AN ABSTRACT OF THE THESIS OF

Elise Shylo Cowley for the degrees of Honors Baccalaureate of Science in Bioresource Research and Honors Baccalaureate of Science in Chemistry presented on May 28, 2013.
Title: Discovery and Characterization of New Cyanobacterial Natural Products from *Moorea* sp.

Abstract approved:_____
Kerry McPhail

Natural products (secondary metabolites) are small organic molecules derived from plants, microbes, and marine organisms that have served as a diverse source of useful compounds pharmaceuticals. New biologically active natural products are critical as anti-infective and cancer drug leads. Cyanobacteria, or blue-green algae, produce diverse cytotoxic metabolites that represent potential anticancer leads.

A Red Sea strain of a *Moorea* cyanobacterium was isolated from field collections made during an exploratory expedition in 2007. A study was conducted to determine unique and known cytotoxic compounds produced by laboratory cultures of this *Moorea* strain.

Large-scale *Moorea* cultures were harvested for chemical extraction and chromatographic purification of compounds by sequential vacuum liquid chromatography, solid phase extraction, and High Pressure Liquid Chromatography. The fractionation was guided by brine shrimp and cancer cell toxicity assays. Spectroscopic methods including mass spectrometry and nuclear magnetic resonance were used to characterize the structures of pure compounds isolated from the active fractions.

The production of the known compounds apratoxin A and lyngbyabellin B was confirmed along with the new apratoxin analogs apratoxin A sulfoxide and apratoxin H.

In addition, three hydrophobic HPLC fractions reduced cancer cell viability by 70%. These results demonstrate the profound ability for *Moorea sp.* to produce an abundance of cytotoxic secondary metabolites, which may serve as pharmaceutical lead compounds or molecular research probes.

Keywords: Cyanobacteria, natural products, cytotoxic

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Discovery and Characterization of New Cyanobacterial Natural Products from *Moorea* sp.

by

Elise Shylo Cowley

A PROJECT

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Toni Doolen, Dean, University Honors College

I understand my project will become part of the permanent collection of Oregon State University, University Honors College, and will become part of the Scholars Archive collection for Bioresource Research. My signature below authorizes release of my project to any reader upon request.

Elise Shylo Cowley, Author

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LIST OF ABBREVIATIONS

Full Term	Abbreviation
Oregon State University	OSU
Methanol	MeOH or M
Ethyl Acetate	EtOAc
Hexanes	Hex
Dichloromethane	DCM
Natural Product	NP
Normal Phase-Vacuum Liquid Chromatography	NP-VLC
Reverse Phase-Solid Phase Extraction	RP-SPE
High Performance Liquid Chromatography	HPLC
Liquid Chromatography-Mass Spectrometry	LC-MS
Mass Spectrometry	MS
Nuclear Magnetic Resonance	NMR
Brine Shrimp Assay	BSA

DEDICATION

This thesis is dedicated to the Cowley Clan, Anita, Mark, Jeannie, Stan, and Kyle, the truest friend a girl could ever ask for. You may never understand what I am doing, but you have always unconditionally supported me. Thank you. Never quit.

Discovery and Characterization of New Cyanobacterial Natural Products from *Moorea* sp.

Introduction

The term "natural product" is synonymous with "secondary metabolite". Secondary metabolites are small organic compounds derived from an organism, which are not necessary for life, but may be critical for survival in specific environments. Secondary metabolites are produced by a majority, if not all, living organisms, the most well known being from terrestrial plants, marine algae, marine invertebrates, bacteria, and fungi. Secondary metabolites are of particular interest to humans because of their wide-ranging applications in human health and agriculture. The structural complexity of secondary metabolites is unrivaled, and they would be difficult to create *de novo* by chemical synthesis in the laboratory without inspiration from nature. Researchers are constantly searching for new sources of unique compounds with unprecedented biological activities, and nature consistently provides a pool of novel compounds.

Natural products have been utilized in the pharmaceutical industry, industrially, and in agriculture. In agriculture, natural products have served as a source of pesticides, herbicides, and fungicides. Of particular interest for this project is the role of natural products as pharmaceuticals. For perspective, over 30% of all FDA-approved pharmaceuticals have been derived from or inspired by natural products. A breakdown of the sources of small molecule drugs is displayed in Figure 1 (Newman, 2012).

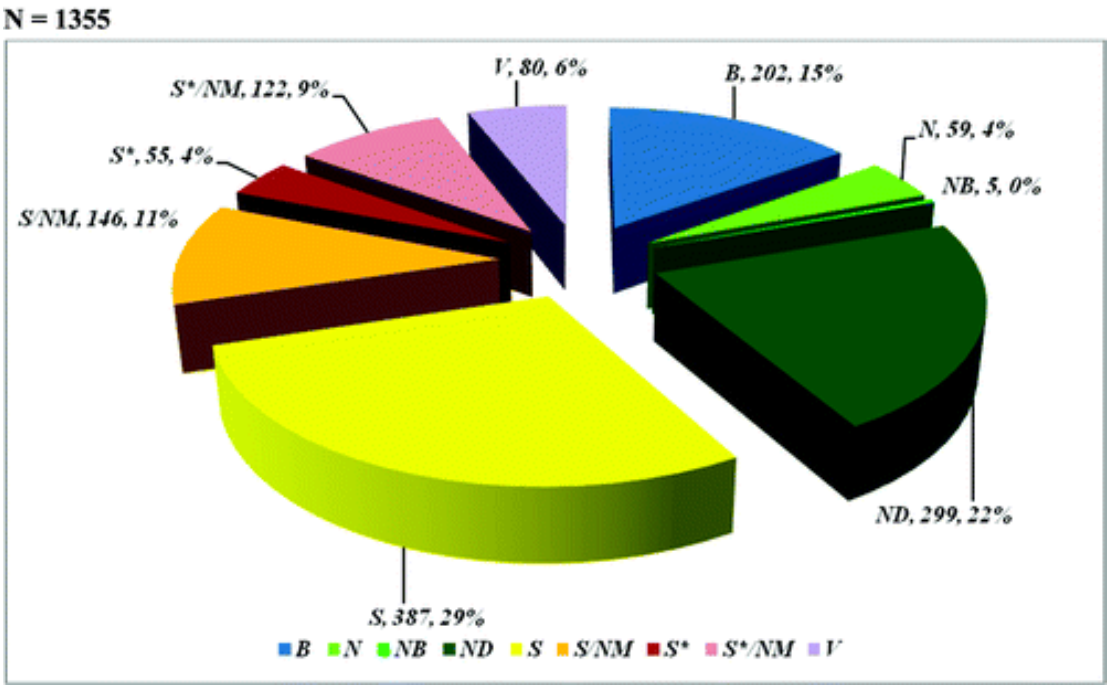


Figure 1. Source of FDA approved small molecule drugs, 1981–2012: major categories, *N* = 1355 (in percentages). Major categories are as follows: N" Natural product. "NB" Natural product "Botanical" (in general these have been recently approved)."ND" Derived from a natural product and is usually a semisynthetic modification. "S" Totally synthetic drug, often found by random screening/modification of an existing agent. "S*" Made by total synthesis, but the pharmacophore is/was from a natural product. "V" Vaccine. (Newman, Cragg, 2012).

Natural products have played a central role in the pharmaceutical industry. Natural products can be used directly as drugs, as inspiration for a drug by providing a scaffold, or as molecular probes to investigate cellular or disease mechanisms. The medicinal use of natural products dates back to the beginning of human history and the first recorded use of a natural product dates to 3500 BCE with the utilization of opium poppies.

A myriad of notable examples of natural products that have led to monumental pharmaceuticals exist and two will be highlighted. Quinine is an alkaloid that is derived from the bark of cinchona trees. Quinine is notable because it was the first effective and

widely used malaria treatment. Quinine is no longer the first option drug to treat or prevent malaria, but it is still used (Trampuz et al., 2003). Another example is the statin drugs. Lipitor, a cholesterol-lowering statin drug, is one of the most widely used drugs in history. Statins were originally discovered in the 1970s and were isolated from fungi. At least two statin analogs were halted in clinical trials, due to unintended side effects. However, development of them as treatments for high cholesterol continued because the issue was viewed as a major human health problem with no mainstream drug treatment options. In 1995, Simvastatin was approved by the FDA and is still in use today. The main role of statin drugs is blocking the enzyme HMG-CoA reductase, which is an enzyme that acts early in the cholesterol biosynthetic pathway (Tobert, 2003).

Natural products discovery underwent a renaissance in the 1970s, but tapered off in the 1990s with the increased use of combinatorial libraries and high throughput assays to screen drug leads. The 2000s saw a renewal of interest in natural products specifically for use as pharmaceuticals. Marine natural products discovery began in the 1960s. Ziconotide was the first marine derived compound approved as a drug. It was isolated form cone snails and is being used to treat pain (Molinski, 2009).

Method development and equipment advances in the last several decades have helped create the natural products discovery renaissance. The improvements in chromatographic methods have helped make isolation of novel compounds quicker and possible with small amounts of biomass. Chromatography is a means of separating a mixture into pure single components based on a physical property of the compounds such as charge, molecular size, or polarity. Preparative, semi-preparative, and analytical chromatography are the three basic chromatography steps for purifying an extract.

Preparative chromatography separates larger volumes of material while analytical chromatography separates smaller volumes, and is commonly used to obtain microgram to low milligram quantities of pure compounds, as well as for analytical profiling of a sample. The mobile phase in liquid chromatography is a solvent and the analytes of interest from the organic extract are partitioned between the solvent and the packing material (stationary phase) of the column being utilized. The partitioning of mixture components back and forth between the mobile and stationary phases (which differ significantly in their polarity) is the mechanism by which compounds are separated. Each of these liquid chromatography methods separates compounds based on polarity.

High performance liquid chromatography (HPLC) is a powerful technique for natural products isolation and the advances in HPLC equipment have helped propel natural products isolation. HPLC collection is based on UV absorption profiles of the injected sample solutions. HPLC uses high pressures (generally 2000-3500 psi), and columns tightly packed with very small (e.g. 5 micron) particles that present a very large surface area to separate mixtures. Using a photodiode array detector, the complete spectrum of wavelengths in a designated range (typically 190-400 nm) is detected over the duration of the chromatographic run. Typically four wavelengths are used for real time viewing as the HPLC run is in progress. These are selected to correspond to different functional groups absorbances: 216 nm for carbonyl groups, 230 nm for carbonyls neighboring electronegative atoms such as in amide or ester bonds, 254 nm for aromatic groups, and 330 nm for highly conjugated (colored) compounds, such as chlorophyll.

