Biologically Active Secondary Metabolites from Red Sea Cyanobacteria

by

Ali A. Alhadab

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in General Science (Honors Associate)

Presented June 1th, 2010

Commencement June 2010

AN ABSTRACT OF THE THESIS OF

Ali A. Alhadab for the degree of Honors Baccalaureate of Science in General Science presented on June 1th, 2010.

Title: Biologically Active Secondary Metabolites from Red Sea Cyanobacteria.

Abstract approved: _____
Kerry L. McPhail

This project describes the investigation of marine cyanobacteria collected from the Red Sea with an emphasis on the identification and prioritization of biologically active fractions for future purification and molecular structure elucidation of medicinally relevant secondary metabolites.

Five crude extracts of taxonomically unidentified Red Sea cyanobacteria supplied by Egyptian collaborators were fractionated by chromatographic means and screened for anti-cancer activities using whole cell biological assays. Only three extracts yielded collectively five cytotoxic active fractions containing chemical compounds with different molecular weights. Further spectrometric and spectroscopic analysis showed that fraction Enq02F-2 contained a compound that had a similar molecular weight, UV absorption properties, and polarity to another bioactive compound isolated previously from a different cyanobacterial crude extract by graduate student Chris Thornburg. This compound is a cyclic depsipeptide closely related to other cyanobacterial natural products derived from Caribbean species of *Lyngbya* cyanobacteria.

Key Words: natural products, secondary metabolites, cyanobacteria, cancer therapeutic agents.

Corresponding e-mail address: Ali.Alhadab@gmail.com

Biologically Active Secondary Metabolites from Red Sea Cyanobacteria

by

Ali A. Alhadab

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in General Science (Honors Associate)

Presented June 1th, 2010

Commencement June 2010

Honors Baccalaureate of Science in General Science project of Ali A. Alhadab presented on June 1th, 2010.

APPROVED:

Kerry L. McPhail

Mentor, representing Pharmacy

Mark Zabriskie

Committee member, representing Pharmacy

Christopher Thornburg

Committee member representing Pharmacy

Dean, University Honors College

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

Ali A. Alhadab, Author

ACKNOWLEDGMENTS

First, I would like to thank Dr. Kerry McPhail, who has been a tremendous mentor throughout my project. Thank you for the opportunity to be a part of this experience, your guidance has been a pleasure. I greatly appreciated your patience and constructive feedback with this work, without you this project would not have been possible. Thank you for also being a great friend.

Secondly, I would like to give praise to Chris Thornburg and Justyna Sikorska who have helped me when Dr. McPhail wasn't there. Your help greatly contributed to this project as well. Thanks for answering all those questions I had.

Thirdly, I would like to thank Rebecca Chávez who provided a large amount of encouragement. Your time and effort in assisting to edit this project is greatly appreciated. Thank you for staying by my side and staying positive. Your positivity gave me energy to finish this thesis! *¡Muchas Gracias!*

Lastly, I would like to thank my family who all has given me great support despite the distance. Their constant phone calls, especially from my mom and oldest brother, gave me the confidence to continue. A special appreciation to my father, who gave me balance by checking in and making sure I kept working hard. Your wise words inspired me to seek perfection.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Words	**Abbreviation**
Oregon State University	OSU
Normal Phase Vacuum Liquid Chromatography	NP-VLC
Hexane	hex
Ethyl Acetate	EtOAc
Methanol	MeOH
Dichloromethane	DCM
Methyl group	Me
Vacuum Liquid Chromatography	VLC
Ethanol	EtOH
Water	H_2O
Reversed Phase Solid Phase Extraction	RP-SPE
High Pressure/Performance Liquid Chromatography	HPLC
Molecular Weight	MW
Ultraviolet	UV
Nuclear Magnetic Resonance	NMR
Ecteinascidin 743	ET-743
Histone deacetylase	HDAC

INTRODUCTION

Human Health

Human life expectancy and quality of life are profoundly influenced by cancer and infectious diseases that are caused by pathogenic microbes. Both have been documented throughout the history of humankind. Before the 20th century, most deaths were attributable to infectious diseases such as pneumonia, malaria, plague, and tuberculosis, limiting the human lifespan to 50 years, while only a small percentage of deaths was known to be due to cancer.[1] Infectious diseases result from the presence of pathogenic agents. They can be viruses, bacteria, fungi, parasite or even aberrant proteins known as prions. Such diseases are prevalent in developing countries of tropic or subtropic regions, in which conditions are favorable for pathogens and health care access is limited.

Since the beginning of the 20th century, cancer has become more prevalent. According to the World Health Organization, it is one of the leading causes of death worldwide.[2] Cancer is an umbrella term commonly used to describe a group of diseases that are characterized by unregulated cellular growth that leads to tumor formation. In advanced stages, the abnormal proliferation of cells spreading throughout the body results in death by interfering with the function of normal organ tissues. Cancer can develop in almost any tissue or organ. It can be caused by both external factors (such as tobacco, infectious organisms, chemicals, and radiation) and internal factors (including inherited mutations, hormones, and immune conditions).[3]

The incidence of cancer affects all age groups. It is less common at early ages, but the incidence rises dramatically with age due to the accumulation of risks (mutations or exposure to carcinogens) for specific cancers. More than three quarters of all cancers are diagnosed in

people 55 years or older.[4] Different types of cancers are basically designated according to the tissue or organ in which they originate. For example, when a tumor first develops in the lungs, it is called lung cancer, stomach cancer when the primary tumor develops in the stomach and so on.

About 13% of all deaths worldwide are due to cancer, which kills approximately 7.4 million people annually. This number is still increasing each year and it is expected to reach 12 million deaths by 2030.[2] The incidence of different cancer types differ between men and women, although lung cancer is associated with the highest mortality in both. Prostate and colorectal cancer are most common among American males, while among American females, breast and colorectal cancer are prevalent.[4] In addition, people in developing nations are more likely to develop types of cancers that are associated with infections, such as hepatitis B virus (liver cancer), human papilloma viruses (cervical cancer) and *Helicobacter pylori* (stomach cancer). However, cancers remain more prevalent in developed western countries due to lifestyle of the populations.[2,5] As one of the developed nations, United States of America has collectively spent about a quarter of a trillion dollars on cancer research since 1971.[6] Despite this substantial investment, only 5% decrease in the cancer death rate was observed between the period of 1950 and 2005.[7] The need to find effective treatments for cancer drives research to fight against this deadly disease.[8]

Natural Products

The term natural product is used to describe chemicals produced by living organisms that produce biological effects on other organisms. Natural products neither play a central role in metabolism and cellular production nor comprise cellular structures critical to the primary metabolism of the organisms. Instead, they may be interchangeably referred to as

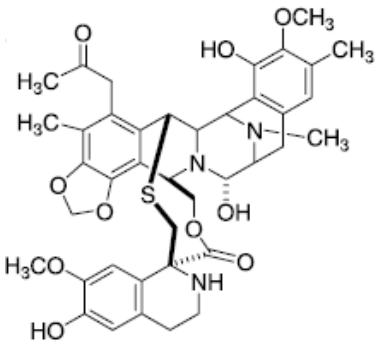
secondary metabolites, and unlike primary metabolites and polymeric macromolecules, are characteristic of only a limited range of species.[9] They were first thought to be waste products but further research has shown they are likely produced for defense, communication and predation purposes.[10] Secondary metabolites have attracted the attention of natural product chemists because of their biological effects on other organisms.[11] For the same reason, many are used to treat and prevent human diseases and have played a role in increasing the average human lifespan from 40 years in the early 20th century to 77 years today.[12]

These natural products have been the main source of new chemical entities for the past two decades and have significantly impacted the drug discovery process and drug design.[13] About half of all new, bioactive chemicals introduced as drugs between the period of 1981 and 2006 are either natural products (5.7%), modified natural products (27.6%), or synthetic products that contain a natural product pharmacophore (17.1%).[14] Historically, terrestrial plant-derived natural products have played a vital role in medicine. A record of 1,000 plant-derived substances in Mesopotamia dated around 2600 BCE documents the uses of these substances in the treatment of coughs, cold, inflammation and parasitic infections, many of which are still used today.[15] The investigation of terrestrial plants has been extremely fruitful and led to the discovery of medicinally useful compounds such as aspirin and taxol.[16]

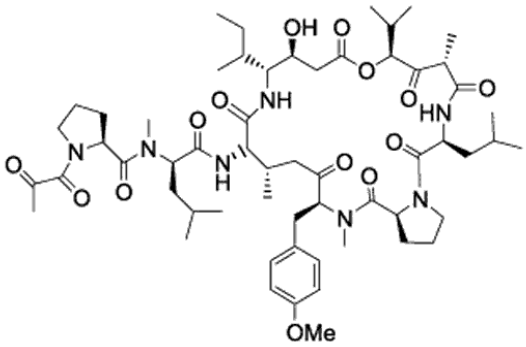
By the 1960s, the attention of natural products chemists shifted to explore the oceans, which cover more than 70% of the Earth's surface, and represented a new untouched source of potential bioactive compounds.[16] As expected, access to marine environments dramatically increased the number of natural products in clinical trials over the past decade.[16] This is due

to the vast chemical and biological diversity of marine environments created by intense competition among species that coexist in limited habitats. In highly competitive environments, species tend to evolve chemical means to defend themselves against predation or to kill their prey.[18] This involves the synthesis of secondary metabolites that differ in their chemical structures from one taxonomic group to another are used in either a defense or attack mechanism.[19]

