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Chemical Investigations of Microbial Isolates from Estuarine and Extreme Marine Environments

Author
Trischman, Jacqueline A.

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Chemical investigations of microbial isolates from estuarine and extreme marine environments

Trischman, Jacqueline Ann, Ph.D.

University of California, San Diego, 1993

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

UNIVERSITY OF CALIFORNIA

SAN DIEGO

Chemical Investigations of Microbial Isolates from Estuarine and Extreme Marine Environments

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Oceanography

by

Jacqueline A. Trischman

Committee in charge:

Professor William H. Fenical, Chair
Professor D. John Faulkner
Professor Douglas H. Bartlett
Professor Daniel F. Harvey
Professor Palmer W. Taylor

1993

The dissertation of Jacqueline A. Trischman is approved, and

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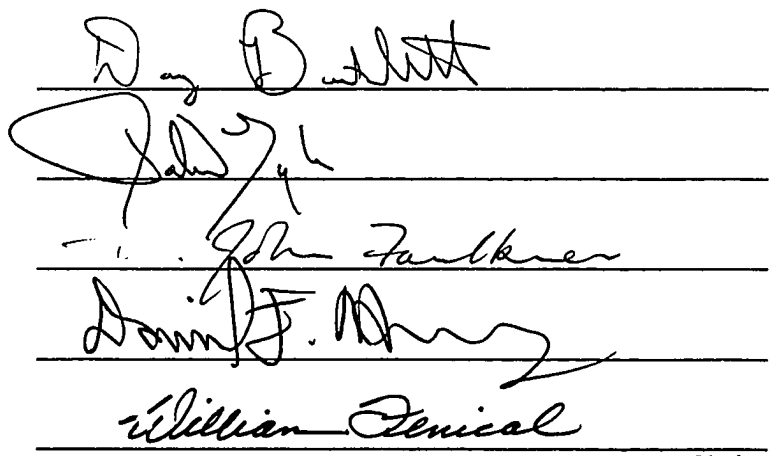

Chair

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1993

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VITA

February 11, 1965	Born, Jacqueline A. Porta, Philadelphia, PA
1988	B. S., Biochemistry, Virginia Polytechnic Institute & State University
1988 - 1989	Research Assistant, Scripps Institution of Oceanography, University of California, San Diego
1989 - 1993	California Sea Grant Trainee, Scripps Institution of Oceanography, University of California, San Diego
1993	Ph.D., University of California, San Diego

PUBLICATIONS

Abramson, S. N., Trischman, J. A., Tapiolas, D. M., Harold, E. E., Fenical, W., and P. Taylor. "Structure/ Activity and Molecular Modeling Studies of the Lophotoxin Family of Irreversible Nicotinic Receptor Antagonists." *J. Med. Chem.*, **34**, 1798-1804 (1991).

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Trischman, J. A., Jensen, P. R., and W. Fenical. "Surfactamide: A Novel Acylpeptide of the Iturin Class Produced by a Deep-Sea Isolate of *Bacillus*." in preparation.

Trischman, J. A., Greaves, M., and W. Fenical. "Antibiotics from Mangrove Isolates: Isolation of *cis*-Cascarillic Acid from a Marine Bacterium." in preparation.

Trischman, J. A., Jensen, P. R., and W. Fenical. "Isolation of the Guaymasols from a Deep-Sea Isolate of *Bacillus*." in preparation.

FIELDS OF STUDY

Major Field: Marine Bio-Organic Chemistry

Studies in Marine Natural Products Chemistry

Professor William H. Fenical, Scripps Institution of Oceanography
Professor D. John Faulkner, Scripps Institution of Oceanography

Studies in Nuclear Magnetic Resonance Spectroscopy

Professor D. John Faulkner, Scripps Institution of Oceanography
Professor William H. Fenical, Scripps Institution of Oceanography
Dr. Susan Pochapsky, Bruker Instruments
Dr. John Wright, University of California, San Diego

Studies in Marine Microbiology

Professor Douglas H. Bartlett, Scripps Institution of Oceanography
Professor Aristedes A. Yayanos, Scripps Institution of Oceanography
Professor Farooq Azam, Scripps Institution of Oceanography
Professor Angelo F. Carlucci, Scripps Institution of Oceanography

ABSTRACT OF THE DISSERTATION

Chemical Investigations of Microbial Isolates from
Estuarine and Extreme Marine Environments

by

Jacqueline Ann Trischman

Doctor of Philosophy in Oceanography

University of California, San Diego, 1993

Professor William H. Fenical, Chair

Microbial inhabitants of the marine environment are poorly understood in terms of chemical interaction. Tremendous taxonomic diversity exists among these microorganisms, encompassing adaptations to the wide range of physical parameters found in marine habitats. Production of secondary metabolites has been studied in several marine bacteria and fungi, primarily isolated from shallow, near-shore sediments. However, most resources for the discovery of new natural products from marine microorganisms remain unexploited, and little is known of the function of microbial secondary metabolites *in situ.*

The study described in this dissertation had two major goals: 1) to identify novel compounds produced by microbial isolates from extreme marine environments, and 2) to identify antibiotic compounds which might act *in situ.* The first investigation resulted in the isolation and structure elucidation of several novel compounds from deep-sea isolates of the genus *Bacillus.* These compounds included guaymasol and epiguaymasol and the iturin class acylpeptide, surfactamide.

In the research focussed on microbial antibiosis, estuaries were chosen as collecting sites, since these environments are rich in nutrients and, thus, high in concentration of microorganisms. In preliminary work, one estuarine isolate produced a series of novel bicyclic depsipeptides, the salinamides, which exhibited moderate activity against Gram (+) bacteria and potent anti-inflammatory activity. Based on the salinamide results and the desire to investigate ecologically-relevant chemical competition, a project was designed to isolate and identify compounds which inhibited growth of microorganisms from the same environment as the producing organism.

The study sites included one tropical mangrove swamp in Belize, Central America, and one temperate lagoon in San Diego County. The antibiotic compounds produced by the lagoon isolates included piericidin, chloramphenicol, nonactin, valinomycin, fumitremorgin C, and the diketopiperazine *cyclo*(L-*trans*-(4-hydroxyprolinyl)-L-Phe). The Belizean mangrove isolates produced one novel compound, cis-cascarillic acid, as well as the known antibiotic compounds: cycloheximide, tirandamycin, *o*-hydroxybenzamide, and several nucleotide bases. Several known secondary metabolites with no recorded bioactivity were also isolated and identified. Culture of the microorganism, isolation and identification of each compound, and the significance of production of the compounds by a marine microorganism are discussed for each isolate.

Chapter I

Introduction

A. Summary of Thesis

This dissertation focuses on the natural products chemistry of unicellular bacteria, actinomycetes and fungi from marine samples. Previous studies have demonstrated that marine microorganisms, primarily sediment isolates, are a prolific and renewable source of bioactive and structurally novel compounds.

The extreme ranges of physical conditions found in marine habitats has selected for tremendous taxonomic diversity among the microbial flora. Few of these taxa have been chemically investigated. In the research presented here, secondary metabolite production was studied in microbial isolates from the extreme environment of the deep-sea floor and the nutrient-rich estuarine environment. The work was focussed on identification of bioactive compounds which may have significance in the natural ecosystem, under the assumption that microbial interaction, especially competitive interaction, is necessarily chemical.

Each project is described biologically and chemically. The biological aspects involved isolation and culture of bacteria and fungi, as well as testing of the crude extracts and pure compounds investigated for antibiotic activity against a panel of microorganisms. This test panel consisted of strains which compete with the producing isolate in the natural environment. The chemical studies involved isolation and structure elucidation of known and novel bacterial products. The possible significance of each compound to humankind and the marine environment is discussed.

B. Microbial Inhabitants of the Marine Environment

The ocean was once thought to be a vast desert as far as bacteria were concerned. Until the 1970's, bacteria were not thought to play much of a role in marine ecology, since they did not grow very densely. With the development of epifluorescence microscopy, however, bacteria were found to be 1000 times more abundant in seawater than previously calculated. The marine environment is now known to harbor 10^6 bacterial cells/ml in sea-water and up to 10^9-10^{12} bacterial cells/ml in sediments and on surfaces. In fact, in oligotrophic regions, bacteria can account for more biomass than all other organisms combined. These bacteria require low nutrients, and thus do not grow well on the high nutrient plates typically used to isolate marine bacteria. Because of this, they were not even recognized until the late 1970's. At this time, many scientists still believed that marine bacteria were only responsible for processing feces that fell into the bottom of the ocean,[1] but Pomeroy found that organisms which pass through a 10 μm filter were responsible for most of the respiration in the ocean.[2] Thus bacteria play a very big role in marine ecosystems.

Sediment and surface habitats in the terrestrial realm vary geographically, especially according to climate. It is easy to imagine what adaptations a bacterium in arid, desert soil would have compared to a bacterium from the soil of coastal Washington state. The desert strain must be able to withstand dessication and powerful UV radiation, while the Washington strain must deal with seasonal variation in sunlight and constant dampness or influx of fresh water. But what do marine bacteria need in their arsenal of adaptations to remain viable in their habitats? To answer this question, we must first look at the incredible diversity in marine habitats which has driven microorganisms to adapt to many niches with no terrestrial

counterparts.

A classical description of the habitats available to marine microorganisms is given by Austin.[3] He lists these habitats as neuston, nekton, seston, plankton, endobiotic, epibiotic, pelagic, and benthic. Each habitat requires different survival strategies. Also, ocean environments are subject to extreme ranges in physical conditions throughout each habitat. Pressure can reach above 1000 atm in the deepest parts of the ocean, since pressure increases by 1 atm for every 10 m of depth. The temperature can range from below 0°C in the deep ocean and polar ice packs to 375°C in the hydrothermal vent regions, which allow such high water temperatures because of the high pressures. Salinity ranges from nearly 0 ‰ in areas with high fresh water run-off, to saturation in hypersaline ocean margins. The pH of most of the ocean is buffered at approximately 8. However, extreme environments exist where the pH can be as low as 2.0 or as high as 11.0. The oxygen level varies from 0 mmol/kg to supersaturation (0.5 mmol/kg) at the surface, and the available organics can be very low (oligotrophic regions) or very high (eutrophic regions, e.g. estuaries). Extreme physical conditions have induced extreme adaptations in the microbes which are no longer capable of living in non-extreme conditions. Often a different set of adaptations has been found in facultative organisms which can grow in both extreme and non-extreme conditions.

Each habitat may contain a range of physical conditions, including several physical extremes, or it may be characterized by one of these extremes. For example, the neuston, or air-water interface, is characterized by supersaturation of air due to wave action. This makes the neuston a good habitat for primary producers which require light radiation and CO_2. It is also rich in polysaccharide and protein complexes due to the high surface tension, so heterotrophs are also concentrated in

this habitat. Total microbial counts can reach 100 times greater than their concentration in the water column below.[4]

High counts of microorganisms, often highly species-specific, can also be found inside other marine organisms. The nekton inludes microbes living in association with large, free-swimming animals. The intestinal tracts of many fish contain associated bacteria which aid in digestion or parasitize the animal. Examination of the gut contents of several species of surgeonfish from the Red Sea and the Great Barrier Reef led to the discovery of an associated microbe which may be the largest known prokaryote. This endosymbiont reached sizes of 576 μm.[5] Another study of the intestinal microbial community of turbot may lead to new methods for control of the pathogen *Vibrio anguillarum* in aquaculture. The secondary metabolite, anguibactin (**1**), produced by this *Vibrio* species, which is now controlled with chemotherapeutic agents and vaccination, may actually contribute to its pathogenicity.[6] However, associated intestinal isolates from the turbot, *Scophthalmus maximus*, have been found to inhibit growth of the *Vibrio* in the host fish. Inocula of these isolates may someday be added to aquaculture pens to support the natural symbiotic defense mechanism.[7]

Another well-studied association found in the nekton involves a fish or squid host that carries a pure culture of luminescent bacteria (e.g. *Vibrio fischeri*) in a specially adapted tissue compartment called a light organ. The advantages of the association to the bacterium are the availability of nutrients and a protected environment supplied by the host, while the fish benefits from attraction of prey or ventral camouflage provided by the light.[8,9]

Endobionts are not limited to swimming organisms. Many benthic organisms harbor phototrophic bacteria or cyanobacteria which supply the macroorganism with

nutrients. The requirements and the specificity of these associations are topics currently being studied by many marine ecologists, physiologists, and chemists. One thing that is certain is that benthic animals can offer environments not found in the surrounding water. For example, phototrophic anaerobic bacteria are in very low concentration in oxic waters. However, these microbes can be found in higher numbers inside sponge tissues. Imhoff and co-workers isolated six strains of the phototrophic *Chromatiaceae* and two strains of *Rhodospirillaceae* from four species of marine sponges collected in Yugoslavia.[10] The advantage of living inside the sponge probably lies in the ability to find an anoxic microhabitat. Other advantages offered by associations with macroorganisms may be exposure to light without risk of being covered by sediment or overgrown, a fixed environment where nutrient-containing water is continuously circulated, or even that specific nutrients are supplied by the macroorganism.

It is not always necessary to live in association with an animal or plant to harvest the nutrients it provides. The seston consists of the organic debris produced by macroorganisms, *e.g.* fish feces. Clear distinction is made between seston and pelagic habitats in most cases, but recent research has shown that the dividing line between these two habitats is not obvious. Concentrations of DNA, protein, and polysaccharide chains are very high in the upper pelagic zone. In fact, if these chains are measured linearly, seawater typically contains 2 km/ml, 310 km/ml, and 5600 km/ml, respectively. Microorganisms are embedded in this polymer matrix. Any of these molecular matrices less than 0.5 μm in size is considered to be dissolved organic carbon (DOC). Until recently, it was thought that a very small percentage of DOC could be utilized by bacteria. However, in 1991, Kirchman *et al* showed that in sea-water, 20 - 40% of the DOC used is taken up by bacteria.[11]

Once molecules and complexes (and cells) reach 0.5 μm, they are considered suspended particulate organic matter (POM). Typically, POM starts to sink when it reaches about 1 μm. The interaction of the DOC, fecal pellets, dead phytoplankton, etc. is now thought to produce POM called "marine snow," as shown in Figure 1.[12]

Marine snow aggregates are not rich in available nutrients. Therefore, since bacteria are the main consumers of DOC,[13] the marine snow must be broken down as it sinks. One hypothesis is that one bacterium attaches to a marine snow particle and produces hydrolytic enzymes.[14] This attached bacterium divides as it works its way quickly along the chain, producing free-living bacteria which consume the released nutrients. The free-living bacteria may produce antibiotic compounds which keep other microbes out of their niche. This marine snow theory accounts for the carbon flow from POM to free bacteria.[15]

Free-living marine bacteria are much smaller than their terrestrial counterparts, only 0.2 - 0.6 μm in diameter, averaging only 3% of the size of *E. coli*. Smaller size would tend to decrease predator pressure, allow the bacteria to better exist within the matrix, and allow better nutrient uptake through an increased surface area/volume ratio. If the marine snow theory proved to be true, high nutrient uptake rates would be very important, since small DOC particles would be quickly swept out of the matrix.

The marine snow hypothesis also agrees with recent conclusions drawn from findings that marine surfaces are rich in bacteria. Epibiotic habitats offer many of the same advantages discussed for the endobiotic habitats, though the environment is typically more exposed to a range of environmental pressures. Several studies have shown that the bacterial flora isolated from the surface of marine plants is different than that of the surrounding sea-water.[16,17,18] The epiphytic bacteria may simply be

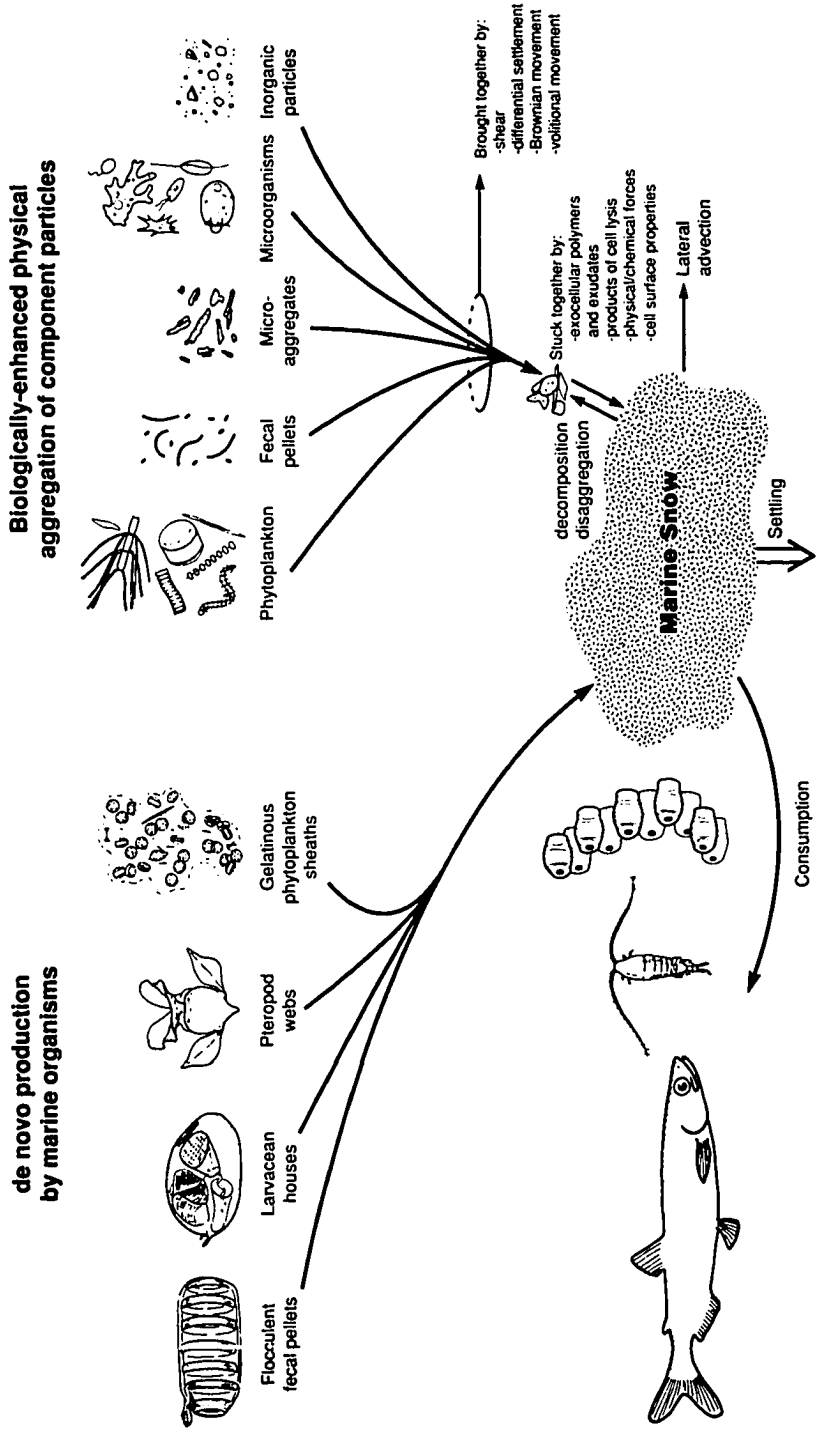


Figure 1. Particle dynamics of marine snow

able to outcompete other organisms when supplied with an increased amount of nutrients, or the host organism may actively select for certain species of bacteria.

The habitat most concentrated with bacteria, and most analogous to the terrestrial soil, is the sediment of the sea floor. Below the photic zone, the surface sediment harbors primarily heterotrophs, while these are intermingled with autotrophs in estuarine and shallow sea sediments. Below the surface, a succession of chemoautotrophs characterizes the sediment. The actual depth of the sediment occupied by each type of microorganism is determined by factors that include particle size, aeration, and geochemical make-up of the sediment. A typical sediment succession is heterotrophs, nitrate reducers, iron and manganese reducers, sulfate reducers and methanogens.[19]

Chemoautotrophic bacteria are not found only in sediments in the marine environment. In 1977, a 2600 m dive by the submersible Alvin, north of the Galapagos Islands, discovered a community of organisms that were completely dependent on sulfur-oxidizing bacteria. These bacteria produce organics from CO_2 using H_2S as an energy source. They exist as free-living microorganisms and in symbioses with the vent macroorganisms. The tube worm, *Riftia pachyptila*, contains vast numbers of bacteria in a thin-walled sac called the trophosome. The tube worm provides the bacteria with CO_2, O_2, and H_2S through its circulatory system, and the bacteria supply the worm with needed organics. Obviously tight symbiotic associations like these hydrothermal vent communities are not common.[20]

C. Chemical Studies of Marine Microorganisms

Chemical studies of marine organisms can provide ecological information which increases the understanding of the diverse marine ecosystem. For example, an

antarctic unicellular alga, *Phaeocystis pouchetii*, has been studied for its unusual manganese binding and releasing ability, and manganese oxidizing bacteria have been found on the surface of the algae. These bacteria may actually produce the interesting acrylic acid complexes known to cause sterility in penguins,[21] a very complex and interesting ecological problem rooted in bacterial production of secondary metabolites. Nonetheless, ecological information is not the only goal for investigation of microbial secondary metabolites. Discovery of new marine microbial products can also contribute to the search for new chemical compounds which may be beneficial to mankind.

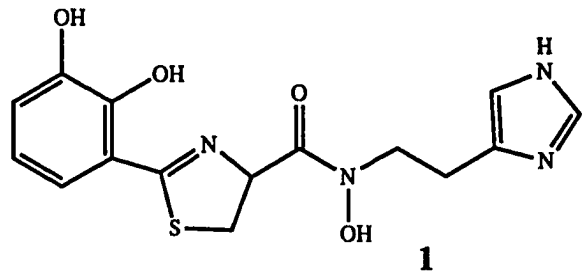
Though soil microorganisms have been exploited for their antibiotic production since the discovery of penicillin in 1929, the marine environment has only recently been recognized as a source of taxonomically-diverse, bioactive compound-producing microbes. Though Rosenfeld and Zobell found marine microorganisms to have activity against the human pathogen *Bacillus anthracis* in 1947,[22] identification of an antimicrobial compound produced by a marine bacterium was not published until 1966. At that time, Burkholder *et al.* identified 2,3,4-tribromo-5-(1'- hydroxy-2',4'-dibromophenyl) pyrrole (**2**), isolated from *Pseudomonas bromoutilis* in a screening survey for antimicrobial products from marine bacteria.[23] Since then, Wratten *et al.* isolated another antibiotic-producing pseudomonad from a tidepool in La Jolla. This strain produced 3-bromoindole carboxaldehyde (**3**) which was not antibiotic as well as the antibiotics 2-n-heptyl-4-quinolinol (**4**) and 2-n-pentyl-4-quinolinol (**5**), the latter being a novel metabolite.[24] Andersen *et al.*, during a similar study of a *Chromobacterium*, isolated several brominated pyrroles (**6a-c**).[25] In a study of the pigments of *Pseudomonas magnesiorubra*, isolated from washings of the green alga *Caulerpa peltata*, the prodigiosin pigments (**7**), n = 4 - 6,

were identified.[26] Though not novel compounds, these pigments have been found to be common in marine bacteria.

In the 1970's, natural products chemists were accustomed to isolating novel compounds from most new biological samples, so bacterial projects which required considerable amounts of screening and dereplication of known compounds were not attractive based on these limited successes in identification of novel compounds. Thus, a gap in research on marine microbial secondary metabolites is noticeable in the 1980's. However, as epifluorescence techniques and hydrothermal vent research regenerated interest in marine microbiology, and high-field NMR instrumentation allowed structure elucidation on smaller samples, marine natural products chemists once again turned to marine microbes in search of novel compounds. In 1991, oncorhyncolide (**8**) was identified from an unidentified marine isolate, and Stierle identified the benzothiazoles, **9a** and **9b**, from a *Micrococcus* species which was consistently isolated from the sponge *Tedania ignis*.[27] One year later, another sponge, *Darwinella rosacea*, was the source of an *Alteromonas* sp. which produced antimicrobial pseudomonic acid derivatives (**10**).[28] Additional work by Stierle has involved the study of diketopiperazines produced by bacteria isolated from sponges. This work, as well as several ecologically-significant compound discoveries, including the macrolactins and isatin, will be discussed in later sections.

Actinomycetes isolated from marine sediments have also been shown to produce many interesting antibiotic compounds. Examples of these are istamycin (**11**), an aminoglycoside antibiotic produced by *Streptomyces tenjimariensis* isolated from sea mud near Tenjin Island in Sagami Bay, Japan.[29] Shallow sea mud from Koajiro inlet, also in Sagami Bay, yielded an actinomycete which produced aplasmomycin

Figure 2. Secondary Metabolites Produced by Unicellular Bacteria from Marine Sources



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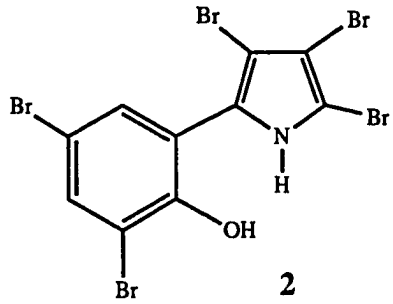

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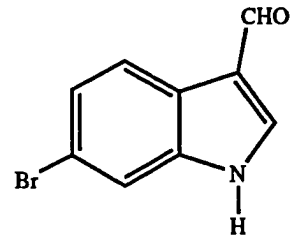

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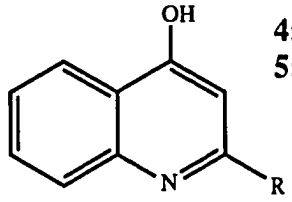

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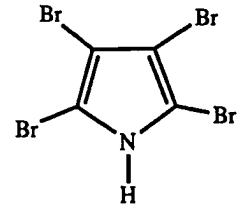
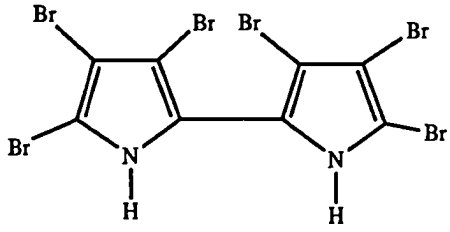
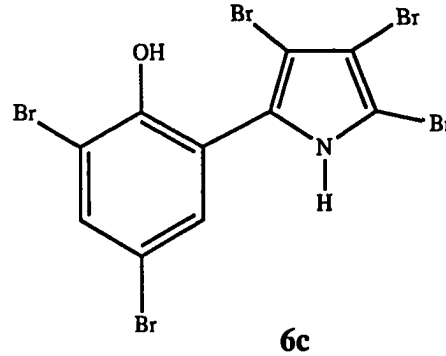
5: R=C_7H_{13}



6a

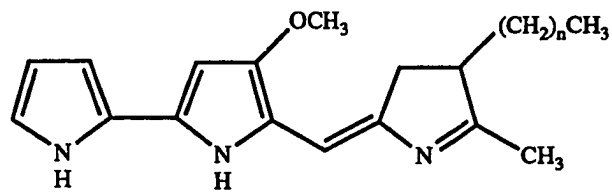


6b



6c

Figure 2. (continued)



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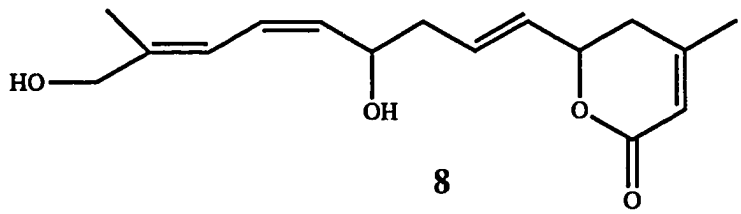

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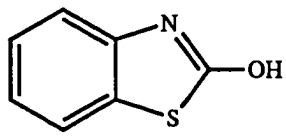
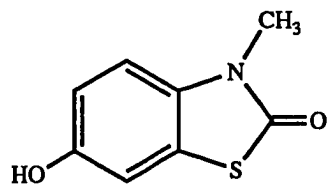
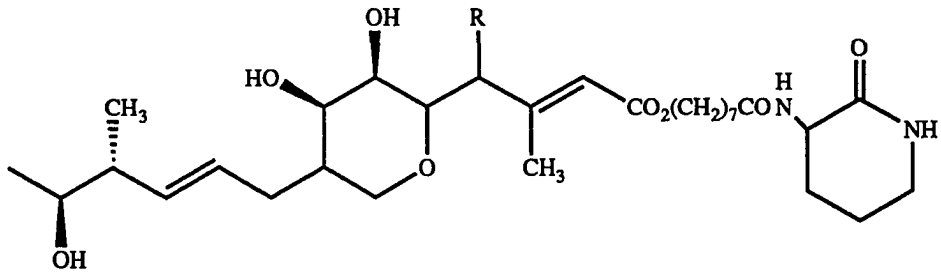


9a



9b

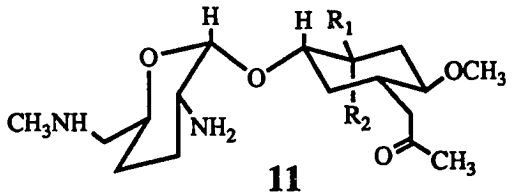


10a: R=OH
10b: R=H

(**12**).[30] Another species, *S. sioyaensis*, isolated by the Okami group from sea mud collected near Gamo, Japan, produced the acaricidal and antitumor alkaloid altemicidin (**13**).[31] Five new series of compounds were added to these by the Fenical group. The octalactins (**14**) are cytotoxic 8-membered ring lactones produced by a *Streptomyces* sp. from the surface of a Sea of Cortez gorgonian. These compounds showed *in vitro* cytotoxicity against murine melanoma and human colon tumor cell lines.[32] The work of Pathirana then added the novel butanolide, **15**, obtained from an actinomycete from a sediment sample taken in the Bahamas Islands,[33] as well as the macrolide glycoside, maduralide (**16**),[34] the antibiotic phenazine L-quinovose esters (**17**),[35] and the antibiotic sesquiterpenoid napthoquinones, marinone (**18**) and debromomarinone (**19**).[36] Maduralide, a weak antibiotic against *B. subtilis*, was produced by an unidentified marine actinomycete. The phenazines came from a species of *Streptomyces* isolated from the shallow sediment of Bodega Bay, CA. These compounds show broad spectrum antibacterial activity at MIC values as low as 1 μg/ml (*Hemophilus influenzae*). Both marinone and debromomarinone show significant antibiotic activity against Gram-positive bacteria.

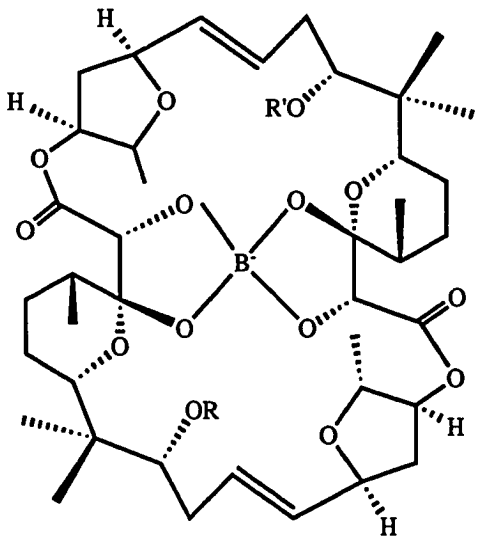
Another phylum of microorganisms found to be a rich source of bioactive compounds are the fungi. Marine fungi were not purposefully isolated in the projects described herein, but several isolates grew on plates containing antifungal compounds. These fungi were often thought to be unicellular bacteria or actinomycetes until they were examined under a microscope. Production of novel compounds by marine fungi has several precedents, including recent isolations of the fellutamides (**20**) and fumiquinazolines (**21**). These cytotoxic compounds were produced by fungi isolated from the gastrointestines of fish. The fumiquinazolines

Figure 3. Secondary Metabolites from Actinomycetes Isolated from Marine Sources



11

A: R_1=H, R_2=NH_2
B: R_1=NH_2, R_2=H



A: R=R'=H
B: R=H, R'=Ac
C: R=R'=Ac

M^+

12

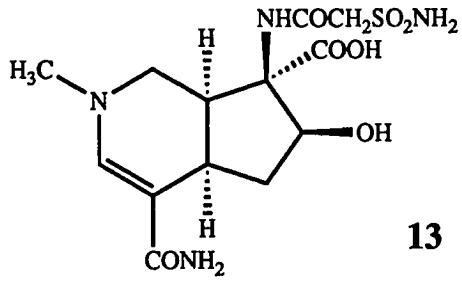


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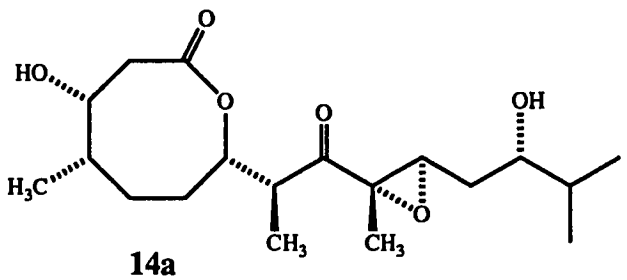
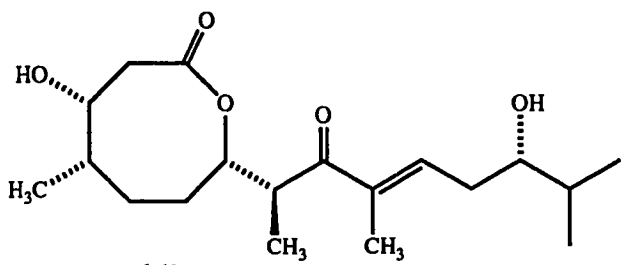
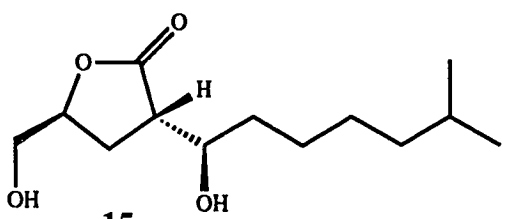
Figure 3. (continued)



14a



14b



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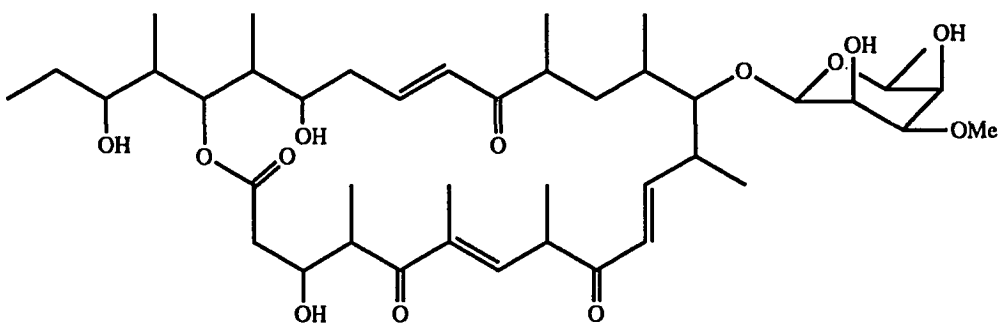

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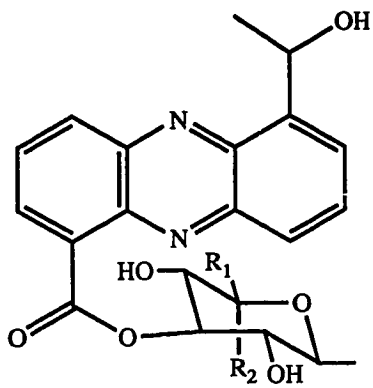
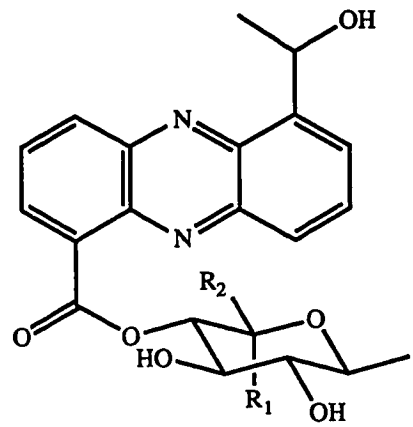
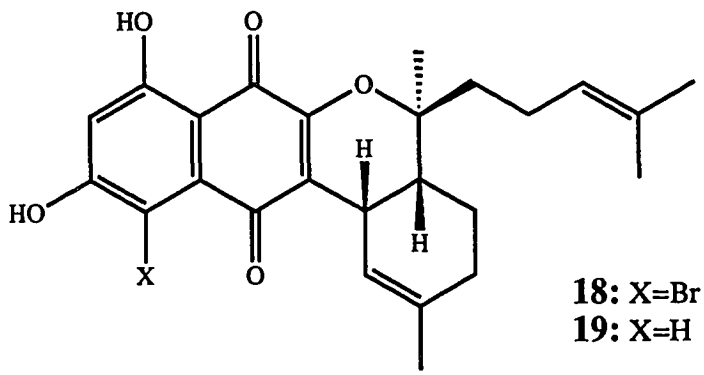
Figure 3. (Continued)



17a: R1=OH, R2=H
17b: R1=H, R2=OH



17c: R1=OH, R2=H
17d: R1=H, R2=OH



18: X=Br
19: X=H

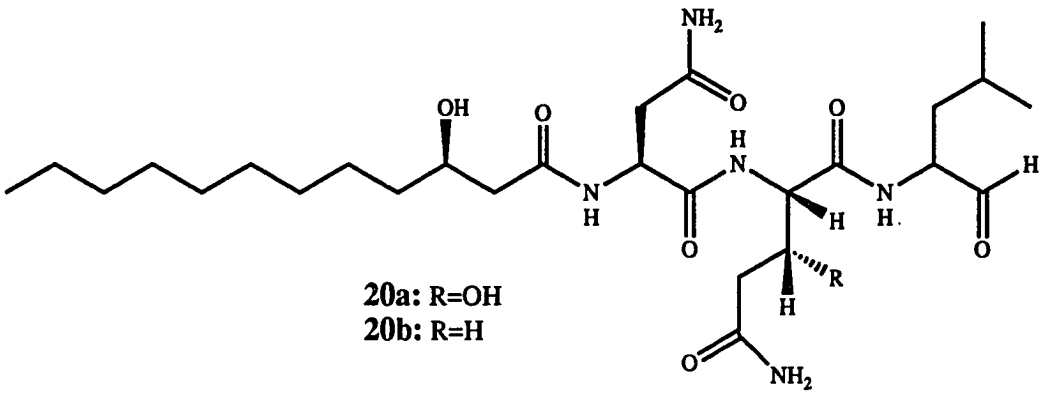
were isolated from the gut isolate *Aspergillus fumigatus*, while the fellutamides came from a fungus isolated from *Apogon endekataenia*.[37] Auranticins (**22**),[38] which are antimicrobial depsidones, and the epimeric δ-lactones, helicascolides A (**23a**) and B (**23b**),[39] were obtained from fungi isolated from mangroves. A halotolerant ascomycete from the surface of coastal marsh grass produced the polyketide obionin A (**24**),[40] while the surface of a crab shell was the source of the *Phoma* sp. which produced phomactin A (**25**).[41] Another surface isolate of *Penicillium* sp. was recently reported to produce the cytotoxic communesins (**26**). The fungal strain was isolated from the surface of the alga *Enteromorpha intestinalis*.[42] Dendryphiellin A (**27**) and a series of eremophilane derivatives were produced by a marine deuteromycete.[43] Also a rich source for novel compounds, the marine fungal isolate *Leptosphaeria oraemaris*, has been found to produce culmorin (**28**),[44] leptosphaerin (**29**),[45] leptosphaerolide (**30a**),[43] and leptosphaerodione (**30b**).

The compounds summarized in Figures 2-4 indicate that marine microorganisms are a valuable new resource for bioactive compounds. Given the extreme taxonomic diversity of microbes in the marine environments, relatively few genera have been chemically investigated. The goal of the work descirbed in this thesis was to identify the natural products of microbes from new, marine sources and to offer some insight into the antibiotic-producing capabilities of marine microorganisms.

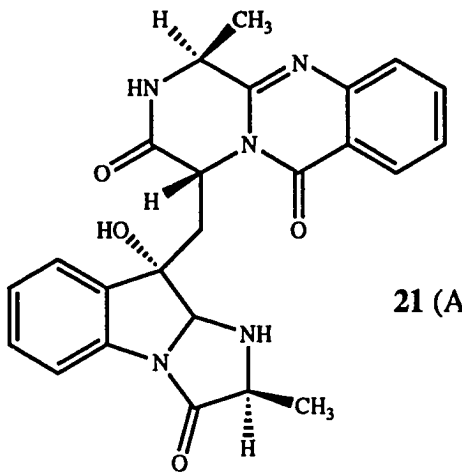
D. General Techniques Used

The study of bacteria required heavy time investment in the isolation and culture of the microorganisms before screening could even take place. Many of the

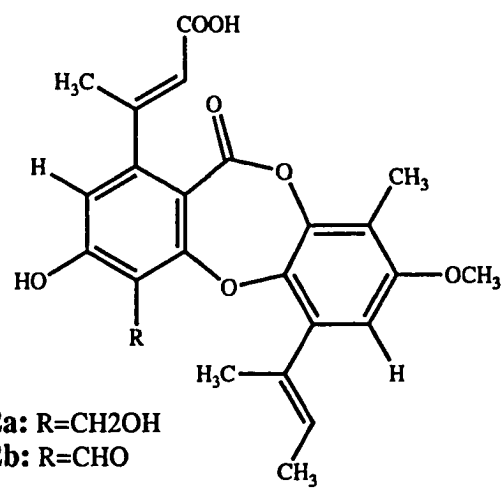
Figure 4. Secondary Metabolites Produced by Fungi from Marine Sources



20a: R=OH
20b: R=H

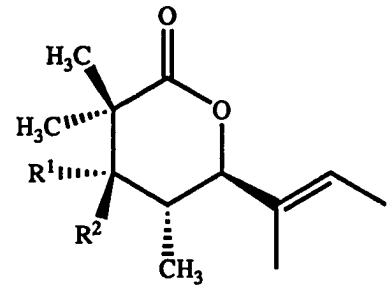


21 (A)

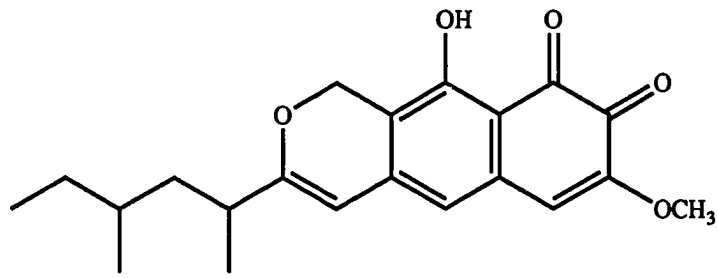


22a: R=CH2OH
22b: R=CHO

Figure 4. (Continued)



23a: R1=OH, R2=H
23b: R1=H, R2=OH



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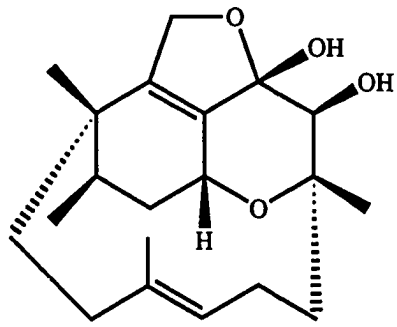

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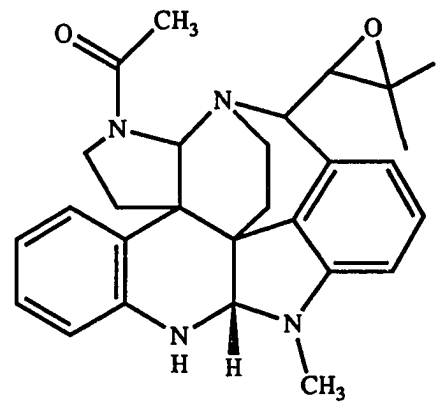

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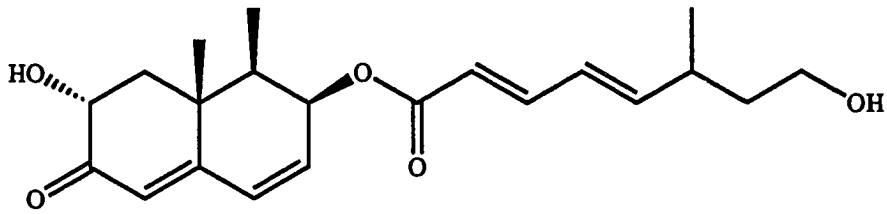
Figure 4. (Continued)



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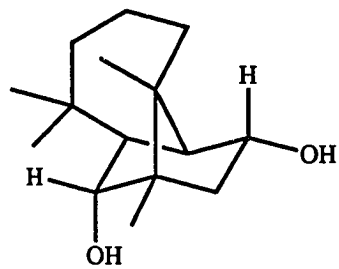

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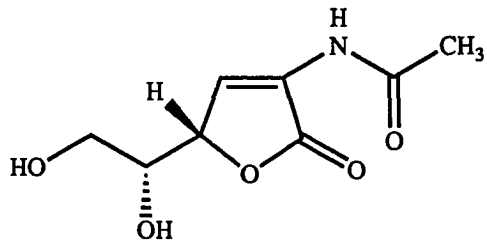

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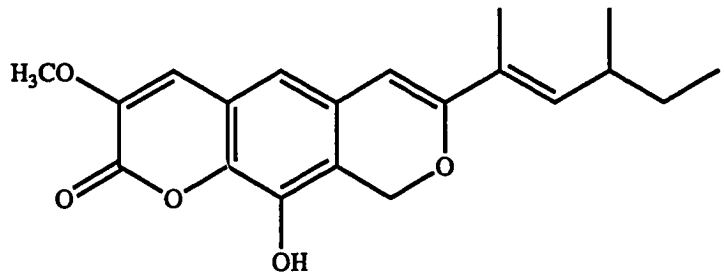
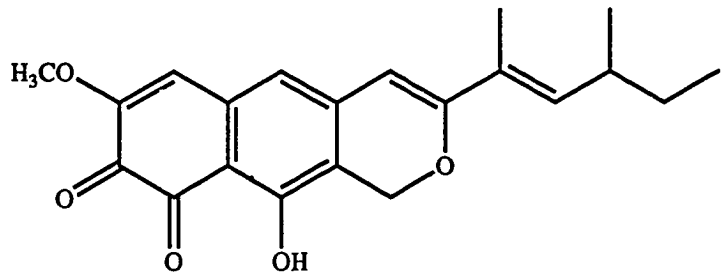
Figure 4. (Continued)



30a



30b

microbiological methods involved use of the following culture media:

Media ingredients per liter:

<u>A-1 media</u>		
Starch	10 g	
Yeast Extract	4 g	
Peptone	2 g	
Tris 1M pH 8.0	10 ml	
Sea-water	750 ml	
Deionized Water	250 ml	
Agar	17 g	(used for plate media only)
Cycloheximide	50 mg	(used for isolation plates only)
<u>B-1 media</u>		
Peptone	2.5 g	
Yeast Extract	1.5 g	
50% Glycerol	3.0 ml	
Sea-water	750 ml	
Deionized Water	250 ml	
Agar	17 g	
Cycloheximide	50 mg	(used for isolation plates only)
<u>B-3 media</u>		
Peptone	10 g	
Yeast Extract	6 g	
50% Glycerol	3.0 ml	
Sea-water	750 ml	
Deionized Water	250 ml	

Once identified as interesting, cultures were stored at -80°C with 10% glycerol added to the usual culture media in 2 ml cryovials. When cultured in liquid media, the contents of a cryovial were thawed for approximately 15 minutes, then transferred to 10 ml of the appropriate media in a 50 ml culture tube. Scale-up typically proceeded to a 100 ml culture in a 500 ml Erlenmeyer flask, then to 1 L in a 3 L Fernbach flask. For large-scale cultures, the 1 L growth was then transferred to a 12 L Virtis fermentor or a 50 L New Brunswick Scientific Fermatron fermentor. All transfers (except into the fermentors) were done aseptically in a sterile laminar flow hood. All tube and flask cultures were grown at room temperature with constant shaking.

Many problems were encountered with the culturing of the microorganisms. These problems included loss of viability of stock cultures, irreproducible production of compound, inconsistent product yield, and long delays in available cultures due to the logistics involved in growth and transfer of microorganisms. In many cases, the fluctuation in compound yield was concluded to be caused by inconsistent scale-up of cultures. Antibiotics are typically produced when a bacterial culture reaches stationary phase. Thus, up to this point, the bacteria are growing exponentially, which is as fast as they can grow. If a culture is scaled-up from 10 ml to 100 ml to 1L, then extracted after 7 days, a proper procedure for the culture of 6 L would be from 10 ml to 100 ml to 1 L to 6 x 1 L using 100 ml inocula (or transfer from 100 ml to 6 x 100 ml using 10 ml inocula). A scale-up from 10 ml to 100 ml to 6 L, grown for 7 days and extracted could only be expected to produce the same amount of compound as the original 1 L culture, since the bacteria cannot multiply any faster in 6 L than they do in 1 L. In fact, changes in scale-up can cause a fermentation to proceed completely differently, sometimes not producing the secondary metabolite at all, sometimes producing it in greater amounts.[46] Scale-up problems caused several long delays in availability of cultures and several months of chemical isolation to be wasted isolating quantities of compounds from large cultures which could have been obtained from extracts of 1 L or smaller cultures.

Cultures were double-extracted with ethyl acetate (EtOAc) in most cases, but large fermentations required use of resins, C_{18} or ion-exchange in this case. Pure metabolites were isolated using vacuum flash chromatography, size-exclusion chromatography (SEC), and high-performance liquid chromatography (HPLC). Structures were elucidated using a combination of chemical and spectral methods: mass spectrometry (MS) and ultraviolet (UV), infrared (IR) and ^{1}H and ^{13}C nuclear

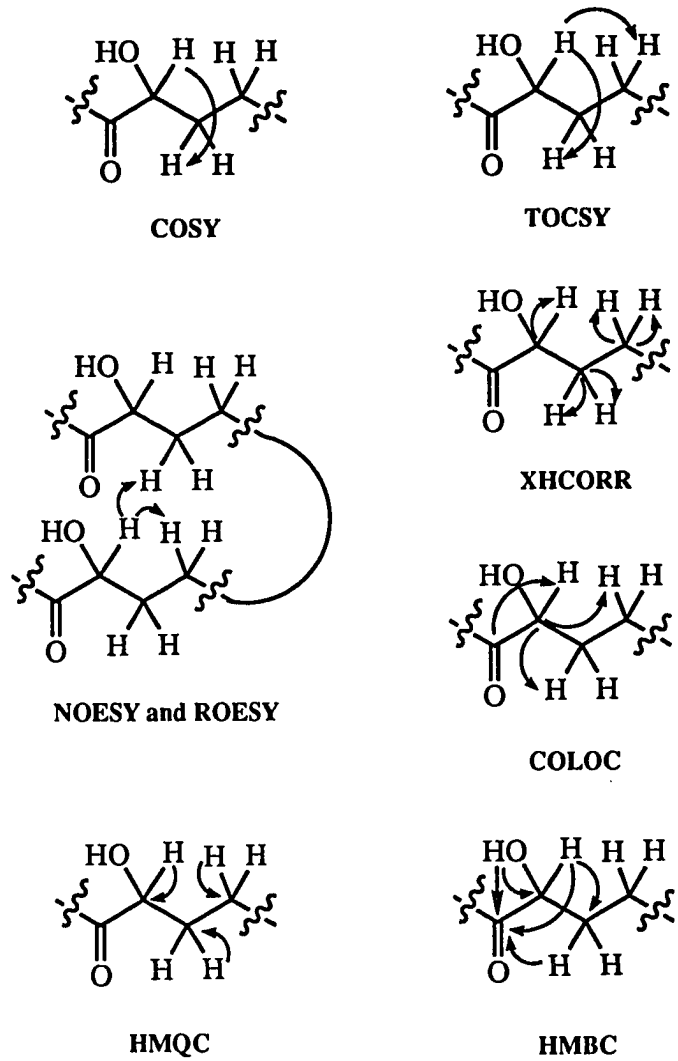
magnetic resonance (NMR) spectroscopy.

Advancements in available NMR techniques have allowed two-dimensional NMR experiments to be run using very small amounts of peptides and other complex molecules. The 2-D NMR experiments used in the described structure elucidations include: COupled SpectroscopY (COSY), TOtally Coupled SpectroscopY (TOCSY), Nuclear Overhauser Enhancement SpectroscopY (NOESY), Rotating-frame Overhauser Enhancement SpectroscopY (ROESY), 1-bond (carbon-detected) X-H CORRelation (XHCORR), multiple-bond (carbon-detected) X-H correlation (COLOC), (1-bond, proton-detected) Heteronuclear Multiple Quantum Coherence (HMQC), and (multiple-bond, proton-detected) Heteronuclear Multiple Bond Coherence. These experiments are used to determine the correlations shown in Figure 5.

Despite the availability of these powerful techniques, a few compounds cannot be solved unambiguously using spectral data. Frequently, lack of protons on a carbon skeleton can cause gaps in NMR correlations which prevent partial structures from being linked, even using the other available spectral data. In cases like these, a compound must be altered to yield a product more amenable to elucidation by spectroscopic means. Alternatively, a crystalline compound can often be identified by X-ray analysis.

In most instances, partial or total identification of the compounds in this thesis was followed by a library search, using the Berdy database or Chemical Abstracts (CAS) on-line database, which allowed dereplication to a known compound or confirmation that a compound was novel. The strategies used to discover these compounds, the elucidation of the structures, and the significance of production by marine isolates is described in the subsequent chapters.

Figure 5. Correlations shown by 2-D NMR experiments[a]



[a](not all possible correlations are indicated)

Chapter II

The Natural Products Chemistry of Bacteria Isolated from the Deep-Sea Floor

A. Introduction to Chapter II

Bacterial adaptations to pressure influence their distribution in the marine environment. In the deep ocean (>1000 m), the environment is cold, dark, nutrient-poor, and at extremely high pressure (add 1 atm for every 10 m depth). Actually, this accounts for 75% of the oceanic environment.[3] Despite these harsh conditions, the concentration of bacteria in sediment surfaces, 3 cm into the sediment, and 15 cm into the sediment at 4000 m have been reported as 4.65×10^8, 8.29×10^8, and 1.7×10^7 bacterial cells/g dry sediment, respectively.[47] An even higher concentration of 3.07×10^{10} bacterial cells/g dry weight on fecal pellets indicates that availibility of nutrients plays a significant role in determining bacterial concentration. Larger-sized food sources, such as dead fish, fall to the sea floor sporadically, while "marine snow," consisting of highly-recalcitrant particles, fecal pellets, phytoplankton, and zooplankton, falls continuously, following the cycles of primary production at the surface. With this marine snow, many surface-water bacteria arrive on the ocean floor. Among strains which are isolated from deep-sea samples, these bacteria are characterized by increased metabolic activity as culture pressure is decreased to atmospheric pressure. However, use of cleverly-designed instruments which bring microbes to the surface at their *in situ* temperature and pressure have allowed isolation of obligate barophiles which will not grow at pressures below several hundred atm. These bacteria show interesting physiological adaptations to the deep ocean, including production of long-chain polyunsaturated fatty acids which were previously not thought to be produced by prokaryotes.[48]

More pertinent to this study are the barotolerant bacteria which can grow at 1

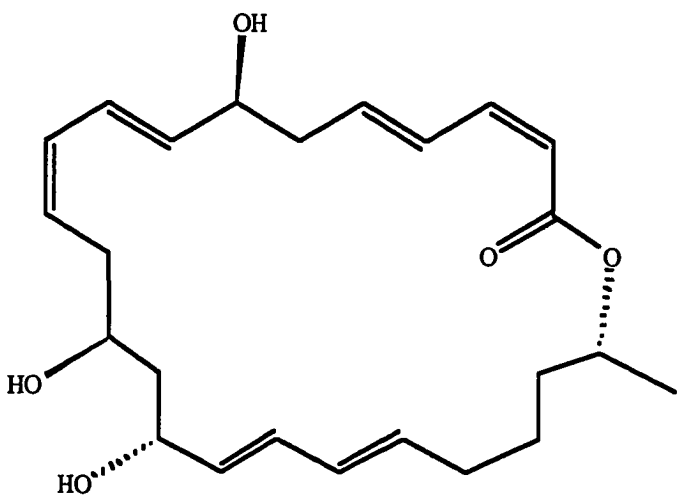
atm, yet survive pressures of 500 atm. The adaptations allowing these organisms to survive appear to include tolerance to starvation, causing cell sizes to be extremely small, with 50-77% of the bacteria found in a deep sediment sample able to pass through a 0.45 μ filter.[49] The upper sediments are oxygenated at >50% saturation levels for air (~4mg/L), but these levels drop rapidly as you move into the dense sediment, with very little aeration at 10 cm. Therefore, anaerobic tolerance would also favor long-term survival in marine sediments, although deposition rates are slow in the deep ocean, reported as 2.3 mm/1000 yrs. for red clay.[3] So, some microorganisms collected from deep in the sediment may have been there as spores or slowly growing cells for millions of years.

In this study, microbes were isolated at 1 atm and room temperature from frozen core samples of deep ocean sediment. Yayanos argues that bacteria growing at 25°C and 1 atm typically show no growth under deep-sea temperature and pressure conditions.[50] With this in mind, the results of these experiments can not be used to draw conclusions about the deep-sea environment without further investigation, especially those dealing with the barotolerance of the isolates. These isolates are valuable, instead, because they come from a new, undeveloped source of microorganisms which may even represent strains that have only existed as spores for the last million years. Though no barophily has been shown, suggestions of metabolic activity at elevated pressure and lowered temperature are included with hypotheses of why compounds may be produced. If nothing else, these ideas indicate that these isolates warrant further study.