Many natural products that were originally attributed to larger organisms are proven or proposed to be produced by bacteria in a symbiotic relationship with the larger organisms. This has fueled the search for bacteria, particularly cyanobacteria. Cyanobacteria are ubiquitous and ancient organisms. They live in both terrestrial and marine environments. Both the marine and terrestrial species have been studied for their production of natural products. Cytotoxic, being toxic to a cell, properties are associated with many compounds isolated from cyanobacteria. Examples of cytotoxic cyanobacterial products include cryptophycins and curacins (Gerwick, 1994). Cyanobacteria have been classified alongside myxobacteria and *Streptomyces* as candidates for pharmaceuticals (Tan, 2010). The ubiquitous nature and demonstrated potential for production of novel secondary metabolites make cyanobacteria excellent candidates for natural product isolation.

With the continued need for new compounds, scientists increasingly look to new and exotic environments for sources of natural products. In May 2007, the McPhail Laboratory collaborated with Professor Diaa Youssef from Suez Canal University in Ismalia, Egypt, to assess the abundance and diversity of cyanobacteria in the northern part of the Red Sea. This exploratory expedition demonstrates attempts to explore new and unique environments for potentially novel compounds. The Red Sea is a saltwater inlet of the Indian Ocean that borders both the Arabian Peninsula and Egypt. The northern portion of the Red Sea includes two gulfs, the Gulf of Aqaba and the Gulf of Suez, that border the Sinai Peninsula.

SCUBA diving and snorkeling were used to collect cyanobacterial samples from reefs and shipwrecks near Hurghada and Sharm El Sheik, as well as around the Sinai

penisula. This collection comprised twelve small field collections and twenty-eight live samples for laboratory culture. Several of the samples were collected from mangroves. Mangroves are saline woodlands and in being so border many coastlines. Most mangroves are centered around the equator. Plants within mangroves grow with roots branching into the water. The roots act as filters and also provide nutrients back into the water. The nutritional density associated with the root systems provides a beneficial ecosystem for diverse marine invertebrates, algae, and cyanobacteria (Lugo, Snedaker, 1974).

Materials and Methods

Collection

The sample that is the subject of this particular project was collected by hand while snorkeling in the Nabq mangroves, which are located in the southern portion of the Sinai Peninsula in the Gulf of Aqaba (Figure 2).

Figure 2. Map of the Sinai Peninsula where the 2007 exploratory expedition focused (sharmelsheik.com).

Isolation and Culturing

In the McPhail lab at OSU, graduate student Christopher Thornburg worked on isolating cyanobacteria for laboratory culture. The collected environmental samples were not one organism, but an assemblage comprising multiple cyanobacterial and heterotrophic bacterial strains. The goal of the isolation step was to separate the environmental sample and identify single cyanobacterial strains for pure monoclonal culture. Separation of cyanobacterial filaments was performed using a dissecting compound microscope. Thornburg classified each organism morphologically with the aid of a contrast microscope. Isolated cyanobacteria were grown in triplicate in 24-well plates at 27 °C in 12 hour light/dark cycles. Each cyanobacterium was grown in triplicate

in four different culture media with different enrichment processes. The four enrichment media included Red Sea Water (RSW) extract, RSW and soil extract, BG-11 medium modified to closely match Red Sea water, called Red Sea Medium (RSM), and a 1:1 combination of RSW and RSM. Monoclonal cultures were established from serial dilutions of the enriched cultures that displayed significant growth. Using this methodology, nineteen of the twenty-eight small live cultures survived and Thornburg was able to establish five monoclonal cultures for larger scale cultivation, harvesting and chemical extraction. The monoclonal cultures were grown in Red Sea Medium (Appendix A).

One of the organisms isolated using the above method was a brown filamentous cyanobacterium. Based on morphological features and 16S rRNA sequencing, the cyanobacterium was identified initially as *Lyngbya majuscula*, which we termed "the Red Sea strain of Black *Lyngbya".* In 2011, Niclas Engene et al. demonstrated that a significant portion of the cyanobacteria in the genus *Lyngbya* had been misclassified, and introduced the new genus *Moorea.* The cyanobacterium for this project, originally identified as a species of *Lyngbya* has since been reassigned as a *Moorea* species (*Moorea* sp.).

Extraction

The first large-scale extraction of the black *Moorea* sp. comprised biomass from 50 liters of medium that was grown over a period of twelve months. The harvested biomass was subjected to organic extraction using a mixture of 2:1 dichloromethane-methanol. This procedure entailed soaking the cyanobacterial mass in fresh solvent in a metal beaker with gentle heating for approximately one hour, allowing it to cool, and then

filtering off the extraction solvent under vacuum through cheese cloth over filter paper to trap the cyanobacterial biomass. The cyanobacterial mass was then returned to the beaker, fresh extraction solvent added, and the procedure repeated five times until the filtered extraction solvent was pale yellow to colorless. The extraction filtrates were combined and concentrated in vacuo by rotary evaporation to remove the solvents and yield a deep green-brown oil (0.85 g).

Chromatography

The organic extract from *Moorea* sp. was subjected to a series of chromatographic techniques with the goal of obtaining pure compounds via iterative separation. Three liquid chromatographic methods were used to separate the organic extract: normal phase-vacuum liquid chromatography (NP-VLC), reversed phase-solid phase extraction (RP-SPE), and high performance liquid chromatography (HPLC).

Normal Phase Vacuum Liquid Chromatography (NP-VLC)

Separation by NP-VLC is based on polarity using a stepped solvent gradient. A 600 mL sintered glass funnel was packed with silica in hexanes using vacuum aspiration. The column was then conditioned with three bed volumes of hexanes before the organic extract was applied carefully to the top of the column in a small amount of hexanes, to produce as thin a band as possible. The column was subsequently eluted with nine different solvent mixtures: 100% hexanes, 10% ethyl acetate/hexanes, 20% ethyl acetate/hexanes, 40% ethyl acetate/hexanes, 60% ethyl acetate/hexanes, 80% ethyl acetate/hexanes, 100% ethyl acetate, 25% methanol/ethyl acetate, and 100% methanol.

Each fraction obtained was a different color because cyanobacteria produce a variety of pigments that have different polarities, and hence elute with a different solvent mixture. Each fraction was designated with a letter A through I (hexanes through methanol) according to the standard VLC fractionation protocol used in the McPhail laboratory. The fractions were concentrated to dryness in vacuo and weighed in glass vials.

Brine Shrimp Assay (BSA)

The NP-VLC fractions obtained were tested for cytotoxicity using a brine shrimp assay (BSA). Brine shrimp (*Artemia salina*), also known as sea monkeys, are used as an indicator species in toxicity tests. A brine shrimp assay was used to guide the fractionation by indicating which fractions contain the components responsible for the initial cytotoxicity observed in the organic extract.

Brine shrimp were hatched from eggs in artificial seawater using a partitioned container with half of the container covered. Eggs were placed in the covered (dark) half of the container, which was left for 48 hours at room temperature to allow the brine shrimp to hatch and move toward the uncovered (light) side, where they could be collected readily in the absence of unhatched eggs. Test solutions of the nine NP-VLC fractions were prepared at a concentration of 1 mg/mL in ethanol, and 50 μL aliquots were added to 4.5 mL sea water in duplicate in each well of a large-well (5 mL) plate. An equivalent amount of 100% ethanol was added to each of two wells as a solvent control. Finally, 10-25 brine shrimp larvae were added to each well. The samples were allowed to incubate for 24 hours after which the live and dead brine shrimp were counted (Table 1) (Meyer et al., 1982). Considering that many of the fractions were highly toxic, another

BSA was performed with test sample concentrations of 0.1 mg/mL, 5 μL aliquots of sample were added (Table 1). The cytotoxicity was recorded as the percent dead brine shrimp of the total number added to each test well. The highly cytotoxic fractions were targeted for the remainder of the project.

Reversed Phase Solid Phase Extraction (RP-SPE)

Based on the BSA of the NP-VLC fractions, seven of the nine fractions (C-H) were considered active. These seven fractions were prioritized for further separation by reversed-phase solid phase extraction (RP-SPE) because of their demonstrated toxic properties. In RP-SPE, the fractions were eluted in the opposite polarity order as the initial NP-VLC separation, with the polar fractions eluting first and the nonpolar, last. A methanol-water stepped solvent gradient was used as mobile phase on pre-packed 2 g Varian C_{18} cartridges. Each column was conditioned with five column volumes of 100% MeOH and equilibrated with five column volumes of 70% MeOH/H_2O, the starting solvent. The VLC parent fractions for separation were each dissolved in a small amount of this starting solvent and transferred in as thin and even a band as possible to the column. The sample was eluted with four solvent systems, 70% MeOH/H_2O, 90% MeOH/H_2O, 100% MeOH, 100% dichloromethane (DCM), and each of these collected as a separate fraction. The fractions were assigned numbers one through four to follow the letter designation of their parent VLC fraction, i.e. C4. The eluted samples were concentrated in vacuo and the masses of each fraction were determined using pre-weighed glass vials.

Reversed-phase High Performance Liquid Chromatography (HPLC)

Reversed phase high performance liquid chromatography (HPLC) was the final chromatographic step to obtaining pure compounds suitable for spectroscopic analysis. Figure 3 displays the HPLC trace for sample F2, as an example. The x-axis represents the time over which the sample was eluted from the column after injection, and the y-axis represents the UV absorbance.

