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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Marine Sponges and Symbionts:

Chemical and Biological Studies

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Oceanography

by

Eric Whitney Schmidt

Committee in charge:

Professor D. John Faulkner, Chair
Professor Joris M. Gieskes
Professor William H. Fenical
Professor Margo G. Haygood
Professor Jay S. Siegel

1999

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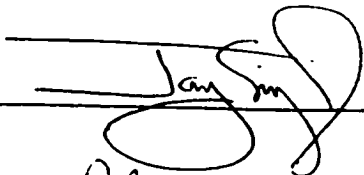
The dissertation of Eric Whitney Schmidt is approved, and it
is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

1999

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LIST OF ABBREVIATIONS

^{1}H	proton
^{13}C	carbon-13
COSY/ GCOSY	correlation spectroscopy/ gradient COSY
DGGE	denaturing gradient gel electrophoresis
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DQCOSY	double quantum COSY
ESIMS	electrospray mass spectrometry
FABMS	fast atom bombardment mass spectrometry
FISH	fluorescent *in situ* hybridization
GC-MS	gas chromatography-mass spectrometry
HMBC/ GHMBC	heteronuclear multiple bond coherence/ gradient HMBC
HMQC/ GHMQC	heteronuclear multiple quantum coherence/ gradient HMQC
HPLC	high-pressure liquid chromatography
IR	infrared
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
rRNA	ribosomal ribonucleic acid
ROESY	rotating frame overhauser effect spectroscopy
SSU	small subunit (rRNA)
TFA	trifluoroacetic acid
TOCSY	total correlation spectroscopy
UV	ultraviolet

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ACKNOWLEDGEMENTS

During my graduate studies. I have had the pleasure of collaborating with, working next to, and consulting with interesting people in a variety of fields, and I am grateful for my interactions with them all. I am most indebted to John Faulkner for taking me in as an undergraduate when I didn't even know how to pipet properly. John then expertly guided my graduate studies and allowed me to chase madly and productively into fields that neither of us knew anything about. Thanks for the opportunities, John.

Margo Haygood has also spent a great deal of time and effort training me in a totally new field. She welcomed me into her lab, even though I knew almost nothing about molecular biology, and I have learned a great deal from her. Doug Bartlett did everything but hold my hand during my early experiments in microbiology, and I thank him for being so encouraging, widening my perspectives, and for great discussions. I have approached Bill Fenical for advice and help numerous times, and he has always been available to me. His input, especially telling me when I was wrong (don't worry, I remember the encouragement, too), definitely helped my development as a scientist. I also thank him for convincing the Coast Guard that we had no evil intentions during a certain Presidential visit. We were, in fact, just happy to see Him. With Joris Gieskes and Jay Siegel, I have had less interaction, but I am grateful for their advice and ever-optimistic dispositions.

From the Analytical Facility, I thank Kevin Walda, Bill Papalawski, and Ron Laborde for putting up with me and training me on half the instruments at SIO. For instrument training, Ralf Goericke also helped with the ESI-MS. In diving, I have been greatly helped by Wayne Pawelek, Ron McConnaughey, Cary Humphries, and Jim

Stewart. Peter Brueggeman at the SIO library performed numerous structure searches that greatly aided my chemical projects.

All of my work has been made possible by collections in foreign waters, due to the generosity of the governments of Palau and the Philippines. In Palau, we have always worked through the Coral Reef Research Foundation, headed by Pat Colin. Over the years, Pat and his wife, Lori, in addition to Larry Sharron and other staff members, have made our stay enjoyable and thoroughly productive. I will never forget seeing the dusk triggerfish spawning with Pat, or getting blasted through Kaibakku Tunnel with Larry. Emilio Basilius and Matt Mesubed also played a large role in our adventures there. I'm glad Emilio knows mud driving, and I'm even happier that Matt can drive a boat at 90 degrees to the water. In the Philippines, we have been aided by Silliman University, Dumaguete. Lori Reymundo was our main contact and a good friend. Her amazing diving skills and keen eye for new sponges have contributed immensely to the Faulkner lab collection over the years.

The students and postdocs that I have worked with for the last four-and-a-half years have made a lot of contributions to my work and ideas, and they have also made my stay at SIO interesting and enjoyable. I would first like to acknowledge all the people from the Faulkner lab, who have generally been interesting, intelligent, and often a bit on edge (in a good way). Mary Kay Harper showed me how to pipet properly as an undergraduate, and it moved on from there. Thanks for all of the help and fun, Ms. Mary. Mother, grandmother, hope for the hopeless, Doris Osborne acted as all of the above during her years with the group. I still can't get over the fact that she's no longer around the lab to give us a bit of sarcasm if we're feeling too self-satisfied or some encouragement when things are rough.

It seems like postdocs just come and go… I can't even remember how long half of them were here. They were all enjoyable to work with, however. David Williams

gave me all of my basic training and showed me by example what it means to be a great scientist. I am much indebted to him for his help with my work leading to Chapters 2-4 of this manuscript. Philippe Kernen is an excellent, well-rounded chemist. Thanks to his sound advice, I will never order a coke in a fondue restaurant. Speaking of incredible chemists, Venkat Reddy is one, and never ceases to inspire with his long hours and great work. Qing Lu's ardent vocalizations were... well, Qing is always ready for a good conversation about almost anything. I don't understand how he keeps up with it all. Cordula Hopmann was truly remarkable at touch-parking. Her wit and appreciation of sarcasm kept me alive through many a slow day. Catherine Woytowicz was my swimming buddy during her year in the lab. It seems like we were almost as fast, or at least as wet, as the seals. Swimming was also a popular pastime when Elmar Müller was around. Besides being a good partner for science discussions, Elmar also infused the lab with a much-needed dose of traditional Manliness, and he certainly let his glassware know it. Scott Mitchell has been of great help, advising me on a range of projects and problems and generally having interesting insights. Don't worry, Scott, I'll keep the "Hammock" and "Frogfish" incidents secret as long as I live. At least secret to my closest few hundred friends. Asfia Qureshi has been a good friend for discussion about issues, both scientific and otherwise. Her grounded enthusiasm has kept me going when I was ready to just give it all up for surfing. Jana Pika had the patience to teach me the basics of 2-D NMR, and her opinions were highly valued. Without her, I would still be begging John Wright to tell me how to get a proper HMBC working.

The tendency of postdocs in this group is to maintain an attitude of perpetual bewilderment, the source of which can inevitably be traced to the graduate students. Chris Blackburn and Christine Salomon, you played no small part in instigating this befuddlement. You both are acknowledged together, despite the fact that I do finally understand you do not share psychic links with each other. I feel that, after all this time

together and experiencing many of the same adventures, we have developed some similar attitudes about science and work in general. I look forward to the time when we can compare notes on how our graduate students are doing at the Gordon Conference. The kids have also played a big role in recent life in the lab. Mike McCoy, I hope you don't lose your youthful attitude about learning and science. Thanks for showing me how a professional diver thinks, as well. Melissa Lerch has definitely perked up the lab with an infusion of zest, of verve, of... well, I don't really know what to call it. But thanks for the enthusiasm and fun attitude. When I got to the lab, Patrick Krug seemed like the only graduate student who would be around for a while, and even though he wasn't, he left an indelible impression on me. At least, it won't come off with repeated rubbing. Patrick's different outlook on life and bizarre (read, "good") approach (at least from the Scripps perspective) kept life fun. Also, Teasdale was the best house in the world, spawning at least one wedding and a permanent horror of frozen lab rats. Ted Evans was also the source of a lot of cajolerie in those early years, and he provided a lot of advice during my trying forays into synthetic chemistry. Needless to say, even if we did have happy hour, it wouldn't last until 10 p.m. now that he's moved on to the real world. Carole Bewley provided me a role model for hard work and goal-chasing. She deserves the best professorship she can get.

During my time in the Faulkner lab, we have also had a number of undergradutates: Dario, Pierre, Christian, Andy, Jason, Andrew, and Soeun. You have all influenced me in some way, and I'm glad I got to know you. Finally, we have had the good fortune to be associated with a number of visiting faculty members, all of whom have been great: John Blunt, Javier Salva, and Reynaldo Compagnone. I would especially like to thank Prof. Blunt for all his help with NMR instrumentation and software.

In the last year and a half, I have had the opportunity to work in the Haygood-Tebo lab and to interact with the many talented members of the group. I would especially like to thank Seana Davidson for her helpful patience with me as a chemist in a biologist's world. I could not have done it without her. Anna Obraztsova worked with me constantly on the culturing project and prodded me to perfect other aspects of the biology work as well. I feel I am lucky to know her, and would gladly work in her company, should she choose to found one. Meriah Arias has been a good friend and of much help in the lab. Grace Lim provided an injection of energy and enthusiasm, and Linda Park helped me with general lab goings-on and with treeing algorithms. I thank Deanne Edwards for sharing a smidgeon of her space with me and for the great diving conversations.

To all my other friends at SIO, you know who you are, thanks for everything.

My father, mother, and fiancé have given me everything, including what I mention here. I am grateful to my bride-to-be Soo Kim for her love and support. I thank my mother, Jessie Knight, for helping me to develop a love for nature and for reminding me that there is more than life to science. I thank my father, Robert Schmidt, for his encouragement and for providing an example of what it means to be an ethical research scientist.

*   *   *

Chapter 7 acknowledgements: We thank Dr. David Newman of the National Cancer Institute for access to the Active Repository and for interest in this project. The sponge sample (C012961) was collected by the Coral Reef Research Foundation with the permission of the government of the Philippines. The sponge taxonomy was identified by Michelle Kelly-Borges, and a voucher specimen is available at the Smithsonian Institute. Bioactivity testing was performed by Bristol-Myers Squibb with the help of Dr. Craig Fairchild. Dr. Jeffrey Bada and Daniel Glavin, Scripps Institution

of Oceanography, offered advice and materials for the derivatization of the Orn-Ser residue.

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PUBLICATIONS

Schmidt, E.W., Harper, M.K, and Faulkner, D.J. "Makaluvamines H-M and damirone C from the Pohnpeian sponge *Zyzzya fuliginosa.*" *Journal of Natural Products,* **1995,** *58,* 1861-1867.

Schmidt, E.W. and Faulkner, D.J. "Palauolol, a new anti-inflammatory sesterterpene from the sponge *Fascaplysinopsis* sp. from Palau." *Tetrahedron Letters,* **1996,** *37,* 3951-3954.

Schmidt, E.W. and Faulkner, D.J. "Absolute configuration of methyl (2*Z*,6*R*,8*R*,9*E*)-3,6-epoxy-4,6,8-triethyl-2,4,9-dodecatrienoate from the sponge *Plakortis halichondrioides.*" *Tetrahedron Letters,* **1996,** *37,* 6681-6684.

Schmidt, E.W., Harper, M.K., and Faulkner, D.J. "Mozamides A and B, cyclic peptides from a theonellid sponge from Mozambique." *Journal of Natural Products,* **1997,** *60,* 779-782.

Schmidt, E.W., Bewley, C.A., and Faulkner, D.J. "Theopalauamide, a bicyclic glycopeptide from filamentous bacterial symbionts of the lithistid sponge *Theonella swinhoei* from Palau and Mozambique." *Journal of Organic Chemistry,* **1998,** *63,* 1254-1258.

Schmidt, E.W. and Faulkner, D.J. "Microsclerodermins C-E, antifungal cyclic peptides from the lithistid marine sponges *Theonella* sp. and *Microscleroderma* sp." *Tetrahedron,* **1998,** *54,* 3043-3056.

Faulkner, D.J., Harper, M.K., Salomon, C.E., and Schmidt, E.W. "Localization of bioactive metabolites in marine sponges. Proceedings, 5th International Sponge Symposium, Brisbane, Australia." *Memoirs of the Queensland Museum.* In press.

Faulkner, D.J., Harper, M.K., Salomon, C.E., and Schmidt, E.W. "Symbiotic bacteria in sponges: Sources of bioactive compounds." In: *Drugs from the Sea,* N. Fusetani, ed, Karger, Basel. In press.

Schmidt, E.W. and Faulkner, D.J. "Scleritodermin A, a cytotoxic cyclic peptide from the marine sponge *Scleritoderma nodosum.*" In preparation.

Schmidt, E.W., Obraztsova, A.Y., Davidson, S.K., Faulkner, D.J., and Haygood, M.G. "The peptide-containing symbiont of the marine sponge *Theonella swinhoei* is a novel δ-subgroup Proteobacterium, '*Candidatus* Entotheonella palauensis'." In preparation.

FIELDS OF STUDY

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Studies in Marine Natural Products Chemistry
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Studies in Molecular Biology and Microbiology
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ABSTRACT OF THE DISSERTATION

Marine Sponges and Symbionts: Chemical and Biological Studies

by

Eric W. Schmidt

Doctor of Philosophy in Oceanography

University of California, San Diego, 1999

Professor D. John Faulkner, Chair

This thesis concerns two quite different types of research that are separated into distinct sections of the thesis, but which seek to answer the same question using diametrically opposite approaches. The first part (Chapters 1-8) covers research leading to novel, bioactive compounds in marine sponges, while the second (Chapters 9-10) involves molecular biological studies of symbiosis between microbes and sponges. Although these topics seem at first glance completely separate, they are in reality intimately tied together through marine natural products chemistry. In some sponges, potential pharmaceuticals or compounds with interesting structures are produced by microbial symbionts, so a more detailed understanding of sponge-microbe symbiosis will lead to new compounds or a better source for new drugs. Conversely, a knowledge of chemistry, particularly biosynthesis and the distribution of compounds, can help in the understanding of sponge-microbe symbioses, leading to information which could never otherwise be obtained on microorganisms that are nearly impossible to culture.

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Chapter 1 outlines chemical research and provides a review of structure elucidation methods. Chapters 2-6 contain reprints of previously published material, describing the isolation and structure elucidation of novel secondary metabolites from marine sponges. Although the first compounds are achiral, most of the work in the remaining chapters pertains to the elucidation of the stereochemistry of new compounds.

The chemistry chapters progress logically into the introduction to biological studies, culminating in the structural solution of a peptide previously isolated directly from symbiotic bacteria of the sponge *Theonella swinhoei* (Chapter 8). With this chemical lead-in, current knowledge of sponge-microbe symbioses is briefly reviewed along with an introductory rationale to my biological studies (Chapter 9). Details of a molecular-biological study of sponge-microbe symbiosis in sponges of the order Lithistida are presented in Chapter 10. It is demonstrated that the symbiotic, peptide-containing bacterium of *T. swinhoei* are new species of δ-subdivision Proteobacteria. Sequences of similar strains from closely related sponges and media used to attempt to culture the symbionts are reported. In conclusion, chemistry and molecular biology are shown to supplement each other in the understanding of sponge-microbe symbiosis in the order Lithistida.

CHAPTER 1
CHEMICAL STUDIES OF MARINE SPONGES:
AN INTRODUCTION

Sponges (Phylum: Porifera) have been the major sources of new marine natural products since the founding of the field. They have usually been studied for biomedical purposes, but a large number of ecological studies of sponge metabolites have also been reported.[1] Several metabolites have been selected for clinical trials,[2] and a large number of compounds that are not drug candidates find use as biomolecular probes.[3] The structures of these compounds are often quite novel, and they require detailed NMR and chemical experiments for their elucidation. Because of these factors, sponge chemistry remains a major topic of research.

The next seven chapters concern the structure elucidation of new marine natural products from sponges, beginning with achiral molecules and proceeding to compounds of increasing stereochemical and conformational complexity. Molecules of several different biosynthetic classes are described, including alkaloids, terpenes, acetogenins, and peptides, and methods of structure elucidation specific to each class are discussed. Most of my thesis work has been on structural elucidation of novel peptides from sponges of the order Lithistida (Class: Demospongiae).[4] These bioactive compounds are replete with stereocenters and novel amino acids, and additionally often exhibit some degree of conformational isomerism. Because much of the work involved in solving a new structure is configurational and conformational analysis, this chapter contains a review of the most elegant and useful methods for stereochemical determination in marine natural products, particularly methods that have been applied to molecules discovered in the course of my dissertation research. In addition, the rationale behind

1

the choice of projects is discussed, including the bioactivity of the compounds studied. The chemistry described in this chapter leads into biological studies of marine sponges, which are introduced in Chapter 9. Since the scope and goals of marine natural products chemistry have been extensively reviewed,[1,5] they will not be discussed here.

Early chemical studies

In my first graduate project, I examined the organic extracts of the Pohnpeian sponge, *Zyzzya fuliginosa*, a coral borer found throughout much of the tropical Pacific (Chapter 2). The sponge was selected for chemical study because its crude extract was active against the Human Colon Tumor (HCT) 116 cell line and because its ^{1}H NMR spectrum had interesting signals in the aromatic region. Seven new alkaloids were isolated (makaluvamines H-M and damirone C), and the structures were determined using a combination of spectroscopic techniques.[6] As the simplest of the compounds I worked with, the makaluvamines provided an ideal introduction to natural products chemistry. Because the compounds were colored, their purification could easily be followed by eye. In addition, these small heterocycles contained no chiral centers, and thus provided no large hurdles to structure elucidation. Once the structure of a known compound in the extract was determined, the others could be generated by comparison of spectral data, although numerous NMR experiments were still required. The heavily substituted nature of the heteroaromatic makaluvamines and damirones required that ^{13}C NMR spectroscopy and [1]H-^{13}C correlation experiments be used as major tools for structural identification. Once chemical studies were completed, some interesting biological data were obtained. Chemotaxonomic evidence from this and other studies was reflected in the reassignment of some members of the *Damiria* and *Zyzzya* genera into the single species, *Z. fuliginosa*.[7]

My second project involved more complicated chemistry, since the new sesterterpene, palauolol (1), contained several chiral centers and required some difficult chemical transformations (Chapter 3).[8] The Palauan sponge *Fascaplysinopsis* sp. used in this study, like the *Z. fuliginosa* specimen decribed in the previous paragraph, was selected based on HCT-116 activity and interesting NMR spectra. The anti-tumor activity was actually caused by known compounds present in the extract, but palauolol (1), like similar butenolide-containing compounds, was found to be mildly anti-inflammatory. Elucidation of the absolute configuration of palauolol (1) required three fairly standard techniques: coupling constant determination, derivatization with Mosher's acid, and CD comparison with known compounds (Figure 1). A number of chemical transformation steps were required to reach compounds which were compatible with these methods. Ultimately, the absolute configuration of most of the molecule was confirmed by conversion of palauolol (1) into a close analog of a degradation product (2) of ilimaquinone (27).

In the third of these early projects, I determined the absolute configuration of the known acetogenin, methyl (2Z,6R,8R,9E)-3,6-epoxy-4,6,8-triethyl-2,4,9-dodecatrienoate (3), from the sponge *Plakortis halichondrioides* (Chapter 4).[9] Chemically, this project presented challenges because of its two isolated stereocenters, which normally would each require separate stereochemical analysis. In addition, the absolute and relative configurations of branched alkyl groups that do not have neighboring functional group handles are notoriously difficult to determine. In most cases, solutions were not even attempted. In this case, a solution that works for alkyl branches adjacent to a double bond was presented. The project originated in an inquiry from another research group about a compound that they thought was the enantiomer, or possibly the diastereomer, of the structure that was originally proposed by Sullivan and
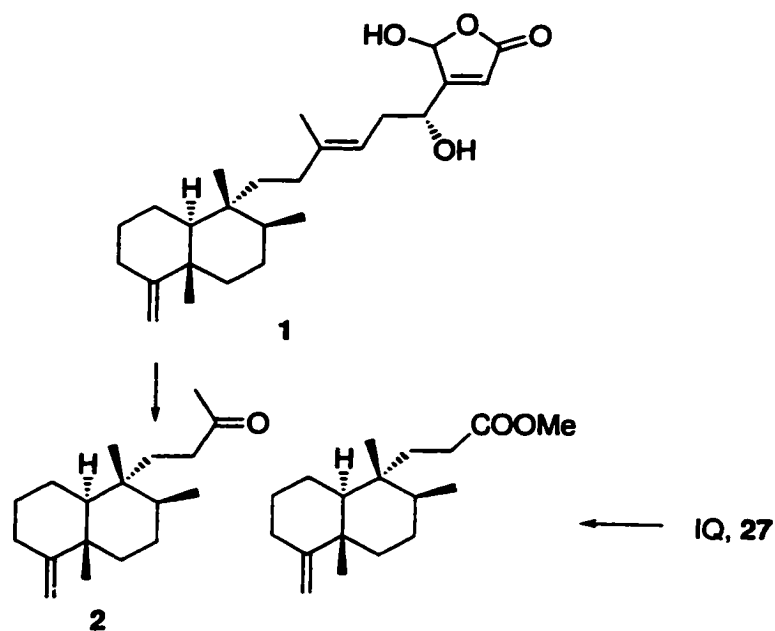
Figure 1. Absolute configuration of palauolol. The hydroxyl stereocenter was determined using the Mosher method, while degradation of palauolol to a ketone (bottom left) and CD comparison with a degradation product of ilimaquinone (bottom right) was used for configurational analysis of the rings.
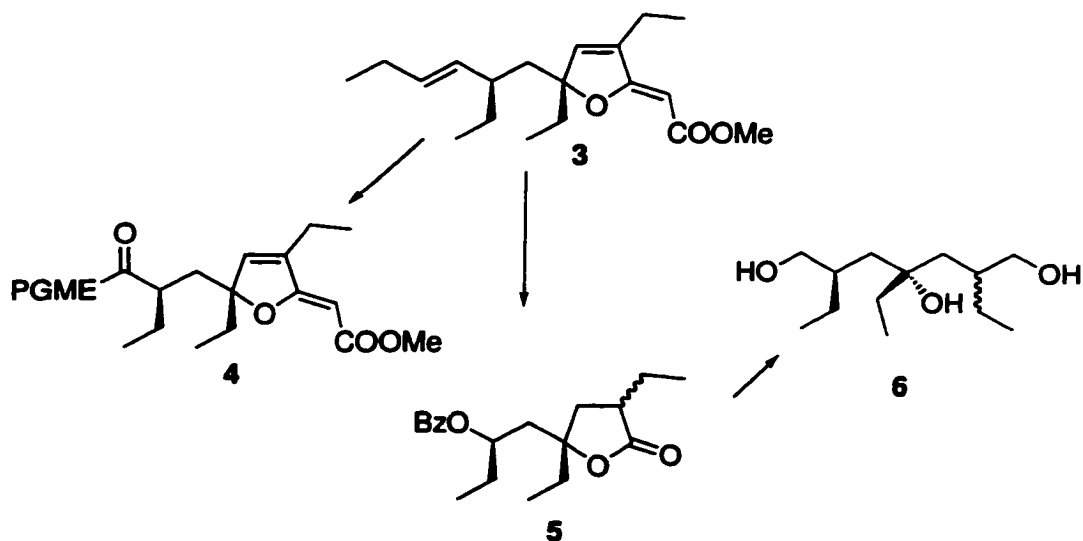
Figure 2. Compound 3 (top middle) methyl ester was converted to the PGME amide 4 (left) for absolute configuration determination. Two compounds 5 were formed and analyzed using 1-dimensional NOE spectra. Finally, conversion into the two compounds 6 (right), one of which was symmetrical, allowed the relative configuration to be determined.

Faulkner because it had the opposite optical rotation. The group isolated the compound from the South China Sea, so I decided to determine whether the Indo-Pacific version of the sponge produced the enantiomer of the compound produced in the Caribbean. Unfortunately, after nearly completing the project, I found an original notebook entry that showed the published α_D for this compound should have been -175°, not +175°. Therefore, the compound actually had the same conformation in both samples. In any case, the methodology worked out in this project was useful in a series of related compounds studied by Dr. M.V.R. Reddy and proved biomedically useful, since some of the compounds were active in assays against the parasite that causes the debilitating tropical disease, leishmaniasis.[10] In addition to derivatization with a chiral reagent to **4**, the project involved a series of chemical transformations leading to two compounds **6**, one of which was symmetrical (Figure 2). With precursors **5**, NOE data and computer models were used to determine the relative configuration of the ring. Coupling constants cannot be used with five-membered rings.

Structural studies of lithistid sponge peptides

Most of my research projects have concerned lithistid sponges, which contain a diverse array of metabolites, especially highly bioactive acetogenins and unusual, modified peptides.[4] The peptide metabolites present challenges in the determination of both two-dimensional structure and stereochemistry, as shown in Chapters 5-8. Much of the difficulty stems from unusual or novel amino acids or polyketide-derived portions, meaning that standard protein NMR procedures are often inapplicable and that standards for absolute configuration determination are not readily available. The peptides are also conformationally labile on a variety of timescales (Figure 3). Structures of lithistid compounds that I have elucidated, represented in Figures 4-5, exemplify all of these challenges. Many of the lithistid compounds have been
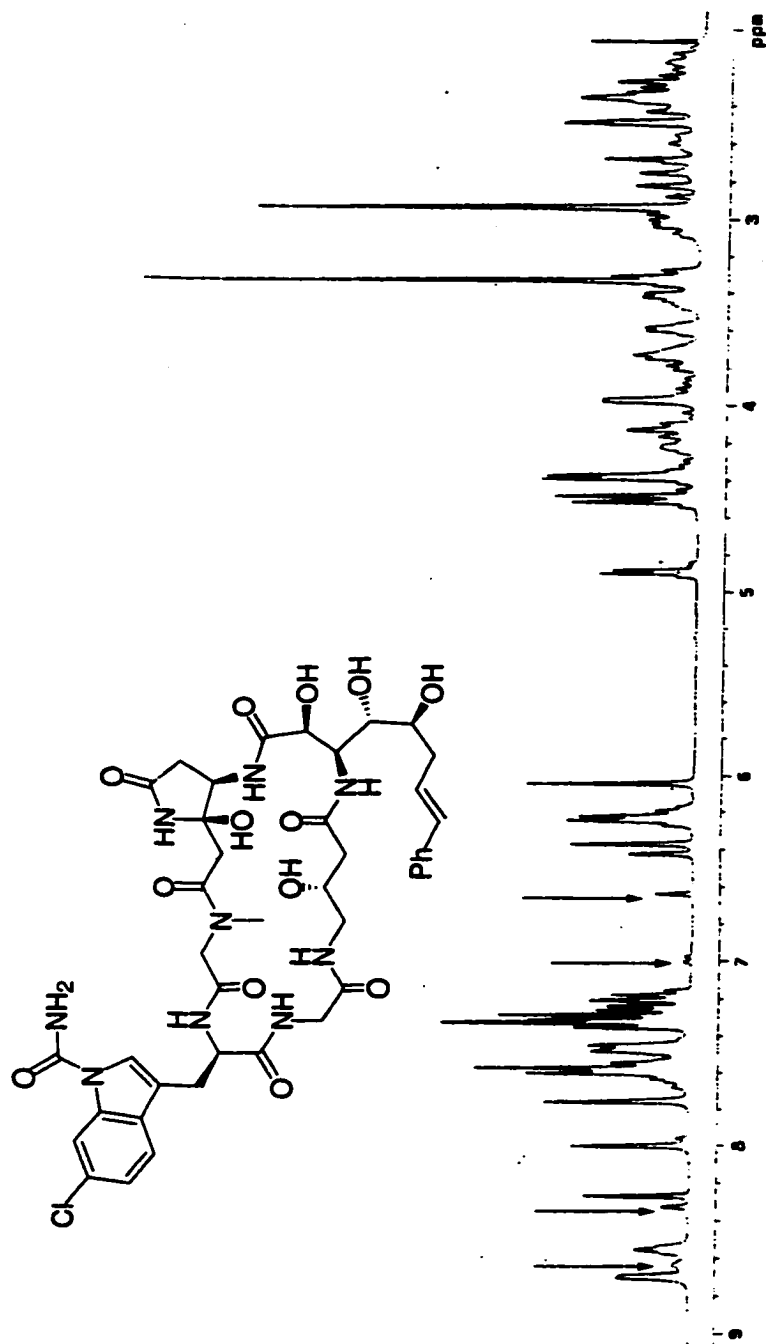
Figure 3. 300 MHz ^{1}H NMR spectrum of microsclerodermin C in DMSO-d_6, showing signal doubling caused by conformational isomerism.

hypothesized (or in one case proven) to be of symbiotic microbial origin, so there is also a biological reason to study the chemistry of the sponge group.[11-13] Details of biological studies on lithistid sponges are given in Chapters 9 and 10. Lithistid peptides are also biologically active, usually showing anti-fungal and anti-tumor activity. Biological activity is described along with structure elucidation in the following chapters.

Some lithistid sponges have interiors stained a bright orange by the presence of aurantosides, and these sponges seem invariably to also contain peptides of the structure type exemplified by mozamides A (7) and B (8) (Chapter 5).[14] These compounds are interesting from the biological point of view because these and similar compounds have been described from two different genera, *Theonella* spp. and *Plakinalopha* spp., which were thought to be quite different but have recently been shown to be closely related.[15] The sponge used in this study, a Mozambique specimen identified as belonging to the family Theonellidae, was selected because of its close resemblance to the marine sponge *Theonella swinhoei*, which is known to harbor a rich array of bioactive and interesting natural products. In this sponge, several peptides with very unusual chemical shifts (several between δ 0.5 and -0.5 ppm) were present in small amounts. In fact, the most difficult part of this study was the establishment of purification procedures leading to the isolation of mozamide A (2.5 mg) and B (0.6 mg). The small amount of peptide recovered also presented some challenges, since many NMR experiments required an unusually long period of time, and many were just not possible. While no bioactivity was found for these compounds, it is probable that we just did not select the right assay system.

At the time that I began to work on the theonellid sponge, I also started to extract a Mozambique sample of *T. swinhoei*, which had a white interior rather than the

7, Mozamide A, R = H
8, Mozamide B, R = Me

9
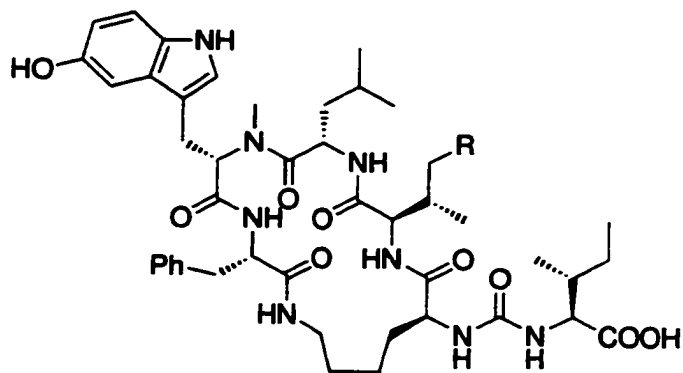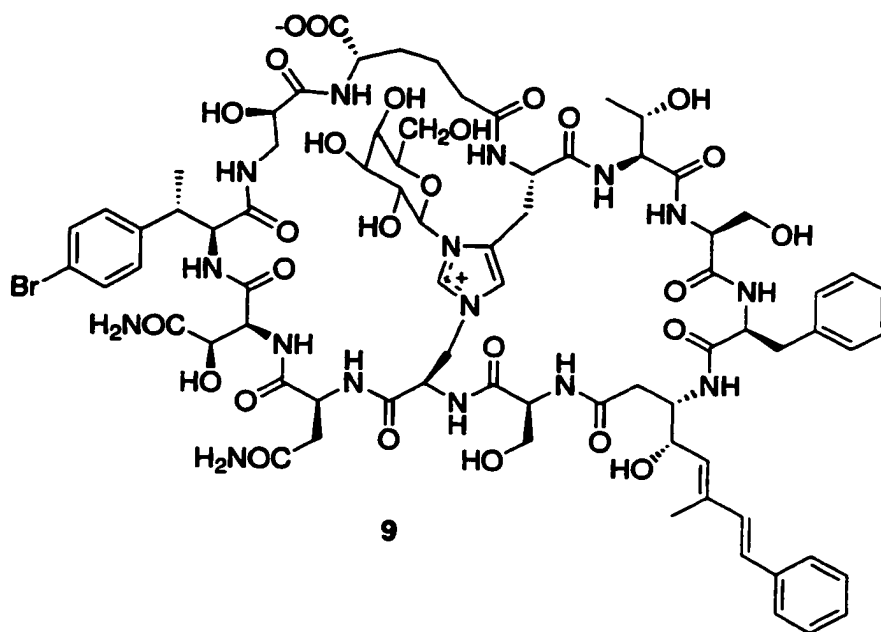
Figure 4. Mozamides A (7) and B (8) and theopalauamide (9).

10, Microsclerodermin C, R = CONH₂
11, Microsclerodermin D, R = H

12

13
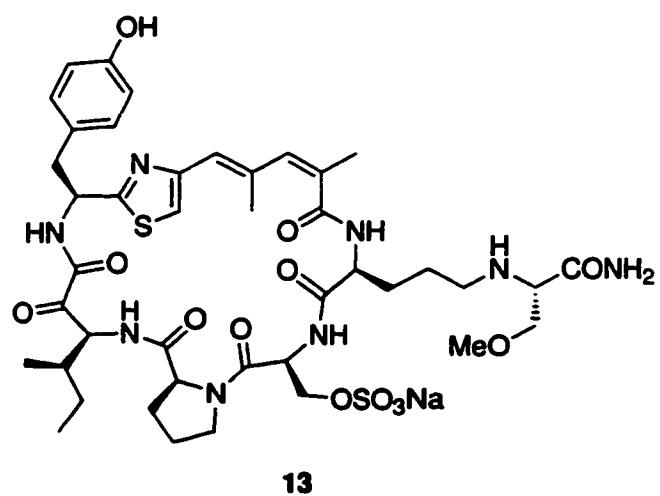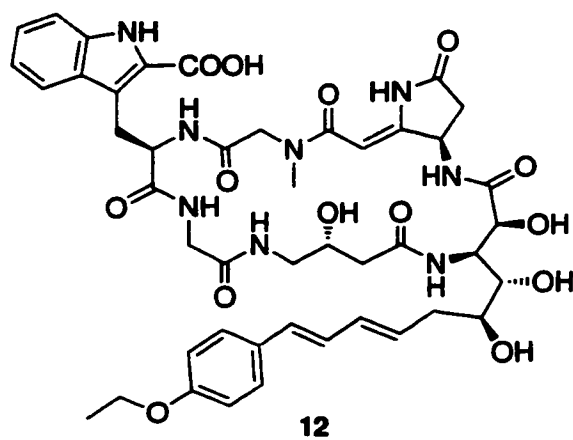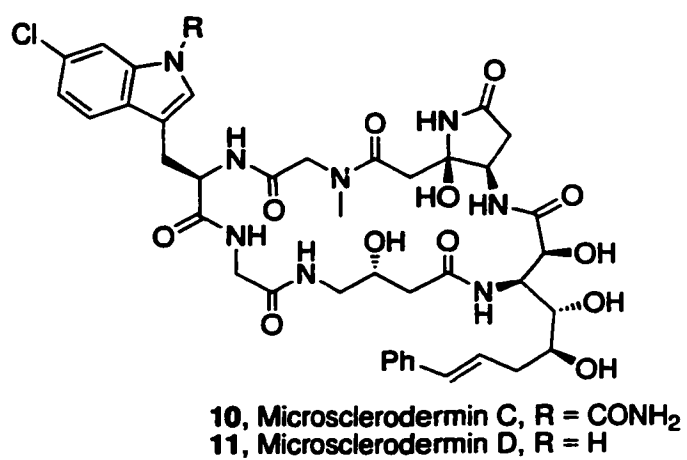
Figure 5. Microsclerodermins C-E (**10-12**) and scleritodermin A (**13**).

orange characteristic of aurantosides (Chapter 8). Several known compounds were isolated, but the major component was the novel bicyclic peptide theopalauamide (9),[16] which differed from a known compound, theonegramide, by an additional CHOH. This difference was localized to a sugar residue, which was determined to be galactose. While it would seem easy to tentatively assign this difference, the actual structure elucidation was quite involved, since a full data set was required to confirm the identity of the compound. The NMR chemical shifts of these peptides are quite variable, depending on concentration, pH, and other factors, so a direct comparison of chemical shift data was impossible.

After solving the structure of theopalauamide (9), I investigated a "pure" peptide obtained by Carole A. Bewley directly from filamentous microbial symbionts of *T. swinhoei* from Palau. It turned out that this was actually a mixture of two peptides, one of which was identical to theopalauamide. The other was identical in nearly all respects, and several degradation experiments showed that the only difference between the two compounds was confined to their conformations. A single bond in the molecule reversibly rotates during column chromatography using acid. This work led to biological studies on *T. swinhoei*, since I began to wonder about the symbiosis that probably produces such a complicated molecule. What was the identity of the bacterium responsible? Was the symbiosis species-specific? Did it evolve over a long timescale? How is it related to other bacteria which may be specific to related sponges such as those containing aurantosides and mozamides? These issues are explored in Chapters 9 and 10.

Probably the most difficult structures to solve from NMR spectra were in the next project, the discovery of microsclerodermins C-E (10-12) from *Theonella* sp. and *Microscleroderma* sp. from the Visayan Islands, Philippines (Chapter 6).[17] These

projects were selected because the sponges were from interesting lithistid genera, and I could not dereplicate them on the basis of their crude extract ^{1}H NMR spectra. Like theopalauamide, microsclerodermins C (**10**) and D (**11**) were conformationally labile, but they were isomerizing on the NMR timescale so that rotamers could not be separated. Because of this problem and extensive overlap of signals, I took a full NMR data set at two different temperatures and compared them to arrive at the final structures. The placement of the indole *N*-amide group on microsclerodermin C (**10**) was also difficult. Microsclerodermins C-E (**10-12**) showed activity against brine shrimp, which often indicates cancer-cell cytotoxicity, but some bioactivity data was not available at the time of publication. In addition to antifungal activity, microsclerodermins C-E (**10-12**) show cancer-cell cytotoxicity in the Bristol-Myers Squibb cell-line panel, with IC_{50}s of 2.4 μM, 4.0 μM, and 9.1 μM, respectively. They are relatively nonselective, with min/max IC_{50} ratios of 10.

In my final peptide project, I screened 13 extracts from the National Cancer Institute's Active Repository (Chapter 7). These extracts were selected because they were from interesting or underrepresented species of Lithistida, and because they were bioactive. Known compounds or metabolites closely related to known compounds were found in all but one extract. From a single sample of *Scleritoderma nodosum*, the novel peptide scleritodermin A (**13**) was isolated, and its structure was elucidated by NMR. Scleritodermin A (**13**) contained two novel amino acids, one of which has a bond unprecedented in marine natural products but commonly used in medicinal chemistry. In that residue, the amino acids ornithine and serine methyl ether were linked through nitrogen, but no amide bond was formed. In effect, this was a linkage of glutamic acid and serine, in which the γ-carbon of glutamic acid has been reduced, making the residue stable to proteolysis. The main challenges of this project were the limited amount of

NMR data obtainable through standard techniques and the need to synthesize and compare the Orn-Ser amino acid for confirmation of structure and configurational analysis. Scleritodermin A (13) was found to be active in Bristol-Myers Squibb's panel of cancer cell lines.

Structural elucidation techniques

The logic of structural elucidation, that proposed structures can only be disproved, means that all natural products are hypotheses subject to challenge and revision. Numerous nuclear magnetic resonance techniques have been developed to make the elucidation of planar structure a relatively speedy process, but there is not enough NMR data to secure a structure without reference to biosynthetic pathways or known compounds. If a biosynthetic hypothesis does not hold in certain instances, as is the case with the recent discovery that mevalonate is not the sole terpenoid precursor,[18] structures that rely on comparison to well established molecules or biosynthetic pathway information may be false. In addition, while elucidations of two-dimensional structures are often routine, stereochemical determination remains a difficult challenge. Solution of relative stereochemistry is sometimes possible using NMR data and computer models alone, but the absolute configuration still must be analyzed with reference to compounds of known configuration. Fortunately, because determination of absolute configuration usually requires degradation to known compounds, it provides additional proof of planar structure as well as evidence of configuration. Structural elucidation should not be considered completed until the absolute configuration has been determined, since this information is crucial to any synthetic effort or to understanding the bioactivity of a compound at the molecular level.

Elucidation of two-dimensional structure

The structural elucidation of makaluvamines H-M and damirone C was the simplest described in this thesis, since the compounds are achiral and are relatively small. The structure elucidation required a reliance on information from ^{13}C NMR chemical shifts and correlations to ^{1}H NMR data. Specifically, the HMQC and HMBC experiments were crucial for the elucidation of these structures. In the Heteronuclear Multiple Quantum Coherence (HMQC) experiment, protons are correlated to the carbons to which they are directly attached. The Heteronuclear Multiple Bond Coherence (HMBC) experiment, on the other hand, filters out the information from the directly attached carbons and instead shows correlations to carbons that are either two or three bonds away. These data allow proton-carbon fragments derived from the HMQC to be attached to each other and to quaternary carbons from the ^{13}C NMR experiment. The experiment still provides challenges to structure elucidation, since the two- and three-bond correlations cannot be differentiated. Recent pulse sequences by Köck et al.[19] provide a way around this, albeit in much less sensitive experiments. HMBC experiments are particularly simplified when methyl groups are directly attached to heteroatoms, since then only three-bond correlations are apparent in the spectra. The use of three-bond couplings from N-Me groups in comparison to NH correlations in the unmethylated analogs allowed the structures of the makaluvamines to be proven unambiguously (Figure 6). The COrrelation SpectroscopY (COSY) experiment was also used in the structure determination, since it shows correlations between all coupled protons. This system is simple enough, however, that ^{1}H-^{1}H spin systems could easily have been elucidated using one-dimensional proton-proton decoupling.

Other compounds required further techniques. In the determination of the planar structures of modified peptides, several additional NMR experiments are important

besides COSY, HMQC, and HMBC. In particular, the TOtal Correlation SpectroscopY (TOCSY) experiment is crucial in sturucture elucidation. Using TOCSY, all coupled protons in an amino acid can be correlated to each other, making it possible to determine the standard amino acids by looking at the TOCSY and HMQC spectra alone. In addition, non-standard amino acids can often be partially or totally elucidated using the TOCSY experiment, although care must be taken and often supporting information from other experiments (HMBC, peptide hydrolysis, etc.) must be used for confirmation. Once the individual amino acid residues have been identified, they must be connected to each other in order to finish the gross structure. The most important sequencing experiments are based on NOE data, since key correlations are often missing from the HMBC spectra of peptides. Distance constraints derived from NOE-type experiments must also be used with caution, since unlike HMBC correlations, they do not necessarily indicate covalent bonds. NOESY spectra (two-dimensional NOE) are most often used with lithistid peptides, which tend to be large and are thus well into the negative NOE region. For some peptides, a different type of distance restraint must be used: Rotating frame nuclear Overhauser Effect SpectroscopY (ROESY). This experiment is not dependent on the correlation time of molecules, giving a theoretical maximum enhancement of +30% for all molecules, regardless of size, solution viscosity, or magnetic field strength. The ROESY, NOESY, and TOCSY experiments provided the key data for the solution of all of the peptide structures described here.

Determination of configuration and conformation of natural products

Except in rare achiral compounds, the most difficult part of structure elucidation is often the determination of the relative and absolute configuration of stereocenters and the identification of conformational isomers. In the elucidation of the relative stereostructure of molecules, NMR experiments which measure coupling constants or

interatom distances are often used, but they can only be helpful when compared with computer-derived or hand-built molecular models. In contrast, the solution of absolute configuration requires one of two basic techniques: degradation to molecules for which standards are available, or derivatization with enantiomerically pure molecules of known configuration. Optical techniques have sometimes been used when no standards are available, but these techniques should be viewed with caution until they have been extensively tested in experiments using model compounds. Once the relative configuration of a molecule has been determined, its conformation can be probed using a variety of NMR and computer modeling methods. In this section, recent examples of stereostructure elucidation from the marine natural products literature will be used to illustrate modern techniques. X-ray crystallography, still the gold standard in conformational and configurational analysis, will not be discussed, since I have never worked with a natural product that was easily crystallized. Lanthanide shift reagents, which are rarely used in modern natural products studies, will also be left out of this section, but see Weinstein et al. for a recent review.[20]

The relative configuration of stereogenic centers in a molecule is usually determined using scalar coupling constants, the nuclear Overhauser effect (NOE), or a combination of the two, although chemical reactions are sometimes employed. These data must then be compared with models of the structure generated either by hand or by computer. A major danger with these techniques is that they are hampered by rotational averaging, necessitating model-building using all possible isomers. NMR experiments to determine scalar couping constants have been reviewed by Eberstadt et al.[21] Basically, they fall into two groups: those that measure ^1H-^1H coupling constants and those that measure ^1H-X atom (usually ^{13}C) coupling constants. Relatively simple examples of the use of ^1H-^1H coupling constants in structure determination are
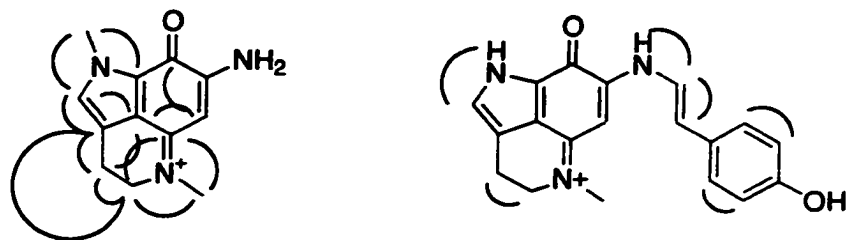
Figure 6. Structure elucidation of the makaluvamines. HMBC correlations of makalauvamine H (left) and COSY correlation of makaluvamine L.

14
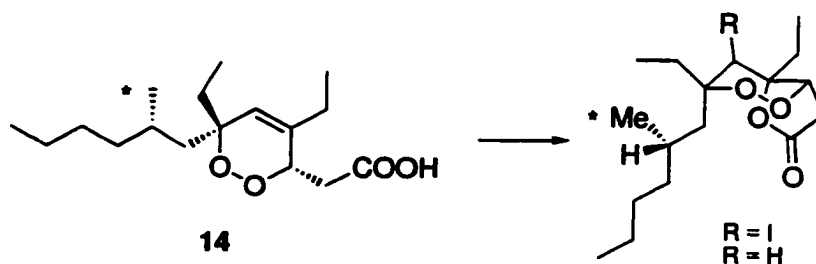
Figure 7. Conversion of the peroxide (left) into bicyclic derivatives (right) allowed NOE correlations to the β-methyl (*), which is fixed in space.

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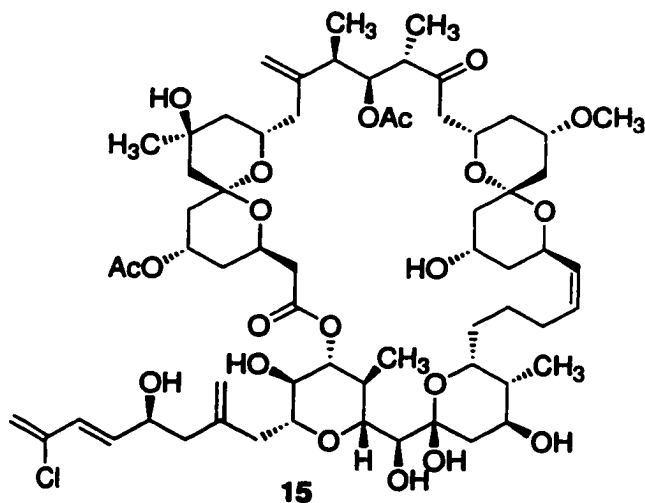
Figure 8. The relative configuration of the entire altohyrtin macrocycle could be fixed using NOE and J-coupling information.

abundant, for instance in the identification of galactose as the sugar residue of theopalauamide (see Chapter 8).[16]

Nuclear Overhauser effect (NOE) experiments, which measure the approximate distance between atoms, are probably the most commonly used techniques in the elucidation of stereostructure. Although it is often claimed that a rigid, cyclic structure is required for NOE measurements, this is not always true. For instance, in the solution of the stereostructure of some cyclic peroxides from the sponge *Plakortis* aff. *angulospiculatus*, M.V.R. Reddy found that a methyl group β to the peroxide ring of 14 was held in a relatively rigid conformation by transannular interactions (Figure 7).[10] By combining chemical reactivity and computer modeling with NOE observations, he conclusively demonstrated the relative configuration of the β-methyl stereocenter. 1,2-Disubstituted ethanes and other highly substituted side chains are also held in a relatively fixed conformation.[22] Still, NOE data are most commonly used with constrained cyclic structures. An excellent example of this is the solution of stereostructure of the altohyrtins (15) from the sponge, *Hyrtios altum*, in which all 24 relative configurations in a complex, 34-membered macrocycle were determined by NOE measurements (Figure 8).[23]

The entire relative and absolute configuration of maitotoxin (16), from the dinoflagellate *Gambierdiscus toxicus*, was elucidated by the Tachibana group using a combination of coupling constants, NOE data, and molecular modeling techniques. The series of publications on maitotoxin (16), one of the largest non-polymeric toxins known, provide an excellent example of the application of these diverse techniques, coupled with chemical synthesis (Figure 9).[23,24] The C1-C14 side chain of maitotoxin (16) contains seven stereocenters on a floppy, acyclic moiety, and Sasaki *et al.* used
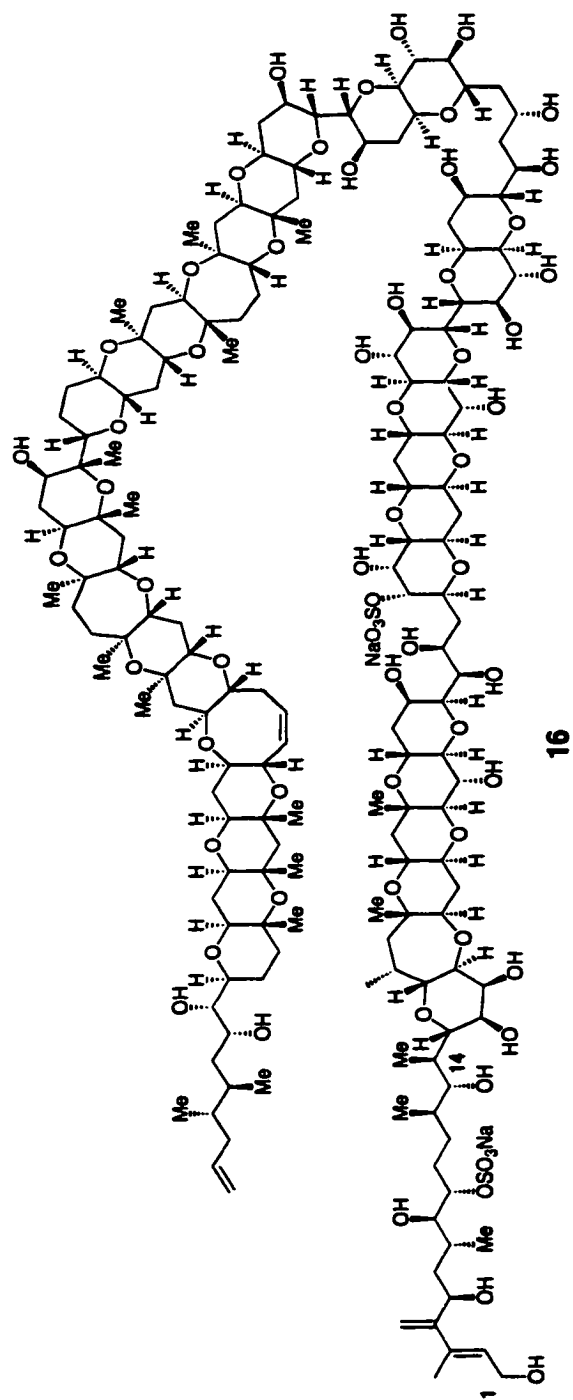
Figure 9. Maitotoxin. The heterocyclic portion stereochemistry was determined mainly by NOE analysis, while the side chains were elucidated using ^{1}H-^{13}C coupling constants and NOE data.

NMR techniques coupled with computer modeling to reduce the number of possible stereoisomers from 128 to 2. NOE and ^{1}H-^{1}H coupling constants were important in the determination, but ^{1}H-^{13}C 2- and 3-bond coupling constants were also used, providing a rare example in the marine natural products literature of the use of proton-carbon coupling constants in stereostructure elucidation. Following the selection of two candidate compounds, both possibilities were synthesized enantio- and diastereoselectively to provide the absolute configuration of the C1-C14 side chain.

Sometimes, chemical reactions are required to modify a structure for the elucidation of relative stereochemistry. Often, as in the case of acetonide formation on 1,2- or 1,3-diols, these chemical modifications are required to lock a structure in a rigid form so that the problem of rotational averaging is attenuated. Once an acetonide is formed, either chemical shift data or NOE measurements can be used to determine the relative configuration of the alcohol stereocenters. With the macrolactins (**17**), from a deep-sea bacterium, the relative configuration of a 1,3-diol was identified by forming an acetonide and measuring the ^{13}C NMR chemical shifts of the acetonide methyls (Figure 10).[25] With the *syn* configuration, two signals are present at δ 19.8 and δ 30.2 ppm. When the configuration is *anti*, on the other hand, there is only one average signal at δ 24.8 ppm. Relative stereochemistry can be determined by formation of an acetonide for NOE measurements, as was used for the 1,2-diol on the peptide, microsclerodermin A (**18**) from the sponge *Microscleroderma* sp. (Figure 11).[26] Chemical reactions can also be used to generate compounds of known relative stereochemistry, for instance in the methylation of a carboxylate anion to determine the configuration of both methylated and unmethylated analogs of **14**.[10] Finally, chemical reactions can be employed in the generation and removal of symmetry for the determination of relative configuration (see Chapter 4).[9]

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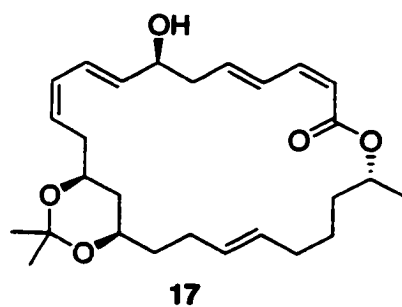
Figure 10. Macrolactin stereochemistry was determined by acetonide formation, and ^{13}C chemical shift data revealed whether the diol was *cis* or *trans*.

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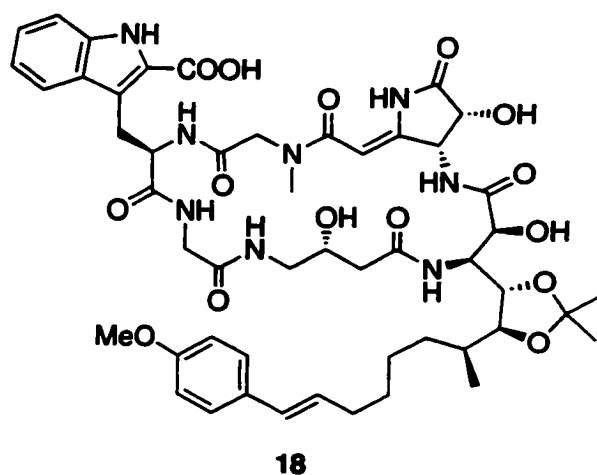
Figure 11. The configuration of the side chain diol of anhydro-microsclerodermin A was determined by acetonide formation and NOE measurements.
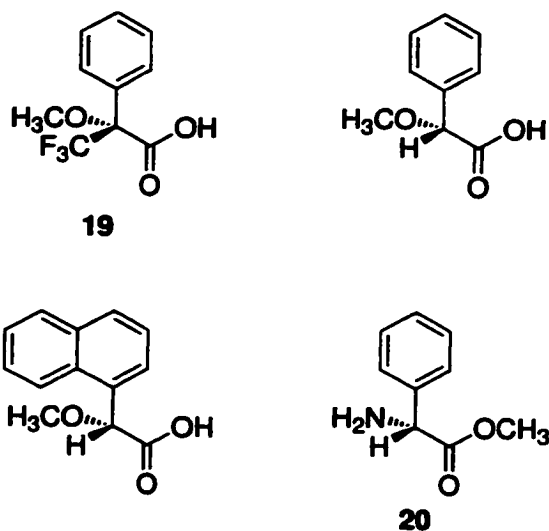
Figure 12. Chiral derivitization agents for absolute configuration determination. Top left: MTPA (Mosher's acid). Top right: MPA. Bottom left: 2-NMA. Bottom right: PGME.

21, R = H
22, R = MTPA
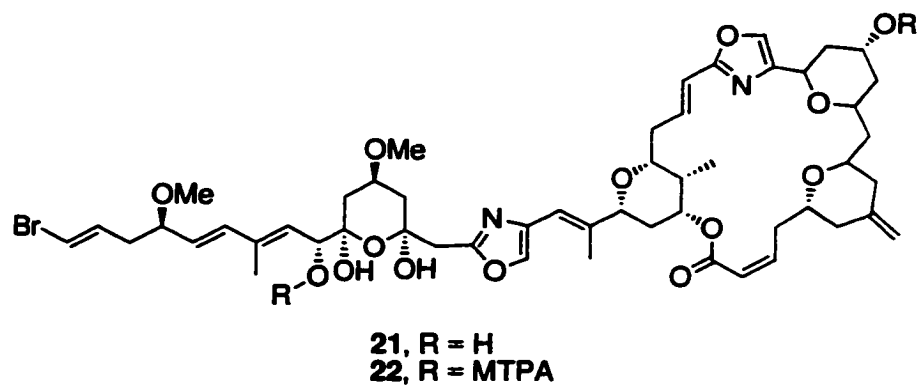
Figure 13. Despite the presence of two secondary alcohols, the absolute configuration of phorboxazole could be determined by the Mosher method because the alcohols were quite distant from each other.