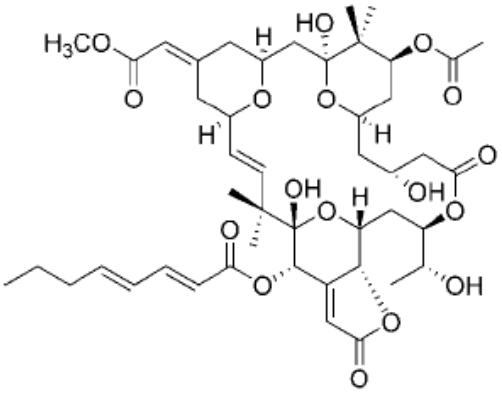
A Newman and Cragg review shows that a large number of drugs, either isolated from marine sources or synthesized because of knowledge gained from marine natural products, are currently in pre-clinical and clinical trials. Not only do these products exhibit anti-cancer potential, but they may also be used in the treatment of a variety of other diseases.[20] No marine natural products have progressed yet to clinical trials for infectious diseases although many show good activity in antimicrobial assays.[21] Examples of current drugs in cancer clinical trials are Ecteinascidin 743 (**1**), Dehydrodidemnin B (**2**) and Bryostatin 1 (**3**) that are all in Phase II trials in the US (Figure 1).[22]



Ecteinascidin 743 **(1)**



Dehydrodidemnin B **(2)**



Bryostatin 1 **(3)**

Figure 1: Molecular structures of some anti cancer drugs

Ecteinascidin[22] 743 (ET-743, Yondelis®) was isolated from the Caribbean tunicate *Ecteinascidia turbinata* and is classified as an alkaloid. It was first reported by Sigel and his colleagues in 1969, yet its structure was not identified until 1990 by Rinehart's team.[23] In 2008, ET-743 was approved in Europe, Russia, and South Korea for the treatment of soft tissue cancer, and is in clinical trials to treat other types of cancer.[24]

Dehydrodidemnin B (Aplidine®) is a cyclic depsipeptide related to didemnin B that is extracted from the Caribbean tunicate *Tridemnum solidum.*[25] Didemnin B was the first marine natural product to enter clinical trials, but was discontinued due to toxicity in phase II trials.[22,26] Its analogue aplidine, isolated from the Mediterranean ascidian *Aplidium albicans,* also showed a broad spectrum of antitumor activities.[27] In 1999, aplidine entered clinical

trials in many European countries to treat solid tumors and Hodgkin's lymphoma, and now it is undergoing studies in combination with other antitumor agents as the basis for possible phase III trials.[20,27] Aplidine acts as an inhibitor of angiogenesis that disrupts blood supply to tumors, and causes apoptosis. It is now obtained by chemical synthesis after the FDA approved the proposal made by PharmMar for the production process of drug.[27]

Another marine-derived anti-cancer agent is the macrocyclic lactone Bryostatin 1, which was isolated by Pettit's team from a collection of the bryozoan *Bugula neretina* from the west coast of Florida in 1982.[20] The isolation of Bryostatin 1 on a large scale is difficult due to its low concentration in bryozoans. For example, about one ton of raw bryozoans is required to extract only one gram of Bryostatin 1. The complexity of the molecular structure also makes it difficult to synthesize, although simpler synthetic analogs with similar biological activity have been prepared recently.[28]

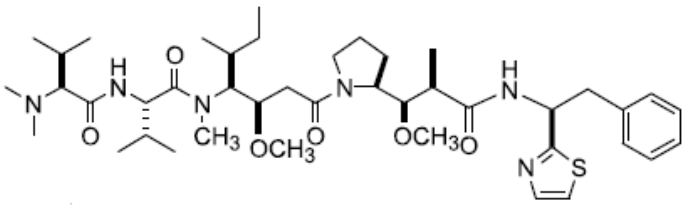
Symbiotic Bacteria

More recent research has revealed overwhelming evidence that many of the bioactive compounds isolated from marine macro-organisms are actually secondary metabolites synthesized by bacteria that are associated with these organisms.[29] Thus, the true origin of several anti-cancer metabolites extracted from tunicates, sponges, mollusks and other marine organisms in clinical trials are produced by different genera of symbiotic marine bacteria. For example, ET-743 isolated from the tunicate *Ecteinascidia turbinata* is originally biosynthesized by the endosymbiont *Endoecteinascidia frumentensis,* aplidine derived from the Mediterranean ascidian *Aplidium albicans* is strongly believed to be originated from a symbiont cyanbacterium species, and bryostatin 1 extracted from bryozoan *Bugula neretina* is produced by the bacterium *Endobugula sertula.*[22,30,31] The ecological significance of these

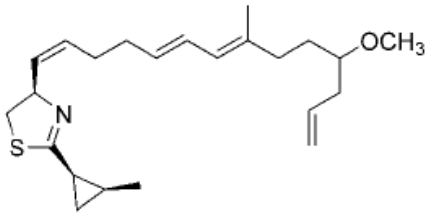
natural products is not well understood but most serve as chemical defense against predation or for food competition.[32] Thus, marine bacteria have captured the attention of natural product researchers for their potential to be a rich source of promising new agents in the treatment of infectious diseases and cancer.[33]

Marine cyanobacteria are prolific producers of bioactive secondary metabolites, providing a great chance of finding a very effective therapeutic agent.[34] It may be that the lack of mechanical defense is compensated for by very powerful chemical defense. This defensive characteristic has evolved over millions of years in which a chemical is produced to fit biological receptors of both invertebrates and vertebrates. These chemicals are cytotoxins that are able to penetrate cell membranes and trigger death of cells. Despite their good activity, no cyanobacterial metabolites have yet been approved by the FDA as treatments for any disease. However, there are a large number of promising compounds that have reached clinical trials, while others are in development.[34]

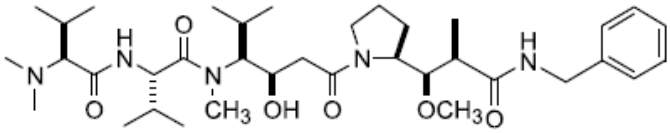
Because of their potent cytotoxic properties, chemicals isolated from marine cyanobacterial are potential anticancer drugs. Best known cyanobacterial-derived compounds that have reached clinical trials are dolastatins 10 (**4**) and 15 (**5**), cryptophycin 1 (**6**), curacin A (**7**) (Figure 2). Coibamide A (**8**), and largazole (**9**) are examples of promising new leads.[10,34]



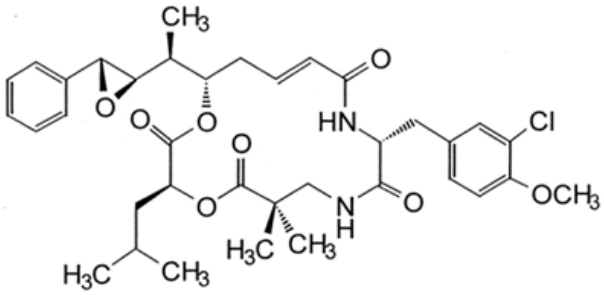
Dolastatin 10 **(4)**



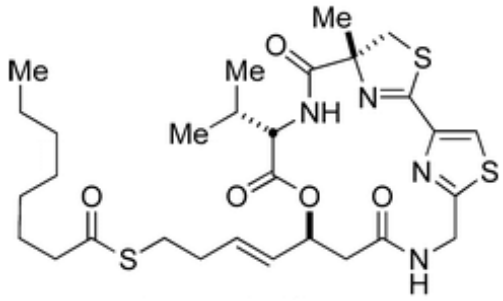
Curacin A **(7)**



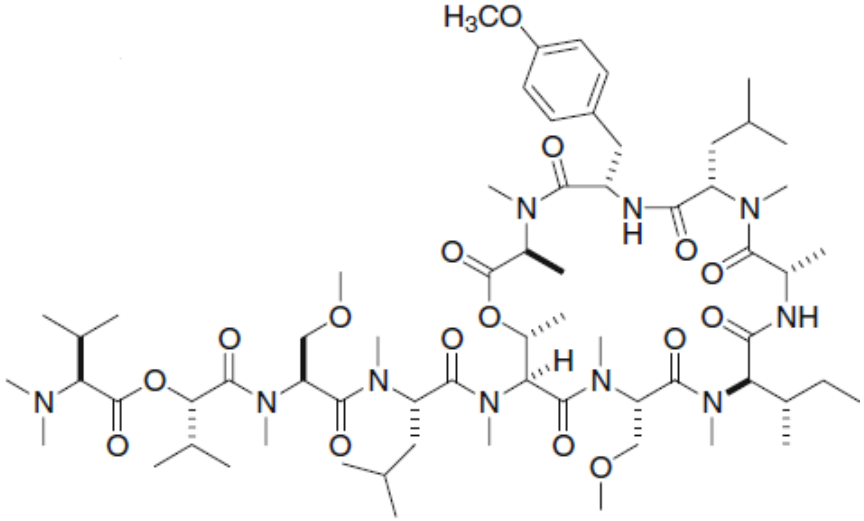
Dolastatin 15 **(5)**



Cryptophycin 1 **(6)**



Largazole **(9)**



Coibamide A **(8)**

Figure 2: Molecular structures of some cyanobacterial-derived compound

Dolastatins 10 and 15 were isolated from the Indian Ocean sea hare *Dolabella auricularia* and both target tubulin.[30] Dolastatin 10, a linear pentapeptide, was discovered in the early 1970s by Pettit's group and structurally identified after 15 years due to its low concentration in sea hares. In the early 1990s dolastatin 10 was introduced to Phase I clinical trials and advanced to Phase II before it was removed because 40% of patients experienced peripheral neuropathy. Dolastatin 15 is also a linear peptide and is closely related to dolastatin 10. It inspired the synthesis of two analogue compounds, cemadotin and synthadotin, with better chemical activity.[22]