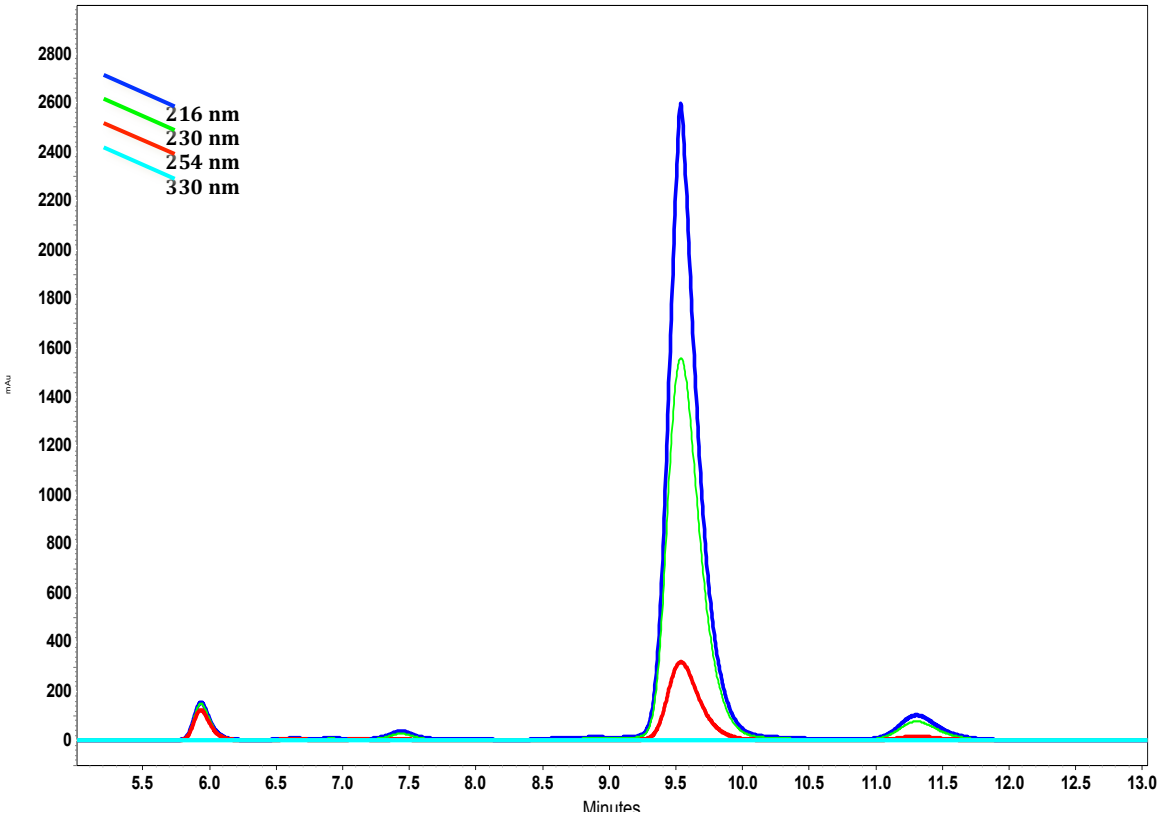


Figure 3. An example HPLC trace for sample F2. The different colored traces correspond to the different wavelengths displayed by the PDA detector.

Three levels of iterative HPLC separation were performed. Initially, a semi-preparative column was used to separate the parent RP-SPE fractions. The hallmark feature of a semi-preparative column is its larger sample capacity (multiple milligrams

depending on sample complexity), and in this project a 10 x 250 mm Phenomenex Synergi Hydro or Synergi Fusion with 5-micron particles was used. Initial method development was performed with each of these columns to determine the column that yielded the best separation. For the majority of the fractions, the Synergi Fusion column provided the cleanest separation. Using a small amount of each fraction, an initial "profile run" was performed using a solvent gradient, in order to determine the best ratio of solvents to perform the bulk of the separation under isocratic conditions. Once the ideal solvent system was determined, the remainder of the semi-preparative separation was performed using an isocratic method in order to provide the best reproducibility for multiple sample injections and to eliminate the time needed to equilibrate the column between each run. Multiple injections were performed for each fraction because the optimal separation was typically achieved for 1 mg sample injections. Injection of amounts larger than this resulted in incomplete separation and saturation of the detector for major peaks. In each case, semi-preparative separation was continued until all the RP-SPE fraction had been injected and the desired peaks had been collected for further separation and final purification of pure compounds as needed.

Semi-preparative HPLC separation was followed by use of an analytical column (4 x 250 nm Phenomenex Synergi Fusion, 5 micron particle size). Separation using the analytical column was performed twice for most of the semi-preparative fractions. Analytical HPLC was necessary because one peak from the semi-preparative column often contained several compounds. The quantity injected and the capacity of the semi-preparative column was so large that the resolution was lower compared to the analytical column. Analytical columns have a smaller capacity and a higher resolution that enabled

the purification of closely related natural products of very similar polarities. Thus, each peak in the analytical column chromatogram corresponded to a single pure compound or a mixture of enantiomeric compounds. A chiral HPLC column with a teicoplanin aglycone (TAG) stationary phase was used to profile the final collected fractions for the presence of enantiomers. Each collected peak from the analytical and chiral columns were reinjected on an analytical column to confirm the presence of only one peak therefore only one compound. In addition, many of the collected peaks were subjected to "clean-up" runs, meaning that they were re-injected and the peak of interest collected again to remove remaining partially overlapping contaminants or degradation products. The parent fractions began HPLC with a label of a letter and a number, corresponding to the VLC and RP-SPE fractions, respectively. With HPLC separation, alternating letters and numbers were added to the name of the compound to indicate the level of separation and the HPLC peak to which the compound corresponded.

All of the collected HPLC peaks were concentrated in vacuo by rotary evaporation and their weights recorded.

Liquid Chromatography-Mass spectrometry (LC-MS)

Liquid Chromatography-Mass Spectrometry (LC-MS) was utilized at each tier of the separation scheme to profile the potential molecular mass ions for the components of each fraction. LC-MS helped to guide the fractionation by revealing mass ions that consistently appeared in the active fraction after each separation, thus enabling putative molecular masses of the active component(s) to be identified.

LC-MS is a hyphenated technique in which chromatography is linked to a mass spectrometric detector in addition to a photodiode array (UV absorbance) detector. It is

useful for analysis of microgram amounts of complex mixtures because of the separation ability granted by LC coupled to the sensitivity of the mass spectrometer. The mixture is injected onto a small analytical column (e.g. Phenomenex Synergi Hydro or Fusion, 2 x 100 mm) using solvent gradient elution. This allows for separation of the mixture and generation of an LC (UV absorbance) profile for separated compounds before they enter the mass spectrometer, where they are ionized and detected. The UV and associated mass ion data facilitate future semi-preparative purification.

LC-MS was performed on NP-VLC fractions and provided a series of interesting mass ions. LC-MS was performed at every chromatography stage in order to map how the compounds responsible for the mass ions of interest were partitioning (in which sub-fraction they were located), and if any previously undetected masses appeared (signaling possible chemical degradation or contamination).

Neuro-2A cell line testing

A second biological assay was utilized to target cytotoxic compounds of interest. The mouse neuroblastoma Neuro-2A cell assay is a cancer cell toxicity model assay. Neuroblastoma is a brain cancer that primarily affects infants. The assay was performed as described previously (Thornburg et al., 2011). Briefly, the cells were cultured and then distributed into 96-well plates with 20,000 cells per well. Candidate pure compounds were added to individual wells at a concentration of 10 μM. As the control, cells were treated with methanol because the compounds being tested were in a solution of methanol (the solvent vehicle). The assay was assessed after a 48-hour incubation period. A standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent at

a concentration of 0.5 mg/mL was introduced to each well at the end of the 48-hour period and the wells incubated for a further two hours. The medium was removed from the wells, leaving behind a colored formazan product. The optical density of the wells was determined at 550 nm with a microplate reader. The optical density of each well was normalized to the control cells as a measure of cell viability. The control cells represented 100% viable cells after incubation, therefore the lower the viability percentage of a well, the more cytotoxic the compound was to the cells and the higher the percentage of neuro-2A cells killed during the treatment. Each candidate pure compound was tested in triplicate and the results were averaged. (Thornburg et al., 2011)

MarinLit Database

The database of published marine natural products, MarinLit, was a critical piece of software used to search the mass data obtained from LC-MS. Mass peaks of *m/z* 200 to 1,500 were considered to be of interest because that is the range of small organic molecules with potential medicinal applications. MarinLit allows searching by molecular mass and taxonomy of producing organisms of published marine natural products. Searches were performed by specifying "*Lyngbya"* or "cyanobacteria" as the taxonomic constraint, and inputting the exact mass value obtained from LC-MS. If there were no hits from this search, additional searches were completed with the LC-MS mass value plus or minus the masses of different functional groups. For example, searches were completed at M+15 and M-15 because 15 mass units corresponds to the mass of a methyl group. The overall goal of the MarinLit searches was to establish if the compounds detected were

potentially known or new metabolites. The MarinLit searches led to a list of hypothetical compounds predicted to be purified by HPLC.

Nuclear Magnetic Resonance (NMR) spectroscopy at 300 and 700 MHz

Once pure compounds were obtained from HPLC, the structures could be confirmed as known or predicted to be new and subsequently identified using NMR spectroscopy. If a compound was available in multiple milligram amounts, the 300 MHz spectrometer was used to collect 1D proton (^{1}H) and carbon (^{13}C) 1D NMR data. If the sample quantity was more limited, the 700 MHz spectrometer was used for all NMR experiments. In addition to 1D ^{1}H and ^{13}C experiments, a suite of 2D NMR experiments was performed on putative new as well as known compounds of interest including: COSY HSQC, HMBC, ROESY, and TOCSY. The suite of NMR experiments was particularly important for characterizing the structures of the unknown compounds. For the determination of absolute configuration, chemical reactions that included degradation, sometimes coupled with derivatization, were completed. These chemical reactions reduced and changed the structure, making it possible to determine and confirm the chirality of the structure.

Results

Culturing

The black *Moorea* sp. was one of the organisms started as a large-scale monoclonal culture in RSM. However, maintaining monoclonal cultures is not an easy task with the cyanobacteria. A brown diatom continually persisted in the pure culture

even after repeated attempts to re-isolate the cyanobacterium and culture it in fresh medium. The diatom was originally thought to be a contaminant and the re-isolation was an attempt to place the cyanobacteria in new medium without contaminants. A large-scale culture was placed at low light on a lower shelf of a culturing rack and after a few weeks, the *Moorea* cyanobacterium was the only organism present in the medium. Thus, the cyanobacterium had out-competed or killed the diatom with toxins. The circuitous and slow culturing process for this *Moorea* sp. led to initial interest in the cyanobacterium because it showed promise for the production of cytotoxic compounds. The slow-growing *Moorea* sp. grew for six months in pure culture before enough biomass was obtained for harvest.

Chromatography Results

The first round of separation was NP-VLC and the resulting masses for each fraction along with the solvent used to extract each particular fraction are summarized in Figure 4.

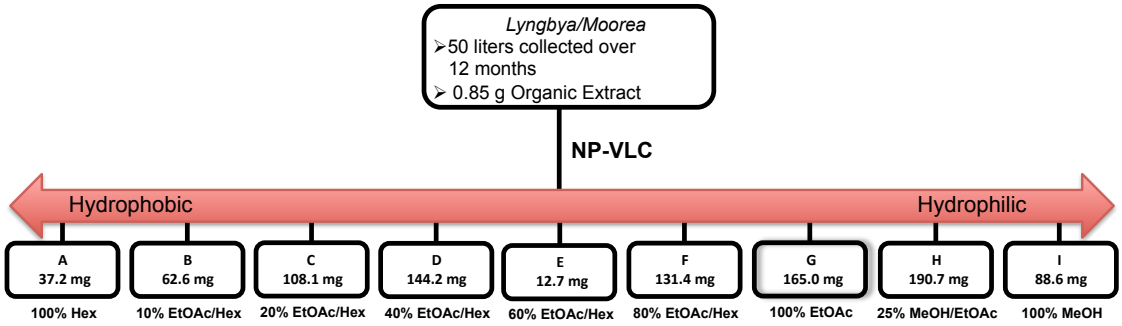


Figure 4. First tier chromatography fractions resulting from NP-VLC of the black *Lyngbya/Moorea* cyanobacterial extract. Corresponding sample masses obtained and the elution solvent are shown. Hex = hexanes, E = EtOAc, and MeOH = methanol.

