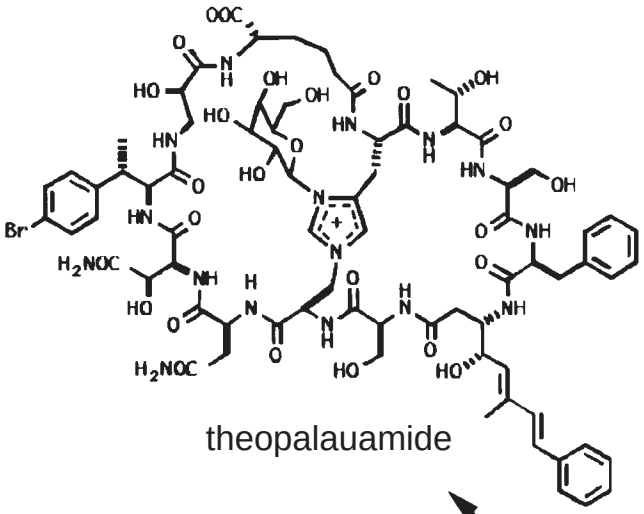
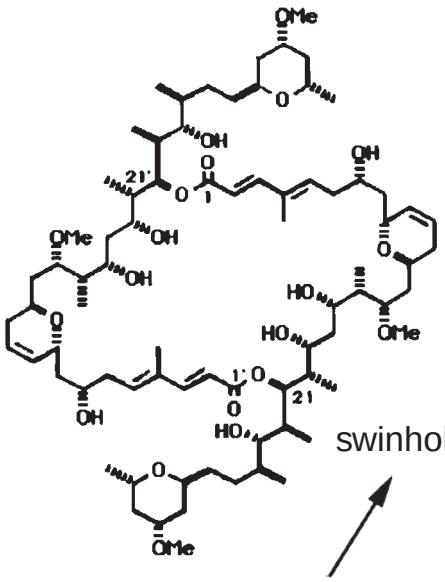
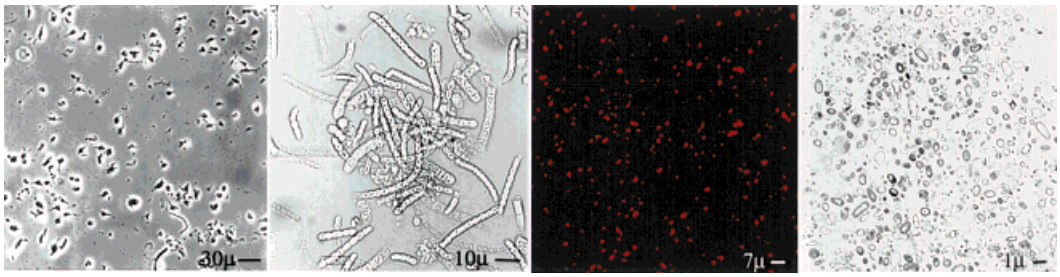


theopalauamide

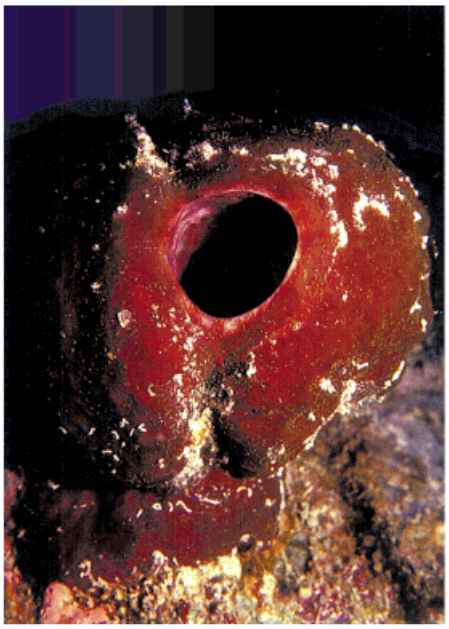


swinholide A

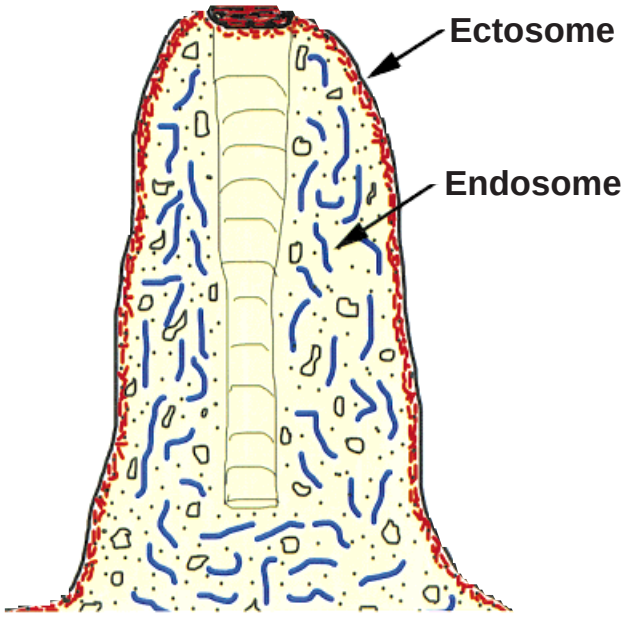
Micrographs of purified cell types. Associated chemistry shown above



Sponge cells | **Filamentous bacteria** | **Unicellular cyanobacteria** | **Unicellular bacteria**



Underwater photo of the lithistid sponge *Theonella swinhoei*



Schematic cross section showing location of bacterial symbionts and sponge cells in *T. swinhoei* (legend shown in center panel)

Lithistid Sponges: Star Performers or Hosts to the Stars

Carole A. Bewley and D. John Faulkner*

The lithistid sponges are almost unique among marine invertebrates because they contain so many different classes of compounds. After defining the term "lithistid", we review the chemistry of the lithistid sponges and illustrate most of the important compounds that have been reported. We then describe the strategies that we have employed in the structural elucidation of cyclic peptides. Whereas most natural product studies end when the structure has been elucidated, we were intrigued by the fact that many lithistid metabolites resembled secondary metabolites from microorganisms and decided to investigate the source of two bioactive metabolites at the cellular level. Using the sponge *Theonella swinhoei*, we were able to separate three types of microorganisms from the sponge cells and analyze the metabolites from each cell type, even after the cells had been fixed. Although there had been speculation that both swinholide A and the bicyclic peptides were produced by cyanobacteria (blue-green algae), our

1. Introduction

Lithistid sponges are renowned among marine organisms for their ability to produce a diverse array of biologically active metabolites. Representative compounds include polyketides, cyclic peptides, alkaloids, pigments, and novel sterols. This extreme diversity of metabolites defied simple chemotaxonomic rationalization until we demonstrated that symbiotic microorganisms were responsible for the production of some representative compounds.[1] In this review we will present a summary of the chemistry attributed to lithistid sponges and outline our research on the chemical relationships between a lithistid sponge and its symbionts.

2. What is a Lithistid Sponge?

The sponge order Lithistida (Porifera, Demospongiae) is an artificial assemblage of species of diverse origins that are grouped together because they have skeletons consisting in large part of fused or interlocked spicules called desmas.[2] The rigid skeleton of fused desmas dictates the shape of the lithistid sponge and also serves to divide the sponge into external (ectosome) and internal (endosome) tissues. From the viewpoint that the lithistid sponges have diverse origins, one might predict the lack of a chemotaxonomic theme among the metabolites. However, one would not expect the dominance of biologically active compounds in sponges that are so well physically protected by a shield of fused siliceous spicules. Lithistid sponges have a quite ancient lineage, being well represented in the Cambrian fossil record. In modern times lithistid sponges are predominantly found in deeper waters, which presents the collector with considerable logistical problems, and which has resulted in chemical investigations being concentrated on those genera that can be found at normal scuba depths. A summary of the taxonomic classification of lithistid sponges[3] is shown in Figure 1.

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3. Chemistry of the Lithistid Sponges

The underlying chemotaxonomic theme of the chemistry of lithistid sponges is that there is no theme. Almost every class of compound is represented in a seemingly random manner. However, the incidence of biologically active compounds with unusual structures is very high and this may indeed be the underlying theme.

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Phylum Porifera
Class Demospongiae
Subclass Tetractinomorpha
Order Lithistida
Family Theonellidae
Genera ***Discodermia, Jereicopsis***, *Racodiscula,* ***Theonella***
Family Phymatellidae
Genera ***Neosiphonia, Reidispongia***
Family Phymaraphinidae
Genus *Kaliapsis*
Family Corallistidae
Genera ***Callipelta, Corallistes,*** *Homophymia, Macandrewia*
Family Neopeltidae
Genus *Neopelta*
Family Pleromidae
Genus ***Pleroma***
Family Dorydermidae
Genus *Anaderma*
Family Scleritodermidae
Genera ***Aciculites***, *Amphibleptula,* ***Microscleroderma***, *Scleritoderma*, *Tabropane*
Family Siphonidiidae
Genera *Gastrophanella, Leiodermatium, Siphonidium*
Family Desmantidae
Genus *Desmanthus*
Family Petromicidae
Genus *Petromica*
Family Vetulinidae
Genus *Vetulina*

Figure 1. Current classification of extant lithistid sponges.[3]

3.1. Sterols

Even the earliest studies of lithistid sponges resulted in the isolation of unusual compounds. In 1981, Djerassi and co-workers identified the 4-methylene sterols conicasterol (**1**) and theonellasterol (**2**) as the major sterols from *Theonella conica* and *T. swinhoei*.[4] Two years later, the same research group reported the isolation of pulchrasterol (**3**) and other Δ^7 sterols from the New Zealand deep water lithistid sponge *Aciculites pulchra*.[5] The Δ^7 sterols are not normal sponge sterols and are normally associated with starfish and sea cucumbers.[6] The 4-methylene sterols appear to be unique to *Theonella*, although 4-methylsterols are often found in dinoflagellates.[6, 7] Subsequently, the corresponding 3-keto-4-methylene sterols, theonellasterone (**4**) and conicasterone (**5**), were isolated from *T. swinhoei*, together with the Diels–Alder adducts, bisconicasterone (**6**) and bistheonellasterone (**7**), and nine minor oxygenated 4-methylene sterols.[8–10] Interestingly, the 3-keto-4-methylene sterols **4** and **5** were obtained as crystals from the tissues of the sponge.[8] More recently, it has been found that in *Jereicopsis graphidiophora*[11] and *Microscleroderma spirophora*[12] the normal 3β-hydroxysterols are replaced by 3β-methoxysterols, which include the bisenol ether **8**.[12]

