Pure & Appl. Chem., Vol. 66, Nos 10/11, pp. 1983–1990, 1994.
Printed in Great Britain.

The chemistry of some sponges and their symbionts

D. John Faulkner, Mia D. Unson and Carole A. Bewley

Scripps Institution of Oceanography, University of California, San Diego
La Jolla, California 92093-0212, USA.

Abstract - From both ecological and biomedical viewpoints, it has become important to know whether the metabolites isolated from sponges are produced by symbiotic micro-organisms. Certain halogenated metabolites from *Dysidea herbacea* are localized in the symbiotic cyanobacterium *Oscillatoria spongeliae*. Recent studies concerning the origin of bioactive metabolites from lithistid sponges, some of which contain filamentous micro-organisms, are presented.

INTRODUCTION

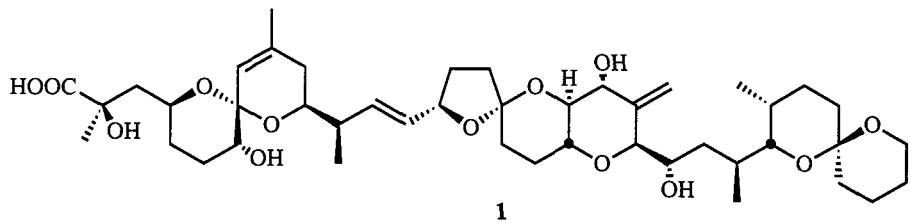
During the past two decades, marine natural products chemists have described a wonderful array of pharmacologically-active metabolites from marine sponges.[1] In fact, statistical analyses confirm that sponges are the most prolific source of biologically-active compounds. Recently, there have been numerous suggestions that these potential pharmaceuticals may be produced by symbiotic micro-organisms rather than by the sponges to which they have been attributed. Unfortunately, most of these proposals are based on circumstantial rather than experimental evidence. It is important from an ecological viewpoint to know the true source of a metabolite, particularly if one wishes to study its biosynthesis, and the information might even be vital for the economic production of pharmaceuticals from marine sources. We have therefore examined some sponges that are known to contain large populations of symbionts and have addressed the question "Do symbiotic micro-organisms produce secondary metabolites previously ascribed to sponges and do these metabolites serve to protect the sponge from predation?"

SYMBIOSIS

There is no single definition of symbiosis that can be universally applied to the wide spectrum of associations that have been observed in nature. In sponges it is probably best to define symbiosis as a consistent association between a micro-organism and a sponge, with the micro-organism distributed within the sponge tissues or cells.[2] This definition requires that every specimen in a particular population of a sponge species should contain the same symbiotic micro-organism(s) distributed in a similar manner within the host. Furthermore, the persistence of a symbiotic relationship implies that the symbiont in some way enhances the evolutionary fitness of the sponge. It is very likely that the sponge provides a special environment for the growth of the symbiont and that the symbionts will not grow in standard culture media. Since sponges eat bacteria and may phagocytose other micro-organisms living within their tissues, they may benefit by maintaining a population of symbionts as a food source. Another possibility is that a symbiont may inhibit the growth of another more harmful micro-organism. However, from the viewpoint of a natural products chemist, these rationales for symbiosis appear rather trivial when compared with the more interesting possibility that the symbionts may produce chemicals that can deter potential predators from eating the sponge.

From the discussion above it can be seen that symbiosis is a rather special situation. We therefore question whether the recent attribution of many marine natural products to "symbiont" sources can be justified for in most cases the supporting evidence is purely circumstantial. In cases where a sponge

