AN ABSTRACT OF THE THESIS OF

Christopher J. Nannini for the degree of Master of Science in Pharmacy presented on June 19, 2002.
Title: Novel Secondary Metabolites from a Madagascar Collection of *Lyngbya majuscula*.

Abstract approved _____

William H. Gerwick

Marine organisms produce a variety of secondary metabolites for defense, communication, and reproduction.[1-4] While these uses are essential for the organisms' survival, marine natural products have demonstrated their value to human society as well. Asian countries used algae for centuries to treat or prevent illnesses as wide-ranging as cough, gout, gallstones, goiter, hypertension, and diarrhea. The Chinese created elixirs from the red alga *Digenea simplex* as a remedy for parasite infections of the intestine.[5] The recognition of their potential as pharmaceuticals has led to extensive investigations. Recently, algae have been screened for anticancer compounds, with several cyanobacteria providing many potential candidates.[6-9]

A Madagascar collection of the marine cyanobacterium *Lyngbya majuscula* yielded two new cyclopropyl-containing fatty acid metabolites, lyngbyamides B and C. The isolation of the lyngbyamides was guided by the brine shrimp

(*Artemia salina*) toxicity assay.[20, 21] The structures were established using spectroscopic methods. Semisynthesis of the lyngbyamides was achieved by coupling the acid chloride derivative of the natural C-13 cyclopropyl fatty acid (3-(2-heptyl-cyclopropyl)-propionic acid), and the respective free amines. Bioactivity profiling was conducted for the natural and semisynthetic products using the brine shrimp toxicity assay.

A novel heterocyclic sulfur-containing compound was isolated from a Madagascar collection of *Lyngbya majuscula* using an antifungal (*Candida albicans*) bioassay-guided fractionation. The structure was established using spectroscopic methods consisting primarily of 1D and 2D NMR experiments. Comparisons are made with other related natural and synthetic products.

Novel Secondary Metabolites from a Madagascar Collection of *Lyngbya majuscula*

by
Christopher J. Nannini

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CHAPTER I

GENERAL INTRODUCTION

Thesis Contents

Our laboratory specializes in the isolation and characterization of novel and bioactive compounds from marine algae and cyanobacteria. This thesis describes some of my research in the area of N-acyl containing lipids and a sulfur-containing heterocyclic compound from *Lyngbya majuscula*.

This thesis contains four chapters. Chapter one provides a general introduction to marine natural products and cyanobacteria. Chapter two discusses the isolation and structure elucidation of two related compounds, lyngbyamides B and C, from the cyanobacteria *Lyngbya majuscula*.

The discovery of a heterocyclic sulfur-containing compound, thiolyngbyan, is described in chapter three. This chapter details the isolation, structure elucidation, antifungal activity, and comparisons with other related natural and synthetic products.

Chapter four summarizes the discoveries of these metabolites from *Lyngbya majuscula* and their biological significance.

Introduction

Marine organisms produce a variety of secondary metabolites for defense, communication, and reproduction.[1-4] While these uses are essential for the organisms' survival, marine natural products have demonstrated their value to human society as well. Asian countries used algae for centuries to treat or prevent illnesses as wide-ranging as cough, gout, gallstones, goiter, hypertension, and diarrhea. The Chinese created elixirs from the red alga *Digenea simplex* as a remedy for parasite infections of the intestine.[5] The recognition of their potential as pharmaceuticals has led to extensive investigations. Recently, algae have been screened for anticancer compounds, with several cyanobacteria providing many potential candidates.[6-9]

As organisms responsible for infectious disease become resistant to antibiotics and other drugs, new methods of discovery will be required to keep up with the demand for novel therapeutic agents. Researchers have developed methods such as combinatorial chemistry to produce more effective compounds. However, many scientists believe that the untapped resources of the ocean will provide leads to the new medicines of the future.[10]

During the last three decades of the twentieth century there have been concentrated efforts to explore the marine environment for useful pharmaceutical agents.[11-14] Marine organisms provided novel compounds that confirmed their potential in several fields, not only as therapeutic agents for disease, but as biochemical tools for research as well. Different species of red algae produce agar

and carrageenan, which are used for the preparation of various gels used in biochemical research.

Marine natural product chemists focus their efforts in search for bioactive compounds. This investigative process requires a large commitment of resources and time. A broad view of this process is depicted in Figure I.1.[15] It begins with sample selection in the collection phase, followed by extraction, bioassay-guided fractionation, isolation and structure elucidation.

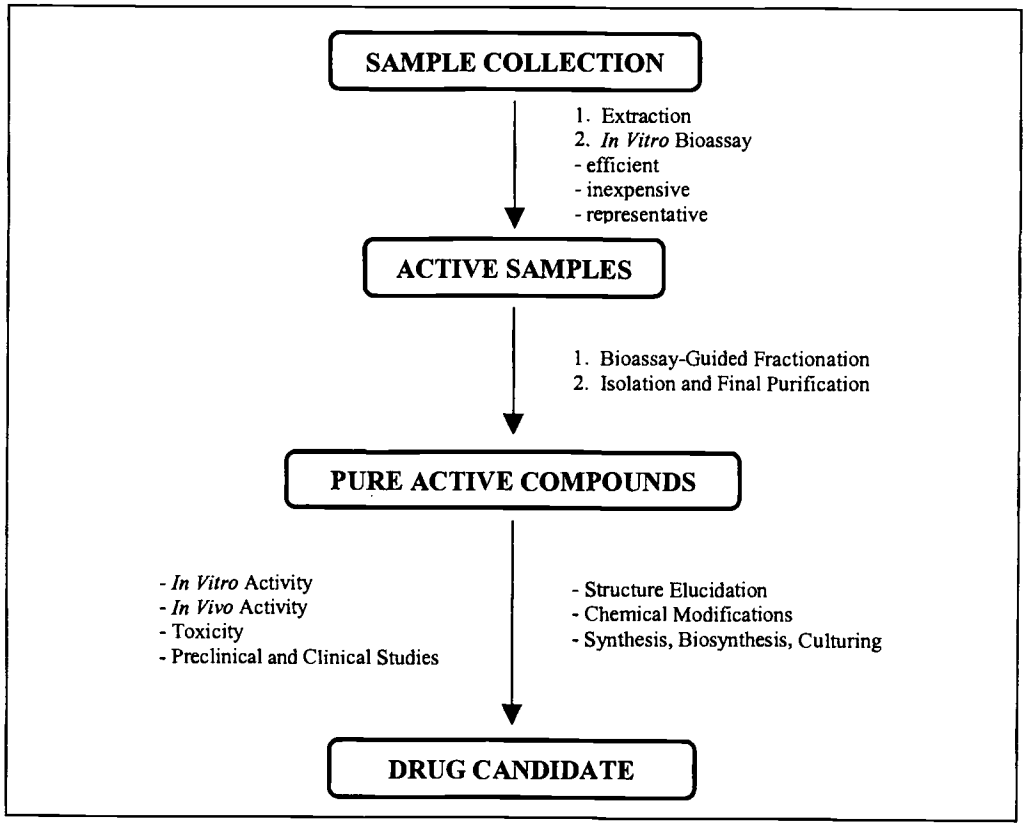


Figure I.1. Investigative Process for Marine Natural Products Chemistry.[15]

Collection, Isolation and Structure Elucidation

Collection is the first step in the investigative process of marine natural products chemistry. The advances in technologies such as scuba diving and studies in remote sensing are overcoming difficulties inherent to making collections in the marine environment.[16,17] Researchers are also dealing with problems in retrieval of sufficient samples by developing diverse research teams and exploring the use of submersibles and autonomous underwater vehicles (AUVs).[18,19]

The likelihood of finding useful bioactive metabolites is reliant on the number of samples screened. Selection of active extracts and fractions is based on fast, economic, and representative initial tests. Our laboratory uses the brine shrimp (*Artemia salina*) toxicity assay and antimicrobial assays (*Bacillus subtilis*, *Candida albicans*, and *Escherichia coli*) to guide us in the selection and fractionation process.[20,21] As our investigations continue, selective fractionations and purification procedures are followed. If the pure compound shows exceptional activity, further pharmacological assays (*in vitro*, *in vivo*) and research (structure modification, preparation of analogs, total synthesis, and cultivation) is explored.

Once individual compounds are isolated in pure form, efforts are focused on structure elucidation. The area of structure elucidation has been enhanced following advances in computers and modern spectrometers. For example, nuclear magnetic resonance (NMR) spectroscopy can define the three-dimensional structure of molecules with as little as 0.1 mg of material.

Research Focus

Our research group specializes in the isolation and characterization of metabolites from marine algae with a focus on marine cyanobacteria. Cyanobacteria provide a rich source of bioactive and structurally unique compounds. This thesis describes my research involving N-acyl containing lipids as well as the isolation of an antifungal heterocyclic sulfur-containing compound.

Marine Natural Products

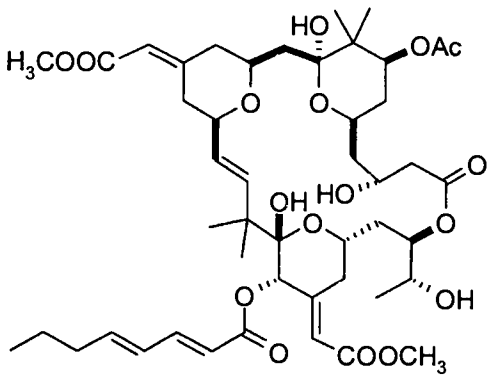
Natural products have formed the foundation for traditional medicine throughout the centuries. China and India, as well as many other countries, have used terrestrial plants to treat illness and disease.[22,23] Approximately 80% of the world's population rely on traditional medicines for their principal resource of health care.[24,25] The earliest use of natural products date back to 2600 BC and are recorded on ancient clay tablets from Mesopotamia. However, the concept and isolation of "pure" compounds as drugs is not recorded until much later in the early 1800s.[26] The history of phytochemistry provided bearing for the early marine natural product chemists of the 1970's. Today, the study of marine natural products combines the fields of marine toxicology, structural chemistry, biochemistry, and marine ecology to create a robust discipline and profession.

Diverse Secondary Metabolites

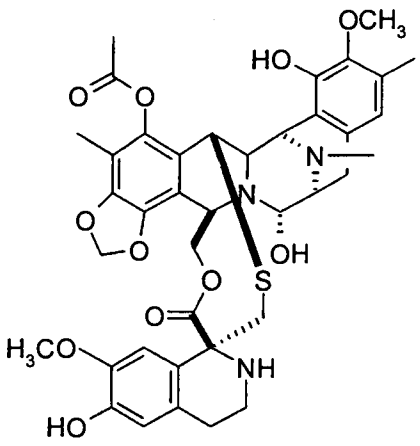
One area of marine natural products involves the study of marine toxins. Several Japanese groups have investigated toxic compounds from marine organisms.[27,28] The necessity for such investigations is supported by the frequency of toxic algal blooms and shellfish poisoning. Another area of concentration lies in the search for bioactive metabolites from marine organisms. During the last thirty years, isolation and structural elucidation of novel bioactive compounds have been the focus of many research groups. Although the pharmaceutical community is still looking for the first medicinal product to reach the consumer shelves, there are many marine natural products under investigation for their drug potential.[29]

There are several anticancer compounds currently under investigation. Bryostatin 1 (**1**) is one such compound isolated from the bryozoan *Bugula neritina* in the 1970's.[30] It is currently in phase 2 anticancer clinical trials. Other marine natural products under investigation include the potential anticancer agents ecteinascidin 743[31,32] (**2**), dehydrodidemnin B[33] (**3**), dolastatin 10[34] (**4**), discodermolide[35] (**5**), halichondrin B[36] (**6**), isohomohalichondrin B[37] (**7**), eleutherobin[38] (**8**), sarcodictyin A[39] (**9**), and curacin A[21] (**10**).

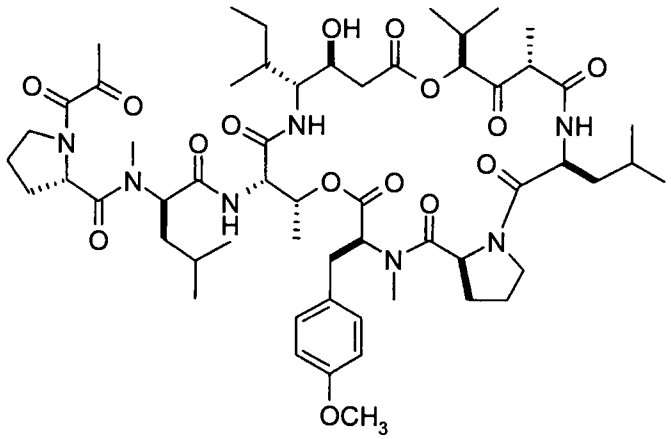
Marine organisms also produce anti-inflammatory agents. Examples of these include pseudopterosins A[40] (**11**) and E[41] (**12**), topsentin[42] (**13**), scalaradial[43] (**14**), and scytonemin[44] (**15**). Manoalide[45,46] (**16**) is another anti-inflammatory agent that has become a standard in inflammation research.



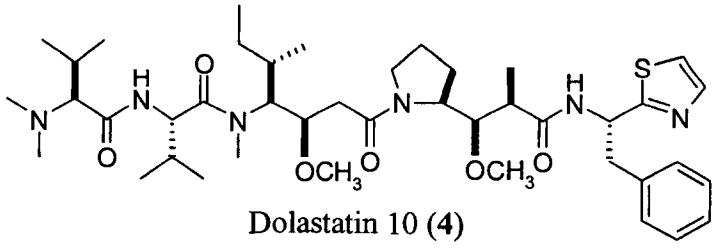
Bryostatin 1 (**1**)



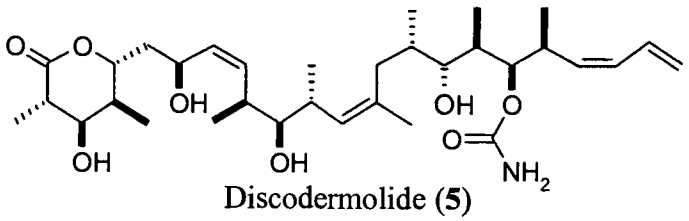
Ecteinascidin 743 (**2**)



Dehydrodidemnin B (**3**)

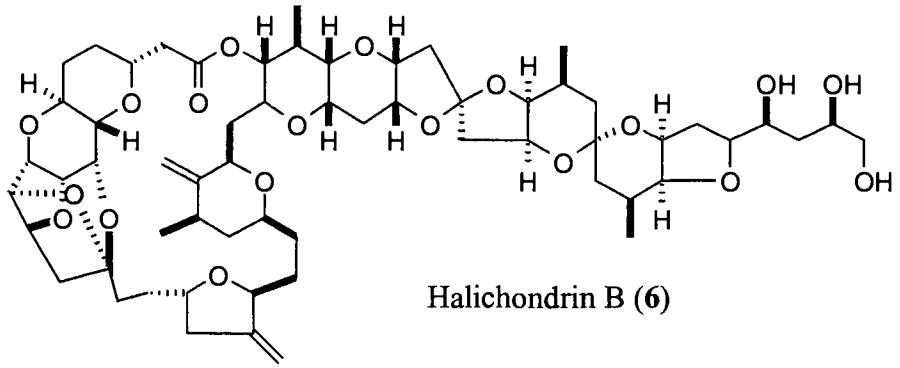


Dolastatin 10 (**4**)

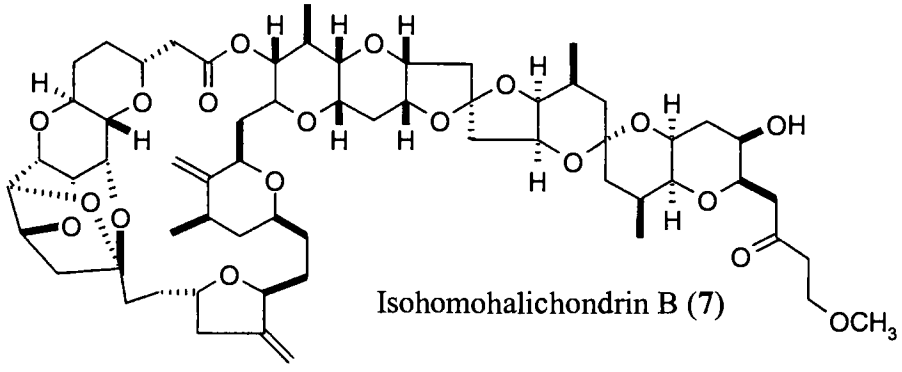


Discodermolide (**5**)

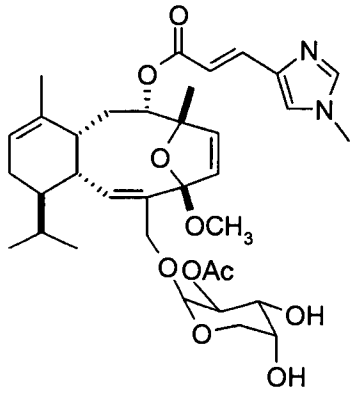
Figure I.2. Anticancer Marine Natural Products.



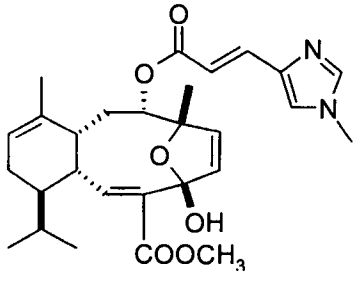
Halichondrin B (**6**)



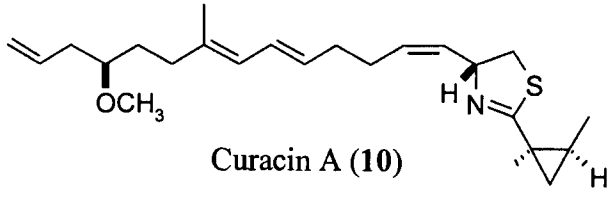
Isohomohalichondrin B (**7**)



Eleutherobin (**8**)

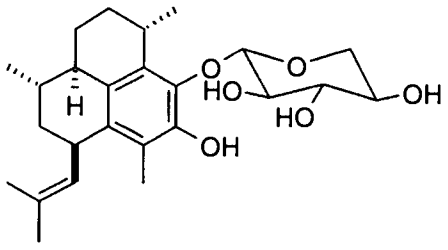


Sarcodictyin (**9**)

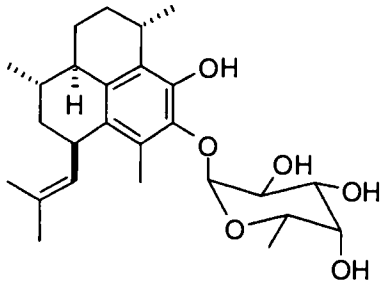


Curacin A (**10**)

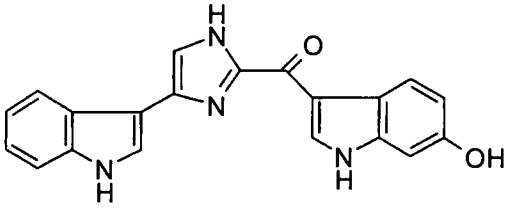
Figure I.3. Anticancer Marine Natural Products.



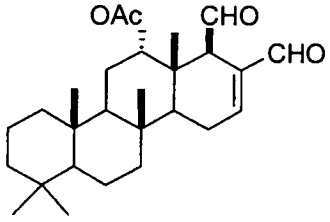
Pseudopterosin A (**11**)



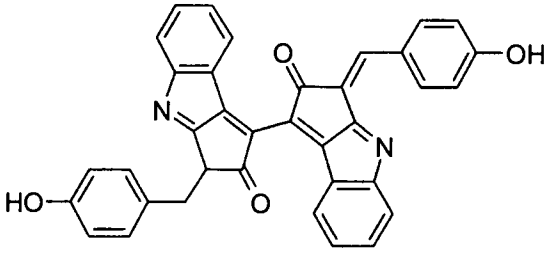
Pseudopterosin E (**12**)



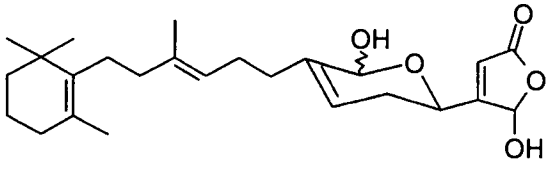
Topsentin (**13**)



Scalaradial (**14**)



Scytonemin (**15**)



Manoalide (**16**)

Figure I.4. Anti-inflammatory Marine Natural Products.

While there are many marine natural products known for their toxic and biomedicinal roles, there are several compounds recognized as signaling molecules that play a part in the control of bacterial biofilm formations.