In the determination of absolute configuration of a natural product, the unknown molecule must be compared in some way to compounds of known absolute configuration. The most common way to do this is by employing a chiral anisotropic reagent that can be attached to functional groups on the natural product. NMR measurements of chemical shifts in the molecule thus formed can be used to determine the absolute configuration. Usually, two enantiomers of known configuration must be attached in separate reactions to the same stereocenter, but this may not be the case with improved computer modeling. The prime example of derivatization for NMR measurements, the Mosher method,[27,28] is used mainly with secondary alcohols, but examples of its utility with tertiary alcohols[29] and primary amines[30] have been reported. In this method, esters or amides of 2-methoxy-2-phenyl-2-(trifluoromethyl)acetate (**19**, MTPA) are formed, using both the R- and the S- isomers of MTPA. The phenyl group creates a difference in chemical shifts because the ring current causes an upfield shift in nearby protons, and the O-Me shifts its neighbors slightly downfield. Because the conformation of the MTPA group is well defined, the difference in chemical shifts, reported in $\Delta\delta$, between the R- and S-MTPA derivates allows the absolute configuration at the alcohol or amine stereocenter to be defined. A large number of variants of this method have been reported in the literature, but they all rely on two basic factors: a relatively predictable conformation of the prosthetic group, and chemical shift anisotropy induced by an aromatic residue. Examples of other chiral shift reagents are found either with increasing aromaticity, such as the use of anthracene instead of benzene rings,[31] or with functionality that allows linking to other functional groups, such as the use of PGME (**20**) with acids (Figure 12).[32] Some authors have questioned the necessity of using both enantiomers in analysis of configuration.[33] Unfortunately, some degree of computer modeling is still usually required with these groups in case the

assumed conformation is incorrect. For instance, the Mosher's ester can be pushed out of its preferred conformation by steric strain,[34] and the PGME amides can lose their predictive value if neighboring H-bond acceptors are present.[35]

Despite occassional problems, derivatization with chiral reagents (especially the MTPA esters) is the most commonly used method to solve absolute configurations in the marine natural products literature. One interesting example that combines complex functionality and use of NMR data with employment of the MTPA reagent is the solution of the absolute configuration of phorboxazole A (21), from the marine sponge *Phorbas* sp. (Figure 13).[36] Two secondary alcohols are present in the phorboxazoles, but they are sufficiently separated that the Mosher method can be employed to determine the absolute stereochemistry at each center without intereference from the other MTPA group (22). The macrocycle's rigid configuration could be elucidated just by coupling with MTPA and using NMR data, but the side chain required synthesis of two possible isomers to confirm the absolute configuration. In Chapter 4, I discuss the use of the PGME amides in the determination of acid stereochemistry in marine natural products.[9]

Besides NMR methods, optical methods such as circular dichroism and optical rotation are often used in marine natural products chemistry. Circular dichroism is best used in comparison with standards that are identical to the natural product in question, but a large number of rules for various classes of molecules, such as diols[37] and lactones,[38] have been proposed. The efficacy of these rules for natural product configurational analysis is largely undetermined, so caution is warranted with these techniques. Often, highly fluorescent groups are used in tandem with CD to increase sensitivity and accuracy. Usually, the group must be chemically placed on the natural product, as was the case of the *p*-bromobenzyl derivative (23) used to solve the absolute configuration of ciguatoxin (24, Figure 14).[39] In one interesting recent case,
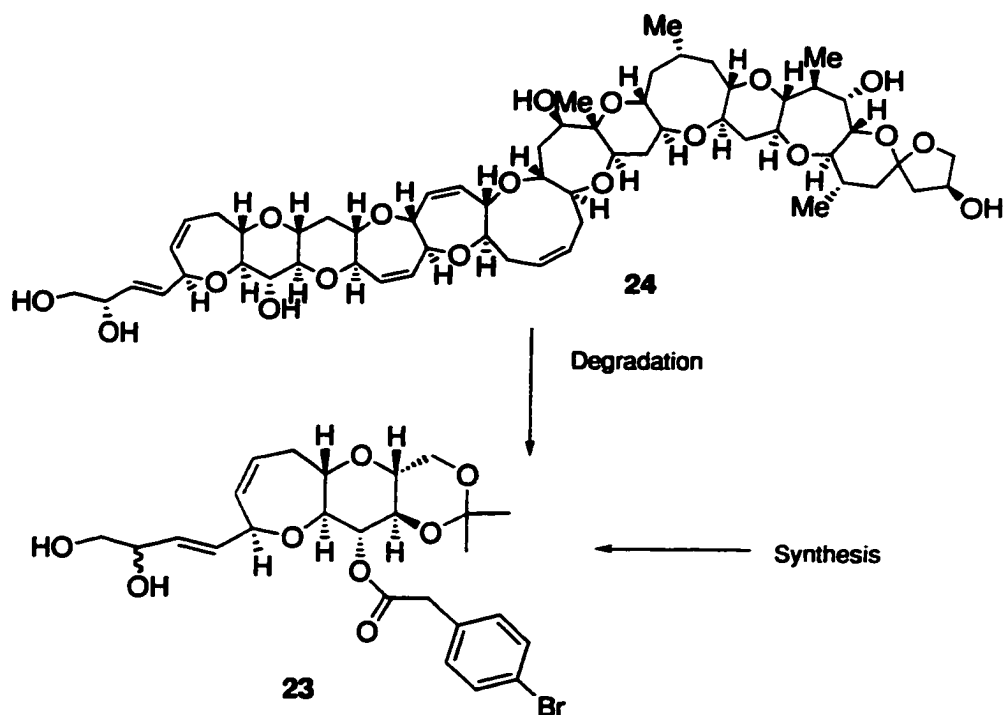
Figure 14. Ciguatoxin. Absolute configuration was determined using circular dichroism on fragments. *p*-Bromobenzoate was used as a chromophore.
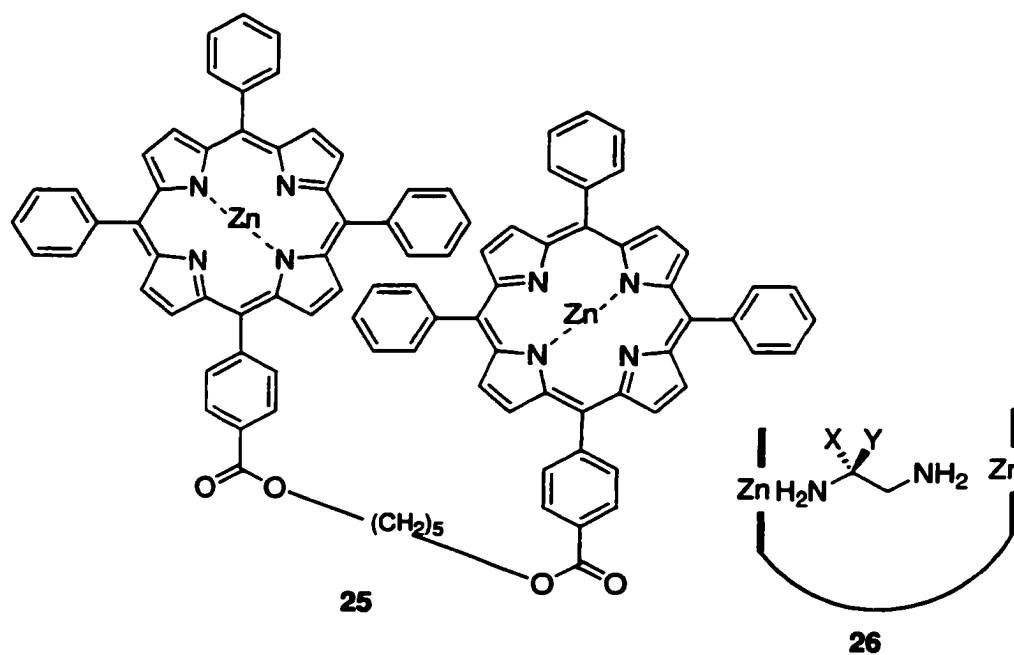
Figure 15. Nakanishi's porphyrin tweezer. A chiral diamine (shown figuratively at left) fits into the porphyrin chromophore, providing a large Cotton effect for circular dichroism analysis.

the Nakanishi group showed that permanent chemical modification can be unneccessary.[40] A zinc-porphyrin "tweezer" (25) was placed in solution with certain diamines (26), and the resulting (very large) Cotton effect was shown to accurately predict the configuration of the chiral amine (Figure 15). Circular dichroism has also been used to compare structures that are very similar, but not identical, in the determination of absolute configuration (see Chapter 3).[8]

Optical rotation measurements have been used extensively. Usually, direct comparison of a natural product with known compounds is not possible, so chemical degradations are often employed to turn natural products into known compounds or close analogs. A good example of this is ilimaquinone (27, from the sponge *Hippospongia metachromia*), the absolute configuration of which was identified by two-step conversion into an ester, which was compared to an identical compound (28) prepared from aureol (Figure 16).[41] A similar approach was taken with the isolated portion of the phorboxazole side chain.[42] Specifically, ozonolytic cleavage of phorboxazole, followed by methylation, afforded the enantionmer of *S*-malic dimethyl ester (29), as shown by optical rotation comparisons (Figure 17). Although degradation to known compounds is commonly used in conjunction with optical rotation, there have been recent reports of extremely accurate optical rotation calculations that could eventually replace the older methods. Polavarapu and Chakraborty calculated the optical rotations of small molecules using *ab initio* methods, so as these computer methods improve, it may be possible to calculate the rotation of more complex natural products in this manner.[43] In one striking example, the absolute configuration of the marine natural product hennoxazole A (30) was determined by *ab initio* calculation of molar rotations of the three stereogenic fragments (31-33) that make up the molecule
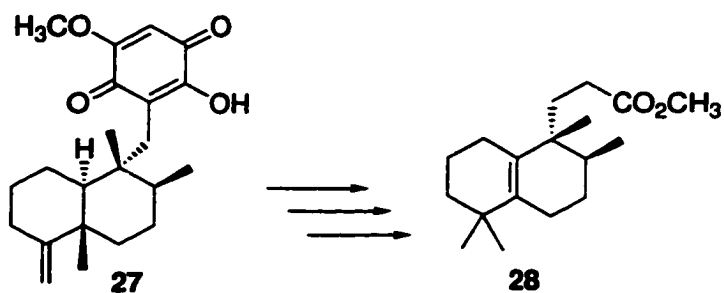
Figure 16. Conversion of ilimaquinone into a degradation product of aureol for optical rotation measurements.
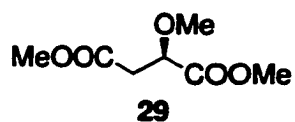
Figure 17. Phorboxazole A was ozonized and methylated to afford this analog, the configuration of which was determined by optical rotation.
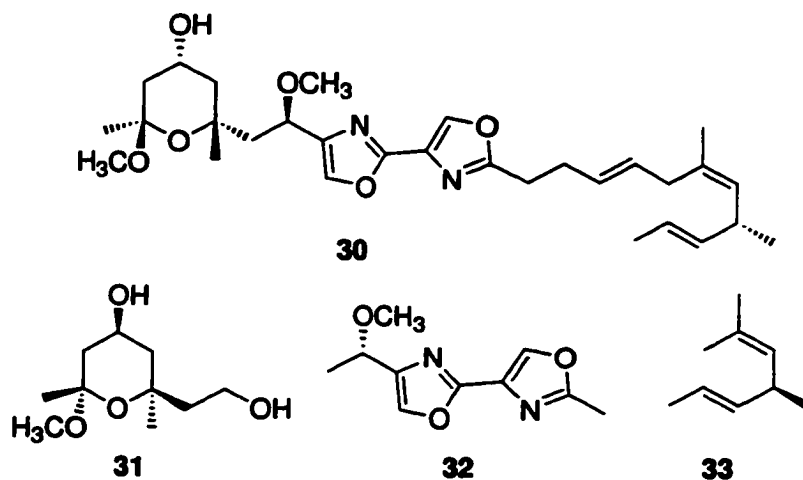
Figure 18. Hennoxazole A (top). Configuration was determined by *ab initio* calculation of optical rotations of three fragments (bottom).

(Figure 18).[44] Excellent correlation was observed between the optical rotations calculated for hennoxazole stereoisomers and synthesized fragments. In order for this technique to work, the fragments used in calculations must interact only weakly.

In addition to the spectroscopic methods discussed above, chromatographic techniques are often used in the elucidation of absolute stereostructure. Most commonly used are thin layer chromatography (TLC), high pressure liquid chromatography (HPLC), and gas chromatography (GC). With these techniques, compounds must be degraded into components for which standards are available. For this reason, they are most commonly used with peptides, which are hydrolyzed in concentrated acid to produce amino acids before chromatography. The solution of stereostructure of the modified peptide discobahamin (34), from *Discodermia* sp., provides a good example of the use of TLC in the solution of stereostructure (Figure 19).[45] The authors first hydrolyzed the peptides, then compared them with both enantiomers of standard amino acids by TLC on plates with chiral backings under a variety of solvent conditions. In studies of marine natural products, the most commonly reported HPLC method is the Marfey method, in which the hydrolysate is derivatized with the Marfey reagent (35) and co-eluted on reversed-phase HPLC with standards of all possible configurations (Figure 20).[46] A large number of HPLC methods have been used to distinguish steroisomers; all employ a chiral unit that has a highly UV-absorbent or fluorescent moiety. For example, chiral reagents for HPLC have been used extensively by the Yasumoto group in studies of complex marine toxins such as ciguatoxin (16).[39] Finally, enantiomeric molecules such as amino acids can be separated on GC columns with chiral packings, such as Alltech's™ Chirasil-Val column. Good separation can be achieved for all standard amino acids and for many unusual ones. An additional
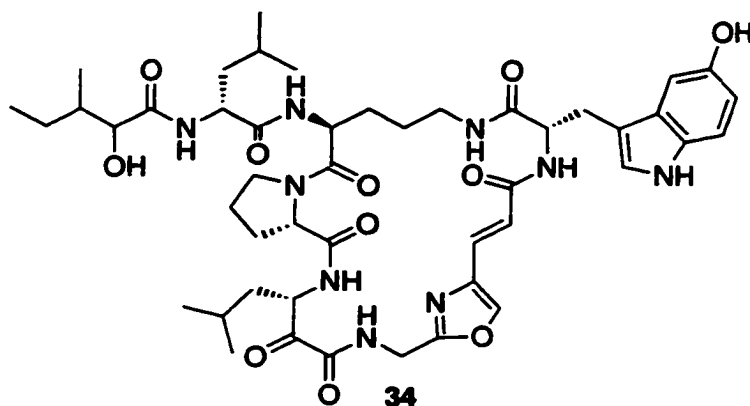
Figure 19. Discobahamin. Absolute configuration was determined by degradation and chiral TLC of resultant amino acids.
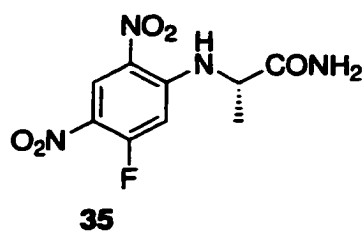
Figure 20. Marfey's reagent.
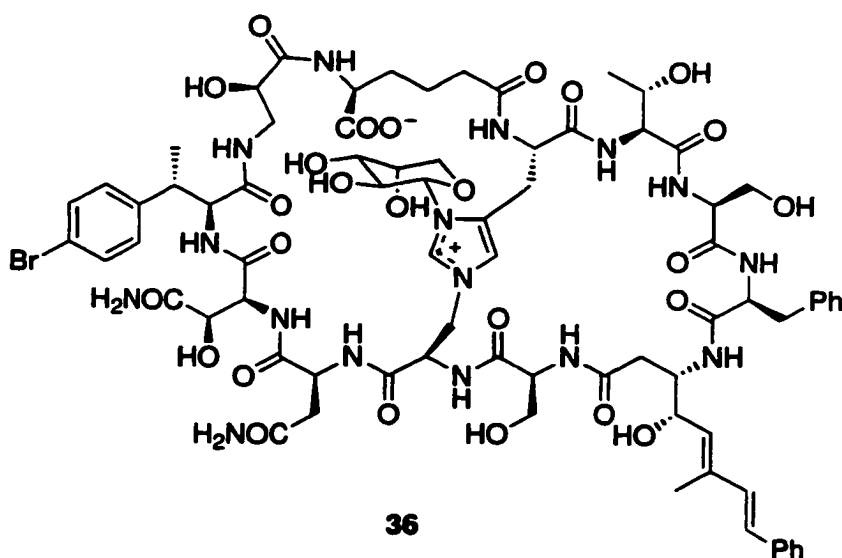
Figure 21. Theonegramide. Configuration was determined by degradation and GC-MS on a chiral GC column.

advantage is that the GC peaks can be analyzed using a mass spectrometer, providing extra sensitivity and confirmation of structure. The main disadvantages are that two derivitization steps are required to increase the volatility of amino acids, and that GC-MS is more expensive than HPLC. Bewley's work on theonegramide (36) provides a good example of the use of GC-MS techniques in the elucidation of a modified peptide structure (Figure 21).[47]

Once the absolute configuration of a natural product has been determined, its conformation can be approached using NMR experiments and computer modeling, often in conjunction with X-ray crystallographic data for related structures. The most commonly used NMR techniques in conformational analysis are NOE and scalar coupling constant experiments. NOE measurements have been applied with great success to rigid systems, but for molecules with fewer conformational restrictions, coupling constant determinations are often the method of choice. The solution conformation of okadaic acid (37), from the sponge *Halichondria okadai*, was proposed based solely on $^{2,3}J_{C,H}$ and $^{3}J_{H,H}$ measurements (Figure 22).[48] Studies of conformation have recently been performed mainly on cyclic peptides, probably because of the availability of a large database on preferred conformations of amino acids in proteins. Also, while studies of other molecules may rely heavily on scalar coupling, conformational analysis of peptides is usually NOE-based and may also involve estimated measurements of intramolecular hydrogen bond strengths. The most common approach is to measure the chemical shift changes of NH-protons with increasing temperature. If the NH-proton chemical shifts exhibit relatively large changes with increasing temperature, the NH-protons are probably more exposed to solvent and have less internal hydrogen bonding. The increasing entropy of the solvent with increasing temperature is expected to have the greatest effect on solvent-exposed protons, while

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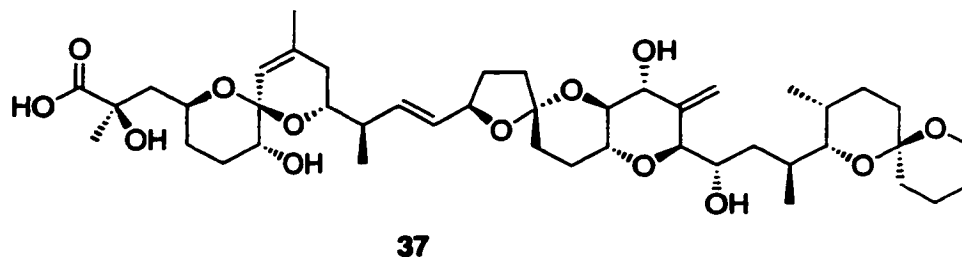
Figure 22. Okadaic acid's solution conformation was determined using homonuclear and heteronuclear coupling constants.

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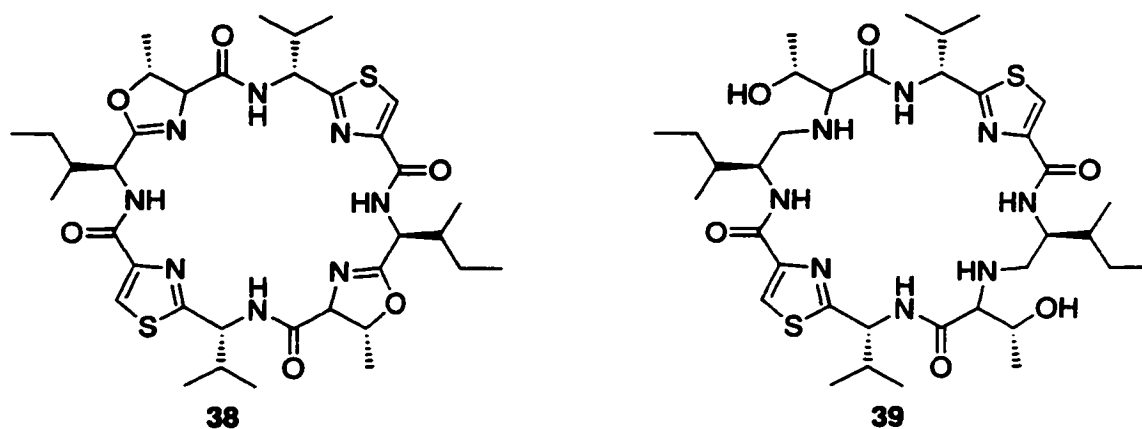
Figure 23. The oxazoline and thiazole rings of the peptide at left impose stringent conformational restrictions. The more flexible compound at right, with oxazoline rings removed, forms a novel, pseudo-chair conformation, as shown using NOE, coupling constant, and NH $\delta\Delta/\delta T$ NMR data.

protons with strong hydrogen bonds will be less affected. Caution is required in the interpretation of such data, however, since other changes in the molecule with temperature can also affect NH-proton chemical shifts. Andersen *et al.* showed that increases in the randomness of peptide structure with increasing temperature have large impacts on NH-proton chemical shifts.[42] To avoid misinterpretation of NH-proton chemical shift data, the measurements should be made on several closely related compounds. Alternatively, the α-proton chemical shift changes can be measured as an indication of a change in the overall structure of the peptide.

Probably the best illustration of peptide conformational studies using a marine natural product is the exhaustive examination of ascidiacylamide derivatives (**28,29**) by Abbenante *et al.* (Figure 23).[50] The authors synthesized a series of peptides based on the natural products from *Lissoclinum patella* to show that the thiazole and oxazoline rings in the compounds impose stringent conformational restrictions. Selective removal of some of the 5-membered rings created novel conformations not present in the natural product. In their analysis of a less constrained analog (**29**), the authors used NOE and coupling constant data to impose restraints on their computer models. Further constraints were derived using NH chemical shift changes with temperature. The removal of the two oxazoline rings created a novel, pseudochair conformation, as shown by the computer-minimized NMR structures. Conformational studies using similar strategies have also often been applied to constrained peptides containing prolines,[51,52] but the techniques have found less use in other highly modified marine metabolites with more degrees of freedom.

Configurational and conformational studies of lithistid peptides

The determination of absolute configuration of lithistid peptides is an important problem, since there are many non-standard amino acids, and even the standard amino

acids often have the D-configuration. In all of my projects, hydrolysis followed by derivatization and GC-MS on a chiral support was the main technique used to determine the absolute configurations. However, in some cases modified reaction conditions were necessary to release standard amino acids. For instance, hydrazinolysis released only the ureido-linked isoleucine of the mozamides (7,8). Ozonolysis was also frequently required to degrade unusual aromatic and unsaturated amino acids into aspartic acid derivatives. This technique was used in the solution of the histido-alanine of theopalauamide (9),[16] of the modified tryptophans in microsclerodermins (10-12)[17] and mozamides (7, 8),[14] of the conjugated amino acids in microsclerodermins (10-12), and of the modified tyrosine in scleritodermin A (13). Basic, oxidative cleavage of a ketoamide was also required in the solution of scleritodermin's structure, and periodate cleavage was used to determine the stereochemistry of the microsclerodermin (10-12) side chain. Hydrogenation was employed in the determination of configuration of the polyketide side chain of theopalauamide (9).

Other chemical modifications were also required for most projects. The side chain configuration of microsclerodermins C, D, and E (10-12) was elucidated by acetonide formation and NOE analysis. In order to determine the stereochemistry of the novel ornithine-serine residue of scleritodermin A (13), standards had to be synthesized. The standards were derivatized using fluorescent chiral and achiral linkers and compared with the derivatized hydrolysate of scleritodermin A (13) using HPLC with fluorescence detection.

Theopalauamide (9) provided some unique challenges. The identity (= stereochemistry) of the sugar residue was determined to be galactose by coupling constant analysis, the only such analysis required during in my peptide projects. A stable isomer of theopalauamide, isotheopalauamide, was found to differ only in

conformation, mainly about one bond. In the analysis, the peptide was hydrogenated and hydrolyzed on the 150 mg scale, then amino acids were derivatized with fluorodinitrobenzene. NOE analysis of isolated amino acids revealed that theopalauamide undergoes conversion into a stable conformational isomer through rotation of a single C-C bond in the large molecule.

NMR techniques have improved so much that few, if any, chemical modifications are required to solve the structures of most natural products. In contrast, stereochemical elucidation still requires a great deal of wet chemistry in most instances. Numerous advances have been made in recent years that have allowed the determination of absolute configurations of the complex molecules detailed in this chapter, but there is still a lot of room for innovation. Highly modified peptides, in particular, are challenging because of their numerous stereocenters and lack of easily defined conformations. The molecules described in Chapters 3-8 exemplify a range of structure elucidation techniques used in marine natural products chemistry.

References

1. Faulkner, D.J. *Natural Products Reports* **1998**, *14*, 113-158 and previous reviews in this series.

2. Hung, D.T.; Chen, J.; Schreiber, S.L. *Chemistry & Biology*, **1996**, *3*, 287-293.

3. Sakowicz, R.; Berdelis, M.S.; Ray, K.; Blackburn, C.L.; Hopmann, C.; Faulkner, D.J.; Goldstein, L.J. *Science*, **1998**, *280*, 292-295.

4. Bewley, C.A.; Faulkner, D.J. *Angew. Chem. Int. Ed. Engl.* **1998**, *37*, 2162-2178.

5. Various authors, *Chem. Rev.* **1993**, *93*, 1671-1944.

6. Schmidt, E.W.; Harper, M.K.; Faulkner, D.J. *J. Nat. Prod.* **1995**, *58*, 1861-1867.

7. VanSoest, R.W.M.; Zea, S.; Kielman, M. *Bijdragen Tot de Dierkunde* **1994**, *64*, 163-192.

8. Schmidt, E.W.; Faulkner, D.J. *Tetrahedron Lett.* **1996**, *37*, 3951-3954.

9. Schmidt, E.W.; Faulkner, D.J. *Tetrahedron Lett.* **1996**, *37*, 6681-6684.

10. Compagnone, R.S.; Piña, I.C.; Rangel, H.R.; Dagger, F.; Suárez, A.I.; Reddy, M.V.R.; Faulkner, D.J. *Tetrahdron* **1998**, *54*, 3057-3068.

11. Kobayashi, J.; Ishibashi, M. *Chem. Rev.* **1993**, *93*, 1753-1769.

12. Fusetani, N.; Matsunaga, S. *Chem. Rev.* **1993**, *93*, 1793-1806.

13. Faulkner, D.J.; He, H.Y.; Unson, M.D.; Bewley, C.A. *Gazz. Chim. Ital.* **1993**, *123*, 301-307.

14. Schmidt, E.W.; Harper, M.K.; Faulkner, D.J. *J. Nat. Prod.* **1997**, *60*, 779.

15. McInerney, J.; Kelly-Borges, M. Origin & Outlook: 5th International Sponge Symposium Abstracts, 1998.

16. Schmidt, E.W.; Bewley, C.A.; Faulkner, D.J. *J. Org. Chem.* **1998**, *63*, 1254-1258.

17. Schmidt, E.W.; Faulkner, D.J. *Tetrahedron* **1998**, *54*, 3043-3056.

18. Piel, J.; Donath, J.; Bandemer, K.; Boland, W. *Angew. Chem. Int. Ed. Engl.* **1998**, *37*, 2478-2481.

19. Köck, M.; Reif, B.; Fenical, W.; Griesinger, C. *Tetrahedron Lett.* **1996**, *37*, 363-366.

20. Weinstein, S.E.; Vining, M.S.; Wenzel, T.J. *Mag. Res. Chem.* **1997**, *35*, 273-280.

21. Eberstadt, M.; Gemmecker, G.; Mierke, D.F.; Kessler, H. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1671-1695.

22. Sasaki, M.; Matsumori, N.; Maruyama, T.; Nonomura, T.; Murata, M.; Tachibana, K.; Yasumoto, T. *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1672-1674.

23. Kobayashi, M.; Aoki, S.; Gato, K.; Kitagawa, I. *Chem. Pharm. Bull.* **1996**, *44*, 2142-2149.

24. Nonomura, T.; Sasaki, M.; Matsumori, N.; Murata, M.; Tachibana, T.; Yasumoto, T. *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1675-1678.

25. Rychnovsky, S.D.; Skalitzky, D.J.; Pathrina, C.; Jensen, P.R.; Fenical, W. *J. Am. Chem. Soc.* **1992**, *114*, 671-677.

24. Bewley, C.A.; Debitus, C.; Faulkner, D.J. *J. Am. Chem. Soc.* **1994**, *116*, 7631.

26. Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092-4096.

27. Dale, J.A.; Dull, D.L.; Mosher, H.S. *J. Org. Chem.* **1969**, *34*, 2543-2549.

28. Izumi, S.; Moriyoshi, H.; Hirata, T. *Bull. Chem. Soc. Jpn.* **1994**, *67*, 2600-2602.

29. Hoye, T.R.; Renner, M.K. *J. Org. Chem.* **1996**, *61*, 8489-8495.

30. Seco, J.M.; Latypov, S.; Quiñoa, E.; Riguera, R. *Tetrahedron Lett.* **1994**, *35*, 2921-2924.

32. Nagai, Y.; Kusumi, T. *Tetrahedron Lett.* **1995**, *36*, 1853-1856.

33. Latypov, S.K.; Seco, J.M.; Quiñoa, E.; Riguera, R. *J. Am. Chem. Soc.* **1998**, *120*, 877-882.

34. Kusumi, T.; Fujita, Y.; Ohtani, I.; Kakisawa, H. *Tetrahedron Lett.* **1991**, *32*, 2923-2926.

35. Sata, U.N.; Matsunaga, S.; Fusetani, N. OR2, Proceedings of the 9th International Symposium on Marine Natural Products, 1998.

36. Searle, P.E.; Molinski, T.F.; Brzezinski, L.J.; Leahy, J.W. *J. Am. Chem. Soc.* **1996**, *118*, 9422-9423.

37. Wielser, W.T.; Nakanishi, K. *J. Am. Chem. Soc.* **1989**, *111*, 9205-9213.

38. Djerassi, C. *Optical Rotatory Dispersion, Application to Organic Chemistry.* McGraw-Hill Co. Inc., 1960.

39. Satake, M.; Morohashi, A.; Oguri, H.; Oishi, T.; Hirama, M.; Harada, N.; Yasumoto, T. *J. Am. Chem. Soc.* **1997**, *119*, 11325-11326.

40. Huang, X.; Rickman, B.H.; Borhan, B.; Berova, N.; Nakanishi, K. *J. Am. Chem. Soc.* **1998**, *120*, 6185-6186.

41. Capon, R.J.; MacLeod, J.K. *J. Org. Chem.* **1987**, *52*, 5060-5061.

42. Molinski, T.F. *Tetrahedron Lett.* **1996**, *37*, 7897-7880.

43. Polavarapu, P.L.; Chakraborty, D.K. *J. Am. Chem. Soc.* **1998**, *120*, 6160-6164.

44. Kondru, R.K.; Wipf, P.; Beratan, D.N. *J. Am. Chem. Soc.* **1998**, *120*, 2204-2205.

45. Gunasekara, S.P.; Pomponi, S.A.; McCarthy, P.J. *J. Nat. Prod.* **1994**, *56*, 293-296.

46. Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591-596.

47. Bewley, C.A.; Faulkner, D.J. *J. Org. Chem.* **1994**, *59*, 4849-4852.

48. Matsumori, N.; Murata, M.; Tachibana, K. *Tetrahedron* **1995**, *51*, 12229-12238.

49. Andersen, N.H.; Neidigh, J.W.; Harris, S.M.; Lee, G.M.; Liu, Z.; Tong, H. *J. Am. Chem. Soc.* **1997**, *119*, 8547-8561.

50. Abbenante, G.; Fairlie, D.P.; Gahan, L.R.; Hanson, G.R.; Pierens, G.K.; van den Brenk, A.L. *J. Am. Chem. Soc.* **1996**, *118*, 10384-10388.

51. Morita, H.; Kayashita, T.; Takeya, K.; Itokawa, H.; Shiro, M. *Tetrahedron* **1997**, *53*, 1607-1616.

52. Tamaki, M.; Komiya, S.; Yabu, M.; Watanabe, E.; Akabori, S.; Muramatsu, I. *J. Chem. Soc., Perkin Trans. 1* **1997**, 3497-3500.

CHAPTER 2

MAKALUVAMINES H–M AND DAMIRONE C FROM THE POHNPEIAN SPONGE ZYZZYA FULIGINOSA

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Abstract.—Seven new pyrroloiminoquinone alkaloids, makaluvamines H–M [19–24] and damirone C [25], together with the known compounds, makaluvamines C [13], D [14], and G [17], were isolated from the sponge Zyzzya fuliginosa collected at Nahpali Island, Pohnpei, Micronesia. The structures of the new compounds were elucidated by interpretation of spectral data. The chemotaxonomic relationships involving the makaluvamines and related pyrroloiminoquinone alkaloids are discussed.

Pyrroloiminoquinone alkaloids have been isolated from several sponges and from an ascidian. The simplest of these alkaloids are batzellines A–C [1–3] and isobatzellines A–D [4–7] from a deep-water Caribbean sponge of the genus *Batzella* (1;2) and damirones A [8] and B [9] from a *Damiria* sp. from Palau (3). The most complex of these alkaloids are the discorhabdins, exemplified by discorhabdin A [10], which have been reported from a New Zealand collection of *Latrunculia* sp. (4–6), *Prianos melanos* from Okinawa (7,8), and *Zyzzya* cf. *marsailis* from Fiji (9). Makaluvamines A–G [11–17] are cytotoxic alkaloids from the Fijian *Zyzzya* cf. *marsailis* (9) and an Indonesian *Histodermella* sp. (10) that are intermediate in complexity and may in fact be intermediates in the biosynthetic route to the discorhabdins. In addition, a related pyrroloiminoquinone alkaloid, wakayin [18], was isolated from an ascidian of the genus *Clavelina* from Fiji (11). From the viewpoint of chemotaxonomy, this very complex situation has recently been simplified by the taxonomic reassignments noted below. We now report the isolation and identification of seven new alkaloids, makaluvamines H–M [19–24], and damirone C [25], together with the known metabolites makaluvamines C [13], D [14], and G [17], from a Pohnpeian specimen of *Zyzzya fuliginosa* (order Poecilosclerida) (Carter, 1879).

RESULTS AND DISCUSSION

Zyzzya fuliginosa was collected by hand using scuba (−13 meters) from a reef at Nahpali Island, Pohnpei, and was kept frozen until it was lyophilized and extracted. A portion of the MeOH-CH$_2$Cl$_2$ (1:1) extract of *Z. fuliginosa* was partitioned between EtOAc and H$_2$O and the H$_2$O-soluble material was chromatographed on Sephadex LH-20, using MeOH as eluent, to obtain six major colored fractions. Further purification of these fractions on either Sephadex LH-20 or on a C$_{18}$ reversed-phase Sep Pak column using a MeOH-H$_2$O (0.1% TFA) gradient, with final purification by hplc on a C$_{18}$ reversed-phase column, yielded makaluvamines C [13] (0.51% dry wt), D [14] (0.07% dry wt), G [17] (0.34% dry wt), H [19] (0.47% dry wt), I [20] (1.03% dry wt), J [21] (0.08% dry wt), K [22] (0.02% dry wt), L [23] (0.15% dry wt), and M [24] (0.03% dry wt), and damirone C [25] (0.54% dry wt). Makaluvamines C [13], D [14], and G [17] were identified by comparison of their spectral data with literature values (9,10). Makaluvamines H–M [19–24] and damirone C [25], which differ from known compounds with respect to the position and number of N-methyl groups, were identified by comparison of their spectral data with those of the known members of these series (3,9,10).

Makaluvamine H [19] was obtained as the TFA salt, which is a red-brown solid. The molecular formula of the protonated base was found by hrms to be C$_{12}$H$_{14}$N$_3$O. The ^{1}H-

	R₁	R₂	R₃	R₄
1	Me	Cl	H	SMe
2	H	Cl	H	SMe
3	Me	Cl	H	H
8	Me	H	Me	H
9	H	H	Me	H
25	H	H	H	H

	R₁	R₂
4	SMe	Cl
5	SMe	H
6	H	Cl

	R₁	R₂	R₃
11	Me	H	H
13	H	Me	H
14	H	H	a
15	Me	H	b
17	Me	Me	b
19	Me	Me	H
20	H	H	H
21	H	Me	a
22	Me	H	a
23	H	Me	b
24	H	H	b
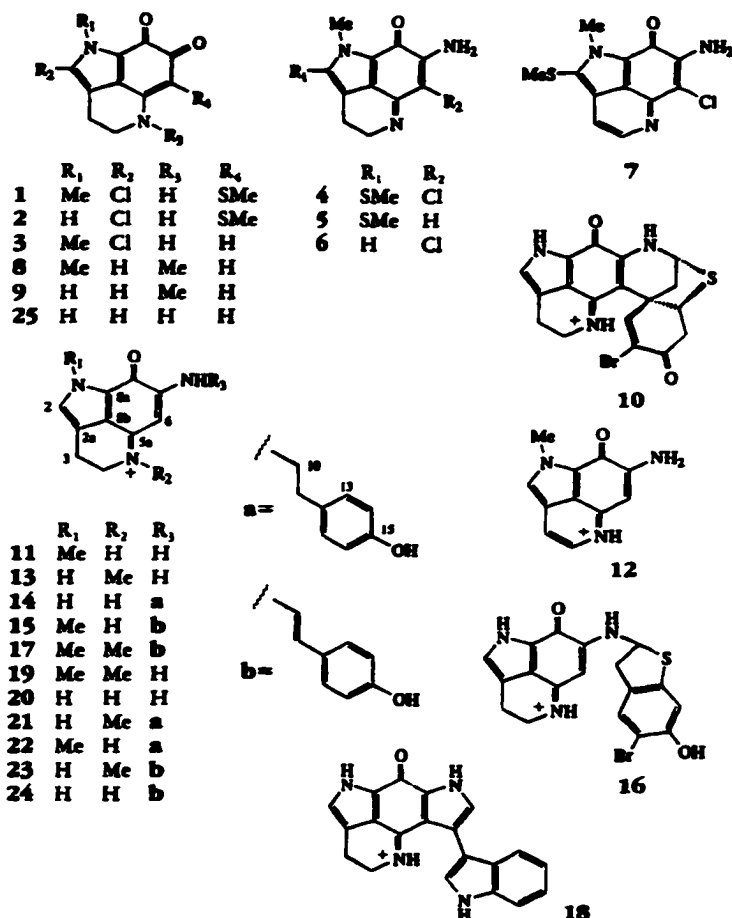
nmr spectrum (Table 1) contained signals at δ 7.26 (1H, s, H-2), 5.65 (1H, s, H-6), 3.81 (2H, t, *J*=7.5 Hz, H-4), and 2.86 (2H, t, *J*=7.5 Hz, H-3), which were assigned by comparison with the spectral data of makaluvamines A [11] and C [13] (9), both of which have one fewer methyl group than makaluvamine H [19]. The two methyl signals at δ 3.83 (3H, s, Me-1) and 3.27 (3H, s, Me-5) correspond in chemical shift to the *N*-methyl signals in makaluvamines A [11] and C [13], respectively. A complete analysis of HMQC and HMBC nmr experiments allowed the assignment of the ¹³C-nmr data (Table 1) and confirmed the positions of the two *N*-methyl groups.

Makaluvamine I [20], obtained as the TFA salt, is a green solid that has the molecular formula C₁₀H₁₀N₃O. The ¹H-nmr spectrum is similar to that of makaluvamine H [19] except that it lacks signals corresponding to *N*-methyl groups. The ¹H- and ¹³C-nmr data of makaluvamine I [20] (Table 1) were compared with those of makaluvamines A [11] and C [13] and the resulting assignments were confirmed using DEPT and HMBC experiments.

The TFA salts of makaluvamine J [21] and makaluvamine K [22] were obtained as red-brown solids. The protonated bases of both compounds have the same molecular formula, C₁₉H₂₀N₃O₂. The ¹H- and ¹³C-nmr spectra of both compounds (Table 2) contain signals that were assigned to a tyramine residue, and the structures must therefore be

TABLE 1. Comparison of the ^{1}H- and ^{13}C-Nmr Data of Makaluvamines H [19] and I [20] with those of Makaluvamines A [11] and C [13].

Position	Makaluvamine H [19]			Makaluvamine I [20]			Makaluvamine A [11]			Makaluvamine C [13]		
	δ_H	mult.,J	δ_C	δ_H	mult.,J	δ_C	δ_H	mult.,J	δ_C	δ_H	mult.,J	δ_C
2	7.26	s	131.1	7.29	s	127.0	7.30	s	131.0	7.28	s	126.6
2a			118.7			118.9			117.8			123.4
3	2.86	t, 7.5	18.8	2.84	t, 7.5	18.5	2.83	t, 7.5	18.0	2.92	t, 7.5	18.9
4	3.81	t, 7.5	52.4	3.76	t, 7.5	42.7	3.75	t, 7.5	42.0	3.90	t, 7.5	52.6
5a			158.1			156.4			156.0			155.7
6	5.65	s	85.6	5.63	s	87.0	5.61	s	86.4	5.73	s	85.5
7			158.8			157.7			156.7			156.5
8			167.7			168.4			168.2			167.4
8a			123.2			124.2			123.0			117.9
8b			122.5			123.0			122.3			123.2
N(1)H				13.02						13.10		
N(1)Me	3.83	s	35.9				3.88	s	35.8			
N(5)H				10.47			10.44					
N(5)Me	3.27	s	39.6							3.31		39.0
N(9)H$_2$	8.61			8.54			8.37			8.65		
	9.40			8.92			9.09			9.53		

TABLE 2. ^{1}H- and ^{13}C-Nmr Data of Makaluvamines J [21], K [22], L [23], and M [24].

Position	Makaluvamine J [21]			Makaluvamine K [22]			Makaluvamine L [23]			Makaluvamine M [24]		
	δH	mult.,J	δC	δH	mult.,J	δC	δH	mult.,J	δC	δH	mult.,J	δC
2	7.31	s	126.8	7.33	s	131.4	7.28	s	126.2	7.33	s	126.5
2a			118.1			118.0			117.6			118.4
3	2.91	t, 7.5	18.9	2.84	t, 7.5	18.0	2.93	t, 7	18.9	2.89	t, 7	18.2
4	3.89	t, 7.5	52.7	3.78	t, 7.5	42.2	3.92	t, 7	52.8	3.87	t, 7	42.8
5a			155.4			156.5			154.7			157.8
6	5.59	s	83.4	5.47	s	84.2	6.28	s	85.6	6.12	s	86.6
7			153.3			152.9			146.7			147.1
8			167.0			167.7			167.3			167.7
8a			123.2			123.0			123.5			122.4
8b			123.4			122.3			123.1			124.0
10	3.59	m	45.0	3.48	m	45.1	7.58	m	121.7	6.99	d, 13.5	121.4
11	2.81	t, 7	32.7	2.77	t, 7	32.4	7.05	d, 13.5	124.2	7.40	d, 13.5	123.5
12			128.3			128.2			126.2			126.2
13,17	7.04	d, 8	129.8	7.03	d, 8	129.6	7.41	d, 8	128.4	7.38	d, 8	128.2
14,16	6.68	d, 8	115.2	6.68	d, 8	115.3	6.76	d, 8	115.8	6.77	d, 8	115.7
15			156.0			156.0			158.0			156.3
N(1)H	13.09	brs					13.10	brs		13.12	brs	
N(1)Me				3.89	s, 3H	35.9						
N(5)H				10.47	brs					10.63	brq	
N(5)Me	3.37	s, 3H	39.3				3.47	s, 3H	39.6			
N(9)H	9.13	t, 6		8.97	t, 6		10.78	d, 11		10.63	brs	
OH	9.1	brs		9.3	brs		9.8	brs		9.8	brs	

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related to that of makaluvamine D [14], which does not contain an *N*-methyl group. The ¹H-nmr signal at δ 3.37 (3H, s) in the spectrum of makaluvamine J [21] was assigned to a methyl group at N-5 and the signal at 3.89 (3H, s) in the spectrum of makaluvamine K [22] was assigned to a methyl group at N-1. The assignments were confirmed by analysis of HMBC and HMQC results.

The TFA salt of makaluvamine L [23] was obtained as a green solid. The molecular formula, $C_{19}H_{18}N_3O_2$, reveals that it is isomeric with makaluvamine E [15], which is also a green solid with a similar uv-visible spectrum. The ¹H-nmr spectrum of 23 contained signals at δ 7.58 (1H, m), 7.41 (2H, d, J=8 Hz), 7.05 (1H, d, J=13.5 Hz), 6.76 (2H, d, J=8 Hz) that were assigned to a *N*-(*p*-hydroxy-*trans*-styryl) group, similar to that found in makaluvamine E [15]. The major differences between the ¹H-nmr data of 23 and 15 were in the chemical shifts of the *N*-methyl signals at δ 3.47 in 23 and 3.97 in 15 indicating that the *N*-methyl group in makaluvamine L [23] was at N-5 rather than N-1. Analysis of HMQC and HMBC data allowed assignment of the ¹³C-nmr signals (Table 2) and confirmed the location of the *N*-methyl group at N-5.

A second green compound, makaluvamine M [24], was obtained as a very minor metabolite. The molecular formula of the protonated base, $C_{18}H_{16}N_3O_2$, coupled with the lack of *N*-methyl signals in the ¹H- and ¹³C-nmr spectra (Table 2), indicated that makaluvamine M [24] is the *N*-demethyl derivative of makaluvamine L [23].

Damirone C [25] was isolated as a red-brown solid of molecular formula $C_{10}H_8N_2O_2$. In contrast with data for the makaluvamines, the ¹³C-nmr spectrum of damirone C [25] contained two signals between δ 170–180, and was similar to the spectra of the damirones (3). The ¹H- and ¹³C-nmr data, assigned using HMQC and HMBC experiments, were fully compatible with the proposed structure, which lacks the *N*-methyl group of damirones A [8] and B [9].

The rather complicated chemotaxonomic picture described in the introductory paragraph has recently been simplified by taxonomic revisions proposed by van Soest *et al*. (12). The Fijian sponge *Zyzzya* cf. *marsailis* (9), which is an incorrect spelling of *Z. massalis*, and the Palauan *Damiria* sp. (3) have been reassigned to *Z. fuliginosa*. The Indonesian *Histodermella* sp. (10) is also now considered to be a species of *Zyzzya* (M. Kelly-Borges, Harbor Branch Oceanographic Institution, personal communication). Thus, all the makaluvamines and damirones described to date have been obtained from sponges of the genus *Zyzzya*.

Some of the makaluvamines (A, C, E, and F) were reported to be cytotoxic against the human colon tumor line HCT-116 and to inhibit topoisomerase II activity (9). Makaluvamines D, G, H, and J–M were inactive against topoisomerase in an assay that employs three genetically engineered yeast strains (13); one strain was least sensitive to DNA damaging agents, one was hypersensitive with a DNA repair gene deleted, and one had its topoisomerase I and DNA repair genes deleted. We recognize that this assay is not comparable with that used previously (9). However, the makaluvamines were cytotoxic against HCT-116 in vitro. In a semi-quantitative assay, makaluvamines I [20] and L [23] were an order of magnitude more active than makaluvamines C [13], G [17], H [19], and K [22], and makaluvamines D [14] and M [24] were the least active. The new makaluvamines showed only mild activity against *Bacillus subtilis* and no activity against other standard test organisms in our panel.

EXPERIMENTAL

EXTRACTION AND ISOLATION.—The sponge was stored frozen until it was lyophilized. The sponge (34

g dry wt) was extracted exhaustively with CH₂Cl₂-MeOH (1:1) to obtain a black extract (7.5 g). A portion (3.8 g) of the extract was partitioned between H₂O and EtOAc. The darkly pigmented aqueous layer was lyophilized and the residue chromatographed on Sephadex LH-20, using MeOH as eluent, to obtain several colored fractions that were further purified by chromatography on a reversed-phase C₁₈ Sep Pak (Waters Boodapak) column using a gradient of 20–70% MeOH in H₂O containing 0.1% TFA as eluent to obtain a mixture of makaluvamines C [13], H [19], and I [20], and damirone C [25], as the TFA salts. The mixture was purified on Sephadex LH-20 using 100% MeOH as eluent to obtain makaluvamines C [13] (88 mg, 0.51% dry wt), H [19] (81 mg, 0.47% dry wt), and I [20] (177 mg, 1.03% dry wt), and damirone C [25] (93 mg, 0.54% dry wt). An impure fraction containing makaluvamines G and I was further purified by hplc on a prep. Dynamax-C₁₈ column using 40% MeOH in 0.1% aqueous TFA solution as eluent to obtain makaluvamine G [17] (59 mg, 0.34% dry wt) and makaluvamine L [23] (26.4 mg, 0.15% dry wt). A second impure fraction was separated on hplc using 30% MeOH in 0.1% aqueous TFA solution as eluent to obtain makaluvamines D [14] (12 mg, 0.07% dry wt), J [21] (14 mg, 0.08% dry wt), and K [22] (3.4 mg, 0.02% dry wt). A third impure fraction was purified on hplc using 22% CH₃CN in 0.1% aqueous TFA solution as eluent to obtain makaluvamine M [24] (5 mg, 0.03% dry wt).

Makaluvamine H [19].—Red-brown solid; ir (film) ν max 3500–3000 (br), 2975, 1675, 1610, 1530, 1200 cm⁻¹; uv (MeOH) λ max (ϵ) 240 (19 400), 345 (13500), 522 (1100) nm, (MeOH+NaHCO₃) 209 (15 700), 241 (21 200), 334 (14 200) nm; ¹H-nmr data (DMSO-d₆), see Table 1; ¹³C-nmr data (DMSO-d₆), see Table 1; hrfabms m/z 216.1101 (C₁₂H₁₄N₃O [M+H]⁺ requires 216.1137).

Makaluvamine I [20].—Green solid; ir ν max (film) 1665, 1660 cm⁻¹; uv (MeOH) λ max (ϵ) 240 (23 900), 340 (13 500), 534 (1000) nm, (MeOH+NaHCO₃) 214 (16 000), 327 (15 500), 458 (1400) nm; ¹H-nmr data (DMSO-d₆), see Table 1; ¹³C-nmr data (DMSO-d₆), see Table 1; hrfabms m/z 187.0737 (C₁₀H₉N₃O [M]⁺ requires 187.0746).

Makaluvamine J [21].—Red-brown solid; ir (film) ν max 3250 (br), 1680, 1620, 1550, 1200 cm⁻¹; uv (MeOH) λ max (ϵ) 220 (11 600), 241 (18 600), 354 (13 900), 534 (1400) nm; (MeOH+NaHCO₃) 217 (9600), 257 (1200), 370 (5600) nm; ¹H-nmr data (DMSO-d₆), see Table 2; ¹³C-nmr data (DMSO-d₆), see Table 2; hrfabms m/z 322.1564 (C₁₉H₂₀N₃O [M+H]⁺ requires 322.1555).

Makaluvamine K [22].—Red-brown solid; ir (film) ν max 3250 (br), 1685, 1675, 1560 cm⁻¹; uv (MeOH) λ max (ϵ) 222 (18 000), 246 (29 800), 347 (19 200), 536 (2600) nm; (MeOH+NaHCO₃) 224 (10 900), 242 (10 200), 335 (8800) nm; ¹H-nmr data (DMSO-d₆), see Table 2; ¹³C-nmr data (DMSO-d₆), see Table 2; hrfabms m/z 322.1568 (C₁₉H₂₀N₃O [M+H]⁺ requires 322.1555).

Makaluvamine L [23].—Green solid; ir (film) ν max 3280, 2970, 1675, 1615, 1545, 1200 cm⁻¹; uv (MeOH) λ max (ϵ) 276 (1050), 344 (11 700), 451 (9200), 638 (10 400) nm, (MeOH+NaHCO₃) 271 (7800), 365 (2900), 608 (5200) nm; ¹H-nmr data (DMSO-d₆), see Table 2; ¹³C-nmr data (DMSO-d₆), see Table 2; hrfabms m/z 320.1387 (C₁₉H₁₈N₃O [M+H]⁺ requires 320.1399).

Makaluvamine M [24].—Green solid; ir (film) ν max 3290 (br), 1675, 1605, 1545, 1205 cm⁻¹; uv (MeOH) λ max (ϵ) 274 (14 900), 330 (11 000), 445 (8400), 623 (9200) nm, (MeOH+NaHCO₃) 217 (11 300), 302 (12 600), 596 (5800) nm; ¹H-nmr data (DMSO-d₆), see Table 2; ¹³C-nmr data (DMSO-d₆), see Table 2; lrms m/z 306 (C₁₈H₁₆N₃O [M+H]⁺ requires 306).

Damirone C [25].—Red-brown solid; ir (film) ν max 1725, 1670, 1650, 1535, 1280, 1120 cm⁻¹; uv (MeOH) λ max (ϵ) 240 (23 200), 330 (10 200), 528 (900) nm, (MeOH+NaHCO₃) 240 (23 900), 330 (10 800), 518 (1100) nm; ¹H nmr (DMSO-d₆) δ 2.72 (2H, t, J=7.5 Hz, H-3), 3.49 (2H, t, J=7.5 Hz, H-4), 5.02 (1H, s, H-6), 7.08 (1H, s, H-2), 8.24 (1H, s, NH-5), 12.39 (1H, s, NH-1); ¹³C nmr (DMSO-d₆) δ 19.3 (C-3), 41.5 (C-4), 92.7 (C-6), 117.0 (C-2a), 124.2 (C-2, C-8b), 125.3 (C-8a), 154.5 (C-5a), 171.3 (C-7), 178.4 (C-8); hreims m/z 188.0587 (C₁₀H₈N₂O₂ [M]⁺ requires 188.0586).

ACKNOWLEDGMENTS

LITERATURE CITED

1. S. Sakemi, H.H. Sun, C.W. Jefford, and G. Bernardinelli, *Tetrahedron Lett.*, **30**, 2517 (1989).
2. H.H. Sun, S. Sakemi, N. Burres, and P. McCarthy, *J. Org. Chem.*, **55**, 4964 (1990).
3. D.B. Stierle and D.J. Faulkner, *J. Nat. Prod.*, **54**, 1131 (1991).

4.   N.B. Perry, J.W. Blunt, J.D. McCombs, and M.H.G. Munro, *J. Org. Chem.*, **51**, 5476 (1986).
5.   N.B. Perry, J.W. Blunt, and M.H.G. Munro, *Tetrahedron*, **44**, 1727 (1988).
6.   N.B. Perry, J.W. Blunt, M.H.G. Munro, T. Higa, and R. Sakai, *J. Org. Chem.*, **53**, 4127 (1988).
7.   J. Kobayashi, J. Cheng, M. Ishibashi, H. Nakamura, Y. Ohizumi, Y. Hirata, T. Sasaki, H. Lu, and J. Clardy, *Tetrahedron Lett.*, **28**, 4939 (1987).
8.   J. Cheng, Y. Ohizumi, M.R. Walchli, H. Nakamura, Y. Hirata, T. Sasaki, and J. Kobayashi, *J. Org. Chem.*, **53**, 4621 (1988).
9.   D.C. Radisky, E.S. Radisky, L.R. Barrows, B.R. Copp, R.A. Kramer, and C.M. Ireland, *J. Am. Chem. Soc.*, **115**, 1632 (1993).
10.   J.R. Carney, P.J. Scheuer, and M. Kelly-Borges, *Tetrahedron*, **49**, 8483 (1993).
11.   B.R. Copp, C.M. Ireland, and L.R. Barrows, *J. Org. Chem.*, **56**, 4596 (1991).
12.   R.M.W. van Soest, S. Zea, and M. Keilman, *Bijdragen tot de Dierkunde*, **64**, 163 (1994).
13.   W.-K. Eng, L. Faucette, R.K. Johnson, and R. Sternglanz, *Mol. Pharmacol.*, **34**, 755 (1988).

Received 31 May 1995

This chapter, in full, is a reprint of the material as it appears in the Journal of Natural Products, 1995, 58, 1861-1867, Schmidt, Eric W., Harper, Mary Kay, Faulkner, D. John. The dissertation author was the primary investigator of this paper.

CHAPTER 3

Palauolol, a New Anti-inflammatory Sesterterpene from the Sponge *Fascaplysinopsis* sp. from Palau

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Abstract: A specimen of *Fascaplysinopsis* sp. from Palau contained palauolide (1), fascaplysin (2), and a new sesterterpene, palauolol (3). The structure and absolute stereochemistry of palauolol (3) were determined using spectroscopic and chemical methods and in doing so, the absolute stereochemistry of palauolide was also determined. Palauolide (1) and palauolol (3) both inactivate bee venom phospholipase A$_2$. Copyright © 1996 Elsevier Science Ltd

In 1982, we reported the isolation of palauolide (1) from a collection of three or more sponges from Palau that had been combined due to a freezer failure.[1] Since that time, we have examined hundreds of sponge specimens from Palau but have only recently located palauolide (1) in a sponge of the genus *Fascaplysinopsis*. Like other *Fascaplysinopsis* species that have been studied,[2-4] this sponge contained the alkaloid fascaplysin (2) in the aqueous extract and a mixture of sesterterpenes in the organic extracts. However, no sesterterpene-alkaloid salts were isolated or observed during fractionation. From the ethyl acetate extract, we have isolated a new sesterterpene, palauolol (3). We suspect that palauolol (3) is a biosynthetic precursor of palauolide (1), which is present in the first crude extract of the sponge and is not formed by dehydration during the separation and purification procedures.

A specimen of *Fascaplysinopsis* sp. was collected by hand using SCUBA (-25 m) at Ngemelis drop-off, Palau. The crude methanol extract of the sponge exhibited antimicrobial activity, which was followed during the purification procedure. The hexane-soluble material from the methanol extract was chromatographed on silica gel to obtain palauolide (1), which was identical in all respects, including optical rotation, to an authentic specimen. Purification of the water-soluble material by reversed-phase chromatography gave fascaplysin (2), which had identical spectral data to those reported earlier.[2] The ethyl acetate soluble fraction was purified using diol and reversed-phase chromatography to obtain palauolol (3).

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1 R = OH
7 R = H

2

3 R₁ = R₂ = OH
5 R₁ = H, R₂ = OH
6 R₁ = H, R₂ = OMTPA

4

9

8 R = CMe₂OH
10 R = COOH
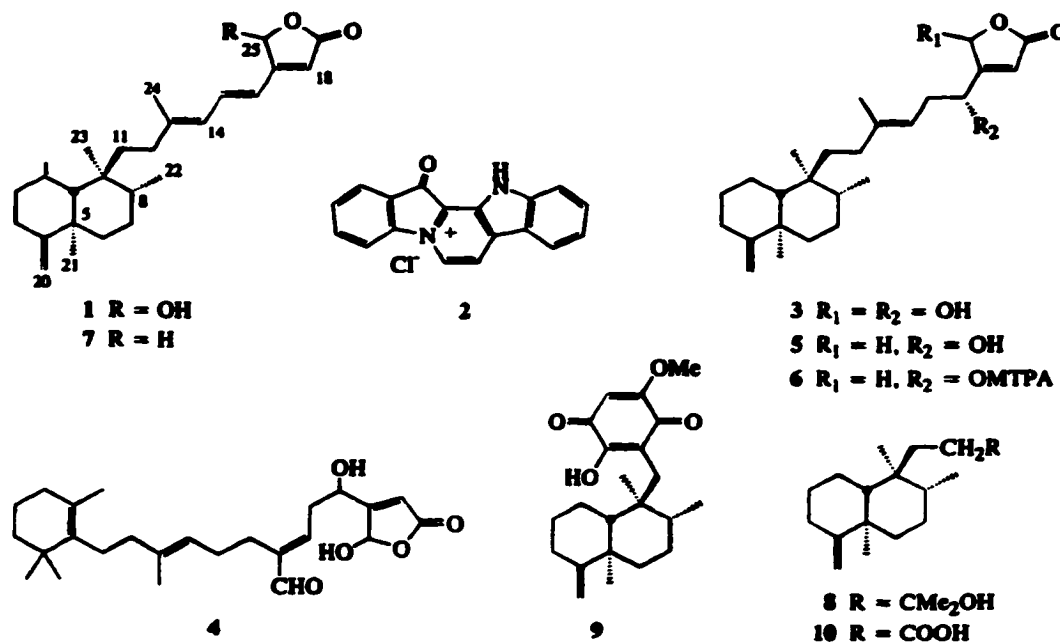
Palauolol (3) was obtained as a clear oil of molecular formula $C_{25}H_{36}O_4$. The molecular formula was determined by mass spectrometry and from the ^{13}C NMR data.[5] Inspection of the ^1H NMR spectrum (Table 1) revealed that palauolol (3) was closely related to palauolide (1) but lacked the conjugated olefinic proton signals and the strong UV absorption at 322 nm.[5] The ^{13}C NMR spectrum (Table 1) of 3 contained a signal at δ 69.1 (C-16), assigned to a carbon bearing oxygen, and one additional methylene carbon signal that replace two of the olefinic carbon signals in 1. In the ^1H NMR spectrum of 3, the signal at δ 4.54 (t, 1H, J = 5 Hz, H-16) is coupled to methylene proton signals at 2.35 (m, 1H, H-15) and 2.45 (m, 1H, H-15), that are in turn coupled to an olefinic proton signal at 5.21 (t, 1 H, J = 6.5 Hz, H-14). The location of the secondary alcohol at C-16 was readily assigned from HMBC correlations (see Table 1). Comparison of the NMR data for the C-15 to C-19 region of palauolol (3) with the corresponding data for secomanoalide (4)[6] showed the expected similarity. Palauolol (3) is therefore a secondary alcohol that on dehydration gives palauolide (1).