Brine Shrimp Assay Results

All of the NP-VLC fractions were tested in brine shrimp assays and the results are listed in Table 1. Six of the fractions were significantly toxic at concentrations 100 times more dilute than the standard concentration used in the BSA, which motivated further investigation.

Table 1. Brine shrimp assay results for the NP-VLC fractions tested. The fractions were tested at 100 and 10 ppm concentrations. The rows shaded in gray indicate the fractions that demonstrated significant cytotoxicity.

NP-VLC Fraction	Brine Shrimp Assay Kill Rate at 1.0 mg/mL (%)	Brine Shrimp Assay Kill Rate at 0.1 mg/mL (%)
A	4.00	5.00
B	0.00	0.00
C	100.00	96.00
D	100.00	97.00
E	89.00	7.00
F	100.00	85.00
G	100.00	94.00
H	89.00	7.00
I	0.00	0.00

Mass Spectrometry Results

Each chromatography step in the purification process provided additional information about the potential cytotoxic compounds that the Red Sea strain of *Moorea* sp. produces. After NP-VLC, mass spectrometry was performed on the six resultant fractions that demonstrated cytotoxic properties. The masses obtained from LC-MS were input into the MarinLit database following the protocol outlined in the Materials and Methods section. Based on the MarinLit searches, seven known compounds were

predicted to be produced by the cyanobacterium, along with two new compounds. The seven putative known compounds were apratoxin A, 30-methyloscillatoxin, bromoaplysiatoxin, a brominated lipid, pitipeptolide, lynbyabellin H, and tasiamide, although it was recognized that the polarities of these known compounds did not necessarily match the NP-VLC fraction in which their putative mass ions were detected. The two predicted molecular mass ions for the hypothetical new compounds were *m/z* 658, detected in VLC fraction E, and 854, detected in both VLC fractions E and F respectively.

One mass ion, *m/z* 658, detected in the initial NP-VLC fraction E, was present in RP-SPE fractions, but was no longer present in LC-MS analyses of subsequent HPLC chromatographic fraction. This may have been a result of the small mass of fraction E, 12.7 mg, and the subsequent HPLC fractions being of small quantity. The new mass may have been present, but not detectable because of low quantities. The other new mass, *m/z* 854, present in NP-VLC fraction F was isolated in HPLC fraction F2D and characterized as apratoxin H.

Summary of Chromatographic and Mass Spectrometry Results

The three iterative stages of chromatography provided less complex mixtures at the end of each separation. The fractions were massed at every stage of separation and also analyzed with LC-MS. The mass ions obtained from LC-MS were searched via MarinLit. The hypothetical compound list became more defined at each chromatographic stage. Table 2 lists the masses for each fraction along with putative compound identifications based on the MarinLit searches.

Table 2. HPLC fractions with masses and corresponding parent SPE and VLC fractions. Mass ions observed by LC-MS (using electrospray ionization, ESI). These putative molecular masses were searched in the MarinLit database and potential matching known compounds, if any, are listed.

NP-VLC-ID (Mass/g)	**RP-SPE-ID (Mass/mg)**	**HPLC-ID (Mass/mg)**	**ESI-MS (*m/z*)**	**MarinLit (*m/z*)**
C (108.1)				
	C1 (1.3)			
	C2 (70.5)			
		C2A (5.3)		
		C2B (1.0)	401	
		C2C (39.5)	403 or 806	
		C2C1 (5.6)		
		C2C2A (12.5)	403	
		C2C2B (9.1)	403	
		C2C3 (5.2)	471	
		C2D (3.5)	419 or 838	
		C2E (4.1)	431	
		C2F (3.4)	471	
		C2G (2.5)	459	
	C3 (33.9)			
		C3Baseline (3.8)		
		C3A (2.5)		
		C3B (0.6)		
		C3C (1.5)		
		C3D		
		C3E		
	C4 (6.8)			
D (144.2)				
	D1 (2.5)			
	D2 (84.6)			
	D3 (55.1)			
		D3A (2.5)		
		D3B (0.9)	433	
		D3C (5.3)	373	
		D3D (3.0)		
		D3E (30.2)	401	
		D3E1		
		D3E2		
		D3F (10.5)		

		D3G (2.3)	452	
		D3H (7.1)	470	
		D3H1		
		D3H2	470	
		D3I (3.3)		
	D4 (8.5)			
E (12.7)				
	E1 (0.2)			
	E2 (7.6)			
		E2A (2.7)		
		E2B (1.2)	489	
		E2C (4.1)		
	E3 (4.8)			
	E4 (5.3)			
F (131.4)				
	F1 (8.2)			
	F2 (91.6)			
		F2Baseline (8.2)		
		F2A (4.6)	679.05	Lyngbyabellin B (679)
		F2B (2.6)		
		F2C (42.5)	840.3	Apratoxin A
		F2C1 (8.0)		
		F2C2 (2.2)		
		F2C3 (17.6)	840.47	
		F2C4 (3.6)	822.47	
		F2C5 (1.8)	880.13	
		F2D (9.9)	854	Methylated Apratoxin A
	F3 (2.7)			
	F4 (2.1)			
G (165.0)				
	G1 (6.5)			
	G2 (7.8)			
	G3 (7.1)			
	G4 (145.7)			
		G4Baseline (6.4)		
		G4A (0.5)		
		G4B (3.2)		
		G4C (2.0)		
		G4D (5.8)		
		G4E (2.2)		
	G5 (2.6)			
H (190.7)				

	H1 (9.8)			
	H2 (18.9)			
	H3 (60.5)			
		H3A (22.2)		
		H3B (4.5)		
		H3C (2.2)	825	
		H3D (3.8)	839	Apratoxin A

Compound Confirmation and Elucidation Results

Known Compounds

Confirmation of two known compounds produced by *Moorea* sp. was completed based on NMR experiments in parallel with high resolution mass data. Chris Thornburg confirmed the presence of lyngabellin B, as opposed to lyngbyabellin H proposed after LC-MS analysis of the NP-VLC fractions. Figures 5 and 6 show the structures of lyngbyabellins B and H. The two have a similar peptide backbone, but a striking difference is the presence of a complex side chain on lyngbyabellin H (Han et al., 2005) that is not present in lyngbyabellin B. MS analysis of lyngbyabellin B, which was isolated from HPLC fraction F2A, provided *m/z* 679.1775 $[M + H]^+$ ($C_{28}H_{41}C_{l2}N_4O_7S_2$).

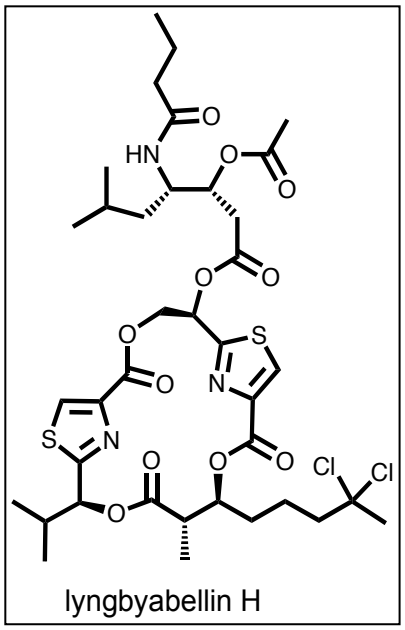


Figure 5. The structure of lynbyabellin H, the original lynbyabellin proposed to be produced by *Moorea* sp.

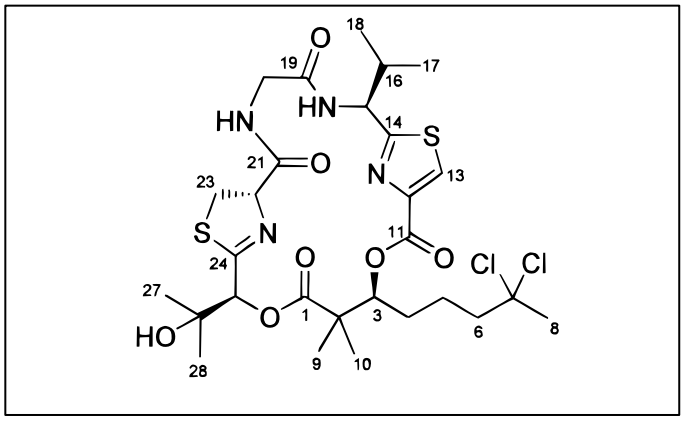


Figure 6. The structure of lyngbyabellin B, the confirmed compound produced by *Moorea* sp.

The other known compound that was confirmed to be present in RP-SPE fraction F, HPLC fraction F2C, was apratoxin A, with *m/z* 840.4927 $[M + H]^+$ ($C_{45}H_{70}N_5O_8S$) (Figure 7). Apratoxin A was present in large quantity in the Red Sea strain of *Moorea* sp.

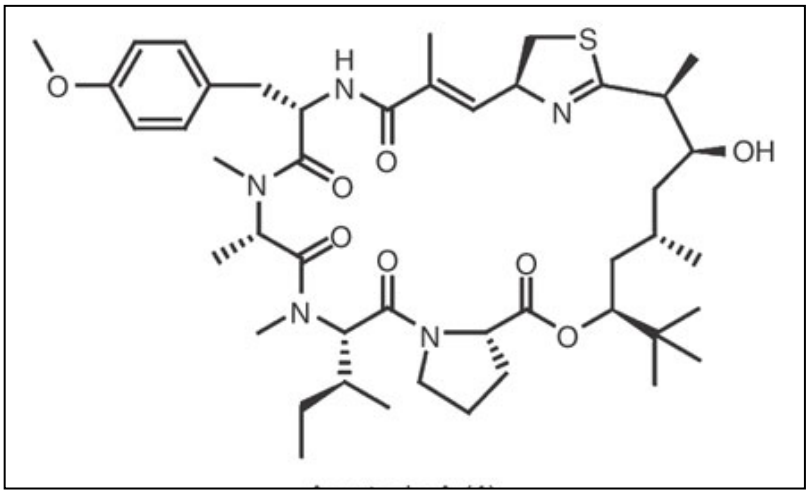


Figure 7. The structure of apratoxin A.