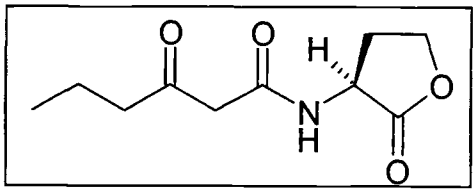


Figure I.5. *N*-3-(oxohexanoyl)-L-homoserine lactone (OHHL)

Researchers have shown that the formation of bacterial biofilms requires communication with acylated homoserine lactone (HSL) cell-to-cell signaling molecules.[1] Understanding the characteristic features of biofilms and the use of signaling molecules for their development will help researchers design new strategies to manage biofilms in medical and industrial environments.[47]

Researchers have shown that furanones from marine algae inhibit swarming *S. liquefaciens* at concentrations found on the surface of the alga.[2,4] Swarming is a coordinated motility that allows bacteria to colonize surfaces in short periods of time. The concentration of AHL determines when the bacterial colony will begin to swarm. Researchers found that incorporating halogenated furanones into swarming media delays the swarming activity of the colony.[48]

The most notable alga that produces furanones is the red seaweed *Delisea pulchra*. *D. pulchra* is a benthic red seaweed common to the southeastern coast of Australia. The alga has been the focus of several chemical, biological activity and ecological studies. These studies have resulted in novel compounds and useful ecological information.[3]

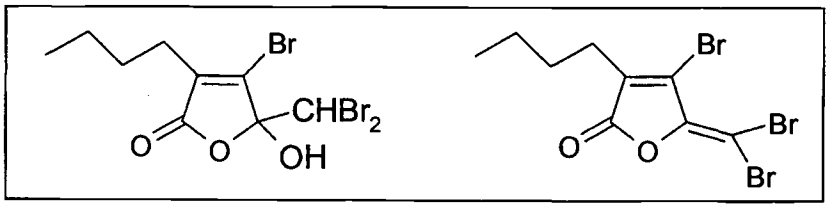


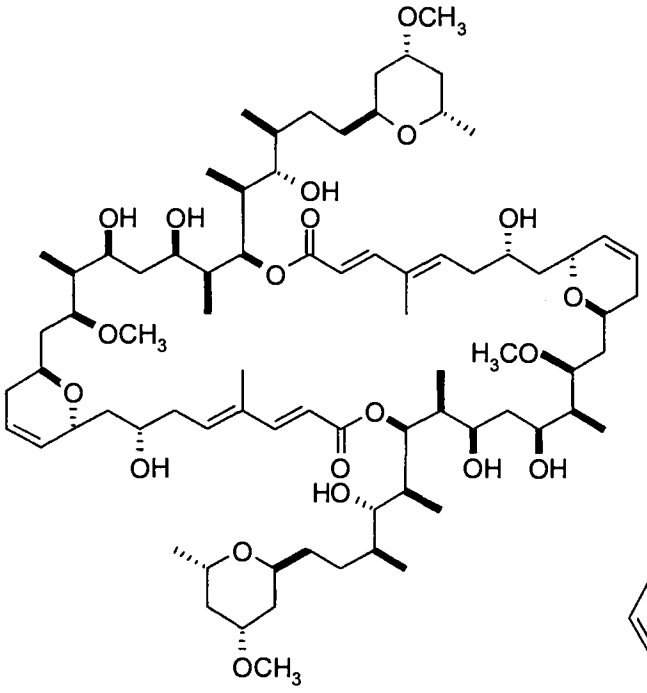
Figure I.6. Brominated Furanones from *Delisea pulchra*.[49]

The furanones found in *D. pulchra* are structurally similar to the acyl HSLs employed by the bacteria in signaling systems. Several of the brominated furanones act as competitive inhibitors to the acyl HSL receptor proteins.[49] In this manner, the molecules prevent bacterial colonization and biofilm formation. In addition, these compounds can deter grazing by marine herbivores.[2,3]

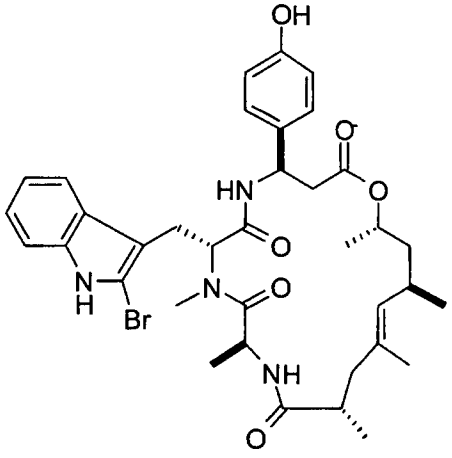
Marine natural product chemists are not only looking for bioactive compounds for their medicinal use. Many secondary metabolites are used as reagents in molecular biology. The sponge metabolites swinholide A[50] (**17**) and jaspamide[51,52] (**18**), which act on actin, ilimaquinone[53] (**19**), which causes vesiculation of the Golgi,[54] and adociasulfate 2[55] (**20**), an inhibitor of motor proteins,[56] are just a few examples of biochemical tools used in research.

Unique Structural Properties

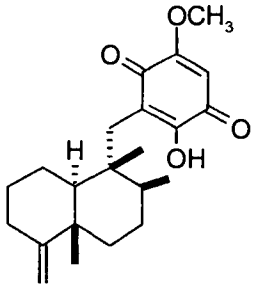
The ancient and recent history has proven natural products to be a rich source of biologically active compounds. They play a significant role in the development of future pharmaceuticals as well as agricultural applications.[57,58]



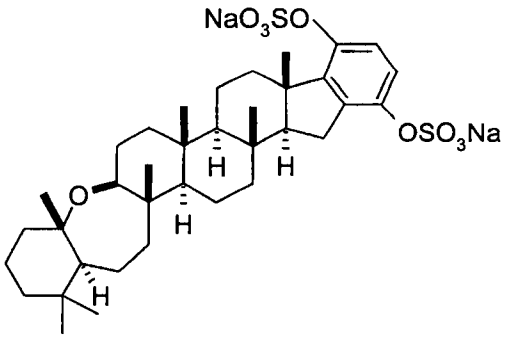
Swinholide A (**17**)



Jaspamide (**18**)



Ilimaquinone (**18**)



Adociasulfate 2 (**20**)

Figure I.7. Marine Natural Products used as Reagents in Molecular Biology.

Emerging technologies drives the future of marine natural products. High throughput screening (HTS) and combinatorial chemistry technologies have opened a new era in drug development. By combining these technologies with innovative methods of sample selection, collection, isolation and structure elucidation, the diverse structures offered by marine natural products will continue to enhance the lead-finding process of drug discovery.

Henkel *et al.*[57] explored the future role of natural products by evaluating whether natural products represent a structurally unique pool of test substances that differ significantly from synthetic compounds. His evaluation was based on the analysis of five databases: *Dictionary of Natural Products* (*DNP*); *Bioactive Natural Product Database* (BNPD); *Available Chemicals Directory* (*ACD*); *Synthetics*; *Drugs*. The databases describe the majority of the published natural products, available chemicals, synthetic test compounds, and pharmaceutical compounds.

Henkel's studies showed that natural products have higher molecular weights than synthetic compounds. While the content of oxygen was higher in natural products, synthetic chemicals had higher numbers of nitrogen, halogen, and sulfur atoms. Additionally, natural products were more complex. They contained a higher number of rings, chiral centers, and sp^3-hybridized bridgehead atoms per molecule (Table I.1).

Table I.1. Structural Properties.

Abundance of selected structural properties from all the individual entries of three representative data bases as well as the average number of structural properties per molecule.[57]

Properties	Drugs	Synthetics	DNP
bridgehead atoms with three ring bonds	25 %	9.0 %	49 %
bridgehead atoms with four ring bonds	4 %	1.4 %	13 %
rotatable C-C bonds	74 %	58.0 %	66 %
rings per molecule	3.0	2.6	3.3
chiral centers per molecule	1.2	0.1	3.2
rotatable bonds per molecule	10.7	8.0	11.1

Table I.2. Pharmacophoric Groups.

Combinations of pharmacophoric groups and their abundance.[57]

Pharmacophoric Groups	Drugs (%)	Synthetics (%)	DNP (%)
alcohol/ether	19	5	41
alcohol/ester	10	3	30
arene/alcohol	24	13	40
arene/alcohol/ether	12	5	27
amine/arene	50	40	15
arene/amide	31	43	12
amine/arene/amide	20	15	5

When comparing the number of pharmacophoric groups per molecule between the synthetic compounds and natural products, Henkel found that they were similar statistically (DNP, 3.2 pharmacophores/molecule compared with Synthetics, 3.3). However, when comparing the abundance of combinations of pharmacophoric groups, he found that the difference between drugs, synthetic compounds and natural products is much greater (Table I.2).

Interestingly, through computer similarity analysis, the occurrence of structural analogs for every type of natural product molecule was determined to be about 85%. Thus, the DNP database can be truncated from 80,000 compounds to 11,500 structurally unique molecules. Forty percent of the natural product structural motifs are not found in the synthetic assemblage.

Henkel also compared the structural properties of individual natural sources. He analyzed the differences between molecular weight and heteroatom

distribution. Results showed the percentage of marine macroorganisms and algae containing various heteroatoms: Oxygen, 95%/90%; Nitrogen, 53%/33%; Sulfur, 10%/8%; Halogens 20%/30% (respectively).

The diverse applications and unique structural properties of natural products demonstrate their importance in ecological studies, biochemical tools, and bioactive metabolites as potential pharmaceutical lead compounds. In the next section, I will discuss some of the natural products isolated from marine cyanobacteria.

Marine Cyanobacteria

Life on earth can be organized into a classification system consisting of five kingdoms: Monera, Protista, Fungi, Plantae, and Animalia. The kingdom Monera includes the phylum cyanobacteria. Cyanobacteria, like other bacteria, are prokaryotes. The term "prokaryote" means "before the nucleus" and refers to the internal organization of the cells. Prokaryotes lack a defined nucleus and organelles that can be found in all other kinds of cells. Although other bacteria may have existed during the Archaean period, cyanobacteria-like creatures are the oldest group of organisms identified in the fossil record. Microfossils found in Australia are more than 3.5 billon years old.[59,60]

Cyanobacteria are distinguished from bacteria by the existence thylakoids, internal membranes that enclose chlorophyll and other structures essential for photosynthesis. Plants have two kinds of chlorophyll called a and b, whereas cyanobacteria contain only chlorophyll a. Cyanobacteria contain secondary

pigments such as c-phycocyanin (blue) and c-phycoerythrin (red), giving the organism varying appearances of color depending on the amount of each pigment.[61]

Cyanobacteria reproduce asexually by binary fission, spore production, or fragmentation. They may be free-living, aggregate into colonies, form fine hair-like strands called filaments, or gelatinous masses. While most lack flagella and are nonmotile, species like *Oscillatoria* have developed alternate methods of movement. Its filamentous forms are able to rotate in a screw like manner, allowing it to move about. Other species that have a gelatinous form incorporate their slippery mucus to glide around their environment.[61]

Early History

Fossilized cyanobacteria have been found in rocks more than three billion years old. The accumulation of cyanobacteria is responsible for Proterozoic oil deposits in sedimentary rock.[62,63] During the Archaean and Proterozoic Eras, cyanobacteria fashioned the course of evolution and ecological change that made the essential atmosphere for life on earth today. Scientists theorize that the photosynthesis of cyanobacteria led to the accumulation of oxygen in the atmosphere.[61]

Another contribution of the cyanobacteria is the origin of plants. The chloroplast within plants is derived from a cyanobacterium living within the plant's cells. During the late Proterozoic or early Cambrian Eras, cyanobacteria formed a symbiotic relationship with some eukaryotic cells. The process of

merging free-living bacteria within phagotrophic eukaryotes to form organelles is called primary endosymbiosis. The genetic material of the bacteria becomes incorporated into the host's nuclear genome. Several eukaryotic algal organisms, such as cryptomonads and dinoflagellates, obtained plastids in subsequent acquisitions of existing endosymbionts known as secondary and tertiary endosymbiosis, respectively. Molecular genetic evidence supports the idea that plants incorporated chloroplasts in a similar fashion.[59,64,65]

Collections of Marine Cyanobacteria

Cyanobacteria are found in typical aquatic and terrestrial habitats, hot springs, glaciers, and some of the most unassuming locations such as tree bark and desert rocks.[66] They also provide significant ecological functions in the environment, such as nitrogen fixation.[61]

Marine natural product chemists collect cyanobacteria from many oceans around the world. Organisms collected from tropical regions lying near the equator provide the bulk of the sources for research. This can be attributed to the rich biodiversity of marine ecosystems consisting of mangroves, seagrass beds and coral reefs. The primary collection sites include the Caribbean, South Pacific, and Indo-Pacific regions.[67]

Secondary Metabolites

Cyanobacteria are beneficial as well as harmful to humans and the environment. Some serve as natural fertilizers in rice paddies and other agricultural crops. Others produce mild to very potent toxins.[68] Mild cyanobacteria toxins in sea water cause a rash known as swimmer's itch, while potent neuromuscular toxins from other cyanobacteria kill fish and animals that share the same water source.

In addition to toxins, cyanobacteria produce many compounds of pharmaceutical interest. Some of these bioactive metabolites demonstrate their potential as cytotoxic, antiviral, fungicidal, and antimicrobial compounds.[67]

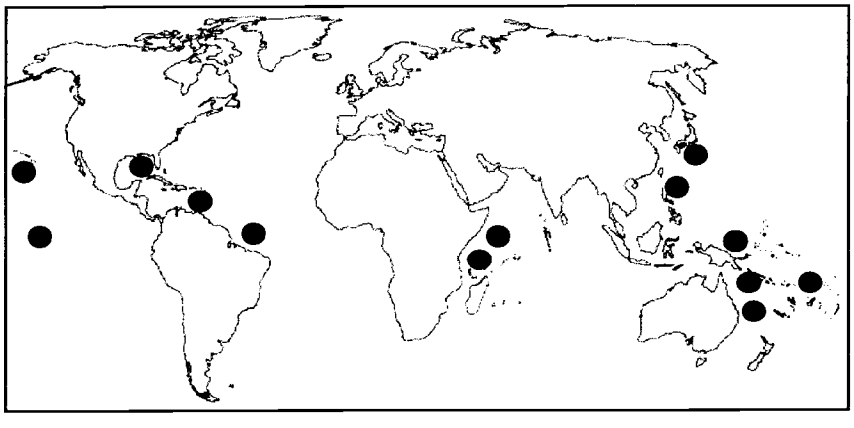
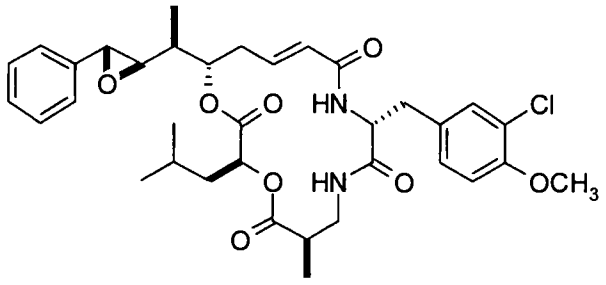


Figure I.8. Primary Collection Sites for Marine Cyanobacteria.

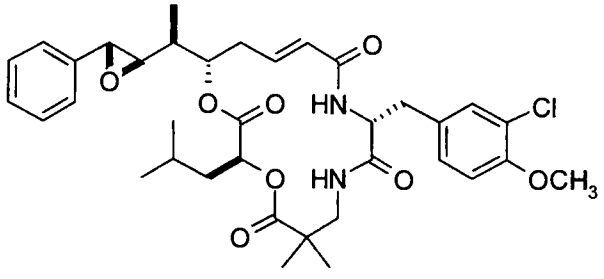
When comparing the cyanobacterial metabolites, we see two developing themes in relation to their biological activity. The first is the high incidence of metabolites that disrupt tubulin and actin formation in eukaryotic cells. The second includes metabolites that impede mammalian ion channels.[67]

Many novel compounds have been elucidated using bioassays for anticancer, antibacterial, antifungal, and protease inhibitory effects.[69,70] The strain *Nostoc* GSV 224 synthesizes cryptophycins which inhibit microtubule assembly; examples of such compounds include cryptophycin A[71] (**21**) and the synthetic derivative, cryptophycin 52 (**22**).[72,73] These compounds demonstrate anticancer activity against a broad spectrum of tumors.[71,72] A variety of other antimitotic and cytotoxic metabolites are produced by *Lyngbya majuscula*, such as the dolastatins, curacin A (**10**), and the lyngbyabellins (**23** and **24**).[74-78]

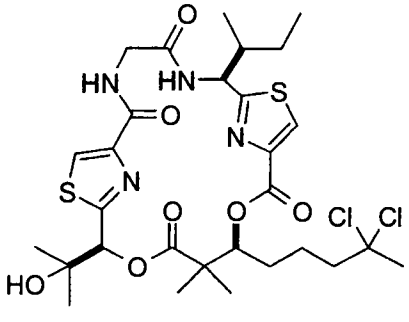
Some important compounds originally isolated from marine animals were later found in cyanobacteria. Examples include compounds such as the dolastatins, which were initially found in the seahare *Dolabella auricularia*. Recent studies support a dietary origin of cyanobacterial compounds in this invertebrate.[79] Other examples of symbiotic relationships exists where the producer organism for a secondary metabolite turned out to be of cyanobacterial origin rather than the marine animal it was originally isolated from, such as tunicates and sponges.[61,67]



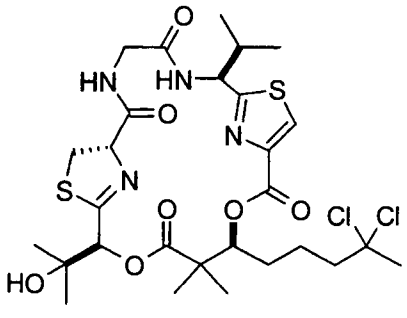
Cryptophycin A (**21**)



Cryptophycin 52 (**22**)



Lyngbyabellin A (**23**)



Lyngbyabellin B (**24**)

Figure I.9. Antimitotic and Cytotoxic Metabolites.

Ecological Functions

Cyanobacteria serve as primary producers by capturing energy from sunlight and in turn are associated with the production of carbohydrates, fats, proteins and other organic compounds in complex aquatic ecosystems. Primary production is sometimes increased through symbiotic associations between organisms.[80] It is not surprising then to see the surfacing of symbiotic relationships between cyanobacteria and other marine organisms.[81]

An interesting discovery concerning the antipredatory functionality of *L. majuscula* compounds indicated that they were selective when it comes to what type of herbivore they deter. Tested extracts inhibited feeding by rabbitfish, a generalist reef herbivore, whereas the same extracts encouraged feeding by the sea hare *Stylocheilus longicauda*.[67]

Biosynthetic Themes

Cyanobacteria produce numerous secondary metabolites, in particular, integrated nonribosomal peptide and polyketide structures in the form of peptides and depsipeptides. Two forms of biosynthetic combinations are seen. In the first, we see the linking of polyketides to amino acids through amide or ester linkages. The second combination takes the form of amino acids used as starter units for polyketide extension, an example being curacin A (**10**). The predominant residues used in polyketide extensions are proline, cysteine, and valine.[67]

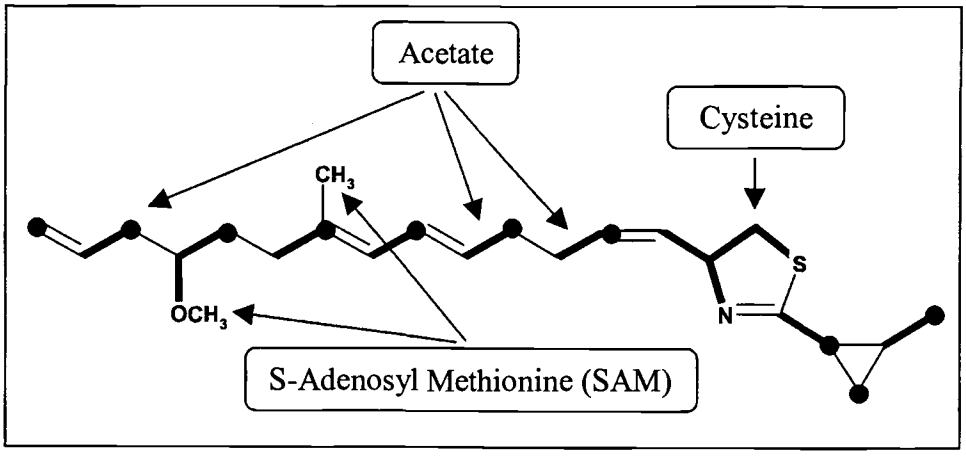


Figure I.10. Biosynthetic Themes Identified in Curacin A.[65]

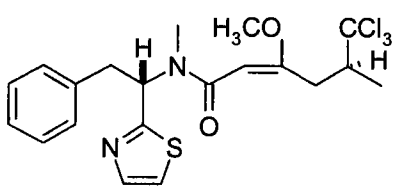
Aliphatic amino acids (Ala, Val, Leu, Ile, Pro, Phe) comprise the majority of the amino acid residues in marine cyanobacterial metabolites. The polar amino acids used most commonly are Gly, Cys, Tyr, and Ser. Generally, we do not see many basic or acidic amino acid residues in the marine cyanobacterial metabolites.[67]

Another identifying characteristic of the amino acid residues that should be considered is their stereochemistry; while most are found in the L-configuration, a substantial number of D-amino acids are present as well. Additionally, it is not uncommon to find N or O-methylated residues. An exception to this trend is cysteine due to the fact that all of the marine cyanobacterial metabolites with this amino acid residue are found in heterocyclized forms. Approximately 83% of these heterocycles are achiral thiazoles. These heterocyclizations occur primarily

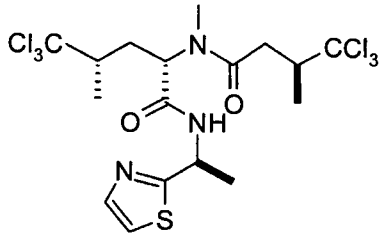
between the cysteine residues and the carbonyl of neighboring glycine, alanine, valine, proline, leucine, isoleucine, and phenylalanine residues.[67]

Marine cyanobacterial metabolites also contain several halogenated functional groups. The trichloromethyl group of barbamide[83] (**25**) and dysidin[84,85] (**26**), the vinyl chloro group found in malyngamide A[86] (**27** and **28**), and the gem-dichloro group of the lyngbyabellins are a few examples.[67]

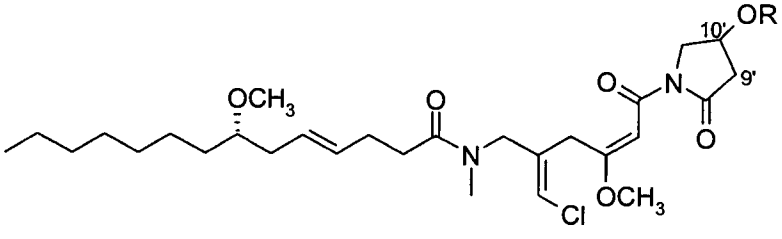
Another common trend is the use of S-adenosyl methionine (SAM) to form the pendant methyl groups on methyl-substituted polyketides. Through various



Barbamide (**25**)



Dysidin (**26**)



Malyngamide A (**27**) R = CH_3, $\Delta^{9',10'}$
Malyngamide B (**28**) R= H

Figure I.11. Halogenated Cyanobacterial Metabolites.

stable and radioactive isotope precursor-feeding studies, this type of biosynthetic theme was identified in the formation of curacin A **(10)**.[67,87,88] S-adenosyl methionine is also responsible for the O-methylation of several cyanobacterial metabolites.

