BIOACTIVE METABOLITES FROM MICROORGANISMS

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ABSTRACT

Secondary metabolites produced by microorganisms were isolated and identified. These metabolites are not essential for the growth of the producing culture, are usually synthesized as closely related members of a chemical family and include compounds that act as hormones, antibiotics and toxins. In the first chapter, actinomycetes were isolated from local marine sediments and cultivated in order to determine bioactive metabolites of interest. Broad spectrum antibiotic compounds produced as constitutional isomers were isolated from a strain identified as *Streptomyces malaysiensis*. Additionally, a family of compounds which selectively inhibited *Mycobacterium smegamatis*, a surrogate test organism used in the discovery of tuberculosis treatments, were isolated from a strain identified as *Streptomyces fimicarius*. In the second chapter, microcystin-LR, a potent liver toxin, and its derivatives were isolated from laboratory cultures of *Microcystis aeruginosa*. During the isolation of these toxic metabolites, additional unrelated peptides belonging to the microginin class of cyanopeptide metabolites were isolated and identified. Structural determination of these five cyanopeptides revealed unique structural features including the amino acid methionine, various sites of methylation on the modified octanoic acid chain and the first tripeptide microginin.

ACKNOWLEDGMENTS

I would like to thank Candace Jones and Kristi Sommer for their help with the sampling and selective isolation of actinomycetes as well as Eve Wright for the mass culture of *Microcystis aeruginosa*. To all the members of the Wright lab, I couldn't ask for a better work environment. A special thank you to Katie Wimmer, working with such a great friend made every day more fun. To all my friends at CMS, I am so lucky to have such a great group of friends and colleagues.

When I started graduate school I had no research experience and I must thank Dr. Wright for taking a chance on me. I am a true believer that all things happen for a reason and I am so grateful for the opportunity to work in his lab. Not only have I developed a lifetime interest in natural products chemistry and a wealth of knowledge and experience to help me on my path, I have also found a role model and a friend.

Lastly, I must thank my family for believing in me and encouraging me. Their endless support has been invaluable.

DEDICATION

This thesis is dedicated to my late father, Douglas J. Drummond. His legacy has so much to do with my successes and although I was almost too young to remember him, his passion for knowledge that has been portrayed to me by all his friends and loved ones will always inspire me.

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CHAPTER 1: BIOACTIVE METABOLITES FROM MARINE SEDIMENT DERIVED ACTINOMYCETES

INTRODUCTION

Microorganisms are a rich source of structurally unique bioactive substances. Since the 1940s, over 30,000 natural products have been discovered from microorganisms, more than 10,000 of which are biologically active (Fenical 1993). Several characteristics of microorganisms make them important sources of bioactive natural products. Microorganisms have a high ratio of surface area to volume, facilitating the rapid uptake of nutrients required to support high rates of metabolism and biosynthesis. Microbes are capable of carrying out a tremendous variety of reactions and can adapt to a range of environments allowing them to be transplanted from nature to the laboratory flask, where they can be grown on inexpensive carbon and nitrogen sources to produce valuable compounds (Demain 2000b). Because of their biological activity, secondary metabolites of microbial origin are extremely important to our health and nutrition and have a tremendous economic importance. The antibiotic market amounts to almost 30 billion dollars a year, with other important pharmaceutical compounds from microbes having world markets of over 1 billion dollars per year. Microorganisms, particularly culturable bacteria and cyanobacteria, are recognized as important renewable sources of pharmaceuticals (Ireland et al 1993). Most notably, bacteria within the order Actinomycetales have been shown to possess an unparalleled ability to produce diverse secondary metabolites with a range of biological activities (Davidson 1995). These morphologically diverse gram positive soil bacteria, known collectively as actinomycetes, are a prolific source of antibiotics and have been the focus of numerous research efforts since the discovery of actinomycin from *Actinomyces antibioticus* by Selman Waksman at Rutgers University in 1940 (Waksman 1940). In fact, actinomycete compounds or derivatives thereof accounted for approximately two thirds of the naturally occurring antibiotics discovered as of 1988, making them the single most

important source of prescription drugs (Jensen et al. 2003). However, new bioactive metabolites from terrestrial actinomycetes are becoming increasingly difficult to discover, due to repeated isolation of common species and subsequent rediscovery of large numbers of previously described metabolites. This along with the increased incidence of naturally resistant pathogens makes it imperative that new sources of bioactive natural products be developed (Demain 2000a, Fenical 1993).

Oceans cover approximately 70% of the planet and contain organisms with broad biodiversity and complex ecology, underscoring the proposal that the marine environment offers a remarkable opportunity as a source of novel compounds. The bactericidal properties of seawater began to be recognized as early as the 1930s (ZoBell 1936), attributed to antibiotic producing bacteria and planktonic algae. The first documented bioactive marine microbial metabolite was isolated by Burkholder and co-workers in 1966 from a marine *Pseudomonas* sp. (Burkholder 1966). The structure of this unique highly brominated pyrrole antibiotic is shown in Figure 1. Since then, extensive chemical studies on marine organisms have yielded over 6,000 unique metabolites featuring new structural classes, functional groups and a relatively high incidence of halogenation (Jensen and Fenical 1994). Early studies, targeting marine invertebrates such as sponges, soft corals, and bryozoans, have demonstrated that marine organisms indeed possess the ability to produce unique secondary metabolites. These sources, however, are limited by supply. When possible, massive collection efforts of these marine invertebrates are not only expensive and time consuming, but are also potentially damaging to tropical habitats (Davidson 1995).

Marine flora and fauna have developed symbiotic relationships with numerous microorganisms, particularly bacteria. Indeed, the importance of bacterial symbiosis has given increasing

recognition to bacteria as the true producers of many compounds isolated from sponges and other marine invertebrates (Fenical 1993). Despite this, relatively little attention was directed towards the study of marine bacterial metabolites, due to a widespread perception that marine bacteria were extremely difficult to isolate and cultivate, with culturability rates as low as 0.1 to 0.01% typically reported. These rates, however, reflect attempted cultivation in standard nutrient conditions and do not consider the adaptations these marine bacteria have acquired, such as the requirement of salt water for growth. Simple media modifications, such as nutrient limitation and pretreatments, have been found to increase culturabilty rates of marine actinomycetes in excess of 50% (Jensen et al. 2003; Piret and Demain 1988).

Early studies of marine bacteria showed that seawater was composed mostly of gram-negative bacteria, with gram positive bacteria representing less than 10% of the total seawater population. Despite the abundance of gram negative microbes in seawater, the majority of marine antibiotic producers were found to be gram positive bacteria. Evidence now suggests that gram positive bacteria exist in high percentages in marine sediments, animate and inanimate surfaces, and internal spaces of invertebrate animals. The best marine sources of actinomycetes studied thus far are sediments (Fenical 1993; Zheng et al 2000). Representative structures of several bioactive actinomycete metabolites isolated from marine sediments are shown in Figure 2. Istamycins, aplasmomycins, and altemicidin, isolated by researchers at the Institute of Microbial Chemsitry in Tokyo, were produced by various actionomycetes isolated from marine sediment samples collected from Sagami Bay, Japan. These metabolites were found to be produced only in selected seawater media containing the Japanese seaweed product “Kobu Cha.” Kobu Cha is a dried powder derived from the brown seaweed *Laminaria*. Interestingly, istamycins are aminoglycoside antibiotics that resemble fortimicins and sporaricins, a closely

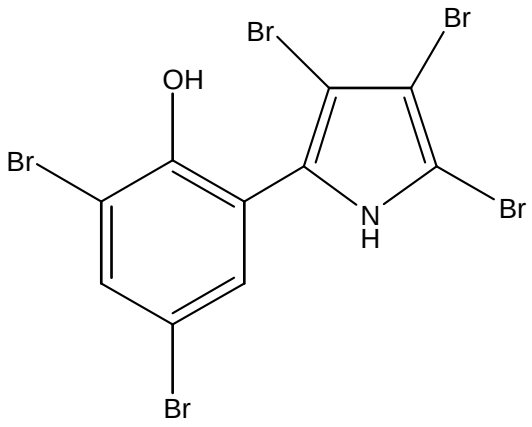
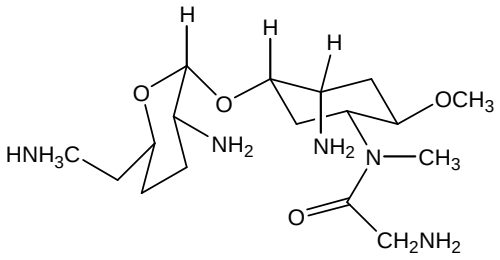


Figure 1. The first reported bioactive metabolite from a marine microorganism

related group of aminoglycosides isolated from terrestrial actinomycetes. Istamycins, however, may be more therapeutically important because of their activity against aminoglycoside-resistant pathogens (Fenical 1993; Okami et al 1979). Asplasmomycins are large boron-containing compounds with antibiotic activity (Nakamura et al 1977), and altemicidin is a structurally novel alkaloid with potent anticancer activity (Takahashi et al 1989). Marinone is a brominated sesquiterpenoid napthoquinone antibiotic isolated from marine sediments collected at Torrey Pines Estuary in La Jolla, California (Pathirana et al 1992).

On one hand, marine sediments have proved to be a prolific resource for the isolation of marine bacteria with the ability to produce structurally unique bioactive secondary metabolites and as new and unusual strains are isolated, it is likely that new metabolites will be discovered. On the other hand, it has been proposed that actinomycetes isolated from marine habitats are not indigenous marine bacteria, but instead originate from spores of terrestrial actinomycetes that have been washed to sea. However, recent studies have shown that the distribution of actinomycetes in marine sediments and the requirements of seawater for growth give conclusive evidence that actinomycetes adapted to the marine environment represent a physiologically unique class of microorganisms (Jensen et al 1991). In fact, researchers at Scripps Institution of Oceanography have identified two new taxa of marine bacteria within the order Actinomycetales discovered from ocean sediments: The genus *Salinospora* and the new marine actinomycete with the proposed genus "*Marinispora*" (Mincer et al 2002; Feling et al 2003; Kwon et al 2006). These strains have been consistently and exclusively isolated from marine sediments, are distinguished by morphological characteristics, share a unique small subunit rRNA signature nucleotide, and display saltwater requirements for growth. *Salinospora* strain CNB-392 was found to produce the unique metabolite, Salinosporamide A, which exhibits potent cancer cell



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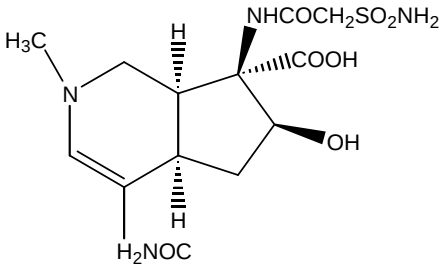

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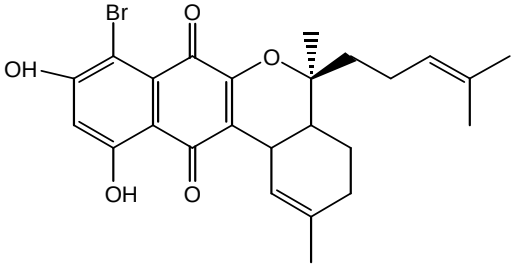

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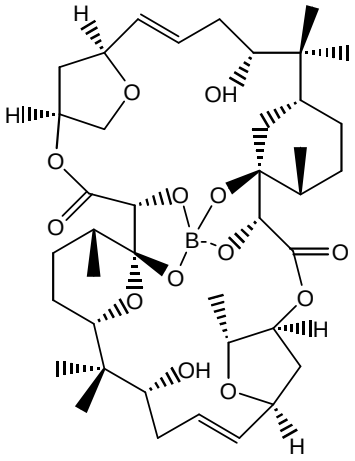

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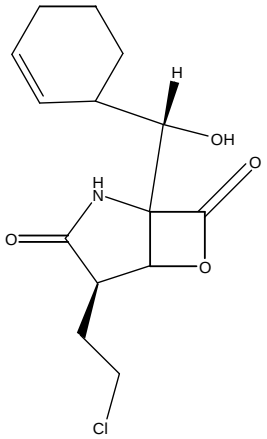
	Metabolite	Producing strain	Source	Activity	Class	Reference
a	Istamycin	*Streptomyces tenjimariensis*	Marine sediment	Antibiotic	Aminoglycoside	Okami et al 1979
b	Altemicidin	*Streptomyces sioyaensis*	Marine sediment	Anticancer	Alkaloid	Takahashi et al 1989
c	Marinone	*Streptomyces sp.*	Marine sediment	Antibiotic	Napthoquinone	Pathirana et al 1992
d	Asplasmomycin	*Streptomyces griseus*	Marine sediment	Antibiotic		Nakamura et al 1977

Figure 2. Selected bioactive compounds from marine actinomycetes: their source, origin and biological activity.

toxicity towards HCT-116 human colon carcinoma (Feling et al 2003). A recent publication describes the isolation of four macrodiolide antibiotics, marinomycins A-D from *Marinispora* strain CNQ-140 that exhibit impressive cancer cell toxicities against eight melanoma lines in the NCI's 60 cell line panel (Kwon et al 2006). The structures of Salinosporamide A as well as Marinomycins A-D can be seen in Figure 3. Additionally, chemical analyses of other *Salinospora* and *Marinispora* isolates have shown the production of a significant number of diverse secondary metabolites that are currently under investigation. Clearly, the evidence points to marine derived actinomycetes as a major source of bioactive metabolites with unique structural features and activities that represent an exciting biomedical resource (Fenical and Jensen 1993).

Infectious diseases are rapidly developing resistance towards traditional antibiotics, perpetuating the need for safer, more potent, and broader spectrum compounds (Demain 2000a). For example, recently observed strains of *Mycobacterium tuberculosis* have developed multiple drug resistance and antibiotics to control this specific bacterium are simply not available (Fenical 1993). *Mycobacterium tuberculosis* requires Biosafety Level 3 containment, making it difficult to perform activity testing in an ordinary lab setting. Recent studies have demonstrated the utility of using *Mycobacterium smegmatis*, a non-pathogenic cousin of *M. tuberculosis*, as a surrogate test organism. *M. smegmatis* grows much faster than *M. tuberculosis* and allows testing in an ordinary lab without extra safety precautions (Reyrat and Kahn 2001).

The focus of the work described in this portion of the thesis was to explore the potential of actinomycetes isolated from the coastal waters of North Carolina as sources of new biologically active compounds of therapeutic interest to the pharmaceutical industry.



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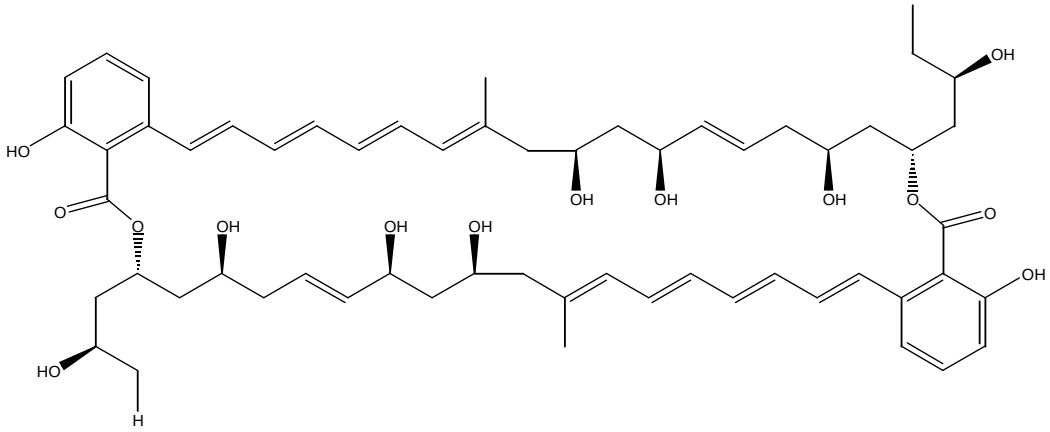

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Figure 3. The structures of bioactive metabolites from new marine actinomycete genera
(a) Salinosporamide A from *Salinospora* ; (b) Marinomycin A from *Marinispora*

EXPERIMENTAL

Collection and Isolation of Actinomycetes from Marine Sediments

Near shore mud samples were collected using a sterile plastic syringe and transferred to a sterile Falcon tube (50 ml). Sediment samples were vigorously shaken by hand to ensure uniformity and then allowed to settle. The overlaying water was decanted off and used for pH and salinity measurements. Sediment samples were diluted with sterilized filtered seawater (1:10) and incubated (55^0C, 1 hr). The dilution was then vortexed (1 min) and mixed using a nutator (30 min). Each sample (100 μl) was plated on duplicate agar plates (See Table 1 for media recipes). Plates were then incubated (25^0C) for 7-45 days. Potential actinomycete colonies, identified by morphological characteristics and gram staining in collaboration with microbiologists at aaiPharma, were continually transferred to fresh agar plates until pure colonies were obtained. Pure colonies of potential actinomycetes were sent to Accugenix (Newark, DE) for phylogenetic analysis using 16S ribosomal DNA sequence comparison. After identification, liquid cultures of positively identified colonies were frozen for storage in DMSO (20% v/v, 0.6 ml) in sterile tubes (1.0 ml) and kept at -70^0C until use.

Culture Methods

Isolates were cultured in various production mediums (See Table 1 for media recipes). Cultures were transferred from slants to production media (5 ml). After 3 days of growth in small tubes, cultures were aseptically transferred to larger tubes (20 ml) and after 5 days, transferred again to flasks (300 ml). Cultures were grown on an orbital shaker in 1 l Erlynmeyer flasks for 7-10 days (200 rpm, 25^0C). Initially, individual isolates were grown six at a time, with two flasks per organism (600 ml total culture) in order to obtain an initial activity profile.

Isolates displaying strong or interesting bioassay activity in the crude extracts were then cultured in larger scale, twelve flasks at a time (3.6 L total culture).

Extraction Methods

Media and cells were filtered using Whatman GF/A filter papers. Cells on the filter paper were extracted once (500 ml 80% MeOH, 24 hrs.). Filtered culture media was partitioned against ethyl acetate (3:1, 3 x) and butanol (3:1, 2 x). The two fractions resulting from the partitioning as well as the cell extract were dried on a rotary evaporator and subjected to antimicrobial testing and TLC analysis.

Chromatography Methods

Thin layer chromatography was performed on normal phase glass backed chromatography plates with fluorescent UV indicator (Silica Gel 60 F_{254}). Extracts and fractions were chromatographed using mobile phase composed of butanol-acetic acid-water (12 : 3 : 5). Plates were visualized with short wavelength UV and sprayed with vanillin (0.5 g) in sulfuric acid-ethanol (4:1, 100 ml). After spraying, plates were heated and observed for color changes.

Initial fractionation was performed using Supelco ENVI-18 SepPak cartridges followed by partitioning on Sephadex LH-20 (25-100 μm). Final purification was achieved by high performance liquid chromatography (HPLC; Waters SunFire C_{18}). LCMS was performed using a Waters Symmetry Shield RPC_{18} column. Solvents used were HPLC grade methanol, acetonitrile, ethyl acetate, 1-butanol, acetic acid and formic acid (FA) purchased from Fisher scientific. Water was purified by Labconco Water Pro Plus water deionization system. Preparative HPLC was performed using Waters pumps (515), gradient controller, and dual wavelength detector (2487).

Isolation Media	
AIA	1.0 L Deionized water (DIW) 22 g Actinomycete Isolatoin Agar (Difco) 5.0 g glycerol
M4	1.0 L Sterile Filtered Seawater (75 ppt) 2.0 g chitin 18 g Agar
M4100	1.0 L Sterile Filtered Seawater (75 ppt) 2.0 g chitin 18 g Agar 100 µg/ml cycloheximide

Production Media	
P3	1.0 L Sterile Filtered Seawater (75 ppt) 10 g starch 1.0 g yeast extract 1.0 g peptone
Marine Broth	1.0 L Deionized water (DIW) 18 g Marine Broth (Difco) 7.5 g tryptone 9 g NaCl
LM	1.0 L Sterile Filtered Seawater (75 ppt) 0.1 g Laminaria (Sigma) 0.1 g glycerol

Table 1. Isolation and production media recipes

Analytical Methods

ESI-MS data was obtained using a Hewlett Packard Series 1100 HPLC connected to a Waters Micromass ZQ with electrospray ionization (capillary voltage 3.0 kV; cone voltages 10V/30 V; source temperature 140; desolvation temperature 350; cone gas flow 100; desolvation gas flow 600). All samples were run in positive ion mode with a mass range of 100-1400 and a scan time of 0.5 sec. Accurate molecular weight data was obtained using a quadrupole time of flight mass spectrometer (Applied Bioscience-MDS Sciex QStar XL).

Nuclear magnetic resonance (NMR) experiments were performed on a Bruker Avance 500 MHz NMR at 293.9 K. Probes used include a 5.0 mm BBO, 5.0 mm TXI, and 1 mm TXI. NMR solvents (d_4-methanol; d_5-pyridine) were purchased from Cambridge Isotopic Laboratories.