New Compounds

Two new compounds produced by the cyanobacterium were isolated and characterized (Thornburg, 2013). These metabolites are both analogues in the apratoxin series. Apratoxin H features a pipecolic acid (6-membered ring) in place of the proline amino acid (5-membered ring) present in apratoxin A, as shown in Figure 8. Apratoxin H was purified from HPLC fraction F2D and yielded a molecular ion at *m/z* 854.5074 $[M + H]^+$ ($C_{46}H_{72}N_5O_8S$) when analyzed by ESIMS. Apratoxin A sulfoxide (Figure 9) was the second new compound isolated and characterized. Apratoxin A sulfoxide gave a molecular ion at *m/z* 856.4897 $[M + H]^+$ ($C_{45}H_{70}N_5O_9S$) and was isolated from HPLC fraction F2C. Apratoxin A sulfoxide differs from apratoxin A in the oxygenation of the thiazoline sulfur atom (attached to C-32). This degree and site of oxygenation is unusual, and required careful analysis of infrared (IR) spectroscopic data and MS/MS fragmentation patterns to confirm that the oxygenation was not on the thiazoline nitrogen atom. These compounds are the subject of a publication to be submitted to the Journal of Natural Products.

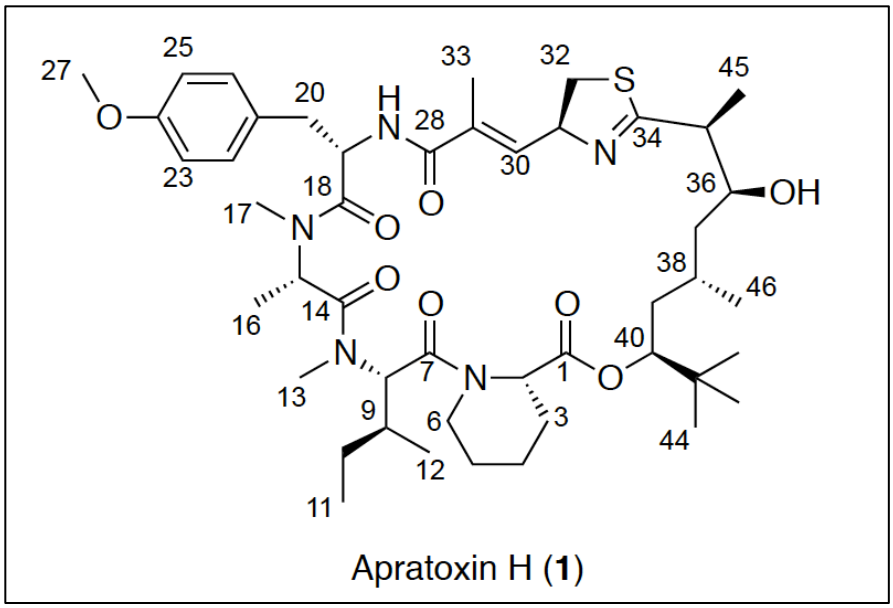


Figure 8. The structure of newly characterized apratoxin H.

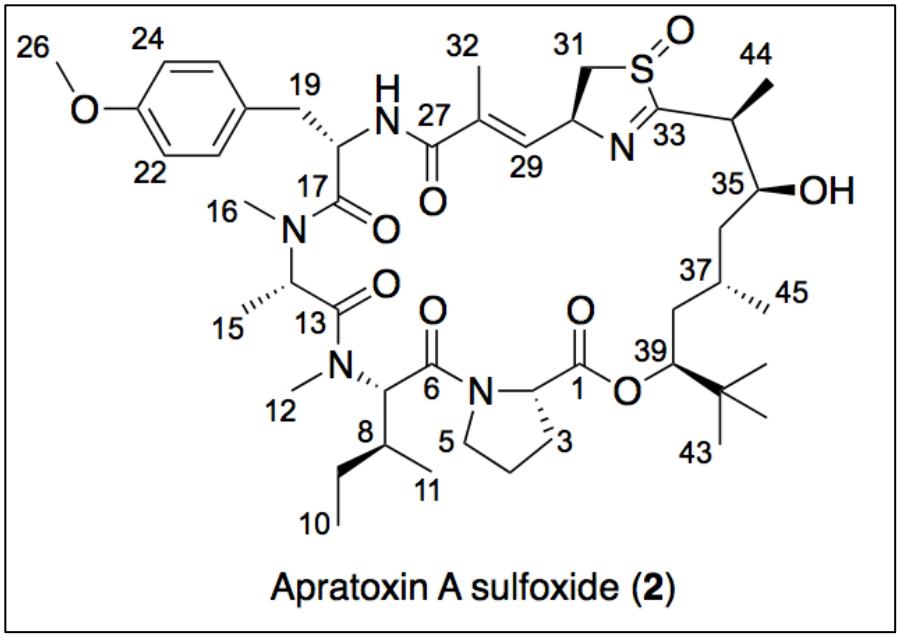


Figure 9. The structure of the new compound apratoxin A sulfoxide.

Cytotoxic Hydrophobic Fraction Results

Fraction C (20% EtOAc-hexanes) from NP-VLC demonstrated significant cytotoxic properties and was also continued through the purification process because of its cytotoxicity. It was separated with RP-HPLC and eleven HPLC fractions were tested against the mouse Neuro 2A neuroblastoma cell line. Table 3 lists the results of the Neuro 2A cell assay and Figure 10 provides a graphical representation of the results. Typically, less than 30% viable cells remained after incubation with the test fraction, indicating a high level of cytotoxicity. Fractions C2C2B, C2E2, and C2F2 were particularly cytotoxic. These three fractions were analyzed with LC-MS and the resulting putative, molecular masses were searched in MarinLit. Three mass ions, which could not be dereplicated as putative known compounds, were present at *m/z* 403, 431, and 471, respectively.

Table 3. Neuro 2A neuroblastoma cell line assay results for HPLC subfractions from VLC fraction C. Each sample was tested in duplicate and the percentage of viable cells remaining were averaged over the two test replicates and normalized to a solvent vehicle control.

HPLC Fraction	Viable Cells-Plate 1 (%)	Viable Cells-Plate 2 (%)	Average Viable Cells (%)	Average Killed Cells (%)	Standard Deviation
C2B	76.72	76.33	76.52	23.48	2.10E-02
C2C2A	82.33	80.21	81.27	18.73	5.09E-03
C2C2B	23.85	36.37	30.11	69.89	7.61E-03
C2D2	103.63	100.82	102.22	-2.22	2.57E-02
C2E	21.58	32.75	27.17	72.84	2.67E-02
C2F2	20.16	31.77	25.97	74.04	1.78E-02
C2F3	94.56	94.26	94.41	5.59	2.46E-02
C2G	90.08	82.89	86.48	13.52	2.48E-02
C3C	118.23	120.59	119.41	-19.41	2.00E-02
C3D	110.69	109.43	110.06	-10.06	2.83E-02
C3E	101.15	106.97	104.06	-4.06	2.46E-02

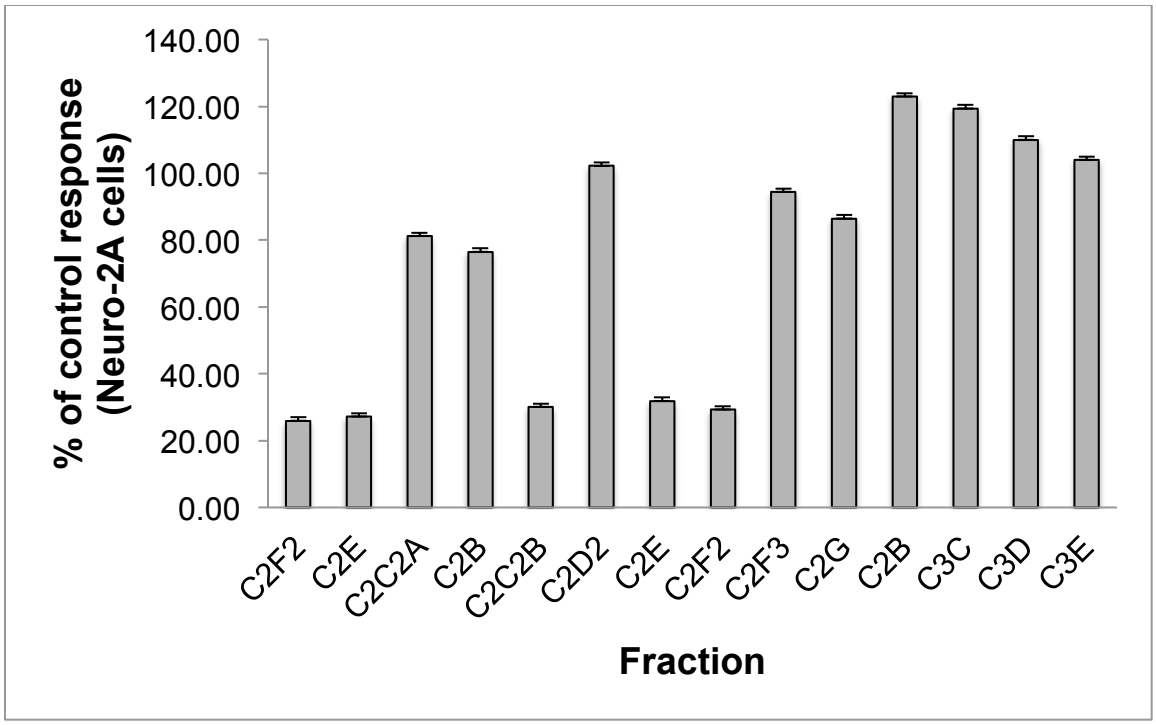


Figure 10. Results of testing *Moorea* extract subfractions from NP-VLC Fraction C against the mouse Neuro 2A neuroblastoma cell line. The average percentage of viable cells remaining, after incubation in duplicate with each test sample, is normalized to a solvent vehicle control.

Discussion

The Red Sea strain of *Moorea* sp. demonstrates an ability to produce a range of biosynthetically unrelated secondary metabolites. Furthermore, it produces significant quantities of known and new compounds in laboratory culture. Although only two known compounds and two novel compounds have been confirmed in this study, more extensive purification would likely lead to additional metabolites based on the mass spectrometric data that revealed the potential for many interesting compounds. In fact, Chris Thornburg also confirmed the presence of hectochlorin (related to lyngbyabellin B) and apratoxins B and C.

From literature reports, lyngbyabellin B demonstrates toxicity against oral carcinoma (KB) and human colon adenocarcinoma (LoVo) cells (Luesch et al., 2000). It is also an antifungal agent and acts as a protease inhibitory compound (Ma et al., 2006). Apratoxin A is also toxic against oral carcinoma and human colon adenocarcinoma cells. It induces G_1 phase cell cycle arrest, but has no effect on microfilament network development or microtubule polymerization or de-polymerization. A lack of selectivity for apratoxin A limited its potential as an antitumor agent when it was originally discovered. However, some natural and semisynthetic analogues reported subsequently have been demonstrated to be much more selective (Luesch, 2000).