metabolite is known to be produced by a free-living or cultured micro-organism, one must consider the possibility that the sponge simply stores a compound that it obtains from the micro-organisms that it consumes. Since sponges are such efficient filter-feeders, it is probably more advantageous for the sponge to concentrate a biologically-active compound from a micro-organism that is abundant in the waters around it than to maintain a symbiotic population of the same micro-organism. This appears to be a logical explanation of the occurrence of dinoflagellate toxins, such as okadaic acid (**1**), in sponges.[3] Chemists have often invoked the existence of symbionts to explain the isolation of the same or similar complex molecules from species belonging to different invertebrate phyla. The best known examples are the pyridoacridine alkaloids that are found in sponges, tunicates and coelenterates.[4] While it is quite possible that these compounds are produced by micro-organisms, the possibility of convergent evolution must not be overlooked. This is particularly true for the pyridoacridine alkaloids because the biosynthesis of shermilamine B (**2**) from aromatic amino-acids has been demonstrated in both *in situ* experiments and in experiments using cell-free extracts of the tunicate *Cystodytes dellechijei*.[5] Therefore, unless a symbiont is observed, food-chain accumulation and convergent evolution must be considered as alternatives to explain the origin of compounds previously assigned to "symbiosis".[6] However, failure to isolate or culture a symbiotic micro-organism does not preclude its existence.



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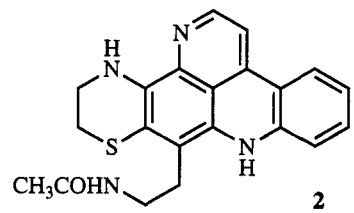

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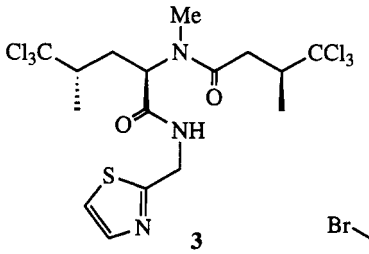
DYSIDEA HERBACEA

There are two chemotypes of *Dysidea herbacea*, which is a relatively common tropical shallow-water sponge. One chemotype contains both polychlorinated amino-acid derived metabolites and sesquiterpenes while the second chemotype contains only polybrominated diphenyl ethers. Both chemotypes support large populations of a symbiotic filamentous cyanobacterium *Oscillatoria spongeliae* and the persistence of this symbiotic relationship has been well established by biological studies.[7] Because some of the polychlorinated metabolites from *D. herbacea* bear a striking resemblance to compounds isolated from free-living cyanobacteria,[8] it had often been suggested that this class of compounds were probably synthesized by the symbiotic cyanobacteria. The sesquiterpenes, which are commonly found in other species of *Dysidea*, were considered to be true sponge metabolites. On the basis of a study described below, the polybrominated diphenyl ethers were thought to be bacterial metabolites. As part of her thesis research,[9] Dr. Mia Unson has studied the cellular localization of selected metabolites in both chemotypes of *D. herbacea*. It is assumed that metabolites are biosynthesized within the cells in which they are localized.

A specimen of *Dysidea herbacea* from Heron Island, Australia, contained 13-demethylisodysidenin (**3**) as the major chlorinated metabolite together with the sesquiterpenes herbadysidolide (**4**) and spirodysin (**5**). In order to separate the cyanobacteria from sponge cells and bacteria, we took advantage of the fluorescence of the cyanobacterial pigments. The dissociated cells from a small sample of fresh sponge were fixed in glutaraldehyde. The fixed cells were then separated on the basis of fluorescence using Becton-Dickinson FACStar Plus cell-sorter to obtain about 2 million cyanobacterial filaments ($> 95\%$ pure) and about 10 million non-fluorescent particles ($< 1\%$ cyanobacteria) that were mainly sponge cells.