The absolute stereochemistry at C-16 of palauolol (3) was determined using the advanced Mosher's method[7] applied to lactone 5 that was prepared by reduction of palauolol (3) with sodium borohydride in methanol.[8] The lactone 5 was converted into both the R- and S-MTPA esters 6, each of which was a single diastereoisomer by ^1H NMR spectroscopy, showing the enantiomeric purity of 3; comparison of the ^1H NMR data for the MTPA esters (Table 2) indicated the 16R absolute stereochemistry. The absolute stereochemistry of the bicyclic ring system of palauolol (3) was

Table 1. ¹H (500 MHz, CD₃OD) and ¹³C NMR (50 MHz, CD₃OD) data for palauolol (3).

C#	¹³C	¹H NMR		HMBC	COSY
1	22.9	1.41	(m)		H-2, H-10
		1.58	(m)		H-2, H-10
2	29.9	1.2	(m)		H-1, H-3
		1.86	(m)		H-1, H-3
3	34.2	2.1	(m)	C-2, C-3, C-4, C-20	H-2
		2.3	(m)	C-2, C-3, C-4, C-20	H-2
4	160.0				
5	41.2				
6	38.7	1.6	(m, 2H)	C-5, C-7, C-8, C-21	H-7
7	28.6	1.43	(m, 2H)		H-6, H-8
8	38.0	1.42	(m)		H-7, H-22
9	40.4				
10	50.0	1.14	(dd, 12, 2.5)	C-1, C-5, C-9, C-23	H-1
11	38.2	1.24	(m)		H-12
		1.34	(m)		H-12
12	34.3	1.74	(m)	C-11, C-13, C-14, C-24	H-11
		1.86	(m)	C-11, C-13, C-14, C-24	H-11
13	140.6				
14	119.7	5.21	(t, 6.5)		H-15
15	36.0	2.35	(m)	C-13, C-14, C-16	H-14, H-16
		2.45	(m)	C-13, C-14, C-16	H-14, H-16
16	69.1	4.54	(t, 5)	C-14, C-15, C-17	H-15
17	173.0				
18	117.8	5.98	(br s)		
19	173.0				
20	103.2	4.49	(br s, 2H)	C-3, C-4, C-5	
21	21.5	1.05	(s, 3H)	C-4, C-5, C-6, C-10	
22	16.4	0.81	(d, 6.5, 3H)	C-7, C-8, C-9	H-8
23	18.7	0.74	(s, 3H)	C-8, C-9, C-10, C-11	
24	16.7	1.62	(s, 3H)	C-12, C-13, C-14	
25	99.7	6.01	(br s)		

Table 2. ¹H NMR data for the R- and S-MTPA esters of lactone 5.

H#	δ_R	δ_S	$\Delta\delta_{R-S}$(ppm)	$\Delta\delta_{R-S}$(Hz)
21	1.090	1.091	+0.001	+0.5
23	0.679	0.684	+0.005	+2.5
14	4.918	4.996	+0.078	+39
15	2.504	2.538	+0.034	+17
18	5.922	5.820	-0.102	-51
25	4.663	4.546	-0.117	-58.5
	4.767	4.696	-0.071	-35.5

established by converting palauolide (1) into the corresponding lactone 7 and comparing its CD spectrum with that of alcohol 8, prepared from ilimaquinone (9)[9] by oxidation of the quinone ring with basic hydrogen peroxide[1] followed by treatment of the resulting acid 10 with methyl lithium. Both 7 and 8 showed positive Cotton effects at λ_{max} 197 nm of almost equal magnitude ($\Delta\epsilon$ +3.5° ± 0.5°). Thus, the absolute stereochemistry of palauolide (1), $[\alpha]_D$ = +1.5 (c = 0.2, CHCl$_3$), is 5S,8S,9R,10S and that of palauolol (3), $[\alpha]_D$ = 0 ± 0.5 (c = 0.2, CHCl$_3$) is 5S,8S,9R,10S,16R.[10]

Both palauolide (1, 85% inhibition @ 0.8 μg/mL) and palauolol (3, 82% inhibition @ 0.8 μg/mL) inactivate bee venom PLA$_2$.[11] Palauolol (3) is mildly antimicrobial against S. aureus and B. subtilis but the antimicrobial activity of the crude extract is primarily due to fascaplysin (2).[12]

References and Notes

1.   Sullivan, B.; Faulkner D.J. Tetrahedron Lett. 1982, 23, 907.

2.   Roll, D.M.; Ireland C.M.; Lu, H.S.M.; Clardy, J. J. Org. Chem. 1988, 53, 3276.

3.   Jiménez, C.; Quiñoà, E.; Adamczeski, M.; Hunter, L.M.; Crews, P. J. Org. Chem. 1991, 56, 3403.

4.   Jiménez, C.; Quiñoà, E.; Crews, P. Tetrahedron Lett. 1991, 32, 1843.

5.   Palauolol (3): oil; 7.3 mg, 0.66% extract weight; $[\alpha]_D$ = 0 ± 0.5 (c = 0.2, CHCl$_3$); IR (film) 3370, 1745 cm^{-1}; UV (MeOH) 206 nm (ϵ 25000); ^{1}H NMR (methanol-d$_4$) see Table 1; ^{13}C NMR (methanol-d$_4$) see Table 1.; LREIMS m/z 402 (M$^+$, 1), 384 (5), 191 (56), 95 (100); HRCIMS, Obsd. m/z = 420.2873, C$_{25}$H$_{40}$O$_3$ [M+H$_2$O]$^+$ requires 420.2875.

6.   de Silva, E.D.; Scheuer, P.J. Tetrahedron Lett. 1981, 22, 3147.

7.   Dale, J.A.; Mosher H.S. J. Am. Chem. Soc. 1973, 95, 512. Ohtani, I.; Kusumi, T.; Kashman, Y., Kakisawa, H. J. Am. Chem. Soc. 1991, 113, 4092.

8.   All new compounds gave satisfactory analytical and spectral data.

9.   Luibrand, R.T.; Erdman, T.R.; Vollmer, J.J.; Scheuer, P.J.; Finer, J.; Clardy, J. Tetrahedron 1979, 35, 609.

10.   Although Capon and MacLeod (J. Org. Chem. 1987, 52, 5060) caution against using weak Cotton effects associated with 4-keto derivatives derived from ilimaquinone, the stronger π-π* Cotton effect used to determine the absolute configuration of avarol (de Rosa, S.; Minale, L.; Riccio, R.; Sodano, G. J. Chem. Soc., Perkin Trans I 1976, 1408) appears reliable.

11.   Potts, B.C.M.; Faulkner, D.J.; Jacobs, R.S. J. Nat. Prod. 1992, 55, 1701.

12.   We thank Mary Kay Harper for identifying the sponge and for performing the antimicrobial assays, Bryan Wylie and Dr. Robert S. Jacobs (UC Santa Barbara) for performing the anti-inflammatory assays, and Barbara Doerner and Behrouz Forood (Torrey Pines Institute for Molecular Studies) for assistance with CD measurements. We thank the Republic of Palau and the State of Koror for collecting permits and Larry Sharron of the Coral Reef Research Foundation, Palau for invaluable assistance with collections. This research was supported by the California Sea Grant College Program (NOAA grant NA36RG0537, project R/MP-60).

This chapter, in full, is a reprint of the material as it appears Tetrahedron Letters, 1996, 37, 3951-3954, Schmidt, Eric W., Faulkner, D. John. The dissertation author was the primary investigator of this paper.

CHAPTER 4

Absolute Configuration of Methyl (2Z,6R,8R,9E)-3,6-epoxy-4,6,8-triethyl-2,4,9-dodecatrienoate from the Sponge *Plakortis halichondrioides*

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Abstract: The absolute stereochemistry of methyl ester 2, which is a major metabolite of the Caribbean sponge *Plakortis halichondrioides*, was determined to be 6R,8R by means of chemical degradation and application of a modification of Mosher's method. The absolute configuration at C-8 was determined by analysis of the ^{1}H NMR spectra of the amides formed from the acid 4 and (R)- and (S)-phenylglycine methyl ester.
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Although advances in NMR technology have greatly simplified the structural elucidation of natural products, it is still difficult to determine the stereochemistry of certain classes of compounds. Foremost among these problems is the determination of the absolute configuration at centers where an alkyl group is attached to a longer alkyl chain. In this paper we present a solution to this problem which can be applied in situations where the alkyl group is allylic.

The earliest reports of cyclic peroxides from sponges, exemplified by the isolation of plakortin (1) from the Caribbean sponge *Plakortis halichondrioides*,[1] contained limited stereochemical assignments.[2] In recent years, some progress has been made in reliably deducing the relative[3] and absolute[4] stereochemistry of stereocenters around the peroxide ring, but the problem of assigning the stereochemistry of the side-chain remained. Our interest in this problem arose from an inquiry[5] about the enantiomeric integrity of the methyl ester 2 that we had earlier isolated from a specimen of *P. halichondrioides*.[6]

Our strategy for assigning the absolute stereochemistry of methyl ester 2 was to first determine the absolute configuration at C-8 and then elucidate the relative stereochemistry at C-6. The latter process is particularly difficult because there are no other chiral centers on the dihydrofuran ring, but the problem can be attacked by hydrogenation of the 4,5-double bond to create an asymmetric center at C-4, which then allows the stereochemistry at C-4 and C-6 to be related. In cyclic peroxides such as plakortin (1), the absolute and relative stereochemistry about the peroxide ring can readily be determined, thus making the procedure described below of general application to the structural determination of cyclic peroxides in this series.

A sample of the methyl ester 2, $[\alpha]_D = -175°$ (c 1.4, CCl$_4$), that had been stored at 4°C for 16 years was shown to be essentially pure by HPLC.[7] Analysis of the ^{1}H NMR data, particularly the NOEDS data, revealed that the compound was unchanged but that the stereochemistry of the 2,3-double bond had been incorrectly

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assigned in 1980 on the basis of the chemical shift of H-2. Careful ozonolysis of the methyl ester 2, followed by oxidation of the resulting aldehyde 3 with sodium chlorate, gave the corresponding acid in 66% overall yield (Scheme 1).[8] The absolute stereochemistry at C-8 was determined by application of the chiral amide method developed by Nagai and Kusumi,[9] in which the acid 4 was reacted with (R)- and (S)-phenylglycine methyl ester (PGME) and the ^{1}H NMR spectra of the resulting amides 5 and 6 were compared (Table 1). The NMR analysis indicated that no racemization had occurred during the oxidation procedure and that the absolute configuration at C-8 was R.
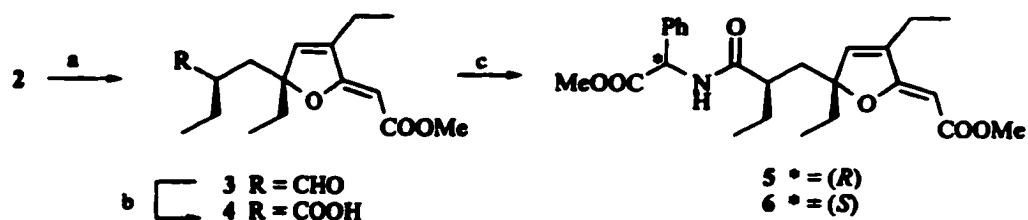
Scheme 1. a. O_3 (5% in Ar), EtOAc, 1 hr., -78°; then DMS (excess) (95%). b. $NaClO_2$, PO_4^{2-} buffer (pH 7), 2-methyl-2-butene, 24 hrs, 4°C (70%). c. (R)- or (S)-PGME, benzotriazolyloxytri(pyrrolidinyl)-phosphonium hexafluorophosphate (PyBoP) (1.5 eq.), 1-hydroxybenzotriazole (HOBT) (1.5 eq), N-methylmorpholine (cat.), 4 hr. (90%).
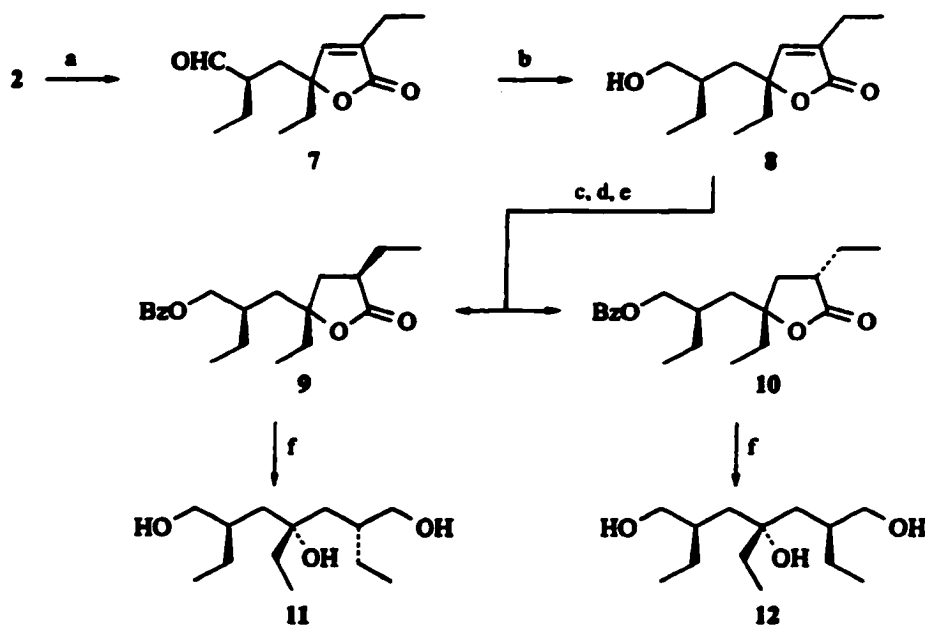
Scheme 2. a.OsO_4 (cat.), $NaIO_4$, 24 hr. (90%). b. $NaBH_4$ (excess), MeOH, 15 min.. c. H_2, Pd-C (4 mol%), MeOH, 3 hrs. d. BzCl (1.5 eq.), TEA (1.5 eq.), DMAP (cat.), DCM, 15 min. e. Silica gel HPLC (25% for b-e). f. $LiBH_4$ (excess), MeOH (1.5 eq.), Et_2O, 1 hr., reflux (95%).

Oxidation of methyl ester 2 with osmium tetroxide/sodium periodate produced the lactone 7 containing an aldehyde at C-9 that was reduced with sodium borohydride to the alcohol 8 (Scheme 2). Hydrogenation of the olefin gave a 55:45 mixture of diastereoisomers at C-2 that could be readily separated by HPLC on silica as the corresponding benzoyl esters 9 and 10. The relative stereochemistry about the five-membered ring was determined using NOEDS experiments to be cis-diethyl for 9 and trans-diethyl for 10. Reduction of the HPLC-pure lactones 9 and 10 with lithium borohydride[10] resulted in the formation of triols 11 and 12, respectively. The relative and hence absolute stereochemistries of the triols 11 and 12 were immediately apparent from examination of the ^{1}H NMR spectra (Figure 1): the C_s symmetry of triol 12 resulted in a relatively simple spectrum compared with the more complex spectrum of the unsymmetrical triol 11.[11] Thus, the methyl ester 2 is defined as methyl (2Z,6R,8R,9E)-3,6-epoxy-4,6,8-triethyl-2,4,9-dodecatrienoate.

Table 1. 500 MHz ^{1}H NMR data for the (R)- and (S)-PGME amides of 7 in CDCl$_3$.

H#	δ_S	δ_R	$\Delta\delta_{(S-R)}$
2	4.63 (s)	4.82 (s)	-0.19
5	6.02 (s)	6.29 (s)	-0.27
7	1.86 (m), 2.33 (m)	1.86 (m), 2.30 (m)	0, +0.03
8	1.99 (br m)	1.99 (br m)	0
9	1.58 (m), 1.82 (m)	1.38 (m), 1.47 (m)	+0.20, +0.35
10	0.85 (t, J = 7)	0.69 (t, J = 7)	+0.16
12	1.55 (m)	2.10 (m)	-0.55
13	0.86 (t, J = 7)	1.08 (t, J = 7)	-0.22
14	1.68 (m), 1.78 (m)	1.72 (m), 1.82 (m)	-0.04, -0.04
15	0.71 (t, J = 7)	0.76 (t, J = 7)	-0.05
16	3.66 (s)	3.69 (s)	-0.03
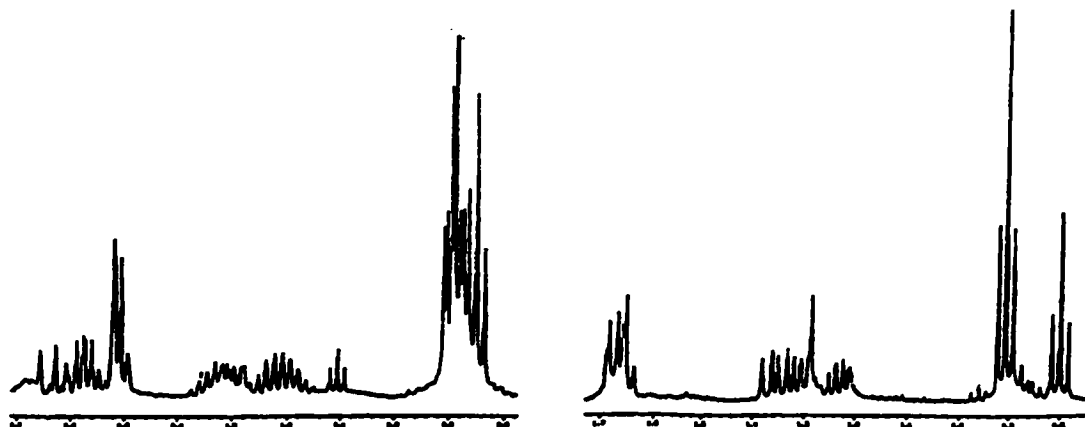
Figure 1. Comparative upfield regions of ^{1}H NMR spectra of 11 (left) and 12 in acetone-d_6.

References and notes

1. Higgs, M.D.; Faulkner, D.J. *J. Org. Chem.* **1978**, *43*, 3454.

2. Faulkner, D.J. *Nat. Prod. Rep.* **1984**, *1*, 513 and subsequent reports in this series.

3. a) De Guzman, F.S.; Schmitz, F.J. *J. Nat. Prod.* **1990**, *53*, 926. b) Patil, A.D.; Freyer, A.J.; Carte, B.; Johnson, R.K.; Lahouratate, P. *J. Nat. Prod.* **1996**, *59*, 219. Relative stereochemistry was determined by NOE experiments.

4. a) Kobayashi, M.; Kondo, K.; Kitagawa, I. *Chem. Pharm. Bull.* **1993**, *41*, 1324. This work, using Mosher's method, represents the first assignment of absolute configuration in this class of compounds. b) Varoglu, M.; Peters, B.M.; Crews, P. *J. Nat. Prod.* **1995**, *58*, 27.

5. We thank Dr. Ying-cai Peng of Zhongshan University, People's Republic of China, for bringing this problem to our attention and for proposing the revision of the double bond geometry at C-2.

6. Stierle, D.B.; Faulkner, D.J. *J. Org. Chem.* **1980**, *45*, 3396. The sign of the optical rotation of methyl ester 2 ($[\alpha]_D = -175°$) was incorrectly reported.

7. The sample had become a very pale yellow during storage.

8. All key intermediates were purified by HPLC and were fully characterized by ^{1}H NMR, HRMS, IR, and UV. NOESY, COSY, and ^{13}C data were used to further substantiate several structures.

9. Nagai, Y.; Kusumi, T. *Tetrahedron Lett.* **1995**, *36*, 1853.

10. Soai, K.; Okawa, A.J. *J. Org. Chem.* **1986**, *51*, 4000.

11. [13]C spectral data also confirmed the stereochemical assignments.

12. We thank Dr. Jay S. Siegel (University of California, San Diego) for helpful comments on this manuscript. The ester 2 was originally isolated in our laboratory by Donald B. Stierle. This work was supported by the California Sea Grant College Program (NOAA grant NA36RG0537, project R/MP-60).

This chapter, in full, is a reprint of the material as it appears in Tetrahedron Letters, 1996, 37, 6681-6684, Schmidt, Eric W., Faulkner, D. John. The dissertation author was the primary investigator of this paper.

CHAPTER 5

Mozamides A and B, Cyclic Peptides from a Theonellid Sponge from Mozambique

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Received April 8, 1997

A sponge of the lithistid family Theonellidae from southern Mozambique contained the known compound aurantoside A (1) together with two new cyclic peptides, mozamides A (2) and B (3). The structures and absolute configurations of mozamides A and B, which contain a rare ureido group, were determined by interpretation of spectral data and chiral GC−MS analysis of the amino acids resulting from acid-catalyzed hydrolysis.

Lithistid sponges have yielded a wide variety of bioactive marine natural products that include a diverse array of cyclic peptides, which are notable for their incorporation of structurally unusual amino acids and nonproteinogenic D-amino acids.[1-3] Structural similarities between lithistid sponge metabolites and those of microorganisms led to the suggestion that the metabolites might be produced by symbiotic bacteria, or more specifically, cyanobacteria.[1,4] We have recently demonstrated that a bicyclic peptide[5] from *Theonella swinhoei* was located in and presumably produced by symbiotic filamentous bacteria, while a macrolide, swinholide A, was isolated from a mixed population of unicellular bacteria.[6] The hypothesis that cyclic peptides from lithistid sponges are produced by filamentous bacteria is of considerable interest because of the biotechnological implications. It has been demonstrated that there is a correlation between the presence of filamentous bacteria and the isolation of cyclic peptides,[7] but more research is required to determine the validity of the hypothesis.

Lithistid sponges identified as *Theonella* sp. are often superficially similar to *T. swinhoei* except that the interior tissue is colored orange or yellow, due to the presence of aurantoside A (1) and/or related polyenes. From one such *Theonella* sp., Fusetani and co-workers isolated both a mixture of aurantosides A and B[8] and the cyclic peptides, cyclotheonamides A and B[9] and orbiculamide.[10] Other cyclic peptides from *Theonella* spp. include keramamide A[11] and konbamide,[12] both of which incorporate a 5′-hydroxytryptophan residue and a ureido linkage joining two amino acid residues. Similar peptides have also been described from freshwater cyanobacteria, as illustrated by the ferintoic acids from *Microcystis aeruginosa*[13] and the anabaenopeptins from *Anabaena flos-aquae* and *Oscillatoria agardhii*.[14,15] We now report the isolation and identification of two new cyclic peptides, mozamides A (2) and B (3), from a theonellid sponge that also contained aurantoside A (1).

Results and Discussion

The theonellid sponge was collected in southern Mozambique. The lyophilized sponge was sequentially extracted with EtOAc, 1:1 EtOAc−Me₂CO, and 4:1 MeCN−H₂O. Reversed-phase chromatography of the

2 R = H
3 R = Me
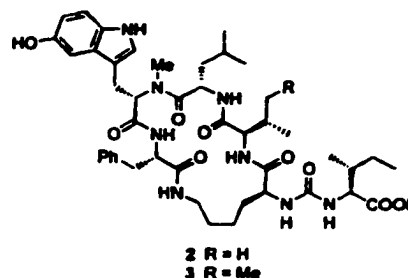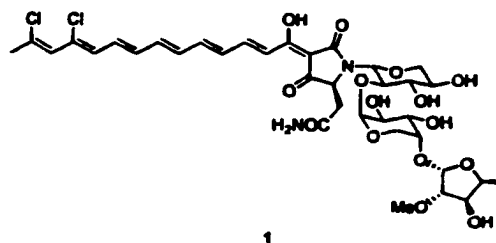
aqueous extract led directly to the isolation of aurantoside A (1), which was identified by comparison of its spectral data with literature values.[8] At first, aurantoside A masked the presence of the cyclic peptides, but after a multistep purification procedure, reversed-phase HPLC was successfully employed to separate mozamides A (2, 2.6 mg, 7 × 10⁻⁴% yield) and B (3, 0.7 mg, 2 × 10⁻⁴% yield).

Mozamide A (2) was isolated as a white powder. The HRFABMS spectrum indicated a molecular formula of C₄₅H₆₄N₈O₉. The IR spectrum appeared typical of a peptide with a broad band between 1640 and 1740 cm⁻¹. The presence in the ¹H-NMR spectrum of several exchangeable protons between δ 6.3 and 8.8 and six signals between 3.8 and 4.65, which may be assigned to the α-protons of amino acids, suggested that mozamide A might be a hexapeptide. A methyl signal at δ 1.85, which is unusually upfield, showed an HMQC correlation to a carbon signal at δ 27.6 and was assigned as an *N*-methyl signal. The ¹H-NMR spectrum also contained several other highfield signals at δ −0.46 (t, 1 H, *J* = 11.5 Hz), 0.22 (d, 3 H, *J* = 6 Hz), 0.36 (d, 3 H, *J* = 6.5 Hz), and 0.90 (m, 1 H) that were assigned to a leucine residue. A TOCSY experiment led to the identification of lysine, valine, leucine, and isoleucine
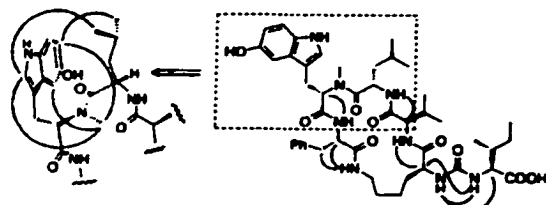
Figure 1. ¹H−¹H ROESY (300 MHz) correlations used to establish the structure of 1.
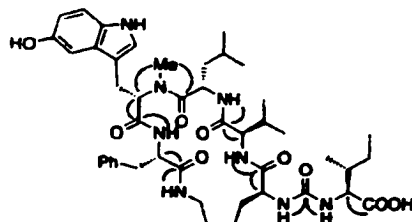
Figure 2. GHMBC (600 MHz) correlations used to establish the structure of 1.

residues, a GCOSY experiment defined the phenylalanine residue, and the 5′-hydroxytryptophan unit was identified by analysis of the GHMQC and GHMBC experiments and by comparison of the ¹H- and ¹³C-NMR data with literature values.[16] A ROESY experiment allowed the determination of the amino acid sequence and discrimination between the two aromatic amino acids (Figure 1). However, the ROESY experiment showed some unusual correlations for the isoleucine residue: the NH signal of isoleucine showed a strong correlation with the α-NH signal of lysine in addition to weak correlations between the α-protons of isoleucine and lysine and the NH protons on the neighboring residues. These data, together with the presence of an unassigned signal at δ 157.3 in the ¹³C-NMR spectrum, suggested that the isoleucine residue was joined to the α-amino group of lysine through a ureido moiety. This assignment was confirmed by long-range carbon−hydrogen NMR correlations and by comparison of the NMR data with the literature values for keramamide A and konbamide.[11,12] All other spectral data, particularly those of the GHMBC experiment (Figure 2), were compatible with the structure proposed for mozamide A (2).

The stereochemistry of mozamide A (2) was determined by chiral GC−MS experiments. Acid hydrolysis of mozamide A, followed by derivatization of the resulting amino acids, gave the isopropyl ester pentafluoropropionamide derivatives that were analyzed by GC−MS using a Chirasil-Val capillary column, leading to the identification of L-leucine, L-lysine, L-phenylalanine, and D-valine. Ozonolysis of mozamide A using an oxidative workup, followed by hydrolysis and derivatization, gave N-methyl-L-aspartic acid,[17] indicating the presence of N-methyl-L-5′-hydroxytryptophan in mozamide A (2). The final isoleucine residue, which was attached to the urea group, was not observed under acid hydrolysis conditions, but treatment of mozamide A (2) with hydrazine[18] produced only one amino acid, which was derivatized and identified as L-allo-isoleucine.

Mozamide B (3) was isolated as a white powder of molecular formula $C_{46}H_{66}N_8O_9$, which differs from that

of mozamide A by the addition of a methylene group. Although insufficient material was available to record a ¹³C-NMR spectrum, analysis of the ¹H-NMR data, including TOCSY, DQCOSY, and GHMQC experiments, indicated that mozamide B contained an isoleucine residue in place of the valine residue in mozamide A. Chiral GC−MS confirmed this assignment and indicated that D-valine had been replaced by D-isoleucine. Comparison of the spectral data of mozamide B (3) with those of mozamide A (2) strongly suggested that the only difference between the compounds was the replacement of D-valine by D-isoleucine but the lack of HMBC and ROESY data prevented complete confirmation.

Although mozamides A (2) and B (3) are similar in structure to keramamide A[11] and konbamide,[12] the latter compounds do not contain any D-amino acid residues. We have not been able to study the cellular localization of metabolites in this sponge but can present two relevant observations at this time. Filamentous cells are evident in the endosome (interior) of this theonellid sponge, while cyanobacteria are present only in the ectosomal layer. The orange color of the endosome appears to be due to the presence of aurantoside A (1). We had insufficient material to allow broad screening of mozamides A (2) and B (3), which at low concentrations showed no antimicrobial activity against Gram-positive and Gram-negative bacteria and a yeast.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Rudolph Research Autopol III polarimeter. IR and UV spectra were recorded on Perkin-Elmer 1600 FT-IR and Lambda 3B instruments, respectively. The ¹H, G(radient)COSY, DQCOSY, TOCSY, and ROESY spectra were recorded on a Varian Inova 300 MHz spectrometer, the ¹³C and DEPT spectra on a Varian Gemini 400 MHz spectrometer, and the GHMQC and GHMBC spectra on a Bruker AMX-600 spectrometer. All NMR data were recorded in DMSO-d_6 solution, and those for mozamide B were recorded using H₂O-suppression sequences. TOCSY spectra were obtained using 80 msec mixing times, and ROESY spin locks were established by continuous pulsing for 300 msec. Absolute configurations were determined using a Hewlett-Packard 5890 GC−MS fitted with an Alltech Chirasil-Val capillary column. HRMS data were obtained from the UC Riverside Regional Mass Spectrometry Facility. All solvents were distilled prior to use.

Animal Material. The lithistid sponge specimen (MOZ95-032) is massive with a reddish brown exterior and a bright yellow-orange interior. The skeleton is composed of nonarticulating tetracrepid-like desmas with strongyles and acanthomicrorhabds. This sponge is comparable to *Placinolopha mirabilis*, described by de Laubenfels in 1954,[19] which is now considered to be a representative of an undescribed genus in the lithistid family Theonellidae.[20] Superficially, this sponge is remarkably similar to a co-occurring species of *Theonella*, but is easily distinguished by its unique desmas and color. A voucher specimen has been deposited in the SIO Benthic Invertebrate Collection, catalog no. P1167.

Collection, Extraction, and Purification. The sponge was collected by hand using scuba at Malangaan reef (26° 46′ S, 32° 54′ E) off southern Mozambique and was maintained frozen until extraction. The sponge

Table 1. ^{1}H-(400 MHz, DMSO-d_6) and ^{13}C-(100 MHz, DMSO-d_6) NMR Data for Moxamides A (2) and B (3)

	moxamide A (2)			moxamide B (3)[a]		
N-Methyl-L-5′-hydroxytryptophan						
1	169.9					
2	60.7	4.64	dd, J = 9, 2.5 Hz	61.6	4.64	dd, J = 8, 2.5 Hz
3	22.3	2.72	dd, J = 15, 9 Hz	23.0	2.78	dd, J = 15, 8 Hz
		3.04	dd, J = 15, 2.5 Hz		3.11	dd, J = 15, 2.5 Hz
NH		10.80			10.56	
2′	124.2	6.81		125.1	6.84	
3′	108.6					
4′	103.2	6.84	d, J = 2 Hz	103.0	6.82	d, J = 2 Hz
5′	150.3					
6′	111.3	6.58	dd, J = 8, 2 Hz	112.1	6.58	dd, J = 8, 2 Hz
7′	111.6	7.10	d, J = 8 Hz	112.5	7.10	d, J = 8 Hz
8′	130.4					
9′	127.9					
OH		8.50			8.50	
NMe	27.6	1.85	s, 3 H	28.2	1.96	s, 3 H
L-Leucine						
1	172.2					
2	47.3	4.24		48.0	4.24	
3	37.0	−0.46	t, J = 11.5 Hz	37.9	−0.42	t, J = 11.5 Hz
		0.90			0.90	
4	23.1	1.39		24.0	1.30	
5	19.5	0.22	d, J = 6 Hz	20.1	0.25	d, J = 6 Hz
6	22.6	0.36	d, J = 6.5 Hz	23.4	0.38	d, J = 6.5 Hz
NH		8.42	d, J = 4 Hz		8.43	d, J = 4 Hz
D-Valine				D-isoleucine		
1	172.3					
2	56.7	3.80	t, J = 7.5 Hz	56.8	3.98	t, J = 7.5 Hz
3	29.9	1.86		38.0	1.72	
4	18.8	0.85	d, J = 6.5 Hz	25.9	1.50	
5	18.9	0.94	d, J = 6.5 Hz	12.3	0.88	
6				16.5	0.85	
NH		6.73	d, J = 7 Hz		6.70	d, J = 7.5 Hz
L-Lysine						
1	172.0					
2	56.7	3.84		55.2	3.86	
3	31.5	1.58		32.2	1.60	
4	20.2	1.10		21.0	1.10	
5	28.3	1.40		29.0	1.42	
6	39.0	2.85		39.1	2.88	
		3.59			3.61	
2-NH		6.44	d, J = 7 Hz		6.48	d, J = 7 Hz
6-NH		7.44	d, J = 7.5 Hz		7.48	d, J = 7.5 Hz
L-Phenylalanine						
1	170.1					
2	54.4	4.58	ddd, J = 9, 8, 2.5 Hz	55.2	4.60	ddd, J = 9, 8, 2.5 Hz
3	39.0	2.71	dd, J = 15, 8 Hz	40.8	2.84	dd, J = 15, 8 Hz
		3.37	dd, J = 15, 2.5 Hz		3.46	dd, J = 15, 2.5 Hz
4	138.4					
5,9	128.8	7.08		129.7	7.10	
6,8	128.3	7.21		129.0	7.25	
7	126.1	7.17		129.5	7.20	
NH		8.79	d, J = 9 Hz		8.75	d, J = 9 Hz
Urea						
1	157.3					
L-allo-isoleucine						
1	173.8					
2	57.7	4.02	dd, J = 9, 5 Hz	57.5	4.06	dd, J = 9, 5 Hz
3	37.7	1.72		37.4	1.75	
4	24.6	1.12		25.8	1.15	
		1.34			1.30	
5	11.5	0.83		12.3	0.84	
6	15.7	0.82		15.5	0.82	
NH		6.30	d, J = 9 Hz		6.34	d, J = 9 Hz

[a] ^{13}C-NMR chemical shifts were derived from the GHMQC experiment (error ± 0.3 ppm).

(364 g) was lyophilized and sequentially extracted with EtOAc (2 × 1 L), 1:1 EtOAc–Me$_2$CO (2 × 1 L), and 4:1 CH$_3$CN–H$_2$O (6 × 1 L). The aqueous MeCN extracts were concentrated under vacuum and chromatographed on Sephadex LH-20 using MeOH as eluent. A small portion of the aqueous MeCN extract was chromatographed on a C$_{18}$ reversed-phase column using a CH$_3$-

CN–H$_2$O gradient as eluent to obtain a sample of aurantoside A (1) that had identical spectral data to those reported in the literature.[6] Peptide-containing fractions, which were still heavily contaminated with aurantoside A (1), were recombined and chromatographed on a CN-SepPak using a CH$_3$CN–H$_2$O gradient as eluent. The peptides were followed first by TLC

using cyano plates and later by ¹H-NMR spectroscopy. Material eluted with 30–40% CH₃CN in H₂O was purified by HPLC on a cyano column using 25% CH₃CN–H₂O as eluent followed by HPLC on a C₁₈ reversed-phase column using 40% CH₃CN–0.01% aqueous TFA to obtain a mixture of mozamides A and B. Final separation was accomplished using C₁₈ reversed-phase HPLC using 35% CH₃CN–0.01% aqueous TFA as eluent to obtain, after a 90-min elution time, mozamide A (2, 2.5 mg, 7 × 10⁻⁴% dry wt) and mozamide B (3, 0.7 mg, 2 × 10⁻⁴% dry wt).

Mozamide A (2): white powder; [α]_D −66° (c 0.024, MeOH); IR (film) 2940, 2920, 1740–1640 (br), 1550, 1260, 1205, 1120, 1080, 1030 cm⁻¹; UV (MeOH) λ_max 208 (ε 19 600), 268 (ε 2600) nm; ¹H NMR, see Table 1; ¹³C NMR, see Table 1; HRFABMS (NBA matrix) m/z 861.4729 [M + H]⁺, calcd for C₄₅H₆₅N₆O₉, 861.4875.

Mozamide B (3): white powder; [α]_D −33° (c 0.018, MeOH); IR (film) 2955, 2925, 1740–1640 (br), 1555, 1260, 1200, 1125, 1075, 1035 cm⁻¹; UV (MeOH) λ_max 208 (ε 19 600), 268 (ε 2600) nm; ¹H NMR, see Table 1; ¹³C NMR, see Table 1; HRFABMS (NBA matrix) m/z 875.4854 [M + H]⁺, calcd for C₄₆H₆₇N₆O₉, 875.5031.

Hydrolysis and Derivatization of Mozamides A (2) and B (3) for Chiral GC–MS Analysis. Mozamides A and B (150 μg of each) were placed in 1-mL conical vials containing 6 N HCl (0.5 mL), and the sealed vials were heated at 110 °C for 15 h. After evaporation of the solvent under nitrogen, isopropyl alcohol (0.4 mL) and acetyl chloride (0.1 mL) were added, and the sealed vials were again heated at 110 °C for 1 h. After evaporation of the reagents under nitrogen, pentafluoropropionic anhydride (400 μL) in CH₂Cl₂ (0.4 mL) was added, and the sealed vials were again heated at 110 °C for 15 min. The derivatives were again dried under nitrogen and redissolved in EtOAc (100 μL). Aliquots (3 μL) were injected onto a Chirasil-Val capillary column, and the temperature was ramped from 50 to 210 °C over a period of 45 min. Elution times were measured by GC–MS and compared with those of standards that had been derivatized in the same manner. In this manner, L-leucine, L-lysine, L-phenylalanine, and D-valine were identified.

Ozonolysis of Mozamide A (2) and Chiral GC–MS Analysis of Hydrolysis Products. Ozone in oxygen was bubbled through a cooled solution of mozamide A (200 μg) in MeOH (4 mL) at −78 °C for 30 min. The reaction was quenched with 50% H₂O₂ (10 drops) and allowed to warm to room temperature. After 1 h, the solvent was removed under nitrogen, and the ozonolysis product was transferred to a 1-mL conical vial and treated exactly as in the hydrolysis experiment above. Chiral GC–MS analysis revealed the presence of an additional peak that was identified as N-methyl-L-aspartic acid. The isopropyl ester of racemic N-methylaspartic acid was synthesized as follows. A solution of aspartic acid (10 mg) in isopropyl alcohol (1

mL) containing acetyl chloride (300 μL) was heated to 110 °C for 1 h. The product was dried under nitrogen then redissolved in MeOH (2 mL), to which was added iodomethane (12 mg) and K₂CO₃ (12 mg), and the solution was stirred overnight at 25 °C.

Hydrazinolysis of Mozamide A (2). A solution of mozamide A (100 μg) in hydrazine (0.5 mL) was heated in a sealed conical vial at 110 °C for 18 h. The hydrazine was removed under high vacuum, and the sample was relyophilized from H₂O to remove the last traces of hydrazine. After the resulting mixture had been derivatized as described above, a single peak was identified by chiral GC–MS as L-allo-isoleucine.

References and Notes

(1) Pusetani, N.; Matsunaga, S. *Chem. Rev.* **1993**, *93*, 1793–1806.
(2) Faulkner, D. J. *Nat. Prod. Rep.* **1997**, *14*, 259–302, and previous reviews in this series.
(3) Bewley, C. A.; Faulkner, D. J. *Angew. Chem. Int. Ed. Engl.* Manuscript submitted.
(4) Kobayashi, J.; Ishibashi, M. *Chem. Rev.* **1993**, *93*, 1753–1770.
(5) The structure of the bicyclic peptide will be reported in the near future (Schmidt, E. W.; Bewley, C. A.; Faulkner, D. J. Manuscript in preparation).
(6) Bewley, C. A.; Holland, N. D.; Faulkner, D. J. *Experientia* **1996**, *52*, 716–722.
(7) Bewley, C. A. Ph.D. dissertation, University of California at San Diego, **1996**; pp 108–113.
(8) Matsunaga, S.; Fusetani, N.; Kato, Y.; Hirota, H. *J. Am. Chem. Soc.* **1991**, *113*, 9690–9692.
(9) Fusetani, N.; Matsunaga, S.; Matsumoto, H.; Takebayashi, Y. *J. Am. Chem. Soc.* **1990**, *112*, 7053–7054.
(10) Fusetani, N.; Sugawara, T.; Matsunaga, S.; Hirota, H. *J. Am. Chem. Soc.* **1991**, *113*, 7811–7812.
(11) Kobayashi, J.; Sato, M.; Ishibashi, M.; Shigemori, H.; Nakamura, T. Ohizumi, Y. *J. Chem. Soc., Perkin Trans. 1* **1991**, 2609–2611.
(12) Kobayashi, J.; Sato, M.; Murayama, T. Ishibashi, M.; Walchi, M. R.; Kanai, M.; Shoji, J.; Ohizumi, Y. *J. Chem. Soc., Chem. Commun.* **1991**, 1050–1052.
(13) Williams, D. E.; Craig, M.; Holmes, C. F. B.; Andersen, R. J. *J. Nat. Prod.* **1996**, *59*, 570–575.
(14) Harada, K.; Fujii, K.; Shimada, T.; Suzuki, M.; Sano, H.; Adachi, K.; Carmichael, W. W. *Tetrahedron Lett.* **1995**, *36*, 1511–1514.
(15) Murakami, M.; Shin, H. J.; Matsuda, H.; Ishida, K.; Yamaguchi, K. *Phytochemistry* **1997**, *44*, 449–452.
(16) Gunasekera, S. P.; Pomponi, S. A.; McCarthy, P. J. *J. Nat. Prod.* **1994**, *57*, 79–83.
(17) Ishibashi, M.; Li, Y.; Sato, M.; Kobayashi, J. *Nat. Prod. Lett.* **1994**, *4*, 293–296.
(18) Akabori, S.; Ohno, K.; Narita, K. *Bull. Chem. Soc. Jpn.* **1952**, *25*, 214–218.
(19) de Laubenfels, M. W. *Oregon State Monographs Zool.* **1954**, *7*, 1–306.
(20) Kelly-Borges, M. Personal communication.

NP970196X

This chapter, in full, is a reprint of the material as it appears in the Journal of Natural Products, 1997, 60, 779–782, Schmidt, Eric W., Harper, Mary Kay, Faulkner, D. John. The dissertation author was the primary investigator of this paper.

CHAPTER 6

Microsclerodermins C - E, Antifungal Cyclic Peptides from the Lithistid Marine Sponges *Theonella* sp. and *Microscleroderma* sp.

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Abstract: Three new cyclic peptides, microsclerodermins C - E (3-5), were isolated from two species of lithistid sponges from the Visayan Islands, Philippines. The sponge *Theonella* sp. contained peptides 3 and 4, while *Microscleroderma* sp. contained peptides 4 and 5. Their structures, which feature three unprecedented amino acids, were elucidated using spectroscopic methods and chemical degradation. © 1998 Elsevier Science Ltd. All rights reserved.

Sponges of the order Lithistida are unparalleled as sources of unusual natural products in the marine environment.[1] Bioactive compounds isolated from these sponges include discodermolide,[2] swinholides,[3] and calyculins;[4] the sponges also contain an arsenal of modified peptides with close structural affinities to cyanobacterial natural products.[1,5] In addition to chemical riches, lithistid sponges often harbor a large number of symbionts, that in *Theonella swinhoei* include a filamentous bacterium which has been shown to contain the peptide theopalauamide[6] and a unicellular bacterium containing swinholide A.[7] We have been studying the peptide chemistry of lithistid sponges because of the bioactivity of many of their metabolites and our interest in understanding the association between the sponges and their bacterial flora.

During field collections in the Philippines in 1996, we encountered several lithistid sponges, including a *Theonella* sp. containing microsclerodermins C (3) and D (4) and a *Microscleroderma* sp. containing microsclerodermins D (4) and E (5), which are related to microsclerodermins A (1) and B (2) from *Microscleroderma* sp.[8] Since these compounds are thought to be synthesized by microbes, the presence of peptide 4 in both genera of sponges brings into question the specificity of symbiosis among lithistid sponges. Filamentous, non-photosynthetic microorganisms were found only in the *Theonella* sp. In this paper, we report the isolation and characterization of microsclerodermins C - E (3-5).

E-mail: jfaulkner@ucsd.edu (John Faulkner), eschmidt@ucsd.edu (Eric Schmidt).

1, R = OH
2, R = H

3, R = CONH$_2$
4, R = H

5

6, R = CONH$_2$

7, R = H

15
16
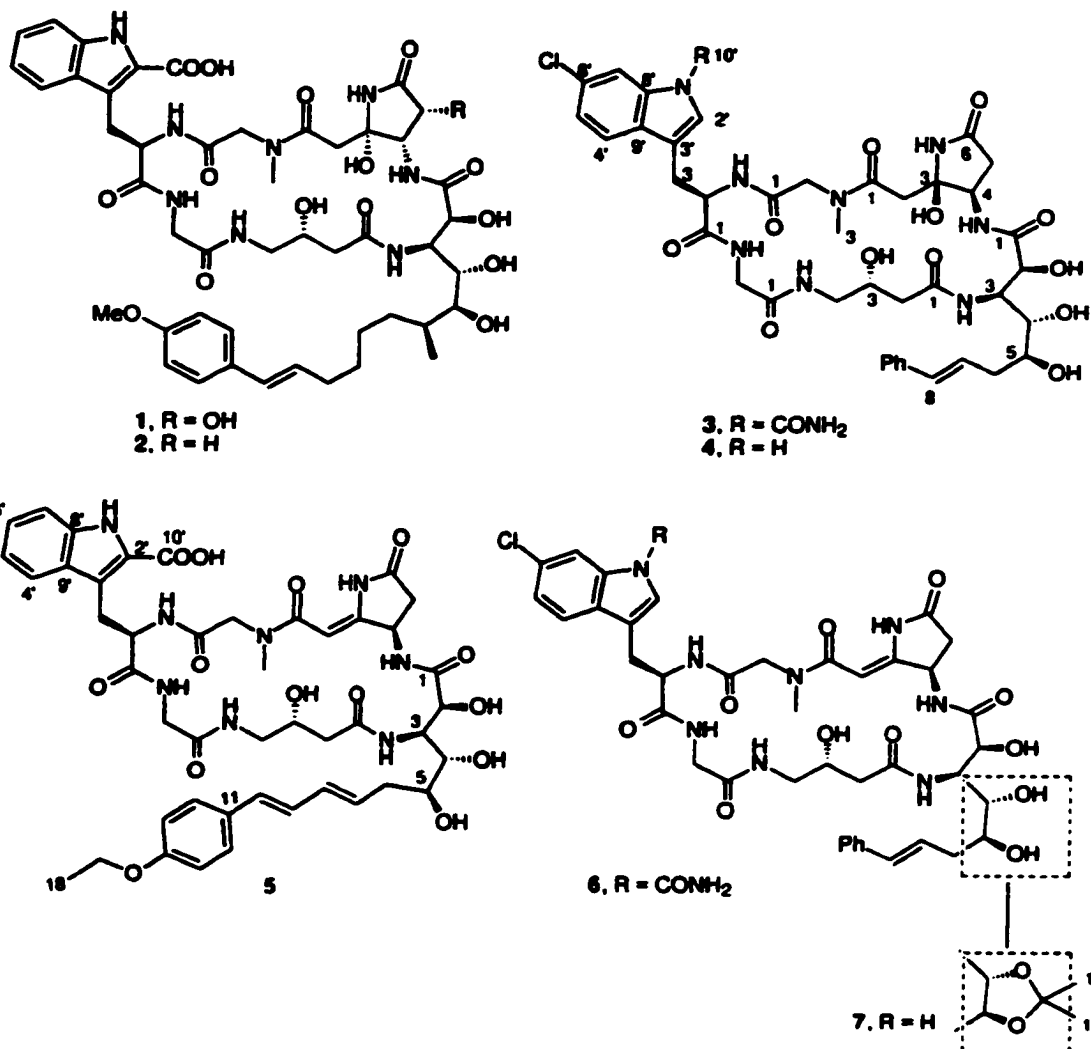
Isolation and Structural Determination of Microsclerodermins C and D

A lithistid sponge, *Theonella* sp., was collected using SCUBA off Olango Island in the Philippines at a depth of about 20 meters. This organism was selected for chemical investigation because light microscopy revealed that the sponge contained an abundance of filamentous bacteria, and the aqueous extract was active against the yeast *Candida albicans*. Microsclerodermins C (3) and D (4) were obtained by sequential extraction of the lyophilized sponge with solvents of increasing polarity. Rotary evaporation of the 1:1 acetonitrile/water extract gave a white precipitate that was subsequently centrifuged and subjected to reversed-phase chromatography. Peptide-containing fractions were lyophilized and resuspended in acetonitrile-water mixtures, which readily dissolved microsclerodermin D (4) but left a precipitate of pure microsclerodermin C

(3). Microsclerodermin D (4) was further purified and separated from dissolved microsclerodermin C (3) by reversed-phase HPLC.

Microsclerodermin C (3) was isolated as a white powder. The molecular formula, $C_{41}H_{50}ClN_9O_{13}$, was determined by high resolution mass measurement. In the ^1H NMR spectrum, a series of NH signals between δ 7.48 and 8.70 coupled to signals in the correct region for α-protons of amino acids suggested that the compound was a peptide. Despite its similarities with microsclerodermins A (1) and B (2), an affinity with those peptides was not immediately obvious from NMR spectral data (see Table 1), and thus the two-dimensional structure was elucidated by NMR methods. The ^1H, ^{13}C, and GHMQC NMR data indicated that there were six amino acids, including one that was N-methylated, and five hydroxyl groups. These experiments also suggested the presence of a monosubstituted phenyl group and an indole substituted at the N' and 6' positions. A DQCOSY spectrum was used to construct the major features of the constituent amino acids, and the resulting assignments were confirmed using the GHMBC spectrum. From these data, glycine, N-methyl glycine, and 4-amino-3-hydroxybutyric acid (GABOB) were readily identified. In addition, these experiments showed that the indole unit was part of tryptophan and that the phenyl group was conjugated to a double bond. GHMBC (see Figure 1) and ROESY (see Figure 3) experiments were used to determine the connectivity between the N-Me-Gly, disubstituted tryptophan, Gly, and GABOB residues. However, the pyrrolidone and 3-amino-8-phenyl-2,4,5-trihydroxyoct-7-enoic acid (APTO) groups could not be completely constructed from these data alone.

Assignment of the APTO structure was complicated by overlapping OH and CH protons in the NMR spectrum. For example, H-2 of this unit could not be distinguished from OH-5 in any two-dimensional NMR experiment, and APTO OH-4 was difficult to differentiate from pyrrolidone H-4. To resolve these overlapping signals, a complete NMR data set was acquired at 40 °C, at which temperature the exchangeable OH signals were overlapping with different CH signals in the ^1H NMR spectrum. Comparison of the two data sets were used to unambiguously construct two halves of the APTO unit, C-12 to C-5 and C-4 to C-1, and to assign resonances belonging to the pyrrolidone group. Unfortunately, a lack of observable coupling between H-4 and H-5 and the absence of any GHMBC correlations between the two parts of the molecule made the assignment of the APTO structure difficult. The APTO unit was postulated on the basis of ROE correlations between the two halves of the amino acid, but was not confirmed until the acetonide 7 was synthesized.

In the pyrrolidone residue, the cyclic portion was established using the GHMBC spectrum, which included correlations from OH-3 to C-2 and C-4, and from NH-3 to C-2, C-3, C-4, C-5 and C-6. The relative stereochemistry of the pyrrolidone was established using the ROESY spectrum. After defining the amino acid

Table 1. NMR data for microsclerodermin C (3) and dehydromicrosclerodermin C (6) in DMSO-d_6

amino acid	assignment	3 δC	3 δH	6 δC	6 δH
APTO	1	172.6		173.5	
	2	69.6	4.38 m	69.5	4.40 m
	3	53.3	4.13 t, 10.0	53.5	4.18 m
	4	70.1	3.29 m	70.2	3.29 m
	5	69.0	3.59 q, 4.5	69.0	3.61 m
	6	36.6	2.35 m	36.6	2.35 m
	7	128.1	6.27 m	128.2	6.32 dt, 16.0, 7.0
	8	130.8	6.40 d, 16.0	130.8	6.40 d, 16.0
	9	137.4		137.4	
	10,14	128.4	7.29 m	128.5	7.30 m
	11,13	125.7	7.33 m	125.8	7.34 m
	12	126.9	7.19 m	126.9	7.20 m
	NH3		7.48 m		7.12 m
	OH2		6.20 d, 6.0		
	OH4		4.49 d, 9.5		
	OH5		4.38 d, 5.0		
GABOB	1	172.9		172.4	
	2	41.1	2.16 m	41.5	2.13 t, 12.0
			2.43 m		2.30 m
	3	67.3	3.71 m	66.8	3.73 m
	4	45.0	2.57 m	45.2	2.60 m
			3.39 m		3.38 m
	NH4		7.48 m		7.38 m
	OH3		4.90 d, 5.5		
Gly	1	168.8		168.8	
	2	42.7	3.37 m	42.8	3.45 m
			3.77 m		3.72 m
	NH2		8.55 d, 6.0		8.40 m
6'-Cl-N'-form.-Trp	1	171.5		170.8	
	2	54.6	4.23 m	54.8	4.09 m
	3	25.8	3.03 m	25.1	2.95 m
					3.22 m
	2'	124.2	7.75 s	124.5	7.75 s
	3'	114.9		115.4	
	4'	120.2	7.60 m	120.2	7.60 m
	5'	121.8	7.23 m	121.9	7.24 m
	6'	not obsd		not obsd	
	7'	114.9	8.27 d, 2.0	114.9	8.27 d, 2.0
	8'	135.9		135.9	
	9'	128.5		128.5	
	10'	152.3		152.4	
	NH2		8.70 d, 3.5		8.71 d, 5.0
N-Me-Gly	1	170.4		170.9	
	2	49.7	3.97 m	50.6	3.45 m
			4.00 m		4.50 m
	3	36.4	2.91 s	37.0	2.98 s
pyrrolidone	1	170.4		168.1	
	2	38.8	2.65 d, 17.0	87.8	5.25 s
			2.89 d, 17.0		
	3	85.6		157.5	
	4	50.5	4.47 m	46.0	5.23 t, 9.5
	5	35.2	2.27 m	34.1	2.48 m
					2.68 m
	6	173.1		174.8	
	NH3		7.98 s		10.38 s
	OH3		6.02 s		—
	NH4		7.56 m		8.38 m

Figure 1. Key GHMBC correlations used to determine the structure of microsclerodermin C (3). The indole portion is shown on the right.

structures, the peptide sequence was fully defined on the basis of GHMBC and ROESY correlations, and the similarity to microsclerodermins A (1) and B (2) was recognized. The substituents on the tryptophan unit were not identified until the structure of microsclerodermin D (4) was established.

Microsclerodermin C (3) was readily dehydrated by addition of a trace amount of TFA to the NMR solvent. The structure of anhydromicrosclerodermin C (6, $C_{41}H_{48}N_9O_{12}Cl$) was elucidated using a full NMR data set to confirm the proposed structure of 3 (see Table 1). Interestingly, several peaks in the ^1H NMR spectrum of 3 appeared to be doubled (~5:1 ratio of peaks), but no signal doubling was visible in the ^1H NMR spectrum of the dehydrated compound 6. Since no evidence could be obtained to indicate that OH-3 of the pyrrolidone residue had two different configurations or that 3 was contaminated by 6, we conclude that the signal doubling must have resulted from different conformers of 3.

Microsclerodermin D (4, $C_{40}H_{47}ClN_8O_{12}$) was isolated as a white powder. Full NMR spectral data (see Table 2), including DQCOSY, GHMQC, GHMBC, and NOESY indicated that 4 was identical to 3 except for the tryptophan unit, which was no longer N-substituted. The molecular formula indicated that a chlorine atom was present in 4 and NMR data established that the chlorine was located on the 6' position of the indole unit.