Cryptophycin 1 was isolated from a *Nostoc* species that was originally extracted from lichen taken from a Scottish Island. It displayed only antifungal properties and it was too toxic to continue its development. Cryptophycin 1 was also isolated from a non-marine cyanobacterium and an Okinawan sponge by two different research teams. It led to the synthesis of a derivative, Cryptophycin 52, that passed Phase I to Phase II before it was withdrawn due to toxicity.[10,20]

Gerwick and his team were able to isolate a cytotoxic lipopeptide, named curacin A, from Caribbean cyanobacteria of the genus *Lyngbya majuscula.* Curacin A is an antimitotic agent that inhibits tubulin polymerization in cells.[34] It was an anticancer lead but its water insolubility hindered its advancement.[10] Researchers at the University of Pittsburgh have produced curacin A analogs to overcome the solubility problem while maintaining biological activity and selectivity. The research team was able to synthesize an analog that is more potent than curacin A but is unfortunately unstable.[35]

Coibamide A is a cancer cell toxin with an unknown mechanism of action derived from a Panamanian *Leptolyngbya* cyanobactrium. It is a cyclic depsipeptide with high

selectivity for breast, central nervous system colon and cancers.[36] Largazole is a histone deacetylase (HDAC) inhibitor that was recently discovered by Luesch and his colleagues from a *Symploca* cyanobactrium. It is a pro-drug activated when its lipophilic tail is metabolically cleaved off to generate a thiol compound that competitively inhibits the HDAC enzyme by chelating the required zinc ions. Structurally, the lipophilic tail helps in both cell penetration and thiol protection.[33]

Red Sea Cyanobacteria

Chemical diversity is proposed to be a function of taxonomic diversity.[33] This means that different molecular structures are synthesized by different phyla in response to a range of biological interactions, and therefore exploring a unique marine ecosystem may result in the discovery of new chemical entities. These may be therapeutically useful in treating and preventing human diseases. Assuming that the Red Sea is a unique marine environment in terms of pH and salinity, which are very different than the oceans and other seas, new molecules may be discovered. In addition, Red Sea organisms are relatively under investigated compared to the large numbers of biologically active secondary metabolites that have been reported from marine organisms. In particular, the chemistry of Red Sea cyanobacteria remains unreported in the literature.[37] Thus, natural products chemists have become more interested in exploring the organisms, especially cyanobacteria, in this ecosystem. To that end, this project is intended to identify biologically active chromatographic fractions from Red Sea cyanobacteria to be prioritized for future purification and molecular structure elucidation of active natural products.

METHODOLOGY

Marine cyanobacteria were collected previously from shipwrecks and coral reefs in the Red Sea (May 2007) by Dr. McPhail as part of a collaboration with Professor Diaa Youssef and other faculty at the Suez Canal University in Ismailia, Egypt. All cyanobacterial collections were processed to produce crude organic extracts at the Suez Canal University, and these were shipped to OSU College of Pharmacy in 2008. For the purposes of this project, five crude organic extracts of taxonomically unidentified Red Sea cyanobacteria were fractionated by chromatographic means and screened for cytotoxic activity in whole cell biological assays. The most active cytotoxic fractions were then prioritized for further fractionation towards isolating pure, active constituents that might be relevant to the treatment of cancer. To accomplish this goal, the project comprised the three distinct major stages of fractionation and biological activity testing, purification of active compounds, and identification or structure elucidation of pure compounds, as described in the following discussion (Figure 3).

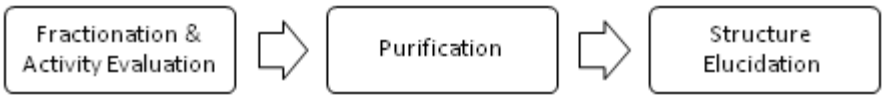


Figure 3: Overview of the main steps of the project

Stage One: Fractionation of Crude Extracts and Evaluation of Biological Activity.

Each crude extract was fractionated according to polarity into smaller sub-fractions in which bioactive compounds should thus be concentrated to a detectable level. The resultant "reduced complexity" samples were then tested for biological activity.

According to our standard protocol, each extract was divided into 8 first-tier fractions using normal phase vacuum liquid chromatography (NP-VLC) in which the silica gel column was eluted with a stepped solvent gradient ranging from 100% hex to 100% EtOAc to 100%

MeOH. Based upon the crude extract weight, an appropriate column size was selected to carry out the chromatographic procedure. The column size was important to avoid sample loss and to optimize separation. Since most crude extract masses were between 1.5 and 3 g, a 350 mL VLC funnel was generally the most suitable, although a smaller column (150 mL) was used for the smallest of the five extract extracts (< 1 g).

The VLC column was first prepared by pouring TLC-grade silica gel into the appropriate size sintered glass vacuum funnel, while applying a vacuum created by water aspiration or a vacuum pump. Once a tightly packed column was achieved, it was then conditioned with hexanes (approximately 300 mL) before the crude extract was applied in a small amount of ether (about 3 to 5 mL) to create a thin and even band of sample at the top of the column. This was then flushed through the column with approximately 300 mL of each solvent and the corresponding eluant collected into a separate clean round-bottom flask. Thus, the column was eluted 8 times, respectively, with roughly one bed volume (300 mL) each of 100% hex, 20% EtOAc/hex, 40% EtOAc/hex, 60% EtOAc/hex, 80% EtOAc/hex, 100% EtOAc, 25% MeOH/EtOAc and finally 100% MeOH. To keep track of the generated fractions, each one was assigned a letter from A to I, appended to the previously assigned crude extract code. Therefore, A always denotes the first (100% hex) fraction collected and I the last (100% MeOH) fraction produced (Figure 4). Note that B is not used since this would represent the 10% EtOAc/hex fraction in the standard laboratory protocol used.

After elution of the VLC column, solvents were removed from the collected fractions by vacuum distillation using a rotary evaporator and the resultant concentrates were each transferred (using ether as a solvent) into 4 mL vials for ease of handling and storage purposes. After concentration to dryness in vials, the fractions were then weighed, clearly

labeled with extract and fraction number and stored at – 20 ^{0}C. The collected data was then recorded in a chemistry notebook for future reference.

To evaluate the biological activities of generated VLC fractions, a brine shrimp toxicity assay was used. This is a popular method that is easy, rapid, reliable, inexpensive and commonly used in natural products research.[38] Because fractions were generally stored in 4 mL vials, a 95% EtOH solution of 100 mg/mL concentration were first prepared for fractions weighing 50 mg, or higher and then 1:10 dilutions were performed to achieve the 10 mg/mL concentration desired for testing. Likewise fractions that weighed 500 mg or more were diluted 1:100 after each 1,000 mg/mL stock solution was made.

Brine shrimp eggs were hatched over 24 hours in a rectangular dish filled with artificial sea water, which was prepared by dissolving a commercially available marine medium ("Instant Ocean") in deionized water. The dish was unequally divided with a perforated plastic divider. The small compartment in which brine shrimp eggs were placed was darkened by a cardboard covering, while the other half of the container was exposed to light. This ensures that the newly hatched brine shrimp swim through the divider towards the light, and can be collected without concomitantly picking up unhatched eggs. After 24 hours, roughly 10-25 hatched shrimps were pipetted into 4 mL of artificial seawater contained in each of the wells of a plastic (5 mL) large-well plate.

Subsequently, 50 µL of a 10 mg/mL test sample solution were added to each well using a micropipette and disposal pipette tips to avoid cross contamination. Duplicate tests of each fraction were performed and compared to duplicate control treatments in which the 50 µL added was 95% EtOH, as a way to examine the effect of the solvent vehicle on the

shrimps. The wells were covered with transparent plastic to prevent the evaporation of H_2O while allowing light in.

After 24 hours, the dead and surviving shrimps were counted microscopically against a light background. In some instances, shrimps had not died but were greatly affected (twitching without directional motion). This was documented and taken into consideration for the prioritization of fractions. The percentage of deaths was calculated as an indication of which fraction had the greatest cytoxicity.

Stage Two: Compound Purification from Active Fractions

Of the bioassay-tested factions, only the fractions that show the highest brine shrimp toxicity were selected to proceed with. Other fractions were stored frozen until further workup. Prioritization of samples is imperative for saving time, and for cost effectiveness in general. At this stage, spectroscopic and more advanced chromatographic methodologies were used to identify relative abundances as well as the approximate molecular weight of active chemical compounds composing the active fractions.

The bioactive VLC fractions were further separated into 4 sub-fractions by reversed phase solid phase extraction (RP-SPE). The selection of cartridge size was primarily determined by the amount of sample available. A small cartridge (100 mg C_{18} stationary phase) was used for small samples to minimize any loss of sample, while large samples required a large cartridge (2g C_{18}) for optimal separation. Each pre-packed C_{18} silica gel cartridge was first primed with 10 bed volumes of MeOH and then equilibrated with up to 10 bed volumes of 70% MeOH-H_2O (the starting solvent). After dissolved in enough ether, the sample was applied on the top of the pre-packed cartridge that was eluted with 70% MeOH-H_2O, 90% MeOH-H_2O, 100% MeOH and 100% dichloromethane (DCM) respectively. The

elutions were collected in 4 separate 50 mL beakers labeled 1 through 4, dried down *in vacuo* as described in stage one and weighed. Again all data were recorded for future reference.

Depending on the masses of the dried sub-fractions, the brine shrimp bioassay could or could not be carried out to identify in which sub-fraction active compounds resided. When sufficient amounts of sample were available, sub-fractions were tested for biological activities. However, the brine shrimp assay was bypassed in the case of low masses to preserve samples for further purification.

Samples of active SPE sub-fractions were diluted to 0.1 mg/mL and directly injected (1-2 µL, using a 10 µL syringe), multiple times into a mass spectrometer in positive mode using 95% MeOH-H_2O as the solvent. Peaks with relatively high abundance were selected and their corresponding molecular masses were compared against known molecular structures in a database (MarinLit) to get an initial sense of what kinds of molecules might be present.