All of the isolates used in this investigation were taken from frozen cores of the Deep-Sea Drilling Project (DSDP). The DSDP was begun by the National Science Foundation in 1968, but soft sediment cores were not obtained until 1979, with the

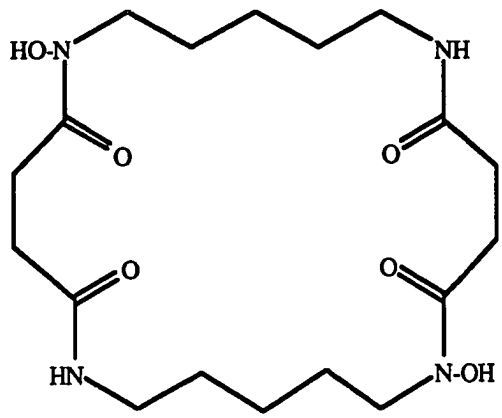
invention of the hydraulic piston corer. With this corer, virtually undisturbed samples of soft sediment layers can be taken, thus allowing study of the bacteria living in the surface layers of sediments from up to 5000 m in this study. The frozen storage of these cores should not greatly affect the microbial populations, since the *in situ* temperature at the collection depths is approximately 2°C. In fact, these cores have proved to be a good resource for producers of novel compounds in the past.

Gustafson *et al.* isolated a novel class of macrolides from a deep-sea isolate from one such DSDP core sample from the North Pacific.[51] One of these compounds, macrolactin A (**31**), was found to have antibacterial, antiviral, and cytotoxic properties. Unfortunately, not enough of **31** was produced to determine its stereochemistry. In later cultures, attempts to isolate **31** failed; the isolate had stopped producing macrolactin A. Thus, the stereochemistry had to be determined using the ß-glucosyl analog, macrolactin B.[52]



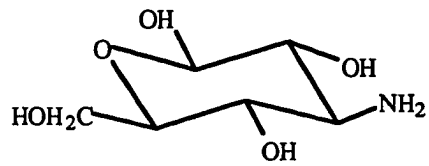
31

The Okami group has also published a novel compound from a deep-sea (3300 m) sediment isolate of *Alteromonas haloplanktis*.[53] The compound, bisucaberin **(32)**, showed activity as a siderophore (a low molecular weight iron chelator), as



32

well as antitumor activity.[53] Another chemical study of 90 deep-sea isolates yielded the carbohydrate **33** from a *Bacillus* sp.[54] Compound **33** showed antimicrobial activity against several medically-relevant strains. An interesting aspect of this study



33

is that mainly Gram (+) organisms were found. Perhaps these microbes, which may exist as spores, are the most common in these isolations because the samples used were box cores or "grabs" which were brought through the water column at ambient temperature and pressure. This report combined with the proven track record of Gram (+) marine isolates producing novel compounds convinced the microbiologists

of the Fenical lab to undertake a more selective isolation approach, looking exclusively for Gram (+) bacteria from one set of the DSDP cores.

Chemical studies were actually performed using two sets of isolates from the DSDP cores. In the first set, bacteria were isolated from the top 50 cm of 4 cores taken at the following locations and depths:

Cabo San Lucas	624 m
Island in the Gulf of California	362 m
Guaymas Basin	1834 m
Sunda Trench	5234 m

Though hydrothermal vent activity has been found in the Guaymas Basin, the samples here are all from cold sediments. Approximately 50 strains were isolated, cultured on 100 ml scale, then extracted with EtOAc. The extracts showed little activity against the three medically-relevant strains *Bacillus subtilis, Escherichia coli,* or *Candida albicans*, so projects were selected based on unique TLC characteristics and ^{1}H NMR spectra. Approximately 10 projects were deemed worthy of further investigation at this point.

In the second approach, the core samples were taken from 5 cm and 15 cm into the core, then heated to 37°C overnight to select for spore-formers. These samples were then serially-diluted, spread-plated, and isolated. The screening procedure was the same as that of the first set. No actinomycetes were isolated, but several *Bacillus* sp. produced interesting compounds.

The isolation techniques in this study only afforded 70 strains for chemical study, but several novel compounds were isolated from these strains. The first of these new structures, the guaymasols, seem to be representative of the type of molecules found in the majority of the deep-sea isolates, small, simple aromatics.

For example, N-acetyl-phenylethylamine was another compound found in one *B. cereus* strain, along with more volatile aromatics. Four other projects were not pursued when the "interesting" TLC bands could be attributed to small aromatics. A more exciting discovery was an analog of the powerful biosurfactant, surfactin. The compound was named surfactamide, since it differed from surfactin in the presence of an amide funtionality in place of the acid moiety of the Glu residue. This represents a significant finding in that no acylpeptides have previously been reported from marine sources. Overall, the deep-sea isolates appear to be a fruitful new source of novel compounds when the limited number of isolates screened is considered.

The majority of the projects pursued involved deep-sea isolates of the genus *Bacillus*. Most likely, these strains exist as spores in the sediments from 1000 m or more in depth. Investigations of the lethality of extreme hydrostatic pressure effects have led to the conclusion that germination is induced in spores of *B. subtilis, B. pumilis,* and *B. cereus* at pressures between 325 and 975 atm.[55] Germination led to inactivation of the cells, because they could not grow at these pressures. Therefore, the isolates used in this study were probably present as spores in the deeper cores which were not heat-treated in addition to the heat-treated core samples from all depths. The cores from less than 1000 m may contain metabollically-active vegetative cells of *Bacillus* species, though the heat-treated samples show that there are some cells present as spores in marine sediments even at pressures of less than 100 atm. Also, since no spores in the studies of Murrell and Wills survived prolonged exposure to pressures higher than 970 atm,[55] any future *Bacillus* isolates from cores taken at depths greater than 9700 m should be suspected of having some special adaptations to the deep-ocean environment.

Once again, the point of chemical examination of deep-sea isolates was to identify a new source, not to prove that the chemicals produced are somehow active in the environment. In the following sections, each identified compound will be discussed in terms of structure elucidation and the significance of its production. A ^{1}H NMR spectrum is shown for each pure compound to aid in subsequent dereplication.

B. The Isolation and Structure Elucidation of Guaymasol (34), Epiguaymasol (35) and Guaymasone (37) from Isolate CNA995

CNA995 is an aerobic, Gram (+) strain isolated from a Deep Sea Drilling Project (DSDP) core. The core was taken in the Guaymas Basin in the Gulf of California at 1834 m. The strain was isolated from 50 cm into the frozen core sample. Since it was still viable in culture, the isolate must have been present as a spore in the core sample (Though it was undoubtedly inactive metabolically at 50 cm under the sediment, it could have been metabolizing when it was on the surface of the sediment.). No bioactivity was seen against *E. coli*, *C. albicans* or *B. subtilis*, but the extract of an 100 ml culture had purple- and yellow-charring bands at R_F = 0.6 and R_F = 0.5, respectively, on a TLC chromatogram developed in EtOAc. Also, the ^1H NMR spectrum contained unusual signals at a shift of 3.8 ppm. The area of the signal indicated that it was present in good quantity, a true concern with unicellular bacterial projects, even with exciting chromatograms.

CNA995 was grown in 6 L of B-3 media in Fernbach flasks with constant shaking. The EtOAc extract yielded 680 mg of crude organics. Using vacuum flash chromatography followed by normal-phase HPLC, three interesting pure compounds were isolated. The TLC of one of the flash column fractions had appeared to be pure purple-charring material; and the ^1H NMR spectrum also looked nearly pure. However, HPLC separated this into two distinct peaks, corresponding to guaymasol (**34**, 19.1% of the crude) and epiguaymasol (**35**, 3.0% of the crude). Both compounds charred purple, and their spectroscopic characteristics were nearly identical.

Because **34** was isolated in higher yield, structure elucidation experiments proceeded with this material. With only the 1-D ^1H and ^{13}C NMR spectra, pieces of

the structure could be assembled, including a *para*-substituted phenol ring and an isopropyl group. With the *para*-methylphenol fragment (*m/z* 107) in the low-resolution EIMS and the HMQC data added, the anisotropic shifts of the benzylic group were assigned. The benzylic carbon had to be adjacent to a carbon with one proton according to the vicinal coupling seen. Finally, the COSY experiment was used to piece the structure together, with a bond added from C2 to C3. The protons on C2 and C3 had the same chemical shift, so they either had to be linked as in **34**, or one had to be left out of the structure. The heteroatoms on C2 and C3 were tentatively assigned as oxygen based on ^{13}C chemical shifts, and this was confirmed with high-resolution FABMS data which required the formula $C_{13}H_{20}O_3$.

Epiguaymasol showed the same coupling patterns as **34**, along with the same molecular formula by high-resolution FABMS. Therefore, the difference must lie in the stereochemistry at the two chiral centers. The relative stereochemistries of **34** and **35** were proved by forming the acetonides and recording the ^{13}C NMR experiments as shown in Table 3. In guaymasol, the methyl shifts of the acetonide are nearly equivalent. This indicates *anti* conformation for the isopropylidene ring, so the stereochemistry is either (*R,R*)or (*S,S*). Epiguaymasol reacted much differently than guaymasol, taking higher temperature and longer time to form the acetonide. In this case, the methyl groups of the ring were shifted by 2.4 ppm, one upfield and one downfield. The *syn* conformation accounts for these shifts. According to previous studies on 1,3-dioxolanes,[56,57] the downfield signal is *cis* to the C2 and C3 substituents and the upfield methyl is *trans*. Therefore, guaymasol is (*R*,R**) and epiguaymasol is (*R*,S**) at C2 and C3, respectively.

The difference in reaction conditions needed can be accounted for by examining the Newman projection along the C2-C3 bond as shown below. With the bulky

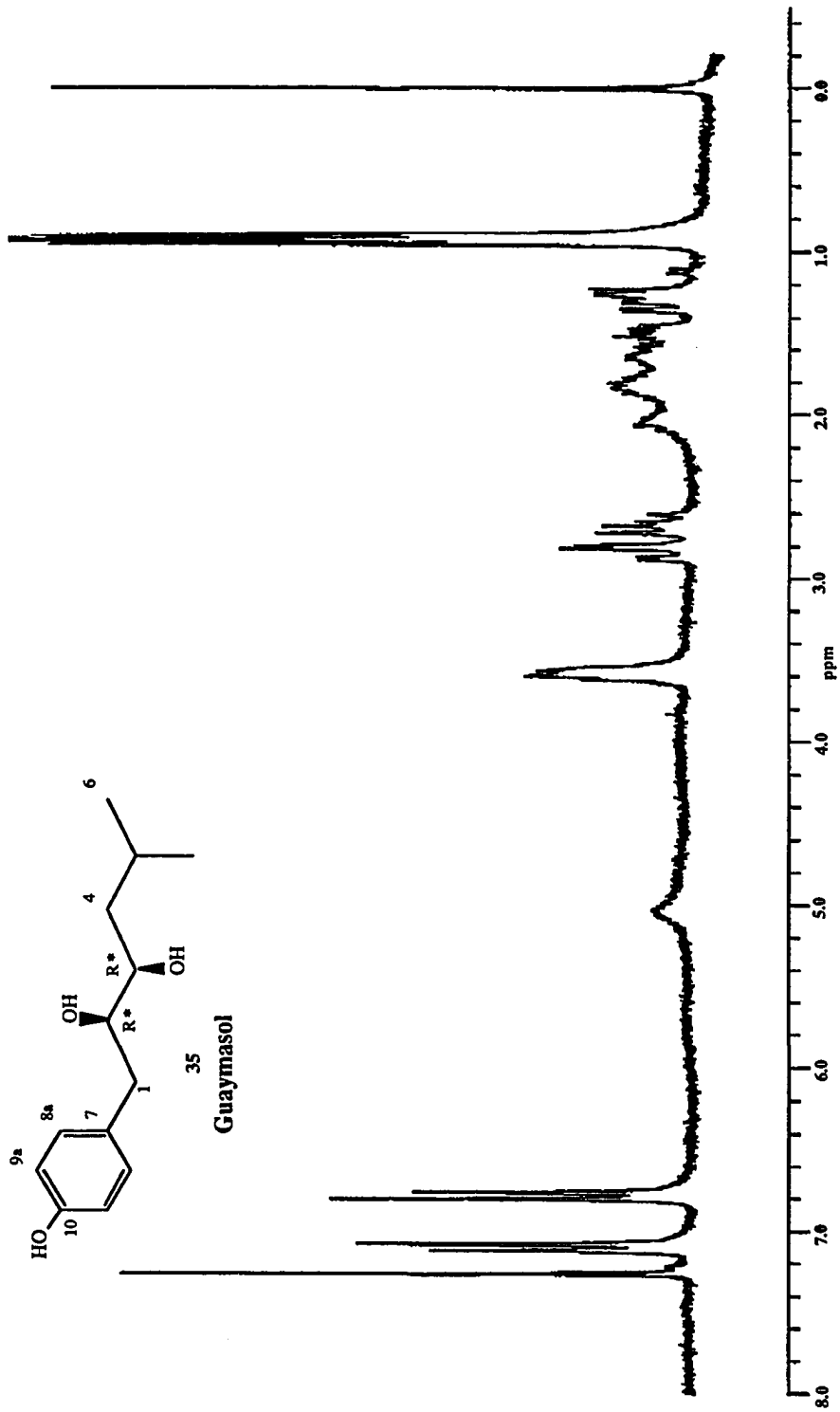
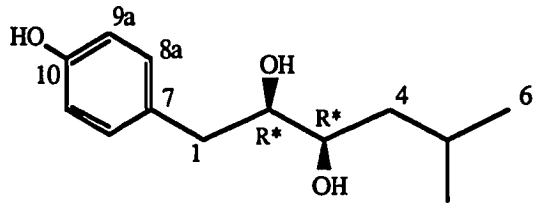


Figure 6. ^1H NMR spectrum of guaymasol (35) in $CDCl_3$ at 200 MHz

Table 1. Spectral data for guaymasol (34)



Source: CNA995, Deep-Sea Drilling Project core, Gram (+) isolate

Molecular formula: $C_{13}H_{20}O_3$

HRFABMS: ($M+H^+$) m/z 225.15050 (obs); 225.14907 (calc), Δ 6.4 ppm

LREIMS: M^+, *m/z* (rel. int.) 107 (100), 77 (46), 43 (61), 91 (34), 119 (11), 136 (6), 224 (4)

IR ($CHCl_3$): 3594 (sharp), 3400 (broad), 3156 (sharp), 2959 cm^{-1}

NMR data [a,b]

C#	$\delta^{13}C$ (ppm)	DEPT	$\delta^{1}H$ (ppm)	mult, J (Hz)	COSY
1	42.8	CH_2	2.65	dd, 7.3, 14.1	2
			2.82	dd, 4.4, 14.2	2
2	75.5	CH	3.60	m	1
3	71.7	CH	3.58	m	4
4	39.2	CH_2	1.25	m	3
			1.48	m	3, 5
5	24.5	CH	1.80	m	6, 11, 4b
6	21.8	CH_3	0.90	d, 7.0	5
7	129.9	C			
8a	130.5	CH	7.05	d, 8.5	9
8b	130.5	CH	7.05	d, 8.5	9
9a	115.5	CH	6.88	d, 8.5	8
9b	115.5	CH	6.88	d, 8.5	8
10	154.4	C			
11	23.6	CH_3	0.90	d, 7.0	5

[a] The ^1H NMR experiments were recorded at 500 MHz in $CDCl_3$. [b] The ^{13}C NMR experiments were recorded in $CDCl_3$ at 125 MHz.

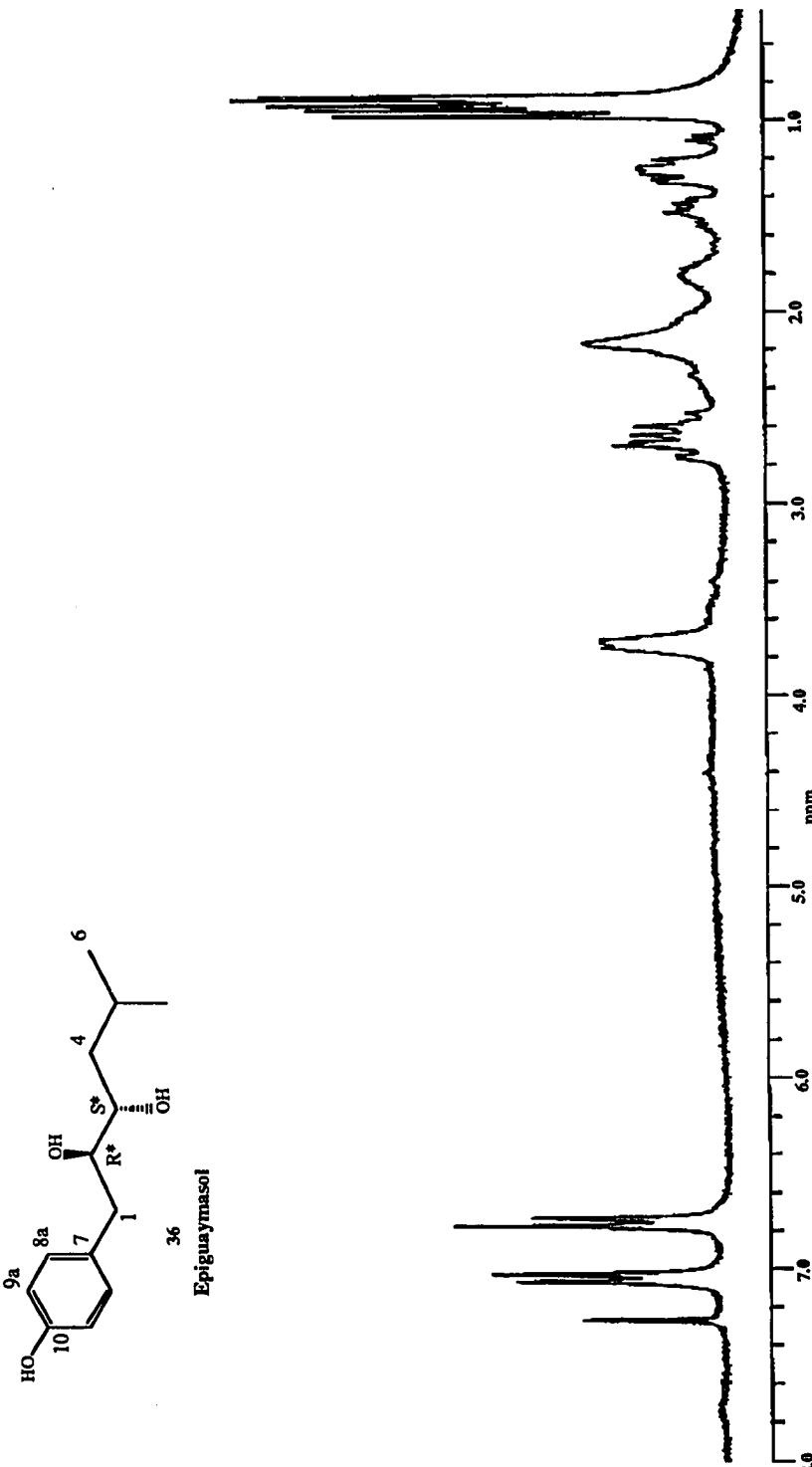
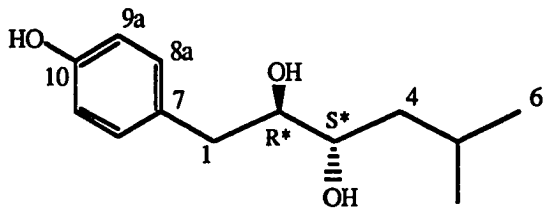


Figure 7. ^{1}H NMR spectrum of epiguaymasol (36) in $CDCl_3$ at 200 MHz

Table 2. Spectral data for epiguaymasol (35)



Source: CNA995, Deep-Sea Drilling Project core, Gram (+) isolate

Molecular formula: $C_{13}H_{20}O_3$

HRFABMS: $(M+H^+)$ m/z 225.15084 (obs); 225.14907 (calc), Δ 7.9 ppm

LREIMS: M^+, *m/z* (rel. int.) 107 (100), 77 (46), 43 (61), 91 (34), 119 (11), 136 (6), 224 (4)

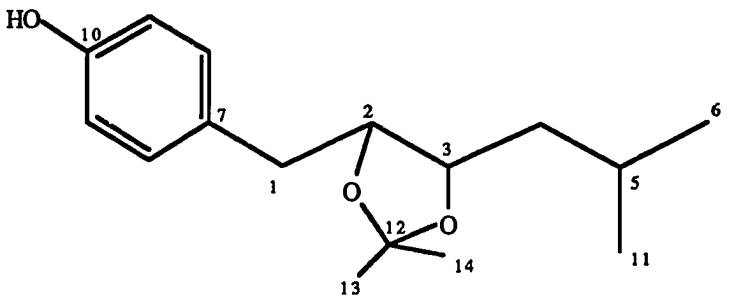
IR ($CHCl_3$): 3596 (sharp), 3400 (broad), 3156 (sharp), 2959 cm^{-1}

NMR data [a,b]

C#	$\delta^{13}C$ (ppm)	DEPT	$\delta^{1}H$ (ppm)	mult, J (Hz)
1	40.2	CH_2	2.62	dd, 7.3, 14.1
			2.74	dd, 4.4, 14.2
2	76.0	CH	3.74	m
3	71.8	CH	3.72	m
4	36.9	CH_2	1.25	m
			1.45	m
5	24.2	CH	1.86	m
6	21.3	CH_3	0.96	d, 7.0
7	129.5	C		
8a	130.0	CH	7.05	d, 8.5
8b	130.0	CH	7.05	d, 8.5
9a	115.0	CH	6.88	d, 8.5
9b	115.0	CH	6.88	d, 8.5
10	154.9	C		
11	23.4	CH_3	0.90	d, 7.0

[a] The ^1H NMR experiments were recorded at 500 MHz in $CDCl_3$. [b] The ^{13}C NMR experiments were recorded in $CDCl_3$ at 125 MHz.

Table 3. ^{13}C chemical shifts of the acetonides of guaymasol and epiguaymasol



Guaymasol acetonide (**36**)

Guaymasol acetonide

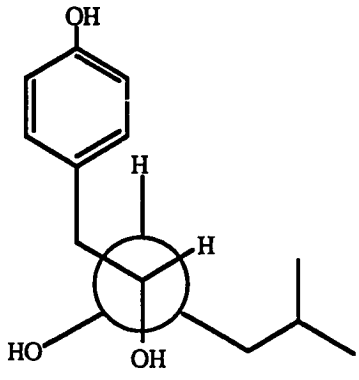
C #	$\delta^{13}C$
1	42.0
2	81.7
3	78.7
4	38.2
5	25.4
6	23.6*
7	129.7
8	130.5
9	115.2
10	154.2
11	21.9*
12	108.1
13	27.2†
14	27.4†

Epiguaymasol acetonide

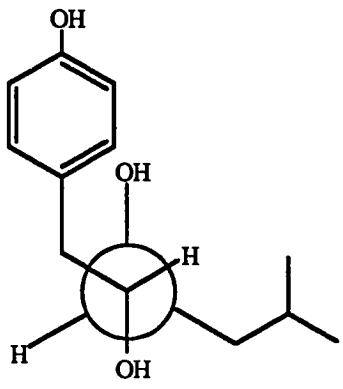
C #	$\delta^{13}C^{a}$
1	38.3
2	78.9
3	75.8
4	35.4
5	25.9
6	23.6**
7	-
8	130.1
9	115.1
10	-
11	21.7**
12	-
13	28.6‡
14	25.0‡

a ^{13}C NMR experiments done in $CDCl_3$ at 200 MHz, guaymasol B acetonide chemical shifts recorded using DEPT experiment. *, **, †, ‡ Shifts may be interchanged

phenylmethylene and isobutyl groups at 180°, the reaction proceeds rapidly. However, in the transition state of the reaction of **35** with dimethoxypropane, the



34



35

phenylmethylene and isobutyl groups must be *cis* to form the desired product, requiring more energy than the reaction of **34** to overcome the steric hindrance.

The yellow-charring compound was identified as guaymasone (**37**, 2.4% of the crude extract) based on the ^{13}C shift at 211 ppm, a strong IR band at 1730 cm^{-1}, a molecular ion at two mass units less than **34** and **35**, and the comparison of the remaining data to those of guaymasol. The ketone was placed at C3, because the shifts of the C4 protons had sharpened to a clear doublet, and the C1 protons appeared unchanged in their coupling pattern when compared to guaymasol.

Guaymasone was reported once previously, from a tissue culture of human uterine cells.[58] Stereochemistry at C2 was not reported. The reported compound showed anti-neoplastic activity against a leukemia cell-line in the screening program of Ikegawa. However, none of the CNA995 compounds showed any activity in the Bristol-Myers Squibb screening against the human colon tumor cell-line, HCT-16.

These compounds do not appear to be antibiotic, and they have no obvious uses in primary metabolism of the microorganism. However, there must be some evolutionary advantage to the production of guaymasol and guaymasone.

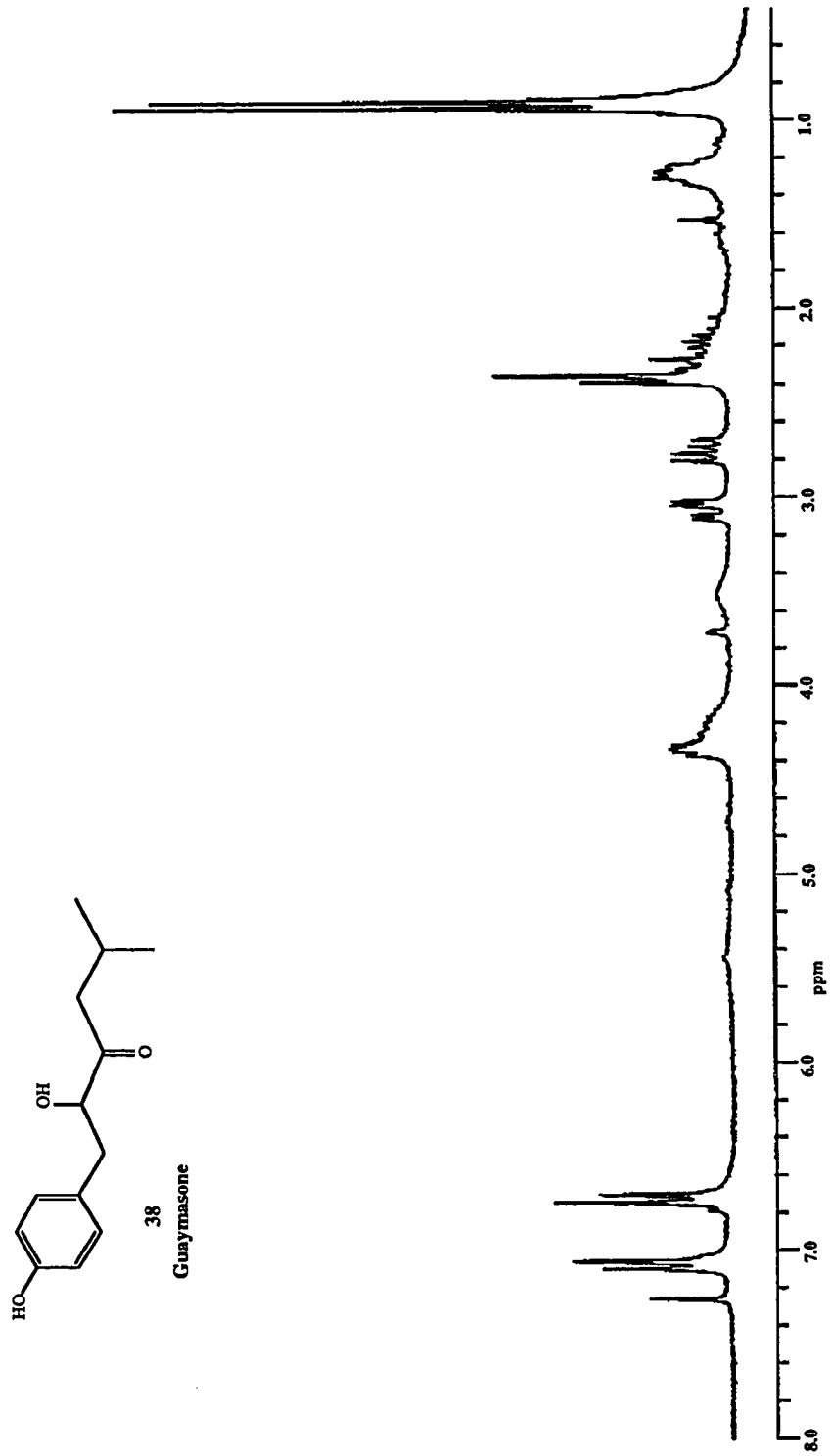
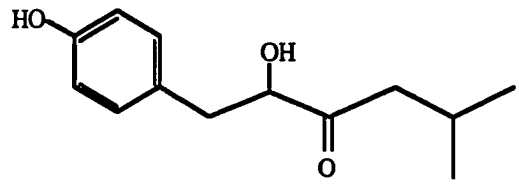


Figure 8. ^{1}H NMR spectrum of guaymasone (37) in $CDCl_3$ at 200 MHz

Table 4. Spectral data for guaymasone (37)



Source: CNA995, Deep-Sea Drilling Project core, Gram(+) isolate

Molecular formula: $C_{13}H_{18}O_3$

HRFABMS: $(M+H^+)$ *m/z* 223.13540 (obs); 223.13342 (calc), Δ 8.9 ppm
LREIMS: M^+, *m/z* (rel. int.) 107 (100), 137 (16), 91 (15), 57 (12), 204 (5)

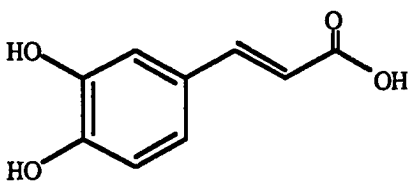
IR ($CHCl_3$): 3594 (sharp), 3468 (broad), 2962, 1731 (strong), 1251 cm^{-1}

NMR data [a,b]

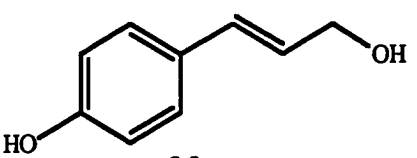
C#	$\delta^{13}C$ (ppm)	DEPT	$\delta^{1}H$ (ppm)	mult, J (Hz)
1	39.1	CH_2	2.76	dd, 7.3, 14.1
			3.08	dd, 4.4, 14.2
2	77.6	CH	4.35	dd, 4.4, 7.4
3	211.4	C	-	
4	47.4	CH_2	2.38	d, 6.8
			2.38	d, 6.8
5	24.6	CH	2.18	m
6	22.5	CH_3	0.91	d, 7.0
7	128.3	C	-	
8a	130.4	CH	7.11	d, 8.5
8b	130.4	CH	7.11	d, 8.5
9a	115.3	CH	6.76	d, 8.5
9b	115.3	CH	6.76	d, 8.5
10	154.6	C	-	
11	22.6	CH_3	0.94	d, 7.0

[a] The ^1H NMR experiments were recorded at 200 MHz in $CDCl_3$. [b] The ^{13}C NMR experiments were recorded in $CDCl_3$ at 50 MHz.

Otherwise, metabolic resources would not be used for the production of these compounds. In examining the poly-hydroxylated guaymasol/guamasone structures, comparisons to the building block of tannins (**38**) and lignins (**39**) can be drawn.



38



39

Research on these phenolics has resulted in several hypotheses for their uses in the natural environment.[59] In a review on tannins and lignins, Swain proposes that phenolics are responsible for retention of nitrogen in the ecosystem. In the marine environment, nitrogen fixation occurs primarily by cyanobacteria. This occurs only in the photic zone. Heterotrophic nitrogen-fixing bacteria are present on the ocean floor, and it would seem that they should have a competitive advantage in this niche. However, it costs nitrogen-fixers 8-12 g glucose to fix 1 g N_2, so these bacteria are carbon-limited while most other microbes are nitrogen-limited on the ocean floor.[60] Thus, nearly all of the fixed nitrogen that reaches the deep ocean floor falls out of the water column.

Once nitrogenous material reaches the sediment, detritovores begin to break down complex molecules into small carbon and nitrogen sources consumable by most heterotrophs. However, these nitrogen sources are water-soluble. Unless a heterotroph can grow extremely fast and use all of the nitrogen as it is released by the detritovores, some material will be lost. Therefore, compounds which retain fixed nitrogen in the sediment would offer a slow-growing microbe a competitive advantage by allowing consumption of nitrogenous material at the desired rate.

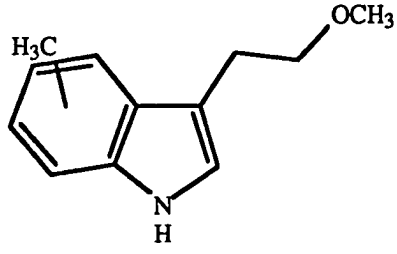
It has been shown that phenolics are produced by plants in response to nitrogen

limitation in soil.[59] And, several microbially-produced polymers have been shown to be excreted in greater quantities under conditions of nitrogen limitation,[61] but the causes of this phenomenon are not known. It is not even known whether or not these polymers are produced *in situ*. Similarly, it would be impossible to determine whether or not guaymasol and guaymasone are being produced in the ocean sediment. The rate of metabolism of typical ocean floor bacteria is very slow, so these compounds would not be present in detectable quantities. However, the first step in hypothesizing that these compounds can be active in the marine sediment would be to show that they are produced in response to nitrogen limitation, and that they can act as protein-binding phenolics in the laboratory.

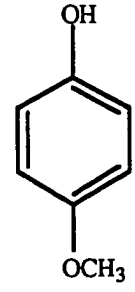
Preliminary study of guaymasol production with nitrogen limitation showed that nearly twice the relative amount of the guaymasols and guaymasone were produced when the available nitrogen source was 1% of its usual value in B-3 media plus starch. However, the actual amounts of the extracts were very different, and the cultures were harvested on the same day of growth, most likely at different points on their growth curves. So, a more controlled experiment must be performed to draw any valid conclusions. The steps of this experiment would involve: 1) finding a defined medium on which guaymasol/guaymasone is produced in good amounts; 2) determining the secondary metabolite production curve for each medium; 3) adding an internal standard to avoid variability caused by extraction procedures; 4) culturing CNA995 in the defined medium with varying percentages of the nitrogen source and harvesting at the same point in the production curve for each medium; and 5) determining the amount of **34**, **35** and **37** in each extract. Increase of guaymasol/ guaymasone production in response to decreases in available nitrogen would support the hypothesis that these compounds are used to retain fixed nitrogen in sediments.

C. The Isolation and Dereplication of N-acetyl-2-phenylethylamine (40) from *Bacillus cereus* Isolate CND009

CND009 is an aerobic, spore-forming, Gram (+) bacterium, isolated from a DSDP core. P. Jensen identified the strain as *Bacillus cereus*. The sample was taken from 0.2 cm beneath the surface of a Guaymas Basin core (1834 m). The extract of CND009 showed no bioactivity against *E. coli*, *C. albicans*, or *B. subtilis*, but again purple- and yellow-charring bands on the TLC and a ^{1}H NMR shift of 3.6 ppm in good concentration were seen in the chemical screening. After having isolated the guaymasols from CNA995, this project appeared worth pursuing. A 5 L culture was extracted with EtOAc. Many compounds were separated using vacuum flash chromatography followed by normal-phase HPLC. The purple-charring TLC band turned out to be a tryptophan derivative, most likely **41**, and the yellow-charring compound was so volatile that it was lost on the rotary evaporator



41



42

once purified. Several more aromatic compounds, including **42**, were isolated and partially identified by ^{1}H NMR and low-resolution EIMS. These small molecules were not pursued.

The compound responsible for the 3.6 ppm shift seen in the screening extract was N-acetyl-phenylethylamine (**40**), identified with ^{1}H, ^{13}C, and COSY NMR experiments combined with a low-resolution EIMS molecular ion of m/z 163.

Only one report of **40** as a natural product was found in a CAS on-line search. N-acetyl-phenylethylamine was found as a component of the aroma of various types of honey.[62] Neither **40** or any of the other small aromatics were active as antibiotics.

Bacillus cereus produces several proteinaceous toxins, and it has been implicated in several food-borne illnesses.[63] This strain is remarkable in its ability to grow well at low temperature (as in the deep ocean) and yet survive the extremely high temperature of pasteurization as spores. No reports of volatile aromatics produced by *B. cereus* were found. However, production of the detectable toxins has been shown to decrease as temperature is lowered,[64] and yet, the culture still shows cytotoxicity.[63] Since the volatile compounds produced by CND009 would be in higher concentration in a cold solution, perhaps some of these compounds are responsible for this phenomenon.

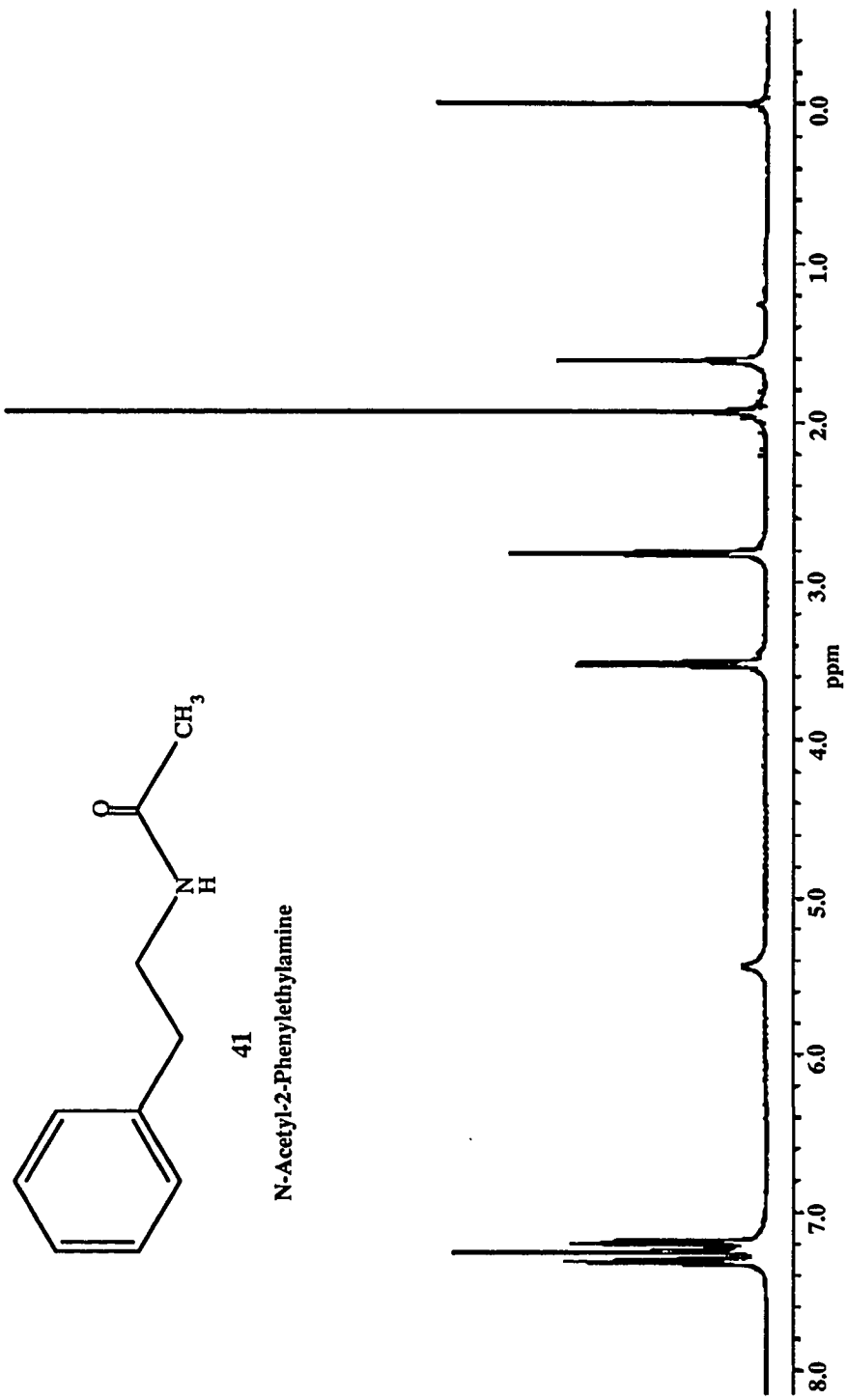


Figure 9. ^{1}H NMR spectrum of N-acetyl-phenylethylamine (41) in $CDCl_3$ at 500 MHz

D. The Isolation and Structure Elucidation of Surfactamide (**43**) from Isolate CND914

CND914 is a *Bacillus* sp. bacterium isolated from an incubated DSDP core from 124 m on the edge of the Guaymas Basin. No antibiotic activity was seen in the original crude extract, but the isolate was selected as a lead project based on a purple-charring TLC band at $R_F = 0.2$ and unusual ^1H NMR signals at 4 ppm. A tryptophan-derivative was expected, since previous work on purple-charring compounds had provided **34**, **35** and **78**. On regrowth, a red- then black-charring baseline spot overshadowed the other TLC bands. A 10 L Virtis fermentation was extracted with EtOAc then fractionated between MeOH and TMP to eliminate the antifoam used. A small amount of broth left after EtOAc extraction was freeze-dried and extracted with MeOH to confirm that the EtOAc fully extracted the red-charring material. Size-exclusion chromatography, reversed-phase vacuum flash chromatography, then reversed-phase HPLC yielded 153 mg (7.2% of crude) of surfactamide (**43**).

In CH_2Cl_2:MeOH, the compound would move off the baseline in the TLC, but it would streak. The band was sharpened on addition of acid, so a carboxyl group was suggested right away. Many of the ^1H NMR shifts of **43** were too broad for 2-D NMR work, especially in a solvent such as DMSO where the exchangeable protons were visible (Figure 10). So, a methylation was carried out using diazomethane, yielding a product that behaved well in the NMR experiments as well as on the TLC (Figure 11). From the 1-D NMR experiments, at least 50 carbons and 90 protons could be distinguished, with tremendous overlapping at 26 to 29 ppm in the ^{13}C spectra and 1.2 ppm region in the ^1H data. These methylenes were thought to represent an alkyl chain.

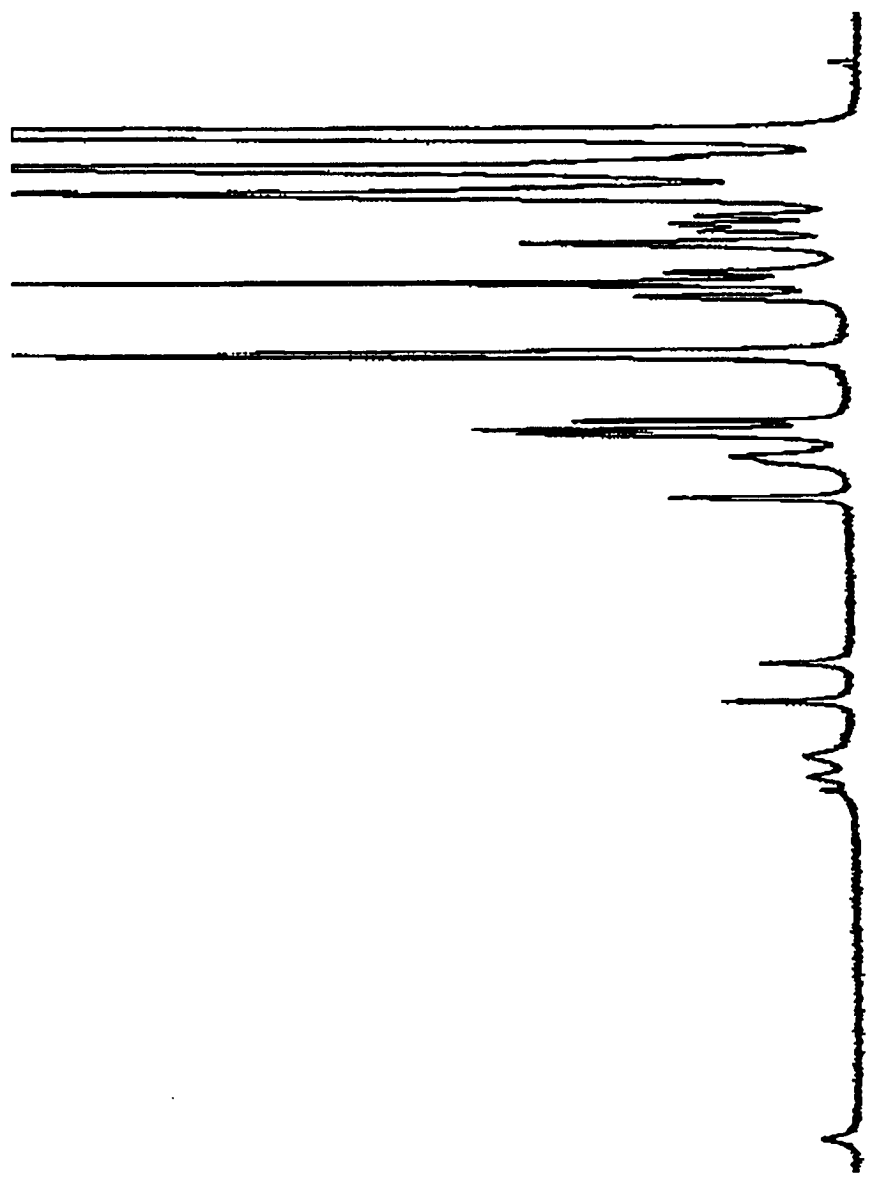


Figure 10. ^{1}H NMR spectrum of surfactamide (44) in DMSO-d_6 at 500 MHz

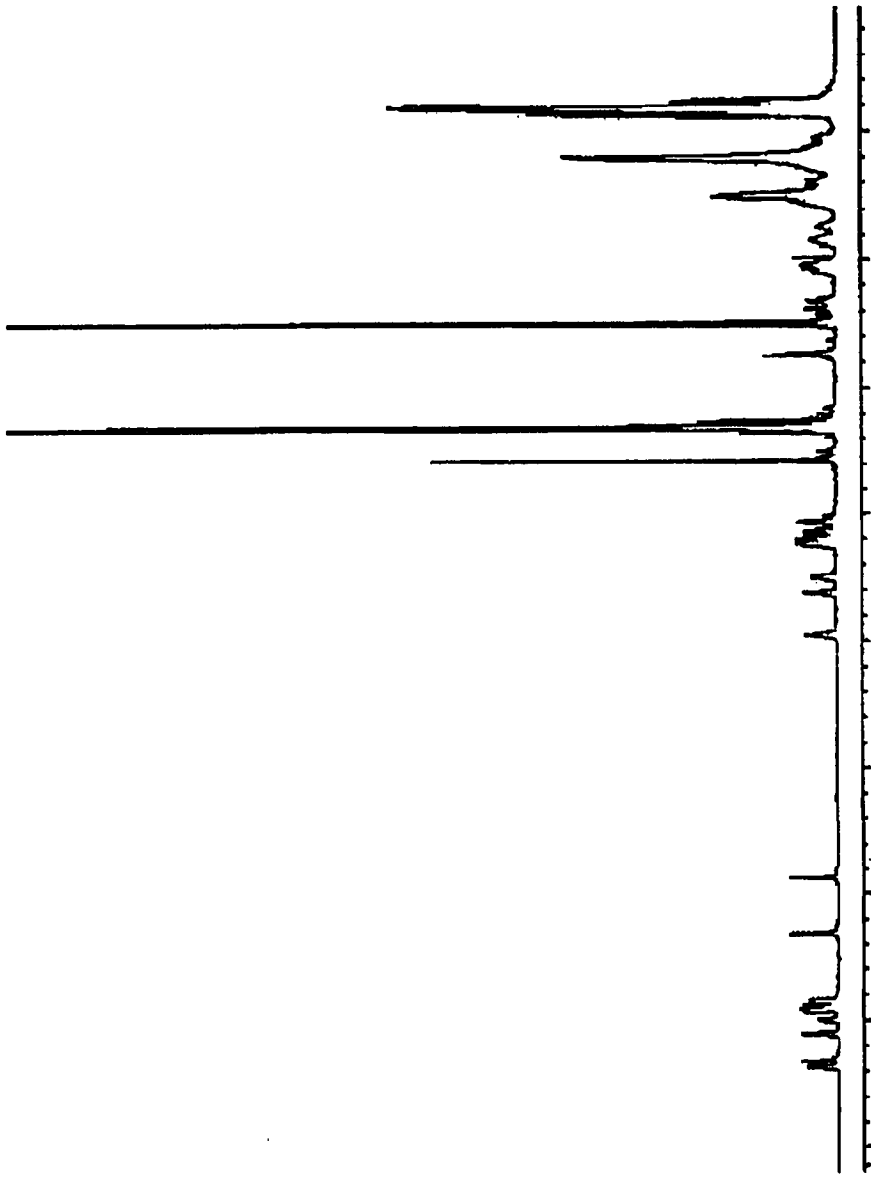


Figure 11. ^{1}H NMR spectrum of methyl-surfactamide (44-me) in DMSO-d_6 at 500 MHz

With the TOCSY data, seven amino acid residues could be pieced together: valine (Val), four leucines (Leu) or isoleucines (Ile), and a combination of one glutamine (Gln)/one aspartic acid (Asp) or one glutamic acid (Glu)/one asparagine (Asn). The HMBC and ROESY data shown in Table 6, allowed the heptapeptide

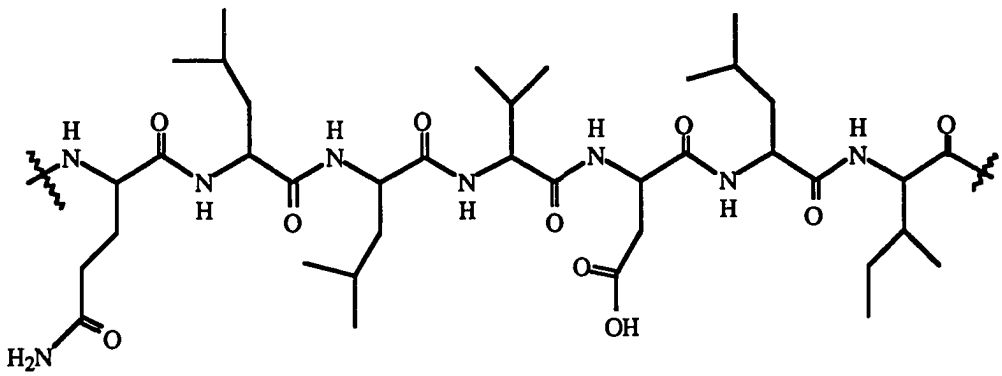
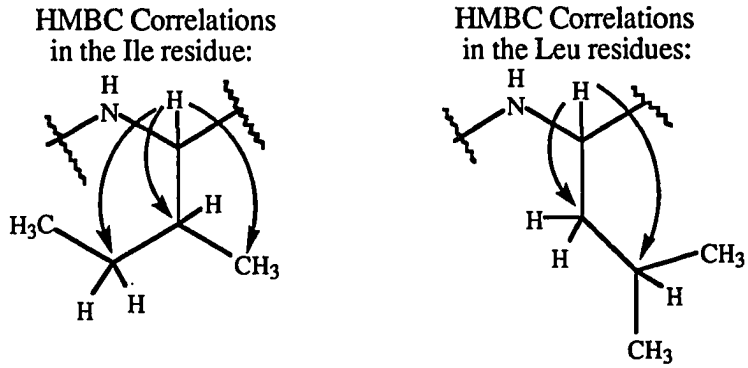


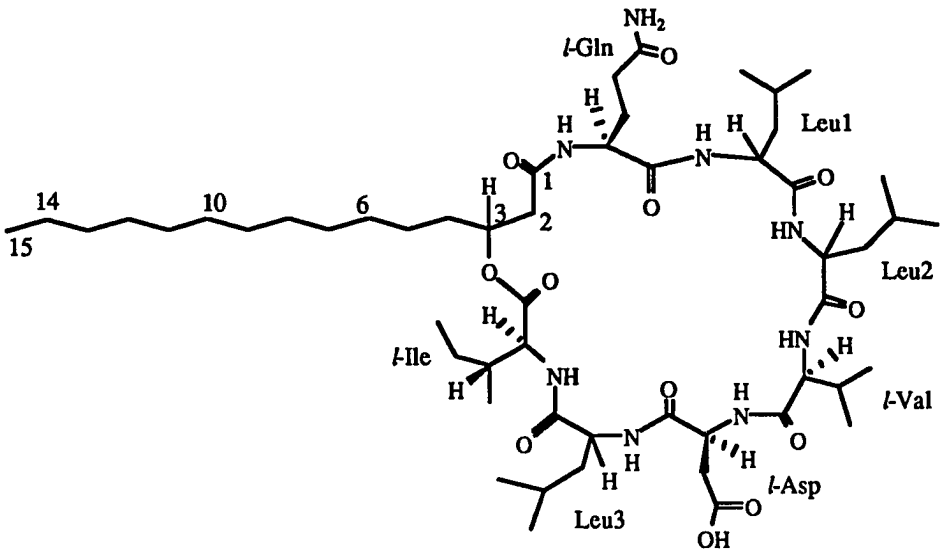
Figure 12. Secondary structure of heptapeptide fragment of 43

to be assembled as shown in Figure 12. The long-range heteronuclear correlation from Hα to Cß, Cγ1, and Cγ2, but not to Cδ, indicated that residue 7 was most



likely an Ile, while the other three residues in question (2,3 and 6) were Leu. The presence of Ile was later confirmed by hydrolysis and GCMS analysis. Also, the

Table 5. Spectral Data for Surfactamide (43)



Source: CND914, Deep-Sea Drilling Project core, *Bacillus* sp.

Molecular formula: $C_{53}H_{94}N_8O_{12}$

HRFABMS: $(M+H^+)$ *m/z* 1035.7076 (obs); 1035.7069 (calc), D -0.6 ppm

Table 6. NMR Data for Me-SURFACTAMINE (44)

Residue/ Position		[a]δ 13C	DEPT	HMQC	TOCSY	HMBC (6Hz)	ROESY[b]
Gln	Cα	52.9	CH	4.13	NH, ß, γ	CO, 1, ß, γ	Leu1NH, Nε1, ß
	Cβ	31.7	CH_2	2.06	NH, α, γ	δ	α, Nε1,2, NH, Chain
				2.01	NH, α, γ	δ	α, Nε1,2, NH, Chain
	Cγ	28.3	CH_2	1.84	NH, α, ß	δ, α, ß	NH
				1.73	NH, α, ß	δ, α, ß	NH
	Cδ	174.1	C	-	-	-	-
	Nε1			7.30	Nε2	δ	α, ß
	Nε2			6.82	Nε1		ß
	NH			8.17	α, ß, γ	α, 1	3, 2, ß, γ
	CO	171.2	C	-	-	-	-
Leu1	Cα	51.6	CH	4.24	NH, ß	CO	NH, ValNH, ß
	Cβ	41.3	CH_2	1.46	NH, α	CO	α, NH
	Cγ	24.0	CH	1.52	NH		
	Cδ1	23.0	CH_3	0.84		δ2, ß	
	Cδ2	22.7	CH_3	0.85		δ2, ß	
	NH			8.08	α, ß, δ2	GlnCO	α, Glnα, ß
	CO	172.2	C	-	-	-	-

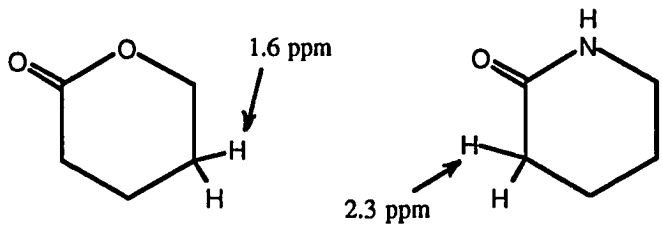
Table 6. (continued)

Residue/ Position		[a]δ ^{13}C	DEPT	HMQC	TOCSY	HMBC (6Hz)	ROESY[b]
Leu2	Cα	52.1	CH	4.22	NH, ß	CO, ß, γ	ValNH, NH, ß
	Cβ	39.9	CH_2	1.51	NH, α		α, NH
	Cγ	24.3	CH	1.35			
	Cδ1	23.0	CH_3	0.88		δ2, ß	
	Cδ2	21.4	CH_3	0.79		δ1, γ	
	NH			8.44	α, ß	α, ß, 3	α, Leu1α, ß
	CO	172.5	C	-	-	-	-
Val	Cα	58.4	CH	4.04	NH, ß	CO, Leu2CO, ß, δ1,2	AspNH, NH, ß
	Cβ	30.2	CH	2.04	NH, α	α, δ1, δ2	α, NH, AspNH
	Cγ1	19.1	CH_3	0.81		α, ß, δ2	
	Cγ2	17.8	CH_3	0.77		α, ß, δ1	
	NH			8.02	α, ß	δ1, Leu2CO	Leu2α, α, ß
	CO	170.8	C	-	-	-	-
Asp	Cα	49.7	CH	4.61	NH, ß	ValCO, CO, ß	Leu3NH, NH, ß
	Cβ	36.05	CH_2	2.75	NH, α	γ, α	α, NH, Leu3NH
	Cγ	170.5	C	-	-	-	-
	OMe	51.5	CH_3	3.56	-	γ	----------
	NH			8.27	α, ß	ß, ValCO	α, Valα, ß, Valß
	CO	169.8	C	-	-	-	-
Leu3	Cα	50.6	CH	4.47	NH, ß	CO, AspCO, ß	IleNH, ß
	Cβ	41.6	CH_2	1.38	NH, α		α
				1.32	NH, α		α
	Cγ	33.8	CH	1.26			
	Cδ1	22.0	CH_3	0.82		γ	
	Cδ2	11.2	CH_3	0.80		ß, γ	
	NH			7.93	α, ß	AspCO	Aspα, Aspß
	CO	171.8	C	-	-	-	-
Ile	Cα	56.5	CH	4.16	NH	CO, Leu3CO, ß, γ1,2	NH, ß, Chain
	Cβ	36.0	CH	1.84	NH		α, NH
	Cγ1	24.5	CH_2	1.29			
	Cγ2	15.5	CH_3	0.82		ß, γ1, α	
	Cδ	21.6	CH_3	0.80		ß	
	NH			8.28	α, ß	α, Leu3CO	Leu3α, α, ß, Chain
	CO	170.7	C	-	-	-	-
Alkyl	1	169.3	C	-	-	-	-
Chain	2	40.7	CH_2	2.36	3, 1.19	1, 4	3, GlnNH, Chain
	3	71.7	CH	4.97	2, 1.19	IleCO, 1, 2	GlnNH, 2, 4, Chain
	4	33.0	CH_2	1.52			3
	5	40.4	CH_2	1.46			
	6	36.1	CH_2	1.21			
	7	31.4	CH_2	1.19			
	8	29.4	CH_2	1.19			
	9	28.9	CH_2	1.19			
	10	28.9	CH_2	1.19			
	11	28.6	CH_2	1.19			
	12	26.5	CH_2	1.19			
	13	38.6	CH_2	1.08			
	14	26.8	CH_2	1.19			
	15	22.6	CH_3	0.84		13, 14	

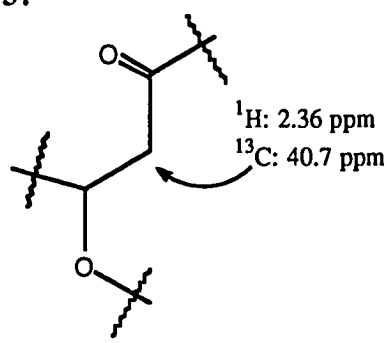
[a] All ^{1}H experiments done at 500MHz; all ^{13}C experiments done at 50 MHz. All NMR experiments (except ROESY) done on 44 in DMSO-d_6 at room temperature. [b] ROESY expt done with natural product (43).