The only question remaining was the identity of the functional group on the indole nitrogen in 3. The molecular mass, established by FABMS measurements, showed that 3 was 43 AMU heavier than 4. Microsclerodermin C (3) also had one more ^{13}C NMR signal than 2, at δ 152.3 ppm. This ^{13}C resonance

Table 2. NMR data for microsclerodermin D (4) in DMSO-d_6

amino acid	assignment	δ_C	δ_H
APTO	1	172.6	
	2	69.5	4.41 d, 6.0
	3	53.2	4.14 m
	4	70.0	3.32 m
	5	69.0	3.61 m
	6	36.4	2.35 m
	7	128.2	6.35 m
	8	130.8	6.42 d, 16.0
	9	136.5	
	10,14	128.5	7.30 m
	11,13	125.8	7.36 m
	12	126.8	7.20 t, 7.5
	NH3		7.44 m
	OH2		6.25 d, 6.0
GABOB	1	172.7	
	2	41.0	2.16 m
			2.43 m
	3	67.1	3.73 m
	4	45.0	2.64 m
			3.39 m
	NH4		7.48 m
	OH3		4.85 d, 4.0
Gly	1	168.8	
	2	42.7	3.35 m
			3.75 m
	NH2		8.54 br t, 4.5
6'-Cl-Trp	1	171.9	
	2	55.5	4.18 m
	3	26.0	2.98 m
	2'	124.9	7.28 s
	3'	110.0	
	4'	119.6	7.55 d, 8.5
	5'	118.6	7.01 d, 8.5
	6'	not obsd.	
	7'	111.0	7.39 m
	8'	134.4	
	9'	125.9	
	NH2		8.65 brd, 3.0
	NH1'		11.05 s
N-Me-Gly	1	170.2	
	2	49.8	3.84 m
			4.10 m
	3	36.7	2.92 s
pyrrolidone	1	170.5	
	2	38.0	2.70 m
			2.86 m
	3	85.6	
	4	50.6	4.47 m
	5	35.1	2.28 m
	6	173.2	
	NH3		7.98 m
	OH3		6.10 s
	NH4		7.54 s

showed a weak correlation to H-2' in the GHMBC spectrum, confirming the placement of this unit on the aromatic nitrogen. Unfortunately, two possible structures could be proposed based on these data: the functional group in question could be either CH_4N_2 (guanidinium salt) or CH_2NO (urea). FABMS measurements could be used to support either answer. While the exact mass of microsclerodermin C (3) was closer to the guanidinium salt, the FABMS spectrum of anhydromicrosclerodermin C (6) matched the urea formula more closely. A ^1H NMR experiment on 3 was employed to discriminate between the two possibilities. Triethylamine (1 µL increments up to 3 µL) was added to an NMR tube containing 6 mg of 6 in DMSO-d_6. The ^1H NMR spectrum of the basic solution, especially in the aromatic region, was identical to that of 6 in neutral or slightly acidic solution, indicating that the N'-substituting group was unaffected by the pH change. Thus, 3 is a urea rather than a guanidinium salt. To the best of our knowledge, 6'-chloro-N'-formamidotryptophan and APTO are unprecedented amino acids.

Anhydromicrosclerodermin C (6) was used in chemical degradation experiments to establish the absolute stereochemistry of 3. Treatment of peptide 4 with periodate, followed by ozonolysis using an oxidative workup and acid-catalyzed hydrolysis gave a mixture of amino acids that were derivatized and analyzed by chiral GC-MS. Comparison with derivatives of amino acid standards allowed the identification of (3R)-3-hydroxy-4-aminobutyric acid, (2S,3S)-3-hydroxyaspartic acid, and (R)-aspartic acid. Although we were unable to cleanly form an acetonide from 6, treatment of anhydromicrosclerodermin D with 2,2-dimethoxypropane using pyridinium p-toluenesulfonate as catalyst gave the acetonide 7 in quantitative yield. A NOESY experiment performed on 7 gave results similar to those found for the acetonide of microsclerodermin A (1),[8] indicating that the stereochemistry of the APTO group was (2S,3R,4S,5S,7E).

Isolation and Structural Determination of Microsclerodermin E

The sponge *Microscleroderma* sp. was collected at a depth of 15 m near Panglao Island, Visayas, Philippines. The sponge, while much different in appearance than the *Theonella* sp., also had antifungal activity but did not appear to contain filamentous microorganisms. Initial extraction and separation procedures were identical to those used to isolate 3 and 4. Following purification through a C_{18} Sep Pak, it was found that the resulting peptide mixture (approximately 70% one compound) was completely insoluble in many solvents and was only moderately soluble in DMSO. The peptide mixture was found to be soluble in mixtures containing acetonitrile and 0.1 N NH_4HCO_3, so this system was used for a reversed-phase HPLC separation that yielded microsclerodermins D (4) and E (5). The identity of microsclerodermin D (4) was readily established by comparing ^1H and ^{13}C NMR and HRMS data with those determined previously. It is possible that microsclerodermin C (3) was also present in the sponge before separation, since mild aqueous

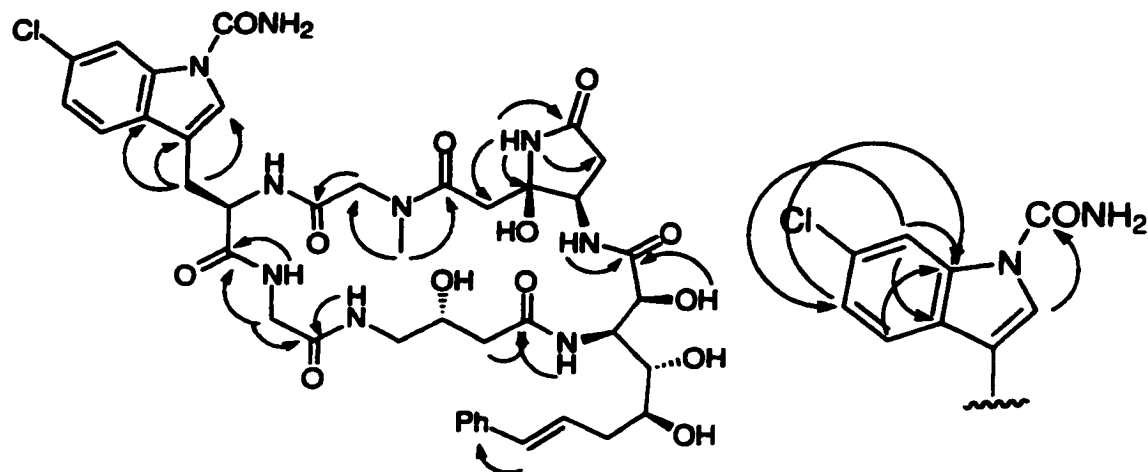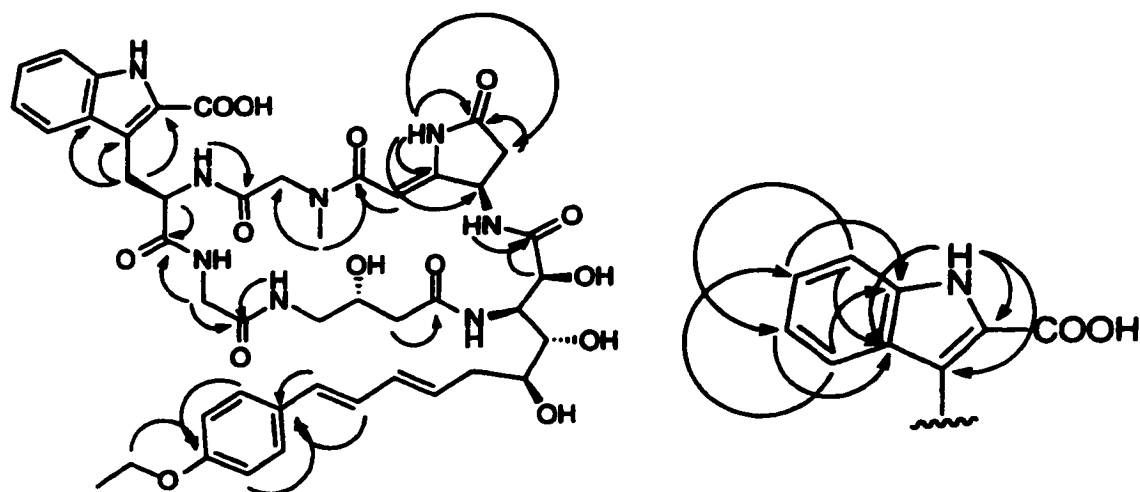**Figure 2.** Key GHMBC correlations used to determine the structure of microsclerodermin E (5). The indole portion is shown on the right.

base hydrolysis rapidly converts 3 to 4.

Microsclerodermin E (5) was isolated as a white powder. Its molecular formula ($C_{45}H_{54}N_8O_{14}$) suggested that it had a close structural affinity to microsclerodermin C (3), with the lack of the chlorine atom and four additional carbon atoms being the major differences. The NMR data, including ^1H, ^{13}C, DEPT, DQCOSY, TOCSY, NOESY (see Figure 3), GHMQC and GHMBC (see Figure 2), established the relationship between the two compounds (see Table 3). From these data, the presence of GABOB, N-Me-Gly, and Gly were readily established and confirmed by comparison with the spectral data of 3. The pyrrolidone residue was established by NMR data, especially GHMBC data from NH-5, and confirmed by comparison with the spectral data of anhydromicrosclerodermin C (6). Interestingly, the pyrrolidone residue in 5 did not appear to dehydrate during separation, since key signals were present in the NMR spectra of the crude mixture of peptides before acid or base treatment. In addition, 4 was isolated from this sponge with its pyrrolidone OH intact, even after HPLC separation.

The two residues which appeared unique to microsclerodermin E (5) were the substituted tryptophan and the amino acid identified as 3-amino-10-(p-ethoxyphenyl)-2,4,5-trihydroxydeca-7,9-dienoic acid (AETD). The AETD carbons bound to oxygen and nitrogen had nearly identical NMR chemical shifts and 2-D NMR data to those of the APTO group in 3 and 4, leading to the proposed structure for C-1 to C-7. The ^1H and DQCOSY NMR spectra indicated that this unit was connected to a diene, rather than the single double bond found in 3. GHMBC correlations were used to connect the diene to the aromatic unit, which was identified as

Table 3. NMR data for microsclerodermin E (5) in DMF-d_7 with trace TFA[9]

amino acid	assignment	δ_C	δ_H
		5[b]	
AETD	1	174.9	
	2	71.1	4.69 s
	3	55.4	4.34 t, 10.0
	4	71.8	3.46 m
	5	70.6	3.72 m
	6	37.7	2.39 m
	7	132.9	5.83 dt, 15.0, 8.0
	8	133.4	6.25 dd, 15.0, 10.5
	9	128.5	6.73 dd, 16.0, 10.5
	10	130.8	6.45 d, 16.0
	11	131.3	
	12,16	128.5	7.41 d, 8.5
	13,15	115.7	6.91 d, 8.5
	14	159.6	
	17	64.3	4.04 q, 5.0
	18	15.4	1.35 t, 5.0
	NH3		7.57 d, 10.5
GABOB	1	174.2	
	2	43.1	2.19 dd, 14.0, 9.0
			2.49 dd, 14.0, 1.0
	3	68.7	3.93 br
	4	46.7	2.92 m
			3.45 m
	NH4		7.35 brt, 9.0
Gly	1	170.5	
	2	44.2	3.62 m
			3.70 m
	NH2		8.45 t, 6.5
Trp-2'-COOH[e]	1	172.4	
	2	58.1	4.24 dt, 9.0, 2.0
	3	26.4	3.60 m
			3.70 m
	2'	126.4	
	3'	119.7	
	4'	121.5	7.72 d, 8.5
	5'	120.9	7.16 t, 8.5
	6'	125.8	7.30 t, 8.5
	7'	113.5	7.54 d, 8.5
	8'	137.5	
	9'	129.3	
	10'	164.9[c]	
	NH2		8.88 d, 2.0[d]
	NH1'		11.58 br s
N-Me-Gly	1	172.4	
	2	51.8	3.47 d, 16.0
			4.64 d, 16.0
	3	37.9	3.09 s
pyrrolidone	1	169.9	
	2	89.0	5.45 s
	3	159.5	
	4	47.4	5.42 dt, 5.0, 9.0
	5	35.0	2.58 dd, 18.0, 5.0
			2.89 dd, 18.0, 9.0
	6	176.1	
	NH3		8.59 d, 9.0
	NH4		10.60 s
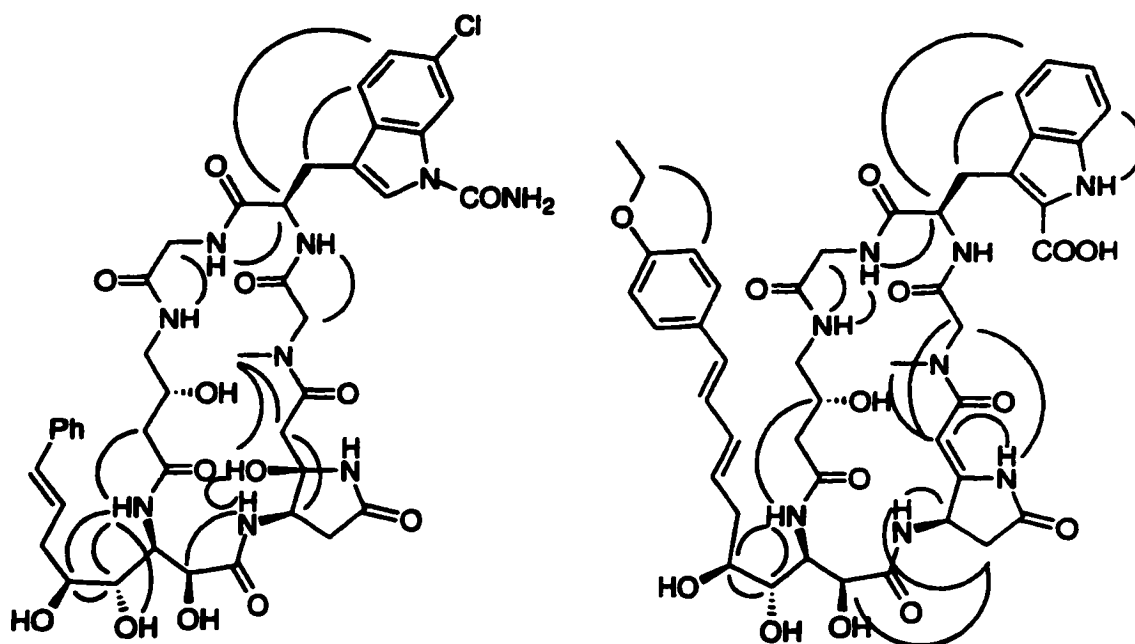
Figure 3. Summary of key NOESY/ROESY correlations used to determine the structure of microsclerodermins C (3, left) and E (5, right).

a p-ethoxy phenyl ring[10] based on ^{1}H and ^{13}C NMR chemical shift data. The ethyl group was not produced during the purification of 5, since the O-ethyl signals were visible in the ^{1}H and ^{13}C NMR spectra of the crude peptides. Thus, the AETD residue accounted for the extra C_4H_7 in 5 compared with 3.

Following the identification of all other residues, it was apparent from the molecular formula that the tryptophan residue was substituted with a carboxylate group. The identification of this residue was complicated by the fact that the α-NH proton was not visible in the NMR spectra of either the original extract or the purified peptide (5). Upon acidification of the NMR solvent with TFA vapor, however, the Trp NH proton signal was revealed at δ 8.84 ppm (d, J = 2 Hz). From the DQCOSY and GHMBC data, the tryptophan residue was substituted adjacent to the aromatic nitrogen. These data confirmed the core structure of the tryptophan unit, but there were no NMR correlations to the substituent adjacent to the aromatic nitrogen to confirm placement of the carboxyl group.

The only remaining unassigned NMR signal, at δ 164.9 in the ^{13}C spectrum, was consistent with a COOH at C-2' based on comparison to simulated NMR spectra. The presence of a carboxylate anion was also indicated by the sensitivity of many chemical shifts of the amino acids, especially tryptophan, to pH changes. In basic or neutral solution, the carboxylate ^{13}C NMR signal resonated at δ 167.9 ppm, but addition of trace

acid significantly shifted the resonance. Low resolution positive ESIMS gave a molecular ion at 953, corresponding to $(M+Na)^+$, while negative ESIMS gave 929 (M^-). In addition, methylation of 5 with diazomethane afforded a mono-methylated compound with a high resolution mass spectrum that further corroborated the proposed structure of 5. Finally, comparison of ^{13}C NMR data from 5 with those of anhydromicroscleroderminin A[8] served to confirm the structure.

The absolute configuration of microscleroderminin E (5) was determined to be identical to that of 3 and 4 by employing the same sequence of reactions used for 6 and comparing GC-MS peaks with those resulting from authentic standards. Only two stereocenters, C-4 and C-5 in the AETD residue, were not determined by chemical means. Because their ^1H and ^{13}C NMR chemical shift data were close to those of known microscleroderminins, C-4 and C-5 were assumed to have the same absolute configurations in all compounds.

Bioactivity

Microscleroderminins C (3), D (4), E (5), and anhydromicroscleroderminin C (6) were active against *Candida albicans* in a paper disk assay. Peptide 3 was most active at 5 μg per disk, followed by 5 at 10 μg, 6 at 50 μg and 4 at 100 μg. Reported concentrations are the minimum at which inhibition was observed.

EXPERIMENTAL SECTION

General Experimental Procedures: ^1H, DQCOSY, GHMQC, and GHMBC, TOCSY and ROESY NMR spectra were recorded on a Varian Inova 300 MHz spectrometer. ^{13}C and DEPT spectra were recorded on a Varian Gemini 2000 400 MHz spectrometer. A NOESY spectrum was recorded on a Varian Unity 500 MHz spectrometer. All NMR data are reported in DMSO-d_6 except for those of peptide 5, which were reported in DMF-d_7 with trace TFA. TOCSY spectra were obtained with 80 msec mixing times, and ROESY spin locks were established by continuous pulsing for 300 msec. High-resolution FABMS data were obtained from the Mass Spectrometry Facility, University of California at Riverside. Optical rotations were measured on an Autopol III polarimeter. UV and IR spectra were recorded on Perkin Elmer Lambda 3B and 1600 FT-IR instruments, respectively. Absolute configurations were determined using an Alltech Chirasil-Val column with a Hewlett Packard 5890 GC-MS system. The temperature was ramped from 50 °C to 210 °C over 45 minutes in all GC-MS experiments. GC-MS retention times were similar to previously reported results.[8]

Isolation of Microscleroderminins C (3) and D (4): A sample of *Theonella* sp. (NCI 2218) was collected at a depth of 20 m near Olango Island, Visayas, Philippines (N 10° 16' 53.2", E 124° 2' 57.6"). The sponge was lyophilized (39.4 g dry weight) and sequentially extracted with solvents of increasing polarity: 2 x DCM; 2 x 1:1 EtOAc/acetone; 5 x 1:1 CH_3CN/H_2O. The CH_3CN was removed from the aqueous extract by rotary

evaporation, and the resulting solution was centrifuged to yield a white precipitate. The solid material was dry loaded on a C_{18} Sep Pak (Waters) and subjected to a gradient of 0-100% CH_3CN (aq). Fractions eluting between 50 and 60% acetonitrile were combined and resuspended in 10 mL of 1:1 CH_3CN/H_2O. The suspension was centrifuged for 10 minutes, yielding a white powder of pure microsclerodermin C (3, 30 mg). The eluant was subjected to HPLC on a C_{18} preparative column using 37% CH_3CN (aq) to yield an additional 43 mg of 3 (total: 73 mg, 0.19% dry weight) and microsclerodermin D (4, 30 mg, 0.077% dry weight).

Isolation of Microsclerodermin E (5): A sample of *Microscleroderma* sp. (NCI 2309) was collected at a depth of 15 m near Panglao Island, Visayas, Philippines (N 9° 23' 37.3", E 123° 43' 80.1"). The sponge was lyophilized (127.5 g dry weight), then extracted with 4 x EtOAc and 5 x 1:1 H_2O/CH_3CN. The aqueous extract was dried by rotary evaporation until the formation of a white precipitate, which was removed by centrifugation. The white powder was dry loaded on a C_{18} Sep Pak and subjected to a gradient of 20-100% CH_3CN (aq). Fractions between 30-40% CH_3CN were pooled and repurified on a C_{18} HPLC column using 73:27 0.1 N NH_4HCO_3/CH_3CN at 3 mL/min. Two major fractions eluted after approximately 1 hour: microsclerodermins D (4, 8.6 mg, 0.007% dry weight) and E (5, 15 mg, 0.012% dry weight).

Microsclerodermin C (3): white powder; $[\alpha]_D$ -24° (c 0.063, 1:1 MeOH/DMSO); UV (MeOH) 202 (ϵ 46,500), 239 (28,000), 250 (27,100), 256 (28,000); IR (AgCl) 3390, 1650-1660, 1540, 1435, 1410 cm^{-1}; ^{1}H NMR (DMSO-d_6) see Table 1; ^{13}C NMR (DMSO-d_6) see Table 1; HRFABMS obsd m/z 934.3270 (M+Na)$^+$, $C_{41}H_{50}ClN_9O_{13}Na$ requires m/z 934.3114.

Microsclerodermin D (4): white powder; $[\alpha]_D$ -56° (c 0.07, 1:1 MeOH/H$_2$O); UV (MeOH) 202 (ϵ 56,000), 224 (34,000), 242 (16,100), 269 (9600), 277 (9000), 287 (3700); IR (AgCl) 3310, 1660, 1545, 1405 cm^{-1}; ^{1}H NMR (DMSO-d_6) see Table 2; ^{13}C NMR (DMSO-d_6) see Table 2; HRFABMS obsd m/z 891.3090 (M+Na)$^+$, $C_{40}H_{49}ClN_8O_{12}Na$ requires m/z 891.3056.

Microsclerodermin E (5): white powder; $[\alpha]_D$ -24° (c 0.19, 1:1 MeOH/0.1 N NH_4HCO_3 (aq)); UV (MeOH) 203 (ϵ 33,100), 206 (34,700), 218 (33,000), 284 (37,200); IR (AgCl) 3310, 2930, 1740, 1660, 1540 cm^{-1}; ^{1}H NMR (DMF-d_7) see Table 3; ^{13}C NMR (DMF-d_7) see Table 3; HRFABMS obsd m/z 931.4026 (M+H)$^+$, $C_{45}H_{55}N_8O_{14}$ requires m/z 931.3838.

Preparation of anhydromicrosclerodermin C (6): A solution of peptide 3 (15 mg) in DMSO-d_6 (750 μL) in an NMR tube was acidified with a trace amount of TFA and immediately observed by ^{1}H NMR. Quantitative dehydration occurred in less than 5 minutes to yield 6 (15 mg, 100% yield): white powder; ^{1}H NMR (DMSO-d_6) see Table 1; ^{13}C NMR (DMSO-d_6) see Table 1; HRFABMS obsd m/z 894.3276 (M+H)$^+$, $C_{41}H_{49}ClN_9O_{12}$ requires m/z 894.3189.

Preparation of acetonide 7: Vapors of TFA were added to a solution of peptide 4 (7 mg) in dry DMF (100 μL) in a conical vial. The vial was left for 5 minutes, then dried *in vacuo* to yield anhydromicrosclerodermin D. The product was added to a solution of dry DMF (100 μL) containing dimethoxypropane (400 μL) and a catalytic amount of pyridinium *p*-toluensulfonate. The solution was stirred overnight, then dried under a stream of nitrogen and *in vacuo* to give the pure acetonide 7 (7 mg, 100% yield): white powder; ^{1}H NMR (DMSO-d_6) APTO: 1.37 (s, 6H, H-15,16), 2.29 (m, 1H, H-6), 2.40 (m, 1H, H-6'), 3.77 (dd, J = 8,10 Hz, 1H, H-4), 4.06 (m, 1H, H-5), 4.22 (s, 1H, H-2), 4.35 (t, J= 10 Hz, 1H, H-3), 6.26 (dt, J = 16,7.5 Hz, 1H, H-7), 6.45 (d, J = 16 Hz, 1H, H-8), 7.12 (m, 1H, NH-3), 7.20 (t, J = 6 Hz, 1H, H-12), 7.30 (m, 2H, H-10,14), 7.42 (m, 2H, H-11,13); GABOB: 2.06 (m, 1H, H-2), 2.25 (m, 1H, H-2'), 2.99 (m, 1H, H-4), 3.19 (m, 1H, H-4'), 3.94 (m, 1H, H-3), 7.38 (m, 1H, NH-4); Gly: 3.62 (d, J = 4 Hz, 1H, H-2), 3.64 (d, J = 4 Hz, 1H, H-2'), 8.37 (t, J = 4 Hz, 1H, NH-2); Trp-6'-Cl: 3.10 (m, 1H, H-3), 4.14 (m, 1H, H-2), 7.02 (d, J = 8.5 Hz, 1H, H-5'), 7.32 (s, 1H, H-2'), 7.33 (m, 1H, H-7'), 7.55 (d, J = 8.5 Hz, 1H, H-4'), 8.79 (d, J = 4.5 Hz, 1H, NH-2), 11.01 (s, 1H, NH-1'); NMeGly: 3.10 (s, 3H, H-3), 3.46 (d, J = 16 Hz, 1H, H-2), 4.53 (d, J= 16 Hz, 1H, H-2'); pyrrolidone: 2.48 (m, 1H, H-5), 2.75 (m, 1H, H-5'), 5.24 (m, 1H, H-4), 5.29 (s, 1H, H-2), 8.36 (d, J = 7.5 Hz, 1H, NH-4), 10.40 (s, 1H, NH-3); HRFABMS obsd m/z 891.3534 (M+H)$^+$, $C_{43}H_{52}ClN_8O_{11}$ requires m/z 891.3444.

Methylation of 5: Peptide 5 (0.6 mg) was suspended in EtOH (1.5 mL) and cooled in an ice bath. Diazomethane was distilled into the solution, which was then brought to room temperature and allowed to react overnight. The solvent was evaporated under nitrogen, and the sample was loaded onto a C_{18} analytical HPLC column using 40% CH_3CN (aq) as eluant. A single major fraction (0.5 mg) was recovered. The ^{1}H NMR spectrum (DMSO-d_6) contained a new peak at δ 3.87; HRFABMS obsd m/z 945.3999 (M+H)$^+$, $C_{46}H_{57}N_8O_{14}$ requires m/z 945.3994.

Degradation and derivatization of 5 and 6 for GCMS: Peptide 6 (300 μg) was dissolved in water (100 μL, acidified to pH = 4 with AcOH), to which was added $NaIO_4$ (300 μg). The reaction was stirred overnight, then lyophilized. The periodate cleavage product was dissolved in MeOH (200 μL) and ozonized at -78 °C for 50 minutes. The reaction was quenched with 50% H_2O_2 (5 drops), brought to room temperature, and allowed to stand for 1 hour. The reaction mixture was dried under a stream of N_2 followed by lyophilization, then hydrolyzed and derivatized as described previously.[8] After drying under nitrogen, the residue was dissolved in 100 μL EtOAc (GC-MS grade) and subjected to GC-MS using a chiral column. Comparison of retention times and MS fragmentation patterns with amino acids that had been derivatized in the same manner gave (3R)-3-hydroxy-4-aminobutyric acid, (2S,3S)-3-hydroxyaspartic acid, and (R)-aspartic acid. Microsclerodermin E (5) was analyzed in an identical manner.

REFERENCES AND NOTES

1. Bewley, C.A.; Faulkner, D.J. *Angew. Chem. Intl. Ed. Engl.* **1997**, in press.

2. Gunasekera, S.P.; Gunasekera, M.; Longley, R.E.; Schulte, G.K. *J. Org. Chem.* **1990**, *55*, 4912-4915.

3. Kobayashi, M.; Tanaka, J.; Katori, T.; Masuura, M.; Kitagawa, I. *Tetrahedron Letts.* **1989**, *30*, 2963-2966.

4. Kato, Y.; Fusetani, N.; Matsunaga, S.; Hashimoto, K. *J. Am. Chem. Soc.* **1986**, *108*, 2780-2781.

5. Fusetani, N.; Matsunaga, S. *Chem. Rev.* **1993**, *97*, 1793-1806.

6. Schmidt, E.W.; Bewley, C.A.; Faulkner, D.J. *J. Org. Chem.* In press.

7. Bewley, C.A.; Holland, N.D.; Faulkner, D.J. *Experientia* **1996**, *52*, 716-722.

8. Bewley, C.A.; Debitus, C.; Faulkner, D.J. *J. Am. Chem. Soc.* **1994**, *116*, 7631-7636.

9. a) Most ^{13}C and ^{1}H NMR signals were highly pH dependent. b) Observed with trace TFA. c) In basic solution, δ 167.9 ppm. d) Not observed in basic or neutral solution.

10. We found no record of natural products containing the *p*-ethoxy phenyl moiety in computer databases.

This chapter, in full, is a reprint of the material as it appears Tetrahedron, 1998, 54, 3043-3056, Schmidt, Eric W., Faulkner, D. John. The dissertation author was the primary investigator of this paper.

CHAPTER 7

SCLERITODERMIN A FROM THE MARINE SPONGE *SCLERITODERMA NODOSUM*: USING THE NCI ACTIVE REPOSITORY

From the National Cancer Institute active repository, I obtained 13 extracts of marine lithistid sponges selected using bioactivity profiles and taxonomic information. These extracts were rapidly dereplicated using taxonomy and ESI-MS as key tools. One of the extracts contained a novel cytotoxic peptide which was selected by Bristol-Myers Squibb for *in vivo* anti-tumor testing. Here, I report the dereplication strategy leading to the isolation and structure elucidation of the novel peptide, scleritodermin A (13) from the marine sponge *Scleritoderma nodosum*.

Introduction

The NCI's Natural Products Branch maintains a collection of marine organisms that numbered over 10,000 as of March, 1997.[1] The samples are available to qualified investigators, and they offer several advantages to natural products chemists. The source organisms have been taxonomically identified by experts, eliminating the vexing process of source identification, allowing faster dereplication, and giving the investigator the ability to choose research samples based on species. The selection of species prior to study is particularly important, since organisms can be selected based on interesting chemistry in their taxonomic group, or on the absence of the species from the marine natural products literature. In addition, the NCI repository offers an advantage because the crude extracts have already been screened against the NCI 60 cell-line panel, allowing choice of extract based on interesting activity profile. The main disadvantage of using the repository for the discovery of new compounds is that only 20% of each

70

extract is available for preliminary studies, making detection of minor constituents unlikely.

The repository is also potentially useful to the field of sponge taxonomy. Compounds do not necessarily need to be isolated from every sample; once the 60 cell-line profile is established, it can be compared to other samples in the database. Unfortunately, the utility of the database to taxonomists is somewhat limited because the locations of sample origin are not readily obtained. Results which may prove useful in the taxonomy of sponges and their symbionts are presented in Chapter 9.

To test the utility of the Active Repository in the discovery of novel compounds, I requested 13 bioactive extracts that were selected based on taxonomy. All 13 were from the order Lithistida, known to be the source of a variety of interesting compounds and promising drug candidates.[2] From the numerous lithistid sponges available in the collection, I selected organisms which I had not previously examined or which had not been extensively studied by the marine natural products community. The most promising sponges from my initial screening of candidates were two samples of the species *S. nodosum*, which had not been previously reported in natural products papers. A variety of theonellid sponges, including different species in the genera *Theonella* and *Plakinolopha*, were selected because, although this family has been well studied, a large number of novel compounds continues to be reported from its members. In addition, *Microscleroderma hardmani* and *Aciculites ciliata* were selected, since each genus had only been previously studied once before.[3,4]

In total, ten organic extracts and three aqueous extracts were requested, but work on the aqueous extracts proved too difficult due to insufficient organic material and high salt content. The organic extracts were partitioned between methanol and hexane, then chromatographed on C_{18} or LH-20 adsorbents to provide fractions that contained

compounds with interesting peaks in the ^{1}H NMR spectra. Rapid dereplication was possible using electrospray mass spectrometry (ESI-MS) to analyze the interesting fractions and then comparing the masses obtained, the sponge taxonomy, and proton spectra with the marine natural products literature. Based on initial promising results, four of the organic extracts were pursued for new compounds, while the remaining six contained mainly known metabolites. Unfortunately, one of the four contained known microsclerodermin derivatives,[4,5] and from two samples identical, but not previously described, theonellapeptolides[6] were isolated. The remaining extract (*S. nodosum*) contained the novel compound scleritodermin A (13), which was subjected to further biological testing. Details of dereplication results are given in Table 1.

Isolation and Structure Elucidation of 13

The organic extract of the marine sponge *S. nodosum* from the National Cancer Institute (C0-12961) was partitioned between hexane and methanol. The methanol fraction was chromatographed on a C_{18} Sep Pak using an acetonitrile/water gradient. HPLC separation with 28% CH_3CN (aq) afforded pure scleritodermin A (13, 6 mg). A further sample containing a carotenoid-like pigment was purified by size exclusion chromatography on LH-20, followed by TLC on a C_{18} prep plate to give an additional 6 mg of 13 (12 mg total; 0.8% extract weight).

Scleritodermin A (13) was isolated as an off-white powder. HRFABMS analysis gave a molecular ion at m/z = 952.3436 [M+H]$^+$, indicating a molecular formula of $C_{42}H_{57}N_8O_{12}$ S_2Na (δ = -0.15 ppm). The ^{1}H and GCOSY NMR spectra exhibited standard proton chemical shifts for amino acids. In particlar, several exchangeable NH proton signals between 7 and 9 ppm and protons between 3.5 and 5 ppm were important in establishing that 13 was a peptide. The proteinogenic amino acids tyrosine, isoleucine, proline, and serine were identified based on NMR data,

Table 1. Dereplication of NCI organic extracts.

Sample Number	Species Name	Compounds
C012961	*Scleritoderma nodosum* (II)	scleritodermin A (13)
C012997	*Theonella* sp. 3	theonellapeptolides (41)
C014177	*Aciculites ciliata*	aciculitins A (42) & B (43) microsclerodermin E (12)
C014321	*Theonella cylindrica*	microsclerodermin E (12)
C014323	*Plakinalopha* sp. 3	aurantosides/mozamides (similar to 7, 44)
C014451	*Theonella* sp. 6	theonellapeptolides (41)
C014537	*Microscleroderma hardmani*	microsclerodermins C (10) and D (11)
C015443	*Scleritoderma nodosum* (I)	pyridinine alkaloids (similar to 45)
C015943	*Theonella invaginata*	aurantosides/mozamides (similar to 7, 44)
C015997	*Plakinalopha mirabilis*	aurantosides/mozamides (similar to 7, 44)

41

42 R = -C₅H₁₁
43 R = -C₆H₁₃

12

44

7

10 R = CONH₂
11 R = H

45
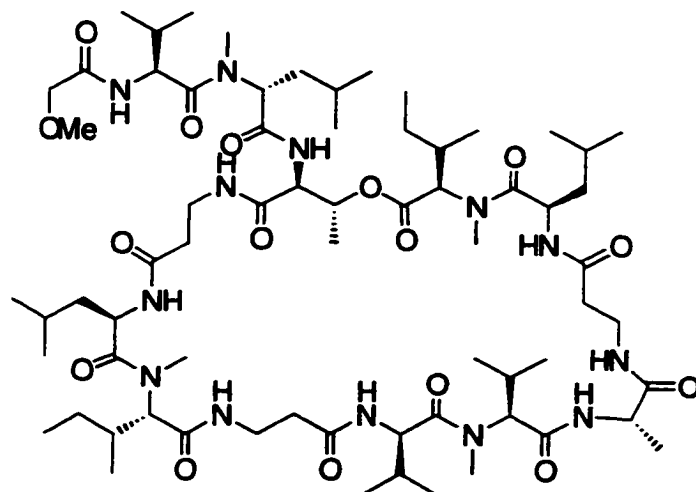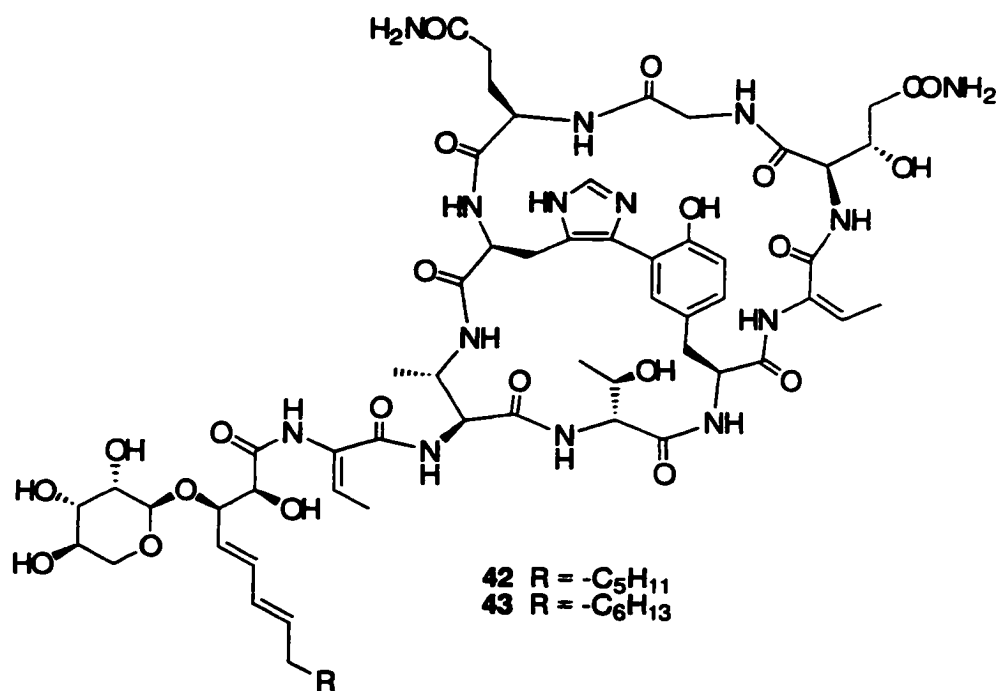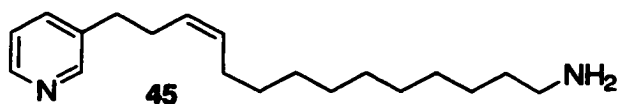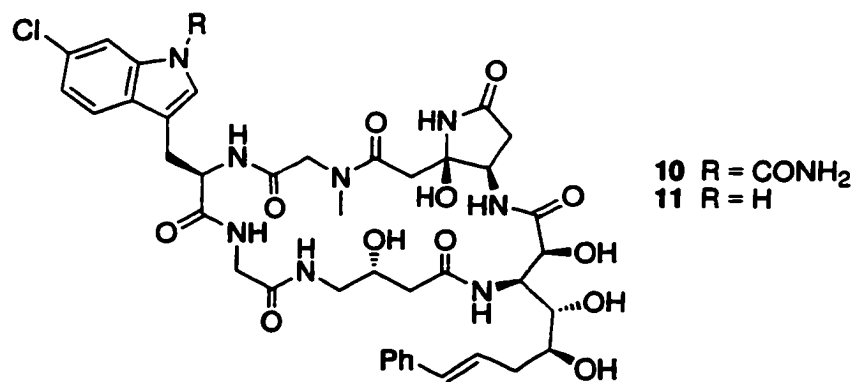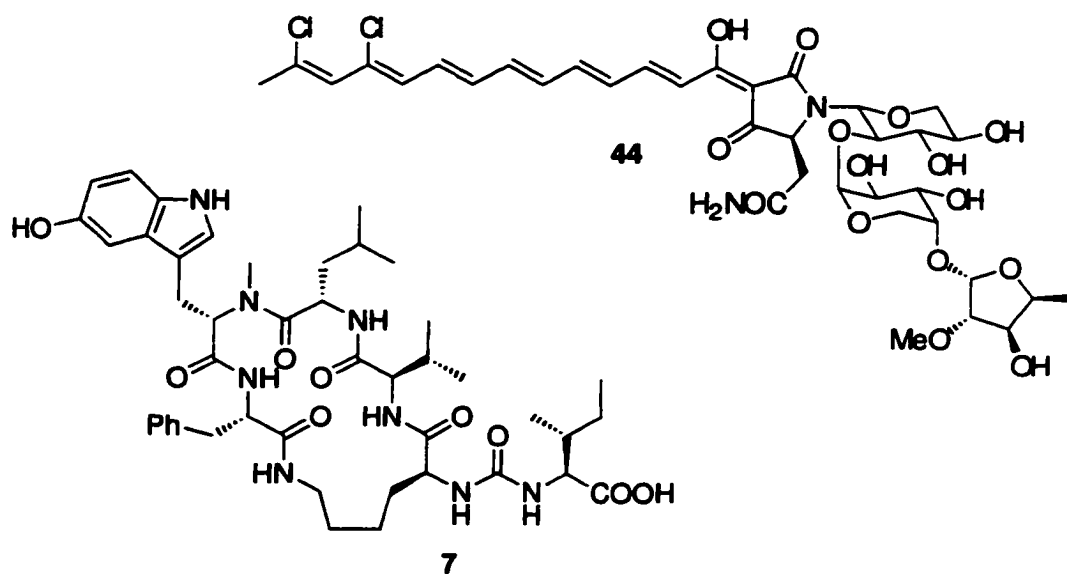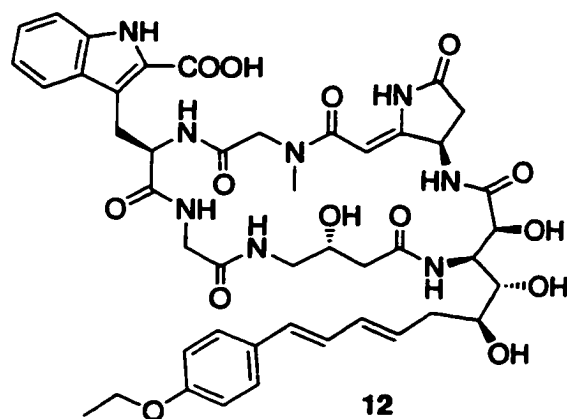
especially the TOCSY spectrum (Figure 4) and comparison of chemical shifts with those of standard amino acids. For instance, in the Ser residue, TOCSY correlations were observed in a single trace connecting the NH proton at δ 8.26 with protons at 4.52, 3.96, and 4.41. By comparing this TOCSY trace with the GCOSY spectrum, in which the NH proton was only correlated to its neighboring proton at δ 4.52, the order of protons in the amino acid could be determined. With the GHMQC (Figure 3) spectrum, it was evident that the latter two protons were covalently bound to a single carbon at δ 62.5 characteristic of an oxygen-bound methylene, while only a single proton was bound to a carbon at 49.4, which is within the characteristic range of amino acid α-carbons. These data are consistent only with a Ser-type amino acid. A GHMBC spectrum gave the chemical shift of the nearest carbonyl (δ 167.2), and other correlations within the amino acid served to reinforce the TOCSY data. The molecular formula and a sharp IR band at 1240 cm^{-1} indicated the presence of a sulfate, and the proton and carbon signals at the Ser β-position were unusually downfield, so the sulfate was placed on the serine residue.

In a similar manner, Ile, Pro, and Tyr were identified. A major difference in the determination of Ile is that TOCSY correlations often either do not go through the β-proton, or the β-proton is not visible in the TOCSY trace. Therefore, the GCOSY is very important in the determination of this residue. Chemical shift differences, GHMBC (Figure 5), and NOESY (Figure 6) experiments (Figure 7) revealed that Ile and Tyr were not normal amino acids, in that they were modified at their carboxyl ends. The presence of keto-Ile was determined by a weak GHMBC correlation between the α-proton and a carbon at 198.2 ppm. The unusually downfield α-carbon of keto-Ile at δ 58.7 and comparison with literature chemical shift values were also used to establish the ketoamide. The modified Tyr was clearly attached to an unsaturated moiety. A broad
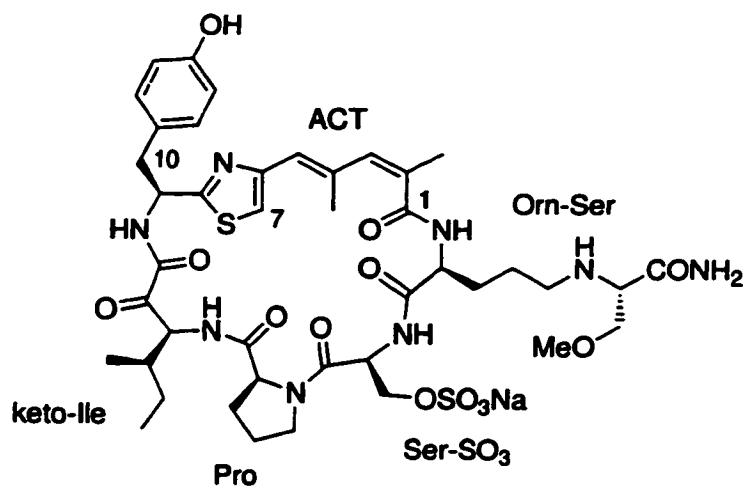
13
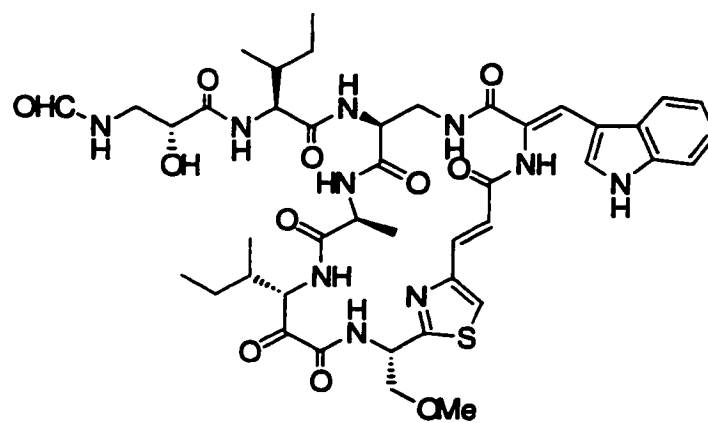
40, keramamide F
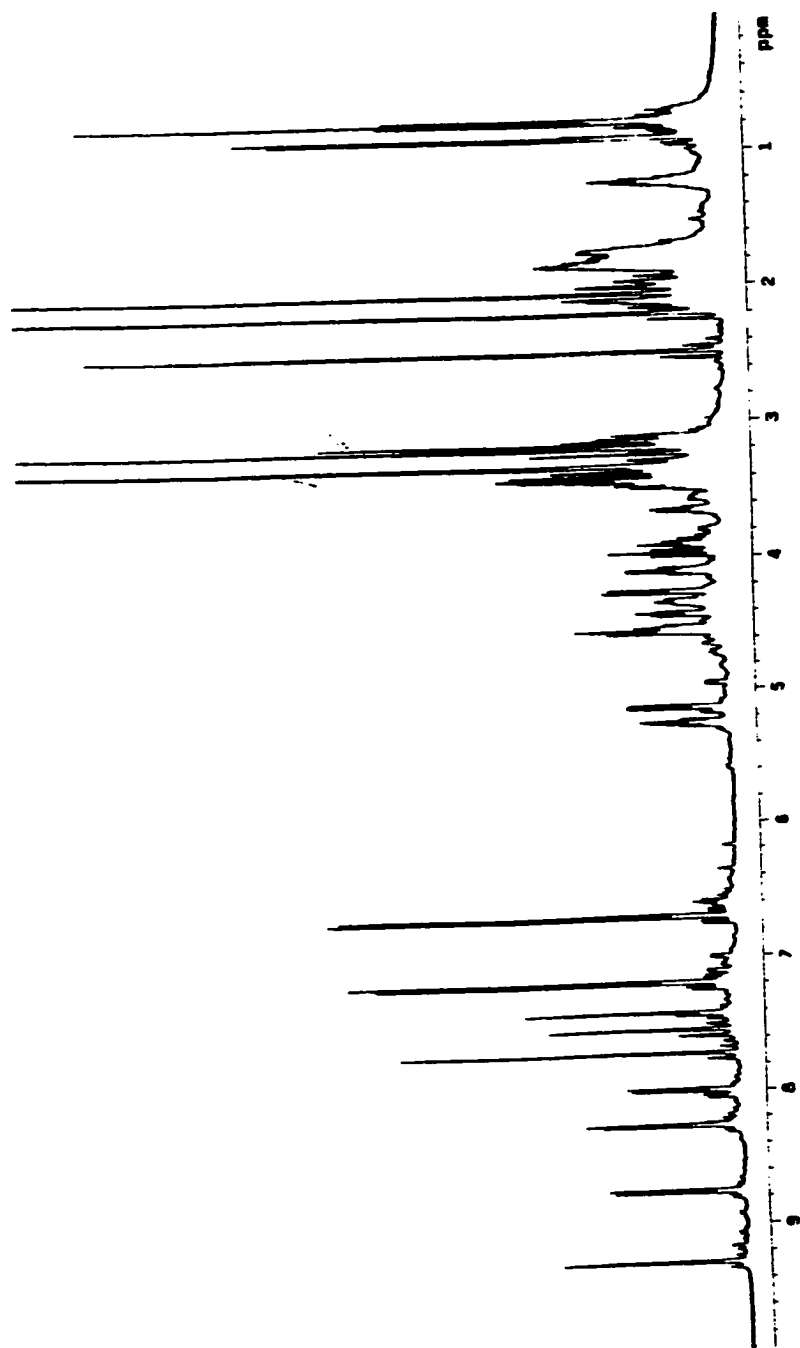
Figure 1. 500 MHz ^{1}H NMR spectrum of scleritodermin A (13) in DMSO-d_6.
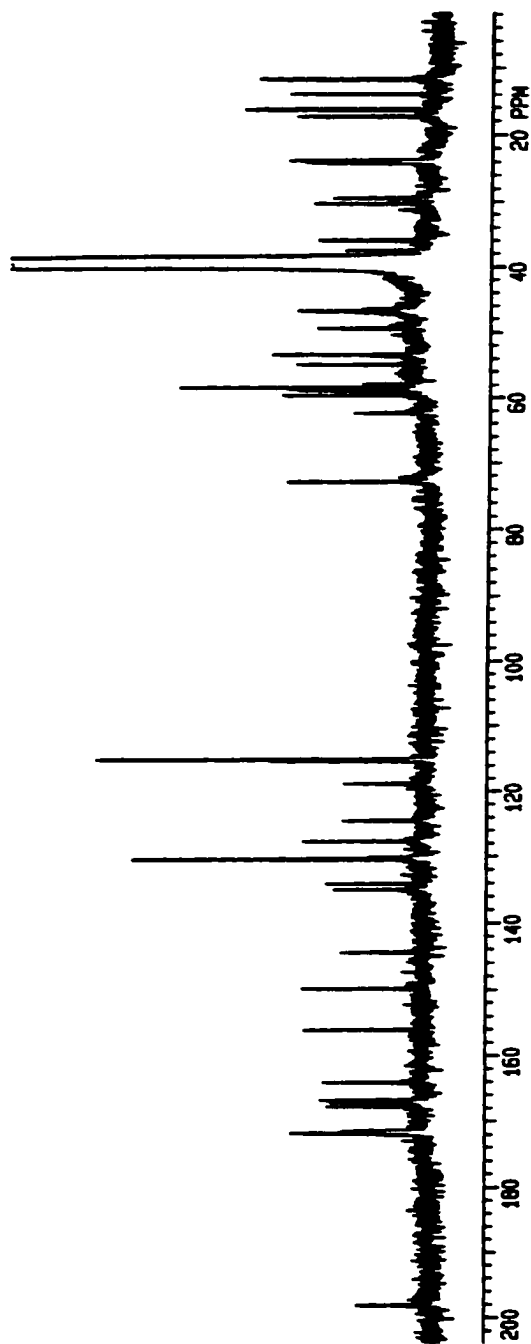
Figure 2. 100 MHz ^{13}C NMR spectrum of scleritodermin A (13) in DMSO-d_6.
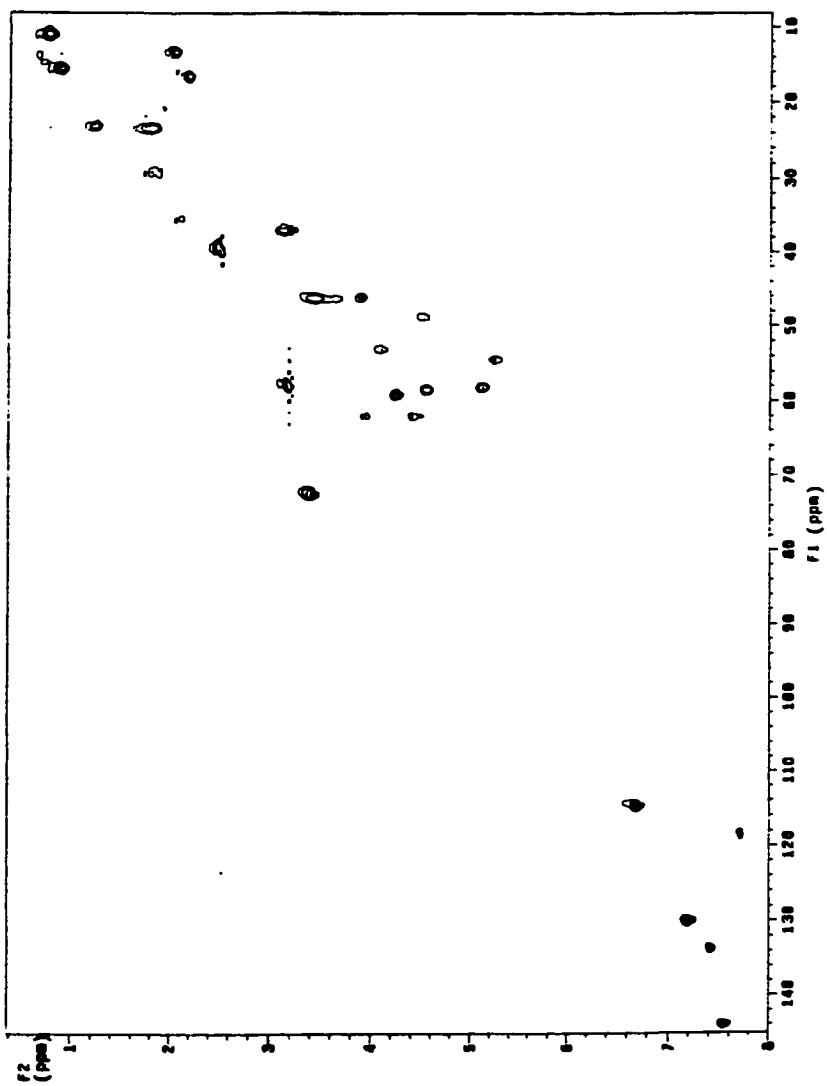
Figure 3. 300 MHz GHMQC spectrum of scleritodermin A (13) in DMSO-d_6.

Table 2. ^1H and ^{13}C NMR assignments for scleritodermin A (13) in DMSO-d_6.

amino acid		δ ^{13}C	^1H	C mult	m, J (Hz)
ACT					
	1	167.9		C	
	2	124.6		C	
	3	144.5	7.54	CH	s
	4	135.1		C	
	5	134.2	7.41	CH	s
	6	149.9		C	
	7	119.0	7.71	CH	s
	8	167.2		C	
	9	55.0	5.25	CH	ddd, 9.5, 9, 6
	10	37.7	3.14	CH_2	m
	11	127.8		C	
	12,16	130.5	7.20	CH	d, 8.5
	13,15	115.4	6.70	CH	d, 8.5
	14	156.2		C	
	17	17.3	2.22	CH_3	s
	18	14.0	2.05	CH_3	s
	NH		8.76		d, 9.5
	OH		9.28		s
keto-Ile					
	1	164.2		C	
	2	198.2		C	
	3	58.7	5.13	CH	dd, 8, 4.5
	4	36.0	2.08	CH	m
	5	23.8	1.26	CH_2	m
	6	16.3	0.91	CH_3	t, 7.5
	7	11.8	0.79	CH_3	d, 6.5
	NH		7.99		d, 8

Table 2. (continued.)

amino acid		δ ^{13}C	δ ^1H	C mult	m, J (Hz)
Pro					
	1	171.7		C	
	2	59.1	4.55	CH	dd, 9, 3
	3	30.4	1.8,2.0	CH_2	m
	4	24.2	1.8	CH_2	m
	5	47.1	3.46	CH_2	m
			3.67		q, 8.5
Ser-OSO₃					
	1	167.2			
	2	49.4	4.52	CH	m
	3	62.5	3.96	CH_2	t, 11
			4.41		dd, 11, 4.5
	NH		8.26		d, 6.5
Orn					
	1	171.8		C	
	2	59.8	4.26	CH	dd, 9, 3
	3	29.6	1.84	CH_2	m
	4	24.0	1.8	CH_2	m
	5	46.9	3.45,3.88	CH_2	m
	NH-γ		4.30		brt, ~9
Ser-OMe					
	1	171.9			
	2	53.5	4.10	CH	dt, 7.5, 5
	3	73.0	3.40	CH_2	m
	4	58.6	3.20	CH_3	s

UV maximum at λ = 305 nm indicated a conjugated system, and unusual NMR chemical shifts and GHMBC correlations led to the elucidation of the conjugated thiazole moiety, 2-(1-amino-2-*p*-hydroxyphenylethane)-4-(4-carboxy-2,4-dimethyl-1*E*,3*Z*-propadiene)-thiazole (ACT). Conjugated thiazoles have precedents in marine cyclic peptides, notably in the keramamides (e.g. **40**) and konbamides,[7] and comparison of chemical shifts in the thiazole moieties aided in structure elucidation. For instance, the proton singlet at δ 7.71 on a carbon at 119.0 ppm is characteristic of a 2,4-disubstituted thiazole, and GHMBC data confirmed the proposal. Despite unusual chemical shifts in the unsaturated ACT side chain, the proposed structure must be correct because all possible GHMBC correlations were observed. Correlations from the methyl singlets were particularly helpful, since they served to constrain the structure. The configuration of the double bonds was evident from the NOESY spectrum. The strong NOESY correlations between CH_3-17 and CH-3, CH-3 and CH-5, and CH_3-18 and CH-7 are only possible if the ACT adopts the indicated configuration.