Bioassay

Crude extracts were tested for antimicrobial activity using a disc diffusion assay. Test organisms used were the gram positive bacteria *Bacillus subtilis* and *Mycobacterium smegmatis*, the gram negative bacteria *Escherichia coli*, the yeast *Candida kefyr* and the filamentous fungus *Aspergillus niger*. Sterile tubes containing media (5ml) were inoculated in duplicate with the bioassay organisms and incubated (24 h). *E .coli*, *B. subtilis*, and *M. smegmatis* were grown in nutrient broth and incubated at 37^0 C, while *C. kefyr* and *A. niger* were grown in potato dextrose broth and incubated at 24^0C. Aliquots of extracts to be tested (20 µl) were loaded in duplicate on to ¼ inch sterile filter paper discs and placed on agar plates containing 100 µl of the test organisms. Plates were inverted and grown under appropriate conditions for 3 days, checking for activity daily.

Bioassay guided fractionation

Isolate 4: *Streptomyces malaysiensis* (Figure 4)

Streptomyces malaysiensis was cultivated in a production media containing 1% starch, 0.1% yeast, and 0.1% peptone in 75 ppt sterile filtered seawater (SFSW). Both the ethyl acetate and butanol crude extracts from this isolate showed activity against all organisms tested. Both extracts were fractionated in the same manner. Fractionation of each active crude extracts began with a Sep-Pak C_{18} cartridge (10 g/60 ml). The crude extracts were resuspended in 20% aqueous methanol (2.0 ml), applied to the column and eluted with an increasing methanol gradient (20 to 100%) to yield a total of 5 fractions. Each fraction was collected separately and tested for antimicrobial activity. Activity was conserved in the 100% methanol fraction in both the ethyl acetate and butanol crude fractionation. These fractions were combined, dissolved in 100% methanol (2.0 ml), loaded onto an LH-20 column (19 x 1.0 cm) and eluted with two column volumes of 100% methanol (50 ml). Fractions (1 ml) were collected using a RediFrac fraction collector. TLC was used to determine the chemical profile of these fractions. Plates were developed and analyzed, and fractions containing common spots were combined and further tested for antimicrobial activity. All fractions displaying biological activity against all the test organisms consistently displayed a purple stain with vanillin, and where appropriate this was used to guide the fractionation process. HPLC-DAD-ESIMS analysis on these combined fractions using a Waters Symmetry Shield RPC_{18} column (2.1 x 150 mm, 20-80% acetonitrile/ 0.05% TFA over 30 min, 0.2 ml/min) revealed two major peaks. These two peaks were purified by HPLC-UV using a Waters SunFire C_{18} column (10 x 250 mm, 30-65% acetonitrile / 0.1% FA; flow rate 2.5 ml/min; detection 232 and 214 nm), yielding **9** (r_t = 14.4 min) and **10** (r_t = 18.3 min). Both peaks were tested for antimicrobial activity and analyzed by NMR in d_5-pyridine

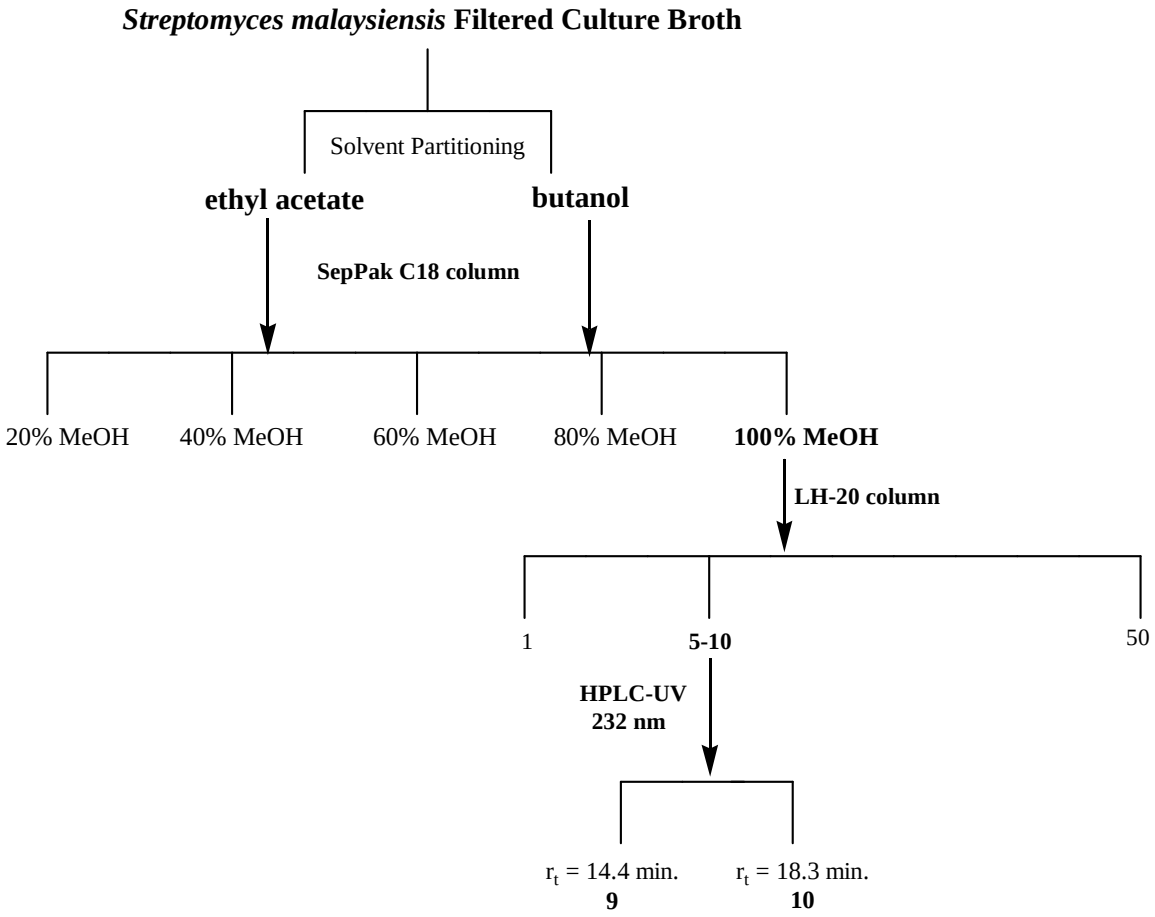


Figure 4. Fractionation scheme for Isolate 4 (*Streptomyces malaysiensis)*

(see Appendix A). Additional biological and analytical data for each compound are reported below.

I4a (**9**): UV λ_{max} (MeOH) 232 nm; ESIMS m/z 1143 $[M+H]^+$; high resolution MS m/z 1142.6935

I4b (**10**): UV λ_{max} (MeOH) 232 nm; ESIMS m/z 1143 $[M+H]^+$; high resolution MS m/z 1142.6935

Isolate 29: *Streptomyces fimicarius* (Figure 5)

Streptomyces fimicarius was cultivated in a production media containing marine broth (Difco), tryptone, and NaCl in deionized water (see Table 1). The crude butanol extract of this isolate showed selective activity against *M. smegmatis*. Fractionation of this active crude extracts began with a Sep-Pak C_{18} cartridge (10 g/60 ml). The crude extract was resuspended in 25% aqueous methanol (2.0 ml), applied to the column and eluted with a methanol gradient (25 to 100%) providing a total of 4 fractions. Each fraction was collected separately and tested for antimicrobial activity. Activity was conserved in the 50% and 75% methanol fractions. These active fractions were combined, dissolved in 100% methanol (2.0 ml) and loaded onto an LH-20 column (19 x 1.0 cm). The mixture was eluted with two column volumes of 100% methanol (50 ml) and fractions (1 ml) were collected using a RediFrac fraction collector. TLC was used to determine the chemical profile of these fractions. Plates were developed and analyzed, and fractions containing common spots were combined and further tested for antimicrobial activity. Activity against *M.smegmatis* was seen only in those combined fractions that stained yellow and contained a UV-quenching spot. LC/MS was performed on the combined active fractions using a Waters Symmetry Shield RPC_{18} column (2.1 x 150 mm, 20-80% acetonitrile/ 0.05% TFA over 30 min; 0.2 ml/min). 1D and 2D homonuclear and heteronuclear NMR experiments were

performed in order to characterize the chemical nature of this active fraction (see Appendices B and C). Additional biological and analytical data for each compound are reported below.

I29a (**11**): UV λ_{max} (MeOH) 270 nm; high resolution MS m/z 1015.6242

I29b (**12**): UV λ_{max} (MeOH) 270 nm; high resolution MS m/z 1031.6151

I29c (**13**): UV λ_{max} (MeOH) 270 nm; high resolution MS m/z 981.6327

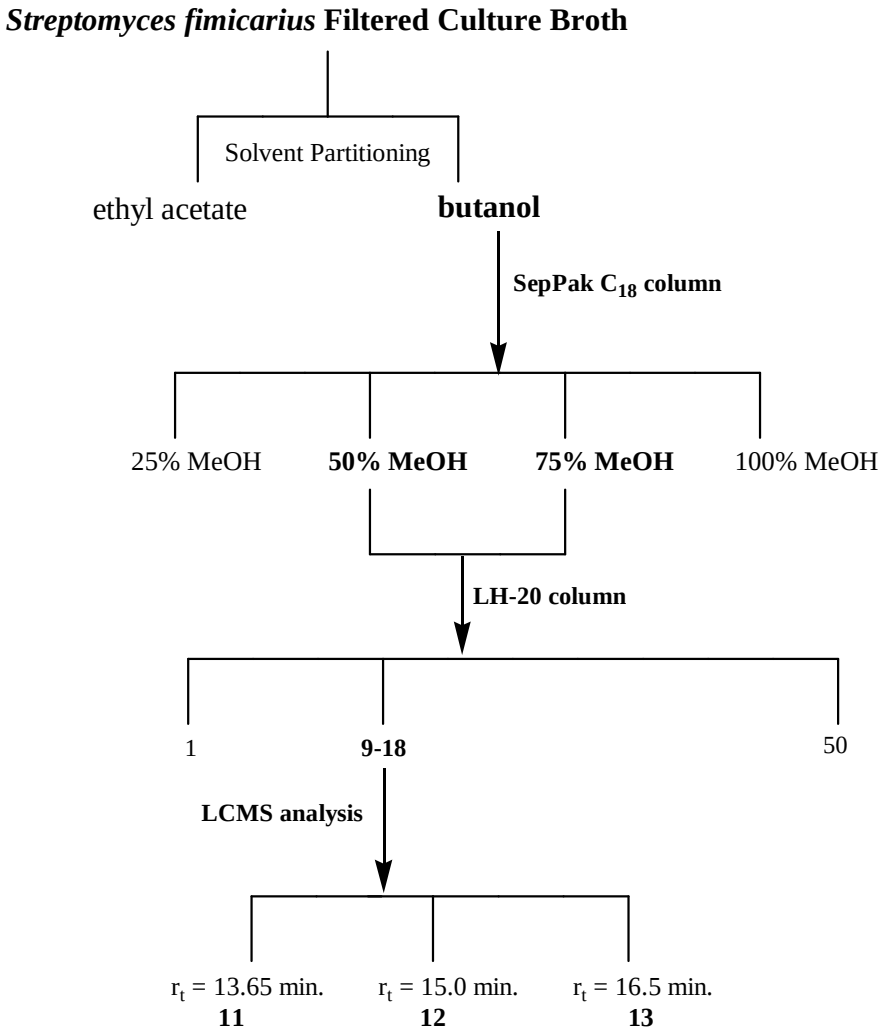


Figure 5. Fractionation scheme for Isolate 29 (*Streptomyces fimicarius*)

Cytotoxicity Assay

Active compounds from isolate 4 (*Streptomyces malaysiensis*) were also tested for cytotoxicity against murine fibroblast cells (NCTC clone L-929) using an MTT based spectrophotometric assay. This assay was performed by Candace Jones at aaiPharma.

RESULTS

Isolation of Actinomycetes from Near Shore Sediments

Sediment samples were taken from the dock at CMS (April 15, 2004 and May 12, 2004), along the Cape Fear River (June 8, 2004), as well as various sites in the Florida Keys (July 15, 2005). A total of 50 clones were isolated and positively identified as actinomycetes, encompassing 20 different species with varying differences in genetic divergence. The distribution, media used in isolation, genetic identification and activity of these marine sediment derived actinomycetes can be seen in Table 2. *Micromonospora* species were dominant in the sediments collected from the Dock at CMS, while *Streptomyces* species were dominant in sediments collected along the Cape Fear River. Sediment samples collected in the Florida Keys resulted in only two actinomycete species, however salinity measurements revealed that these samples originated from a much more saline environment than other sampling locations.

Initial Activity Testing

26 of the 50 isolates, representing one of each identified species as well as some replicates with larger differences in genetic divergence, were grown in 600 ml scale. The cell extract as well as ethyl acetate and butanol extracts of the culture broth of each were tested for activity in a series of antimicrobial disc diffusion assays. 10 of these 26 isolates (38.5%) showed activity against one or more test organism. Two of these isolates, *Streptomyces malaysiensis* (isolate 4) and *Streptomyces fimicarius* (isolate 29) were chosen for large scale cultivation. Isolate 4 showed broad spectrum antimicrobial activity whereas isolate 29 selectively inhibited *Mycobacterium smegmatis*.

Bioactive Metabolites from *Streptomyces malaysiensis*

This isolate (isolate 4) was obtained from sediment samples collected in June of 2004

Isolate	Sampling Site	Isolation Media	Identification	Genetic divergence	Activity
1a	A1	AIA	*Streptomyces albidoflavus*	0.80% Difference	
1b	A1	AIA	*Streptomyces albidoflavus*	0.80% Difference	+
2	A1	AIA	*Micromonospora halophytica*	1.40% Difference	
3	A1	AIA	*Micromonospora aurantiaca*	0.40% Difference	
4	B1	AIA	*Streptomyces malaysiensis*	1.19% Difference	+
5	B1	AIA	*Streptomyces albidoflavus*	0.80% Difference	
6	B1	AIA	*Streptomyces albidoflavus*	0.80% Difference	
7	B1	AIA	*Streptomyces lienomycini*	1.00% Difference	+
8	B1	AIA	*Streptomyces griseoplanus*	0.00% Difference	
9	B1	AIA	*Streptomyces griseoplanus*	0.40% Difference	+
10	A2	AIA	*Micromonospora aurantiaca*	0.00% Difference	
11	A2	AIA	*Micromonospora aurantiaca*	0.40% Difference	
12	A2	M4100	*Streptomyces morookaensis*	2.89% Difference	+
13	A2	M4100	*Micromonospora olivasterospora*	2.10% Difference	+
14	A2	M4100	*Micromonospora aurantiaca*	0.40% Difference	
15	A2	M4100	*Micromonospora aurantiaca*	0.50% Difference	
16	A2	M4100	*Micromonospora aurantiaca*	0.40% Difference	
17	B1	M4100	*Micromonospora chalcea*	0.00% Difference	
18	A2	AIA	*Micromonospora chalcea*	0.00% Difference	+
19	A2	M4100	*Micromonospora olivasterospora*	0.80% Difference	
20	A2	M4100	*Micromonospora aurantiaca*	0.40% Difference	
21	B1	AIA	*Streptomyces malaysiensis*	1.09% Difference	
22	B1	AIA	*Micromonospora fusca*	0.60% Difference	
23	B1	AIA	*Micromonospora chalcea*	0.00% Difference	
24	B1	AIA	*Streptomyces griseoaurantiacus*	0.80% Difference	
25	B1	AIA	*Streptomyces malaysiensis*	0.20% Difference	
26	B1	M4	*Streptomyces albidoflavus*	0.00% Difference	
27	B1	M4	*Streptomyces griseorubens*	0.00% Difference	+
28	B1	M4	*Micromonospora halophytica*	0.00% Difference	
29	B1	M4100	*Streptomyces fimicarius*	0.80% Difference	+
30	B1	M4	*Streptomyces albus albus*	1.49% Difference	+
31	B1	M4	*Streptomyces lavendulae*	2.00% Difference	
32	B1	M4100	*Streptomyces griseinus*	0.00% Difference	
33	B1	M4100	*Streptomyces lavendulae*	2.00% Difference	
34	B1	M4	*Streptomyces fimicarius*	0.80% Difference	
35	A2	AIA	*Micromonospora chalcea*	0.00% Difference	
36	A2	AIA	*Micromonospora chalcea*	0.20% Difference	
37	B1	AIA	*Micromonospora aurantiaca*	0.40% Difference	
38	B1	AIA	*Micromonospora chalcea*	0.00% Difference	
39	B1	AIA	*Micromonospora chalcea*	0.00% Difference	
40	A2	M4100	*Micromonospora aurantiaca*	0.40% Difference	
41	A2	M4100	*Micromonospora globosa*	0.00% Difference	
42	A2	M4100	*Micromonospora chalcea*	0.80% Difference	
43	A3	AIA	*Streptomyces albidoflavus*	0.80% Difference	
44	B1	AIA	*Nocardiopsis lucentensis*	1.56% Difference	
45	B1	M4	*Streptomyces sampsonii*	0.80% Difference	
46	B1	M4	*Micromonospora olivasterospora*	0.00% Difference	
47	B1	M4	*Streptomyces citreofluorescens*	0.40% Difference	
48	B1	M4	*Micromonospora aurantiaca*	2.40% Difference	
49	C1	AIA	*Micromonospora chalcea*	0.20% Difference	
50	C1	M4	*Micromonospora aurantiaca*	2.20% Difference	

Table 2. Distribution, isolation media, genetic identification and activity of marine sediment derived actinomycetes

along the Cape Fear River. Original sediment samples were plated onto agar plates of Actinomycetes Isolation Agar (AIA, see Table 1 for recipe) and continual transfer yielded a pure leathery colony with a powdery white surface identified as *Streptomyces malaysiensis* (1.19% difference). After cultivation and harvest, crude extracts were tested using a disc diffusion assay. Crude ethyl acetate and butanol extracts of filtered culture media were found to inhibit all organisms tested. Bioassay guided fractionation using reversed phase and gel permeation chromatography was used to isolate the compound(s) responsible for activity (Figure 4). Further clean up by preparative HPLC resulted in two major peaks, both of which displayed broad spectrum activity as observed in the original crude extracts. HPLC-DAD-ESIMS analysis revealed that both peaks had the same UV absorbance (λ_{max} =232 nm; Figure 6) and molecular weight (m/z 1143, $[M+H]^+$). The HPLC chromatogram as well as the electrospray positive mass spectra are shown in Figure 7. Final purification of the products has not yet been achieved, but initial NMR analysis suggests that these compounds contain a polyhydoxy- or polyether-polyene structure, as evidenced by numerous protons under oxygen (δ 3.0 -δ 4.0) and several olefinic resonances (δ 5.2, δ 5.4, δ 5.6, and δ 6.0). Furthermore, the UV chromophore (λ_{max} 232 nm) is consistent with a conjugated diene system. The absence of low field resonances below δ 6.5 makes the presence of any aromatics unlikely. Additionally, the ESI-MS isotopic pattern of the parent ion does not suggest the presence of any halogenation. Cytotoxicity activity of this extract was determined using an MTT based spectrophotometric assay and revealed activity comparable with Triton-X.