Selected bioactive RP-SPE sub-fractions were taken to the next step of High Pressure Liquid Chromatography (HPLC) to isolate the pure active molecules. First, sub-fractions needed to be diluted to an approximate concentration before HLPC could actually be performed. Then, the selection of the appropriate column type and size that best separates component chemical compounds of the sub-fraction was necessary. As the instrument was running, peaks observed due to the ultraviolet (UV) light absorption property of each chemical compound were collected in separate round-bottom flasks. After drying down the solutions of collected peaks, the same process was repeated with a smaller column to get a greater degree of purification until pure compounds were obtained.

Stage Three: Structure Elucidation

Once the active compounds are isolated in sufficiently pure form (~95% pure), they are ready to be characterized and structurally identified. NMR spectroscopy is necessary to establish the connectivity of atoms in a molecule, while mass spectrometry (MS) may help to establish molecular size and to identify functional groups present. Advanced 2D NMR and also mass spectrometric experiments are usually necessary to assign the exact structure, and each piece of analytical data for a pure compound obtained throughout the structure elucidation process must be interpreted. Knowledge of the molecular structure may provide insight into the pharmacophore of a bioactive compound. Since it requires more knowledge, time and the use of advanced techniques than were within the scope of this project, the structure elucidation step was left to be done by more advanced researchers.

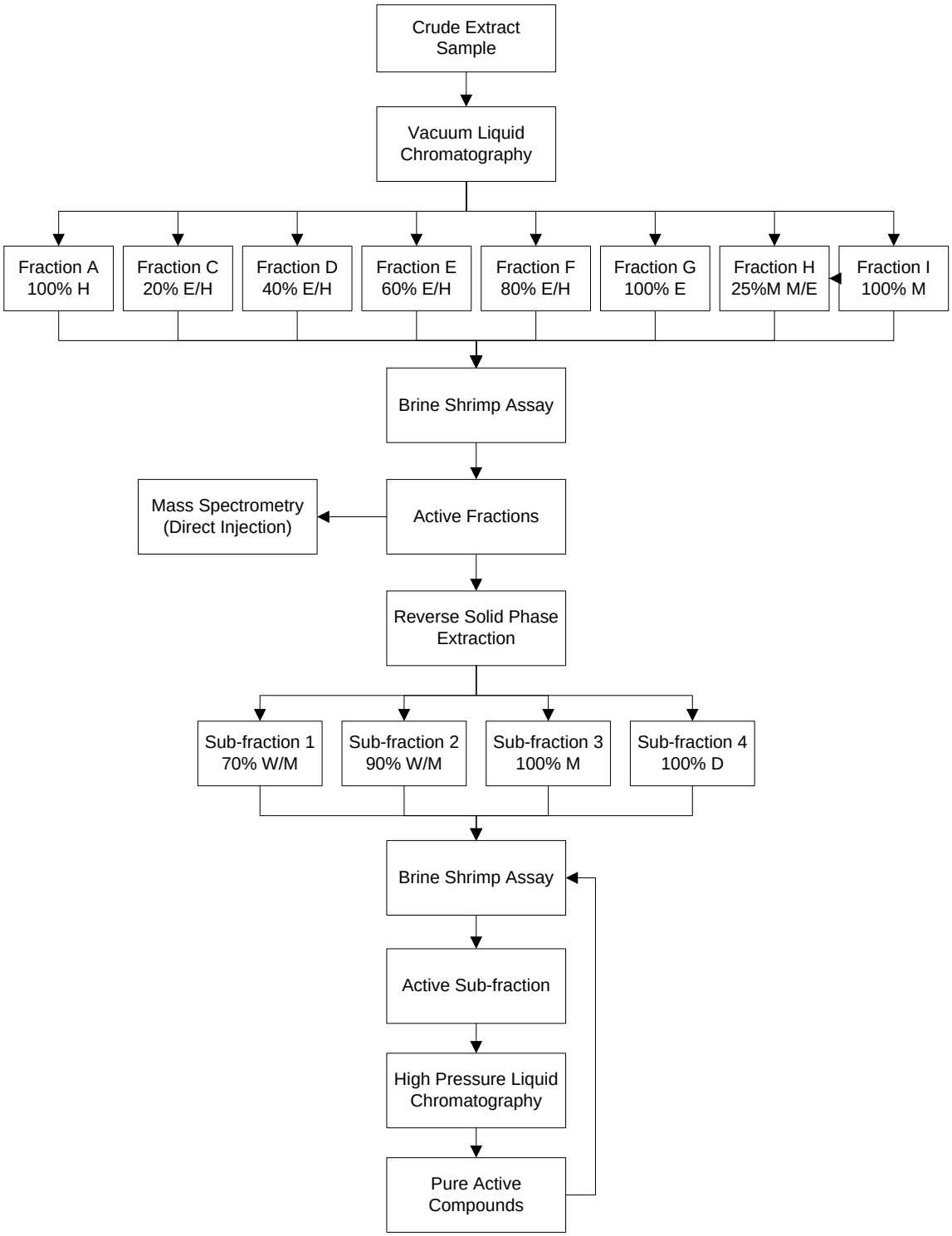


Figure 4: General VLC fractionation scheme

RESULTS

I. Vacuum Liquid Chromatography

For each crude extract, eight first-tier fractions were produced by NP-VLC, resulting in a total of 40 fractions. These samples were dried, weighed and reconstituted in 95% EtOH to be tested for any anti-cancer or anti-microbial activities in the brine shrimp and bacterial assays (not yet performed) at 100 ppm, respectively. The brine shrimp assay results showed that only 5 fractions had average percent mortality greater than 50%. These samples caused the death of less robust, young brine shrimps and significantly decreased the motility of the rest. Therefore, these fractions were considered active and were prioritized for our further attention. Listed from the most active to least active, the fractions were Enq02G with 100%, Ehu01G with 94%, Enq02H with 91%, Enq02F with 84% and Ehu02E with 74% average mortality at 100 ppm (Table 1). To readily distinguish these in the table, they are highlighted in grey.

Other fractions had a profound effect on the motility of shrimps but they were not cytotoxic enough to cause death. The shrimps were mainly twitching or otherwise barely moving, making them appear as though dead and thus easily counted. These fractions are marked with double asterisks in Table 1. The impact of the remaining fractions varied from no effect, making it hard to count the shrimps unless they were treated with MeOH to induce death at the end of the experiment, to very little effect that did not reduce the shrimps' movement significantly.

Table 1: Result of brine shrimp bioassays of crude extract fractions resulting from NP-VLC

Sample code		Fraction total Mass (mg)	Treatment 1		Treatment 2		Average mortality
	Fraction		Shrimp #	% death	Shrimp #	% death	
Schizothrix from Nabq Mangroves	Enq-05/31/07-2						
	A	34.3	13	8	18	6	7
	C	126.5	10	0	20	10	5
	D	103.2	10	0	11	0	0
	E	26.8	17	6	13	15	11
	F	20.4	9	89	10	80	84
	G	6.2	8	100	12	100	100
	H	36.5	14	93	18	89	91
	I	125.9	10	10	14	14	12
Bright orange mat from Wreck Elmena	Ehu-05/28/07-2						
	A	4.2	10	0	10	0	0
	C	4.8	13	0	9	11	6
	D	5.5	15	20	12	0	10
	E	2.6	18	72	16	75	74
	F**	0.7	30	27	20	30	28
	G	2	6	17	17	29	23
	H**	2.2	11	9	12	25	17
	I	42.5	10	10	14	21	16
Dark brown filamentous cyanobacteria from Nabq Mangroves	Enq-05/31/07-3						
	A	8.8	19	0	14	0	0
	C	9.7	8	25	9	11	10
	D	44.3	13	31	12	42	36
	E	5.6	8	25	12	33	29
	F	4.2	8	13	20	20	16
	G	1	11	18	10	0	9
	H	11.8	17	12	32	22	17
	I	26.6	12	0	15	0	0
Shaggy green/brown Scytonema	Enq-05/31/07-1						
	A	5.2	6	0	17	0	0
	C	1	18	0	20	5	3
	D	1.6	8	25	14	14	20
	E	1.2	16	0	21	0	0
	F	1	20	0	11	0	0
	G*	0.4	12	0	17	6	3
	H*	0.4	29	3	22	5	4.0
	I	10.6	16	0	11	0	0
Spirocoleus/ Microcoleus cyanobacteria from Wrek Elmena	Enq-05/31/07-2						
	A	435.7	12	8	18	17	13
	C	127	8	13	19	26	19
	D	456.7	14	36	15	40	38
	E	138.9	15	13	9	11	12
	F	80.4	10	0	14	14	7
	G	43	14	100	8	88	94
	H	368.1	12	0	15	0	0
	I	774.6	10	0	14	0	0

All fractions were tested at 100 ppm and shaded fractions were prioritized active; ** the motility of brine shrimps was greatly affected but they were not dead; * fractions tested at lower concentrations (10 ppm) due to limited quantities

II. Reversed Phase SPE

A total of 40 sub-fractions resulted from the separation of active VLC fractions by reversed phase SPE. The SPE sub-fractions of Enq02F, Enq02G, and Ehu01G showed lower cytotoxicities than the parent fractions when tested at 100 ppm. A few shrimps were dead while the rest were twitching. Table 2 summarizes the results of shrimp assay as well as the masses of each sub-fraction recovered from the SPE separation.