The final amino acid, 5-*N*-(1-carboxy-2-methoxyethane)-ornithine, referred to hereafter as Orn-Ser, was elucidated with GCOSY, TOCSY, and GHMBC data and confirmed by chemical synthesis. The ornithine α-NH signal was not seen in any NMR spectrum, either in a series of solvents, including DMF, CD_3OH, and DMSO, or at various temperatures. It is likely that this proton exchanges on the NMR timescale. However, good TOCSY correlations were observed along the length of the Orn and Ser sides of the residue. TOCSY correlations were not observed through the γ-NH group, but weak COSY correlations were seen from both sides to the same NH at 4.30 ppm. This upfield chemical shift indicated that the NH was not an amide, confirming the COSY correlations from δ-CH_2 of Orn and α-CH of Ser. In addition, NOESY and GHMBC signals across the γ-NH bond were observed. The methyl ether on Ser was
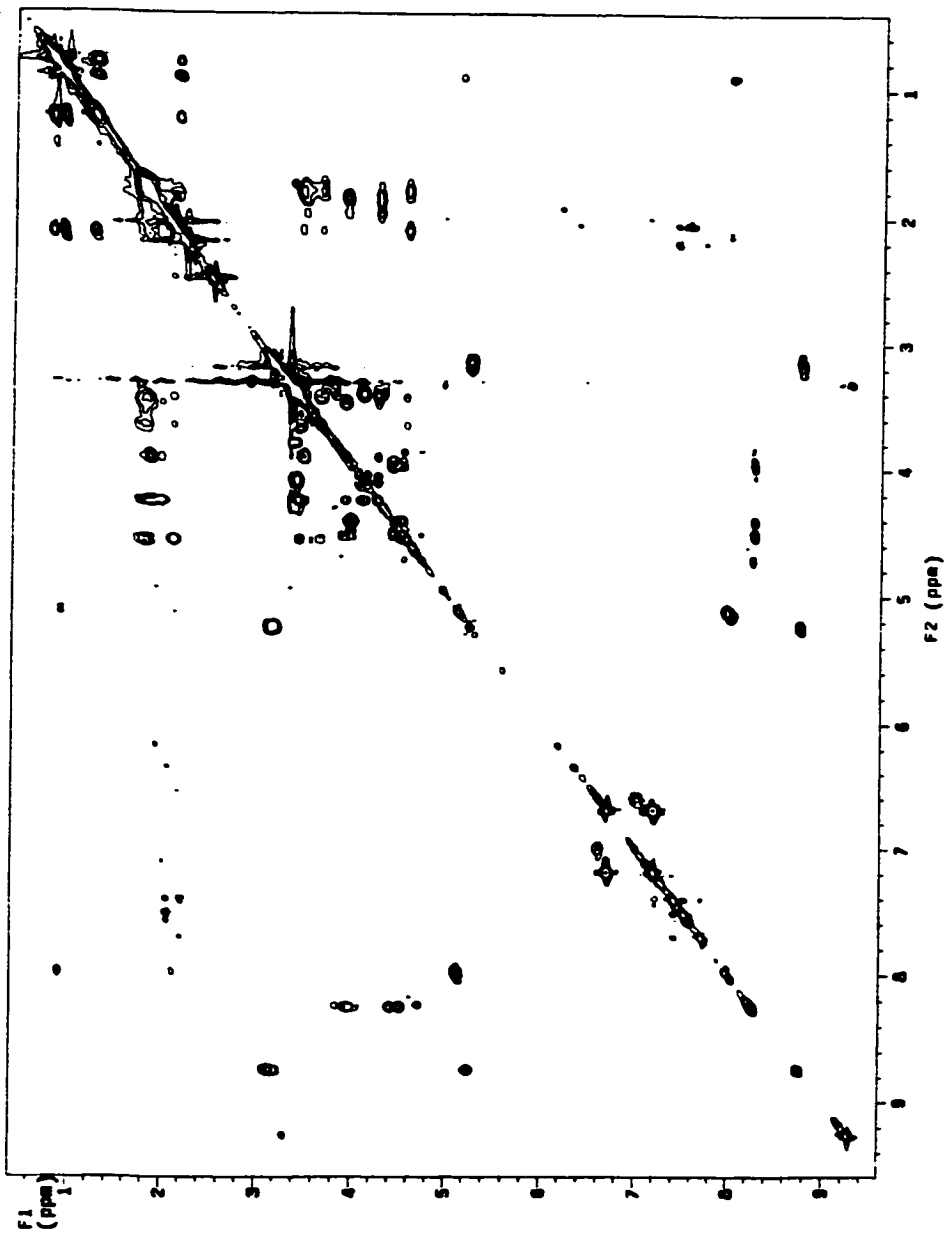
Figure 4. 300 MHz TOCSY spectrum of scleritodermin A (13) in DMSO-d_6 with an 80 msec mixing time.
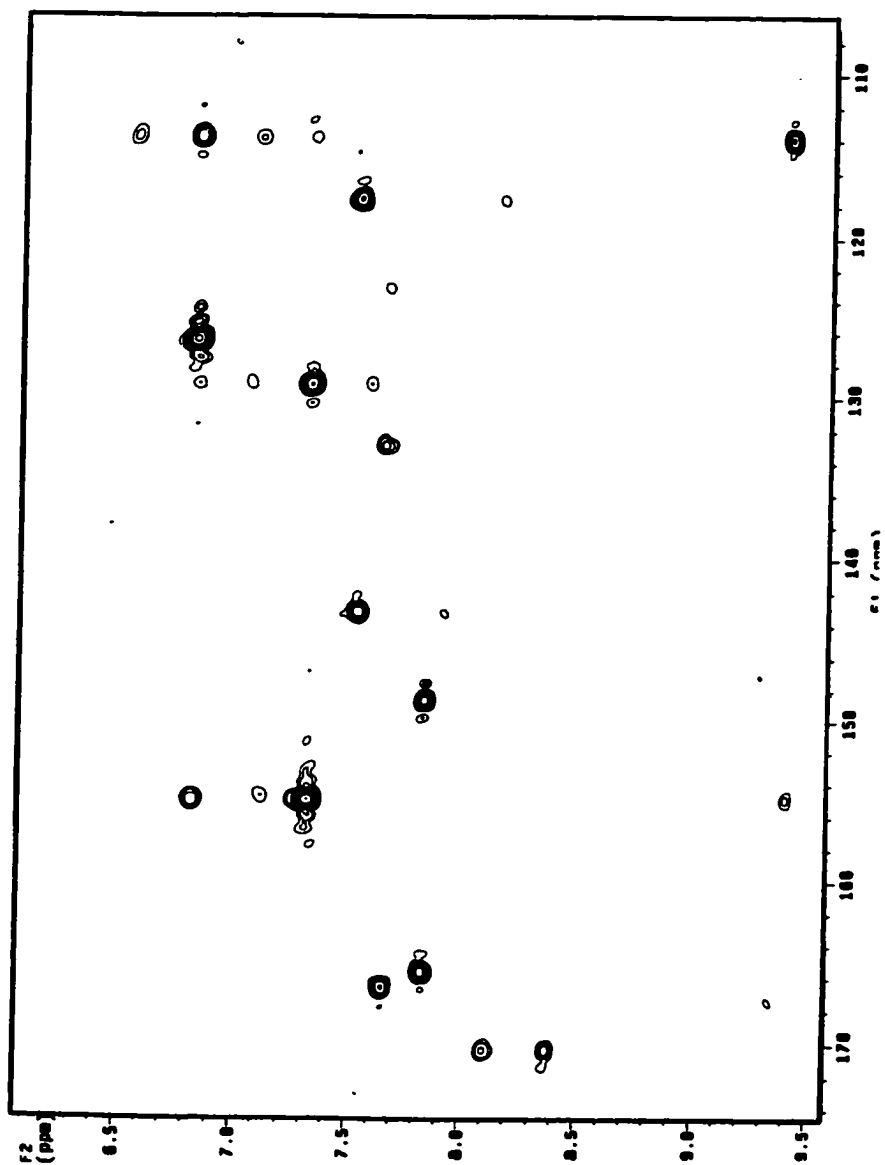
Figure 5. 300 MHz GHMBC spectrum of aromatic/ unsaturated region of scleritodermin A (13) in DMSO-d_6.
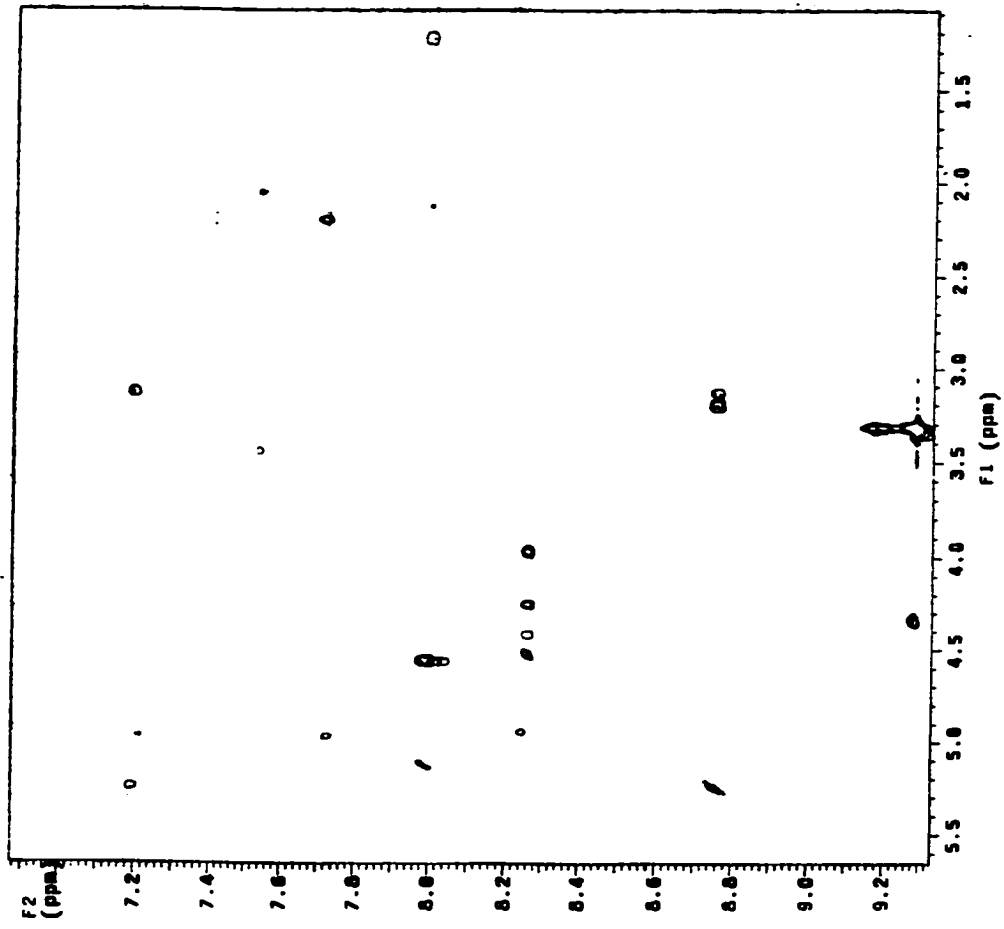
Figure 6. Portion of 300 MHz NOESY spectrum of scleritodermin A (13) in DMSO-d_6.
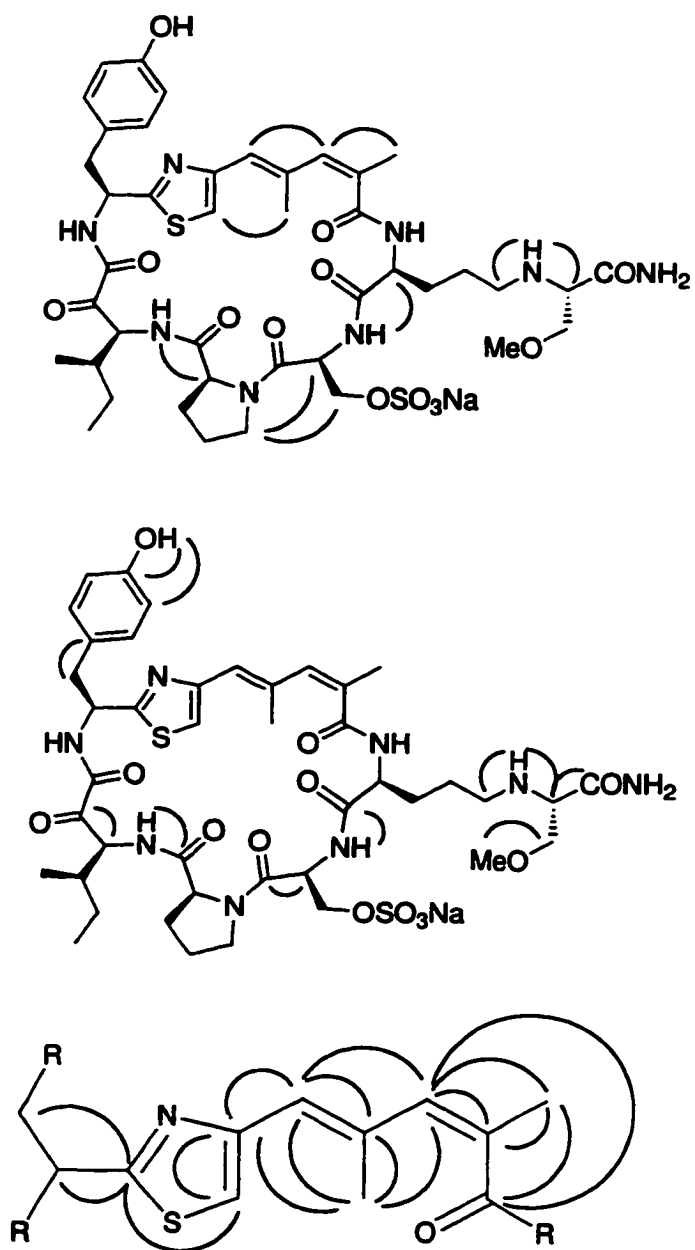
Figure 7. Key NOESY (top) and GHMBC (bottom, middle) correlations for scleritodermin A (13).

unambiguously established by a strong GHMBC correlation across the oxygen and reinforced by the extremely downfield shift of the Ser β-carbon at 73.0 ppm.

The amino acid sequence of scleritodermin A (13) was achieved mainly through GHMBC and NOESY correlations (Figure 7). The proposed sequence was confirmed by a series of MS experiments (Figure 9). The negative ion ESI-MS[n] (Figure 8) spectrum was particularly valuable in establishing the connectivity unambiguously, since a key HMBC correlation was missing from the NMR spectra. No HMBC correlations were seen to convincingly establish the ketoamide portion of the molecule, so this piece was deduced by comparison of chemical shift data with those of the keramamides[7] and confirmed by MS fragmentation patterns.

Because the ornithine α-NH proton could not be observed by NMR, there were still two possible structures: either a linear peptide with the serine-ornithine cyclized in an 8-membered ring, or a cyclic peptide with an amidated serine methyl ether terminus. FABMS gave only two major fragment ions: loss of sulfate, and loss of sulfate plus portions of the serine-ornithine amino acid. These ions supported the proposed structure for scleritodermin A (13) because they are consistent with a linear, and not a cyclized, Orn-Ser amino acid. Negative ion ESI-MS[n] provided a series of fragment ions which could only be explained by the proposed structure of scleritodermin A (13). In particular, the ion at $m/z = 378$ could only be generated from the structure as drawn.

The absolute configurations of the Ile, Ser, Tyr, and Pro residues were determined by chemical degradation, followed by chiral GC-MS (Figure 10). First, the peptide was ozonized, then hydrolyzed and derivatized. Comparison with authentic standards of DL-Asp, -Pro, and -Ser showed that the Tyr, Pro, and Ser were all in the L configuration. The stereochemistry of Ile was determined by reacting scleritodermin A
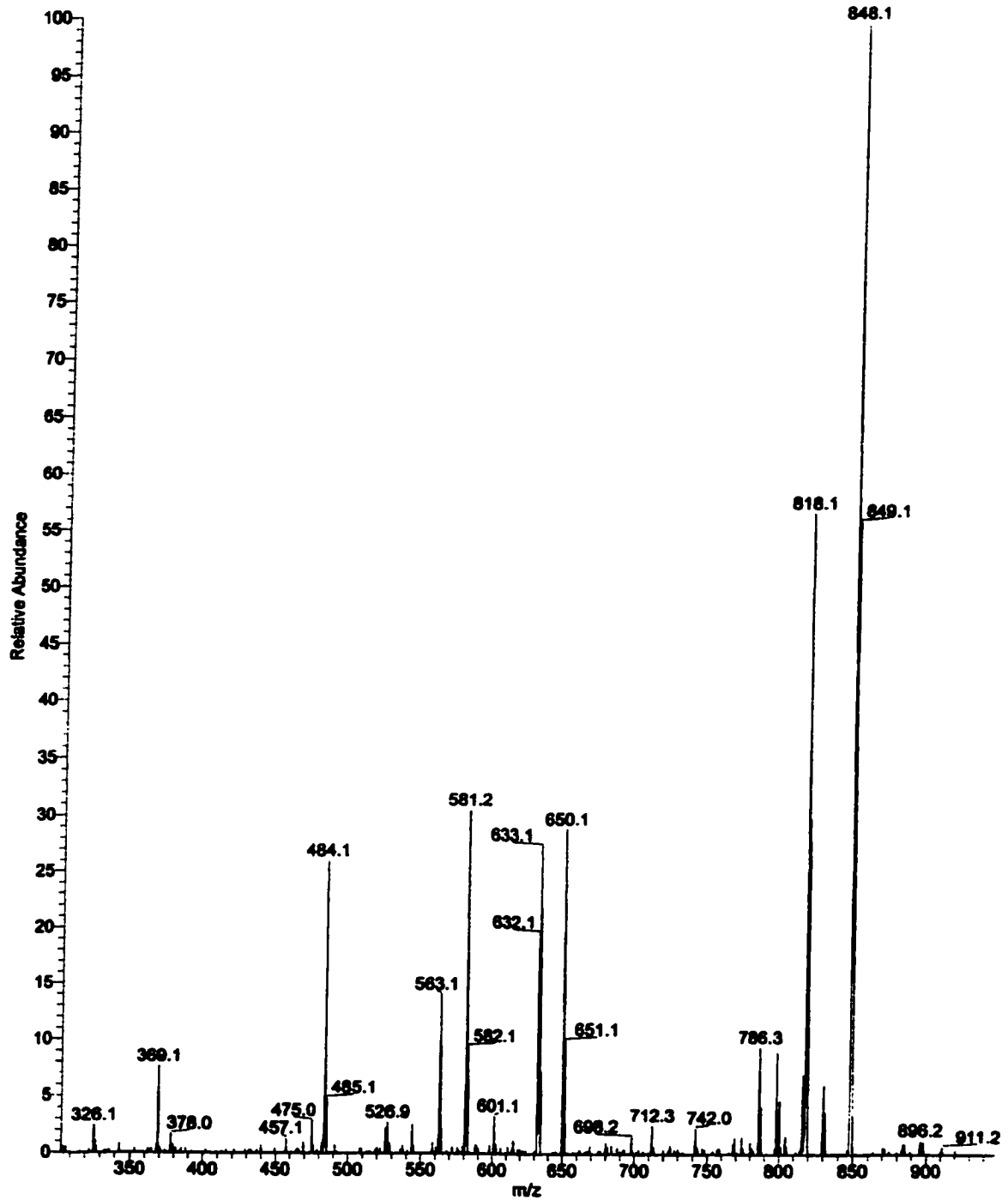
Figure 8. Negative ESI-MS spectrum of scleritodermin A (13).
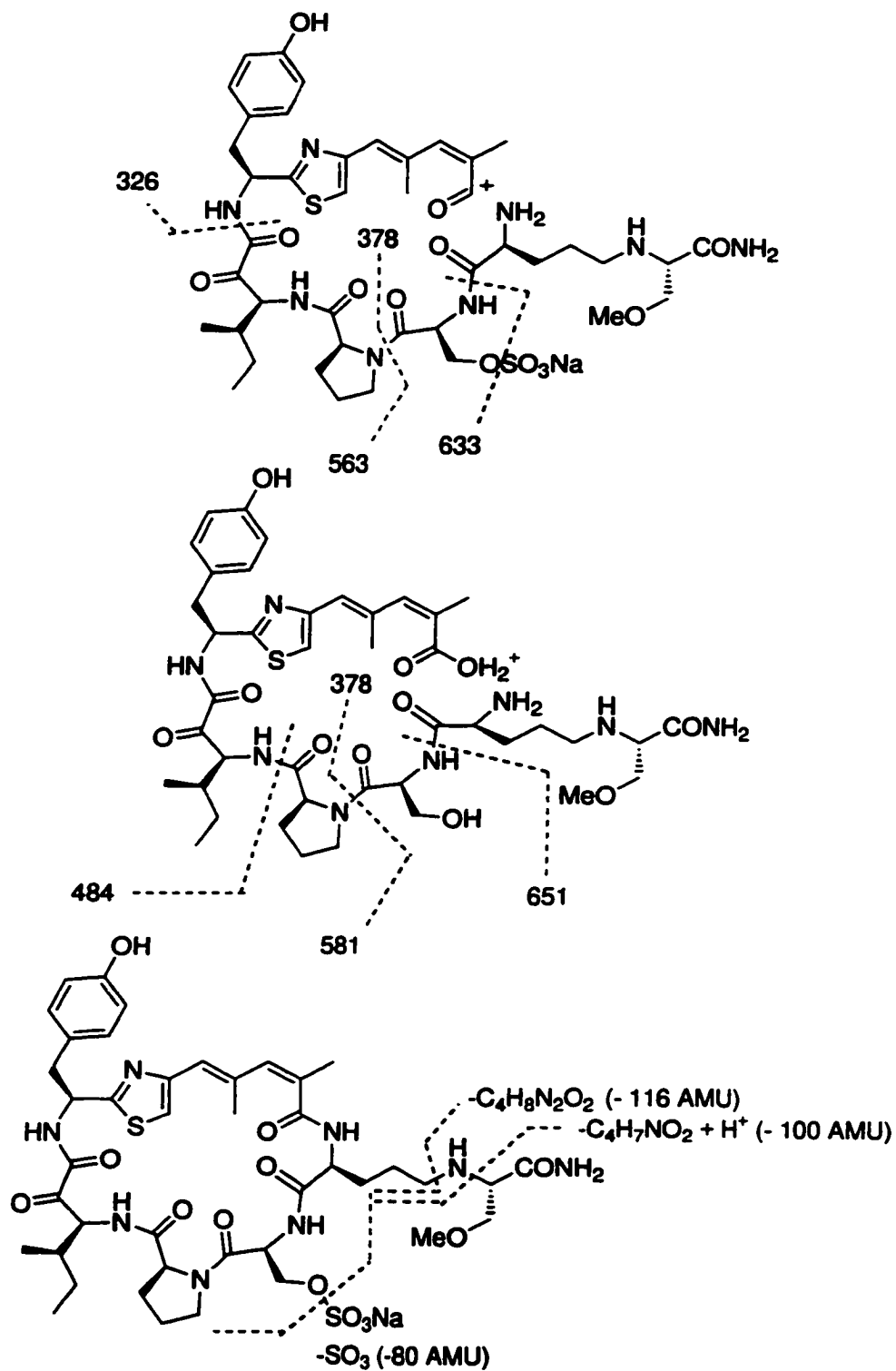
Figure 9. ESI-MS (top, middle) and FAB-MS fragmentation patterns.
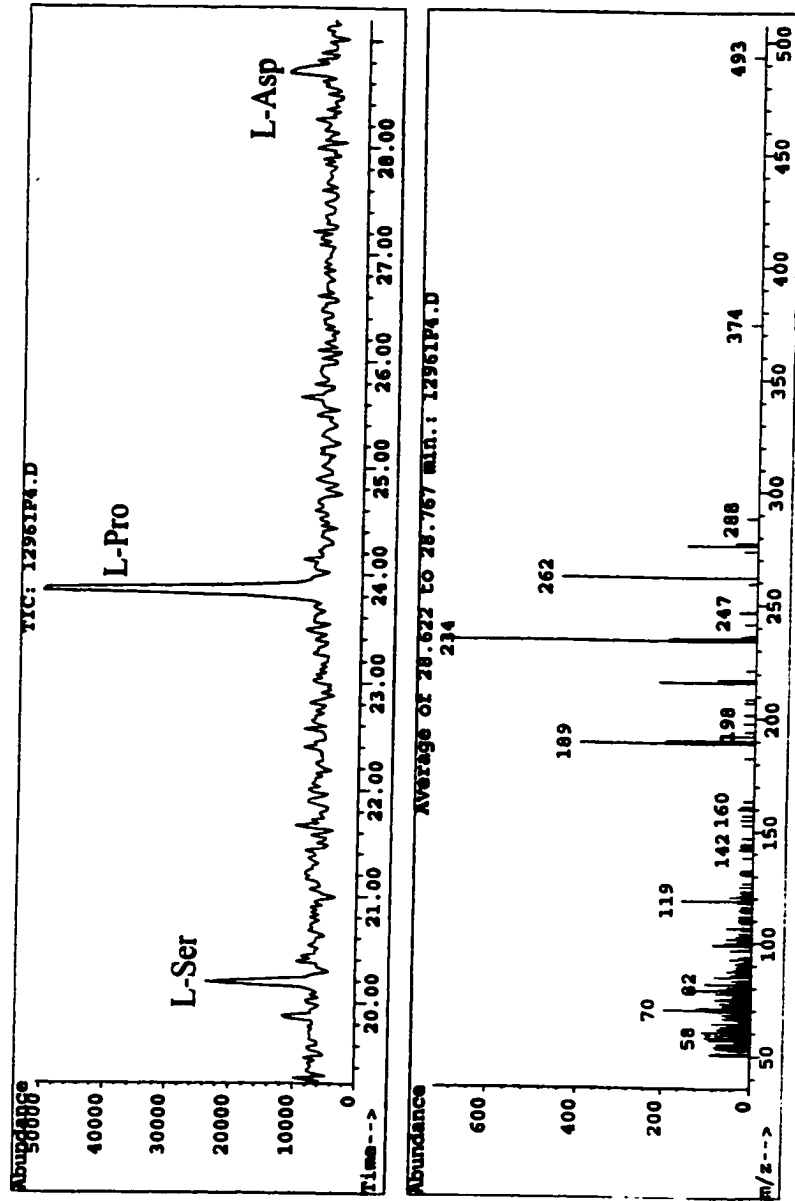
Figure 10. GC-MS spectrum of the hydrolysate from the ozonolysis product of scleritodermin A (13).

(13) with H_2O_2/NaOH[7] followed by hydrolysis and derivatization. Chiral GC-MS showed one new amino acid, corresponding to L-*allo*-Ile.

The determination of the Orn-Ser stereochemistry was complicated by the lack of a suitable standard or degradation reaction to produce known compounds. In order to unambiguously confirm the existence of the novel amino acid, and to elucidate its absolute configuration, two analogs were synthesized following the method of Bavetsias *et al.*[8] First, N-*t*-BOC-L-glutamic α-*t*-butoxy ester was converted to a mixed anhydride and reduced to the alcohol. Mesylation, followed by nucleophilic substitution with L-serine methyl ether afforded **45**, which was purified by column chromatography (Scheme 1). The same procedure was used beginning with N-*t*-BOC-D-glutamic α-benzyl ester to give a similar product. Both the LL- and DL-Orn-Ser derivatives were deprotected by acid hydrolysis. GC-MS was inappropriate for detection of the Orn-Ser residue, since the compounds were not volatile enough to run through the column at the maximum column temperature (210 °C). To analyze the Orn-Ser stereochemistry, an alternative HPLC method was chosen in which the amino acids were detected at micromolar concentrations by formation of a fluorescent derivative.[9] Briefly, aliquots of standards and hydrolyzed peptides were mixed with a solution containing *o*-phthalaldehyde (OPA) and 3-mercaptopropionic acid. The total reaction time was one minute, at which point acetate buffer was used to dilute the reagents and the sample was loaded directly onto a C_{18} analytical HPLC column. An amino acid from the peptide hydrolysate co-eluted with the LL-Orn-Ser standard, but not with the DL-standard, indicating that the compound was either LL or DD. Repetition of the same procedure, but replacing 3-mercaptopropionic acid with the chiral derivative, N-Ac-L-cysteine, showed that the compound was probably LL, since it still co-eluted with the LL standard.

Scheme 1

achiral, R = CH₂CH₂COOH
chiral, R = S-CH₂CH(COOH)NH-Ac
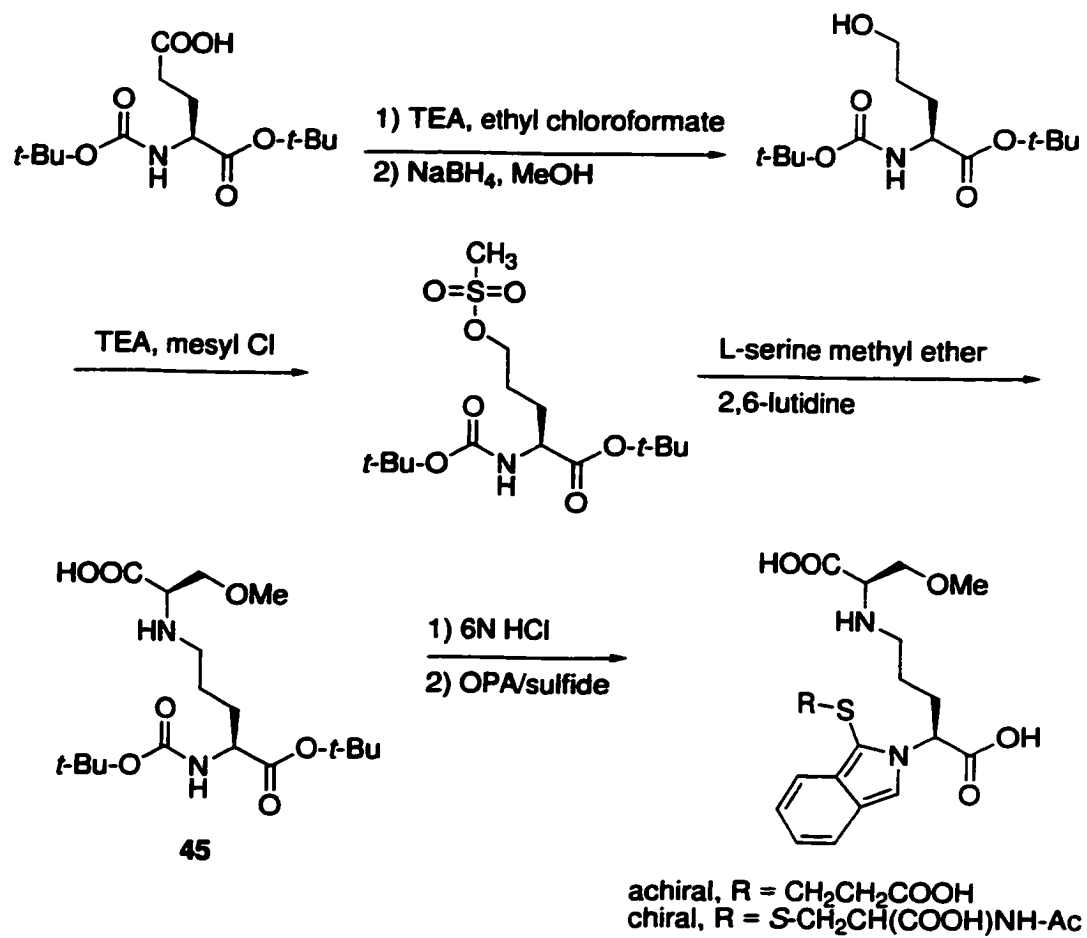
Scheme 2
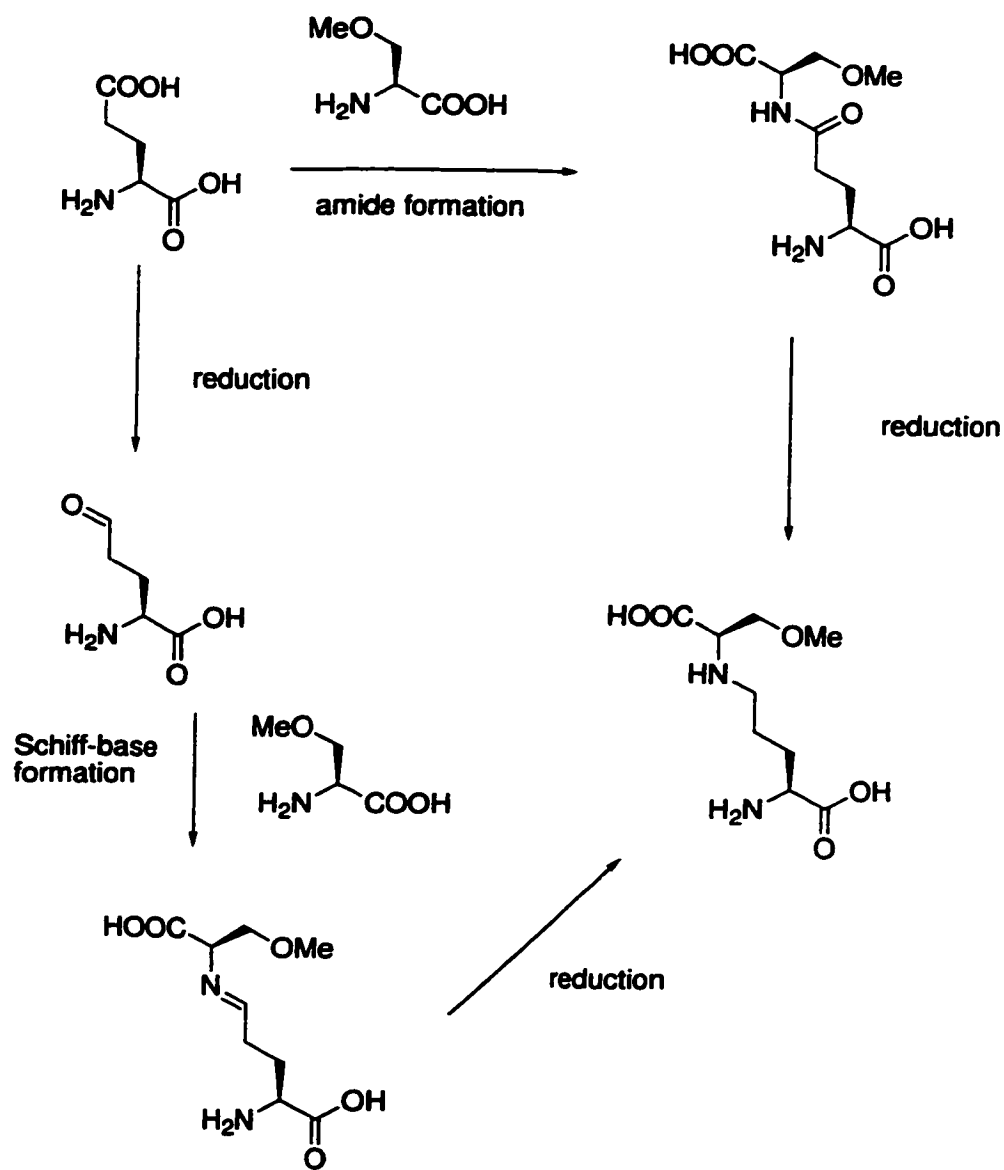
Molecular modeling (PCModel™) of the conjugated thiazole moiety shows that it should be very sterically hindered, and this is reflected in unusual chemical shifts and extremely strong NOE correlations between neighboring groups. The Orn-Ser portion is unusual, since it is the first report of this type of link in the natural products literature, at least as far back as computer databases reach. The bond between the amino acids arises from a reduced glutamate coupled to serine, but it could be formed in two different ways. The δ-carboxyl group of glutamate could be reduced to the aldehyde, followed by Schiff base formation and reduction. Alternatively, the peptide bond could be formed before reduction of the carbonyl group (Scheme 2). The former process has an analog in the biosynthetic pathway leading to ornithine, in which glutamic acid is reduced to glutamic-5-semialdehyde, then an amino transferase transfers an ammonium ion to the aldehyde.[13]

Bioactivity

Scleritodermin A (13) has been determined to be the constituent responsible for the anti-tumor activity of the crude extract. In the Bristol-Myers Squibb cancer cell panel, the compound had an IC_{50} of 1.5 μM, with an IC_{50} maximum/minimum ratio of 10. Mechanistic studies at BMS showed that the compound inhibits tubulin polymerization with about one-tenth the potency of the known chemotherapeutic vinblastine. Insufficient material was available for *in vivo* testing; the compound was neither active nor toxic at 6 mg/kg mouse per day over 5 days. If additional material becomes available, *in vivo* testing should be carried out at higher concentrations until the maximum tolerated dose is reached.

Conclusions

The NCI Active Repository proved to be a valuable tool for drug discovery and chemotaxonomy. Ten extracts were rapidly dereplicated over a two-week period, and

the most promising samples were pursued. From this study, some insight into chemotaxonomy was obtained (see Chapter 11), and a novel and potent anti-tumor metabolite was isolated from the collection. As it becomes increasingly difficult to find new natural products in the marine environment, the repository will grow in importance because samples can be picked based on taxonomy and bioactivity. This will greatly simplify the pursuit of novel compounds.

Scleritodermin A (13) possesses a number of interesting functional groups, two of which are unprecendented in marine natural products. Portions of the molecule are reminiscent of other structures, particularly of the keramamides and konbamides, with which it shares a conjugated thiazole-amino acid-diketoisoleucine group. However, the conjugated portion of the ACT residue is without precedent, as is the ornithine-serine side chain.

The Orn-Ser residue is particularly resistant to proteolytic digestion. Terminal amide groups are well known to prevent proteolysis.[10] In addition, medicinal chemists use the reduced peptide bond equivalent to fashion peptides that are based on natural bioactive substances which are otherwise too labile for pharmaceutical use.[8] It would be interesting to determine whether or not the molecule would retain its bioactivity with a glutamate-serine in place of ornithine-serine. Retention of activity could indicate that the amide bond in scleritodermin A (13) has been reduced to prevent proteolysis.

Experimental

General Experimental Procedures. ^{1}H, DQCOSY, GHMQC, GHMBC, and NOESY NMR spectra were recorded on a Varian Inova 300 MHz spectrometer. ^{13}C and DEPT NMR spectra were recorded on a Varian Gemini 400 MHz spectrometer. All NMR data for **13** are reported in DMSO-d_6. NMR data for **45** and **46** were recorded in

CDCl$_3$. TOCSY spectra were obtained with 80 msec mixing times. ESI-MS was performed on a Finnigan LCQ system. High-resolution FABMS data were obtained from the Mass Spectrometry Facilities at the University of Minnesota and the University of California at Riverside. Optical rotations were measured on an Autopol III polarimeter. UV and IR spectra were taken on Perkin Elmer instruments. Absolute configurations were determined using an Alltech Chirasil-Val column with a Hewlett Packard 5890 GCMS system. The temperature was ramped from 50 °C to 210 °C over 45 minutes in all GC-MS experiments, and derivatized amino acids were used as internal standards. Fluoresence detection HPLC was performed using a Shimadzu RF-530 Fluorescence HPLC monitor.

Dereplication of Compounds from the NCI Collection. Three aqueous extracts were observed by ^{1}H NMR in CD$_3$OD and in D$_2$O and determined to be too dilute for chemical study. Ten organic extracts were partitioned between methanol and hexane, and each fraction was tested for lethality against brine shrimp and observed by ^{1}H NMR. In most cases, the activity could be correlated with interesting NMR spectra, but not in the case of a single species of *S. nodosum* that contained scleritodermin A (**13**). This extract was not lethal to brine shrimp at 50 μg/mL despite its anti-tumor activity in the 60 cell-line panel. Three of the extracts (*Plakinolopha mirabilis*, *P.* sp. 3, *Theonella invaginata*) were observed to contain ^{1}H NMR peaks similar to those observed in the mozamides/ aurantosides mixture from other sponges studied. While this does not preclude the existence of other bioactive compounds in the extracts, they are not major components. The remaining organic extracts were chromatographed, and samples were observed by ^{1}H NMR. Interesting fractions were used for negative ion ESI-MS[n] spectroscopy and dereplicated using computer databases. The following samples were fractionated by C$_{18}$ Sep Pak using CH$_3$CN/H$_2$O gradients: *Scleritoderma*

nodosum I, *Aciculites ciliata*, *T.* sp. 3, *T. cylindrica*, *T.* sp. 6, and *Microscleroderma hardmani*. *S. nodosum* II was fractionated by C_{18} and LH-20. Results of dereplication are given in Figure 1.

Isolation of Scleritodermin A (13). A sample of *S. nodosum* was collected by the Coral Reef Research Foundation at 50 m depth at Olango Island, Visayas, Philippines (10° 16'25" N, 124° 2'19" E) in 1994 and sent to the NCI. 1.46 g of organic extract was partitioned between hexane (3 x 100 mL) and methanol (100 mL) to give a MeOH extract (950 mg) and a hexane extract (600 mg). The methanol fraction was chromatographed on a C_{18} Sep Pak using a gradient from 10-100% CH_3CN in 10% steps. Fractions eluting in 30-40% CH_3CN were pooled and further purified by C_{18} HPLC using 28% CH_3CN (aq, λ = 220 nm detection) to give 6 mg scleritodermin A (**13**). Fractions eluting between 40-50% CH_3CN were combined and run on an LH-20 column (MeOH). High-molecular-weight fractions were pooled and purified on a C_{18} prep plate with 80% CH_3CN (aq) to give additional scleritodermin (**13**, 6 mg). In an improved preparation, the MeOH extract was dried and partitioned between 15% MeOH (aq) and dichloromethane, leading to a much cleaner sample that did not require LH-20 chromatography

Scleritodermin A (13): Off-yellow powder; $[\alpha]_D$ = -41.0° (c = 0.1, MeOH); UV (MeOH) 200 (ε 26 500), 305 (ε 11 300); IR (thin film) 3470, 3290, 2660, 1630-1700, 1520, 1450, 1240, 1210, 1155, 1125, 1040, 830, 750, 620, 555 cm^{-1}; ^1H NMR (DMSO-d_6) see Table 2; ^{13}C NMR (DMSO-d_6) see Table 2; HRFABMS, m/z = 952.3436 (M+H)$^+$, $C_{42}H_{57}N_8O_{12}NaS_2$ requires m/z = 952.3435.

Determination of Tyr, Pro, Ile, and Ser-OSO$_3$ configurations. The peptide (250 µg) was dissolved in 5% (wt.) NaOH (2 mL), and 50% H_2O_2 (200 µL) was added dropwise to the reaction at 60 °C following the method of Kobayashi *et al.*[6] The

reaction was stirred for 30 minutes, then 5% AcOH (2 mL) was added and the suspension was lyophilized, redissolved in MeOH, filtered through Celite, and dried. The resulting compound was dissolved in MeOH (4 mL), and a stream of ozone from 100% O_2 was bubbled through the solution at -78 °C for 20 minutes. The reaction was quenched with 50% H_2O_2 (8 drops), brought to room temperature, and left for 30 minutes. The solution was dried by rotary evaporation, and the compound was redissolved in 6N HCl (500 μL) and heated to 100 °C for 16 hours. The hydrolysate was dried under N_2, dissolved in isopropanol (400 μL), and acetyl chloride (100 μL) was added. After 1 hour at 100 °C, the reaction vial was dried under N_2, and DCM (400 μL) and pentafluoropropionic anhydride (400 μL) was added. After 15 minutes at 100 °C, the reagents were removed under N_2, and the sample was subjected to GC-MS analysis with standards that had been derivatized in an identical manner to give L-Asp (= L-Tyr), L-Ser, L-*allo*-Ile, and L-Pro.

Synthesis of mesylates. *S*-2-(*t*-BOC-amino)-5-O-mesyl valeric *t*-butyl ester was prepared by the method of Bavetsias *et al.* Briefly, α-*t*-butyl-*N*-(*t*-BOC)-L-glutamate (300 mg) in freshly distilled THF (3 mL) at -10 °C was mixed with triethylamine (200 μL) followed by ethyl chloroformate (105 μL). The reaction was stirred for 10 minutes, at which time it was flushed with N_2, and of $NaBH_4$ (105 mg) was added, followed by dropwise addition of dry MeOH (300 μL) over 15 minutes. The reaction was brought up to 0 °C and left stirring for 30 minutes. Remaining borohydride was quenched with several drops of 2N HCl, and the product was evaporated to give an aqueous suspension that was partitioned against ethyl acetate. Purification of the organic extract on silica using 40-60% EtOAc (hexane) gave *S*-2-*N*-(*t*-BOC)-5-hydroxy valeric *t*-butyl ester (192 mg, 67% yield). The purified alcohol was dissolved in dry DCM (3 mL), TEA (180 μL), and mesyl chloride (70 μL) and stirred for 25 minutes at -10 °C to give

the crude mesylate, which was further purified by silica column chromatography (0-50% EtOAc in hexane) to give S-2-N-(t-BOC)-amino-5-mesyloxy valeric t-butyl ester (184 mg, 75%), with NMR data matching those of the known compound. The 2R analog of the mesylate was prepared in the same manner, but starting from α-benzyl-N-(benzyloxycarbonyl)-D-glutamate.

Synthesis of Orn-Ser standards. L-Serine methyl ether (55 mg) and S-2-N-(t-BOC)-amino-5-mesyloxy valeric t-butyl ester (55 mg) were dissolved in dry DMF (1 mL), and 2,6-lutidine (70 μL) was added. The reaction was brought to 65 °C and stirred over molecular sieves for 48 hours, after which it was quenched by partitioning in 1N HCl and EtOAc. The organic extract was dried under vacuum and chromatographed on a C_{18} Sep Pak using a 0-100% MeOH gradient (aq). Fractions between 60-80% MeOH were combined to give the protected L-Orn-L-Ser standard (**45**, 17.2 mg, 30% yield from the mesylate). The D-Orn-L-Ser standard (**46**) was prepared in the same way.

1-N-t-butoxycarbonyl-5-N-(S-1-carboxy-2-methoxyethane)-S-ornithine t-butyl ester (45): ^{13}C NMR (CDCl$_3$): 169.6, 160.7, 155.5, 82.0, 79.8, 72.1, 59.3, 53.1, 51.2, 29.8, 29.4, 28.3 (3C), 28.0 (3C), 24.5. ESI-MS m/z = 441 for $[C_{18}H_{32}N_2O_7 \cdot HCl + OH]^+$.

1-N-t-butoxycarbonyl-5-N-(S-1-carboxy-2-methoxyethane)-R-ornithine benzyl ester (46): ^{13}C NMR (CDCl$_3$): 169.6, 160.7, 155.4, 135.2, 128.6 (2C), 128.5 (2C), 128.3, 80.0, 72.1, 67.2, 59.2, 52.9, 51.2, 30.0, 28.3 (3C), 27.8, 24.4. ESI-MS m/z = 475 for $[C_{21}H_{30}N_2O_7 \cdot HCl + OH]^+$.

Determination of stereochemistry of the Orn-Ser residue. Scleritodermin (100 μg) and the Orn-Ser standards (100 μg) were separately hydrolyzed in 6N HCl (500 μL) at 110 °C for 16 hours. The solvent was then removed under nitrogen, and

the samples were taken up in Fischer HPLC water (1 mL). Derivatization proceeded by the method of Bada.[9] Each sample (10 µL) was mixed in an Eppendorf tube with 0.4M borate buffer (pH = 9.4, 10 µL). OPA/thiol reagent (5 µL) was added, and the mixture was left standing for 1 minute before quenching with acetate buffer (475 µL, pH = 5.5) and immediate injection on an analytical C_{18} HPLC column. The OPA/thiol reagent consisted of the following:o-phthalaldehyde (4 mg) in MeOH (300 µL), borate buffer (250 µL), doubly distilled water (435 µL), and a 1M solution of either N-acetyl-L-cysteine (chiral, 15 µL) or 3-mercaptopropionic acid (achiral, 15 µL). The OPA/thiol reagent was stored at 4 ºC for no more than 2 weeks. HPLC was performed using a fluorescence detector (emission: 450 nm; excitation: 340 nm). The HPLC acetate buffer was composed of NaAc (13.6 g) in dd H_2O (2 L), titrated to pH = 5.5 with AcOH. HPLC method for the chiral derivatization was as follows: 1) 100% acetate buffer, 4 minutes; 2) gradient to 37% MeOH over 10 minutes; 3) gradient to 42% MeOH over 10 minutes; 4) gradient to 60% MeOH over 5 minutes. With these conditions, a peak corresponding to D-Orn-L-Ser eluted after 19.8 mins., while both the L-Orn-L-Ser standard and a peak in the peptide hydrolysate eluted after 19.7 mins. HPLC with the achiral thiol was performed as above, except that the time of step 3 was doubled to 20 minutes. The L-Orn-L-Ser standard and the scleritodermin hydrolysate eluted after 20.8 minutes, while the DL standard eluted after 20.9 minutes. Thus, scleritodermin contains L-Orn-L-Ser.

References

1. Information available through the NCI Active Repository web page: http://epnws1.ncicrf.gov:2345/dis3d/natprod/np_repo.html/.

2. Bewley, C.A.; Faulkner, D.J. *Angew. Chem. Int. Ed. Engl.* **1998**, *37*, 2162-2178.

3. Bewley, C.A.; He, H.; Williams, D.H.; Faulkner, D.J. *J. Am. Chem. Soc.* **1996**, *118*, 4314.

4. Bewley, C.A.; Debitus, C.; Faulkner, D.J. *J. Am. Chem. Soc.* **1994**, *116*, 7631.

5. Schmidt, E.W.; Faulkner, D.J. *Tetrahedron* **1998**, 54, 3043-3056.

6. Kobayashi, M.; Lee, N.K.; Shibuya, H.; Momose, T.; Kitagawa, I. *Chem. Pharm. Bull.* **1991**, *39*, 1177-1184.

7. Kobayashi, J.; Itagaki, F.; Shigemori, H.; Takao, T.; Shimonishi, Y. *Tetrahedron*, **1995**, *51*, 2525.

8. Bavetsias, V.; Bisset, G.M.F.; Kimbell, R.; Boyle, F.T.; Jackman, A.L. *Tetrahedron*, **1997**, *53*, 13383-13396.

9. Bada, J.L.; Glavin, D.D.; McDonald, G.D.; Becker, L. *Science*, **1998**, *279*, 362-365.

10. Creighton, T.E. *Proteins: Structures and Molecular Properties*. W.H. Freeman and Company, New York, 1998.

11. Matsunaga, S.; Fusetani, N.; Kato, Y. *J. Am. Chem. Soc.* **1991**, *113*, 9690.

12. Schmidt, E.W.; Harper, M.K.; Faulkner, D.J. *J. Nat. Prod.* **1997**, *60*, 779.

13. Luckner, M. *Secondary metabolism in microorganisms, plants, and animals*. Springer-Verlag, New York, 1990, pp. 300.

CHAPTER 8

THEOPALAUAMIDE AND ITS CONFORMATIONAL ISOMER FROM BACTERIAL SYMBIONTS OF THE MARINE SPONGE, *THEONELLA SWINHOEI*

The structure of theopalauamide (**9**), a glycopeptide isolated from symbionts of the marine sponge *Theonella swinhoei*, was elucidated by spectroscopic methods, and its stereochemistry was determined by chemical degradation and chiral GC-MS analysis. Theopalauamide (**9**) is identical to theonegramide (**36**) except that it contains β-D-galactose instead of β-D-arabinose. It was shown that a major degradation product of **9** is a stable conformational isomer, isotheopalauamide (**47**), formed during chromatographic purification.

Introduction

In 1996, we received two sponge samples from Professor Mike Davies-Coleman (Rhodes University) and SmithKline Beecham from their Mozambique collection. Both sponges were tentatively identified as *Theonella swinhoei*, but they were stored separately because one had an off-white interior while the inside of the other was bright orange. The latter, which more closely resembled sponges of the genus *Plakinalopha*, contained aurantoside A and compounds that were later named the mozamides (see Chapter 5). The sponge with the white interior, which proved to be *T. swinhoei*, contained swinholide A and an interesting peptide with an unprecedented molecular formula. The planar structure of the peptide was elucidated entirely by 1- and 2-dimensional NMR spectroscopy and was shown to be closely related to theonegramide (**36**),[1] from a sample of *T. swinhoei* collected in the Philippines. This peptide, it later turned out, was identical to a compound isolated by Carole Bewley in

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9, 47

36
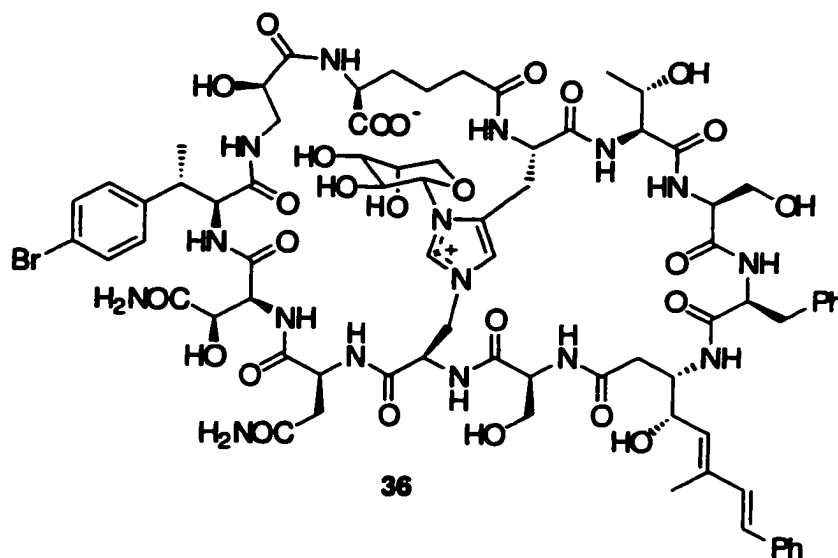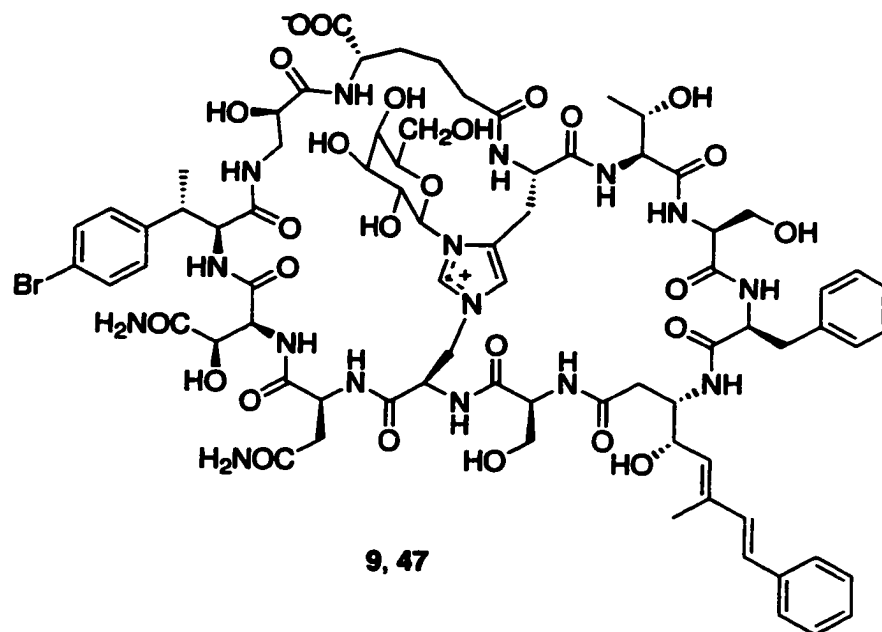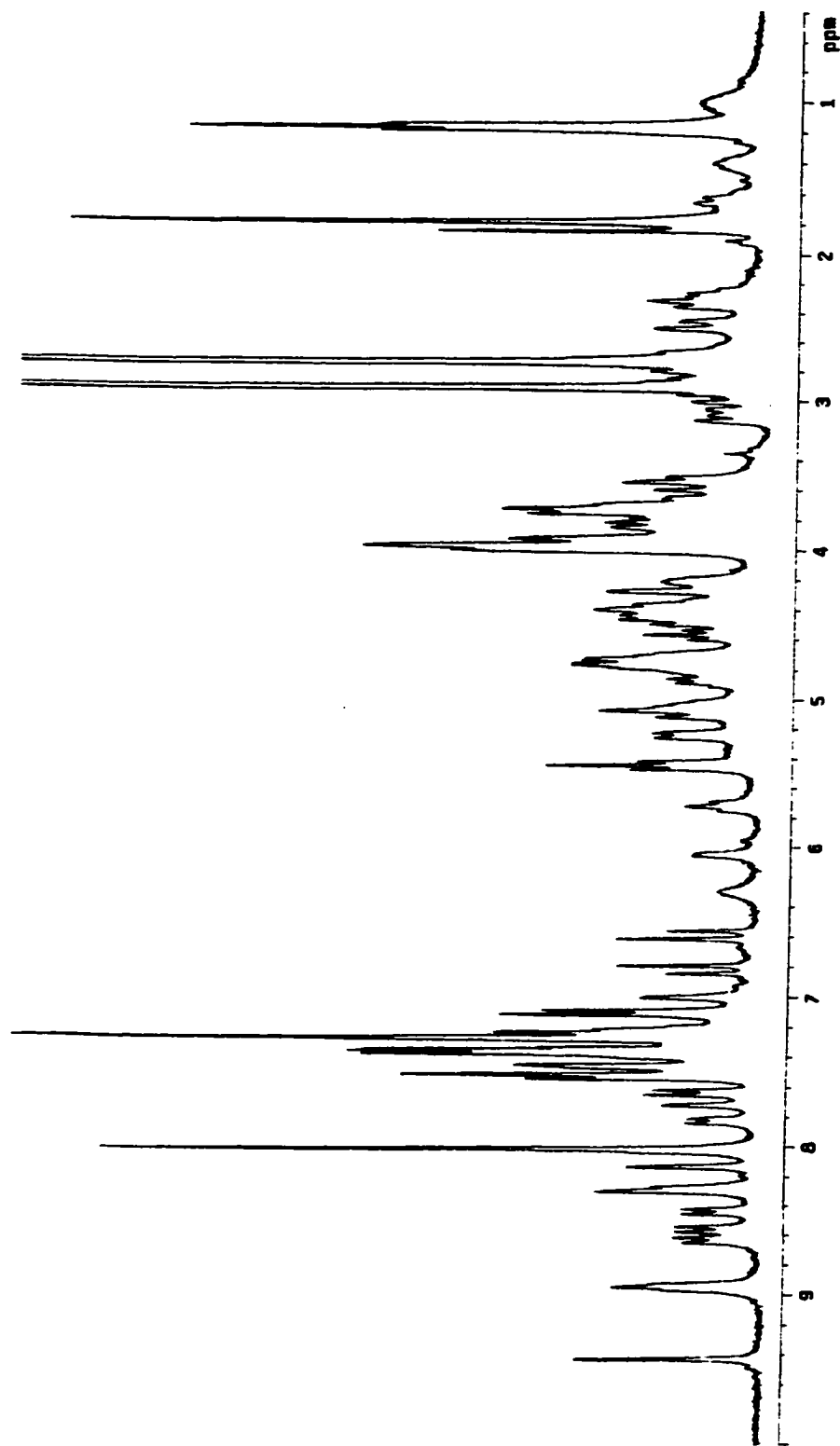
Figure 1. 300 MHz ^{1}H NMR spectrum of theopalauamide (9) in DMF-d_7.

this laboratory from filamentous microorganisms living within a Palauan specimen of *T. swinhoei*.[2] She was not able to elucidate the structure of the peptide, however, since it appeared to be a mixture of two compounds based on its NMR spectra. In my studies of this sponge metabolite, I found conditions for the separation of the two compounds and determined that they were actually interconverting when TFA was used in conjunction with column chromatography. In the absence of TFA, the compounds were stable and isolable. The non-natural isomer was much less bioactive than the natural product. Through a series of chemical degradation experiments, I proved that the only difference between the peptides was one of conformation; a single C-C bond was rotating to produce the two conformational isomers. To the best of my knowledge, this is the first case of such an interconversion in the marine natural products literature.

Structure Elucidation of Theopalauamide (9)

The structural elucidation of theopalauamide (9), isolated by Bewley as previously described from bacterial symbionts of *T. swinhoei*, was complicated by the fact that many of the signals in the ^{13}C NMR spectrum were doubled, although the sample at first appeared to be pure by HPLC and FABMS. It was clear from the molecular formula, $C_{76}H_{100}BrN_{16}O_{27}$, and from 1-dimensional NMR spectra that 9 shared many features with the known peptide theonegramide (36), also from *T. swinhoei*, except that the molecular formula differed by an additional CH_2O group.

Fortunately, a compound with an identical molecular formula but lacking any NMR signal doubling was discovered in a sample of *T. swinhoei* from Mozambique, and it was this peptide that was used for most structural studies. Once the structure of theopalauamide (9) was established, it was realized that the signal doubling in the original sample was due to a 1:1 mixture of two HPLC-separable compounds, one of

which was identical to **9** in all respects. The second compound, isotheopalauamide (**47**), was shown to be a conformational isomer of **9**.