The next chapter describes the isolation and characterization of two new N-acyl containing lipids. The compounds represent examples of compounds formed from fatty acids linked via an amide bond to amino acid residues from the cyanobacteria *Lyngbya majuscula*.

CHAPTER II

LYNGBYAMIDES B AND C, N-ACYL CONTAINING LIPIDS FROM THE MARINE CYANOBACTERIUM *LYNGBYA MAJUSCULA*

Abstract

A Madagascar collection of the marine cyanobacterium *Lyngbya majuscula* yielded two new cyclopropyl-containing fatty acid metabolites, lyngbyamides B (**30**) and C (**31**), and the re-isolation of grenadamide (lyngbyamide A) (**29**), previously reported from a Grenada collection of *L. majuscula*.[89] The isolation of the lyngbyamides was guided by the brine shrimp (*Artemia salina*) toxicity assay.[20, 21] The structures were established using spectroscopic methods. Semisynthesis of the lyngbyamides was achieved by coupling the acid chloride derivative of the natural C-13 cyclopropyl fatty acid (3-(2-heptyl-cyclopropyl)-propionic acid) (**34**), and the respective free amines. Bioactivity profiling was conducted for the natural and semisynthetic products using the brine shrimp toxicity assay.

Introduction

Cyanobacteria are a rich source of chemically and biologically interesting compounds. A significant focus for our laboratory involves the characterization of metabolites isolated from this source. This chapter describes the investigation of two N-acyl containing lipids isolated from a brine shrimp active crude extract of *Lyngbya majuscula*. We employed bioassay-guided fractionation during this project to facilitate the isolation of these interesting compounds.

Lyngbya majuscula is a filamentous marine cyanobacterium found in tropical and sub-tropical estuarine and marine environments worldwide.[66,90] The species acquired its name after a Danish botanist H.C. Lyngbye.[90] In the 1930's, the German phycologist Lothar Geitler identified nearly 100 *Lyngbya* species.[91] *Lyngbya* grows as thin filaments 10-30 cm long attached to seagrass, rocks and macroalgae. The organism forms mats that can cover the seafloor. In extended periods of sunlight and warm weather, increased photosynthesis promotes the formation of bubbles caused by increased oxygen. The bubbles become trapped in the microalgae mats causing them to float to the surface. In bloom conditions, toxin levels increase in the surrounding water and may remain in sun dried *Lyngbya* that collects on the beach.

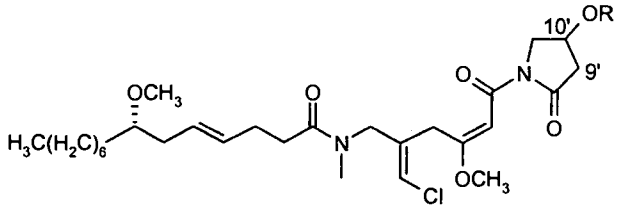
The toxins in algal blooms cause dermatitis, eye and nose irritations, and respiratory problems like asthma. The rash that forms on unfortunate beachgoers and divers due to cyanobacterial toxins is known as "swimmer's itch".

The marine cyanobacterium *Lyngbya majuscula* produces many biologically active and structurally unique secondary metabolites. The majority of the natural products isolated from *L. majuscula* have polyketide-derived subunits that are combined with amino acid-derived components. Examples of these compounds include the malyngamides, and the hermitamides.[92-104]

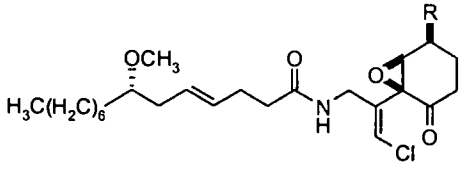
The malyngamides consist of several compounds demonstrating unique structural variations (Figure II.1 and Figure II.2). The compounds exhibit a methoxy fatty acid and an assortment of functionalized amines that are connected through an amide bond representing a typical metabolic theme of cyanobacteria. The malyngamides show modest potency in fish, brine shrimp, and cancer cell toxicity ranging from 1-50 μM concentrations.[67]

Another set of malyngamide-type compounds was isolated from a Papua New Guinea collection of *L. majuscula*. Named after their collection site from the coral reefs at Hermit Island Village, the hermitamides A (**27**) and B (**28**) displayed an LD_{50} for brine shrimp toxicity at 5 μM and 8 μM, respectively. Additionally, the IC_{50} to Neuro-2a neuroblastoma cells was 2.2 μM for A and 5.5 μM for B.[67]

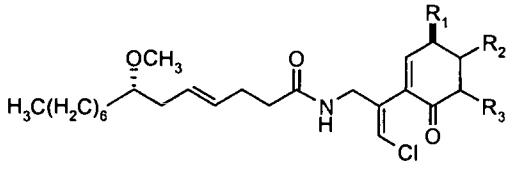
A recent isolation of natural products revealed grenadamide (lyngbyamide A) (**29**) from a Grenada collection of *L. majuscula*.[89] The nitrogen-containing compound displayed an amide linkage similar to the metabolites described above. This structural trend is found in other compounds that demonstrate important physiological activity in mammalians, such as the endocannabinoid, anandamide (**33**).[67,105,106]



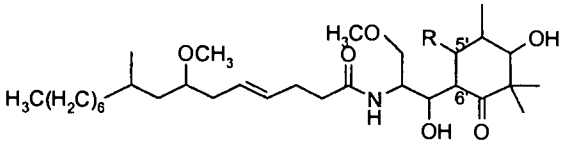
Malyngamide A (**1**) R = CH_3, $\Delta^{9',10'}$
Malyngamide B (**2**) R = H



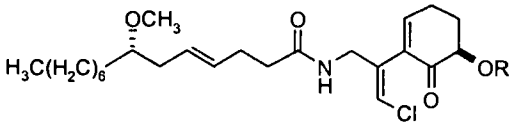
Malyngamide C (**3**) R = OH
Malyngamide C Acetate (**4**) R = OAc
8'-Deacetoxymalyngamide C (**5**) R = H



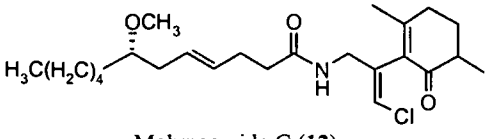
Deoxymalyngamide C (**6**) R_1 = OH, R_2 = R_3 = H
Dideoxymalyngamide C (**7**) (= Malyngamide K) $R_{1,2,3}$ = H
Malyngamide L (**8**) R_1 = H, R_2 = OH, R_3 = CH_3



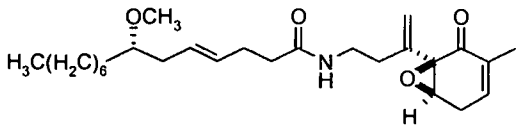
Malyngamide D (**9**) R = OH
Malyngamide E (**10**) R = H, $\Delta^{5',6'}$



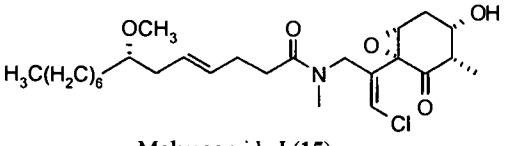
Malyngamide F (**11**) R = H
Malyngamide F Acetate (**12**) R = Ac



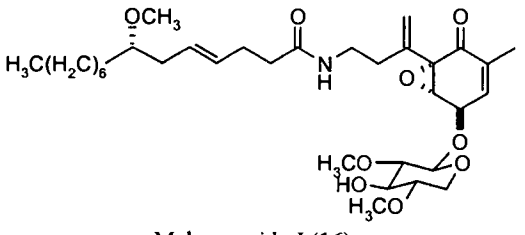
Malyngamide G (**13**)



Malyngamide H (**14**)

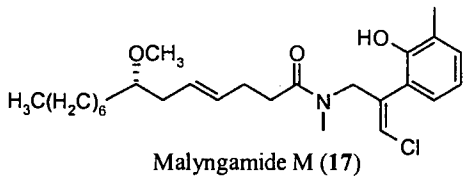


Malyngamide I (**15**)

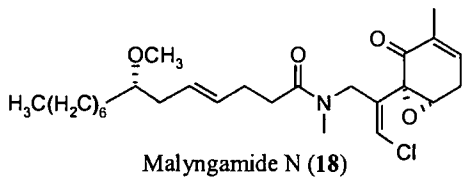


Malyngamide J (**16**)

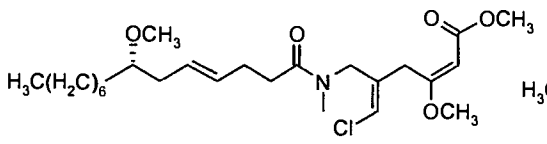
Figure II.1. Malyngamides from Marine Cyanobacteria.



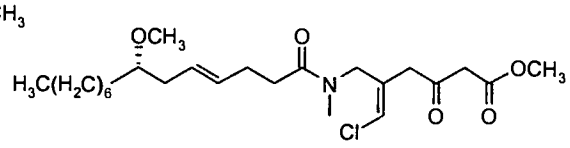
Malyngamide M (**17**)



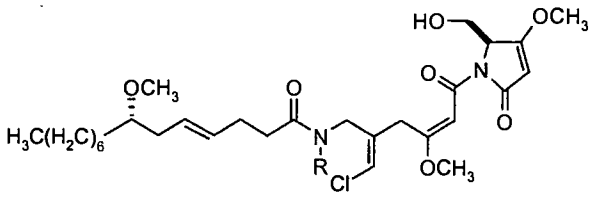
Malyngamide N (**18**)



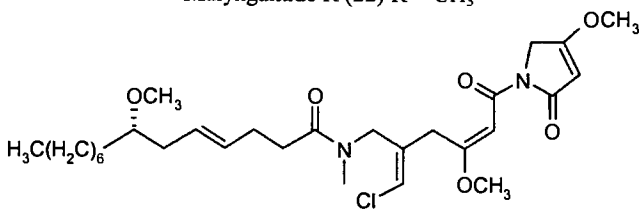
Malyngamide O (**19**)



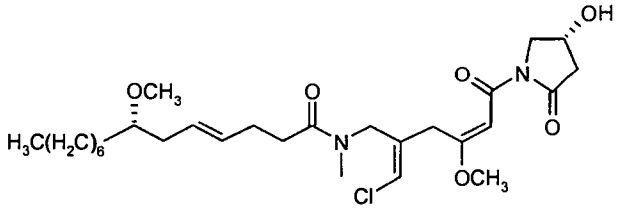
Malyngamide P (**20**)



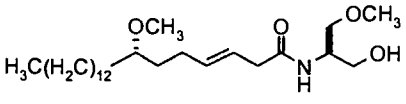
Malyngamide Q (**21**) R = H
Malyngamide R (**22**) R = CH_3



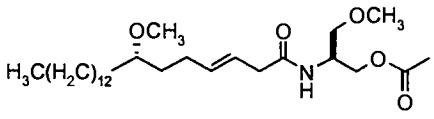
Isomalyngamide A (**23**)



Isomalyngamide B (**24**)



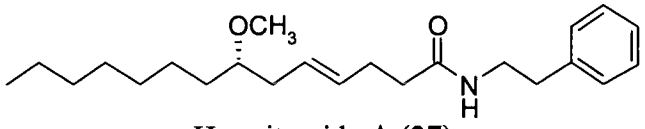
Serinol-derived Malyngamide (**25**)



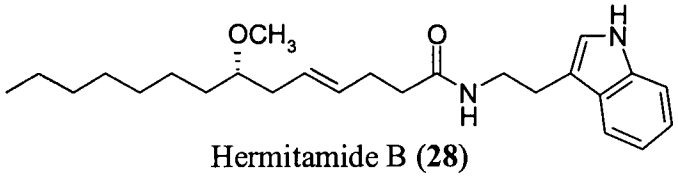
Serinol-derived Malyngamide (**26**)

Figure II.2. Malyngamides from Marine Cyanobacteria.

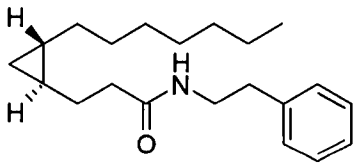
Recognizing the structural theme of these compounds as a hallmark of *Lyngbya majuscula* metabolites, we rename grenadamide as lyngbyamide A (**29**) and herein report these compounds as the "lyngbyamides". Lyngbyamide A (**29**) contains a β-phenylethylamine motif, which is associated with sympathomimetic agents. Lyngbyamide A (**29**) exhibited cannabinoid receptor-binding activity (K_i = 4.7 μM) and brine shrimp toxicity (LD_{50} = 5 μg/mL).[89]



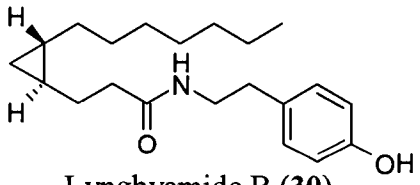
Hermitamide A (**27**)



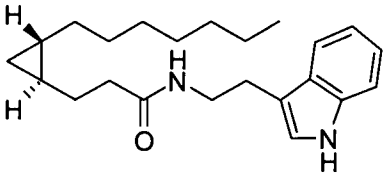
Hermitamide B (**28**)



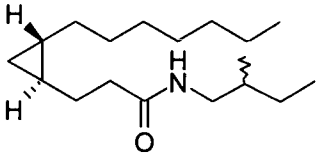
Lyngbyamide A
(Grenadamide) (**29**)



Lyngbyamide B (**30**)



Lyngbyamide C (**31**)



Lyngbyamide D (**32**)

Figure II.3. N-acyl Containing Lipids from *Lyngbya majuscula*.

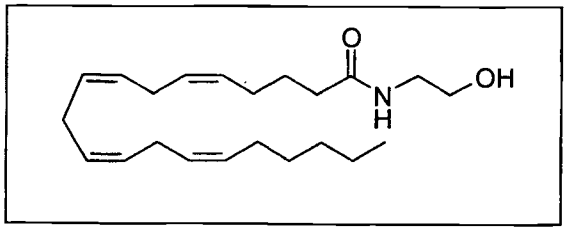


Figure II.4. Anandamide (**33**)

Results and Discussion

In our continued search for pharmaceutically useful agents from marine cyanobacteria, we have isolated two new secondary metabolites, lyngbyamides B (**30**) and C (**31**), from *L. majuscula* collected from exposed supratidal rocks on the island Nosy Tanikely, Madagascar. The collection was kept cold in isopropanol until extracted. A portion of the crude organic extract (9 g) was subjected to silica gel vacuum liquid chromatography (VLC) using a mixture of hexanes and EtOAc as eluent (Figure II.5). Preliminary bioassay of the organic extract showed activity in the brine shrimp toxicity assay at 10 ppm. Guided by the brine shrimp toxicity assay and ^{1}H NMR profiling, the natural products **30** and **31** were isolated as pale yellow oils by sequential solid phase extraction columns and RP-HPLC (Experimental Section).

Lyngbyamide B (**30**) showed an $[M + H]^+$ peak at *m/z* 332.2583 for a molecular formula of $C_{21}H_{33}NO_2$ by HRCIMS (Figure II.6). The molecular formula indicated six degrees of unsaturation. One was identified as an amide carbonyl from ^{13}C NMR (δ 173.6) and IR ($\nu_{C=O}$ 1640 cm^{-1}).

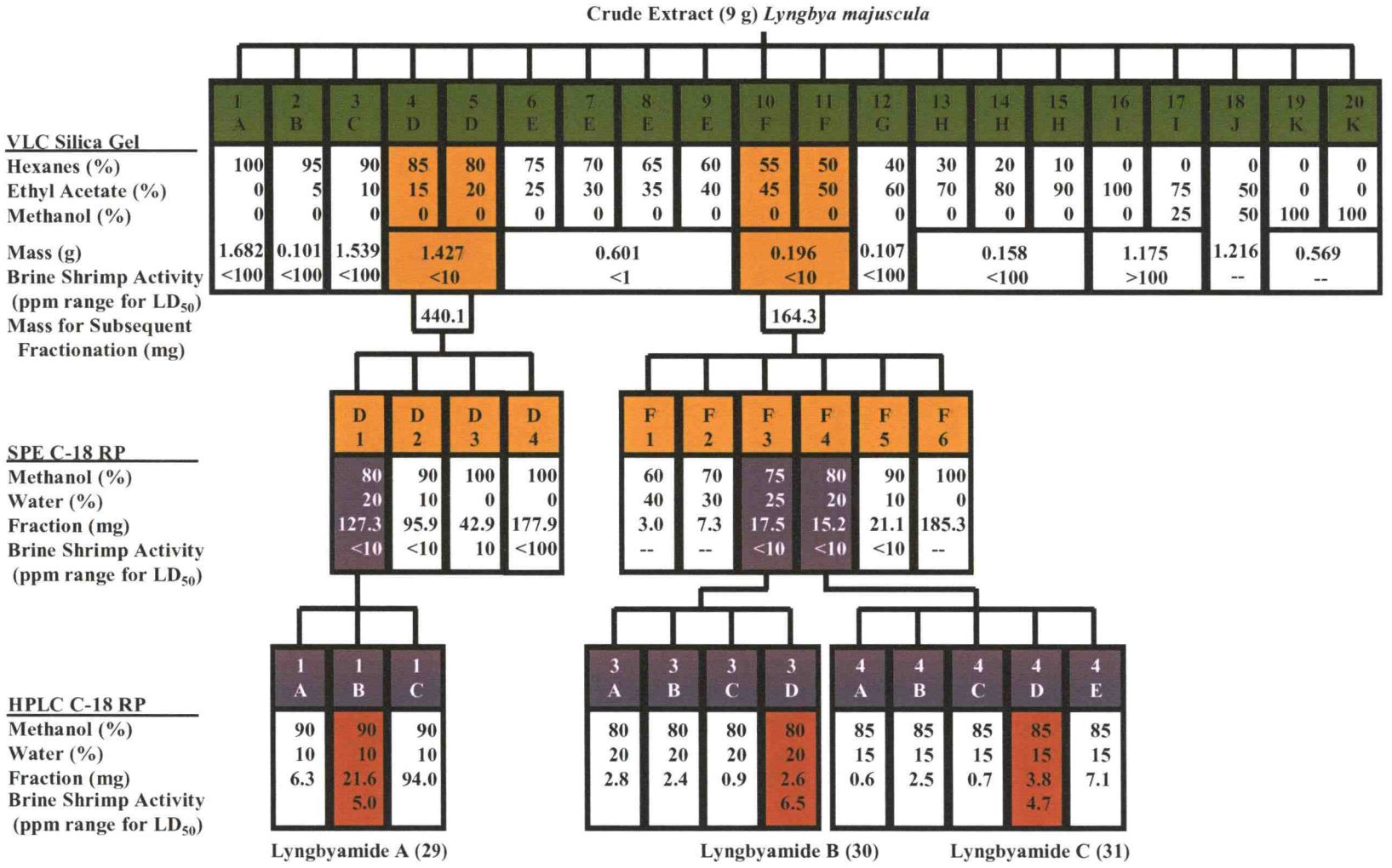


Figure II.5. Fractionation of the Lyngbyamides.