Streptomyces malaysiensis was additionally cultured in a production media containing the brown seaweed *Laminaria* (LM, see Table 1). The crude extracts of the filtered culture media retained inhibition against all organisms tested. Additionally, LCMS analysis revealed that the active

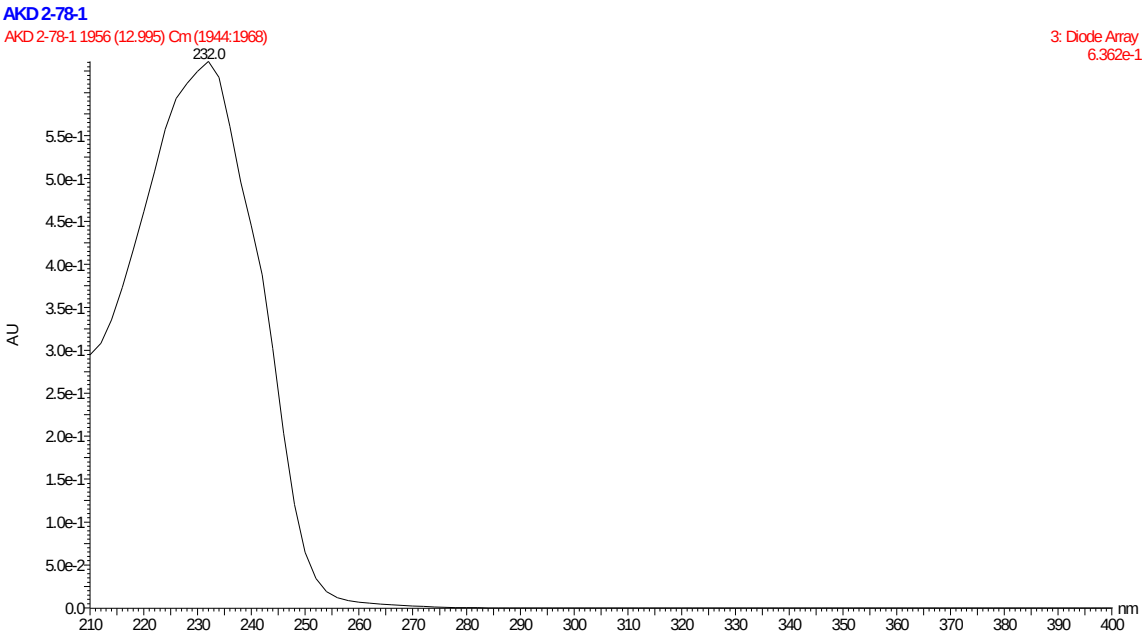


Figure 6. UV data for *Streptomyces malaysiensis* active compounds

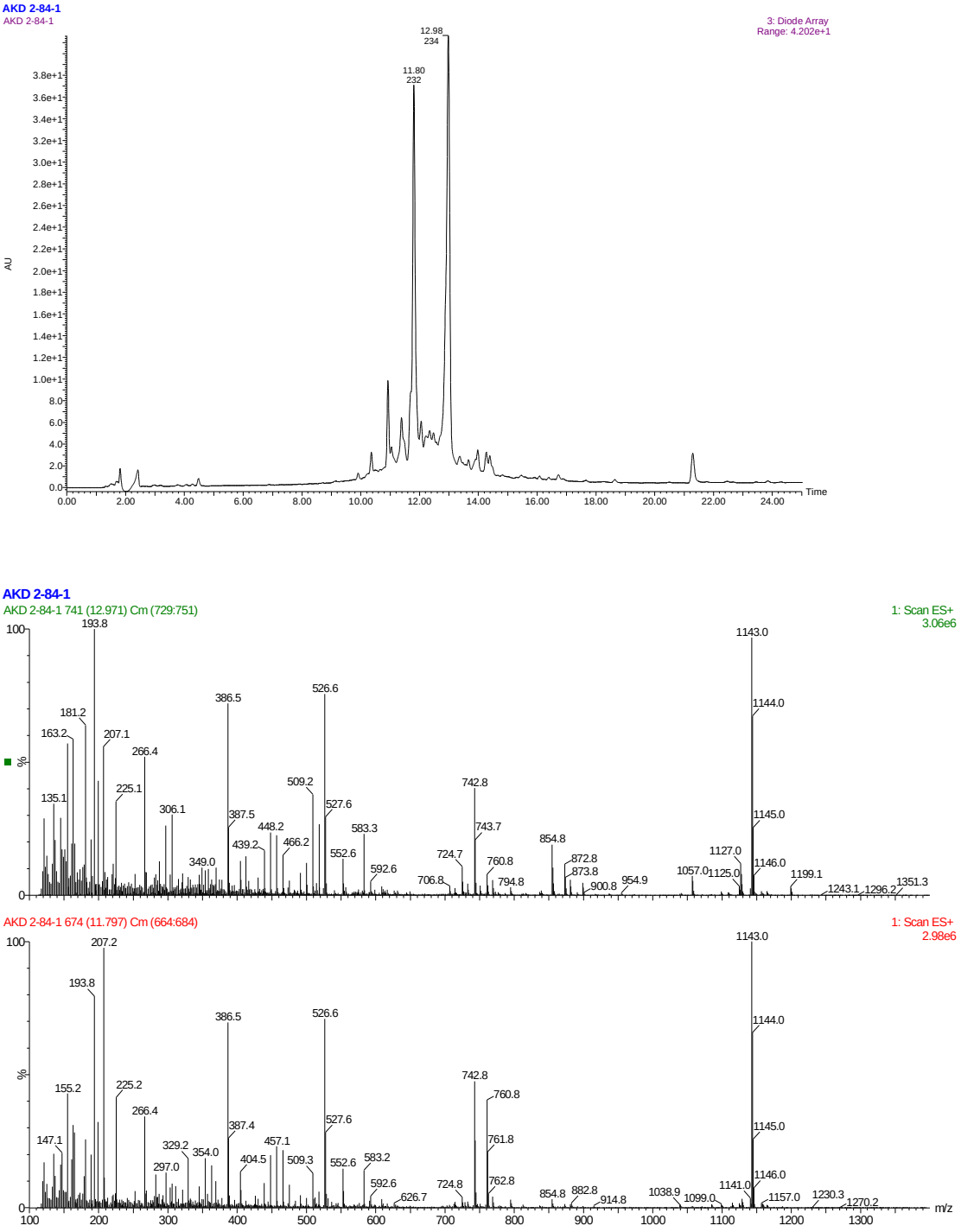


Figure 7. Diode Array (above) and ESIMS (below) data for *Streptomyces malaysiensis* active compounds

metabolites (m/z 1143) were still produced.

Bioactive metabolites from *Streptomyces fimicarius* (Isolate 29)

Isolate 29 was also obtained from sediment samples collected in June of 2004 along the Cape Fear River. Original sediment samples were plated onto agar plates of a chitin containing media supplemented with 100 μg/ml cyclohexamide (M4100, see Table 1 for recipe). Continual transfer yielded a pure leathery concentric colony, greenish gray in color that was identified by genetic analysis as *Streptomyces fimicarius* (0.80% difference). After cultivation and harvest, crude extracts were tested using a disc diffusion assay. The crude butanol extract was found to selectively inhibit *Mycobacterium smegmatis*. Once again, bioassay guided fractionation was used to determine the compound(s) responsible for activity (Figure 5). In this case, gel permeation clean-up resulted in the isolation of three related compounds that collectively inhibited *Mycobacterium smegmatis*. HPLC-DAD-ESIMS analysis revealed that these three metabolites all share a common UV absorbance (λ_{max} =270 nm; Figure 8) and contain common fragment ions ($[M+H]^+$, m/z 337, 319, 295, 241, 227, 159, 126)(Figure 9). The parent ions have been difficult to identify. When these three compounds were analyzed by ESIMS at various cone voltages and mobile phase modifiers, fragment ions at m/z 462, m/z 488, and m/z 502 were always dominant. In contrast, Q-tof HRMS analysis yielded parent ions at m/z 981, m/z 1015, and m/z 1031, confirmed by isotopic pattern and the presence of sodium adducts. It is possible that ESIMS results in doubly charged ions, or that the molecule is a symmetrical dimer that is cleaved under ESIMS conditions. Regardless, at the moment it is not certain how these compounds fragment to the resulting masses. Preliminary NMR studies (in d_4-methanol) were performed on this mixture as a preliminary step to characterize the nature of these active

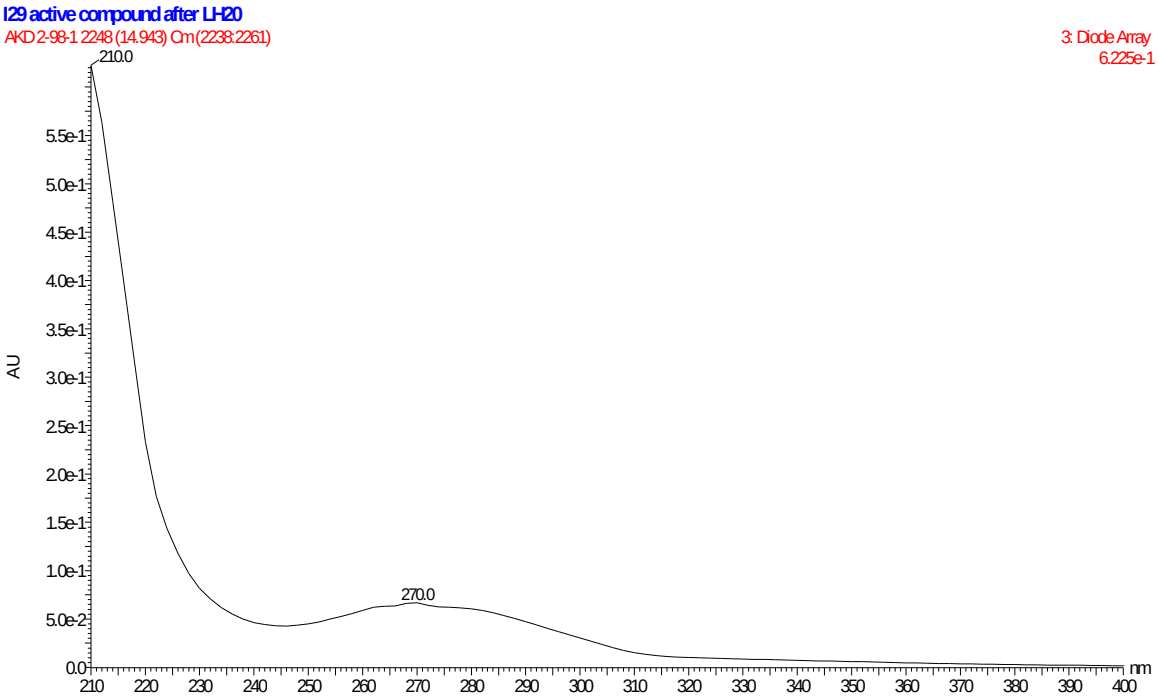


Figure 8. UV data for *Streptomyces fimicarius* active compounds

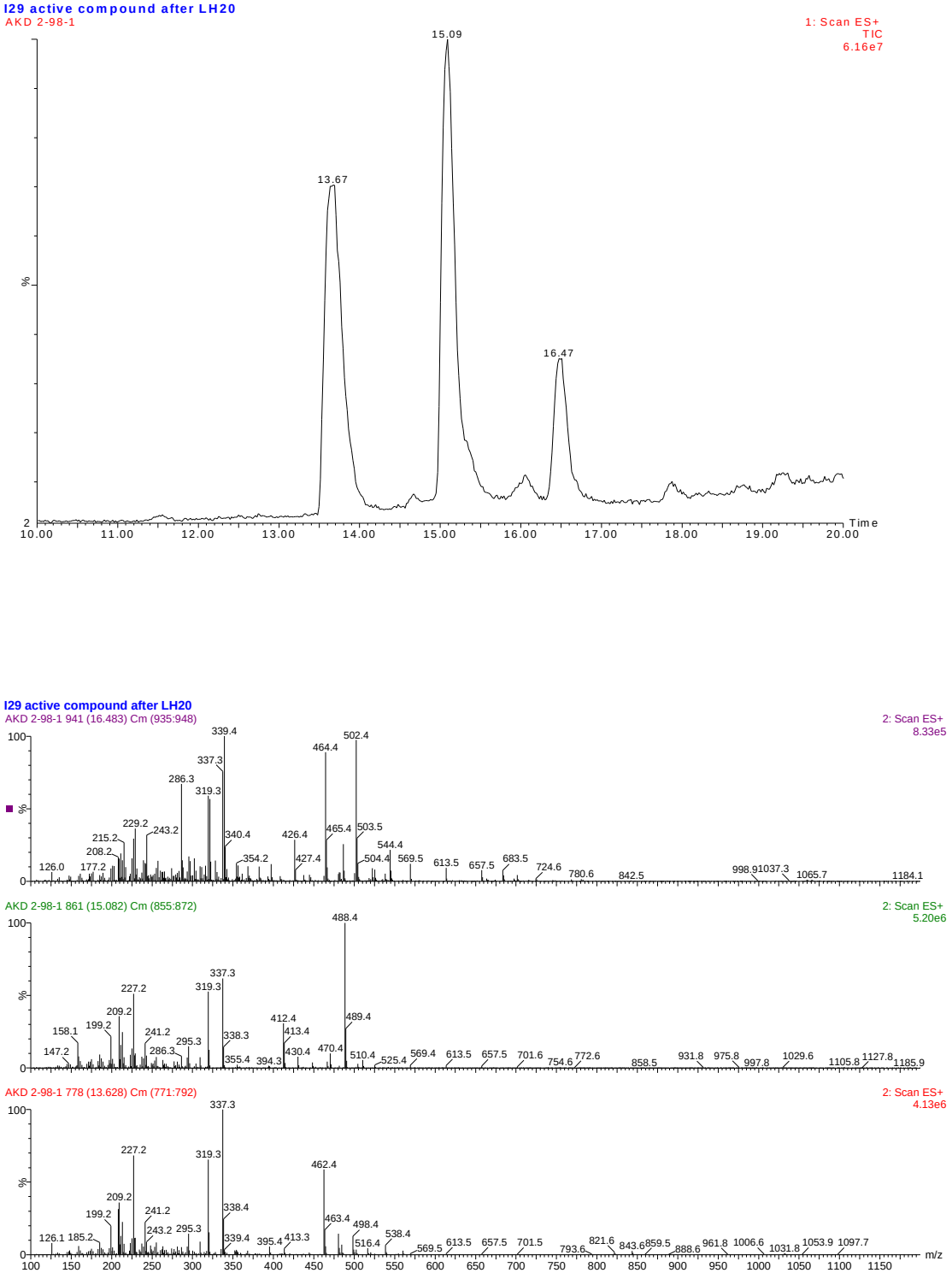


Figure 9. Diode Array (above) and ESIMS (below) data for *Streptomyces fimicarius* active compounds

metabolites. ^{1}H resonances from δ .7- δ 1.35 showed correlations with ^{13}C resonances in the region δ 12-30 and could be attributed to a long acyl chain. Additionally, HSQC correlations between ^{1}H shifts of δ 3.6, δ 4.0 and δ 4.2 with ^{13}C shifts of δ 68, δ 71 and δ 72 respectively, were consistent with protonated carbons linked to oxygen. The lack of olefinic resonances as well as the weak UV absorbance indicates no conjugation in this acyl chain. Additional ^{1}H aromatic resonances at δ 7.05 and δ 6.75 as well as δ 7.25 were consistent with the presence of aromatic rings and the HSQC correlations (δ 7.05, δ 132; δ 6.75, δ 116) suggested the presence of the aromatic amino acid tyrosine as well as phenylalanine (δ 7.25, δ 129). Furthermore, ^{1}H resonances at δ 4.22 and δ 4.1 which were correlated with carbons resonating at δ 59 and δ 61, were consistent with α-hydrogens of an amino acid, supporting the presence of amino acids in the structure.

DISCUSSION

This project began in the spring of 2004. Since then more than fifty actinomycete strains have been isolated from local and tropical marine sediments many of which display interesting and potent biological activity. Two of the most promising strains (*Streptomyces malaysiensis* and *Streptomyces fimicarius*) have been mass cultured and analyzed in detail for their metabolites and many more active strains are waiting to be explored. This project encompasses many aspects of science, involving microbiological methods for the selective isolation of actinomycetes and subsequent cultivation of isolated strains in appropriate production mediums as well as chemical methods for the bioassay guided fractionation of organic extracts of actinomycete cultures and structural determination of the active compounds they produce.

Isolation of actinomycetes from marine sediments

To begin with, the focus of the project was to collect actinomycetes from marine sediments. Sediment samples were treated in order to select for actinomycete colonies. Heat treatment (55^0C, 1 hour) as well as isolation mediums containing complex carbon sources (chitin) and the antibiotic cylcohexamide in order to eliminate competing bacteria and fungi were used for selective isolation. Additionally gram staining and analysis of colony morphology were done by microbiologists at aaiPharma in order to select and isolate potential actinomycete colonies from the highly competitive isolation plate. Once pure colonies of potential actinomycetes were obtained, plates were sent to Accugenix (Newark, DE) for phylogenetic analysis using sequence comparisons of the 16S ribosomal subunit. One disadvantage of outsourcing the genetic identification of these marine actinomycete strains, concerns new studies giving evidence of physiologically unique actinomycetes isolated exclusively from marine sediments. For example, the unique 16S sequences for the two new obligate marine genera

Salinaspora and *Marinaspora* are likely to not exist in the database used for these analyses and those strains with large variations in genetic divergence may in fact belong to these new taxa. This is important because it is more likely that novel metabolites with unique chemistries will be produced by less common actinomycete species, especially those with adaptations to the marine environment.

Media conditions have been found to be very important in the successful isolation and cultivation marine actinomcyetes (Jensen et al 2003). The salinity of sampling sites should be considered when plating sediment samples. The distribution of genera among sampling sites revealed very few actinomycetes in the July 2004 sampling in the Florida Keys. Interestingly, salinity data revealed that these samples originate from a drastically more saline environment than other sampling sites. It could be possible that the isolation conditions were not optimal and increasing the salinity of the media could result in a more accurate depiction of the actinomcyete genera represented.

Isolation and characterization of bioactive metabolites

Fermentation conditions must also be optimized in order to increase the likelihood of isolating new and interesting secondary metabolites. There are several examples of metabolites produced only when conditions specific to the marine environment are provided. Japanese researchers have continuously reported on selective metabolite production in media containing the brown seaweed *Laminaria* (Okami et al 1979; Nakamura et al 1977; Takahashi et al 1989). *Streptomyces malysiensis* (isolate 4) was experimentally cultured in a *Laminaria* containing media and found to retain production of the broad spectrum antibiotics produced in a starch, yeast and peptone containing media. Interestingly, the crude extracts from cultures grown in *Laminaria* containing media were found to be much simpler than those cultured in more standard

nutrient conditions, which could simplify the isolation process. Natural products are produced in low concentrations and continuous cultivation and extraction is required in order to accumulate sufficient amounts of material for analysis. The presence of less complex crude extracts and subsequent simplification of the purification process without a loss in production of the active metabolites could have a tremendous impact on the accumulation of material and should be studied further for both *Streptomyces malysiensis* as well as other actinomycete species. In addition to carbon sources and salinity, parameters such as agitation and hydrostatic pressure should also be considered as the production of new metabolites has been reported by variations in these parameters (Okami and Hotta 1988).

Although only two strains were chosen for large scale cultivation and analysis, several additional actinomycete species isolated from local marine sediments displayed antimicrobial activity in the disc diffusion bioassay. Extracts from cultures of *Streptomyces albidoflavus* and *Micromonospora olivasterospora* were active against fungal cultures, whereas extracts from cultures of *Streptomyces lienomycini* and *Micromonospora chalcea* showed inhibition against bacterial test organisms. Cultures of *Streptomyces griseoplanus* and *Streptomyces griseorubens* showed broader activity, inhibiting 2-3 test organisms each. Slight activity against *M.smegmatis* was seen in extracts from cultures of *Streptomcyes albus albus* and *Streptomyces morookaensis*. Further analysis and determination of these bioactive metabolites is required. Antimycobacterial activity against *M.smegmatis* displayed by other strains was very slight and not nearly as impressive as the activity displayed by extracts of *Streptomyces fimicarius*. Additionally, *Streptomcyes malaysiensis* was the only strain that resulted in extracts inhibiting all test organisms. The potency of the two groups of compounds produced by the selected actinomcyete strains was not suprising due to the marine origin of these cultures. These metabolites are most

likely used as chemical defenses in the highly competitive ocean environment and as these compounds are released they are immediately diluted, requiring high potency in order to have any effect (Haefner 2003).

The active metabolites produced by *Streptomyces malaysiensis* showed activity in the disc diffusion assay against all test organisms as well as impressive cytotoxic activity against murine fibroblast cells. These metabolites are produced as two active forms presumed to be constitutional isomers. The producing organism has been mass cultured five times resulting in 18.0 L of culture media that has been extracted and analyzed for the presence of these potent antimicrobial compounds. Only 10% of this material has been purified by HPLC-UV and crude material processed through the gel permeation chromatography method (100 mg) is awaiting purification. Pure material will be accumulated and analyzed by extensive NMR and MS studies in order to fully characterize the chemical structure of these isomers. The increased incidence of resistant pathogens, which now includes all major bacterial pathogens as well as all classes of antibiotic compounds, has stimulated the need for safer, more potent and broader spectrum antibiotics (Payne and Tomasz 2004; Barrett 2005). The metabolites described here may be of interest in the development of new antibiotics or may be used for other purposes, as many secondary metabolites with antibiotic activity are used for numerous pharmacological purposes, including immunosuppressant and anticancer agents (Demain 2000b).

The active metabolites produced by *Streptomyces fimicarius* exhibit selective inhibition against *Mycobacterium smegmatis*. These compounds were found to be produced as a family of three related compounds but because purity has not been achieved, it is unclear if one or all of these derivatives are responsible for biological activity. The use of *Mycobacterium smegmatis* as a surrogate test organism for the discovery of anti-tuberculosis agents in this project began in

November of 2005. The antimycobacterial properties of *Streptomyces fimicarius* were only recognized recently and since then this strain has only been mass cultured twice, resulting in 7.2 L of culture media. The isolation procedure for these selective *M.smegmatis* inhibitors is still in the developmental stage. Crude material will continue to be isolated from mass culture of *Streptomyces fimicarius* and a method for the purification of these three products will be developed. As pure material is acquired, extensive NMR and MS studies will be used to characterize the structural features as well as bioassay testing to determine relative activity of these three metabolites. Multidrug resistant strains of *Mycobacterium tuberculosis* continue to become dominant and reported tuberculosis cases were in excess of 8 million last year, resulting in over 2 million deaths. Additionally, of the several hundred natural products discovered with antimycobacterial properties none have moved forward in drug development, attributed to low yields, relative structural complexity and lack of selectivity (Pauli 2005). The new antimycobacterial metabolites described in this thesis show strong and selective inhibition against *M.smegmatis*, making them of high interest in the much needed discovery of TB drugs.