Structural elucidation of theopalauamide (**9**) began with the ^{1}H (Figure 1), ^{13}C, HMQC, and DEPT NMR spectra. Through these experiments, the number and substitution patterns of the 76 carbons were established. DQCOSY and TOCSY spectra were used to determine the identity of most amino acids. An HMBC experiment confirmed these identities, and it also gave correlations to every quaternary carbon in theopalauamide (**9**). The structures of the aromatic amino acids were elucidated primarily using the HMBC spectrum, and it was this experiment that was particularly important to confirm of the bromine atom location. While the HMBC experiment was useful in the confirmation of the amino acid sequence, the ROESY spectrum was the most crucial in the establishment of the total peptide structure. In fact, the entire molecule could be pieced together from the constituent amino acids by following ROE correlations between NH protons and the neighboring α-protons. Using these experiments, the peptide portion of theopalauamide (**9**) was shown to be identical to that of theonegramide (**36**).

The presence of galactose was postulated on the basis of the NMR data and proven by chemical degradation. DQCOSY and TOCSY spectra revealed the presence of a hexose, and coupling constants observable in the ^{1}H NMR spectrum allowed identification of the sugar as galactose. The anomeric proton is a doublet, $J = 8.5$ Hz, indicating that it and its neighboring proton are both axial. The third proton on the sugar is a doublet of doublets, $J = 11.5, 2.0$ Hz, so it must be axial, and the fourth proton is equatorial (Figure 2). Of the possible hexoses, only galactose satisfies these data.

The stereochemistry of theopalauamide (**9**) was determined using a series of degradation experiments, followed by GC-MS using a chiral stationary phase. In the

Table 1. ^{1}H and ^{13}C NMR data for theopalauamide (9) in DMF-d_7.

amino acid		δ		mult	J (Hz)
		^{13}C	^{1}H		
allo-Thr					
	1	173.4			
	2	59.1	4.58	t	10
	3	69.1	3.86	m	
	4	21.7	1.16	d	5.5
	OH		6.08		
	NH		7.87	d	8
Ser-1					
	1	170.1			
	2	57.8	4.80	m	
	3	62.3	3.70	m	
			3.84	m	
	NH		8.97	d	10
Phe					
	1	172.4			
	2	54.9	4.88	q	8
	3	39.8	2.77	m	
			2.97	dd	7,13.5
	4	137.8			
	5,9	128.9	7.29	s	
	6,8	130.0	7.29	d	8
	7	127.2	7.23	d	8
	NH		8.28	d	8
AHMP					
	1	172.7			
	2	37.9	2.34	brd	13.5
			2.70	m	
	3	53.7	4.41	m	
	4	68.9	4.48	m	
	5	134.1	5.43	d	8.5
	6	138.3			
	7	134.1	6.83	d	15.5
	8	128.2	6.60	d	15.5
	9	135.7			
	10,14	127.0	7.54	d	7
	11,13	131.6	7.38	t	8
	12	128.0	7.26	d	8
	15	13.0	1.79	s	
	NH		8.49	d	9.5
Ser-2					
	1	171.9			
	2	57.5	4.00	m	
	3	61.5	3.71	m	
			3.94	m	
	NH		8.18	br	

Table 1. continued.

| amino acid | δ | | mult | J (Hz) |
	¹³C	¹H		
Ala				
1	169.8			
2	51.4	5.26	brd	10
3	50.7	4.43	m	
		5.10	brd	9
NH		8.61	d	10.5
Asp				
1	172.1			
2	52.6	4.70	m	
3	37.1	2.49	brd	13.5
4	171.3			
NH		8.30	d	
NH2		6.97	br s	
		7.70	br s	
β-OHAsp				
1	171.3			
2	54.6	5.73	brt	5
3	72.2	4.21	br	
4	170.8			
NH		8.69	d	10
NH₂		7.46	brs	
OH		4.79		
BMPA				
1	174.2			
2	59.1	4.76	m	
3	38.7	3.75	m	
4	17.9	1.19	d	5
5	141.6			
6,10	129.3	7.37	d	8
7,9	131.6	7.23	d	8
8	120.6			
NH		8.06	d	
i-Ser				
1	171.9			
2	70.4	4.29	br	
3	44.3	3.12	brd	10
		4.00	m	
NH		7.44	d	7.5

Table 1. continued.

amino acid		δ		mult	J (Hz)
		¹³C	¹H		
α-AAA					
	1	173.7			
	2	35.1	1.82	m	
			2.27	m	
	3	22.2	1.00	m	
			1.40	m	
	4	31.8	1.66	m	
			1.82	m	
	5	51.9	4.36	m	
	6	174.1			
	NH		7.68	d	8.5
His					
	1	170.1			
	2	54.9	5.05	m	
	3	27.2	3.05	dd	3,16
			3.60	t	16
	4	131.7			
	5	124.8	7.47	br	
	7	131.5	9.49	br	
	NH		8.92	d	8
Gal					
	1	90.1	5.47	d	8.5
	2	70.9	3.76	m	
	3	74.6	3.53	dd	2,11.5
	4	71.1	4.01	m	
	5	78.9	3.93	m	
	6	63.5	3.98	m	

GC-MS experiments, an authentic sample of theonegramide (**36**) was degraded in an identical manner and used as the internal standard, and all amino acids in **9** were shown to have identical absolute configurations to those in **36**. Hydrolysates were derivatized with PFPA/isopropanol prior to analysis. A hydrolysis experiment gave L-*allo*-threonine, (2*S*,3*R*)-3-hydroxyasparagine, L-phenylalanine, (2*S*,3*S*)-3-methyl-*p*-bromophenylalanine, and two L-serine residues. Hydrogenation followed by hydrolysis yielded, in addition to the amino acids already established, (3*S*,4*S*)-3-amino-4-hydroxy-6-methyl-8-phenyl-octa-5,7-dienoic acid (AHMP). Finally, ozonolysis followed by hydrolysis established the stereochemistry of the histidine-alanine amino acids, yielding additional L-aspartic acid and (*R*)-diaminopropionic acid. The presence of D-galactose was proven by mild hydrolysis of theopalauamide (**9**) followed by comparison of the resulting pure sugar sample with standards of D- and L-galactose.

Structure Determination of Isotheopalauamide (47)

Isotheopalauamide (**47**) had an identical molecular formula to **9** and shared most spectroscopic characteristics, but several ^{1}H and ^{13}C NMR signals in the AHMP and phenylalanine residues were at significantly different chemical shifts from those in **9** (Table 2). The most noticeable differences in the ^{1}H NMR spectrum were the vinylic methyl group at δ 1.92 (versus 1.79 in **9**) and the olefinic protons on the AHMP residue (Figure 3). The structure of the **47** was elucidated using the two-dimensional NMR methods already described, and its 2-dimensional structure was shown to be identical to that of **9**. In addition, both compounds could be interconverted during chromatography with TFA, indicating that **47** was either a conformational or stereochemical isomer of **9**.

The stereochemical identity of **47** was established with the same GC-MS methods used for theopalauamide, and the only difference lay in the AHMP residue. Unlike **9**, which gave only one peak for the AHMP residue by GC-MS after
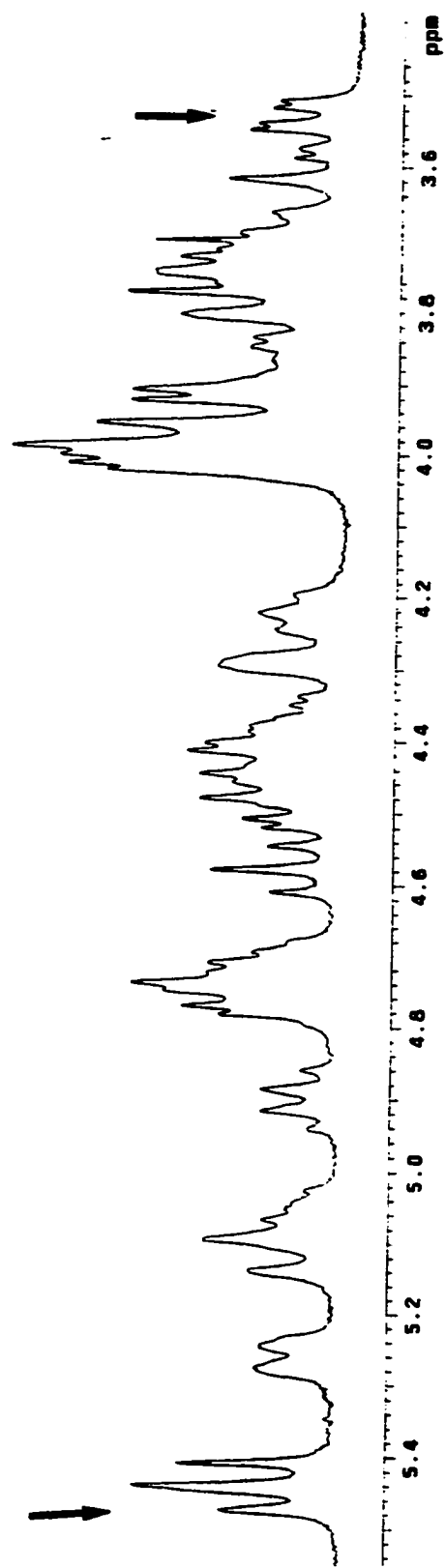
Figure 2. ^{1}H NMR coupling constants used in determination of galactose.

Table 2. Comparison of NMR data for **9** and **47** in DMF-d_7.

amino acid	Theopalauamide (9)			Isotheopalauamide (47)		
	δ_C	δ_H	J (Hz)	δ_C	δ_H	J (Hz)
Ser-1						
1	170.1			170.4		
2	57.8	4.80	m	57.9	4.70	m
Phe						
1	172.4			172.2		
2	54.9	4.88	q, 8	55.1	4.73	m
3	39.8	2.77	m	39.7	2.70	m
		2.97	dd, 13.5,7		2.81	m
4	137.8			138.3		
5,9	128.9	7.29	s	129.9	7.23	s
6,8	130.0	7.29	d, 8	128.8	7.24	m
7	127.2	7.23	t, 8	128.0	7.25	m
NH		8.28	d, 8		8.26	d, 9.5
AHMP						
1	172.7			173.1		
2	37.9	2.34	br d, 13.5	37.8	2.50	m
		2.70	m		2.84	m
3	53.7	4.41	m	53.8	4.43	m
4	68.9	4.48	m	70.4	4.42	m
5	134.1	5.43	d, 8.5	134.4	5.64	d, 8.5
6	135.7			136.4		
7	134.1	6.83	d, 15.5	134.0	6.92	d, 15.5
8	182.2	6.60	d, 15.5	128.6	6.65	d, 15.5
9	138.3			138.3		
10, 14	127.0	7.54	d, 7	127.0	7.54	m
11, 13	131.6	7.38	t, 8	131.6	7.38	m
12	128.0	7.26	t, 8	128.2	7.26	m
15	13.0	1.79	s	13.3	1.92	s
NH		8.49	d, 9.5		8.53	d, 12
Ser-2						
2	57.5	4.00	m	57.7	3.95	m
His						
7	137.5	9.49	br s	137.8	9.32	br s
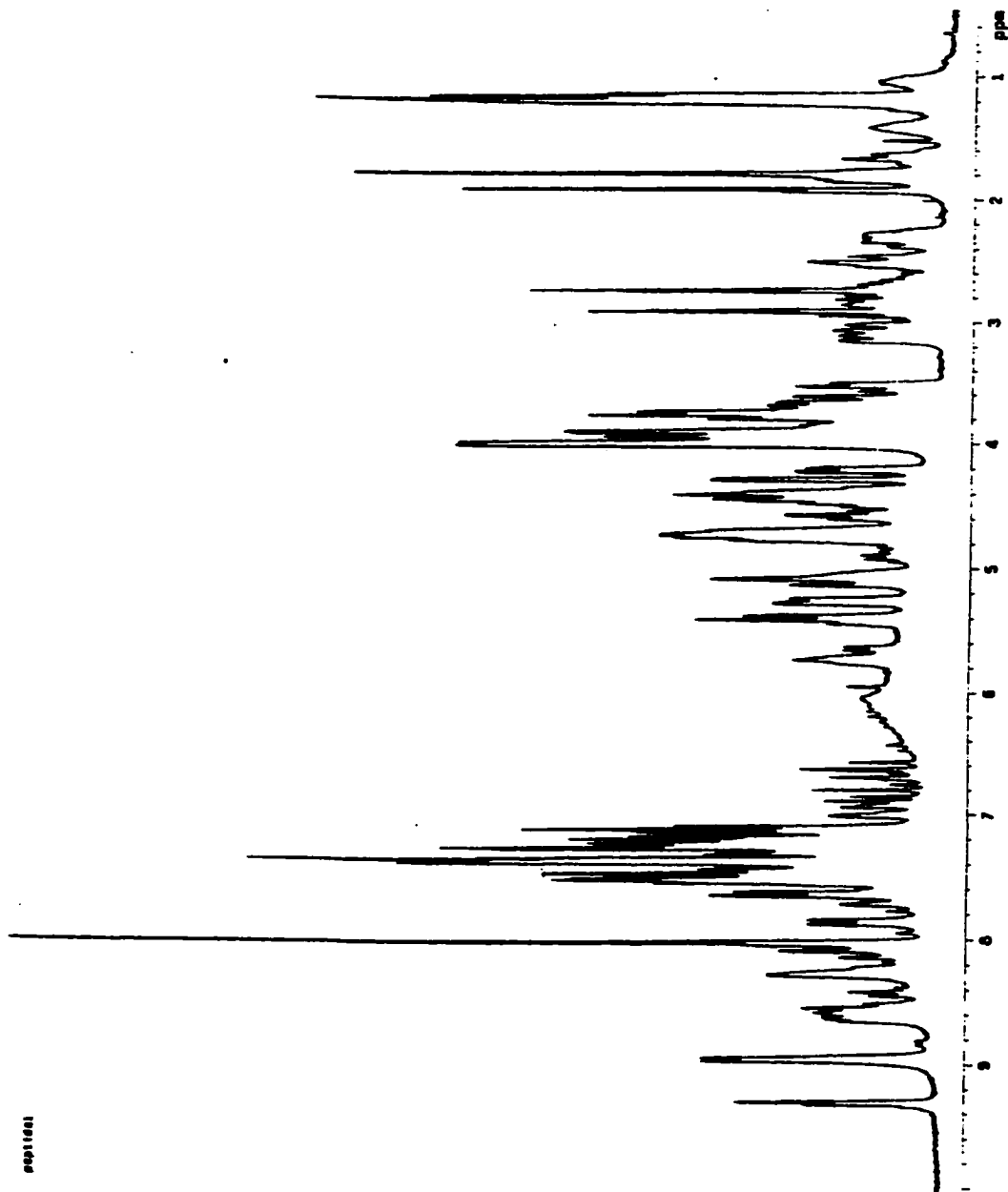
Figure 3. 300 MHz ^{1}H NMR spectrum of a mixture of **9** and **47**.

hydrogenation and hydrolysis, **47** contained two amino acids with the same MS-fragmentation profile as the product from **9** in a 1:1 ratio (Figure 4). One of the amino acids co-eluted with the amino acid from **9**, but the major compound eluted earlier. Two possibilities were immediately apparent: the allylic hydroxyl group could be racemizing in an acid-catalyzed process, or a difference in the side-chain of **47** could be making both faces of the diene susceptible to hydrogenation. It seemed reasonable that a change in stereochemistry of the hydroxyl group could induce a change in preference of hydrogenation due to chelation with the palladium catalyst as well. Since NOE difference spectra showed that the configuration of the double bond was identical in both **9** and **47**, the carbon bearing the hydroxyl group could have racemized, or the conformation of the peptide could have changed. Since the hydrogenation of the AHMP diene introduces a third stereocenter and acid hydrolysis decomposes the unsaturated AHMP residue, GC-MS could not be used to rule out either possibility. Ozonolysis of the AHMP would eliminate the hydrogenation problem, but unfortunately an internal standard for the ozonolysis product of AHMP could not be conveniently synthesized.

To determine the difference between **9** and **47**, a 1:1 mixture of **9** and **47** was hydrogenated and hydrolysed, then derivatized with 2,4-dinitrofluorobenzene in the method of Matsunaga *et al.*[3] to produce a mixture of lactones **48** and **49** in a 1.7:1 ratio. The lactones could be separated by HPLC and were nearly identical, except for chemical shift differences about the methyl group (Figure 5). In addition, NOESY experiments performed on both compounds were similar, especially about the lactone ring, indicating that **48** and **49** were identical except for the stereochemistry of the methyl group. These data are only consistent with the hypothesis of configurational isomerism, not with racemization of the hydroxyl group. In the ROESY spectra of theopalauamide (**9**), a correlation was observed between the AHMP NH and Phe α-H protons, characteristic
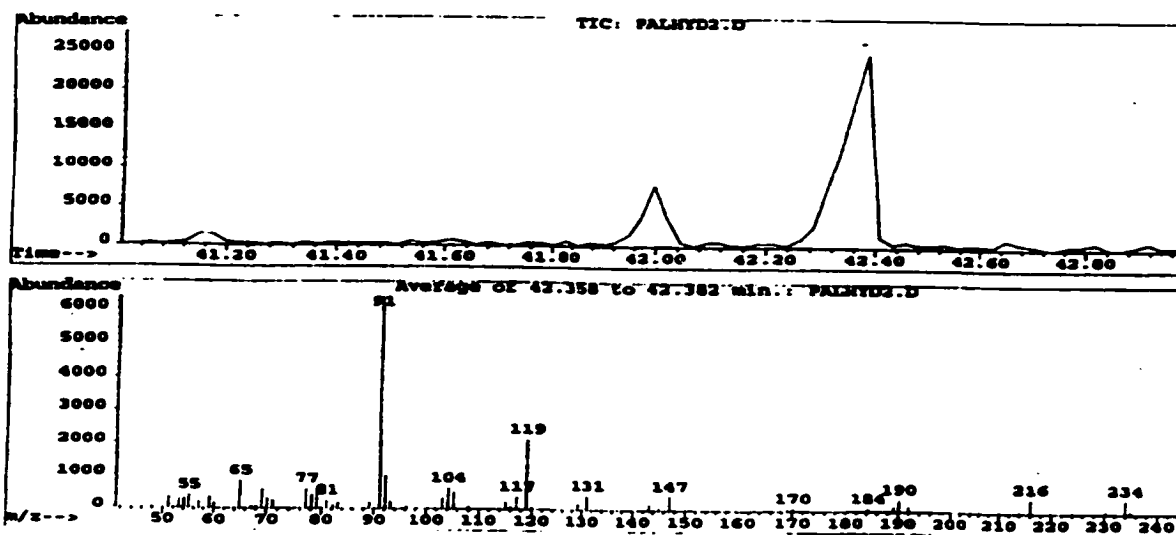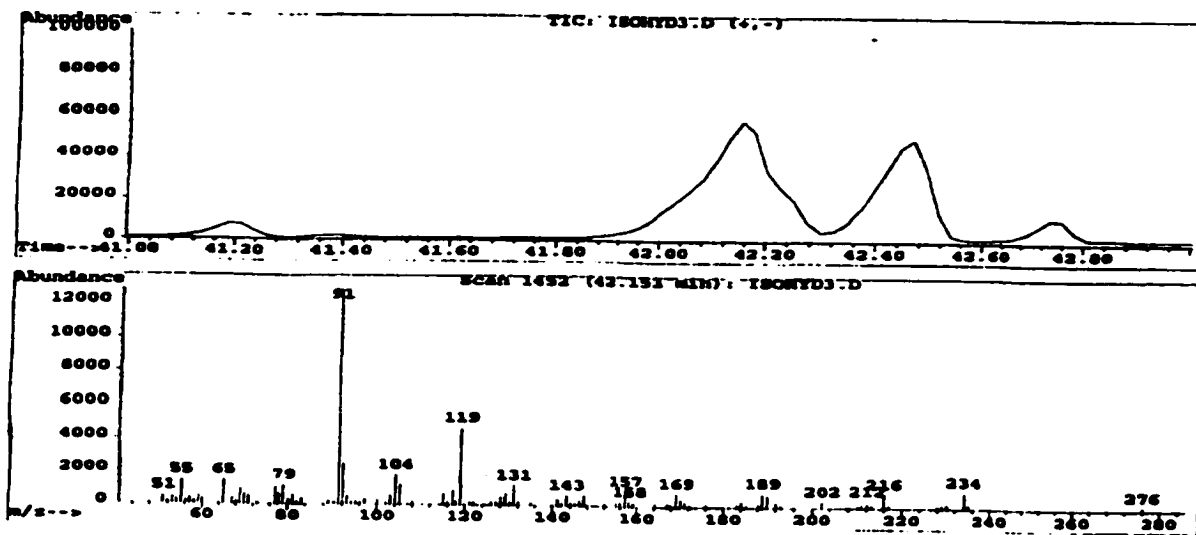
Figure 4. GC-MS of reduced AHMP residue of theopalauamide (9, top) and isotheopalauamide (47, bottom).

of a pseudo-α-helical conformation. In **47**, on the other hand, a correlation was observed between the NH protons of both AHMP and Phe (Figure 6). This is consistent with a bending of the carbonyl-C-α-C bond of Phe to produce a conformation similar to a β-turn (Figure 7).

Because the change in NH proton chemical shift with temperature can sometimes be used to indicate the degree of hydrogen bonding,[4,5] several ^{1}H NMR spectra of **9** and **47** were acquired between 25 and 40 °C. Analysis of the chemical shift differences showed that the NH protons on both the AHMP and Phe residues were more strongly hydrogen bonded in **47** than in **9**, consistent with the flipping of the aromatic residues from buried to more exposed positions. In **9** the AHMP-NH and Phe-NH proton signals had $\Delta\delta/\Delta T$ (ppb/K) values of 6.5 and 5.1, respectively, while the same protons in **47** had $\Delta\delta/\Delta T$ values of 3.1 and 1.5, respectively. The NH-CHα coupling constants for Phe and AHMP were slightly larger in **47** than in **9**, but not enough to indicate a major change in geometry about these bonds. Thus, it appears that the major conformational difference between **9** and **47** is a rotation of the bond between C-1 and C-2 residue. Molecular modeling was performed on the AHMP-Phe residues of **9** and **47** using ROE data as a constraint. The models were minimized using PCModel,™ using molecular mechanics calculations, and compared with a hand-built space-filling model. While the models were only qualitative, they revealed that the conformational switch would move the AHMP side chain out of a sort of π-stack with Phe, in which both side chains were in proximity, into a more solvent-accessible position (Figure 7). The model reflects the hydrogenation results: the conformational change allows both sides of AHMP to be hydrogenated. In addition, when a mixture of the two compounds was hydrogenated, most of the ^{1}H and ^{13}C NMR shift differences except in the
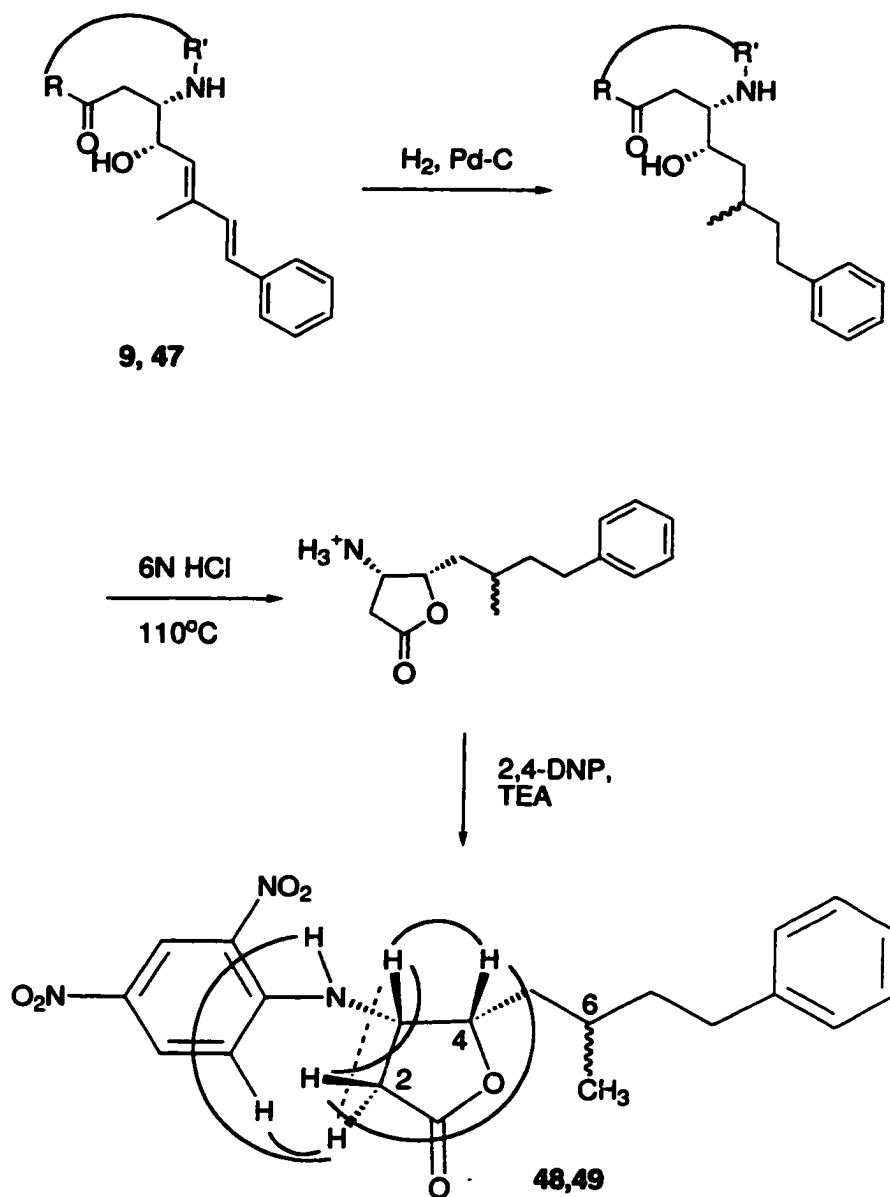
Figure 5. Formation of lactones **48** and **49**, and NOESY data for **48** and **49** (bottom).
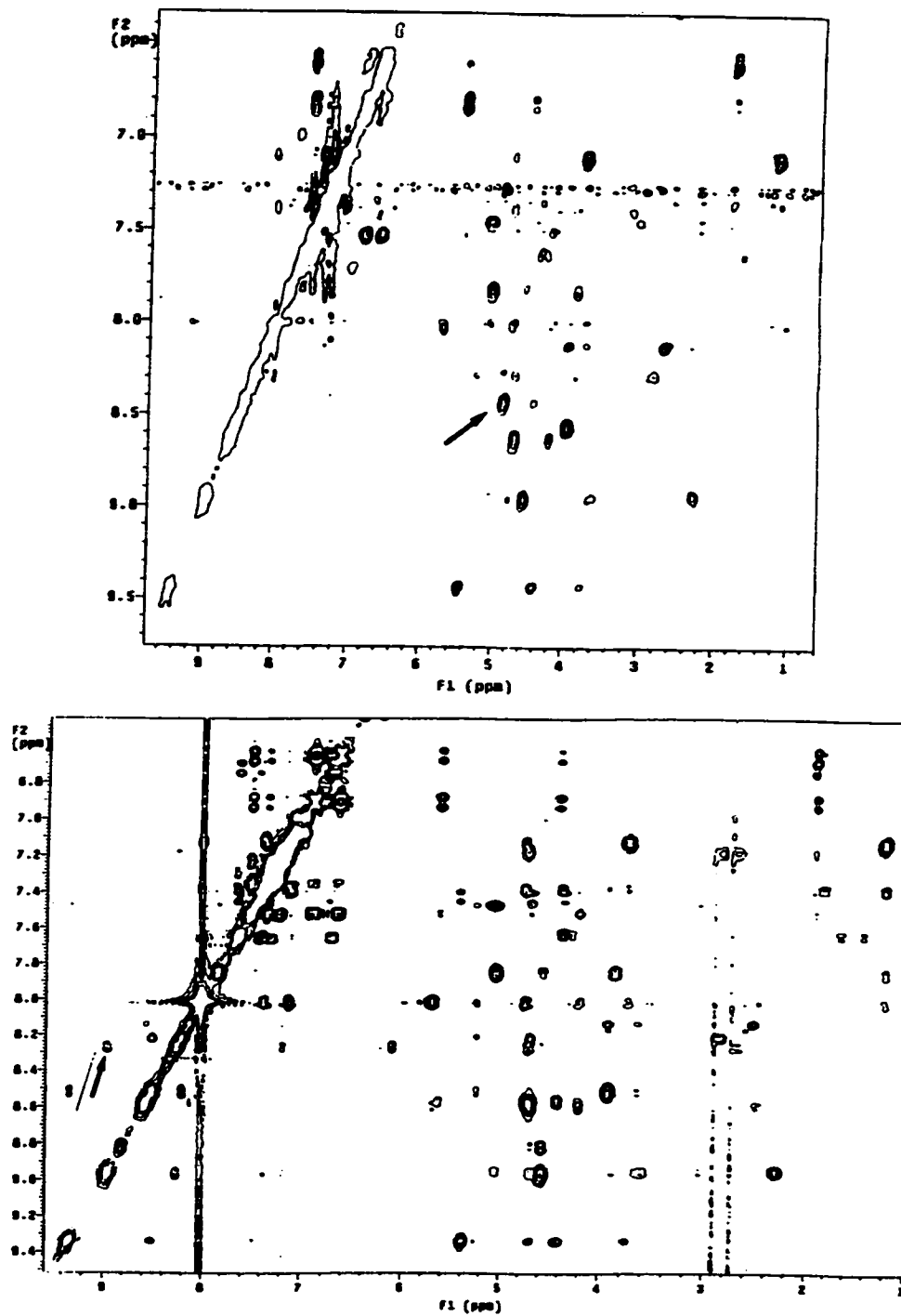
Figure 7. Comparative ROESY spectra (300 MHz) of **9** (top) and **47**, showing NOE correlations between AHMP NH and Phe α-H (top) or Phe NH (bottom).
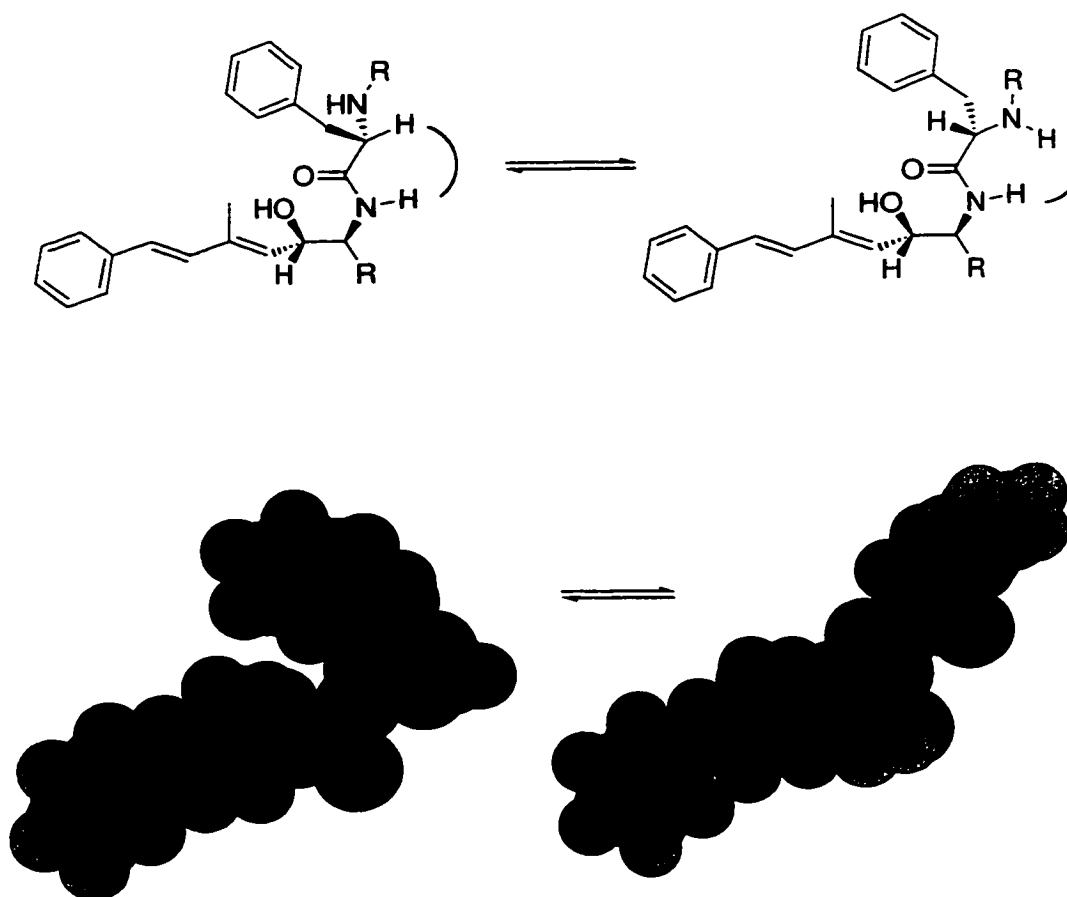
Figure 7. AHMP-Phe residues of theopalauamide (9, left) and isotheopalauamide (47).

immediate vicinity of the new methyl stereocenter, indicating that only a single conformational isomer was probably present (Figure 8). In fact, the only doubled signals were in the immediate vicinity of the formerly vinylic methyl.

Bioactivity and Biological Data

Theopalauamide (9) and isomer 47 were shown to be active against *Candida albicans*. Theopalauamide (9) was more active in the disc diffusion assay (10 mm inhibition at 10 μg/disk) than 47 (14 mm inhibition at 50 μg per disc), but the exact difference in activity was difficult to measure because preparations of 47 always contain a small amount of 9.

The known compounds swinholide A (50)[6] and theonellasterol (51)[7] were also isolated from the Mozambique specimen of *T. swinhoei* and identified by MS and NMR data. Swinholide A (50) has also been shown to be found in unicellular symbiotic microbes living within *T. swinhoei*. While this specimen had numerous filamentous bacterial symbionts by light microscopy, it had far fewer spicules than comparable samples from the Pacific Ocean. Theonellasterol (51) was present in several cell separation fractions produced by Carole Bewley, so the identity of the producing organism is unknown.

Conclusion

Several marine natural products papers on unusual peptides report a problem with NMR signal doubling that is caused by conformational isomerism, but in no case so far have the conformations been isolable.[8,9] In each case, they occur in solution on a relatively fast timescale, and thus they cannot be resolved. The conformations of marine peptides in different solutions or complexed with different cations have been studied, but the changes found so far are relatively minor, differing only slightly depending on

95-08SMIXEDHYDROGPROD
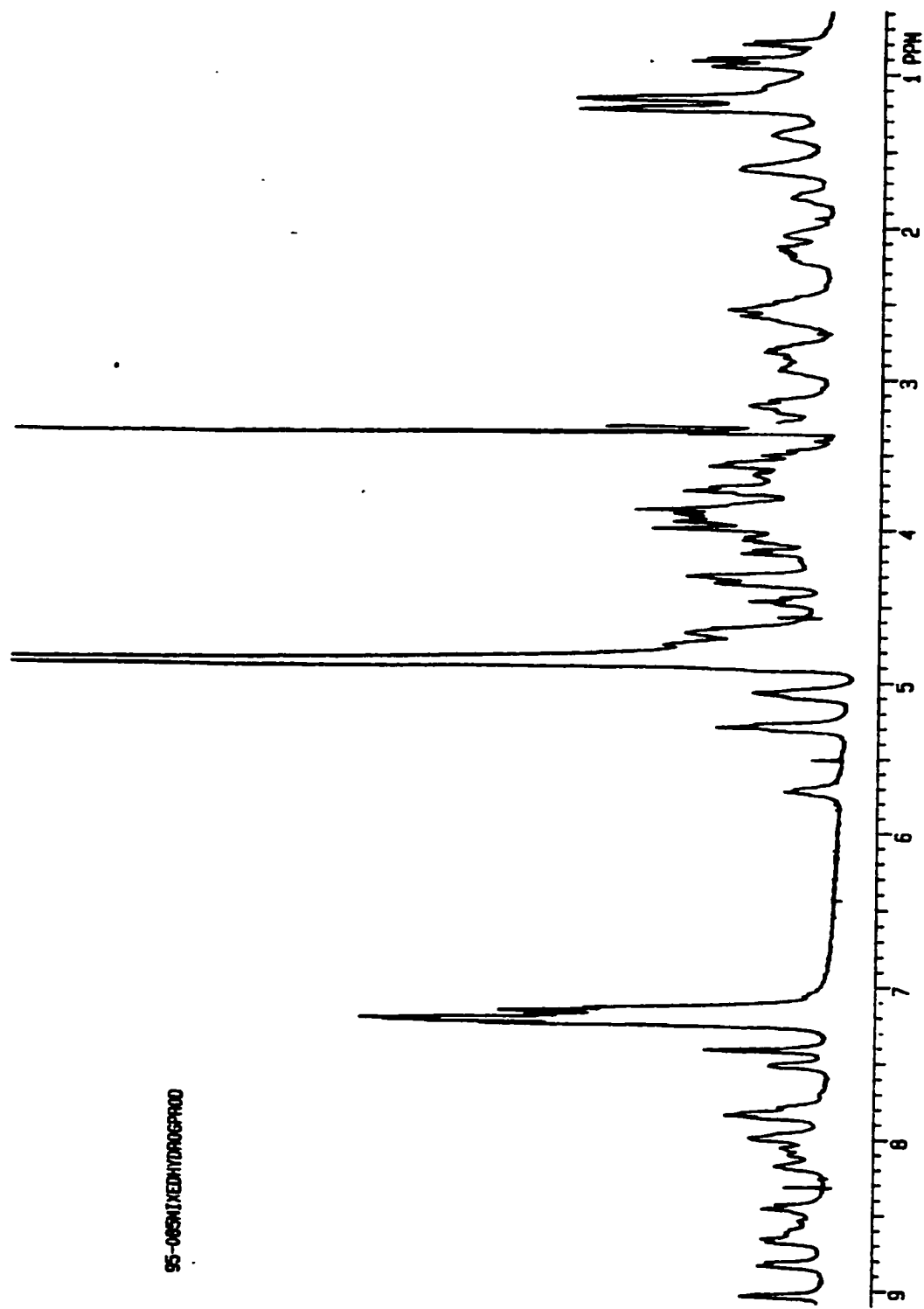
Figure 8. 400 MHz ^{1}H NMR spectrum of the hydrogenation product of a mixture of **9** and **47** in CD_3OD.

environment and not changing significantly from crystal structures.[10] Another case in which the conformation of peptides often changes on a rapid scale occurs when the amino acid proline is present in otherwise unconstrained structures.[4,11] The small difference in energy between *cis*- and *trans*-peptide bonds associated with the proline nitrogen often creates two conformations which can be observed by NMR.

Unlike other examples of stable conformational isomerism in peptides, theopalauamide (9) contains no proline residues. Instead, it would appear that the natural conformation of the aromatic side chains is preserved by relatively weak intramolecular hydrogen bonds and by π-stacking of the aromatic amino acids. This general structure is in agreement with the model for theonellamide F proposed by Matsunaga and co-workers,[3] which was based on some key NOE signals. In particular, the δ-Me group of threonine showed an NOE correlation to all three aromatic residues, indicating the possibility of a cage-like structure with aromatic groups in proximity. If this conformation of the peptide is correct, the three aromatic "bars" of the cage are separated in the unnatural isomer 47. This indicates that conformational effects are not responsible for the clearer NMR signals present in DMF-d_7 solution over DMSO-d_6, as proposed by Bewley. Instead, better solvation is more likely for the clean NMR spectrum obtained in DMF-d_7 solution. This is supported by the observation that small amounts of 9 give good spectra in DMSO-d_6.

The occurrence of theopalauamide (9) in *T. swinhoei* specimens from such distant locales (Mozambique and Palau) indicates a stability of symbiosis, since the producing filamentous microorganism must be present in both instances. Similar peptides have been reported from locales throughout the tropical Indo-Pacific, from Australia to Japan and the Red Sea. However, sponges that look morphologically very similar, and host filamentous symbionts, but are of a different genus do not contain
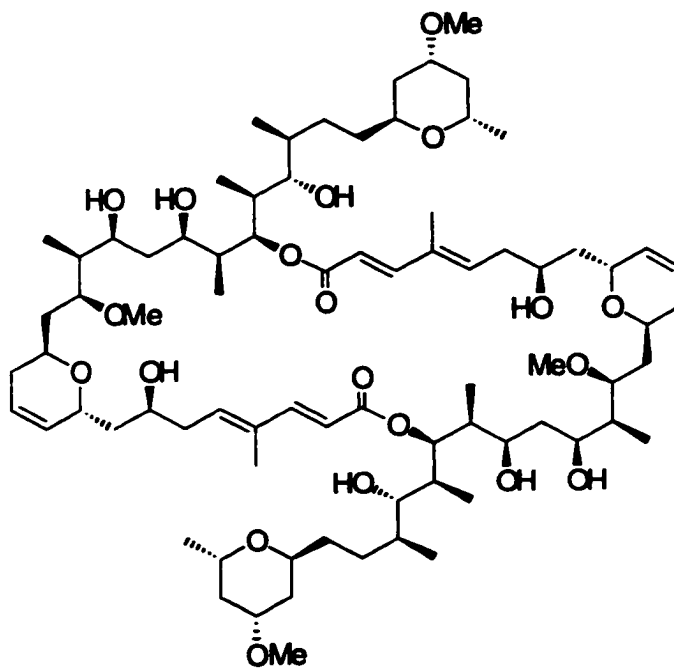
50
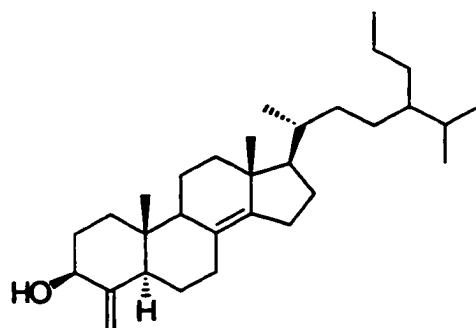
51

these peptides. The results of my chemical studies of the mozamides and
theopalauamide led me to think about this symbiosis, including how it evolved, how
specific it was, and its age. As the next chapters detail, I chose molecular biological
methods as a compliment to these studies in an attempt to answer these questions.

Experimental Section

General Experimental Procedures. ^{1}H, DQCOSY, HMQC, and HMBC NMR
spectra were recorded on a Varian Unity 500 MHz spectrometer. [13]C, DEPT, and NOE
difference NMR spectra were recorded on a Varian Inova 300 MHz spectrometer. All
NMR data for **9** and **47** are reported in DMF-d_7. NMR data for **48-51** were recorded
in $CDCl_3$. TOCSY spectra were obtained with 80 msec mixing times, and ROESY spin
locks were established by continuous pulsing for 300 msec. High-resolution FABMS
data were obtained from the Mass Spectrometry Facility, University of California at
Riverside. Optical rotations were measured on an Autopol III polarimeter. UV and IR
spectra were taken on Perkin Elmer instruments. Absolute configurations were
determined using an Alltech Chirasil-Val column with a Hewlett Packard 5890 GCMS
system. The temperature was ramped from 50 °C to 210 °C over 45 minutes in all GC-
MS experiments, and theonegramide **(36)** derivatives were used as internal standards.
GC-MS retention times were similar to previously reported results.[1]

Isolation of theopalauamide (9), conformer 47, and swinholide A (50).
The mixture of **9** and **47** from symbiotic bacteria was isolated as previously described.[2]
The mixture was separated by RP HPLC on a C_{18} preparative column using 35%
acetonitrile (aq, 0.01% TFA), detection at $\lambda = 215$ nm.

Theopalauamide **(9)** and swinholide A were obtained from the Mozambique
specimen (MOZ 95-004) as follows. The sponge (211 g dry weight) was lyophilized
and sequentially extracted 3 times with approximately 1 L of each of the following

solvents: 1:1 hexanes/CH_2Cl_2; EtOAc; 1:1 EtOAc/acetone. The sponge was then

exhaustively extracted with 1:1 acetonitrile/water until no further peptide was detected in

the crude extracts by TLC. The aqueous extracts were dried by rotary evaporation until

most CH_3CN was removed and a white precipitate had formed. The solvent was

centrifuged, and the supernatant removed. The precipitate was applied directly to a C_{18}

Sep Pak (Waters) and subjected to reversed-phase chromatography using an

acetonitrile/water gradient (0-100% CH_3CN in 10% increments). Fractions eluting in

80% acetonitrile were combined to yield impure swinholide A, which was subsequently

repurified by silica flash chromatography (100% EtOAc) to give swinholide A (**50**, 21.3

mg, 0.01% dry weight). Fractions eluting in 40-60% acetonitrile were enriched with a

single peptide, which was purified by reversed-phase HPLC (38% CH_3CN, 0.01%

TFA) to give theopalauamide (**9**, 55.7 mg, 0.026% dry weight).

Isolation of theonellasterol (51). The hexane/DCM extract from the Mozambique

T. swinhoei was partitioned between hexane and methanol (8 mL each). The hexane

fraction was dried under vacuum and purified by flash chromatography on silica gel

using a hexane/ethyl acetate gradient (20-100% EtOAc). Pure theonellasterol (**51**, 28.2

mg, 0.013% dry weight) was isolated from the 30% EtOAc fraction.

Theopalauamide (9): white powder; $[\alpha]_D$ = +19° (c = 0.4, MeOH); UV (MeOH)

203 (ϵ 19 600), 276 (ϵ 9 400), 285 (ϵ 9 900), 304 nm (ϵ 4 500); IR (AgCl) 3300, 2920,

1660, 1540 cm^{-1}; ^{1}H NMR (DMF-d_7) see Table 1; ^{13}C NMR (DMF-d_7) see Table 1;

HRFABMS, m/z = 1769.5944 (M+Na)$^+$, $C_{76}H_{99}{}^{79}BrN_{16}O_{27}Na$ requires m/z =

1769.5947.

Isotheopalauamide (47): $[\alpha]_D$ = +35° (c = 0.04, MeOH); UV and IR data were

identical to those of **9**; HRFABMS, m/z = 1747.6233 (M+H)$^+$, $C_{76}H_{100}{}^{79}BrN_{16}O_{27}$

requires m/z = 1747.6127; ^{1}H and ^{13}C NMR: identical to **9** within the limits of error except for some resonances, listed in Table 2.

Hydrolysis experiments. Between 100 and 500 μg of **9, 36,** or **47** were used for all hydrolysis experiments. The glycopeptides were dissolved in 6N HCl (500 μL) and heated to 110 °C in tightly sealed conical vials for 15 hours. HCl was removed under a stream of nitrogen.

Derivatization of hydrolysates. The hydrolysed peptides in 1 mL conical vials were dissolved in isopropanol (400 μL), to which were added acetyl chloride (100 μL). The vials were quickly capped, and the solutions were heated to 100 °C for 1 hour. Excess reagents were removed under nitrogen, and the peptides were redissolved in DCM (400 μL). Pentafluoropropionic anhydride (400 μL) was added, and the reaction mixture was heated to 100 °C for 15 minutes. Reagents were removed under nitrogen, and the derivatized hydrolysates were redissolved in EtOAc for GC-MS experiments.

Hydrolysis of galactose. Both theopalauamide (**9**) and **36** were analyzed by comparison with a standard sample of D- and L-galactose. Each peptide (500 μg) was dissolved in 4N HCl (500 μL) and heated to 70 °C for 12 hours. Removal of solvent and derivatization as described above led to single peaks in the GC-MS corresponding to D-galactose.

Ozonolysis of 9 and 36. The peptides (1.2 mg) and a standard of theonegramide were dissolved in 0.5 mL MeOH and ozonized with 100% oxygen at -78 °C for 15 minutes. To the resulting ozonides, 10 drops of 50% H_2O_2 were added and the mixture was brought to room temperature for 1 hour. Solvent was removed under a stream of nitrogen, and the ozonolysis products were hydrolysed and derivatized as described above.

Hydrogenation of 9, 47 and 36 for GC-MS. 9, 47, and **36** (1.2 mg) were hydrogenated in MeOH with 5% Pd-C (3-10 mg) under hydrogen gas. The resulting peptides were filtered through Celite, dried under vacuum, and subjected to the hydrolysis/derivatization sequence described above.

Hydrogenation and hydrolysis to produce 48 and 49. A 1:1 mixture of **9** and **47** (120 mg) was dissolved in MeOH (10 mL). 5% Pd-C (250 mg) was added, and the mixture was stirred over hydrogen gas for 24 hours. The product was filtered, dried by rotary evaporation, and shown to be the debrominated, hydrogenated compound by ^{1}H NMR. Hydrolysis of the compounds in 6N HCl (6 mL) for 15 hours at 110 °C, followed by lyophilization and extraction between EtOAc and water, yielded a mixture of lactones (5.6 mg). The lactones were dissolved in ether (0.1 mL) containing 2,4-dinitrofluorobenzene (14 µL) and TEA (4 µL). The mixture was stirred for 2 h at room temperature, then filtered through silica gel using 3:2 EtOAc-hexane as eluant to obtain pure samples of lactone **48**, which had identical ^{1}H NMR data to literature values, and lactone **49**.

Lactone 48: ^{1}H NMR (CDCl$_3$) δ 9.17 (d, 1 H, J =2.5 Hz), 8.80 (d, 1 H, J = 7.5 Hz), 8.33 (dd, 1 H, J = 9.5, 2.5 Hz), 7.1-7.25 (m, 5 H), 6.79 (d, 1 H, J = 9.5 Hz), 4.83 (dt, 1 H, J = 10, 5 Hz), 4.40 (dddd, 1 H, J = 7.5, 6.5, 5,3 Hz), 3.04 (dd, 1 H, J = 17.5, 7 Hz), 2.70 (m, 1 H), 2.64 (dd, 1 H, J = 17.5, 3 Hz), 2.56, (m, 1 H), 1.95 (ddd, 1 H, 15, 10, 5 Hz), 1.77 (m, 1 H), 1.67 (m, 2 H), 1.43 (ddd, 1 H, J = 15, 10, 5 Hz), 1.02 (d, 3 H, J = 6.5 Hz).

Lactone 49: ^{1}H NMR (CDCl$_3$) δ 9.17 (d, 1 H, J = 2.5 Hz), 8.81, (d, 1 H, J = 7.5 Hz), 8.33 (dd, 1 H, J = 9.5, 2.5 Hz), 7.1-7.3 (m, 5 H), 6.80 (d, 1 H, J = 9.5 Hz), 4.83, dt, 1 H, J = 9, 5 Hz), 4.44 (dddd, 1 H, J = 7.5, 6.5, 5, 3 Hz), 3.04 (dd, 1 H, J

= 17.5, 6.5 Hz), 2.70 (m, 1 H), 2.66 (dd, 1 H, J = 17.5, 3 Hz), 2.54 (m, 1 H), 1.88 (m, 1 H), 1.73 (m, 1 H), 1.69 (m, 2 H), 1.48 (m, 1 H), 1.07 (d, 3 H, J = 6.5 Hz).

This work originally appeared in the *Journal of Organic Chemistry* **1998**, *63*, 1254-1258 with co-authors Carole A. Bewley and D. John Faulkner. It has been rewritten here so that my contribution is clarified.

References

1.   Bewley, C.A.; Faulkner, D.J. *J. Org. Chem.* **1994**, *59*, 4849.

2.   Bewley, C.A.; Holland, N.D.; Faulkner, D.J. *Experientia* **1996**, *52*, 716-722.

3.   Matsunaga, S.; Fusetani, N; Hashimoto, K.; Wälchli, M. *J. Am. Chem. Soc.* **1989**, *111*, 2582.

4.   Morita, H.; Kayashita, T.; Takeya, K.; Itokawa, H.; Shiro, M. *Tetrahedron* **1997**, *53*, 1607-1616.

5.   Andersen, N.H.; Neidigh, J.W.; Harris, S.M.; Lee, G.M.; Liu, Z.; Tong, H. *J. Am. Chem. Soc.* **1997**, *119*, 8547-8561.

6.   Kitagawa, I.; Kobayashi, M.; Katori, T.; Yamashita, M.; Tanaka, J.; Doi, M.; Ishida, T. *J. Am. Chem. Soc.* **1990**, *112*, 3710.

7.   Kho, E.; Imagawa, D.K.; Rohmer, M.; Kashman, Y.; Djerassi, C. *J. Org. Chem.* **1981**, *46*, 1836.

8.   Toske, S.G.; Jensen, P.R.; Kauffmann, C.A.; Fenical, W. *Tetrahedron* **1998**, *54*, 13459-13466.

9.   Schmidt, E.W.; Faulkner, D.J. *Tetrahedron* **1998**, *54*, 3043-3056.

10.   Abbenante, G.; Fairlie, D.P.; Gahan, L.R.; Hanson, G.R.; Pierens, G.K.; van den Brenk, A.L. *J. Am. Chem. Soc.* **1996**, *118*, 10384-10388.

11.   Tamaki, M.; Komiya, S.; Yabu, M.; Watanabe, E.; Akabori, S.; Muramatsu, I. *J. Chem. Soc. Perkin Trans. 1* **1997**, 3497-3500.

CHAPTER 9

NATURAL PRODUCTS AND MOLECULAR BIOLOGY: TOOLS FOR THE STUDY OF SPONGE-MICROORGANISM CO-EVOLUTION

In the early Cambrian Period, before the rise of scleractinian corals, sponges thrived as reef-builders living in close association with bacterial mats.[1-4] These simple metazoans have maintained their prokaryotic ties into the modern world, filtering bacteria from seawater as food[5] and harboring large and diverse microbial populations.[6] In many sponges, the bacteria have been shown to play a role in the lives of their host, either through processing of waste products,[7] transfer of nutrients to their hosts,[8] or production of secondary metabolites.[9] However, despite several studies, the evolutionary age and species-specificity of symbiosis is undetermined, leaving open the intriguing possibility that some symbioses may have originated early in the histories of the sponge hosts.[10] In the polyphyletic sponge order Lithistida, natural products may be used, in concert with biological tools, to probe the evolution of bacterial-poriferan symbiosis. The order contains a large number of diverse, bioactive molecules that have been ascribed microbial origins, and in one case a bacterial source has been proven.[11] Because it is relatively easy to determine the presence of known compounds in a sponge, the distribution and specificity of metabolites in this order can be mapped. By comparing this data with sponge taxonomy and molecular phylogeny of sponge symbionts, hypotheses of the nature of these symbioses can be advanced.

Introduction to sponge-microbe symbiosis

As the simplest of metazoans, sponges (Phylum: Porifera) have an extremely long evolutionary history, with a fossil record that indicates a Precambrian origin for

the phylum.[2] The history of sponges is tightly coupled to microorganisms, which they filter from seawater with high efficiency (75-99%).[5] In addition to their role as a primary food source for poriferans, bacteria have also interacted with sponges to produce reef-like structures.[1-4] These 5-15 million year reef-building episodes repeated themselves every 70-100 million years, beginning with the Early Cambrian and ending in the Late Jurassic age.[1] The structures of sponge-microbe reefs were highly variable, from shallow, clear water formations much like tropical coral reefs, to deeper mounds. Sponges, either demosponges or hexactinellids, were involved in reef formation in a number of ways, including building the primary reef structure or supporting the microbial reef-builders. The roles of the associated prokaryotes were no less diverse. Microorganisms, like sponges, helped to build or stabilize reef structures in widely varying ways. The microorganisms also led quite diverse lifestyles and may not have been closely related. Shallower reefs were mainly composed of photoautotrophic bacteria, while deeper reefs probably contained mainly heterotrophic bacteria. Despite the probable heterotrophy of the deep-sea microbes, they still formed calcified thrombolites or stromatolites of filaments similar to algae. Sponge-microbe associations in ancient reefs have been reviewed in detail by Brunton and Dixon.[2]

The sponges that formed ancient reefs or are well documented in the Phanerozoic Era fossil record tend to have strongly reinforced skeletons. For that reason, the major known sponge fossils are hexactinellids or from the demosponge order Lithistida, both of which have strong siliceous spicules and occur widely in the deep ocean today. Unfortunately, the order Lithistida is polyphyletic, with a taxonomy based only on a spicule type (desma), and thus does not reflect the true evolutionary relationship of these sponges.[12] Since many fossilized lithistids are

extinct or have not yet been discovered in the living fauna, it is impossible to make evolutionary assumptions based on their good fossil record. Making the situation more complicated, the earliest known sponge fossils from the microbial reefs are archaeocyaths, a long-extinct group that may not have been true sponges at all. In at least one instance, though, lithistids and hexactinellids have been found together in Early Cambrian formations.[3] Because of the uncertain relationship between fossil and living sponges, it is difficult to determine the importance of the early sponge-microbe associations in the evolution of living sponges.

Despite the lack of a clear co-evolution of sponges and microbes in the fossil record, numerous associations between microbes and extant sponges have been described. Sponges feed on microbes, and they also harbor large populations (up to 40% of body mass) of microorganisms, both intra- and extra-cellularly.[13] Bacterial density is particularly high in massive sponges with a large mesohyl, and cyanobacteria often coat the outer layers of shallow, exposed sponges. The literature on the ecological significance of microbial sponge symbionts through 1987 has been reviewed by Wilkinson.[10] Until that time, sponges were studied primarily for their relationship with photosynthetic organisms such as cyanobacteria and dinoflagellates, and nutrient transfer relationships were demonstrated in a number of cases. Nitrogen fixation and the presence of facultative and obligate anaerobes in marine sponges were also areas of active research. In some of these studies, it was shown that sponges derive a significant amount of their nutrients from microbial symbionts, although in one recent study the transfer of photosynthate from cyanobacteria to sponges was shown to be of minor importance.[7] Possibly the most novel trophic transfer from microbe to sponge, reported by Vacelet *et al.*, was a symbiosis between the deep-sea carnivorous sponge *Cladorhiza* sp. and a methane-oxidizing bacterium.[14] The

sponges live near mud volcanoes that leach methane, and they seem to derive a significant amount of nutrition from their microbial symbionts. However, without culturable bacteria or taxonomic data derived from molecular biology, the evolutionary significance of the symbiosis is uncertain.