3.2. Macrolides and Acetogenins

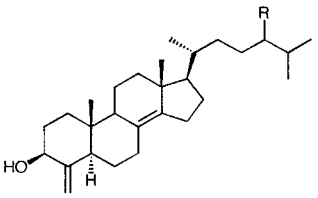
The isolation and identification of swinholide A (**9**) provided natural product chemists with a very valuable lesson in the art of structural elucidation. Swinholide A, which is a potent cytotoxic agent from *T. swinhoei*, was first reported to be a monomeric 22-membered lactone,[13] while misakinolide A from *Theonella* sp. was first identified as a monomeric

D. John Faulkner received his B. Sc. and Ph. D. degrees from Imperial College, London, where he studied synthetic organic chemistry under the guidance of Sir Derek Barton. After receiving postdoctoral training from R. B. Woodward at Harvard University and W. S. Johnson at Stanford University, he joined the faculty of the Scripps Institution of Oceanography, University of California, San Diego, where he is now Professor of Marine Chemistry. At the urging of his colleagues to "do something more marine", he turned his attention to marine natural products chemistry and has never regretted making the change in research emphasis. His research interests include the structural elucidation of marine natural products, their biomedical potential, and their role in the life cycles of the marine environment.

D. J. Faulkner

C. A. Bewley

Carole Ann Bewley, born October 27, 1963, received her B. Sc in Chemistry from San Diego State University in 1985. After working as a research associate at the Salk Institute for Biological Studies in the group of Professor Jean Rivier, she joined the graduate program at Scripps Institution of Oceanography, University of California, San Diego, and completed her doctoral studies in 1995 under the supervision of Professor D. John Faulkner. She is currently a Cancer Research Institute postdoctoral fellow at the National Institutes of Health in the laboratory of Dr. G. Marius Clore.



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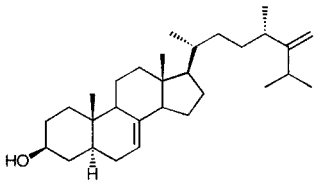
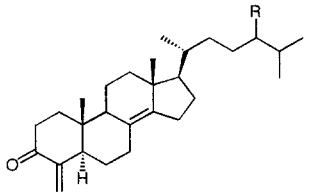
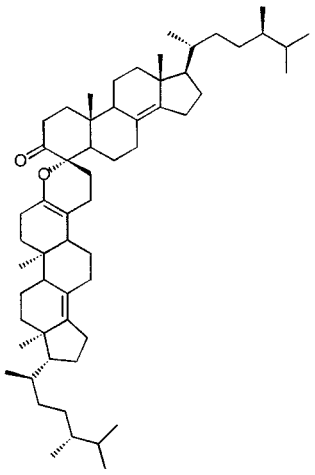
2, R = α-Et, theonellasterol



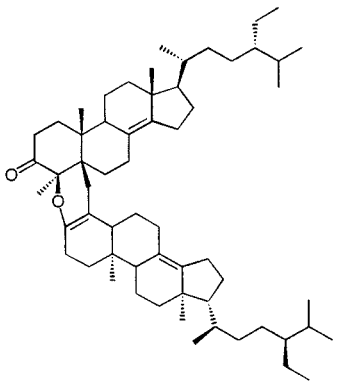
3 pulchrasterol



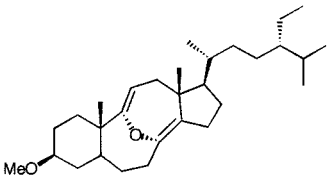
4, R = α-Et, theonellasterone
5, R = β-Me, conicasterone



6 bisconicasterone



7 bistheonellasterone



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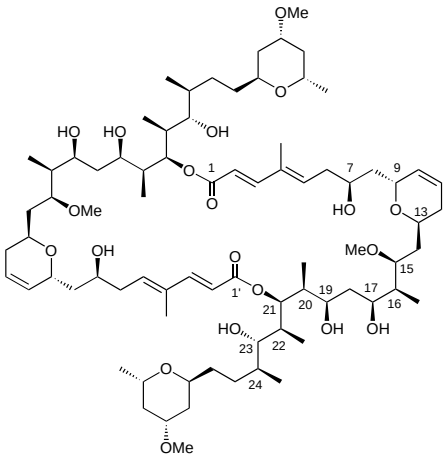
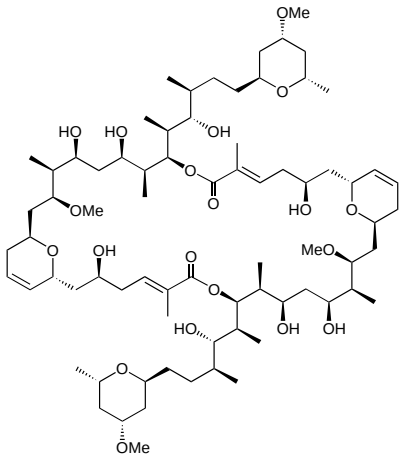
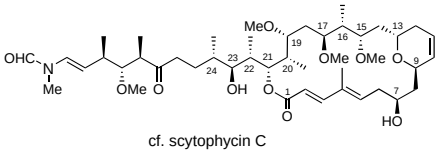


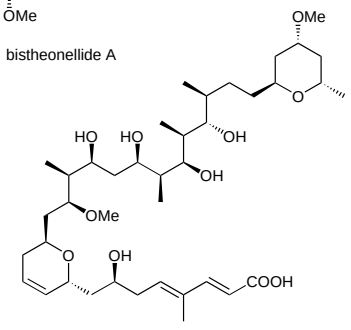
9 swinholide A



10 bistheonellide A



cf. scytophycin C

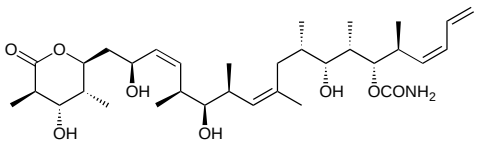


11 preswinholide A

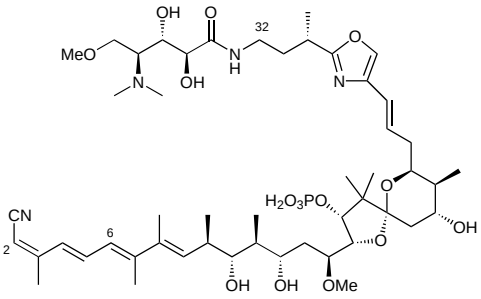
20-membered lactone that lacked a disubstituted olefin in the lactone ring.[14] Both compounds gave the correct elemental analytical results and misakinolide A even gave a strong $[M^{+}+H]$ peak for the monomer in the mass spectrum. The symmetrical dimeric structures of both swinholide A and misakinolide A first became apparent during the structural elucidation of bistheonellides A (**10**) and B from *Theonella* sp., when it was discovered that bistheonellide A (**10**), which was spectroscopically identical to misakinolide A,[15, 16] gave an unsymmetrical tribenzoate as one of its esterification products and a "dimeric" molecular ion in the mass spectrum. The dimeric 44-membered ring structure and absolute stereochemistry of swinholide A (**9**) were established unambiguously by X-ray crystallographic analysis.[17] Since then, a number of dimeric macrolides of the swinholide[18, 19] and bistheonellide[20] families, together with preswinholide A (**11**),[21] the monomer of swinholide A (**9**), have been reported. Of these compounds, swinholide A (**9**) was shown to be the most cytotoxic[22] and has, therefore, been the target of both conformational[23] and synthetic studies that culminated in two excellent total syntheses.[24, 25]

The X-ray structure of discodermolide (**12**), an immunosuppressive and cytotoxic agent from *Discodermia dissoluta*, revealed a novel "polypropionate" structure.[26] Owing to its unusual and potentially useful biological profile, which includes the ability to stabilize microtubules more potently than taxol,[27] and the real or perceived difficulty in obtaining large supplies of the drug from natural sources, discodermolide (**12**) has become a popular target for synthetic programs.[28, 29] The calyculins, exemplified by calyculin A (**13**),[30] are a family of eight potent antitumor agents from *D. calyx* that inhibit protein phosphatases 1 and 2A. Calyculins B, E, and F are geometrical isomers of calyculin A (**13**) with respect to the C2–C3 and C6–C7 bonds while calyculins C, D, G, and H are a homologous series with a methyl group at C-32.[31–34] *D. polydiscus* contained the cytotoxic and antifungal macrocyclic lactam discodermide (**14**).[35]

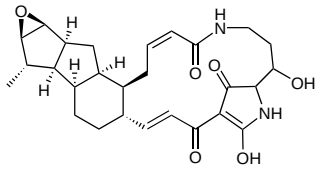
In 1988, onnamide A (**15**) was isolated from an Okinawan species of *Theonella*.[36] Onnamide A (**15**), whose structure strikingly resembled that of the insect toxin pederin,[37] was shown to possess antiviral properties and was subsequently synthesized by Hong and Kishi.[38] An additional nine compounds in the onnamide series were subsequently reported together with their cytotoxicities against the P388 cell line.[39, 40] The theopederins, exemplified by theopederin A (**16**), are cytotoxins from *Theonella* sp. that



