	8.0			
25	[e]	Unknown	No	3.6	3.0			
26	[e]	Absolute	No	2	>10			
27	13	Absolute	Total synthesis	8.0	>10			
28	26	Unknown	No	3.3	5.3			
29a	20	Absolute	Fragment				0.002	0.003
29b	20	Absolute	No				0.9	
29c	20	Unknown	No				0.002	0.004
30	15	Absolute	No				0.08	0.05
31	26	Unknown	Analog			0.001		

[a] 50% inhibition concentration.
[b] Murine lymphoma cell.
[c] Human epidermoid carcinoma cells.
[d] Macrodiolide.
[e] Linear polyketide.

2.09.2.1.4 Amphidinolides B, G, H, and L and their related macrolides

Amphidinolide B (**2a**) is a 26-membered macrolide isolated from *Amphidinium* sp. (Y-5 strain) with an allyl epoxide and an *s-cis* diene moiety and shows potent cytotoxicity (IC_{50} 0.000 14 and 0.004 2 μg ml^{-1} against L1210 and KB cells, respectively).[65,66]

Shimizu and coworkers isolated three amphidinolide B (**2a**) congeners, amphidinolides B1 (**2a**), B2 (**2b**), and B3 (**2c**) from a free-swimming dinoflagellate *A. operculatum* ver nov *Gibbosum.* The relative stereochemistry of nine chiral centers in amphidinolide B1 (**2a**) was determined by X-ray analysis.[67] Amphidinolide B1 (**2a**) was shown to be identical with amphidinolide B (**2a**) by comparison of HPLC retention times, ^{1}H NMR data, and optical rotations. The absolute stereochemistry of amphidinolide B (**2a**) was assigned as 8*S*, 9*S*, 11*R*, 16*R*, 18*S*, 21*R*, 22*S*, 23*R*, and 25*S* on the basis of chiral HPLC analyses of the C-22–C-26 segment obtained by oxidation with $NaIO_4$, reduction with $NaBH_4$, and acetylation followed by HPLC separation of amphidinolide B (**2a**), and the synthetic C-22–C-26 segments prepared from (2*S*,4*S*)- and (2*R*,4*R*)-pentanediol.[68] Shimizu and coworkers reported that amphidinolides B2 (**2b**) and B3 (**2c**) were C-18 and C-22 epimers of amphidinolide B (**2a**), respectively. The ^{1}H NMR spectra of amphidinolide B2 (**2b**) and D (**4**),[66] the latter of which was assigned as a C-21 epimer of amphidinolide B (**2a**) by us, were quite similar to each other, indicating that the two compounds were identical. Recently, the proposed structure of amphidinolide B2 (**2b**) has been synthesized by Carter and coworkers; however, the NMR data of synthetic amphidinolide B2 (**2b**) did not agree with those reported for natural amphidinolide B2.[57] Amphidinolide B (**2a**) causes a concentration-dependent increase in the contractile force of skeletal muscle skinned fibers.[69]

Amphidinolides G (**7a**) and H (**8a**), 27- and 26-membered macrolides, respectively, were isolated from the extracts of the cultured cells of the Y-25 strain separated from the marine flatworm *A. vreviviridis* collected off Sunabe beach, Okinawa.[70] These macrolides are regioisomers at C-26 and C-25, respectively, and are also different in the position of a hydroxy group (C-16 and C-26, respectively). Amphidinolide H (**8a**) was crystallized from hexane–benzene as colorless needles. The relative stereochemistry of nine chiral centers in amphidinolide H (**8a**) was obtained from a single crystal X-ray diffraction analysis. The X-ray structures of amphidinolides H (**8a**) and B (**2a**) were close to each other. Both conformations had an intramolecular hydrogen bond (amphidinolide B (**2a**), 2.02 Å) between the hydroxy group at C-21 and the epoxide oxygen atom, and their macrocyclic skeletons overlapped well with each other. The absolute stereochemistry of amphidinolide H (**8a**) was concluded to be 8*S*, 9*S*, 11*R*, 16*S*, 18*S*, 21*R*, 22*S*, 23*R*, and 25*R* on the basis of comparison of the ^{1}H NMR data of the *tris*-(*S*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) ester of the C-22–C-26 segment derived from natural amphidinolide H (**8a**) by a four-step conversion (reduction with $NaBH_4$, oxidation with $NaIO_4$, reduction with $NaBH_4$, MTPA esterification, and HPLC separation) with those of *tris*-(*S*)- and *tris*-(*R*)-MTPA esters of the C-22–C-26 segment synthesized from methyl (2*S*)-3-hydroxy-2-methylpropionate.[71] On the other hand, treatment of amphidinolide H with K_2CO_3 in EtOH at 4 °C for 18 h yielded a 1:1 mixture of amphidinolides G (**7a**) and H (**8a**). All spectral data of amphidinolide G (**7a**) isolated from this mixture were identical to those of the natural product. Thus, the absolute configurations of amphidinolide G (**7a**) were concluded to be the same as those of amphidinolide H (**8a**).[71] Solution conformations of amphidinolide H (**8a**) in $CDCl_3$ and DMSO-d_6 were investigated on the basis of NMR data, distance geometry calculation, and restrained energy minimization. Three-dimensional conformations in $CDCl_3$ were suggested to be close to the X-ray structure of amphidinolide H (**8a**), whereas those in DMSO-d_6 were indicated to be different from those in both $CDCl_3$ and the X-ray structure.[72]

Amphidinolide H-congeners – amphidinolides H2–H5 (**8b–8e**) – and two amphidinolide G-congeners – amphidinolides G2 (**7b**) and G3 (**7c**) – were obtained from the extracts of the cultured cells of the Y-42 strain isolated from the marine acoel flatworm *Amphiscolops* sp. collected off Sunabe beach, Okinawa.[73] The structures of these macrolides were deduced from the detailed analyses of spectroscopic data including *J*-based configuration analysis as well as distance geometry calculation based on NOESY data. Amphidinolide H2 (**8b**) was originally assigned as 16,18-epimer of amphidinolide H (**8a**). However, the total synthesis of 16,18-epimer, 16,18,21,22-epimer, and 18,21,22-epimer of amphidinolide H (**8a**) revealed that the natural amphidinolide H2 (**8b**) is 18,21,22-epimer of amphidinolide H.[35] The structure of amphidinolide H3 (**8c**) was assigned as 16,22-epimer of amphidinolide H (**8a**), whereas amphidinolides H4 (**8d**) and H5 (**8e**) were assigned as the 6,7-dihydro form of amphidinolide H (**8a**) and 16,18-epimer of amphidinolide H (**8a**), respectively. Amphidinolides G2 (**7b**) and G3 (**7c**) were assigned as 16,18-epimer and 6,7-dihydro form of amphidinolide G (**7a**), respectively.

The cytotoxicity of 5 derivatives of amphidinolide H (**8a**) and 6 amphidinolides B- and H-related macrolides was examined (**Table 3**). Amphidinolide H4 (**8d**), the 6,7-dihydro form of amphidinolide H (**8a**) (0.18 and 0.23 $\mu g\ ml^{-1}$ against L1210 and KB cells, respectively), was 300 and 400 times less potent than amphidinolides H (**8a**). An epoxide ring-opened form of amphidinolides H (**33**) showed no cytotoxicity at 3 $\mu g\ ml^{-1}$. Reduction of the ketone group at C-20 (**34 and 35**) resulted in a remarkable reduction of the activity. Cytotoxicity of the 26-*O*-(α-methoxy)isopropyl derivative (**36**) against L1210 and KB cells (IC_{50} 0.002 1 and 0.006 4 $\mu g\ ml^{-1}$, respectively) was 4–12 times less potent than that of amphidinolide H (**8a**). Oxidation of the *s-cis* diene moiety as peroxide (**37**) led to a 400-fold decrease of cytotoxicity (IC_{50} against L1210 and KB cells, 0.2 and 0.26 $\mu g\ ml^{-1}$, respectively). These results indicate that the presence of an allyl epoxide, an *s-cis* diene moiety, and the ketone at C-20 is important for the cytotoxicity of amphidinolide H-type macrolides.[73] Amphidinolide H (**8a**) exhibits antitumor activity against murine leukemia P388 mice (T/C: 140% at a dose of 0.2 $mg\ kg^{-1}$).

The molecular target of amphidinolide H (**8a**), which shows potent cytotoxicity against L1210 and KB cells *in vitro* (IC_{50} 0.000 48 and 0.000 52 $\mu g\ ml^{-1}$, respectively), has been investigated. Amphidinolide H (**8a**) induced multinucleated cells by disrupting actin organization in the cells and the hyperpolymerization of purified actin into filaments of apparently normal morphology *in vitro*. Amphidinolide H covalently bound on actin, and the binding site was determined as Tyr200 of actin subdomain 4 by mass spectrometry and the halo assay using the yeast harboring site-directed mutagenized actins. Time-lapse analyses showed that amphidinolide H (**8a**) stimulated the formation of small actin patches, followed by F-actin rearrangement into aggregates through the retraction of actin fibers. These results indicate that amphidinolide H (**8a**) is a novel inhibitor that covalently binds on actin.[74,75]

Table 3 Cytotoxicity of amphidinolides B (**2a**), D (**4**), G (**7a**), H (**8a**), G2 (**7b**), G3 (**7c**), H2 (**8b**), H3 (**8c**), H4 (**8d**), and H5 (**8e**) and five derivatives of amphidinolide H (**33–37**)

	IC_{50} $\mu g\ ml^{-1}$	
Compounds	*L1210*	*KB*
2a	0.000 14	0.004 2
4	0.019	0.08
7a	0.005 4	0.004 6
7b	0.3	0.8
7c	0.72	1.3
8a	0.000 48	0.000 52
33	>3	>3
34	0.3	0.2
35	0.2	0.2
36	0.002 1	0.006 4
37	0.2	0.26

33

34 : 20α, **35** : 20β

36

37

Amphidinolide L (**11**), a 26-membered macrolide, was isolated from the extracts of the cultured cells of the Y-25 strain.[76] Amphidinolide L (**11**) possesses a tetrahydropyran moiety, which corresponds to a 20-dihydro-21-dehydro derivative of amphidinolide G (**7a**). The absolute configurations at C-21, C-22, C-23, and C-25 in amphidinolide L (**11**) were assigned by comparison of the ^{1}H NMR data of the C-21–C-26 segment derived from a natural specimen by a four-step degradation (reduction with $NaBH_4$, oxidation with $NaIO_4$, reduction with $NaBH_4$, acetylation, and HPLC separation) with those of the C-21–C-26 segment synthesized from methyl (2*S*)-3-hydroxy-2-methylpropionate.

Synthetic studies of amphidinolides B and H were carried out by some groups including our group. Total synthesis of amphidinolide H (**8a**) has been completed by Professor Fürstner's group using 1,4-*anti*-aldol reaction, the modified Stille coupling under chloride-free conditions, and the ring-closing metathesis by Grubbs catalyst as key steps (**Scheme 1**).[34,35]

Two new cytotoxic 26-membered macrolides, amphidinolides B4 (**2d**) and B5 (**2e**), have been generated from the marine dinoflagellate *Amphidinium* sp. (strain Y-100), which was isolated from the marine acoel flatworm *Amphiscolops* sp. collected off Ma'eda Cape, Okinawa.[77] *Amphidinium* sp. (strain Y-100) was cultured in the seawater medium with 1% $NaH^{13}CO_3$ to give ^{13}C-enriched samples of amphidinolides B4 (**2d**) and B5 (**2e**). Amphidinolide B4 (**2d**), showed a pseudomolecular ion peak at m/z 569.5 $(M + Na)^+$ in the electrospray ionization mass specrometry (ESIMS), and the ^{13}C-enrichment was estimated as 32% by the pattern of the pseudomolecular ion peak. Detailed analyses of the heteronuclear multiple quantum coherence (HMQC) and incredible natural abundance double quantum transfer experiment (INADEQUATE) spectra of amphidinolide B4 (**2d**) established the carbon chain from C-1–C-26 and six C1 branches including five methyl groups and an *exo*-methylene. Heteronuclear multiple bond correlation (HMBC) correlations revealed that C-25 is involved in an ester linkage with C-1; thus, the gross structure of amphidinolide B4 (**2d**) was assigned. Amphidinolide B5 (**2e**) was demonstrated to have the same molecular formula as amphidinolide B4 (**2d**) by high resolution electrospray ionization mass spectrometry (HRESIMS). Profiles of the ^{1}H and ^{13}C NMR spectra of amphidinolide B5 (**2e**) were reminiscent of those of amphidinolide B4 (**2d**). The gross structure of amphidinolide B5 (**2e**) was elucidated to be the same as that of amphidinolide B4 (**2d**) from the analyses of the HMQC, HMBC, and INADEQUATE spectra. Comparison of the ^{13}C NMR data and CD spectra of amphidinolides B4 (**2d**) and B5 (**2e**) with those of amphidinolides B (**2a**), H (**8a**), H2 (**8b**), and H3 (**8c**) revealed that amphidinolides B4 (**2d**) and B5 (**2e**) were the 16-deoxy and 16-deoxy-16,18-epi forms of amphidinolide B (**2a**), respectively. Amphidinolides B4 (**2d**) and B5 (**2e**) exhibited potent cytotoxicity against L1210 cells (IC_{50} 0.000 12 and 0.001 4 $\mu g\ ml^{-1}$, respectively) and KB cells (IC_{50} 0.001 and 0.004 $\mu g\ ml^{-1}$, respectively).

Scheme 1 Retrosynthetic analysis of amphidinolide H (8a) by Professor Fürstner.

Amphidinolides B6 (2f) and B7 (2g) have been isolated from a marine dinoflagellate *Amphidinium* sp. (strain HYA002), which was isolated from the marine acoel flatworm *Amphiscolops* sp. collected off Sunabe beach, Okinawa.[78] The planer structures of amphidinolides B6 (2f) and B7 (2g) were elucidated from the detailed analyses of 2D NMR spectra. Relative stereochemistries of these macrolides were deduced from NOESY data

and comparison of ^1H and ^{13}C chemical shifts and ^1H–^1H coupling constants with those of amphidinolides H4 (**8d**) and H5 (**8e**). The CD spectra for these macrolides matched those for amphidinolide H (**8a**). Therefore, the absolute configuration of amphidinolides B6 (**2f**) and B7 (**2g**) were proposed to be 2*S*, 3*S*, 8*S*, 9*S*, 11*R*, 16*S*, 18*S*, 21*R*, 22*S*, 23*R*, and 25*S*, and 8*S*, 9*S*, 11*R*, 16*S*, 18*S*, 21*R*, 22*S*, 23*R*, and 25*S*, respectively. Amphidinolide B6 (**2f**) is the first oxygenated congener at C-3 in the amphidinolides B- and H-type macrolides, whereas amphidinolide B7 (**2g**) is a 6,7-dihydro form of amphidinolide B4 (**2d**).

2f

2g

Amphidinolactone B (**28**), a new 26-membered macrolide possessing a tetrahydrofuran ring, a keto carbonyl, four hydroxy groups, and six branched methyl groups, was isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-25).[79] The gross structure and partial relative stereochemistry of amphidinolactone B (**28**) was elucidated from 2D NMR data and deuterium-induced shifts of oxymethine carbons measured in C_6D_6/CD_3OD (95:5) and C_6D_6/CD_3OH (95:5), respectively. Amphidinolactone B (**28**) has the same carbon skeleton as amphidinolide B-type macrolide. Amphidinolactone B (**28**) showed cytotoxicity against L1210 and KB cells (IC_{50} 3.3 and 5.3 μg ml^{-1}, respectively) *in vitro*.

28

2.09.2.1.5 Amphidinolides C, F, and U and their related macrolides

Amphidinolide C (**3a**) is a 25-membered macrolide isolated from *Amphidinium* sp. (strain Y-5), possessing an *s-cis* and an *s-trans* dienes, two *exo*-methylenes, two tetrahydrofuran rings, a 1,2-diol, and four branched methyl groups.[80] The relative stereochemistry of the C-1–C-8 and C-20–C-23 portions has been elucidated by NOESY correlations of amphidinolide C (**3a**) and its 7,8-*O*-isopropylidene derivative. Application of the *J*-based configuration analysis revealed the *erythro*-relation for the C-12–C-13 bond and the *threo*-relation for the C-23–C-24 bond. The absolute configurations of two oxymethine carbons at C-13 and C-29 were determined by the modified Mosher's method. The C-1–C-7 segment was obtained by degradation of amphidinolide C (**3a**). The ^1H NMR data of the *bis*-(*S*)-MTPA ester derived from a natural specimen were identical with those of the synthetic *bis*-(*S*)-MTPA ester. Therefore, the absolute configurations at C-3, C-4, and C-6 were established to be *S*, *R*, and *R*, respectively. The absolute configurations at C-7, C-8, and C-24 were elucidated by application of the modified Mosher's method for linear methyl ester. Furthermore, from comparison of the ^1H NMR chemical shifts of MTPA esters of each diastereomer of the C-1–C-10 and C-17–C-29 segments with those of linear methyl ester, the absolute configurations at C-7, C-8, C-20, C-23, and C-24 in amphidinolide C (**3a**) were confirmed to be all *R*. To determine the absolute configuration at C-16, a Baeyer–Villiger degradation was applied for amphidinolide C (**3a**) to afford a 1,3-butanediol corresponding to the C-16–C-18 segment of amphidinolide C (**3a**). The ^1H NMR data of *bis*-(*R*)-MTPA ester of the C-16–C-18 segment derived from a natural specimen were identical with those of authentic *bis*-(*R*)-MTPA ester of (*S*)-1,3-butanediol, indicating that the absolute configuration at C-16 of amphidinolide C (**3a**) was *S*. Therefore, the absolute configurations at 12 chiral centers in amphidinolide C (**3a**) were determined to be 3*S*, 4*R*, 6*R*, 7*R*, 8*R*, 12*R*, 13*S*, 16*S*, 20*R*, 23*R*, 24*R*, and 29*S*.[81,82]

Amphidinolide F (**6**) is an analog of amphidinolide C (**3a**), isolated from *Amphidinium* sp. (strain Y-26) obtained from a flatworm *A. magniviridis* collected at Zampa, Okinawa.[83] The structural difference between amphidinolides F (**6**) and C (**3a**) was found in the length of the alkyl side chain. Since the ^{1}H and ^{13}C chemical shifts of amphidinolide F (**6**) were close to those of amphidinolide C (**3a**), the relative stereochemistry of 11 chiral centers in amphidinolide F (**6**) was suggested to be the same as that of amphidinolide C (**3a**).

Amphidinolide U (**20**) is a 20-membered macrolide possessing a tetrahydrofuran ring, two *exo*-methylenes, three branched methyl groups, two ketones, two hydroxy groups, and a C_{10} linear side chain, isolated from *Amphidinium* sp. (strain Y-56) obtained from a flatworm *Amphiscolops* sp. collected at Cape Zanpa, Okinawa.[84] The absolute configurations at both C-8 and C-24 were assigned as *S* on the basis of the modified Mosher's method. The gross structure of the C-9–C-29 unit in amphidinolide U (**20**) corresponds to that of C-14–C-34 in amphidinolide C (**3a**), whereas the carbon skeleton of the C-1–C-8 unit in amphidinolide U (**20**) is close to that of C-1–C-8 in amphidinolide A (**1**). This observation suggests that amphidinolide U (**20**) may be biogenetically related to amphidinolides C (**3a**) and A (**1**).

Amphidinolide C2 (**3b**) has been purified from the marine dinoflagellate *Amphidinium* sp. (strain Y-71), which was isolated from the marine acoel flatworm *Amphiscolops* sp. collected off Sunabe beach, Okinawa.[85] The ^{1}H–^{1}H COSY and total correlation spectroscopy (TOCSY) spectra revealed that the gross structure of amphidinolide C2 (**3b**) was 29-*O*-acetyl form of amphidinolide C (**3a**). Amphidinolide C2 (**3b**) was converted into its 7,8,13-*O*-triacetate, the spectroscopic data of which were identical with those of the tetraacetate of amphidinolide C (**3a**). Therefore, the absolute configurations at all 12 chiral centers in amphidinolide C2 (**3b**) have been elucidated to be 3*S*, 4*R*, 6*R*, 7*R*, 8*R*, 12*R*, 13*S*, 16*S*, 20*R*, 23*R*, 24*R*, and 29*S*.

3a: R = OH
3b: R = OAc

6

20

2.09.2.1.6 Amphidinolide E

Amphidinolide E (**5**), a 19-membered macrolide possessing a diene chromophore, two *exo*-methylenes, a tetrahydrofuran ring, a 1,2-diol, and two branched methyl groups, was isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-5).[86] The relative stereochemistry of C-7 and C-8 was elucidated to be *threo* on the basis of NOESY data of the 7,8-isopropylidene derivative of amphidinolide E (**5**), whereas the relative stereochemistry of C-13 and C-16–C-19 was assigned as H-13/H-16-*syn*, C-16/C-17-*threo*, C-17/C-18-*threo*, and C-18/C-19-*erythro* by a combination of the *J*-based configuration analysis of amphidinolide E (**5**) and the detailed analyses of NOESY data of the 7,8,17,18-di-*O*-isopropylidene derivative of amphidinolide E (**5**). The absolute stereochemistry was determined to be 7*R* and 8*R* by application of the exciton chirality method using 7,8-*bis*-*O*-*p*-methoxycinnamate. The 17*R*-configuration was assigned by the modified Mosher's method for a hydroxy group at C-17 in amphidinolide E (**5**). To elucidate the absolute configurations at the remaining chiral centers, a five-step oxidative degradation of amphidinolide E (**5**) was performed. The absolute configuration at C-2 was elucidated to be *R* on the basis of chemical shift differences and signal patterns of the two geminal protons at C-1

of the MTPA esters of C-1–C-7 segment, whereas the absolute configurations at C-13 and C-16 were elucidated by comparison of NMR data of MTPA esters of C-8–C-17 segment with those of their corresponding synthetic enantiomers. Therefore, the absolute configurations at eight chiral centers in amphidinolide E (**5**) were assigned as 2*R*, 7*R*, 8*R*, 13*S*, 16*S*, 17*R*, 18*R*, and 19*R*.[87] Total syntheses of amphidinolide E (**5**) have been achieved by two groups to prove the proposed structure of amphidinolide E (**5**) (**Schemes 2** and **3**).[49,53,54]

Scheme 2 Retrosynthetic analysis of amphidinolide E (**5**) by Professor Lee.

Scheme 3 Retrosynthetic analysis of amphidinolide E (**5**) by Professor Roush.

2.09.2.1.7 Amphidinolides J, R, and S

Amphidinolide J (**9**), a 15-membered macrolide possessing two hydroxy groups, an *exo*-methylene, and three branched methyl groups, was isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-5). The absolute stereochemistry of amphidinolide J (**9**) was determined to be 3*R*, 9*S*, 10*R*, 13*R*, 14*R*, and 15*R* by comparison of NMR data and optical rotation of three segments, C-1–C-7, C-8–C-11, and C-12–C-16, obtained by ozonolysis with those of their corresponding synthetic enantiomers, respectively.[88] Total synthesis of amphidinolide J (**9**) has been succeeded through organozinc-mediated coupling between C-1–C-12 and C-13–C-20 subunits followed by macrocyclization by using the Yamaguchi procedure.[26]

Amphidinolides R (**17**) and S (**18**) are minor congeners of amphidinolide J (**9**). The structure of amphidinolide R (**17**) was assigned as a regioisomer of amphidinolide J (**9**) having a 14-membered macrolactone ring, since treatment of amphidinolide J (**9**) and amphidinolide R (**17**) with sodium methoxide yielded an identical linear methyl ester. On the other hand, amphidinolide S (**18**) was concluded to be the 9-didehydro form of amphidinolide J (**9**) by spectroscopic data.[89]

9 **17** **18**

2.09.2.1.8 Amphidinolide K

Amphidinolide K (**10**) is a 19-membered macrolide possessing an *s-trans* diene, two *exo*-methylenes, a tetrahydrofuran ring, an epoxide, and three branched methyl groups, isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-5).[90] The relative stereochemistry of the C-9–C-15 portion was proposed on the basis of NOESY data and coupling constants. The stereoisomers of the proposed structure of amphidinolide K have been synthesized by Williams' group (**Scheme 4**). The NMR data of a stereoisomer (2*S*, 4*S*, 9*R*, 10*R*, 11*R*, 12*S*, 15*S*, and 18*R*) were identical with those of natural specimen of amphidinolide K (**10**); however, the sign of $[\alpha]_D$ value was the opposite of that measured for amphidinolide K (**10**). Therefore, the absolute stereochemistry of amphidinolide K (**10**) was concluded to be 2*R*, 4*R*, 9*S*, 10*S*, 11*S*, 12*R*, 15*R*, and 18*S*.[28]

2.09.2.1.9 Amphidinolide M

Amphidinolide M (**12**) is a 29-membered macrolide with two tetrahydrofuran rings, an epoxide, two diene moieties, and two vicinally located methyl and *exo*-methylenes, isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-5).[91] The stereochemistry of amphidinolide M (**12**) remains undetermined, although the angular hydrogens of two tetrahydrofuran portions were both implied as trans-relations.

2.09.2.1.10 Amphidinolide N and its related macrolides

Amphidinolide N (**13**) is a 26-membered macrolide containing a 6-membered hemiacetal ring, an epoxide, a ketone carbonyl, four C_1 branches, and seven hydroxy groups, isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-5).[92] This macrolide was extremely cytotoxic against L1210 and KB cells (IC_{50} 0.000 05 and 0.000 06 μg ml^{-1}, respectively). Although the relative stereochemistry of C-14, C-15, C-16, and C-19 was elucidated as shown, the absolute stereochemistry of amphidinolide N (**13**) remains to be defined. Shimizu and coworkers isolated an amphidinolide N-type macrolide, named caribenolide I (**31**), from a

Scheme 4 Retrosynthetic analysis of enantiomer of amphidinolide K (57) by Williams' group.

free-swimming dinoflagellate *A. operculatum* ver nov *Gibbosum*.[93] Caribenolide I (31) was reported to show potent cytotoxicity against human colon tumor cells HCT116 and its drug-resistant strain HCT116/VM46 (IC_{50} both 0.001 μg ml^{-1}). Caribenolide I (31) showed antitumor activity against murine leukemia P388 (T/C: 150% at a dose of 0.03 mg k g^{-1}) *in vivo*.

Recently, *iso*-epoxy-amphidinolide N and *des*-epoxy-caribenolide I have been synthesized by Nicolaou *et al.*,[59,60] whereas a diastereomer of C-13–C-29 of caribenolide I (**31**) has been synthesized by Figadère and colleagues.[94]

2.09.2.1.11 Amphidinolides O and P

Amphidinolides O (**14**) and P (**15**) were isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-5).[95] Amphidinolide O (**14**) is a 15-membered macrolide possessing a tetrahydropyran ring, an epoxide, α,β-unsaturated ketone, an *exo*-methylene, and three branched methyl groups. The structure of amphidinolide P (**15**) is almost analogous to that of amphidinolide O (**14**). The structural difference between amphidinolides O (**14**) and P (**15**) is at the C-11 position, a ketone group for amphidinolide O (**14**) and an *exo*-methylenes for amphidinolide P (**15**). The relative stereochemistry of amphidinolides O (**14**) and P (**15**) was proposed on the basis of NOESY cross peaks, *J*-value, and molecular mechanics. The total synthesis of an enantiomer of the proposed structure for amphidinolide P (**15b**) was achieved by Professor Williams *et al.*[27] The optical rotation of the synthetic enantiomer was opposite to that of the natural amphidinolide P (**15**), thus indicating that the absolute stereochemistry of amphidinolide P (**15**) was concluded to be 3*R*, 4*S*, 7*R*, 8*S*, 9*R*, 14*R*, and 15*S* (**Scheme 5**). Total synthesis of natural amphidinolide P (**15**) has been accomplished by Professor Trost's group using Ru-catalyzed alkene–alkyne coupling.[42,45]

15b

64

63

65

66

Scheme 5 Retrosynthetic analysis of enantiomer of amphidinolide P (**15b**) by Williams' group.

2.09.2.1.12 Amphidinolide Q

Amphidinolide Q (**16**), $C_{21}H_{34}O_4$, is a 12-membered macrolide possessing four branched methyl groups, an *exo*-methylene, and a ketone carbonyl, isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-5).[96] The relative stereochemistry of amphidinolide Q (**1**) was elucidated on the basis of *J*-based configuration analysis method and NOESY correlations. The absolute configurations of amphidinolide Q (**16**) were concluded to be 4*R*, 7*R*, 9*S*, 11*R*, and 13*R* by application of the modified Mosher's method to amphidinolide Q (**16**) and the linear methyl ester derivatives of amphidinolide Q.[97]

2.09.2.1.13 Amphidinolide T1 and its related macrolides

Amphidinolide T1 (**19a**), a 19-membered macrolide possessing a tetrahydrofuran ring, one *exo*-methylene, three branched methyl groups, one ketone, and one hydroxy group, was isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-56). The absolute configurations at four (C-2, C-13, C-14, and C-18) of the seven chiral centers were determined to be *S*, *S*, *R*, and *R*, respectively, by the modified Mosher's method for C-1–C-12 and C-13–C-21 segments.[98]

The absolute configurations at C-7, C-8, and C-10 were elucidated to be *S*, *S*, and *R*, respectively, by comparison of the ^{1}H NMR data of C-1–C-12 segments with those of synthetic model compounds for the tetrahydrofuran portion.[99] The structure of amphidinolide T1 (**19a**) has been confirmed by a single crystal X-ray analysis.[100] Amphidinolide T1 (**19a**) possesses an odd-numbered macrocyclic lactone ring but has no vicinally located one-carbon branches.

Amphidinolide T2 (**19b**) is a congener of amphidinolide T1 (**19a**) with one-carbon elongation at C-21 isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-71).[99] Amphidinolides T3–T5 (**19c–19e**) are 12-hydroxy-13-oxo isomers of amphidinolide T1 (**19a**) isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-56).[99,100] The absolute stereochemistry of amphidinolides T2–T5 (**19b–19e**) was elucidated by chemical methods similar to those applied for the determination of amphidinolide T1 (**19a**).[99] The structure of amphidinolide T5 (**19e**) was assigned by the interconversion of amphidinolide T4 (**19d**) to amphidinolide T5 (**19e**) with K_2CO_3.[100]

Synthetic studies on the amphidinolide T series have attracted a great deal of attention. Several groups have reported the total syntheses of T1 (**19a**) and T3–T5 (**19c–19e**).[12] Fürstner's group reported the first total syntheses of T4 (**19d**) in 2002,[29] and T1 (**19a**) and T3–T5 (**19c–19e**) in 2003[30] by utilizing an efficient ring-closing metathesis to obtain the macrocycles. The syntheses of amphidinolides T1 (**19a**) and T4 (**19d**) were also reported by Jamison and cowokers in 2004 and 2005 using a nickel-catalyzed alkyne–aldehyde reductive coupling reaction to form the 19-membered ring.[51,52] Amphidinolides T1 (**19a**) and T3 (**19c**) were synthesized through a Yamaguchi macrolactonization reaction by the Ghosh group[38] in 2003, Zhao group[48] in 2006, and Yadav group[58] in 2009, respectively.

A linear polyketide, amphidinin B (**26**), was isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-56), obtained from a marine acoel flatworm *Amphiscolops* sp. collected off Cane Zanpa, Okinawa.[101] Amphidinin B (**26**) exhibited infrared (IR) absorptions at 3400–2500 and 1714 cm^{-1}, indicating the presence of carboxylic acid functionality. The gross structure of amphidinin B (**26**) was assigned by the inspection of 2D NMR spectra. The ^{1}H NMR data of the *bis*-(*S*)-MTPA esters of the C-1–C-9 and C-11–C-21 segments, which were obtained by the successive treatment of amphidinin B (**26**) with TMS-CHN_2, $LiAlH_4$, and (*R*)-MTPACl, were identical with those of the *bis*-(*S*)-MTPA esters of the C-13–C-21 and C-1–C-12 segments obtained from the natural amphidinolide T1 (**19a**), respectively. Thus, the absolute configurations at six chiral centers in amphidinin B (**26**) were elucidated as 2*R*, 6*R*, 11*S*, 16*S*, 17*S*, and 19*R*. Amphidinin B (**26**) is a new polyketide consisting of two linear carbon-chain units of C-1–C-9 (C-22 and C-23) and C-10–C-21 (C-24 and C-25) through an ester linkage (C-6 and C-10), possessing a tetrahydrofuran ring, one *exo*-methylene, three branched methyl groups, and two carboxylic acid groups. The backbone framework of amphidinin B (**26**) was the same as that of amphidinolides T1 (**19a**) and T3–T5 (**19c–19e**). Biogenetically, amphidinin B (**26**) may be related to amphidinolides T1 (**19a**) and T3–T5 (**19c–19e**).

19a **19b**

26

19c: $R_1 = H$, $R_2 = OH$, $R_3 = CH_3$, $R_4 = H$
19d: $R_1 = OH$, $R_2 = H$, $R_3 = CH_3$, $R_4 = H$
19e: $R_1 = OH$, $R_2 = H$, $R_3 = H$, $R_4 = CH_3$

2.09.2.1.14 Amphidinolide V

Amphidinolide V (**21**) is a 14-membered macrolide possessing four *exo*-methylenes and an epoxide, isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-5).[102] The relative stereochemistry of amphidinolide V was deduced from the combination of the molecular mechanics calculation and the analyses of $^1H–^1H$ coupling constants and NOESY data. Recently, a total synthesis of the proposed structure of amphidinolide V (**21**) has been achieved by Fürstner group (**Scheme 6**).[33] Comparison of the NMR data and CD spectrum of the

21 **67** **68** **64** **69** **70**

Scheme 6 Retrosynthetic analysis of amphidinolide V (**21**) by Professor Fürstner.

synthetic material with those of the natural amphidinolide V (21) allowed to determine the absolute configuration of amphidinolide V as 8*R*, 9*S*, 10*S*, and 13*R*.[33,36]

2.09.2.1.15 Amphidinolide W

Amphidinolide W (22), a 12-membered macrolide possessing four branched methyl groups and no *exo*-methylene, was isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-42).[103] The gross structure was elucidated on the basis of the spectroscopic data including ^{13}C–^{13}C NMR correlations obtained from an INADEQUATE spectrum. The absolute configurations at C-11, C-12, and C-14 were elucidated by combination of the *J*-based configuration analysis and the modified Mosher's method. Originally, the absolute configuration at C-6 was proposed to be *S* on the basis of ^{1}H NMR data of 6,11,12-*tris*-MTPA esters of C-6–C-20 segment obtained by the Baeyer–Villiger degradation of amphidinolide W (22). However, the total synthesis of the proposed structure of amphidinolide W (22) and its C-6 epimer revealed that the absolute configuration at C-6 was *R* (**Scheme 7**). Thus, the absolute configurations at all chiral centers in amphidinolide W (22) were assigned as 2*S*, 6*R*, 11*S*, 12*S*, and 14*R*.[39,40]

Scheme 7 Retrosynthetic analysis of amphidinolide W (22) by Professor Ghosh.

2.09.2.1.16 Amphidinolides X and Y

Amphidinolides X (23) and Y (24) were isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-42).[104,105] Amphidinolide X (23) is a 16-membered macrodiolide possessing a tetrahydro ring, four branched methyl groups, and no *exo*-methylene. The gross structure of amphidinolide X (23) was elucidated on the basis of spectroscopic data including one-bond and long-range ^{13}C–^{13}C correlations obtained from 2D distortionless enhancement by polarization transfer (DEPT) C–C relay and 2D DEPT C–C long-range relay experiments. The relative stereochemistry of C-10/C-11 was elucidated to be *erythro* by the *J*-based configuration analysis, whereas the relative stereochemistry of the tetrahydrofuran portion was assigned on the basis of NOESY data. The absolute configurations at C-10 and C-17 were elucidated to be *S* and *R*, respectively, by the application of the modified Mosher's method for the C-8–C-22 segments, which were produced together with the C-1–C-6 segments by reduction of amphidinolide X (23) with $LiAlH_4$. A 4*S*-configuration was deduced from comparison of ^{1}H NMR data of MTPA esters of the C-1–C-6 segments with those of the synthetic 1,6-*bis*-(*R*)-MTPA ester.[104]

Amphidinolide Y (24) is a 17-membered macrolide obtained together with amphidinolide X (23), and it was elucidated to exist as a 9:1 equilibrium mixture of 6-keto and 6(9)-hemiacetal forms on the basis of 2D NMR data. The structure and absolute stereochemistry of the 6-keto form were assigned on the basis of spectroscopic data and chemical conversion of amphidinolide Y (24) into amphidinolide X (23) by $Pb(OAc)_4$ oxidation. The 6-keto form of amphidinolide Y (24) is a 17-membered macrolide possessing a tetrahydrofuran ring, five branched methyl groups, a ketone, and two hydroxy groups.[105]

Total synthesis of amphidinolides X (23) and Y (24) have been accomplished by Professor Fürstner's group using a powerful iron-catalyzed process (Scheme 8),[31,32,37] while amphidinolide Y (24) has been synthesized by Professor Dai's group using the formation of trisubstituted (*E*)-double bond through ring-closing metathesis of densely functionalized alkenes (Scheme 9).[46]

2.09.2.1.17 Amphidinin A

A cytotoxic linear polyketide, amphidinin A (25), was isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-5). The structural features of amphidinin A (25) include vicinally located one-carbon branches, which are one of the unique structural features of the amphidinolides.[106]

2.09.2.1.18 Amphidinolactone A

Amphidinolactone A (27) is a new 13-membered macrolide consisting of a C_{20} carbon chain possessing four disubstituted double bonds and two hydroxy groups, isolated from the marine dinoflagellate *Amphidinium* sp. (strain Y-25).[107] Amphidinolactone A (27) is the first macrolide without a branched methyl or an *exo*-methylene among all the macrolides isolated from the dinoflagellates *Amphidinium* sp. so far. Amphidinolactone A (27) showed cytotoxicity against L1210 cells (IC_{50} 8 μg ml^{-1}). The carbon framework of amphidinolactone A (27) indicates that the macrolide might be derived from C_{20}

23 ⟹ 80 + 79 ⟹ 81

Scheme 8 Retrosynthetic analysis of amphidinolide X (23) by Professor Fürstner.

Scheme 9 Retrosynthetic analysis of amphidinolide Y (**24**) by Professor Fürstner.

unsaturated fatty acid. Recently, we have accomplished the asymmetric total synthesis of amphidinolactone A (**27**) to determine its absolute stereochemistry as 8*R*, 11*S*, and 12*R*.[108]

27

2.09.2.1.19 Iriomoteolides

Recently, four new macrolides, iriomoteolides 1a (**29a**), 1b (**29b**), 1c (**29c**), and 3a (**30**) were isolated from the marine dinoflagellate *Amphidinium* sp. (strain HYA024), which was monoclonally separated from sea sand collected off Iriomote Island, Okinawa.[109,110]

Iriomoteolide 1a (**29a**) is a 20-membered macrolide having four hydroxy groups, five methyl groups, an *exo*-methylene branch, three endocyclic double bonds, and a tetrahydropyran ring. Iriomoteolides 1b (**29b**) and 1c (**29c**) are new 20-membered macrolides structurally related to iriomoteolide 1a (**29a**).[109] Iriomoteolide 1b (**29b**) has a hydroxy group at C-9 and a ketone at C-13 conjugated with an *E*-double bond at C-11–C-12, whereas the corresponding part for iriomoteolide 1a (**29a**) is a six-membered hemiacetal ring and an *exo*-methylene group. Although iriomoteolide 1b (**29b**) might be an artifact generated from iriomoteolide 1a (**29a**), interchange between iriomoteolides 1a (**29a**) and 1b (**29b**) in solvents such as $CHCl_3$ or MeOH was not observed. On the other hand, iriomoteolide 1c (**29c**) is a homolog of iriomoteolide 1a (**29a**) with a 4-hydroxy-3-methylpentyl side chain.[110]

Iriomoteolide 3a (**30**) is a 15-membered macrolide having three hydroxy groups and an allyl epoxide. Iriomoteolide 3a (**30**) exhibited potent cytotoxicity against human B lymphocyte DG-75 (IC_{50} 0.08 μg ml^{-1}) and Raji cells (IC_{50} 0.05 μg ml^{-1}), the latter of which was infected with Epstein–Barr virus (EBV).[111]

29a

29b

29c

30

2.09.2.2 Amphezonol A

Amphezonol A (87), a polyhydroxy linear carbon-chain metabolite, has been obtained from the cultured marine dinoflagellate *Amphidinium* sp. (strain Y-72), which was isolated from a marine acoel flatworm *Amphiscolops* sp. collected off Cane Zanpa, Okinawa.[112] Amphezonol A (87) possesses one tetrahydrofuran ring, two tetrahydropyran rings, and 21 hydroxy groups on a C_{60} linear aliphatic chain with one *exo*-methylene and one methyl branch. The structure of amphezonol A (109) was elucidated by detailed analyses of the 2D NMR spectra including heteronuclear single quantum coherence (HSQC)–TOCSY and INADEQUATE. The presence of a tetrahydrofuran and two tetrahydropyran rings was deduced from deuterium-induced shift analysis of the oxymethine carbon signals in the ^{13}C NMR spectra of amphezonol A (87), observed in CD_3OD and CD_3OH, respectively. The relative stereochemistry of a tetrahydrofuran ring (C-3–C-6) and two tetrahydropyran rings (C-13–C-17 and C-39–C-43) in amphezonol A (87) was elucidated on the basis of rotating frame nuclear Overhauser effect spectroscopy (ROESY) correlations of amphezonol A (87). The successive hydroxylated moiety of the carbon chain (C-23–C-33) is a characteristic of amphezonol A (87). Amphezonol A (87) exhibited inhibitory activity against DNA polymerase α (IC_{50} 15 $\mu mol\,l^{-1}$).

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2.09.2.3 Amphidinoketides

Amphidinoketide I (**88**) and an isomeric compound amphidinoketide II (**89**) were isolated with amphidinolides B1 (**2a**), B2 (**2b**), and B3 (**2c**), and caribenolide I (**31**), from a dinoflagellate *Amphidinium* sp. S1-36-5, obtained from the water at Brewers Bay, St. Thomas, US Virgin Islands.[113] The structures of the amphidinoketides are very unusual since they bear carbonyl groups in a 1,4-relationship. In preliminary screening, amphidinoketide I (**88**) showed cytotoxicity against the human colon tumor cell line HCT-16 and its drug-resistant strain (IC_{50} 4.98 μg ml^{-1}). The deconjugated isomer, amphidinoketide II (**89**), showed much weaker activity (IC_{50} 73 μg ml^{-1}). The relative and absolute stereochemistries of amphidinoketide I (**88**) have been determined by total synthesis of four diastereoisomers.[114] Molecular modeling was used to infer that the natural product is not the thermodynamically preferred diastereoisomer.

88

89

2.09.2.4 Amphidinols and their Related Compounds

Amphidinols are a series of long-chain polyhydroxypolyene compounds eliciting potent antifungal and hemolytic activities. The structures of amphidinols were clarified by extensive analyses of 2D NMR and MS/MS data. Amphidinol 1 (**90**) (synonymous to amphidinol) was isolated from cultures of the dinoflagellate *Amphidinium klebsii* collected at Ishigaki Island in Okinawa by Yasumoto and coworkers,[115] and subsequently amphidinols 2–7 (**91–96**) and 9–15 (**97–103**) were isolated from a different strain of *Amphidinium* spp.[116–120] The structure of amphidinol 1 (**90**) was assigned as a polyhydroxypolyene compound consisting of C_{69} aliphatic chain with a sulfate ester, 2 tetrahydropyrans, 21 hydroxy groups, 1 *exo*-methylene, and 2 branched methyl groups. Amphidinols 2–7 (**91–96**) were isolated from *A. klebsii*, obtained from a surface wash of a seaweed collected at Aburatsubo Bay, Japan. Amphidinol 7 (**96**) has a sulfate group on the shortest carbon backbone of any of the known amphidinols. Amphidinols 9–13 (**97–101**) were isolated from *Amphidinium carterae* collected at Kauauroa, New Zealand. Amphidinols 14 (**102**) and 15 (**103**) were isolated from the dinoflagellate *A. klebsii*, obtained from the surface wash of several species of seaweed that were abundant at the collection site near the shore of Aburatsubo Bay. Their weak membrane-disrupting activity indicates that the hydrophobic polyene chain is essential for the potent biological activities.

Structure–activity relationship for the polyhydroxy part was then examined with the use of amphidinol homologs possessing various chain lengths, indicating that the pore size of the channel/lesion formed by amphidinols was not greatly affected by the length of the polyhydroxy chain.

90: R_1 = R_2 =

91: R_1 = R_2 =

92: R_1 = R_2 =

93: R_1 = R_2 =

94: R_1 = R_2 =

95: R_1 = R_2 =

96: R_1 = R_2 =

97: R_1 = R_2 =

98: R_1 = R_2 =

99: R_1 = R_2 =

100: R_1 = R_2 =

101: R_1 = R_2 =

102: R_1 = R_2 =

103: R_1 = R_2 =

Amphidinols have common structural features characterized by 2 ether rings, 6 double bonds involving a conjugated triene and an *exo*-methylene, a branched methyl, and 14 hydroxy groups. Murata and coworkers elucidated the absolute stereochemistry of amphidinol 3 (**92**) by combination of J-based configuration analysis (JBCA) method, modified Mosher's method, and chiral gas chromatography-mass spectrometry (GC-MS) analysis of the degradation product of natural specimen with authentic samples.[121,122] Amphidinol 3 (**92**) exhibited the most potent antifungal and hemolytic activities among amphidinols, and its mode of action has been studied intensively.[123]

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Luteophanols A–D (**104–107**) have been isolated from the Y-52 strain of the dinoflagellate *Amphidinium* sp., which was a symbiont of acoel flatworm *Pseudaphanostoma luteocoloris*.[124–126] The gross structure of luteophanol A (**104**) was elucidated to be a C_{57} aliphatic chain with a sulfate ester, 2 tetrahydropyrans, 19 hydroxy groups, 1 *exo*-methylene, and 2 branched methyl groups by NMR data as well as fast atom bombardment tandem mass spectrometry (FAB-MS/MS) data. Luteophanols B–D (**105–107**) possess 2 tetrahydropyrans and 23 hydroxy groups on a linear aliphatic chain with three C_1 branches. The structures of the central portions of luteophanols A–D (**104–107**) are common to those of the amphidinols.

104: R_1 = ; R_2 =

105: R_1 = ; R_2 =

106: R_1 = ; R_2 =

107: R_1 = ; R_2 =

A cytotoxic long-chain polyhydroxy compound, lingshuiol (**108**), was isolated from the cultured marine dinoflagellate *Amphidinium* sp., obtained from the surface wash of seaweeds collected at Lingshui Bay, Hainan Province, China.[127] The same culture yielded lingshuiols A (**109**) and B (**110**).[128]

Karatungiols A (**111**) and B (**112**) were isolated from the cultured marine dinoflagellate *Amphidinium* sp., which was isolated from an unidentified marine acoel flatworm collected at Karatung Island, Indonesia. The structures of karatungiols A (**111**) and B (**112**) were elucidated by spectroscopic analysis and degradation reactions. Karatungiols were amphidinol analogs with a ketone group, a 3,4,5-trihydroxy-tetrahydropyran ring, and a terminal saturated alkyl chain moiety. Karatungiol A (**111**) exhibited potent antifungal activity against NBRC4407 *Aspergillus niger* and antiprotozoan activity against *Trichomonas foetus*.[129]

Carteraol E (**113**) was isolated from the cultured marine dinoflagellate *A. carterae* (AC021117009), which was isolated from the wash-off epiphytes of seaweeds collected at the southern coast of Taiwan in 2002. Carteraol E (**113**) possessed 3 tetrahydropyrans, and 19 hydroxy groups on a C_{69} linear aliphatic chain with a ketone moiety, an *exo*-methylene, and 3 methyl branches. The structure was elucidated by extensive analyses of 2D NMR spectra. Carteraol E (**113**) exhibited potent ichthyotoxicity with LD_{50} value of 0.28 $\mu mol\,l^{-1}$ and antifungal activity against *A. niger*.[130]

113

2.09.2.5 Colopsinols

Colopsinols A–E (**114–118**) are the first members of a new class of polyketide natural products that possess a glucoside moiety and a sulfate ester.[131–133] Colopsinols were isolated from more polar fractions than the macrolide-containing fraction of *Amphidinium* sp. (Y-5 strain). The gross structures were elucidated on the basis of extensive spectroscopic analyses including the CH_2-selected editing HSQC spectra as well as FAB-MS/MS experiments and chemical means. The polyketide moiety of colopsinol A (**114**) consists of a C_{56} linear aliphatic chain with three C_1 branches (one *exo*-methylene and two methyl groups), two ketones, five hydroxy groups, and a tetrahydropyran and two epoxide rings. Colopsinol A (**114**) exhibited potent inhibitory activity against DNA polymerase α and β with IC_{50} values of 13 and 7 $\mu mol\,l^{-1}$, respectively.[131] On the other hand, colopsinols B (**115**) and C (**116**) are new polyhydroxy compounds possessing a C_{53} linear carbon chain including three C_1 branches as well as a tetrahydropyran, a tetrahydrofuran, and an epoxide ring, six hydroxy groups, a glucoside moiety, and a sulfate ester. Colopsinol C (**116**) exhibited cytotoxicity against L1210 cells *in vitro* with the IC_{50} value of 7.8 $\mu g\,ml^{-1}$, whereas colopsinol B (**115**) did not show such activity (IC_{50} > 10 $\mu g\,ml^{-1}$).[132] Colopsinols D (**117**) and E (**118**) are congeners of colopsinol A (**114**). Colopsinol D (**117**) has a tetrahydrofuran ring at C-9–C-12, whereas colopsinol E (**118**) is the monodeglucosyl form of colopsinols A (**114**). Colopsinol E (**118**) exhibited cytotoxicity against L1210 cells (IC_{50} 7 $\mu g\,ml^{-1}$), whereas colopsinol D (**117**) did not show such activity (IC_{50} > 20 $\mu g\,ml^{-1}$).[133] Biosynthetically, it is interesting that quite different types of polyketides such as colopsinols and amphidinolides are produced from the same dinoflagellate strain.

114: R = β-D-glucosyl
118: R = H

115: R = β-D-glucosyl
116: R = H

117

2.09.3 *Alexandrium* sp.

2.09.3.1 Goniodomin A

Goniodomin A (**119**), a 25-membered polyether macrolide, has been isolated by Murakami and coworkers from the dinoflagellate *Alexandrium hiranoi* (formerly *Goniodoma pseudogoniaulax*) in 1988, and recently detected from *Alexandrium monilatum* as well.[134,135] In addition to potent antifungal activity, this macrolide inhibits cell division in the fertilized sea urchin and was found to modulate rabbit skeletal actomyosin ATPase activity by altering actin conformation. Moreover, goniodomin A (**119**) acts as an antiangiogenic agent to inhibit endothelial cell migration and tube formation induced by basic fibroblast growth factor.[136–140] Recently, the absolute stereochemistry of goniodomin A (**119**) was established from the analysis of ROESY experiments and coupling constants, synthesis of suitable model compounds for NMR spectroscopic comparisons, degradation experiments, and correlation with synthetic reference compounds.[141]

119

2.09.3.2 Saxitoxin

Saxitoxin (**120**), a paralytic shellfish poisoning alkaloid, was obtained from cultures of a red tide dinoflagellate *Alexandrium* sp. The toxin is also used for studies on ion channels. The biosynthesis of saxitoxin (**120**) involves arginine as a precursor of the guanidinio groups, whereas the perhydropurine skeleton is derived from Claisen condensation of acetate with arginine. The carbon atom in the side chain is derived from *S*-adenosyl methionine.[142]

120

2.09.3.3 Spirolides (Pinnatoxin)

The spirolides were first isolated from Nova Scotian mussels (*Mytilus edulis*) and scallops, and ultimately shown to originate from the dinoflagellate *Alexandrium ostenfeldii*. The structure of spirolides B (**122**) and D (**124**), the major metabolites in shellfish digestive gland extracts, were the first to be characterized. Two ring A hydrolysis products, biologically inactive spirolides E (**125**) and F (**126**), were subsequently reported. However, spirolides E (**125**) and F (**126**) were not observed as culture products of *A. ostenfeldii*, suggesting that these nontoxic analogs are mollusc-hydrolyzed detoxification products. Cultured dinoflagellates provided spirolides A (**121**) and C (**123**) and 13-desmethyl spirolide C (**127**) in sufficient quantity for characterization. The relative stereochemistry of 13-desmethyl spirolide C (**127**) has been established to be the same as that found in pinnatoxin A (**131**), which share rings A–C and E with the spirolides. Subsequently, 13,19-didesmethyl spirolide C (**128**), 27-hydroxy-13,19-didesmethyl spirolide C (**129**), and 13-desmethyl spirolide D (**130**) have been isolated from *A. ostenfeldii*. The bis-spiroacetal ring system (rings B, C, and D) has been the subject of synthetic efforts in both the spirolides and the pinnatoxins.[143–150]

121: R_1, R_4 = H, R_2, R_3 = CH_3, $\Delta^{2,3}$
122: R_1, R_4 = H, R_2, R_3 = CH_3
123: R_1, R_2, R_3 = CH_3, R_4 = H, $\Delta^{2,3}$
124: R_1, R_2, R_3 = CH_3, R_4 = H
127: R_1, R_3 = CH_3, R_2, R_4 = H, $\Delta^{2,3}$
128: R_1 = CH_3, R_2, R_3, R_4 = H, $\Delta^{2,3}$
129: R_1, R_3 = CH_3, R_2 = H, R_4 = OH, $\Delta^{2,3}$
130: R_1, R_3 = CH_3, R_2, R_4 = H

125: $\Delta^{2,3}$
126

131

2.09.4 *Dinophysis* sp.

2.09.4.1 Pectenotoxins

Pectenotoxins are a family of polyether macrolides, which were first isolated from scallops and greenshell mussels as a toxin. The structure of pectenotoxin-1 (**132**) was first characterized by X-ray crystallography. The biogenetic origin of pectenotoxins has been identified in 1996, since pectenotoxin-2 (**133**) was isolated from the dinoflagellate *Dinophysis fortii*. Pectenotoxin-1 (**132**), pectenotoxin-3 (**134**), and pectenotoxin-6 (**135**) are producd from pectenotoxin-2 (**133**) by the oxidation of the methyl group at C-43, while the lactone ring-opened analog, pectenotoxin-2 seco acid (**136**), is generated by enzymatic hydrolysis from pectenotoxin-2 (**133**) in some species of mussel and scallop. Pectenotoxin-11 (**137**), pectenotoxin-12 (**138**), and several analogs have been isolated from the dinoflagellates *D. fortii* and *Dinophysis acuta* so far. Pectenotoxin-2 seco acid (**136**) has also been isolated from *D. acuta* as a natural component.[151–168]

132: R_1 = CH_2OH, R_2 = H
133: R_1 = CH_3, R_2 = H
134: R_1 = CHO, R_2 = H
135: R_1 = COOH, R_2 = H
137: R_1 = CH_3, R_2 = OH

136

138

2.09.5 *Durinskia* sp.

2.09.5.1 Durinskiols

A long carbon-chain polyoxygenated polyketide, durinskiol A (**139**), was isolated from the cultured dinoflagellate *Durinskia* sp., which was separated from the sea slug *Chelidonura fulvipunctata*. The gross structure was elucidated by inspection of 2D NMR data and MS/MS analysis. The relative stereochemistries of ether rings and sugar moieties were elucidated from 2D NMR analysis and molecular modeling studies. Conformation of the 6,5,6-bis-spiroacetal ring was analyzed by using *ab initio* methods. Durinskiol A (**139**) caused a short body length, abnormal pigment pattern, and pericardiac and yolk-sac edema in zebrafish.[169,170] Subsequently, the structure of durinskiol B (**140**), a congener of durinskiol A (**139**), was reported.[171]

139: l + m + n = 4, R = H
140: l + m + n = 3, R = CH_3

2.09.6 *Gambierdiscus toxicus*

2.09.6.1 Ciguatoxins

Ciguatera toxin is the most famous seafood poisoning and an important medical issue in the tropical and subtropical Pacific and Indian Ocean regions and in the tropical Caribbean. Ciguatoxin (**141**), the principal causative agent of ciguatera, was first isolated from the viscera of moray eels (*Gymnothorax javanicus*), and the structure was elucidated by NMR analysis to be a huge ladderlike polycyclic ether with the 13 ether rings ranging from five- to nine-membered. Subsequently, ciguatoxin 4B (**142**) was isolated from the benthic dinoflagellate *Gambierdiscus toxicus* collected at the Gambier Islands. More than 20 ciguatoxin congeners have been reported and 6 of which, ciguatoxin 3C (**143**), ciguatoxin 4A (**144**), ciguatoxin 4B (**142**), 51-hydroxy ciguatoxin 3C (**145**), 2,3-dihydroxy ciguatoxin 3C (**146**), and 52-*epi*-54-deoxy ciguatoxin (**147**), have been isolated from the cultured *G. toxicus*. Ciguatoxins bind to the site 5 of the voltage-sensitive sodium channel in neurons and inhibit depolarization to allow inward Na^+ influx to continue. Total synthesis of ciguatoxin 3C (**143**) was accomplished by Hirama and coworkers in 2001.[172–180]

141: R_1 = R_2 =

142: R_1 = R_2 =

144: R_1 = R_2 =

147: R_1 = R_2 =

143: R_1 = R_2 =

145: R_1 = R_2 =

146: R_1 = R_2 =

148

2.09.6.2 Maitotoxin

Maitotoxin (**148**) is the most toxic and largest natural product ($C_{164}H_{256}O_{68}S_2Na_2$, MW 3422) except for biopolymers. The toxin consists of 142 carbon chain containing 32 ether rings, 28 hydroxy groups, and 2 sulfate esters. Maitotoxin (**148**) was first isolated from the ciguateric fish *Ctenochaetus striatus*, which was called 'maito' in Tahiti, and later shown to be a metabolite of the dinoflagellate *G. toxicus*. The structure of maitotoxin (**148**) was elucidated by a combination of degradative methods and extensive NMR experiments such as 3D pulsefield gradient nuclear Overhauser enhancement and exchange spectroscopy-heteronuclear multiple quantum coherence (PFG NOESY-HMQC) using a 10–20 mg of the sample and 9 mg of the ^{13}C-enriched sample. Owing to the slow growth and low toxin productivity of the organism, it took more than 10 years to obtain the sample from 6000-l cultures. The relative stereochemistry of the ring systems and the acyclic portions of the molecule were elucidated by JBCA method and comparison of NMR data with synthetic models. Maitotoxin (**148**) elicits Ca^{2+} influx in virtually all cells and tissues, which usually occurs concomitantly with depolarization of the membrane potential.[181–190]

2.09.6.3 Gambieric Acids

Gambieric acids A–D (**149–152**) were potent antifungal compounds isolated from the culture medium of *G. toxicus* that produces ciguatoxin precursors (**142–147**) and maitotoxin (**148**). The absolute stereochemistry of gambieric acids A–D (**149–152**) were assigned by the modified Mosher's method, conformational analysis based on *J* values and NOE correlations, chiral fluorimetric HPLC analysis, and synthesis of A/B-ring models of gambieric acids A–D (**149–152**).[191–195]

149: R_1 = H, R_2 = H

150: R_1 = CH_3, R_2 = H

151: R_1 = H, R_2 =

152: R_1 = CH_3, R_2 =

2.09.6.4 Gambierol

Gambierol (**153**) was isolated as a toxic constituent of cultured cells of the ciguatera causative dinoflagellate cells of *G. toxicus* collected at the Rangiroa Atoll in French Polynesia. The structure of gambierol (**153**) including its relative stereochemistry was established on the basis of extensive NMR studies, and the absolute configuration was determined by derivatization and application of the modified Mosher's method. Total synthesis of gambierol has been achieved by three groups. Recent investigations revealed that gambierol (**153**) acts as a functional antagonist of neurotoxin site 5 on voltage-gated sodium channels in cerebellar granule neurons.[196–203]

153

2.09.7 *Karenia* sp.

2.09.7.1 Brevetoxins and Its Related Compounds

Brevetoxins A (**154**) and B (**155**) were the first members of a structurally varied series of cyclic polyether compounds, possessing a characteristic structural feature of a ladderlike skeleton consisting of trans-fused polyether rings. Brevetoxin A (**154**) was isolated from the dinoflagellate *Karenia brevis* (*Gymnodinium breve*), and the structure was first elucidated by Shimizu and coworkers by X-ray crystallography and independently determined by Nakanishi through spectroscopic studies. Brevetoxin B (**155**) was also isolated from the same microorganism and the structure including the absolute stereochemistry was determined by X-ray crystallography. Brevetoxins A (**154**) and B (**155**) possess a lactone ring, an α,β-unsaturated aldehyde side chain, and 9 and 10 consecutive ether rings, respectively. Total syntheses of brevetoxins A (**154**) and B (**155**) have been achieved by some groups. In addition, several brevetoxin analogs (**156–162**) possessing a variety of side chain with brevetoxins A (**154**) and B (**155**) skeletons have been isolated from the dinoflagellate and the structures were clarified by comparing the spectra with those of brevetoxins A (**154**) and B (**155**). Brevetoxins bind to site 5 of the voltage-sensitive sodium channel in neurons and inhibit channel inactivation. Hemibrevetoxin B (**163**) is the smallest cyclic polyether compound produced by *K. brevis*, which contains only four fused cyclic ether rings (7/7/6/6). Brevenal (**164**) contains five fused ether rings (7/7/6/7/6), a terminal conjugated aldehyde, and a conjugated diene. The side chain and the 7/7/6 rings of brevenal (**164**) are similar to hemibrevetoxin B (**163**).[204–221]

154: R = CHO **161**: R = OH **162**: R = OH

155: R = , **156**: R = , **157**: R = , 37-acetoxy

158: R = , 27,28-epoxy **159**: R = **160**: R =

163 **164**

Further examination of organic extracts of *K. brevis* has uncovered another unprecedented cyclic ether alkaloid, named brevisamide (**165**), which consists only of a single tetrahydropyran ring furnished with a methyl and a hydroxy substituent, a 3,4-dimethylhepta-2,4-dienal side chain, and an acetylated terminal amine. Brevisamide (**165**) can be regarded as a truncated analog of brevenal (**164**) containing an ether ring portion and the dienal side chain.[222] The absolute stereochemistry of brevisamide (**165**) has been established by total synthesis.[223]

165

Brevisin (**166**) is an unprecedented polycyclic ether isolated from *K. brevis*, which consists of two separate fused polyether ring assemblies linked by a methylene group. One of the polycyclic moieties contains a conjugated aldehyde side chain similar to brevisamide (**166**). The interrupted polyether structure of brevisin (**166**) is novel and provides further insight into the biogenesis of such fused ring polyether systems. Brevisin (**166**) inhibits the binding of brevetoxin 3 (**156**) to its binding site on the voltage-sensitive sodium channels in rat brain synaptosomes.[224]

166

2.09.7.2 Gymnocins

Gymnocins A (**167**) and B (**168**) are a series of cytotoxic polyether compounds isolated from the notorious red tide dinoflagellate *Karenia mikimotoi*. These toxin molecules are rare polycyclic ethers that exhibit potent *in vitro* cytotoxic activity against P388 murine leukemia cells. The structures gymnocins A (**167**) and B (**168**) were established on the basis of extensive 2D NMR analysis and collision-induced MS/MS experiments.[225–227] The structure of gymnocin A (**167**) was characterized by 14 contiguous saturated ether rings (5/7/6/7/6/6/7/6/6/6/6/7/6/6), whereas gymnocin B (**168**) consists of 15 contiguous saturated ether rings (5/7/6/6/6/6/7/7/6/7/

6/6/6/6/7). The absolute stereochemistry of gymnocin A was clarified by combining the analysis of NOE data and the modified Mosher method. The total syntheses of gymnocins A (167) and B (168) have been accomplished by Sasaki and coworkers.[228–235]

167

168

2.09.7.3 Gymnodimine

Gymnodimine (169), a complex pentacyclic derivative incorporating a C_{24} carboxylic acid and a fused azine, was originally isolated as a toxic substance from oysters. The unique structure of gymnodimine (169), containing butenolide, a 16-membered carbocycle, and cyclic imine moieties, was elucidated by extensive 2D NMR experiments and the absolute stereochemistry was deduced from X-ray crystallography. The biogenetic origin of gymnodimine (169) was assigned as the dinoflagellate *Karenia selliformis*. Two analogs, gymnodimines B (170) and C (171), have been isolated from the dinoflagellate.[236–239]

169

170: $R_1 = H$, $R_2 = OH$
171: $R_1 = OH$, $R_2 = H$

2.09.8 *Karlodinium veneficum*

2.09.8.1 Karlotoxins

Karlotoxins 1 (172) and 2 (173) are a group of potent amphipathic ichthyotoxins produced by the dinoflagellate *Karlodinium veneficum*. The structure of karlotoxin 1 (172), determined using extensive 2D NMR spectroscopy, is very similar to that of the amphidinols and related compounds, although karlotoxin 1 (172) features unique structural modifications of the conserved core region. Karlotoxin 2 (173) consists of the C_{63} linear aliphatic chain, which is shorter than that of karlotoxin 1 (172) by two methylene groups and possesses a chlorine atom at

the terminal of its hydrophobic arm.[240]

172: R =

173: R =

2.09.9 *Ostreopis siamensis*

2.09.9.1 Ostreocins (Palytoxin)

Palytoxin (**174**) is one of the most fascinating natural products. Although palytoxin (**174**) was first isolated from the zoanthid *Palythoa tubercurosa*, the toxin was subsequently found in various kinds of organisms such as algae, crabs, herbivorous fish, and a surgeon fish. The toxin contents markedly fluctuated both seasonally and regionally. Therefore, the biogenetic origin of palytoxin (**174**) has not been identified for a long time. However, the wide distribution of palytoxin (**174**) suggested that the toxin is produced by a microorganism. Ostreocins were isolated from the marine dinoflagellate *Ostreopsis siamensis*, and the structure of the major component in this strain, ostreocin D (**175**), was elucidated to be 42-hydroxy-3,26-didemethyl-19,44-dideoxy palytoxin by detailed 2D NMR analyses of intact ostreocin D (**175**) and its ozonolysis products. The structure of ostreocin D (**175**) was supported by FAB-MS/MS experiments using its 2-sulfobenzoylated derivative. Thus, the dinoflagellate *O. siamensis* was presumed to be one of the biogenetic origins of palytoxin (**174**). Palytoxin (**174**) has been proposed to convert Na^+/K^+ ATPase into a cation-selective ion channel.[241–245]

174: R_1, $R_3 = CH_3$; R_2, $R_5 = OH$, $R_4 = H$
175: R_1, R_2, R_3, $R_5 = H$, $R_4 = OH$

2.09.10 *Prorocentrum* sp.

2.09.10.1 Belizeanolide

Belizeanolide (**176**) and its secoacid form, belizeanolic acid (**177**), were isolated from a benthic marine dinoflagellate *Prorocentrum belizeanum.* Belizeanolide has a backbone with 66 carbon atoms that includes a unique 54-membered lactone ring containing two furan-type rings. Belizeanolide (**176**) and belizeanolic acid (**177**) showed antiproliferative activity against ovarian (A2780), lung (SW1573), breast (HBL100, T47D), and colon (WiDr) human solid tumor cells *in vitro.* The GI_{50} (μM) values for belizeanolic acid (**177**) were 0.26 (A2780), 0.31 (SW1573), 0.32 (HBL100), 0.40 (T47D), and 0.41 (WiDr). Belizeanolide (**176**) is 10 times less potent than belizeanolic acid (**171**).[246]

176

2.09.10.2 Formosalides

Recently, two cytotoxic 17-membered macrolides, formosalides A (**178**) and B (**179**), were isolated from the cultured marine dinoflagellate *Prorocentrum* sp., which was obtained from the wash-off epiphytes of seaweeds at South Bay, southern Taiwan. Their gross structures, including partial relative stereostructures, were seen by extensive spectroscopic studies. Formosalides A (**178**) and B (**179**) possess all-*cis* tetraenes, a tetrahydropyran ring, a tetrahydrofuran ring, two branched methyl groups, and a C_{14} linear side chain. Formosalides A (**178**) and B (**179**) exhibited cytotoxicity against CCRF-CEM human T-cell acute lymphoblastic leukemia cells and/or DLD-1 human colon adenocarcinoma cells *in vitro* (LD_{50} values of **178**: 0.54 and >40 μg ml^{-1}, respectively; LD_{50} values of **179**: 0.43 and 2.73 μg ml^{-1}, respectively).[247]

178: R = H
179: R = CH_3

2.09.10.3 Hoffmaniolide

Hoffmaniolide (180) has been isolated from *Prorocentrum hoffmannianum*.[248] The gross structure of hoffmaniolide (180) was obtained by analyzing the liquid secondary ion mass spectrometry (LSIMS)-MS/MS spectrum as well as 2D NMR such as ^{1}H–^{1}H COSY, TOCSY, and HMBC, and the relative stereochemistry of a tetrahydropyran ring was obtained from *J* coupling data. Isolation of hoffmanniolide (180) from *P. hoffmannianum* suggested a biosynthetic capability of this genus to produce either linear or macrocyclic polyethers.

180

2.09.10.4 Okadaic Acid and Its Related Compounds

Although okadaic acid (181) has been first isolated from the marine sponges *Halichondria okadai* and *Halichondria melanodocia*, it was found that okadaic acid (181) and its related polyethers, such as the dinophysis toxins (DTX), DTX-1 (182), DTX-2 (183), DTX-3 (184), DTX-4 (185), DTX-5a (186), DTX-5b (187), and DTX-6 (188); acanthifolicin (189); and okadaic acid diol esters (190 and 191) were produced by several dinoflagellates that belong to the genera *Prorocentrum* (*Prorocentrum lima*, *Prorocentrum concavum*, *Prorocentrum maculosum*, and *Prorocentrum acuminate*) and *Dinophysis* (*Dinophysis fortii*).[249–254] Okadaic acid (181) is a protein phosphatase PP-1 and PP-2A inhibitor and has been used extensively as a probe to identify cellular processes that are regulated by phosphorylation and dephosphorylation and for the identification of phosphatases.[255,256] The ester derivatives showed relatively potent activity, indicating that a free carboxylate is required to inhibit phosphatase. Recently, two metabolites, 19-*epi*-okadaic acid (192)[257] and belizeanic acid (193),[258] belonging to the okadaic acid class of protein phosphatase inhibits, have been isolated from cultures of the dinoflagellate *P. belizeanum*.

181: $R_1 = CH_3$, R_2, R_3, $R_4 = H$
182: R_1, $R_2 = CH_3$, R_3, $R_4 = H$
183: R_1, R_3, $R_4 = H$, $R_2 = CH_3$
184: R_1, $R_2 = CH_3$, $R_3 =$ acyl (typically palymitoyl), $R_4 = H$
185: $R_1 = CH_3$, R_2, $R_3 = H$, $R_4 =$ see below

186: $R_1 = CH_3$, R_2, $R_3 = H$, $R_4 =$ see below

187: $R_1 = CH_3$, R_2, $R_3 = H$, R_4 = see below

188: $R_1 = CH_3$, R_2, $R_3 = H$, R_4 = see below

189: $R_1 = CH_3$, R_2, R_3, $R_4 = H$, 9,10-episulfide

190: R_1, $R_3 = H$, $R_2 = CH_3$, R_4 = see below

191: R_1, $R_3 = H$, $R_2 = CH_3$, R_4 = see below

192: $R_1 = CH_3$, R_2, R_3, $R_4 = H$, 19-*epi*

193

2.09.10.5 Prorocentrolides

Prorocentrolide (**194**) has been isolated from *P. lima*, whereas prorocentrolide B (**195**) was isolated as a fast-acting toxin from *P. maculosum*.[259,260] Both macrolides possess a cyclic imine moiety and are lethal in the mouse bioassay. The specific mode of action of the prorocentrolides, however, has not been elucidated.

Spiro-prorocentrimine (**196**) is a polar lipid-soluble toxin isolated from a laboratory-cultured benthic *P. lima* collected in Taiwan.[261] It was crystallized in methanol and X-ray diffraction analysis of spiro-prorocentrimine (**196**) revealed a spiro-linked cyclic imine with the 1,1,2,4-tetrasubstituted 3-cyclohexene in addition to its macrolide skeleton. Biogenetically, prorocentrolides and spiro-prorocentrimine might be related to each other.

194 **195**

196

2.09.10.6 Prorocentin

Prorocentin (**197**), a C_{35} polyketide with four pendant methyl groups, possessing an all-*trans* triene moiety, an epoxide, a furan ring, and the 6/6/6-trans-fused/spiro-linked tricyclic ether rings, was isolated from an okadaic acid-producing strain of *P. lima*.[262] The relative stereostructure was elucidated on the basis of spectral data. Both spiro-linked ethers of prorocentin (**197**) (C/D rings) and okadaic acid (**181**) (A/B rings) shared the same backbone. Prorocentin (**197**) exhibited inhibitory activity against human colon adenocarcinoma DLD-1 and human malignant melanoma RPMI7951 with IC_{50} values of 16.7 and 83.6 μg ml^{-1}, respectively.

197

2.09.11 *Protoceratium reticulatum*

2.09.11.1 Yessotoxins

Yessotoxin (YTX, **198**), a disulfated polyether toxin, was first isolated from the digestive gland of Japanese scallop, *Patinopecten yessoensis*, and subsequently the biogenetic origin was identified to be the dinoflagellate *Protoceratium reticulatum*. About 100 YTX analogs have been reported from shellfish and dinoflagellate, and the structures of about 40 of them have been elucidated by NMR and MS/MS analyses so far. 45,46,47-Trinor-YTX (**199**), homo-YTX (**200**), noroxo-YTX (**201**), 40-*epi*-41-keto-YTX (**202**), 41-keto-YTXenone (**203**), 41a-homo-YTX (**204**), 9-methyl-41a-homo-YTX (**205**), 9-methyl-41-keto-YTXenone (**206**), 44,55-dihydroxy-YTX (**207**), 44,55-dihydroxy-41a-homo-YTX (**208**), 44,55-dihydroxy-9-methyl-41a-homo-YTX (**209**), 41a-homo-YTXamide (**210**), 9-methyl-41a-homo-YTXamide (**211**), 45-OHdinor-YTX (**212**), 44-oxotrinor-YTX (**213**), 41a-homo-44-oxotrinor-YTX (**214**), and 45,46,47-trinorhomo-YTX (**215**) have been isolated from dinoflagellate. In addition, four nor-ring-A-YTX analogs, nor-ring-A-YTXs (**216**), nor-ring-A-41-keto-YTX (**217**), nor-ring-A-40-*epi*-41-keto-YTX (**218**), and nor-ring-A-41-keto-YTXenone (**219**), and glycosylated YTX analogs, protoceratins II (**220**), III (**221**), and IV (**222**), and 32-*O*-mono-arabinosyl-YTX (**223**), and 32-*O*-di- and 32-*O*-tri-arabinofuranosyl-YTXs (**224**) were isolated from dinoflagellate.[263–281]

R₁
O
H
H
O
H
O
OH
H
H
O
H
H
H
O
HO_3SO
n
O
H
R_2
O
H
H
O
O
HO_3SO
O
O
H
H
H
H
H

198: R_1 = (OH, H) R_2 = H, n = 1
199: R_1 = (OH, H) R_2 = H, n = 1
200: R_1 = (OH, H) R_2 = H, n = 2
201: R_1 = (O, H) R_2 = H, n = 1
202: R_1 = (O, H) R_2 = H, n = 1
203: R_1 = (O) R_2 = H, n = 1
204: R_1 = (OH, H) R_2 = H, n = 1
205: R_1 = (OH, H) R_2 = CH_3, n = 1
206: R_1 = (O) R_2 = CH_3, n = 1
207: R_1 = (OH, H, OH, HO) R_2 = H, n = 1
208: R_1 = (OH, H, OH, OH) R_2 = H, n = 1
209: R_1 = (OH, H, OH, OH) R_2 = CH_3, n = 1
210: R_1 = (OH, H, OH, O, N, H, OH, OH) R_2 = H, n = 1
211: R_1 = (OH, H, OH, O, N, H, OH, OH) R_2 = CH_3, n = 1
212: R_1 = (OH, H, OH) R_2 = H, n = 1
213: R_1 = (OH, H, O) R_2 = H, n = 1
214: R_1 = (OH, H, O) R_2 = H, n = 1
215: R_1 = (OH, H) R_2 = H, n = 1

HO$_3$SO
HO$_3$SO
H H H H H H H H H H H H H H H H H
O O O O O O O O O
R
OH

216: R =
H OH

217: R =
H O

218: R =
H O

219: R =
O

HO$_3$SO
HO$_3$SO
HO
OR
n

220: R$_1$ = HO OH HO OH H H O O O OH — $n = 2$

221: R$_1$ = HO OH H O OH — $n = 2$

222: R$_1$ = HO OH HO OH HO OH H H H O O O O O OH — $n = 2$

223: R_1 = n = 2

224: R_1 = n = 2

225: R_1 = n = 2

2.09.12 *Symbiodinium* sp.

2.09.12.1 Symbioimine

Symbioimine (**226**) and neosymbioimine (**227**), two amphoteric iminium metabolites, were isolated from a cultured symbiotic marine dinoflagellate *Symbiodinium* sp. isolated from an acoel flatworm *Amphiscolops* sp. collected at Sesoko Island, Okinawa. Symbioimine (**226**) and neosymbioimine (**227**) have a characteristic 6,6,6-tricyclic iminium ring structure and an aryl sulfate moiety. The absolute stereochemistry of symbioimine (**226**) was determined by X-ray analysis. The plausible biogenetic pathways for symbioimine (**226**) and neosymbioimine (**227**) can be explained by an intramolecular Diels–Alder reaction followed by imine cyclization. Symbioimine (**226**) inhibited the differentiation of the murine monocytic cell line RAW264 cells into osteoclasts ($EC_{50} = 44\,\mu mol\,l^{-1}$) and significantly inhibited the cyclooxygenase-2 activity at $10\,\mu mol\,l^{-1}$.[282–285]

226: R_1, R_2 = H
227: R_1, R_2 = CH_3

2.09.12.2 Symbiospirols

Three long carbon-chain compounds, symbiospirols A (**228**), B (**229**), and C (**230**), were isolated from the culture of *Symbiodinium* sp., which was isolated from the marine acoel flatworm *Amphiscolops* sp. collected at Sesoko Island, Okinawa. Their planar structures and partial relative stereochemistries were elucidated based on NMR spectra and a degradation reaction. Symbiospirols consist of a C_{67} linear chain with a β,β'-dihydroxy ketone moiety, eight hydroxy groups, one tetrahydropyran ring, and two 1,6-dioxaspiro[4,4]nonane rings. Symbiospirol A (**228**) exhibited an inhibitory effect against L-phosphatidylserine-induced PKC activation.[286]

228

229

230

2.09.12.3 Zooxanthellabetaine A

Zooxanthellabetaine A (231) was isolated from *n*-BuOH-soluble portion of 70% EtOH extract of cultured *Symbiodinium* sp., which produce zooxanthellatoxins. The structure of zooxanthellabetaine A (231) was seen as 4-(4-hydroxybenzoyloxy)-3-(trimethylammonio)-butyrate.[287]

231

2.09.12.4 Zooxanthellamine

Zooxanthellamine (232), a C_{30} alkaloid, was obtained from the EtOAc-soluble portion of 70% EtOH extract of cultured *Symbiodinium* sp., together with zooxanthellabetaine A (231) and zooxanthellatoxins. The structural

similarity of zooxanthellamine (**232**) and zoanthamine (**233**), an alkaloid isolated from zoanthid, suggested that the real producer of those alkaloids is a dinoflagellate. Biogenetically, zooxanthellamine (**232**) might be derived from a polyketide chain presumably started from a glycine unit.[287]

232 **233**

2.09.12.5 Zooxanthellatoxins and Its Related Compounds

Zooxanthellatoxins A (**234**) and B (**235**) have been isolated from cultures of the symbiontic dinoflagellate *Symbiodinium* sp. isolated from an acoel flatworm *Amphiscolops* sp. collected at Okinawa. The 62-membered lactone rings of zooxanthellatoxins A (**234**) and B (**235**) are highly oxygenated and possess cyclic ethers, a spiroketal moiety, and a sulfate ester at C-59. In addition, they contain an amide linkage. The structures of the zooxanthellatoxins A (**234**) and B (**235**) were elucidated by a combination of spectroscopic analysis and comparison of spectral data of degradative products with synthetic fragments. These toxins elicit contractile responses in rat aorta that may be abolished in the absence of Ca^{2+} or in the presence of verapamil, indicating that they enhance calcium influx in smooth muscle.[288–291]

X =
HO 64 62
HO 66 OH
R_1 = H, R_2,R_3 = OH
234

X =
H (62)
HO (64) OH
HO (66)
R_1 = OH, R_2,R_3 = H
235

Symbiodinolide (**236**), a structural congener of zooxanthellatoxins, was isolated from the symbiotic dinoflagellate *Symbiodinium* sp. Symbiodinolide (**236**) exhibited a potent voltage-dependent N-type Ca^{2+} channel-opening activity at 7 nmol l^{-1} and immediately ruptured the tissue surface of the acoel flatworm *Amphiscolops* sp. at 2.5 μmol l^{-1}. The gross structure of symbiodinolide (**236**) was clarified from spectroscopic analysis and chemical degradations using the second-generation Grubbs' catalyst. The relative stereochemistries of C26–C32, C44–C51, and C64–C66 parts, and the absolute stereochemistries of C-69–C-73, C-83–C-103, and C-3′–C-18′ parts in symbiodinolide (**236**) were established.[292]

236

Zooxanthellamides A (**237**) and B (**238**) were isolated from a cultured marine dinoflagellate *Symbiodinium* sp. (strain HA3-5). In contrast with zooxanthellatoxins, zooxanthellamides A (**237**) and B (**238**) do not possess bisepoxide and *exo*-methylene. An amide and a sulfate groups exist in zooxanthellatoxins, whereas there is a pair of both of these groups in zooxanthellamides A (**237**) and B (**238**). The resemblance of the partial structure suggested that zooxanthellatoxins and zooxanthellamides might be synthesized from a similar biosynthetic pathway. Because zooxanthellamides A (**237**) and B (**238**) showed no vasoconstrictive activity, the existence of a lactone structure in zooxanthellatoxin might be important for vasoconstrictive activity.[293,294]

237: R =

238: R =

239

Zooxanthellamide Cs (**239**) was obtained from a cultured marine dinoflagellate *Symbiodinium* sp. as an inseparable isomeric mixture of polyhydroxylated 61- to 66-membered macrolides. The detailed analysis of 2D NMR data revealed that zooxanthellamide Cs (**239**) were macrolactonized analogs of zooxanthellamide A (**237**). Lability of zooxanthellamide Cs (**239**) suggests that zooxanthellamide A (**237**) is an artifact derived from zooxanthellamide Cs (**239**) during the isolation steps. Three of the components possess the largest ring sizes (63-, 64-, and 66-membered) found to date among the natural macrolides. Zooxanthellamide Cs (**239**) exhibited higher vasoconstrictive activity than that of the zooxanthellatoxins A (**234**) and B (**235**). The structure–activity relationship suggested that the huge macrolactone ring is important for biological activity.[295]

Zooxanthellamide D (**240**), a polyhydroxy amide consisting of a C_{22} acid part and a C_{32} amine part, and which furnishes three tetrahydropyran rings and six isolated butadiene chromophores, was isolated from a cultured marine dinoflagellate *Symbiodinium* sp. The relative stereochemistry of the tetrahydropyran ring was revealed by NMR data. This metabolite showed moderate cytotoxicity against two human tumor cell lines.[296]

240

2.09.13 Biosynthesis of Dinoflagellate Polyketides

In our studies of the biosynthesis of amphidinolides, incorporation patterns of ^{13}C-labeled acetate for amphidinolides B (**2a**), C (**3a**), G (**7a**), H (**8a**), J (**9**), T1 (**19a**), W (**22**), X (**23**), and Y (**24**) were investigated. The incorporation patterns for amphidinolides revealed that the main chain of these macrolides were generated from unusual units derived only from C-2 of acetates in addition to successive polyketide chains. The experiments also indicated that all C_1 branched carbons were derived from C-2 of acetates and attached to C-1 of intact acetate or isolated C-2 of acetate. These unusual incorporation patterns, which might be generated from nonsuccessive mixed polyketide biosynthesis, could be found in most dinoflagellate polyketides (**93,119,155,181,198,** and **232**) in which their biosyntheses have been studied so far.[8,9,15,16,297]

2a

3a

8a **9**

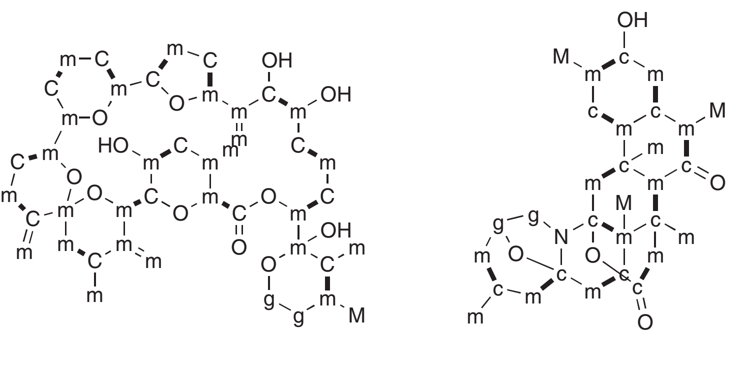
19a **22**

23 **24**

CH_3CO_2Na

2 1
m—c

Acetate incorporation patterns of amphidinolides B (2a), C (3a), H (8a), J (9), T1 (19a), W (22), X (23), and Y (24).



119 **232**

198

181

93

155

CH_3CO_2Na

2 1
m—c

Acetate incorporation patterns of amphidinol 4 (**93**), goniodomin (**119**), brevetoxin B (**155**), okadaic acid (**181**), yessotoxin (**198**), and zooxanthellamine (**232**).

Although incorporation patterns of ^{13}C-labeled carbon and ^{18}O-labeled oxygen for dinoflagellate polyketides have been reported, studies on the biosynthesis gene and the enzyme for dinoflagellate polyketides have rarely been reported. Rein and coworkers amplified approximately 700 bp DNA fragments homologous with ketoacyl synthase (KS) domains in known type I polyketide synthase (PKS) from seven different species of dinoflagellates, including *K. brevis*, using polymerase chain reaction (PCR). Rein and colleagues reported the localization of PKS encoding genes using a combination of flow cytometry/PCR and fluorescence *in situ*

hybridization (FISH). The results indicated that some genes were localized exclusively in *K. brevis* cells, and some genes were localized in both *K. brevis* and associated bacteria. However, these genes have not yet been linked to toxin production.[298,299]

We have attempted to clone the PKS gene for amphidinolide biosynthesis from a dinoflagellate *Amphidinium* sp. (strailn Y-42). Fourteen KS gene fragments were obtained by PCR amplification from degenerated primer sets designed on the basis of the conserved amino acid sequences of KS domains in known type I PKSs. The PCR analyses using primer sets designed from these 14 KS gene fragments revealed that these DNA sequences exist only in the dinoflagellates producing amphidinolides. The DNA sequence of the positive clone, which was isolated from the genomic DNA library of *Amphidinium* sp. (strain Y-42), was analyzed by shotgun sequencing. The deduced gene products in the positive clone showed similarity to KS, acyl transferase (AT), dehydratase (DH), ketoreductase (KR), acyl carrier protein (ACP) in known type I PKSs, and thioesterase (TE).[300]

Recently, Van Dolah and coworker identified eight PKS transcripts in *K. brevis* by using high-throughput cDNA library screening. Full-length sequences obtained through 3′ and 5′ RACE demonstrated the presence of polyadenylation, 3′-UTRs, and an identical dinoflagellate-specific spliced leader sequence at the 5′ end of PKS transcripts. Six transcripts encoded for individual KS domains, one KR, and one transcript encoded both ACP and KS domains. Baysian phylogenetic analysis of the KS domains placed them well within the prokaryotic type I PKS clade. However, the presence of individual catalytic domains on separate transcripts suggests that protein structures are similar to type II PKSs, in which each catalytic domain resides on an individual protein.

To date, no cloning of of the biosynthetic gene cluster of dinoflagellate-derived polyketides has been reported.[301] The huge and unusual genome of dinoflagellate prohibits traditional approaches such as the generation and screening of genomic libraries or construction of gene-disrupted mutants. Although Professor Miller and coworker reported that the dinoflagellates *Symbiodinium microadriaticum* and *Amphidinium* sp. were transformed with plasmid constructs by agitation with silicon carbide whiskers, it is still difficult to transform dinoflagellates.[302] This is one of the reason that makes it difficult to analyze and confirm the function of the putative polyketide gene by gene disruption. However, such an approach is important for understanding the mechanisms of the unique biosynthesis of dinoflagellate-derived polyketides.

2.09.14 Prospects

Dinoflagellates produce structurally diverse and bioactive compounds such as macrolides, long-chain polyketides, and polyethers. As described above, many dinoflagellate metabolites have been recognized as useful bioprobes. For example, okadaic acid (**181**), a protein phosphatase PP-1 and PP-2A inhibitor, has been used as a probe to identify cellular processes that are regulated by phosphorylation and dephosphorylation and to identify phosphatases. Saxitoxin (**120**) and polyether toxins such as brevetoxins and ciguatoxin are very useful tools for understanding the structure and function of the Na^+ channel. Goniodomin A (**119**) is used as a probe for studying relationships between structure and function of contractile proteins as well as interaction between actin and myosin in cardiac muscle. On the other hand, some bioactive dinoflagellate metabolites have potential as new drug leads. Among marine dinoflagellates, the genus *Amphidinium* has undoubtedly proven to be a good source of cytotoxic and antitumor macrolides (amphidinolides, carbenolide I, etc.) and bioactive long-chain polyketides (amphidinols, luteophanols, colopsinols, etc.). Among all the amphidinolides, amphidinolide N (**13**) exhibits remarkably potent cytotoxicity against human tumor cell lines and is expected to be a lead compound for new anticancer drugs.

However, due to poor production of these secondary metabolites and difficulty in large-scale cultivation of these dinoflagellates, biological tests of these bioactive substances remain insufficient. For further biological testing, such poor productivity of these bioactive metabolites needs to be improved, although total syntheses of many bioactive dinoflagellate metabolites have been achieved. Large-scale synthesis of them might be possible due to development of synthetic strategy. Through structure–activity relationship studies of complicated bioactive natural products, it might be possible to discover more simplified analogs like the halichondrin B analog (E7389).[303] Another approach is to identify and clone the PKS gene from the dinoflagellate and to express the biosynthetic genes in a heterologous host. To clarify mechanisms of multiplication of this unicellular alga is also important. For example, it is known that toxic dinoflagellates

(*P. lima*, *K. brevis*, *Hetereocapsa circularisquama*, etc.) are responsible for algal blooms (red tides) through extraordinary multiplication, whereas in cultivation of the dinoflagellate *Amphidinium* sp. multiplication of the microalga automatically ceases after limited cell division. Further development of the cell biology and molecular biology of dinoflagellates is required for biomedical and pharmaceutical applications of dinoflagellate metabolites.[304,305]

Glossary

ciguatera A food poisoning in humans caused by eating marine fish whose flesh is contaminated with toxins that are produced by microorganisms.

dinoflagellate A large group of unicellular protists that exhibit a great diversity of form. Although most of them inhabit the ocean, they can be found in freshwater as well. About half of the dinoflagellates are photosynthetic, and these make up the largest group of eukaryotes aside from the diatoms, and are recognized as important primary producers of the aquatic food chain. Some species are endosymbionts of other marine organisms and play an important part in the biology of coral reefs.

flatworm The simplest of the worm groups. There are about 20 000 species in this group. They are found in both marine and freshwater, and can be free-living or parasitic. The algal symbionts, such as dinoflagellates, are found among cells of the peripheral parenchyma in some cases.

macrolides Macrocyclic lactones with a ring of 12 or more members.

polyketide Natural compounds containing alternating carbonyl and methylene groups, biogenetically derived from acetyl-CoA and malonyl-CoA, and usually generated from repeated condensation of them.

red tide A common name for blooms of algae, more specifically phytoplankton such as dinoflagellate, which can form dense, visible patches near the water surface. These phytoplanktons contain photosynthetic pigments that vary in color from green to brown or red.

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2.10 Marine Invertebrates: Sponges

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2.10.1 Introduction

Sponges (phylum Porifera), often called 'living fossils' or 'most primitive multicellular animals', are the first metazoans evolved from a common ancestor termed Urmetazoa[1] about 600 million years ago, which is evident from molecular data.[2] More than 8000 extant species of sponges have been described,[3] and they are grouped into three classes, Hexactinellida (glass sponges), Demospongiae (demosponges), and Calcarea (calcareous sponges), of which demosponges are most abundant (~95% of sponge species). Evolutionally, glass sponges appeared first, followed by demosponges, and calcareous sponges are believed to be more close to cnidarians.[4]

Sponges are sessile and filter feeders, some of them are able to filtrate their body volumes of water every 5 s to extract edible materials.[5] Their bauplan (body plan) is very simple, consisting of only a few different cell types, namely pinacocytes (epithelial cells), choanocytes (collar cells), sclerocytes, and amoebocytes, the first two of which are most abundant and form 'ectoderm' and 'endoderm', respectively. Sponges live from pole to pole and from intertidal shores to abyssal depths (more than 8500 m deep). They are also found in rivers, ponds, and lakes; approximately 200 species of freshwater sponges were recorded.[6]

Since Bergmann's pioneering discovery[7] of unprecedented nucleosides, for example, spongouridine (**1**), from the Caribbean marine sponge *Cryptotethya crypta*, sponges have been the prime target for drug discovery research on marine organisms, which resulted in the isolation of more than 6700 new compounds (MarinLit, a database of the marine natural products literature, lists 6668 compounds from 1185 species of sponges[8]). These compounds consist of polyketides, nonribosomal peptides, terpenoids, alkaloids, sugars, and metabolites of mixed biogenesis as shown below.

O
HN
O
HO
O
HO
OH
1

2.10.1.1 Sponge Systematics

Sponge systematics are difficult mainly due to the simple bauplan as mentioned above; definitive (true) synapomorphies are yet to be established.[9] Traditionally, spicules made of silicate (or calcium carbonate in the case of calcareous sponges) and proteins have been the most fundamental synapomorphy; size, type, shape, and combination of spicules, and their skeletal arrangements are currently used for taxonomical analysis. Also used are various morphological characters such as shape, surface, texture, or color, but these characters vary with habitat conditions or season. Cytological features might not be sufficient for higher sponge taxonomy. Indeed, the sequence data of 28S rDNA (fragments of the nuclear rDNA) are inconsistent with morphological classification. Perhaps a combination of molecular data and morphological characteristics can make sponge systematics more reliable.[10]

Chemical taxonomy of sponges is also problematic partly due to misidentification of sponges. Contamination of other sponges, mainly overgrowth of other sponges, and, more significantly, the presence of symbiotic microbes lead to inconsistent results, the latter of which is very serious from the viewpoint of chemotaxonomy. Demospongic acids thought to be the specific molecular marker for demosponges were also found in glass sponges, which is likely due to the presence of common bacterial community in the sponges examined.[11] Nevertheless, more than a dozen classes of compounds are considered to be biochemical markers for certain orders, families, genera, or species as shown in **Table 1**.[12,13]

2.10.1.2 Bacterial Symbiosis

Since Vacelet[14] first showed the presence of bacteria in sponges, it has become evident that most sponges live in association or symbiosis with bacteria. Surprisingly, bacterial biomasses reach 50% in some sponges.[15] Therefore, marine natural product chemists became aware of the real origin of 'sponge metabolites'. In fact, considerable

Table 1 Chemotaxonomical markers for sponge taxa

Taxon	*Compound class*
Homosclerophorida	
Plakina, *Corticium* (Plakinidae)	Aminosterols
Spirophorida	
Cinachyrella (Tetillidae)	Hydroxyiminosterols
Astrophorida	
Rhabdastrella (Ancorinidae)	Isomalabaricane triterpenes
Penares (Ancorinidae)	Penaresidins
Hadromerida	
Suberites (Suberitidae)	Suberitane-derived sesquiterpenes
Halichondrida	Isocyanoditerpenes
Axinellidae	Hydroxymethyl-A-nor-sterols; oroidin derivatives
Desmoxyidae	Cyanthiwigin diterpenes
Myrmekioderma	Linear diterpenes
Agelasida	Oroidin derivatives
Agelasidae	Hypotaurocyamines
Poecilosclerida	
Crambeidae	Polycyclic guanidine alkaloids
Haplosclerida	Peroxy sesterterpenes; pyrroloquinoline alkaloids
Xestospongia (Haliclonidae)	Bromoacetylenes
Dictyoceratida	Spongian diterpenes
Spongiidae, Thorectidae, Irciniidae	Scalarane sesterterpenes
Dendroceratida	Spongian diterpenes

numbers of compounds isolated from sponges were similar to metabolites of terrestrial bacteria.[16] Furthermore, more closely related or identical compounds were found in different phyla, and it was natural for chemists to propose that those compounds might be produced by the same or similar microorganisms endemic to hosts. In this context, lithistid sponges are particularly interesting and the prize organisms for marine natural products; they produce a diverse array of bioactive metabolites, including polyketides, nonribosomal peptides, alkaloids, novel steroids, and metabolites of mixed biogenesis, many of which are reminiscent of actinomycete metabolites.[17] Among lithistid sponges, those of the genus *Theonella* are most productive; the most recent version of MarinLit lists 373 compounds isolated from lithistid sponges, of which 237 compounds were from *Theonella* spp.

Faulkner was the first marine natural product chemist to challenge this problem using a classical approach, which led to the discovery of an unculturable new δ-proteobacterium '*Candidatus* Entotheonella palauensis' from *Theonella swinhoei* that was believed to produce novel bicyclic peptides.[18] Later, Piel[19] isolated gene clusters involved in the synthesis of potent cytotoxins of the pederin class using a metagenomic technique. These two cases dealt with uncultured bacteria, but isolation of sponge metabolites from cultured bacteria has been reported, including manzamine A from actinomycetes of the genus *Micromonospora* isolated from an Indonesian sponge *Acanthostrongylophora* sp.[20] However, no evidence has been obtained whether endophytic bacteria found in sponges are symbiotic or associates.[20]

This chapter describes typical sponge metabolites according to the biosynthetic classification, with references to their important roles in drug discovery as well as in marine ecosystems. To avoid duplication, those compounds described in *Comprehensive Natural Products Chemistry* are not described in detail here. We do not deal with freshwater sponges, from which only lipids and steroids were reported, except for okadaic acid from the Baikalian sponge *Lubomirskia baicalensis*.[21]

2.10.2 Structure and Bioactivities of Metabolites Characteristic to Marine Sponges

This section deals with metabolites characteristic to marine sponges and their biological activities, focusing on those reported after *Comprehensive Natural Products Chemistry* (CONAP-I), published in 1999. However, some important compounds described in CONAP I are included so that it will be easy to follow the trends of marine natural products chemistry.

2.10.2.1 Polyketides

Diverse polyketides ranging from simple oxylipins to highly complex polyethers and macrolides have been reported from marine sponges.

2.10.2.1.1 Fatty acid–derived compounds

Antiviral and cytotoxic fatty acid–derived cyclic peroxides are often found in marine sponges of the genus *Plakortis*; the first example was chondrillin (**2**). 1,2-Dioxane and 1,2-dioxolane carboxylates were also discovered from *Plakortis* sponges.[22] These cyclic peroxides show a range of biological activities, for example, antimicrobial, cytotoxic, and antimalarial activities. Sponges also contain a variety of bioactive fatty acid derivatives including glycoceramides.[23]

MeO O–O CO_2H

2

2.10.2.1.2 Open-chain polyketides

Discodermolide (**3**) is a polypropionate-based unique compound isolated from the Caribbean deep-sea sponge *Discodermia dissoluta*. It is immunosuppressive as well as highly cytotoxic. More significantly, it stabilizes microtubules more potently than taxol.[24] Attempts at large-scale synthesis were carried out for clinical trials as an anticancer agent but the clinical trials were discontinued in 2005 due to its toxicity.[25]

Callystatin A (**4**) is a higly potent cytotoxic polyketide isolated from *Callyspongia truncata*.[26] Its absolute structure was determined by chemical method[27] and confirmed by total synthesis.[28] Its potent cytotoxicity ($IC_{50} = 0.022\ nmol\ l^{-1}$ against human pharyngeal carcinoma KB cells) was presumed to be due to the inhibition of nuclear export signal (NES)-dependent export of nuclear proteins.[29,30]

O O OH OH OH OH $OCONH_2$

3

O O O OH

4

2.10.2.1.3 Macrolides

Sponges contain a diverse array of macrolides with intriguing activities. The first sponge macrolide, latrunculin A (**5**), was isolated from the Red Sea collection of *Latrunculia magnifica* as an ichthyotoxic compound and was found to inhibit polymerization of G-actin allosterically. Swinholide A (**6**) is a macrodiolide originally isolated from the Red Sea sponge *T. swinhoei*. It is highly antifungal and cytotoxic, but its primary target is G-actin. Recently, *T. swinhoei* was discovered from cyanobacteria, although its eubacterial origin was predicted.[19] Another class of macrolides that inhibit actin polymerization is the tris-oxazole–containing macrolides, namely kabiramides and mycaolides.[31]

Halichondrin B (**7**), which is a polyether macrolide isolated from *Halichondria okadai*, shows promising antitumor activity and has entered phase I clinical trials.[24,32] Halichondrins have been found in several species of sponges, indicating their microbial origin.[19] Spongistatins/hyrtiostatins/cinachyrolide A (spongistatin-1 (**8**)) are highly unusual, 35-membered macrolides isolated from several sponges.[31] They inhibit growth of tumor cells at subnanomolar levels by binding to the vinca domain of tubulin. Their low yields and occurrence in several different sponges suggest their microbial origin. Spirastrellolide B (**9**) was reported recently as an antimitotic agent from the sponge *Spirastrella coccinea*. It is actually a potent and selective inhibitor of protein phosphatase 2A.[33] Peloruside A (**10**), which is a 16-membered, highly oxidized macrolide from the sponge *Mycale hentscheli*, induces tubulin polymerization.[34] Salarin A (**11**) and tulearin A (**12**) are nitrogenous

5

7

6

8

10

9

11

12

macrolides isolated from the Madagascar *Fascaplysinopsis* sp., showing micromolar level antiproliferating activity against two different human leukemic cell lines of K562 and UT7. Biosynthetic pathways for both compounds remain to be clarified.[35]

13-Deoxytedanolide (**13**) isolated from Japanese sponges of the genus *Mycale* shows promising anti-tumor activity. It inhibits protein synthesis by binding to a 70S large subunit of eukaryotic ribosome.[36] Tedanolide C (**14**) isolated from Papua New Guinean *Ircinia* sp. caused a strong S phase arrest as well as potent cytotoxicity against HCT-116 cells with IC_{50} value of 9.53×10^{-8} mol l^{-1}.[37] An Australian sponge of newly described genus *Candidaspongia* yielded a mixture of candidaspongiolides (**15**), which have a tedanolide-type skeleton with acylating modification at two hydroxy groups in the molecule, acetylated at OH-7 and esterified wth longer fatty acids (C_{13}–C_{17}) at the hydroxy group on C-28.[38] Candidaspongiolides showed potent cytotoxicity in the National Cancer Institute (NCI) human disease-oriented 60-tumor cell line assay with a mean GI_{50} of 14 ng ml^{-1}. It also inhibited protein synthesis and potently induced apoptosis in both U251 and HCT116 cells, in part by a caspase 12-dependent pathway.[39] Myriaporones 1–4 (**16–19**), smaller analogs isolated from the Mediterranean bryozoan *Myriapora truncate*, share the southern hemisphere of the tedanolide family.[40] Myriaporone was shown to promote a rapid, reversible, and p-21-independent activity to block S phase progression in mammalian cells; the C18–19 epoxide and the C14 hydroxymethyl group (tedanolide numbering) were essential for the activity.[41]

13

14

15: R = $C_{13}H_{27}$; $C_{14}H_{29}$; $C_{15}H_{31}$; $C_{16}H_{33}$; $C_{17}H_{35}$; $C_{17}H_{33}$

16

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2.10.2.2 Amino Acids and Peptides

There are many amino acids and peptides possessing highly functionalized or unique chemical skeletons from marine sponges. In many cases, such compounds also show a range of unique and potent biological activities. Recent examples of unusual amino acids, bromotyrosines, and nonribosomal peptides containing unusual or unprecedented amino acids are described here. Although medium-sized peptides composed of 20–30 usual amino acids have been rarely reported from marine sponge, examples of such 'rare' sponge peptides with bioactivities are also introduced.

2.10.2.2.1 Amino acid derivatives

Dysiherbaine (**20**), a novel betaine isolated from a Micronesian collection of *Dysidea herbacea* (it was recently identified as *Lendenfeldia chondrodes*)[42–47] along with neodysiherbaine (**21**)[48] and dysibetaines (**22–25**).[49,50] Dysiherbaine (**20**) and neodysiherbaine A (**21**) are remarkable excitatory amino acids with potent convulsant activity; they actually are selective agonists for (*S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) and kinate class of receptors but not for *N*-methyl-D-aspartate (NMDA) receptors,[51] whereas dysibetaines showed only weak affinities for NMDA and kinate receptors.[50] Cribronic acid (**26**), a metabolite of a Micronesian *Cribrochalina olemda*, showed an agonistic effect for NMDA receptors but not for AMPA or kinate receptors.[52] Recently, the origin of dysiherbaine was suggested to be an endosymbiotic cyanobacterium *Synechocystis* sp.;[53] however, in contrast to the biosynthesis of polyketide synthetase (PKS)/nonribosomal peptide synthetase (NRPS) products, genetic approach to their biosynthesis remains difficult because of a lack of information about biosynthetic genes for such small unusual molecules.

20 **21** **22**

23 **24** **25** **26**

2.10.2.2.2 Bromotyrosine derivatives

Sponges of the families Aplysinidae and Pseudoceratidae, in particular *Pseudoceratina* (=*Psammaplysilla*) *purpurea*, are known to be a rich source of bromotyrosine-derived metabolites. Bromotyrosine derivatives show a variety of biological activities including antimicrobial, enzyme inhibitory, and antifouling activities. Psammaplysin A (**27**) is antimicrobial, cytotoxic, and antifouling, whereas psammaplin A (**28**) is an inhibitor of histone deacetylase (HDAC) and DNA methyltransferase (DNMT).[54,55] The marine sponge *Ianthella basta* synthesizes at least 25 bastadins that are linear or cyclic peptides composed of four bromotyrosine residues and that show antimicrobial, antiangiogenic, cytotoxic, and enzyme-inhibitory activities as well as interaction with ryanodine receptor 1 (RyR1)/FKBP12 Ca^{2+} channel complex. Bastadin 5 (**29**) enhances release of Ca^{2+} from stores within the sarcoplasmic reticulum (SR) of muscle and nonmuscle cells by modulating ryanodine binding to the RyR1/FKBP12 complex. Structure-activity relationship (SAR) study using simplified analogs of bastadin 5 revealed that the essential structural element for its binding activity is not oxime moiety but the bromocatechol ether unit.[56] Bastadin 6 exerts antiangiogenic effects *in vitro* and *in vivo* through the selective induction of apoptosis of endothelial cells. In this case, oxime moiety plays a crucial role for its potent antiangiogenic activity, indicating that the target of bastadin 6 (**30**) should not be the RyR1/FKBP12 complex.[57]

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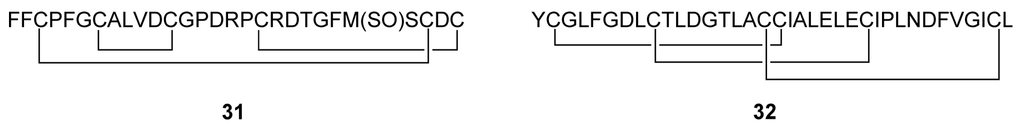
2.10.2.2.3 Medium-sized peptides

Apart from the NRPS products that contain unusual amino acids, sponges also produce medium-sized (20–40 residues) peptides consisting of usual L-amino acids. Neopetrosiamides A and B (**31**), which differ only by stereochemistry at the sulfoxide functionality of the methionine sulfoxide (M(SO)) residue derived from a methionine, were isolated from Papua New Guinean *Neopetrosia* sp. They inhibited amoeboid invasion by human tumor cells at 6 μg ml^{-1}.[58] Asteropine A (**32**) is a peptide consisting of 36 usual L-amino acids isolated from *Asteropus simplex*. The structure of asteropine contains three disulfide bonds between Cys residues to form a rigid cystine knot scaffold. Asteropine A is the first cystine knot of sponge origin showing potent and competitive inhibition against bacterial sialidases (from *Clostridium perfringens*, *Vibrio cholerae*, and *Salmonella typhimurium*) with K_i values of 36.7, 340, and 350 nmol l^{-1}, respectively.[59]



31

32

2.10.2.2.4 Nonribosomal peptides

Nonribosomal peptides of sponge origin have been reviewed.[60,61] To avoid duplication, those that were discovered thereafter are briefly described here. Microsclerodermins A (**33**) and B (**34**) are antifungal cyclic peptides originally isolated from a New Caledonian deep-sea lithistid *Microscleroderma* sp.[62] Related analogs were also isolated from several lithistid sponges collected from the Philippines[63] and Palau,[64] suggesting involvement of symbiotic microbes in their production. These compounds are highly antifungal and cytotoxic. Nagahamide A (**35**) isolated as an antibacterial depsipeptide from Japanese *Theonella swinhoei* contains an unusual amino acid, 4-amino-3-hydroxybutanoic acid, which was also contained in microsclerodermins.[65] The polyketide portion of this compound, 8,10-dimethyl-9-hydroxy-7-methoxytrideca-2,4-dienoic acid, which also shares the partial structure with YM47522, an antifungal metabolite of *Bacillus* sp., except for the geometry of one double bond.[66,67] Phoriospongin A (**36**), a nematocidal depsipeptide, was isolated from sponges of the orders of Poescilosclerida and Haplosclerida.[68] Because the related cyclolithistid A was found in the other taxonomic order of Lithistida, it suggested their origin for symbiotic microbes.[69] Callipeltin A (**37**) isolated from New Caledonian Lithistida sponge *Callipelta* sp. is an anti-HIV cyclic depsipeptide containing three new amino acids β-methoxytyrosine, (2*R*,3*R*,4*S*)-4-amino-7-guanidino-2,3-dihydroxyheptanoic

acid, and (3*S*,4*R*)-3,4-dimethyl-L-glutamine. Callipeltin A protected cells from HIV infection at ED_{50} of 0.01 μg ml^{-1} (selectivity index ratio of 29 over its cytotoxicity).[70] Similar depsipeptides named papuamides A (**38**) and B (**39**) were isolated from the sponge *T. mirabilis* and *T. swinhoei* collected in Papua New Guinea.[71] Recently, the mode of action for papuamide B was disclosed to compromise yeast cell membrane integrity through a direct interaction with phosphatidylserine (PS). Because there is evidence that PS in the outer leaflet of the HIV-1 membrane is required for HIV infection, it is suggested that papuamide B inhibits HIV infection through its interaction with PS.[72] The other example of the related depsipeptide, homophymine A (**40**), was isolated from New Caledonian *Homophymia* sp. Homophymine A (**40**) effectively inhibited the production of HIV-1 infection with an IC_{50} of 75 nmol l^{-1}, while its cytotoxicity against the host cells (PBMC cell line) remains at TC_{50} 1.19 μmol l^{-1}. Because **40** lacking β-methoxytyrosine in its molecule showed the equivalent anti-HIV activity as callipeltin A, papuamides, and neamphamide A, this residue turned out to be not essential for the anti-HIV activity.[73]

33 R = OH
34 R = H

35

36

37

38 R = CH_3
39 R = H

40

Dysinosin A (**41**) is an inhibitor of factor VIIa and thrombin isolated from the Australian sponge belonging to the family Dysideidae.[74] Related dysinosins B–D were isolated from another species of Australian sponge *Lamellodysidea chlorea*.[75] They belong to the aeruginosin family produced by freshwater cyanobacteria, many of which strongly inhibit serine proteases. The structural similarity of the dysinosins to the aeruginosins implied that they may be biosynthesized by associated microbes.[76] Miraziridine A (**42**), a cathepsin B inhibitor isolated from Japanese *T.* aff. *mirabilis*, contains three unusual amino acid residues, including particularly an unusual vinylogous arginine and aziridine-2,3-dicarboxylic acid units.[77] Because miraziridine A contains three inhibitory elements of aziridine-2,3-dicarboxylic acid (cysteine protease), statine (aspartyl protease), and vinylogous arginine (serine protease), its inhibitory activities against cathepsin L, pepsin, and trypsin were tested as well as that against cathepsin B; as a result, it showed inhibitory activities against all these enzymes.[78] Azumamides A–E, cyclic tetrapeptides, isolated from *Mycale izuensis*, contain unusual β-amino acids. The major compound azumamide A (**43**) showed inhibitory activity against deacetylation of Ac-H3 (Lys9 and Lys14) and Ac-H4 (Lys8) in a dose-dependent manner in a cell-based assay using K562 cells, as well as antiangiogenic activity.[79,80] Later, azumamide E (**44**) was found to be more active than **43** and was also antiangiogenic in an *in vitro* vascular organization model using mouse iPS cells.[81] Halipeptin A (**45**), a depsipeptide isolated from *Haliclona* sp. collected in Vanuatu, consists of two Ala residues and 3-hydroxy-2,2,4-trimethyl-7-methoxydecanoic acid (HTMMD), as well as two unusual amino acids such as *N*-methyl-δ-hydroxyisoleucine (NMeOHILe) and 2-[1-(*S*)-t-butoxycarbonylaminoethyl]-4-(*R*)-methyl-4,5-dihydrothiazole-4-carboxylic acid,[82] the latter of which was formerly misassigned as 1,2-oxazetidine-4-methyl-4-carboxylic acid (OMCA).[83] Halipeptine A showed potent anti-inflammatory activity *in vivo*, causing 60% inhibition of edema in mice at the dose of $300\,\mu g\,kg^{-1}$ (i.p.).[76] Bengamide A (**46**) was isolated originally as anthelmintic agents from a Fijian *Jaspis* sp.[84] and was later found to inhibit growth of tumor cells as well as methionine aminopeptidases.[24]

Polytheonamide B (**47**), the largest nonribosomal peptide of marine origin isolated from *T. swinhoei*, showed the potent cytotoxicity by forming transmembrane ion channels.[85–87]

41

42

45

43 R = NH_2
44 R = OH

46

47

2.10.2.2.5 PKS/NRPS metabolites

Salicylihalamide A (**48**) is a salicylic acid-containing macrolide enamide isolated from *Haliclona* sp., and it inhibits V-ATPases at a low nanomolar concentration. Members of this family have been isolated from sponges, tunicates, and bacteria.[33] Phorboxazole A (**49**) is an unusual oxazole-containing antifungal and cytostatic macrolide isolated from a sponge *Phorbas* sp.[88] An Australian collection of *Chondropsis* sp. contained chondropsins A (**50**) and B (**51**),[89] potent cytotoxic compounds that inhibit V-ATPase.[90] Dysidazirine (**52**) is a cytotoxic azacyclopropene isolated from Fijian *Dysidea fragilis* showing cytotoxicity against L1210 cells at 0.27 μg ml^{-1} and inhibiting the growth of Gram-negative bacteria (*Pseudomonas aeruginosa*) and yeast (*Candida albicans* and *Saccharomyces cerevisiae*) at a minimum inhibitory concentration (MIC) of 4 μg per disk in a standard paper disk assay.[91] Halogenated azacyclopropenes (**53–54**) were also isolated from *D. fragilis*.[92] Another specimen of *D. fragilis* collected in Pohnpei yielded heterogeneous terminal halogenation products (**54**). Biosynthetic pathways to these long-chain 2*H*-azirine compounds remains unknown; however, a common theme of halogenation followed by dehydrohalogenation at each of the termini may explain the formation of ω-vinyl halides and azirines.[93]

Marine natural products of the pederin class (mycalamides, onnamides, and theopederins) isolated from sponges are mixed biogenesis metabolites of polyketide synthase and nonribosomal peptide synthase.[31] In fact, biosynthesis gene clusters of this class have been cloned recently using metagenomic techniques from the marine sponge *T. swinhoei*.[19] These compounds are potently cytotoxic; theopederin A (**55**) inhibits protein synthesis in a similar mode of action to that of 13-deoxytedanolide. Psymberin/irciniastatin A (**56**), isolated from *Psammocinia* sp., collected in Papua New Guinea[94], and *Ircinia ramosa*, collected in Malaysia[95], represents a new skeleton as pederinlike compounds lacking the A-ring oxane and the 1,3-dioxane ring. Psymberin showed selective cytotoxicity against several melanoma (MALME-3M, SK-MEL-5, and UACC-62 at $LC_{50} < 2.5\ \text{nmol}\ l^{-1}$), breast cancer (MDA-MB-435 at $LC_{50} < 2.5\ \text{nmol}\ l^{-1}$), and colon cancer cell lines (HCT-116 at $<2.5\ \text{nmol}\ l^{-1}$), whereas irciniastatin A showed potent cytotoxicity against BXPC-3 (pancreas), MCF-7 (breast), SF268 (CNS), NCI-H460 (lung), KM20L2 (colon), DU-145 (prostate), and P-388 (leukemia) with GI_{50} values of less than $5\ \text{nmol}\ l^{-1}$. Interestingly, psymberin with the same planar structure as irciniastatin A showed only a moderate cytotoxicity ($LC_{50} > 25\ \mu\text{mol}\ l^{-1}$) against the same MCF-7 cells to which irciniastatin A showed strong cytotoxicity (GI_{50} $5\ \text{nmol}\ l^{-1}$). The only possible explanation for this inconsistent activity between the same planar structures of psymberin and irciniastatin A is due to the difference in stereochemistry between both compounds, although both compounds possess the identical spectral properties.[94] Calyculin A (**57**) is an extraordinary metabolite composed of C_{28} fatty acid and two amino acids isolated from the sponge *Discodermia calyx*. It is not only highly antifungal and antitumor but also a potent cancer promoter that was found to be caused by potent inhibition of protein phosphatases 1 and 2A.[31] More than 15 calyculin derivatives were isolated from several marine sponges, which indicates the involvement of symbiotic microorganisms in the production of calyculins.[19] Pateamine (**58**) is a macrolide isolated from a New Zealand *Mycale* sp. Its potent cytotoxicity was attributed to inhibition of transcriptional initiation.[96]

2.10.2.3 Alkaloids

Marine sponges produce a wide range of alkaloids with potent bioactivities, which include such specific classes as 3-alkylpiperidine, guanidine, indole, polyamine, pyridoacridine, and pyrrole-imidazole alkaloids. Their biological activities vary from antimicrobial to neurological.

2.10.2.3.1 Alkylpiperidines

A variety of 3-alkylpiperidine-derived compounds have been obtained from sponges belonging to five families of the order Haplosclerida.[97] They show a range of bioactivities, for example, cytotoxic, antimalarial, and antifouling. It is likely that 3-alkylpiperidines are produced by sponge cells but not by symbiotic microorganisms. The first 3-alkylpiperidine derivative reported is halitoxin (**59**), which was isolated from *Haliclona* sp.[98] Similar polymeric alkylpyridines are also known from several sponges. In addition to the polymers, various types of metabolites of 3-alkylpyridines or 3-alkylpiperidines have been isolated, namely, macrocyclic bis-3-alkylpiperidine, cyclostellettamine A (**60**), which showed a HDAC-inhibitory activity.[99] Bis-quinolizadine, petrosin A (**61**),[100] and bis-1-oxaquinolizadine macrocycles, xestospongin C (**62**),[101] isolated from *Petrosia seriata* and *Xestospongia* spp., respectively, also belong to this group. The former is ichthyotoxic, whereas the latter is a potent vasodilator as well as an inhibitor of IP_3 receptor. Halicyclamine (**63**), isolated from *Haliclona* sp. is another group of macrocyclic bis-3-alkylpiperidines,[102] whereas sarain A (**64**) isolated from *Reniera sarai* has a more complex polycyclic core.[103] These compounds are moderately cytotoxic. The most well-known group of 3-alkylpiperidine alkaloids is the manzamines. Manzamine A (**65**), the first member of this group, was isolated from Okinawan *Haliclona* sp.[104] More than 20 manzamines have been isolated from sponges of eight different genera. The biosynthetic route for manzamines from reductive coupling product of two C_3 and two C_{10} units with two ammonias through Diels–Alder adduct of resulting bis-dihydropyridine was proposed by Baldwin and Whitehead.[105] Manzamine A is highly cytotoxic, antitubercular, and antimalarial, but its mode of action remains unknown.[106] Polycyclic alkaloids (njaoamines A–H (**66–73**)) were isolated from two Tanzanian sponge collections of *Reniera* sp.[107] and *Neopetrosia* sp.[108] Njaoamines A-F (**66–71**) showed cytotoxicity against A-549, HT-29, and MDA-MB-231 cells with GI_{50} values ranging from 1.5 to 7.2 μmol l^{-1}, whereas G (**72**) and H (**73**) showed potent toxicity in the brine shrimp (*Artemia salina*) assay with IC_{50} values of 0.17 and 0.08 μg ml^{-1}, respectively.