Table 2: Brine shrimp bioassay of the sub-fractions resulting from reversed SPE

Sample code Sub-Fraction	Mass (mg)	Treatment 1 Dead/Total	# Twitch	Treatment 2 Dead/Total	# Twitch	Average % of mortality
Enq02F						
1	6.5	0/15	0	1/23	0	3
2	3.1	1/11	10	2/15	4	12
3	2.9	0/17	0	0/13	0	0
4	5.8	0/8	0	0/7	1	0
Enq02G						
1	0.7	1/14	0	0/8	1	5
2	0.4	0/8	0	1/8	0	6
3	0.8	4/12	0	4/10	0	18
4	0.8	0/8	1	0/10	0	0
Ehu01G						
1**	16.1	0/10	0	CL	CL	-
2**	5.5	1/14	0	0/9	0	0
3	20.1	0/16	1	0/14	0	0
4	13.2	0/13	0	0/15	0	0

Sub-fractions were tested at 100 ppm; CL means the content was lost due to a crack in the well; ** the motility of brine shrimps was greatly affected but they were not dead.

The other subfractions of (Enq02H and Ehu02E) were not tested because Enq02H (36.5 mg) was believed to have the same bioactive molecules already found in Enq02F and Enq01G and it was saved in the refrigerator for later work. However, a small sample of Ehu02E (2.6 mg) was available for SPE separation and doing the brine shrimp assay might cause unnecessary loss of the sample.

III. Mass Spectrometry

The mass spectra of diluted samples of Enq02F-2 and Enq02G-3, directly injected into the mass spectrometer, showed that both subfractions contained the same bioactive

compounds (Figure 5 & 6). The mass peaks observed were *m/z* 881 and 1138, and the latter could be the compound responsible for the cytotoxic activity. Compared to structurally well-known bioactive compounds in the MarinLit database, the 1138 Da molecule could have a similar structure or be identical to 15,34-Dissulfatobastadin 7 ($C_{34}H_{24}Br_4N_4Na_2O_{14}S_2$, MW 1141.6 Da), Didemnin B ($C_{58}H_{91}N_7O_{15}$, MW 1112.35 Da), or Lyngyblastatin 4 ($C_{53}H_{68}N_8O_{18}S$, MW 1136 Da).

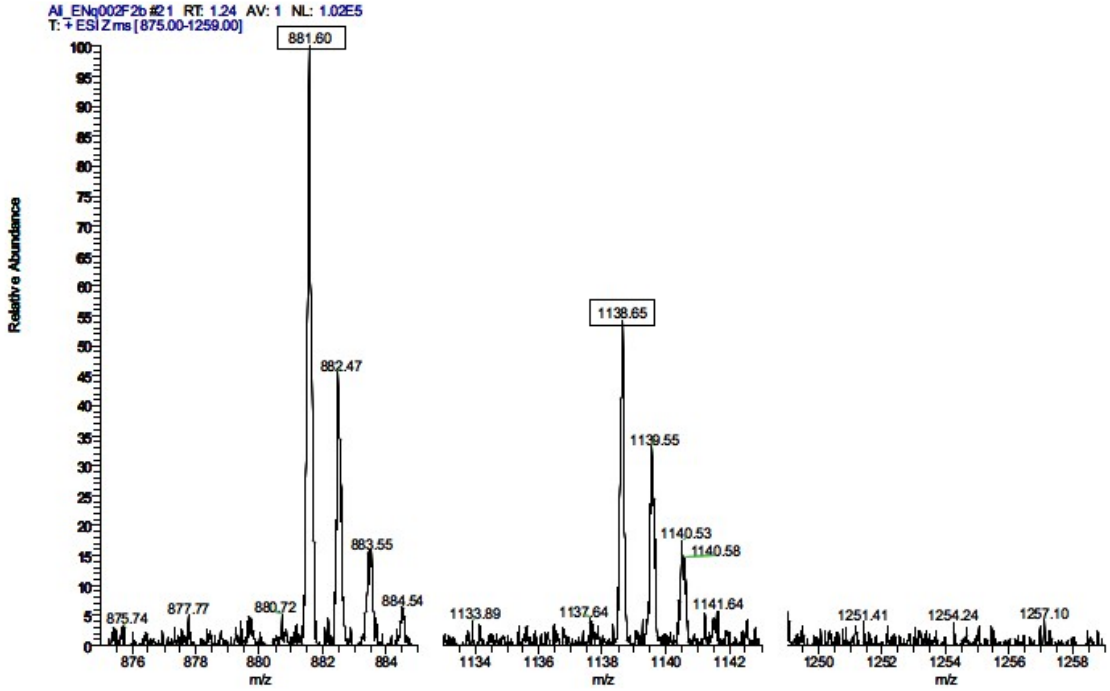


Figure 5: Mass spectrum for Enq02F-2

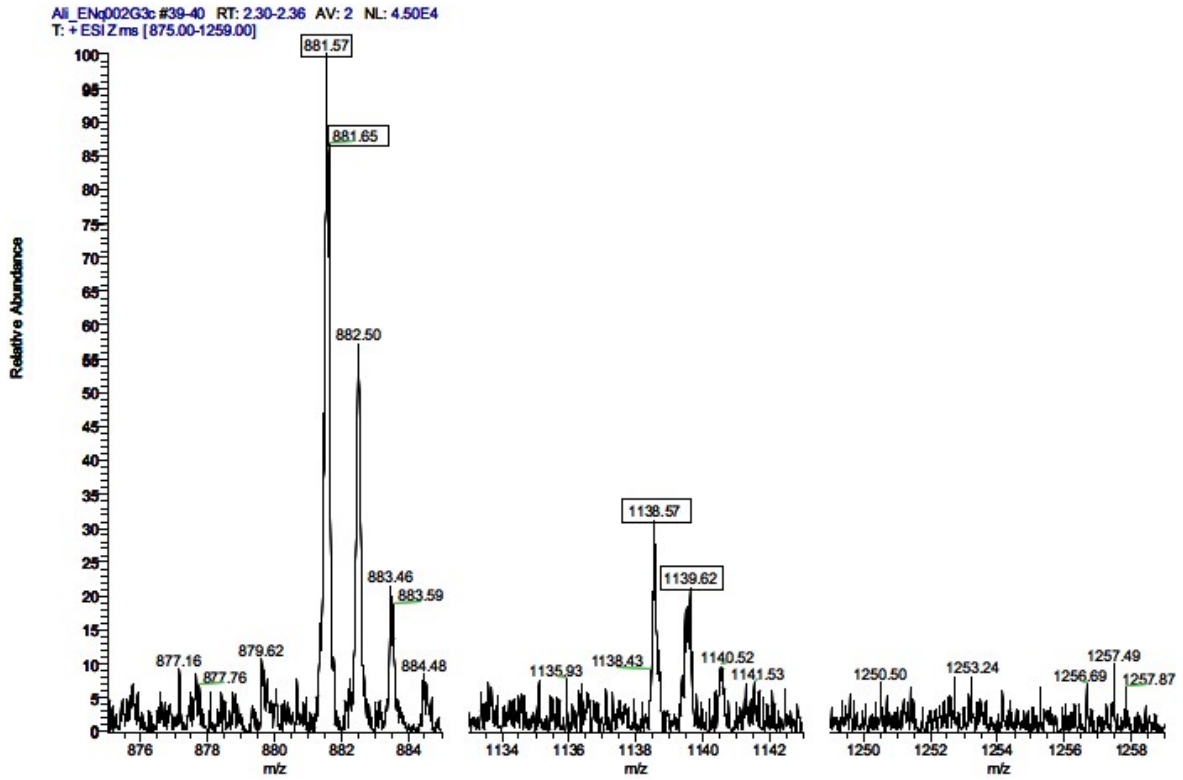


Figure 6: Mass spectrum for Enq02G-3

On the other hand, the abundant molecules in Ehu01G-1 and Ehu01G-2 sub-fractions had different masses. The spectrum of Ehu01G-1 showed one compound that gave *m/z 7*25 (Figure 7). Ehu01G-2 had two major compounds: one with the same peak found earlier in Ehu001G-1 (*m/z 7*25) and the other constituent with *m/z* 803 (Figure 8). When searched, the MarinLit database of bioactive natural products shows many molecules with the molecular masses of 725 and 803 Da. However, it should also be noted that the peak at *m/z* 803 could represent the sodium adduct of dioctyl phthalate dimer. Dioctyl phthalate is a common contaminant of organic solvents.