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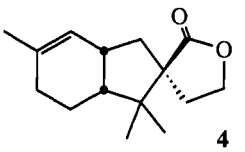

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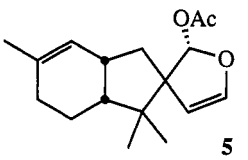

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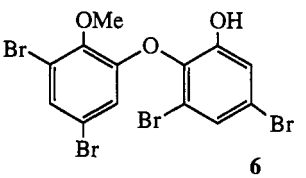

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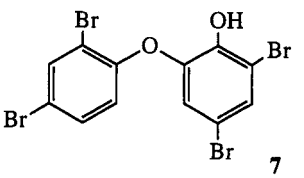

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The two fractions were extracted and the extracts analyzed by 500 MHz ^{1}H NMR spectroscopy and GC-MS analysis. Signals due to 13-demethylisodysidenin (**3**) were observed in the ^{1}H NMR spectrum of the cyanobacterial extract but no signals associated with the sesquiterpenes could be observed in the ^{1}H NMR spectra of either the sponge or cyanobacterial extracts. However, GC-MS analysis of the extracts unambiguously showed that the sesquiterpenes herbadysidolide (**4**) and spirodysin (**5**) were localized in the sponge cell fraction while 13-demethylisodysidenin (**3**) was localized in the cyanobacterial cells.[10]

In 1991, Elyakov *et al.*[11,12] reported that a brominated diphenyl ether (**6**), that had previously been isolated from a *Dysidea* species, was produced by a cultured marine bacterium of the genus *Vibrio* that had been isolated from a specimen of *Dysidea*. Although the quantity of brominated diphenyl ether (**6**) produced by the bacterial cultures was pitifully small compared with the large amounts (2-10% dry wt.) of brominated diphenyl ethers typically associated with the sponge, we nonetheless expected the brominated metabolites to be associated with the non-fluorescent cell fraction. However, microscopic examination of the sponge tissues and analysis of the fluorescent and non-fluorescent cell fractions revealed a very different picture.[13]

A specimen of *Dysidea herbacea* from a shallow lagoon near the Hotel Nikko in Palau contained 2-(2',4'-dibromophenyl)-4,6-dibromophenol (**7**) as the major metabolite (6.0% dry weight). Three other *Dysidea* species were found in the same location: two of these contained different polybrominated diphenyl ethers while the third contained only sesquiterpenes. The nudibranch *Chromodoris funerea*, which is common in that location, contains a complex mixture of polybrominated diphenyl ethers and sesquiterpenes that are all obtained from the *Dysidea* spp.[14]

A preliminary examination of frozen unfixed sections of the sponge tissue by light microscopy revealed abundant needle-like objects that were brightly refractive under cross-polarized light. Since the needles dissolved in organic solvents but not in water and contained bromine as the major element determined by X-ray emission spectroscopy, we concluded that they were crystals of 2-(2',4'-dibromophenyl)-4,6-dibromophenol (**7**). The crystals were often observed in contact with sponge cells and cyanobacterial filaments but were not arranged within the sponge tissues in any consistent pattern, except that they were more abundant in the ectosomal layer where they were arranged in feather-like patterns similar to those obtained by recrystallization. Since the polybrominated diphenyl ethers possess anti-microbial properties, it is not surprising that few eubacteria were observed in the sponge tissue.

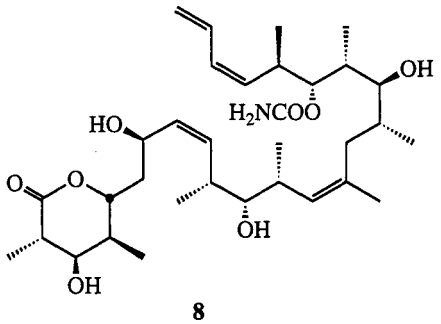
Flow-cytometric sorting of the glutaraldehyde-fixed dissociated cells gave about 500,000 fluorescent particles that consisted of >95% cyanobacterial filaments and about a million non-fluorescent particles that were mainly sponge cells, with relatively few heterotrophic bacteria. ^{1}H NMR spectroscopy of the crude extracts of the two fractions clearly showed signals due to 2-(2',4'-dibromophenyl)-4,6-dibromophenol (**7**) in the cyanobacterial fraction with just a trace in the sponge fraction. This observation was confirmed by GC-MS analysis, except that the brominated diphenyl ether could not be detected in the sponge cell extract. The finding that 2-(2',4'-dibromophenyl)-4,6-dibromophenol (**7**), is localized in the cyanobacterial cells contradicts the earlier report by Elyakov *et al.*[11]