59 *n* = 3, 4, 5, 6

60

61

62

63

64

65

66 $R^1 = OH$, $R^2 = Me$, $R^3 = H$
67 $R^1 = OH$, $R^2 = R^3 = Me$
68 $R^1 = R^2 = R^3 = H$
69 $R^1 = R^3 = H$, $R^2 = Me$

70

71

72 R = H
73 R = OH

2.10.2.3.2 Indole and quinoline alkaloids

Many indole-containing metabolites have been reported from marine organisms, some of which were already mentioned. This section focuses on important indole-containing substances that belong to structural classes not mentioned above. These alkaloids show antimicrobial, antiparasitic, antitumor, and enzyme-inhibitory activities.[78] Dragmacidin (**74**) is a novel bis-indole isolated from a deep-sea sponge *Dragmacidon* sp.,[109] whereas another deep-sea sponge, *Spongosorites ruetzleri*, contains a similar bis-indole, topsentin (**75**).[110] Aaptamine (**76**),[111] a cytotoxic benzonaphthyridine alkaloid isolated from the sponge *Aaptos aaptos*, induces differentiation in chronic leukemia cells.[112] Schulzeine A (**77**) is a novel dihydroquinoline alkaloid isolated from the sponge *Penares schulzei* that inhibits glycosidases.[113] Recently, 77 syntheses were achieved; its stereochemistry at C-20′ was revised from 20′*S* to 20′*R*.[114]

Sponges and tunicates contain tetrahydroisoquinoline alkaloids.[115] Renieramycin A (**78**), distilled from the sponge *Reniera* sp., represents the first example of this particular class of alkaloids isolated from marine organisms. Exiguaquinol (**79**) is a pentacyclic hydroquinone isolated from Australian *Neopetrosia exigua*. Exiguaquinol inhibited *Helicobacter pylori* MurI, a glutamate racemase essential for the survival of *H. pylori* with an IC_{50} value of 4.4 $\mu mol\, l^{-1}$.[116] The Papua New Guinean *N. exigua* yielded an indoleamine-2,3-dioxygenase (IDO) inhibitor, exiguamine A (**80**). IDO is thought to be responsible for providing immune protection and is also known to be overexpressed in most tumors leading to immune escape by solid tumors. Therefore, inhibiting this enzyme can be an attractive approach to cancer treatment. Exguamine A showed IDO inhibition with a K_i value of 210 $nmol\, l^{-1}$, revealing it to be the most potent inhibitor to date.[117] Recently, biomimetic synthesis of exguamines A and B was also achieved.[118]

2.10.2.3.3 Pyridoacridines and pyrroloiminoquinones

Pyridoacridines are highly colored polycyclic alkaloids mainly isolated from sponges and tunicates.[119] They are classified into four groups according to the ring numbers of 4, 5, 6, and 8. From marine sponges, members of 5 and 6 rings were obtained. The first pyridoacridine identified is pentacyclic amphimedine (**81**) isolated from *Amphimedon* sp. collected in Guam.[120] Amphimedine was not cytotoxic, while neoamphimedine (**82**) isolated from *Xestospongia* sp. collected in the Philippines and Micronesia[121] showed topoisomerase II-mediated cytotoxicity with IC_{50} values of 0.83–7.6 $\mu mol\ l^{-}$.[122] Dercitin (**83**) is a thiazole-containing pentacyclic pyridoacridine[123], whereas cyclodercitin (**84**) is a member of the hexacyclic group.[124] Both compounds were isolated from a deepwater *Dercitus* sp. from the Bahamas, and their structures were revised in 1992.[125] Detailed evaluation for the biological activities of **83** indicated that it inhibits proliferation of several cancer cell lines at IC_{50} values of 63–240 $nmol\ l^{-1}$ as well as shows *in vivo* antitumor activity. It was also indicated that **83** disrupted DNA and RNA synthesis with less effects on protein synthesis, similar to the effects of known DNA intercalators.[126] Biological activities of pyridoacridines are well summarized in the review by Marshall and Barrows.[127]

Marine pyridoacridines show a wide range of biological activities, such as antimicrobial, antiviral, antiparasitic, insecticidal, antitumor, and enzyme inhibitory.[128] Discorhabdin C (**85**), the first marine pyrroloiminoquinoline alkaloid, was isolated from *Latrunculia* sp. collected in New Zealand as a highly cytotoxic pigment.[129] An additional example of this family is batzelline A (**86**) from a deep-sea sponge of the genus *Batzella*.[130] Batzelline A was evaluated in the cell line panel at the NCI and found to express selective cytotoxicity against several melanoma cell lines.[131] The other pyrroloiminoquinone, makaluvamine A (**87**), isolated from Fijian sponges of the genus *Zyzzya*, showed topo II inhibition with IC_{90} values of 41 $\mu mol\ l^{-1}$, respectively, whereas the

structurally related makaluvone and damirone B were not active. Makaluvamine A (87) exhibited *in vivo* antitumor activity against the human ovarian carcinoma Ovcar3 implanted in athymic mice.[132]

85 **86** **87**

2.10.2.3.4 Guanidine alkaloids

Many guanidine-containing compounds have been reported from diverse marine organisms.[133] Ptilomycalin A (88) is a novel polycyclic guanidine alkaloid isolated from the sponge *Ptilocaulis spiculifer*.[134] It is highly cytotoxic, antifungal, and antiviral. The related alkaloids were reported from the Mediterranean sponge *Crambe crambe* that also contains other types of guanidine alkaloids such as crambescin A (89). Batzelladines are a similar class of alkaloids isolated from *Batzella* sp.; batzelladine A (90) shows anti-HIV activity. Variolin B (91), which is a pyridopyrropyrimidine alkaloid isolated from the sponge *Kirkpatrichia varialosa*, is strongly cytotoxic, antifungal, and antiviral.[115] It inhibits cyclin-dependent kinases.[12]

A wide variety of oroidin class compounds derived from two components such as 3-amino-1-(2-aminoimidazolyl)-prop-1-ene (92) and 4,5-dibromopyrrole-2-carboxylic acid (93) are well summarized in the review by Mourabit and Potier.[135] The parent compound, oroidin (94), was initially isolated from *Agelas oroides* in 1971.[136,137] Girolline (95), which is a potent cytotoxin isolated from the sponge *Pseudaxinyssa cantharella*, inhibits protein synthesis by acting preferentially on the termination step rather than the intiation or elongation steps. Girolline proceeded to phase I clinical trials, but the trials were stopped due to a significant side effect of hypertension.[138] There are also dimeric derivatives such as ageliferin,[139] sceptrin,[140] axinellamine,[141] or mauritiamine.[142] Palau'amine (96) is a cytotoxic and immunosuppressive compound originally isolated from the Palauan *Stylotella agminata*.[143] More than 10 years after it was first publicized, the revised structure (97) for palau'amine was suggested by three groups almost coincidentally.[144–146] The detailed overview of the structural revision was documented by Köck *et al.*[147] Ageladine A (98) is a matrix metalloproteinase (MMP)-inhibiting fluorescent alkaloid isolated from the sponge *Agelas nakamurai*. Unlike other MMP inhibitors, ageladine A (98) was not capable of chelating Zn^{2+} and is not a competitive inhibitor of MMP2.[148] Due to those interesting aspects of its fluorescent nature, mode of inhibition, as well as antiangiogenic activity, 98 became a target for total synthesis[149,150] or was evaluated as the potential fluorescent pH sensor, which can be used for detection of intracellular pH changes.[151]

Stellettamide A (99), an unusual indolizidine alkaloid originally isolated from *Stelletta* sp. as an antifungal and cytotoxic compound,[152] was found to inhibit Ca^{2+}/calmodulin-dependent phosphodiesterase and (Ca^{2+}–Mg^{2+})-ATPase with IC_{50} values of 52 and 100 μmol l^{-1}, respectively.[153] Three related alkaloids stellettazole A (100)[154] and bistellettadines A (101) and B (102)[155] from the same sponge showed moderate inhibitory activity against Ca^{2+}/calmodulin-dependent phosphodiesterase (stellettazole A: 45% inhibition at 100 μmol l^{-1}, bistellettadines A and B: 40% inhibition at 100 μmol l^{-1}).

2.10.2.3.5 Polyamines

Interesting biological functions have been revealed for polyamines of sponge origin. Penaramides (103) were isolated as a mixture of acylating variants from *Penares* aff. *incrustans*. Penaramides are the first example of nonpeptidic natural products that inhibit binding of ω-conotoxin GVIA to N-type Ca^{2+} channels, which is involved in the regulation of cytosolic Ca^{2+} concentration in neurons. To confirm their characteristic structural features and biological activity, the simplest derivative, penaramide A, containing two linear C11 fatty acids was synthesized. Penaramides and the synthetic penaramide A both inhibited the binding of ^{125}I-ω-CgTx GVIA to N-type Ca^{2+} channels with IC_{50} values of 1.3 and 5.8 μmol l^{-1}, respectively.[156] Spermatinamine (104) is an alkaloid isolated from Australian *Pseudoceratina* sp. with a bromotyrosyl–spermine–bromotyrosyl sequence. It inhibited isoprenylcysteine carboxyl methyltransferase catalyzing the carboxyl methylation of oncogenic proteins in the final step of a series of posttranslational modification with an IC_{50} value of 1.9 μmol l^{-1}.[157] The other examples are the long-chain polyamines (LCPAs, 105) from *Axinyssa aculeata*. It was suggested that these LCPAs were involved in silica deposition and spicule formation in sponges.[158]

88

89

90

91

92 + 93 X,Y = H or Br

94

95

96 (incorrect)

97 (correct)

98

99

100

101

102

103 R, R′ =

104

105 $n/2\ SO_4^{2-}$ $n = 4–14$

2.10.2.4 Terpenoids and Steroids

Although terpenoids that are similar to terrestrial ones are found commonly in marine organism, particularly in algae, several terpenoids with new or modified skeletons have been isolated frequently from algae, sponges, and cnidarians. Halogenated terpenoids are often found in algae, whereas sulfated terpenoids and steroids are distributed widely in sponges.

2.10.2.4.1 Isocyanoterpenoids

Isocyanide-containing natural products are rare; they have been reported only from cyanobacteria, *Penicillium* fungi, marine sponges, and nudibranchs.[159,160] Sesquiterpenoid and diterpenoid isocyanides are found in a limited species of sponges and nudibranchs that prey on these sponges. Axisonitrile-1 (**106**) isolated from *Axinella cannabina* is the first isocyanide-containing marine natural product.[161] 9-Isocyanopupukeanane (**107**) was isolated originally from the Hawaiian nudibranch *Phyllidia verucosa* and later from its prey, a sponge, *Hymeniacidon* sp later reclassified as a Ciocalypta sp.[162] Kalihinol A (**108**) and 7,20-diisocyanoadocane (**109**) were isolated from the sponges *Acanthella carvenosa*[163] and *Adocia* sp.,[164] respectively. Isocyanoterpenes are often accompanied by thiocyanates, isothiocyanates, and formamides. These terpenoids show a wide range of bioactivities, which include antimicrobial, cytotoxic, ichthyotoxic, antifouling, and antimalarial activities. The carbonimidic dichloride group is considered to be equivalent to isocyanide, and the first sesquiterpenoid that contains this moiety (**110**) was isolated from the sponge *Pseudoaxinyssa pitys*. Terpenoids that contain carbonimidic dichloride show similar bioactivities to those of isocyanide counterparts.[165]

106 **107** **108** **109** **110**

2.10.2.4.2 Spongian diterpenes

Spongian diterpenes are a chemical marker for dictyoceratid and dendroceratid sponges.[166] The first example of a spongian diterpene was isoagatholactone (**111**) isolated from *Spongia officinalis*.[167] In addition to those possessing a basic spongian skeleton, a wide variety of rearranged spongian diterpenoids have been reported, which include gracilin B (**112**) from *Spongionella gracilis*.[168] Many spongian diterepenes are antimicrobial and cytotoxic. Agelasine A (**113**), which is a 9-methyladenine derivative of diterpene isolated from a sponge *Agelas* sp., is antimicrobial and inhibitory against Na, K-ATPase.[169]

111 **112** **113**

2.10.2.4.3 Sesterterpene peroxides

Sigmosceptrellin A (**114**), a norsesterterpene peroxide, was isolated as a methyl ester from the Papua New Guinean *Sigmosceptrella laevis*. It consists of the ichthyotoxic fraction of this sponge along with its stereoisomers B and C.[170,171] Mycaperoxide A (**115**) was related to sigmosceptrellins, in which 13-Me was rearranged to C-14 and an additional hydroxy group on C-18. Mycaperoxide was isolated from the Thai sponge *Mycale* sp. and showed significant cytotoxicity, antimalarial activity, and *in vitro* antiviral activity against vesicular stomatitis virus and herpes simplex virus type-1 (HSV-1).[172] Red Sea *Diacarnus erythraenus* yielded a cytotoxic monocyclic peroxide, aikupikoxide A (**116**)[173] as well as three bicyclic peroxides, tasnemoxide A (**117**)–C.[174] Peroxides including fatty acids and terpenes from marine source were reviewed by Casteel[175] and Dembitsky.[176]

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2.10.2.4.4 Triterpenoid polyethers

Only few sponges are also known to produce triterpenes, such as squalene-derived cyclic ethers consisting of two separate polycyclic systems connected with different types of linkers. The first is sipholenol (118), isolated from the Red Sea sponge *Siphonochalina siphonella*.[177] Structurally related sodwanones F (119),[178] yardenone (120),[179] or abudinols (121)[180] have been isolated from the marine sponge *Axinella weltneri*, *A.* cf. *bidderi*, or *P. spiculifer*.

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2.10.2.4.5 Terpenoids of mixed biogenesis

Furanosesterterpenes are frequent sponge metabolites with a wide variety of bioactivities. Palinurin (122) is an aldose reductase–inhibiting linear furanosesterterpene isolated from Mediterranean *Ircinia variabilis*.[181] Later, it was shown to be biocatalytically converted to the corresponding lactam, palinurine A (123) by the fungus *Cunninghamella* sp. NRRL 5695.[182] This result suggested that the production of pyrrolsesterterpenes such as 124 and 125 found in Korean *Sarcotragus* sp. might also be converted biocatalytically by the symbiotic microbes.[183,184]

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Meroterpenoids represented by avarone (**126**),[185] ilimaquinone (**127**),[186] or halenaquinone (**128**)[187] are also known as rich sponge metabolites with a wide variation of chemical modifications. The C-2 epimer of nakijiquinone C (**130**) was designed based on the receptor kinase inhibiting nakijiquinones isolated from the Okinawan sponge of the family Spongiidae. This compound showed potent and selective inhibition (IC_{50} 21 $\mu mol\,l^{-1}$) against tyrosine kinase VEGFR2, the receptor for the vascular endothelial growth factor (VEGF) deeply involved in tumor angiogenesis.[188,189] A first naturally occurring meroterpenoid–nucleoside conjugate, avinosol (**132**) was isolated as an anti-invasive compound along with the related 3′-aminoavarone (**129**) and 3′-phenethylaminoavarone (**131**) from *Dysidea* sp., collected in Papua New Guinea.[190]

2.10.2.4.6 Steroidal alkaloids

Although steroidal alkaloids are well-known metabolites of certain terrestrial plants, only a limited number of this class of compounds have been reported from a marine source. The first examples of marine steroidal alkaloids are plakinamines A (**133**) and B (**134**) isolated from Micronesian *Plakina* sp. These compounds showed antimicrobial and antifungal activity against *Staphylococcus aureus* and *Candida albicans*, respectively.[191] Ten years later, closely related lokysterolamines A (**135**) and B (**136**) were reported from Indonesian *Corticium* sp. as metabolites possesssing antimicrobial activity against *Bacillus subtilis* and *C. albicans*, cytotoxicity against several cancer cell lines such as P-388, A-549, HT-29, and MEL-28 (IC_{50} values 0.5 to $>2\,\mu g\,ml^{-1}$), as well as immunomodulatory activity (LcV/MLR > 187).[192] Cortistatins (**137**), antiangiogenic steroidal alkaloids isolated from *Corticium simplex*, showed extremely potent and highly selective growth inhibition against human umbilical vein endothelial cells (HUVECs).[193] Characteristic features of cortistatins include a seven-membered ring and an isoquinoline unit and make its structure striking, but the basic skeleton is common to those of plakinamines and lokysterolamines, the latter of which were isolated from the sponge of the same genus. This suggests that there may be common biosynthetic precursors. The structure–activity relationship and biological property of cortistatins were also evaluated to show that cortistatin A did not inhibit VEGF-induced phosphorylation of ERK1/2 and p38, one of the signaling pathways for migration and tube formation,

but instead inhibited phosphorylation of the unidentified 110 kDa protein in HUVECs.[194] Total synthesis of cortistatin A was achieved in 2008.[195] Clionamines A–D (**138–141**) are the other type of amino sterols that lack nitrogen atoms in the side chains. Clionamines were isolated from the South African *Cliona celata* as autophagy-modulating agents stimulating autophagy, particularly in starvation conditions.[196]

133: $R^1 = R^2 = R^3 = H$
135: $R^1 = R^2 = Me$, $R^3 = OH$
136: $R^1 = Ac$, $R^2 = H$, $R^3 = OH$

134

137

138 Δ^{22}
140 Δ^{25}

139

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2.10.2.5 Sugars

Several unusual polysaccharides have been isolated from sponges,[197] as well as triterpene oligoglycosides such as eryloside D (**142**) from New *Caledonian Elyrus* sp.[198] Latrunculinoside A (**143**) was a decalactone-containing glycoside isolated from Red Sea *Latrunculia corticata*. This compound showed no activity in antibacterial, antifungal, brine shrimp toxicity, or sea urchin eggs assays, but showed antifeeding activity against gold fish at concentrations of 10–100 μg ml^{-1}.[199] Caminoside A (**144**) isolated from Dominican *Caminus sphaeroconia* showed inhibition against bacterial type III secretion at IC_{50} 20 μmol l^{-1} along with *in vitro* inhibition against methicillin-resistant *S. aureus* (MIC 12 μg ml^{-1}) and vancomycin-resistant *Enterococcus* (MIC 12 μg ml^{-1}).[200] Among polysaccharides obtained from sponges, the most unusual is axinelloside A (**145**), which has been isolated recently as a potent telomerase inhibiter from *Axinella infundibula*[201] and resembles bacterial lipopolysaccharides.

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X = SO_3Na

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2.10.3 Sponge Metabolites as Drug Leads

No drug obtained directly from marine sources has yet made it to the commercial sector in spite of their wide availability of unique chemical skeletons and biological activities. However, there are significant numbers of interesting compounds originating from marine natural products that are in clinical or advanced preclinical stages.[24,102] Trends of developing useful small molecules utilizing marine natural products as the starting leads will be more popular because of the advancement of chemical synthetic strategies or drug design methodologies. It is also an appropriate and practical way to determine the chemistry of marine natural products when we think about the prospects of the future from the viewpoints of sustainable supply and conservation of marine natural resources.

2.10.3.1 Anticancer Agents

2.10.3.1.1 Halichondrin B

Halichondrin B (**7**), a highly cytotoxic metabolite isolated from the Japanese sponge *H. okadai*[202,203] is a tubulin assembly inhibitor,[204] which binds to the colchicine domain. The macrocyclic portion seems to be essential for the activity.[24] Eribulin (E7389: **146**) is a drug candidate designed on the basis of the macrocyclic portion of halichondrin B and is now under phase II/III trials for breast cancer conducted by Eisei Co. Ltd., Tokyo.[205]

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2.10.3.1.2 Hemiasterlin

Hemiasterlin (**147**), originally isolated from the South African *Hemiasterella minor*[206] followed by the isolation from the Papua New Guinean *Cymbastela* sp., is a potent cytotoxic compound that inhibits tublin assembly.[207] HTI-286 (**148**) was the most promising synthetic analog and was submitted to the phase II clinical trial by Wyeth. Further development by Wyeth was discontinued in 2005, but Esai's phase I clinical trial is still ongoing.[25]

147 **148**

2.10.3.1.3 KRN-7000

Agelasphins (**149**) are a group of α-galactosyl ceramides with various chain lengths originally isolated from *Agelas mauritiana*.[208] KRN-7000 (**150**), the optimized analog obtained by the detailed structure–activity relationship study of agelasphins,[209] has been in clinical trials as an immunostimulator expected for the treatment of cancer and hepatitis B and C. Although the development, sponsored by Kirin Brewery Co. Ltd. was discontinued, clinical trials are still continuing.[210]

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2.10.3.1.4 NVP-LAQ824

Histone acetylation and deacetylation are catalyzed by histone acetyltransferases (HATs) and HDACs, respectively, and play important roles in transcriptional regulation.[211] Inhibitors of these enzymes are known to induce cell cycle arrest,[212,213] p53-independent induction of the cyclin-dependent kinase inhibitor p21,[214–216] tumor-selective apoptosis,[217,218] and differentiation of normal and malignant cells.[219,220] HDAC inhibitors were shown to have antiangiogenic effects through the alteration of VEGF signaling.[221] These direct and indirect effects on tumor growth and metastasis have indicated the HDAC inhibitors as potential anticancer agents. Psammaplins (**28**) and its dimerized product bisaprasin (**151**), isolated from *P. purpurea* and collected in Papua New Guinea (IC_{50} 2.1–327 nmol l^{-1}) and cell-based p21 promoter activity (AC_{50} 0.7–15 μmol l^{-1}), as well as inhibitory activity against DNA methyltransferase (DNMT).[54,55] NVP-LAQ824 (**152**), which was developed on the basis of the structures of HDAC inhibitors including the psammaplins,[222] entered phase I clinical trials in patients with solid tumors or leukemia.[223]

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2.10.3.2 Anti-inflammatory Agents

2.10.3.2.1 Manoalide

Manoalide (**153**), initially isolated as an antibacterial metabolite from *Luffariella variabilis*,[224] was later found to be a potent inhibitor of phospholipase A_2 (PLA_2) with an IC_{50} value of 1.7 μmol l^{-1}.[225,226] Although more than 100 analogs were synthesized and evaluated for anti-inflammatory activity, none were developed as drugs. Three congeners of manoalide were also obtained from the same sponge,[227] of which secomanoalide (**154**) is more potent against bovine pancreatic PLA_2.[228] These sesterterpenes are known to covalently and specifically bind to PLA_2 by Schiff base formation between the Lys56 residue of PLA_2 and the hemiacetal or aldehyde functionality.[229,230] On the basis of this mechanism, Katsumura designed several PLA_2 inhibitors, and one of them potently and selectively inhibited bovine pancreas PLA_2.[231]

153 154

2.10.3.2.2 Contignasterol

Contignasterol (**155**) is a highly oxygenated steroid having an unusual 14β-configuration isolated from *Petrosia contignata*.[232,233] Contignasterol inhibited antigen-induced bronchial responsiveness in ovalbumin-sensitized guinea pigs and release of histamine from lung tissue slices from the sensitized guinea pigs. Despite their steroidal frameworks, contignasterols do not exhibit PLA_2 inhibition, the target for classical glucocorticosteroides. Their anti-inflammatory effects are mediated through the inhibition of the release of histamine from leukocytes but did not seem to be done by either phospholipase C (PLC) or PLA_2 inhibition.[234] Due to the structural complexity and the potential pharmacokinetic instability, derivatives such as IPL576,092 (**156**) or IPL512,602 (**157**) had been developed and submitted to clinical trials as antiasthma agents under the collaboration between Inflazyme Pharmaceuticals Ltd. and Aventis Pharma. Although this collaboration was terminated because of the difficulty in proving the efficacy of IPL512,602 during a phase IIa study, the development of IPL512,602 is still being continued by Inflazyme.[235]

155 156 157

2.10.3.3 Anti-infectious Disease Agents

Because of emerging resistance against traditional medications, infectious diseases such as malaria or tuberculosis still remain serious threats, especially in subtropical areas. New drug leads with new chemical skeletons or new modes of actions are highly desirable for the development of medications against such infectious diseases. In this context, marine sponge-derived manzamines are of significant interest as promising candidates for new anti-infectious disease drugs.

Manzamine A (**65**) was first isolated from Okinawan *Haliclona* sp. as a cytotoxic compound against P-388 mouse leukemia cells,[236] and over 50 analogs have been discovered up to now.[237] Significant *in vitro* and *in vivo* antimalarial activities of manzamine A and (−)-8-hydroxymanzamine A were reported in 2000[238]; both compounds were also potently antimicrobial against *Mycobacterium tuberculosis*.[239,240] With very long half-life and low plasma clearance, manzamine A is one of the most promising antimalarial drug leads from a marine source.[241] Manzamine A has also been found in other genera of marine sponges such as *Pellina*,[242] *Pachypellina*,[243] *Xestospongia*,[244,245] *Ircinia*,[246] and *Amphimedon*[247] suggesting symbiotic microbes as its origin. In fact, manzamine A was successfully obtained in culture from the bacterium *Micromonospora* sp. of the deepwater Indonesian sponge *Acanthostrongylophora* sp.,[248,249] suggesting the possibility of supplying the compound by fermenting this bacterium.

2.10.4 Roles of Sponge Metabolites in Marine Ecosystems

Because marine sponges adhere to the bottom of the sea and cannot escape attacks by predators, parasites, or biofouling, the primary objective of most marine natural products are presumed to be as a chemical defense.[250] However, recent progress in the research area of marine chemical ecology has indicated that understanding the ecological roles of marine natural products is not straightforward, especially in complex ecosystems where microbes produce secondary metabolites used as a chemical defense for their hosts.[251]

Direct evidence for the involvement of sponge metabolites in chemical defense was first provided by Walker *et al.*[252], who showed exudation of aerothionin (**158**) and homoaerothionin (**159**) into the surrounding seawater from the intertidal sponge *Aplysina fistularis*. These bromotyrosine derivatives are antimicrobial, antifeedant, and antifouling. Importantly, aerothinins were found to be localized in spherulous cells, which are concentrated under the exopinacoderm (peripheral layer),[253,254] suggesting their sponge origin. Similarly, the Mediterranean sponge *Crambe crambe* secretes guanidine derivatives, for example, crambescin **A** (**89**) and crambescidins, into seawater; these compounds are also localized in spherulous cells.[255,256] The compounds are highly toxic to various marine organisms. Poly-APs (**160**) that show a wide range of bioactivities, including ichthyotoxic and antifouling activities, are also secreted by the Adriatic sponge *Reniera (Haliclona) sarai*.[257]

158 $n = 4$
159 $n = 5$

160

In contrast, formoside (**161**), antifeedant, antifouling, and overgrowth-inhibiting triterpenoid glycoside, was not exudated by or concentrated in the outermost layer of the sponge *Erylus formosus*; it was distributed uniformly through the remaining sponge.[258]

161

Another interesting feature is wound-activated chemical defense in sponges. *Aplysina aerophoba* is known to contain such bromotyrosine derivatives (**162**) as aerophobin-2, aplysinamisin-1, and isofitsularin-3, which were bioconverted into the dienone (**164**) via aeroplysinin-1 (**163**) within less than 1 min upon mechanical damage of the sponge.[259] The dienone showed stronger deterrent activity against fish. Recently, wound-activated conversion of psammaplin A sulfate (**165**) into psammaplin A (**28**) was reported in the tropical sponge *Aplysinella rhax,* in which the conversion was very rapid (only seconds).[260] Antifeeding activity against fish was increased after conversion. These wound-activated conversions were mediated by enzymes. Wound-activated defense is thought to be more common in sponges.

Kalihinol A (**108**) was originally isolated as an antimicrobial compound from *Acanthella* sp. collected in Guam.[163] Kalihinol A, and other isocyanides, including kalihinenes X (**166**), Y (**167**), and Z (**168**), as well as 10-formamidokalihinene (**169**) – all of which were isolated from the Japanese *Acanthella cavernosa* – were shown to inhibit the larval settlement and metamorphosis at concentrations less than $0.1\,\mu g\,ml^{-1}$, whereas toxicity toward the barnacle cyprids was very low (LD_{50} values $>100\,\mu g\,ml^{-1}$).[261] Antimalarial activity was also reported for other biological activities of kalihinol A.[262]

2.10.5 Conclusions

There is no doubt that sponges (Porifera) are one of the most productive organisms of bioactive because they are antimicrobial, antitumor, and enzyme inhibitory. Especially interesting are highly unusual macrolides, peptides, and alkaloids that exhibit potent biological activities, many of which are important as drug leads and molecular probes for life science research. It should be emphasized that sponge metabolites targeting specific molecules continue to play increasingly more important roles in elucidating biological processes.

Perhaps the most intriguing and important problem is the origin of sponge metabolites, especially PKS and NRPS (PKS/NRPS) metabolites. Increasing data that support the roles of symbiotic bacteria in producing such metabolites have been accumulated by metagenomic approaches. However, it seems that additional breakthroughs are required for the complete identification of PKS metabolite-producing bacteria and heterogeneous expression of biosynthetic genes in fast-growing bacteria such as *E. coli* for large-scale production of bioactive compounds, while development of the methodology to grow uncultured bacteria found in sponges should be pursued. Strangely, there is no information of the origin of bioactive shikimates; Some sponge-specific terpenoids are believed to be of sponge origin.

Another important issue to be solved is the roles of unusual sponge metabolites, although their defensive roles have been considered most frequently. Many sponge metabolites show antimicrobial, antifouling, antifeeding, and toxic properties, but their real roles remain to be elucidated. For example, terpenoid isocyanides specifically found in certain sponges show a wide array of biological activities, but their origin and roles are unknown, although many researchers believe in their defensive roles in sponges.

Sponges will continue to be a productive source of novel and bioactive compounds. We need to learn much more about sponges after all.

Abbreviations

NRPS nonribosomal peptide synthetase
PKS polyketide synthetase

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Biographical Sketches

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2.11 The Natural Products Chemistry of the Gorgonian Genus Pseudopterogorgia (Octocorallia: Gorgoniidae)

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2.11.1 Introduction

SCUBA (self-contained underwater breathing apparatus) divers and snorkelers have long marveled at the beauty of coral reefs found in the Caribbean and other exotic locations. Caribbean reefs are teeming with unique organisms, many of which do not occur on land. Gorgonian corals, for instance, are the most conspicuous sessile organisms on these reefs, and if one ever finds oneself submerged in the warm and translucent waters of the Caribbean, one will certainly have a close encounter with gorgonians of the genus *Pseudopterogorgia* (Kükenthal, 1919). As presently recognized, this taxonomically complex octocorallian genus comprises of two geographically distinct clades or groups of taxa and is further organized into finer groupings totaling 22 species and subspecies (**Figure 1**).[1,2] *Pseudopterogorgia* species can also be found in Indo-Pacific reefs, but occur at their greatest diversity and abundance in the tropical northwestern Atlantic Ocean and in the Caribbean Sea (**Figure 2**).[2] Biologists who survey the abundance and size

(a)

Kingdom: Animalia
Phylum: Cnidaria
Class: Anthozoa
Subclass: Octocorallia (Alcyonaria)
Order: Alcyonacea
Suborder: Holaxonia
Family: Gorgoniidae
Genus: *Pseudopterogorgia*

(b)

*Pseudopterogorgia acerosa** (Pallas, 1766)
Pseudopterogorgia albatrossae (Bayer, 1961)
*Pseudopterogorgia americana** (Gmelin, 1791)
Pseudopterogorgia anceps (Pallas, 1766)
*Pseudopterogorgia australiensis** (Ridley, 1884)
*Pseudopterogorgia bipinnata** (Verrill, 1864)
Pseudopterogorgia blanquillensis (Stiasny, 1941)
*Pseudopterogorgia elisabethae** (Bayer, 1961)
Pseudopterogorgia formosa (Nutting, 1910)
Pseudopterogorgia fredericki (Williams & Vennam, 2001)
*Pseudopterogorgia hummelincki** (Bayer, 1961)
Pseudopterogorgia hystrix (Bayer, 1961)
*Pseudopterogorgia kallos** (Bielschowsky, 1918)
Pseudopterogorgia luzonica (Kükenthal, 1919)
Pseudopterogorgia marcgravii (Bayer, 1961)
Pseudopterogorgia navia (Bayer, 1961)
Pseudopterogorgia oppositipinna (Ridley, 1888)
Pseudopterogorgia pinnata (Nutting, 1910)
*Pseudopterogorgia rigida** (Bielschowsky, 1929)
Pseudopterogorgia rubrotincta (Thompson & Henderson, 1905)
Pseudopterogorgia thomassini (Tixier–Durivault, 1972)
Pseudopterogorgia torresia (Wright & Studer, 1889)

*Chemically investigated species. The gorgonians highlighted in blue are from the Indo-West Pacific and are rare to infrequently encountered azooxanthellate species with mostly colored sclerites. The remaining gorgonians are commonly or frequently encountered zooxanthellate species from the western Atlantic with mostly colorless sclerites.

Figure 1 (a) Phylogeny of the genus *Pseudopterogorgia*. (b) Current list of documented *Pseudopterogorgia* species.

distribution of Caribbean gorgonian faunas report that *Pseudopterogorgia* spp. are abundant in most outer, middle, and inner shelf coral reefs.[3–5] These soft-bodied reef dwellers are best known as sea plumes for the pinnate or feather-like appearance of their branches. Colonies of different *Pseudopterogorgia* species are morphologically similar in their highly branched nature but are quite variable in sclerites, polyp size, color, texture, growth form, mucus production, and geographical distribution (**Figure 3**).[1,2,6–9]

Despite their relative abundance and the fact that they inhabit an environment noted for nutrient scarcity and intense predation, gorgonian corals of the genus *Pseudopterogorgia* appear to have few predators.[10,11] While a definite explanation for the low predation rates on these gorgonian species is still lacking, researchers have been able to demonstrate that the soft tissues of many gorgonian corals contain lipid-soluble feeding deterrents, which act as a defense mechanism against predation.[10–14] Thus, as in sponges, secondary metabolites are the primary means of defense against fish predators. The genus *Pseudopterogorgia* is in fact the most highly chemically defended of all Caribbean gorgonians.[10,11] Starting in 1968, studies of the natural products chemistry of gorgonian octocorals of the *Pseudopterogorgia* genus have yielded a wealth of novel metabolites, which occur in many of its species at astonishingly high concentrations.[15–19] Since then, a number of research laboratories have maintained a very strong interest in the applications of these defensive metabolites as prototype molecules in the development of new therapeutic agents.[15] The basis for this fascination rests mainly on the observation that *Pseudopterogorgia* secondary metabolites possess novel structures, which are largely unprecedented from

Figure 2 Distribution of the genus *Pseudopterogorgia* in the northwestern Atlantic Ocean (top) and in the Indo-West Pacific Ocean (bottom). Based in part on information provided by G. C. Williams; J. S. Vennam, *Bull. Biol. Soc. Wash.* **2001**, *10*, 71–95.

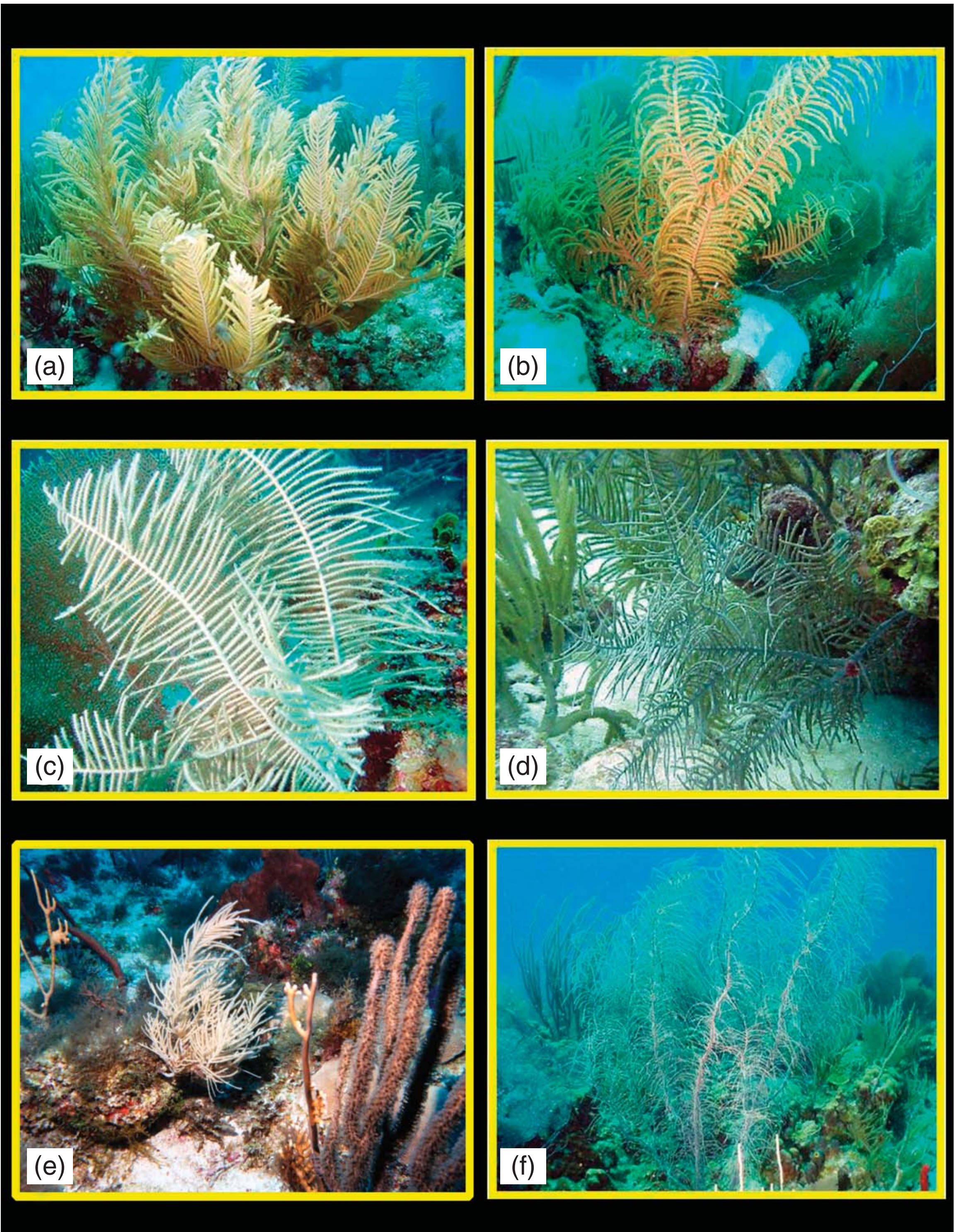


Figure 3 The most representative species of the West Indian genus *Pseudopterogorgia*: (a) *P. acerosa*; (b) *P. americana*; (c) *P. bipinnata*; (d) *P. elisabethae*; (e) *P. kallos*; (f) *P. rigida*.

terrestrial sources. This review covers the literature on marine natural products from *Pseudopterogorgia* spp. published during the last 40 years of research starting from 1968 up to early 2009. It focuses on marine natural products from this source with unusual structures and interesting pharmacological activities, and is organized loosely by gorgonian species followed by structural class. Studies of the chemical ecology aimed at the elucidation of the defensive roles of secondary metabolites of *Pseudopterogorgia* spp. and the importance of secondary metabolite composition as a chemotaxonomic tool are also discussed. Since *Pseudopterogorgia* natural products have often become the target of synthetic and biosynthetic chemists, we include synthetic and biosynthetic works, where available, on novel compounds isolated from this exceptionally productive source for novel structure types. At this point, we would like to emphasize that, unlike the early days in marine natural products chemistry, the discovery of new classes of diterpenoids from *Pseudopterogorgia*, since the late 1980s, has generally been followed by a comprehensive assessment of the biomedical potential and, in some instances, commercialization of this unique faunal resource.[20] The molecular structural diversity found among the many terpenoid natural products isolated from this gorgonian genus is truly remarkable. During the last decade alone, after chemically examining only five *Pseudopterogorgia* species, our research group has discovered over 30 novel carbon skeletal families of compounds, many displaying a wide spectrum of biological activities. This observation, plus the fact that barely 36% of the documented species have been chemically scrutinized (**Figure 1**), suggest that this prolific and taxonomically complex genus is, not only the most chemically inventive, but also the most highly biodiversified of all Caribbean gorgonians. Furthermore, it is our desire to stimulate future research on *Pseudopterogorgia* gorgonian octocorals by indicating the extent of knowledge gaps and by citing case examples, which show that many exciting novel natural products with relevant biological activity can still be discovered from this prolific natural resource.

2.11.2 Natural Products from *Pseudopterogorgia* spp.

2.11.2.1 *Pseudopterogorgia elisabethae* (Bayer, 1961)

Common names: sea plume, purple sea whip, purple frilly gorgonian
Geographic division: Western Atlantic Ocean
Distribution: The Bahamas; Florida Keys; Bermuda; Cuba; and San Andrés Archipelago (Colombia)

Brief description: This species is <1 m tall. Its side branches may be pinnate (paired on opposite sides of the main branches) but often are not pinnate. The distinguishing features of *Pseudopterogorgia elisabethae* are its short, stout branchlets, large, moderately pointed scaphoids with nearly or quite smooth convex surface, and large anthocodial rods. It is moderately slimy, but not as much as is the case with *Pseudopterogorgia americana*. The colonies may be either yellow or purple.

2.11.2.1.1 Diterpene glycosides

2.11.2.1.1(i) Pseudopterosins (compounds reported = 30) In June 1982, an expedition of the University of Miami's research vessel *Calanus* took place during which time numerous habitats in the Florida Keys, the Grand Bahama Island, and the central Bahama Islands were investigated. During this expedition Professor William Fenical and coworkers (Scripps Institution of Oceanography, La Jolla, CA, USA) found that the deeper water and highly branched *P. elisabethae* was of particular interest since shipboard chemical and biological assays revealed this animal to contain large quantities of unknown polar lipid metabolites. The field-oriented antimicrobial and cytotoxicity assays employed revealed that these secondary metabolites were apparently coupled with strong inhibitory activities. A subsequent collection, extraction, and fractionation of Bahamian specimens of *P. elisabethae* led to the isolation of the highly bioactive diterpenoid glycosides pseudopterosins A–D (**1–4**). Their molecular structures and potent bioactivities were subsequently disclosed in two separate accounts each published in 1986.[21,22] The specimens of *P. elisabethae* investigated were collected near Crooked Island in the Bahama Islands between −15 and −35 m depth. The freshly collected specimen was stored frozen and subsequently extracted, first with $CHCl_3$ and then with EtOAc. Pseudopterosins A–D (**1–4**) were isolated by rapid-elution chromatography of the combined crude extracts (using TLC (thin-layer

chromatography)-grade Florisil with solvent mixtures that ranged from 20% CH_2Cl_2 in isooctane to 100% CH_2Cl_2 and then through mixtures of CH_2Cl_2 with increasing portions of EtOAc) and were finally purified by normal-phase HPLC (high-performance liquid chromatography). Pseudopterosin C (3) was the major component, comprising 7.5% of the lipid extract, while pseudopterosins A, B, and D each represented less than 1% of the organic extract. The structure of the only crystalline metabolite, pseudopterosin C (3), was determined by X-ray crystallography. Isolation and identification of the pentose portion of 3 as D-xylose allowed the assignment of the absolute configuration of all centers in 3. Since the relative stereochemistry of all centers was elucidated from the X-ray studies, and the sugar was shown to be 3-*O*-acetyl-β-D-xylopyranose, the absolute configuration of all chiral centers in pseudopterosin C (3) could be assigned: C1(*R*), C3(*S*), C4(*R*), C7(*S*), C1′(*S*), C2′(*R*), C3′(*S*), C4′(*R*). The structures of pseudopterosins A, B, and D (1, 2, and 4) were subsequently defined by spectral analyses and by conversion into the same tetraacetate derivative produced from 3.

These diterpene glycosides have been reported to inhibit pancreatic phospholipase A_2 (PLA_2, inhibition concentration – $IC_{50} = 0.5–4.0\ mmol\ l^{-1}$) and are pharmacologically distinct from typical cyclooxygenase inhibiting NSAIDs (nonsteroidal anti-inflammatory drugs).[21] Pseudopterosin A (1) inhibits cell division in fertilized sea-urchin eggs with an IC_{50} of 25 $\mu mol\ l^{-1}$, and has also been found to significantly inhibit phorbol myristate acetate (PMA)-induced topical inflammation in mice.[23,24] When administered subcutaneously to mice, it was found that pseudopterosin A is several times more potent, as an analgesic, than the industrial anti-inflammatory drug indomethacin (i.e., the ED_{50} (effective dose) for pseudopterosin A is approximately 3.12 $mg\ kg^{-1}$ versus approximately 10 $mg\ kg^{-1}$ for indomethacin) in blocking the stretch–reflex response in mice induced by intraperitoneal injection of phenyl-quinone. Further studies performed *in vitro* suggested that, as an anti-inflammatory agent, pseudopterosin A (1) appears to modify the arachidonic acid cascade by an as-yet undefined mechanism of pharmacological action.[21] A semisynthetic pseudopterosin (Ps) derivative currently in phase II of clinical trials, pseudopterosin A methyl ether (TMO), can substantially improve tissue repair and healing for severe burn victims, and has shown promise as a treatment for contact dermatitis. Additional Ps ether derivatives such as pseudopterosin A 4-hydroxybutyl ether can be useful in the treatment of pain and inflammation.[25,26] A *C*-glycoside analogue of pseudopterosin A methyl ether has been synthesized and assayed for its anti-inflammatory activity. Since its bioactivity is maintained despite the presence of the C-linked sugar, it is believed that the Pss are not prodrugs.[27]

A subsequent chemical investigation of *P. elisabethae* collected at Bermuda at −35 m depth led to the discovery of pseudopterosins E–J (5–10).[28] Pseudopterosins E and F (5, 6) were the major components of the polar fractions while the monoacetates, pseudopterosins H–J (8–10), were present in minor quantities. Pseudopterosin G (7), on the other hand, was found in the extract only in trace quantities. From the relatively nonpolar fractions of the same extract some of the previously encountered pseudopterosins A–D (1–4) were also isolated. The structure of pseudopterosin F (6) was confirmed by X-ray crystallographic analysis which yielded its relative stereochemistry only, and several Pss coisolated during the same investigation were chemically converted into the same derivatives generated from pseudopterosin F. The absolute configuration of pseudopterosin F was later confirmed by comparison of the methylated aglycon derivatives from pseudopterosins A (1) and F (6).[28] Interestingly, *P. elisabethae* collected at Great Abaco Island in the Bahamas, and extracted in the same manner, was found to contain exclusively pseudopterosins K and L (11, 12) without any trace of the previously discussed metabolites.[28] The aglycon portion of pseudopterosins E and F are identical with that of pseudopterosins A–D (1–4), but both metabolites have sugar moieties (α-L-fucose for pseudopterosin E and α-D-arabinose for pseudopterosin F) attached at the C10 hydroxyl. Pseudopterosin G (7) and its monoacetates (pseudopterosins H–J) are C9 α-L-fucose glycosides. The diterpene aglycon of the latter molecules is an isobutenyl epimer at the C1 chiral center, in relation to the aglycons derived from pseudopterosins E and F. Pseudopterosins K and L (11, 12), on the other hand, are α-L-fucosides with the same diterpene skeleton as pseudopterosins E and F, but with the sugar attached to the C9 hydroxyl. However, the aglycons of pseudopterosins K and L were found to be enantiomeric to those from pseudopterosins E and F (5, 6). It should be noted that pseudopterosins E and F appear to be metabolites unique to the Bermuda collections of *P. elisabethae.* In summary, the diterpene skeletons of pseudopterosins A–F are identical and only differ from the diterpene moiety of pseudopterosins G–J in the orientation of the alkyl group at C1.

Ps A (**1**) $R^1 = R^2 = R^3 = H$
Ps B (**2**) $R^1 = Ac$, $R^2 = R^3 = H$
Ps C (**3**) $R^2 = Ac$, $R^1 = R^3 = H$
Ps D (**4**) $R^3 = Ac$, $R^1 = R^2 = H$

Ps G (**7**) $R^1 = R^2 = R^3 = H$
Ps H (**8**) $R^1 = Ac$, $R^2 = R^3 = H$
Ps I (**9**) $R^2 = Ac$, $R^1 = R^3 = H$
Ps J (**10**) $R^3 = Ac$, $R^1 = R^2 = H$

Ps K (**11**) R = H
Ps L (**12**) R = Ac

Ps E (**5**)

Ps F (**6**)

Ps M (**13**) $R^1 = Ac$, $R^2 = R^3 = H$
Ps N (**14**) $R^1 = R^3 = H$, $R^2 = Ac$
Ps O (**15**) $R^1 = R^2 = H$, $R^3 = Ac$

Pseudopterosin E (**5**) was found to be a superior anti-inflammatory agent in comparison to pseudopterosin A (**1**). Pseudopterosin E showed very low acute toxicity in mice ($LD_{50} > 300\ mg\ kg^{-1}$) and appeared to act by a novel mechanism of pharmacological action.[28] Subsequent pharmacological characterization of pseudopterosins A (**1**) and E (**5**) suggested that the Pss may mediate their anti-inflammatory effects by inhibiting eicosanoid release from inflammatory cells in a concentration and dose-dependent manner. There is also recent evidence suggesting that Pss may serve as antioxidants.[29–32] Because of the improved pharmacological properties of pseudopterosin E, larger quantities of this less abundant compound were needed. This encouraged Fenical and coworkers to design an efficient semisynthetic pathway to convert the more abundant pseudopterosins A–D (**1– 4**) into pseudopterosin E (**5**).[28] Unfortunately, the yields of several key steps used in their synthetic scheme were always very low, and subsequent modification of catalysts, solvents, bases, and leaving groups failed to improve the reaction yields. Since then, several enantiospecific total syntheses of pseudopterosin A (**1**) and pseudopterosin E (**5**) have been reported,[33–36] and syntheses for the aglycon component of these Pss and its methylated derivatives have also been outlined.[37,38] An impressive number of elegant synthetic approaches to the Pss and their aglycons have been described, and to this date, the fascination with this endeavor continues unabated.[39–52] These syntheses provided the first compelling evidence that pseudopterosins G–J (**7–10**) are diastereomeric at C1 relative to the pseudopterosins A–F (**1–6**), *not* at C7 as originally reported.[48] Owing to their excellent anti-inflammatory and analgesic activity, partially purified extracts containing Pss are currently

incorporated into skin care preparations (anti-irritants) that are widely marketed.[53–55] The exact mechanism of action of the active components remains elusive but may involve membrane stabilization.[23,24]

In 2003, Kerr and coworkers reported the structures of pseudopterosins M–O (**13–15**) from *P. elisabethae* collected at a depth of −25 m off Long Key, Florida.[56] After complete structure determination of the diterpene portion of pseudopterosin M (**13**) using routine spectroscopic methods, the carbohydrate moiety was identified as an arabinose derivative by comparison of the ^{1}H and ^{13}C-NMR (nuclear magnetic resonance) chemical shifts of the sugar moieties with literature values. The D-configuration of the sugar was established by comparing the optical rotation of the sugar solution that was obtained by hydrolysis of compound **13** using an established protocol. The negative optical rotation of the arabinose sugar solution generated from **13** was identical to that of a standard sample of D-arabinose, which was treated with a similar amount of acid. After interpretation of ^{1}H, ^{13}C-NMR, COSY (correlation spectroscopy) −45°, and HMQC (heteronuclear multiple quantum coherence) spectral data of pseudopterosins M–O the Florida-based authors concluded that these compounds have the same amphilectane-type diterpene aglycon characteristic of the pseudopterosins G–J (**7–10**), but with their monoacetylated arabinose residue appended at C10. It bears mentioning that even though Kerr and coworkers described in their article pseudopterosins M, N, and O as *O*-acetyl-arabinosides (see above), the structures drawn (**13–15**) are in fact *O*-acetyl-xyloside derivatives.[56,57] (In articles published subsequently, two research groups have remarked that the reported ^{1}H- and ^{13}C-NMR data for the aglycon component of pseudopterosins M–O (**13–15**) might not be consistent with the structures published by the Kerr group; see Duque *et al.*[58] and Rodríguez *et al.*[59]) Upon evaluation in a mouse ear anti-inflammatory assay, pseudopterosins M (**13**), N (**14**), and O (**15**) exhibited significant percent inhibitions (68, 88, and 69%, respectively). The ED_{50} of the most active compound, pseudopterosin N (**14**), was 9.7 μg per ear indicating that the latter compound exhibited superior potency than pseudopterosin A (**1**) and pseudopterosin E (**5**) (ED_{50} values 14.6 and 41.0 μg per ear, respectively).

Ps P (**16**) $R^1 = R^2 = R^3 = H$
Ps Q (**17**) $R^1 = Ac$, $R^2 = R^3 = H$
Ps R (**18**) $R^1 = R^3 = H$, $R^2 = Ac$
Ps S (**19**) $R^1 = R^2 = H$, $R^3 = Ac$
3′-*O*-acetyl-Ps Q (**23**) $R^1 = R^2 = Ac$, $R^3 = H$
2′-*O*-acetyl-Ps Q (**24**) $R^1 = R^3 = Ac$, $R^2 = H$

Ps T (**20**) $R^1 = R^2 = R^3 = H$
Ps U (**21**) $R^1 = Ac$, $R^2 = R^3 = H$
Ps V (**22**) $R^1 = R^3 = H$, $R^2 = Ac$
3′-*O*-acetyl-Ps U (**25**) $R^1 = R^2 = Ac$, $R^3 = H$
2′-*O*-acetyl-Ps U (**26**) $R^1 = R^3 = Ac$, $R^2 = H$

Ps W (**27**)

Ps X (**28**) R = H
Ps Y (**29**) R = Ac

iso-Ps E (**30**)

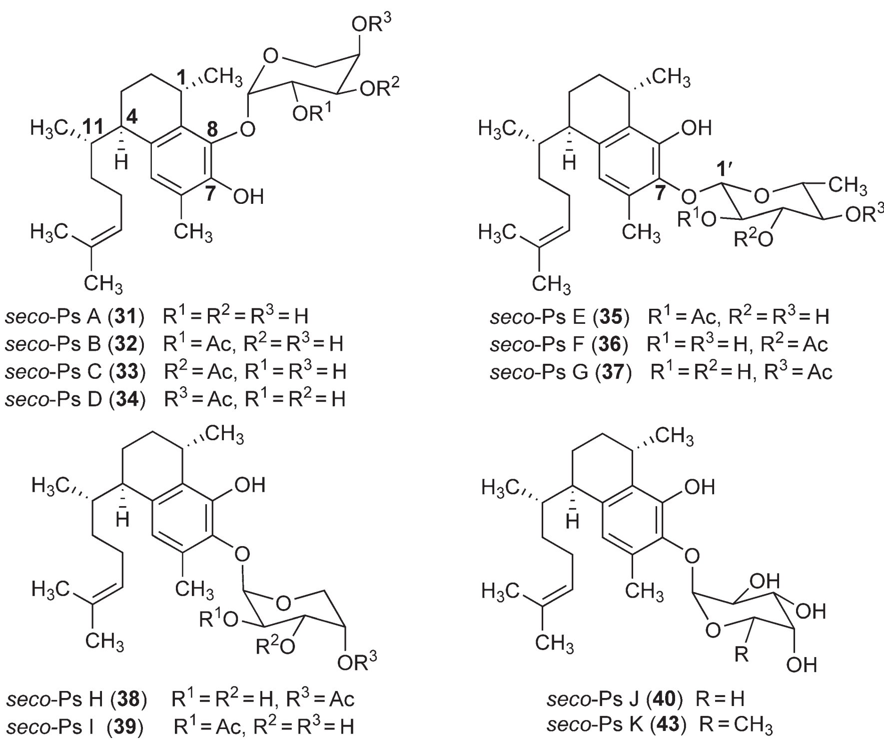
During 2004, in separate but nearly synchronous works, the research groups of Ata (Manitoba, Canada), Duque (Bogota, Colombia), and Rodríguez (San Juan, Puerto Rico) reported 14 new diterpene glycosides from *P. elisabethae* specimens collected in the Bahamas and Providencia Island, Colombia, namely pseudopterosins P–Y (**16–29**). Such coincidence unwittingly led to duplication in structures and trivial names. (To rectify matters, an agreement leading to the revision of many of the trivial names originally assigned to compounds **16–29** was reached among these authors.[58–61] The revised names are used throughout this review.) As part of an investigation to determine Ps composition and concentration in colonies of *P. elisabethae* from the islands of San Andrés and Providencia,[60] the Colombian group described the isolation and structure elucidation of seven new compounds, pseudopterosins P–V (**16–22**), along with the known pseudopterosins G (**7**) and K (**11**),[58] whereas the Puerto Rican group reported exactly the same new compounds, except for pseudopterosin S (**19**), from *P. elisabethae* specimens collected at the same location.[59] Additionally, Rodríguez and coworkers described the isolation, structure characterization, and biological evaluation of five distinct metabolites, namely, 3′-*O*-acetyl-pseudopterosin Q (**23**), 2′-*O*-acetyl-pseudopterosin Q (**24**), 3′-*O*-acetyl-pseudopterosin U (**25**), 2′-*O*-acetyl-pseudopterosin U (**26**), and pseudopterosin W (**27**), without any trace of the previously described pseudopterosins A–O (**1–15**).[59] The structures of all the new compounds discovered in Puerto Rico, including absolute stereochemistry, were proposed on the basis of comprehensive spectral analyses, chemical transformations, specific rotation, and TLC chromatographic analyses. On the other hand, the Ata group reported two new diterpene glycosides from the MeOH extract of *P. elisabethae* collected from the Bahamas, pseudopterosins X and Y (**28, 29**), along with the known pseudopterosins A–E (**1–5**) and K (**11**).[61] The structures of the new compounds were established through routine spectroscopic analyses. The sugar component of pseudopterosins X (**28**) and Y (**29**) was determined as β-L-xylose and 3′-*O*-acetyl-β-L-xylose, respectively, by comparing the ^{1}H- and ^{13}C-NMR data of the sugar moiety of **28** with the literature values for pseudopterosins A–D (**1–4**), which each has a xylose moiety. The L-configuration of the xylose was established by comparing the negative optical rotation of the sugar solution obtained by the hydrolysis of compound **28** following a described protocol with the sugar solution of commercially available L-xylose, which was previously treated with a similar amount of acid. The coupling constant ($J_{1',2'} = 9.0$ Hz) determined the β-L-configuration of the sugar.

Pseudopterosin R (**18**) inhibited thromboxane B_2 (TXB_2) ($IC_{50} = 4.7\ \mu mol\ l^{-1}$) and superoxide anion ($O_2^-$) ($IC_{50} = 11.2\ \mu mol\ l^{-1}$) generation from *Escherichia coli* lipopolysaccharide (LPS) activated rat neonatal microglia *in vitro*.[59] In contrast, pseudopterosins Q (**17**), U (**21**), V (**22**), 3′-*O*-acetyl-pseudopterosin U (**25**), and 2′-*O*-acetyl-pseudopterosin U (**26**) demonstrated minimal effects on both TXB_2 and O_2^- release. In addition, many of the new Pss displayed strong antituberculosis, antiviral, antimalarial, and anticancer activity. Critically, pseudopterosin P (**16**) exhibited the strongest inhibitory activity (76%) against *Mycobacterium tuberculosis* at $6.25\ \mu g\ ml^{-1}$, whereas pseudopterosin V (**22**) showed the most potent *in vitro* antimalarial activity, with an IC_{50} of $1\ \mu g\ ml^{-1}$. Pseudopterosin Q (**17**) was shown to be very toxic against the Herpes simplex viruses HSV-1 and HSV-2 (effective concentration – $EC_{50} = 2.9\ \mu mol\ l^{-1}$), with SI (selectivity index) values of <2.4. Considering the potential ability of some of these diterpene glycosides to inhibit inflammation, pseudopterosins Q (**17**), R (**18**), U (**21**), V (**22**), and 3′-*O*-acetyl-pseudopterosin U (**25**) were also evaluated in a three-cell line panel consisting of MCF-7 (breast cancer), National Cancer Institute – NCI-H460 (nonsmall cell lung cancer), and SF-268 (central nervous system – CNS) cells. Results from the one dose primary anticancer assay showed Pss **17, 21, 22, and 25** to have significant cytotoxicity. In each case, the percent of growth of the treated cells when compared to the untreated cells was 0%. On the other hand, pseudopterosin R (**18**) indicated a lack of significant cytotoxicity in the three-cell line panel, as the percent of growth of the treated cells was 100%.[59] The *in vivo* anti-inflammatory effects of extracts and fractions, and *in vitro* anti-inflammatory activity of pure compounds isolated by the Colombian group, were reported in 2008.[62] Pseudopterosins X and Y (**28, 29**) showed antibacterial activity selectively against the

Gram-positive bacteria *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Enterococcus faecalis* with minimum inhibitory concentration (MIC) values ranging from 0.8 to 2.3 μg ml^{-1}.[61]

A chemical investigation of raw material isolated from the feathers of *P. elisabethae* from an undisclosed location, led recently to the discovery of iso-pseudopterosin E (**30**), an α-L-fucoside with the same diterpene skeleton as pseudopterosin E (**5**), but with the sugar attached to the C9 hydroxyl. The structure of iso-pseudopterosin E was confirmed by X-ray crystallographic analysis, which yielded its relative stereochemistry only. However, the absolute configuration of **30** was established after measuring the specific rotation of its methylated aglycon derivative and comparing it with the optical rotation values obtained for the methylated aglycon derivatives of pseudopterosins A (**1**) and K (**11**). Similar to its relatives, iso-Ps E (**30**) inhibits PMA-induced inflammation of a mouse ear with an ED_{50} of 27 μg per ear and significantly decreases basal levels of phagocytosis in cultured tetrahymena cells.[63]

2.11.2.1.1(ii) seco-Pseudopterosins (compounds reported = 13) In June 1980, as part of a NSF(National Science Foundation)-sponsored expedition to the Florida Keys on-board the research vessel *Calanus*, Fenical and coworkers collected a gorgonian coral near Cosgrove Shoal, which they tentatively identified as *Pseudopterogorgia kallos* and whose crude extract displayed modest cytotoxicity and antimicrobial activity. From this gorgonian coral, they isolated four new bicyclic diterpenoid glycosides in roughly 1.5% of dry gorgonian weight, which they christened *seco*-pseudopterosins A–D (**31–34**).[64] The compounds turned out to be arabinose glycosides (derivatives **32–34** are monoacetate positional isomers) possessing aglycons of the serrulatane class. These metabolites are related to the Ps series by bond cleavage at the C1–C12 positions (i.e., amphilectane numbering system). *seco*-Pseudopterosin A (**31**) was isolated as the major metabolite, while *seco*-pseudopterosins B–D were all found in lesser quantities. The molecular structures of **31–34** were deduced on the basis of comprehensive spectral analyses and upon chemical transformations. The *seco*-pseudopterosins displayed potent anti-inflammatory and analgesic activities equivalent to commercial anti-inflammatory drugs. *seco*-Pseudopterosin A (**31**), for example, at doses of approximately 50 μg per ear, showed 69% reduction of inflammation in the mouse ear-edema assay. The *seco*-pseudopterosins also showed antimicrobial activity against a wide variety of bacterial and fungal pathogens. For instance, compound **31** displayed an MIC against *S. aureus* of 8 μg ml^{-1}.[64]



Amphilectosin A (**41**) Amphilectosin B (**42**)

From specimens of *P. elisabethae* collected at −25 m off Long Key, Florida during August 1999, the Kerr group isolated three new serrulatane-based diterpenoid glycosides, namely, *seco*-pseudopterosins E–G (35–37).[56] This report was also the first to describe the co-occurrence in *P. elisabethae* of Ps and *seco*-pseudopterosin diterpene glycosides, suggesting that both the amphilectane and serrulatane families of diterpene are derived from the same geranylgeranyl diphosphate cyclase product. The identity of the carbohydrate in compound 35 was determined to be a fucose by comparing the ^{1}H and ^{13}C NMR chemical shifts of the sugar residue with that of Pss containing a fucose. The diaxial coupling constant (8.9 Hz) of H-1′ and H-2′ indicated the β-linkage between C1′ and C7. The L-configuration of the fucose was assigned on the basis of a strong negative optical rotation of the sugar obtained by the hydrolysis of 35. The two additional *seco*-pseudopterosins F and G (36, 37) were found to have very similar UV, IR, MS, ^{1}H- and ^{13}C-NMR spectra to those of 35. Thus, compounds 36 and 37 were found to differ only in the point of attachment of the acetoxy group in the fucose residue. (The reported ^{1}H- and ^{13}C-NMR data for the aglycon component of *seco*-pseudopterosins E–G (35–37) might not be consistent with the structures published. Furthermore, in their report Kerr and coworkers[56] describe the sugar components of compounds 35–37 as *O*-acetyl-L-fucoses even though the structures drawn are in fact 6-deoxy-β-D-glucopyranoses (β-D-quinovoses).) Upon evaluation in a mouse ear anti-inflammatory assay, *seco*-pseudopterosins E (35), F (36), and G (37) exhibited significant percent inhibitions (88, 65, and 74%, respectively). The ED_{50} of the most active compound, *seco*-pseudopterosin E (35), was 10.4 μg per ear indicating that it possesses superior potency than pseudopterosin A (1) and pseudopterosin E (5).[56]

As part of an extensive program to explore the chemical constituents of Caribbean marine invertebrates, Rodríguez *et al.*[59] reported two new compounds present only in trace amounts, *seco*-pseudopterosins H and I (38 and 39), from a specimen of *P. elisabethae* collected at the Colombian Southwestern Caribbean Sea near the island of San Andrés. *seco*-Pseudopterosins H and I were found to have similar IR, MS, UV, and ^{1}H- and ^{13}C-NMR spectra, and comparable optical rotations, to those of previously reported *seco*-pseudopterosins A–D (31–34). The identities of the pentose portion in compounds 38 and 39 were determined to be 4′-*O*-acetyl-α-arabinose and 2′-*O*-acetyl-α-arabinose, respectively, from ^{1}H–^{1}H COSY and NOESY (nuclear Overhauser effect spectroscopy) NMR experiments. At a concentration of 6.25 $\mu g\,ml^{-1}$, compounds 38 and 39 did not significantly inhibit the growth of *M. tuberculosis* $H_{37}Rv$. These compounds also demonstrated minimal effects on both TXB_2 and O_2^{-} release, although they also appeared to be rather toxic (i.e., lactate dehydrogenase − LDH_{50} 3.6–10 $\mu mol\,l^{-1}$) to microglia cells *in vitro*.[59]

Ferns and Kerr reported in 2005 the isolation of known pseudopterosins F (6) and T (20), along with three new diterpene glycosides, 40–42, from samples of *P. elisabethae* collected at a depth of −80 ft off Long Key, Florida. The structures of the new compounds, *seco*-pseudopterosin J (40), amphilectosin A (41), and amphilectosin B (42) were established through spectral analyses and chemical degradation studies.[65] To further confirm the structure of amphilectosin A (41), a small sample of this compound was transformed to pseudopterosin T (20) upon treatment with acid and heat. Interestingly, amphilectosin B (42) was transformed to pseudopterosin F (6) when treated under the same conditions. These transformations suggest that the α/β stereochemistry for the isobutenyl group at C1 in the Pss arises from the selective ring closure of the *cis*- and *trans*-amphilectosins, thus implying a plausible biosynthetic link between the *seco*-pseudopterosins and Pss.

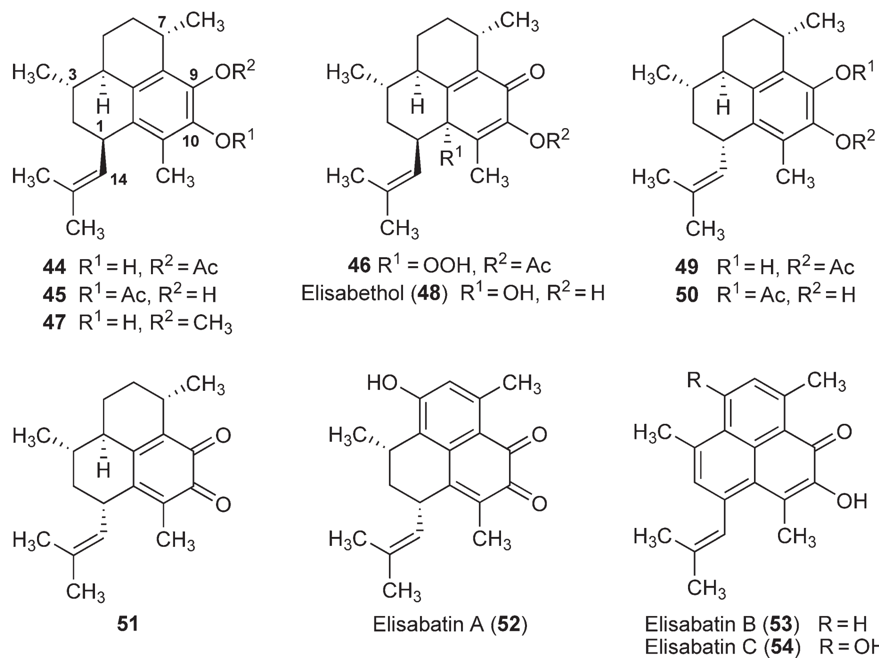
The structurally new *seco*-pseudopterosin K (**43**), together with the previously isolated *seco*-pseudopterosin J (**40**) and amphilectosins A and B (**41 and 42**), were reported by Duque *et al.*[66] in 2006 from the MeOH–CH_2Cl_2 extract of *P. elisabethae* specimens collected from the islands of San Andrés and Providencia. Interpretation of the ^{1}H- and ^{13}C-NMR data for **43** indicated that the signals for the aglycon moiety were in good agreement with those reported for *seco*-pseudopterosin J (**40**), while those for the sugar moiety were essentially identical with the data of α-fucopyranosyl moiety found in pseudopterosin P (**16**), which was coisolated during an earlier investigation of the same organism.[58]

Similar to their carbotricyclic congeners, the *seco*-pseudopterosins also represent challenging target molecules for chemical synthesis and thus, have attracted considerable attention from synthetic and medicinal chemists alike because they function as anti-inflammatory and analgesic agents with potencies substantially greater than the common market drug indomethacin.[44] Their limited availability from natural sources and the recent commercialization of pseudopterosin C (**3**) by the internationally well-known brand, Estée Lauder as the active principle of the topical skin cream *Resilience*, has done much to popularize these molecules as synthetic targets.[67,68] Eye balm creams listing extracts of *P. elisabethae* as one of its key ingredients are being sold by US-based companies such as SkinCeuticals Inc. and Revision SkinCare as rehabilitative emollients for aging skin and to reduce dark circles under the eyes. Their effect on preventing sun damage and nourishing the skin represent their key selling point. A popular skin cleanser by Renee Rouleau is based on the same sea whip extract. Interestingly, the results of recent investigations have demonstrated that greatly simplified structural analogues of the *seco*-pseudopterosins are still capable of maintaining several of the important bioactivities that characterize the natural products.[69] At present, *P. elisabethae* is being actively harvested from reefs off the coast of Bermuda and in the Bahamas with a reported market value of \$3–\$4 million a year.[70] The colony growth responses of *P. elisabethae* to disturbances such as harvesting, grazing, and storm damage, the relationship between recruitment and adult distribution in the Bahamas, and its high fertilization success, have been documented by Lasker and coworkers.[71–73]

2.11.2.1.2 Nonglycosylated diterpenes

2.11.2.1.2(i) Amphilectanes (compounds reported = 13) A series of structurally interesting compounds, which are members of the amphilectane diterpenes, have been isolated from specimens of *P. elisabethae* collected throughout the Western Atlantic Ocean. For instance, compounds **44–46**, which were isolated from a gorgonian coral of the genus *Pseudopterogorgia* collected by the Fenical group near Highborne Cay, Bahamas Islands in 1982, possess tricyclic ring skeletons related to the aglycon component of the Pss.[74] Compounds **44 and 45** were obtained as an inseparable equimolar mixture, which appear to be composed of interconverting acetates. Through some routine chemical interconversions, it was demonstrated that **44 and 45** possess the 1*R*, 3*S*, 4*R*, 7*S* absolute configurations. Fortuitously, hydroperoxide **46**, which was recognized as a cross-conjugated cyclohexadienone by its spectral properties, was found to spontaneously decompose at 25 °C in a matter of weeks to give a mixture of monoacetates **44 and 45**. The methylated aglycon from pseudopterosin E (**47**) was isolated by Fenical and coworkers from the same Bermuda collection that led to pseudopterosins E–J (**5–10**).[28] On the other hand, extracts of *P. elisabethae* from the Florida Keys have been shown to contain elisabethol (**48**).[56] The multiplicity of H-14 revealed that compound **48** was an amphilectane-type diterpene, rather than a serrulatane derivative. This multiplicity difference can be used as an important tool to differentiate between these two classes of diterpenes. Compounds **49 and 50**, the C1 epimers of **44/45**, were also isolated as an interconverting monoacetate mixture along with *o*-quinone **51**, from the same species collected in San Andrés Island. Thus, similar to **44/45**, compounds **49/50** were characterized in the form of the 9,10-diacetoxy derivative. It is very likely that compounds **49/50** have the 1*S*, 3*S*, 4*R*, 7*S* absolute stereochemistry on the basis of its positive sign of optical rotation.[66] The absolute configuration of **51**, a sensitive material that gradually decomposed even at low temperatures, was established as 1*S*, 3*S*, 4*R*, 7*S*. An earlier chemical study of the same coral species from San Andrés Island had led to the isolation of three highly conjugated amphilectane-type diterpenes, elisabatins A–C (**52–54**).[75,76] The molecular structures of these orange to red pigments were carefully established by spectroscopic methods and subsequently confirmed by X-ray crystallographic studies.[77] (The results of the X-ray crystallographic study of elisabatin A (**52**) are as yet unpublished.) Although elisabatin A (**52**) is a very stable substance, elisabatins B and C (**53, 54**) decompose slowly in $CDCl_3$ solution upon

prolonged exposure to air and light at 25 °C. Biological screening of elisabatin A indicated weak *in vitro* cancer cell cytotoxicity and no anti-HIV (Human immuno deficiency virus) activity. *In vitro* testing of elisabatins A–C at 6.25 $\mu g\,ml^{-1}$ revealed no meaningful antitubercular activity. On the other hand, compounds **52–54** inhibited the growth of *Plasmodium falciparum* with IC_{50} values of 3, 14, and 10 $\mu g\,ml^{-1}$, respectively. Though elisabatin A proved to be a weak anticancer agent, elisabatins B and C showed a combination of selectivity and potent growth inhibition (GI) against several CNS, melanoma, and leukemia cell lines (**53**: SF-268 (GI_{50} = 8.56 E-8), SK-MEL-28 (GI_{50} = 9.33 E-7); **54**: CCRF-CEM (GI_{50} < 1.00 E-8), RPMI-8226 (GI_{50} = 2.57 E-8)). Elisabatin A displayed strong antiviral activity against hepatitis B virus (HBV) (EC_{50} = 0.115 $\mu g\,ml^{-1}$). Follow-up screening revealed an antagonistic interaction in a combination assay with the known nucleoside reverse transcriptase inhibitor 3TC (commercially known as EPIVIR). Interestingly, a complex range of interactions (synergic and antagonistic) were observed when **52** was combined with ADV (Adefovir dipivoxil; commercially known as Hepsera), depending on the concentration of **52**. Unfortunately, further testing at the NIAID (National Institute of Allergy and Infectious Diseases) using HBV animal models has been hampered by the paucity of material. The potential anti-inflammatory activity of elisabatins A–C was also explored using the TBX_2 and O_2^- generation from *E. coli* LPS-activated rat microglia *in vitro*. Elisabatin C (**54**) potently inhibited TBX_2 (IC_{50} = 0.7 $\mu mol\,l^{-1}$) with concomitant low toxicity, measured as LDH release, while elisabatin A (**52**) also inhibited TBX_2, but at a higher concentration (IC_{50} = 9.8 $\mu mol\,l^{-1}$). Meanwhile, elisabatin B (**53**) did not show activity against either the inhibition of O_2^- or TBX_2 release. During the bioassay-guided fractionation of the crude hexane extract of the same gorgonian specimen, Rodríguez and coworkers discovered two novel amphilectane-based diterpene alkaloids containing the uncommon benzoxazole moiety, pseudopteroxazole (**55**) and homopseudopteroxazole (**56**).[78,79] Their structures were deduced from interpretation of combined spectroscopic data, including extensive 1D (one-dimensional) and 2D (two-dimensional) NMR measurements, and NMR spectral comparisons with known amphilectane models. Biological screening studies indicated that benzoxazoles **55 and 56** are strong growth inhibitors of *M. tuberculosis* $H_{37}Rv$. Several total syntheses for pseudopteroxazole have been reported, the first one of which led to a revision of the original stereochemical assignments proposed for the stereogenic centers C1 and C7.[80–83]



Pseudopteroxazole (**55**) R = H
Homopseudopteroxazole (**56**) R = $–CH_2(CH_2)_3CH_3$

2.11.2.1.2(ii) Serrulatanes (compounds reported = 16) Elisabethatriene (**57**), whose molecular structure was proposed based on spectroscopic data alone, was isolated and purified using a radioactivity-guided isolation protocol from a specimen of *P. elisabethae* collected in May 1999 at Sweetings Cay in the Bahamas. The isolation of this metabolite, the first committed intermediate in Ps biosynthesis, suggests that the Pss and *seco*-pseudopterosins are derived from a common bicyclic intermediate.[84] Isoelisabethatriene (**58**) was originally obtained as a semisynthetic product upon treatment of **57** with SeO_2 in the presence of trimethylsilyl polyphosphate (PPSE),[84] but was later identified from an extract of *P. elisabethae* collected in the Florida Keys.[85] The structure of **58** was proposed on the basis of UV, ^{1}H-NMR, and HRMS data alone. A chemical investigation of this gorgonian coral from San Andrés Island led to the isolation of the closely related bicyclic diterpene, elisabethatrienol (**59**).[66] Mosher's ester method was applied to determine the absolute configuration of this molecule as 1*S*, 4*R*, 7*S*, 9*S*, 11*S*. Elisabethatriene, isoelisabethatriene, and elisabethatrienol are interesting, since these structures can be considered as biosynthetic intermediates leading to erogorgiaene (**60**), presumably via dehydrogenation and aromatization. The strongly antitubercular hydrocarbon **60** was reported in 2001, along with its hydroxylated congener 7-hydroxyerogorgiaene (**61**), by the Rodríguez group during a cursory chemical examination of the hexane extract of *P. elisabethae* from San Andrés Island.[86] A number of laboratories have described studies on the total synthesis of erogorgiaene.[87–90] The subsequent coisolation of 7-hydroxyerogorgiane (**61**) and 7,8-dihydroxyerogorgiaene (**62**) from an extract of Floridian *P. elisabethae* led Ferns and Kerr to propose the intermediacy of these compounds in Ps biosynthesis.[91] Elisabethadione (**63**) and the structurally related diterpenoids **64** and **65** were isolated as yellow or orange materials of either gummy or oily consistency, from specimens collected in the Florida Keys,[56] the Bahamas,[74] and San Andrés Island, respectively.[76] Analysis of mass spectral, UV/VIS (ultraviolet/visible), and ^{13}C-NMR data indicated that compounds **63–65** were bicyclic *p*-benzoquinones with an *ortho* hydroxyl functionality. Comparison of these data with those of *seco*-pseudopterosins A–D (**31–34**) showed that these compounds possessed the same aglycon skeleton and had identical relative stereochemistries (α-orientation for the C4 methine, C18, and C20 methyl groups). The versatility of serrulatanes **64** and **65** as putative biosynthetic intermediates in Ps biosynthesis is discussed later. Anti-inflammatory assays indicate that elisabethadione (**63**) is more potent than the related and commercially used natural products, the Pss.[56] Because elisabethadione was in very short supply, a new effort was made to reisolate it from a new harvest of *P. elisabethae* procured at the Florida Keys. Unfortunately, the desired material could not be found, but two closely related natural products, *O*-methylelisabethadione (**66**) and *O*-methyl-*nor*-elisabethadione (**67**), were identified.[92] The enantioselective total syntheses of the assigned structure of (+)-elisabethadione (**63**), (+)-*p*-benzoquinone (**65**), and the two related natural products **66** and **67**, have been described.[92,93] Interestingly, the spectral data of synthetic and natural (+)-*p*-benzoquinone **65** were identical, but the reported ^{1}H- and ^{13}C-NMR data for the natural product (+)-elisabethadione, while similar, were different from the synthesized compound. On the basis of these data, it was concluded that either the assigned structure of the natural product or that of the synthetic material is incorrect. Notwithstanding, after careful reanalysis of the synthetic scheme to **63**, it was concluded that the synthetic sample of (+)-elisabethadione was assigned the correct structure.[92] Assuming that the natural products **65–67** are correctly assigned, these results imply that the assigned structure of (+)-elisabethadione is incorrect or its reported spectral data contain errors. During the course of these synthetic studies, questions arose about the assigned structure of another natural product, elisabethamine (**68**), isolated by the Kerr group from the methanolic extracts of *P. elisabethae* collected from the Florida Keys, which was proposed to be an aminohydroquinone. The structure of elisabethamine was

established with the aid of extensive spectroscopic studies.[94] Attempts at the synthesis of this compound revealed that the aminohydroquinone structure was unstable in air as it readily oxidized to the quinone.[92] This finding raises doubts about the assigned structure of (+)-elisabethamine. Compound **68** exhibited cytotoxicity against lung (LNCap) and prostate (Calu) cancer cell lines, with observed IC_{50} values of 10 and 20 $\mu g\ ml^{-1}$ as determined by an MTT assay.[94] An unusual diterpenoid alkaloid named *seco*-pseudopteroxazole (**69**), which belongs to the serrulatane class of diterpenes, was reported as a minor constituent from a coral specimen collected in San Andrés Island. A biological screening study revealed that this compound, which contains the uncommon benzoxazole functionality, displays moderate to strong *in vitro* inhibitorial activity against *M. tuberculosis* $H_{37}Rv$.[78] The original assignment of the C1 stereogenic center of *seco*-pseudopteroxazole has been revised to that depicted in structure **69**.[81,86] Work toward the total synthesis of *seco*-pseudopteroxazole is currently in progress.[95] In subsequent work with the same gorgonian extract, Rodríguez and coworkers recently reported on the isolation and structure characterization of three additional serrulatane-type diterpenes named elisabethadienol (**70**), 7-hydroxyerogorgiaenone (**71**) and 7,14-erogorgiaenediol (**72**). All three compounds, whose structural characterization was based exclusively on the results of chemical and spectroscopic analysis, turned out to be weak antitubercular agents.[96]

Elisabethatriene (**57**) Isoelisabethatriene (**58**) Elisabethatrienol (**59**) Erogorgiaene (**60**)

7-Hydroxyerogorgiaene (**61**) R = H
7,8-Dihydroxyerogorgiaene (**62**) R = OH

Elisabethadione (**63**) $R^1 = R^2 = H$
64 $R^1 = OH$, $R^2 = H$
O-Methylelisabethadione (**66**) $R^1 = H$, $R^2 = CH_3$

65

O-Methyl-*nor*-elisabethadione (**67**) Elisabethamine (**68**) *seco*-Pseudopteroxazole (**69**)

Elisabethadienol (**70**) 7-Hydroxyerogorgiaenone (**71**) 7,14-Erogorgiaenediol (**72**)

2.11.2.1.2(iii) Elisabethanes and related metabolites (compounds reported = 11) In 1998, a routine inspection of the ^{1}H- and ^{13}C-NMR data of the hexane solubles of *P. elisabethae* collected near San Andrés Island revealed that the majority of the compounds present in that specimen possessed distinctively different carbon frameworks quite unlike those previously described for the aglycon portion of the Pss and *seco*-pseudopterosins. Moreover, unlike the latter families of diterpene glycosides, the terpenoid metabolites found lacked a sugar moiety.[97] The structures and relative configurations of metabolites elisabethins A–C (73–75) were elucidated by interpretation of overall spectral data, chemical reactions, and X-ray crystallographic analyses. The prototype compound, elisabethin A (73), is a diterpene based on a new class of carbon skeleton trivially named elisabethane, whereas elisabethins B and C (74, 75) are, respectively, *nor*- and *bisnorseco*-diterpenoids based on most unusual carbocyclic skeletons. Biological screening of elisabethin B in the NCI 60 cell-line tumor panel indicated significant *in vitro* cancer cell cytotoxicity with concentrations of 10^{-5} mol l^{-1} eliciting significant differential responses at the GI_{50} level from all the renal, CNS, and leukemia cancer cell lines. One nonsmall cell lung cancer line, that is, NCI-H226, was substantially more sensitive than the average. Elisabethin B was also screened as a possible topical anti-inflammatory agent using an *in vivo* assay. However, doses of 74 below 0.3 mmol l^{-1} per ear were not significantly effective against bee venom PLA_2 arachidonic acid and croton oil-induced inflammation in mouse ears. In subsequent works with the same gorgonian extract, three closely related derivatives of elisabethin A were discovered and named elisabethin A acetate (76), elisabethin D (77), and elisabethin D acetate (78).[96,98] Follow-up biological screening of elisabethin D, whose structure was also confirmed by X-ray diffraction analysis, indicated no significant *in vitro* cancer cell cytotoxicity in the NCI's three-cell-line tumor panel, nor was it active in the NCI's test for agents active against the HIV. From an extract of *P. elisabethae* collected from the Bahamas, Ata *et al.*[61] reported two additional metabolites of the elisabethane class of diterpenes named elisabethins E and F (79, 80), each containing a conjugated homoannular diene π system. Curiously, while the authors claim that compounds 79 and 80 have similar configurations at C3, C7, and C9 as those reported for elisabethins A (73) and D (77) on the basis of similar optical rotation and NOESY data, the isobutenyl chain at C9 of 79 and 80 was drawn with opposite relative configuration. (The ^{1}H- and ^{13}C-NMR data for compounds 79 and 80 are not entirely consistent with the proposed structures, suggesting the need for further revision of these structures.) Subsequent chemical studies of the hexane solubles of the San Andrés species led to the isolation of elisabetholide (81), a novel β-keto-δ-lactone that is structurally reminiscent of elisabethin D (77) but has a distinct carbon skeleton,[99] a rare *bisnor*-diterpene named elisabethin G (82),[96] and a unique *pentanor*-diterpene, elisabethin H (83).[96] The structures of these metabolites were determined by interpretation of the 1D and 2D NMR, IR, UV, and HR-MS (high-resolution mass spectrometry) spectra. Although the biosynthesis of these compounds remains to be demonstrated, elisabetholide (81), for instance, can be considered as derived from the chemical degradation of elisabethin D through oxidation and concomitant cleavage of the C16–C17 bond.[99] Circumstantial evidence, on the other hand, suggests that elisabethin H (83) is actually a chemically degraded congener of elisabethin A (73) and not a regular sesquiterpene.[96] Albeit not without controversy, two research groups led by Mulzer and Rawal, have tackled the task of the total synthesis of the prototype elisabethane diterpene, elisabethin A (73).[100–103] The tricyclic *cis*, *trans*-fused 5,6,6 ring system of elisabethin A embodies a fully substituted enedione functionality and six contiguous stereogenic centers, of which one, at the junction of the three rings, is quaternary. Certainly, its intricate structure represents a formidable synthetic challenge for the organic chemist.[104] The total synthesis and absolute configuration of elisabethin C (75) has also been accomplished.[105]

Elisabethin A (**73**) $R^1 = R^2 = H$
Elisabethin A acetate (**76**) = $R^1 = H$, $R^2 = Ac$
Elisabethin D (**77**) $R^1 = OH$, $R^2 = H$
Elisabethin D acetate (**78**) $R^1 = OH$, $R^2 = Ac$

Elisabethin B (**74**)

Elisabethin C (**75**) R = H
Elisabethin G (**82**) R = OH

Elisabethin E (**79**)

Elisabethin F (**80**)

Elisabetholide (**81**)

Elisabethin H (**83**)

Elisapterosin A (**84**) R = OH
Elisapterosin E (**88**) R = H

Elisapterosin B (**85**)

Elisapterosin C (**86**)

Elisapterosin D (**87**)

Colombiasin A (**89**)

2.11.2.1.2(iv) Elisapteranes (compounds reported = 5) Another interesting family of secondary metabolites that appears to be derived from a common biosynthetic pathway was also recovered by Rodríguez and coworkers from the hexane solubles of the same Colombian species, namely, elisapterosins A–E (84–88). The cage-like tetracyclic elisapterane carbon skeleton of the elisapterosins A–E was previously not described, and thus, they represented a new class of C_{20} rearranged diterpenes. The structures and relative configurations of 84–88 were elucidated after interpretation of their combined spectroscopic data, chemical reactions, and in the case of elisapterosin B (85), X-ray diffraction analysis.[76,98,106] Elisapterosin B was found to effect strong inhibitory activity (79%) against *M. tuberculosis* $H_{37}Rv$ at a concentration of 12.5 μg ml^{-1}. The latter metabolite also exhibited moderately strong antiplasmodial activity ($IC_{50} = 10$ μg ml^{-1}) against *P. falciparum*, the parasite responsible for the most severe forms of malaria. In the NCI's three-cell-line tumor panel elisapterosins A and B indicated no significant *in vitro* cancer cell cytotoxicity. Similar to the elisabethanes, the elisapterane family of marine natural products have attracted considerable interest from the synthetic community, due in significant

part to the challenge presented by their fascinating molecular architecture.[102,107–110] The possible biogenetic relationship between these two skeletal classes of diterpenes has been recently demonstrated by the Rawal group.[102] Due to the common biosynthetic ancestry of these natural products, all have three distinctive stereocenters (C1–C3). From a synthetic perspective, these three stereocenters have represented formidable challenges because there are no convenient neighboring functional groups available to assist in their stereocontrol. A novel C–H functionalization strategy that has the potential to be a universal solution of the stereochemical issues associated with the synthesis of the three stereocenters in these natural products has been devised recently by Davies *et al.*[111] and Davis and Manning.[112]

2.11.2.1.2(v) Colombianes (compounds reported = 1) Motivated by the strong inhibitorial activity against *M. tuberculosis* exhibited by the benzoxazole alkaloids pseudopteroxazole (**55**) and *seco*-pseudopteroxazole (**69**), Rodríguez and Ramírez undertook the acquisition of additional quantities of *P. elisabethae* from waters near San Andrés Island, Colombia for further biological evaluation. In the course of purifying larger quantities of the hexane extract for additional bioassays, they isolated and identified smaller quantities of a new metabolite possessing an unprecedented carbon skeleton, colombiasin A (**89**).[113] Structure elucidation by interpretation of 2D-NMR spectroscopic data, IR, UV, and accurate mass measurements (HREI-MS – high-resolution electron-impact mass spectrometry) revealed that colombiasin A belonged to a distinctively different class of C_{20} rearranged diterpenes based on an intricate tetracyclic framework, named colombiane. To date, colombiasin A remains as the only member of this intriguing family of marine natural products. Colombiasin A exhibited moderately strong antiplasmodial activity ($IC_{50} = 10\,\mu g\,ml^{-1}$) against the malaria-causing parasite, *P. falciparum*, but essentially no antituberculosis activity was detected (17% GI of *M. tuberculosis* at $6.25\,\mu g\,ml^{-1}$). Although colombiasin A was not active against the FLU-A (influenza A virus) and HSV-1 viruses, in a primary assay against HBV, it exhibited potent activity with an $EC_{50} = 1.3\,\mu mol\,l^{-1}$. Follow-up studies revealed that in combination with the antiviral drug 3TC, an overall antagonistic interaction was observed. The combination assay with ADV resulted in a generally additive interaction (depending on the relative concentration of **89** to ADV, antagonistic to synergistic interactions were observed). Colombiasin A was also tested in anti-inflammatory, anticancer, protease inhibition, and cyclin B kinase assays, but no activity was found. Colombiasin A has proved to be a popular synthetic target, with total syntheses being reported firstly by Nicolaou *et al.*[114–118] and then by Kim and Rychnovsky.[110] Since their pioneering work, other research groups have pursued this challenging endeavor.[107–109,119,120] Jacobsen and coworkers have also demonstrated that colombiasin A readily isomerizes to elisapterosin B (**85**). While the mechanism of this remarkable transformation has yet to be determined, it is stereoselective and high-yielding (94%), and above all, it underlines the close structural relationship between these natural products.[108,109] It is precisely this confluence of structural complexity and interesting biological activities that has made these diterpenes such attractive targets for chemical synthesis.

2.11.2.1.2(vi) Cumbianes and seco-cumbianes (compounds reported = 3) Perhaps, the most enigmatic family of C_{20} rearranged diterpenoids thus far to be extracted from *P. elisabethae* is that comprised of the three polycyclic compounds christened by Rodríguez *et al.*[121] as cumbiasins A–C (**90–92**). The complex tetracyclic carbon core of cumbiasins A and B (**90 and 91**), dubbed cumbiane, and the carbotricyclic framework of cumbiasin C (**92**), named *seco*-cumbiane, are unprecedented and thus represent novel structure types. (*Cumbia* is originally a Colombian folk dance and dance music, and is Colombia's representative national dance and music.) The structures and relative configurations of metabolites **90–92** were elucidated by interpretation of overall spectral data, which included 2D NMR correlation methods, IR, and accurate mass measurements. Cumbiasin B, which possesses five contiguous oxygen-bearing carbon atoms, could be envisioned as a precursor for cumbiasin C via an enzyme-mediated 1,2-glycol oxidation that leads to cleavage of the C15–C16 bond followed by two synchronous ketalization steps. The remarkably stable dioxabicyclo[2.2.1]heptane core thus formed requires that the relative configuration at C2 of the proposed biosynthetic precursor **91** be S^* rather than R^* (i.e., 2-OH *cis* to Me-18). When screened for biological activity, cumbiasins A and B displayed essentially no *in vitro* antituberculosis activity (at a concentration of $6.25\,\mu g\,ml^{-1}$, compounds **90 and 91** induced 17% GI of *M. tuberculosis*). Cumbiasin C, however, displayed mild antitubercular activity with a calculated $MIC = 114.6\,\mu g\,ml^{-1}$. Cumbiasin A showed weak antimalarial activity ($IC_{50} = 17\,\mu g\,ml^{-1}$) against *P. falciparum*. As far as we have been able to ascertain, no member of the cumbiane or *seco*-cumbiane family of diterpenes has

yet succumbed to total synthesis. Notwithstanding, the tetracyclic ring system of the cumbiasins has been synthesized by a Diels–Alder reaction followed by tandem ring-forming reactions from an α-keto radical. These sequential cyclization reactions rapidly generate three rings with four new stereogenic centers.[122]

2.11.2.1.2(vii) Elisabanes (compounds reported = 2) Two novel *nor*-diterpenes, elisabanolide and 3-*epi*-elisabanolide (93, 94), were coisolated as minor metabolites by Rodríguez *et al.*[97,98] during the same investigation of *P. elisabethae* that led to the discovery of the elisabethane- and elisapterane-type diterpenes. The molecular structures of these crystalline solids were established by spectroscopic and X-ray diffraction studies. The name elisabane was proposed for the structurally unique carbon framework found in lactones 93 and 94. The close structural relationship between these compounds and the elisapterane diterpenoids was demonstrated when it was discovered that the thermally induced decomposition of elisapterosin A (84) leads primarily to *nor*-diterpenes 93 and 94 upon loss of (presumably) formaldehyde.[98] Biological screening of elisabanolide (93) revealed no significant activity against *M. tuberculosis* $H_{37}Rv$ or against the HIV.

Cumbiasin A (**90**) R = H
Cumbiasin B (**91**) R = OH

Cumbiasin C (**92**)

Elisabanolide (**93**)

3-*epi*-Elisabanolide (**94**)

Ileabethin (**95**)

Ileabethoxazole (**96**)

Sandresolide A (**97**) R = H
Sandresolide B (**98**) R = OH

Sandresolide C (**99**)

Caribenol A (**100**)

Caribenol B (**101**)

Amphilectolide (**102**) R = H
4-(acetyl)-amphilectolide (**103**) R = $COCH_3$

Amphiphenalone (**104**)

2.11.2.1.2(viii) Ileabethanes (compounds reported = 2) Ileabethin (95) was obtained as a pale yellow oil from *P. elisabethae* collected in San Andrés Island, whereas the structurally related diterpene alkaloid ileabethoxazole (96) was obtained as a light yellow oil from the same organism collected near Providencia Island.[123,124] The structures of these scanty secondary metabolites were elucidated by extensive spectroscopic data interpretation. These novel diterpenes are based on the previously not described carbon skeleton, named ileabethane, which appears to be biosynthetically related to the serrulatane (biflorane) skeleton. Ileabethin slowly decomposes at 25 °C upon prolonged storage in $CDCl_3$, which precluded probing its biological properties. Ileabethoxazole, on the other hand, is a stable material that displays strong inhibitory activity (92%) against *M. tuberculosis* $H_{37}Rv$ at the concentration range of 128–64 μg ml^{-1}. From these results it was determined that compound 96 has an MIC value of 57.4 μg ml^{-1}.

2.11.2.1.2(ix) Sandresanes, caribanes, and related skeletal systems (compounds reported = 8) A chemical study of the hexane extracts of *P. elisabethae* collected off San Andrés Island led Rodríguez *et al.*[125] to the isolation of three novel *nor*-diterpenes, sandresolides A, B, and C (97–99).[96] The structures of γ-butyrolactones 97–99 were determined by 1D and 2D NMR (^{13}C, ^{1}H, ^{1}H–^{1}H COSY, HMQC, HMBC (heteronuclear multiple bond correlation), and NOESY) and IR, UV, and HR-MS studies. The relative stereochemistry for the stereocenters in the bicyclic nuclei of the sandresolides (i.e., C1, C3, C4, C5, C8, and C11) were assigned primarily on the basis of NOESY NMR data acquired with sandresolide A (97). The sandresolides are based on a unique class of *nor*-diterpene carbon skeleton known as sandresane, which biogenetically, could stem from the adventitious rearrangement of an amphilectane-based precursor involving loss of a carbon atom and a concomitant 1,2-alkyl shift with ring expansion. Another pair of interesting *nor*-diterpenes isolated from this gorgonian is that comprised of caribenols A and B (100 and 101).[126] Caribenol A was found in specimens collected off Providencia Island whereas caribenol B originated from specimens collected near San Andrés Island. Their molecular structures were established by a combination of single-crystal X-ray analysis and comprehensive 2D NMR measurements. Caribenol A (100) and caribenol B (101), each possessing a new carbon skeleton, are the first examples in nature of such structural classes. Compounds 100 and 101 were found to have strong inhibitory activity (61 and 94%, respectively) against *M. tuberculosis* ($H_{37}Rv$) at a concentration range of 128–64 μg ml^{-1}. However, at lower concentrations, their inhibitory activities become significantly diminished. Caribenol A also demonstrated weak *in vitro* antiplasmodial activity against chloroquine-resistant *P. falciparum* W2 (IC_{50} 20 μg ml^{-1}). A rare *trisnor*-diterpene, amphilectolide (102),[99] was isolated from the same coral species from San Andrés Island, along with the closely related *nor*-diterpene, 4-(acetyl)-amphilectolide (103).[76] Their complete molecular structures, including relative stereochemistry, were established by interpretation of spectral data. Amphilectolide can be considered as derived from an amphilectane-based precursor by a series of oxidations and cleavages leading ultimately to the loss of carbons C10, C11, and C20 (i.e., amphilectane numbering system). Amphilectolides 102 and 103 induced, respectively, 42 and 9% GI of *M. tuberculosis* $H_{37}Rv$ at a concentration of 6.25 μg ml^{-1}. Structurally, amphiphenalone (104) can be classified as a novel *tetrisnor*-diterpene of composition $C_{16}H_{20}O_4$. The structure of this unusual metabolite, which was obtained from the same gorgonian specimens as lactones 102 and 103, was secured from spectroscopic methods including comprehensive 2D NMR studies.[76] Interestingly, although it appears that compound 104 contains an unprecedented carbon skeleton, the overall NMR evidence indicated that it possessed some structural features reminiscent of the amphilectane skeleton found in the aglycon component of the Pss. Notwithstanding, comparison of their molecular formulae showed that amphiphenalone lacked the four carbons typically ascribed to the isobutenyl chain at C1. In its place, there is now a ketone carbonyl suggesting loss of the C_4 alkenyl side chain by oxidative cleavage of the C1,14 bond of an amphilectane-based precursor.

2.11.2.1.3 Miscellaneous terpenoids

2.11.2.1.3(i) Sesquiterpenes (compounds reported = 1) The known sesquiterpene (+)-aristolone (105) was isolated by Rodríguez *et al.*[125] in significant amounts from the hexane extract of *P. elisabethae* from San Andrés Island. The structure of 105 was determined by spectral and X-ray crystallographic analyses.

2.11.2.1.3(ii) Steroids (compounds reported = 1) A tetrahydroxylated steroid isolated by the Schmitz group from specimens of *P. elisabethae* collected in Puerto Rico was shown by spectral analyses and degradative studies to be 5α-cholestane-3β,5,6β,9-tetrol (**106**).[127]

2.11.2.1.3(iii) Pseudopteranes (compounds reported = 1) A chemical study on the nonpolar fraction of the methanolic extract of *P. elisabethae* collected in the central Florida keys by Ata and Kerr yielded 12-acetoxypseudopterolide (**107**), a secondary metabolite of the pseudopterane class of diterpenes.[128] The molecular structure of **107** was established through spectroscopic analysis. This compound was shown to exhibit mild anticancer activity against a human prostate cancer cell line (LnCap) with an IC_{50} value of 47.9 $\mu g\,ml^{-1}$ using an MTT assay.

2.11.2.1.3(iv) Bisditerpenes (compounds reported = 1) While working with specimens of *P. elisabethae* from San Andrés Island, Rodríguez and Ramírez[86] isolated a novel bisditerpene of composition $C_{40}H_{58}O_2$, bis-7-hydroxyerogorgiaene (**108**). The NMR spectra and the ultraviolet (UV) and optical properties established that the two C_{20} units of **108** were structurally and configurationally identical. Since the serrulatane-based diterpene 7-hydroxyerogorgiaene (**61**) was coisolated during the same investigation, they assumed the latter metabolite to be a logical precursor to **108** upon undergoing further de-hydrogen coupling with another molecule through C8.

2.11.2.1.3(v) seco-Dolastanes (compounds reported = 1) The known algal metabolite isolinearol (**109**),[129] which belongs to the *seco*-dolastane class of diterpenoids, was isolated by Rodríguez and coworkers from the hexane extract of *P. elisabethae* from Providencia Island (unpublished results). The feeding deterrant effect of isolinearol (and its isomer linearol) from the seaweed *Dictyota cervicornis* toward the herbivorous gastropod *Astraea latispina* has been described (**Scheme 1**).[130,131]

(+)-Aristolone (**105**)   5α-Cholestane-3β,5,6β,9-tetrol (**106**)   12-Acetoxypseudopterolide (**107**)

Bis-7-hydroxyerogorgiaene (**108**)   Isolinearol (**109**)

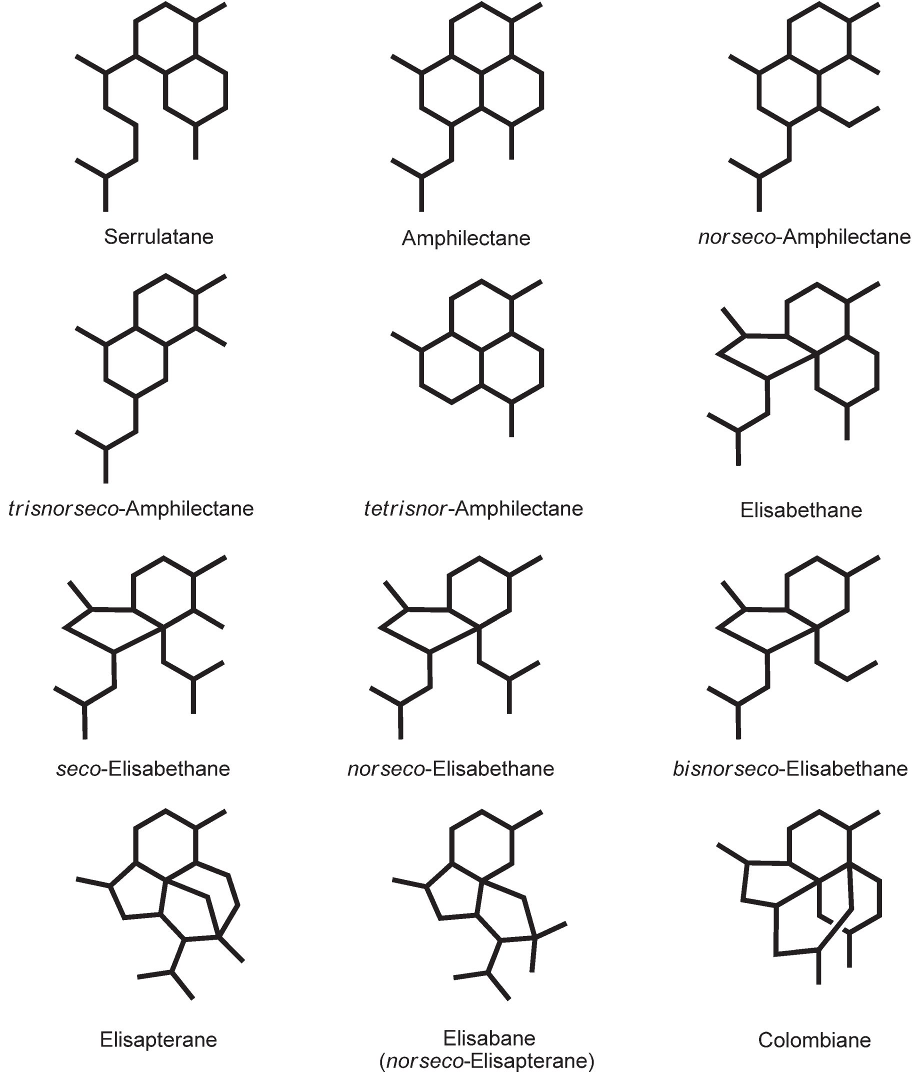
2.11.2.2 *Pseudopterogorgia bipinnata* (Verrill, 1864)

Common names: bipinnate sea plume, forked sea feather, and purple frilly gorgonia
Geographic division: Western Atlantic Ocean
Distribution: The Bahamas; Florida Keys; Caribbean islands; Antilles; and northern coast of South America

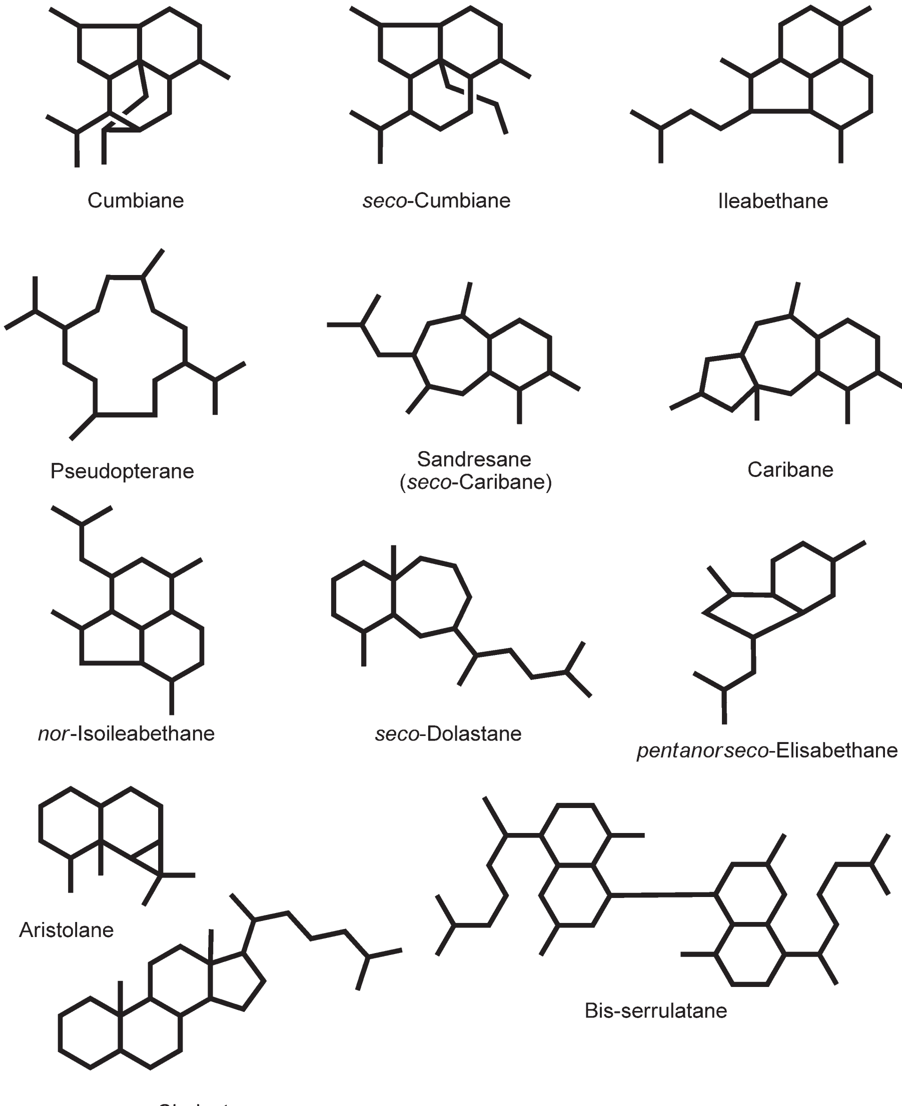
Brief description: This species of plume-like seafans differs in having more spaced branching off the main trunks, and the branches are stiff, not floppy. Both the primary and secondary branches all have branchlets emerging off their sides. These branchlets emerge mostly in matched pairs, on opposite sides of the stems. This species has a more open and 'sea fan' appearance than other species of this genus. The color is usually violet, occasionally yellow or whitish; spicules clear violet, colorless, or pale yellow; anthocodial rods colorless.

2.11.2.2.1 Diterpenes

2.11.2.2.1(i) Cembranes (compounds reported = 19) Diterpenoids of the cembrane class are common metabolites of several gorgonian genera, especially *Eunicea* and *Lophogorgia*. This is also true for gorgonians of the genus *Pseudopterogorgia* but only in a much more restricted sense.[15] Although cembrane-based diterpenoids occur only in three of the recognized species of *Pseudopterogorgia*, by far the major producer of cembrane derivatives is *P. bipinnata*. The most salient characteristics of *Pseudopterogorgia* cembranolides are their high level of oxygenation and their diverse pharmacological properties. Crude shipboard extracts of *P. bipinnata* collected off Jamaica Cay, Acklins Island, Bahamas exhibited *in vitro* inhibition of P338 murine tumor cell replication. Bioassay-guided purification led Wright *et al.* to the isolation of four compounds, bipinnatins A–D (**110**–**113**).[132] Their structures were determined through a combination of spectroscopic and X-ray crystallographic methods. Bipinnatins A (**110**), B (**111**), and D (**113**) were active against the P388 murine tumor cell line with IC_{50}'s of 0.9, 3.2, and 1.5 $\mu g\,ml^{-1}$, respectively. Bipinnatin C (**112**), which lacks the α,β-unsaturated carbonyl functionality at C15–C17, was much less active with an IC_{50} of 46.6 $\mu g\,ml^{-1}$ suggesting that the latter functionality enhances the activity of bipinnatins A, B, and D. As both bipinnatins B and D showed similar



Scheme 1 (Countinued)



Scheme 1 Terpenoid carbon skeletons from *Pseudopterogorgia elisabethae*.

activities, the epoxide found at C11, 12 may not be essential for activity. Bipinnatins A (**110**), B (**111**), C (**112**), E (**114**), and F (**115**) were isolated by Fenical and coworkers from *P. bipinnata* collected also in the Bahamas.[133] On the other hand, bipinnatins G–I (**116–118**) were isolated from an unidentified species of *Pseudopterogorgia* in the Bahamas.[134] Purportedly, the structural assignments of the latter cembranes were established by spectroscopic, X-ray, and chemical methods.[15] Some of the bipinnatins isolated by the Fenical group irreversibly inhibit nicotinic acetylcholine receptors by forming a covalent bond with a tyrosine residue at position 190 in the α-subunit of the receptor.[135–140] From the same gorgonian extracts they also identified bipinnatolide B (**127**) and bipinnatolide E (**128**), along with the unnamed derivative **129**.[15,134] These compounds are cyclic hemiketals possessing cross-conjugated dienone functionalities that are potentially derived by hydrolysis of a furanoid precursor. In all, more than 15 cembrane derivatives were isolated by the Fenical group from *P. bipinnata*, but the structures, physical properties, and spectral data of most of them are as yet unpublished. (Neither the structures nor the physical and chemical data for bipinnatolides A, C, and D have been described in the mainstream literature.)

Bipinnatin A (**110**) R = CO_2CH_3
Bipinnatin B (**111**) R = CHO
Bipinnatin F (**115**) R = CH_3

Bipinnatin D (**113**) R = CHO
Bipinnatin G (**116**) R = CH_3
Bipinnatin I (**118**) R = CO_2CH_3

Bipinnatin C (**112**)

Bipinnatin E (**114**)

Bipinnatin H (**117**)

Bipinnatin J (**119**)

Bipinnatin K (**120**)

Bipinnatin L (**121**)

Bipinnatin M (**122**)

Bipinnatin N (**123**) $R^1 = CH_3$, $R^2 = H$
Bipinnatin O (**124**) $R^1 = CO_2CH_3$, $R^2 = OAc$

Bipinnatin P (**125**)

Bipinnatin Q (**126**)

During a 1996 expedition to the San Andrés Archipelago, Colombia, Rodríguez and Shi[141] collected *P. bipinnata*, extracts of which were found to contain significant amounts of the known pseudopterane kallolide A (136), and smaller amounts of new bipinnatin J (119). The structure of furanocembranolide 119 was established spectroscopically and was subsequently confirmed by an X-ray study. Bipinnatin J was efficiently photoisomerized to kallolide A, thus definitively establishing the long-suspected biogenetic relationship between the cembrane and pseudopterane families of diterpenes. Follow-up biological screening of kallolide A and

bipinnatin J in the NCI 60 cell line tumor panel indicated no significant *in vitro* cancer cell cytotoxicity. During 2006, four stereoselective total syntheses of bipinnatin J were described.[142–145]

From the same gorgonian extracts, Rodríguez *et al.*[146,147] later reported the coisolation of known bipinnatin A (**110**), bipinnatin C (**112**), and bipinnatins G–I (**116–118**), along with the highly oxygenated bipinnatolides F–K (**130–135**). Their chemical structures including relative stereochemistry were established by detailed analysis of the spectral data in addition to X-ray diffraction analysis. During this investigation, the physical and spectral data for bipinnatins G–I were described for the first time. Follow-up biological screening of bipinnatin J (**119**), bipinnatolide F (**130**), and bipinnatolide G (**131**) in the NCI 60 cell-line cancer panel indicated no significant *in vitro* cancer cell cytotoxicity. Bipinnatins H and I (**117**, **118**), on the other hand, displayed strong cytotoxic action. At a concentration of 12.5 $\mu g\,ml^{-1}$, neither bipinnatin J, bipinnatolide F, or bipinnatolide K displayed significant antituberculosis activity.

Bipinnatolide B (**127**) R = CH_3
Bipinnatolide E (**128**) R = CO_2CH_3

129

Bipinnatolide F (**130**)

Bipinnatolide G (**131**)

Bipinnatolide H (**132**)

Bipinnatolide I (**133**)

Bipinnatolide J (**134**)

Bipinnatolide K (**135**)

2.11.2.2.1(ii) Pseudopteranes (compounds reported = 11) Although first encountered in the gorgonian corals *Pseudopterogorgia acerosa* and *P. kallos*, almost as many pseudopterane diterpenes have been found in extracts from *P. bipinnata*. For instance, in addition to large amounts of kallolide A (**136**), the pseudopterane bipinnapterolide A (**147**) was isolated from the chloroform extract of specimens collected in San Andrés Island. The structure of this metabolite, which has the uncommon 2,3-epoxy-1,4-dione moiety, was established by detailed analysis of the spectral data in addition to X-ray diffraction analysis.[146] On the other hand, extracts of *P. bipinnata* from Providencia Island were found to contain the pseudopteranes kallolide A acetate (**137**), kallolide C (**139**), kallolide C acetate (**140**), gersemolide (**149**), kallolides D–G (**141–144**), and bipinnapterolides A and B (**147, 148**).[148–150] The molecular structures of these compounds were deduced from spectral and X-ray diffraction studies. Curiously, kallolide E (**142**)[149] and bipinnapterolide B (**148**)[150] are the only pseudopterane diterpenes reported thus far, in which the C1 isopropylene group is β (pointing upward). The spontaneous 2-*C*-alkoxylation and 2-*C*-acyloxylation of kallolide A (**136**) in some solvents to yield solvolysis products that display net retention of configuration has been described by Rodríguez *et al.*[151] Solvolysis of kallolide A in [^{18}O]-labeled solvent demonstrated that the C2 alkoxyl of the solvolysis products originated from the solvent, suggesting that these conversions may proceed through an S_N1 mechanism with generation of a carbocation intermediate. In an *in vitro* antituberculosis screen none of these metabolites inhibited the growth of *M. tuberculosis* significantly (at 128 μg ml^{-1} the percent inhibition of the two most-active compounds, bipinnapterolide B and gersemolide, were 66 and 60.8%, respectively).[149,150] Similarly, none of the pseudopteranes isolated displayed relevant antiplasmodial activity against *P. falciparum*.

Kallolide A (**136**) R = OH
Kallolide A acetate (**137**) R = OAc
Kallolide B (**138**) R = H
2-*O*-Ethylkallolide A (**150**) R = OEt

Kallolide C (**139**) $R^1 = R^2 =$ OH
Kallolide C acetate (**140**) R^1 = OAc, R^2 = OH
Kallolide H (**145**) R^1 = OAc, R^2 = H
Gersemolide (**149**) $R^1 = R^2 =$ H

Kallolide D (**141**)

Kallolide E (**142**)

Kallolide F (**143**)

Kallolide G (**144**)

Kallolide I (**146**) R = OAc
Bipinnapterolide A (**147**) R = OH

Bipinnapterolide B (**148**)

2.11.2.2.1(iii) Gersolanes (compounds reported = 5) An interesting family of five highly functionalized polycyclic lactones featuring a unique bicyclo[11.1.0] carbon skeleton joined in a *trans* fashion was reported in 1998 by Rodríguez *et al.*[152] from the chloroform extract of *P. bipinnata* from San Andrés Island. These rare marine diterpenoids, named pinnatins A–E (**151–155**), can be regarded as representatives of the uncommon gersolane ring system. Structural assignments were accomplished through extensive spectroscopic analysis including 2D NMR, accurate mass measurements, X-ray crystallography, and chemical interconversions. During this work conclusive evidence was also presented which demonstrated unambiguously the biogenetic relationship between the cembrane and gersolane classes of diterpenes. When screened for *in vitro* cytotoxicity, pinnatins A and B displayed only moderate activity against a small number of tumor cell lines at the 10^{-4}–10^{-5} mol l^{-1} concentration range.

Pinnatin A (**151**)

Pinnatin B (**152**) $R^1 = H$, $R^2 = OAc$
Pinnatin C (**153**) $R^1 = H$, $R^2 = OH$
Pinnatin D (**154**) $R^1 = OH$, $R^2 = H$
Gersolide (**156**) $R^1 = R^2 = H$

Pinnatin E (**155**)

Caucanolide A (**157**)

Caucanolide B (**158**)

Caucanolide C (**159**)

Caucanolide D (**160**)

Caucanolide E (**161**) $R^1 = CH_3$, $R^2 = OCH_3$
Caucanolide F (**162**) $R^1 = OCH_3$, $R^2 = CH_3$

seco-Bipinnatin J (**163**)

2.11.2.2.1(iv) *seco-Cembranes and seco-pseudopteranes (compounds reported = 7)* Six rare diterpenoids, named caucanolides A–F (157–162), were isolated from specimens of this animal collected from shallow reef waters off Providencia Island.[153] Their molecular structures were elucidated by comprehensive analysis of spectroscopic data. Caucanolides A–C (157–159) represent the first examples of a new structural class of diterpenes, namely, the $\Delta^{2,3}$-*seco*-pseudopteranes, whereas caucanolides D–F (160–162) can be regarded as $\Delta^{2,3}$-*seco*-cembranes. (Compounds 157–162 were trivially named after the Cauca River, a mighty river in Colombia that flows into the Caribbean Sea.) While several marine *seco*-cembrane terpenoids have been reported before, there are no other examples of such natural products arising through oxidation cleavage of the C2/C3 bond. Caucanolide B (158) constitutes the only example from nature of a secondary metabolite possessing the N^1,N^1-dimethyl-N^2-acylformamidine functionality. Only caucanolides A and D demonstrated significant *in vitro* antiplasmodial activity against the malaria parasite, *P. falciparum* (IC_{50}'s 17 and 15 μg ml^{-1}, respectively). Caucanolide A moderately inhibited growth of some human cancer cells and marginally inhibited mycobacterial growth by 21% at a concentration of 6.25 μg ml^{-1}. When subjected to *in vitro* antiviral and anti-inflammatory testings, the compounds tested were found to be inactive.[153] From a specimen of *P. bipinnata* collected in 1996 near San Andrés Island, Rodríguez and Shi[147] isolated the novel *seco*-furanocembranolide, *seco*-bipinnatin J (163). Similar to bipinnatin J (119), this compound, whose structure was determined by a combination of spectroscopic methods, was devoid of relevant antituberculosis activity.

2.11.2.2.1(v) *Verrillanes (compounds reported = 1)* In 2000, Rodríguez and Shi[154] reported the isolation and structure determination of verrillin (164), a highly oxygenated diterpene based on an unprecedented carbon skeleton. Verrillin, a hexacyclic diterpene structurally related to some bipinnatins, was isolated as a minor constituent from the chloroform extract of *P. bipinnata* from San Andrés Island. Its structure was deduced exclusively from spectral studies. Although not yet proven, the carbobicyclic ring system of 164 could be produced by subsequent transannular cyclization of a suitable cembranoid via [$C_7 \rightarrow C_{11}$] bond formation. At 6.25 μg ml^{-1}, verrillin caused 0% GI of *M. tuberculosis*. Unfortunately, scarcity of material has precluded more extensive probing of the biological properties of this interesting natural product.

Verrillin (**164**)

Bisgersolanolide (**165**)

Biskallolide A (**166**)

Kallosin A (**167**)

Providencin (**168**)

Ciereszkolide (**169**)

Bielschowskysin (**170**)

Intricarene (**171**)

2.11.2.2.2 Miscellaneous terpenoids

2.11.2.2.2(i) Bisditerpenes (compounds reported = 2) A chemical study of the hexane extracts of *P. bipinnata* collected in San Andrés Island led Rodríguez and Shi[155] to the isolation of an unprecedented heptacyclic C_{40} bisditerpenoid named bisgersolanolide (**165**). The structure of this novel secondary metabolite, which was established by spectroscopic studies that included 2D NMR correlation methods, IR, UV, and accurate mass measurements, was subsequently confirmed by synthesis through Diels–Alder dimerization of two molecules of pinnatin C (**153**). The novel bisditerpenoid ether, biskallolide A (**166**), was isolated from the chloroform extracts of the same gorgonian specimens. Its structural assignment was mainly based on 1D and 2D NMR and MS spectral data, and was further confirmed by synthesis from kallolide A (**136**).[151] Biskallolide A did not display relevant inhibitorial activity against *M. tuberculosis* $H_{37}Rv$ (**Scheme 2**).

2.11.2.3 *Pseudopterogorgia kallos* (Bielschowsky, 1918)

Common names: None
Geographic division: Western Atlantic Ocean
Distribution: Florida Keys; The Bahamas; Dry Tortugas; Cuba; and San Andrés Archipelago (Colombia)

Brief description: *Pseudopterogorgia kallos* is very close to the shrubby examples of *P. bipinnata* but can, in summary, be recognized by: (1) the bushy habit of growth, with steeply ascending twigs not strictly in one plane and not always opposite; (2) the scaphoids with the spaces between the crests of the convex side filled in except for the median constriction; and (3) the short anthocodial rods with noticeably clubbed ends.

Cembrane Pseudopterane Gersolane

7,8-*seco*-Cembrane **2,3**-*seco*-Pseudopterane Verrillane

2,3-*seco*-Cembrane

bis-Gersolane

Scheme 2 Terpenoid carbon skeletons from *Pseudopterogorgia bipinnata*.