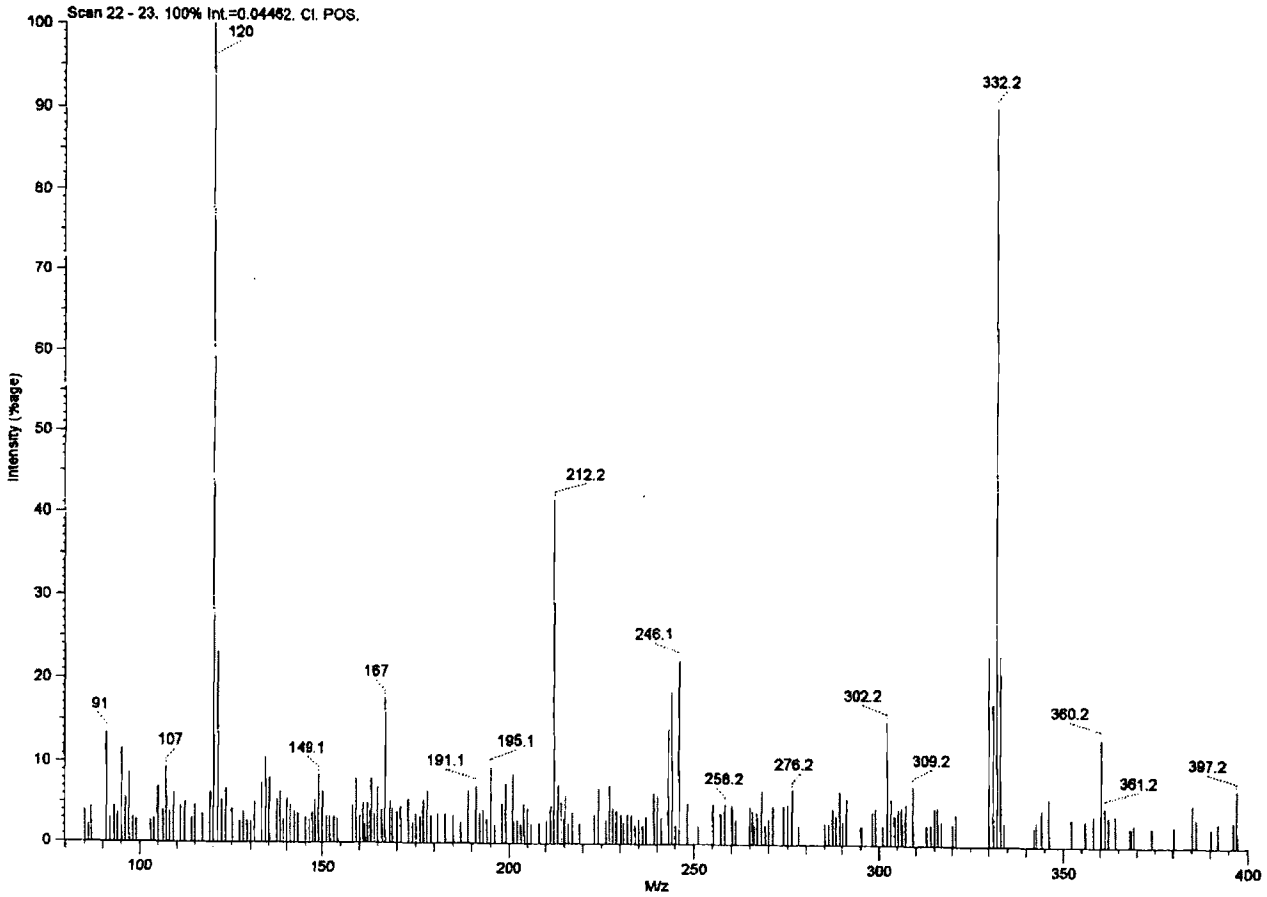


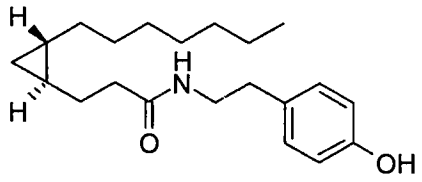
Figure II.6. LRCI (CH_4) – Mass Spectrum of Lyngbyamide B (**30**).

Table II.1. ^{1}H and ^{13}C NMR Assignments for Lyngbyamide B (**30**) in $CDCl_3$.

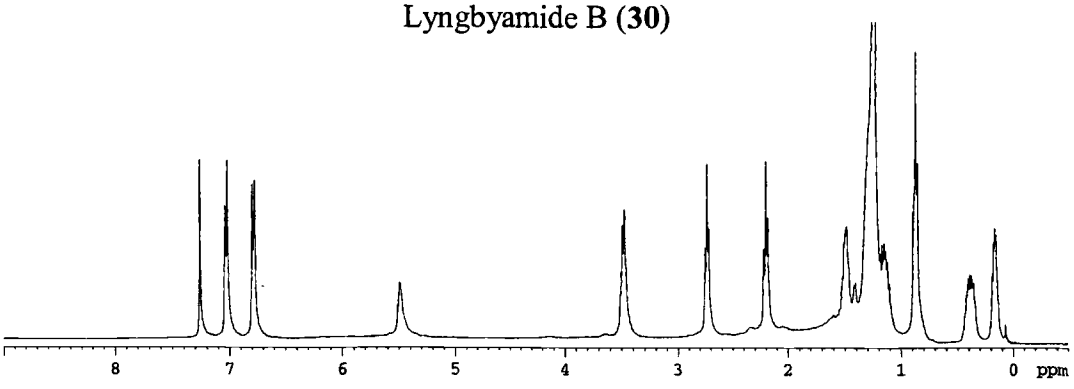
Position	^{1}H	J (Hz)	^{13}C	HMBC
1			173.6	
2	2.21 t	7.5	37.2	173.6, 30.6, 18.2
3	1.50 br q	7.0	30.6	173.6
4	0.38 m		18.4	
5	0.18 m		12.0	30.6, 34.3
6	0.41 m		19.1	
7	1.16 m		34.3	29.8, 19.1
8-10	1.27 m		29.8	29.8, 32.1
11	1.26 m		32.1	29.8
12	1.28 m		22.9	32.1, 29.8
13	0.88 t	6.9	14.3	32.1, 22.9
1'	3.49 q	6.1	41.0	173.6, 130.7, 35.0
2'	2.75 t	6.8	35.0	130.7, 130.0, 41.0
3'			130.7	
4', (8')	7.04 d		130.0	154.9, 130.7, 35.0
5', (7')	6.80 d		115.8	154.9, 130.7, 115.8
6'			154.9	
NH	5.50 br s			

The observed carbon peaks at δ 115.8 (d), 130.0 (d) 130.7 (s), and 154.9 (s) in the ^{13}C NMR spectrum combined with the presence of the downfield ^{1}H NMR doublets at δ 6.80 and 7.04 indicated a para substituted phenyl moiety, accounting for four additional degrees of substitution. The sixth degree of substitution was satisfied by the shielded ^{1}H NMR shifts at δ 0.18 and δ 0.38, demonstrating a 1,2-disubstituted-cyclopropyl ring.

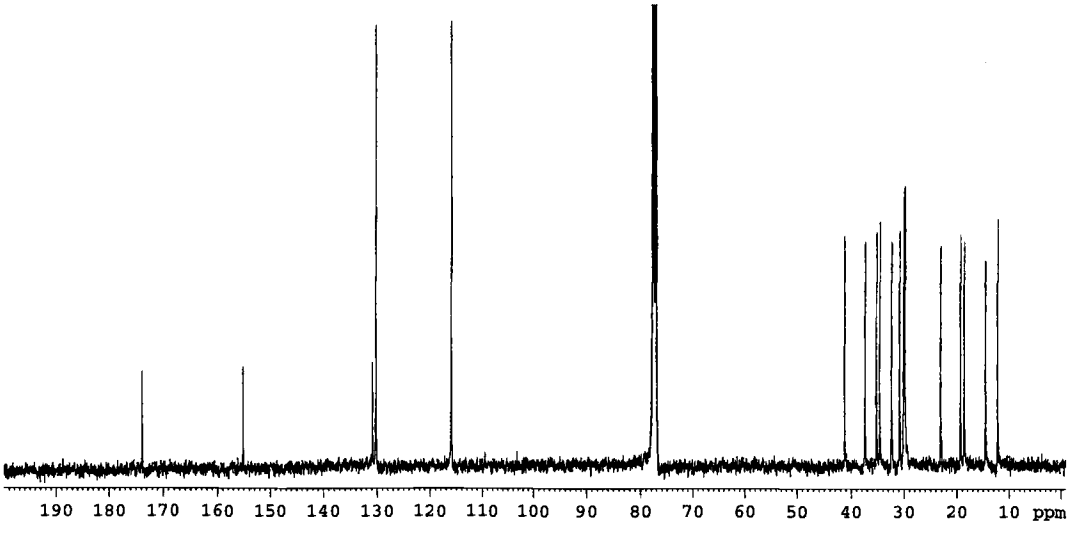
Structure elucidation of **30** was established mainly from interpretation of 1D (^{1}H NMR and ^{13}C NMR (Figure II.7)) and 2D NMR (COSY, HSQC, and HMBC) spectra. Analyzing the ^{1}H and ^{13}C NMR (Table II.1), ^{1}H-^{1}H COSY and HSQC data (Figures II.8 and II.9) led to the generation of two partial structures.



Lyngbyamide B (**30**)

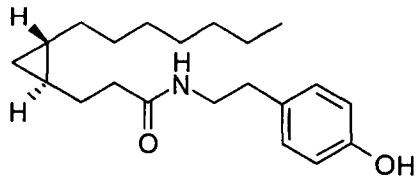


A.



B.

Figure II.7. A. ^{1}H NMR and **B.** ^{13}C NMR of Lyngbyamide B (**30**).



Lyngbyamide B (**30**)

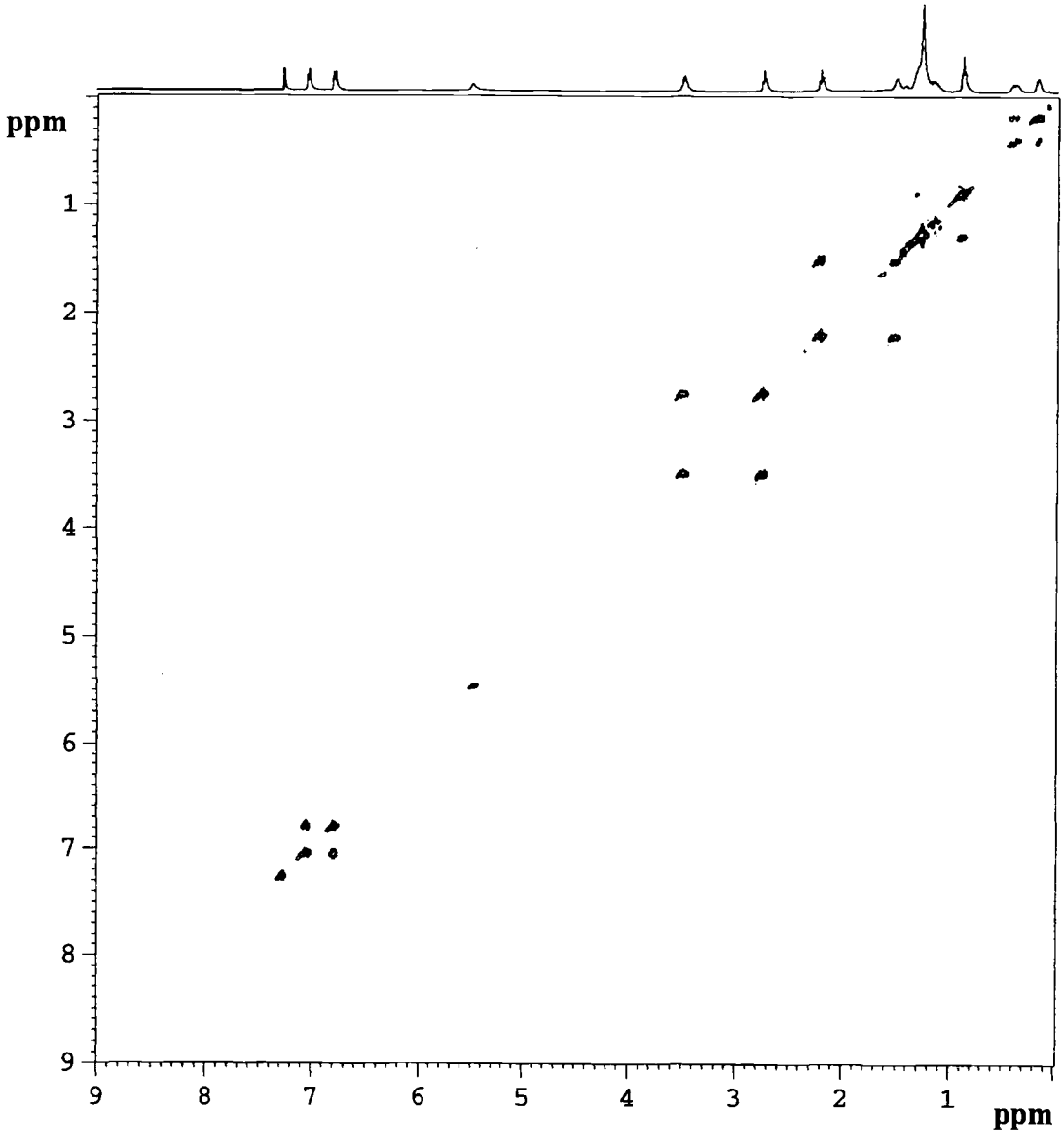
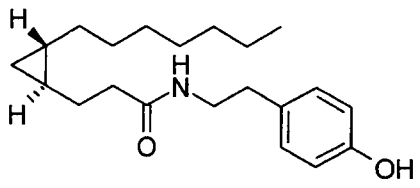


Figure II.8. ^{1}H-^{1}H COSY90 Spectrum of Lyngbyamide B (**30**).



Lyngbyamide B (**30**)

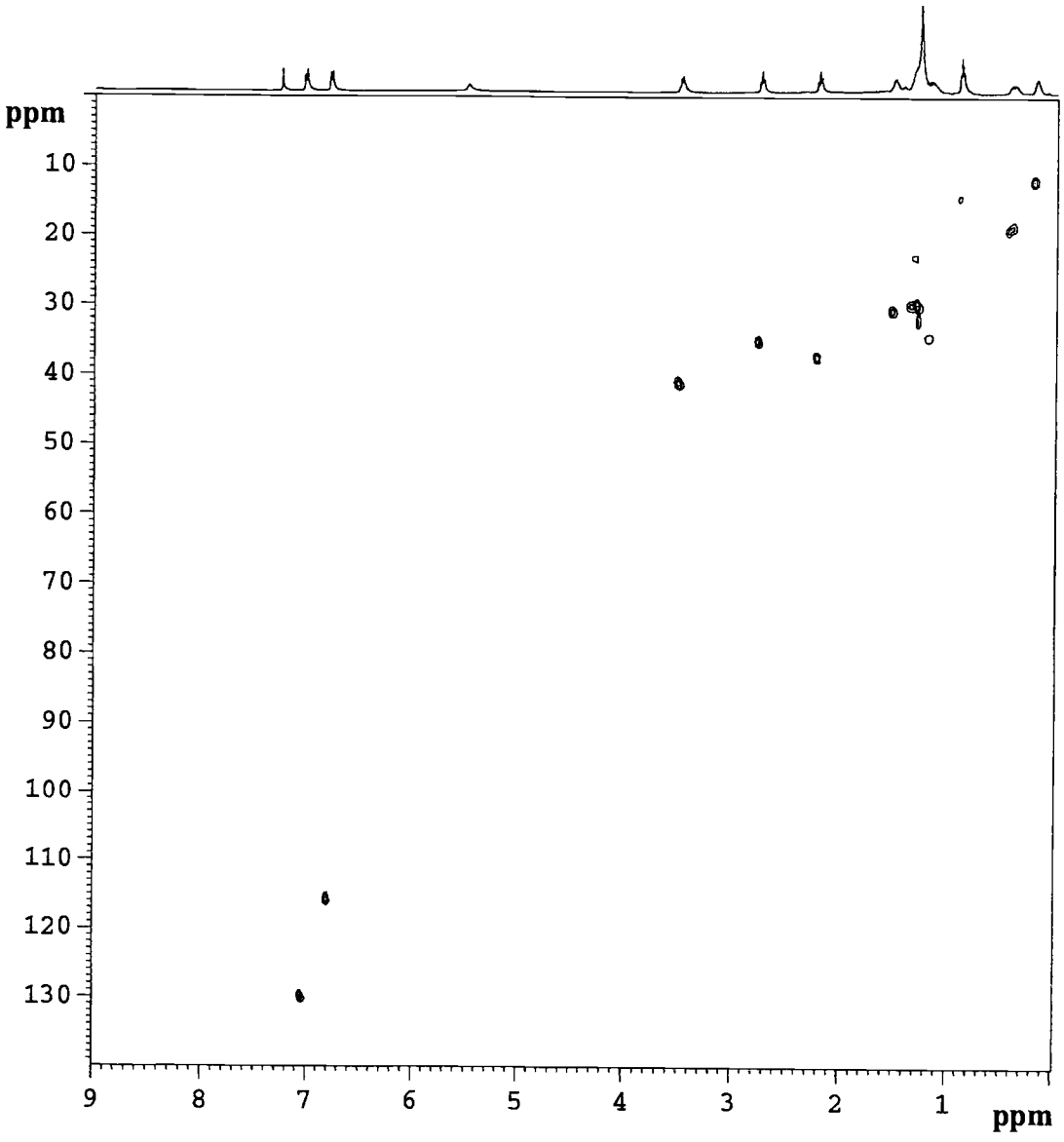
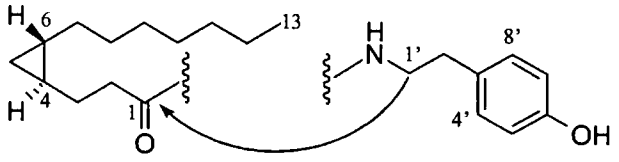


Figure II.9. HSQC Spectrum of Lyngbyamide B (**30**).

Partial structure **30a** is a cyclopropyl-containing fatty acid. The second spin system identified for **30b** consisted of a tyramine moiety. The HMBC spectral data (Figure II.12) showed correlations from the H_2-1' protons to the amide carbonyl, connecting the two partial structures to form lyngbyamide B (**30**) (Figure II.10).

The similar chemical shifts observed for the cyclopropyl ring methylene protons at δ 0.18 indicated that they were in a similar chemical environment. The ^{1}H NMR chemical shifts of the methylene protons at H_2-5 were compared with those of synthetic reference compounds, confirming that the relative stereochemistry of the ring is trans-1,2-disubstituted (Figure II.11).[107,108]



30a **30b**

Figure II.10. Partial Structures **30a** and **30b** of Lyngbyamide B (**30**).

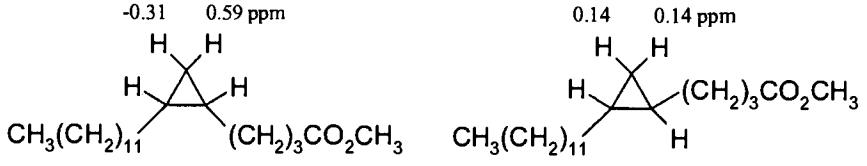
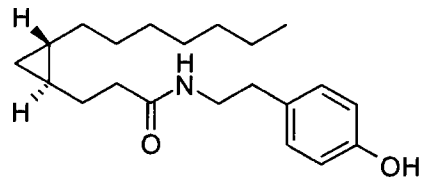


Figure II.11. Synthetic Reference Compounds Indicating the Relative Stereochemistry of the Cyclopropyl Ring in Lyngbyamide B (**30**) is Trans-1,2-Disubstituted.[107,108]



Lyngbyamide B (**30**)

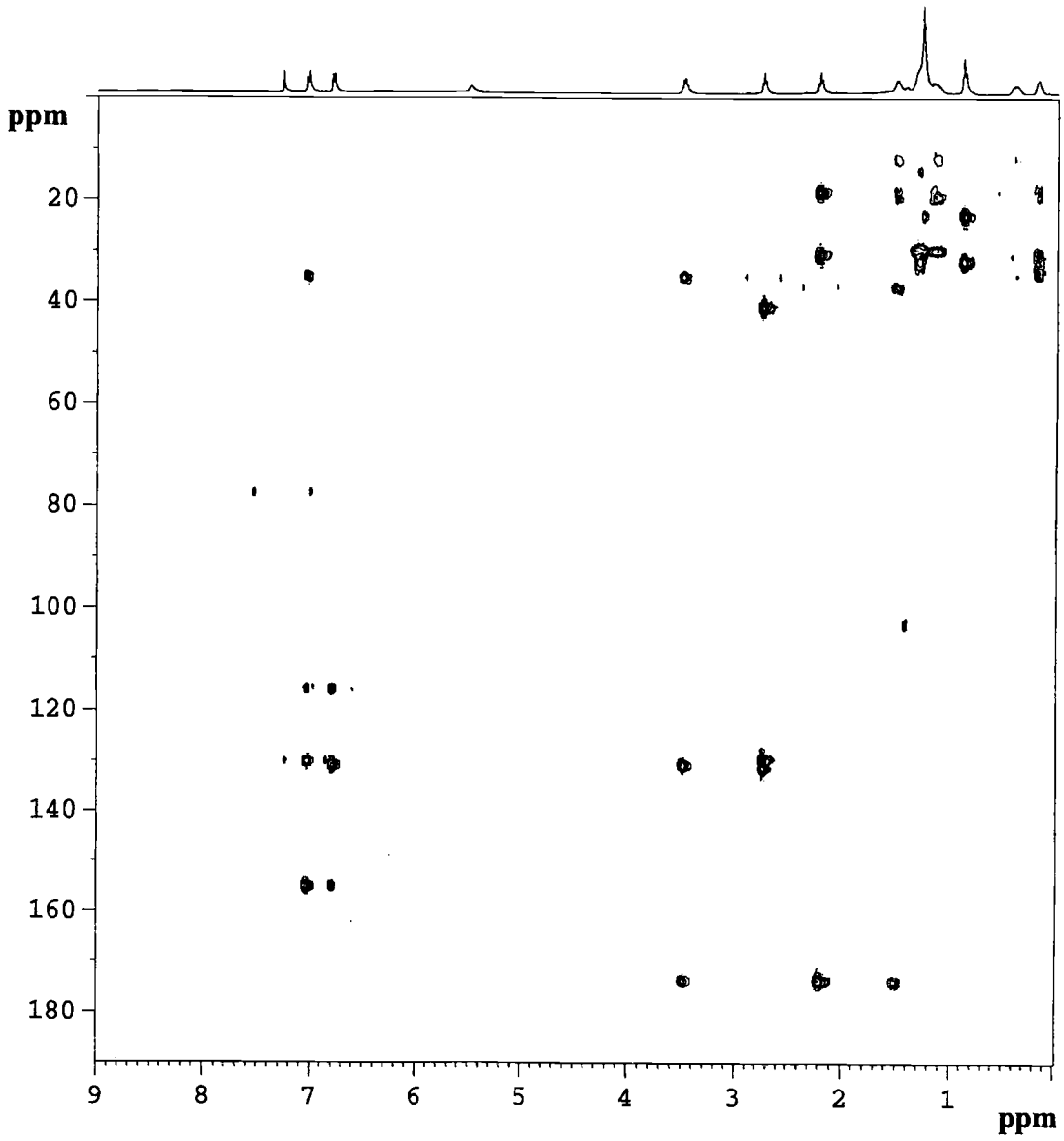


Figure II.12. HMBC (Optimized for 8 Hz) Spectrum of Lyngbyamide B (**30**).