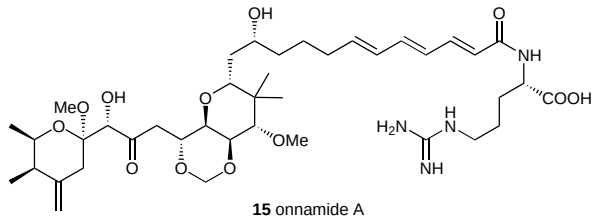
12 discodermolide



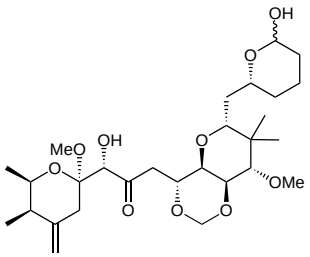
13 calyculin A



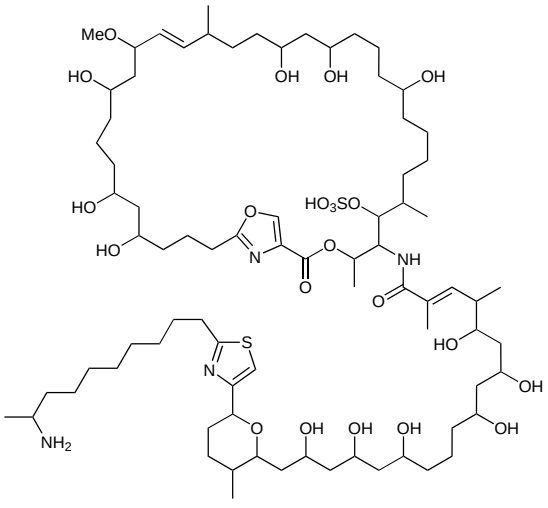
14 discodermide



15 onnamide A



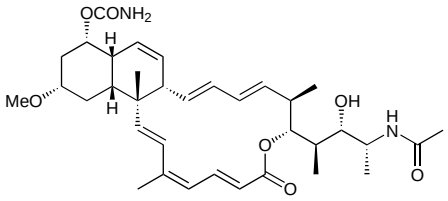
16 theopederin A



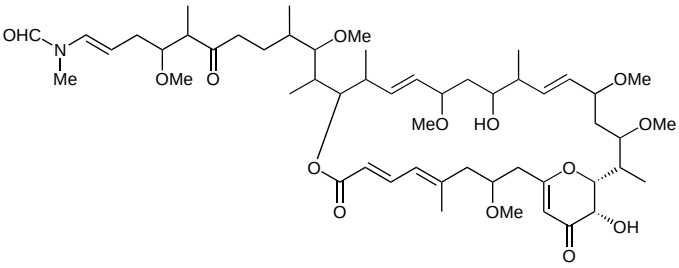
17 theonezolide A

have slightly simpler structures.[41] The most complex of the macrolides from *Theonella* sp. are the cytotoxic theonezolides A (**17**), B, and C, for which there is limited stereochemical data.[42, 43]

Neosiphonia superstes is a deep water lithistid sponge that was obtained by dredging. It is the source of superstolides A (**18**)[44] and its 24,25-dehydro derivative superstolide B,[45] as well as sphinxolides B (**19**), C, and D,[46] all of which are potent cytotoxic macrolides. Sphinxolide itself was originally ob-



18 superstolide A



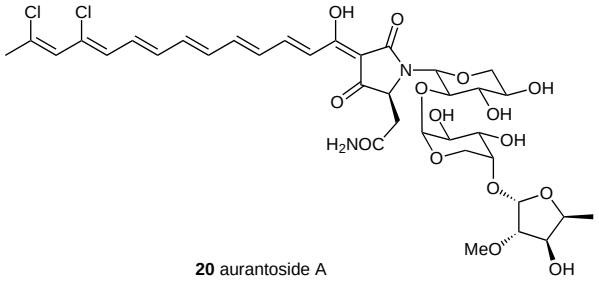
19 sphinxolide B

tained from an unidentified Pacific nudibranch (sea slug) which had evidently consumed a sponge that contained the macrolide.[47] The sphinxolides B (**19**) and D were also found, together with two related cytotoxic macrolides, reidispongiolides A and B, from *Reidispongia coerulea*, which was also dredged from a depth of 500 m in the same region south of New Caledonia.[48]

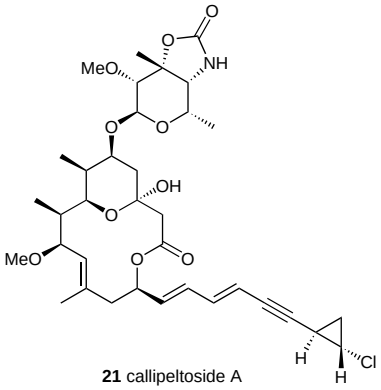
There are two examples of lithistid sponges that contain chlorinated acetogenins with added sugar substituents. Aurantosides A (**20**) and B are cytotoxic orange pigments from a Japanese *Theonella* sp.[49] A New Caledonian species of *Callipelta* contains callipeltosides A (**21**), B, and C, which are most unusual cytotoxic macrolides that contain a chlorocyclopropane group.[50,51]

3.3. Alkaloids and Other Nitrogenous Bases

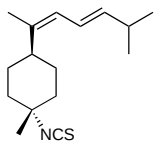
Seven bisabolene sesquiterpenes exemplified by theonellin isocyanate (**22**) from *Theonella swinhoei* from Okinawa[52] and aminobisabolene (**23**) from an Okinawan *Theonella* sp.[53] were among the earliest nitrogenous metabolites to be attributed to lithistid sponges. Theonelladin A (**24**) is one of four antineoplastic (tumor inhibiting) pyridine alkaloids from an Okinawan specimen of *T. swinhoei*.[54] Examples of both of the preceding classes of alkaloids are commonly found in non-lithistid sponges. One of the most unusual natural products from *Theonella* sp. is the mildly antimicrobial and cytotoxic isoquinoline alkaloid theoneberine (**25**),[55] which



20 aurantoside A



21 callipeltoside A



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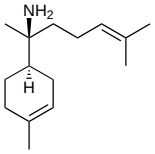

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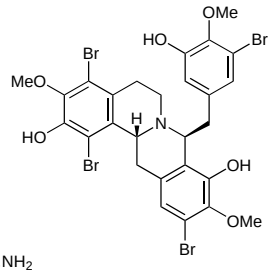
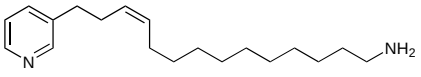
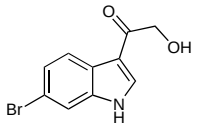


25 theoneberine



24 theonelladin A



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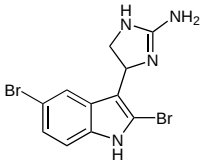
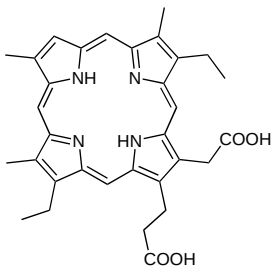


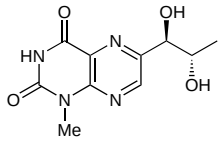
27 discodermindole

resembles a terrestrial alkaloid. The relatively simple metabolites ethyl 6-bromo-3-indolcarboxylate and 3-hydroxyacetal-6-bromoindole (**26**) were isolated from the deep water lithistid *Pleroma menoui*[56] while *D. polydiscus* contained the moderately cytotoxic brominated indole derivative discodermindole (**27**).[57]

The natural function of five cytotoxic porphyrin pigments, exemplified by corallistin A (**28**), from a New Caledonian species of *Corallistes* is something of a mystery since there is almost no light at the depth of 300 m. from which the sponge was collected.[58, 59] Other *Corallistes* species contain pteridines, such as 1-methylpteridine-2,4-dione from *C. fulvodesmus* and the 6-dihydroxypropyl derivative **29** from *C. undulatus*,[60] and corallistine (**30**) also from *C. fulvodesmus*.[61] Finally, there is the chlorinated nucleoside kumusine (**31**) that was isolated from a *Theonella* sp. and shows moderate immunosuppressive activity and cytotoxicity.[62]



28 corallistin A



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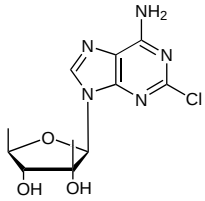
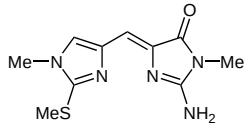


31 kumusine



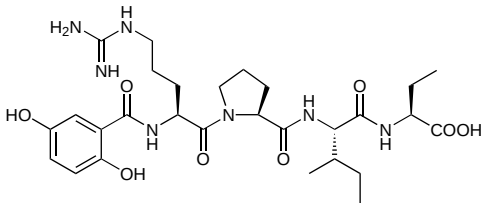
30 corallistine

3.4. Peptides

In 1993, Fusetani and Matsunaga wrote an excellent review of "Bioactive Sponge Peptides" that included detailed accounts of the peptides from lithistid sponges.[63] Rather than duplicate their review, we will simply emphasize the broad range of peptide structures found in lithistid sponges and provide an update to the information in that review. The most remarkable features of the peptides from lithistid sponges are the unusual range of bioactivities and the almost ubiquitous inclusion of nonstandard as well as D-amino acids.