2.11.2.3.1 Diterpenes

2.11.2.3.1(i) Pseudopteranes (compounds reported = 12) In 1985, Fenical and coworkers reported the structure elucidation of four pseudopteranes, kallolides A, A acetate, B, and C (**136–139**), isolated from *P. kallos* collected at Channel Cay, Little Harbor Cay, and Club Cay in the Bahama Islands. Kallolide A (**136**) was the major metabolite isolated from the gorgonian, comprising 2% of the organic extract. Small quantities of metabolites **137–139** were isolated, which together accounted for less than 2% of the crude lipid extract. The structure of the sole crystalline metabolite, kallolide A acetate (**137**), was provided by single-crystal X-ray diffraction analysis. Structures for kallolides A–C were subsequently established on the basis of spectral analyses and chemical interconversions.[156] Pharmacological testing showed that kallolide A inhibits phorbol ester-induced inflammation (PMA, 4-β-phorbol-12-myristate-13-acetate) in the mouse ear assay at concentrations and with efficacies equivalent to the potent nonsteroidal anti-inflammatory drug indomethacin.

In a 2006 report, Rodríguez and coworkers described another chemical study of *P. kallos* that led to the isolation of additional pseudopteranes.[149] Thus, the lipophilic extracts of the gorgonian collected near Providencia Island afforded the known kallolide A (**136**), kallolide A acetate (**137**), kallolide C (**139**), 2-*O*-ethylkallolide A (**150**), gersemolide (**149**), and bipinnapterolide A (**147**), along with the pseudopterane analogs kallolide C acetate (**140**), kallolide E (**142**), and kallolides G–I (**144–146**). The identities of the known compounds isolated were established by comprehensive spectral data comparisons with data previously reported in the literature. The chemical structures of the new metabolites were established on the basis of spectral analyses, X-ray diffraction analyses, and chemical interconversions. None of the pseudopterane diterpenes isolated during this work revealed significant antitubercular or antiplasmodial activity. Several synthetic approaches to the pseudopterane ring system culminating with the stereoselective syntheses of kallolides A and B, were described from 1988 to 2003.[157–164]

2.11.2.3.1(ii) Gersolanes (compounds reported = 3) The previously known compounds pinnatin B (**152**), pinnatin D (**154**), and gersolide (**156**), all representatives of the rare gersolane class of diterpenes, were isolated from *P. kallos* found near Providencia Island.[149,165] The chemical structures of these compounds were established from careful spectral data comparisons with the data already available in the literature. These metabolites did not show significant inhibitorial activity against the pathogenic microbes *M. tuberculosis* $H_{37}Rv$ or *P. falciparum.*

2.11.2.3.1(iii) Kallanes (compounds reported = 1) Kallosin A (**167**), a novel diterpene possessing several unusual structural features, was isolated from the chloroform solubles of *P. kallos* from Providencia Island, Colombia.[166] The structural assignment of **167** was based mainly on 1D and 2D NMR spectral data and was further supported by accurate mass measurements and single-crystal X-ray diffraction analysis. Kallosin A has been envisioned as a rearranged pseudopterane diterpenoid based on the novel kallane skeleton. Although the biosynthesis of this new class of diterpenoids remains unknown, it would appear reasonable, on structural grounds, that the kallane ring system could be produced from a ring contraction reaction of a pseudopterane precursor such as kallolide A (**136**). At 500 $\mu g\,ml^{-1}$, kallosin A displayed no *in vivo* cytotoxicity in the brine shrimp lethality bioassay, whereas kallolide A showed a 64% death response at the same concentration after a 24 h count period.

2.11.2.3.1(iv) Providencianes (compounds reported = 1) The highly oxygenated hexacyclic structure of providencin (**168**), a naturally occurring crystalline solid isolated from *P. kallos* from Providencia Island, is based on a previously not described bicyclo[12.2.0]hexadecane ring system, trivially named providenciane, that was established through spectroscopic analysis and X-ray crystallographic analysis.[167] Biosynthetically, providencin could originate from the known furanobutenolide-based cembranoid bipinnatin E (**114**) through a photochemically mediated intramolecular C–H insertion reaction (Norrish type II or Norrish–Yang reaction) from the α,β-unsaturated aldehyde precursor. Synthetic studies toward the total synthesis of providencin are currently in progress.[168–170] Moreover, a biogenetically patterned synthetic approach to the unusual furan methylenecyclobutanol moiety in providencin has been described recently by Bray and Pattenden.[171] Providencin displays modest *in vitro* cytotoxicity against MCF7 breast cancer, NCI-H460 nonsmall cell lung cancer, and SF-268 CNS cancer (the percent of growth of the treated cells was 57, 39, and 94%, respectively).

2.11.2.3.1(v) Ciereszkanes (compounds reported = 1) An investigation of the chloroform extract of a Colombian specimen of *P. kallos* led to the isolation of a novel rearranged cembrane, ciereszkolide (**169**). The structure of **169** is based on a new 13-membered carbocyclic skeleton, named ciereszkane, which possesses several unusual structural features. The structure of ciereszkolide was resolved by interpretation of 1D and 2D NMR spectroscopic data supported by HRFAB-MS, IR, UV, and a single-crystal X-ray diffraction analysis.[172] From a structural viewpoint, ciereszkolide appears to be related to the *Pseudopterogorgia*-derived cembrane family of diterpenes by a ring contraction process requiring the overall migration of the C2–C3 σ-bond of a suitable cembrane precursor to the C4 position. Ciereszkolide did not display *in vivo* cytotoxicity in the brine shrimp lethality bioassay, even at the highest concentration (500 $\mu g\,ml^{-1}$), after a 24 h count period. During an *in vitro* antituberculosis screen against *M. tuberculosis* $H_{37}Rv$ at 6.25 $\mu g\,ml^{-1}$, compound **169** caused 0% inhibition.

2.11.2.3.1(vi) Bielschowskyanes (compounds reported = 1) Bielschowskysin (**170**) was isolated as a colorless crystalline solid from the ethyl acetate extract of *P. kallos* collected near Providencia Island. Its highly oxygenated hexacyclic structure is based on a previously not described tricyclo[9.3.0.0^{2,10}]tetradecane ring system that was established through spectroscopic analysis and X-ray crystallographic analysis.[173] Although still unproven, the bielschowskyane ring system might be synthesized *in vivo* by subsequent cyclization of a suitable cembranoid precursor via successive [C7 → C11] and [C6 → C12] bond formation. Bielschowskysin showed antiplasmodial activity ($IC_{50} = 10\ \mu g\,ml^{-1}$) when tested against *P. falciparum*, and it was also found to display strong and specific *in vitro* cytotoxicity against the EKVX nonsmall cell lung cancer cell ($GI_{50} < 0.01\ \mu mol\,l^{-1}$) and CAK-1 renal cancer ($GI_{50} = 0.51\ \mu mol\,l^{-1}$). Due to its intriguing architecture and promising biological properties, it is not surprising that bielschowskysin is a current popular target for

total synthesis. A concise and stereocontrolled assembly of the tetracyclic core of bielschowskysin has been achieved by Doroh and Sulikowski.[174] Lear and coworkers have also recently reported a biomimetically inspired strategy to form the polycyclic core of **170** by virtue of a substrated-controlled [2 + 2] photocycloaddition of an allene-butenolide.[175]

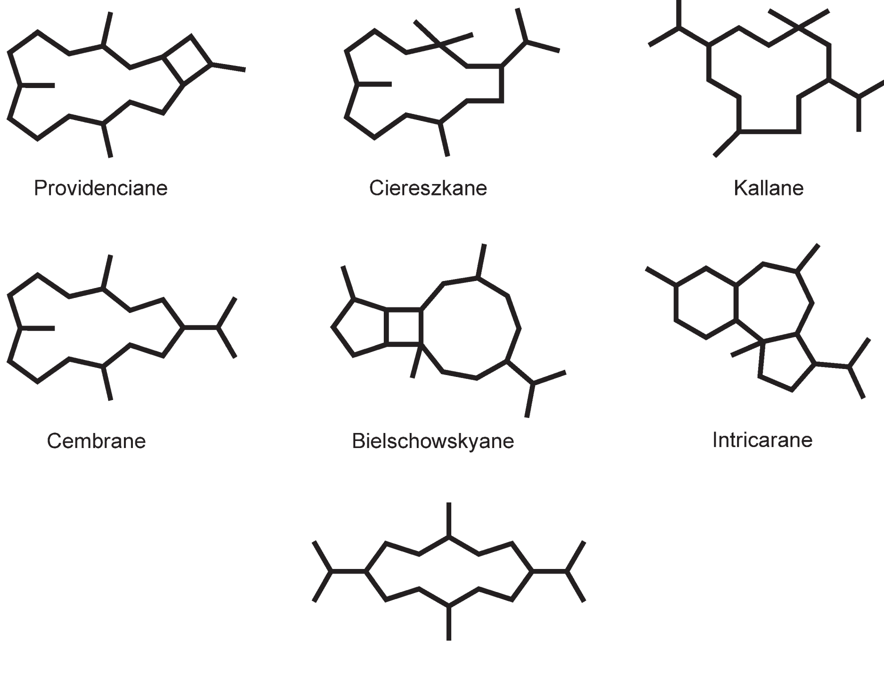
2.11.2.3.1(vii) Intricaranes (compounds reported = 1) During an investigation to screen marine invertebrate extracts for antitubercular metabolites with low cytotoxicity, the novel trispiropentacyclic diterpene, intricarene (**171**), was isolated as a white crystalline solid from the hexane extract of *P. kallos* from Colombia.[176] Its highly entangled structure was established by interpretation of NMR, IR, UV, and HREI-MS data and subsequently confirmed by X-ray diffraction analysis. Intricarene has an unprecedented regular diterpenoid skeleton that can be formally derived from the cembrane skeleton found in many *Pseudopterogorgia* metabolites by sequential cyclization through [C6 → C11] and [C2 → C12] bond formation. The co-occurrence of **171** with various furanobutenolide-based cembranes within the same organism (see below) supports such biogenetic pathway. At a concentration of 128 μg ml^{-1}, compound **171** inhibited the growth of *M. tuberculosis* $H_{37}Rv$ by only 15%. Unfortunately, the paucity of this compound has prevented its further biological screening. Two elegant asymmetric total syntheses of (−)-bipinnatin J (**119**) and its conversion into (+)-intricarene through a transannular 1,3-dipolar cycloaddition, have been described recently by the Pattenden and Trauner groups.[142,145]

2.11.2.3.1(viii) Cembranes (compounds reported = 8) An extensive chemical study of the secondary metabolites found in the crude organic extract of *P. kallos* from Colombia led recently to the isolation of seven new cembranolides, bipinnatins K–Q (**120–126**), and one known compound, bipinnatin E (**114**).[177] The molecular structures of compounds **120–126**, many of which possess unique structural features, were assigned mainly by 2D NMR spectroscopic methods and X-ray crystallographic analyses. Interestingly, this is the only report thus far that describes the isolation of cembranoid diterpenes from this gorgonian species. The discovery of these cembranoid lactones in *P. kallos* may lend support to previously proposed mechanisms for the biosynthesis of other natural products, typically referred to as 'rearranged cembranes', isolated from the same gorgonian specimen (i.e., providencin (**168**), ciereszkolide (**169**), bielschowskysin (**170**), and intricarene (**171**)). The *in vitro* cytotoxicity of bipinnatins M (**122**), N (**123**), and Q (**126**) against the NCI tumor cell lines MCF breast cancer, NCI-H460 nonsmall cell lung cancer, and SF-268 CNS cancer were evaluated, but only bipinnatin Q displayed significant cytotoxic activity. Some of the bipinnatins isolated during this investigation also proved to be inhibitors of the acetylcholine receptors with calculated IC_{50} values ranging from 0.23 to 0.83 μmol l^{-1}. Interestingly, the most potent inhibitory diterpenoid in this series, bipinnatin E (**114**), lacks a C2 acetate group and possesses both the furanyl epoxy array and the α,β-unsaturated aldehyde moiety in the C1 position. The striking similarity of the secondary metabolites isolated from *P. kallos* and *P. bipinnata* attests to their close taxonomic relationship. Thus, the presence of identical metabolites within these organisms supports the theory that these gorgonian species are intimately related taxonomically (**Scheme 3**).[1]

2.11.2.4 *Pseudopterogorgia acerosa* (Pallas, 1766)

Common names: purple sea plume, smooth sea feather, and red sea whip
Geographic division: Western Atlantic Ocean
Distribution: Bermuda; south and west Florida; Florida Keys; The Bahamas; Gulf of Mexico; Caribbean islands; and Antilles

Brief description: This is one of the two common West Indian sea plumes. Although this species may be confused with *P. americana*, which is very similar, the two species can be clearly distinguished. This species is a large plume-like gorgonian with long floppy main and secondary branches; surface not slimy in life. It forms some spectacular and gigantic 'ostrich feathers'. It has secondary branches, which emerge from opposite sides of the main stem, though usually not at the same level. It is characterized by its acute, slender, gently curved, smooth, or nearly smooth scaphoids. Colonies may reach to nearly 2 m tall and broad.



Scheme 3 Terpenoid carbon skeletons from *Pseudopterogorgia kallos*.

2.11.2.4.1 Diterpenes

2.11.2.4.1(i) Pseudopteranes (compounds reported = 24) During a June 1980 Caribbean expedition on board the University of Miami research vessel *Calanus*, Fenical and coworkers found that extracts of the red sea whip *P. acerosa* collected near the Florida Keys, possessed considerable cytotoxic properties. Cytotoxicity was assayed by utilizing the fertilized egg of the California sea urchin *Lytechinus pictus*. Subsequent extraction and purification of this extract using conventional chromatographic methods led to the isolation of the irregular diterpenoid, pseudopterolide (**172**), as a major metabolite of *P. acerosa* (0.7% dry weight).[178] The structure elucidation of **172**, including its absolute stereochemistry, was based on conventional spectroscopic methods and on an X-ray investigation of a suitably prepared crystalline urethane derivative (obtained from **172** in two steps by reaction with H_2SO_4/MeOH followed by treatment of the pseudopterolide–methanol adduct formed with *p*-bromophenyl isocyanate in benzene/pyridine). Pseudopterolide inhibits overall cell cleavage but does not inhibit nuclear division in the fertilized urchin egg assay. Although pseudopterolide is a highly bioactive compound, it has not been shown to produce the ichthyodeterrent effect found in the crude extract of the gorgonian.[10–13] In a 1990 report, Chan and coworkers reported that specimens from this gorgonian species collected at Man-of-War Bay, Tyrrel's Bay, and Culloden Bay, on the north coast of Tobago, contained the rare dimethylamino pseudopterane derivative, tobagolide (**173**). Analysis of standard IR, UV, and NMR spectroscopic data revealed all the major functional and structural features in the molecule.[179,180] Interestingly, marked seasonal variations in the distribution of metabolites in this species were observed; tobagolide was the major constituent isolated from July collections of *P. acerosa*, but it was not found during March collections of the species. Curiously, one of the dominant constituents in the March harvest was an acidic diterpenoid, which upon reaction with diazomethane, was converted into deoxypseudopterolide (**174**).[180] Ester **174** was isolated directly (i.e., without diazomethane treatment) from the July collection, which also afforded tobagolide. The structural assignment of ester **174** depended heavily on 2D-NMR spectroscopy. The stereochemistry of **174**, although not formally assigned, probably corresponds with that of previously

known models based on NMR spectral comparisons.[180] Paquette and Astles[181] synthetically interconverted tobagolide (**173**) and pseudopterolide (**172**), unequivocally establishing the stereochemical relationship, both relative and absolute, between the two compounds. Later on, the same researchers reported the isolation of another series of pseudopteranoids from *P. acerosa* collected at Lau's reef (–10 m), Tobago. The isolated pseudopteranoids corresponded to about 0.2% of the dry weight of the specimen.[182] The most abundant (43.2%) was identified as the same pseudopterolide–methanol adduct **175** of Fenical and coworkers,[178] but pseudopterolide (**172**) itself was not isolated. The second and third most abundant constituents, named gorgiacerone (**176**) and gorgiacerodiol (**177**), were isolated in 21.9 and 14.8% yields, respectively. Their molecular structures were resolved primarily on the basis of NMR data alone. The fourth compound (10.5%), named methoxygorgiacerol, was assigned structure **178** from the NMR data, whereas the structure of the next constituent (6.5%), diepoxygorgiacerone (**179**), was assigned by single-crystal X-ray crystal structure analysis.[183] The least abundant pseudopteranoid isolated (1.7%), isogorgiacerodiol, was assigned structure **180** on the basis of the NMR data. A 1994 collection of *P. acerosa* from yet another location in Tobago (Buccoo Reef) was later shown to contain the new pseudopterane diterpene 11-gorgiacerol (**181**), along with the previously known pseudopterolide and gorgiacerodiol.[184] The molecular structure of 11-gorgiacerol was established by 2D NMR spectroscopy.

Pseudopterolide (**172**)

Tobagolide (**173**)

Deoxypseudopterolide (**174**)

175 R = OCH_3
Isogorgiacerodiol (**180**) R = OH

Gorgiacerone (**176**)

Gorgiacerodiol (**177**) R = H
Methoxygorgiacerol (**178**) R = Me

Diepoxygorgiacerone (**179**)

11-Gorgiacerol (**181**)

Pseudopteradiene (**182**) R = CH_3
Pseudopteradienoic acid (**183**) R = H

11-Pseudopteranol (**184**) Pseudopteranoic acid (**185**) Diepoxygorgiacerodiol (**186**)

The isolation and structural assignment of five pseudopteranoid diterpenes, pseudopteradiene (**182**), pseudopteradienoic acid (**183**), 11-pseudopteranol (**184**), pseudopteranoic acid (**185**), and diepoxygorgiacerodiol (**186**) were described by Rodríguez and Soto[185] during a routine chemical investigation of Puerto Rican specimens of *P. acerosa* collected near La Parguera, Puerto Rico in December 1994. Detailed analysis of the spectral data and chemical methods were used to establish their structures and to define the relative stereochemistry. During this investigation, the previously reported pseudopteranoids **172, 174, 175, 177, and 180** were coisolated. Further chemical scrutiny of the same gorgonian extracts by Rodríguez and Soto[186,187] led subsequently to the discovery of the tetracyclic pseudopterane alkaloids alanolide (**187**) and aceropterine (**188**). Alanolide is a novel *nor*-diterpenoid, whereas aceropterine is a pseudopterane diterpene with a unique transposed lactone moiety. Both of these metabolites appear to be biogenetically related to tobagolide (**173**). The molecular structures of these nitrogenous metabolites were determined by 2D-NMR experiments and supported by extensive mass spectral analysis. Neither pseudopterolide (**172**) nor alanolide (**187**) was active against three human tumor cell lines as they exhibited no detectable cytotoxicity at 50 μg ml^{-1} in any of the cell lines (HCT 116, CCRF-CEM, and MCF-7). Moreover, pseudopterane **172** proved inactive in the NCI's test for agents active against HIV.[186] However, another representative of the pseudopterane family of diterpenes possessing the uncommon 3,4;5,6 diepoxyfuran moiety was also isolated from the same octocoral extracts from Puerto Rico.[188] The structure of β,β-diepoxypseudopterolide–MeOH adduct (**189**) was established by detailed analysis of the spectral data in addition to NMR spectral comparisons with relevant pseudopterane models. Synthetic strategies for the total synthesis of pseudopterolide and some of its structurally related congeners (such as 11-pseudopteranol (**184**) and gorgiacerone (**176**)) have been reported by Paquette and coworkers.[189–191] Many examples of elegant synthetic approaches to the 2,5-furanocyclic ring system of the pseudopterane family of natural products along with prototype pseudopterane and furanocembrane systems have been described.[157–164,192–205]

A 2008 report by Kerr and coworkers[206] described for the first time a rare family of pseudopterane diterpenes with a fatty acid moiety. These lipidyl pseudopteranoids, named lipidyl pseudopteranes A–F (**190–195**), were isolated from specimens of *P. acerosa* collected from Sweetings Cay, Bahamas. Structure elucidation of these compounds was based on 1D and 2D NMR data and MS. A biomimetic synthesis of lipidyl pseudopterane A (**190**) from pseudopterolide (**172**) was used to establish its absolute configuration. The relative configuration of **193** was established on the basis of comparison with **190–192**; however, the relative configuration of C11 could not be unambiguously established. Lipidyl pseudopteranes A and D exhibited modest yet selective inhibitory activity against protein tyrosine phosphatase 1B.

2.11.2.4.1(ii) Cembranes (compounds reported = 3) Acerosolide (**196**), obtained naturally from *P. acerosa* collected on the north coast of Tobago as a carboxylic acid that was later esterified with diazomethane, was purified by chromatography and obtained as a gum that appeared to be homogeneous but to undergo a slow degradation on storage.[180] The reported structural assignment of acerosolide was based primarily on 2D-NMR spectroscopy and was devoid of relevant stereochemistry. The relative stereochemistry shown for **196** was tentatively established by total synthesis.[207,208] A rare norcembranolide diterpene, gorgiacerolide (**197**), was isolated as a minor metabolite from extracts of *P. acerosa* collected in Puerto Rico. Its structure, including relative stereochemistry, was established primarily by careful analysis of the spectral data.[188] Because the researchers did not use EtOH during the isolation and purification procedures, nor was the pure material stored

in EtOH, the possibility that gorgiacerolide is an artifact of the extraction or purification process was ruled out. Quite recently, the organic extract of *P. acerosa* collected in Saint Pierre (Martinique), was subjected to solvent partition followed by chromatographic purification to yield acerolide (**198**) along with pseudopterolide (**172**).[209] The structure of acerolide was determined on the basis of detailed spectroscopic analysis. The coexistence of isomeric lactones **172** and **198** within the same gorgonian species clearly indicates the close biogenetic relationship between the cembrane and pseudopterane skeletons. Thus, acerolide must likely be the immediate biogenetic precursor to pseudopterolide upon undergoing a photochemically induced ring contraction reaction.[141] Compounds **172** and **198** showed moderate *in vitro* cytotoxicity against a small panel of 14 tumor cell lines.[209]

Alanolide (**187**)

Aceropterine (**188**)

189

Lipidyl pseudopterane A (**190**) R = palmityl
Lipidyl pseudopterane B (**191**) R = oleoyl
Lipidyl pseudopterane C (**192**) R = stearyl

Lipidyl pseudopterane D (**193**) R = palmityl

Lipidyl pseudopterane E (**194**) R = palmityl

Lipidyl pseudopterane F (**195**) R = palmityl

Acerosolide (**196**)

Gorgiacerolide (**197**)

Acerolide (**198**)

12-Hydroxy-*E*-γ-bisabolene (**199**)

Bis(gorgiacerol)amine (**201**)

Peridinin (**202**)

(−)-Curcuhydroquinone (**200**)

Acerosterol (**203**)

2.11.2.4.2 Sesquiterpenes

2.11.2.4.2(i) Bisabolanes (compounds reported = 2) From a collection of *Pseudopterogorgia* sp. gathered in Belize whose spicule analysis placed it as closely related to the abundant Caribbean sea whip *P. acerosa*, Look *et al.*[210] isolated exclusively the sesquiterpene alcohol 12-hydroxy-*E*-γ-bisabolene (**199**). The molecular structure of this alcohol was established based upon spectral analyses and through chemical interconversions. Alcohol **199** was isolated by repetitive chromatography as 20% of the organic extracts of *Pseudopterogorgia* sp. The extraction of samples of *P. acerosa* collected off the west coast of Barbados led Miller *et al.*[211] to the isolation of significant amounts of a previously known bisabolane sesquiterpene, (−)-curcuhydroquinone (**200**). Assays conducted on coral reefs have demonstrated that the crude lipid extract of *P. acerosa*, and the purified sesquiterpenoid **200**, deter natural predators at concentrations below their normal levels in the gorgonian tissue.[10–13]

2.11.2.4.3 Miscellaneous terpenoids

2.11.2.4.3(i) Bisditerpenes (compounds reported = 1) Tinto *et al.*[182] reported in 1991 that the least abundant (1.5%) component of the seven pseudopteranoids isolated from their *P. acerosa* collection consisted of the rare bisditerpenoid amine bis(gorgiacerol)amine (**201**). The molecular structure of **201**, whose MS data revealed its molecular formula to be $C_{42}H_{47}O_{12}N$, was deduced mainly by 2D NMR spectroscopy. The NMR spectra and optical activity established that the two C_{21} units conforming **201** were structurally and configurationally identical. The broadening of the ^1H signals observed was attributed to the reduced rate of tumbling of this rather large molecule in solution.

2.11.2.4.3(ii) Carotenoids (compounds reported = 1) The well-known carotenoid peridinin (**202**) was isolated by Tinto and coworkers from a sample of *P. acerosa* collected in March 1987 on the north coast of Tobago. After preliminary characterization by NMR, UV, IR, and HR-MS, they went on to achieve the first complete ^1H and ^{13}C spectral assignment of the red pigment.[212] Peridinin was first isolated over 100 years ago and has since been isolated from a variety of marine and fresh water dinoflagellates and from clams, sea anemones, and corals. Peridinin has been synthesized by several research groups.[213–217] Recent investigations expanding on the biological properties of peridinin and other peridinin-related analogs have been recently described in the literature.[218,219]

2.11.2.4.3(iii) Steroids (compounds reported = 1) The tetrahydroxysterol acerosterol (**203**) was isolated from specimens of *P. acerosa* collected at Lau's reef (−10 m), Tobago by the Tinto group. The structure of **203** was determined to be 4α,23,24(*R*) -trimethyl-5α-cholest-22*E*-ene-1α,3β,6β,11α-tetraol on the basis of 2D NMR spectroscopy.[220] Since dinosterol and other derivatives bearing a 4α-methyl group are characteristic sterols of dinoflagellates, it seems likely that acerosterol might have originated from symbiotic zooxanthellae present in the gorgonian coral (**Scheme 4**).

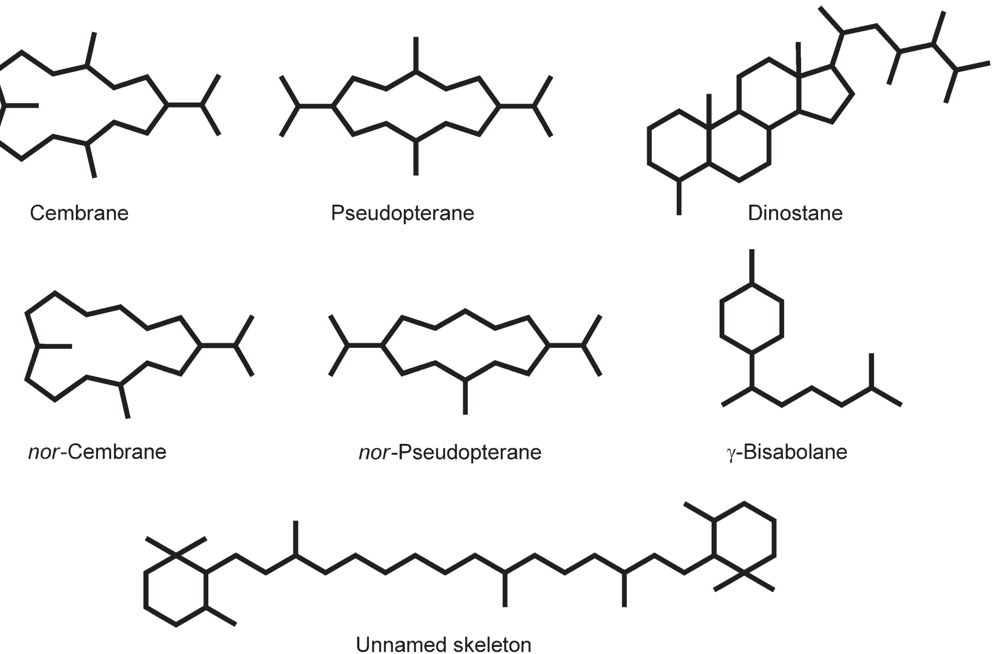
2.11.2.5 *Pseudopterogorgia americana* (Gmelin, 1791)

Common name: slimy sea plume
Geographic division: Western Atlantic Ocean
Distribution: Bermuda; Florida Keys; The Bahamas; Caribbean islands; and Antilles

Brief description: This large plume-like gorgonian has secondary branches, which emerge from opposite sides of the main stem, usually in opposing pairs, that is, at the same level. Colonies reach 1 m in height. This species produces substantial quantities of mucus, so it feels slimy to the touch. Distinct differences in spiculation serve to distinguish *P. americana* from *P. acerosa*. Scaphoids are strongly curved with the sharp ends often recurved outward; convex profile being distinctly echinulate; spindles acute; and no flat rods in anthocodiae.

2.11.2.5.1 Sesquiterpenes

2.11.2.5.1(i) Bicyclic and Tricyclic Hydrocarbons (compounds reported = 8) The sesquiterpene hydrocarbon mixture occurring in the gorgonian *P. americana* collected in shallow waters at Bermuda and the Florida Keys was examined in detail by Weinheimer *et al.*[221] in 1968. It was found to consist primarily (96%) of four components, namely, 9-aristolene (**204**; 8%), 1(10)-aristolene (**205**; 25%), (+)-γ-maaliene (**206**; 8%), and (+)-β-gorgonene (**207**; 55%). The hydrocarbon mixture (a gift from the late Professor L. S. Ciereszko) was isolated by chromatography (Florisil) of the nonsaponifiable fraction of the cold hexane extract of the gorgonian. The identities of compounds **204 and 205** followed from comparisons with



Scheme 4 Terpenoid carbon skeletons from *Pseudopterogorgia acerosa*.

reported properties, particularly their NMR spectra. The structures of 206 and 207 were determined by chemical conversion into the corresponding diol and using single-crystal X-ray diffraction, respectively. During an investigation of the volatiles obtained by steam distillation from the *n*-hexane extracts of powdered air-dried *P. americana* collected off Havana, Cuba, Rosado and coworkers reported the isolation and identification of a variety of known sesquiterpenoid hydrocarbons, namely, 9-aristolene (**204**), 1(10)-aristolene (**205**), (+)-γ-maaliene (**206**), β-epibourbonene (**208**), α-chamigrene (**209**), and calamenene (**210**) (the latter compound was suspected to be an artifact formed during the distillation of the hydrocarbon fraction). All of the known compounds were identified by means of GC–MS measurements. During this research a new sesquiterpene hydrocarbon, caridiene (**211**), was also discovered. The structure of **211** was established spectroscopically.[222] Syntheses for isocaradiene, an isomeric compound of natural caridiene (**211**), and (±)-α-chamigrene (**209**) have been reported.[223,224]

2.11.2.5.1(ii) Germacranes and elemanes (compounds reported = 7) The furanogermacrene derivative furanotriene (**212**), was first isolated by Fenical and coworkers from extracts of *P. americana* collected at Carrie Bow Cay, Belize. From nonpolar fractions, this moderately unstable furan was successfully purified by normal-phase HPLC.[225] The originally proposed structure for this interesting furanogermacrane derivative, established on the basis of spectroscopic analysis, was later revised by Chan *et al.*[226] to the structure shown herein. A synchronous chemical investigation of *P. americana* and a *Pseudopterogorgia* sp. closely related to the former species, both harvested at Man-of-War Bay, Tobago, led Chan *et al.*[226] to the isolation of sesquiterpenes **212–218**. The structures of the new compounds discovered (**214** and **218**) were carefully established by spectroscopic methods. Germacranes **212** and **214** were quite unstable when exposed to air and rapidly decomposed. Isofuranotriene (**214**) and furanodiene (**215**) were isolated as a mixture, but were separated subsequently after preparative TLC using $AgNO_3$ (15%) impregnated silica gel with light petroleum-EtOAc (9:1) as eluant. Curiously, the relative stereochemistry of the stereogenic centers in elemanes **217–218** was not defined during the investigation.[226]

9-Aristolene (**204**) 1(10)-Aristolene (**205**) (+)-γ-Maaliene (**206**) (+)-β-Gorgonene (**207**)

β-Epibourbonene (**208**) α-Chamigrene (**209**) Calamenene (**210**) Caridiene (**211**)

Furanotriene (**212**) (+)-Germacrene D (**213**) Isofuranotriene (**214**) **215** R = CH_3 **216** R = CO_2CH_3

217

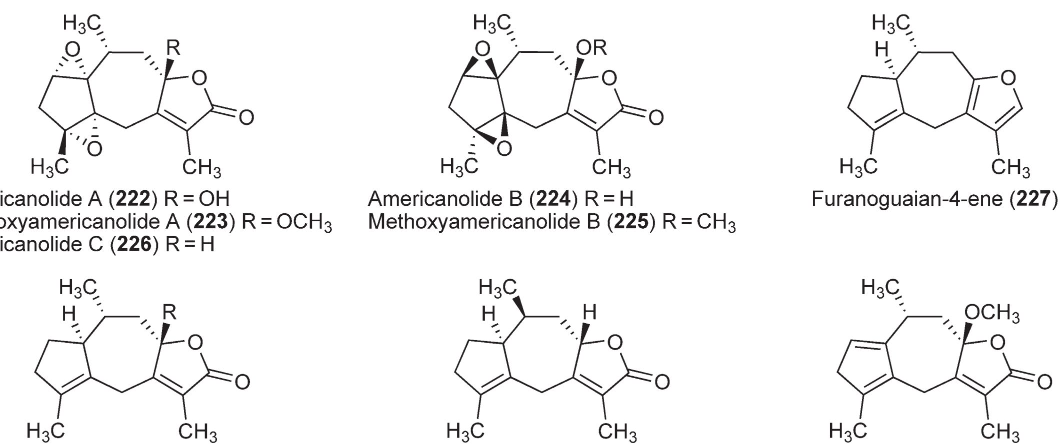
218

219 $R^1 = Ac$, $R^2 = OH$
200 $R^1 = H$, $R^2 = OH$
220 $R^1 = Ac$, $R^2 = H$

221

2.11.2.5.1(iii) Bisabolanes (compounds reported = 4) A family of four bisabolene sesquiterpenes, isolated from Barbadian *P. americana*, was reported by Miller *et al.* in 1995. The gorgonian extracts yielded the new compound (−)-curcuhydroquinone-1-monoacetate (219), and the known compounds (−)-curcuhydroquinone (200), (−)-curcuphenol acetate (220), and (−)-curcuquinone (221).[211] The structures of all compounds were confirmed by the use of HMQC and HMBC NMR experiments.

2.11.2.5.1(iv) Guaianes (compounds reported = 17) During the course of an investigation of the biologically active constituents of *P. americana* collected in La Parguera, Puerto Rico, Rodríguez and Boulanger encountered a family of five metabolites belonging to the guaiane class of sesquiterpene lactones. The structures of prototype lactones 222–226, which were trivially designated americanolides, were established by spectroscopic methods, mainly NMR and MS.[227] The molecular structure of methoxyamericanolide B (225), including relative stereochemistry, was later confirmed by single-crystal X-ray crystallography.[228] Further investigations with extracts of this coral species allowed the Puerto Rico group to eventually isolate many additional metabolites of the same structural class, including furanoguaian-4-ene (227), a short-lived furanoguaiane precursor that gradually decomposed on standing.[229,230] In all, 17 guaiane metabolites were isolated and purified, and their structures, including relative stereochemistry, deduced as 222–238 on the basis of interpretation of spectroscopic data in combination with X-ray crystallographic analyses. Methoxyamericanolide A (223) is a strong and selective inhibitor of MOLT-4 leukemia cells with an IC_{50} of 0.1 μg ml^{-1}. Although americanolide D (228) exhibited very modest levels of cytotoxicity to HeLa and CHO-K1 cells (ED_{50} s = 30 and 100 μg ml^{-1}, respectively), it showed strong cytotoxicity against a human colon (KM-12) cancer cell line (IC_{50} = 0.1 μg ml^{-1}). Methoxyamericanolide G (232), on the other hand, was not cytotoxic to any of the human cancer cell lines in the NCI panel. Unfortunately, the biological properties of these metabolites could not be adequately ascertained as many of them were found to be quite labile and decomposed slowly under normal spectral measurement conditions.



10-*epi*-Methoxyamericanolide A (**233**) R = OCH_3
10-*epi*-Americanolide C (**234**) R = H

8-*epi*-Methoxyamericanolide A (**235**) R = OCH_3
8-*epi*-Americanolide C (**236**) R = H

Methoxyamericanolide H (**237**)

Methoxyamericanolide I (**238**)

2.11.2.5.2 Miscellaneous compounds

2.11.2.5.2(i) Steroids (compounds reported = 9) *Pseudopterogorgia americana* from an undisclosed location was reported by Enwall *et al.*[231] to contain known gorgosterol (**239**) along with 9(11)-*seco*-gorgosterol (**240**). The structure of *seco*-sterol **240** was determined by a single-crystal X-ray diffraction study of the prepared 3-(*p*-iodobenzoyl)-11-acetate derivative. Another sterol possessing a 9,11-*seco* dinostane nucleus was reported by Miller *et al.*[232] in 1995 from *P. americana* collected in Tobago. The structure of 9,11-*seco*-24-hydroxydinosterol (**241**) was determined by a combination of 2D NMR experiments. The relative stereochemistry at C24 could not be established from the spectroscopic data recorded. Also in 1995, *seco*-sterols **240–241** were reisolated by He *et al.*[233] from an unknown *Pseudoptergorgia* sp. collected at Long Keys, Florida, along with new 9(11)-*seco*-sterols **242–243**. The structures of **240–243** were determined by the analysis of NMR and MS data. The relative stereochemistry at C24 in compounds **241 and 242** remains to be determined. *seco*-Sterols **241–243** exhibited moderate inhibitory activity against protein kinase C (PKC) with IC_{50} values in the range 12 and >50 $\mu mol\,l^{-1}$. Interestingly, a fortified enzyme preparation of *P. americana* was developed by Kerr *et al.*,[234,235] which efficiently transforms a variety of sterols to their 9(11)-*seco*-steroid derivative in high yield. NAD, NADP, and glutamate dehydrogenase are key additives in this enzymatic conversion. The yield of protein precipitate generated was typically 0.5 g from 25 g of gorgonian. In addition to naturally occurring metabolites, a series of novel *seco*-steroids were prepared through this enzyme preparation. (Similarly, Corey *et al.*[236] had earlier demonstrated that a homogenate and acetone powder from *P. americana* converted arachidonate into 8-*R*-HPETE and thence to a mixture of preclavulone-A and the 9,12-*trans* isomer of preclavulone-A.) The chemical defense of *P. americana* was investigated by Epifanio *et al.*[237] using feeding assays performed in aquaria and *in situ* with a natural assemblage of predatory fishes. Using a bioassay-guided scheme, crude extracts, fractions, and pure compounds were incorporated into palatable foods and tested. Only the *seco*-sterol fraction deterred fish feeding. Two *seco*-sterols were identified from the bioactive fraction by spectroscopic methods and comparison with literature data: 9(11)-*seco*-gorgosterol (**240**) and 9(11)-*seco*-dinosterol (**243**). Their results suggest that symbiont-produced gorgosterol and dinosterol are oxidized by the coral host to C-ring *seco*-sterols, which then provide a chemical defense to both coral and alga.[10,11,237] A study of the hexane extracts of *P. americana* from Puerto Rico led to the isolation of two new polyhydroxydinostate sterols **244–245**, in addition to previously known 9(11)-*seco*-gorgosterol (**240**). The structures of 1α,9α,11α-trihydroxydinosterol (**244**) and 1α-hydroxy-9(11)-*seco*-dinosterol (**245**) were established by spectroscopic and chemical derivatization studies.[238] Two additional 9(11)-*seco*-gorgosterol derivatives containing oxirane rings at the C5/C6 position (**246 and 247**) were isolated by the Kerr group from Floridian specimens of *P. americana* along with the known *seco*-gorgosterol **240**.[239] The structures were elucidated after detailed spectroscopic studies. Compound **246** exhibited moderate activity against prostate cancer (LnCap) and lung cancer cell lines (Calu-3) with observed IC_{50} s of 15.5 and 11.0 $\mu g\,ml^{-1}$, respectively. Compound **247** behaved similarly when tested against the same cancer cell lines (observed IC_{50} values 18.4 and 12.0 $\mu g\,ml^{-1}$, respectively). Interestingly, compound **240** exhibited IC_{50} values of 41.0 and 38.1 $\mu g\,ml^{-1}$ against the LnCap and Calu-3 cell lines, respectively, suggesting that the epoxy ring is

at least partly responsible for the observed activity. A synthetic study describing the synthesis of the AB-ring portion of 9,11-*seco*-sterols such as 240, 242, 246, and 247 has been described.[240]

Gorgosterol (**239**)

9(11)-*seco*-Gorgosterol (**240**)

9,11-*seco*-24-Hydroxydinosterol (**241**)

9(11)-*seco*-24-Hydroxygorgosterol (**242**)

1α,9α,11α-Trihydroxydinosterol (**244**)

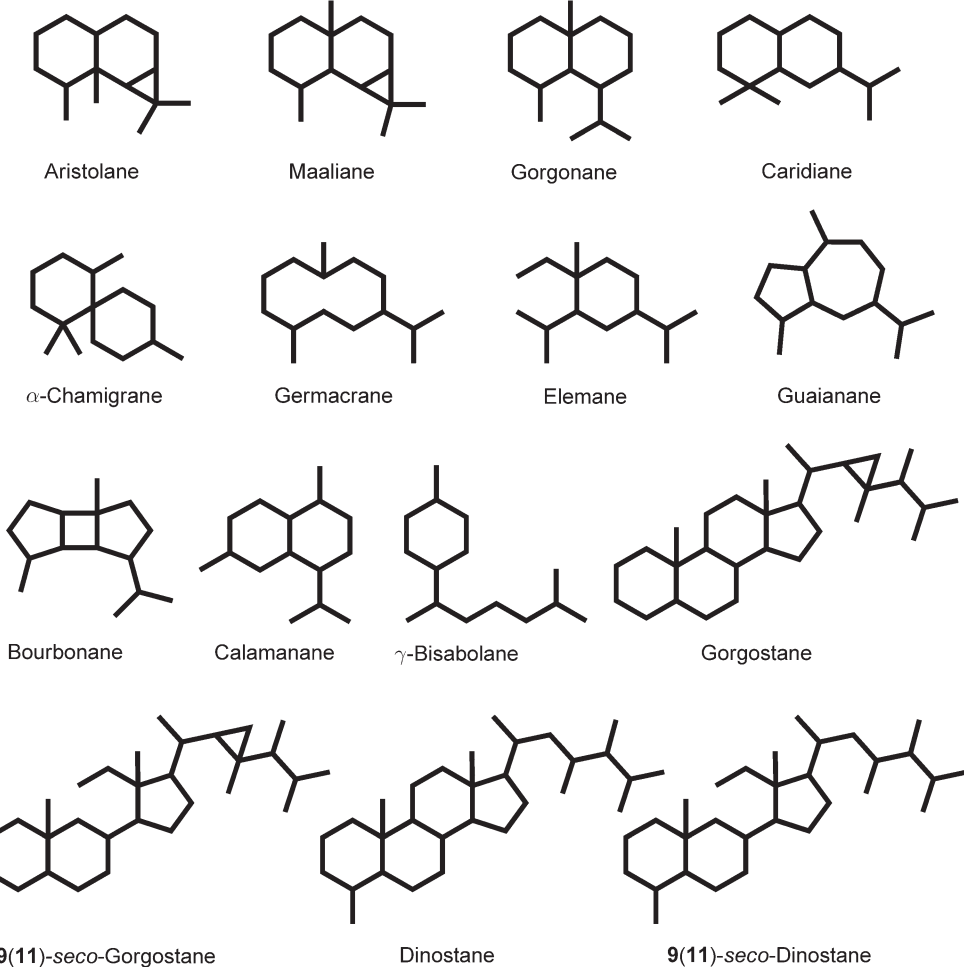
9(11)-*seco*-Dinosterol (**243**) R = H
1α-Hydroxy-9(11)-*seco*-dinosterol (**245**) R = OH

246 R = OH
247 R = H

2.11.2.5.2(ii) Betaines (compounds reported = 3) In addition to the new betaine norzooanemonin (248), Weinheimer *et al.*[241] reported the isolation of known trigonelline (249) and homarine (250) from specimens of *P. americana* collected in the Florida Keys. Each of these betaines is widely distributed in marine invertebrates. The structure of norzooanemonin was proposed on the basis of its spectroscopic data and was confirmed by total synthesis (Scheme 5).

2.11.2.6 *Pseudopterogorgia rigida* (Bielschowsky, 1929)

Common name: None
Geographic division: Western Atlantic Ocean
Distribution: Southern Florida and the Keys; Greater Antilles; and Lesser Antilles



Scheme 5 Terpenoid carbon skeletons from *Pseudopterogorgia americana*.

Brief description: This uncommon species is appropriately named, consisting of upright central stems, which are much stiffer and less flexible than the other *Pseudopterogorgia* species. Secondary branches emerge from all round the central stems, and are relatively short. Color is variable, ranging from pink to nearly black on the central stems, though the secondary branches are usually pale. *Pseudopterogorgia rigida* has a very distinctive spiculation including smooth, blunt scaphoids, and stubby spindles. In external form the colonies are quite variable, but the branchlets usually are almost cylindrical, with the polyps in multiple rows along the sides. While *P. rigida* is virtually inseparable morphologically from most other *Pseudopterogorgia* species, particularly the more abundant *P. americana*, it can readily be recognized based upon its rather distinct lemon-like odor.

2.11.2.6.1 Sesquiterpenes

2.11.2.6.1(i) Bisabolanes (compounds reported = 6) In 1978, McEnroe and Fenical reported that the antibacterial properties of extracts of *P. rigida* collected near Carrie Bow Cay, Belize could be explained by the presence of three derivatives of the aromatic sesquiterpene α-curcumene. Thus, (−)-curcuphenol (**251**), (−)-curcuquinone (**221**), and (−)-curcuhydroquinone (**200**) were isolated in high yield (30% extract) and structurally defined by interconversion to the parent hydrocarbon (−)-α-curcumene. In this work, a high-yielding synthesis of (−)-curcuphenol (**251**), the most active antibacterial metabolite, was also described.[242] During a search for novel biologically active compounds in the macrophage scavenger receptor (MSR) assay, the ethyl acetate extract of *P. rigida* collected at Chub Cay, Bahamas, led Freyer *et al.* to the discovery of

rigidone (**252**).[243] During the bioassay-guided fractionation of the extract (−)-curcuhydroquinone (**200**), (−)-curcuquinone (**221**), and (−)-curcuphenol (**251**) were also isolated. The absolute stereochemistry of **252** was determined by comparison of its optical rotation with that of previously isolated models. Although rigidone showed reasonable potency in the MSR assay ($IC_{50} = 5.6\ \mu mol\ l^{-1}$) it was found to be inactive in the functional assay, and therefore, was not considered as an MSR lead. In addition to the known compounds (−)-curcuhydroquinone (**200**), (−)-curcuquinone (**221**), (−)-curcuphenol (**251**), and (−)-α-curcumene (**253**), a group of investigators led by Mootoo reported from this gorgonian coral a modified sesquiterpene containing an additional C-alkylated group at C2, mochiquinone (**254**).[244] The animal material was collected at a depth of −7 m in Mochima Bay, Sucre State, Venezuela. The structure of this unusual homosesquiterpene was determined by spectroscopic analysis of its diacetylated derivative and comparisons with known model compounds. Several enantiocontrolled total syntheses for several of these monocyclic bisabolane-type sesquiterpenoids, including (−)-curcuhydroquinone (**200**), (−)-curcuquinone (**221**), and (−)-curcuphenol (**251**), have been described.[245–252] A hydrocarbon mixture consisting mainly of monoacetate **219** and (−)-α-curcumene (**253**), which are minor components of *P. rigida*, did not deter natural predators significantly. On the other hand, (−)-curcuhydroquinone (**200**) and (−)-curcuquinone (**221**), which exist in higher concentration at the edges of the colony, a region more susceptible to predation, had significant deterrent effects.[10–13]

Norzooanemonin (**248**) Trigonelline (**249**) Homarine (**250**)

(−)-Curcuphenol (**251**) R = OH
(−)-α-Curcumene (**253**) R = H

(+)-Rigidone (**252**) Mochiquinone (**254**)

255 **256** **257**

2.11.2.7 *Pseudopterogorgia hummelincki* (Bayer, 1961)

Common name: None
Geographic division: Western Atlantic Ocean
Distribution: Known only from the type locality; Belize, Anguilla; and Upper Prickly Pear Island

Brief description: Small colonies spread in one plane; branching pinnate; branches occasionally subdivided to the third order. The colony is cream white, tinged with violet toward the base. In growth form, *P. hummelincki*

resembles the *bipinnata* species but may readily be distinguished from them by the scaphoids, which lack any trace of high, transverse crests around the convex side. The spicules of *P. hummelincki* are similar to those of *P. americana*, which has quite a different growth form.

2.11.2.7.1 Triterpenes

2.11.2.7.1(i) Steroids (compounds reported = 1) The previously known 9(11)-*seco*-gorgosterol (**240**) was isolated from *P. hummelincki* collected off the coast of Belize by Schultz *et al.*[253] Compound **240** was recovered from this gorgonian by homogenization and solvent extraction, followed by chromatography of the crude extract on silica gel. X-ray analysis confirmed the structure proposed on the basis of spectral evidence, primarily NMR (**Scheme 6**).

2.11.2.8 *Pseudopterogorgia australiensis* (Ridley, 1884)

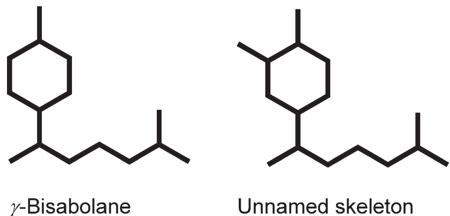
Common name:	None
Geographic division:	Indian Ocean
Distribution:	Northern Australia and Tuticorin Coast, India. Records of existence available only at country level

Brief description: Branching planar, pinnate, and plumose. Short and very narrow lateral branches arise from the main stem. The lateral branches do not branch further. Sclerites range in length from 0.05 to 0.16 mm, and are straight or slightly curved spindles with three to six transverse whorls of tubercles. Some scaphoids are distinct and have smooth tubercles on the convex side, while others are less distinct and have ornamented tubercles on both the convex and concave sides. The sclerites are yellow or red.[2]

2.11.2.8.1 Sphingolipids

2.11.2.8.1(i) Sphingosines (compounds reported = 3) Two new sphingosines (2*S*,3*R*)-2-(docosanoyl amino)nonadecane-1,3-diol (**255**) and (2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-hydroxynonadecanoylamino]nonadecane-1,3,4-triol (**256**), along with the known (2*S*,3*R*,4*E*)-2-(heptadecanoylamino)octadec-4-ene-1,3-diol (**257**) were isolated from *P. australiensis* collected from the Tuticorin Coast, India at a depth of about −10 m. The structures of

Carbon skeletons from *P. rigida*



γ-Bisabolane   Unnamed skeleton

Carbon skeletons from *P. hummelincki*

9(11)-*seco*-Gorgostane

Scheme 6 Terpenoid carbon skeletons from *Pseudopterogorgia rigida* and *P. hummelincki*.

255–257 were deduced from spectral and chemical methods. Compounds 255–257 showed moderate anti bacterial activity at 1 mg ml^{-1} concentration, against Gram-positive bacteria *Bacillus pumilis*, *Bacillus subtilis*, and *S. aureus* and Gram-negative bacteria *E. coli*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*. None of the compounds showed antifungal activity against *Candida albicans* or *Aspergillus niger*.[254]

2.11.3 Aspects of Diterpenoid Biosynthesis and Chemical Ecology

2.11.3.1 *Pseudopterogorgia elisabethae*

In 1999, Kerr and coworkers developed *in vivo* and *in vitro* techniques to test putative intermediates in the biosynthetic pathway to the Pss. The specific data obtained indicated that pseudopterosin A (1) is a precursor to pseudopterosins B, C, and D (2–4), and that during Pss biosynthesis, the glycosylation involves the addition of a xylose residue to a diterpene skeleton to produce pseudopterosin A. Thereafter, the xylose is acetylated to produce pseudopterosins B–D.[255] In continuation of these metabolic studies, Coleman and Kerr[84] set out to identify the diterpene cyclase product in *P. elisabethae*. Their data suggest that geranylgeranyl diphosphate (GGPP) is transformed to elisabethatriene (57) through a series of carbocation rearrangements typical of terpene metabolism (Schemes 7 and 8). However, detailed mechanistic studies to elucidate the precise mechanism of cyclization of GGPP to elisabethatriene have not been conducted. The conversion of 57 into the Pss 1–4 confirmed the intermediacy of elisabethatriene in the biosynthesis of this important family of diterpene glycosides, and for the first time suggested a common biosynthetic origin of the Pss and *seco*-pseudopterosins. Soon thereafter, the same group described the partial purification and preliminary characterization of the Ps diterpene cyclase, elisabethatriene synthase (ELS), responsible for the cyclization of GGPP to elisabethatriene, from a crude coral extract.[256] Further, a revised purification procedure to a homogeneous preparation was developed by Brück and Kerr[257] in order to investigate the kinetic properties of ELS. Interestingly, elisabethatriene synthase also catalyzed the conversion of C_{10} and C_{15} isoprenyldiphosphate analogs into monoterpene and sesquiterpene olefins, respectively. Additional biosynthetic experiments designed by Kohl and Kerr[85] to test the utilization of isoelisabethatriene (58) and erogorgiane (60) in Ps production revealed that only erogorgiaene, not 58, is transformed to pseudopterosins A–D (1–4). These data suggested that in Ps biosynthesis, erogorgiaene is produced from elisabethatriene by a dehydrogenation and spontaneous aromatization. It is conceivable, however, that isomers of elisabethatriene, other than 58, could be involved in this pathway.[66] In continuation of pathway elucidation studies, Ferns and Kerr[91] later demonstrated the intermediacy of 7-hydroxyerogorgiane (61) and 7,8-dihydroxyerogorgiaene (62) in Ps biosynthesis by *in vitro* incubation experiments with these metabolites in radiolabeled form. The conversion of 61 into 62 was also confirmed by these researchers upon conducting additional incubation experiments. A recent review by Kerr *et al.*[258] outlines the completed biosynthesis of the Pss (Scheme 9).

As part of an effort to identify factors responsible for the induction of Pss in *P. elisabethae*, Thornton and Kerr[259] found evidence to suggest that the biosynthesis of these natural products can be significantly increased in response to high levels of predation by the flamingo-tongue snail, *Cyphoma gibbosum*, and in response to decreased levels of UV/VIS radiation.[260] The induction in response to predation by the mollusk is somewhat comparable to insect-induced terpene biosynthesis in vascular plants. In a subsequent investigation by the Kerr group, direct evidence was presented for the first time of the presence of a Ps biosynthetic pathway in the cells of *Symbiodinium* sp.,[261] the dinoflagellate symbiont of *P. elisabethae*.[262,263] The chemical analysis of the purified *Symbiodinium* sp. from the gorgonian indicated a high level of the anti-inflammatory Pss (11% of lipid content) and the presence of the diterpene cyclase product, elisabethatriene (57). A very high correlation between the number of *Symbiodinium* sp. cells and Ps concentration per coral weight provided evidence that the *Symbiodinium* sp. cells are directly involved in Ps biosynthesis. The biosynthetic data from ^{14}C-labeled CO_2 confirmed that the algal cells possess all the biosynthetic machinery required for biosynthesis. The accumulation of Pss within the symbiont cells indicates that the compounds may have a physiologically important role and may be stored for cell signaling purposes.[261] As an extension of their research, Kerr and coworkers examined the terpene content of the dinoflagellate symbiont following a decrease in UV/VIS radiation and in response to the addition of

Scheme 7 Proposed mechanism of formation of elisabethatriene (57) (pathway A).

methyl jasmonate, salicylic acid, and gibberellic acid.[264] The researchers found that Ps biosynthesis can be increased approximately 100% by decreasing the UV radiation on specimens of *P. elisabethae* and through the addition of the abovementioned plant growth factors.[265] They also demonstrated that, while the terpene content of *P. elisabethae* increases in response to decreased UV/VIS light, this is due primarily to an increase in the concentration of the dinoflagellate rather than simply an induction of terpene biosynthesis. After a comparison of two total RNA extraction protocols using *P. elisabethae* and its symbiont *Symbiodinium* sp., Kerr and coworkers concluded that both protocols are suitable for RNA isolation. Trizol is recommended if higher yields are the primary concern, but RNeasy is recommended if time is an issue. These methods can also be used with other corals and their symbionts with similar results.[266] This research group also designed four different freezing protocols to preserve the dinoflagellate symbiont of *P. elisabethae*, thus demonstrating that *Symbiodinium* sp. isolated from a gorgonian can be cryopreserved for relatively long periods of time (3 months) and successfully reinoculated into culture medium.[267] A general outline of a suggested production method utilizing a combination of recombinant enzyme technology and synthetic methods/biocatalysis to produce well-known and newly identified anti-inflammatory agents from *P. elisabethae* has been described by Kohl *et al.*[268]

Scheme 8 Proposed mechanism of formation of elisabethatriene (**57**) (pathway B).

2.11.3.2 *Pseudopterogorgia bipinnata*

A chemical analysis of the dinoflagellate symbiont (the zooxanthellae) of *P. bipinnata* conducted by Kerr and coworkers revealed higher concentrations of kallolides and related diterpenes than are present in the intact holobiont.[269] This observation suggests that the symbiont produces these compounds. Analyses of *P. bipinnata* colonies indicated significant chemical variations within this species, and four distinct chemotypes were identified (chemotypes A–D). To test the hypothesis that the dinoflagellate symbiont of *P. bipinnata* is indeed a biosynthetic producer of the diterpenes, experiments were carried out with radiolabeled geranylgeranyl diphosphate (GGPP), the ubiquitous diterpene producer. As the recovered kallolide A (**136**) was radioactively pure, the Florida-based investigators concluded that the kallolide family of diterpenes is not only localized, but is also produced within the algal symbiont of *P. bipinnata* chemotype A. These studies also determined that all chemotypes are predominantly populated by the symbiont *Symbiodinium* sp. clade B.[262,263,270]

GGPP

Elisabethatriene (**57**)

Erogorgiaene (**60**)

61

62

seco-Pseudopterosin J (**40**)

Amphilectosin A (**41**)

Amphilectosin B (**42**)

Pseudopterosin T (**20**)

Pseudopterosin F (**6**)

R^1, R^2, or R^3 = H or Ac

R^1, R^2, or R^3 = H or Ac

Scheme 9 Elucidation of the biosynthetic origin of the Pss.

2.11.4 Selected Synthetic Transformations Suggesting Plausible Biogenetic Relationships between Different Families of *Pseudopterogorgia* Diterpenes

Encouraged in part by the challenge presented by their interesting molecular architectures and the enticing notion that *Pseudopterogorgia* diterpenoids share a common biosynthetic ancestry, several investigators have undertaken the task of uncovering plausible biogenetic interrelationships among the distinct skeletal classes of diterpenes produced by this chemically prolific clan of marine animals. The fact that different skeletal classes of compounds coexist within the same organism, and the usual confluence of structure complexity and interesting biological activity among the more intricate members, suggest that some of these natural products could be derived from a common intermediate by different cyclization pathways. For instance, in 1998, Rodríguez and Shi[141] demonstrated the biogenetic relationship between two diterpenoids of the cembrane and pseudopterane classes, bipinnatin J (**119**) and kallolide A (**136**) (**Scheme 10**). This high-yielding facile cycloisomerization involves a photochemical suprafacial [1,3]-sigmatropic rearrangement with retention of configuration at the migrating α,β-unsaturated-γ-butenolide group. Later on, the same team of investigators demonstrated the biogenetic relationship between the cembrane and gersolane skeletal classes.[152] They found evidence to suggest that the bipinnatin J (**119**)–pinnatin A (**151**) rearrangement proceeds antarafacially with retention of configuration at the shifting α,β-unsaturated-γ-butenolide group and (presumably) inversion at the other end of the reacting sigma bond, consistent with a [1,2]-sigmatropic rearrangement (**Scheme 10**). All three compounds, bipinnatin J, kallolide A, and pinnatin A were coisolated from extracts of the same *P. bipinnata* collection.

Cembrane → Pseudopterane + Gersolane

Bipinnatin J (**119**) —a→ Kallolide A (**136**); Pinnatin A (**151**) + Pinnatin C (**153**)

Compound	Relative ratio
136	120
151	1
153	6

Reaction conditions: a) $h\nu$, CH_3CN, 1 h, 25 °C in a Pyrex tube (100%).

Scheme 10 Cembrane–pseudopterane and cembrane–gersolane skeletal photoisomerizations.

The coisolation of elisapterosin A (84) and elisabanolides 93 and 94 from the same collection of *P. elisabethae* also encouraged Rodríguez *et al.*[98] to investigate their plausible biogenetic relationship. During a GC–MS analysis, they found that complete thermal decomposition of 84 (presumably by loss of formaldehyde) takes place to produce *nor*-diterpenes 93 and 94 in a 1:2 ratio. Alternatively, upon refluxing a mixture of elisapterosin A and $Pb(OAc)_4$ in dry benzene/pyridine at 80 °C for 22.5 h, the same product mixture is obtained in a 1:1 ratio (Scheme 11). The precise reaction mechanism for this seemingly novel oxidative rearrangement is unknown.

In 2003, Rawal and coworkers reported the stereocontrolled asymmetric synthesis of the enantiomer of elisapterosin B (85), by a route that featured (a) a pinacol-type ketal rearrangement to transfer chirality, (b) an intramolecular Diels–Alder (IMDA) reaction of an *E,Z*-diene to construct the elisabethin A (73) skeleton, and (c) a biosynthesis-inspired oxidative cyclization of the elisabethin precursor to *ent*-elisapterosin B.[102] The latter elisabethane–elisapterane cycloisomerization reaction (Scheme 12) clearly establishes a probable biogenetic connection between these intricate skeletal classes of diterpenes. The oxidative cyclization of 2-epi-*ent*-elisabethin A to *ent*-elisapterosin B took place smoothly and in high yield upon treatment with $Ce(NH_4)_2(NO_3)_6$, followed by the addition of pyridine and triethylamine, to enolize the presumed diketone intermediate.

An elegant unified strategy involving [5 + 2] and [4 + 2] intramolecular cyclizations of a serrulatane skeleton as biomimetic routes to (−)-elisapterosin B (85) and (−)-colombiasin A (89), respectively, was reported by Kim and Rychnovsky[110] in 2003 (Scheme 13). These syntheses provide support for the biosynthetic proposal developed by the Rodríguez group that the uncommon cagelike skeleton of elisapterosin B might arise by cyclization of a serrulatane diterpene such as 65, which was isolated from the same organism. Nicolaou *et al.*[114–118] had reported earlier a synthesis of colombiasin A by an IMDA cyclization of a serrulatane diene. In 2005 and 2006, respectively, the Harrowven and Davies[107,111,112] groups also described total syntheses

Elisapterane

Elisabane

Elisapterosin A (84)

a or b

Elisabanolide (93)

3-*epi*-Elisabanolide (94)
+
other minor products

Reaction conditions: (a) 250 °C during GLC–MS analysis
(b) $Pb(OAc)_4$, benzene, pyridine, 80 °C, 22.5 h.

Scheme 11 Thermally induced conversion of elisapterosin A (84) into elisabanolide (93) and 3-*epi*-elisabanolide (94).

Elisabethane → Elisapterane

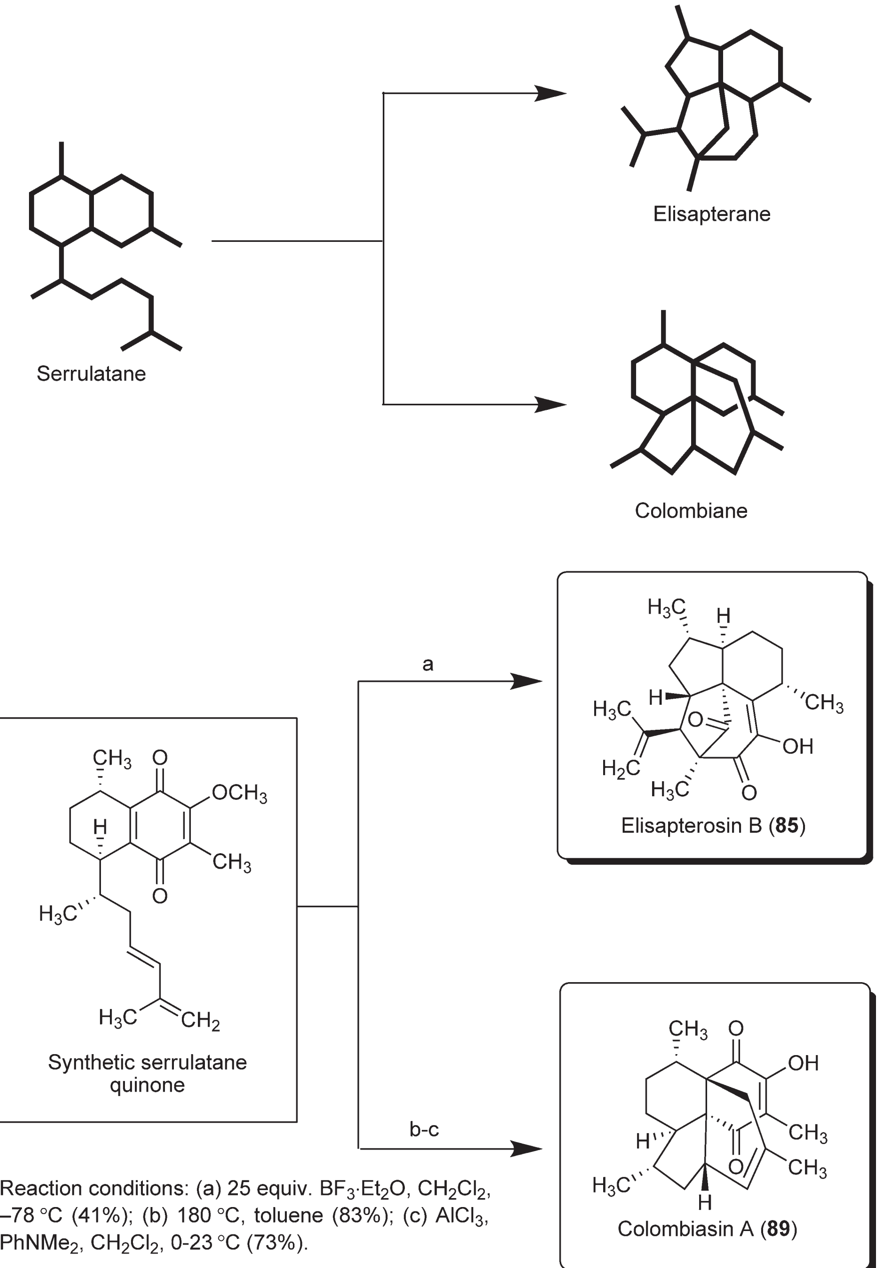
2-*epi*-*ent*-Elisabethin A → (CAN, MeCN, 0 °C, 10 min; Pyridine, Et_3N, 50 °C (84%)) → *ent*-Elisapterosin B

Scheme 12 Oxidative cyclization of 2-*epi*-*ent*-elisabethin A to *ent*-elisapterosin B.

for (−)-elisapterosin B and (−)-colombiasin A whereby [5 + 2] and [4 + 2] cycloaddition reactions of the same serrulatane quinone core were employed as the finishing step.

In 2005, during the course of an investigation into an alternate synthesis of colombiasin A (**89**), Jacobsen and coworkers discovered that treatment of **89** with superstoichiometric quantities of $BF_3 \cdot Et_2O$ resulted in smooth conversion into (−)-elisapterosin B (**85**) (**Scheme 14**).[108,109] This reaction may proceed by a fragmentation reaction, affording an allylic cation that undergoes subsequent cyclization, or by a retro [4+2] cycloaddition followed by a [5+2] cycloaddition. While the precise mechanism of this reaction has yet to be determined, it is stereoselective and high-yielding (94%), and underlines the close structural relationship between these intriguing skeletal classes of *Pseudopterogorgia* metabolites.

Recently, the laboratories of Trauner, Rawal, and Pattenden have independently drawn attention to the biosynthetic interrelationships between furanobutenolide cembranes and the polycyclic diterpenes bielschowskysin (**170**) and intricarene (**171**), and all have reported total syntheses of either racemic or enantiomerically enriched bipinnatin J (**119**).[142–145] Intrigued by these possible biosynthetic relationships, the groups of Trauner and Pattenden[142,145] reported independently the conversion of (−)-bipinnatin J into (+)-intricarene through oxidation of **119** followed by acetylation/elimination thus yielding a transient oxidopyrylium species, which upon heating, undergoes transannular 1,3-dipolar cycloaddition to afford **171** (**Scheme 15**). Aroused by the possibility that such a cycloaddition may also occur in nature during the biosynthesis of (+)-intricarene, Wang and Tantillo have examined this transformation in detail using quantum chemical calculations (B3LYP). They concluded that although enzymatic intervention may be required to generate the oxidopyrylium zwitterion, its subsequent cycloaddition would not require additional intervention.[271] Further biomimetic studies by these groups are in progress to probe links between other families of structurally intriguing and biologically important *Pseudopterogorgia* natural products, including bielschowskysin (**170**).



Scheme 13 Synthesis of (–)-elisapterosin B (85) and (–)-colombiasin A (89) by IMDA cyclization of a serrulatane diene.

2.11.5 Summary and Conclusions

An impressive array of natural products with interesting pharmacological properties and unprecedented molecular structures and functionality has been isolated from *Pseudopterogorgia* octocorals during the last four decades (1968–2008). Of the 257 unique structures accounted for in this report, a total of 251 (97.7%) are terpenoidal in origin. The principal terpenoids manufactured by *Pseudopterogorgia* gorgonians are diterpenes and sesquiterpenes. The approximate breakdown by terpene class is as follows: 196 (76%) are diterpenes (or substances purportedly stemming from them); 40 (16%) are sesquiterpenes; 20 (8%) are triterpenes (sterols); and 1 (<1%) compound is a tetraterpene (carotenoid). Only 16 compounds (6.2%) contain nitrogen and none

Scheme 14 Conversion of colombiasin A (89) into elisapterosin B (85).

(0%) contain chlorine or bromine. The diterpenoids, the largest division, are in turn further analyzed for each gorgonian species, and the distribution by skeleton classes of compounds in this group is shown in **Table 1**. **Schemes 1–6** summarize the impressive structural variety of terpenoid carbon skeletons found in these animals. The diterpene skeleton most frequently elaborated by the species *P. acerosa* and *P. kallos* is the pseudopteranoid system, which contains a 12-membered carbocycle. Although highly functionalized cembranoids are the most commonly isolated diterpenes from *P. bipinnata*, many pseudopteranoids have also been reported. There is a conspicuous dominance of sesquiterpene metabolites isolated from *P. americana*, and to date, not a single diterpenoid has been isolated from this species. From the point of view of biosynthetic capability, over the previous 40 years *P. elisabethae* has been the most versatile species, accounting for the discovery of over 20 diterpenoid skeletal variants (**Figure 4**). By far, metabolites based on the amphilectane and serrulatane skeletons represent, respectively, the first (39%) and second (27%) most frequently found types of diterpenoids in this species. Any mention of source organisms brings up the question of the possible role of microorganisms in producing or influencing the production of metabolites isolated from the macroscopic organism.[272] Dinoflagellates are now a confirmed source of amphilectane (Pss), serrulatane (*seco*-pseudopterosins) and pseudopterane (kallolides) diterpenes, which were originally extracted from corals.[261,269] These dinoflagellate diterpenes resemble other polycyclic (more intricate) diterpenes found in *Pseudopterogorgia* suggesting that dinoflagellates may be the ultimate source of many of these compounds. While Kerr and coworkers have presented a few examples in which bioactive *Pseudopterogorgia* metabolites are produced by symbiotic microorganisms, it is conceivable that these are probably the exception rather than the rule. The majority of *Pseudopterogorgia* gorgonian species do not appear to contain such large populations of symbiotic microorganisms and the metabolites of these gorgonians, particularly those found in >0.01% dry weight, are unlikely to be produced by symbionts.[2,262,263]

As we have discussed above, a significant fraction of *Pseudopterogorgia* metabolites have been evaluated in human disease-oriented *in vitro* testing programs. For instance, several major anti-inflammatory leads have emerged from work on *Pseudopterogorgia* corals. Most prominent are pseudopterosin A (**1**),[21] pseudopterosin E (**5**),[28] *seco*-pseudopterosin E (**35**) and relatives,[56] and elisabethadione (**63**).[56] Pseudopterosins A and E exhibit potent anti-inflammatory and analgesic activities and act as reversible inhibitors of lipooxygenase and PLA_2.[29–32] A semisynthetic Ps derivative, pseudopterosin A methyl ether (TMO), can substantially improve tissue repair and healing for severe burn victims, and has shown promise as a treatment for contact dermatitis.[25–27] TMO has recently completed a study on wound repair in 20 patients undergoing reconstructive surgery and has proved safe to apply to open wounds. The results are promising thus far and the drug is expected to advance

Cembrane → Intricarane

(–)-Bipinnatin J (**119**) —a-b→ … —c→ [Oxidopyrylium ion] —[5 + 2] cycloaddition→ (+)-Intricarene (**171**)

Reagents and conditions:
Pattenden: (a) $VO(acac)_2$, ^tBuOOH, CH_2Cl_2, –20 °C; (b) Ac_2O, Et_3N, DMAP (cat.), CH_2Cl_2, RT(30%) (c) DBU, CH_3CN, reflux, 1h (10%).
Trauner: (a) *m*CPBA, CH_2Cl_2; (b) Ac_2O, Py, DMAP (cat.), CH_2Cl_2, RT (81%); (c) TMP, DMSO, 150 °C (26%).

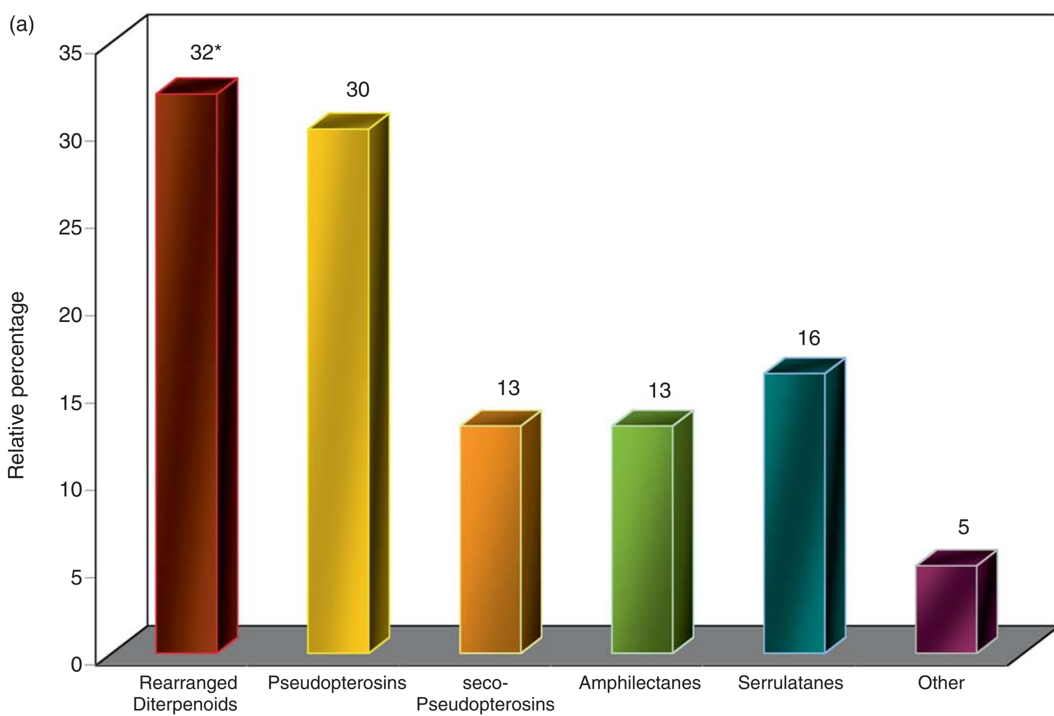
Scheme 15 Biomimetic conversion of (–)-bipinnatin J (**119**) into (+)-intricarene (**171**).

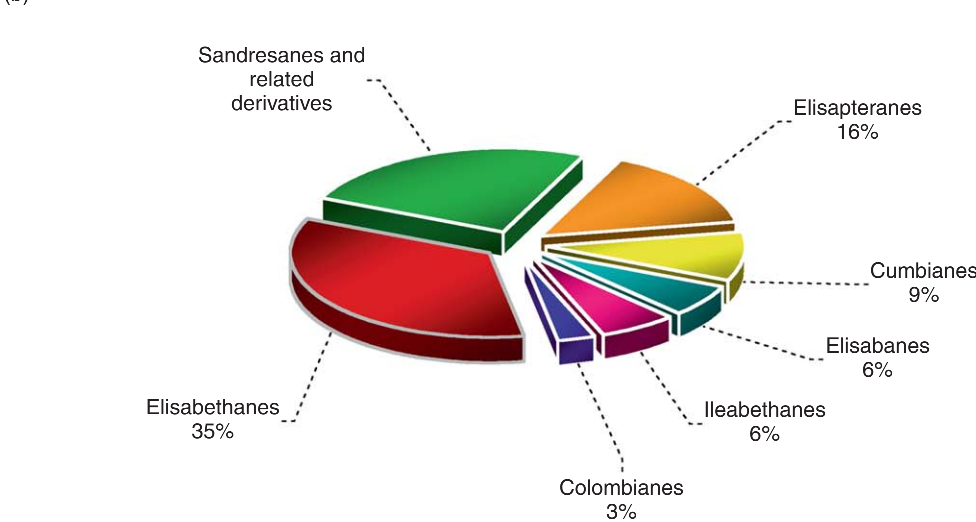
to human clinical testing (phase III) as a therapeutic agent for inflammation and wound healing.[273] Supplies for preclinical and clinical testing have come from widespread natural collections, but it is generally agreed that the scale of collections, which would be necessary to support commercial drug sales on a year-to-year basis would be both environmentally damaging and difficult to manage.[20,70] As the harvesting of wild populations of *P. elisabethae* in the Bahamas and off the coast of Bermuda seems inconsistent with modern goals of biodiversity conservation, far more success is being reported in growing *Symbiodinium* sp. cells in culture media that produce pharmacologically active metabolites.[268] These cultured symbionts can provide a renewable resource and thus preserve natural populations of the gorgonian. Many *Pseudopterogorgia* diterpenoids (16, 55, 56, 60, 69, 85, 96, and 101) have been found to possess significant antitubercular properties, but thus far none is comparable in potency to the standard drug rifampin. That notwithstanding, some of these compounds show enough potency to become important research tools in the fight against tuberculosis. Natural products obtained from this coral genus could also become important models for new antiplasmodial substances. Several examples of important antimalarial agents ($IC_{50} \leq 10\,\mu g\,ml^{-1}$) found in *Pseudopterogorgia* corals are compounds 22, 52, 89, and 170. Clearly, many diterpenes that show some degree of cytotoxicity have been identified from this source over the past 40 years. The levels of inhibition of growth of various tumor cell lines *in vitro* range from 10^{-9} to

Table 1 Distribution of marine natural products by skeletal class isolated from the five most chemically prolific *Pseudopterogorgia* spp. (total = 262 compounds)

Species (total no. of compounds)	*Structure class*	*No. of compounds per class*	*Distribution of compound per class (%)*	*Overall distribution (%)*
P. elisabethae (109)	Pseudopterosins	30	28	11
	seco-Pseudopterosins	13	12	5
	Amphilectanes	13	12	5
	Serrulatanes	16	15	6
	Elisabethanes	11	10	4
	Elisapteranes	5	5	2
	Colombianes	1	1	<1
	Cumbianes	3	3	1
	Elisabanes	2	2	<1
	Ileabethanes	2	2	<1
	Sandresanes and related derivatives	8	7	3
	Sesquiterpenes	1	1	<1
	Steroids	1	1	<1
	Pseudopteranes	1	1	<1
	Bisditerpenes	1	1	<1
	seco-Dolastanes	1	1	<1
P. bipinnata (45)	Cembranes	19	42	7
	Pseudopteranes	11	24	4
	Gersolanes	5	11	2
	seco-Cembranes and *seco*-Pseudopteranes	7	16	3
	Verrillanes	1	2	<1
	Bisditerpenes	2	4	<1
P. kallos (28)	Pseudopteranes	12	43	5
	Gersolanes	3	11	1
	Rearranged Cembranes	5	18	2
	Cembranes	8	29	3
P. acerosa (32)	Pseudopteranes	24	75	9
	Cembranes	3	9	1
	Bisabolanes	2	6	<1
	Bisditerpenes	1	3	<1
	Carotenoids	1	3	<1
	Steroids	1	3	<1
P. americana (48)	Polycyclic	8	17	3
	hydrocarbons	–	–	–
	Germacranes and elemanes	7	15	3
	Bisabolanes	4	8	2
	Guaianes	17	35	6
	Steroids	9	19	3
	Betaines	3	6	1

approximately 50 μg ml^{-1}. For some compounds, only toxicity to brine shrimp or inhibition to sea urchin egg development, has been reported. As far as we can ascertain, only a handful of *in vivo* active compounds from *Pseudopterogorgia* corals have been described in the literature. This is quite unfortunate as *in vivo* testing is an obvious prerequisite for any attempt to introduce a pharmacologically active agent into the clinic. (The most likely reasons to explain this scenario are (1) paucity of the natural product, (2) lack of access to an *in vivo* testing facility, and (3) absence of a sufficient level of *in vitro* activity to warrant such studies.) Some of the metabolites initially discovered through cytotoxicity screenings have subsequently been found to display other interesting activities. An example is pseudopterosin Q (**17**), which was shown to be very toxic against HSV-1 and HSV-2 (EC_{50} = 2.9 μmol l^{-1}), with SI values of <2.4. Another is colombiasin A (**89**), which during a primary assay against HBV, displayed potent antiviral activity with an EC_{50} = 1.3 μmol l^{-1}. Yet another case in





*Distribution of Rearranged Diterpenoids from *P. elisabethae* by Skeletal Class

Figure 4 (a) Distribution of all of the *Pseudopterogorgia elisabethae* diterpenoid variants by carbon skeletal class (total = 109 compounds). (b) Approximate breakdown of all the rearranged diterpenoids from *P. elisabethae* by skeletal type.

point is that of elisabatin A (**52**), which proved to be a weak anticancer but turned out to be a potent growth inhibitor against HBV ($EC_{50} = 0.115\ \mu g\ ml^{-1}$). These few examples illustrate the desirability of evaluating novel cytotoxins from this source for a broad range of biological activities even if they do not display outstanding activities during *in vitro* antitumor assays. All told, researchers in this field have shown that

metabolites isolated from *Pseudopterogorgia* corals also display antibacterial, antifungal, neurotoxic, antiathero-sclerotic, ichthyotoxic, feeding deterrent (antipalatable), and wound-healing properties.

The complex molecular architecture and rich functionalization found among the many terpenoid natural products isolated from *Pseudopterogorgia* octocorals, and the ample spectrum of biological activities exhibited by many of these compounds, have made them irresistible synthetic targets. Diterpenes isolated from *P. elisabethae* and *P. kallos* are of intense current interest. From these efforts have spawned a number of useful synthetic methods. An example is a Diels–Alder-elimination-intramolecular Diels–Alder (DA-E-IMDA) sequence that allows rapid access to the complex tetracyclic core of colombiasin A (**89**).[120] Such a sequence may have additional applications, for example, in diversity-orientated synthesis based on structurally complex, natural product-like templates. Another is a Diels–Alder/sequential radical cyclization sequence that constructs the tetracyclic ring system of cumbiasins A and B (**90, 91**) in a few steps.[122] The completion of these total syntheses have firmly established the structures of these interesting bioactive marine products and in some instances have also demonstrated biomimetic cyclization pathways, which provide a basis for understanding the origin of the structural and stereochemical diversity of these diterpenes. Many of these syntheses are noteworthy for their directness, the involvement of interesting and novel methodology, and the potential to provide numerous structural analogues for the development of potent new therapeutic agents.

The increasing number of pharmacologically active new compounds currently being reported from *Pseudopterogorgia* corals, the unabated interest for these compounds as worthwhile synthetic targets, and the seemingly unrealized fact that only about 36% of all the known *Pseudopterogorgia* species have been systematically scrutinized for their secondary metabolite content, suggest that the excitement engendered by this fascinating research field is not likely to fade away any time soon. Clear evidence for this longstanding fascination with *Pseudopterogorgia*-related natural products are the 300 or so scientific peer-reviewed publications and the approximately 15–20 patents issued between 1968 and 2008 (most of them having being issued during the last 15 years). It should be noted that essentially all of the *Pseudopterogorgia* species studied chemically have originated from the tropical northwestern Atlantic region, and that so far only one species from the Indo-Pacific region appears to have been scrutinized from the natural products standpoint. The as-yet uninvestigated corals from the latter region, representing 64% of all the available *Pseudopterogorgia* species, offer special opportunities for advancing basic science and will lay a foundation for new research. Thus, future research programs of those already involved in the isolation of new compounds from this gorgonian genus and those who may choose to enter this field, should aspire to foster collaborations with scientists from countries of the Indo-West Pacific Rim. Such combined efforts will significantly improve the opportunities for discovery of additional novel marine natural products with desirable pharmacological properties from these rarely encountered species. (The knowledge gained from the systematic chemical scrutiny of Indo-Pacific coral species should be taken into account in future discussions on whether or not generic separation of the Indo-Pacific and Atlantic clades of *Pseudopterogorgia* is justifiable.) If this occurs, it should only strengthen our case in point, and perhaps substantiate our belief that gorgonian corals of the genus *Pseudopterogorgia* stand up among the most chemically inventive marine organisms.

Several articles relevant to this chapter became available to us after completion of the original manuscript. An expeditious entry to the hexahydro-1*H*-phenalene core with the relative configuration of natural Pss at C3–C4 starting from readily available starting materials was developed in 2000 by Plumet and coworkers.[274] A formal total synthesis of the strongly antitubercular natural product pseudopteroxazole (**55**) has recently been described by Harmata *et al.*[275] In addition, Mondal *et al.*[276] reported a concise approach towards the synthesis of the highly biologically active terpenoid caribenol A (**100**) involving sequential aldol condensation ring opening ring closing metathesis of a norbornene derivative. Chemical analysis of the terpene metabolites of *Pseudopterogorgia elisabethae* from San Andrés Island, Colombia resulted in the discovery of a weak antimarial agent belonging to the elisapterane class of diterpenes, namely, elisapterosin F (**258**).[277] Fractionation of the $CHCl_3$ extract of *Pseudopterogorgia bipinnata* collected near Providencia Island, Colombia led Ospina and Rodríguez to the discovery of corallolides A (**259**) and B (**260**). These metabolites, which are based on an unprecedented carbon skeleton named corallolane, were shown to exhibit antiparasitic and antituberculosis activity, respectively.[278] The structures of metabolites **258–260** were established through detailed spectroscopic analyses. Preliminary results stemming from

Kerr's laboratory have provided strong evidence that the diterpene biosynthesis in *Pseudopterogorgia acerosa* is due to bacterial symboints rather than the coral tissues.[279] A recent investigation of a sample of *P. acerosa* collected at Sweetings Cay, Bahamas led the Kerr group to the isolation of bis(pseudopterane) amine (**261**). The structural assignment of **261** was achieved by 1D and 2D NMR and mass spectrometry analysis. Bis(pseudopterane) amine showed selective growth inhibition activity against cancer cell lines with IC_{50} values of 4.2 mmol l^{-1} (HCT116) and 42 mmol l^{-1} (HeLa).[280] A new metabolite based on a previously undescribed carbon skeleton, aberrarone (**262**), was reported by the Rodríguez group as a natural product from *Pseudopterogorgia elisabethae* collected from deep reef waters off San Andres Island, Colombia. The molecular structure of the crystalline metabolite was established by spectral analysis and subsequently confirmed by X-ray crystallographic analysis. Aberrarone showed moderate *it vitro* antimalarial activity against a chloroquine-resistant strain of the protozoan parasite *P. falciparum*.[281]

Elisapterosin F (**258**) Corallolide A (**259**) Corallolide B (**260**)

Bis(pseudopterane) amine (**261**) Aberranone (**262**)

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Abbreviations

1D	one dimensional
2D	two dimensional
CNS	central nervous system
COSY	correlation spectroscopy

DA–E–IMDA	Diels–Alder elimination intramolecular Diels–Alder
EC_{50}	effective concentration
ED_{50}	effective dose
FLU-A	influenza A virus
GI_{50}	growth inhibition
HBV	hepatitis B virus
HIV	human immunodeficiency virus
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum coherence
HPLC	high-performance liquid chromatography
HREI–MS	high-resolution electron-impact mass spectrometry
HR–MS	high-resolution mass spectrometry
HSQC	heteronuclear single-quantum coherence
HSV	herpes simplex virus
IC_{50}	inhibition concentration
IMDA	intramolecular Diels–Alder
IR	infrared
LDH_{50}	lactate dehydrogenase
LPS	lipopolysaccharide
MIC	minimum inhibitory concentration
MS	mass spectrometry
MSR	macrophage scavenger receptor
NCI	National Cancer Institute
NIAID	National Institute of Allergy and Infectious Diseases
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
NSAID	nonsteroidal anti-inflammatory drug
NSF	National Science Foundation
PLA_2	phospholipase A_2
PMA	phorbol 12-myristate 13-acetate
Ps	pseudopterosin
SCUBA	self-contained underwater breathing apparatus
SI	selectivity index
TLC	thin-layer chromatography
TXB_2	thromboxane B_2
UV	ultraviolet
UV/VIS	ultraviolet/visible

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2.12 Exploiting Genomics for New Natural Product Discovery in Prokaryotes

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2.12.1 Introduction

Prokaryotes, which constitute the earliest forms of life, are remarkably adaptable to diverse environments. They are almost ubiquitous on Earth and found in the bodies of all living organisms. Some bacteria are important pathogens but many produce antibiotics, or other bioactive natural products, which are therapeutically invaluable.

Until very recently, the identification of unknown natural products from bacteria mainly relied on bioassays, very often based on assessing antibiotic activity. After interesting metabolites were identified in this way, they were isolated and structurally characterized, and then their biosynthesis was investigated. Owing to such biosynthetic studies, a large number of gene clusters, each directing the biosynthesis of a specific natural product, has been identified.

Recent advances in DNA sequencing technology have generated a huge amount of DNA sequence data from a wide variety of organisms. Most of these data are globally shared through the World Wide Web and represent an invaluable pool of somewhat processed information. Coding sequences (CDSs) are automatically determined and the putative functions of encoded proteins are assigned based on sequence comparisons. These analyses are included as annotations along with the deposited DNA sequences. Additional annotations (based or not on experimental data) can be added to the sequences deposited in publicly accessible databases. To date, more than 635 complete microbial genomes have been sequenced, assembled, and deposited in the GenBank database. However, this number is increasing in an exponential manner with an impressive 971 microbial genome sequencing projects in progress, among which the contiguous DNA sequences of 480 have already been assembled (http://www.ncbi.nlm.nih.gov/).

Concurrently, detailed genetic and biochemical investigations of natural product biosynthetic systems have uncovered a high degree of mechanistic logic, in particular, for modular polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) systems.[1–5] In these systems, the essential enzymatic elements responsible for a specific catalytic function or substrate specificity have been determined. This deep understanding of the most common biosynthetic machineries together with the constant development of specific bioinformatics tools have now opened the way for the discovery of new metabolites from old sources, as well as from noncultivable organisms by the exploitation of genomic sequence data.

2.12.2 Overview of the Genetics and Enzymology of Natural Product Biosynthesis

2.12.2.1 Enzymatic Logic of Natural Product Biosynthetic Systems

The understanding of natural product biosynthetic systems has been extensively developed during the past 20–30 years. Details of both the biochemical mechanisms and the genetic organization of these systems have been investigated. Prokaryotes contain dedicated genes that direct the biosynthesis of natural products. With few exceptions, all the biosynthetic genes required to assemble one particular natural product lie adjacent to each other on prokaryotic chromosomes, that is, they are clustered.

The differing biosynthetic origins of natural products, which depend on the enzymatic systems catalyzing their biosynthesis, have resulted in the classification of natural products into diverse groups. Two of the most common groups are nonribosomal peptides and polyketides. The former as well as many of the latter are assembled by modular multienzyme systems termed NRPSs and modular PKSs, respectively. These multienzymes operate in a similar way to assembly lines where each harbors one or more modules, each responsible for the incorporation of a primary metabolic building block into the final structure. Within each module, there are several individual catalytic domains, each of which executes a specific enzymatic reaction as part of the overall process to select and covalently tether a building block, condense it with the nascent chain residing on the upstream module, and modify it to generate a specific substructure. Numerous genetic investigations have established that a correspondence usually exists in such systems between the number of modules and the number of building blocks incorporated into the natural product, as well as the presence of optional domains and the structural modifications of building blocks that occur during biosynthesis. However, several examples of natural product biosynthetic systems have recently been reported that disobey this 'colinearity rule'.[6] In both PKS and NRPS systems, some modules can be either skipped or iteratively used. Furthermore, not all the domains encoded at a genetic level appear to have enzymatic functions.

2.12.2.2 Modular Polyketide Synthases and Nonribosomal Peptide Synthetases

Polyketide natural products are assembled by PKSs which catalyze the decarboxylative polymerization of malonyl-CoA, as well as the derivatives of malonyl-CoA with different functional groups at C-2, including alkyl, methoxy, hydroxy, and amino groups. PKSs can be classified into different types depending on whether individual catalytic activities are used once (modular) or multiple times (iterative) during the chain assembly process and whether the catalytic activities are part of a multienzyme (type I) or reside within separate monofunctional proteins (types II and III). In practice, many systems do not fit perfectly into these classifications, as they are often hybrids between the idealized mechanistic extremes. Type I modular PKSs generally demonstrate a well-defined enzymatic logic and, as a consequence, the substrate specificity of many of their modules can usually be predicted (**Figure 1**) (see Chapter 1.02).

Many microbial peptide natural products are assembled by ribosome-independent pathways by modular multienzymes called NRPSs. NRPSs catalyze activation of specific amino acids by conversion into their corresponding aminoacyl thioesters and subsequent peptide bond formation between the activated amino acids. Both proteinogenic and nonproteinogenic amino acids are incorporated by NRPSs into natural products and specific enzymes involved in the biosynthesis of nonproteinogenic amino acid substrates of NRPSs are often encoded by genes clustered with the NRPS-encoding genes. Our understanding of NRPS enzymatic logic is also well developed (**Figure 2**), even though the diversity of precursors that can be incorporated by NRPSs is far greater than in modular PKS systems (see Chapter 5.20).

In both modular PKS and NRPS systems, the specificity for the building blocks incorporated in each round of chain elongation is mainly determined by the specificity of a particular domain found in every module. In PKSs, the acyl-CoA:acyl carrier protein transferase (AT) domain governs the building block incorporated by each module (**Figure 1**). However, the AT domain does not govern the stereochemistry of the stereogenic centers derived from the building block it selects. Indeed, once transferred onto the acyl carrier protein (ACP), the substrate of the AT can undergo a series of subsequent transformations on the PKS.[7] In NRPSs, the adenylation (A) domain governs the amino acid incorporated by each module (**Figure 2**). Domains are usually specific for L-amino acids (although a few exceptions have emerged recently) and can selectively recognize and activate the proteinogenic amino acids, as well as many nonproteinogenic amino acids and several other carboxylic acids such as aryl acids. Subsequent inversion of the α-carbon stereochemistry sometimes occurs before condensation of the aminoacyl thioester formed by the A domain with the nascent chain attached to the upstream module. This results from the action of the downstream epimerization (E) and condensation (C) domains. Epimerization domains are optional and are only present in some modules, and condensation domains are highly stereoselective toward the α-carbon epimer of the aminoacyl thioester found in the natural product.

Interestingly, hybrid PKS–NRPS systems are also found in microbes. The knowledge and understanding of modular PKS and NRPS systems also allow structural features of the metabolic products of these hybrid systems to be predicted (see Chapter 1.11).

The paradigm for both modular PKSs and NRPSs is that each module incorporates one building block into the natural product (**Figures 1 and 2**). Within each module there are different domains and each domain catalyzes a specific reaction in the assembly of the metabolic product. Model examples of this colinearity between module and domain organization and metabolite structure for type I modular PKS and NRPS systems are respectively represented by erythromycin and tyrocidine biosyntheses.[8,9] This colinearity is an important feature that underpins our ability to predict structural features of the metabolic products of novel modular PKS and NRPS systems discovered by genomics.

2.12.2.3 Other Biosynthetic Systems

For biosynthetic systems other than modular PKSs and NRPSs, the relationship between the structures of biosynthetic enzymes and the structure of the product(s) formed is often much less clear. Consequently, predicting the structures or structural features of metabolic products of novel biosynthetic systems uncovered by genomics can be challenging. Nevertheless, some structural parameters of such compounds can often be inferred. Indeed, sequence analyses of some classes of biosynthetic enzyme can lead to predictions of substrate specificity.

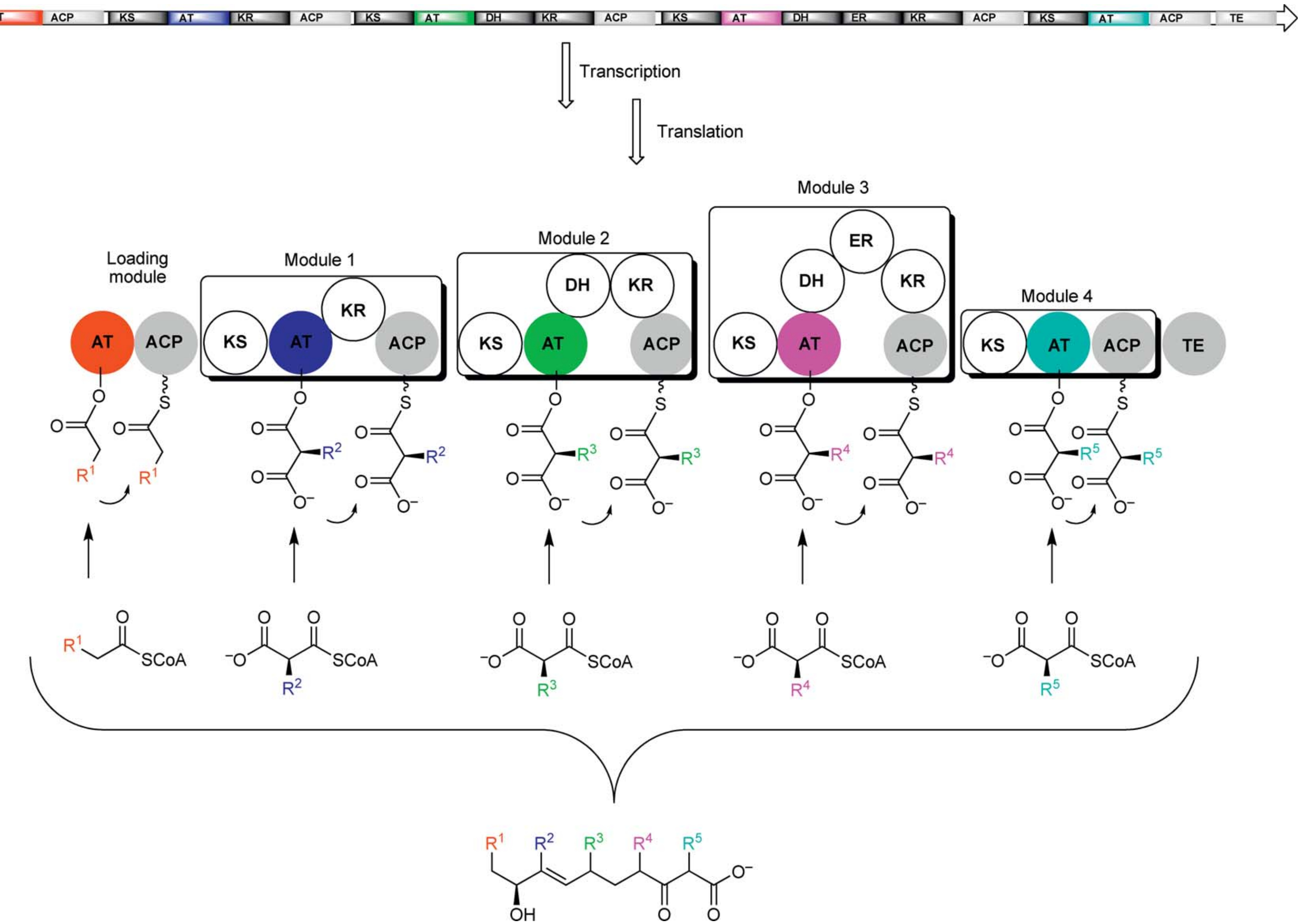

Figure 1 Hypothetical pentaketide biosynthetic system, which illustrates the enzymatic logic of type I modular polyketide synthases (PKSs) and the catalytic role of acyl transferase (AT) domains. Each AT domain selects substrates from the cellular pool and tethers them as thioesters to acyl carrier protein (ACP) domains. In a typical PKS module, the AT and ACP domains are present in all modules. The ketosynthase (KS) domain is present in all chain extension modules. The dehydratase (DH), enoyl reductase (ER), and ketoreductase (KR) domains are optional domains. The final thioesterase (TE) domain catalyzes the release of the product from the PKS.

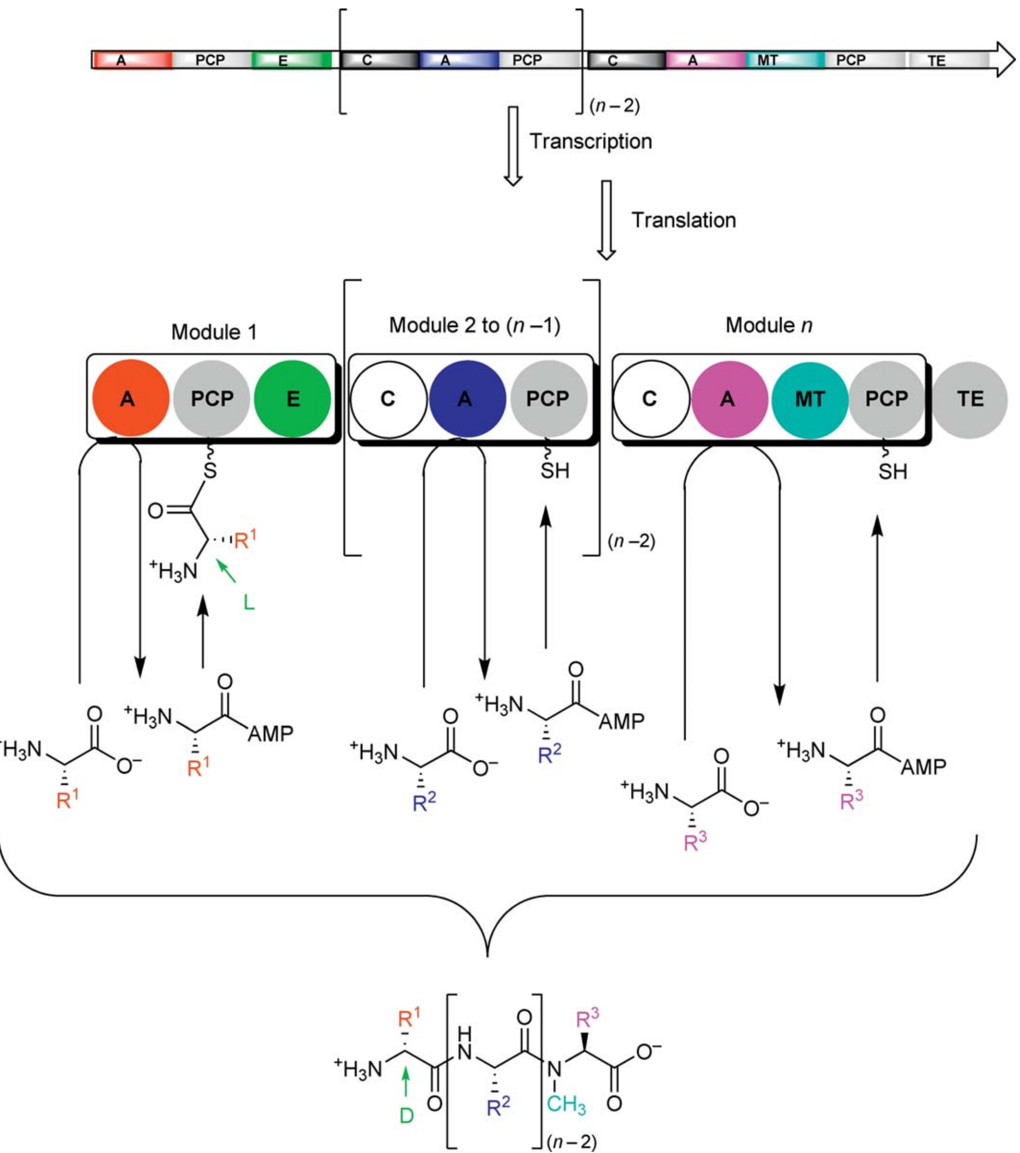

Figure 2 Enzymatic logic of nonribosomal peptide synthetases (NRPSs) and catalytic role of adenylation (A) domains. The A domain selects substrates from the cellular pool and tethers them as thioesters to peptidyl carrier protein (PCP) domains. In a typical NRPS, the A and PCP domains are always present. The condensation (C) domain is present in all chain extension modules. The epimerization (E) and the methyltransferase (MT) domains are optional. A final thioesterase (TE) domain generally catalyzes the release of the peptide from the NRPS.

For instance, terpene cyclases are known to catalyze the conversion of oligomeric isoprenoid pyrophosphate substrates to polycyclic hydrocarbon products.[10] Sequence comparisons of terpene cyclases with different known specificity can allow them to be classified into monoterpene, sesquiterpene, and diterpene synthases, which utilize the 10-carbon substrate geranyl pyrophosphate, the 15-carbon substrate farnesyl pyrophosphate, and the 20-carbon substrate geranyl–geranyl pyrophosphate, respectively, on the basis of sequence criteria.

Similarly, sequence analyses of iterative PKSs can allow the nature of the expected metabolite(s) to be inferred, even though accurate structural predictions are not yet possible. Many catalyze polymerization of malonyl-CoA to make poly-β-ketomethylene thioester intermediates that undergo a series of aldol cyclization and dehydration reactions to yield aromatic products. Others catalyze the assembly of more reduced polyketide

chains from malonyl-CoA such as polyenes that are precursors to enediyne antibiotics.[11] Sequence-based criteria can be used to discriminate between these different types of iterative PKS system, thus providing some insight into the nature of the products of cryptic systems uncovered by genomics.

NRPS-independent siderophore (NIS) synthetases constitute another class of biosynthetic enzymes that can be divided into three types according to their amino acid sequence.[12] These types are proposed to be specific for different substrates. Thus, type A enzymes are specific for citric acid, type B enzymes are proposed to be specific for α-ketoglutaric acid, and type C enzymes are specific for derivatives of citric or succinic acid. The type C enzymes are further divided into modular and iterative subtypes depending on whether they catalyze one or multiple condensation reactions. Thus for novel NIS synthetase systems uncovered by genome sequencing, structural features of their metabolic products can often be predicted.

2.12.2.4 Tailoring Reactions

As mentioned above, in the overwhelming majority of cases, all the genes that direct the biosynthesis of a particular natural product are clustered within the genome of microbes. As a consequence, genes encoding potential tailoring enzymes such as glycosyltransferases, monooxygenases, halogenases, and prenyltransferases are often found clustered with genes encoding NRPSs, PKSs, and other types of ubiquitous natural product biosynthetic system. This implies that the initially formed NRPS, PKS, or other product undergoes modification by tailoring reactions. While it is often possible to predict what structural unit is added in the tailoring reaction, it is seldom possible to predict the site of modification. Thus, in these cases, an additional element of uncertainty is added to the prediction of metabolic product structures from sequence data.

2.12.3 Development of Bioinformatics Tools for Natural Product Discovery by Genome Mining

2.12.3.1 The Discovery of New Natural Product Biosynthetic Gene Clusters by Genomics and Implications for New Natural Product Discovery

Genome mining involves the identification of previously uncharacterized natural product biosynthetic gene clusters within the genomes of sequenced organisms, sequence analysis of the enzymes encoded by these gene clusters, and the experimental identification of the products of the gene clusters. It has already resulted in the identification of many novel natural product biosynthetic gene clusters in the genomes of sequenced microbes.[13–15] In some cases, these gene clusters have been experimentally associated with the production of known metabolites. However in many cases, sequence analyses of the gene clusters predicted that they direct the production of new metabolites or known metabolites not previously identified as products of the organism in question.[16–18] Such biosynthetic gene clusters are often referred to as 'cryptic'. There are several possible reasons why the metabolic products of cryptic biosynthetic gene clusters were not detected in these microbes before genome sequencing. Perhaps, the production of metabolites belonging to certain structural classes has not been examined in the organism, or perhaps the metabolite is produced in quantities too small to detect under standard laboratory culture conditions. Alternatively, perhaps certain metabolites are too difficult to pinpoint in the large mixture of compounds typically present in a microbial culture broth. For this last scenario, prediction of a metabolite's structural features from sequence analyses of its biosynthetic gene cluster constitutes a powerful tool to search for such natural products. Structural predictions from sequence analysis can also indicate the degree of structural novelty of the corresponding metabolic product of a cryptic biosynthetic gene cluster. An alternative approach to whole genome sequencing for the identification of cryptic biosynthetic gene clusters is 'genome scanning'. This relies on the fact that most natural product biosynthetic gene clusters are relatively large and, as a consequence, only a few hundred Sanger sequencing reads on randomly selected clones from a genomic library are required to identify the majority of biosynthetic gene clusters in a given microbial genome. Once a gene in each cluster has been identified, clones containing most of or the entire gene cluster can be isolated from cosmid or bacterial artificial chromosome (BAC) genomic libraries and fully sequenced. Cryptic biosynthetic gene clusters can also be identified by sequence analysis of metagenomic libraries.

2.12.3.2 Role of Bioinformatics in Natural Product Discovery by Genome Mining

Genome mining is entirely dependent on computing technology and bioinformatics tools. A huge amount of data, represented by DNA sequences and their annotations, has to date been deposited in publicly accessible databases. The storage and handling of these ever-expanding resources relies on the continued development of computers and the networks that interconnect them. Indeed, the complete genome of a single prokaryote is described by a unique sequence of the four nucleotides (A, T, C, and G) constituted of a minimum of about 500 000 bp (for symbiotic organisms) up to over 10 000 000 bp (for saprophytic microbes). Once all the CDSs within a genome sequence have been identified, they can be compared with CDSs encoding proteins of known function in the public databases. Since the sequences of many CDSs encoding characterized enzymes involved in natural product biosynthesis are deposited in these databases, it is relatively straightforward to identify the majority of putative natural product biosynthetic gene clusters in a microbial genome sequence by such sequence comparisons. Both raw and annotated genomic data, as well as bioinformatics tools, for sequence comparisons are freely available through the World Wide Web. It is now a mandatory publication prerequisite of most scientific journals that sequence data from any research involving novel DNA sequences is deposited in a publicly accessible database such as GenBank (http://www.ncbi.nlm.nih.gov/).[19] The freely available bioinformatics tools include the programs ClustalX (http://www.clustal.org/), which align multiple DNA or protein sequences with each other; and the basic local alignment and search tool (BLAST: http://www.ncbi.nlm.nih.gov/), which finds regions of local similarity between DNA and protein sequences.[20] These tools enable comparative analyses, which not only facilitate the inference of functional relationships between genes and proteins but also underpin the prediction of substrate specificity, stereospecificity, and other catalytic properties of biosynthetic enzymes. The use of comparative sequence analyses in both these contexts is discussed in the following sections.

2.12.3.3 Functional Assignment of Biosynthetic Enzymes

Once a putative biosynthetic gene cluster has been identified *in silico* using the methods discussed above, detailed analyses of the putative function of each protein encoded by the gene cluster are undertaken. BLAST searches allow a putative function to be assigned for each protein encoded by the gene cluster that shows similarity to a protein of known function.

For multienzymes like modular PKSs and NRPSs, a conserved domain (CD search: http://www.ncbi.nlm.nih.gov/) allows the majority of functional domains within each polypeptide to be identified.[21] Occasionally, functional domains are missed by such analyses and they have to be identified by manual searches for conserved sequence motifs that are characteristic of particular domains. Even if the sequence similarity between a domain being analyzed and a domain of known function is very high, amino acid residues known to be critical for catalytic activity are sometimes missing from the domain being analyzed, suggesting that the domain may be inactive. A clear understanding of the mechanism of each catalytic domain is required to discriminate active ones from inactive ones. Sometimes, large deletions in a domain clearly indicate that it is not functional. Accurately predicting for the majority of PKS and NRPS domains in a biosynthetic system whether they are active or not is particularly important when the strategy for isolating the metabolic product of the system relies on a structural prediction. Once the general function of each catalytic domain in a biosynthetic assembly line has been predicted, more detailed comparative analyses can lead to the prediction of substrate specificity, stereospecificity, or some other catalytic property of particular domains. Examples of this are discussed in the following sections.

2.12.3.4 Predicting Modular Polyketide Synthase Acyl Transferase Domain Substrate Specificity

Every module of a type I modular PKS uses an AT domain that specifically selects a substrate from an acyl-CoA pool and covalently binds it to the downstream ACP domain within the same module (**Figure 1**). Amino acid sequences of large numbers of AT domains of known substrate specificity have been compared and characteristic sequence motifs have been found to be associated with particular substrate specificities.[7,22] For

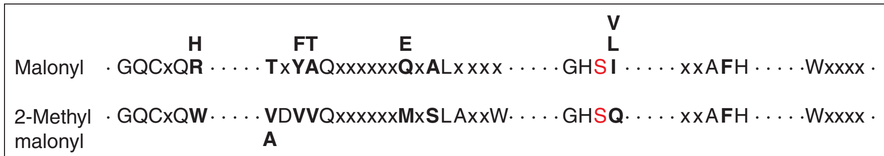

Figure 3 Consensus sequences of acyl transferase (AT) domains specific for malonyl-CoA or 2-methylmalonyl-CoA. Bold letters indicate significant differences. The residue shown in red represents the catalytic serine residue that binds to malonyl-CoA or 2-methylmalonyl-CoA.

instance, malonyl-CoA-specific AT domains can be discriminated from 2-methylmalonyl-CoA-specific AT domains using the consensus sequence presented in **Figure 3**. This method has proved to be invaluable for predicting which building blocks are incorporated into the polyketide chains assembled by cryptic modular PKS systems.

The first example of the discovery of novel polyketide natural products, the halstoctacosanolides, by a genome mining approach involved the prediction of AT domain substrate specificities within the loading module and eight-chain extension modules of a partial cryptic modular PKS encoded within the genome of *Streptomyces halstedii* (**Figure 4**). This analysis was a key component in predicting that the strain had the capability to produce novel polyketides.[23] However, the role of the optional ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) β-carbon processing domains in each module also had to be analyzed to draw this conclusion. A functional KR domain was found in each of the eight-chain extension modules, indicating that each of the nascent β-ketoacylthioester intermediates produced during chain assembly were reduced to a hydroxyl group. Modules 4, 5, and 8 were also found to contain a DH domain, suggesting that water was eliminated from the nascent β-hydroxythioester intermediates produced in these three modules to yield the corresponding α,β-unsaturated thioesters. Module 5 was also found to contain an ER domain, indicating that the nascent α,β-unsaturated thioester produced in this module is reduced to the corresponding saturated thioester. These predictions are summarized in **Figure 4**. The resulting predicted partial structure of the metabolic product of this PKS had never been seen in any known natural product. This provided the impetus to isolate and structurally characterize the halstoctacosanolides, the products of this cryptic modular PKS in *S. halstedii*.[23] More recently, the complete halstoctacosanolide biosynthetic gene cluster has been cloned, sequenced, and analyzed, and gene disruption experiments have confirmed its involvement in the biosynthesis of these novel polyketides.[24]

2.12.3.5 Predicting Ketoreductase Domain Stereospecificity in Modular Polyketide Synthase Systems

In the above example, prediction of catalytically active KR domains was an important factor in predicting the existence of a novel structural fragment in the halstoctacosanolides. However, the stereochemical outcomes of the ketoreduction reactions catalyzed by these domains were not considered. There are two possible stereochemical outcomes for this reaction, as illustrated in **Figure 5**. Sequence comparisons of a series of KR domains, for which the stereospecificity had been previously determined, permitted a correlation between specific KR domain sequence motifs and ketoreduction stereospecificity to be established (**Figure 5**).[25] A-type alcohol stereochemistry is generated by KR domains containing a conserved tryptophan residue (W141). In contrast, B-type alcohol stereochemistry results from KR domains containing an LDD motif together with highly conserved proline (P144) and asparagine (N148) residues. This predictive tool should prove particularly useful for the final steps in the structural elucidation of new modular PKS products discovered by genome mining approaches, because the configuration of stereocenters in complex polyketides can be very challenging to determine by spectroscopic methods and it is often not possible to obtain crystals of such metabolites for relative stereochemistry determination by X-ray crystallography.

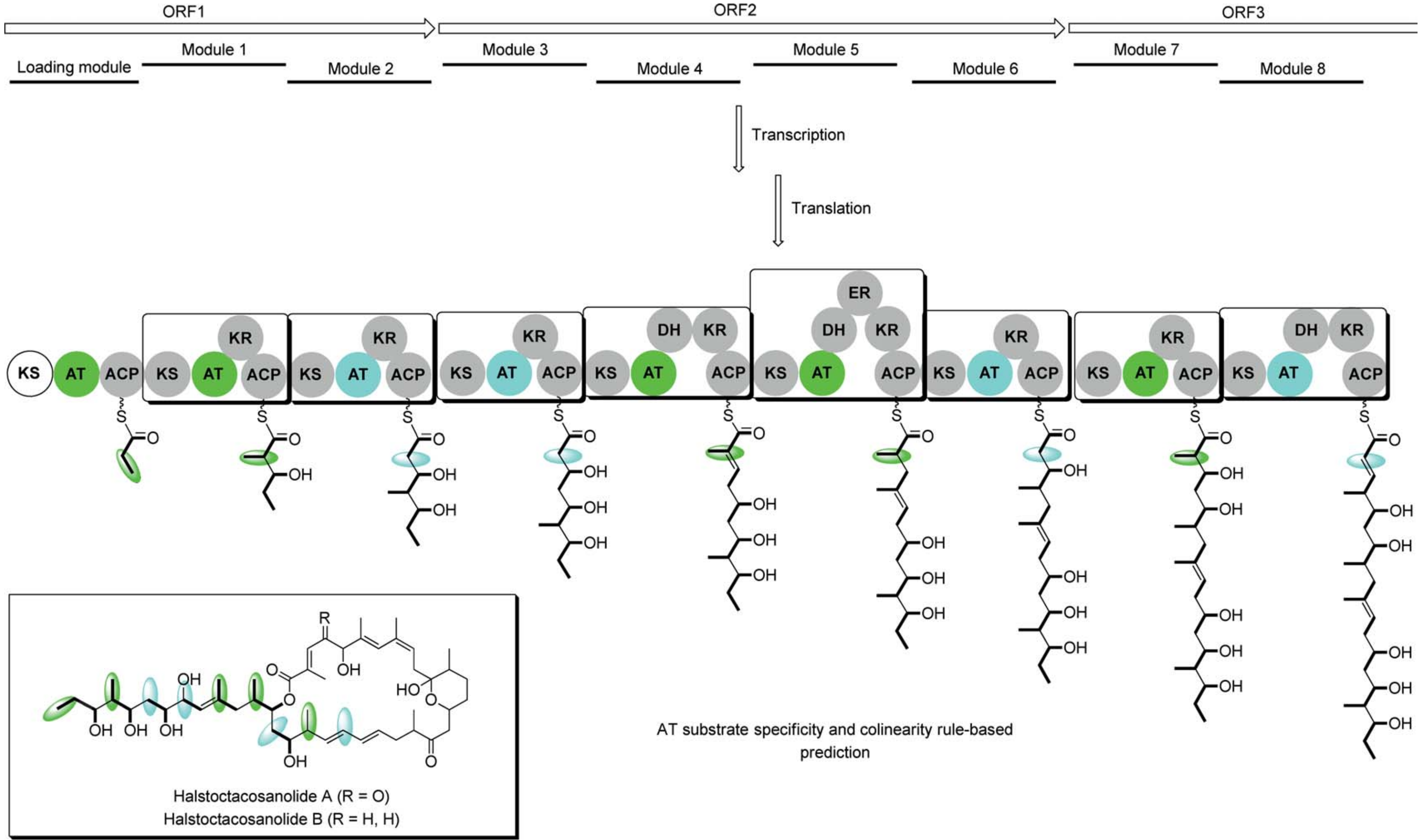

Figure 4 Partial organization of the polyketide synthase (PKS) system for which AT domain substrate specificity and colinearity rule-based prediction has led to the discovery of the halstoctacosanolides. AT domains highlighted in green were predicted to specifically load a methylmalonyl-CoA unit whereas the ones highlighted in blue were predicted to load malonyl-CoA. The actual structure of the halstoctacosanolides isolated from *Streptomyces halstedii* is represented in the bottom left corner.

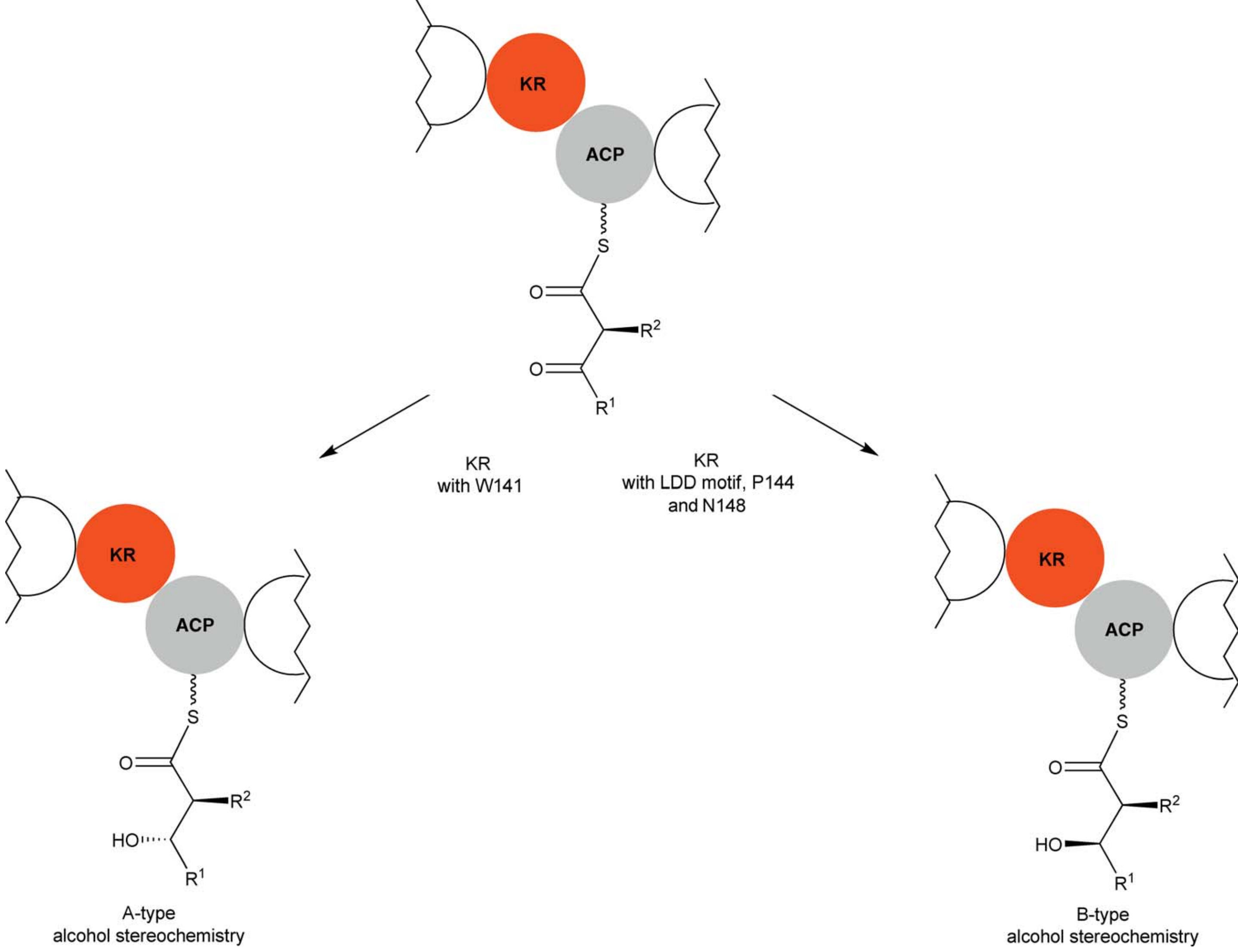

Figure 5 Prediction of ketoreduction stereospecificity of KR domains of modular polyketide synthases (PKSs).