We believe that the location of large quantities of 2-(2',4'-dibromophenyl)-4,6-dibromophenol (**7**) in the ectosomal layer of the sponge, coupled with preliminary data on their biological activity suggests that these compounds may serve a role in the chemical defense of the sponge against both potential predators and bacterial invasion. The brominated phenol **7** is active against representative Gram-positive and Gram-negative eubacteria, including a marine *Vibrio* sp., as well as test strains of the unicellular marine cyanobacterium *Synechococcus* sp. but did not inhibit the growth of two yeasts. Paul has reported that 2-(2',4'-dibromophenyl)-4,6-dibromophenol (**7**) deters feeding by generalist fishes at or below natural concentrations.[15] It therefore appears that a chemical produced by the symbiotic cyanobacterium contributes significantly to the survival of *Dysidea herbacea*.

There are still some outstanding questions concerning this symbiotic relationship. Why are there two chemotypes of *Dysidea herbacea*? Since the sponges are microscopically indistinguishable, it seems logical to suggest that there might be different strains of the cyanobacterium *Oscillatoria spongelliae* producing different metabolites. Why do the *Dysidea* specimens that contain brominated diphenyl ethers not produce the sesquiterpenes that are normally found in members of that genus? Perhaps the production of sesquiterpenes has been suppressed or made redundant by the cyanobacterial metabolites. These and other questions related to the origin and maintenance of the symbiotic relationships will require considerable future research.

LITHISTID SPONGES

From an ecological viewpoint, the lithistid sponges (Demospongiae, Tetractinomorpha, Lithistida) are an unusual order because they are physically well-endowed to resist predation and they also produce some of the most biologically-active marine natural products. Structurally, lithistid sponges contain a high proportion of inorganic material in the form of spicules called desmas. The desmas are fused together to form a solid skeleton that gives the sponge a firm to rock-like consistency, depending on the amount of sponge tissue present. This makes them rather difficult to study by light and electron microscopy. The chemistry of lithistid sponges includes some of the most complex and interesting marine natural products. Lithistid sponges of the genus *Discodermia* produce many cyclic peptides[16] as well as such unusual metabolites as the calyculins[17] and discodermolide (**8**), a powerful immunosuppressive agent.[18] *Theonella swinhoei* is the source of a variety of interesting metabolites including swinholide, a potent cytotoxic macrodiolide,[19] cyclotheonamide A (**9**), a small cyclic peptide possessing potent inhibitory activity against thrombin, trypsin, and plasmin,[20] and many other small peptides.[16] Other species of *Theonella* produce a wide range of small peptides, that often contain unusual amino-acid residues.[16] One fascinating example of this group of compounds is theonellamide F (**10**), an antifungal and cytotoxic dodecapeptide.[21] Our interest in this group of sponges lies not only in the complexity of the chemical structures that they produce but also because they possess symbiotic filamentous micro-organisms.[22]



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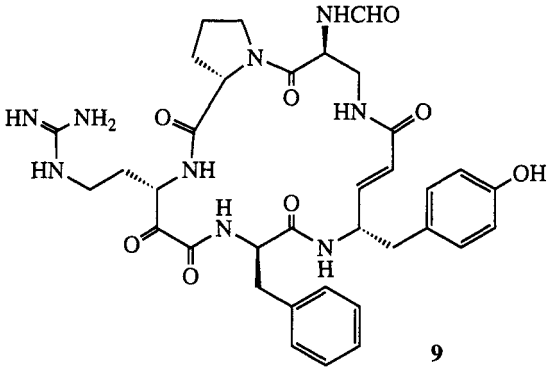