3.4.1. Linear Peptides

The same *Theonella* sp. that produced the cyclotheonamides (see Section 3.4.2) also contained the thrombin inhibitor nazumamide A (**32**), which is a relatively simple tetrapeptide in which the N-terminus is capped as a 2,5-dihydroxybenzamide group.[64] In contrast, polytheonamides

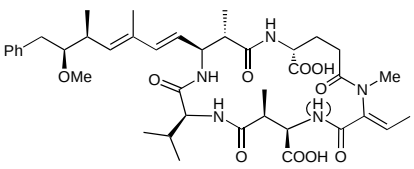


32 nazumamide A

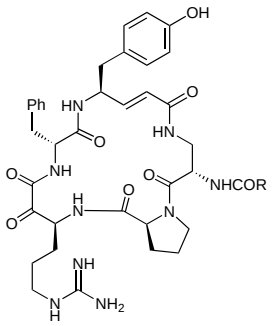
A – C from *T. swinhoei* are highly cytotoxic linear 48-residue peptides that are characterized by an unusually high number of *tert*-leucine residues.[65]

3.4.2. Cyclic Peptides

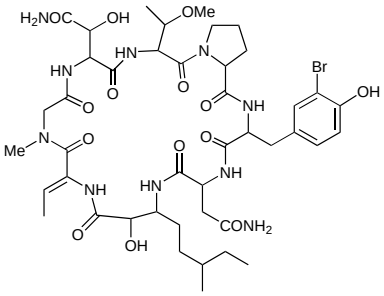
Two of the more spectacular cyclic peptides to be found in lithistid sponges are cyclotheonamides A (**33**) and B (**34**) from a Japanese species of *Theonella*, which were described in 1990 as potent thrombin inhibitors.[66] The presence of



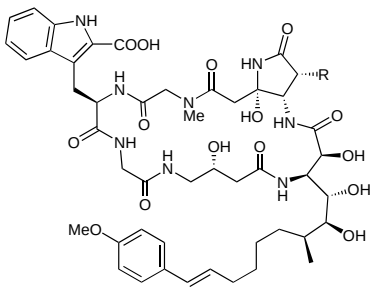
35 motuporin



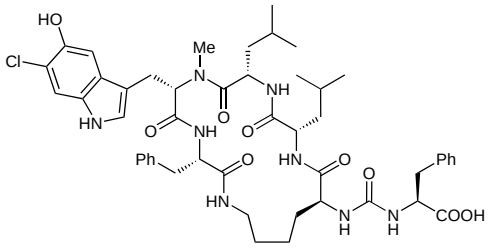
33, R = H, cyclotheonamide A
34, R = Me, cyclotheonamide B



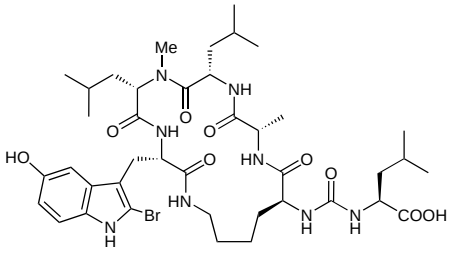
36 perthamide B



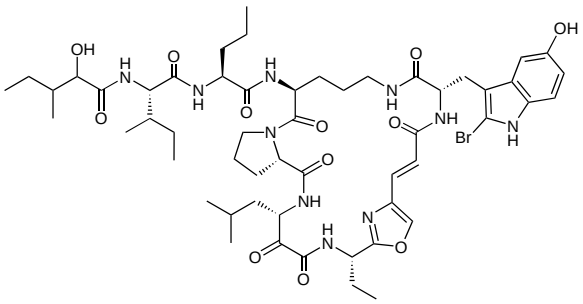
37, R = OH, microsclerodermin A
38, R = H, microsclerodermin B



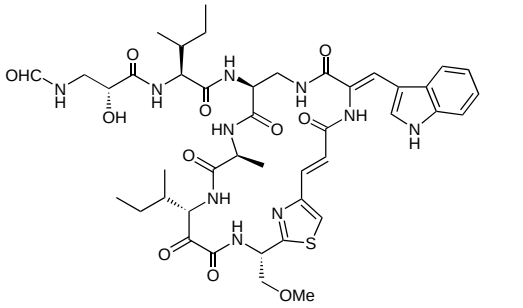
39 keramamide A



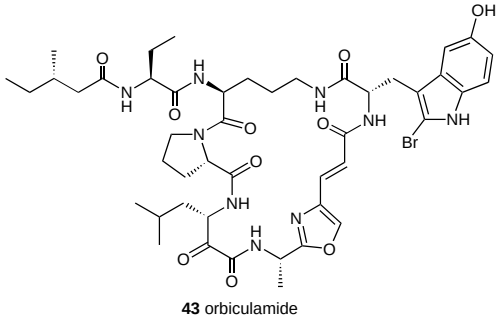
40 konbamide



41 keramamide B



42 keramamide F



43 orbiculamide

the α-ketoamide functionality in the cyclotheonamides attracted the attention of those working on FK506 and cyclosporin, and cyclotheonamide was soon synthesized, which resulted in the reassignment of the stereochemistry.[67] This synthesis was followed by a number of studies to define the molecular basis for the mechanism of action of the cyclotheonamides as serine protease inhibitors,[68] additional synthetic studies,[69] and the structural elucidation of three more analogues, cyclotheonamides C–E.[70] Motuporin (**35**) is a potent inhibitor of protein phosphatase 1 and cytotoxin that was isolated from a specimen of *T. swinhoei* from Papua New Guinea.[71] The structure of the pentapeptide motopurin (**35**) differs from that of the cyanobacterial metabolite nodularin[72] by the substitution of (*S*)-arginine by (*S*)-valine. The cytotoxic octapeptide perthamide B (**36**), which weakly inhibits the binding of interleukin-1β to EL4.1.6 cells is a cyclic octapeptide from an Australian *Theonella* sp. [73] A deep water *Microscleroderma* species from New Caledonia contained two antifungal cyclic peptides, microsclerodermins A (**37**) and B (**38**), which possess a very complex β-amino acid moiety.[74]

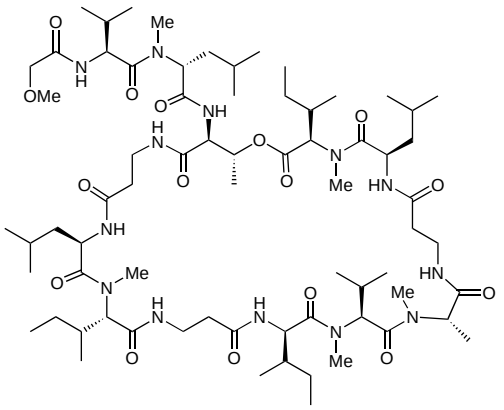
The sarcoplasmic reticulum Ca^{2+}-TPase inhibitor keramamide A (**39**), which was isolated from an Okinawan *Theonella* sp., is a cyclic hexapeptide that contains an unusual urea functionality in the side chain.[75] Konbamide (**40**), which is a calmodulin antagonist, is a very similar cyclic hexapeptide with one urea group that was isolated by the same research group from a different specimen of *Theonella* sp. from Okinawa. The proposed structure of konbamide (**40**) has recently been questioned as a consequence of the results from a synthetic study.[76] Although from the same sponge, the structures of keramamides B (**41**) and C–H are quite different from that of keramamide A (**39**), with all containing an α-ketoamide functionality and either an oxazole (keramamides B (**41**), C, D, and E) or a thiazole (keramamides F (**42**), G and H) moiety in the cyclic peptide ring system.[77–79] Orbiculamide (**43**) is a cytotoxic peptide from a Japanese

Theonella sp. that is closely related to keramamide D.[80] The structures of the discobahamins A and B obtained from a deep water specimen of *Discodermia* sp. collected off the Bahamas differ from that of keramamide D only in the nature of the side chain.[81]

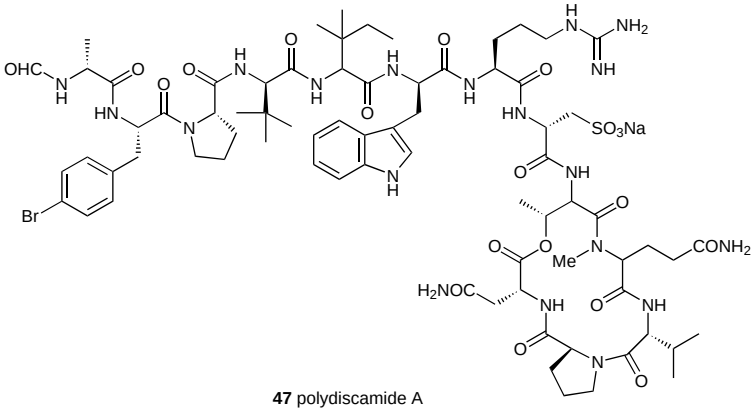
3.4.3. Cyclic Depsipeptides

In 1984, Fusetani's group described discodermin A as an antimicrobial cyclic tetradecapeptide lactone from *Discodermia kiiensis*.[82] Subsequent papers reported the structures of discodermins B – H,[83–86] but in the process the structures of discodermins A (**44**) and B – D were revised.[86] The range of biological activities of this series of compounds has also been expanded: discodermin A (**44**) inhibited the tumor promotion activity of okadaic acid,[87] discodermins A – D are potent inhibitors of phospholipase A_2,[88] and all the members of the series are cytotoxic. Discokiolides A (**45**) and B – D are cytotoxic depsipeptides from *D. kiiensis* that have a unique oxazole-containing β-hydroxy acid forming the side chain.[89]

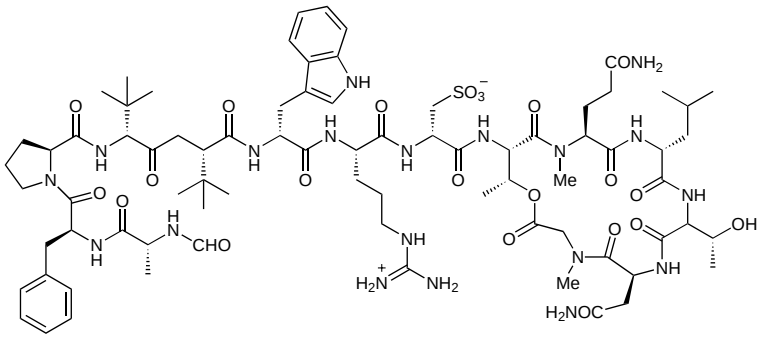
In 1986, Kitagawa's group described the isolation of the theonellapeptolides, which inhibited cell division in fertilized sea urchin eggs, and showed that theonellapeptolide Id (**46**) was a cyclic depsipeptide.[90] Shortly after, Nakamura et al. reported the same compound as theonellamine B from an Okinawan species of *Theonella* and recorded its activity as a Na^+,K^+-ATPase inhibitor.[91] In due course, five more theonellapeptolides (Ia, Ib, Ic, Ie, and IId) were described as cytotoxic tridecapeptide lactones from *T. swinhoei*.[92–95] Polydiscamide A (**47**), which was isolated from *D. polydiscus*, is also a cytotoxic tridecapeptide cyclic lactone albeit with a smaller ring size.[96]