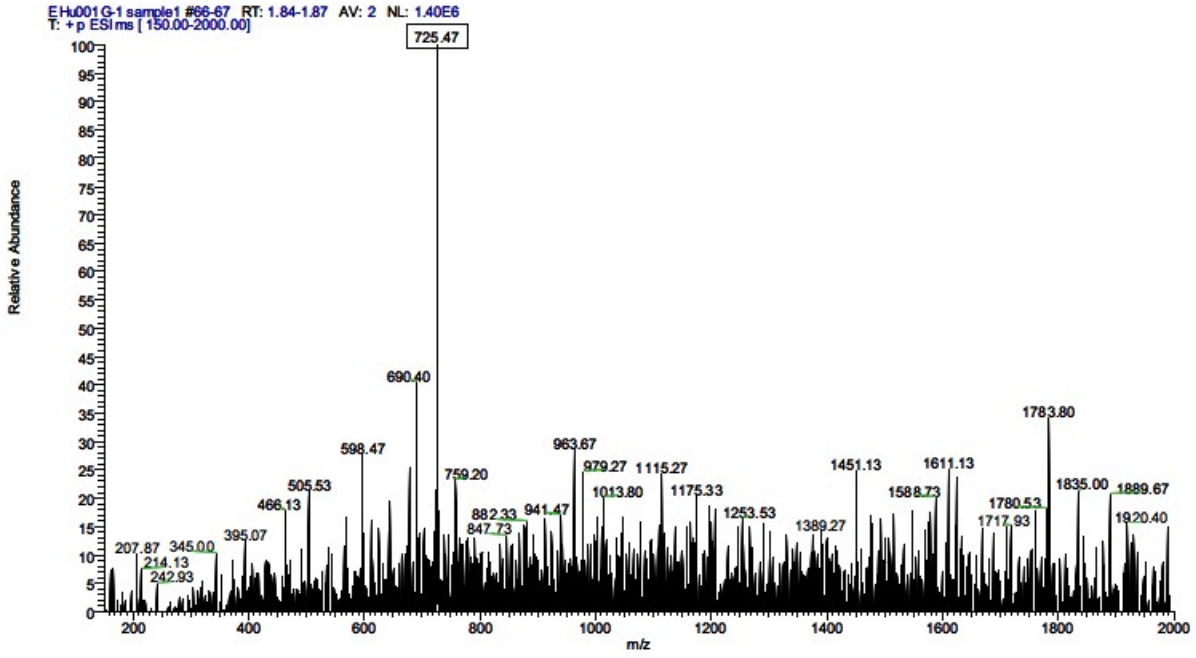


Figure 7: Mass spectrum for Ehu01G-1

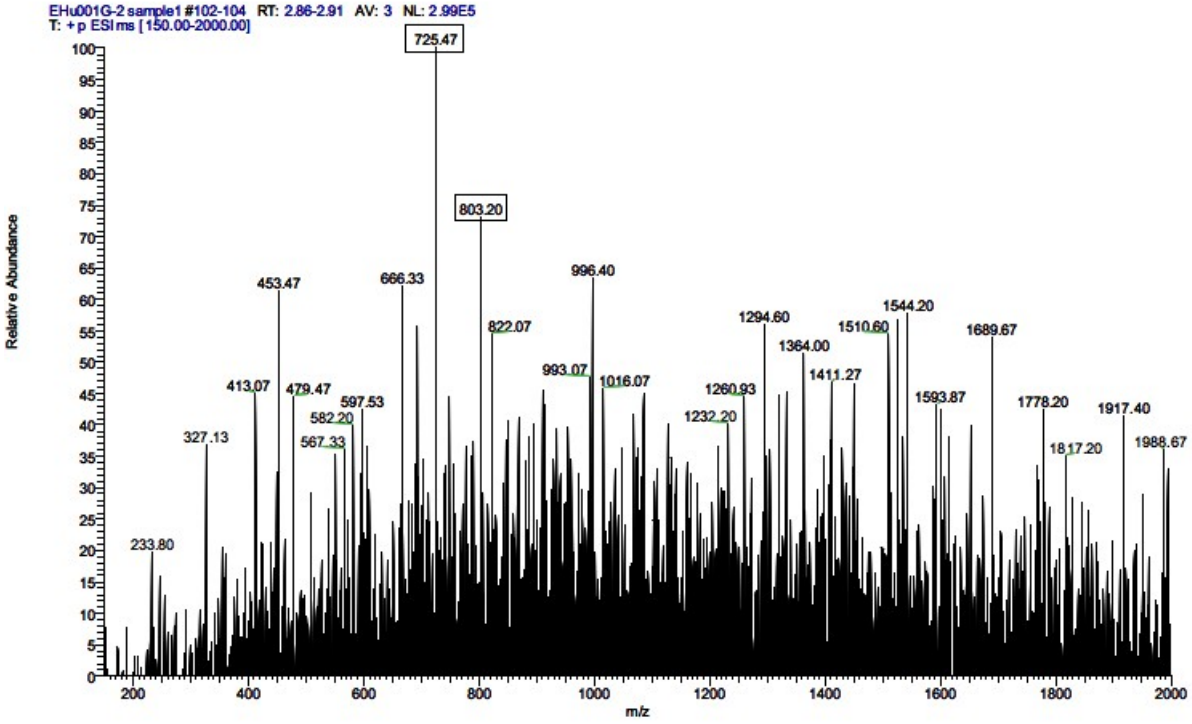


Figure 8: Mass spectrum for Ehu01G-2

Since the mass-limited Ehu02E sub-fractions were not evaluated in the biological assays, the first three fractions (Ehu02E-1, Ehu02E-2, and Ehu02E-3) were analyzed by mass spectrometry. Ehu02E-4 was excluded because it was the non-polar DCM fraction that could clog the mass detector. The data showed that Ehu02E-1 contained two major compounds with *m/z* 505 and 933, while Ehu02E-2 was a mixture of constituents giving *m/z* 431, 673, 803, 997, and an *m/z* 436 peak was predominant in Ehu02E-3 (Figure 9, 10 &11). Too many proposed molecules with similar masses were found in the MarinLit database to list here.