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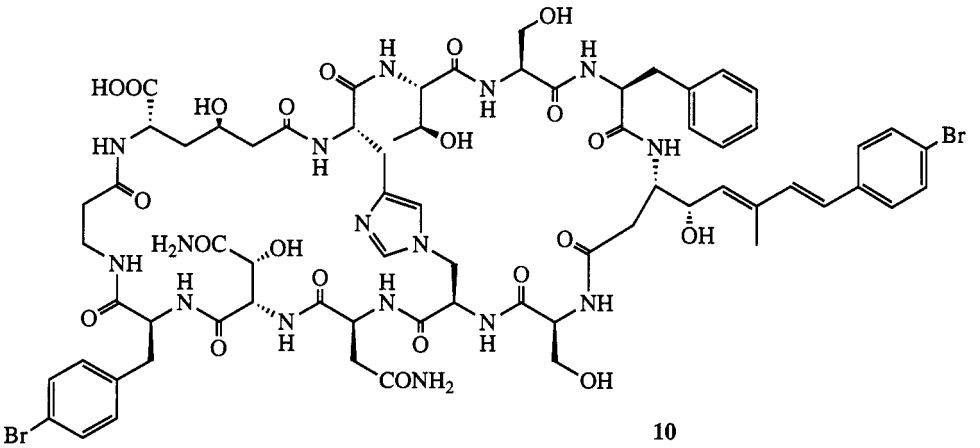

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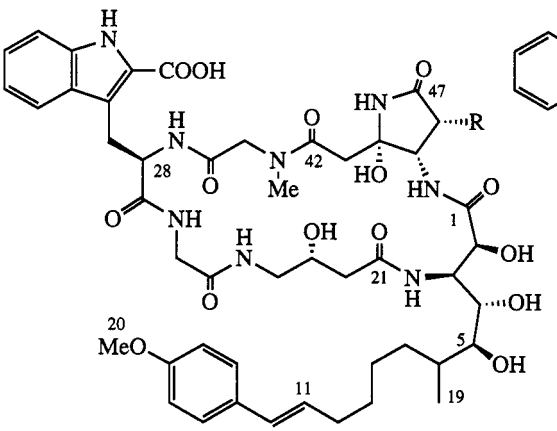
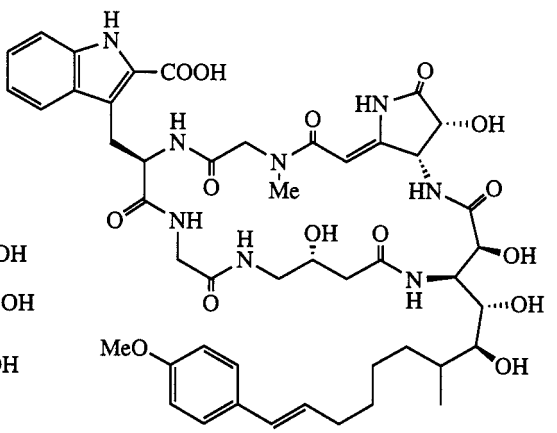
A specimen of the lithistid sponge *Microscleroderma* sp., collected by dredging at a depth of 1000' off Norfolk Rise, New Caledonia, was provided by Dr. Cécile Debitus, who reported that the crude extract showed antifungal activity. We have isolated two polar antifungal metabolites, microsclerodermins A (**11**) and B (**12**) in low yields (0.005% and 0.001% dry wt., respectively). Since we had access to only a limited amount of material and there was almost no hope of recollection, we were forced into using micro-analytical techniques in combination with NMR spectroscopy to tackle the structural elucidation. The structural elucidation was performed on microsclerodermin A (**11**) and dehydromicrosclerodermin A (**13**), which was obtained by mild acid-catalyzed dehydration. Since it is not possible to give a full account of the structural elucidation in this review paper, we will concentrate on the methods and techniques used and present the full spectral data elsewhere.