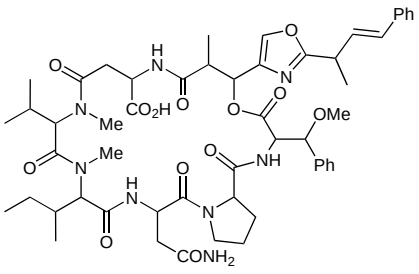
46 theonellapeptolide I d



47 polydiscamide A



44 discodermin A

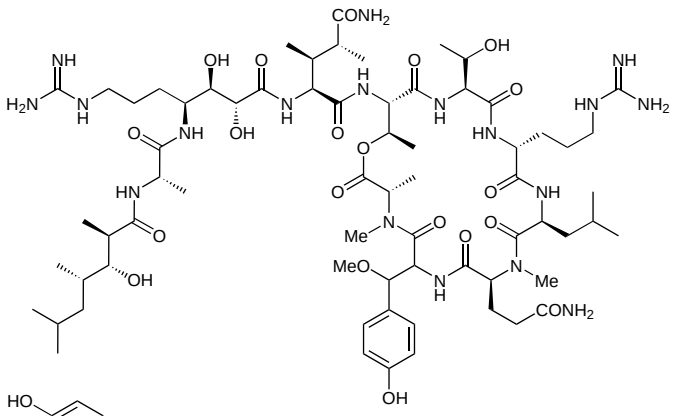


45 discokiolide A

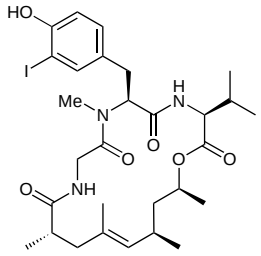
Recently, Minale's group has reported a series of cyclic depsipeptides from a New Caledonian species of *Callipelta*. The structural elucidations of callipeltins A (**48**) B, and C, which possess anti-HIV, antifungal, and cytotoxicity activities, revealed a side chain that terminated with an unusual polypropionate moiety.[97, 98] Along with the sphinxolides reviewed above, *Neosiphonia superstes* contained the small cyclic depsipeptide neosiphoniamolide A (**49**), which is remarkably similar in structure to the geodiamolides and jaspamide that had previously been isolated from nonlithistid sponges.[99]

3.4.4. Bicyclic Peptides

The 1989 paper by Matsunaga et al. on the structural elucidation of the antifungal bicyclic peptide theonellamide F (**50**) from a Japanese species of *Theonella* was a landmark paper in the history of marine natural products chemistry because it presented an elegant solution to a structural problem that was a degree of difficulty beyond the research of the time.[100] This paper inspired us to examine a Philippine specimen of *Theonella*



48 callipeltin A

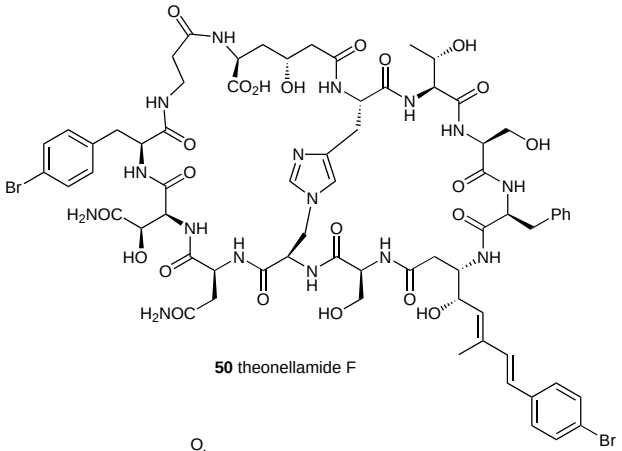


49 neosiphoniamolide A

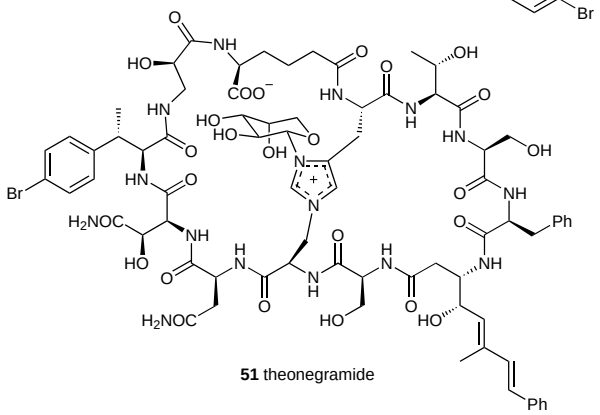
swinhoei, from which we isolated theonegramide (**51**), the first example of a glycopeptide from a lithistid sponge.[101] Matsunaga and Fusetani have subsequently reported the structures of the cytotoxic bicyclic peptides theonellamides B and C and the cytotoxic glycopeptides theonellamides A, D, and E.[102] The structural elucidation of the cytotoxic and antifungal bicyclic lipoglycopeptides aciculitins A – C (**52** – **54**) and the artifacts aciculitamides A (**55**) and B from *Aciculites orientalis* is undoubtedly the most complex problem that has been tackled in our laboratory and is featured below.[103]

4. Structural Elucidation of Cyclic Peptides from Lithistid Sponges

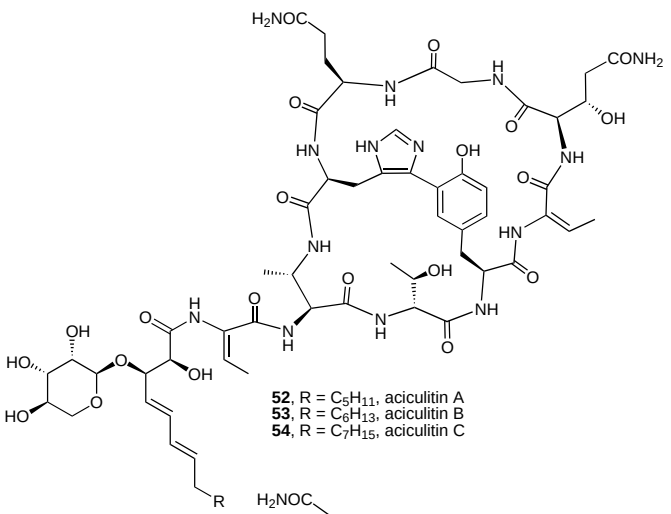
The majority of the techniques used in the structural elucidation of cyclic peptides, depsipeptides, and glycopeptides are standard and are quite familiar to marine natural products chemists. However, there are still some lessons to be learned and improvements to be made. We regard our preferred isolation method as one such improvement. When we extracted *A. orientalis* with methanol, we obtained a crude extract that showed strong antifungal activity but gave very poor NMR spectra. As the purification proceeded, with methanol as the eluent in most chromatographic procedures, the antifungal activity decreased and the spectra improved until the artifacts, aciculitamides A (**55**) and B, were isolated. Both compounds contained a methoxy group that had resulted from addition of the solvent to an autoxidation product. When we obtained a new collection of the sponge, we devised an extraction procedure that avoided the use of methanol and have since found that it gives superior results in most cases.[103] The sponge is diced and lyophilized, then extracted sequentially with hexane and ethyl acetate at room



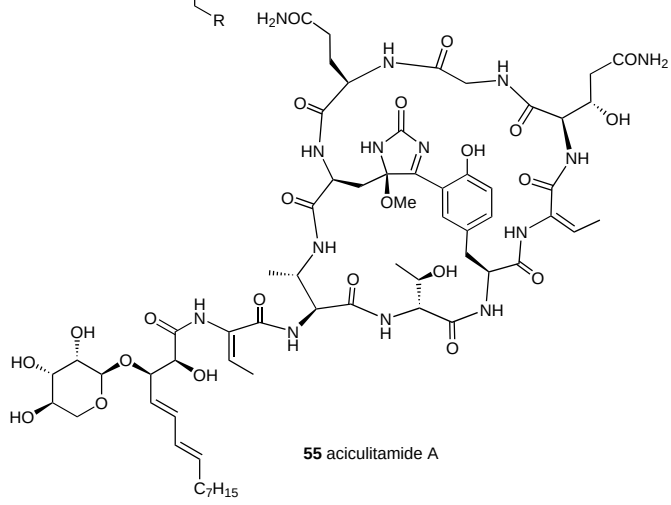
50 theonellamide F



51 theonegramide



52, R = C_5H_{11}, aciculitin A
53, R = C_6H_{13}, aciculitin B
54, R = C_7H_{15}, aciculitin C

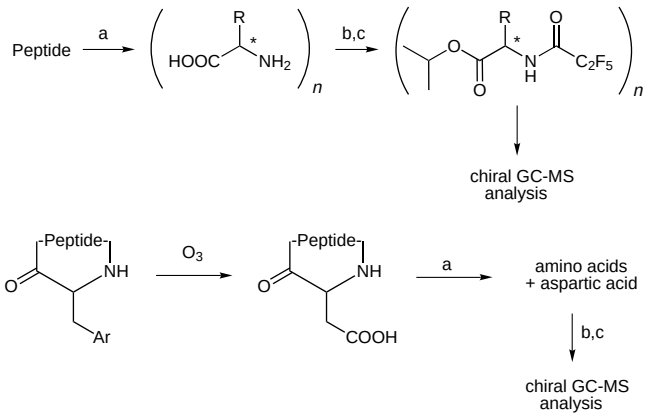


55 aciculitamide A

temperature. After removal of residual organic solvents, the sponge is then extracted with water:acetonitrile (1:1) at room temperature until the extracts no longer show antifungal activity and/or a positive color reaction for the peptide on thin-layer chromatography. The acetonitrile is evaporated on a rotatory evaporator and the remaining water lyophilized to obtain a powder that is of much higher purity than a corresponding methanol extract.