HMBC correlations from Hε to Cδ and from Hγ and Hß to Cδ proved the amide to be in Gln, with the methyl ester being formed with Asp.

Surfactamide had only one free carboxylic acid and no free amine, so it was concluded to be cyclic. The alkyl chain appeared to be attached to the peptide portion of the molecule through an amide bond between C1 and the Gln residue and a lactone linkage between C3 and the Ile residue. The aliphatic acid ester linkage was placed at the β-position of the fatty acid based on chemical shift and coupling arguments. The 40.7 ppm ^{13}C shift of C2 is between the calculated values of 38.2 ppm for the position ß to the carbonyl and 42.6 ppm for the α position.[65] However, the 2.36 ppm shift of H2 is more characteristic of a system adjacent to a carbonyl, rather than an alkyl carbon, as shown in the two examples given below. Add this to the broad doublet coupling pattern of H2, and the position is confirmed



43:



to be between the C1 carbonyl and ester-linked C3. By subtracting the molecular

weight accounted for by the amino acid residues from the molecular ion of *m/z* 1034, given by high-resolution FABMS, and knowing there were two oxygens, the formula of the fatty acid was deduced to be $C_{15}H_{28}O_2$. This eliminated the ambiguity in the ^{13}C NMR spectrum; two methylenes were present at 28.9 ppm. Thus, the molecular formula for **43** was determined to be $C_{53}H_{94}N_8O_{12}$.

The stereochemistry of the amino acids was solved by acid hydrolysis followed by derivatization to the pentafluoropropyl isopropyl (PFP-IPA) esters and GCMS analysis with a chiral capillary column. The GC traces of the derivatized hydrolysate alone, then mixed with the *d*-amino acid standard derivatives, and then the *l*-amino acid standard derivatives are shown in Figure 13. From these experiments, it was concluded that the amino acids present in **43** are *l*-Val, *l*-Asp, *l*-Gln, *l*-Ile, and *d*-Leu and *l*-Leu in a 2:1 ratio. Placement of the *d*- and *l*- isomers of Leu and the stereochemistry at C3 remained to be solved. However, a method for these determinations is proposed below.

One method of breaking a similar lactone, pumilacidin, used 0.5N NaOH in MeOH to yield a mixture of the linear hydroxyacid peptide and the α,β-unsaturated acid peptide.[66] The stereochemistry of the hydroxyacid could then be determined using the first product with the modified Mosher method.[67] Hydrolysis of the α,β-unsaturated product with papain, followed by methylation of the free acid, should yield acylglutamic acid and the hexapeptide with a free amine. According to Kusumi,[68] the modified Mosher method could also be used to determine the configuration of the N-terminal amino acid. If this terminal Leu is indeed analogous to that in surfactin and pumilacidin, it should be the *l*-residue. If this proves to be the case for surfactamide, the other two Leu residues will be assigned as *d*-, thus solving the final stereochemical question.

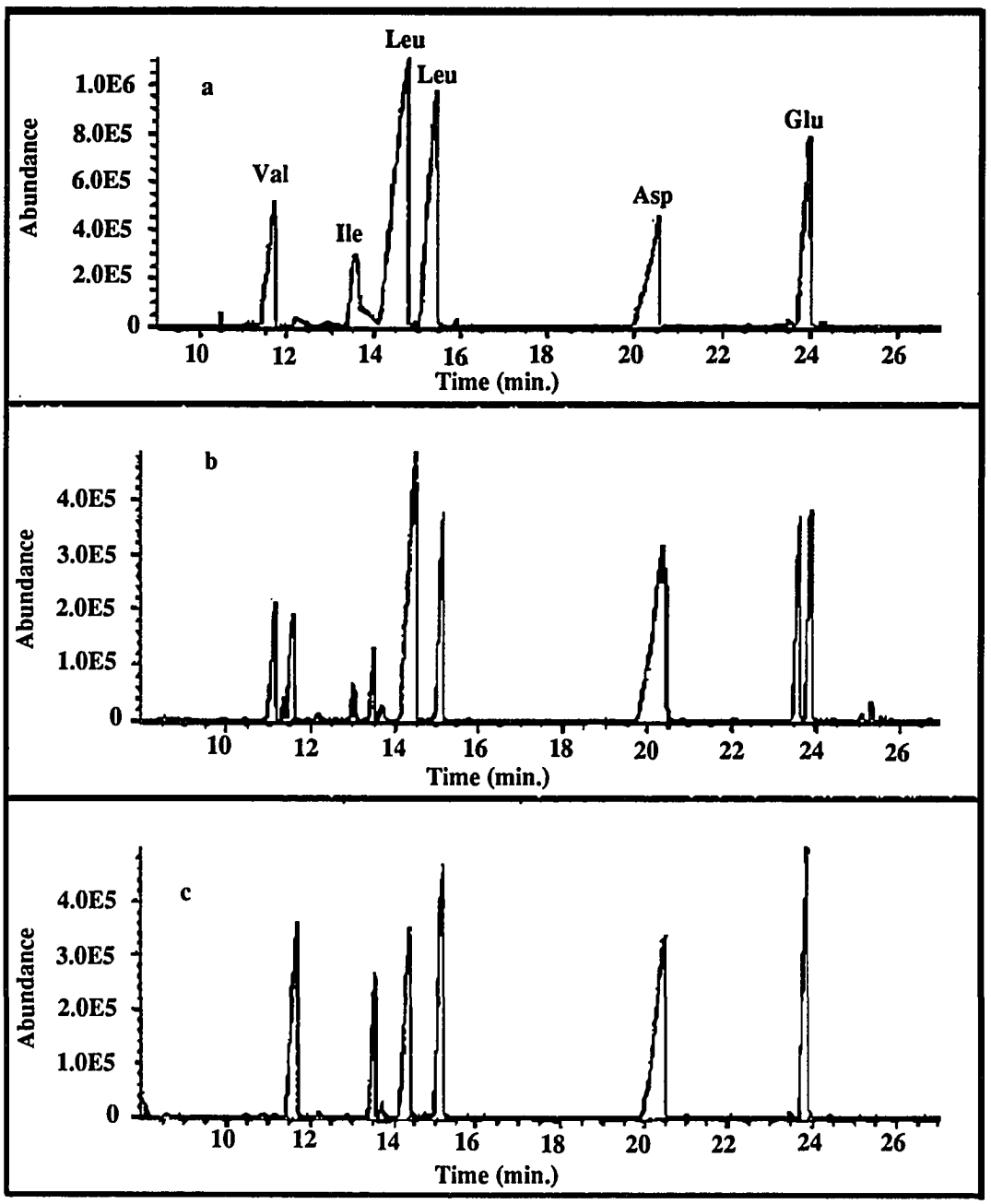


Figure 13. Gas Chromatogram of PFP-IPA Derivatives of a) Hydrolysate of 43, b) Hydrolysate of 43 plus d-Leu, d-Ile, d-Glu, d-Asp, and d-Val, and c) Hydrolysate of 43 plus l-Leu, l-Ile, l-Glu, l-Asp, and l-Val. Abundance is measured in ions on an EI Mass Spectrometer.

A thorough literature search for previously isolated acylpeptides (also referred to as lipopeptides) proved to be very useful in solving the structure of surfactamide and proposing reactions to solve the stereochemistry at C13, though this stereochemistry has not been published for any of the acylpeptides with the lactone linkage at the β-hydroxy group of the fatty acid. Surfactamide is an analog of surfactin (**45**), with a Gln replacing the Glu residue and Ile was involved in the lactone linkage. This difference may seem insignificant in looking at the chemical structure, but the activity of surfactin as an inhibitor of cAMP phosphodiesterase varies greatly even with replacement of one Leu residue by Ile.[69] Therefore, substitution of Glu by Gln might alter the biological activity significantly.

Acylpeptides have been isolated from a variety of microorganisms, as shown on the following pages. They have exhibited many different bioactivities, including inhibtion of cAMP phosphodiesterase, inhibition of fibrin clot formation, and antitumor (surfactin),[70] antiviral and antiulcer (pumilacidin),[66] antifungal (surfactin, iturins),[71] antibiotic (esperin,[72] mycosubtilin),[73] and inhibition of porcine pancreatic PLA_2 (plipistatins).[74] Interestingly, globomycin is active against Gram (-) bacteria,[75] while brevistin is active exclusively against Gram (+) bacteria.[76] Biological activity data has not been received for **43**, but with this wide range of activities with only small variations in structure of acylpeptides, surfactamide is seen as a promising new compound.

Figure 14. Acylpeptide structures and source organisms

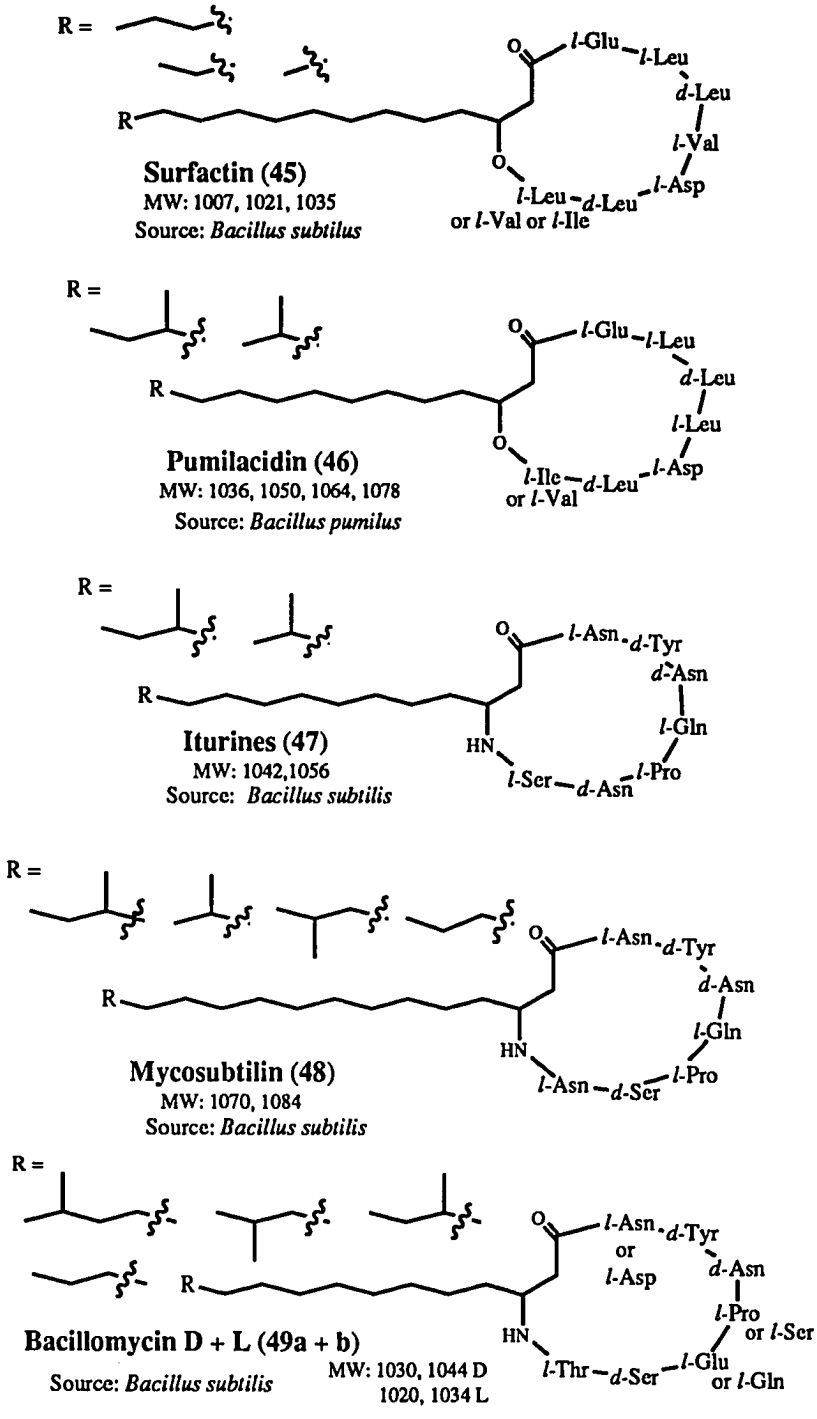
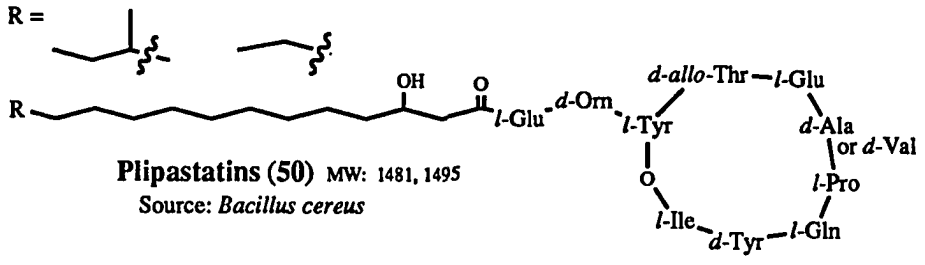
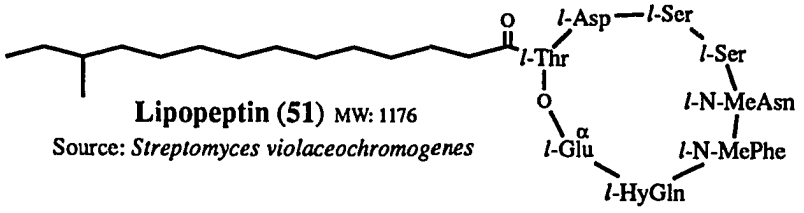


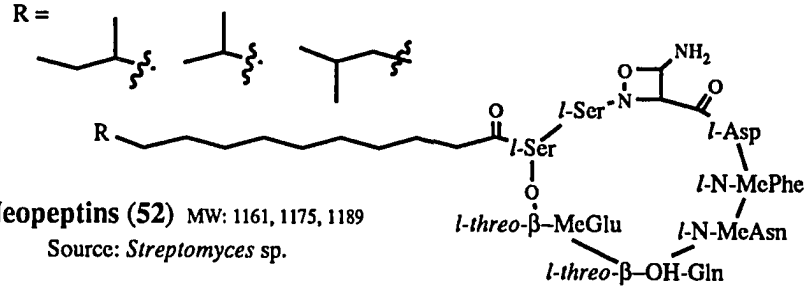
Figure 14. (continued)



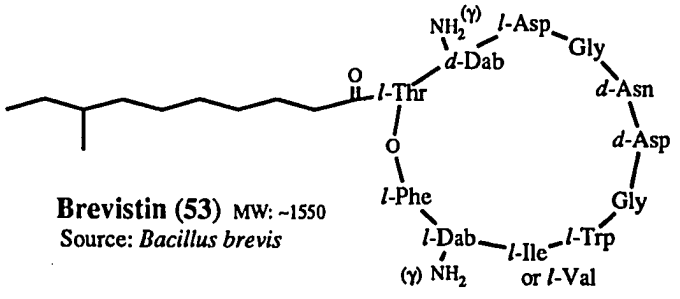
Plipastatins (50) MW: 1481, 1495
Source: *Bacillus cereus*



Lipopeptin (51) MW: 1176
Source: *Streptomyces violaceochromogenes*

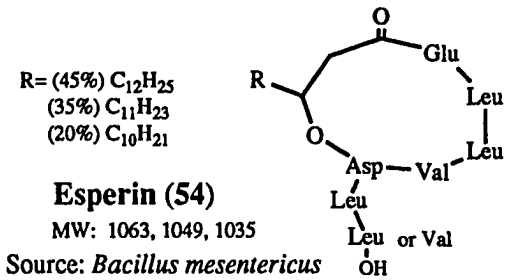


Neopeptins (52) MW: 1161, 1175, 1189
Source: *Streptomyces* sp.

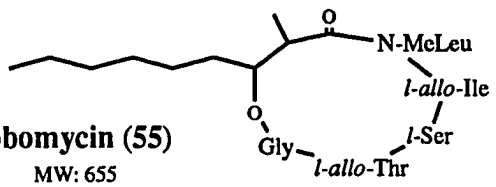


Brevistin (53) MW: ~1550
Source: *Bacillus brevis*

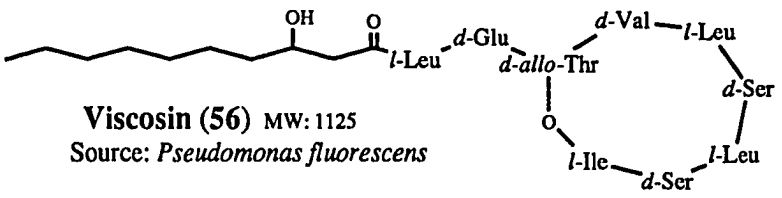
Figure 14. (continued)



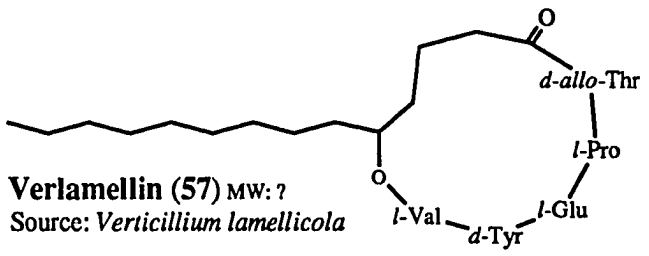
Esperin (54)
MW: 1063, 1049, 1035
Source: *Bacillus mesentericus*



Globomycin (55)
MW: 655
Source: 4 Actinomycete sp.



Viscosin (56) MW: 1125
Source: *Pseudomonas fluorescens*



Verlamellin (57) MW: ?
Source: *Verticillium lamellicola*

E. Experimental, Chapter II

CNA995. Collection, Isolation and Morphology. CNA995 was isolated from 50 cm into the frozen DSDP core LPAZ, P4, from 1834 m in the Guaymas Basin. On an agar plate with B-3 media, the colony was white, irregular, opaque, dull, pasty. The Gram (+) bacterium was stored in 10% glycerol and B-3 media at -80°C.

Culture and Extraction. The 6 L culture of CNA995 was scaled-up from the frozen tube inoculum in 10 ml tube of B-3 media (2 days) to 100 ml B-3 in a 500 ml Erlenmeyer flask (3 days) to 1 L B-3 (2 days), then transferred to 6 x 1 L B-3 media in a Fernbach flask (5 days) with constant shaking at room temperature at all steps. The 6 x 1 L cultures were then combined and extracted with 2 x 6 L EtOAc.

Isolation of Guaymasol (34), Epiguaymasol (35), and Guaymasone (37). The EtOAc extract was dried over Na_2SO_4, filtered, and evaporated *in vacuo* to yield 680 mg crude organics. Most of the extract (400 mg) was separated using a 60 ml silica gel (60Å) flash column with 80 ml mixtures of 100:0 to 0:100 TMP:EtOAc in 10% steps. The 50:50 and 40:60 TMP:EtOAc fractions were combined, then reseparated on normal-phase HPLC (Dynamax silica, 10 mm x 250 mm) with 60:40 EtOAc:TMP at 2.5 ml/min. This yielded 36.5 mg (19.1% of crude) **34** and 12.0 mg (3.0% of crude) **35**. The 60:40 TMP:EtOAc flash fraction was further separated on a normal-phase HPLC with 58:42 EtOAc:TMP at 1.5 ml/min, yielding 9.6 mg (2.4% of crude) **37**.

Gauymasol (34) isolated from CNA995. Compound **34** showed the following spectral characteristics: IR ($CHCl_3$): 3594, 3400, 3156, 2959 cm^{-1}; HRFABMS: $M+H^+$, *m/z* 225.15050, Δ 6.4 ppm, requiring $C_{13}H_{21}O_3$; LREIMS: M^+, *m/z* (rel. int.) 107 (100), 77 (46), 43 (61), 91 (34), 119 (11), 136 (6), 224 (4); ^1H NMR (500 MHz, $CDCl_3$): δ (ppm) 7.05 (2H, d, J=8.5), 6.88 (2H, d, J=8.5), 3.60 (1H,

m), 3.58 (1H, m), 2.82 (1H, dd, J=4.4, 14), 2.65 (1H, dd, J=7.3, 14), 1.80 (1H, m), 1.48 (1H, m), 1.25 (1H, m), 0.90 (6H, d, J=7.0); ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) 154.4, 130.5, 129.9, 115.5, 75.5, 71.7, 42.8, 39.2, 24.5, 23.6, 21.8.

Epigauymasol (**35**) isolated from CNA995. Compound **35** showed the following spectral characteristics: IR ($CHCl_3$): 3596, 3400, 3156, 2959 cm^{-1}; HRFABMS: $M+H^+$, *m/z* 225.15084, Δ 7.9 ppm, requiring $C_{13}H_{21}O_3$; LREIMS: M^+, *m/z* (rel. int.) 107 (100), 77 (46), 43 (61), 91 (34), 119 (11), 136 (6), 224 (4); ^1H NMR (500 MHz, $CDCl_3$): δ (ppm) 7.05 (2H, d, J=8.5), 6.88 (2H, d, J=8.5), 3.74 (1H, m), 3.72 (1H, m), 2.74 (1H, dd, J=4.4, 14), 2.62 (1H, dd, J=7.3, 14), 1.86 (1H, m), 1.45 (1H, m), 1.25 (1H, m), 0.96 (3H, d, 7.0), 0.90 (3H, d, J=7.0); ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) 154.9, 130.0, 129.5, 115.0, 76.0, 71.8, 40.2, 36.9, 24.2, 23.4, 21.3.

Guaymasone (**37**) isolated from CNA995. Compound **37** showed the following spectral characteristics: IR ($CHCl_3$): 3594, 3468, 2962, 1731 (strong), 1251 cm^{-1}; HRFABMS: $M+H^+$, *m/z* 223.13540, Δ 8.9 ppm, requiring $C_{13}H_{19}O_3$; LREIMS: M^+, *m/z* (rel. int.) 107 (100), 137 (16), 91 (34), 57 (12), 204 (5); ^1H NMR (200 MHz, $CDCl_3$): δ (ppm) 7.11 (2H, d, J=8.5), 6.76 (2H, d, J=8.5), 4.35 (1H, dd, J=4.4, 7.4), 3.08 (1H, dd, J=4.4, 14), 2.76 (1H, dd, J=7.3, 14), 2.38 (2H, d, J=6.8), 2.18 (1H, m), 0.94 (3H, d, 7.0), 0.91 (3H, d, J=7.0); ^{13}C NMR (50 MHz, $CDCl_3$): δ (ppm) 211.4, 154.6, 130.4, 128.3, 115.3, 77.6, 47.4, 39.1, 24.6, 22.6, 22.5.

Formation of 1,3-dioxolane **36**. Compound **34** (6.9 mg) was stirred in 1.5 ml DMF at room temperature. 100 μl dimethoxypropane(DMP) and 2 mg *p*-TsOH were added, and the temperature was raised to 40°C for 1 hr. Reaction of 5.2 mg

compound **35** followed the same procedure, except the mixture was heated to 50°C for 8 hr. Work-up in both cases was neutralization with $Na_2(CO_3)_2$ followed by partitioning between CH_2Cl_2 and H_2O. Reactants and products were separated by normal-phase HPLC in 55:45 EtOAc:TMP at 2.0 ml/min. Reaction of **34** yielded 100% product **36**, while reaction of **35** yielded 14% product (and 27% reactant).

Anti 1,3-dioxolane from reaction of **34**. ^{13}C NMR (50 MHz, $CDCl_3$): δ (ppm) 154.2, 130.5, 129.7, 115.2, 108.1, 81.7, 78.7, 42.0, 38.2, 27.4, 27.2, 25.4, 23.6, 21.9.

Syn 1,3-dioxolane from reaction of **35**. ^{13}C NMR (using DEPT135 experiment, 50 MHz, $CDCl_3$): δ (ppm) 130.1, 115.1, 78.9, 75.8, 38.3, 35.4, 28.6, 25.9, 25.0, 23.6, 21.7.

CND009. Collection, Isolation and Morphology. CND009, a strain of *B. cereus* idenitifed by P. Jensen, was isolated from 0.2 cm into the frozen DSDP core LPAZ, P4, from 1834 m in the Guaymas Basin, Gulf of California. On an agar plate with B-1 media, the colony was white and rhizoidal. The strain was stored in 10% glycerol and B-3 media at -80°C.

Culture and Extraction. The 5 L culture of CND009 was scaled-up from the frozen tube inoculum in 10 ml tube of B-3 media to 5 x 1 L B-3 media in a Fernbach flask (5 days) with constant shaking at room temperature at all steps. The exact procedure was not recorded. The 5 x 1 L cultures were then combined and extracted with 2 x 5 L EtOAc.

Isolation of N-acetyl-phenylethylamine (**40**). The EtOAc extract was dried over Na_2SO_4, filtered, and evaporated *in vacuo* to yield 2.7 g crude organics. The extract was separated using a 150 ml silica gel (60Å) flash column with 100 ml mixtures of 90:10 to 60:40 TMP:EtOAc in 10% steps, then to 0:100 in 5% steps, followed by

90:10 EtOAc:MeOH. The 25:75 to 5:95 TMP:EtOAc fractions contained small aromatic compounds. Compound **40** was isolated from this fraction by Sephadex LH-20 (2.5 cm x 40 cm, 50:50 CH_2Cl_2:MeOH) and normal-phase HPLC (Dynamax 60A silica, 10 mm x 250 mm, 95:5 CH_2Cl_2:MeOH at 2.0 ml/min) separations as 0.2% of the crude.

CND914. Collection, Isolation and Morphology. CND914 is a *Bacillus* sp. isolated from the frozen DSDP core LPAZ, G012, from 126 m on the edge of the Guaymas Basin, Gulf of California. The core sample, taken from 15 cm into the sediment, was incubated at 37°C overnight, then serially-diluted and plated on A-1/agar media with cycloheximide added. On an agar plate with A-1 media, the colony was white, irregular, raised, with vertical growth. The strain was stored in 10% glycerol and A-1 media at -80°C.

Culture and Extraction. The 10 L culture of CND914 was scaled-up from the frozen tube inoculum in 10 ml tube of A-1 media (1 day) to 1 L A-1 (4 days) with constant shaking at room temperature at all steps, then transferred to 10 L A-1 media in a 12 L Virtis glass fermentor (2 days), with aeration setting of 5. Impellor speed was not recorded. The 10 L culture was double extracted with 10 L EtOAc.

Isolation of Surfactamide (**43**). The EtOAc extract was dried over Na_2SO_4, filtered, and evaporated *in vacuo* to yield 2.12 g crude organics. The extract was separated using a 4 x 40 cm Sephadex LH-20 column in MeOH. The second fraction (1.02 g) was then separated using a 60 ml reversed-phase vacuum flash chromatography column with 50 ml volumes of the following solvent mixtures: 20:80, 50:50, 75:25, 90:10, 100:0 MeOH:H_2O, then 50:50 MeOH:EtOAc. The final fraction was further separated by reversed-phase HPLC (Dynamax C18, 10 mm x 250 mm) with 94:6 MeOH:H_2O at 2.0 ml/min. This yielded 153 mg (7.2% of

crude) **43**.

Surfactamide (**43**) isolated from CND914. Compound **43** showed the following spectral characteristics: HRFABMS: M+H$^+$, *m/z* 1035.7076, Δ -0.6ppm, requiring $C_{53}H_{95}N_8O_{12}$; ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) 12.40, 8.34, 8.18, 7.92, 7.30, 6.82, 4.94, 4.54, 4.47, 4.24, 4.16, 4.08, 2.64, 2.36, 2.04, 1.88, 1.82, 1.72, 1.50, 1.44, 1.40, 1.30, 1.19, 1.08, 0.87, 0.85, 0.84, 0.82, 0.81, 0.80, 0.79, 0.75

Methylation of (**43**). Compound **43** (50 mg) was dissolved in 2 ml 7:3 acetone:ether and placed in the bottom of a diazomethane reactor with stirring. Approximately 200 mg 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) was added and the reactor was sealed. One ml of 6N KOH was added slowly to the MNNG. After 2 h, the reaction vessel was opened to let any excess CH_2N_2 evaporate. The product was purified using a normal-phase preparative thick-layer plate in 95:5 EtOAc:MeOH. The methylated material moved to R_F~0.4, yielding 23 mg (46%) **44**. Compound **44** exhibited the following spectral characteristics: ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 8.44, 8.28, 8.27, 8.17, 8.08, 8.02, 7.93, 7.30, 6.82, 4.97, 4.61, 4.47, 4.24, 4.22, 4.16, 4.13, 4.04, 3.56, 2.75, 2.36, 2.06, 2.04, 2.01,1.84, 1.73, 1.52,1.51, 1.46, 1.38, 1.35, 1.32, 1.29, 1.26, 1.21, 1.19, 0.88, 0.85, 0.84, 0.82, 0.81, 0.80, 0.79, 0.77; ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) 174.1, 172.5, 172.2, 171.8, 171.2, 170.8, 170.7, 170.5, 169.8, 169.3, 71.7,58.4, 56.5, 52.9, 52.1, 51.6, 51.5, 50.6, 49.7, 41.6, 41.3, 40.7, 40.4, 39.9, 38.6, 36.1, 36.1, 36.0, 33.8, 33.0, 31.7, 31.4, 30.2, 29.4, 28.9, 28.9, 28.6, 28.3, 26.8, 26.5, 24.5, 24.3, 24.0, 23.0, 23.0, 22.7, 22.6, 22.0, 21.6, 21.4, 19.1, 17.8, 15.5, 11.2.

Hydrolysis of **43** and GCMS analysis. Compound **43** (9 mg) was dissolved in

2 ml 6N HCl and stirred overnight at 110°C in a sealed tube. The product and all amino acid standards used were derivatized using the Alltech PFP-IPA amino acid derivatization kit. The steps for this procedure were as follows: 1) Place 1 ml of 0.2M HCl in the vial with the desired amino acid mixture, heat solution to 110°C for 5 minutes, and dry under N_2; 2) In a dry ice bath, slowly add the acetyl chloride to the isopropanol. Add about 0.5 ml of this solution to the dry amino acids, cap vial, and heat to 100°C for 45 minutes. Dry at 115°C under a stream of N_2; 3) Mix one ampule of PFPA with 3 ml CH_2Cl_2. To the cooled amino acid vial, add 0.5 ml of this solution, cap, and heat vial to 100°C for 15 minutes; 4) Evaporate excess reagent under N_2, and redissolve in hexane. The HP 5988A GCMS was run using an Alltech Chirasil-Val capillary column at a head pressure of 8 psi, He flow rate of ~1 ml/s, and a heating rate of 3°C/min. from 50°C to 125°C then 5°C/min. to a maximum temperature of 220°C.

Chapter III

Natural Products Chemistry of Estuarine Microbial Isolates

A. Introduction to Chapter III

Because many populations of microorganisms inhabit any given environment, these microbes must compete for space, nutrients, and other resources in that environment. According to Fredrickson and Stephanopoulos,[77] microbial competition can be divided into two types: indirect competition and direct competition. Indirect competitors limit the growth of other populations by reducing available environmental resources. These resources are usually used in the metabolism of the competing microbe, and they include chemicals, particulate matter, and light. Direct competition, also called interference or antibiosis, is characterized by the release of toxic or inhibitory substances by one microbial population to limit the population of another microbe.

The ecological aspect of antibiosis has been studied since Nakhimovskaia (1937) found that many species of actinomycetes isolated from Russian soil inhibited the growth of bacteria from the same soil samples.[78] Ecologists have used the terms "antagonism" and "amensalism" to describe the interspecific relationship when one microbial population produces a substance inhibitory to another in its natural environment. However, these words are not properly used when referring to studies with sterilized soil or with cultured microorganisms, because the natural environment no longer exists. Therefore, the term "antibiosis" is used to describe the production of inhibitory substances active against another microorganism in culture.

Historically, antibiotic producers have been isolated primarily from soil, so many studies of the ecological significance of antibiotics have been done using soil.

Waksman *et al.* [79] showed that 43% of their isolates, primarily actinomycetes, from soils and composts showed antibiotic effects against other soil microorganisms. Although greater than 10,000 bioactive agents have already been isolated from microbial sources, this high percentage of antibiotic activity indicates that there are likely to be many microbial products yet to be isolated and identified. In general, antibiotic synthesis has been found in a high proportion of actinomycetes, and to a lesser extent in fungi and non-filamentous bacteria. In fact, over 5000 antibiotic compounds have been produced by terrestrially-isolated bacteria in this single taxonomic group, the Actinomycetales. Soil isolates have been studied since the discovery of penicillin in 1929, but researchers have only begun to investigate marine environments as sources of antibiotic-producing bacteria, especially actinomycetes. Examples of bioactive compounds from actinomycetes isolated from marine sediments include istamycin (**11**),[30] aplasmomycin (**12**),[29] altemicidin (**13**),[31] and O-*l*-quinovovsyl saphenate (**17**).[35]

One explanation for interest only recently turning toward marine producers of antibiotics is that researchers have questioned whether actinomycetes are actually metabolically active in the ocean or are spores which have run off from the land. In some cases, researchers have claimed support for the latter opinion in the fact that marine microbes have been shown to produce the same antibiotics as some terrestrial isolates (e.g. Wratten *et al.*, 1977).[24] However, Jensen *et al.* have shown that actinomycetes are active members of the marine microbial community,[80] so marine actinomycetes are indeed a new, virtually untapped resource for antibiotic screening.

For these reasons, initial examination of marine microorganisms for production of novel compounds was focused on actinomycetes from the Fenical Lab collection. One of the bacteria, CNB091, had been isolated from a surface swab of a jellyfish

collected in a mangrove in the Florida Keys in 1987. CNB091 produced a series of depsipeptides, the salinamides, which show moderate antibiotic activity and potent anti-inflammatory activity. Discovery of the salinamides prompted further investigation of antibiosis among marine estuarine isolates.

One tropical estuary, a mangrove in Belize, Central America, and one warm-temperate estuary, Batiquitos Lagoon in Carlsbad, CA, were selected as sources for studies of microbial antibiosis which may occur *in situ*. Isolates were screened for antibiotic activity not against medically-relevant microorganisms, but against a panel of isolates from the same environment, *i.e.* ecologically-relevant microorganisms. They were also screened chemically using TLC and ^{1}H NMR methods. Only one novel antibiotic compound, *cis*-cascarillic acid, was isolated. All other leads were dereplicated to known compounds or could only be partially identified.

Although the estuarine isolates yielded primarily known compounds, it is still considered a valuable resource for further investigations searching for novel compounds. However, screening for novel compounds should also involve new methods for bioactivity screening, since antibiotic screening has been used for over 60 years. Hopefully the results given here will stimulate further research into the role of antibiotic production in the marine environment. The machinery for antibiosis may lie dormant while the microbe is in an environment where primary metabolism is highly stressed, but it would not be present at all if it was not used to provide a competitive advantage at some stage. Now that producers of certain antibiotics are known to be present in estuarine sediments, perhaps further studies could shed some light on when and why they are produced *in situ*.

B. The Isolation and Structure Elucidation of the Salinamides from Isolate CNB091

An unknown species of the genus *Streptomyces* was isolated from a surface swab of a jellyfish taken from a mangrove in the Florida keys in 1987. The actinomycete was initially cultured on a 10 L scale in a 20 L glass Virtis fermentor for compound isolation. The culture was double-extracted with EtOAc, and the extract was dried over Na_2SO_4, filtered and evaporated *in vacuo* to yield 1.2 g crude material. Vacuum flash chromatography with silica gel in a 150 ml column, eluted with 100:0 to 0:100 TMP:EtOAc in 10% steps, then 80:20 and 0:100 EtOAc:MeOH, yielded 220 mg of the active and chemically-interesting fraction. Further purification with reversed-phase HPLC using 80:20 MeOH:H_2O yielded 170 mg of pure salinamide A (**58**) as a pale yellow non-crystalline solid and very little of any other similar compounds.

Salinamide A was identified primarily by spectroscopic means. With olefinic, aromatic, and ester/amide shifts, the 1-D ^1H and ^{13}C NMR spectra (Figures 15b and 16b) were very exciting. The IR data confirmed the presence of hydroxyl groups, esters, amides, and olefinic bonds. High-resolution FABMS data required a molecular formula of $C_{51}H_{69}N_7O_{15}$. Not all of these carbons and protons could be seen at 200 MHz, presumably due to overlapping signals. Since no higher field instruments were available for 2-D work at Scripps or UC-San Diego at this time, several experiments were performed by D. Tapiolas in collaboration with T. McKee in the Ireland group at the University of Utah. Higher field 1-D NMR results were initially disappointing, because several carbons and protons were still not accounted for in the spectra. As shown in Figures 15 and 16, experiments performed at -30°C were needed to lock the molecule into one conformation in order to sharpen all of the

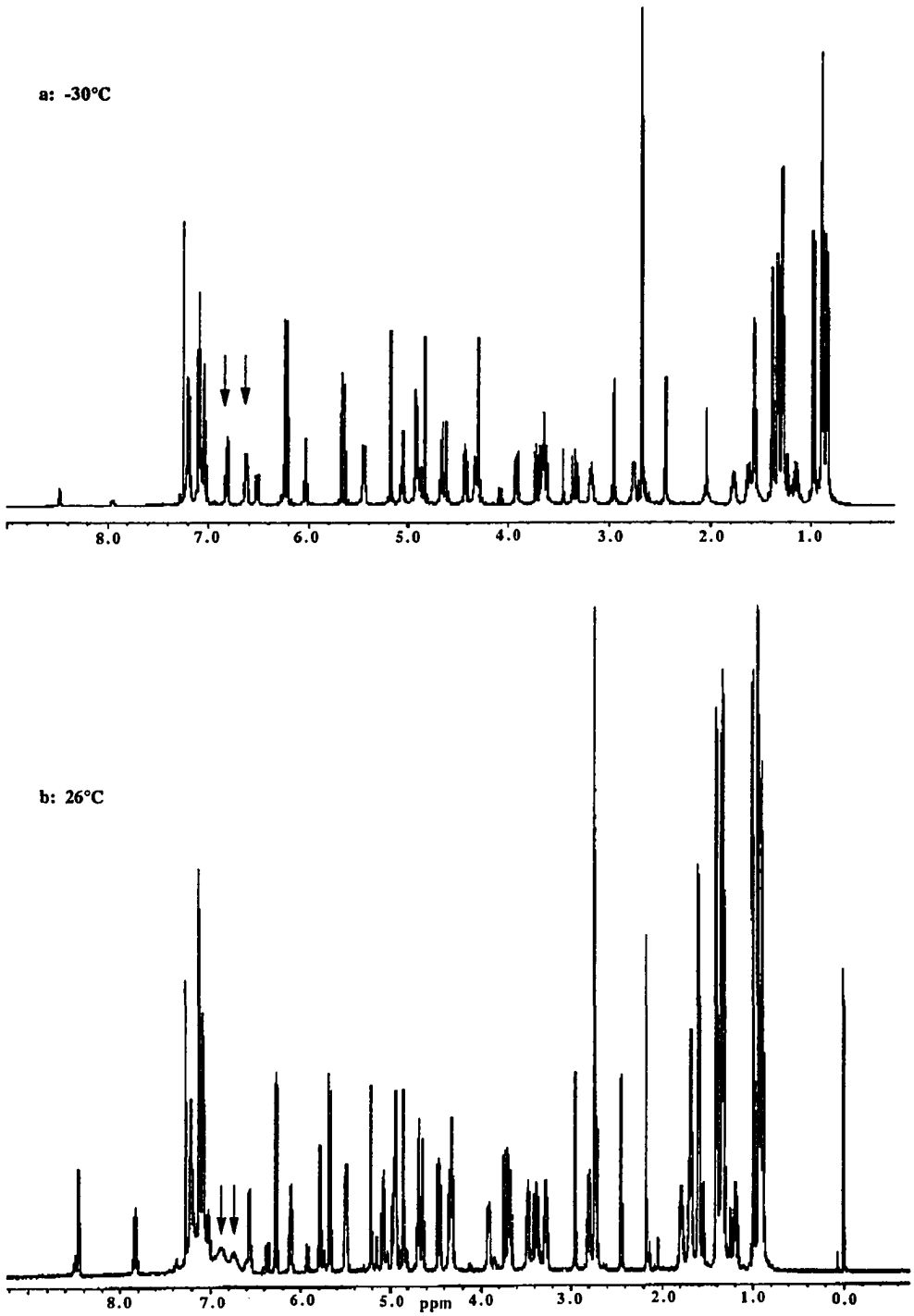


Figure 15. ^{1}H NMR spectra for salinamide A (58) in $CDCl_3$ at 500 MHz at a) -30°C and b) 26°C

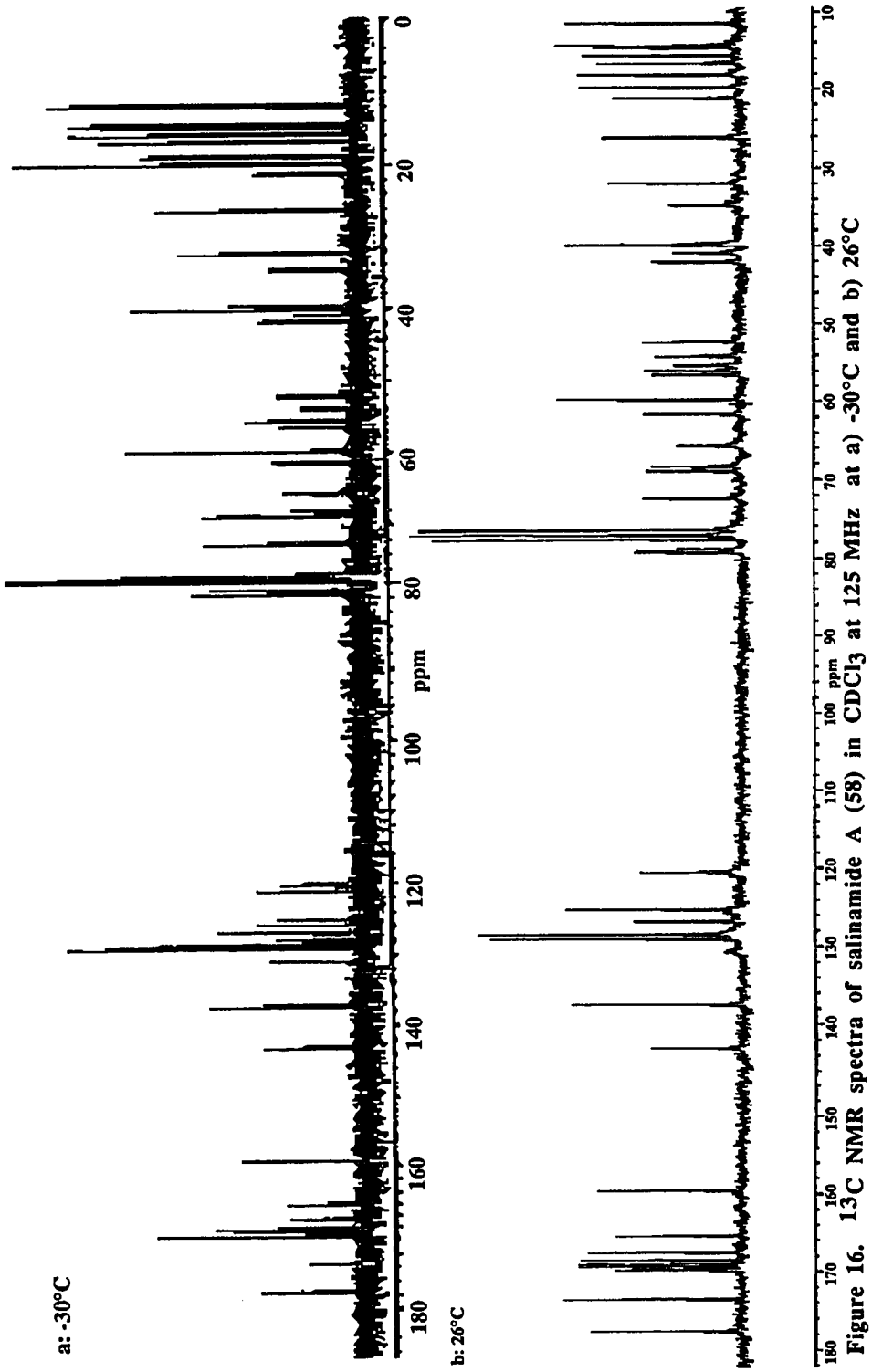
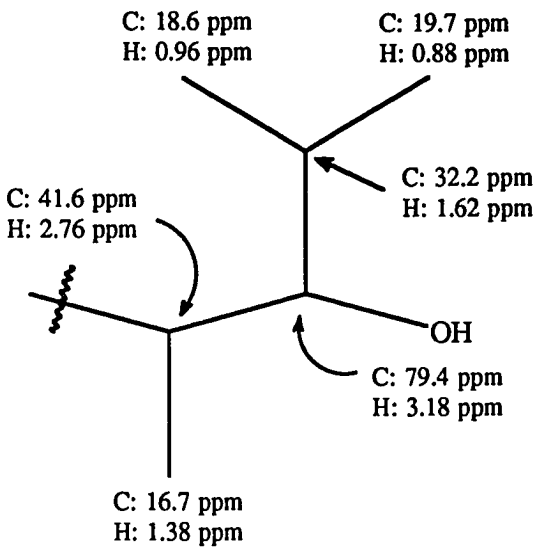


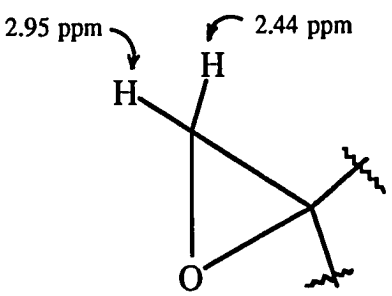
Figure 16. ^{13}C NMR spectra of salinamide A (58) in $CDCl_3$ at 125 MHz at a) -30°C and b) 26°C

NMR signals.

With the 125 MHz ^{13}C and 500 MHz ^{1}H NMR data at -30°C and the COSY and TOCSY results, several amino acid residues could be assigned: Gly, Leu/Ile, 2 Thr, Ser, (N-me)Phe, and one additional substituted glycine. The N-methyl was assigned to Phe because it was the only residue without an amide correlation. Also, one non-peptide partial structure could be constructed:



The 5.4 Hz splittings of the highly anisotropic protons of C40 were indicative of an epoxide with no protons on the neighboring carbon:



Long-range XHCORR experiments at 125 MHz allowed some of the peptides to be

linked, but the complete secondary structure could only be assigned through rotating-frame nOe (ROESY) and inverse-detected heteronuclear multiple bond correlation (HMBC) experiments. The data for these experiments are shown in Table 8. The structure shown in Figure 17 was incomplete only in stereochemical assignments and in the regiochemistry of the Thr connections to C50 and C25, with one very weak HMBC correlation from H49 to C50 supporting the bonds as shown.

Thus, the amino acid not previously assigned was an α-phenylglycine (Phg) residue. Restricted rotation of this ring system probably accounts for the broadened NMR signals at room temperature. A ROESY experiment supported this structure with through-space correlations from the amide proton of the Ile to the C57 proton and from the N49 proton to the proton on C51. The ROESY experiment also offered some insight into the three-dimensional orientation of the molecule in solution. As seen in Figure 18, the C57 methyl group in close proximity with the bridge proton H16, and the *d*-Thr side chain. This structure could be accounted for by hydrogen bonding between N17-O38, N32-O48, and N49-O61, which are also brought into close proximity in this structure

With 100 mg of **58** in Utah for NMR work, more compound was needed for chemical work and bioactivity testing. The subsequent fermentation was run with all variables as close to those in the first fermentation as possible (though no settings had been recorded), then extracted and processed exactly as it had been previously. Approximately 190 mg of **58** was purified from this extract, and 120 mg of a second depsipeptide, salinamide B (**59**),was also isolated. The final pH of this fermentation was several units lower than the original fermentation. Perhaps this was responsible for the production of **59** by addition of HCl, since no other variables were thought to have changed. High-resolution FABMS indicated the

Figure 17. Structure of Salinamide A (58)

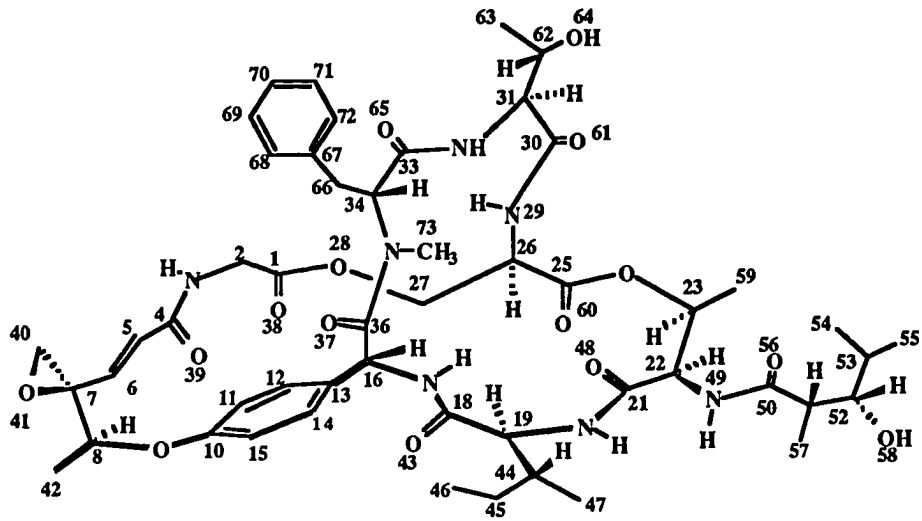


Table 7. Spectral Data for Salinamide A (58)

Source: CNB091, a *Streptomyces* sp. from a surface swab of a jellyfish

$[\alpha]_D$ -26° (c 0.97, $CDCl_3$) **mp** 221-225°C decomposes

Molecular Formula: $C_{51}H_{69}N_7O_{15}$

HRFABMS: ($M+H^+$) m/z 1020.492 (obs), 1020.493 (calc), Δ 1.0 ppm

IR ($CDCl_3$): 3436, 3379,1745, 1735, 1682, 1657, 1636 cm^{-1}

UV (MeOH): 281, 267, 224, 208 nm

Table 8: NMR Data for Salinamide A (58)

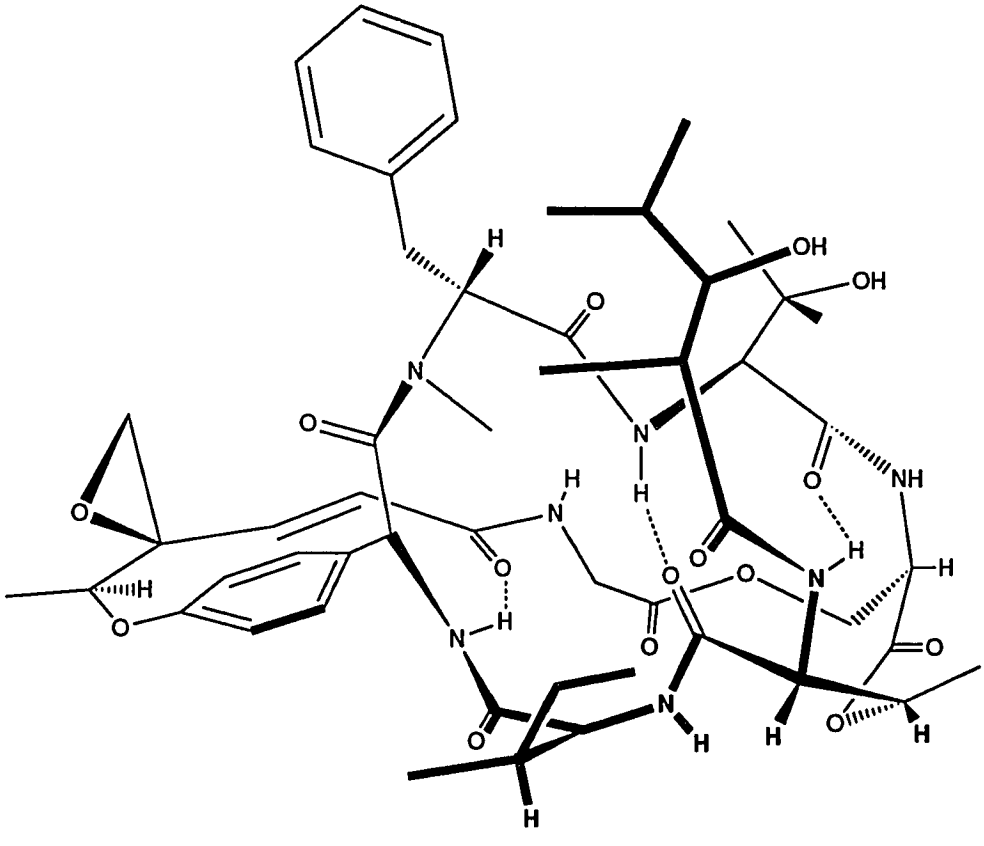
C/X #	δ $^{13}C^a$	δ $^{1}H^b$	COSY[c]/ TOCSY[d]	HMBC[d]	ROESY[d]
1	169.7 (C)				
2	40.8 (CH2)	3.66 (d, 19.1)	3	1, 4	19, 3, (4.90)
		4.90 (dd, 19.1,19.7)	3	4	3, (3.66)
3		6.23 (d, 9.0)	2	5	2, 5
4	165.3 (C)				
5	120.1 (CH)	5.64 (d, 14.8)	6	4, 6, 7	
6	142.9 (CH)	6.21 (d, 14.8)	5	4, 5, 7	5, 8, 40
7	59.6 (C)				
8	78.9 (CH)	4.91 (q, 9.7)		7,10,42,68,72	6, 42, 40, 11
10	159.3 (C)				
11	125.0†(CH)	6.62 (br, 8)			
12	127.9‡(CH)	6.82 (br, 8)			
13	120.6 (C)				
14	130.8‡(CH)	6.82 (br, 8)			
15	125.6†(CH)	6.62 (br, 8)			
16	56.4 (CH)	5.17 (d, 3.2)	17	11-15, 36	73, 57, 17, 32
17		8.41 (d, 3.2)	16	18	19, 16, 44, 57
18	173.6 (C)				
19	53.6 (CH)	5.05 (dd, 10.4, 4.0)	44, 20	18,21,45,47,50	2,47,45,20,17
20		6.51 (bd, 10.4)	19	21	22, 19, 47, 57, 49
21	167.5 (C)				
22	55.6 (CH)	4.83 (dd, 9.7, 2.2)	23, 49	50	59, 49, 20
23	72.4 (CH)	5.45 (dq, 6.1,2.2)	22, 59		59
25	168.6 (C)				
26	52.0 (CH)	4.64 (m)	27, 29		27
27	65.6 (CH_2)	4.66 (d, 10.8)	(4.42)	25, 26	26, 29, (4.42)
		4.42 (dd, 10.8, 3.2)	(4.66)		26, 29, (4.66)
29		7.24 (bd, 6.1)	26	1, 26, 27, 30	27
30	169.1 (C)				
31	61.3 (CH)	4.32 (d, 7.2)	32, 64		33, 45, 73, 5
32		7.21 (bd, 7.6)		33	16, 45, 73
33	168.9 (C)				
34	68.7 (CH)	3.92 (dd, 11.2, 3.6)	66		73, 66 (3.34)
36	169.9 (C)				
40	55.4 (CH_2)	2.44 (d, 5.4)	(2.95)	5, 6, 7	5, 6, 8
		2.95 (d, 5.4)	(2.44)	7	8, 42
42	14.7 (CH_3)	1.32 (d, 6.1)		7, 8	8, 40 (2.95)
44	39.5 (CH)	1.77 (m)	19, 47, 45		19, 47
45	26.1 (CH_2)	1.14 (m, 7.0)	44, 46, (1.28)	19, 44	
		1.28 (m, 6.5)	46, (1.14)		19, 31, 62, 32
46	11.5 (CH_3)	0.87 (d, 7.9)	45	45	
47	14.2 (CH_3)	0.83 (d, 7.2)		19, 44, 45	19, 44, 20
49		7.95 (bd, 9.7)	22	50	22, 51, 20
50	177.6 (C)				

Table 8. NMR Data for Salinamide A (58) (continued)

C/X #	δ $^{13}C^a$	δ $^{1}H^b$	COSY[c]/ TOCSY[d]	HMBC[d]	ROESY[d]
51	41.6 (CH)	2.76 (dq, 6.8,4.3)	52, 57		52-55, 57, 63, 49, 64, 58
52	79.4 (CH)	3.18 (bd, 7.0,3.5)	51, 53, 54, 55, 57, 58		51, 54, 55, 57, 31, 58
53	32.2 (CH)	1.62 (ddd,7,4.3,3.2)	52, 54, 55	52	51, 52, 54, 55 64, 58
54	18.6 (CH_3)	0.96 (d, 6.8)	52, 53	52, 53	51, 52, 53, 58
55	19.7 (CH_3)	0.88 (d, 6.5)	52, 53	52, 53	51, 52, 53
57	16.7 (CH_3)	1.38 (d, 6.8)	51, 52	68, 72	16, 31, 62, 51, 52, 11, 20,17, 64, 58
58		3.46 (bd, 8.6)	52		51-54, 57, 63, 66, 64
59	15.6 (CH_3)	1.30 (d, 6.1)	23	23	23, 22
62	68.1 (CH)	4.32 (m)	63, 64		45, 63, 57, 32, 64
63	21.1 (CH_3)	1.56 (d, 6.1)	62, 64	31, 62, 66	62, 51, 64, 58
64		5.84 (bd, 1.8)	31, 62, 63	63	31, 73, 62, 63, 51, 53, 57, 58
66	34.6 (CH_2)	3.34 (dd, 14.4, 3.6)	34	34	34, 27, 66, 68, 58
		3.63 (dd, 14.4,3.6)	34	34	34, 66, 15
67	137.1 (C)				
68,72	129.0 (CH)	7.09 (bd, 5.0)	66	34	
69,71	128.4 (CH)	7.09 (bd, 5.0)			
70	126.8 (CH)	7.09			
73	40.1 (CH_3)	2.67 (s)		36, 34	16, 34, 31, 68, 32, 64

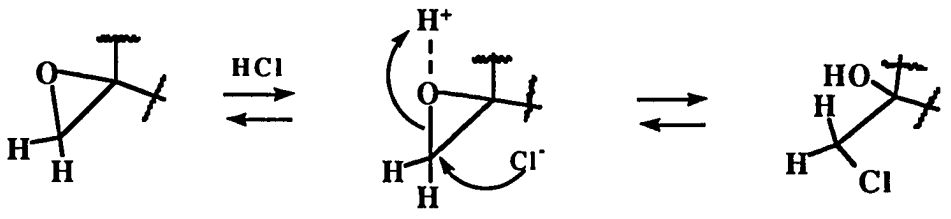
[a] Recorded in $CDCl_3$ at -30°C on a VXR500 NMR at 125MHz. Chemical shifts are reported with reference to internal TMS at 0.00 ppm. [b] Recorded in $CDCl_3$ at -30°C on a VXR500 NMR at 500MHz. Chemical shifts are reported with reference to internal TMS at 0.00 ppm. [c] Recorded in $CDCl_3$ at 26°C on a Nicolet 360 MHz NMR. [d] Recorded in $CDCl_3$ at 26°C on a VXR500 500 MHz NMR. Chemical shifts are reported with reference to $CDCl_3$ at 77.0 ppm for ^{13}C shifts and internal TMS at 0.00 ppm for ^{1}H shifts. †, ‡ Shifts may be interchanged.