11 R = OH
12 R = H



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Microsclerodermin A (**11**) has the molecular formula $C_{47}H_{62}N_8O_{16}$, as determined by HRMS [*m/z* = 995.4395 $(M+1)^+$], and loses one molecule of water on dehydration. The peptidal nature of the compound was indicated by the FTIR and NMR spectra. Methylation of **11** with diazomethane in methanol produced a mono-methyl ester (*m/z* = 1008) while acetylation of either **11** or **13** with acetic anhydride in pyridine containing DMAP gave the same penta-acetate [*m/z* = 1187 $(M+1)^+$], indicating the presence of one carboxylic acid and six hydroxyl groups. The ^1H NMR spectrum revealed six amide NH signals, an N-methyl signal and an indole NH signal, accounting for all of the nitrogen atoms. The ^{13}C NMR spectrum revealed eight carbonyls, seven involved in amide linkages and one carboxylic acid, which together with an O-methyl group and the six hydroxyl groups, accounts for all of the oxygen atoms.

Standard amino-acid analysis revealed that glycine was the only common amino-acid in **11**. N-Methyl glycine and 4-amino-3-hydroxybutyric acid were easily identified by interpretation of the NMR data. Analysis of the aromatic region of the ^{1}H and ^{13}C NMR spectra provided evidence for an indole ring system that was substituted at both the 2- and 3-positions. The HMBC data required alkyl substitution at the 3-position in the manner of a tryptophan residue. The assignment of a carboxylic acid residue at the 2-position was based on comparison of the ^{13}C chemical shifts with those of model compounds. The data fully support the presence of a tryptophan-2-carboxylic acid residue. The presence of the pyrrolidone ring system within the C-42 to C-47 portion of **11** was elucidated by analysis of the NMR spectra, particularly the HMBC data, and by comparing the NMR data of **11** with those of **13**, which contains signals typical of a 3-hydroxy-4-amido-5-vinylpyrrolidone. The aromatic regions of the NMR spectra also contained signals for a *p*-substituted benzene ring and a disubstituted olefin that, on further analysis, were shown to constitute a *p*-methoxy-*trans*-styrene moiety. The C-1 to C-6(C-19) portion of the molecule was difficult to establish by NMR spectroscopy because H-2 and H-3 are orthogonal and are not coupled. However, the ROESY spectrum clearly indicated the proximity of these hydrogens. The remaining signals in the ^{13}C spectrum were assigned to the C-7 to C-10 methylene chain. At this stage we had identified six amino-acid residues and, by analysis of the unsaturation equivalents, had determined that **11** was a cyclic peptide. Linkages between the amino-acid residues were established by interpretation of the HMBC and ROESY data to obtain a planar structure for microsclerodermin A (**11**).

The stereochemistry of **11** was determined by a series of degradation experiments coupled with analysis of nOe data. In each of the sub-milligram scale degradation reactions, the amino-acids were analyzed as their N-pentafluoro-propionamide isopropyl ester derivatives by GC-MS using a chiral capillary column. Hydrolysis of **11** using 6N hydrochloric acid at 100°C in a sealed tube gave racemic 3-hydroxy-4-aminobutyric acid but treatment of **11** with neat methanesulphonic acid at 110°C in a sealed tube gave (3*R*)-3-hydroxy-4-aminobutyric acid. The *R*-stereochemistry at the α-carbon in the tryptophan-2-carboxylic acid residue was established by ozonolysis of **11**, followed by an oxidative work-up and hydrolysis of the product with 6N hydrochloric acid to obtain *R*-aspartic acid. Ozonolysis of **13** followed by hydrolysis of the reaction product with 6N hydrochloric acid gave (2*S*,3*R*)-3-hydroxyaspartic acid, indicating that the stereochemistry in the pyrrolidone ring must be 45*S*,46*R*. The 44*S*-stereochemistry was determined by the observation of ROESY correlations between H-43, H-45, and H-46. Cleavage of the C-4, C-5 diol with sodium periodate, followed by oxidative work-up and hydrolysis with 6N hydrochloric acid, gave (2S,3S)-3-hydroxyaspartic acid, which requires the (2*S*,3*R*)-stereochemistry. The stereochemistry about the C-4, C-5 diol was established by treating **13** with dimethoxypropane in DMF to obtain the C-4, C-5 acetonide (alternative acetonides were ruled out by analysis of the ^{1}H NMR spectrum). A ROESY experiment on the acetonide indicated a *threo* relationship between the hydroxyl groups at C-4 and C-5. Furthermore, examination of molecular models showed that the observed nOe correlations were consistent with the (2*S*,3*R*,4*S*,5*S*)-stereochemistry but not the (2*S*,3*R*,4*R*,5*R*)-stereochemistry (see Figure). We analyzed potential experimental routes to determine the stereochemistry at C-7 but concluded that all of these would require more material than was available.