The new apratoxin A sulfoxide demonstrates a similar level of cytotoxicity to apratoxin A, except that it is slower acting. Apratoxin H also demonstrates similar cytotoxic properties as Apratoxin A. In addition, it is highly toxic to NCI-H460 human lung cancer cells. The two new apratoxins isolated may be beneficial by providing selectivity and toxicity not characteristic of the previously isolated apratoxins, and this possibility warrants further biological characterization.

Along with the confirmed compounds discussed above, *Moorea* sp. demonstrated production of multiple other natural products based on the HPLC traces and the mass spectrometric data. The potential for novel NPs in fraction C is of particular interest. Fraction C is in the hydrophobic portion of the NP-VLC profile and is typically ignored during natural products isolation because the hydrophobic fractions contain the lipid components of the cells, including fatty acids and lipid membrane molecules. These types of molecules are common to a majority of living organisms and are often nuisance compounds in natural products investigations because they can show unwanted

cytotoxicities that are not relevant for pharmaceutical development. However, the significant cytotoxicity observed for fraction C in the BSA was deemed unusual for the hydrophobic NP-VLC fractions from cyanobacteria.

As presented in the results, three of the HPLC subfractions for NP-VLC fraction C demonstrated significant cytotoxicity in the Neuro 2A assay, with less than 30% of the neuroblastoma cells viable after incubation with the test fractions. As with other *in vitro* cell line assays, this assay is used to screen new compounds for cytotoxic properties and does not signify that the compounds are selectively toxic to cancerous versus healthy cells. Although the assay does not indicate differentiation between cell types, the potent toxicity results for the three hydrophobic compounds are promising. In general, the hydrophobic NP-VLC fractions of cyanobacterial extracts are neglected, and this neuroblastoma cell assay demonstrates the potential existence of new cytotoxic compounds in the ignored portion of the chromatographic spectrum. Three new putative molecular masses were present in two subfractions, C2 and C3, of the hydrophobic NP-VLC fraction C. Further spectroscopic analysis is necessary on the HPLC separations from fraction C in order to determine if these compounds are new natural products or "nuisance" compounds closely related to primary metabolites.

Curacin A (Figure 11) is an example of a hydrophobic natural product that served as a potential anticancer lead compound. It was isolated by William Gerwick's group, and demonstrated an ability to interact with tubulin. It possessed poor aqueous solubility and therefore could not be tested for anticancer activity in animal models. Numerous variants of curacin A were synthesized that proved to be more soluble, and some of these

entered preclinical trials. However, unacceptable toxicity was observed and testing of these analogues was discontinued (Gerwick et al., 1994).

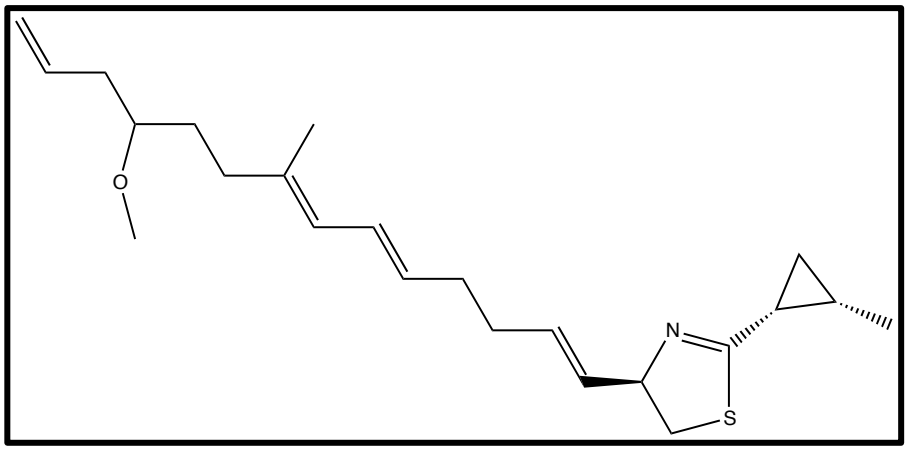


Figure 11. The molecular structure of Curacin A.

The Neuro 2A blastoma cell assay yielded intriguing results for the hydrophobic fraction C obtained in this project. Only two bioassays were implemented throughout the course of this natural products isolation project. Only focusing on two assays limits the potential to discover other biological properties of the cyanobacterial extract components. It is unrealistic to complete all of the potential bioassays with the many different fractions and pure compounds. However, it is critical to be aware that the fractions not investigated fully may have had substantial effects in other biological assays. This is one significant limitation in this natural products isolation project. Fractions that were not cytotoxic to brine shrimp may contain interesting novel compounds with other biological activities, but the overall goal of this project was to use brine shrimp toxicity as an indicator of potential cytotoxicity to human cancer cells.

It is interesting to note that the cyanobacterium produced the myriad of natural products in laboratory culture. *Moorea* sp. produced secondary metabolites in culture

even though it is an incredibly energy intensive process. This medium is interesting in that it is not autoclaved before growing cyanobacteria. The water is autoclaved before adding the additional components, but the cyanobacteria were found to grow best in the medium if it was not autoclaved after all of the components were added. (Thornburg et al., 2011).

The cyanobacterial culture was monoclonal (comprised one cyanobacterial species), but not axenic (free of heterotrophic bacteria) because of the lack of autoclaving once the culture medium was prepared. The presence of heterotrophic bacteria in the culture may have induced the cyanobacterium to produce NPs. The presence of heterotrophic bacteria in the culture with the *Moorea* sp. also makes the biosynthetic origin of the NPs isolated somewhat uncertain. Could the heterotrophic bacteria be the source of the natural products? Or does their presence elicit expression of the biosynthetic pathways in the cyanobacterium to cause production of the wide array of NPs observed? As noted in the method section for cyanobacterial culturing, early in the method development, a brown diatom grew with the cyanobacterium until it was eventually out-competed. The diatom may have served as a source of stress to induce secondary metabolite biosynthetic pathways.

It would be interesting to investigate what NPs the cyanobacterium produced in its native environment of mangroves, and how that compares with the compound identities and quantities produced in laboratory culture. Different environments and stress conditions could trigger different biosynthetic pathways and production of a varied subset of secondary metabolites. This is a potential area of expansion for natural products investigations.

This project and most microbial natural products isolation projects utilize organisms that can be grown in pure culture in a laboratory setting so that a large enough quantity of biomass can be grown for chemical extraction. Relying on cultured microorganisms is a large disadvantage for natural products isolation because the majority of the microbes on earth are not yet able to be cultured and this has earned the term the 'unculturable majority'. A recent direction for NP investigations that is gaining momentum is to address the unculturable majority and to develop techniques to investigate the secondary metabolites being produced by organisms that are difficult to culture.

The unidentified molecular mass ion at *m/z* 658 present in the original LC-MS analysis of the NP-VLC fractions that subsequently disappeared after HPLC may have decomposed after initially being detected by MS.

This project pursued a method for NP isolation based on culturing, extraction, and chromatography to obtain pure compounds. Genomics and proteomics approaches are also relevant for NP isolation. Future directions for studying *Moorea* sp. further could include genomics approaches. This project focused on one particular species of cyanobacterium and demonstrated that a Red Sea strain of *Moorea* sp. is a prolific producer of secondary metabolites. The astounding NP production demonstrates the importance of continuing to turn to nature for inspiration for useful and structurally intriguing novel compounds.

References

1. Carballo, J., Hernandez-Inda, Z., Perez, P., & Garcia-Gravalos, M. (2002). A comparison between two brine shrimp assays to detect in vitro cytotoxicity in marine natural products. *BMC Biotechnol., 2*(1), 17.
2. Engene, N., Rottacker, E. C., Kastovsky, J., Byrum, T., Choi, H., Ellisman, M. H., Komárek, J., & Gerwick, W. H. (2012). Moorea producens gen. nov., sp. nov. and Moorea bouillonii comb. nov., tropical marine cyanobacteria rich in bioactive secondary metabolites. *Int. J. Syst. Evol. Microbiol., 62*(Pt 5), 1171-1178.
3. Gerwick, W., Proteau, P., Nagle, D., Hamel, E.,Blokhin, A., Slate, D., (1994). Structure of Curacin A, a novel antimitotic, antiproliferative and brine shrimp toxic natural product from marine cyanobacterium Lyngbya majuscule. *J. Org. Chem.,* 59 (6): 1243-1245.
4. Han, B., McPhail, KL., Gross, H., Goeger, D., Mooberry, S., Gerwick, W., (2005). Isolation and structure of five lyngbyabellin derivatives from a Papua New Guinea collection of the marine cyanobacterium *Lynbya majuscule. Tetrahedron, 61*(49): 11723-11729.
5. Luesch, H., Yoshida, W. Y., Moore, R. E., & Paul, V. J. (2002). New apratoxins of marine cyanobacterial origin from guam and palau. *Bioorg. Med. Chem., 10*(6), 1973-1978.
6. Luesch, H., Yoshida, W. Y., Moore, R. E., Paul, V. J., & Corbett, T. H. (2001). Total Structure Determination of Apratoxin A, a Potent Novel Cytotoxin from the Marine Cyanobacterium *Lyngbya majuscula*. *J. Am. Chem. Soc., 123*(23), 5418-5423.
7. Luesch, H., Yoshida, W. Y., Moore, R. E., Paul, V. J., (2000). Isolation and structure of the cytotoxin Lyngbyabellin B and absolute configuration of Lyngbyapeptin A from the Marine Cyanobacterium *Lyngbya majuscula*. *J. Nat. Prod., 63*(10), 1437-1439.
8. Lugo, A., Snedaker, S., (1974). The ecology of mangroves. *Ann. Rev. of Ecol. and System.*, 5:39-64.
9. Ma, D., Zou, B., Cai, G., Hu, X., & Liu, J. O. (2006). Total Synthesis of the Cyclodepsipeptide Apratoxin A and Its Analogues and Assessment of Their Biological Activities. *Chem. Euro. J., 12*(29), 7615-7626.
10. Molinski, T., Dalisay, D., Lievens, S., Saludes, J. (2009). Drug Development from marine natural products. *Nature Rev.* 8:69-85.
11. Meyer, B.N., N.R. Ferrigni, J.E. Putnam, L.B. Jacobsen, D.E. Nichols and J.L. McLaughlin, (1982). A convenient general bioassay for active plant constituents. *Planta Medica*, 45: 31-34.
12. Newman, D (2008). Natural Products as Leads to Potential Drugs: An Old Process or the New Hope for Drug Discovery? *Journal of Medicinal Chemistry*,*51* (9), 2589-2599.
13. Newman, D., Cragg, G., (2012). Natural Products as Source of New Drugs over the 30 Years from 1981 to 2010. *Journal of Natural Products*, *75* (3), 311-335.
14. Tan, LK., (2010). Filamentour tropical marine cyanobacteria: a rich source of natural products for anticancer drug discovery. *J. App. Phycology 22*(5):659-676.
15. Thornburg, C. C., Thimmaiah, M., Shaala, L. A., Hau, A. M., Malmo, J. M., Ishmael, J. E., Youssef, D. T. A., & McPhail, K. L. (2011). Cyclic

Depsipeptides,Grassypeptolides D and E and Ibu-epidemethoxylyngbyastatin 3, from a Red Sea *Leptolyngbya* Cyanobacterium. *J. Nat. Prod., 74*(8), 1677-1685.
16. Thornburg, C. "Investigation of Unique Marine Environments for Microbial Natural Products". Ph.D. dissertation, Oregon State University, 2013.
17. Tobert, J, (2003). Lovastatin and BeyondLThe History of the HMG-CoA reductase inhibitors. *Nature Rev.* 2:517-526.
18. Trampuz, A., Jereb, M., Muzlovic, I., Prabhu, R. (2003). Clinical review: Severe Malaria. *Critical Care,* 7, 315-323.