Figure 18. A three-dimensional view of salinamide A in solution



incorporation of a chlorine atom in the structure, with a molecular formula of $C_{51}H_{70}N_7O_{15}Cl$. Additionally, the epoxide protons were missing, as is evident in Figure 19. Since 15 oxygens were still present in the formula, this suggested a chlorohydrin derivative at C7 and C40. Compound **59** was crystalline, and its relative stereostructure was established by T. Stout and Professor J. Clardy at Cornell University using single-crystal X-ray diffraction analysis. The X-ray drawing is shown in Figure 21. The same restricted bicyclic form was suggested by the similarity of the $^1H/^{13}C$ NMR behavior of **58** and **59**.

Upon HCl addition, **58** was converted to a halohydrin which showed an optical rotation and ^1H NMR spectrum identical to **59**. This acid-catalyzed ring-opening



reaction indicates **58** and **59** have the same absolute stereochemistry, differing only in the bacterial conversion of the epoxide to a chlorohydrin in the later fermentation. The absolute stereochemistry was determined by hydrolysis and derivatization of **58** followed by ^1H NMR and chiral GC analysis of the 2**R**, 3**S** (*d*-)Thr and 2**S**, 3**S** (*l-allo*-)Thr residues, yielding the stereochemistry shown in Figures 17 and 20 .

A third fermentation produced 220 mg of a single compound (**59a**) which appeared to be different than salinamides A and B judging from the ^1H NMR data

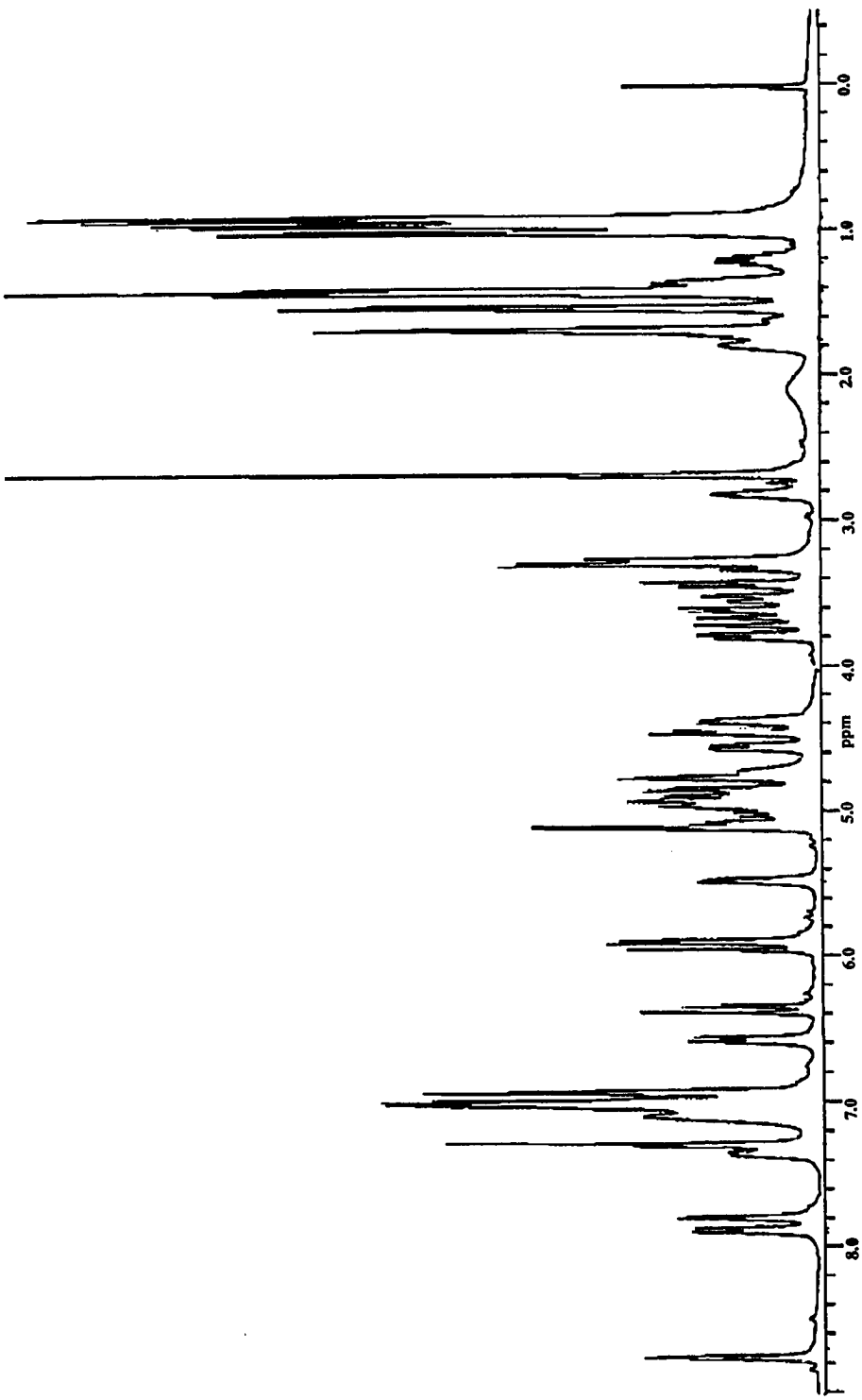


Figure 19. ^{1}H NMR spectrum of salinamide B (59) in $CDCl_3$ at 360 MHz at 26°C

Figure 20. Structure of Salinamide B (59)

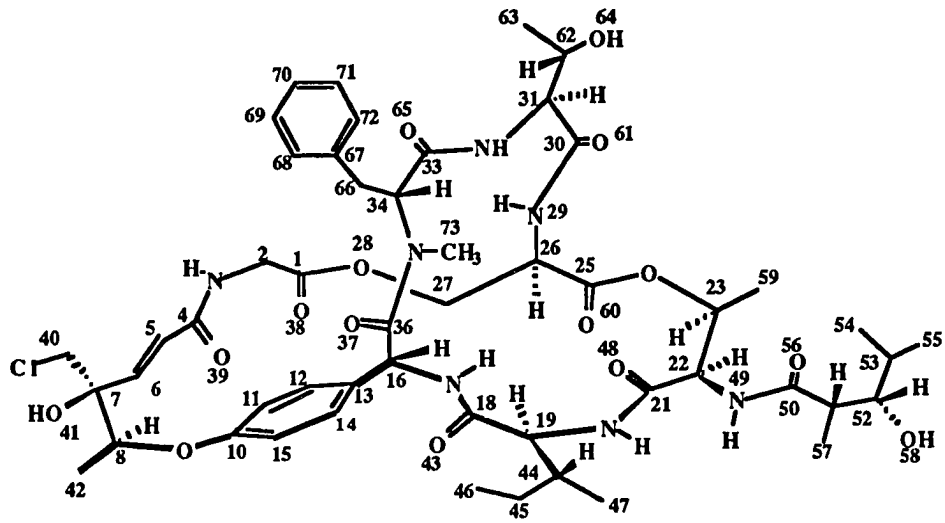


Table 9. Spectral Data for Salinamide B (59)

Source: CNB091, a *Streptomyces* sp. from a surface swab of a jellyfish

$[\alpha]_D$ -65° (c 0.59, $CDCl_3$) **mp** 239-241°C melts

Molecular Formula: $C_{51}H_{69}N_7O_{15}Cl$

HRFABMS: ($M+H^+$) m/z 1056.470 (obs), 1056.468 (calc), Δ 1.9 ppm

IR ($CDCl_3$): 3445, 3347,1745, 1734, 1683, 1651, 1636 cm^{-1}

UV (MeOH): 282, 268, 227, 209 nm

Table 10. NMR data for salinamide B (59)

C/X #	δ[a] ^{13}C	δ[b] ^{1}H	TOCSY[c]
1	170.2 (C)		
2	40.7 (CH_2)	4.90 (dd, 18.8, 10.0)	3, 3.62
		3.62 (d, 18.8)	3, 4.90
3		7.02 (bd)	2
4	165.2 (C)		
5	118.1 (CH)	5.92 (d, 15.6)	6
6	146.9 (CH)	6.30 (d, 15.6)	5
7	81.3 (C)		
8	79.7 (CH)	4.78 (m)	42
10	160.9 (C)		
11	123.3†(CH)	6.9-7.1 (br)	
12	128.4‡(CH)	6.9-7.1 (br)	
13	123.0 (C)		
14	131.1‡(CH)	6.9-7.1 (br)	
15	124.0†(CH)	6.9-7.1 (br)	
16	56.6 (CH)	5.05 (d, 2.0)	17
17		8.71 (bs)	16
18	173.7 (C)		
19	54.3 (CH)	4.95 (dd, 9.6, 3.2)	20, 44, 47
20		6.50 (d, 10.8)	19, 44, 47
21	167.8 (C)		
22	56.2 (CH)	4.84 (m)	23, 49, 59
23	73.6 (CH)	5.44 (dq, 6.4, 2.0)	22, 49, 59
25	168.9 (C)		
26	53.1 (CH)	4.68 (bs)	27, 29
27	65.5 (CH_2)	4.75 (q, 8.6, 6.0)	26, 28, 4.50
		4.50 (bd, 10.0)	26, 28, 4.75
29		7.73 (bs)	26, 27
30	170.1 (C)		
31	61.6 (CH)	4.38 (d, 7.6)	32, 62, 63
32		7.23 (bd, 9.6)	31

C/X #	δ[a] ^{13}C	δ[b] ^{1}H	TOCSY[c]
33	170.1 (C)		
34	69.3 (CH)	3.73 (dd, 11.6, 2.0)	66
36	170.2 (C)		
40	47.9 (CH_2)	3.37 (d, 11.2)	3.20
		3.20 (m)	3.37
42	14.5 (CH_3)	1.47 (d, 5.6)	8
44	39.8 (CH)	1.72 (m)	19, 20, 45, 46, 47
45	26.3 (CH_2)	1.28 (m)	44
		1.14 (m)	44
46	11.5 (CH_3)	0.88 (d, 8.4)	44, 45
47	14.4 (CH_3)	0.83 (d, 6.8)	19, 20, 44
49		7.84 (d, 8.6)	22, 23
50	177.9 (C)		
51	42.1 (CH)	2.74 (m)	52, 57, 58
52	80.8 (CH)	3.19 (m)	51, 54, 55, 57, 58
53	32.4 (CH)	1.65 (q, 6.4)	52, 54, 55, 58
54	18.5 (CH_3)	0.94 (d, 6.4)	52, 53, 55, 58
55	19.9 (CH_3)	0.88 (d, 6.6)	52, 53, 54, 58
58		3.51 (m)	51, 52, 53, 54, 55
59	15.8 (CH_3)	1.38 (d, 6.4)	22, 23
62	68.9 (CH)	4.34 (bq, 7.5)	31, 63, 64
63	21.4 (CH_3)	1.64 (d, 6.0)	31, 62, 64
64		5.90 (bs)	63, 64
66	34.8 (CH_2)	3.42 (m)	34, 3.23
		3.25 (m)	34, 3.42
67	137.7 (C)		
68,72	129.2 (CH)	6.91 (d, 6.8)	
69,71	128.5 (CH)	6.97 (t, 6.8)	
70	128.4 (CH)	6.99 (d, 6.8)	
73	40.0 (CH_3)	2.60 (s)	

[a] ^{13}C NMR experiments done at -30°C in $CDCl_3$ on a VXR500 NMR at 125 MHz, relative to TMS. [b] ^{1}H NMR experiments done at -30°C (1-D) or room temperature (2-D) in $CDCl_3$ on a VXR500 NMR at 500 MHz, relative to TMS. †,‡ Shifts may be interchanged.

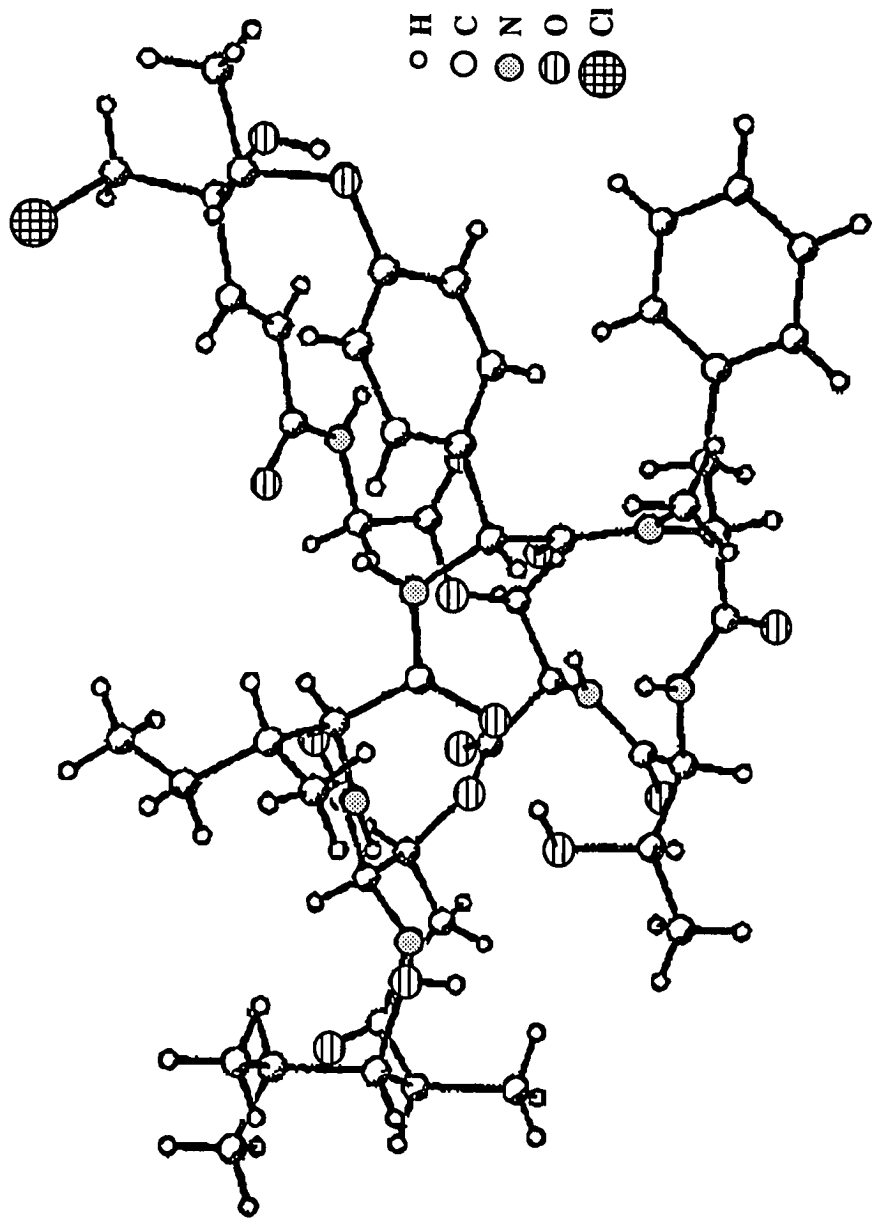


Figure 21. X-ray drawing of salinamide B (59), courtesy of Professor Jon Clardy, Cornell University

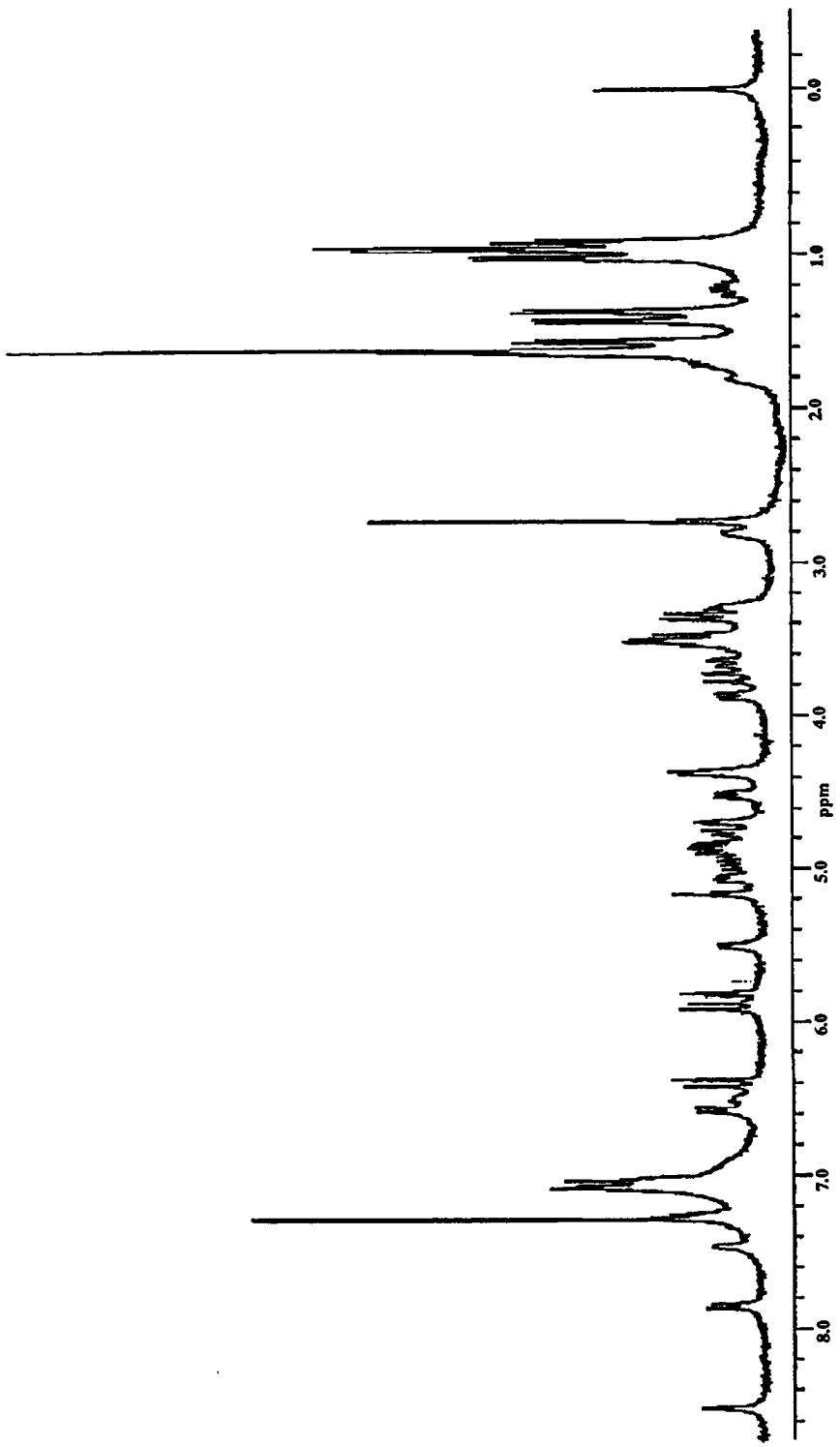


Figure 22. ^{1}H NMR spectrum of salinamide B-concentrated (59a) in $CDCl_3$ at 360 MHz

(Figure 22). Clear differences were seen in the shifts of several exchangeable protons. The proton on N49, at a chemical shift of 7.84 ppm in the original ^{1}H NMR spectrum of salinamide B, was at 7.42 ppm in the spectrum shown in Figure 22, and H64 was at 5.80 ppm, rather than 5.90 ppm. Also, the spectral region from 4.3 to 5.2 ppm looked different in the Figure 22 ^{1}H NMR spectrum than in that of salinamide B shown in Figure 19. However, the molecular formula and ^{13}C NMR spectrum were identical to **59**. The compound was finally determined to be salinamide B through a simple experiment. When mixed in ratios of 10:1 to 1:10 in 20% steps, the ^{1}H NMR spectra of the mixtures of **59** and **59a** appeared to be one clean compound, **59**, in every case. Apparently, **59** takes on a different solution conformation or interacts differently with the solvent at high concentration in $CDCl_3$, causing the ^{1}H environments to change enough to see the several small changes in the NMR spectrum.

A small amount (~5 mg) of a third depsipeptide, salinamide C (**60**), was isolated in one of the fermentations where **59** was produced. The high-resolution FABMS indicates a molecular formula $C_{52}H_{73}N_7O_{14}$. Combined with the additional olefinic signals and the lack of epoxide protons, the structure was thought to be further reduced between C6 and C8. The most notable feature of the NMR data of **60**, however, was its clarity at room temperature. The restricted ring appears to be gone, with a methoxyl group at the *para* position of the Phg residue.

The structure elucidation of **60** was held for two months while a new 500 MHz instrument was installed at UC San Diego. During this period, **60** was stored frozen in benzene. Unfortunately, the compound broke down, and only 1.5 mg could be salvaged. With this small amount, the HMQC and HMBC results were not

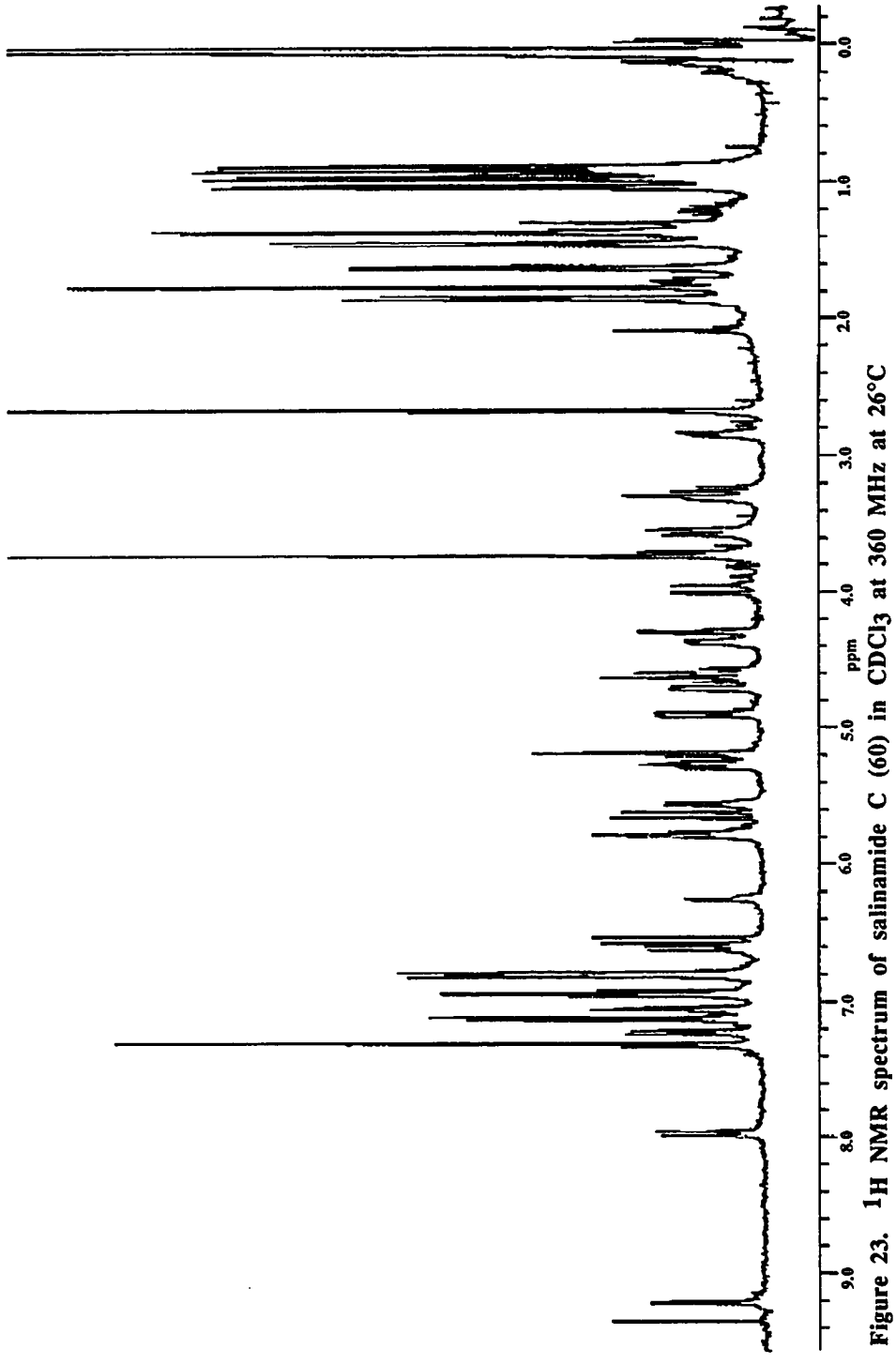


Figure 23. ^{1}H NMR spectrum of salinamide C (60) in $CDCl_3$ at 360 MHz at 26°C

Table 11. ^{13}C NMR data for salinamides A, B, C, E

C #	A δ^a	B δ^a	C δ^b	E δ^b
1	169.7	170.2	170.3	-
2	40.8	40.7	41.3	-
4	165.3	165.2	167.1	-
5	120.1	123.3	?	-
6	142.9	146.9	147.0	-
7	59.6	81.3	?	-
8	78.9	80.8	?	-
10	159.3	160.9	160.3	161.0
11	125.0†	118.1‡‡	114.6	115.1
12	127.9‡	128.4††	130.2	130.0
13	120.6	124.0	122.9	122.9
14	130.8‡	131.1‡‡	130.2	130.0
15	125.6†	123.0††	114.6	115.1
16	56.4	56.6	56.7	56.1
18	173.6	173.7	174.4	174.4
19	53.6	54.3	54.2	55.0
21	167.5	167.8	168.0	168.7
22	55.6	56.2	56.2	56.6
23	72.4	73.6	72.6	71.4
25	168.6	168.9	168.7	169.4
26	52.0	53.1	52.5	55.2
27	65.6	65.5	65.7	62.7
30	169.1	170.1	169.2	169.2
31	61.3	61.6	61.6	61.6
33	168.9	170.1	169.5	168.8
34	68.7	69.3	70.0	70.2
36	169.9	170.2	170.7	171.8
40	55.4	47.9	?	-
42	14.7	14.5	14.5	-
44	39.5	39.8	40.0	39.7
45	26.1	26.3	26.5	26.2
46	11.5	11.5	11.6	11.7
47	14.2	14.4	14.2	14.1
50	177.6	177.9	177.9	177.7
51	41.6	42.1	42.2	42.1
52	79.4	79.7	79.5	79.5
53	32.2	32.4	32.3	32.2
54	18.6	18.5	18.3	18.1
55	19.7	19.9	19.9	19.7
57	16.7	16.7	16.8	16.6
59	15.6	15.8	15.9	15.9
62	68.1	68.9	68.6	68.3
63	21.1	21.4	21.2	21.3
66	34.6	34.8	34.2	33.5
67	137.1	137.7	138.0	137.5
68,72	129.0	129.2	128.4	128.9
69,71	128.4	128.5	129.0	128.5
70	126.8	128.4	126.4	126.5
73	40.1	40.0	40.1	40.4
-OMe	-	-	54.9	55.6

[a] Recorded in $CDCl_3$ at -30°C on a VXR500 125 MHz NMR. Chemical shifts are reported with reference to $CDCl_3$ at 77.0 ppm. †,††,‡,‡‡ Carbon resonances may be interchanged. [b] Recorded in $CDCl_3$ at 26°C on a WP200SY 50 MHz NMR.

Table 12. ^{1}H NMR data for the salinamides

C/X #	A δ^a	B δ^a	C δ^b	D δ^b	E δ^b	F δ^b
2	3.66	3.64	3.98	4.85	-	-
	4.90	4.94	5.22	3.70	-	-
3	6.23	7.06	?	6.18	-	-
5	5.64	5.92	?	5.72	-	-
6	6.21	6.34	6.52	6.26	-	-
8	4.91	4.82	?	4.92	-	-
11	6.62	6.9-7.1	6.75	6.80	7.02	7.04
12	6.82	6.9-7.1	7.09	6.90	7.35	7.10
14	6.82	6.9-7.1	7.09	6.90	7.35	7.10
15	6.62	6.9-7.1	6.75	6.80	7.02	7.04
16	5.17	5.10	5.20	5.22	5.17	5.16
17	8.41	8.74	?	8.50	7.02	7.12
19	5.05	5.04	5.25	4.84	4.81	4.82
20	6.51	6.58	6.58	6.61	6.75	6.62
22	4.83	4.90	4.86	4.95	4.74	4.74
23	5.45	5.48	5.52	5.50	5.61	5.68
26	4.64	4.76	4.67	4.70	4.33	4.32
27	4.66	4.82	4.57	4.64	4.17	4.24
	4.42	4.56	4.57	4.45	3.97	3.99
29	7.24	7.79	7.28	7.20	7.07	7.07
31	4.32	4.42	4.24	4.32	4.29	4.32
32	7.21	7.23	7.20	7.08	7.09	7.09
34	3.92	3.78	3.67	3.92	3.73	3.72
40	2.44	3.24	?	2.45	-	-
	2.95	3.42	?	2.96	-	-
42	1.32	1.51	1.81	1.35	-	-
44	1.77	1.81	1.73	1.98	1.66	1.92
45	1.14	1.22	1.20	-	1.09	-
	1.28	1.32	?	-	1.37	-
46	0.87	0.85	0.86	0.96	0.87	0.87
47	0.83	0.82	0.84	0.95	0.80	0.80
49	7.95	7.88	7.94	7.77	7.70	7.64
51	2.76	2.85	2.80	2.80	2.82	2.82
52	3.18	3.23	3.28	3.30	3.39	3.39
53	1.62	1.74	1.69	1.70	1.68	1.68
54	0.96	0.99	0.99	0.99	0.99	0.99
55	0.88	0.88	0.94	0.98	0.95	0.95
57	1.38	1.42	1.37	1.41	1.37	1.37
58	3.46	3.62	3.69	3.46	3.53	3.54
59	1.30	1.40	1.32	1.33	1.27	1.28
62	4.32	4.39	4.30	4.28	4.29	4.30
63	1.56	1.64	1.58	1.58	1.56	1.56
64	5.84	5.85	5.12	5.76	5.13	5.11
66	3.34	3.31	3.21	3.38	3.52	3.50
	3.63	3.52	3.51	3.68	3.15	3.14
68,72	7.09	6.91	6.90	7.10	6.62	6.61
69,71	7.09	6.97	6.74	7.10	6.90	6.88
70	7.09	6.99	7.02	7.10	7.02	7.02
73	2.67	2.68	2.63	2.72	2.73	2.73
-OMe	-	-	3.80	-	3.90	3.90

[a] Recorded in $CDCl_3$ at -30°C on a VXR500 500MHz NMR. [b] Recorded in $CDCl_3$ at 26°C on a VXR500 500MHz NMR. Chemical shifts are reported with reference to internal TMS at 0.00 ppm.

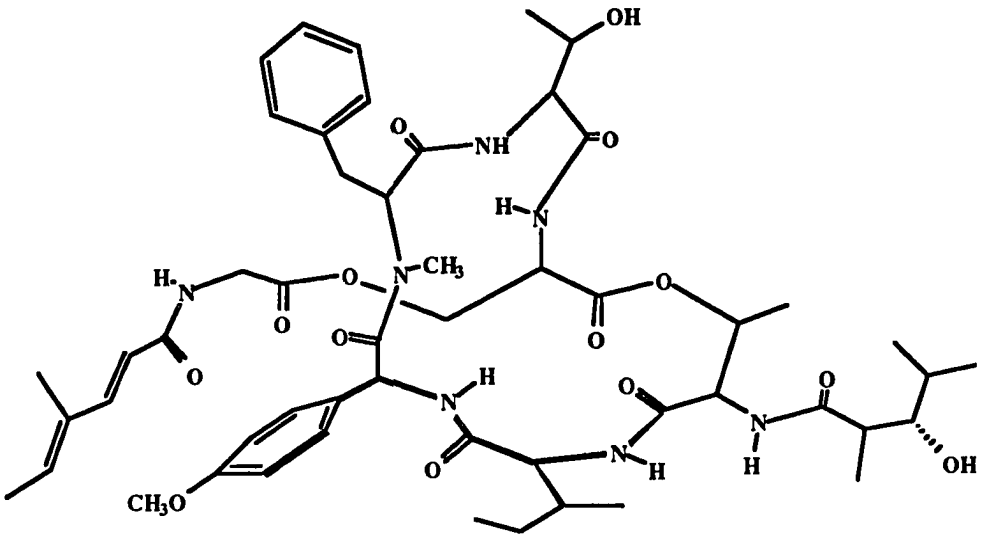


Figure 24. Proposed structure of salinamide C (60)

Table 13. Unassigned NMR chemical shifts of salinamide C

^{13}C:	^{1}H:
136.9 ppm	9.18 ppm
133.1 ppm	6.22 ppm
116.4 ppm	5.75 ppm
	5.60 ppm
	5.25 ppm
	0.86 ppm

very clear, although the assignments shown in Tables 11 and 12 could be made, tentatively. The unassigned shifts are shown in Table 13. A weak HMBC correlation from H42 to the 133.1 and 135.9 ppm carbons, allowed the structure to be proposed as shown in Figure 24. Several additional experiments are needed to confirm this structure, but further cultures of CNB091 have not produced salinamide C again.

Recently, a large (75 L) New Brunswick Scientific Fermatron fermentor was set-up in the Fenical group. Looking for more of compound **60**, a 40 L fermentation of CNB091 was obtained. Ethyl acetate extraction was not feasible on this scale, so C18 resin was used (1 L resin/5 L culture broth) to extract the Celite-filtered broth. The resin was rinsed twice with deionized water to remove the salts, then the organics were eluted with MeOH. The extract (20 L) was dried to 500 ml, then partitioned between H_2O and $CHCl_3$. All of the depsipeptides were in the $CHCl_3$ fraction. This fraction was dried with Na_2SO_4, filtered, and evaporated *in vacuo*, then brought up in MeOH and partitioned with TMP to remove the antifoam. The MeOH fraction was dried again to yield 3.4 g crude.

A 350 ml vacuum flash column was eluted with 200 ml fractions of TMP:EtOAc:MeOH mixtures. The salinamide fraction was then separated using a preparative reversed-phase HPLC column with 75:25 MeOH: H_2O at 6.0 ml/min. This yielded 276.0 mg of salinamide A, 81.0 mg salinamide B, and several new analogs, including salinamide D (**61**), salinamide E (**62**) and salinamide F (**63**).

The spectral data for salinamide D (14.9 mg isolated) were very similar to **58**, with a molecular formula of (M^+) $C_{50}H_{67}N_7O_{15}$ by high-resolution FABMS. Careful examination of the ^1H NMR spectrum revealed a change in the chemical

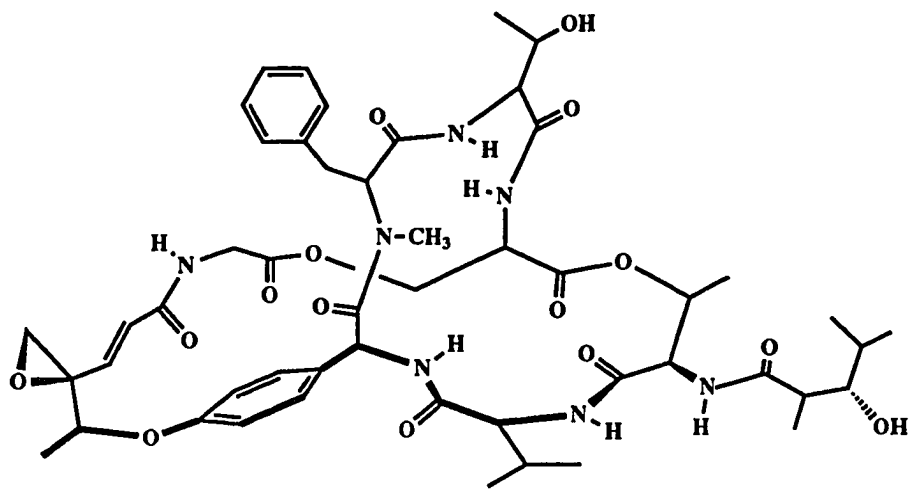


Figure 25. Structure of salinamide D (61)

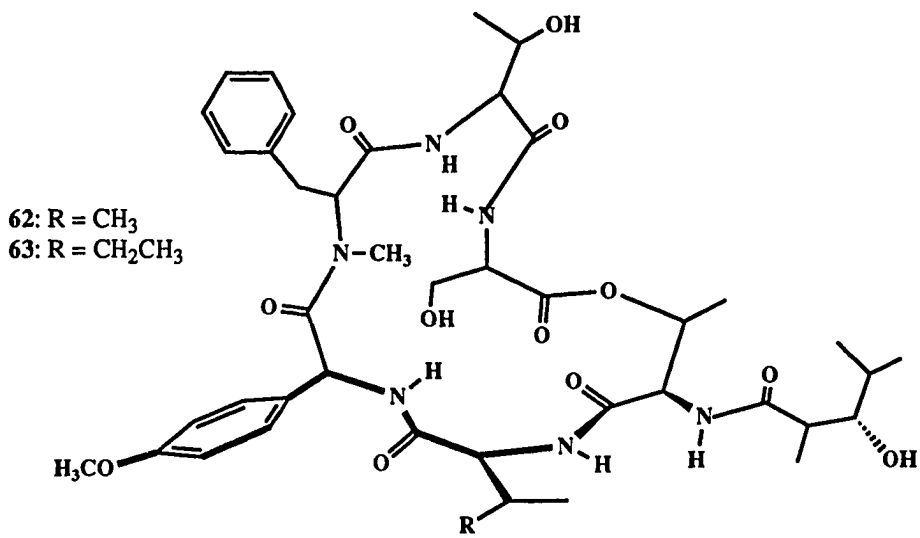


Figure 26. Structure of salinamide E (62) and salinamide F (63)

shifts of H19 from 5.05 ppm to 4.85 ppm and H44 from 1.77 ppm to 1.99 ppm. The protons of C45 were completely missing, leading to the conclusion that the Ile in **58** had been replaced by a Val residue in **61**.

The 40 L fermentation also yielded 20.3 mg of salinamide E (**62**), another monocyclic depsipeptide. Several features were immediately noticeable in the NMR spectra: no epoxide protons were present; the aromatic region was clear, with a methoxy group most likely located on the phenylglycine ring; and the olefinic protons and carbons were missing. The high-resolution FABMS data ($M+H^+$, *m/z* 855.4535) analyzed for $C_{43}H_{63}N_6O_{12}$. By this point, a 500 MHz NMR instrument was available at UC San Diego, so an HMBC experiment was performed to determine the connectivities. Data for the 1-D NMR experiments done are shown in Tables 11 and 12. Most of the chemical shifts and long-range correlations were analogous to those of **58** and **59**. The correlation from the extra exchangeable proton at 4.60 ppm to C26 confirmed its position attached at C27. Thus, the entire section from C1 to C8, including the glycine residue and the 7-carbon α,ß-unsaturated amide, is missing, and the structure is as shown in Figure 26.

One of the other depsipeptides isolated in small amounts (8 mg) and still impure appears to be the Val analog of **62**. The ^1H NMR spectrum of this compound, salinamide F (**63**), shows the same change of shifts relative to **62** that **61** shows relative to **58**. Further purification work is needed to confirm the structure as shown in Figure 26 and to decide if any **60** was produced in the large fermentation.

In addition to their significance to marine natural products chemistry as complex novel carbon skeletons, the salinamides show biomedically-relevant activities. Salinamides A and B exhibit moderate antibiotic activity against Gram (+) bacteria. The most potent *in vitro* activity is seen against *Streptococcus pneumoniae* and

Table 14. Antibiotic activity of salinamides A and B.

Organism	Primary MIC Values (μg/ml) A	B	Standard*
1. *S. pneumoniae*/ Todd Hewitt	4	4	.003
2. *S.pyogenes*/ Todd Hewitt	4	2	.003
3. *E. faecalis*	128	128	1
4. *E. faecium*	>128	>128	4
5. *S. Aureus*/ Pen. -	8	8	.007
6. *S. aureus*/ 50% human serum	-	-	-
7. *S. aureus*/ NCCLS strain	8	8	.015
8. *S. aureus*/ Pen. +	8	8	.015
9. *S. aureus*/ Pen. +	8	8	.015
10. *S. aureus*/MR28°C+NaCl	32	32	32
11. *S. epidermis*	8	16	.015
12. *S. epidermis*/ MR28°C+NaCl	32	32	.03
13. *S. haemolyticus*	8	8	.015
14. *E. coli*	32	32	.125
15. *E. coli*/ NCCLS strain	?	?	.125
16. *K. pneumoniae*	128	128	.125
17. *E. cloacae*	32	32	2
18. *P. mirabilis*	128	128	1
19. *P. vulgaris*	64	128	2
20. *M. morganii*	128	128	1
21. *P. rettgeri*	128	128	1
22. *P. stuartii*	128	>128	2
23. *S. marcescens*	128	128	.5
24. *P. aeruginosa*	64	64	1
25. *P. aeruginosa*/ NCCLS strain	128	128	1
26. *P. aeruginosa*	64	64	.5
27. *P. aeruginosa*/ Imipenem MIC=8	64	64	8
28. *P. aeruginosa*/Imipenem MIC=32	128	128	32
29. *P. cepacia*	64	64	4
30. *X. maltophilia*	16	16	>64
31. *H. influenzae*/ P- w/1%sup.C	?	.5	.25
32. *H. influenzae*/ P+ w/1%sup.C	?	1	.125

* All testing done by Bristol-Myers Squibb, using Imipenem as a standard.

Staphylococcus pyogenes with MIC values of 4 μg/ml for salinamide A and 4 μg/ml and 2 μg/ml respectively for salinamide B, as shown in Table 14. Though these activities are not clinically significant, their specificity for Gram (+) bacteria may indicate a mode of action similar to vancomycin,[81] an antibiotic used in treatment of persistent Gram (+) bacterial infections. Vancomycin inhibits mucopeptide biosynthesis in bacterial cell walls by binding to *d*-alanine residues in peptidoglycan precursors. The restricted chlorine-containing and phenylglycine rings are important in forming the binding pocket of vancomycin. Similarly, the restricted phenylglycine ring may be important in the mechanism of action of the salinamides, eventhough no aromatic halides are present. T. Stout and J. Clardy, the collaborators at Cornell University responsible for the X-ray structure determination, have attempted to show solution binding to *d*-alanine dipeptides through NMR spectroscopy, but results to date have been inconclusive.

From a biomedical standpoint, more significant activity is seen in the area of anti-inflammatory testing, where R. Jacobs at UC Santa Barbara has found the salinamides to be active in the mouse-ear edema assay. Further testing is needed in this area. Isolation of significant amounts of salinamides C-F would be valuable for biotesting, since the restricted ring is not present in several of the analogs. With these data, speculation could be made as to the features of the salinamide structure most important in causing the anti-inflammatory and antibiotic activity.

C. Antibiosis in Estuarine Microbial Isolates.

1) Introduction to Antibiosis Projects

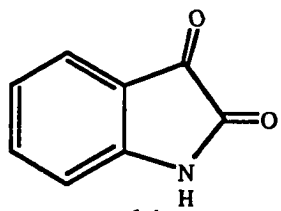
The salinamides showed activity against clinically-important Gram (+) bacteria, but why were they produced? One hypothesis is that antibiotic compounds like the salinamides favor colonization and survival of the producing organism *in situ*. The first step in proving this theory would be to determine the activity of the compounds against microorganisms isolated from the same environment as the producer. This same technique could be used to screen bacterial isolates for antibiotic activity that might be expressed in the natural environment. A literature search found only two studies which screened for antibiosis in this manner. Waksman laid the groundwork in 1941 by isolating microorganisms from soils and composts and testing these isolates against other soil microorganisms,[79] and further pioneering work was done by Landerkin and Lochhead.

Landerkin and Lochhead studied extensively the chemical microbial competition in bacteria isolated from soil. Their first general study of antibiosis involved the purchase or culture of 50 strains of soil actinomycetes shown to be active against *Escherischia coli* using the flood plate technique.[82] These actinomycetes were then spotted on Conn's asparagine agar plates, 5 cultures per plate, grown for 5 days, then flooded with agar seeded with 1% of a test soil microorganism. Fourteen strains of microorganisms were used as a test panel. All of the actinomycetes were active against at least two members of the test panel. Nineteen (38%) were active against all members of the test panel. The antibiotics responsible for these results were never isolated.

The second study of Landerkin, Smith, and Lochhead included 660 actinomycetes isolated on a non-selective basis from soils in 5 different areas of

Northern Canada.[83] Eight test organisms, including 5 bacteria and 3 common pathogenic soil fungi, were used as a panel. At least one of the test microbes was inhibited by 404 (61%) of the actinomycetes. Six of the eight were inhibited by 25 (4%) of the actinomycetes. *Bacillus subtilis* and *Staphylococcus aureus* were especially susceptible to inhibition, 101 (15%) of the actinomycetes showing activity against these two species. Again, no attempts were made to isolate the chemical compounds responsible for these activities.

Studies like these formed a foundation for further work on microbial ecology in soil. Since microbial ecology in marine environments is an emerging field, these experiments suggest a strategy for chemical study of marine sediments. Several microbially-produced antibiotic compounds had been found to be ecologically important in the marine environment. Gil-Turnes *et al.* showed that an epibiotic bacterium, a member of the genus *Alteromonas* and associated with the embryos of the shrimp *Palaemon macrodactylus*, produces an antifungal compound, 2,3-indolinedione (**64**), which inhibits the marine pathogenic fungus *Lagenidium callinectes*.[84] Similar cases where external symbionts appear to be producing protective chemicals were studied with Lobster eggs and blue-green algae.[85] Also, several other marine bacteria have been found to be antibiotic producers in culture.



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The first marine microbial antibiotic identified, 2,3,4-tribromo-5-(1'-hydroxy-2',4'-dibromophenyl) pyrrole (**2**), was isolated from *Pseudomonas bromoutilis* in the

first published screening survey for antimicrobial products from marine bacteria.[23,86] Add to these the secondary metabolites isolated from marine unicellular bacteria, actinomycetes and fungi previously mentioned, including the salinamides, and the marine microbial products shown in Figures 2 - 4, and it is evident that the marine environment is a promising source of antibiotics and microbial products in general.

But, perhaps the best approach is not simply screening for chemical compounds or activities alone. Another approach is to try to learn something about the environment being studied while screening for these interesting compounds. The approach taken in this study is an attempt to develop the same basic information for a marine environment that Landerkin and Lochhead developed in soil and identify interesting antibiotic compounds at the same time. The primary goal was to identify novel compounds. Therefore, an environment where the most antibiotic activity would be expected was desired.

If we again consider the hypothesis that antibiotic production is a significant factor in the colonization of a microenvironment, that is, the space in contact or a few microns away from a microorganism, then it follows that antibiotic production would be favored where continuous competition for resources occurs, as in places of high environmental transition and in environmental margins. Environmental transition means a change in the physical conditions of the environment, such as change in salinity, oxygen level, surface tension, turbidity or suspended solids, turbulence, and nutrients, whereas margins refer to longlasting differences in the physical properties of adjacent areas. Estuarine systems are one of the few environments predictably characterized by high rates of transition and an abundance of natural margins. Salinities range from 0% to 3.5% in a typical estuary, with

hypersaline margins in many cases. Turbidity and nutrient levels are changed by river run-off and influx of seawater. Turbulence and oxygen levels are at the mercy of the weather and activity of estuarine organisms, and surface tension is most affected by plant products and man-made pollution.[87] Because of these characteristics, the recent isolation of several marine microbial products from mangrove isolates, and the fact that estuaries are very rich in microbial populations, this environment appears to be a good place to look for antibiotic-producing bacteria and actinomycetes.

Definitions of the word estuary abound. One of the most inclusive, yet still understandable, is that of Day:[87]

> An estuary is a partially enclosed coastal body of water which is permanently or periodically open to the sea and within which there is a measurable variation of salinity due to the mixture of sea water with fresh water derived from land drainage.

Of the many types of estuarine environments this would include, intertidal wetlands seemed to be the most promising. Intertidal wetlands, including salt marshes and sloughs, mangrove swamps and tidal lagoons, are characterized by high transition and/or many environmental margins. Also, several tidal lagoons, sloughs and salt marshes were accessible locally: Bahia San Quintin and Bahia de Todos Santos in Baja California, Mexico and Tijuana Slough, Chula Vista Wildlife Reserve, Mission Bay Estuary, Torrey Pines Slough, Del Mar Slough, San Elijo Lagoon, Batiquitos Lagoon, Agua Hedionda Lagoon, Buena Vista Lagoon, Newport Back Bay, and Seal Beach National Wildlife Refuge in Southern California. The choice was quickly narrowed to San Elijo and Batiquitos Lagoons, because all of the other choices, even the reserves and refuges, were too polluted, too influenced by humans in other ways, or had too much terrestrial run-off. San Elijo Lagoon had little interaction with the ocean at the desired time of collection; it had become anoxic.

Since culture of anything other than aerobic, heterotrophic bacteria would have made the study too complex microbiologically, Batiquitos Lagoon was chosen as the first sampling site for the antibiosis study.

Very little run-off enters Batiquitos Lagoon, because the flower growers along one of the three sides of the lagoon and La Costa Country Club along another side (Figure 27) are required to control their run-off to keep any fertilizer from entering the lagoon. Also, Batiquitos is not affected by daily tides, but extremely high tides cause frequent input from the Pacific. The salinity is close to that of the ocean, with variation between 26 and 30‰ in the areas tested. Vegetation was dominated by grasses and algae, and all of the visible inhabitants, including birds, plants, and worms, appeared to be healthy, in contrast to several of the other wetlands visited along the California coast.

The other environment chosen to study was a mangrove embayment in Belize, Central America. Mangrove swamps, also called tidal forests, are another estuarine environment rich in organics. Also, isolation of the salinamides proved that mangroves are a promising environment for the isolation of producers of novel compounds. In June, 1991, the opportunity arose to participate in a research cruise to Belize, Central America. The extensive mangroves on the islands of Belize provided an excellent second study site. The area selected was on Big Cay Bokel, in the Turneffe Islands. Samples were taken in a grid pattern from the mangrove embayment, and from a variety of other identifiable habitats.

After sample site selection, the investigations were similar to those of Landerkin and Lochhead, except the test panels were to be selected from the actual isolates, and the active compounds were to be identified. The actual steps of the experiments consisted of: sampling the lagoon or mangrove, isolating as many bacteria as

possible, selecting a test panel from these isolates, culturing the isolates on small scale, extracting the cultures and testing them against the test panel isolates, selecting leads, and culturing on larger scale for purification and structure elucidation of the active compound(s). Each step will be described in detail.

2) Antibiotic Compounds from Batiquitos Lagoon Isolates

A map of Batiquitos Lagoon is shown in Figure 27, with the sampling areas expanded in Figure 27a and Figure 27b. The "A" samples were collected in sterile tubes from the surface of the sediment or the water column. The sampling region offered a wide range of habitats, including exposed mud and sediment under stagnant water, under a strong current, surrounding plant roots, and riddled with worms. Serial dilutions were made of each sample and spread plates were made from the 10^{-3} to 10^{-6} dilutions. Cycloheximide was added to the B-3/agar plate media to avoid overwhelming fungal contaminants. Colonies began to show up on these plates after only 24 hours, and actinomycetes after about 5 days. The colonies were then isolated from each of the dilution plates containing 1-100 colonies based on colony morphology. Between 10 and 12 colonies were selected from each dilution series in most cases, yielding 223 isolates, including very few actinomycetes.

In order to increase the number of actinomycetes obtained, the B series of samples (Figure 27b) were collected. These samples were heated to 40°C for 1 hr. to select for spore-formers, then diluted and plated out as described for the A series, selecting only actinomycetes for isolation. This brought the total number of isolates up to 240. Each isolate was kept on an agar plate and an agar slant until activity testing was complete. Due to limited freezer space, only active isolates were stored at -80°C in 10% glycerol/B-1.