2.12.3.6 Ketosynthase Domain Specificity Predictions in the '*trans*-AT' Class of Modular Polyketide Synthases

A subgroup of type I modular PKSs is the so-called '*trans*-AT class', in which AT domains lack individual modules.[26] As a consequence, the ACP domain in each module is specifically loaded with malonyl-CoA by a separately encoded, *trans*-acting acyl transferase (AT) enzyme. In these systems, the stand-alone AT enzymes act iteratively to load each of the ACP domains in the synthase with the requisite extender unit. Predicting the metabolic products of cryptic systems belonging to this class can be very challenging because the modules in these systems frequently contain extra domains with no obvious function, or appear to lack domains that would be expected to be required to produce structural features in the known products that result from β-carbon processing reactions during chain assembly. These missing activities are provided in *trans* by separately encoded enzymes and it is not easy to predict on which intermediate in the chain assembly process these might act. Thus, the good correlation between domains found in a module and the substructure generated by that module, observed in the modular PKS systems discussed above, breaks down.

A recent study of the phylogenetic relationship between the ketosynthase (KS) domains in such *trans*-AT PKSs in the context of the nature of the structural unit supplied by the upstream module to each domain resulted in an important discovery; a strong correlation between the sequence of the KS domain and the chemical structure of the substrate that is elongated by reaction with a malonyl thioester in the KS-catalyzed reaction was found. As a consequence, this analysis is able to predict what postcondensation processing reactions occur within the PKS module preceding the KS domain being analyzed. These predictions do not rely on the analysis of the functional role of each catalytic domain present in the different modules, which can be difficult to infer in *trans*-AT PKS systems, as discussed above. This new tool adds significantly to the armory of bioinformatics methods for the discovery of novel polyketides by genome mining. The power of this tool has been exemplified by the discovery of the thailandamides (**Figure 6**), products of a cryptic hybrid *trans*-ATPKS/NRPS system identified in *Burkholderia thailandensis*.[26]

2.12.3.7 Substrate Specificity Prediction of Nonribosomal Peptide Synthetase Adenylation Domains

In NRPS systems, A domains present within each module of a synthetase have been found to specifically recognize amino acids from the cellular pool and catalyze their chemical activation by adenylation and subsequent condensation with the phosphopantetheine thiol of the adjacent peptidyl carrier protein (PCP) domain (**Figure 2**). A domains are therefore considered to be the primary determinant of substrate specificity in NRPSs.[27] However other domains within NRPSs, such as condensation (C) domains, also exert a certain degree of substrate selectivity. On the basis of the crystal structure of the A domain PheA, which selectively activates L-Phe in the gramicidin S synthetase GrsA,[28] and A domain amino acid sequence alignments, two independent studies established an empirical correlation between the substrate activated and the amino acid residues in each A domain that correspond to the 10 residues in direct contact with L-Phe in PheA.[29,30] Since then, sequence analyses of new NRPSs and biochemical characterization of A domains have contributed to the development and refinement of models for A-domain selectivity. These models have been used to predict the substrate specificity of newly discovered NRPSs and consequently the structures of novel nonribosomal peptides. A pertinent example is a novel gene cluster identified by analysis of the partial genome sequence of the model antibiotic-producing bacterium *Streptomyces coelicolor* A3(2), which contains a gene encoding a new trimodular NRPS system.[31] Analysis of the domain organization of this NRPS coupled with prediction of the substrate specificity of its three A domains led to two alternative possible novel tripeptide structures for the metabolic product of this cryptic gene cluster (**Figure 8**).[31] These structural predictions suggested culture conditions likely to induce expression of the gene cluster and also indicated a likely physicochemical property of the metabolic product of the cluster, thus greatly facilitating its identification in culture supernatants of *S. coelicolor*. As a consequence the product of this cryptic gene cluster was easily identified.[32] Detailed spectroscopic analyses showed that this metabolite, named coelichelin, was derived solely from the predicted substrates of the A domains in the three modules of the NRPS. Surprisingly, however, they showed that coelichelin was a tetrapeptide, rather than the expected tripeptide, arising from the incorporation of two units

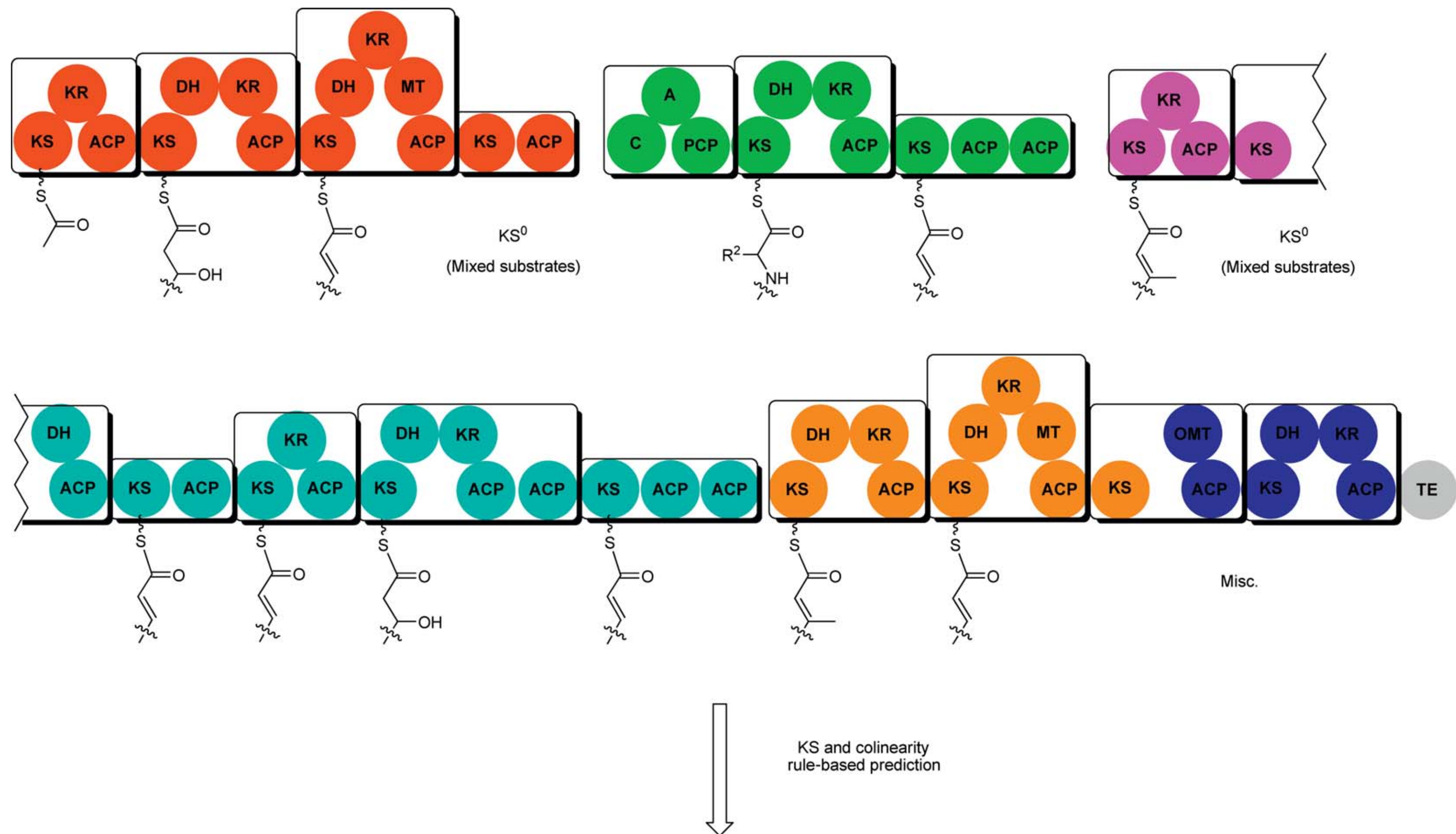

Figure 6 (Continued)

Colinearity rule-based prediction only

Thailandamide A

Figure 6 Organization of the polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) system for which ketosynthase (KS) and colinearity rule-based predictions have assisted the discovery of the thailandamides from *Burkholderia thailandensis*. The different proteins are highlighted in different colors and each module is boxed. The experimentally determined structure of thailandamide A is shown at the bottom of the figure.

of one of the predicted precursors. Further genetic experiments confirmed that no NRPS other than the trimodular one encoded within the cryptic gene cluster was required for assembly of this tetrapeptide. This was the first example of a tetrapeptide assembled by a trimodular NRPS and nonlinear enzymatic logic, including iterative module use and module skipping are implied in the chain assembly process. Details of the experimental strategy used to identify coelichelin are described later on in the chapter.

2.12.3.8 Predicting the Mode of Chain Release by Thioesterase Domains of Polyketide Synthases

Typically, the last domain in the final module of modular PKS and NRPS systems is a thioesterase (TE) domain. This domain catalyzes the release of the assembled polyketide or peptide chain from carrier protein domain within the last module of the PKS or NRPS. Separately encoded, stand-alone TE enzymes are also found in some systems, such as the coelichelin biosynthetic system. TE domains catalyze two related types of chain release reactions. The first type is the hydrolysis or intermolecular condensation with a soluble amine and the second is intramolecular amide or ester bond formation. These chain release reactions result in distinct metabolic products. The intermolecular reactions lead to linear products with a carboxyl-terminus, whereas the intramolecular reactions lead to cyclic products. Sequence comparisons of TE domains from various modular PKSs that assemble known metabolic products have established a correlation between the phylogenetic relatedness of the domains and the type of chain release reaction catalyzed.[33,34] However, this predictive tool, which has been developed by Ecopia BioSciences, is not yet publicly accessible.

2.12.4 Experimental Strategies for the Isolation of New Natural Products by Genome Mining

2.12.4.1 An Important Prerequisite: Establishing that the Cryptic Biosynthetic Gene Cluster is Expressed

Once a cryptic gene or gene cluster has been discovered, the bioinformatics tools previously described can be used to analyze the biosynthetic proteins encoded by the gene(s). The results of such analyses may allow structural characteristics of the metabolic product(s) to be inferred. Before deciding which strategy is the best for identifying the metabolic product(s), it is important to establish whether the cryptic biosynthetic genes are expressed. Obviously, if the genes are not expressed the metabolic product(s) of the cryptic gene(s) will not be biosynthesized. The mRNA transcripts of specific genes can be detected using standard molecular biology techniques such as reverse transcription–polymerase chain reaction (RT–PCR) and Northern blot. Alternatively, DNA microarrays can be used to simultaneously detect the majority of mRNA transcripts present in the cell in a particular medium at a particular point of growth. These methods can be used to ascertain whether cryptic gene cluster(s) are expressed and are consequently useful for determining an optimum set of growth conditions (fermentation medium, incubation time) in which to embark on the search for the metabolic product(s) of the cryptic cluster(s). However, transcriptional analyses may not be required if the cryptic biosynthetic gene cluster(s) are constitutively expressed. A direct search for the metabolic products of such clusters may prove fruitful in such cases.

2.12.4.2 Choosing the Right Approach for Identifying the Products of Cryptic Biosynthetic Gene Clusters

Identifying natural products of interest in a bacterial culture supernatant can be very challenging. The use of bioassays to detect compounds with specific biological activities is a classical approach that has proved to be very effective. In the context of genome mining for new natural product discovery, such bioassay-guided approaches are often of little value because the likely biological function of metabolic products of cryptic biosynthetic gene clusters often cannot be predicted using the bioinformatics tools described above. This has necessitated the development of new strategies for identifying the metabolic products of cryptic biosynthetic gene clusters. Some of these strategies rely on comparative metabolic

profiling of genetically engineered bacteria, where cryptic biosynthetic genes have been disrupted, genes that regulate the expression of the cryptic gene cluster have been manipulated, or the cryptic gene cluster has been heterologously expressed. These approaches require no prior structural information about the metabolic product(s) of a cryptic biosynthetic gene cluster. They rely solely on the ability to detect differences in the profile of metabolites in culture supernatants, culture extracts, or biomass extracts, and are especially useful when the bioinformatics approaches described above yield few or no clues about the likely structure of the metabolic product(s) of a cryptic biosynthetic gene cluster. For cases where the bioinformatics approaches do provide significant insights into the substrate specificity of key biosynthetic enzymes encoded within the cryptic cluster or the structural features of the metabolic product(s), a range of more targeted approaches can be applied. These include *in vitro* reconstitution of the cryptic biosynthetic pathway, focusing analyses on only compounds that have the predicted physicochemical properties of the putative metabolic product(s) of the cryptic gene cluster, or the so-called 'genomisotopic approach'. Obviously, complimentary approaches can also be combined to produce powerful multifaceted strategies. Each of the abovementioned strategies is discussed in the following sections with reference to pertinent examples of their application to natural product discovery.

2.12.4.2.1 Manipulation of biosynthetic gene expression coupled with comparative metabolic profiling

Using transcriptional analyses, several cryptic biosynthetic gene clusters have been found to be silent (not expressed) under a variety of growth conditions. Such clusters may represent a rich source of new metabolites, which cannot be accessed unless expression of the genes can be activated. Several approaches have been developed to overcome the problem of silent cryptic biosynthetic gene clusters.

One empirical approach involves growing the organism of interest in a wide variety of conditions (in particular in diverse media) and assessing the production of new metabolites in the different conditions. This so-called 'one-strain-many-compounds (OSMAC)' approach relies on the fact that the metabolic profile of an organism can be significantly affected by the environment in which it grows.[35,36] Sometimes, it is possible to utilize the information gleaned from bioinformatics analyses of cryptic biosynthetic gene clusters to predict growth conditions under which the gene cluster should be expressed. This was the case for the cryptic coelichelin biosynthetic gene cluster of *S. coelicolor*, discussed above, where the sequence analyses identified putative iron-dependent repressor binding sites in intergenic regions within the cluster, suggesting that expression of the cluster would be induced under iron-deficient conditions and repressed under iron-replete conditions. This guided the selection of appropriate culture conditions to ensure expression of the cryptic gene cluster, which proved to be an important component of the overall strategy for identifying coelichelin in *S. coelicolor* supernatants.[22]

Another approach has been pioneered in the search for metabolic products of cryptic biosynthetic gene clusters in fungi. The expression of genes encoding both global- and pathway-specific regulators of secondary metabolism (identified by sequence comparisons) has been manipulated to induce expression of silent cryptic gene clusters in *Aspergillus nidulans*.[37,38] This has led to the identification of novel products of a cryptic hybrid PKS–NRPS system by comparative metabolic profiling of the strains in which the cryptic gene cluster is silent and expressed.[37] However, this elegant approach has yet to be applied to the discovery of new natural products from prokaryotes by genome mining.

2.12.4.2.2 Biosynthetic gene inactivation coupled with comparative metabolic profiling

This approach involves inactivation of one or more genes predicted to be essential for metabolite biosynthesis in the cryptic gene cluster and comparison of the profile of metabolites produced by the wild-type organism and the constructed mutant(s) using an appropriate analytical technique such as liquid chromatography–mass spectrometry (LC–MS). The metabolite(s) present in the wild-type strain but lacking in the mutant(s), identified by this analysis, are likely products of the cryptic biosynthetic gene cluster. An important advantage of this method is that it establishes an experimental link between the metabolites identified and the cryptic biosynthetic genes. Another important advantage is that no prior structural insight into the metabolic product(s) of the cryptic gene cluster is required for this method to be successful. This gene knockout/comparative metabolic profiling approach was another key facet of the experimental strategy used to identify coelichelin as the metabolic product of a cryptic NRPS-encoding gene cluster in *S. coelicolor*, discussed above (**Figure 7**).[32]

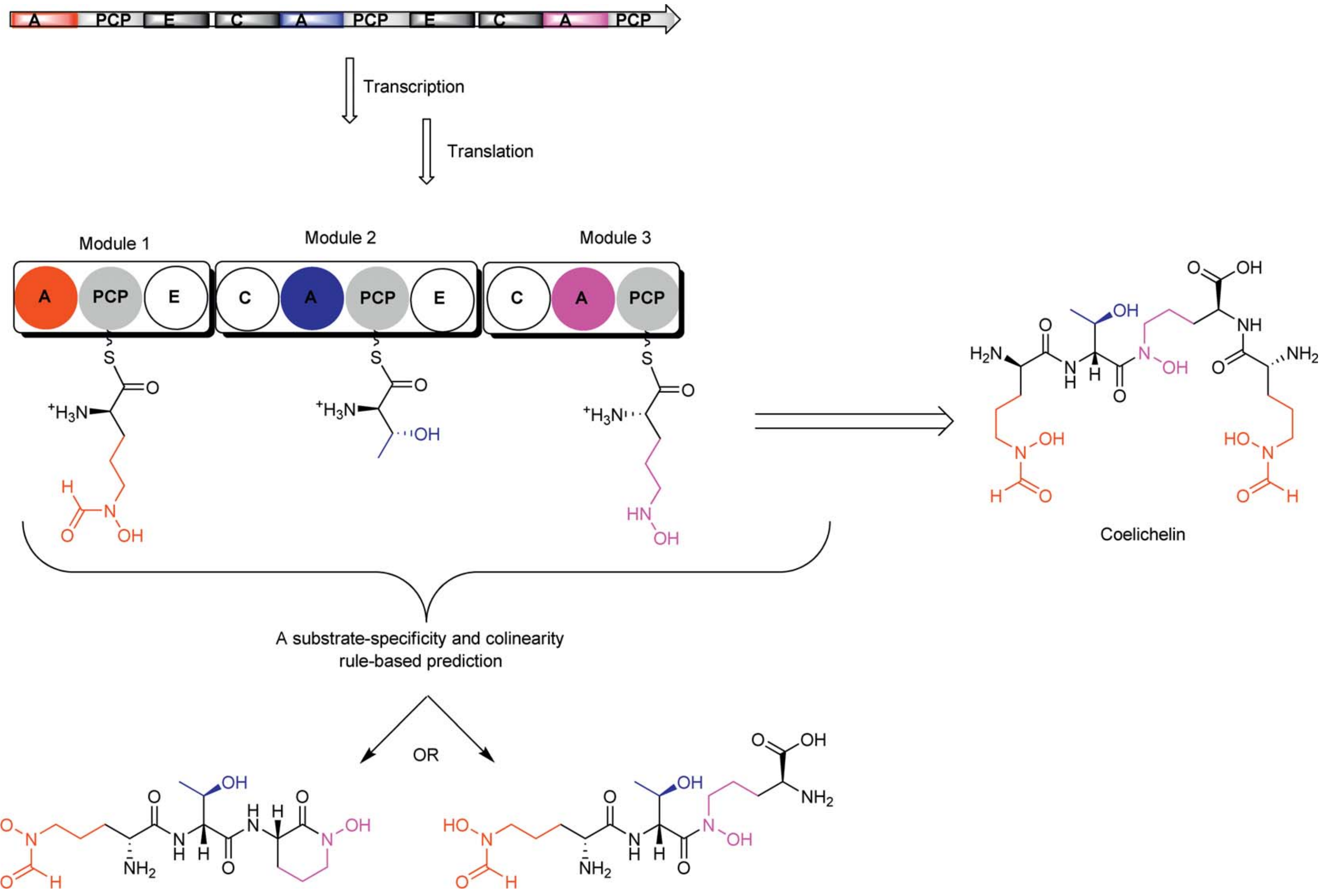

Figure 7 Predicted chemical structures of the nonribosomal peptide coelichelin based on A domain substrate specificity and colinearity rule (at the bottom) and experimentally determined structure of coelichelin (on the right).

This approach has also been applied to the identification of the metabolic products of other cryptic NRPS systems. Genome scanning identified NRPS-encoding fragments not associated with the production of known metabolic products in *Stigmatella aurantiaca*. Application of the gene disruption/comparative metabolic profiling approach led to the identification and structural characterization of the novel cyclic pentapeptide myxochromides S^1–S^3 as novel products of one of the cryptic NRPS systems identified (**Figure 10**).[39,40] In this example, no structural insight into the putative metabolic products of the cryptic biosynthetic system was apparently available. A similar approach was used to identify bacillaenes as novel products of a cryptic hybrid *trans*-AT modular PKS/NRPS system identified by analysis of the complete genome sequence of *Bacillus subtilis* (**Figure 10**).[41] Known and novel germicidins were also identified as wholly unanticipated metabolic products of a gene encoding a cryptic type III PKS in *S. coelicolor* (**Figure 8**).[42] In both these cases, little or no structural insight into the metabolic products of the cryptic biosynthetic systems could be gleaned from comparative bioinformatics analyses.

2.12.4.2.3 Heterologous biosynthetic gene cluster expression coupled with comparative metabolic profiling

A related approach to the gene knockout/comparative metabolic profiling one described above involves introducing the cryptic biosynthetic gene cluster into a different but usually related organism (a heterologous host) to the one in which the cryptic gene cluster was identified. If the gene cluster is expressed, comparison of the metabolite profiles in the heterologous host containing and lacking the gene cluster should result in the identification of new compounds in the former.

There are several advantages of this approach. First, no prior structural insight from bioinformatics analyses is required to identify the metabolic product(s) of the cryptic biosynthetic gene cluster. Second, the identification of the cryptic metabolic product can be easier in the heterologous host than the wild-type producer, for example, because better or faster growth of the heterologous host results in higher titers of metabolite, or because the heterologous host produces fewer secondary metabolites and thus offers a cleaner background. Third, this approach can allow an experimental link between the biosynthetic gene cluster and its product to be established and finally, it can be used to identify the metabolic products of cryptic biosynthetic gene clusters identified in metagenomic libraries or in organisms that are difficult to cultivate.

The main disadvantage of this approach is that, in order to be successful in the discovery of the true metabolic product(s) of the cryptic biosynthetic gene cluster rather than merely an intermediate or shunt metabolite from the cryptic pathway, the entire set of genes required for production of the metabolite(s) needs to be introduced into the heterologous host, usually within a single vector. The fact that many natural product biosynthetic gene clusters can be significantly larger than 40 kb necessitates the use of BAC or similar vectors capable of carrying large inserts. There are many technical challenges associated with the handling of large DNA fragments and their stable insertion into BAC vectors. Another potential pitfall is that the cryptic biosynthetic gene cluster may not be expressed in the heterologous host because it has been taken out of its natural regulatory context. This is the reason why a host similar to the organism in which the cryptic gene cluster was identified is normally used. This pitfall can be overcome by reconstruction of the biosynthetic gene cluster to place it under the control of a constitutive or inducible promoter.

This approach has been applied to the discovery of CBS40 as the novel chlorinated metabolic product of a cryptic iterative (type II) PKS system discovered by genome scanning approach that targets gene clusters containing genes encoding chlorinase enzymes, which are associated almost exclusively with secondary metabolic pathways (**Figure 8**).[43] Very recently, it has also been applied to the discovery of a new structural class of antibiotic biosynthesis inducers as the products of a three gene operon within the methylenomycin biosynthetic gene cluster that resides on the giant linear plasmid SCP1 of *S. coelicolor* (**Figure 10**).[44] In both these examples, little structural insight into the products of the cryptic biosynthetic gene clusters could be derived from bioinformatics analyses.

2.12.4.2.4 In vitro *reconstitution*

This approach is the first of three that rely on accurate prediction of the substrate(s) of enzyme(s) encoded by cryptic biosynthetic gene clusters. Putative biosynthetic gene(s) within the cryptic cluster are overexpressed (usually in *Escherichia coli*) and the resulting overproduced recombinant enzyme(s) are purified to homogeneity.

ECO-02301

E-837: R^1 = Me, R^2 = R^3 = H
E-492: R^1 = Me, R^2 = OH R^3 = Me
E-975: R^1 = Et, R^2 = OH R^3 = Me

ECO-0501

Figure 8 (Continued)

CBS40

Isogermicidin A: $R^1 = R^2 = R^4 = Me$, $R^3 = H$
Isogermicidin B: $R^1 = R^3 = H$, $R^2 = R^4 = Me$
Germicidin C: $R^1 = R^4 = H$, $R^2 = R^3 = Me$

Salinilactam A

Figure 8 Novel polyketide natural products discovered by genome mining.

The predicted substrate(s) are incubated with the purified enzyme(s) and their consumption is monitored. If the substrate(s) are consumed, comparative analyses using an appropriate technique such as LC–MS of the incubation mixture with a control reaction where enzyme is omitted can be used to identify the new product(s) formed, which can be isolated and structurally characterized.

This approach can be useful for silent cryptic biosynthetic systems because overexpression of the biosynthetic genes in *E. coli* removes the regulatory constraints placed on the system by the natural host and places them under the control of a heterologous, inducible promoter. The main drawback of this approach is that many systems contain several biosynthetic genes and reconstitution of the entire pathway *in vitro* to produce its true metabolic product can therefore be very time consuming and laborious. Another potential drawback is the problems associated with the overproduction of heterologous biosynthetic proteins in *E. coli* or another host in soluble, active form. This can be very challenging and it is not easy to predict which genes will and which will not lead to the production of soluble active proteins.

This method has been applied to the identification of the metabolic product of a cryptic sesquiterpenoid biosynthetic pathway discovered by analysis of the *S. coelicolor* genome sequence, although it turned out that it was involved in the biosynthesis of a natural product known to be produced by other *Streptomyces* species.[45,46] It has also been used to identify the products of a cryptic gene cluster identified in the complete genome sequence of *Bacillus halodurans* C-125 that was predicted to direct the biosynthesis of a two-component lantibiotic named haloduracin, which was identified in the culture supernatants of *B. halodurans* as well (**Figure 9**) (see Chapter 5.08).[47]

2.12.4.2.5 *Prediction of physicochemical properties*

Insight into the structural features of metabolic products of cryptic biosynthetic gene clusters derived from bioinformatics analyses can lead to the prediction of physicochemical properties of the putative compounds, such as molecular masses or mass ranges and specific UV–Vis absorbance profiles. If such properties can be predicted with a reasonable degree of confidence, they can be exploited to greatly simplify the analytical challenge associated with identifying the metabolic products of cryptic biosynthetic gene clusters. However, this method is limited to only those cases where reasonably confident predictions can be made. It should also be noted that, when used on its own, this approach does not afford a direct experimental link between the cryptic gene cluster and the metabolic products that are identified on the basis that they posses the predicted physicochemical properties. Thus, this method is often more powerful when combined with the other strategies discussed above that do establish a direct experimental link between the cryptic genes and the identified metabolites.

Trichamide

α-Component

β-Component

Haloduracin

Figure 9 Novel ribosomally biosynthesized peptide natural products discovered by genome mining.

Predicted UV–Vis absorbance profiles for putative metabolic products of several cryptic biosynthetic gene clusters have been used in several cases to identify and guide purification of the metabolites. In the example of coelichelin, which has been discussed above, it was predicted that this metabolite contains hydroxamic acid functional groups, which make strong complexes with ferric iron that exhibit specific UV–Vis absorbance profiles due to ligand-to-metal charge transfer bands. This prediction was exploited by adding ferric iron to culture supernatants before high-performance liquid chromatography (HPLC) analyses targeting the expected absorbance maxima for ferric *tris*-hydroxamate complexes. When combined with the gene knockout/comparative metabolic profiling approach, this allowed coelichelin to be very rapidly identified, because the metabolite profiles being compared were extremely simple. Polyene structural motifs have been predicted to occur within putative metabolic products of two cryptic modular PKS systems identified by analysis of the complete genome sequence of the marine actinomycete *Salinispora tropica* CNB440 and by genome scanning of *Streptomyces aizunensis*.[48,49] The characteristic UV–Vis absorbance profiles associated with polyenes allowed the putative metabolic products of these gene clusters, salinilactam and ECO-02301 (**Figure 8**), to be rapidly identified in culture supernatants of *Sal. tropica* and *S. aizunensis*, respectively.

Analysis of the complete genome sequence of the bloom-forming marine cyanobacterium *Trichodesmium erythraeum* ISM101 identified a cryptic gene cluster predicted to direct the production of a novel ribosomally biosynthesized cyclic peptide. Bioinformatics analyses led to the prediction that the metabolic product of this system should have a molecular weight between 1079 and 1157 Da. Electrospray ionization–mass spectrometry (ESI–MS) analysis of crude culture extracts of the cyanobacterium revealed a significant compound with m/z 1099.[50] This compound was subsequently shown to be trichamide (**Figure 9**), the likely metabolic product of the cryptic gene cluster. Interestingly, *T. erythraeum* was not known to produce secondary metabolites before this study. Genome scanning has identified novel cryptic NRPS systems in *Streptomyces* sp., one of which was predicted to assemble a depsipeptide with a structure that is similar to several known antitumor compounds. A combination of bioactivity screening with LC–MS analyses that targeted a specific predicted mass range led to the identification of ECO-7942 as the product of this system (**Figure 10**).[17]

ECO-0501 and a family of related alkenylfuranones (**Figure 8**) are other metabolites identified as the putative metabolic products of other cryptic biosynthetic gene clusters discovered by genome scanning by exploitation of their predicted physicochemical properties.[33,34]

2.12.4.2.6 The genomisotopic approach

This approach relies on the accurate prediction of substrates of enzymes encoded within a cryptic biosynthetic gene cluster and monitoring the incorporation of the stable isotope-labeled predicted substrates into metabolites of the organism in which the cryptic cluster was identified. It has been applied to the identification of orfamides (**Figure 10**) as the products of a cryptic NRPS system identified by analysis of the complete genome sequence of *Pseudomonas fluorescens* Pf-5.[51,52] Bioinformatics analyses of the cryptic NRPS, including A domain substrate specificity predictions, led to the hypothesis that this system assembles a novel lipopeptide containing four leucine residues. This hypothesis was exploited by using ^{1}H–^{15}N heteronuclear multiple bond correlation experiment (HMBC) NMR spectroscopy to guide the purification of the lipopeptides from cultures of *P. fluorescens* fed with ^{15}N-labeled leucine.[52] A bioassay-guided fractionation process was used in parallel to identify the lipopeptides because they were predicted to possess antifungal activity by comparison of the predicted structures with closely related compounds. This approach can be extended to other biosynthetic systems where specific precursors are predicted to be incorporated. ^{1}H–^{13}C HMBC NMR spectroscopy could be used as an alternative to ^{1}H–^{15}N HMBC experiments in combination with feeding of ^{13}C-labeled precursors.[53]

2.12.5 Concluding Remarks

Genomics has revealed that a potentially vast and largely untapped reservoir of novel bioactive natural products may remain undiscovered in prokaryotes. Novel natural product discovery through traditional methods is a challenging, expensive, and time-consuming process. The application of genomics-based tools and technologies to new natural product discovery promises to greatly speed up and simplify this process. The

ECO-7942

Myxochromides

S^1 R = Me
S^2 R = Et
S^3 R = CH=CH-Me

Orfamide A

MMF1

MMF2

Bacillaene

MMF3

MMF4

MMF5

Figure 10 Other novel bacterial natural products discovered by genome mining.

current state of knowledge of the genetics and enzymology of natural product biosynthesis is such that, for several natural product classes, it is possible to make reasonable predictions of likely structural features of a yet-to-be-discovered natural product by analyzing the sequences of the genes that direct its biosynthesis. This benefits the natural product discovery process in many ways: it can give insight into the likelihood of structural

novelty; it can lead to the prediction of a distinctive physicochemical property, which may be exploited to simplify identification and isolation; it can lead to potential bioactivity predictions by structural comparisons with known bioactive natural products; and it can greatly aid the assignment of spectroscopic data and as a consequence structure elucidation. Nevertheless, current bioinformatics tools for predictive analysis of natural product biosynthetic gene clusters are far from perfect and continual development is needed that keeps pace with our rapidly developing knowledge of natural product biosynthesis. We are still a long way away from tools that can reliably predict the majority of structural features of metabolic products of cryptic biosynthetic systems. This is true even for the most amenable systems such as NRPSs and modular PKSs, where predicting the site and nature of post-PKS/NRPS modifications catalyzed by tailoring enzymes, and inferring whether nonlinear enzymatic logic may be in operation, remain challenging. Other types of biosynthetic system, such as terpene synthases and iterative PKSs, are less amenable and the development of predictive bioinformatics tools for these is currently difficult. Despite these challenges, the proofs-of-principle that exciting new natural products can be discovered in prokaryotes by the exploitation of genomics already appears to be rekindling interest in the inclusion of novel natural product discovery as a rational part of the new drug and agrochemical discovery process.

Abbreviations

A	adenylation
ACP	acyl carrier protein
AT	acyl transferase
C	condensation
DH	dehydratase
E	epimerization
ER	enoyl reductase
KR	ketoreductase
KS	ketosynthase
MT	methyltransferase
NRPS	nonribosomal peptide synthetase
PCP	peptidyl carrier protein
PKS	polyketide synthase
S.	*Streptomyces*
Sal.	*Salinispora*

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Biographical Sketches

Dr. Christophe Corre was born in Nimes, France, in 1974. He received his education in Biochemistry/Molecular Biology at the University of Nice Sophia Antipolis, France. He then carried out work on antibiotic biosynthesis in *Streptomyces* for which he was awarded a Ph.D. from the School of Chemistry at the University of Exeter, UK, in 2004. He is currently a postdoctoral research fellow with Professor G. L. Challis in the Department of Chemistry at the University of Warwick, UK. During his postdoctoral studies, he has worked on elucidating antibiotic biosynthetic pathways in *Streptomyces coelicolor* and the discovery of novel bioactive natural products by genome mining.

Professor Gregory L. Challis graduated from Imperial College London with a first class in B.Sc. honors degree in Chemistry, following which he was awarded a D.Phil. by the University of Oxford in 1998 for research in organic chemistry under the supervision of Professor Sir Jack Baldwin FRS. He then spent 2 years as a Wellcome Trust International Prize Travelling Research Fellow in the Department of Chemistry at Johns Hopkins University in Baltimore, after which he returned to the United Kingdom to work in the Department of Genetics at The John Innes Centre in Norwich for one year on the same fellowship. In 2001 he was appointed as a lecturer in Chemical Biology in the Department of Chemistry at the University of Warwick. In 2003 he was promoted to senior lecturer and in 2006 he was promoted to his current position of professor of chemical biology. He has received several prizes in recognition of his research achievements including the 2002 Royal Society of Chemistry Meldola Medal and Prize, the 2007 Society for General Microbiology Fleming Prize Lecture, and the 2007 Wain Medal Lecture Award of the University of Kent. His research interests reside at the interface of chemistry and biology, specifically the discovery, biosynthesis, and biological function of bioactive microbial natural products.

2.13 Unlocking Environmental DNA Derived Gene Clusters Using a Metagenomics Approach

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2.13.1 Introduction

In the 1940s, Selman Waksman helped launch the golden age of antibiotic discovery with the characterization of numerous antibiotics produced by bacteria cultured from soil samples. More than half a century after the isolation of streptomycin from a cultured actinomycete the methods used to identify biologically active natural products from bacteria have remained largely unchanged. Although bioassay-guided fractionation of extracts derived from cultured bacteria has uncovered many of the most important pharmacophores known today, the continued screening of easily cultured bacteria for the production of novel biologically active small molecules has, in recent years, shown diminishing returns due in large part to the re-isolation of known compounds.[1] One promising new source of bioactive natural products is uncultured bacteria. A single gram of soil is predicted to contain more than 10 000 unique bacterial species, and by most estimates, less than 1% of these have been cultured in the laboratory.[2–5] Uncultured bacteria are likely the largest remaining pool of genetic diversity that has not been examined for the production of biologically active and pharmacologically useful small molecules.

For much of the last century light microscopy studies suggested that there was a large discrepancy between the number of bacteria present in an environmental sample and the number of colonies that could be cultured from the environment.[4,6,7] Molecular phylogenetic techniques, which use single-gene sequences instead of entire organisms grown in pure culture to assess phylogenetic diversity, later conclusively demonstrated that pure culture methods only provided access to a small fraction of the microbes present in the environment.[2,8–12] Microbial diversity analyses based on 16S rRNA genes isolated from environmental samples now suggest that more than 80 major bacterial divisions exist and that fewer than half of these divisions contain isolates that have been cultured in the laboratory.[13–17]

To circumvent the challenges of culturing environmental bacteria, a culture-independent discovery strategy has been developed, which relies on the extraction of DNA from environmental samples (environmental DNA (eDNA)) and the cloning of this DNA into easily cultured model bacterial hosts (**Figure 1**). The culture-independent analysis of natural microbial communities is now known as metagenomics. The term metagenome

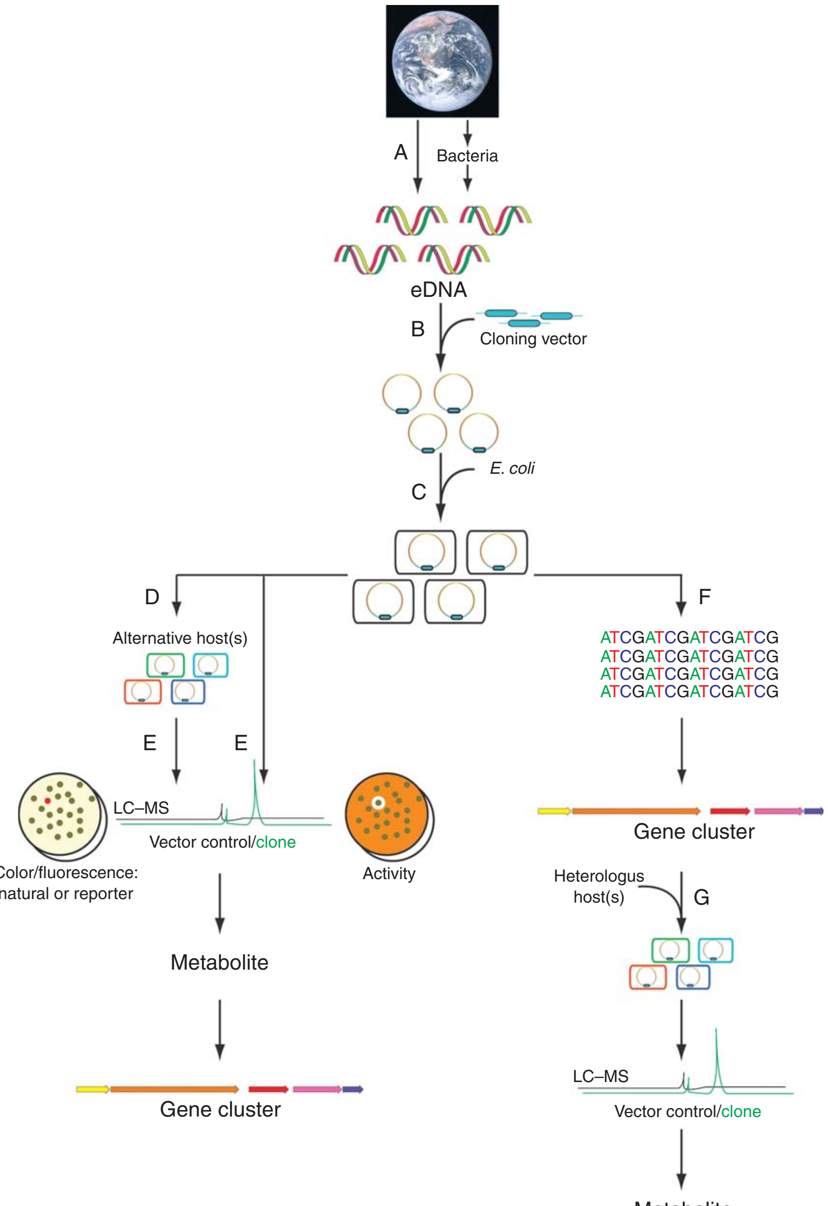

Figure 1 Metagenomic library creation begins with the extraction of eDNA from an environmental sample. (A) This involves either direct DNA isolation or whole-cell isolation followed by DNA extraction. After purification, the DNA is either digested or end repaired, (B) ligated into a vector (BAC, cosmid, or fosmid), and (C) introduced into *Escherichia coli*. At this point the library can either be screened in *E. coli* or (D) shuttled into another bacterial host. Both functional and DNA-based screens are used to examine eDNA libraries. (E) Functional screening strategies rely on activity-based, colorimetric, chromatographic, or reporter gene readouts to detect the production of secondary metabolites. (F) Sequence-based screens generally use degenerate primers to identify clones that contain conserved natural product biosynthetic gene sequences. (G) Gene clusters isolated with DNA-based screening methods are then tested for the ability to confer the production of novel metabolites in a heterologous host.

was originally coined in 1998 to describe the collection of microbial genomes present in an environmental sample.[18] Functionally accessing the genomes of uncultured bacteria using a metagenomics approach has been of particular interest to the natural products community because all of the genes required for the biosynthesis of a bacterial natural product, including genes that code for biosynthetic enzymes, regulatory enzymes, and resistance enzymes, are often found clustered on bacterial chromosomes. Large-insert eDNA clones therefore have the potential to contain functionally intact natural product biosynthetic gene clusters that can confer the production of new metabolites to a heterologous host. This chapter includes an overview of the current metagenomic methods used for natural product discovery and a discussion of molecules that have been characterized using this approach.

2.13.2 Methodologies

2.13.2.1 Bacterial Diversity and Sample Selection

A number of new culturing strategies including consortia-culturing, single-cell gel microdroplet encapsulation, environmental nutrient diffusion, and very low nutrient growth have been used to cultivate previously uncultured bacteria from environmental samples.[8,9,12,19–21] While these methods represent important advances, they do not provide a general solution for culturing a significant portion of the bacteria present in natural microbial populations. The vast majority of environmental bacteria remain inaccessible using culture-based methods and for most of these microbes, little is known about them beyond their 16S rRNA gene sequences. Although 16S rRNA-based phylogenies provide a simple means of cataloging the diversity of bacteria in environmental samples, these phylogenies do not provide insights into the biosynthetic 'capacity' of an environmental sample. High-throughput sequencing has begun to provide a more detailed biochemical picture of these microbial communities. Large-scale sequencing of both terrestrial and marine samples suggests that the environment contains a huge reservoir of previously uncharacterized biosynthetic potential.[22–26] Almost 70 000 novel genes were found in the large-scale sequencing of DNA obtained from microbes found in the Sargasso Sea alone, and more the 1700 new protein families were identified by The Global Ocean Sampling Expedition.[25,26] Owing to the complexity of microbial communities, only a handful of complete or near-complete bacterial genomes have been successfully reconstructed from environmental sequencing efforts.[22–24] Complete sequencing of even the most dominant species in a soil sample is predicted to require 2–5 billion base pairs of sequencing data.[11] Until sequencing on this scale becomes more practical, studying large *cis*-linked gene clusters from uncultured bacteria will likely require the use of metagenomic cloning techniques.

Most environments, including marine, freshwater, soil, and the human gut, have been shown to contain diverse microbiomes that could serve as rich sources of metagenomic DNA. However, with only a rudimentary picture of the biosynthetic diversity present in most environmental samples, it is difficult to prioritize environmental samples for metagenomic analysis. Soils are predicted to contain the largest collection of uncultured microbes. In fact, the bacterial diversity present in as little as 1 ton of soil has been predicted to exceed the total diversity present in the sea.[27] Owing to this extraordinary bacterial diversity, soil has been a particularly attractive starting point for metagenomic studies designed to identify natural products. A comparison of microbial diversity in different soils, using a combination of fluorescence microscopy and 16S rRNA diversity analysis, found that the number of detectable species can vary by more than three orders of magnitude among different terrestrial sources.[4,28] High-throughput sequencing also indicates that there are often significant phylogenetic differences among what appear to be similar environmental samples.[23] Small changes in temperature, water content, particle size, soil type, heavy metal contamination, and pH have all been shown to affect the microbial diversity present in a soil sample.[29–33] Microarray technology has been used to assess the diversity of both individual genes and organisms in environmental samples and may be useful for rapidly profiling environmental samples for natural product biosynthetic genes.[34–36] Future small-molecule discovery efforts will clearly benefit from comparative metagenomic analyses that shed light on which environments and environmental characteristics correlate well with a rich diversity of novel secondary metabolite gene clusters.

2.13.2.2 eDNA Isolation Strategies

Two distinct approaches, direct DNA extraction and whole-cell isolation followed by DNA extraction, have been used to extract DNA from environmental samples. Numerous direct DNA isolation protocols have appeared in the literature.[37–40] In almost all cases the methods are very similar: bacteria present in an environmental sample are lysed *in situ* (using a mixture of heat, detergents, enzymes, organic solvents, and physical manipulation), DNA is collected by alcohol precipitation from a centrifuge-clarified crude lysate, and 'pure' eDNA is obtained from the crude precipitate by either gel or affinity matrix purification (silica, Sephadex). Attempts to optimize these methods for soil DNA extraction have revealed that chelating agents such as cetyltrimethylamminobromide and ammonium acetate, which help remove contaminating humic acids, increase the likelihood of obtaining DNA that can be enzymatically manipulated in downstream experiments and that the inhibition of contaminating nucleases with formamide or calcium carbonate increases the size of the recovered DNA.[33,41,42] Direct DNA isolation strategies routinely yield 30–50 kb eDNA that is well suited for both cosmid- and fosmid-based cloning strategies. While these methods generally yield microgram quantities of eDNA ($\sim$50 $\mu g\, g^{-1}$ soil), this DNA is often too short to be useful for constructing very-large-insert metagenomic libraries.[41] Prefractionation of bacteria from environmental samples prior to cell lysis has been used to obtain higher-molecular-weight DNA from an environmental sample; however, because bacterial enrichment strategies are inefficient, this approach often yields 10–100-fold less DNA than direct DNA isolation methods.[43,44] In whole-cell isolation experiments, cells are initially separated from environmental contaminants by differential centrifugation, filtration, or high-speed differential density centrifugation through a Nycodenz polymer. The bacteria are then embedded in an agarose plug prior to cell lysis. eDNA fragments in excess of 1 Mb in size have been obtained using this approach; however, low yields have made the construction of large libraries challenging.[33]

One promising approach that may address the difficulty of isolating large quantities of high-molecular-weight eDNA is multiple displacement amplification (MDA) with phi29 polymerase. phi29 is capable of efficiently amplifying up to 70 kb fragments of DNA and can be used to produce milligram quantities of amplified DNA from a single DNA fragment.[45–50] While these methods still need optimization to reduce chimeric artifacts, MDA-generated DNA from low-abundance organisms has been used to successfully construct metagenomic libraries.[49,51]

2.13.2.3 Cloning Strategies

Natural product biosynthetic gene clusters can range from a few kilobases to more than a hundred kilobases in length. Most efforts to clone gene clusters for natural product discovery have therefore used cosmid, fosmid, or bacterial artificial chromosome (BAC) vectors that are capable of stably replicating large fragments of cloned DNA. Cosmid libraries with more than 100 000 members are routinely constructed with DNA isolated using direct isolation methods. Cosmid- and fosmid-based cloning systems, which rely on a lambda phage packaging step, can accept 35–40 kb DNA inserts. Although some complete biosynthetic gene clusters can be captured on cosmid-sized clones, many are too large to be captured on a single cosmid. BACs, however, do not have an insert size limit and therefore should be useful for constructing larger-insert libraries. Unfortunately, because the isolation of very high-molecular-weight DNA from environmental samples is still not routine, the average insert size for most large metagenomic BAC libraries has rarely exceeded 50 kb.[33]

2.13.2.4 Functional Screening and Host Selection

Both functional and sequence-based screens have been used to identify metagenomic clones that produce novel small molecules. Functional screening requires the successful expression of a biosynthetic gene cluster in the specific host bacterium under the precise culture conditions used for a particular assay. In addition, the recombinant clone must generate sufficient quantities of a metabolite to be detected in the chosen assay. Owing to the low frequency at which this is likely to happen, functional screening of metagenomic libraries for small-molecule production has primarily relied on simple colony-based assays that can be easily run on a large number of clones. Antimicrobial activity, color production, liquid chromatography–mass spectrometry

(LC−MS) analysis of culture broth extracts, and reporter gene activation have all been successfully used as readouts in high-throughput assays designed to find small-molecule-producing clones (see Section 2.13.4). The most frequently used functional assays have been color production and antibacterial activity. While any assay strain can be selected for an overlay assay, *Bacillus subtilis* is commonly used due to its sensitivity to most known classes of antibiotics. Using simple functional assays, clones that produce new natural products have been recovered from both *Escherichia coli*- and *Streptomyces lividans*-based eDNA libraries (see Section 2.13.4).

In comparison to screening extracts from cultured bacteria, hit rates for functional screens of metagenomic libraries are low. Hit rates in antibacterial and colored screens rarely exceed 0.01% (see Section 2.13.4). Heterologous expression barriers likely prevent the functional expression of most genes captured in a metagenomic library. The remarkable phylogenetic diversity present in environmental samples makes selecting a host for phenotypic screening difficult. *E. coli* remains the most efficient system to use for the creation of large genomic DNA libraries and therefore it has been the preferred host to use for the construction and screening of metagenomic libraries. Although libraries initially constructed in *E. coli* have now been shuttled into a number of different hosts including *S. lividans, Rhizobium leguminosarum*, and *Pseudomonas putida*,[52–54] metagenomic studies designed to access new small molecules from the heterologous expression of eDNA have largely focused on the use of just two model systems, *E. coli* and *S. lividans*, as heterologous hosts. A meta-analysis of soil metagenomic libraries revealed that the majority of bacteria present in environmental samples reside in five major divisions of bacteria: α-, β-, γ-proteobacteria, acidobacteria, and actinobacteria.[55] Developing genetically tractable heterologous hosts from each of these bacterial divisions should expand the repertoire of natural products that can be accessed using functional screens.

2.13.2.5 Sequence-Based Screening

Sequence-based screening is initially expression independent and therefore has the potential of providing access to a more diverse collection of gene clusters than functional screening. In this approach, degenerate primers based on conserved regions in natural product biosynthetic genes are used to PCR-amplify novel homologues from cloned eDNA. Probes based on these amplicons are then used to recover large-insert clones from a metagenomic library. In contrast to phenotypic screens where functionally intact gene clusters must be captured on individual clones, sequence-based screening can be used to recover multiple overlapping clones that contain portions of gene clusters too large to be captured on a single clone. The development of Red/ET recombination should greatly facilitate the reconstruction of large gene clusters captured on different, multiple metagenomic clones.[56–58] One of the major challenges of DNA-based screening is identifying heterologous expression systems in which recovered gene clusters can be functionally expressed. In metagenomic studies, this challenge is compounded by the fact that neither the molecule produced by the gene cluster nor the source organism is generally known. Both polyketide and nonribosomal peptide biosynthetic gene clusters have been heterologously expressed in *E. coli* using strains engineered to produce holo thiolation domains and nonnative polyketide synthase (PKS) substrates.[58] Even with these advances, *E. coli* is not an ideal host for the heterologous expression of most gene clusters derived from phylogenetically unrelated bacteria. Promoter activation, ribosome binding site recognition, and differences in primary metabolite diversity are just some of the factors that can impede heterologous expression. As with phenotypic screening, the development of a large number of phylogenetically diverse model bacterial systems that can be used for heterologous expression studies should significantly increase the number and diversity of new metabolites discovered from eDNA-derived gene clusters.

Some estimates, which take into account rare members of soil communities, indicate that more than 10^{11} BAC clones with an average insert size of 100 kb would be required to capture the diversity present in just 1 g of soil.[18,59] The largest metagenomic libraries constructed to date capture only a fraction of the genetic diversity present in most environmental samples. Despite this size discrepancy, large gene clusters from many of the dominant uncultured species present in soil samples can likely be recovered and reconstructed using DNA-based screening strategies. Owing to the immense microbial diversity found in environmental samples, the target genes in a metagenomic library represent only a small fraction of the DNA captured in the library. Preenrichment methods provide an attractive means of increasing the frequency of a desired sequence in a library, thereby reducing the size of the metagenomic library that must be screened. A number of methods have

been successfully used for gene enrichment in genomic DNA libraries. These include stable isotope labeling, subtractive hybridization, fluorescence *in situ* hybridization combined with cell sorting, affinity capturing, and phage display.[60–64] Phage display has been used to specifically preenrich genomic DNA libraries for natural product biosynthetic gene clusters.[64] In this study, the authors designed a system to specifically biotinylate thiolation domains in order to affinity-capture thiolation domain fusion proteins displayed on the surface of a phage. The authors demonstrated the utility of this enrichment scheme using genomic DNA from two model, cultured bacteria. Other enrichment and screening strategies could be adapted to metagenomic studies including biotinylated nucleotide hybridization and magnetic capture, gene targeting with selectable markers using homologous recombination, and any combination of fluorogenic gene-specific reporters in conjunction with fluorescence activated cell sorting (FACS) purification.[23,63,65–74] These represent only a few examples of techniques that may prove useful for screening and enriching metagenomic libraries in the future.

2.13.3 Type II PKS KS$_\beta$ from Soil Multigenomic DNA

The first attempt to functionally explore natural product biosynthesis in uncultured bacteria involved the PCR amplification and heterologous expression of soil-derived type II PKS genes.[75] Type II PKS gene clusters are responsible for the biosynthesis of a large number of structurally diverse aromatic polyketides and are found in phylogenetically diverse bacteria. Minimal type II PKS systems are composed of three proteins, two β-ketoacyl synthases (KS$_\alpha$ and KS$_\beta$) that catalyze sequential Claisen condensation reactions and control the polyketide chain length and one acyl carrier protein (ACP) that provides a covalent anchor for the nascent polyketide during the elongation process.[76] In many actinomycete-derived type II PKS gene clusters, the minimal PKS has a conserved organization, with the KS$_\alpha$ and ACP genes located on either side of the KS$_\beta$ gene (KS$_\alpha$-KS$_\beta$-ACP). Armed with the knowledge that environmental samples contain large numbers of uncultured bacteria and that many type II PKS gene clusters are organized in this conserved manner, Davies and colleagues formulated a strategy to access type II polyketide biosynthetic machinery from environmental samples using PCR-based methods. In this study, degenerate PCR primers designed to recognize a conserved C-terminal region in KS$_\alpha$ sequences and a conserved active site sequence in the downstream ACP gene (**Figure 2**) were used to amplify full-length KS$_\beta$ sequences from eDNA.

Using the KS$_\alpha$- and ACP-specific primer sets, PCR amplicons were obtained from both control *Streptomyces* genomic DNA and DNA isolated directly from soil. Two unique sequences that showed similarity to known KS$_\beta$ genes were cloned from the eDNA-derived amplicon. These putative KS$_\beta$ genes displayed high G + C content (~70%), which is typical for coding regions in cultured actinomycetes. To understand better the function of these genes, hybrid PKS expression cassettes in which the KS$_\beta$ gene from an existing minimal PKS was replaced with one of the two new eDNA-derived sequences were constructed. Different combinations of actinorhodin and tetracenomycin ACP and KS$_\beta$ genes were used in the hybrid constructs. These hybrid constructs were introduced into *S. lividans* or *S. glaucescens* WMH1077 for functional expression studies. Isolation and characterization of the resultant metabolites yielded several octa- and decaketide PKS products (**1**–**4**) (**Figure 2**). While compounds **1**–**4** are molecules previously described in the literature,[77] this study also identified two compounds that appeared novel; however, their molecular structures were not determined due to the small quantities recovered. Based on chromatographic and spectroscopic analyses, one of the novel metabolites appeared to be related to SEK4 (**1**) and SEK4b (**2**). The other uncharacterized metabolite did not appear to resemble any of the observed metabolites and was presumed by the authors to be novel. Even though no new metabolites were structurally characterized in this study, it does represent the first example of the heterologous expression of novel biosynthetic genes from metagenomic DNA to yield small molecules.

A large number of both PCR and functional studies have now been carried out to identify new enzymes from eDNA. A variety of industrially relevant enzymes and biocatalysts have been recovered from metagenomic libraries. These include various zenobiotic degradation enzymes such as esterases (lipases), alcohol dehydrogenases, amidases, and β-lactamases.[78–80] A number of studies exploring the diversity of natural product-associated enzymes have also appeared in the literature, including studies on type I PKS genes, nonribosomal peptide synthetase genes, and aminoglycosides and antibiotic resistance genes.[81–84] PCR-based discovery strategies are generally limited to the discovery of single genes or even parts of genes, which in and of

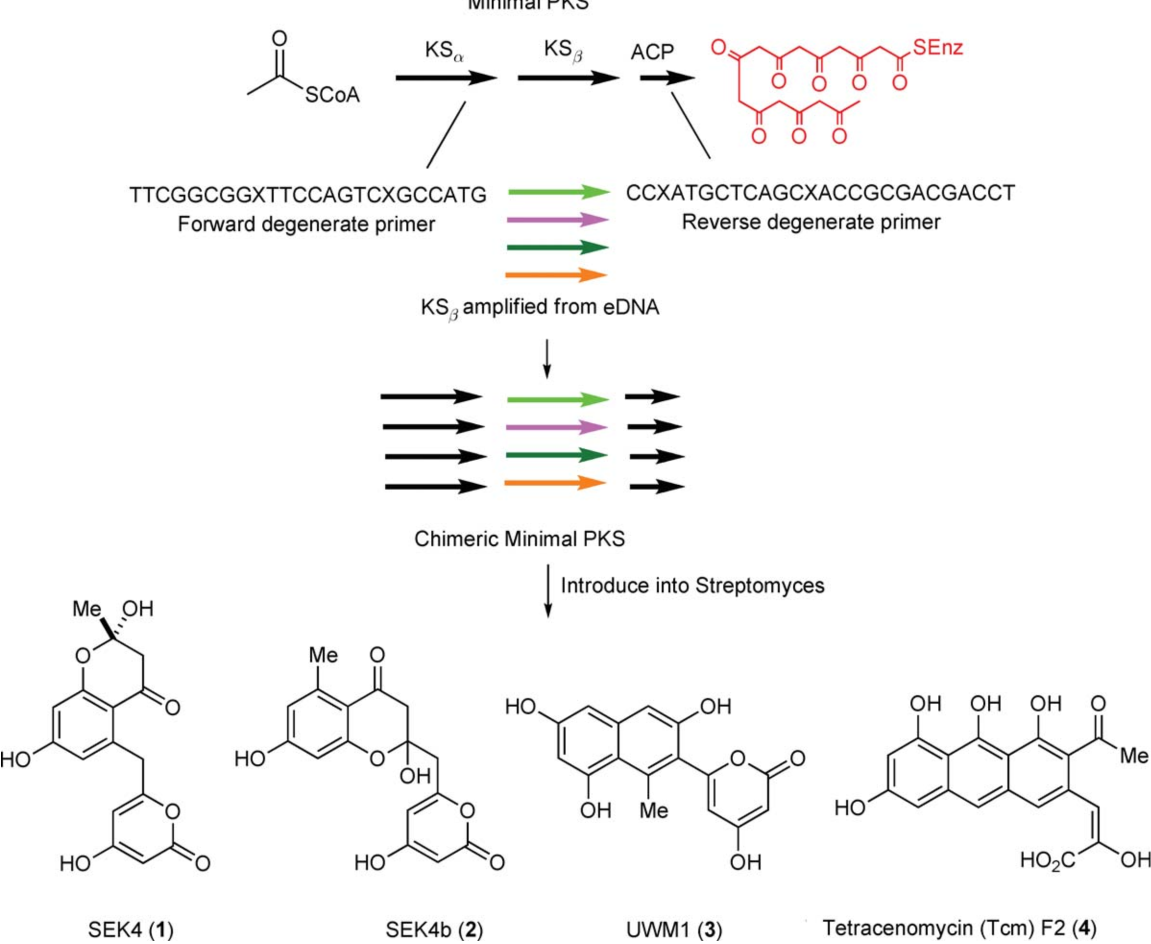

Figure 2 In the first attempt to functionally explore natural product biosynthesis in uncultured bacteria PCR amplified KS_β sequences were cloned into hybrid minimal PKS systems, introduced into *Streptomyces*, and four metabolites (**1–4**) were subsequently characterized in heterologous expression studies.[75]

themselves are not sufficient to generate novel metabolites. As Davies and colleagues noted in their early KS_β study, this approach could be extended to 'final products of such biosynthetic pathways by use of cloned genes as specific probes to clone the rest of the gene cluster from cosmid or BAC libraries of the multigenomic DNA sample'.[75] Metagenomic approaches used in subsequent attempts to access the chemical diversity of uncultured bacteria would, indeed, employ this large-insert cosmid and BAC cloning strategy.

2.13.4 Molecules and Their Biosynthetic Genes Isolated from Metagenomic Libraries

2.13.4.1 Early Libraries and Functional Screening

In a study published in 2000, a collaboration between academic and industrial scientists took the functional screening of DNA extracted from soil a step further with the construction of ~30 000 BAC clones containing eDNA from soil collected in Wisconsin.[85] Plasmid-based metagenomic libraries had been constructed prior to this study;[86] however, these libraries contained small inserts (average 5–8 kb) that would preclude the cloning of all but a few small natural product gene clusters. The Wisconsin soil BAC libraries reported in this study had average inserts between 27–44.5 kb, representing the first example of an eDNA library with inserts large enough

to capture complex natural product biosynthetic gene clusters. While no small-molecule-producing clones were reported in this study, the suite of assays used to functionally survey these libraries did uncover clones with antibacterial, lipase, amylase, nuclease, and hemolytic activities. The identification of clones with an array of activities represented a major advance in the development of practical techniques for the construction and screening of functional metagenomic libraries.

2.13.4.2 Terragines

The first example of the functional, heterologous expression of a secondary metabolite from an environmental clone appeared in March 2000.[54] A collaboration between the Anderson and Davies groups at the University of British Columbia and TerraGen Discovery Inc. resulted in the discovery of five structurally novel compounds, the terragines A–E (**5–9**) in addition to the known microbial metabolite norcardamine (**10**) (Figure 3). Using DNA isolation methods developed earlier by Davies and coworkers,[87] genomic DNA from soil collected in British Columbia was used to construct a cosmid library in *E. coli.* The cosmid clones used in this study were constructed in *E. coli–Streptomyces* shuttle vectors to permit the facile transfer of clones from *E. coli* into *S. lividans*. The initial chemical analysis and screening of the *S. lividans* clones were done by high performance liquid chromatography electrospray ionization mass spectrometry (HPLC–ESIMS) and two recombinants found to produce clone-specific metabolites were selected for further investigation. Following a 10-day fermentation period, cultures of these two clones were subjected to organic extraction and liquid chromatographic separation. Extensive spectroscopic structure elucidation studies resulted in the identification of

Terragine A (**5**), R =

Terragine B (**6**), R =

Terragine D (**8**), R =

Terragine C (**7**), R =

Terragine E (**9**), R = H
Norcardamine (**10**), R = OH

Figure 3 Molecular structures of terragines A–E (**5–9**) and norcardamine (**10**) are shown. Genomic DNA from soil collected in British Columbia was used to construct a cosmid library in *Escherichia coli* that was subsequently transferred to *Streptomyces lividans*. Eighteen of the ∼1000 recombinant *S. lividans* clones examined in this study were found to produce either terragines or norcardamine.[14]

terragines A–E (**5–9**) (**Figure 3**) as well as the reisolation of the known microbial siderophore norcardamine (**10**). Terragines A–D were isolated from cultures of recombinant 436-s4-5b1 while terragine E was isolated from cultures of the second recombinant, 446-s3-102g1. Recombinants that produced members of this family of metabolites were subsequently found at a high frequency in the metagenomic library. Eighteen of the ~1000 unique *S. lividans* recombinants examined were found to produce members of the terragine/norcardamine families. The DNA inserts in these clones were not sequenced; therefore, it is not known if the terragines are produced by modifications to host pathways or by entirely new biosynthetic systems.

Despite the absence of sequencing data, the authors were able to infer a possible biosynthetic scheme that links the production of terragines A–D (**5–8**) with the production of terragine E (**9**) and norcardamine (**10**). All six compounds contain diaminopentane and succinyl subunits. Terragines A–D (**5–8**) also contain a phenylacetamide subunit and terragines A, B (**5**, **6**) contain terminal succinimide functional group. Neither norcardamine (**10**) nor terragine E (**9**) contains these latter substructures. The biosynthesis of norcardamine (**10**), and presumably all of the terragines, begins with the decarboxylation of L-lysine to form 1,5-diaminopentane. The proposed biosynthetic scheme for terragines A–D (**5–8**) then diverges through the *N*-phenylacetylation and *N*-hydroxylation of a one terminal amine of the 1,5-diaminopentane precursor. Acetylation of the second amino group would yield terragine C (**7**), while succinylation of the second amino group followed by the cyclization of this succinyl group would yield terragine B (**6**). Terragine A (**5**) can be generated by succinylation of the second amino group on the original 1,5-diaminopentane starter unit followed by coupling of this first succinyl group to a second succinyl group that undergoes cyclization. Alternatively, terragine D (**8**) can be generated if succinylation of the second amino group on the original 1,5-diaminopentane starter unit is followed by the coupling of this succinyl group to a second 1,5-diaminopentane subunit, *N*-hydroxylation of the new amide nitrogen, and acetylation of the terminal amino group. The biosynthesis of norcardamine (**10**) and terragine E (**9**) could arise from the alternate coupling of three 1,5-diaminopentanes and three succinoyl residues, followed by *N*-oxidation and cyclization. The authors suggested that uncharacterized enzymes encoded by the eDNA insert from the clone responsible for producing terragines A–D (**5–8**) catalyzed the novel phenylacetylation and succinimide modifications found in these molecules. When examined for biological activity, none of the terragines displayed any significant antibacterial activity against either *E. coli* or *B. subtilis*.

2.13.4.3 *N*-Acyl Amino Acids

The first paper to detail the discovery of novel, bioactive small molecules from eDNA describes the heterologous production and structure elucidation of a family of long-chain *N*-acyl amino acids with antibacterial activity.[88] In this work, a library of ~700 000 recombinant cosmid clones was generated in *E. coli* and of these, 65 were found to exhibit antibacterial properties. The production of antibiotic activity by members of this library was noted by the appearance of zones of growth inhibition around recombinant *E. coli* in a top-agar overlay assay using *B. subtilis* as the assay strain. With this simple screen, thousands of colonies at a time could be assessed for antibacterial activity, and the active ones could be easily recovered from the screen for further characterization. A single clone that produced an antibacterially active organic extract was selected for large-scale fermentation, organic extraction, and sequence interrogation. Ethyl acetate extraction followed by reversed phase high performance liquid chromatography (RP-HPLC) purification yielded 13 novel long-chain *N*-acyl-L-tyrosines (**11**) that contained both saturated and monounsaturated acyl side chains (**Figure 4**). Derivatives containing saturated and monounsaturated C_{13}–C_{16} side chains were the most potent antibiotics. A marked reduction in activity was seen for compounds with either longer or shorter acyl side chains.

The heterologous expression approach to the discovery of natural products automatically couples a natural product to its cloned biosynthetic genes. In most cases, it is therefore possible to rapidly identify the biosynthetic machinery responsible for an observed phenotype using a combination of subcloning, transposon mutagenesis, and small-scale sequencing experiments. In this study, a single open reading frame (ORF) was found to be necessary and sufficient for the biosynthesis of these metabolites (**Figure 4**). Neither the long-chain *N*-acylated antibiotics nor their biosynthetic enzyme (*N*-acyl amino synthase (NAS)) characterized in this study had been previously reported from cultured bacteria. Since the initial characterization of long-chain *N*-acyltyrosine antibiotics and the first eDNA-derived NAS, a number of additional antibacterially active eDNA clones that produce similar structures have been identified.[89–91] These include clones that produce

Gene	Predicted function
feeA	lipase
feeB	esterase
feeC	membrane fusion protein
feeD	ABC transporter
feeE	ABC transporter
feeF	fatty acid desaturase
feeG	decarboxylase
feeH	*N,O*-acyltransferase
feeI	putative fatty acid activation
feeJ	acyl acyl carrier protein (AACP)
feeK	phosphopantethienyl transferase (PPT)
feeL	acyl carrier protein (ACP)
feeM	*N*-acyl amino acid synthase (NAS)

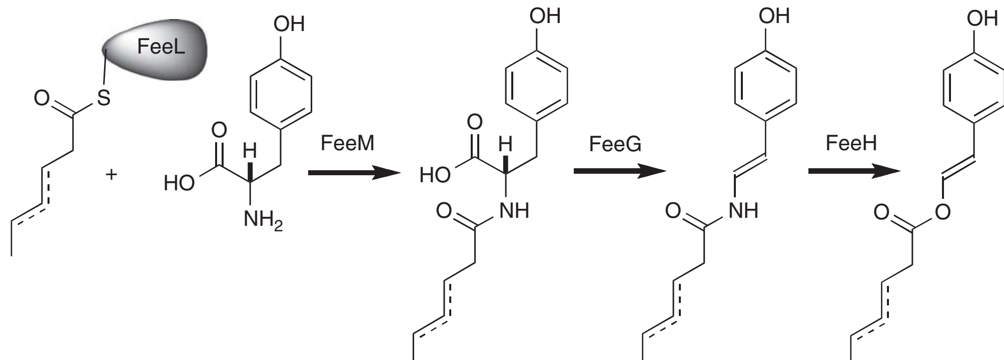

Figure 4 Molecular structures of long-chain *N*-acyl amino acid antibiotics and their derivatives that have been characterized from antibacterially active eDNA clones are shown. In each case, a family of metabolites with saturated and monounsaturated side chains of different lengths is produced by the antibacterially active recombinant *Escherichia coli*. The eDNA-derived *fee* gene cluster encodes the biosynthesis of long-chain *N*-acyltyrosine (**11**), long-chain *N*-acyl eneamide (**16**), and long-chain *N*-acyl enol ester (**17**)-based metabolites (GenBank accession No AY128669). The proposed biosynthetic scheme for this collection of metabolites was inferred from bioinformatics and transposon mutagenesis studies.[88]

long-chain *N*-acylphenylalanines (**12**), long-chain *N*-acyltryptophans (**13**), long-chain *N*-acylarginines (**14**), and long-chain *N*-acylputrescines (**15**).[89–92] In each case, a single ORF was reported to be responsible for the biosynthesis of these metabolites (GenBank accession Nos AY214919, AY214920, DQ224236, AY632377).

A detailed analysis of the organic extracts obtained from a second long-chain *N*-acyltyrosine-producing clone indicated the presence of two additional families of compounds, long-chain *N*-acyl eneamides (**16**) and long-chain *N*-acyl enol esters (**17**).[55] Sequencing of the eDNA captured in this clone indicated that the NAS responsible for the formation of the long-chain *N*-acyltyrosine antibiotics was part of a 13 ORF biosynthetic gene cluster (FeeA–M) that encodes the biosynthesis of all three families of natural products (**Figure 4**). The biosynthetic scheme for these compounds was inferred from transposon mutagenesis studies and bioinformatics. In the proposed biosynthetic scheme, an ACP (either the ACP FeeL from the pathway or the native *E. coli* ACP) is charged with a fatty acid that is then transferred to tyrosine by the NAS (FeeM) to produce long-chain *N*-acyltyrosine intermediates.[93] The *N*-acyltyrosines are then oxidatively decarboxylated by FeeG, and the resulting long-chain eneamides then undergo an *N*–*O* acyl transfer carried out by FeeH to give the corresponding enol esters. The role of these *N*-acylated metabolites is not known. However, based on their structural relationship to many known signaling molecules (acylhomoserine lactones in bacteria and long *N*-acylethanolamines in animals), it was speculated in one study that these metabolites might function as signaling molecules in the native producers.[89–91]

Although the DNA cloned directly from the environment cannot be easily attributed to the original organism from which it was derived, the sequencing of large eDNA inserts can provide some insight into the origin of this DNA. A full-length 16S rRNA gene sequence found on the same eDNA insert as the NAS that confers the production of long-chain *N*-acylphenylalanines to *E. coli* indicates that the eDNA captured in this clone is derived from an uncultured group of proteobacteria.[92] This 16S rRNA gene is most closely related to other 16S rRNA gene sequences characterized directly from eDNA. The closest 16S rRNA gene sequences from cultured bacteria are from ammonia-oxidizing β-proteobacteria *Nitrosospira* spp. and *Nitrosomonas* spp. Although the exact organism from which the eDNA fragment was obtained is not known, the 16S rRNA gene sequence found in this clone indicates that it very likely originated from a previously uncultured microbe.

2.13.4.4 Violacein

The presence of color in microbial cultures is often an indication of small-molecule biosynthesis. Color production can therefore be used as a very simple screen for identifying eDNA clones that might contain natural product biosynthetic gene clusters. In one example, transposon mutagenesis studies and sequencing of the cosmid isolated from a faint blue clone found in a library constructed from New York soil DNA indicated that four genes (*vioA–D*) were necessary for the production of the blue color (**Figure 5**).[94] Both subcloning of this gene cluster and transposon insertions upstream of *vioA*, the first gene in the cluster, led to a significant increase in color production, which permitted the characterization of the colored metabolites produced by this clone. The two colored metabolites isolated from *E. coli* transformed with these overproducing constructs were found to be spectroscopically identical to the known amino acid dimers violacein (**18**) and deoxyviolacein (**19**). The organization of the eDNA-derived violacein biosynthetic gene cluster is identical to that of the violacein biosynthetic gene cluster that had been previously sequenced from the cultured bacterium *Chromobacterium violaceum*.[95] While the gene organization of the two clusters is identical, the individual violacein biosynthetic enzymes (VioA–D) from the two pathways only show 48, 62, 71, and 69% amino acid identity, respectively. Although *E. coli* is not known for producing large numbers of complex secondary metabolites, this study demonstrated that *E. coli*-based metagenomic libraries could be used to access complex natural products from uncultured microorganisms.

2.13.4.5 Turbomycins

Turbomycin A (**20**) and B (**21**) offer another example where the characterization of a colored clone led to the identification of heterologously produced small molecules and their biosynthetic enzymes (**Figure 6**).[96] Turbomycin A and B are triaryl cation antibiotics that were isolated from the cell-free culture broth of a brown clone found in a soil DNA BAC library.[85,96] The production of a dark brown color by bacterial cultures is often suggestive of the

Violacein (**18**) Deoxyviolacein (**19**)

vioA vioB vioC vioD

7.0 kb

48% 62% 71% 69%

% Identity to *C. violaceum* gene cluster

Figure 5 Violacein (**18**) and deoxyviolacein (**19**) are produced by a blue clone that was found in a soil DNA library hosted in *Escherichia coli*. A four-gene cluster (*vioA–D*) captured on this clone is responsible for the biosynthesis of both metabolites (GenBank accession No AF367409). The eDNA-derived biosynthetic gene cluster and the violacein biosynthetic gene cluster sequenced from the cultured bacterium *Chromobacterium violaceum* have the same gene organization but show low amino acid sequence identity.[94]

Turbomycin A (**20**) Turbomycin B (**21**)

Figure 6 Molecular structures of turbomycin A (**20**) and B (**21**). These two triaryl cation antibiotics were isolated from the cell-free culture broth of a brown clone found in an *Escherichia coli*-based BAC library.[96]

production of melanin or a melanin-type polymer, which can be collected from spent culture broth by acid precipitation. During the characterization of the melanin-like material produced by this clone, two colored metabolites consistently appeared in the acid precipitate at elevated levels compared to the acid precipitates from identically treated vector control cultures. Extensive spectroscopic, synthetic, and crystallographic efforts were used to confirm the structures of these two colored metabolites. While turbomycin A had been previously characterized as a fungal metabolite, turbomycin B had not previously been described as a natural product.[96]

A single ORF that shares extensive sequence similarity with members of the 4-hydroxyphenylpyruvate dioxygenase (4HPPD) family of enzymes was found to be necessary and sufficient to confer the production of both the melanin-like material and the triaryl cations to *E. coli* (GenBank accession No AF511570). In some bacteria, 4HPPD-related enzymes catalyze the production of homogentisic acid (HGA), which, under aerobic conditions has been shown to undergo spontaneous polymerization into HGA–melanin.[97] The proposed biosynthetic scheme for the turbomycins involves a chimeric pathway where indole biosynthesis from the host *E. coli* is combined with the HGA biosynthesis introduced by the eDNA insert (**Figure 6**). It was proposed that in this biosynthetic scheme, the majority of the HGA undergoes spontaneous oxidation and polymerizes to melanin, which then, by some unknown mechanism, enhances the formation of the turbomycins in cultures of the eDNA clone.[96]

2.13.4.6 Type I PKS Clones

In 2003, French and American scientists screened a 5000-member soil-derived eDNA cosmid library using both functional and DNA-based techniques.[30] For this study, recombinants were assayed for the production of antibiotic activity and the DNA isolated from these clones was screened with PCR primers targeting both 16S rRNA and type I PKS genes. Even with this relatively small library, 47 unique 16S sequences were identified, representing many new candidate species. Using two sets of primers designed to conserved regions of actinomycete type I PKS ketoacyl synthase (KS) domains, 11 unique sequences resembling known KSs were amplified from the library. Three of the KS sequences were found to arise from a single cosmid. This cosmid was completely sequenced to reveal six large ORFs (**Figure 7**). ORFs 3, 4, and 5 contained PKS modules, ORF 2 encoded a mixed nonribosomal peptide synthetase (NRPS)−PKS module, and ORFs 1 and 6, although incomplete, were predicted to encode additional NRPS and PKS modules. Despite being an incomplete gene cluster, the information derived from sequencing this clone suggested that clones identified in the PCR screen for KS domains were likely to contain complex natural product gene clusters that might, under the appropriate heterologous expression conditions, yield novel secondary metabolites. The library used in this study was constructed in an *E. coli*−*S. lividans* shuttle vector (pOS700I) making it possible to easily shuttle clones from *E. coli* into *S. lividans* for phenotypic screening. Each clone found to contain a KS sequence was introduced by conjugation into *S. lividans* TK24. In an initial screen for antibiotic activity, no antibacterially active S. *lividans* clones were detected; however, when extracts derived from cultures of the recombinant *S. lividans* were analyzed by HPLC for the presence of UV-active clone-specific peaks, two *S. lividans* clones were found to produce clone-specific metabolites. The novel aliphatic dienic alcohol isomers, **22** and **23**, were isolated and spectroscopically characterized from culture extracts of these two recombinant *S. lividans* clones (**Figure 7**).

2.13.4.7 Isocyanide-Functionalized Antibiotic

The novel isocyanide-functionalized antibiotic **24** is produced by an antibacterially active eDNA clone found in a cosmid library constructed from soil collected in Boston, Massachusetts.[98] Isocyanide functional groups appear in a number of metabolites produced by cultured bacteria; however, the biosynthesis of this functional group, in particular, identifying the source of the nitrogen and carbon atoms, had not yet been elucidated by studying cultured bacteria. Saturating transposon mutagenesis of an antibacterially active subclone of the original cosmid indicated that two predicted eDNA-derived ORFs, *isnA* and *isnB* (GenBank accession No DQ084328), were necessary for the production of compound **24** (**Figure 8**).

The cloning and heterologous expression of an isonitrile biosynthetic enzyme in *E. coli* made it possible to perform well-controlled feeding experiments that allowed the origin of both the nitrogen and carbon in the isonitrile functional group to be determined.[99] In a normal feeding study, labeled precursors are added to an unlabeled background to decipher the origin of individual atoms in a molecule; however, because of the abundance of possible isonitrile precursors, an 'inverse labeling' strategy was used in this study. With this approach ^{12}C precursors are added to a ^{13}C background, eliminating the need to synthesize any ^{13}C precursor. Extensive 'inverse' feeding studies, using *E. coli* strains with mutations in key primary metabolic steps were carried out to interrogate systemically the *E. coli* metabolome for the source of the isocyanide carbon. These feeding studies showed that the isocyanide carbon was derived from the C2 carbon of regio- and stereochemical five-carbon sugar isomers found in the pentose phosphate pathway. Additional feeding studies suggested tryptophan as the source of the nitrogen. *In vitro* reconstitution experiments using purified IsnA, IsnB, tryptophan, and ribulose-5-phosphate confirmed the source of both isocyanide atoms (**Figure 8**). The

x y OH x y HO

22 **23**

Figure 7 Molecular structures of two novel aliphatic dienic alcohol isomers ($x + y = 12$) isolated from *Streptomyces lividans* transformed with a soil DNA cosmid clone are shown. Each of the eDNA cosmid clones examined in this study was shown by DNA-based screening techniques to contain PKS I KS genes.[30]

Figure 8 Compound **24** is a C3-isocyanide functional indole antibiotic that was isolated from an antibacterially active eDNA clone containing soil DNA. The active clone was identified in a top-agar overlay screen using *Bacillus subtilis* as the test organism. The isonitrile in this metabolite is biosynthesized in a single enzymatic step by IsnA using tryptophan and ribulose-5-phosphate. IsnB oxidatively decarboxylates the isonitrile-functionalized tryptophan intermediate to produce **24**.[98,99]

characterization of this antibacterially active eDNA clone led to the identification of not only the novel isocyanide-functionalized antibiotic **24**, but also the first reported isonitrile synthase, IsnA.

In a subsequent DNA-based screening study, additional *isnA*-containing gene clusters from both cultured and uncultured bacteria were identified.[100] Degenerate primers based on the eDNA-derived *isnA* sequence and predicted *isnA* homologues found in a basic local alignment search tool (BLAST) search were used to PCR-amplify *isnA*-related sequences from the DNA cloned in four eDNA cosmid libraries. Ultimately, 12 clones with predicted *isnA* homologues were recovered from the 400 000 cosmid clones that were screened. The region surrounding each of the predicted *isnA* homologues was sequenced to reveal a number of unique *isnA*-containing biosynthetic operons. The *isnA*-containing operons discovered in either a BLAST search of sequenced bacteria or the eDNA screen were PCR-amplified, cloned into a variety of bacterial protein expression vectors (pGEX-3X, pMAL-C2, or pMMB67), and then transformed into either *E. coli* or *Pseudomonas aeruginosa* for expression studies. A detailed analysis of ethyl acetate extracts derived from these cultures led to the characterization of nine clone-specific metabolites. The identification of new metabolites from the induced expression of this family of operons suggests that using standard *E. coli* protein expression vectors to activate even simple biosynthetic operons recovered from the environment could be a rewarding and straightforward strategy for the discovery of structurally novel metabolites.

2.13.4.8 Antifungal Activity from a PKS Gene Cluster

In February 2008, Korean scientists reported the construction of two metagenomic fosmid libraries containing 113 700 total clones.[101] Top-agar overlay bioassays employing the fungus *Saccharomyces cerevisiae* as the target organism provided evidence for the production of antifungal activity by a single clone in this library. Sequence analysis of the 40 kb insert from this clone revealed 39 ORFs many of which encoded putative bacterial type II PKS elements. All transposon insertions that disrupted the production of antifungal activity by the recombinant *E. coli* fell into the PKS associated gene. These included an ACP, four β-ketoacyl ACP synthases, an aminotransferase, an ACP reductase, and a ketoreductase (**Figure 9**). Two transcription factors (a LysR-type response regulator and a IclR-type response regulator) located adjacent to the predicted biosynthetic genes

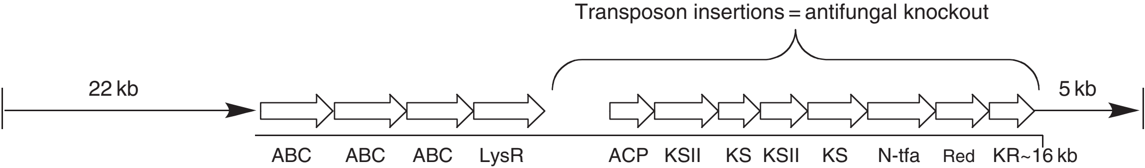

Figure 9 Transposon mutagenesis of a soil DNA cosmid clone isolated from an antifungally active recombinant *Escherichia coli* indicated that eight genes were required for the observed antifungal activity (GenBank accession No DQ000460). The LysR transcription factor adjacent to the biosynthetic genes positively regulates the observed antifungal activity. Despite exhaustive efforts, no metabolites with antifungal activity were characterized in this study.[101]

enhanced the production of antifungal activity when cloned onto high copy number vectors. Unfortunately, and despite exhaustive efforts, no small molecules with antifungal activity were reported in this work. Based on the transposon mutagenesis results and the genes that appear in the sequenced gene cluster the product is presumed to be a polyketide containing at least one nitrogen atom. While this work did not yield a tangible small molecule with biological activity, the study demonstrated the successful application of activity-based screening against a broader range of microbes than had been previously investigated.

2.13.4.9 Indigo/Indirubin

A number of metagenomic libraries hosted in *E. coli* have now been screened for clones that produce either antibacterial activities or color. In addition to hits that are unique to each library, these studies have encountered four common hits. These include the antibacterially active long-chain *N*-acyl amino acid-producing clones described earlier in this section, red antibacterially active clones that express aminolevulinic acid synthases (*hemA*), brown clones that produce melanin-like polymers, and blue clones that produce mixtures of indigo (**25**) and indirubin (**26**).[90,96,102,103]

Three independent metagenomic studies have reported the discovery of either indigo- or indirubin-producing clones (**Figure 10**). The first of these clones was found in a 12 000-membered BAC library generated from New England soil, the second was found in a collection of 110 000 fosmid clones constructed from Korean soil, and the third was found in an 800 000-membered plasmid library constructed from DNA extracted from the midguts of gypsy moth larvae.[104–106] The rich biosynthetic diversity present in metagenomic samples is highlighted by the fact that three different biosynthetic systems were identified in these studies (GenBank

Indigo (**25**) Indirubin (**26**) Isatin (**27**)

DNA source	Vector	Screen	Indigo	Indirubin	Isatin	Additional uncharacterized
New England soil	BAC	Color		X		Yes
Korean soil	Fosmid	Antibiotic	X	X		
Gypsy moth	Plasmid	METREX	X	X	X	QSM-1

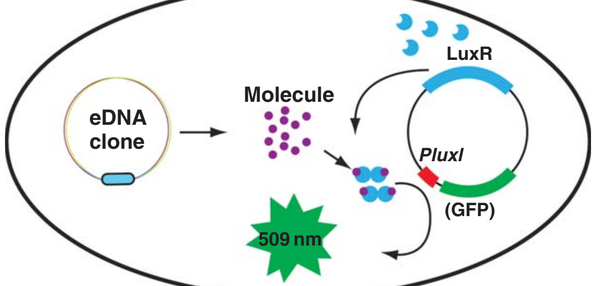

Figure 10 Clones that produce either indigo (**25**) or indirubin (**26**) have been identified in three different metagenomic studies. The key features of these three studies and the molecules characterized in each study are outlined here. Two of the indigo-producing clones were identified in screens for color and antibacterial activity while the third was found in an assay for quorum-sensing mimics (METREX). In this intracellular assay for quorum-sensing inducers, activated LuxR (LuxR bound to an AHL or an AHL mimic) induces the expression of GFP.[107]

accession Nos DQ000460, EF569599, AR053980). The BAC clone from New England soil contains two predicted indole dioxygenases, each of which is sufficient to produce the observed blue color. Color production by the fosmid clone from Korean soil requires a monooxygenase that is regulated by an adjacent TetR-like response regulator. The gypsy moth midgut plasmid clone was found to contain a two-component flavin-dependent monooxygenase system (MoxZ/Y) in which MoxY alone is sufficient for color production but the color production is enhanced by MoxZ, a predicted NADH:flavin oxidoreductase.

Two of these indigo-producing clones were initially identified in commonly used screens for color and antibacterial activity while the third was found in a reporter gene assay designed to detect quorum-sensing inducers. Bacteria use quorum-sensing systems to regulate a wide range of behaviors in response to changes in cell density. Gram-negative quorum-sensing systems are often composed of an *N*-acyl homoserine lactone (AHL) and a LuxR-type regulatory protein that responds to the AHL. This conserved architecture was used as the basis for the development of an intracellular screen for small-molecule quorum-sensing mimics produced by metagenomic clones (metabolite-regulated expression (METREX)) (**Figure 10**).[107] In this assay, activated LuxR (LuxR bound to an AHL or an AHL mimic) induces the expression of green fluorescent protein (GFP). METREX screening of metagenomic libraries has so far uncovered two small-molecule-producing clones. In the first METREX-active clone reported, the quorum-sensing inducer was reported to be an AHL that most closely resembles *N*-(3-oxohexanoyl)-L-HSL (homoserine lactone).[107] The second clone is the blue gypsy moth midgut clone described above. Unfortunately none of the metabolites purified from extracts of this clone (indigo (**25**), indirubin (**26**), and isatin (**27**)) were found to be quorum-sensing mimics and all attempts to purify a quorum-sensing mimic (QSM-1) from extracts of this clone were unsuccessful. The authors of this study did however find that air oxidation of a known indigo biosynthetic intermediate, indoxyl, yielded an unstable METREX-active compound. QSM-1 was therefore proposed to be an unstable indoxyl oxidation product. One of the unique features of the METREX assay is that the entire screen, from molecule production to fluorescent readout, occurs intracellularly. Interestingly, nearly all of the clones (10 out 11) that were active in the intracellular METREX screen did not activate the GFP expression when the biosensor was used as an overlay.[107] Thus, intracellular assays may provide a more sensitive alternative to screening metagenomic libraries.

2.13.4.10 Symbionts

Metagenomic approaches have been used to study metabolites produced by bacterial symbionts of marine animals, insects, and nematodes. The literature now provides several examples where DNA-based strategies targeting specific genetic loci have enabled the isolation and cloning of biosynthetic gene clusters encoding for the biosynthesis of important symbiont-associated molecules such as pederin, onnamide A, bryostatins, trunkamide, theopalauamide, and the patellamides.[108] These studies are the focus of another chapter in this series and therefore will not be discussed here.

2.13.5 Conclusions

One of the key revelations to arise from molecular phylogenetic analyses of environmental samples is that only a small fraction of bacteria present in the environment is easily cultured in the laboratory. Uncultured bacteria are therefore likely the largest pool of genetic diversity that has not been examined for the production of biologically active and pharmacologically useful metabolites. The advent of ultra high-throughput sequencing has the potential to make large-scale eDNA sequencing routinely available to most laboratories. While this technology is still not able to provide the complete sequence of most metagenomes, it will provide an additional way to access previously inaccessible eDNA-derived natural product biosynthetic gene cluster sequences. Sequenced bacterial genomes contain anywhere from a few to over 20 secondary metabolite biosynthetic pathways, suggesting that a single 100 000-membered eDNA cosmid library (~4000 Mb or 1000 bacterial genome equivalents of eDNA) may contain as many as 20 000 natural product biosynthetic gene clusters, most of which will have originated from bacteria that have never been observed in culture-dependent laboratory settings. Further development of methods to functionally access this cornucopia of previously inaccessible

biosynthetic pathways should significantly increase the number and diversity of natural products that are available as therapeutic agents or as probes for interrogating biological systems. Collaborative efforts involving individuals from many disparate fields including bacterial genetics, sequencing, bioinformatics, robotics, synthetic biology, and natural products chemistry will be necessary to effectively address this large-scale but potentially rewarding problem. Although the molecules that have been characterized to date from eDNA are structurally simple, many are structurally novel. These initial studies clearly support the hypothesis that uncultured bacteria are likely to be a rich source of novel biologically active small molecules. Whether gaining functional access to the genomes of previously uncultured bacteria will usher in a new golden age of small-molecule discovery remains to be seen.

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2.14 The Chemistry of Symbiotic Interactions

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2.14.1 Introduction

Symbiosis is an intimate, long-term, and specific association between organisms of two or more species. This definition is largely identical to the original concept of symbiosis coined by Anton de Bary in 1879 and can be broadly applied to a multitude of relationships of beneficial, neutral, or harmful nature. Accordingly, a symbiosis beneficial to both partners is called 'mutualism,' a relationship harmful to one partner is termed 'parasitism,' and 'commensalism' is beneficial to one partner and largely neutral for the other. There also exists an alternative definition of symbiosis, frequently employed by European scientists, that exclusively refers to mutualistic relationships. In this chapter, the original broad concept is taken into consideration, since for many associations the effect on the organisms is unknown, and often there are no clear borderlines between mutualism, commensalism, and parasitism/pathogenicity but rather an interactive continuum. Moreover, the mechanisms underlying establishment and maintenance of different types of associations can be based on very similar molecular mechanisms.

One of the main functions of secondary metabolites is communication. It is therefore not surprising that small molecules can play a crucial role in symbiotic systems, which use highly developed signaling and recognition mechanisms to establish and maintain the interaction. In symbionts, natural products are employed as chemical cues for partner localization, as invasive toxins, as developmental triggers, or as protective agents to eliminate competitors or to increase the host's fitness. Owing to these highly specialized roles, many of which are unique for symbiosis, selection conditions during the evolution of natural product pathways can differ from those in free-living organisms. This might explain why the chemistry of symbionts is often highly distinct even if closely related nonsymbiotic species exist. Thus, research on symbiotic systems not only reveals unique mechanisms in chemical ecology and metabolic evolution, but is also rewarding in terms of natural product discovery.

This chapter reviews secondary metabolites isolated from symbionts, organized by host taxonomy. It provides an overview of the different roles of these compounds in the chemical ecology of the interaction, if known, and discusses experimental techniques to study symbiotic systems as well as present gaps of knowledge.

2.14.2 Protists

Protists are a taxonomically inhomogeneous group of mostly unicellular eukaryotic microorganisms. Dinoflagellates (superphylum Alveolata) are protists and are the most important source of natural products (see Chapter 2.09).[1] Several species form massive blooms in the ocean, known as red tides, and many are notorious producers of neurotoxins, mainly complex polyketides, that may accumulate in food chains. Besides playing important roles as photosynthesizing endosymbionts in corals and other marine invertebrates (see the following sections), many dinoflagellates are also hosts for bacteria, raising questions about the actual origin of the toxins. Classical cultivation studies and culture-independent approaches, such as fluorescence *in situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and 16S ribosomal RNA (rRNA) analysis, revealed a remarkable diversity of taxa and loci of attachment within or outside the eukaryotic cell.[2–6] The majority of the identified strains belonged to the phyla Proteobacteria and Bacteroidetes, but members of Cyanobacteria and Actinobacteria have also been detected. Most studies on symbiotic toxin producers centered on saxitoxin (**1**) and related compounds (**Scheme 1**), which are paralytic shellfish poisons (PSPs) of nonpolyketidic origin. Several free-living cyanobacteria of the genera *Anabaena*,[7] *Lyngbia*,[8] and *Aphanizomenon*[9] have been discovered that produce saxitoxins, suggesting a bacterial origin in the dinoflagellate. However, reports on the true producer have been contradicting. They include (1) the isolation of a large number of unrelated bacterial PSP producers from *Alexandrium* spp.,[10] among them an intracellular γ-proteobacterium from surface-sterilized *Alexandrium tamarense*,[11] (2) the drop of PSP production in dinoflagellates treated with antibacterial agents,[12] (3) the occurrence of toxin production in axenic dinoflagellate cultures,[13] and (4) a Mendelian inheritance scheme of biosynthetic capabilities, suggesting the location of biosynthetic genes on the dinoflagellate genome.[14] Several scenarios could explain these discrepancies. It has been pointed out that the methods most commonly used for PSP detection, that is, high-performance liquid chromatography (HPLC) and mouse neuroblastoma assay, are not specific enough and might generate false-positive results.[15,16] A second possibility is that PSP biosynthetic genes are present in dinoflagellates as well as

1 **2** **3** **4** **5** **6**

Scheme 1

in bacteria, either due to horizontal gene transfer or convergent evolution, but that not all strains produce the compounds. Modern techniques such as high-sensitivity nuclear magnetic resonance (NMR) analysis or the localization of biosynthetic genes will very likely resolve this issue.

Several researchers have addressed the question whether dinoflagellate polyketides might be of bacterial origin. In immunofluorescence assays, okadaic acid **2** did not colocalize with bacteria, providing indication for host production (unless metabolites are transported between cells).[17] In addition, several lines of evidence, such as FISH on separated cells[18] and the generation of reverse-transcript polymerase chain reaction (RT-PCR) amplicons from polyadenylated, that is, eukaryotic, RNA,[19] demonstrated that dinoflagellate genomes can harbor polyketide synthase (PKS) genes. In the light of these results and the uniqueness of dinoflagellate polyketides regarding structure and biosynthesis,[1] a host origin is very likely.

A small number of compounds have been isolated from bacterial cultures obtained from dinoflagellates. In the case of *Pfiesteria* spp. dinoflagellates, signaling between symbiotic partners has been studied in more detail. Members of the *Roseobacter* clade are attracted to these hosts by using the dinoflagellate-derived substance dimethylsulfoniopropionate (**3**) as chemical cue.[20] The bacteria colonize the host, catabolize the compound, and incorporate a part of the assimilated sulfur into an antibiotic, tropodithietic acid (**4**).[21] In another study, species of *Marinobacter* that are required for the growth of the bloom-former *Gymnodinium catenatum* produce the siderophore vibrioferrin (**5**),[22] a known natural product first reported from pathogenic *Vibrio parahemolyticus*.[23] In addition to iron, **5** also binds borate, which is common in marine but not terrestrial environments. **5** is therefore suspected to mediate signaling processes or boron transport. A further example of signaling chemistry is quorum sensing (QS; sensing of the cell density within a population) mediated by long-chain acylhomoserine lactones, such as **6**, that have been isolated from the dinoflagellate-associated γ-proteobacterium *Dinoroseobacter shibae*.[24]

2.14.3 Brown Algae

Lobophorolide (7) (**Scheme 2**) is a complex polyketide with antifungal activity that is isolated from the brown alga *Lobophora variegata*.[25] It belongs to a group of structurally similar mono- and dimeric macrolides isolated from several unrelated organisms, including swinholide A that has been reported from marine sponges[26] as well as cyanobacteria[27] (see Section 2.14.6). The resemblance strongly suggests that a bacterium is the actual source of 7 in the alga. Since 7 has been consistently detected at deterrent concentrations at different locations in the Bahamas, it is not the product of an accidental contamination. The existence of a symbiosis based on antimicrobial defense, unprecedented for macroalgae, is therefore very likely (see Chapter 2.03).

2.14.4 Green Plants

The association between plants and nitrogen-fixing bacteria is one of the economically most important and best-studied symbiotic interactions.[28–30] These include the mutualism between legumes and α-Proteobacteria of the order Rhizobiales, but also other bacteria such as *Frankia* spp.,[31] *Azospirillum* spp.,[32] and cyanobacteria[33] undergo symbioses with a range of plants. They are capable of inducing and colonizing specialized root structures, termed nodules, in the plants. Within these tissues, they fix elemental nitrogen into amino acids

7

Scheme 2

Scheme 3

that are provided to the plants. A wide range of secondary metabolites are involved in the mutual recognition of symbiotic partners and the maintenance of symbiosis (**Scheme 3**). Luteolin (**8**) and other flavonoids, betaines, such as stachydrine (**9**) and trigonelline (**10**), tetronic acid (**11**), erythronic acid (**12**), acetosyringone (**13**) and related shikimate derivatives, jasmonic acid (**14**),[34,35] and other compounds present in the root exudates serve free-living rhizobia as chemical cues and trigger the biosynthesis of bacterial signals termed Nod (nodulation) factors. These in turn induce root nodule formation in the plant. Nod factors feature a tri- to hexameric β-1,4-linked *N*-acetyl-D-glucosamine core structure with a fatty acyl residue at the terminal nonreducing unit, but there is extensive variation in the substitution pattern and this has a marked influence on host selection and the degree of specificity.[36] It has been found that closely related plants are colonized by bacteria belonging to different groups. This may appear surprising, as it would indicate a low degree of coevolution of host and endosymbiont. However, loosely related rhizobia that are specific for the same plant taxon often produce Nod factors with similar structures,[37] suggesting that horizontal transfer of nodulation genes between rhizobial species is extensive. The pattern of Nod factors produced can vary considerably in the same strain depending

on the environmental conditions. For example, *Rhizobium tropici* CIAT899 synthesizes 52 compounds at acidic and 29 at neutral pH with only 15 factors in common.[38] Treatment of roots with Nod factors alone can already induce nodulation,[39] but for invasion a second signal type, rhizobial exopolysaccharides, is required.[40] These molecules are responsible for initiation and extension of a plant structure termed infection thread.[41]

A third chemical player in the plant–rhizobial interaction is bacterial small molecules that act as QS signals.[42] QS activates the transcription of genes if the signal concentration, correlating to the number of cells in a bacterial population, reaches a threshold level. In *Rhizobium leguminosarum* bv. *viciae*, four QS systems based on *N*-acyl homoserine lactones have been identified.[43] These participate in a complex regulatory network governing the transfer of a plasmid harboring symbiosis genes to nonsymbiotic rhizobia, inhibition of bacterial growth, and nodulation efficiency. To complicate matters even more, the host plant can produce substances that interfere with the QS system. An example is L-canavanine (**15**) from alfalfa that inhibits QS-mediated exopolysaccharide synthesis in *Sinorhizobium meliloti*.[44] An unusual QS autoinducer identified in *Bradyrhizobium japonicum* is bradyoxetin (**16**),[45] which represses nodulation genes at high cell densities.[46] Another nonlactonic QS signal is 3-hydroxy palmitic acid methyl ester **17** from *Ralstonia solanacearum*, which controls exopolysaccharide production similar to the *S. meliloti* autoinducers.[47]

Some *Rhizobium* strains produce a further class of molecules important for symbiosis, the rhizopines *scyllo*-inosamine (**18**) and 3-*O*-methyl-*scyllo*-inosamine (**19**).[48] These compounds are produced by the nodule-colonizing bacteroids and used by the free-living stages as energy, carbon, and nitrogen source. The genes for rhizopine biosynthesis and catabolism are closely linked,[49] which ensures that the compounds benefit only synthesizing strains and thus provide a competitive advantage to the nodulating bacterium.[50] A similar phenomenon of interdependent biosynthesis and catabolism of unusual metabolites, here called opines, is also known from the plant pathogen *Agrobacterium tumefaciens* (see below). A wide range of additional metabolites have been isolated from rhizobia. This includes rhizobitoxine (**20**),[51] an inhibitor of plant defense responses. **20** blocks 1-aminocyclopropane-1-carboxylate synthase, which catalyzes a key step in the biosynthesis of the defensive plant hormone ethylene.[52] Siderophores such as vicibactin (**21**) and rhizobactin 1021 (**22**) are used for iron sequestration under iron-limiting conditions, such as pathogenesis and symbiosis. The benefit of these compounds has been demonstrated in competition experiments using strains defective in siderophore biosynthesis.[53] It has been pointed out[54] that invasive mechanisms used by rhizobia closely parallel those employed by plant pathogens: both types of organisms often rely on siderophores, QS systems, and two-component regulators, and both have to suppress plant defense responses by inhibiting ethylene biosynthesis and employing surface polysaccharides, antioxidant systems, and virulence genes. This indicates a close evolutionary relationship between seemingly distinct types of associations.

Pseudomonas fluorescens Pf5 is a plant commensal of the rhizosphere that suppresses the growth of various plant pathogens.[55] The sequenced genome was found to harbor nine secondary metabolite gene clusters.[56,57] Six of these could be attributed to known biosynthetic pathways for pyoluteorin (**23**) (**Scheme 4**), 2,4-diacetylphloroglucinol (**24**), pyrrolnitrin (**25**), and HCN, which all exhibit antifungal activities, the siderophores pyochelin (**26**) and salicylic acid (**27**) (the latter also a precursor of the defensive plant hormone methyl salicylate), and polyketides of the rhizoxin[58] series, such as rhizoxin (**28**), which also play an important role in a fungal symbiosis (see Section 2.14.5). A further compound, the previously unknown nonribosomal peptide orfamide A (**29**), was identified using a novel 'genomisotopic approach.'[59] The method consisted of predicting the structure from gene sequences, feeding one of the predicted amino acid building blocks carrying a ^{15}N-label to the bacterium, and isolating compounds enriched in the nitrogen isotope from the culture medium.

A similarly high diversity of natural products was revealed during genome sequencing of the biocontrol strain *Bacillus amyloliquefaciens* FZB42,[60] a plant-associated bacterium that suppresses pathogens and in addition promotes plant growth (**Scheme 5**).[61,62] It has been shown previously that the strain produces the plant growth hormone indole-3-acetic acid (**30**) when fed with tryptophan.[62] The sequencing study revealed that more than 8.5% of its genome is devoted to secondary metabolism. Giant gene clusters for the biosynthesis of seven types of antibiotics (**Scheme 5**), bacillaene (**31**),[63] difficidin (**32**),[63] macrolactin (**33**),[64] surfactin (**34**), fengycin (**35**), bacillomycin D (**36**), and bacilysin (**37**),[65] were identified. In addition, FZB42 harbors genes for the siderophore bacillibactin (**38**) and a nonribosomal peptide with as yet unknown structure and function.[60]

Scheme 4

The occurrence of complex polyketides in macroorganisms, as described above for lobophorolide (**7**), is usually a strong indication of a microbial origin. Examples from plants are macrolides of the maytansine group,[66] such as the parent compound maytansine (**39**) (**Scheme 6**). They are potent tubulin binders[67] first reported[68] from *Maytenus* spp. plants (Celastraceae) and later also from other plant groups, including mosses.[69] Intriguingly, natural products with closely related or even identical structures are produced by actinomycete bacteria, some of which had been isolated from plant rhizospheres.[70] Since it is highly unlikely that the biosynthesis of highly complex macrolides has independently evolved several times in unrelated organisms, the best candidates for maytansin production are bacterial symbionts. An experiment targeting the gene for 3-amino-5-hydroxybenzoic acid (AHBA) synthase, a key enzyme in maytansine biosynthesis,[71] by Southern hybridization and PCR, produced no signal in preparations of pure cell cultures of the plant *Putterlickia verrucosa*, but a PCR product was obtained from the DNA of a plant-derived mixed bacterial culture.[72] Cloning of the entire biosynthetic gene cluster from the DNA will provide information whether it indeed encodes maytansine biosynthesis or another metabolite derived from AHBA.

Virtually every plant participates in symbioses with fungi. Most widespread are fungal endophytes,[73,74] which are found ubiquitously in plant tissues and also in marine algae. A single plant can harbor hundreds of distinct fungal species[75] with widely differing degrees of host specificity. The nature of symbiosis can be similarly diverse. Some associations exhibit characteristics of a contained pathogenic relationship.[76] For example, an examination of axenic cultures of separated plant cells and fungi revealed increased endophyte growth by host substances, but herbicidal effects of metabolites excreted by the fungi.[77] In other cases, the symbiosis shows all hallmarks of a mutualism: *Acremonium* (*Epichloë*/*Neotyphodium*) spp. are intimately associated with various grass species, such as perennial ryegrass (*Lolium perenne*) and tall fescue (*Festuca arundinacae*), and can be transmitted only via seeds in some cases.[78] The fungi produce a range of alkaloids that are toxic to herbivores and in this way protect the host plant. Examples are the insecticides peramine (**40**) and loline (**41**), tremorgenic indol alkaloids, such as paxilline (**42**) and lolitrem B (**43**), and ergoline alkaloids, such as ergovaline (**44**),

Scheme 5

which are a common cause of livestock toxicosis (Scheme 6). Ergoline alkaloids are found in plants of the genus *Ipomoea* (morning glory).[79] Seeds of these species have been employed as hallucinogens for religious purposes by Mexican Native Americans and are today consumed in many countries as recreational drugs. Since

Scheme 6

treatment with antifungal agents results in elimination of alkaloids,[80] biosynthesis by a fungus is likely. 18S rRNA analysis indeed revealed the presence of an as yet unculturable clavicipitaceous fungus that is transmitted via seeds.[81] Ergoline alkaloid biosynthesis in this fungal group is very common, a notorious example being the rye pathogen *Claviceps purpurea*, the source of ergotamine (**45**) and other compounds.[82] Consumption of infected grains can cause ergotism, a painful disease known as St. Anthony's Fire that during the Middle Ages could result in the shedding of limbs. Hundreds of additional compounds from endophytic fungi have been reported, including alkaloids, terpenoids, polyketides, peptides, and lipids.[73,74] **Scheme 7** provides a small impression of the structural diversity of the isolated compounds.

Another important fungal–plant symbiosis is the formation of mycorrhiza, a usually mutualistic association of fungi with plant roots. The most widespread is the arbuscular mycorrhiza (AM) consisting of members of the fungal phylum Glomeromycota[83] and diverse plants.[84,85] At least 80% of angiosperm species participate in this kind of mutualistic interaction. AM is a very ancient association, and it is suspected that it has once facilitated land colonization by plants. The fungal symbiont, a part of which forms intracellular branched structures, provides the plant through its extended mycelial network with mineral nutrients, in particular phosphate, in exchange for receiving carbohydrates to meet its complete carbon requirement. AM fungi depend on the plant for survival, exist in the soil as spores, and have not yet been grown in pure culture. There is evidence for a sophisticated signaling system at the presymbiotic (unassociated) stage similar to that found in rhizobial–plant interactions. Germination of spores is stimulated by plant root exudates.[86,87] Exudates also stimulate respiratory activity[88] and hyphal growth and branching in the vicinity of the plant root. One class of germination and branching factor was identified as strigolactones, for example, 5-deoxystrigol (**53**)[89] and sorgolactone (**54**) (**Scheme 8**).[90,91] These signaling compounds also play an important role in some plant–plant interactions (see below).[91] Flavonoids present in root exudates, including 5,6,7,8-hydroxy-4′-methoxy flavone (**55**), quercetin (**56**), and luteolin (**8**), also display highly differentiated effects (reviewed in Shaw *et al.*[92]) on germination, hyphal growth, and the number of entry points. The compounds were often found to be highly specific in terms of compound structure, fungal

Heptelidic acid (**46**) Chokol A (**47**) Gamahorin (**48**)

Sequoiatone A (**51**)

Preussomerin N_1 (**49**) Cryptocandin (**50**) (+)-Cryptosporiopsin (**52**)

Scheme 7

species, and developmental stage. In addition to these signals perceived by the fungus, a diffusible fungal factor was found to induce plant gene expression.[93] This provides evidence that chemical recognition at the presymbiotic stage is mutual, although the exact nature of the signal(s) is so far unknown.

A second variant of mycorrhizal interactions is found in the ectomycorrhiza.[94] Here the fungus forms extracellular sheaths around fine roots and is responsible for water and mineral supply while obtaining photosynthetic assimilates from the plant host. Rutin (**57**) (**Scheme 8**), present in eucalyptus exudates, has been shown to stimulate hyphal growth of *Pisolithus* strains,[95] and the plant hormones jasmonic acid (**14**) and *trans*-zeatin (**58**) trigger accumulation of the *Pisolithus* compound hypaphorine (**59**) in hyphae.[96] **59** in turn inhibits the elongation of root hairs in its host[97] as well as in the model plant *Arabidopsis thaliana*[98] by counteracting the activity of the host hormone indole-3-acetic acid (**30**).[99] There is evidence that plant-derived flavonoids, which also play a role in AM and rhizobial symbiosis, participate in ectomycorrhizal chemical communication. For example, rutin (**57**) in *Pinus densiflora* and eucalyptus exudates triggered spore germination of *Suillus bovinus*[100] and stimulated the growth of *Pisolithus* hyphae[95] at micro- and picomolar concentrations, respectively. Intriguingly, formation of ectomycorrhizae can be markedly influenced by prokaryotes termed mycorrhiza helper bacteria.[101] These taxonomically diverse organisms can promote the growth of symbiotic fungi and inhibit mycopathogens and plant defense responses.[102] In one study, the growth-promoting activity of the helper bacterium *Streptomyces* sp. AcH 505 has been traced to the compound auxofuran (**60**).[103,104] In addition, the strain also produced antibiotics, such as WS-5995 B (**61**).

An economically relevant example of symbiosis between two plant species is root parasitism. *Striga* (witchweed) and *Orobanche* spp. (broomrape) are facultative parasites of the family Scrophulariceae that die within a short time after germination if no contact with a photosynthesizing plant is established. After successful attachment to the host roots, they obtain water and assimilates from the host, which can be weakened to such a degree that entire harvests are lost. The strategies used by these parasites to locate host plants are remarkably similar to those of microorganisms.[105,106] Germination of *Striga* spp. is specifically triggered by

Scheme 8

compounds present in root exudates. Sorgoleone (**62**),[107] sesquiterpene lactones, such as dihydroparthenolide (**63**),[108] and strigolactones, for example, strigol (**64**)[109] and sorgolactone (**54**) (**Scheme 8**),[110] have all been shown to induce a germination response, although there has been some debate whether **62** is too hydrophobic to diffuse in the aqueous medium of natural environments.[111] Examples of germination stimulants of *Orobanche* spp. are the strigolactones alectrol (**65**) and orobanchol (**66**).[112] Some of these compounds are identical to those used as host clues by AM fungi (see above).[89,113,114] The parasites might therefore have evolved to exploit an ancient signaling system that cannot be easily abandoned by parasitized plants, since it is used in an important and widespread mutualism. In addition to germination stimulants, xenognosins also play a role in the development of Scrophulariacean parasites. These are structurally diverse molecules that induce the formation of haustoria, that is, parasite structures that allow attachment to the host. Among the characterized compounds are xenognosin A (**67**),[115] peonidin (**68**), and 2,6-dimethoxybenzoquinone (**69**).[116] The fact that these are all

quinones or hydroquinones with similar redox potentials suggests a common mechanistic basis for xenognostic (i.e., modifying growth and development of other organisms) activity.[117]

Plant galls are abnormal outgrowths of tissues induced by invading parasites. Galling organisms include wasps, sawflies, midges, beetles, thrips, coccids, aphids, psyllids, mites, nematodes, fungi, bacteria, and other plants. The chemical and biochemical signals triggering the formation of galls are, with the exception of bacteria, in most cases not well understood. The most extensively studied gall former is the bacterial plant pathogen *A. tumefaciens*, the causative agent of crown gall disease.[118] Strains of this bacterium as well as the related species *A. rhizogenes* possess a remarkable ability to genetically engineer host plants by injecting a conjugative plasmid into their cells (Ti plasmid for *A. tumefaciens*, Ri plasmid for *A. rhizogenes*). This mechanism has been widely exploited for biotechnological purposes to introduce DNA into plants and other organisms. The *A. tumefaciens* DNA, termed T-DNA, integrates into the plant genome and triggers tumor-like growth after transcription. The bacterium then feeds on compounds produced by the tumor. Similar to rhizobia, which belong to the same family as *A. tumefaciens*, an intricate signaling system governs cell–cell recognition and symbiosis. Substances that have been reported as plant-derived inducers of bacterial chemotaxis toward the host include acetosyringone (**13**) and other small phenolic compounds.[119,120] These metabolites and related substances,[121] such as coniferin (**70**) (**Scheme 9**),[122] as well as various sugars[123] also induce the transcription of virulence (*vir*) genes present on the Ti plasmid. The products of these genes mediate plasmid transfer into the host cell, resulting in tumorigenesis. In the plant, further genes present on the T-DNA direct the biosynthesis of plant growth hormones, for example, indole-3-acetic acid (**30**)[124] and the cytokinins *trans*-zeatin (**58**) and *trans*-ribosylzeatin (**71**),[125,126] which disturb the hormonal balance of the host and cause tumor formation. In addition, the plant is reprogrammed to synthesize unusual amino acids and sugar derivatives termed opines[127] that are used as a specific carbon, nitrogen, and sometimes phosphorous source. Depending on the *A. tumefaciens* strain, different opines might be generated, but the presence of degradation pathways is usually highly specific to the compounds produced. Representative opines are octopine (**72**), nopaline (**73**), mannopine (**74**), and agrocinopine A (**75**), after which four of the main opine families are named. Some opines have a signaling function and

72 R = CH_2-COOH
73 R = H

Corynetoxin S15α (**77**)

Scheme 9

induce the conjugal transfer of plasmid subsets belonging to specific opine families.[118,127] Opine-like molecules, the rhizopines, have also been reported from rhizobia (see above).

Plant growth hormones are also synthesized and/or manipulated by other gall formers (see Chapter 2.02).[128] The bacterium *Rhodococcus fascians* induces leafy galls in a wide range of plants and has been shown to produce **30** as well as **58**, isopentenyladenine (**76**) (**Scheme 9**), *cis*-zeatin, and other cytokinins. Other examples of demonstrated bacterial hormone producers in the context of gall formation are *Pseudomonas savastanoi*[129] and *Erwinia herbicola*.[130] All three bacterial species do not genetically modify the plants, and in contrast to *A. tumefaciens*, their gall tissue cannot be propagated in *in vitro* cultures in the absence of bacteria unless supplied with exogenous growth hormones.[128] In addition to bacteria, cytokinins are also secreted by gall-inducing nematodes, such as *Meloidogyne* spp. generating root knots, and are at least in part responsible for the production of giant cells on which the parasites feed.[131,132] As with other galls, the metabolic profile in nematode-modified plant tissue is different from adjacent parts of the parasitized plants. In one remarkable example, production of a toxin is the result of an association of four organisms: *Rathayibacter* (*Clavibacter*) *toxicus* is a bacterium that may attach to the cuticle of *Anguina* spp. nematodes, the causative agent of seed galls in rye grass. When introduced into the gall, it produces corynetoxins, such as **77**. The compound is occasionally responsible for the poisoning of cattle that feed on parasitized grass, but is biosynthesized only if *R. toxicus* is infected with a bacteriophage.[133]

The mechanism of gall formation by numerous other organisms, including insects, is not fully understood. In a number of cases, gall induction could be achieved by applying the contents of insect glands to plants.[134] Elevated cytokinin levels in gall tissues have been reported in several studies, but there is little direct evidence that insects actually produce these compounds. In one report, the highest concentration of **58** and **71** in galls was detected in the developing larvae of the chalcid wasp *Erythrina latissima*,[135] suggesting that they are the source of the compounds. Another study demonstrated that triacylglycerides containing (*E,E,E*)-octa-2,4,6-trienoic acid, such as **78**, extracted from the aphid *Colopha moriokaensis*, induced hypertrophy in cultivated cells of the host plant *Zelkova serrata*.[136] In the light of the sometimes spectacular plant structures induced by the presence of galling insects, further research on the induction mechanisms will be highly rewarding.

Insects can also manipulate plant chemistry for purposes other than nutrition. For example, *Pontania* spp. sawflies increase the tannin content preferentially at the outer gall regions for defensive purposes,[137] and the gall wasp *Anistrophus rufis* modifies the pattern of volatile terpenes emitted by Asteraceae host plants to generate sex pheromone-like substances that serve as localization cues for male wasps.[138]

2.14.5 Fungi

Fungi form intimate associations with either cyanobacteria or algae to generate lichens (see Chapter 2.08). The latter partner is called phycobiont and provides the fungal symbiont (termed mycobiont) with photosynthetic assimilates and, if the phycobiont is a cyanobacterium, the fungal symbiont is provided with fixed nitrogen. In turn, it receives water, minerals, and protection from the fungus. The fossil record indicates that lichen formation existed already 600 million years ago before the advent of vascularized plants.[139] More than 1000 secondary metabolites have been isolated so far from lichens,[140–143] many of which exhibit interesting cytotoxic, anti-inflammatory, antiviral, and other pharmacological activities. In addition, lichen substances are used as pH indicators (litmus), fragrances, and antioxidants. Examples illustrating the structural and pharmacological diversity of these compounds are vulpinic acid (**79**) (anti-inflammatory, feeding deterrent), atranorin (**80**) (an anti-inflammatory depside), stictic acid **81** (an antifeedant), usnic acid **82** (antibiotic, cytotoxic, antiviral, antifeedant, analgetic), the antiviral anthraquinone derivatives emodin (**83**) and 7,7′-dichlorohypericin (**84**), scabrosin esters, such as **85** (cytotoxic), protolichesterinic acid (**86**) (an anti-inflammatory member of the paraconic acid group), divarinol (**87**) (skin whitening agent), dasypoga-lactone (**88**), scytalidin (**89**), and nostoclide I (**90**) (cyto- and phytotoxin) (**Scheme 10**).[141,143] The ecological function of such metabolites is likely to protect the slow-growing organism against pathogens, lichenivors, and overgrowth by plants. In addition, many of the aromatic molecules, which are usually of polyketide origin, strongly absorb UV light and thus provide protection in the exposed habitats that are often colonized by lichens. The symbiotic partners can often be separated and grown in pure culture for chemical analysis,[143] and in cases where the same compounds as in lichens were produced, the mycobiont was usually found to be the actual source. However, often the isolated organism generates different metabolites than the

Scheme 10

93

Scheme 11

lichens, and the metabolic profile can drastically change under modified culture conditions.[143] So far, very little is known about chemical signaling in the life cycle of lichens and about their biosynthetic enzymology, both being areas that clearly warrant further research.

Among the few known examples of secondary metabolites synthesized by the phycobiont are the nostoclides (e.g., **90**),[144] hepatotoxic microcystins (e.g., [ADMAdda^5]microcystin-LR, **91**), and the cytotoxic cryptophycins[145] from cyanobacteria. The cryptophycin analogue cryptophycin 52 (**92**) reached clinical trials for cancer chemotherapy but was later abandoned.[146]

An example of a taxonomically very specialized symbiosis of lichens and bacteria is the *Rhizopus–Burkholderia* interaction. The phytopathogen *Rhizopus microsporus*, the causative agent of rice seedling blight, uses the polyketide rhizoxin (**28**) as a virulence factor. When applied to rice seedlings alone, **28** induces root swelling characteristic of the disease. The compound is a potent cytotoxin and inhibits mitosis by binding to β-tubulin. A study on its biosynthesis revealed that the true producer is an intracellular γ-proteobacterium, that is, either *Burkholderia rhizoxinica* or *Burkholderia endofungorum* depending on the *Rhizopus* strain.[147–150] Proof was obtained by successful cultivation of producing bacteria and by introducing them into axenic *R. microsporus* strains, which resulted in rhizoxin-positive chemotypes. Interestingly, removal of endosymbionts also generated fungal strains that were not able to form sporangia and spores, and conversely, vegetative reproduction was re-initiated by introducing bacteria into the hyphae.[151] Thus, the endosymbiont is indispensable to the fungus not only for pathogenesis but also for completion of the life cycle. Isolation of the rhizoxin biosynthesis genes revealed that the bacterial plant commensal *P. fluorescens* Pf5 (see above) harbors a virtually identical gene cluster, and rhizoxin production was subsequently proven also for this strain.[58] The rhizoxin PKS is rather unusual in that it lacks acyltransferase (AT) domains, which are normally integrated into the multidomain enzyme and select the polyketide building blocks. Acyltransfer is instead performed by monofunctional ATs encoded on isolated genes.[152] This type of enzyme has been termed trans-AT or AT-less PKS and has evolved independently from 'standard' modular PKSs as known from erythromycin biosynthesis. Trans-AT PKSs are common in symbionts (see below), but almost absent in actinomycetes, which are the source of most known complex polyketides.