During the 1970s and 1980s, investigators also studied the type and specificity of sponge-microbe associations, often using microscopy to show the presence of specific symbiont morphologies within specific sponges. The molecular basis of symbiosis was also probed, albeit to a lesser extent. Wilkinson showed that bacteria, which live symbiotically with sponges, can be passed through their feeding chambers without being digested and suggested some sort of encapsulation or recognition process.[10] Müller et al. showed that in the demosponge Halichondria panicea, an association with the microbe Pseudomonas insolita may have been lectin-based.[15] Wilkinson found an immunological basis for symbiosis in some sponges, which he claimed as evidence of a Precambrian origin for many symbioses.[6]

A major problem with the early studies on sponge-microbe symbiosis was that most microorganisms were uncultured or unculturable, so descriptions of symbioses usually relied either on morphology of symbionts or chemical measurements of nutrient transfer. Even in the cases where putative symbionts could be cultured, the ecological relevance of symbiosis could not be determined. The period following Wilkinson's review has been marked by the ascendance of molecular biological techniques, which have allowed investigators to focus on uncultured microorganisms. Using PCR and DNA-RNA or DNA-DNA hybridization together with molecular databases, investigators have begun to determine the phylogentic affinity of associated microorganisms, a necessary step in establishing the evolutionary history of symbiont and host. The most commonly targeted gene encodes the RNA from the small subunit

of ribosomes. Since in bacteria this subunit has a sedimentation constant of 16S, the RNA is termed 16S or SSU rRNA. The 16S rRNA gene is used for bacterial taxonomy for three major reasons: it encodes an essential cellular function (protein synthesis) and therefore evolves at a rate assumed to be nearly constant in all organisms and is not likely to be subject to lateral transfer; it has regions that do not appear to be very crucial to function and so evolve more quickly; it contains very highly conserved regions, so that PCR is usually presumed to amplify all bacterial sequences using "universal" primers. Large, computerized databases of bacterial SSU rRNA sequences and algorithms for determining taxonomy are readily available over the world wide web, allowing newly obtained sequences to be rapidly compared with those from known strains.

The application of molecular biology to sponge-microbe symbiosis is yielding results that could not have been obtained by classical microbiological methods. Probably the most unusual monograph concerned the discovery of a member of the Archaea living specifically within a sponge similar to *Axinella mexicana*.[16] The archaeal microorganism, dubbed *Cenarchaeum symbiosum* (P: Crenarchaeota), lives at a relatively cold 10 °C and is therefore considered psychrophilic (cold-loving). Preston *et al.* applied universal archaeal PCR primers to DNA extracts from several tropical and temperate sponges, but archaeal sequences were amplified only from *A. mexicana*. Subsequent *in situ* hybridization experiments showed which microorganism in the sponge was archaeal and allowed localization of the symbiont. Another molecular biological study of demosponges aimed to elucidate the major microorganisms within the marine sponges *Chondrosia reniformis* and *Petrosia ficiformis*.[8] Schumann-Kindel *et al.* used *in situ* hybridization probes specific to Archaea, the subclasses of Proteobacteria, Flavobacteria-Cytophaga, and sulfate-

reducing bacteria. Both sponges contained mainly gamma-sublcass Proteobacteria, according to the authors, with sulfate-reducing (delta-subclass) Proteobacteria also being present in significant numbers. γ-Proteobacteria were also the only bacterial isolates obtained using aerobic enrichment media. However, the authors did not present details of their methodology, including the presence or absence of control probes and the use of PCR-cloning-sequencing as confirmation, so it is difficult to evaluate the ecological relevance of the study. Sulfate-reducing bacteria were postulated to play a role in the mineralization of dead sponge tissue.

In some cases, the picture of symbiosis that is emerging is markedly different from studies using cultured organisms. For instance, in the same species, *Halichondria panicea*, used by Müller *et al.* in lectin studies, PCR-cloning-sequencing led to microorganisms wholly different from the cultured *Pseudomonas* sp. described earlier.[17] SSU rRNA sequences from all individuals of *H. panicea* collected led to the identification of strains of *Rhodobacter* (α-Proteobacteria) as the dominant species. A symbiotic relationship between the sponge and these microbes was suggested based on their ubiquitous occurrence in *H. panicea*. This study is limited by the lack of data supporting a role for the bacteria, but the common presence of this genus of bacteria would not have been detectable using classical methodology.

Although PCR-based methods widely expand the possibilities for research in sponge-microbe symbiosis, some caveats need to be applied. PCR readily generates chimeric sequences from closely related microbes, giving an overestimate of the actual diversity in environmental samples. In one study, 30% of cloned sequences were shown to be chimeric.[18] Computer programs are used to screen for potentially chimeric sequences, but their accuracy dwindles when sequences are very novel. PCR is also prone to errors from contamination, and the assumption of the "universal"

nature of some primers may not be true. Cloning of PCR products to generate a library also carries an element of uncertainty because cloning efficiency may differ in a sequence-dependent manner. These and other uncertainties with PCR make the quantification of individual bacteria and species diversity difficult. Nonetheless, PCR and allied techniques, when properly used, are powerful tools in the study of sponge-microbe interactions. The problems and benefits of the use of molecular biological techniques in environmental microbiology have been reviewed by Head *et al.*[19]

Natural products in sponge-microbe symbiosis

Natural products chemists have been interested in sponge-microbe symbioses because of the diverse, bioactive chemical structures found in sponges.[20,21] To chemists, such symbioses are worthy of study both from a basic scientific and a biotechnological perspective. Chemists have sought to aid biologists in defining complicated taxonomy of sponges by using chemical markers, but such markers will be more complicated to apply if the chemicals actually arise from microbial symbionts. The compound source also affects theories and studies of biosynthesis, and thus chemical evolution. Finally, the scientific goal of classifying nature is left unfinished if the true sources of interesting compounds remain undetermined. From the biotechnological perspective, the source of potential drugs is a crucial financial question, since compounds are often found only in small amounts from animals that are difficult to obtain. If the compound proves to have a microbial origin, the microorganism could potentially be cultured, providing a reliable and cheaper source of the drug.

Unfortunately, despite the scientific and technological benefits of determining the source organisms of interesting and bioactive metabolites, a microbial origin of sponge compounds has been demonstrated only twice.[9,11] Most of the literature is

purely speculative, based on similarities that are often slight between compounds from sponges and those from cultivated microorganisms, especially cyanobacteria.[20] Several researchers have attempted to culture microorganisms from invertebrates in the hopes of obtaining some of these bioactive compounds.[22] Although they have been successful in the discovery of novel natural products, this research has not demonstrated the presence of sponge metabolites in the microbial isolates. These results demonstrate yet again the intractability of traditional culturing approaches to the environmental problems of sponge-microbe symbiosis. One research group has potentially accomplished this feat by finding the same compound in a *Hyatella* sp. sponge as in a *Vibrio* sp. cultured from that sponge.[23] There are several obvious problems with speculation about the source of compounds. Similar and identical compounds can arise from parallel or convergent evolution, or they can be simple digestive degradation of standard compounds, such as the dioxopiperazines formed by hydrolysis of peptides and proteins. Some sort of convergent evolution appears to explain the appearance of pyridoacridines in sponges and ascidians of various species.[24] Another potential problem, as reviewed by Hopwood, is transfer of genes.[25] Many biosynthetic enzymes are modular, and it is reasonable to assume that pieces of their genes, as well as entire gene clusters, may be moved by lateral gene transfer. Specialized genes encoding unusual functions could be swapped into the biosynthetic apparatus of wholly different organisms. A high frequency of plasmid transfers in the marine environment, as demonstrated by Dahlberg *et al.*,[26] amplifies the possibility of lateral gene transfer. Finally, it is premature to declare that because a compound has only previously been found in a single source such as a species of microbe, related compounds must come from the same microbe. It is more likely that other sources

have simply not yet been found, and in any case any speculation of source for a compound must be investigated in each instance.

The two demonstrated cases of symbiont production of sponge compounds both originate in work completed in the Faulkner laboratory. Both studies relied on cell fixation and physical separation techniques, bypassing the problem of culturing symbiotic microorganisms. Unson separated cyanobacterial symbionts from sponge *Dysidea herbacea* by flow cytometry and showed that chlorinated amino acid derivatives could only be found in the cyanobacterial fraction, while terpenes were localized in the sponge cell fraction.[9] The symbiont was typed as *Oscillatoria spongeliae* based on morphological characteristics. In another study, Bewley determined the cellular locations of two major bioactive metabolites, theopalauamide (9) and swinholide A (50), from the sponge *Theonella swinhoei*.[11] Contrary to the expectation that the metabolites would be produced by cyanobacteria, it was found that the modified peptide theopalauamide (9) was found only in filamentous microorganisms, while the polyketide swinholide A (50) was located solely in the unicellular bacterial fraction. *T. swinhoei* is a massive sponge that is packed with bacteria of many different species, so the exact microorganism producing swinholide A (50) could not be determined, but in TEM pictures the filamentous microorganism was thought to resemble *Beggiatoa* sp., which are γ-Proteobacterial sulfide oxidizers. Such bacteria are not known for their ability to synthesize complicated molecules, but because of their unusual metabolic requirement (reduced sulfur) they also have not been extensively studied by natural products chemists.

Despite a good deal of speculation on the microbial origin of sponge metabolites, relatively little has been conclusively demonstrated. The number of cell separation studies on sponges is increasing, but a complete understanding of the

source organisms of sponge compounds is still a distant goal. Molecular biological techniques, specifically the location of biosynthetic genes within certain cell types, are just beginning to be applied to the source question. However, no studies have addressed the evolution and ecological importance of sponge-microbe symbioses in the production of natural products. A key to starting to model these symbioses is to address the taxonomy of chemically important microbes in sponges. By 16S rRNA analysis of these microbes, phylogeny can be determined by statistical techniques. In addition, probes can be developed so that the presence or absence of these and related microbes in related sponges can be shown. This can lead to a better understanding of the meaning and evolution of particular symbioses and allow models to be advanced to explain less-studied sponge-microbe interactions.

The case for Lithistida as the model

Like the light emitted by bacteria (although more commonly not by symbionts) in some fishes and squid,[27] natural products can provide a convenient signal of bacterial-sponge symbiosis, provided that the tracer compounds are proven to be bacterially derived. Compounds from the order Lithistida are particularly good markers, since they are usually present in relatively large amounts, often have unusual bioactivities, and can be relatively easily purified. Many lithistid compounds are probably of microbial origin,[28] and in one case there is evidence supporting bacterial production.[11] In some cases, production of certain compounds seems species-specific, while other compounds are found in a number of totally unrelated lithistids, or even in whole other sponge orders. This opens the possibility of a large number of types, ages, and plasticities of symbiotic interactions. Finally, the polyphyletic diversity of the order may actually be of help in determination of the actual age and

Table 1. Distribution of selected "lithistid" metabolites.

compound type/name	source organism	location/source	filamentous microorganisms[*]
swinholides			
swinholide A	*Theonella swinhoei*	Indo-Pacific tropical[39,40]	Yes
	Ircinia sp.	Palau[31]	?
swinholide H	*Lamellomorpha strongylata*[†]	New Zealand[32]	Yes
	Tedania diversirapihidiphora	New Zealand[32]	?
ω-phenyl-β-amino acids I: theonellamide-type			
theonegramide	*Theonella swinhoei*	Philippines[41]	Yes
theonellamides	*Theonella* sp.	Japan, Philippines[42,43]	Yes
theopalauamide	*Theonella swinhoei*	Palau, Mozambique[40]	Yes
ω-phenyl-β-amino acids II: microsclerodermin-type			
microsclerodermins A and B	*Microscleroderma* sp.	Philippines, Hawaii[43,44]	Yes
microsclerodermins C and D	*Microscleroderma hardmani*	Philippines, NCI[45,46]	No, ?
	Theonella sp.	Philippines[45]	Yes
	Microscleroderma sp.	Philippines[45]	No
microsclerodermin E	*Theonella cylindrica*	? (NCI)[46]	?
	Aciculites ciliata[‡]	? (NCI)[46]	?
calyculin-type			
calyculin A	*Discodermia calyx*	Japan[33]	?
calyculins	*Lamellomorpha strongylata*[†]	New Zealand[32]	Yes
clavosines	*Myriastra clavosa*	Fiji[37]	?

Table 1 (continued). Distribution of selected "lithistid" metabolites.

compound type/name	source organism	location/source	filamentous microorganisms*
aurantoside-mozamide mixture	Theonella sp.	Japan[36]	?
	Theonellid	Mozambique[35]	Yes
	Plakinalopha spp.	Philippines[43]	Yes
		? (NCI)[46]	?
aciculitins	Aciculites orientalis	Philippines[47]	No
	Aciculites ciliata⧧	? (NCI)[46]	No
theonellapeptolides	Theonella sp.	Japan[48]	?
		? (NCI)[46]	?
	Lamellomorpha strongylata†	New Zealand[32]	Yes

* Filamentous, non-photosynthetic bacteria as seen by light microscopy.
† These compounds were all found in the same sample.
⧧ Compounds from the same sample.

specificity of symbiosis. For instance, a physical or biochemical factor common to unrelated lithistid sponges, rather than a species-specific factor, may promote the growth of certain types of bacteria. By attempting to construct molecular biological probes for the bacteria that produce lithistid compounds, symbiosis in this group of sponges can be characterized as a possible model for other sponge groups. The status of chemical research in the order lithistida as of early 1997 was reviewed by Faulkner and Bewley.[29]

Lithistid compounds often share characteristics with microbial metabolites, particularly with those of the cyanobacteria.[28,29] Once Bewley *et al.* showed that the major metabolites of *Theonella swinhoei*, a lithistid sponge, were localized in symbiotic bacteria, the hypothesis that many compounds in the order are microbe-derived gained some experimental weight. Only a massive series of cell-separation experiments on a large number of lithistid sponges could prove the hypothesis, but it is nonetheless a reasonable assumption when choosing targets of molecular biological research. It could be stated that Bewley only showed the existence of the compounds in the bacteria and not bacterial production, but it is reasonable to presume that large metabolites are not completely transferred from one cell type to another. Research on the biosynthesis of *T. swinhoei* metabolites is underway in the laboratory of Dr. Brad Moore (University of Washington) to answer this question definitively.

Bewley hypothesized that lithistid peptides containing ω-phenyl-β-amino acids were produced by filamentous microorganisms similar to the producer of theopalauamide (9) in *T. swinhoei* based on her observation of the coexistence of the two in a large number of lithistid sponges. The results of her survey are documented in a table in her thesis.[30] Thus, gross morpholgy of the symbiont as well as chemistry could be used as an entry in the selection of targets for molecular biology studies.

7, Mozamide A, R = H
8, Mozamide B, R = Me
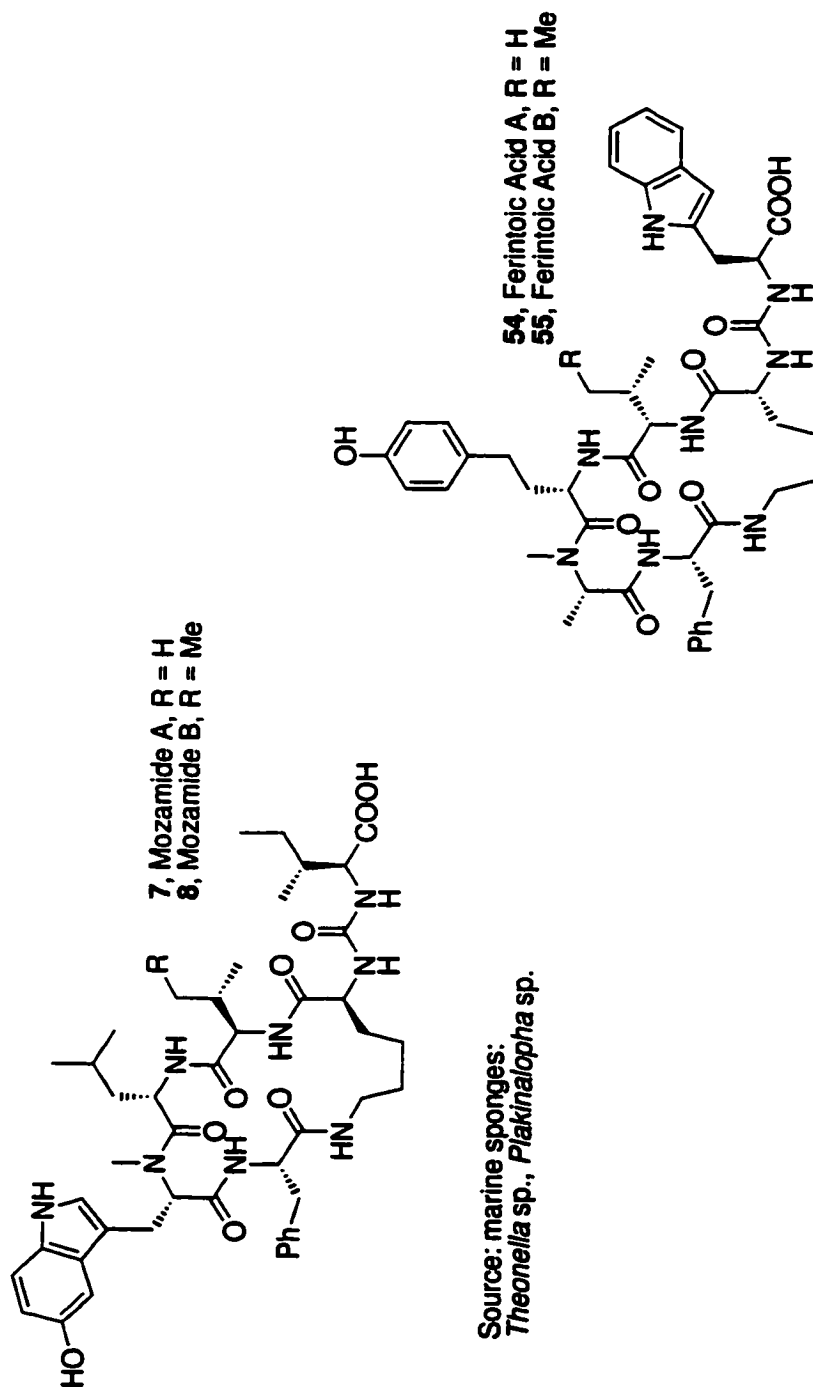
Source: marine sponges:
Theonella sp., Plakinalopha sp.

54, Ferintoic Acid A, R = H
55, Ferintoic Acid B, R = Me

Source: freshwater cyanobacterium:
Microcystis aeruginosa

Figure 1. Comparison of sponge compounds mozamides[35] with bacterial products ferintoic acids.[37]

44, Aurantoside A
Source: marine sponge
Theonella sp.

56, Streptolydigin
Source: eubacterium
Streptomyces sp.
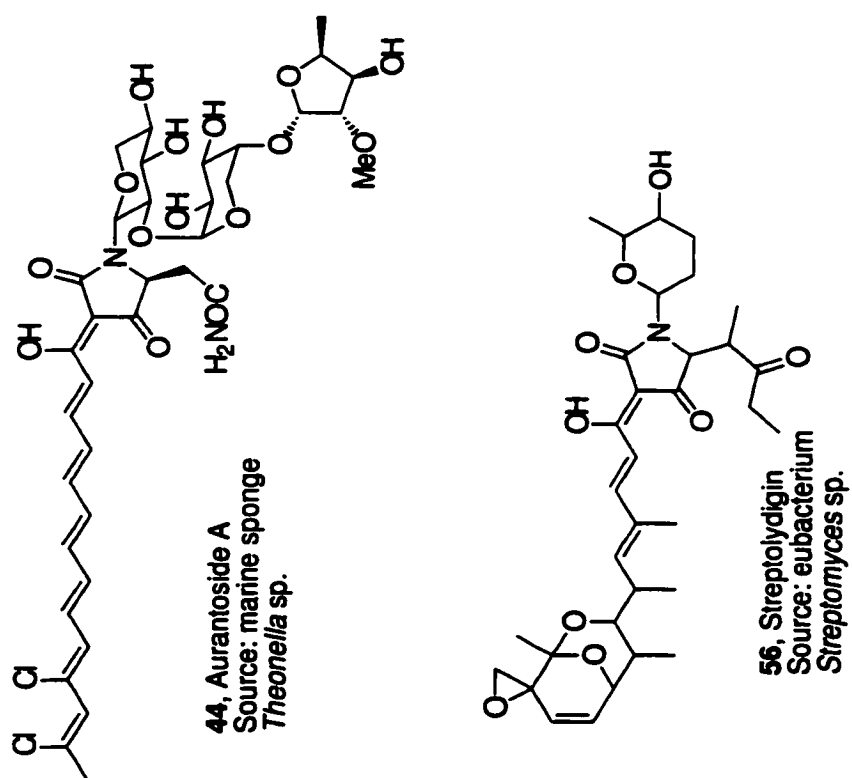
Figure 2. Comparison of sponge compound aurantoside A[36] with bacterial product streptolydigin.[38]
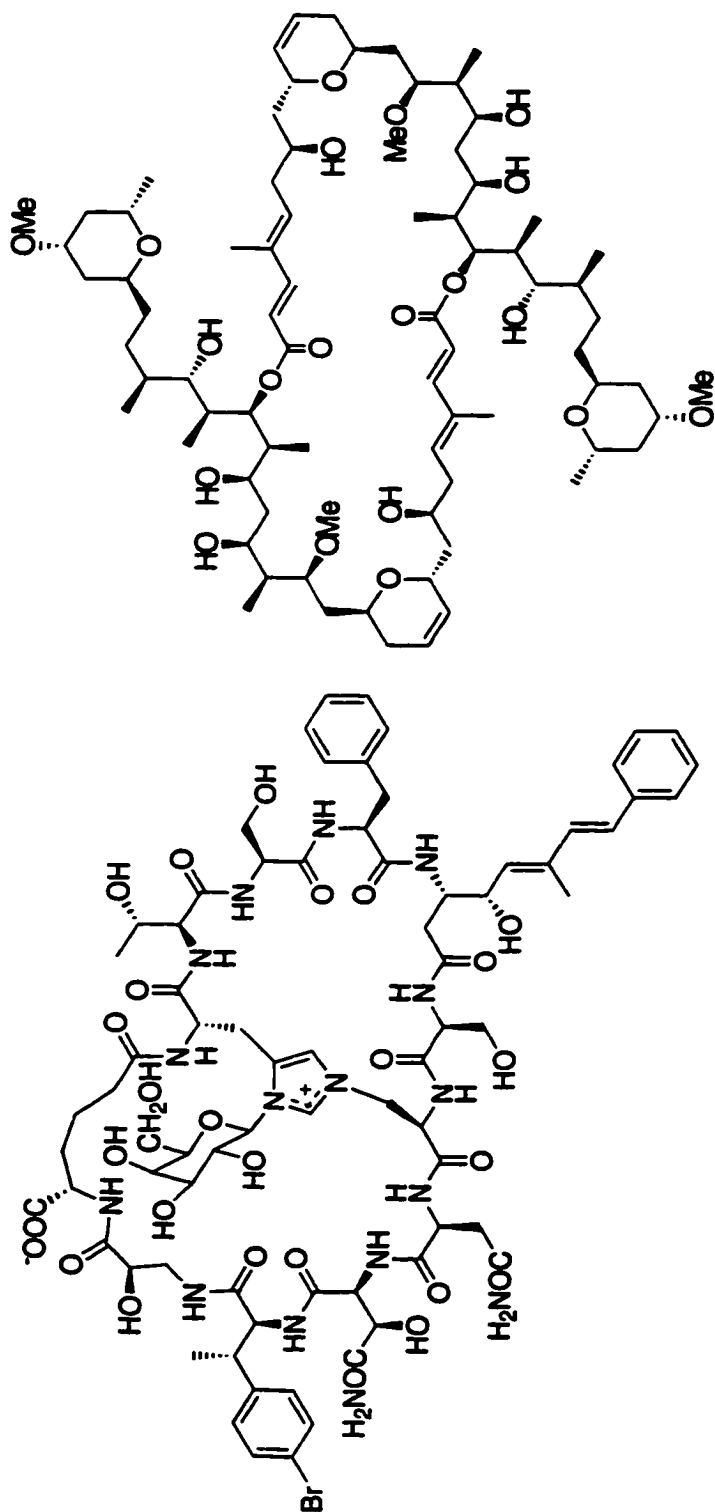
Figure 3. Metabolites from microbial symbionts of *Theonella swinhoei*. Theopalauamide (left) from filamentous microorganisms and swinholide A from unicellular microbes.

However, I have found that peptide-producing sponges do not always contain filamentous microorganisms (Table 1). For example, I have found representatives of *Microscleroderma* sp. containing microsclerodermins that are wholly lacking in filamentous microorganisms. In addition, as Bewley first noted, samples of *Aciculites* sp. containing compounds in the aciculitin family (**42, 43**) do not contain filamentous microorganisms. The potential for a specific symbiosis in this group is revealed by chemistry, since the bacteria lack unusual morphologies like the filaments of *T. swinhoei*.

Another reason for choosing chemical rather than biological markers is the large repository of sponge extracts available through the National Cancer Institute (see Chapter 7). Once the taxonomy of organisms producing certain compound classes are determined, the degree of specificity can be checked against the NCI database, and hypotheses of specificity of symbiosis can be ruled out or possibilities of lateral gene transfer can be revealed. A particular advantage of using this system is that once pure compounds can be screened in the NCI's 60 cell-line panel, the pattern of activity can be used to detect similar compounds in other lithistids or in the whole database. If a sponge metabolite has been localized to a bacterial symbiont, the specificity of the metabolite to the host species can be checked by its activity profile in the database or by ordering the extracts and isolating compounds. This would be particularly useful for closely related organisms with similar bioactivity profiles. For example, if the microsclerodermins are ever localized to bacteria, their existence in sponges of the genera *Microscleroderma* sp., *Aciculites* sp., and *Theonella* sp., revealed through the NCI database (Chapter 7), may indicate a relatively non-specific symbiosis based on some common physical or biochemical factor. This possibility could be probed in part by comparing SSU rRNA sequences obtained from the different sponges, looking for
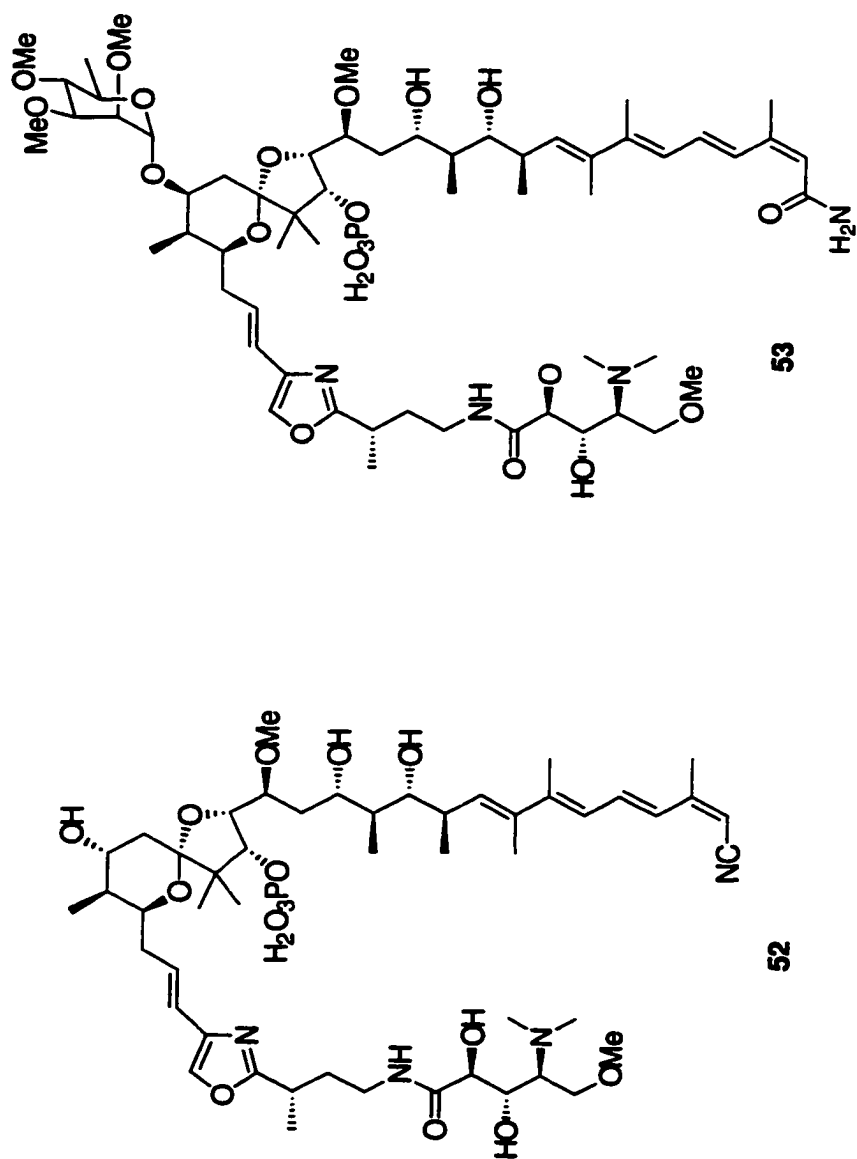
Figure 4. Comparison of the lithistid metabolite calyculin A (left) and clavosine A from *Myriastra clavosa*.

identical RFLP patterns and thus attempting to identify the microsclerodermin-producing organism. The organisms in the NCI repository have also been taxonomically identified by experts, which could save the investigator a significant amount of effort. In return, the NCI can be provided with pure, bioactive compounds for screening. Using chemical markers, results of symbiosis studies can also be compared with those reported in the literature, assuming that the producing macroorganism is correctly identified.

As with compounds that were thought to be produced by filamentous microorganisms, the picture obtained for other metabolites (mainly polyketides) becomes more confused as more data is gathered. The key example in this group of compounds is swinholide A (50), which was localized by Bewley in unicellular bacterial fractions of *T. swinhoei*. The compound and a related chemical were subsequently found in two sponges which are unrelated: *Ircinia* sp. and *Tedania diversiraphidiphora* from the order Dictyoceratida[31,32] and *Lamellomorpha strongylata* from the order Epipolasida.[32] In the absence of lateral transfer of biosynthetic genes or in the unlikely event that the complex structure could be reached by convergent evolution, these results indicate that the symbiosis leading to the production of swinholide is not species- or even order-specific. Unfortunately, it is difficult to track down which bacterial species is actually producing swinholide. Because of the problem of specificity in the production of swinholide and the difficulty in determining its producing organism, peptides are the obvious first choice for studying symbiosis. Another instance of polyketide metabolites found in many different species occurs with calyculins (52)[33] and the clavosines (53)[34] from *Discodermia calyx* and *Myriastra clavosa*, respectively, which are sponges from

different orders entirely. These similar compounds and related metabolites are found in a number of apparently unrelated sponges. It would be interesting to determine if similar compounds are always produced by similar bacteria, or if convergent evolution or lateral gene transfer are possibilities. Studies of these hypotheses become accessible through the combination of chemical and biological data. Ultimately, such studies could lead to a better understanding of the evolutionary importance of these compounds and to better culturing methods for non-specific "symbionts".

The polyphyletic nature of the order Lithistida may confound early attempts at rationalizing the evolution of symbiosis. A clearer picture of sponge phylogeny could emerge with increasing understanding of symbiotic and chemical specificity, but it is more likely that symbiosis will not be understood until the phylogeny of the sponges is well defined. Michelle Kelly-Borges is currently heading a massive effort involving 23S rDNA analysis, preliminary results of which are already available.[12] For instance, sponges from the genera *Plakinalopha* and *Theonella*, formerly thought to be completely unrelated, are actually closest relatives: some *Theonella* spp. are more closely related to certain *Plakinalopha* spp. than to other theonellids. This clarifies chemical observations that some members of both *Theonella* sp. and *Plakinolopha* sp. contain aurantosides (**44**) and mozamides (**7, 8**) or related peptides as their major bioactive metabolites (see Chapter 7). The compound classes are very similar to compounds produced by streptomyces and cyanobacteria, respectively, and it would be interesting to determine whether or not the compounds are actually produced by bacteria and whether such a symbiosis holds in members of both sponge genera.

Proposal for a pilot study

Lithistid sponges are clearly preferred candidates for the study of sponge-microbe symbiosis. A pilot study using this order requires a representative for which

the producing microorganism has been identified and found to have distinct morphology. *T. swinhoei*, in which filamentous microbes are known to produce theopalauamide (9), meets these requirements. A starting point for investigation into this symbiosis is the phylogenetic identification of the bacterial symbiont using 16S rRNA analysis. If the bacterium is closely related to known strains from other sources, then the sponge-microbe interaction would probably be non-specific or evolving over a short timescale. Conversely, if the sequence is relatively unique and seems to be confined to certain sponges, then the symbiosis could be hypothesized to be species-specific, although related microbes could be found elsewhere at any time, immediately disproving the hypothesis. Such specificity indicates a long-term co-evolution, especially if it occurs only in members of the same family, which may be highly diverged from each other.

The next step, once the 16S rRNA molecule of the filamentous microorganisms has been sequenced and compared with database sequences, is probing the specificity of the symbiosis. Sequence-specific probes can be applied to sponges containing theopalauamide (9) and related compounds, as well as those with different chemistry. Increasing sequence divergence with changing chemistry or sponge hosts will reveal the specificity of the sponge-microbe relationship. In this way, chemistry and molecular biology can be used together to define the age and specificity of the symbiosis in *T. swinhoei*.

References

1. Riding, R.; Zhuravlev, A. Y. *Geology*, **1995**, *23*, 649-652.

2. Brunton, F.R.; Dixon, O.A. *Palaios* **1994**, *9*, 370-387.

3. Zhang, X-g.; Pratt, B.R. *Geology* **1994**, *22*, 43-46.

4. De Freitas, T.A. *Can. J. Earth. Sci.* **1991**, *28*, 2042-2061.

5. Turon, X.; Galera, J.; Uriz, M.J. *J. Exp. Zoology* **1997**, *278*, 22-36.

6. Wilkinson, C.R. *Proc. R. Soc. Lond. B* **1984**, *220*, 509-517.

7. Beer, S.; Ilan, M. *Mar. Biol.* **1998**, *131*, 613-617.

8. Schumann-Kindel, G.; Bergbauer, M.; Manz, W.; Szewzyk, U.; Reitner, J. *Facies* **1997**, *36*, 268-272.

9. Unson, M.D.; Holland, N.D.; Faulkner, D.J. *Mar. Biol.* **1994**, *119*, 1-11.

10. Wilkinson, C.R. *Symbiosis* **1987**, *4*, 135-146.

11. Bewley, C.A.; Holland, N.D.; Faulkner, D.J. *Experientia* **1996**, *52*, 716-722.

12. McInerney, J.; Kelly-Borges, M. Origin & Outlook: 5th International Sponge Symposium Abstracts, 1998.

13. Vacelet, J.; Donadey, C. *J. Exp. Mar. Biol. Ecol.* **1977**, *30*, 301-314.

14. Vacelet, J.; Fiala-Médioni, A.; Fisher, C.R.; Boury-Esnault, N. *Mar. Ecol. Prog. Ser.* **1996**, *145*, 77-85.

15. Müller, W.E.G.; Zahn, R.K.; Kurelec, B.; Lucu, C.; Müller, I.; Uhlenbruck, G. *J. Bact.* **1981**, *145*, 548-558.

16. Preston, C.M.; Wu, K.Y.; Molinski, T.F.; DeLong, E.F. *Proc. Natl. Acad. Sci. USA*, **1997**, *93*, 6241-6246.

17. Althoff, K.; Schütt, C.; Steffen, R.; Batel, R.; Müller, W.E.G. *Mar. Biol.* **1998**, *130*, 529-536.

18. Wang, G.C.Y.; Wang, Y. *Appl. Env. Microb.* **1997**, *63*, 4645-4650.

19. Head, I.M.; Saunders, J.R.; Pickup, R.W. *Microb. Ecol.* **1998**, *35*, 1-21.

20. Kobayashi, J.; Ishibashi, M. *Chem. Rev.* **1993**, *93*, 1753-1769.

21. Faulkner, D.J.; He, H.Y.; Unson, M.D.; Bewley, C.A. *Gazz. Chim. Ital.* **1993**, *123*, 301-307.

22. Pietra, F. *Nat. Prod. Reports* **1997**, *14*, 453-464.

23. Oclarit, J.M.; Tamaoka, Y.; Kamimura, K.; Ohtan, S.; Ikegami, S. in *Sponge Sciences: Multidisciplinary Perspectives*. Watanabe, Y.; Fusetani, N., eds. Springer-Verlag, Tokyo, 1998, pp. 391-398.

24. Salomon, C.E., personal communication.

25. Hopwood, D.A. *Chem. Rev.* **1997**, *97*, 2465-2497.

26. Dahlberg, C.; Bergström, M.; Hermansson, M.; *Appl. Environ. Microbiol.* **1998**, *64*, 2670-2675.

27. Haygood, M.G.; Distel, D.L. *Nature* **1993**, *363*, 154-156.

28. Fusetani, N.; Matsunaga, S. *Chem. Rev.* **1993**, *93*, 1793-1806.

29. Bewley, C.A.; Faulkner, D.J. *Angew. Chem. Int. Ed. Engl.* **1998**, *37*, 2162-2178.

30. Bewley, C.A. Ph.D. Thesis, pp 112. University of California, San Diego, 1995.

31. Faulkner lab, unpublished data.

32. Dumdei, E.H.; Blunt, J.W.; Munro, M.H.G; Pannell, L.K. *J. Org. Chem.* **1997**, *62*, 2636-2639.

33. Kato, Y.; Fusetani, N.; Matsunaga, S.; Hashimoto, K. *J. Am. Chem. Soc.* **1986**, *108*, 2780-2781.

34. Fu, X.; Schmitz, F.J.; Kelly-Borges, M.; McCready, T.L.; Holmes, C.F.B. *J. Org. Chem.* **1998**, *63*, 7957-7963.

35. Schmidt, E.W.; Harper, M.K.; Faulkner, D.J. *J. Nat. Prod.* **1997**, *60*, 779-782.

36. Matsunaga, S.; Fusetani, N.; Kato, Y.; Hirota, H. *J. Am. Chem. Soc.* **1991**, *113*, 9690-9692.

37. Williams, D.E.; Craig, M.; Holmes, C.F.B.; Andersen, R.J. *J. Nat. Prod.* **1996**, *59*, 570-575.

38. Rinehart, K.L.; Beck, J.R.; Borders, D.B.; Kinstle, T.H.; Krauss, D. *J. Am. Chem. Soc.* **1963**, *85*, 4038-4039.

39. Kitagawa, I.; Kobayashi, M.; Katori, T.; Yamashita, M.; Tanaka, J.; Doi, M.; Ishida, T. *J. Am. Chem. Soc.* **1990**, *112*, 3710.

40. Schmidt, E.W.; Bewley, C.A.; Faulkner, D.J. *J. Org. Chem.* **1998**, *63*, 1254-1258.

41. Bewley, C.A.; Faulkner, D.J. *J. Org. Chem.* **1994**, *59*, 4849.

42. Matsunaga, S.; Fusetani, N; Hashimoto, K.; Wälchli, M. *J. Am. Chem. Soc.* **1989**, *111*, 2582-2588.

43. unpublished data

44. Bewley, C.A.; Debitus, C.; Faulkner, D.J. *J. Am. Chem. Soc.* **1994**, *116*, 7631.

45. Schmidt, E.W.; Faulkner, D.J. *Tetrahedron* **1998**, 54, 3043-3056.

46. This structure was introduced in Chapter 7.

47. Bewley, C.A.; He, H.; Williams, D.H.; Faulkner, D.J. *J. Am. Chem. Soc.* **1996**, *118*, 4314.

48. Kobayashi, M.; Lee, N.K.; Shibuya, H.; Momose, T.; Kitagawa, I. *Chem. Pharm. Bull.* **1991**, *39*, 1177-1184.

CHAPTER 10

THE PEPTIDE-CONTAINING SYMBIONT OF

THE MARINE SPONGE *THEONELLA SWINHOEI* IS A NOVEL

δ-SUBDIVISION PROTEOBACTERIUM, "*CANDIDATUS*

ENTOTHEONELLA PALAUENSIS"

The SSU rRNA gene sequence of a filamentous eubacterium specific to the Palauan marine sponge *Theonella swinhoei* was isolated using cell separation, denaturing gradient gel electrophoresis (DGGE) and DNA sequencing. Computer database searches and phylogenetic analysis programs showed that the bacterium, which contains the complex bicyclic glycopeptide theopalauamide, is novel at the genus level and is most closely related to the myxobacteria (Proteobacteria; δ-subdivision). Specific primers derived from the sequence found in the Palau specimen were used to amplify closely related sequences from other members of *T. swinhoei* that contain slightly different peptides, indicating that slightly different chemistry is correlated with different bacterial strains. The sequence was shown to originate in the filamentous bacteria by *in situ* hybridization.

Introduction

Sponge-microbe symbioses are common,[1] and some may have ancient origins, dating back to before the differentiation of modern sponge orders more than 500 MYA.[2] The symbioses have been widely studied, but the significance of individual bacterial species associated with sponges has been difficult to probe. The key questions of the age, stability, and specificity of sponge-microbe symbiosis remain largely unanswered. In traditional symbiosis studies , bacteria were either cultured[3] or classified according to their morphologies. [4] Unfortunately, pure culture isolation is

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often difficult for many obligate symbionts, and morphological characteristics can be misleading. More recently, a number of studies have relied on SSU rRNA analysis to determine the phylogeny and specificity of bacteria in sponges,[5-7] but rRNA sequences rarely provide enough information about the ecological functions of these bacteria. A major problem is that no convenient marker is available for significant symbiosis within sponges that would allow biochemical processes to be attributed to specific bacteria, in contrast to bioluminescent symbioses or clear nutrient-transfer interactions such as those found with corals.

With the discovery that the glycopeptide theopalauamide from the sponge *Theonella swinhoei* is located in filamentous bacteria, it became possible to probe this symbiosis using natural products chemistry in addition to morphology.[8] *T. swinhoei* is a representative of the polyphyletic sponge order Lithistida, which has many members that could also be useful in probing symbiosis if their compounds are microbially derived, as predicted based on chemical structure.[9-11] Several of the compounds in this sponge order resemble microbial chemicals, and sometimes similar chemicals also exist in other, unrelated sponges or in bacteria (see Chapter 9 for a full discussion). As the first sponge in this order for which a microbe was shown to contain a natural product, *T. swinhoei* provides a good model to test the usefulness of the chemical marker in sponge-microbe symbiosis studies.

The first step in understanding this symbiosis is identifying the phylogenetic affiliation of the bacterial strain. On the basis of morphology, some investigators assumed that the filaments were cyanobacteria,[9] but Bewley *et al.* [2,13] demonstrated that the filaments did not contain photosynthetic pigments and did not require light for growth and hypothesized their placement in the family Beggiatoaceae on the basis of morphology only. Since the bacteria could be readily enriched by centrifugation and

were easily recognized by morphology and chemistry, SSU rRNA analysis was chosen to determine the phylogenetic affiliations of the bacteria. Once PCR primers specific to the strain were in hand, the specificity and extent of symbiosis could be studied as an additional benefit to this method. Based on the retrieved sequence, several culture media were also tried.

Collection of sponge material and isolation of filamentous bacteria

Representatives of *T. swinhoei* were collected from 10-30 m depth at four different locations along the eastern side of Palau, Western Caroline Islands, in 1997. The presence of theopalauamide and filamentous bacteria in the sponges was assayed by thin layer chromatography and light microscopy, respectively, in laboratories at the Coral Reef Research Foundation, Koror, Palau. Most of the sponge was used for the separation of filamentous bacteria from sponge material and other bacterial cells following a method similar to that of Bewley *et al.*,[2] except that fixatives were not used. First, the cyanobacteria-containing ectosome of the sponge was removed using a razor blade, then the sponge interior was cut up and either put through a juicer or diced with a razor blade. The resulting cell suspensions were centrifuged several times at ~200 g to purify the filamentous bacteria.

In 1998, several *T. swinhoei* samples containing the related peptide theonegramide were collected from northern Panay and Boracay islands, Philippines. A single sample of the lithistid sponge *Aciculites* sp., containing aciculitin B, was also collected. The ectosome of these samples was removed, and sponge pieces were fixed either in paraformaldehyde, followed by an ethanol dehydration series, or immediately placed in ethanol for DNA preservation. A single sample of *T. swinhoei* was collected, and pieces were immediately added to sterile culture tubes for culture experiments.

Several more representative sponges (*T. swinhoei*) were recollected in Palau in 1998, and these sponges were purified through 200 micron and 40 micron sieves for improved enrichment of filamentous cells. These preparations were used in further culture experiments and were observed at the time of collection to have a morphology consistent with a reproductive process (Figure 1). The cells were placed in either sterile seawater, 1/10 Difco marine broth diluted in seawater, or dilutions of aqueous sponge extract (sterile filtered). Within two days, the material was brought back to San Diego and used in further experiments.

Storage of sponge tissue and purified filaments

Fresh sponge tissue was fixed in 4% paraformaldehyde (MOPS-buffered) for several hours, then dehydrated to 95% ethanol in a dehydration series. Both sponge tissue and filamentous bacterial preparations were frozen for later analysis. DNA from both whole sponge tissue and enriched filaments was fixed in 100% ethanol or guanidinium isothiocyanate buffer. Finally, DNA was extracted immediately after sponge collection or filament enrichment using a QIAamp tissue kit™ (QIAGEN).

Chemical analysis

Sponge samples used in this project were analyzed using previously reported techniques (Chapter 7). Known peptides (~0.1-10 mg) were purified to at least 90% purity by extraction and column chromatography and identified by comparing their ^{1}H NMR spectra and electrospray ionization mass spectra to literature data. These methods do not preclude the possibility that some compounds may be isomers of known metabolites, although the differences are probably minor. Natural products relevant to this study are shown in Figure 2.

PCR amplification, purification, and sequencing

The SSU rRNA genome was amplified from samples collected in 1997 using the universal bacterial primers 27f and 1492r and standard PCR techniques.[14] PCR purification was performed using the QIAQuick kit™ (Qiagen). Standard PCR techniques used in this study are: 100 nM each primer, 1 unit Taq, 1 x Mg buffer, 400 nM dNTP solution; 30 cycles of 94 °C, 1 min; 50 °C, 1 min; 72 °C 1 min. Cloning was attempted using the TA cloning kit,™ but no majority sequence emerged from the first 12 clones screened despite abundant filaments (Figure 1). Denaturing gradient gel electrophoresis (DGGE), which separates gene fragments on the basis of sequence instead of size, was used to find the majority sequence in the enriched filamentous preparation.[15,16] Using universal bacterial primers (1055f, 1392r) designed by Ferris et al.,[17,18] ~350 bp fragments of SSU rDNA from whole sponge and enriched filament DNA extracts were amplified by PCR. Two strategies were employed: the DGGE primers were used either on the crude DNA extract or on SSU rDNA sequences that had been previously amplified with 27f-1492r bacterial primers (Figure 3). Bands corresponding to different sequences were separated on a denaturing gradient acrylamide gel using either 0-100% denaturant or 35-65% denaturant solution. Bands were visualized with Sybr-green™ fluorescent stain and excised from the gel with a razor blade. DNA was re-extracted overnight in 1 x SSC buffer at 37 °C, and gel fragments were removed by centrifugation. The DNA was then precipitated at -20 °C for 15 minutes in 10 M LiCl (1/10 vol/vol) and 95% EtOH (2 vols). DNA was re-amplified by PCR and sequenced using the DGGE primers and ABI-Prism™ dye terminator cycle-sequencing. Forward and reverse sequences were aligned using the Sequencher™ and AssemblyLIGN™ programs and verified by eye.

Figure 1. A) Enriched filamentous bacteria (200x) used in this study.

Figure 1. B) Epifluorescence and light micrograph of purified filamentous bacteria (1000x) used in this study, showing a single (rare) fluorescing filamentous cyanobacterium among the enriched unpigmented symbionts.

Theopalauamide R_1 = CH$_3$, R$_2$ = OH, R$_2$ = H, X = galactose
Theonegramide R_1 = CH$_3$, R$_2$ = OH, R$_2$ = H, X = arabinose
Theonellamide A R$_1$ = H, R$_2$ = H, R$_3$ = OH, X = galactose
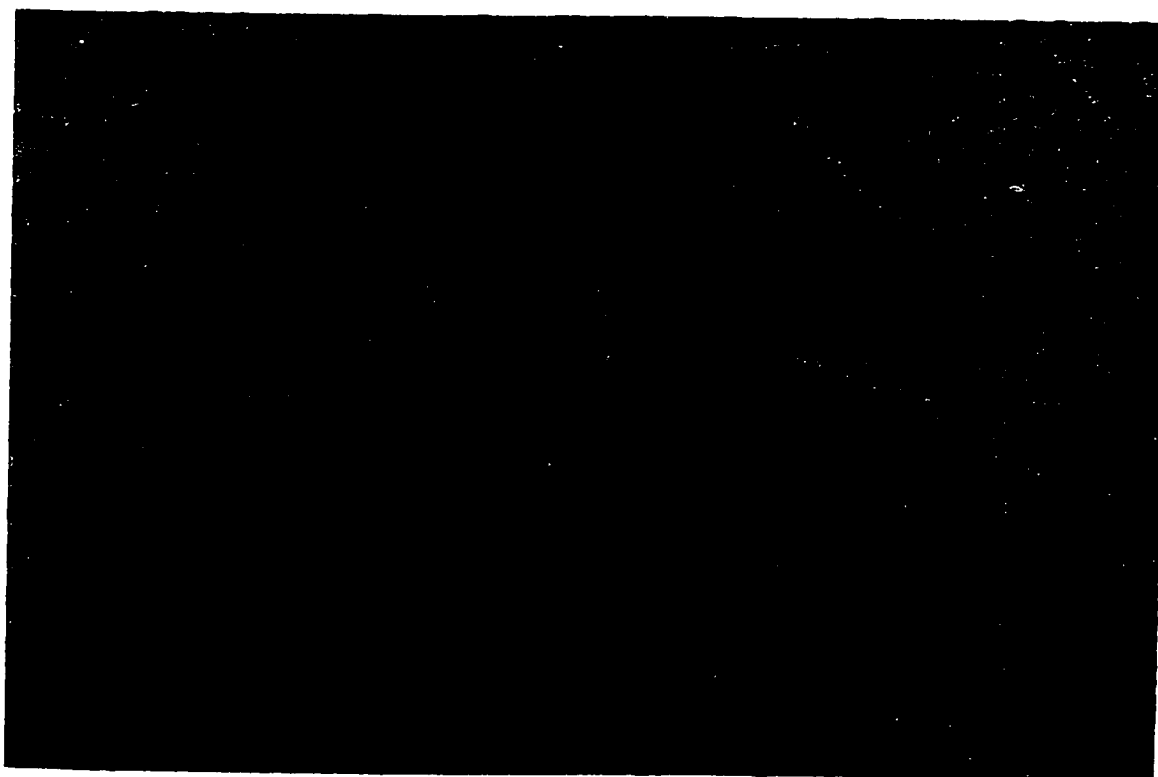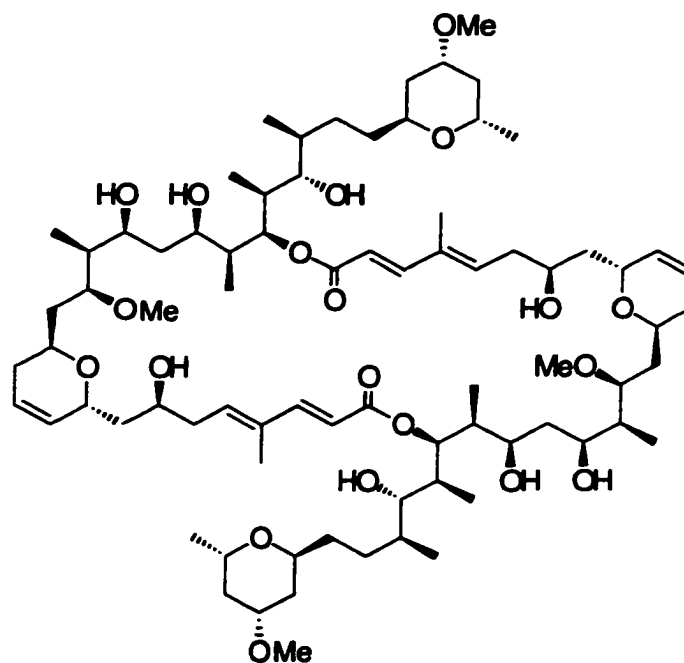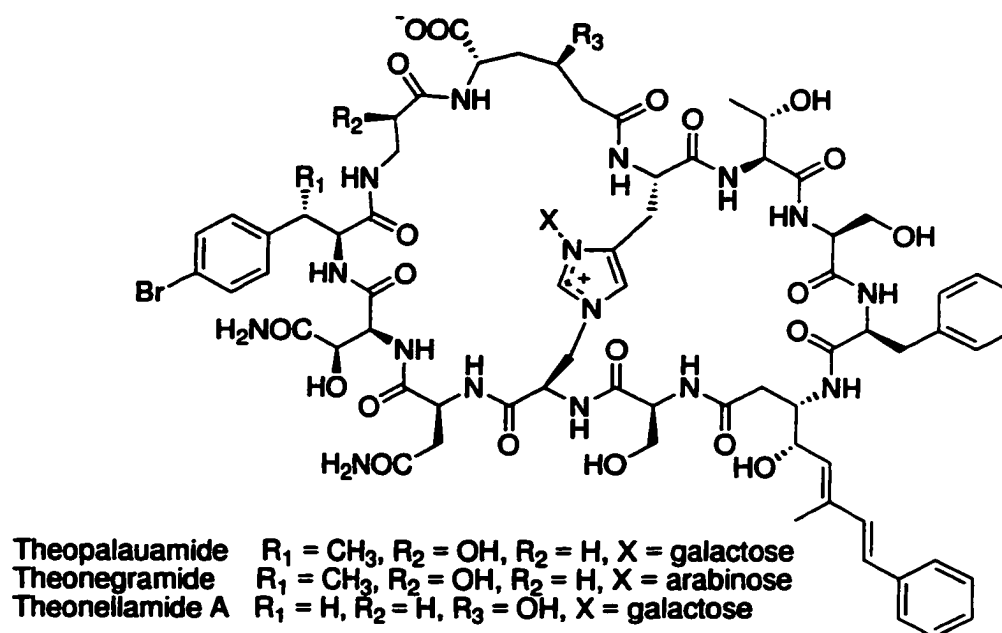
Figure 2. Natural products of *Theonella swinhoei*: theopalauamide, theonegramide, and theonellamide A (top); swinholide (bottom).
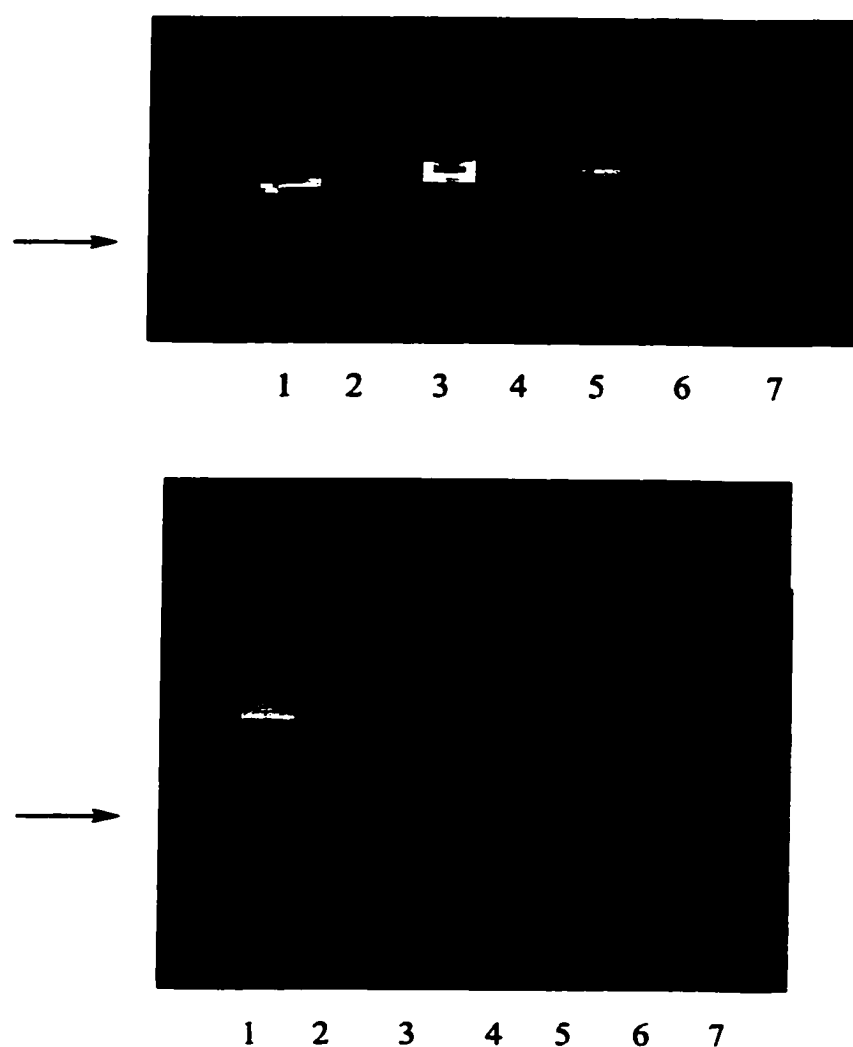
Figure 3. Upper: DGGE of positive control (lanes 1,3,5), purified filamentous (lanes 2,4) and whole sponge (lanes 6,7) DNA extracts which were twice amplified by PCR (left). Lower: DGGE of positive control (lane 1), bacterial DNA from *Bugula neritina* larvae (lane 2), no DNA control (lane 3), and purified filamentous (lanes 4,5) and whole sponge (lanes 6,7) DNA extracts which had been amplified once by PCR using DGGE primers (right). Positive control: *Vibrio fischeri* SSU rRNA.

Specific primer design

From a sequence obtained from a major DGGE band amplified from the filamentous bacterial DNA extract, a 16-bp region of variable DNA was identified and shown to be different by at least two base pairs from all other known sequences using CheckProbe (Ribosomal Database Project).[19] A primer for PCR and an *in situ* hybridization probe was based on this sequence and named 1247r (Figure 4).

Amplification and sequencing of other regions of the SSU rRNA gene

PCR products corresponding to 27-1492 (*E. coli*) from filamentous and whole sponge DNA extracts were purified and amplified using the primers 27f and 1247r. When variable region primers were used, an annealing temperature of 54 °C was used instead of 50 °C. A single, pure sequence was obtained from the filamentous preparation amplified with 27f-1247r, while the whole sponge preparation did not give a clean sequence with 27f-1247r (Table 1). The product from the filament preparation was sequenced with the 1247r primer, and the following universal bacterial sequencing primers were used: 27f, 342r, 530f, 690r, 926f, and 1101r. Clean sequence was obtained from every primer, as judged by alignment using Sequencher™ and AssemblyLIGN™ and by eye. The sequence was unlikely to be a chimera due to the way in which it was obtained, but the sequence was also screened using ChimeraCheck (RDP).[20] The final edited sequence of 1335 bp corresponds to positions 28-1391 of the *E. coli* sequence. This sequence was derived from the 27f-1247r and purified DGGE symbiont band (1055f-1392r) PCR products.