Table II.2. ^{1}H and ^{13}C NMR Assignments for Lyngbyamide C (**31**) in $CDCl_3$.

Position	^{1}H	*J* (Hz)	^{13}C	HMBC
1			173.3	
2	2.19 t	7.6	37.2	173.3, 30.6, 18.4
3	1.50 br q	7.4	30.6	173.3, 37.2, 18.4, 11.9
4	0.37 m		18.4	
5	0.16 m		11.9	34.3, 30.6, 18.4
6	0.40 m		19.1	
7	1.16 m		34.3	29.8, 19.1
8-10	1.28 m		29.8	
11	1.28 m		32.1	29.8, 14.3
12	1.28 m		22.9	29.8, 14.3
13	0.89 t	6.5	14.3	32.1, 22.9
NH	5.54 br s			
1'	3.62 dd	12.7, 6.7	39.9	173.3, 113.4, 25.6
2'	2.99 t	6.7	25.6	127.6, 122.2, 113.4, 39.9
3'			113.4	
4'			127.6	
5'	7.62 br d	7.9	118.9	136.6, 113.4, 122.5
6'	7.14 td	7.9, 0.4	119.7	127.6, 111.4
7'	7.22 td	7.9, 0.4	122.5	136.6, 118.9
8'	7.39 br d	8.1	111.4	127.6, 119.7
9'			136.6	
10'	7.05 br d	1.6	122.2	136.6, 127.6, 113.4
NH	8.13 br s			

Lyngbyamide C (**31**) showed a $[M+H]^+$ peak at *m/z* 355.2583 for a molecular formula of $C_{23}H_{34}N_2O$ by HRCIMS (Figure II.13). The molecular formula indicated eight degrees of unsaturation. Similar to **30**, structure elucidation of **31** was established from the interpretation of 1D (^{1}H NMR and ^{13}C NMR (Table II.2)) and 2D NMR (COSY, HSQC, and HMBC) experiments.

The ^{1}H NMR spectrum of **31** was indicative of a lyngbyamide-type metabolite. The proton signals indicated a cyclopropyl-containing fatty acid portion. These included the 1,2-disubstituted cyclopropyl ring protons at δ 0.16 and δ 0.37, the protons of a long aliphatic chain in the δ 1.16 - δ 1.28, and a terminal $-CH_3$ triplet signal at δ 0.89 (Figure II.14).

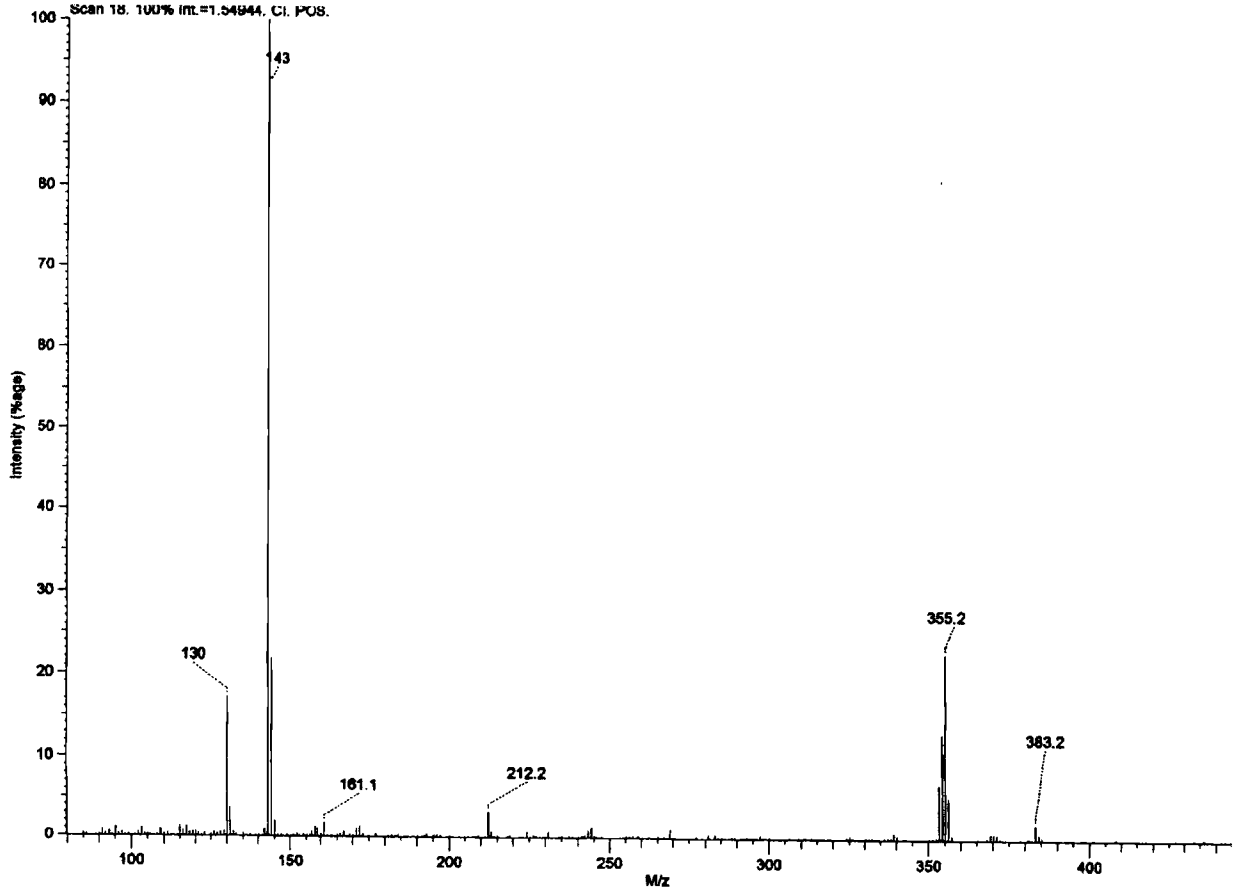
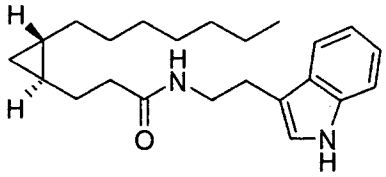
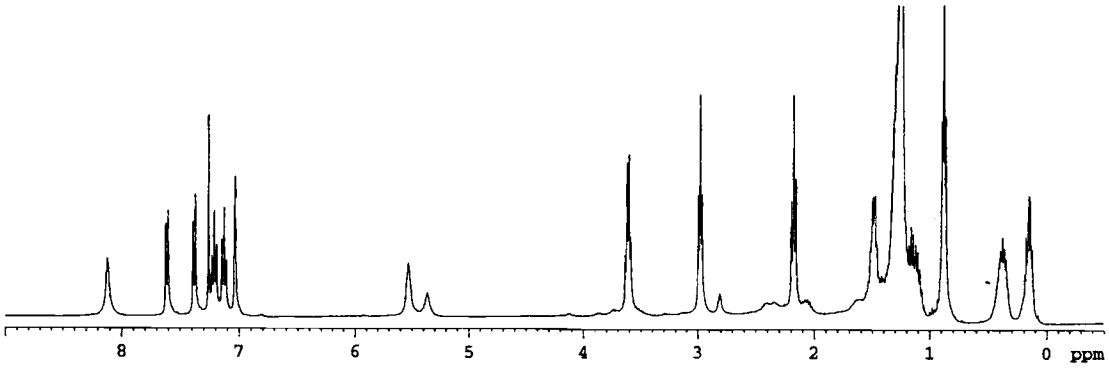


Figure II.13. LRCI (CH_4) – Mass Spectrum of Lyngbyamide C (**31**).



Lyngbyamide C (**31**)



A.

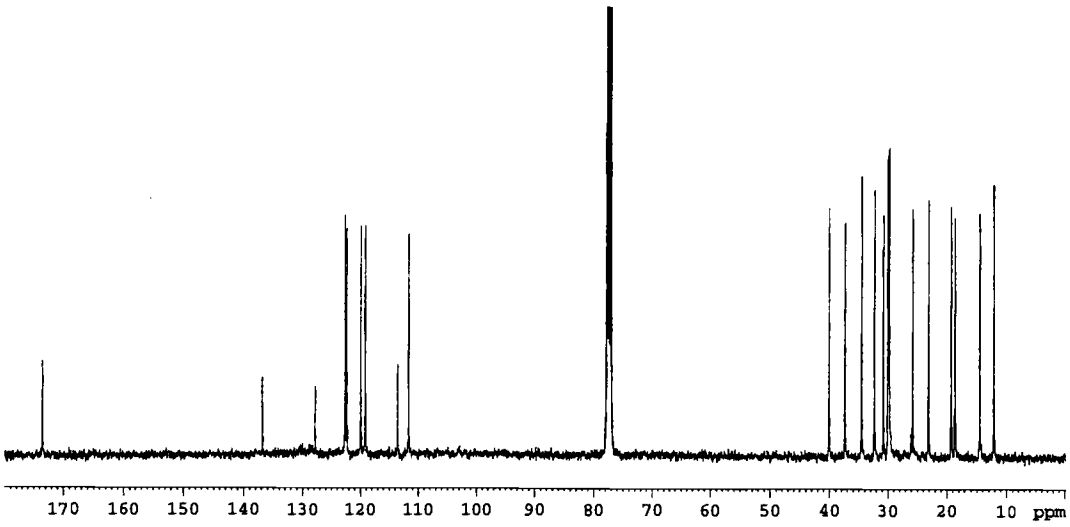
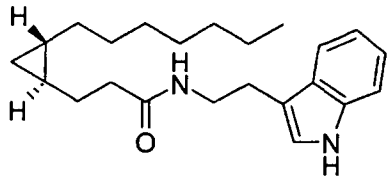


Figure II.14. **A.** ^{1}H NMR and **B.** ^{13}C NMR of Lyngbyamide C (**31**).

Recognizing the proton signals of the cyclopropyl-containing fatty acid portion facilitated the structure elucidation. The unique characteristics of the downfield peaks allowed us to distinguish the tryptamine moiety of lyngbyamide C (**31**) from the tyramine moiety of lyngbyamide B (**30**). The tryptamine moiety of **31** was confirmed through analysis of the low-field multiplicity patterns in the ^{1}H NMR [δ 7.62 br d (H-5'), δ 7.39 br d (H-8'), δ 7.05 br d (H-10'), δ 7.22 td (H-7'), and δ 7.14 td (H-6')]. The COSY spectrum of **31** showed cross-peaks between H-5' (δ 7.62)/H-6' (δ 7.14) and H-7' (δ 7.22)/H-8' (δ 7.39) (Figure II.15). The position of the tryptamine functionality in **31** was determined from HMBC data, which showed correlations from H_2-1' (δ 3.62) and H_2-2' (δ 2.99) to C-3' (δ 113.4) of the indole ring (Figure II.16). The cross peak between H_2-1' (δ 3.62) and the amide proton (δ 5.54) observed in the COSY, combined with the correlation from H_2-1' to the amide carbonyl signal at C-1 (δ 173.3) in the HMBC spectrum, established the position of the tryptamine moiety in **31** (Figure II.18).

As it was for lyngbyamide B (**30**), the similar chemical shifts observed for the cyclopropyl ring methylene protons at δ 0.16 indicated that they were in a similar chemical environment. The ^{1}H NMR chemical shifts of the methylene protons at H_2-5 were compared with those of synthetic reference compounds, confirming that the relative stereochemistry of the ring is trans-1,2-disubstituted for lyngbyamide C (**31**).[107,108]



Lyngbyamide C (**31**)

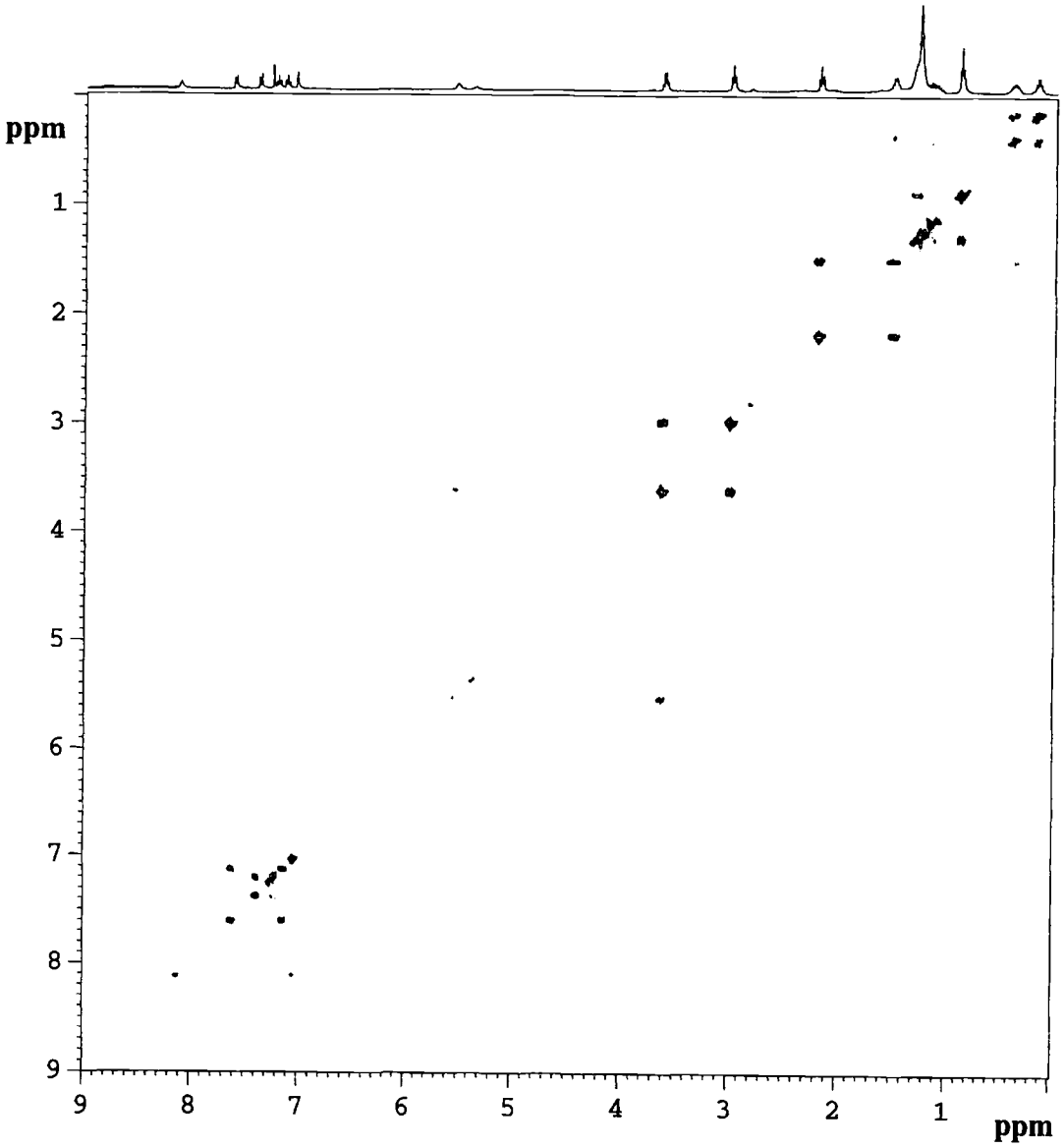
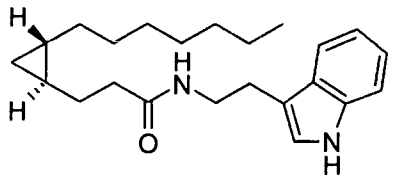


Figure II.15. ^{1}H-^{1}H COSY90 Spectrum of Lyngbyamide C (**31**).



Lyngbyamide C (**31**)

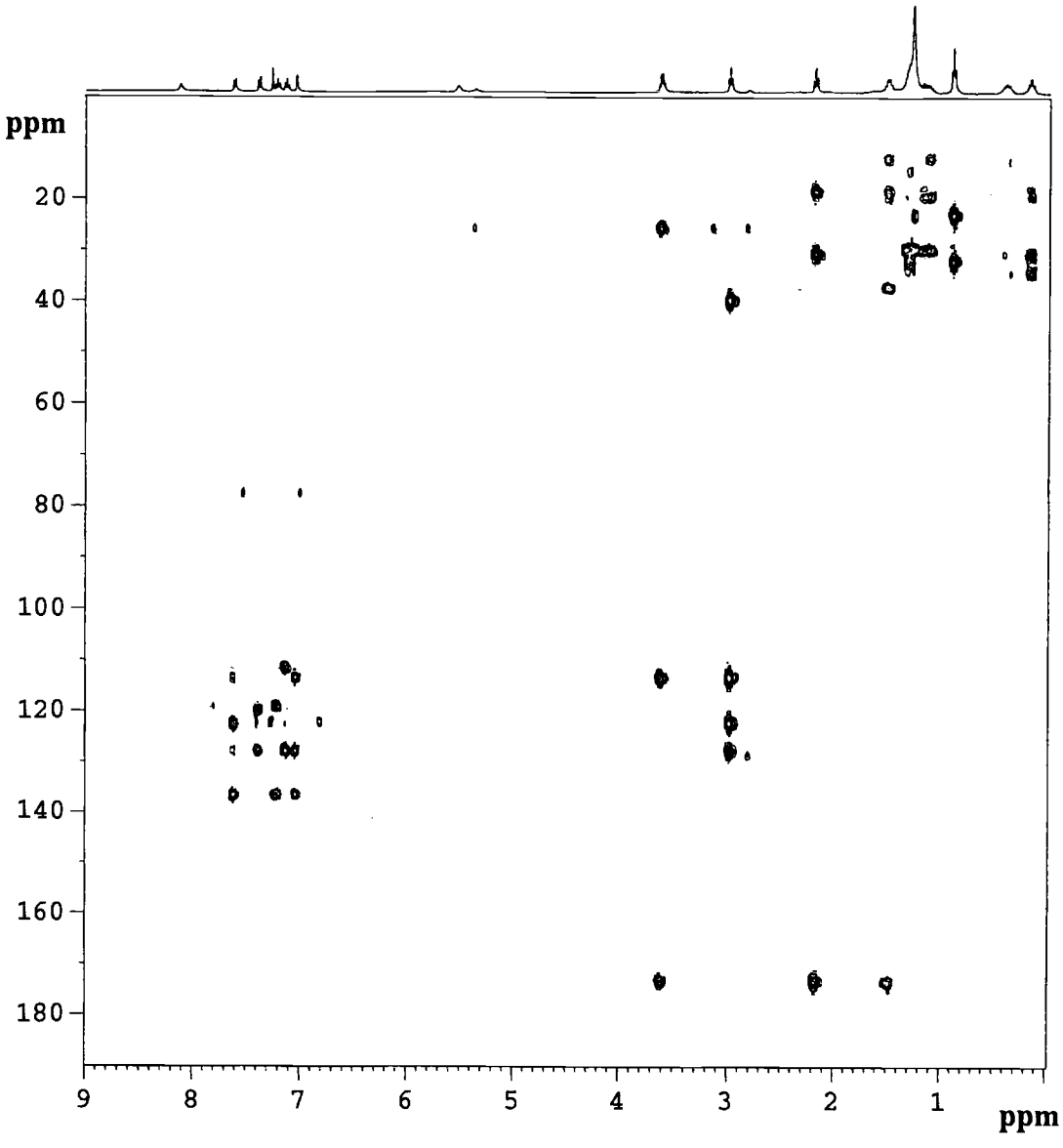
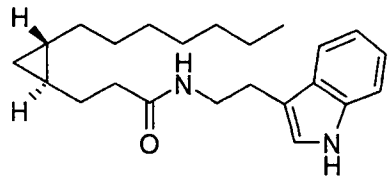


Figure II.16. HMBC (Optimized for 8 Hz) Spectrum of Lyngbyamide C (**31**).