An attractive feature of the peptides from lithistid sponges is the presence of unusual amino acids. The identification of unusual amino acids can usually be accomplished from the ^{1}H and ^{13}C NMR data together with the COSY, TOCSY, HMBC, and HMQC nmr experiments. Problems arise when the amino acids are not joined through standard peptide bonds, but instead with unusual linkages such as those between histidine and alanine in theonellamide F (**50**) and theonegramide (**51**) and between histidine and tyrosine in the aciculitins (**52**–**54**). In such cases, and particularly for aciculitamide A (**55**), a combination of HMBC and ROESY data must be used to establish the location of nonpeptidal bonding between the residues. The sequence in which individual amino acids are linked through peptide or lactone bonds is determined by C–H correlations through two and three bonds. However, in some bicyclic peptides the required correlation data may be missing when the molecule either adopts a conformation in which the dihedral angles are inappropriate for scalar coupling or is undergoing motion that is fast on the NMR time scale. This occurred in the aciculitins (**52**–**54**) when no long-range coupling was observed across the peptide bonds adjacent to the bicyclic ring junctions. The solution was provided by a ROESY experiment that indicated the proximity of hydrogen atoms on adjacent amino acid residues.[103]

The peptides from lithistid sponges often contain both D- and L-amino acids, which makes the determination of the absolute configuration of each individual amino acid an essential part of every structural elucidation. We selected mass spectrometry coupled to gas chromatography on a chiral phase as the method of choice for the determination of the absolute configuration of the amino acids that resulted from acid hydrolysis of the peptides since it requires the least amount of material and, more importantly, the identity of the peaks can be confirmed by MS (Scheme 1, top). The method depends on having reference standards for both D- and L-amino acids and thus determination of the absolute configuration of unusual amino acids can be problematic. There are two potential solutions. One can often obtain standards of unusual amino acids through the generosity of colleagues who have isolated or synthesized the required compounds or by in house synthesis. Alternatively, one can chemically modify the peptide prior to hydrolysis to convert the unusual amino acid residue into one of the standard amino acids, and then determine the absolute configuration of the newly formed amino acid. For example, aromatic amino acids that are substituted on the aromatic rings can be converted by ozonolysis into aspartic acid residues, which are then analyzed by GC-MS on a chiral phase (Scheme 1, bottom). In the aciculitins (**52**–**54**) the two aromatic residues, histidine and tyrosine, were differentiated by varying the ozonolysis reaction time to take advantage of the fact that the heteroaromatic ring is oxidized more rapidly than the phenolic ring. We have examined the possibility of determining the absolute configuration of an individual amino acid residue by using molecular modeling and ROESY data but we do not recommend this method for small peptides unless the peptide has a nearly rigid ring system.



Scheme 1. Determination of the absolute configuration of amino acids in a peptide. Top: Hydrolysis of a peptide and derivatization of the resulting amino acids for chiral GC-MS analysis. Bottom: Ozonolysis of a peptide containing a substituted aromatic amino acid to obtain a peptide containing aspartic acid, which is then analyzed using GC-MS with a chiral phase. a) 5N HCl, 100 °C, 16 h; b) CH_3COCl, isopropyl alcohol, 100 °C, 45 min; c) $(C_2F_5CO)_2O$, CH_2Cl_2, 100 °C, 45 min.

5. Many Lithistid Metabolites Resemble Compounds from Other Sources!

While recognizing that the degree of similarity between chemical structures is a subjective matter, Table 1 illustrates some of the more obvious similarities between lithistid metabolites and natural products from other sources. There is a small possibility that theonellin isocyanate (**22**) and theonelladin A (**24**) were incorrectly attributed to *T. swinhoei* and were in fact biosynthesized by other sponges. This may arise if the taxonomist identifies only one specimen selected from a mixed collection or if the lithistid sponge has absorbed the compound from seawater or from another sponge during the collection process. In other cases, we know that certain lithistid metabolites have been found in other sponges: for example, we have isolated swinholide A (**9**) from an *Ircinia* sp. from the Philippines. However, even when we eliminate the problematic examples, we are still confronted by Kitagawa's observation[17] that part of the structure of swinholide A (**9**) bears a striking resemblance to a portion of scytophycin C from the filamentous cyanophyte *Scytonema pseudohofmanni*,[104] even down to the level of having identical absolute configurations at all of the twelve stereocenters that are common to both molecules. This degree of similarity cannot be ignored.

In 1984, Fusetani and co-workers were the first to observe that discodermin A (**44**) contained amino acids that previously had been found only in peptides from bacteria and speculated, without any real evidence, that the peptides might be of bacterial origin.[82] By 1993 and perhaps earlier, that

Table 1. Some compounds from lithistid sponges that are similar to compounds from other sources.

Species	Compound	Related compound	Source of related compound
Theonella sp.	theonellasterol (**2**)	3-methylsterols	dinoflagellates[7]
T. swinhoei	swinholide (**9**)	scytophycin C	cyanobacterium (*Scytonema pseudohofmanni*)[104]
Theonella sp.	onnamide A (**15**)	pederin	blister beetle (*Paederus* spp.)[37]
Theonella sp.	theopederin A (**16**)	pederin	blister beetle (*Paederus* spp.)[37]
Neosiphonia superstes	sphinxolide B (**19**)	scytophycin C	cyanobacterium (*Scytonema pseudohofmanni*)[104]
Neosiphonia superstes	sphinxolide B (**19**)	rhizopodin	myxobacterium (*Myxococcus stipitatus*)[105]
Reidispongia coerulea	reidispongiolides (cf.**19**)	halichondramide	sponge (*Halichondria* sp.)[106]
T. swinhoei	theonellin isothiocyanate (**22**)	3-isocyanotheonellin	sponge (*Halichondria* cf. *lendenfeldi*)[107]
T. swinhoei	theonelladin A (**24**)	niphatesines	sponge (*Niphates* sp.)[108]
Corllistes sp.	corallistin A (**28**)	chlorophylls	plants and algae
T. swinhoei	motuporin (**35**)	nodularin	cyanobacterium (*Nodularia spumigena*)[109]
Theonella sp.	keramamide A (**39**)	ferintoic acid	cyanobacterium (*Microcystis aeruginosa*)[110]
Neosiphonia superstes	neosiphoniamolide A (**49**)	chondramides	myxobacterium (*Chondromyces crocatus*)[111]
Neosiphonia superstes	neosiphoniamolide A (**49**)	geodiamolide	sponge (*Geodia* sp.)[112]
Discodermia dissoluta	discodermide (**14**)	alteramide A	marine bacterium (*Alteromonas* sp.)[113]
Discodermia dissoluta	discodermide (**14**)	ikarugamycin	soil bacterium (*Streptomyces* sp.)[114]

hypothesis had undergone a subtle change that resulted in many authors attributing the metabolites in lithistid sponges to cyanobacterial symbionts.[115] Two factors influenced this change. The first was the similarities between cyanobacterial metabolites and certain compounds from lithistid sponges, as discussed above. The second was the presence of filamentous bacterial symbionts in lithistid sponges that, when viewed by scanning electron microscopy, physically resemble the filamentous cyanobacterial symbiont *Oscillatoria spongeliae* found in the sponge *Dysidea herbacea*.

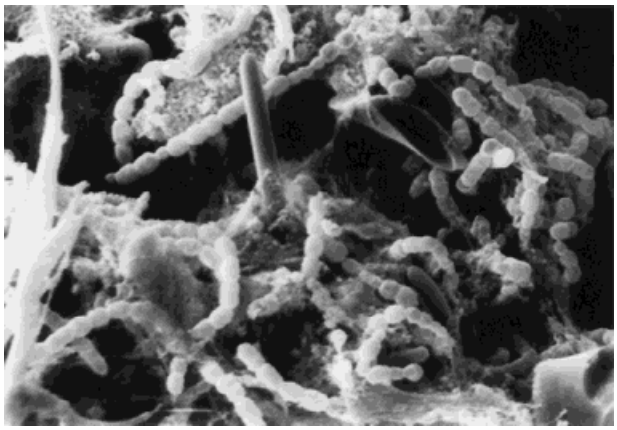


Figure 2. Scanning electron micrographs of *Theonella swinhoei*; filamentous bacteria in the interior of the sponge are visible.

Not all lithistid sponges have filamentous microorganisms interspersed among the interior tissues. We performed an analysis of lithistid sponges in our collection and found that the presence of filamentous microorganisms (Figure 2) correlated with antifungal activity of a crude extract of the sponge and, where the metabolites were known, with the presence of peptides containing aromatic β-amino acids (Table 2). Aromatic and aliphatic β-amino acids were well known as constituents of cyclic peptides from cyanobacteria (Table 3), but we could not accept the hypothesis that the filaments were cyanobacteria because photosynthetic organisms such as cyanobacteria do not prosper in the absence of light and lithistid sponges are often found at great depths, in caves and in other shaded environments. Furthermore, the filaments are found only in the interior of the sponge where they are situated within a skeleton of fused siliceous desmas that exclude light (Figure 2). This does not mean that all lithistid sponges are devoid of cyanobacteria: shallow water lithistid sponges have symbiotic unicellular cyanobacteria within the outer tissues but they were considered insufficient in density to account for the relatively high yields of cyclic peptides obtained from lithistid sponges. In order to solve this conundrum, we decided to investigate the cellular location of metabolites in a shallow water specimen of *Theonella swinhoei* from Palau.