Once isolated and on slants, the bacteria were Gram tested using the KOH method described by Buck,[88] and their colony morphologies on B-3/ agar plates were described. This allowed each isolate to be placed in one of four broad taxonomic groups: 1) Gram-positive, filamentous (order Actinomycetales);

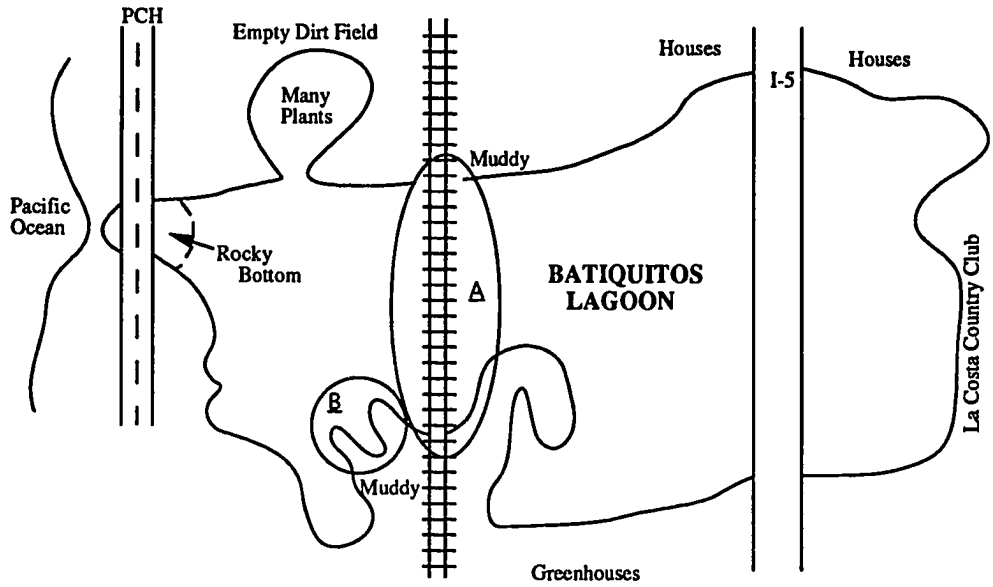


Figure 27. Batiquitos Lagoon Collecting Site (not drawn to scale)

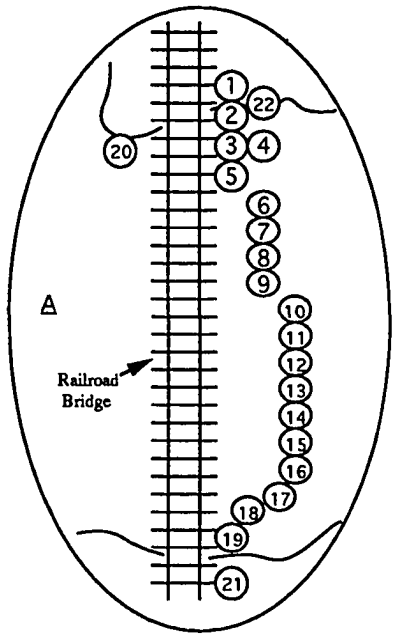


Figure 27a. Sampling site A, expansion of Figure 27

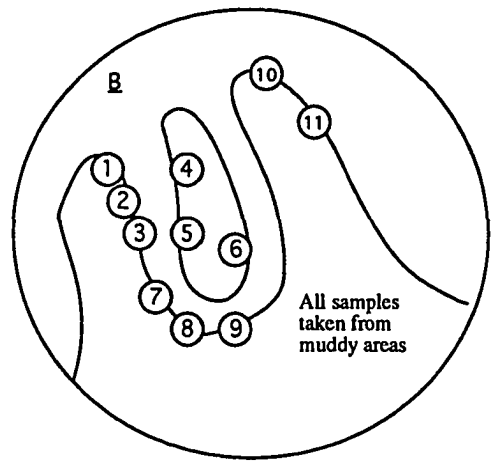


Figure 27b. Sampling site B, expansion of Figure 27

2) Gram-positive, unicellular; 3) Gram-negative, unicellular; and 4) fungus. The final group was surprising, since a broad-spectrum antifungal agent had been used in the original plate media. The five fungi isolated must be cycloheximide-resistant strains.

After this crude separation into groups, a test panel was selected. The criteria set for the test panel were: 1) it should contain representatives from each taxonomic group; 2) it should consist of the most commonly isolated bacteria for the unicellular bacteria; and 3) it should reflect the same Gram-negative:Gram-positive ratio as the isolates, excluding the actinomycetes from the B group. The Gram testing resulted in a ratio of 138:62 Gram -/+, with 23 isolates giving questionable results or lost

Table 15. Batiquitos Lagoon project test panel

Strain #	Gram (±)	Colony Description
CNB739	+	Sporulating actinomycete
CNC098	NA	Orange fungus
CNF029	+	Orange, irregular, dull
CNF028	-	Opaque, irregular, swarmer, flat, shiny
CNF017	+	Salmon, round
CNF018	-	Light yellow, irregular, shiny
CNF019	+	Opaque, round, granular, umbonate
CNF020	+	White creamy, semi-translucent, irregular, dull
CNF021	-	Mauve-brown, tear-drop shape
CNF022	-	Greenish-tan, round, entire
CNF023	-	Orange, round, entire, flat
CNF024	-	Nearly opaque, irregular, undefined
CNF025	-	Beige, round, slightly lobate
CNF026	-	Translucent orange, round, entire, convex
CNF027	-	Small, brown, irregular, rhizoidal, umbonate

before Gram testing. Thus, the Gram -/+ ratio of the test panel was 9:5, excluding the actinomycete. The test panel strains were as shown in Table 15.

After isolation, each culture was grown either in 1 L (actinomycetes and fungi) or 100 ml (unicellulars) scale, then double extracted with EtOAc. The extracts were then dissolved in 1 ml of solvent and screened for activity against the test panel using the paper disk technique. An antibiotic "profile" was recorded for each isolate. Any extract showing activity (≥ 8 mm zones) against 2 or more test panel strains was then subjected to chemical screening. This included TLC analysis on a normal-phase silica plate in EtOAc and recordin of the ^{1}H NMR spectrum in $CDCl_3$/MeOD. Any interesting projects, from a biological or chemical standpoint, were regrown if needed for identification of the active compound(s).

2) a. Overall Results of Microbial Isolation, Activity Testing, and Search for Antibiotic Compounds

The Gram testing ratio was the first interesting result of this investigation. Reports of the percent of marine isolates which were Gram (+) have been as low as 5%.[89] Recently, however, Jensen has found that the Gram (+) bacteria make up closer to 30% of the culturable bacteria from sediment and surface samples.[80] In the Batiquitos Lagoon samples, 31% of the isolates (Collection A only) were Gram (+), agreeing with the results of Jensen. Of course, heterotrophic, aerobic culturable bacteria represent a small fraction of the total viable bacteria in marine sediments,[90] so these percentages may not be indicative of the natural populations in marine sediments.

The activity results showed much better agreement with the literature than the Gram testing results. As expected, the actinomycetes showed tremendous activity, with, 68.4% of the extracts having ≥8 mm zones of inhibition against at least two

test panel strains. Two of the five (40%) fungi and 25.8% of the unicellular bacteria produced active extracts. The activity of the unicellular bacteria was approximately equal for the Gram (+) (24.4%) and Gram (-) (26.3%) isolates. Only two Gram (-) isolates were active against the test panel fungus, while one Gram (+) and four actinomycete extracts inhibited the test panel actinomycete. The most sensitive test panel strain was CNF021. Over 23% of the extracts showed activity against this common Gram (-) isolate.

These 55 active microbial extracts were then subjected to chemical screening. The results of the TLC and ^{1}H NMR analyses are considerably more subjective than the biological screening. The unicellular extracts all showed UV-active TLC bands at the baseline and at R_F values of 0.2, 0.4, and 0.75. Only about 25% of the extracts were identified as chemical leads by having UV-active or charring compounds at different R_F values. The ^{1}H NMR spectra proved to be nearly useless for screening of the unicellular extracts, because there was too little material in most cases. However, the ^{1}H NMR spectra combined with the TLC data for the actinomycete isolates allowed immediate dereplication of several compounds and classification of the remaining active extracts based on chemical interest.

The final goal was to identify new antibiotic compounds from these active extracts. Unfortunately, several strains lost the production of compounds on regrowth, including CNB748, CNB762, and CNB785. On examining records of these regrows, it is important to note that the scale-up procedures varied from the original procedure. Upon following the exact scale-up process for CNB785, activity was regained, though it was not pursued due to time demands. Also, CNF923 and CNF896 showed excellent activity in the antibiotic screening, but the cultures were lost through storage at 0°C rather than -80°C. CNB744 and CNB746

were fractionated, but the only active fraction appeared to be indole, so these were dropped. The majority of the activity pursued could be attributed to known bacterial metabolites. Four known antibiotics were isolated from the actinomycete projects that were pursued, four known compounds resulted from the examination of the fungal extracts, and two known compounds were identified from the unicellular leads. However, CNC100, a fungus, yielded an antibiotic compound which could not be identified due to rapid decomposition in NMR solvents. CNF816, a Gram (-), unicellular bacterium, produced a very polar compound which was not identified due to low yield. The partial identification of these unknown compounds, dereplication of the knowns, and the significance of their isolation follows. A ^{1}H NMR spectrum of all of the known compounds is included for use in future dereplication.

2) b. The Isolation and Dereplication of Piericidin (65) and Chloramphenicol (66) from Isolate CNB765

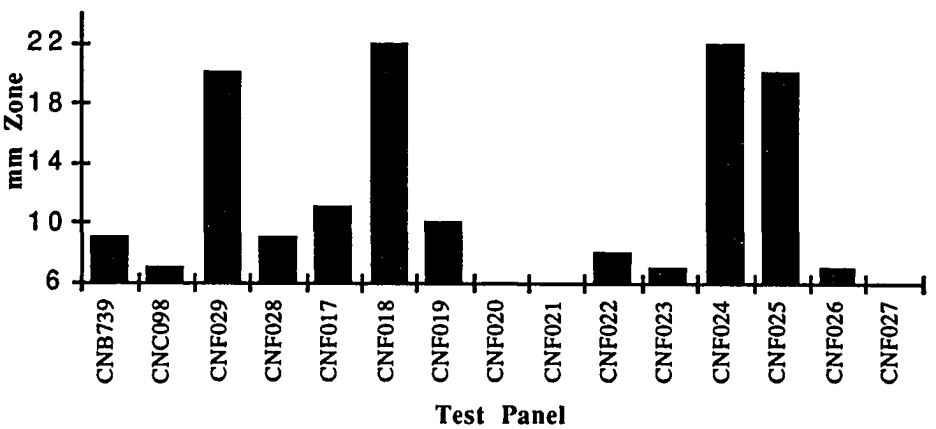


Figure 28. Activity profile of CNB765 crude extract

CNB765 was selected as a lead project based on its potent activity against the Batiquitos Lagoon panel, showing activity against nine of eleven strains tested, as shown in Figure 28. The extract was inactive against two Gram-negative strains. The silica TLC in ethyl acetate was interesting, with red/brown-charring, UV-active and orange-charring, UV-active spots at an R_F of 0.2 and 0.7, respectively. The ^1H NMR of the crude 1-L extract also showed promise, with several unique signals between 5.0 and 6.0 ppm.

CNB765 is an actinomycete isolated from exposed mud on the south shore of Batiquitos Lagoon. It was cultured in scales between 1 L and 55 L in A-1 media. Piericidin (**65**) was purified by flash column chromatography of the crude organic extract. Identification of **65** was based on comparison of NMR data (see Figure 29) with a reference ^1H NMR spectrum of Dr. I. C. Pathirana and with literature NMR data.[91]

Piericidin A was first identified in 1965.[92] Subsequently, many analogs have been isolated and identified from several species of *Streptomyces*. Piericidins are insecticidal agents which have been shown to inhibit respiration at very low concentrations.[91] In addition to this activity, Bristol-Myers Squibb has found the crude extract of a piericidin-producer to be cytotoxic at a concentration on the order of 1 ng/ml.

The flash column fraction eluting immediately after piericidin contained a compound which did not have the substituted pyridine ring of the piericidins, yet still had tremendous activity. Upon further purification by HPLC, chloramphenicol (**66**) was isolated as a pale yellow crystalline solid. Chloramphenicol was much more difficult to dereplicate, because early mass spectrometric attempts, including positive-ion high-resolution FABMS, failed to yield a parent ion. The nitrobenzyl

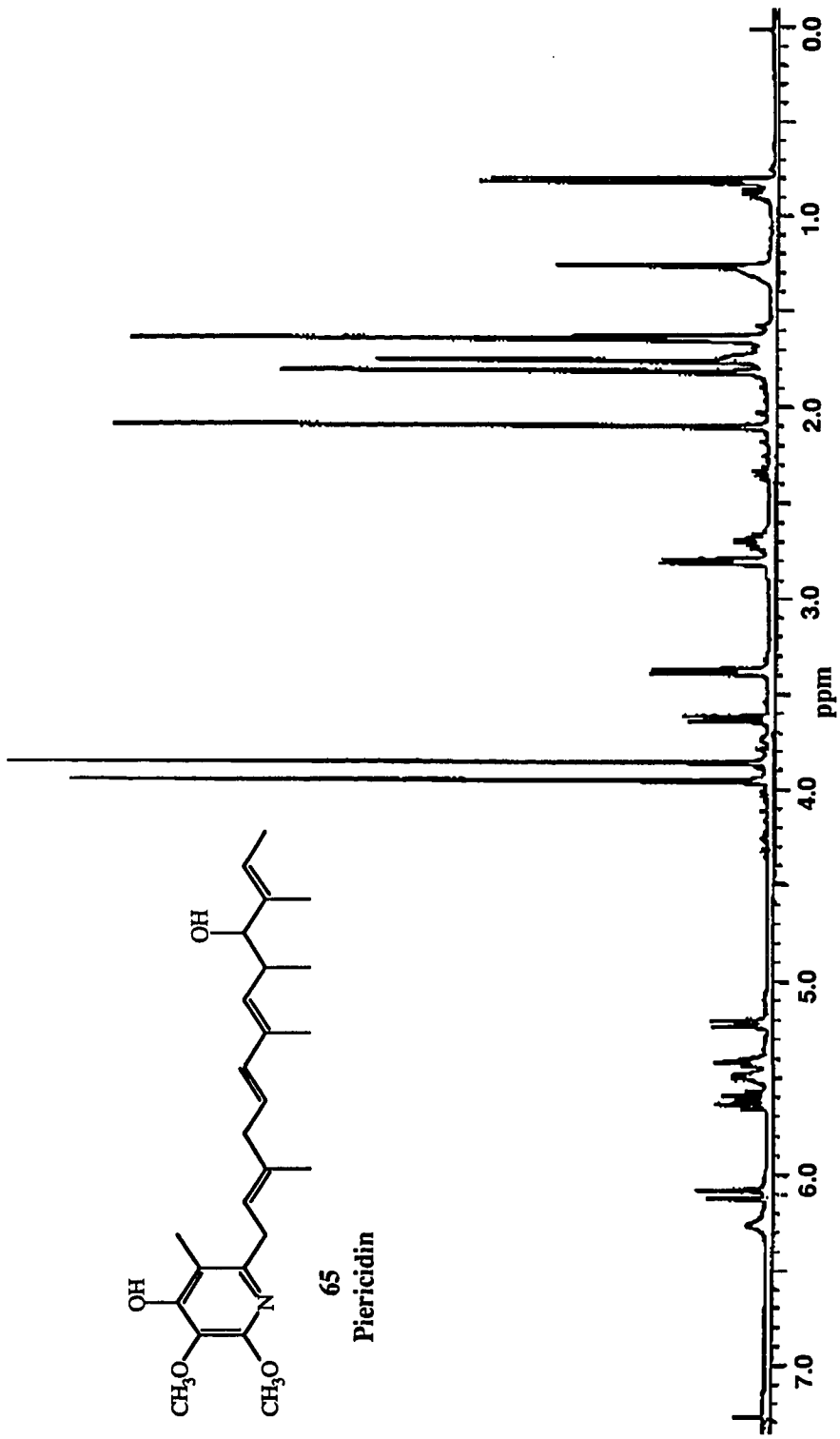


Figure 29. ^{1}H NMR spectrum of piericidin (65) in $CDCl_3$ at 500 MHz

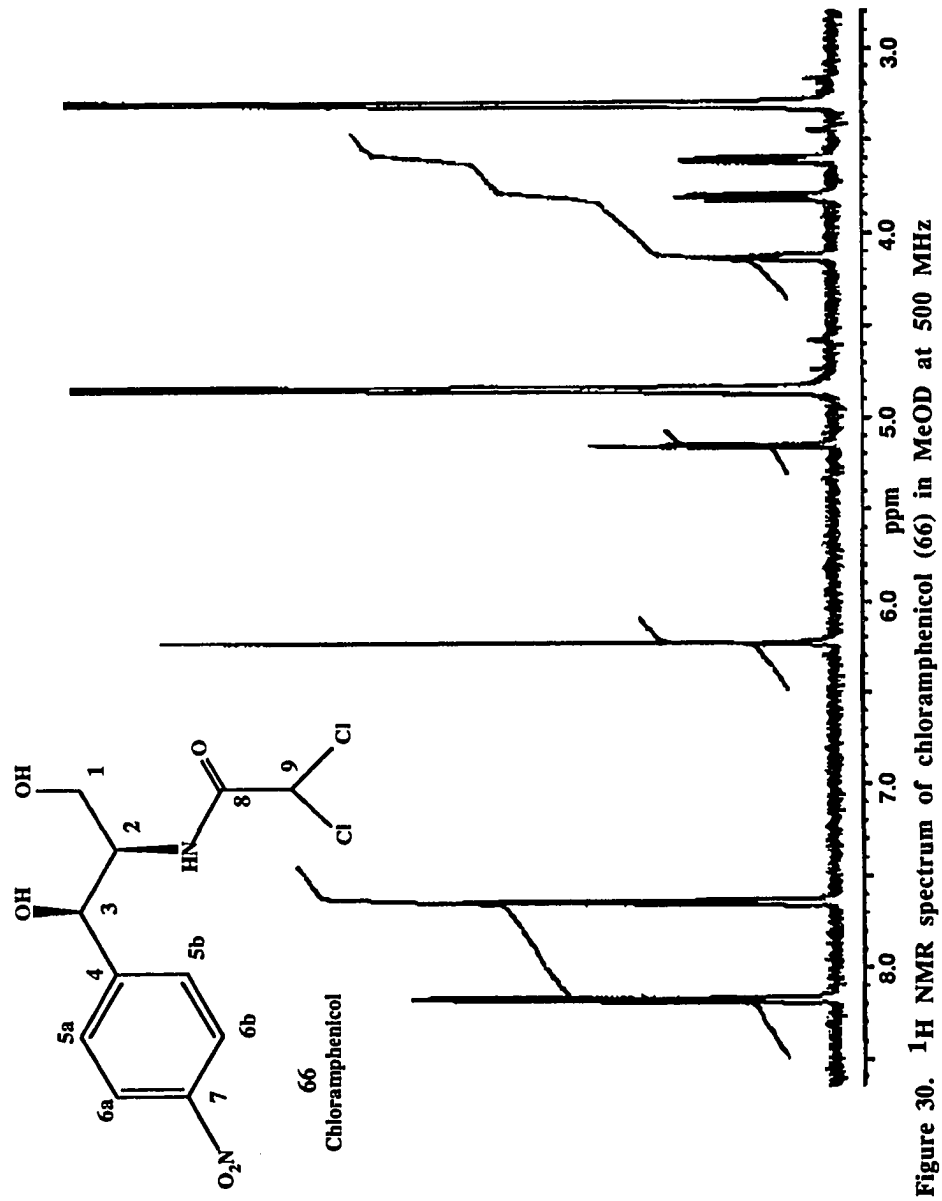


Figure 30. ^{1}H NMR spectrum of chloramphenicol (66) in MeOD at 500 MHz

group was indicated by the strong IR bands at 1518 and 1349 cm^{-1}, and the dichloroacetamide functionality was proved by a combination of 2-D NMR experiments, X-ray fluorescence to show the halogen was a chlorine, and analysis of the C-H coupling constant of C9 using the proton NMR spectrum, the $J_{C\text{-}H}$ value of 181 Hz corresponding to two chlorine atoms on one carbon. Final confirmation that the molecule was indeed chloramphenicol came from negative-ion high-resolution FABMS, yielding the formula (M^+-H) $C_{11}H_{11}N_2O_5Cl_2$. Because only one of the four possible isomers is active, the structure of compound **66** is most certainly as shown in Figure 30.

Chloramphenicol, the first broad-spectrum antibiotic discovered,[93] contains the rare combination of the nitrobenzene and a halogen in the structure. It acts by inhibiting 50S ribosomal protein synthesis in bacteria, with MIC values of 0.5 - 2.0 μg/ml against the strains for which it is used commercially. Chloramphenicol was first used to treat rickettsial infections, though it is no longer the treatment of choice. Side effects can include a form of aplastic anemia which is 50% fatal once detected, so chloramphenicol is only used for life-threatening infections in modern clinical settings. It remains the treatment of choice in life-threatening cases of typhoid fever, influenza, and meningitis, often in conjunction with ampicillin.[94] Synthetic routes are now used to produce **66** commercially.

Chloramphenicol is interesting in the context of marine science because it has been isolated from the moon snail, *Lunatia heros*, the only source other than an actinomycete ever referenced.[95] The compound may indeed be produced by the moon snail, but it is more likely of dietary origin or is being produce by associated bacteria. The significance of the latter would be that the compound is actually being produced in the marine environment in quantities suffcient for isolation. Previous

studies of amensalism have shown that *Trichoderma viride* produces extractable quantities of gliotoxin on wheat straw that has been added to soil during tillage.[96] Another study showed that gliotoxin was present in the seed coats of pea seeds sown in soil that contained *T. viride* naturally.[97] A completely different approach was used by Mazzola *et al.*,[98] comparing the ecological competence of phenazine-producing (Phz+) Pseudomonads and non-phenazine-producing (Phz-) mutants. The strains used, *Pseudomonas fluorescens* 2-79 and *P. aureofaciens* 30-84, were known to control the suppression of take-all in wheat by *Gaeumannomyces graminis* var. *tritici*, a natural wheat pathogen, through production of phenazine antibiotics in the rhizosphere. The study found reduced survival in the Phz- strains in natural soil and especially, in soil infested with *G. graminis* var. *tritici*. However, the Phz+ and Phz- strains competed equally well in sterilized soil. These results are cited as proof that the phenazine antibiotics are produced and active in the natural environment, and that antibiotic production increases the ecological competence of these strains. Though evidence like this is generally accepted as proof of antibiotic production and function in the environment, some scientists refuse to accept this as proof, demanding isolation of the antibiotic from the undisturbed environment and proof that natural concentrations inhibit surrounding microflora.[97] Given chloramphenicol's potency, the concentration in the snail (4 mg per 100 mg wet weight of minced snail) is surely enough to act as an antibiotic *in situ*. If this could be shown, it would be definitive proof that antibiotics can be active in the natural environment.

2) c. The Isolation and Dereplication of Nonactin (67) from Isolate CNB741

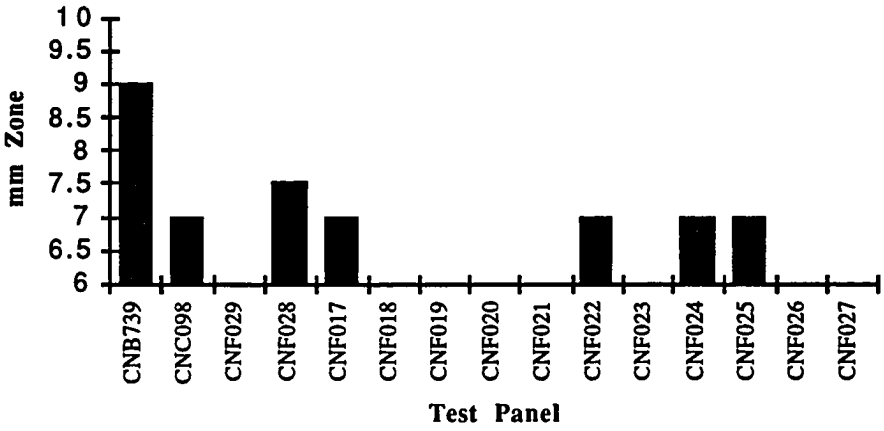


Figure 31. Activity profile of CNB741 crude extract

Though CNB741 showed little activity against the Batiquitos Lagoon test panel, it was still selected as a lead project, based on its interesting ^{1}H NMR spectrum. The extract also showed cytotoxicity at 0.3 μg/ml in the Bristol-Myers Squibb screening program.

CNB741 is an actinomycete isolated from shallow mud off the north shore of Batiquitos Lagoon. The 1 L screening extract was enough to dereplicate the active component using size exclusion chromatography and HPLC. The active fraction was shown to be the macrolide antibiotic, nonactin, by data comparison (see Figure 32) with ^{1}H NMR spectra of nonactin previously isolated and identified by Dr. H. Hagmann and with ^{13}C NMR data in the literature.[99] Nonactin (**67**), produced by several strains of *Streptomyces*, is made up of 4 units of nonactic acid (**68**), which is in turn biosynthesized from 2 acetate, 1 succinate, and 1 propionate unit.[100]

Nonactin by itself is biologically inert, but acts as a transmembrane ion carrier in

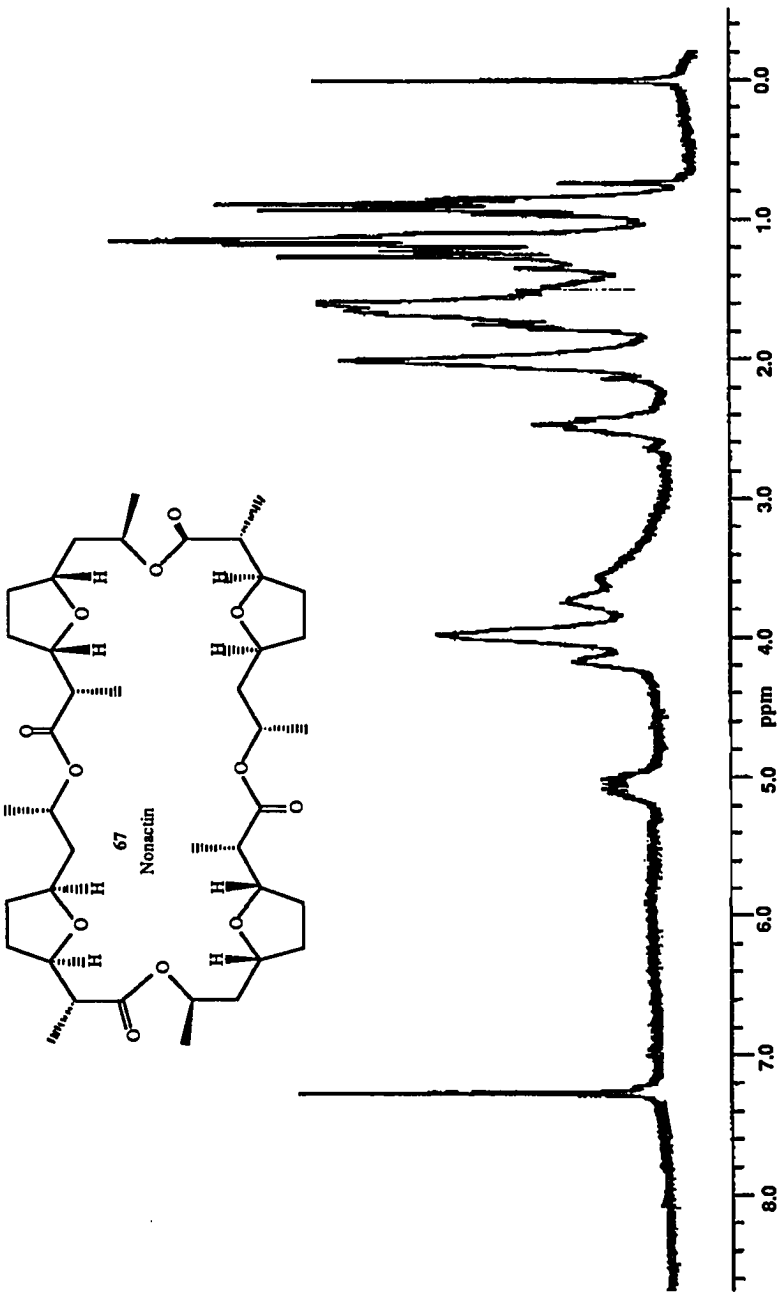
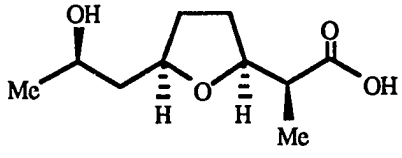


Figure 32. ^{1}H NMR spectrum of nonactin (67) in $CDCl_3$ at 200 MHz

the presence of Na^+ or K^+ ions. It is especially effective against Gram-positive bacteria. This argues for selection of projects of chemical interest even with mild activity, since pure nonactin is not very active without Na^+ or K^+ present but is highly antibacterial in combination with these ions. Another consideration is that highly active secondary metabolites may be so diluted by common metabolites, such as diketopiperazines, or by the antifoam used in large-scale culture, that only mild activity is seen in the screen. Several unicellular projects were followed for this reason.



(+)-(2S, 3S, 6R, 8R)
Nonactic Acid

68

2) d. <u>The Isolation and Dereplication of Valinomycin (69) from Isolate CNB761</u>

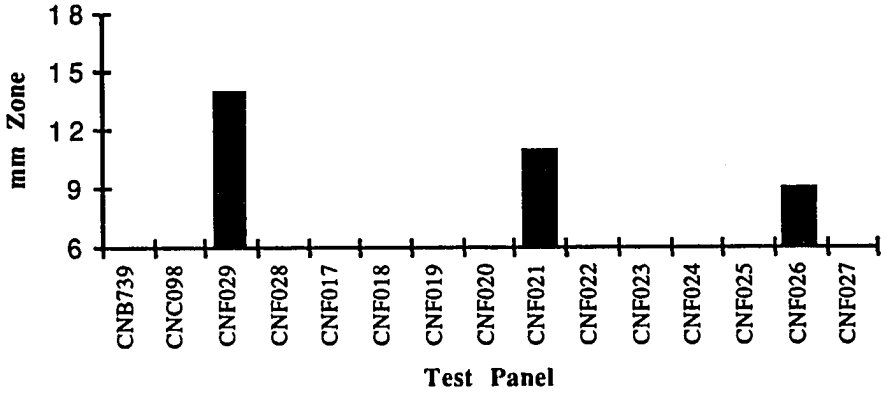


Figure 33. Activity profile of CNB761 crude extract

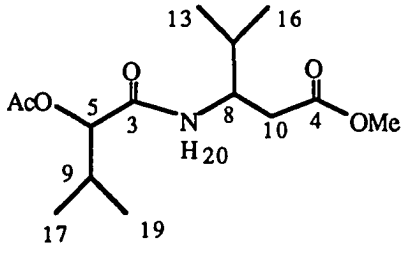
CNB761 was only tested against three test panel strains, but it was active against all of them. It also had several unique bands in the TLC, including a large

UV-active non-polar area. The ^1H NMR spectrum looked like there was a high concentration of one or two small compounds.

CNB761 is an actinomycete isolated from exposed mud on the south shore of Batiquitos Lagoon. A 4 L culture extract was used to dereplicate the antibiotic, valinomycin (**69**), using vacuum flash chromatography followed by HPLC and several reaction steps.

The ^1H NMR spectrum of the active fraction was deceptively simple (Figure 34). From the NMR data shown in Table 16, it was possible to piece together fragments **70** and **71**. With strong bands at 1656 and 1735 cm^{-1}, the IR data confirmed the presence of amide and ester bonds in the molecule. At this point, the only mass spec data available (low-resolution EI and CI) showed a false molecular ion at m/z 526. This made the linking of the fragments impossible without further experiments.

Changing NMR solvents cleared up one ambiguity, the signal at δ 1.26 ppm in the ^1H NMR spectrum was a solvent impurity, and its weak correlation to the proton on C8 in the COSY experiment was simply noise. A methanolysis of the esters yielded the compounds shown below:



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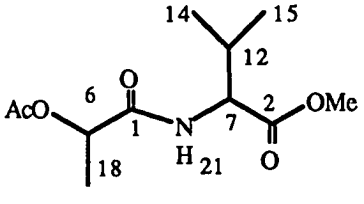

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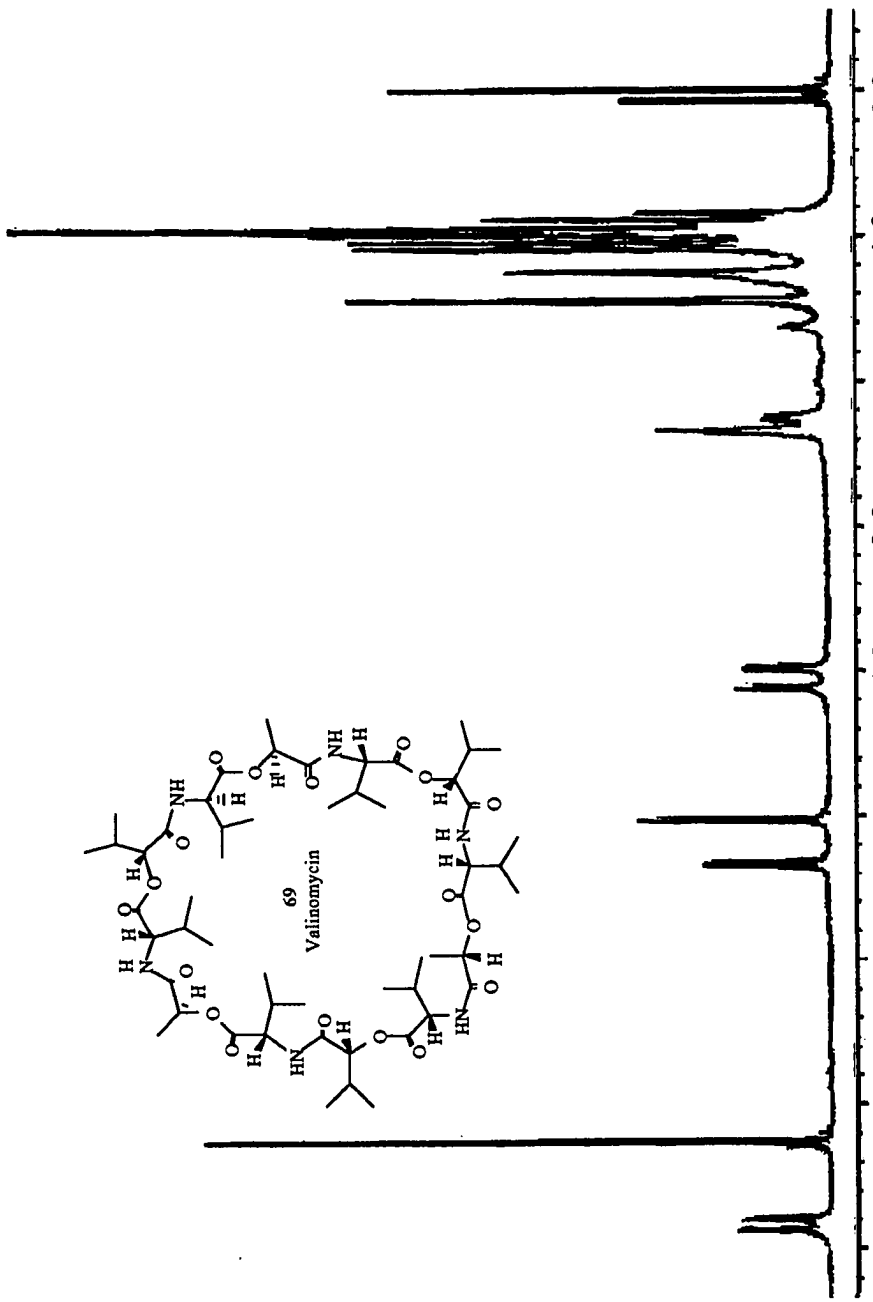
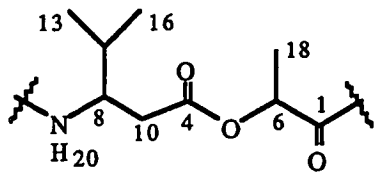
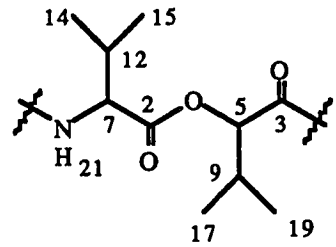


Figure 34. ^{1}H NMR spectrum of valinomycin (69) in $CDCl_3$ at 500 MHz



Fragment 70



Fragment 71

Table 16. NMR data for compound 69[a,b]

C#	$\delta^{13}C$ (ppm)	DEPT	$\delta^{1}H$ (ppm)	mult, J (Hz)	COSY	HMBC
1	172.4	C				
2	171.7	C				
3	170.7	C				
4	170.0	C				
5	78.5	CH	5.02	d, 2.9	9	2, 3, 9, 17, 19
6	70.2	CH	5.32	q, 6.3, 6.8	18	1, 4, 18
7	60.4	CH	3.99	dd, 9.8, 6.4	21, 12	2, 12, 14, 15
8	58.8	CH	4.14	dt, 7.3	20, 11, 10	4, 11
9	30.2	CH	2.34		8, 5, 16[c]	14, 15, 8
(10)	29.7	CH_2	1.26	t, 7.3	8	
11	28.5	CH	2.34		8, 5, 16[c]	14, 15, 8
12	28.3	CH	2.26		7, 14, 15	14, 15, 7
13	19.6	CH_3	0.99			
14	19.4	CH_3	1.07	d, 6.8	12	7, 12
15	19.4	CH_3	0.96	d	12	12
16	19.1	CH_3	1.03	d, 6.4	11	8, 13, 11
17	19.0	CH_3	0.98	d		5, 9, 19
18	17.1	CH_3	1.45	d, 6.3	6	1, 6
19	16.6	CH_3	0.97	m		17
20(N)			7.85	d, 8.3	8	8
21(N)			7.76	d, 5.9	7	7

[a] All ^{1}H experiments were done at 500 MHz in $CDCl_3$ at room temperature. [b] All ^{13}C experiments were done at 50 MHz in $CDCl_3$ at room temperature. [c] COSY and HMBC data may be interchanged.

Structures were assigned based on NMR data and confirmed with unambiguous EIMS results. Finally, high-resolution FABMS data, (M^++H) *m/z*=1111, were combined with these results to yield the structure **69**. Compound **69** proved to be valinomycin after a CAS on-line search on the molecular formula $C_{54}H_{90}N_6O_{18}$. The formula could not be derived from the FABMS data earlier, since greater than 100 matches were listed in the peak match results.

Valinomycin was discovered in 1955[101] and synthesized in 1963.[102] The antibiotic depsipeptide acts by complexing with alkali metal ions, especially K^+, Rb^+, and Cs^+, to enhance their transport across membranes, including mitochondrial, erythrocyte, and chloroplast membranes. Valinomycin has been important in the chemical study of cyclopeptides. It was the first biologically-important peptide whose spatial structure was not established by X-ray analysis.[103] Ovchinnikov and Ivanov found that the solution structure is an equilibrium of 3 major conformers: A) six hydrogen bonds forming six condensed 10-membered cycles; B) triple H-bonding forming a propeller-like conformation, more flexible than A; and C) no H-bonding in the 36-membered ring. In the final conformer, the structure is actually an equilibrium mixture of a large number of energetically similar conformers. Another interesting point is that the symmetry of the molecule is so complete that there is no splitting of signals in the ^1H NMR even at -95 °C.[104]

2) e. <u>The Isolation and Dereplication of Fumitremorgin C (74) and Bisdethiobis(methylthio)gliotoxin (75) from Isolate CNF895</u>

CNF895 showed activity against only 3 Gram-negative panel strains, but these 3 zones were large enough that it was considered activity worth pursuing. The TLC showed unique bands in the non-polar region, and the crude ^1H NMR looked very

interesting in the olefinic and aromatic regions.

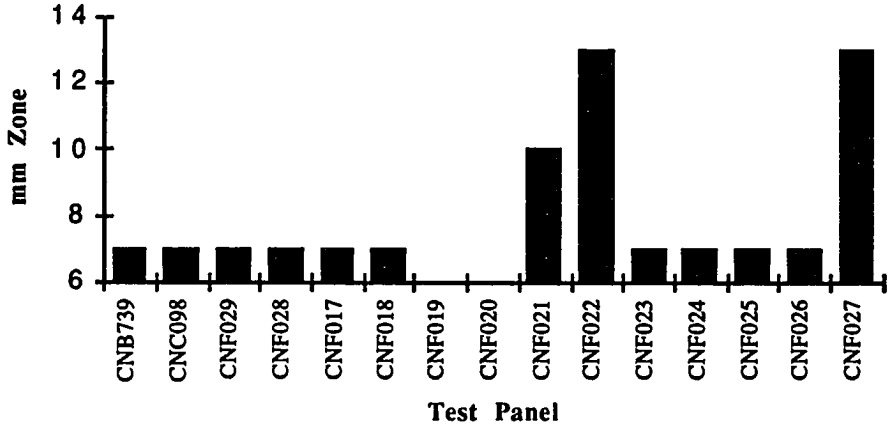


Figure 35. Activity profile of CNF895 crude extract

CNF895 was originally thought to be an unicellular bacterium. However, microscopic analysis revealed large, nucleated cells, indicating CNF895 was actually a fungus. It was cultured in the 1 L scale using B-1 media. The extract of the cell and broth mixture was separated by flash chromatography followed by HPLC of one of the fractions to yield 5.2 mg (0.9% of crude) of fraction 4-3-3 and 1.3 mg (0.2% of crude) of fraction 4-3-2. Both of these fractions were spectroscopically identical to fumitremorgin C (**74**). Identification of fumitremorgin C was based on the complete structure elucidation of 4-3-3 using 1-D and 2-D NMR techniques and low-resolution EIMS, followed by an on-line search of the structure in the CAS database. Fractions 4-3-2 and 4-3-3 were separated by HPLC as 2 distinct peaks, and they showed 2 distinct peaks when mixed for EIMS. Given this separation, epimers at C12 or C3 were suspected. However, a comparison of the ^{1}H NMR assignments of the various epimers in Table 17 reveals that both fractions (Figures 36 and 37) appear to be 12α,3α fumitremorgin C.

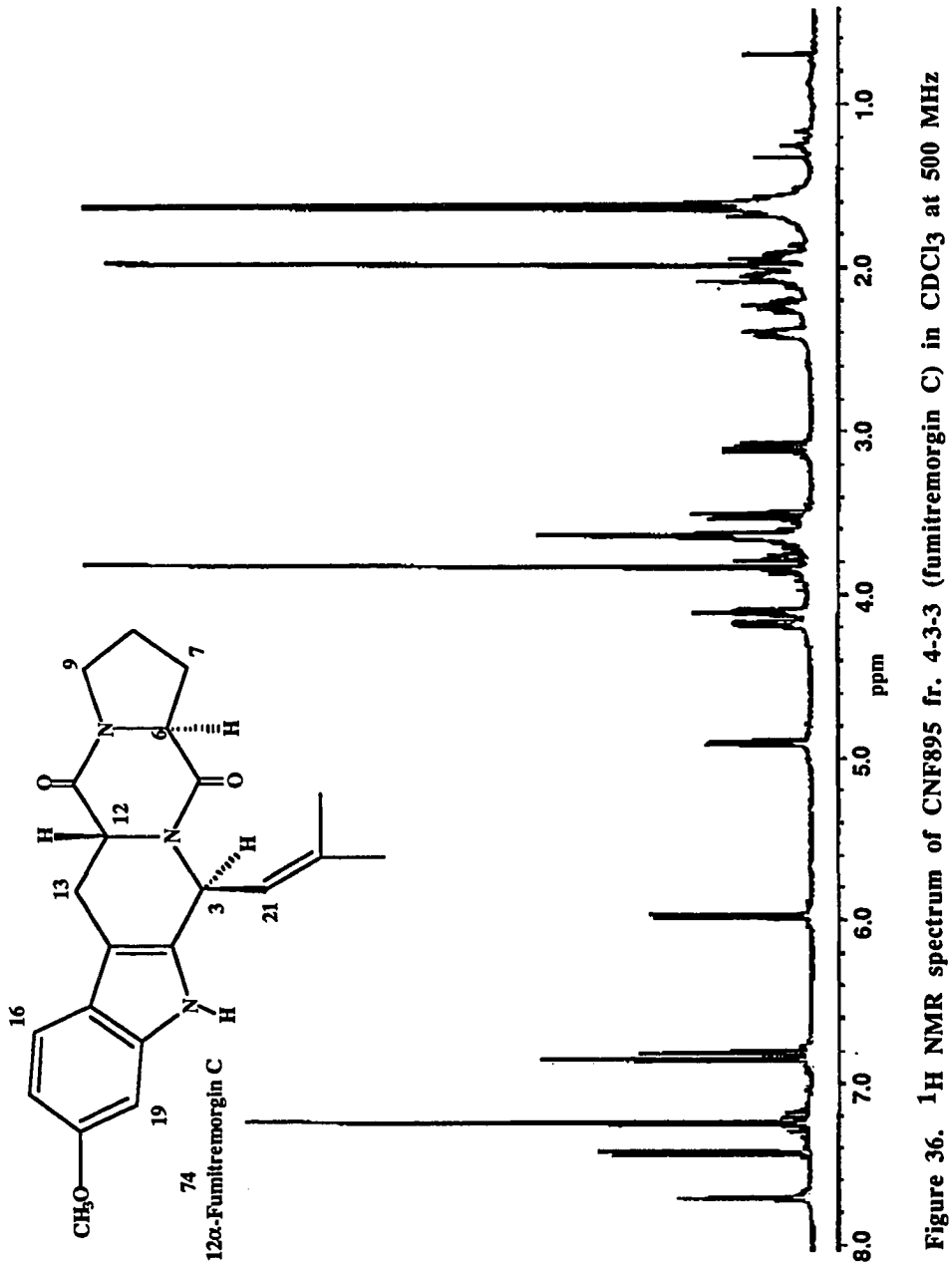


Figure 36. ^{1}H NMR spectrum of CNF895 fr. 4-3-3 (fumitremorgin C) in $CDCl_3$ at 500 MHz

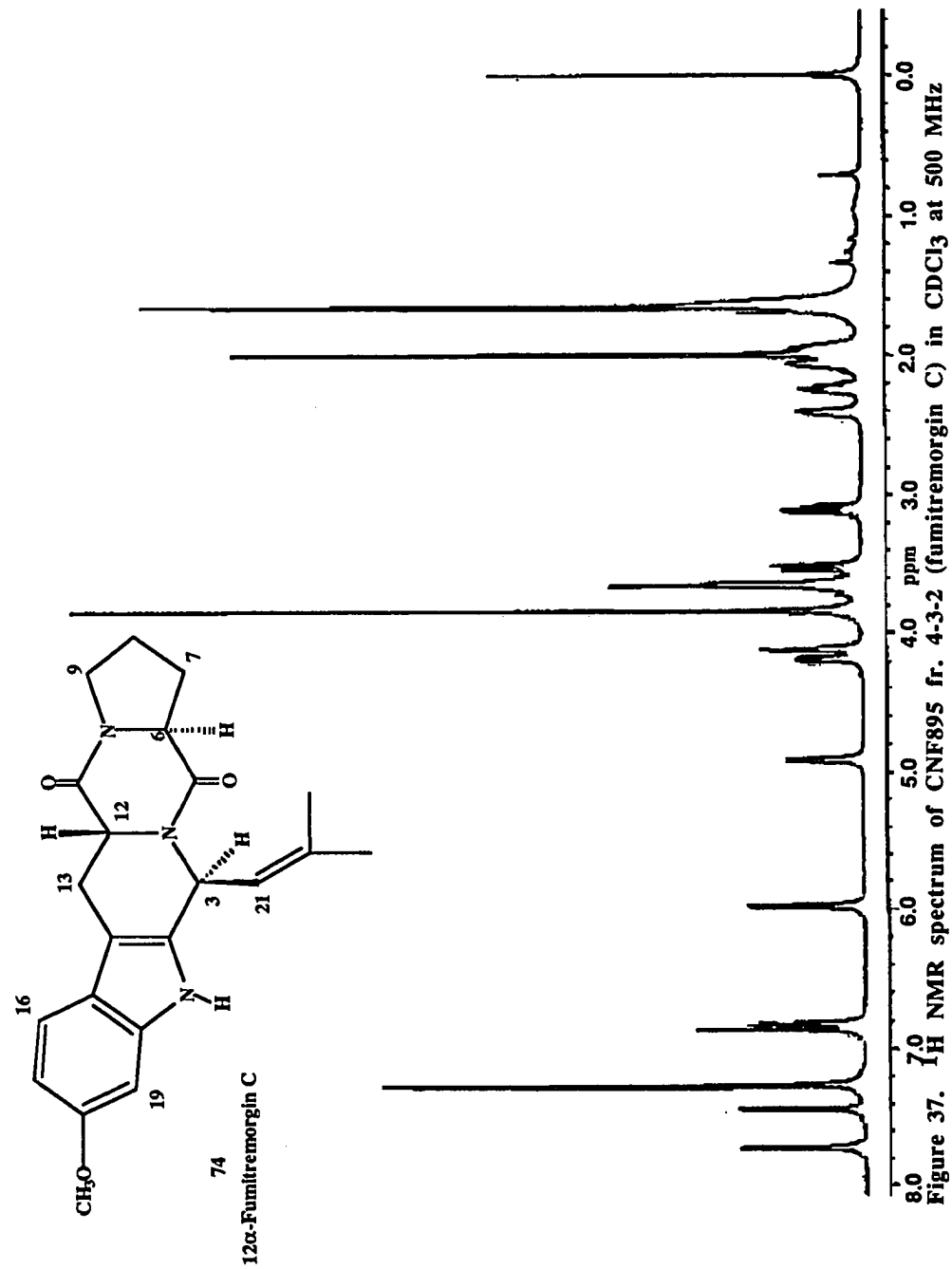


Figure 37. ^1H NMR spectrum of CNF895 fr. 4-3-2 (fumitremorgin C) in $CDCl_3$ at 500 MHz

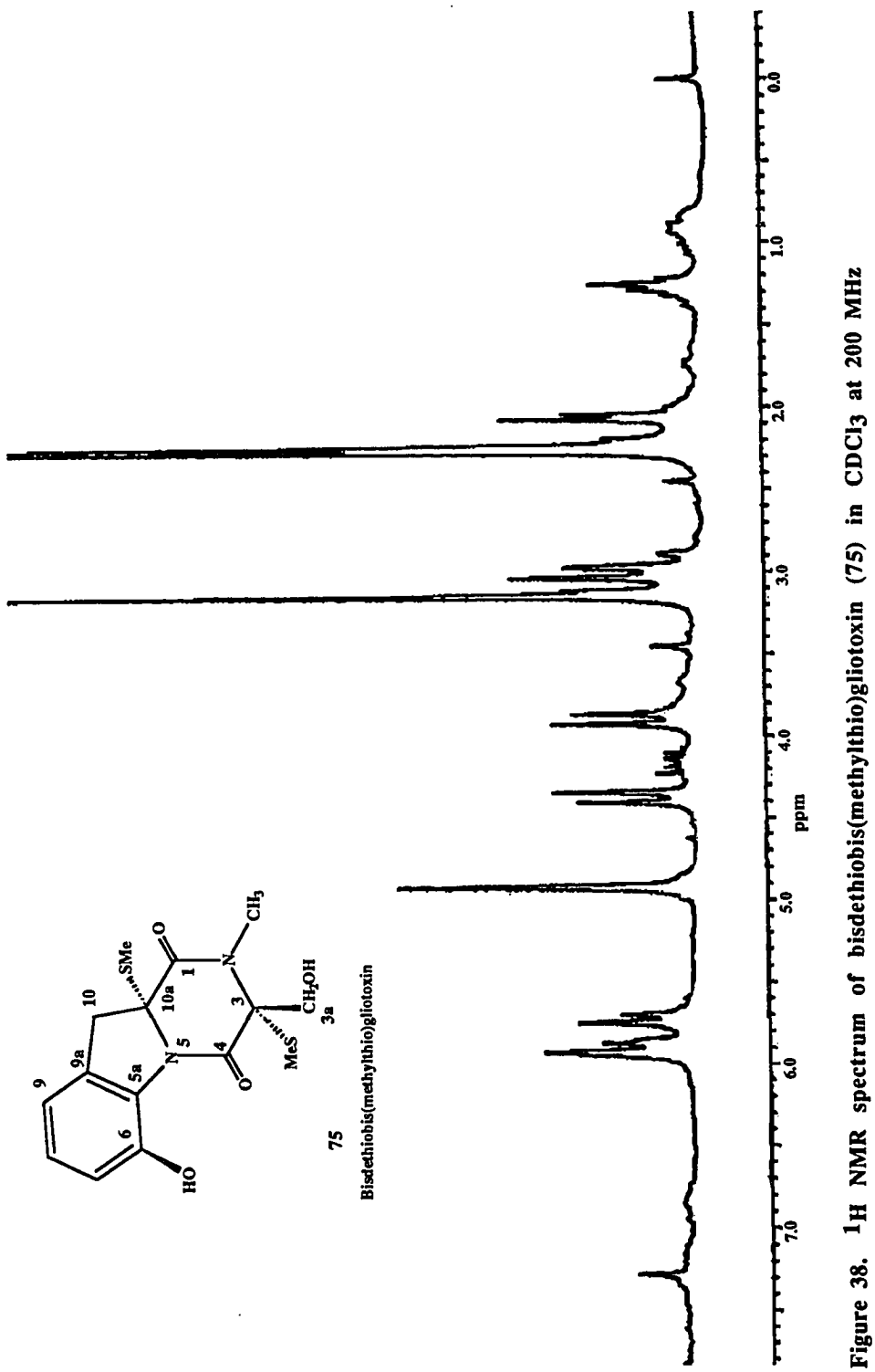


Figure 38. ^{1}H NMR spectrum of bisdethiobis(methylthio)gliotoxin (75) in $CDCl_3$ at 200 MHz

Table 17. ^{1}H NMR shifts of fumitremorgin C and its epimers

Epimer	12α, 3α (74)	12β, 3α	12α, 3β	12β, 3β	4-3-2	4-3-3
C12a	~ 4.15	4.40	4.44	4.20	4.19	4.19
C3a	5.98	6.44	6.38	5.54	5.89	5.89

Fumitremorgin C, first isolated from *Aspergillus fumigatus* Fres. in 1977,[105] elicits tremors in vertebrate animals. The stereochemistry at C3 and C12 were determined by synthetic routes.[106] The fumitremorgin-verrucalogen group is one of four groups of tremorgenic mycotoxins, all having in common the tryptophan-derived indole. No reports of isolation of **74** from a *Penicillium* species were found, though *Penicillium, Aspergillus, and Claviceps* are the 3 genera known to produce tremorgenic mycotoxins.[107]

One other flash column fraction looked like it would warrant further investigation. In comparing this fraction with the compounds which were reported to be co-produced with **74**, the spectral characteristics were found to be similar to gliotoxin. However, there were 2 extra methyl signals and differences in the shifts of the protons on C3a. Further comparison with literature ^{1}H NMR data confirmed this structure as bisdethio-bis(methylthio)gliotoxin (**75**).

Sulphur-containing dioxopiperazines like gliotoxin have been implicated in the virulence of *Candida*, a major cause of death in immuno-compromised human hosts.[108] Bisdethiobis(methylthio)gliotoxin (**75**) acts as a specific inhibitor of the platelet activating factor (PAF). Gliotoxins have been isolated from many different genera of fungi, including *Aspergillus* ,[109] *Candida* ,[108] *Dichotomomyces* ,[110] and as in this case, *Penicillium* .[109] The biosynthesis of gliotoxin followed by conversion to **75** has been studied in a species of *Gliocladium.*

One chemical point of interest in the study of gliotoxin compounds is the large coupling of the hydroxyl proton and the methylene (3a) protons through the oxygen atom, J = 9.5 and 6.1 Hz in gliotoxin.[110] This coupling is not seen in the methylene protons in **75**, the signals being a 4.39 ppm doublet (J = 13 Hz) and a 3.90 ppm doublet (J = 13 Hz) (see Figure 38).

2) f. The Isolation and Dereplication of Ergosterol (**76**) and Ergosterol Peroxide (**77**) and the Partial Structure Elucidation of Unknown A from Isolate CNC100

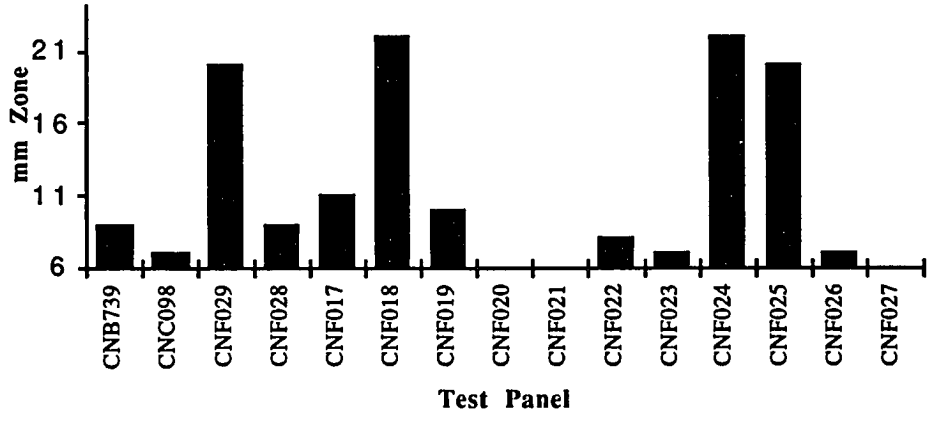


Figure 39. Activity profile of CNC100 crude extract

The crude extract of CNC100 showed large zones of inhibition against several test panel strains, and it had many unique TLC bands compared to the other isolates. The ^{1}H NMR spectrum had interesting, but weak signals from 4.0 ppm to 9.0 ppm.