CONCLUSION

A diverse collection of actinomycetes has been isolated from local marine sediments. Cultivation of isolated strains and analysis of organic extracts have revealed many interesting biological activities. Although this project is still in its infancy, preliminary results show marine sediments to be a new source of actinomcyete strains with the ability to produce unique compounds. More than 50 actinomycete strains have been isolated and cultivated, 38.5% of which show biological activity against one or more test organism. Two strains (*Streptomyces malaysiensis* and *Streptomyces fimicarius*) have been mass cultured and analyzed in detail for the compounds they produce. Broad spectrum antimicrobial metabolites were isolated from cultures of *Streptomyces malaysiensis* and a family of antimycobacterial metabolites was isolated from cultures of *Streptomcyes fimicarius*. Although the structures of these metabolites have not yet been determined, they display potent biological activity and appear to contain unique structures. This collection of actinomycetes will continue to be explored in search of new and unique bioactive substances and has the potential to yield a variety of new and interesting bioactive secondary metabolites.

CHAPTER 2: BIOACTIVE METABOLITES FROM THE CYANOBACTERIUM *MICROCYSTIS AERUGIONSA*

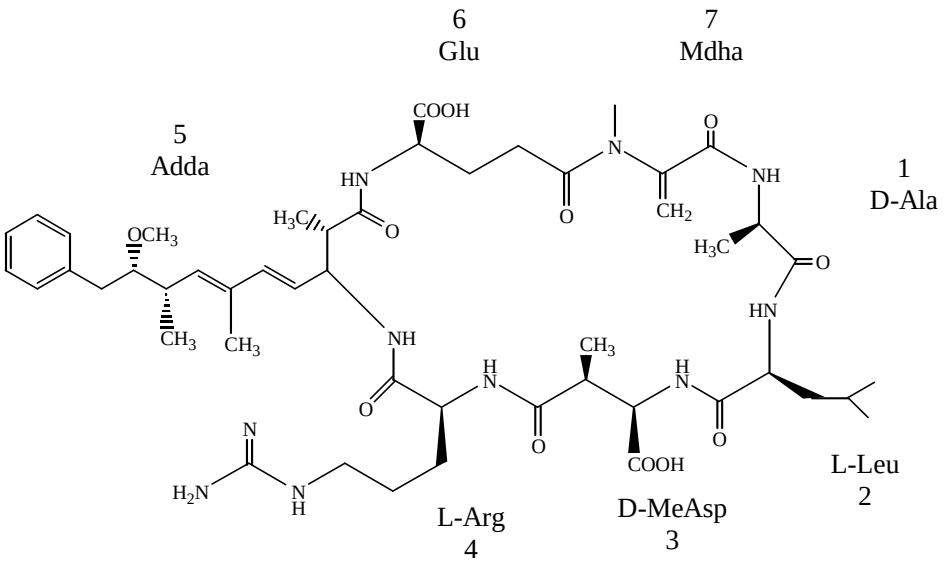
INTRODUCTION

Cyanobacteria are prokaryotic, single celled organisms formerly called blue-green algae. These photosynthetic prokaryotes get their name from a unique accessory pigment phycocyanin, a bluish phycobilin that gives these organisms their blue-green color (Chorus 2000). Blooms of cyanobacteria are a natural phenomenon and can occur in inland and coastal waters worldwide (Reynolds and Walsby 1975). Cyanobacteria have the ability to produce a variety of secondary metabolites, many of which are unique peptide-based compounds. In most instances, the ecological role of such secondary metabolites is unclear, though some of these peptide compounds are extremely potent toxins which can kill a variety of organisms. Consequently, it has been suggested that the production of intracellular toxins discourages grazing and consumption by herbivores, though other biological roles could exist. Regardless of the physiological basis of toxin production, the poisonous health effects reported among higher invertebrates and vertebrate aquatic and terrestrial biota constitute serious environmental and economic problems in both coastal and marine habitats (Paerl 1988). Such cyanobacterial blooms have been predicted to proliferate in both size and duration due to increased eutrophication of water bodies worldwide by pollution with nutrients from agriculture and domestic waste water (Carmichael 1992).

Microcystis is the most common bloom-forming genus of cyanobacteria (Carmichael 1989). *Microcystis* blooms commonly produce liver toxins, known as microcystins. The microcystins are cyclic heptapeptides consisting of (2S,3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid (β-amino acid referred to as Adda) connected to D-glutamic acid (D-Glu), N-methyldehydroalanine (Mdha) and D-alanine (D-Ala) followed by two variable L-amino acids X and Z linked through a D-methylaspartic acid (D-MeAsp) (Kubwabo

2005). Adda, a C-20 β-amino acid residue unique to cyanobacterial metabolites, contains a conjugated diene responsible for the UV chromophore of microcystins which absorbs at 238 nm (McElhiney and Lawton 2005). The toxicity of microcystins also appears to be associated with this Adda residue (Harada et al 1990). Substitutions of variable L-amino acids at positions 2 and 4 give rise to more than 20 primary analogs (Figure 10). The most common and most toxic microcystin contains leucine (L) and arginine (R) at these positions, and is designated microcystin-LR (Sivonen and Jones 1999). Numerous additional analogues result from various methylation and demethylation patterns, as well as modifications in the Adda component (Zurawell et al. 2005). More than 70 analogs of microcystins have been described; these variations have consequences for the chemical properties, geometry, and conformation of the molecule and result in pronounced differences in toxicity as well as hydrophobic/hydrophilic properties (Chorus 2000). Microcystins cannot cross cell membranes, but can be actively transported by the bile acid transporter mechanism and hence preferentially attack the liver. The toxic effects of microcystins are due to their inhibition of eukaryotic protein phosphatases 1 and 2a (PP1 and PP2a). PP1 and PP2a are major factors in the control of several cellular processes including carbohydrate metabolism, muscle contraction, and cell division. As these toxins are preferentially taken up by hepatocytes (the functional cells of the liver), they are responsible for acute liver failure as well as tumor promotion (Chorus 2000, Lawton and Codd 1991).

Increased concern about the acute toxic effects of microcystins has led to the World Health Organization to establish a drinking water guideline level of 1 μg/ L for MCLR (Humpage et al. 2000). No analytical standards for microcystins exist and only few variants are available commercially (McElhiney and Lawton 2005). Rapid detection methods are needed to



Microcystin-LR

Microcystin	2	4	MW (Da)
AR	Ala	Arg	952
FR	Phe	Arg	1028
HilR	Hil	Arg	1008
HtyR	Hty	Arg	1058
LA	Leu	Ala	909
LF	Leu	Phe	985
LL	Leu	Leu	951
LR	Leu	Arg	994
LW	Leu	Trp	1024
LY	Leu	Tyr	1001
RA	Arg	Ala	953
RR	Arg	Arg	1037
WR	Trp	Arg	1067
YA	Tyr	Ala	959
YR	Tyr	Arg	1044
YM(O)	Tyr	Met(O)	1035
M(O)R	Met(O)	Arg	1028

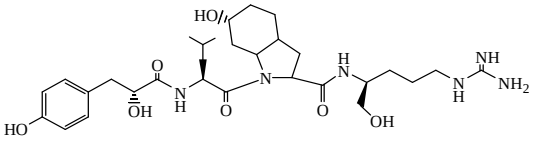
Figure 10. Microcystin structure and some structural variants

test for the presence of these toxins in water supplies, requiring a steady supply of reference material (Carmichael 1981, Lawton and Codd 1991). The North Carolina Department of Health and Human Services (NCDHHS) requires a reliable source of purified microcystin-LR as a reference standard for the development of methods to test environmental samples. The purpose of this section of the thesis was to develop a method for the isolation and purification of quantities (2-10 mg) of microcystin-LR from laboratory cultures of *Microcystis aeruginosa*.

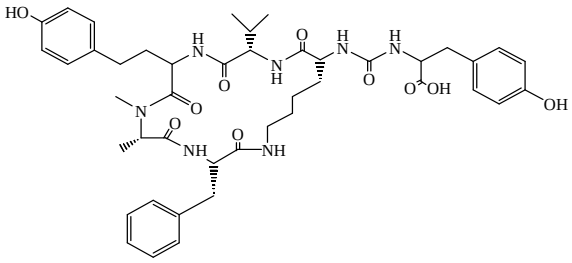
In addition to the hepatotoxic microcystins, several other families of non-hepatotoxic peptides have been identified from *Microcystis*, as well as other genera of cyanobacteria (Namikoshi and Rinehart 1996). The majority of peptides that have been isolated from *Microcystis* can be grouped into six classes according to their structural features (see Table 3 and Figure 11 for representative examples) (Welker et al. 2004a). Aeruginosins and microginins are linear polypeptides containing 3-6 amino acids. These cyanopeptides are protease inhibitors, responsible for such activities as thrombin inhibition and angiotensin converting enzyme (ACE) inhibition. Anabaenopeptins, another class of cyanopeptide protease inhibitors, are cyclic hexapeptides. Microcystins, as mentioned, are cyclic heptapeptide hepatotoxins containing a unique C-20 β-amino acid known as Adda. Cyanopeptolins are cyclic polypeptides possessing various enzyme inhibitory activities, including tyrosinase and plasmin inhibition. Microviridins are large multicyclic peptides which also possess inhibitory activity towards proteases such as trypsin and chemotrypsin. Similar to the Adda residue in microcystins, several other classes of cyanopeptides contain unusual β-amino acids that are unique to each class of cyanopeptide and aid in their identification by the presence of indicative fragment ions in mass spectrometry (Kubwabo et al. 2005; Welker et al. 2004b). The protease inhibitory activity of these peptides provides no known function for the producing organism, but is most likely directed against

Peptide class	General structure	Amino acids	Known variants	Characteristics	Taxonomic distribution
Aeruginosins	Linear	4	19	2-Carboxy-6-hydroxy-octahydroindole (Choi); Arg derivative at C-terminus; hydroxyphenyllactic acid (Hpla) at N-terminus	*Microcystis, Planktothrix, Anabeana*
Microginins	Linear	3 to 6	38	Modified deacnoic or octanoic acid at N-terminus, Tyr at C-terminus	*Microcystis, Planktothrix*
Anabaenopeptins	Cyclic	6	29	Lys with secondary amino bond; ureido bond from ring (Lys) to side chain	*Microcystis, Planktothrix, Anabeana*
Cyanopeptolins	Cyclic	7 to 9	68	3-Amino-6-hydroxypiperidone (Ahp); ester bond of Thr in ring; side chain of variable length	*Microcystis, Planktothrix, Anabeana, Nostoc, Lyngbya*
Microcystins	Cyclic	7	90	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda)	*Microcystis, Planktothrix, Anabeana, Nostoc*
Microviridins	Multicyclic	14	10	Secondary amino and ester bonds of Lys, Ser, Thr; tri-or tetracyclic peptide	*Microcystis, Planktothrix,*

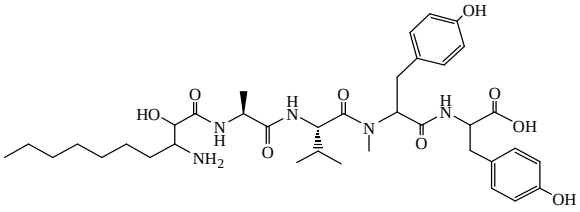
Table 3. Structural characteristics of six classes of cyanobacteria peptides (Welker *et al*. 2000)



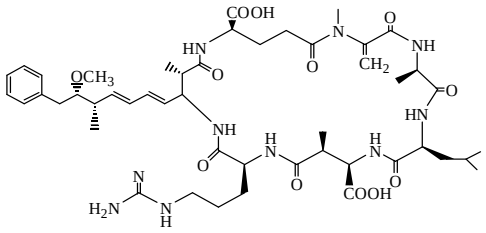
Aeruginosin 98-A



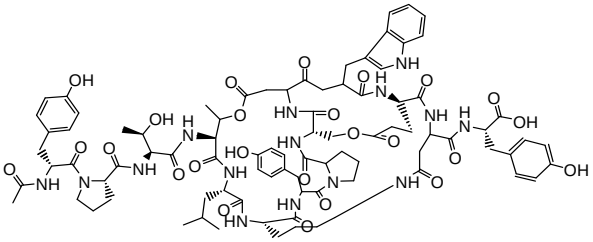
Anabaenopeptin A



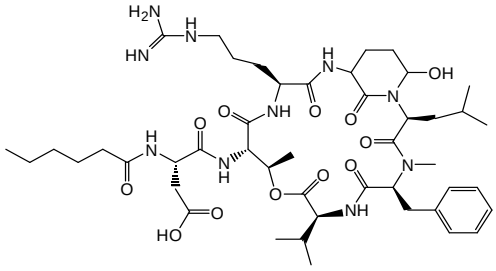
Microginin



Microcystin LR



Microviridin I



Cyanopeptolin A

Figure 11. Representative structures of 6 major cyanopeptide classes

digestive proteases of crustacean grazers (Agrawal et al 2005).

In a 5 year study at the University of Hawaii and supported by the National Cancer Institute and the Eli Lilly Research Laboratories, the discovery rate for bioactive compounds within cyanobacteria for field collected and then laboratory grown cultures was about 7%, very comparable to other groups of microorganisms. However in contrast to the other groups tested, cyanobacteria had a significantly lower rediscovery rate of known bioactive compounds. Thus, cyanobacteria have the potential to provide useful pharmacological substances that do not duplicate structures already discovered from other natural sources (Carmichael 1992). During the large-scale growth, isolation and purification of MCLR from laboratory cultures of *Microcystis aeruginosa* (UTEX LB2385), some other peptide metabolites of interest were isolated and characterized. These peptides were found to contain structural features consistent with the microginin class of cyanopeptide metabolites. The first microginin was described in 1993 and since then more than 20 derivatives have been described (Okino et al.1993). These cyanopeptides contain 4-6 amino acids, usually including a modified β-amino acid as well as a C-terminus tyrosine. The structural features as well as biological activities of known structural variants can be seen in Table 4. Previously characterized microginins have shown angiotensin converting enzyme (ACE) inhibition as well as leucine aminopeptidase M (APM) inhibition, making them potent vasodilators. Consequently, these compounds are of interest as lead compounds in the discovery of novel antihypertensive agents as well as treatments for congestive heart failure. This thesis describes the isolation and structural determination of five new microginin peptides containing previously undescribed structural features including the amino acid methionine, various methylation on the modified octanoic acid chain and the first tripeptide microginin. Biological activity of these new microginin congeners has not yet been determined.

Cyanobacterium	Microginin	R1	R2	R3	R4	R5 (R6)	Activity	References
Microcystis aeruginosa NIES-100	microginin	Ahda	Ala	Val	MeTyr	Tyr	ace i, apm i	Okino *et al*., 1993 Ishida *et al*., 2000
Microcystis aeruginosa TAC-51	microginin 51-A microginin 51-B	Ahda MeAhda	Tyr Tyr	MeVal MeVal	MeTyr MeTyr	Pro (Tyr) Pro (Tyr)	ace i	Ishida *et al*., 2000
Microcystis aeruginosa TAC-91	microginin 91-A microginin 91-B microginin 91-C microginin 91-D microginin 91-E	Ahda-Cl Ahda-Cl_2 Ahda Ahda-Cl Ahda-Cl_2	Ile Ile Ile Ile Ile	MeLeu MeLeu MeLeu MeLeu MeLeu	Pro Pro Pro Pro Pro	 Tyr Tyr Tyr	 apm i apm i apm i	Ishida *et al*., 2000
Microcystis aeruginosa NIES-99	microginin 99-A microginin 99-B	Ahda-Cl Ahda-Cl_2	Tyr Tyr	Leu Leu	MeTyr MeTyr	Pro Pro		Ishida *et al*., 1998
Microcystis aeruginosa NIES-299	microginin 299-A microginin 299-B	Ahda-Cl Ahda-Cl_2	Val Val	MeVal MeVal	MeTyr MeTyr	Pro (Tyr) Pro (Tyr)	lap i lap i	Ishida *et al*., 1997
Microcystis aeruginosa NIES-299	microginin 299-C microginin 299-D	Ahda Ahda-Cl_2	Val Val	MeVal MeVal	MeTyr MeTyr	Pro (Tyr)	lap i lap i	Ishida *et al*., 1998
Microcystis aeruginosa NIES-478	microginin 478	MeAhda	Val	MeVal	MeTyr	Tyr	ace i, apm i	Ishida *et al*., 2000
Microcystis sp. (bloom)	microginin FR1	Ahda	Ala	MeLeu	Tyr	Tyr	ace i, apm i	Neumann *et al*., 1997
Microcystis aeruginosa (bloom)	microginin SD755	MeAhoa	Val	MeIle	MeTyr	Tyr	apn i	Reshef & Carmeli, 2001
water bloom water bloom	microginin T1 microginin T2	Ahda-Cl Ahda	Ala Ala	Pro Pro	Tyr Tyr	Tyr Tyr	ace i, lap i ace i, lap i	Kodani *et al*., 1999
Nostoc sp. (bloom)	Nostoginin BN578 Nostoginin BN471	Ahoa Ahoa	Val Val	NMeIle NMeIle	NMeTyr NMeTyr	Tyr		Pluotmo and Carmeli, 2002
Oscillatoria agardhii NIES-610	Oscillaginin A Oscillaginin B	Ahda-Cl Ahda	Ser Ser	NMeVal NMeVal	Hty Hty			Sano and Kaya, 1997

[a] Order of the residues is from the N-terminus to the C-terminus. Ahda = 3-amino-2-hydroxydecanoic acid; Ahda-$Cl_{(2)}$ = 10-(di)chloro-3-amino-2-hydroxydecanoic acid; MeAhda = N-methyl-Ahda; MeAhoa = N-methyl-3-amino-2-hydroxyocanoic acid; MeVal = N-methyl-valine; MeTyr = N-methyl-tyrosine

[b] Activities reported in the references. ace = angiotensin-converting enzyme; apm = aminopeptidase M; apn = bovine aminopeptidase N; lap = leucine aminopeptidase; i= inhibition

Table 4. Structure, origin, and activity of microginins isolated from freshwater cyanobacteria (Welker et al. 2004a).

EXPERIMENTAL

Culture Methods

Microcystis aeruginosa cells (UTEX LB2385) were obtained from the University of Texas Culture Collection and grown at CMS in B3N media in a 14/10 light-dark cycle for 4 weeks before harvest.

Chromatography Methods

Stationary phases used in open column chromatography were Supelco ENVI-18 (reversed phase) and Sephadex LH-20. High performance liquid chromatography (HPLC) stationary phases used were Waters SunFire C_{18}, Supelco Discovery Amide C_{16}, and Waters Symmetry Shield RPC_{18}. Solvents used were HPLC grade methanol and acetonitrile purchased from Fisher Scientific and trifluoroacetic acid (TFA) purchased from Alfa Aesar. Water was purified by Labconco Water Pro Plus water deionization system. Preparative HPLC was performed using Waters pumps (515), automated gradient controller, and dual wavelength detector (2487).

Analytical Methods

ESI-MS data was obtained using a Hewlett Packard Series 1100 HPLC connected to a Waters Micromass ZQ with electrospray ionization (capillary voltage 3.0 kV; cone voltages 30V/50 V; source temperature 140; desolvation temperature 350; cone gas flow 100; desolvation gas flow 600). All samples were run in positive ion mode with a mass range of 100-1400 and a scan time of 0.5 sec. Accurate molecular weight data was obtained using a quadrapole time of flight mass spectrometer (Applied Bioscience-MDS Sciex QStar XL). Daughter ion experiments were performed using an ion trap mass spectrometer (Applied Bioscience-MDS-Sciex QTrap).

Nuclear magnetic resonance (NMR) experiments were performed on a Bruker Avance 500 MHz NMR at 293.9 K. Probes used include a 5.0 mm BBO, 5.0 mm TXI, and 1 mm TXI. NMR solvents (d_6-DMSO, d_4-methanol) were purchased from Cambridge Isotope Laboratories.

Harvest and Extraction

Cultures were filtered (Whatman GF/A filter paper) and collected cells were frozen until extraction (-20^{0}C). Filter paper discs containing cells were extracted with 80% methanol overnight (800 ml). This procedure was repeated, the extracts combined and partitioned against hexane to remove any pigments and non-polar compounds. The hexane layer was discarded and the aqueous methanol layer was dried and weighed.