6. Cellular Location of Swinholide A and Cyclic Peptides Isolated from *Theonella Swinhoei*

6.1. Background

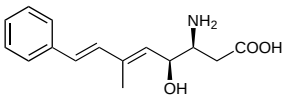
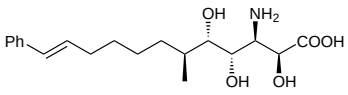
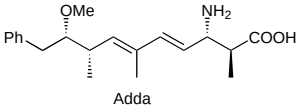
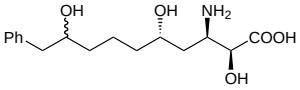
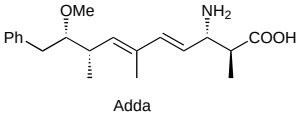
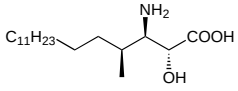
There are several techniques that can be used to determine the cellular location of metabolites in sponges. In 1983, we reported that the brominated metabolites aerothionin (**56**) and homoaerothionin (**57**) of *Aplysina fistularis* were located in sponge cells called spherulous cells and not in bacteria. The cellular location of bromine was detected with an energy dispersive X-ray analyzer in conjunction with transmission electron microscopy.[118] One might also use preferential staining to detect chemicals in cells observed by light microscopy. These methods rely on direct observation of a specific chemical class but do not allow the identity of the individual chemicals to be determined.

A significant breakthrough in cellular localization studies came when we found that metabolites could be recovered in good yield from cells that had been fixed with formalin or glutaraldehyde. This enabled us to separate the cell types in *Dysidea herbacea* by disrupting the sponge into individual cells, fixing the cells, and then separating the cyanobacterial cells from all other cell types by using a cell sorter to detect and separate those cells that contained fluorescent photosynthetic pigments. The fluorescent cell fraction contained better than 99% filamentous cyanobacteria, as judged by

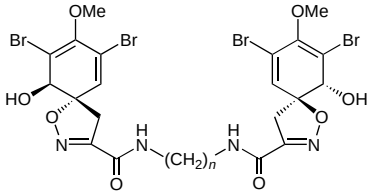
Table 2. Correlations between the presence of symbiotic filamentous bacteria, antifungal activity, and secondary metabolites.

Collection number	Identification	Collection site	Filamentous bacteria	Antifungal	Presence of β-amino acids	Chemistry
NCI-007	*Theonella swinhoei*	Negros Island	+	+	+	theonegramide (**51**)
NCI-013	*Aciculites orientalis*	Negros island	−	+	−	aciculitins (**52**–**54**)
NCI-036	*T. swinhoei*	Negros island	+	+	+	theonegramide (**51**)
NCI-141	*T. swinhoei*	Siquijor	+	+	+	theonegramide (**51**)
NCI-154	*Theonella* sp.	Siquijor	−	+	−	aurantosides (cf. **20**)
NCI-1010	*Theonella* sp.	Siquijor	+	+	+	theonegramide (**51**)
NCI-1919	*Theonella* sp.	Siquijor	+	+	+	theonegramide (**51**)
NCI-1933	*Scleritoderma* sp.	Cuyo Island	−	−	−	unknown
NCI-1934	*Discodermia* sp.	Cuyo Island	+	+	+	unknown peptide
NCI-1935	*Discodermia* sp.	Cuyo island	+	+	+	unknown peptide
93-085	*T. swinhoei*	Palau	+	+	+	theopalauamide (**62**), swinholide A (**9**)
93-087	*T. swinhoei*	Palau	+	+	+	theopalauamide (**62**), swinholide A (**9**)
95-001	*T. swinhoei*	Palau	+	+	+	theopalauamide (**62**), swinholide A (**9**)
R-1385	*Corallistes* sp.	New Caledonia	−	−	−	corallistins (cf. **28**)
R-1401	*Corallistes* sp.	New Caledonia	−	−	−	pyrroles
R-1407	*Reidispongia coerulea*	New Caledonia	−	−	−	reidispongiolides (cf. **19**)
R-1408	*Neosiphonia superstes*	New Caledonia	−	−	−	sphinxolides (cf. **19**)
R-1466	*Microscleroderma* sp.	New Caledonia	+	+	+	microsclerodermins (**37**, **38**)
L1978-279	*T. conica*	Red Sea	−	−	−	sterols (cf. **1**)
L1979-541	*T. swinhoei*	Red Sea	+	?	+	swinholide A (**9**)

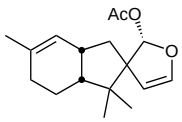
Table 3. β-Amino acids found in peptides from lithistid sponges and cyanophytes.

β-Amino acid	Source
	theonegramide (**51**) *Theonella swinhoei* sponge[101]
	microsclerodermin (**37** and **38**) *Microscleroderma* sp. sponge[74]
	motuporin (**35**) *Theonella swinhoei* sponge[71]
	scytonemin A *Scytonema* sp. U-3-3 cyanobacterium[116]
	nodularin *Nodularia spumigena* cyanobacterium[72]
	calophycin *Calothrix fusca* EU-10-1 cyanobacterium[117]

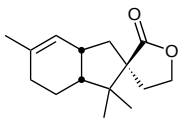
Adda = (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid.



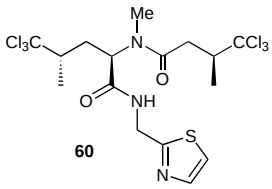
56, $n = 4$, aerothionin
57, $n = 5$, homoaerothionin



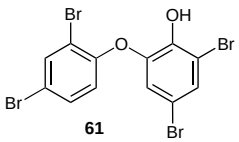
58 spirodysin



59 herbadysidolide



60



61

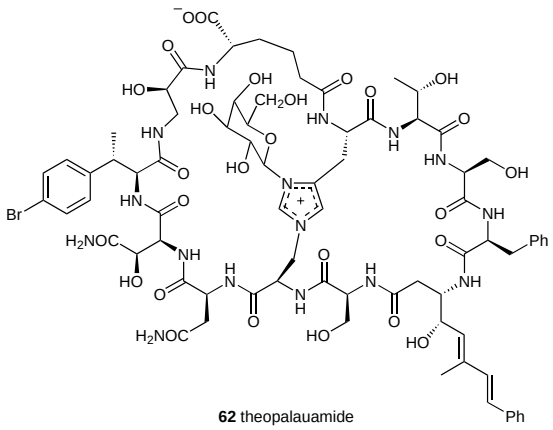
light, fluorescence, and electron microscopy. After washing the cells repeatedly to remove all traces of fixative, the cells were disrupted in methanol and the contents analyzed by GC-MS or NMR spectroscopy. In a specimen of *D. herbacea* from the Great Barrier Reef that contained a very high proportion of cyanobacterial cells, the sesquiterpenes spirodysin (**58**) and herbadysidolide (**59**) were found in the cell fraction containing mainly sponge cells and some non-photosynthetic bacteria, while the chlorinated amino acid derivative 13-demethylisodysidenin (**60**) was extracted from the filamentous cyanobacterium *Oscillatoria spongeliae*.[119] A specimen of *D. herbacea* from Palau contained the brominated biphenyl ether **61** that was located in the cyanobacterial cells and not in the sponge cells. However, examination of sponge sections by polarized light microscopy revealed crystals of the bromin-

ated biphenyl ether **61** situated just below the surface of the sponge. This suggested that the cyanobacterium had been actively excreting the brominated metabolite, which crystallized on exposure to seawater.[120]

In order to examine the cellular location of the metabolites from *T. swinhoei* we could not use the techniques outlined above because there were no specific reagents to stain for swinholide A (**9**) or cyclic peptides and we could not use fluorescence spectroscopy to separate the cells on a cell sorter. This led to experimenting with techniques to separate cell types on the basis of density.

6.2. Metabolites of a Palauan Specimen of *T. swinhoei*

An important step in any investigation of the localization of metabolites in a sponge is to identify and record the spectral characteristics of the metabolites in the sponge. Three specimens of *T. swinhoei* were collected in Palau, Western Caroline Islands, and were immediately subjected to cell separation procedures. Representative samples of the sponges were frozen for chemical studies, which showed that the chemical constitutions of the three sponges were identical. The freeze-dried sponges were extracted as described above. The hexane and dichloromethane extracts contained large amounts of theonellasterol (**2**), while the ethyl acetate extract contained appreciable quantities of swinholide A (**9**). The aqueous acetonitrile extract contained what initially appeared to be a single cyclic peptide but has now been resolved into two interconverting cyclic peptides.[122] The major bicyclic peptide theopalauamide (**62**), which was earlier called P951,[121] is



62 theopalauamide

closely related to theonegramide (**51**, previously isolated from a specimen of *T. swinhoei* from Negros Island in the Philippines)[101] and differs only by the replacement of the sugar D-arabinose by D-galactose.[122] Although we were able to identify the new sugar in a straightforward manner, confirmation that the structure of the peptide was identical in both compounds was both tricky and time-consuming. Details of the structural elucidation of theopalauamide (**62**) and the interconverting isomer have been reported.[122]

6.3. Cellular Location Studies

In retrospect, one of the greatest advantages of working with the lithistid sponge *T. swinhoei* was that the presence of the layer of fused desmas that separates the ectosome from the endosome enabled us to simply and cleanly separate the true unicellular cyanobacterium *Aphanocapsa feldmanni*, that occurs only in the ectosome, from the filamentous bacterium that was found only in the endosome. Sponge cells and unicellular eubacteria were found in both ectosome and endosome. After the gross dissection to separate ectosomal and endosomal tissues of three replicate sponges, each tissue sample was dissociated using an Omega 1000 juicer.[123] The tissues collected on the filter were suspended in a mixture of 1.25% glutaraldehyde and 1.9% formaldehyde in filtered (0.2-μm) seawater (pH 8.15). After passing the suspension through a 42-μm nylon filter, the resulting cell suspensions were separated by differential centrifugation to obtain cell pellets, each 1 mL in volume, of sponge cells, filamentous bacteria, unicellular bacteria and, from the ectosome, unicellular cyanobacteria (see Frontispiece). Each of the pellets, which were prepared in triplicate, was washed three times by resuspension in sterile filtered seawater followed by centrifugation.[121]

Each pellet was examined by transmission electron microscopy and by two methods of chemical analysis. For the chemical analyses, the pellets were extracted with methanol and the resulting extracts dried under nitrogen and redissolved in $CDCl_3$. Extracts of the sponge cells and cyanobacteria were completely soluble in $CDCl_3$ but extracts of the filamentous bacteria and unicellular bacteria were incompletely dissolved so that the insoluble material from these fractions had to be dried under nitrogen and dissolved in $[D_6]$DMSO. Each fraction was examined by ^{1}H NMR spectroscopy using a 500 MHz spectrometer and required 512 scans to produce satisfactory spectra. The spectra were compared with spectra of solutions (1 mM) of authentic swinholide A (**9**) and theopalauamide (**62**) recorded on the same instrument using 128 scans. The resulting spectra (Figure 3) clearly indicated that swinholide A (**9**) was present only in the lightest fraction that contained a mixed population of heterotrophic unicellular bacteria and that theopalauamide (**62**) was localized in the filamentous bacteria. These results were confirmed by HPLC analyses (Figure 4), which also confirmed that the sponge cell fractions and the ectosomal fractions of *A. feldmanni* were devoid of the bioactive metabolites. The three replicates for each cell type gave identical results and thus fortified the validity of the experimental data. The semi-quantitative manner in which the study was performed allows us to predict that the quantities of the bioactive compounds produced by the symbionts is not trivial.