Lyngbyamide C (**31**)

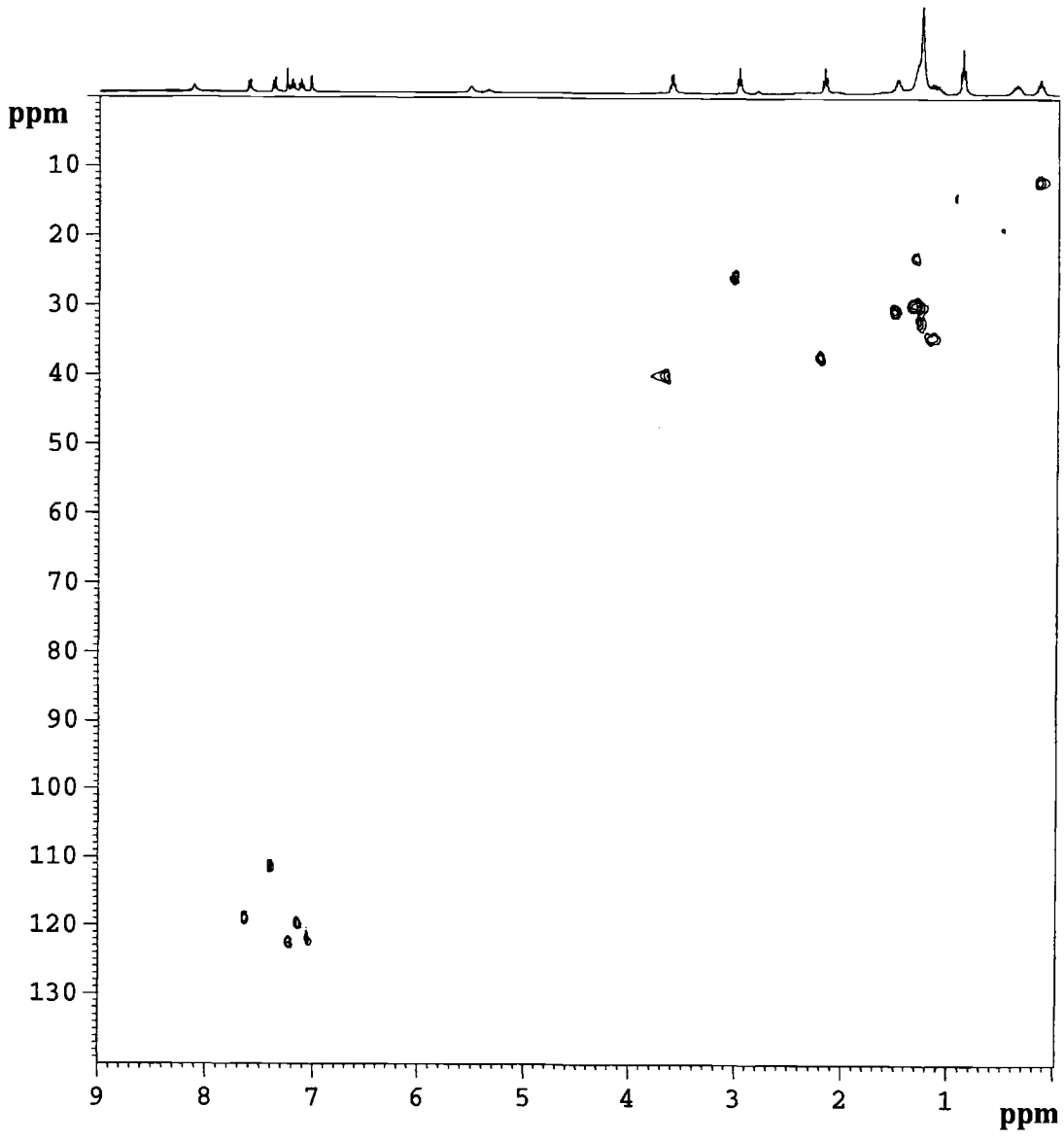


Figure II.17. HSQC Spectrum of Lyngbyamide C (**31**).

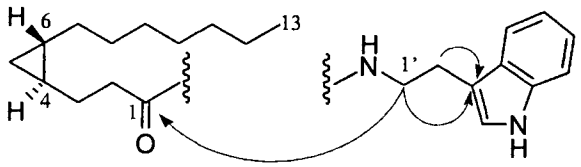


Figure II.18. HMBC Correlations Involving the Tryptamine Moiety of Lyngbyamide C (**31**).

McPhail previously isolated an isoleucyl derivative of the lyngbyamides from a Curaçao collection of *Lyngbya majuscula* (Unpublished). This new metabolite is named herein as lyngbyamide D (**32**). Lyngbyamide D (**32**) was isolated using antifungal (*Candida albicans*) bioassay-guided fractionation. The interesting bioactivity of this compound and assemblage of reported lyngbyamide derivatives warrants further investigation and biological profiling. In order to embark on this investigation, as well as to confirm structures, we semi-synthetically reproduced the natural products.

The cyclopropyl fatty acid, 3-(2-heptyl-cyclopropyl)-propionic acid (**34**), from a Curaçao collection of *Lyngbya majuscula* was used in the semisynthesis of lyngbyamides B (**30**) and C (**31**). We used thionyl chloride for the conversion of the free acid to the corresponding acid chloride, followed by the addition of the respective free amine to produce the natural product. One-dimensional ^{1}H NMR

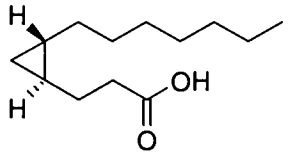
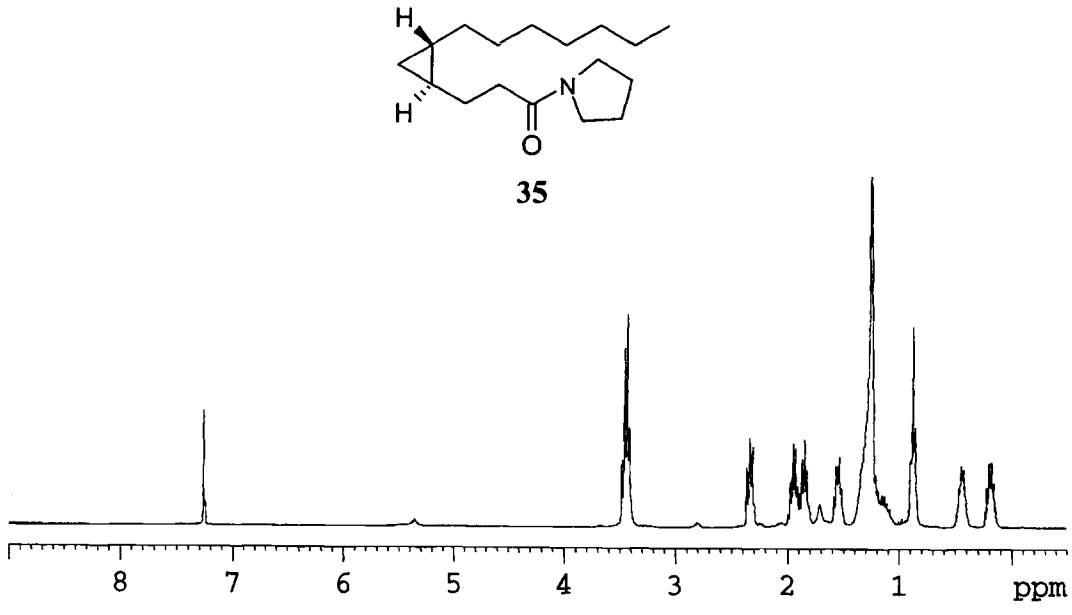


Figure II.19. 3-(2-heptyl-cyclopropyl)-propionic acid (**34**).

and LR/HRCIMS were used to confirm that the planar structures of the semi-synthetic compounds were identical to the natural products **30** and **31**. The optical rotation values were $[\alpha]_D$ - 16.0° ($CHCl_3$, c = 0.15) for semi-synthetic lyngbyamide B and $[\alpha]_D$ - 17.6° ($CHCl_3$, c = 0.22) for semi-synthetic lyngbyamide C. The optical rotations for the previously isolated grenadamide[89] (=lyngbyamide A) and the cyclo-propyl fatty acid (**34**) used in the semi-synthesis reactions were also negative. These values are in contrast to the positive values obtained for lyngbyamide A ($[\alpha]_D$ + 15.0° ($CHCl_3$, c = 0.24)), lyngbyamide B ($[\alpha]_D$ +10.4° ($CHCl_3$, c = 0.26)) and lyngbyamide C ($[\alpha]_D$ + 3.2° ($CHCl_3$, c = 0.125)) isolated from the Madagascar collection. The difference in the optical rotation values of the compounds indicates that the relationship between the fatty acid moieties from the different collections is enantiomeric.

After successfully creating the semi-synthetic natural products of lyngbyamide B (**30**) and C (**31**), we decided to make a pyrrolidine derivative (**35**) using the same coupling reaction employed above. We intended to use this unnatural semi-synthetic product to explore the structure activity relationship of the amino acid residues in the brine shrimp toxicity assay. One-dimensional ^1H NMR



35

Figure II.20. ^{1}H NMR of Pyrrolidine Derivative (**35**).

(Figure II.20) and LR/HRCIMS (Figure II.21) were used to confirm the structure of the pyrrolidine derivative (**35**).

We conducted the brine shrimp (*Artemia salina*) toxicity assay for the natural lyngbyamides as well as the semi-synthetic products. The natural and semisynthetic lyngbyamides A (**29**), B (**30**), and C (**31**) demonstrated brine shrimp toxicity (LD_{50}) between 4.7 - 6.5 ppm (Figure II.22). The pyrrolidine derivative (**35**) showed significantly more potent activity at LD_{50} = 0.3 ppm. This increase in activity could be attributed to the size of the pyrrolidine ring in relation to the tryptamine and tyramine functionalities.

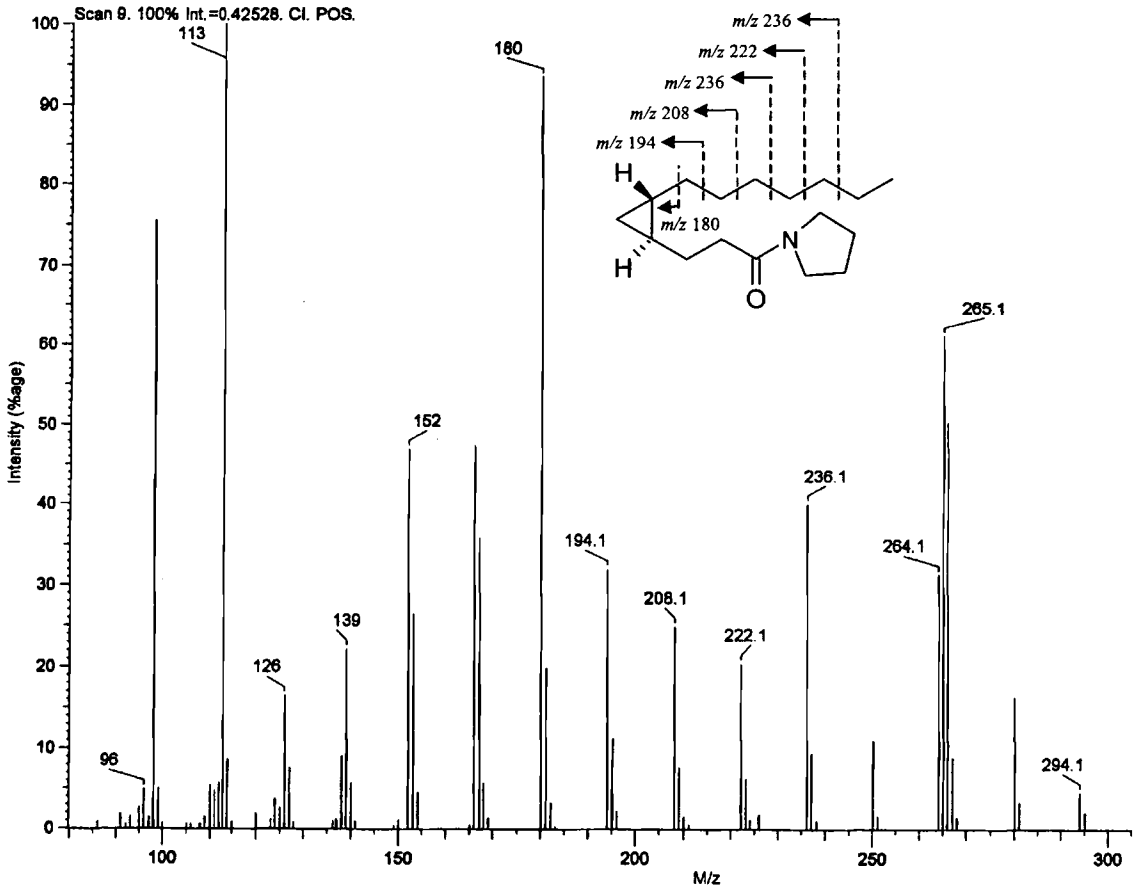


Figure II.21. LRCI (CH_4) – Mass Spectrum of Pyrrolidine Derivative (**35**).

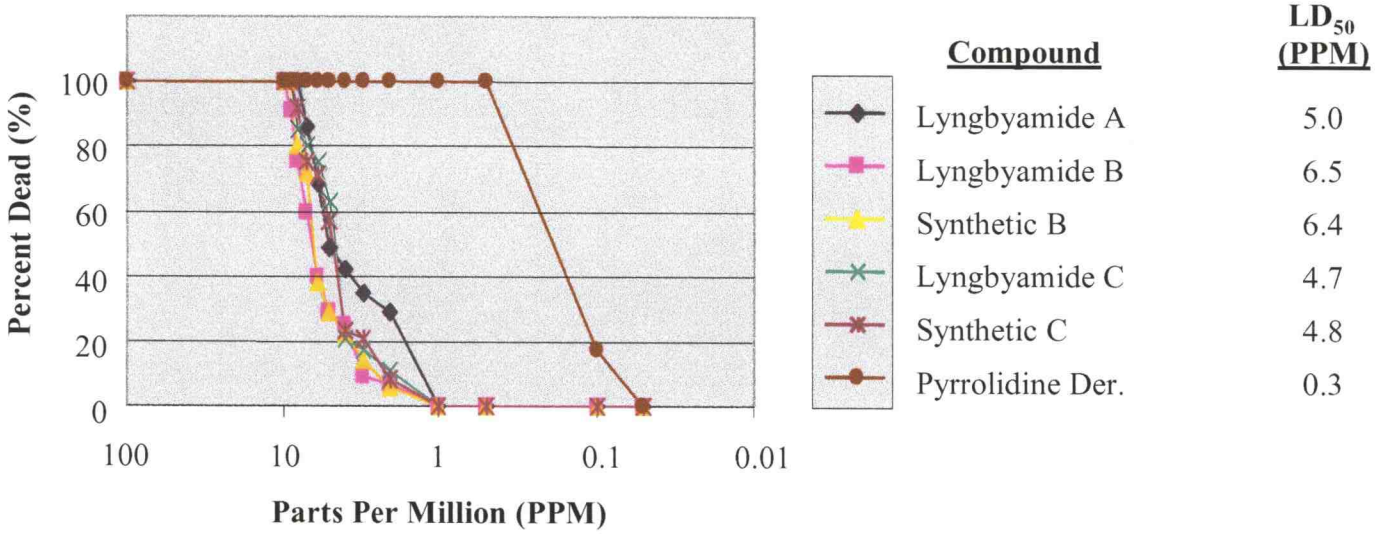


Figure II.22. Brine Shrimp Toxicity Assay of the Lyngbyamides.

The lyngbyamides represent interesting examples of N-acyl containing lipids from the cyanobacterium *Lyngbya majuscula*. The compounds exhibit a cyclopropyl fatty acid and an assortment of functionalized amines that are connected through an amide bond. This characteristic represents a typical metabolic theme of cyanobacteria. Similar compounds sharing the same motif such as the malyngamides and hermitamides demonstrate biological activity in several systems.

Experimental

General. ^{1}H and ^{13}C NMR spectra were measured on a Bruker AM 400 spectrometer operating at a proton frequency of 400 MHz and a carbon frequency of 100 MHz with the solvent used as an internal standard ($CDCl_3$ at δ 7.26 and δ 77). Mass spectral data was recorded on a Kratos MS50TC mass spectrometer. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter. UV and IR were recorded on Hewlett-Packard 8452A UV-vis and Nicolet 510 spectrophotometers, respectively. HPLC separations were performed with a Waters 515 pump and a Waters 996 Photodiode Array Detector. Merck aluminum-backed thin-layer chromatography sheets were used for TLC.

Collection. The marine cyanobacterium, *L. majuscula* (MNT-17/APR/00-1), was hand-collected from exposed supratidal rocks on the island Nosy Tanikely (*S* 12°51.185', *E* 48°25.153'), Madagascar on April 17, 2000. The specimens were preserved in isopropanol upon collection and stored at low

temperature until workup. The natural C-13 cyclopropyl fatty acid (3-(2-heptyl-cyclopropyl)-propionic acid) (**34**) $[\alpha]_D$ – 7.59° ($CHCl_3$, c = 1.08) used in the semisynthesis was extracted from *L. majuscula* (NAC 9SEP97-01/02) collected by hand using scuba (20-40 ft depth) in Carmabi, Curaçao on September 9, 1997.

Extraction and Isolation of Lyngbyamide A (Grenadamide) (29) and Lyngbyamides B (30) and C (31). The thawed algal material (1091 g, dry wt) was homogenized in CH_2Cl_2/MeOH (2:1) and filtered. The solvents were removed *in vacuo* to yield a residue that was partitioned between CH_2Cl_2 and H_2O. The residue was extracted repeatedly with CH_2Cl_2, and the combined CH_2Cl_2 layers were reduced *in vacuo* to yield a dark green crude extract (11.2 g). A portion of the crude extract (9 g) was fractionated using vacuum liquid chromatography (VLC) on Si gel with a stepwise gradient of hexanes/EtOAc and EtOAc/MeOH. Eluted material was collected in 20 fractions and monitored by TLC. Similar fractions were combined to give eleven fractions (A-K, Figure II.5). Fraction D (1.4 g, eluted with hexanes/EtOAc (4:1)) showed brine shrimp toxicity (LD_{50} < 10 ppm). It was further fractionated using Mega Bond Elut C18 12 cc and a final purification on RP-HPLC (Varian Microsorb MV 100-5 C18 250 x 4.6 mm; detection at 254 nm) with MeOH/H_2O (9:1) to give lyngbyamide A (**29**, t_R = 13-14 min, 21.6 mg). Fraction F (196 mg, eluted with hexanes/EtOAc (5:5)) showed brine shrimp toxicity (LD_{50} < 10 ppm). It was further fractionated using Mega Bond Elut C18 12 cc to give six sub-fractions: sub-fraction F3

(17.5 mg, eluted with MeOH/H_2O (3:1)) and sub-fraction F4 (15.2 mg, eluted with MeOH/H_2O (4:1)) showed brine shrimp toxicity (LD_{50} < 10 ppm). Sub-fractions F3 and F4 were purified on RP-HPLC (Phenomenex Sphereclone 5μ ODS 250 x 10 mm; detection at 254 nm) with MeOH/H_2O (4:1) and MeOH/H_2O (85:15) respectively. Sub-fraction F3D gave lyngbyamide B (**30**, t_R = 23-25 min, 2.6 mg). Sub-fraction F4D gave lyngbyamide C (**31**, t_R = 19-21 min, 3.8 mg).

Lyngbyamide A (grenadamide) (29). Pure lyngbyamide A was a pale yellow oil; $[\alpha]_D$ + 15.0° ($CHCl_3$, *c* = 0.24); LRCIMS *m/z* 315 (54), 296 (18), 281 (73), 252 (32), 224 (44), 210 (27), 196 (84), 182 (33), 163 (31), 129 (100), 104 (62), 88 (13); HRCIMS $[M]^+$ *m/z* 315.2563 (calculated for $C_{21}H_{33}NO$, 354.2562).

Lyngbyamide B (30). Pure lyngbyamide B was a pale yellow oil; $[\alpha]_D$ +10.4° ($CHCl_3$, *c* = 0.26); UV (MeOH) λ_{max} 285 nm (ε 1000), 230 nm (ε 4800), 217 nm (ε 4000); IR (neat) ν_{max} 3307, 2989, 2921, 2853, 1706, 1640, 1550, 1454, 722 cm^{-1}; LRCIMS *m/z* 331 (17), 246 (22), 212 (41), 120 (100); HRCIMS $[M]^+$ *m/z* 331.2583 (calculated for $C_{21}H_{33}NO_2$, 331.2590).

Lyngbyamide C (31). Pure lyngbyamide C was a pale yellow oil; $[\alpha]_D$ + 3.2° ($CHCl_3$, *c* = 0.125); UV (MeOH) λ_{max} 297 nm (ε 2100), 288 nm (ε 2400), 227 nm (ε 14700), 212 nm (ε 14100); IR (neat) ν_{max} 3406, 3296, 3059, 2924, 2855, 1711, 1647, 1534, 1456, 742 cm^{-1}; LRCIMS *m/z* 354 (13), 212 (3), 143 (100), 130 (17); HRCIMS $[M]^+$ *m/z* 354.2583 (calculated for $C_{23}H_{34}N_2O$, 354.2590).

Semisynthesis of Lyngbyamide B (30). The acid chloride of C-13 cyclopropyl fatty acid was prepared by adding 5 μl of pyridine to 13.5 mg of 3-(2-heptyl-cyclopropyl)-propionic acid (**34**, 0.064 mmol) dissolved in dry THF (250 μl) in a 10 ml 2-necked pear flask with a reflux condenser (and a magnetic stirrer bar) under argon. The flask was heated with an oil bath to 60 °C. $SOCl_2$ (92.9 μl) was added drop-wise. After 50 min., the THF and excess $SOCl_2$ were removed by venting the reaction flask under argon with a needle inserted into the septum-sealed neck of the of the pear flask (see semisynthesis of lyngbyamide C below). This was followed by addition of 3 molar equiv of tyramine (30.6 mg) dissolved in DMSO. The reaction was left to stir for 1 h at room temperature. The DMSO was removed from the crude mixture *in vacuo* and successively washed with 5% HCl (2 x 20 ml), 5% $NaHCO_3$ (2 x 20 ml), and H_2O (2 x 20 ml). The washed mixture was extracted 3x with CH_2Cl_2. Semisynthetic lyngbyamide B (**30**, 9.6 mg, 45 %) was purified using HPLC (Phenomenex Sphereclone 5μ ODS 250 x 10 mm; detection at 254 nm) with MeOH/H_2O (9:1) to give semisynthetic lyngbyamide B (**30**, t_R = 9-10 min) showing $[\alpha]_D$ - 16.0° ($CHCl_3$, c = 0.15); LRCIMS *m/z* 331 (17), 246 (17), 212 (26), 120 (100); HRCIMS $[M]^+$ *m/z* 331.2593 (calculated for $C_{21}H_{33}NO_2$, 331.2590).