Isolation and purification of cyanopeptides (Figure 12)

The aqueous methanol extract was resuspended in 20% methanol (2 ml), applied to a Sep-Pak cartridge (10 g/60 ml) and eluted with a methanol step gradient (20-100% MeOH). Compounds of interest eluted in the 40% and 60% methanol fractions and were combined and applied to an LH-20 column (19 cm x 1 cm). The column was eluted with two column volumes of 100% methanol (50 mL), and fractions (1 ml) were collected using a RediSep fraction collector. Fractions were analyzed for the presence of microcystin-LR using HPLC-UV in comparison to the retention time of an MCLR standard purchased from Sigma (Vydac RPC_{18}, 2 x 250 mm; 2-100% aqueous acetonitrile / 0.05% TFA over 10 min; flow rate 1 ml/min; detection 238 and 214 nm; MCLR standard r_t = 8.5 min.) Microcystin- and microginin-containing fractions were combined and subjected to preparative HPLC-UV using a Waters SunFire C_{18} column (19 x 250 mm; 40-100% aqueous methanol / 0.05% TFA over 30 min; flow rate 10 ml/min; detection 238 and 214 nm). Five peaks (**5** r_t 16.1 min, **3** r_t 18.0 min., **1, 2, 4** r_t 20.0 min., **6,7** r_t 22.0 min., and **8** r_t 23.0 min.) were collected and analyzed by HPLC-ESI-MS. Final

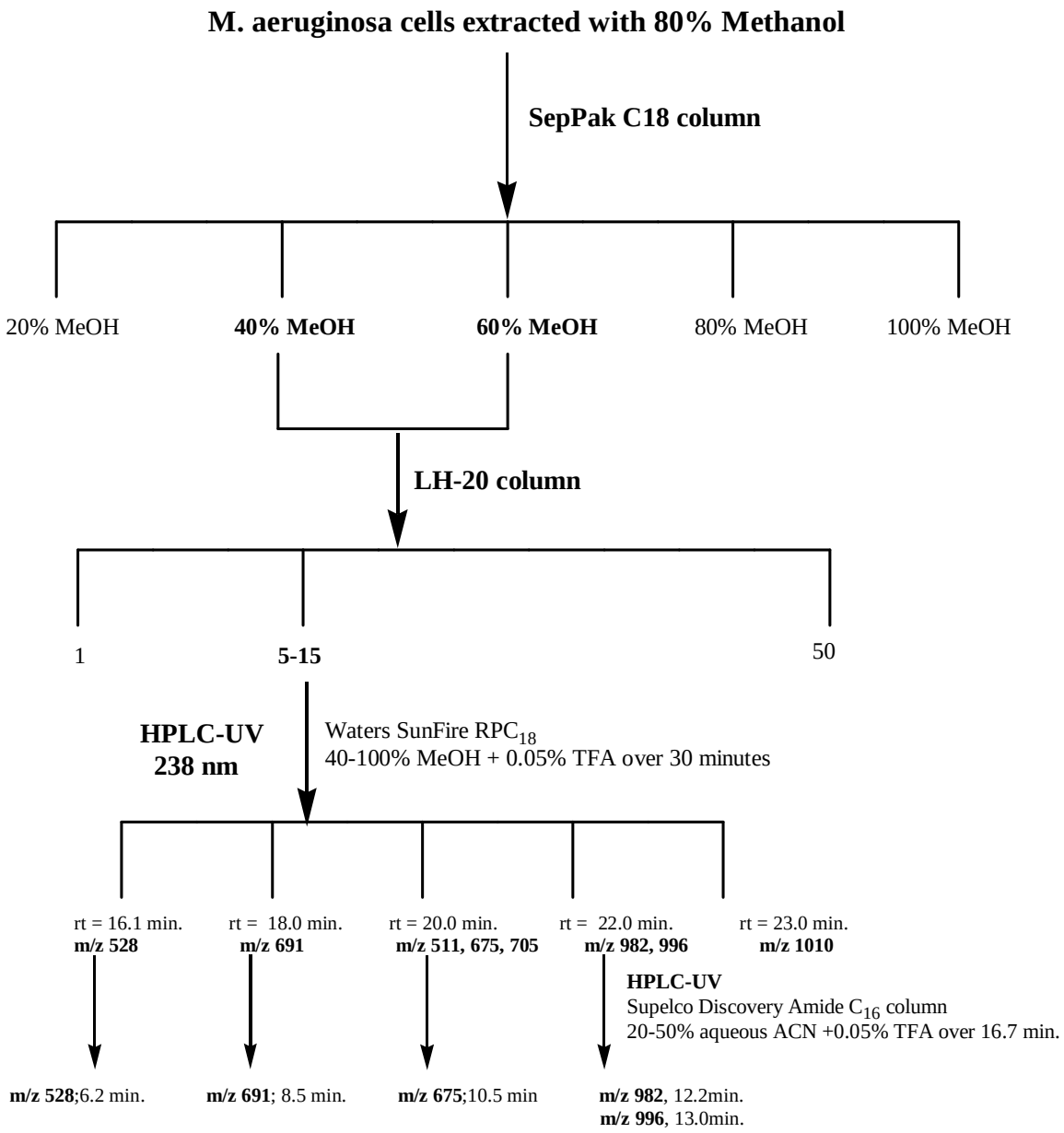


Figure 12. Fractionation scheme for the isolation of peptides from organic extracts of *Microcystis aeruginosa*

purification by HPLC-UV of peaks was subsequently performed using a Supelco Discovery Amide C_{16} column (4.6 x 250 mm, 20-50% acetonitrile/0.05% TFA over 16.7 min; flow rate 1.0 ml/min), yielding purified **5** r_t 6.2 min, **3** r_t 8.0 min., **1** r_t 10.5 min**, 7** r_t 12.2 min., and **6** r_t 13.0 min. These purified compounds were analyzed by NMR using d_6-DMSO and d_4-methanol. LC/MS analysis was performed using a Waters Symmetry Shield RPC_{18} column (2.1 x 150 mm, 3.5 μm; 20-80% acetonitrile/0.05%TFA over 30 min; flow rate 0.2 ml/min). Additional biological and analytical data for each compound are reported below.

Microginin 674(**1**): UV λ_{max} (MeOH) 224, 276 nm; ESIMS m/z 675 $[M+H]^+$ (fragmentation data in Figure 17); high resolution MS m/z 675.3253 ($C_{34}H_{50}N_4O_8S$); ^1H and ^{13}C-NMR see Table 5. ^1H, TOCSY and HSQC NMR spectrum see Appendices D-F

Microginin 511(**2**): UV λ_{max} (MeOH) 224, 276 nm; ESIMS m/z 512 $[M+H]^+$; high resolution MS m/z 511.8786 ($C_{25}H_{41}N_3O_6S$).

Microginin 690(**3**): UV λ_{max} (MeOH) 224, 276 nm; ESIMS m/z 691 $[M+H]^+$ (fragmentation data in Figure 18); high resolution MS m/z 691.3205 ($C_{34}H_{50}N_4O_9S$); ^1H and ^{13}C-NMR see Table 6. ^1H, TOCSY and HSQC NMR spectrum see Appendices G-I

Microginin 704(**4**): UV λ_{max} (MeOH) 224, 276 nm; ESIMS m/z 705 $[M+H]^+$; high resolution MS m/z 705.1430 ($C_{35}H_{51}N_4O_9S$).

Microginin 527(**5**): UV λ_{max} (MeOH) 224, 276 nm; ESIMS m/z 528 $[M+H]^+$; high resolution MS m/z 527.8912 ($C_{25}H_{41}N_3O_7S$).

Microcystin-LR(**6**): UV λ_{max} (MeOH) 238 nm; ESIMS m/z 996 $[M+H]^+$ (fragmentation data in Figure 14); high resolution MS m/z 995.5469 ($C_{49}H_{73}N_{10}O_{12}$)

[D-Asp^3] Microcystin-LR(**7**): UV λ_{max} (MeOH) 238 nm; ESIMS m/z 982 $[M+H]^+$; high resolution MS m/z 981.7139 ($C_{48}H_{71}N_{10}O_{12}$).

Microcystin-L(NMe)R (**8**): UV λ_{max} (MeOH) 238 nm; ESIMS m/z 1010 $[M+H]^+$; high resolution MS m/z 1009.7587 ($C_{50}H_{75}N_{10}O_{12}$)

	Position	**^{1}H**	**^{13}C**
2 O-Me, 3-N,N-dimethyl amino-octanoic acid	1 2 2-OMe 3 3-NMe (2) 4 5 6 7 8	 4.0 3.18 3.01 2.63 1.41 1.24 1.22 1.35 .85	171.5 71 49 54 32.5 26 30 30 23 15
Met (O)	1 2 3 4 5 NH	 5.0 2.3 1.8 2.0 8.4	169.5 57 31 29.5 16
Tyrosine	1 2 3 4 5, 9 6, 8 7 7-OH NH	 4.3 2.95 2.85 7.02 6.64 9.2 7.83	172.5 55 37 131 116 158
Tyrosine	1 2 3 4 5, 9 6, 8 7 7-OH NH	 4.7 2.87 2.77 7.0 6.62 9.2 8.23	174 52 37 131 115 158

Table 5. Assignment of ^{1}H and ^{13}C NMR data for microginin 674 (**1**) in d_6-DMSO

	Position	**^{1}H**	**^{13}C**
2 O-Me, 3-N,N-dimethyl amino-octanoic acid	1 2 2-OMe 3 3-NMe (2) 4 5 6 7 8	 4.0 3.18 3.01 2.63 1.41 1.24 1.22 1.35 .85	171.5 71 49 54 32.5 26 30 30 23 15
Met (O)	1 2 3 4 5 NH	 5.0 2.4 2.55 2.5 8.4	169.5 57 30 51 39
Tyrosine	1 2 3 4 5, 9 6, 8 7 7-OH NH	 4.3 2.95 2.85 7.02 6.64 9.2 7.83	172.5 55 37 131 116 158
Tyrosine	1 2 3 4 5, 9 6, 8 7 7-OH NH	 4.7 2.87 2.77 7.0 6.62 9.2 8.23	174 52 37 131 115 158

Table 6. Assignment of ^{1}H and ^{13}C NMR data for microginin 690 (**3**) in d_6-DMSO

RESULTS

Isolation and Purification of Microcystin-LR and Derivatives

Compound **6** (m/z 996, $[M+H]^+$)was isolated as a white powder. The UV data (λmax 238 nm, Figure 9) as well as MS data (Figure 10) confirmed the identity of this peak as microcystin-LR. The desmethyl derivative [Asp^3] microcystin-LR (**7,** m/z 982, $[M+H]^+$) as well as the N-methyl derivative microcystin-L(NMe)R (**8,** m/z 1010, $[M+H]^+$) were also isolated by this procedure. Location of these desmethyl and methyl variants was established by comparison of the ESIMS fragment ions with those assigned in the literature. For example, in determination of the desmethyl variant, analysis of the ESIMS data revealed that the fragment ion of m/z 286 corresponding to $[H+Arg\text{-}MeAsp]^+$ as well as the fragment ion of m/z 399 corresponding to $[H+Leu\text{-}MeAsp\text{-}Arg]^+$ observed in **6** were both 14 Da less in the ESIMS of **7**. The fragment ion of m/z 174 corresponding to $[Arg\text{-}NH_2\text{-}2H]^+$ was seen in the spectra of both compounds. These data indicate that the MeAsp (residue 3) in **6** has been replaced by Asp in **7** resulting in [Asp^3] microcystin-LR. In determination of the methylated derivative, the characteristic ESIMS fragment ions at m/z 553 ($[H+Mdha+Ala+Leu+MeAsp+Arg]^+$), m/z 470 ($[H+Ala+Leu+MeAsp+Arg]^+$), m/z 399 ($[H+Leu\text{-}MeAsp\text{-}Arg]^+$) and m/z 268 ($[H+Mdha+Ala+Leu]^+$) in the spectra of **6**, were all 14 Da more in the HRMS of **8**. However, the fragment ion of m/z 599 ($[H+MeAsp+Arg+Adda]^+$) was unchanged, indicating that this methylation existed on the leucine residue (residue 2). ^1H NMR analysis of **8** revealed a singlet at δ 2.62 not seen in the ^1H spectra of **6**, indicating that **8** contained an NMe-Leu in place of Leu in residue 2.

Isolation and Structural Determination of Microginin Peptides

Compound **1** (m/z 674, $[M+H]^+$) was isolated as a white powder. The HRMS and NMR

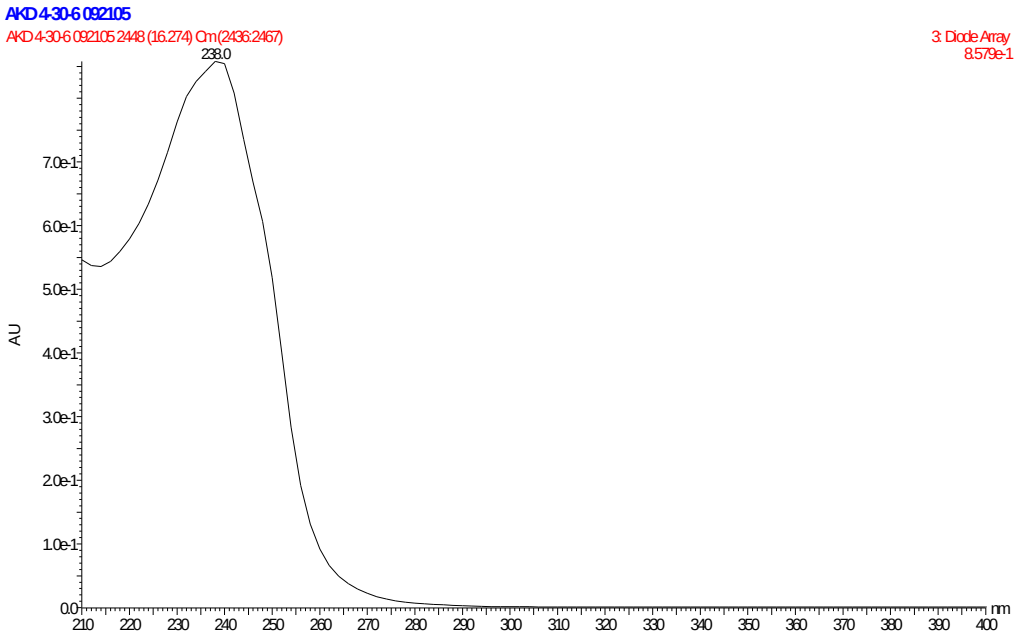


Figure 13. UV data for microcystin-LR

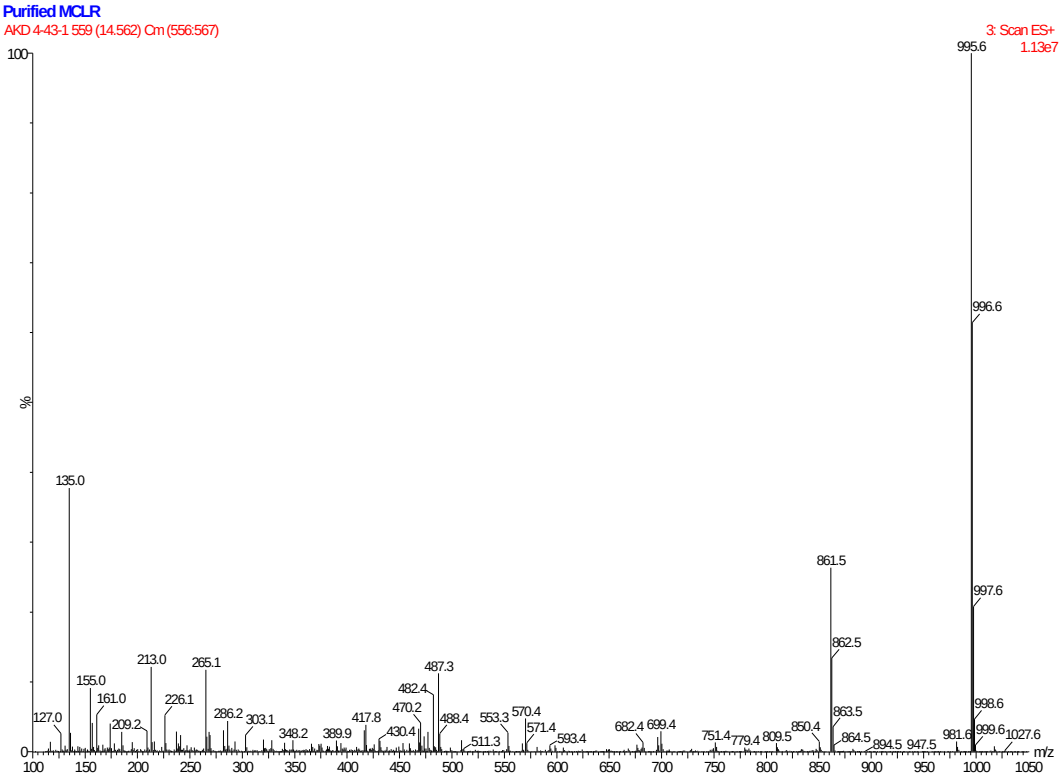
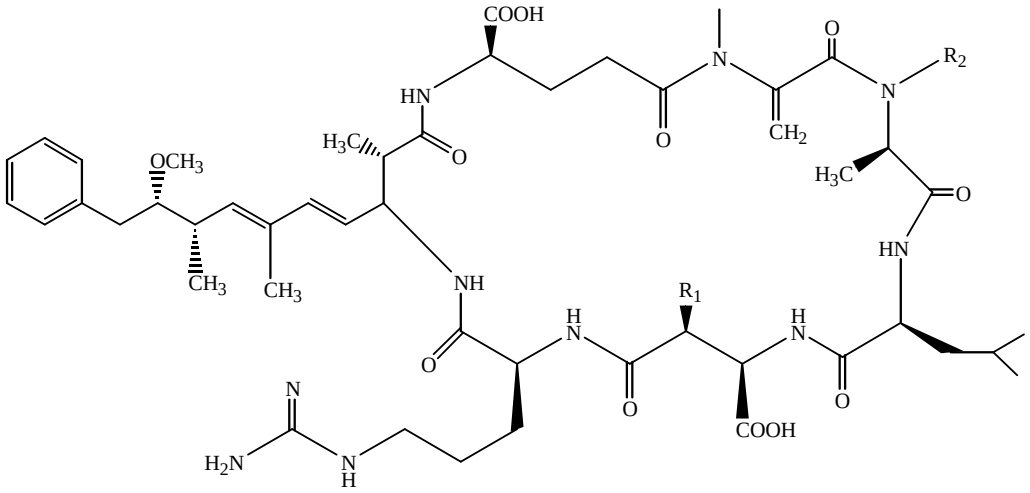


Figure 14. ESIMS of purified microcystin-LR



6: R_1 =CH_3 , R_2 = H
7: R_1 = H, R_2 = H
8: R_1 = CH_3, R_2 = CH_3

Figure 15. Three microcystin variants isolated from *Microcystis aeruginosa* (UTEX LB2385)

data revealed a molecular formula of $C_{34}H_{50}N_4O_9S$, requiring 12 degrees of saturation. Four carbonyl carbons revealed by the ^{13}C NMR data as well the presence of four isolated spin systems in the TOCSY and COSY NMR spectra confirmed the tetrapeptide nature of this compound (Table 5). The UV data (λmax 224, 276 nm) (Figure 16) indicated the presence of an aromatic amino acid. Indeed, the ESI-MS data (Figure 17) revealed a strong fragment ion at $[M+H -180]^+$ (m/z 494) consistent with a C-terminus tyrosine moiety. Another fragment ion at m/z 327, characterized as the loss of a di-tyrosine C-terminus, revealed a second tyrosine residue adjacent to the C-terminal tyrosine. The presence of two tyrosine residues was confirmed by NMR data (Table 5). The two rings from the tyrosine residues and the four carbonyl carbons satisfied all the degrees of saturation. The fragment ion at m/z 128 suggested an N-terminus α-amino-β-hydroxy decanoic acid (Ahda), common in microginin peptides. ^1H and TOCSY NMR data was consistent with a modified β-amino acid; however none of the observed amide resonances correlated to resonances in this chain. HSQC data revealed two methyl resonances at δ 2.63 correlated to a carbon resonance at δ 32.5, indicating two methyl groups attached to nitrogen. It was therefore determined that the β-amino acid was a 3-N, N-dimethyl amino-octanoic acid, instead of Ahda. Furthermore, the methyl resonance at δ3.18 which correlated with a carbon at δ 49 was consistent with a methyl attached to oxygen. This N-terminus moiety was finally determined to be 2 O-methyl, 3-N, N-dimethyl amino-octanoic acid. A moiety corresponding to 147 Da remained unaccounted for. The HSQC spectra of **1** revealed a correlation between a proton at δ 2.0 and a carbon at δ 16, consistent with a methyl group attached to sulfur. Analysis of the TOCSY and COSY spectra revealed a spin system correlating the amide resonance at δ 8.4 with the methine resonance at δ 5.0, which in turn was correlated to the resonances of δ 2.3, 2.0, and 1.8. It was therefore determined that the unknown residue was a