Rhizopus microsporus strains have also been described as a source of food mycotoxins. Rhizonins, such as **93** (**Scheme 11**), which were the first-described toxins from zygomycetes, are strongly hepatotoxic nonribosomal peptides isolated from moldy peanuts in Mozambique. Examination for the presence of bacteria again showed that a *Burkholderia* sp. endosymbiont is the true biosynthetic source.[153] As with the rhizoxin (**28**) producer, the establishment of a pure symbiont culture was successful.

2.14.6 Sponges

Among all groups of organisms found in the ocean, sponges, the oldest extant metazoans, so far yielded the largest number of bioactive natural products (see Chapter 2.10).[154,155] This remarkable richness might be in part a consequence of the fact that sponges are largely unprotected, sessile filter feeders that often rely on chemical

defenses for survival.[156–158] The chemistry of sponges is exceptionally diverse and includes alkaloids, lipids, terpenes, carbohydrates, ribosomal and nonribosomal peptides, and polyketides. Since the last two groups of metabolites are commonly found in microorganisms, it has often been speculated that bacterial symbionts could be the actual producers.[159–161] Many sponges indeed harbor extraordinarily large numbers of prokaryotes, sometimes accounting for up to 60% of the biomass.[162] As cultivation of these bacteria has failed in almost all cases, they have so far been characterized mostly by cultivation-independent studies, such as 16S rRNA analysis, FISH, and DGGE.[163–165] The microbial consortia present in sponges can be extremely complex, yet unrelated sponges often harbor highly similar communities quite distinct from those of the surrounding habitats. Major bacterial taxa for which larger numbers of sponge-specific 16S rRNA sequences have been recovered are Proteobacteria, Cyanobacteria, Chloroflexi, Actinobacteria, and Gemmatimonadetes.[166] In addition, at least one entirely sponge-specific candidate phylum has been described, the Poribacteria, which can be readily recognized by the presence of an unusual 'nuclear' membrane surrounding the chromosomal DNA.[167,168] In contrast, several bacterial groups known as rich natural product sources from terrestrial drug screening programs, such as filamentous actinomycetes or myxobacteria, are underrepresented or even absent. At least in some sponges a large portion of the community is vertically transmitted to the next generation via larvae, suggesting that coevolution occurs.[169–173] This is supported by phylogenetic analyses of the bacterial 16S rRNA and the sponge cytochrome oxidase genes.[174,175] However, horizontal transmission of symbionts between sponges and enrichment from the water column have also been discussed as additional mechanisms that account for the bacterial diversity.[166] Thus, while some of the associations might be very ancient, perhaps even dating back to Precambric times, others seem to be of more recent origin, and further studies are needed to systematically address the issue of 'ancientness.'

Besides the largely descriptive taxonomic work, little is known about the biology and chemistry of sponge-inhabiting bacteria. In the earliest chemical studies, cell types were separated by flow cytometry or by centrifugation based on differences in cell densities. Fractions enriched in sponge or bacterial cells could then be analyzed for natural product contents. In a study on the sponge *Dysidea herbacea*, chlorinated compounds, such as dysidin (**94**), neodysidenin (**95**), and dihydrodysamide C (**96**), were localized to the cyanobacterium *Oscillatorium spongeliae* (**Scheme 12**).[176,177] An epimer of **95**, pseudodysidenin (**97**), and structurally related metabolites are also known from free-living cyanobacteria.[178] Catalyzed reporter deposition-FISH (CARD-FISH) demonstrated that *O. spongeliae* contains a halogenase gene similar to a gene involved in chlorination during biosynthesis of barbamide (**98**) from a free-living cyanobacterium, as would be expected for the production of structurally related metabolites.[179] Another group of natural products that were localized to *O. spongeliae* are brominated biphenyl ethers,[180] such as **99**, which occur in a distinct chemotype of *D. herbacea* devoid of chlorinated peptides.[181,182] Genetic analyses of different sponges that contain *O. spongeliae* revealed the presence of distinct strains and showed that difference in the chemistry (chlorinated peptides, brominated biphenyl ethers, or no halogenated compounds) is reflected in symbiont taxonomy.[174,183] In another study, chemical variation in one sponge was traced to morphologically distinct bacteria. The antifungal cyclic peptide theopalauamide (**100**) and the polyketide swinholide A (**101**) were detected after cell separation in two different bacterial preparations of the sponge *Theonella swinhoei* collected in Palau.[184,185] While **101** was found only in the unicellular heterotrophic fraction in the Palauan sponge, **100** was obtained from '*Candidatus* Entotheonella palauensis,' an interesting filamentous γ-proteobacterium distantly related to the myxobacteria.[186] By 16S rRNA analysis, this bacterium was also detected in other *T. swinhoei* specimens that contain compounds structurally related to **100**, for example, theonellamide F (**102**).[186] *Theonella swinhoei* with unrelated chemistry did not contain this symbiont. Intriguingly, **101** was recently also reported from free-living cyanobacteria, thus lending additional evidence to the hypothesis that a sponge symbiont is the true producer.[27] That **101** was detected in the heterotrophic instead of the cyanobacterial symbiont fraction is not necessarily a contradiction, since horizontal transfer of biosynthetic genes between unrelated bacteria is rather common. It should be noted that not all sponge-derived metabolites have been localized to bacterial cells. Some examples of compounds detected in sponge cells are aerothionin (**103**),[187] avarol (**104**),[188,189] diisocyanoadociane (**105**),[190] dercitamide (**106**),[191] and latrunculin (**107**) (**Scheme 13**).[192]

With all cell separation studies, the possibility that secondary metabolites might be excreted by the producing organism and taken up by a different cell type needs to be kept in mind. Localizing a compound

Scheme 12

in a cell fraction is therefore no unequivocal proof that it is also biosynthesized there. If the producer cannot be cultivated, a more rigorous approach would consist of isolating biosynthetic genes and determining their location. However, so far, this is a technically very demanding task, since sponge metagenomes (i.e., the sum of all genomes) are highly complex and contain a multitude of homologous genes. There are a handful of studies that have focused on PKSs responsible for the biosynthesis of complex polyketides. Numerous PKS genes were found in various sponges, such as *T. swinhoei*, *Aplysina aerophoba*, and *Discodermia dissoluta*.[165,193–195] However, most of these do not resemble the multimodular PKSs catalyzing complex polyketide biosynthesis but belong to

Scheme 13

a peculiar enzymatic type that is so far known only from sponge symbionts and architecturally resembles type I fatty acid synthases. Based on the sequence data, it has been suggested that these PKSs are involved in the biosynthesis of methyl-branched fatty acids commonly found in sponges.[165] In one case, the structural similarity of a complex polyketide to a natural product from another source could be exploited for the targeted isolation of specific PKS genes.[196] *Theonella swinhoei* from Japan contains onnamide A (**108**) and related compounds, which are highly similar to pederin (**109**) (**Scheme 13**), a defensive compound of *Paederus* spp. rove beetles (see Section 2.14.8).[197] From both sources, architecturally almost identical PKS genes of the unusual trans-AT type were isolated.[196,198–200] In the case of the sponge, this involved screening 400 000 clones of a metagenomic fosmid library using a newly developed semiliquid gel-based method.[201] Sequence analysis of the isolated DNA

region revealed that it did not belong to the sponge but to an as yet uncharacterized bacterial symbiont.[196] The confirmation that at least some polyketides in sponges are of bacterial origin should have important biotechnological implications. Development of bacterial production systems, either by cultivating the producer or by expressing symbiont-derived biosynthetic genes in suitable bacterial hosts, is a promising strategy to generate sustainable supplies of rare drug candidates from sponges and other marine animals. Understanding the basic mechanisms underlying symbiosis in sponges will be an important prerequisite for reaching this goal.

Several studies reported natural products from bacteria that had been isolated from sponges. However, in almost all cases, these microbes are taxonomically different from what can be detected in sponges with culture-independent methods. This suggests that the presently available cultivation strategies mainly yield easily cultivable contaminations or food bacteria prior to digestion. In most cases, the compounds from such bacteria are also different from what has been previously isolated from the animals. However, there are a few exceptions (**Scheme 13**): the diketopiperazines **110–112** from the sponge *Tedania ignis* and an associated *Micrococcus* sp.,[202] brominated biphenyl ethers, such as **99**, from *D. herbacea* and a *Vibrio* sp.,[203] and andrimid (**113**) from a *Hyatella* sp. sponge and a *Vibrio* sp. bacterium.[204] Another example, so far only published in a patent, is the cultivation of an actinomycete belonging to the genus *Micromonospora* that produces manzamine A (**114**), an antimalarial alkaloid.[205]

Sponges can harbor eukaryotic organisms in addition to bacteria and archaea. Among these, dinoflagellates have been detected in a number of sponges from marine and freshwater habitats.[206–212] Some sponges also contain natural products similar or even identical to polyether toxins from dinoflagellates. The diarrhetic shellfish poison okadaic acid (**2**) was first isolated from the sponge *Halichondria okadai*[213] and later detected in the free-living dinoflagellate *Prorocentrum lima*.[214] In the Mediterranean sponge *Suberites domuncula*, this polyketide exerts an apoptotic effect against annelids colonizing the animal,[215] and in the freshwater sponge *Lubomirskia baicalensis* from Lake Baikal, which lives under an ice cover during winter, it augments the expression of heat-shock proteins.[209] Since **2** readily accumulates in marine food chains, a similar sequestration mechanism instead of symbiosis could also account for its presence in sponges. However, in *L. baicalensis*, this does not seem to be the case. The sponge exhibits a bright green color due to the presence of dinoflagellates related to *Gymnodinium sanguineum*, and antibodies specific to **2** exclusively react with these endosymbionts.[209] In contrast, a related study using a similar assay reported that bacteria are the source of **2** in *S. domuncula*.[215] Experimental approaches such as cell separation[207] might resolve this apparent contradiction and shed additional light on the biosynthetic source.

Similar to the compounds mentioned above, the identification of the true producer of dinoflagellate-type polyketides might lead to interesting pharmacological applications. One biomedically important natural product that resembles dinoflagellate polyethers is halichondrin B (**115**) (**Scheme 13**), a highly promising antitumor drug candidate first discovered from *H. okadai* (also the source of **2**).[216] Another sponge that contains **115** is *Lyssodendoryx* sp. from New Zealand. One metric ton of this sponge was collected to obtain just 300 mg of halichondrins for preclinical anticancer trials.[217] It has been calculated that if we were to use the natural source to produce a halichondrin-based drug, for only a single year of production the number of *Lyssodendoryx* sp. required would exceed the global biomass of this species.[217] So far, nothing is known about the true producer that could aid in the design of alternative biotechnological production systems. Cloning and expression of PKS genes from dinoflagellates is currently an extremely challenging task, but might be more straightforward in future.

Numerous natural products have been reported from fungi isolated from marine sponges.[218] However, only in rare cases have fungi been observed to actually colonize these animals.[219] The cultivated strains might therefore be accidental contaminations rather than true symbionts.

2.14.7 Cnidarians

Many members of the phylum Cnidaria (sea anemones, corals, jellyfish, and hydrozoans) form symbioses with photosynthesizing dinoflagellates and/or green algae, termed zooxanthellae and zoochlorellae, respectively.[220–222] In reef-building corals, *Symbiodinium* spp. zooxanthellae provide the host with oxygen as well as carbon and nitrogen compounds and contribute to calcification. In the gorgonian *Pseudopterogorgia elisabethae*, a *Symbiodinium* sp. was reported to be involved in the biosynthesis of diterpene glycosides of the pseudopterosin series.[223] The substances, for example, pseudopterosin A (**116**) (**Scheme 14**), possess potent anti-inflammatory and analgesic

120

116

117

118

121

119

Scheme 14

properties and are commercially used as additives in several skin creams.[224,225] Although the dinoflagellate symbiont has not been cultivated yet, its isolation by mechanical means was successful.[223] Such symbiont preparations were shown to incorporate ^{14}C-labeled inorganic carbon as well as tritiated geranylgeranyl pyrophosphate (GGPP) into pseudopterosins, strongly suggesting that the coral host is not the true producer. Elisabethatriene synthase, the diterpene cyclase generating elisabethatriene (**117**) from GGPP, has been isolated from the preparation of entire animals, and its sequence information might now be exploited to localize the gene and determine whether the producer is the dinoflagellate itself or a symbiotic bacterium associated with the *Symbiodinium* sp.[226]

Cultivated members of the genus *Symbiodinium* are the source of a wide range of highly complex natural products, including the vasoconstrictive zooxanthellatoxin A (**118**),[227,228] cytotoxic and vasoconstrictive zooxanthellamides, such as zooxanthellamide B (**119**) and zooxanthellabetaine A (**120**),[229] the osteoclastogenesis inhibitor symbioimine (**121**) (**Scheme 14**),[230] the Ca^{2+}-channel activator symbioramide-C16 (**122**),[229] and zooxanthellamine (**123**) (**Scheme 15**).[229] The last compound is structurally related to zoanthamine (**124**) and related alkaloids from zoanthid corals,[231] suggesting a dinoflagellate origin of these alkaloids. With the

	R_1	R_2	R_3	R_4	R_5
125	CH_3	OH	CH_3	H	OH
126	H	H	H	OH	H

Scheme 15

exception of the zooxanthellamides, which were obtained from a free-living *Symbiodinium* sp., all compounds were isolated from dinoflagellates associated with flatworms (not related to cnidarians).

Another zoanthid-derived polyketide that is likely produced by a dinoflagellate is palytoxin (**125**), an extremely potent neurotoxin from *Palythoa toxica* and several other unrelated animals.[232] The closely related ostreocins, for example, ostreocin D (**126**), have been reported from a free-living dinoflagellate *Ostreopsis siamensis*, indicating a similar source in the animal.[233]

2.14.8 Arthropods

Insects belong to the most intensely studied animal groups regarding associations with microbial symbionts, yet due to the vast diversity of this taxon (more than 750 000 described species), most symbiotic interactions likely remain undiscovered. Many insects harbor intracellular bacteria in specialized tissues termed bacteriomes.[234] Symbiosis with such bacteria, called primary endosymbionts, can be very ancient, and some symbiont genomes belong to the smallest known among prokaryotes due to extensive loss of genetic information during evolution.[235] The record keeper in terms of genomic minimalization is currently *Carsonella ruddii*, a psyllid symbiont with a genome of only 160 kb.[236] One possible mechanism that would enable the maintenance of basic cell functions in such organelle-like bacteria might involve the import of proteins, which are encoded on the host genome, into the symbiont cell. Cultivation of primary symbionts usually fails, and their removal often results in significantly reduced host fitness. In some cases, the latter phenomenon has been traced back to provision of basic nutrients by the symbiont. Examples are aphids and sharpshooters that feed on plant saps and are provided with amino acids and vitamins,[237–239] and *Wigglesworthia glossinidia*, a symbiont of tsetse flies that synthesizes vitamin B_{12}.[240]

Several cases have been reported where symbionts generate secondary metabolites that the insect host uses for chemical protection. In the examples known so far, the bacteria differ from primary endosymbionts in that they are extracellular or even reside on the insect cuticle and do not seem to be obligate for host survival. Rove beetles of the cosmopolitan genera *Paederus* and *Paederidus*, which together encompass ca. 700 species, use the complex polyketide pederin (**109**) as chemical defense.[197] **109** is highly similar to a wide range of polyketides isolated from marine sponges, such as onnamide A (**108**) mentioned above. It has been shown that **109** confers effective protection against wolf spiders to the beetle larvae.[241] The compound is highly cytotoxic and generates a blistering inflammation if beetles are crushed on the human skin.[242] In warm habitats, the insects can swarm in large numbers and cause notorious outbreaks of dermatitis among inhabitants.[243,244] It has even been speculated that three of the 10 biblical plagues, which consisted of large numbers of insects followed by blisters on humans and cattle, were caused by a *Paederus* epidemic.[245] Recent studies demonstrated that the true producer of **109** is a bacterial endosymbiont closely related to *Pseudomonas aeruginosa*.[198,199,246,247] The bacterium is present in ca. 90% of the female beetles and is transmitted via the eggs to the next generation.[248] Thus, males and a small portion of the females lack the symbiont, and the progeny of these females do not contain **109**. Upon egg deposition, pederin-positive females distribute a portion of their stored pederin to each egg along with the symbiont. Therefore, all progeny is protected by the compound even if some do not contain multiplying symbionts. So far, all attempts to cultivate the symbiont have failed. Isolation of the biosynthetic genes from the metagenomic DNA of *Paederus fuscipes* revealed that **109** is produced by enzymes that are almost identical to the PKS responsible for the biosynthesis of sponge-derived onnamides.[196,198,249] Curiously, during sequencing of the gene cluster, gene remnants were found that correspond to the arginine-containing polyketide terminus of onnamide A (**108**), although this moiety is absent in pederin (**109**).[198] This indicates a close evolutionary link between both pathways despite the fact that they occur in extremely distantly related animals from different habitats. Inspection of the biosynthetic locus for pederin (**109**) revealed a large number of pseudogenes involved in gene mobility, suggesting that horizontal gene transfer might be the underlying mechanism for the spread of pederin-like genes in nature.[199] Indeed, compounds related to pederin are present in at least seven marine sponge species in addition to rove beetles.[250] In spite of the highly conspicuous activity of the compounds, so far no free-living bacterial producer has been discovered, indicating that the polyketides provide an evolutionary advantage only in a symbiotic context.

Another example of natural product symbiosis in insects is found in leaf-cutting ants of the tribe attini (see Chapter 2.04). The insects collect fresh leaves as nutrient source for a fungus that they cultivate within their

colony for food.[251] The fungus garden is occasionally attacked by a pathogenic fungus of the genus *Escovopsis*. To prevent infection, *Atta* spp. and *Acromyrmex* spp. ants cultivate yet another symbiont that resides on the ventral part of their cuticle.[252] It has been identified as an actinomycete of the family Pseudonocardiaceae[253] and is thus related to streptomycetes, bacteria that are a prolific source of bioactive compounds. Successful cultivation of the bacterium showed that it produces an antifungal substance against the pathogen and is able to promote the growth of the garden fungus. Conversely, removal of the bacterial symbiont resulted in increased infection rates within the garden.[254] Distinct strains of the symbionts are maintained by individual ant colonies and vertically transmitted by the founding queens, and it has been shown that ant workers can distinguish these colony-specific strains from closely related other bacteria.[255–257] Thus, these fascinating findings suggest that leaf-cutting ants were engaged in agriculture and the use of pesticides long before humans discovered these practices. The structure of the active compound(s) has yet to be reported.

A functionally related and rather bizarre symbiosis with actinomycetes is known from bee wolves, wasps of the genus *Philanthus*. These animals have evolved an unusual feeding behavior. They prey on bees, paralyze them, and place them in subterranean brood chambers as food for the developing larvae. Since the food is kept in the chambers for long periods, contamination by microbes is a serious threat to larval development. To prevent infections, the wasp cultivates a *Streptomyces* sp. bacterium, '*Candidatus* Streptomyces philanthi,' in specialized glands of its enlarged antennae, which is squeezed out and applied to the larval brood chamber prior to oviposition onto the bee.[258,259] The bacterium grows on the walls of the cocoon and effectively suppresses fungal contaminants by producing one or several antibiotics.[260] Cultivation is not yet successful, and nothing is known about the nature of the secondary metabolites involved. The brood chambers of *Philanthus triangulum* are sometimes parasitized by another hymenopteran, the cuckoo wasp *Hedychrum rutilans*. This insect evades recognition by the bee wolf by using host-like hydrocarbons in its cuticular wax.[261]

The desert locust *Schistocerca gregaria* forms enormous swarms consisting of up to tens of millions of individuals that can fly over large distances and cause devastating crop losses. Members of the gut microflora influence the biology of the locust in several interesting ways.[262] Locusts are protected from bacterial infection by the compounds **127, 128, and 129** (**Scheme 16**) present in the gut fluids and fecal pellets.[263] These metabolites are not present in axenic insects, but **127** is formed again after reinfection with *Pantoea agglomerans*, a predominant member of the microbial gut community. Moreover, the feces of axenic specimens do not contain guaiacol (**130**), a component of the aggregation pheromone involved in swarm formation.[264] Intriguingly, *P. agglomerans* and other gut bacteria produce **130** directly from constituents present in the fecal pellet or from vanillic acid (**131**), which is formed from plant lignins by degradation, suggesting that bacteria are an important factor during generation of locust swarms.[264]

An example of symbioses between insects and fungi is parasitism by ascomycete fungi of the genus *Cordyceps*.[265] The microbes invade the insect via their tracheae, form an extensive mycelium, and eventually lead to death of the host, at which stage the fruiting bodies sprout out and produce spores. Some fungal species even modify the behavior of the insect host by causing them to climb plants prior to their death, thereby improving spore dispersal.[266] The fruiting bodies of several *Cordyceps* spp. belong to the most prized agents used in traditional Chinese medicine.[265] Numerous bioactive natural products have been isolated from fruiting bodies and mycelia of *Cordyceps* spp. (**Scheme 16**),[267] for example, the antimicrobial, insecticidal, and antitumoral cordycepin (**132**),[265] cordyheptapeptide A (**133**),[268] the cytotoxic cordycedipeptide A (**134**),[269] the antibiotic cicadapeptin I (**135**),[270] the macrolide (**136**),[271] the bioxanthracene (**137**),[272] cordyol C (**138**) with anti-HSV-1 and cytotoxic activity,[273] the antimalarial cordyformamide (**139**),[274] and the antifungal ophiocordin (**140**).[275] However, the compound with the highest biomedical relevance is cyclosporin A (**141**), an immunosuppressive agent widely used in organ transplants to minimize risks of rejection.[276] The industrial producer of **141** is *Tolypocladium inflatum*, which has been isolated as a free-living fungus. Only much later after its initial discovery it was found that the sexual state of the fungus (teleomorph) is identical to that of the beetle-invading fungus *Cordyceps subsessilis*.[277] As with most microbial natural products, virtually nothing is known about the ecological function of compounds from *Cordyceps* spp.

A number of symbioses exist in which insects use natural products to parasitize insect colonies. Meloid beetles, which contain cantharidin (**142**) (**Scheme 17**) as a blistering repellant,[278] spend one part of their complicated life cycle in nests of hymenopterans.[279] The first larval stage is the mobile triungulin form. In *Meloë* and other genera, these larvae climb flowers and assemble to form multispecimen aggregates with bee-like appearance and an attractive effect on male bees. In *Meloë franciscanus*, the parasite of the solitary bee *Habropoda pallida*, it has been

127 128 129 130 131

132 133 134

138 136

135 137

139

140 141

Scheme 16

demonstrated that the triungulins also produce a chemical cue that mimics the sex pheromone of the female bee.[280] Tricked by these visual and chemical cues, the male bee attempts to copulate, upon which larvae attach to the host and are transported into the nest. In the brood chamber, the meloid larva commences metamorphosis into a second,

142 143 144 145 146 147 148 149 150 151 152 153 154

Scheme 17

maggot-like stage that feeds on eggs, pollen, and nectar. Ultimately, adults emerge from the nest after pupation to mate outside. In addition to meloids, further social parasites of colony-forming insects are known that employ various strategies of chemical communication and deceit. Chemical mimicry and camouflage,[281] such as the use of host-like cuticular hydrocarbons as described above for the cuckoo wasp, is a common strategy adopted by organisms such as beetles,[282–285] crickets,[286] butterflies,[287–289] wasps,[290] ants,[291] and spiders[292] invading the nests of ants, bees, wasps, and termites. Butterfly larvae of the family Lycaenidae, of which hundreds of species live associated with ants, not only chemically mimic the host brood, but also produce alarm pheromones when in distress and nutrient droplets for appeasement. Moreover, Lycaenid pupae can generate various sound signals for communication.[289] There is even evidence that fungi can parasitize termites by adopting an egg-like shape and copying the host's chemistry, which elicits tending behavior.[293] To obtain their cuticular chemistry, some parasites synthesize the compounds themselves (chemical mimicry), while others sequester the hydrocarbons from the host or nest material (chemical camouflage). In the latter situation, the parasites either use an odorless stealth strategy to enter the nest or are sufficiently defended by mechanical means to endure the initial attack.[281] Another strategy of nest-invading parasites is the employment of chemical deterrents. Both chemical camouflage and deterrency can enable the invader to have a wider host range than chemical mimicry. For example, while some myrmecophilous *Formicoxenus* spp. ants are highly host-specific and rely on mimicry, *Formicoxenus nitidulus* can parasitize over 11 different ant hosts by using repulsive compounds preliminarily identified as alkadienes.[294] Slave-making ants, which can usurp entire ant colonies, use camouflage strategies to gain control over nests of a wider range of species.[295]

Many parasites and parasitoids (parasite-like organisms that ultimately kill their host) of solitary insects are known to use host sex pheromones as localization cues. Examples are hexadecanal (**143**) and (*Z*)-7-, (*Z*)-9-, and (*Z*)-11-hexadecenal (**144–146**), from the pheromone blend of *Helicoverpa* (=*Heliothis*) *zea*, the corn earworm moth (**Scheme 17**).[296] These substances attract not only mating partners but also *Trichogramma* spp. wasps, which lay their tiny eggs into the eggs of the hosts. Although there is a significant time lag between copulation and egg deposition, it has been proposed that pheromones are being released long after mating from the leaves of the mating site. A different strategy is used by the wasp *Telonomus calvus*. Attracted by sex pheromones of *Podisus* spp. bugs, they wait at the mating site, ride on the attracted female, and wait until oviposition.[297] In addition, in *Podisus maculiventris* the male sex pheromones (*E*)-2-hexenal (**147**), benzyl alcohol (**148**), linalool (**149**), terpinen-4-ol (**150**), α-terpineol (**151**), and piperitol (**152**) attract tachinid flies that parasitize the

adults.[297] In yet another variant, signals of plants attacked by herbivores are exploited. Feeding by caterpillars induces a series of host reaction including the release of volatile substances by the leaves. The blend of compounds, mainly consisting of terpenoids, indol, and fatty acid degradation products, serves as an alarm call to attract parasitic wasps. An example of such a tripartite interaction is the wasp *Cotesia marginiventris*, which reacts to several compounds emitted by maize, using them as cue to locate and deposit eggs into *Spodoptera* spp. caterpillars.[298] Volicitin (**153**) present in the saliva of these caterpillars has been shown to trigger volatile release.[299,300] While the fatty acid portion of the compound is derived from plant fatty acids, amide formation and hydroxylation occur within the feeding larva.[301] Interestingly, of 23 bacteria isolated from the gut of *Spodoptera exigua* and two other species, 10 were able to catalyze the formation of the proposed intermediate **154** from the amide building blocks.[302] Bacteria might therefore be another partner in this intricate symbiosis.

2.14.9 Nematodes

Nematodes of the genera *Steinernema* and *Heterorhabditis* form a fascinating association with endosymbiotic *Xenorhabdus* and *Photorhabdus* spp. bacteria, respectively.[303,304] The nematodes infect insects and inject the bacteria into their hemolymph. There the bacteria multiply and produce a range of low-molecular-weight compounds[303] and insecticidal proteins.[305,306] The insect usually dies within 48 h and serves the nematodes as nutrient source and habitat for reproduction. After acquisition of new endosymbionts, the nematodes leave their host to search for other prey. The symbionts can be cultivated, and an astonishing diversity of natural products has been isolated from these bacteria. Examples of compounds isolated from *Xenorhabdus* are the antibiotics xenorhabdin (**155**), the indol derivative **156**, and xenocoumacin 1 (**157**) and the cytotoxics phenethylamide (**158**) and xenofuranone A (**159**).[307] Representative compounds from *Photorhabdus* strains are the antibacterial siderophore photobactin (**160**), the antibacterial, antifungal, and nematicidal hydroxystilbene (**161**),[308] and anthraquinones, such as **162**, which are biosynthesized by a type II PKS (**Scheme 18**).[309] There is also good genetic and functional evidence that *Photorhabdus* spp. are able to synthesize carpapenem antibiotics.[310] In addition, genome sequencing of *Xenorhabdus* and *Photorhabdus* spp. strains has revealed a very high number of gene clusters involved in secondary metabolism,

155 **156** **158** **157** **160** **159** **161** **162**

Scheme 18

many of which encode PKSs and nonribosomal peptide synthetases (NRPSs), along with genes for insecticidal proteins, suggesting that these bacteria represent an even richer natural product source than previously expected.[311] The antibiotic activity of many of the isolated compounds indicates that they serve as preservation agents by preventing other organisms from consuming the insect carcass. However, two recent studies showed that some metabolites counteract the insect immune system. *Xenorhabdus nematophilus* produces a chemically uncharacterized inhibitor of phospholipase A_2 and thereby inhibits hemocyte phagocytosis in the caterpillar *S. exigua*.[312] In *Photorhabdus luminescens*, the antibiotic **161** was demonstrated to also suppress similar host defense reactions in *Manduca sexta* through inhibition of phenoloxidase.[308] Interestingly, **161** is so far the only stilbene known from bacteria and is assembled via an entirely different biosynthetic mechanism from plants.[313]

2.14.10 Flatworms

Marine flatworms of the genus *Amphiscolops* contain intracellular *Amphidinium* spp. dinoflagellates, which have been cultivated. These microorganisms produce a large variety of cytotoxic macrolides of the amphidinolide series, for example, amphidinolide A (**163**) (**Scheme 19**).[314] The compounds **118** and **120–123**, discussed in Section 2.14.7, have been described from *Symbiodinium* spp. isolated from *Amphiscolops* sp. flatworms.[227–230] Nothing is known so far about the biological relevance of the substances and the nature of the symbiosis.

2.14.11 Bryozoans

The bryozoan *Bugula neritina* is the source of complex polyketides of the bryostatin series.[315] Particularly high concentrations are present in the larvae and juveniles, where they provide protection against fish predators.[316–319] Bryostatins are potent activators of protein kinase C and exhibit anticancer properties.[315] Bryostatin 1 (**164**) (**Scheme 20**) has reached phase II clinical trials for cancer chemotherapy and gained orphan drug status for

Scheme 19

Scheme 20

combination therapy with taxol. To make clinical tests possible, 13 tons of *B. neritina* were harvested to yield 18 g of **164**.[320] In a search for alternative production methods, a bacterial symbiont was discovered to be consistently present in the pallial sinus, a ring-shaped structure of the larvae.[321] The symbiont was classified by 16S rRNA analysis as a novel member of the γ-proteobacteria, '*Candidatus* Endobugula sertula.' Using a protein kinase C-binding assay, bryostatins were colocalized with microcolonies of 'E. sertula' in buds of the first zooid forming from the settled larva.[319] This study also provided detailed insights into the fate of bryostatins and the symbiont throughout the animal life cycle. In order to achieve vertical transmission to daughter generations of the bryozoan, the symbiont is transferred into the ovicell via funicular cords, structures that connect individual zooids in the colony and play a role in nutrient transport. Bryostatins are deposited on the entire exterior of the larvae and ultimately shed after larval settlement and metamorphosis, while the symbiont migrates to developing buds and finally into the funicular cords of rhizoids, where bryostatin levels rise again. There is further compelling evidence that this bacterium is the source of bryostatins. In one of the few successful examples of FISH using biosynthetic gene probes, rRNA encoding a modular PKS was localized in 'E. sertula.'[322] By treating *B. neritina* with antibiotics, bryostatin levels as well as cell numbers of the symbiont and the strength of the PKS signal decreased. Two versions of a PKS locus were finally isolated from the metagenomic DNA, enriched in symbiont preparations, of two different animal hosts.[323] One contained an intact multimodular PKS gene cluster spanning 77 kb, and the other harbored a virtually identical but physically disconnected gene set on two distinct DNA regions. Isolation of these genes is a biotechnologically significant result, since as with sponge-derived polyketides, expression of the PKS genes in a culturable bacterium could in future provide long-term supplies of bryostatins.

2.14.12 Molluscs

Kahalalide F (**165**) (**Scheme 21**) is a depsipeptide isolated from the sacoglossan mollusc *Elysia rufescens*.[324] The potent antitumor agent has reached phase II clinical trials for a range of cancer types. The compound alters the lysosomal and mitochondrial membranes and induces cell death by oncosis. **165** was also isolated from a *Bryopsis* sp. green alga. The fact that *E. rufescens* feeds on *Bryopsis* sp. suggests that **165** is diet-derived. Indeed, similar cases of sequestration have also been discussed or demonstrated for other natural products in molluscs, such as dolastatin 10 (**166**) from the sea hare *Dolabella auricularia* and the cyanobacterium *Symploca* sp. VP462. However, there is a patent claiming the isolation of a bacterial producer of kahalalides, including **165**, from both *Symploca* sp. and *E. rufescens*.[325] The bacterium was identified as a *Vibrio mediterranei* strain. The inventors speculate that the mollusc acquires the symbiont by feeding on the alga and subsequently maintains it in its body.

The complex polyol durinskiol A (**167**) has been isolated from a *Durinskia* sp. dinoflagellate associated with the sea snail *Chelidonura fulvipunctata*.[326]

2.14.13 Tunicates

A wide range of cyclic peptides with heterocyclic moieties are known from marine didemnid tunicates. More than 60 compounds of this family are known today, and many co-occur in the same animal specimen. The structures of patellamide A (**168**), patellamide C (**169**), ulithiacyclamide (**170**), and lissoclinamide 3 (**171**) (**Scheme 22**) from *Lissoclinum patella* provide an impression about the chemical diversity of this group. The animals establish a symbiosis with as yet unculturable *Prochloron* spp. cyanobacteria that appear as green patches on the host. Two independent studies[327,328] have shown that the symbiont of the tunicate *L. patella* is the true producer of patellamides. Both exploited the fact that the relatively pure symbiont preparations can be obtained by mechanical separation. In one case, the entire genome of the symbiont was sequenced and screened for potential peptide biosynthetic genes.[327] This revealed the presence of a gene, *patE*, portions of which corresponded to the amino acid sequences of **168 and 169**. This suggested that in spite of their unusual structures, the patellamides are not generated by an NRPS but by a ribosomal pathway via excision of the two peptide precursors for **168 and 169** from the same pre-propeptide PatE. The *patE* gene was predicted to be part of a 10 kb gene cluster encoding genes for further peptide processing, and the entire *pat* cluster was successfully

165

166

167

Scheme 21

expressed in *Escherichia coli* to yield the two patellamides.[327] In the second approach to pinpoint the biosynthetic pathway, a BAC library of *Prochloron* sp. DNA was prepared in *E. coli*, and one of the clones was demonstrated to produce two members of the patellamide family.[328] Another publication by the same group that performed the genomic study provided fascinating insights into the evolutionary origin of the peptide diversity found in

168 169 170

171 172

173 174

Scheme 22

tunicates.[329] An analysis of 46 *Prochloron* spp.-containing animals from different species showed that the symbionts contain gene clusters that are virtually identical to each other with the notable exception of the short regions in *patE* encoding the cyclic peptide precursors. Thus, these portions of *patE* represent hypervariable cassettes generated by evolutionary diversification that account for the broad range of peptide structures found in the animals. The study further suggested that the different variants of *pat* gene clusters are present not on the same genome but belong to distinct *Prochloron* spp. strains that colonize the tunicates. Interestingly, evolution was mimicked by introducing an artificial pre-propeptide cassette into *patE*. Subsequent expression in *E. coli* resulted in the production of the expected peptide eptidemnamide (**172**) with a novel structure.[329] Gene

clusters similar to the *pat* system were found to be widespread in tunicate-associated as well as free-living cyanobacteria, where they direct the biosynthesis of numerous cyclic peptides with diverse structures.[330,331] For this novel and biosynthetically distinct group of metabolites the term cyanobactins was proposed.[331]

Similar to sponges, tunicates are the source of numerous further bioactive natural products, many of which resemble bacterial metabolites and have been suggested to be of symbiont origin.[332] However, with the exception of the patellamide-like compounds, their biosyntheses have been poorly studied. Particularly suggestive of a bacterial source is the structure of ecteinascidin 743 (**173**) (**Scheme 22**),[333] a DNA-alkylating agent from *Ecteinascidia turbinata* that has recently been approved for the treatment of cancer. **173** is closely related to a range of substances isolated from free-living bacteria,[332] and one of these, safracin B (**174**) from a pseudomonad,[334] is in fact used as a starting point for semisynthetic preparation of **173**. There is only one study so far on the microbiology of *E. turbinata*.[335] Diverse bacteria have been detected by 16S rRNA analysis and FISH, dominated by the intracellular γ-proteobacterium '*Candidatus* Endoecteinascidia frumentensis.' It is so far unknown whether one of these microbes might be the producer of **173**.

2.14.14 Conclusions

Secondary metabolites play a key role in a myriad of organismal interactions. This chapter can only provide a limited impression about the remarkable diversity of ecological functions, structures, and biosynthetic pathways involved. What is known today very likely represents only a small fraction of the actual extent of symbiotic chemistry. Natural product research is becoming an increasingly interdisciplinary science by embracing fields such as molecular biology, ecology, and molecular evolution, and the present chemo- and bioanalytic methods allow one to characterize and trace molecules with unprecedented sensitivity. With this arsenal of tools, in particular those applicable to the study of uncultivated organisms, the coming years of symbiont research will reveal numerous new and fascinating ways about how small molecules are used in, and even shape, interactions between species.

Abbreviations

AHBA	3-amino-5-hydroxybenzoic acid
AM	arbuscular mycorrhiza
AT	Acyltransferase
CARD-FISH	catalyzed reporter deposition-fluorescence hybridization
DGGE	denaturing gradient gel electrophoresis
FISH	fluorescence *in situ* hybridization
GGPP	geranylgeranyl pyrophosphate
HPLC	high-performance liquid chromatography
NMR	nuclear magnetic resonance
NRPS	nonribosomal peptide synthetase
PCR	polymerase chain reaction
PKS	polyketide synthase
PSP	paralytic shellfish poison
QS	quorum sensing
rRNA	ribosomal RNA
RT-PCR	reverse-transcript PCR

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Biographical Sketch

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2.15 Natural Peptide Toxins

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2.15.1 Introduction

Venomous predatory animals, such as snakes, spiders, scorpions, sea anemones, and cone snails, produce a plethora of peptidic scaffolds that target specific ion channels and receptors as part of their neurochemical and biochemical strategy to capture their prey. In addition to predation, these animals might use their venom for defense. Most of the peptidic components found in the venom are ribosomally expressed as larger protein precursors that are subsequently processed and modified to produce the mature toxins. The genes that are involved in these processes have evolved for exogenous purposes, more specifically, to effectively interact with prey organisms by immobilization, tissue degradation, or by causing deterrence. The mature peptidic components of the venom are naturally devised to possess extraordinary stability and biological potency, as they have to rapidly and effectively interact with the prey's systems to secure survival. Although proteins, including

proteases and lipases, are among the necessary ingredients for prey envenomation, it is the smaller peptidic components that have captured the attention of the natural products community. Their 'small-molecule'-like properties, such as selectivity toward cellular targets, synthetic accessibility, and stability under a variety of conditions, make them suitable as therapeutic agents or as molecular probes to outline cellular processes.

Generally speaking, the peptide toxins from marine animal sources are more 'natural product-like' than their terrestrial counterparts, as on average they are smaller in size and more heavily modified. For this reason, in this chapter, we will concentrate on the peptide toxins expressed by cone snails and sea anemones as quintessential examples of natural peptide toxins suitable for therapeutic uses.

2.15.2 Cone Snails: Distribution, Diversity, Behavior, Feeding, and Defense

Venomous marine gastropods belonging to the genus *Conus* (cone snails) are among the most prolific and versatile peptide engineers known in nature. Cone snails use venom as part of a biochemical strategy to immobilize and capture their prey. These unique marine organisms deliver their complex venom through a specialized radular tooth that serves as both harpoon and disposable hypodermic needle. The venom of cone snails is predominantly composed of highly modified peptidic components (conopeptides) that effectively interact with the prey animals (**Figure 1**).

The fossil record indicates that cone snails have been around for 55 million years, during which evolution and extinction cycles have resulted in the current *Conus* living stock. There are over 700 species of living cone snails described to date.[1] These animals mostly inhabit tropical and subtropical marine habitats around the world in a variety of habitats, ranging from reef formations to deep sandy areas. They are predominant in the Indo-Pacific region; however, they can be found in the Americas in the eastern Pacific ranging from southern California to Peru, and the western Atlantic ranging from Florida to Rio de la Plata (northern Argentina), including the Caribbean Sea. Cone snails are also found in the west coast of Africa and one species inhabits the Mediterranean Sea. They are highly adaptable animals as they can live as deep as 500 m or as shallow as the intertidal areas. Cone snails can be as large as 250 mm (*Conus pulcher*, a western African species) and as small as 4 mm (*Conus Sahlbergi*, a Bahamian species).

Cone snails have been classified into three subgroups according to their prey preference. Some species preferably feed on marine worms (vermivorous) including polychaetes, echiuroids, and hemichordates. The second group primarily hunts mollusks (molluscivorous) and the third group primarily hunts fish (piscivorous). The venom composition is highly optimized to the prey preference of the snail and the environmental variables

Figure 1 *Conus purpurascens*, the only fish-hunting species of the Eastern Pacific region. (a). The animal showing the siphon, eyes, and mouth. (b) The animal swallowing a fish (photograph from the bottom of the animal displaying its muscular foot). Photos courtesy of Nicole Vanderweit and Storm Stilmann, Boca Raton, FL.

of the habitat of a cone snail colony. Thus, conopeptides found in the venom of fish-hunting cones have been evolutionarily optimized for these slow-moving snails, which must immobilize and ingest fast-moving fish for survival in an environment rich in potential competitors. Consequently, these species have evolved a different complement of conopeptides from their worm-hunters and molluscivorous counterparts. The current published database of conopeptides is heavily biased toward components of the venom of fish-hunting cone snails, as bioassay-guided fractionations of the conopeptides were based on vertebrate systems, where the venom of piscivorous cone snails was thought to be most effective. However, the vast majority of *Conus* species are worm-hunters. Several components of the venom of worm-hunting and mollusk-hunting cone snails have been shown to elicit unique neurophysiological responses in vertebrates.

Defensive uses of the venom of cone snails have not been well documented. In a few instances, human fatalities have resulted from the stings of these animals;[2] it can be argued that this is the result of defensive maneuvers of the snail. However, most *Conus* species are not sufficiently aggressive to display such behavior. Nevertheless, these animals should not be handled by nonexperts and all precautions must be taken when handling live cone snails.

2.15.3 Molecular Diversity of Cone Snail Venom

2.15.3.1 General Features

The precise composition of cone snail venom is species-specific[3–5] with significant intraspecies variability.[6,7] The venom is an extremely complex concoction (20–200 components) of modified peptides (conopeptides) that elicit a wide range of strong neurophysiological responses in a variety of organisms.[3–5,8,9] The development of such extremely potent and biochemically diverse venom is likely to be an evolutionary adaptation designed to compensate for the lack of mobility of cone snails when compared to other marine predators.

The enormous molecular diversity of the venom components of cone snails ranges from small molecules[10] to larger protein toxins.[11] The first active molecular component identified from *Conus* venom was serotonin in 1972.[10] Although serotonin appears to be involved in the envenomation mechanism of some *Conus* species,[12] the bulk of cone snail venom is a complex mixture of conopeptides with unique characteristics. Unlike the larger neurotoxin peptides found in other venomous animals (such as snakes, spiders, and scorpions) (see Chapter 5.10), most conopeptides vary in length from 6 to 30 amino acids. Some conopeptides are among the smallest polypeptide-based compounds with a well-defined three-dimensional structure. Conopeptide sequences have very little homology with the existing sequences of peptides and proteins of non-*Conus* sources. Most conopeptide sequences and the rest of their covalent structures have been established by traditional Edman degradation protocols along with biochemical and spectrometric determinations. However, by extracting the mRNAs from the venom and producing their corresponding cDNA libraries, genes encoding for the venom precursors can be outlined and several 'unmodified' conopeptide sequences can be deduced via mRNA-based libraries.[13–17]

2.15.3.2 Posttranslational Modifications

Ribosomally expressed peptide toxins are, by default, posttranslationally modified, as modifications are inherently required for the production of the mature toxin. Cleavage of the precursor during processing typically does not result in unusually modified amino acids *per se*. However, the action of processing enzymes can lead to modified amino acids in the mature toxins that impart stability or target selectivity that characterizes a particular class of peptide toxin. Inherently, most venomous predatory animals produce peptidic scaffolds constrained by disulfide bonds in a diversity of patterns that define highly stable miniprotein scaffolds. This is through the action of the enzyme protein disulfide isomerase, which can catalyze the oxidative folding of cysteine residues.

However, in addition to the presence of disulfide bonds, most conopeptide sequences exhibit a high degree of 'additional' posttranslational modifications (PTMs), which include carboxylation of glutamate to form carboxyglutamate and hydroxylation of proline, lysine, and D-valine to form γ-hydroxyproline (Hyp), δ-hydroxylysine, and D-γ-hydroxyvaline, respectively. Other PTMs include epimerization of L-amino acids to form their respective D-amino acid counterparts, halogenation of tryptophan to produce 5-Br-tryptophan

(Br-Trp), sulfation of tyrosine to produce sulfotyrosine (Tyr-SO_3), glycosylation of serine and threonine, and N- and C-terminal modifications to produce pyroglutamate and amidated C-termini, respectively.[3,4,8,18,19]

The presence of so many different types of PTMs in the venom of one genus is not surpassed by any other predatory venomous animals. In fact, some of these modifications are unique to cone snails. These modifications are found in all conopeptide scaffolds, usually as combinations of PTMs. For example, bromocontryphan-R has been described as having the highest density of PTMs among known gene-encoded polypeptides,[20] as this eight-residue peptide has five different modified amino acids within its structure. The biosynthesis of this particular conopeptide closely resembles that of classical natural products, as it is produced through a specialized biosynthetic pathway comprising several enzyme-catalyzed steps, in spite of its ribosomal origin.

The carboxylation of glutamate to produce γ-carboxyglutamate (Gla) is particularly notable. Polycarboxylated linear conopeptides, such as the conantokins (**Table 2**), are capable of binding Ca^{2+} ions and acquiring highly stable helical secondary structures. In this particular case, polycarboxylation is the defining feature of this family of conopeptides. Single and double carboxylations sparingly occur in several conopeptide families (**Table 2**) without having the same defining effect as in the conantokin family. Carboxylation as a PTM has been observed in humans,[53,54] *Aplysia californica*[55] (D-peptide from the insulin prohormone), and in cone snails (in an assortment of conopeptides in the venom).

2.15.3.3 Cystine-Constrained Scaffolds

Cystine-constrained peptidic scaffolds are recurrent features found in the venom of all predatory animals.[56] The number of constraints ranges from a single disulfide bond to beyond six disulfide bonds. Conotoxins are operationally defined by peptidic scaffolds in the venom of cone snails with two or more disulfide bonds. Conopeptides with one disulfide bond have been found in the venom of cone snails, and they are given trivial names such as contryphans and conopressins.

In general, peptides with one disulfide bond tend to be extremely stable and resistant to digestion. In these compounds, the flexibility of the main chain is dramatically reduced by the presence of the disulfide bond. Additionally, these peptides exhibit a high degree of intramolecular hydrogen bonding, which confers them significant structural stability and target selectivity.[57] Peptides with one disulfide bond can serve as antibiotics, toxins, ion-transport regulators, protein-binding inhibitors, enzyme inhibitors, hormones, and immunosuppressants. Just as other conopeptides, one-disulfide conopeptides inherently contain high degrees of modified amino acids (usually combinations of them), such as γ-carboxyglutamate, Br-Trp, D-Trp, D-Leu,[18] pyroglutamate at the C-terminus, N-terminus amidation, and hydroxyproline.[58]

Conopressins are vasopressin/oxytocin-related peptides found in the venom of cone snails (**Table 2**).[59,60] Conopressins were originally discovered by bioassay-guided fractionation of venom, where upon intracerebroventricular (ICV) injections in mice, these compounds induced an 'intense scratching' effect.[59] The molecular target of conopressins has remained unclear; however, very recently it was found that conopressin-T acts as a selective antagonist at the human V_{1a} receptor.[61] Furthermore, the L7P analogue of conopressin-T has an increased affinity for the V_{1a} receptor and a weak V_2 receptor binding. Through structure–activity relationship (SAR) studies on conopressin-T, it was surprisingly found that when Gly9 was replaced with Val9 in oxytocin and vasopressin, these hormones can undergo an agonist/antagonist switch at the V1a receptor.[61] The discovery and characterization of a uniquely modified conopressin from the venom of *Conus villepinii*,[62] a vermivorous cone snail species from the western Atlantic Ocean, has been described. This novel peptide, designated γ-conopressin-vil (as it has γ-carboxyglutamate in the eight residue position), undergoes structural changes in the presence of calcium. This suggests that the peptide binds calcium, and the calcium-binding process is mediated by the carboxyglutamate residue.

The contryphan family of cyclic peptides, isolated from various species of cone snails, have the conserved sequence motif H_3N^+-X^1COD-WX^5PWC-NH_2, where X^1 is either Gly or absent, O is 4-*trans*-hydroxyproline, and X^5 is Glu, Asp, or Gln. The contryphans possess a distinctive number of PTMs that include tryptophan bromination, proline hydroxylation, glutamate carboxylation (γ-carboxyglutamate), C-terminal amidation, and leucine and tryptophan, L to D, isomerization.[63]

The molecular target of contryphans has not been determined. Some of the modulatory effects of these compounds have been described. For example, contryphan-Vn, a member of the contryphan family, modulates

Table 1 Conotoxin classification by superfamilies and frameworks

Superfamily	*Cystine arrangement*	*Frame*	*Families*	*Examples*
A	CC-C-C	I/II	α, ρ	GI, TIA
	CC-C-C-C-C	IV	αA, κA	EIVA, SVIA
F	C-C-C-C	14	TBD	vil14a
J	C-C-CXC	14	κ, α	pl14a
L	C-C-CXXC	14	α	lt14a
M				
m-1	CC-C-CXCC	TBD		
m-2	CC-C-CXPCC	12/III		mr3a
m-3	CC-C-CXXPCC	TBD		
m-4	CC-C-C----CC	III	μ, ψ	PIII, PIIIE
	C-C-CC	16		Lt16.1
	CCC-C-C-C	TBD		Vx2
O	C-C-CC-C-C	VI/VII	δ, μO, ω, κ, γ	TxVIA, MrVIB, MVIIA, PVIIA
	C-C-CC-CXC-C-C	15		Lt15.1
P	C-C-C-CXC-C	IX	Spastics	Tx9a
	C-C-C-CXC-C			reg9a-c, bru9a
TBD	C-C-C-CCXC	17		flf17a-c
TBD	CC-CC-CC	18		pri18a
TBD	C-CC-C-CXC	19		flf19a
TBD	C-C-C-CC-C-C-C	13		De13a
S	C-C-C-C-C-C-C-C	VIII	σ	GVIIIA
T	CC-CC	V/X	τ, χ	TxIX, MrI
I	C-C-CC-CC-C-C	XI		κBtX
	C-C-CSC-CC-C-C			GlaMrIV
D	C-CC-C-CC-CXC-CXC	XII	αD	VxXIIA-C
E	CC-C-C-C-CC-C-C-C	21		pur21a
V	C-C-CC-C-C-CXC	15		vi15a

TBD = to be determined.

the activity of both voltage-gated and calcium-dependent K^+ channels.[64] Glacontryphan-M, a contryphan isolated from *Conus marmoreus* and which contains carboxyglutamate residues, possesses calcium-binding properties. Glacontryphan-M blocks L-type voltage-gated calcium ion channel activity in a calcium-dependent manner.[23]

The premier components of venom of cone snails are the conotoxins. Most conotoxins contain either two or three disulfide bonds. Several conotoxin frameworks constrained in a variety of patterns (including knotting) have been identified (**Table 1**). Multiple disulfide bonds provide conotoxins with unusual structural stability that makes them difficult to degrade, as they typically cannot unfold with the same ease as their nonconstrained counterparts. Furthermore, location of Cys residues along the sequence and disulfide pairing impart additional structural diversity, which allows conotoxins to effectively target a vast array of different ion channels and G-protein-coupled neuronal receptors present in their prey organisms.

α-Conotoxins[65] and ρ-conotoxins,[47] which belong to the A-superfamily, ε-conotoxins[45] and χ-conotoxins,[48] which are members of the T-superfamily, and framework 14 (F14) conotoxins are examples of two-disulfide conotoxins with different selectivities. Among the determining factors of conotoxin selectivity are loop size variability and disulfide pairing. For example, the α-conotoxins contain two disulfide bridges (first to third Cys and second to fourth Cys) forming two loops of variable size and specifically inhibit either the muscular or the neuronal nicotinic acetylcholine receptors (nAChRs)[66] depending on their loop size subtype. $\alpha 3/5$-Conotoxins block the muscular subtype nAChRs, whereas the $\alpha 4/7$-conotoxins block the neuronal subtype nAChRs. α-, ε-, and ρ-conotoxins have a ^{1}Cys-^{3}Cys/^{2}Cys-^{4}Cys connectivity and they target different receptors. In contrast, χ-conotoxins have a ^{1}Cys-^{4}Cys/^{2}Cys-^{3}Cys connectivity and they target the NE transporter. The F14 conotoxins have a ^{1}Cys-^{4}Cys/^{2}Cys-^{3}Cys pairing (the same connectivity as χ-conotoxins); however, unlike the other four-cystine conotoxins, the F14 conotoxins have all four cystine residues spaced out by loops (no vicinal Cys residues).

Several conotoxin frameworks with three disulfide bonds have been described (**Table 1**). These frameworks also differ in the distribution of Cys residues along their sequence, the length of the inter-Cys loops, and the pairing of cystine residues. However, given the number of Cys residues, there are 15 possible combinations for pairing, most of which have not been observed to date. The most common pairing of Cys residues in conotoxin frameworks is the 'sequential' pairing where ^{1}Cys is bonded to ^{4}Cys, ^{2}Cys to ^{5}Cys, and ^{3}Cys to ^{6}Cys; however, other parings are commonly observed in the mini-M conotoxin subclass. Cystine knotting,[67] where at least one disulfide bond loops through the other two to form a 'knot', has been described for O-superfamily (frameworks VI and VII), P-superfamily (framework IX), and M-superfamily (framework III). These ultrastable scaffolds have been observed in the peptide toxins of other animals such as spiders and sea anemones and even in plant toxins such as the cyclotides.

Conotoxin frameworks VI and VII have a C-C-CC-CC arrangement of Cys residues with a sequential disulfide pairing, which is also found in spider toxins[68] and in the venom of scorpions.[69] These particular frameworks form stable cystine knots with variable loop lengths. The sequence of amino acids within the loops defines the targeting and pharmacology of a particular framework VI/VII conotoxin.

Conotoxins belonging to framework III have a CC-C-C-CC arrangement of Cys residues with a diverse disulfide pairing. This particular arrangement of Cys residues is almost unique to conotoxins. Several subclasses within framework III have been described. The maxi-M subclass is the most studied, and they have a sequential disulfide pairing, with four or more residues in their last loops. Several targets have been identified in the maxi-M subclass: voltage-gated sodium channels for the μ-conotoxins,[70] voltage-gated potassium channels for the κM-conotoxins,[43] and nAChR for the ψ-conotoxins.[32] Mini-M conotoxins, also designated framework III (in spite of the differences compared to the maxi-M subclass), share the same arrangement of Cys in their sequence as the maxi-M or the M4 subclass. However, the loops in the mini-M are much shorter and the Cys pairing can be different from the classical Cys knot observed in the maxi-M subclass. The Cys pairing of the m2 mini-M conotoxins determined so far is different from the m4/maxi-M subtype (^{1}Cys-^{4}Cys, ^{2}Cys-^{5}Cys, ^{3}Cys-^{6}Cys) and the m1 mini-M subtype (^{1}Cys-^{5}Cys, ^{2}Cys-^{4}Cys, ^{3}Cys-^{6}Cys).[71] The shorter lengths in the loops and the different disulfide pairing in the m2 mini-M conotoxins will dictate a scaffold that is substantially different from the m4/maxi-M conotoxins, that is, shorter loops in the mini-M and different Cys pairing hinder the formation of a classical Cys knot observed in the maxi-M subclass. Likewise, the mini-M conotoxins of the m1 and m3 subtypes differ from the m2 subtype because of their shorter loop lengths.

Another group of three-disulfide conotoxins can be found within the A-superfamily. The αA- and κA-conotoxins contain three disulfide bonds with three loops but different disulfide pairing and loop sizes. They also have different molecular targeting: nAChR for the αA-conotoxins versus potassium channels for the κA-conotoxins.[72]

2.15.3.4 Conopeptide Gene Superfamilies

The genes that express conopeptide precursors are divided into three segments: the signal region, the propeptide region, and the mature toxin. As with other protein precursors, the signal region is a highly hydrophobic segment initiated with a codon for Met. The signal region defines members of a conopeptide superfamily, as this region is highly conserved among conopeptide families that show the same structural scaffold; that is, ω-, μ-, δ-, and κ-conotoxins all belong to the O-superfamily (regardless of their pharmacology) as they all share the same signal sequence. They also share a characteristic C-C-CC-C-C arrangement of Cys residues with a sequential disulfide pairing as the structural framework. However, signal sequences within a superfamily can also encode for different structural frameworks, such as in the case of the A- and M-superfamilies.

The conserved signal sequence in conopeptide superfamilies allows the discovery of conopeptides from a single snail specimen. cDNA libraries can be produced by extracting the mRNA from the *Conus* venom duct and amplifying the corresponding cDNA by PCR using primers based on the known signal sequences or conopeptide sequences (in the case of new superfamilies). The procedures to generate these libraries are standard protocols; however, the efficiency of the approach using genomic analysis of cDNA libraries can be less effective (as it does not address PTMs and it could be costly and more labor-intensive) than the

direct bioanalytical screening approach. However, these approaches are complementary and they have been widely adapted to conopeptide discovery processes. Unlike native conopeptides, cDNA-generated sequences require synthetic material for their biochemical characterization.

2.15.4 Neuropharmacology of Cone Snail Toxins

2.15.4.1 α-Conotoxins

The first toxins to be isolated from the venom were the α-conotoxins, so-called because they have the same site of action as α-neurotoxins isolated from snake venom that target the muscular subtype nAChR. Their structure and function have been extensively reviewed.[73,74] The nAChR is part of the ligand-gated ion channel superfamily that includes the $GABA_A$, serotonin, and glutamate receptors. Nicotinic receptors are composed of five subunits; each consists of four transmembrane helices. Although some are homopentameric receptors assembled from one subtype, the majority are heteromeric structures generally consisting of two α-subunits and three non-α-subunits (δ, γ, or ε). The ACh ligand-binding site is located at the interface between α- and non-α-subunits, in the case of homopentameric receptors between two α-subunits, and requires two ACh molecules to bind before opening (**Table 2**).

α-Conotoxins show selectivity toward different subtypes of nAChRs, including both the neuromuscular and neuronal types.[65] Several subfamilies of α-conotoxins have been characterized, the most common of which is the α4/7-conotoxins that target every class of nicotinic receptor, from the muscle subtype, to both homomeric and heteromeric neuronal subtypes. The α3/5 subfamily, found in the majority of fish-hunting species, includes paralytic toxins that target the muscle nicotinic receptor subtype, with many able to differentiate between the two binding sites.[73] The binding of only one toxic molecule is sufficient to block function.[74] The α4/3 subfamily is found primarily in *Conus* species that prey on polychaete worms and may specifically target neuronal nicotinic receptors.[28,75]

The α-conotoxin GIC from *Conus geographus* has a 4/7 spacing and in contrast to the α3/5-conotoxin GI isolated from the same mollusk shows no paralytic activity in mice or fish.[76] The peptide has no detectable effect on human muscle nicotinic receptors. GIC however has the highest known selectivity for neuronal subtype compared with muscle subtype potently blocking the $\alpha3\beta2$ subtype. It is not clear why a fish-hunting mollusk would produce a nonparalytic toxin that targets the neuronal nAChR when it is attempting to paralyze the prey. However, $\alpha3\beta2$ receptors exist in autonomic ganglia where they aid in the fight-or-flight response; therefore, this toxin may act to suppress this system.[76]

Six α-conotoxins have been isolated from *C. geographus*, two of which target the neuronal nAChRs, suggesting that both muscle and neuronal subtypes are important in prey capture. Often multiple α3/5-conotoxins are found within the venom of one mollusk, although the reason for having multiple toxins targeting one receptor is not clear.

Small changes in the amino acid sequence can alter the subtype specificity and can result in toxins that target the same receptor subtype at different sites.[77] For example, ImI and ImII, which share 9 out of 12 amino acids, both inhibit the α7 nAChRs; however, only ImI prevents the binding of α-bungarotoxin, suggesting that the functional binding sites of the two toxins are different.[75] The presence of a proline at position 6 of the peptide appears to play an important role in the ability of ImI to prevent α-bungarotoxin binding.[77] PnIA and PnIB isolated from *Conus pennaceus* have 14 out of 16 amino acids in common but block $\alpha3\beta2$ and α7 receptors, respectively.[78] The α-conotoxins present in the venom target not only distinct receptor subtypes but also distinct sites on a single nAChR subtype.

PTMs are often observed in the conotoxin sequence and are thought to contribute to the diversity of α-conotoxins. One such PTM is the sulfation of a tyrosine residue, found in EpI, PnIA, PnIB, AnIA, AnIB, and AnIC.[79–81] Although the function of this tyrosine sulfation is currently not known, it may improve the stability or solubility of the protein.[79] Tyrosine sulfation and amidation improve the activity of AnIB at the α7 nAChR subtype, indicating that PTMs may aid subtype selectivity.[79]

Another α-conotoxin found in *C. geographus* has a previously unseen N-terminus tail that includes the four amino acids Ile, Arg, Asp, and a posttranslationally modified γ-carboxyglutamate.[82] The structure of GID is thought to enable the peptide to target a broader range of nAChR subtypes, with the N-terminus thought to

Table 2 Sequences of selected conopeptides isolated from cone snail venom

Name	*Sequence*	*Species*	*Prey*	*Target*
Nondisulfide-rich single S–S				
Contryphans				
Contryphan-R[21]	GCOWEPWC*	***Conus radiatus***	p	TBD
Leu-contryphan-P[22]	GCVLLPWC	***Conus purpurascens***	p	TBD
Glacontryphan-M[23]	NγSγCPWHPWC*	***Conus marmoreus***	m	Ca^{2+} channel (L-type)
Conopressins				
Lys-conopressin-G[24]	CFIRNCPKG*	***Conus geographus***	p	Vasopressin receptor
Arg-conopressin-S[24]	CIIRNCPRG*	***Conus striatus***	p	Vasopressin receptor
Nondisulfide-rich linear conopeptides				
Contulakins				
Contulakin-G[25]	ZSEEGGSNAŦKKPYIL	***C. geographus***	p	Neurotensin receptor
Conantokins				
Conantokin-T[26]	GEγγYQKMLγNLRγAQVKKNA*	***Conus tulipa***	p	NMDA receptor
Conantokin-G[26]	GEγγLQγNQγLIRγKSN*	***C. geographus***	p	NMDA receptor
Conorfamide				
Conorfamide-Sr1[27]	GPMGWVPFYRF*	***Conus spurius***	p	RFamide receptor
Disulfide-rich (two or more disulfides): conotoxins				
A-superfamily				
α-Conotoxins				
α4/6				
AuIB[28]	--GCCSYPPCFATNPD-C*	***Conus aulicus***	m	nAChR (α3β4)
α-4/4				
BuIA	--GCCSTPPCAVLYC*	***Conus bullatus***	(*)	nAChR(α6/α3β2)
α-3/5				
GI[29]	--ECCNPA-CGRHYS-C*	***C. geographus***	p	nAChR(α/δ)
MI[30]	-GRCCHPA-CGLNYS-C*	***Conus magus***	p	nAChR
αA-Conotoxins				
PIVA[31]	GCCGSYONAACHOCSCKDROSY-CGQ*	***C. purpurascens***	p	nAChR (α/δ;α/γ)
EIVA[32]	GCCGPYONAACHOCGCKVGROOYCDROSGG*	***Conus ermineus***	p	nAChR (α/δ;α/γ)
κA-Conotoxins				
SIVA[33]	ZKSLVPSVITTCCGYDOGTMCOOCRCTNS*	***C. striatus***	p	K^+ channel
MIVA[34]	AOγLVVŦAŦTNCCGYNOMTICOOCMCTYSCOOKRKO*	***C. magus***	p	K^+ channel

O-superfamily				
ω-Conotoxins				
GVIA[35]	**C**KSOGSS**C**SOTSYN**CC**-RS**C**NHWTKR**C**Y*	***C. geographus***	p	Ca^{2+} channel
MVIIA[36]	**C**KGKGAK**C**SRLMYD**CC**TGS**C**RS--GK**C***	***C. magus***	p	Ca^{2+} channel
CVID[37]	**C**KSKGAK**C**SKLMYD**CC**SGS**C**SGTVGR**C***	***Conus catus***	p	Ca^{2+} channel
κ-Conotoxin				
PVIIA[38]	**C**RIONQK**C**FQHLDD**CC**SRK**C**NRFNK-**C**V	***C. purpurascens***	p	K^{+} channel
δ-Conotoxin				
PVIA[39]	EA**C**YAOGTF**C**GIKOGL**CC**SEF-**C**LPGV**C**FG*	***C. purpurascens***	p	Na^{+} channel
μO-Conotoxins				
MrVIA[40]	A**C**RKKWEY**C**IVPIIGFIY**CC**PGLI**C**GPFV**C**V	***C. marmoreus***	m	Na^{+} channel
M-superfamily				
μ-Conotoxins				
GIIIA[41]	RD-**CC**TOOKK**C**KDRQ**C**KOQR-**CC**A*	***C. geographus***	p	Na^{+} channel
Mini-M conotoxins				
Mr3a[42]	G**CC**GSFA**C**RFG**C**VO**CC**V*	***C. marmoreus***	m	TBD
κM-Conotoxins				
RIIIK[43]	LOS**CC**SLNLRL**C**OVOA**C**KRNO**CC**T*	***C. radiatus***	p	K^{+} channel
ψ-Conotoxins				
PIIIE[32]	HOO**CC**LYGK**C**RRYOG**C**SSAS**CC**QR*	***C. purpurascens***	p	nAChR
S-superfamily				
σ-Conotoxins				
GVIIIA[44]	G**C**TRT**C**GGOK-**C**TGT**C**T**C**TNSSK**C**G**C**RYNVHPSGWG**C**G**C**A**C**S*	***C. geographus***	p	5-HT_3R
T-superfamily				
TxIX[45,46]	γ**CC**γDGW-**CC**~~T~~AAO	***C. textile***	m	TBD
χ-Conotoxins				
MrIA[47–49]	NGV**CC**GYKL**C**HO**C**	***C. marmoreus***	m	NE transporter
P-superfamily				
Tx9a[50]	G**C**-NNS**C**QγHSD**C**γSH**C**I**C**TFRG-**C**GAVN*	***Conus textile***	m	TBD
I-superfamily				
ViTx[51]	SR**C**FPPGIY**C**TSYLP**CC**WGI--**CC**ST-----**C**RNV------- **C**HLRIGK	***C. virgo***	V	K^{+} channel
rg11a[52]	**C**QAYGES**C**SAVVR**CC**DPNAV**CC**QYPEDAV**C**VTRGY----- **C**RPPATVLT	***Conus regius***	V	TBD

p = piscivorous; m = molluscivorous; v = vermivorous; W = bromotryptophan; Ŷ = (SO4); γ = Gla; * = amidated; O = hydroxyproline; strikethrough = glycosylated; Z = pyroglutamate; TBD = to be determined.

stabilize the binding at $\alpha7$ and $\alpha3\beta2$ receptors at low nanomolar potency, and also contribute to the $\alpha4\beta2$ activity of the peptide.[82] The sequence of GID contains another PTM, namely hydroxyproline.

When studying the target specificity of α-conotoxins, the expression of single nAChR isoforms in the *Xenopus* oocytes has been vital for the advancement of the field.[73] The system is suitable due to the high efficiency in protein expression, absence of endogenous nAChR subunits, and the ease in which subunit combinations are formed.[83] However, the functional properties of the expressed channels in the oocytes can differ from those found in mammalian cell lines.[83] For example, α-conotoxin EpI shows no significant activity at the oocyte-expressed $\alpha3\beta4$ and $\alpha3\beta2$ subtypes, but blocks $\alpha7$ subtypes.[83] These results differ from those previously found in the native intracardiac ganglion neurons system.[80]

The variability in these results may be due to the different membrane lipid composition of the oocytes as well as differences in maturation and folding events.[74] Even nAChRs found in mammalian cell lines differ from native receptors. Interactions with other membrane proteins, adapter proteins, or cytoskeletal elements, which may not be present or sufficiently expressed in the nonneuronal oocytes, may be important in modulating receptor activity.[74]

Neuronal nAChR subtypes have an active role in neuronal growth, development and plasticity,[84] and neuronal signal modulation.[85] When nicotine, a nonselective nAChR agonist, is administered, anxiolytic and cytoprotective effects are observed.[74] High doses of nicotine have been shown to be beneficial to individuals with cognitive and attention deficits, Parkinson's disease, Tourette's syndrome, and ulcerative colitis and schizophrenia.[85]

2.15.4.2 μ-Conotoxins

The first μ-conotoxin isolated from a *Conus* species was μ-GIIIA, from the fish-hunting *C. geographus*.[86] The μ-conotoxins block receptor site I of the voltage-gated sodium channel, preventing the influx of sodium into the cell.[87] The voltage-gated Na^+ channels are extremely important in action potential generation, with Na^+ influx being responsible for the upstroke during depolarization. Site 1 is the site of interaction of the classically characterized Na^+ channel toxin, tetrodotoxin (TTX). However, in contrast to TTX, μ-conotoxins are thought to bind more superficially to the ion channel, physically blocking the channel pore. The importance of positively charged amino acids for the biological activity of this particular class of conotoxins has previously been demonstrated, with the Arg13 thought to be particularly crucial in GIIIA.[88–90]

The μ-conotoxins have been used substantially as probes to gain greater understanding of Na^+ channel structure–function relationships.[73] Studies using GIIIA demonstrated that the four repeats within the α-subunit of all Na^+ channels have a clockwise orientation.[91]

Mutant versions of μ-conotoxins also provide further information about the structure and function of Na^+ channels. A mutant μ-conotoxin GIIIA was used to demonstrate that a conformational change in the P-loop of domain IV occurs during the activation of the channel.[92] Modification of remote parts of the peptide, away from the active center of μ-conotoxin GIIIA, such as the addition of functional tags to Cys5-GIIIA, also provides additional useful information, with new analogues aiding analysis of the importance of vestibular structure of sodium channels.

2.15.4.3 ω-Conotoxins

ω-Conotoxins inhibit neuronal voltage-sensitive calcium channels (VSCCs), which are important in a large number of mammalian physiological processes.[93] They have desirable therapeutic potential because of the high level of selectivity they exhibit for specific calcium channel types. A number of these, ω-conotoxin MVIIA, TVIA and GVIA, have been shown to be highly selective for N-type calcium channels, whereas others such as ω-conotoxin MVIIC selectively inhibit both P- and Q-type calcium channels.[94] The presence of a number of basic residues within their structure is important for their biological activity.[95]

There is a great pharmaceutical interest in ω-conotoxins such as ω-conotoxin MVIIA that selectively inhibit N-type calcium channels. The N-type VSCCs play integral roles in the release of neurotransmitters in the spinal cord, the inhibition of which causes attenuation of neuropathic pain response[96] with subjects less prone to tolerance when compared to morphine.[97]

Nonpeptide analogues of ω-conotoxin MVIIA have demonstrated comparable N-type VSCC interaction efficiency.[98] A better understanding of the pharmacophore of ziconotide may lead to the development of truncated peptides or peptidomimetic analogues.[99]

Another ω-conotoxin CVID isolated from *Conus catus* has been shown to have similar potency as ω-conotoxin MVIIA but lower levels of toxicity at comparable therapeutic levels,[100] and has been shown to have potential as an antinociceptive agent.[37] The highly selective ω-conotoxin GVIA, isolated from *C. geographus*, demonstrated 3–4 times greater *in vivo* potency when compared to MVIIA and CVID although it dissociates slowly and may be difficult to administer clinically.[100–102]

2.15.4.4 Conantokins

Conantokins are linear conopeptides 17–27 residues in length that contain multiple γ-carboxyglutamate residues in their sequence. Gla is produced by the γ-carboxylation of glutamate, which is catalyzed by vitamin K-dependent γ-glutamyl carboxylase.[103] Conantokins Con-G, Con-R, and Con-T have been isolated from fish-hunting species, and they typically contain four or five Gla residues. Because of the lack of disulfide bonds in the sequence of conantokins, the presence of Gla is important for the formation of a helical structure. The binding of calcium ions to these peptides leads to a conformational change in their structure thought to be important for their bioactivity.[104]

Conantokins bind to the *N*-methyl-D-aspartate (NMDA) receptor, one form of ionotropic glutamate receptor. The therapeutic potential of clinically available NMDA receptor antagonists is currently limited because of the prevalence of undesirable side effects, thought to be a result of a lack of specificity.

2.15.4.5 Other Conopeptides

Several groups of conopeptides that are homologous to endogenous neuropeptide families have been isolated from the venom of cone snails. First, a family of one-disulfide conopeptides initially discovered in the venom of *C. geographus* and *Conus striatus* were found to be invertebrate vasopressin/oxytocin homologues. It was determined that conopressins are ubiquitous endogenous peptides in a host of invertebrates. Conopressins act as vasopressin receptor agonists.[59] However, the functional role of conopressins in the venom of cone snails is currently unclear.

The next class of endogenous peptides discovered in *Conus* venom was the contulakins,[25] which are peptide homologues to neurotensin. The contulakins are currently the only member of the neurotensin family to have been isolated from an invertebrate and bind to three known neurotensin receptor subtypes with a significantly higher potency than neurotensin itself and therefore have therapeutic potential for pain prevention.

The conorfamides, isolated from *Conus spurius*, belong to the RFamide neuropeptide family and may act as an agonist of the FMRF-amide-gated ion channels.[27] In invertebrates, this peptide family has many diverse functions, whereas in the mammalian system they moderate opioid function in the CNS,[105] modulate epithelial Na^+ channels,[27] have important cardiovascular effects, and stimulate pancreatic somatostatin secretion.[106]

Conomap-Vt is an excitatory peptide that has significant sequence homology to peptides of the myoactive tetradecapeptide (MATP) family. MATPs are important endogenous neuromodulators in mollusks, annelids, and insects. It has been found that Conomap-Vt is the first member of the MATP family with a D-amino acid. The isomerization of L-Phe to D-Phe enhances the biological activity of the peptide, suggesting that this posttranslationally modified conopeptide may have evolved for prey capture.

Conolysin-Mt is a linear amphoteric conopeptide with cytolytic properties.[107] The mechanism whereby this peptide disrupts cellular membranes has been compared to that of melittin, a well-characterized component of bee venom. However, conolysin-Mt is capable of selectively disrupting eukaryotic membranes as opposed to bacterial ones.

Other conopeptides include the bromotryptophan-containing σ-conotoxin GVIIA, a high-affinity antagonist of the 5-hydroxytryptamine (5-HT_3) receptor,[44] and the contryphans, whose neuronal target has yet to be determined[21] although contryphan-Vn has been shown to modulate Ca^{2+}-activated K^+ channel activity.[108] The recently isolated ρ-conopeptide TIA, from *Conus tulipa*, and χ-MrIA and MrIB, isolated from *C. marmoreus*, conotoxins are the first examples of peptides that selectively target the α1-adrenoceptors and the noradrenaline

transporter, respectively.[47] They have therapeutic potential as they do not inhibit Na^+ channels, unlike most α1-adrenoceptor antagonists, or antagonize the muscarinic AChR, unlike many neuronal norepinephrine transporter (NET) inhibitors.

Given the enormous library of peptidic natural products found in the venom of cone snails, the discovery of conopeptides is a task that will occupy researchers for decades to come. Only a small fraction of the existing *Conus* peptides library has been characterized; nevertheless, new peptidic arrangements with novel bioactivities are constantly described in the literature.

2.15.5 Structure and Activity of Cone Snail Toxins

There is an intimate relationship between the conopeptide type, its structure, and its selectivity; different types of conopeptides target different neuronal receptor types. However, with the same conopeptide type (or subtypes), selectivity varies tremendously, as different sites within the same receptor can be targeted by the conopeptides whose difference is only one amino acid. This is illustrated by the selectivity of α-conotoxins (**Table 3**) toward the nAChR.[65,66] This is further augmented by a new level of conotoxin diversity, as a nonnative disulfide bond connectivity in α-conotoxin AuIB reduces structural definition but increases biological activity.[109]

The selectivity of the conotoxin family can be attributed to differences in bridging structure and loop variability. For example, the α-conotoxins contain two disulfide bridges (first to third Cys and second to fourth Cys) forming two loops of variable size and specifically inhibit either muscular or neuronal nAChR.[66] The ω-conopeptides contain three disulfide bridges and selectively inhibit certain classes of calcium channels.[110] The μ-conotoxins, which selectively block sodium channel subtypes, also contain three disulfide bridges; however, their bridging pattern differs from that of the ω-conotoxins.[111] Conotoxin subtypes are not uniquely associated with specific neuronal receptors. For example, α-conotoxin (two-disulfide bridges) inhibits neuromuscular nicotinic receptors through competitive inhibition at acetylcholine-binding sites,[66] whereas the unrelated ψ-conotoxin (three-disulfide bridges) inhibits the same receptors through a noncompetitive mechanism.[32] Other conotoxins, such as ε-TxIX,[45] have two disulfide bridges, just as the α-conotoxin; however, ε-TxIX incorporates an unparallel level of modification that includes two Gla, one Hyp, and two sugar units (GalNAc-Gal) attached to Thr and brominated Trp. This conotoxin selectively reduces neurotransmitter release by reducing the presynaptic influx of Ca^{2+} in a slow and reversible manner.[45]

Sequence homology within conotoxin subtypes appears not to be related to receptor selectivity. For example, the ω-conotoxins found in several piscivorous cone snail species all paralyze fish and target the presynaptic calcium channels, yet they have up to 70% difference in their sequences.[19] The only amino acids that are conserved are the Cys residues that provide the basic framework of these particular conotoxins. These findings indicate that the non-Cys amino acids are crucial in the physiological role of conotoxins. The King-Kong peptide (named from the aggressive response that it causes in lobsters) isolated from *Conus textile* has retained the

Table 3 Comparison of the amino acid sequences and known targets of α-conotoxins

α-Conotoxin	*Sequence*	*Source*	*Subtype*	*nAChR*
EI	RDO**CC**YHPT**C**NMSNPQI**C***	*Conus ermineus*	4/7	$\alpha1\beta1\delta\gamma$
PnIA	G**CC**SLPP**C**AANNPDY**C***	*Conus pennaceus*	4/7	Molluscan
PnIB	G**CC**SLPP**C**ALSNPDY**C***	*C. pennaceus*	4/7	Molluscan
EpI	G**CC**SDPR**C**NMNNPDY**C***	*Conus episcopatus*	4/7	$\alpha3\beta2$,$\alpha3\beta4$
MII	GR**CC**SNPV**C**HLEHSNL**C***	*Conus magus*	4/7	$\alpha3\beta2$
GIA	E**CC**NPA-**C**GRHYS--**C**GK*	*Conus geographus*	3/5	$\alpha1\beta1\delta\gamma$
GII	E**CC**HPA-**C**GKHFS--**C***	*C. geographus*	3/5	$\alpha1\beta1\delta\gamma$
SI	I**CC**NPA-**C**GPKYS--**C***	*Conus striatus*	3/5	$\alpha1\beta1\delta\gamma$
ImI	G**CC**SDPR**C**AWR----**C***	*Conus imperialis*	4/3	$\alpha7$,$\alpha9$

Disulfide bonding: first Cys to the third and second Cys to the fourth. A single asterisk indicates that the C-terminus is amidated. O = 4-hydroxyproline.

ω-conotoxin framework, but does not target the calcium channel.[19] Conotoxin variability in sequence and selectivity within the same subtype can be developed by retaining a given disulfide framework or backbone structure and mutating loop regions between Cys residues or specific amino acids in a mature peptide. This variability is manifested by species to species variations in amino acid sequence (**Table 2**), which results in distinct differences in potential pharmacological applications. Nonetheless, numerous conotoxin families across a variety of peptidic scaffolds have been described, including α, ρ, λ, αA, κA, μ, ψ, δ, μO, ω, κ, γ, σ, τ, χ, and π.[9,19,49]

Conotoxin families have uniquely stable scaffolds, which are optimal for presenting critical residues within a pharmacophore. Some of these conopeptides, while targeting different receptors, share a common structural scaffold. For example, members of the O-superfamily possess a structural motif composed of a cystine knot and a triple-stranded β-sheet.[19] This is also the case found in the structure of conopeptides belonging to the α4/7-conotoxin subfamily; although these conopeptides can target either the neuronal or the muscular subtypes of nAChR, their structure shows a similar scaffold where helical loop is stabilized by a disulfide bond and a turn (**Figure 2**). Differences in receptor selectivity of the different α4/7-conotoxins can be attributed to difference in the surface charge distribution among members of this conopeptide subfamily.[112,113]

A novel family of conotoxins that are characterized by a four-cystine/three-loop arrangement has been recently described; it has been designated F14 conotoxins. Sequence-specific nanoNMR analysis of the F14 conotoxins at room temperature revealed a well-defined helix–loop–helix tertiary structure that resembles that of the Cs α/α scorpion toxins κ-hefutoxin, κ-KTx1.3, and Om-toxins, which adopt a stable three-dimensional fold where the two α-helices are linked by two disulfide bridges. One of these conotoxins (vil14a) has a Lys/Tyr dyad, separated by approximately 6 Å, which is a conserved structural feature in K^+ channel blockers. It appears that cone snails are utilizing the same biochemical strategy as scorpions in targeting ion channels through this particular Cs α/α framework in order to capture their prey.

2.15.6 Cone Snail Toxins as Therapeutics and Drug Leads

The ability of conotoxins to selectively block ion channels and neuronal receptors has led to their development into therapeutic agents. So far, most conotoxin applications as therapeutics have been concentrated on the treatment of different forms of pain. The first drug of marine origin is based on the ω-conotoxin MVIIA for the treatment of chronic pain (see below). Other therapeutic applications of conotoxins include treatment of schizophrenia, epilepsy, neuromuscular disorders, certain types of cancer, urinary dysfunction, Parkinson's disease, Alzheimer's disease, stroke, and related brain injuries. Other uses include muscle relaxants, anesthetics, and antiseizure compounds.[3] As the demand for new painkillers and other neuropharmacological agents is expected to increase, the value of the discovery and testing of new conotoxins is expected to continue to expand.

2.15.6.1 Prialt/Ziconotide/SNX-111

A synthetic form of ω-conotoxin MVIIA, initially isolated from *Conus magus*, under the generic name ziconotide (Elan Pharmaceuticals) underwent extensive human clinical trials and has been approved by the US Food and Drug Administration (FDA) as an analgesic for chronic pain.[114] By blocking neurotransmitter release from primary nociceptive afferents, ziconotide prevents the propagation of pain signals to the brain.[115] Ziconotide underwent a second round of phase III clinical trials under the trade name Prialt,[116] and it finally gained FDA approval in the United States in December 2004.[117]

Ziconotide is neuroprotective in rat models of ischemic neuronal damage[118] and after intrathecal administration, antinociception is observed in rats with limited toxicity.[119] The neuroprotective effects observed in rat models are thought to be due to a reduction in body temperature.[120] Analgesic effects are observed in cancer and AIDS patients whose pain was not relieved after opioid administration[121] and in neuropathic conditions.[122] Intrathecal administration of ziconotide prevents mechanical and cold allodynia[97,123] and heat hyperalgesia[124] in neuropathic rats. The use of N-type VSCC inhibitors in both ischemic brain injury and pain treatment is complicated by their important role in the synapse.[125] Adverse effects are observed in patients but they are managed through dose reduction or symptomatic treatment, although serious supraspinal and systemic adverse effects have been seen.[125]

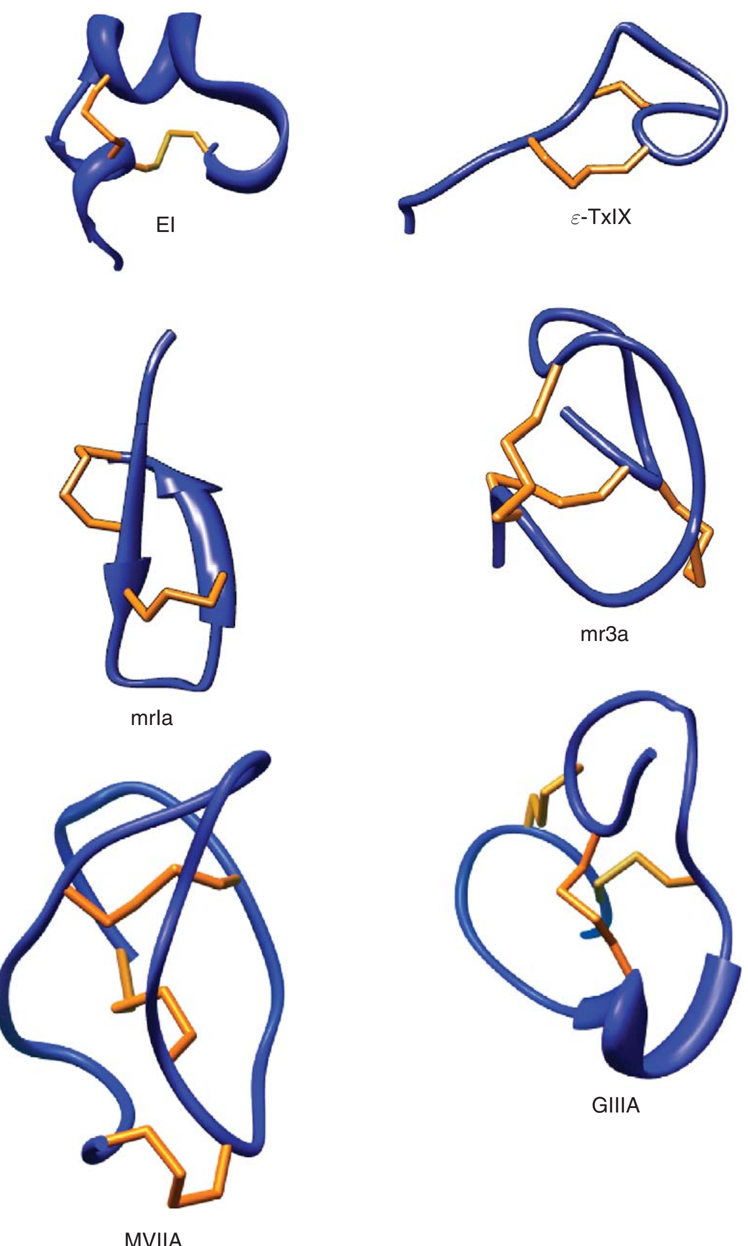

Figure 2 Three-dimensional structures of the α-conotoxin EI, T-superfamily conotoxins ε-TxIX and χ-mrIA, mini-M mr3a, ω-conotoxin MVIIIA, and μ-conotoxin GIIIA. The ribbon representation of the backbone is in blue. Disulfide bridges are shown in orange.

Being a peptide, the available dosage of ziconotide is poor and sophisticated delivery techniques are required even when the compound is delivered intrathecally.[126] The toxin fared poorly in phase III trials for cerebral ischemia when administered intravenously, with brain concentrations measured at only 0.005% of the administered dose per gram of tissue, although it should be noted that ziconotide is therapeutically active at the picomolar to low nanomolar range.[127] Diffusion into the brain may be impeded by the molecular weight and the positive charge of the peptide. The Medtronic infusion system is currently being utilized for Prialt/ziconotide.

2.15.6.2 Xe2174

Xenome Ltd., Australia, is developing strategies for the management of certain types of pain, for which there is currently a lack of effective treatment, using analogues of χ-conotoxins Mr-IA/B from *C. marmoreus*, which target the NET.[47,128] The NET is a validated target for the treatment of pain.[129] NETs are located on nerve endings that release norepinephrine after nerve stimulation. Thus, NET regulates norepinephrine levels in the CNS. Since Xe2174 blocks NETs, the increase in norepinephrine levels leads to suppression of pain signals. Just as Prialt, the mode of administration of Xen2174 is intrathecal. Phase I/II clinical trials on cancer patients suffering from chronic pain have completed, indicating that Xe2174 can relieve pain quickly and for a sustained period of time. The drug is currently in phase II clinical trials and the results so far are promising and encouraging for the development of Xe2174 as a new class of pain therapeutics.

2.15.6.3 Vc1.1

ACV1, which is derived from the α-conotoxin Vc1.1 from *Conus victoriae*, reached clinical trials in Australia under the auspices of Metabolics Ltd. The compound was administrated subcutaneously for the treatment of neuropathic pain and for speeding the rate of functional recovery after a nerve injury.[130] ACVI in principle represented a novel pain management strategy, as it is a neuronal-type nicotinic receptor antagonist, which might overcome the drawbacks of agents targeting the N-type Ca channels.[130] However, it was found that Vc1.1 (and not the actual Vc1a, expressed by the snails with PTMs) specifically blocks the $\alpha9\alpha10$ nAChR subtype. This surprising finding precipitated the withdrawal of Vc1.1 from clinic trails as it would require exceedingly copious amounts of the drug to treat patients. It was later determined that Vc1.1 can act as an agonist of $GABA_B$ G-protein-coupled receptors (GPCRs) that can modulate N-type Ca^{2+} channels, which are implicated in pain.[131] It is likely that some form of Vc1.1 or a related α-conotoxin may be revisited as potential pain therapeutics.

2.15.6.4 CVIA

ω-Conotoxin CVIA isolated from *C. catus* underwent clinical trials under the sponsorship of AMRAD Operations Australia, under license from the University of Queensland for severe morphine-resistant pain. For trial purposes, CVIA was designated AM336. AM336 has higher selectivity than Prialt for N-type over P/Q-type calcium channels and similar potency in inhibiting current through central splice variants of the rat N-type calcium channels.[37,132,133] AM336 represents the same type of therapeutics as Prialt, with added advantages in selectivity and stability. However, given the limited market for such 'specialty' pain killers, the development of AM336 has remained on hold.

2.15.6.5 Contulakin-G

Contulakin-G is a 17-residue O-glycosylated linear peptide isolated from the venom of *C. geographus* that targets the neurotensin receptor.[25] Contulakin-G has an incompletely defined mechanism of action.[134] When delivered as a bolus intrathecally in rats, it significantly decreased paw flinching produced by intradermal formalin. In dogs, intrathecal administration of contulakin-G produced a dose-dependent increase in the thermally evoked skin-twitch latency by 30 min after administration. However, no physiologically significant dose-dependent changes in motor function, heart rate, arterial blood pressure, or body temperature were found after the administration of contulakin-G. Contulakin-G can be a potent antinociceptive drug when delivered intrathecally with no apparent negative side effects in rats or dogs. Contulakin-G can be considered as an alternative to opioid spinal analgesics.[134]

2.15.6.6 Conantokin-G

Conantokin-G (Con-G), a 17-residue linear conopeptide that contains five γ-carboxyglutamate (Gla) residues, is an antagonist of the NMDA receptor. Conantokin-G has been shown to be an effective antiepileptic agent in several animal models of seizure.[135]

Con-G is a selective competitive antagonist for the NMDA NRB2 site and displays an improved therapeutic ratio when compared to noncompetitive NMDA antagonists such as MK-801.[135] The residue at position 5 of the peptide sequence is thought to play a particularly important role in the subunit specificity of the toxin.[136] Con-T has been shown to inhibit both the NR2A and NR2B subunits of the NMDA receptor.[136] Spinal delivery of both Con-G and Con-T produces antinociceptive effects at doses 10 times lower than those associated with motor impairment and 20 times lower than those associated with side effects in models of injury-invoked pain.[137]

The NMDA receptor is thought to be involved in a number of neuropsychological processes, such as learning and memory, and has been implicated in a number of neuropathological disorders such as Parkinson's disease, Alzheimer's disease, epilepsy, and ischemic cell death.[136] The overexcitation of the NMDA receptor by glutamate results in an elevation of intracellular calcium and an increase in necrotic and apoptotic cell death.[138] Conantokins exhibit neuroprotection in rat models of transient focal brain ischemia[139] and may also be suitable anticonvulsants[135] and anti-Parkinsonian agents.[140] NMDA receptor antagonists have also been shown to prolong the effects of L-3,4-dihydroxyphenylalanine (L-DOPA) in animal models of Parkinson's disease[141] as well as reduce side effects associated with prolonged usage and high doses.[142] Administration of Con-G has also been shown to significantly reduce levels of brain infarction with observed improvements in neurological recovery and electroencephalography (EEG) power scores after a middle cerebral artery occlusion.[139]

2.15.6.7 Conopeptide Druggability and other Uses

As with most bioactive peptides, the delivery of these compounds to the site of action is a major concern when considering the druggability of a conopeptide lead. Improvement in the bioavailability of conotoxins can be achieved with modified peptides with improved potency and lipid solubility. The synthesis of lipo- and liposaccharide conjugates of α-conotoxin, such as MII,[143] may improve the oral availability and stability of the peptide and facilitate the crossing of the blood-brain barrier, thus improving the pharmaceutical suitability of these compounds.

Besides their therapeutic applications, conotoxins have exhibited great potential for the development of neurological probes.[73] Conotoxins are currently being used in hundreds of research laboratories for a wide variety of physiological investigations. Some conotoxins have become well-established neurobiological tools. They can also be used as antagonists of specific subtypes in functional studies; for example, ω-conotoxin MVIIA is used as a specific N-type calcium channel blocker. Conotoxins are also utilized in research to provide information on the role and distribution of different receptor subtypes. Undoubtedly, these research applications will continue to expand.

2.15.7 Sea Anemones: Distribution, Diversity, Behavior, Feeding, and Defense

Sea anemones are ocean-dwelling members of the phylum Cnidaria. They are invertebrates belonging to the class of Anthozoa. The name Cnidaria (with a silent 'c') refers to the cnidae, or nematocysts, that is, the cellular entity of the venom apparatus, which all Cnidarians possess. The phylum Cnidaria includes anemones, corals, jellyfish (including box jellyfish), and hydras. Sea anemones, named after a terrestrial flower, have a basic radial symmetry with tentacles that surround a central mouth opening. The tentacles are used to catch food and transfer it to their mouth. Each stinging capsule in the tentacles, and other parts of the sea anemone, contains a coiled hollow filament, usually barbed, heavily loaded with venom. This is used to immobilize smaller organisms, for defense against predators, and to fight territorial disputes. When triggered by mechanical or chemical stimulation, the capsule 'explodes' and drives the filament into its prey, discharging its venom.

2.15.8 Sea Anemone Venom

Cnidaria venom contains a variety of peptidic compounds, including potent toxins affecting several targets. The characterization of sea anemone toxins began as early as 1968 when Shapiro purified a toxin that he called Condylactis toxin from the sea anemone *Condylactis gigantea*[144] (**Figure 3**). This toxin appeared to cause an increase in action potential duration in lobster giant axons. Several years later, in 1975, Beress and coworkers isolated three toxins from *Anemonia sulcata* venom,[145] which became widely used as tools to study voltage-gated Na^+ channels. The fact that sea anemone toxins affect voltage-gated ion channels seems logical since these targets are an important component of the action potential in the signal transduction process of both vertebrates and invertebrates. In fact, an encounter with some species (e.g., *Chironex fleckeri* and *Phyllodiscus semoni*) can even be lethal to humans. However, in most cases, a sting by the nematocysts will cause local inflammations, pain, and sometimes edema. Together with toxins targeting voltage-gated Na^+ channels, actinoporins have also been reported to be highly toxic to fish and crustaceans, which may be the natural prey of sea anemones. In addition to their role in predation, it has been suggested that actinoporins could act, when released in water, as efficient repellents against potential predators.

Historically, the toxins can be divided into the following three classes (the original idea of Norton[146] and an excellent review by Honma and Shiomi[147] are referred here): (1) 20-kDa pore-forming cytolysins inhibitable by sphingomyelin (also called actinoporins) and protease inhibitors,[145,148,149] (2) 3–5-kDa neurotoxins acting on voltage-gated Na^+ channels, and (3) 3.5–6.5-kDa neurotoxins acting on voltage-gated K^+ channels. At present, also novel peptide toxins from sea anemones have emerged that target different ion channels, for example, BDS-I and BDS-II acting on $K_v3.4$ channels, APETx1 on *human ether-a-go-go*-related gene K^+ channels (*hERG*), and APETx2 on acid-sensing ion channels.[150–152]

Figure 3 Purple-tipped giant sea anemone – *Condylactis gigantea*. *Condylactis gigantea* is a common Caribbean anemone that is not always 'purple-tipped'. Several organisms can find refuge by inhabiting near a sea anemone, as the anemone shrimp (*Periclimenes brevicarpalis*) shown in the picture. Photo courtesy of Susan Davis, Key Largo, FL.

2.15.8.1 Sodium Channel-Acting Toxins

Na^+ channel peptide toxins can be classified into at least three types based on the amino acid sequences: type 1 and type 2 toxins that are composed of 46–49 amino acid residues with ApA (*Anthopleura xanthogrammica*) and RTX I (*Radianthus (Heteractis) macrodactylus*) as prototype toxins, respectively.[153,154] They are stabilized by 3 disulfide bridges and 10 residues (including the 6 Cys residues) are completely conserved between type 1 and type 2 toxins. It has been noticed that the distribution of type 1 and type 2 toxins seems to be related to the taxonomical position of sea anemones: members of the family Actiniidae contain only type 1 toxins, whereas those belonging to the family Stichodactylidae contain either type 1 or type 2 toxins, or both types. The third class of toxins, type 3 peptide toxins, are somewhat shorter and contain 27–32 amino acid residues, but still with an impressive number of Cys residues, six or eight, thus strongly stabilizing these peptides by three or four disulfide bridges. As a representative member, PaTX can be quoted from *Entacmaea (Parasicyonis) actinostoloides* (**Table 4**).[155]

From a functional point of view and similar to α-peptide toxins from scorpion venom, sea anemone type 1–3 toxins bind to receptor site 3 of voltage-gated Na^+ channels. As such, they prolong the open state of the channels during the depolarization phase by slowing the macroscopic inactivation of the channel.[156] Since this discovery, this unique feature has advertised these toxins as valuable pharmacological tools for high-throughput systems in drug discovery and pharmaceutical research in general.

2.15.8.2 Potassium Channel-Acting Toxins

Sea anemone peptide toxins that target voltage-gated K^+ channels were discovered almost 20 years after their Na^+ channel toxin counterparts. They can also be grouped into three classes: (1) Type 1 toxins are composed of 35–37 amino acid residues and cross-linked by three disulfide bridges. They block K_v1 (*Shaker*)-type channels and as a representative toxin ShK from *Stichodactyla helianthus* can be taken.[157] A dyad of two residues, Lys22 and Tyr23 that are completely conserved in this class of peptides, has been discovered to play a pivotal role in the binding to K_v1-type channels. Interestingly, a similar dyad has been found in scorpion toxins blocking K_v1-type channels, like charybdotoxin and margatoxin.[158] (2) Type 2 voltage-gated K^+ channel toxins are composed of 58 or 59 amino acids and also display K_v1-type inhibition, albeit with much lower potency. The kalicludines 1–3, abbreviated AsKC 1–3, possess the intriguing feature of sharing their K^+ channel inhibitory properties with Kunitz-type protease inhibitors, such as bovine pancreatic trypsin inhibitor (BPTI).[159] (3) Type 3 toxins, like BDS-I and BDS-II from *A. sulcata*[150] and APETx1 from *Anthopleura elegantissima*,[151] may look at first sight similar to type 1 Na^+ channel toxins. However, they possess a unique pharmacology as BDS-I and BDS-II have been shown to be the first specific blockers of $K_v3.4$ (*Shaw*-type) channels, and APETx1 a selective blocker of *hERG*.

2.15.8.3 Miscellaneous Peptide Toxins from Sea Anemones

In the extensive review by Honma and Shiomi,[147] other structurally novel peptide toxins are described. Some of these toxins, such as AETx-II and AETx-III from *Anemonia erythraea*,[160] contain as many as 10 Cys residues and they are described as being lethal to crabs. Others, such as gigantoxin I from *Stichodactyla gigantea*, have surprising structural resemblance to epidermal growth factors (EGFs) from mammals and can have agonistic activity on TRPV1 receptors (Tytgat *et al.*, unpublished data).

2.15.9 Structure and Activity of Sea Anemone Toxins

The first three-dimensional structures of sea anemone toxins active on voltage-gated Na^+ channels, determined by NMR spectroscopy, were AP-A in 1988[161] and ATX-I in 1989 (**Figure 4**).[162] Both molecules contain a core of four strands of antiparallel β-sheets connected by two loops. The structures of ShI,[163] ATX-III,[164] and AP-B[165] have also been published. AP-B seems to exist in multiple conformations in solution as a result of *cis–trans* isomerization about the Gly40–Pro41 peptide bond. Three loops connect the four β-sheets, the longest and least well-defined being the first loop, extending from residues 8 to 17. Mutagenesis of

Table 4 Sequences of selected peptides isolated from sea anemone venom

Name	*Sequence*	*Species*
Sodium channel modulators		
Type 1		
ApA	GVS**C**L**C**DSDGPSVRGNTLSGTLWLYPSG**C**PSGWHN**C**KAHGPTIGW**CC**KQ	***Anthopleura xanthogrammica***
ATX-I	GAA**C**L**C**KSDGPNTRGNSMSGTIWVFG**C**PSGWNN**C**EGRAIIGY**CC**KQ	***Anemonia sulcata***
BgII	GAS**C**R**C**DSDGPTSRGNTLTGTLWLIGR**C**PSGWHN**C**RGSGPFIGY**CC**KQ	***Bunodosoma granulifera***
Type 2		
RTX-I	AS**C**K**C**DDDGPDVRSATFTGTVDFAY**C**NAGWEK**C**LAVYTPVAS**CC**RKKK	***Heteractis crispa***
ShP-I	AA**C**K**C**DDEGPDIRTAPLTGTVDLGS**C**NAGWEK**C**ASYYTIIAD**CC**RKKK	***Stichodactyla helianthus***
RpII	AS**C**K**C**DDDGPDVRSATFTGTVDFWN**C**NEGWEK**C**TAVYTPVAS**CC**RKKK	***Radianthus paumotensis***
Type 3		
PaTX	AGGKST**CC**P**C**AM**C**KYTAG**C**PWGQ**C**AHH**C**G**C**S	***Parasicyonis actinostoloides***
Potassium channel blockers		
Type 1		
ShK	RS**C**IDTIPKSR**C**TAFQ**C**KHSMKYRLSF**C**RKT**C**GT**C**	***Stychodactyla helianthus***
Type 2		
AsKC-I	INKD**C**LLPMDVGR**C**RASHPRYYYNSSSKR**C**EKFIYGG**C**RGNANNFHTLEE**C**EKV**C**GVR	***Anemonia sulcata***
Type 3		
APETx-I	GTT**C**Y**C**GKTIGIYWFGTKT**C**PSNRGYTGS**C**GYFLGI**CC**YPVD	***Anthopleura elegantissima***
BDS-I	AAP**C**F**C**SGKPGRGDLWILRGT**C**PGGYGYTSN**C**YKWPNI**CC**YPH	***Anemonia sulcata***

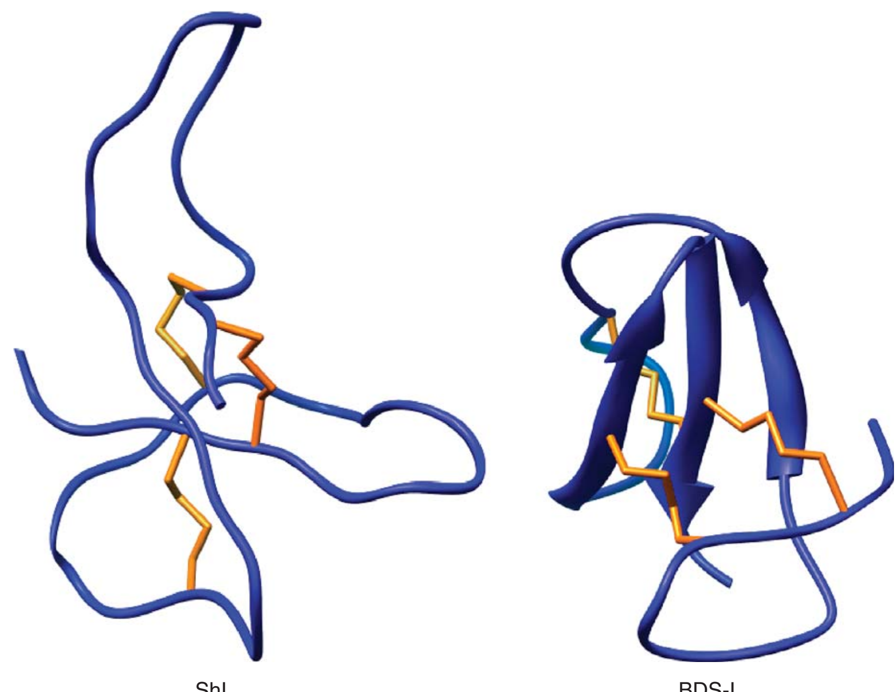

Figure 4 Three-dimensional structures of the sea anemone toxins ShI and BDS-I. The ribbon representation of the backbone is in blue. Disulfide bridges are shown in orange.

AP-B resulted in the identification of a flexible loop in the region of residues 8–17 (Arg14 loop) as being important for binding to voltage-gated Na^+ channels[166,167] Furthermore, the Blumenthal lab has shown that Leu18 is an absolute requirement for the binding of AP-B to its target.[147,168] Neighboring residues are either less sensitive or their sensitivity is dependent on the nature of the mutation; particularly, the introduction of negative charges at these positions, is poorly tolerated. In addition, Arg12, Ser19, and Lys49 are reported to be important for toxin affinity and channel isoform specificity of AP-B.[169,170] It should be noted that AP-B has no selectivity between neuronal and cardiac voltage-gated Na^+ channels, whereas AP-A is selective for the latter. Only two residues located outside the flexible loop region (Trp33 and Lys37) seem to be indispensable for pharmacological activity. It has been shown that Lys37 can interact directly with Asp1612 of $rNa_v1.5$ (i.e., the cardiac isoform of voltage-gated Na^+ channel).[147,171]

In comparison to scorpion α- and β-toxins,[172,173] not much work has been done on the insect or mammalian specificity of sea anemone toxins.[174] Early work on ATX-I has indicated its preferential toxicity against crabs, rather than mice.[146,175] The proof of the link between insects and crustaceans has been described on a genetic level.[176] Therefore, since both crabs (crustaceans) and insects belong to the same phylum (Arthropoda), one could hypothesize that ATX-I preferentially targets insects rather than mice. The same argument could be made for ShI and Rp-II. In fact, a study by Salgado and Kem[177] in 1992 investigated the membrane actions of ShI, CgII, and CpI on action potentials and voltage clamp membrane currents of the giant axon from the crayfish *Procambarus clarkii*. ShI and CgII were also tested on the cockroach (*Periplaneta americana*) giant axon. Both toxins were particularly lethal to crustaceans, moderately toxic to an insect (cockroach), and essentially nontoxic to a mammal (mouse). Both toxins prolonged crayfish giant axon action potentials by selectively slowing voltage-gated Na^+ channel inactivation without affecting activation. However, more experiments on cloned voltage-gated Na^+ channels (called in general '$Na_v1.x$') of insects and mammals should be carried out.

ATX-II is toxic to crabs but the activity on mice via ICV injection is still pronounced.[146,175] However, ATX-II is also very effective on the 'para' insect channel and binds with high affinity to cockroach neuronal membranes, whereas its binding affinity for rat brain synaptosomes is low.[175,178–180] Mutagenesis of ATX-II has provided an insight into the pharmacologically important epitopes of this toxin; either acetylation or

fluorescamine treatment of ATX-II that destroyed the positive charges of the three ε-amino groups of residues Lys35, Lys36, and Lys46, and of the α-amino function of Gly1, produced an almost complete loss of toxicity and a considerable decrease in binding activity.[181] Furthermore, it was shown that carbethoxylation of His32 and His37 provoked a significant decrease of both toxicity and binding activity, and it was also found that modification of the guanidine side chain of Arg14 could destroy both toxicity and binding of the toxin to voltage-gated Na^+ channels. Additionally, Barhanin *et al.* concluded that modification of the carboxylate functions of Asp7, Asp9, and Gln47 with glycine ethyl ester in the presence of a soluble carbodimide completely abolished the toxicity but left the affinity for the sea anemone toxin receptor unchanged. Nevertheless, ATX-II displays a high potency when tested on the mammalian cardiac $Na_v1.5$ channel.[182]

Two toxins from the *Bunodosoma granulifera*, BgII and BgIII, have been thoroughly tested on mice, cloned channels, dorsal root ganglia, and rat brain synaptosomes.[183–186] Both toxins seem to have a preference for insects. BgII in particular has a 100-fold higher potency on the insect channel, para, as compared to other mammalian channels when expressed in *Xenopus laevis* oocytes. However, BgII does affect rat brain synaptosomes, with a K_d of 9 nmol l^{-1}, but no experiments have yet been carried out on insect preparations. It should be stressed that BgII and BgIII differ only in one residue (Asn16 to Asp16), which is situated in the aforementioned Arg14 loop. Yet, their potencies toward voltage-gated Na^+ channels are remarkably different. Another trademark of BgII and BgIII is that they have a particularly devastating effect on the inactivation of the insect Na^+ channel, para. This is in sharp contrast to vertebrate channels. The inactivation is extremely slowed such that the channel simply does not inactivate any more. This removal of inactivation is also seen when ATX-II is applied on *para*.[175,178–180] Wang *et al.*[187] have studied four 'naturally occurring mutant or isoform' toxins from an *Anthopleura* sp. that were expressed in *Escherichia coli* and tested in contractile force studies. They suggest that residues at positions 14, 22, 25, and 37 (with an emphasis on Arg14) are important to explain the isoform-specific features of the toxins toward their pharmacological effects.

More recently, a new type 1 peptide toxin with a strong paralytic activity on Crustacea ($LD_{50} \sim 1$ mg kg^{-1}) was isolated from the sea anemone *C. gigantea*.[188] This new toxin, CgNa, increased action the potential duration in dorsal root ganglia neurons under current clamp conditions. CgNa also prolonged the cardiac action potential duration and enhanced contractile force albeit at 100-fold higher concentrations than ATX-II. The action on voltage-gated Na^+ channel inactivation and cardiac excitation–contraction coupling resembles previous results with compounds obtained from this and other sea anemones. In a recent paper by Moran *et al.*,[189] the authors try to resolve the bioactive surface of ATX-II. To this end, they established an efficient expression system for this toxin and mutagenized it throughout. Six residues were found to constitute the anti-insect bioactive surface of ATX-II (Val2, Leu5, Asp19, Asn16, Leu18, and Ile41). Further analysis of nine ATX-II mutants on $Na_v1.5$ indicated that the bioactive surfaces interacting with insect and mammalian channels practically coincide but differ from the bioactive surface of AP-B. All residues important for activity excluding Arg12 and Lys49 appear in both ATX-II and AP-B. Yet, Ser19 seems to be important for the antimammalian activity; however, it only has a small effect on the activity of ATX-II toward insects. This suggests that this residue is not a major contributor to the insecticidal activity of this toxin (as opposed to AP-B). The authors also investigated a major variation between the bioactive surfaces of AP-B and ATX-II that consists of Trp33 and Lys37 in AP-B as compared to Trp31 and Lys35 in ATX-II. Substitution of these residues in AP-B using their ATX-II equivalents had no effect on the insecticidal activity and only a slight effect on $Na_v1.5$. These conspicuous disparities in bioactive surfaces imply that despite similarities in the Arg14 loop, the interaction site of ATX-II with site 3 is different from that of AP-B. Support for this conclusion can be found in the report that the binding sites of AP-A and AP-B on $Na_v1.4$ and $Na_v1.5$ are also slightly different.[190]

2.15.10 Sea Anemone Toxins as Insecticides

Abundant use of one of the most commonly used insecticides in crop protection, pyrethroids, has led to the development of resistance in many insect species.[191] One of the most important mechanisms is that of knock-down resistance (kdr), caused by several mutations in the *para* gene (L1014F and M918T), which confers cross-resistance to the entire class of pyrethroids.[192,193] Another problem is that most insecticides cause toxicity in organisms other than insects because of the general conservation of the voltage-gated Na^+ channel structure

throughout the animal kingdom. Nevertheless, reports of toxins that show selectivity toward insects or mammals are being published.[183] In fact, projects to replace classical chemical insecticides have already been undertaken.[194] Already in 1988, Bloomquist and Soderlund studied the effects of saturating concentrations of DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane) and the pyrethroid insecticides cismethrin and deltamethrin on veratridine-dependent activation of voltage-gated Na^+ channels using measurements of $^{22}Na^+$ uptake into mouse brain synaptosomes.[195] They also conducted additional experiments to assess the interactions of insecticides and ATX-II as modifiers of alkaloid-dependent uptake. DDT and ATX-II acted synergistically to increase uptake stimulated by veratridine. Moreover, DDT shifted the potency of ATX-II for enhancing veratridine-dependent uptake to fivefold lower concentrations. In contrast, DDT and subsaturating concentrations of ATX-II acted independently in their enhancement of voltage-gated Na^+ channels' activation by batrachotoxin (BTX). Combining several insecticidal synergetic peptides like insect-selective sea anemone and scorpion toxins (e.g., Regev *et al.*[196]) in a baculovirus could also prove to be valuable. This virus by itself is already insect-selective (for a general overview on baculovirus biology, see Zlotkin *et al.*[194] and Inceoglu *et al.*[197]). However, its natural kill-rate is too slow and its host range is limited (which could also be interpreted as advantageous). In 1993, Hammock *et al.*[198] developed recombinant baculovirus insecticides using two approaches. In one approach, an insect-specific scorpion neurotoxin (AaHIT1) was expressed by the virus leading to a dramatic reduction in time to death (as compared to the native baculovirus). In the second approach, an insect juvenile hormone esterase was expressed, which led to a significant reduction in feeding time, indicating that an increase in lethality is not always necessary. Despite this promising application, research on characterizing the differences in binding sites of insect- and mammalian-specific toxins toward voltage-gated Na^+ channels is progressing slowly.

The identification and comparison of the components that are involved in the interaction of sea anemone toxins with their targets is an absolute requirement to design novel insecticides. Beyond this, insect-selective toxins could be truncated (engineered) in order to make them more stable when administering them. Importantly, recent reports suggest that some insect-selective peptide toxins, such as ω-atracotoxin-Hv1a, might be orally active in certain species.[199] Surprisingly, a ω-atracotoxin-Hv1a fusion protein was also topically effective.[200] Not only do arthropods destroy about 20–30% of the world's food supply,[201] but they are also responsible for the transmission of many human diseases. This should be an incentive to explore the sea anemone toxin world more carefully and by doing so it could mean the beginning of a new era in insect pest control. Also from an evolutionary point of view, the existence of crustacean-selective toxins in sea anemones is very interesting. It may help us to understand why we can encounter insect-selective toxins in sea anemones, even when insects and sea anemones in an everyday life will never 'encounter one another'. Indeed, Zrzavy and Stys[202] have proposed a taxon, the so-called 'Pancrustacea', comprising all crustaceans and hexapods. It should hereby be explained that Hexapoda is a subphylum of the phylum Arthropoda and comprises the class Insecta, in addition to some wingless arthropods such as Collembola, Protura, and Diplura. It is known that the taxonomy of Insecta is very extensive with the majority of invertebrates being classified as insects ($\sim$1 million extant species) and 95% of the earth's animals being invertebrates. Furthermore, a monophyletic Pancrustacea taxon has been supported by several molecular studies,[203–205] in which most of the subphylum Crustacea is paraphyletic with respect to insects. This means that insects are derived from crustacean ancestors and that by definition crustacean-selective toxins found in sea anemones may be considered as 'lead compounds' for molecules with an anti-insect profile, that is, insecticides.

2.15.11 Conclusions

Natural peptide toxins from marine organisms provide a remarkable combinatorial library of modified peptides that have been refined and perfected for millions years to target exquisitely a vast array of neuronal receptors, including human ones. Although the original intention of these marvelous peptide engineers was securing their next meal or defending themselves from predation, they have provided us with novel therapeutic agents that have only just began to be explored. The exploration of vast natural peptide toxin libraries offers many opportunities for the discovery of valuable bioactive molecules. However, this process can challenge, current peptide technologies. Although significant advances have been made in the past 30

years, these represent just the beginning as tens of thousands of natural peptide toxins from marine organisms remain to be investigated.

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Biographical Sketches

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Marine Institute, for his research in the area of *Cnidaria* intoxications). He gives frequent national and international lectures, is a member of diverse scientific societies and editorial boards, and is a consultant for pharmaceutical and biotech companies. He is also heading a forensic toxicology laboratory in Belgium at the request of the Ministry of Justice. Dr. Tytgat has been elected as President of the European Section of the International Society on Toxinology (IST).

2.16 Cyanobactins – Ubiquitous Cyanobacterial Ribosomal Peptide Metabolites

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2.16.1 Introduction

Beyond nonribosomal peptides and polyketides, the ribosomal peptides comprise one of the major groups of bioactive natural products from cyanobacteria. A large number of these peptides contain related features and biosynthetic pathways that warrant their inclusion in a new family, the cyanobactins. These features include N–C terminal cyclization and derivatization of cysteine, serine, threonine, and tyrosine by heterocyclization or isoprenylation. More than 100 cyanobactins are known from a diverse range of free-living and symbiotic cyanobacteria, including obligate symbionts of sponges and ascidians in the marine environment. Many different, often potent, biological activities have been identified, and in addition potential ecological roles have been assessed for some representatives. For example, many cyanobactins are known to bind metals selectively *in vitro* under defined conditions, while others are allelochemicals that inhibit the growth of competing cyanobacteria. Because of these potent bioactivities and diverse roles, cyanobactins have been the subject of numerous synthetic studies and total syntheses. Finally, biosynthetic pathways to cyanobactins have been cloned and functionally examined, leading to new observations about natural product pathway evolution and engineering.

2.16.2 Cyanobactin Structures

Cyanobactins are defined as ribosomally derived, N–C terminally cyclized peptides from cyanobacteria (Figure 1).[1] Known cyanobactins have a second feature, either prenylation by dimethylallylpyrophosphate (DMAPP) or by heterocyclization of Cys, Ser, or Thr. Cyanobactins include potently bioactive molecules such as dolastatin 3,[2–4] ulithiacyclamide,[5] and trunkamide;[6] selectively metal-binding natural products such as haliclonamides,[7,8] patellamide C,[8] and westiellamide;[9,10] and molecules with other activities such as nostocyclamides,[11–13] which are anticyanobacterial allelochemicals. These elaborate structures and activities have attracted a large number of synthetic and biosynthetic research groups to these molecules and at least in one case have led to preclinical development.

The recent cloning of nearly 40 cyanobactin biosynthetic gene clusters[1,14–17] has provided genetic data firmly relating cyanobactins to each other, enabling the proposal of the cyanobactin group.[1] A number of N–C cyclic peptides without further modification could be cyanobactins as well, but these are not included in the current definition because genetic data are lacking. Not considering the unmodified cyclic peptides, more than

cis, *cis*-Ceratospongamide

Trunkamide

Microcyclamide 7806A

Leucamide A

Wewakazole

Ulithiacyclamide

Patellin 2

Patellamide A

Westiellamide A

Figure 1 Representative cyanobactins.

100 cyanobactins have been isolated and many more have been identified by genome mining and metagenomics, making them one of the most important classes of cyanobacterial natural products (**Table 1**). Biosynthetically, cyanobactins are a subset of the microcin ribosomal natural products,[18] which in turn are part of the bacteriocin superfamily.[19] Cyanobactins share some features with compounds in these groups, but they can be clearly discerned as a subgroup based upon both chemical structures and conserved biosynthetic pathway genes. The designation 'cyanobactin' is thus a practical term to link compounds that are in fact closely related but do not currently share common search terms. It should be emphasized that genetic information is available only for a subset of proposed cyanobactins, as clearly defined in **Table 1**. However, based upon bioinformatic analysis it is highly probable that the compounds in **Table 1** are all biosynthetically related.

As can be seen in **Table 1**, cyanobactins share a number of features in addition to the N–C cyclization:

1. The C-terminal amino acid appears to be universally heterocyclic. The heterocycle is either Pro or derived from Cys, Ser, or Thr by an enzyme-catalyzed process.
2. Nearly all cyanobactins are 6, 7, or 8 amino acids in length with a few rare exceptions of 9–12 amino acids.
3. Cys is nearly always heterocyclized with the sole exception of the ulithiacyclamide group, in which two of the four Cys residues form a disulfide bridge and the other two are thiazole heterocycles.
4. Ser, Thr, and Tyr are often modified, either by heterocyclization to oxazole/oxazoline or by prenylation with DMAPP. The ease of spontaneous ring opening of oxazoline residues[20] could explain the prevalence of unmodified Ser/Thr residues. Alternatively, enzyme specificity could explain this variability. The prenyl group is often in the 'reverse prenyl' position, in which DMAPP has added at the 3-carbon instead of the 1-carbon.
5. Residues adjacent to thiazoline or oxazoline are often epimerized to the nonproteinogenic D form. Current data indicate that this is likely a spontaneous reaction due to the labile nature of this position and not due to enzymatic activity.[21,22]
6. Heterocyclic residues are commonly oxidized to thiazole/oxazole although there is much variability in this oxidation even within single groups of compounds.

Even with these shared features there is an enormous amount of diversity that arises from unique genetic features of this compound class (described below). The actual amino acids in the cyanobactins are hypervariable; that is, each position is subject to numerous amino acid substitutions, leading to a wide diversity in nature. Cyanobactins actually form a series of overlapping families of natural products. Within each family, there are conserved features as well as numerous hypervariable positions. The genetic basis for these changes are now understood, leading to an easy transition from genes to chemical structures and from chemical structures to genes.

Finally, there are other classes of ribosomal peptides found in cyanobacteria, as exemplified by the microviridin group.[23,24] These classes are structurally and biosynthetically interesting in their own right but are outside the scope of this manuscript (for more details on Cyanobactin Structures, see Chapter 2.06).