7. Conclusions

Although we have demonstrated that the bioactive metabolites swinholide A (**9**) and theopalauamide (**62**) are localized in symbiotic unicellular bacteria and filamentous bacteria,

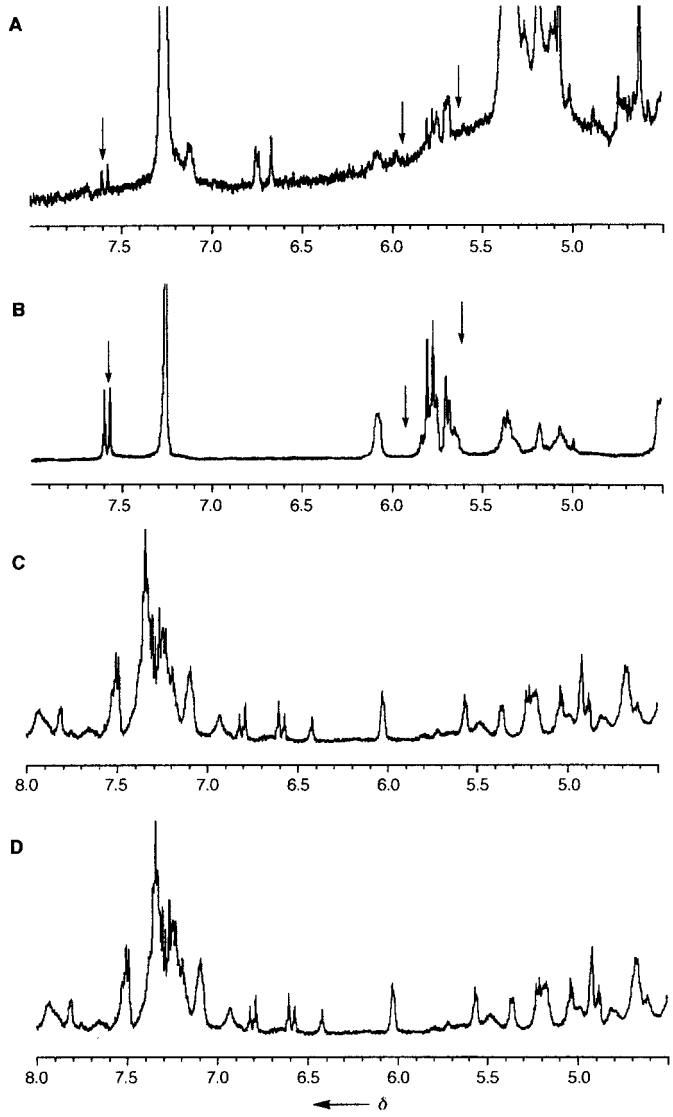


Figure 3. ^{1}H NMR spectra of an extract of purified unicellular eubacteria (A), an authentic sample of swinholide A (**9**, B), an extract from purified filamentous non-photosynthetic bacteria (C), and an authentic sample of 1 mM theopalauamide (**62**, D). Spectra were recorded on a 500 MHz spectrometer (Varian Unity). Spectra of cell extracts were recorded with 512 scans and the standards with 128 scans.

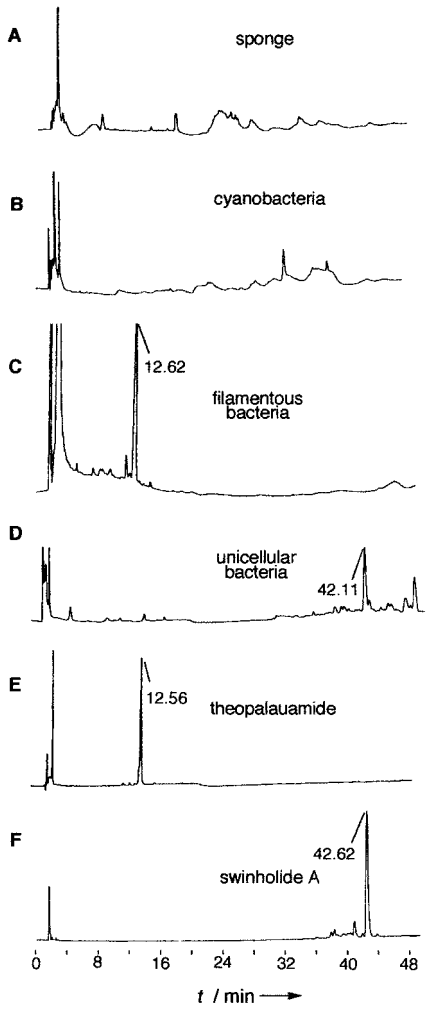


Figure 4. HPLC traces of extracts from purified sponge cells (A), purified unicellular cyanobacterial cells (B), purified filamentous non-photosynthetic bacteria (C), and the mixed population of unicellular heterotrophic eubacteria (D). HPLC traces of authentic standards of theopalauamide (**62**) and swinholide A (**9**) are shown in (E) and (F), respectively. Co-injections of swinholide A and the unicellular eubacterial extract, and theopalauamide and the filamentous bacterial extract, gave single peaks eluting from the HPLC column (data not shown).

respectively, and are presumably produced by the symbionts, we would caution against making generalized statements about the biosynthetic origin of other metabolites until additional studies suggest a trend. The statistical correlation between the presence of cyclic peptides that contain aromatic β-amino acids and the presence of filamentous bacteria appears strong enough to suggest a real correlation but this remains to be confirmed. However, we cannot propose that all peptides from lithistid sponges are produced by filamentous bacteria because we could not detect filaments in some sponges that produce peptides devoid of aromatic β-amino acids.

One of the more curious discoveries of this research, as determined by transmission electron microscopy, is that the filamentous bacteria that were earlier presumed to be cyanobacteria are in fact non-photosynthetic and lack the cellular constituents (thylakoids) necessary for photosynthesis. We have compared the transmission electron micrographs of the filamentous bacteria from the Palauan *T. swinhoei* with micrographs showing the ultrastructure of other filamentous non-photosynthetic prokaryotes and have noted a strong resemblance to filamentous gliding bacteria of the Family *Beggiatoaceae.* However, we do not have any definitive evidence that the filamentous symbionts are *Beggiatoa-like*, and their taxonomic classification remains to be determined by molecular techniques used in phylogenetic studies. While there are no published reports of chemistry from *Beggiatoa*, it is interesting to note that chemical studies of other distant non-photosynthetic gliding bacteria (myxobacteria) have resulted in the isolation of some metabolites that are similar to those described from lithistid sponges.[124]

The research described above may have a damaging impact on the validity of applying chemotaxonomy to sponges. In principle, one cannot place any faith in chemotaxonomic

relationships between sponges if the metabolites are not biosynthesized by the sponge. Yet it is possible that the associations between symbiotic bacteria and host sponges are so stable and long-lasting that the metabolites produced by the symbionts are truly representative of the host sponge. In that case, we can treat any sponge that has unique symbionts as a single entity for chemotaxonomic purposes. The evolution of secondary metabolites is a subject for speculation because individual compounds do not leave a fossil record. A simple application of Darwin's principles leads to the suggestion that secondary metabolites evolved from primary metabolites by means of a series of random mutations that served to produce, in a step-wise manner, new metabolites that increased the fitness (ability to survive) of the producing organism.[125] If symbionts evolve to produce metabolites that increase the fitness of the host and the association is stable, then the host may also be considered to have evolved accordingly. Thus the application of chemotaxonomy to lithistid sponges depends on the stability of the host–symbiont relationships. From that viewpoint, the isolation of swinholide A (**9**) from an *Ircinia* sp. from the Philippines, and the absence of swinholides in all samples of *T. swinhoei* thus far examined from the Philippines, are problematic and suggest that the symbiotic association between the producing bacterium and *T. swinhoei* is less stable or less well evolved. On the other hand, the fact that all specimens of *T. swinhoei* from the Indo-Pacific that we have examined to date contained very similar bicyclic peptides, exemplified by theopalauamide (**62**) and theonegramide (**51**), together with large populations of filamentous bacteria, provides some evidence of stability in host–symbiont relationships (see Table 2), but more data is required.

Our research has raised as many questions as it has answered. We do not know the identity of the filamentous bacteria in which theopalauamide (**62**) was localized. The situation is even worse for swinholide A (**9**), which was localized to a cell fraction containing a mixture of unicellular bacteria. The obvious solution to this problem is to culture the individual bacteria and determine which isolate contains swinholide A (**9**). We are aware of at least one unsuccessful attempt to culture symbiotic bacteria from *T. swinhoei* that produces swinholide A, but we are not really surprised that the desired symbiont could not be cultured. It seems quite logical to expect that a microorganism that has adapted to life as a symbiont will not readily accept routine culture conditions.

The good news for marine natural product chemists is that there are probably many more populations of lithistid sponges that contain new biologically active metabolites. Each specimen must be carefully sampled and compounds purified, for one cannot assume that every specimen of a particular species of lithistid sponge will contain exactly the same array of metabolites. We anticipate that the lithistid sponges will remain in the forefront of chemical studies and will provide some of the best experimental systems for future studies of symbiosis in sponges.

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