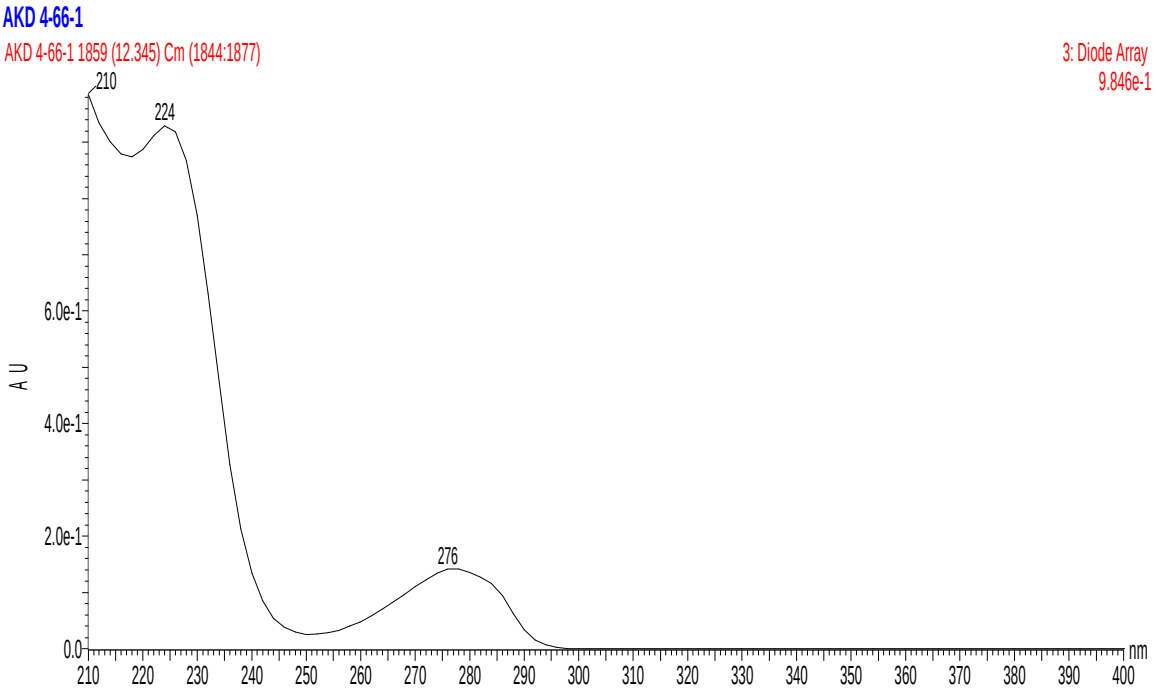


Figure 16. UV data for microginins (**1-5**)

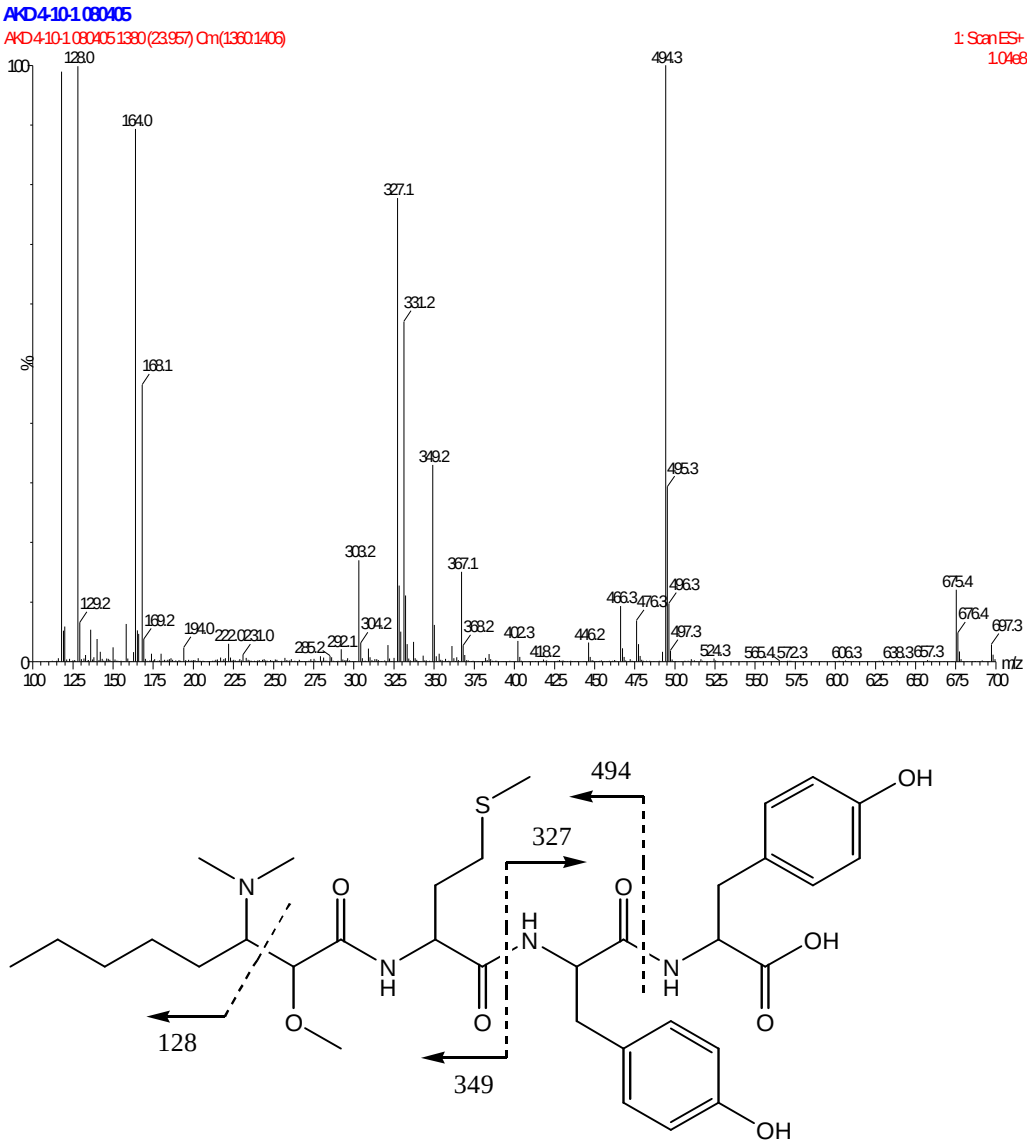


Figure 17. ESIMS data and proposed fragmentation of microginin 674 **(1)**

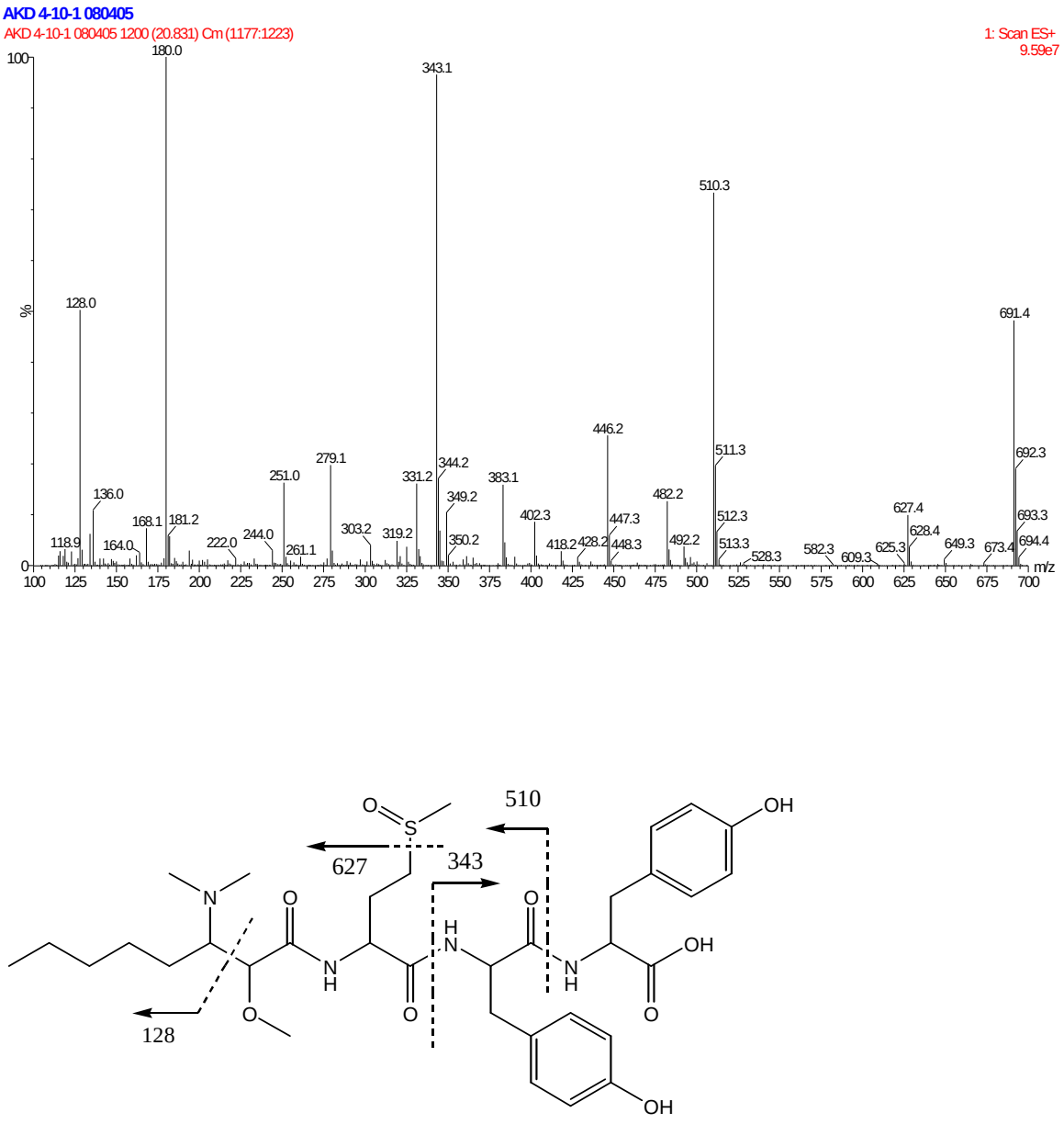
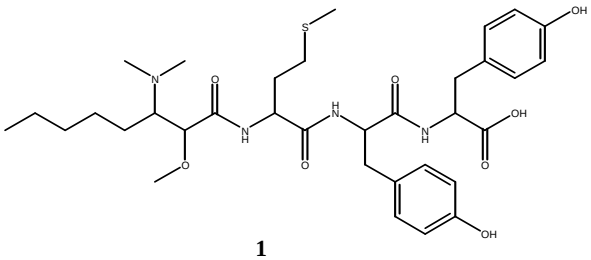
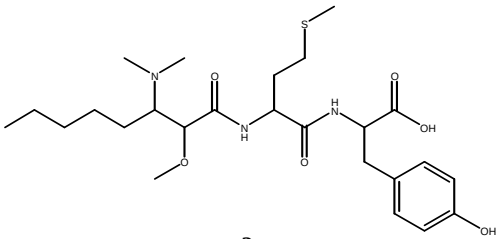


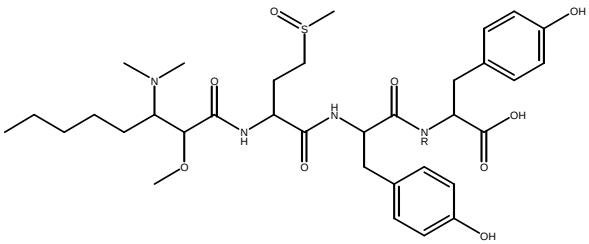
Figure 18. ESIMS data and proposed fragmentation of microginin 690 (**3**)



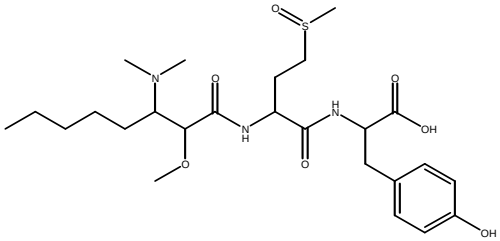
1



2



3 R=H**, 4** R=CH_3



5

Figure 19. Microginin peptides isolated from *Microcystis aeruginosa* (UTEX LB2385)

methionine. NMR analysis of compound **3** (m/z 691, $[M+H]^+$)(Table 6), showed the di-tyrosine C-terminus and N-terminus modified octanoic acid to be unchanged. The HSQC spectra of **3** revealed that the methyl group resonances attached to sulfur in methionine had moved downfield (^1H and ^{13}C resonances at δ 2.5 and δ 39, respectively). Furthermore, an intense fragment ion of $[M-64]^+$ in the ESI-MS of **3** was consistent with a loss of a CH_3SOH entity, previously not seen in the ESI-MS of **1** (Figure 18). It was therefore determined that **3** was the methionine sulfoxide derivative of **1**.

Compound **2** (m/z 511, $[M+H]^+$)was also isolated as a white powder. This compound had the same UV absorbance as compound **1** and shared the diagnostic fragment ion of m/z 128 but had a molecular weight of 511.27 Da, 163 Da less than 1. NMR studies confirmed the presence of the modified octanoic acid chain as well as the methionine of **1**, however **2** contained a single C-terminus tyrosine. Compound **5** (m/z 528, $[M+H]^+$), shared the UV absorbance of **2** and NMR shifts corresponding to the modified octanoic chain and a single tyrosine residue. HSQC correlations revealed the same downfield shift of the methionine resonances as seen in **3** compared to **1**. **5** was therefore determined to be the methionine sulfoxide derivative of **2.** Compound **4** (m/z 705, $[M+H]^+$) had a parent ion 14 Da more than compound **3**. NMR analysis revealed the same spectra of **3**, with the exception of a new ^1H shift at δ 2.55, consistent with N-methylation. ESIMS analysis of **4** showed similar fragmentation to **3** however the fragment ion at m/z 510, corresponding to the loss of the terminal tyrosine residue was seen in both spectra. **4** was determined to have the same structure of **3** with the exception of an N-methyl tyrosine at the C-terminus.

DISCUSSION

Isolation and purification of microcystins

Concern regarding the presence of microcystins in drinking water lakes and reservoirs has resulted in the need for reliable methods for their detection and accurate quantification. One of the main difficulties associated with the detection of these toxins is the lack of standardized reference material. Of the more than 70 variants of microcystins that have now been reported, only a few are commercially available and although many studies refer to commercially supplied microcystins as standards, none are actually sold as such and the amounts provided cannot be guaranteed (McElhiney and Lawton 2005). Purified microcystins are utilized in a range of research applications including toxicological and biochemical studies, development of detection systems and the investigation of water treatment strategies (Lawton and Edwards 2001).

In a typical microcystin purification process, cyanobacterial cells are extracted, the resultant extract concentrated and the microcystins purified by a range of sample separation techniques, though the number of steps and methods employed vary greatly. A recent study by Lawton and Edwards (2001) examined the optimal methods for purification of microcystins. Aqueous methanol (50-80%) was found to be most suitable for the extraction of microcystins, typically followed by reversed phase flash chromatography. Size exclusion chromatography, usually Sephadex LH-20, was also found to be useful in the isolation of microcystins for the removal of pigments and large interfering molecules. For HPLC chromatography, trifluoroacetic acid is often used as a modifier in the mobile phase. The addition of TFA not only maintains a low pH in order to protonate carboxylic acid groups in peptides, it also acts as an ion pairing agent, increasing the retention of poorly retained peptides. Analytes with minor structural differences often overlap in HPLC separations, especially desmethyl variants. Amide C_{16}

stationary phase was found to be optimal for the separation of desmethylated toxins from their non-desmethylated forms (Spoof et al 2001).

Microcystins are frequently isolated from both field collected and laboratory cultured cyanobacteria. Although field samples have the advantage of biomass availability with no initial investment of time required for the preparation of bulk cultures, these field samples are rarely composed only of cyanobacteria, let alone a single species, making it difficult to determine which organisms is responsible for metabolite production. In contrast, laboratory cultured cyanobacteria display consistent metabolic profiles and many are maintained in culture collections providing a reliable and renewable source. Additionally, extracts from laboratory harvested cells tend to be less complex, making purification of microcystins simpler (Lawton and Edwards 2001).

In the course of this work, the method developed for the isolation and purification of microcystin-LR has been automated and optimized. The first fractionation step, initially performed using a C_{18} reversed phase SepPak column, has been automated to involve the use of a CombiFlash companion. This automated solid phase extraction system decreases time and effort by the ability to detect compounds at specified wavelengths but also by automated mixing of mobile phases. Additionally in this method the preparative HPLC stage has been scaled up. The same stationary phase is utilized in this scaled up method, but a larger size column (19 x 250 mm) is used, allowing up to 1 mg of material to be loaded and separated at a time.

Microcystin-LR and its derivatives have been isolated from collected cells of more than 300 L of *M. aeruginosa* cultures. In the final stage of purification the microcystin-LR still contains traces of [Asp^3]microcystin-LR which can be separated using an amide C_{16} HPLC column. So far not all of this material has been purified by Amide C_{16} HPLC but to date a total

of 4.6 mg of microcystin-LR has been obtained and half of this (2.3 mg) was sent to the NCDHHS. More than 15 mg of microcystin-LR still containing the desmethyl variant are awaiting purification and an additional 9 mg of microcystin-LR is expected to be recovered from this.

Isolation and structure determination of microginin peptides

Of the more than 20 microginin congeners already described, the five new peptides described here are the only variants that contain a methionine residue. Additionally, these derivatives contain a unique N-terminus β-amino acid with a higher degree of methylation than any previously described congener. It was fortunate that the indicative fragment ion of m/z 128 was still a product of this unusual β-amino acid. Without this characteristic fragment, the identification of these metabolites as being microginin derivatives as well as subsequent structural determination would have been more difficult.

The existence of the methionine sulfoxide is most likely an artifact of the isolation process and not a product of biosynthesis. Initial analysis revealed one major peak (m/z 691) eluting before the microcystins, with the other microginin derivatives being produced in much lower concentrations. However, extracts that were analyzed more rapidly contained two major peaks eluting before the microcystins (m/z 675 and m/z 691) as well as the other microginin derivatives still present in low concentrations. As these compounds were found to be related by the existence of common fragment ions and UV absorbance, the most abundant derivative (m/z 691) was chosen for analysis of structural features. Structural determination of the methionine sulfoxide (m/z 691) microginin derivative was difficult due to the doubling and tripling of ^{1}H and ^{13}C NMR signals. This phenomenon was resolved when the other major peak (m/z 674), the non-sulfoxide congener, was analyzed by NMR. The NMR data for the non-sulfoxide

tetrapeptide revealed a much cleaner spectrum and a distinctive ^{1}H-^{13}C correlation that indicated the presence of a sulfur atom (δ 2.0, δ 16). Until the presence of a sulfur atom was considered, required double bond equivalence made identification of the unknown amino acid extremely difficult. The presence of fragment ions representing the loss of 64 Da, later determined to be due to a loss of CH_3SOH, as well as the doubling and tripling of NMR signals could not be explained until this methionine residue was identified. Other methionine sulfoxide containing metabolites from cyanobacteria have been reported and problems in structural determination due to the doubling and tripling of NMR signals were also described. Dendroamides are a family of cyclic hexapeptides isolated from the cyanobacterium *Stigonema dendroideum*. These cyanopeptides exhibit multidrug resistance reversing activity, making them of interest as treatments for multidrug resistant tumors. Dendroamide B contains a methionine residue and dendroamide C is the methionine sulfoxide form of dendroamide B. The ^{1}H and ^{13}C NMR signals of dendroamide C were found to be doubled, suggesting that the compound actually exists as a mixture of R and S sulfoxides (Ogino et al 1996). Symplostatin 2, a cytotoxic cyclic depsipeptide containing a methionine sulfoxide residue, was isolated from the marine cyanobacterium *Symploca hydnoides*. Once again ^{1}H and ^{13}C NMR signals were found to be tripled and this was assigned to the presence of a sulfone conformer in addition to the R and S sulfoxides (Harrigan et al. 1999). Almost all of the methionine sulfoxide signals of **3, 4** and **5** were doubled or tripled, suggesting that these derivatives exist as both R- and S- forms as well as the sulfone conformer. The sulfone conformer in these new microginin derivatives is indicated by the presence of a $[M+Na+16]^{+}$ fragment in the ESIMS. The ratios of the extra NMR signals reveal that the R and S sulfoxides exist in different concentrations, however until separation of these conformers is achieved, the dominant form can not be determined.

In addition to the unique methionine residue and high degree of methylation on the octanoic acid chain, this is the first report of tripeptide microginin derivatives. These smaller derivatives are produced in much lower quantities than the tetrapeptide methionine containing congeners, however continuous isolation and purification will accumulate sufficient amounts for activity testing.