Isolate CNC100 is a cycloheximide-resistant fungus obtained from exposed mud on the south shore of Batiquitos Lagoon. The culture was grown on several scales, with and without shaking. However, the majority of the elucidation work

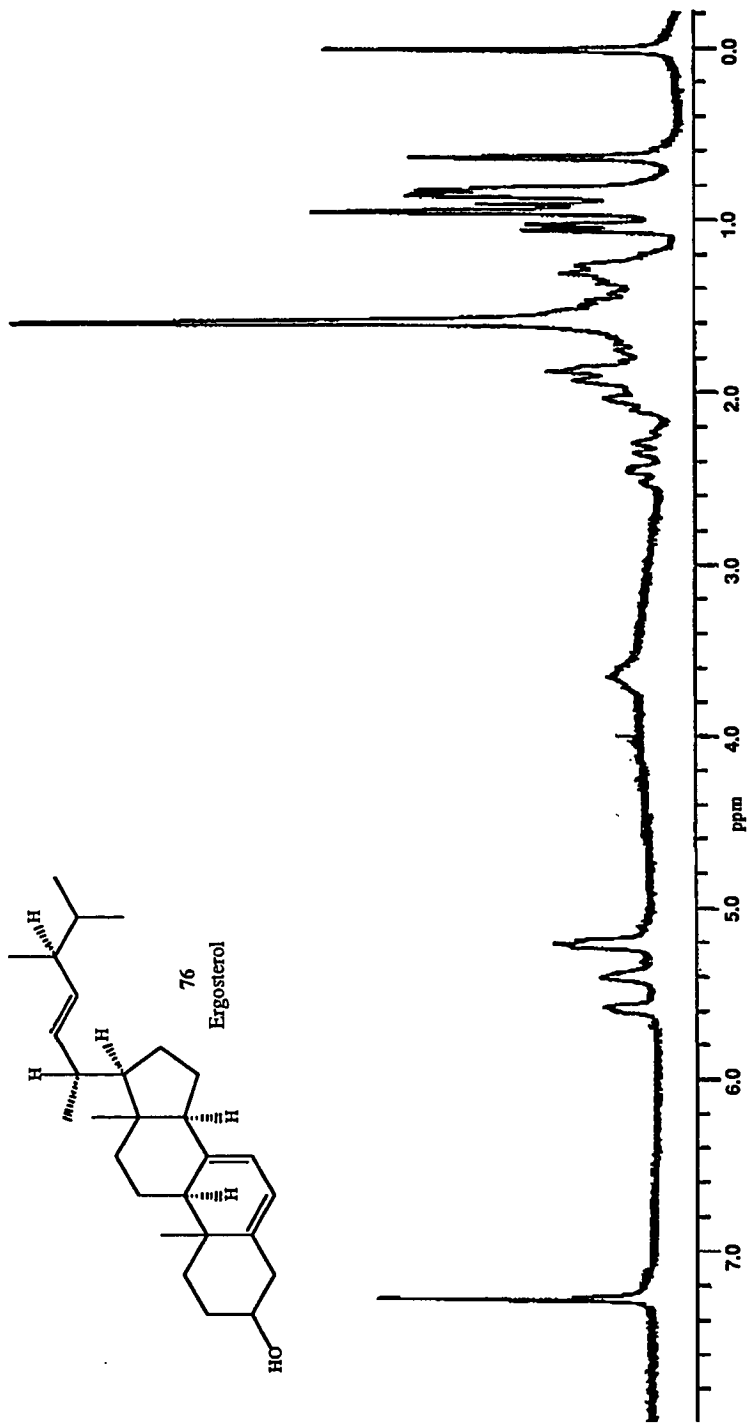


Figure 40. ^{1}H NMR spectrum of ergosterol (76) in $CDCl_3$ at 200 MHz

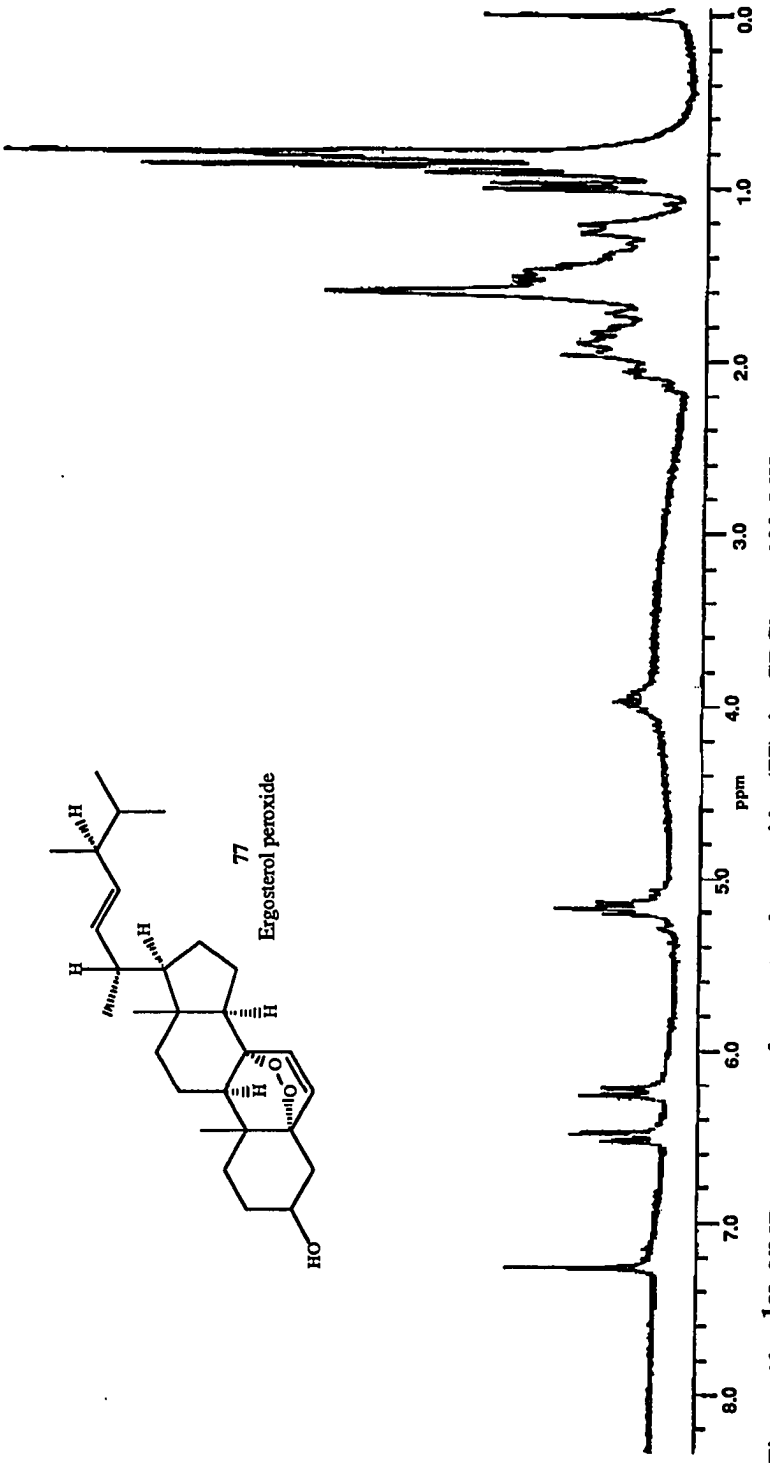


Figure 41. ^{1}H NMR spectrum of ergosterol peroxide (77) in $CDCl_3$ at 200 MHz

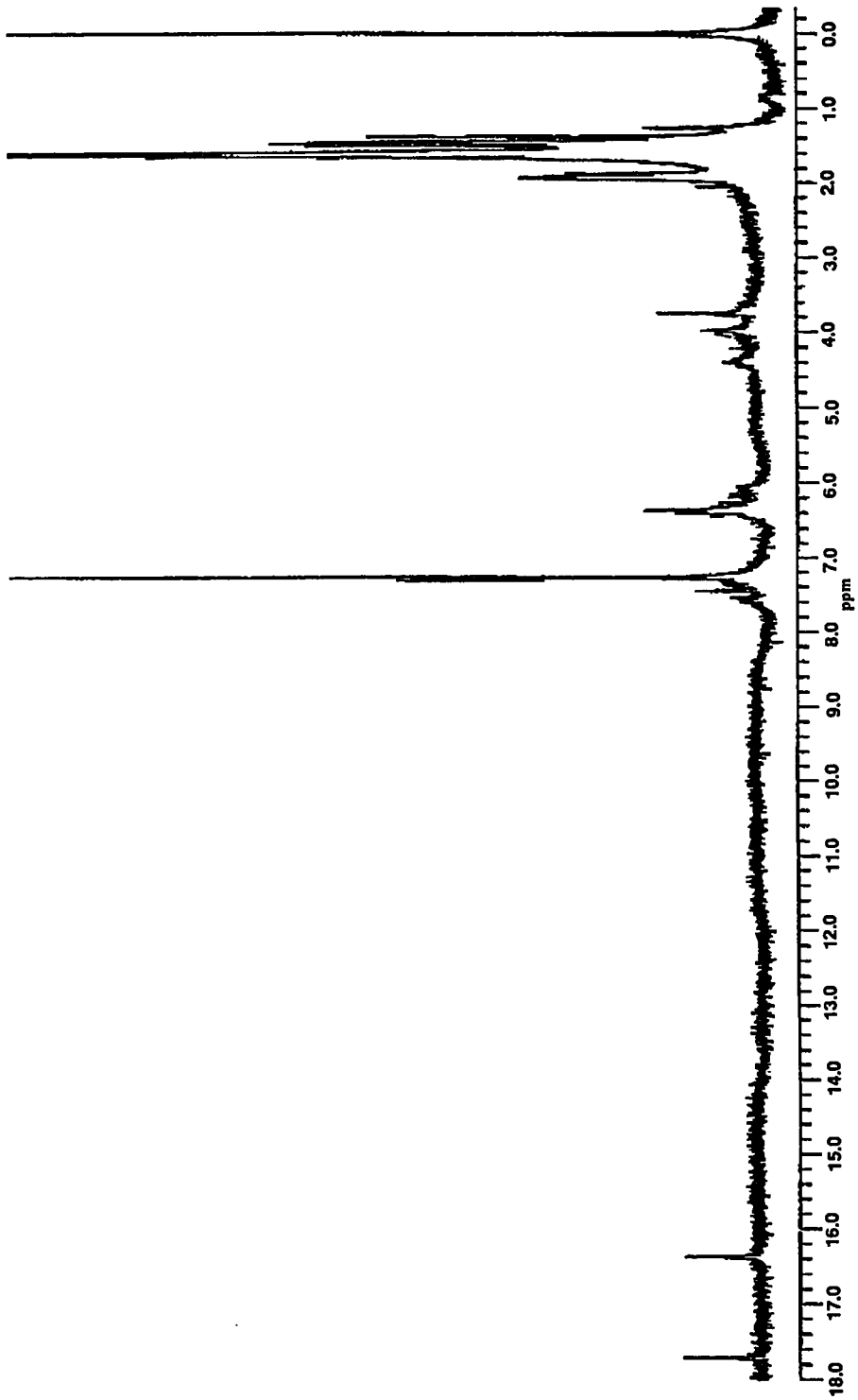
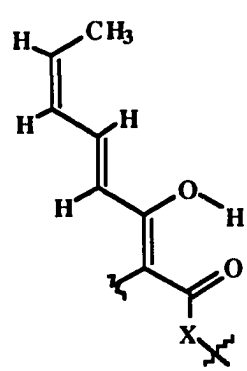


Figure 42. ^{1}H NMR spectrum of unknown A from CNC100 in $CDCl_3$ at 200 MHz

was done on the original 1 L screening culture. The original extract yielded 14.4 mg of compound A, along with ergosterol (**76**) and ergosterol peroxide (**77**). Previous to the isolation of the sterols, isolate CNC100 was thought to be an actinomycete (aka CNB747) rather than a fungus. At this point, the strain was sent to Professor Jan Kohlmeyer's lab for taxonomic identification, where it was determined that CNC100 is a *Penicillium* species.

Upon addition of D_2O, the resonances at 0.4 and 0.9 ppm in the original ^1H NMR spectrum of unknown A both disappeared. Exchangeable protons are not common in this region, so it was suspected that they were wrapped around and actually have chemical shifts below the sweep width limit used (14 ppm). In fact, these signals were found at 16.4 and 17.8 ppm, as shown in Figure 42! Strong hydrogen bonding was needed for protons to move to such low-field resonances. Extended conjugation was indicated by the UV absorbance at 365 nm. The partial structures shown in Figure 43 were constructed based on the NMR data shown in Table 18. The enol form of the ß-dicarbonyl accounts for the downfield shift of 16.4 ppm, and this partial structure also fits the UV data very well, using the UV absorbance calculations described in Scott.[111]



UV Absorbance Calculation:

215	Base carbonyl value
30	} 3 conjugated double bonds
30	
30	
10	a-alkyl group
18	g and higher alkyl groups
30	ß-hydroxyl group
363	calculated absorbance
365	observed

Unfortunately, a large part of the structure of compound A consists of quaternary carbons which show no correlations in the 2-D NMR experiments. Two approaches could be taken to try to see further couplings. The first approach was simple derivatization to be able to observe correlations through the hydroxyl-bearing carbons. Approximately 5 mg of the unknown was dissolved in ether and reacted with diazomethane. After 1 hour, at least 4 bands were seen by TLC analysis, and the only product isolated in sufficient yield for acquisition of a ^{1}H NMR spectrum showed methylation at several of the double bonds. Due to the instability caused by multiple unsaturations, the next proposed step was to reduce the olefins to increase stability and attempt the structure elucidation. However, the compound decomposed in storage, probably due to light sensitivity. More compound was needed to proceed.

The second approach would be to isolate sufficient compound (200 mg) for an INADEQUATE (C-C correlation) experiment. Simple scale-up to 10 - 20 L was expected to afford this amount. As was the case with several projects, the compound was produced in much lower yield upon regrowth. Another approach was needed.

One proven method to yield more or different compounds from fungal cultures is to change the aeration rate. When CNC100 was grown in shaking and static cultures (all other variables constant), a very small amount of the original compound was produced by both cultures, as detected by TLC and diode-array HPLC. However, a second, more polar, yellow compound with the same UV spectrum was produced in good quantity in the static culture.

The second compound appears to be more stable than the original unknown, but a large growth (>40 L) will be required to produce enough compound for chemical

Figure 43. Proposed partial structures of unknown A

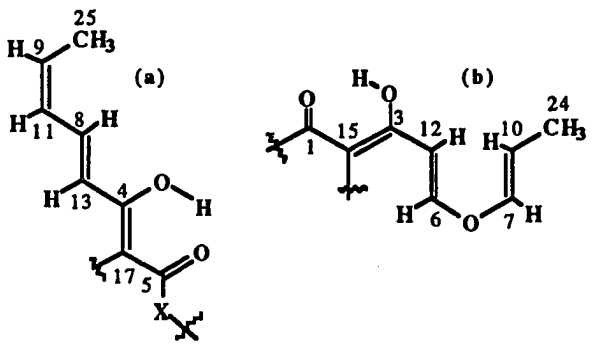


Table 18. NMR data for CNC100-A[a,b]

C#	δ ^{13}C (ppm)	DEPT	δ ^{1}H (ppm)	Mult	J (Hz)	COSY	HMBC (6 Hz)
1	199.8	C					
2	191.2	C					
3	185.6	C					
4	170.3	C					
5	163.6	C					
6	148.5	CH	7.57	dd	15, 9.8	12, 24, 7	3, 12
7	144.2	CH	6.36	d	8.3	6	6
8	139.7	CH	7.32	dd	15, 11	11, 13	4
9	137.7	CH	6.14	m	7	11, 25	8
10	131.3	CH	6.35	m		24	
11	131.0	CH	6.35	m		8, 9	13
12	121.8	CH	7.39	d	15	6, 24	3, 10, 6
13	120.0	CH	6.42	d	16	8	4, 8, 11
14	111.0	C					
15	107.2	C					
16	104.1	C					
17	99.8	C					
18	79.8	C					
19	79.0	C					
20	59.8	C					
21	54.4	CH	3.77	s		24?	1, 2, 4, 5, 17, 18, 20, 22, 24?
22	25.7	CH_3	1.47				
23	23.0	CH_3	1.39	s			16, 19
24	19.2	CH_3	1.91	d	5.4	6, 10, 12	7, 10
25	18.8	CH_3	1.88	d	6.4	9	9, 11, 13
26	18.6	CH_3	1.47				
27	7.0	CH_3	1.47	d	6.4		2, 5, 14
		OH	17.78	s			3, 12, 15
		OH	16.35	s			4, 13, 17
		XH	6.62	br			
		OH	4.75	br			
		OH	4.46	br			

[a] All ^{1}H NMR experiments, including HMQC, COSY, and HMBC, done at 500 MHz in $CDCl_3$ with TFA vapor added, room temperature. [b] All ^{13}C NMR experiments done at 125 MHz in $CDCl_3$ with TFA vapor added.

work. These compounds show potent antibiotic activity against several of the test panel strains, and the extremely low-field proton chemical shifts are very intriguing. Also, the partial structure (a) showed no matches when searched in the CAS on-line database. Therefore, this project appears worthy of the effort required for such a large-scale fungal culture.

2) g. The Isolation and Dereplication of a Tryptophan Derivative (78) from Isolate CNF917

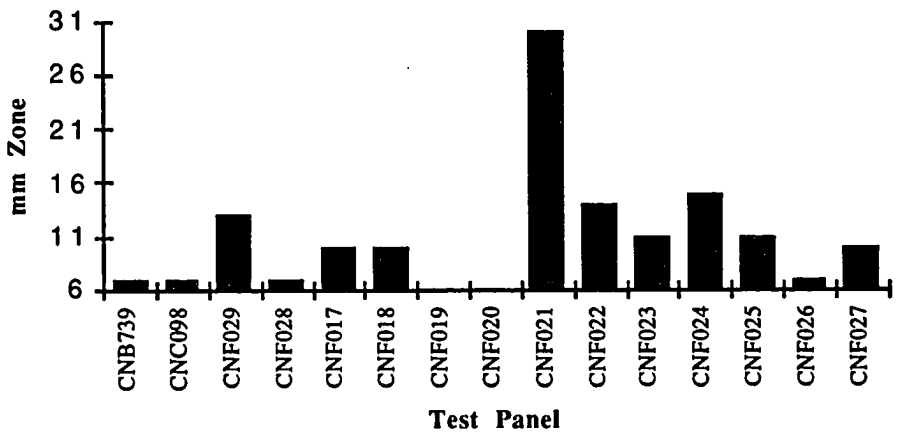


Figure 44. Activity profile of CNF917 crude extract

Isolate CNF917 showed very good activity against both Gram-negative and Gram-positive strains, so it was selected as a lead project. However, the TLC showed only a polar compound and the ^{1}H NMR sample was too dilute to see much except the largest media signals.

Isolate CNF917 is a Gram-negative, swarming, rod-shaped bacterium obtained from a sediment sample taken at a depth of 7 feet in the Batiquitos Lagoon. It was cultured in 100 ml for screening and 4 L scale for isolation work. Fractionation of

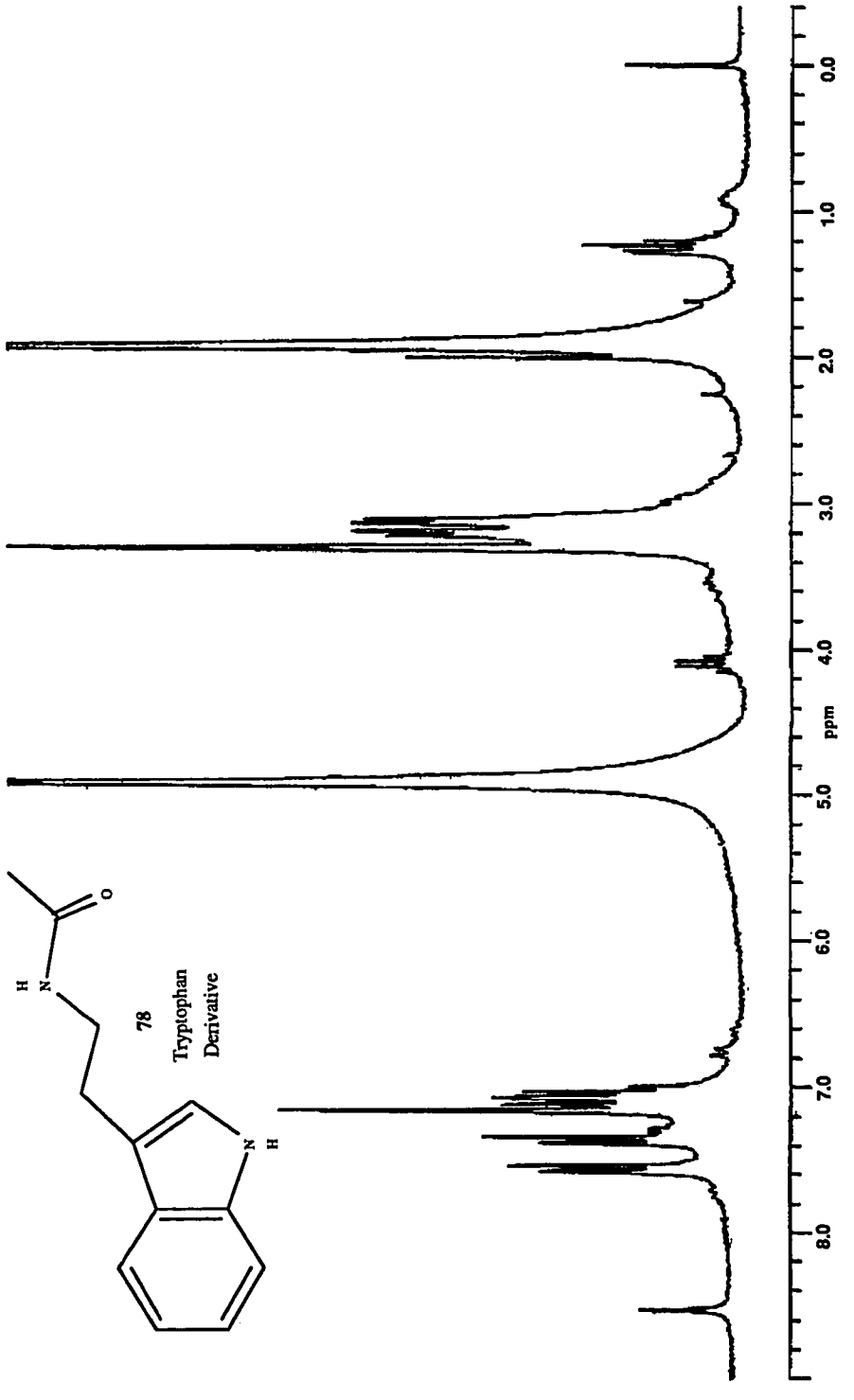


Figure 45. ^{1}H NMR spectrum of tryptophan derivative (78) from CNF917 in MeOD/$CDCl_3$ at 200 MHz

the 4 L EtOAc extract by size exclusion chromatography yielded 26 mg of an active fraction that consisted of at least 5 compounds by TLC and ^{1}H NMR. With this small amount, the activity was not pursued further, although the potent activity would warrant further study if large-scale growth and extraction operations for unicellular bacteria were available. Before a large-scale culture, the separated, freeze-dried cells should be extracted to see if a more polar compound or a compound that is stored inside the cells is responsible for the activity. A later fraction from the LH-20 column showed several signals in the aromatic region of the ^{1}H NMR spectrum and a deep purple-charring TLC band at the baseline on a silica plate in EtOAc. Upon purification by thick-layer plate, the compound was determined to be an N-acetyl derivative of tryptamine based on the ^{1}H NMR and ^{13}C NMR spectrum. The ^{1}H NMR spectrum of the impure fraction is shown in Figure 45. The ^{13}C chemical shifts included an acid signal at 177 ppm, indole signals, and three non-indole shifts at 41.2, 24.6 and 22.7 ppm. Compound **78** was interesting because it was a common, inactive secondary metabolite of many cultures in the Fenical lab collection.

2) h. The Isolation and Dereplication of Phe-hyPro Diketopiperazine (79) and the Isolation of Unknown B from Isolate CNF816

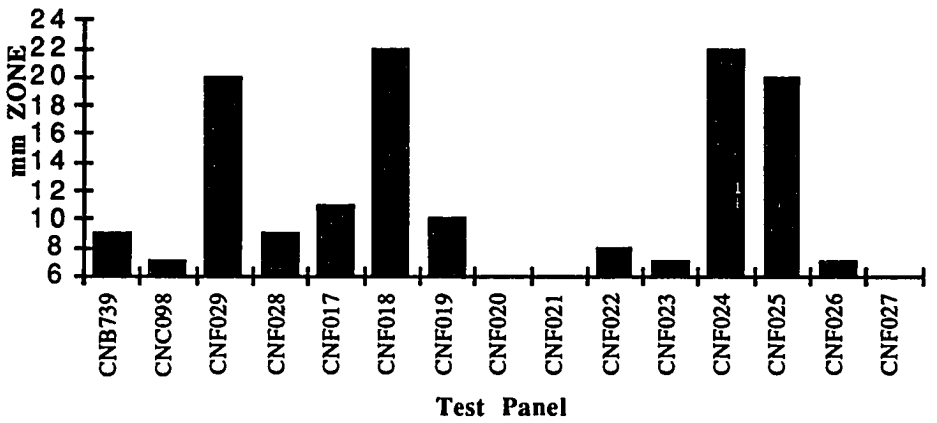


Figure 46. Activity profile of CNF816 crude extract

Isolate CNF816 produced one of the most active extracts from unicellular bacteria, with activity against six test panel strains. The TLC showed a long, brown-charring streak above the baseline, but the ^{1}H NMR showed nothing unusual.

Isolate CNF816 is a Gram-negative bacterium isolated from a sediment sample taken at a depth of 2.5 feet in the Batiquitos Lagoon. It was cultured in 100 ml for screening and 4 L scale for isolation work. Fractionation of the 4 L EtOAc extract by vacuum flash chromatography resulted in isolation of the activity in the most polar fraction. This fraction was further purified by size exclusion chromatography, then normal-phase HPLC, yielding 0.9 mg of the active fraction (Unknown B) and 2.7 mg of Phe-hyPro diketopiperazine (**79**).

Because the active compound(s) was in the most polar fraction, a regrow was requested with separation of the cells and broth to isolate the activity. The regrow

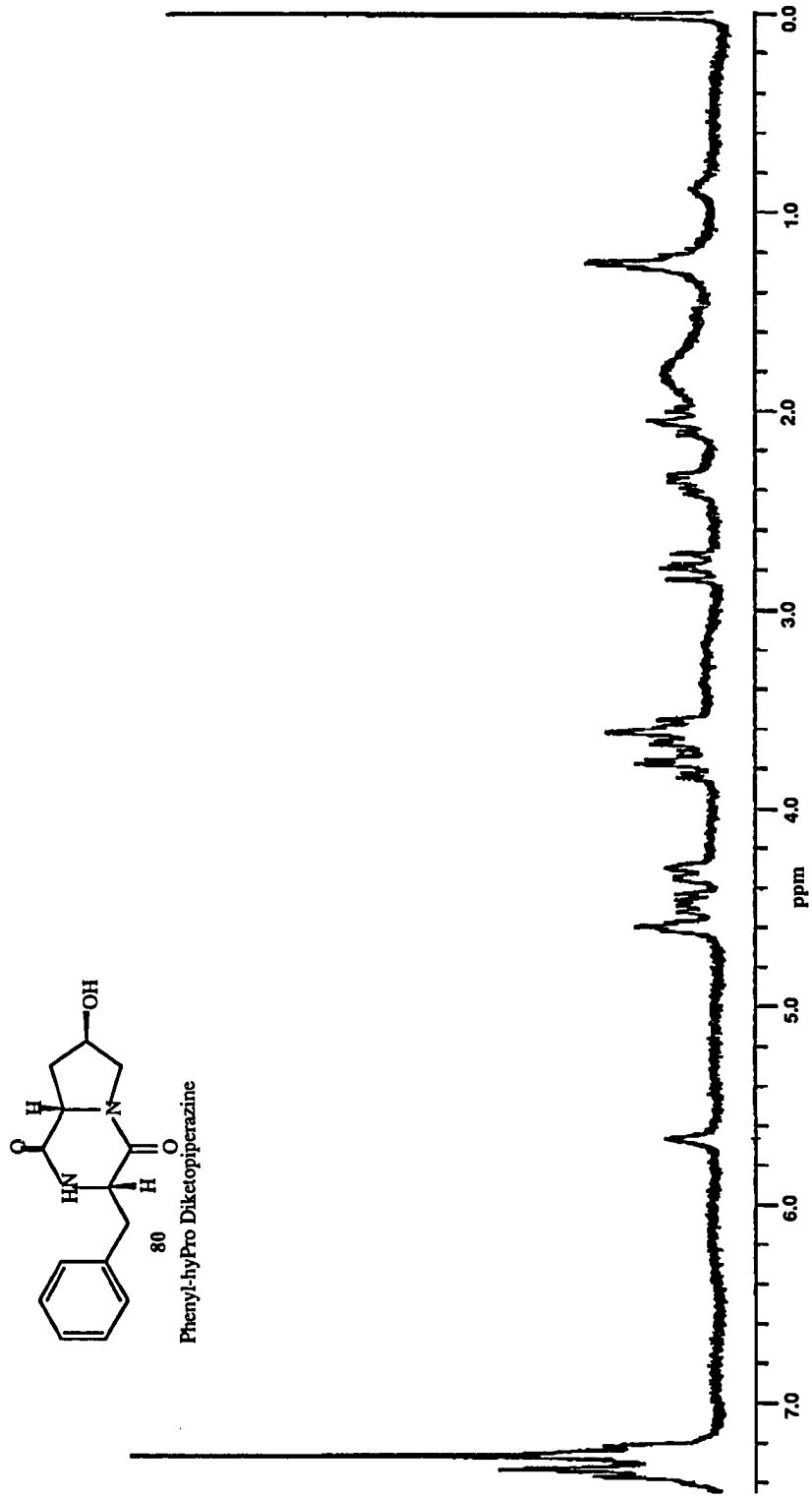


Figure 48. ^{1}H NMR spectrum of phe-hypro diketopiperazine (80) in $CDCl_3$ at 200 MHz

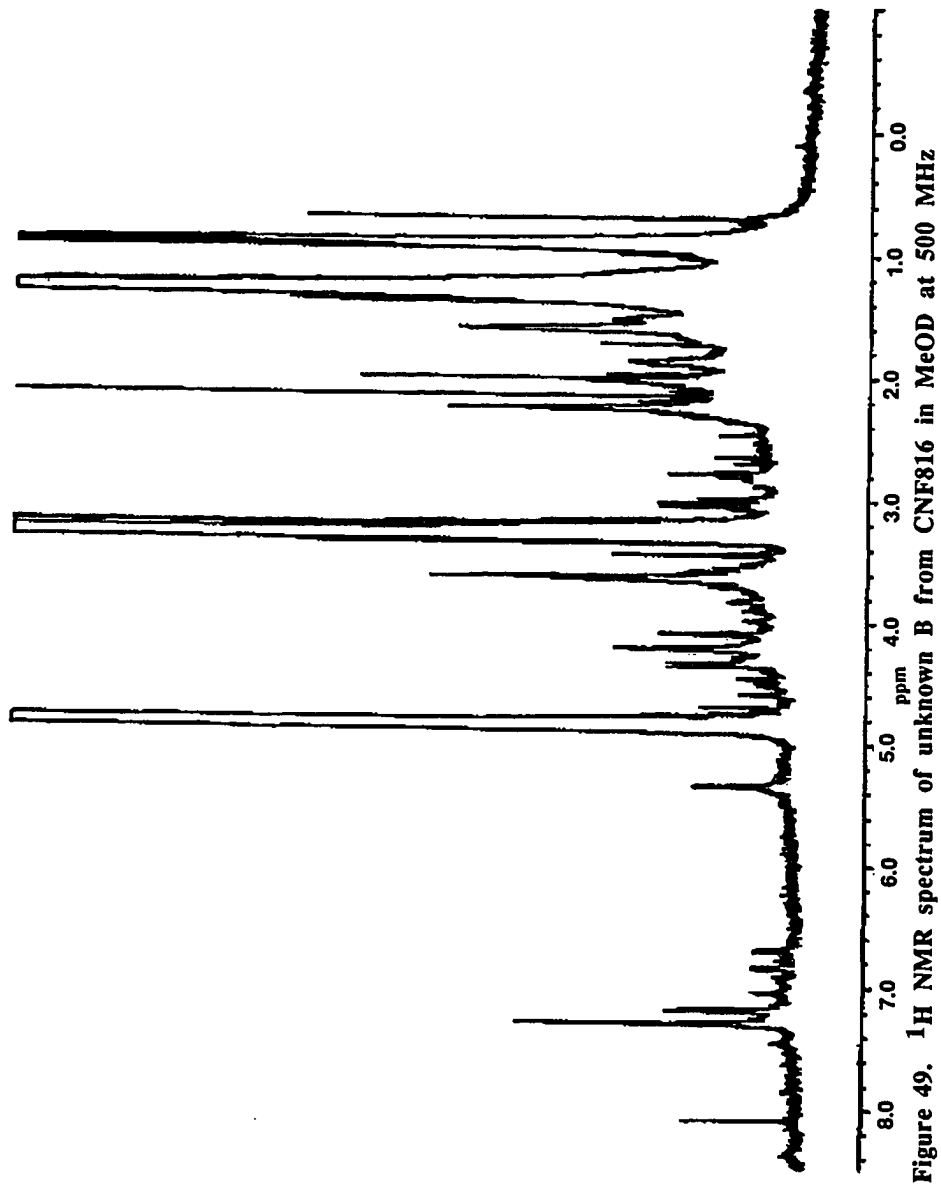


Figure 49. ^{1}H NMR spectrum of unknown B from CNF816 in MeOD at 500 MHz

extract was received nine months after being requested, effectively too late to risk working on such a low-yield project. However, all of the activity was isolated in the freeze-dried cells at a test concentration of 5 mg extract/ml. This makes the project worthy of further study, since the biologically-active compound may be in feasible concentration in the cell extract.

The diketopiperazine (**79**) was mentioned as a natural product only once before in the literature, from a sponge.[112] It is possible that the sponge, an undescribed Jaspidae species, is producing **79**, but it is much more likely that a bacterium is the source. This argument is based on the fact that nearly all of the cultures screened in the Fenical lab contain diketopiperazines, while they are not commonly isolated from sponges. Previous work by Stierle,[113] indicated that a certain bacterial isolate was a symbiont of the sponge *Tedania ignis*, because the bacterium produced a diketopiperazines (Ala-Pro, Val-Pro, and Leu-Pro) which had been previously isolated from the sponge.[114] At least two alternative hypotheses must be offered in light of this prevalence of diketopiperazine production: 1) the diketopiperazines are of dietary origin. Because sponges filter feed, bacteria are digested continuously, giving the sponge access to the diketopiperazines a bacterium had produced. As the bacteria are digested, these diketopiperazines may be sequestered and stored by the sponge; or 2) the diketopiperazines isolated from the sponge were actually isolated from the extract of the many different species of bacteria which were present in the sponge at the time of collection, but which are not necessarily associated with the sponge except as a chance food source. Virtually any of these bacteria would have been able to produce these compounds in culture, given the proper media.

3) Antibiotic Compounds from Belizean Mangrove Isolates

The second site selected for an antibiosis study was in Belize, Central America, in the Turneffe Islands. Samples were taken in a grid pattern from the mangrove embayment, and from a variety of other identifiable habitats, as shown in Figure 50. Each sample was serially diluted, and the 10^{-2} to 10^{-6} dilutions were spread on B-3/ agar plates with cycloheximide added. The 10^{-2} to 10^{-4} dilutions were heated for 1 hr. at 40°C to select for spore-formers, then spread on A-1/agar plates with cycloheximide added. Colonies began to show up on these plates after only 24 hours, and actinomycetes after about 5 days. The colonies were then isolated from each of the dilution plates containing 1-100 colonies based on colony morphology. Between 8 and 10 colonies were selected from each dilution series in most cases, yielding 288 isolates from the unheated and five additional filamentous strains from the heated samples. Over 100 of these isolates were lost during isolation, leaving a total of 94 unicellular and 26 filamentous bacteria and one fungus.

Once transferred to a slant, each isolate was Gram tested and described. Test panel selection was based on the same criteria as the lagoon project test panel: 1) it should contain representatives from each taxonomic group; 2) it should consist of the most commonly isolated bacteria for the unicellular bacteria; and 3) it should reflect the same Gram-negative:Gram-positive ratio as the isolates. This ratio was approximately 7:3, so the test panel selected was as shown in Table 19.

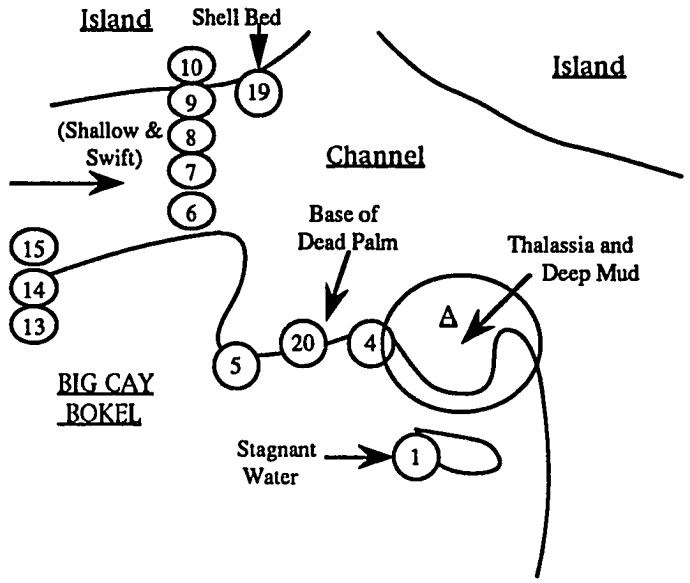


Figure 50. Big Cay Bokel, Belize Collecting Site

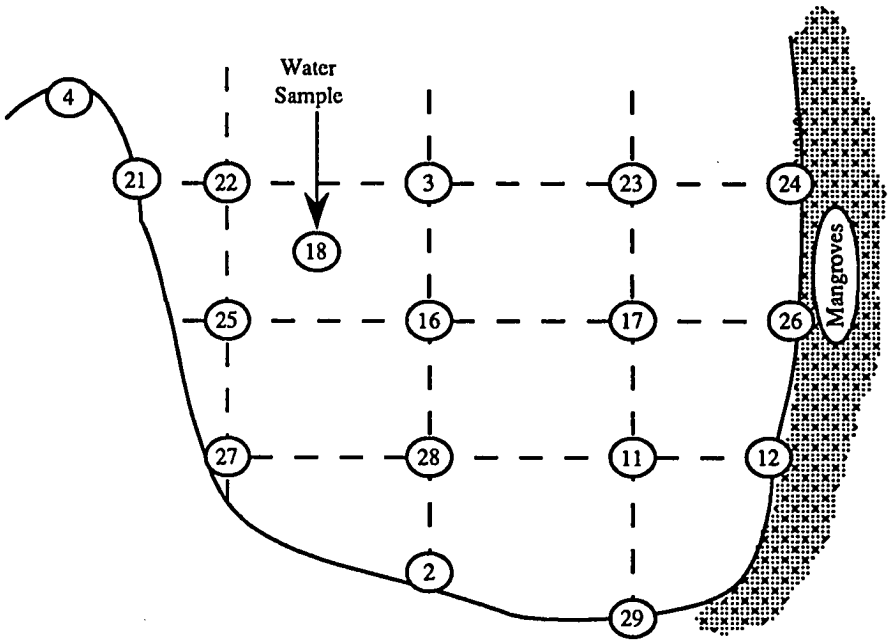


Figure 50a. Expansion of Mangrove Embayment Site

Table 19. Big Cay Bokel project test panel

Strain #	Gram (±)	Colony Description
CNB774	+	Beige-brown, dug into agar, white spores
CNC102	NA	Beige, round, dull, pasty, spores (fungus)
CNF939	-	Beige, swarmer, irregular, lobate, flat
CNF940	-	Yellow-brown, swarmer, irregular, lobate
CNF941	-	Cream, small, slightly convex, dent in center
CNF942	-	White, irregular, lobate, shiny, elevated
CNF943	-	Beige, small, translucent, entire, round
CNF944	-	Salmon, round, entire, translucent edges
CNF945	+	Beige-yellow, irregular, lobate, flat
CNF946	-	Beige/tan center, irregular, lobate, translucent
CNF947	+	Cream, irregular, lobate, punched into agar
CNF948	+	Beige, round, lobate, flat, dull, pasty

After isolation, each culture was grown either on 1 L (actinomycetes and fungi) or 100 ml (unicellulars) scale, then double extracted with EtOAc. The extracts were then dissolved in 1 ml of solvent and screened for activity against the test panel using the paper disk technique. An antibiotic "profile" was recorded for each isolate. Any extract showing activity (≥ 8 mm zones) against 2 or more test panel strains was then subjected to chemical screening. This included TLC analysis on a normal-phase silica plate in EtOAc and ^1H NMR in $CDCl_3$/MeOD. Any interesting projects, from a biological or chemical standpoint, were regrown if needed for identification of the active compound(s).

3) a. Overall Results of Microbial Isolation, Activity Testing, and Search for Antibiotic Compounds

The Gram testing results once again supported the findings of Jensen and Fenical,[80] with Gram (+) strains comprising 31% of the sediment isolates. However, the activity results were much different than the Batiquitos Lagoon results. Of the non-filamentous bacteria, 56.4% of the isolates had ≥8 mm zones of inhibition against two or more members of the test panel. The percentage of active actinomycete extracts (57.7%) was 10% less than those of the lagoon actinomycetes. Once again, the Gram test result had no correlation to activity; 57.1% of the Gram (+) isolates and 56.1% of the Gram (-) isolates were active.

These increased percentages of active extracts may be explained by increased test panel sensitivity compared to the lagoon test panel. However, the actinomycete extracts showed a decreased activity. Therefore, this explanation could only be true if the test panel members are more sensitive to compounds produced by unicellular bacteria, but not by actinomycetes. One such compound is indole. Indole chars yellow to red with acid at an RF of ~0.75 on the silica TLC in EtOAc, and many unicellular extracts show this TLC band. In contrast, actinomycete extracts infrequently produce indole in detectable quantities. Another factor supporting this argument is that CNF944, a Gram (-) test panel strain, was killed by 70% of the extracts, and CNF923 was killed by 44%. Without these two strains on the test panel, the percent of non-filamentous extracts which were active falls to 23%, while the actinomycetes only fall to 54%. These percentages are closer to the results found in the Batiquitos Lagoon study.

The 68 active extracts were screened by TLC and ^{1}H NMR and leads were selected. The antifungal agents, staurosporine and cycloheximide, could be

dereplicated with the screening extracts, and 17 other extracts were chosen as lead projects based on the combination of the biological and chemical screening. No activities were lost when regrown. One novel compound, *cis*-cascarillic acid, was identified from these leads, along with several known compounds and a partial identification of an unknown antifungal compound. Also, three of the lead extracts were dropped upon dereplication to anthracycline antibiotics by Bristol-Myers Squibb. The complete or partial identification of the novel compound, dereplication of the knowns, and the significance of their isolation follows. A ^{1}H NMR spectrum of all of the known compounds is included for use in future dereplication.

3) b. The Isolation and Structure Elucidation of *cis*-Cascarillic Acid (**81**) from Isolate CNF994

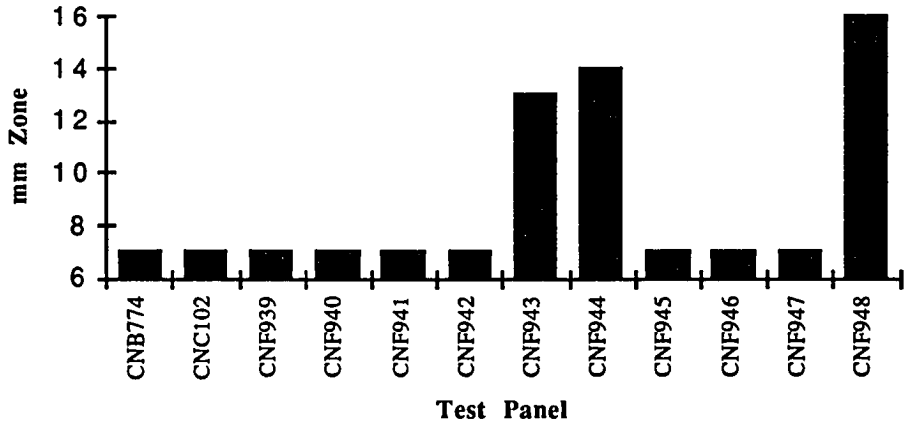


Figure 50. Activity profile of CNF994 crude extract

Isolate CNF994 was selected as a lead project, because the extract produced large zones of inhibition against 3 isolates, a good activity for a unicellular bacterium. The TLC also looked interesting in the non-polar region, and the ^{1}H NMR spectrum had faint signals above the TMS standard.

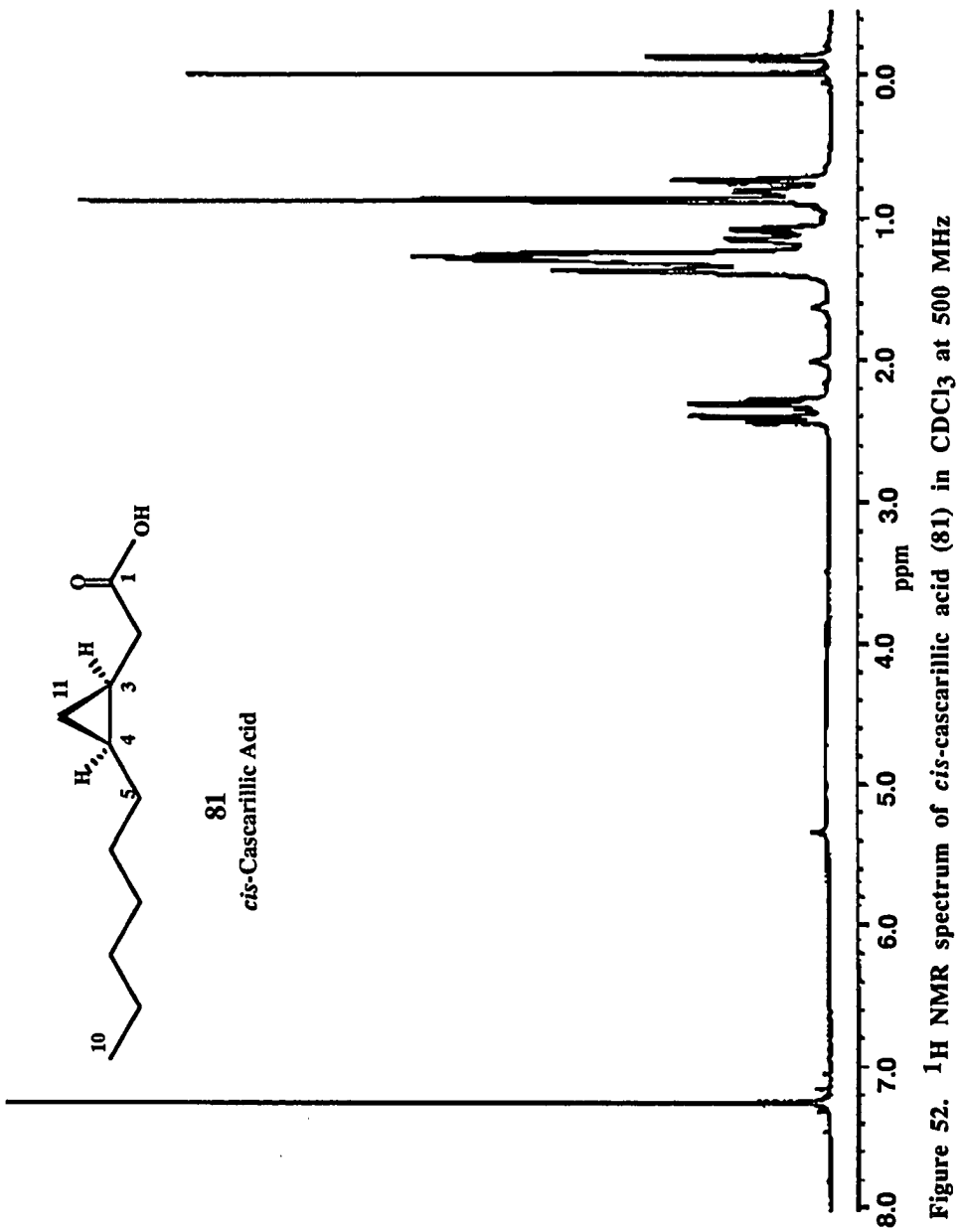


Figure 52. ^{1}H NMR spectrum of *cis*-cascarillic acid (81) in $CDCl_3$ at 500 MHz

Isolate CNF994 is a unicellular bacterium obtained from a sediment sample taken in 1.5 feet of water in a mangrove embayment on Big Cay Bokel. It was cultured in 100 ml for screening and subsequently on a 4 L scale. *cis*-Cascarillic acid (**81**) was purified from the 4 L extract by vacuum flash chromatography followed by normal-phase HPLC. It was found to have potent activity against the 3 test panel strains at 1 mg/ml:

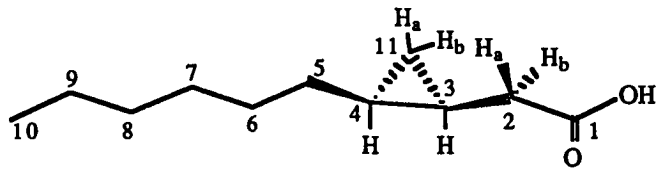
Table 20. Activity of 81 at 1 mg/ml

Panel Strain	CNF943	CNF994	CNF948
Zone of Inhibition	20 mm	14 mm	16 mm

The structure was elucidated using ^{1}H, ^{13}C, HMQC, and COSY NMR experiments, EIMS and IR data. The HMQC experiment allowed the C-H units to be assigned. The COSY correlations then joined all of the fragments together except at the C6-C7 bond, due to overlapping of C6 and C8 protons and the C1-C2 bond due to lack of protons on C1. These linkages were assigned based on chemical shift arguments, the C2 protons being downfield of the alkyl chain protons because they are deshielded by the carboxyl group. The molecular formula of $C_{11}H_{20}O_2$ was deduced from EIMS data (M^+, *m/z* 184) combined with ^{13}C NMR data which showed 11 strong carbon signals. The cyclopropane and the carboxyl moieties were also indicated by the IR bands at 3250, 2928 and 1711 cm^{-1}.

The stereochemistry was determined by comparison with literature data for synthetic *cis*- and *trans*- isomers. These data were ambiguous due to the low-field NMR instruments used, so a NOESY experiment was used to prove a *cis* conformation of the cyclopropane ring. The key correlations in the experiment were from H11b to H2a, H2b and H5 and H11a to H3 and H2b only, requiring a *cis*

Table 21. Spectral data for *cis*-cascarillic acid (81)



Source: CNF994, Big Cay Bokel, Gram(-) isolate

Molecular formula: $C_{11}H_{20}O_2$

EIMS: M+, m/z (rel. int.) 184 (0.6), $C_{11}H_{20}O_2$; 124 (52.4), C_9H_{16}; 83 (80.7), C_5H_7O; 69 (100), C_5H_9

IR ($CHCl_3$): 3250, 2929, 1711 cm^{-1}

NMR data[a,b]

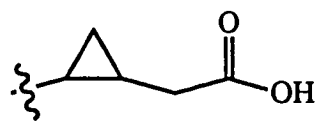
C#	$\delta^{13}C$ (ppm)	DEPT	$\delta^{1}H$ (ppm)	Mult	J (Hz)	COSY	NOESY
1	179.1						
2a	33.6	CH_2	2.42	dd	11.5	3	11b
2b			2.30	dd	11.5	3	3, 11a,b
3	11.0	CH	1.08	q	5.86	4, 2, 11	2b, 11a
4	15.4	CH	.81	m		5, 3, 11	11b
5	22.5	CH_2	1.14	m		6, 4	6, 11b
6	28.7	CH_2	1.40	m		5	5
7	29.1	CH_2	1.30	m		8	
8	29.7	CH_2	1.38	d	4.88	9, 7	
9	31.7	CH_2	1.26	m		10, 8	10
10	14.0	CH_3	.86	t	6.84	9	9
11a	10.7	CH_2	.72	td	4.55	4, 11b, 3	3, 2b
11b			-.12	q	4.90	4, 11a, 3	2a,b, 4, 5

[a] The ^1H NMR experiments were recorded at 500 MHz in $CDCl_3$. [b] The ^{13}C NMR experiments were recorded in $CDCl_3$ at 125 MHz.

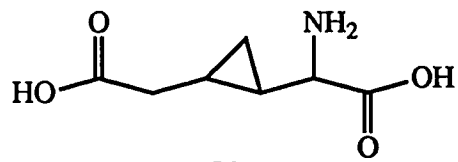
conformation for both sets of protons to be within 4Å of each other.

Cascarillic acid was first isolated in 1900 as the major component of cascarilla essential oil, the steam distillate from the bark of *Croton eleuteria*. The Eleuthera bark is used throughout the West Indies to make a tonic used for intermittent fevers, dyspepsia, and chronic diarrhea. It is also used as a flavoring addition to smoking tobacco in Cuba.[115] Its structure was finally established in 1972,[116] and the stereochemistry of the natural product was determined to be *trans* several years later.[117] This is the first report of *cis*-cascarillic acid as a natural product.

The 3,4-methylene-butanoic acid functionality (**81a**) of cascarillic acid is found in very few other natural products. The seeds of plants in the family Sapindaceae have amino acids containing **81a**, primarily *trans*-α-(2-carboxycyclopropyl)glycine

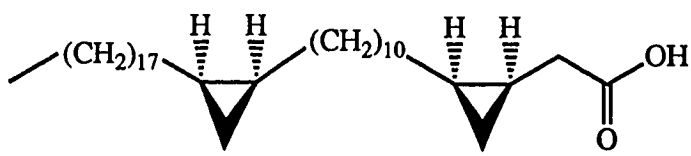


81a



82

(**82**).[118] Also, **81a** is found in urinary acids.[119] The *cis* configuration has been published only once as a natural product, when *cis, cis*-3,4-15,16-dimethylenetetratriacontanoic acid (**83**) was purified from fatty acids of *Mycobacterium tuberculosis*, the causative agent of tuberculosis. This fatty acid



83

is thought to be a precursor of mycolic acid, the major constituent of mycobacterial cell wall.[120]

3) c. The Isolation and Dereplication of Cycloheximide (84) from Isolate CNB773

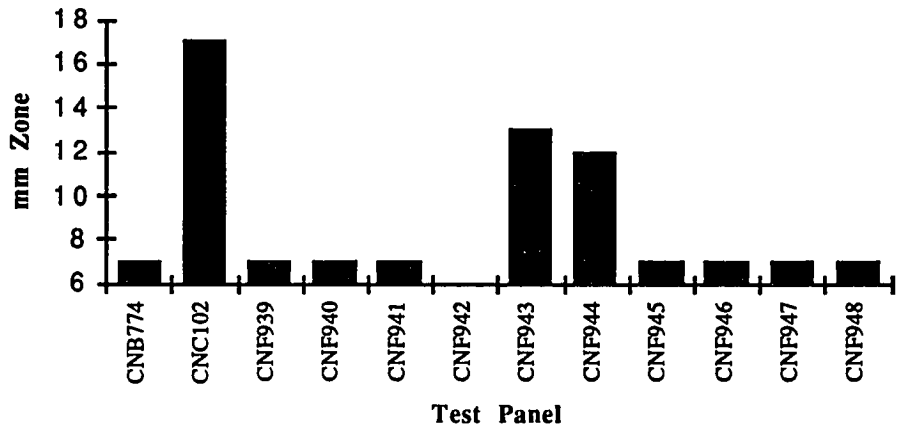


Figure 52. Activity profile of CNB773 crude extract

Isolate CNB773 was selected as a lead based on its potent antifungal activity, along with an interesting mid-polarity, brown-charring band on the TLC. Interesting ^{1}H NMR signals were recorded at 6.3 ppm and 6.8 ppm. As the compound was purified and run in different solvents, these exchangeable signals moved in the ^{1}H NMR spectrum, finally having shifts of 5.7 ppm and 8.6 ppm in the pure compound (see Figure 54).

Isolate CNB773 is an actinomycete obtained from a sand-water interface sediment sample taken on Big Cay Bokel. The 1 L screening extract was enough to dereplicate the active component using a preparative thick-layer plate. The active fraction was proved to be cycloheximide (**84**) by comparison of the ^{1}H NMR spectrum (Figure 54) with previous isolations in the Fenical lab. Cycloheximide producers are common isolates using the described sampling and isolation techniques.