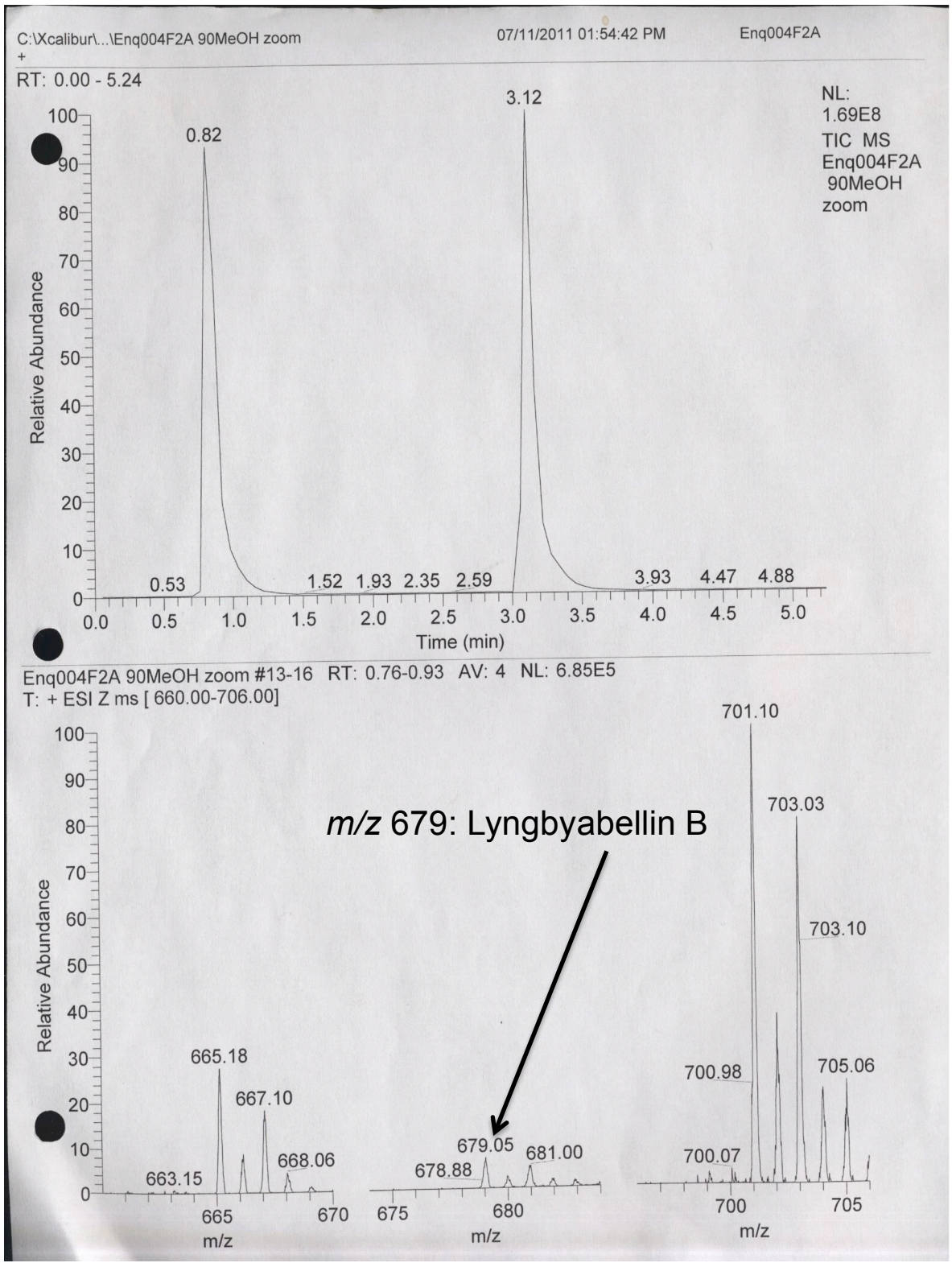
Appendices

Appendix A-Red Sea Medium Recipe

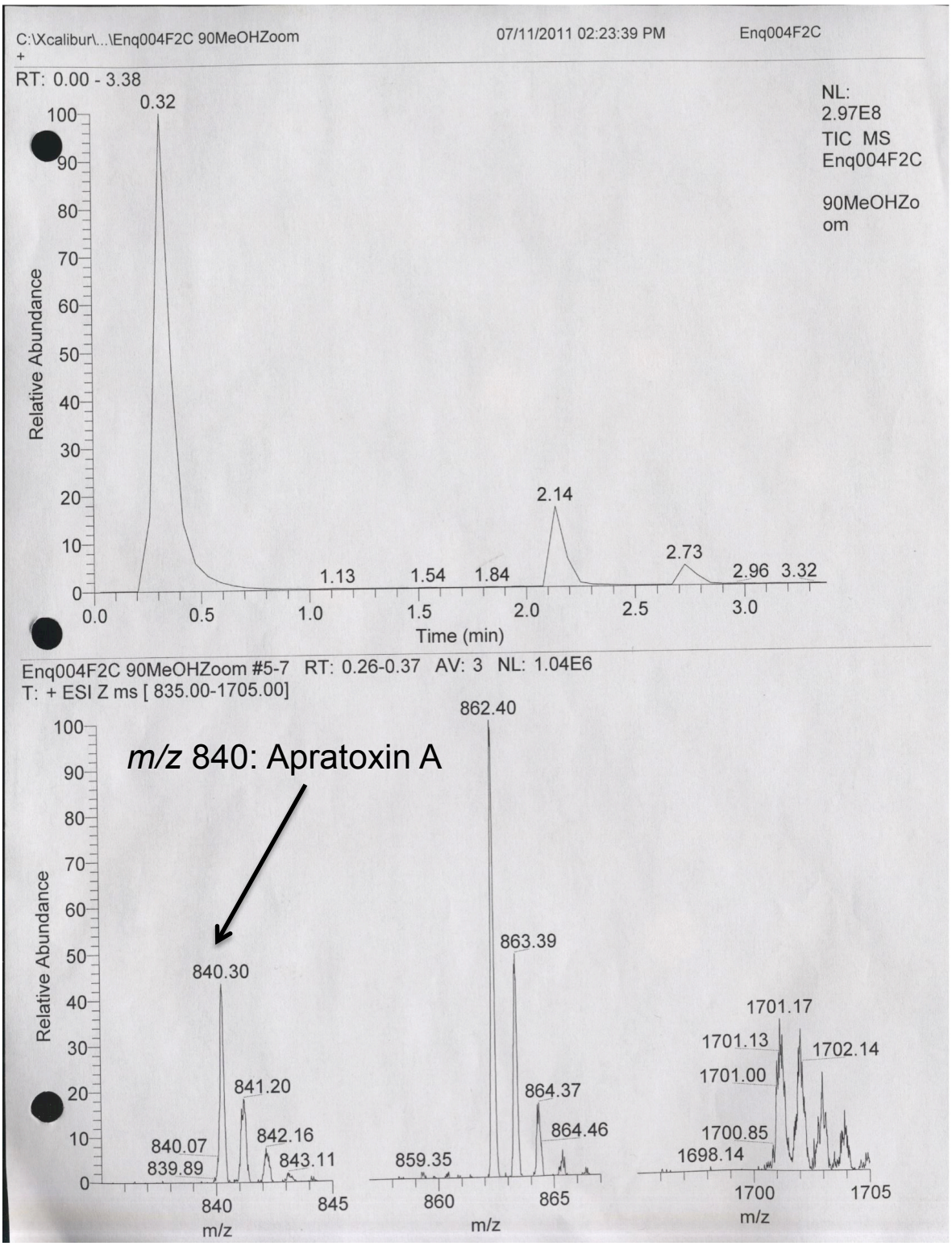
Ingredient	Conc. (mg/L)	15 L
$NaNO_3$	1.5 g	22.5 g
K_2HPO_4 $3H_2O$	40 mg	0.6 g
$MgSO_4$ $7H_2O$	75 mg	1.125 g
$CaCl_2$ $2H_2O$	36 mg	0.54 g
$Na_2CO_3(H_2O)$	20 mg	0.30 g
SL-8 Micronutrients		15 mL
DN Vitamin Mix		15 mL
Na_2-EDTA	1 mg	15 mg
Citric Acid	6 mg	90 mg
Ferric ammonium citrate	6 mg	90 mg
Instant Ocean	41 g	615 g
dH_2O (pyrogen free)	998 mL	14,970 mL

*Adjust pH to 8.4 with 1M NaOH or HCl. Filter sterilize with 0.4 μm pore size filter.