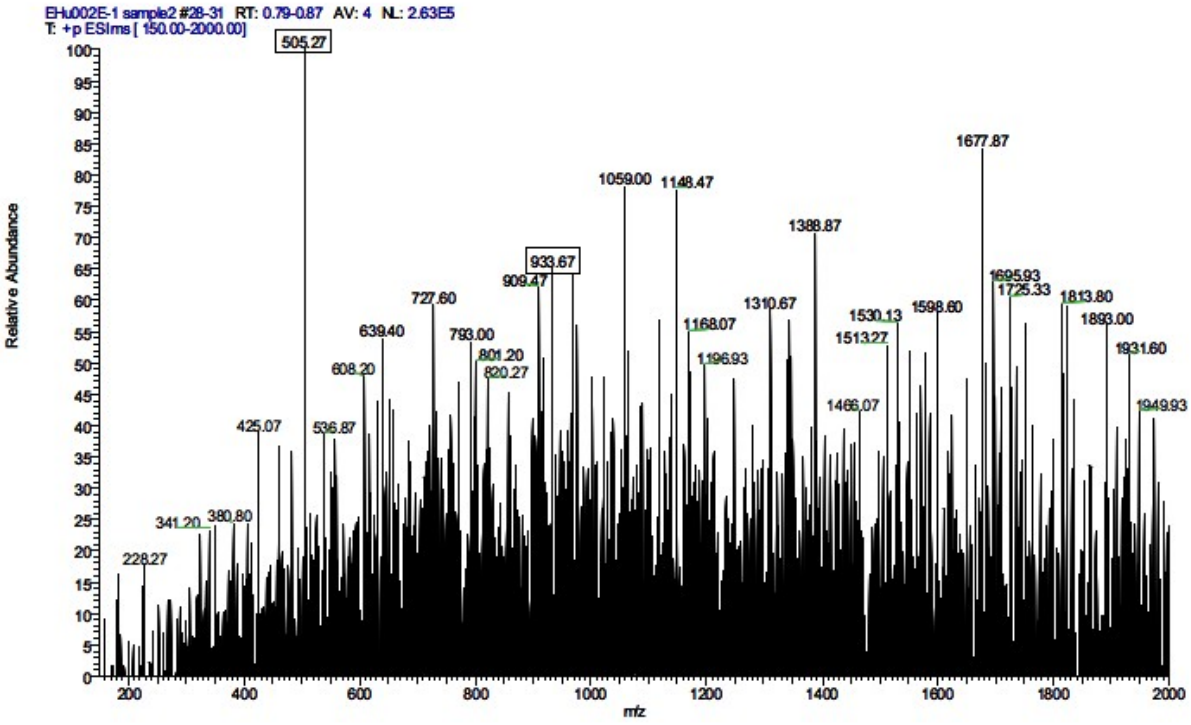


Figure 9: Mass spectrum for Ehu02E-1

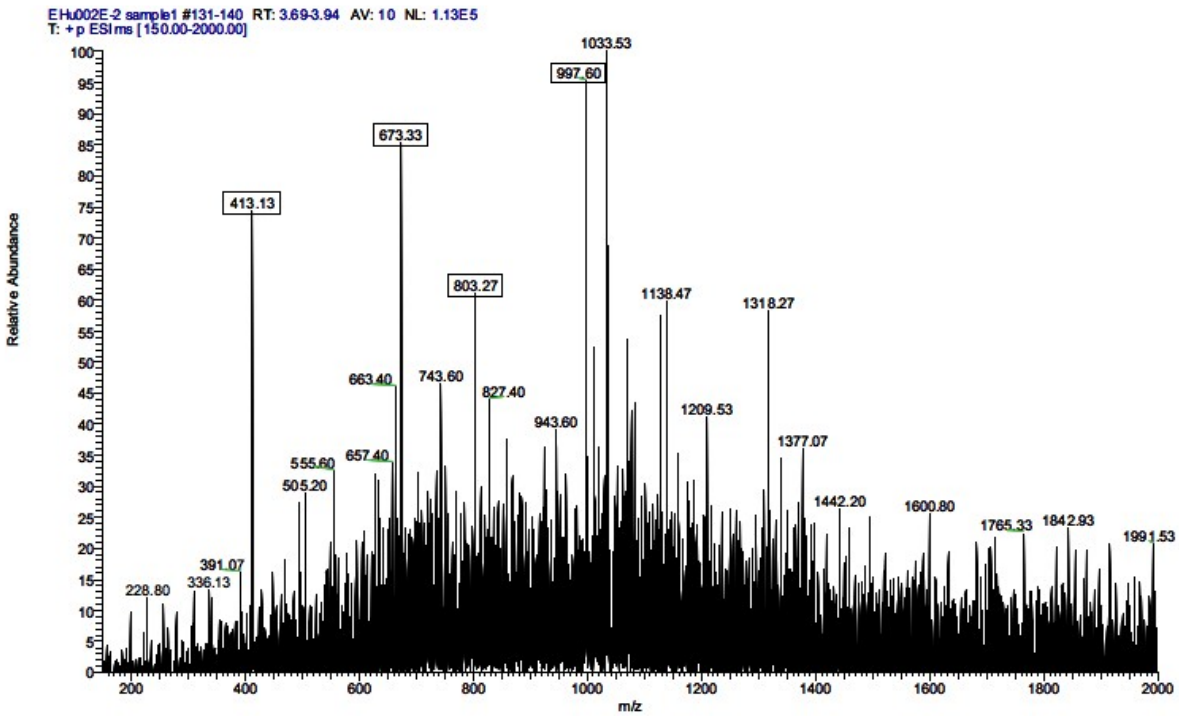


Figure 10: Mass spectrum for Ehu02E-2

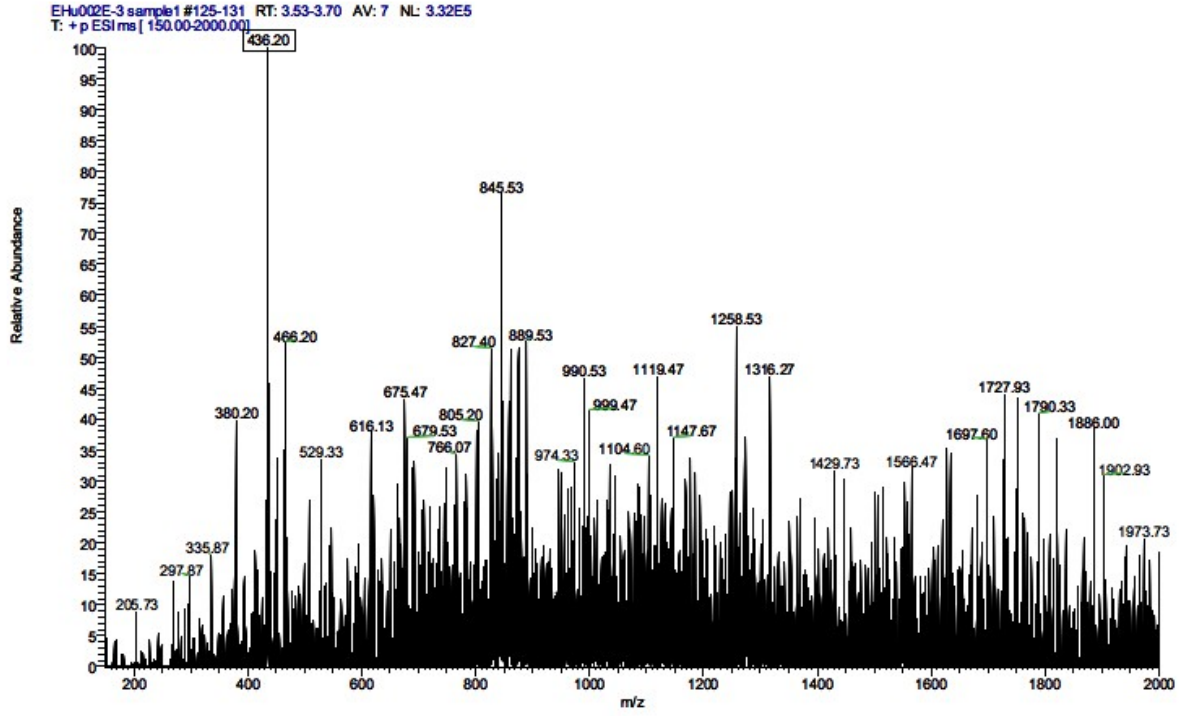


Figure 11: Mass spectrum for Ehu02E-3

Overall, 10 unknown compounds that gave mass peaks ranging from *m/z* 431 to 1138 were extracted from three of the five crude samples investigated. The three crude samples were Enq-05/31/07-2, Ehu-05/28/07-2, and Ehu-05/31/07-1. The numbers of bioactive compounds found in each sub-fraction and their corresponding mass peaks are summarized in Table 3.

Table 3: Summary of putative molecular masses observed in each sub-fraction

Sub-Fraction	# of compounds	Mass peak (*m/z*)
Enq02F-2	2	881, 1138
Enq02G-3	2	881, 1138
Ehu01G-1	1	725
Ehu01G-2	2	725, 803
Ehu02E-1	2	505, 933
Ehu02E-2	4	431, 673, 803, 997
Ehu02E-3	1	436

IV. HPLC Chromatography

Of all the sub-fractions, only Enq02F-2 was profiled using HPLC chromatography because of the time limitation. As seen in Figure 12, the UV chromatogram for Enq02F-2 shows that there are three overlaping peaks that absorb light at the monitored wavelengths 216 (blue), 230 (green), and 254 nm (red). The data collected for Enq02F-2 including its mass, UV absorption properties, and the parent fraction polarity indicate that it contains a compound (or close analog) indicated by an arrow in figure 12 recently isolated by Chris Thornburg (Ph.D. student in Pharmaceutical Sciences) from a different cyanobacterial crude extract (EHu5). On the basis of these data, there is a good chance that the two compounds are structurally similar if not identical. Chris identified the bioactive natural product as a cyclic depsipeptide (Figure 13), related to other cyanobacterial metabolites isolated from a Caribbean collection of *Lyngbya* species.

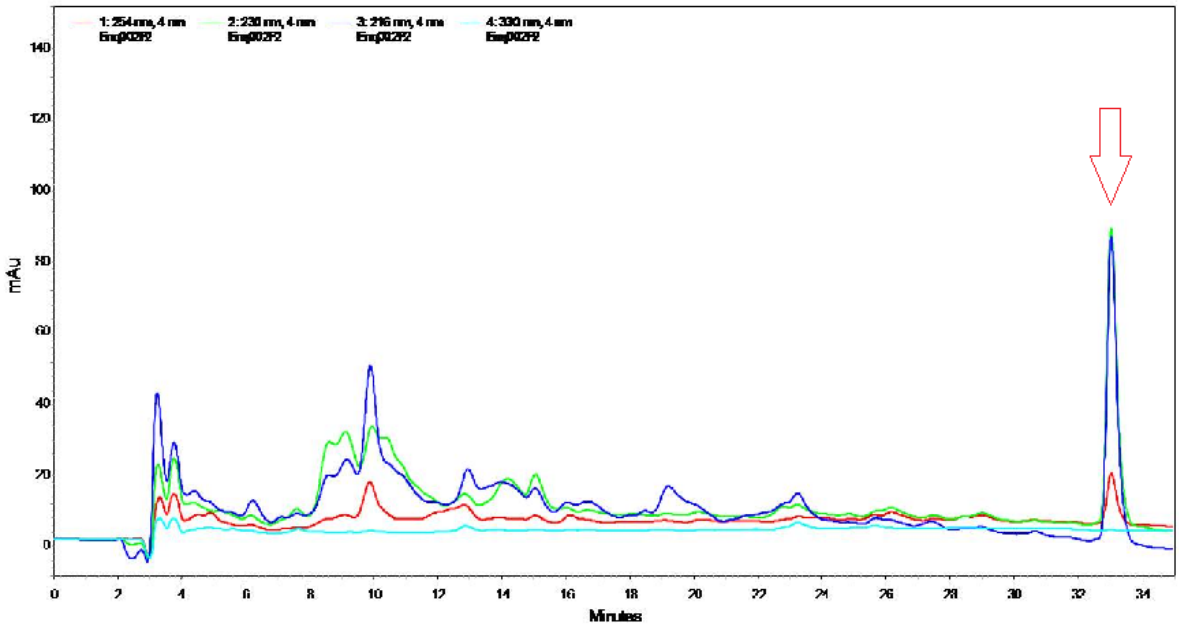


Figure 12: HPLC chromatogram for Enq02F-2

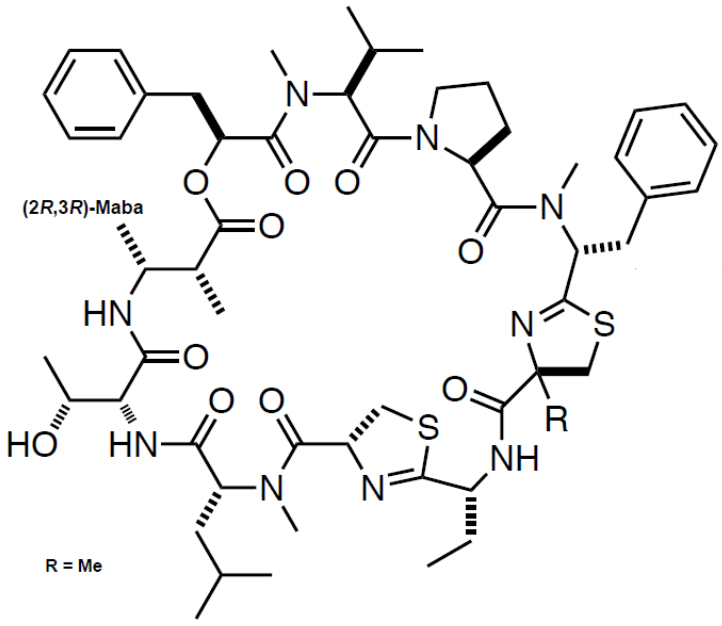


Figure 13: Molecular structure of the compound isolated from Ehu5H-2 and present in Enq02F-2

DISCUSSION

Of 40 first-tier VLC fractions, 5 showed varying levels of toxicity to the brine shrimp *Artemia salina*. Other biological activities of these VLC fractions still need to be evaluated because marine cyanobacteria have also been a source of anti-inflammatory, anti-viral, antibiotic, antifungal, and immunomodulatory agents.[16] In addition, single, pure natural products may have more than one therapeutic indication.