Semisynthesis of Lyngbyamide C (31). The acid chloride of C-13 cyclopropyl fatty acid was prepared by adding 5 μl of pyridine to 11.0 mg of 3-(2-heptyl-cyclopropyl)-propionic acid (**34**, 0.052 mmol) dissolved in dry THF (250 μl)

in a 10 ml 2-necked pear flask with a reflux condenser (and a magnetic stirrer bar) under argon. The flask was heated with an oil bath to 60 °C. $SOCl_2$ (75.9 μl) was added drop-wise. The mixture was stirred and heated (60 °C) for 1 hour. THF and excess $SOCl_2$ evaporated during the initial reaction since no cooling water was running through the condenser. This action prompted the future use of venting the reaction flask under argon with a needle after 50 min. during subsequent coupling reactions in order to remove THF and excess $SOCl_2$ prior to the addition of the free amine (see semisynthesis of lyngbyamide B above). This was followed by addition of 3 molar equiv of tryptamine (24.9 mg) in dry THF (250 μl). The reaction was left to stir for 1 h at room temperature. The THF was removed from the crude mixture *in vacuo* and successively washed with 5% HCl (2 x 20 ml), 5% $NaHCO_3$ (2 x 20 ml), and H_2O (2 x 20 ml). The washed mixture was extracted 3x with CH_2Cl_2. Semisynthetic lyngbyamide C (**31**, 5.6 mg, 30 %) was purified using HPLC (Phenomenex Sphereclone 5μ ODS 250 x 10 mm; detection at 254 nm) with MeOH/H_2O (85:15) to give semisynthetic lyngbyamide C (**31**, t_R = 20-22 min) showing $[\alpha]_D$ - 17.6° ($CHCl_3$, c = 0.22); LRCIMS *m/z* 354 (10), 212 (6), 143 (100), 130 (22); HRCIMS $[M]^+$ *m/z* 354.2673 (calculated for $C_{23}H_{34}N_2O$, 354.2671).

Semisynthesis of Pyrrolidine Derivative (35). The acid chloride of C-13 cyclopropyl fatty acid was prepared by adding 5 μl of pyridine to 13.8 mg of 3-(2-heptyl-cyclopropyl)-propionic acid (**34**, 0.065 mmol) dissolved in dry

THF (250 μl) in a 10 ml 2-necked pear flask with a reflux condenser (and a magnetic stirrer bar) under argon. The flask was heated with an oil bath to 60 °C. $SOCl_2$ (92.9 μl) was added drop-wise. After 50 min., the THF and excess $SOCl_2$ were removed by venting the reaction flask under argon with a needle inserted into the septum-sealed neck of the of the pear flask. This was followed by addition of 3 molar equiv of pyrrolidine (12 μl) and THF (240 μl). The reaction was left to stir for 1 h at room temperature. The THF was removed from the crude mixture *in vacuo* and successively washed with 5% HCl (2 x 20 ml), 5% $NaHCO_3$ (2 x 20 ml), and H_2O (2 x 20 ml). The washed mixture was extracted 3x with CH_2Cl_2. The semisynthetic pyrrolidine derivative (**35**, 10.1 mg, 59 %) was purified using HPLC (Phenomenex Sphereclone 5μ ODS 250 x 10 mm; detection at 254 nm) with MeOH/H_2O (95:5) to give semisynthetic pyrrolidine derivative (**35**, t_R = 10.5 – 11.5 min) showing $[\alpha]_D$ –12.4° ($CHCl_3$, c = 0.51); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.44 (4H, m, H-1'–2'), 2.33 (2H, t, J = 7.6 Hz, H-2), 1.93 (2H, m, H-3'), 1.85 (2H, m, H-4'), 1.54 (2H, br q, J = 6.9 Hz, H-3), 1.25 (10H, m, H-8–12), 1.15 (2H, m, H-7), 0.88 (3H, t, J = 6.6 Hz, H-13), 0.44 (2H, m, H-4,6), 0.18 (2H, m, H-5); LRCIMS *m/z* 265 (61), 236 (40), 222 (20), 208 (25), 194 (32), 180 (93), 166 (48), 152 (48),139 (22), 126 (16), 113 (100), 98 (75); HRCIMS $[M]^+$ *m/z* 265.2400 (calculated for $C_{17}H_{31}NO$, 265.2406).

Brine Shrimp Bioassay.[20,21] Brine shrimp (*Artemia salina*) eggs were added to a shallow rectangular pan filled with artificial seawater (Instant Ocean®,

Aquarium Systems, Inc.) and incubated at 28 °C for 24 hours. The hatching chamber contained a plastic divider with numerous 2 mm holes and eggs were added to the half of the chamber that was kept in the dark. After 24 hours, the phototropic nauplii were gathered from the illuminated side of the chamber and used for assays. About 30 brine shrimp in ca. 0.5 ml seawater were added to each well containing different concentrations of sample in 50 μl EtOH and 4.5 ml artificial seawater to make a total volume of ca. 5 ml. Samples and controls were run in triplicate. After 24 hours at 28 °C, the brine shrimp were observed and counted using a dissecting light microscope to calculate LD_{50} values.

CHAPTER III

DISCOVERY OF A NOVEL HETEROCYCLIC SULFUR-CONTAINING COMPOUND WITH ANTIFUNGAL ACTIVITY FROM *LYNGBYA MAJUSCULA*

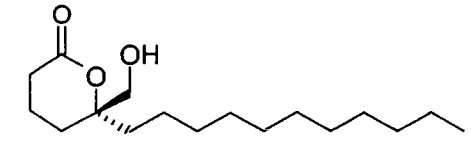
Abstract

A Madagascar collection of the marine cyanobacterium *Lyngbya majuscula* yielded a novel heterocyclic, sulfur-containing compound, thiolyngbyan (**44**), with antifungal activity. The compound was isolated using antifungal (*Candida albicans*) bioassay-guided fractionation. The structure of the thiolyngbyan is proposed based on 1D and 2D NMR and mass spectral data.

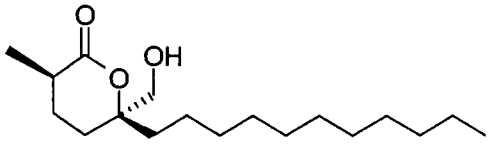
Introduction

Cyanobacteria are a prehistoric and assorted group of photosynthetic microorganisms which inhabit many diverse and extreme environments. This ability indicates a high degree of adaptation which has enabled these organisms to live and contend effectively in nature. The mat-forming cyanobacterium, *Lyngbya majuscula*, produces many promising cytotoxic and antifungal agents.[21,109]

A collection of *Lyngbya majuscula* from Tanikely Island, Madagascar yielded the brine-shrimp toxic and antifungal compound tanikolide (**35**). Tanikolide (**35**) gave a 13 mm diameter zone of inhibition (100 μg/disk) to *Candida albicans* using paper disk-agar plate methodology.[110] Another compound of related structure, malyngolide (**36**), is also reported from *L. majuscula*.[111] The stereochemistry at C-5 in malyngolide (2*R*,5*S*) is the opposite of tanikolide (5*R*). (–)-Malyngolide does not show activity to *C. albicans*.



Tanikolide (**36**)



Malyngolide (**37**)

Figure III.1. Tanikolide (**36**) and Malyngolide (**37**).

Several terrestrial plants produce sulfur-containing compounds, some of which demonstrate antifungal activity.[112-115] Raw garlic (*Allium sativum*) produces allicin (**38**) when the plant is crushed or injured. Cavallito *et al.* first discovered allicin in 1944.[113] Although allicin demonstrates potent antifungal activity it was never developed into a drug or commercial product due to its instability, lack of bioavailability, and unpleasant odor.[116-118]

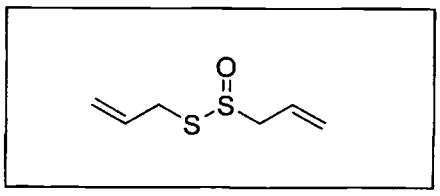
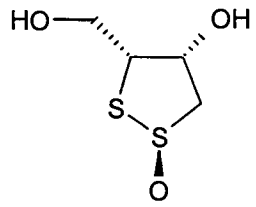
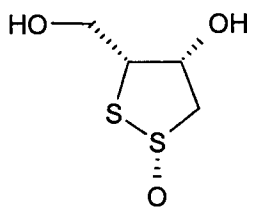


Figure III.2. Allicin (**38**).

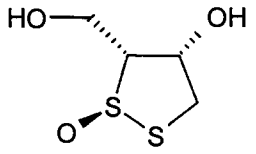
Several cyclic thiosulfinates, zeylanoxides **39–42**, were isolated from the tropical weed *Sphenoclea zeylanica*.[112] *S. zeylanica* grows in seasonal swamps in Asia, Africa and America. In the United States, it is known as "gooseweed". The zeylanoxides were investigated for their allelopathic properties. Compounds **39–42** completely inhibited root growth in rice seedlings at 3 mM. Whereas thiosulfinates such as allicin demonstrated antimicrobial activity, (3*R*,4*R*)-**39–42** did not inhibit growth of fungi or bacteria. It is important to keep in mind that while (3*R*,4*R*)-**39–42** did not demonstrate antimicrobial activity, their stereoisomers were not tested. As was the case for tanikolide (**36**) and malyngolide (**37**), stereoconfiguration seemed to play a role in their ability to inhibit the growth of *C. albicans*.[111] Stereochemistry did affect the inhibitory activities of a synthetic derivative of the zeylanoxides.



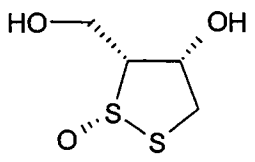
Zeylanoxide A (**39**)



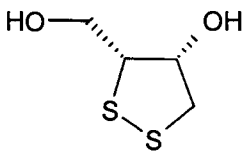
epi-Zeylanoxide A (**40**)



Zeylanoxide B (**41**)



epi-Zeylanoxide B (**42**)



Synthetic Derivative (**43**)

Figure III.3. Allelochemicals from the Tropical Weed *Sphenoclea zeylanica.*

Dithiolane (3*R*,4*R*)-**43** completely inhibited root growth of rice seedlings at 1 mM, but (3*S*,4*S*)-**43** required 3 mM for complete inhibition. The methodology used for the bioassay could be another factor in not detecting antifungal activity in the zeylanoxides. For the antifungal assay, a pulp disk containing the test compound was placed on potato-dextrose-agar medium containing *C. albicans*, and incubated at 25 °C for the fungi in the dark. The diameter of the zone showing growth inhibition was measured after 7 days. The time of incubation may be too long to

maintain a zone of inhibition against the microorganism due to instability of the compound or metabolic degradation. In our studies, zones of inhibition for test compounds in similar assays diminished on the agar plate after 24-48 hrs.

Results and Discussion

We isolated a novel secondary metabolite, thiolyngbyan (**44**), from *Lyngbya majuscula* collected from exposed supratidal rocks on the island Nosy Tanikely, Madagascar. The collection was kept cold in isopropanol until extracted. A portion of the crude organic extract (9 g) was subjected to silica gel vacuum liquid chromatography (VLC) using a mixture of hexanes and EtOAc as eluent (Figure III.4). A preliminary bioassay using 2 mg of organic extract showed a 30 mm diameter inhibitory zone in the antifungal toxicity assay (*Candida albicans*).[110] Guided by the antifungal toxicity assay, the natural product **44** was isolated in small yield (3.0 mg) as a white crystal by sequential solid phase extraction columns and RP-HPLC (Experimental Section).

Thiolyngbyan (**44**) showed an M^+ peak at *m/z* 225.9122 for a molecular formula of $C_5H_7S_2BrO$ by HRCIMS (Figure III.5). The molecular formula indicated two degrees of unsaturation. One of these was accounted for with the two olefinic carbons at δ 123.7 (C_4) and δ 128.0 (C_6). The second degree of unsaturation was attributed to a heterocyclic ring.

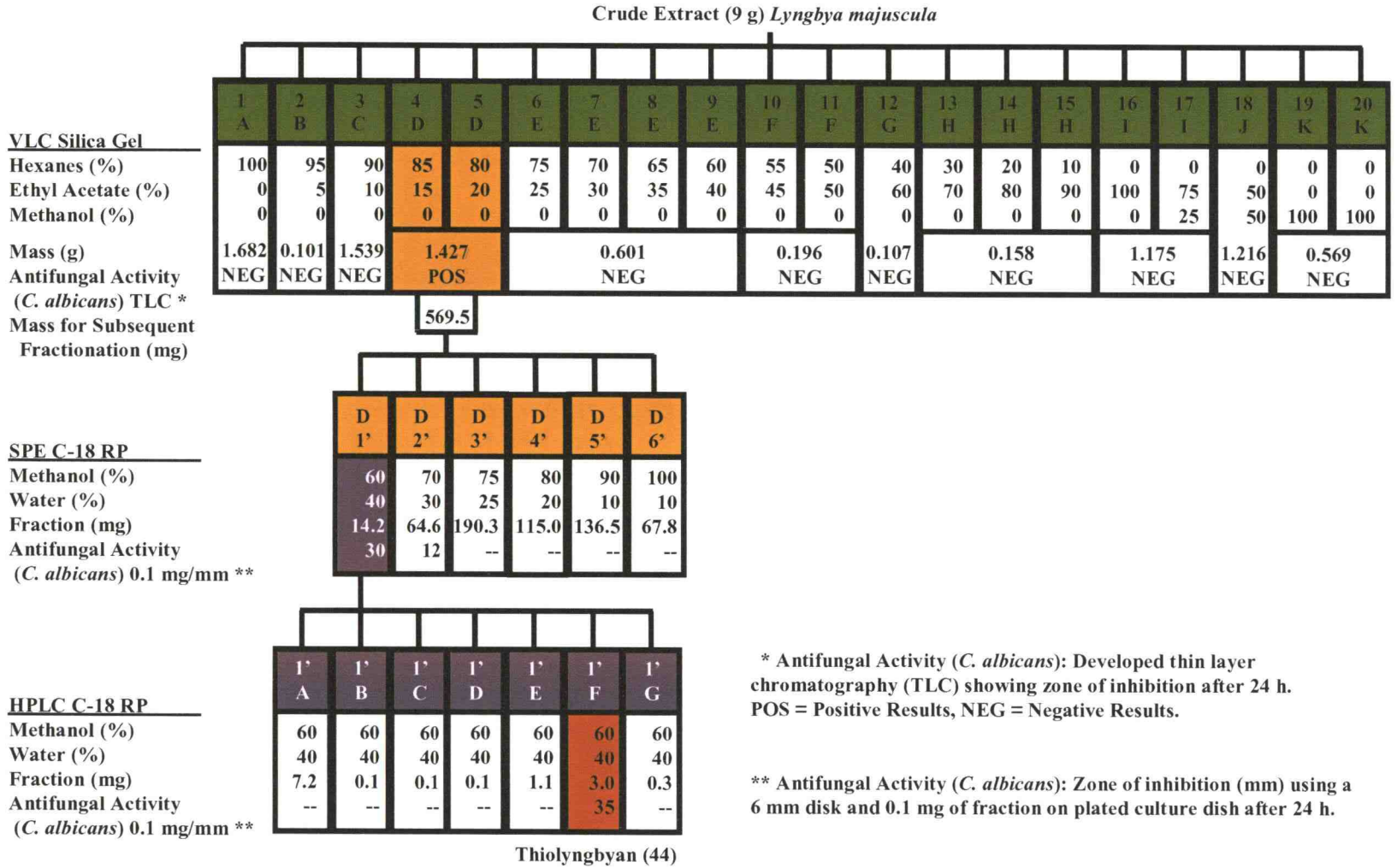


Figure III.4. Fractionation of Thiolyngbyan (**44**).

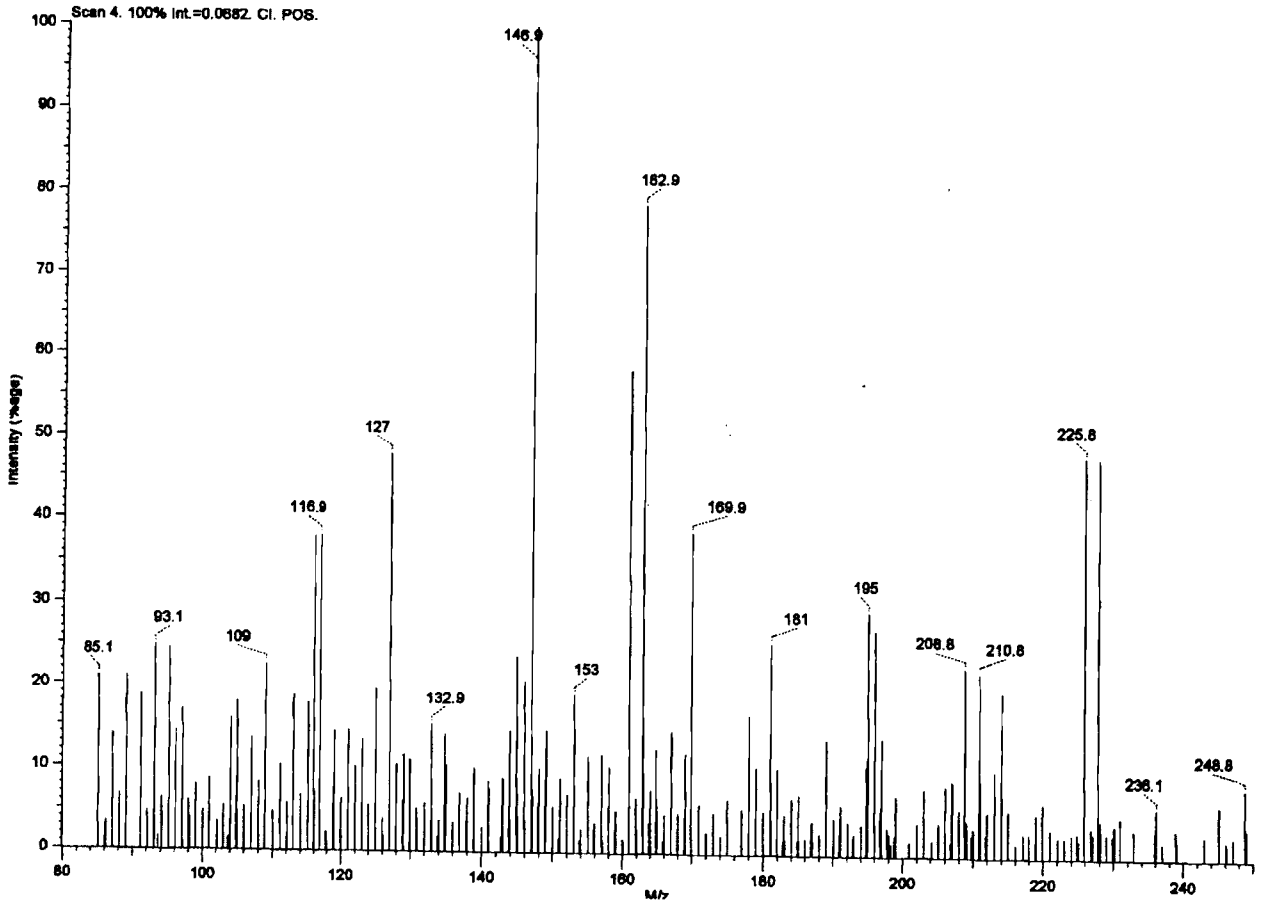
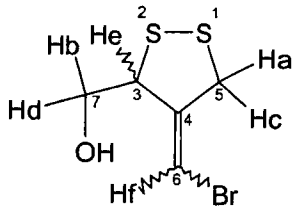
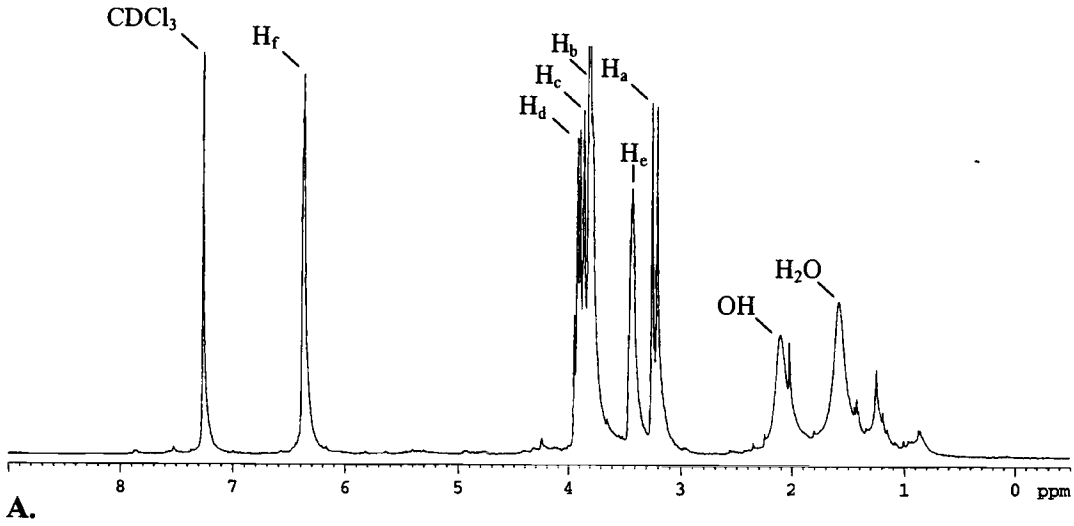


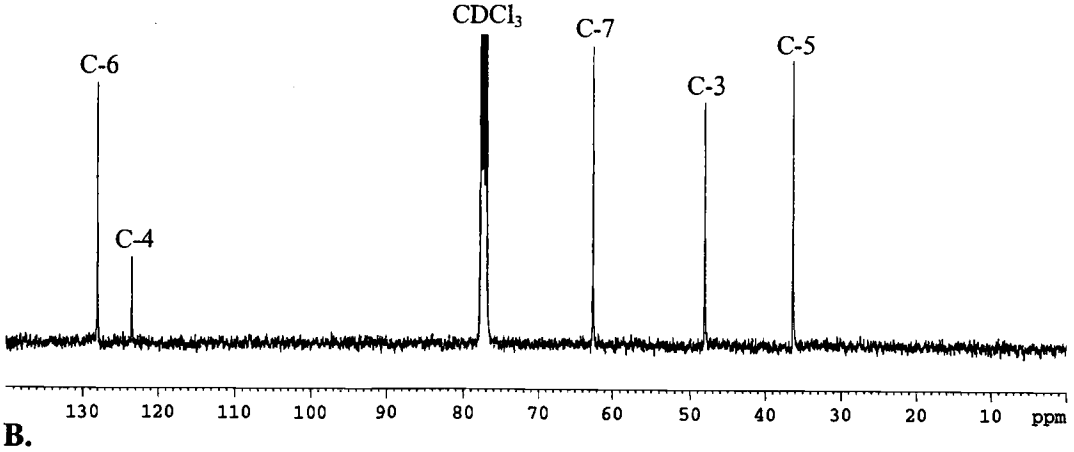
Figure III.5. LRCI (CH_4) – Mass Spectrum of Thiolyngbyan (**44**).