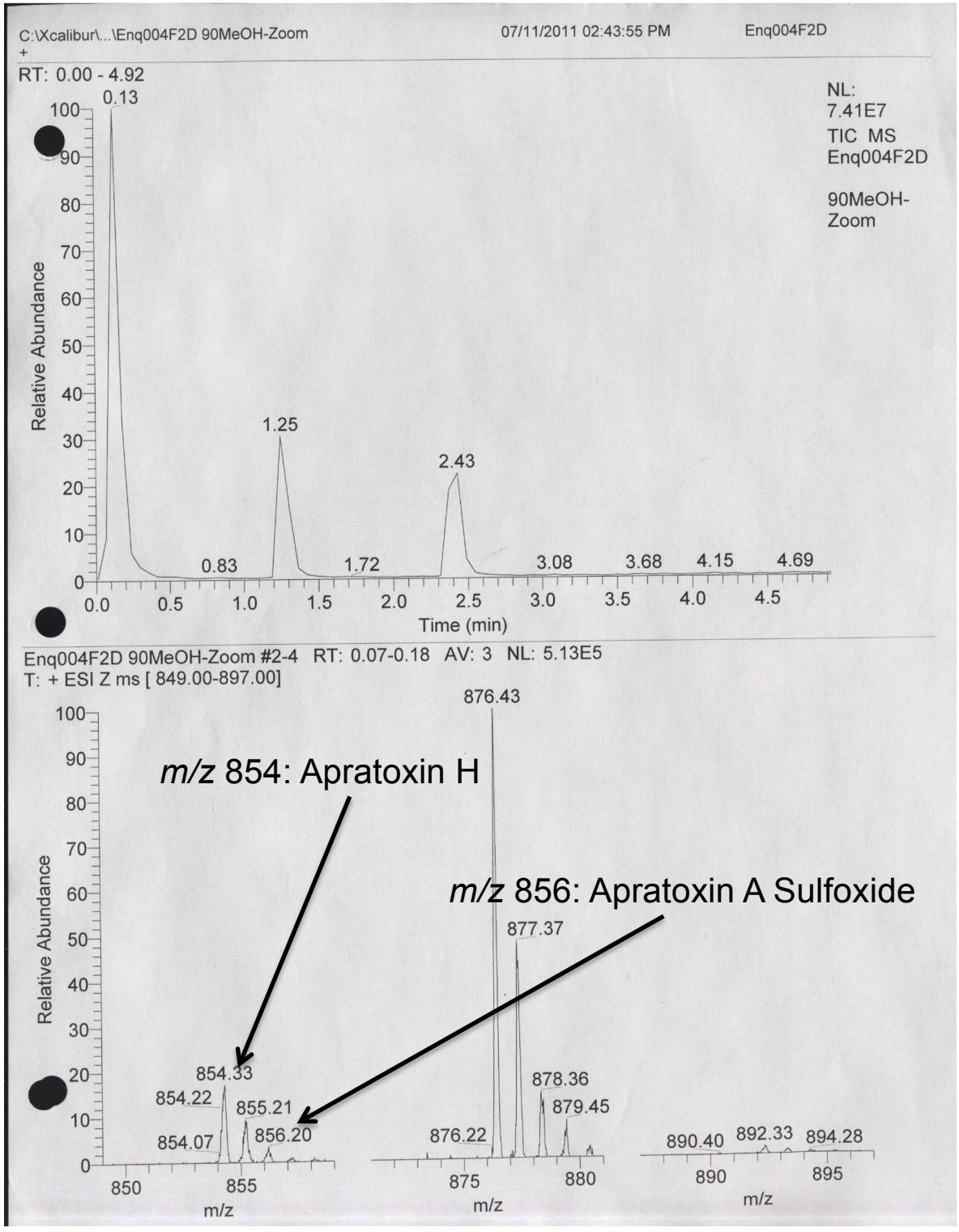
Appendix B-Mass Spectra for New and Known Confirmed Compounds



LC-MS Spectrum for Lyngbyabellin B from RP-HPLC Fraction F2A

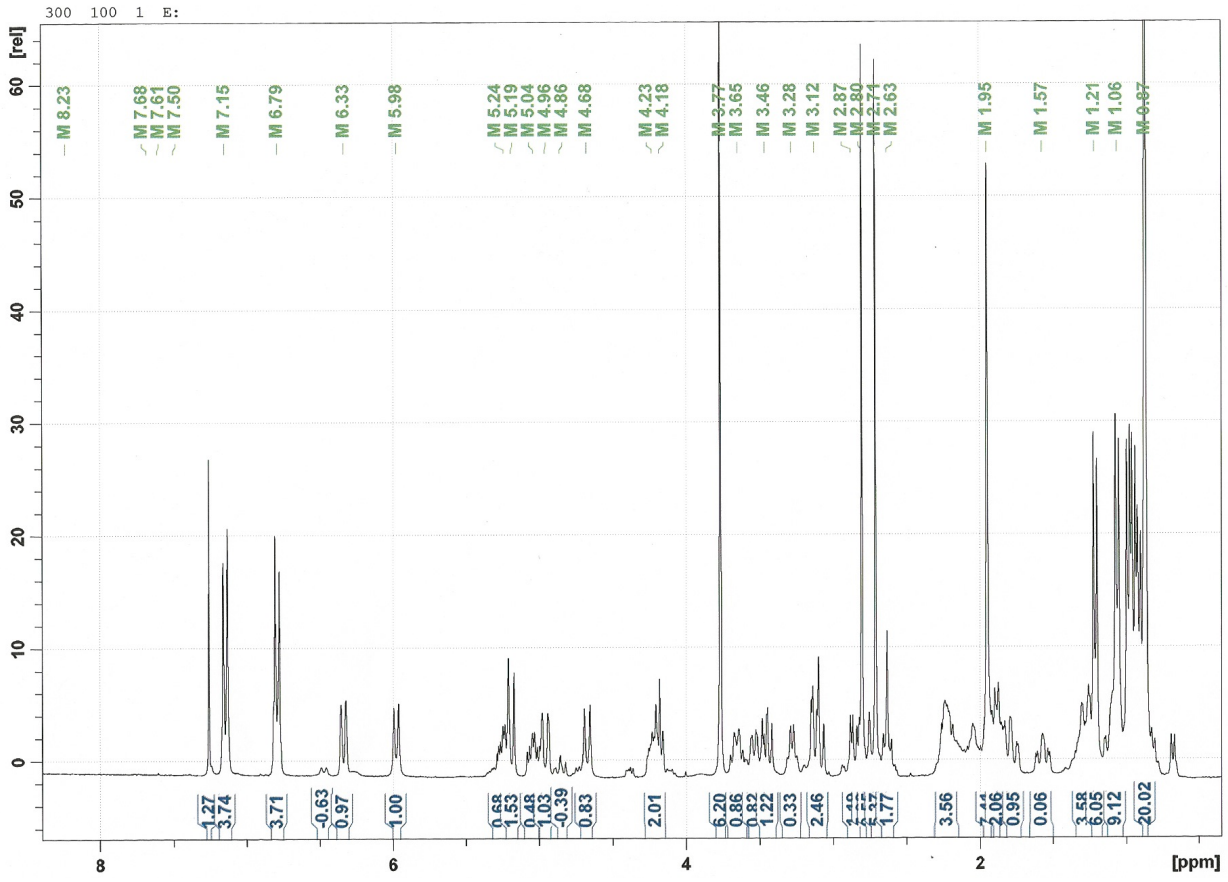


LC-MS Spectrum for Apratoxin A from RP-HPLC Fraction F2C

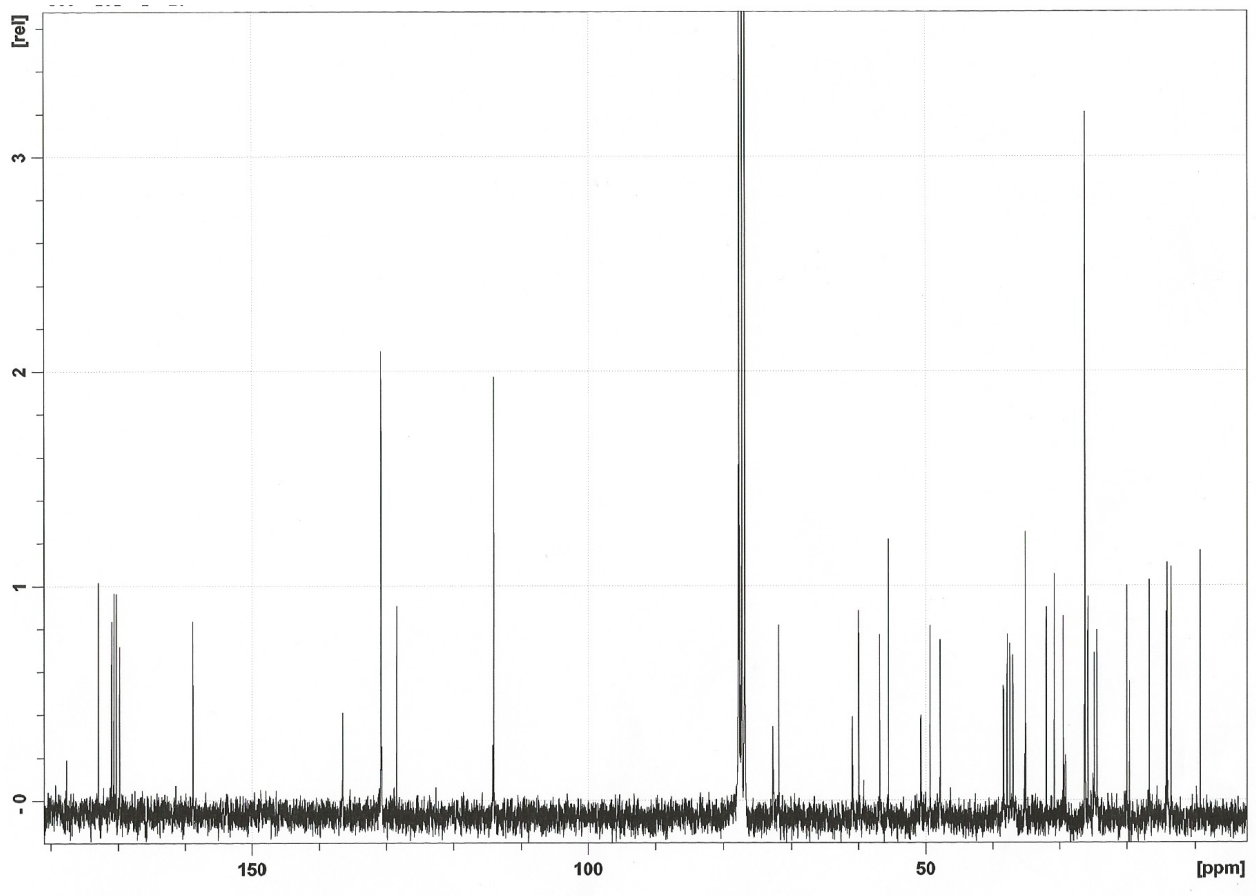


LC-MS Spectrum for Apratoxin H and Apratoxin A Sulfoxide from RP-HPLC Fraction F2D

Appendix C-NMR spectra for -Apratoxin A



^{1}H NMR Spectrum for Isolated Apratoxin A (300 MHz, $CDCl_3$)



^{13}C NMR Spectrum for Isolated Apratoxin A (75 MHz, $CDCl_3$)

Appendix D-University Honors College Copyright Release Form

University Honors College Copyright Release Form

We are planning to release this Honors Thesis in one or more electronic forms. I grant the right to publish **my thesis** entitled, "Discovery and Characterization of New Cyanobacterial Natural Products from *Moorea* sp.", in the Honors College OSU Library 's Digital Repository (D-Space), and its employees the nonexclusive license to archive and make accessible, under conditions specified below.

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