It was also observed that the most bioactive fractions (ranging from E to H) were of medium polarity. Given the purpose of these chemicals and the environments in which cyanobacteria live, this could be expected because very polar compounds may be unable to penetrate the plasma membrane, whereas non polar molecules are insoluble in aqueous solutions of the blood and cell cytoplasm, as well as sea water through which the compounds must diffuse if they are actively secreted by the producing organism. Thus, the medium polarity of these natural products may be favorable for their role as defensive chemicals in marine cyanobacteria because it allows them to reach other organisms in competition for space and other resources, and also to cross the lipid bilayer of cell membranes to exert their biological effects on an organism. Therefore, medium polarity fractions should be prioritized for isolating active natural products from marine organisms. However, although this approach is most practical and convenient, it should be kept in mind that potently bioactive secondary metabolites can be very lipophilic, residing in the non-polar VLC fractions (A-D). One of these classes of secondary metabolites is lipopeptides, which have fatty acid portions derived from condensation of acetate units combined with peptide portions.[9] Lipophilicity of compounds in this class is a significant disadvantage that hinders their development as anti-cancer leads because poor water solubility limits their effect in vivo. The best representative

of this class is curacin A which was first reported in 1994.[34] Its bioactivity could not be demonstrated in vivo animals models,[10] and as a result, synthetic strategies to produce more potent and stable analogs have been used and these structure-activity relationship studies remain ongoing.[35]

In addition, it was observed that the level of cytotoxicity decreased when VLC fractions were further separated into 4 sub-fractions by reversed phase SPE. Since the normal phase VLC fractions were somewhat non-polar, it is possible that some amount of bioactive compound could have been lost during reversed phase chromatography due to incomplete solubility in the aqueous mobile phases or irreversible binding to the C_{18} silica gel stationary phase of SPE cartridge. Another plausible explanation is the phenomenon of synergistic biological activity. The greater cytotoxicity of the cruder fractions could be caused by a synergy between several closely related compounds, which individually were not very active, but when combined, contribute to the activity level, which then becomes significant.

Interestingly, the biologically active metabolite in Enq02F-2 was found to have a similar analytical profile to a bioactive compound isolated by graduate student Chris Thornburg from a different cyanobacterial extract from the Red Sea. Both had the same molecular weight, UV absorption properties, and were from parent fractions of the same polarity. This biologically active metabolite has been identified as a cyclic depsipeptide by Chris Thornburg. When compared to other active cyanobacterial metabolites, the compound was structurally related to those found in cyanobacteria from Florida, the Caribbean and elsewhere. Based on this fact, it is believed that Red Sea cyanobacteria are not different from those collected from other tropical regions. Therefore, according to our limited investigations to date, the unique marine environment of the Red Sea, with its significantly different pH and

salinity, does not give rise to *Lyngbya* species that produce substantially different natural products. However, there are numerous other genera of Red Sea cyanobacteria that have not been investigated.

REFERENCES

1. Cooper, G. M.; Hausman, R. E. The Cell: A Molecular Approach, 4th ed.; **2007,** 721.
2. World Health Organization. Programmes and Projects: Key Facts. http://www.who.int/mediacentre/factsheets/fs297/en/ (accessed Mar 31, **2010**)
3. American Cancer Society. Cancer Facts & Figures 2009. http://www.cancer.org/downloads/STT/500809web.pdf (accessed Mar 31, **2010**)
4. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun M. J. Cancer Statistics, **2008**. *Cancer J Clin*. **2008**, 58, 71-96
5. Adami, H.O., Hunter, D., Trichopoulos, D., Ed. Textbook of Cancer Epidemiology; Oxford University Press: New York, **2008**, 379.
6. Begley, Sharon. We Fought … And Cancer Won. Newsweek. [Online] **2008**. http://www.newsweek.com/2008/09/05/we-fought-cancer-and-cancer-won.html
7. Kutt, Phyllis. *Advances elusive in the drive to cure cancer*, April **2009,** pp A1.
8. Abou-Jawde, R; Cheouseiri, T.; Alemany, C., Mekhail, T. An overview of targeted treatments in cancer. *Clin Ther.* **2003**, 25, 2121-2137.
9. Hanson, J. R. Natural Products: the Secondary Metabolites; Royal Society of Chemistry, **2003**, 1-3.
10. Cragg, G. M.; Grothaus, P. G.; Newman, D. J. Impact of natural products on developing new anti-cancer agents. *Chem Rev.* **2009,** 30, 1, A-AF.
11. Newman, D. J.; Cragg G. M.; Snader, K. M. *Nat. Prod. Rep.* **2000**, 17, 215-234.
12. Li, J W.-H.; Vederas, J. C. Drug Discovery and Natural Products: End of an Era or an Endless Frontier? *Science*. **2009**, 325,161-165.
13. Newman, D. J.; Cragg G. M. Natural Products as sources of new drugs over the last 25 years. *J. Nat. Prod.* **2007**, 70, 461-477.

14. Newman, D. J. Natural Products as leads to potential drugs: an old process or the new hope for drug discovery? *J. Med. Chem*. **2008**, 51, 2589-2599.

15. Borchardt, J.K. The beginnings of drug therapy: ancient Mesopotamian medicine. *Drug News Perspect* **2002**, 15, 187.

16. Burja, A. M.; Banaigs, B.; Abou-Mansour, E.; Burgess, G.; Wright, P. C. Marine cyanobacteria – a prolific source of natural products. *Tetrahedron*. **2001**, 57, 9347-9377.

17. Butler, M. S. Natural products to drugs: natural product derived compounds in clinical trials. *Nat Prod Rep.* **2005**, 22, 162-195.

18. Williams D. H.; Stone M. J.; Hauck, P. R.; Rahman; S. K. Why are secondary metabolites (natural products) biosynthesized. *J Nat Prod*. **1989**, 52, 1189-1208.

19. Firn, R. D.; Jones C. G. Natural products - a simple model to explain chemical diversity. *Nat. Prod. Rep.* **2003**, 20, 382-391.

20. Newman, D. J.; Cragg G. M. Marine Natural Products and related compounds in clinical and advanced preclinical trials. *J. Nat. Prod.* **2004**, 67, 1216-1238.

21. Donia, M.; Hamann, M. T. Marine natural products and their potential applications as anti-infective agents. *Lacent infect. Dis.* **2003**, 3, 338-348

22. Simmons, T. L.; Andrianasolo, E.; McPhail, K.; Flatt, P.; Gerwick, W. H. Marine natural products as anticancer drugs. *Mol. Cancer Ther.* **2005**, 4, 333-342.

23. Wright, A. E.; Forleo, D. A.; Gunawardana, G. P.; Gunasekera, S. P.; Koehn, F. E.; McConnell, O. J. Antitumor tetrahydroisoquinoline alkaloids from the colonial ascidian *Ecteinascidia turbinata*. *J Org Chem***. 1990**, 55, 4508-4512.

24. Reuters. Article. S.Korea approves Zeltia cancer drug Yondelis. http://www.reuters.com/article/idUSB73735320080508 (accessed Mar 31, 2010)

25. Rinehart, K. L. Jr.; Gloer, J. B.; Cook, J. C.; Mizsak, S. A.; Scahill, T. A. Structures of the didemnins, antiviral and cytotoxic depsipeptides from a Carribean tunicate. *J. Am. Chem. Soc.* **1981,** 103, 1857–1859.

26. Nuijen B.; Bouma, M.; Manda, C. et al. Pharmaceutical development of anticancer agents derived from marine sources. *Anticancer Drugs*. **2000**, 11, 793-811.

27. Medical News Today. Heath News. The FDA Approves Production Process Strategy Of Aplidin(R), New Marine-Derived Anti-Tumor Agent, Proposed By PharmaMar. http://www.medicalnewstoday.com/articles/126754.php (accessed Mar 31, **2010**)

28. Wender, P. A.; Baryza, J. L.; Bennett, C. E.; Bi, F. C.; et al. The Practical Synthesis of a Novel and Highly Potent Analogue of Bryostatin. *J Am Chem Soc.* **2002**, 124, 13648-13649.

29. Piel, J. Metabolites from symbiotic bacteria. *Nat. Prod. Rep.* **2004**, 21, 519-38.

30. Sudek, S.; Lopanik, N. B.; Waggoner, L. E.; el at. Identification of the Putative Bryostatin Polyketide Synthase Gene Cluster from "*Candidatus* Endobugula sertula", the Uncultivated Microbial Symbiont of the Marine Bryozoan *Bugula neritina*. *J Nat Prod*. **2007**, 70, 67-74.

31. Walsh, P. J.; Smith, S. L.; Felming, L. E.; Solo-Grabriele, H. M.; Gerwick, W. H. Eds. Oceans and Human Health: Risks and Remedies from the Seas; Academic Press: London, **2008.**

32. Simmons, T. L.; Coates, R. C.; Clark, B. R; el at. Biosynthetic origin of natural products isolated from marine microorganism-invertebrate assemblages. *Proc Nat Acad Sci USA.* **2007**, 105, 4587-4594.

33. William, P. G. Panning for chemical gold: marine bacteria as a source of new therapeutics. *Trends Biotechnol.* 2009, 27, 45-52.

34. Gerwick, W. H.; Proteau, P. J.; Nagle; D. G.; et al Structure of Curacin A, a Novel Antimitotic, Antiproliferative and Brine Shrimp Toxic Natural Product from the Marine Cyanobacterium Lyngbya majuscule. *J Org Chem*. **1994**, 59, 1243-1245.

35. Borman, S. A Pharmaceutical Company Would Never Do This. *Chem. Eng. News.* **2002**, *80*, 52.

36. Medina, R. A.; el at. Coibamide A, a Potent Antiproliferative Cyclic Depsipeptide from the Panamanian Marine Cyanobacterium Leptolyngbya sp. *J Am Chem.* **2008**, 130, 6324-6325.

37. Red Sea Report

38. Meyer, B. N.; ferrigni, N. R.; Putnam, J. E.; Jacobsen, L. B.; Nichols, D. E.; McLaughlin, J. L. Brine shrimp: a convenient general bioassay for active plants constituents. *Planta Med.* **1982**, 45, 31-34