Numerous microginin analogs have shown angiotensin converting enzyme (ACE) inhibition as well as aminopeptidase M (APM) inhibition as seen in Table 2. It is not clear how the presence of a methionine residue, the size of the tripeptide derivatives or the methylation of the N-terminus will affect activity. Structure activity relationships have shown that the dityrosine C-terminal structure is important for ACE inhibition. Additionally, tetrapeptide microginins were found to be more active than pentapeptide microginins and fragments thereof (Ishida et al. 2000). Although biological activity has not yet been determined in these microginin derivatives, both the proven track record of these peptides for vasodilative properties as well as structure activity relationships make bioactivity of these new congeners probable. ACE inhibitors are one of the most effective treatments for hypertension and congestive heart failure. Additionally, APM inhibitory activity makes the vasodilatory activity of these peptides two-fold. Consequently, compounds exhibiting dual APM/ACE inhibitory activity, like microginins, might prove invaluable as novel vasodilative therapies (Ishida et al. 2000).

CONCLUSION

Microcystis aeruginosa (UTEX LB2385) was mass cultured and analyzed for the presence of toxic peptides. During the isolation and purification of the heptapeptide hepatotoxin microcystin-LR, other unrelated peptide metabolites were found and characterized. These new peptide metabolites were determined to be tri- and tetrapeptide microginin congeners with unique structural features including a methionine residue and a highly methylated N-terminus β-amino acid. Cyanobacteria are routinely analyzed for the production of toxins but are rarely studied for the production of new and interesting metabolites, however increased concern regarding the presence of producing species in drinking water lakes and reservoirs has prompted new studies of cyanobacterial metabolites resulting in the isolation of new and interesting bioactive metabolites, particularly peptide based compounds with enzyme inhibitory activity. Cyanobacteria are now recognized as an important renewable source of pharmaceuticals.

REFERENCES

Agrawal, M.K.; Zitt, A.; Bagchi, D.; Weckesser, J.; Bagchi, S.; von Elert, E. (2005) Characterization of proteases in guts of *Daphnia magna* and their inhibition by *Microcystis aerugionsa* PCC 7806. *Environmental Toxicology,* 20, 314-322

Barrett, J. (2005) Can biotech deliver new antibiotics? *Current Opinion in Microbiology,* 8, 498-503

Burkholder, P.R.; Pfister, R.M.; Leitz, F.H. (1966) Production of a pyrrole antibiotic by a marine bacterium. *Applied Microbiology,* 14, 649-653

Carmichael, Wayne W. (1981) Freshwater blue-green algae (cyanobacteria) toxins--a review. *Environmental Science Research,* 20, 1-13

Carmichael, Wayne W. (1989) Freshwater cyanobacteria (blue-green algae) toxins. *Proceedings of the 9th World Congress on Animal, Plant and Microbial Toxins*, 3-16

Carmichael, W.W. (1992) Cyanobacteria secondary metabolites--the cyanotoxins. *Journal of Applied Bacteriology,* 72, 445-459

Chorus, Ingrid. (2000) Health risks caused by freshwater cyanobacteria in recreational waters. *Journal of Toxicology and Environmental Health, Part B,* 3, 323-347

Davidson, B.S. (1995) New dimensions in natural products research: cultured marine microorganisms. *Current Opinions in Biotechnology*, 6, 284-291

Demain, A. L. (2000a) Microbial biotechnology. *Trends in Biotechnology*, 18, 26-31

Demain, A.L. (2000b) Small bugs, big business: the economic power of the microbe. *Biotechnology Advances*, 18, 499-514

Feling, R.H.; Buchanan, G.O.; Mincer, T.J.; Kauffman, C.A.; Jensen, P.R.; Fenical, W. (2003) Salinosporamide A: a highly cytotoxic proteasome inhibitor from a novel microbial source, a marine bacterium of the new genus, *Salinopora*. *Angewadte Chemie International Edition,* 42, 355-357

Fenical, W. (1993) Chemical studies of marine bacteria: developing a new resource. *Chemical Reviews*, 93, 1673-1683

Fenical, W. and Jensen, P. R. (1993) Marine Microorganisms: A New Biomedical Resource in Marine Biotechnology, Volume I: Pharmaceutical and Bioactive Natural products, eds. David H. Attaway and Oskar R. Zaborsky. Plenum Press, New York

Haefner, B. (2003) Drugs from the deep: marine natural products as drug candidates. *Drug Discovery Trends*, 8, 536-544

Harada, K.; Matsura, K.; Suzuki, M.; Watanabe, M.; Oishi, S.; Dahlem, A.; Beasley, V.R.; Carmichael, W.W. (1990) Isolation and characterization of the minor components associated with microcystin LR and RR in the cyanobacterium (blue-green algae). *Toxicon*, 28, 55-64

Harrigan, George, G.; Luesch, Hendrik; Yoshida, Wesley Y.; Moore, Richard E.; Nagle, Dale G.; Paul, Valerie J. (1999) Symplostatin 2: a dolastatin 13 analogue from the marine cyanobacterium *Symploca hydnoides*. *Journal of Natural Products*, 62, 655-658

Humpage, Andrew R.; Hardy, Stephen J.; Moore, Emma J.; Froscio, Suzanne M.; Falconer, Ian R. (2000) Microcystins (cyanobacterial toxins) in drinking water enhance the growth of aberrant crypt foci in the mouse colon. *Journal of Toxicology and Environmental Health, Part A*, 61, 155-165

Ireland, C.M.; Copp, B.R.; Foster, M.P.; McDonald, L.A.; Radisky, D.C.; Swersey, J.C. (1993) Biomedical Potential of Marine Natural Products in Marine Biotechnology, Volume I: Pharmaceutical and Bioactive Natural products, eds. David H. Attaway and Oskar R. Zaborsky. Plenum Press, New York.

Ishida, Keishi; Matsuda, Hisashi; Murakami, Masahiro; Yamaguchi, Katsumi. (1997) Microginins 299-A and –B, leucine aminopeptidase inhibitors from the cyanobacterium *Microcystis aeruginosa* (NIES-299). *Tetrahedron*, 53, 10281-10288

Ishida, Keishi; Matsuda, Hisashi; Murakami, Masahiro. (1998) Four new microginins, linear peptides from the cyanobacterium *Microcystis aeruginosa*. *Tetrahedron*, 54, 13475-13484

Ishida, Keishi; Kato, Taku; Murakami, Masahiro; Watanabe, Masayuki; Watanabe, Mariyo. (2000) Microginins, zinc metalloprotease inhibitors from the cyanobacterium *Microcystis aeruginosa. Tetrahedron*, 56, 8643-8656

Jensen, P.R.; Dwight, R.; Fenical, W. (1991) Distribution of Actinomycetes in near shore tropical marine sediments. *Applied and Environmental Microbiology*, 57, 1102-1108

Jensen, P.R.; Fenical, W. (1994) Strategies for the discovery of secondary metabolites from marine bacteria: ecological perspectives. *Annual Reviews in Microbiology*, 48, 559-584

Jensen, P.R.; Mincer, T.J.; Fenical, W. (2003) The true potential of the marine microorganism. *Current Drug Discovery*, 17-19

Kodani, Shinya; Suzuki, Shingo; Ishida, Keishi; Murakami, Masahiro. (1999) Five new cyanobacterial peptides from water bloom materials of lake Teganuma (Japan). *FEMS Microbiology Letters*, 178, 343-348

Kubwabo, Cariton; Vais, Natalia; Benoit, Frank M. (2005) Characterizations of microcystins using in-source collision induced dissociation. *Rapid Communication in Mass Spectrometry*, 19, 597-604

Kwon, H.C.; Kauffman, C.A.; Jensen, P.R.; Fenical, W. (2006) Marinomycins A-D, antitumor-antibiotics of a new structure class from a marine actinomycete of the recently discovered genus "*Marinispora*." *Journal of the American Chemical Society,* 128, 1622-1632

Lawton, Linda A.; Codd, G.A. (1991) Cyanobacterial (blue-green algal) toxins and their significance in UK and European waters. *Journal of the Institution of Water and Environmental Management*, 5, 460-465

Lawton, Linda A.; Edwards, Christine. (2001) Purification of microcystins. *Journal of Chromatography A*, 912, 191-209

McElhiney, Jacqui; Lawton, Linda A. (2005) Detection of the cyanobacterial hepatotoxins microcystins. *Toxicology and Applied Pharmacology,* 203, 219-230

Mincer, T.J.; Jensen, P.R.; Kaufmann, C.A.; Fenical, W. (2002) Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments. *Applied and Environmental Microbiology*, 68, 5005-5011

Nakamura, H.; Iitaka, Y.; Kitahara, T.; Okazaki, T.; Okami, Y. (1977) Structure of asplasmomycin. *Journal of Antibiotics*, 30, 714-719

Namikoshi, M.; Rinehart, K.L. (1996) Bioactive compounds produced by cyanobacteria. *Journal of Industrial Microbiology*, 17, 373-384

Neumann, U.; Forchert, A.; Flury, T.; Weckesser, J. (1997) Microginin FR1, a linear peptide from a water bloom of *Microcystis* sp. *FEMS Microbiology Letters*, 153, 475-478

Ogino, Junishi; Moore, Richard E.; Patterson, Gregory M.L.; Smith, Charles D. (1996) Dendroamides, new cyclic hexapeptides from a blue-green alga. *Journal of Natural Products,* 59, 581-586

Okami, Y.; Hotta, K.; Yoshida, M.; Ikeda, D.; Knodo, S.; Umezawa, H. (1979) New aminoglycoside antibiotics, istamycins A and B. *Journal of Antibiotics*, 32, 964-966

Okami, Y.; Hotta, K. (1988) Search and discoveryof new antibiotics in Actinomcyetes in Biotechnology, eds. Goodfellow, M; Williams, S.T., Mordarski, M. Academic Press, San Diego

Okino, T.; Matsuda, H.; Murakami, M.; Yamaguchi, K. (1993) Microginin, an angiotensin-converting enzyme inhibitor from the blue-green alga *Microcystis aeruginosa. Tetrahedron Letters*, 34, 501-504

Paerl, Hans W. (1988) Nuisance phytoplankton blooms in coastal, estuarine, and inland waters. *Limnology and Oceanogrpahy*, 33: 823-847

Pathirana, C.; Jensen, P.R.; Fenical, W. (1992) Marinone and debromomarinone, antibiotic sesquiterpenoid napthoquinone of a new structural class from marine bacterium. *Tetrahdron Letters*, 33, 7663-7666

Pauli, G.F.; Case, R.J.; Inui, T.; Wang, Y.; Cho, S.; Fischer, N.H.; Franzblau, S.G. (2005) new perspectives on natural products in TB drug research. *Life Sciences*, 78, 485-494

Payne, D.; Tomasz, A. (2004) Antimicrobials: The challenge of antibiotic resistant pathogens: the medical need, the market and prospects for new antimicrobial agents. *Current Opinion in Microbiology*, 7, 435-438

Piret, J.M.; Demain, A.L. (1988) Actinomycetes in Biotechnology: an overview in Actinomcyetes in Biotechnology, eds. Goodfellow, M; Williams, S.T., Mordarski, M. Academic Press, San Diego

Pluotno, Alexei; Carmeli, Shmuel. (2002) Modified peptides from a water bloom of the cyanobacterium *Nostoc* sp. *Tetrahedron*, 58, 9949-9957

Reyrat, J.M.; Kahn, D. (2001) *Mycobacterium smegmatis*: an absurd model for tuberculosis? *Trends in Microbiology*, 9, 472-473

Reshef, Vered; Carmeli, Shmuel. (2001) Protease inhibitors from a water bloom of the cyanobacterium *Microcystis aeruginosa*. *Tetrahedron*, 57, 2885-2894

Reynolds, C.S.; Walsby, A.E. (1975) Water-Blooms. *Biological Reviews*, 50, 437-481

Sano, Tomoharu; Kaya, Kunimitsu. (1997) A 3-amino-10-chloro-2-hydoxydecanoic acid containing tetrapeptide from *Oscillatoria agardhii*. *Phytochemistry*, 44, 1503-1505

Spoof, Lisa; Karlsson, Krister; Meriluoto, Jussi. (2001) High-performance liquid chromatographic separation of microcystins and nodularin, cyanobacterial peptide toxins, on C_{18} and amide C_{16} sorbents. *Journal of Chromatography A*, 909, 225-236
Carmichael, Wayne W. (1989) Freshwater cyanobacteria (blue-green algae) toxins. *Proceedings of the 9th World Congress on Animal, Plant and Microbial Toxins*, 3-16

Takahashi, A.; Ikeda, D.; Nakamura, H.; Naganawa, H.; Kurasawa, S.; Okami, Y.; Takeuchi, T. (1989) Altemicidin, a new acaricidal and antitumor substance. *Journal of Antibiotics*, 11, 1562-1566

Waksman, S.A. and H.B. Woodruff. 1940. Bacteriostatic and bacteriocidal substances produced by soil actinomycetes. *Proc. Soc. Exp. Biol. Med*. 45: 609-614

Welker, Martin; Christiansen, Guntram; von Dohren, Hans. (2004a) Diversity of coexisting *Planktothrix* (cyanobacteria) chemotypes deduced by mass spectral analysis of microcystins and other oligopeptides. *Arch Microbiology*, 182, 288-298

Welker, Martin; Brunke, Matthias; Preussel, Karine; Lippert, Indra; von Dohren, Hans. (2004b) Diversity and distribution of *Microcystis* (cyanobacteria) oligopeptide chemotypes from natural communities studied by single-colony mass spectrometry. *Diversity of oligopeptide chemotypes,* 150, 1785-1796

Weyland, H. (1981) Characteristics of actinomycetes isolated from marine sediments in Actinomycetes, eds. Schaal and Pulverer, Gustav Fischer Verlag, New York

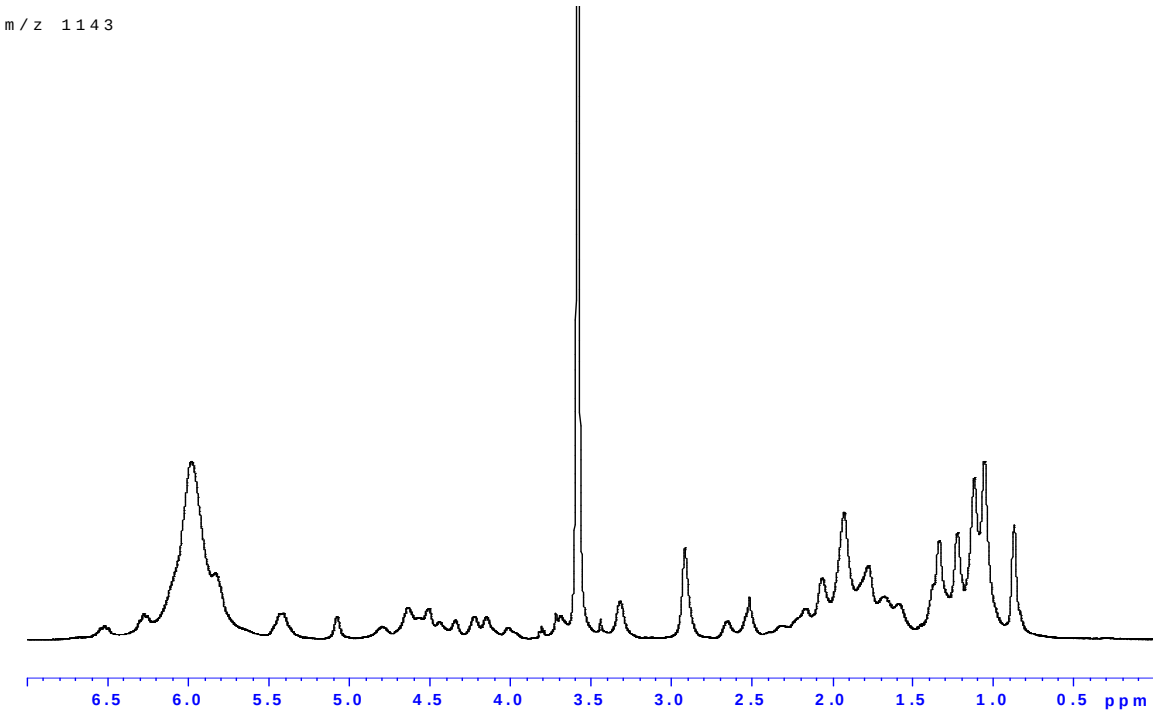
Zheng, Z.; Zeng, W.; Huang, Y.; Yang, Z.; Li, J.; Cai, H.; Su, W. (2000) Detection of antitumor and antimicrobial activities in marine organism associated actinomycetes isolated from Taiwan Strait, China. *FEMS Microbiology Letters*, 188, 87-91

ZoBell, C.E. (1936) Bactericidal Action of Seawater. *Proceedings of the Society for Experimental Biology and Medicine*, 34, 113-116

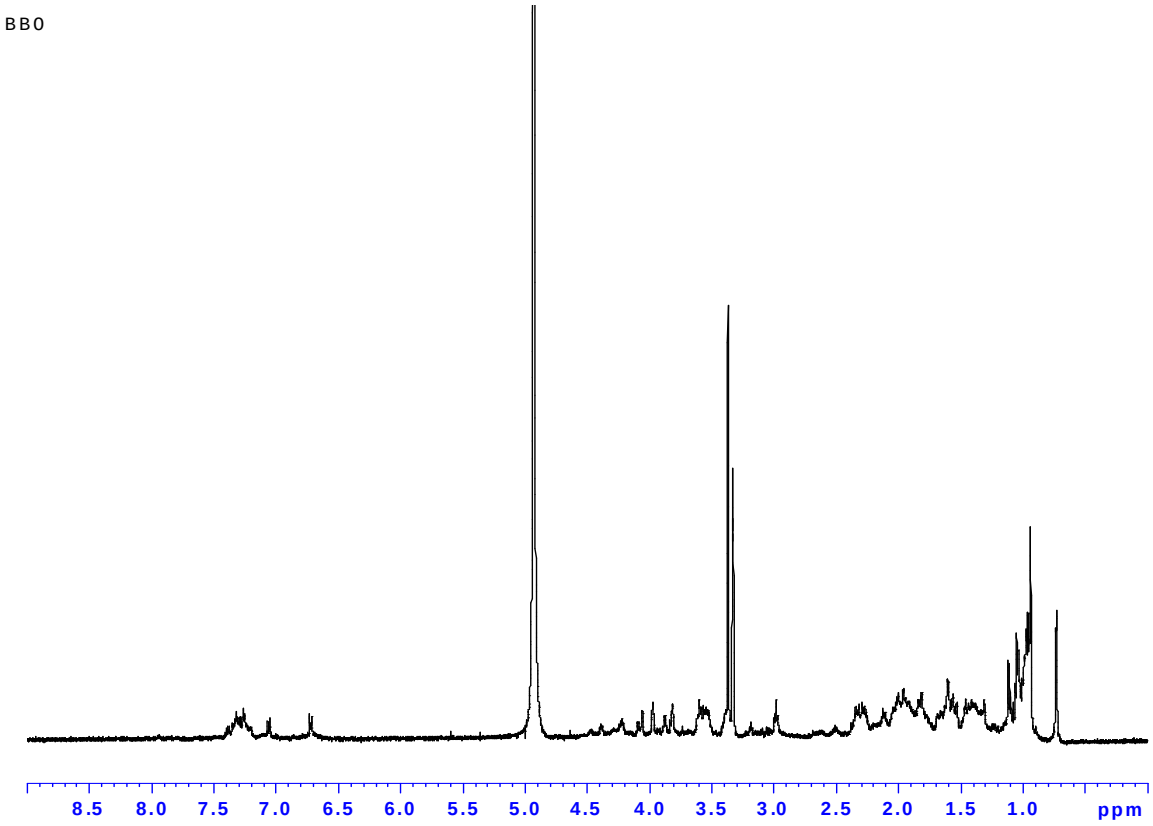
Zurawell, Ronald W.; Chen, Huirong; Burke, Janice M.; Prepas, Ellie E. (2005) Hepatotoxic cyanobacteria: A review of the biological importance of microcystins in freshwater environments. *Journal of Toxicology and Environmental Health, Part B*, 8, 1-37