2.16.3 Sources and Symbiosis

Cyanobactins originate from a variety of sources, including marine animals (sponges, ascidians, and mollusks). However, it is probable that in every case cyanobacteria are the ultimate sources of the compounds. Thus, the cyanobactin group provides a good experimental platform to trace symbiotic cyanobacteria in marine animals. The free-living cyanobactin producers are found in freshwater, the ocean, and even terrestrial habitats. The ubiquity of cyanobactins within cyanobacteria from a variety of environments and their absence from other bacteria indicates that the molecules probably perform a function of particular importance to cyanobacteria. Although no universal role has been defined, possibilities based upon current data are discussed in Section 2.16.8.

The evidence for the production of cyanobactins by free-living cyanobacteria is clear: axenic cultures produce the compounds, and cyanobactins have also been isolated from cyanobacterial mats in the environment (**Table 1**).[25] The best evidence for the production of cyanobactins by uncultivated symbiotic bacteria comes from work with didemnid ascidians.[14,26] These ascidians commonly harbor symbiotic cyanobacteria, *Prochloron* spp., which have so far eluded cultivation.[27] The cyanobacterial symbionts are obvious even from a distance. *Prochloron* contains the

Table 1 Cyanobactins from diverse cyanobacteria

Compound	*Cyanobacterial strain*	*Predicted/known sequence*	*Activity*	*Synthesis*	*Genes*
Compounds isolated from free-living cyanobacteria					
Aerucyclamide A,B	*Microcystis aeruginosa*	ITGCIC	Modest anticrustacean toxicity	No	Yes
Banyascyclamide A,B,C	*Nostoc* sp.	FTACAC	Not reported	No	No
Dendroamide A	*Stigonema dendroideum*	ATVCAC	Multidrug resistance reversal	Yes	No
Dendroamide B,C	*S. dendroideum*	ATACMC	None detected	No	No
Dolastatin 3 (also from a mollusk)	*Lyngbya majuscula*	GCVPLQC	Potent cytotoxin HIV integrase inhibition	Yes	No
Homodolastatin 3	*L. majuscula*	GCIPLQC	HIV integrase inhibition	No	No
Kororamide	*L. majuscula*	ILYCNPSLC	Not reported	No	No
Lyngbya prediction 2	*Lyngbya aestuarii*	VCMPCYP	Predicted structure	No	Yes
Lyngbya prediction 1	*L. aestuarii*	ACMPCYP	Predicted structure	No	Yes
Microcyclamide	*M. aeruginosa*	HCATIC	Modest cytotoxin	Yes	Yes
Microcyclamide 7806A,B	*M. aeruginosa*	ATVSIC	None detected	No	Yes
Microcyclamide prediction 1	*M. aeruginosa*	FTGCMC	Predicted structure	No	Yes
Nostocyclamide	*Nostoc* sp.31	ATGCVC	Anticyanobacteria	Yes	No
Nostocyclamide M	*Nostoc* sp.31	ATGCMC	Anticyanobacteria	No	No
Prenylagaramide A	*Oscillatoria agardhii*	YGTGEFFNP	None detected	No	No
Prenylagaramide B	*O. agardhii*	YLYPINP	None detected	No	No
Raocyclamide A,B	*Oscillatoria raoi*	ISASFC	Sea urchin toxicity	Yes	No
Tenuecyclamide C,D	*Nostoc spongiaeforme* var. *tenue*	ATGCMC	Sea urchin embryo toxicity	Yes	Yes
Tenuecyclamide A,B	*N. spongiaeforme* var. *tenue*	ATGCAC	Sea urchin embryo toxicity	Yes	Yes
Trichamide	*Trichodesmium erythraeum*	GDGLHPRLCSC	None detected	No	Yes
Venturamide A	*Oscillatoria* sp.	ATACVC	Antimalarial	No	No
Venturamide B	*Oscillatoria* sp.	TTACVC	Antimalarial	No	No
Westiellamide A	*Westiellopsis prolifica*	VTVTVT	Modest cytotoxin	Yes	No
Wewakazole	*L. majuscula*	FPISAPPGVTFS	Not reported	No	No
Compounds isolated from ascidians					
Ascidiacyclamide	*Prochloron* spp.	ITVCITVC	Modest cytotoxin	Yes	No*
Bistratamide A,B	*Prochloron* spp.	VTACFC	Modest cytotoxin	Yes	No
Bistratamide C	*Prochloron* spp.	VSACVC	Not reported	Yes	No
Bistratamide D,F,G,I	*Prochloron* spp.	VSVTVC	Mouse sluggishness	Yes	No
Bistratamide E,H,J	*Prochloron* spp.	VCVTVC	Modest cytotoxin	No	No
Comoramide A,B	*Prochloron* spp.	ITFTAC	Modest cytotoxin	No	No
Cyclodidemnamide	*Prochloron* spp.	FTVPCVC	Modest cytotoxin	Yes	No

Cyclodidemnamide B	*Prochloron* spp.	ITVPCLC	Not reported	Yes	No
Cycloxazoline (identical to westiellamide above)	*Prochloron* spp.	VTVTVT	G2/M block Modest cytotoxin	Yes	No
Didmollamide A,B	*Prochloron* spp.	ATFCAC	Modest cytotoxin	Yes	No
Keenamide A (also from a mollusk)	*Prochloron* spp.	LSGPIC	Modest cytotoxin	No	No
Lissoclinamide 1	*Prochloron* spp.	ICFPTVC	Not reported	No	No
Lissoclinamide 10	*Prochloron* spp.	ICFPTIC	Not reported	No	No
Lissoclinamide 2,3, ulicyclamide	*Prochloron* spp.	ACFPTIC	Modest cytotoxin DNA/RNA synthesis inhibitor	Yes	Yes
Lissoclinamide 4–8	*Prochloron* spp.	FCFPTVC	Cytotoxin	Yes	Yes
Lissoclinamide 9	*Prochloron* spp.	VCFPTIC	Not reported	No	No
Mayotamide A	*Prochloron* spp.	ICPVCMC	Modest cytotoxin	No	No
Mayotamide B	*Prochloron* spp.	VCPVCMC	Modest cytotoxin	No	No
Mollamide	*Prochloron* spp.	IPISFPC	Modest cytotoxin RNA synthesis inhibitor	Yes	No
Mollamide B	*Prochloron* spp.	VTPFVC	Modest cytotoxin	No	No
Mollamide C	*Prochloron* spp.	LSGPIC	Modest cytotoxin	No	No
Nairaiamide A	*Prochloron* spp.	VTIPIIP	Not reported	No	No
Nairaiamide B	*Prochloron* spp.	ITIPIIP	Not reported	No	No
Patellamide A	*Prochloron* spp.	ITVCISVC	Modest cytotoxin	Yes	Yes
Patellamide B	*Prochloron* spp.	LTACITFC	Anti-MDR Modest cytotoxin	Yes	Yes
Patellamide C	*Prochloron* spp.	VTACITFC	Anti-MDR Modest cytotoxin	Yes	Yes
Patellamide D	*Prochloron* spp.	ITACITFC	Modest cytotoxin	Yes	No*
Patellamide E	*Prochloron* spp.	VTVCITFC	Modest cytotoxin	No	No
Patellamide F	*Prochloron* spp.	VTVCVTFC	Modest cytotoxin	No	No
Patellamide G	*Prochloron* spp.	ITACLTFC	Not reported	No	No
Patellin 2 (1?)	*Prochloron* spp.	TVPTLC	Not detected	No	Yes
Patellin 3	*Prochloron* spp.	TLPVPTLC	Not detected	No	Yes
Patellin 4	*Prochloron* spp.	TLPVPTVC	Not detected	No	No
Patellin 5	*Prochloron* spp.	TVPVPSFC	Not detected	No	No
Patellin 6	*Prochloron* spp.	TFPVPTVC	Modest cytotoxin	No	Yes
Tawicyclamide A, dehydrotawicyclamide A	*Prochloron* spp.	VPVCFCIC	Modest cytotoxin	No	No
Tawicyclamide B, dehydrotawicyclamide B	*Prochloron* spp.	VPVCLCIC	Modest cytotoxin	No	No
Trunkamide	*Prochloron* spp.	TSIAPFC	Potent cytotoxin	Yes	Yes
Ulithiacyclamide A	*Prochloron* spp.	CTLCCTLC	Cytotoxin Protein synthesis inhibitor	Yes	Yes
Ulithiacyclamide B,E-G	*Prochloron* spp.	CTLCCTFC	Cytotoxin	No	Yes

(*Continued*)

Table 1 (Continued)

Compound	*Cyanobacterial strain*	*Predicted/known sequence*	*Activity*	*Synthesis*	*Genes*
Compounds isolated from sponges					
Ceratospongamide	Unknown	FPISFPC	Potent PLA_2 inhibitor Modest cytotoxin	Yes	No
Haliclonamide A–E	Unknown	PASYPTIP PASYPTIP PASYPTIP	Antifouling	No	No
Haligramides A,B	Unknown	FPMPPMC	Modest cytotoxin	No	No
Leucamide A	Unknown	VPLSATC	Modest cytotoxin Antiviral	Yes	No
Waiakeamide	Unknown	FPMPPMC	Antifouling	No	No
Compounds isolated from mollusks					
Dolastatin 3 (also from cyanobacteria)	Unknown	GCVPLQC	Potent cytotoxin HIV integrase inhibition	Yes	No
Dolastatin E	Unknown	ASACIC	Modest cytotoxin	Yes	No
Keenamide A (also from an ascidian)	Unknown	LSGPIC	Modest cytotoxin	No	No

Nearly all cyanobactins are listed here, with the exception of ~30 patellamide relatives identified by metagenome mining. Linear amino acid sequences of these N–C terminal cyclic peptides are presented either on the basis of known gene sequence or by prediction using alignment with the closest-known sequenced relative. The sequence is highlighted in yellow (heterocycle) or green (prenylated). Underlined residues are oxidized to oxazole or thiazole and residues in italics are variable, being oxidized in some cases and not in others. Some individual sequences are listed with multiple names. This multiplicity is due to either epimerization adjacent to thiazoline or oxazoline or to variable oxidation. * indicates genes reported in the literature, but without publicly available sequence data. Synthesis indicates whether a total synthesis has been reported, while the genes column indicates whether the biosynthetic pathway has been sequenced for the molecule in question (as of August 2008).

Table references: aerucyclamide;[28] ascidiacyclamide, lissoclinamide, patellamide, and ulithiacyclamide group;[5,8,14–17,20,26,29–106] banyascyclamides;[107] bistratamides;[9,90,93,108–125] ceratospongamide;[126] comoramides;[127] cyclodidemnamides;[128–132] dendroamides;[53,109,112,119,123,133–137] dolastatins;[2–4,58,64,71,72,74,83,97,111,138–159] haliclonamides;[7,160] haligramides;[161] keenamide;[162] leucamide;[90,163–166] lyngbya prediction;[1] mayotamide;[127] microcyclamides;[17,167–171] mollamides;[172–175] nairaiamides;[176] nostocyclamides;[11–13,133,177–179] patellins and trunkamide group;[1,6,21,22,102,180–183] prenylagaramides;[184,185] raocyclamides;[186,187] tawicyclamides;[126] tenuecyclamides;[1,13,188,189] trichamide;[16] venturamides;[53] westiellamide/cycloxazoline;[9,10,42,80,81,90,190–194] waiakeamide;[161,168,169,195,196] wewakazole.[197]

unusual (for cyanobacteria) pigment chlorophyll *b*, casting a spinach-green shade over their host ascidians. Numerous studies demonstrate that *Prochloron* and host ascidians are tightly coupled and exchange nutrients such as fixed carbon and reduced nitrogen. These associations are also renowned for their production of diverse, bioactive cyanobactins. It was unambiguously demonstrated using genetic methods that *Prochloron* spp. are the actual producers of cyanobactins.[14] A large number of (nearly 40) related pathways have since been identified within *Prochloron*, confirming the role of these organisms in generating the enormous cyanobactin diversity of ascidians.[1,14–17] In fact, 6% of all known ascidian natural products can be classified as cyanobactins derived ultimately from symbiosis with *Prochloron* cyanobacteria.

Mollusks also contain cyanobactins.[2,138,162] The mollusks in question consume either cyanobacteria or *Prochloron*-containing ascidians, and thus the source of these compounds is probably dietary.[139,172,198] This is a common theme for the soft-bodied gastropods, which often sequester metabolites from dietary sources. There are a few examples of cyanobactins in calcareous sponges and demosponges.[7,160,161,195,199] Currently, there are no data addressing which organism(s) produce these sponge cyanobactins, but it is likely that they are also derived from cyanobacterial symbionts. Cyanobacteria are very commonly found in sponges and are known to contribute to the natural product diversity of the animal assemblage (for more details on Sources and Symbiosis, see Chapter 2.14).[200]

2.16.4 Shape and Metal Binding

The presence of azole nitrogens pointing toward the center of a ring has long led to speculation that metal binding may be a role of cyanobactins in nature. Experimental evidence in favor of metal binding has been achieved in several instances (**Table 2**) although there is as yet no evidence of the importance of metal binding to *in vivo* activity. Since this topic has been the subject of excellent reviews,[29,201] key features of metal binding will be just briefly touched upon here.

The first characterized metal binder was ascidiacyclamide, which bound two Cu(II) atoms coordinated to carbonate.[30] Basic conditions and organic solvents were required to stabilize this complex for crystallization. Another early case involves coordination of westiellamide to four Ag(I) atoms in a relatively tight-binding complex ($K_{assoc} > 2.8 \times 10^{13}\,mol^{-5}\,l^{5}$).[190] This binding property could be greatly improved by synthesis of a tethered westiellamide dimer.[191] Beyond these two groups, most of the metal-binding studies have focused on cyclic hepta- and octapeptides of the patellamide and lissoclinamide group.[7,20,29–40,133,177,201] Some of these molecules have shown preferential binding to Cu(II) and Zn(II) and not to other ions. For the most part, binding constants are on the order of $\sim 10^4$–10^7 in organic solvents such as methanol or acetonitrile, sometimes in the addition of stoichiometric base. Haliclonamides were shown to bind specifically to Fe(III) and Cr(III), which is similar to the action of siderophores, although with $\sim 10^{11}$ lower binding affinity.[7]

Table 2 Metal-binding cyanobactins

Compound	*Metal*
Ascidiacyclamide	Cu(II) Synthetic variants: Ca^{2+}, K^{+}
Haliclonamides	Fe(III), Cr(III)
Lissoclinamide 10	Cu(II)-selective
Mayotamide A	Cu(II), Zn(II)
Patellamide A	Cu(II), Zn(II)
Patellamide B	Cu(II), Zn(II)
Patellamide C	Cu(II)-selective
Patellamide D	Cu(II) Synthetic variants: Ca^{2+}
Patellamide E	Cu(II), Zn(II)
Ulithiacyclamide	Cu(II)-selective
Westiellamide	$4 \times$ Ag(I), Cu(II)

Most of the work on metal-binding cyanobactins has focused on Cu(II) and Zn(II) binding with the patellamides. Patellamides A, B, C, D, and E, as well as ulithiacyclamide, have been shown to bind Cu(II) and Zn(II).[32,35,38–40] Strikingly, patellamide C was shown to selectively bind Cu(II) even in the presence of Zn(II).[35] Molecular shape is clearly related to metal-binding activity. Patellamide C adopts a 'figure eight' conformation when uncomplexed and in the presence of Zn(II). However, when Cu(II) is added, the conformation switches to a 'square' form that can accommodate Cu(II) preferentially. When one or two of the oxazoline rings in patellamide A were synthetically opened, Ca^{2+} bound with low affinity.[34,41] Similarly, synthetic ester analogues of ascidiascylcamide led to the formation of a relatively stable K^+ complex.[20] More recently, westiellamide has also been shown to bind Cu(II) in mononuclear and dinuclear clusters.[42]

Overall cyanobactin shapes are important to metal binding and other properties and have been examined in detail in a number of systems (**Figure 2**). When heterocycles are introduced to macrocyclic structures, as in cyanobactins, flexibility is greatly reduced and often only a single conformation is observed.[22,32–36,38,43–47,108,126,128,140–142,199,202–205] The 6-amino acid cyanobactins assume 'triangle' shapes, while the octapeptides are either 'saddle'/'square' or 'figure eight'. The heptapeptide lissoclinamide 7 adopts a fold that is somewhat similar to the 'twisted figure eight' conformation of patellamide D, except that the prolyloxazoline moiety of the former adopts a type II β-turn motif.[33] The above findings are probably overgeneralized given the extreme diversity of cyanobactin structures. For example, the 'sponge' cyanobactin ceratospongamide has two Pro residues that are *cis*,*cis* in the natural product but *trans*,*trans* in the thermodynamic decomposition product.[199,204,206] The *cis* configuration, which is critical to the potent phospholipase A_2 activity of the molecule, has a very compact structure while the inactive *trans* variant adopts a more rounded, flat shape. Shape also strongly influences the configuration of the α-proton adjacent to thiazoline. In lissoclinamide 7, for example, this proton could not be epimerized using base treatment for several days. By contrast, other peptides are readily epimerized to their more stable forms. Trunkamide was shown to undergo epimerization

Figure 2 Shape of cyanobactins. These molecules adopt a number of conformations that are important to metal binding and activity, including (clockwise from upper left) triangle, square, lissoclinamide turn, and figure eight. This figure was adapted from a previous figure, reprinted with permission from P. Wipf; P. C. Fritch; S. J. Geib; A. M. Sefler, *J. Am. Chem. Soc*. **1998**, *120*, 4105–4112, copyright 1998 American Chemical Society.

in solution from the natural L-form to give a mixture of D- and L-isomers, with the lower energy configuration being the D-Phe epimer.[21,22] Trunkamide is the only prenylated cyanobactin for which 'shape' information is available and as with lissoclinamide 7 the Pro and thiazoline heterocycles dominate the conformation by dictating the presence of turns.[22,180,181] The shape of cyanobactins thus influences a number of properties, including metal binding, stereochemistry, and biological activity.

It is now commonly stated that the 'purpose' of the patellamides and related molecules is almost definitely to bind metals. As will be seen in Section 2.16.8, this is only one of many possible scenarios, and there is much data in support of alternatives. There are several caveats in that the binding constants are rather low (in most cases, at the same level as ligands such as free histidine) in comparison to physiologically relevant molecules and that they have been obtained in organic solvents. These considerations do not mean that one should discount the elegant work on metal binding; it is merely that more experimental evidence is required in a natural system prior to making firm conclusions. It should be kept in mind that metal binding may be related to the mode of action in any case. A good analogy would be bleomycin, which binds both DNA and iron, causing radical damage. However, the iron-binding constant of bleomycin is at least several orders of magnitude better than the best cyanobactin metal-binding constant, that of silver-westiellamide. Finally, it has been speculated that metals could actually 'template' the assembly of cyanobactins *in vivo*.[201] Based upon the characterized biosynthetic pathway to these compounds,[14] that role for metals has been firmly ruled out.

Even in the absence of *in vivo* relevance, metal binding is of importance for biotechnological reasons. For example, it may be possible to design synthetic analogues that have extremely high affinity and could function as sequestering or detection reagents. The tethered westiellamide silver-binder is one example of such an approach.[191] In another report, it proved possible to design patellamide analogues that specifically recognized pyrophosphate in water, although this was due to a backbone modification.[207] These structures have also inspired approaches to construct other molecular receptors, to make templates for chirality transfer,[143] and to mimic protein loops. In the third case, a patellamide-like structure was modified to mimic the interhelical loops of cytochrome *b562*.[186] The constrained nature of thiazole and oxazole may allow cyanobactins and their analogues to precisely mimic constrained portions of proteins (for more details on Shape and Metal Binding, see Chapter 8.15).

2.16.5 Bioactivity

Cyanobactins are often potently bioactive in biological assays, leading to wide-ranging interest in the discovery, synthesis, development, and biosynthesis of these compounds. Many of the cyanobactins isolated from marine animals were found through cancer drug discovery programs and therefore many of them have been characterized for their cytotoxic properties. In addition, several compounds are reported to be antimalarial, allelopathic against competing strains, antifouling, antigrazer, antiviral, and anti-multidrug resistance (MDR). In a few cases, further mechanistic studies have shed light on the potential mechanisms of these activities, but in no case has mechanism of action been strictly defined. A general rule is that cyanobactins have been found to be active only against metazoans (animals) such as malaria, grazing or settling marine or freshwater animals, and human cell lines, but not against fungi. The compounds so far do not exhibit antibacterial activities except against cyanobacteria.

Most of the interest in cyanobactins as potential pharmaceuticals has focused on anticancer activity. The molecules could be of anticancer use in two different ways: (1) many of the molecules are moderate to potent cytotoxins and (2) some of the molecules have been shown to inhibit the MDR phenotype, which is the bane of many chemotherapeutic regimens. Probably the most potent cytotoxic cyanobactins are dolastatin 3, trunkamide, ulithiacyclamide, and lissoclinamide 7, which have low- to mid-nanomolar activities against human cell lines.[2,5,6,48] The most advanced agent was trunkamide, a low-nanomolar cytotoxin, which was reported to be highly selective for the human renal UO-31 cell line and to have a favorable COMPARE profile at the National Cancer Institute.[180] Trunkamide was listed as a preclinical candidate at the marine natural products pharmaceutical company, PharmaMar, but no further information has been available in the past 7 years.

Most other cytotoxin cyanobactins are active in the clinically irrelevant micromolar range. However, their MDR profiles may be of interest nonetheless. Patellamide D was the first example of this activity, and the molecule was shown to be a selective antagonist to MDR, improving the potency of certain drugs in human cell lines.[49] Dendroamides and patellamides B and C were later shown to have similar properties.[50,109]

Relatively little data are available concerning the anticancer mechanism of action of cyanobactins. Ulithiacyclamide-treated mouse leukemia L1210 cells were inhibited with an IC_{50} of 40 ng ml^{-1}.[51] A steep decrease in initial growth followed by a moderate decrease was interpreted to indicate that ulithiacyclamide directly interacts with cellular constituents, causing lethality. Evidence was obtained that the compound inhibits growth in a self-destructive manner, but does not interact with membrane constituents. While the compound did not greatly affect DNA synthesis, RNA synthesis and especially protein synthesis were greatly impacted. By contrast, treatment of the same cell line with ulicyclamide inhibited DNA and RNA synthesis, with about 100–1000-fold less cytotoxicity than ulithiacyclamide.[208] Treatment of human HL-60 cells with westiellamide (also known as cycloxazoline) led to accumulation of cells in G2/M.[192] Polyploid and multinuclear cells accumulated after about 24 h of treatment with westiellamide. After 48 h at high doses, most cells underwent apoptosis. In these cases, the actual mechanisms underlying the effects were not determined.

Antimalarial activity of several cyanobactins has been assessed. Some of the patellamides are antimalarial, but at a dose that is 10-fold greater than the cytotoxic dose.[52] Venturamides A and B have the opposite therapeutic index, being at least 10-fold selective for malaria than human cells.[53] Several cyanobactins have been demonstrated to be antiviral. Dolastatin 3 and homodolastatin 3 were reported to inhibit HIV integrase although these molecules are also potent cytotoxins.[139] Mice injected intracranially with bistratamides became sluggish.[108] Ceratospongamide was shown to be potently anti-inflammatory, inhibiting phospholipase A_2 selectively in the low nanomolar range.[199]

Some of the more interesting cyanobactin biological activities may be related to their ecological roles. Several cyanobactins from sponges, including haliclonamides and waiakeamide, were shown to inhibit settlement by the mussel *Mytilus edulis*.[160,196] This may be relevant in potentially keeping the sponge free from fouling organisms. A number of cyanobactins from free-living cyanobacteria have also been shown to be allelopathic against competing cyanobacteria and small grazing organisms, as discussed in Section 2.16.8. Finally, in many cases no biological activity has been detected or reported. It is probable that many more potential applications will be discovered for these bioactive compounds (for more details on Bioactivity, see Chapter 2.20).

2.16.6 Total Synthesis

Considering the relatively small size of the cyanobactin family, an enormous synthetic effort has gone into the construction of representatives of nearly all subfamilies.[3,4,14,21,37,54–81,110–122,129,130–137,140–142,144–154,163–165,173,174,177–183,187,188,191,193,194,202,204–206,209–216] A large number of analogues with various shapes and properties, especially aimed at metal chelation, have also been synthesized. Many of the syntheses are claimed to be 'biomimetic'. With the accumulation of biosynthetic experiments on this compound class, it is clear that these reactions, while elegant, do not imitate the natural processes. As a complete discussion of cyanobactin synthesis would require its own review, we list synthesized cyanobactins in **Table 1**.

2.16.7 Biosynthesis

The first cyanobactin biosynthetic pathways were discovered in ascidians and firmly attributed to production by *Prochloron* symbiotic cyanobacteria using genome sequencing (**Figure 3**).[15] These pathways could also be directly expressed in *Escherichia coli* in the absence of sequence data.[26] Since then, about 40 new cyanobactin pathways have been discovered in free-living and symbiotic cyanobacteria, providing a great deal of genetic data for the elucidation of biosynthetic routes. Symbiosis has played a key role in this process, allowing unique evolutionary insights to be obtained. These insights have informed genetic engineering approaches to obtain new peptides.

Prochloron spp. synthesize patellamides using a bacteriocin-like process.[14] The final patellamide structures are directly encoded on a precursor peptide, PatE. This linear, ribosomally encoded peptide must be modified and cleaved to yield heterocyclized, macrocyclized patellamides. In particular, the patellamides must be cut out of the precursor peptide from both their C- and N-termini, a property not yet observed in any other cyclic

Hypervariable Hypervariable

N$_{29}$ LSEEALGDAGLEASVTACITFCAYDGVEPSITVCISVCAYDGE

PatE

PatD/TruD
PatF/TruF1/TruF2

PatG (oxidase)
$-2H_2$

Figure 3 (Continued)

PatA/PatG
Proteases

Figure 3 Proposed generic biosynthesis of cyanobactins. Enzymes act to heterocyclize and then macrocyclize a precursor peptide, yielding cyanobactins.

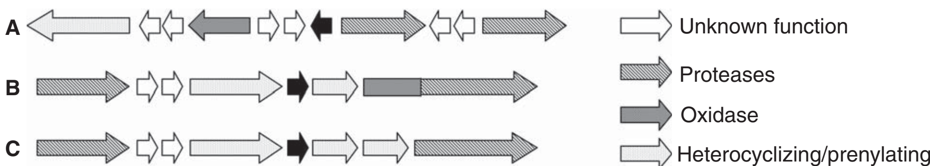

Figure 4 Cyanobactin gene clusters. (a) Trichamide cluster. (b) Patellamide gene cluster. Syntenic gene clusters were identified for tenuecyclamides and microcyclamides. (c) Trunkamide gene cluster leading to prenylation.

peptide of the bacteriocin group. In addition to *patE*, the patellamide gene cluster consists of six other genes, *patABCD* and *patFG* (**Figure 4**). By transferring *patABCDEFG* to *E. coli* and detecting patellamide A and C production, it was demonstrated that these genes are necessary and sufficient for patellamide production. Coexpression of individual genes from this cluster led to the identification of PatA, PatD, PatF, and PatG as the essential modifying enzymes.[15] *Prochloron* spp. have not yet been cultivated, so all experiments are performed in *E. coli*.

Based upon the predicted protein sequence of these peptides, a biosynthetic pathway was proposed.[14,15] The PatE precursor peptide encodes two patellamides, which are flanked with repeating motifs that were thought to be recognition elements for modifying enzymes. PatD contains regions that are functional, but not sequence, homologues of genes required for heterocyclization of Cys, Ser, and Thr in the microcin pathway from *E. coli*.[217,218] PatA and the C-terminus of PatG harbor domains that are homologous to subtilisin serine proteases. Therefore, it is probable that PatD and the C-terminus of PatG are responsible for thiazole and oxazoline formation in the patellamides. Two proteins could be responsible for macrocyclization. PatA and the N-terminus of PatG harbor domains that are homologous to subtilisin serine proteases. Because patellamides must be cut twice, and there are two different putative recognition elements, it seemed probable that PatA would recognize one cleavage site while PatG would recognize the other. Serine proteases are mechanistically related to thioesterases from nonribosomal metabolism, so it was proposed that one of these proteases would also act as a macrocyclization catalyst to yield the final patellamides. A mystery remained in that PatF was not homologous to any known protein, yet it was required for the synthesis of these compounds.

Cloning of a related biosynthetic pathway shed light on the potential function of PatF.[1,16,17] Trunkamide and its relatives from ascidians are heterocyclized at Cys, yet Ser and Thr are prenylated. It was thus anticipated that the trunkamide biosynthetic pathway might contain a new prenyltransferase enzyme. However, when the trunkamide/patellin pathway was cloned, no new enzyme class was identified. In fact, the *tru* gene cluster was highly similar to the *pat* cluster. The genes were nearly identical, except in a central region of the gene cluster. The C-terminus of the PatD homologue, TruD, was quite different. In addition, TruG did not have an N-terminal oxidase, unlike PatG. This made sense, since the trunkamide/patellin group lacks thiazole, having only thiazoline. Between TruD and TruG, the TruE precursor peptide was relatively similar to PatE, but there were two PatF homologues, TruF1 and TruF2, that were only ~40% identical to each other and to PatF. Thus, there were no new enzyme types in this cluster, despite the presence of prenylation. *truABCDEF1F2G* were expressed in *E. coli*, leading to production of patellins, so one of these genes had to be responsible for the prenylation activity. Because the major differences between *pat* and *tru* were the PatF homologues, it was proposed that this new protein class controlled prenylation vs. heterocyclization of Thr and Ser. An alternative pathway to that described above has also been proposed on the basis of molecular modeling.[82]

The strong resemblance of the *pat* and *tru* clusters and the clear presence of a variable region within these clusters leading to functional divergence could be observed because the producing bacteria are closely related symbionts of animals. Another striking observation from the symbiotic systems was that, within the patellamide class, only the precursor peptide varies, while other enzymes and genes are nearly 100% identical.[15] For example, in the lissoclinamide, patellamide, and ulithiacyclamide families, all enzymes are identical, and in fact even the precursor peptide PatE is nearly identical. Only the region within PatE that directly encodes products is varied, leading to the discovery of 30 PatE variants. The same story holds true with the trunkamides: pathways are identical, and only a small region directly encoding products is hypervariable. This observation,

seen with symbiotic bacteria, has not been reported from any other system in natural products. Hypervariability within a small region directly affecting the products indicates that these small molecules are phenotypically important, as discussed in Section 2.16.8.

Cyanobactin biosynthetic pathways have also been found in a number of free-living bacteria.[219] The producing genes are homologous to those for patellamides and trunkamide. Based upon this observation and an alignment of all known small-molecule relatives, the cyanobactin class was proposed. With free-living cyanobacteria, transcriptional analysis was performed, demonstrating that at least one pathway is constitutively expressed (for more details on Biosynthesis, see Chapters 1.14 and 5.08).[11,12]

2.16.8 Ecology and Purpose

There are relatively few experiments assessing the direct ecological impact of cyanobactins, and most proposals are therefore based upon circumstantial evidence. Possibly the best experiments to be performed on this group involves the nostocyclamides from the freshwater cyanobacteria *Nostoc* sp.31. This strain was identified from 65 cultivated cyanobacterial strains as having an especially potent anticyanobacterial activity.[13] In addition, six other strains exhibited anticyanobacterial activity. A later study relied on bioassay-guided fractionation to identify the cyanobactin nostocyclamide as the active anticyanobacterial metabolite.[19] Nostocyclamide potently inhibited the growth of *Anabaena* sp. cyanobacteria, at $100\,\mathrm{nmol\,l^{-1}}$, but it did not affect other nonphotosynthetic bacteria tested. Additionally, the compound was a $12\,\mu\mathrm{mol\,l^{-1}}$ inhibitor of a rotiferan predator. Later, the related molecule nostocyclamide M was also isolated from this strain and shown to be similarly anticyanobacterial.[12] The data were interpreted to indicate that the molecules were required for competition with other cyanobacteria.

This hypothesis of allelopathy is intriguing, especially in light of the massive amount of data regarding other bacteriocins.[15] Many bacteriocins have been shown, through elegant experiments, to be toxic only to bacteria that are closely related to the producing organism and not to more distantly related organisms. Mathematical modeling as well as bacterial competition experiments with colicin-producing *E. coli* clearly demonstrates that bacteriocins actually promote diversity by increasing the number of niches available to otherwise nearly identical bacteria. Cyanobactins could thus be true bacteriocins in every sense, performing a function analogous to that of the bacteriocins in human intestinal flora. Nostocyclamide was somewhat more broad-spectrum than the traditional bacteriocins, however.

If the cyanobactins are indeed targeted against closely related cyanobacteria in general, several questions remain: what is the molecular target that allows cyanobacterial specificity? Why are these molecules often potently active against mammalian cell lines and other higher eukaryotes? Have these molecules been co-opted to other purposes in symbiotic organisms? In addition, a necessary cautionary note is that these experiments have only been performed with one strain of cyanobacteria and may not be universally applicable.

Experiments to define the mechanism of anticyanobacterial action of nostocyclamide indicated that the compounds also inhibit chlorophyceans, although less potently.[15] It was shown that *Anabaena* cyanobacteria were inhibited in the synthesis of chlorophyll *a*, carotenoids, and protein after 36 h. These filamentous bacteria also formed much shorter filaments than normal, and the cells appeared swollen, with a greater than normal diameter. However, nostocyclamide did not inhibit electron flow through photosystem II, indicating that the compound did not directly target photosynthesis *per se*.

Data with *Prochloron* spp. symbionts of ascidians indirectly support a possible allelopathic role for cyanobactins.[16] Numerous strains of *Prochloron* spp. inhabit individual ascidians. Data indicate that each strain has 0 or 1 cyanobactin pathways, and in addition the pathways do not reflect the taxonomy of the producing strains but appear to be possibly horizontally transferred. It could be asked, how can many different strains occupy a relatively closed environment in a single niche over time? The allelopathy hypothesis provides one possible explanation. Like *E. coli* in the human intestine, *Prochloron* in ascidians may produce small molecules that allow the proliferation of diverse strain types within a single ecological niche.

The rapid evolution of 'cassettes' that encode patellamides seems to support this possible role for cyanobactins.[15] The fact that evolution takes place essentially solely at the site encoding the final products indicates that these molecules are undergoing strong selection. Many variants can be detected at low abundance in whole animals, yet a

few cyanobactin subtypes are currently prevalent in ascidians across vast swaths of the world's tropical oceans. This type of observation is highly reminiscent of observed distributions of bacteriocins in terrestrial mammals although the evolutionary swaps observed in *Prochloron* have not yet been observed in mammal symbiotic bacteria.

However, the prevalence of cytotoxic cyanobactins within ascidian associations indicates that allelopathy may not be the 'purpose' of these metabolites, or possibly not the sole purpose. For example, they could have been co-opted by the animal itself for use in chemical defense against predation or settlement of competing organisms. There is a large literature concerning chemical defense of sessile marine invertebrates, and didemnids often inhabit predation-intensive reef environments where chemical defense is common.[220] Certain sponge cyanobactins have even been shown to inhibit the settlement of mussels, albeit at relatively high concentrations.[160,182] The molecular mechanisms of cytotoxicity, antifouling, and anticyanobacteria allelopathy remain unknown. It is possible that these actions are related in some unknown way.

Metal binding may also play an ecological role, as summarized in a review.[29] A number of potential roles for metal binding have been proposed. Carbonate is part of a number of cyanobactin–metal complexes, leading to the idea that the molecules could function to immobilize CO_2 or as a carbonic anhydrase. Another possibility is that the molecules could act catalytically using oxygen and/or small organic molecules. Finally, patellamides could be involved in copper detoxification within ascidians. It could also be suggested that metal binding may be important to mode of action of the compounds even in the absence of catalytic or sequestering action. For example, cyanobactins seem to affect metazoans and cyanobacteria, seemingly distantly related groups. Could metals tie these actions together? These speculations, if validated, would greatly impact understanding of natural product roles in the environment.

It is possible in light of the above considerations that cyanobactins have diverse roles in nature. Experiments are lacking to raise these possible roles above the level of sheer speculation, unfortunately. The unusual evolutionary pathway to these molecules should prompt and enable ecological experiments to define the role of these ubiquitous cyanobacterial metabolites.

2.16.9 Genome Mining and Modification

The addition of genetic and genomic technology for cyanobactins has led to new applications for this group. One of the early examples of natural product structure prediction from genome sequence was found with trichamide, a cyanobactin produced by free-living cyanobacteria, *Trichodesmium erythraeum*.[16] Since then, there have been a number of cyanobactins discovered or predicted from genome sequence and it is also possible to predict and clone cyanobactin pathways from a starting structure.[1,17] The ability to express *pat* genes in *E. coli* has led to engineering approaches to synthesize analogues.[15]

Trichodesmium erythraeum is a global cyanobacterium that produces massive blooms in the ocean and is also one of the most important nitrogen fixers on earth. No natural products were known from *T. erythraeum*, yet its genome sequence contained a cluster of genes homologous to *pat*.[16] The structure of trichamide was predicted and validated by fourier transform mass spectrometry. Microcyclamide variants were predicted from the genome sequence of *Microcystis aeruginosa*.[17] In addition, the microcyclamide gene cluster was identified on the basis of sequence prediction. The tenuecyclamide gene cluster from *Nostoc spongiaeformae* was cloned on the basis of structure, and in addition cyanobactin structures were predicted on the basis of the *Lyngbya aestuarii* genome.[1]

Genetic engineering methods have allowed the rapid exploitation of genome-mined clusters. For example, the patellin pathway was cloned in a single polymerase chain reaction (PCR) into an *E. coli* vector for expression.[1] In order to produce trunkamide, a small piece from an environmental metagenome was PCR-amplified and crossed into the expression vector by yeast recombination. Rapid mutagenic techniques yield cyanobactin analogues *in vivo*. This technology will allow rare pathways from small amounts of biomass to be functionally expressed and analyzed, bypassing sample limitations. Another approach to obtain cyanobactin diversity involves genetic engineering to make unnatural analogues. Using the patellamide biosynthetic pathway, a wholly new cyclic peptide, eptidemnamide, was 'synthesized' in *E. coli* using genetic engineering.[15] This type of methodology could allow synthesis of large libraries of cyanobactin analogues at the phage-display scale for purposes such as discovery of drugs and new metal-binding materials (for more details on Genome Mining and Modification, see Chapters 2.12–2.13).

2.16.10 Summary and Conclusions

Cyanobactins are currently one of the biggest groups of cyanobacterial natural products and it is likely that many more of the compounds will be discovered by genetic and chemical means. Since their discovery in 1980, the cyanobactins have been the subject of hundreds of reports, yet key questions have yet to be answered and many potential applications remain. Particularly intriguing unsolved questions include the potential role of metals in ecology and mode of action. The prevalence of cyanobactins and their importance in a variety of processes should make answering these questions a high priority. It is also of particular note that many cyanobactins are part of symbiotic interactions with marine animals. What is it about these molecules that makes them so common in animal associations? There are many possibilities, including that they act as typical allelopathic bacteriocins or that their initial allelopathic activity has been co-opted for defense. Understanding the molecular mode of action of certain compounds in humans might help to answer this question and will also be important for developing the compounds or their relatives into useful agents. Finally, biosynthetic questions remain. In particular, the evolutionary question is intriguing. How does the patellamide pathway evolve by replacing small cassettes within an absolutely conserved genetic background? This process is somewhat reminiscent of the way the human acquired immune system functions, but that is very distant from bacteria in many ways. Clearly, interaction between organisms is driving this hypervariable diversity, but what are those interactions? Answering these and many other questions concerning the cyanobactins will have a large biotechnological impact in the discovery, design, and synthesis of new pharmaceuticals and materials.

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2.17 The Role of Synthesis and Biosynthetic Logic

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2.17.1 Introduction

A significant number of natural products exhibit pharmacological activities that are beneficial to human health. There are many examples of natural product compounds that are in clinical use, including antibacterial penicillins, cephalosporins, immunosuppressive cyclosporine A, and the cholesterol-lowering HMG-CoA reductase inhibitors best known as the 'statins'.[1] These natural products, which have achieved their functions over the course of millions of years of evolution, offer chemical scaffolds for development of new analogues with improved or altered functions. New bioactive analogues that contain novel structural elements may be generated by both semisynthesis and total synthesis efforts.[2,3] In fact, over two-thirds of newly introduced drugs worldwide were natural products or natural product derivatives in the past two decades.[4]

The number of natural products that function in biological systems is large but represents only a small fraction of the total possible number of small carbon-based compounds, indicating the importance of stereochemistry and functional groups in natural product functions.[5] Modern synthetic chemistry has encountered difficulty in preparation of complex, high-molecular-weight natural products containing a great number of reactive groups and stereocenters for the generation of drug leads in the pharmaceutical industry. Incorporating the use of biocatalysts during natural product synthesis represents a promising strategy for the production of compounds that are desperately needed for pharmaceutical development.[6,7] Although enzymes in living cells have been extensively used as biocatalysts in the food and beverage industry, isolated enzymes play critical roles in performing chemical transformations on organic compounds in many areas.[8–11] Often, these remarkable catalysts are able to perform a wide array of reactions on structurally diverse compounds. Furthermore, enzymes can also selectively catalyze reactions with chiral (enantio-) and positional (regio-) selectivities.[11] With these advantages, enzymes are applied in organic synthesis to avoid tedious protection and deprotection steps commonly required for enantio- and regioselective synthesis. The inherent selectivity of enzymes generates few by-products, making it an environmentally friendly alternative to chemical catalysts. Enzymes used in organic synthesis include acyltransferases (e.g., lipases, esterases, peptidases, amidases, and acylases), carbohydrate-processing enzymes (e.g., glycosidases, glycosyltransferases), hydrolytic enzymes (e.g., nitrilases, nitrile hydratases), reductases, oxidases and oxygenases, aldolases, and oxynitrilases.[8]

Polyketides (PKs), nonribosomal peptides (NRPs), and PK/NRP or NRP/PK hybrids represent three large subclasses of highly diverse natural products with various bioactivities.[12] These natural products are produced by large megaenzymes, polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs). Type I PKSs consist of multiple modules, with each module minimally containing three core domains: acyltransferase (AT) domain, ketosynthase (KS) domain, and thiolation (T) domain (also called acyl carrier protein (ACP) domain). Typically, one type I PKS module catalyzes a single elongation cycle for PK production (**Figure 1**). During elongation, the AT domain serves as the gatekeeper for specificity, responsible for selecting the

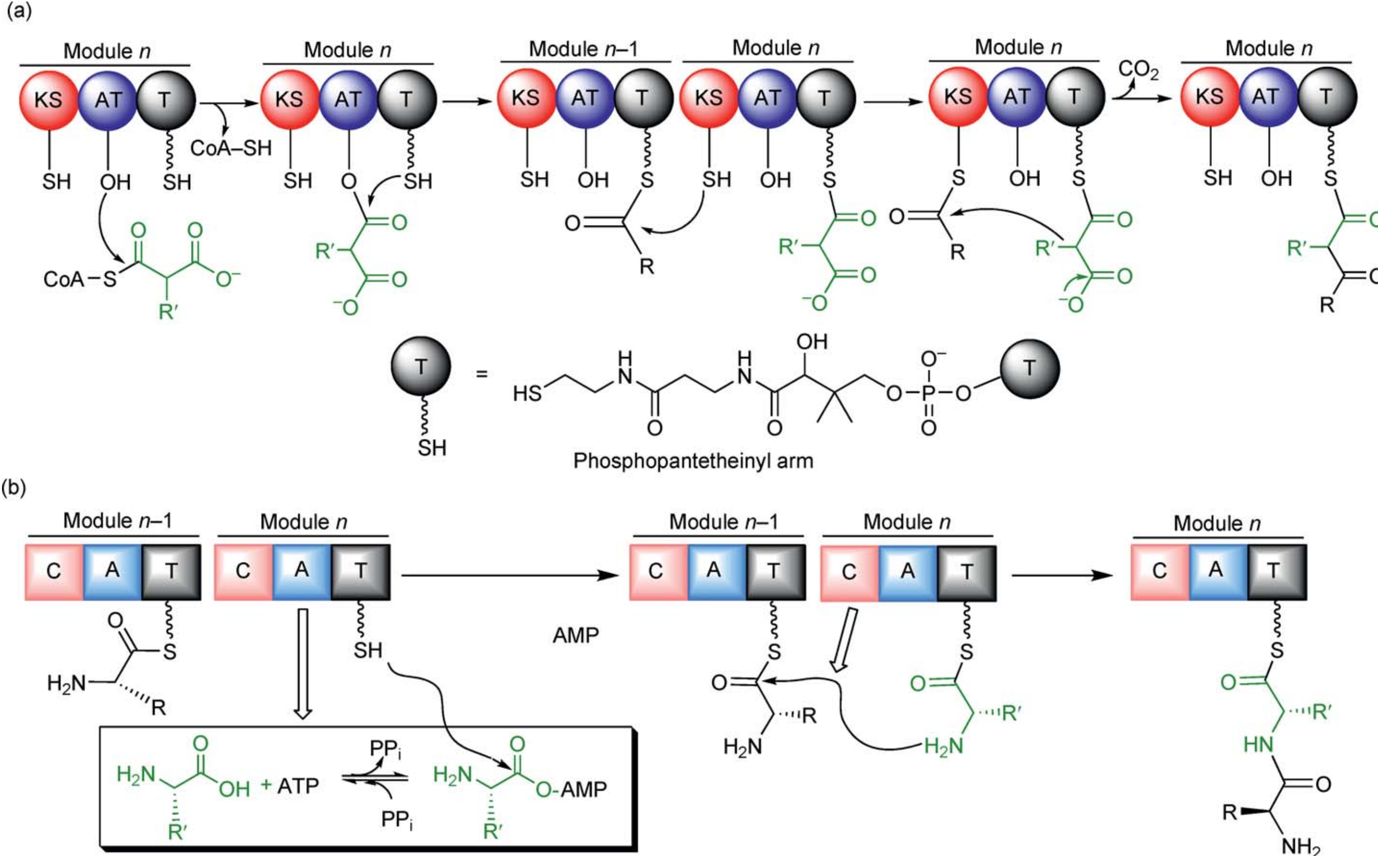

Figure 1 Schematic representations of one elongation cycle catalyzed by the minimal module of PKS (a) and NRPS (b). A common phosphopantetheinyl arm is found in the holo-T domain of both PKS and NRPS as shown in (a).

appropriate CoA extender unit (e.g., malonyl-CoA, methylmalonyl-CoA) and transferring the extender unit to the sulfhydryl terminus of the phosphopantetheinyl arm on the T domain.[13] The KS domain catalyzes the decarboxylation of acyl-*S*-T to generate a carbanion that reacts with the PK intermediate linked to the T domain generated in the previous elongation cycle. The resulting α-ketoacyl-*S*-T becomes the substrate for the next cycle of elongation catalyzed by the subsequent module. In addition to type I PKSs, there are two other PKS classes, type II PKSs and type III PKSs.[14,15] Unlike the type I class, type II PKSs consist of discrete enzymes that are organized as a multicomponent system.[15] The type III PKSs are distinguished from the others by lack of an AT and T domain. Type III PKS systems typically use CoA substrates (i.e., malonyl-CoA), but there is precedent for their ability to accept acyl-*S*-T substrates.[14,16] Similar to the type I PKSs, NRPSs are composed of multifunctional enzymes that are arranged into modules. Each NRPS module contains three core domains: adenylation (A), condensation (C), and thiolation (T) (also called peptidyl carrier protein (PCP) domain)[17] (**Figure 1**). The A domain is responsible for selecting and activating the natural or modified amino acid monomer. The activated amino acid monomer is covalently attached via a thioester bond to the cysteamine group of a phosphopantetheinyl arm in the holo-T domain. The condensation (C) domain catalyzes the formation of the peptide bond between the amino acid monomer and the peptidyl intermediate tethered to a T domain in an adjacent module. Similar to type I PKS modules, each NRPS module performs a single elongation step of the growing peptidyl chain. In both NRPSs and PKSs, there are several additional domains that contribute to natural product structural diversity. Ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), and methyltransferase (MT) domains are commonly found in PKS modules while *N*-MT and epimerase (E) domains are generally embedded within NRPS modules. These additional domains contribute significantly to the diversity and bioactivity of PKs and NRPs. Thioesterase (TE) domains, typically found at the C-terminus of the final elongation module in both PKSs and NRPSs are responsible for terminating biosynthesis. In most cases, TE domains catalyze intramolecular macrocyclization or hydrolysis of the thioester bond between the final T domain and the PK or NRP intermediate.[18] The structures of the nascent PK and NRP products are often further modified through oxidation, glycosylation, acylation, alkylation, and halogenation reactions catalyzed by tailoring enzymes in natural product biosynthetic pathways.[19,20]

Here, we highlight recent advances in chemoenzymatic approaches to synthesize natural products. Particular attention is given toward the application of TE domains in macrocyclization of PK and NRP natural products. The utilization of tailoring enzymes such as glycosyltransferase, P-450 hydroxylase, and P-450 epoxidase to diversify and functionalize natural products in synthesis is also discussed. Preparation of PKs and NRPs with multifunctional enzymes is a new direction in chemoenzymatic synthesis and will be included as well.

2.17.2 Chemoenzymatic Approaches to Pikromycin Synthesis

Pikromycin (**1**) is a 14-membered ring macrolide antibiotic produced by *Streptomyces venezuelae* (**Figure 2**).[21,22] This naturally occurring ketolide binds to the 23S bacterial rRNA and inhibits protein synthesis by interfering with channeling of the nascent peptide product. Although its anti-infective ability is relatively weak, the structure of pikromycin (**1**) does offer a chemical scaffold that can be further modified by semisynthesis to produce novel ketolide compounds. Moreover, the enzymes responsible for pikromycin biosynthesis provide biocatalytic tools that may be employed for the chemoenzymatic generation of diverse libraries of ketolide-type analogue structures. The biosynthetic machinery has been extensively investigated over the past decade, and as discussed below these studies have provided significant new insights into the assembly and modification of novel macrolide antibiotics using chemoenzymatic approaches.

2.17.2.1 Pikromycin Biosynthesis

Details of pikromycin biosynthesis were first elucidated following the cloning and sequencing of the pikromycin biosynthetic gene cluster from *S. venezuelae* ATCC 15439 in 1998.[23] Within an approximate 60 kb region of DNA, 18 discrete pikromycin biosynthetic genes were identified. Based on their encoded protein function, these genes were assigned into five separate loci: PKS (*pikA*), desosamine biosynthesis (*des*), cytochrome P-450

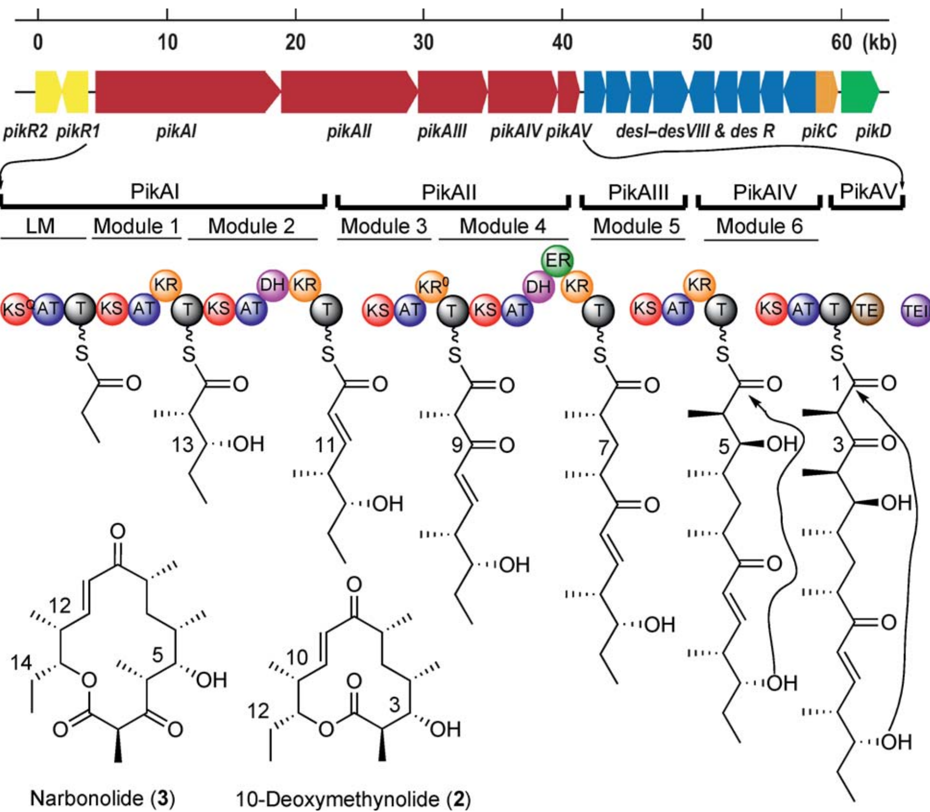

Figure 2 Schematic organization of the pikromycin biosynthetic gene cluster and its elongation and processing pathway. The pikromycin biosynthetic gene cluster is composed of 18 discrete genes, clustered into five separate loci. The pikromycin type I PKS is composed of a single loading module (LM) and six elongation modules that span four polypeptide chains (PikAI–PikAIV). Both 12-membered and 14-membered ring products, 10-deoxymethylnolide (**2**) and narbonolide (**3**), respectively, are generated by this unique type I PKS assembly line.

hydroxylase (*pikC*), transcriptional activator (*pikD*), and resistance locus (*pikR*) (**Figure 2**).[24] The *pikA* gene locus encodes a prototypical type I modular PKS composed of five polypeptide chains (PikAI–PikAV) (**Figure 2**). A single loading module (LM) and two elongation modules reside in PikAI, which act in concert with three or four additional elongation modules, spanning PikAII–PikAIV, to assemble a hexaketide- (elongation through PikAIII) or heptaketide-T domain intermediate (elongation through PikAIV, **Figure 2**). The linear PK intermediate is subsequently macrolactonized by the terminal TE domain of PikAIV to generate either a 12-membered ring macrolactone, 10-deoxymethynolide (**2**), or a 14-membered ring macrolactone, narbonolide (**3**), generated by one additional elongation through PikAIV. PikAV is a type II thioesterase that serves to remove aberrantly decarboxylated extender units from PKS T domains to prevent blockage of the PKS assembly line.[25]

Sugar anchoring is essential for macrolide antibiotic interaction with the bacterial 23S rRNA subunit.[26] A desosamine sugar moiety is tethered to the C-5 position of pikromycin (**1**) and appears to be critical for its bioactivity. Within the pikromycin biosynthetic gene cluster, a complete set of genes is encoded (*desI–desVI)* whose protein products convert the common primary metabolite D-glucose-1-phosphate to thymidine diphosphate D-desosamine (TDP-desosamine) (**Figure 3**).[27] Also included is *desVII*, a gene that encodes the glycosyltransferase that is responsible for attachment of the desosamine aminosugar to the polyketide backbone.[27] In addition, *desVIII* encodes an enzyme of unknown function but this polypeptide was shown to be indispensable for DesVII activity *in vitro*.[28] Finally, a putative β-glucosidase encoded by *desR* is proposed to reactivate the antibiotic during or after cellular secretion by removing the glucose group from a desosaminyl–glucosyl disaccharide moiety, which is possibly linked to the same position as desosamine in the natural products.[29]

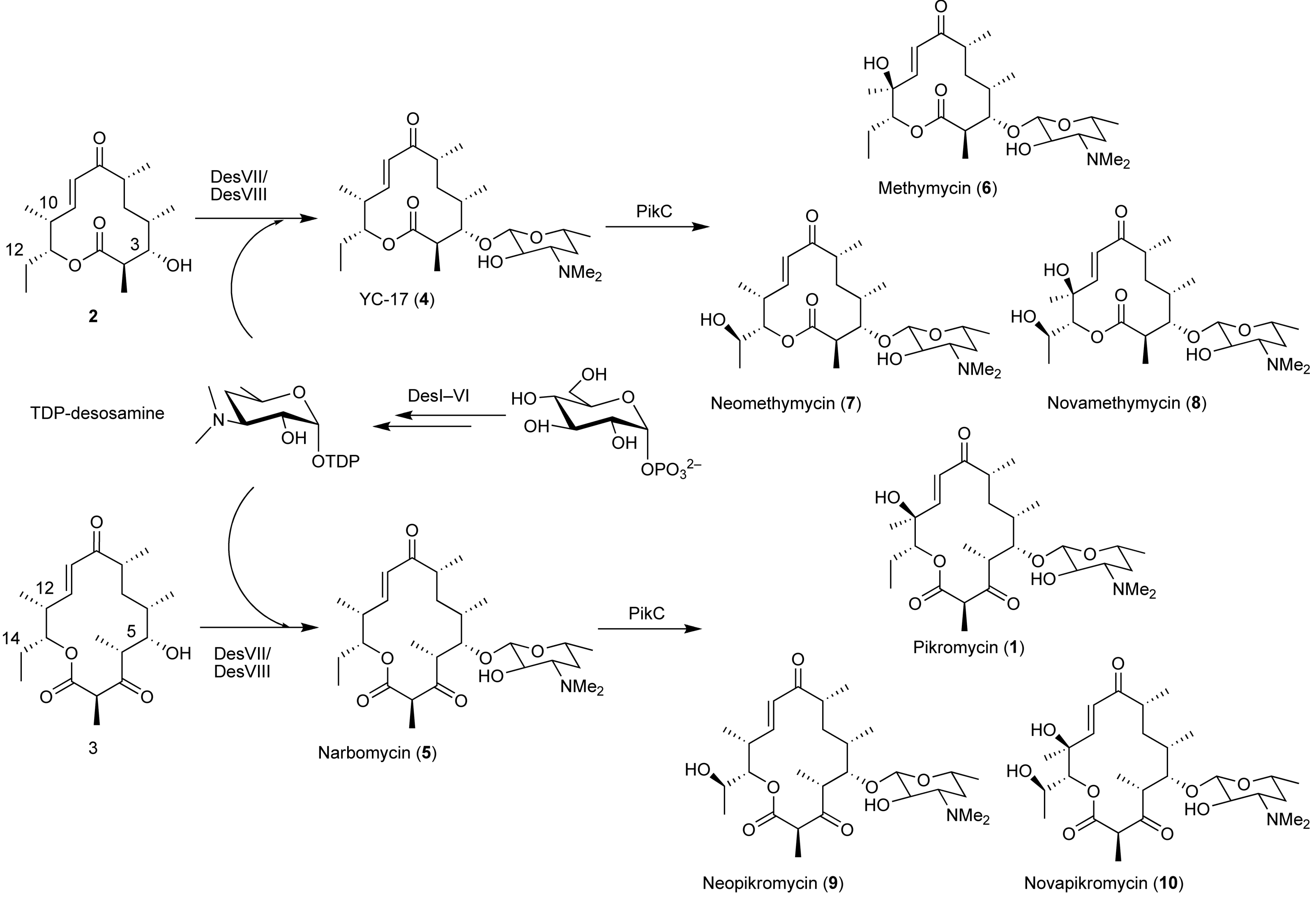

Figure 3 Post-PKS modification reactions catalyzed by tailoring enzymes in pikromycin biosynthesis. TDP-desosamine is biosynthesized by DesI–DesVI with D-glucose-1-phosphate as the starting substrate and is used to decorate both 12-membered and 14-membered ring aglycones by DesVII/DesVIII to produce two glycosylated macrolides, YC-17 (**4**) and narbomycin (**5**). These two natural products are further modified *in vivo* by P-450 PikC to generate six different hydroxylated natural products, including pikromycin (**1**). PikC is capable of producing compound (**1**) and (**6**) with spinach ferredoxin reductase, ferredoxin, NADPH, and O_2 during *in vitro* analysis. The engineered RhFRED-PikC catalyzes the same reactions only with NADPH and O_2.[31]

PikC, the P-450 hydroxylase in the pikromycin gene cluster catalyzes hydroxylation of the initial macrolide products (YC-17/narbomycin) and displays unparalleled flexibility toward the macrolactone core of its macrolide substrates. Both 12-membered ring YC-17 (**4**) and 14-membered ring narbomycin (**5**) are produced after glycosylation with desosamine. Interestingly, PikC catalyzes hydroxylation at C10 or C12 of the 12-membered ring (**4**) to yield methymycin (**6**) or neomethymycin (**7**) (**Figure 3**).[30] Novamethymycin (**8**) occurs from hydroxylation at both the C10 and C12 positions of compound (**4**).[31] Similarly, pikromycin (**1**), neopikromycin (**9**), and novapikromycin (**10**) are produced after hydroxylation at C12, C14, and both positions of 14-membered ring macrolide (**5**), respectively (**Figure 3**).[32]

In summary, the enzymes responsible for pikromycin biosynthesis represent powerful tools for the generation of novel macrolide antibiotic compounds with the unique biosynthetic capabilities of the pikromycin PKS and the substrate flexibility of the respective tailoring enzymes. In the next section, we highlight work directed at investigating and exploiting the biosynthetic enzymes encoded by *pikA*, *des*, and *pikC* for the production of ketolide compounds through chemoenzymatic approaches.

2.17.2.2 Synthesis of Pikromycin with Application of Its PKS and Tailoring Enzymes

The manipulation of type I PKS systems represents an appealing strategy for the generation of numerous structurally diverse polyketide natural products in combinatorial biosynthesis.[33] These enzymes can also be employed in chemoenzymatic approaches toward natural product synthesis. In a recent application, PikAIII and PikAIV, two monomodular PKS proteins, were selected for detailed investigation due to their involvement in mediating the partitioning of the different-sized macrolactone products in *S. venezuelae*. Both PikAIII and PikAIV have each been individually coexpressed with the phosphopantetheinyl transferase from *Bacillus subtilis* (*sfp*) in *Escherichia coli* BL21 (DE3) and soluble holo-enzymes have been purified.[34,35] Supplying methylmalonyl-CoA and diketide *N*-acetylcysteamine (NAC) thioesters led to triketide and tetraketide lactone structures from PikAIII and the combination of PikAIII and PikAIV, suggesting their ability to produce novel PKs in chemoenzymatic synthesis (**Figure 4(a)**).[34,35] The known 10-deoxymethynolide (**2**) and narbonolide (**3**) were also effectively produced in the combination of PikAIII and PikAIV using the synthetic NAC-pentaketide thioester and NAC-hexaketide thioester substrates, respectively. This work represented the first reported chemoenzymatic synthesis of either of these macrolactone products (**Figure 4(a)**).[36,37] The biosynthesis of both macrolactones relies on proper cyclization of their respective linear chain elongation intermediates, and is catalyzed by the TE domain embedded in PikAIV, thus stimulating efforts to investigate this remarkably flexible domain.[24]

The macrocyclic core structures of natural products dramatically reduce the number of possible conformations, resulting in proper orientation of bioactive molecules for specific interaction with target proteins.[18] Although macrolactones of eight atoms or more can be generated with several synthetic strategies,[38] factors such as complex protecting group strategies, poor regioselectivity, and intermolecular oligomerizations significantly limit their general application. To overcome these complications, nature has employed a TE catalytic domain for the termination of macrocyclic polyketide biosynthesis. TE domains efficiently catalyze cleavage and subsequent regiospecific cyclization of the fully extended linear intermediate, a characteristic that positions these enzymes as attractive candidates for development into useful chemoenzymatic tools. The TE domain from the pikromcyin type I PKS system was excised from *pikAIV*, heterologously overexpressed and purified.[39,40] This recombinant catalyst exhibited broad substrate specificity toward a series of NAC-diketide thioesters, suggesting a relaxed substrate specificity.[39] Later, the natural hexaketide chain elongation intermediate was synthesized as an NAC thioester and chemoenzymatically transformed by the excised TE domain to the 12-membered ring macrolactone product, 10-deoxymethynolide (**2**) (**Figure 4(b)**).[37,41] Interestingly, reduction of the C7-carbonyl of the NAC-hexaketide substrate to the allylic alcohol resulted in exclusive hydrolysis to produce a *seco*-acid following TE reaction, demonstrating that despite its natural substrate tolerance for chain length variation (i.e., hexaketide and heptaketide), Pik TE is sensitive to minor functional group modifications of its natural substrates. The recent high-resolution X-ray crystal structure of the pikromycin TE domain offered detailed information regarding its substrate specificity and catalytic mechanism.[37,42] Interestingly, two well-ordered water molecules, residing near the exit of the substrate channel, appear to effectively reroute the hydrophobic substrate back toward the active site for macrocyclization. Furthermore,

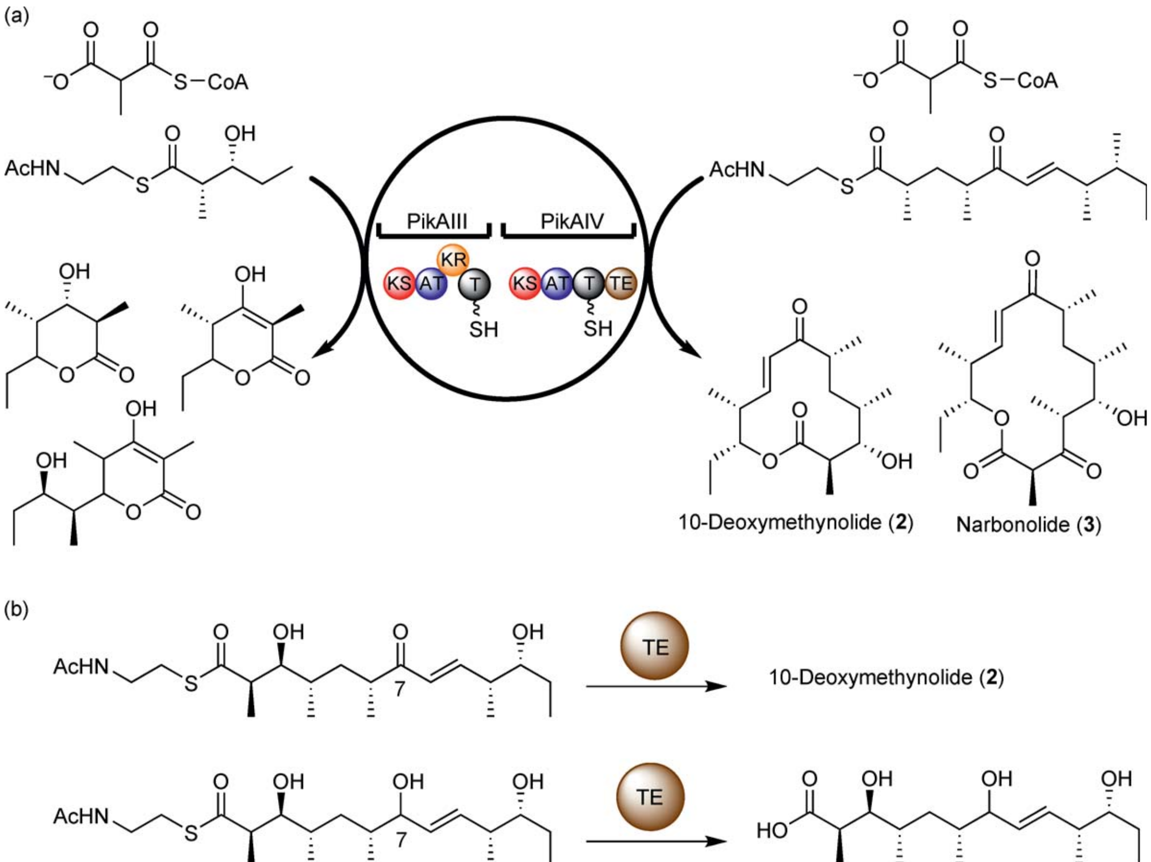

Figure 4 (a) Representative examples to apply PikAIII and PikAIV in the preparation of natural and unnatural products through chemoenzymatic synthesis. PikAIII and PikAIV alone or their combination are capable of producing a diverse series of products.[35–38] (b) Two reactions catalyzed by excised Pik TE domain. The excised TE domain efficiently catalyzes macrolactonization of NAC-hexaketide but produces the linear hydrolytic product when the substrate was modified with a hydroxyl group at the C7 position.

the distal hydroxyl is directed to the proper position for attack of the acyl-enzyme intermediate due to conformational restrictions imposed by the α,β-unsaturated ketone.[42] Current efforts are focused to further extend understanding, exploration, and expansion of the substrate tolerance of this versatile PKS catalytic domain.

Carbohydrates include diverse structures and fulfill numerous physiological roles for normal cell function and survival.[43] The presence of a sugar moiety in natural products not only enhances their chemical diversity but also imparts remarkable influence on their pharmacology and pharmacokinetic properties and cellular distribution.[5,44–46] The desosamine sugar that is a component of pikromycin effectively contributes to its bioactivity due to interactions with the bacterial 23S rRNA subunit. As such, efforts have been focused on varying the chemical structure of this sugar as a means to generate novel pikromycin analogues.[28,47,48] The attachment of sugar moieties to aglycones during natural product biosynthesis is catalyzed by glycosyltransferases.[49] In narbomycin (**5**) biosynthesis, the DesVII glycosyltransferase is responsible for transferring the TDP-desosamine that is produced from D-glucose-1-phosphate by DesI–DesVI to the aglycone narbonolide (**3**) (**Figure 3**). This versatile enzyme is also involved in producing YC-17 (**4**) from 10-deoxymethynolide (**2**). The Liu group first characterized DesVII *in vitro* after successfully cloning and purifying it from *E. coli*.[28] Interestingly, the presence of DesVIII provided a 20-fold enhancement to DesVII glycosyltransferase activity, demonstrating DesVII/DesVIII pairing in chemoenzymatic synthesis of glycosylated natural products (**Figure 3**).[28] In order to further investigate the substrate specificity of the DesVII/DesVIII glycosyltransferase system and to expand

their application in chemoenzymatic synthesis, a small sugar library was prepared. This was made possible by improvements in chemical methods for NDP-sugar synthesis and in particular developments in glycodiversification.[47,49,50] In total, six nonnatural sugar moieties were utilized to decorate both 10-deoxymethynolide (**2**) and narbonolide (**3**), suggesting that DesVII/DesVIII is tolerant of fairly broad structural variation in 6-deoxyhexose substrates (**Figure 5(a)**).[47] Moreover, the DesVII/DesVIII combination installed TDP-desosamine and six nonnatural sugar substrates to 16-membered ring tylactone (**11**) and two hydroxylated 10-deoxymethynolide (**2**) analogues, methynolide (**12**) and neomethynolide (**13**) to generate 21 products, many of which were novel.[47] These experiments demonstrated that DesVII/DesVIII displays relaxed substrate selectivity toward TDP-deoxyhexose donor and aglycone acceptor, suggesting that this glycosyltransferase system could be useful in chemoenzymatic strategies for the generation of novel macrolide antibiotics. Remarkably, DesVII/DesVIII was able to regioselectively attach desosamine to the C3–OH group of two linear NAC-hexaketides, expanding the application of this system toward linear aglycones along with cyclic compounds (**Figure 5(b)**).[48] The apparent plasticity of this system toward unnatural substrates offers the exciting possibility of generating novel macrolide antibiotics through chemoenzymatic glycodiversification. It is evident that DesVII/DesVIII and many other glycosyltransferases found in secondary metabolic biosynthetic systems are poised to find wide application in the preparation of new analogues in the search for valuable medicinal agents.

P-450s are a superfamily of heme-thiolate enzymes composed of more than 6000 members. Their catalytic activity depends on the consumption of NADPH/NADH and O_2. This family of enzymes is widely utilized in the biosynthesis of antibiotics and other bioactive natural products by catalyzing a variety of reactions such as hydroxylation and epoxidation. The functional groups introduced by P-450s not only enhance the biological activities of natural products but also increase their chemical diversity and provide reactive sites that are amenable for additional chemical modification. The difficulties encountered in synthetically installing hydroxyl or epoxide functionality into natural products have motivated researchers to utilize recombinant P-450s in organic synthesis. PikC is a P-450 hydroxylase that exhibits broad substrate selectivity during pikromycin biosynthesis. In a series of studies, PikC has been heterologously expressed and purified from *E. coli* to address important issues relating to its selectivity and reactivity. The purified recombinant enzyme was able to hydroxylate both C10 and C12 positions of the 12-membered ring macrolide YC-17 (**4**) and convert 14-membered ring macrolide narbomycin (**5**) into three different products by adding a hydroxyl group to C12 and C14 positions (**Figure 3**).[23,31,51] Further insight into PikC substrate flexibility was unveiled in an *in vivo* analysis that utilized novel 12-membered ring macrolides that were linked with unnatural sugar moieties.[52–54] Given its broad substrate specificity, PikC is an attractive candidate to be developed into a useful biocatalyst for the hydroxylation of novel macrolide compounds containing an appropriately linked desosamine sugar. Recently, PikC was engineered into a self-sufficient hydroxylation catalyst by fusing with the RhFRED reductase domain.[55] The presence of this additional domain effectively eliminates PikC dependence on spinach ferredoxin reductase (FNR) and ferredoxin (Fer) for its activity and enhances significantly catalytic efficiency in hydroxylation of YC-17 (**4**) and narbomycin (**5**) compared to wild-type enzyme (**Figure 3**).[55]

While only enzymes involved in pikromycin biosynthesis have been discussed in this section, they represent excellent examples for application of secondary metabolite pathway enzymes to produce novel natural products through chemoenzymatic approaches. In the upcoming sections, additional enzymes involved in structurally diverse natural product biosyntheses will be included to further examine biocatalysts functioning in drug discovery and development.

2.17.3 Chemoenzymatic Approaches to Tyrocidine Synthesis

Tyrocidine is an NRP antibiotic that is isolated from *Bacillus brevis*.[56] This natural product is able to permeate the lipid phase of the membrane and perturb the lipid bilayer of a Gram-positive inner cell membrane.[57,58] Although four tyrocidine analogues have been identified, they are assembled by the same biosynthetic machinery that is able to incorporate different amino acids of structural similarity at specified sites.[59] The tyrocidine biosynthetic system consists of three tyrocidine (Tyc) NRPSs, Tyc A–C (**Figure 6**). In total, 10

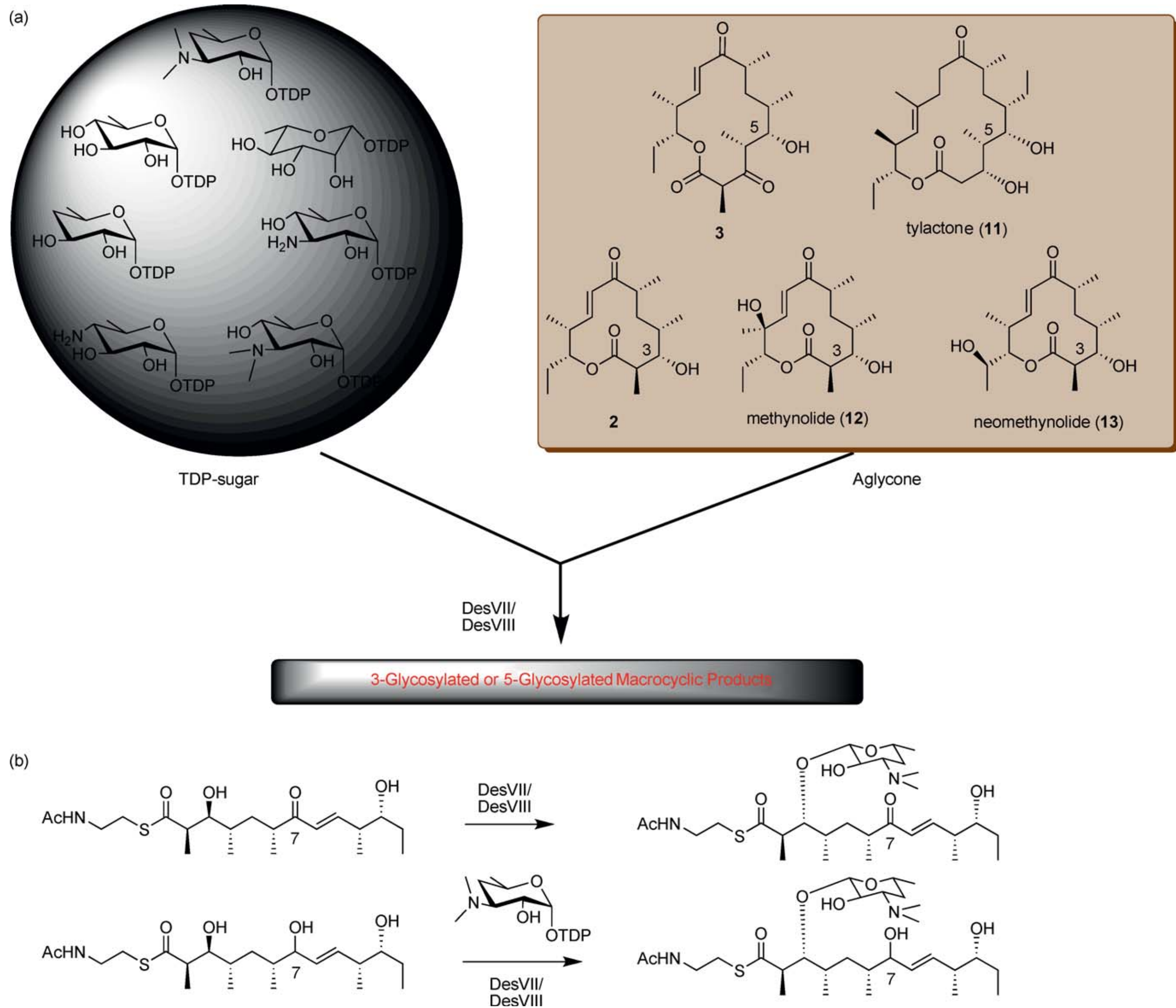

Figure 5 (a) Chemoenzymatic synthesis of glycosylated macrocyclic products with DesVII/DesVIII. The enzyme system successfully transfers natural and unnatural TDP-sugars to C3–OH or C5–OH groups in the natural and unnatural aglycone substrates. (b) Two hexaketide NAC thioesters utilized by DesVII/DesVIII as substrates.

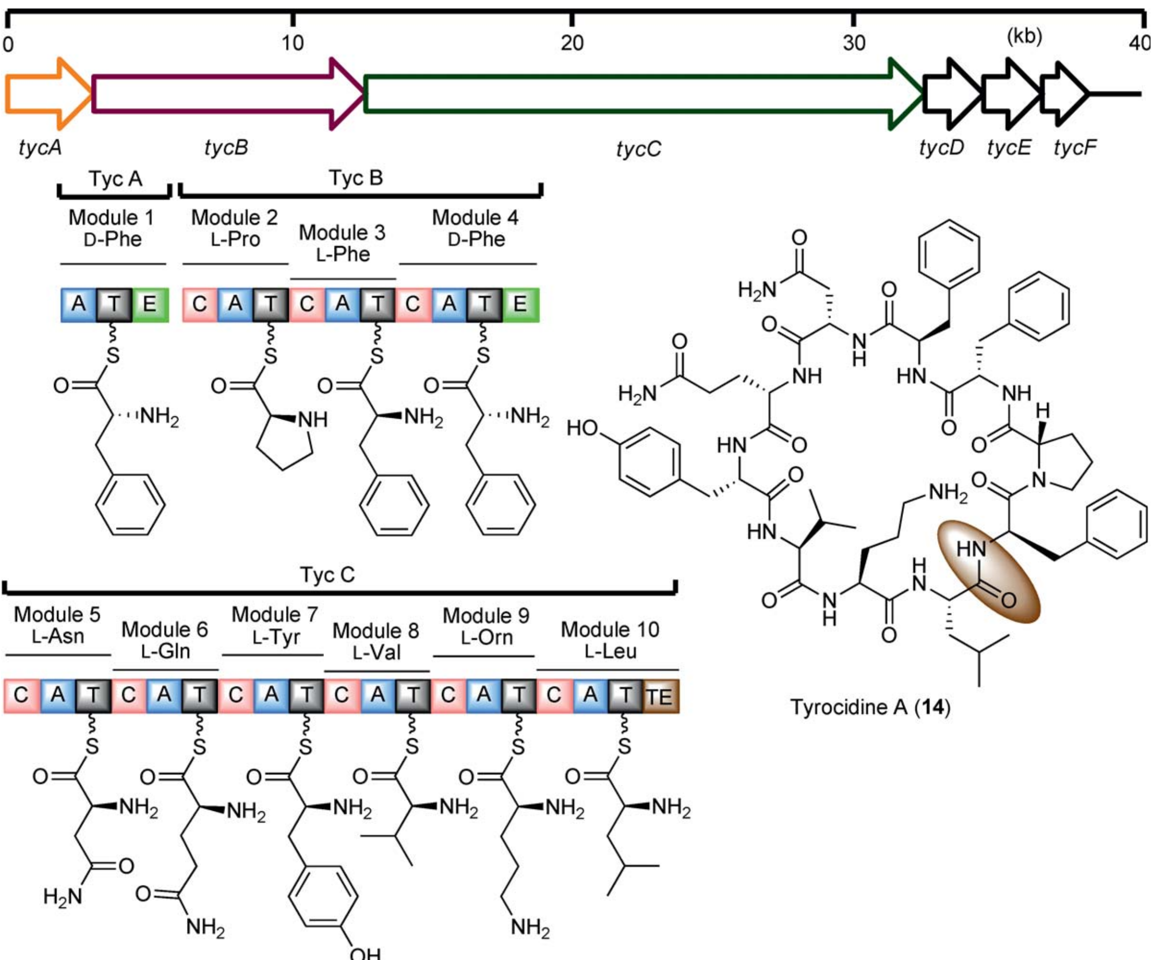

Figure 6 Schematic organization of tyrocidine gene cluster and TycA, Tyc B, and TycB domain components. The tyrocidine gene cluster extends ~38 kb of DNA and consists of three NRPS genes (*tycA*, *tycB*, and *tycC*), two ABC transporter genes (*tycD* and *tycE*), and one putative TE gene (*tycF*). Tyc A is a single module enzyme responsible for activating D-Phe. Tyc B and Tyc C have three and six modules, respectively, and nine natural and modified amino acid residues activated by these modules are also included. Tyrocidine A (**14**) is generated after forming the macrolactam catalyzed by Tyc TE domain in the last NRPS module.

modules that span three megasynthetase enzymes are responsible for specifically activating and incorporating 10 natural and modified amino acid residues into a linear decapeptide intermediate bound to a T domain. For example, the production of tyrocidine A (**14**), relies on the ordered addition of D-Phe, L-Pro, L-Phe, D-Phe, L-Asn, L-Gln, L-Tyr, L-Val, L-ornithine (L-Orn), and L-Leu. The TE domain located at the C-terminus of Tyc C catalyzes the macrocyclization of the linear intermediate in a head-to-tail fashion.

Despite the ease and convenience of synthesis of various linear peptides by solid-phase peptide synthesis, there are limited chemical methods that can be employed to cyclize the linear peptide products.[60] Thus, TE domains from NRPS biosynthetic systems represent a promising tool to overcome these synthetic challenges. The Tyc TE has been widely studied for its substrate tolerance and stability in different circumstances.[7,18] The Tyc TE domain was cloned and overexpressed as an excised recombinant enzyme, representing the first *in vitro* investigation of an NRPS TE domain.[61] The decapeptide-*S*-T, the native substrate of Tyc TE domain, was replaced with a synthetic peptide NAC thioester for analysis of Tyc TE reactions. The NAC-activated peptide was effectively converted into the macrocyclic tyrocidine A (**14**) along with a lower extent of substrate hydrolysis (**Figure 7**). Although the nature of TE-catalyzed hydrolysis is unknown, nonionic detergents

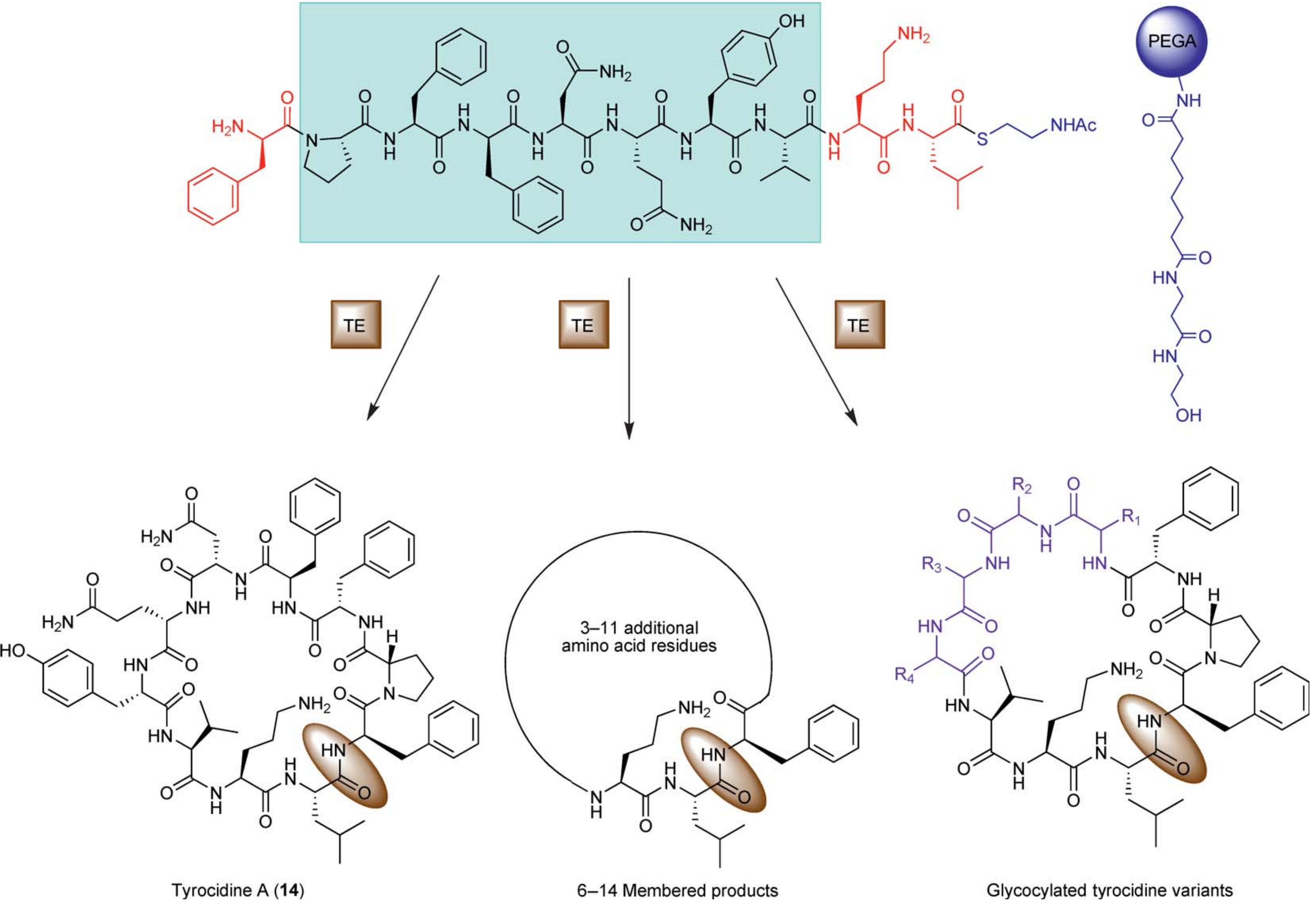

Figure 7 Chemoenzymatic synthesis of macrolactams catalyzed by Tyc TE. The excised Tyc TE is capable to produce tyrocidine A (**14**) with the linear decapeptide NAC thioester or PEGA resin (in blue) with a biomimetic linker. Tyc TE tolerates various modifications to the residues in the rectangle box. However, D-Phe, L-Orn, and L-Leu (in red) are required in all substrates. With modified substrates, Tyc TE generates both macrocyclic peptides with 6–14 amino acids and tyrocidine analogues with different sugar moieties attached to position 4, 5, 6, and 7.

present in the reaction mixture significantly improved the cyclization-to-hydrolysis ratio.[62] The successful production of compound (**14**) in this chemoenzymatic approach demonstrated Tyc TE macrocyclization ability and prompted the search for novel bioactive cyclic peptides. Detailed substrate specificity of the Tyc TE domain clearly revealed that this powerful enzyme not only cyclized a series of decapeptide-NACs with D-Phe, L-Orn, and L-Leu conserved in position 1, 9, and 10 but also formed 6–14 residue cyclic peptides (**Figure 7**).[61,63,64] The effective use of this protein in chemoenzymatic synthesis led to the generation of a cyclic artificial integrin-binding peptide, which had 7 out of 10 amino acid residues different than tyrocidine A and had a high affinity for the integrin receptor.[65] The versatility of Tyc TE domain was further exemplified in the production of glycosylated tyrocidine variants with improved bioactivity and macrocyclization of a library of decapeptides tethered to PEGA resin through a biomimetic linker (**Figure 7**).[66–68] In summary, the Tyc TE domain is a versatile enzyme with general utility to generate improved therapeutic agents in chemoenzymatic synthesis, coupled with other organic tools such as solid-phase combinatorial chemistry, carbohydrate synthesis, and 'click' chemistry.

There are other mechanisms to release NRP intermediates from NRPSs, although TE domains are mostly utilized. For example, cyclosporine A is possibly released and macrocyclized by one unusual N-terminal C domain in its synthetase.[69] Besides cyclosporine A, thaxomin A may also employ the same strategy in its biosynthesis.[70] The another uncommon method to release NRP intermediates is to reduce the final carboxy group with the consumption of NAD(P)H by reduction domain, exemplified by nostocyclopeptide biosynthetic system.[71,72] Nonetheless, TE-catalyzed macrocyclization is the favorable mechanism for product proteolytic stability and possibly improved bioactivity.

2.17.4 Chemoenzymatic Approaches to Cryptophycin Synthesis

Cryptophycins are isolated from the cyanobacterial symbiont *Nostoc* sp. ATCC 53789 and *Nostoc* sp. GSV 224.[73,74] They are potent anticancer agents due to their ability to stimulate cellular microtubule instability, inhibit microtubule assembly, and induce tubulin self-association, resulting in a G2/M phase transition block in the cell cycle.[75,76] The cryptophycin mode of action and cellular target resembles both the vinca alkaloids and taxol.[77,78] Significantly, the cryptophycins are not active substrates of P-glycoprotein and/or multiple drug resistance-associated protein, making them viable chemotherapeutic alternatives for treatment of vinca alkaloid- and taxol-resistant cancers.[79,80] The clinical potential, validated mode of action, and synthetically challenging structure of cryptophycins, as well as the lack of large-scale fermentation methods for their production have stimulated the development of synthetic methods to provide suitable amounts of material and new analogues with improved physiochemical properties for clinical studies.

The cryptophycin gene cluster from *Nostoc* sp. ATCC 53789 and *Nostoc* sp. GSV 224 was recently identified in a 40 kb region of DNA, offering potential chemoenzymatic tools for cryptophycin production (**Figure 8**).[81] The gene cluster is composed of two type I PKS genes, *crpA* and *crpB*, two NRPS genes, *crpC* and *crpD*, and four tailoring enzyme genes including a P-450 epoxidase gene (*crpE*), a putative 2-ketoglutarate iron-dependent hydroxylase gene (*crpF*), a decarboxylase gene (*crpG*), and a flavin-dependent halogenase gene (*crpH*). CrpA and CrpB each contain two elongation modules that are hypothesized to generate the δ-hydroxy phenyloctenoic acid polyketide moiety in cryptophycin 1 (**15**) from one molecule of phenylacetate or its derivative as a starter unit, and three molecules of malonyl-CoA as extender units. CrpC is a monomodular NRPS containing a single elongation module that activates and epimerizes L-tyrosine to D-tyrosine and subsequently condenses the activated D-tyrosine with the PK acyl intermediate tethered to the CrpB T domain. The activated amino acid may also be methylated by a single MT domain in CrpC. CrpD is a bimodular NRPS enzyme. Its first module activates methyl-β-alanine that is converted from L-aspartic acid by CrpG, a β-methylaspartate-α-decarboxylase.[82] The second module of CrpD assembles an activated α-ketoisocaproate to produce the final PK/NRP hybrid intermediate. The immediate source of α-ketoisocaproate remains unknown, but may result from transformation of L-leucine. Interestingly, this NRPS module contains one KR domain, which may convert α-ketoisocaproate into α-hydroxyisocaproate. The final domain of CrpD is a thioesterase that catalyzes release of the linear intermediate from the final T domain of CrpD and generates a cyclic 16-membered ring natural product. This cyclic depsipeptide is further structurally diversified by tailoring

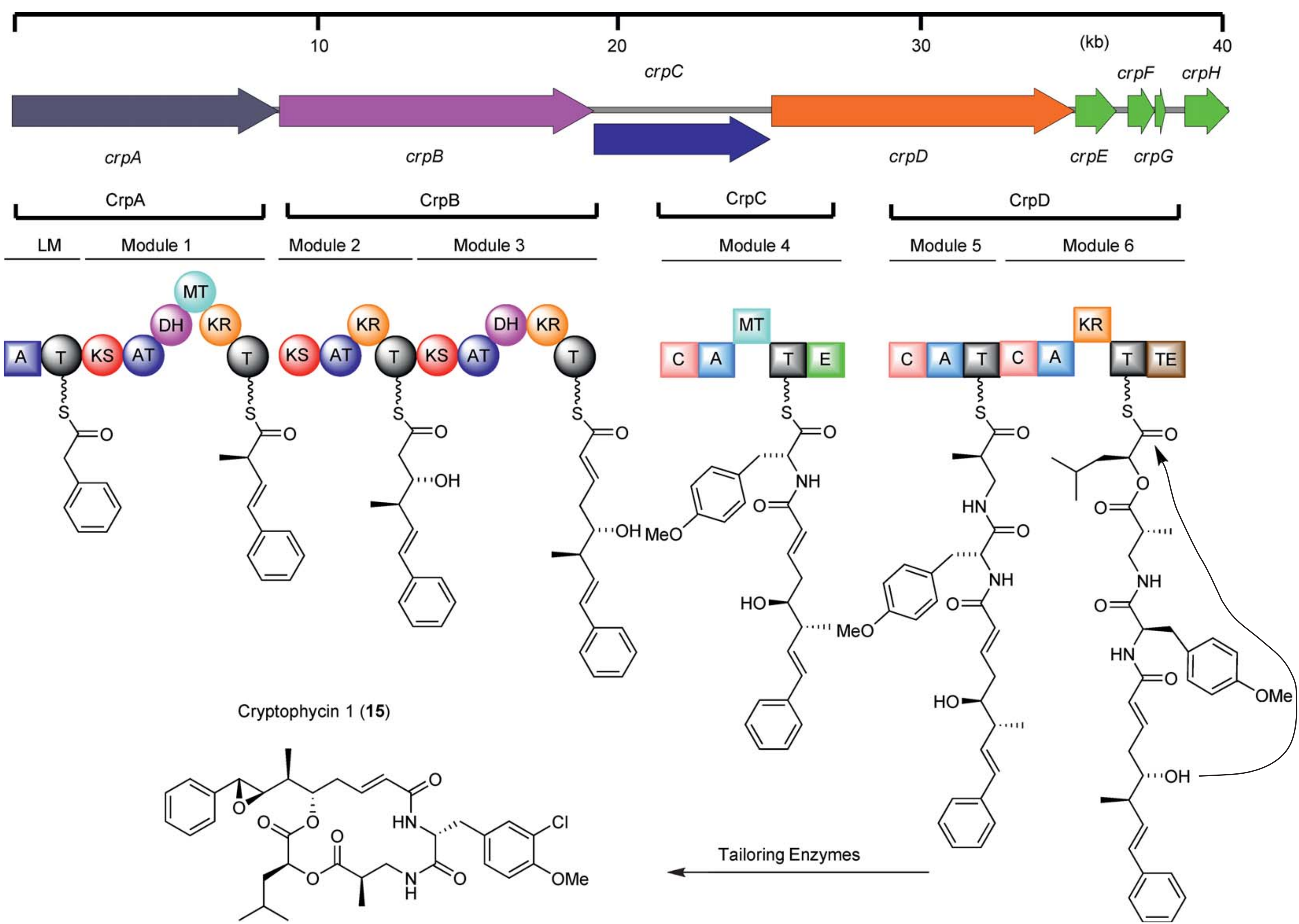

Figure 8 Schematic organization of cryptophycin gene cluster (*crp*) and its biosynthetic pathway with CrpA, CrpB, CrpC, and CrpD. The *crp* system encompasses about 40 kb of DNA region and consists of two PKS genes (*crpA* and *crpB*), two NRPS genes (*crpC* and *crpD*), and four tailoring enzyme genes (*crpE*, *crpF*, *crpG*, and *crpH*). CrpA is composed of one LM and one elongation module. CrpB is a bimodular PKS and produces PK unit along with CrpA. CrpC and CrpD are two NRPSs responsible for activation and assembly of three amino acid residues. The PK/NRP intermediate is released by intramolecular macrocyclization catalyzed by Crp TE. Cryptophycin 1 (**15**) is generated after post-PKS/NRPS modifications catalyzed by tailoring enzymes. The cryptophycin biosynthetic pathway represents a prototypical route to produce a diverse series of PK/NRP natural products.

enzymes whose genes are encoded within the cryptophycin biosynthetic gene cluster. More than 25 naturally occurring cryptophycin analogues are produced by this biosynthetic machinery in *Nostoc* sp. ATCC 53789,[81] with the major isolate being cryptophycin 1 (**15**) (**Figure 8**).

The large number of cryptophycins produced by *Nostoc* sp. ATCC 53789 is indicative of the flexibility of the cryptophycin biosynthetic system, including PKSs, NRPSs, and tailoring enzymes. The versatility of this assembly line was first assessed using a precursor-directed biosynthesis approach.[81] In total, 22 unnatural amino acids and halogen sources were introduced to the *Nostoc* culture and 44 unnatural cryptophycin analogues including cryptophycin 52, the synthetic lead molecule that later advanced to clinical trials, were isolated and identified. This result highlighted the exciting possibility that the cryptophycin biosynthetic machinery could be employed to generate and identify new bioactive cryptophycin analogues as anticancer leads.[81]

The extended growth period of *Nostoc* sp. ATCC 53789 and GSV224 limits their ability to produce cryptophycin analogues in large-scale fermentation. Alternatively, total synthetic strategies have been developed to provide adequate supplies for clinical evaluation.[83] Several effective synthetic approaches have been employed for the generation of cryptophycins, including sufficient quantities of cryptophycin 52 required for clinical trials. However, proper macrocyclization and efficient epoxidation are the two most challenging issues for cryptophycin chemical synthesis, and chemical reagents to address these issues add considerable time and expense to achieve the final desired products. As described with pikromycin and tyrocidine chemoenzymatic synthesis approaches above, cryptophycin (Crp) TE has the potential to solve the problem of proper macrocyclization in both an environmentally friendly and economical manner. In a recently described approach, the Crp TE was excised and heterologously overexpressed as a recombinant enzyme.[84] Four NAC-activated *seco*-cryptophycins were chemically synthesized and utilized to interrogate the *in vitro* activity and substrate specificity of Crp TE (**Figure 9**). Naturally occurring cryptophycin 4 (**16**), cryptophycin 24 (**17**), and an unnatural C6 *gem*-dimethyl analogue, cryptophycin B (**18**), were produced as the major products in the chemoenzymatic reactions, suggesting the termination enzyme is robust in its ability to produce both natural and unnatural products and tolerant to variants occurred at the cryptophycin β-alanine moiety. TE specificity was further investigated using a substrate lacking the phenyl group in its PK moiety. Interestingly, Crp TE was also capable of generating the cyclic product (**19**) with significantly increased levels of hydrolysis (cyclization:hydrolysis = 1:8.3).[84]

Solid-phase peptide synthesis is widely used in the preparation of NRP substrates for NRPS TE domain studies. Recently, this approach was applied to PK/NRP natural product studies.[85] Several *seco*-cryptophycin analogues were synthesized on safety-catch PEGA resin and subjected to chemoenzymatic reactions with Crp TE (**Figure 9**). Similar to the previously described Tyr TE studies, Crp TE also recognized these substrates and catalyzed macrocyclization to produce cryptophycin 29 (**20**) and the unnatural des-epoxy cryptophycin 24 (**21**).[85] Since the ester bond between methyl-β-alanine and α-ketoisocaproate moieties in cryptophycins is labile to hydrolysis, Crp TE was probed for its tolerance regarding replacement of this ester with an amide linkage. Generation of a novel cyclic compound bearing the amide (**22**) in the Crp TE reaction demonstrates the unusual versatility of this enzyme. Thus, Crp TE is an attractive tool to overcome the macrocyclization challenge in chemical synthesis, and to generate an array of new cryptophycin analogues in sufficient quantity for bioactivity analysis.

The most significant challenge in cryptophycin chemical preparation is the late-stage regio- and stereospecific installation of the epoxide moiety due to the labile and highly reactive nature of this functionality.[83] Initial efforts with mCPBA or dimethyl dioxirane (DMD) resulted in conversion of 25% of the starting material into the unfavorable α-epoxy cryptophycin. The potency of this isomer is at least 100 times less than the β-epoxy product.[86,87] Although several other stereospecific epoxidation methods have been developed, there remains significant need for improvement in yield and selectivity.[88,89] This demand may be fulfilled with the successful development of CrpE from the cryptophycin biosynthetic system as a chemoenzymatic tool. In a recent study, CrpE was generated as a soluble recombinant enzyme whose N-terminus was tagged with a maltose-binding protein.[81,90] The proper folding of this enzyme is facilitated by coexpression with chaperone enzymes within the bacterial host. Spectral analysis of the recombinant enzyme revealed it to be an authentic P-450. Subsequently, its activity and substrate specificity was characterized using a small library of NAC-activated *seco*-cryptophycin analogues and cyclic des-epoxy analogues that included all possible structural variation in both the tyrosine and methyl-β-alanine moieties (**Figure 10(a)**). Although the native redox

16, R^1 = Ph, R^2 = Me, R^3 = H
17, R^1 = Ph, R^2 = H, R^3 = H
18, R^1 = Ph, R^2 = Me, R^3 = Me
19, R^1 = H, R^2 = H, R^3 = H

20, X_1 = OMe, X_2 = Cl, X_3 = O
21, X_1 = OMe, X_2 = H, X_3 = O
22, X_1 = H, X_2 = H, X_3 = NH

Figure 9 Chemoenzymatic synthesis of cryptophycin analogues using the excised Crp TE domain. Recombinant TE from this prototypical PKS/NRPS system tolerates structural modifications on its native *seco*-cryptophycin substrates. Two different tags are used to mimic the T domain to which the PK/NRP intermediate is tethered *in vivo*.

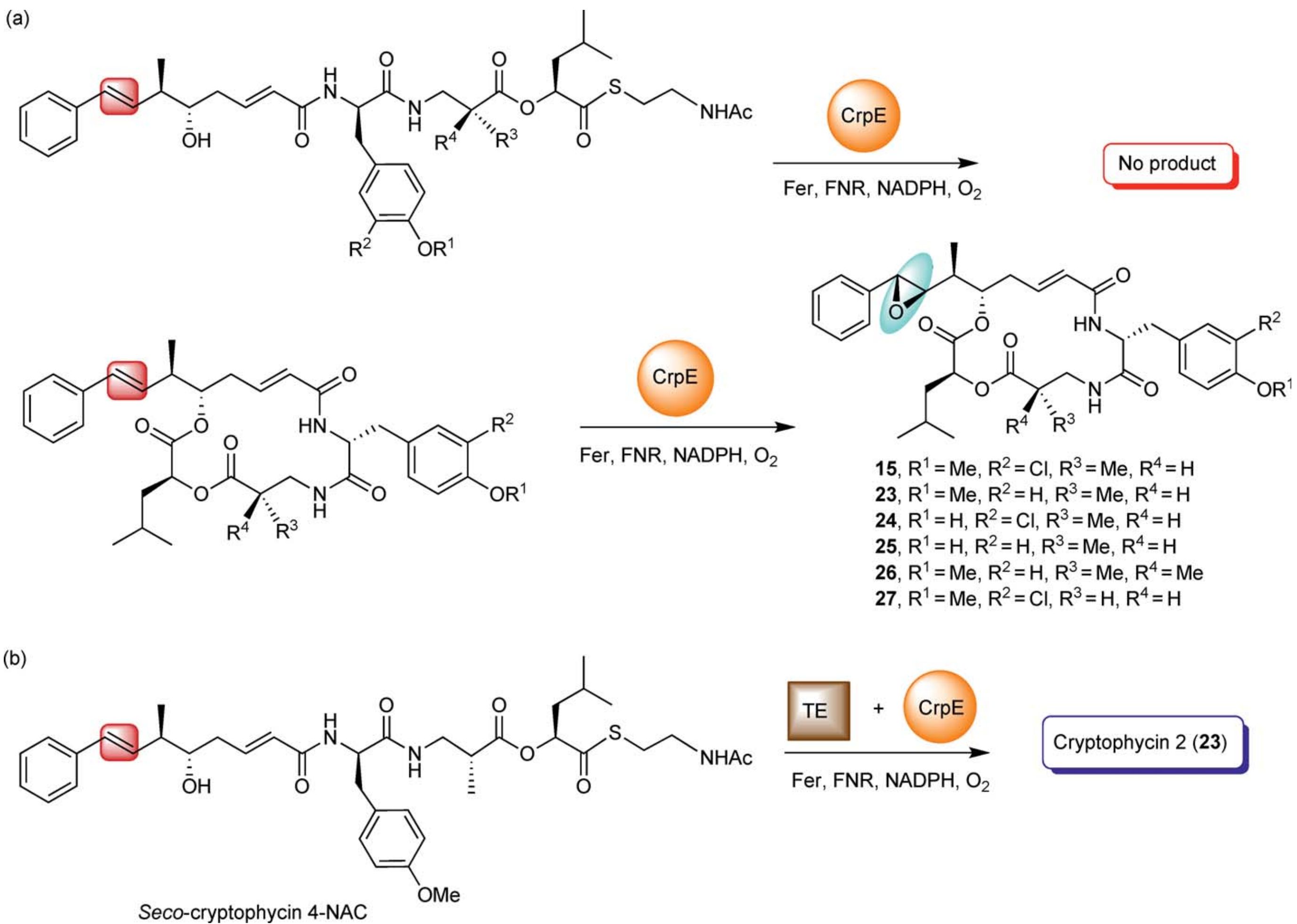

Figure 10 (a) Epoxidation reactions on des-epoxy cryptophycin analogues catalyzed by CrpE. Recombinant CrpE catalyzes regio- and stereospecific installation of the β-epoxide toward cyclic substrates to generate both natural and unnatural cryptophycin analogues. No *seco*-substrate is utilized by CrpE. (b) *In vitro* tandem reactions toward *seco*-cryptophycin 4 catalyzed by enzymes TE and CrpE to yield cryptophycin 2 (**23**).

Figure 11 Chemoenzymatic synthesis of epothilone C with pathway-specific TE domain. This TE was excised from the last PKS module involved in epothilone biosynthesis. Its function in production of epothilone C (**28**) further suggests the versatility of TE domains from PKS, NRPS, PKS/NRPS hybrid, and NRPS/PKS hybrid systems.

partners remain unknown, CrpE activity was achieved with spinach Fer and FNR cofactors. Using this reconstituted system, cryptophycin 1 (**15**) was produced from cryptophycin 3 in the CrpE reaction (**Figure 10(a)**). Interestingly, all cyclic des-epoxy substrates were converted into both natural and unnatural cryptophycin analogues while none of the *seco*-crytophycin substrates were utilized, suggesting that macrocyclization occurs prior to epoxidation in cryptophycin biosynthesis (**Figure 10(a)**). The natural epoxy cryptophycin analogues produced include cryptophycin 1 (**15**), cryptophycin 2 (**23**), and cryptophycin 16 (**24**) while the unnatural ones include cryptophycin 41 (**25**), cryptophycin BE (**26**), and cryptophycin 539 (**27**). The regio- and stereospecificity of CrpE was verified by NMR analysis of cryptophycin 2 (**23**) isolated from a large-scale *in vitro* CrpE reaction.[81] The production of known and novel cryptophycin analogues in CrpE-catalyzed reactions indicates that this P-450 holds substantial promise as a valuable biocatalyst to generate a multitude of novel cryptophycin analogues in the search for improved anticancer drug leads. Furthermore, cryptophycin 2 (**23**), was produced in a single reaction vessel containing both Crp TE and CrpE as biocatalysts and *seco*-cryptophycin 4 NAC thioester as the substrate (**Figure 10(b)**).[81] This novel chemoenzymatic synthesis route effectively bypassed the two most challenging steps in normal chemical synthesis of cryptophycin analogues and holds immediate promise for efficient access to new compounds.

Chemoenzymatic studies of cryptophycins provide representative examples about how to explore biosynthetic tools in biosynthesis of PK/NRP hybrid to organic synthesis. Similarly, enzymes in NRP/PK hybrid biosynthesis are also capable of being applied to the discovery of natural products with increased bioactivity. For example, epothilones are potent anticancer agents that are generated through the action of one NRPS and five PKSs.[91] The excised epothilone TE domain from the EpoF multifunctional protein was heterologously overexpressed and the purified enzyme effectively converted *seco*-epothilone C NAC thioester into epothilone C (**28**), further demonstrating the effectiveness and versatility of TE domains of NRPSs and PKSs in chemoenzymatic synthesis (**Figure 11**).[92] Moreover, several unnatural epothilone biosynthetic intermediates were produced by purified EpoA, EpoB, and EpoC *in vitro*, establishing the ability to reengineer the epothilone biosynthetic pathway to produce novel analogues and confirming this application of combinational biosynthesis in the search for new anticancer drugs.[93]

2.17.5 Conclusions

Many natural products or their derivatives have been and are being developed into valuable pharmaceuticals. However, new disease targets and rapidly emerging drug resistance support the urgent need to identify and develop novel therapeutics. Although hundreds of new bioactive natural products have been identified from terrestrial and marine organisms every year, isolation from natural sources can result in insufficient amounts for clinical evaluation, making chemical synthesis one if not the only way to further develop them.[1,94] However, most natural products have very complex chemical structures, which inevitably hinder economical large-scale synthetic approaches. Enzymes often have a high level of chemoselectivity, regioselectivity, and stereoselectivity. These features make them attractive biocatalysts in organic synthesis to generate pure and structurally diverse products. However, there are still many challenges related to chemoenzymatic synthesis such as limited use of certain reaction solvents, enzyme stability, and control of side reactions.

The utility of intact PKS, NRPS, excised TE domain, and tailoring enzymes in natural product biosynthesis has great potential to facilitate the preparation of chemical libraries consisting of bioactive analogues. Chemoenzymatic approaches have been demonstrated to be an efficient way to generate various structural scaffolds. However, despite some limitations associated with enzymes in organic synthesis, their flexibility toward various substrates will motivate future research through traditional *in vitro* approaches, as well as through structure-based protein engineering or directed protein evolution.[95] We expect that novel chemoenzymatic routes to important therapeutically active natural product drug leads and diverse analogue libraries will continue to expand in scope and depth.

Abbreviations

A	adenylation
ACP	acyl carrier protein
AT	acyltransferase
C	condensation
CoA	coenzyme A
DH	dehydratase
DMD	dimethyl dioxirane
E	epimerase
ER	enoyl reductase
Fer	ferredoxin
FNR	ferredoxin reductase
KR	ketoreductase
KS	ketosynthase
LM	loading module
MT	methyltransferase
NAC	*N*-acetylcysteamine
***N*-MT**	*N*-methyl transferase
NRP	nonribosomal peptide
NRPS	nonribosomal peptide synthase
PCP	peptidyl carrier protein
PK	polyketide
PKS	polyketide synthase
T	thiolation
TDP-desosamine	thymidine diphosphate D-desosamine
TE	thioesterase

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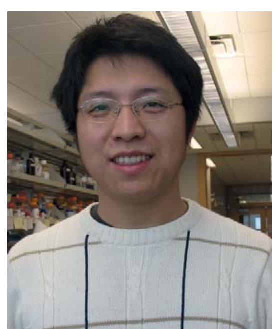
Biographical Sketches



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2.18 Missasigned Structures: Case Examples from the Past Decade

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2.18.1 Introduction

Total structure elucidation of complex natural products has become more straightforward over the past several decades. Contributing factors for unambiguous structure elucidation stem from further advances in the newly developed NMR probes, novel MS technology including ultra-high-resolution MS (HRMS) and tandem MS (MS/MS), and the application of synchrotrons for X-ray structure analysis. Classical structure

Morphine ($C_{17}H_{19}NO_3$)
Isolation: 1806
2D Structure: 1925
3D Structure: 1955

Tetrodotoxin ($C_{11}H_{17}N_3O_8$)
Isolation: 1907
3D Structure: 1964

Penicillin G ($C_{16}H_{18}N_2O_4S$)
Discovery: 1928
Isolation: 1941
Structure elucidation: 1945

Figure 1 Selected examples of challenging structure elucidations on classical natural products from the last century.

elucidation prior to the development of these techniques (around 1960) was quite challenging, and required tremendous effort and time even for small organic molecules (MW < 500). The structures shown in **Figure 1** are classical natural products which illustrate the difficulty of structure elucidation without the techniques mentioned above. Structure elucidation of morphine ($C_{17}H_{19}NO_3$), which is perhaps the first secondary metabolite isolated in pure form, took almost 150 years (isolation: 1806, two-dimensional (2D) structure: 1923, three-dimensional (3D) structure: 1952).[1] The puffer fish toxin, tetrodotoxin ($C_{11}H_{17}N_3O_8$), required more than 50 years for its structure to be fully elucidated (isolation: 1907, 3D structure: 1964).[2] Compared to these two examples, the structure elucidation of the antibiotic penicillin G ($C_{16}H_{18}N_2O_4S$) was relatively fast, but still needed more than 15 years (discovery: 1929, isolation: 1941, 3D structure: 1945).[3] Although it had been suggested that penicillin possessed a β-lactam core, the total structure of penicillin G was directly determined by the application of X-ray structural analysis.[4] The structural motifs of these molecules continue to intrigue chemists, notably the fascinating ring systems.

Modern structure elucidation using one of, or a combination of, the abovementioned analytical techniques has enabled chemists to investigate large and complex biomolecules (MW > 1000). Some outstanding total structure analysis utilizing limited samples in the past 15 years for marine-derived macromolecules are shown in **Figure 2**: (1) polyether toxin, maitotoxin ($C_{164}H_{256}O_{68}S_2Na_2$), (discovery: 1979,[5] 2D structure: 1993,[6] 3D structure: 1996[7,8]), (2) polyketide, amphidinol 3 ($C_{70}H_{118}O_{23}$), (3D structure: 1999,[9] structure revision: 2008[10]), lipopolysaccharide, axinelloside A ($C_{137}H_{219}O_{117}S_{19}Na_{19}$), (3D structure: 2005[11]) and (3) polypeptide, polytheonamide A ($C_{219}H_{376}N_{60}O_{72}S$), (2D structure: 1994,[12] 3D structure plus structure revision[13]). However, it is also true that incorrectly assigned structures for small biomolecules continue to appear in the literature. This issue was emphasized in a recent review, which suggested that more than 300 errors in proposed structures were disclosed from 1990 to early 2004.[14] It is important to learn and analyze the outstanding structure elucidation examples mentioned above. However, considering the number of recently revised structures and stereochemical reassignments, it is more practical and significant to study why structure elucidation goes wrong. This chapter discusses a subset of misassigned molecules derived from marine sources and is composed of incorrect 2D and 3D structures. It should be mentioned that the object of this chapter is not to criticize the structure misassignments based on the original works, but to provide clues to avoid the pitfalls for future structure elucidation works.

Maitotoxin ($C_{164}H_{256}O_{68}S_2Na_2$)
Discovery: 1979
2D Structure: 1993
3D Structure: 1996

Amphidinol 3 ($C_{70}H_{118}O_{23}$)
3D Structure: 1993
Stereochemical reassignment: 2008

Figure 2 (Continued)

Axinelloside A: R = SO_3Na ($C_{137}H_{219}O_{117}S_{19}Na_{19}$)
3D Structure: 2005

Polytheonamide A ($C_{219}H_{376}N_{60}O_{72}S$)
2D Structure: 1994
Structure revision and 3D structure: 2005

Figure 2 Selected marine natural products illustrating the triumph of modern structure determination.

2.18.2 Structure Diversity of Marine Natural Products

Prior to the discussion of the misassigned marine-derived molecules, it is instructive to consider the diversity of marine natural products. The structures collected in **Figure 3** are several marine natural products either revised or possessing incomplete stereochemistry and represent each biosynthetic class. This short list of compounds demonstrates that marine-derived secondary metabolites have characteristic structural diversity when compared with terrestrial-derived molecules. Unique carbon skeletons and functional groups are present in the structures of suvanine,[15,16] ecteinascidine 759B,[17,18] spirastrellolide A,[19–21] and yendolipin[22,23] with a dimethyl guanidinium cation, sulfoxide, bis-spiroketal, and quaternary amine cation, respectively. The characteristic polyketide/peptide side chain of iejimalide A[24,25] is often observed in secondary metabolites found in marine invertebrate-derived metabolites. The structure of haplosamate A[26,27] with a molecular formula (MF) $C_{29}H_{49}Na_2O_{12}PS$ is unique in terms of atom diversity. In other words, these structural moieties contained within marine natural products are often the cause of structure misassignments. In addition, since many marine natural products are isolated in minute amounts, and possess complex and/or remote chiral centers within the molecules, the completion or process of determining correct stereochemical assignments is often very challenging. Some recently reported compounds such as usneoidone[28,29] and batzellaside A[30] have yet to be fully characterized due to the challenges of chiral centers on the aliphatic chains. The correct assignment of natural products is critical to many researchers since potent bioactive marine natural products such as spirastrellolide A,[21] kahalalide F,[31–34] and pericosine A[35,36] often become not only synthetic targets but also pharmaceutical leads and molecular probes to characterize biological functions of proteins and enzymes.

2.18.3 Misassigned Marine Natural Products

By the end of 2008, more than 200 structure revisions for marine natural products had been reported. In the 1970s and early 1980s, the structure revisions were essentially made based on NMR analysis. This was mostly due to the improvement of NMR sensitivity via more powerful magnetic fields and the development of new pulse sequences. Many structure revisions have also arisen from total syntheses since the middle of the 1980s when marine natural products with potent bioactivity and complex carbon skeleton stimulated the synthetic community. In the past decade, GIAO (gauge-independent atomic orbital)[37] methods such as density functional theory (DFT) calculations to estimate quantum mechanical-derived NMR chemical shifts have been conspicuously applied to structure elucidation. This calculation method has proven to be a very useful structure elucidation tool especially to confirm structures with low hydrogen-to-carbon (H/C) ratio cores that make other NMR methods nearly useless. Recently, some structure revisions including TAEMC161[38] (identical to viridiol)[39] and the *Brosimum* allene[40] (identical to mururin C[41]) have resulted from DFT calculations. Described below are case examples of misassigned marine natural products categorized by the types of errors as well as tables that will be beneficial for researchers who encounter the same difficulties of structure elucidation.

2.18.4 Difficulties of Molecular Formula Assignments

Secure structure elucidation begins with obtaining the correct MF. This step is the most important part in structure elucidation since an incorrect MF guides structure elucidation down the wrong path, resulting in misassignment. However, it is sometimes very difficult to finalize the MF based on MS results. All the examples collected in the following section were given an incorrect MF in the original structure. Analysis of the examples categorized the pitfalls into four groups: (1) functional groups, (2) monomeric or dimeric forms, (3) hydroxy or hydroperoxide groups, and (4) diols versus ethers. Each subsection discusses these groups individually using several examples.

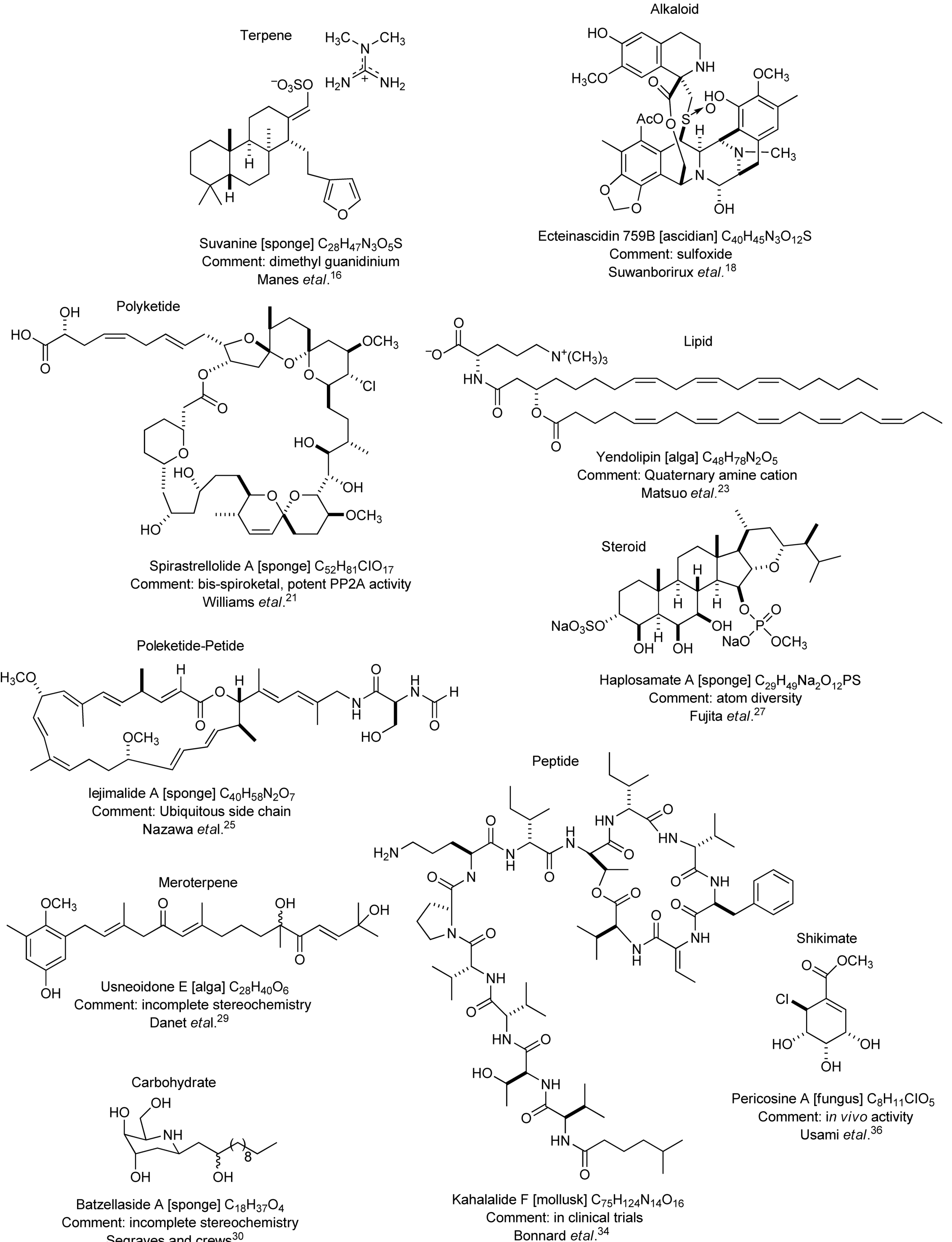


Figure 3 Selected examples, by biosynthetic type, of marine natural products illustrating a range of past and current assignment difficulties.