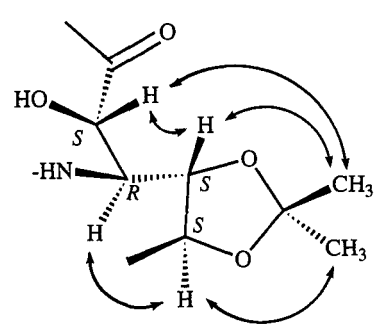

Figure. A representation of the C-1 to C-6 region of the acetonide derivative of **12** showing the observed nuclear Overhauser enhancements.

Microsclerodermin B (**12**) has the molecular formula $C_{47}H_{62}N_8O_{15}$. Analysis of the NMR data revealed that the hydroxyl group at C-46 had been replaced by a hydrogen. The similarity between the spectral data of **11** and **12** strongly suggests that the stereochemistry of microsclerodermin B (**12**) is the same as that of microsclerodermin A.

Carole Bewley has examined a number of lithistid sponges from both shallow-water and deep-water environments and found that not all lithistid sponges contain the symbiotic filaments. However, it may be significant that, in the relatively small sample of lithistid sponges that we have examined, there is a correlation between antifungal activity and the presence of the filamentous symbionts. Because of the similarities between compounds reported from lithistid sponges and those found in certain cyanobacteria, it had been assumed that the symbionts were filamentous cyanobacteria. However, we became suspicious of this assignment because the filaments do not auto-fluoresce and are found only in the interior of the sponge. In addition, lithistid sponges are often found in environments where there is little light and their crude extracts do not appear to contain chlorophyll. Examination of the filaments by scanning electron microscopy does not allow one to differentiate between cyanobacteria and other filamentous non-photosynthetic bacteria. Examination of the filaments by transmission electron microscopy clearly reveals the absence of the thylakoid structures that occur in all cyanobacteria. Analysis of the TEM pictures suggests that the filamentous bacteria may be most closely related to those belonging to the family Beggiatoaceae,[23] such as *Beggiatoa* sp., a filamentous bacterium that occurs as mats in deep-water environments, such as the deep-sea vents.

ACKNOWLEDGMENT

We thank M. K. Harper for assistance with sponge taxonomy and antimicrobial screening, J. Nordberg for assistance with cell sorting, Dr. N. D. Holland for his advice and assistance on all aspects of microscopy, and C. Graham for help with scanning electron microscopy. The specimen of *Microscleroderma* sp. was generously provided by Dr. C. Debitus and was identified by Dr. C. Lévi. We thank Dr. M. Garson, Dr. D. C. Manker, Dr. T. F. Molinski, and A. F. Dexter for their assistance with field research at Heron Island that was made possible by an Australian Research Council grant to Dr. Garson. This research was generously supported by the National Science Foundation (CHE 92-04647), the Graduate Department of the Scripps Institution of Oceanography (for a travel grant to MDU), and an unrestricted gift from SmithKline Beecham.

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22. For photographs of the filamentous symbionts, see references 6 (p. 306) and 16 (p. 1799).

23. TEM pictures were compared with those in: "Bergey's manual of systematic bacteriology," Kreig, N.R., ed., Williams & Wilkins, Baltimore, 1984, vol. 3, pp. 2089-2097, and Lawry, N.H.; Jani, V.; Jensen, T.E. *Curr. Microbiol.* **1981**, *6*, 71.