APPENDIX

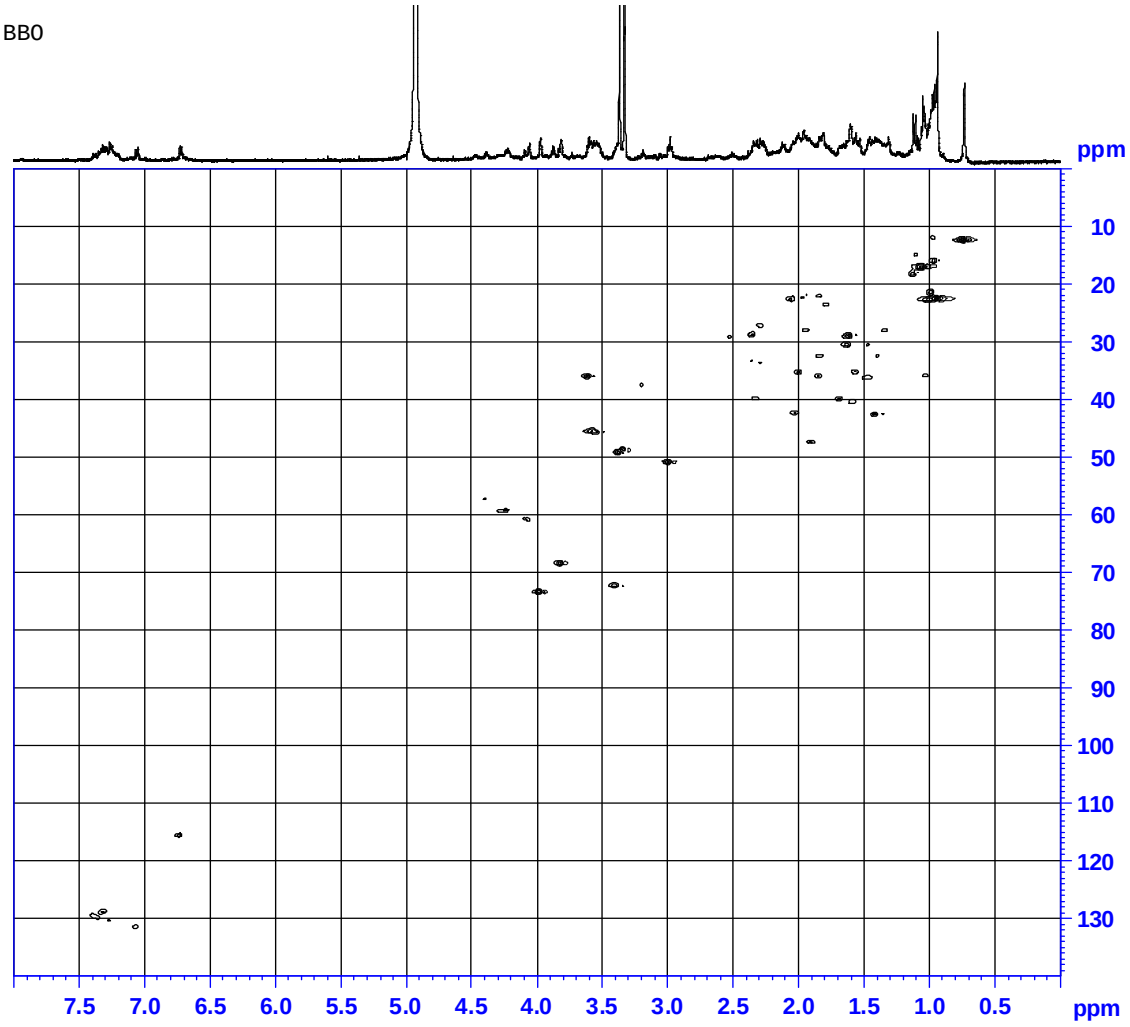
Appendix A. ^{1}H NMR data for I4a (**9**) (d_5-pyridine; 1 mm TXI probe)



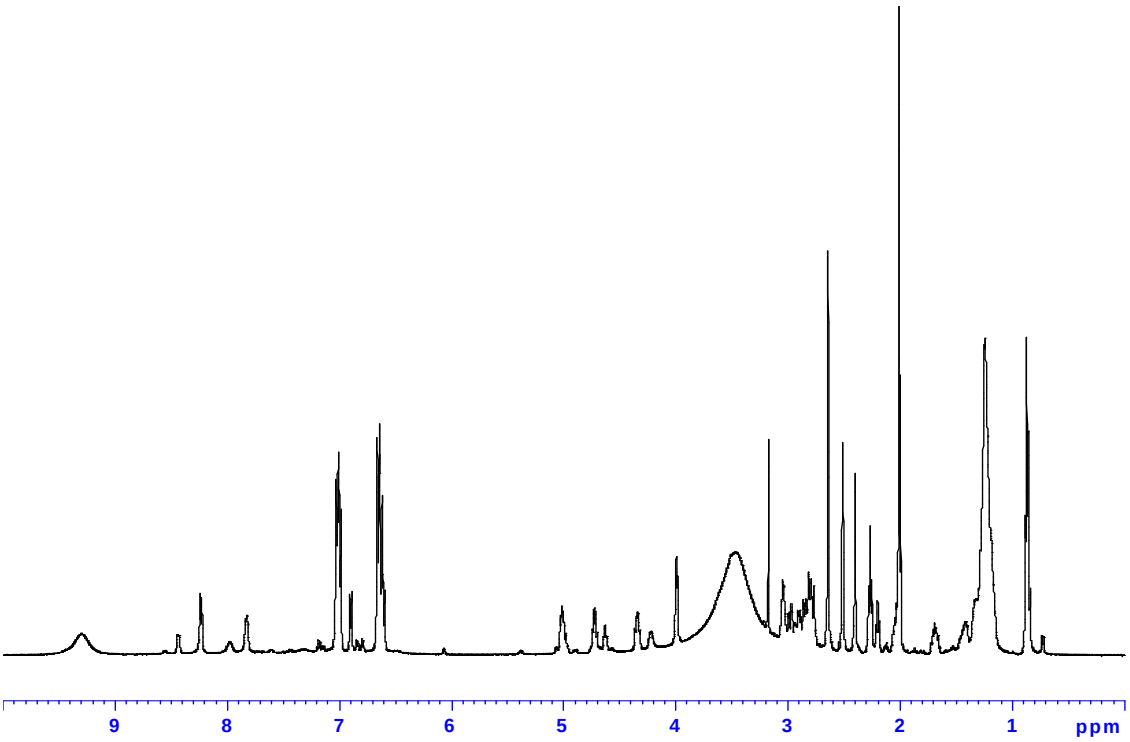
Appendix B. ^{1}H NMR data for I29 active compounds (**11-13**) (d_4-methanol; 5 mm BBO probe)



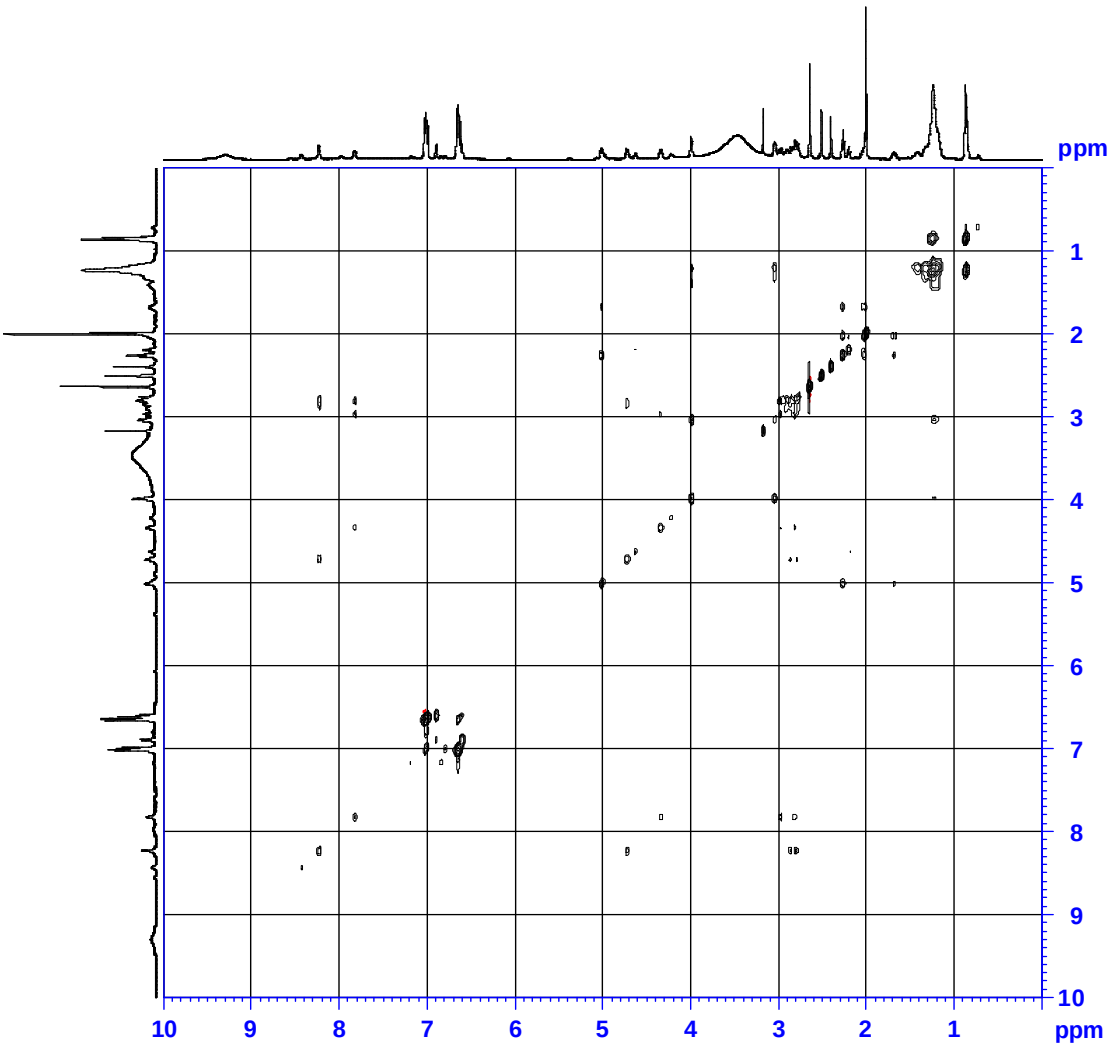
Appendix C. HSQC NMR data for I29 active compounds (**11-13**) (d_4-methanol; 5 mm BBO probe)



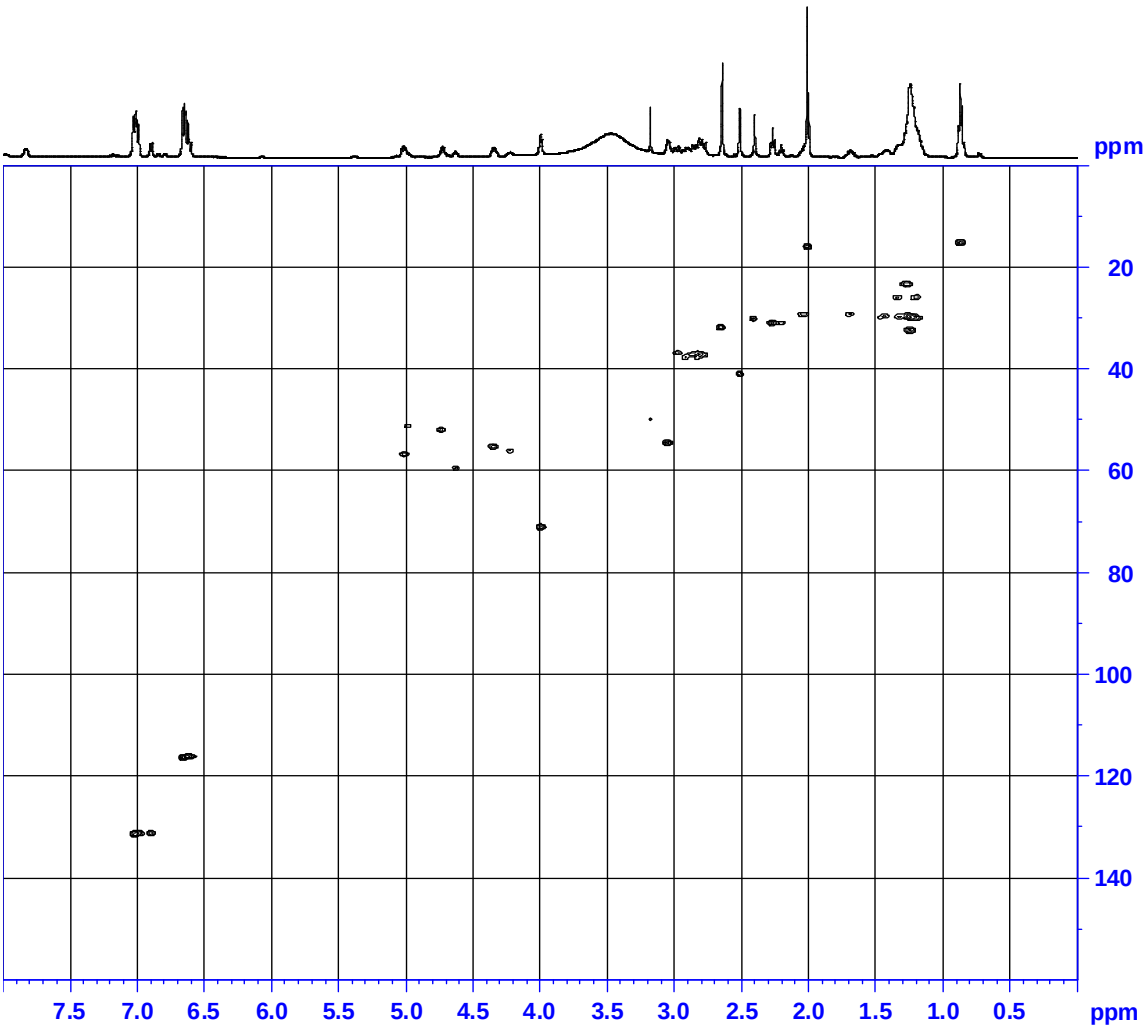
Appendix D. ^{1}H NMR data for microginin 674 (**1**) (d_6-DMSO; 1 mm TXI probe)



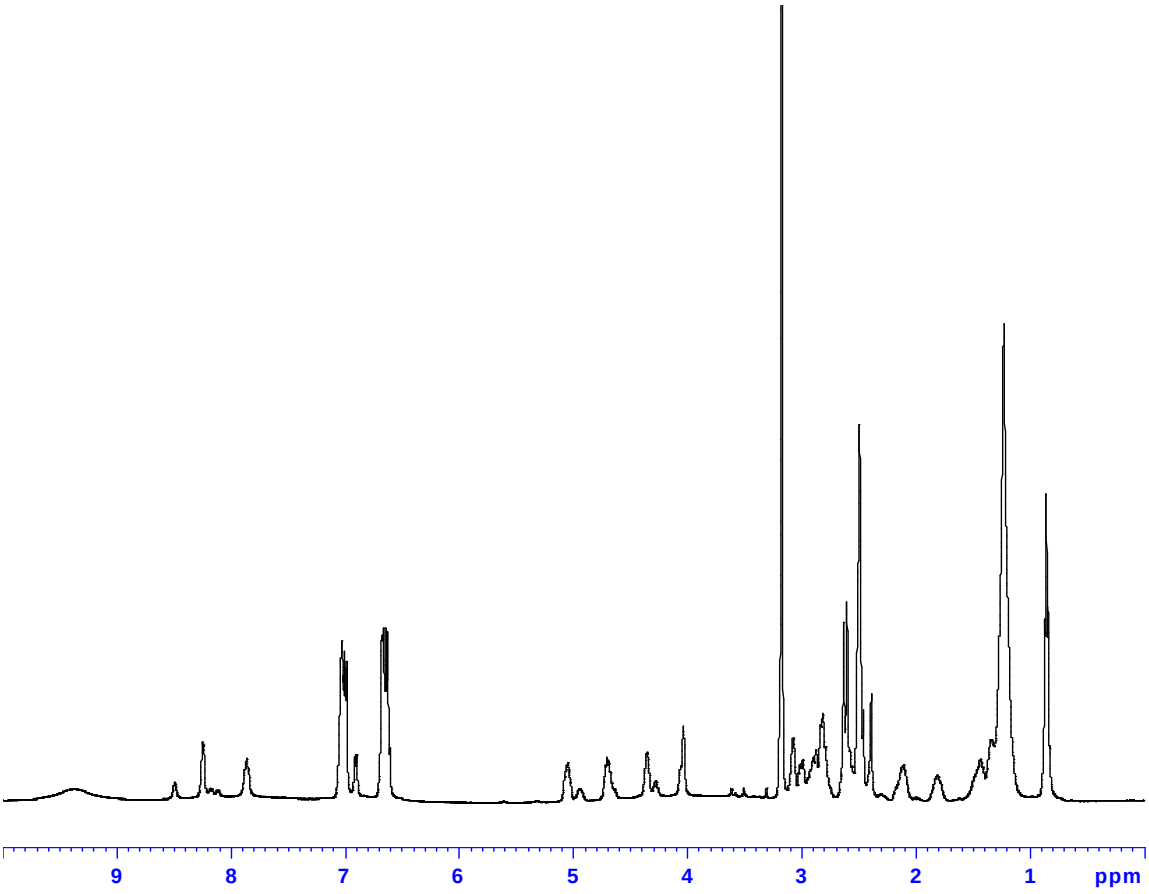
Appendix E. TOCSY NMR data for microginin 674 (**3**) (d_6-DMSO; 1 mm TXI probe)



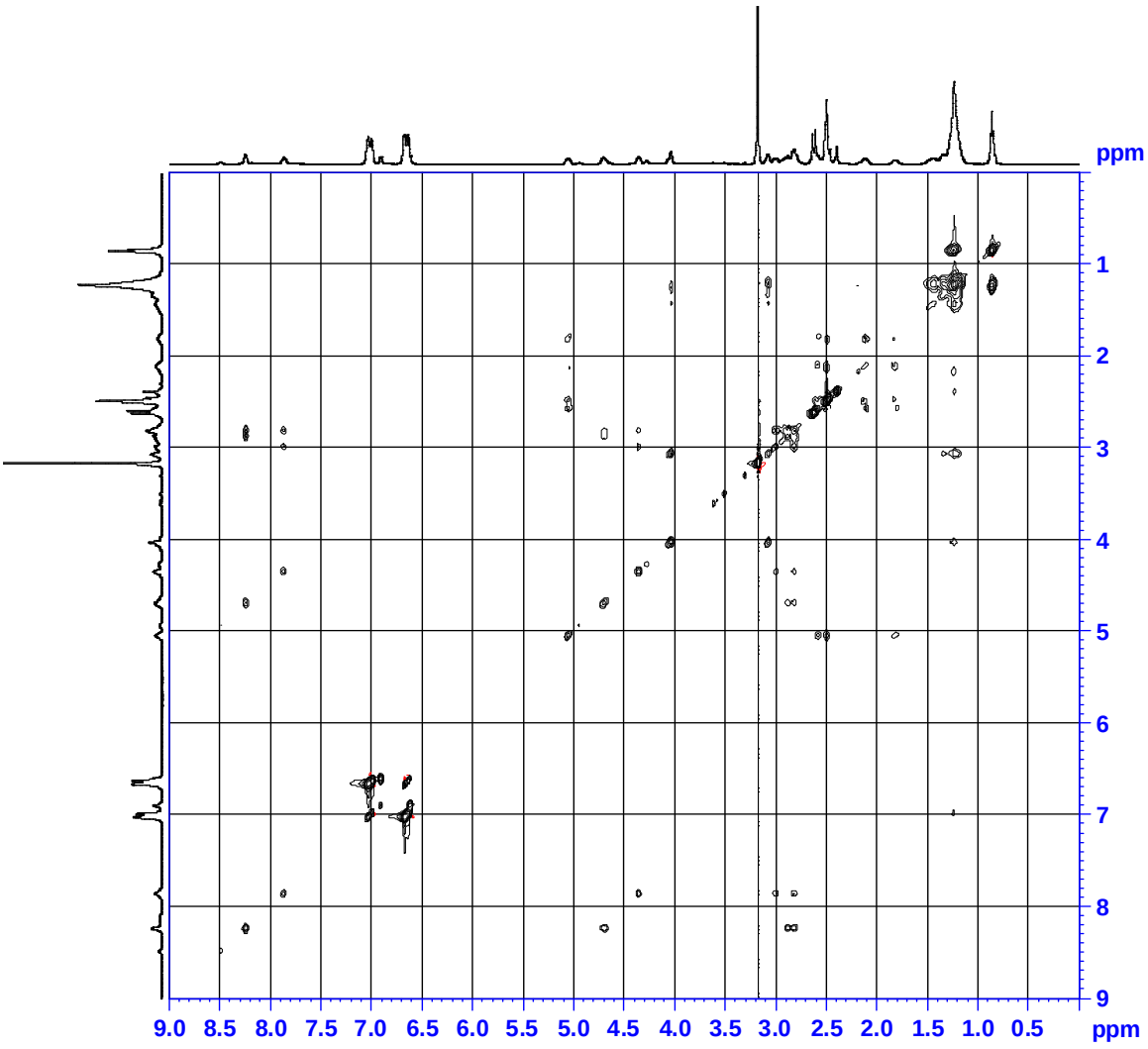
Appendix F. HSQC NMR data for microginin 674 (**1**) (d_6-DMSO; 1 mm TXI probe)



Appendix G. ^{1}H NMR data for microginin 690 (**3**) (d_6-DMSO; 1 mm TXI probe)



Appendix H. TOCSY NMR data for microginin 690 (**3**) (d_6-DMSO; 1 mm TXI probe)



Appendix I. HSQC NMR data for microginin 690 (**3**) (d_6-DMSO; 1 mm TXI probe)