2.18.4.1 Incorrect Functional Groups Derived from Misassigned Molecular Formula

Each example collected here possesses a different factor that can lead to MF misassignment (**Table 1**). One of the best ways for establishing an accurate MF is to confirm its isotope pattern by simulation. It is also important to reconsider the working MF when it possesses more than 5 milli mass unit (mmu) error from the exact mass. Furthermore, if ^{1}H and ≠ or ^{13}C NMR signals are overlapped, due prudence requires establishing a correct MF. It should also be noted that in some cases, such as diazonamides, X-ray structure analysis does not always provide a secure 3D structure.

2.18.4.1.1 Spirastrellolide A

Spirastrellolide A, isolated from the Dominican sponge *Spirastrella coccinea*, was reported as an antimitotic macrolide in 2003.[19] Structure elucidation of spirastrellolide A was performed using its side chain methyl ester. The MF of the methyl ester was originally assigned to be $C_{53}H_{86}O_{19}$ based on an *m/z* 1027.580 5 as $[M+H]^+$ by high resolution chemical ionization mass spectrometry (HRCIMS), which had an error of −3.8 mmu from the exact MS. The original structure consisting of an unprecedented polyketide macrolide with a 47-carbon skeleton, two spiroketals, and eight hydroxy groups was determined based on one-dimensional (1D) and 2D NMR techniques. Although this structure possesses 21 stereocenters, only seven stereogenic centers on the spiroketal rings were proposed due to the limited amount of the sample (6.2 mg as methyl ester from 2.6 kg sponge). A year later, this proposed structure was revised by the same research group using a new sample (46.1 mg as methyl ester from 19 kg sponge).[20] First, the structure revision began with the number of hydroxy groups. Acetylation of the methyl ester gave not only an octaacetate, but also a pentacetate, which was confirmed based on the observation of a 5 amu increase when the low resolution electrospray ionization mass spectrometry (LRESIMS) for the methyl ester was measured in mono-deuteromethanol (MeOD). Second, the actual MF was determined to be $C_{53}H_{83}ClO_{17}$ based on the $[M+Na]^+$ ion observed at *m/z* 1049.520 26 (Δ −0.84 mmu) obtained by ultra-high-resolution fourier transform mass spectrometry (FTMS), which was confirmed by the agreement of the isotope pattern between experimental and simulated values. This new MF required the subtraction of H_3O and addition of one chlorine atom and one unsaturation degree. These differences accounted for the ether linkage between C-35 and C-38 and the addition of chlorine on C-28 in the revised structure. Although this relatively large quantity of methyl ester enabled this group to determine the relative stereostructure of the macrocyclic ring by comprehensive rotating-frame overhauser effect spectroscopy (ROESY) analysis, the remote stereochemistry at C-46 remained unsolved. The absolute stereostructure of the macrocyclic core was determined later based on X-ray structure analysis of the *p*-bromobenzoate derivative of spirastrellolide B (= 15,16-dihydro-28-dechloro-spirastrellolide A).[42] In 2007, five more derivatives were reported (spirastrellolides C–G) and the remaining stereocenter on C-46 has been finally determined to be *R* configuration by conversion of the side chain (C-44–C-47) of spirastrellolide D (= 4-chloro-spirastrellolide A) to dimethylmalate.[21] Although many research groups have taken up the challenge to synthesize spirastrellolide A since its initial isolation report in 2003, the first total synthesis was achieved in 2008.[43,59] Spirastrellolide A is expected to be a promising anticancer lead due to its potent selective activity against protein phosphatase 2A ($IC_{50} = 1\ nmol\,l^{-1}$).[20]

2.18.4.1.2 Plakevulin A

Plakevulin A was reported in 2003 as a new oxylipin DNA polymerase inhibitor from the sponge *Plakortis* sp. collected in Okinawa.[44] The MF of plakevulin A was proposed to be $C_{28}H_{48}O_6$ based on both the low-resolution and the high-resolution MS data: *m/z* 480 $[M]^+$ (field desorption mass spectrometry (FDMS)), *m/z* 503 $[M+Na]^+$ (fast atom bombardment mass spectrometry (FABMS)), and *m/z* 480.342 7 $[M]^+$ (Δ −2.4 mmu). The presence of the two structural cores, a levulinyl group and oxylipin was confirmed based on both 2D NMR data and electron impact mass spectrometry (EIMS) fragment peaks (*m/z* 381 $[M–CH_3CO(CH_2)_2CO]^+$, *m/z* 157 $[M–CH_3CO(CH_2)_2CO–C_{16}H_{33})$. The planar structure of plakevulin was confirmed by an hetero-nuclear multiplebond correlation (HMBC) correlation (H-1/C-1′). The stereochemistry of the cyclopentene ring was determined by detailed analysis of the NMR data of three synthetic derivatives from plakevulin A (**Figure 4**). Finally, the absolute stereostructure was determined by the application of a modified Mosher's method[60] for the synthetic alcohol derivative (**Figure 4**). In 2004, however, this structure was revised based on the total synthesis and repurification of the natural product.[45] The spectral data of the synthetic plakevulin A were not identical to those of the natural product. Differences were observed in the carbon signals of C-1, C-2, C-3, C-5, and C-1′. The

Table 1 Problematic assignments of functional groups

Original structure	*Revised structure*	*Features*
Spirastrellolide A (sponge) MF: $C_{52}H_{84}O_{19}$ Williams *et al*.[19]	MF: $C_{52}H_{81}ClO_{17}$ Williams *et al*.[20]	Revision rationale: (a) NMR analysis of the pentacetate methyl ester, (b) absolute structure of the macrocyclic ring by X-ray structure analysis.[42] Remaining undefined: none. Critical data: (a) ultra-HRMS provided correct MF (*m/z* 1049.520 26 $[M+Na]^+$ (Δ −0.84 mmu, $C_{54}H_{83}O_{17}ClNa$ as methyl ester); original MF: HRCIMS, *m/z* 1027.580 5 $[M+H]^+$ (Δ −3.8 mmu, $C_{53}H_{86}O_{19}$ as methyl ester), (b) five exchange protons: *m/z* 1049 $[M+Na]^+$ in $MeOH/CH_2Cl_2$, *m/z* 1054 $[M+Na]^+$ in $MeOD/CH_2Cl_2$. Other issues: stereochemistry for OH on side chain was determined as *R* by conversion of the side chain to dimethyl malate.[21] Total synthesis of spirastrellolide A methyl ester has been achieved.[43]
Plakevulin A (sponge) MF: $C_{28}H_{48}O_6$ Tsuda *et al*.[44]	MF: $C_{23}H_{42}O_4$ + $C_5H_8O_3$ Saito *et al*.[45]	Revision rationale: total synthesis. Remaining undefined: absolute structure. Critical data: (a) the synthetic product ≠ the natural product, (b) H1 = 6.04 ppm (synthetic product), 5.34 ppm (natural product), (c) NMR data: the oxylipin alcohol moiety of the synthetic product = the natural product except levulinic acid, and (d) the oxylipin alcohol was obtained from the natural sample by repurification. Other issues: the natural product is a 1:1 mixture of the oxylipin alcohol and levulinic acid and small amount of the ester with the original structure but it is an artifact.

Siphonodictyal C (sponge)

MF: $C_{22}H_{30}O_4$

Sullivan *et al*.[46]

MF: $C_{22}H_{29}NaO_7S$

Mukku *et al*.[47]

Revision rationale: reassessment of NMR.
Remaining undefined: absolute structure.
Critical data: (a) (NMR) the revised was nearly identical to those of the proposed, (b) the loss of $-SO_3Na$ was confirmed by EI and FABMS, Revised MW; HRESIMS *m/z* 437.162 9 [M–Na]$^-$ (calcd for $C_{22}H_{29}O_7S$), (c) location of the sulfate group was determined by the calculated shift.
Other issues: Original MS came from HREIMS, *m/z* 358.212 2 (calcd for 358.214 4, $C_{22}H_{30}O_4$).

Halipeptin A (sponge)

MF: $C_{31}H_{54}N_4O_9$

González *et al*.[50]

MF: $C_{31}H_{54}N_4O_7S$

Kiyota *et al*.[51]

Revision rationale: reassessment of NMR.
Remaining undefined: none.
Critical data: (a) revised MF ($C_{31}H_{54}N_4O_6SNa$) = HRESIMS, *m/z* 649.361 1 [M+Na]$^+$ (Δ +2.3 mmu), (b) the methyl thiazoline unit = synthesis of model compounds and GIAO calculated chemical shifts.
Other issues: (a) original MF possessed large error (Δ +10.4 mmu), (b) two synthetic groups have achieved the total synthesis of halipeptin A in 2005.[49,52]

Barettin (sponge)

MF: $C_{16}H_{14}BrN_3O_2$

Lidgren *et al*.[53]

MF: $C_{17}H_{19}BrN_6O_2$

Solter *et al*.[54]

Revision rationale: reassessment of NMR.
Remaining undefined: none.
Critical data: (a) the synthetic product ≠ the natural product, (b) the spectral data of the revised structure = the original structure.
Other issues: total synthesis of barettin has been achieved, and the stereochemistry has been determined to be 12*S*.[55]

(*Continued*)

Table 1 (Continued)

Original structure	*Revised structure*	*Features*
Haplosamates A and B (sponge) MF: $C_{29}H_{50}NNa_{11}S_2$ (hap A: R = H) MF: $C_{29}H_{49}NNa_2O_{14}S_3$ (hap B: R = SO_3Na) Qureshi and Faulkner[26]	MF: $C_{29}H_{49}Na_2O_{12}PS$ (hap A: R = H) MF: $C_{29}H_{48}Na_4O_{15}P_2S$ (hap B: R = PO_3Na_2) Fujita *et al*.[27]	Revision rationale: reassessment of HRMS and NMR. Remaining undefined: none. Critical data: (a) δ_H 3.61 (3H, d, $^3J_{H,P} = 10.4$ Hz), δ_C 53.2 ($^2J_{C,P} = 12.9$ Hz), (b) HRFABMS (revised) *m/z* 653.276 6 (calcd for 653.276 0, $C_{29}H_{50}O_{12}PS$), HRFABMS *m/z* 653.289 8 (calcd for 653.290 4, $C_{29}H_{51}NO_{11}S_2$). Other issues: none.
Diazonamides A and B (ascidian) MF: $C_{40}H_{36}Cl_2N_6O_7$ (diazonamide A) MF: $C_{35}H_{25}BrCl_2N_5O_6$ (diazonamide B) Lindquist *et al*.[56]	MF: $C_{40}H_{34}Cl_2N_6O_6$ (diazonamide A) MF: $C_{35}H_{25}BrCl_2N_6O_4$ (diazonamide B) Li *et al*.[57]	Revision rationale: total synthesis. Remaining undefined: none. Critical data: (a) the synthetic diazonamide A ≠ the natural product, (b) amine proton in valine residue appeared one-proton sharp doublet, (c) NMR data at C-37 in diazonamide A (δ_H 3.75, δ_C 76.9), (d) downfield shift of H-37 by acetylation to δ_H 5.11, (e) the original MF of diazonamide B (*m/z* 743.059 0 $[M+H-H_2O]^+$, calcd for $C_{35}H_{24}N_5O_5Cl_2Br$) appeared to be miscalculated by 1 amu (the exact MS = 744.041 6), (f) the synthetic diazonamide A did not lose hemiacetal OH by MS experiments. Other issues: the revised structure of diazonamide A has been synthesized.[58]

HREIMS, high resolution electron impact mass spectrometry; HRFABMS, high resolution fast atom bombardment mass spectrometry.

Plakevulin A

H_2/Rd-Al_2O_3 | mCPBA oxidation | LAH

NOESY analysis modified Mosher's method | NOESY analysis unsuccessful | Formation of acetonide unsuccessful

Figure 4 Synthetic derivatives from plakevulin.

proton signal of the synthetic product of C-1 appeared at δ 6.04 whereas the corresponding signal of the natural product was observed at δ 5.34. These observations suggested that plakevulin A possessed a free hydroxy group on C-1. The NMR data of the alcohol obtained by hydrolysis from the synthetic plakevulin A were identical to those of the natural product except the signals related to levulinic acid. In fact, the oxylipin alcohol was obtained as a pure white solid from the natural product by a column chromatography. Thus, plakevulin A was hypothesized to be a nearly 1:1 mixture of the oxylipin alcohol and levulinic acid or an artifact of these compounds.

2.18.4.1.3 Siphonodictyal C

Siphonodictyal C was initially reported in 1986 as an antibacterial sesquiterpene quinol from the sponge *Siphonodictyon coralliphagum* collected from Ponape and Kwajalein in the Pacific Ocean.[46] The original MF assignment of $C_{22}H_{30}O_4$ came from HREIMS data (*m/z* 358.2122, $[M]^+$, calcd for $C_{22}H_{30}O_4$, 358.2144). The relative structure of siphonodictyal C was determined based on the NMR data comparison to the same class of known compounds. In 2003, the structure of siphonodictyal C was revised by another group, which placed a sulfate group on C-17, since both the structures had nearly identical NMR data.[47] The new MF was established as $C_{22}H_{29}NaO_7S$ by HRESIMS data (*m/z* 437.1629 $[M–Na]^-$, calcd for $C_{22}H_{29}O_7S$). The presence of the sulfate group was proposed by the MF and the 46 amu difference between positive and negative electrospray ionization mass spectrometry (ESIMS). The location of the sulfate group was proposed by the calculation of the carbon chemical shift. It is very interesting in regard to the original report that siphonodictyal D, an isomer of the revised siphonodictyal C with a sulfate group on the aromatic ring, had already been reported. The MF of siphonodictyal C was initially determined by HREIMS as mentioned above, whereas HRFABMS was applied to establish the MF of siphonodictyal D. In fact, the report proposing the revised structure proved that only FABMS and ESIMS detected the sulfate group in siphonodictyal C but EIMS did not.

2.18.4.1.4 Halipeptin A

Halipeptin A was originally reported in 2001 as an anti-inflammatory cyclic depsipeptide with an extremely rare amino acid residue from the sponge *Haliclona* sp. collected in Vanuatu together with its desmethyl analogue, halipeptin B.[48] The MF of halipeptin A was proposed to be $C_{31}H_{54}N_4O_9$ based on the HRFABMS peak at *m/z* 627.4073 $[M+H]^+$ with an error of +10.4 mmu from the exact mass (calcd for $C_{31}H_{55}N_4O_9$, 627.3969). The original structure was assembled based on comprehensive 2D NMR techniques including double-quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), heteronuclear multiple quantum coherence (HMQC), and HMBC. Importantly, ^1H-^{15}N HMBC played a pivotal role in establishing the 1,2-oxazetine-4-methyl-4-carboxylic acid (OMCA) residue. Although the authors indicated that ^{15}N chemical shifts for hydroxamic acid derivatives were expected to be in the range of −190 to −170 ppm, the chemical shift observed at −89.3 ppm for the OMCA nitrogen was proposed to be a consequence of ring strain on the oxazetidine ring. The absolute structure of halipeptin A except positions 3 and 4 in 3-hydroxy-2,2,4-trimethyl-7-methoxydecanoic acid (HTMMD) was determined by a combination of modified Mosher's method and Marfey's method.[61] The remaining stereocenters in the HTMMD moiety turned out to be a *threo* relative configuration by

application of Murata's method.[9] In 2002, the structures of halipeptins A and B were revised by the same group, together with the new derivative halipeptin C.[62] The new MF for halipeptin A was established as $C_{31}H_{54}N_4O_6S$ based on the HRESIMS ion peak at *m/z* 649.361 1 $[M+Na]^+$ with +2.3 mmu difference from the exact mass (calcd for $C_{31}H_{54}N_4O_6SNa$, 649.378 8). This MF suggested that the OMCA moiety was incorrectly assigned and was replaced by methylthiazoline. The presence of the methylthiazoline unit was confirmed by the NMR chemical shifts of a synthetic thiazoline unit and the comparison of the GIAO calculated chemical shifts for both oxazetidine and thiazoline units to those of the natural products. The stereochemistry of the α-position of the methylthiazoline was proposed to be *R* since the calculation results slightly favored the *R* configuration and all the amino acid residues found in this group of compounds possess L configuration. In 2005, two synthetic groups achieved total synthesis of halipeptin A and confirmed the absolute structure with a 3*S*,4*R*,7*S*-HTMMD moiety.[49,52] The latter group also synthesized halipeptin D and claimed that the potent cytotoxic properties of halipeptin D were not observed in the synthetic material. Thus, the potent cytotoxic properties of halipeptin D were concluded to come from residual impurities.

2.18.4.1.5 Barettin

Barettin, a new indole diketopiperazine derivative with inhibitory activity of electrically induced contractions of an isolated guinea pig ileum, was intially reported in 1986 from a northern Swedish deepwater sponge *Geodia baretti*.[53] The MF of barettin was proposed to be $C_{16}H_{14}BrN_3O_2$ based on the HREIMS peak at *m/z* 359.028 0 $[M]^+$ (calcd for $C_{16}H_{14}BrN_3O_2$, 359.027 0). The planar structure was deduced by comparison of the NMR to a synthetic diketopiperazine (cyclo-L-propyl-L-trptophy). At that point, the geometry of the olefin at C-8 and C-9 was unclear. A year later, the original structure of barettin with *S* configuration on the proline residue was synthesized by another group.[63] However, the spectral data of the synthetic compound were completely different from those of the natural compound. This synthetic group suggested that the structure of barettin could be the cyclic dimer. In 2002, another natural product group found a new diketopiperazine (cyclo[6-bromo-8*Z*-entryptophan]arginine) from the Norwegian deepwater (−300 m) sponge, *G. baretti*, in which the proline residue in barettin was replaced by arginine.[54] The MF of the new diketopiperazine was established to be $C_{17}H_{19}BrN_6O_2$ from the HRESIMS ion peak at *m/z* 419.083 3 $[M+H]^+$ (calcd for $C_{17}H_{20}BrN_6O_2$, 419.083 1). Interestingly, all the spectral data were completely identical with those of barretin. Thus, the structure of barettin was revised to be that of the new diketopiperazine with 8*Z* configuration. In the revised structure, the carbon chemical shifts at δ 127.45 and 127.55 recorded at 101 MHz were assigned as C-2 and C-3a, respectively, whereas these carbons showed up as an overlap signal at δ 127.4 recorded at 90 MHz in the original report. Further, the carbon signal of the guanidine sp^2 quaternary carbon at δ 158.61 in the revised structure was interpreted to be C-9 in the original structure. Although the absolute stereochemistry of the revised structure remained unsolved, the total synthesis of barettin was achieved in 2004, with the data from the synthetic product indicating an *S* configuration for the arginine residue.[55]

2.18.4.1.6 Haplosamate A

Haplosamate A was originally reported as a new steroidal sulfate ester isolated from the Philippines sponges *Xestospongia* sp. and an unidentified haplosclerid sponge in 1999, together with one more derivative haplosamate B with an additional sulfate group on C-7.[26] These new steroids were reported as the first example of marine sterols with sulfamate functional group and tetrahydropyran E ring. In the original report, the MF of haplosamate A was established as $C_{29}H_{51}NaNO_{11}S_2$ based on the HRFABMS and NMR data. The HRFABMS data observed at *m/z* 653.289 8 $[M-Na]^-$ showed an excellent match for the MF (calcd for $C_{29}H_{51}NaNO_{11}S_2$, 653.290 4). The presence of the sulfamate group was deduced from the ESI–MS/MS results and ^1H NMR data. The MS/MS result for *m/z* 675 provided several key fragment signals including *m/z* 573 $[M-Na-SO_3]^-$, *m/z* 559 $[M-Na-SO_2-NHCH_3]^-$, and *m/z* 543 $[M-Na-SO_3-NHCH_3]^-$, which supported the presence of the sulfamate group. On the other hand, two proton signals at 3.59 and 3.63 ppm with an integration of 1.5 protons on each signal (recorded in CD_3OD at 300 MHz) were initially accounted to be a *N*-methyl doublet (J = 10 Hz). However, since these signals correlated to two different carbon signals (δ_C 53.2 and 53.3) in the HMQC experiment and *N*-methyl sulfamates normally appear as methyl singlets, these signals were concluded to be two isomers based on the orientation of *N*-methyl group, which required the hydrogen bond to form a stable ring in the molecule (**Figure 5**). In 2001, two new phosphorylated sterol sulfates isolated as membrane-type matrix metalloproteinase

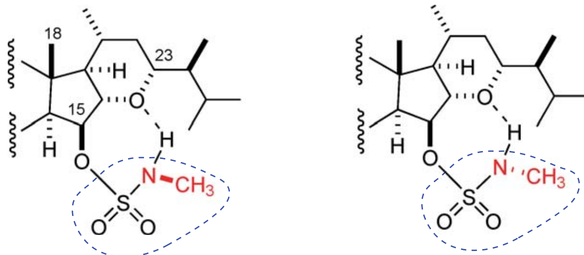


Figure 5 Two stereoisomers of the sulfamate group.

(MT-MMP) inhibitors from a Japanese marine sponge *Cribrochalina* sp. turned out to be identical with haplosamates A and B due to the same ^{1}H and ^{13}C NMR spectra as those previously reported.[27] The MF was assigned to be $C_{29}H_{49}Na_2O_{12}PS$ based on the HRFABMS (m/z 653.276 6 [M+H–Na$_2$]$^-$, Δ +0.6 mmu). The methylphosphate functionality in the revised structure was explained by the NMR data. The characteristic *O*-methylphosphate NMR signals were observed at δ_H 3.61 (3H, d, $^3\mathcal{J}_{H,P}=10.4$ Hz) and δ_C 53.2 ($^2\mathcal{J}_{C,P}=12.9$ Hz), which was further confirmed by a signal at δ 2.12 in the ^{31}P NMR spectrum. The same group also determined the absolute stereochemistry by the application of a modified Mosher's method.[60]

2.18.4.1.7 Diazonamides A and B

Diazonamides A and B were reported in 1991 as potent cytotoxic metabolites separated from the colonian ascidian *Diazona chinensis* collected in the Philippines.[56] The MFs of diazonamide A ($C_{40}H_{36}Cl_2N_6O_7$) and B ($C_{35}H_{25}BrCl_2N_5O_6$) were proposed based on the HRFABMS data observed at m/z 765.199 8 [M+H–H$_2$O]$^+$ (Δ −0.1 mmu) and m/z 743.059 0 [M+H–H$_2$O]$^+$ (Δ +25.0 mmu), respectively. As suggested from the MFs, the structure elucidation for these new compounds was extremely difficult due to a low H/C ratio core in the molecules. Generally, a structural core with a H/C ratio <1 makes structure elucidation challenging. In this case, the original structures for diazonamides A and B possessed an extremely low H/C core (A–D ring: $C_{14}H_4Cl_2N_3O_2$, H/C = 0.29). In fact, the structure elucidation was performed directly by X-ray structure analysis of the *p*-bromobenzoate derivative of diazonamide B (**Figure 6**). The X-ray structure, however, had a problem on the furofuran moiety since diazonamide B had a hemiacetal group on C-11 (δ_H 6.46, δ_C 106.6) to which the proton signal coupled with an exchangeable proton at δ_H 7.36. The original structure of diazonamide B was proposed with a hemiacetal group on the F ring due to the observation of a HMBC correlation between H-11 and C-17 on the E ring. The structure of diazonamide A was straightforwardly assigned based on the architecture of diazonamide B, possessing a terminal valine residue on the C-2 amine and hydrogen on C-6 instead of bromine. Diazonamide A showed potent cytotoxicity *in vitro* against both human (HCT-116) and murine (B-16) cancer cell lines with IC_{50} <15 nmol l^{-1}.

Diazonamides have evoked much interest in the synthetic community due to both their interesting architecture and potent cytotoxic properties. It took a decade to achieve the total synthesis of diazonamide A and the *p*-bromobenzoate derivative of diazonamide B.[64] However, the synthetic materials turned out to be not identical to the natural products. This synthetic group then elicited the revised structures of diazonamides A and B based on verification of the spectral data.[57] The major spectral data differences between the synthetic and

Figure 6 X-ray structure of diazonamide B *p*-bromobenzoate.

natural products in diazonamide A were observed in the terminal valine residue. The amine in the valine appeared as a sharp one-proton doublet at δ 5.46 coupled with the H-37 methine at δ 3.75 ppm. In the synthetic compound, this methine proton was observed at δ 3.16 as a broad singlet. The H-37 methine proton of the triacetate of natural diazonamide A shifted downfield to δ 5.11. Further, the carbon chemical shift of C-37 in the natural compound was observed at δ 76.9. As a result of these observations, the amine group in the valine residue was replaced by a hydroxy group, which was supported by the fact that acid hydrolysis of diazonamide A did not provide valine. However, this change required replacing an OH to NH_2 in the macrocyclic core established by X-ray analysis with a 1 amu increase. On the other hand, the MF of diazonamide B $C_{35}H_{24}N_5O_5Cl_2Br$ as $[M+H–H_2O]^+$ established from the HRMS ion peak at *m/z* 743.059 0 appeared to be miscalculated by 1 amu and the exact MS of the MF required was 744.041 6 amu. The best MF derived from incorporating an additional nitrogen atom for diazonamide B was $C_{35}H_{25}BrCl_2N_6O_4$ with the exact mass 743.057 6 as $[M+H]^+$. This MF has one less hydrogen than the original MF and is more reasonable with the observed HRESIMS data (*m/z* 743.059 0). Thus, the structure revision was considered on the hemiacetal moiety since the synthetic diazonamide A with a hemiacetal group ionized well to provide the molecular ion peak $[M+H]^+$. Finally, the revised structures for diazonamides A and B with a furopyrrole moiety were supported by the analysis of the bond length of the crystal structures, which were also confirmed by an ^{15}N-^{1}H heteronuclear single quantum coherence (HSQC) experiment for natural diazonamide A. In 2002, the revised structures were confirmed by the total synthesis of diazonamide A.[58] Although X-ray structure analysis has been recognized as the most secure structure elucidation method, it should be emphasized that X-ray structure analysis does not always provide the correct structure. An additional example of X-ray structure not corresponding to the true structure occurred in the structure elucidation of aminosamides A and B isolated from a marine-derived actinomycete.[65] It is interesting to note that a new diazonamide congener diazonamide C reported in 2008 possesses an amine group instead of a hydroxy group on C-37 like the original reported structure of diazonamide A.[66] However, this minor structure change makes the cytotoxicity (GI_{50}) considerably weaker from nanomolar to micromolar level. The cytotoxic effect of diazonamide A turned out to be the disruption of mitosis in the cell cycle.[67] Its mode of action has recently been suggested to be due to inhibition of the mitochondrial enzyme ornithine δ-amino transferase (OAT).[68,69] This enzyme has been suggested not to be essential in cell division for normal cells but to be critical for cancer cells. Moreover, the synthetic analogue (di-dechlorodiazonamide A) showed significant *in vivo* activity against xenograft mice with the same effectiveness as paclitaxel and vinblastine without side effects. Consequently, diazonamide A and its analogues are expected to be a new anticancer drug with significant cancer cell selectivity.

2.18.4.2 Challenge in Distinguishing Dimers from Monomers

The structures discussed in this section demonstrate the difficulty of distinguishing between dimeric and monomeric forms (**Table 2**). Only a few such examples have been reported among misassigned marine natural products. Caution is required when soft ionization methods such as ESIMS are used to measure molecular weight since these MS techniques frequently generate double charge ion peaks and/or dimeric ion peaks. Furthermore, selection of ionization method is also important since some of the ionization methods including EI (electron impact), CI (chemical ionization), and APCI (atmospheric pressure chemical ionization) are not appropriate to measure large molecule over 1000 Da.

2.18.4.2.1 Zamamistatin (aeroplysinin-1)

Zamamistatin was originally reported in 2001 as an antibacterial bromotyrosine dimer composed of a unique azaoxa-spiro[6.5] ring connected with an exo-double bond from the Okinawan sponge *Pseudoceratina purpurea*.[69] The MF of zamamistatin was established as $C_{18}H_{18}Br_4N_2O_6$ by HRESIMS (*m/z* 696.776 6 $[M+Na]^+$, Δ -2.9 mmu). The symmetrical form of zamamistatin was indicated based on nine carbon signals in the ^{13}C NMR (carbon NMR) spectrum. The carbon skeleton was straightforwardly established based on the NMR data. The isoxazolidine moiety was suggested from the observed chemical shift data and similarities with those of the aerothionin derivatives containing isoxazoline that are often seen in marine natural products. The geometry of

Table 2 Distinguishing monomeric forms from dimeric structures

Original structure	*Revised structure*	*Features*
Zamamistatin (sponge) MF: $C_{18}H_{18}Br_4N_2O_6$	Aeroplysinin 1 (sponge) MF: $C_9H_9Br_2NO_3$	Revision rationale: MS analysis, IR data, and NMR reassessment. Remaining undefined: none. Critical data: (a) the quintet peak centered at *m/z* 700.8 ($C_{18}H_{18}{}^{79}Br_2{}^{81}Br_2N_2NaO_6$) disappeared when measured with 0.15 $\mu mol\,l^{-1}$ concentration, (b) IR peak at 2262 cm^{-1} indicated nitrile group, (c) NMR data: zamamistatin = aeroplysinin-1. Other issues: (a) the endoform was proposed by analysis of ^{13}C NMR data of the model structures, (b) aeroplysin-1 was earlier reported.[71]
Takada *et al*.[69]	Kita *et al*.[72]	
MF: $C_{18}H_{18}Br_4N_2O_6$		
Hayakawa *et al*.[70]		

(*Continued*)

Table 2 (Continued)

Original structure	*Revised structure*	*Features*
Swinholide A (sponge)		Revision rationale: reassessment of MS and NMR data. Remaining undefined: none. Critical data: (a) FABMS *m/z* 1411 $[M+H]^+$, *m/z* 1388 $[M-H]^-$, (b) swinholide A tri-*p*-bromobenzoate derivative gave the complex ^1H NMR spectrum, (c) the monomeric methyl ester was obtained by treatment with NaOMe. Other issues: the absolute structure has been determined by X-ray structure analysis.[75] Swinholide A has also been isolated from marine cyanobacteria.
MF: $C_{39}H_{66}O_{10}$	MF: $C_{78}H_{132}O_{20}$	
Carmely and Kashman[73]	Kobayashi *et al*.[74]	

the exo-double bond was determined to be *trans* based on the nuclear overhauser effect spectroscopy (NOESY) correlation between H-7′ and NH, which furnished the original structure. In 2006, the second isolation of this compound from the Okinawan sponge *P. purpurea* by another research group led to the structure revision of zamamistatin as an endo-type dimer with an azaoxo-spiro[6.6] ring.[70] The determination of this alternative structure was made based on comparison of the natural product NMR data to those of the synthetic analogues. The carbon chemical shifts of the spiro carbons of the synthetic analogue with a 4,5-dihydroisoxazole appeared at δ_C 92.4 whereas the carbon chemical shifts of the spiro carbon of the synthetic 5,6-dihydro-4*H*-1,2-oxazine was observed at δ_C 74.3. These carbon chemical shifts indicated that the structure of zamamistatin possessed a 5,6-dihydro-2*H*-1,2-oxazine ring instead of isoxazolidine since the spiro carbon of zamamistatin was observed at δ_C 74.3. Despite the detailed discussion of the NMR chemical shifts, the MS data of the second isolation of zamamistatin were not described in the article.[70] Subsequently, the same research group that revised zamamistatin as the endo-type form concluded that zamamistatin was identical to aeroplysinin-1, which was isolated as an antibacterial dibromophenylpyruvic acid derivative from the sponge *Verongia aerophoba* in 1972,[71] based on simple MS experiments.[72] Zamamistatin gave two indicative ESIMS ion peaks: the quintet peak centered at m/z 700.8 ($C_{18}H_{18}^{79}Br_2^{81}Br_2N_2NaO_6$) and the triplet peak centered at m/z 361.9 ($C_9H_9{}^{79}Br^{81}BrNNaO_3$) when measured with a concentration of 15 μmol l^{-1}, which were originally assigned to be the molecular ion peak $[M+Na]^+$ and a doubly charged peak $[(M/2)+Na]^+$, respectively. However, the quintet ion peak (m/z 700.8) disappeared when ESIMS analysis was performed with 0.15 μmol l^{-1} concentration, suggesting that the MF of zamamistatin must be $C_9H_9BrNO_3$ and the quintet peak was due to a dimeric ion peak $[2M+Na]^+$. Furthermore, the IR signal at 2262 cm^{-1} suggested that zamamistatin possesses a nitrile group. This structure revision was confirmed based on the above observations plus the identical spectral data (NMR, IR, and optical rotation) between zamamistatin and aeroplysinin-1.

2.18.4.2.2 Swinholide A

Swinholide A was first reported in 1985 as a polyketide macrolide isolated from the Red Sea sponge *Theonella swinhoei*.[73] The structure elucidation of swinholide A was performed without MS data since this compound did not provide any molecular ion peak in EIMS, chemical ionization mass spectrometry (CIMS), FDMS, secondary ion mass spectrometry (SIMS), and FABMS. The original structure was proposed based on the 1D and 2D NMR experiments of the tetraformate derivative. A year later from this report, the same group reported the complete ^{1}H and ^{13}C NMR assignments for swinholide A.[77] In 1989, another research group reported that swinholide A has a dimeric form based on the MS and NMR analysis for both the natural product newly isolated from the Okinawan *T. swinhoei* and its synthetic derivatives.[74] First, swinholide A showed the molecular ion peak in both positive and negative FABMS at m/z 1411 $[M+Na]^+$ and m/z 1388 $[M–H]^-$, respectively. The molecular formula, $C_{78}H_{132}O_{20}$, obtained from the MS and NMR data was confirmed by combustion analysis. Since the ^{1}H and ^{13}C NMR data of the material turned out to be identical to those of the published values, swinholide A appeared to possess a dimeric form rather than the original structure. To break the symmetrical form, swinholide A was converted into a di-acetonide followed by *p*-bromobenzoylation (**Figure 7**). The reactions gave a di-*p*-bromobenzoate, tri-*p*-bromobenzoate, and tetra-*p*-bromobenzoate derivative with masses at m/z 1858 $[M+Na]^+$, m/z 2041 $[M+Na]^+$, and m/z 2224 $[M+Na]^+$, respectively. The dimeric form was proven by the more complicated ^{1}H NMR spectrum of the tri-*p*-bromobenzoate than those of the symmetric di- and tetra-*p*-bromobenzoates. Further reactions have been performed to elucidate the total structure of swinholide A (**Figure 7**). NMR analysis of the monomeric methyl ester (m/z 727 $[M+H]^+$) obtained by the treatment of NaOMe in MeOH provided the partial structure from C-1 to C-23 and C-27 to C-32. The remaining part (C-24–C-26) and its location in the monomer were confirmed based on the NMR analysis of the 1,2,4-triazepin-3-one converted from the methyl ester, which enabled the assignment of swinholide A as a 44-membered dilactone. In 1990, the same research group also reported the absolute stereostructure of swinholide A by X-ray structure analysis of the di-*p*-bromobenzoate.[75] It is interesting to note that swinholide A has also been isolated from two different marine cyanobacteria, together with its glycosylated derivatives.[76] This evidence strongly suggests that marine cyanobacteria are the true producers of the swinholide class of compounds. It is also noteworthy that swinholide A is a known actin stabilizer, used widely as a molecular probe.[78]

Swinholide A

(1) 2,2- Dimethoxypropane, p-TsOH
(2) *p*-Benzoyl chloride, pyridine

di-*p*-bromobenzoate: $R^{1'} = R^{1'} = p$-BrBz, $R^2 = R^{2'} = H$
tri-*p*-bromobenzoate: $R^1 = R^{1'} = R^2 = p$-BrBz, $R^{2'} = H$
tetra-*p*-bromobenzoate: $R^1 = R^{1'} = R^2 = R^{2'} = p$-BrBz

NaOMe

Monomeric methylester

(1) O_3, $(CH_3)_2S$
(2) Semicarbazide, NaOAc
(3) Ac_2O

1,2,4-Triazepin-3-one derivative

Figure 7 Synthetic strategy to solve the 2D structure of swinholide A.

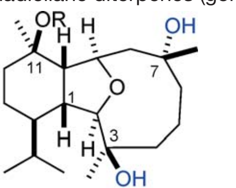
2.18.4.3 Challenges in Distinguishing Diols from Ethers

MS experiments frequently provide ion peaks following the loss of H_2O, especially in EIMS. Misreading these ion peaks as molecular ion peaks $[M]^+$ has the potential to cause structure misassignment when the molecules have more than two hydroxy groups. This section describes three examples with regard to this issue: the first two of which are structure revisions from ethers to diols and the last example is a reassignment from diol to ether (**Table 3**). These examples suggest that EIMS analysis alone may not be suitable for molecules with multiple hydroxy groups.

2.18.4.3.1 Peribysins C and D

Peribysins C and D were initially reported in 2004 as diastereomers that functioned as potent cell adhesion inhibitors from the fungus *Periconia byssoides* separated from the sea hare *Aplysia kurodai*.[79] These compounds

Table 3 Distinguishing diols from ethers

Original structure	*Revised structure*	*Features*
Peribysins C and D (marine-derived fungus) MF: $C_{15}H_{22}O_2$ Yamada *et al*.[79]	MF: $C_{15}H_{24}O_3$ Koshino *et al*.[80]	Revision rationale: (a) CAST/CNMR prediction, (b) new HREIMS data, (c) conformation search by *ab initio* calculations plus NOESY data. Remaining undefined: absolute stereo. Critical data: (a) inconsistent ^{13}C NMR shifts: peribysin C, C6 (δ 69.98), C8 (δ 84.12), peribysin D, C6 (δ 85.95), C8 (δ 63.69), (b) HREIMS *m/z* 252.171 9 $[M]^+$ (calcd for $C_{15}H_{24}O_3$) for perybysin C, (c) relative stereo = *ab initio* calculations plus original NOE data. Other issues: original MS data from HREIMS.
Prevezol B (alga) MF: $C_{20}H_{31}BrO_2$ Mihopoulos *et al*.[81]	MF: $C_{20}H_{33}BrO_3$ Iliopoulou *et al*.[82]	Revision rationale: reassessment of NMR data. Remaining undefined: absolute structure.Critical data: (a) prevezol B = prevezol C (14-epimer) except for C9, 14, and 18, (b) NOEs (H9/H11a, H9/H18, H14/H12b), $^3J_{H14,9} = 11.2$ Hz. Other issues: FABMS = $[M–2H_2O+H]^+$, EIMS = $[M–H_2O+H]^+$, original MS data from HREIMS.
Cladiellane diterpenes (gorgonian)  MF: $C_{22}H_{38}O_5$ (R = Ac) MF: $C_{20}H_{36}O_4$ (R = H) Bowden *et al*.[83]	Polyanthellin A (gorgonian) MF: $C_{22}H_{36}O_4$ (polyanthellin A: R = Ac) MF: $C_{20}H_{34}O_3$ (synthetic derivative: R = H) Ospina *et al*.[84]	Revision rationale: reassessment of NMR data. Remaining undefined: absolute structure. Critical data: (NMR) polyanthellin A, deacetyl-polyanthellin A = cladiellane diterpenes, but opposite optical rotation values. Other issues: total synthesis of (+)-polyanthellin A has been achieved.[85]

NOE, nuclear overhauser effect.

possessed the same MF $C_{15}H_{22}O_2$ from the HREIMS data observed at *m/z* 234.161 9 $[M]^+$ (Δ 0.0 mmu) for peribysin C and *m/z* 234.161 8 $[M+]^+$ (Δ −0.1 mmu) for peribysin D. The planar and relative structures for these two compounds were proposed from the NMR data. The unique 1,3,4,6-tetrahydrofuro[3,4-*c*]furan moiety in peribysins C and D was proposed as a consequence of HMBC correlations (H_2-12/C-7, C-8, C-11, C-13, H_2-13/C-6, C-7, C-11, C-12). These structures were revised in 2006[80] by the carbon chemical shift prediction tool CAST (canonical-representation of stereochemistry)/CNMR.[86,87] This software predicted similar carbon chemical shifts on C-12 and C-13 for both peribysins C and D since these carbons exist in similar environments. However, the reported carbon chemical shifts for peribysins C and D showed approximately 20 ppm difference between C-12 and C-13. Furthermore, similar carbon chemical shifts were expected on C-6–C-8 and C-11–C-13 between these two natural products, but high-field carbon shifts were observed on C-6 (δ 69.98, Δ −15.97 ppm) and C-13 (δ 56.03, Δ −20.33 ppm) on peribysin C, and C-8 (δ 63.69, Δ −20.43 ppm) and C-12 (δ 55.68, Δ −20.69 ppm) on peribysin D. This evidence suggested that peribysins C and D were a 6,13-diol and an 8,12-diol, respectively, which was supported by the weak ion peaks at *m/z* 252 found in the original EIMS data for peribysins C and D. Later the new MF $C_{15}H_{24}O_3$ was confirmed by HREIMS data (*m/z* 252.171 9 $[M]^+$, Δ −0.7 mmu). *Ab initio* calculations using the program Spartan 04 finalized the relative stereostructures for peribysins C and D as 8α,12-epoxy-7(11)-eremophilin-6β,13-diol and 6β,13-epoxy-7(11)-eremophilin-8α,12-diol, respectively.

2.18.4.3.2 Prevezol B

Prevezol B together with prevezol A was reported in 2001 as brominated diterpenes from the red alga *Laurencia obtusa* collected in Greece (**Figure 8**).[81] The MF of prevezol B was established as $C_{20}H_{31}BrO_2$ based on the HREIMS data observed at *m/z* 382.150 7 $[M]^+$ (Δ +0.6 mmu). The structure of prevezol B with unique oxetane ether linkage was proposed essentially by comparison of the NMR data to those of prevezol A. The major difference between prevezols A and B was that the epoxide carbons (δ 59.2 and δ 62.2) were replaced by oxygen bridge carbons (δ 72.9 and δ 75.9). The structure of prevezol B was proposed as a 1,2-methyl shift followed by ether linkage formation. The relative stereostructure was deduced based on Monte Carlo conformational analysis and the proposed structure with the lowest energy conformation was supported by NOE correlations. In 2003, the same research group revised the structure of prevezol B as a stereoisomer of the new analogue prevezol C whose structure was firmly assigned since the spectral data of prevezol C closely resembled those of prevezol B.[82] The number of hydroxy groups in prevezol B was confirmed by MS data of the methylation product (data not provided). Furthermore, the oxymethine H-14 turned out to be an axial proton ($\mathcal{J}_{9,14} = 11.20$ Hz), which eradicated the possibility of an ether bridge between C-12 and C-14. Finally, NOE correlations allowed assignment of the relative stereostructure of prevezol B as 2*R*,3*S*,6*R*,9*R*10*S*,13*S*,14*S**or 2*R*,3*S*,6*R*,9*S*10*R*,13*R*,14*R**.

2.18.4.3.3 Cladiellane diterpenes

Two new cladiellane class of diterpenes, (1*R**,4*R**,5*S**,6*R**,8*R**,12*R**,13*R**,14*R**)-cladiellane-4,8,12-triol and its 4-acetoxy derivatives were isolated in 1989 as secondary metabolites from the Australian gorgonian *Briareum* species.[83] The MF of the cladiellane triol was established as $C_{20}H_{36}O_4$ based on the HREIMS data observed at *m/z* 322.250 $[M–H_2O]^+$ (Δ −1 mmu). Both the 2D and 3D relative structures of this triol were determined based on 2D NMR data, NOE correlations, and comparison of the NMR data to those of the similar known compounds cladiellin and eunicellin, both of which had firmly determined structures via X-ray structure

Figure 8 Structure of prevezol A.

analysis.[88,89] The structure of the acetate was determined from nearly identical NMR data, with the exception of the acetyl group signals and downfield shift of the C-4 carbon chemical shift by 13 ppm (the MS data of the acetate are not provided). In 2003, a new cladiellane derivative polyanthellin A was reported from the gorgonian *Briareum polyanthes* collected in Puerto Rico.[84] HREIMS experiment did not provide a molecular ion peak for polyanthellin A (m/z 304.250 2 $[M\text{–}AcOH]^+$, Δ +10.0 mmu). However, the secure MF $C_{22}H_{36}O_4$ was obtained from HRFABMS experiment (m/z 387.2512 $[M+Na]^+$, Δ +0.1 mmu). It was suggested that this molecule had no alcohol groups based on the IR spectrum. The planar and relative structures of polyanthellin A were straightforwardly determined by the analysis of the 1D and 2D NMR data. Treatment of this compound with lithium aluminum hydride provided a triol on C-3, C-7, and C-11. The ^1H and ^{13}C NMR data of both polyanthellin A and the synthetic triol turned out to be identical to those of the cladiellane triol and the triol from the Australian specimens. However, the compounds previously assigned possessed opposite optical rotation values (polyanthellin A = $[\alpha]_D$ −9.9° vs the acetate = $[\alpha]_D$ +8.9°, the synthetic triol = $[\alpha]_D$ −11.0° vs the cladiellane triol = $[\alpha]_D$ +19.4°). Thus, the cladiellane diterpene derivatives previously assigned were revised as the enantiomers of polyanthellin A and the triol. The total synthesis of (+)-polyanthellin A (polyanthellin A enantiomer) was achieved in 2006,[85] proving the structure of polyanthellin A with the ether linkage.

2.18.4.4 Challenges in Distinguishing Hydroxy Groups from Hydroperoxides

This section illustrates the difficulties that can arise when deciding between hydroxy and hydroperoxide groups. Caution is required when establishing the MF since these functional groups are easily cleaved from the molecule in MS experiments (especially EIMS) and the corresponding fragment peak can no longer give significant information to distinguish these two functionalities. Two structure revisions regarding this issue are reviewed below and both examples are reassigned from a hydroxy to a hydroperoxide group (**Table 4**). These revisions suggest that the key to distinguishing these functional groups is the downfield shift of the hydroperoxide carbon.

2.18.4.4.1 12S-Hydroxybromosphaerodiol

12*S*-Hydroxybromosphaerodiol was reported in 1987 as a new brominated diterpene from the red alga *Sphaerococcus coronopifolius* collected in the Mediterranean Sea.[90] The MF $C_{20}H_{32}Br_2O_3$ was established based on the HREIMS data (m/z 478.071 85 $[M]^+$, Δ −0.06 mmu). The presence of three hydroxy groups in the molecule was proposed based on the EIMS peaks observed at m/z 478 $[M]^+$, m/z 460 $[M\text{–}H_2O]^+$, m/z 442 $[M\text{–}2H_2O]^+$, and 424 $[M\text{–}3H_2O]^+$. The total structure including its absolute stereostructure was determined by the analysis of ^1H NMR data and chemical conversion to a common diol obtained from 12*S*-hydroxybromosphaerol, of which the absolute stereostructure had already been determined. In 2008, 1*S*-hydroperoxy-12*S*-bromosphaerodiol was reported as a cytotoxic diterpene isolated from the Greek *S. coronopifolius* algae.[91] The MS and NMR data of this hydroperoxide compound turned out to be identical to those of the previously reported 12*S*-hydroxybromosphaerodiol (CIMS: m/z 477 $[MH\text{–}H_2O]^+$, m/z 459 $[MH\text{–}2H_2O]^+$, m/z441 $[MH\text{–}3H_2O]^+$). Thus, the structure of 12*S*-hydroxybromosphaerodiol was revised to be the peroxide, which was also confirmed by X-ray structure analysis. Moreover, it was disclosed that the hydroperoxide carbon on C-1 (δ_C 79.2) possessed a downfield carbon shift by 13 ppm when compared to the hydroxy carbon on C-1 (δ_C 66.2) in the known compound bromosphaerodiol, whose structure had previously been determined by X-ray structure analysis.[91]

2.18.4.4.2 Briarellin A

In 1995, briarellin A was reported as a new cytotoxic eunicellin diterpene isolated from the gorgonian *Briareum asbestinum* collected in Puerto Rico.[92] The MF of briarellin A was established as $C_{28}H_{44}O_7$ based on the HREIMS peak observed at m/z 492.307 79 $[M]^+$ (Δ −0.8 mmu). The presence of two alcohol groups was proposed based on several additional HREIMS fragment peaks including m/z 474.297 43 $[M\text{–}H_2O]^+$ (Δ −0.9 mmu) and m/z 312.171 63 $[M\text{–}2H_2O\text{–}C_8H_{16}O_2]^+$ (Δ −0.9 mmu). The structure including its relative stereostructure was assembled by basic comparison of the NMR data to the related compound asbestinin-7 and the NOESY data. In 2003, the same group found several additional new briarellin derivatives from the

Table 4 Distinguishing hydroxy groups from hydroperoxide groups

Original structure	*Revised structure*	*Features*
12*S*-Hydroxy bromosphaerodiol (alga) MF: $C_{20}H_{32}Br_2O_3$ Cafieri *et al*.[90]	1*s*-hydroperoxy-12 *s*-hydroxybromosphaerol B MF: $C_{20}H_{32}Br_2O_4$ Smyrniotopoulos *et al*.[91]	Revision rationale: reassessment of MS and NMR and X-ray structure analysis. Remaining undefined: none. Critical data: (a) MS and NMR data: 12*S*-hydroxy-bromosphaerodiol = hemigaran E, (b) downfield shift of the hydroperoxide carbon C-1 (δ_C 79.2) compared to that of the hydroxy carbon (δ_C 66.2), (c) the structure of hemigran E was confirmed by X-ray structure analysis. Other issues: none.
Briarellin A (gorgonian) MF: $C_{28}H_{44}O_7$ Rodriguez and Cobar[92]	MF: $C_{28}H_{44}O_8$ Ospina *et al*.[84]	Revision rationale: reassessment of NMR. Remaining undefined: absolute structure. Critical data: (a) the NMR data (C6; d 4.67, 82.9 ppm) correspond to the C6-peroxide analogues (briarellin K hydroperoxide (C6; d 4.65, 85.6 ppm), briarellin D hydroperoxide (C6; d 4.62, 85.5 ppm)), but not to the C6-hydroxy analogue (briarellin K (C6; d 4.28, 72.6 ppm)), (b) MS data; original HREIMS *m/z* 492.307 79 (calcd for 492.308 67 $C_{28}H_{44}O_7$), revised LRFABMS *m/z* 509 (calcd for $C_{28}H_{45}O_8$), *m/z* 531 (calcd for $C_{28}H_{44}O_8Na$). Other issues: none.

Figure 9 Structural relationship and the chemical shift differences between hydroxy and hydroperoxide carbons.

B. polyanthes collected in Puerto Rico.[84] The structural pair briarellin K and its hydroperoxide among the new compounds gave a clue to revise the structure of briarellin A (**Figure 9**). The major differences between these two derivatives were the following three spectral data points: (1) Δ 16 amu (briarellin K, HREIMS *m/z* 392.2175 (calcd for $C_{22}H_{32}O_6$, Δ −2.3 mmu), HRFABMS *m/z* 399.2366 (calcd for $C_{22}H_{32}O_6Li$, Δ +0.7 mmu); briarellin K hydroperoxide, HRFABMS *m/z* 431.2040 (calcd for $C_{22}H_{32}O_7Na$, Δ −0.6 mmu)); (2) $\Delta\delta_{C\text{-}6}$ 13 ppm (briarellin K (δ 72.6), briarellin K hydroperoxide (δ 85.5)); and (3) $\Delta\delta_{H\text{-}6}$ 0.3 ppm (briarellin K (δ 4.28), briarellin K hydroperoxide (δ 4.62)). Similar observations were also present between briarellin D and its hydroperoxide. The spectral data of C-6 in briarellin A was almost identical to those of the hydroperoxides (δ_H 4.67, δ_C 82.9), strongly suggesting that briarellin A was a hydroperoxide at C-6. This assumption was confirmed later by MS data (FABMS *m/z* 509 $[M+H]^+$, *m/z* 531 $[M+Na]^+$).

2.18.5 Difficulties in Assembling Planar Structures

In this section, attention is shifted to 2D structure misassignment including double bond geometry, which arises from the interpretation of NMR data. The topics discussed below focus on the three factors leading to incorrect 2D structures: (1) C═Z assignment, (2) C═C assignment, and (3) assignment of either exo or endo form. Among the case examples described below, assignment of C═Z functional groups must be made particularly carefully since misassignment of C═Z functionalities provides completely different structures from the true structure.

2.18.5.1 Assessment of C═Z Assignments

Analyzing the examples discussed in this section demonstrates that it is very difficult to assign C═Z functional groups in a molecule when it possesses a low H/C ratio core (**Table 5**). All the examples collected here possess cores bearing a meager number of H atoms (H/C < 1) that make general ^{1}H and ^{13}C NMR techniques inadequate for structure elucidation. These examples show that it is sometimes quite difficult to distinguish C═Z functionalities that have similar carbon chemical shift, such as amide C═O versus ester C═O and guanidine C═N versus urea C═O. It is also important to assign C═Z carbons correctly when the molecule has multiple C═Z functionalities.

2.18.5.1.1 Spiroleucettadine

Spiroleucettadine was reported in 2004 as a new imino-imidazolidine alkaloid isolated from the sponge *Leucetta* sp. collected in Fiji.[93] The proposed structure of spiroleucettadine was very unique in terms of possessing a rare amino hemiketal functional group. This amino-hemiketal moiety was placed on the 5,5-*trans* bicyclic ring system that possessed three characteristic quaternary carbons. The first carbon signal at δ 159.5 (C-1) was assigned to be a guanidine carbon in the imino-imidazolidine ring. The other two carbons observed at δ 102.5 (C-4) and δ 82.5 (C-5) were accounted to be an amino-hemiketal carbon and nitro carbon, respectively, both of which were embedded as ring carbons between the 2-imino-imidazolidine and tetrahydrofuran moieties. These assignments for the characteristic carbons were essentially supported by an analogy to the alkaloid

Table 5 Assessing C = Z functional groups

Original structure	*Revised structure*	*Features*
Spiroleucettadine (sponge) Ralifo and Crews[93]	White *et al*.[94]	Revision rationale: DFT calculations, X-ray structure. Remaining undefined: absolute structure. Critical data: (a) HMBC correlation (H_2-8/C-6) = the $^1J_{CH}$ cross peak of CD_3OD, (b) the ROESY correlation (OH/H-19) was very weak, (c) the revised structure was supported by DFT calculations and X-ray structure. Other issues: the crystals of spiroleucettadine were a racemic mixture.
Kasarin (fungus) Suenaga *et al*.[95]	Kita *et al*.[96]	Revision rationale: reassessment of NMR. Remaining undefined: none. Critical data: (a) OCH_3/N-4 ^{15}N HMBC correlation was reassigned as OCH_3/N-1, (b) the synthetic model compound with a 5-hydroxyprozin-2(1*H*)-one ring showed the similar spectral data to the natural product. Other issues: none.
Aspernigrin A (fungus) Hiort *et al*.[97]	Ye *et al*.[98]	Revision rationale: NOE and X-ray structure analysis. Remaining undefined: none. Critical data: (a) NOEs (NH/$H_2$7, NH/H2), (b) the revised structure with 1*H*-pyridin-4-one ring was confirmed by the X-ray structure analysis. Other issues: none.

Calcaridine A   Spirocalcaridine A

Figure 10 Related amino-imidazole secondary metabolites from a *Leucetta* sponge.

spirocalcaridine isolated from the same sponge specimen (**Figure 10**). The relative structure of spiroleucettadine was deduced based on the analysis of the 2D NMR data. The key 2D correlations to establish the *trans* 5,5-bicyclo ring with the amino-hemiketal moiety were the HMBC correlation from H_2-8 to C-6 and the ROESY correlation between OH and H-19. After this report regarding the structure of spiroleucettadine, three synthetic groups failed to synthesize the *trans* fused bicyclic core and concluded that this alkaloid required structural revision.[99–101] The last synthetic group also suggested a possible revised structure for spiroleucettadin using DFT calculations.[101] In 2008, the structure revision of spiroleucettadine has been reported using a pure sample newly isolated by the original natural product team.[94,95] First, the *trans* 5,5-bicyclo ring system with amino-hemiketal functionality turned out to be assigned incorrectly since the key HMBC correlation (H_2-8/C-6) was actually due to the $^1J_{CH}$ cross peak of the NMR solvent CD_3OD and the ROESY correlation (OH/H-19) was very weak. The proposed revised structure was selected by DFT calculations among 16 candidate structures and later confirmed by X-ray structure analysis. It is interesting that the crystals obtained for X-ray structure analysis were a racemic mixture and thus spiroleucettadine has been proposed to exist as a scalemic mixture due to its optical activity (original sample: $[\alpha]_D$ −27.1, new sample: $[\alpha]_D$ −5.1). In the revised structure, the carbon signal observed at δ 159.5 (C-1) was assigned to be the urea carbon.

2.18.5.1.2 Kasarin

Kasarin was first described in 2000 as a new alkaloid from the marine bacterium *Hyphomycetes* sp., which was separated from the zoanthid *Zoanthus* sp.[95] The original structure of kasarin had a characteristic feature of a low H/C ratio azetinone core. This structure was proposed based on the 1D and 2D NMR techniques including ^{15}N HMBC experiments. Although direct evidence of a connection between C-2 and N(3) was not observed, the azetinone core was supported by its MF and the IR data (1760 cm^{-1} as β-lactam). In 2007, the structure of kasarin was revised based on reevaluation of the NMR data between the natural product and the synthetic analogues and degradation products.[96] The key ^{15}N HMBC correlation (OCH_3/N-4) in the original assignment actually turned out to be the correlation between OCH_3 and N-1. Furthermore, three sp^2 quaternary carbons on C-3, C-5, and C-1′ were reassigned as follows: (1) the amide carbonyl carbon (δ 161.5, C-3) connected to the isopropyl group in the original structure was assumed to be an imine carbon attached to the carbonyl carbon (δ 152.0, C-2), (2) the imine carbon (δ 134.9, C-5) was accounted as an sp^2 quaternary carbon bearing both nitrogen and oxygen, and (3) the amide carbonyl (δ 166.0, C-1′) was proposed to be an ester carbonyl. These changes provided two alternative structures: the oxygenated pyrazinone and the oxazolone derivative (**Figure 11**). The revised structure with the oxygenated pyrazinone for kasarin was chosen based on

Figure 11 Candidate structure for kasarin.

the spectral similarity including ^{13}C NMR, IR, and UV spectra between the natural product and a synthetic pyrazinone derivative, and was also supported by the spectral data of one of the degradation products. It should be mentioned that the structure elucidation of the pyrazinone core is especially challenging since the core ring has no hydrogen atoms (C_4N_2O, $H/C = 0$).

2.18.5.1.3 Aspernigrin A

Aspernigrin A was reported in 2004 as a new alkaloid from the fungus *Aspergillus niger* separated from the Mediterranean sponge *Axinella damicornis*.[97] The structure elucidation of aspernigrin A was performed based on the 1D and 2D NMR data. The two amide carbonyl carbons were observed at δ 177.6 and δ 165.5, which were assigned to be the carboxy amide and lactam carbonyl carbon, respectively. The planar structure including the placement of the benzyl group and the carboxy amide moiety was proposed based on the HMBC correlations of the two protons on the lactam ring. In 2005, another research group isolated aspernigrin A from the endophytic fungus *Cladosporium herbarum* possessing almost identical spectral data to the first isolated sample.[98] It was necessary first to examine the amide groups since the carbon chemical shift of the conjugated carboxy amide (δ 177.6) appeared significantly more downfield than as usual (δ_C ~165). This inconsistency was solved by swapping the amide carbons between the carboxy amide and the lactam. This carbon reassignment and NOEs observed between NH/H_2-7 and NH/H-2 indicated that the lactam core in the original structure must be revised to a 4-pyridone. The revised structure proposed based on both the above observations and the HMBC data has subsequently been proven by X-ray structure analysis.

2.18.5.2 Assessment of C=C Assignments

The examples described below detail incorrect C=C assignments including geometry and placement (**Table 6**). Misassignment regarding this issue often occurs when multiple C=C functionalities exist in aliphatic chain and/or polyketide macrocyclic ring. This situation causes signal overlapping for sp^2 protons, which makes secure structure elucidation intensely difficult.

2.18.5.2.1 Mycothiazole

Mycothiazole was reported in 1988 as a novel polyketide/alkaloid with anthelmintic activity isolated from the Vanuatu sponge *Spongia mycofijiensis* (also known as *Cacospongia mycofijiensis*).[102] The planar structure possessing four double bonds in the polyketide chains was proposed based on 1D and 2D NMR data. Two of the double bonds were assigned as terminal olefins ($\Delta^{4,19}$ and $\Delta^{18,19}$) and the others were assigned as a *Z* olefin ($\Delta^{5,6}$, $J = 12$ Hz) and an *E* olefin ($\Delta^{14,15}$, $J = 18$ Hz). The original structure of mycothiazole was confirmed by total synthesis in 2000.[109] The synthetic mycothiazole with 8*R* configuration showed identical spectral data including ^1H and ^{13}C NMR, IR, and HRMS. However, differences in the optical rotation values between the synthetic material and natural product were conspicuously inconsistent (synthetic $= [\alpha]_D$ -26.0, natural $= [\alpha]_D$ -3.8), but this was rationalized by contamination or degradation of the natural product sample. In 2006, the structure of mycothiazole was revised by the original natural product team.[103] The key difference between the synthetic product and the natural product appeared to be not only the specific rotation values but also the carbon chemical shifts of two allylic methylenes. The carbon chemical shifts of C-13 and C-16 in the synthetic product showed a downfield shift by ca. 2 ppm compared to those in the natural product (synthetic: δ 34.7 (C-13), δ 36.6 (C-16); natural: δ 29.4 (C-13), δ 31.5 (C-16)). This evidence corresponded to an upfield shift of an allylic position in a disubstituted *Z* olefin rather than *E* configuration. Thus, the structure of mycothiazole was suggested to possess C-14/C-15 *Z* configuration, which was confirmed by strong NOEs between H-13/H-16 and the newly observed coupling constant ($J_{14,15} = 10.7$ Hz). Mycothiazole has shown selective cytotoxicity against human lung cancer cell lines (DMM-114 and NCI-H23) and further biological evaluations are being continued.

2.18.5.2.2 Lasonolide A

Lasonolide A was reported in 1994 as a cytotoxic polyketide macrolide isolated from the sponge *Forcepia* sp. collected in the British Virgin Islands.[104] The structure of lasonolide A containing seven double bonds was proposed from the NMR data. Although all the double bonds were disubstituted olefins, one of those was

Table 6 Problematic assignments of C=C bonds

Original structure	*Revised structure*	*Features*
Mycothiazole (sponge) Crews *et al*.[102]	Sonnenschein *et al*.[103]	Revision rationale: reassessment of NMR. Remaining undefined: none. Critical data: (a) upfield carbon chemical shift on C-13 and C-16 of the synthetic product comparing to those of the natural product (synthetic: δ 34.7 (C-13), δ 36.6 (C-16); natural: δ 29.4 (C-13), δ 31.5 (C-16)) was accounted for 14*Z*-isomer, (b) NOE between H13/H16, (c) $J_{14,15} = 10.7$ Hz. Other issues: none.
Lasonolide A (sponge) Horton *et al*.[104]	Lee *et al*.[105] Song *et al*.[106]	Revision rationale: total synthesis. Remaining undefined: none. Critical data: (a) the 28*S* and 28*R* synthetic materials ≠ the natural products, (b) the proton chemical shift of C-19 in the synthetic products = 0.4 ppm downfield comparing to the natural products, suggested 17*E* configuration, (c) the diastereomers, 17*E*,28*S* and 17*E*,28*R* ≠ the natural products, (d) the 17*E*,25*Z*,28*R* isomer = the natural product. Other issues: none.

(*Continued*)

Table 6 (Continued)

Original structure	*Revised structure*	*Features*
Pyrinodemin A (sponge) Tsuda *et al*.[107]	Ishiyama *et al*.[108]	Revision rationale: total synthesis. Remaining undefined: none. Critical data: (a) the synthetic product ≠ the natural product, (b) HPLC retention time; the $\Delta^{14,15}$ isomer = the natural product, (c) ESIMS peak (*m/z* 241) of one of the degradation products obtained by treatment of OsO_4 and $NaIO_4$ = the C-15′ aldehyde with the C7′-C-15′ alkyl chain and pyridine. Other issues: the HPLC study suggested that pyrinodemin A exists in a 1:1 racemic mixture.

HPLC, high performance liquid chromatography.

assigned as a terminal olefin ($\Delta^{31,40}$) on the side chain, five double bonds were embedded in the macrocyclic ring ($\Delta^{2,3}$, $\Delta^{4,5}$, $\Delta^{12,13}$, $\Delta^{14,15}$, $\Delta^{16,17}$), and the last one ($\Delta^{25,26}$) was placed in the side chain. The geometries of the olefins were directly determined from the NMR data. NOEs (H-2/H-4, H-3/H-5, H-12/H-37, H-15/H-37) and large coupling constants ($J_{2,3} = 15.4$ Hz, $J_{4,5} = 15.3$ Hz, $J_{14,15} = 15.7$ Hz, $J_{25,26} = 18.0$ Hz) suggested C-2/C-3 *E*, C-4/C-5 *E*, C-12/C-13 *Z*, C-14/C-15 *E*, and C-25/C-26 *E* geometries. The geometry of the remaining C-17/C-18 double bond was determined as *Z* even though the proton signals of H-17 and H-18 were partially overlapped due to no coupling over 15 Hz, and NOE between H_2-16 and H-20α that could be observed when C-17 and C-18 were in a *cis* configuration. While the relative stereostructure excepting the configuration of C-28 for lasonolide A was proposed based on the NOE data, the structure of lasonolide A was revised based on the total synthesis of the natural product in 2002.[105,106] First, the spectral data of both synthetic 28*R* and 28*S* lasonolide A were not identical to those of the natural product. Second, the same group synthesized two lasonolide A diastereomers (28*R* and 28*S* lasonolide A with an enantiomeric B ring), but neither diastereomer exhibited the same spectral data as the natural product. The most significant spectral difference of the synthetic materials was the proton signal of H-19 centered at δ 4.70 ppm for the synthetic product and δ 4.30 ppm for the natural product. This chemical shift difference was quite similar to two geometric isomers of one of the ambruticin synthesis intermediates. In that case, the methine proton chemical shift on the C-2 position of tetrahydropyran with a *trans* double bond appeared 0.4 ppm upfield compared to that of a *cis* double bond. Thus, the geometry of C-17/C-18 double bond was deduced to be of *E* configuration. However, both the 17*E*,28*R* and 17*E*,28*S* synthetic materials were not spectrally identical to the natural product. The chemical shift differences were found primarily in the $\Delta^{25,26}$ olefin and thus the *E* olefin of C-25/C-26 was suspected. Finally, the synthetic diastereomer with 17*E*,25*Z*,28*R* configuration was confirmed as the natural product. Lasonolide A is expected to be a new anticancer lead due to its potent cytotoxicity against human cancer cells A-549 (human carcinoma) and Panc-1 (human pancreatic carcinoma) with an IC_{50} of 8.6 and 89 nmol l^{-1}, respectively.

2.18.5.2.3 Pyrinodemin A

Pyrinodemin A was reported in 1999 as a cytotoxic pyridine alkaloid isolated from the sponge *Amphimedon* sp. collected in Okinawa.[107] The planar structure of this alkaloid was composed of a unique cyclopent[*c*]isoxazolidine connected with a bis-3-alkylpyridine based on the MS and NMR data. Location of the *Z* olefin [δ_C 129.3 (2C), C-16′ and C-17′] was assigned from the ^{13}C chemical shift of the two vinyl carbons (C-15′ and C-18′, δ_C 27.1 each) on the detailed analysis of the EIMS fragment peaks. The relative stereostructure of the bicyclic ring was proposed from rigorous examination of the NOESY data. In 2001, pyrinodemin A was synthesized by two different synthetic groups.[110,111] However, upon completion of the synthesis, both groups concluded that the C-16′/C-17′ double bond was incorrectly assigned due to the observation of two carbon signals for C-16′ and C-17′ separated by 1 ppm in the synthetic product. Based on the comparison of the NMR data of the synthetic materials to those of the natural products, one of the synthetic groups suggested that the revised structure of pyrinodemin A was the $\Delta^{15'16'}$ isomer[110] while the other group proposed the $\Delta^{14'15'}$ isomer.[112] In 2005, the natural product research group that reported the original structure of pyrinodemin A settled dispute by characterizing pyrinodemin A as the $\Delta^{15'16'}$ isomer based on the total synthesis of the isomers and HPLC analysis.[108] The HPLC retention time of natural pyrinodemin A corresponded to that of the synthetic $\Delta^{15'16'}$ isomer. This conclusion was supported by an ESIMS ion peak (*m*/*z* 242 $[M+Na]^+$) of one of the degradation products obtained by treatment of the natural product with OsO_4 and $NaIO_4$, which yielded the C-15′ aldehyde with the pyridine ring and C-7 to C-15′ moiety. Interestingly, chiral HPLC analysis concluded that the natural product exists as a 1:1 racemic mixture.

2.18.5.3 Challenges in Distinguishing Exo Forms from Endo Forms

This section describes two examples, in which it was very hard to distinguish a compound as either exo-type form or endo-type form by NMR analysis (**Table 7**). One structure was revised from endo-type form to exo-type form and the other vice versa. The first structure revision in this section was one of the most challenging examples of structural reassignment since the original (proposed) and the revised (alternative) structures show essentially the same 2D NMR correlation pattern. Thus, either total synthesis or NMR chemical shift simulations are required to confirm the structure. The first structure revision of zamamistatin

Table 7 Distinguishing endo forms from exo forms

Original structure	*Revised structure*	*Features*
Elatenyne and the enyne (alga) Hall and Reiss[113] Wright *et al*.[114]	Sheldrake *et al*.[115]	Revision rationale: total synthesis, NMR assessment. Remaining undefined: absolute structure. Critical data: (spectroscopic data) natural products ≠ synthetic products, ^{13}C NMR < δ 76 (ring CHs for pyrano[3.2-*b*]pyran), ^{13}C NMR > δ 76 (2,2-bifuranyl compound). Other issues: the relative stereo of elatenyne was elucidated by DFT calculations.[116]
4′-Chloroaurone (alga) Atta Ur *et al*.[117]	3-(4′-Chlorophenyl)-isocoumarin Venkateswarlu *et al*.[118]	Revision rationale: total synthesis, reassessment of NMR. Remaining undefined: none. Critical data: (a) the synthetic products both *Z* and *E* isomers of 4′-chloroaurones ≠ the natural products, (b) the 1H NMR data; the natural product = 3-(4′-chlorophenyl)-isocoumarin. Other issues: none.

(Section 2.18.4.2.1) from exo-type form to endo-type form is also categorized into this group, with the final proposed structure resulting from synthetic effort.

2.18.5.3.1 Elatenyne and the related enyne

Elatenyne was reported in 1986 as a new vinylacetylene from the alga *L. elata* collected at Victoria.[113] The structure of elatenyne was first proposed based on the MS and NMR data. The presence of the C_5H_5 side chain (*Z*-pent-3-ene-1-yne) was suggested based on the following spectral information: (1) MS data (m/z 325 $[M–C_5H_5]^+$), (2) IR absorption (terminal acetylene = 3300, 2120 cm^{-1}, *Z* olefin = 3035, 1620, 750 cm^{-1}), (3) UV absorption (enyne = 224, 233 nm), and (4) proton and carbon coupling constants ($^3J_{3,4}$ = 11.0 Hz, J_{CH} = 251 Hz (acetylene)]. The detailed analysis of the ^{1}H and ^{13}C NMR data including distortionless enhancement by polarization transfer (DEPT) and shift reagent experiments gave the planar structure of elatenyne. The relative stereostructure of the pyrano[3,2-*b*]pyran ring was proposed based on the proton coupling constants obtained by treatment of the natural product with a shift reagent. In 1993, a new elatenyne derivative isolated from the Australian *L. majuscula* was reported.[114] The linear carbon skeleton C-1 to C-15 was established from the ^{1}H-^{1}H and ^{1}H-^{13}C COSY data. The major difference in the new derivative was the presence of an *E* olefin ($^3J_{3,4}$ = 15.9 Hz) in the side chain. Determination of the stereostructure of the new derivative was made by the similarity of the coupling constants to that of elatenyne. Although these two compounds were synthesized in 2006,[115] the spectral data of the synthetic products were not identical to those of the natural products. The synthetic groups carefully analyzed the carbon chemical shifts of a number of the pyrano[3,2-*b*]pyran and 2,2′-bifuranyl compounds synthesized in this work. This analysis elucidated the trends of the carbon chemical shifts for these two ring systems. The central oximethine carbons in the pyrano[3,2-*b*]pyran compounds were observed at <δ 76 whereas those in the 2,2′-bifuranyl compounds occurred at >δ 76. The oximethine carbons in elatenyne and the derivative were observed at

δ 71.3 and δ 71.4 (elatenyne) and δ 73.9 and δ 70.5, respectively. Thus, the planar structures of these two compounds were revised as 2,2′-bifuranyl derivatives. At this point, the stereostructures remained unsolved. In 2008, the relative stereostructure of elatenyne was suggested based on DFT calculations of all possible 32 diastereomers plus the original structure.[116]

2.18.5.3.2 4′-Chloroaurone

4′-Chloroaurone was reported in 2001 as one of two new aurone derivatives from the marine brown alga *Spatoglossum variabile* collected in Pakistan.[117] The structure of 4′-chloroaurone was deduced by comparison of its MS and NMR data to that of the other new compound, 4′-chloro-2-hydroxyaurone, of which the structure was assembled by analysis of the spectral data. This spectral comparison including the ^{1}H and ^{13}C NMR data as well as the MS data indicated that the structure of 4′-chloroaurone was the dehydration product of 4′-chloro-2-hydroxyaurone on C-2 and C-10. The geometry of the $\Delta^{2,10}$ double bond was proposed to be a *Z* olefin based on the calculation results using the Austin model 1 (AM1) method. In 2007, aurone derivatives including 4′-chloroaurone and its *E* isomer were synthesized to investigate their antioxidant and antibacterial activities.[118] The spectral data of both the synthetic *Z* and *E* isomers of 4′-chloroaurone were not identical to those of the natural product. In fact, the synthetic group revealed that the ^{1}H NMR data of the natural product corresponded to those of the reported value of 3-(4′-chlorophenyl)-isocoumarin. Since the ^{13}C NMR data of the isocoumarin has not been published, the structure of 4′-chloroaurone was tentatively revised as 3-(4′-chlorophenyl)-isocoumarin and further investigation is necessary to confirm the revised structure.

2.18.5.4 Challenges in Stereochemical Assignments

Stereochemical reassignments occur much more frequently than structure revisions and account for about two-thirds of all misassigned marine natural products. Several standard methods to determine stereochemistry have been routinely applied to compounds to finalize the structure elucidation. However, these methods provide incorrect results (opposite configurations) when applied inappropriately. Stereochemical assignments of the structures collected in **Table 8** have recently been revised from those that were originally made by standard methods. In the table, harzialactone A[119] and peribysin E,[120] both of which had their absolute structure established by a modified Mosher's method,[60] turned out to be an antipode by total synthesis.[121,127] In the case of harzialactone A, a modified Mosher's method was incorrectly applied and the chemical shift differences between *R*- and *S* α-Methoxy-α-trifluoromethylphenylacetic acid ester were only measurable from one side of the MTPA ester group. Even when Mosher's method provides the right results, the absolute structure can be incorrect, such as the case of peribysin E. This case was a simple erroneous labeling of the *R*- and *S*-MTPA ester. The stereochemistry of D-*N*-Me-Phe in yanucamides A and B[123] proposed by Marfey's method[61] was later revised to be L configuration by total synthesis.[124] A similar error in assigning *N*-Me-Phe is found in kulokekahilide-2,[125] which was later revised from L to D configuration.[126] These examples show that even the standard stereochemical assignment methods have the potential to lead to structural misassignment. It goes without saying that careful application and interpretation of the results obtained are important to reach the correct stereostructure.

Stereochemical reassignment for the structure with more than three incorrect chiral centers is extremely difficult due to 2^{n-1} potential relative stereostructures. In this situation, synthetic effort, biosynthesis considerations, and X-ray structure analysis are essential to verify the correct stereostructure. The structures depicted in **Table 9** are the examples of the compounds that have had more than three stereocenters revised. The stereochemical reassignment of nakiterpiosin[128] was conducted by a succinct method without synthesizing multiple diastereomers. First, the proposed and revised structures were predicted based on biosynthesis analysis including structure comparison to the same class of compounds (C-nor-D-homosteroid) cyclopamine and veratramine. This new proposed structure was confirmed by agreement of the spectral data between the synthetic material and the natural product. It is remarkable that the original assignment was made using a modified Mosher's method and bioassay performed with only 0.2 mg of the pure material from 30 kg of sponge. Also, in the case of dolastatin 19,[130] the revised structure was proposed prior to the synthesis work by dereplication,[131] whereas the stereostructure of seragakinone A[132] was directly revised based on X-ray structure analysis.[133]

Table 8 Revision of absolute stereochemistry by standard methods

Original structure	*Revised structure*	*Features*
Harzialactone A (fungus) Amagata *et al*.[119]	Mereyala and Gadikota *et al*.[121]	Assignment: modified Mosher's method. Revision rationale: total synthesis. Remaining undefined: none. Critical data: (a) *ent*-harzialactone A = the natural product, (b) $[\alpha]_D$ +33.50 (synthetic product), $[a]_D$ +38.0 (natural product). Other issues: none.
Peribysin E (fungus) Yamada *et al*.[120]	Angeles *et al*.[122]	Assignment: modified Mosher's method Revision rationale: total synthesis Remaining undefined: none Critical data: (a) NMR data; the synthetic product = the natural product, (b) big difference in the optical rotation value, $[\alpha]_D$ −262.2 (synthetic product), $[\alpha]_D$ −52.17 (natural product), (c) the optical rotation of the diacetate, $[\alpha]_D$ −34.78 (synthetic product), $[\alpha]_D$ +35.00 (natural product), (d) the synthetic enantiomer diacetate; $[\alpha]_D$ +37.49 Other issues: none
Yanucamides A and B (cyanobacterium) R = CH_3 (yanucamide A) R = *S*-C_2H_5 (yanucamide B) Sitachitta *et al*.[123]	R = CH_3 (yanucamide A) R = *S*-C_2H_5 (yanucamide B) Xu *et al*.[124]	Assignment: Marfey's method. Revision rationale: total synthesis. Remaining undefined: none. Critical data: (a) both synthetic 3*S*- and 3*R*-yanucamide A ≠ the natural product, (b) major diference; shielded doublet Me (Hiv) at δ_H 0.30 in the synthetic materials vs δ_H 0.66 in the natural product, (c) 3*S*,22*S*-yanucamide A = the natural product. Other issues: none.

Kulokekahilide-2 (mollusk)

Nakao *et al*.[125]

Takada *et al*.[126]

Assignment: Marfey's method (amino acid), chiral HPLC analysis (Hica), synthesis (polyketide chain).
Revision rationale: total synthesis.
Remaining undefined: none.
Critical data: (a) the synthetic product ≠ the natural product, (b) C-21 and C-24 had opposite stereo comparing to those of the related depsipeptide, aurilide, (c) position 43 easily racemize, (d) the synthetic. 21*S*,24*R*,43*R* isomer = the natural product.
Other issues: none.

Table 9 Stereochemical reassignment based on biosynthetic considerations

Original structure	*Revised structure*	*Features*
Nakiterpiosin (sponge) Teruya *et al*.[128]	Chiang *et al*.[129]	Revision rationale: total synthesis. Remaining undefined: absolute structure. Critical data: (a) the synthetic product ≠ the natural product, (b) opposite C-20 stereo, the original structure vs the related compound, (c) the synthetic 6*R*,20*S*,25*S* isomer = the natural product. Other issues: none.
Dolastatin 19 (mollusk) Pettit *et al*.[130]	Paterson *et al*.[131]	Revision rationale: total synthesis. Remaining undefined: none. Critical data: (a) the revised structure was proposed by comparison of the ^{1}H NMR data to those of callipeltoside A and aurisides A and B with same macrocyclic ring system, (b) the spectral data of the revised structure by the total synthesis were identical to those of the natural product, (c) $[\alpha]_D$ +2.2 (synthetic product), $[\alpha]_D$ +7.5 (natural product). Other issues: none.
Seragakinone A (fungus) Shigemori *et al*.[132]	Komatsu *et al*.[133]	Revision rationale: X-ray structure analysis. Remaining undefined: absolute structure. Critical data: the revised structure was proposed by the X-ray structure analysis. Other issues: none.

2.18.6 Completion of Structure Elucidation

Structure elucidation for chiral molecules is complete when the absolute stereostructure is determined. Stereochemical assignment is an important task to connect chemistry and biology since the orientation of functional groups and the ring conformations are key to understanding the mechanisms of biological activities. However, there are many natural products whose 3D structures cannot be solved based only on spectroscopic analysis. In most of these cases, the presence of tiny amount of pure sample makes it very difficult to complete stereochemical assignment. The structures in **Table 10** are two selected examples of marine natural products whose 3D structures have been completed via total synthesis. The first example is a sponge-derived cytotoxic compound, psymberin[134] (also known as irciniastatin A[135]). In 2004, two independent research groups reported a potent cytotoxic pederin class of compound named psymberin and irciniastatin A, which were the C-8 epimers. At that time, psymberin had one uncharacterized stereocenter while five stereocenters of irciniastatin A remained undetermined. Although the ^{1}H and ^{13}C NMR spectra of these compounds were measured in a different solvent, irciniastatin A was expected to be identical to psymberin since the stereochemistries of pedrin class of compounds such as pedrin and mycalamide A corresponded to those of psymberin. The total synthesis of psymberin was completed in 2005 and the remaining chiral center was determined to be 4*S* configuration.[136] In addition, this synthesis demonstrated that irciniastatin A was identical to psymberin. Further synthetic study disclosed that the stereochemistries of two methoxy groups on the side chain are critical to demonstrate its potent cytotoxicity. A second example is the tunicate-derived protein kinase C (PKC) isoform δ inhibitor bistramide A whose original structure with a 19-membered lactam was reported in 1989.[137] The 2D structure of bistramide A was revised in 1992 by 2D incredible natural abundance double quantum transfer experiment (INADEQUATE) experiments.[138] The revised structure possessed a unique acyclic nature containing two polyketide chains with a spiroketal moiety and a substituted tetrahydropyran connected by a central γ-amino acid. At this point, all 11 chiral centers remained unsolved, leaving the absolute stereostructure of bistramide A as one out of 2048 (2^{11}) possible isomers. In 2002, the complete assignment of bistramide C[141] (39-keto-bistramide A) was made by a combination of chiroptic analysis and synthesis of 34-epi-bistramide C.[139] Finally, the absolute stereostructure of bistramide A was confirmed in 2005 by total synthesis.[142]

Total synthesis remains one of the most powerful structure elucidation tools as many structure revisions and stereochemical assignments have been made by total synthesis. In the future, a combination of DFT calculations and total synthesis is expected to be a more important structure elucidation tool to finalize 3D structures since natural products chemistry has been shifting to focus to more minor secondary metabolites, which often have complex cores and multiple chiral centers. For example, amphezonol A,[143] which was recently reported as a C_{60} polyhydroxy linear polyketide with a tetrahydrofuran ring and two tetrahydropyran rings isolated from a dinoflagellate, possesses 19 unassigned stereogenic centers – meaning that the absolute structure of amphezonol A is one of 524 288 (2^{19}) possible stereoisomers (**Figure 12**).

Figure 12 Structure of amphezonol A.

Table 10 Completion of stereochemical assignments via synthesis

Original structure	*Complete structure*	*Features*
Psymberin (sponge) Cichewicz *et al*.[134] Irciniastatin A (sponge) Bistramide A (tunicate) Pettit *et al*.[135]	Psymberin Jiang *et al*.[136]	Stereochemistry determination: total synthesis. Remaining undefined: none. Critical data: none. Other issues: none.

Degnan *et al*.[137]

Statsuk *et al*.[141]

Revision rationale: 2D INADEQUATE.
Stereochemical determination: total synthesis.
Remaining undefined: none.
Critical data: (a) the carbon skeleton was confirmed by the result of 2D INADEQUATE experiment,[138] (b) a combination of chiroptic analysis and synthesis work led to the assignment of bistramide C (39-keto-bistramide A),[139] (c) spectral data (NMR, $[\alpha]_D$, MS); the synthetic product = the natural product.
Other issues: mode of action of cytotoxicity shown by bistramides = actin.[140]

2.18.7 Conclusions

The case examples introduced in this chapter demonstrate that pitfalls leading to misassignment lie in each step of structure elucidation, from MF assignment to determination of the absolute stereostructure. Analysis of the misassigned molecules shows that assignment errors occur frequently when NMR signals are overlapped, or in molecules which have a low H/C ratio core. The former case can be avoided using several different NMR solvents, whereas simulated NMR chemical shifts obtained by calculation such as DFT methods are useful for the latter case. Furthermore, it is often necessary to reconsider the MF again when high-resolution MS data has greater than ± 5 mmu error. Another impediment to structure elucidation is the bias of the individual, which leads to misassignments. To overcome this prejudice, it is necessary to analyze and validate all possible alternative structures against the working structure.

Abbreviations

APCI	atmospheric pressure chemical ionization
CAST	canonical-representation of stereochemistry
CI	chemical ionization
DFT	density functional theory
EI	electron impact
GIAO	gauge-independent atomic orbital
HRMS	high-resolution MS
HTMMD	3-hydroxy-2,2,4-trimethyl-7-methoxydecanoic acid
MF	molecular formula
mmu	milli mass unit
MS/MS	tandem MS
MT-MMP	membrane-type matrix metalloproteinase
OAT	ornithine δ-amino transferase
OMCA	1,2-oxazetine-4-methyl-4-carboxylic acid
PKC	protein kinase C

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Biographical Sketch

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2.19 Natural Products of Therapeutic Importance

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2.19.1 Introduction

2.19.1.1 The Role of Traditional Medicine in Drug Discovery

The history of natural products from a variety of sources, namely, plants, microbes, and marine organisms as medicinal agents in man and other animals, is long and varied, with significant 'early literature' in the case of plants. It should be noted, however, that in a significant number of cases, the actual sources are now only being recognized as often being associated microbes rather than the organism from which they were first reported.

The first records, written on clay tablets in cuneiform, were from Mesopotamia and date from about 2600 BCE, while Egyptian medicine dates from about 2900 BCE, with the best known Egyptian pharmaceutical record being the *Ebers Papyrus* (1500 BCE).[1,2] The Chinese *Materia Medica* has been extensively documented

over the centuries, with the first record (Wu Shi Er Bing Fang), containing 52 prescriptions, dating from about 1100 BCE[3,4] though records from the *Pent'sao* are reputed to be even earlier (~2700 BCE) and documentation of the Indian Ayurvedic system dates from about 1000 BCE (Susruta and Charaka).[5,6]

In the ancient Western world, the Greeks contributed substantially to the rational development of the use of herbal drugs. The philosopher and natural scientist, Theophrastus (~300 BCE), in his *History of Plants*, dealt with the medicinal qualities of herbs and Dioscorides, a Greek physician (100 CE), during his travels with Roman armies, recorded the collection, storage, and use of medicinal herbs. Galen (130–200 CE), who practiced and taught pharmacy and medicine in Rome, published no less than 30 books on these subjects and is well known for his complex prescriptions and formulas used in compounding drugs, sometimes containing dozens of ingredients ('galenicals').

During the Dark and Middle Ages (fifth to twelfth centuries), it was the Arabs who were responsible for the preservation of much of the Greco-Roman expertise and for expanding it to include the use of their own resources, together with Chinese and Indian herbs unknown to the Greco-Roman world. The Arabs were the first to establish privately owned drug stores in the eighth century and the Persian pharmacist, physician, philosopher, and poet, Avicenna, contributed much to the sciences of pharmacy and medicine through works such as *Canon Medicinae*, regarded as 'the final codification of all Greco-Roman medicine'. For the interested reader a comprehensive review of the history of medicine may be found on the National Library of Medicine's History of Medicine homepage.[7]

In this particular chapter, we will give information on some of the 'drugs from nature' that are still in use in human medicine and whose structures have led to novel agents in use today or in clinical trials at the moment, but we will not cover in detail the anticancer drugs isolated from plants (i.e., the taxanes, camptothecins, podophyllotoxin-derived, and the vinca alkaloids) as these will be covered in a later chapter by different authors. What is extremely interesting, however, is that in the cases of these four 'basic' structures, all isolated from plants that have between them 13 approved antitumor drugs to date, all have now been identified as secondary metabolites of endophytic fungi isolated from the 'nominally producing plants'.[8–16] How many more such discoveries are yet to be made is unknown, but it does raise some interesting hypotheses as to how plants respond to attack by predators as one can invoke a version of quorum sensing as a possible response.

However, we will cover the important areas of anti-infectives (bacterial, fungal, parasitic (though not antimalarials as those will be covered in a later chapter), and viral), anticholestemics, and some of the anticancer agents that have come from microbes and from marine organisms. In general, these areas will include both current and past agents in use and some of the materials that are currently in preclinical and clinical trials. The coverage will not be exhaustive due to space limitations but will be designed to show the utility of natural products as both human use medicinals, and in particular, as novel structures that have been, and may be further utilized as scaffolds upon which to discover new therapeutic entities. Four recent review articles are very relevant in this regard and should be consulted by the interested reader.[17–20]

In order to discuss the utility of natural product scaffolds, we will discuss by therapeutic area rather than use the customary 'by structure' format, since over the years, various structural classes have moved into therapeutic areas far beyond where the original discoveries were made.

2.19.2 Anti-infectives

2.19.2.1 Antibacterials

The first usage of natural products as true antibacterials rather than as surface sterilants (use of thymol and other essential oils for example) can be traced to the usage of microbial-derived secondary metabolites in the early part of World War II (WWII), though as is now recognized, the use of Prontosil (**1, Figure 1**) led to the introduction of synthetic antibacterials with the first clinical efficacy report in 1933, and ultimately leading to the award of the Nobel Prize for Medicine in 1938 to Domagk. This could also be thought of as the first formal prodrug in the antibiotic field as the active principle, sulfanilamide (**2, Figure 1**), is a structural analogue of *para*-aminobenzoic acid (PABA), an essential nutrient of many bacteria and in particular, the cocci. PABA competitively inhibits dihydropteroate synthase, thus leading to inhibition of folic acid and bacterial death. So although synthesized in the absence of such knowledge, and for an entirely different purpose, it was in retrospect, an isostere of a natural product.[21]

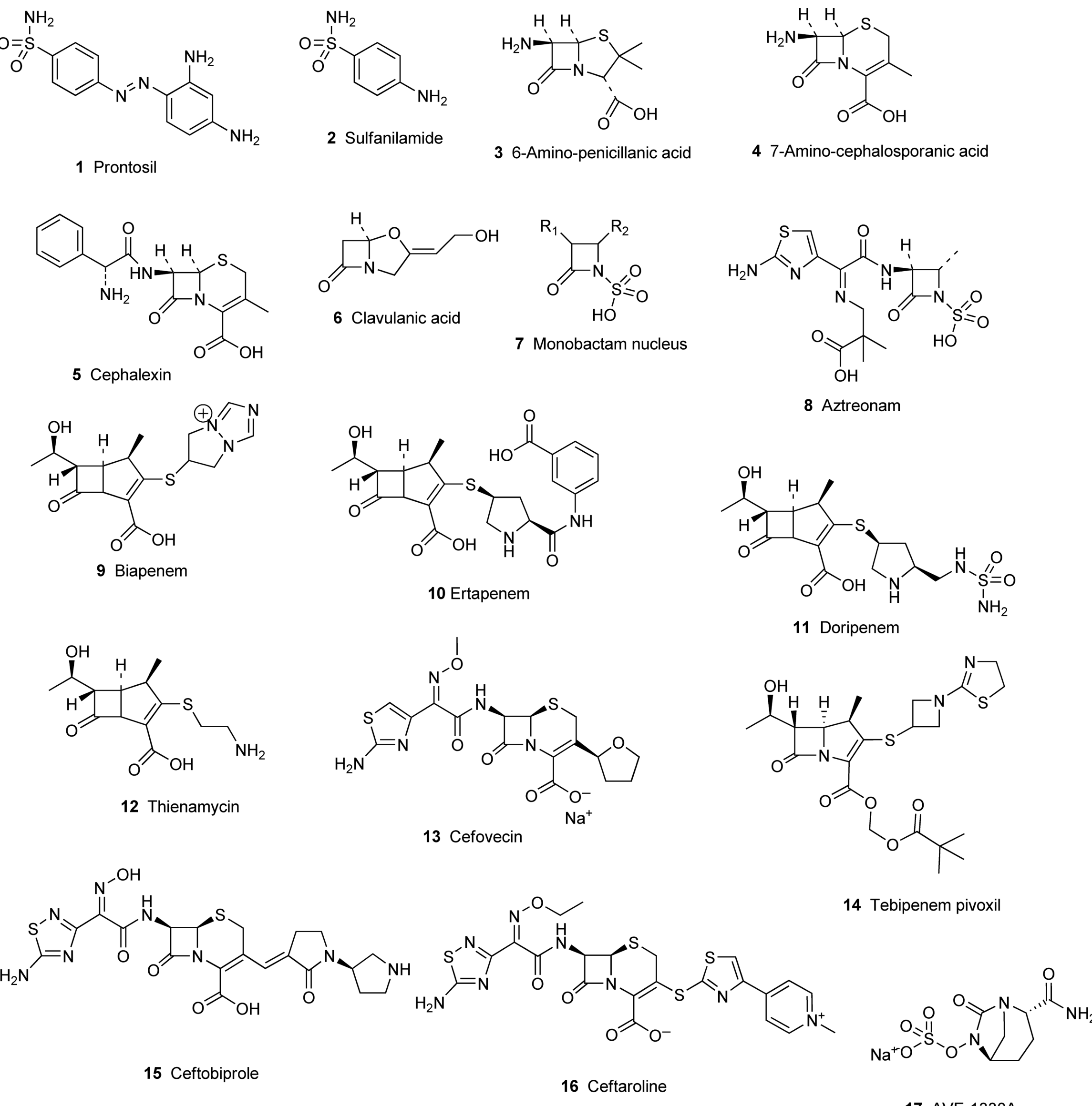

Figure 1 β-Lactams and β-lactamase inhibitors.

Though there were anecdotal reports of scientists such as Tyndall, Roberts, and Pasteur in the 1870s recognizing antagonism between various bacteria, it was the recognition by Fleming in the late 1920s of the activity of penicillin that led ultimately to the well known and documented use of penicillins G and V[22] and streptomycin in the early 1940s by troops of the Western Allies in WWII. What is perhaps not quite as well known is that Russian forces were reported using Gramicidin S as an antibiotic for wound treatments in the same time frame, a product of work by Gause and coworkers in Moscow.[23]

2.19.2.1.1 β-Lactams of all classes

The number of penicillin and cephalosporin-based molecules produced by semisynthesis and total synthesis to date is well in excess of 20 000. Most started with modification of the fermentation product, 6-amino-penicillanic acid (**3, Figure 1**) or the corresponding cephalosporin, 7-amino-cephalosporanic acid (**4, Figure 1**), both of which can be produced by simple chemical or biochemical deacylation from penicillin or cephalosporin C. The above

number is only approximate as a significant proportion of materials from industry were never published, particularly if they had only marginal or no significant activity over those that had previously been reported.

In 1948, the ring-expanded version of penicillin, cephalosporin C, was reported from *Cephalosporium* sp. by Brotzu and its structure determined in 1961 by the Oxford group.[24,25] As with the penicillin nucleus, this ring-expanded molecule also served as the building block (as its 7-aminocephalosporanic acid homologue) for many thousands of cephalosporins, with the first orally active molecule, cephalexin (5, Figure 1), being introduced in 1970. Since that time, a multitude of cephalosporins have been synthesized with the aim of producing molecules that are more resistant to β-lactamases.

In order to give further 'medicinal life' to β-lactams that were no longer resistant to the common β-lactamases, efforts were made in the late 1960s and early 1970s, particularly by Beecham (now part of GlaxoSmithKline (GSK)) and Pfizer, to find molecules that would have similar pharmacokinetics to the β-lactams but would inhibit the 'regular' β-lactamases that were part of the pathogenic microbe's defense systems. Beecham discovered the clavulanate family with clavulanic acid (6, Figure 1) being incorporated into the combination known as Augmentin, a 1:1 mixture of amoxicillin and clavulanic acid (6, Figure 1) launched in 1981, thus extending the franchise of this particular β-lactam well beyond its original patent date.

Along with the search for the β-lactamase inhibitors, efforts were underway to produce the simplest β-lactam, the monobactam. Following many years of unsuccessful research at major pharmaceutical houses, predominately in the synthetic areas, in 1981 Imada *et al.*[26] and a Squibb group led by Sykes,[27] both reported the same basic monobactam nucleus (7, Figure 1). What is important to realize is that no synthetic route attempted before the discoveries of these NPs involved stabilizing the ring by addition of a sulfonyl group to the lactam nitrogen. Since that time, a significant number of variations upon that theme have been placed into clinical trials and in some cases, such as Aztreonam (8, Figure 1), into commerce. Recently, this compound as the lysinate salt, has been submitted for approval in the European Union (EU) and the United States for the inhalation treatment of *Pseudomonas aeruginosa* in cystic fibrosis under an orphan drug category.

That these base structures and others discovered after the early 1940s are still valid as scaffolds upon which to base drugs is shown by the following data on current compounds. Since 2000, three penems, biapenem (9, Figure 1), ertapenem (10, Figure 1), and doripenem (11, Figure 1), which though produced synthetically, are modeled on the NP thienamycin (12, Figure 1), and one cephalosporin, cefovecin (13, Figure 1), a veterinary drug, have been approved for commerce. Currently, there is one penem, tebipenem pivoxil (14, Figure 1), which is effectively a prodrug ester of the ex-Wyeth penem, tebipenem, and one cephalosporin, ceftobiprole (15, Figure 1), at the preregistration stage.

In addition to these, the lesson of Augmentin has been well learned since Forest Pharmaceuticals recently announced that the cephalosporin ceftaroline (16, Figure 1), which is currently in phase III clinical trials, has been combined with Novexel's synthetic β-lactamase inhibitor,[28] NXL-104 (AVE-1330A, 17; Figure 1), and has entered phase I trials.

2.19.2.1.2 Tetracycline derivatives

Even though the base molecule or its better known chloro-derivative, aureomycin, and later the dimethyl amino derivative, doxycycline, have been stalwart members of the physician's armamentarium for between 40 and 50 years, in 2005, Wyeth had the glycyl derivative of a modified doxycycline molecule, tigecycline (18, Figure 2), approved for complicated skin and soft tissue infections; it now has been reported to have broad-spectrum activity, including both Gram-positive and Gram-negative bacteria and methicillin-resistant

N N H H OH H N O N H NH2 OH O OH OH O O

18 Tigecycline

Figure 2 Tetracyclines.

Staphylococcus aureus (MRSA). Thus, by what are effectively relatively simple chemical modifications, even very old base structures can have a new lease on life and provide activity against clinically important infections.

2.19.2.1.3 Lipopeptide antibacterials

That directly searching for novel antibiotics from microbes is still a viable method in certain cases can be seen from the data on daptomycin (**19, Figure 3**), which, although having a somewhat chequered career in moving from Lilly to Cubist, was developed by Cubist and approved in 2003 by the Food and Drug Administration (FDA) as a Gram-positive active antibiotic now known to act on potassium channels. By use of modern genetic techniques coupled to chemistry, it is now possible to produce biosynthetically modified scaffolds (the so-called 'nonnatural natural products') that widen the chemical universe for antibiotic discovery. Using such techniques, Baltz' group at Cubist have recently published their results, which included the production of one variant that had activity against an *Escherichia coli* strain with an outer membrane modification that rendered it more permeable.[29] This paper should be read in conjunction with their earlier review in 2005 covering both daptomycin and earlier lipopeptides.[30]

Another rather old antibiotic that is still being optimized for use is ramoplanin, which as currently utilized is a lipopeptide antibiotic complex, consisting of small amounts of factors A1, A′1, A′2, A3, and A′3[31,32] and factor A2 ('ramoplanin') (**20, Figure 3**) isolated from *Actinoplanes* sp. ATCC33076. Factor A2 is the major component of the complex and is being evaluated in phase III trials by Oscient Pharmaceuticals for the treatment of *Clostridium difficile*-associated diarrhoea (CDAD).[33,34] Ramoplanin (**20, Figure 3**) exerts its antibacterial activity by binding to the peptidoglycan intermediate Lipid II (C_{35}-MurNAc-peptide-GlcNAc) rather than to the vancomycin tripeptide, thus disrupting bacterial cell wall synthesis.[32,35,36] The compound has received orphan drug status in the EU for the treatment of vancomycin-resistant *Enterococci* (VRE) but no trials are yet reported.

2.19.2.1.4 Glycopeptide antibacterials

In a similar fashion, even vancomycin, which was first approved in 1955, is still the prototype for variations around the same mechanism of action. In most cases, the compounds are semisynthetic modifications of the same structural class (glycopeptides) as the prototype, thus following in the 'chemical footsteps' of the β-lactams. Currently, there are a number of such molecules in clinical trials with three semisynthetic glycopeptides, oritavancin (**21, Figure 4**), dalbavancin (**22, Figure 4**), and telavancin (**23, Figure 4**) all in preregistration status with the FDA. In all cases, their antibacterial mechanism is through inhibition of cell wall production, initially via the vancomycin target though the exact mechanisms can vary with the individual agent. Thus in the case of oritavancin, it would appear from recent data, that the agent is comparable to vancomycin in its inhibition of transglycosylation, but more effective as a transpeptidation inhibitor.[37] As mentioned above, all are semisynthetic derivatives of vancomycin-like natural products, with oritavancin being a modified chloroeremomycin (a vancomycin analogue), dalbavancin being based on the teicoplanin relative, B0-A40926, and telavancin (TD-6424) is directly based on chemical modification of vancomycin.[38]

That one may combine the characteristics of two separate agents working at different targets within the same basic biological area is shown by the work of Theravance (also the originator of telavancin), who have successfully combined a cephalosporin with vancomycin itself to produce TD-1792 (**24, Figure 4**), which is currently in phase II trials against complicated skin and soft tissue infections. Thus two old antibiotic classes can produce novel agents, underscoring the possibilities of reworking older structures.

2.19.2.1.5 Macrolidic antibiotics

Following on the track of novel modifications of old structures, since 2000 there have been three molecules based on the erythromycin molecule that have either been approved (telithromycin in 2001, **25; Figure 5**), or entered clinical trials, cethromycin (ABT-773) (**26, Figure 5**), and EDP-420 (EP-013420, S-013420) (**27, Figure 5**). Cethromycin (**26, Figure 5**) is currently in phase III trials against community acquired pneumonia (CAP) and is being evaluated as an antianthrax agent (and against other biodefense targets) by the National Institute of Allergy and Infectious Diseases (NIAID) and the US Army. Another modification of the base erythromycin structure is the 'bicyclolide' EDP-420 (**27, Figure 5**), a novel, bridged bicyclic derivative originally designed by Enanta Pharmaceuticals,[39,40] currently in phase II trials for treatment of CAP by both

19 Daptomycin

20 Ramoplanin A2

Figure 3 Lipopetide antibacterials.

Figure 4 Glycopeptide antibacterials.

Enanta and Shionogi. Interestingly, this molecule is also quite active in a murine model of *Mycobacterium avium*, a common infection in immunosuppressed patients,[41] which may well expand its usage in future trials.

2.19.2.1.6 Pleuromutilin derivatives

Demonstrating yet again that older antibiotic structures have significant validity for today's diseases, GSK received approval in 2007 for a modified pleuromutilin, retapamulin (**28**, **Figure 6**), for the treatment of impetigo in pediatric patients. The base structure, pleuromutilin (**29**, **Figure 6**), was first reported in 1951 from the basidiomycetes *Pleurotus mutilis* (FR.) Sacc and *Pleurotus passeckerianus* Pilat.[42] In the mid-1970s a significant amount of work was reported on the use of derivatives of the base molecule as veterinary antibiotics;[43] thus, the subsequent utilization of the base molecule as a source of human antibiotics is very

25 Telithromycin

26 Cethromycin

27 EDP-420

Figure 5 Macrolidic antibiotics.

28 Retapamulin

29 Pleuromutilin

Figure 6 Pleuromutilin antibiotics.

reminiscent of the work that led to the approval of Synercid in the late 1990s, as the base molecules in that case were also used extensively in veterinary applications.

It is possible that a number of human use antibiotics based on this 'elderly' structure will enter later human trials. Currently, there are four 'mutulins' in phase I clinical trials all against Gram-positive infections, two from GSK under the code numbers 565154 and 742510 for oral use and two, BC-3205 and BC-7013 from Nabriva in Vienna, Austria, with the former for oral use and the latter for topical use. As yet, their structures have not been released.

2.19.2.1.7 New screens and novel agents from old sources

Although we have shown how medicinal chemists have taken older structures and modified them to produce novel agents based on those scaffolds, the use of novel screens and old (meaning stockpiled) microbial extracts is

Figure 7 Novel agents from old sources.

also a very valid way of discovering new natural product scaffolds with potential. Perhaps the best example reported in the last few years is the work by Merck scientists in Rahway, NJ. Utilizing a very clever screen looking for selective inhibition of bacterial cellular lipid biosynthesis, in 2006, the group led by Singh[44,45] reported the identification of platensimycin (**30, Figure 7**), a representative of a previously unreported structural class, as a specific inhibitor of the FabF/B condensing enzymes, with activity against MRSA and vancomycin-resistant enterococci. Over the last 2 years, significant numbers of reports have been published demonstrating that once a novel scaffold has been identified, synthetic chemists will rapidly devise novel synthetic methods to produce both the original structure and be able to proceed toward relatively easy modifications as a result of the synthetic designs.[46–49]

Subsequent to the report of platensimycin, the same group reported the identification of another inhibitor of the same basic target, also from *Spirulina platensis*, but this time with a subtly different structure that they named platencin (**31, Figure 7**).[50,51] To date, no synthetic methods have yet been published, but they will not be long in appearing. However, both the biosynthetic pathway to platensimycin[52] and the chemistry of the compound have been published,[53] and it was reported at the 2007 Society of Industrial Microbiology (SIM) Meeting that the genetic pathway to the same molecule has been cloned from the producing organism (B. Chen, personal communication). As a result of these discoveries, this particular pathway has now begun to be of interest to a series of investigators with an excellent short review just published by Wright and Reynolds that is worth consulting.[54]

2.19.2.2 Antifungal Antibiotics

2.19.2.2.1 Introduction to NP-derived antifungal antibiotics

Since fungi are eukaryotes, the number of potential targets where there are significant differences between the microbe and its host (the human or animal/plant) are much smaller than those in bacteria where there are intrinsic differences in primary metabolic processes, and also in the architecture of their cell walls and membranes. In general, the valid fungal targets are the cell wall components leading to glucans or chitins, the biosynthesis of some of the membrane sterol precursors, and utilization of subtle differences in membrane structure.

Although a very considerable amount of time and effort was expended in the early days of antibiotic discovery, only three general use antifungal agents entered clinical practice as a result. Perhaps the best known is the heptaene polyene, amphotericin B (**32, Figure 8**), originally isolated from *Streptomycetes nodosus* and reported in 1956, but whose full structure was not known until 1970 when it was determined by X-ray crystallography.[55] The absolute configuration was determined using the iodo-derivative and by mass spectroscopy,[56] with a recent review giving the highlights of the chemistry around the compound.[57]

Though many polyenes with varying numbers of conjugated double bonds have been reported since those early days, only the first identified (in 1950) of this general structural class, the tetraene nystatin (**33, Figure 8**), has gone into general clinical use, and like amphotericin B (**32, Figure 8**), its primary indication is for candidiasis. It was first reported from *Streptomyces noursei* and, as with amphotericin, its formal structure was reported in the 1970 time frame by two groups, one using classical chemical degradation plus proton nuclear magnetic resonance (NMR)[58]

32 Amphotericin B

33 Nystatin A1

34 Griseofulvin

35 5-Fluorocytosine

36 5-Fluorouracil

37 Caspofungin

38 Micafungin

39 Anidulafungin

40 Aminocandin

41 SPK-843

Figure 8 Antifungals.

and the other via mass spectroscopy.[59] Further confirmation of the proposed hemiketal structures of amphotericin B (**32, Figure 8**) and nystatin (**33, Figure 8**) was published by the Rinehart laboratory in 1976.[60]

Probably the first clinically used antifungal NP, launched in 1958 but originally reported in 1939, was griseofulvin (**34, Figure 8**), whose nonpolyene structure was defined in a series of papers in 1952 using classical techniques.[61] Even today, almost 70 years after it was first described, griseofulvin (**34, Figure 8**) is still in clinical use against dermatophytes; this is the only class of fungi that it is active against and long-term treatment is necessary due to its insolubility.

One other NP-derived molecule that is also in clinical use predominately against yeasts is the modified pyrimidine nucleoside, 5-fluorocytosine (flucytosine) (**35, Figure 8**), which although made synthetically, can be considered to be derived from a NP. Following transport into *Candida* or *Cryptococcus* via a cytosine permease, 5-fluorocytosine (**35**) undergoes deamination yielding 5-fluorouracil (**36, Figure 8**), which interferes with RNA and DNA metabolism. This is an example of fungal cell selectivity as a result of the lack or very low levels of cytosine deaminase in mammalian cells.

2.19.2.2.2 Current status of NP-derived antifungal antibiotics

Since 2000, there have been three NP-derived antifungal drugs from the echinocandin/pneumocandin class of glucan inhibitors class approved as human use agents.[62,63] In temporal order, these were caspofungin (2001, Merck) (**37, Figure 8**), which recently has been shown to function successfully in both invasive candidiasis and in candidaemia,[64] micafungin (2002, Astellas) (**38, Figure 8**), which is currently in clinical trials for pediatric disease,[65] and anidulafungin (2006, Pfizer) (**39, Figure 8**).[66,67] Finally, another echinocandin, aminocandin (HMR-3270) (**40, Figure 8**), a semisynthetic derivative of deoxymulundocandin, is currently in phase I clinical trials with phase II reported as being scheduled.[68]

Although the heptaene polyene SPK-843 (**41, Figure 8**) was in phase II clinical trials as an antifungal agent, it was reported by Kaken as being discontinued in late 2007. Even close to 70 years after the introduction of amphotericin, the basic polyene scaffold is still being used as the basis for new agents, in spite of the synthetic azoles in current use.

2.19.2.3 Antiparasitic Antibiotics (Except Antimalarials)

2.19.2.3.1 Strongyloidiasis and onchocerciasis

Analogous to the derivation of Synercid from a veterinary antibiotic, another famous example of the use of a modified veterinary product in man as an antiparasitic is the oral use of ivermectin (Mectizan, Stromectol) (**42a** (80%), **42b** (20%); **Figure 9**) in the treatment of strongyloidiasis, onchocerciasis (river blindness), and for the treatment of filariasis. In addition to these uses (predominately in Africa), in 2006 Banyu received approval of the product in Japan for the treatment of scabies.[69]

2.19.2.3.2 Other antiparasitics: Current status

Fumagillin (**43a, Figure 9**) was isolated from *Aspergillus fumigatus* in 1949 and used shortly after its discovery to treat intestinal amebiasis. It was registered by Sanofi-Aventis in France in 2006 (Flisint) for the treatment of intestinal microsporidiosis caused by chronic *Enterocytozoon bieneusi* infection in immunocompromised patients.[70] The antimicrosporidiosis and angiogenic activities of fumagillin and related compounds such as TNP-470 (**43b, Figure 9**) are thought to be due to their strong methionine aminopeptidase 2 (MetAP2) binding, and it has also been shown with zebra fish models that inhibition of MetAP-2 blocks noncanonical *Wnt* signaling, a process that plays a critical role in cell differentiation, development, and tumorigenesis.[71–73]

2.19.2.4 Antiviral Agents

2.19.2.4.1 Introduction to NP-derived antiviral agents

It can be argued quite successfully (and has been a number of times) that the derivation of the nucleoside-based antiviral agents can be traced back to the time frame 1950–56, when Bergmann and Feeney[74,75] and Bergmann and Burke[76] reported on two compounds that they had isolated from marine sponges, spongouridine (**44, Figure 10**) and spongothymidine (**45, Figure 10**). What was significant about these materials was that

42a Ivermectin B1a

42b Ivermectin B1b

43a Fumagillin

43b TNP-470

Figure 9 Antiparasitics.

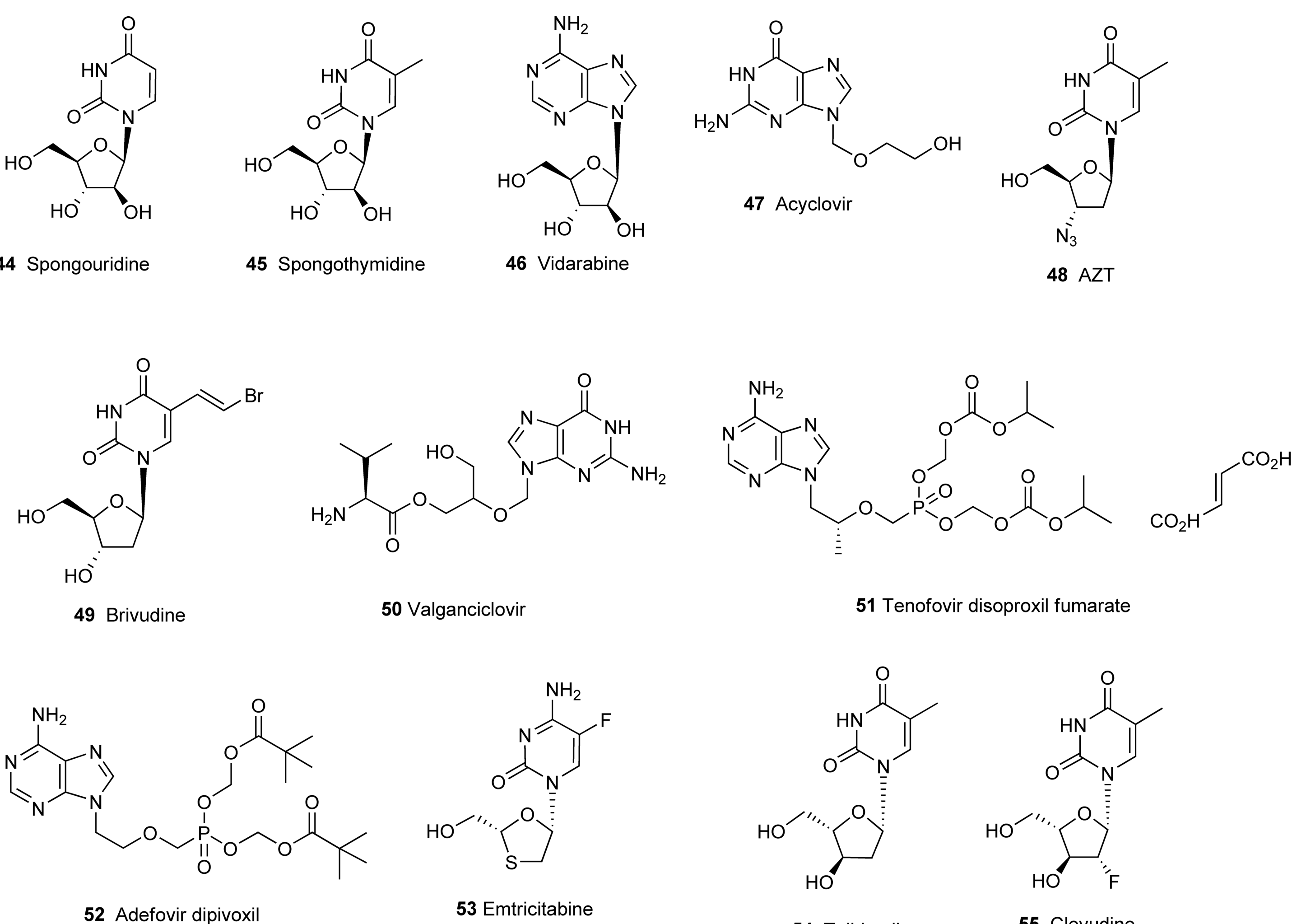

Figure 10 Antivirals.

they demonstrated, for the first time, that naturally occurring nucleosides with biological activity could be found containing sugars other than ribose or deoxyribose. These two compounds can be thought of as the prototypes of all of the modified nucleoside analogues made by chemists that have crossed the antiviral and antitumor stages since then.

Once it was realized that biological systems would recognize the base and not pay too much attention to the sugar moiety, chemists began to substitute the 'regular pentoses' with acyclic entities, and with cyclic sugars with unusual substituents. These experiments led to a vast number of derivatives that were tested extensively as antiviral and antitumor agents over the next 30+ years. Suckling, in a 1991 review,[77] showed how such structures evolved in the (then) Wellcome laboratories, leading to AZT and incidentally to Nobel Prizes for Hitchens and Elion, though no direct mention was made of the original arabinose-containing leads from natural sources.

Showing that 'Mother Nature' may follow chemists rather than the reverse, or conversely that it was always there but the natural products chemists were 'slow off the mark', arabinosyladenine (Ara-A or Vidarabine, **46, Figure 10**) was synthesized in 1960 as a potential antitumor agent[78] but was later produced by fermentation[79] of *Streptomyces antibioticus* NRRL3238 and isolated, together with spongouridine, from a Mediterranean gorgonian (*Eunicella cavolini*) in 1984.[80]

Building on from these original discoveries, medicinal chemists over the next 40+ years made a very large number of 'substituted nucleosides' varying the base and the sugar moieties, including molecules that were acyclic, leading to the very well-known antiviral agents, acyclovir (**47, Figure 10**) and its later prodrug derivatives and AZT (**48, Figure 10**).

2.19.2.4.2 Current antiviral agents

Although a very significant number of antiviral vaccines have either been approved or are in clinical trials for a variety of viral diseases, small molecules based on 'modified nucleosides' are still being approved by either the FDA or the European Medicines Agency (EMEA) (EU). As in earlier days, agents originally approved as antiviral agents may later be shown to have potential utility as antitumor agents.

Since 2000, seven such agents have been approved for antiviral treatments covering anti-HIV, hepatitis B, and cytomegalovirus (CMV). In 2001, brivudine (**49, Figure 10**) was approved as an antiherpes drug and is currently in phase II trials for pancreatic cancer in conjunction with gemcitabine. In the same year, valganciclovir hydrochloride (**50, Figure 10**), a synthetic guanine derivative, was launched by Roche in a tablet formulation for the oral treatment of CMV retinitis in patients with acquired immunodeficiency syndrome (AIDS), and was later approved in 2003 for the treatment of CMV retinitis and CMV infection in transplant patients.

Also, 2001 was the year that tenofovir disoproxil fumarate (**51, Figure 10**), a prodrug of tenofovir, was approved for treatment of HIV, subsequently being preregistered in the United States for treatment of hepatitis B. What is of import is that this compound is unique in antiviral therapy since it is part of approved two drug (tenofovir disoproxil fumarate/emtricitabine; Truvada) and three drug (tenofovir disoproxil fumarate/emtricitabine/efavirenz; Atripla) fixed dose combination therapies for treatment of HIV. Adefovir dipivoxil (**52, Figure 10**), an acyclic AMP analogue, was launched in 2002 as an antihepatitis B agent, though originally tested as an anti-HIV agent, and the following year, 2003, emtricitabine (**53, Figure 10**) was launched as an anti-HIV agent and is now in phase III trials as an antihepatitis B agent. In the past 2 years, telbivudine (**54, Figure 10**) was launched by Idenix and Novartis in 2006 as an antihepatitis B drug functioning as a DNA polymerase inhibitor, and in 2007 Eisai launched clevudine (**55, Figure 10**) in Korea as a treatment for hepatitis B.

Thus, even 50+ years after Bergmann's discovery of bioactive arabinose nucleosides, small molecules synthesized as a result of his discoveries are still in clinical use and in clinical trials for treatment of viral diseases.

2.19.3 Anticholestemics

2.19.3.1 Introduction to NP-Derived Anticholestemics

In Section 2.19.2 we demonstrated the invaluable role that natural products have played in the discovery and development of a wide range of anti-infective drugs, which are essential components of the physicians' armamentarium. In this section we wish to highlight how nature, once more, has provided indispensable

models for the development of an important class of drugs for the treatment of serious ailments afflicting populations mainly residing in the more industrialized nations.

The physical blockage of the arteries by plaques of cholesterol/lipoproteins (atherosclerotic plaque) is a major cause of elevated blood pressure. Since humans usually synthesize about 50% of their cholesterol with the rest coming from diet, if the synthesis can be inhibited, a reduction in overall cholesterol levels may reduce its deleterious effects.

A potential site for inhibition of cholesterol biosynthesis in eukaryotes (both fungi and higher organisms) is at the rate limiting step in the system, the reduction of hydroxymethylglutaryl coenzyme A (HMG-CoA) to produce mevalonic acid. By using fungal fermentation broths as the source of NPs and measuring the inhibition of sterol production, Endo first reported the inhibitory activity of a fungal metabolite, mevastatin (**56, Figure 11**), in 1975.[81] Mevastatin (as compactin) was reported very shortly thereafter by Brown *et al.*[82] as an antifungal agent and was shown to be a competitive inhibitor of the enzyme with K_i's in the nanomolar range but was not further developed due to toxicity. Endo, using a similar assay, reported the isolation of the 7-methyl derivative as monacolin K (mevinolin, now known as lovastatin) (**57, Figure 11**) from *Monascus ruber*.[83,84] This agent was patented in Japan but without a structure. Concomitantly, workers at Merck in the United States discovered the same material from *Aspergillus terreus*, using an isolated HMG-CoA reductase assay and microbial broths as their source of agents (having tried their synthetic library to no avail). After submission of both structure and findings to the US Patent Office, a US patent was issued in late 1980 and lovastatin (Mevacor) (**57, Figure 11**) subsequently became the first commercialized HMG-CoA reductase inhibitor in 1987.[85] Further work by Sankyo and Merck led to the entry of two more slightly modified versions. Firstly, the 2-methylbutanoate side-chain of lovastatin (**57, Figure 11**) was converted to 2,2-dimethyl butanoate, which was launched in 1988 as simvastatin (Zocor) (**58, Figure 11**) by Merck. Secondly, biotransformation of mevastatin led to the production of a lactone ring-opened, 7-hydroxy derivative, pravastatin (**59, Figure 11**), which was launched in 1989 by Sankyo and subsequently licensed to Bristol-Myers Squibb.

Following the success of these agents, other small and large pharmaceutical companies used the information from mevastatin (**56, Figure 11**) and lovastatin (**57, Figure 11**) (effectively the NP's 'warhead plus a varying lipophilic attachment') to develop a series of molecules, some of which have been approved for use. These include the best-selling drug of all time, atorvastatin (Lipitor) (**60, Figure 11**), whose sales in 2005, 2006, 2007, and 2008 were US$12.2, US$12.9, US$12.7, and US$13.4 billion worldwide, respectively. There are three further agents similar in concept to atorvastin that either have the lactonized or ring-opened form of the 'warhead from nature' coupled to different lipophilic entities in clinical use today.