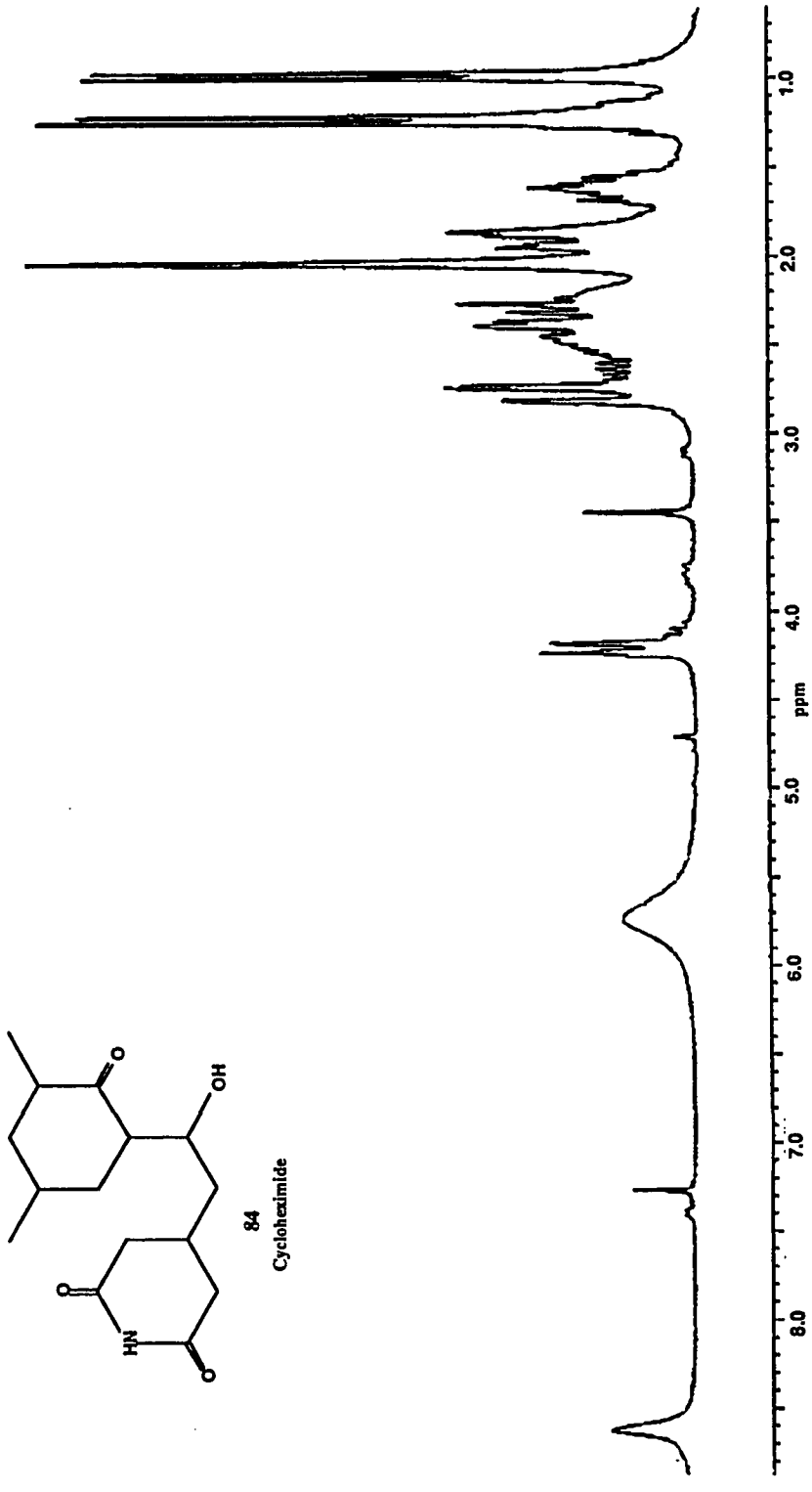


Figure 54. ^{1}H NMR spectrum of cycloheximide (84) in $CDCl_3$ at 200 MHz

Cycloheximide (aka Naramycin A or Actidione) was first isolated from *Streptomyces griseus* in 1947.[121] It has shown activity as an antifungal agent, plant growth regulator, and in inhibition of protein synthesis on eukaryotic ribosomes.[122]

3) d. The Isolation and Dereplication of Staurosporine (85) and the Structure Elucidation of an Analog (86) of Tirandamycin from Isolate CNB766

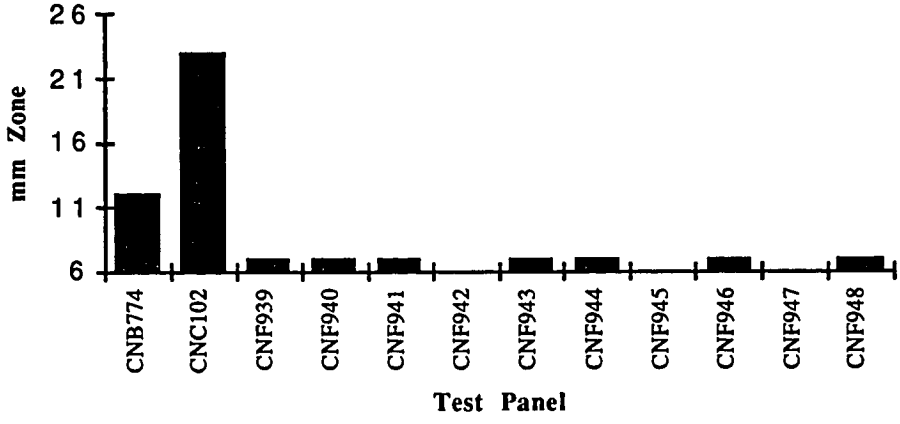


Figure 54. Activity profile of CNB766 crude extract

Isolate CNB766 showed excellent antifungal activity as well as 5 unique TLC bands spread out in polarity, so it was selected as a lead. The ^{1}H NMR spectrum had several interesting aromatic signals.

Isolate CNB766 is an actinomycete obtained from a sand-water interface sediment sample taken on Big Cay Bokel. The 1 L screening extract was enough to dereplicate one active component, staurosporine (**85**), with a reversed-phase preparative thick-layer plate. The non-polar fraction that contained **85** was bright blue-charring on the TLC and was found to be identical to staurosporine by ^{1}H

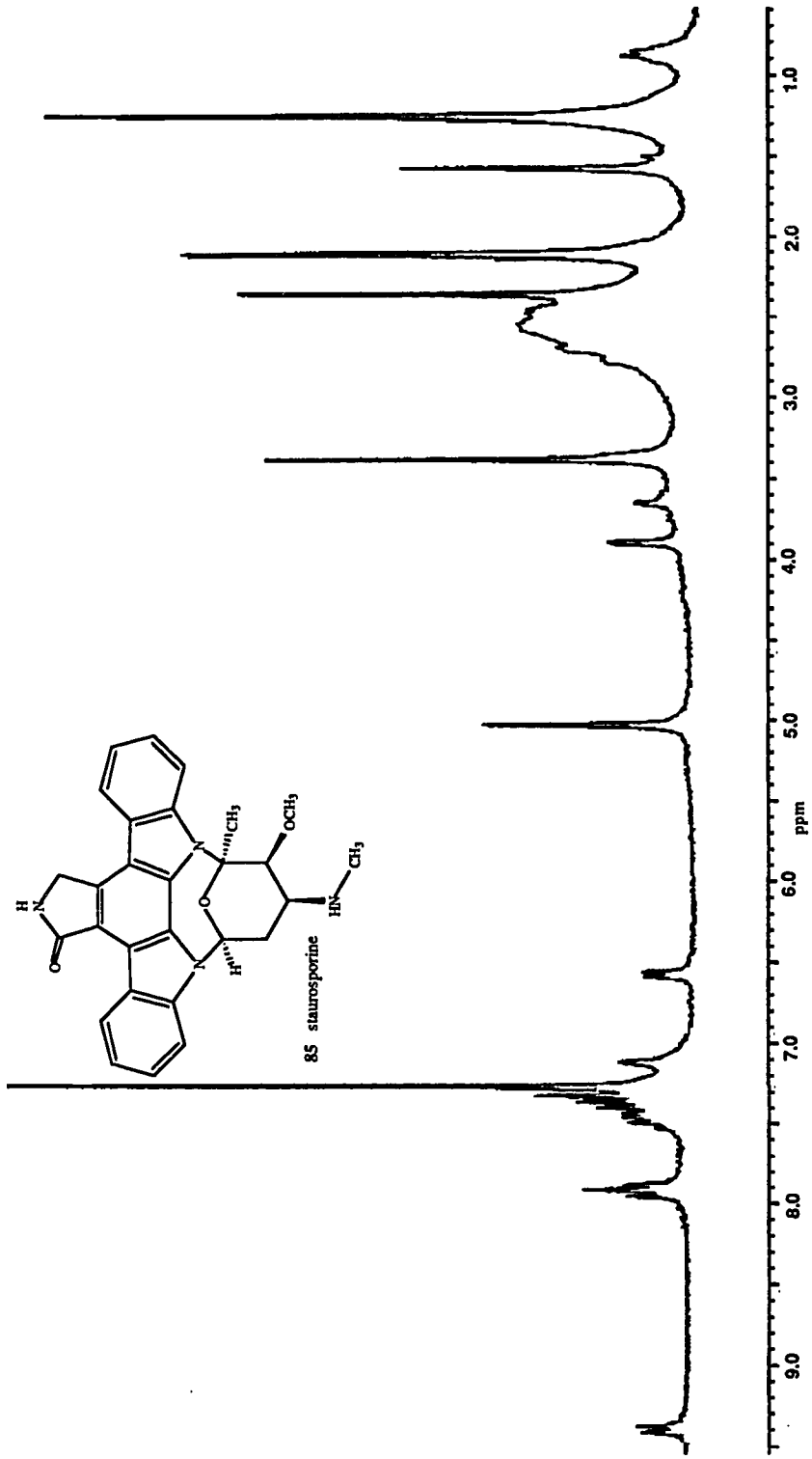


Figure 56. ^{1}H NMR spectrum of staurosporine (85) in $CDCl_3$ at 200 MHz

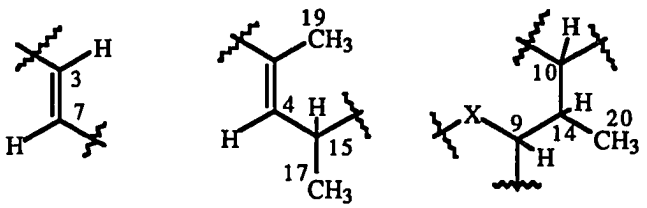
NMR (see Figure 56).[123] The antifungal properties and x-ray structure of staurosporine were reported in 1978,[124] but the fully functionalized core of the staurosporine structure was only recently synthesized.[125]

The tirandamycin analog (**86**) was isolated from a 6 L and an 8 L culture which were separated by vacuum flash chromatography followed by amino HPLC. From the NMR data shown in Table 22, the following partial structures in Figure **57** could be constructed. With these data, the carbon at 131.0 ppm could not be placed. The high-resolution FABMS gave a molecular ion of M^+, *m/z* 420.19780, requiring $C_{20}H_{28}N_4O_6$ or $C_{26}H_{28}O_5$. There was sufficient noise in the ^{13}C NMR to examine both possibilities. A search of these formulae included no compounds with NMR chemical shifts similar to the unknown. The IR showed bands at 1660, 1613, 1565, 1452 and 1002 cm^{-1}, indicating amide, imine, and/or ether bonds rather than esters. Subsequent NMR experiments revealed a gradual change in the compound, yielding a parent ion of M^+, *m/z* 418 rather than 420. In the ^1H NMR spectrum, signals which originally appeared as small amounts of impurity grew as decomposition occured. All further attempts at purification and reactions only caused more breakdown of the compound.

At this point, the partial structures were searched (overnight) in the CAS on-line data base, yielding a match to a compound which nearly fit the spectral data, tirandamycin (**87**). Adding two protons to the tirandamycin formula yields a molecular formula of $C_{22}H_{29}NO_7$, which fit the high-resolution FABMS data (Δ 10.5 ppm). It is unclear why this formula was not included as a possibility in the original peak match results. The 2-D NMR results were now recognized as ambiguous, because **86** was converted to **87** in the NMR tube, most likely by air oxidation, although small amounts of TFA added to sharpen the spectral

Figure 56. Partial structures of 86

Partial Structures from COSY/TOCSY Data



Partial Structures with HMBC Data Added

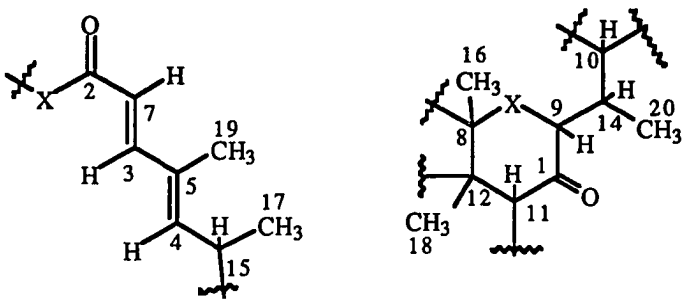


Table 22. NMR data for tirandamycin analog 86[a,b]

C #	δ ^{13}C (ppm)	DEPT	δ ^{1}H (ppm)	mult, J (Hz)	DQ COSY	TOCSY	HMBC (4, 8 Hz)
1	203.5	C					
2	178.0	C					
3	149.6	CH	7.57	d, 16	7	7	2, 19, 4, 5
4	144.3	CH	6.21	d, 10	15	17, 15, 19	19, 3, 10, 17, 13
5	135.9	C					
6	131.0	C					
7	117.5	CH	7.18	bd, 14	3	3	2, 5
8	97.8	C					
9	79.9	CH	3.96	d, 5.9	14	20, 10, 14, 11	1, 8, 14, 10, 11, 13
10	77.8	CH	3.75	d, 5.8	14	20, 14, 9, 11	4, 9, 14, 15, 17
11	61.9	CH	3.30	m		10, 13, 9, 17, 14	1, 9, 18, 12, 13
12	57.9	C					
13	51.9	CH_2	3.74	d, 5.8?			
14	35.6	CH	1.90	d	9,10, 20	10, 13, 20, 9	1, 20, 9, 10
15	35.5	CH	2.95	m	17	17, 4, 19	17, 4, 5
16	22.7	CH_3	1.51	s			8, 12
17	17.1	CH_3	1.14	d, 6.8	15	4, 15	15, 10, 4
18	15.6	CH_3	1.45	s			12, 8, 11
19	12.2	CH_3	1.90	s		4, 17, 15	5, 4, 3
20	11.4	CH_3	0.71	d, 7.3	14	10, 14, 9	14, 10, 9

[a] All ^{1}H NMR experiments were done at 500 MHz in MeOD at room temperature. [b] All ^{13}C NMR experiments were done at 50 MHz in MeOD at room temperature.

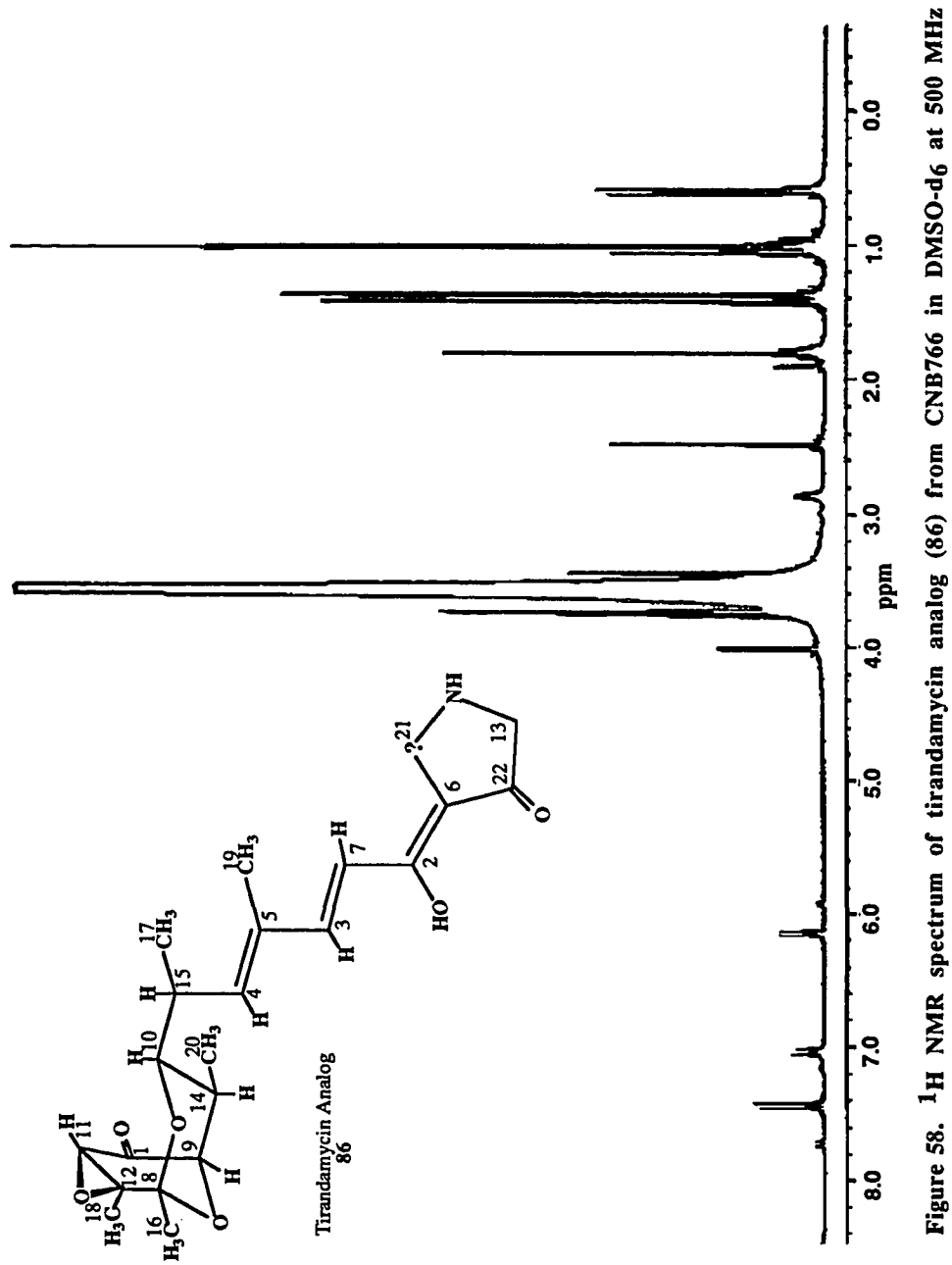
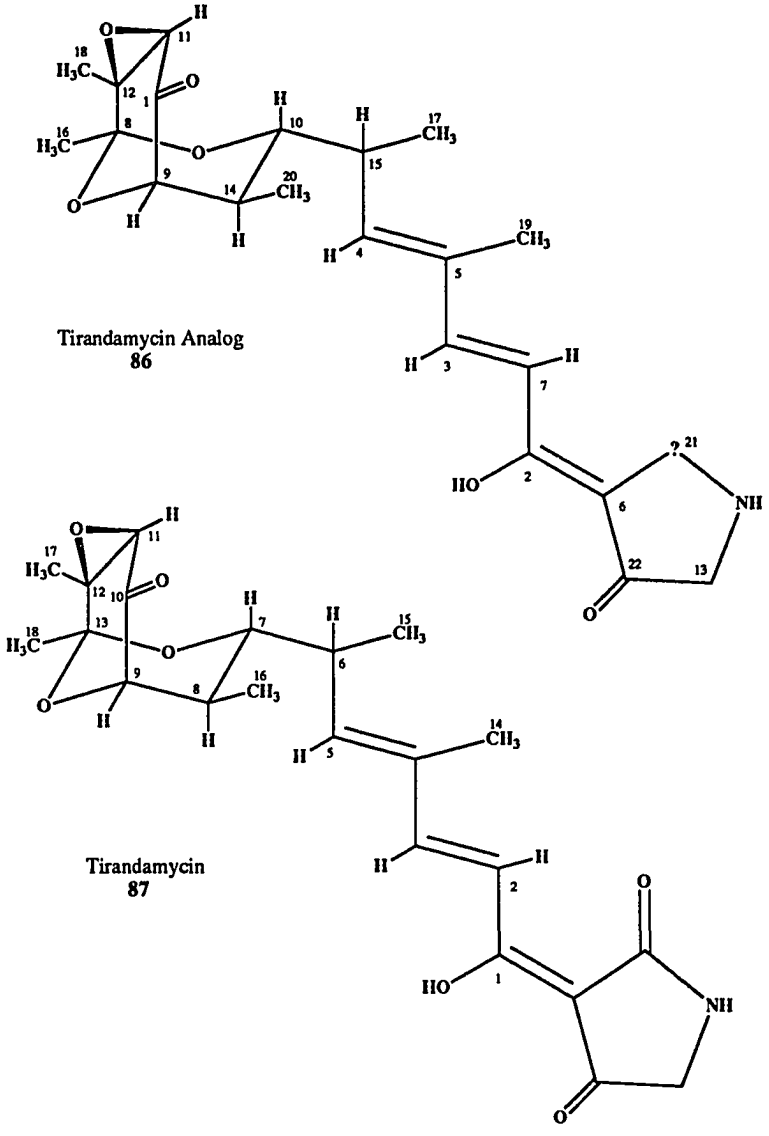


Figure 58. ^{1}H NMR spectrum of tirandamycin analog (86) from CNB766 in DMSO-d_6 at 500 MHz

Figure 59. Structure of tirandamycin (87) and proposed partial structure of its analog, 86



characteristics may have been responsible, also. Hence, the correlations to 102.0 and 195.0 ppm, first thought to be noise, were now seen as evidence for the presence of the ketone in the tetramic acid moiety. The carbon at a shift of 102 ppm can not be placed using the available data. Two hydroxyl protons are seen as a broad hump at 5.20 ppm in the NMR spectrum in DMSO-d_6, leaving one additional unassigned proton in the structure. Solution of the structure will require manipulation of the 5-membered, tetramic acid ring. The remainder of the molecule fit the spectral data of **87** perfectly.

The structure of tirandamycin was solved by X-ray analysis after its isolation from *Streptomyces tirandis*.[126] The compound shows antibiotic activity, especially against Gram (+) bacteria, as well as potent activity as an inhibitor of RNA polymerase. Compound **86** has shown potent activity against the test panel fungus, warranting further investigation.

3) e. The Isolation and Dereplication of *o*-Hydroxybenzamide (**88**) from Isolate CNF974

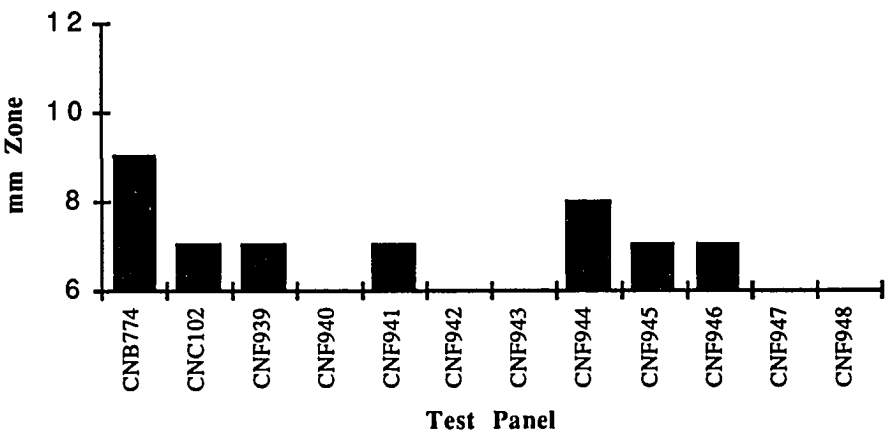
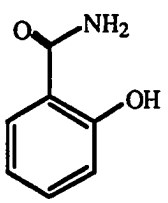


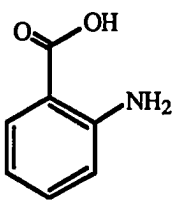
Figure 59. Activity profile of CNF974 crude extract

Isolate CNF974 was selected because it was one of the only extracts to show activity against the actinomycete on the test panel. It also had an unique yellow-charring band on the TLC. The ^1H NMR spectrum was acquired with a sample that was not concentrated enough to be very useful in the selection process.

Isolate CNF974 is a unicellular bacterium obtained from a sediment sample taken in 2 feet of water in a mangrove embayment on Big Cay Bokel. It was cultured in 100 ml for screening and subsequently on a 4 L scale. *o*-Hydroxybenzamide (**88**) was purified from the 4 L extract by vacuum flash chromatography followed by HPLC as 0.6% of the crude. It was found to be the active component of the extract.



88



89

The structure was determined to be either o-hydroxybenzamide (**88**) or anthranilic acid (**89**) based on the ^1H (Figure 61) and ^{13}C NMR data and low-resolution EIMS data, which were combined to give a molecular formula of $C_7H_7NO_2$. Final conclusion that the active compound was **88** came from the IR data. For an aromatic acid as in **89**, a band at 1730-1715 cm^{-1} would be expected, while a free amide should have bands at 1690 and 1610 cm^{-1}.[65] The isolated

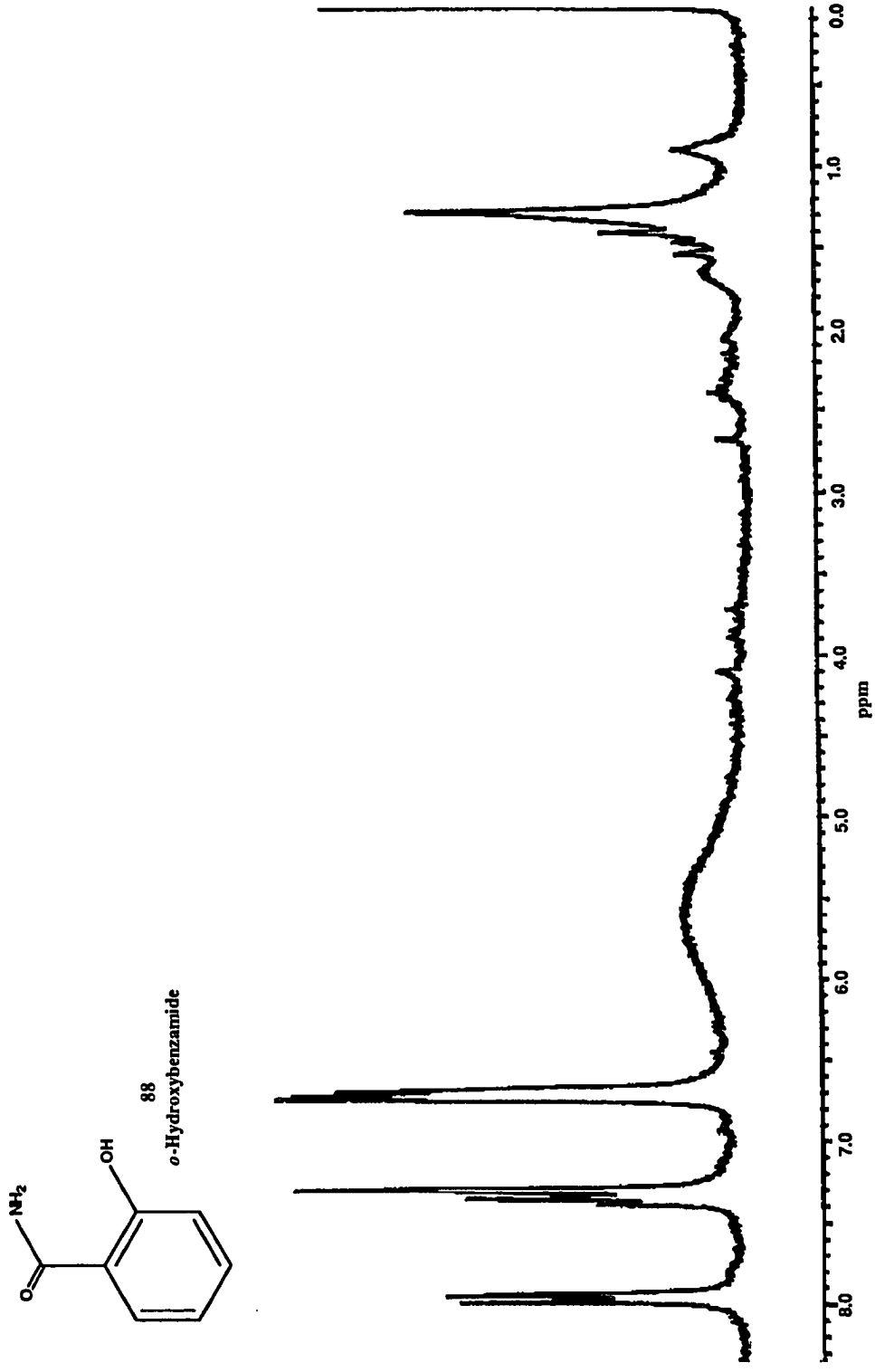


Figure 61. ^{1}H NMR spectrum of *o*-hydroxybenzamide (88) in $CDCl_3$ at 200 MHz

compound had IR bands at 1694 and 1616 cm^{-1}, therefore, it was decidedly *o*-hydroxybenzamide rather than **89**.

From this investigation, it can not be concluded that **88** is being produced *in situ*. However, the isolate certainly has the capability to produce **88** given the proper nutrients. The significance of finding **88** as a metabolite produced by an estuarine isolate is that benzamides are commonly considered man-made water pollutants.[127] An estuary is a pollution-sensitive area, because it is a breeding ground for so many organisms. For this reason, estuarine waters must be protected. But, before assigning blame for hazardous chemical run-off or dumping of compounds like benzamides in an estuary, the possibility of a natural source should be considered.

3) f. The Isolation and Dereplication of Val-Pro (**90**), Tyr-Pro (**91**) and Leu-Pro (**92**) Diketopiperazines, Adenine (**93**), and Another Purine (**94**) and the Isolation of Unknown D from Isolate CNF962

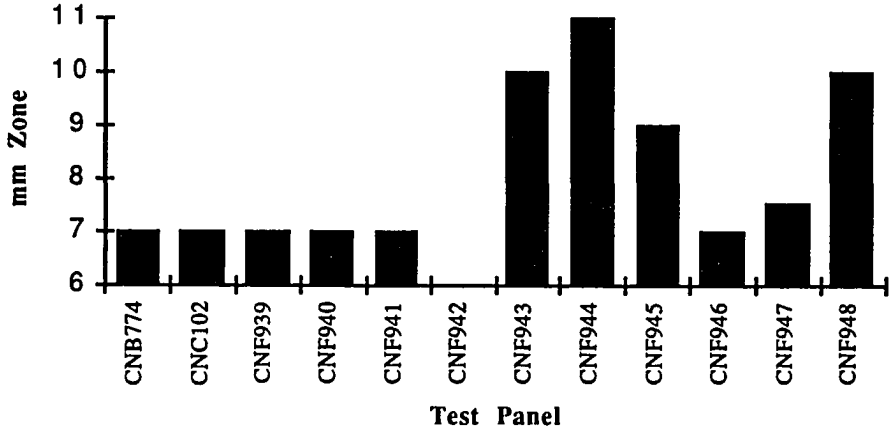


Figure 61. Activity profile of CNF962 crude extract

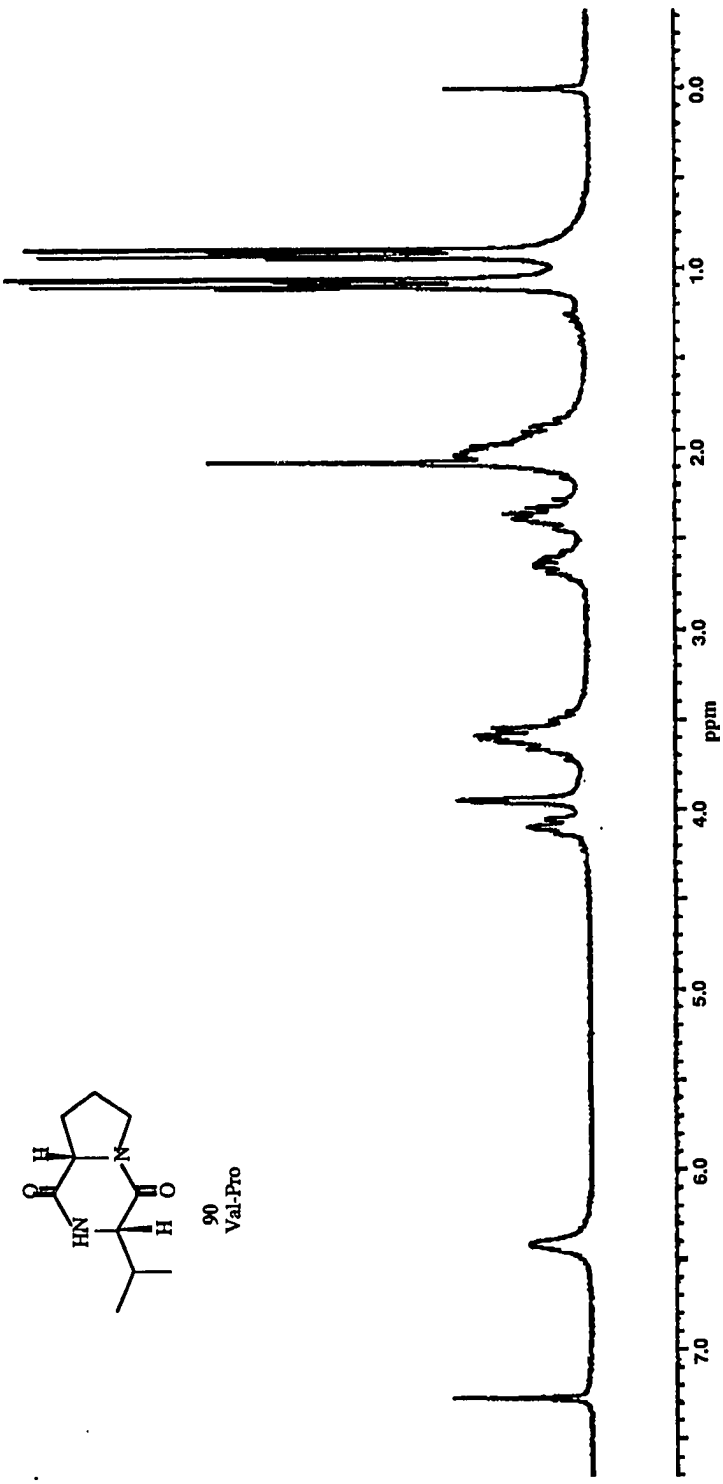


Figure 63. ^{1}H NMR spectrum of val-pro diketopiperazine (90) in $CDCl_3$ at 200 MHz

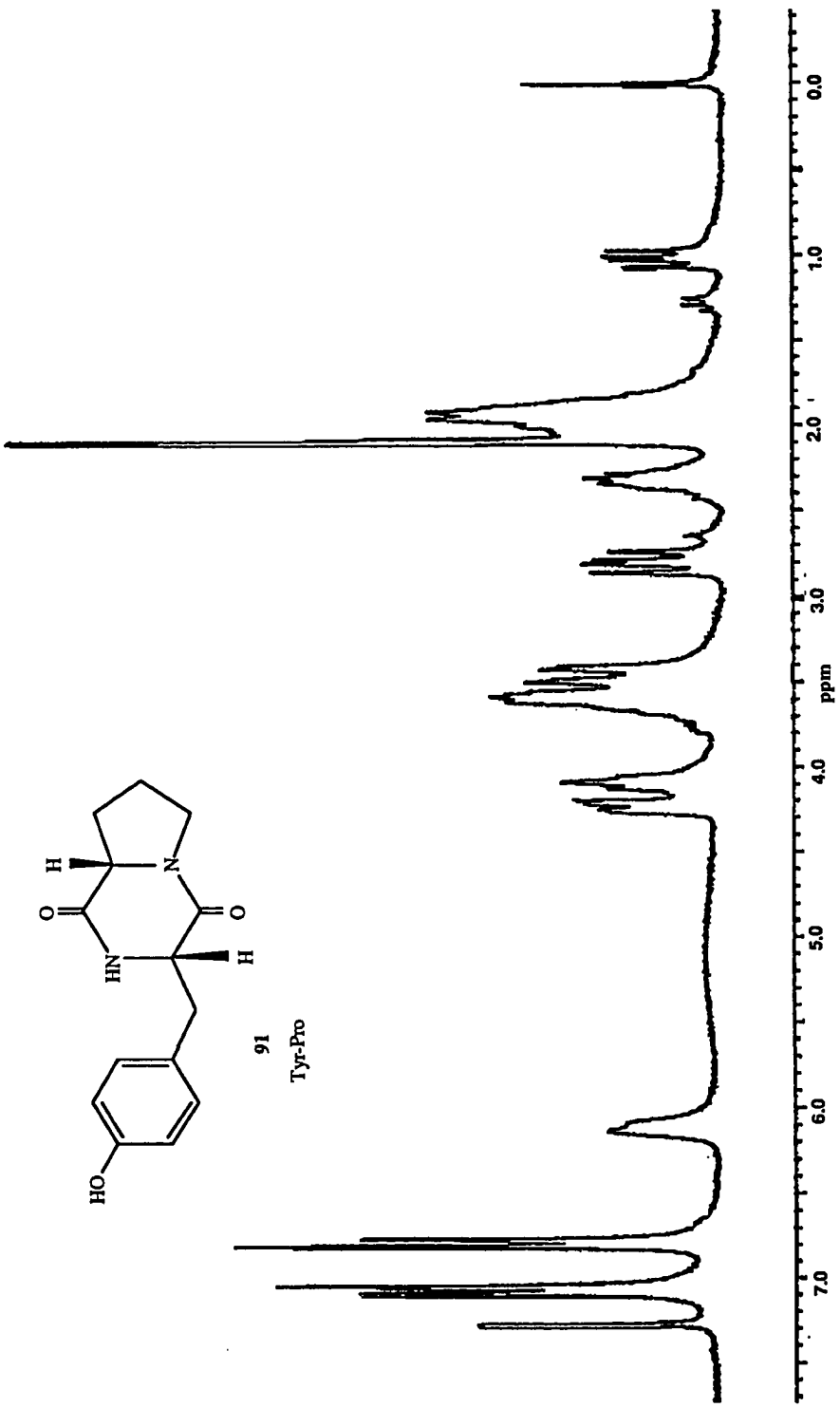


Figure 64. ^{1}H NMR spectrum of tyr-pro diketopiperazine (91) in $CDCl_3$ at 200 MHz

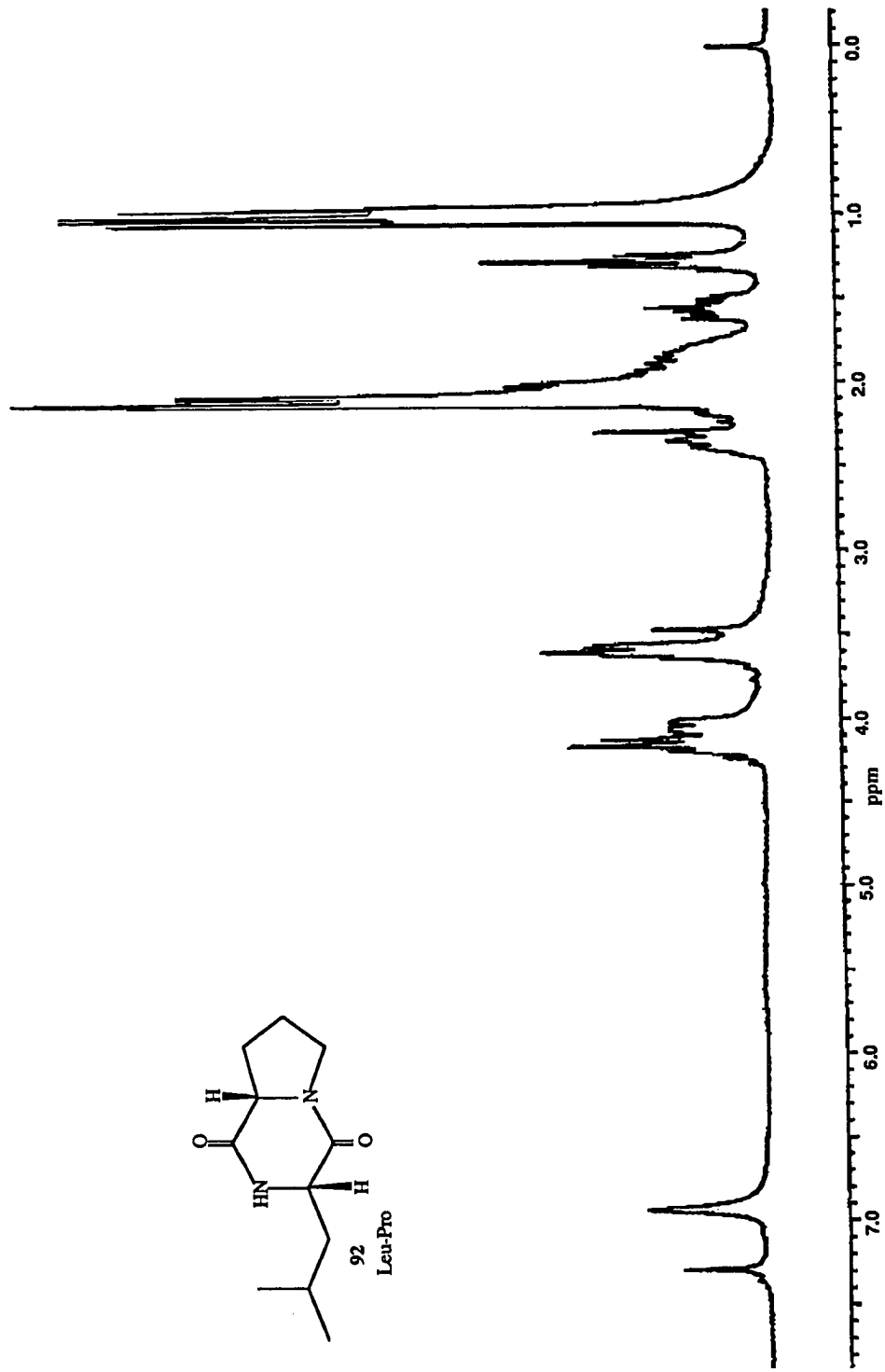


Figure 65. ^{1}H NMR spectrum of leu-pro diketopiperazine (92) in $CDCl_3$ at 200 MHz

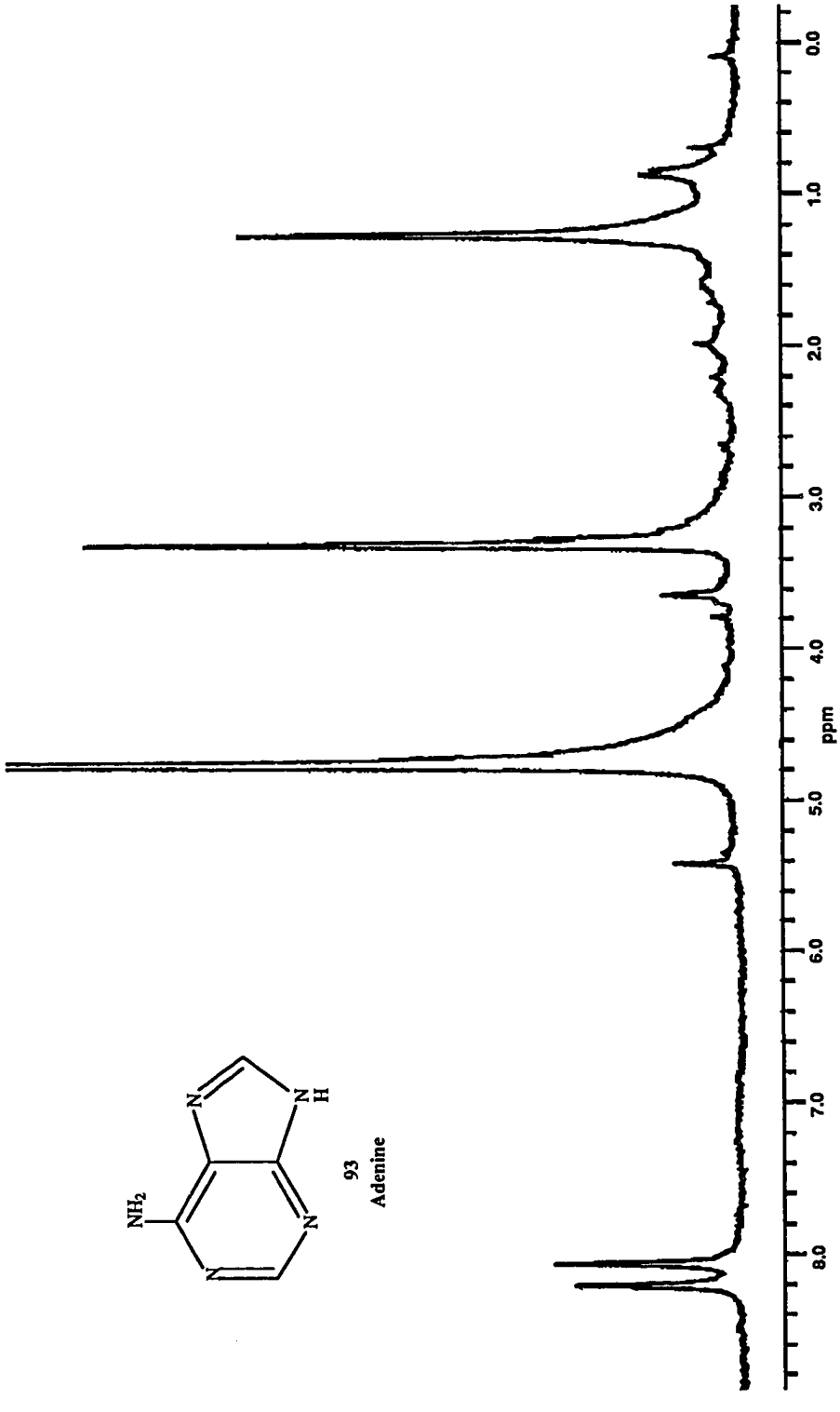


Figure 66. ^{1}H NMR spectrum of adenine (93) in MeOD at 200 MHz

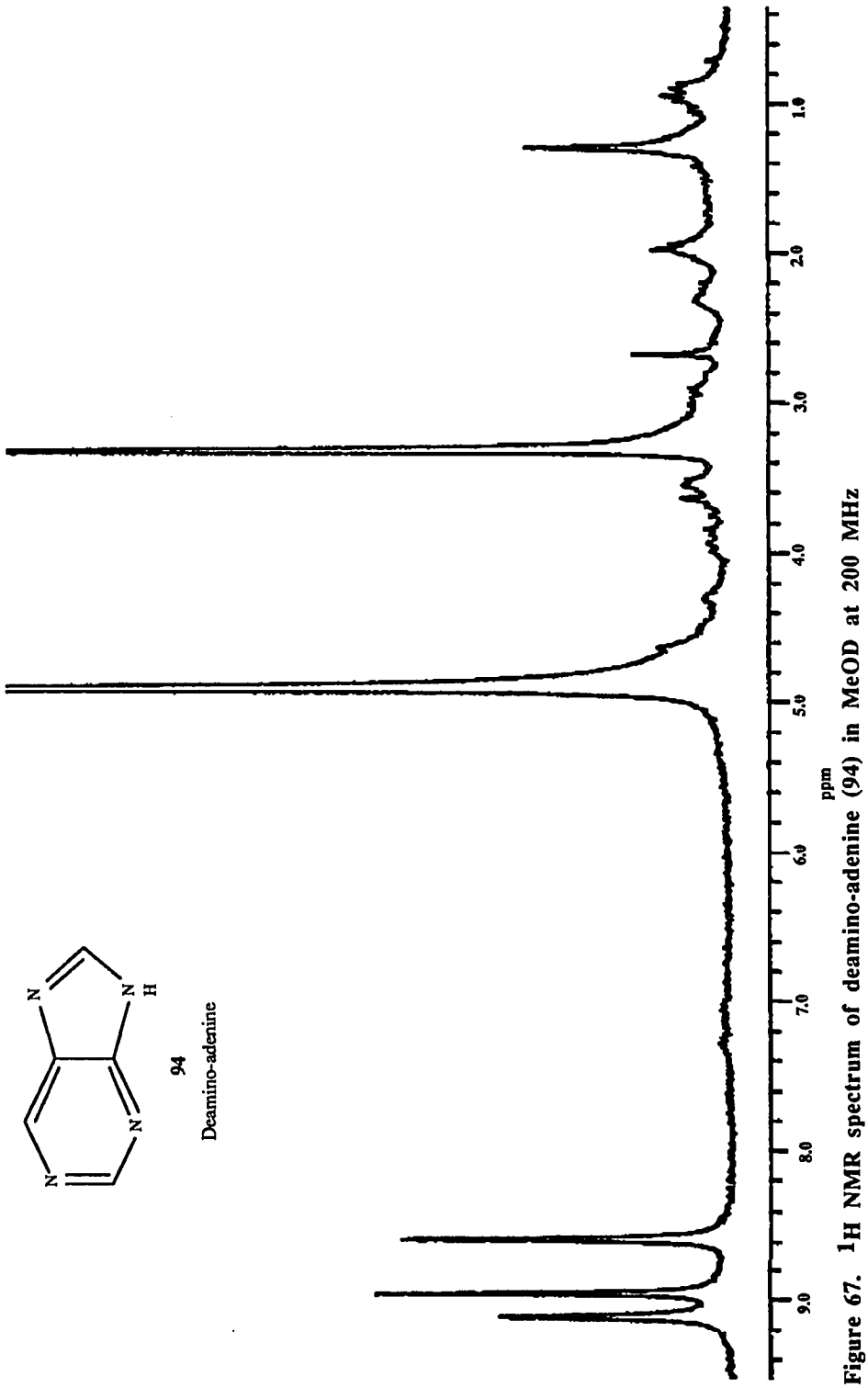


Figure 67. ^{1}H NMR spectrum of deamino-adenine (94) in MeOD at 200 MHz

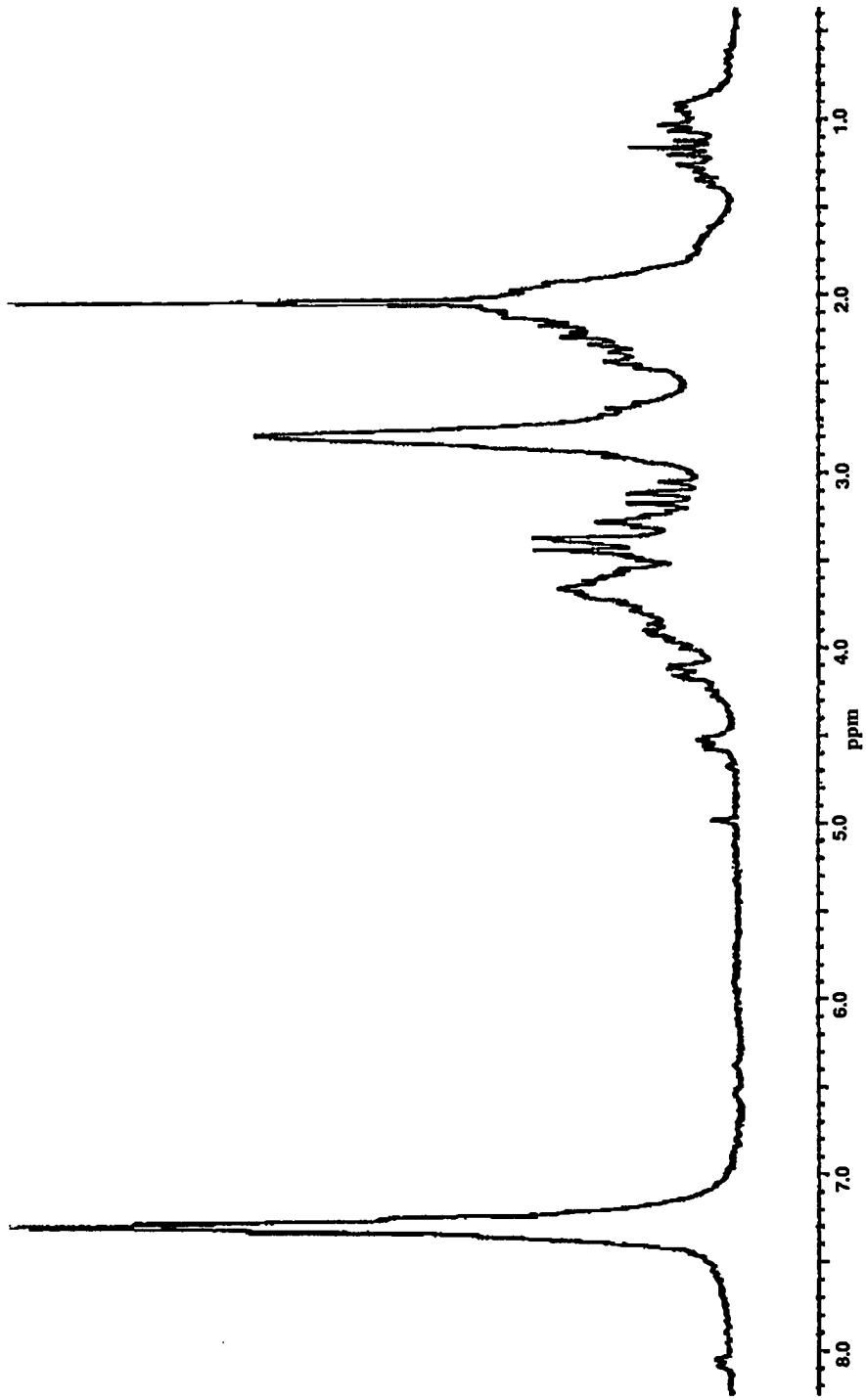


Figure 68. ^{1}H NMR spectrum of unknown D from CNF962 in $CDCl_3$ at 200 MHz

An extract of CNF962 was active against 5 test panel strains, a definite lead for a unicellular extract. The TLC had a deep purple-charring band of medium polarity, as well as UV-active compounds that stayed close to the baseline. The ^{1}H NMR spectrum had a strong 6.6 ppm multiplet and unusual complexity low-field of 7 ppm.

Isolate CNF962 is a Gram-positive unicellular bacterium obtained from a shell bed at the sand-water interface on Big Cay Bokel. The isolate was cultured in 100 ml for screening and on a 6 L scale for isolation work. Fractionation of the 6 L extract by vacuum flash chromatgraphy followed by normal-phase HPLC yielded 7.8 mg of the active fraction, 9.1 mg Tyr-Pro (**91**), 7.7 mg Leu-Pro (**92**), and 2.0 mg Val-Pro (**90**). The active fraction appeared to be a peptide by ^{1}H NMR (Figure 68), based on the shifts in the 3.5 to 5.0 ppm region where α-C protons are found. However, the limited amount made it difficult to proceed until further cultures were grown and extracted. The diketopiperazines were very common metabolites in both unicellular and actinomycete extracts. In fact, proline-containing diketopiperazines were seen in 90% to 95% of the cultures extracted.

While waiting for a regrow, a second interesting flash column fraction was investigated. The compounds were polar by TLC, but they would not dissolve in solvent mixtures with greater than 70% water. Separation was achieved by linking two reversed-phase HPLC columns together. This yielded 4.1 mg **93** and 3.1 mg **94**, identified in each case based on ^{1}H and ^{13}C NMR and low-resolution EIMS experiments followed by comparison with literature[65] and calculated values.

All living organisms, except some species of bacteria, are able to synthesize purine bases such as **93** and **94** from simpler precursors. Normally, the precursors are constructed on a sugar residue, not independently, so isolation of the purines

without a sugar attached is not common.[128] The explanation for the isolation of **93** and **94** is most likely one or more of the following: 1) the bacterium synthesizes the purines without the sugar attached via some pathway not yet discovered; 2) the bacterium has an excess of hydrolytic enzymes that break down nucleotides; 3) the bacterium is missing the salvage enzymes responsible for recovery of free purines and pyrimidines resulting from this hydrolytic breakdown of nucleotides; or 4) the isolation process was responsible for cleaving the sugar moiety.

D. Experimental, Chapter III

Section B.

Isolation and Morphology of CNB091. This *Streptomyces* sp. was isolated from a surface swab of a jellyfish collected in the Florida Keys in 1987. It was cultured on seawater agar plates and transferred to liquid seawater based media. After growing for one day, the culture was frozen with glycerol at -80°C for subsequent culture work. On an A-1/agar plate, the colony was green with a folded center and light green spores.

Culture and Extraction. Fermentation 1: The isolate CNB091 was cultured in a 12 L Virtis glass fermentor at 25°C for 3 days in A-1 media. The entire fermentation broth was then double extracted with EtOAc. The extracts were combined and dried over Na_2SO_4, filtered, and evaporated *in vacuo* to yield 1.2 g crude organics. Fermentation 2: The isolate CNB091 was scaled-up from 10 ml A-1 (4 days) to 1 L (1 day) to 50 L (1 day) in A-1 media in a New Brunswick Scientific FM-75 Fermatron fermentor with the following settings: Air 1.8 L/min., Impellor Speed 200 rpm. The culture was then filtered with Celite to remove the cells. The broth was extracted using C18 resin, 1 L resin/5 L broth. The resin was mixed with the broth, placed in a Buchner funnel under vacuum, rinsed twice with deionized water, then eluted with a total of 20 L MeOH. The MeOH was concentrated *in vacuo* to ~500 ml, then partitioned between H_2O and $CDCl_3$. The $CDCl_3$ fraction was then dried over Na_2SO_4, filtered and evaporated *in vacuo*. The extract was partitioned again between MeOH and TMP, and the MeOH fraction was evaporated *in vacuo* to yield 3.4 g crude organics.

Isolation of salinamides A-F from a CNB091 extract. The crude extract was separated on a silica vacuum flash column, eluted with TMP:EtOAc mixtures

followed by EtOAc:MeOH mixtures. The salinamide-containing fractions (100% EtOAc and 95:5 EtOAc:MeOH) were then separated by reversed-phase HPLC (C18 Dynamax, 10 mm x 250 mm or 25 mm x 500 cm) with 75:25 MeOH:H_2O. From the large fermentation (3.4 g crude), 276.0 mg **58**, 81.0 mg **59**, 14.9 mg **61**, 20.3 mg **62** and 8.0 mg **63** were isolated. Compound **60** (~5 mg) was isolated only once from a 12 L fermentation (1.2 g crude).