Phlyogenetic analysis

Initial comparisons of the 1335 bp sequence from the filamentous bacteria were performed using the Ribosomal Database Project's (RDP) similarity rank and the National Center for Biotechnology Information BLAST search.[20] BLAST searches

Domain Bacteria oligonucleotides

Eub338[25]   5'-GCT GCC TCC CGT AGG AGT-3' (5'-biotinylated for FISH)
1055f[17]   5'-ATG GCT GTC GTC AGC T-3'
1392r[17]   5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG
CCC CAC GGG CGG TGT GTA C-3'

"E.palauensis"-specific oligonucleotides

238f   5'-CCG GTC TGA GAT GAG CTT GC-3'
1247r   5'-AAG TAG CGG CCC TTT GTC-3' (also 5'-biotinylated for FISH)

Control primer

Bncontrol[25]   5'-ACG TCA CCG TCC AGC CTC T-3' (5'-biotinylated for FISH)

Figure 4. PCR primers and FISH probes used in this study. Universal bacterial primers (top), symbiont-specific primers (middle), and control, primer (bottom).

revealed closest similarity with members of the delta-subdivision Proteobacteria (85% of 1176 alignable bps), with an unidentified delta-subdivision Proteobacterium from deep sea sediments (AB015560) and *Pelobacter acetylenicus* (PAC16SRNA) ranking highest. RDP also showed that Proteobacteria were the closest relatives, with the Sequence Align program giving *P. acetylenicus* as the highest match. Using Sequence Match, the highest similarity score for a classified bacterium was 0.526 for a member of the Pedomicrobium assemblage (α-subdivision Proteobacteria). Further analysis was performed using the PAUP 4.01 program, with heuristic, branch and bound, and exhaustive searches using maximum parsimony and maximum likelihood algorithms.[21] The Phylip™ program was employed with distance data using neighbor joining and maximum likelihood programs to construct trees.[22-24] An initial evaluation of relationships between the filament sequence and representatives of the domain Bacteria was performed using Phylip neighbor joining and bootstrapping, and a series of trees were obtained in which the symbiont sequence was most closely related to the δ-subdivision Proteobacteria. In PAUP, a maximum parsimony tree was then constructed with the branch and bound search algorithm using *Aquifex pyrophilus* (outgroup), *Acidobacter capsulatum*, *Fusobacterium nucleatum*, and ten members of the α-, β-, and γ-Proteobacteria. Eleven members of the δ-subdivision of Proteobacteria were also used: *Bdellovibrio stolpii*, *Desulfovibrio desulfuricans*, *Myxococcus xanthus*, *Stigmatella aurantiaca*, *Desulfobacter postgatei*, *Nannocystis exedens*, *Chondromyces crocatus*, *Geobacter metallireducens*, *Pelobacter acetylenicus*, *Desulfuromonas acetoxidans*, and *Desulfovibrio baarsii*. With these taxa, the symbiont was joined as the sister taxon to the order Myxococcales. Bootstrapping was performed using either 1126 alignable characters or 512 informative characters and using a 2:1 transversion:transition cost matrix. In the

former case, the symbiont was grouped as a sister taxon to Myxococcales with a bootstrap value (100 replicants) of 88%; in the later case, the value was 77%. RI and CI index values for the best tree were 0.40 and 0.38, respectively, for a tree containing 25 taxa with 2640 steps without the transition/transversion cost matrix. The length of the tree using the transversion cost matrix was 3857 steps (Figure 5). An exhaustive search (PAUP) using maximum parsimony with just *E. coli*, *A. pyrophilus*, and the δ-subgroup Proteobacteria placed the symbiont in the same position neighboring the myxobacteria (Figure 6).

In situ hybridization

Sponge tissue collected and fixed in the Philippines in 1998 was used for *in situ* hybridization, following protocols outlined by Haygood and Davidson.[25] The following 5'-biotinylated probes were used: a universal bacterial probe (positive control),[25] a random probe (negative control), [25] and a 1247r sequence-specific probe. *T. swinhoei* tissue, stored in ethanol at 4 °C, was diced into small pieces (approximately 2 mm^3) using a razor blade, and the pieces were placed in phosphate buffered saline (PBS) solution (500 μL) in Eppendorf tubes. The PBS was removed, then PBS-0.1% Tween-20 (500 μL) was added, and tissue was treated with Proteinase K (4 μg) for 10 minutes. The digestion was quenched with 10% glycine solution (aq, 40 μL) and an additional 600 μL PBS-Tween. After removal of the solution, 4% paraformaldehyde solution (500 μL) was added, and the tissue was refixed for 1 hour. The solution was decanted, and triethanolamine solution (500 μL) was added and left for five minutes. The solution was decanted, and triethanolamine solution (500 μL) was added and left for 10 minutes, after which acetic anhydride (1.25 μL) was added. After 10 minutes, a TEA/Ac$_2$O solution was added and left for 10 more minutes. The solution was decanted and rinsed in 500 μL PBS-TWEEN 3 times for 10 minutes

each. The tissue was then rinsed in the hybridization buffer (200 µL) briefly. The solution was decanted, and an additional 200 µL of hybridization buffer was added and heated to 55 °C for two hours. Each probe solution (8 µL of 50 ng/µL) was added to tissue in separate tubes. The hybridization solutions were incubated at 43, 47, 50, and 55 °C for 5 hours, after which time the solutions were decanted. Wash buffer 1 (see "solutions and media" section of this chapter) was briefly rinsed over each sample, then decanted, then 500 µL of buffer 1 was incubated with each sample at the hybridization temperature for 30 minutes. After decanting, wash buffer 2 (500 µL) was added and left at 37 °C for 30 minutes and decanted. Samples were incubated with wash buffer 3 at room temperature for 30 minutes. Signal was amplified using the TSA GREEN-FISH kit,[TM26-27] following the maunfacturer's recommendations, and the samples were crushed with a razor blade and mounted on glass slides using Vectashield.[TM] Samples were viewed by microscopy under epifluorescence. The sample containing the filament-specific probe only hybridized with the filamentous microorganisms and not other microbes in the samples at 47 °C and 50 °C, while results were less clear at other temperatures. With the positive control probe, all bacterial cell types fluoresced, while with the negative control probe only a dim background fluorescence was visible. Best results were obtained with a six-hour hybridization at 48.5 °C (Figure 7).

PCR using other sponge DNA

To test the specificity of the derived sequence, filament-specific primers were used in PCR with several closely and distantly related lithistid sponge species. Ethanol-preserved sponge tissue (*T. swinhoei, Aciculites* sp.) from the 1998 Philippines collection was processed using the QIAamp tissue kit.[TM] Voucher specimens of lithistid sponges from the Philippines (*Theonella swinhoei*,
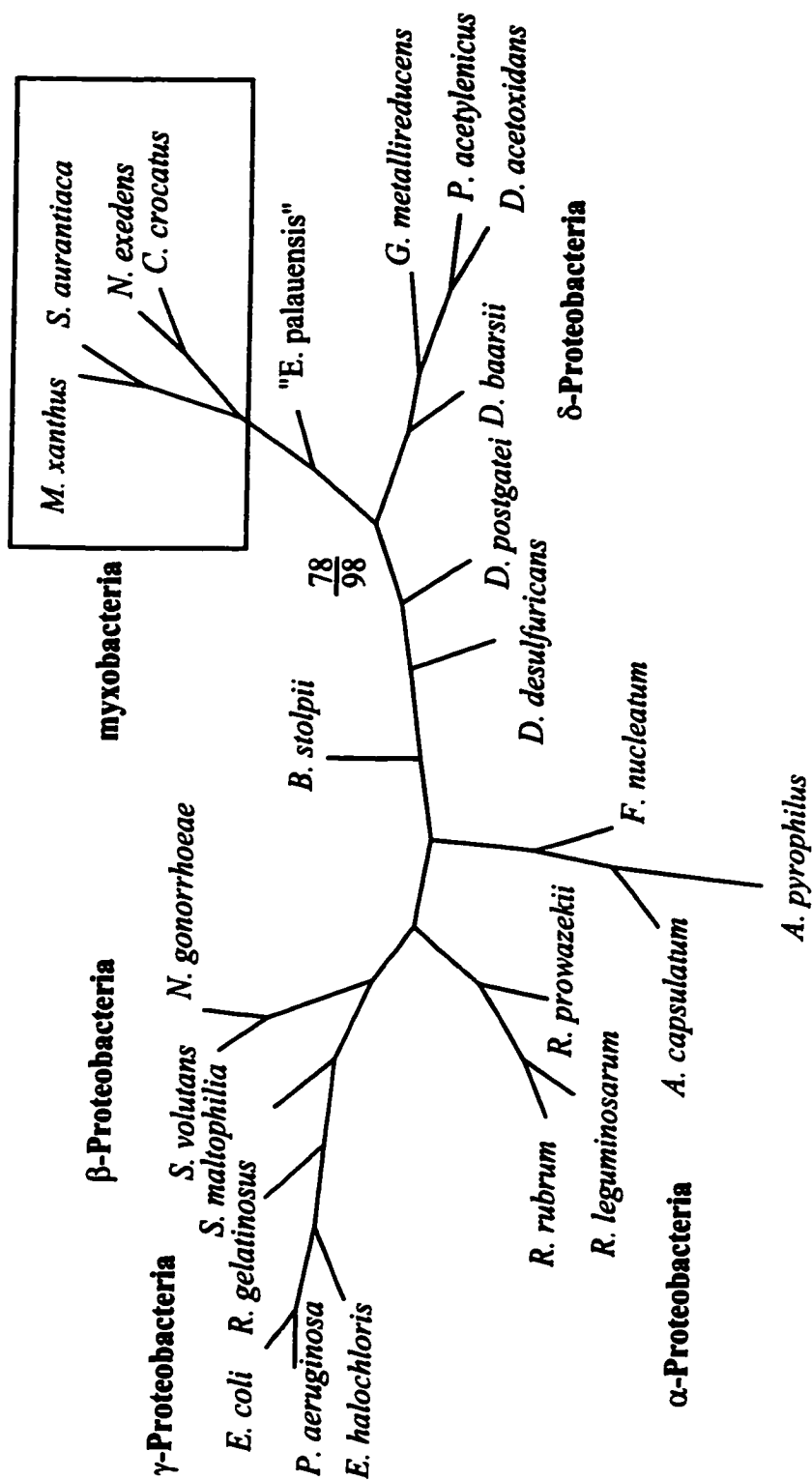
Figure 5. Cladogram of Proteobacteria and "*Candidatus* E. palauensis". Numbers indicate bootrap values using maximum parsimony/maximum likelihood algorithms (100 replications).

Chondromyces crocatus

Nannocystis exedens

Stigmatella aurantiaca

Myxococcus xanthus

"Entotheonella palauensis"

Pelobacter acetylenicus

Desulfovibrio baarsii

Desulfobacter postgatei

Escherichia coli

Desulfovibrio desulfuricans

Aquifex pyrophilus
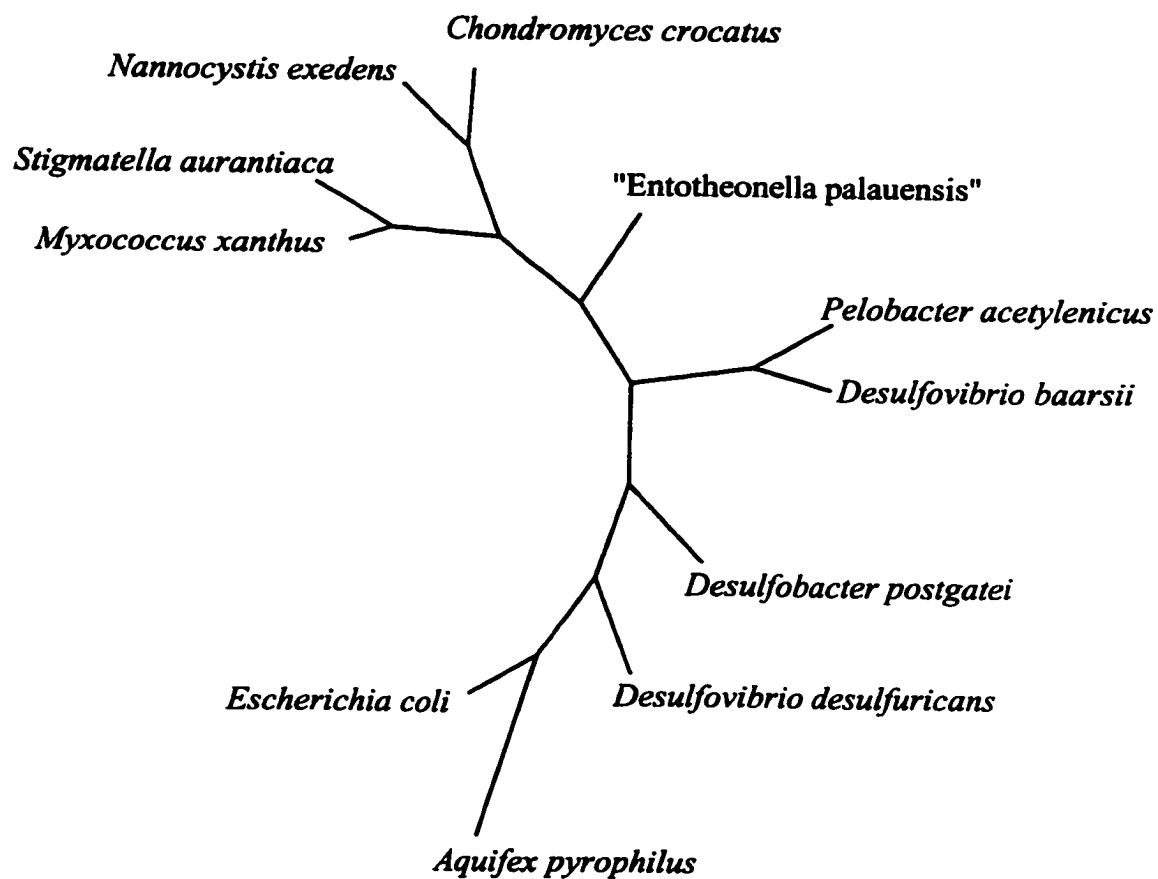
Figure 6. Cladogram of δ-subdivision Proteobacteria using an exhaustive search.

Figure 7. *In situ* hybridization. A) Fluorescence micrograph of unicellular bacteria, universal probe (600x, left); B) Fluorescence micrograph of filamentous bacteria, universal probe (1200x, right).

Figure 7. *In situ* hybridization. C) Light micrograph, "E. palauensis"-specific probe (600x); D) Fluorescence micrograph, "E. palauensis"-specific probe (600x).
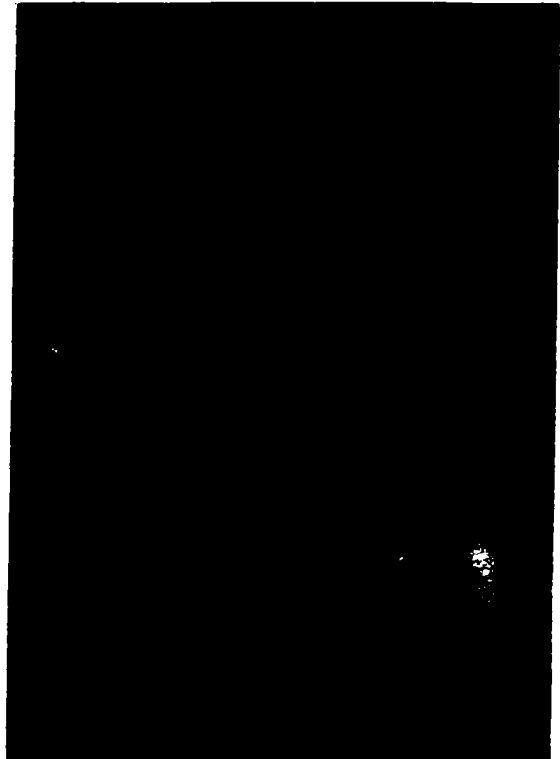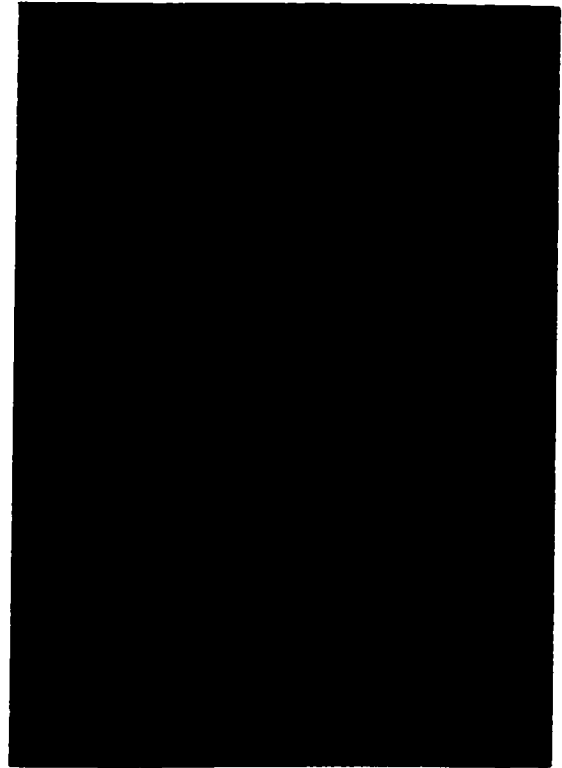
Scleritoderma? sp., NCI 2302 and 2260) were also extracted using the QIAamp kit. Although these voucher specimens were initially frozen, then stored in aqueous methanol for 1-3 years, they appeared to have well preserved DNA based on gel electrophoresis. The SSU rDNA of the samples was amplified using 27f-1492r primers and previously described methods, then by reamplification with two primers specific to the filamentous microorganism: 238f and 1247r. The PCR products were sequenced primarily with the specific primers using the ABI-Prism Big Dye Terminator™ kit and aligned as previously described. The ethanol-preserved *T. swinhoei* specimen contained theonegramides, while the same species from the voucher collection contained primarily theonellamides A and B. *Aciculites* sp. contained mainly aciculitin B, while the *Scleritoderma*? sp. had other novel peptides (work in progress). Only the *T. swinhoei* specimens' DNA was amplified using the specific primers, and the resulting 729 bp sequences were each 1.9-2.7% different from each other and from the previously described Palau sequence, with the theonegramide sponge symbiont the most divergent (Table 1).

Culture experiments

Despite knowledge of the phylogeny and a hint of the specificity of symbiosis, several major questions remain which could be answered by culturing the filamentous symbionts. Metabolic requirements of the bacteria and possible exchange of nutrients between sponge and microbes or between microbes themselves could be determined with a pure culture of bacteria. Proof that the bacteria actually make the peptides could also be obtained, and a pure culture would allow easier access to biosynthetic genes responsible for peptide production. Finally, from the biotechnological perspective, the methods of culturing these symbionts could serve as a model for a

better, more economical source of natural products that could be applied to other, more pharmaceutically important organisms.

In total, more than 100 different culture conditions were employed to grow the filamentous symbionts of *T. swinhoei*. Based on the phylogenetic affiliations proposed by previous authors[10,12] and early BLAST search results for 16S sequence data, several media combinations were tried. After the failure of those attempts, media were based on guesses about environmental conditions on the inside of the sponges.

Previously, it was proposed that the filamentous symbionts could be cyanobacteria or *Beggiatoa* sp., so Chu's No. 11 cyanobacterial medium and *Beggiatoa-Thiothrix* medium recommended by Atlas and Parks[28] were used in several combinations. While cell growth (both numbers and types) in cyanobacterial medium was highly variable because of different combinations of light/no light and nutrient additions, a single species of bacteria appeared to grow in *Beggiatoa* medium. By DGGE, the bacterium did not correspond to major representatives in the sponge tissue, nor were any significant secondary metabolites observed by ^{1}H NMR, so the culture were not pursued further. No filamentous microorganisms were observed in either medium.

The 16S rRNA sequence obtained from the filamentous microorganisms indicated (by BLAST) that the symbionts may have been related to sulfate-reducing bacteria or *Pelobacter* spp. Based on these BLAST results, Widdel's and *Pelobacter* media[29,30] were placed in Balch tubes, which were treated using standard anaerobic techniques and closed with rubber stoppers and aluminum crimp seals. A piece of *T. swinhoei* was collected at 8 m depth in Boracay Island, Philippines in 1998. The sponge was placed in a plastic bag underwater and brought on deck in the tied plastic bag. The sponge was immediately cut with a sterilized razor into pieces of varying

size (0.5-4 cm length), and pieces were placed into numerous tubes with sterile seawater or media for *Beggiatoa, Pelobacter,* or sulfate-reducing bacteria. Residual oxygen was removed by addition of reductant (Na_2S) by syringe after the sponge tissue had been added to the tubes. The culture tubes were maintained at room temperature until arrival at San Diego 3 days later, when they were incubated at 30 °C. Transfer from sulfate-reducing bacterial media tubes eventually led to two cultures that looked fairly clean, and a mixed culture of two cell types was obtained. By DGGE, the media contained mainly two bacteria that matched major bands in the whole sponge DNA lane. The ~350 bp DGGE sequences were identical to *Vibrio vulnificus* and *Desulfovibrio acrylicus* in a BLAST search.

Finally, the filaments were cultured on agar plates with high and low nutrient media (K-, J_{NO3}, J_{S2O3}, see "solutions and media" section and Table 2). To these plates were added all possible combinations of reductant (20 μL of 20 mM Na_2S/ CysNa), sodium silicate solution, and aqueous sponge extract, based on possible environmental requirements for growth. Silicate (20 μL of 20 mM per plate) was selected because the symbionts grow on the interior of a sponge with a silicate skeleton, while sponge extract (20 μL per plate) was used because of a possible symbiont requirement for a specific nutrient or signal from the sponge. The aqueous sponge extract was prepared by stirring sliced pieces of frozen sponge tissue in distilled water for about 30 minutes, centrifuging the liquid, then sterile filtering the supernatant. The plates were incubated at 30 °C for 1 month, after which they were checked for filamentous cell growth. Several plates had colonies containing cells which looked like the filaments from natural sponge and seemed to be going through a growth cycle (Figure 8). Filaments were only seen to grow on J_{S2O3} plates containing sodium silicate and aqueous sponge extract. The filaments have not yet been shown to

be genetically identical to the symbionts of *T. swinhoei*, nor have they been found in pure cultures.

PCR amplifications from other Theonellid sponges

Previously described methods were used to amplify SSU rDNA using the symbiont-specific primers 238f and 1247r. The following samples were tested from the NCI collection: 2167, 2178, 2208, 2309, 2336, and 2413. Preliminary data indicate that some members of the genus *Plakinalopha* may contain symbionts that are related to those in the *T. swinhoei* specimens, but with sequences that are about 10% divergent. However, more work is required to verify the new sequences and to probe the extent of their occurrence.

Proposal of "*Candidatus* Entotheonella palauensis"

The filamentous microorganisms inhabiting *T. swinhoei* are morphologically unusual, and they can be recognized by their co-occurrence with the sponge and with peptides of the theonellamide class. In addition, the Palau filaments have a unique 16S rDNA sequence. For these reasons, I propose that the filamentous symbionts of *T. swinhoei* from Palau be accorded a name under the designation *Candidatus*.

"*Candidatus* Entotheonella palauensis" [(δ-Proteobacteria) NC; G-; F; NAS (GenBank Number AF130847), oligonucleotide sequence complementary to the unique region of 16S rRNA 5'-AAGTAGCGGCCCTTTGTC-3'; S (*Theonella swinhoei*, mesohyl); M]

Discussion

Sequence data from this analysis indicate that the bacterium, "*Candidatus* Entotheonella palauensis", previously shown to contain theopalauamide in the marine sponge, *Theonella swinhoei*, is a novel δ-subdivision Proteobacteria, and the data are consistent with a specific association between sponge and symbiont. Different

chemotypes of *T. swinhoei* contain slightly different symbionts, even when sponges were collected in the same area, showing that there is probably a strain-specific basis for chemistry. The uniqueness of the sequence is shown by the complete absence of closely related bacteria from databases and by the inability of specific primers to amplify DNA from other sponges.

This project began with the idea that, if filamentous microorganisms can be purified using physical separation techniques, it should be possible to directly sequence the resulting enrichment. However, it was impossible to obtain homogeneous fractions of bacteria without using fixatives, which were employed in early chemical studies. Although the filamentous microorganisms appeared greatly enriched (Figure 1), the first 12 cloned SSU rDNA genes examined all had different sequences.

While cloning and sequencing of PCR products was first attempted with this study, it was soon abandoned because of the number of clones and steps needed to get a meaningful picture of the symbiosis. I chose instead to apply another commonly used tool of environmental microbiology, denaturing gradient gel electrophoresis.[31] DGGE has the advantage that a rough estimate of major strains can be made by eye. Bands can be prioritized for sequencing rather than sequencing all clones. Unfortunately, it also only yields short (300-400 bps) fragments of DNA, meaning that more sequence is required for a good phylogenetic study. Typically, the number of base pairs required is thought to be about 1000. DGGE also suffers from the formation of heteroduplexes of DNA, which add bands that do not correspond to different bacteria. As an advantage, isolation of chimeric products is less worrisome, since individual chimeras are probably never the major products of a single PCR reaction.[32] In this study, a majority sequence was clearly visible by DGGE of the

filamentous bacterial preparations, and from this sequence a specific primer, 1247r, was designed. In tandem with the 27f universal bacterial primer, most of the gene (1335 bps) could be amplified from enriched filamentous DNA extracts, although this only worked if the whole SSU rRNA gene had been previously amplified by 27f-1492r universal primers. In addition, two specific primers were required to retrieve the sequence from whole sponge extracts.

It was difficult to prove the sequence origin, since *in situ* hybridization[33] experiments initially failed with positive, negative, or filament-specific probes. Indeed, no signal was seen above background until fresh samples were obtained. Even then, signal amplification was required for sufficient signal, despite over 10 attempts with varying protocols. Others who have worked on Porifera have encountered similar difficulties, especially with sponges containing a large amount of spicules.[34] During the hybridization, several steps bear explanation. Whole sponge pieces were used because dissociated sponge cells fell apart during centrifugation steps. The proteinase K digestion of the cells was crucial, since it broke up the cell-wall proteins and proteins surrounding the 16S rRNA, allowing the probe access to the hybridization site. The acetic anhydride treatment was used to block remaining cationic sites that could bind with the negatively charged DNA probe, and this step had the added benefit that it further broke up sponge tissue. A temperature series was used in the hybridization in order to optimize the specific annealing of the probes, according to their melting temperatures with their RNA targets. All wash and rinse steps were crucial to remove background noise, which was extensive in early experiments with fewer wash steps. Using the described protocol, it was possible to eliminate nearly all signal, but just enough was visible to be assured that the hybridization was working, albeit at low intensity. Using the Tyramide Signal

Amplification kit, excellent signal was observed, confirming the origin of the sequence. With the TSA-Direct kit, biotinylated probe is hybridized to its target, and then the samples are incubated with streptavidin-horseradish peroxidase, which binds to biotin. Finally, tyramide-fluorescein is added, and the horseradish peroxidase reacts with the tyramide to produce radicals, which bind to neighboring DNA. Because this reaction repeats itself many times, an otherwise small signal can be greatly amplified, up to 1000 times according to the manufacturer. A more realistic figure is probably about 10 times, according to several studies using this method.[26,27] With the symbiont-specific probe, only the filamentous microorganisms showed fluorescence above background level.

Treeing the sequence was also time-consuming, since it was highly divergent from its closest relatives. Using Phylip algorithms and a large number of bacterial representatives, the sequence always fell close to myxobacteria, or at least within the δ-Proteobacteria, but when only δ-Proteobacteria were used, the sequence fell closer to the outgroup. The more δ-Proteobacterial sequences used, however, the more the sequence tended to stay in that subdivision, and addition of myxobacterial sequences especially strengthened the correlation. Placement of ε-subdivision Proteobacteria in the tree was problematic because this subdivision has SSU rRNA sequences that are highly diverged from other Proteobacterial sequences. A more rigorous proof of phylogeny was obtained by using the best neighbor joining tree as a starting tree in maximum parsimony and maximum likelihood searches in PAUP and MacClade,[35] looking for the shortest tree and the best consistency index (CI) and retention index (RI) values. Both CI and RI were relatively low (0.38 and 0.40, respectively), but they are actually respectable considering the number of taxa (25) used in the tree. Other studies have similar or lower values when this large a group was analyzed.[23] These

statistical values are used as indicators of the degree of homoplasy, or convergent evolution of characters. Many DNA sequences have a high degree of homoplasy, since characters can only be in one of 4 possible states. Therefore, an individual base in a sequence has a high probability of representing a random change toward convergence rather than an evolutionary step reflecting phylogeny. In addition, bootstrap values indicate that the derived tree is correct. In bootstrapping, a minimization of tree length is repeated a certain number of times (often 100) with a different random sampling of the data set. The number of times that a certain taxon falls beyond the same node in a tree despite random starting points indicates the strength of the node. For instance, my sequence falls within the node containing the myxobacteria 88% of the time, indicating that these sequences are grouped together in 88% of minimization experiments. This is somewhat analogous to minimization of molecular structure by computer; whichever structures appear the greatest amount of the time are selected by the computer as the probable model. The symbiont is not closely related to known myxobacteria (only ~80% sequence identity), but the tree shows that the symbiont and myxobacteria probably both descend from the same ancestor within the δ-subdivision Proteobacteria. The possibility that these bacteria are related to myxobacteria may seem controversial, since marine representatives from this group are not well known. However, perhaps the first molecular evidence of cultured marine myxobacteria was recently revealed,[36] and indeed marine myxobacteria have been studied previously in the course of dissertation research.[37] Although the 16S sequence of "E. palauensis" is not very close to those of other myxobacteria, it apparently has a complex life cycle, at least based on preliminary cell culture experiments (Figure 7), somewhat reminiscent of the complex cycles of the

myxobacteria.[38] Myxobacteria are renowned for their production of complex, bioactive secondary metabolites in culture.[39]

With a phylogenetic tree and proof of sequence in hand, the specific primers were applied to other sponges. Two of the sponges were other *T. swinhoei* samples from the Philippines, which had theonegramide or theonellamides in place of theopalauamide. These samples were different by about 1.9-2.7% both from each other and from the "E. palauensis", with the sequence from the theonegramide-containing sample the most divergent. Sequence was not amplified from two sponges outside the theonellid group. Thus, the slightly different chemicals found in the three specimens are probably produced by three slightly different bacterial strains, although preferably a larger number of sequences should be obtained to prove the correlation.

Conclusions and future prospects

The bacterial symbiont of *T. swinhoei* containing theopalauamide has a SSU rDNA sequence about 20% different from its closest known relative, which is consistent with the hypothesis that the sponge and symbiont have co-evolved. To further prove the hypothesis, sponge sequences would have to be compared with the bacterial sequences, and preferably a larger number of samples would have to be analyzed. The symbiont is novel at least at the class level, and may constitute a new order outside of myxobacteria (δ-subdivision Proteobacteria). These results stand in contrast to educated guesses based on morphology (i.e. *Beggiatoa* or cyanobacteria), reinforcing the danger of over-interpreting phylogeny based on microscopy. Specific PCR primers designed for this study amplified DNA sequences from only theonellid sponges, further indicating a relationship based on sponge phylogeny, not on physical characteristics of the sponges. With these primers, it will be possible to secure relationships between symbionts in the entire theonellid family, and with less specific

primers other sponges may also be probed. By comparing chemistry to sequence, it will be possible to understand the chemotaxonomy of the bacteria and to propose hypotheses on the origin of the metabolites. Thus, these biological studies will eventually return to their chemical origins.

From the viewpoint of biotechnology, this research is most valuable because it may lead to more economical sources for potential pharmaceuticals from marine invertebrates. For instance, knowing the phylogeny of the symbiont could allow appropriate expression vectors for the metabolites to be chosen, leading to production of the compound in cultured transgenic microbes. Another possibility is that knowing phylogeny could allow culture conditions to be selected to grow the actual symbionts themselves. At least one other group of investigators has tried repeatedly to culture the filaments from *T. swinhoei* with no success, but they used a shotgun approach to culturing.[40] To address the possibility that phylogeny could provide better culture conditions, various media were tried based on the sequence and cell morphology. While the filamentous microorganisms did not survive in the anaerobic conditions first tried, other microbes thrived, including *Vibrio vulnificus* and *Desulfovibrio acrylicus* that appeared by DGGE to make up a significant population within the sponge. Both species are known to grow well in anaerobic conditions. *Beggiatoa* medium was also used, based on an early idea that the bacteria may be sulfide oxidizers due to their morphology. Later, we decided to try cyanobacterial media, with and without added amino acids that make up theopalauamide. Unfortunately, these experiments failed, but this was not surprising since the symbiont is only distantly related to known groups.

In our most recent experiments, we abandoned the approach of following the phylogeny and used low and high nutrient complex media fortified with various

mixtures of silicate, reductant, and aqueous sponge extract chosen to imitate conditions in the interior of *T. swinhoei*. On one set of plates containing thiosulfate, aqueous sponge extract, and silicate, we saw dividing filamentous microorganisms that may be the symbiont, but they were not in pure culture and their identity was not confirmed. The doubling time for these cells is apparently on the order of one month under the current conditions, so it will take time to purify the cells and confirm that they are indeed symbionts. Production of theopalauamide in pure culture, or using probes for biosynthesis genes on the cultures, will prove that the symbionts make the molecules. The rationale behind our final culture experiments has recently been eloquently explained in Ward *et al.*:

> Incongruity between plate counts and direct microscopic counts or between populations sampled by culture or molecular methods has led some investigators to the opinion that many (most?) of the bacteria in natural environments are "uncultivatable." We think the more likely explanation is our inability to understand and reproduce the real microenvironmental niches defined by physiochemical and also biotic features (e.g., in symbiotic relationships) that influence naturally occurring bacteria. Hence, we prefer the term "uncultivated," which conveys the more optimistic view that microbiologists will eventually be able to more accurately reproduce the features of natural microenvironments in the laboratory. [41]

Figure 8. Light micrographs of reproducing filamentous microorganisms in culture (600x).
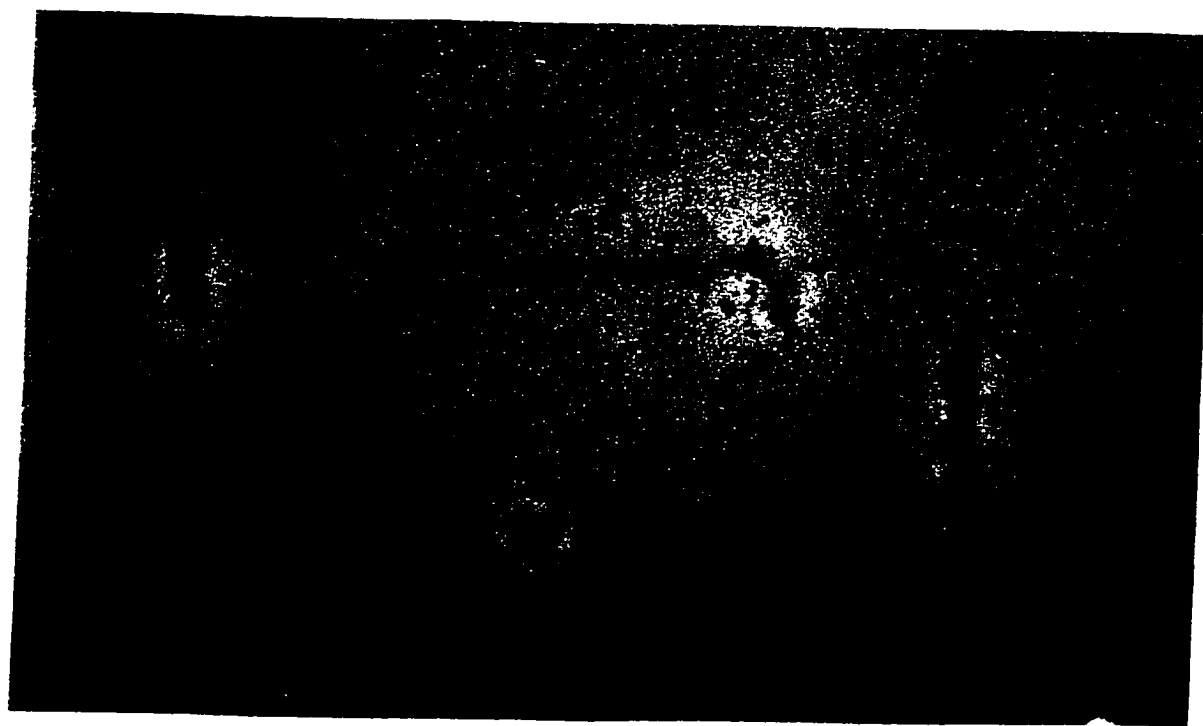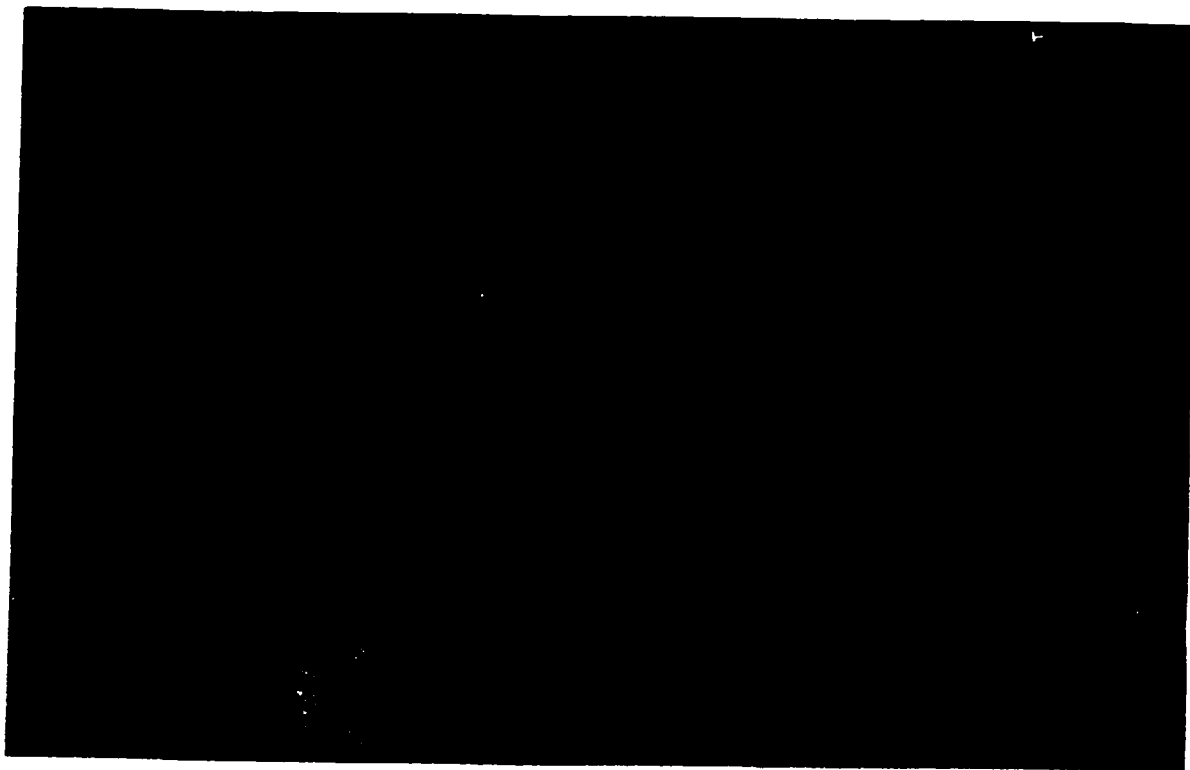
Solutions and Media

Paraformaldehyde: Add paraformaldehyde (8 g) to H_2O (90 mL), and two drops of NAOH and heat <60°C until dissolved. Adjust to a final volume of 100 mL. Add MOPS (8.37 g) and NaCl (11.69 g) to 200 mL H_2O, and mix 100 mL with the paraformaldehyde solution.

PBS: Sodium phosphate (20 mM), pH = 7.4 in 0.15 M NaCl.

Triethanolamine: 0.1 M, pH = 8.0.

20 x SSC: NaCl (17.5 mg), trisodiumcitrate (88.5 g) in H_2O (1 L) pH = 7.4.

Denhardt's solution: Ficoll type 400 (200 mg), polyvinyl pyrrolidone (200 mg), bovine serum albumen (200 mg), bring to 10 mL with H_2O and filter sterilize.

Hybridization buffer (10 mL): deionized formamide (1 mL), 20 x SSC (2.5 mL), Tween-20 (10 μL), 0.5 M EDTA (100 μL), Denhardt's solution (100 μL), RNA (10 mg total), heparin (1 mg).

Wash buffer 1: formamide (5 mL), 20 x SSC (5 mL), 10% SDS (5 mL), H_2O (27.5 mL).

Wash buffer 2: formamide (5 mL), 20 x SSC (5 mL), H_2O (35 mL), 10% SDS (5 mL).

Wash buffer 3: formamide (5 mL), 20 x SSC (5 mL), H_2O (40 mL), Tween-20 (50 μL).

Medium 1 (rich medium; K⁻ medium): dd H_2O (250 mL), natural seawater (750 mL), yeast extract (500 mg), peptone (2 g), agar (15 g), Hepes buffer (20 mL).

Medium 2 (low-nutrient medium): 2 x artificial seawater (500 mL), dd H_2O (500 mL), 8% NH_4Cl (1 mL), agar (15 g), 1M Hepes pH = 7.7 (20 mL), vitamin mix (10 mL), 1M $KHCO_3$ (2 mL), 1M $MnCl_2$ (1 mL), 10% KH_2PO_4 (0.1 mL), ferric ammonium nitrate (3 μg/mL, 0.1 mL).

J_{NO3} medium: Medium 2 + 10% KNO_3 (5 mL).

J_{S2O3} medium: Medium 2 + 10% S_2O_3 (20 mL).

Table 1. SSU rDNA sequence of "Entotheonella palauensis", aligned with bacterial sequences from theonellamide- and theonegramide-containing sponges (*E. coli* 20-1398).

```
                        20              40              60
           TGGCTCAGAACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGGAAAGGCTGC

E.palauensis ◀         TGGCTCAGAACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGGAAAGGCTGC
Theonellamides ◀
Theonegramide ◀


                        80             100             120
           TTCGGCGGCTGAGTAGAGTGGCGAACGGGTGCGTAACACGTGAGTAATCTGCCTCAGACC

E.palauensis ◀         TTCGGCGGCTGAGTAGAGTGGCGAACGGGTGCGTAACACGTGAGTAATCTGCCTCAGACC
Theonellamides ◀
Theonegramide ◀


                       140             160             180
           CTGGGATAACATCTCGAAAGGGGTGCTAATACCGGATAGGTCGCTTGGGTGATGCCCAAG

E.palauensis ◀         CTGGGATAACATCTCGAAAGGGGTGCTAATACCGGATAGGTCGCTTGGGTGATGCCCAAG
Theonellamides ◀
Theonegramide ◀


                       200             220             240
           CGGGAAAGGTTCGCCGGTCTGAGATGAGCTTGCGGCCCATTAGCTAGTTGGCGGGGTAAG

E.palauensis ◀         CGGGAAAGGTTCGCCGGTCTGAGATGAGCTTGCGGCCCATTAGCTAGTTGGCGGGGTAAG
Theonellamides ◀
Theonegramide ◀


                       260             280             300
           CGCCCACCAAGGCGACGATGGGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGCACTG

E.palauensis ◀         CGCCCACCAAGGCGACGATGGGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGCACTG
Theonellamides ◀       GCCCACCAAGGCGACGATGGGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGCACTG
Theonegramide ◀        GCCCACCAAGGCGACGATGGGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGCACTG


                       320             340             360
           AGACACGGGCCAGACTCCTACGGGAGGCAGCAGTG GGAATTTTGCGCAATGGGCGAAAG

E.palauensis ◀         AGACACGGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGGGCGAAAG   .
Theonellamides ◀       AGACACGGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGGGCGAAAG
Theonegramide ◀        AGACACGGGCCAGACTCCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGGGCGAAAG


                       380             400             420
           CCTGACGCAGCAACGCCGCGTGAAGGATGAAGGCT TCG GTCGTAAACTTCTG CTGGG

E.palauensis ◀         CCTGACGCAGCAACGCCGCGTGAAGGATGAAGGCTCTCGAGTCGTAAACTTCTGTCTGGG
Theonellamides ◀       CCTGACGCAGCAACGCCGCGTGAAGGATGAAGGCTCTCGGGTCGTAAACTTCTGTCTGGG
Theonegramide ◀        CCTGACGCAGCAACGCCGCGTGAAGGATGAAGGCTTTTCGAGTCGTAAACTTCTG CTGGG
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Table 1. continued.

```
                              440           460           480
        GGAAAGAT██TGACGGTACCCCCAAAG█AAGCCCCGGCTAATTCCGTGCCAGCAGCCGCG
                 ┣━━━━━━━━━━━┫━━━━━━━━━━━┫━━━━━━━━━━━┫
E.palauensis ◀   GGAAAGATGATGACGGTACCCCCAAAGCAAGCCCCGGCTAATTCCGTGCCAGCAGCCGCG
Theonellamides ◀ GGAAAGATGGTGACGGTACCCCCAAAGCAAGCCCCGGCTAATTCCGTGCCAGCAGCCGCG
Theonegramide ◀  GGAAAGATAATGACGGTACCCCCAAAG█AAGCCCCGGCTAATTCCGTGCCAGCAGCCGCG


                              500           520           540
        GTAATACGGAAGGGGCAAGCGTTGTTCGGAAT█ATTGGGC█TAAAGGGCGTGTAGGCGGC
                 ┣━━━━━━━━━━━┫━━━━━━━━━━━┫━━━━━━━━━━━┫
E.palauensis ◀   GTAATACGGAAGGGGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGGGCGTGTAGGCGGC
Theonellamides ◀ GTAATACGGAAGGGGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGGGCGTGTAGGCGGC
Theonegramide ◀  GTAATACGGAAGGGGCAAGCGTTGTTCGGAATCATTGGGCATAAAGGGCGTGTAGGCGGC


                              560           580           600
        █TCATATGTCTG█GGTGAAAG█AT█C█GCTCAAC█C█ATTAAGCCGTGGAAACTGTG██G
                 ┣━━━━━━━━━━━┫━━━━━━━━━━━┫━━━━━━━━━━━┫
E.palauensis ◀   TTCATATGTCTGCGGTGAAAGGATGCGGCTCAACTGCATTAAGCCGTGGAAACTGTGGAG
Theonellamides ◀ CTCATATGTCTGCGGTGAAAGCATACAGCTCAACTGTATTAAGCCGTGGAAACTGTGAGG
Theonegramide ◀  TTCATATGTCTG█GGTGAAAGCAT█CGGCTCAAC█CG█ATTAAGCCGTGGAAACTGTGGAG


                              620           640           660
        CTTGAGGCTGGGAGGGGCTGGTGGAATTCCCTGTGTAGCGGTGAAATGCGTAGAGATGGG
                 ┣━━━━━━━━━━━┫━━━━━━━━━━━┫━━━━━━━━━━━┫
E.palauensis ◀   CTTGAGGCTGGGAGGGGCTGGTGGAATTCCCTGTGTAGCGGTGAAATGCGTAGAGATGGG
Theonellamides ◀ CTTGAGGCTGGGAGGGGCTGGTGGAATTCCCTGTGTAGCGGTGAAATGCGTAGAGATGGG
Theonegramide ◀  CTTGAGGCTGGGAGGGGCTGGTGGAATTCCCTGTGTAGCGGTGAAATGCGTAGAGATGGG


                              680           700           720
        █GAGAACACTCGTGG█CGAAGGCGGCCAGCTGGACCAGTTCT█ACGCT██AG█CGCGAAA
                 ┣━━━━━━━━━━━┫━━━━━━━━━━━┫━━━━━━━━━━━┫
E.palauensis ◀   GGAGAACACTCGTGGGCGAAGGCGGCCAGCTGGACCAGTTCTGACGCTGTAGGCGCGAAA
Theonellamides ◀ GGAGAACACTCGTGG.CGAAGGCGGCCAGCTGGACCAGTTCTGACGCTA.AGGCGCGAAA
Theonegramide ◀  █GAGAACACTCGTGG.CGAAGGCGGCCAGCTGGACCAGTTCTAACGCTG.AG█CGCGAAA


                              740           760           780
        GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGGCACTA
                 ┣━━━━━━━━━━━┫━━━━━━━━━━━┫━━━━━━━━━━━┫
E.palauensis ◀   GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGGCACTA
Theonellamides ◀ GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGGCACTA
Theonegramide ◀  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGGCACTA


                              800           820           840
        GGTGTCGGCGGTTCTTAATCCGTCGGTG█CGGCGCTAACGCAGTAAGTGCCCCGCCTGGG
                 ┣━━━━━━━━━━━┫━━━━━━━━━━━┫━━━━━━━━━━━┫
E.palauensis ◀   GGTGTCGGCGGTTCTTAATCCGTCGGTGTCGGCGCTAACGCAGTAAGTGCCCCGCCTGGG
Theonellamides ◀ GGTGTCGGCGGTTCTTAATCCGTCGGTGCCGGCGCTAACGCAGTAAGTGCCCCGCCTGGG
Theonegramide ◀  GGTGTCGGCGGTTCTTAATCCGTCGGTGCCGGCGCTAACGCAGTAAGTGCCCCGCCTGGG
```

Table 1. continued.

```
                          860              880              900
          GAGTACGGTCGCAAGGCTGAAACTCAAACGAATTGACGGGGGCCCGCACAAGCGGTGGAG   .
E.palauensis ◄  GAGTACGGTCGCAAGGCTGAAACTCAAACGAATTGACGGGGGCCCGCACAAGCGGTGGAG
Theonellamides ◄  GAGTACGGTCGCAAGGCTGAAACTCAAACGAATTGACGGGGGCCCGCACAAGCGGTGGAG
Theonegramide ◄  GAGTACGGTCGCAAGGCTGAAACTCAAACGAATTGACGGGGGCCCGCACAAGCGGTGGAG


                          920              940              960
          CATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTAGGTTTGACATG■A■■GA■■
E.palauensis ◄  CATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTAGGTTTGACATGGATGGGACA
Theonellamides ◄  CATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTAGGTTTGACATGCATAGGACA
Theonegramide ◄  CATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTAGGTTTGACATGGACCAGATC


                          980             1000             1020
          ■C■GGTGAAAGTCCGTCTTCCTTCGGGACCCATTCACAGGTGCTGCATGGCTGTCGTCAG
E.palauensis ◄  GCGGGTGAAAGTCCGTCTTCCTTCGGGACCCATTCACAGGTGCTGCATGGCTGTCGTCAG
Theonellamides ◄  GCCGGTGAAAG
Theonegramide ◄  ■CCGGTGAAAG


                         1040             1060             1080
          CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGCCCTCTGTTGC
E.palauensis ◄  CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGCCCTCTGTTGC
Theonellamides ◄
Theonegramide ◄


                         1100             1120             1140
          TACCGGGTCAAGCCGGGCACTCTGAGGGGACTGCCTCGGTTAACGGGGAGGAAGGTGGGG
E.palauensis ◄  TACCGGGTCAAGCCGGGCACTCTGAGGGGACTGCCTCGGTTAACGGGGAGGAAGGTGGGG
Theonellamides ◄
Theonegramide ◄


                         1160             1180             1200
          ATGACGTCAAGTCCTCATGGCCTTTATGCCTAGGGCTACACACGTGCTACAATGGTCAGG
E.palauensis ◄  ATGACGTCAAGTCCTCATGGCCTTTATGCCTAGGGCTACACACGTGCTACAATGGTCAGG
Theonellamides ◄
Theonegramide ◄


                         1220             1240             1260
          ACAAAGGGCCGCTACTTCGCAAGGAGACGCCAATCCCAAAAACCTGGCCCCAGTTCGGAT
E.palauensis ◄  ACAAAGGGCCGCTACTTCGCAAGGAGACGCCAATCCCAAAAACCTGGCCCCAGTTCGGAT
Theonellamides ◄
Theonegramide ◄
```

Table 1. continued.

```
                                         1280           1300                1320
                   GGTCGGCTGCAACTCGTCGGCTTGAAGCTGGAATCGCTAGTAATCGGAGATCAGCACGCT
E.palauensis ◀     GGTCGGCTGCAACTCGTCGGCTTGAAGCTGGAATCGCTAGTAATCGGAGATCAGCACGCT
Theonellamides ◀
Theonegramide ◀


                                  1340              1360              1380
                   CCGGTGAATACGTTCCCGGGCCTTGTAC
E.palauensis ◀     CCGGTGAATACGTTCCCGGGCCTTGTAC
Theonellamides ◀
Theonegramide ◀
```

Code Key:
A = adenosine   S = G/C
C = cytidine   W = A/T
G = guanine   B = G/T/C
T = thymidine   D = G/A/T
R = G/A   H = A/C/T
Y = T/C   V = G/C/A
K = G/T   N = A/G/C/T
M = A/C   - = gap

Table 2. Successful media conditions (shown in bold) for growth of filamentous bacteria (by visual inspection and DGGE confirmation).

Plate #	Medium 1	Medium 2	NaNO₃	Na₂S₂O₃	Peptone/yeast	Silicate	Reductant	Sponge extract
1	X				X			
2	X				X	X	X	X
3	X				X	X	X	
4	X				X	X		X
5	X				X		X	X
6	X				X	X		
7	X				X		X	
8	X				X			X
9		X	X					
10		X	X			X	X	X
11		X	X			X	X	
12		X	X			X		X
13		X	X				X	X
14		X	X			X		
15		X	X				X	
16		X	X					X
17		X		X				
18		X		X		X	X	X
19		X		X		X	X	
20		X		X		X		X
21		X		X			X	X
22		X		X		X		
23		X		X			X	
24		X		X				X

References

1. Wilkinson, C.R. *Symbiosis* **1987**, *4*, 135-146.

2. Wilkinson, C.R. *Proc. R. Soc. Lond. B* **1984**, *220*, 509-517.

3. Müller, W.E.G.; Zahn, R.K.; Kurelec, B.; Lucu, C.; Müller, I.; Uhlenbruck, G. *J. Bact.* **1981**, *145*, 548-558.

4. Vacelet, J.; Fiala-Médioni, A.; Fisher, C.R.; Boury-Esnault, N. *Mar. Ecol. Prog. Ser.* **1996**, *145*, 77-85.

5. Preston, C.M.; Wu, K.Y.; Molinski, T.F.; DeLong, E.F. *Proc. Natl. Acad. Sci. USA*, **1997**, *93*, 6241-6246.

6. Althoff, K.; Schütt, C.; Steffen, R.; Batel, R.; Müller, W.E.G. *Mar. Biol.* **1998**, *130*, 529-536.

7. Diaz, M.C., personal communication.

8. Schmidt, E.W.; Bewley, C.A.; Faulkner, D.J. *J. Org. Chem.* **1998**, *63*, 1254-1258.

9. Fusetani, N.; Matsunaga, S. *Chem. Rev.* **1993**, *93*, 1793-1806.

10. Kobayashi, J.; Ishibashi, M. *Chem. Rev.* **1993**, *93*, 1753-1769.

11. Faulkner, D.J.; He, H.Y.; Unson, M.D.; Bewley, C.A. *Gazz. Chim. Ital.* **1993**, *123*, 301-307.

12. Bewley, C.A.; Holland, N.D.; Faulkner, D.J. *Experientia* **1996**, *52*, 716-722.

13. Bewley, C.A. Ph.D. Thesis, University of California, San Diego, 1995.

14. Sambrook, J.; Fritsch, E.F.; Maniatis, T. *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory, New York, 1989.

15. Muyzer, G.; De Waal, E.C.; Uitterlinden, A.G. *Appl. Environ. Microbiol.* **1993**, *59*, 695-700.

16. Abrams, E.S.; Murdaugh, S.E.; Lerman, L.S. *Genomics* **1990**, *7*, 463-475.

17. Ferris, M.J.; Ward, D.M. *Appl. Environ. Microbiol.* **1997**, *63*, 1375-1381.

18. Ferris, M.J.; Muyzer, G.; Ward, D.M. *Appl. Environ. Microbiol.* **1996**, *62*, 340-346.

19. Maidak, B.L.; Olsen, G.J.; Larsen, N.; Overbeck, R.; McCaughey, M.J.; Woese, C.R. *Nucleic Acid Res.* **1997**, *25*, 109-111.

20. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. *J. Mol. Biol.* **1990**, *215*, 403-410.

21. Program by Swofford, D.L.

22. Feilstein, J. *Cladistics* **1989**, *5*, 164-166.

23. Givnish, T.J.; Sytsma, K.J. *Molecular evolution and adaptive radiation.* Cambridge University Press, New York, 1997.

24. Forey, P.L.; Humphries, C.J.; Kitching, I.J.; Scotland, R.W.; Siebert, D.J.; Williams, D.M. *Cladistics: A practical course in systematics.* Clarendon Press, Oxford, 1992.

25. Haygood, M.G.; Davidson, S. *Appl. Eviron. Microbiol.* **1997**, *63*, 4612-1616.

26. Schönhuber, W.; Fuchs, B.; Juretschko, S.; Amann, R. *Appl. Eviron. Microbiol.* **1997**, *63*, 3268-3273.

27. Lebaron, P.; Catala, P.; Fejon, C.; Joux, F.; Baudart, J.; Bernard, L. *Appl. Environ. Microbiol.* **1997**, *63*, 3274-3278.

28. Atlas, R.M.; Parks, L.C. *Handbook of Microbiological Media.* CRC Press, Inc., Boca Raton, 1993.

29. Widdel, F.; Bak, F. in *The Prokaryotes, Vol IV.*, pp 3352-3378. Balows, C.A.; Trüper, H.G.; Dworkin, M.; Harder, W.; Schlerfer, K.H. eds. Springer-Verlag, Berlin, 1992.

30. Schink, B. *Arch. Microbiol.* **1984**, *137*, 33-41.

31. Head, I.M.; Saunders, J.R.; Pickup, W. *Microbial Ecol.* **1998**, *35*, 1-21.

32. Wang, G. C.-Y.; Wang, Y. *Appl. Environ. Microbiol.* **1997**, *63*, 4645-4650.

33. Amman, R.I.; Krumholz, L.; Stahl, D.A. *J. Bact.* **1990**, *172*, 762-770.

34. Preston, C.M.; Matsumoto, B.; DeLong, E.F. American Society for Microbiology 98th General Meeting Abstract N-50, 1998.

35. Maddison, W.P.; Maddison, D.R. *Folia Primatologica* **1989**, *53*, 190-202.

36. Iizuka, T.; , Y.; Fudou, R.; Yamanaka, S. *FEMS Microbiol. Letts.* **1998**, *169*, 317-322.

37. Starr, T.J. Ph.D. Thesis, University of Washington, 1953.

38. Dworkin, M. *Microbiol. Revs.* **1996**, *60*, 70-102.

39. Reichenbach, H.; Höfle, G. *Biotech. Adv.* **1993**, *11*, 219-277.

40. Kobayashi, M.; Kitagawa, I. *in Sponge Sciences: Multidisciplinary Perspectives*. Springer-Verlag, New York, 1998, pp 379-390.

41. Ward, D.M.; Ferris, M.J.; Nold, S.C.; Bateson, M.M. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 1353-1370.