Over the years, a number of similar molecules have entered clinical trials but are currently not under development, with only one, crilvastatin (**61, Figure 11**), not being based on the mevinolin warhead.

2.19.3.2 Current Status of NP-Derived Anticholestemics

2.19.3.2.1 Combination therapies

A very interesting combination compound was approved in 2004 for the treatment of this disease complex. It was interesting from at least two aspects, one commercial and the other scientific. The commercial aspect was the joining together of Schering-Plough with Merck to develop and then commercialize the Niemann–Pick C1-like protein 1 inhibitor, ezetimibe (**62, Figure 11**), itself based on the monobactam nucleus though not designed as an antibiotic, and the then generic Merck compound simvastatin (**58, Figure 11**) in a fixed ratio medication.

Inspection of the Prous Integrity database shows that this method of linking a cholesterol synthesis inhibitor and another agent(s) with a different mechanism of action but related to lipid metabolism is definitely 'alive and well' as currently there are at least four combinations of different drugs at various stages of clinical trials. In phase III, Merck have two different sets of drugs with the first being MK-524A/simvastatin where MK-524A[86] (laropiprant, **63, Figure 11**) is combined with niacin as well as simvastatin. The role of MK-524A is as an inhibitor of the niacin-induced increase in plasma levels of prostaglandin D2 (PGD2), due to 'flushing from the skin of the vasodilatory prostanoid after niacin treatment'. Thus if an inhibitor of either of the PGD2 receptors DP1 or DP2 could be identified, then a combination of such an agent plus niacin and a HMG-CoA reductase inhibitor might well be efficacious in raising high-density lipoprotein (HDL)-C and lowering triglycerides.

56 Mevastatin

57 Lovastatin

58 Simvastatin

59 Pravastatin

60 Atorvastatin

61 Crilvastatin

62 Ezetimibe

63 Laropiprant (MK-524A)

Figure 11 Anticholestemics.

Merck also have the combination of atorvastin (60, Figure 11) and ezetimibe (62, Figure 11) in phase II clinical trials, an interesting adjunct to Vytorin.

In addition to these newer agents, there are currently three 'fibrate-based' combinations with HMG-CoA reductase inhibitors in clinical trials. In phase III, Sciele has fenofibrate/pravastatin combination and Astra-Zeneca and Abbott have rosuvastatin/choline fenofibrate (ABT335), while in phase II, Life Cycle Pharma have atorvastatin/fenofibrate.

2.19.3.2.2 HMG-CoA reductase inhibitors

Finally, compounds based on the original warhead were still being tested until recently, with PF-3052334 from Pfizer now announced as being discontinued after phase I trials, and a modification of the pitavastatin structure (NK-104-LH) was in phase II trials in late 2007 with Kowa though no formal identified structure had been published.

Thus even 30+ years after the first identification of natural products with HMG-CoA reductase inhibitory activity and their use as lead scaffolds into semisynthetic variants, these agents are still being investigated as compounds in their own right, as partners with other agents with different pharmacologic functions and also in experimental treatments in quite different areas, including cancer and neuropharmacology.

2.19.4 Microbial Products in Cancer

2.19.4.1 Introduction

The use of natural products from microbes in cancer is extremely well described in a series of chapters in a recent book[87] so we will not be describing any of the classes covered in that compendium except for an update on the epothilones and the halichondrin B-derived E7389. Instead, we will show how over the past few years, the actual source(s) of many agents in clinical use, in clinical trials, or in preclinical investigations directed toward clinical trials, are now thought to involve microbes of one 'type' or another, rather than to be solely the product of the organism(s) from which they were first isolated and identified. We will describe selected compounds demonstrating some directly from microbes, some now known to be from microbes, and some where microbes could well be involved.

One may well ask why is the identification of the actual source (so) important? The answer is usually that if the source is microbial in nature, then it may well aid in the production of an important lead compound or even of a clinical candidate via fermentation, rather than requiring heroic measures to collect and process massive amounts of raw material from a source that may itself be scarce or endangered, or both.

2.19.4.1.1 Epothilones

With the identification of the myxobacterial products epothilones A and B (64, 65; Figure 12) as tubulin stabilizers (a similar mechanism to that of paclitaxel) by Bollag *et al.*[88] in 1995 came a veritable avalanche of modifications of the base structure by chemical, biochemical, and even genomic means in order to further explore the utility of the base skeleton. This culminated in the approval in October 2007 by the FDA of the semisynthetic epothilone, 16-aza-epothilone B, known generically as ixabepilone (66, Figure 12; Ixempra) for treatment of breast cancer.

Currently, there are four other epothilones in active development as anticancer agents listed in Integrity. The natural product epothilone B (65, Figure 12; patupilone) is in phase III trials in conjunction with Novartis and the original discoverers. A totally synthetic derivative, though very close to the base skeleton, Sagopilone (ZK-EPO, 67; Figure 12) is in phase II trials under Bayer-Schering, with much fuller details of this and the opportunities for synthesis of other agents being given in three recent reviews that should be consulted for the finer details.[89–91] Finally, there are two agents derived from work originating in Danishefsky's laboratory at Memorial Sloan-Kettering being developed by Kosan. The first is (*E*)-9,10-didehydroepothilone D (68, Figure 12), currently in phase II, and the second is isoxazolefludelone[92] (69, Figure 12), currently in preclinical evaluation but scheduled for an Investigational New Drug Application (IND) filing in early 2009. Now that the genetic sequence of the original producing organism has been reported, we can expect not just modifications of the base skeleton, but also a number of different secondary metabolites from the same microbe may well be reported in the literature in the near future.[93–95]

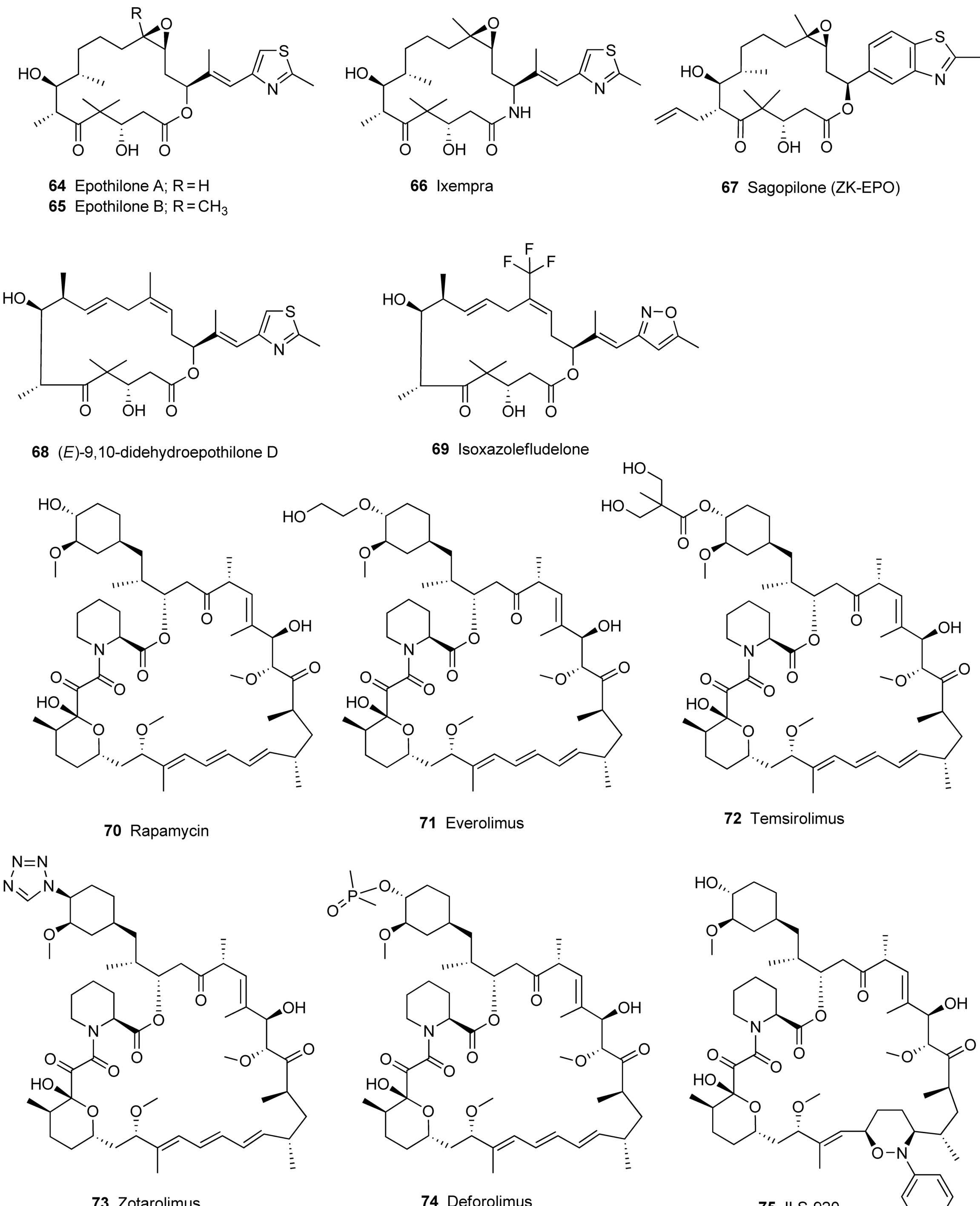

Figure 12 Microbial products in cancer.

2.19.4.1.2 Rapamycin and derivatives

Though not solely active in cancer, the molecules based on the rapamycin skeleton (70, Figure 12) show the capability of a single skeleton to produce compounds that are active in a variety of pharmacologic areas, including cancer. Initially, modifications were at one site and led to four clinical drugs, with the rapamycin base

molecule being approved as sirolimus in 1999 initially as an immunosuppressive and now in clinical trials in various cancers. Similarly, everolimus (**71, Figure 12**) was launched in 2004 as an immunosuppressive and it is also in clinical trials for cancers in both the EU and Japan. The third variation temsirolimus (CCI-779, **72; Figure 12**) was approved as a treatment for renal carcinoma in the United States in 2007, and in another method of drug use and delivery a fourth, zotarolimus (**73, Figure 12**), was launched in the United States in 2005 for treatment of restenosis as part of a drug-eluting stent.

Currently, deforolimus (A23573, **74; Figure 12**) is in phase III clinical trials for cancer, and two 'prodrugs' of rapamycin, Abraxis' ABI-009 (which is a nanoparticle encapsulated formulation of rapamycin) and Isotechnika's TAFA-93 (structure not yet published) are in phase I cancer trials. All of these are either the base molecule or have been modified at only one site, the C-43 alcoholic hydroxyl group that avoids both the FKBP-12 and the target of rapamycin (TOR) binding sites, since modifications in other areas were thought to negate the basic biological activity of this molecule.[96]

There is also one rapamycin derivative that is modified in the ring structure that is currently in phase 0 clinical trials, the Wyeth compound known as ILS-920 (**75, Figure 12**). ILS-920 has a modification in the triene portion of the molecule designed to disrupt the mammalian target of rapamycin (mTOR) binding, and appears to have a different target as it is a nonimmunosuppressive, neurotrophic rapamycin analogue that demonstrated over a 200-fold higher binding affinity for FKBP52 over FKBP12, promoted neuronal survival and outgrowth *in vitro*, and bound to the β1 subunit of L-type calcium channels (CACNB1).[97]

2.19.4.1.3 Scaffolds from marine microbes

2.19.4.1.3(i) Cryptophycins These compounds were reported from two nonmarine blue-green algae (cyanobacteria), initially by Merck in 1990 from a lichen-associated *Nostoc* species (ATCC 53789) and by Moore's group at the University of Hawaii who identified the same compound from the cyanophyte, *Nostoc* sp., strain GSV-224. Although the original cryptophycins came from terrestrial cyanophytes, and the clinical candidate (cryptophycin 52, **76; Figure 13**) came from semisynthetic modifications of the natural product, in 1994 Kobayashi *et al.*[98] reported that an acetone extract of the Okinawan sponge, *Dysidea arenaria*, had potent cyctoxicity and on purification, the compound arenastatin A (**77, Figure 13**) subsequently turned out to be identical to cryptophycin 24 (**77, Figure 13**) reported by Moore's group in 1995.[98,99] A later report from the Japanese group[100,101] demonstrated that arenastatin A and synthetic analogues also are tubulin interactive agents similar in activity to the other cryptophycins reported by Moore *et al.*

The University of Hawaii and Wayne State University licensed the natural and synthetic cryptophycin derivatives to the Lilly Company for advanced preclinical and clinical development. This led to the selection of cryptophycin 52 (LY355703) (**76, Figure 13**) as a phase I clinical candidate in the mid-1990s, with a single publication[102] in late 2002 giving the phase I and pharmacological results from a variety of schedules, with an intermittent schedule being chosen for phase II studies.

The routes, both chemical and pharmacological, leading to the choice of this particular derivative were described by Shih and Teicher[103] of the Lilly Research Laboratories. The compound progressed toward phase II trials but in 2002 cryptophycin 52 (**76, Figure 13**) was withdrawn from trial. A full report on the molecules derived from these studies was given by Al-Anwar and Shih of Lilly Research Laboratories in 2005,[104] and in 2006, a report on activity against platinum-resistant ovarian carcinoma in phase II trials was published.[105]

Recently, there have been some significant reports on the identification of the biosynthetic gene products that led to the production of cryptophycins in the source cyanophytes,[106–109] methods of total synthesis of the base molecules,[110,111] and on synthetic cryptophycin-epothilone hybrids though these compounds had no tubulin activity.[112] However, the original molecules may now have received a new lease on life as they have been licensed to Sanofi-Aventis for further development (G. Patterson, personal communication).

2.19.4.1.3(ii) Dolastatins There was always a potential question with the dolastatins, a group of linear and cyclic peptides originally isolated from *Dolabella auricularia*, as to whether or not they were microbial in origin, as peptides with unusual amino acids had been well documented in the literature as coming from the Cyanophyta. In the past few years, this supposition has been shown to be the fact. Thus in 1998, workers at the Universities of Guam and Hawaii reported the isolation and purification of symplostatin 1(**78, Figure 13**) from the marine cyanobacterium *Symploca hynoides*.[113] This molecule differed from dolastatin 10 (**79, Figure 13**)

76 Cryptophycin 52

77 Arenastatin A, Cryptophycin 24

78 Simplostatin 1

79 Dolastatin 10

80 Dolastatin 16

81 Homodolastatin 16

Figure 13 Scaffolds from marine microbes I.

by the replacement of the isopropyl group by a sec-butyl group on the first N-dimethylated amino acid. Subsequently, in 2001, the same groups reported the direct isolation of dolastatin 10 (79, Figure 13) from another marine cyanobacterium that was known to be grazed on by *D. auricularia*.[114] Dolastatin 10 (79, Figure 13) was in fact isolated from the nudibranch following feeding of the cyanophyte, thus confirming the original hypothesis (V. J. Paul, personal communication).

Subsequently, two further examples of dolastatin-like peptides isolated from different collections of the ubiquitous cyanophyte, *Lyngbya majuscula*, were later reported in the literature, namely, dolastatin 16 (80, Figure 13) from a Madagascan collection by Nogle and Gerwick[115] and homodolastatin 16 (81, Figure 13) from a Kenyan collection by Davies-Coleman *et al.*,[116] further evidence for the microbial source of these peptidic cytotoxins.

2.19.4.1.3(iii) Kahalalide F This cyclic depsipeptide (82, Figure 14) was isolated from the Sacoglossan mollusk, *Elysia rufescens*, following grazing by the mollusk on the macroalga, *Bryopsis* sp. Following isolation and identification, it was discovered that the depsipeptide also occurs in the alga, but on a wet weight basis, the mollusk concentrated the depsipeptides significantly.[117] The compound was licensed to PharmaMar by the University of Hawaii in the 1990s and it is currently in phase II clinical trials. Other variations on the base molecule have been reported from the same genus but a different species of the mollusk, collected in the Indian Ocean rather than the Pacific Ocean,[118] though a later paper implies that the mollusk and/or another symbiotic microbe may also be performing some modification of the base structure as new derivatives were detectable by mass spectroscopy.[119]

The only published information on a microbial involvement in the production of this compound is in a 2005 PCT International Application filed by Hill *et al.*[120] claiming production of Kahalalide F and other derivatives from a *Vibrio* species isolated from *Bryopsis* and also *E. rufesens*, implying that the invertebrate obtains the producing microbe from the alga and then maintains the microbe(s) as symbionts. Thus there is a potential

82 Kahalalide F

83 Salinosporamide A

84 Cinnabaramide A

Figure 14 Scaffolds from marine microbes II.

renewable source of these agents by use of fermentation, though significant modifications are also being made by chemical synthesis of the base molecule and these new compounds are demonstrating activity against infections due to *Fusarium*.[121]

2.19.4.1.3(iv) Salinosporamide A (NPI-0052) This particular compound (**83, Figure 14**), in addition to having an unusual structural motif, is also the first of what may well be a future wave of compounds to enter clinical trials, and without question it was produced by a marine-derived streptomycete of an entirely new genus and species, *Salinispora tropica*. Over the past 20 years or so, there have been many comments and presentations implying that a number of the agents found in marine invertebrates had 'microbe(s) in their background'; such comments have been based on genomic information in the cases of bryostatin and ET743, and on direct isolation of microbes from the *Bryopsis* alga in the case of Kahalalide F. However, it was the thesis of Fenical and Jensen that there were deep sea free-living microbes that could be cultivated and novel agents produced utilizing modifications of methods used for other microbial flora.

Salinoporamide A (**83, Figure 14**) was first reported[122] from a marine streptomycete of a new genus and species named initially as *Salinospora tropica*. The genus name was subsequently changed to *Salinispora* but the compound retained its original name. The structure was reminiscent of the terrestrial bacterial rearrangement product, omuralide, a known proteasome inhibitor and on further testing, that activity was reported for the new compound in the original publication.[122] The compound had an unusual chlorine substitution and within a year or so of the publication, two academic groups had synthesized the base molecule.[123,124] These reports were followed by a synthetic paper from Nereus scientists,[125] and subsequently many groups have reported improved syntheses with an excellent review in 2007 covering almost all of these.[126] However, in addition to providing synthetic methods, Nereus scientists in concert with the now defunct fermentation group at Industrial Research Limited (IRL) in New Zealand, were able to produce the necessary current Good Manufacturing Practices (cGMP) product for clinical trials by fermentation in a saline environment, the first time that this task had been successfully performed on any scale with a marine-sourced microbe. During these runs, a significant number of other salinosporamide derivatives were also isolated and other secondary metabolites were further explored.[127]

In the past year or so, some very interesting papers have been published on both the isolation of similar molecules from a terrestrial microbial source, the cinnabaramides A–G (**84, Figure 14**),[128] and on the genomic aspects of the producing organism.[129,130] In the case of the genomic information, inspection of the sequence of *S. tropica* demonstrates that these marine streptomycetes, as with their terrestrial cousins, have many more 'currently unexpressed' secondary metabolite clusters in their genomes, thus demonstrating that they are as biochemically diverse as the quintessential terrestrial microbe, *Streptomyces coelicolor*, and that the genome also encodes for a very unusual chlorinase that can be substituted by other halogens.[131] What is also of interest for the future is that initial chemogenomic studies on the genus *Salinispora* have indicated that there are species-specific secondary metabolites, as well as metabolites that appear to be produced by all species of this genus so far investigated.[132]

2.19.5 Nonmicrobial Products in Cancer

2.19.5.1 Marine Scaffolds (Halichondrin B-Derived)

There is one agent currently in phase III trials that is a prime example of what can be accomplished by synthetic chemistry based on a marine-derived structure (not yet proven to be microbial in origin but the source may ultimately turn out to involve microbe–sponge interactions). This is the fully synthetic compound known as eribulin (**85, Figure 15**, E7389), which was modeled from the naturally occurring antitubulin compound, halichondrin B (**86, Figure 15**).

It arose from a synthetic *tour de force* by utilizing the synthetic method for halichondrin B (**86, Figure 15**) first reported by Kishi's group in 1992,[133] and the subsequent realization by Kishi that the active part of the molecule resided in the macrolide ring (~600 MW) and not in the 'tail' (the remaining ~400 of the over ~1000 MW). Chemists at the Eisai Research Institute in Massachusetts, working very closely with the Kishi group at Harvard, synthesized over 200 molecules and, in conjunction with the Developmental Therapeutics Program

85 Eribulin; E7389

86 Halichondrin B

Figure 15 Marine scaffolds (nonmicrobe).

(DTP) at the National Cancer Institute (NCI), chose the modified truncated macrocyclic ketone, eribulin (85, Figure 15, E7389), as the candidate compound.

This molecule, like its parent, is a tubulin interactive agent with very potent activity at the nanomolar level in *in vitro* studies[134] and binding at or close to the vinca site from recent modeling studies.[135] Much fuller details of the synthetic and base biological information were published by the leaders of the studies in 2005,[136] and recently a short article covering the basic details but including the latest clinical citations was published by Wang.[137] These two articles should be read by the interested reader for fuller details of the evolution of this compound.

2.19.6 Summary and Future Prospects

We have shown, albeit in abbreviated form, that secondary metabolites from a variety of nominal sources (though probably a significant number are microbial in nature, or have a microbe in their background) are still very relevant as drug molecules, and, perhaps more importantly, as scaffolds from 'privileged structures' upon which to build both semisynthetic and totally synthetic molecules thus expanding the armamentarium of drugs available for treatment of important diseases.

In particular, the necessity for novel agents against infectious diseases is now becoming obvious even to the layman. Hopefully, the modification of older molecules and the discovery of new scaffolds are now on the horizon of small and large pharmaceutical houses, as well as in academia. The potential shown for the discovery of novel secondary metabolites through genomic analyses, where well over a dozen previously unrecognized secondary metabolites appear to be present in each genus/species in the actinomycetes alone, bodes well for the identification of such agents in due course,[130,138–140] and hopefully their ultimate entry into treatment regimens in a number of diseases, and as leads to novel potent molecules via a variety of chemical, biochemical, and genetic manipulations.

87a Zanamivir

87b Oseltamivir

88 Shikimic Acid

Figure 16 Shikimic acid scaffold.

Finally, the recent emergence (2009) of the H1N1 subtype of the influenza A virus as a potential global health threat illustrates the importance of natural products scaffolds in the efficient production of effective drugs. The neuraminidase inhibitors, zanamivir (Relenza, 87a; Figure 16) and oseltamivir (Tamiflu, 87b; Figure 16), are recommended as treatments. Though they were developed through rational drug design utilizing the X-ray crystallographic structure of the neuraminidase on which to model potential inhibitors based on the normal substrates, when it came to synthesis, the natural product shikimic acid (88, Figure 16), isolated from the star anise (*Illicium religiosum*), provided a useful scaffold for the synthesis of Tamiflu.[141] Further information on the derivation of both of these agents can be found by consulting the relevant full papers, rather than the first reports as abstracts of presentations, on the methods used by the original investigators.[142–144]

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Biographical Sketches

David J. Newman is the current chief of the Natural Products Branch (NPB) in the Developmental Therapeutics Program at the National Cancer Institute in Frederick, MD. He was born in Grays, Essex, UK, in 1939. In 1963, he received an M.Sc. in synthetic organic chemistry from the University of Liverpool working under Prof. George Kenner, FRS, on pyrrole and porphyrin syntheses. After spending 18 months a synthetic chemist at Ilford, Ltd., he joined the ARC's Unit of Nitrogen Fixation at the University of London and then Sussex, as a research assistant in metallo-organic chemistry with Prof. J. Chatt, FRS, transferring to the microbial biochemistry group in early 1966 as a graduate student under Prof. John Postgate, FRS, and was awarded a D.Phil. in 1968 for work on microbial electron transport proteins from *Desulfovibrio*. Following a move to the United States in September 1968, he spent 2 years as a postdoc at the Biochemistry Department at the University of Georgia

working on protein sequencing of *Desulfovibrio* ferredoxins, and then in 1970 joined SK&F in Philadelphia as a biological chemist. At SK&F, most work was related to biological chemistry and antibiotic discovery and he left SK&F in 1985 when the antibiotic group was dissolved. For the next 6 years, he worked in marine and microbial discovery programs (Air Products, SeaPharm, and Lederle) and then in 1991, joined the NPB as a chemist responsible for marine and microbial collection programs. He was given the NIH Merit Award in 2003 for this work and following Gordon Cragg's retirement from the position of Chief, NPB, at the end of 2004, he was acting chief until appointed chief in late 2006. He has been the author or coauthor of over 110 papers, reviews, book chapters (and an editor, with Gordon Cragg and David Kingston of *Anticancer Agents from Natural Products*), and holds18 patents.

Gordon M. Cragg completed his undergraduate training in chemistry at Rhodes University, South Africa, and his D.Phil. (organic chemistry) from Oxford University in 1963. After 2 years of postdoctoral research at the University of California, Los Angeles, he returned to South Africa to join the Council for Scientific and Industrial Research. In 1966, he joined Chemistry Department at the University of South Africa, and transferred to the University of Cape Town in 1972. In 1979, he returned to the United States to join the Cancer Research Institute at Arizona State University. In 1985, he moved to the National Cancer Institute (NCI) in Bethesda, MD, and was appointed Chief of the Natural Products Branch in 1989. He retired in December 2004, and is currently serving as an NIH Special Volunteer. His major interests lie in the discovery of novel natural product agents for the treatment of cancer and AIDS, with an emphasis on multidisciplinary and international collaboration. He has given over 100 invited talks at conferences in many countries worldwide, and has been awarded NIH Merit Awards for his contributions to the development of taxol (1991), leadership in establishing international collaborative research in biodiversity and natural products drug discovery (2004), and contributions to developing and teaching NIH technology transfer courses (2004). In 1998–99 he was president of the American Society of Pharmacognosy, and was elected to honorary membership of the Society in 2003. In November 2006, he was awarded the 'William L. Brown Award for Plant Genetic Resources' by Missouri Botanical Garden at a 2-day symposium entitled 'Realizing Nature's Potential: The Once and Future King of Drug Discovery' held in his honor. Missouri Botanical Garden also named a recently discovered Madagascar plant in his honor, *Ludia craggiana*. He has established collaborations between the NCI and organizations in many countries promoting drug discovery from their natural resources. He has published over 150 papers related to these interests.

2.18 Missasigned Structures: Case Examples from the Past Decade

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2.18.1 Introduction

Total structure elucidation of complex natural products has become more straightforward over the past several decades. Contributing factors for unambiguous structure elucidation stem from further advances in the newly developed NMR probes, novel MS technology including ultra-high-resolution MS (HRMS) and tandem MS (MS/MS), and the application of synchrotrons for X-ray structure analysis. Classical structure

Morphine ($C_{17}H_{19}NO_3$)
Isolation: 1806
2D Structure: 1925
3D Structure: 1955

Tetrodotoxin ($C_{11}H_{17}N_3O_8$)
Isolation: 1907
3D Structure: 1964

Penicillin G ($C_{16}H_{18}N_2O_4S$)
Discovery: 1928
Isolation: 1941
Structure elucidation: 1945

Figure 1 Selected examples of challenging structure elucidations on classical natural products from the last century.

elucidation prior to the development of these techniques (around 1960) was quite challenging, and required tremendous effort and time even for small organic molecules (MW < 500). The structures shown in **Figure 1** are classical natural products which illustrate the difficulty of structure elucidation without the techniques mentioned above. Structure elucidation of morphine ($C_{17}H_{19}NO_3$), which is perhaps the first secondary metabolite isolated in pure form, took almost 150 years (isolation: 1806, two-dimensional (2D) structure: 1923, three-dimensional (3D) structure: 1952).[1] The puffer fish toxin, tetrodotoxin ($C_{11}H_{17}N_3O_8$), required more than 50 years for its structure to be fully elucidated (isolation: 1907, 3D structure: 1964).[2] Compared to these two examples, the structure elucidation of the antibiotic penicillin G ($C_{16}H_{18}N_2O_4S$) was relatively fast, but still needed more than 15 years (discovery: 1929, isolation: 1941, 3D structure: 1945).[3] Although it had been suggested that penicillin possessed a β-lactam core, the total structure of penicillin G was directly determined by the application of X-ray structural analysis.[4] The structural motifs of these molecules continue to intrigue chemists, notably the fascinating ring systems.

Modern structure elucidation using one of, or a combination of, the abovementioned analytical techniques has enabled chemists to investigate large and complex biomolecules (MW > 1000). Some outstanding total structure analysis utilizing limited samples in the past 15 years for marine-derived macromolecules are shown in **Figure 2**: (1) polyether toxin, maitotoxin ($C_{164}H_{256}O_{68}S_2Na_2$), (discovery: 1979,[5] 2D structure: 1993,[6] 3D structure: 1996[7,8]), (2) polyketide, amphidinol 3 ($C_{70}H_{118}O_{23}$), (3D structure: 1999,[9] structure revision: 2008[10]), lipopolysaccharide, axinelloside A ($C_{137}H_{219}O_{117}S_{19}Na_{19}$), (3D structure: 2005[11]) and (3) polypeptide, polytheonamide A ($C_{219}H_{376}N_{60}O_{72}S$), (2D structure: 1994,[12] 3D structure plus structure revision[13]). However, it is also true that incorrectly assigned structures for small biomolecules continue to appear in the literature. This issue was emphasized in a recent review, which suggested that more than 300 errors in proposed structures were disclosed from 1990 to early 2004.[14] It is important to learn and analyze the outstanding structure elucidation examples mentioned above. However, considering the number of recently revised structures and stereochemical reassignments, it is more practical and significant to study why structure elucidation goes wrong. This chapter discusses a subset of misassigned molecules derived from marine sources and is composed of incorrect 2D and 3D structures. It should be mentioned that the object of this chapter is not to criticize the structure misassignments based on the original works, but to provide clues to avoid the pitfalls for future structure elucidation works.

Maitotoxin ($C_{164}H_{256}O_{68}S_2Na_2$)
Discovery: 1979
2D Structure: 1993
3D Structure: 1996

Amphidinol 3 ($C_{70}H_{118}O_{23}$)
3D Structure: 1993
Stereochemical reassignment: 2008

Figure 2 (Continued)

Axinelloside A: R = SO_3Na ($C_{137}H_{219}O_{117}S_{19}Na_{19}$)
3D Structure: 2005

Polytheonamide A ($C_{219}H_{376}N_{60}O_{72}S$)
2D Structure: 1994
Structure revision and 3D structure: 2005

Figure 2 Selected marine natural products illustrating the triumph of modern structure determination.

2.18.2 Structure Diversity of Marine Natural Products

Prior to the discussion of the misassigned marine-derived molecules, it is instructive to consider the diversity of marine natural products. The structures collected in **Figure 3** are several marine natural products either revised or possessing incomplete stereochemistry and represent each biosynthetic class. This short list of compounds demonstrates that marine-derived secondary metabolites have characteristic structural diversity when compared with terrestrial-derived molecules. Unique carbon skeletons and functional groups are present in the structures of suvanine,[15,16] ecteinascidine 759B,[17,18] spirastrellolide A,[19–21] and yendolipin[22,23] with a dimethyl guanidinium cation, sulfoxide, bis-spiroketal, and quaternary amine cation, respectively. The characteristic polyketide/peptide side chain of iejimalide A[24,25] is often observed in secondary metabolites found in marine invertebrate-derived metabolites. The structure of haplosamate A[26,27] with a molecular formula (MF) $C_{29}H_{49}Na_2O_{12}PS$ is unique in terms of atom diversity. In other words, these structural moieties contained within marine natural products are often the cause of structure misassignments. In addition, since many marine natural products are isolated in minute amounts, and possess complex and/or remote chiral centers within the molecules, the completion or process of determining correct stereochemical assignments is often very challenging. Some recently reported compounds such as usneoidone[28,29] and batzellaside A[30] have yet to be fully characterized due to the challenges of chiral centers on the aliphatic chains. The correct assignment of natural products is critical to many researchers since potent bioactive marine natural products such as spirastrellolide A,[21] kahalalide F,[31–34] and pericosine A[35,36] often become not only synthetic targets but also pharmaceutical leads and molecular probes to characterize biological functions of proteins and enzymes.

2.18.3 Misassigned Marine Natural Products

By the end of 2008, more than 200 structure revisions for marine natural products had been reported. In the 1970s and early 1980s, the structure revisions were essentially made based on NMR analysis. This was mostly due to the improvement of NMR sensitivity via more powerful magnetic fields and the development of new pulse sequences. Many structure revisions have also arisen from total syntheses since the middle of the 1980s when marine natural products with potent bioactivity and complex carbon skeleton stimulated the synthetic community. In the past decade, GIAO (gauge-independent atomic orbital)[37] methods such as density functional theory (DFT) calculations to estimate quantum mechanical-derived NMR chemical shifts have been conspicuously applied to structure elucidation. This calculation method has proven to be a very useful structure elucidation tool especially to confirm structures with low hydrogen-to-carbon (H/C) ratio cores that make other NMR methods nearly useless. Recently, some structure revisions including TAEMC161[38] (identical to viridiol)[39] and the *Brosimum* allene[40] (identical to mururin C[41]) have resulted from DFT calculations. Described below are case examples of misassigned marine natural products categorized by the types of errors as well as tables that will be beneficial for researchers who encounter the same difficulties of structure elucidation.

2.18.4 Difficulties of Molecular Formula Assignments

Secure structure elucidation begins with obtaining the correct MF. This step is the most important part in structure elucidation since an incorrect MF guides structure elucidation down the wrong path, resulting in misassignment. However, it is sometimes very difficult to finalize the MF based on MS results. All the examples collected in the following section were given an incorrect MF in the original structure. Analysis of the examples categorized the pitfalls into four groups: (1) functional groups, (2) monomeric or dimeric forms, (3) hydroxy or hydroperoxide groups, and (4) diols versus ethers. Each subsection discusses these groups individually using several examples.

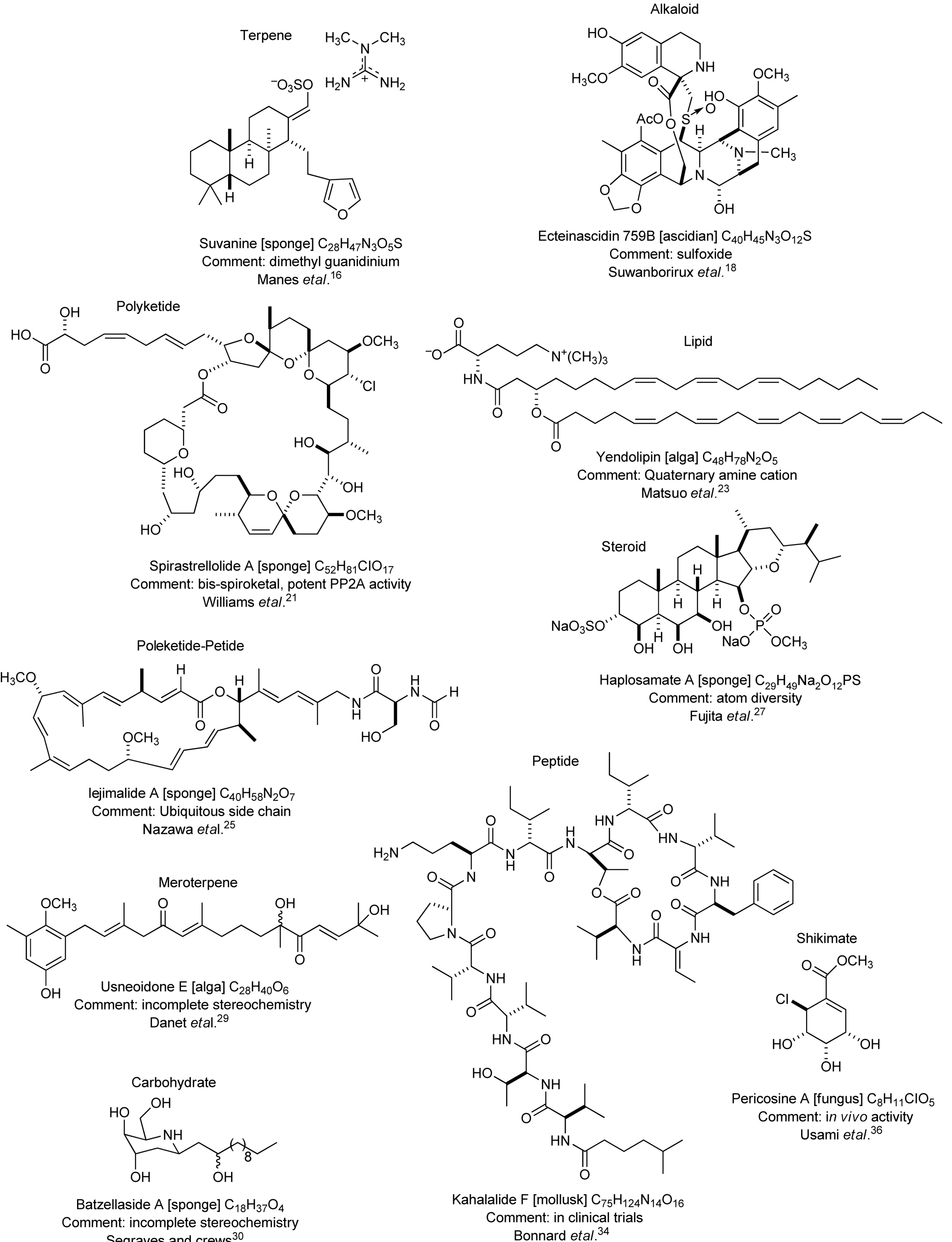


Figure 3 Selected examples, by biosynthetic type, of marine natural products illustrating a range of past and current assignment difficulties.

2.18.4.1 Incorrect Functional Groups Derived from Misassigned Molecular Formula

Each example collected here possesses a different factor that can lead to MF misassignment (**Table 1**). One of the best ways for establishing an accurate MF is to confirm its isotope pattern by simulation. It is also important to reconsider the working MF when it possesses more than 5 milli mass unit (mmu) error from the exact mass. Furthermore, if ^{1}H and ≠ or ^{13}C NMR signals are overlapped, due prudence requires establishing a correct MF. It should also be noted that in some cases, such as diazonamides, X-ray structure analysis does not always provide a secure 3D structure.

2.18.4.1.1 Spirastrellolide A

Spirastrellolide A, isolated from the Dominican sponge *Spirastrella coccinea*, was reported as an antimitotic macrolide in 2003.[19] Structure elucidation of spirastrellolide A was performed using its side chain methyl ester. The MF of the methyl ester was originally assigned to be $C_{53}H_{86}O_{19}$ based on an *m/z* 1027.580 5 as $[M+H]^+$ by high resolution chemical ionization mass spectrometry (HRCIMS), which had an error of −3.8 mmu from the exact MS. The original structure consisting of an unprecedented polyketide macrolide with a 47-carbon skeleton, two spiroketals, and eight hydroxy groups was determined based on one-dimensional (1D) and 2D NMR techniques. Although this structure possesses 21 stereocenters, only seven stereogenic centers on the spiroketal rings were proposed due to the limited amount of the sample (6.2 mg as methyl ester from 2.6 kg sponge). A year later, this proposed structure was revised by the same research group using a new sample (46.1 mg as methyl ester from 19 kg sponge).[20] First, the structure revision began with the number of hydroxy groups. Acetylation of the methyl ester gave not only an octaacetate, but also a pentacetate, which was confirmed based on the observation of a 5 amu increase when the low resolution electrospray ionization mass spectrometry (LRESIMS) for the methyl ester was measured in mono-deuteromethanol (MeOD). Second, the actual MF was determined to be $C_{53}H_{83}ClO_{17}$ based on the $[M+Na]^+$ ion observed at *m/z* 1049.520 26 (Δ −0.84 mmu) obtained by ultra-high-resolution fourier transform mass spectrometry (FTMS), which was confirmed by the agreement of the isotope pattern between experimental and simulated values. This new MF required the subtraction of H_3O and addition of one chlorine atom and one unsaturation degree. These differences accounted for the ether linkage between C-35 and C-38 and the addition of chlorine on C-28 in the revised structure. Although this relatively large quantity of methyl ester enabled this group to determine the relative stereostructure of the macrocyclic ring by comprehensive rotating-frame overhauser effect spectroscopy (ROESY) analysis, the remote stereochemistry at C-46 remained unsolved. The absolute stereostructure of the macrocyclic core was determined later based on X-ray structure analysis of the *p*-bromobenzoate derivative of spirastrellolide B (= 15,16-dihydro-28-dechloro-spirastrellolide A).[42] In 2007, five more derivatives were reported (spirastrellolides C–G) and the remaining stereocenter on C-46 has been finally determined to be *R* configuration by conversion of the side chain (C-44–C-47) of spirastrellolide D (= 4-chloro-spirastrellolide A) to dimethylmalate.[21] Although many research groups have taken up the challenge to synthesize spirastrellolide A since its initial isolation report in 2003, the first total synthesis was achieved in 2008.[43,59] Spirastrellolide A is expected to be a promising anticancer lead due to its potent selective activity against protein phosphatase 2A (IC_{50} = 1 nmol l^{-1}).[20]

2.18.4.1.2 Plakevulin A

Plakevulin A was reported in 2003 as a new oxylipin DNA polymerase inhibitor from the sponge *Plakortis* sp. collected in Okinawa.[44] The MF of plakevulin A was proposed to be $C_{28}H_{48}O_6$ based on both the low-resolution and the high-resolution MS data: *m/z* 480 $[M]^+$ (field desorption mass spectrometry (FDMS)), *m/z* 503 $[M+Na]^+$ (fast atom bombardment mass spectrometry (FABMS)), and *m/z* 480.342 7 $[M]^+$ (Δ −2.4 mmu). The presence of the two structural cores, a levulinyl group and oxylipin was confirmed based on both 2D NMR data and electron impact mass spectrometry (EIMS) fragment peaks (*m/z* 381 $[M–CH_3CO(CH_2)_2CO]^+$, *m/z* 157 $[M–CH_3CO(CH_2)_2CO–C_{16}H_{33})$. The planar structure of plakevulin was confirmed by an hetero-nuclear multiplebond correlation (HMBC) correlation (H-1/C-1′). The stereochemistry of the cyclopentene ring was determined by detailed analysis of the NMR data of three synthetic derivatives from plakevulin A (**Figure 4**). Finally, the absolute stereostructure was determined by the application of a modified Mosher's method[60] for the synthetic alcohol derivative (**Figure 4**). In 2004, however, this structure was revised based on the total synthesis and repurification of the natural product.[45] The spectral data of the synthetic plakevulin A were not identical to those of the natural product. Differences were observed in the carbon signals of C-1, C-2, C-3, C-5, and C-1′. The

Table 1 Problematic assignments of functional groups

Original structure	*Revised structure*	*Features*
Spirastrellolide A (sponge) MF: $C_{52}H_{84}O_{19}$ Williams *et al*.[19]	MF: $C_{52}H_{81}ClO_{17}$ Williams *et al*.[20]	Revision rationale: (a) NMR analysis of the pentacetate methyl ester, (b) absolute structure of the macrocyclic ring by X-ray structure analysis.[42] Remaining undefined: none. Critical data: (a) ultra-HRMS provided correct MF (*m/z* 1049.520 26 $[M+Na]^+$ (Δ −0.84 mmu, $C_{54}H_{83}O_{17}ClNa$ as methyl ester); original MF: HRCIMS, *m/z* 1027.580 5 $[M+H]^+$ (Δ −3.8 mmu, $C_{53}H_{86}O_{19}$ as methyl ester), (b) five exchange protons: *m/z* 1049 $[M+Na]^+$ in MeOH/CH_2Cl_2, *m/z* 1054 $[M+Na]^+$ in MeOD/CH_2Cl_2. Other issues: stereochemistry for OH on side chain was determined as *R* by conversion of the side chain to dimethyl malate.[21] Total synthesis of spirastrellolide A methyl ester has been achieved.[43]
Plakevulin A (sponge) MF: $C_{28}H_{48}O_6$ Tsuda *et al*.[44]	MF: $C_{23}H_{42}O_4$ + $C_5H_8O_3$ Saito *et al*.[45]	Revision rationale: total synthesis. Remaining undefined: absolute structure. Critical data: (a) the synthetic product ≠ the natural product, (b) H1 = 6.04 ppm (synthetic product), 5.34 ppm (natural product), (c) NMR data: the oxylipin alcohol moiety of the synthetic product = the natural product except levulinic acid, and (d) the oxylipin alcohol was obtained from the natural sample by repurification. Other issues: the natural product is a 1:1 mixture of the oxylipin alcohol and levulinic acid and small amount of the ester with the original structure but it is an artifact.

Siphonodictyal C (sponge)

MF: $C_{22}H_{30}O_4$

Sullivan *et al*.[46]

MF: $C_{22}H_{29}NaO_7S$

Mukku *et al*.[47]

Revision rationale: reassessment of NMR.
Remaining undefined: absolute structure.
Critical data: (a) (NMR) the revised was nearly identical to those of the proposed, (b) the loss of $-SO_3Na$ was confirmed by EI and FABMS, Revised MW; HRESIMS *m/z* 437.162 9 $[M-Na]^-$ (calcd for $C_{22}H_{29}O_7S$), (c) location of the sulfate group was determined by the calculated shift.
Other issues: Original MS came from HREIMS, *m/z* 358.212 2 (calcd for 358.214 4, $C_{22}H_{30}O_4$).

Halipeptin A (sponge)

MF: $C_{31}H_{54}N_4O_9$

González *et al*.[50]

MF: $C_{31}H_{54}N_4O_7S$

Kiyota *et al*.[51]

Revision rationale: reassessment of NMR.
Remaining undefined: none.
Critical data: (a) revised MF ($C_{31}H_{54}N_4O_6SNa$) = HRESIMS, *m/z* 649.361 1 $[M+Na]^+$ (Δ +2.3 mmu), (b) the methyl thiazoline unit = synthesis of model compounds and GIAO calculated chemical shifts.
Other issues: (a) original MF possessed large error (Δ +10.4 mmu), (b) two synthetic groups have achieved the total synthesis of halipeptin A in 2005.[49,52]

Barettin (sponge)

MF: $C_{16}H_{14}BrN_3O_2$

Lidgren *et al*.[53]

MF: $C_{17}H_{19}BrN_6O_2$

Solter *et al*.[54]

Revision rationale: reassessment of NMR.
Remaining undefined: none.
Critical data: (a) the synthetic product ≠ the natural product, (b) the spectral data of the revised structure = the original structure.
Other issues: total synthesis of barettin has been achieved, and the stereochemistry has been determined to be 12*S*.[55]

(*Continued*)

Table 1 (Continued)

Original structure	*Revised structure*	*Features*
Haplosamates A and B (sponge) MF: $C_{29}H_{50}NNa_{11}S_2$ (hap A: R = H) MF: $C_{29}H_{49}NNa_2O_{14}S_3$ (hap B: R = SO_3Na) Qureshi and Faulkner[26]	MF: $C_{29}H_{49}Na_2O_{12}PS$ (hap A: R = H) MF: $C_{29}H_{48}Na_4O_{15}P_2S$ (hap B: R = PO_3Na_2) Fujita *et al*.[27]	Revision rationale: reassessment of HRMS and NMR. Remaining undefined: none. Critical data: (a) δ_H 3.61 (3H, d, $^3J_{H,P} = 10.4$ Hz), δ_C 53.2 ($^2J_{C,P} = 12.9$ Hz), (b) HRFABMS (revised) *m/z* 653.276 6 (calcd for 653.276 0, $C_{29}H_{50}O_{12}PS$), HRFABMS *m/z* 653.289 8 (calcd for 653.290 4, $C_{29}H_{51}NO_{11}S_2$). Other issues: none.
Diazonamides A and B (ascidian) MF: $C_{40}H_{36}Cl_2N_6O_7$ (diazonamide A) MF: $C_{35}H_{25}BrCl_2N_5O_6$ (diazonamide B) Lindquist *et al*.[56]	MF: $C_{40}H_{34}Cl_2N_6O_6$ (diazonamide A) MF: $C_{35}H_{25}BrCl_2N_6O_4$ (diazonamide B) Li *et al*.[57]	Revision rationale: total synthesis. Remaining undefined: none. Critical data: (a) the synthetic diazonamide A ≠ the natural product, (b) amine proton in valine residue appeared one-proton sharp doublet, (c) NMR data at C-37 in diazonamide A (δ_H 3.75, δ_C 76.9), (d) downfield shift of H-37 by acetylation to δ_H 5.11, (e) the original MF of diazonamide B (*m/z* 743.059 0 $[M+H-H_2O]^+$, calcd for $C_{35}H_{24}N_5O_5Cl_2Br$) appeared to be miscalculated by 1 amu (the exact MS = 744.041 6), (f) the synthetic diazonamide A did not lose hemiacetal OH by MS experiments. Other issues: the revised structure of diazonamide A has been synthesized.[58]

HREIMS, high resolution electron impact mass spectrometry; HRFABMS, high resolution fast atom bombardment mass spectrometry.

Figure 4 Synthetic derivatives from plakevulin.

proton signal of the synthetic product of C-1 appeared at δ 6.04 whereas the corresponding signal of the natural product was observed at δ 5.34. These observations suggested that plakevulin A possessed a free hydroxy group on C-1. The NMR data of the alcohol obtained by hydrolysis from the synthetic plakevulin A were identical to those of the natural product except the signals related to levulinic acid. In fact, the oxylipin alcohol was obtained as a pure white solid from the natural product by a column chromatography. Thus, plakevulin A was hypothesized to be a nearly 1:1 mixture of the oxylipin alcohol and levulinic acid or an artifact of these compounds.

2.18.4.1.3 Siphonodictyal C

Siphonodictyal C was initially reported in 1986 as an antibacterial sesquiterpene quinol from the sponge *Siphonodictyon coralliphagum* collected from Ponape and Kwajalein in the Pacific Ocean.[46] The original MF assignment of $C_{22}H_{30}O_4$ came from HREIMS data (*m/z* 358.212 2, $[M]^+$, calcd for $C_{22}H_{30}O_4$, 358.214 4). The relative structure of siphonodictyal C was determined based on the NMR data comparison to the same class of known compounds. In 2003, the structure of siphonodictyal C was revised by another group, which placed a sulfate group on C-17, since both the structures had nearly identical NMR data.[47] The new MF was established as $C_{22}H_{29}NaO_7S$ by HRESIMS data (*m/z* 437.162 9 $[M–Na]^-$, calcd for $C_{22}H_{29}O_7S$). The presence of the sulfate group was proposed by the MF and the 46 amu difference between positive and negative electrospray ionization mass spectrometry (ESIMS). The location of the sulfate group was proposed by the calculation of the carbon chemical shift. It is very interesting in regard to the original report that siphonodictyal D, an isomer of the revised siphonodictyal C with a sulfate group on the aromatic ring, had already been reported. The MF of siphonodictyal C was initially determined by HREIMS as mentioned above, whereas HRFABMS was applied to establish the MF of siphonodictyal D. In fact, the report proposing the revised structure proved that only FABMS and ESIMS detected the sulfate group in siphonodictyal C but EIMS did not.

2.18.4.1.4 Halipeptin A

Halipeptin A was originally reported in 2001 as an anti-inflammatory cyclic depsipeptide with an extremely rare amino acid residue from the sponge *Haliclona* sp. collected in Vanuatu together with its desmethyl analogue, halipeptin B.[48] The MF of halipeptin A was proposed to be $C_{31}H_{54}N_4O_9$ based on the HRFABMS peak at *m/z* 627.4073 $[M+H]^+$ with an error of +10.4 mmu from the exact mass (calcd for $C_{31}H_{55}N_4O_9$, 627.396 9). The original structure was assembled based on comprehensive 2D NMR techniques including double-quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), heteronuclear multiple quantum coherence (HMQC), and HMBC. Importantly, ^1H-^{15}N HMBC played a pivotal role in establishing the 1,2-oxazetine-4-methyl-4-carboxylic acid (OMCA) residue. Although the authors indicated that ^{15}N chemical shifts for hydroxamic acid derivatives were expected to be in the range of −190 to −170 ppm, the chemical shift observed at −89.3 ppm for the OMCA nitrogen was proposed to be a consequence of ring strain on the oxazetidine ring. The absolute structure of halipeptin A except positions 3 and 4 in 3-hydroxy-2,2,4-trimethyl-7-methoxydecanoic acid (HTMMD) was determined by a combination of modified Mosher's method and Marfey's method.[61] The remaining stereocenters in the HTMMD moiety turned out to be a *threo* relative configuration by

application of Murata's method.[9] In 2002, the structures of halipeptins A and B were revised by the same group, together with the new derivative halipeptin C.[62] The new MF for halipeptin A was established as $C_{31}H_{54}N_4O_6S$ based on the HRESIMS ion peak at *m/z* 649.361 1 $[M+Na]^+$ with +2.3 mmu difference from the exact mass (calcd for $C_{31}H_{54}N_4O_6SNa$, 649.378 8). This MF suggested that the OMCA moiety was incorrectly assigned and was replaced by methylthiazoline. The presence of the methylthiazoline unit was confirmed by the NMR chemical shifts of a synthetic thiazoline unit and the comparison of the GIAO calculated chemical shifts for both oxazetidine and thiazoline units to those of the natural products. The stereochemistry of the α-position of the methylthiazoline was proposed to be *R* since the calculation results slightly favored the *R* configuration and all the amino acid residues found in this group of compounds possess L configuration. In 2005, two synthetic groups achieved total synthesis of halipeptin A and confirmed the absolute structure with a 3*S*,4*R*,7*S*-HTMMD moiety.[49,52] The latter group also synthesized halipeptin D and claimed that the potent cytotoxic properties of halipeptin D were not observed in the synthetic material. Thus, the potent cytotoxic properties of halipeptin D were concluded to come from residual impurities.

2.18.4.1.5 Barettin

Barettin, a new indole diketopiperazine derivative with inhibitory activity of electrically induced contractions of an isolated guinea pig ileum, was intially reported in 1986 from a northern Swedish deepwater sponge *Geodia baretti*.[53] The MF of barettin was proposed to be $C_{16}H_{14}BrN_3O_2$ based on the HREIMS peak at *m/z* 359.028 0 $[M]^+$ (calcd for $C_{16}H_{14}BrN_3O_2$, 359.027 0). The planar structure was deduced by comparison of the NMR to a synthetic diketopiperazine (cyclo-L-propyl-L-trptophy). At that point, the geometry of the olefin at C-8 and C-9 was unclear. A year later, the original structure of barettin with *S* configuration on the proline residue was synthesized by another group.[63] However, the spectral data of the synthetic compound were completely different from those of the natural compound. This synthetic group suggested that the structure of barettin could be the cyclic dimer. In 2002, another natural product group found a new diketopiperazine (cyclo[6-bromo-8*Z*-entryptophan]arginine) from the Norwegian deepwater (−300 m) sponge, *G. baretti*, in which the proline residue in barettin was replaced by arginine.[54] The MF of the new diketopiperazine was established to be $C_{17}H_{19}BrN_6O_2$ from the HRESIMS ion peak at *m/z* 419.083 3 $[M+H]^+$ (calcd for $C_{17}H_{20}BrN_6O_2$, 419.083 1). Interestingly, all the spectral data were completely identical with those of barretin. Thus, the structure of barettin was revised to be that of the new diketopiperazine with 8*Z* configuration. In the revised structure, the carbon chemical shifts at δ 127.45 and 127.55 recorded at 101 MHz were assigned as C-2 and C-3a, respectively, whereas these carbons showed up as an overlap signal at δ 127.4 recorded at 90 MHz in the original report. Further, the carbon signal of the guanidine sp^2 quaternary carbon at δ 158.61 in the revised structure was interpreted to be C-9 in the original structure. Although the absolute stereochemistry of the revised structure remained unsolved, the total synthesis of barettin was achieved in 2004, with the data from the synthetic product indicating an *S* configuration for the arginine residue.[55]

2.18.4.1.6 Haplosamate A

Haplosamate A was originally reported as a new steroidal sulfate ester isolated from the Philippines sponges *Xestospongia* sp. and an unidentified haplosclerid sponge in 1999, together with one more derivative haplosamate B with an additional sulfate group on C-7.[26] These new steroids were reported as the first example of marine sterols with sulfamate functional group and tetrahydropyran E ring. In the original report, the MF of haplosamate A was established as $C_{29}H_{51}NaNO_{11}S_2$ based on the HRFABMS and NMR data. The HRFABMS data observed at *m/z* 653.289 8 $[M-Na]^-$ showed an excellent match for the MF (calcd for $C_{29}H_{51}NaNO_{11}S_2$, 653.290 4). The presence of the sulfamate group was deduced from the ESI–MS/MS results and ^{1}H NMR data. The MS/MS result for *m/z* 675 provided several key fragment signals including *m/z* 573 $[M-Na-SO_3]^-$, *m/z* 559 $[M-Na-SO_2-NHCH_3]^-$, and *m/z* 543 $[M-Na-SO_3-NHCH_3]^-$, which supported the presence of the sulfamate group. On the other hand, two proton signals at 3.59 and 3.63 ppm with an integration of 1.5 protons on each signal (recorded in CD_3OD at 300 MHz) were initially accounted to be a *N*-methyl doublet (J = 10 Hz). However, since these signals correlated to two different carbon signals (δ_C 53.2 and 53.3) in the HMQC experiment and *N*-methyl sulfamates normally appear as methyl singlets, these signals were concluded to be two isomers based on the orientation of *N*-methyl group, which required the hydrogen bond to form a stable ring in the molecule (**Figure 5**). In 2001, two new phosphorylated sterol sulfates isolated as membrane-type matrix metalloproteinase

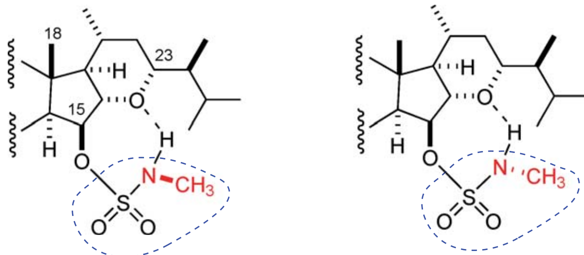


Figure 5 Two stereoisomers of the sulfamate group.

(MT-MMP) inhibitors from a Japanese marine sponge *Cribrochalina* sp. turned out to be identical with haplosamates A and B due to the same ^{1}H and ^{13}C NMR spectra as those previously reported.[27] The MF was assigned to be $C_{29}H_{49}Na_2O_{12}PS$ based on the HRFABMS (m/z 653.276 6 $[M+H-Na_2]^-$, Δ +0.6 mmu). The methylphosphate functionality in the revised structure was explained by the NMR data. The characteristic *O*-methylphosphate NMR signals were observed at δ_H 3.61 (3H, d, $^3J_{H,P}=10.4$ Hz) and δ_C 53.2 ($^2J_{C,P}=12.9$ Hz), which was further confirmed by a signal at δ 2.12 in the ^{31}P NMR spectrum. The same group also determined the absolute stereochemistry by the application of a modified Mosher's method.[60]

2.18.4.1.7 Diazonamides A and B

Diazonamides A and B were reported in 1991 as potent cytotoxic metabolites separated from the colonian ascidian *Diazona chinensis* collected in the Philippines.[56] The MFs of diazonamide A ($C_{40}H_{36}Cl_2N_6O_7$) and B ($C_{35}H_{25}BrCl_2N_5O_6$) were proposed based on the HRFABMS data observed at m/z 765.199 8 $[M+H-H_2O]^+$ (Δ −0.1 mmu) and m/z 743.059 0 $[M+H-H_2O]^+$ (Δ +25.0 mmu), respectively. As suggested from the MFs, the structure elucidation for these new compounds was extremely difficult due to a low H/C ratio core in the molecules. Generally, a structural core with a H/C ratio <1 makes structure elucidation challenging. In this case, the original structures for diazonamides A and B possessed an extremely low H/C core (A–D ring: $C_{14}H_4Cl_2N_3O_2$, H/C = 0.29). In fact, the structure elucidation was performed directly by X-ray structure analysis of the *p*-bromobenzoate derivative of diazonamide B (**Figure 6**). The X-ray structure, however, had a problem on the furofuran moiety since diazonamide B had a hemiacetal group on C-11 (δ_H 6.46, δ_C 106.6) to which the proton signal coupled with an exchangeable proton at δ_H 7.36. The original structure of diazonamide B was proposed with a hemiacetal group on the F ring due to the observation of a HMBC correlation between H-11 and C-17 on the E ring. The structure of diazonamide A was straightforwardly assigned based on the architecture of diazonamide B, possessing a terminal valine residue on the C-2 amine and hydrogen on C-6 instead of bromine. Diazonamide A showed potent cytotoxicity *in vitro* against both human (HCT-116) and murine (B-16) cancer cell lines with IC_{50} <15 nmol l^{-1}.

Diazonamides have evoked much interest in the synthetic community due to both their interesting architecture and potent cytotoxic properties. It took a decade to achieve the total synthesis of diazonamide A and the *p*-bromobenzoate derivative of diazonamide B.[64] However, the synthetic materials turned out to be not identical to the natural products. This synthetic group then elicited the revised structures of diazonamides A and B based on verification of the spectral data.[57] The major spectral data differences between the synthetic and

Figure 6 X-ray structure of diazonamide B *p*-bromobenzoate.

natural products in diazonamide A were observed in the terminal valine residue. The amine in the valine appeared as a sharp one-proton doublet at δ 5.46 coupled with the H-37 methine at δ 3.75 ppm. In the synthetic compound, this methine proton was observed at δ 3.16 as a broad singlet. The H-37 methine proton of the triacetate of natural diazonamide A shifted downfield to δ 5.11. Further, the carbon chemical shift of C-37 in the natural compound was observed at δ 76.9. As a result of these observations, the amine group in the valine residue was replaced by a hydroxy group, which was supported by the fact that acid hydrolysis of diazonamide A did not provide valine. However, this change required replacing an OH to NH_2 in the macrocyclic core established by X-ray analysis with a 1 amu increase. On the other hand, the MF of diazonamide B $C_{35}H_{24}N_5O_5Cl_2Br$ as $[M+H–H_2O]^+$ established from the HRMS ion peak at *m/z* 743.059 0 appeared to be miscalculated by 1 amu and the exact MS of the MF required was 744.041 6 amu. The best MF derived from incorporating an additional nitrogen atom for diazonamide B was $C_{35}H_{25}BrCl_2N_6O_4$ with the exact mass 743.057 6 as $[M+H]^+$. This MF has one less hydrogen than the original MF and is more reasonable with the observed HRESIMS data (*m/z* 743.059 0). Thus, the structure revision was considered on the hemiacetal moiety since the synthetic diazonamide A with a hemiacetal group ionized well to provide the molecular ion peak $[M+H]^+$. Finally, the revised structures for diazonamides A and B with a furopyrrole moiety were supported by the analysis of the bond length of the crystal structures, which were also confirmed by an ^{15}N-^{1}H heteronuclear single quantum coherence (HSQC) experiment for natural diazonamide A. In 2002, the revised structures were confirmed by the total synthesis of diazonamide A.[58] Although X-ray structure analysis has been recognized as the most secure structure elucidation method, it should be emphasized that X-ray structure analysis does not always provide the correct structure. An additional example of X-ray structure not corresponding to the true structure occurred in the structure elucidation of aminosamides A and B isolated from a marine-derived actinomycete.[65] It is interesting to note that a new diazonamide congener diazonamide C reported in 2008 possesses an amine group instead of a hydroxy group on C-37 like the original reported structure of diazonamide A.[66] However, this minor structure change makes the cytotoxicity (GI_{50}) considerably weaker from nanomolar to micromolar level. The cytotoxic effect of diazonamide A turned out to be the disruption of mitosis in the cell cycle.[67] Its mode of action has recently been suggested to be due to inhibition of the mitochondrial enzyme ornithine δ-amino transferase (OAT).[68,69] This enzyme has been suggested not to be essential in cell division for normal cells but to be critical for cancer cells. Moreover, the synthetic analogue (di-dechlorodiazonamide A) showed significant *in vivo* activity against xenograft mice with the same effectiveness as paclitaxel and vinblastine without side effects. Consequently, diazonamide A and its analogues are expected to be a new anticancer drug with significant cancer cell selectivity.

2.18.4.2 Challenge in Distinguishing Dimers from Monomers

The structures discussed in this section demonstrate the difficulty of distinguishing between dimeric and monomeric forms (**Table 2**). Only a few such examples have been reported among misassigned marine natural products. Caution is required when soft ionization methods such as ESIMS are used to measure molecular weight since these MS techniques frequently generate double charge ion peaks and/or dimeric ion peaks. Furthermore, selection of ionization method is also important since some of the ionization methods including EI (electron impact), CI (chemical ionization), and APCI (atmospheric pressure chemical ionization) are not appropriate to measure large molecule over 1000 Da.

2.18.4.2.1 Zamamistatin (aeroplysinin-1)

Zamamistatin was originally reported in 2001 as an antibacterial bromotyrosine dimer composed of a unique azaoxa-spiro[6.5] ring connected with an exo-double bond from the Okinawan sponge *Pseudoceratina purpurea*.[69] The MF of zamamistatin was established as $C_{18}H_{18}Br_4N_2O_6$ by HRESIMS (*m/z* 696.776 6 $[M+Na]^+$, Δ −2.9 mmu). The symmetrical form of zamamistatin was indicated based on nine carbon signals in the ^{13}C NMR (carbon NMR) spectrum. The carbon skeleton was straightforwardly established based on the NMR data. The isoxazolidine moiety was suggested from the observed chemical shift data and similarities with those of the aerothionin derivatives containing isoxazoline that are often seen in marine natural products. The geometry of

Table 2 Distinguishing monomeric forms from dimeric structures

Original structure	*Revised structure*	*Features*
Zamamistatin (sponge) MF: $C_{18}H_{18}Br_4N_2O_6$	Aeroplysinin 1 (sponge) MF: $C_9H_9Br_2NO_3$	Revision rationale: MS analysis, IR data, and NMR reassessment. Remaining undefined: none. Critical data: (a) the quintet peak centered at *m/z* 700.8 ($C_{18}H_{18}{}^{79}Br_2{}^{81}Br_2N_2NaO_6$) disappeared when measured with 0.15 $\mu mol\,l^{-1}$ concentration, (b) IR peak at 2262 cm^{-1} indicated nitrile group, (c) NMR data: zamamistatin = aeroplysinin-1. Other issues: (a) the endoform was proposed by analysis of ^{13}C NMR data of the model structures, (b) aeroplysin-1 was earlier reported.[71]
Takada *et al*.[69]	Kita *et al*.[72]	
MF: $C_{18}H_{18}Br_4N_2O_6$		
Hayakawa *et al*.[70]		

(Continued)

Table 2 (Continued)

Original structure	*Revised structure*	*Features*
Swinholide A (sponge) [chemical structure] MF: $C_{39}H_{66}O_{10}$ Carmely and Kashman[73]	[chemical structure] MF: $C_{78}H_{132}O_{20}$ Kobayashi *et al*.[74]	Revision rationale: reassessment of MS and NMR data. Remaining undefined: none. Critical data: (a) FABMS *m/z* 1411 $[M+H]^+$, *m/z* 1388 $[M–H]^-$, (b) swinholide A tri-*p*-bromobenzoate derivative gave the complex ^1H NMR spectrum, (c) the monomeric methyl ester was obtained by treatment with NaOMe. Other issues: the absolute structure has been determined by X-ray structure analysis.[75] Swinholide A has also been isolated from marine cyanobacteria.

the exo-double bond was determined to be *trans* based on the nuclear overhauser effect spectroscopy (NOESY) correlation between H-7′ and NH, which furnished the original structure. In 2006, the second isolation of this compound from the Okinawan sponge *P. purpurea* by another research group led to the structure revision of zamamistatin as an endo-type dimer with an azaoxo-spiro[6.6] ring.[70] The determination of this alternative structure was made based on comparison of the natural product NMR data to those of the synthetic analogues. The carbon chemical shifts of the spiro carbons of the synthetic analogue with a 4,5-dihydroisoxazole appeared at δ_C 92.4 whereas the carbon chemical shifts of the spiro carbon of the synthetic 5,6-dihydro-4*H*-1,2-oxazine was observed at δ_C 74.3. These carbon chemical shifts indicated that the structure of zamamistatin possessed a 5,6-dihydro-2*H*-1,2-oxazine ring instead of isoxazolidine since the spiro carbon of zamamistatin was observed at δ_C 74.3. Despite the detailed discussion of the NMR chemical shifts, the MS data of the second isolation of zamamistatin were not described in the article.[70] Subsequently, the same research group that revised zamamistatin as the endo-type form concluded that zamamistatin was identical to aeroplysinin-1, which was isolated as an antibacterial dibromophenylpyruvic acid derivative from the sponge *Verongia aerophoba* in 1972,[71] based on simple MS experiments.[72] Zamamistatin gave two indicative ESIMS ion peaks: the quintet peak centered at m/z 700.8 ($C_{18}H_{18}^{79}Br_2^{81}Br_2N_2NaO_6$) and the triplet peak centered at m/z 361.9 ($C_9H_9{}^{79}Br^{81}BrNNaO_3$) when measured with a concentration of 15 μmol l^{-1}, which were originally assigned to be the molecular ion peak $[M+Na]^+$ and a doubly charged peak $[(M/2)+Na]^+$, respectively. However, the quintet ion peak (m/z 700.8) disappeared when ESIMS analysis was performed with 0.15 μmol l^{-1} concentration, suggesting that the MF of zamamistatin must be $C_9H_9BrNO_3$ and the quintet peak was due to a dimeric ion peak $[2M+Na]^+$. Furthermore, the IR signal at 2262 cm^{-1} suggested that zamamistatin possesses a nitrile group. This structure revision was confirmed based on the above observations plus the identical spectral data (NMR, IR, and optical rotation) between zamamistatin and aeroplysinin-1.

2.18.4.2.2 Swinholide A

Swinholide A was first reported in 1985 as a polyketide macrolide isolated from the Red Sea sponge *Theonella swinhoei*.[73] The structure elucidation of swinholide A was performed without MS data since this compound did not provide any molecular ion peak in EIMS, chemical ionization mass spectrometry (CIMS), FDMS, secondary ion mass spectrometry (SIMS), and FABMS. The original structure was proposed based on the 1D and 2D NMR experiments of the tetraformate derivative. A year later from this report, the same group reported the complete ^{1}H and ^{13}C NMR assignments for swinholide A.[77] In 1989, another research group reported that swinholide A has a dimeric form based on the MS and NMR analysis for both the natural product newly isolated from the Okinawan *T. swinhoei* and its synthetic derivatives.[74] First, swinholide A showed the molecular ion peak in both positive and negative FABMS at m/z 1411 $[M+Na]^+$ and m/z 1388 $[M–H]^-$, respectively. The molecular formula, $C_{78}H_{132}O_{20}$, obtained from the MS and NMR data was confirmed by combustion analysis. Since the ^{1}H and ^{13}C NMR data of the material turned out to be identical to those of the published values, swinholide A appeared to possess a dimeric form rather than the original structure. To break the symmetrical form, swinholide A was converted into a di-acetonide followed by *p*-bromobenzoylation (**Figure 7**). The reactions gave a di-*p*-bromobenzoate, tri-*p*-bromobenzoate, and tetra-*p*-bromobenzoate derivative with masses at m/z 1858 $[M+Na]^+$, m/z 2041 $[M+Na]^+$, and m/z 2224 $[M+Na]^+$, respectively. The dimeric form was proven by the more complicated ^{1}H NMR spectrum of the tri-*p*-bromobenzoate than those of the symmetric di- and tetra-*p*-bromobenzoates. Further reactions have been performed to elucidate the total structure of swinholide A (**Figure 7**). NMR analysis of the monomeric methyl ester (m/z 727 $[M+H]^+$) obtained by the treatment of NaOMe in MeOH provided the partial structure from C-1 to C-23 and C-27 to C-32. The remaining part (C-24–C-26) and its location in the monomer were confirmed based on the NMR analysis of the 1,2,4-triazepin-3-one converted from the methyl ester, which enabled the assignment of swinholide A as a 44-membered dilactone. In 1990, the same research group also reported the absolute stereostructure of swinholide A by X-ray structure analysis of the di-*p*-bromobenzoate.[75] It is interesting to note that swinholide A has also been isolated from two different marine cyanobacteria, together with its glycosylated derivatives.[76] This evidence strongly suggests that marine cyanobacteria are the true producers of the swinholide class of compounds. It is also noteworthy that swinholide A is a known actin stabilizer, used widely as a molecular probe.[78]

Swinholide A

(1) 2,2- Dimethoxypropane, p-TsOH
(2) *p*-Benzoyl chloride, pyridine

NaOMe

di-*p*-bromobenzoate: $R^{1'} = R^{1'} = p$-BrBz, $R^2 = R^{2'} = H$
tri-*p*-bromobenzoate: $R^1 = R^{1'} = R^2 = p$-BrBz, $R^{2'} = H$
tetra-*p*-bromobenzoate: $R^1 = R^{1'} = R^2 = R^{2'} = p$-BrBz

Monomeric methylester

(1) O_3, $(CH_3)_2S$
(2) Semicarbazide, NaOAc
(3) Ac_2O

1,2,4-Triazepin-3-one derivative

Figure 7 Synthetic strategy to solve the 2D structure of swinholide A.

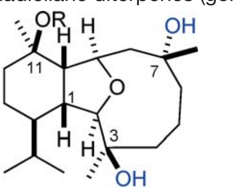
2.18.4.3 Challenges in Distinguishing Diols from Ethers

MS experiments frequently provide ion peaks following the loss of H_2O, especially in EIMS. Misreading these ion peaks as molecular ion peaks $[M]^+$ has the potential to cause structure misassignment when the molecules have more than two hydroxy groups. This section describes three examples with regard to this issue: the first two of which are structure revisions from ethers to diols and the last example is a reassignment from diol to ether (**Table 3**). These examples suggest that EIMS analysis alone may not be suitable for molecules with multiple hydroxy groups.

2.18.4.3.1 Peribysins C and D

Peribysins C and D were initially reported in 2004 as diastereomers that functioned as potent cell adhesion inhibitors from the fungus *Periconia byssoides* separated from the sea hare *Aplysia kurodai*.[79] These compounds

Table 3 Distinguishing diols from ethers

Original structure	*Revised structure*	*Features*
Peribysins C and D (marine-derived fungus) MF: $C_{15}H_{22}O_2$ Yamada *et al*.[79]	MF: $C_{15}H_{24}O_3$ Koshino *et al*.[80]	Revision rationale: (a) CAST/CNMR prediction, (b) new HREIMS data, (c) conformation search by *ab initio* calculations plus NOESY data. Remaining undefined: absolute stereo. Critical data: (a) inconsistent ^{13}C NMR shifts: peribysin C, C6 (δ 69.98), C8 (δ 84.12), peribysin D, C6 (δ 85.95), C8 (δ 63.69), (b) HREIMS *m/z* 252.171 9 $[M]^+$ (calcd for $C_{15}H_{24}O_3$) for perybysin C, (c) relative stereo = *ab initio* calculations plus original NOE data. Other issues: original MS data from HREIMS.
Prevezol B (alga) MF: $C_{20}H_{31}BrO_2$ Mihopoulos *et al*.[81]	MF: $C_{20}H_{33}BrO_3$ Iliopoulou *et al*.[82]	Revision rationale: reassessment of NMR data. Remaining undefined: absolute structure.Critical data: (a) prevezol B = prevezol C (14-epimer) except for C9, 14, and 18, (b) NOEs (H9/H11a, H9/H18, H14/H12b), $^3J_{H14,9}$ = 11.2 Hz. Other issues: FABMS = $[M–2H_2O+H]^+$, EIMS = $[M–H_2O+H]^+$, original MS data from HREIMS.
Cladiellane diterpenes (gorgonian)  MF: $C_{22}H_{38}O_5$ (R = Ac) MF: $C_{20}H_{36}O_4$ (R = H) Bowden *et al*.[83]	Polyanthellin A (gorgonian) MF: $C_{22}H_{36}O_4$ (polyanthellin A: R = Ac) MF: $C_{20}H_{34}O_3$ (synthetic derivative: R = H) Ospina *et al*.[84]	Revision rationale: reassessment of NMR data. Remaining undefined: absolute structure. Critical data: (NMR) polyanthellin A, deacetyl-polyanthellin A = cladiellane diterpenes, but opposite optical rotation values. Other issues: total synthesis of (+)-polyanthellin A has been achieved.[85]

NOE, nuclear overhauser effect.

possessed the same MF $C_{15}H_{22}O_2$ from the HREIMS data observed at *m/z* 234.161 9 $[M]^+$ (Δ 0.0 mmu) for peribysin C and *m/z* 234.161 8 $[M+]^+$ (Δ −0.1 mmu) for peribysin D. The planar and relative structures for these two compounds were proposed from the NMR data. The unique 1,3,4,6-tetrahydrofuro[3,4-*c*]furan moiety in peribysins C and D was proposed as a consequence of HMBC correlations (H_2-12/C-7, C-8, C-11, C-13, H_2-13/C-6, C-7, C-11, C-12). These structures were revised in 2006[80] by the carbon chemical shift prediction tool CAST (canonical-representation of stereochemistry)/CNMR.[86,87] This software predicted similar carbon chemical shifts on C-12 and C-13 for both peribysins C and D since these carbons exist in similar environments. However, the reported carbon chemical shifts for peribysins C and D showed approximately 20 ppm difference between C-12 and C-13. Furthermore, similar carbon chemical shifts were expected on C-6–C-8 and C-11–C-13 between these two natural products, but high-field carbon shifts were observed on C-6 (δ 69.98, Δ −15.97 ppm) and C-13 (δ 56.03, Δ −20.33 ppm) on peribysin C, and C-8 (δ 63.69, Δ −20.43 ppm) and C-12 (δ 55.68, Δ −20.69 ppm) on peribysin D. This evidence suggested that peribysins C and D were a 6,13-diol and an 8,12-diol, respectively, which was supported by the weak ion peaks at *m/z* 252 found in the original EIMS data for peribysins C and D. Later the new MF $C_{15}H_{24}O_3$ was confirmed by HREIMS data (*m/z* 252.171 9 $[M]^+$, Δ −0.7 mmu). *Ab initio* calculations using the program Spartan 04 finalized the relative stereostructures for peribysins C and D as 8α,12-epoxy-7(11)-eremophilin-6β,13-diol and 6β,13-epoxy-7(11)-eremophilin-8α,12-diol, respectively.

2.18.4.3.2 Prevezol B

Prevezol B together with prevezol A was reported in 2001 as brominated diterpenes from the red alga *Laurencia obtusa* collected in Greece (**Figure 8**).[81] The MF of prevezol B was established as $C_{20}H_{31}BrO_2$ based on the HREIMS data observed at *m/z* 382.150 7 $[M]^+$ (Δ +0.6 mmu). The structure of prevezol B with unique oxetane ether linkage was proposed essentially by comparison of the NMR data to those of prevezol A. The major difference between prevezols A and B was that the epoxide carbons (δ 59.2 and δ 62.2) were replaced by oxygen bridge carbons (δ 72.9 and δ 75.9). The structure of prevezol B was proposed as a 1,2-methyl shift followed by ether linkage formation. The relative stereostructure was deduced based on Monte Carlo conformational analysis and the proposed structure with the lowest energy conformation was supported by NOE correlations. In 2003, the same research group revised the structure of prevezol B as a stereoisomer of the new analogue prevezol C whose structure was firmly assigned since the spectral data of prevezol C closely resembled those of prevezol B.[82] The number of hydroxy groups in prevezol B was confirmed by MS data of the methylation product (data not provided). Furthermore, the oxymethine H-14 turned out to be an axial proton ($J_{9,14}$ = 11.20 Hz), which eradicated the possibility of an ether bridge between C-12 and C-14. Finally, NOE correlations allowed assignment of the relative stereostructure of prevezol B as 2*R*,3*S*,6*R*,9*R*10*S*,13*S*,14*S**or 2*R*,3*S*,6*R*,9*S*10*R*,13*R*,14*R**.

2.18.4.3.3 Cladiellane diterpenes

Two new cladiellane class of diterpenes, (1*R**,4*R**,5*S**,6*R**,8*R**,12*R**,13*R**,14*R**)-cladiellane-4,8,12-triol and its 4-acetoxy derivatives were isolated in 1989 as secondary metabolites from the Australian gorgonian *Briareum* species.[83] The MF of the cladiellane triol was established as $C_{20}H_{36}O_4$ based on the HREIMS data observed at *m/z* 322.250 $[M–H_2O]^+$ (Δ −1 mmu). Both the 2D and 3D relative structures of this triol were determined based on 2D NMR data, NOE correlations, and comparison of the NMR data to those of the similar known compounds cladiellin and eunicellin, both of which had firmly determined structures via X-ray structure

Figure 8 Structure of prevezol A.

analysis.[88,89] The structure of the acetate was determined from nearly identical NMR data, with the exception of the acetyl group signals and downfield shift of the C-4 carbon chemical shift by 13 ppm (the MS data of the acetate are not provided). In 2003, a new cladiellane derivative polyanthellin A was reported from the gorgonian *Briareum polyanthes* collected in Puerto Rico.[84] HREIMS experiment did not provide a molecular ion peak for polyanthellin A (*m/z* 304.250 2 $[M–AcOH]^+$, Δ +10.0 mmu). However, the secure MF $C_{22}H_{36}O_4$ was obtained from HRFABMS experiment (*m/z* 387.2512 $[M+Na]^+$, Δ +0.1 mmu). It was suggested that this molecule had no alcohol groups based on the IR spectrum. The planar and relative structures of polyanthellin A were straightforwardly determined by the analysis of the 1D and 2D NMR data. Treatment of this compound with lithium aluminum hydride provided a triol on C-3, C-7, and C-11. The ^{1}H and ^{13}C NMR data of both polyanthellin A and the synthetic triol turned out to be identical to those of the cladiellane triol and the triol from the Australian specimens. However, the compounds previously assigned possessed opposite optical rotation values (polyanthellin A = $[\alpha]_D$ −9.9° vs the acetate = $[\alpha]_D$ +8.9°, the synthetic triol = $[\alpha]_D$ −11.0° vs the cladiellane triol = $[\alpha]_D$ +19.4°). Thus, the cladiellane diterpene derivatives previously assigned were revised as the enantiomers of polyanthellin A and the triol. The total synthesis of (+)-polyanthellin A (polyanthellin A enantiomer) was achieved in 2006,[85] proving the structure of polyanthellin A with the ether linkage.

2.18.4.4 Challenges in Distinguishing Hydroxy Groups from Hydroperoxides

This section illustrates the difficulties that can arise when deciding between hydroxy and hydroperoxide groups. Caution is required when establishing the MF since these functional groups are easily cleaved from the molecule in MS experiments (especially EIMS) and the corresponding fragment peak can no longer give significant information to distinguish these two functionalities. Two structure revisions regarding this issue are reviewed below and both examples are reassigned from a hydroxy to a hydroperoxide group (**Table 4**). These revisions suggest that the key to distinguishing these functional groups is the downfield shift of the hydroperoxide carbon.

2.18.4.4.1 12S-Hydroxybromosphaerodiol

12*S*-Hydroxybromosphaerodiol was reported in 1987 as a new brominated diterpene from the red alga *Sphaerococcus coronopifolius* collected in the Mediterranean Sea.[90] The MF $C_{20}H_{32}Br_2O_3$ was established based on the HREIMS data (*m/z* 478.071 85 $[M]^+$, Δ −0.06 mmu). The presence of three hydroxy groups in the molecule was proposed based on the EIMS peaks observed at *m/z* 478 $[M]^+$, *m/z* 460 $[M–H_2O]^+$, *m/z* 442 $[M–2H_2O]^+$, and 424 $[M–3H_2O]^+$. The total structure including its absolute stereostructure was determined by the analysis of ^{1}H NMR data and chemical conversion to a common diol obtained from 12*S*-hydroxybromosphaerol, of which the absolute stereostructure had already been determined. In 2008, 1*S*-hydroperoxy-12*S*-bromosphaerodiol was reported as a cytotoxic diterpene isolated from the Greek *S. coronopifolius* algae.[91] The MS and NMR data of this hydroperoxide compound turned out to be identical to those of the previously reported 12*S*-hydroxybromosphaerodiol (CIMS: *m/z* 477 $[MH–H_2O]^+$, *m/z* 459 $[MH–2H_2O]^+$, *m/z*441 $[MH–3H_2O]^+$). Thus, the structure of 12*S*-hydroxybromosphaerodiol was revised to be the peroxide, which was also confirmed by X-ray structure analysis. Moreover, it was disclosed that the hydroperoxide carbon on C-1 (δ_C 79.2) possessed a downfield carbon shift by 13 ppm when compared to the hydroxy carbon on C-1 (δ_C 66.2) in the known compound bromosphaerodiol, whose structure had previously been determined by X-ray structure analysis.[91]

2.18.4.4.2 Briarellin A

In 1995, briarellin A was reported as a new cytotoxic eunicellin diterpene isolated from the gorgonian *Briareum asbestinum* collected in Puerto Rico.[92] The MF of briarellin A was established as $C_{28}H_{44}O_7$ based on the HREIMS peak observed at *m/z* 492.307 79 $[M]^+$ (Δ −0.8 mmu). The presence of two alcohol groups was proposed based on several additional HREIMS fragment peaks including *m/z* 474.297 43 $[M–H_2O]^+$ (Δ −0.9 mmu) and *m/z* 312.171 63 $[M–2H_2O–C_8H_{16}O_2]^+$ (Δ −0.9 mmu). The structure including its relative stereostructure was assembled by basic comparison of the NMR data to the related compound asbestinin-7 and the NOESY data. In 2003, the same group found several additional new briarellin derivatives from the

Table 4 Distinguishing hydroxy groups from hydroperoxide groups

Original structure	*Revised structure*	*Features*
12*S*-Hydroxy bromosphaerodiol (alga) MF: $C_{20}H_{32}Br_2O_3$ Cafieri *et al*.[90]	1*s*-hydroperoxy-12 *s*-hydroxybromosphaerol B MF: $C_{20}H_{32}Br_2O_4$ Smyrniotopoulos *et al*.[91]	Revision rationale: reassessment of MS and NMR and X-ray structure analysis. Remaining undefined: none. Critical data: (a) MS and NMR data: 12*S*-hydroxy-bromosphaerodiol = hemigaran E, (b) downfield shift of the hydroperoxide carbon C-1 (δ_C 79.2) compared to that of the hydroxy carbon (δ_C 66.2), (c) the structure of hemigran E was confirmed by X-ray structure analysis. Other issues: none.
Briarellin A (gorgonian) MF: $C_{28}H_{44}O_7$ Rodriguez and Cobar[92]	MF: $C_{28}H_{44}O_8$ Ospina *et al*.[84]	Revision rationale: reassessment of NMR. Remaining undefined: absolute structure. Critical data: (a) the NMR data (C6; d 4.67, 82.9 ppm) correspond to the C6-peroxide analogues (briarellin K hydroperoxide (C6; d 4.65, 85.6 ppm), briarellin D hydroperoxide (C6; d 4.62, 85.5 ppm)), but not to the C6-hydroxy analogue (briarellin K (C6; d 4.28, 72.6 ppm)), (b) MS data; original HREIMS *m/z* 492.307 79 (calcd for 492.308 67 $C_{28}H_{44}O_7$), revised LRFABMS *m/z* 509 (calcd for $C_{28}H_{45}O_8$), *m/z* 531 (calcd for $C_{28}H_{44}O_8Na$). Other issues: none.

Briarellin K: R = H (δ_{C6} 72.6)
Briarellin K hydroperoxide: R = OH (δ_{C6} 85.6)

Briarellin D: R = H(δ_{C6} 72.6)
Briarellin D hydroperoxide: R = OH (δ_{C6} 85.5)

Figure 9 Structural relationship and the chemical shift differences between hydroxy and hydroperoxide carbons.

B. polyanthes collected in Puerto Rico.[84] The structural pair briarellin K and its hydroperoxide among the new compounds gave a clue to revise the structure of briarellin A (**Figure 9**). The major differences between these two derivatives were the following three spectral data points: (1) Δ 16 amu (briarellin K, HREIMS *m/z* 392.2175 (calcd for $C_{22}H_{32}O_6$, Δ −2.3 mmu), HRFABMS *m/z* 399.236 6 (calcd for $C_{22}H_{32}O_6Li$, Δ +0.7 mmu); briarellin K hydroperoxide, HRFABMS *m/z* 431.204 0 (calcd for $C_{22}H_{32}O_7Na$, Δ −0.6 mmu)); (2) $\Delta\delta_{C\text{-}6}$ 13 ppm (briarellin K (δ 72.6), briarellin K hydroperoxide (δ 85.5)); and (3) $\Delta\delta_{H\text{-}6}$ 0.3 ppm (briarellin K (δ 4.28), briarellin K hydroperoxide (δ 4.62)). Similar observations were also present between briarellin D and its hydroperoxide. The spectral data of C-6 in briarellin A was almost identical to those of the hydroperoxides (δ_H 4.67, δ_C 82.9), strongly suggesting that briarellin A was a hydroperoxide at C-6. This assumption was confirmed later by MS data (FABMS *m/z* 509 $[M+H]^+$, *m/z* 531 $[M+Na]^+$).

2.18.5 Difficulties in Assembling Planar Structures

In this section, attention is shifted to 2D structure misassignment including double bond geometry, which arises from the interpretation of NMR data. The topics discussed below focus on the three factors leading to incorrect 2D structures: (1) C=Z assignment, (2) C=C assignment, and (3) assignment of either exo or endo form. Among the case examples described below, assignment of C=Z functional groups must be made particularly carefully since misassignment of C=Z functionalities provides completely different structures from the true structure.

2.18.5.1 Assessment of C=Z Assignments

Analyzing the examples discussed in this section demonstrates that it is very difficult to assign C=Z functional groups in a molecule when it possesses a low H/C ratio core (**Table 5**). All the examples collected here possess cores bearing a meager number of H atoms (H/C < 1) that make general ^1H and ^{13}C NMR techniques inadequate for structure elucidation. These examples show that it is sometimes quite difficult to distinguish C=Z functionalities that have similar carbon chemical shift, such as amide C=O versus ester C=O and guanidine C=N versus urea C=O. It is also important to assign C=Z carbons correctly when the molecule has multiple C=Z functionalities.

2.18.5.1.1 Spiroleucettadine

Spiroleucettadine was reported in 2004 as a new imino-imidazolidine alkaloid isolated from the sponge *Leucetta* sp. collected in Fiji.[93] The proposed structure of spiroleucettadine was very unique in terms of possessing a rare amino hemiketal functional group. This amino-hemiketal moiety was placed on the 5,5-*trans* bicyclic ring system that possessed three characteristic quaternary carbons. The first carbon signal at δ 159.5 (C-1) was assigned to be a guanidine carbon in the imino-imidazolidine ring. The other two carbons observed at δ 102.5 (C-4) and δ 82.5 (C-5) were accounted to be an amino-hemiketal carbon and nitro carbon, respectively, both of which were embedded as ring carbons between the 2-imino-imidazolidine and tetrahydrofuran moieties. These assignments for the characteristic carbons were essentially supported by an analogy to the alkaloid

Table 5 Assessing C = Z functional groups

Original structure	*Revised structure*	*Features*
Spiroleucettadine (sponge) Ralifo and Crews[93]	White *et al*.[94]	Revision rationale: DFT calculations, X-ray structure. Remaining undefined: absolute structure. Critical data: (a) HMBC correlation (H_2-8/C-6) = the $^1J_{CH}$ cross peak of CD_3OD, (b) the ROESY correlation (OH/H-19) was very weak, (c) the revised structure was supported by DFT calculations and X-ray structure. Other issues: the crystals of spiroleucettadine were a racemic mixture.
Kasarin (fungus) Suenaga *et al*.[95]	Kita *et al*.[96]	Revision rationale: reassessment of NMR. Remaining undefined: none. Critical data: (a) OCH_3/N-4 ^{15}N HMBC correlation was reassigned as OCH_3/N-1, (b) the synthetic model compound with a 5-hydroxyprozin-2(1*H*)-one ring showed the similar spectral data to the natural product. Other issues: none.
Aspernigrin A (fungus) Hiort *et al*.[97]	Ye *et al*.[98]	Revision rationale: NOE and X-ray structure analysis. Remaining undefined: none. Critical data: (a) NOEs (NH/$H_2$7, NH/H2), (b) the revised structure with 1*H*-pyridin-4-one ring was confirmed by the X-ray structure analysis. Other issues: none.

Calcaridine A   Spirocalcaridine A

Figure 10 Related amino-imidazole secondary metabolites from a *Leucetta* sponge.

spirocalcaridine isolated from the same sponge specimen (**Figure 10**). The relative structure of spiroleucettadine was deduced based on the analysis of the 2D NMR data. The key 2D correlations to establish the *trans* 5,5-bicyclo ring with the amino-hemiketal moiety were the HMBC correlation from H_2-8 to C-6 and the ROESY correlation between OH and H-19. After this report regarding the structure of spiroleucettadine, three synthetic groups failed to synthesize the *trans* fused bicyclic core and concluded that this alkaloid required structural revision.[99–101] The last synthetic group also suggested a possible revised structure for spiroleucettadin using DFT calculations.[101] In 2008, the structure revision of spiroleucettadine has been reported using a pure sample newly isolated by the original natural product team.[94,95] First, the *trans* 5,5-bicyclo ring system with amino-hemiketal functionality turned out to be assigned incorrectly since the key HMBC correlation (H_2-8/C-6) was actually due to the $^1\mathcal{J}_{CH}$ cross peak of the NMR solvent CD_3OD and the ROESY correlation (OH/H-19) was very weak. The proposed revised structure was selected by DFT calculations among 16 candidate structures and later confirmed by X-ray structure analysis. It is interesting that the crystals obtained for X-ray structure analysis were a racemic mixture and thus spiroleucettadine has been proposed to exist as a scalemic mixture due to its optical activity (original sample: $[\alpha]_D$ −27.1, new sample: $[\alpha]_D$ −5.1). In the revised structure, the carbon signal observed at δ 159.5 (C-1) was assigned to be the urea carbon.

2.18.5.1.2 Kasarin

Kasarin was first described in 2000 as a new alkaloid from the marine bacterium *Hyphomycetes* sp., which was separated from the zoanthid *Zoanthus* sp.[95] The original structure of kasarin had a characteristic feature of a low H/C ratio azetinone core. This structure was proposed based on the 1D and 2D NMR techniques including ^{15}N HMBC experiments. Although direct evidence of a connection between C-2 and N(3) was not observed, the azetinone core was supported by its MF and the IR data (1760 cm^{-1} as β-lactam). In 2007, the structure of kasarin was revised based on reevaluation of the NMR data between the natural product and the synthetic analogues and degradation products.[96] The key ^{15}N HMBC correlation (OCH_3/N-4) in the original assignment actually turned out to be the correlation between OCH_3 and N-1. Furthermore, three sp^2 quaternary carbons on C-3, C-5, and C-1′ were reassigned as follows: (1) the amide carbonyl carbon (δ 161.5, C-3) connected to the isopropyl group in the original structure was assumed to be an imine carbon attached to the carbonyl carbon (δ 152.0, C-2), (2) the imine carbon (δ 134.9, C-5) was accounted as an sp^2 quaternary carbon bearing both nitrogen and oxygen, and (3) the amide carbonyl (δ 166.0, C-1′) was proposed to be an ester carbonyl. These changes provided two alternative structures: the oxygenated pyrazinone and the oxazolone derivative (**Figure 11**). The revised structure with the oxygenated pyrazinone for kasarin was chosen based on

Figure 11 Candidate structure for kasarin.

the spectral similarity including ^{13}C NMR, IR, and UV spectra between the natural product and a synthetic pyrazinone derivative, and was also supported by the spectral data of one of the degradation products. It should be mentioned that the structure elucidation of the pyrazinone core is especially challenging since the core ring has no hydrogen atoms (C_4N_2O, H/C = 0).

2.18.5.1.3 Aspernigrin A

Aspernigrin A was reported in 2004 as a new alkaloid from the fungus *Aspergillus niger* separated from the Mediterranean sponge *Axinella damicornis*.[97] The structure elucidation of aspernigrin A was performed based on the 1D and 2D NMR data. The two amide carbonyl carbons were observed at δ 177.6 and δ 165.5, which were assigned to be the carboxy amide and lactam carbonyl carbon, respectively. The planar structure including the placement of the benzyl group and the carboxy amide moiety was proposed based on the HMBC correlations of the two protons on the lactam ring. In 2005, another research group isolated aspernigrin A from the endophytic fungus *Cladosporium herbarum* possessing almost identical spectral data to the first isolated sample.[98] It was necessary first to examine the amide groups since the carbon chemical shift of the conjugated carboxy amide (δ 177.6) appeared significantly more downfield than as usual (δ_C ~165). This inconsistency was solved by swapping the amide carbons between the carboxy amide and the lactam. This carbon reassignment and NOEs observed between NH/H_2-7 and NH/H-2 indicated that the lactam core in the original structure must be revised to a 4-pyridone. The revised structure proposed based on both the above observations and the HMBC data has subsequently been proven by X-ray structure analysis.

2.18.5.2 Assessment of C=C Assignments

The examples described below detail incorrect C=C assignments including geometry and placement (**Table 6**). Misassignment regarding this issue often occurs when multiple C=C functionalities exist in aliphatic chain and/or polyketide macrocyclic ring. This situation causes signal overlapping for sp^2 protons, which makes secure structure elucidation intensely difficult.

2.18.5.2.1 Mycothiazole

Mycothiazole was reported in 1988 as a novel polyketide/alkaloid with anthelmintic activity isolated from the Vanuatu sponge *Spongia mycofijiensis* (also known as *Cacospongia mycofijiensis*).[102] The planar structure possessing four double bonds in the polyketide chains was proposed based on 1D and 2D NMR data. Two of the double bonds were assigned as terminal olefins ($\Delta^{4,19}$ and $\Delta^{18,19}$) and the others were assigned as a *Z* olefin ($\Delta^{5,6}$, J = 12 Hz) and an *E* olefin ($\Delta^{14,15}$, J = 18 Hz). The original structure of mycothiazole was confirmed by total synthesis in 2000.[109] The synthetic mycothiazole with 8*R* configuration showed identical spectral data including ^{1}H and ^{13}C NMR, IR, and HRMS. However, differences in the optical rotation values between the synthetic material and natural product were conspicuously inconsistent (synthetic = $[\alpha]_D$ −26.0, natural = $[\alpha]_D$ −3.8), but this was rationalized by contamination or degradation of the natural product sample. In 2006, the structure of mycothiazole was revised by the original natural product team.[103] The key difference between the synthetic product and the natural product appeared to be not only the specific rotation values but also the carbon chemical shifts of two allylic methylenes. The carbon chemical shifts of C-13 and C-16 in the synthetic product showed a downfield shift by ca. 2 ppm compared to those in the natural product (synthetic: δ 34.7 (C-13), δ 36.6 (C-16); natural: δ 29.4 (C-13), δ 31.5 (C-16)). This evidence corresponded to an upfield shift of an allylic position in a disubstituted *Z* olefin rather than *E* configuration. Thus, the structure of mycothiazole was suggested to possess C-14/C-15 *Z* configuration, which was confirmed by strong NOEs between H-13/H-16 and the newly observed coupling constant ($J_{14,15}$ = 10.7 Hz). Mycothiazole has shown selective cytotoxicity against human lung cancer cell lines (DMM-114 and NCI-H23) and further biological evaluations are being continued.

2.18.5.2.2 Lasonolide A

Lasonolide A was reported in 1994 as a cytotoxic polyketide macrolide isolated from the sponge *Forcepia* sp. collected in the British Virgin Islands.[104] The structure of lasonolide A containing seven double bonds was proposed from the NMR data. Although all the double bonds were disubstituted olefins, one of those was

Table 6 Problematic assignments of C=C bonds

Original structure	*Revised structure*	*Features*
Mycothiazole (sponge) Crews *et al*.[102]	Sonnenschein *et al*.[103]	Revision rationale: reassessment of NMR. Remaining undefined: none. Critical data: (a) upfield carbon chemical shift on C-13 and C-16 of the synthetic product comparing to those of the natural product (synthetic: δ 34.7 (C-13), δ 36.6 (C-16); natural: δ 29.4 (C-13), δ 31.5 (C-16)) was accounted for 14*Z*-isomer, (b) NOE between H13/H16, (c) $J_{14,15} = 10.7$ Hz. Other issues: none.
Lasonolide A (sponge) Horton *et al*.[104]	Lee *et al*.[105] Song *et al*.[106]	Revision rationale: total synthesis. Remaining undefined: none. Critical data: (a) the 28*S* and 28*R* synthetic materials ≠ the natural products, (b) the proton chemical shift of C-19 in the synthetic products = 0.4 ppm downfield comparing to the natural products, suggested 17*E* configuration, (c) the diastereomers, 17*E*,28*S* and 17*E*,28*R* ≠ the natural products, (d) the 17*E*,25*Z*,28*R* isomer = the natural product. Other issues: none.

(*Continued*)

Table 6 (Continued)

Original structure	*Revised structure*	*Features*
Pyrinodemin A (sponge)		Revision rationale: total synthesis. Remaining undefined: none. Critical data: (a) the synthetic product ≠ the natural product, (b) HPLC retention time; the $\Delta^{14,15}$ isomer = the natural product, (c) ESIMS peak (*m/z* 241) of one of the degradation products obtained by treatment of OsO_4 and $NaIO_4$ = the C-15′ aldehyde with the C7′-C-15′ alkyl chain and pyridine. Other issues: the HPLC study suggested that pyrinodemin A exists in a 1:1 racemic mixture.
Tsuda *et al*.[107]	Ishiyama *et al*.[108]	

HPLC, high performance liquid chromatography.

assigned as a terminal olefin ($\Delta^{31,40}$) on the side chain, five double bonds were embedded in the macrocyclic ring ($\Delta^{2,3}$, $\Delta^{4,5}$, $\Delta^{12,13}$, $\Delta^{14,15}$, $\Delta^{16,17}$), and the last one ($\Delta^{25,26}$) was placed in the side chain. The geometries of the olefins were directly determined from the NMR data. NOEs (H-2/H-4, H-3/H-5, H-12/H-37, H-15/H-37) and large coupling constants ($J_{2,3} = 15.4$ Hz, $J_{4,5} = 15.3$ Hz, $J_{14,15} = 15.7$ Hz, $J_{25,26} = 18.0$ Hz) suggested C-2/C-3 *E*, C-4/C-5 *E*, C-12/C-13 *Z*, C-14/C-15 *E*, and C-25/C-26 *E* geometries. The geometry of the remaining C-17/C-18 double bond was determined as *Z* even though the proton signals of H-17 and H-18 were partially overlapped due to no coupling over 15 Hz, and NOE between H_2-16 and H-20α that could be observed when C-17 and C-18 were in a *cis* configuration. While the relative stereostructure excepting the configuration of C-28 for lasonolide A was proposed based on the NOE data, the structure of lasonolide A was revised based on the total synthesis of the natural product in 2002.[105,106] First, the spectral data of both synthetic 28*R* and 28*S* lasonolide A were not identical to those of the natural product. Second, the same group synthesized two lasonolide A diastereomers (28*R* and 28*S* lasonolide A with an enantiomeric B ring), but neither diastereomer exhibited the same spectral data as the natural product. The most significant spectral difference of the synthetic materials was the proton signal of H-19 centered at δ 4.70 ppm for the synthetic product and δ 4.30 ppm for the natural product. This chemical shift difference was quite similar to two geometric isomers of one of the ambruticin synthesis intermediates. In that case, the methine proton chemical shift on the C-2 position of tetrahydropyran with a *trans* double bond appeared 0.4 ppm upfield compared to that of a *cis* double bond. Thus, the geometry of C-17/C-18 double bond was deduced to be of *E* configuration. However, both the 17*E*,28*R* and 17*E*,28*S* synthetic materials were not spectrally identical to the natural product. The chemical shift differences were found primarily in the $\Delta^{25,26}$ olefin and thus the *E* olefin of C-25/C-26 was suspected. Finally, the synthetic diastereomer with 17*E*,25*Z*,28*R* configuration was confirmed as the natural product. Lasonolide A is expected to be a new anticancer lead due to its potent cytotoxicity against human cancer cells A-549 (human carcinoma) and Panc-1 (human pancreatic carcinoma) with an IC_{50} of 8.6 and 89 nmol l^{-1}, respectively.

2.18.5.2.3 Pyrinodemin A

Pyrinodemin A was reported in 1999 as a cytotoxic pyridine alkaloid isolated from the sponge *Amphimedon* sp. collected in Okinawa.[107] The planar structure of this alkaloid was composed of a unique cyclopent[*c*]isoxazolidine connected with a bis-3-alkylpyridine based on the MS and NMR data. Location of the *Z* olefin [δ_C 129.3 (2C), C-16′ and C-17′] was assigned from the ^{13}C chemical shift of the two vinyl carbons (C-15′ and C-18′, δ_C 27.1 each) on the detailed analysis of the EIMS fragment peaks. The relative stereostructure of the bicyclic ring was proposed from rigorous examination of the NOESY data. In 2001, pyrinodemin A was synthesized by two different synthetic groups.[110,111] However, upon completion of the synthesis, both groups concluded that the C-16′/C-17′ double bond was incorrectly assigned due to the observation of two carbon signals for C-16′ and C-17′ separated by 1 ppm in the synthetic product. Based on the comparison of the NMR data of the synthetic materials to those of the natural products, one of the synthetic groups suggested that the revised structure of pyrinodemin A was the $\Delta^{15'16'}$ isomer[110] while the other group proposed the $\Delta^{14'15'}$ isomer.[112] In 2005, the natural product research group that reported the original structure of pyrinodemin A settled dispute by characterizing pyrinodemin A as the $\Delta^{15'16'}$ isomer based on the total synthesis of the isomers and HPLC analysis.[108] The HPLC retention time of natural pyrinodemin A corresponded to that of the synthetic $\Delta^{15'16'}$ isomer. This conclusion was supported by an ESIMS ion peak (*m*/*z* 242 $[M+Na]^+$) of one of the degradation products obtained by treatment of the natural product with OsO_4 and $NaIO_4$, which yielded the C-15′ aldehyde with the pyridine ring and C-7 to C-15′ moiety. Interestingly, chiral HPLC analysis concluded that the natural product exists as a 1:1 racemic mixture.

2.18.5.3 Challenges in Distinguishing Exo Forms from Endo Forms

This section describes two examples, in which it was very hard to distinguish a compound as either exo-type form or endo-type form by NMR analysis (**Table 7**). One structure was revised from endo-type form to exo-type form and the other vice versa. The first structure revision in this section was one of the most challenging examples of structural reassignment since the original (proposed) and the revised (alternative) structures show essentially the same 2D NMR correlation pattern. Thus, either total synthesis or NMR chemical shift simulations are required to confirm the structure. The first structure revision of zamamistatin

Table 7 Distinguishing endo forms from exo forms

Original structure	*Revised structure*	*Features*
Elatenyne and the enyne (alga) Hall and Reiss[113] Wright *et al*.[114]	Sheldrake *et al*.[115]	Revision rationale: total synthesis, NMR assessment. Remaining undefined: absolute structure. Critical data: (spectroscopic data) natural products ≠ synthetic products, ^{13}C NMR < δ 76 (ring CHs for pyrano[3.2-*b*]pyran), ^{13}C NMR > δ 76 (2,2-bifuranyl compound). Other issues: the relative stereo of elatenyne was elucidated by DFT calculations.[116]
4′-Chloroaurone (alga) Atta Ur *et al*.[117]	3-(4′-Chlorophenyl)-isocoumarin Venkateswarlu *et al*.[118]	Revision rationale: total synthesis, reassessment of NMR. Remaining undefined: none. Critical data: (a) the synthetic products both *Z* and *E* isomers of 4′-chloroaurones ≠ the natural products, (b) the 1H NMR data; the natural product = 3-(4′-chlorophenyl)-isocoumarin. Other issues: none.

(Section 2.18.4.2.1) from exo-type form to endo-type form is also categorized into this group, with the final proposed structure resulting from synthetic effort.

2.18.5.3.1 Elatenyne and the related enyne

Elatenyne was reported in 1986 as a new vinylacetylene from the alga *L. elata* collected at Victoria.[113] The structure of elatenyne was first proposed based on the MS and NMR data. The presence of the C_5H_5 side chain (*Z*-pent-3-ene-1-yne) was suggested based on the following spectral information: (1) MS data (m/z 325 $[M–C_5H_5]^+$), (2) IR absorption (terminal acetylene = 3300, 2120 cm^{-1}, *Z* olefin = 3035, 1620, 750 cm^{-1}), (3) UV absorption (enyne = 224, 233 nm), and (4) proton and carbon coupling constants ($^3J_{3,4} = 11.0$ Hz, $J_{CH} = 251$ Hz (acetylene)]. The detailed analysis of the ^{1}H and ^{13}C NMR data including distortionless enhancement by polarization transfer (DEPT) and shift reagent experiments gave the planar structure of elatenyne. The relative stereostructure of the pyrano[3,2-*b*]pyran ring was proposed based on the proton coupling constants obtained by treatment of the natural product with a shift reagent. In 1993, a new elatenyne derivative isolated from the Australian *L. majuscula* was reported.[114] The linear carbon skeleton C-1 to C-15 was established from the ^{1}H-^{1}H and ^{1}H-^{13}C COSY data. The major difference in the new derivative was the presence of an *E* olefin ($^3J_{3,4} = 15.9$ Hz) in the side chain. Determination of the stereostructure of the new derivative was made by the similarity of the coupling constants to that of elatenyne. Although these two compounds were synthesized in 2006,[115] the spectral data of the synthetic products were not identical to those of the natural products. The synthetic groups carefully analyzed the carbon chemical shifts of a number of the pyrano[3,2-*b*]pyran and 2,2′-bifuranyl compounds synthesized in this work. This analysis elucidated the trends of the carbon chemical shifts for these two ring systems. The central oximethine carbons in the pyrano[3,2-*b*]pyran compounds were observed at $<\delta$ 76 whereas those in the 2,2′-bifuranyl compounds occurred at $>\delta$ 76. The oximethine carbons in elatenyne and the derivative were observed at

δ 71.3 and δ 71.4 (elatenyne) and δ 73.9 and δ 70.5, respectively. Thus, the planar structures of these two compounds were revised as 2,2′-bifuranyl derivatives. At this point, the stereostructures remained unsolved. In 2008, the relative stereostructure of elatenyne was suggested based on DFT calculations of all possible 32 diastereomers plus the original structure.[116]

2.18.5.3.2 4′-Chloroaurone

4′-Chloroaurone was reported in 2001 as one of two new aurone derivatives from the marine brown alga *Spatoglossum variabile* collected in Pakistan.[117] The structure of 4′-chloroaurone was deduced by comparison of its MS and NMR data to that of the other new compound, 4′-chloro-2-hydroxyaurone, of which the structure was assembled by analysis of the spectral data. This spectral comparison including the ^{1}H and ^{13}C NMR data as well as the MS data indicated that the structure of 4′-chloroaurone was the dehydration product of 4′-chloro-2-hydroxyaurone on C-2 and C-10. The geometry of the $\Delta^{2,10}$ double bond was proposed to be a *Z* olefin based on the calculation results using the Austin model 1 (AM1) method. In 2007, aurone derivatives including 4′-chloroaurone and its *E* isomer were synthesized to investigate their antioxidant and antibacterial activities.[118] The spectral data of both the synthetic *Z* and *E* isomers of 4′-chloroaurone were not identical to those of the natural product. In fact, the synthetic group revealed that the ^{1}H NMR data of the natural product corresponded to those of the reported value of 3-(4′-chlorophenyl)-isocoumarin. Since the ^{13}C NMR data of the isocoumarin has not been published, the structure of 4′-chloroaurone was tentatively revised as 3-(4′-chlorophenyl)-isocoumarin and further investigation is necessary to confirm the revised structure.

2.18.5.4 Challenges in Stereochemical Assignments

Stereochemical reassignments occur much more frequently than structure revisions and account for about two-thirds of all misassigned marine natural products. Several standard methods to determine stereochemistry have been routinely applied to compounds to finalize the structure elucidation. However, these methods provide incorrect results (opposite configurations) when applied inappropriately. Stereochemical assignments of the structures collected in **Table 8** have recently been revised from those that were originally made by standard methods. In the table, harzialactone A[119] and peribysin E,[120] both of which had their absolute structure established by a modified Mosher's method,[60] turned out to be an antipode by total synthesis.[121,127] In the case of harzialactone A, a modified Mosher's method was incorrectly applied and the chemical shift differences between *R*- and *S* α-Methoxy-α-trifluoromethylphenylacetic acid ester were only measurable from one side of the MTPA ester group. Even when Mosher's method provides the right results, the absolute structure can be incorrect, such as the case of peribysin E. This case was a simple erroneous labeling of the *R*- and *S*-MTPA ester. The stereochemistry of D-*N*-Me-Phe in yanucamides A and B[123] proposed by Marfey's method[61] was later revised to be L configuration by total synthesis.[124] A similar error in assigning *N*-Me-Phe is found in kulokekahilide-2,[125] which was later revised from L to D configuration.[126] These examples show that even the standard stereochemical assignment methods have the potential to lead to structural misassignment. It goes without saying that careful application and interpretation of the results obtained are important to reach the correct stereostructure.

Stereochemical reassignment for the structure with more than three incorrect chiral centers is extremely difficult due to 2^{n-1} potential relative stereostructures. In this situation, synthetic effort, biosynthesis considerations, and X-ray structure analysis are essential to verify the correct stereostructure. The structures depicted in **Table 9** are the examples of the compounds that have had more than three stereocenters revised. The stereochemical reassignment of nakiterpiosin[128] was conducted by a succinct method without synthesizing multiple diastereomers. First, the proposed and revised structures were predicted based on biosynthesis analysis including structure comparison to the same class of compounds (C-nor-D-homosteroid) cyclopamine and veratramine. This new proposed structure was confirmed by agreement of the spectral data between the synthetic material and the natural product. It is remarkable that the original assignment was made using a modified Mosher's method and bioassay performed with only 0.2 mg of the pure material from 30 kg of sponge. Also, in the case of dolastatin 19,[130] the revised structure was proposed prior to the synthesis work by dereplication,[131] whereas the stereostructure of seragakinone A[132] was directly revised based on X-ray structure analysis.[133]

Table 8 Revision of absolute stereochemistry by standard methods

Original structure	*Revised structure*	*Features*
Harzialactone A (fungus) Amagata *et al*.[119]	Mereyala and Gadikota *et al*.[121]	Assignment: modified Mosher's method. Revision rationale: total synthesis. Remaining undefined: none. Critical data: (a) *ent*-harzialactone A = the natural product, (b) $[\alpha]_D$ +33.50 (synthetic product), $[a]_D$ +38.0 (natural product). Other issues: none.
Peribysin E (fungus) Yamada *et al*.[120]	Angeles *et al*.[122]	Assignment: modified Mosher's method Revision rationale: total synthesis Remaining undefined: none Critical data: (a) NMR data; the synthetic product = the natural product, (b) big difference in the optical rotation value, $[\alpha]_D$ −262.2 (synthetic product), $[\alpha]_D$ −52.17 (natural product), (c) the optical rotation of the diacetate, $[\alpha]_D$ −34.78 (synthetic product), $[\alpha]_D$ +35.00 (natural product), (d) the synthetic enantiomer diacetate; $[\alpha]_D$ +37.49 Other issues: none
Yanucamides A and B (cyanobacterium) R = CH_3 (yanucamide A) R = *S*-C_2H_5 (yanucamide B) Sitachitta *et al*.[123]	R = CH_3 (yanucamide A) R = *S*-C_2H_5 (yanucamide B) Xu *et al*.[124]	Assignment: Marfey's method. Revision rationale: total synthesis. Remaining undefined: none. Critical data: (a) both synthetic 3*S*- and 3*R*-yanucamide A ≠ the natural product, (b) major diference; shielded doublet Me (Hiv) at δ_H 0.30 in the synthetic materials vs δ_H 0.66 in the natural product, (c) 3*S*,22*S*-yanucamide A = the natural product. Other issues: none.

Kulokekahilide-2 (mollusk)

Nakao *et al*.[125]

Takada *et al*.[126]

Assignment: Marfey's method (amino acid), chiral HPLC analysis (Hica), synthesis (polyketide chain).

Revision rationale: total synthesis.

Remaining undefined: none.

Critical data: (a) the synthetic product ≠ the natural product, (b) C-21 and C-24 had opposite stereo comparing to those of the related depsipeptide, aurilide, (c) position 43 easily racemize, (d) the synthetic. 21*S*,24*R*,43*R* isomer = the natural product.

Other issues: none.

Table 9 Stereochemical reassignment based on biosynthetic considerations

Original structure	*Revised structure*	*Features*
Nakiterpiosin (sponge) Teruya *et al*.[128]	Chiang *et al*.[129]	Revision rationale: total synthesis. Remaining undefined: absolute structure. Critical data: (a) the synthetic product ≠ the natural product, (b) opposite C-20 stereo, the original structure vs the related compound, (c) the synthetic 6*R*,20*S*,25*S* isomer = the natural product. Other issues: none.
Dolastatin 19 (mollusk) Pettit *et al*.[130]	Paterson *et al*.[131]	Revision rationale: total synthesis. Remaining undefined: none. Critical data: (a) the revised structure was proposed by comparison of the ^{1}H NMR data to those of callipeltoside A and aurisides A and B with same macrocyclic ring system, (b) the spectral data of the revised structure by the total synthesis were identical to those of the natural product, (c) $[\alpha]_D$ +2.2 (synthetic product), $[\alpha]_D$ +7.5 (natural product). Other issues: none.
Seragakinone A (fungus) Shigemori *et al*.[132]	Komatsu *et al*.[133]	Revision rationale: X-ray structure analysis. Remaining undefined: absolute structure. Critical data: the revised structure was proposed by the X-ray structure analysis. Other issues: none.

2.18.6 Completion of Structure Elucidation

Structure elucidation for chiral molecules is complete when the absolute stereostructure is determined. Stereochemical assignment is an important task to connect chemistry and biology since the orientation of functional groups and the ring conformations are key to understanding the mechanisms of biological activities. However, there are many natural products whose 3D structures cannot be solved based only on spectroscopic analysis. In most of these cases, the presence of tiny amount of pure sample makes it very difficult to complete stereochemical assignment. The structures in **Table 10** are two selected examples of marine natural products whose 3D structures have been completed via total synthesis. The first example is a sponge-derived cytotoxic compound, psymberin[134] (also known as irciniastatin A[135]). In 2004, two independent research groups reported a potent cytotoxic pederin class of compound named psymberin and irciniastatin A, which were the C-8 epimers. At that time, psymberin had one uncharacterized stereocenter while five stereocenters of irciniastatin A remained undetermined. Although the ^{1}H and ^{13}C NMR spectra of these compounds were measured in a different solvent, irciniastatin A was expected to be identical to psymberin since the stereochemistries of pedrin class of compounds such as pedrin and mycalamide A corresponded to those of psymberin. The total synthesis of psymberin was completed in 2005 and the remaining chiral center was determined to be 4*S* configuration.[136] In addition, this synthesis demonstrated that irciniastatin A was identical to psymberin. Further synthetic study disclosed that the stereochemistries of two methoxy groups on the side chain are critical to demonstrate its potent cytotoxicity. A second example is the tunicate-derived protein kinase C (PKC) isoform δ inhibitor bistramide A whose original structure with a 19-membered lactam was reported in 1989.[137] The 2D structure of bistramide A was revised in 1992 by 2D incredible natural abundance double quantum transfer experiment (INADEQUATE) experiments.[138] The revised structure possessed a unique acyclic nature containing two polyketide chains with a spiroketal moiety and a substituted tetrahydropyran connected by a central γ-amino acid. At this point, all 11 chiral centers remained unsolved, leaving the absolute stereostructure of bistramide A as one out of 2048 (2^{11}) possible isomers. In 2002, the complete assignment of bistramide C[141] (39-keto-bistramide A) was made by a combination of chiroptic analysis and synthesis of 34-epi-bistramide C.[139] Finally, the absolute stereostructure of bistramide A was confirmed in 2005 by total synthesis.[142]

Total synthesis remains one of the most powerful structure elucidation tools as many structure revisions and stereochemical assignments have been made by total synthesis. In the future, a combination of DFT calculations and total synthesis is expected to be a more important structure elucidation tool to finalize 3D structures since natural products chemistry has been shifting to focus to more minor secondary metabolites, which often have complex cores and multiple chiral centers. For example, amphezonol A,[143] which was recently reported as a C_{60} polyhydroxy linear polyketide with a tetrahydrofuran ring and two tetrahydropyran rings isolated from a dinoflagellate, possesses 19 unassigned stereogenic centers – meaning that the absolute structure of amphezonol A is one of 524 288 (2^{19}) possible stereoisomers (**Figure 12**).

Amphezonol A
$C_{62}H_{114}O_{24}$

Figure 12 Structure of amphezonol A.

Table 10 Completion of stereochemical assignments via synthesis

Original structure	*Complete structure*	*Features*
Psymberin (sponge) Cichewicz *et al*.[134]	Psymberin Jiang *et al*.[136]	Stereochemistry determination: total synthesis. Remaining undefined: none. Critical data: none. Other issues: none.
Irciniastatin A (sponge) Bistramide A (tunicate) Pettit *et al*.[135]		

Degnan *et al*.[137]

Statsuk *et al*.[141]

Revision rationale: 2D INADEQUATE.
Stereochemical determination: total synthesis.
Remaining undefined: none.
Critical data: (a) the carbon skeleton was confirmed by the result of 2D INADEQUATE experiment,[138] (b) a combination of chiroptic analysis and synthesis work led to the assignment of bistramide C (39-keto-bistramide A),[139] (c) spectral data (NMR, $[\alpha]_D$, MS); the synthetic product = the natural product.
Other issues: mode of action of cytotoxicity shown by bistramides = actin.[140]

2.18.7 Conclusions

The case examples introduced in this chapter demonstrate that pitfalls leading to misassignment lie in each step of structure elucidation, from MF assignment to determination of the absolute stereostructure. Analysis of the misassigned molecules shows that assignment errors occur frequently when NMR signals are overlapped, or in molecules which have a low H/C ratio core. The former case can be avoided using several different NMR solvents, whereas simulated NMR chemical shifts obtained by calculation such as DFT methods are useful for the latter case. Furthermore, it is often necessary to reconsider the MF again when high-resolution MS data has greater than ± 5 mmu error. Another impediment to structure elucidation is the bias of the individual, which leads to misassignments. To overcome this prejudice, it is necessary to analyze and validate all possible alternative structures against the working structure.

Abbreviations

APCI	atmospheric pressure chemical ionization
CAST	canonical-representation of stereochemistry
CI	chemical ionization
DFT	density functional theory
EI	electron impact
GIAO	gauge-independent atomic orbital
HRMS	high-resolution MS
HTMMD	3-hydroxy-2,2,4-trimethyl-7-methoxydecanoic acid
MF	molecular formula
mmu	milli mass unit
MS/MS	tandem MS
MT-MMP	membrane-type matrix metalloproteinase
OAT	ornithine δ-amino transferase
OMCA	1,2-oxazetine-4-methyl-4-carboxylic acid
PKC	protein kinase C

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Biographical Sketch

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