Thiolyngbyan (**44**)



A.



B.

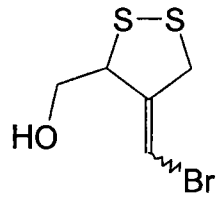
Figure III.6. A. ^{1}H NMR and **B.** ^{13}C NMR Spectra of Thiolyngbyan (**44**).

Table III.1. ^{1}H and ^{13}C NMR Assignments Thiolyngbyan (**44**) in $CDCl_3$.

Position	^{1}H	J (Hz)	^{13}C	HMBC
1 S				
2 S				
3	(H_e) 3.42 br m		48.1	62.6, 123.4, 128.0
4			123.4	
5	(H_a) 3.23 d	17.5	36.3	123.4, 128.0
	(H_c) 3.82 d	17.5		123.4, 128.0
6	(H_f) 6.37 br s		128.0	36.3, 48.1, 123.4
7	(H_b) 3.80 m		62.6	123.4
	(H_d) 3.90 m			48.1

The ^{1}H NMR spectrum (Figure III.6) showed signals of four methylene protons at δ 3.23 (H_a), δ 3.80 (H_b), δ 3.84 (H_c), δ 3.90 (H_d), and two methine protons at δ 3.42 (H_e) and δ 6.37 (H_f) (Table III.1). This suggested that all protons were bound to carbons bonding to heteroatoms such as oxygen, bromine, and sulfur.

The ^{13}C NMR spectrum showed four carbons at δ 36.3 (C_5), δ 48.1 (C_3), δ 62.6 (C_7), and δ 128.0 (C_6) (Figure III.6). The HSQC showed that these carbons bonded to $H_{a,c}$, H_e, $H_{b,d}$, and H_f, respectively (Figure III.7). In the HMBC spectrum, correlations were observed between C_3, and $H_{b,d}$, and H_f, between C_4, and $H_{a,c}$, H_b, H_e, and H_f, between C_5, and H_f, between C_6, and $H_{a,c}$, and H_e, and between C_7, and H_e (Figure III.8). Observing the ^{13}C NMR shifts of C_5 (δ 36.3),



Thiolyngbyan (**44**)

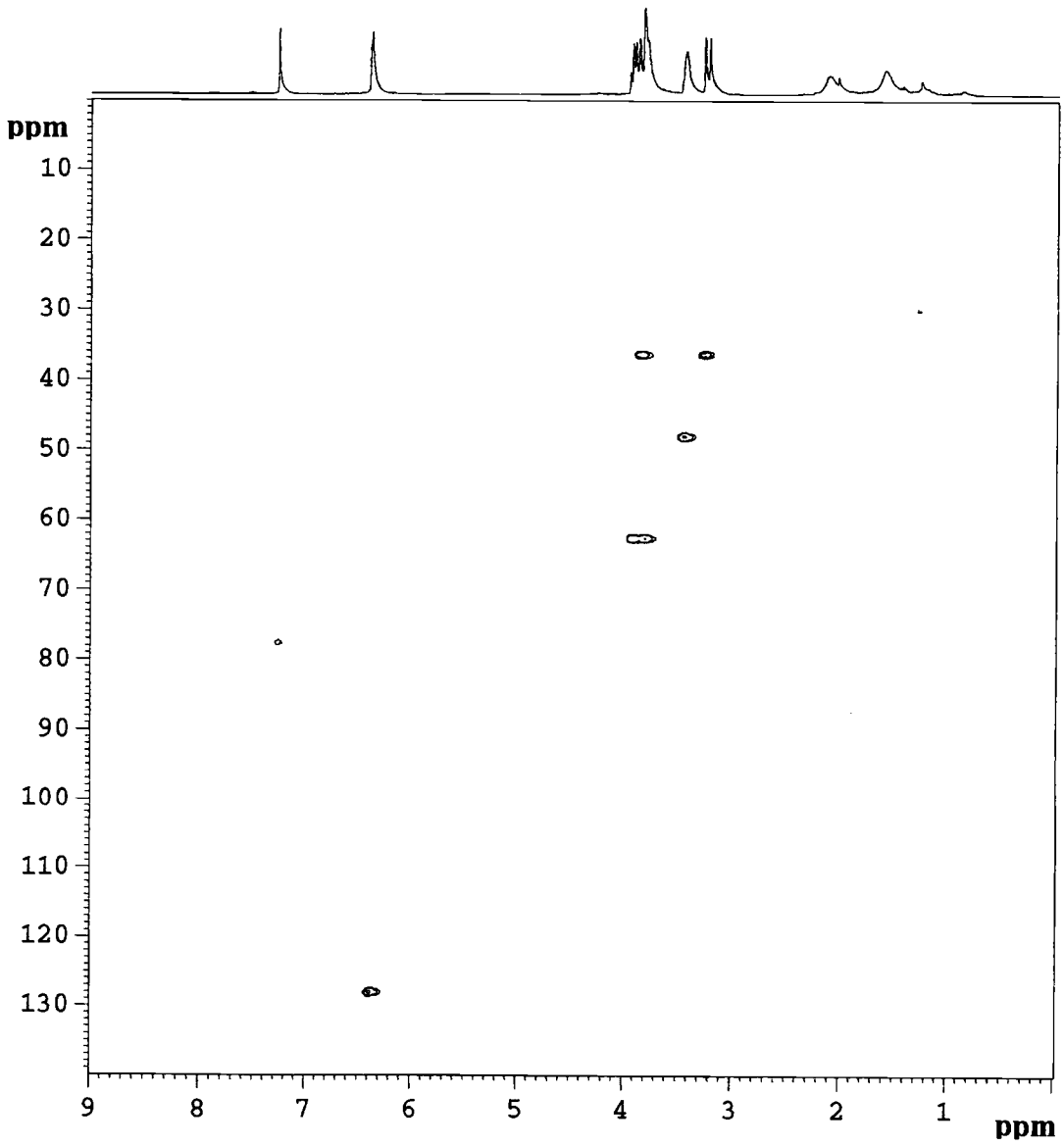
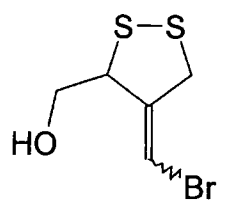


Figure III.7. HSQC Spectrum of Thiolyngbyan (**44**).



Thiolyngbyan (**44**)

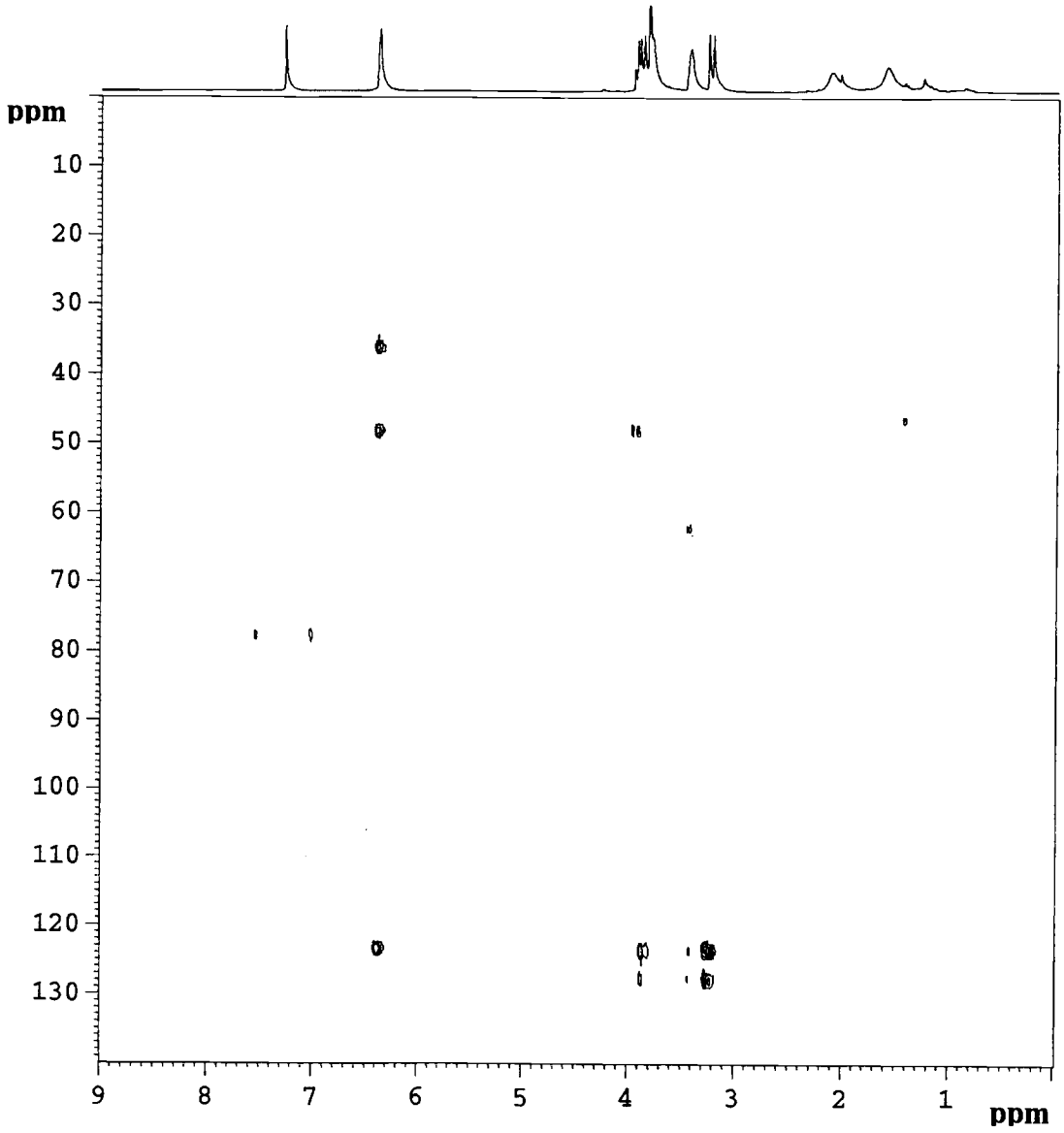
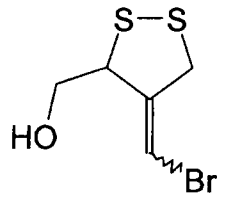


Figure III.8. HMBC (Optimized for 8 Hz) Spectrum of Thiolyngbyan (**44**).



Thiolyngbyan (**44**)

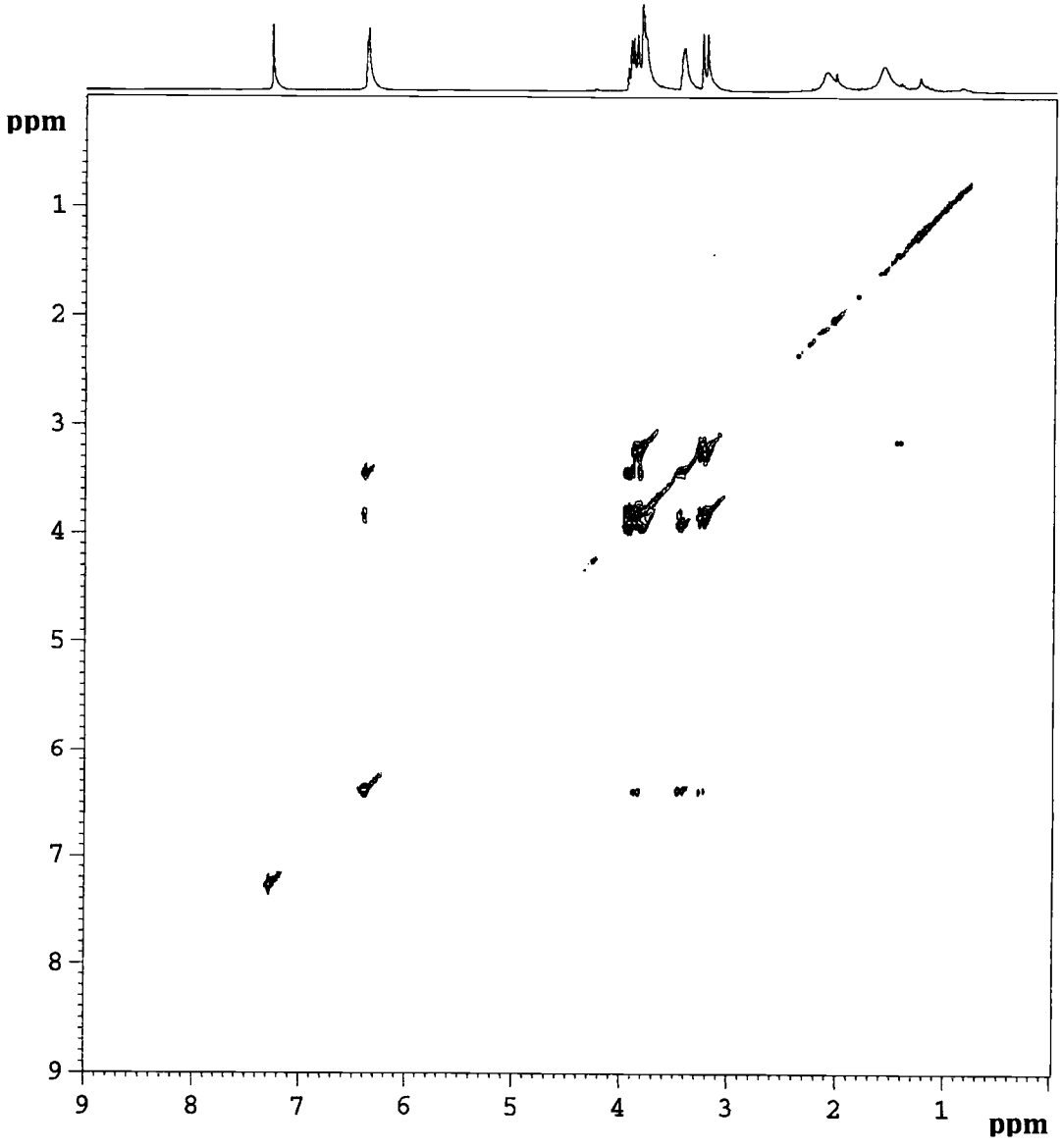


Figure III.9. ^{1}H-^{1}H COSY90 Spectrum of Thiolyngbyan (**44**).

C_3 (δ 48.1), and C_7 (δ 62.6) indicated that they were bonded to heteroatoms consisting of the disulfide functionality for C_3 and C_5, and a hydroxyl group (IR 3300 cm^{-1}) for C_7. The analysis was further supported by the ^1H-^1H COSY, showing correlations between H_e and $H_{b,d}$ and long range W coupling between H_f and H_e, and H_f and H_a. These correlations led to the suggested planar structure of compound **44**. Thiolyngbyan (**44**) (0.1 mg) showed a 35 mm zone of inhibition using a 6 mm disk on a plated culture dish (*Candida albicans*) after 24 h. The control disk (Amphotericin B, 2 mg) showed a 19 mm zone of inhibition after 24 h. Shortly after acquiring the 1D and 2D NMR and mass spectral data sets, thiolyngbyan (**44**) decomposed completely. The decomposition was noted using ^1H NMR during further attempts to acquire additional spectral data.

Experimental

General. ^1H and ^{13}C NMR spectra were measured on a Bruker AM 400 spectrometer operating at a proton frequency of 400 MHz and a carbon frequency of 100 MHz with the solvent used as an internal standard ($CDCl_3$ at δ 7.26 and δ 77). Mass spectral data was recorded on a Kratos MS50TC mass spectrometer. IR was recorded on a Nicolet 510 spectrophotometer. HPLC separations were performed with a Waters 515 pump and a Waters 996 Photodiode Array Detector. Merck aluminum-backed thin layer chromatography sheets were used for TLC.

Collection. The marine cyanobacterium, *Lyngbya majuscula* (MNT-17/APR/00-1), was hand-collected from exposed supratidal rocks on the island Nosy Tanikely (*S* 12°51.185', *E* 48°25.153'), Madagascar on April 17, 2000. The specimens were preserved in isopropanol upon collection and stored at low temperature until workup.

Extraction and Isolation of Thiolyngbyan (43). The thawed algal material (1091 g, dry wt) was homogenized in CH_2Cl_2/MeOH (2:1) and filtered. The solvents were removed *in vacuo* to yield a residue that was partitioned between CH_2Cl_2 and H_2O. The residue was extracted repeatedly with CH_2Cl_2, and the combined CH_2Cl_2 layers were reduced *in vacuo* to yield a dark green crude extract (11.2 g). A portion of the crude extract (9 g) was fractionated using vacuum liquid chromatography (VLC) on Si gel with a stepwise gradient of hexanes/EtOAc and EtOAc/MeOH. Eluted material was collected in 20 fractions and monitored by TLC. Similar fractions were combined to give eleven fractions. Fraction D (1.4 g, eluted with hexanes/EtOAc (4:1)) showed antifungal activity on TLC. It was further fractionated using Mega Bond Elut C18 12 cc into six sub-fractions. Sub-fraction D1' (14.2 mg, eluted with MeOH/H_2O (3:2)) showed a 30 mm diameter inhibitory zone in the antifungal assay at 0.1 mg. The sub-fraction was further purified on RP-HPLC (Varian Microsorb MV 100-5 C18 250 x 4.6 mm; detection at 254 nm) with MeOH/H_2O (3:2) to give thiolyngbyan (**44**, t_R = 21-23 min, 3.0 mg). Pure thiolyngbyan showed a 35 mm diameter inhibitory zone in the antifungal toxicity assay at 0.1 mg.

Thiolyngbyan (44). Thiolyngbyan was a white crystal; HRCIMS $[M]^+$ *m/z* 225.9122 (calculated for $C_5H_7S_2BrO$, 225.9124). IR (neat) ν_{max} 3300, 2810, 2660, 1630, 1550, 1500, 1450, 1375, 1250, 1190,1075, 820, 730 cm^{-1}.

Antimicrobial Assay. Antimicrobial activity of the fractions and thiolyngbyan (**44**) was evaluated using standard paper sensitivity disk-agar plate methodology (disk diameter, 6 mm).[110] Fractions and sample dissolved in ethanol were transferred to a paper disk with a micropipette and dried. The disks were placed on an agar plate streaked with *Candida albicans* (ATCC# 14053). The plate was incubated at 37 °C for 24 hr. Zones of inhibition were identified and the diameters measured in millimeters. Controls were prepared on 6 mm disks with Amphotericin B (2 mg).

CHAPTER IV

CONCLUSION

The marine cyanobacterium *Lyngbya majuscula* produces many biologically active and structurally unique secondary metabolites. This investigation demonstrated the diversity of secondary metabolites produced by *L. majuscula*. From a single collection off the coast of Nosy Tanikely, Madagascar (April 17, 2000), we isolated two related compounds, lyngbyamides B and C, and the antifungal compound, thiolyngbyan.

The majority of the natural products isolated from *L. majuscula* have polyketide-derived subunits that are combined with amino acid-derived components.[92-104] The lyngbyamides represent interesting examples of N-acyl containing lipids from the cyanobacteria *Lyngbya majuscula*. The compounds exhibit a cyclopropyl fatty acid and an assortment of functionalized amines that are connected through an amide bond. This characteristic represents a typical metabolic theme of cyanobacteria. Similar compounds sharing the same motif such as the malyngamides and hermitamides demonstrate biological activity in several systems.[67,101,106]

Lyngbya majuscula produces many promising cytotoxic and antifungal agents.[21,109] Thiolyngbyan was isolated using antifungal (*Candida albicans*) bioassay-guided fractionation. Reviewing similar compounds in the literature

identified the structurally similar zeylanoxides isolated from the tropical weed *Sphenoclea zeylanica*.[112] Thiolyngbyan possess a bromine functional group which is indicative of the unique tailoring enzymes known to modify cyanobacterial metabolites.[67] Further characterization of thiolyngbyan, such as its stereochemistry at the C_5 position, was interrupted by its unfortunate and untimely decomposition!

Lyngbya majuscula continues to demonstrate its ability to provide novel metabolites consistent with metabolic themes as well as distinctive bioactive compounds such as thiolyngbyan. This trend will allow marine natural product chemists to investigate and learn more about the ecological and biosynthetic processes of this amazing organism.

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