Salinamide A (**58**) isolated from CNB091. Salinamide A, obtained as a pale yellow non-crystalline solid (12 mg/L fermentation yield), showed: $[\alpha]_D$ -26° (c 0.97, $CDCl_3$); mp 221-225 °C (decomposes); IR ($CDCl_3$) 3436, 3379, 1745, 1735, 1682, 1657, 1636 cm^{-1}; UV (MeOH) 281, 267, 224, 208 nm; HRFABMS *m/z* 1020.492 (M^++H), calcd. for $C_{51}H_{70}N_7O_{15}$ 1020.493; ^1H NMR ($CDCl_3$) δ 8.41, 7.95, 7.24, 7.21, 7.09, 6.82, 6.62, 6.51, 6.23, 6.21, 5.84, 5.64, 5.45, 5.17, 5.05, 4.91,4.90, 4.83, 4.66, 4.64, 4.42, 4.32, 3.92, 3.66, 3.63, 3.46, 3.34, 3.18, 2.95, 2.76, 2.67, 2.44, 1.77, 1.62, 1.56, 1.38, 1.32, 1.30, 1.28, 1.14, 0.96, 0.88,. 0.87, 0.83; ^{13}C NMR ($CDCl_3$) δ 177.6, 173.6, 169.9, 169.7, 169.1, 168.9, 168.6, 167.5, 165.3, 159.3, 142.9, 137.1, 130.8, 129.0, 128.4, 127.9, 126.8, 125.6, 125.0, 120.6, 120.1, 79.4, 78.9, 72.4, 68.7, 68.1, 65.6, 61.3, 59.6, 56.4, 55.6, 55.4, 53.6, 52.0, 41.6, 40.8, 40.1, 39.5, 38.6, 32.2, 26.1, 21.1, 19.7, 18.6, 16.7, 15.6, 14.7, 14.2, 11.5.

Salinamide B (**59**) isolated from CNB091. Salinamide B, obtained as an off-white crystalline solid (12 mg/L fermentation yield), showed: $[\alpha]_D$ -65° (c 0597, $CDCl_3$); mp 239-241 °C ; IR ($CDCl_3$) 3445, 3347, 1745, 1734, 1683, 1651, 1636 cm^{-1}; UV (MeOH) 282, 268, 227, 209 nm; HRFABMS *m/z* 1056.470 (M^++H), calcd for $C_{51}H_{70}N_7O_{15}Cl$ 1056.468; ^1H NMR ($CDCl_3$) δ 8.74, 7.88, 7.79, 7.23, 7.06, 6.9-7.1, 6.99, 6.97, 6.91, 6.58, 6.34, 5.92, 5.85, 5.48, 5.10, 5.04, 4.94, 4.90,

4.82, 4.76, 4.56, 4.42, 4.39, 3.78, 3.64, 3.62, 3.52, 3.42, 3.31, 3.24, 3.23, 2.85, 2.68, 1.81, 1.74, 1.64, 1.51, 1.42, 1.40, 1.32, 1.22, 0.99, 0.88, 0.85, 0.82; ^{13}C NMR ($CDCl_3$) δ 177.9, 173.7, 170.2, 170.1, 168.9, 167.8, 165.2, 160.9, 146.9, 137.7, 131.1, 129.2, 128.5, 128.4, 124.0, 123.3, 123.0, 118.1, 81.3, 80.8, 79.7, 73.6, 69.3, 68.9, 65.5, 61.6, 56.6, 56.2, 54.3, 53.1, 47.9, 42.1, 40.7, 40.0, 39.8, 34.8, 32.4, 26.3, 21.4, 19.9, 18.5, 16.7, 15.8, 14.5, 14.4, 11.5.

Salinamide C (60) isolated from CNB091. ^1H NMR ($CDCl_3$) δ 9.18, 7.94, 7.28, 7.20, 7.09, 7.02, 6.90, 6.75, 6.74, 6.58, 6.52, 6.22, 5.75, 5.60, 5.52, 5.25, 5.22, 5.20, 5.12, 4.86, 4.67, 4.57, 4.30, 4.24, 3.98, 3.80, 3.69, 3.67, 3.51, 3.28, 3.21, 2.80, 2.63, 1.81, 1.73, 1.69, 1.58, 1.37, 1.32, 1.20, 0.99, 0.94, 0.86, 0.84; ^{13}C NMR ($CDCl_3$) δ 177.9, 174.4, 170.7, 170.3, 169.5, 169.2, 168.7, 168.0, 167.1, 160.3, 147.0, 138.0, 136.9, 133.1, 130.2, 129.0, 128.4, 126.4, 122.9, 116.4, 114.6, 79.5, 72.6, 70.0, 68.6, 65.7, 61.6, 56.7, 56.2, 54.9, 54.2, 52.5, 42.2, 41.3, 40.1, 40.0, 34.2, 32.3, 26.5, 21.2, 19.9, 18.3, 16.8, 15.9, 14.5, 14.2, 11.6.

Salinamide D (61) isolated from CNB091. ^1H NMR ($CDCl_3$) δ 8.50, 7.77, 7.20, 7.10, 7.08, 6.90, 6.80, 6.61, 6.26, 6.18, 5.76, 5.72, 5.50, 5.22, 4.95, 4.92, 4.85, 4.84, 4.70, 4.64, 4.45, 4.32, 4.28, 3.92, 3.70, 3.68, 3.46, 3.38, 3.30, 2.96, 2.80, 2.72, 2.45, 1.98, 1.70, 1.58, 1.41, 1.35, 1.33, 0.99, 0.98, 0.96, 0.95.

Salinamide E (62) isolated from CNB091. ^1H NMR ($CDCl_3$) δ 7.70, 7.35, 7.09, 7.07, 7.02, 6.90, 6.75, 6.62, 5.61, 5.17, 5.13, 4.81, 4.74, 4.33, 4.29, 4.17, 3.97, 3.90, 3.73, 3.53, 3.52, 3.39, 3.15, 2.82, 2.73, 1.68, 1.66, 1.56, 1.37, 1.27, 1.09, 0.99, 0.95, 0.87, 0.80; ^{13}C NMR ($CDCl_3$) δ 177.7, 174.4, 171.8,

169.4, 169.2, 168.8, 168.7, 161.0, 137.5, 130.0, 128.9, 128.5, 126.5, 122.9, 115.1, 79.5, 71.4, 70.2, 68.3, 62.7, 61.6, 56.6, 56.1, 55.6, 55.2, 55.0, 42.1, 40.4, 39.7, 33.5, 32.2, 36.2, 21.3, 19.7, 18.1, 16.6, 15.9, 14.1, 11.7.

Salinamide F (63) isolated from CNB091. ^{1}H NMR ($CDCl_3$) δ 7.64, 7.12, 7.10, 7.09, 7.07, 7.04, 7.02, 6.88, 6.62, 6.61, 5.68, 5.16, 5.11, 4.82, 4.74, 4.32, 4.30, 4.24, 3.99, 3.90, 3.72, 3.54, 3.50, 3.39, 3.14, 2.82, 2.73, 1.92, 1.68, 1.56, 1.37, 1.28, 0.99, 0.95, 0.87, 0.80.

Acid-catalyzed Ring Opening of Salinamide A. Salinamide A (9.5 mg) was dissolved in THF and H_2O and stirred under N_2. Hydrochloric acid (6N) was added dropwise until products were seen by TLC. The reaction was stopped when nearly all of **58** appeared to be consumed by TLC analysis (2 hr.). The organic products were back-extracted with $CHCl_3$, then purified by RP HPLC. The rotation ($[\alpha]_D$ -56° (c .05, $CDCl_3$)) and the ^{1}H NMR spectrum of the single purified product were identical to **59** (20% overall with 80% starting material).

Section C.

Sample Collection. Batiquitos Lagoon. The samples shown in Figure 27 consisted of approximately 5 mg sediment or 10 ml water; they were collected, by hand or by snorkeling, in sterile tubes from the sediment surface or water column. The B samples were heated to 40 °C for 1 h. Each sample was serially diluted, and the 10^{-3} to 10^{-6} dilutions were spread-plated on B-3/agar plates with cycloheximide. Colonies were isolated from plates containing 1-100 colonies. Like colony morphologies were not selected from the same sample. Actinomycetes were transferred to A-1/agar plates for isolation work; all other isolates were isolated on B-3/agar plates. Each isolated colony was then transferred to a slant.

Sample Collection. Big Cay Bokel. The samples shown in Figures 50

consisted of approximately 5 mg sediment or 10 ml water; they were collected, by hand or by snorkeling, in sterile tubes from the sediment surface or water column. Each sample was serially diluted, and the 10^{-2} to 10^{-6} dilutions were spread-plated on B-3/agar plates with cycloheximide. The 10^{-2} to 10^{-4} dilutions were then heated to 40°C for 1 hr. and spread-plated on A-1/agar plates with cycloheximide. Colonies were isolated from plates containing 1-100 colonies. Like colony morphologies were not selected from the same sample. Actinomycetes were transferred to A-1/agar plates for isolation work; all other isolates were isolated on B-3/agar plates. Each isolated colony was then transferred to a slant.

Gram testing. 24-hr old cultures were placed on a drop of KOH with an inoculating loop and mixed well. Sticky threads as the loop was raised indicated a Gram (-) isolate, while lack of stickiness indicated Gram (+). Gram (+) strains were transferred to another plate and retested the following day.

Culture and extraction. The unicellular isolates were transferred from slant to 10 ml B-1 media in a culture tube using a sterile inoculating loop. The actinomycetes were grown in 10 ml A-1 media using the same procedure. The unicellular cultures generally became cloudy as they grew, while the actinomycete and fungal cultures grew in small spheres of mycelia. After 24-48 hr., depending on density of the culture, the 10 ml was transferred to 100 ml of the proper media and shaken for 3-7 days. The unicellular cultures were double extracted with EtOAc at this point, while the fungi and actinomycetes were transferred to 1 L A-1 media and shaken for 7 days, then double extracted with EtOAc. All extracts were dried over Na_2SO_4, filtered, evaporated *in vacuo*, and weighed before activity testing. All transfers were carried out aseptically, and all liquid cultures were shaken at room temperature.

Activity testing. Each extract was diluted with 0.5 ml EtOAc and 0.5 ml MeOH. The side of a 7 mm diameter filter paper disk was placed in the extract to adsorb 25 µl of the solution. The paper disk was dried in the hood. A B-3/agar plate, or A-1/agar plate for the actinomycete, was spread with 0.1 ml of a 1-2 day old 10 ml culture of the desired test panel strain. The liquid media, B-1 for unicellulars and the fungus and A-1 for the actinomycete, was allowed to dry. The filter paper disks were then placed on the plate at regular intervals with no more than 18 disks per plate. The plates were sealed with parafilm and placed in the grow room for 24 hr. Diameters of the zones of inhibition were recorded in mm, with the letter 'h' after the number if the zone was hazy. 'NR' indicates no result; the test strain did not grow. 'NA' indicates no activity.

CNB765. Activity. The crude extract was tested at 19.2 mg/ml. The activity against the test panel was: CNB739, 9 mm; CNC098, NA; CNF029, 9 mm; CNF028, 20 mm; CNF017, 11 mm; CNF018, 22 mm; CNF019, 10 mm; CNF020, NR; CNF021, NR; CNF022, 8 mm; CNF023, NA; CNF024, 22h mm; CNF025, 20 mm; CNF026, NA; CNF027, NR.

Collection, Isolation and Morphology. CNB765 was isolated from Batiquitos Lagoon sample B7, 10^{-3} dilution after heating. On an agar plate with A-1 media, the colony was beige, dull, irregular, nearly round, punched into agar, with little white spores, indicating it was an actinomycete. The strain was stored frozen in 10% glycerol and A-1 media at -80°C.

Culture and Extraction. The screening culture was scaled-up from the frozen tube inoculum in 10 ml tube of A-1 media (1 day) to 100 ml A-1 in a 500 ml Erlenmeyer flask (2-3 days) to 1 L A-1 media in a Fernbach flask (7 days) with constant shaking at room temperature. This 1 L culture was then extracted with

ethyl acetate. The 55 L culture was scaled-up similarly, except the 1 L growth was transferred to 10 L of A-1 in a 12 L Virtis fermentor. Ethyl acetate extraction of a 55 L culture was not practical, so a resin technique was used. The cells were filtered out with Celite, and the broth was loaded onto C18 resin, 0.5 L resin per 1 L broth.

Isolation of Piericidin (65) and Chloramphenicol (66). The C18 resin was rinsed twice with deionized water, then the organics were eluted with 15 L of methanol. The extract was evaporated *in vacuo* to 300 ml, then fractionated between CH_2Cl_2 and H_2O. The CH_2Cl_2 fraction was dried over Na_2SO_4, filtered, and evaporated to yield 2.09 g crude extract. The extract was separated using a 350 ml vacuum flash chromatography column, packed with 100 g of 250 Å silica gel, which was eluted with 200 ml solutions of 60:40 TMP:EtOAC to 100% EtOAc in 5% steps, then 80:20 EtOAc:MeOH and 100% MeOH. Piericidin was isolated in 2.0% yield in the 25:75 TMP:EtOAc fraction. The 20:80 to 10:90 TMP:EtOAc fractions were combined and further separated by normal phase HPLC (Dynamax 60A, silica, 10 mm x 250 mm) using 90:10 EtOAc:TMP at 2.5 ml/min., yielding 16.8 mg (0.8% of the CH_2Cl_2-soluble extract) chloramphenicol.

CNB741. Activity. The crude extract was tested at 4.6 mg/ml. The activity against the test panel was: CNB739, 9 mm; CNC098, NA; CNF029, NR; CNF028, 7 mm; CNF017, NA; CNF018, NR; CNF019, NR; CNF020, NR; CNF021, NR; CNF022, NA; CNF023, NR; CNF024, NA; CNF025, NA; CNF026, NR; CNF027, NR.

Collection, Isolation and Morphology. CNB741 was isolated from Batiquitos Lagoon sediment sample A6, approximately 20 feet from the north shore, 20 feet east of the railroad bridge at 1.5 feet in depth. On an agar plate with A-1 media, the colony was uneven, raised, wrinkled, and granular with grey-green spores,

indicating it was an actinomycete. The strain was stored in 10% glycerol and A-1 media at -80°C.

Culture and Extraction. The screening culture was scaled-up from the frozen tube inoculum in 10 ml tube of A-1 media (1 day) to 100 ml A-1 in a 500 ml Erlenmeyer flask (2-3 days) to 1 L A-1 media in a Fernbach flask (7 days) with constant shaking at room temperature. This 1 L culture was then extracted with 2 x 1 L ethyl acetate.

Isolation of Nonactin (67). The EtOAc extract was dried over Na_2SO_4, filtered, and evaporated *in vacuo* to yield 166 mg crude organics. The crude extract was separated on a Sephadex LH-20 column (2 cm x 60 cm) using 1:1 CH_2Cl_2:MeOH. The fractions were combined into 10 fractions based on TLC results. The interesting fraction was then separated on reversed-phase HPLC (μsorb, 3 μm, 10 mm x 100 mm) using 70:30 MeOH:H_2O at 2 ml/min, yielding 2.4 mg pure nonactin (1.4% of the crude).

CNB761. Activity. The crude extract was tested at 10 mg/ml. The activity against the test panel was: CNB739, NR; CNC098, NR; CNF029, 14 mm; CNF028, NR; CNF017, NR; CNF018, NR; CNF019, NR; CNF020, NR; CNF021, 13 mm; CNF022, NR; CNF023, NR; CNF024, NR; CNF025, NR; CNF026, 9.5 mm; CNF027, NR.

Collection, Isolation & Morphology. Isolate CNB761 was isolated from heat-treated Batiquitos Lagoon mud sample B7, taken on the south shore, 50 ft. west of the railroad bridge. On an agar plate with A-1 media, the colony was round, flat but uneven, and dry with heavy green-grey spores and aerial hyphae, indicating it was an actinomycete. The strain was stored in 10% glycerol and A-1 media at -80°C.

Culture and Extraction. The screening culture and the dereplication culture were

scaled-up from the frozen tube inoculum in 10 ml tube of A-1 media (1 day) to 100 ml A-1 in a 500 ml Erlenmeyer flask (2 days). At this point, the screening culture was transferred to 1 L A-1 media in a Fernbach flask and grown for 5 days with constant shaking at room temperature. The later culture was transferred from 100 ml to 4 x 100 ml (4 days) using 10 ml inocula, then to 4 x 1 L (5 days). Both cultures were double extracted with ethyl acetate.

Isolation of Valinomycin (69). The EtOAc extract of the 4 L culture of CNB761 was dried over Na_2SO_4, filtered, and evaporated *in vacuo* to yield 698 mg crude extract. Half of the extract was then separated on a 60 ml silica gel (60Å) flash column using 100 ml mixtures of 100:0 to 0:100 TMP:EtOAc in 10% steps followed by 80:20 EtOAc:MeOH. Fractions were combined based on TLC, and the interesting fraction (70:30 and 60:40 TMP:EtOAc) was further purified by normal-phase HPLC (Dynamax silica column, 10 mm x 250 mm) in 80:20 TMP:EtOAc at 2 ml/min. to yield 37 mg pure valinomycin (10.6% of the crude extract).

Saponification/Acetylation of 69. Compound **69** (10 mg) was stirred in 1 ml MeOH at 0°C. Methanolic KOH (1%) was added dropwise (35 drops total). After 22 hr., the reaction was neutralized with 1 N HCl and evaporated under N_2 to give a white powder. The reaction mixture was redissolved in MeOH, filtered, and evaporated again. The products were then dissolved in 1 ml pyridine. Acetic anhydride (0.5 ml) was added, and the reaction was stirred for 16 hr. in a sealed vial. The vial was then placed in an ice bath and several drops of distilled H_2O were added. The solvents were azeotroped with toluene and evaporated *in vacuo*. Upon separation by normal-phase HPLC in 70:30 TMP:EtOAc at 2.5 ml/min., 1.3 mg **72** and 2.3 mg **73** were isolated.

CNF895. Activity. The crude extract was tested at 17.7 mg/ml. The activity

against the test panel was: CNB739, NA; CNC098, NA; CNF029, NA; CNF028, NA; CNF017, NA; CNF018, NA; CNF019, NR; CNF020, NR; CNF021, 10 mm; CNF022, 13 mm; CNF023, NA; CNF024, NA; CNF025, NA; CNF026, NR; CNF027, 13 mm.

Collection, Isolation and Morphology. CNF895 was isolated from Batiquitos Lagoon sediment sample A7, approximately 25 feet from the north shore, 20 feet east of the railroad bridge at 2.5 feet in depth. On an agar plate with B-1 media, the colony was tan, translucent, irregular, lobate, flat, and shiny. At 97x under the oil-immersion microscope, the cells were large, long chains with dark inclusions. The strain was stored in 10% glycerol and B-1 media at -80°C.

Culture and Extraction. The screening culture was scaled-up from the frozen tube inoculum in 10 ml tube of B-1 media (2 days) to 100 ml B-1 in a 500 ml Erlenmeyer flask (2 days). The 1 L culture used for dereplication was cultured similarly to this point, then transferred to 1 L B-1 media in a Fernbach flask (9 days) with constant shaking at room temperature. This 1 L culture was then extracted with 2 x 1 L ethyl acetate.

Isolation of Bisdethiobis(methylthio)gliotoxin (**75**) and Fumitremorgin C (**74**). The 1 L crude extract (590 mg) of CNF895 was separated by flash chromatography using 80:20 TMP:EtOAc to 100% EtOAc in 20% steps, then 90:10 and 50:50 EtOAc:MeOH in 100 ml fractions on a 350 ml column of 60Å silica gel. The 10:90 TMP:EtOAc fraction afforded 34.4 mg of partially purified **75**. The 100% EtOAc and 90:10 EtOAc:MeOH fractions were combined and further purified by normal-phase HPLC in EtOAc on a Dynamax silica column at 2.5 ml/min. The interesting HPLC fraction was separated into 2 distinct peaks on reversed-phase HPLC in 60:40 MeOH:H_2O (μsorb C18 column) at 1.8 ml/min. These fractions were called

4-3-2 (1.3 mg) and 4-3-3 (5.2 mg) and both appeared to be identical to 12α-fumitremorgin C.

CNC100. Activity. The crude extract was tested at 71.3 mg/ml. The activity against the test panel was: CNB739, 7.5 mm; CNC098, NA; CNF029, NA; CNF028, 20 mm; CNF017, NA; CNF018, 8 mm; CNF019, NA; CNF020, NR; CNF021, NR; CNF022, 18 mm; CNF023, 8 mm; CNF024, NA; CNF025, NR; CNF026, 11 mm; CNF027, NR.

Collection, Isolation and Morphology. CNC100 is a *Penicillium* species, isolated from Batiquitos Lagoon mud sample B3, on the south shore, approximately 50 feet west of the railroad bridge. On an agar plate with A-1 media, the colony was round, rippled, entire, and elevated with white spores. The strain was stored in 10% glycerol and A-1 media at -80°C.

Culture and Extraction. The screening culture was scaled-up from the frozen tube inoculum in 10 ml tube of A-1 media (1 day) to 100 ml A-1 in a 500 ml Erlenmeyer flask (2-3 days) to 1 L A-1 media in a Fernbach flask (7 days) with constant shaking at room temperature. This 1 L culture was then extracted with 2 x 1 L ethyl acetate.

Isolation of Ergosterol (76), Ergosterol Peroxide (77) and Unknown A. The EtOAc extract was dried over Na_2SO_4, filtered, and evaporated *in vacuo* to yield 347 mg crude organics. The crude extract was separated on two preparative (1 mm) thick-layer silica plates in EtOAc, and the activity was isolated in the most non-polar fraction. This active fraction was separated on normal-phase HPLC using 60:39:1 EtOAc:TMP:HOAc at 2.0 ml/min., yielding 14.4 mg (4.1% of crude) compound A. Ergosterol (2.6 mg, 0.7% of crude) and ergosterol peroxide (6.1 mg, 1.8% of crude) were purified from the second prep-plate fraction by normal-phase HPLC in

50:50 TMP:EtOAc, 0.1% HOAc at 2.5 ml/min.

Unknown A isolated from CNC100. Compound A, a bright yellow powder, exhibited the following spectral characteristics: IR ($CHCl_3$): 3419, 1606, 1557, 1412, 1347 cm^{-1}; HRFABMS: ($M+H^+$) *m/z* 513.2090 ; UV ($CDCl_3$) 365 (41000), 270 (31000), 266 (31000), 225 (33000) nm; ^1H NMR (500 MHz, $CDCl_3$): δ 17.08 (s), 16.35 (s), 7.57 (dd, 15, 9.8), 7.39 (d, 15), 7.32 (dd, 15, 11), 6.62 (br), 6.36 (d, 8.3), 6.35 (2H, m), 6.14 (m, 7), 4.75 (br), 4.46 (br), 3.77 (s), 1.91 (d, 5.4), 1.88 (d, 6.4), 1.47 (4H, m), 1.47 (d, 6.4), 1.39 (s); ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) 199.8, 191.2, 185.6, 170.3, 163.6, 148.5, 144.2, 139.7, 137.7, 131.3, 131.0, 121.8, 120.0, 111.0, 107.2, 104.1, 99.8, 79.8, 79.0, 59.8, 54.4, 25.7, 23.0, 19.2, 18.8, 18.6, 7.0.

CNF917. Activity. The crude extract was tested at 18.4 mg/ml. The activity against the test panel was: CNB739, NA; CNC098, NA; CNF029, 13 mm; CNF028, NA; CNF017, 10 mm; CNF018, 10 mm; CNF019, NR; CNF020, NR; CNF021, 30 mm; CNF022, 14 mm; CNF023, 11 mm; CNF024, 15 mm; CNF025, 11 mm; CNF026, NA; CNF027, 10 mm.

Collection, Isolation and Morphology. Isolate CNF917 was isolated from Batiquitos Lagoon sediment sample A15, approximately 30 feet from the south shore, 20 feet east of the railroad bridge at 7 feet in depth with swiftly moving water above the sediment. On an agar plate with B-3 media, the colony was yellow, swarming, irregular, lobate, flat and shiny. At 97x with an oil-immersion microscope, the Gram-negative bacterium was small and rod-shaped. The strain was stored in 10% glycerol and B-1 media at -80°C.

Culture and Extraction. The screening culture was scaled-up from the frozen tube inoculum in 10 ml tube of B-1 media (2 days) to 100 ml B-1 in a 500 ml

Erlenmeyer flask (2 days). In a subsequent 4 L growth, inoculae (10 ml each) were taken from the 100 ml culture at this stage to 4 x 100 ml B-1 (2 days) and these were transferred to 4 x 1 L B-1 media in a Fernbach flask (9 days) with constant shaking at room temperature. This 4 x 1 L culture was then combined and extracted with 2 x 4 L EtOAc.

Isolation of Tryptamine Derivative (78). The EtOAc extract was dried over Na_2SO_4, filtered, and evaporated *in vacuo* to yield 1.6 g crude organics. The extract was separated using a 3 cm x 80 cm Sephadex LH-20 column in MeOH. Fractions were combined based on TLC, and the 5th fraction, containing the purple-charring compound, was separated further using a 1 mm preparative (thick-layer) plate in 60:40 EtOAc:MeOH. The tryptamine derivative was isolated from just above the baseline (12.6 mg, 0.8% of the crude).

CNF816. Activity. The crude extract was tested at 15.8 mg/ml. The activity against the test panel was: CNB739, NA; CNC098, NA; CNF029, 10 mm; CNF028, NA; CNF017, 11 mm; CNF018, 11 mm; CNF019, NR; CNF020, NR; CNF021, 23 mm; CNF022, 12 mm; CNF023, NA; CNF024, NA; CNF025, NA; CNF026, NA; CNF027, 13 mm.

Collection, Isolation and Morphology. Isolate CNF816, a Gram-negative bacterium, was obtained from Batiquitos Lagoon sediment sample A7, approximately 40 feet from the north shore, 20 feet east of the railroad bridge at 4.5 feet in depth with slowly moving water above the sediment. On an agar plate with B-3 media, the colony was beige/pink, round, slightly lobate, umbonate, and shiny. The strain was stored in 10% glycerol and B-1 media at -80°C.

Culture and Extraction. The screening culture was scaled-up from the frozen tube inoculum in 10 ml tube of B-1 media (2 days) to 100 ml B-1 in a 500 ml

Erlenmeyer flask (2 days). Scale-up for a subsequent 4 L growth was not recorded. This 4 x 1 L cultures were combined and extracted with 2 x 4 L EtOAc.

Isolation of the Unknown B and Phe-hyPro (**80**). The EtOAc extract of CNF816 was dried over Na_2SO_4, filtered, and evaporated *in vacuo* to yield 537 mg crude organics. The extract was separated using a vacuum flash column eluted with 100:0 to 0:100 TMP:EtOAc in 20% steps then 95:5, 90:10, and 0:100 EtOAc:MeOH. This was followed by reversed-phase HPLC of the most polar fraction with 93:7 CH_2Cl_2:MeOH at 2.5 ml/min. to yield 2.7 mg (0.5% of crude) **80** and 0.9 mg of the impure active fraction.

CNF994. Activity. The crude extract was tested at 16.6 mg/ml. The activity against the test panel was: CNB774, NA; CNC102, NA; CNF939,NA; CNF940, NA; CNF941, NA; CNF942, NA; CNF943, 13 mm; CNF944, 14 mm; CNF945, NA; CNF946, NA; CNF947, NA; CNF948, 16 mm.

Collection, Isolation and Morphology. Isolate CNF994 was obtained from Big Cay Bokel sediment sample 28, taken in 1.5 ft. of water on a sand bottom in the middle of the mangrove embayment. On an agar plate with B-3 media, the colony was round, white, entire, shiny, smooth, and slightly umbonate. The Gram-negative bacterium was stored in 10% glycerol and B-1 media at -80°C.

Culture and Extraction. The culture used for identification of **81** was scaled-up from the frozen tube inoculum in 10 ml tube of B-1 media (1 day) to 100 ml B-1 in a 500 ml Erlenmeyer flask (3 days) to 4 x 100 ml B-1 (10 ml inocula, 3 days), then transferred to 4 x 1 L B-1 media in a Fernbach flask (12 days) with constant shaking at room temperature at all steps. The 4 x 1 L cultures were then combined and extracted with 2 x 4 L EtOAc.

Isolation of *cis*-Cascarillic Acid (**81**). The EtOAc extract was dried over

Na_2SO_4, filtered, and evaporated *in vacuo* to yield 745 mg crude organics. The extract was separated using a 150 ml silica gel (60Å) flash column with 100 ml mixtures of 100:0 to 0:100 TMP:EtOAc in 20% steps followed by 50:50 and 0:100 EtOAc:MeOH. The 60:40 TMP:EtOAc fraction was further separated on a normal-phase HPLC (Dynamax silica, 10 mm x 250 mm) with 35:65 EtOAc:TMP at 1.5 ml/min, yielding 8.6 mg (1.2% of crude) **81**.

cis-Cascarillic Acid (**81**) isolated from CNF994. Compound **81** showed the following spectral characteristics: IR ($CHCl_3$): 3250, 2928, 1711 cm^{-1}; EIMS: M^+, *m/z* 184; ^1H NMR (500 MHz, $CDCl_3$): δ 2.42 (1H, dd, J=11.5), 2.30 (1H, dd, J=11.5), 1.40 (2H, m), 1.38 (2H, d, J=4.9), 1.30 (2H, m), 1.26 (2H, m), 1.14 (2H, m), 1.08 (1H, q, J=5.9), 0.86 (3H, t, J=6.8), 0.81 (1H, M), 0.72 (2H, td, J=4.8), - 0.12 (1H, q, J=4.8); ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) 179.1, 33.6, 31.7, 29.7, 29.1, 28.7, 22.5, 15.4, 14.0, 11.0, 10.7.

CNB773. Activity. The crude extract was tested at 10.9 mg/ml. The activity against the test panel was: CNB774, NA; CNC102, 17 mm; CNF939,NA; CNF940, NA; CNF941, NA; CNF942, NR; CNF943, 13 mm; CNF944, 12 mm; CNF945, NA; CNF946, NA; CNF947, NA; CNF948, NA.

Collection, Isolation and Morphology. Isolate CNB773 was obtained from Big Cay Bokel sediment sample 27, taken at the sand-water interface of the mangrove embayment. On an agar plate with A-1 media, the colony developed white spores quickly, making it difficult to define other morphological characteristics.. The strain was stored in 10% glycerol and A-1 media at -80°C.

Culture and Extraction. The screening culture was scaled-up from the frozen tube inoculum in 10 ml tube of A-1 media (1 day) to 100 ml A-1 in a 500 ml Erlenmeyer flask (2-3 days) to 1 L A-1 media in a Fernbach flask (7 days) with

constant shaking at room temperature for all steps. This 1 L culture was then extracted with 2 x 1 L ethyl acetate.

Isolation of Cycloheximide (84). The EtOAc extract was dried over Na_2SO_4, filtered, and evaporated *in vacuo* to yield 109 mg crude organics. The extract was separated using a silica preparative thick-layer plate (1 mm) developed in EtOAc, yielding 18.7 mg (17.1% of crude) cycloheximide.

CNB766. Activity. The crude extract was tested at 233 mg/ml. The activity against the test panel was: CNB774, 12 mm; CNC102, 23 mm; CNF939,NA; CNF940, NA; CNF941, NA; CNF942, NR; CNF943, NA; CNF944, NA; CNF945, NR; CNF946, NA; CNF947, NR; CNF948, NA.

Collection, Isolation and Morphology. Isolate CNB766 was obtained from Big Cay Bokel sediment sample 10, taken in 2" to 6" of water at the sand-water interface. On an agar plate with A-1 media, the colony was round, yellow, flat, and punched into the agar, with white spores, indicating it was an actinomycete. The strain was stored in 10% glycerol and A-1 media at -80°C.

Culture and Extraction. The screening culture was scaled-up from the frozen tube inoculum in 10 ml tube of A-1 media (2 days) to 100 ml A-1 in a 500 ml Erlenmeyer flask (2 days) to 1 L A-1 media in a Fernbach flask (7 days) with constant shaking at room temperature for all steps. In subsequent cultures, the 100 ml culture was used to inoculate (10 ml each) 6 or 8 x 100 ml (2 days). This was transferred to 6 or 8 x 1 L (11 days), then combined and double extracted with EtOAc in each case.

Isolation of Staurosporine (85). The EtOAc extract of CNB766 was dried over Na_2SO_4, filtered, and evaporated *in vacuo* to yield 233 mg crude organics. The extract was separated using a C18 preparative thick-layer plate (1 mm) developed in

95:5 MeOH:H_2O, yielding 17.4 mg (7.5% of crude) staurosporine.

Isolation of Tirandamycin Analog (86). The 8 L extract was dried over Na_2SO_4, filtered, and evaporated *in vacuo* to yield 1.1 g crude material. The extract was separated in 3 parts on a 3.2 cm x 100 cm Sephadex LH-20 column eluted with MeOH. Fractions were combined based on TLC analysis and separated on an amino HPLC column (Dynamax 60A-NH_2, 10 mm x 250 mm) in 32:15:53 H_2O:Iso:MeOH at 1.5 ml/min. to yield approximately 40 mg (3.6% of crude) of the interesting compound.

Tirandamycin Analog (86) isolated from CNB766. Compound **86** exhibited the following spectral characteristics: IR (thin film): 3370, 3250, 1660, 1613, 1565, 1452, 1002 cm^{-1}; HRFABMS: M^+, *m/z* 420.19780 requires $C_{22}H_{29}NO_7$, Δ 10.5 ppm; ^1H NMR (500 MHz, MeOD-d_4): δ 7.57 (1H, d, J=16), 7.18 (1H, d, J=14), 6.21 (1H, d, J=10), 3.96 (1H, d, J=5.9), 3.75 (1H, d, J=5.8), 3.74 (2H, d?), 3.30 (1H, s), 2.95 (1H, m), 1.90 (4H), 1.51 (3H, s), 1.45 (3H, s), 1.14 (3H, d, J=6.8), 0.71 (3H, d, J=7.3); ^{13}C NMR (50MHz, MeOD): δ (ppm) 203.5, (195), 178.0, 149.6, 144.3, 135.9, 131.0, 117.5, (102), 97.8, 79.9, 77.8, 61.9, 57.9, 51.9, 35.6, 35.5, 22.7, 17.1, 15.6, 12.2, 11.4.

CNF974. Activity. The crude extract was tested at 8.9 mg/ml. The activity against the test panel was: CNB774, 9 mm; CNC102, NA; CNF939,NA; CNF940, NR; CNF941, NA; CNF942, NR; CNF943, NR; CNF944, 8 mm; CNF945, NA; CNF946, NA; CNF947, NR; CNF948, NR.

Collection, Isolation and Morphology. Isolate CNF974 was obtained from Big Cay Bokel sediment sample 16, taken in 2 ft. of water on a sand bottom in the middle of the mangrove embayment. On an agar plate with B-3 media, the colony was beige, swarming, irregular, lobate, flat, and shiny. At 97x with an oil-

immersion microscope, the Gram-negative bacterium was very small, motile, and rod-shaped. The strain was stored in 10% glycerol and B-1 media at -80°C.

Culture and Extraction. The culture used for identification of **88** was scaled-up from the frozen tube inoculum in 10 ml tube of B-1 media (2 days) to 100 ml B-1 in a 500 ml Erlenmeyer flask (1 day) to 4 x 100 ml B-1 (10 ml inocula, 3 days), and these were transferred to 4 x 1 L B-1 media in a Fernbach flask (9 days) with constant shaking at room temperature at all steps. The 4 x 1 L cultures were then combined and extracted with 2 x 4 L EtOAc.

Isolation of *o*-Hydroxybenzamide (88). The EtOAc extract of CNF917 was dried over Na_2SO_4, filtered, and evaporated *in vacuo* to yield 900 mg crude organics. The extract was separated using a 60 ml silica gel (60Å) flash column with 50 ml mixtures of 100:0 to 0:100 TMP:EtOAc in 10% steps followed by 90:10, 80:20, and 50:50 EtOAc:MeOH. The 40:60 TMP:EtOAc fraction was further separated on a normal-phase HPLC (Dynamax silica, 10 mm x 250 mm) with 55:45 EtOAc:TMP at 3.0 ml/min. *o*-Hydroxybenzamide was isolated just after the solvent front (3.2 mg, 0.36% of crude).

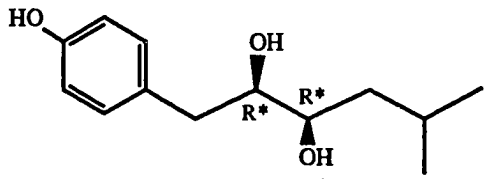
CNF962. Activity. The crude extract was tested at 56.4 mg/ml. The activity against the test panel was: CNB774, NA; CNC102, NA; CNF939,NA; CNF940, NA; CNF941, NA; CNF942, NR; CNF943, 10 mm; CNF944, 11 mm; CNF945, 9 mm; CNF946, NA; CNF947, 7.5 mm; CNF948, 10 mm.

Collection, Isolation and Morphology. Isolate CNF962 is a Gram-positive, unicellular bacterium, obtained from Big Cay Bokel sediment sample 19. The sample was taken from a shell bed at the sand-water interface. On an agar plate with B-1 media, the colony was white, irregular, lobate, flat, and dull. The strain was stored in 10% glycerol and B-1 media at -80°C.

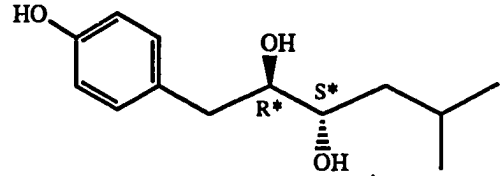
Culture and Extraction. The culture used for identification of **90-94** was scaled-up from the frozen tube inoculum in 10 ml tube of B-1 media (1 day) to 100 ml B-1 in a 500 ml Erlenmeyer flask (2 days) to 6 x 100 ml B-1 (10 ml inocula, 3 days), then transferred to 6 x 1 L B-1 media in a Fernbach flask (7 days) with constant shaking at room temperature at all steps. The 6 x 1 L cultures were then combined and extracted with 2 x 6 L EtOAc.

Isolation of Compounds **90**, **91**, **92**, **93**, **94**, and Unknown D. The EtOAc extract was dried over Na_2SO_4, filtered, and evaporated *in vacuo* to yield 2.2 g crude organics. The extract was separated using a 150 ml silica gel (60Å) flash column eluted with 100 ml mixtures of 85:15 to 0:100 CH_2Cl_2:MeOH in 5% and 10% steps followed by: 1) repeated normal-phase HPLC (Dynamax silica, 10 mm x 250 mm) in 98:2 EtOAc:MeOH at 2.0 ml/min. to yield **90**, **91**, and **92**; and 2) reveresed-phase HPLC (2 columns linked together, Dynamax C18, 10 mm x 250 mm) at 1.5 ml/min. in 70:20:10:1 H_2O:ACN:MeOH:Isopropanol to yield **93** and **94**.

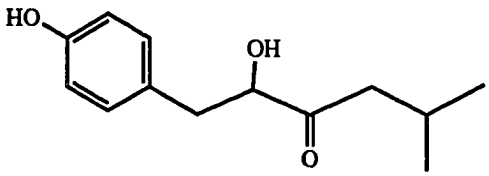
LIST OF COMPOUNDS ISOLATED DURING THIS INVESTIGATION



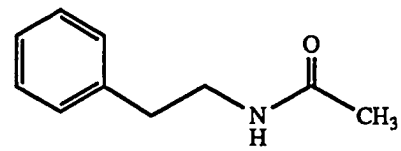
34 guaymasol†



37 epiguaymasol†



37 guaymasone



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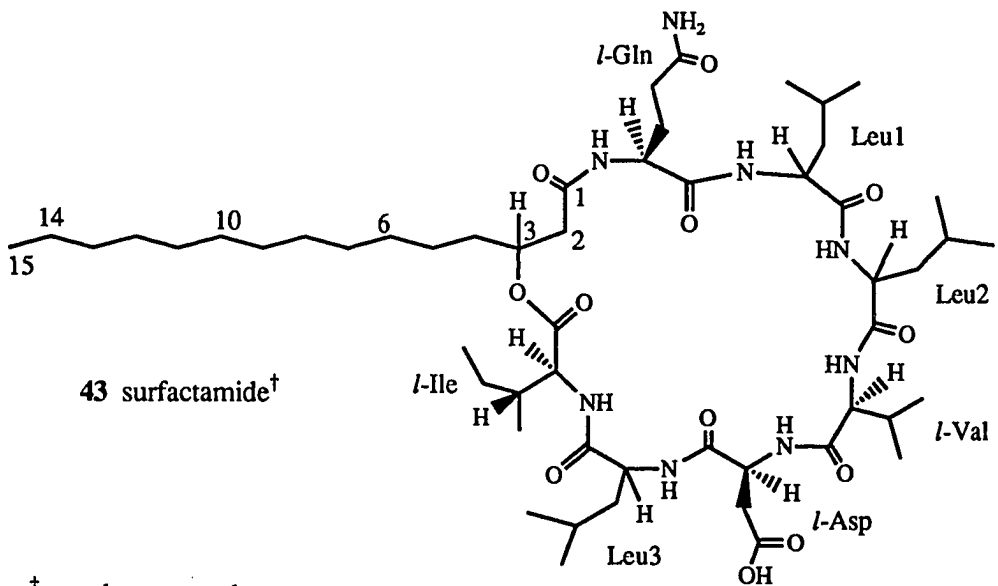
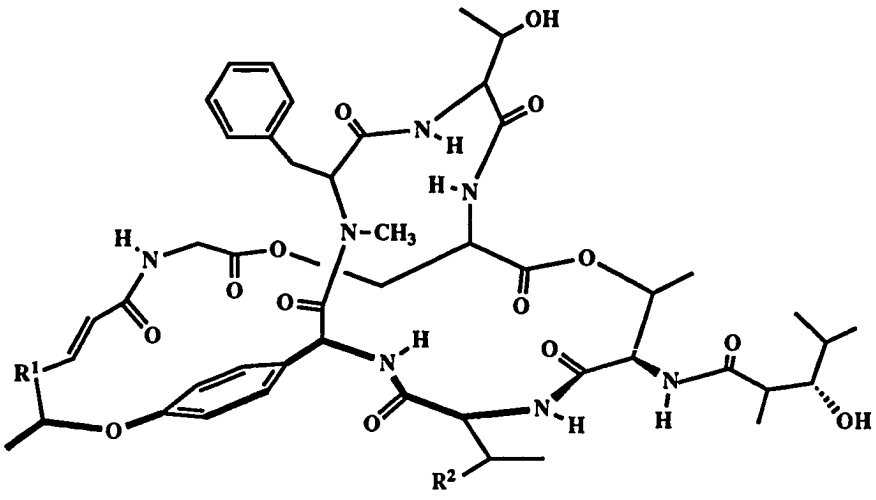


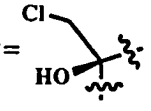
43 surfactamide†

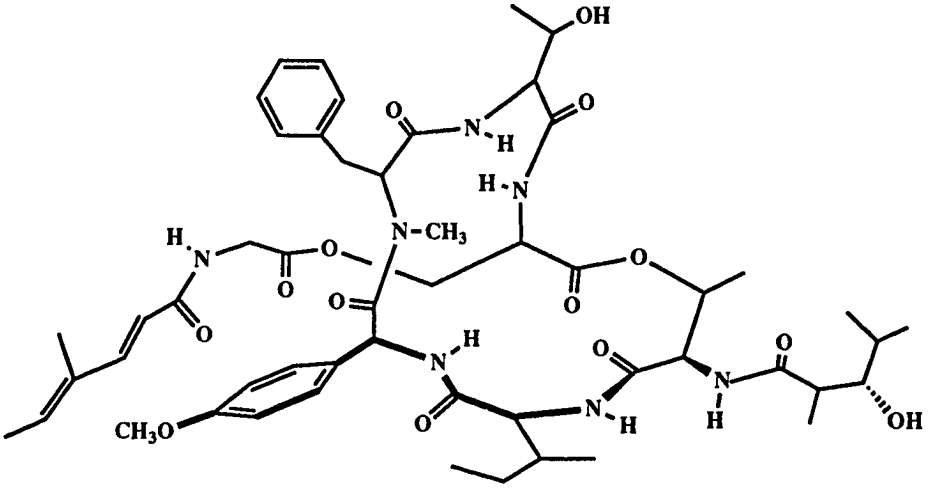
† novel compound



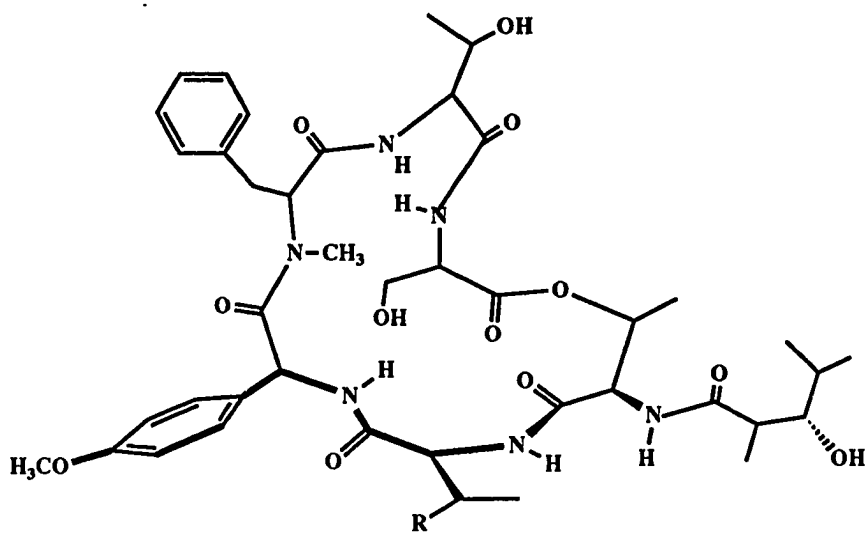
58 salinamide A†: R^1=  , R^2= CH_2CH_3

61 salinamide D†: R^1=  , R^2= CH_3

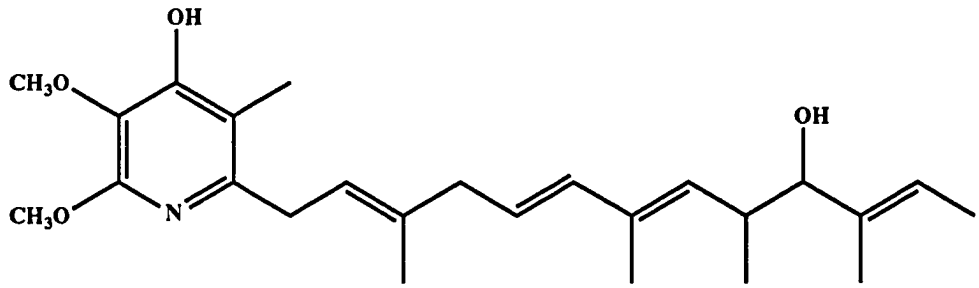
59 salinamide B†: R^1=  , R^2= CH_2CH_3



60 salinamide C†



62 salinamide E†: R= CH_2CH_3
63 salinamide F†: R= CH_3



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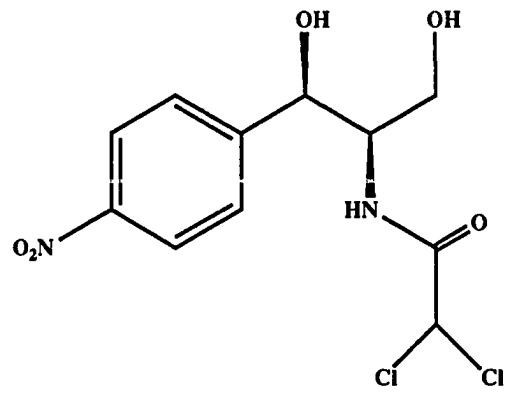
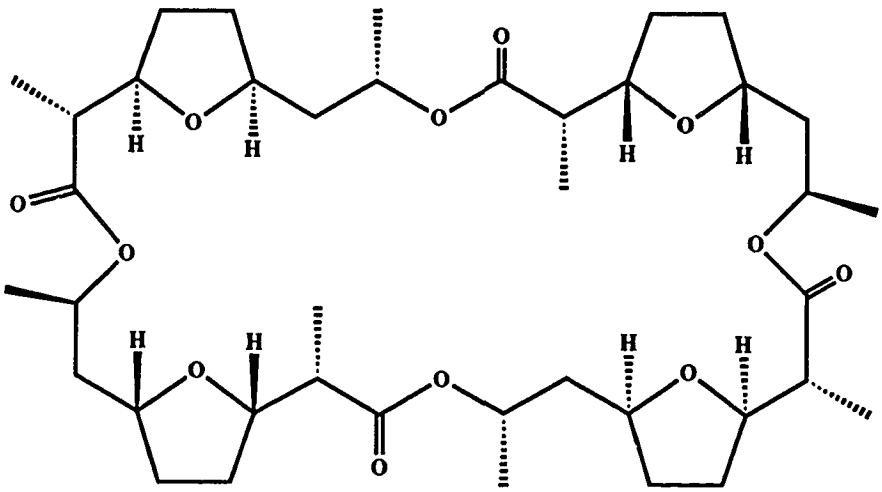


66 Chloramphenicol



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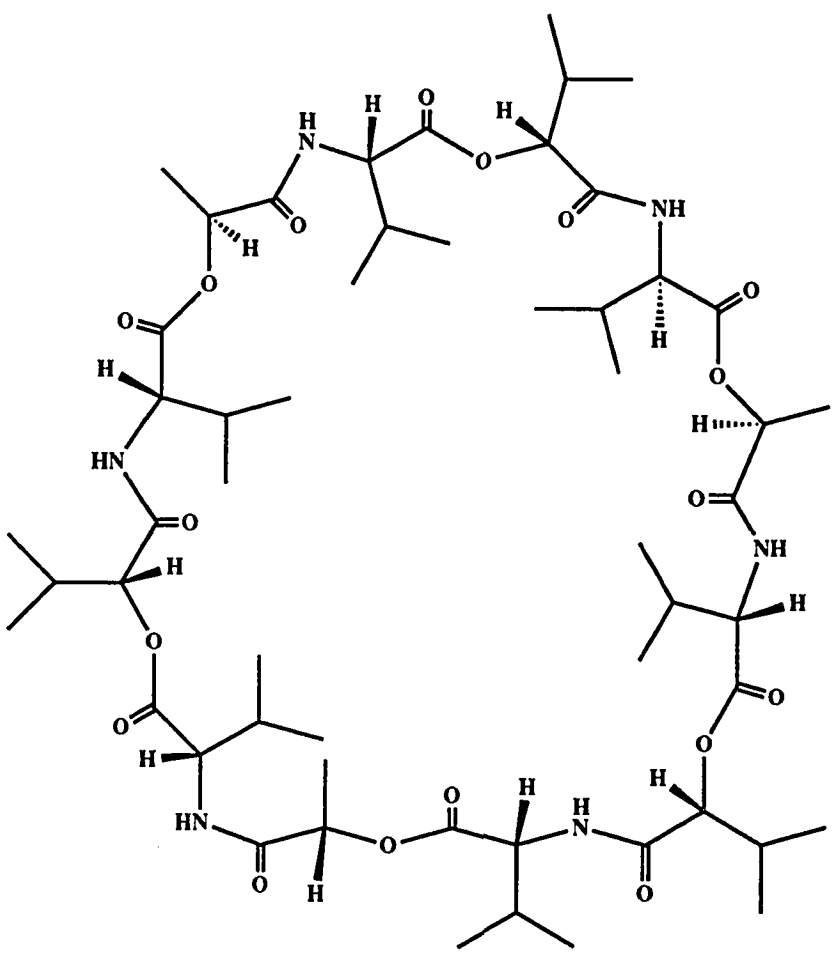

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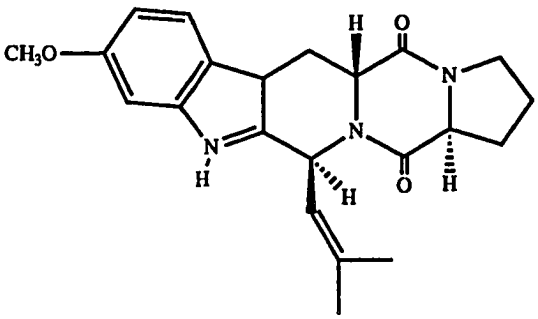
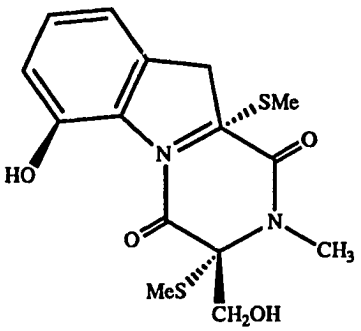
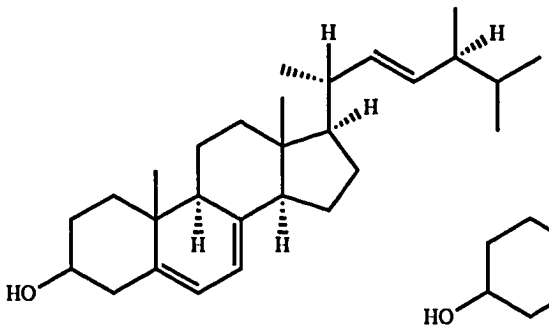


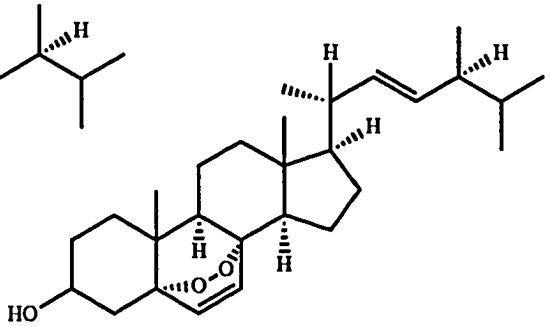
74 fumitremorgin C



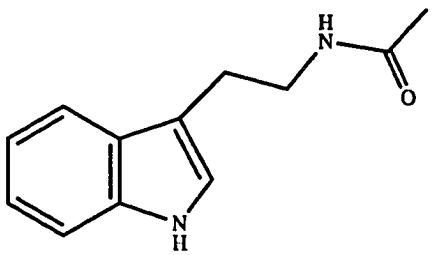
75 bisdethiobis(methylthio)gliotoxin



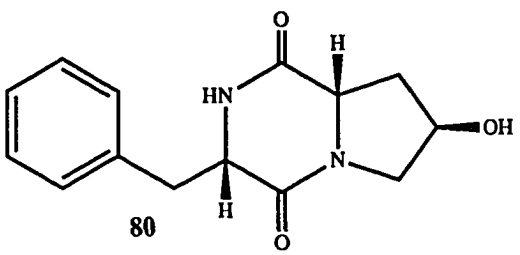
76 ergosterol



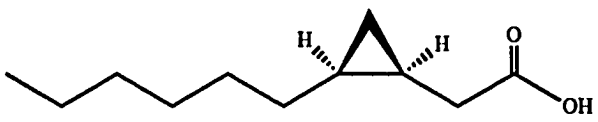
77 ergosterol peroxide



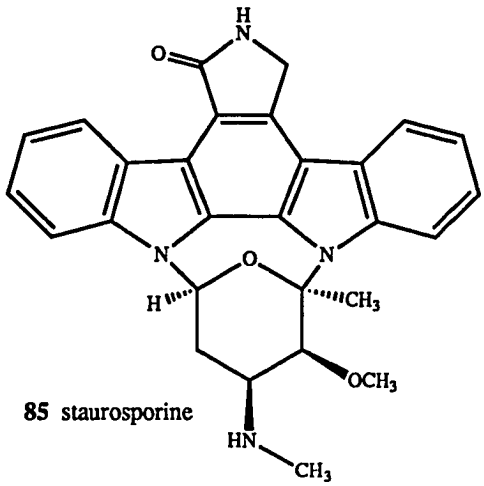
78 N-acetyl-tryptamine



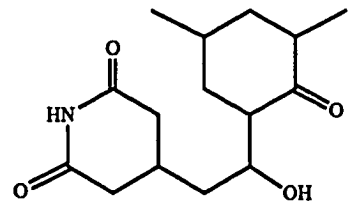
80 phe-hypro diketopiperazine



81 *cis*-cascarillic acid†



85 staurosporine



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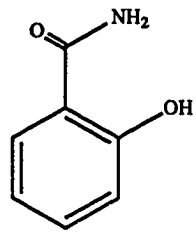
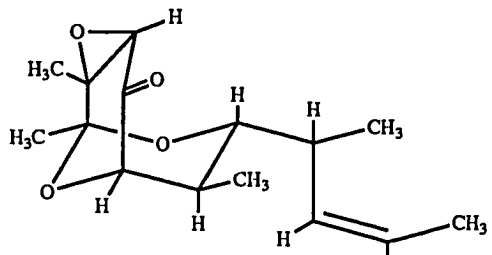
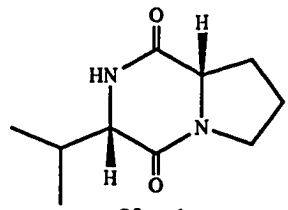


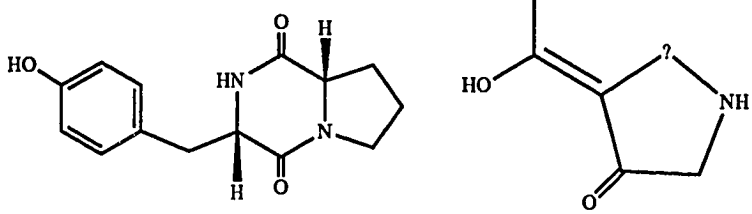
88 *o*-hydroxybenzamide



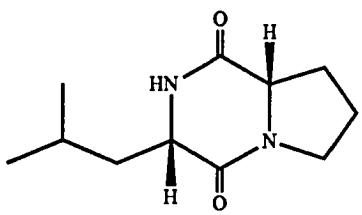
86 tirandamycin analog



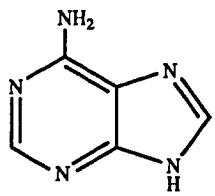
90 val-pro



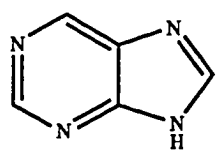
91 tyr-pro



92 leu-pro



93 adenine



94 deamino-adenine

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